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Cryopreservation and Freeze-Drying Protocols

Second Edition

Edited by
John G. Day
Glyn N. Stacey

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Cryopreservation and Freeze-Drying Protocols

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Preface

The preservation of biological material in a stable state is a fundamental requirement in biological/medical science, agriculture, and biotechnology. It has enabled standardization of experimental work over time, has secured lifesaving banks of cells and tissue ready for transplantation and transfusion at the time of need and has assured the survival of critical germ plasm in support of programs for the conservation of species. Cryopreservation and freeze-drying are widely accepted as the preferred techniques for achieving long-term storage, and have been applied to an increasingly diverse range of biological materials. Although the basis for many methodologies is common, many laboratories lack expertise in applying correct preservation and storage procedures and many apply outdated or inappropriate protocols for storing samples or cultures.

Cryopreservation and Freeze-Drying Protocols, *Second Edition,* in addition to outlining the fundamental principles associated with the conservation of biological resources, freeze-drying, and cryopreservation, is a compilation of cryopreservation and freeze-drying methodologies applicable to different biological materials, which have been developed by expert laboratories. The protocols are reproducible, robust, and most have been transferred successfully to other laboratories. Our intended readers are those proposing to establish, or improve, biostorage systems in their laboratory, whether concerned with biological resource centers, animal husbandry, aquaculture, horticulture, medicine, or human fertilization programs.

Because the emphasis of this volume is on methodology it is intended that practical progress can be made without reference to other sources. Each chapter deals with a discrete biological resource: a short introduction on the status of biostorage development is followed by a detailed description of materials required and methodological protocol to be followed, with explanatory notes on key technical issues.

This second edition expands on the range of materials covered in the 1995 edition and includes many novel approaches and protocols for biological materials that were not preservable in 1995. However, there are still many materials that remain preservation-recalcitrant, we hope and trust that future editions will contain cryopreservation and freeze-drying protocols that can be used to preserve biological resources that are at present recalcitrant to successful preservation.

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Glossary of Specialized Terms

Cell Packing Effect: The effect on the survival of cells when frozen at a high, rather than a low, packing density.

Chilling Injury: Injury that occurs as a result of a reduction in temperature.

Colligative Effect: A physical property of a system that depends on the number of molecules and not their nature.

Cryopreservation: The storage of a living organism, or a portion thereof, at an ultra-low temperature (typically colder than –130°C) such that it remains capable of survival upon thawing.

Cryoinjury: Damage caused by reduction in temperature irrespective of the mechanism.

Cryoprotectant or Cryoprotective Agent (CPA): A substance that protects a living system against injury due to reduction in temperature.

Cryostorage: The storage of a living organism, or a portion thereof, at an ultra-low temperature (typically colder than –130°C) such that it remains capable of survival upon thawing.

Eutectic Temperature: The lowest temperature (for a crystallizing solute) at which the existence of a liquid phase for a given system is possible.

Freezing: The crystallization of liquid water to form ice.

Freeze-Drying: A controllable method of dehydrating labile products by vacuum desiccation (also termed as lyophilization).

Glass Transition Temperature (Tg'): The temperature (for an amorphous solute) where the residual liquid vitrifies in the presence of ice.

Intracellular Freezing: The formation of ice crystals within cells.

Lyophilization: A controllable method of dehydrating labile products by vacuum desiccation (also termed "freeze-drying").

Melting Point: The temperature during the warming of an aqueous system at which the last ice melts. This temperature is equal to the equilibrium freezing point.

Nucleation: The formation of a nucleus upon which an ice crystal can grow; this may be an appropriate arrangement of water molecules or a foreign particle.

Solution Effects: Damage to cells that is a result of the increase in solute concentration that occurs as a secondary effect of freezing.

Super-Cooling: Reduction of temperature below the equilibrium freezing point but without freezing, hence, an unstable situation. Note: supercooling is often not hyphenated.

Vitrification: The conversion of an aqueous system to an amorphous, noncrystalline solid solely by increase in viscosity.

1

Long-Term *Ex Situ* Conservation of Biological Resources and the Role of Biological Resource Centers

Glyn N. Stacey and John G. Day

Summary

The establishment and maintenance of biological resource centers (BRCs) requires careful attention to implementation of reliable preservation technologies and appropriate quality control to ensure that recovered cultures and other biological materials perform in the same way as the originally isolated culture or material. There are many types of BRC that vary both in the kinds of material they hold and in the purposes for which the materials are provided. All BRCs are expected to provide materials and information of an appropriate quality for their application and work to standards relevant to those applications. There are important industrial, biomedical, and conservation issues that can only be addressed through effective and efficient operation of BRCs in the long term. This requires a high degree of expertise in the maintenance and management of collections of biological materials at ultra-low temperatures, or as freeze-dried material, to secure their long-term integrity and relevance for future research, development, and conservation.

Key Words: Biological resource centers; preservation; microorganisms; cell lines; tissues.

1. Introduction

Collecting examples of different types of organisms has been the pursuit of scientists and amateur collectors for centuries. This activity was originally stimulated by man's curiosity regarding the natural diversity of "his" environment, but for well over a century scientists have been collecting strains of animals, plants, and microorganisms with specific scientific and technical aims relating to taxonomy, infectious disease, and biochemistry. The first collection of microorganisms for industrial use was established by Kral in 1869 and collections of plants and other organisms developed based on the maintenance of examples of each strain or species under controlled laboratory or field conditions.

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However, such collections of actively growing cultures suffered from complications, such as adaptation of the organism to the in vitro environment, genetic mutations, contamination, and accidental loss of cultures. Clearly a mechanism for arresting growth to reduce such risks was needed.

The 1800s also saw tremendous expansion in our scientific knowledge and engineering capabilities with the consequent development of new techniques, including the compression of gases to the liquid state, which enabled the field of cryobiology to develop rapidly. The ability to use ultra-low temperatures to prevent degradation of biological materials has probably been utilized by man for millennia, and further scientific observations from the 17th and 18th centuries paved the way for discoveries in cyropreservation technology in the 20th century. These led to successful and reliable methods for the preservation of both prokaryotic and eukaryotic organisms so they could be stored indefinitely in a viable and unchanging state of "suspended animation." The preservation of bacteria and fungi had been established by pioneers from the 19th and early 20th centuries, subsequent work of people like Polge et al. ***(1)*** for preservation of animal cells, and Sakai ***(2)*** for plant cells can be viewed as key milestones in the development of cryopreservation processes. This pioneering work has been improved and refined with new approaches and fundamental research into cryobiology that has enabled the preservation of diverse and complex cell and tissue cultures as exemplified in the protocols provided in this book.

Today, culture collections, or more broadly, biological resource centers (BRCs), are a mixture of academic, public service, private, government and commercial activities that deliver important characterized cultures as "seed" stocks:

1. For the development of industrial processes.
2. As reference strains for biological assays and published scientific literature.
3. As type strains for taxonomical studies.
4. As centers for conservation of biodiversity.

In this chapter, we shall outline some of the important principles and challenges involved in the establishment and long-term maintenance of collections of cryopreserved biological materials and cultures.

2. Fundamental Principles for BRCs

There are three fundamental features of collections of biological materials that must be sustained to establish the value of stored material: (1) purity (freedom from contaminant organisms); (2) authenticity (correct identity of each strain), and (3) stability, including correct functional characteristics. Purity of a strain is critical to avoid erroneous data. However, in some situations purity

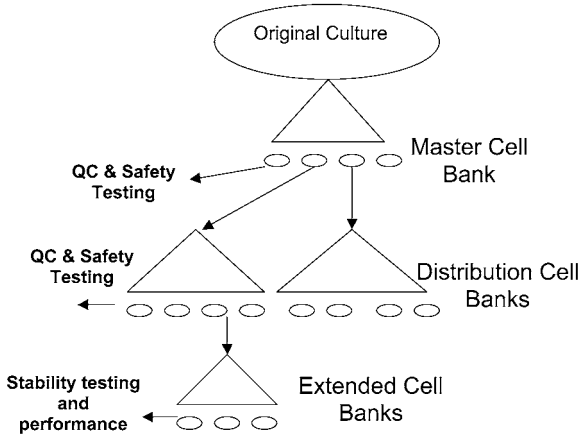

Fig. 1. Scheme of the "master" and "working" (or "distribution") bank system. Each "bank" comprises a number of containers (⬭) each containing a sample of cryopreserved cells from a homogenously mixed single batch of harvested cells. Extended cell banks can also be established to enable testing of culture characteristics at different passages in parallel experiments. This banking scheme can be used to ensure that cultures are subjected to minimum passages in vitro before distribution to researchers. It can also act as the basis for a process map that can be used to establish a quality assurance system with appropriate traceability for bank preparation and quality control ***(9)***.

can be very difficult to establish as the organism may have a saprophytic relationship with other organisms and cannot survive as a pure culture (e.g., certain protists, saprophytic fungi), or can only be preserved within other cells, e.g., malaria ***(3)***. Authenticity is usually based on certain stable phenotypic or genotypic characteristics and will vary for each group of organisms. However, some DNA profiling (e.g., multilocus DNA fingerprinting, short tandem repeat analysis ***[4]***, or gene sequencing techniques (e.g., cytochrome *c* oxidase sequencing ***[5]***, "ribotyping" ***[6]***) provide some generic approaches that can be applied across a broad range of organisms in some cases. For cultures maintained in vitro, failure to maintain their phenotypic and genotypic features on serial passage is a serious concern as cultures (notably viruses, pathogenic bacteria, and cell lines ***[7]***) are known to undergo irreversible changes if maintained in culture for long periods.

Accordingly, it is important that BRCs maintain practices to ensure that the samples of organisms that they hold and distribute sustain these important characteristics. This can be achieved through the adoption of working practices including establishment of master and distribution banks for each organism (**Fig. 1**) ***(8)***, and a robust quality control system to provide assurance that each

bank prepared meets the criteria outlined. The specific quality control regime will vary depending on the particular characteristics of the type of culture. A risk assessment will need to be performed relating to the history of each culture and the more general scientific experience with that culture type. Examples of such considerations is given in **Table 1**.

These requirements have been enshrined in best practice guidelines for culture collections such as the World Federation for Culture Collections' Standards ***(14)***. These identify the minimum standards to ensure an appropriate level of quality of materials available from BRCs that generally seek to exceed them. In addition, there may be specialist guidance for the maintenance of the certain types of cell culture and there are also legally binding regulations and ethical issues for some BRCs as outlined in **Table 2**.

3. Important Long-Term Roles for BRCs

The provision of cultures from BRCs is of little value unless these cultures are accompanied by information on their identity, provenance, and characteristics. Culture collections are important store houses of information on cultures that can be accessed by direct contact with individual collections or contacting microorganism database organizations such as MIRCEN at http://wdcm.nig.ac.jp/. This information resource can grow with time as work on cultures from BRCs is published. BRCs also engage in research and development on the technologies involved in the banking of cultures, and are very often the only locations where this type of specialist research and development is maintained. Accordingly, BRCs are often valuable repositories for up-to-date information topics such as biosafety, shipment, characterization, preservation, and taxonomy (*see* www.wfcc.info). They are also frequently the drivers for training and education in such areas.

Many BRCs provide vital services for *ex situ* conservation of biodiversity through the preservation, storage, and documentation of endangered plant tissues (Chapter 12), seeds (Chapter 13), and animal gametes and embryos (Chapters 21–23). Such resource centers could potentially regenerate species that are lost to their natural habitat. The field of biotechnology and biomedicine is also reliant on long-term storage of viable cells to secure reliable seed stocks for the manufacture and testing of products. Furthermore, such long-term storage is critical to secure intellectual property by supporting patent applications where the preserved cells must remain viable for at least 30 yr ***(16,21)***.

4. Assuring the Success of Long-Term Storage

4.1. Experience and Critical Issues

It is often assumed that once an organism has been successfully preserved in a viable state in liquid nitrogen that it will remain viable indefinitely at ultra-low

Table 1
Standards That May Apply to Biological Resource Centers

Characteristic	Typical methods for bacterial strains	Typical methods for plant germplasm	Typical methods for animal cell lines
Purity	Broth and agar culture to reveal pure growth	Broth and agar culture to reveal absence of bacteria and fungi	Broth and agar culture to show no growth of bacteria and fungi ***(12)*** Tests for mycoplasma ***(12)*** and viral contamination (e.g., electron microscopy, molecular detection, inoculation of animals or test cell lines) ***(13)***
Authenticity	Gram stain reaction, colony morphology, biochemical genotyping, biochemical reactions, ribosomal gene sequence	Morphology of cells/structures, genotype, expression profile of "secondary products," DNA genotype ***(10)***	DNA fingerprint, karyology, isoenzyme analysis, cytochrome *c* oxidase sequence ***(4)***
Stability/functional capacity (cultures recovered from cell banks reproducibly show key functions)	Antibiotic susceptibility, absence of mutations by gene sequencing	Retain ability to regenerate plants, sustained expression of "secondary products," absence of "sports" ***(10,11)***	Antibody secretion by hybridomas, susceptibility to viruses, karyology

Table 2
Standards for Cell Banking

Type of culture/application	Standards that may be applicable
Research and development	National regulations for handling, containment, and disposal of pathogenic strains National regulations on genetically modified organisms Good cell culture practice ***(15)***
Organisms deposited in support of patent applications	Budapest treaty ***(16)***
Plant cells and tissues	National and international regulations on import and export of pathogens (http://www.wfcc.info) and endangered species (http://www.cites.org/)
Animal cells and tissues	National and regulations on appropriate records of licenses, procedures, and staff training National and international regulations on import and export of pathogens and endangered species (*see* above)
Human cells and tissues for research	National regulations on ethical procurement and project approval
Human cells and tissues for transplantation and therapy	National and international regulations on ethical procurement, processing and storage ***(17)***
Organisms and cell lines for manufacture of products for human therapy	International regulations for cell substrates ***(18,19)*** International regulations on proliferation of biological warfare
Organisms and cell lines for testing purposes	International regulations and protocols for product testing (e.g., OECD Good Laboratory Practice ***(20)***, Pharmacopeia monographs (http://www.usp.org/ or http://www.pheur.org/)

storage temperatures. Many of the authors in this book have commented on long-term storage and a review of the literature gives many examples of successful storage of cryopreserved organisms for periods of up to many decades **(Table 3)**.

However, statements regarding sustained viability of cryopreserved cells is often based on the assumption that their storage temperature will be reliably maintained at that of liquid nitrogen (–196°C). Clearly, material stored in the vapor phase will never achieve this temperature and the actual storage temperature may well fluctuate because of variation in the levels of liquid nitrogen in storage vessels over time. Critically the storage temperature should not rise above the melting point of any cryopreserved cell suspensions or above the glass transition temperature of any vitrified material (*see* Chapters 2 and 3). Individuals searching for stored material may also move preserved preparations into ambient temperatures for brief periods, which if repeated over time could lead to loss of viability. In order to assure the long-term viability of stored cells and tissues it is therefore important to ensure that storage areas are well controlled with regular checks on liquid nitrogen levels and vessel-filling rotas, as well as appropriate staff training ***(18)***.

4.2. Storage Facilities

Having invested time and resources in the quality of banks of cell cultures it is wise to provide a secure, clean, and stable environment for long-term storage that is also safe for laboratory staff to use ***(15)***. Security for stored material is assured through adoption of appropriate management systems to restrict access to authorized personnel, appropriate alarms for nitrogen storage vessels, and documented procedures for filling and maintenance of nitrogen storage. Monitoring in the form of temperature alarm systems and auditing to ensure correct maintenance and documentation are also important activities for BRC operation.

It is important to establish whether storage will be in the liquid or vapor phase of nitrogen or if electrical freezers (–100°C or below) are to be considered. In theory, the liquid phase of nitrogen provides the lowest and most stable storage temperature and is the method of choice for long-term storage. However, the risks of transmission of pathogenic virus should be considered as highlighted in past cases of patient deaths from bone marrow contaminated in storage ***(35)***. Vapor-phase storage may increase the risk of temperature cycling in stored materials but is generally more convenient and safer for regular access to stored material than liquid-phase storage. Some manufacturers (e.g., CBS) supply vapor-phase storage systems where liquid nitrogen is retained in the vessel walls, thus improving safety for laboratory staff. Electrical storage systems provide a very practical and maintenance-free low temperature storage solution. However, materials stored in such systems in a

Table 3
Examples of Long-Term Viability of Preserved Organisms

Taxa/material	Stored	Storage duration	Comment	Reference
Bacteria	LN	5–35 yr	Survival	***22***
19 yeast strains	LN	long term	No reduction in viability	***23***
Yeast	FD	up to 30 yr	Survival	Chapter 6
Fungi	FD	>30 yr	Good survival	***24***
Schizo-saccharomyces pombe	LN	10 yr	Unchanged genetically	Chapter 7
Various microalgae	LN	>20 yr	No reduction in viability	***25***
Various cyanobacteria	LN	>20 yr	No obvious loss of viability	Rippka (personal communication)
Lettuce seeds	LN	>10 yr	No loss viability half-life c. 3400 yr	***26***
Stem cells	LN	15 yr	High efficiency of recovery	***27***
Human and sheep red cells	LN	12 yr	No deterioration in function	Chapter 20
Human hematopoietic stem cells	LN	14 yr	Retain engraftment potential	***28***
Bovine sperm	LN	37 yr	Normal motility and successful fertilization	***29***
Human sperm	LN	5 yr	No statistical reduction in quality	***30***
Human sperm	LN	21 yr	Successful fertilization and live birth	***31***
Canine islets of langerham	LN	6 mo	No reduction in insulin secretion	***32***
Sheep embryos	LN	5 yr	No reduction in live births vs 2 wk storage	***33***
Sheep embryos	LN	13 yr	No reduction in live births vs 1 mo storage	***34***

FD, freeze dried; LN, liquid nitrogen storage.

multiuser environment may suffer from regular disturbance to access material with the risk that critical materials may be lifted into ambient temperatures on a regular basis. Electrical freezers are also at risk where power supplies may not be reliable and even if power is reliable manufacturers often recommend liquid nitrogen or carbon dioxide back-up systems to cope with emergencies.

Appropriate facilities, equipment, and training in handling liquid nitrogen are vitally important for staff safety in cryostorage environments. In addition to the risks of frostbite from contact with ultra-low temperature equipment and liquids, staff should also be made aware of the risks of ampoule explosion (from trapped nitrogen liquid) and asphyxiation resulting from displacement of oxygen. The latter hazard is particularly important in enclosed storage areas and staff may need to be issued with personal oxygen monitors. Hazardous materials and those in quarantine should be stored separately, and it is also useful to separate material intended for archive storage and other material that will be accessed regularly.

4.3. Documentation of Stored Materials

Accurate records of stored materials are vital to enable retrieval of ampoules of cells efficiently. They may also be a legal requirement where genetically modified, infectious, or other hazardous materials are stored. Numerous commercial database systems are available specially designed for this purpose, but it is important to select a system which is flexible to the full range of user requirements. It is wise to have up-to-date hard copy printouts or back-up electronic copies of these, and to ensure that amendments to storage records for additions or withdrawals can be made at the storage site to avoid transcriptional errors.

4.4. Other Issues for Long-Term Storage

Over time liquid nitrogen freezers become clogged with a build up of ice sludge, which can accumulate microbial contamination from environmental sources ***(36)***. Thus, long-term storage vessels will benefit from periodic cleaning to remove the ice sludge and it is also helpful to carefully disinfect recovered ampoules. The use of double-sealing methods for ampoules or storage boxes will also help to provide protection against microbial contamination. Serious and lethal viral cross-infection of cells for transplantation from damaged containers in liquid nitrogen has also been reported ***(35)***, which emphasizes the importance of such procedures for all stored biological materials.

Natural radiation has also been considered a potential cause of loss of viability or mutation in stored cells and tissues. However, there does not appear to be any evidence for the adverse effects of long-term storage in well-maintained nitrogen vessels even for biological systems that might be expected to be more sensitive to such effects, such as embryos ***(37)***.

Table 4
Typical Storage and Transport Conditions for Different Kinds of Cryopreserved Materials

Material	Storage conditions	Shipping conditions
Freeze-dried proteins, bacteria, and fungi	Generally stored at or below 4°C depending on thermal stability	Can be stored at ambient temperature depending on stability
Cryopreserved cells and organisms in vials or ampoules	Generally stored at temperatures below –100°C in electric freezers (–100 to –150°C) or in the liquid or vapor phase of liquid nitrogen (–160 to 196°C). Storage is possible at –80°C but viability will decrease with time ***(41)***	Shipment can be made in dry-ice packages (–80°C). It is vital to ensure sufficient dry ice is included to keep the material frozen until receipt. Good shipping companies will ensure that packages are topped up with dry ice
Cryopreserved tissues and cells for transplantation	Generally stored in the vapor phase (not submerged in liquid) of liquid nitrogen to avoid contamination	Special shipment containers ("dry shippers") are often used to maintain temperature of vapor-phase LN
Cells preserved by vitrification (*see* Chapters 3 and 12)	Stored below the glass transition temperature (*see* Chapters 2 and 3) for the particular preservation method and cells	Shipment must also be below the glass transition temperature (e.g., in dry shippers containing adsorbed nitrogen liquid). Higher temperatures, such as dry ice, will cause devitrification and loss of viability

LN, liquid nitrogen storage.

For important archive material it is wise to split such material between separate storage vessels and ideally to have an additional off-site storage location. It is clearly vital to monitor the quality of the storage environment for long-term sustained viability of stored cultures. Where much of the stored materials is of one type in terms of its potential stability in cryostorage, it may be helpful establish "sentinel" banks of representative cells stored in locations prone to temperature cycling (e.g., the upper regions of storage inventory systems) that are recovered periodically to detect any trends in viability levels ***(38)***. However, alternative physical temperature monitoring methods for key locations will generally be adequate, and RFID technology ***(39)*** is now delivering devices that could record and report the temperature experienced by individual vials or ampoules of cells in storage over time. In some circumstances accelerated thermal stability studies are used to predict the survival of stored biological materials at ultra-low temperature (*see* Chapters 4 and 13 ***[40]***). A variety of storage conditions can be obtained for biological materials and **Table 4** shows a simplified summary of suitable storage and transport conditions for different kinds of preserved biological materials.

5. Conclusion

The availability of quality controlled and authentic biological materials and cultures through professional BRCs is a significant advantage for science promoting standardization, efficiency, and laboratory safety. Culture strains become established in professional standards for industrial and biomedical work and may need to be stored stably for decades to support conservation programs, production processes, testing methods, and patents. BRCs may be challenged to provide cryostorage for large numbers of strains of organisms even when cryopreservation methods are not optimized for all organisms to be preserved. BRCs will continue to provide sources of specialist advice and training in the skills of preservation and culture that are increasingly needed with the development and expansion of cell-based in vitro experimentation.

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2

The Principles of Freeze-Drying

Gerald Adams

Summary

This chapter provides an up-to-date overview of freeze-drying (lyophilization) with particulars relevance to stabilizing live cells or viruses for industrial applications as vaccines or seed culture. The chapter discusses the importance of formulation, cycle development, validation, and the need to satisfy pharmaceutical regulatory requirements necessary for the commercial exploitation of freeze-dried products.

Key Words: Freeze-drying; lyophilization; lyoprotectants; secondary drying; sublimation.

1. Introduction

Water is essential to life, providing a universal solvent supporting biochemical activities within cells, which enables metabolism to continue and sustains all living processes. Quite simply, in the absence of water, life as we define it will cease, resulting in a state of death or dormancy in live cells or inhibiting biochemical activity in cellular extracts. Water also plays a major role in the degradation of stored material, providing conditions that potentiate autolysis, or promote the growth of spoilage organisms ***(1)***.

In order to stabilize labile products, it is therefore necessary to immobilize or reduce the water content of stored samples.

Vaccines, other biological materials, and microorganisms can be stabilized by chilling or freezing. However, maintaining and transporting samples in the frozen state is costly, whereas freezer breakdown may result in the complete loss of valuable product ***(2)***.

Alternatively, bioproducts can be dried in air using high processing temperatures. Traditional drying typically results in marked changes in the physical and chemical properties of the product by high solute concentration or thermal

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inactivation, and is more appropriate for dehydrating low-cost products such as foodstuffs.

Freeze-drying combines the benefits of both freezing and drying to provide a dry, active, shelf-stable, and readily soluble product ***(3,4)***.

1.1. Defining Freeze-Drying

Freeze-drying or lyophilisation describe precisely the same process. The term "lyophilization," which means "to make solvent loving," is less descriptive than the alternative definition "freeze-drying." Several alternative definitions have been used to describe freeze-drying. Operationally we could define freeze-drying as a controllable method of dehydrating labile products by vacuum desiccation.

Earlier accounts of freeze-drying suggested that ice was only removed by sublimation and defined this step as primary drying. The cycle was then described as being extended by secondary drying or desorption. Although these definitions are applicable to ideal systems, they incompletely define the process for typical systems that form an amorphous matrix or glass when cooled ***(5)***.

Technically, freeze-drying may be defined as:

1. Cooling of the liquid sample, followed by the conversion of freezable solution water into ice; crystallization of crystallizable solutes and the formation of an amorphous matrix comprising noncrystallizing solutes associated with unfrozen moisture.
2. Sublimation of ice under vacuum.
3. "Evaporation" of water from the amorphous matrix.
4. Desorption of chemiabsorbed moisture resident in the apparently dried cake.

1.2. History

The method can be traced back to prehistoric times and was used by the Aztecs and Eskimo for preserving foodstuffs. Toward the end of the 1880s the process was used on a laboratory scale and the basic principles understood at that time. Practically, the method remained a laboratory technique until the 1930s when there was the need to process heat-labile antibiotics and blood products. At this time, refrigeration and vacuum technologies had advanced sufficiently to enable production freeze-dryers to be developed, and since then the process has been used industrially in both the food and pharmaceutical industries ***(3,6)***.

Freeze-drying has a number of advantages over alternative stabilizing methods. These may be summarized by the following criteria ***(6)***:

1. The need to stabilize materials for storage or distribution.
2. The product may demand to be freeze-dried and there may be no suitable alternative available.

3. There may be a legal requirement to freeze-dry the product to satisfy regulatory demands.
4. Freezing will reduce thermal inactivation of the product and immobilize solution components.
5. Concentration effects such as "salting out" of proteins, alterations in the distribution of components within the drying and dried product, and so on, may be minimized by freeze-drying.
6. The water content of the dried product can be reduced to low levels, and in general samples are more shelf-stable when dried to low moisture contents, although overdrying may reduce shelf stability in sensitive biomaterials.
7. Because the product is normally sealed under vacuum or an inert gas, oxidative denaturation is reduced.
8. Loss of water equates to a loss of product weight and this may be important where transport costs are significant.
9. Sample solubility, shrinkage, unacceptable appearance, or loss of activity may all be improved when freeze-drying is used rather than an alternative technique.
10. Dispensing accuracy may be facilitated when the sample is dispensed as a liquid rather than a powder.
11. Particulate contamination is often reduced when samples are freeze-dried rather than spray or air-dried.
12. The need to compete with competitors supplying similar products.
13. The requirement to launch a product on the market while less costly drying techniques are being developed.
14. The production of intermediate bulk or requirement to remove solvents such as ethanol.
15. The need to maximize investment in drying plants by freeze-drying a minor product rather than invest in an alternative and costly drying process.
16. The need to separately dry two or more components that would be incompatible if dispensed together within a single container.

1.3. Types of Freeze-Dried Products

Freeze-dried products may be classified as:

1. Nonbiologicals, where the process is used to dehydrate or concentrate reactive or heat-sensitive chemicals.
2. Nonliving bioproducts. These comprise the major area of application and include enzymes, hormones, antibiotics, vitamins, blood products, antibiotics, inactivated or attenuated vaccines, and so on. This subgroup includes pharmaceuticals, which may be used diagnostically or therapeutically.
3. Bone and other body tissues for surgical or medical use; foods where organoleptic properties are important; industrial bioproducts.
4. Living organisms for vaccine or seed culture use, which must grow and multiply to produce new progeny after drying and reconstitution.
5. Miscellaneous, for example flood-damaged books, museum artifacts, and so on.

However, freeze-drying is less appropriate for:

1. Oily or sugar-rich materials where the medium does not freeze.
2. Products that form impervious surface skins, thereby preventing vapor migration from the drying sample during processing.
3. Eukaryote cells, which are able to retain viability when frozen only in the presence of additives, may be incompatible with the freeze-drying process.

2. The Process of Freeze-Drying

2.1. Description of Process

For convenience, the freeze-drying process may be divided into a number of discrete steps that may be summarized as ***(7,8)***:

1. For the processing of cell or other bioproducts a variety of preparatory processing steps may be required, e.g., vaccine preparation, extraction, purification, and formulation in a suitable medium for freeze-drying.
2. Sample freezing, which reduces thermal denaturation of product, immobilizes solution components, and prevents foaming when the vacuum is applied. Freezing also induces a desired ice-crystal structure within the sample, which facilitates drying.
3. Primary drying (sublimation) where conditions must be maintained in the drying chamber to sustain water migration from the sample ice during drying. During primary drying the sample temperature (strictly freeze-drying interface temperature) must be maintained below the eutectic, glass transition, collapse, or melt temperature as appropriate to minimize sample damage during drying.
4. A secondary drying stage during which resident moisture adsorbed to the apparently dry structure is removed by desorption.
5. Sealing the dried sample in a vacuum or under an inert gas at the end of the process, both of which exclude the entry of reactive, destabilizing, atmospheric gases such as oxygen or carbon dioxide into the dried sample and prevent the ingress of damp air into the freeze-dried sample. (Note that a freeze-dried product will have a vastly expanded dry surface area and is therefore particularly sensitive to air denaturation or moisture readsorption.)
6. The samples are then removed from the freeze-dryer, stored and/or distributed for use prior to reconstitution for injection, application, or regrowth.

2.2. Processing Principles

Freeze-drying is a complex process during which drying may proceed more or less rapidly within individual samples throughout the process batch, such that parts of the product will be frozen, whereas other areas are drying or will have dried depending on the nature of the sample and stage in the cycle. The precise freezing and drying behavior will be determined by the interrelationship between the sample and shelf temperature, system pressure, extent of product dryness, and variations in drying conditions throughout the cycle.

Often regarded as a gentle method of drying materials, freeze-drying is in reality a potentially damaging process where the individual process stages should be regarded as a series of interrelated stresses, each of which can damage sensitive bioproducts. Damage sustained during one step in the process may be exacerbated at succeeding stages in the process chain and even apparently trivial changes in the process, such as a change in container, may be sufficient to transform a successful process to one which is unacceptable ***(8)***.

Freeze-drying will not reverse damage incurred prior to formulation and care must be exercised when selecting an appropriate cell type or technique used to culture or purify the cell or its extracts prior to freeze-drying. The essence of the formulation exercise should be to minimize freeze-drying damage, loss of viability, or activity. To ensure minimal losses of activity, the sample may require dilution in a medium containing protective additives, specifically selected for the product or application. Although frequently described as "protectants" these additives may not be effective at all stages of the process but may protect only during particular steps in the drying cycle. At other stages, the additive may fail to protect the active component and indeed may be incompatible with the process. It is also important to appreciate that individual stages in the process can result in damage, which initially remains undetected, becoming evident only when the dried sample is rehydrated. Particular attention must be applied to the selection and blending of the additive mixes in the formulation and the importance of formulation will be discussed at greater length later ***(1,9–12)***.

Freeze-dried products should be:

1. Minimally changed by the process.
2. Dry.
3. Active.
4. Shelf stable.
5. Clean and sterile (for pharmaceutical applications).
6. Ethically acceptable.
7. Pharmaceutically elegant.
8. Readily soluble and simple to reconstitute.
9. Process should be economically practicable.

Products should be formulated to ensure batch product uniformity, whereas there may be particular requirements relating to product use. In this context, vaccines freeze-dried for oral or aerosol delivery may require the inclusion of excipients that minimize damage when the dried product is exposed to moist air ***(11)***.

A wide range of containers can be used to freeze-dry vaccines, microorganisms, and others, including all glass ampoules, rubber stoppered vials, double

chambered vials, and prefilled syringes that hold both dried vaccine and diluent, bifurcated needles, and so on. Alternatively, vaccines can be dried in bulk in stainless steel or plastic trays and the resultant powder tableted, capsulated, sachet filled, or dispensed into aerosol devices for lung or nasal delivery.

2.3. Sample Freezing

Regarded as the first step in the process, the formulated product must be frozen before evacuating the chamber to induce sublimation ***(13,14)***. Freezing will:

1. Immobilize the components in the solution and prevent foaming as the vacuum is applied.
2. Reduce thermal inactivation of the dispensed product.
3. Induce a specific ice-crystal structure within the frozen mass, which will facilitate or inhibit vapor migration from the drying cake. In short, the ice structure formed during freezing will dictate subsequent freeze-drying behavior and the ultimate morphology of the dried cake.

Ideally freezing should minimize solute concentration effects and result in a sample where all the components are spatially arranged as in the dispensed solution. However, it may not be possible to achieve this ideal when typical solutions or suspensions are frozen. When addressing the freezing of aqueous solutions or suspensions, there is the need to consider both the solvent (water in the case of aqueous solutions) and solute(s) in the formulation.

Frequently, the terms cooling and freezing are erroneously interchanged and confusion in understanding the process may occur and may be compounded by failing to distinguish between shelf or product cooling and freezing. Cooling refers to the reduction of temperature of the freeze-dryer shelves, the diatherm fluid circulating through the shelves, the vial and tray mass, interior of the freeze-dryer, and the dispensed solution or suspension. Cooling does not assume a change in state from liquid to solid and strictly should be used to describe reducing temperature during the initial stage of freeze-drying. Freezing refers to the abrupt phase change when water freezes as ice. Except for very complex biomolecules or cold sensitive cells, cooling in the absence of freezing (chilling) is generally not damaging to biomaterials.

When solutions or suspensions are frozen, they may cool appreciably below their measured freezing point prior to ice formation, a phenomenon defined as supercooling (undercooling or subcooling). The extent of supercooling depends on cooling rate, sample composition and cleanliness, dispensed fill volume, container type, method of sample cooling, and so on. Even when a simple solution is repeatedly cooled or warmed, the onset and extent of supercooling will vary from cycle to cycle. In the supercooled state, while the composition of the solution remains unchanged, the cooled liquid is thermodynamically unstable and sensitive to ice

formation. As the solution is cooled to lower temperatures, the probability of ice crystallization will correspondingly increase. For optimized freeze-drying, the intention should be to induce supercooling in the suspension to encourage uniform cooling and freezing throughout the sample contents ***(15–17)***.

Sample freezing may be defined as the abrupt conversion of the suspension into a mixture of ice and solute concentrate. Freezing is a two-step process during which water initially nucleates, followed by the growth of the ice crystals that pervade the solute phase resulting in a mixture of ice and solute concentrate. Under typical processing conditions, ice nucleates heterogeneously around microscopic particles within the suspension and is encouraged by reducing temperature and agitating the supercooled suspension to increase the probability of contact between nucleating foci and water clusters. Nucleation depends on the number and physical nature of particulate impurities within the suspension or solution. Ice is a particularly effective nucleation focus and cryobiologists may deliberately seed samples with ice to induce nucleation. Other effective ice nucleators include glass shards and specifically formulated nucleation promoters. Whereas nucleation aids can be added to experimental systems, deliberate attempts to add ice inducers to pharmaceutical materials would be at variance with Good Pharmaceutical Manufacturing Practice ***(18)***.

In contrast to nucleation, ice growth (proliferation) is encouraged by raising the temperature, thereby decreasing the suspension viscosity. Ice nucleation and proliferation are inhibited at temperatures below the glass transition temperature (Tg′), whereas above the melting temperature (Tm) the suspension or solution will melt. The consequences and measurements of these parameters are important elements in the formulation exercise ***(14,18,20,21)***.

To facilitate the sublimation of water vapor from the drying mass, the ice crystals should be large, wide, and contiguous, extending from the product base toward its surface, thereby providing an optimized structure for vapor migration. Crystal structures commonly observed during freeze-drying when solutions are frozen in trays or vials include dendritic structuring, where the ice crystal branches continuously from the nucleating focus and the spherulite form, and where subbranching is discouraged because the solution viscosity is high, or fast rates of cooling are used.

Cooling or freezing rates are defined as slow (suboptimal), rapid (superoptimal), or optimal as assessed by criteria such as postfreezing cell survival or biopolymer activity, and is ambiguous unless conditions are more precisely defined. Cooling rates may be defined in terms of:

1. The rate at which the shelf temperature is cooled per unit time.
2. The rate at which the solution cools per unit time.
3. The depth of liquid within the vial (in mm) which cools per unit time.

