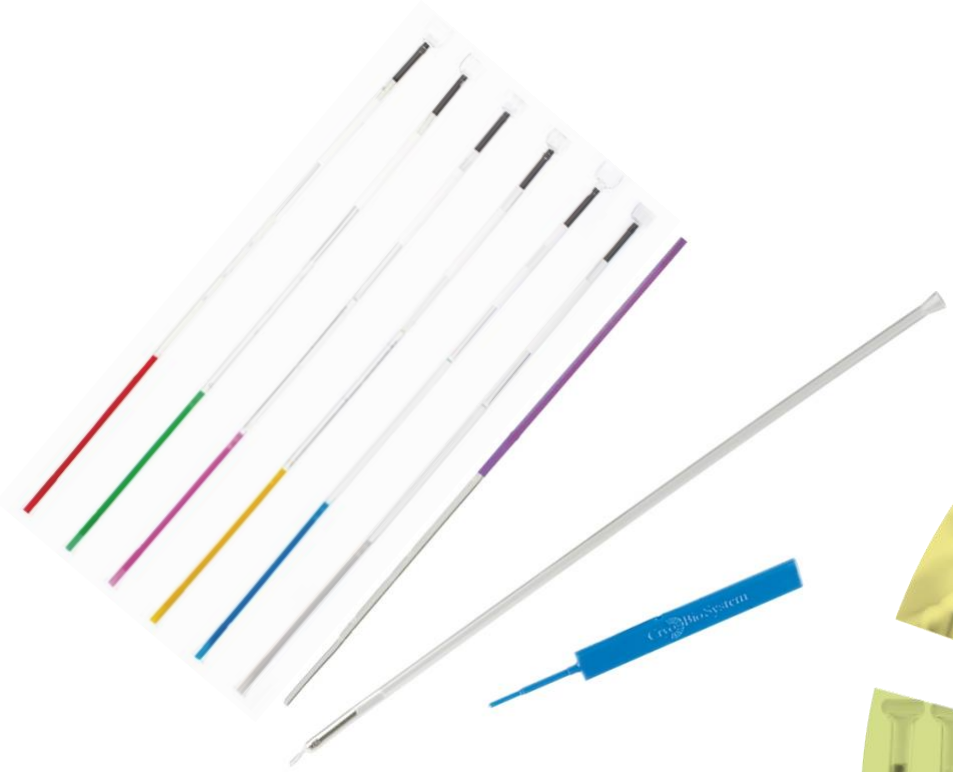
Cryo Bio System

CBS™ HIGH SECURITY CRYOBANKING SYSTEMS PRODUCT MONOGRAPH



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INTRODUCTION

This Product Monograph for the CBS™ range of High Security systems for cryobanking brings together, for the first time, a comprehensive background on cryobiology, cryopreservation (slow freezing) and vitrification systems, along with considerations of biocontainment that are fundamental to "safe cryobanking".

A Brief History of Cryobanking

Certainly the most significant, albeit accidental, discovery relating to sperm cryopreservation was the effectiveness of glycerol as a cryoprotective agent for bovine spermatozoa by Polge and colleagues in 1949.At that time the emphasis in semen cryopreservation was focussed upon farm animals (mainly bulls), but soon afterwards Sherman reported the successful cryopreservation and storage of human spermatozoa ondry ice (-78.5°C) with the production of normal offspring. Sherman also first described the use of liquid nitrogen vapour freezing in the early 1960s, along with the first normal births from this method. The still commonly used glycerol-egg yolk-citrate (GEYC) cryoprotectant medium for human spermatozoa was originally described by Ackerman in the late 1960s. Detailed historical reviews of this period have been published by Sherman (1973, 1977, 1980, 1990). For more recent reviews, see Royere (1996), Gao et al. (1997), Critser (1998), Leibo & Bradley (1999), Nijs & Ombelet (2001), Leibo et al. (2002), Pegg (2002) and Mortimer (2004a).

In the early 1970s the first commercial human semen cryobanks opened in the USA, in the face of unfounded and misleading concerns over the possible dangers in genetic and functional instability of cryopreserved spermatozoa. The perceived market was to provide "fertility insurance" for the anticipated millions of men who would have vasectomies. However, poor growth in that market considerably slowed the expansion of commercial semen banks until the second half of the 1980s when the absolute need for quarantined donorsemen to obviate the risk of AIDS transmission was established. Cryobanking human semen is now an established procedure worldwide.

Probably the most organized country for human sperm banking is France where the Fédération Française des Centres d'Étude et de Conservation du Sperme Humain (Fédération CECOS) was created in 1981 (David, 1989; Federation CECOS et al., 1989). From the original centre established at Le Kremlin-Bicêtre just outside Paris in 1973, CECOS grew to 14 centres by 1979 and 20 by 1989. In 1989 the name was modified to consider the possible cryopreservation of both sexes of gametes and became the Federation of Centres d'Étude et de Conservation des Oeufs et du Sperme Humain. Cumulative results from CECOS now exceed 50,000 live births (Le Lannou et al., 1998).

It is now accepted that semen cryopreservation maintains the reproductive potential of spermatozoa during effectively indefinite storage at -196°C. Frozen bovine spermatozoa have been used to produce normal blastocysts by IVF after 37 years in cryostorage (Leibo et al., 1994).

A Brief History of IMV and CBS™

IMV was founded by Robert Cassou, inventor of the famous "Cassou straw" or "French straw", in 1963. This followed his creation of the first cattle artificial insemination centre in 1946, and his being named Directorof the first cattle artificial insemination centre in France, at l'Aigle (Normandy) in 1952.

The French straw or paillette very quickly became the universally recognized method and de facto world standard for animal semen packing and preservation, and placed IMV Technologies firmly on the international stage. Today IMV Technologies – the artificial insemination pioneer in France – is the leading company in reproductive biotechnology, and continues to demonstrate the same passion for innovation as it did 30 years ago, constantly striving to perfect new technologies.

Timeline

1963 Creation of the "straw" which enabled bull semen to be stored in liquid nitrogen at -196°C, along with other artificialinsemination instrumentation, leading to the founding of the IMV Company.

1968 Birth of the calf Victoire as a result of IMV techniques, 10 years after the death of its sire.

1969 The French straw and French insemination techniques, as well as artificial genetic improvement in cattle, becomeworld standards.

1978 Development and launch of robotic machines for straw filling, sealing, labelling and freezing in liquid nitrogen.

1980 Creation of a US subsidiary.

1981 Creation and opening of the "la Sapaie" farm in l'Aigle and the international centre for training in cattle, sheep andother species artificial reproduction methods, as well as insemination and embryo transfer.

1982–89 Development of specific instrumentation for other animal species (poultry, sheep, goats, rabbits, horses, etc).

1992 The CBS™ straw is used in the first large scale epidemiological study carried out with the International Agency forResearch on Cancer in Lyon, involving 350,000 Europeans.

1993 Launch of a revolutionary product range (Cochette and Goldenpig) in the pig breeding sector.

1994 IMV is taken over by Jean-Gérard SAINT-RAMON.

1995 Opening of a clean room and laboratory for the definition and manufacture of biological media (in particular gametepreservation).

Launch of BIOCIPHOS, a major biotechnological innovation in cattle semen preservation.

1996 Launch of a pig semen preservation media product range, perfected in association with an American VeterinaryUniversity.

1997 Perfection of highly innovative poultry semen preservation media brings further refinements to the practice of artificialinsemination.

Perfection of a range of human artificial insemination products.

Perfection of new types of straw for new applications, including cryogenic preservation of live cell vaccines, genome resource banking for endangered species).

1998 IMV Technologies is introduced to the Paris Stock Exchange Second Market.

IMV India becomes a full subsidiary of IMV Technologies.

1999 Launch of DEC, the first bovine electronic Heat Detector.

IMV Technologies is awarded the Gold Trophy in the national contest, as well as the West Regional Trophy, at the10th International Trophy of French Small and Medium-sized Companies organized by «Les Echos» with the CréditLyonnais bank.

2000 Launch of the DEEPGOLDENPIG device for porcine intra-uterine insemination. This insemination process maximizesthe use of elite boars.

Launch of INNOV'IA, insemination synchronized with uterine

contractions.Tibet: The first AI stud for Yaks is set up by IMV

Technologies.

Partnership with the Wildlife Breeding Resource Center (Pretoria, South Africa) in Biological Resource Banking forAfrican Wildlife Conservation.

2001 The CBS™ straw is cleared by the FDA for marketing as a medical device in

the USA.Launch of Sacol, the on-farm AI Straw, for swine semen

packaging.

Launch of the TBS Straw for Bovine Semen Production: a productivity gain for semen production laboratories.

2002 Opening of IMV China, IMV Technologies' representative office in Beijing.

2003 CBS™ obtains ISO 13485 certifcation.

Launch of the "All in One Goldenbag" bio-secured swine semen packaging semen

system.Launch of "IS4" our latest semen processing integrated system.

Partnership with Hamilton Thorne Research (a division of Hamilton Thorne Bioscience, Inc.), the world leader inComputer-Aided Sperm Analysis (CASA).

2004 IMV Technologies receives ISO 9001 : 2000 certification

2006 Launch of the HSV High Security Vitrification Straw

An Overview of "Safe Cryobanking"

In 1995 a cluster of six cases of acute Hepatitis B virus (HBV) infection occurred among multiply-transfused patients undergoing cytotoxic treatment (Tedder et al., 1995). This event – the only reported case of cross-infection via LN2 storage (which was apparently due to a poor quality product combined with imperfect technique) – created enormous concern among cryobank operators and customers alike. For cross- contamination to occur, the infectious organisms must not only be released into the liquid nitrogen but also enter a "clean" unit and contaminate the material within.

Fortunately, the problem of such "cross-contamination" has not proven to be a major issue:

- Liquid nitrogen contaminated with pathogenic viruses did not contaminate specimens stored in properly sealed cryovials or straws immersed in it (Bielanski et al., 2000).
- Micro-organisms contained in properly-sealed straws neither leaked out in the liquid nitrogen nor contaminated "clean" samples in the same tanks (Bielanski et al., 2003).
- Semen from an HIV-positive donor was unknowingly used to inseminate several women who became infected (Stewart et al., 1985) but there were no infections in the many insemination cycles where donor semen that had been stored contemporaneously in the same cryotank was used (JPP Tyler, cited by Mortimer, 2004a).
- Various papers have reported that there is no direct evidence of any cross-contamination in a cryobank within a fertility clinic or sperm bank setting (Kuleshova & Shaw, 2000; Tomlinson & Sakkas, 2000; Centola, 2002).
- World-wide enquiries using reproductive biology professional groups' Internet list servers returned no reports from reproductive cryobanks of any occurrence of cross-contamination using straws or cryovials (Mortimer, 2004a).

Nonetheless, although the risk of cross-contamination is certainly unquantifiable (Tomlinson & Sakkas, 2000), it cannot be ignored. The risk cannot be presumed to be theoretical, and everyone involved in gamete and embryo cryobanking must take all available steps to minimize the risk in their banks.

While some authorities have stated that "straws are microbiologically hazardous" and consequently recommended screw-capped cryotubes for storage of semen and embryos (UK Royal College of Pathologists, see McLaughlin et al., 1999 or Wood, 1999), as well as recommending that both secondary seals and vapour phase storage should be used, some experts (e.g. Rall, 2003, Mortimer, 2004a) consider that properly-sealed straws are the more secure packaging system.

The Table on the following page is adapted from Mortimer (2004a) and lists the various mechanisms, in decreasing order of likelihood, by which liquid nitrogen and cryogenic storage tanks can become contaminated.

The use of "quarantine tanks" to store specimens pending their release after re-testing of the donor for infectious organisms is fraught with issues, and an increasing number of human sperm and embryo cryobanks now operate on the principle of "universal contamination" (see Mortimer, 2004a). Certainly, cryopreserved samples from patients who are known to carry an infection (e.g. HIV- or hepatitis-positive men) can be stored in separate "dirty" tanks (Tomlinson & Sakkas, 2000) – but, of course, there must be a separate tank for each combination of recognized pathogenic organisms.

Mechanisms (in decreasing order of likelihood in each category) by which liquid nitrogen can become contaminated.

Contamination of liquid nitrogen with pathogens	Packaging issues	Use of quarantine tanks
Semen contaminating the outside ofthe packaging unit, particularly straws (Russell et al., 1997).	Using unpackaged material (e.g.“pellets” as with ram semen, Piasecka-Serafin, 1972).	Moving "cleared" samples into a long-term “clean” storage tank froma quarantine tank that has previously held specimens known tohave tested positive for pathogens.
Split or broken straws.	Non-sealed packaging devices (aparticular problem with many vitrification devices, e.g. cryo- loops).	Moving "cleared" samples into a long-term “clean” storage tank froma quarantine tank that is subsequently found, upon retesting,to have held specimens positive for pathogens.
Using LN2 from a contaminated cryotank to handle units being frozen (e.g. during seeding) or while being transferred from the freezing machine to the cryobank, between cryotanks within the bank,or to fill a dry shipper.		
Room air or the operators' exhaledbreath (the "fogging" when a cryotank is opened). This is the major source of the white (ice) "sludge" that accumulates at the bottom of cryotanks.	Frozen material from imperfectlysealed cryovials or straws.	Re-tasking of a cryotank previouslyused to store known-contaminatedspecimens, without adequate sanitization.
Skin commensals from operatorswhile leaning over an opened cryotank.	Directly through the wall of intact,properly sealed plastic straws (no evidence exists for this mechanism).	Semen from unscreened men (e.g."rush" freezes for oncology patients).
The liquid nitrogen itself, at point ofmanufacture.		
Via liquid nitrogen that escapes from an imperfectly sealed cryovialor straw that holds contaminated material (although there is no physical reason why this would happen during storage).		Semen from screened men in the case of currently unknown pathogens (e.g. new strains of thehepatitis virus, see Bahadur & Tedder, 1997b; Clarke, 1999; Tomlinson & Sakkas, 2000).

CRYOBIOLOGY BASICS

Basic Principles of Cryobiology

As a general rule, the challenge when it comes to preserving tissue boils down to being able to block, or at least slow down, intracellular functions at the same time as preserving the physicochemical structures on which these functions depend. The laws of nature determine that as a living organism is submitted to decreasing temperature, its activity slows down and eventually stops altogether. Although the chemical reactions of life can be suspended for an unlimited duration in simple biological organisms, the same is nottrue for organisms with high-level complexity. Moreover, for any given complex life form, each constituentcell type has a certain degree of resistance which is, in every case, greater than that of the organism as awhole.

1) General conditions for the preservation of ultrastructure and cellular function

Resistance to cold – and freezing – depends on a number of different factors. Sudden cooling can adversely affect almost all cells; a form of thermal shock can occur both above and below the freezing point. Also, the formation of ice crystals both inside and outside cells can cause serious physical damage to the cells. Another consequence of ice formation is a change in the physical environment of the cells. The freezing of water results in an increase in the concentration of salt in the solution up to a specific level which corresponds tothe eutectic point. Such an increase in electrolyte concentration leads to cellular dehydration. It is worthnoting that the cells which are most resistant to dehydration are the same ones that are most resistant tolow temperature.

2) Thermal shock

Thermal shock can occur when cells are cooled too quickly, even in the absence of ice crystallization. The critical range is between +15°C and 0°C (between 59 and 32°F), although thermal shock can also occur between 0°C and -80°C (between 32 and -112°F). Thermal shock damage begins at the plasma membraneas a result of:

- differential shrinkage of various membrane components;
- mechanical shearing; and
- conformational changes in membrane topography.

The damage inflicted is not significantly dependent on the cation profile in the extracellular fluid; the anionprofile is far more important. Important anions are, in order from the least dangerous to the most dangerous: acetate, chloride, nitrate, iodide, sulphate.

Thermal shock can be mitigated by:

- cryprotective agents, both permeating and non-permeating;
- the presence of certain phospholipids (phosphatidyl serine);
- slow cooling; and
- pre-conditioning in a high salt medium.

3) Dehydration threshold

Most vertebrate cells are susceptible to even partial dehydration (at either normal or low temperature). Depending on the specific cell type and the species, cells cannot tolerate losing more than 20–80% of their baseline water content. Every cell type has a dehydration threshold beyond which cellular structures suffer irreversible damage. The correlation between a cell's resistance to dehydration and its resistance to low temperature stems from effects on ionic balance, with the increase in salt concentration that accompanies crystallization being responsible for the worst effects. Such an increase induces the precipitation of proteins, resulting in serious damage to membrane lipoprotein structures. At the same time, the crystallization of

buffer salts can lead to major pH changes, which can also result in protein damage. The increase in the concentration of certain compounds and ions can lead to toxic effects inside the cell. Freezing can also affect the colloidal nature of the intracellular milieu – an effect which may be reversible or irreversible. The colloidal system may separate out into its two distinct phases, with associated water molecules being stripped away from the organic macromolecules so that the latter aggregate and become vitrified. However, the vitrification theory, although consistent with many experimental observations, cannot be reconciled with the rates of cooling and warming that are in routine use.

4) Rate of cooling and warming

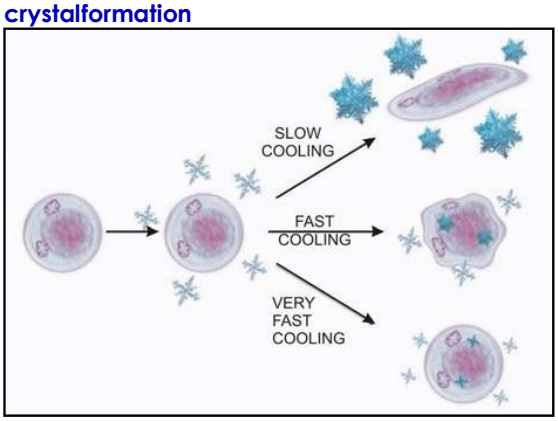
There are no definitive rules when it comes to freezing mammalian cells and tissues. The ideal method of freezing and optimum rate of cooling depend on many, sometimes conflicting, factors – many of which remain poorly understood. Ideal cooling conditions are often determined by trial and error, resulting in protocols stipulating the rate of cooling, whether the temperature should be lowered in timed steps, which cryoprotectant agents to use, etc. The general aim of such protocols is to prevent thermal shock, the adverse effects of excessive salt concentrations, and damage to the cell's colloidal milieu. Although it is difficult tostate rules, it is possible to make certain general comments about cooling rates and associated cellular damage.

At the cooling rates in routine usage, seeding and crystallization always begin in the extracellular compartment. This means that the cell's osmotic balance is disturbed resulting in nascent dehydration, resulting in increased electrolyte concentrations that can lead to irreversible damage. Once ice has begun to form, an important factor with respect to keeping the cells viable during the subsequent freezing is therate of temperature change. This determines:

- the length of time the cells spend above the eutectic point, i.e. the length of time that they areexposed to a high salt concentration;
- the rate of intracellular freezing; and
- the size and shape of any ice crystals formed.

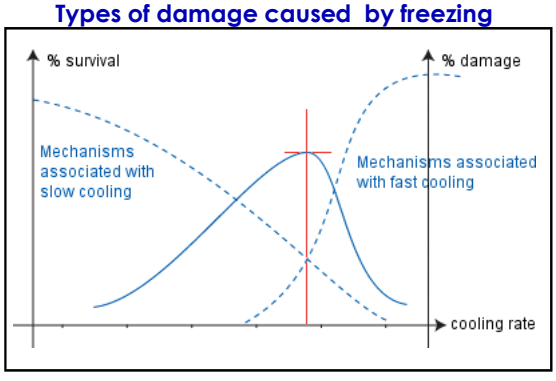
If cooling is slow, ice crystals grow in the extracellular compartment so the cells shrink and are exposed to very high salt concentrations. Subsequently, the fluid phase freezes at the eutectic temperature. But if cooling is rapid, the water inside the cells forms small, irregularly-shaped ice crystals which are relatively unstable. If the cells are subsequently thawed out too slowly, these crystals will aggregate to form larger, more stable crystals which can cause damage.

Cooling rate and intracellular and extracellular crystalformation



The increase in salt concentration that accompanies the freezing process causes shrinkage of the cells which, if it continues beyond a certain threshold, leads to permeabilization of the membrane to ions. Cell survival therefore depends on the rate of cooling: survival peak at a certain cooling rate, and then falls as the rate increases further. The ideal cooling rate is low enough to allow the cell to lose sufficient water to prevent premature intracellular freezing. However, if the rate is too slow the cells will be exposed to a high salt concentration for too long a time.

Types of damage caused by freezing



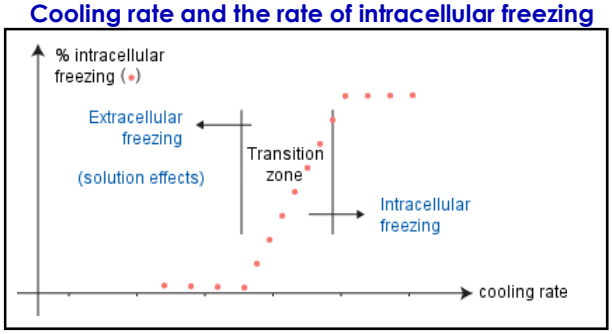
In addition, the ideal rate of cooling depends on the cell's **critical volume** which is defined by:

- the permeability of the plasma membrane to water;
- the surface area of the membrane; and
- the cell's surface to volume ratio.

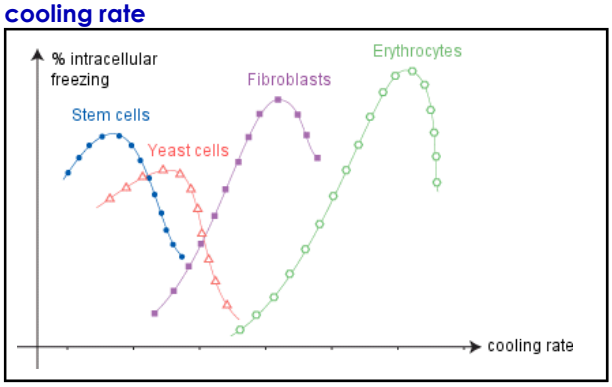
In summary, it could be said that the simplest explanation of why an ideal cooling rate exists at all is because cell survival depends on two conflicting factors, both of which depend on the cooling rate. The main factor which causes loss of viability is the formation of intracellular ice crystals, and especially the aggregation of these crystals during thawing. At a slow rate of cooling, the resultant high salt concentration exercises adverse effects when intracellular water – which has a high chemical potential – diffuses out of the cell to freeze in the extracellular compartment. The highest survival rates are obtained at a range of cooling rates referred to as the transition zone, in which the combined effect of both mechanisms is at a minimum.

As a general rule, the rate of thawing is closely associated with the rate of freezing, with rapid thawing typically being preferable to slow thawing. Although the rate of thawing does not have as great an effect on cells which were frozen slowly, it has a major impact on cells that were frozen more rapidly. These observations tend to show that at least some of the intracellular damage caused by ice occurs duringthawing and not just when the crystals are initially formed in the freezing process.

Cooling rate and the rate of intracellular freezing



Survival rate of different types of cells as a function of cooling rate



5) Low temperature phenomena

There is a great deal of information about the biophysical and biochemical changes that occur in living cells at temperatures of below the freezing point of water, i.e. below 0°C (32°F). Many bacteria and yeast cells can survive and multiply at -8°C (17.6°F) despite the frozen state of the surrounding environment. It is wellknown that food (meat, fish, fruit and vegetables) lose some of their flavour when stored at -25°C (-13°F):at this temperature, microorganisms are quite inactive so this indicates that there remains some enzyme activity at such low temperatures. It can be concluded that, unless the storage temperature is very low indeed, cells will continue to age and their survival time will depend on their residual metabolic rate whichwill be slowed down to a greater or lesser extent.

At temperatures of between 0°C and -40°C (between 32°F and -40°F), which is not low enough for the efficient preservation of certain types of cell, diffusion proceeds and can modify the ionic balance in the medium. At lower temperatures, the electrolyte solutions are solidified in the form of eutectics.

