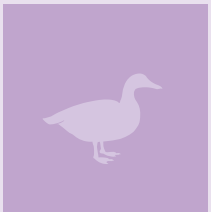
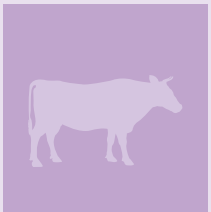
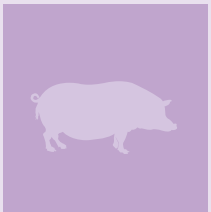
SECTION 7

Basic principles of cryopreservation

Basic principles of cryopreservation

Spermatozoa were the first mammalian cells to be cryopreserved successfully (Polge *et al.,* 1949). This success was due to the serendipitous discovery by Polge and co-workers of the cryoprotective effect of glycerol. Since then, many methods have been developed for various types of cells, tissues and organs. Much progress in the field has come from empirical work as well as from fundamental cryobiology. Increased understanding of the causes of cryo-injury has continually helped to improve cryopreservation methods. Research into fundamental cryobiology has provided the basis for new cryopreservation methods such as vitrification.

The two most commonly used cryopreservation methods for animal germplasm are slow-freezing and vitrification. These are quite different methods, but relate to the same physico-chemical relationships. The differences between the two can be explained by first describing what happens during slow freezing.

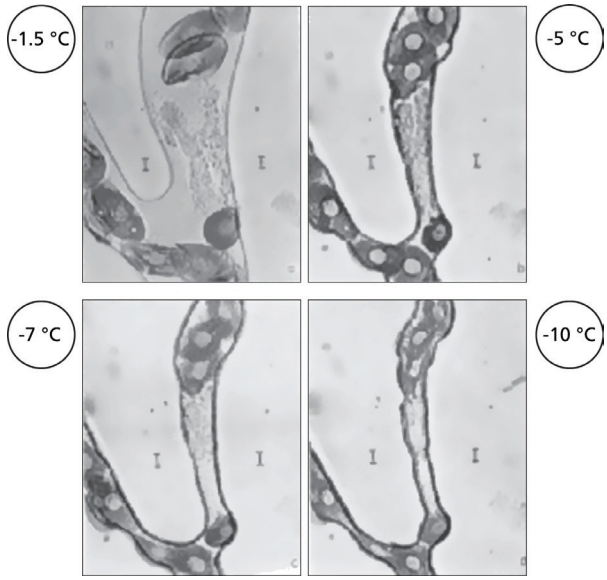
SLOW FREEZING

In slow-freezing, cells in a medium are cooled to below freezing point. At some stage, ice masses containing pure crystalline water will form. What remains between the growing ice masses is the so-called unfrozen fraction, in which all cells and all solutes are confined (see Figure 7). The concentrations of sugars, salts and cryoprotectant (e.g. glycerol) increase, while the volume of the unfrozen fraction decreases. The increase in osmotic strength causes an efflux of water from the cells. Slow cooling is needed in order to allow sufficient efflux of water to minimize the chance of intracellular ice formation. As cooling continues, the viscosity of the unfrozen fraction ultimately becomes too high for any further crystallization. The remaining unfrozen fraction turns into an amorphous solid that contains no ice crystals.

Chilling injury and cold shock

The first challenge in cryopreserving cells from homeotherm (warm-blooded) animals is in cooling the cells below body temperature. Cells may be damaged by very rapid cooling (cold shock) or be damaged by low temperature *per se* (chilling injury). Behaviour and function of membrane lipids and proteins may be affected by temperature. For example, membrane lipids that are normally in a liquid crystalline state may solidify at non-physiological temperatures, which can change their function and begin processes such as cryocapacitation of the production of reactive oxygen species that increase damage to membranes. Decreasing the temperature may cause an imbalance in cellular processes because the rate of one process may be affected more strongly than that of another. One example is the disintegration of the metaphase spindle of oocytes caused by a change in the dynamic equilibrium of the association/dissociation of the tubulin filaments.

FIGURE 7
Frog erythrocytes in the "unfrozen fraction", which is enclosed by growing masses of ice



Source: Rapatz and Luyet (1960).