2.3.1. Shelf-Cooling Rate

The shelf-cooling rate ***(22)*** is the simplest parameter to control and programmed rates of cooling are standard options on research and production freeze-dryers. Because shelf temperature and product responses are not identical, defining shelf-cooling rate will not fully define product behavior. Although we are concerned with the cooling rate achievable within each vial, this parameter is less easy to monitor compared with shelf cooling, and freeze-drying cycles generally are controlled by programmed shelf cooling rather than feedback control from the sample. Cooling rates of the product/cell suspension will vary considerably from vial and throughout the sample within the vial and, consequently, measuring the temperature of vial contents at a fixed position will give only an approximation of the sample temperature variation.

Observing the freezing pattern of a number of vials arranged on a shelf will demonstrate that while the contents of some vials will freeze slowly from the vial base, neighboring vials may remain unfrozen and supercool appreciably before freezing instantly. This random freezing pattern will reflect differences in ice structure from vial to vial and translated into different drying geometries from sample-to-sample vials. In summary, freezing patterns will be related to:

1. The ice forming potential within each vial.
2. The relative position of the vial on the shelf causing exposure of individual vials to cold or hot spots.
3. Edge effects where samples in vials on the periphery of each shelf will be subjected to heat transmitted through the chamber walls or door.
4. The insertion of temperature into the sample, which will induce ice crystallization.
5. The evolution of latent heat as samples freeze, which will tend to warm adjacent containers.
6. Variations in container base geometry, which may impede thermal contact between sample and shelf.

The ice and solute crystal structure resulting from sample freeze has a major impact on subsequent freeze-drying behavior, encouraging the sample to dry efficiently or with defects such as melt or collapse depending on freezing rate used. The preferred ice structure comprising large contiguous ice crystals is induced by freezing the sample at a slow rate of c. 0.2–1.0°C/min. Slow cooling will also induce the crystallization of solutes reluctant to crystallize when faster rates of cooling are used. However, a slow rate of cooling may exacerbate the development of a surface skin, which inhibits sublimation efficiency (*see* **Subheading 3.6.2.**). Slow cooling can also inactivate a bioproduct by prolonging sample exposure to the solute concentrate biomolecules. However, a fast rate of cooling can result in the formation of numerous, small, randomly

orientated ice crystals embedded in an amorphous solute matrix, which may be difficult to freeze-dry. Complicating the choice of freezing regimes is the fact that the optimal cooling rate cannot be sustained where the sample fill depth exceeds 10 mm. In short, defining cooling rates often requires a compromise in sample requirements.

2.3.2. Ice Structure and Freeze Consolidation **(13)**

A period of consolidation (defined as the hold time), is necessary at the end of sample cooling to ensure that all the vial contents in the sample batch have frozen adequately, although excessive hold times will increase the time of sample freeze and impact on the overall cycle time. It is a fallacy to assume that the ice structure induced remains unchanged during this consolidation period and an ice structure comprising a large number of small ice crystals, induced by rapid cooling, is thermodynamically less stable than an ice structure comprising fewer, larger crystals. The thermodynamic equilibrium can be maintained by recrystallization of ice from small-to-large crystals, a process termed grain growth. Although ice structure changes take place randomly from vial to vial, the hold period is a major factor in ice recrystallization resulting in significant variation in crystal structure and subsequent sublimation efficiency from sample to sample the longer the hold period is employed.

As an alternative to increasing the length of the hold time to encourage ice recrystallization, a more controlled and time-efficient method of inducing recrystallization is to heat anneal the frozen sample ***(23)***. Essentially heat annealing is achieved by:

1. Cooling the product-to-freeze solution water and crystallized solutes.
2. Raising the product temperature during the freezing stage to recrystallize ice from a small to a large ice crystal matrix. (**Note**: this warming phase may also crystallize solutes that are reluctant to crystallize by cooling [*see* **Subheading 2.3.3.**].)
3. Cooling the product to terminal hold temperature prior to chamber evacuation.

Heat annealing (also defined as tempering) is particularly useful to:

1. Convert an ice structure to a crystalline form, which improves sublimation efficiency.
2. Crystallizing solutes that are reluctant to crystallize during cooling.
3. Provide a more uniform, dry structure throughout the product batch.
4. Integrated with rapid cooling, heat annealing may minimize the development of a surface skin on the sample thereby facilitating sublimation.
5. Because heat annealing induces a more porous cake structure with improved drying efficiency, a lower dried sample moisture content may be achieved, with improved solubility.

Although heat annealing will increase the length of the freezing stage of the cycle, overall freeze-drying cycle times may be significantly reduced because of improvements in drying efficiency resulting from heat annealing (*see* **Fig. 1**).

Care should be exercised when selecting temperatures and hold times for heat annealing, particularly when defining the upper temperature for sample warming (*see* **Subheading 2.3.3**). Subjecting a labile product, such as a vaccine, to temperatures above the eutectic temperature will expose the sample to hypertonic solution concentrates as the sample partially melts, which can damage sensitive biomolecules.

2.3.3. Freezing Solute Behavior **(6,24–27)**

Regardless of the precise freezing pattern, the formation of ice will concentrate the remaining solution within the container. As the proportion of ice increases within the mixture, solute concentration will correspondingly increase. In the case of an aqueous 1% (w/v) saline solution, this concentration effect will be considerable, increasing to approx 30% (w/v) just prior to freezing, and damage to biomolecules results as a consequence of solute concentration exposure rather than direct damage by ice crystals. The behavior of the solute(s) within the solute concentrate depends on the nature, concentration, cooling rate, and interactions between individual solutes present in the medium and forms the basis for experimental review during a formulation development exercise.

Overall, four patterns of solute response are observed during freeze-drying:

1. Solute crystallizes readily, regardless of cooling rate or freezing conditions, to form a mixture of ice and solute crystals (this behavior is termed eutectic freezing).
2. Solute crystallizes, but only when the solution is subjected to a slow rate of cooling.
3. Solute crystallizes only after the solution has been heat annealed.
4. Solute fails to crystallize regardless of cooling rate or regime adopted, and solute remains associated with unfrozen water as a metastable amorphous mass or glass.

For a crystallizing solute, the eutectic point is the lowest temperature in a system in which a residual liquid phase and solid phase are in equilibrium. Above the eutectic point, ice and solute concentrate persist, whereas below the eutectic point, a mixture of ice and solute crystals is formed. Eutectic temperatures for aqueous solutions containing crystallizing salts are characteristic for each solute and are significantly below the freezing point of water (for example, eutectic temperature for sodium chloride = –21.4°C). Exposing cells or proteins for prolonged periods to a eutectic solution comprising hypertonic salt concentrations can cause damage by plasmolysis or precipitation by "salting out" ***(28)***.

The eutectic zone is the range of temperatures encompassing all the eutectic temperatures within the system. For a two-part water/solute system, the eutectic temperature is a discrete, quantifiable temperature in contrast to multisolute systems where a eutectic zone may be observed that represents a range of

Fig. 1. Samples of freeze-dried products freeze-dried using an unsuitable process cycle (sample A) compared with an identically formulated sample (sample B) where processing conditions have been optimized to provide an acceptable product. Both samples are formulated in an identical medium and filled to a depth of 60 mm, which greatly exceeds the recommended fill depth maximum for freeze-drying of 10 mm. Although such excessive fill depths are often required for commercial or marketing reasons, such fills greatly impede sublimation resulting in prolonged cycles, collapsed product, poor sample solubility, reduced product activity and shelf stability. In addition, such samples often exhibit high-moisture content, unacceptable pharmaceutical elegance, and display cake fracture and physical loss of sample from the vial as drying progresses (defined as ablation and of particular concern when live vaccines or cytotoxic drugs are freeze-dried). Sample A was freeze-dried using a conventional freezing and drying cycle and resulted in a cycle time in excess of 10 d with extensive vial breakage, in addition to the unacceptable features noted previously. Sample B was dried using a cycle designed to ensure satisfactory freezing to induce an optimized frozen structure conducive to rapid rates of sublimation. In addition, the author was able to accelerate drying by adjusting shelf temperature and chamber pressure so that drying times were reduced to 3 d. Product quality was assured by maintaining sample temperatures sufficiently below collapse (Tc) or glass transition (Tg′) temperature, defined during formulation and process development.

temperatures where the minimum eutectic temperature is lower than that of any individual eutectic temperatures in the medium.

Typical freeze-dried vaccine formulations fail to crystallize completely when cooled, and a proportion of the solutes in the sample persist as an amorphous,

noncrystalline, glass. When exposed to temperatures above their glass transition (Tg′) or collapse temperature (Tc), these samples may warm during sublimation causing the amorphous mass to soften so that freeze-drying progresses with sample collapse to form a sticky, structure-less residue within the vial. Less severe collapse will result in the formation of a shrunken, distorted, or split cake ***(20,29–33)*** (*see* **Fig. 1**).

Collapsed cakes are not only cosmetically unacceptable but may be poorly soluble, exhibit reduced activity, or compromised shelf stability. Collapse may be exacerbated by the formation of a surface skin, which impedes vapor migration from the drying structure. To avoid sample collapse, it is necessary to maintain the sublimation interface below Tg′ or Tc throughout primary drying and to include excipients in the formulation, which reduce the severity of collapse. It is therefore essential to characterize formulations experimentally during the process development program. Although collapse may cause operational difficulties during freeze-drying, the induction and maintenance of the amorphous state may be essential for protecting labile biomolecules during freezing, drying, and storage ***(23,34,35)***.

2.3.4. Freezing in Practice

Samples may be frozen in a variety of ways depending on operational requirements:

1. Samples may be frozen in a freezer or a cooling tunnel prior to transfer to the freeze-dryer for desiccation. Advantages include increased annual sample throughput because the freeze-dryer is used only for drying. Disadvantages include the greater risk of sample melt or contamination resulting from the need to transfer samples from the freezer into the drier.
2. Samples may be frozen in the absence of cooling by evacuating the container and relying on evaporative cooling to freeze the sample. However, the need to prevent sample foaming when the dryer is evacuated precludes the widespread use of the method.
3. Pellet freezing. Strictly this is not a method of freezing but can be useful when bulk products, including vaccines for subsequent powder filling, are processed. The suspension is sprayed into a cryogenic liquid or onto a cold surface to form frozen sample droplets, which are then be placed into trays or flasks for freeze-drying. Under these conditions, sublimation rates are typically very high because the thickness of the dry layer is restricted only by the pellet radius, and drying proceeds in a virtually unimpeded manner from each pellet.
4. The most widely used technique is to freeze the samples directly on the freeze-dryer shelf. Although this method has the disadvantage that the drier is used for part of the cycle as freezer, freezing and drying samples within a single machine eliminates the need to transfer samples from freezer to drier, and therefore improves sample cleanliness while reducing product vulnerability.

3. The Process of Water Removal

3.1. Sublimation and Drying (34)

Under atmospheric conditions, liquid water is converted into vapor by warming, a process defined as evaporation. However the three states of water ice, liquid, and vapor coexist at the triple point and illustrates that at subatmospheric pressures ice can convert directly to vapor by sublimation. Ice sublimation from a frozen sample results in an open, porous, dry structure where solutes are spatially arranged as in the original solution or suspension. In contrast to evaporation, where components are concentrated as drying progresses, sublimation under vacuum minimizes concentration effects providing a dry product that is active and readily soluble.

Having frozen the solution, the next step is to dry the sample by subliming ice directly into water vapor. In order to maintain freeze-drying conditions, it is essential to lower the partial pressure of water below the triple point (approx 800 mBar at 0°C) , to ensure the direct conversion of ice into water vapor and prevent sample melt. Vacuum will (1) reduce the air concentration above the product and encourage sublimation, and (2) ensure that air leaking into the system is removed.

3.2. Sublimation Rate and Chamber Pressure Conditions

Decreasing the chamber pressure below 0.8 mBar will increase the rate of sublimation by reducing the gas/vapor concentration above the sample to provide minimal resistance to water molecules migrating from the sample. Sublimation rate continues to increase, reaching a maximum at approximately mBar. Reducing system pressure further will not increase sublimation rate and indeed, contrary to expectations, at very low system pressures the sublimation rate will decrease.

This apparent paradox can be explained by assuming that two separate factors influence sublimation efficiency:

1. System pressure reduction sufficient to “thin” the chamber atmosphere and facilitate vapor migration from the sample.
2. A system pressure containing sufficient gas or vapor molecules in the chamber to conduct heat energy from the shelf into the sample. Essentially, under high-vacuum conditions a thermos flask effect is induced in the chamber, which inhibits heat transfer from the shelf. Under high-pressure (poor vacuum) conditions, heat transfer from the shelf to the sample is gas/vapor conduction in contrast to high-vacuum conditions where heat transfer by conduction is reduced and product heat is predominantly by radiation, which is a relatively inefficient mechanism.

Industrial and development freeze-dryers are routinely operated at a constant chamber pressure to facilitate heat transfer into the sample, and system pressure can be controlled by (1) bleeding air into the chamber, condenser, or vacuum

system, or (2) isolating the pump from the chamber to raise chamber pressure by increasing the number of water molecules migrating from the sample into the chamber.

Either method is equally effective. It is important to appreciate that improving the drying rate by bleeding air into the chamber does not "blow" water molecules from the product ***(36)***.

3.3. Vapor Differential Pressure and Drying Efficiency (36,37)

To sustain freeze-drying it is necessary to establish a pressure gradient from a sample (highest pressure), to condenser, and finally vacuum pump (lowest pressure) so that water migrates from the sample as drying progresses. Although the temperature of the sample must be higher than that of the condenser to ensure a net migration of water from the sample, the system driving force represents the difference in vapor pressure (VP) rather than the difference in temperature between sample and condenser, and can be calculated as the difference in VP between the two. For example, sample at –20°C has a VP = 0.78 torr and with the condenser at –40°C (equivalent to a VP of 0.097 torr), driving force will be 0.78–0.097 or 0.683 torr. Little improvement in driving force is achieved by operating the condenser at –70°C. (VP = 0.002 torr, providing a VP differential of 0.78 [sample] – 0.002 [condenser] of 0.778 torr.)

The example illustrates that greater sublimation efficiency is derived by increasing sample temperature rather than reducing condenser temperature, and the selection of suitable excipients that enable high processing temperatures to be used during freeze-drying without compromising sample quality plays an important role in process and cycle development.

3.4. Heat and Mass Transfer (36,37)

The essence of the freeze-drying process depends on maintaining a critical balance between the conversion of ice into water vapor by sublimation under vacuum and the removal of that vapor from the frozen mass. To maintain sublimation, heat energy is applied to the product to compensate for sublimation cooling. However, the heat extracted from the drying sample as water vapor must carefully balance the amount of energy added to the sample. Unless this equilibrium can be maintained, the product temperature will either decrease thereby reducing drying efficiency or increase, which may compromise product quality by inducing melt or collapse. This critical balance between sample warming to increase drying rate and vapor extraction is defined by the heat and mass transfer equation. In the early stages of sublimation the equilibrium is simple to maintain because the dry structure offers minimal resistance to vapor flow. However, as drying progresses and the depth of the dry layer increases, impedance to vapor flow will also increase and the sample

may warm sufficiently to melt or collapse unless the process temperature is reduced. One consequence of reducing the energy input will be to reduce drying rate and prolong cycle times, but this may be unavoidable if sample quality is to be preserved.

3.5. Cooling and Warming the Product (7,14,22)

The shelves fitted into the freeze-dryer to support sample containers may be alternatively cooled to initially freeze the sample or maintain shelf at a constant temperature throughout the drying cycle or warmed to provide energy for drying. Basically two systems may be fitted:

1. An independent cooling coil is embedded in the shelf through which cold refrigerant is supplied (this system is termed direct expansion) and a heating element is bonded into or onto the base of the shelf. Shelf control is maintained by alternately operating either the heater or cooler. Direct expansion systems are relatively inexpensive but fail to achieve temperature control much better than ±5°C.
2. For industrial or development activities, where shelf control to ±1°C is necessary to meet good manufacturing practice (GMP) requirements, a diatherm fluid, which is invariably silicone fluid, is circulated through the shelves and a separate refrigerator/heat exchanger maintains the diatherm fluid at a preset temperature.

The mechanism and the relative quantities of heat entering the product will depend on:

1. The nature of the product, its fill depth, consistency, and so on.
2. The dimensions and geometry of the sample container and whether the container rests directly on a shelf or is supported in a tray.
3. The freeze-dryer design.
4. Chamber vacuum conditions.

Product temperature can be maintained by either raising or reducing shelf temperature or by alternating system pressure which has the effect of improving; or reducing heat transfer efficiency as outlined in **Subheading 3.1.** Regardless of the precise system incorporated into the freeze-dryer, shelf temperature conditions may be controlled manually or programmed using a PC or microprocessor control.

3.6. The Drying Cycle

For clarity it is usual to separate the drying cycle into primary drying (the sublimation stage) and secondary drying or desorption.

3.6.1. Primary Drying

The first step in the drying cycle is defined as primary drying and represents the stage where ice, which constitutes between 70 and 90% of the sample

moisture, is converted into water vapor. Sublimation is a relatively efficient process although the precise length of primary drying will vary depending on the sample formulation, cake depth, and so on. During primary drying, the sample dries as a discrete boundary (the sublimation interface), which recedes through the sample from surface to base as drying progresses.

3.6.2. The Sublimation Interface **(7,8,14,32,33)**

Variously described as the drying front, freeze-drying front, and so on, macroscopically the sublimation interface can be observed as a discrete boundary that moves through the frozen sample to form an increasingly deeper layer of dried sample above the frozen sample. Heat is conducted from the shelf through the vial base and the frozen sample layer to the sublimation front where ice is converted into water vapor. Several consequences result from this progressive recession of the sublimation front through the dry layer, which include:

1. The maintenance of the frozen zone at a low temperature because of sublimation cooling.
2. An increase in the resistance to vapor migration and a decrease in sublimation rate as the dry layer increases in thickness.
3. Because the sublimation interface represents a zone representing maximum change of sample temperature and moisture content, the interface represents the zone over which structural softening or collapse is likely to occur.
4. Water migrating from the sublimation front can reabsorb into the dried material above the sublimation interface.

Because the sublimation interface is the region where freeze-drying takes place, temperature monitoring of the interface is of paramount importance for product monitoring. However, because the sublimation front is constantly moving through the sample, interface temperature cannot be effectively monitored using traditional temperature probes. Although the sublimation interface is defined as a discrete boundary, this is true only for ideal eutectic formulations, where ice crystals are large, open, and contiguous with each other. For typical amorphous formulations, such as vaccines, the sublimation front is much broader and comprises individual ice crystals imbedded in the amorphous phase. Under these conditions, although ice sublimes within the isolated crystals, the water vapor must diffuse through the amorphous phase (which is itself progressively drying) until it can migrate freely from the drying sample matrix. Under these conditions, sublimation rates are much lower than those anticipated from data derived using eutectic model systems. Complicating a precise prediction of sublimation rate is the fact that fractures in the dry cake between the ice crystals can improve drying efficiency. All of these factors, including system impedances caused by the development of a surface skin on the sample, have to

be considered during sample formulation and cycle development programs. Notwithstanding these complications in precisely defining primary drying, sublimation is nevertheless a relatively efficient process and conditions used for primary drying include the use of shelf temperatures high enough to accelerate sublimation without comprising sample quality by inducing collapse or melt, combined with high system pressures designed to optimize heat conduction from shelf into product. Removing the product when sublimation has been judged as complete will provide a vaccine which appears dry but which displays a high-moisture content that is invariably too high (7–10%) to provide long-term storage stability, and the drying cycle is extended to remove additional moisture by desorption or secondary drying.

3.6.3. Secondary Drying

In contrast to primary drying, which is a dynamic process associated with high vapor flow rates, secondary drying is much less efficient with secondary drying times representing 30–40% of the total process time but only removing 5–10% of the total sample moisture. Under secondary drying conditions, the sample approaches steady-state conditions where moisture is desorbed or absorbed from or into the sample in response to relative humidity and shelf temperatures. Desorption is favored by increasing shelf temperature, using high-vacuum conditions in the chamber, thereby reducing the system vapor pressure or relative humidity. Conversely, when the shelf temperature is reduced and the vapor pressure in the system increased by warming the condenser, dried samples will reabsorb moisture and exhibit an increase in moisture content. Although sample collapse during secondary drying is generally less likely than collapse during primary drying, it is possible to induce collapse in the dried matrix by exposing the sample to temperature above its glass transition temperature (Tg).

3.6.4. Stoppering the Product

A freeze-dried product is both hygroscopic and has an enormously exposed surface area. Consequently, exposing the dried product to atmosphere will result in reabsorption of damp air into the product. Both water and air are damaging to a dried sample, causing degradative changes resulting in poor stability and it is therefore prudent to stopper samples within the freeze-dryer prior to removal. Stoppering under a full vacuum provides ideal conditions for ensuring product stability because reactive atmospheric gases are reduced to a minimum. However, injecting water into a sample in a fully evacuated vial can induce foaming, which can be reduced by filling vials with an inert gas, such as nitrogen, before stoppering.

4. Reconstituting the Product

It is often supposed that because freeze-drying only removes water, then all products will be fully active by rehydrating only with water. This may not be the case and freeze-dried products often exhibit enhanced activity when reconstituted in an isotonic medium, such as saline, rather than water.

5. Freeze-Dryer Design *(6,22,38–40)*

The need to operate the freeze-dryer under low-pressure conditions to convert ice directly into water vapor (a process termed sublimation) adds to the complexity and cost of dryer because the chamber holding the sample must withstand the differential pressure from vacuum to atmosphere. Although a suitable vacuum pump is essential for initially evacuating the chamber and eliminating air that may leak into the dryer during operation, vacuum pumps are not capable of continuously removing water vapor subliming from the sample and a refrigerated trap (termed the process condenser) must be placed between the sample and the pump to condense the moisture migrating from the drying sample. In reality, it is the condenser that comprises the "pumping force" of the system. Process condensers may be incorporated into the drying chamber (referred as an internal condenser) or located in a separate chamber between the sample chamber and pump (external condenser). Each geometry has advantages and disadvantages although either design may be used. Stainless steel is typically used to fabricate research or production dryers because this metal can be cleaned by a wide range of sanitizers including steam. For GMP manufacture the freeze-dryer is invariably sterilized by pressurized steam and this adds to the complexity and expense of the dryer because it has to conform to the requirements to operate under subatmospheric and pressure conditions. Modern freeze-dryers are also fitted with internal stoppering devices for sealing vials at the end of the cycle, valves and monitoring devices for assessing drying efficiency, and are typically computer or microprocessor controlled so that cycles can be reproduced and evaluated for regulatory purposes. When freeze-drying vaccines, it may be necessary to incorporate protective devices and introduce processing protocols that ensure both safe operation and prevent product cross-contamination.

6. Sample Damage During Freeze-Drying *(13,23,27,31–33)*

Damage to a freeze-dried product may occur:

1. When the solution is cooled (described as cold or chill shock).
2. During freezing as the ice forms and the unfrozen solute phase concentrates.
3. During drying, particularly when the sample collapses as drying progresses.
4. By protein polymerization when high shelf temperature are used for secondary drying.

5. During drying and storage because of damage by reactive gases, such as oxygen, and it is important to appreciate that even in a vacuum, sufficient gas molecules will be present in the sealed sample to cause inactivation.
6. During storage by free radical damage or Maillard reactions.
7. During reconstitution, particularly if the sample is poorly soluble.

6.1. Chill Damage (Cold Shock)

Reducing temperature in the absence of ice formation is generally not damaging to biomolecules or live organisms, although sensitive biopolymers may be damaged by cold shock ***(39)***.

6.2. Freezing Damage (9,28)

Reducing temperature in the presence of ice formation is the first major stress imposed on a biomolecule. Direct damage by ice is not generally damaging except when living cells are frozen at very fast rates, which may induce the formation of intracellular ice within the cell. Biomolecules are more likely to be damaged by an increase in solute concentration as ice forms. We have described how bacteria can remain fully viable when cooled to –18°C in the absence of ice formation, but when frozen to this temperature, viability was reduced to 60%. Freezing will result in:

1. Ice formation ***(40)***.
2. A rise in solute concentration (this effect can be appreciable and a 1% solution of sodium chloride will increase to 30% by freeze concentration as ice forms) ***(41)***.
3. Changes in solution tonicity ***(42)***.
4. Concentration of all solutes, including cells and biomolecules that are encouraged to aggregate ***(43,44)***.
5. An increase in solute concentration that may result in "salting out" of protein molecules ***(43)***.
6. Differential crystallization of individual buffer salts resulting in marked changes in solution pH as the solution freezes ***(44)***.
7. Concentration of potentially toxic impurities above a toxic threshold sufficient for the impurities to become toxic ***(44)***.
8. Disruption of sulfur bonds.
9. Generation of anaerobic conditions as freezing progresses.

7. Factors Effecting Dried Products

Freeze-dried vaccines should be formulated to minimize storage decay and should tolerate storage at ambient temperatures for distribution purposes. However, it is a fallacy to suppose that a freeze-dried product remains immune to damage during storage and factors which damage freeze-dried products include:

1. Temperature. Whereas a freeze-dried product is more shelf stable than its solution counterpart, freeze-dried materials are sensitive to thermal decay and will be influenced by storage temperature ***(45)***.
2. Moisture content ***(46–53)***.
3. Reactive gases ***(54)***.
4. Light.
5. Free radical damage ***(55)***.
6. Background nuclear radiation.
7. Specific chemical reactions including Maillard reactions ***(56)***.

The interrelationship between sample formulation, dried cake moisture cake, storage conditions, and glass transition temperature (Tg) are complex. In general terms, any physical distortion of the dry cake during storage will often result in a much more rapid loss of sample activity than predicted using the Arrhenius equation for reviewing similar samples ***(21,29,32,33,57–59)***.

7.1. Influence of Suspending Medium Composition on Survival of Live Cells to Freeze-Drying

Attempts to freeze-dry cells in water or a simple salt solution typically result in poor survival. A wide range of protective media has been developed for preserving freeze-dried vaccines, including augmented growth media or sugar solutions. Carbohydrates are widely used as freeze-drying protectants either individually or in combination with other solutes. They should be chosen on the basis of experimentally determining their freeze-drying characteristics rather than on a pragmatic basis. Monosaccharides, such as glucose, provide good bioprotection during freezing and freeze-drying but exhibit low glass transition (Tg′) or collapse temperatures (Tc) and dry with collapse when orthodox freeze-drying cycles are used. Disaccharides are effective freeze-drying protectants, and because they display higher collapse than monosaccharides, freeze-dry successfully when conventional drying cycles are used. Reducing sugars may induce damaging Maillard reactions, thereby compromising stability, and for this reason nonreducing disaccharides, such as sucrose or trehalose, are preferred to reducing sugars such as lactose ***(62)***. The addition of salts to formulations containing sugars will markedly depress Tg′ or Tc. ***(1,3,20,23,31,58,60)***.

Although presenting technical difficulties such as sample collapse, during freeze-drying the amorphous phase may be an essential prerequisite for stabilizing biomaterials, such as vaccines and live cells, by providing an integration of the protective additive, and biomolecule thereby minimizing damage level during freeze-drying and drying.

It is not possible in a review of this length to provide anything other than an overview of freeze-drying relevant to the freeze-drying of labile biomolecules and both inactivated or attenuated vaccines. In producing this chapter, the

author nevertheless has attempted to examine all aspects of freeze-drying, because even a basic understanding of this complex technology requires an appreciation of all the factors in the process that can influence the acceptability or unacceptability, safety, and efficacy of the preparation.

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3

Principles of Cryopreservation

David E. Pegg

Summary

Cryopreservation is the use of very low temperatures to preserve structurally intact living cells and tissues. Unprotected freezing is normally lethal and this chapter seeks to analyze some of the mechanisms involved and to show how cooling can be used to produce stable conditions that preserve life. The biological effects of cooling are dominated by the freezing of water, which results in the concentration of the solutes that are dissolved in the remaining liquid phase. Rival theories of freezing injury have envisaged either that ice crystals pierce or tease apart the cells, destroying them by direct mechanical action, or that damage is from secondary effects via changes in the composition of the liquid phase. Cryoprotectants, simply by increasing the total concentration of all solutes in the system, reduce the amount of ice formed at any given temperature; but to be biologically acceptable they must be able to penetrate into the cells and have low toxicity. Many compounds have such properties, including glycerol, dimethyl sulfoxide, ethanediol, and propanediol.

In fact, both damaging mechanisms are important, their relative contributions depending on cell type, cooling rate, and warming rate. A consensus has developed that intracellular freezing is dangerous, whereas extracellular ice is harmless. If the water permeability of the cell membrane is known it is possible to predict the effect of cooling rate on cell survival and the optimum rate will be a tradeoff between the risk of intracellular freezing and effects of the concentrated solutes. However, extracellular ice is not always innocuous: densely packed cells are more likely to be damaged by mechanical stresses within the channels where they are sequestered and with complex multicellular systems it is imperative not only to secure cell survival but also to avoid damage to the extracellular structure. Ice can be avoided by vitrification—the production of a glassy state that is defined by the viscosity reaching a sufficiently high value (~10^{13} poises) to behave like a solid, but without any crystallization. Toxicity is the major problem in the use of vitrification methods.

Whether freezing is permitted (*conventional cryopreservation*) or prevented (*vitrification*), the cryoprotectant has to gain access to all parts of the system. However, there are numerous barriers to the free diffusion of solutes (membranes), and these can result in transient, and sometimes equilibrium, changes in compartment volumes and these can be damaging. Hence, the processes

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of diffusion and osmosis have important effects during the introduction of cryoprotectants, the removal of cryoprotectants, the freezing process, and during thawing. These phenomena are amenable to experiment and analysis, and this has made it possible to develop effective methods for the preservation of a very wide range of cells and some tissues; these methods have found widespread applications in biology and medicine.

Key Words: Cryopreservation; cryoprotectants; intracellular freezing; solution effects; supercooling; vitrification.

1. Freezing Injury and Cryoprotection

1.1. The Discovery of the Cryoprotective Effect of Glycerol

Writers of science fiction have been greatly attracted by the concept of "suspended animation," whereby the biochemistry of life could be reversibly suspended for long periods of time and then restored. Although such phenomena do occur in nature, though rarely, it is unfortunately a fact that freezing is normally lethal. In order to understand the effects of very low temperatures, we have to recognize that many structures and processes are temperature dependent and, consequently, cooling has extraordinarily complex effects that produce conditions that are far removed from normal physiology. When we cool below 0°C the biological effects are dominated by the freezing of water, which typically constitutes at least 80% of the tissue mass. Freezing is the conversion of liquid water to crystalline ice, which results in the concentration of dissolved solutes in the remaining liquid phase and the precipitation of any solutes that exceed their solubility limit. It was not until 1948 that a general method was discovered that permitted the freezing of many types of animal cells with subsequent restoration of structure and function. In 1949, Polge, Smith, and Parkes published their landmark paper ***(1)*** in which they showed that the inclusion of 10–20% of glycerol enabled the spermatozoa of the cock to survive prolonged freezing at –80°C. The theories of freezing injury then extant envisaged ice crystals piercing or teasing apart the cells and intracellular structures, destroying them by direct mechanical action. Glycerol, simply by increasing the total solute concentration, would reduce the amount of ice formed in the same way that antifreeze (ethanediol) reduces the amount of ice forming in the cooling system of an automobile engine. But it was also recognized very early on that one effect of freezing an aqueous solution was to increase the concentration of solutes in the dwindling volume of the remaining solution, and that this could be a fundamental cause of injury. In a series of classical papers published in the 1950s, James Lovelock ***(2,3)*** provided strong evidence that salt concentration, rather than ice, is the cause of freezing injury to cells, and that glycerol protects against this damage only to the extent that it modulates the rise in salt concentration during

freezing. It follows that the effectiveness of glycerol, or of any similar cryoprotectant, depends on a number of properties: (1) the compound must be highly soluble in water and remain so at low temperatures in order to produce a profound depression of the freezing temperature; (2) it must be able to penetrate into the cells; and (3) it must have a low toxicity so that it can be used in the high concentrations that are required to produce these effects. Many compounds have these properties. Those in common use include glycerol, dimethyl sulfoxide, ethanediol, and propanediol.

1.2. The Effect of Rate of Change of Temperature

That degree of understanding provided a starting point for the development of practical freeze-preservation techniques for a range of cells, but it soon became clear that reality was considerably more complex. First cooling rate, and then warming rate, was found to be important determinants of survival and Lovelock's theories did not account for such kinetic effects. In 1963, Mazur discovered that the rate of change of temperature was important because it controlled the transport of water across the cell membrane, and hence, indirectly, the probability of intracellular freezing ***(4)***. In general, intracellular freezing is lethal. Mazur argued that the rate of cooling controls the rate at which water is converted to ice; hence it controls the rate at which the concentration of the solution surrounding the cells changes; therefore, by controlling the osmolality of the surrounding fluid, the rate of change of temperature also influences the rate at which water is transported out of the cells during cooling and into the cells during warming. Providing water can leave the cells rapidly to maintain thermodynamic equilibrium across the cell membrane, the cytoplasm will not cool below its freezing point (supercool), and all the ice will be external to the cells. On the other hand, if the cooling rate is too rapid for the membrane of the cell in question to transport sufficient water out of the cell, then the protoplasm will become supercooled, and the greater the extent of supercooling, the more likely is the cell to freeze internally (**Fig. 1**). The combination of these two factors, solution effects and intracellular freezing, causes each cell to show maximal survival at a characteristic cooling rate; as the cooling rate increases from very low rates so does survival because the deleterious effects of exposure to high salt concentrations are reduced, but eventually survival drops off because intracellular freezing supervenes. Each cell has an optimum cooling rate (**Fig. 2**), although absolute survival is usually extremely low unless a cryoprotectant is present to reduce the damage at low cooling rates. Cryoprotectants, like glycerol, have the effect of reducing the solution effects, resulting in a lower optimal cooling rate and an increase in the maximum survival obtained (**Fig. 3**). We

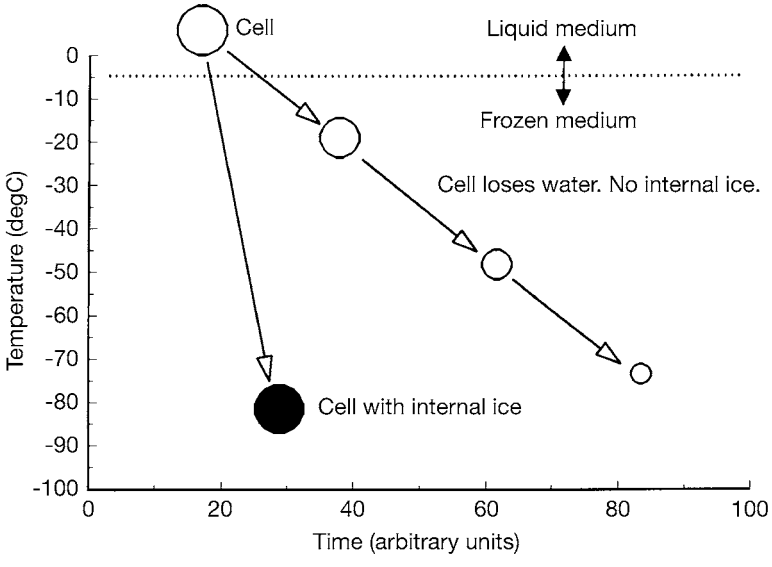

Fig. 1. Schematic representation of cells being cooled rapidly, and freezing internally or sufficiently slowly to lose water and avoid intracellular ice.

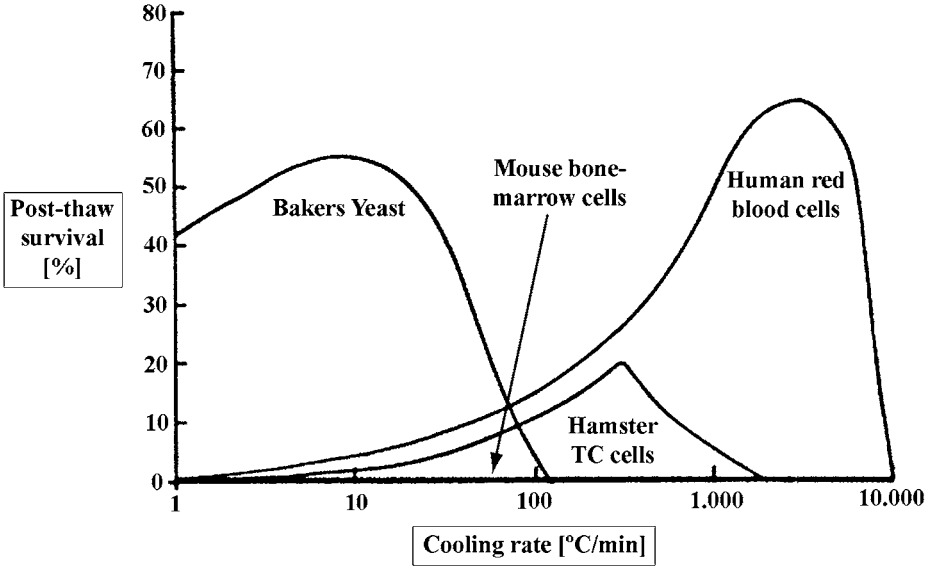

Fig. 2. The effect of cooling rate on the survival following freezing of four types of cell. (Based on **ref. 5**.)