The crystalline structure of the ice may also change (with the growth of crystals or re-crystallization) resulting in serious physical damage to important biological structures.

6) Low temperature preservation

In the world of the biological sciences, -70°C (-94°F) is considered the threshold temperature below which no life process is sustained. When contemplating long-term or very long-term storage, temperatures belowthis threshold have to be employed. Most observers hold that cells stored in the presence of some cryoprotective agent (e.g. dimethylsulphoxide [DMSO], glycerol or propanediol) do not undergo any damage as long as they are kept at a temperature of below -132°C (-205.6°F), the transition point of water. Some particularly labile cells might need to be stored at extremely low temperatures, such as that of liquid nitrogen
-196°C (-320.8°F). Although it was estimated that a seed with a lifetime of 1 year when stored at between 10°C and 20°C (Becquerel, 1950) would still be able to germinate after over 71 trillion years if it were stored at a temperature of -270°C, Ashwood-Smith & Grant (1977) pointed out that indefinite preservation at -196°C is a practical impossibility because, as a result of ambient ionizing radiation, the cells would absorb a crippling dose of 600 rads over a period of just 32,000 years.

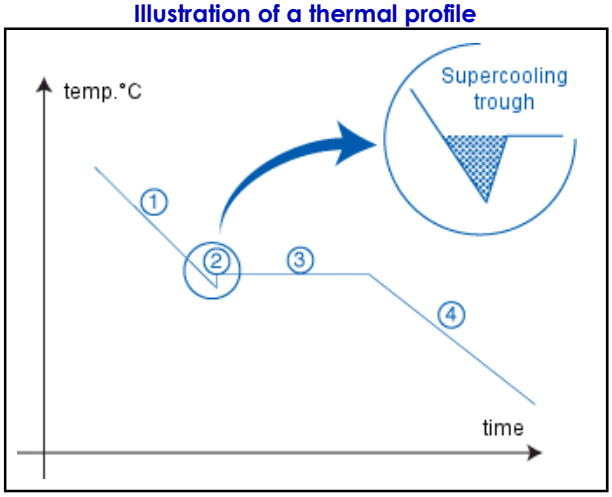
7) Temperature measurements

Because the rates of freezing and thawing can have a major impact on the viability of cells and tissues, they need to be measured in a quantitative fashion. The best way of doing this is to generate complete curves describing the processes of freezing and the thawing. From these, the following can be determined:

- the cooling before freezing begins;
- the time of super-cooling and its parameters;
- the onset of crystallization and the duration of the phase-change plateau; and
- the rate at which the temperature drops after the phase change.

This "cooling curve" is referred to as the thermal profile of the system under consideration and can be normalized to a reference measurement for ease of manipulation. The most suitable parameter to estimate the cooling rate is the time it takes for the temperature to fall from 5°C (41°F) – i.e. the point at which the major part of the phase change proceeds – to below -50°C (-58°F). Over this range, temperature tends to become a linear function of time in such a way that a single mean value for the cooling or thawing rate can be used meaningfully.

Illustration of a thermal profile



During the freezing process, super-cooling (i.e. cooling below a solution's freezing point) constitutes an important and critical phase of the thermal profile: it can determine how the seeding of ice crystals occurs and hence the mode of crystallization.

8) Cryoprotective agents

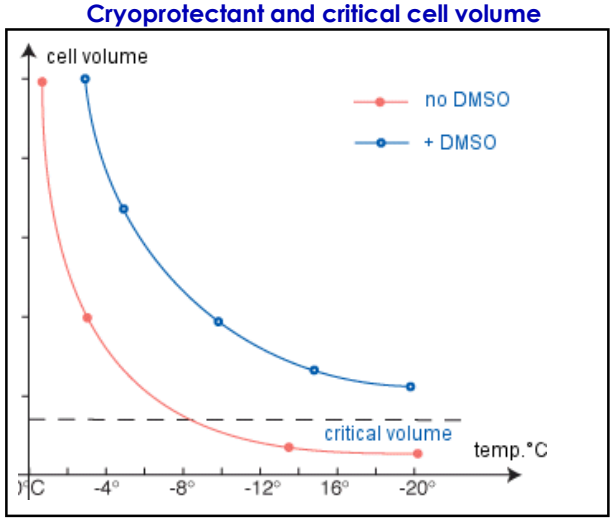
The main causes of cell death are:

- the formation of intracellular ice; and
- exposure to high salt concentrations.

The challenge during freezing is therefore to maintain – in both the intracellular and the extracellular compartments – a sufficient volume of water in the liquid phase (i.e. uncrystallized) to keep the electrolytes in solution.

Seeding and subsequently crystallization can be inhibited by inactivating potential condensation points by means of chemical "poisoning". The agents used to this end are capable of binding to water molecules through hydrogen bonds and are referred to as cryoprotectants. These are diverse in chemical structure but all work in more or less the same way.

Cryoprotectant and critical cell volume



What are cryoprotectors?

Whatever their molecular structure, all cryoprotective agents ("cryoprotectants") are highly soluble in water. By virtue of their capacity to form stable hydrogen bonds with water molecules, they decrease the freezing point of any solution in which they are included. Their second key property is that they are non-toxic to the cells that they are supposed to be "protecting". Toxicity in this case is a rather difficult concept to define because external conditions – as opposed to the nature of the product itself – may be important.

In practice, toxicity depends on:

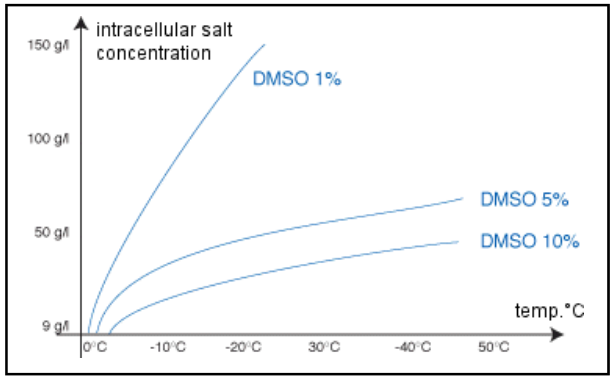
- the concentration of the cryoprotective agent;
- the tonicity of the medium; and
- how the cells are in contact with the agent.

To describe the biophysical and biochemical interactions between a cryoprotector and the cells, the term intrinsic toxicity is more suitable. The other variables that determine the extent to which a particular additive will be able to protect the cells of a given biological system depend on factors such as the compound's molecular weight and its ability to cross the plasma membrane of the cells being frozen. Only agents of relatively low molecular weight (below 400 Da) are able to penetrate into cells. How higher molecular weight agents (of the order of thousands) which cannot cross the plasma membrane exert their protective effect ispoorly understood. How cryoprotective agents are added and removed is itself a whole separate issue in the field of cryopreservation because it is vital that the cell's critical volume limits are not exceeded.

What cryoprotectors do?

In general terms, cryoprotectors modulate the eutectic properties of a solution so that the amount of ice formed and, in consequence, the concentrations of salts, are reduced. By lowering the freezing point of the extracellular fluid, they inhibit the efflux of intracellular water thereby preventing the cell shrinking to its minimum critical volume. By reducing cellular retraction, these agents attenuate hyper concentration of the intracellular fluid and thereby inhibit the precipitation of protein. In practice, in the presence of 1% DMSO an isotonic saline solution (9 g/l NaCl) willreach a concentration of 50 g/l at a temperature of -5°C (23°F). In the presence of 5% DMSO, this concentration will not be reached until the temperature has dropped to -20°C (-4°F), and at 10% DMSO, notuntil -50°C (-58°F). At lower temperatures, protein isdenatured to a significantly lesser extent.

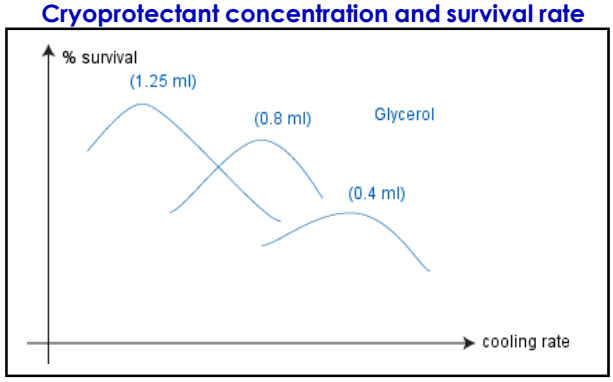
Starting DMSO concentration and salt concentrationduring freezing



Cryoprotectors and cooling rates

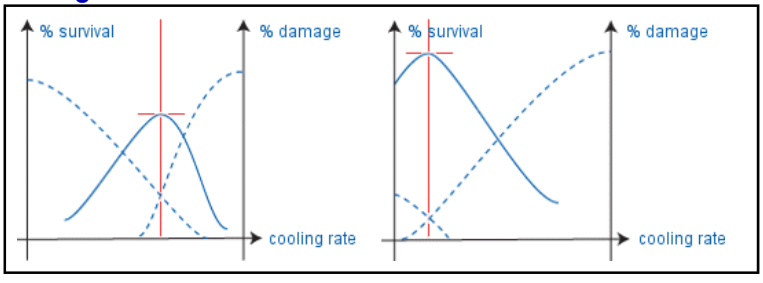
The amount of damage induced in cells during freezing depends on two conflicting factors, both of which depend on the rate of cooling: at rates that are too slow, high salt concentrations will be generated; and at rates that are too fast, ice will form inside the cells. Maximum viability is obtained by cooling at a rate in a transition zone in which the combined effect of both these mechanisms is minimized. As a general rule, cryoprotective agents appear to protect cells at slow cooling rates at which damage associated with "solution effects" is believed to predominate.

Cryoprotectant concentration and survival rate



Based on empirical observations, it has been shown that the result of increasing the concentration of a cryoprotectant (e.g. glycerol or DMSO) in cell suspen- sions being cooled at different rates is toshift the optimum rate downward coupled with an enhancement of the overall survival rate. It has been documented that the problems associated with intra-

Effect of cryoprotective agent on survival rate and shifting of the cell damage "transition zone"



cellular freezing are not affected by the presence of a cryoprotectant.

With reference to the transition zone concept described above, cryoprotectants present during freezing shift the entire zone in the direction of lower survival by lowering the threshold associated with damage causedby the combination of high salt and intracellular ice.

Controlled Rate Freezing and Vitrification Compared

Cryopreservation, or "controlled rate" freezing is achieved by slow cooling with induction of ice crystallizationin the medium outside the cells or embryos, causing a progressive dehydration of the cells. Vitrification, onthe other hand, uses ultra-rapid cooling of the medium containing the cells or embryos so that the medium does not form ice crystals but rather increases so greatly in viscosity that it becomes a "glass" at low temperatures. Basically, the solution solidifies so swiftly the molecules do not have sufficient time to rearrange themselves into a crystal structure, retaining an amorphous structure. Dehydration is achievedin the brief period of exposure to the very high osmolarity vitrification solution just before the ultra-rapid cooling step. While the cryoprotective agents used for both cryopreservation and vitrification are composed of permeating (e.g. ethylene glycol and/or DMSO) and non-permeating (e.g. sucrose or trehalose) agents, vitrification formulations differ in their use of highly concentrated solutions of permeating cryoprotectant(s), typically 30–50% (v/v). Recent reviews of vitrification include Nawroth et al. (2005), Mortimer & Vanderzwalmen (2006) and Vajta & Nagy (2006).

The exposure of embryos to high concentrations of cryoprotectants required for vitrification has meant thatmany IVF laboratories are cautious about using vitrification clinically. Certainly vitrification is quick, and does not require a controlled rate freezer or involve the technical issues surrounding proper seeding, but it has its own problems: the high concentrations of permeating cryoprotectants are toxic to most types of cell, andthe very rapid dehydration of the cells will result in them exceeding their critical volume limits – so one set of problems has simply been exchanged for another. Cryoprotectant toxicity can be mitigated to some extent by using two different cryoprotectants in the vitrification solution. Additionally, in many vitrification protocols the solutions are supplemented with macromolecules such as polyethylene glycol (PEG; MW 8000), Ficoll (MW 70,000 or 400,000) or polyvinylpyrrolidone (PVP; MW 360,000) to increase the viscosity; thesepolymers are generally less toxic and can protect the embryos against cryo-injury, while also of the solution.By increasing the viscosity, the macromolecules support vitrification with lowered concentrations of cryoprotectants (Lieberman & Tucker, 2002; Lieberman et al., 2003). The viscous matrix that encapsulates the cells also prevents water crystallization during cooling and warming. Also, increased survival of oocytes after vitrification using solutions supplemented with macromolecules has been reported (Kuleshova et al.,2001). Toxicity of the vitrification solution can also be reduced by shortening the length of time the embryos are exposed to it, or by pre-cooling it, since the rate of cryoprotectant penetration is temperature dependent (Vanderzwalmen et al., 1988).

In contrast to the slow cooling rates employed for cryopreservation, vitrification requires cooling rates of upto 2000°C/min (Vanderzwalmen et al., 1997, 2002). Achieving such ultra-rapid cooling rates is hamperedby the following factors, all of which slow the effective cooling rate:

- the thickness of the wall of the packaging;
- the relative volume of liquid surrounding the gametes or embryos; and
- boiling of the liquid nitrogen, since the vapour layer formed around the specimen insulates it from thecold liquid nitrogen (the "Leidenfrost Effect").

The Leidenfrost Effect can be reduced by decreasing the temperature of the liquid nitrogen to –210°C, thereby eliminating vapour formation and achieving even higher cooling rates (e.g. the Vitmaster device, see Arav et al., 2000). High cooling rates (approaching 10,000°C/min) are also achieved by employing "minimalist" carrier devices for the cells being vitrified: minimizing both the specimen volume, the packaging mass, and maximizing the surface area:volume ratio of the specimen. See the section on "Packaging devices for vitrification" for further discussion of these devices.

Effect of Packaging on Cooling and Warming Rates

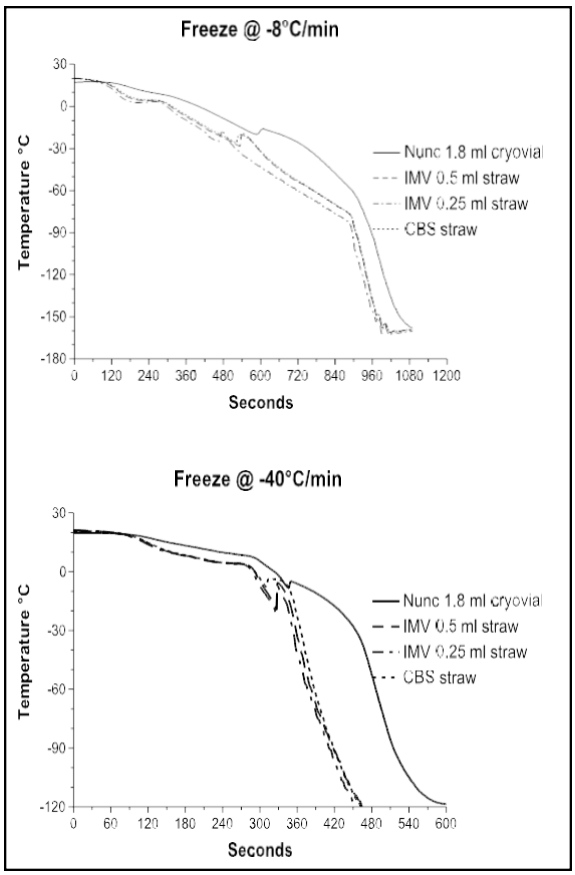
Simple physics dictates that a larger radius impedes heat transfer so that the cooling rate achieved lags behind the desired cooling curve, and there is uneven heat exchange throughout the specimen (Morris, 2002). This issue was discussed at length by Mortimer (2004a), whose analysis showed this to be a marked disadvantage of cryovials, and likely to lead to impaired cryosurvival of specimens frozen in such large diameter packages.

Practical measurements of the cooling curves achieved in all sizes of straws (IMV 0.5 and 0.25 ml and CBS™ 0.5 ml High Security Straws) confirmed that specimens packaged in each type of straw experience very similar cooling curves.

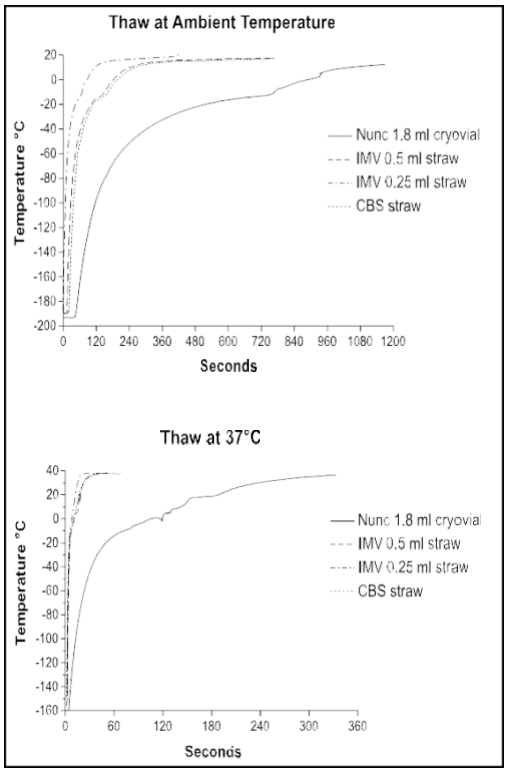
The other side of this issue is the effective warming rate that can be achieved, impacting not only the effective thawing of specimens but also the risk of their warming during handling for brief periods outside the cryogenic storage tank. Here the ability to achieve rapid warming rates in straws is a double-edged sword as it leads to an increased risk of damage: a 0.25-ml straw will warm to -80°C within 15 seconds in air at ambient temperature (Tyler et al., 1996).

Other techniques and devices have been developed in order to achieve the ultra-rapid cooling rates of the order of 2000°C to 10,000°

Cooling curves measured by thermocouple inside various packaging systems (see Mortimer, 2004a).



Warming curves measured by thermocouple inside various packaging systems (see Mortimer, 2004a).



per minute that are required for vitrification (see "Packaging devices for vitrification", below).

Storage Temperatures

To maintain biological integrity, specimens must be maintained below the glass transition temperature of water, i.e. below ca. -132°C in order to stop all biological activity (Mazur, 1984). Because some biological activity might continue even at -80°C in an "ultracold" mechanical refrigerator, or at -79°C on dry ice, degradation of cryobanked biological material accumulates over time. Human spermatozoa stored at -79°C show a progressive decline in post-thaw motility, an effect that is accelerated when stored above -75°C (Ackerman, 1968). Parallel storage of human sperm specimens in a mechanical freezer at -70°C and in liquid nitrogen at -196°C showed lower cryosurvival at -70°C, an effect that was amplified with longer storage time (Trummer et al., 1998). Bovine spermatozoa might be more hardy than human spermatozoa since functional survival has been demonstrated following 4 years of storage at -79°C, as well as after 33 years at -196°C (Leibo, 1999).

Of particular importance to the storage and handling of all cryobanked materials is what happens to water as it warms from cryogenic temperatures. The glass transition of a frozen aqueous solution is not a sudden event at exactly -132°C, glass transition occurs progressively between this temperature and about -90°C; so that by -80°C substantial change would have already occurred. During warming, energy is returned to thewater molecules, allowing them to resume their natural orientation: because very small ice crystals are unstable, with a large surface area:volume ratio, they tend to fuse together to reduce that ratio. It is therefore essential that cryopreserved material be kept below -132°C, and hence storage in liquid nitrogenvapour (-150°C) or in liquid nitrogen (-196°C) must be employed. The lower the temperature the greater the margin of safety, for example when a specimen is removed briefly to check its identity.

The report of cross-infection of bone marrow and blood stem cells during storage in liquid nitrogen (Tedderet al., 1995) led to several calls for storage in liquid nitrogen to be abandoned in favour of storage in the vapour or gaseous phase either above a layer of liquid nitrogen ("vapour phase" storage) or in newer design freezers that enclosed the cryogenic liquid nitrogen inside a sealed vessel so that storage was in super-coldair (e.g. Isothermal Vapor Storage models from Custom BioGenic Systems, Shelby Township, MI, USA). Mechanical cryogenic freezers (e.g. Ultima II Series from Revco, Asheville, NC, USA: Burden, 1999) are very expensive to run and provide no security if the mains power fails without a substantial emergency generatorsystem (typically in excess of 4 kW, with many models requiring 3-phase power).

A major concern is that liquid nitrogen vapour and super-cold air are poor conductors of heat and have verylow thermal capacity, so they cool specimens poorly and heat up very quickly in the presence of a warm object, even ambient air. Because every second spent above -132°C (and especially above -80°C, see above) causes accumulation of irreversible damage to the frozen cells extreme care must be taken to ensure that specimens are kept below -132°C whenever they are they are manipulated, e.g. during transfer into the cryobank, during storage audits, and when they are being retrieved (Simione, 1999).

Specimen Identification

Labelling

Modern accreditation standards require that at least two unique identifiers be used to label each clinical specimen, and within Europe, labelling will be regulated under the EU Tissues and Cells Directive 2004/23/EC (European Union, 2004), as detailed in an as-yet unpublished second Technical Directive that will include aunified European Coding System. Certainly the inclusion of all proposed information on packaging as small as straws might be problematic, but it is clearly essential that every specimen must be labelled both unambiguously and as informatively as is practically possible. While location identifiers within a storage dewar are very helpful in locating a specimen, they cannot replace direct labelling of each packaging unit. This is obviously impossible with, for example, (ram) semen frozen in pellets and stored "naked" in the liquid nitrogen inside plastic tubes, and such a system can have no place in any commercial or clinical cryobank. Similarly, some of the early vitrification devices offered very limited secure labelling options.

Bar codes have been used for many years in cryobanking but cannot be employed exclusively as some form of human-readable identification must also be used in case of machine failure. Similarly, radio frequency identification devices ("RFIDs") have been proposed as an extra layer of secure labelling, but most currently- available RFIDs are too large to be used with straws, and there remain several issues with reading them, especially more than one at a time, at cryogenic storage temperatures.

Historically, many methods were used to label straws that are now considered unsafe, e.g. wrapping a sticky label around the straw as a "flag" (these are easily broken off when handled at cryogenic temperatures). Also, writing on some types of straws could lead to contamination of the insidesof the straw by the solvent base of the marker pen.

Adhesive labels are commonly used in IVF labs and sperm banks, and it is important to verify that the labels will not come off during immersion in liquid nitrogen. Probably the most widely-used labels are those from BradySystems (Brady Corporation: Milwaukee, WI, USA; see www.bradyid.com), generated using a Brady label printer such as the LabPal (see Figure, right).

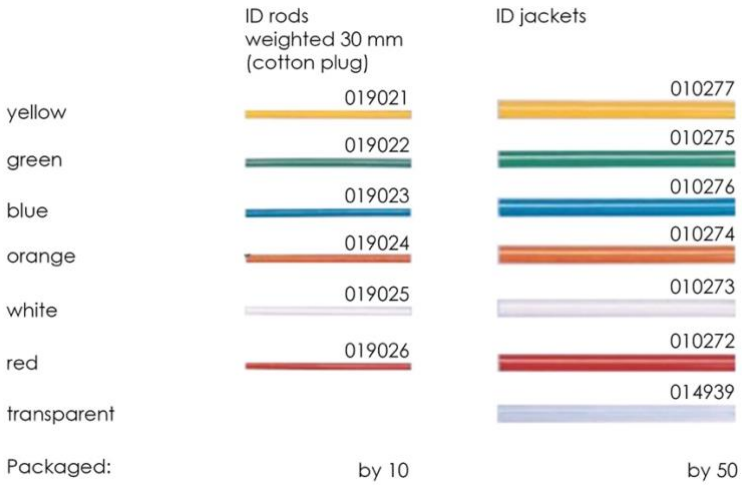
Labelling is an important component of the CBS™ "High Security" cryobanking packaging systems:
For 0.3 ml straws

- Their two-compartment design separates the specimen and identification compartments, allowing identifying information to be sealed inside the straw itself, making identification tamper-proof. (Thisoption is not available on certain special purpose products.)