Supercooling

In slow-freezing methods cells are brought into a suitable freezing medium and cooling is continued below the freezing point of the medium. Ice formation does not necessarily start at the freezing point. Small ice crystals have a lower melting/freezing point than "bulk" ice, due to their large surface tension. Spontaneous ice nucleation will in most cases occur after the solution is supercooled to a temperature between -5 and -15 °C. Thereafter, ice will grow rapidly in all directions, and the release of the latent heat of fusion will cause the sample to warm up abruptly until the freezing/melting temperature of the solution (i.e. of the remaining unfrozen fraction) is reached. At this point, the ice formation will stop, or will proceed at a rate governed by the rate at which the heat of fusion is transported from the sample. Finally, the sample can "catch up" again with the lower temperature in the freezing apparatus. From a practical perspective, this means that the cells undergoing cryopreservation in a typical semen straw have to withstand a series of large and abrupt temperature changes.

Conditions in the unfrozen fraction

Cells are faced with very high concentrations of solutes in the unfrozen fraction. Dehydration and high salt concentration may result in loss of stability in the membranes or denaturation of proteins (Tanford, 1980; Crowe and Crowe, 1984; Hvidt and Westh, 1992; Lovelock, 1953). Moreover, high salt concentrations may cause extracellular salts to enter the cells, a process known as "solute loading" (Daw *et al.,* 1973; Griffiths *et al.,* 1979).

The fast efflux of water causes a rapid decrease in the volume of the cells to approximately 50 percent of their original volume. This leads to structural deformation of the cells. Further mechanical stress may be caused by cells being confined in very narrow channels of unfrozen solution and squeezed between growing masses of ice (Rapatz and Luyet, 1960).

The influence of cryoprotectants

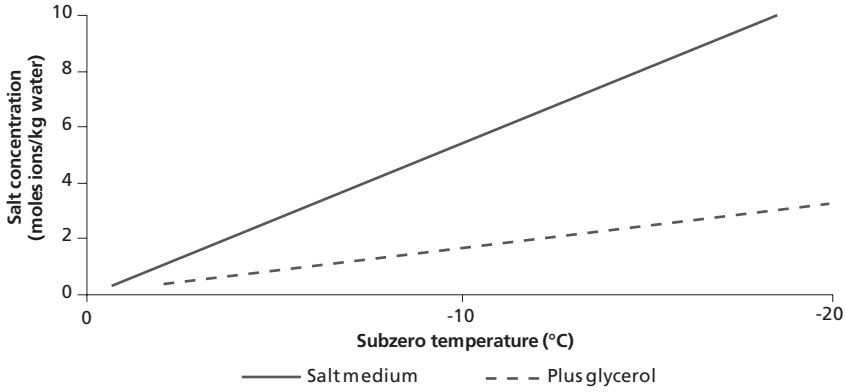
At all practical cooling rates, the total solute concentration (which is measured in moles per kg water) is determined only by the subzero temperature (Figure 8). When the initial freezing medium contains only salts (electrolytes), salt concentrations in the unfrozen fraction will reach extremely high levels as the temperature decreases. In contrast, in a medium that contains a large proportion of non-electrolytes, the total solute concentration at each subzero temperature will be the same as that found at the equivalent temperature in a medium containing only salts; however, the salt concentration will be much lower.

Sugars can be used as non-electrolyte solutes, but they will only affect the extracellular salt concentration. Moreover, high concentrations of impermeable solutes impose osmotic stress on the cells already before freezing. This is much less the case when a membrane permeable solute, such as glycerol, is used rather than a non-permeable solute. When cells are brought into a hypertonic glycerol medium, water will leave the cells because of the osmotic pressure difference. However, at the same time, glycerol will enter the cells. After a short period of equilibration, the cells will have regained their original volume. The osmotic stress imposed by a hypertonic glycerol solution is therefore much smaller than that imposed by a hypertonic sugar solution. Hence, glycerol can be used at greater concentrations than sugars without damaging the cells. A substantial initial glycerol concentration in the medium means that part of the extracellular and intracellular water is replaced by the glycerol. Hence, the amount of ice formed is lower, the unfrozen fraction remains larger, the degree of shrinkage of the cells is limited, and the electrolyte concentration in the unfrozen solution and in the cells will be relatively small (see Figure 8). The mechanisms through which other membrane permeable substances, such as ethylene glycol and dimethyl-sulfoxide (DMSO), provide cryoprotection are similar to those involving glycerol.