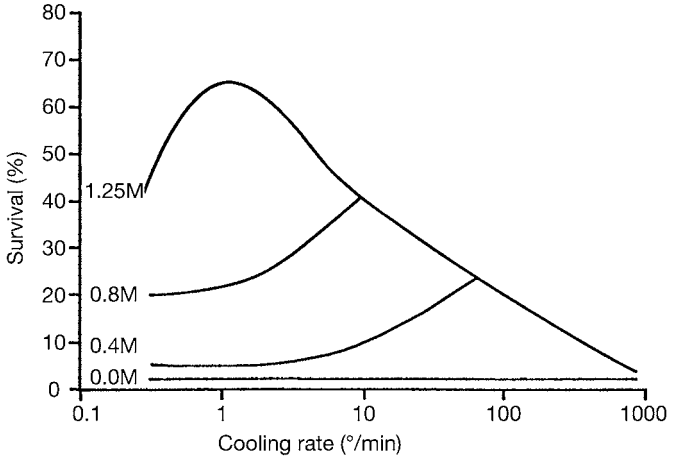

Fig. 3. The effect of cooling rate on the cryopreservation of mouse hemopoietic stem cells cooled in the presence of the indicated molar concentrations of glycerol. (Reprinted with permission from **ref. *6***.)

will now examine these mechanisms of cryoinjury and cryoprotection in a little more detail.

1.3. Solution Effects

Lovelock ***(2)*** had actually shown that the extent of hemolysis that occurred when a saline suspension of erythrocytes was cooled to and thawed from a given subzero temperature was similar to that suffered by a cell suspension that was exposed to the concentration of sodium chloride produced by freezing to that temperature and then returning to isotonic saline (**Fig. 4**). Lovelock also demonstrated ***(3)*** that when glycerol was present, hemolysis started at the (lower) temperature at which the same critical concentration of salt was produced (**Fig. 5**). Correlation does not prove causation, but in this case, if the solution changes were not causative of freezing injury, then the correspondence would be a remarkable coincidence indeed. It was these studies that led to the consensus that extracellular ice is harmless to cells and that freezing injury is caused by indirect effects of the formation of ice. However, the salt in the suspending medium is not the only solute to be concentrated—the cryoprotectant is concentrated to the same degree. Pegg and Diaper ***(7)*** showed that red blood cells actually suffer more damage when exposed to a given salt concentration in the presence of glycerol than in its absence, and this effect is dependent on the concentration of glycerol (**Fig. 6**). When red blood cells were frozen and thawed in the presence of a range of

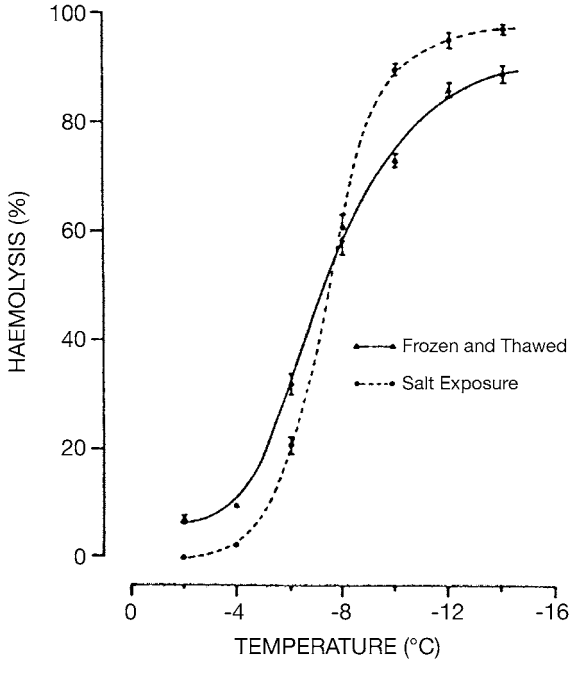

Fig. 4. Human erythrocytes were frozen to the indicated temperatures and then thawed (triangles) compared with exposure to equivalent salt concentrations and then being returned to isotonic conditions (circles). (Reprinted with permission from **ref. *8***.)

concentrations of glycerol, they demonstrated that the correspondence between the effects of salt exposure and of freezing was retained. This observation is important for two reasons: it shows that cryoprotectants are not innocuous—overall they are protective but at a price; second, the observation adds powerful support to the solution-effect theory.

1.4. Intracellular Freezing

If the water permeability of the cell membrane is known, and the temperature coefficient of water permeability can be estimated, then it is possible to predict the effect of cooling rate on cell survival (**Fig. 7**). The calculated degree of supercooling for different rates of cooling shows that intracellular freezing is unlikely at 1°C/min but is highly probable at a cooling rate of 10°C/min. For hepatocytes a cooling rate around 1°C/min will essentially eliminate the risk of intracellular freezing and faster cooling will be preferred only if solution effects are a problem. The optimum rate will be a tradeoff between those two factors. Of course, other cells have different water permeabilities and it has been shown by direct experiment that the cooling rate that

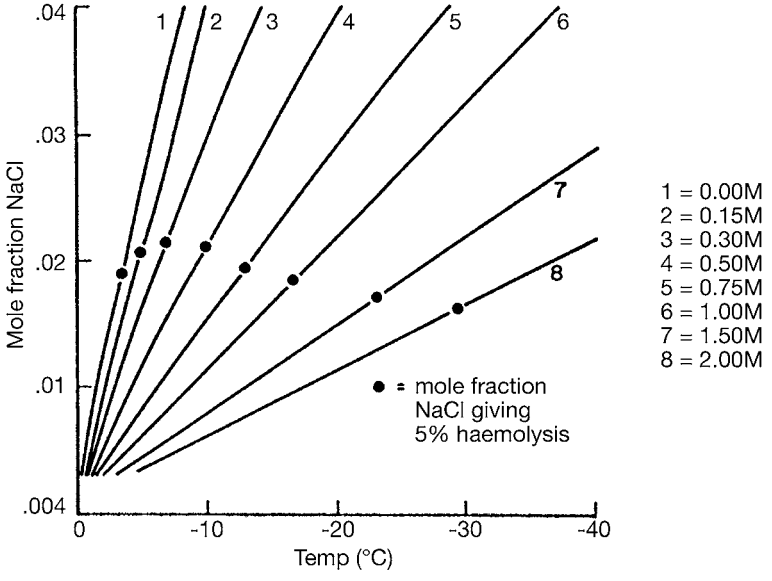

Fig. 5. The increase in mole fraction of NaCl in solutions that have the indicated molality of glycerol and are isotonic with respect to NaCl. Five percent hemolysis was observed at a mole fraction of NaCI = 0.016 – 0.021. (Reprinted with permission from **ref. *9***.)

produces intracellular freezing on a cryomicroscope corresponds with the cooling rate that produces significant intracellular supercooling (***[10]***; **Fig. 8**). In fact, very small amounts of intracellular ice are compatible with recovery, and this is one reason why the warming rate has a profound effect. The behavior of very small intracellular ice crystals differs between slow and rapid warming: slow warming allows the crystals to recrystallize, to coalesce, and to grow. This has been demonstrated to damage the cells in which it occurs; however, during rapid warming there is insufficient time for this to happen and the ice simply melts. Because the cooling rate influences the formation of intracellular ice, while warming rate controls what happens to that ice subsequently, and because cells differ in their water permeability and probably also in their susceptibility to intracellular ice, then it follows that cells will differ in their cooling and warming requirements and cooling rate will interact with warming rate.

1.5. The Cell Packing Effect

Most studies of freezing injury have been carried out with relatively dilute cell suspensions, whereas the cells are quite densely packed in some systems that have to be preserved, for example red blood cells for transfusion, and

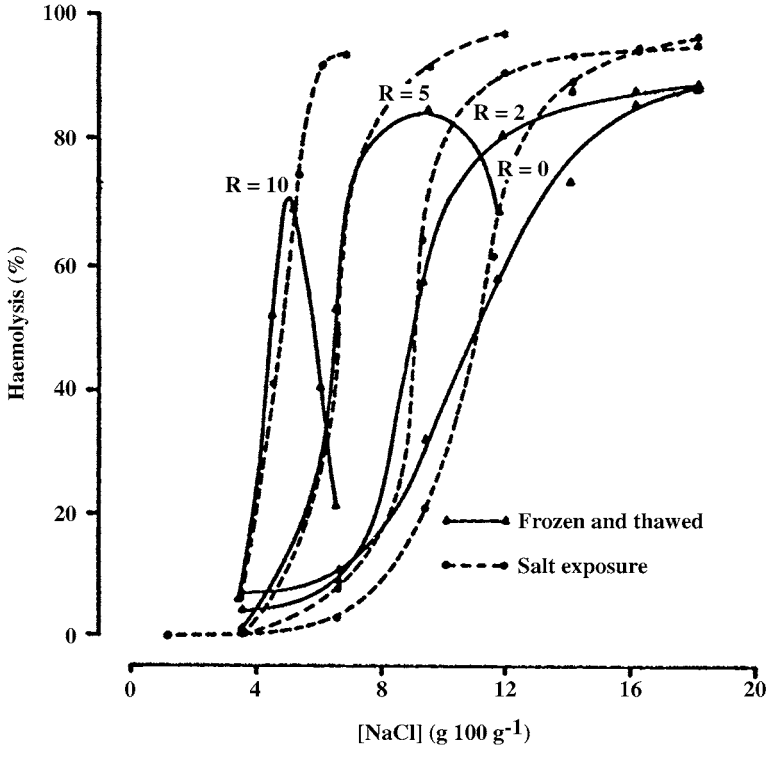

Fig. 6. Hemolysis observed when human erythrocytes were frozen to temperatures that produce the indicated concentrations of NaCl, compared with exposure to equivalent salt concentrations followed by return to isotonic conditions. The R values are the weight ratio of glycerol to NaCl in each solution. (Reprinted with permission from **ref. *8***.)

particularly in tissues and organs. Experiment has shown that the proportion of red blood cells suspended in 2.5 *M* glycerol solution that are hemolyzed during freezing and thawing is strongly dependent on hematocrit (the percentage of cells by volume) when the hematocrit exceeds 50%. The increase in hemolysis as the hematocrit is increased is ameliorated by increasing the glycerol concentration. At 2 *M* glycerol concentration, hemolysis is inversely dependent on warming rate when the cooling rate is less than 1000°C/min and is directly dependent on cooling rates at higher cooling rates ***(11)***.

These observations cannot be accounted for by the classical mechanisms of cryoinjury—solution effects and intracellular freezing. The most likely explanation is that densely packed cells are more likely to be damaged by mechanical stresses when the channels within which they are sequestered change shape as a result of recrystallization of the ice that form their boundaries.

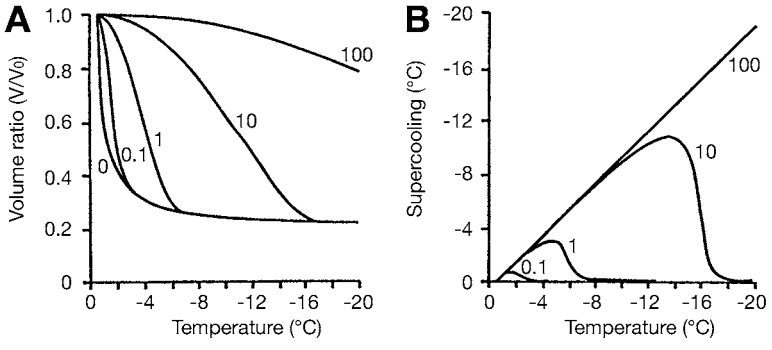

Fig. 7. The calculated effect of cooling rate on the volume of hepatocytes and the extent of supercooling of the cell contents. **(A)** Relative volume (v/v_0) of cells cooled at the indicated rates (°C/min). The line labeled 0 is the equilibrium line. **(B)** From the same calculations as in **A**, the calculated degree of supercooling of the cell contents at the indicated cooling rates. At 10°C/min the cells are supercooled by 10°C and therefore likely to freeze internally. (Reproduced with permission from **ref.** ***12***.)

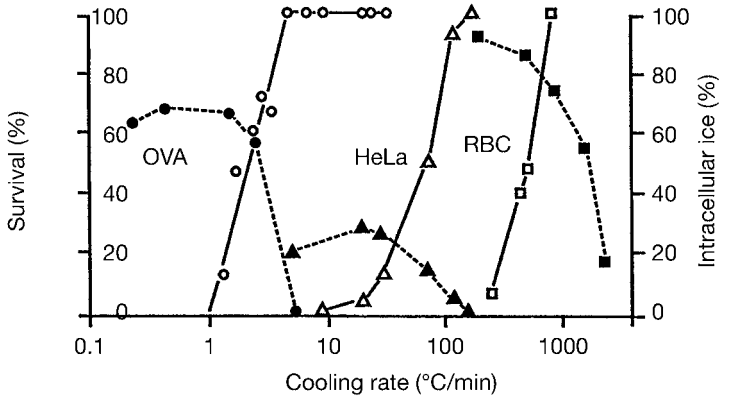

Fig. 8. The survival of three types of cells plotted against cooling rate and correlated with the observed occurrence of intracellular freezing. (Reproduced with permission from **ref.** ***10***.)

2. Cyroprotection

2.1. General Considerations

Cryoprotection usually involves treatment of the cells or tissues with cryoprotectant solutes, often in high concentration, and this produces a driving force for the movement of water by osmosis and of solutes by diffusion. Freezing involves changes in the concentration and composition of aqueous solutions and this also produces driving forces for the movement of water and

solutes. Biological systems contain numerous barriers to the free diffusion of solutes (membranes), and these can result in transient, and sometimes equilibrium, changes in compartment volumes; if excessive, these changes can be damaging. Hence, the processes of diffusion and osmosis are very important for cryopreservation. Fortunately, the quantitative description of mass transfer processes is well developed ***(13,14)***.

The driving force for flow is pressure. Thus, the flow of water, J_v, through a membrane is given by

$$J_v = k.P$$

where k is a constant that is characteristic of the membrane-and-water-combination and P is the pressure difference across the filter. J is given the subscript v to signify volumetric flux. When the driving force for the flow of water through a membrane is osmotic pressure rather than hydrostatic pressure, flow can be described by the same equation if osmotic pressure, π, is substituted for hydrostatic pressure, thus,

$$J_v = k.\pi$$

The constant k has the same value in the two equations, providing only that the membrane and the solvent, water in this case, remain the same. Thus, both hydrostatic and osmotic pressure differences can be incorporated into a single equation. The constant k is then known as the hydraulic conductivity L_p. (The units are cm/atm/s.)

$$J_v = L_p.(P + \pi)$$

Under the conditions prevailing in cryobiology the hydrostatic pressure term will normally be zero and π can be calculated from concentration by multiplication by the product of the universal gas constant R, and the absolute temperature T. If the area of the membrane is A, and internal and external osmolalities are denoted by C_i and C_e, then we obtain

$$J_v = L_p.A.R.T.(C_i - C_e)$$

The solute flux J_s is described by

$$J_s = \omega_s.A.R.T.(S_e - S_i)$$

This equation states that flux across unit area of membrane is proportional to the solute permeability ω_s, and the difference in concentration of the solute across the membrane. The more familiar solute permeability P_s (units are cm/s) is equal to $\omega_s RT$. The constant RT is 23,235 atm.cm^3/mole. The convention for the direction of flux is that outside $\rightarrow$ inside is positive. A somewhat more complex formalism was elaborated by Kedem and Katchalsky ***(13)*** in 1958 and their equations are often used in cryobiology where they are usually referred to as the

K–K equations. Kedem and Katchalsky assumed that the solvent and solute used a common channel through the membrane and they therefore added a solvent/solute interaction term, σ, known as the reflection coefficient. This led to modification of the equations for J_v and J_s, as shown

$$J_v = L_p.A.R.T.[(C_i - C_e) + \sigma(C^p_i - C^p_e)]$$

$$J_s = \omega_s.A.R.T.[(S_e - S_i) + J_v(1 - \sigma)c_s]$$

In the equation for J_v the solutes are partitioned between impermeant solutes (C) and permeating solutes (C^p), the latter interacting with water in the common flow channel. The equation for J_s has an additional term that represents solvent drag on the permeant solute, which is present in the membrane at concentration c_s. Clearly, the K–K is more complex and curve fitting routines can lead to uncertain results because of the lack of independence of the parameter, σ. Kleinhans ***(14)*** has discussed these problems in detail and moreover he has argued that the K–K formalism is often invalid because of the presence of separate channels for water and solute. In practice, the simpler formalism is adequate for the current needs of cryobiologists. The two equations are solved simultaneously by numerical methods and programs to carry out these calculations can be run on an ordinary PC.

Several methods are available for the determination of permeability parameters in cryobiology. If the solute under study can be radiolabeled, the time-course of isotope uptake is easily measured but the calculation of concentration requires the additional measurement of water content at each time-point. Permeating solutes can be extracted after known times of exposure and high-performance liquid chromatography methods are often suitable for their assay. The Karl Fischer method, using a backtitration scheme, is a convenient method for water ***(15)***. If the compound under study has a distinctive nuclear magnetic resonance spectrum, nuclear magnetic resonance can be used to determine the time-course of both solute and water content simultaneously, so this technique yields concentration directly. A commonly used indirect method for isolated cells is to record the time-course of cell volume following exposure to a known concentration of the compound by Coulter counter- or light-scattering methods; the equations described previously are then used to model the experimental data and derive estimates of L_p and P_s. We will now consider in more detail some situations in which these permeability parameters are relevant to cryopreservation.

2.2. Introduction of Cryoprotectants

The exposure of cells to a high concentration of cryoprotectant causes osmotic dehydration. If the cryoprotective compound permeates, the cells then increase in volume, water entering along with the cryoprotectant until the cells

reach their final volume. The extent of shrinkage and the rate of change in cell volume are determined by the permeability parameters. The final equilibrium volume depends on the concentration of impermeant solutes in the solution and is the same as the normal volume only if the concentration of impermeant solutes is isotonic in molar (per liter) terms. This is because of the fact that the cryoprotectant occupies space within the cells and the volume of water must therefore be lower than the physiological water content if the total volume is to be normal ***(16)***. The rate of change of volume, and particularly the equilibrium volume, are both important and must be optimized in cryopreservation procedures.

2.3. Removal of Cryoprotectants

When a permeating cryoprotectant is removed by exposing the cells to a lower concentration of the compound, the osmotic uptake of water causes the cells to swell above their initial volume. They then shrink as the cryoprotectant moves out, accompanied by sufficient water to maintain osmotic equilibrium; they return to physiological volume only if nonpermeating solute has neither been lost nor gained during the process. Because cells are generally more sensitive to swelling than to shrinkage, removal of cryoprotectants tends to be more hazardous than their addition. Again, both the rate of change of volume and the final volume must be considered when designing protocols for the recovery of cryopreserved cells.

2.4. Freezing and Thawing

Freezing causes the solution surrounding the cells to concentrate, and as a consequence the cells shrink at a rate that depends upon the rate of formation of ice, the cell's Lp and its temperature coefficient, and temperature itself. This phenomenon is an extremely important determinant of intracellular freezing. The final extent of shrinkage depends on the cryoprotectant concentration.

2.5. Exposure to Nonpermeating Solutes

Cells immersed in a solution of nonpermeating solute reach an equilibrium volume that is an inverse function of the osmolality of the solution; ideally,

$$V_{rel} = \frac{1}{M_{rel}}$$

where V_{rel} is the volume of intracellular water relative to the physiological water content and M_{rel} is the external osmolality relative to its physiological value.

This relationship requires that a plot of V_{rel} against M_{rel}, which is known as a Boyle van't Hoff plot, is a straight line of slope = 1, which must pass through the origin because water content is zero at infinite osmolality ***(17)***. In reality there is always an intercept on the *y*-axis—the so-called nonosmotic water

volume or V_{inf}. This probably represents a physically distinct portion of the cell water that is so structured that it does not participate in solution phenomena. (Alternatively, it could reflect the nonideal behavior of the intracellular solutes such that osmolality increases with concentration more than in linear proportion.) The value of V_{inf}, determined by a Boyle van't Hoff plot, is needed to interpret volume/time data for the cells in question and to calculate intracellular concentrations of permeating solutes. Experimentally, the collection of such data can usefully be combined with determining the upper and lower volume limits that the cells will tolerate without damage.

3. Preservation of Cells and Tissues

3.1. Preservation of Cells

The basic cryobiological knowledge reviewed here has made it possible to develop effective methods for the preservation of a very wide range of cells, and these have found widespread applications in biology and medicine. Examples include the long-term preservation of spermatozoa of many species, including cattle, laboratory animals, and man, very early embryos and ova, red and white blood cells, hemopoietic stem cells, tissue culture cells, and so on (*see* following chapters in this volume). For each type of cell there is a set of conditions that is optimal for preservation, determined by the interaction of the particular properties of the cell in question with the cryobiological factors that have been discussed. If the characteristics of the cell are known, it is usually possible to predict with reasonable precision the conditions that will provide effective cryopreservation.

3.2. Preservation of Multicellular Systems

The situation becomes much more difficult when we move from single cells to complex multicellular systems. Cell survival is still required, of course, but tissues and organs contain a heterogeneous collection of cells, which may have quite different optimum requirements for preservation, unlike the situation in cell preservation where one is usually dealing with a single type of cell. Yet it is necessary to find a method that will secure adequate survival of all the cells that are important for the function of that tissue. Fortunately, the use of high concentrations of cryoprotectant results in a flattening of the bell-shaped survival curve and a broadening of its peak: with sufficiently high concentrations of cryoprotectant it is possible to secure overlapping survival curves for many different cells. Another problem is that it is not sufficient to obtain high levels of survival for the various types of cell that are present in tissues and organs; it is also imperative to avoid damage to important extracellular structures and to retain normal interconnections between the cells and their attachments to basement membranes ***(18)***. Ice that forms outside the cells when a cell suspension

is frozen is outside the system that it is desired to preserve, and it can damage the cells only by indirect means (solution effects) or by exerting a shear or compressive force on them externally. The situation is quite different for organized tissues; here, extracellular ice is still within the system that is to be preserved and can disrupt the structure of the tissue directly. The first evidence of such an effect was provided by Taylor and Pegg ***(17)*** when they showed that smooth muscle, frozen to –21°C by cooling at 2°C/min in the presence of 2.56 *M* dimethyl sulfoxide was functionally damaged, whereas exposure to the solution conditions produced by freezing that solution to that temperature, at the same temperature, was innocuous. Structural studies using freeze substitution showed that ice formed within the muscle bundles ***(20)***. If cooling was slowed to 0.3°C/min, freezing produced less damage and ice was shown to form only between the muscle bundles. This showed that extracellular ice damaged this tissue, but the extent of such damage was dependent on the site at which the ice formed. Damaging effects of extracellular ice have also been demonstrated in kidneys and livers, where it has been shown to cause rupture of the capillaries. Rubinsky and Pegg ***(12)*** have proposed a mechanism for this effect; ice forms within the vessel lumens, drawing in water from the surrounding tissue until the volume of intraluminal ice exceeds the elastic capacity of vessel and rupture ensues. In organs and tissues that require an intact vasculature for function, vascular rupture is lethal, even if many cells survive, and this mechanism provides the major barrier to effective cryopreservation of such systems. The avoidance of freezing, or at least limitation of the amount of ice to very small quantities in the least susceptible locations, seems to be the only way to avoid this problem. Attempts to cryopreserve complex multicellular systems simply by adapting techniques from single-cell systems have generally been unrewarding. In the medical field, the situation may be more favorable with tissues that can be transplanted without revascularization; it all depends on the precise requirements for surgical acceptability. For example, the primary requirement for heart valve grafts is that the collagen structure is intact, and it is unclear whether the survival of donor fibroblasts has any useful effect. Similarly, human skin can be cryopreserved by methods similar to those used for cell suspensions and will then retain significant numbers of viable keratinocytes, although it is questionable whether these influence the clinical results when skin grafts are used as a temporary covering on seriously burned patients. For other tissues, such as small elastic arteries, satisfactory methods have only been developed relatively recently ***(21)***. For corneas and cartilage and for whole vascularized organs there are no effective methods.

3.3. Vitrification Methods

Much of the very early work in cryobiology, notably by Luyet ***(22)***, had been based on the assumption that freezing damaged cells directly and, consequently,

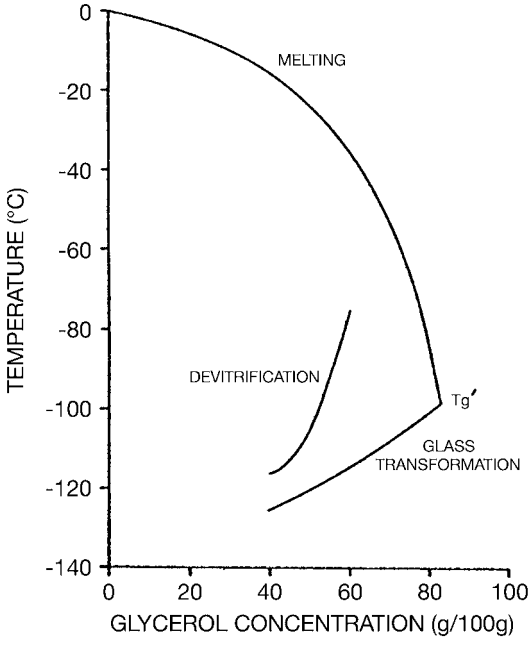

Fig. 9. Supplemented phase diagram for glycerol/water. The intersection of the melting curve and the glass transition curve at Tg´ indicates the lowest concentration of glycerol that, in theory, will vitrify. In practice, the lower temperatures on the melting curve are unlikely to be reached owing to the high viscosity preventing the crystallization of ice. (Reproduced with permission from **ref. *23***.)

that effective preservation would require a technique that completely prevented the crystallization of ice. Luyet devoted a great deal of effort to the search for conditions that would produce a vitreous or glassy state with biological systems and that living cells could survive. Vitrification is defined by the viscosity of the solution reaching a sufficiently high value (~10^{13} poises) to behave like a solid but without crystallization. In conventional cryopreservation, the concentration of solute in the remaining liquid increases during progressive freezing, and a temperature (Tg) is eventually reached with many systems where the residual liquid vitrifies in the presence of ice (**Fig. 9**). Cells can survive this situation, they do so in conventional cryopreservation, but they will not tolerate exposure to the necessary concentration for vitrification without freezing (~ 80g% [w/w]) at temperatures above 0°C. Some other solutes will vitrify at lower concentrations, for example butane-2,3-diol at around 35% (w/w), but unfortunately this compound is more toxic than glycerol. Luyet knew that it was possible to vitrify solutions that are less concentrated than this if sufficiently rapid cooling

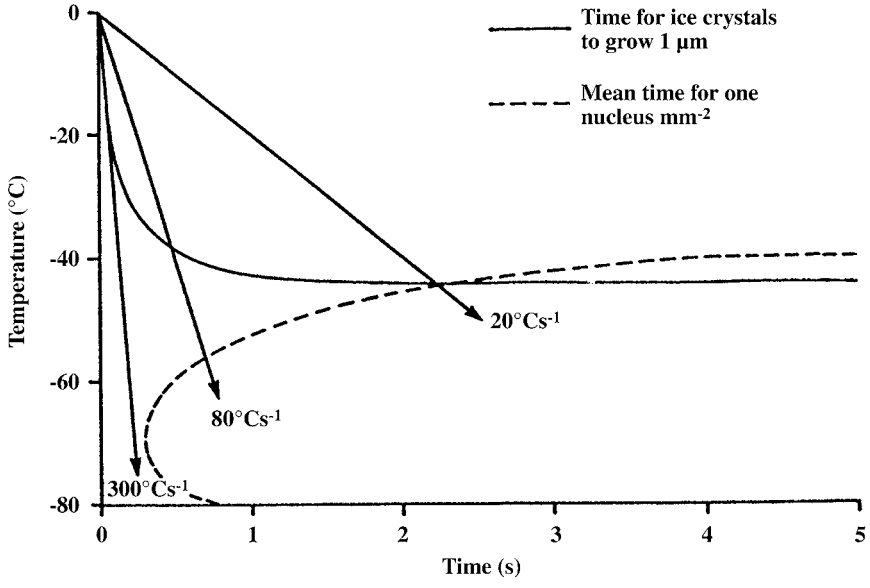

Fig. 10. Diagram constructed from data by Luyet showing the time- and temperature dependence of nucleation and ice crystal growth in a thin film of a 50% (w/v) solution of polyvinylpyrrolidone. The arrows indicate cooling trajectories that avoid nucleation (300°C/s), nucleate without crystal growth (80°C/s) and produce ice crystals (20°C/s). (Reprinted with permission from **ref. *8***.)

was employed; the reason is that ice crystals form by the accretion of water molecules onto a nucleus. Both the formation of the nuclei and the subsequent growth of ice crystals are temperature dependent. Nucleation is unlikely just below the equilibrium freezing point (hence the phenomenon of supercooling), but it becomes more probable as the temperature falls, reaches a maximum rate, and then decreases as the movement of water is limited by viscosity. However, the growth of ice crystals is maximal just below the freezing point and is progressively slowed, and eventually arrested, by cooling. The interaction of these two processes creates three possibilities for a cooled sample (**Fig. 10**); if it cools rapidly it may escape both nucleation and freezing; if it cools sufficiently slowly it will nucleate and then freeze; and at an intermediate cooling rate it will nucleate but not freeze. Upon warming, however, there are only two possibilities; if heated sufficiently rapidly it will escape both nucleation and freezing during warming; the alternative is that the trajectory passes through both the nucleation and the ice crystal growth zones and, therefore, it will nucleate (if it is not already nucleated) and the ice crystals will then grow before eventually melting. Therefore, unless a sufficient concentration of cryoprotectant has been used to ensure that no ice can form under any circumstances, there is

a risk that freezing will occur during warming. The problem is that bulky tissues and organs cannot be cooled much more rapidly than a few degrees per minute in practice. For small samples it is more feasible to cool rapidly, as was demonstrated by the successful vitrification of *Drosophila melanogaster* embryos ***(24)***. These were complex organisms comprising some 50,000 cells with advanced differentiation into organ systems, and they cannot be preserved by conventional freezing methods. The successful method required careful permeablization of the waxy vitelline membrane to allow penetration of the cryoprotectant, exposure to 8.5 *M* ethanediol, cooling at 100,000°C/min., storage at approx –200°C, and warming at 100,000°C/min. The extremely high rate of warming was far more critical than the rate of cooling, which is consistent with the crucial importance of maintaining the vitreous state.

The demonstration that ice forming in tissues produces so much damage has created renewed interest in the possibility of using vitrification with very high concentrations of appropriate cryoprotectants to avoid the formation of ice completely. Current research aims to identify materials that will inhibit the formation of ice crystals during warming ***(25,26)***, and one interesting possibility is the antifreeze proteins that some polar fish and overwintering insects have evolved to avoid freezing in nature. One effect of such compounds is to reduce the warming rate required to prevent ice crystallization to more manageable rates. This approach is being used in conjunction with electromagnetic heating ***(27,28)*** to achieve more rapid and more uniform heating. However, despite progress in the design of vitrification cocktails with reduced toxicity, the major problem remains cryoprotectant toxicity. One approach to this problem is to increase the concentration of cryoprotectant progressively during cooling so that the tissue concentration follows the liquidus curve: ice does not form but the cells do not experience any greater concentration of cryoprotectant than occurs during freezing. This has recently proved to be practical and very effective for the cryopreservation of articular cartilage, an otherwise recalcitrant tissue ***(29)***. The same method may potentially be effective for other resistant tissues and perhaps even for organs.

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4

Lyophilization of Proteins

Paul Matejtschuk

Summary

This chapter describes the methods that can be applied to successfully freeze-dry proteins. Laboratory applications are given at small scale, typified by the purification of a protein intermediate as part of the analytical characterization of a protein, and at intermediate scale, as illustrated by the pilot development of a lyophilized protein reference material such as for use in bioassay or immunoassay. Advice on common problems with freeze-drying of proteins is also given.

Key Words: Lyophilization; freeze-drying; thermal analysis; freeze-drying microscopy; stabilizer; glass transition temperature; formulation.

1. Introduction

Lyophilization, the removal of the majority of the water in a sample under conditions of low temperature and vacuum, is a widely used technique in the areas of protein purification, protein reagent preparation, and the manufacture of protein biomolecules for therapeutic and diagnostic applications. It can be performed on a sample in order to:

1. Increase stability by minimizing the available water residual in the sample, which facilitates degradative processes such as peptide bond hydrolysis, deamidation, and the like.
2. Reduce the volume of the sample so facilitating a further processing step in a purification process.
3. Store a protein sample in a convenient format requiring less storage space and permitting storage under ambient conditions.
4. Simplify transport of the protein material with minimal loss of activity between geographic locations—obviating the need for cold chain shippage.
5. Remove volatile buffer/solvent components facilitating reformulation.

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Proteins were some of the earliest biomolecules to be lyophilized—indeed the need for bulk preparations of plasma proteins in the second World War was a driving force in the development of lyophilization as an industrial process. Proteins are highly diverse and whereas a single-chain protein with a highly ordered tertiary structure may freeze-dry with little difficulty, a multimeric protein with multiple domains and hydrophobic proteins (such as a membrane receptor complex) will pose a far more demanding challenge to achieve successful lyophilization.

1.1. Theory of Freeze-Drying

The theoretical basis of freeze-drying is discussed elsewhere in this volume (*see* Chapter 2) and so space will not be given here to reiterate. Freeze-drying is split into three process stages, freezing, primary drying at lower temperatures when most of the water is removed, and secondary drying at ambient or higher temperatures to minimize the final unbound water content. To maintain their biological activity during freeze-drying, conservation of the higher order structure of proteins is vital. Proteins found in aqueous solution are hydrated and so removal of the water shell during lyophilization will have serious thermodynamic implications for the maintenance of their correctly folded structure when that water shell is in part or wholly removed by desiccation. The optimum residual water content for proteins should be determined on a case-by-case basis, but several publications have illustrated that an optimum water content is required to maintain the activity of proteins under study and that drying beyond that level may also be detrimental ***(1)***, whereas high water content may well result in excessive degradative changes on storage ***(2)***.

1.2. Impact of Freezing on Proteins

During the freezing process, water crystallizes to ice and the excluded excipient salts concentrate to local concentrations far higher than those in the original liquid state. This may in itself have implications for the stability of the protein(s) present, which may be destabilized and denature because of the change in ionic strength. In addition, if buffer salts, such as mixed phosphate buffer are present, the selective crystallization or precipitation of one of these salts at a higher temperature to the other may result in localized pH shifts, which again may induce denaturation of the proteins ***(3)***. Such denaturation may lead to exposure of normally buried residues and an increase in aggregation, some of which may be irreversible on reconstitution. Other proteins may be satisfactorily immobilized in the lyophilized state but undergo changes that result in aggregation on reconstitution ***(4)***. Membrane-bound proteins may pose special problems when undergoing lyophilization, cell membranes are particularly prone to disruption during the dehydration process and so membrane-associated proteins will also be at risk.

In order to successfully lyophilize proteins to yield a product of cosmetically acceptable appearance, all of the water in the sample must first be fully immobilized, either as ice crystals or bound with the nonvolatile and amorphous components of the sample, in the superviscous state known as a glass. The temperature to which samples must be cooled to ensure this amorphous state is discussed elsewhere (*see* Chapters 2 and 3). Practically, the glass transition (Tg′) or collapse temperature (Tc) for the formulation should be determined and temperature probes used in the samples to ensure that this temperature is not exceeded in the product during drying.

Finally, it must not be assumed that, although slower, degradative reactions will be entirely absent in a reduced water environment, and they should be considered and assessed during short or longer term storage in the lyophilized states ***(5)***. Saccharides are often chosen as stabilizers for lyophilization processes, but covalent modification of proteins in the presence of reducing sugars is well known, especially at elevated temperatures ***(6)*** and the need to control this should be considered when choosing excipients and storage conditions. All of these issues must be addressed by the selection of suitable formulation and processing conditions if stable lyophilized protein preparations, which retain full biological activity, are to be achieved.

1.3. Formulation/Stabilizer Choice for Proteins

When considering freeze-drying of proteins, their formulation is particularly important, for instance:

1.3.1. pH/Buffers

Buffers should be kept to a minimum concentration to maintain pH in the desired application and in particular, mixed buffer systems, such as phosphate, should be used cautiously as pH shifts can occur on freezing, histidine, citrate, or Tris buffers being preferable.

1.3.2. Salts

Inorganic salts reduce the glass transition temperature (Tg′) of the formulation and so limit the temperature at which primary drying can be performed. Therefore, salt concentrations should be reduced to a minimum and where alternative osmotic modulators can be selected that have less impact on the Tg′ should be preferred.