For 0.5 ml straws

- Secure external identification sleeves or "jackets" that cannot slide off the straw once sealed (due tothe flat "tabs" formed at each end when the straw is welded shut) can also be used.

The different configuration of High Security Straws requires slightly different internal identification rods ("IDrods"). ID rods are weighted so as to prevent the filled straws from floating when immersed in liquid nitrogen due, for example, to the extra air bubbles that are included when loading embryos. Specific 30 mm ID rods are used when straws are filled using the MAPI or PACE automated systems. The complete range of the identification devices is shown in the Figure, below.



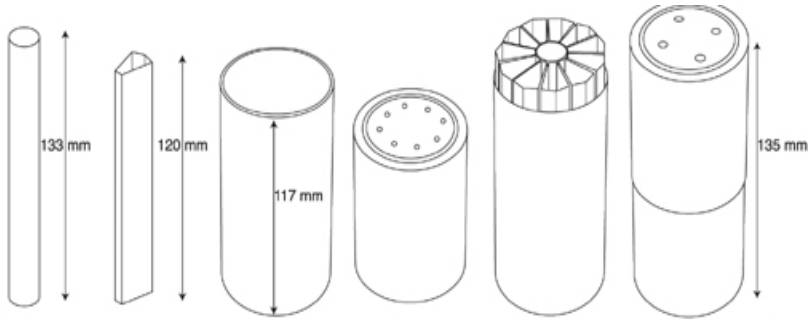
Brady or other labels can be attached to ID rods, and the ID jackets can be written-on directly using a suitable marker pen, or printed using a custom printer such as the MAPI device from Cryo Bio System (see separate product information at www.cryobiosystem.com).

Inventory systems

Inventory systems for organizing storage in cryotanks are very important in running a secure and efficient cryobank. Straws are best stored using goblets inside the long-handled metal or plastic canisters that are located inside the dewars. Goblets are sub-divided using mini-goblets or visotubes – which come in a variety of shapes and colours to facilitate the rapid location of specimens. A comprehensive description of these inventory system components is provided on the following page.

CBS™ High Security Straws in a daisy goblet

Range of goblets and visotubes available from Cryo Bio System

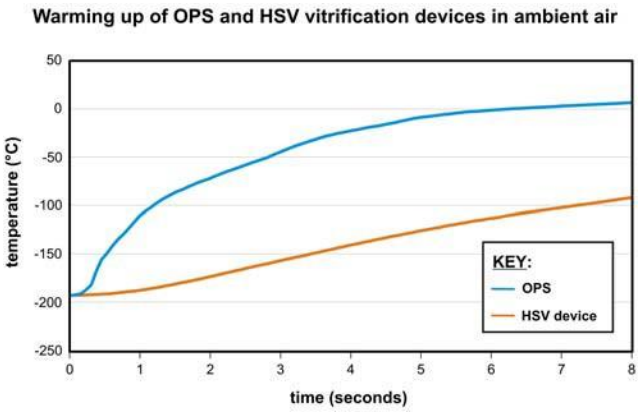


ITEM Specifications		Round Visotube Ø 17.5	Triangular Visotube	Perforated Goblet Ø 65 mm	Cover (Ø 67 mm) for Goblet	Daisy Goblet* (bag of 1)	Daisy Goblet* (bag of 5)
COLORS	Clear	006924	-	006995	006321	015144	015152
	Black	-	018153	-	-	-	-
	Brown	-	018147	-	-	-	-
	Red	-	018146	006997	-	015146	015154
	Green	-	018145	006998	-	015147	015155
	Blue	-	018148	007001	-	015151	015159
	Grey	-	018144	-	-	018141	018142
	Purple	-	018152	006999	-	015150	015158
	Yellow	-	018143	007000	-	015148	015156
	Pink	-	018149	-	-	-	-
	Orange	-	018150	-	-	-	-
	Pistachio	-	018151	006996	-	015149	015157
PACKAGING		100	100	10	10	1	5
CAPACITY	0.25 ml classical Straws	45	45	820	-	540	540
	0.5 ml classical Straws	20	20	365	-	240	240
	0.3 ml & 0.5 ml CBS™ Straw	12	12	225	-	144	144
* With colored visotubes and white cover							

Name Caracteristics	Polygonal visotube	Hexagonal visotube	Round visotube ø 7.1 mm	Round visotube ø 9.2 mm	Round visotube ø 10 mm	Round visotube ø 12 mm	Round visotube ø 13 mm	Goblet ø 35 mm	Goblet ø 65 mm
White	006401	006875	006658	006421	005561	006677	006420	006934	006418
Red	006622	006398	006441	006668	006392	006408	006404	-	006397
Green	006623	006394	006659	006390	006406	006679	006413	-	006754
Blue	006620	006409	006399	006400	006422	006678	006395	-	006755
Purple	-	-	-	-	-	-	-	-	006757
Yellow	006624	006414	006412	006423	006402	006695	006411	-	006756
Orange	006619	006405	006662	006667	006673	006683	006689	-	-
Pistachio	-	-	-	-	-	-	-	-	006753
Unit packaging, minimum quantity 10.									

Auditing

Extreme care must be taken whenever cryobanked specimens are handled, both from the perspective of the safety of the operator andto protect the specimen from the damage that will accumulate, irreversibly, whenever it warms to above -132°C. This problem is greatest for material frozen in straws, in particular for the older 0.25-ml IMV straws, and especially for vitrification devices. For example, a open-pulled straw ("OPS") warms from -196°C to the glass transition point of water (-132°C) in about 0.6 seconds when exposed to ambient temperatureair (see Figure, right).



Many cryobanks carry out periodic reviews of the status of all stored gametes and embryos in order to ensure that the cryobank's records reconcile with the material actually in storage (and also perhaps with patients'medical records). While such an "audit" is often perceived as necessary, it must be recognized that it could put the specimens at risk due to the accumulation of latent cryodamage during (repeated) transient warming of specimens above the glass transition point of water (-132°C). Efficient and safe auditing of a cryobank relies upon the following factors (taken from Mortimer, 2004a):

- An inventory system that allows for easy and quick access to specimens within the cryogenic storagetanks.
- Unambiguous (and secure) labelling systems that facilitate the rapid and accurate identification of eachspecimen.
- Skilled staff who can handle material at cryogenic temperatures quickly, safely and securely. This isprobably the biggest single area of weakness in the performance of any audit.
- Proper maintenance of records, either in paper or electronic form, so that the records do reconcile withthe material actually in the cryobank.

Therefore, in order to establish the true value and importance of cryobank audits, each cryobank must undertake a risk assessment to balance the potential deterioration of cryopreserved material during handling against the likelihood of identifying a discrepancy between their records and a cryotank's actual contents.If it can demonstrate that the cryobank has accurate records, and that its systems and procedures minimizeall opportunities for discrepancies occurring, then the negative risk of potential cryodamage will outweigh any positive "verification" benefit, and the need for an audit should be rejected.

SELECTING THE IDEAL PACKAGING SYSTEM

Packaging Devices for Controlled Rate Freezing

Four main types of packaging container have been used over the years for cryopreserving animal and human spermatozoa and embryos.

Glass ampoules

The most common brand of these traditional packaging devices is the Wheaton Cryule® vials (see www.wheatonsci.com) made of borosilicate glass. After filling using a fine pipette or capillary the glass vialis heat-sealed using a Bunsen burner or spirit lamp. However, use of these vials in clinical laboratories hasbeen strongly discouraged for many years on safety grounds due to their fragility during cryostorage, andthe risk of injury during their opening post-thaw.

Cryotubes / cryovials

Plastic screw-top vials or "cryovials", primarily the NUNC™ CryoTube® range of products (Nunc A/S, Roskilde, Denmark and Nalge Nunc International, Naperville, IL, USA), are made from polypropylene with either polypropylene or polyethylene screw caps. There are considerable questions about their integrity during cryostorage (Byers, 1998; Wood, 1999; Mortimer, 2004a), even for the cryovials with internal threads which, in conjunction with the silicone gasket, provide the best possible seal. It should be noted that in both the Nunc catalogues and the Nunc Cryopreservation Manual (Nalge Nunc International, 1998) it is stated that storage of cryovials immersed in liquid nitrogen is not advised. Moreover, for storage under such "extreme" conditions CryoTube® vials must be correctly sealed in Nunc CryoFlex™ tubing. CryoFlex tubing is, however, rarely used because it hinders the secure attachment of cryovials to canes. It has been reported that, in spite of strict laboratory technique when filling and sealing cryovials, 45% of Nunc cryovials without an O-ring (Nalge Nunc product no.340711) and 85% of Iwaki cryovials with an O-ring (Asahi Techno Glass Corporation Scitech Division, Tokyo, Japan) allowed leakage up to 1 ml of liquid nitrogen (Clarke, 1999).

Mortimer (2004a) summarized the arguments in favour of and against secondary containment (sometimesreferred to as "double bagging") as follows:

PRO	CON
When cryovials are immersed in liquid nitrogen the air inside contracts as it cools, resulting in reduced pressure that will draw liquid nitrogen into the air spaceif there is a faulty seal. Upon thawing this liquid nitrogen rapidly turns into nitrogen gas with a 700-fold volume expansion, which can cause the cryovial to explode.	The extra layer of plastic adversely affects the cooling and warming rates of the specimen, an effect that is exacerbated if air is trapped inside the secondary seal.
	The extra layer of plastic, with or without a layer of trapped air, will create difficulties when seeding.
A non-hermetic seal will constitute a breach of biocontainment between the specimen and the liquidnitrogen inside the cryotank.	A loose-fitting secondary containment sheath makeshandling specimens more cumbersome and also causes difficulties in attaching cryovials to canes.

Classic straws

French straws or "paillettes" (Cassou, 1964) were originally made of polyvinyl chloride (PVC) and commercialized by IMV. However, PVC straws were withdrawn in 1998 because they could not be sterilized by irradiation without damaging them, and all "classic" straws are now made of polyethylene terephthalate glycol (PETG). The small radius of straws allows for excellent correspondence between the desired coolingcurve and the actual thermal profile achieved for the specimens being frozen.

One concern that has been expressed regarding straws is that they are more fragile than cryovials at -196°C, because at cryogenic storage temperatures they become extremely rigid and inflexible (a problem that is worse with the 0.25-ml straws compared to the 0.5-ml straws). However, if an appropriate inventory system is used – one that does not expose straws to any bending stress during handling at cryogenic storage temperatures – straw breakage should be extremely rare.

After filling, PVC or PETG straws are sealed either by:

- Occluding the open end with polyvinyl alcohol (PVA) powder which polymerizes upon contact with moisture. The PVA powder will form a poor seal unless it is fully cured: original IMV documentation indicated that, after tamping in the PVA powder, straws should be immersed in water "to allow theplugs to become firmer".
- Using either solid plastic (nylon) plugs, plastic spheres, or steel balls.
- With haematocrit tube sealant.
- By ultrasonic welding.
- By heating. Crude heat sealing devices such a flame-heated forceps should not be used with classicstraws because flattening of the cylinder creates stress fractures at the corners, making the straws more liable to crack during freezing, leak during storage, and explode upon thawing.

In all cases an air space must be left inside the straw to allow for the expansion of the aqueous material as it cools (since water's volume increases as it freezes: by about 9% at 0°C). Early documentation from IMV stated that "the air bubble or space at the open end of the paillette is essential so that the column of semen in the paillette can extend [expand] during the freezing process". Without the air space the plug can be ejected as the column of specimen expands, but the air space also represents an opportunity for liquid nitrogen to enter the straw if the seal is faulty. Upon warming this liquid nitrogen will undergo a 700-fold expansion in volume as it turns into gas, creating a serious explosion hazard,

High Security Straws

The High Security Straws from Cryo Bio System (commonly referred to as "CBS™ straws") are made from an ionomeric resin which has a number of significant advantages over PETG. These straws are sealed by thermal soldering using a special heat sealing device, the SYMS, from Cryo Bio System (see photo on right). Only using this device (or the automated MAPI or PACE instruments) can they be sealed properly, with a seal guaranteed according to specifications. The internal plug is a composite of powder between two cotton wadding. However, in High Security Straws the PVA powder has been replaced by alginate.

For further information see the section **CBS™ High Security Straws**, below.

Packaging devices for vitrification

As mentioned above, in order to achieve the necessary high cooling rates, specimen packaging for vitrification requires the use of "minimalist" carrier devices, minimizing both the specimen volume, the packaging mass, and maximizing the surface area:volume ratio of the specimen. However, a persistent problem with many of the earlier devices was the direct exposure of the specimen being vitrified to the cryogenic coolant and storage medium (liquid nitrogen).

Capillary-based devices

An early concept for vitrifying embryos was to use straws with very thin walls, in order to decrease the thermal gradient. Vajta et al. (1998a) described the Open Pulled Straw ("OPS") system, and Liebermann et al. (2002) described a method using Flexipet-denuding pipettes with bovine and murine oocytes. A sterile technique for the OPS method has also been described (Vajta et al., 1998b).

Miscellaneous proprietary devices

These vitrification devices allowed direct contact between a small volume of vitrification solution (containing the cells) and liquid nitrogen:

- copper electron microscopy grids (Martino et al., 1996);
- the "Cryoloop" (Lane et al., 1999), the "CryoLeaf" and the "CryoTop" (Kuwayama et al., 2005) devices; and
- the "Hemi-Straw" system (Vanderzwalmen et al., 2000).

There is also a more recent "closed" version of the "CryoTop" device, called the "CryoTip" (Kuwayama et al., 2005).

CBS™ HSV Straw

The **H**igh **S**ecurity **V**itrification Straw is an adaptation of Cryo Bio System's high security straw concept to allow for absolute biocontainment of vitrified specimens. For further information on these modern packaging devices, see the section **CBS™ High Security Vitrification Straw**, below.

Sanitization of packaging devices

During filling of cryovials or CBS™ straws there should be no contamination of their outsides with the biological material, but when filling the "classic" straws the open end of the straw is immersed in the semen+CPM mixture or embryo culture medium as it is be aspirated into the straw. After sealing it is essential that residual material on the outside of these straws be removed, usually by wiping with a paper tissue. This removes liquid that would "cement" adjacent straws together at cryogenic storage temperatures, under which conditions attempts to separate the straws carry grave risk of breakage due to their fragility at such temperatures. Although many laboratories also disinfect the outside of the straws with an alcohol wipe before cooling them, IMV and CBS™ recommend that straws be disinfected using a less volatile disinfecting agent such as hypochlorite, followed by rinsing with sterile water.

Upon thawing, the outside of all containers will be contaminated with whatever organisms were present inthe liquid nitrogen, even if vapour storage was used (Fountain et al., 1997). Disinfecting the outside of specimens after thawing should be common to all cryobank standard operating procedures ("SOPs"). Safelaboratory practice and risk management requires avoiding all risks of "finger stick" or similar injuries, andhence sterile disposable suture scissors rather than sterile scalpel blades should be used (Clarke, 1999). CBS™ have a custom-designed, easily sterilized device, the"StrawCut", which operates on the hot-wire principle, for opening High Security straws. Because they cannot be disinfected without damaging the contained specimen, any straws that are broken, obviously cracked, or have lost their seal at either end, should be discarded (although for "precious" material, additional patient information and consenting could permit their clinical use).

Obviously unsealed packaging devices can neither be sanitized before freezing/vitrification or after removalfrom cryostorage. This is a very serious concern that can, in many situations where there is concern regarding proper biocontainment during cryobanking, preclude the use of such packaging devices. In particular, until the arrival of sealed vitrification systems, notably the **CBS™ HSV Straw**, many IVF laboratories or donor cryobanks have been unable to consider vitrification as a viable, safe option.

Sanitizing the controlled rate freezer

If there is any risk of contaminating the inside of the controlled rate freezing machine with biological material that might contain pathogens, then a cryobank must have a protocol in place to sterilize the contaminatedsurfaces; not only the freezer, but of all units that are frozen using it. As an extra level of safety, when processing samples from pathogen-infected individuals (even using effective biocontainment packaging) it would be good practice to sterilize the cooling chamber – or at least the contact surfaces – afterwards.

Contamination of liquid nitrogen and cross-contamination

The report of a cluster of six cases of acute Hepatitis B virus (HBV) infection occurred among patients undergoing cytotoxic treatment (Tedder et al., 1995) brought to the fore the issue of contamination of liquid nitrogen and the possible problem of cross-contamination of specimens during storage immersed in liquid nitrogen. Although this event remains the only reported case of cross-infection during cryogenic storage, the concern it has created among cryobank operators has led to enormous effort being expended on risk management. Even though the risk of cross-contamination is certainly unquantifiable, it cannot be ignored: the risk cannot be presumed to be theoretical, and everyone involved in gamete and embryo cryobanking must take all available steps to minimize the risk in their banks.

In order for cross-contamination to occur, the infectious organisms must not only be released into the liquidnitrogen but also enter a "clean" unit and contaminate the material within. Fortunately, the problem of such "cross-contamination" has not proven to be a major issue, with there being no direct evidence of any cross-contamination in a cryobank within a fertility clinic or sperm bank setting (Bielanski et al., 2000, 2003; Kuleshova & Shaw, 2000; Tomlinson & Sakkas, 2000; Centola, 2002; Mortimer, 2004a).

After the "blood bag incident" (Tedder et al., 1995) there were calls for storage in liquid nitrogen to be abandoned in favour of storage in the vapour or gaseous phase either above a layer of liquid nitrogen

("vapour storage") or in newer design freezers that enclosed the cryogenic liquid nitrogen inside an outervessel so that cold was transferred either through the walls of the secondary vessel or via of heat shunt devices ("cold fingers") to minimize temperature gradients. In these latter systems, specimens are actuallystored in super-cold air. However, the very low thermal capacity and poor heat transfer rates of both liquidnitrogen vapour and super-cold air are major concerns because such storage environments heat up rapidlyin the presence of a "warm" object, even ambient temperature air.

While vapour storage does go a long way to reducing the risks of cross-contamination via liquid nitrogen, especially with leaky specimen packaging, it is not an absolute solution because pathogens have been isolated from liquid nitrogen vapour (Fountain et al., 1997) and it should be expected that the same will be true ofthe super-cold air systems where organisms will be frozen out of the air that enters the storage chamber every time the lid is opened. Environmental organisms and skin commensals are common, low level, contaminants of liquid nitrogen and liquid nitrogen vapour, along with occasional enteric contaminants. However, high levels of a potential pathogen (Aspergillus spp) have been reported in liquid nitrogen vapour (Fountain et al., 1997).

These concerns only amplify the value of the CBS™ High Security range of packaging devices for secure cryostorage since they not only provide total biocontainment for the specimens, they also permit externalsanitization of units at thawing.

Risk analyses for packaging devices

The Tables on the following pages employ the "Failure Modes and Effects Analysis" (FMEA) approach to evaluate what is the safest form of packaging device for gametes and embryos that are being cryobanked,either by controlled rate freezing or vitrification.

For further explanation on the FMEA technique, see Mortimer & Mortimer (2005).

For further details on the CBS™ High Security Straws and High Security Vitrification Straw, see the respectivesections that follow the Tables.

Risk analysis table for controlled rate freezing packaging devices

Table adapted from Mortimer (2004b).

R = risk rating or consequence of the risk happening, **L** = likelihood and **C** = "Criticality" (= R×L). Superscript symbolsdenote conditions assuming a device is used correctly according to the manufacturer's instructions* and/or best practicelaboratory procedure has been followed.† For numeric superscripts see Notes, below.

Risk	Consequence		Packaging types							
			Cryovial		IMV 0.25-ml straw		IMV 0.50-ml straw		CBS™ HS Straw	
	Description of failure	R	L	C	L	C	L	C	L	C
Contamination of the outside of the deviceduring filling	Will carry contamination into thecryogenic storage vessel	4	7	**28**	8†	**32**	8†	**32**	0*	**0**
Microbial transmission through the device wall	Outward contamination of cryo-genic storage vessel or inward contamination of specimen	4	1	**4**	1	**4**	1	**4**	0*	**0**
Fragility of the device at -196°C	Risk of breakage during handlingwhile in storage (e.g. audits)	7	0	**0**	3†1	**21**	2†1	**14**	0*	**0**
2° containment needed for safe use of device under "extreme" storage conditions (i.e. -196°C)	Ability to provide reasonable expectation of hermetic integrityof the specimen	6	9*	**54**	4†	**24**	4 †	**24**	0*	**0**
Adverse practical sequelae of the 2° containment system	Handling difficulties in attachingdevices to canes	5	5*	**25**	0	**0**	0	**0**	0*	**0**
Cooling curve of the specimen does not follow the programmedrate closely	Proper cooling rate is not experienced by the specimen, orrate is variable throughout the specimen	7	8	**56**	1	**7**	2	**14**	2	**14**
Warming rate of specimen does not follow ambient temperature closelyduring thawing	Proper (rapid) warming ratecannot be achieved during thawing	6	6†	**36**	1†	**6**	2†	**12**	2†	**12**
Risk of inadvertent warming during handlingof cryobanked device	Risk of ice recrystallization due tospecimen warming above the glass transition temperature of water (ca. -132°C)	7	2	**14**	6†2	**42**	4†2	**28**	4†2	**28**
Explosion hazard whenthawing specimen	Explosive over-pressure due toevaporation of liquid nitrogen trapped inside the device	5	4†	**20**	2†	**10**	2†	**10**	0*	**0**
ID information can be lost or smudged duringcryostorage	Integrity of identifying informationof each unit stored	8	1	**8**	2†	**16**	1†	**8**	0*†	**0**
Total Criticality Scores				**245**		**162[3]**		**146[3]**		**54[3]**

Notes: 1. These risk likelihood ratings reflect the typical practice in many human IVF cryobanks of storing straws in narrow visotubes attached to canes, rather than following the manufacturer's instructions to use visotubes inside goblets (which would merit reducing these ratings by 1 rank). When attached to canes there is a risk of (inadvertent) bending of straws during their removal from the visotubes.

2. These risk likelihood ratings reflect the typical practice in many human IVF cryobanks of handling straws in isolation, rather than inside visotubes where the surrounding LN2 would guarantee their remaining at -196EC. If straws were only handled in visotubes, as per the manufacturer's recommendations, (except when removing for thawing) these risk likelihood ratings could each be reduced to 1

3. If the correct practices described in Notes 1 and 2 were followed, these Total Criticality Scores would be reduced to 113, 118and 33 respectively.

Risk analysis table for vitrification packaging devices

This Table does not include all vitrification packaging devices, some proprietary devices will need to be evaluated by consideration against the closest comparable device (e.g. the CryoTip with OPS).

R = risk rating or consequence of the risk happening, **L** = likelihood and **C** = "Criticality" (= R×L). Superscript symbolsdenote conditions assuming a device is used correctly according to the manufacturer's instructions* and/or best practice laboratory procedure has been followed.† Table based on Mortimer (2004b), for numeric superscripts see Notes, below.