There are additional mechanisms through which polyols, such as like glycerol and several sugars, provide cryoprotection. These substances can stabilize lipid membranes by hydrogen bonding with the polar head groups of membrane lipids (Crowe and Crowe 1984; Crowe *et al.,* 1985), which is especially important under severely dehydrated conditions. In addition, these substances may affect the mechanical properties of the unfrozen fraction, especially its viscosity and glass-forming tendency.

The degree to which cells shrink and re-swell after addition of a membrane-permeable cryoprotectant depends on the concentration of the cryoprotectant and the relative permeability of the membrane to water and to the cryoprotectant (Kleinhans, 1998). For instance, bull sperm shrink very little when brought into a freezing medium with glycerol (Chaveiro *et al.,* 2006), whereas bovine embryos react much more strongly. Upon thawing, removal of the cryoprotectant has the opposite effect on cells: they first swell and then they shrink again. This may lead to damage if the cells expand too much. Damage due to over-swelling of cells can be prevented by stepwise removal of the cryoprotectant.

FIGURE 8
Effect of subzero temperature on salt concentration in the presence and absence of glycerol



Note: The total solute concentration (salts plus non-electrolytes) is a function of subzero temperature. Thus, the presence of non-electrolytes, such as glycerol, results in a lower salt concentration in the unfrozen fraction and inside the cells (Mazur and Rigopoulos 1983).

The influence of cooling rate

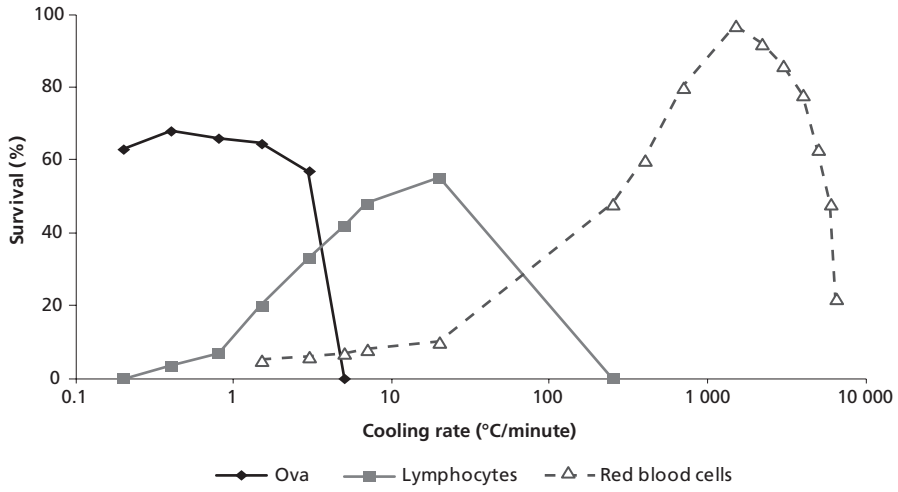
A general observation in the cryopreservation of cells and other biological systems is that each system has a specific optimal cooling rate, with decreased survival at cooling rates that are too low (slow-cooling damage) or too high (fast-cooling damage) (Mazur *et al.,* 1972).

Ice growth is a rapid process, but transport of water through the cell membrane is relatively slow, because the membrane acts as a resistance barrier. Therefore, as cooling and extracellular ice growth continue, the liquid water of the unfrozen fraction remains very close to equilibrium with the ice, but the intracellular water lags behind. This means that the water concentration (i.e. the chemical potential of water) is too high for thermodynamic equilibrium, and there may be a risk of intracellular ice formation.

The optimal cooling rate falls in a range that is neither too fast nor too slow. When cells are cooled very slowly, the intracellular water lags behind only a little, and the risk of intracellular ice formation is minimal. However, it also means that the dehydration of the cells is maximal, which is not desired. At higher cooling rates, intracellular dehydration, intracellular solute concentration and shrinkage of the cells is less excessive. Moreover, the cells are exposed to the unfavourable conditions for a shorter period of time. However, when cooling rates are increased too much, the dehydration may not be fast enough to prevent intracellular ice nucleation (Mazur, 1963, 1985; Mazur *et al.,* 1972). Fast-cooling damage can also be caused by other factors. For instance, it has been proposed that rapid water flow through membrane pores could lead to an uneven distribution of pressure on the membrane (Muldrew and McGann, 1993, 1994). Fast-cooling damage could also result from the very sudden changes in size, shape and ultrastructure, caused by the rapid efflux of water (Woelders *et al.,* 1997).