1.3.3. Stabilizers

Hydrogen bond-forming sugars, such as trehalose or sucrose, are well documented as excellent stabilizers for freeze-drying of proteins. Other stabilizers may be more specific in their effects (such as metal ions, e.g., zinc ***[7]***), whereas

others, such as mannitol or glycine, are valuable as matrix formers without offering the stabilizing benefits of sucrose/trehalose.

1.3.4. Dry Weight

In order to produce a robust lyophilized cake it is important that the dry weight of nonvolatiles be sufficient (for example, 10 mg/container).

1.3.5. Protein Concentration

Proteins themselves are often common choices as stabilizers for use in formulating for freeze-drying. Albumin has a high Tg′, is a good cake-former, and also blocks nonspecific binding of proteins to glass surfaces, making it an ideal choice of stabilizer for low concentrations of recombinant proteins.

2. Materials

2.1. Protocol 1: Lab-Scale Lyophilization on Reverse-Phase Fractions From High-Performance Liquid Chromatography Run

1. A lab-scale freeze-dryer (e.g., Edwards Modulyo, Virtis Advantage, or similar unit) (**Fig. 1**) (*see* **Notes 1** and **2**).
2. Suitable containers for freeze-drying the product, e.g., round-bottomed glass flasks, glass vials, plastic microcentrifuge tubes, microtiter plates (e.g., from VWR Ltd., Poole, UK) (*see* **Note 3**).
3. Parafilm™.
4. Freezing system, e.g., liquid nitrogen or dry-ice acetone, or a –70°C freezer.

2.2. Protocol 2: Pilot Scale Freeze-Drying

2.2.1. Freeze-Drying of a Dilute Concentration of a Purified Protein as a Reference Preparation for Laboratory Assay

1. Ampoules or vials (preferably type I glass formed from cylindrical tubes (suppliers, e.g., Adelphi Tubes, Haywards Health, UK).
2. Closures (e.g., Adelphi Tubes).
3. Hot air oven or autoclave.
4. Pipets (e.g., Gilson, Anachem, Luton, UK) or autodispensing syringe (M-Lab, Hamilton Bonaduz, Switzerland).

2.3. Differential Scanning Calorimetry

1. Modulated differential scanning calorimeter with cooling accessory (e.g., TA Instruments (Surrey or Perkin Elmer [Beaconsfield, Crawley, UK]).
2. Sample pans (e.g., part no. 900825.902 TA Instruments) and crimper.
3. Dry nitrogen gas.
4. Liquid nitrogen for cooling accessory.

2.4. Freeze-Drying Microscopy

1. A commercially available freeze-drying microscope is available from Biopharma (Biopharma Technology Limited, Winchester, UK), or Linkam (Epsom Downs, UK).

Fig.1. Typical benchtop freeze-dryer suitable for drying from flasks via the manifold or from trays of vials placed on the chamber shelf. (Photo Courtesy of Biopharma, UK.)

2. Quartz glass crucible, 13-mm glass cover slips, and semicircular metal shims (Linkam).
3. Ethanol.
4. Liquid nitrogen.

3. Methods

Sodium azide must *not* be present in samples to be dried on the majority of freeze-dryers because of the risk of forming explosively reactive metal azides at brazed tubing connections, though some dryers are marketed as "azide friendly." Although samples need not necessarily be fully solubilized, a homogeneous suspension should be produced before freezing commences.

The process of freeze-drying may be divided into the following stages:

1. Freezing. Lab-scale freeze-drying is often performed in general laboratory glassware items such as round-bottomed glass flasks, glass vials, plastic microcentrifuge tubes, or even microtiter plates (*see* **Note 3**). Containers must not be more than one-third filled or sudden volume and thermal changes may result in the container shattering, with catastrophic loss or cross-contamination of the valuable samples.
2. Drying temperature. Simple units are different to production freeze-dryers in that usually there is no control of the product temperature and the product warms to ambient temperature in an uncontrolled manner over several hours. Conversely, the condenser is temperature controlled usually at –60°C or less and a vacuum

maintained typically at 10–100 µbar. Lyophilization is rapid, usually completed overnight, it can be even hours dependent on the drying system and solvent being removed.

Product is usually removed by breaking vacuum to air or inert gas. Individual samples may be removed from a manifold without losing vacuum on the system as a whole, although some laboratory systems may offer precooled sample shelves in which case samples may only be removed at one time with back fill with air/inert gas. Simple stoppering mechanisms may be available, allowing more sophisticated lyophilization conditions to be modeled with individual vials being fully sealed by lyophilization closures prior to the system being opened to atmosphere. No stoppering is possible for samples lyophilized in flasks on a manifold (*see* **Note 4**).

3.1. Lab-Scale Lyophilization of High-Performance Liquid Chromatography Protein Fractions

The following protocol describes a method for freeze-drying protein fractions from a reverse-phase high-performance liquid chromatography fractionation, prior to further analysis for example by peptide mapping.

1. Switch on condenser and vacuum pump on the benchtop freeze-dryer, assemble the manifold or place samples on shelf, and ensure that a good vacuum (100 µbar or less is achieved) and that the condenser is down to –50°C or lower.
2. Dispense fractions into microcentrifuge tubes or other suitable containers ensuring that the microcentrifuge tubes are not more than 33% full. Pierce holes in the lid or remove lid and replace with Parafilm pierced with several holes.
3. Snap freeze microcentrifuge tubes by dipping until partially submersed in liquid nitrogen or dry/acetone (beware cold hazard), alternatively freezing in a suitable spark-proof deep freezer (–40°C or lower).
4. Place frozen microcentrifuge tubes into a Quickfit style round-bottom flask (neck wide enough to allow easy loading and removal of the tubes). Freeze outside of glass to –60°C or below and attach on manifold. Apply vacuum by opening the manifold valve so as to connect the flask to the condenser. To avoid shattering of the flasks place nylon meshing around the glass spheres. Alternatively, place the sample containers in a suitable tray within the chamber of the dryer (for the Modulyo type) or within the chamber on the shelf (for a unit such as the Virtis Advantage). Check to ensure that the vacuum achieved returns to below 100 µbar.
5. Allow the samples to completely warm to room temperature (usually overnight [16 h] but may be less for volatile solvents).
6. Carefully allow the vacuum to be released by switching the manifold valve slowly to prevent material ablating from the microcentrifuge tubes.
7. The system can be left on and fractions can be dried over several days before the condenser needs thawing out. If multiple flasks on a manifold are used, different flasks can be removed at different times depending on when they have completed drying.

A number of common problems may arise during freeze-drying and **Notes 5–10** give some helpful tips for trouble-shooting and quality control (*see* **Note 11**).

3.2. Pilot Scale Freeze-Drying

3.2.1. Freeze-Drying of a Dilute Concentration of Purified Protein as a Reference Preparation for In-House Standard

This protocol describes the lyophilization of a purified protein as a small batch (50–500 vials), suitably formulated so as to stabilize the biological activity, prevent the degradative processes possible in the liquid state, to facilitate storage as an in-house reference material, and to help distribution to other laboratories for analysis. Pilot scale freeze-drying is applicable to formulation development and product development environments, where a more rigorous and yet still easily affordable lyophilization solution is required and where multiple containers are required, of identical product composition and concentration, for stability study, repeat assay, or distribution.

1. Selection and preparation of glassware and closures. Glassware (preferably type I glass formed from cylindrical tubes) should be washed without detergents and baked/autoclaves to sterilize if low bioburden is required (*see* **Note 12**). To prepare such a protein it is most convenient to use small stoppered or screw-cap vials (usually glass) or to use ampoules that can be flame sealed after lyophilization. Closures could be siliconized to ease stoppering and should be baked to reduce residual moisture content (e.g., 16 h at 116°C) as otherwise this moisture may transfer to the lyophilized product on storage.
2. Formulation choice. A range of formulations should be assessed to allow evaluation of the choice of buffers and the impact of freezing and of dehydration on the target protein, based on the points outlined (*see* **Subheading 1.3.**).
3. Dispensing of sample. The method of dispensing the product will depend on the number of containers to be filled and the accuracy and consistency of fill volume required. Options range from a simple pipet, an autodispensing syringe, to a peristaltic pump.
4. Freezing. The timescale of freezing may be important (*see* **Note 13**). Freezing temperature can be derived from study of the critical glass transition or collapse temperatures by thermal analysis or freeze-drying microscopy experiments (respectively), and the length of the freezing step should be such to ensure that the contents of all of the containers have frozen completely.
5. Lyophilization cycle design. For primary drying, conditions of vacuum and temperature must be set so as to permit drying without collapse of the cake structure, but at a sufficiently warm temperature that sublimation occurs quickly to achieve drying in a practical time period (*see* **Note 14**).
6. Temperature probes to monitor process. Setting a simple thermocouple into the solution to be dried before freezing will allow the freezing and drying process to be monitored. Although the data acquired will apply to the container monitored

alone, and the probe will itself introduce differences to the nucleation point and tell little about the process in neighboring containers, it is still a useful and widely applied measure of the progress of a freeze-drying cycle.

7. Back-filling/stoppering. Once lyophilization is completed product containers can be stoppered under vacuum, or under an inert atmosphere depending on the design specification of the product (*see* **Note 15**).
8. Assessment of product parameters. The lyophilized product should be assessed against frozen but undried material (frozen baseline) taken from the fill in order to assess the recovery of functional activity. Other parameters, such as appearance and residual moisture content, should be assessed.

3.3. Experimental Determination of Glass Transition/Collapse Temperature

3.3.1. Determination of Glass Transition Temperature by Modulated Differential Scanning Calorimetry

The principle of modulated differential scanning calorimetry and its application to developing freeze-drying conditions is described elsewhere ***(8)***.

1. Aliquots of the sample in the intended lyophilization buffer or a range of buffers are prepared and an 80-µL aliquot is dispensed per pan and the pans crimped with an appropriate crimper.
2. The sample pan and an empty crimped reference pan are placed within the oven of the modulated differential scanning calorimetry machine.
3. Apply a programmed freeze to –70°C and rewarming to ambient temperature using proprietary software, recording the heat flow profile, and the reversing heat flow profile. Use a range of heating rates between 0.5 and 3°C/min with a modulating heating rate such that the overall thermal trend is heating only.
4. Data analysis: from the profile note any glass transition (an inverse sigmoidal event in the reversing heat flow profile in the thermal region below the melting temperature. Proprietary software will help to set this and identify the glass transition temperature.

Repeat analyses are recommended to establish robustness of the Tg′ determination.

Temperature calibration may be made using materials of well-characterized melting points such as indium (156.6°C) and *n*-decane (–29.6°C).

3.3.2. Determination of Collapse Temperature by Freeze-Drying Microscopy

1. Assemble the sample by placing the metal shim onto a cleaned quartz glass crucible. Dispense an aliquot (5 µL) of the sample within the shim and place a 13-mm cover slip over the sample.
2. Insert the crucible into the metal sample holder and place this into the microscope cryostage.

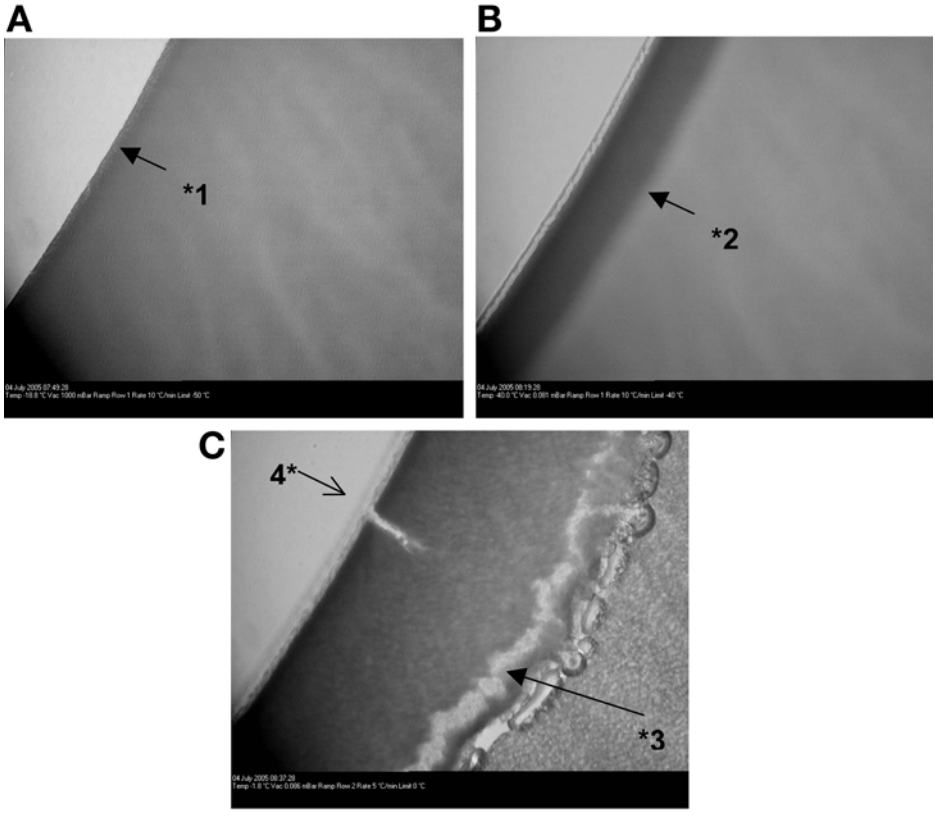

Fig. 2. Freeze-drying microscopy profile of an albumin solution showing **(A)** sample frozen with air/liquid interface (*1), **(B)** primary drying under vacuum with drying front advancing (*2), and finally **(C)** collapse of freeze-drying front (*3) and rupture of sample skin at surface (*4) as temperature was raised past the collapse temperature. Data produced by Ms. Kiran Malik, NIBSC.

3. Focus the microscope on the interface between the edge of the liquid sample and air using a ×4 or ×10 objective. Polarized light and differential interference techniques significantly improve the quality of the image obtained.
4. Freeze the sample to –50°C at 5°C/min.
5. Hold unit frozen then apply vacuum to a selected vacuum level (25–100 μbar).
6. Ramp the temperature at a rate of 1–5°C/min and monitor physical appearance during the warming phase. Once collapse has been observed the temperature may be reduced again, the frozen state restored, and the collapse event revisited with a slower ramp rate to refine the collapse temperature evaluation.
7. Once the collapse temperature is established, raise the temperature to ambient and clean the cell and slide thoroughly, removing any biological materials with neat ethanol.

8. The collapse temperature is determined by repeated cycling through the temperature range that results in structural collapse of the ordered drying front (*see* **Fig. 2**). The calibration of the stage temperature probe can be checked by performing an initial study on each day of operation using a sample with well-characterized glass transition or eutectic (e.g., sucrose at –32°C or NaCl at –21.5°C).

4. Notes

1. Lyophilization is a process that is widely used in biotechnology in a range of different situations. It may be performed on a small benchtop dryer with little process control or in a multimillion pound industrial lyophilizer whose operation is carefully monitored and controlled by computer systems and described in detailed written operating protocols. Laboratory-scale lyophilization differs from the production process commonly applied to the industrial preparation of biological/biotechnology materials in the scale of the operation, the degree of process and parameter control available, and the requirements in terms of the properties of the lyophilized product.
2. A lab-scale freeze-dryer may be of simple design where solvent is removed from products frozen in round-bottom flasks or in containers such as microcentrifuge tubes contained within such flasks. There is no product temperature control or monitoring, no controlled shelf temperature, and the drying process is driven merely by the uncontrolled warming of the sample to ambient temperature over a period of hours while the condenser remains below –50°C to trap the subliming water vapor.
3. Containers used should be capable of withstanding rapid snap freezing (either by immersion in liquid nitrogen or dry-ice acetone. Beware cryogenic liquid and asphyxiant hazards) or freezing within a freezer at –70°C without cracking or shattering. The containers must then also be capable of withstanding exposure to deep vacuum conditions. Plastic microcentrifuge tubes, frozen by careful incubation in cryogenic liquid, are particularly suited for column fractions such as those obtained from preparative peptide mapping, although microtiter plates may be equally useful providing cross-contamination is avoided, in such cases freezing on the shelf of a freezer would be more practicable. The caps must be pierced with a syringe needle or other suitable means, to allow escape of subliming water vapor.

   Such samples may well dry down to leave no visible solid material but reconstitute, often in only a few microliters of diluent, to produce samples suitable for MALDI–mass spectrometry or further enzymatic degradation procedures.

   Standard glass vials designed for lyophilization are ideally suited for operation within such a lab-scale machine providing either a shelf or sample plate is available. More complex laboratory dryers may allow for these vials to be part-stoppered with rubber closures and then be fully stoppered at the end of lyophilization. In small numbers they can be placed suitably sealed (with Parafilm or equivalent material, again carefully punctured in several places to allow subliming vapors to escape) within larger glass flasks.

Larger volumes (up to 100 mL) may be lyophilized on simple lab-scale dryers by freezing in flasks at not more than one-quarter filled, frozen by immersion in a cryogenic bath (beware star-cracking of reused flasks that may implode when placed under vacuum) and attached to a manifold (*see* **Fig. 1**). Such flasks should be contained within plastic netting to minimize risk were the flask to implode under vacuum.

4. Laboratory-scale dryers have proven themselves to be very versatile equipment, and adaptations have allowed stoppering to be achieved using a manual screw mechanism, manifolds that can accommodate six or more quick-fit flasks of up to 500 mL, specialist adaptors that allow for several dozens of ampoules to be dried and then flame sealed individually for the drying of microbial cultures and the like.
5. Poor appearance: Though appearance is not usually a critical factor in laboratory-scale applications, on occasions an oily or sticky residue may result after freeze-drying rather than a dry powder. This is due to product temperature exceeding the glass transition or collapse temperature. The sample may need to be frozen to a lower temperature, or salts may need to be further diluted or the samples reformulated, especially if excipients such as glycerol are present, which will not readily undergo sublimation. For larger scale experiments in vials/ampoules the product should have an acceptable appearance, homogeneous across the batch. Poor appearance (collapse) may be caused by shelf temperature being too high during primary drying resulting in the Tg' being exceeded. Wispy cakes result from insufficient dry weight mass and so this should be addressed by use of bulking agents (glycine or mannitol are common examples, but other proteins, sugars and polymers such as dextran or polyvinyl pyrollidone have been used). Products that are removed from the dryer with a satisfactory appearance but which show collapse of structure or transformation to a glassy gel on storage may have too high a residual water content. Discoloration on storage at ambient or higher temperatures of lyophilized product formulated with reducing sugars such as glucose or hydrolyzable sugars, may be because of browning reactions whereby amino groups in the product undergo a Schiff base-type reaction with the carbonyl group on the sugar.
6. Product boil-out. For microcentrifuge tubes ensure that caps are secure and do not overfill the containers. Release vacuum gently to prevent disturbing fine powders. Product loss from vials/ampoule is less common, but cannot be assumed not to occur to some degree. It is worse where the product cake is less robust.
7. Incomplete removal of liquid. Samples that return to a liquid or gelatinous state after removal have not undergone sufficient drying and the period on the dryer should be extended or samples reformulated as in **Note 5**.
8. Cracking of container. Star cracks in round-bottom flasks will prevent formation of a good vacuum and may result in catastrophic implosion of the flask, with subsequent loss of valuable sample and possible injury. Glass flasks should always be contained within plastic netting to prevent such accidental injury.
9. Loss of bioactivity, where activity is lost on drying, may be a result of pH or salt concentration shifts during freezing resulting in loss of biological activity and

protein denaturation. If this is the case, trial freeze/thaw the material and see if activity is lost. If activity remains on freeze-thawing but is lost on freeze-drying then it is more likely that the material can not withstand dehydration, to the degree to which the sample has been dried. Try lyophilizing for a shorter period to remove less water and/or add stabilizers that are known to be lyoprotecting, such as sucrose or trehalose ***(9,10)***. Loss of activity on storage may be because of too high a residual moisture content, as many degradative reactions of proteins require water.

10. Vacuum pumps and maintenance. Inability to draw a sufficiently low vacuum may be because of problems with the door seal on the chamber and/or condenser, which require regular inspection and cleaning if contaminated. Another possible cause is contamination of the vacuum pump oil or leaks within the pump tubing, fittings, or equipment.
11. Quality control:
    a. Vial-to-vial variability. Visually inspect all vials in the batch and assess the appearance and biological activity across the fill. Heterogeneity should be mapped to see whether it correlates particular location. Appearance is more important in large-scale freeze-drying than for laboratory applications as above. However, heterogeneity should be avoided and poor appearance may indicate suboptimal drying.
    b. Moisture determination. It may be necessary to make some assessment of this so as to predict suitability for longer term storage. Commonly used methodology includes colourometric Karl Fischer and thermogravimetric analysis ***(11)***.
    c. Storage stability analysis. Stress studies using elevated temperatures and humidities over time can indicate the likely storage stability of the lyophilized material. The trends in activity loss can be analyzed using Arrhenius or similar kinetic models to predict the stability expected at storage temperature ***(12)***.
    d. Activity recovery. The recovered biological activity should be measured against controls taken before lyophilization, so the choice of formulation and stabilizer that gives best activity recovery can be identified and then applied at scale up.
12. Closures are available in a variety of formats and compositions ***(13)*** and selection should be based on the specific application.
13. Some have recommended rapid freezing rates, especially if the target protein loses activity rapidly in the liquid state or if the selected formulation results in marked pH shifts during freezing and denaturation. However, rapid freezing may result in small ice crystals and slower drying times; hence if rapid freezing is necessary then an annealing (thermal tempering) step ***(14)*** may be needed to correct the effects.
14. Vacuum conditions can be set by selecting the atmospheric pressure based on the vapor pressure of ice at the shelf temperature ***(3)***, and then experimenting with different shelf temperatures until the highest temperature is identified that does not result in the critical collapse temperature being exceeded in the product.

    During secondary drying, shelf temperature is set to ambient or higher temperature with hard vacuum applied to maximize the loss of remaining water.

The typical length of the step has been approximated at one-third of the primary drying ***(15)*** but should be sufficient but not exceed that which yields product of the target residual moisture content.

15. Sealing under vacuum will allow reconstitution fluid to be drawn in at a greater rate and may aid dissolution of lyophilized products. If an atmosphere is required, then inert gas is preferable to minimize oxidation of the proteins over prolonged storage. Moisture content of the product may increase over time, with water equilibrating from that residual in the closure, and for this reason closures should be pretreated to minimize residual water content.

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5

Vacuum-Drying and Cryopreservation of Prokaryotes

Brian J. Tindall

Summary

Traditionally, the *ex situ* study of prokaryotes (members of Archaea and Bacteria) has required access to living cultures. Although it may be possible to maintain a small number of strains by serial transfer, such methods are not the best, particularly for long-term storage or for keeping a large collection. One of the major problems is dealing with the vast range of organisms that have been isolated to date. Particularly in the case of methods of cultivating prokaryotes, it is almost impossible to give a simple list of media to use. However, general principles are outlined that should cover most of the currently known prokaryotes in pure culture. The methodologies used for the long-term storage of prokaryotes may be divided into those that involve freezing and those that involve vacuum-drying. The methods described are those that are either used in the Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH (http://www.dsmz.de/index.htm) or in other national culture collections.

Key Words: Prokaryotes; bacteria; archaea; freezing; freeze-drying; long-term storage.

1. Introduction

The long-term storage of strains is an issue that few scientists pay much attention to. Strictly speaking the reproducibility of every scientific publication that involves the use of a particular strain depends on the availability of that strain. In addition, databases such as EMBL/GenBank are plagued by the accumulation of sequences attributed to organisms that have been wrongly labeled, "mixed up," or contaminated. It is impossible to estimate how much of the published literature is affected by the nonavailability of strains to subsequent researchers either to check the identity of the strains concerned or to reproduce the results. Serial transfer of strains over long periods of time is not the best way of maintaining strains. Some form of

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storage in a fashion that keeps the strain in an inert, but potentially viable state, is recommended.

Over the decades a number of methods have been developed to store strains. The choice of methods to be used often depends on the time period over which strains are to be maintained, as well as whether strains are to be shipped frequently. The goal of a research group may be to maintain strains during the duration of a 5-yr research project, whereas a national culture collection is interested in storing the strains for decades. In some cases, it may be wise to use two different methods, one for working stocks and one for long-term "back ups." Storage in liquid nitrogen, if available, is probably a fairly good "all round" method. In particular, the capillary method currently used in the Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH (DSMZ) has a number of advantages. Storage on beads or in glycerol in mechanical freezers may be more suitable for maintaining strains for the duration of research projects.

One cannot stress enough the importance of not only the proper storage of strains long term, but the fact that viability of stored batches must be checked at the time they are prepared, as well as some form of authenticity/identity check. The largest national collections check the viability of all stocks are they are prepared, which comes as a surprise to many customers when a strain fails to subculture in their laboratory. In many cases, long-term storage of anywhere between 5–35 yr being documented for freeze-dried cultures ***(1)***. Data on the longevity of storage in liquid nitrogen is still missing, simply because the method has only been comparatively recently introduced. Experience in the DSMZ (founded in 1969) indicates that the vast majority of the original stocks have survived 30 yr.

Although it is the goal of the present chapter to give detailed methods for the storage of prokaryotes, with the exception of cyanobacteria (*see* Chapter 10), each laboratory will find that some of the consumables used will vary slightly, and that the equipment used will depend on the local suppliers. The second greatest variable is the sheer diversity of prokaryotes that are being isolated. The DSMZ currently has a list of well over 1000 different media that are required for their holdings and will certainly continue to grow. Some methods of storing strains can be applied to a wide variety of strains, but there will always be one or two strains that may behave differently. Sometimes the literature may emphatically declare that a particular group of organisms cannot be frozen and/or dried. However, adapting standard protocols and studying the mechanisms organisms use to survive various forms of "dehydration" may help to overcome some of these problems. The present chapter attempts to bridge the gap between some general principles and some detailed protocols. Apart from

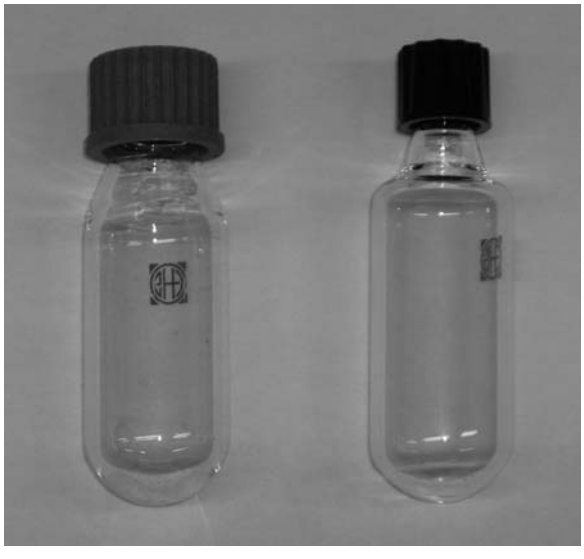

Fig. 1. Glass centrifuge tubes made by Ochs (Bovenden, Germany). To the left, a glass centrifuge tube used for aerobic strains. To the right, a centrifuge tube used for anaerobic strains. The narrow neck is sealed with a rubber stopper, which fits inside the screw cap, and has a hole in the top.

hands-on experience with a variety of methods, two books have been particularly useful, Lapage and Redway ***(2)*** and Kirsop and Doyle ***(3)***.

2. Materials

2.1. Standard Materials Used for All Techniques

1. Laminar flow cabinet and appropriate facilities for following good microbiological practice.
2. All glassware should be sterilized, and all media and suspending liquids sterilized by filtration or autoclaving as appropriate.
3. Use a liquid culture or agar slope culture grown to the late log or early stationary phase.
4. Custom-made glass centrifuge tubes (Ochs, Bovenden, Germany) in which liquid cultures of strains may be grown and harvested (**Fig. 1**).

2.2. Cryopreservation Methods

2.2.1. Cryopreservation in Glycerol

1. Glycerol. This is best dispensed if it is warmed gently to allow it to flow better.
2. Sterilized glass screw-cap bottles. 15 mm (outside diameter) × 50-mm bottles are used in the DSMZ. The bottles should not be too small and should be easy to handle and open without the risk of contamination.

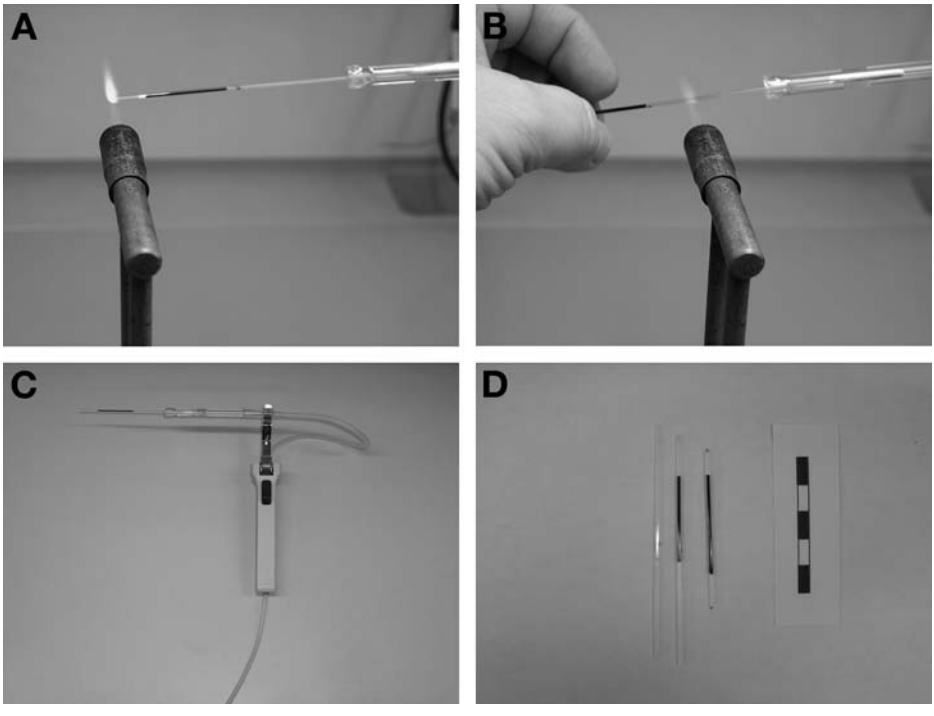

Fig. 2. The process of making a glass capillary. **(A)** Heating the open end of the capillary to seal it. Note the fine hot flame that only comes into contact with the very tip of the capillary. **(B)** Once the open end is sealed the capillary may be cut from the adapter by heating about 25–30 mm from the top end (to the right). Note the use of the fine hot flame, which only comes into contact with a very limited length of the capillary. **(C)** The capillary held in the glass adapter, which is fitted to the "microclassic" (Brand) pipetting aid. The region where the capillary is filled has been made visible with dyed water (capillary to the left). **(D)** Capillaries. To the left, empty, in the middle filled (top toward the adapter), right sealed vials.

2.2.2. Cryopreservation on Glass of Ceramic Beads

1. Small glass or ceramic beads (in different colors) can be obtained from a variety of sources, ranging from hobby shops to those selling embroidery products. Two to three millimeter external diameter is the ideal size, but care should be taken that larger sizes do not hinder their removal from the glass bottle.
2. Screw-capped glass bottles, about 10–15 mm (outside diameter) × 30–50 mm.

2.2.3. Cryopreservation in Glass Capillaries

1. Glass capillaries 1.4 mm (outside diameter) × 90 mm, wall thickness 0.261 mm (article no. 1400290 from Hilgenberg GmbH, Malsfeld, Germany) (**Fig. 2D**).
2. Micropipetting aid (microclassic from Brand) (**Fig. 2C**).

3. A custom-made adapter for fitting into the micropipetting aid in order to hold the capillary (*see* **Note 1**).
4. Aluminium miniature pipet canisters are used to store and sterilize the glass capillaries (top section approx 18 × 40 mm, lower section, which slides into the upper section, approx 16.5 × 80 mm). Usually 20 or 40 are placed in the canister at one time.
5. 11 mm × 43.5-mm small glass vial fitted with a cotton plug (the type of plug used in the DSMZ can be obtained from suppliers of dental consumables) (*see* **Fig. 3B**,**C**). This is sterilized and used to hold the concentrated cell suspension. In practice, a number of such vials are sterilized by placing them in a glass beaker of suitable size, covering the mouth of the beaker with a lint cover, and covering that with aluminium foil. The beaker is then autoclaved.
6. An ice bath.
7. A glass Petri dish.
8. A 10-mL glass, screw-capped test tube filled with ethanol and cooled on ice.
9. Paper tissue for drying the finished capillaries.
10. A gas burner that produces a fine hot flame that can be adjusted. Some natural gas-compressed air miniature torches may be suitable. Soldering torches, such as those manufactured by Camping Gaz, are also suitable.
11. Cryoprotectant: glycerol or dimethyl sulfoxide (DMSO) may be used. The final concentration of glycerol is usually 10–15% (v/v). DMSO is used at a final concentration of 5% (v/v). DSMO may be either filter sterilized or autoclaved under a nitrogen atmosphere at 115°C for 15 min.

2.3. Drying/Freeze-Drying Methods

2.3.1. Materials Needed for All Methods

1. Constrictor (*see* **Note 2**).
2. Freeze-drier (*see* **Note 3**).
3. Gas burner for sealing the ampoules.

2.3.2. Cryopreservation and Drying or Drying From a Predried Plug

1. –20°C Freezer.
2. 20% (w/v) skimmed milk, sterilized by autoclaving at 115°C for 13 min. 5 mL will produce 25 ampoules. The skimmed milk is best sterilized in 5-mL portions in glass screw-capped tubes.
3. A Pasteur pipet may be used, but sterile multistepper pipetting aids such as those made by Eppendorf are more accurate and easier to handle. A multistepper delivering 200 µL is used in the DSMZ.
4. 11 mm × 43.5-mm small glass vials, fitted with a cotton plug (the type of plug used in the DSMZ can be obtained from suppliers of dental consumables) (**Fig. 4B**).
5. Suitable material for labeling the outside of the inner vial.
6. 14.5 mm × 100-mm soda glass tubes, into which has been placed a few pellets of a silica-based humidity indicator and a layer of cotton wool (**Fig. 4B**).

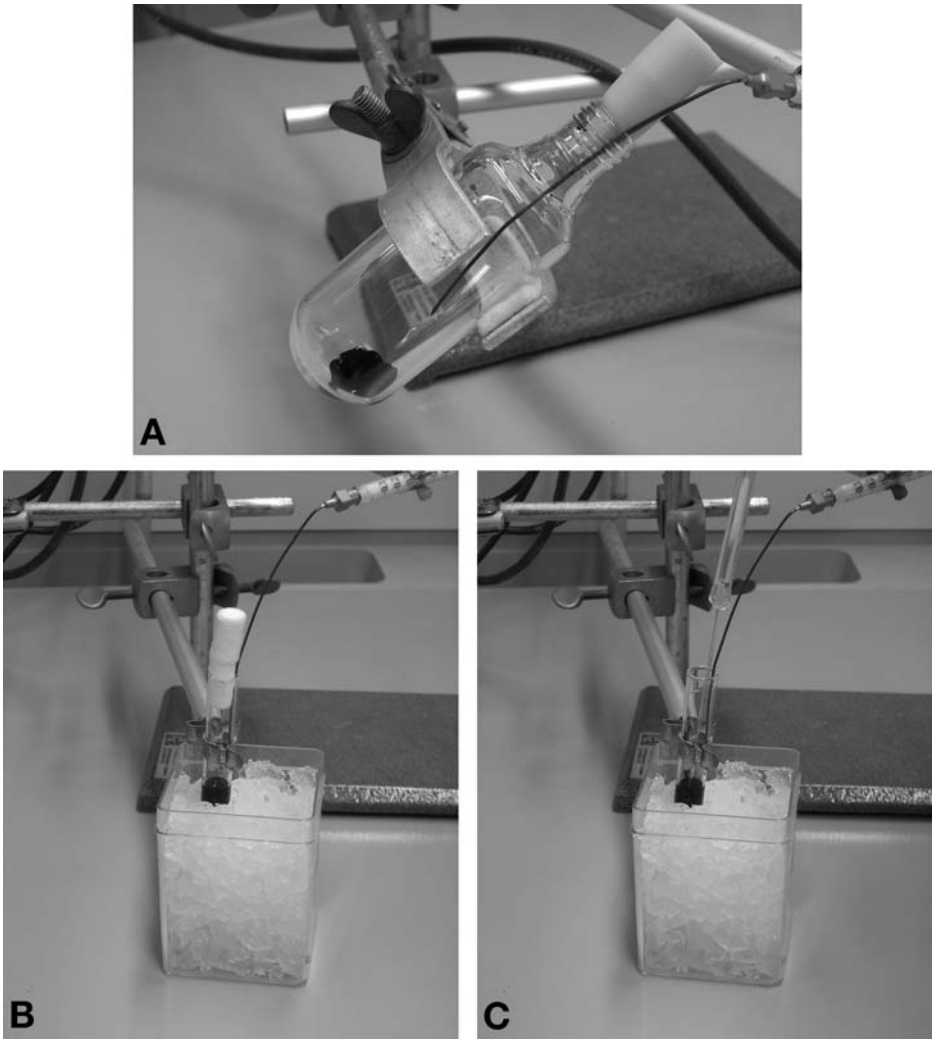

Fig. 3. The process of handling anaerobic or oxygen-sensitive prokaryotes for capillary freezing. **(A)** The centrifuge tube with the rubber stopper removed and replaced by a soft silicon stopper. The supernatant has been removed and the cell suspension is gassed via a sterile syringe needle (that enters the tube from the right and extends to just above the cell suspension). **(B)** The cell suspension is transferred to a small (sterile) vial, fitted with a cotton stopper. The vial is gassed with a sterile syringe needle (that enters from above). The vial is stored on ice. **(C)** The cell suspension is drawn into the capillary using the adapter and pipetting aid. The cell suspension is gassed continually with a flow of sterile nitrogen.