Risk	Consequence		Packaging types							
			OPS		CryoLoop		0.25-ml PETG straw		CBS™ HSV	
	Description of failure	R	L	C	L	C	L	C	L	C
Contamination of the packaging device outersurface during loading	Will carry contamination into thecryogenic storage vessel	4	10	**40**	10	**40**	8†	**32**	0*	**0**
Microbial transmission through the device wall(no wall = L of 10)	Outward contamination of cryo-genic storage vessel or inward contamination of specimen	4	5	**20**	10	**40**	1	**4**	0*	**0**
Fragility of the device at -196°C	Risk of breakage during handlingwhile in storage (e.g. audits)	7	3	**21**	3	**21**	2†1	**14**	0*	**0**
2° containment needed for safe use of device under "extreme" storage conditions (i.e. -196°C)	Ability to provide reasonable expectation of hermetic integrityof the specimen	6	10	**60**	10	**60**	4†	**24**	0*	**0**
Adverse practical sequelae of the 2° containment system	Handling difficulties, e.g. in attaching devices to canes orretrieving specimens	5	5	**25**	5	**25**	0	**0**	0*	**0**
Cooling curve of the specimen does not follow the intended rateclosely	Proper cooling rate is not experienced by the specimen, orrate is variable throughout the specimen	8	0	**0**	0	**0**	4‡	**32**	1	**8**
Warming rate of specimen does not follow ambient temperature closelyduring thawing	Proper (rapid) warming ratecannot be achieved during thawing	6	0	**0**	0	**0**	1	**6**	0	**0**
Risk of inadvertent warming during handlingof cryobanked device	Risk of ice recrystallization due tospecimen warming above the glass transition temperature of water (ca. -132°C)	7	8	**56**	10	**70**	4†2	**28**	4†2	**28**
Explosion hazard whenthawing specimen	Explosive over-pressure due toevaporation of liquid nitrogen trapped inside the device	5	0	**0**	0	**0**	2†	**10**	0*	**0**
ID information can be lost or smudged duringcryostorage	Integrity of identifying informationof each unit stored	8	1	**8**	2	**16**	1†	**8**	0*†	**0**
Total Criticality Scores				**230**		**272**		**1583**		**363**

Notes: 1. These risk likelihood ratings reflect the typical practice in many human IVF cryobanks of storing straws in narrow visotubes attached to canes, rather than following the manufacturer's instructions to use visotubes inside goblets (which would merit reducing these ratings by 1 rank). When attached to canes there is a risk of (inadvertent) bending of straws during their removal from the visotubes.

2. These risk likelihood ratings reflect the typical practice in many human IVF cryobanks of handling straws in isolation, rather than inside visotubes where the surrounding LN2 would guarantee their remaining at -196EC. If straws were only handled in visotubes, as per the manufacturer's recommendations, (except when removing for thawing) these risk likelihood ratings could each be reduced to 1.

3. If the correct practices described in Notes 1 and 2 were followed, these Total Criticality Scores would be reduced from 158 and36 to 123 and 12 respectively.

CBS™ HIGH SECURITY STRAWS

The High Security Straws from Cryo Bio System (commonly referred to as "CBS™ straws") are made from an ionomeric resin which has a number of significant advantages over PETG. They are sealed by thermal soldering using a special heat sealing device, the SYMS, from Cryo Bio System. Only using this device (or the automated MAPI or PACE instruments) can they be sealed properly, with a seal guaranteed according to published specifications.

CBS™ High Security Straws have the following features:

- Heat sealable using a special thermal welding device (the SYMS sealer). Properly-sealed CBS™ straws are guaranteed to be absolutely leakproof at pressures of up to 150 kg/cm^{-2}.
- Mechanically resistant, shatterproof even at -196°C, and can be subjected to substantial bending even while frozen.
- Bacteria and virus proof (see below).
- A special filling nozzle so that none of the material being loaded into the straw ever comes into contact with the outside of the straw (see Russell et al., 1997).
- Every batch undergoes extensive biocompatibility testing to verify that they are non-toxic (low endotoxin) and free of bacterial and viral contamination. CBS™ High Security Straws are guaranteed to cause no specific damage to human or bovine spermatozoa or mammalian embryos (MEA test).
- Sterile, by irradiation at 25 kGy in accordance with European Pharmacopoeia standards.
- Two-compartment straws allow identifying information (identification tubes or weighted rods) to be sealed inside the straw itself, making identification tamper-proof.
- Secure external identification labelling option using identification jackets ("sleeves") that cannot slide off the straw once sealed (due to the flat "tabs" formed at each end when the straw is welded shut).
- Manufactured according to GMP standards in facilities certified to EN 46002, ISO 13485 and ISO 9002.
- Approved for human applications by the US Food and Drugs Administration with a 510(k) pre-market notification clearance K002595.
- CE-marked as a medical device according to EU Directive 93/42/CE.

Since their original introduction into non-assisted reproduction markets in 1990, CBS™ High Security Straws have been used in a wide variety of applications, including (but not limited to):

- Preservation of cell lines and microbiological material at the Pasteur Institute (Paris, France).
- Epidemiological research projects (in areas including cancer, nutrition, diabetes, AIDS) in numerous countries, including France, Italy, Spain, Greece, Norway, Netherlands, Sweden, UK, Germany, USA, China and Korea (e.g. Bingham & Riboli, 2004).
- The French National Blood Transfusion Agency uses them to store blood components of each blood donation for medico-legal purposes.
- The Mérieux Foundation (France) uses them to store pathogen containment level 4 ("P4") organisms such as Ebola and Marburg viruses.

Following their launch into the assisted reproduction market in 1998, CBS™ High Security Straws were adopted by the French national CECOS network (Centres d'Étude et de Conservation des Oeufs et du Sperme Humain) in 2000, by the Danish donor sperm bank CRYOS which has frozen more than 400,000 units, and the Wildlife Breeding Resource Centre (Pretoria, South Africa) which uses them in its biodiversity and genome resource banking activities to preserve gametes and embryos of endangered species throughout South Africa and neighbouring countries.

Finally, Thammavongs et al. (2004) reported improved cryosurvival of some bacterial and fungal species using CBS™ High Security Straws.

Product Family Overview

CBS™ High Security Straws family of products includes 0.3 ml straws for embryos and sperm and 0.3 & 0.5 ml straws for sperm and blood fractions. Sterile filling nozzles are sold separately (box of 100 individually packaged units, cat. no. 024856). The following Table summarizes the most commonly-available formats:

Volume (ml)	Compartments and plug type	Dimensions (mm)			Purpose	Package	Cat. No.
		L	OD	ID			
0.3	2 – cotton/aliginate powder plug	133	3.15	2.55	Embryos & sperm	20 × 1	025292
0.3	2 – cotton/aliginate powder plug				sperm	20 [4×5]	010287
						100 [5×20]	010288
0.5	1 – cotton/alginate powder plug				sperm	20 [4×5]	014651
						100 [5×20]	014650*
					blood fractions	300 (non-sterile)	019052

*Also available in packs of 100 with the external part of the cotton plug coloured: yellow 016611; green 016612; blue 016613;orange 016614; grey 016615; and red 016584.

Note : For tissue (and large volumes), CBS™ High Security Tubes, also fully secured for storage in liquid nitrogen, are now available under Cat. No. 022252 (bag of 20 sterile blisters by 1 unit each) and Cat. No. 022251 (non sterile by 100 for non IVF biobanking purpose).

Validation Studies

Effective cryopreservation

CBS™ High Security Straws have been widely used for cryopreserving gametes and embryos of various mammalian species since their introduction to the assisted reproduction market in the late 1990s. No studies reporting reduced cryosurvival compared to classic straws have been published.

Biocontainment / sanitary safety

Studies on the sanitary safety of CBS™ High Security Straws were carried out by Dr Bernard Guérin of the Laboratoire pour le Contrôle des Reproducteurs of the Ministère de l'Agriculture (Maisons-Alfort, France) in1998. Straws were either loaded with infectious organisms and placed in sterile medium, or vice versa, and then the assemblies were frozen in liquid nitrogen. After thawing in a 37°C water bath the straws that hadbeen immersed in contaminated medium were decontaminated before opening using sterile scissors and recovery of the contents using sterile Pasteur pipettes; straws that contained contaminated medium wereopened using sterile scissors and the contents recovered using sterile Pasteur pipettes.

The bacterial contamination study was conducted on six bacterial strains: Campylobacter fetus venerealis (CFV), Actinomyces pyogenes bovis (APB), Pseudomonas aeruginosa (PA), Escherichia coli (EC), Staphylococcus aureus (SA) and Haemophilus somnus (HS) with testing performed after 2, 5, 6, 9, 15 and 23 days of storage, as well as after 9 months (5 straws per bacterium per time point per configuration).

The viral studies employed bovine hepatitis virus type 1 (BHV-1) at titres of 10^3 and 10^7 $TCID_{50}$/ml either inside the straws or in the outside medium, and using bovine viral diarrhea virus (BVDV) in a separate series in side the straws. All testing was performed after 18 hours of storage (10 straws per test).

In all cases the original infected medium (inside or outside the straws) retained its infectious status. Results of the bacterial studies are summarized in the following Tables: 0 = test strain culture negative; c = contamination by one or several bacterial strains different from the experimental ones.

STERILE STRAWS IN CONTAMINATED MEDIUM							
Bacterium	**2 days**	**5 days**	**6 days**	**9 days**	**15 days**	**23 days**	**9 months**
CFV	0	0	0	0	0	0	0
HS	0	0	0	0	0	0	0
APB	0	0	0	0	0	0	0
SA	0	0	0	0	0	0	0
EC	0	0	0	0	0	0	0
PA	0	0	0	0	0	0	0

CONTAMINATED STRAW CONTENTS IN STERILE MEDIUM							
Bacterium	**2 days**	**5 days**	**6 days**	**9 days**	**15 days**	**23 days**	**9 months**
CFV	0	0	0	0	0	0	0
HS	0 c	0	0	0	0	0	0
APB	0 c	0	0 c	0	0	0 c	0 c
SA	0	0	0	0	0	0	0
EC	0	0	0	0	0	0	0
PA	0	0	0	0	0	0	0

In all the viral studies the original infected medium (inside or outside the straws) retained its infectious status.Results of these studies are summarized in the following Table.

VIRUS	**INSIDE-TOWARD-OUTSIDE STUDY**		**OUTSIDE-TOWARD-INSIDE STUDY**	
	Titre in straws	**Testing of outside medium**	**Titre in outside medium**	**Testing in the straws**
BHV-1	10^3 $TCID_{50}$/ml	Virus absent	10^3 $TCID_{50}$/ml	Virus absent
	10^7 $TCID_{50}$/ml	Virus absent	10^7 $TCID_{50}$/ml	Virus absent
BVDV	10^3 $TCID_{50}$/ml	Virus absent	not investigated	
	10^7 $TCID_{50}$/ml	Virus absent		

The conclusions drawn from these studies were that:

- There was no release of contaminant bacterial or viral agents from within the CBS™ High Security Straws.
- There was no contamination of the straws' contents even when exposed to high or very highcontaminated environmental conditions.

Put simply, CBS™ High Security Straws provided total biocontainment both in terms of protecting their contentsfrom external contamination, and for cryostoring infectious agents.

Step-by-step Protocols for Using HS Straws

Instructional videos/animations are available on the Cryo Bio System website (www.cryobiosystem.com).

Semen

1. Register the semen specimen in the Laboratory Specimen Register and assign a Laboratory Reference Number (LRN). Place the specimen jar on the orbital mixer platform inside the Andrology incubatorat 37°C to liquefy. Initiate a Sperm Freeze Report laboratory form.
2. Make sure that sufficient cryoprotectant medium ("CPM") has been placed in the incubator to warmup. The volume required is equal to the ejaculate volume less the aliquot required for semen analysis(typically 0.2 ml).
3. a) Prepare the controlled rate freezer system and select the correct program.
   b) Switch on the SYMS heat sealer unit.
4. As soon as the semen is liquefied perform a semen analysis (see appropriate SOP).
5. Label the appropriate number of ID rods for CBS™ semen High Security Straws with the man's ***NAME***,the ***LRN*** and the ***DATE***. The number of ID rods required is the same as the number of straws that will be used, which is estimated using the formula: [(semen volume in ml – 0.2) × 3.3]

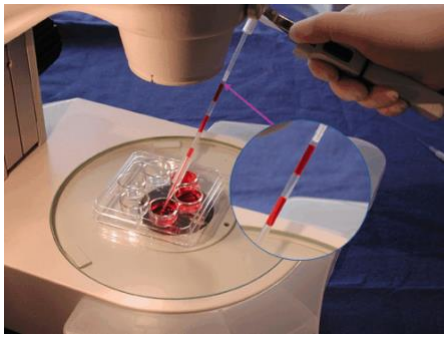
   Any fraction of a straw is considered as another ID rod to be labelled.
6. Draw up the required volume of CPM in a sterile serological pipette. The volume is calculated as thesemen volume less 0.2 ml that will be used for the semen analysis.
7. Add the CPM drop-wise over 10 minutes with constant swirling to ensure thorough mixing. The first drops must be added singly, the next few in twos, then in threes; beyond this the volume per additioncan increase but must not exceed 1/20th of the semen+CPM mixture thus far.
8. a) Attach a sterile filling nozzle to the lower end of the first straw (the open end of the larger compartment) and load the semen+CPM mixture into the straw through the loading nozzle by aspiration, this will ensure that the outside of the straw is not contaminated and that a 15 mm airgap is left at the lower end of the straw.
   b) Remove the filling nozzle and seal the lower end of the straw using the SYMS heat sealer.
   c) Insert the ID rod into the upper compartment of the straw and seal using the SYMS heat sealer.
   d) Repeat steps 8a–8c (using the same filling nozzle) until all the semen+CPM mixture has been loaded into straws.
   e) The last straw, usually only partly filled, will be the Test-Thaw. Using a cryopen, identify this straw with a ring around the top end of the straw.
9. Disinfect the outside of each straw.
10. Load all the straws into the controlled rate freezer and begin the freezing run.
11. When the freezing is complete, remove the straws from the controlled rate freezer's chamber and transfer them, into a dewar or styrofoam box containing liquid nitrogen. This transfer of straws through the air must be extremely quick. If straws are allowed to warm up above -132°C (the glass transition temperature of water) ice recrystallization can occur within the frozen material and causedamage to the spermatozoa.
12. Keep the Test-Thaw straw aside and transfer the remaining straws into the cryobank. See the appropriate SOP describing how the cryobank is organized.
13. Update the cryobank inventory records as per the appropriate SOP.
14. Later: Perform the Test-Thaw as per the appropriate SOP.

Note: Upon thawing, always sanitize the outside of the straw before cutting it open.

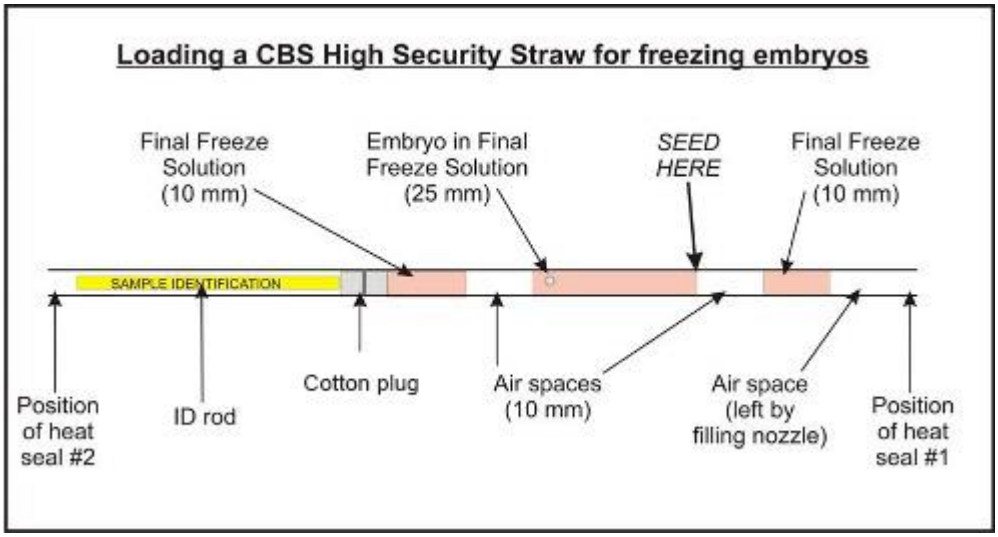
Embryos

1. a) Prepare the controlled rate freezer system and select the correct program (see Note #2).
   b) Switch on the SYMS heat sealer unit.
2. Prepare a series of culture dishes with the sequence of freezing solutions. The first dish is placed in a 37°C incubator to equilibrate while the remainder are left at ambient temperature (ca. 20°C).
3. Check the correct Patient Data Sheet for the number of embryos to be frozen.
4. Label one ID rod for each embryo being frozen with the following identifying information:
   - Identity of the embryo = the Chart Number, female partner's name and the freeze number;
   - Date of the freeze;
   - The embryo's number (i.e. 1, 2, 3, etc).
5. Process the embryos through the series of freezing solutions as per the manufacturer's instructions and/or the appropriate SOP. Always observe the embryos under the microscope during this step.
6. Immediately begin loading the embryo(s), individually, into CBS™ straws. To do this open the individually-packed straw and verify that the filling nozzle is firmly attached (but do not remove thenozzle while doing this). Then attach the aspiration device tubing to the upper end of the straw and aspirate, in the following sequence (see photo below right, and Figure, below):

   a) 10 mm of the final freeze solution followed by 10 mm air space then <10 mm of the final freeze solution + EMBRYO
   + more of the final freeze solution to a total of ca. 25 mm, followed by a 10 mm air space and finally a further 10 mmof the final freeze solution.

   b) Remove the filling nozzle and seal the open end of the straw using the SYMS heat sealer.
   c) Insert an ID rod into the upper compartment of the strawand seal that end using the SYMS heat sealer.

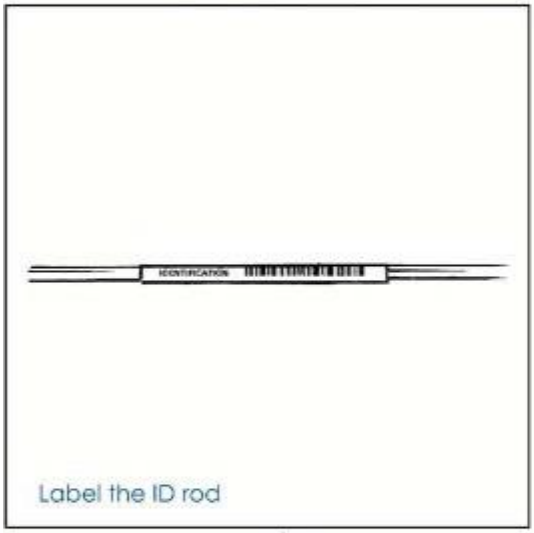


   d) Place the straw on a holding rack (not on the bench surface as that could cause temperature shiftsin the straw)
   e) Repeat steps (a) through (d) until all the embryos have been loaded into straws and sealed.
7. Disinfect the outside of each straw.

8. Load the straws into the chamber of the controlled rate freezer and begin the freezing run.
9. At the appropriate point in the freezing program seeding must be performed:
   a) Cool the tips of a pair of seeding forceps in liquid nitrogen.
   b) Withdraw a straw from the freezing chamber just until the upper meniscus of the top air bubble inthe straw can be reached by the seeding forceps.
   c) Touch the tips of the cooled seeding forceps to the upper meniscus of the top bubble. When icebegins to form in the straw release the seeding forceps.
      Note: If no ice forms, replace the straw in the chamber, re-cool the seeding forceps and try again.
   d) Replace the straw back in the chamber.
   e) Repeat steps (a) through (d) for each straw.
   f) As soon as all the straws have been seeded, close the freezing chamber and allow the program tocontinue.
10. When the freezing is complete, remove the straws from the controlled rate freezer's chamber and transfer them, into a dewar or styrofoam box containing liquid nitrogen; ensure that the straws arecompletely immersed. This transfer of straws through the air must be extremely quick. If straws are allowed to warm up above -132°C (the glass transition temperature of water) ice recrystallization canoccur within the frozen material and cause damage to the embryo.
11. Transfer the straws into the cryobank storage tank. See the appropriate SOP describing how the cryobank is organized.
12. Update the cryobank inventory records as per the appropriate SOP.

Note: Upon thawing, always sanitize the outside of the straw before cutting it open.

Simplified Procedure Chart for Using HS Straws for Sperm



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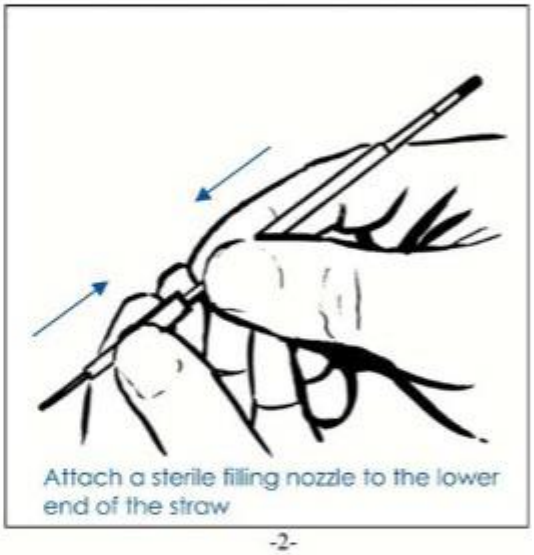

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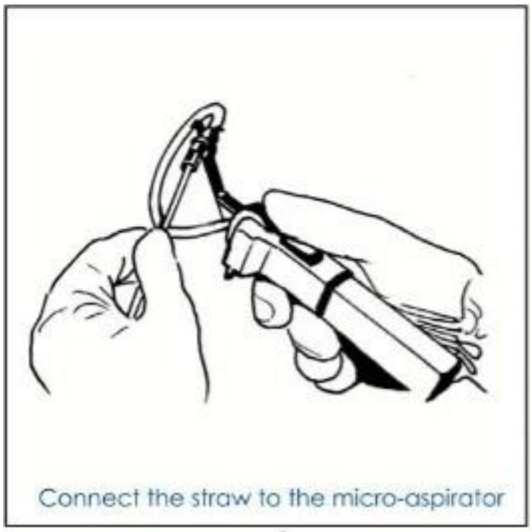

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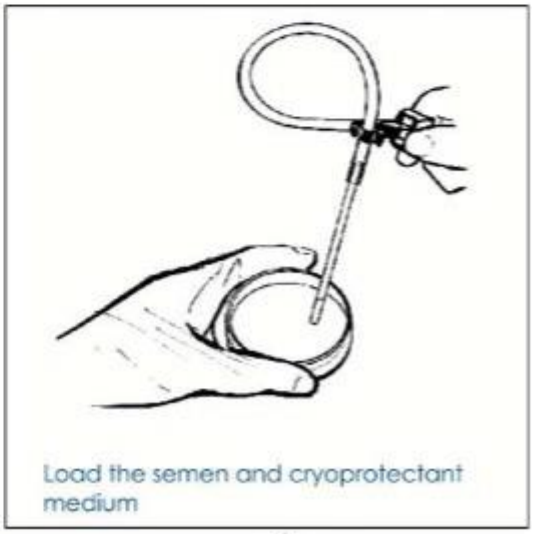

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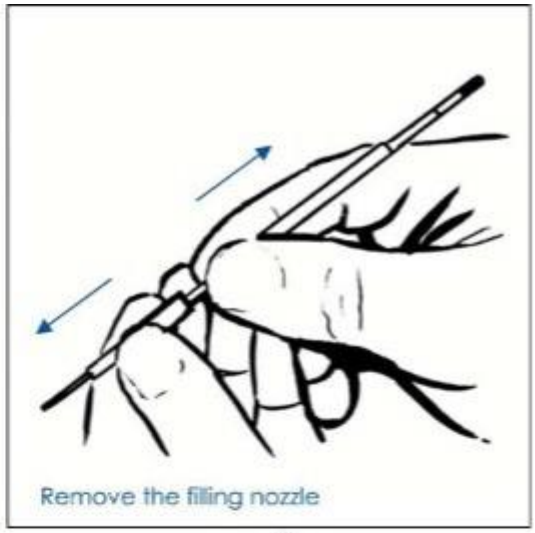
Sealing

Cryo Bio System



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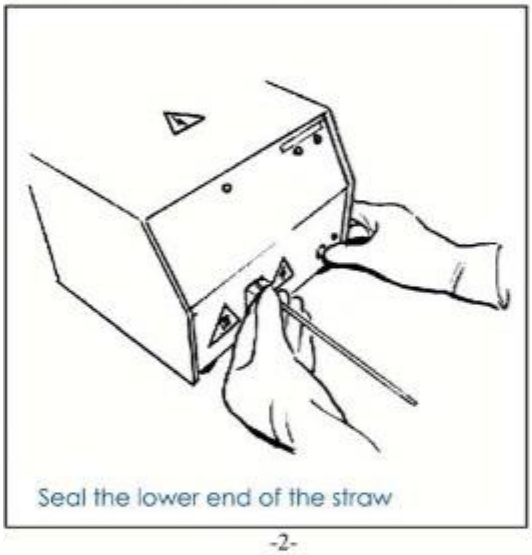