Different cells or other biological materials (embryos, tissue pieces) may have different optimal cooling rates. The optimal cooling rate of cells is largely determined by their volume

FIGURE 9
Effect of cooling rate on survival of different kinds of cells



Note: Cells may have a specific optimal cooling rate, i.e. they have a lower survival rate at cooling rates that are too low (slow-cooling damage) and at a cooling rates that are too high (fast-cooling damage) (Mazur, 1985).

and their membrane surface area (volume to surface area ratio), and by the permeability of the membrane to water and to cryoprotectant (see Figure 9).

Interactions of cooling rate with thawing rate and cryoprotectant concentration

The optimal cooling rate may depend on various other factors, such as the cryoprotectant concentration and the thawing rate. It has been observed in semen from a number of species that the combination of fast cooling and slow thawing is particularly damaging to the cells. (Rodriguez *et al.,* 1975; Fiser, 1991; Henry *et al.,* 1993; Woelders and Malva 1998). If intracellular ice nucleation occurs at a low temperature and cooling proceeds rapidly, it may be that the cytoplasm turns into glass before the intracellular ice crystals grow to a significant size, thus causing only sublethal, or no, damage. During slow thawing, the small crystals can grow and subsequently damage the cells (Rall *et al.,*1984). In addition, cells may be damaged by extracellular restructuring of ice masses, a process known as "recrystallization" (Bank, 1973).

Programmable and non-programmable freezers

Biological material can either be frozen using quite simple, non-programmable, freezers or using more sophisticated, programmable, freezers (see Figure 10). Although programmable freezers are more expensive, they do not necessarily yield more satisfactory results, especially for experienced technicians and cryobiologists. Therefore, the choice between

FIGURE 10
Example of a programmable freezer

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Note: the freezer is about 1 metre in height.

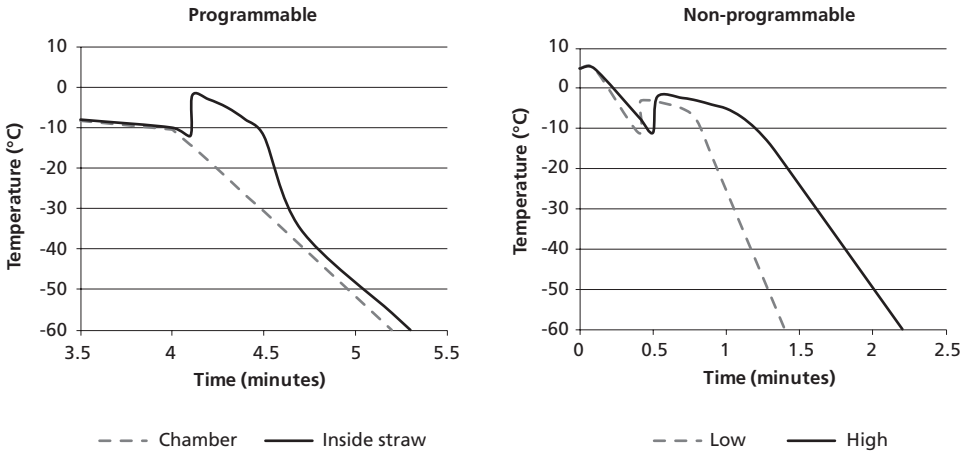
programmable and non-programmable systems will depend on the financial resources available and the experience of the technicians. In some cases, even the most experienced technicians prefer the operating simplicity of programmable models.

In most programmable freezers, the straws or vials are cooled by cold nitrogen vapour. The temperature inside the cooling chamber can be accurately controlled and the time course of the temperature can be programmed. However, the time course of temperature inside the straws may be different due to the generation of heat of fusion (Figure 11).

In non-programmable freezers, the straws may be cooled by being exposed to vapour (or a cold surface) at a constant low temperature. An example of a simple system is the freezing of straws placed on a rack in a Styrofoam box partially filled with liquid nitrogen without ventilation. The height of the straws above the liquid nitrogen determines the rate of heat exchange. Alternatively, straws can be placed on a piece of Styrofoam that floats on the liquid nitrogen (e.g. Dong *et al.,* 2009). The thickness of the Styrofoam piece determines the rate of heat exchange.