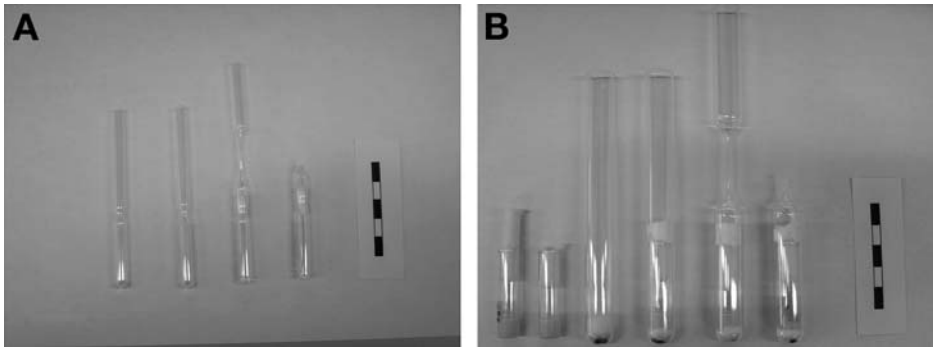

Fig. 4. Processes in the making of freeze-dried vials. **(A)** 7–8 × 100-mm vials. (Left to right) The original vial, a vial fitted with a cotton plug, a constricted vial, and a finished vial. **(B)** Double-vial system used in the DSMZ. (Left to right) The inner vial containing sterile, freeze-dried skimmed milk, the vial with the cotton stopper shortened ready to be placed in the outer tube, the outer tube with a few crystals of a silica gel-based humidity indicator and a layer of cotton-wool, the inner vial packed into the outer tube held in place by a glass wool plug, the constricted ampoule, and the finished ampoule.

7. Glass wool (depending on the product this may cause respiratory problems or other health hazards if inhaled—take appropriate precautions) and a plastic "ramrod" that fits loosely into the soda glass tubes.

2.3.3. Centrifugal Drying

1. "Mist desiccans."
   a. 100 mL horse serum.
   b. 33 mL oxoid nutrient broth (CM1).
   c. Mix the two solutions and add 10 g glucose.
   d. Dissolve the glucose by slow, steady shaking.
   e. Sterilize by over pressure filtration through a membrane filter.
2. Blotting paper, bearing the strain designation and date of preparation of the ampoule.
3. Freeze-drier (fitted with a centrifuge) (*see* **Note 3**).
4. Gas burner for sealing the ampoules.
5. Sterile Pasteur pipets.
6. 7 mm × 100-mm glass vials. Plug with cotton wool, sterilize in covered racks or containers, with the labels (**Fig. 4A**).

3. Methods

3.1. Cultivation of Strains Prior to Preservation

Each strain to be either stored frozen or in a dried state should be cultivated on the medium on/in which it grows best. The list of possible media and growth

conditions is far too extensive to be dealt with here but the following sections deal with some general principles.

3.1.1. Cultivation of Aerobes

Aerobic strains are usually grown either on agar slopes or in liquid media. It is impossible to generalize as to how many agar slopes or what volume of liquid culture is to be prepared, and one has to rely on experience. Generally either three to five agar slopes (in 16 mm × 180-mm test tubes) or about 20 mL of liquid culture are inoculated and used as the source of cell material for the preparation of a concentrated cell suspension. Some strains grow under special gas mixtures, such as hydrogen-oxidizing bacteria or methanotrophs (*see* **Note 4**).

Organisms that are difficult to remove from the agar surface may be better cultivated in liquid culture. Some organisms, such as filament-forming actinomycetes, may form aggregates in liquid culture, which may be difficult to break up under normal conditions. Strains should be grown under optimal conditions and harvested in the late exponential/early stationary phase. In the case of organisms that produce spores, they should be grown under conditions that enhance the numbers of spores produced.

3.1.2. Cultivation of Oxygen-Sensitive Organisms

Some microaerophiles or anaerobes may be grown on agar slopes in tubes that are filled with the appropriate gas mixtures and sealed with appropriate rubber stoppers ***(4)***. However, in laboratories, such as the DSMZ, where the Hungate technique ***(5)*** is a routine method, it has been found more convenient to grow such strains in liquid culture. It is normally necessary to harvest cells by centrifugation and it may be easier to cultivate the strains directly in suitable, custom-made glass centrifuge tubes, fitted with a screw cap and a suitable, gas-tight rubber stopper. Strains should be grown under optimal conditions and harvested in the late exponential/early stationary phase. In the case of organisms that produce spores, they should be grown under conditions that enhance the numbers of spores produced.

3.1.3. Harvesting Aerobic/Oxygen-Tolerant Strains From Agar Slopes

1. Cells that can be easily removed from the agar are removed from the surface by repeated gentle washing with a small volume of the appropriate suspending solution (usually about 1–5 mL) (*see* **Note 5**).
2. Because of the different methods that can be used for either freezing or drying cells, the suspending medium may be either fresh growth medium, fresh growth medium containing cryoprotectants (for freezing only), skimmed milk, or other suitable protective agents (*see* **Note 6**).

3.1.4. Harvesting Aerobic/Oxygen-Tolerant Strains by Centrifugation

1. Oxygen-tolerant strains may be either grown in separate vessels (e.g., conical flasks or serum bottles for those requiring special gas mixtures) or the organisms may be grown directly in the appropriate glass centrifuge tubes. Custom-made glass centrifuge tubes (Ochs) in which the strains may be grown and harvested, or the liquid cultures are transferred to them in order to be harvested are illustrated in **Fig. 1** (*see* **Note 7**).
2. Cells are generally harvested in a swinging bucket rotor, running at about 3000 rpm for 20 min (*see* **Notes 8** and **9**).
3. The rotor should be braked at a speed that will not cause the sedimented cell pellet to resuspend.
4. The supernatant is carefully removed using a suitably sized pipet and the remaining cell pellet resuspended in the appropriate medium (*see* **Note 6**).

3.1.5. Harvesting Anaerobic/Oxygen-Sensitive Strains From Agar Slopes

This method is included for completeness, but such organisms are generally not grown on slopes in the DSMZ. All manipulations during the process of harvesting must be carried out under the appropriate gas mixture. Cells that can be easily removed from the agar are removed from the surface by repeated gentle washing with a small volume of the appropriate suspending solution (usually about 1–5 mL) (*see* **Notes 5** and **6**).

3.1.6. Harvesting Anaerobic/Oxygen-Sensitive Strains by Centrifugation

1. Oxygen-sensitive or anaerobic strains may be either grown in separate vessels (e.g., serum bottles), or the organisms may be grown directly in the appropriate glass centrifuge tubes (*see* **Note 10**).
2. All manipulations must be carried out under oxygen-free (or limiting) conditions (*see* **Note 7**).
3. Cells are generally harvested in a swinging bucket rotor, running at about 3000 rpm for 20 min (*see* **Notes 8** and **9**).
4. The rotor should be braked at a speed that will not cause the sedimented cell pellet to resuspend.
5. The supernatant is carefully removed (under an appropriate gas atmosphere) using a suitably sized pipet and the remaining cell pellet resuspended in the appropriate medium (*see* **Note 6**).

If precautions are not taken then no survival will be observed after "reviving" preserved material. Certain organisms may be difficult to manipulate, grow, and subsequently preserve if not dealt with in the proper fashion (*see* **Notes 11–15**).

3.2. Cryopreservation-Based Preservation/Stabilization Techniques

3.2.1. Storage by Cryopreservation in Mechanical Freezers

3.2.1.1. Cryopreservation in Glycerol

1. Dispense the glycerol in 1-mL portions into the glass bottles and sterilize in an autoclave.
2. Once cooled, the glass bottles may be labeled on the sides or by painting the top of the cap with Tippex and writing the strain designation with a fine, water-insoluble, felt-tipped pen.
   a. To prepare the strain for storage remove 0.6 mL of the grown culture and add it to the glycerol; mix gently to give a homogeneous solution.
3. Add a freshly grown liquid culture to sterilized glycerol in appropriate screw-capped glass bottles.
4. Freeze the bottles at –20°C and store in a conventional freezer.
5. When samples are withdrawn, it is not necessary to thaw the bottles and they may be conveniently held in a precooled wax block containing appropriate sized holes. It should be noted that every time that a sample is withdrawn that there is always a chance of contamination.

3.2.1.2. Freezing Using Glass/Ceramic Beads

This method is essentially that developed by Feltham et al. *(7)* and may be used to store organisms either in mechanical freezers (–20 to –80°C), or using liquid nitrogen (*see* **Note 16**). If commercially available systems are to be employed then one simply follows the manufacturer's instructions.

1. Wash the beads in detergent, followed by dilute HCl (to neutralize the alkalinity of the detergent). The beads are then washed several times with tap water, and finally with distilled water. The beads are then dried at 40–50°C.
2. Add 10–20 beads to the glass bottles, loosely fit the caps, and sterilize them. Different colored beads may be used to distinguish different groups/categories of organisms.
3. Cell suspensions may be obtained either by washing the strains from agar slopes, or by centrifugation of liquid cultures.
4. Resuspend the culture in the same growth medium (in broth form) containing 15% (v/v) glycerol (*see* **Note 17**).
5. Pipet the cell suspension into the glass vials, using a Pasteur pipet, so that the beads are fully covered.
6. Introduce the end of the pipet to the bottom of the vial and use the pipet to empty and recover the beads several times. Gently agitate the beads to dislodge any air bubbles. Once the beads are fully wetted, the vial is held at a slight angle and the excess cell suspension removed from the lowest point of the bottom of the vial. Excess liquid will only cause the beads to stick together.
7. Place the vials in the freezer (usually –60 to –80°C).

The same method may be used for the storage of strains in liquid nitrogen. However, it is generally recommended that plastic, screw-cap vials are used and that they be stored in the gas phase. In addition, the approach may be used for the storage of anaerobes (*see* **Note 18**).

*3.2.2. Storage Above or in Liquid Nitrogen (*see ***Notes 19–21****)*

3.2.2.1. Freezing in Glass Capillaries

The method described here is that which was developed in the DSMZ during the 1970s in order to cope with the growing number of "exotic" strains that were being deposited, and is an adaptation of the method described by Hippe ***(5)*** for anaerobes.

1. DMSO is generally added to freshly prepared, sterile growth medium to give a final concentration of 5% (v/v). DMSO is always added just before the medium is used, and the mixture is never stored. Cool the medium containing DMSO in an ice bath.
2. Harvesting the cells. Use strains grown to the late exponential to early stationary phase.
   a. If grown on slopes the cells are washed off using about 1 mL of fresh, sterile medium containing 5% DMSO. If several slopes are used then the washing medium, containing an increasing concentration of cells, is transferred from tube to tube. The final, concentrated cell suspension is then transferred to the small, cotton-plugged vial and placed on ice.
   b. If cells are harvested by centrifugation of liquid media, then the supernatant is carefully removed and the cell pellet resuspended in 1 mL of fresh, sterile medium containing 5% (v/v) DMSO. In some cases it is not easy to obtain a compact pellet and in such cases the cells may be diluted 1:1 with fresh sterile medium containing 10% (v/v) DMSO (the final concentration of DMSO is 5% v/v).
3. The final, concentrated cell suspension is then transferred to the small, cotton-plugged vial and placed on ice.
4. Place the adapter in the pipetting aid using normal microbiological techniques to avoid contaminating the end into which the capillary will be inserted.
5. Insert a capillary (under aseptic conditions) into the adapter (**Fig. 2C**).
6. Remove the cotton plug and dip the tip of the capillary into the concentrated cell suspension held in the small vial and slowly withdraw sufficient cell suspension to fill the capillary to about 40 mm from the lower end (**Fig. 3C**). Remove the end of the capillary from the cell suspension and gently draw the cell suspension further up the capillary so that the lower end of the cell suspension is about 10 mm from the lower (open) end. Remove the capillary from the vial and replace the cotton stopper.
7. Place the open end of the capillary in the fine gas flame, just above the hottest part of the flame (**Fig. 2A**). The end of the capillary should melt very quickly and seal in a slightly rounded drop. Holding the capillary too long in the flame will only

heat the culture and kill the suspension. Excessive heating of the end may also cause the gas held between the closed end and the suspension to expand, causing the end to form a thin-walled glass ball—this is fragile and will usually break.

8. Remove the end of the capillary from the flame and place the region between the upper surface of the cell suspension and the adapter about 25–30 mm from the upper end of the capillary in the tip of the flame (**Fig. 2B**). The glass should melt and collapse, sealing the capillary, and at this point the capillary is separated by pulling the two ends apart. The capillary is usually about 60-mm long, with a 40-mm long cell suspension 10 mm from each end.
9. Place the sealed capillary in the glass Petri dish that is kept in the ice bath. Remove the short end of the capillary that is still held in the adapter with forceps. (**Warning**: the end will still be hot.)
10. Repeat **steps 1–9** until sufficient capillaries have been made (*see* **Note 22**).
11. The finished capillaries may be immersed in the cold alcohol to sterilize the outside. All capillaries are then dried, by rubbing them gently between absorbent tissue paper, and placed in their storage container (*see* **Fig. 5A**).
12. The capillaries, in their storage container are then placed in a labeled plastic Petri dish and laid in the gas phase of the liquid nitrogen tank. Once frozen, the capillaries in their storage container may be placed in their storage position (*see* **Notes 23** and **24**).

The method may also be used to store anaerobic or oxygen sensitive-strains in capillaries in liquid nitrogen. The methods used in the DSMZ for the storage of anaerobes or oxygen-sensitive organisms generally uses the Hungate technique for handling the strains ***(5)***, which employs additional precautions to ensure that the strains are not exposed to oxygen.

1. Strains are either cultivated in suitable anaerobic containers or directly in specially made centrifuge tubes, which may be maintained under the appropriate gas mixture.
2. Harvest the cells by centrifugation; remove the supernatant under a stream of nitrogen gas that is led into the tube via a long, sterile (sterilized by flaming) syringe needle. The gas should be passed through a sterile cotton plug to prevent contamination from the nitrogen gas lines (**Fig. 3A**).
3. Resuspend the cell pellet in anaerobic medium containing DSMO, which is added just before use. The points mentioned in **Subheading 2.2.** are to be followed, remembering that all work is to be carried out under a flow of sterile nitrogen gas (**Fig. 3B**,**C**). The final concentrated cell suspension is then transferred to the small, cotton-plugged vial, gassed with nitrogen, and placed on ice (**Fig. 3B**,**C**) (*see* **Note 25**).
4. Follow **steps 4–12**, with the exception that in **step 6** the tip of the capillary is first held above the cell suspension, and nitrogen is drawn into the capillary and adapter before the capillary is filled with the cell suspension and sealed out outline in the subsequent steps.

This method is routinely used in the DSMZ for storing some of the most fastidious anaerobes.

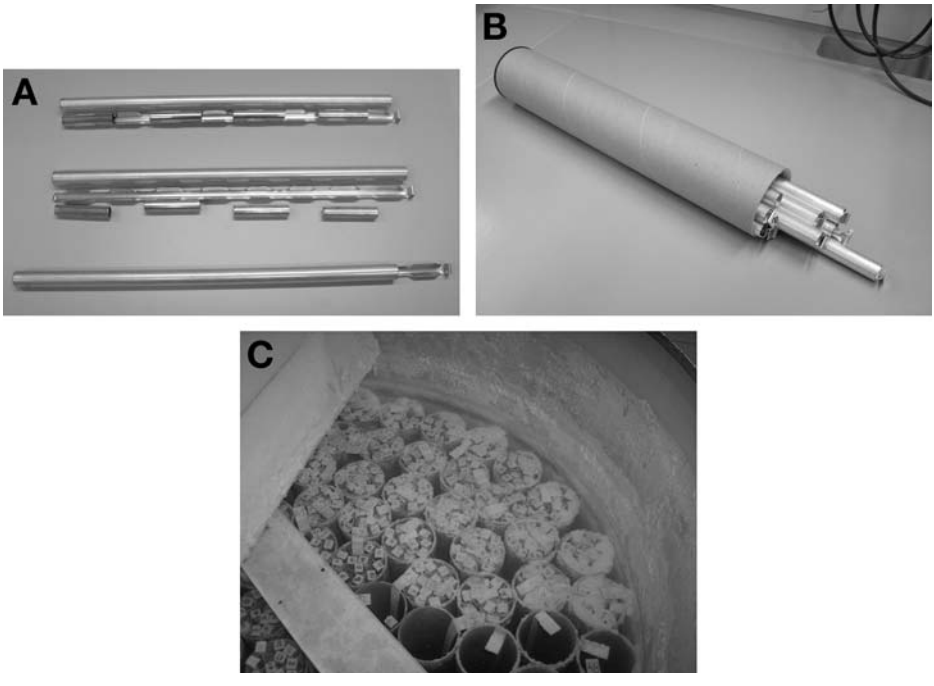

Fig. 5. **(A)** The system used for storing capillaries in the liquid phase in the DSMZ. Bottom in photo the aluminium cane, holders, and sleeve fully assembled. Middle of photo the individual components, holder, cane, and sleeve. Top of photo the holders clipped into the cane, ready to be inserted into the sleeve. **(B)** The aluminium containers held in the cardboard canister. The bottom of the tube is strengthened by a metal plate that has holes drilled in it. **(C)** View into a partially packed tank, showing the empty cardboard canisters (toward the bottom of the photo) and the filled canes and sleeves. Both the canisters and the canes are given a numerical code.

3.3. Drying/Freeze-Drying-Based Preservation/Stabilization Techniques (see Note 3 and Chapter 2)

3.3.1. Freezing and Vacuum-Drying

In this method, a cell suspension in an appropriate suspending fluid is first frozen and then quickly placed under vacuum before the vials have time to thaw. Ice contained in the samples is then removed under vacuum by sublimation.

The method described is that used in the DSMZ and is based on a 10% (w/v) sterile, skimmed milk suspension.

1. Label the outside of the inner vials with suitable details—strain designation, date of preparation, and so on.

2. Either wash the cells from the surface of agar slants using the sterile skimmed milk (20% [w/v]), or resuspend the cell pellet of a centrifuged liquid culture using the skimmed milk.
3. Transfer the skimmed milk suspension into the multistepper and dispense 200 µL of the homogeneous suspension into the stopper vials. Freeze at –20°C.
4. Quickly transfer the frozen material to the freeze-drier and apply the vacuum overnight (primary drying).
5. Once dry, remove the inner vials from the vacuum chamber, cut off the overhanging tops of the cotton stoppers, and place each inner vial in a soda glass tube (**Fig. 4B**).
6. Pack the inner vial into the soda glass tube by firmly packing a layer of about 5 mm of glass wool on top of the vial (**Fig. 4B**).
7. Constrict the ampoules (*see* **Notes 2** and **26**) (**Fig. 6A–D**).
8. Place the ampoules on the freeze-drier, using the "tree" for "secondary drying," and wait until the vacuum has built up again—usually 2–3 h (**Fig. 7A**,**B**).
9. Cut off the ampoules at the narrowest point using the gas torch. Practice is needed to ensure that the glass collapses evenly and forms a uniform seal. The end should be rounded off to give a more stable tip (**Fig. 4B**).
10. Store the ampoules in the dark at reduced temperature.

3.3.2. Vacuum-Drying Using a Predried Plug or Absorbent Material

In this method a relatively large volume of a suitable material (dextran, starch, peptone, skimmed milk) is sterilized and freeze-dried. A small volume of the cell suspension to be dried is then dropped onto this predried plug and then placed under vacuum. It is unclear whether the cell suspension freezes under these conditions. The DSMZ uses a skimmed milk plug, whereas Dando and Bousfield ***(6)*** have described a variation of the centrifugal freeze-drying method that uses a thicker, more absorbent filter paper to soak up the cell suspension, dispensing with the centrifugation step.

1. Dispense the 20% (w/v) skimmed milk solution in 500-µL portions into the stoppered glass vials. Sterilize by autoclaving at 115°C for 13 min.
2. Freeze the sterile skimmed milk-containing vials at –20°C.
3. Quickly transfer the frozen, sterile, skimmed milk to the vacuum chamber of a freeze-drier and apply the vacuum until dry. This may take more than 1 d, depending on the size of the batch. The dried, sterile plugs can be stored covered in suitable containers for some weeks.
4. Label the outside of the inner vials with suitable details—strain designation, date of preparation, and so on.
5. Either wash the cells from the surface of agar slants using 1 mL fresh medium, or resuspend the cell pellet of a centrifuged liquid culture using 1 mL fresh medium.
6. Transfer the cell suspension into the multistepper and dispense 20 µL of the homogeneous suspension into the stopper vials so that the suspension drops onto the middle of the sterile skimmed-milk plug.

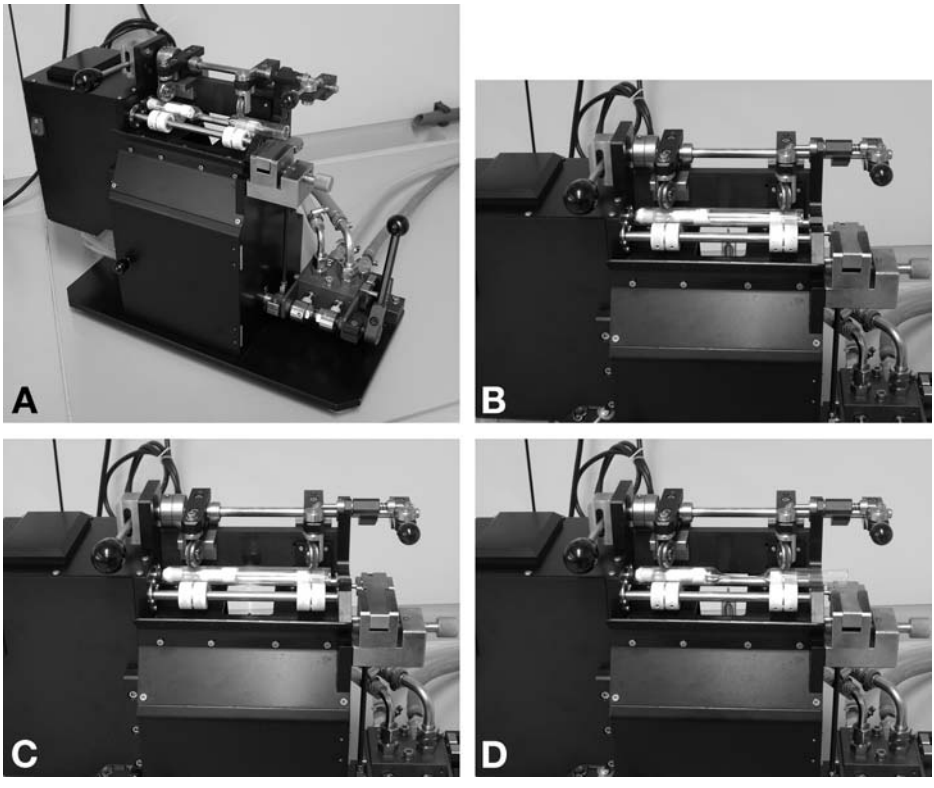

Fig. 6. The process of constricting the DSMZ double vial. **(A)** The constrictor model currently in use in the DSMZ. **(B)** The packed ampoule laying in the bed of the constrictor. The fishtailed burner lies behind the ampoule (middle of photo). **(C)** The packed ampoule laying in the bed of the constrictor, being heated by the fishtailed burner (middle of photo). **(D)** The constricted ampoule laying in the bed of the constrictor. The fishtailed burner has been returned to its position behind the ampoule (middle of photo).

7. Take the inner vials, cut off the overhanging tops of the cotton stoppers, and place each inner vial in a soda glass tube (**Fig. 4B**).
8. Pack the inner vial into the soda glass tube by firmly packing a layer of about 5 mm of glass wool on top of the vial (**Fig. 4B**).
9. Constrict the ampoules (*see* **Notes 2** and **26**) (**Fig. 6A–D**).
10. Place the ampoules on the freeze-drier, using the "tree," and dry overnight (**Fig. 7 A**,**B**).
11. Cut off the ampoules at the narrowest point using the gas torch. Practice is needed to ensure that the glass collapses evenly and forms a uniform seal. The end should be rounded off to give a more stable tip (**Fig. 4B**).
12. Store the ampoules in the dark at reduced temperature.

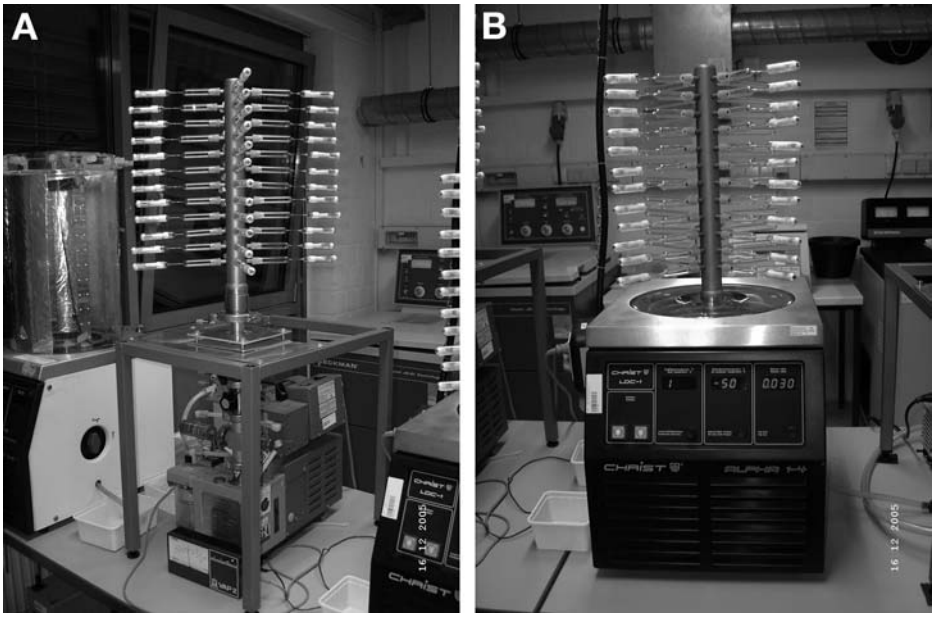

Fig. 7. Two freeze-driers used in the DSMZ. **(A)** An RC4 chemical hybrid pump from Vacuubrand. **(B)** An Alpha 1-4 from Martin Christ.

Dando and Bousfield ***(6)*** have described a variation of this method using thick, absorbent filter paper. This may be substituted for the skimmed-milk plug in this method or used in combination with narrower vials. The authors also used "mist descicans," rather than growth medium, as the suspending solution.

3.3.3. Centrifugal Freeze-Drying

Using this method a cell suspension, in an appropriate suspending fluid, is placed in a low-speed centrifuge that can be placed under vacuum ***(1,2,6)***. The centrifuge is first switched on and once running the vacuum is then applied. Under these conditions the cell suspension freezes from the application of the vacuum. In the initial stages it is important that the centrifuge remains switched on until the cell suspension is completely frozen, otherwise the cell suspension with froth and bubble. This method is particularly useful for drying organisms that tolerate oxygen for a limited amount of time.

1. Either wash the cells from the surface of agar slants using "Mist desicans," or resuspend the cell pellet of a centrifuged liquid culture using "Mist desicans."
2. Transfer the cell suspension into the Pasteur pipet and dispense 100–200 µL of the homogeneous suspension into the bottom of the sterile vials so that the suspension does not contaminate the upper part of the vial.

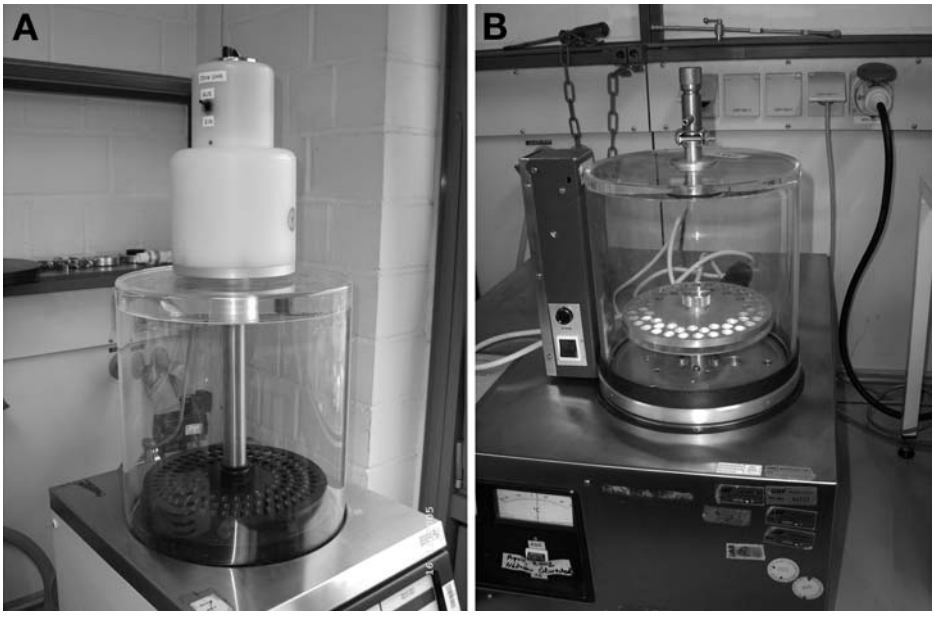

Fig. 8. Two centrifugal drying systems. **(A)** A centrifuge head from Martin Christ, fitted to an Alpha 1–4. **(B)** A centrifuge head made by Edwards High Vacuum, fitted to an Edwards Modulyo freeze-drier. Neither company currently manufactures these parts.

3. Place the stoppered, filled vials in the centrifuge rotor. Start the rotor, and once running at full speed turn on the vacuum. Alternatively, the vials may be covered with close-fitting gauze or cotton caps. "Primary drying" (*see* Chapter 2) continues for a minimum of 3 h (**Fig. 8A**,**B**).
4. Stop the centrifuge and replug the vials with sterile cotton wool.
5. Constrict the ampoules (*see* **Notes 2** and **26**) (*see* **Fig. 6A–D**).
6. Place the ampoules on the freeze-drier, using the "tree" for secondary.
7. Once secondary drying is complete cut off the ampoules at the narrowest point using the gas torch. Practice is needed to ensure that the glass collapses evenly and forms a uniform seal. The end should be rounded off to give a more stable tip (**Fig. 4A**).
8. Store the ampoules in the dark at reduced temperature.

Hippe ***(5)*** described a variation of this method for anaerobes (using a suspending fluid containing 1 mg/mL amorphous iron [II] sulfide) and the DSMZ "double-vial" method whereby the inner vial is placed in a vacuum centrifuge and later packed into the outer vial. The vacuum is released by flushing the vacuum chamber with nitrogen.

3.4. Quality Control of Frozen and Dried Cultures

The viability of all strains stored in the DSMZ is checked by comparing the viability of the cell suspension used to prepare the frozen or vacuum-dried

stocks with that of the stocks after they have been frozen or vacuum dried. The method is a simple 10-fold dilution series viable count.

1. Viability counts before storage: take the same volume of cell suspension used in preparing the vials/ampoules capillaries (or a known volume of the glycerol/strain mixture or a single bead) and prepare a 10-fold dilution series in the liquid media. Aerobes may be plated out by dropping one drop from a sterile Pasteur pipet from each tube onto the same medium solidified with agar. The drop may be gently distributed using a sterile loop or sterile glass rod. Typically an agar plate may be divided into either three or six equal sectors and one drop from the Pasteur is placed in that sector, beginning at the lowest dilution. Incubate the strain at the appropriate temperature until growth occurs.
2. Viability counts after storage: this requires that a frozen vial/capillary or vacuum-dried ampoule is opened.
   a. Screw-cap vials may be transported to the lab in specially prepared containers that have holes into which the vials may be placed. Alternatively, wax is poured into a suitably stable container and a hole is drilled large enough to take the vial being used. The blocks/containers are routinely stored in the freezer. Open the vials and either remove a known portion of the material or a single bead. Proceed as in **step 1**.
   b. Vacuum-sealed ampoules can be opened in one of two ways, depending on their diameter. The smaller diameter (7–8 mm "single" ampoules) are scored with a diamond or glass cutter in the middle of the region where the cotton plug is located. Sterilize the outside by wrapping it in an alcohol-soaked cloth. Carefully break open the vial, making sure that the cotton plug remains in the section where the dried cell material is located. The larger "double vials" (such as those supplied by the DSMZ) are opened by placing the tip of the vial in a Bunsen flame. The vial is removed from the flame and a drop of distilled water placed on the glass to crack it. Wear goggles if working outside of a safety cabinet. The tip should crack but otherwise remain intact. Hold the tip over a suitable container (beaker) and gently tap the end with a hard object, such as the end of a set of forceps (do not use a hammer). The end should fall off. Remove the upper glass wool plug with care, using forceps, and dispose of it carefully (the glass wool may be a health hazard). Remove the inner vial.
3. Hydrate the contents of the vials using 0.2–0.5 mL freshly prepared growth medium. Oxygen sensitive strains should be handled in a way that reduces exposure to oxygen (e.g., Hungate technique). In the case of predried plugs they may take 15–30 min to fully rehydrate. Remove all of the contents of the vial and inoculate the first tube of liquid medium. Prepare a dilution series as in **step 1**.
4. Capillaries are removed from their storage position and immersed in a small water bath. Pyschrophiles may need to be thawed in iced water. Using forceps, remove the capillary from the water bath and place it on dry, absorbent paper. Dry the capillary. Using a sharp, fine diamond lightly score the capillary at both ends (about 5 mm from each end). Holding the capillary in self-closing forceps dip the ends of

the capillary in 100% ethanol so that the score mark is immersed, remove the capillary from the ethanol, and ignite the end with a small, cool flame (e.g., a lighter). Sterilize both ends in the same way. Using flamed forceps break off the ends of the capillary by breaking *away* from the score mark. Be careful not to contaminate the open ends. The contents of the capillary may be removed with either a suitably sized sterile syringe and needle (that fits into the capillary), or using finely drawn out Pasteur pipets. Anaerobes are best removed using a sterile syringe and needle, which has been flushed with sterile, oxygen-free nitrogen. Inoculate the first tube of liquid medium and prepare a dilution series as in **step 1**.

3.5. Authenticity/Identity Checks

There is no standard method for confirming the authenticity/identity of a culture. The methods used for a collection of strains of different species will differ from those used to distinguish a collection of mutant strains of the same species. In some cases, the organisms may not even be fully characterized and the sole goal is to make sure that strains are not swapped or contaminated. The methods available to a national culture collection may also differ to those available to a research laboratory.

4. Notes

1. The adapter used in the author's laboratory is made from the top of a 1-mL pipet, which has a wide top to take a cotton plug (inside diameter approx 5.3 mm) (**Fig. 2C**). Using a very hot flame (a glass blower's torch would be ideal) heat the pipet about 50–60 mm from the top end. When the glass softens gently pull the two ends apart to give a conical constriction. Allow to cool and separate the two halves of the pipet with a glass cutter. Round off the sharp ends of the shorter (top) end in a hot flame. Three to five millimeters above the region where the cotton plug narrows to join the main bore of the pipet, score the glass with a glass cutter and break off the section with the wider bore. Round off the sharp ends of the remaining section in a hot flame. Insert a piece of cotton wool into the bore so that it forms a plug midway between the two ends of the glass adapter. Insert a short piece (5–6 mm, 3-mm o.d., 0.8-mm wall thickness) of silicon rubber tubing (3-mm o.d., 0.8-mm wall thickness) into the bore of the pipet where the wider bore constricts into the narrower (main bore of the pipet). Wetting the silicon tube with water may help the tubing to slide into place. The combination of the constriction in the glass bore and the silicon rubber tubing forms a seal when the end of the capillary is inserted into the adapter (similar in principle to the way most pipetting aids function). The pipetting aids are sterilized by placing them in short, glass, screw-capped tubes (16×100 mm). A different, sterile adapter is used for each strain to be handled.
2. Vacuum drying in glass; constriction and sealing of the ampoules: when drying strains under vacuum there are two important steps that are crucial in preparing the ampoules. The first is to create a constriction (narrowing of the diameter) of the

ampoule, without which it would be impossible to seal the glass under vacuum. The second important stage is sealing the ampoule under vacuum.