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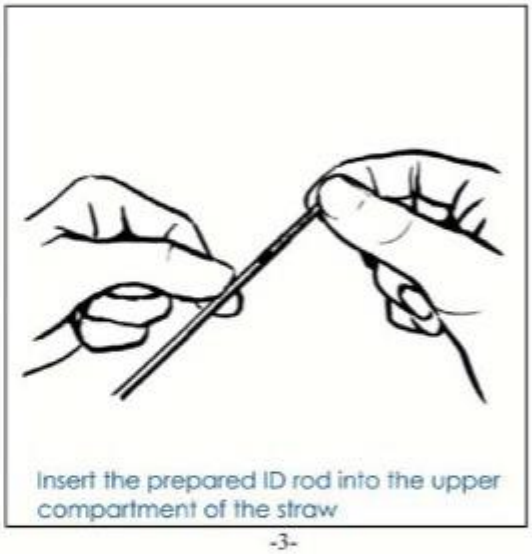

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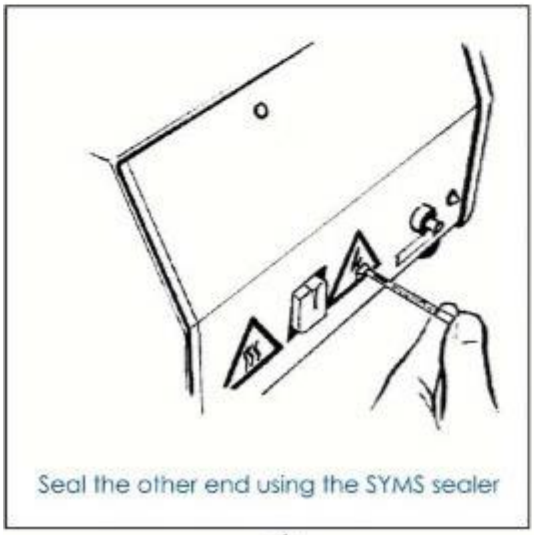

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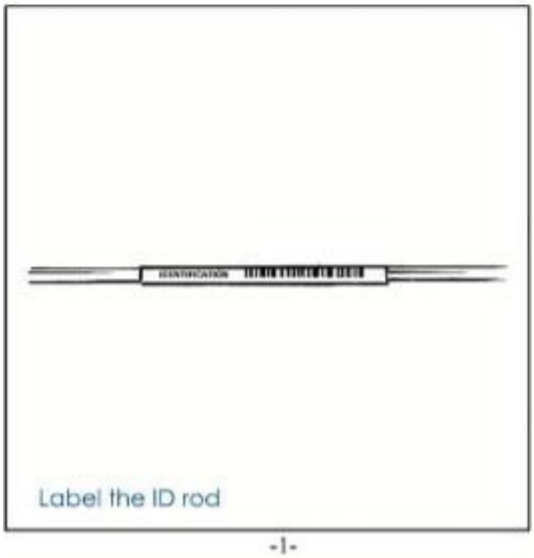
Cryo Bio System

www.cryobiosystem.com

Simplified Procedure Chart for Using HS Straws for Embryos



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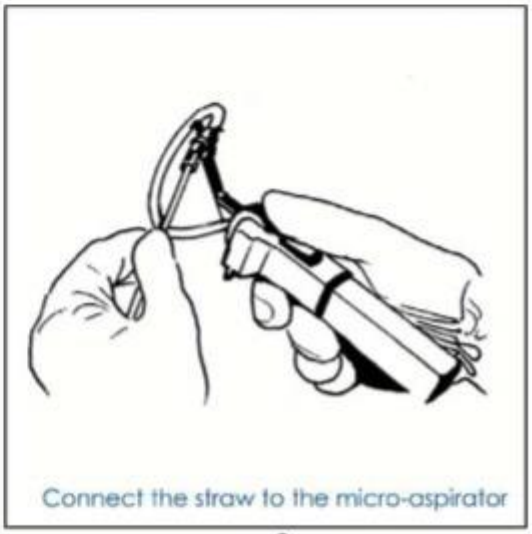


-2-

-3-

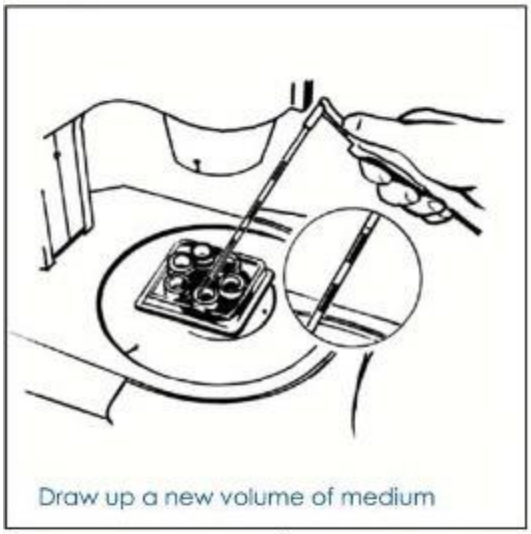
-4-


Load the embryo

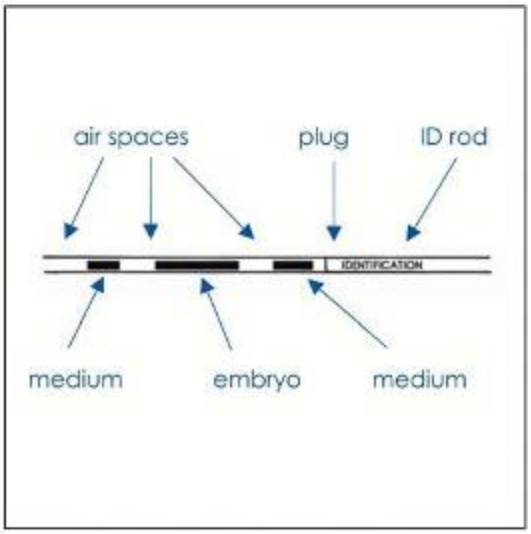
-5-


Create another air bubble

-6-


Draw up a new volume of medium

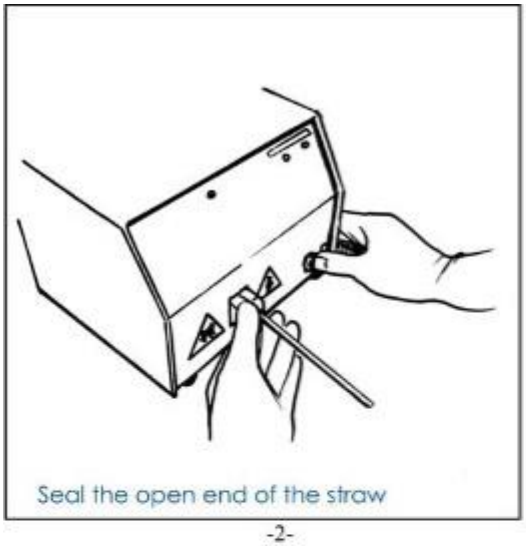
-7-


air spaces
plug
ID rod
IDENTIFICATION
medium
embryo
medium


Sealing
Cryo Bio System


Remove the filling nozzle

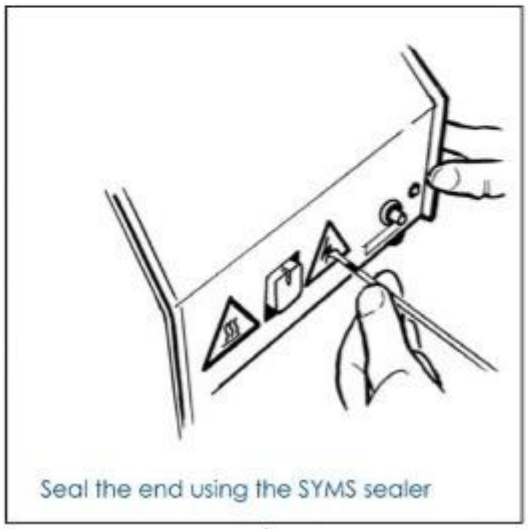
-1-


Seal the open end of the straw

-2-


Insert the prepared ID rod into the upper compartment of the straw

-3-


Seal the end using the SYMS sealer

-4-


Cryo Bio System
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CBS™ HIGH SECURITY VITRIFICATION STRAW

Product Overview

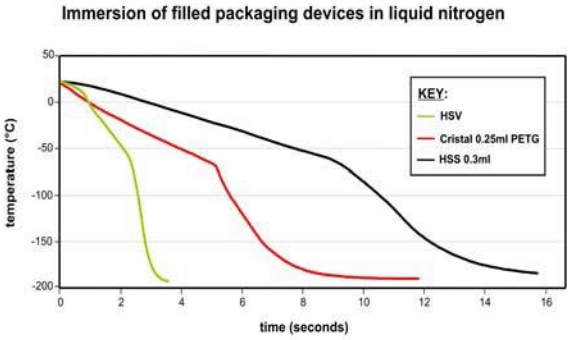
The **H**igh **S**ecurity **V**itrification Straw is an adaptation of Cryo Bio System's high security straw concept to allowfor absolute biocontainment of vitrified specimens. The system comes as a 3-part straw, comprising:

- A capillary tube specimen holder with a pre-formed gutter at one end where the specimen (<1 µl) islocated. The other end serves as a handling rod, and is coloured (white, blue, red, green, yellow orpurple) for easy specimen identification.
- A plastic insertion and removing device. The long end of the device inserts the capillary tube specimen holder into the HSV straw (see below) at the optimal depth, about 3 mm from the clear resin plugthat isolates the weighted rod.
- An HSV straw which is open and flared at one end, allowing for the safe introduction of the capillary tube specimen holder. The other end of the HSV straw is factory sealed and weighted with a medical grade stainless steel rod, to prevent buoyancy when immersed in liquid nitrogen; the metalrod is isolated by a clear resin plug. After loading, the HSV straw is thermally sealed using the SYMS Heat Sealer.

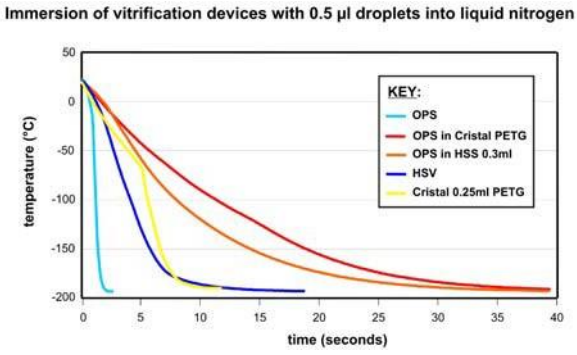
Validation Studies

Cooling Rate Study

In one experiment, the cooling rates inside filled HSV straw ("HSV"), 0.25-ml PETG "Cristal" straw and 0.3-ml High Security Straw ("HSS") devices were measured using a thermocouple after plunging into liquid nitrogen (see Figure, right). In each case the device was held using forceps and held vertically in the liquid nitrogen until the inside temperature reached -196°C. The steps in the cooling curves observed forthe straws were due to the insulating effect of nitrogen vapour bubbles forming around the straws, due to their greater thermal mass.



In a second experiment, the cooling curve achieved for a filled HSV device was compared with those obtained for Open-Pulled Straws ("OPS") both naked and enclosed within either a 0.25-ml PETG "Cristal" straw or a 0.3-ml High Security Straw ("HSS") as secondary containment; filled Cristal and HS straws were also runas controls (see Figure, right). While the HSV device did not cool as quickly as the naked OPS, its cooling curvewas much faster than for an OPS enclosed in either type of straw. The slower cooling curve for an enclosed OPS than a normally-filled PETG straw was due to theinsulating effect of the air around the OPS.



The conclusion from this study is that the HSV device provides a cooling rate only slightly slower than thatobtained with an OPS, and is far preferable to an OPS enclosed inside another straw in an attempt to achieve biocontainment. The problem caused by the insulating layer of air could probably be extrapolated to other vitrification devices when enclosed inside secondary packaging devices.

Embryo Survival

A study comparing the survival of human embryos following vitrification using the "Hemi-Straw" (Vanderzwalmen et al., 2003) and a "mini-Hemi-Straw" devices was carried out using both Day 3 embryos (derived from zygotes with 1 or 3 pronuclei) and "spare" Day 5 blastocysts that were ineligible for transfer or cryopreservation (P. Vanderzwalmen et al., unpublished data). Vitrification solutions and procedures were as described for the "Hemi-Straw" system (Vanderzwalmen et al., 2003), which was used as the control, with vitrification being induced by direct plunging of the naked "Hemi-Straw" into liquid nitrogen. Aseptic vitrification was achieved by either inserting the "Hemi-Straw" into a 0.5-ml High Security Straw, or by using a mini-Hemi-Straw inside a special 0.25-ml High Security Straw (developed for the HSV Straw) before immersing in liquid nitrogen. A further evaluation considered the HSV Straw itself, but only using Day 5 blastocysts. Survival was defined as cleavage to the morula stage for thawed Day3 embryos, and the re-expansion of thawed blastocysts. The results of this study are summarized in the first Table, below.

Vitrification device	Day 3 embryos	Day 5 blastocysts
Naked Hemi-Straw (aseptic)	82% (23/28)	73% (58/80)
Hemi-Straw in 0.5-ml High Security Straw	73% (11/15)	62% (33/53)
Mini-Hemi-Straw in 0.25-ml High Security Straw	79% (15/19)	79% (26/33)
HSV device	not determined	70% (26/35)

A secondary analysis of the results obtained with blastocysts revealed that expanded blastocysts might be more sensitive to the reduced cooling rate than early blastocysts (and perhaps also Day 3 embryos) whenenclosed in the larger volume straw (see Table, below).

Vitrification device	Early blastocysts	Expanded blastocysts
Naked Hemi-Straw	81% (22/27)	74% (14/19)
Hemi-Straw in 0.5-ml High Security Straw	83% (15/18)	27% (3/11)
Mini-Hemi-Straw in 0.25-ml High Security Straw	75% (12/15)	60% (6/10)

The results of this study showed that post-thaw survival rates of Day 3 and day 5 embryos vitrified at a lower cooling rate inside a hermetically sealed straw are comparable to the control non-aseptic "Hemi-Straw" technique, although expanded blastocysts are more sensitive to packaging in a device with a larger insulating air space that will reduce the effective cooling rate.

These investigators concluded that the HSV Straw was simple to handle, and allowed complete isolation of theembryos from the liquid nitrogen.

Step-by-step Protocol for the Using HSV Straw

1. Switch on the SYMS heat sealer unit.

2. Prepare culture dishes with the appropriate equilibration and vitrification solutions for the method being employed. Equilibration is usually performed at ambient temperature (ca. 20°C) for cleavage stage and early or artificially-shrunken blastocysts, but at 37°C for expanded blastocysts.

3. a) Check the correct Patient Data Sheet for the number of embryos to be vitrified.
   b) Collapse the blastocoels of expanded blastocyst using a sterile glass pipette (Vanderzwalmen et al., 2002).

4. a) Prepare a liquid nitrogen resistant label for each embryo being frozen with the following identifyinginformation:
   - Identity of the embryo = the Chart Number, female partner's name and the freeze number;
   - Date of the freeze;
   - The embryo's number (i.e. 1, 2, 3, etc).

   b) Attach the labels to the HSV straws approximately 15 mm (0.6") from the flared end of the straw. This free space at the open end of the straw is where it will be sealed.

For each embryo:

5. a) Connect the longer end of the blue plastic insertion device to the coloured end of the handling rod.
   b) Using a micropipette, carefully deposit the embryo into the gutter a few millimetres from the end. The drop of medium must be <1 µl. (A maximum of 2 embryos can be vitrified per device.)
   c) Process the embryo through the series of equilibration / vitrification solutions as per the protocolfor the method being employed.
   d) Immediately place the capillary rod and handler into the HSV straw and push until the flat rectangular portion of the handler comes into contact with the flared end of the straw.
   e) Slightly pinch the straw between your thumb and forefinger to restrain the end of the handling rodfurthest from the specimen gutter. Remove the blue plastic insertion device.
   f) While still holding the straw in the same place, seal the open end of the HSV straw using the SYMS heat sealer.

Notes: **1) The amount of time between the last vitrification solution and immersion of the specimen into liquid nitrogen must not exceed 60 s.**

2) Follow all established safety procedures for handling liquid nitrogen.

6. a) Hold the loaded HSV straw in the region of the handling rod using a pair of forceps.
   b) Quickly plunge the entire HSV straw into a bath of liquid nitrogen (or other vitrification device) vertically.
   c) Gently stir the HSV straw in the liquid nitrogen for s few seconds to prevent the formation of an insulting layer of nitrogen vapour around the straw.

7. When the specimen has been vitrified, remove the straw from the liquid nitrogen (or vitrification device) and transfer it **IMMEDIATELY** into a dewar or styrofoam box containing liquid nitrogen; ensure that the straw is completely immersed. This transfer of the straw through the air must be extremely quick: if the straw is allowed to warm up above -132°C (the glass transition temperature of water) ice recrystallization can occur within the frozen material and cause damage to the embryo.

8. Repeat steps 5 through 7 for each of the embryos to be vitrified.

9. Transfer the straws into the cryobank storage tank. See the appropriate SOP describing how the cryobank is organized. **There is no need to sanitize the outside of the straw prior to transferring itinto the cryobank because the specimen never comes into contact with the outside of the HSV straw.**

10. Update the cryobank inventory records as per the appropriate SOP.

Thawing

11. Prepare the thawing/dilution solutions for the method being employed.

12. Identify the correct unit to be thawed, and verify that it is the correct specimen. (See the appropriateSOP describing how the cryobank is organized.)

13. Transfer the HSV straw from the storage container into a transport dewar filled with liquid nitrogen using forceps. This transfer of the straw through the air must be extremely quick: if the straw is allowed to warm up above -132°C (the glass transition temperature of water) ice recrystallization canoccur within the frozen material and cause damage to the embryo.

14. When you are ready to proceed:
    a) Lift the straw out of the liquid nitrogen only sufficient to expose the coloured handling rod. **Make sure that the end of the straw where the specimen is located remains immersed in the liquid nitrogen.**
    b) Cut the HSV straw just above the handling rod using scissors or specific CBS™ opening device (a scalpel blade is not recommended for this due to the risk of injury to the operator's fingers).
    c) While grasping the straw firmly just below the now open end (so as to hold the handling rod in place) insert the short end of the plastic insertion/removal device into the coloured handling rod.Then pull the capillary rod containing the embryo out of the straw. It is harmless to the specimen should the gutter region of the capillary rod come into contact with the clear resin plug during thisprocedure.
    d) **IMMEDIATELY** (within 2 seconds) plunge the gutter into the first of the thawing/dilution solutions.
    e) Process the embryo through the series of thawing/dilution solutions according to the protocol forthe method being employed.

15. Repeat step #14 for each embryo to be thawed.

16. Update the cryobank inventory records as per the appropriate SOP.

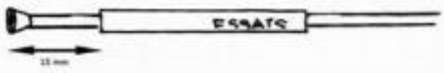
Note: There is no need to sanitize the outside of the straw before cutting it open since the capillary rodnever comes into contact with the outside of the HSV straw.

Simplified Procedure Chart for Using the HSV Straw

HSV High Security
Vitrification Kit

Cryo Bio System

Filling
Sealing
Vitrification

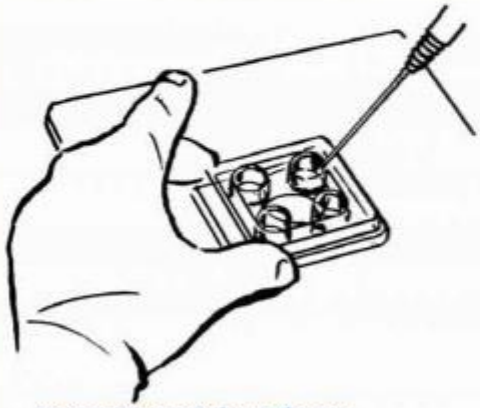


Apply an identification label approximately 15 mm (2/3 of an inch) from the flared end of the straw.

-1-

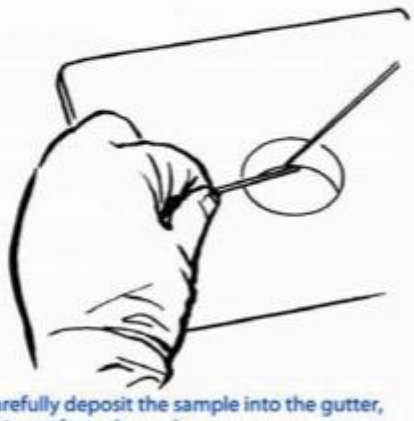
Connect the longer end of the blue plastic insertion device to the colored end of the handling rod.

-2-



Prepare the sample for vitrification.

-3-

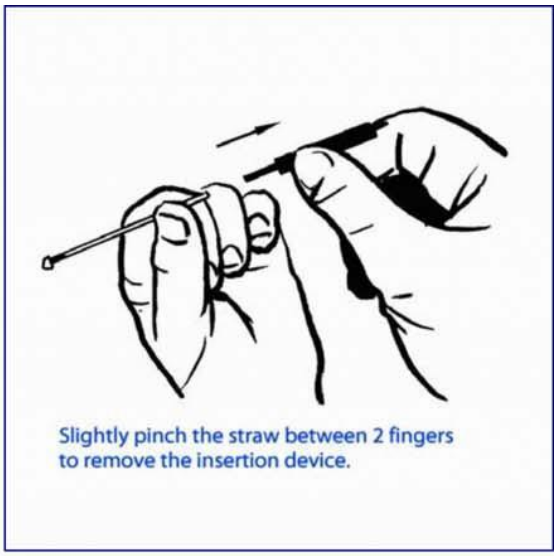


Carefully deposit the sample into the gutter, at 1 mm from the end.

-4-

Immediately place the capillary rod and handler into the straw.

-5-



Slightly pinch the straw between 2 fingers to remove the insertion device.

-6-

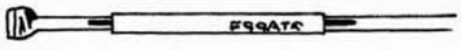
Seal the open end of the HSV High Security Vitrification straw using a SYMS sealer.

-7-

Quickly plunge the entire straw in liquid nitrogen. Gently stir the straw in liquid nitrogen for a few seconds.

-8-

Thawing



Identify the HSV High Security Vitrification straw.

-1-

Lift the straw enough to expose
the colored handling rod.

-2-

Cut the straw with scissors (or bistouries).

-3-

Insert the short end of the plastic removal device
into the colored handler in order to pull
the capillary rod / handling device out of the straw.

-4-

Immediately plunge the gutter into
the first dilution media.

-5-

Cryo Bio System

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RESOURCES

Frequently Asked Questions

Abbreviations used in this section

CPA cryoprotective agent
CPM cryoprotectant medium
DMSO dimethyl sulphoxide, a permeating CPA used in early work on embryo
freezingGEYC glycerol-egg yolk-citrate, a commonly-used CPM formulation
for human semen
HEPES N-(2-hydroxyethyl)piperazine-N'-(2-ethanesulphonic acid), a zwitterionic pH buffer with a useful range of pH 6.8–8.2 (pKa@ 25°C = 7.5)
HSPM human sperm preservation medium, a CPM formulation used for washed human
spermatozoaIUI intra-uterine insemination
LN2 liquid nitrogen
LNV liquid nitrogen vapour
MOPS 3-(N-morpholino)propanesulphonic acid, a zwitterionic pH buffer with a useful range of pH 6.5–7.9 (pKa @
25°C = 7.2)PBS phosphate buffered saline, a base solution used in the original CPMs for freezing embryos
PETG polyethylene terephthalate glycol, a plastic used to make traditional straws that are to be gamma-irradiated
PrOH propanediol or propylene glycol, the most commonly-used permeating CPM for human oocytes, zygotes and cleavage stageembryos
PVA polyvinyl alcohol, a powder that polymerizes upon contact with water, used to seal
traditional straws PVC polyvinyl chloride, a plastic used to make the original straws
ROS reactive oxygen species or oxygen free radicals
TES N-[Tris(hydroxymethyl)methyl]-2-aminoethanesulphonic acid, a zwitterionic pH buffer with a useful range of pH 6.8–8.2(pKa @ 25°C = 7.5)
TEST the combination of TES and TRIS, a commonly-used buffer system n CPM formulations for human
spermatozoa TRIS Tris(hydroxymethyl)aminomethane, a zwitterionic pH buffer with a useful range of pH 7–9
(pKa @ 25°C = 8.1) TYG TEST-yolk-glycerol, the most commonly-used CPM formulation for human semen

Cryoprotectants

What is a cryoprotective agent (CPA)?