Generally in such systems, the rate of heat exchange is governed by the temperature difference between the inside and the outside of the straw, and by the extent of heat conduction. The latter depends strongly on the volume to surface ratio of the straw or vial and the rate of (forced) ventilation. Therefore, it is difficult to compare one type of non-programmable freezer with another, or to know the actual freezing rate obtained with any given non-programmable apparatus. Optimal conditions have to be determined by experimentation.

FIGURE 11
Examples of freezing curves in programmable and non-programmable freezers



Left panel: A typical freezing programme in a programmable freezer (dotted line) and the corresponding freezing curve measured inside the straw. Note that the temperature inside the straw doesn't necessarily follow the programmed chamber temperature and that in parts of the curve the cooling rate may be higher than anticipated.
Right panel: In a non-programmable freezer (Styrofoam box), the constant temperature outside the straw results in sigmoidal freezing curves. The overall steepness of the freezing curve can be changed by choosing a different vapour temperature, for example, by changing the height of the straws above the liquid nitrogen (Woelders and Zuidberg, personal communication).

Non-programmable systems do, however, have an advantage. The cooling curve (the time course of cooling and freezing) is, by default, of the form theoretically predicted to be optimal for slow freezing (Woelders and Chaveiro, 2004), with relatively low cooling rates directly after ice formation begins and higher cooling rates later. The bulk of the ice formation happens in the temperature range between the freezing point and -10 °C, and consequently most of the water efflux from the cells must also take place in this temperature range. Thus, the heat of fusion liberated during ice formation slows the cooling exactly at the point when cells need extra time to export intracellular water. The overall steepness of the freezing curve can be adjusted in non-programmable systems by choosing the height of the straws above the liquid nitrogen, which is proportional to the temperature of the vapour around the straws (Figure 11). In more sophisticated systems with forced ventilation and adjustable preset vapour temperatures, the rate of heat exchange can be adjusted by choosing the preset vapour temperature.

VITRIFICATION

The term "vitrification" refers to any process resulting in "glass formation", the transformation from a liquid to a solid in the absence of crystallization. According to this definition, cells that are properly slow frozen become "vitrified".

If, in slow-cooling methods, cells ultimately become vitrified, how do so-called vitrification methods differ? Vitrification methods involve the use of a medium that has a very high solute concentration to begin with. Thus, ice cannot form in any part of the sample. As no ice forms, cooling does not have to be slow. In fact, it may be beneficial to cool very rapidly. The vitrified state and the associated physico-chemical conditions obtained using vitrification methods, are to some extent similar to those obtained by slow cooling, but the way of reaching this point is quite different.

Chilling injury and cold shock

As in the case of slow-freezing methods, vitrification methods can damage cells or tissues through cold shock and chilling injury. Depending on the material and the protocol used, however, cells or tissues may be rapidly cooled from a temperature at which chilling injury and cold shock play no role (e.g. room temperature). Extremely high rates of cooling from such a temperature to the vitrified state seem to be able to "outrun" cold shock and chilling injury. For example, rapid cooling seems to prevent disintegration of the metaphase spindle of oocytes.

Cryoprotective agents

In vitrification methods, cells or tissues are brought into a medium that has a very high concentration of cryoprotective agents, also known as cryoprotectants. If the concentration of solutes is high enough, vitrification solutions will solidify to a glass without any risk of intracellular or extracellular ice formation during cooling or warming, independently of the cooling and warming rates used. However, the very high concentrations of cryoprotective agent needed for vitrification may cause damage due to abrupt osmotic changes, extremely low water potential or chemical toxicity. According to the description provided by Rall (1987), the embryos are first equilibrated with 25 percent vitrification solution at room temperature. Then the embryos are cooled to 4 °C and transferred to 50 percent vitrification solution and then to 100 percent vitrification solution. They are then rapidly packed and transferred into liquid nitrogen. The stepwise increase of cryoprotective agent concentration reduces osmotic effects, while the low temperature and rapid transfer help prevent damage by chemical toxicity. In addition, chemical toxicity may be reduced by using mixtures of various permeant CPAs, or addition of non-permeant CPAs (60 g/litre polyethylene glycol) (Rall, 1987) or 60 g/litre bovine serum albumin (BSA) (van Wagtendonk-de Leeuw *et al.,* 1997).