Many culture collections use simple glass ampoules, which are 7 × 100 mm (wall thickness approx 1 mm). In the past, Edwards used to supply a machine (a constrictor) to help produce a uniform narrowing of the ampoule prior to the final evacuation of the vial. This machine is no longer available and several culture collections have had their own machines custom made. It is possible to produce a constriction in the vials by turning the ampoules in a fine hot flame. The constriction being produced by gentle pulling with the ampoule is rotated. This requires some skill and practice and is often the limiting factor is determining whether one embarks on the task of freeze drying a collection of strains.

3. General considerations on the type of freeze-drier to be used: over the decades various freeze-driers have been used for the vacuum-drying of cultures. Some publications centers on a particular model used in a particular collection, but few articles deal with some of the general principles. The following information may be helpful:
   a. The size of the freeze-drying unit should fit the needs of the laboratory. In some cases a single, large freeze-drier may not be the best option when handling many different strains with a variety of growth requirements and where different methods of drying are to be used. In many cases, simple apparatus may suffice if properly designed and implemented (**Fig. 7A**,**B**).
   b. Under normal vacuum-drying conditions a relatively high vacuum should be obtained, which means that oil rotary vacuum pumps are essential.
   c. During the course of vacuum-drying water must be removed from the sample and should not be allowed to collect in the oil of the rotary pump. There are a number of ways to prevent water collecting in the pump oil.
      i. Some systems used a phosphorus pentoxide trap between the vials and the vacuum pump. The old Edwards 5PS freeze-drying machine (that may still be in use) was such an example. Phosphorus pentoxide is, of course toxic, will cause burns, and must be handled with care.
      ii. Most modern freeze-driers use a refrigerated cold trap to remove the water by freezing in a chamber that is placed between the vials and the vacuum pump. Such systems were the Edwards Modulyo series and the machines currently produced by Martin Christ (**Fig. 7B**).
      iii. Experiments in the DSMZ have shown that pumps made by Vacuubrand (Wertheim, Germany) for use in chemical laboratories, such as the RC4 (**Fig. 7A**) and RC5, chemical hybrid pumps (both have been replaced by the RC6) can be used without the need for either phosphorus pentoxide or a cold trap. Essentially these pumps are a rotary vacuum pump, to the exhaust of which is added a membrane vacuum pump. Oil pumps get hot when running, which means that if the oil is put under additional vacuum (with a membrane pump) then any water collecting in the oil will also be removed. This type of pump also has the advantage that the large "dead space" needed by either the phosphorus pentoxide reservoir or cold trap is not required.

iv. Finding combined centrifugal driers of the type produced by Edwards (5PS or the centrifuge head for the Modulyo) or by Martin Christ in the past is becoming difficult (**Fig. 8A**,**B**). Photographs of the Edwards Modulyo centrifuge and the unit that used to be produced by Martin Christ are included for reference. Both use a lightweight plate in which suitably sized angled (it is important that the holes are at an angle) holes have been drilled. The plate is spun directly by a suitable motor. In the case of the Edwards unit, the motor is below the centrifuge plate (and fits into the cold trap chamber being fixed with screws), whereas the Martin Christ unit is driven by a motor placed on top of the vacuum chamber. High rotation speeds are not needed; they should be sufficient to prevent foaming and frothing during the initial stages of centrifugal drying. The alternative is to use a separate vacuum centrifuge.

4. These organisms are best grown in liquid culture, but once the cells are harvested, and there is no indication that oxygen may be toxic, they may be handled as normal aerobes in all subsequent steps.
5. Some organisms may be particularly fragile (e.g., many members of the family Halobacteriaceae) and great care should be taken not to lyse such cells.
6. Because of the different methods that can be used for either freezing or drying cells, the suspending medium may be either fresh growth medium, fresh growth medium containing cryoprotectants (for freezing only), skimmed milk, or other suitable protective agents. It should be noted that some experimentation may be needed with the organisms concerned simply because there is no single universal methods that will work well for all prokaryotes.
7. Should the strains be grown in a medium that contains particulate matter (e.g., sulfur or cellulose) it is best to grow the strain in a separate vessel, allow the particulate matter to settle out by standing the culture for about 30 min, and then transfer the supernatant to the centrifuge tubes.
8. Some strains may be difficult to harvest if they produce large amounts of extracellular polysaccharide or similar material, and it may be necessary to increase the length of time in the centrifuge or to increase the speed of rotation. However, the glass centrifuge tubes should never be used in centrifuges where the speed of rotation may be increased to 10,000 rpm or more.
9. Under normal conditions the sealed glass tubes will prevent the formation of aerosols. However, when working with organisms of risk group 2 it is a wise precaution to use swinging buckets that are themselves contained in a rotor that may be sealed.
10. If the strains need to be transferred from the original cultivation vessel to the centrifuge tube, the gas mixture in the centrifuge tube must be the same as in the culture vessel.
11. The following cover a number of special cases where the use of the wrong technique can result in the organisms not surviving:
    a. Obligate acidophiles, such as members of the genera *Thermoplasma* or *Sulfolobus*, should be grown in liquid media at low pH. Prior to harvesting the

cultures by centrifugation, cool the cultures to room temperature and an excess of sterile, powdered calcium carbonate should be added to the liquid cultures. This has the effect of increasing the pH to about 6.0. The excess calcium carbonate should be allowed to settle out and the supernatant collected and cells harvested by centrifugation. If the medium is not neutralized in this fashion, members of these two genera will not survive freezing or vacuum-drying.

12. When working with obligate psychrophiles it may be necessary to precool all media and, occasionally, all glassware. Some psychrophiles that could not be vacuum-dried at room temperature, have been successfully dried using a freeze-drier operating in a cold room at 8°C.
13. Members of the family Halobacteriaceae require high salt concentrations. If suspended in low-salt solutions ("mist desiccans," or skimmed milk) they will lyse. These organisms also do not seem to tolerate freezing and drying ***(9)***.
14. Members of the genus *Thermus* may be grown on a variety of media, but they do not always freeze-dry successfully if resuspended in their fresh growth medium. Most strains seem to prefer to be resuspended in Oxoid nutrient broth when the DSMZ predried skimmed milk plug is used.
15. Dando and Bousfield ***(6)*** indicate that methylotrophs do not store well for long periods when glycerol is used as a cryoprotectant. DMSO may be more suitable (Tindall, unpublished).
16. The original version used at the University of Leicester was "homemade," with glass/ceramic beads being purchased from a hobby shop, washed and cleaned before being sterilized and used. However, a number of commercial systems are also available that provide everything needed in kit form (e.g., Cryobank from Mast, CryoLine products from Nunc). An important factor is that the cell suspension either coats the bead, or the cell suspension is contained in the hole in the bead. Theoretically, this makes removing individual beads easier. However, every time a bead is removed there is always a risk (however small) of the stored material being contaminated.
17. Feltham et al. ***(7)*** and Jones et al. ***(8)*** recommend the use of Oxoid nutrient broth containing 15% (v/v) glycerol with work at the University of Leicester centered on medically important organisms.
18. Storage of anaerobic strains on glass/ceramic beads: Jones et al. ***(8)*** have indicated that glass beads may also be used to store anaerobes. However, it should be remembered that exposure of strains to oxygen on the large surface area of the beads may reduce the viability of the strains.
19. There are a number of risks associated with storage or cell suspensions in glass or plastic vials using liquid nitrogen. If screw-capped glass vials are used, the sealing rubber gasket will become brittle and hard at the low temperatures in the liquid nitrogen tank. This will cause the seals to leak and they will fill with cold nitrogen gas (if stored above the gas phase), or will fill with liquid nitrogen if stored in the liquid phase. On removal from the liquid nitrogen tank the unopened vial will explode violently. The alternative is to use plastic vials. However, they too have a seal, which may, if not handled properly, also leak. Leaks may be a potential source of contamination. However, exploding vials are also a potential health

risk, as well as leading to the loss of valuable biological material. Wherever possible the methods used in the DSMZ for the storage of strains in liquid nitrogen avoids the use of glass or plastic vials. Instead a glass capillary system has been developed ***(5)***.

20. The question of whether to use glass or plastic from freezing strains in the temperature range from –20 to –80°C is not given enough attention. Glass has a number of advantages, including the fact that it can be easily sterilized without distortion. Plastic containers, such as Eppendorf caps or similar products with a flip lid, are to be avoided at all costs. They may be suitable for storing small portions of chemical reagents, but are *not* suitable for storing strains. The flip-lid action is probably the best way of contaminating the contents.

    When storing strains using liquid nitrogen, screw-cap glass vials are problematic because it is very difficult to ensure a good seal, and if nitrogen enters the vial on removal from the tank they will explode violently. Several plastic vials are available, specifically designed for use in liquid nitrogen tanks, although it is generally recommended that they be used in the gas phase. Again, improperly sealed plastic vials will explode violently when removed from the liquid nitrogen tank. In this respect the capillary system developed in the DSMZ is one of the best and safest methods of storing strains in liquid nitrogen.
21. The advantages of capillaries vs plastic cryovials include: a single capillary is used to inoculate the medium and other capillaries prepared in parallel are left unopened, reducing the risk of subsequent contamination of the stored material to zero; the samples are sealed in glass, which is largely impermeable to gases, making the system ideal for the long-term storage of strains that are sensitive to oxygen; the capillaries are dense enough to be stored in the liquid nitrogen phase—they also do not leak.
22. The method used is the same as that given in **step 26**, except that 15% (v/v) glycerol should be mixed with a suitable, freshly prepared anaerobic medium, preferably the same medium as the growth medium.
23. Experience usually indicates when capillaries are not properly sealed. However, it is a wise precaution (and certainly to be recommended for beginners) to check that the ends of the capillaries are properly sealed and formed by examining them using a binocular dissecting microscope. The first end sealed may be slightly bulbous and rounded, whereas the other end is slightly constricted.
24. In the DSMZ all strains are stored in the liquid phase of liquid nitrogen. The storage containers used in the DSMZ are aluminium tubes that are clipped onto "canes" (that can normally hold appropriately sized plastic vials) (**Fig. 5A**). Depending on the depth of the tank, four or five containers may be clipped to the canes, each being filled with up to 40 capillaries. The whole is then placed in an aluminium sleeve. Up to 12 such sleeves and canes are contained in cardboard inner canisters (**Fig. 5B**). A large 350-L liquid nitrogen tank will hold approx 100 of these cardboard inner containers (**Fig. 5C**). If each aluminium tube on a "cane" contains 1 strain, then a single 350-L liquid nitrogen tank will hold 4800–6000 strains. In addition, capillaries may also be color coded by placing them in closely fitting plastic sleeves. If only 10 capillaries per strain are prepared and each strain color coded, then up to

three strains per aluminium tube may be stored. This increases the capacity of a single 350-L liquid nitrogen tank to 14,400–18,000 strains.

25. At the final stage nitrogen is the gas of choice; using nitrogen:carbon dioxide mixtures causes the sealed capillaries to crack when frozen.
26. The DSMZ uses a double-vial system. The inner vial is 11 × 43.5 mm and is contained within a soda glass tube, initially 14.5 × 140 mm. It is almost impossible to constrict these by hand. The constrictor model that was constructed in cooperation with the DSMZ is illustrated in the accompanying photos (**Fig. 6A**). The combined inner vial and outer tube are placed in the bed of the machine, and rotates on the two pairs of ceramic wheels (**Fig. 6B**). A gas–air fishtail burner initially lies behind the tube and runs on a small flame. A lever is pulled forward and brings the burner into position directly below the tube, at the same time allowing the gas–air flow to reach their set maximum (**Fig. 6C**). As the tube rotates in the flame and softens, the two upper angled guide wheels begin to draw the tube apart. Experience is needed to know when the glass is hot/soft enough to be properly constricted. The burner lever is pushed back, the flame is reduced in intensity, and is moved away from the hot tube. The upper lever to the right is then moved to the right, increasing the angle of the right wheel, and rapidly draws the outer tube to the right, forming a narrow constriction (**Fig. 6D**). Care must be taken that the constriction is not too wide, nor so narrow that it is closed. The width of the bore of the constriction and the thickness of the glass all contribute to the stability of the final ampoules when they are sealed.

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I would like to thank the various members of the scientific staff and technicians of the DSMZ, past and present, who have contributed to the development and routine use of some of these methods in the DSMZ. I would also like to thank those scientists in other institutions that have either passed on their knowledge in the written word or verbally. This work underlies the maintenance of our microbiological scientific heritage, which can be passed on to the next generation in the knowledge that they are working with the same strains as their predecessors.

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6

Freeze-Drying of Yeast Cultures

Chris Bond

Summary

A method is described that allows yeast species to be stored using a variation on the standard freeze-drying method, which employs evaporative cooling in a two-stage process. Yeast cultures are placed in glass ampoules after having been mixed with a lyoprotectant. Primary drying is carried out using a centrifuge head connected to a standard freeze-dryer. Once the centrifuge head is running, air is removed and evaporated liquid is captured in the freeze-dryer. Centrifugation continues for 15 min and primary drying for a further 3 h. The ampoules are constricted using a glass blowing torch. They are then placed on the freeze-dryer manifold for secondary drying under vacuum overnight, using phosphorus pentoxide as a desiccant. The ampoules are sealed and removed from the manifold by melting the constricted section.

Although the process causes an initial large drop in viability, further losses after storage are minimal. Yeast strains have remained viable for more than 30 yr when stored using this method and sufficient cells are recovered to produce new working stocks. Although survival rates are strain specific, nearly all National Collection of Yeast Cultures strains covering most yeast genera, have been successfully stored with little or no detectable change in strain characteristics.

Key Words: Yeast; lyophilization; freeze-drying; lyoprotectant; trehalose; skimmed milk; horse serum; ampoules; evaporative cooling.

1. Introduction

Freeze-drying is a method that is commonly used to preserve a wide variety of microorganisms and has been long used to preserve yeast cultures ***(1–3)***. The term "freeze-drying" generally refers to the technique of the removal of water by sublimation of a frozen culture sample under vacuum (*see* Chapter 2). The method used by the National Collection of Yeast Cultures (NCYC) (http://www.ncyc.co.uk/) is more correctly described as "air-drying" as it dispenses with the initial freezing of the samples to be stored and relies on evaporative cooling by the application of a vacuum during the primary drying process to remove water from the sample.

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The removed water vapor is trapped and the dried material is stored under vacuum in ampoules sealed after the secondary drying stage.

Although viability levels are not as high as those seen in cultures stored under liquid nitrogen, the product has the advantages of only requiring low maintenance, protecting the cultures from contamination during storage, and providing an easy method for distribution of cultures by mail that is particularly valuable to culture collections. The NCYC has successfully stored a wide variety of yeast species by this method and has found that after the initial drying process viability levels tend to remain static, and cultures are recoverable after periods greater than 30 yr. Furthermore, changes in the characteristics of yeast stored are either very minor or undetectable.

2. Materials

1. Growth medium: YM broth/agar. Dissolve 3 g each yeast and malt extracts, 5 g peptone, and 10 g glucose in 1 L of distilled water (pH not adjusted). Autoclave for 15 min at 121°C and dispense aseptically into suitable bottles for storage. Final pH should be approx 6.2. Alternatively, dissolve 21 g of Becton Dickinson Franklin Lakes, NJ. YM Broth (271120) in glass distilled water and make up to 1 L. Autoclave and dispense as above. Agar (Oxoid agar no. 1, OXOID Ltd. Basingstoke, Hampshire, UK), when required, is added to a final concentration of 1.5–2% (w/v).
2. Lyoprotectant/suspending medium (*see* **Note 1**).
   a. Horse serum + glucose: dissolve 7.5 g glucose in 80 mL horse serum (heat inactivated, mycoplasma screened, analytical grade) and make up to 100 mL. Filter-sterilize using 0.22-µm filters and dispense into sterile 1-oz plastic-capped bottles. Store at 4°C.
   b. Skimmed milk + trehalose + monosodium glutamate (sodium glutamate): dissolve 10 g skimmed milk, 10 g trehalose, and 5 g sodium glutamate (all food grade) in 80 mL distilled water and make up to 100 mL. Dispense into 1-oz universal bottles and sterilize by autoclaving for 5 min at 121°C.
3. Incubator: temperature controlled at 25°C.
4. Centrifuge: suitable for 1-oz bottles containing approx 10 mL YM broth culture, capable of approx 2500 rpm (900*g*) for up to 10 min.
5. Ampoules: 0.5 mL neutral glass ampoules for freeze-drying, approx 8-mm outer diameter and 10-cm long. Wash before use with detergent and rinse with demineralized water.
6. Cotton wool: nonabsorbent, white.
7. Filter paper: Whatman no. 1 or similar.
8. Freeze-dryer: freeze-dryer with centrifuge, cooling chamber, and manifold (Edwards Whatman: Florham Park, NJ; BOC/Edwards Wilmington, MA, Modulyo or similar model).
9. Glass blowing torch: butane/oxygen torch with single tip suitable for constriction of ampoules.

10. Ampoule sealing torch: butane/propane hand-held blowtorch suitable for producing a small directional flame for sealing and removing ampoules from freeze-dryer manifold.
11. Dessicant: phosphorus pentoxide.
12. High-frequency spark tester.
13. Sapphire or metal glass cutter/scorer.
14. Fume cupboard.
15. Ampoule storage area: preferably temperature controlled at 1°C.

3. Methods

1. Preparation of ampoules: Labels for the ampoules are prepared from strips of Whatman No. 1 filter paper or similar. The number of the yeast strain and the date of the freeze-drying are printed on the label, either with a stamp using nontoxic ink or with a laser printer (*see* **Note 2**). The label is folded in half lengthwise and inserted into the ampoule so that the writing faces outward. The ampoule is loosely plugged with nonabsorbent cotton-wool and placed in a tin or suitable container with a lid for oven sterilization. Ampoules for different yeast strains should be placed in separate tins. Oven-sterilize for 4 h at 180°C.
2. Preparation of inoculum: The culture to be freeze-dried is grown without aeration (*see* **Note 3**) in YM broth at 25°C for 72 h; the amount needed depends on the number of ampoules to be prepared (*see* **Note 4**).
3. Lyoprotectant/suspending medium. Use either horse serum + glucose as detailed in **Subheading 2.**, **item 2a**, or skimmed milk + trehalose + monosodium glutamate as detailed in **Subheading 2.**, **item 2b** (*see* **Note 5**).
4. Filling of ampoules. The bottles containing the yeast culture and YM broth should be centrifuged for approx 10 min at 2500 rpm (900*g*) to ensure that the yeast culture has formed a pellet in the bottom of each bottle. Once removed from the centrifuge, all the liquid should be aseptically removed from one of the duplicate bottles and approximately half the liquid from the other, taking care not to disturb the pellet. The yeast culture pellet in the bottle with the liquid should then be resuspended in the liquid and transferred aseptically to the other bottle, where it is mixed with the second pellet to give a cell suspension double the concentration of the original culture in a single bottle. Equal volumes of this suspension and the lyoprotectant are then mixed aseptically in a separate sterile bottle; the volume prepared depending on the number of ampoules to be freeze-dried.

   A sterile 30-dropper Pasteur pipet is used to transfer six drops (approx 0.15 mL) of the mixture to each ampoule, after which the cotton-wool plug is replaced. Care should be taken not to touch the insides of the ampoule with the pipet so that each of the drops from the pipet reaches the bottom of the tube and is not allowed to run down the inside of the ampoule (*see* **Note 6**). The open plug end of the ampoule is briefly flamed to sterilize it before the cotton-wool is replaced. The remaining lyoprotectant/culture mixture is retained for viability estimation. If this is not to be done immediately, the culture should be cooled in a refrigerator to slow further cell division.

Fig. 1. Centrifuge head with filled ampoules awaiting primary drying.

5. Primary drying. The filled ampoules are placed immediately in the centrifuge head of the freeze-dryer with the writing on the labels facing the center of the centrifuge head so as not to be obscured by the dried yeast after centrifugation (*see* **Fig. 1**). The centrifuge head is placed on the spindle in the drying chamber. The refrigeration mechanism of the freeze-dryer should be switched on sufficiently early to allow the temperature of the vapor collection chamber in the freeze-dryer to be lowered to –50°C before primary drying begins (*see* **Note 7**).

   The centrifuge is switched on 10 s before starting the vacuum pump, allowing any droplets of the mixture still on the inside of the ampoule to be forced to the bottom of the tube before drying commences. Centrifugation should continue for 10 min. Primary drying is continued for 3 h, after which time the vacuum is released and the centrifuge head and ampoules are removed from the drier. The yeast in the ampoules should appear completely dry and should have formed a slope of suspended material against the wall in each of the ampoules.
6. Ampoule constriction. Trim the projecting ends of the cotton-wool plugs with scissors that have been sterilized by flaming with ethanol. The remainder of each plug is then pushed into the ampoule with a plunger that has also been sterilized by flaming with ethanol (*see* **Note 8**). The plug should be pushed sufficiently far into the ampoule so that the flame does not scorch it during the constriction process.

   Using the butane/oxygen glass blowing torch, constrict the ampoules at a point above the level of the plug. Once heated, the glass wall of the ampoule should melt forming a thicker walled central section to the ampoule with a narrow

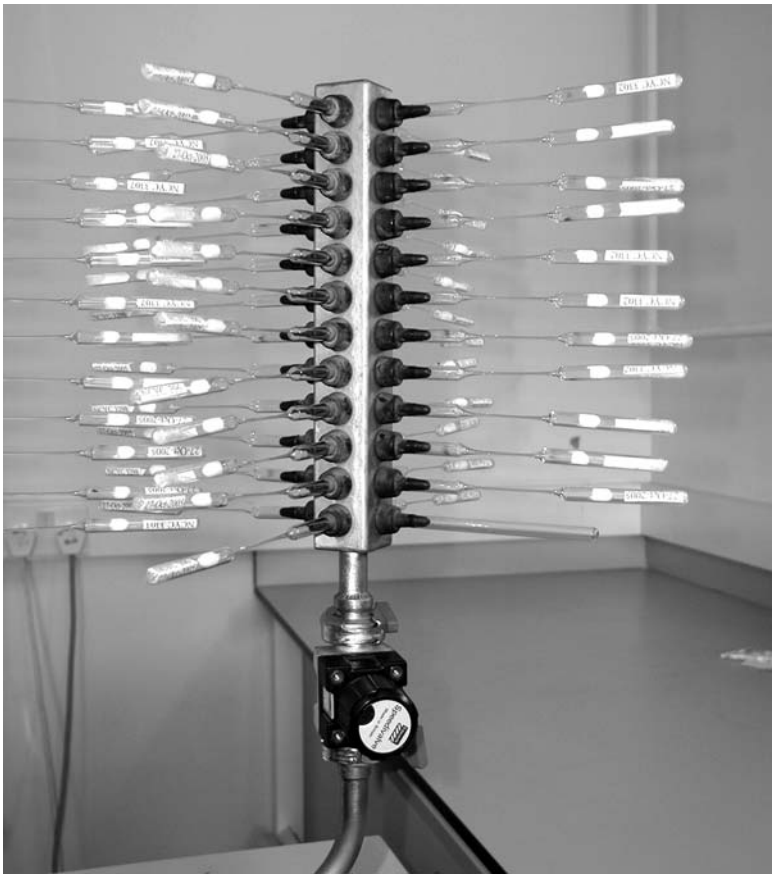

Fig. 2. Constricted ampoules on manifold during secondary drying.

central bore. The ampoule should be constantly rotated as it is heated to keep the heating even and to keep the two ends of the ampoule in alignment. The central constricted section of the ampoule should be thick enough to support the weight of the end section when it is placed on the manifold for secondary drying (*see* **Note 9**).

7. Secondary drying. Ensure that the vapor collection chamber is completely dry, fill the desiccant trays with phosphorus pentoxide, and place them in the chamber (*see* **Note 10**). Place the manifold over the chamber and place the constricted ampoules on the manifold taking care not to damage the manifold nipples or crack the necks of the ampoules. Any unused spaces on the manifold can be filled with unconstricted ampoules. Once all the spaces have been filled the vacuum pump can be started. For secondary drying the bleed valve on the vacuum pump should be shut.

   Secondary drying is normally continued overnight, although it may be terminated after 2 h, allowing the whole process to be completed in a single day.

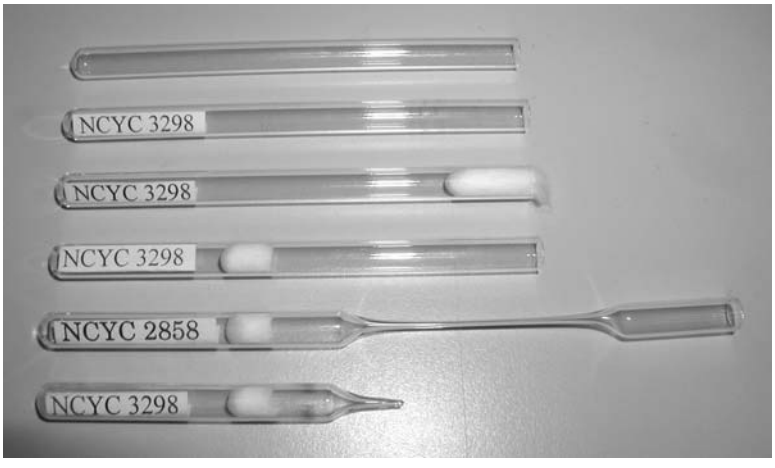

Fig. 3.

8. Removal of ampoules from the manifold. The ampoules can be sealed and removed with the use of a small blowtorch. The flame should be applied to the constricted part of the ampoule while the end containing the dried yeast is supported to prevent it drooping. The central bore of the constriction should close, seal, and then separate from the end attached to the manifold if the end of the ampoule is slowly and gently pulled away from the manifold. Any sharp spikes, either on the detached ampoule or the end still attached to the manifold, should be rounded off by melting with the blowtorch.

   Once all the ampoules have been removed the vacuum pump can be switched off and the chamber valve opened. Spent phosphorus pentoxide should be covered with water in a fume cupboard and then discarded (*see* **Note 11**). The remaining part of the ampoules, still attached to the manifold, should be removed, placed in a "discard-pot" for sterilization by autoclaving, and subsequently discarded.
9. Testing. The ampoules should be tested to make sure they contain a vacuum by using a high-frequency spark tester. The end of the spark tester should be briefly touched on the surface of each ampoule. Ampoules containing a vacuum will glow blue/violet when the spark is applied. Ampoules containing air will not glow and should be discarded (*see* **Note 12**).
10. Quality control and viability counts. On cultures prior to drying: add 1 mL of the mixed lyoprotectant/yeast suspension to 9 mL of sterile glass-distilled water. Prepare further logarithmic dilutions to 10^{-6}. Transfer three drops from a 30-dropper pipet (0.1 mL) of dilutions 10^{-6} to 10^{-3} onto YM agar. Incubate the plates at 25°C for 72 h or longer, if necessary (*see* **Note 13**). One or two drops from the mixture are also placed on an agar plate and streaked across the surface as per standard "streak-plate" procedure. The streak plate is incubated with the serial dilution plate and should be used to check for the presence of contaminants, aberrant colonies or any other anomalies when compared with the postfreeze-drying streak plate.

On cultures after freeze-drying. The count is carried out as soon as possible after freeze-drying. Open the ampoule as outlined (*see* **Note 14**). An aliquot (1 mL) of YM broth is transferred to a sterile bijoux bottle (7 mL or one-fourth ounce bottles). Using a Pasteur pipet the entire contents of the bijoux are transferred aseptically to the opened ampoule. The yeast is thoroughly resuspended and mixed with the pipet contents, taking care not to allow the YM broth to overflow the opened ampoule. One or two drops of the resuspended yeast are also placed on an agar plate and streaked across the surface as per standard "streak-plate" procedure, incubated as previously mentioned, and used as a comparison with the predried culture. All the remaining suspension is returned to the bijoux. This suspension is a 10^{-1} dilution. Prepare further logarithmic dilutions to 10^{-5}. Transfer three drops from a 30-dropper pipet (0.1 mL) of dilutions 10^{-5} to 10^{-2} onto YM agar. Incubate the plates at 25°C for 72 h, or longer if necessary.

The number of colonies formed by each dilution of pre- and postdrying suspensions is counted and the viability of the culture is calculated and recorded. Any ampoules without vacuum, containing mixed or contaminated cultures, or those that have low viability should be discarded.

11. Storage of ampoules. Storing ampoules at low temperatures (between 1 and 4°C) in the dark is believed to prevent deterioration of the dried yeast (*see* **Note 15**).

4. Notes

1. Several different substances have been shown to be effective as lyoprotectants and have significantly increased the survival rates of freeze-dried cultures. The NCYC successfully used a mixture of horse serum and glucose for many years, but now uses a mixture based on skimmed milk and other food-grade ingredients similar to that described by Berny and Hennebert ***(4)*** (*see* **Note 5**). If only a few yeast strains are to be maintained it may be possible to establish the optimum lyoprotectant for each strain. The NCYC has chosen the two lyoprotectants listed as ones that have worked well with a wide range of strains covering most yeast genera and giving acceptable survival rates.
2. Ampoules may be labeled on the outside using a labeling machine. However, the NCYC prefers to use internal labels as these cannot become separated from the ampoule, or be rendered unreadable without destroying the ampoule. Furthermore, studies performed at the NCYC have suggested that the viability of cultures originating from ampoules with internal labels was higher that that of those with no filter paper inside. This indicates that the presence of the filter paper may have a beneficial effect during the drying process.
3. The NCYC has observed that the degree of oxygenation provided during growth of the culture may affect survival during drying. Although response to oxygen is strain specific, in general, higher survival levels are obtained following growth in nonoxygenated media ***(3)***. It was also noted that cultures of sensitive strain grown on nutritionally poor medium before freeze-drying had higher survival levels.
4. Standard NCYC procedure is to inoculate two 1-oz bottles each containing 10 mL YM broth for each set of 16 ampoules to be filled. The bottles and media should be of equal weight so as to be balanced when centrifuged.

5. Both lyoprotectants listed have been used successfully over many years by the NCYC. Because of considerations of health and safety, as well as customer acceptability, it is standard NCYC policy to use the skimmed milk-based lyoprotectant with any cultures that are likely to be used as ingredients for, or in preparation of, any beverages or foodstuffs, as the ingredients can all be found as "food-grade" products.
6. When the ampoule is heated during constriction, plug any of the dried yeast suspension on the inside of the glass above the level of the will burn, releasing fumes that may lower viability and blacken the inside of the ampoule. A significant percentage of the yeast culture can be lost in this way.
7. The freeze-dryer vents should be shut in preparation to produce the vacuum, although it may be preferable to allow a small amount of air to flow through the vacuum pump bleed valve in order to let air from the atmosphere pass through the oil to reduce the amount of dissolved vapor retained in it.
8. The plunger should be flamed and sterilized when moving between batches of ampoules containing different strains, so as to prevent cross-contamination.
9. Ultraviolet-proof tinted safety glasses should be worn during the constriction process and appropriate care taken to prevent burns when using the glass blowing torch.
10. Phosphorus pentoxide desiccant is harmful and can cause burns on skin contact. Desiccant trays should be filled in a fume cupboard to avoid inhalation of the powder, and appropriate safety clothing and goggles should be worn.
11. Phosphorus pentoxide reacts vigorously with water. This operation only should be performed in a fume cupboard and appropriate safety clothing and goggles should be worn.
12. The spark tester can cause electric shock and burns. Care should be taken not to bring the electrical discharge from the tester into contact with flesh. There is some evidence to suggest that prolonged exposure to the electrical discharge can lower the viability of the yeast in the ampoules. The amount of time the spark tester is in contact with the ampoules should be kept as short as possible. Carrying out the testing away from bright light sources can make the "glow" in the ampoules easier to see.
13. The plates are kept horizontal at all times to ensure the drops remain discrete. Dilutions containing 20–30 colonies are used for estimating viability. The number of cells per milliliter inoculated into the ampoule is equal to the number of colonies in three drops multiplied by 10 times the dilution factor.
14. Score the ampoule with an ampoule cutter or file just above the level of the cotton-wool plug (toward the pointed end of the ampoule). Sterilize the ampoule with 70% (v/v) ethanol. Apply the end of a red-hot glass rod to the file mark to crack the glass. Remove the tip of the ampoule and extract the plug with sterile forceps and place them in a container for subsequent sterilization before disposal.
15. NCYC cultures are stored in the dark at 1°C. The NCYC holds strains that are still viable 30 yr after being freeze-dried. Other laboratories hold strains that have proven viable after longer periods though it has been noted that occasionally strains suddenly lose viability ***(3)***. If possible viability should be monitored on a regular basis.

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7

Cryopreservation of Yeast Cultures

Chris Bond

Summary

A method is described that allows a wide range of yeast species to be stored in liquid nitrogen while maintaining a high level of viability. Yeast cultures are sealed in commercially available polypropylene straws after having been mixed with a glycerol-based cryoprotectant. Once placed in a secondary cryotube the temperature of the sealed straws is reduced slowly to –30°C in a methanol bath over a period of up to 3 h. The straws are then transferred directly to the liquid nitrogen and placed in a racking system for long-term storage.

Key Words: Yeast; cryopreservation; liquid nitrogen; cryoprotectant; glycerol; straws; dehydration.

1. Introduction

Yeast cultures are held in long-term storage in the National Collection of Yeast Cultures (NCYC) (http://www.ncyc.co.uk/) by two methods: (1) freeze-dried in glass ampoules, and (2) under liquid nitrogen using glycerol as a cryoprotectant. Freeze-drying is a generally accepted method for yeast storage, having the advantages of conferring longevity and genetic stability, as well as being suitable for easy worldwide postal distribution of the cultures in glass ampoules. However, preservation by freeze-drying tends to be much more labor intensive than storage in liquid nitrogen and requires a higher level of skill to produce an acceptable product. Strain viabilities are generally low, typically being between 1 and 30%, as compared with more than 30% for those of yeast preserved by being frozen in liquid nitrogen. There are also several yeast genera, including *Lipomyces, Leucosporidium*, and *Rhodosporidium* that have particularly low survival levels and frequently cannot be successfully freeze-dried by the standard method.

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However, some improvements were made in the 1990s using trehalose as a protectant ***(1,2)*** and these were incorporated into NCYC procedures. A technique used by the NCYC for freeze-drying yeasts can be found in Chapter 6 of this volume.

Storage of cultures in liquid nitrogen, although technically simple, can involve relatively high running costs because of the necessity of regular filling of the containers. The initial cost of the equipment is comparable to that used for freeze-dying, but the costs and problems associated with the handling of liquid nitrogen have led some collections to seek alternatives ***(3)***. However, for most workers, the technique of liquid nitrogen storage is convenient, well tried and tested, and unlikely to be superseded in the near future.

The method presented here uses heat-sealed straws and is a miniaturized version of that commonly used. This enables a considerable reduction in storage space and extra protection against contamination by liquid nitrogen leakage. Storage in straws was first described in 1978 by Gilmour et al. ***(4)*** using artificial insemination straws and variations on the original method are now in use in a large number of laboratories around the world. Work on refining methods of storage in liquid nitrogen is continuing, as is research into the effects of the freezing process on the cells. The following paragraphs give an outline of the current understanding.

During the process of liquid nitrogen storage, certain changes take place in the cells and their immediate environment ***(5)***. As the straws are cooled, extracellular ice formation results in an increase in the solute concentration around the cells causing them to lose water and shrink ***(6,7)*** (*see* Chapter 3 for a review). This freeze-induced dehydration causes the cell wall to decrease in surface area and increase in thickness. As the maximum packing density of the lipids in the cell membrane bilayer is reached its normal structure changes. Membrane invaginations occur to allow the cells to shrink further as water is removed. This process is reversible, provided that none of the membrane material becomes lost within the cytoplasm, and upon thawing, the cell will return to its normal volume.

Cell shrinkage during freezing is vital to prevent cellular damage, hence the need to select the correct cooling rate. If the cooling rate is too rapid there is insufficient time for the cells to lose water and intracellular ice formation then occurs that causes damage to cell organelles ***(7)***.

Genetic damage may occur if the nucleus becomes disrupted. Plasmids have been found to be lost from strains of bacteria by the freezing and thawing processes ***(8)***. Similar loss of plasmids has been observed in *Saccharomyces cerevisiae*, along with mitochondrial damage that results in respiratory-deficient cells giving rise to petite colonies ***(5)***.

2. Materials

The procedures described involve use of potentially hazardous materials. The relevant local safety regulations (e.g., COSHH regulations in the UK) should be consulted prior to implementation of these procedures.