Cryoprotective agents or "CPAs" are molecules that protect cells or tissues during cryopreservation, by preventing damage caused by ice formation inside the cells. Whatever their molecular structure, all CPAs are highly soluble in water. By virtue of their capacity to form stable hydrogen bonds with water molecules, CPAsdecrease the freezing point of any solution in which they are included.

What is a permeating (or penetrating) cryoprotectant?

These are cryoprotective agents (CPAs) that are able to cross the plasma membrane of the cells being frozen and replace water molecules inside them – thereby preventing the formation of intracellular ice crystals. Permeating cryoprotectants are characterized by:

- being able to cross the plasma membrane easily;
- being able to replace the water inside a cell;
- being very water-soluble (there is a positive correlation between solubility and cryoprotective ability);
- possibly being able to organize their neighbouring water molecules, thereby creating "structured" waterwhich is less likely to freeze, but can still act as a solvent; and
- being able to remain in solution as the temperature decreases, and not crystallize out of solution as thefirst ice crystals form.

What is a non-permeating (or non-penetrating) cryoprotectant?

Non-permeating CPAs are macromolecules or sugar molecules that increase the extracellular

osmolarity and aid in dehydrating the cells during slow freezing, but do not enter the cell (e.g. sucrose and trehalose). Sucrose is a commonly-used non-permeating cryoprotectant for embryos. It is not protective when used alone, but confers additional protection against freezing-induced damage when used with a permeating cryoprotectant. Because it does not cross the plasma membrane, the sucrose acts to create an osmotic gradient that assists in cellular dehydrating during the equilibration and cooling processes, and protects against excessive swelling and rupture of cells during the thawing and rehydration processes. Other non-permeating cryoprotectants include high molecular weight polymers such as polyvinyl pyrrolidone (PVP), which might act to bridge cell membrane defects or breaches; and albumin or serum which could repair damaged membranes during thawing. Hen's egg yolk, a common component of media for freezing human spermatozoa, is also a non-permeating cryoprotectant, and helps maintain sperm plasma membrane fluidity.

What is a cryoprotectant medium (CPM)?

A cryoprotectant medium or "CPM" is a solution that is used to treat the cells or tissues being cryopreserved.It is a buffered medium that contains a permeating CPA and, usually, one or more non-permeating CPAs. For semen or sperm suspensions a CPM is the medium used to dilute the sperm suspension. For animal semen the medium used for this dilution step is often referred to as an "extender" because the relative dilution of the original sample is often quite substantial.

Which buffer should I use in a CPM?

Buffering of the pH of the cryoprotectant medium during freezing is essential to avoid damaging the cells or tissue being cryopreserved. For human spermatozoa this buffering was achieved by glycine and citrate in the traditional Ackerman's GEYC (glycerol-egg yolk-citrate) medium, but more modern recipes employ a combination of the zwitterionic buffers TES and TRIS. TES-TRIS (usually abbreviated to "TEST") is most often used. CPMs based on phosphate buffered saline are not recommended due to the poor pH buffering provided by such solutions at lower temperatures [van den Berg & Rose, 1959] and ones containing high concentrations of bicarbonate should be avoided as their buffering capacity could be unstable in the absence of elevated carbon dioxide concentrations.

What is the difference between a CPA and a CPM?

CPA refers only to the cryoprotective agent while CPM refers to the actual cryoprotectant medium used to protect cells or tissues during cryopreservation. [See What is a cryoprotectant medium?]

What cryoprotectant should I use?

This decision will vary according to the permeability of the plasma membranes of the cells or tissues being frozen.

- Semen: Glycerol is by far the most widely used permeating CPA for human spermatozoa; a final concentration of 6.0 to 7.5% (v/v) seems to be optimum. Dimethylsulphoxide (DMSO) not only has direct deleterious effects on human spermatozoa but also exposes them to cold shock during processing. Propanediol (PrOH) has rarely been used with spermatozoa, while ethylene glycol has received little attention despite being proposed as a suitable CPA for human spermatozoa. Some workers have reported that adequate cryosurvival can be obtained with egg yolk in the absence of glycerol but nowadays such an approach is rare.
- Sperm (washed): Glycerol has been used almost exclusively for the cryopreservation of washed human spermatozoa. Hen's egg yolk should be avoided as such spermatozoa are often inseminated either into the uterine cavity post-thaw or are used in ICSI, at which time the entire sperm plasma membrane – along with the intercalated egg yolk phospholipids – is introduced into the ooplasm, an environment which they would not enter under normal biological circumstances.
- Oocytes: Protocols reported for cryopreserving human oocytes have all been modifications of ones used for zygotes, and hence propanediol has been the permeating CPA used, typically in conjunction with sucrose and a non-permeating CPA. The most successful protocols employ higher concentrations of sucrose that those typically used for cryopreserving zygotes [Critser et al., 1997; Fabbri et al., 2001].

- Zygotes: Zygote cryopreservation protocols have been derived from ones used for cleavage stage embryos (see below), and hence employ propanediol as the permeating CPA inconjunction with sucrose an a non-permeating CPA.
- Cleaving embryos: Although early workers used DMSO, the most successful protocols for cryopreserving cleavage stage mammalian embryos employ propanediol as the permeating CPA in conjunction with sucrose an a non-permeating CPA [Lassalle et al., 1985].
- Blastocysts: Again although some early workers used DMSO, the great majority of studies have employed glycerol as the permeating CPA, used in conjunction with sucrose an a non-permeating CPA [Ménézo et al., 1992].
- Testicular tissue: The great majority of current protocols employ glycerol as the permeating CPA in conjunction with sucrose as a non-permeating CPA. Both TYG and HSPM have been used for this purpose.
- Ovarian tissue: There is still debate as to the optimum permeating CPA for ovarian tissue slices [e.g. Newton et al., 1998; Oktay et al., 1998; Gosden, 2000].

What are IUI-ready" sperm samples?

"IUI-ready" sperm samples are ones where the spermatozoa have been separated from the seminal plasma prior to cryopreservation so that the frozen sample could be inseminated directly into the uterine cavity without the need to wash the spermatozoa free from those seminal plasma components that can cause cramping of the myometrium (e.g. prostaglandins) [Larson et al., 1997; Wolf et al., 2001]. Ideally this washing step also separates likely functional spermatozoa from the dead and non-/dysfunctional spermatozoa in the semen samples as well as eliminating the other cells and debris that contaminate semen. This is bestachieved using density gradient centrifugation; simple centrifugal washing should be avoided as it can cause iatrogenic damage to the spermatozoa that will impair their fertilizing ability. Also, hen's egg yolk should not be used in CPM formulations for this application since it contains xenologous proteins that should not be introduced into the uterine cavity with the inseminate. "Safe" IUI-ready specimens should therefore be prepared using a validated density gradient method to avoid iatrogenic damage to the spermatozoa and then cryopreserved using a CPM formulation that does not contain any xenoproteins.

Is there a "best" cryoprotectant medium formulation that I should use?

Clearly this question must be answered according to the type of cells or tissues being frozen.

- Semen: No one CPM formulation (or freezing regimen) has been proven to be better than others on a population basis. This has been largely because of the great inter- andintra-individual variability that exists in the cryosurvival of spermatozoa between different CPMs and cryopreservation methods [e.g. Friberg & Gemzell, 1977]. Over the past decade or so there has been a substantial shift away from the traditional Ackerman's GEYC medium to TEST-yolk-glycerol (TYG) medium which has now become the de facto standard CPM for most laboratories freezing human semen.
- Sperm (washed): Almost all work to date has used either "HSPM" [Mahadevan & Trounson, 1983 – but see the FAQ on CPM buffering since HSPM contains 31mM sodium bicarbonate] or TYG. However, there are some commercially available products designed for this purpose, e.g. Tardigrade (Cryo Bio System, Paris).
- Oocytes: Although this is still generally considered a research technique, the method reportedby Fabbri et al. (2001) seems to give the best success currently.
- Zygotes: The standard propanediol + sucrose method [Lassalle et al., 1985].
- Cleaving embryos: The standard propanediol + sucrose method [Lassalle et al., 1985].
- Blastocysts: The protocol described by Dr Yves Ménézo [Ménézo et al., 1992] is currently the most widely used.
- Testicular tissue: Glycerol, although the actual CPM formulations vary widely.
- Ovarian tissue: DMSO is probably the most widely-used permeating CPA but others have been used and there is probably no real consensus yet as to the best technique based on post-thaw studies [e.g. Newton et al., 1998; Oktay et al., 1998; Gosden, 2000].

Why use hen's egg yolk in sperm freezing?

Hen's egg yolk is rich in phospholipids that intercalate into the plasma membrane of spermatozoa during preparation for cryopreservation and seem to create improved membrane fluidity that enhances sperm cryosurvival. It might also contain other macromolecules that could stabilize cell surface molecules duringthe freezing and thawing process.

Is hen's egg yolk essential for sperm freezing?

No. However, the general levels of cryosurvival seen when freezing semen from unselected populations ofmen do seem to be higher if hen's egg yolk is included in the CPM formulation, e.g. TEST-yolk-glycerol or"TYG".

Why shouldn't I use hen's egg yolk in my "IUI-ready" sperm samples?

Hen's egg yolk contains many xenoproteins that could induce immune reactions within the female reproductive tract. As a simple matter of biosafety, introduction of such molecules into the female tract above the level of the cervix should be avoided.

What is the role of sucrose in oocyte/embryo freezing?

Sucrose is a non-permeating CPA, therefore it remains outside the cells where it creates an osmotic gradientthat increases the removal of water from the cells (i.e. dehydration) during slow freezing. The intracellularwater is replaced by the permeating CPA.

What is trehalose and why is it used in some cryoprotectant media?

Trehalose is a disaccharide, similar to sucrose, that has been reported not only to facilitate dehydration of cells during slow freezing, and possibly to help stabilize cell surface molecules during freezing and thawing. However, its application in human gamete and embryo cryopreservation has seen little success to date.

Why must I add/remove cryoprotectant slowly (or step-wise)?

When a permeating CPA is added to cells they undergo substantial dehydration as water leaves the cells dueto the osmotic gradient – and hence they shrink. Then, as the permeating CPA enters the cells (which it does more slowly than water leaves due to the higher membrane permeability coefficient for water than CPAs) the cells return to their isotonic volume. Upon removal of the permeating CPA by diluting the post-thaw specimen, water enters the cells quickly along the osmotic gradient and the CPA leaves the cells more slowly, hence the cells swell before equilibrium is restored. For a detailed recent discussion of this topic, readersshould refer to work from Dr John Critser's laboratory [Gao et al., 1997].

Consequently, CPM addition must be stepwise, and most protocols involve a drop-wise addition with constant mixing over several minutes. Upon thawing, if insemination is to be intra-cervical (or into the uterine cavitywith "IUI-ready" spermatozoa) then CPA removal occurs as the spermatozoa migrate from the semen/spermatozoa suspension + CPM mixture into the fluids of the female reproductive tract. However, if the spermatozoa are to be washed in any way, the thawed specimen must be diluted slowly using stepwise addition of a HEPES-buffered culture medium because too rapid dilution can damage cryopreserved spermatozoa. Adding a 10-times volume of sperm buffer slowly will enable optimum yields using density gradients.

What are "critical volume limits"?

All cells have critical volume limits which, if exceeded during these volume excursions, result in irreversibledamage to the cell, presumably via the integrity of its cytoskeleton. Only with extreme swelling will cells burst, so cells can have been damaged but still be alive and, in the case of spermatozoa, will still probablybe motile.

- Spermatozoa: Using a 5% loss of motility as the criterion for damage, the upper and lower critical volume limits for human spermatozoa are 110% and 75% of their isotonic volume [Gao et al., 1995].
- Oocytes: Not yet determined for human oocytes.

- Zygotes: Not yet determined for human zygotes.
- Cleaving embryos: Not yet determined for human cleavage stage embryos (might differ according tostage).
- Blastocysts: Unknown, could be different for trophoblast and inner cell mass cells.
- Testicular tissue: Unknown (multiple cell types).
- Ovarian tissue: Unknown (multiple cell types).

Why don't we remove the glycerol before cervical insemination of cryobanked semen or "IUI-ready" sperm samples?

Because of the relatively low volume of female tract fluids with which the inseminate is mixed immediatelyafter insemination there is no opportunity for rapid removal of the permeating CPA (usually glycerol), and hence the spermatozoa will not be exposed to the risk of large volume excursions that might exceed their critical volume limits. The permeating CPA is lost relatively slowly as the spermatozoa migrate through thefemale tract.

Packaging systems

What are the pros and cons of the various packaging systems?

- Straws ("traditional"): Plastic straws (sometimes referred to as "French straws" or "paillettes") were first described by Robert Cassou in 1963 [Cassou, 1964]. He created the company Instruments de Médicine Vétérinaire or IMV based in L'Aigle (France) to commercialize this technology. The original straws were made from polyvinyl chloride (PVC), and later polyethylene terephthalate glycol (PETG). PVC straws were withdrawn in 1998 because they could not be sterilized by irradiation without compromising their mechanical integrity. Other companies have also made similar products to Cassou's paillettes.
- Straws ("high security"): Straws made from a special ionomeric resin were described in 1992 for special applications that required higher standards of mechanical integrity and biocontainment than the traditional paillettes. These "high security straws" are manufactured and distributed exclusively by the medical subsidiary of IMV called Cryo Bio System (Paris, France) for use in human assisted reproduction and other areas of application. These CBS™ High Security Straws are often referred to simply as "CBS™ straws", but must not be confused with other straws made from PETG that the company also sells.
- Cryovials: Plastic screw-top vials or "cryovials" used in human gamete and embryo cryobanking have typically been the NUNC™ CryoTube® range of products (Nunc A/S, Roskilde, Denmark and Nalge Nunc International, Naperville, IL, USA) made from polypropylene with either polypropylene or polyethylene screw caps. Similar products are also available from other manufacturers but there have been anecdotal reports of variable degrees of endotoxin contamination of some of these alternate products.
- Glass ampoules: These have been strongly discouraged for many years on safety grounds dueto their fragility.

Why should I use straws instead of cryovials?

Aspects of historical and personal preference will not be considered here. However, technical arguments in the straws vs cryovials debate involve issues concerning the effective cooling and warming rates that will be experienced by specimens packaged in devices of different radius, the mechanical fragility of the various devices at liquid nitrogen temperature (i.e. -196°C), their effective sealing or leakiness, and biocontainment. These issues are tightly inter-connected because they are all governed by the physical characteristics of thepackaging systems.

Although studies directly comparing cryosurvival of the human gametes and embryos in the different packaging devices have not been carried out, Whittingham reported that 8-cell mouse embryos frozen in plastic cryotubes showed significantly lower cryosurvival and post-thaw blastocyst development rates compared to straws [see McLaughlin et al., 1999 or Wood, 1999]. Also, it has been known for almost 40 years that bull spermatozoa frozen in ampoules has lower fertility compared to that frozen in straws [see Watson, 1979]. Finally, there is circumstantial evidence that the fecundity of human spermatozoa cryopreserved in cryovials could be lower than that obtained when using straws [Mortimer, 2004a].

How do I fill straws?

When filling the traditional IMV straws the open end of the straw is immersed in the semen+CPM mixture as it is be aspirated into the straw. After sealing it is essential that residual material on the outside of the straw be removed, usually by wiping with a paper tissue (otherwise adjacent straws during freezing become "cemented" together at LN2 temperatures and cannot be separated without grave risk of breaking them.

Because traditional straws are filled by immersing their open end into the specimen their outsides are contaminated – this represents a major source of contamination of the LN2 in storage cryotanks [Russell et al., 1997]. However, there is no such risk with the CBS™ High Security Straw system since, while aspiration is also used, they have a special nozzle attached to their open end through which material is loaded into the straw at a distance of several millimetres along the straw's lumen. This means that the outside of the straw never comes into contact with the sample.

Why must I leave an air space inside straws?

An air space must be left inside a straw to allow for expansion of the semen+CPM mixture as it cools (since water has its maximum volume at +4°C). Without the air space the plug can be ejected as the column of semen+CPM expands. (As a corollary, the air space also represents an opportunity for LN2 to enter the straw if the seal is faulty.) These issues are of less importance when using the CBS™ High Security Straws that have been properly sealed using the SYMS welder, but an air gap must still be present so as to avoid contaminating the sealer.

How do I seal straws?

The open ends of PVC or PETG straws are sealed either by:

- tamping in polyvinyl alcohol (PVA) powder which polymerizes upon contact with moisture;*
- inserting solid plastic (nylon) plugs;
- inserting plastic spheres or steel balls;
- using haematocrit tube sealant;
- ultrasonic welding; or
- heating.**
- CBS™ ionomeric resin straws must be sealed using the special SYMS thermal soldering device.

*The PVA powder that is tamped into the open end of the filled straw might only be partially cured by moisture lining the straw, and will form a poor seal unless it is fully cured. It is important that, after tamping in the PVA powder, straws should be immersed in water to ensure complete curing of the PVA plug.

**Crude heat sealing using forceps heated in a Bunsen burner flame is especially dangerous with the traditional IMV straws because the flattened cylinder has stress fractures at its corners, rendering the straws more liable to crack during freezing, leak during storage and explode upon thawing.

Why do the plugs or balls come out of straws?

There are two main reasons for this to happen:

1. If the ball or plug contracts then it could no longer be a tight fit inside the straw lumen and simply fall out.
2. If the seal is not hermetic then LN2 will enter the straws past the plug or ball due to the reduced pressure inside the air space caused by contraction of the air at cryogenic storage temperatures. Then, upon warming (even transitory warming during transfer between cryotanks), this LN2 rapidly turns from liquid to vapour – with a 700-fold increase in volume – and the sealing plug or ball is ejected (often very forcefully).

What is the white substance that "puffs" out of straws when they're removed from liquid nitrogen?

In the traditional straws the upper end of the straw is sealed by a quantity of PVA powder located between two plugs of cotton wadding. The cryoprotectant medium is aspirated until it passes through the inner wadding plug and comes into contact with the PVA powder – which polymerizes upon contact with the water. However, the outer wadding plug retains trapped air which, once immersed in LN2, contracts and sucks LN2into the interstices of the wadding. Upon even transitory warming (such as removing a goblet of straws from LN2 to either remove a straw or to move the goblet to another cryotank) this LN2 very rapidly turns from liquid to vapour – with a 700-fold increase in volume – and the entire wadding plug explodes out of the endof the straw (typically accompanied by a quiet "puff" sound). While the white cotton material is harmless, is does contribute to the "sludge" that accumulates at the bottom of cryotanks. However, the seal of the straw will remain intact so long as the PVA powder had been properly polymerized.

Does heat sealing straws damage the sperm/embryos?

No. Plastic is a very poor conductor of heat (hence its use as a thermal insulator) and the localized application of heat to seal a straw does not cause heating beyond a very short distance from the area of application of the heat. Since embryos and spermatozoa will be many millimetres from that point they will not be adversely affected. Moreover, the air gap will act as a further thermal brake, since air is a much poorer conductor of heat than water.

What are "High Security Straws"?

These are a new range of products developed by IMV that are manufactured and marketed by the company's human medical subsidiary, Cryo Bio System (Paris, France). They are made of a unique ionomeric resin and their design confers several highly important benefits upon them for use in critical cryopreservation applications.

- Heat sealable using a special thermal welding device (the SYMS sealer). Properly-sealed CBS™ straws areguaranteed to be absolutely leakproof at pressures of up to 150 kg.cm^{-2}.
- Mechanically resistant, i.e. shatterproof even at -196°C.
- Bacteria and virus proof.
- A special filling nozzle so that none of the material being loaded into the straw ever comes into contactwith the outside of the straw.
- Extensive biocompatibility testing of every batch to verify that the straws are non-toxic (low endotoxin)and free of bacterial and viral contamination. The straws are guaranteed to cause no specific damage tohuman or bovine spermatozoa or mammalian embryos.
- Sterile (after irradiation at 25 kGy in accordance with European Pharmacopoeia standards).
- Two-compartment design that allows identifying information to be sealed inside the straw itself, makingidentification tamper-proof.
- Secure external identification labelling option using sleeves that cannot slide off the straw once it has beensealed (due to the flat "tabs" that are formed by the welds).
- Approved for human applications by the US FDA with a 510(k) pre-market notification clearance K002595and CE marked as a medical device according to CE 93/42.

Why must I use the SYMS unit to seal High Security Straws?

Proper thermal soldering of the ionomeric resin requires the application of heat at a specific temperature,under a specific pressure, and for a specific time, in order to achieve a guaranteed weld. These conditionscannot be met by any manual methods.

How do I fill cryovials?

Cryovials are filled by transferring the specimen directly into the lumen using either a (sterile) glass Pasteur pipette, a volumetric (e.g. serological) pipette, or any other liquid handling device with disposable tips. Great care must be taken not to allow any of the specimen to contaminate either the threads where the cap willscrew into the vial or the upper rim of the cryovial where the silicone O-ring will be compressed to form aseal.

Disinfection

Should I disinfect the outside of straws/cryovials after filling (i.e. before freezing)?

Definitely yes – if there is any risk of contamination of the outside of the straw or cryovial with non-sterilespecimen (e.g. semen+CPM mixture). Also, if the straw or cryovial has been handled in such a way that itmight be contaminated by skin commensals or other micro-organisms that could contaminate the cryostorage tank, then again yes.

Should I disinfect the outside of straws/cryovials after thawing?

It is now well-known that LN2 is not sterile, and can be contaminated by many airborne micro-organisms,skin commensals and even pathogens. Moreover, even vapour storage systems can be contaminated by micro-organisms [Fountain et al., 1997]. Consequently, it must be assumed that the outside of any packaging device that has been stored in a cryotank is contaminated – and therefore if there is any risk thatthe outside of the packaging could come into contact with its contents during their removal, best practice considerations require that the outside of all specimens be disinfected prior to opening them.

What should I use to disinfect straws/cryovials?

A non-volatile disinfectant should be used, such as a solution of hypochlorite or stabilized chlorine dioxide(Expel). Toxic reagents such as those containing aldehydes and alcohol (ethanol or isopropyl alcohol) should not be used, especially if it is uncertain whether the material of the packaging device might be permeableto them.

Are there any risks when disinfecting straws/cryovials?

Obviously great care must be taken to avoid any risk of the disinfectant coming into contact with the specimen inside the cryovial or straw. Therefore, broken or cracked straws, or cryovials that are not completely sealed cannot be disinfected – and hence ought to be discarded.

Should I sterilize my programmable freezer between runs?

Provided that every precaution has been taken to ensure that the outside of the straws or cryovials containing specimens being cryopreserved was not contaminated there should be no need to sterilize the chamber or other surfaces of a controlled rate freezer. However, if a laboratory is freezing specimens containing knownpathogens (e.g. hepatitis B or C virus, HIV), a best practice protocol might well include a requirement to perform such a sterilization step. Very few programmable freezer chambers are amenable to such sterilization and users should consider this aspect carefully when choosing which particular instrument to purchase according to their specific needs.

Should I sterilize my heat sealer between samples?

Provided that every precaution has been taken to ensure that the outside of the straws or cryovials containing specimens being cryopreserved was not contaminated there should be no need to sterilize the heat sealer. However, if a laboratory is working with specimens containing known pathogens (e.g. hepatitis B or C virus,HIV), a best practice protocol might well include a requirement to perform such a sterilization step. The same disinfecting agent as used for the outside of the straws should be used. Because of the heat source, flammable agents such as alcohols must not be used.