Reduction of cryoprotective agent concentration at high cooling rates

Solutions that have a solute concentration lower than that of classical vitrification solutions have freezing points below which there is a significant tendency to form ice crystals. But when the solution is cooled very rapidly, there is simply no time for ice formation. Below a certain temperature, the solution becomes so viscous and stiff that ice formation becomes impossible, and the solution turns into "metastable" glass. The solute concentration needed for metastable vitrification decreases as a function of increasing cooling rate. The most recent vitrification procedures, therefore, make use of high cooling rates in order to reduce the concentration of CPAs and thereby decrease the damage caused by osmotic stress and chemical toxicity.

The cooling rate can be increased in several ways. One is to reduce the volume of the sample to be vitrified. An early example of this approach is the open pulled straw method (often abbreviated OPS) (Vajta *et al.,* 1998, 2000a,b). Even smaller sample volumes have been used on electron microscope grids, so-called hemi-straws, nylon loops (cryoloops) or polypropylene strips (Cryotop® – Kitazato Supply Co., Fujinomiya, Japan) (Kuwayama, 2007). The Cryotop system allows a volume of 0.1 µl to be vitrified.

In addition to reducing the sample volume, a faster cooling rate can be achieved by heat transfer to a liquid that does not boil. Liquid nitrogen at its boiling point (-196 °C) will generate nitrogen gas when it absorbs heat. This will create a film of gas that insulates the sample from the liquid nitrogen. Liquid nitrogen at its freezing point (also known as "nitrogen slush") doesn't have this disadvantage. It can be produced with an apparatus called Vit Master® (IMT Ltd, Ness Ziona, Israel) (Arav *et al.,* 2002).

In metastable vitrification procedures, it is also essential that the warming (i.e. thawing) of the sample is very rapid. If warming is slow, ice crystals can form while the temperature is between the vitrification temperature and the freezing point of the vitrification solution.

Most recent vitrification protocols make use of these ultra-rapid approaches in order to reduce cryoprotective agent concentrations and prevent cold shock and chilling injury. Current vitrification solutions (Liu *et al.,* 2008; Morató *et al.,* 2008) have much lower solute concentrations than those used in classical vitrification solutions (e.g. VS3, Rall 1987). As described in Section 4, very good results are currently obtained when using these approaches for vitrification of oocytes and embryos. Recent studies with pig and cattle oocytes have indicated that the Cryotop system gives better results than the open pulled straw system (Liu *et al.,* 2008; Morató *et al.,* 2008).

FREEZE DRYING

Storage of freeze-dried biological material is extremely cost efficient, as no expensive and bulky liquid nitrogen containers are necessary. Furthermore, it is safe. The material may be stored at ambient temperature and, unlike cryogenic storage, there is no risk of equipment malfunction or of personal injury from liquid nitrogen. On the negative side, however, freeze drying generally reduces cell viability. Therefore, standard insemination procedures generally cannot be used for freeze-dried sperm. However, freeze-dried sperm have been successfully used to produce live offspring using ICSI in mice and rabbits (Wakayama and Yanagimachi 1998; Liu *et al.,* 2004). In addition, freeze-dried somatic cells have been successfully used to produce apparently healthy embryos using SCNT (Loi *et al.,* 2008a, 2008b). However, there have so far been no reports of cloned offspring produced by SCNT using freeze-dried somatic cells. Thus, while freeze drying is potentially useful for gene banking of genetic resources with the objective of regenerating live animals and recovering lost breeds, this would require further development and optimization of procedures. Conversely, freeze-dried gametes and somatic cells can already be used for conservation of germplasm intended for use in (genetic) research.

The key to freeze-drying is to bring the material to a vitrified glass state in which the glass transition temperature is higher than ambient temperature. The first step is to bring the biological material to a vitrified state. The next step is to apply a vacuum to the material,

which results in sublimation of any ice that may be present and further decreases the water content of the vitrified material. This increases the glass transition temperature, which ultimately reaches a level higher than the ambient temperature. Thus, at the end of the process the material can be stored at ambient temperature while remaining in the stable glass state. Obviously, the initial freezing/vitrification procedure, and the medium used, should be optimized so as to ensure the survival of the germplasm throughout this phase. In addition, the medium composition must be optimized so as to prevent the cells from being damaged by the effects of the further dehydration of the material.