1. 21 g/L YM broth (Becton Dickinson/DIFCO Franklin Lakes, NJ): Difco dehydrated YM broth (cat. no. 271120).
   Alternatively:

YM media	Per liter
Yeast extract	3 g
Malt extract	3 g
Peptone	5 g
Glucose	10 g

   After mixing the pH should be between 5.0 and 6.0. Dispense in 10-mL volumes into suitable bottles. Sterilize by autoclaving for 15 min at 121°C.
2. YM agar: add 2% (w/v) agar to Difco YM broth or YM media before sterilization. After mixing the pH should be between 5.0 and 6.0. Sterilize by autoclaving for 15 min at 121°C. Dispense in 20-mL aliquots into sterile Petri dishes and leave to cool.
3. 10% (v/v) glycerol cryoprotectant solution: dissolve 10 mL glycerol in 90 mL distilled water. Sterilize by filtration through a 0.22-µm filter and dispense in 15-mL aliquots into suitable bottles. Store at room temperature.
4. Straws: cut colored polypropylene drinking straws to 2.5-cm lengths (*see* **Note 1**). Seal one end of each straw by holding firmly with nonridged forceps 2 mm from the end to be sealed, bringing the projecting end l cm from the flame of a Bunsen burner (*see* **Fig. 1**; **Note 2**). Once the polypropylene has melted sufficiently to close the end of the straw (usually within 1–2 s) the straw should be removed from the heat and allowed to cool while still firmly held with the forceps. Once the polypropylene has solidified, the straw can be released.

   Place the straws in a glass Petri dish and sterilize by autoclaving at 121°C for 15 min. For ease of handling, the straws should be evenly spaced around the edge of the dish with all the open ends pointing in the same direction. Two long, unsealed straws should also be prepared for use as rests for straws awaiting final sealing (*see* **Fig. 2**; **Note 3**). These should also be placed in glass Petri dishes and sterilized. Ensure that the straws are dry before use, using a moderate-temperature (40–60°C) drying cabinet if necessary.
5. Nunc cryotubes: 1.8-mL plastic screw-cap ampoules are available sterilized from the manufacturer (Nunc International, Rochester, NY; Fisher Scientific UK, Loughborough, Leicester, UK; VWR International Ltd., West Chester, PA).
6. A refrigerated methanol bath precooled to –30°C (*see* **Note 4**).
7. Liquid nitrogen containers: cryogenic storage containers with liquid-phase storage racks and dividers to store 2-mL cryotubes (e.g., Jencons Scientific Ltd.) (*see* **Fig. 3**).
8. Safety equipment: cryogloves, goggles, and so on (e.g., Jencons Scientific Ltd., East Grinstead, West Sussex, UK).

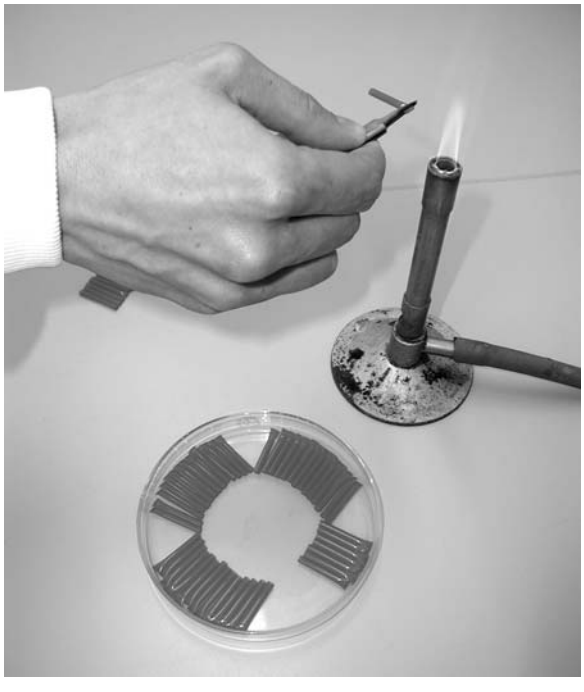

Fig. 1. Sealing polypropylene straws.

Fig. 2. Filled polypropylene straws sealed at one end awaiting final sealing and (lower center) sealed straws in a cryotube awaiting transfer to methanol bath.

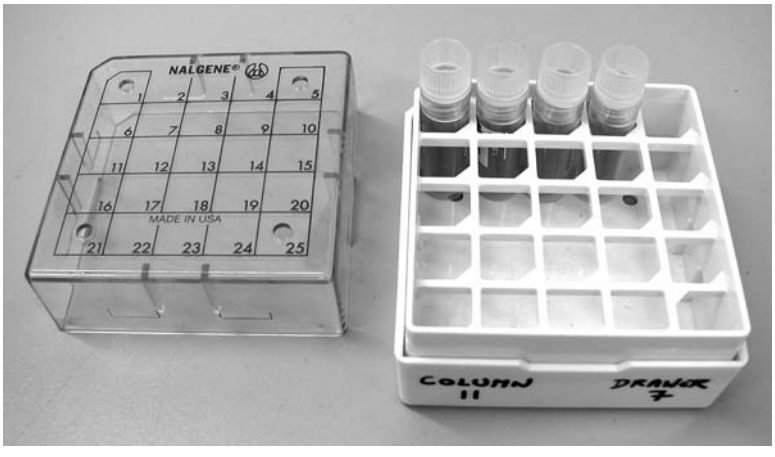

Fig. 3. Filled cryotubes in numbered racking system.

3. Methods

Follow good microbiological practice and aseptic techniques throughout.

1. Grow the culture to be frozen in 10 mL of YM broth for 72 h at 25°C on a reciprocal shaker.
2. Mix equal amounts of inoculum and glycerol cryoprotectant solution aseptically in a sterile bottle (*see* **Note 5**). Use forceps, sterilized by immersion in alcohol followed by flaming, to remove a single straw from the Petri dish. Gently grip the straw about halfway along its length to allow the insertion of the end of a Pasteur pipet containing the inoculum. Insert the pipet until the pipet tip is at the sealed end of the straw and then withdraw it as the inoculum fills the straw. Fill the straw to approximately two-thirds of its capacity. On withdrawing the pipet from the straw, any excess inoculum can be sucked back into the pipet (*see* **Notes 6** and **7**).
3. The open end of the straw is clamped shut with the forceps and sealed as described (*see* **Subheading 2.**, **item 4**). Care should be taken not to allow any of the contents of the straw to be forced out of the open end of the straw as it is clamped with the forceps, as this will prevent a good seal from being made.
4. Test the straws for leaks by holding them with forceps above the surface of a suitable disinfectant in a high-sided beaker and then squeezing them. Any liquid forced out of the seals should be safely contained within the beaker. Discard leaking straws and autoclave them.
5. Place six straws in each 1.8-mL cryotube. If post-thaw cell viability counts are required, a single straw is placed in a separate ampoule for ease of recovery. Mark each straw and cryotube with the relevant strain designation and date of freezing using a black Pentel (Pentel Co. Ltd., Swindon, Wiltshire, UK) permanent marker pen.
6. Place the filled cryotubes in the methanol bath, which has been precooled to –30°C, for 2 h to allow dehydration (*see* **Note 8**).

7. Transfer the cooled cryotubes to the liquid nitrogen containers (*see* **Note 9**) and place in the racking (inventory) system (*see* **Notes 10** and **11**). Note the position of the cryotubes (*see* **Note 12**). Remove excess methanol from the outside of the cryotubes to prevent it freezing the tubes to the racking system once immersed in the liquid nitrogen (*see* **Notes 9**, **13**, and **14**).
8. After storage: locate the cryotube and remove it from the racking system, check the strain number and date of freezing written on the tube. Remove a single straw and replace the cryotube in the racking system (*see* **Note 15**). Rapidly transfer the straw to a water bath, incubate at 35°C, and agitate to ensure that rapid and even thawing takes place (*see* **Note 16**).
9. Remove the straw from the water bath and dry it. Grip one end of the straw and sterilize the other end by wiping with 70% (v/v) alcohol. Cut off the sterile end with scissors that have been flamed with alcohol. Then remove the contents using a Pasteur pipet inserted into the open mouth of the straw. Mix the contents by repeated pipetting before transferring as an inoculum to suitable growth media (*see* **Note 17**).
10. Viability counts.
    a. On cultures prior to freezing: add 1 mL of the original cell suspension to 9 mL of sterile, glass-distilled water. Prepare further logarithmic dilutions to 10^{-6}. Transfer three drops from a 30-dropper pipet (0.1 mL) of dilutions 10^{-6} to 10^{-3} onto YM agar. Incubate the plates at 25°C for 72 h, or longer if necessary (*see* **Note 18**).
    b. On cultures after freezing: add two drops from a Pasteur pipet of the thawed cell suspension (0.06 mL) to 0.54 mL of sterile, glass-distilled water. Transfer 0.5 mL of this 10^{-1} dilution to 4.5 mL of sterile, glass-distilled water, prepare additional logarithmic dilutions to 10^{-6} as detailed previously, then inoculate YM agar plates, incubate, and count as in **step 10**. The percentage viability of the culture is calculated and recorded.

4. Notes

1. The NCYC uses polypropylene drinking straws in preference to insemination straws because of their low cost, robust nature, and ability to withstand autoclaving. Straws of approx 3- to 4-mm diameter were found to be the most suitable to use with this method.
2. Polypropylene straws should melt quickly and will form a strong seal that will set firm within 3–4 s of being removed from the heat. Care should be taken not to burn the polypropylene or deform the rest of the straw. A standard Bunsen burner may be used although "fishtail" will give a more controllable flame. Occasionally, leaks occur at the corners of the straw; particular attention should be paid to these areas to ensure they are properly sealed. Some workers have used impulse heat sealers to seal polypropylene straws. In our experience these tend to produce weak seals and do not produce the characteristic "lip" formed by the melted polypropylene that is useful when removing straws from cryotubes.
3. If several straws are being filled with the same inoculum it is convenient to transfer each straw to a sterile Petri dish before it is sealed. The straws should be leaned with the open end against a second longer straw to allow their easy removal for sealing and to prevent leaking from the opening (*see* **Fig. 2**). The straws are then

sealed and placed in the cryotube. This is more efficient than filling and sealing each straw separately.

4. **Caution**: methanol is toxic and flammable. Avoid skin contact.
5. Other workers have successfully used cryoprotectants other than glycerol. Some have been used with a wide range of yeasts, others with single strains only. Substances used include 10% (v/v) each of dimethyl sulfoxide ***(4)***, ethanol ***(9)***, methanol ***(10)***, YM broth, and 5% (w/v) hydroxyethyl starch.
6. Care should be taken not to allow droplets to remain on the open end of the straw because these can prevent proper sealing.
7. The final cell concentration in the straws once the inoculum and cryoprotectant are mixed is between 10^6 and 10^7 cells/mL. Final glycerol concentration in the straws is 5% (v/v).
8. Experiments on two test strains of *S. cerevisiae* at the NCYC showed no significant variation in viability for cells frozen to –20, –30, or –40°C for 1, 2, or 3 h during primary freezing. The NCYC uses the intermediate range that has so far been successful for all NCYC strains. A study carried out by Pearson et al. ***(5)*** indicated that cooling rates greater than 8°C/min result in a sharp drop in cell viability and cause irreversible genetic damage. Cells have been found to be more sensitive to cooling rates than glycerol concentration ***(5)***. Survival of cells appears to be related to the avoidance of intracellular ice formation and excessive cryodehydration ***(7)***.
9. If there is a large distance between the methanol bath and liquid nitrogen containers, a precooled Dewar should be used to transport the tube to prevent warming.
10. Care should be taken to provide adequate ventilation where liquid nitrogen is in use as a buildup of nitrogen can cause asphyxiation. Workers should not travel in lifts with containers of liquid nitrogen. Goggles and gloves should be worn while carrying out operations using liquid nitrogen.
11. Several models of liquid nitrogen storage container are available. Some offer greater amounts of storage space by reducing the level of liquid nitrogen above the racking system to a minimum. In order to ensure that the top racks of these containers are always completely submerged, they must be filled more regularly than those with less height of racking. These containers are best avoided unless an automatic "top-up" system is available.
12. Because stock levels cannot be easily checked once immersed in liquid nitrogen, accurate record keeping of both stock levels and the position of each strain in the racking system is important. Computerized stock control systems are ideal for storage of this information. The position of each cryotube in a rack may be mapped and recorded in the following way, for example:

Strain number:	240
LN_2 container:	A
Section:	3
Tray:	2
Row:	1
Position:	6
Total no. of straws:	6

If only a small number of strains are to be stored, different colored caps can be used for color coding of cryotubes.

13. Cultures can be stored successfully in the vapor phase of liquid nitrogen. However, because changes may still occur in the stored cells at temperatures greater than –139°C, storage in the liquid phase at –196°C is preferable. While the cultures are submerged in the liquid nitrogen temperature stability is guaranteed. If storage is only required for short periods higher temperatures may be adequate.
14. The long-term survival of yeasts held in liquid nitrogen has not been well documented, but all evidence suggests that losses during storage are insignificant. The NCYC has found no drop in viability of cultures stored for up to 10 yr. Work with mutant strains of *S. cerevisiae* and *Schizosaccharomyces pombe* at the NCYC has also demonstrated that genetic stability is also very good.
15. If the racking system used holds many cryotubes they will be exposed to higher temperatures when the racking system is removed from the liquid nitrogen to recover a straw. Care should be taken to minimize this time as much as possible. Work done at the NCYC has suggested that using straws sealed inside cryotubes offers considerable protection against short exposure to higher temperatures. No significant drop in viability has been recorded in straws held in a racking system that has been repeatedly removed briefly from the liquid nitrogen.
16. Nunc recommends that cryotubes should not be used for freezing in liquid nitrogen unless correctly sealed in Cryoflex because trapped nitrogen can expand and cause the tubes to explode once they are removed from the liquid. Because, in this method, the cryotube is only being used for secondary containment, the screw cap should not be firmly tightened. This will allow trapped nitrogen to leave the tube safely as its temperature increases. However, tubes should always be held at arm's length when being removed and safety goggles and gloves should be worn. Very occasionally straws will rupture on thawing from poor seals letting in liquid nitrogen. Provision should be made for sterilizing benchtops and equipment if they become contaminated with the contents of the ruptured straw.
17. Cells removed from liquid nitrogen storage should be transferred initially to small aliquots (10 mL) of suitable growth media. Larger volumes of media can be inoculated from this culture once sufficient growth has occurred.
18. The plates are kept horizontal at all times to ensure the drops remain discrete. Dilutions containing 20–30 colonies are used for estimating viability. The number of cells per milliliter inoculated into the straw is equal to the number of colonies in three drops multiplied by 10 times the dilution factor.

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8

Freeze-Drying Fungi Using a Shelf Freeze-Drier

C. Shu-hui Tan, Cor W. van Ingen, and Joost A. Stalpers

Summary

Lyophilization, the removal of water by freezing and then volatilization at low pressure and temperature, has been employed as a standard long-term preservation method for many filamentous fungi. The method outlined involves the use of standard shelf freeze-drying and skimmed milk as a suspending solution/lyoprotectant. This approach has been employed to freeze-dry the majority of the 50,000 fungal strains that have been successfully lyophilized at the Centraal bureau voor Schimmelcultures (CBS) culture collection (http://www.cbs.knaw.nl/).

Key Words: Freeze-drying; fungi; lyophilization.

1. Introduction

Lyophilization is the preferred method for cultures that must be dispatched because, unlike cryopreserved cultures, these cultures do not need to be revived prior to dispatch. In the cooling step preceding drying, the extracellular water crystallizes, consequently the cells dehydrate and, surrounded by the highly viscose lyoprotectant, they become embedded in a feathered pattern of the ice crystals. During primary drying the temperature is reduced to such extent that the lyoprotectant becomes so viscose that it transforms into a glass. A glass is a liquid in which the molecules are immobilized ***(1)***. In the primary drying phase the ice crystals are evaporated, leaving a glass interwoven with channels. By slowly increasing the temperature to that of the secondary drying phase, water is sublimated from this glass and escapes through these channels, making the glass even more viscose. Finally, at the end of the secondary drying phase, when only 1–2% (w/v) moisture is left, the suspending medium is so viscose that it is in the glass phase at room temperature. As in cryopreservation, a glass

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is an ideal formulation in which to store the dehydrated organisms (*see* Chapters 2 and 3), because there is no enzyme or chemical activity and the molecules are arranged in an unordered structure, allowing protection of the membranes and the proteins ***(2)***.

Skimmed milk is an ideal lyoprotectant, which is cheap (also standard skimmed milk, available from any foodstore, may be employed). The glass transition temperature (Tg) of frozen skimmed milk is –18°C, which facilitates freeze-drying and contains a mixture of macromolecules (lactalbumine and casein) and a saccharide. The macromolecules serve as bulking agents, the amino acids contained in the macromolecules help to repair sublethal damage and provide the energy during revival. The saccharide, lactose, helps to diminish transitions of the membrane during dehydration by replacing the dipoles of the water ***(3–5)***. Furthermore, denaturation of the proteins during cooling is diminished by saccharides ***(6)***. They repel the hydrophobic parts of the amino acids, thus preventing unfolding of the proteins (preferential exclusion) ***(7–9)***. To improve survival, 7% (w/v) trehalose can be added to the skimmed milk. This disaccharide "fits best" within the membrane structure ***(10)*** and produces a stable glass at a relatively high Tg value ***(11)***. Trehalose is actually produced in the spores and conidia of fungi and yeasts to protect their membranes, and proteins at the low water activity Aw values present in these propagules ***(12,13)***. Survival levels might also be increased by adding 1% (w/v) Na-glutamate to the lyoprotectant. Like the saccharides, amino acids diminish protein denaturation during freeze-drying by the mechanism of preferential exclusion.

2. Materials

1. Biological safety cabinet and facilities for carrying out microbiological methods safely.
2. Cultures should be healthy and sporulating, exhibiting all characters to be preserved both morphologically and physiologically.
3. Growth medium as required for cultivating different fungi.
4. A lyoprotectant of 12% (w/v) Tyndalized skimmed milk (*see* **Note 1**).
5. Cooling system, for example, an insulated container with a mixture of solid carbon dioxide and acetone, or alternatively a controlled-rate cooler.
6. 0.5-mL neutral borosilicate glass ampoules. These are heat sterilized at 180°C for 3 h.
7. Metal racks for supporting 0.5 mL glass ampoules.
8. Shelf freeze-drier.
9. Nonabsorbent cotton wool sterilized at 180°C for 3 h.
10. Air/gas-constricting torch.
11. Air/gas-sealing torch.
12. Heat-resistant mat.

3. Methods

3.1. Preparation Suspension in Lyoprotectant

All culture transfers should be carried out using aseptic techniques in an appropriate microbiology safety cabinet for the organisms to be preserved. It is important that all local safety requirements are adhered to.

1. Make a spore or conidia suspension in 12% (w/v) Tyndalized skimmed milk (*see* **Note 1**).
2. Add 50–200 µL spore suspension, depending on the concentration to a borosilicate freeze-drying ampoule (*see* **Note 2**).
3. Close the ampoule with a cotton plug.
4. Cool the propagule suspension to at least –45°C (*see* **Note 3**).

3.2. Freeze-Drying

To avoid "collapse," the whole freeze-drying process must be performed below the Tg. Tg is the temperature at which a glass melts during warming. When the temperature is raised above the Tg, the frozen suspension melts and is liquid- instead of freeze-dried resulting in a tremendous protein denaturation. Tg can be estimated with the aid of a differential scanning calorimeter ***(14)***. Alternatively, most manufacturers of freeze-drying devices recommend the use of a eutectic monitor to establish the temperature of complete solidification (eutectic temperature) (*see* **Note 4**).

1. Load the freeze-drying device at a shelf temperature of –35°C to avoid melting during loading.
2. Dry suspension in the primary drying phase at least 5°C below Tg of the frozen suspension (*see* **Note 1**).
3. Consult a phase transition diagram to establish the vacuum of the primary drying phase (*see* **Note 5**).
4. Choose the vacuum that corresponds with a product temperature of 10°C below Tg (*see* **Table 1**) (e.g., skimmed milk: 0.470 mbar; skimmed milk + 7% (w/v) trehalose: 0.220 mbar; skimmed milk + 7% (w/v) trehalose + 1% (w/v) Na-glutamate: 0.140 mbar (*see* **Table 2**) (*see* **Note 5**).
5. Choose a safety vacuum that corresponds with the Tg temperature (*see* **Note 1**) (e.g., skimmed milk: 0.1.250 mbar; skimmed milk + 7% [w/v] trehalose: 0.630 mbar; skimmed milk + 7% [w/v] trehalose + 1% [w/v] Na-glutamate: 0.420 mbar [*see* **Note 5**]).
6. The end of the primary drying phase is detected by a pressure rise test. In this procedure the valve between the condenser and the drying chamber is closed. A pressure increase after closure of the valve indicates that not all the frozen water is evaporated. When a pressure rise test cannot be performed, primary drying should last approx 16 h when the thickness of the layer of material to be dried is 1–2 mm (*see* **Note 6**).

Table 1
Eutectic Temperature, Glass-Transition Temperature, and Temperature Primary Drying Phase of Various Lyoprotectants

Lyoprotectant	Teut (°C)	Tg (°C)	T prim. dr. phase (°C)
12% (w/v) sm	–16	–18	–23
12% (w/v) sm + 7% (w/v) tr	–17	–25	–30
12% (w/v) sm + 7% (w/v) tr + 1% (w/v) Na-gl	–24	–29	–34

Na-gl, Na-glutamate; sm, skim milk; Teut, eutectic temperature; Tg, glass-transition temperature; T prim. Dr. phase, primary drying phase; tr, trehalose.

7. Increase temperature to 25°C to reach the secondary drying phase. Increase the temperature slowly (maximum rate 1°C/min) to avoid collapse (*see* **Note 7**).
8. Apply maximum vacuum during the secondary drying.
9. Continue secondary drying until a residual moisture content of 1–2% is reached. This will take 6–9 h (*see* **Note 8**).
10. Close the ampoules after secondary drying, while still being under vacuum (*see* **Note 9**).
11. Heat-seal glass ampoules while still being under vacuum to avoid leakage during storage. To guarantee axenicity during opening, the ampoules should be sealed above the cotton plug.
12. Store freeze-dried ampoules at 4°C (*see* **Note 10**).

3.3. Revival

1. Open the ampoule just above the cotton plug by scoring the glass wall with a glass cutter or a sharp file, and break it at the scored mark.
2. Flame the opening and remove the cotton plug with sterile forceps. Suspend the material by pouring it into a tube containing 1–2 mL sterile water (*see* **Note 11**).
3. Pour the suspension over a suitable agar medium and incubate at optimal conditions.

4. Notes

1. Optionally 7% (w/v) trehalose and/or 1% (w/v) Na-glutamate can be added to the 12% (w/v) skimmed milk to improve survival rates, however, Na-glutamate decreases Tg substantially (*see* **Table 1**). Consequently, when this component is added to the protectant, low temperatures must be applied during drying and increased drying periods. When a new protocol is developed, estimate Tg of the dried product by differential scanning calorimeter to establish the storage temperature.
2. This glass quality is needed because the ampoules must be heat-sealed after drying.
3. Cool one-celled (<4 µm), thin-walled propagules, e.g., those from *Aspergillus* ssp., *Penicillium* ssp., *Trichoderma* ssp., *Acremonium* ssp., *Verticillium* ssp. instantaneously to avoid protein denaturation. Propagules can be cooled instantaneously by immersing them in a mixture of solid carbon dioxide and acetone (–75°C). Cool septate fungi (>4 µm in diameter), e.g., *Alternaria* ssp., *Curvularia* ssp., *Fusarium* ssp.,

Table 2
Phase-Transition Data Showing Relation Between Pressure Above Ice and Temperature of Ice

°C	mbar	°C	mbar	°C	mbar	°C	mbar
0	6.110	–20	1.030	–40	0.120	–60	0.011
–1	5.620	–21	0.940	–41	0.110	–61	0.009
–2	5.170	–22	0.850	–42	0.100	–62	0.008
–3	4.760	–23	0.770	–43	0.090	–63	0.007
–4	4.370	–24	0.700	–44	0.080	–64	0.006
–5	4.020	–25	0.630	–45	0.070	–65	0.0054
–6	3.690	–26	0.570	–46	0.060	–66	0.0047
–7	3.380	–27	0.520	–47	0.055	–67	0.0041
–8	3.010	–28	0.470	–48	0.050	–68	0.0035
–9	2.840	–29	0.420	–49	0.045	–69	0.0030
–10	2.560	–30	0.370	–50	0.040	–70	0.0026
–11	2.380	–31	0.340	–51	0.035	–71	0.0023
–12	2.170	–32	0.310	–52	0.030	–72	0.0019
–13	1.980	–33	0.280	–53	0.025	–73	0.0017
–14	1.810	–34	0.250	–54	0.024	–74	0.0014
–15	1.650	–35	0.220	–55	0.021	–75	0.0012
–16	1.510	–36	0.200	–56	0.018	–76	0.0010
–17	1.370	–37	0.180	–57	0.016	–77	
–18	1.250	–38	0.160	–58	0.014	–78	
–19	1.140	–39	0.140	–59	0.012	–79	

and one-celled thick-walled (*Periconia* ssp.) propagules slowly at –1°C/min to avoid production of intracellular ice crystals ***(15)*** in a controlled-rate cooler.

4. In this measurement, a drop in electrical resistance of the product indicates when the solid phase changes to liquid. However, when some of the components of the lyoprotectant, such as the disaccharide trehalose solidified as a glass instead of a eutectic, eutectic temperature deviates substantially from Tg (*see* **Table 1**).
5. In a phase-transition diagram, the relation between the temperature of ice and the pressure above this ice is shown (*see* **Table 2**).
6. When 1% (w/v) Na-glutamate is added, primary drying should be at least 48 h to dry a layer with thickness of 1–2 mm.
7. When 1% (w/v) Na-glutamate is added to the protectant the temperature should be increased to 25°C at a maximum rate of 0.5°C/h.
8. When 1% (w/v) Na-glutamate is added to the protectant, secondary drying at 30°C instead of 25°C is recommended, and the secondary drying phase is prolonged to 16 h (layer thickness, 2 mm).
9. Dried membranes, proteins, and even DNA are very vulnerable to damage caused by free radicals. Moreover, the half-life of free radicals is increased in dehydrated material.
10. Check viability immediately after drying (to check whether the organisms survive the freeze-drying process), after 5-yr storage, or 1-wk storage at 37°C to check stability of the dried product.
11. The suspension can be poured as soon as the pellet is dissolved. However, to improve the recovery process, it is advised to incubate the suspension 16 h at 20–24°C. Alternatively 2 mL malt-peptone solution can be used to restore sublethal damage caused to the membranes and proteins by freezing and drying, and to restore the energy charge.

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9

Cryopreservation and Freeze-Drying of Fungi Employing Centrifugal and Shelf Freeze-Drying

Matthew J. Ryan and David Smith

Summary

The aim of preserving a fungus is to maintain it in a viable state without change to its genetic, physiological, or anatomical characters. There are numerous methodologies available to preserve a fungus, but the two methods widely used by culture collections (biological or genetic resource centers) to achieve successful preservation are cryopreservation with liquid nitrogen using controlled-rate freezing and centrifugal freeze-drying. Generic methods are often used, but specific variations of a method may be required in order to achieve optimal stability. No single method can be applied to all fungi. More recently, techniques such as vitrification and encapsulation cryopreservation have been used to preserve recalcitrant fungi. The protocols described within this chapter have been developed over many years at one of the world's largest culture collections of filamentous fungi.

Key Words: Cryopreservation; cryoprotectant; encapsulation; fungus; lyophilization; recalcitrant; vitrification.

1. Introduction

The need for well-characterized, preserved, and authenticated fungal cultures for taxonomy, genomic, and proteomic research programs, industrial processes, conservation, testing, and education is well established ***(1)***. Cultures must retain their physiological and genetic characteristics to be suitable for their intended use. The best methodologies available to achieve this are cryopreservation and lyophilization, which can be optimized for specific species of fungi ***(1,2)***. However, the characteristics of the fungus must be monitored before, during, and after preservation procedures to ensure that preservation procedures do not compromise the integrity of the fungus ***(3)***.The main requirement

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of a preservation technique is to maintain the fungus in a viable and stable state without morphological, physiological, or genetic change until it is required for further use. Therefore, conditions of storage should be selected to minimize the risk of change. Culture preservation techniques range from continuous growth methods that reduce the rate of metabolism, to an ideal situation where metabolism is considered suspended, or nearly so ***(4,5)***. No single technique has been applied successfully to all fungi, although storage in or above liquid nitrogen is considered to be the optimum method for many fungal strains. A decision-based key is available to help researchers select the best method available for the preservation of a fungus and considers logistical, economic, and scientific criteria ***(6)***. More recently, preservation regimes have been developed for more preservation-recalcitrant fungi that otherwise could not be preserved, these techniques include encapsulation and vitrification-based cryopreservation approaches ***(1,2)***.

One factor common to all methods is the need to start with a well-characterized, healthy culture in order to obtain the best results. Optimal growth conditions including temperature, aeration, humidity, illumination, and media must be established ***(4)***. The growth requirements vary from strain to strain, although different strains of the same species and genera usually grow best on similar media. A medium that induces good sporulation and minimal mycelium formation is desirable for successful freeze-drying ***(4)***, although this is not necessary if the culture is to be cryopreserved. In some cases it is often better to grow the organism under osmotically stressful conditions, as chemicals that accumulated in the cytoplasm under these conditions may also protect the organism during cooling.

1.1. Cryopreservation

Lowering the temperature of biological material reduces the rate of metabolism until all internal water is frozen and no further biochemical reactions occur ***(7)***. This is quite often lethal and at the very least causes cellular injury. Although fungi are quite often resistant to ice-induced damage, cooling must be controlled to achieve optimum survival. The avoidance of intracellular ice and the reduction of solution, or concentration, effects are necessary ***(8)***. Little metabolic activity takes place less than –70°C. However, recrystallization of ice can occur at temperatures greater than –139°C ***(9)*** and this can cause structural damage during storage. Consequently, cooling protocols have to be carefully designed for cells in order to inflict the least damage possible. The cryopreservation of microfungi at the ultra-low temperature of –196°C in liquid nitrogen or the vapor above is currently regarded as the best method of preservation ***(1,10,11)***. It can be widely applied to sporulating and nonsporulating cultures. Initial work with fungi was undertaken by Hwang ***(12)*** who employed a method

designed for freezing avian spermatozoa ***(13)***. Similar methods have been used successfully ***(11,14,15)***. Provided adequate care is taken during freezing and thawing, the culture will not undergo any change either phenotypically or genotypically. Optimization of the technique for individual strains has enabled the preservation of organisms that have previously been recalcitrant to successful freezing ***(14)***.

The choice of cryoprotectant is a matter of experience and varies according to the organism. Glycerol gives very satisfactory results but requires time to penetrate the organism; some fungi are damaged by this prolonged incubation step. Dimethyl sulfoxide penetrates rapidly and is often more satisfactory ***(16,17)***, but is often toxic to sensitive organisms. Sugars and large molecular substances such as polyvinylpyrrolidine ***(18,19)*** have been used but, in general, have been found to be less effective.

At CAB International (CABI) (http://www.cabi-bioscience.org/bioresources.asp) over 4000 species belonging to 700 genera have been successfully frozen in 10% (v/v) glycerol. Few morphological or physiological changes have been observed in material that has been revived after successful cryopreservation ***(20)***. Finding the optimum cooling rate has been the subject of much research ***(8,12,14,15)***. Slow cooling at 1°C/min over the critical phase has proved most successful ***(15,20,21)***, but some less-sensitive isolates respond well to rapid cooling, some preferably without protectant. Slow warming may cause damage because of the recrystallization of ice, therefore, rapid thawing is recommended. Slow freezing and rapid thawing generally give high recoveries for fungi ***(22)***. Storage at –196°C in the liquid nitrogen, or at slightly higher temperatures in the vapor phase, is employed at CABI.

Occasionally, alternative techniques for the cryopreservation of recalcitrant fungi are required. Recalcitrant fungi include organisms such as *Halophythophthora*, *Saprolegnia*, and *Aphanomyces* spp., microcylic rust fungi, some Basidiomycota, and the Glomeromycota. The techniques utilized to preserve strains of these taxa include encapsulation cryopreservation and vitrification-cryopreservation techniques ***(1,23,24)***.

Vitrification of fungi ***(25)*** involves the application of very highly concentrated cryoprotectant solutions and has been applied to organisms of many cell types, especially plant cells (***24;*** *see also* Chapter 12). The technique does not require the use of controlled-rate cooling; samples are plunge cooled, with the vitrification solution forming an amorphous glass that prevents the onset of "concentration effects" or ice damage (*see* Chapters 3 and 12). On "resuscitation" from the frozen state, care must be taken to ensure that samples do not "crack," which could cause physical damage to the mycelium. Samples must be immediately washed to remove the vitrification solution, because on prolonged exposure it is toxic to the fungi. The technique has been applied to a number of

fungi with some success ***(1)***. However, vitrification solutions are extremely toxic, so routine use may not be advantageous. The use of encapsulation (immobilization) cryopreservation ***(23)***, the entrapment of mycelium/spores in calcium alginate beads prior to preservation, is well documented for fungi, for example, *Serpula lacrymans* ***(26)*** and monoxenically produced spores of *Glomeromycota* ***(27)***. Essentially, encapsulation has two main functions; it allows cells to be easily manipulated by providing a suitable suspending matrix and provides a mechanism for the water content of cells to be reduced by osmotic treatment or drying that decreases the prospects of ice damage or concentration effects during the cooling step(s) of the cryopreservation procedure. Encapsulation and vitrification cryopreservation techniques have significant potential for preserving recalcitrant fungi that could otherwise not be stored in the long term. However, the use of these techniques (for fungi) is still relatively underresearched and therefore not broadly tested.

Approaches to cryopreservation include when organisms are preserved with their host, or alternative growth substrate have been applied in various semblances for many years. The technique has potential for obligate organisms, mutualists, parasites, and yet-unculturable fungi that are otherwise not maintained in a living state by genetic resource centers. This approach has been used for recalcitrant organisms such as the microcyclic rust fungus, *Puccinia spegazzini*, where the teliospores were preserved on stem or petiole tissue ***(28)***. A similar approach incorporating encapsulation cryopreservation was used for the basidomycete fungus *Ceratobasidium cornigerum* ***(29)***, when seeds of the green-winged (*Anacamptis morio*) and common spotted (*Dactylorhiza fuchsii*) orchids were encapsulated in alginate beads with hyphae of the fungus with no adverse effects to the fungus postcryopreservation.

1.2. Freeze-Drying (Lyophilization)

Lyophilization (preservation by drying under reduced pressure from the frozen state by sublimation of ice) of fungi was first reported by Raper and Alexander ***(30)***. Improvements in methods and equipment over the years have led to a reliable and successful preservation technique for sporulating fungi ***(4)***. The technique is not suitable for preserving nonsporulating fungi, although some workers have reported limited success when lyophilizing mycelium ***(31)***. The advantages of freeze-drying over other methodologies include good stability of characters, long shelf-life, convenient storage of ampoules in the laboratory environment under ambient conditions, and easy distribution ***(22,30–32)***.

A freeze-drying system incorporates freezing the suspension, generating a vacuum, and removing the water vapor that evolved. The protectant used, rate of cooling, final temperature, rate of heat input during drying, residual moisture,

and storage conditions all affect the viability and stability of fungi ***(3,34,35)***.The suspending medium should give protection to the spores from freezing damage and during storage. Media often used are skimmed milk, serum, peptone, various sugars, or mixtures of these.

The rate of freezing is a very important factor, which must be optimized to achieve the best recovery. Slow freezing rates are employed, 1°C/min is the rate normally quoted ***(4,22)***. The technique of evaporative cooling can be used successfully for the storage of many sporulating fungi ***(4)***.

2. Materials

2.1. Liquid Nitrogen Storage

1. Biological safety cabinet and facilities for carrying out microbiological methods and aseptic techniques (*see* **Note 1**).
2. Cultures should be healthy and exhibit all characters to be preserved, both morphologically and physiologically (*see* **Note 2**).
3. Growth medium as required for cultivating different fungi ***(4)***.
4. 2 mL cryovials (System 100, Nalgene, Rochester, NY) (*see* **Note 3**).
5. Cryoprotectant: 10% (v/v) glycerol in distilled water dispensed in 10-mL aliquots, autoclaved at 121°C for 15 min.
6. Liquid nitrogen wide-necked storage tank with drawer rack inventory control system.
7. Safety equipment: should include cryogloves (cold resistant), face shield, forceps, and oxygen monitor.