Cooling

What is thermal shock?

Thermal shock is the general term used to describe damage to cells that occurs when they are cooled tooquickly, even if ice formation does not occur. The primary critical range is between +15°C and 0°C, although there is a second range between 0°C and -80°C (also known as the "lethal zone"). Thermal shock beginswith damage to the plasma membrane as a result of one or more of the following effects: (a) differential shrinkage of membrane components; (b) mechanical shearing; and (c) conformational changes in membrane topography. The occurrence of thermal

shock can be mitigated by employing one or more of the following: (a) cryoprotective agents; (b) certain phospholipids (especially phosphatidyl serine); (c) slow cooling; and (d) pre-conditioning in high-salt conditions.

What is the lethal zone?

This term is sometimes used to describe that part of the cooling curve between the freezing point of the CPM and -30°C. In this range if the formation of ice crystals is not carefully controlled then it can be lethal to thecells.

Are there differences between the various models of programmable freezers?

There are four general approaches to designing programmable controlled rate freezers:

1. Where LN2 vapour is injected into the freezing chamber so as to cool the vapour inside the chamber and hence the specimens contained therein (e.g. Planer Kryo10 series, Cryo Bio System Nano-Digitcool & Digitcool range).
2. Where the specimen is lowered into a static vapour phase above a reservoir of LN2, with lower levels being colder (e.g. Cryogenetic Technologies).
3. Where LN2 vapour is pulled through a chamber where the straws are located; the fan can operate at fixed or variable speed (e.g. Air Liquide Nicool LM10).
4. Where the specimens are in contact with a cold mass of metal that is cooled by contact with LN2, usuallyemploying electrical heating to regulate the rate of cooling. Examples include the Biotronics DB1 and theCryoLogic series of models from Freeze Control.

What happens during slow freezing?

As the temperature falls below the freezing point of the medium, ice formation begins. Pure water forms small crystals, leaving an increased concentration of solutes in the remaining liquid medium. The cell membrane prevents ice crystals from entering the cell, and so the intracellular contents become supercooled (i.e. the remaining liquid inside the cell is at a temperature below its freezing point). This causes a differencein the chemical potential of the water inside and outside the cells (the intracellular water is now "metastable"), so water leaves the cell, going down the osmotic gradient. With a sufficiently slow cooling rate water is able to leave the cells, which therefore dehydrate; these cells will be viable upon thawing. However, too fast a cooling rate does not allow the water to leave the cells quickly enough and there will be increased super-cooling within the cell which requires the intracellular water to freeze so that the equilibrium can be regained. This development and growth of ice crystals within the cells causes damage and even celldeath.

As the amount of extracellular ice increases the concentration of solutes in the remaining liquid aqueous phase increases, causing more water to leave the cells along the osmotic gradient and more permeating CPAto enter the cells down the concentration gradient. This process needs to continue until all the water hasbeen drawn out of the cells, thereby preventing intracellular ice formation. After this point the permeatingCPA inside the cell becomes supercooled and eventually freezes. If the cooling rate is too high then ice might form inside the cells and/or larger ice crystals will form outside the cells – in both situations these ice crystals can damage, even destroy, the cells.

Why must cells dehydrate during slow freezing?

If the water is not removed from inside cells it will freeze at some point during the cooling and the intracellular ice crystals will disrupt structures and organelles inside the cells, leading ultimately to death ofthe cell.

Is there a "best" cooling rate that I should use?

The following cooling protocols represent the ones most commonly used for each type of cell. The expression "freefall" denotes uncontrolled cooling, usually at quite a high rate (e.g. -30°C to -50°C per minute), oncethe specimens are well below the melting point of the permeating CPA inside the cells. Upon completion of the freezing program the specimens must be transferred rapidly – i.e. as quickly as possible – into LN2 at
-196°C (often described as "plunging").

Note: The cooling curves are what the specimens are expected to experience, although there can be lags between the specimen and the program if the freezing chamber is being cooled using LN2 vapour. These lags will be greater for specimens with larger mass, and especially if the specimen has a larger radius. It is vital that a "hold" or "soak" period is included in the program to ensure that specimens are at the correct temperature before seeding.

- Semen: a) For spontaneous ice nucleation (i.e. no "seeding"): Cool at -5°C/min from roomtemperature to +4°C, then at -10°C/min down to -80°C and freefall to at least -120°C.
  b) For seeding of ice crystal formation: Cool at -3°C/min from room temperature to -5°C, then hold for 10 minutes to ensure equilibration at that temperature beforeinducing seeding. Subsequent cooling is at -10°C/min down to -80°C and freefallto at least -120°C.
- Sperm (washed): Use the same cooling program as for semen.
- Oocytes: Use the same cooling program as for cleaving embryos.
- Zygotes: Use the same cooling program as for cleaving embryos.
- Cleaving embryos: Cool at -3°C/min from room temperature to -7°C, then hold for 10 minutes to ensureequilibration at that temperature before inducing seeding and a further hold of 5–10minutes to allow time to perform the seeding. Subsequent cooling is at -0.3°C/mindown to -80°C and then -50°C to -150°C.
- Blastocysts: Use the same cooling program as for cleaving embryos.
- Testicular tissue: Because the cells of interest are the spermatozoa, most reports have used the samecooling program as for semen.
- Ovarian tissue: Equilibration with the DMSO-based CPM is performed on ice. Load the specimens into the programmable freezer chamber at 0°C and then cool at -2°C/min to -7°C. Hold at -7°C for 10 minutes to ensure equilibration before seeding. Subsequent cooling isat -3°C/min to -40°C and then -10°C/min to -40°C [Oktay et al., 1998].

What is the "actual" cooling curve, and why does it differ from the programmed one?

The "actual" cooling curve is what the specimen itself actually experiences. It can lag behind the programmed curve due to a combination of factors that include:

a) The efficiency of the specimen chamber cooling – particularly if it has a large volume and is being cooled using LN2 vapour.
b) The radius of the specimen – this will affect the rate of heat transfer through the specimen so that larger specimens might experience different cooling rates at different positions through the "depth"of the specimen.
c) The total mass of specimen(s) being frozen if the chamber cooling system has a maximum heat transfer capacity.

If straws are held in bundles, those on the inside will experience different cooling rates to those on the outside, and this will contribute to differential survival and quality post-thaw.

Is the cooling rate the same inside straws and cryovials?

According to the laws of physics, heat transfer through objects will be impeded as a direct result of increased radius. Consequently, inside cryovials (diameter 6 mm) not only will the cooling rate lag further behind theprogrammed rate than in a straw (radii ranging from 1.0 to 1.55 mm), there will also be less effective, uneven cooling throughout the sample. This problem will be exacerbated when using controlled rate freezers thatoperate via the temperature of the vapour inside the cooling chamber (e.g. Planer Kryo-10) compared to those where the specimens are cooled by direct contact with a mass of metal (because cold air or liquid nitrogen vapour has a lower thermal capacity).

Do I have to use a programmable freezer for spermatozoa?

No. There are many published techniques for achieving adequate cooling curves for spermatozoa packagedin straws or cryovials, typically using the "static vapour freezing" approach although some use a combination of refrigerator / -20°C or -30°C mechanical freezer / LN2 vapour.

Very successful static vapour methods are available from the French Federation CECOS and collaborators and employ large, wide-mouthed dewars vessels (hence the common nickname of "garbage can" methods).

a) For 0.25 ml straws, hold at 25 cm above LN2 for 10 minutes before plunging.
b) For 0.5 ml straws, hold at 35 cm above LN2 for 15 minutes and then at 15 cm for a further 15 minutes before plunging.

Because a temperature gradient is established through a static vapour phase, straws frozen by this method must be placed horizontally in the vapour so that they will experience the same cooling effect along their length. Moreover, it is vital that straws are arranged in a monolayer, not in bundles or multiple layers, sothat all of the straws will experience the same cooling effect. Straws in bundles will experience different cooling rates and this will contribute to poor reproducibility between units in their post-thaw survival and quality. A particularly poor technique is to place straws in a bundle, usually inside a visotube, in the neck ofan open storage dewar vessel for a certain period of time. This method results in very unreliable cryopreservation with differences between straws on the outside and inside of the bundle, and probably evenin variations in cryosurvival along the length of each straw.

Why don't we use the "alcohol bath" type of programmable freezer in human IVF labs?

Many research laboratories have used this technology for freezing spermatozoa and embryos, but it isunpopular in clinical laboratories for a combination of reasons:

a) Safety hazard due to the large volume of flammable/explosive alcohol.
b) Concern that the alcohol might permeate the walls of straws, or the seals of cryovials as they cool andcontract, thereby perhaps adversely affecting the specimen.
c) Many labelling systems used for straws and cryovials are either soluble in alcohol or might be adverselyaffected by the solvent.

At what temperature should I transfer specimens from the programmable freezer into the cryotank?

Cryopreserved material must be kept below the glass transition temperature of water (-132°C), and water recrystallization damage will accumulate with every moment that a frozen specimen is at temperatures higher than this. Therefore, the lower the temperature the greater the safety margin when a specimen is removedfrom a controlled rate freezer and transferred into cryogenic storage.

Vitrification

What is vitrification?

Vitrification uses ultra-rapid cooling of the medium containing the cells or embryos, e.g. -2000°C/min. The medium increases so greatly in its viscosity that it becomes a solid at low temperatures: ice crystal formation is bypassed and the whole sample turns directly to "glass". Basically, the solution solidifies so quickly that the molecules do not have sufficient time to arrange themselves into a crystal structure.

What is the difference between cryopreservation and vitrification?

Like cryopreservation media, vitrification solutions commonly are composed of permeating (ethylene glycol and/or DMSO) and non-permeating (sucrose or trehalose) cryoprotective agents. In some protocols, the vitrification medium is also supplemented with macromolecules such as polyethylene glycol (PEG; 8 kD), Ficoll (70or 400 kD) or polyvinylpyrrolidone (PVP; 360 kD). However, in vitrification solutions the permeating CPAs are highly concentrated in order to dehydrate the cells just before cooling. But to achieve solidification ofa solution at low temperature without ice crystal formation there must be a combination of both high CPA concentration and an extremely high cooling rate.

What are the advantages of vitrification?

Vitrification is a very quick, technically simple process – assuming that a technique for achieving theultra-rapid freezing rate is available. Also, vitrification does not require a controlled rate freezer.

What are the problems with vitrification?

Problems with vitrification fall into two general categories: (a) toxicity of the high concentrations of permeating CPAs in the vitrification solutions; and (b) the only means whereby the very high cooling rates required for successful vitrification can be achieve require direct exposure of the specimen to the refrigerant (typically LN2 or LN2 slurry). While the toxicity issues can be dealt with using very rapid specimen processing (see below), packaging systems that do permit biocontainment for vitrified specimens represent a major obstacle to the safe application of vitrification within a modern clinical system founded on the principles of best practice and risk management. This is why the CBS™ HSV system was developed.

The necessity for exposing embryos to high concentrations of cryoprotectants, e.g. 30–50% (v/v), has made many people cautious about using vitrification clinically. However, it is possible to limit cryoprotectant toxicity by using a solution of two different cryoprotectants, thereby decreasing the relative concentration of each. An alternative approach involves the addition of macromolecules to the cryoprotectant solution; these polymers are generally less toxic and can protect the cells against cryo-injury, and also increase the viscosity of the solution. By increasing viscosity, the macromolecules support vitrification with lowered concentrations of cryoprotectants. It is also possible to reduce the toxicity of the vitrification solution by reducing the length of time the cells are exposed to it, or by pre-cooling it (although the speed of cryoprotectant penetration is influenced by temperature). The balance between the prevention of intracellular ice formation and the prevention of toxic injury must be determined for each protocol for each cell type.

Seeding

What is “seeding”?

When cells are cooled in a medium containing high concentrations of CPAs, the medium will super-cool to 15°C to 20°C below its true freezing point (which is around -5°C to -8°C depending on the actual formulation) before spontaneous ice crystallization occurs. Ice crystallization will only occur at the true freezing point if there is a “nucleus” around which the ice crystals can form.

As water transforms to ice, the molecules go from being in a relatively disordered state, with moderate levels of free energy, to being a highly-ordered structure with substantially less free energy. The energy that is lost from the water when it goes from a liquid to a solid state is largely released as heat – the “latent heat of fusion”. This release of heat increases the temperature of the medium towards its true freezing point until ice crystals form and then, after the phase transition from liquid to solid is completed, the sample cools rapidly back to the temperature of the freezing chamber. Therefore, if there was no facilitated ice crystallization, the cells could be cooled to, perhaps -20°C before spontaneous ice formation occurred. This would be accompanied by a rapid increase in temperature, to just below 0°C (due to release of the latent heat of fusion), followed by an uncontrolled drop in temperature back to -20°C. However, cells have significantly better cryosurvival if they are cooled slowly after the phase transition, and so they are extremely susceptible to damage during the rapid cooling phase which follows phase transition. If this can be minimized, cell survival is increased.

Seeding provides this protection against large temperature excursions around the phase transition. It is performed when the temperature of the sample reaches about -7°C (i.e. just below the freezing point of the CPM), and is accomplished by touching the side of the straw or cryovial with a very cold object, e.g. metal forceps which have been cooled in LN2. Once ice formation is induced and the preparation starts to freeze, the temperature differential caused by the ongoing release of the latent heat of fusion is minimized.

How do I induce seeding?

Some controlled rate freezers have “auto-seeding” capabilities, although very few people freezing human embryos trust them to perform correctly. Manual seeding typically employs a “Q-tip” or “cotton bud” soaked in LN2 or forceps that have been super-cooled by immersion in LN2. Because ordinary forceps only contact the straw at two tangential points, special forceps that encircle the straw with a thickened mass of metal have been designed so as to ensure maximum contact between a larger cold mass and the wall of the straw. With cryovials, effective seeding requires the Q-tip approach or larger metal instruments.

What temperature must I seed at?

The correct temperature for seeding depends on the formulation of the CPM. For glycerol-based

CPMs intended for human spermatozoa this is around -5°C, and for propanediol-based CPMs for cleavage stageembryos it is -6°C or -7°C. If seeding is induced "late", i.e. at a lower a temperature, the initial ice crystalsmight form too quickly, i.e. be too large. If seeding is attempted too soon, i.e. at too high a temperature,the thermal mass of the specimen might warm up the area where seeding was induced and the nucleatedice crystals will melt, potentially leading to super-cooling and its attendant problems.

What is the "latent heat of fusion"?

As water transforms to ice, the molecules go from being in a relatively disordered state, with moderate levelsof free energy, to being a highly-ordered structure with substantially less free energy. The energy that is lost from the water when it goes from a liquid to a solid state is largely released as heat – the "latent heat of fusion". The greater the mass of water crystallizing at a point in time, the greater the amount of heat released. The release of this heat can be seen clearly as a "blip" in recordings made inside specimens during cryopreservation. Ideally, this heat should be removed from the specimen by the cooling system: this is relatively easy is the specimens are in contact with a mass of cold metal, but rather more difficult if coolingis being effected via LN2 vapour.

Why don't we seed spermatozoa?

The vast majority of sperm cryobanks do not induce ice nucleation during cooling. This is because seedingof spermatozoa has not been shown to confer any significant improvement in their cryosurvival. Icenucleation will occur somewhere between -5°C and -8°C depending on the CPM formulation being used. However, it is also affected by the very nature of its being "spontaneous", it might happen in one straw at -7°C and in another at -16°C, quite arbitrarily, and this could contribute to between-straw variability incryosurvival.

What do I use to seed my specimens?

Manual seeding typically employs a "Q-tip" ("cotton bud") soaked in LN2 or forceps that have been super-cooled by immersion in LN2. Because ordinary forceps only contact the straw at two tangential points, special forceps that encircle the straw with a thickened mass of metal have been designed so as to ensure maximum contact between a larger cold mass and the wall of the straw. With cryovials, effective seeding requires the Q-tip approach or larger metal instruments.

What is "automatic seeding"?

Some controlled rate freezing machines have an automated seeding device. This is simply a cold bar or rodof metal that is brought into contact with the specimens at the appropriate point in the cooling program.

Should I use automatic seeding?

The great majority of clinical embryologists do not trust automatic seeding devices and prefer to perform seeding manually. However, this is personal decision based upon experience and trust in each particular machine's ability to perform seeding reliably.

Where should I seed my embryo straws?

Seeding must be induced in a portion of the sample remote from the embryos. The recommended location is at the opposite meniscus of the air bubble above the column of medium that contains the embryos, i.e. the meniscus of the column of medium that touches the upper seal of the straw. Ice crystal formation creeps across the air bubble by virtue of the thin layer of moisture that remains behind after the straw was filled.The importance of the seeding step in embryo freezing cannot be overemphasized, as incorrect seeding is probably a major contributor to failure of cryosurvival.

Some workers have commented that when straws are frozen vertically instead of horizontally, ice formationdoes not always cross the air bubble. This finding might be due slightly different surface characteristics ofPETG compared to PVC.

Warming/Thawing

Why must spermatozoa/embryos be warmed/thawed rapidly?

Because frozen water will undergo recrystallization at temperatures above the glass transition temperatureof water (-132°C) cryopreserved specimens must pass through this zone where they can suffer lethal damage as quickly as possible.

How do I achieve rapid warming during thawing?

The rate of warming is proportional to the temperature difference between the specimen and its environment. But, because the specimen starts at -96°C, thawing temperatures of 22°C, 30°C or 37°C make very little difference in the theoretical rate. However, because air has poor thermal transfer characteristics, thawingin air is much slower than in specimens which are immersed in a material with greater thermal capacity and better thermal transfer characteristics, e.g. water. Consequently, optimum thawing rates are achieved usinga water bath – but because some CPAs exhibit greater toxicity at 37°C compared to, say, 22°C, care mustbe taken not to warm the specimens to too high a temperature. Specimens of larger mass, e.g. cryovials,will benefit more from thawing in a water bath than straws as they require substantially more heat to complete the thawing of the entire specimen.

Is the warming rate the same inside straws and cryovials?

Because the rate of warming of an object is proportional to the temperature differential between it and theenvironment, as well as to its thermal capacity, larger objects of greater radius (in this case, cryovials) willwarm more slowly than straws. In this case the laws of physics create a double-edged sword: while rapidthawing is required for optimum cryosurvival of gametes and embryos cryopreserve using slow freezing protocols, we do not want small objects, which will follow changes in ambient temperature very quickly, towarm up during transfer between freezing machine and storage cryotank. When a cryovial is removed fromcryostorage its contents will thaw more slowly and less uniformly than those of a straw, even if immersedin a 30°C or 37°C water bath. But transferring a 0.25 ml straw from -196°C to 22°C will achieve a warmingrate of 400°C/min [Henry et al., 1993] and its contents will thaw within a very few seconds. Therefore, straws will allow more effective thawing of their contents than will cryovials, but straws are at greater riskof accumulating ice recrystallization damage during transfer into and between cryotanks and during audits.

Why must I take great care when handling frozen specimens?

Cryopreserved material must be kept below the glass transition temperature of water (-132°C) otherwise water recrystallization damage will accumulate with every moment that a frozen specimen is at temperatures higher than this. In addition, specimens with a smaller radius will warm faster, making 0.25 ml straws highly temperature labile, and OPS extremely so – hence specimens stored in either of these devices are very susceptible to recrystallization damage.

What is recrystallization?

During warming, energy is returned to the frozen water molecules, allowing the molecules to resume theirnatural orientation. Very small ice crystals are unstable due to their large surface area:volume ratio, and hence they will fuse together to reduce that ratio. Therefore, ice crystals will grow within the frozen sampleand these larger ice crystals can cause physical damage to the frozen cells.

Is there a special way to wash spermatozoa after thawing?

When a permeating CPA leaves cells during their dilution post-thaw, water enters the cells quickly along theosmotic gradient and hence the cells swell before equilibrium is restored. Because all cells have critical volume limits, if these are exceeded during such volume excursions the cell will suffer irreversible damage. (Onlywith extreme swelling will cells burst, so cells can have been damaged but still be alive and, in the case ofspermatozoa, will still probably be motile.)

Upon thawing, if insemination is to be intra-cervical (or into the uterine cavity with "IUI-ready" spermatozoa) then CPA removal occurs as the spermatozoa migrate from the semen/spermatozoa suspension + CPM mixture into the fluids of the female reproductive tract. However, if the spermatozoa are to be washed in any way, the thawed specimen must be diluted slowly using

stepwise addition of a HEPES-buffered culturemedium (to avoid pH shifts that could occur with a bicarbonate-buffered medium under an air atmosphere). Slowly adding a 10-times volume of medium enables optimum yields using density gradients [Mortimer, 2000].

Cryosurvival

How is cryosurvival defined?

The following definitions of cryosurvival are the measures most commonly used to describe the perceivedsurvival of the various cell types.

- Semen: Percentage return of motility, usually based on assessments of progressive motility. The proportion of (progressively) motile spermatozoa post-thaw is expressed as percentage of the proportion of (progressively) motile spermatozoa before freezing.
- Sperm (washed): The same as for semen.
- Oocytes: The single cell of the oocyte remains intact after thawing. Not all workers include consideration of whether the thawed oocytes are able to undergo fertilization or not, but if fertilization occurs at a lower rate than with fresh oocytes, a proper assessmentof cryosurvival should take this into account.
- Zygotes: The single cell of the zygote remains intact after thawing. Not all workers include consideration of whether the thawed zygotes under successful cleavage at the samerate as fresh zygotes (typically in excess of 95%), but unless a frozen zygote undergoes normal cleavage it cannot be considered to have survived the cryopreservation process.
- Cleaving embryos: Cleaving embryos are generally considered to have survived cryopreservation if at least half their blastomeres remain intact after their return to an appropriate culture medium. Many workers report this rate of cryosurvival as well as the "100% intactblastomere" cryosurvival rate.
- Blastocysts: For a blastocyst to survive cryopreservation it must undergo re-expansion upon its return to an appropriate culture medium.
- Testicular tissue: Since testicular tissue is typically cryopreserved only to obtain live spermatozoa afterthawing, survival is determined only in a qualitative sense according to the finding ofmotile spermatozoa post-thaw.
- Ovarian tissue: Because robust, effective protocols for the use of cryopreserved ovarian tissue are still the subject of extensive research, there are no standard criteria for assessing cryosurvival of this tissue at present.

What is the difference between fertility and fecundity of spermatozoa?

Fertility of a couple is defined as whether they have achieved a pregnancy, while fecundity describes theirchance of achieving a pregnancy per attempt or per unit of time. For spermatozoa, fertility can be considered in terms of whether they are capable of effecting fertilization post-thaw, while their fecundity defines the likelihood of a couple achieving a pregnancy per insemination using the spermatozoa post-thaw.

Do cryopreserved spermatozoa have a lower fecundity/fertility than fresh spermatozoa?

Because the success of a clinical insemination protocol is dependent upon a wide variety of factors this is a difficult question to answer. However, it is generally considered that more motile cryopreserved spermatozoa are required in order to achieve a given fecundity rate than when using fresh spermatozoa. An alternativeconsideration is not to look at a given fecundity rate, but to identify how many motile spermatozoa are required to achieve the plateau of the fecundity curve. The difference in functionality of cryopreserved spermatozoa compared to fresh spermatozoa is considered to reflect changes in other aspects of sperm function beyond simple motility.

Do cryopreserved embryos have a lower implantation rate than fresh ones?

Again because the success of a clinical embryo transfer procedure is dependent upon a wide variety of factors this is a difficult question to answer. Certainly in many IVF centres it seems that cryopreserved embryos show a substantially lower rate of implantation than the centre achieves with fresh embryos. The difference is considered to be due to damage suffered by cryopreserved embryos that cannot be identified visually, but direct comparisons are confounded because the best embryos are typically transferred fresh, although the endometrium might be considered to be more receptive in a frozen transfer cycle when there will have been much lower circulating levels of oestradiol than in the stimulation cycle when the fresh embryos were transferred.

Do straws give a higher cryosurvival of spermatozoa?

While the cryosurvival of human spermatozoa frozen in straws and cryovials appears to be the same in terms of the percent return of motility, there is circumstantial evidence that the fecundity of spermatozoa packaged in straws might be higher than that of spermatozoa packaged in cryovials, although direct experimental or clinical evidence is not available [Mortimer, 2004a].

Do cryovials give a lower cryosurvival of spermatozoa?

While the cryosurvival of human spermatozoa frozen in straws and cryovials appears to be the same in terms of the percent return of motility, there is circumstantial evidence that the fecundity of spermatozoa packaged in cryovials might be lower than that of spermatozoa packaged in straws, although direct experimental or clinical evidence is not available [Mortimer, 2004a].

Storage

At what temperature must I store cryopreserved (or vitrified) spermatozoa/embryos?

All biological material must be stored below the glass transition temperature of water (about -132°C) in order to stop all biological activity [Mazur, 1984]. Even at -80°C in an "ultra-cold" mechanical refrigerator, or at
-79°C on dry ice, degradation accumulates over time.

Of particular relevance to the storage and handling of cryopreserved materials is what happens as water warms from cryogenic storage temperatures. The glass transition temperature of an already frozen aqueous solution is not a sudden event at exactly -132°C; glass transition will occur progressively between this temperature and about -90°C, so that by, say, -80°C there is a great risk of substantial change having occurred. Damage occurs primarily because during warming energy is returned to the system, energy that allows molecules to resume their natural orientation. Very small ice crystals are unstable due to their large surface area:volume ratio, and hence they will fuse together to reduce that ratio.

Consequently, cryopreserved material must be kept below -132°C, and hence storage temperatures of -150°C (the often reported temperature of LNV) or -196°C (the temperature of LN2) are employed. Clearly the lower the temperature the greater the margin of safety when a specimen is removed briefly to check its identity. This is also why it is better to run a controlled rate freezer to as low a temperature as possible before removing the specimens and transferring them into cryogenic storage: it will reduce the extent of glass transition-induced damage during the brief exposure to warmer temperatures during the transfer.

What is the "glass transition temperature" of water?

The glass transition temperature of water is the temperature at which water that was super-cooled and entered the "glass" state will begin to form a crystal lattice, even within the frozen state. This temperature is -132°C, and the transition occurs gradually at temperatures between this value and the melting point of the solution. Consequently, cryopreserved specimens must be kept below this temperature in order to maintain maximum cryosurvival.

Why can't I use a -80°C mechanical freezer for storage?

Because -80°C is above the glass transition temperature of water (-132°C), cells are not properly cryopreserved at that temperature. Human spermatozoa stored in a mechanical freezer at -70°C showed

lower cryosurvival than did parallel samples stored at -196°C, a detrimental effect that was greater after 3months of storage compared to 7 days [Trummer et al., 1998].

There are some mechanical cryogenic freezers that achieve storage temperatures below -132°C (e.g. UltimaII Series from Revco, Asheville, NC, USA) although they are very expensive to operate, typically requiring inexcess of 4 kW [Burden, 1999]. Another important consideration is that, unlike systems employing LN2 asthe cryogenic refrigerant, there is little security with these systems if the mains power fails without a substantial emergency generator system (many models also require 3-phase power).

Should I use LN2 or vapour phase (LNV) storage?

Cryopreserved material must be kept below -132°C, the glass transition temperature of water, and hencestorage temperatures of -150°C (the generally reported temperature of LNV) or -196°C (the temperature of LN2) are used for cryopreserved (or vitrified) specimens. The lower the temperature the greater the margin of safety when a specimen is removed briefly to check its identity.

Subsequent to the report that cross-infection of bone marrow and blood stem cells had occurred via the LN2in which they were being stored [Tedder et al., 1995] there were calls for storage in LN2 to be abandonedin favour of storage in the vapour or gaseous phase either above a layer of LN2 (LNV storage) or in newerdesign freezers that enclosed the cryogenic LN2 inside an outer vessel so that cold was transferred eitherthrough the walls of the secondary vessel or via of heat shunt devices ("cold fingers") to minimize temperature gradients. In these latter systems, specimens are actually stored in super-cold air. A major concern is that both LNV or super-cold air have very low thermal capacity and poor heat transfer rates, as a consequence of which they heat up rapidly in the presence of a "warm" object, even ambient air.

While LNV storage does go a long way to reducing the risks of cross-contamination via LN2, especially with leaky specimen packaging, it is not an absolute solution because pathogens have been isolated from LNV [Fountain et al., 1997] and presumably the same will be true of the super-cold air systems for organisms that are frozen out of the air that enters the storage chamber every time the lid is opened.

What is "isothermal vapour storage"?

This system uses a new type of cryogenic storage freezer where the cryogenic LN2 in enclosed in a secondary vessel so that cold is transferred through the walls of the vessel, sometimes with the aid of heat shunt devices ("cold fingers") to minimize temperature gradients, and material is therefore stored in what is, in reality, super-cold air (e.g. Isothermal Vapor Storage or "IVS" models from Custom BioGenic Systems, Shelby Township, MI, USA).

Why should I monitor the LN2 level in my cryotanks?

If the vacuum insulation of a cryogenic storage tank or Dewar vessel starts to be lost, e.g. due to a minute leak in the seal or a defect in the metal of the inner or outer shell of the tank, its insulation capacity will reduce and the amount of LN2 that is lost by ongoing evaporation will increase. It is therefore possible to gain early warning of a tank's impending failure if it starts to consume more LN2 per unit time, a trend thatwill also likely increase with time. Consequently, the LN2 level in each tank should be monitored, and recorded, regularly and any changes in the amount lost noted. Allowance must, of course, be made for anyunusual activity involving the tank during any given observation period, e.g. an unusual number of times the tank is accessed, perhaps for an audit of specimens. Monitoring the amount of LN2 lost is easier than noting the amount that needs to be added to top-up a tank since it is difficult to measure, and impossible for tanks on "auto-fill".

How can I monitor the LN2 level in my cryotanks?

All cryogenic storage tank suppliers provide, or sell, a plastic measuring scale – a long plastic ruler. Insertthe warm measuring scale into the tank and wait for the bubbling sound to stop (i.e. the scale has cooledto -196°C). Remove the scale and watch the formation of frost on its surface (due to freezing out of moisture in the air) – if the room is very dry then blowing gently onto the scale will provide moisture to create the frost. Simply note the measurement where the frost stops and that is the depth of LN2 in the tank. Recordthis measurement in a log book and plot it on a graph.

How do I know if one of my cryotanks is developing a problem/failing?

The level of LN2 measured on a regular basis will start to decrease over time; an effect that will, in all likelihood, also increase with time.

What is a "catastrophic failure" of a cryotank?

A catastrophic failure is one where there is no warning that a tank's vacuum insulation is starting to fail, e.g. caused by sudden damage to the "nipple" where the air was removed from the tank wall during manufacture (this is usually protected under a welded-on cover on the upper shoulder of the tank), or by severe damage to the wall of a tank caused by collision with a hard/sharp object. In the most extreme case the loss of vacuum is so sudden that the LN2 inside the tank will boil very quickly and likely eject some of the LN2 and the tank's contents out through the neck of the tank. If this happens, do not try and save anything, there is a severe danger of oxygen depletion in the room which can result in asphyxiation.

What is an "auto-fill" system?

Some larger designs of cryogenic storage tanks can be attached to a large LN2 supply tank or manifold (which must itself be vacuum insulated) and have a sensor installed in the storage tank that triggers the opening of a solenoid valve when the LN2 level in the tank falls below a predetermined limit. LN2 then enters the tank until the LN2 level reaches another sensor that then closes the solenoid valve. These systems ensure that the LN2 level in a storage tank does not fall below a pre-set safety limit. [See also Should I have low level alarms on my cryotanks?]

What are the benefits of "auto-fill" systems?

The user can be assured that the LN2 level in such cryogenic storage tanks will never fall below a pre-set safety limit. Also, there is no need to manually transfer LN2 from a supply tank into the storage tank, a task that can be physically demanding. [See also Should I have low level alarms on my cryotanks?]

What are the risks of "auto-fill" systems?

If the low level sensor were to fail then the auto-fill system would not open the solenoid valve and the LN2 level would continue to fall due to evaporation. If the high level sensor were to fail then the auto-fill system would continue allowing LN2 into the tank until the supply tank was empty. This would cause the tank to overflow and likely cause damage to the laboratory, as well as a severe oxygen depletion risk. [See also Should I have low level alarms on my cryotanks?]

Should I have low level alarms on my cryotanks?

Yes, in all cases. Even if a system has built-in sensors as part of an auto-fill system, an independent low level sensor and alarm should be used to protect against malfunctioning of the auto-fill system. [See also What are the risks of an auto-fill system?]

What is the "static holding time" of a cryotank?

The static holding time of a cryogenic storage tank or Dewar vessel is the period of time that a tank which was filled with LN2 will maintain a safe storage temperature, assuming that the tank is not opened, and that samples are neither put into, nor taken out of the tank. Obviously this is a "theoretical" operational time limit, but it does give some idea as to the typical operating performance of a tank. Note that when a tank is used as a vapour storage vessel, its static holding time will be much less than if it was used as a liquid storage vessel.

Do cryotanks have a maximum useful life?

There is no simple answer to this question. Many cryogenic storage tanks continue to function perfectly well for many years, while others fail perhaps after only a few months. Some manufacturers provide a certain guarantee period – but this does not mean that their tanks will never fail within this period, just that they will replace one that does. Also note that any such guarantee will almost certainly contain a clause that excludes any liability for the samples that were stored in the tank.

Why should I keep a spare empty cryotank available?

If a cryogenic storage tank starts to fail, the time before it ceases to be useful cannot be predicted. Theproblem might continue at a minimal level for many weeks or months, but it might deteriorate at an unpredictably slow or rapid rate. Therefore, as soon as a fault is discovered all the samples in the affectedtank should be removed to a new storage tank as soon as possible. But the order/delivery time for a new tank could exceed the safe working limit for the failing tank. Consequently, proper risk management of a cryobank requires that a spare tank, equal in capacity to the largest tank in current use, should be maintained cold and ready for immediate use.

Cryobanking

What information should I use to identify cryobanked specimens?

There is no specific answer to this, and requirements might be included in licensing regulations or accreditation schemes that govern the operation of different cryobanks. However, good laboratory practicewould require the use of at least two, and preferably three, unique identifiers that permit the unambiguousidentification of every single straw or cryovial in storage.

How can I put identifying information on cryobanked specimens?

Depending on the material of the straws or cryovials being used, and the nature of the marking pen (i.e. the solvent used in its ink), information could be written directly onto the straw or cryovial. Various types of labels have been used over the years, but before any label is used it must be checked for resistance to extreme cold under normal conditions of use (as must the permanence of the inks). If labels are wrapped around straws then tests must be performed to ensure that the labels will not become detached at -196°C, or even unwrapped, as that would complicate removal of straws from the inventory system. Using labels as "flags" on straws is not recommended as these flags become very fragile at -196°C and can be broken off during careless handling of straws.

With CBS™ High Security Straws there are two very safe options for labelling: (1) labels on which identifying information can be written or printed can be wrapped around ID rods and inserted into the upper compartment of the 2-compartment straws before sealing them; and (2) the information can be written or printed on ID sleeves that fit outside the straw but cannot be removed after sealing due to the extra widthof the flat "tab" created by the welded seal.

How safe are labels on straws/cryovials when immersed in LN2?

So long as any particular type of label is known not to detach from straws (or cryovials) over time at -196°C then they can be deemed safe. The cryogenic storage labels supplied by Brady Labelling Systems (www.bradyid.com) are guaranteed to remain attached during immersion in LN2.

Are there special labels I should use when storing in LN2/LNV?

The cryogenic storage labels supplied by Brady Labelling Systems (www.bradyid.com) are guaranteed to remain attached during immersion in LN2. However, so long as any particular type of label is known not to detach from straws (or cryovials) over time at -96°C then they can be deemed safe. Likewise the ink to be used must also be checked for permanence under the same conditions.

Are there special pens that I should use when writing on straws/cryovials?

Various companies sell "cryo pens", whose ink is designed to be indelible at -196°C, and which are intendedfor writing on cryovials (and straws – but remember to check the material for permeability to the solvent used in the ink).

What is an "inventory system"?

This term is used to describe the physical system of racks & boxes, canisters & canes or goblets, etc, thatare used inside a cryogenic storage tank to organize the samples that are stored there.

How do I decide which inventory system to use?

This is partially dependent on the type of packaging being used for your samples (e.g. cryovials can be stored on canes in canisters, or in drawers in special racks; straws are either stored on canes or in visotubes insidegoblets within canisters) and is, to a large extent, a matter of user preference.

What is the purpose of a quarantine tank?

Some cryobanks will not put samples into their main storage tanks until the person(s) from whom they came are known to not carry serious infectious diseases such as hepatitis or AIDS. In this case, the samples areheld in smaller tanks awaiting the results of laboratory tests, which are often repeated after a period of 3 to6 months to allow time for seroconversion in case the person was infected soon before cryobanking their samples. Once the samples are known to be "clear" they are moved into the main cryobank. But this practice has been called into question because of the often illogical classification of a sample as "clear" if itwere to have been kept in a quarantine tank where another sample were found to have come from an infected individual. Given our current knowledge about the risks of cross-contamination during cryostorage, and especially the risks of contamination of samples by organisms in LN2, the use of quarantine tanks except on a strict individual basis, must be questioned.

What is the principle of "universal contamination"?

This concept came into use in Australia in the mid-/late-1990s in recognition of the risks of cross-contamination during cryostorage, and especially the risks of contamination of samples by organismsin LN2. Basically, the assumption is that all samples stored in a cryobank are potentially infectious, and all samples are potentially contaminated on their outside. Therefore, the expectations are that all samples will be packaged carefully and sealed properly, and will be disinfected after removal from the cryotank beforebreaching the seal or wall of the packaging.

What is "divided storage"?

As a consequence of a thorough risk assessment of the long-term security of their cryostorage services, some cryobanks have elected to divide specimens between two or more separate storage tanks. A further refinement is where part of a specimen is sent to an entirely different site, as a precaution against fire, earthquake, etc. While this might seem excessive, for those living in high risk areas such as near to the SanAndreas fault in California, or perhaps somewhere with a high risk of terrorist activities, it would seem a wise precaution.

Are there any risks associated with auditing a cryobank?

Extreme care must be taken whenever a cryopreserved specimen is handled, not just from the perspective of the safety of the operator, but to protect the specimen from damage that will accumulate, irreversibly, whenever the temperature of even part of it exceeds -132°C [Simione, 1999]. This problem is greatest for material frozen in straws, especially the older 0.25-ml IMV straws.

Efficient and safe auditing of a cryobank relies upon the following factors:

- An inventory system that allows for easy and quick access to specimens within the cryogenic storage tanks.
- Unambiguous (and secure) labelling systems that facilitate the rapid and accurate identification of eachspecimen.
- Skilled staff who can handle material at cryogenic temperatures quickly, safely and securely. This is probably the biggest single area of weakness in the performance of any audit.
- Proper maintenance of records, either in paper or electronic form, so that the records do reconcile withthe Therefore, it is recommended that a risk assessment must be undertaken to determine the balancebetween the potential deterioration of cryopreserved material during handling against the likelihood of identifying a discrepancy between the cryobank records and a tank's actual contents. If a cryobank candemonstrate that it (a) has accurate records and (b) its standard operating procedures minimize all opportunities for discrepancies to occur, then the negative risk of potential cryodamage will likely outweigh any positive "verification" benefit and the "need" for an audit should be rejected. Obviously each cryobank must undertake its own risk assessment on this question and establish its own level of confidence.

Shipping

What is a "dry shipper"?

A "dry" shipper is a cryogenic storage tank that, instead of being entirely empty inside, has space for a single canister at its core with the surrounding space inside the vessel being filled by an absorbent material that takes up the LN2 refrigerant and prevents it from running out if the tank is tipped onto its side. If properly filled, i.e. without any free LN2 that can run out, these tanks are considered non-hazardous by IATA for the transportation of cryogenically frozen material.

How do I fill/prepare a dry shipper?

Because it takes a long time for the LN2 to be absorbed into the material inside the tank, preparing a dry shipper properly from ambient temperature is a long process. The process involves adding small amounts of LN2 to the dry shipper and waiting for the inside of the tank to equilibrate. The process cannot be speeded up or curtailed. Attempting to fill a dry shipper too rapidly can cause irreversible damage that will likely shorten its useful life, if not ruin the tank. Before samples are loaded into a dry shipper it must be topped up a last time with LN2 and then, immediately prior to loading the specimens to be transported, any remaining free liquid is poured out of the dry shipper so that it is "dry".

Is it better to keep my dry shipper cold all the time?

Because it takes so long to prepare a dry shipper tank from warm, and keeping one cold requires very little LN2, it is best to always keep dry shipper tanks cold. A simple solution is to top them up regularly with LN2 so that the tank, while being empty of samples, is full of LN2. Then, when it is required for shipping samples, any remaining free liquid can be poured out of the dry shipper so that it is "dry", and the samples then loaded into it for transportation.

Why can't I ship samples on dry ice?

Because -79°C is above the glass transition temperature of water (-132°C), cells are not properly cryopreserved at that temperature. Human spermatozoa stored at -79°C showed a progressive decline in post-thaw motility, an effect that was accelerated at storage temperatures above -75°C [Ackerman, 1968].

Biocontainment

What is "biocontainment"?

Biocontainment is the storage of biological specimens so that they neither contaminate their surroundings nor can they be contaminated by their surroundings. Clearly it is important that cryobanked specimens are protected from contamination by either other specimens stored alongside them ("cross-contamination") or by the cryogenic refrigerant (i.e. LN2, LNV or super-cold air).

What is the risk of cross-contamination during storage in LN2?

In 1995 six cases of acute Hepatitis B virus (HBV) infection occurred among patients undergoing cytotoxic treatment (Tedder et al., 1995). All had been HBV negative at the start of treatment and subsequent investigations revealed that the only common factor linking the patients, who had all been treated at different times and/or locations, was that the harvested bone marrow or peripheral blood stem cells had been stored in the same cryogenic tank. The source of the contamination was found to be due to splitting of the heat seals of the blood bags in which the cells had been frozen and stored in LN2, allowing entry of LN2 into the bags. Although this was at the time, and still is today, the only reported case of cross-infection via LN2 storage, it has caused enormous concern and substantial expense for many organizations.

There is no direct evidence of any cross-contamination in a cryobank within a fertility clinic or sperm bank setting (Kuleshova & Shaw, 2000; Tomlinson & Sakkas, 2000; Centola, 2002) and most experts consider the risk to be extremely small. While the risk of cross-contamination is certainly unquantifiable, rendering robust risk analysis to ascertain its real likelihood impossible, it cannot be ignored. The risk cannot be presumed to be theoretical, and all workers involved in human gamete and embryo cryobanking must understand its origins and be able to take every available practical step to minimize the risk in their banks.

Why do straws leak?

This can be due to either a faulty seal, or to the expulsion of a seal during freezing. Proper sealing of straws is essential [see How do I seal straws?] because as the air inside the straw contracts during cooling it willcreate negative pressure that will suck LN2 into the air space through a faulty seal. Also, an air space mustbe left inside a straw to allow for expansion of the semen+CPM mixture as it cools (since water has its maximum volume at +4°C) – without the air space the plug can be ejected as the column of semen+CPMexpands, thereby breaching the containment of the straw.

Why do straws break?

Traditional straws made from PVC or PETG are very fragile at -196°C and if they are subjected to any lateralmechanical stress they will very likely snap. However, CBS™ straws are made from an ionomeric resin that is not fragile at -196°C and, indeed, such straws can even be bent immediately after removing from LN2.

Why should I discard broken straws?

Because broken straws have been exposed to the LN2 cryogenic refrigerant their contents must be considered contaminated by the organisms that are known to be present in LN2 or LNV [Fountain et al., 1997]. Also see Why is liquid nitrogen contaminated?

Why do cryovials leak?

When cryovials are immersed in LN2, the air space inside them is under greatly reduced pressure due to contraction of the cooled air and LN2 will be drawn into the air space if there is a faulty seal. Serious concerns have been expressed about the frequently incorrect use of cryovials [Byers, 1998]. Even Nunc CryoTube® cryovials with internal threads and a silicone gasket, which achieve the best possible seal, should not be stored under "extreme" conditions immersed in LN2 unless they are protected by a secondary containment system such as CryoFlex tubing [Nalge Nunc International, 1998]. Experimental evidence hasconfirmed that cryovials both with and without an O-ring have a high propensity to allow entry of LN2 evenduring a short 3 h immersion [Clarke, 1999].

What is "secondary containment"?

Secondary containment is the application of a second layer of sealing material around a straw or cryovial tohelp protect the material inside from being exposed to the cryogenic refrigerant, or vice versa. [See Whatcan I use for secondary containment?] Secondary containment is considered essential for cryovials that are to be stored under "extreme" conditions (i.e. immersed in LN2), an opinion that comes from their manufacturer, Nunc (Nunc Nalgene International, 1998). However, secondary containment is not required for CBS™ High Security Straws and CBS™ High Security Tubes that have been sealed properly using the SYMS/SYMS III welder.

What can I use for secondary containment?

For straws there is no effective secondary containment other than sealing small straws inside larger ones. But the air gap between the two walls creates a major problem for effective cooling of the specimens. Because CryoFlex tubing hinders the secure attachment of cryovials to canes, very few banks use it routinely and a UK survey revealed that no sperm banks or IVF Centres were using secondary containment even though many used cryovials [Wood, 1999]. An alternative secondary sealing method using a product calledNescofilm has also been reported (Bahadur & Tedder, 1997).

Must I use secondary containment with cryovials?

According to their manufacturers, Nunc CryoTube® vials that are to be stored under "extreme" conditions,
i.e. immersed in LN2, must be must be correctly sealed in Nunc CryoFlex™ tubing (Nunc Nalgene International, 1998).

Are there any problems or difficulties with using secondary containment?

Many workers have reported that CryoFlex tubing hinders the secure attachment of cryovials to canes, veryfew banks use it routinely and a UK survey revealed that no sperm banks or IVF Centres were using secondary containment even though many used cryovials [Wood, 1999].

Why is liquid nitrogen contaminated?

There are various mechanisms by which LN2 might become contaminated with (pathogenic) organisms,including:

1) From semen contaminating the outside of the packaging unit, particularly straws [Russell et al., 1997].
2) From split or broken straws.
3) By removing LN2 from a contaminated cryotank to handle units being frozen (e.g. for seeding) or while being transferred from the freezing machine to the cryobank, between cryotanks within the bank, or tofill a dry shipper. This is very poor practice, especially since all cryotanks should be considered as likelybeing contaminated by pathogens [e.g. Rall, 2003].
4) From room air or the exhaled breath of operators. This is what causes the "fogging" when a cryotank is opened, and hence constitutes the major source of the material that forms the "sludge" at the bottom ofcryotanks.
5) By skin commensals from operators while leaning over an opened cryotank.
6) At point of manufacture.
7) From frozen material out of imperfectly sealed cryovials or straws.
8) Directly through the wall of intact, properly sealed plastic straws, although there is no evidence for thismechanism.
9) By LN2 that escapes from an imperfectly sealed cryovial or straw that had been contaminated by the material stored in that cryovial or straw. There is no physical reason why this would happen and available evidence does not support such an event.

Can I sterilize liquid nitrogen?

Suggestions that sterile LN2 can be created either by ultrafiltration or u.v. irradiation [Vajta et al., 1998b;Kuleshova & Shaw, 2000] have not been demonstrated experimentally. Therefore it is best to assume thatLN2 will have likely been contaminated during its manufacture [Rall, 2003].

What is the white sludge at the bottom of my cryotanks?

This is mostly ice, formed when water vapour in the air inside the neck of the cryotank freezes. A major source of such humidity is the exhaled air of operators working over or near the tank. It can also includesome of the cotton wadding from the upper plug of PVA-sealed straws [see What is the white substance that "puffs" out of straws when they're removed from liquid nitrogen?]. It will also typically contain a wide variety of micro-organisms, both non-pathogenic and pathogenic [Fountain et al., 1997].

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