2.2. Freeze-Drying

1. Biological safety cabinet and facilities for carrying out microbiological methods safely (*see* **Note 1**).
2. Cultures should be healthy and sporulating, exhibiting all characters to be preserved both morphologically and physiologically (*see* **Note 4**).
3. Growth medium as required for cultivating different fungi ***(4)***.
4. 10% (w/v) skimmed milk and 5% (w/v) inositol in distilled water dispensed in 10-mL aliquots in glass universal bottles. These are autoclaved at 114°C for 10 min (*see* **Note 5**).
5. High-voltage vacuum spark tester.
6. 0.5-mL neutral glass ampoules (Adelphi tubes, West Sussex, UK) labeled with the culture number and covered with lint caps to prevent aerial contamination (*see* **Note 6**). These are heat sterilized at 180°C for 3 h.
7. Metal racks for supporting 0.5-mL neutral glass ampoules.
8. Freeze-drier with spin freeze and manifold drying accessories.
9. Nonabsorbent cotton wool sterilized in a dry oven at 180°C for 3 h.
10. Air/gas-constricting torch.
11. Air/gas-sealing torch.
12. Heat-resistant mat.
13. Diphosphorus pentoxide general purpose reagent (GPR) (*see* **Note 7**).

2.3. Shelf Freeze-Drying (2-mL Vials)

1. 2-mL flat-bottomed preconstricted glass (ampoules) vials labeled with the culture number. These are covered with aluminium foil to prevent aerial contamination then heat sterilized in an oven at 180°C for 3 h.
2. Metal racks for holding 2-mL flat-bottomed glass vials.
3. Grooved rubber bungs sterilized by autoclaving at 121°C for 15 min then placed in 70% (v/v) industrial spirits (*see* **Note 8**).
4. Shelf freeze-dryer with programmable shelf temperature control.

3. Methods

3.1. Liquid Nitrogen Storage

All culture work should be carried out using aseptic techniques in a microbiology safety cabinet (*see* **Note 1**).

1. Grow cultures under optimal growth conditions and on suitable medium (*see* **Subheading 2.1.**, **items 2** and **3**; **Notes 2** and **9**).
2. Prepare a spore or mycelial suspension in sterile 10% (v/v) glycerol; mechanical damage must be avoided (*see* **Note 10**).
3. Add 0.5-mL aliquots of suspension to each cryovial, label with the culture number using a permanent ink cryomarker or barcode.
4. Allow at least l h for the cells to equilibrate in the glycerol (*see* **Note 11**).
5. Place the cryovials in racks and cool at approx 1°C/min in a controlled-rate cooler (*see* **Notes 12** and **13**). Alternatively, place the cryovials in the neck of the nitrogen refrigerator at –35°C for 45 min (*see* **Note 12**).
6. Transfer the cryovials into storage racks held in the vapor phase of the liquid nitrogen, this cools them to below –150°C.
7. Record the location of each culture in the inventory control system.
8. After at least 1 d retrieve an ampoule from the refrigerator to test viability and purity of the fungus.
9. Warm the cryovial rapidly by immersion in a water bath at 37°C. Remove immediately on completion of thawing and do not allow it to warm up to the temperature of the bath. Alternatively, thaw the vials in a controlled-rate cooler on a warming cycle (*see* **Note 14**).
10. Opening of the cryovial and the transfer to media should be carried out in an appropriate level microbiological safety cabinet. Surface sterilize the cryovials by immersion or wiping with 70% (v/v) alcohol. Aseptically transfer the contents using a Pasteur pipet and transfer on to suitable growth medium.

3.2. Spin Freeze-Drying

All culture work should be carried out in an appropriate microbiological safety cabinet (*see* **Note 1**).

1. Grow cultures under the optimal growth conditions for the species and on suitable media (*see* **Subheading 2.2.**, **items 2** and **3**; **Note 4**).

2. Prepare a spore suspension in sterile 10% (w/v) skimmed milk and 5% (w/v) inositol mixture.
3. Dispense 0.2-mL aliquots of the spore suspension into the sterilized and labeled ampoules ensuring the suspension does not run down the inside of the ampoule (*see* **Note 15**). Then cover the ampoules with lint caps.
4. Transfer the ampoules to the spin freeze-drier, and spin while the chamber is evacuated. Cool the suspensions at approx 10°C/min (this is uncontrolled, the actual rate depends on the amount of water and the pressure in the system).
5. After 30 min switch off the centrifuge; the spore suspension will have frozen into a wedge tapering from the base of the ampoule. This gives a greater surface area for evaporation of the liquid.
6. Dry for 3.5 h, at a pressure of between 5×10^{-2} and 8×10^{-2} mbar, then raise the chamber pressure to atmospheric pressure and remove the ampoules.
7. Plug the ampoules with a small amount of sterilized cotton wool, in a laminar air flow cabinet or a suitable microbiological safety cabinet (*see* **Note 16**).
8. Compress the plugs (aseptically) to 10 mm in depth with a glass or metal rod, and push down to just above the tip of the slope of the freeze-dried suspension.
9. Constrict the plugged ampoules using an air/gas torch just above the cotton wool plug. (The object is to ensure the glass is not drawn too thinly at the constriction and there is a sufficiently large bore left for the evacuation of air and the passage of water vapor.)
10. Hold the ampoule at each end and rotate in a narrow hot flame of the air/gas torch so it is heated evenly around an area 10 mm above the cotton wool plug; ensure that the ampoule is turned back and forth through 360°. When the glass begins to become pliable allow the flame to blow the glass in toward the center of the ampoule while rotating the ampoule slowly. Then stretch the ampoule to give no more than a 10-mm increase in total length. This is performed by moving the open-end section back and forward no more than 5 mm with equal and opposite movement of the closed end of the ampoule. When the outer diameter of the constriction is approximately one-third of its original diameter the constriction should be complete.
11. Place the ampoule down onto a heat-resistant mat while it is still slightly pliable, and roll it on the mat so the ampoule returns to a straight alignment.
12. Attach the constricted ampoules to the secondary drier and evacuate. Incubate the secondary drying stage for about 17 h, this leaves a residual moisture content of 1–2% by dry weight (*see* **Note 17**). The evolved water is absorbed by phosphorus pentoxide placed in the chamber below the manifolds of the secondary drier (*see* **Note 7**).
13. Seal the ampoules across the constriction while still attached to the manifold and under the vacuum (*see* **Note 18**) using an air/gas cross-fire burner. Support the ampoule. Project the two flames onto opposite sides of the constriction. The flames seal and subsequently cut through the glass (*see* **Note 19**). The ampoule must not be allowed to pull away from the molten seal until it has separated, or a long thin extension to the ampoule will be made. Use the flame of the torch to melt the glass top of the ampoule so it flows to form a thickened seal.

14. Before storage, test the sealed tubes with a high-voltage spark tester to ensure the seal is intact. A purple-to-blue illumination will appear inside the ampoule indicating the pressure is low enough and the seal is intact.
15. Store the ampoules under appropriate conditions (*see* **Note 20**).
16. After 2 d storage, open sample ampoules for viability and purity tests in an appropriate microbiological safety cabinet. Score the ampoule midway down the cotton wool plug with a serrated-edged, glass-cutting blade. Then heat a glass rod in a Bunsen until red hot, and press it down onto the score; the heat should crack the tube around the score.
17. Reconstitute the dried suspension and revive by adding three to four drops of sterile distilled water aseptically with a Pasteur pipet. Allow 15–20 min for absorption of the water by the spores (*see* **Note 21**).
18. Streak the contents of the ampoule onto suitable agar medium and incubate at an appropriate growth temperature.
19. It is advisable to rehydrate and check viabilities at regular intervals.

3.3. Shelf Freeze-Drying (2-mL Vials)

All culture work should be carried out in the appropriate microbiological safety cabinet (*see* **Note 1**).

1. Grow cultures under the optimal growth conditions for the species and on suitable media (*see* **Subheading 2.2.**, **items 2** and **3**; **Note 4**).
2. Prepare a spore suspension in sterile 10% (w/v) skimmed milk and 5% (w/v) inositol mixture (*see* **Note 5**).
3. Dispense 0.5-mL quantities into sterile 2-mL ampoules.
4. Aseptically insert sterile, grooved, butyl rubber bungs into the necks of the ampoules to the premolded rim so that the groove opening is above the vial lip.
5. Place the ampoules on the precooled shelf (–35°C) of the freeze-dryer (*see* **Note 22**).
6. Place the sample temperature probe into an ampoule containing the skimmed milk and inositol mixture only. When the temperature of this reaches –20°C, evacuate the chamber, this reduces the temperature of the sample to –145°C as the latent heat of evaporation is removed and rises again to the shelf temperature.
7. Maintain the shelf temperature at –35°C for 3 h and then raise to 10°C at 0.08°C/min (*see* **Note 23**).
8. After 24 h drying, from the time the temperature of the sample reaches –45°C, lower the shelf base to push the bungs into the neck of the ampoules to seal them.
9. Raise the chamber pressure to atmospheric pressure and heat seal the ampoules above the constriction using an air/gas torch ready for storage. Retain a final vacuum of approx 4×10^{-2} mbar.
10. When cool, test the sealed tubes with a high-voltage spark tester. A purple-to-blue illumination will appear inside the ampoule indicating the pressure is low enough and the seal is intact.
11. Store the ampoules in appropriate conditions (*see* **Note 20**).

12. After 4 d storage, open sample ampoules for viability and purity tests. Ampoules are snapped open at the constriction in an appropriate microbiological safety cabinet. Add 0.3 mL of sterile distilled water and then temporarily plug the ampoule with sterile cotton wool.
13. After 30 min remove the cotton wool plug and transfer the contents of the ampoule onto suitable agar medium using a Pasteur pipet. Incubate at an appropriate growth temperature.
14. It is advisable to rehydrate and check viabilities at regular intervals.

4. Notes

1. All exposure to microorganisms must be reduced to a minimum. This entails the containment of many procedures, particularly those that may create aerosols. Although good aseptic technique will contain organisms during simple transfers it is essential, where more intricate (for example, those liable to create aerosols) procedures are carried out a suitable microbiological safety cabinet is used. The latter becomes essential when hazard group 2 organisms are being handled. Hazard groups and the containment level necessary for handling them are defined in the Advisory Committee for Dangerous Pathogens, The Approved List of Biological Agents ***(33)***. The Control of Substances Hazardous to Health regulations (2002) and their subsequent amendments enforce these requirements in the United Kingdom. Fungi may also produce volatile toxins, which may be harmful to man. These too must be contained or disposed of and a suitable cabinet should be used. A class II microbiological safety cabinet is recommended as this not only protects the worker, but also protects the cultures from contamination. Good practice should ensure that appropriate containment facilities are also used for hazard group 1 organisms. Fungi of the higher hazard group 3 require total containment in a class III microbiological cabinet or use of a glove box.
2. Sterile cultures survive the technique well but it is often best to allow full development before preservation. Sporulating cultures give better recovery. Poor isolates will not be improved by this method and may be more sensitive to the process, giving rise to preservation failures.
3. Unless stated by the manufacturer, plastic cryovials should only be stored in the vapor phase to avoid seepage of nitrogen through the cap seal.
4. Only sporulating fungi seem to survive centrifugal freeze-drying, though some sterile ascocarps, sclerotia, and other resting stages have been processed successfully. However, the method of shelf freeze-drying (*see* Chapter 8) can be much more successful for these than the spin-freeze method. Cooling rate, drying temperature, and rate of heat input can be optimized for the organism. Organisms have survived this method, which have failed spin freeze-drying ***(34)***.
5. The skimmed milk and inositol mixture is sensitive to heat and denatures easily. The sugars are caramelized, therefore the temperature and the time of exposure to high temperatures must be controlled. Autoclave at 114°C for 10 min.
6. Lint caps (fluffy side innermost) can be made to go over individual tubes or batches of tubes. At CABI, 15 replicate ampoules are covered by one cap. The tubes are placed in metal racks covered with aluminium foil and sterilized.

7. The phosphorus pentoxide desiccant is harmful and caustic, all contact must be avoided. Desiccant trays should be filled carefully to avoid bringing the powder into the air. Goggles, gloves, and particle masks should be worn.
8. Sterilization of the butyl rubber bungs by autoclaving at 121°C for 15 min will introduce water into the bung that will be liberated during the freeze-drying process or storage afterwards. Immersion in sterile industrial methylated spirits will help remove the water and can be evaporated away. Alternatively, surface sterilization with industrial methylated spirits without autoclaving can be sufficient to prevent contamination of the freeze-dried product.
9. Cold hardening of the cultures prior to freezing may prove beneficial. Pregrowth of cultures in the refrigerator (4–7°C) can improve post-thaw viabilities of some fungi, though others cannot grow at low temperatures. For those isolates that are sensitive, a short exposure to these temperatures may be tolerated, or this stage can be omitted altogether.
10. Various precautions can be taken to prevent mechanical damage. Fungi on slivers, or blocks, of agar can be placed in the ampoules. Alternatively, the fungus can be grown on small amounts of agar in the ampoule before the cryoprotectant is added (plastic cryotubes are more suitable for this). An alternative is to grow cultures on plant seeds in liquid culture or on small inanimate particles. These can then be transferred to the cryotube and frozen.
11. Cryoprotectants protect in several different ways but they must be allowed to come to equilibrium with the cells. The permeable protectants must be given time to enter the cell and this time depends on the permeability of the cell membrane. Generally the cell membrane of fungi is more permeable to dimethyl sulfoxide than to glycerol. Larger molecular weight substances, such as sugars and polyvinylpyrrolidone, do not penetrate the cell and either protect by reducing the amount of water in the cell through exosmosis, or impede ice crystal formation. Normally a period of at least 1 h is necessary for equilibration (*see* Chapter 3).
12. Liquid nitrogen is considered to be a hazardous substance; being extremely cold it will produce injuries similar to burns and it is also an asphyxiant gas at room temperature. It is important that it is handled with care. Contact must be avoided with the liquid or anything it has come into contact with. A face shield should be worn to prevent splashes hitting the face. Cold-resistant gloves will prevent direct contact but care must be taken not to allow nitrogen to splash into them.

    Liquid nitrogen can penetrate incompletely sealed vials during storage. On retrieval of the vial from liquid nitrogen the liquid will expand and, if it unable to escape, may cause the ampoule to explode.

    The liquid nitrogen and culture storage vessels must be stored in a well-ventilated area, ideally with low-level extractor fans installed. It is recommended that the level of oxygen in the area be monitored. If the level of oxygen in the atmosphere falls below 18% (v/v) anyone present will suffer drowsiness and headache. If the level falls to 16% (v/v) this is potentially lethal. As the generated nitrogen is initially cold there will be a higher concentration closer to the ground. If a person faints they would be in danger of asphyxiation. At CABI the liquid nitrogen storage

area has a low-level oxygen alarm and in other areas where liquid nitrogen is used staff are issued with an individual oxygen monitor.

13. A reproducible method of cooling to the holding temperature can be achieved in a controlled-rate cooler. CABI uses a Kryo 16 programmable cooler (Planer Products Ltd., Sunburg, UK). The control temperature is measured in the chamber wall and therefore the sample temperature can vary quite widely from the programmed protocol. The program must take this into account and be adjusted until linear cooling of the sample is achieved.
14. A controlled-rate cooler can be programmed to thaw the frozen fungi. The ampoules are heated at about 200°C/min raising the chamber temperature to 50°C. The sample is removed before its temperature reaches 20°C.
15. When filling the ampoules it is important not to allow the suspension to run down the length of its inner surface. When the ampoule is heated during constriction, the suspension burns releasing fumes that may be toxic to the freeze-dried material and leaves residues that could interfere with the eventual sealing of the ampoule.
16. The period when ampoules are kept at atmospheric pressure between drying stages must be as short as possible, as the exposure to the atmosphere of the partly dried material can cause deterioration ***(35)***.
17. Excessive drying will be lethal to cells, or at least may induce mutation by damaging the DNA ***(36)***. Also, having too high a residual moisture will result in rapid deterioration during storage ***(34)***. A residual water content of between 1 and 2% by dry weight proves successful for fungi.
18. At CABI evacuation continues while sealing after the second stage drying to ensure low pressure levels in the ampoule and therefore good storage conditions. An alternative method is to back fill with a dry inert gas such as nitrogen or argon.
19. If the flame is allowed to heat the ampoule on either side of the constriction during sealing, the molten glass will be pushed in by atmospheric pressure and the ampoule may implode.
20. Storing the ampoules at a low temperature is thought to give greater longevities, and 4°C seems to be favored by many workers in the field ***(34)***. At CABI the ampoules are stored at temperatures between 15 and 20°C and fungi have survived over 30 yr ***(4)***.
21. Rehydration of the fungi should be carried out slowly giving time for the absorption of moisture before plating onto a suitable medium. It is sometimes necessary to rehydrate preserved specimens in a controlled environment for some very sensitive strains ***(37)***.
22. The shelf temperature of a shelf freeze-drier can be controlled to cool at a particular rate. This enables the cooling stage of the freeze-drying process to be optimized for individual fungal strains. However, it may be preferable to precool the ampoules in a programmable cooler and then transfer them onto precooled shelves.
23. The temperature of the sample must be kept below its melting point during drying. The shelf temperature is therefore kept low (–35°C) during the initial stages of the process. The freezing point of fungal cytoplasm is quite often between –15

and –20°C ***(34)*** and, therefore, the temperature must remain below this until all unbound water is removed. The suspension reaches 5% moisture content after 3 h, and the warming protocol takes over 6 h to rise to –20°C.

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10

Cryopreservation of Microalgae and Cyanobacteria

John G. Day

Summary

Most culturable cyanobacteria and soil microalgae can be cryopreserved with relatively high viability. Furthermore, many freshwater and marine eukaryotic algae can also be cryopreserved, but typically with lower post-thaw viability levels. However, to date, most dinoflagellates, cryptophytes, synurophytes, and raphidophytes cannot be successfully cryopreserved. Marine diatoms can be cryopreserved, and often have high viability, although freshwater diatoms have thus far proven more problematic. Large numbers of strains have been examined, most notably at the four major protistan collections: Culture Collection of Algae and Protozoa (CCAP) (UK), The Provasoli-Guillard National Center for Culture of Marine Phytoplankton (CCMP) (USA), Sammlung von Algenku Huren Göttingen (SAG) (Germany), and The Culture Collection of Algae at the University of Texas at Austin (UTEX) (USA), and it has been observed that chlorarachniophytes, eustigmatophytes, pelagophytes, phaeothamniophytes, and ulvophytes also have very high success rates, comparable with the other green algae and cyanobacteria. It has been noted that virtually all algae with a large cell size, as well as most filamentous strains, cannot as yet be cryopreserved. There are no known fundamental reasons why large and more complex algae cannot be successfully cryopreserved. Thus, it is anticipated that further research on the basic mechanisms of freezing damage and the empirical development of improved protocols will continue to expand the number and diversity of algal taxa that can be successfully cryopreserved.

Key Words: Algal cryopreservation; cryoinjury; cryostorage; cyanobacteria freezing; microalgal storage; culture collection.

1. Introduction

Unlike most other groups of microorganisms, prokaryotic cyanobacteria (blue-green algae) and eukaryotic microalgae have traditionally been maintained by routine serial subculture, with the frequency of transfer being largely determined by the growth characteristics of the strain. However, continuous maintenance of actively growing algal and cyanobacterial strains over long periods of time is relatively complex, time consuming, and, when it involves

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large numbers of cultures, costly ***(1)***. In contrast, where cultures are kept "alive" in an arrested or retarded metabolic state they generally require a minimum of attention. Resting spores or other dormant stages of some species can be maintained at ambient or cool temperatures for many years without attention. For example, *Haematococcus pluvialis* aplanospores were recovered from air-dried soil after 27 yr ***(2)***, and the cyanobacterium *Nostoc commune* was revived from herbarium specimens after 107 yr of storage ***(3)***. However, the viabilities of resting stages generally decrease with time, and many aquatic algae do not have any dormant/resting stages. Freeze-drying has not been found to be a successful biostorage method for microalgae, with very low levels of viability (<1% of original population) ***(4)*** and a further reduction in viability on prolonged storage ***(5,6)***. The successful lyophilization of the cyanobacterium *Nostoc muscorum*, using a method similar to that detailed in Chapter 6, has been reported, with no observed reduction in viability after 5-yr storage ***(7)***. This technique has been adopted by a small number of researchers to preserve selected cyanobacterial strains. Where appropriate, effective methodologies have been developed; cryopreservation allows living algae that do not have any normal resting stage to be maintained indefinitely in an arrested state. High levels of viability are required to minimize the possibility of selecting an unrepresentative freeze-tolerant subpopulation. Furthermore, the relatively slow growth rate of algae (generation times in the range of 8 to 72 h are not uncommon), along with other problems associated with low viable cell density (*see* **Note 1**), can result in practical difficulties in reestablishing a viable culture.

Cryopreservation is employed at most of the major algal collections, and protocols adapted or developed empirically at these facilities typically employ relatively simple procedures that cryopreserve a broad range of algal species. The greatest degree of success has been achieved on cryopreserving cyanobacteria, marine pennate diatoms, and unicellular green algae, most of which are small and not morphologically complex ***(8)***. The methods described in this chapter have been applied successfully to a broad range of microalgae, including over 1400 strains in the CCAP and approx 1300 strains at UTEX ***(8)***. These procedures can be categorized as two-step freezing protocols. Two-step protocols require addition of a cell-permeating cryoprotectant to the algal culture, then the culture is cooled to a specified subzero temperature (step 1) to facilitate dehydration/cryodehydration of the sample. Next, it is cooled rapidly to the final storage temperature (step 2). The culture can be maintained at the storage temperature for an indefinite period of time. The cooling devices employed for algal cryopreservation, as for other cell types (*see* Chapters 5, 7, 9–23) generally fall into two categories: passive freezing systems and controlled cooling rate freezers. Three standard cryopreservation protocols using these approaches

are outlined in **Subheading 3**. Alternative strategies have been developed that may be employed to cryopreserve taxa that are recalcitrant to conventional methods ***(9,10)***; however, these are not outlined here (for further information on encapsulation/vitrification methods applicable to algae, *see* Chapter 12).

2. Materials

1. Laminar flow cabinet and appropriate facilities for following good microbiological practice.
2. Culture: use late log or early stationary phase cultures if available (*see* **Note 2**).
3. Medium: this is dependent on the nutritional requirements of the alga. For strains currently cryopreserved, the most commonly employed media are detailed in **items 4–6**. In all cases Analar-grade chemicals should be used.
4. BG 11 medium (**Table 1**), used for freshwater cyanobacteria and nonaxenic algae.
5. *Euglena gracilis*: Jaworski's medium (**Table 2**) used for most axenic freshwater algae.
6. Guillard's (f/2) medium (**Table 3**) used for marine algae.
7. Cryoprotectant: 10% (v/v) dimethyl sulfoxide (Sigma-Aldrich) (DMSO) in the appropriate medium. DMSO is cytotoxic and care should be taken when handling. Alternative cryoprotectants are also regularly employed for some strains (*see* **Notes 3** and **4**).
8. Cryovials: 1.8-mL presterilized plastic screw-cap cryovials (*see* **Note 5**).
9. Refrigeration systems:
   a. Refrigerated methanol bath precooled to –40°C. **Note**: methanol is toxic and flammable.
   b. Passive freezing unit (Mr. Frosty, Nalge Nunc International, Rochester, NY) and –80°C freezer (*see* **Note 6**).
   c. A controlled-rate cooler (*see* **Note 7**).
10. Liquid nitrogen Dewar: small 1- to 2-L wide-neck Dewar.
11. Safety equipment: long forceps, cryogloves, cryoapron, and goggles.
12. Storage system: cryogenic storage containers with appropriate storage racks and inventory system suitable for holding cryovials (*see* **Note 8**).
13. Water bath.
14. Fluorescein diacetate (FDA) stain stock solution: 25 mg FDA in 25 mL of methanol (*see* **Note 9**).
15. Microscope equipped with fluorescent lamp and ×400 magnification.
16. Dissecting microscope (×50 magnification).

3. Methods

All culture manipulations should be carried out following good microbiological practice/aseptic technique in a laminar flow cabinet if possible.

1. Grow cultures in the appropriate medium (*see* **Subheading 2.**, **items 3–6**) under controlled environmental conditions. Flasks (50 mL) containing 30 mL medium should be incubated static at 15°C under a photofluence rate of 25–100 μmol m^2/s

Table 1
BG 11 Medium

Stock solutions	Per L
$NaNO_3$	15.0 g[a]
$K_2HPO_4 \cdot 3H_2O$	4.0 g
$MgSO_4 \cdot 7H_2O$	7.5 g
$CaCl_2 \cdot 2H_2O$	3.6 g
Citric acid	0.6 g
Ferric ammonium citrate	0.6 g
EDTA (disodium salt)	0.1 g
Na_2CO_3	2.0 g
Trace metal mixture:	
H_3BO_3	2.86 g
$MnCl_2 \cdot 4H_2O$	1.81 g
$ZnSO_4 \cdot 7H_2O$	0.222 g
$Na_2MoO_4 \cdot 2H_2O$	0.39 g
$CuSO_4 \cdot 5H_2O$	0.079 g
$Co(NO_3)2 \cdot 6H_2O$	0.0494 g

[a]May be omitted for nitrogen-fixing cyanobacteria.

Add 100 mL solution 1, 10 mL each of solutions 2–8, and 1 mL solution 9 to distilled or deionized water to obtain a total volume of 1 L. pH is adjusted to 7.8 prior to sterilization.

(Based on the medium described by Rippka et al. ***[11]***.)

(*see* **Note 10**). Light should be provided on a light:dark cycle; 16:8 h is generally regarded as optimal. Incubate cultures until they reach stationary phase; 30 d is used as a standard culture interval at CCAP.

2. Aseptically transfer sedimented cells with 15 mL medium into a presterilized universal tube. Alternatively, for uniform cell suspensions, centrifuge at 500*g* for 10 min then decant the supernatant and resuspend the algae in 15 mL fresh sterile medium. Remove a 5-mL aliquot for use as a control for viability assays (*see* **step 11**).
3. Add 10 mL medium containing 10% (v/v) DMSO in culture medium (bacterial contaminants removed by passing it through a 0.45-µm filter) to the remaining 10 mL of dense culture and mix thoroughly to give a final concentration of 5% (v/v) DMSO (*see* **Notes 3**, **4**, and **11**).
4. Dispense the mixture in 1-mL aliquots into sterile, prelabeled cryovials (*see* **Note 12**). Seal the vials and incubate at room temperature for 5 min (*see* **Notes 13** and **14**).
5. First cooling phase (*see* **Note 15**).
   a. Passive freezing system method 1: refrigerated alcohol bath. Transfer the filled cryovials to a precooled refrigerated bath (–40°C) and incubate for 15 min (*see* **Note 16**).

Table 2
***Euglena gracilis*: Jaworski's Medium**

Stock solutions	Per 200 mL
$Ca(NO_3)_2 \cdot 4H_2O$	4.0 g
KH_2PO_4	2.48 g
$MgSO_4 \cdot 7H_2O$	10.0 g
$NaHCO_3$	3.18 g
$CaCl_2$	2.00 g
EDTA FeNa	0.45 g
EDTA Na_2	0.45 g
H_3BO_3	0.496 g
$MnCl_2 \cdot 4H_2O$	0.278 g
$(NH_4)_6Mo_7O_{24} \cdot 4H_2O$	0.2 g
Cyanocobalamin (vitamin B_{12})	0.008 g
Thiamine HCl (vitamin B_1)	0.008 g
Biotin	0.008 g
$NaNO_3$	16.0 g
$Na_2HPO_4 \cdot 12H_2O$	7.2 g

Add 0.5 mL solutions 1–10 to distilled or deionized water to obtain a total volume of 1 L medium. Then add 0.5 g sodium acetate (trihydrate), 0.5 g Lab-Lemco powder (Oxoid L29), 1.0 g Tryptone, and 1.0 g yeast extract. (Based on the medium described by Thompson et al. ***[12]***.)

Table 3
Guillard's (f/2) Medium

Stock solutions	Per L
$NaNO_3$	7.5 g
$NaH_2PO_4 \cdot 2H_2O$	5.65 g
Na_2EDTA	4.36 g
$FeCl_3 \cdot 6H_2O$	3.15 g
$CuSO_4 \cdot 5H_2O$	0.01 g
$ZnSO_4 \cdot 7H_2O$	0.022 g
$CoCl_2 \cdot 6H_2O$	0.01 g
$MnCl_2 \cdot 4H_2O$	0.18 g
$Na_2MoO_4 \cdot 2H_2O$	0.006 g
Cyanocobalamin (vitamin B_{12})	0.5 mg
Thiamine HCl (vitamin B_1)	100.0 mg
Biotin	0.5 mg
$Na_2SiO_3 \cdot 9H_2O$	40.18 g

Add 10 mL solution 1 and 1 mL each of solutions 2–4 to filtered natural seawater obtained from a clean offshore/nonestuarine source, to obtain a total volume of 1 L. Adjust pH to 8.0 prior to sterilization. Add 1 mL of solution 5 to the medium prior to pH adjustment and sterilization, if the medium is to be used for diatoms. (Based on the medium described by Thompson et al. ***[12]***.)

b. Passive freezing system method 2: "Mr. Frosty" (*see* **Note 6**). Place isopropanol, as per instructions, in the reservoir adjacent to the chamber containing cryogenic vials of a "Mr. Frosty" and place in a refrigerator overnight to equilibrate at approx 4°C. Quickly transfer the cryovials containing cryoprotectant-treated algae into the "Mr. Frosty" and immediately transfer to a –80°C freezer. Leave the container undisturbed for 1.5 h, by which time the temperature of the contents of the vial is less than –50°C (*see* **Note 17**).
c. Controlled-rate cooling. A simple protocol calls for cooling the chamber at 1°C/min from 20 to –40°C, then dwelling at –40°C for 30 min prior to transferring cryogenic vials to liquid nitrogen (*see* **Notes 18** and **19**).

6. Second cooling phase. On completion of the first cooling phase as outlined above (*see* **step 5a–c**). Transfer the vials rapidly, using forceps, to a wide-necked Dewar containing liquid nitrogen. Then, transport the vials in the Dewar to the storage system.
7. Transfer the vials rapidly, using long forceps, to the storage/racking system. Storage is generally in the liquid phase of liquid nitrogen (*see* **Note 20**).
8. A full inventory/stocklist including the locations of the vials within the storage system should be maintained. This can most easily be retained either on a computer database as card indices or on any other appropriate systems.
9. Thawing. Transfer stored cryovials (as in **step 6**) to a Dewar containing liquid nitrogen for transport and temporary storage. When ready, place the vials in a preheated water bath (40°C) and agitate until the last ice crystal has begun melting (*see* **Note 21**). On completion of the thawing remove immediately and transfer to the laminar flow cabinet (*see* **Note 22**).
10. Wipe the cryovial with 70% (v/v) ethanol to sterilize the outer surface of the vial. Aseptically transfer the contents into 30 mL of appropriate sterile medium and incubate as detailed in Method section 1 (*see* **Notes 23** and **24**).
11. Viability assays.
    a. Vital staining (*see* **Note 25**). It is necessary to dilute out cryoprotectant by transferring the thawed culture into 9 mL of appropriate sterile medium and incubating for up to 24 h prior to staining (*see* **Note 25**). Add 50 µL of FDA stain stock solution to 1 mL culture, incubate at room temperature for 5 min, and observe by blue-light fluorescence microscopy (*see* **Note 26**). Viable cells fluoresce green (FDA positive) and nonviable cells appear red or colorless. Viability is expressed as a percentage of control (nontreated unfrozen culture; **step 2**) vs FDA-positive cells.
    b. Colony formation in agar (*see* **Note 27**). Dilute control cultures, i.e., the untreated unfrozen starting culture (**step 2**) 1:1 with sterile medium. Transfer 1-mL aliquots of logarithmic dilutions of control and freeze/thawed cultures (*see* **Note 28**) into sterile Petri dishes (50-mm diameter). Add approx 2.5–4.0 mL of 1.0% (w/v) agar in the appropriate medium at 40°C; agitate gently to mix culture and agar (*see* **Note 29**). When the agar has gelled, seal the Petri dishes with Parafilm™ and incubate under standard growth conditions as detailed in **step 1** (*see* **Note 30**). Colonies are counted using a dissecting microscope (magnification ×50) and the viability of thawed cultures expressed as a percentage of control culture.

4. Notes

1. At low cell densities photoinhibition may occur as a result of the lack of self-shading by other algal cells. This, in turn, may result in the death of cells that have survived cryopreservation.

   Where cultures are not axenic, low post-thaw viabilities may result in the overgrowth of the alga by the competing associated microbial flora.
2. Stationary-phase cultures are denser, thus reducing the time required to reestablish a viable culture. Also, stationary-phase cultures may contain intracellular storage products, including lipids, which may act as additional cryoprotectants.
3. DMSO may also be used at 10% (v/v) final concentration. Alternatively, glycerol at either 5 or 10% (v/v) or methanol at 5 or 10% (v/v) may be used. Glycerol is normally sterilized by autoclaving at double the final concentration in culture medium. DMSO and methanol are sterilized by filter sterilizing through an alcohol-stable 0.2-µm filter.
4. Choice of cryoprotectant is largely dependent on its effectiveness and its cytotoxicity to the algal strain being frozen. Concentrations of methanol or DMSO less than 2% (v/v) are seldom effective as cryoprotectants, whereas concentrations higher than 12% (v/v) are often toxic. Within this range the most effective concentration varies greatly among species, sometimes even among closely related strains.
5. Costar vials (Cambridge, MA) have an internal rubber O-ring that appears to reduce the likelihood of liquid nitrogen leaking into the vial. Nitrogen leakage may result in contamination of algal cultures when stored under liquid nitrogen. Leakage can also lead to the possible rupturing of the vial on thawing, which has obvious safety implications (*see* **Note 22**). It is important not to overtighten or undertighten the caps, as this will result in leakage. An additional step that may be employed to minimize the risk of leakage is to use Cryoflex (Nunc). This will form a seal and prevent leakage into the vial.
6. Commercially available controlled-cooling canisters, such as "Mr. Frosty" and "Handi-Freeze" (Taylor Wharton, Nottingham, UK), are inexpensive and when used correctly have highly reproducible cooling rates. However, care should be taken on transferring them to/from the freezer and placing them in the freezer to avoid any loss of isopropanol, as this will alter the cooling rates of the unit.
7. A variety of commercial instruments (e.g., Biotronics, Leominster, UK; Planer Products, Sunbury, UK; Cryomed, USA; Gordinier Electronics, Roseville, MI; CryoLogic, Musgrave, Victoria, Australia) allow accurate control and manipulation of the cooling regime.
8. Vials must be stored at less than –135°C. This is achieved by storage either in the liquid or vapor phase in an insulated Dewar, or alternatively, in a more sophisticated autofill crystore. At CCAP, liquid-phase storage is preferred as this ensures that stored material is always maintained at below –135°C.
9. Prepare a standard FDA stock solution (0.001% [w/v]) by first dissolving 25 mg FDA crystals in a few drops of acetone and making up to the final volume (25 mL) with methanol.

10. Cyanobacteria and red algae, particularly when physiologically stressed, should be cultured at relatively low light levels. Light intensity is less critical for most other eukaryotic algal groups.
11. A few small, unicellular Chlorophyceae, such as *Chlorella protothecoides* Krüg, can withstand direct immersion into liquid nitrogen without a cryoprotectant or any control over the rate of cooling ***(8)***. Some strains of *Chlorella* can be preserved by incubating a culture in 5% (v/v) DMSO at ambient temperature for 5 min in a cryogenic vial, and then plunging the vial directly into liquid nitrogen.
12. Cryogenic vials are best labeled using a fine-tip, alcohol-resistant permanent marker. Despite limited space, the algal name or identifying number and the date of freezing should be labeled on each frozen vial. If multiple batches of vials are frozen, a batch number should also be written on each cryogenic vial and a permanent record should be kept of each batch.
13. For some slow and nonpenetrating cryoprotectants, e.g., glycerol, cultures are incubated for 30 min in the presence of cryoprotectant prior to cooling. For some sensitive strains it has been suggested that cryoprotectants should be added at 0°C by incubating on ice ***(10)***.
14. Many algae can be cryopreserved directly on an agar slant. A small volume (0.3–0.5 mL) of sterile agar medium is aseptically transferred to a sterile 2-mL cryogenic vial and allowed to solidify as a slant. An algal culture is spread or streaked on the surface of the solidified agar and incubated under normal growth conditions. The culture is ready for cryopreservation after it has grown into a heavy streak or a lawn, and many cyanobacteria and unicellular chlorophytes can be cryopreserved after 2–3 wk. In preparation for cryopreservation, growth medium containing an appropriate cryoprotectant solution is added slowly to the cryogenic vial in order to minimize the disturbance of algae growing on the agar surface. This preparation of algae can then be cryopreserved like liquid cultures. To revive the culture, rapidly thaw the vial, gently decant the liquid, and add fresh culture medium to the vial. If the alga adheres to the agar surface, then the liquid medium can again be decanted and the cryogenic vial incubated under normal culture conditions. Viable algae remaining on the agar surface typically grow into a lawn within 2–3 wk ***(13)***.
15. For most protocols a Step 1 terminal temperature of –40°C is sufficient. However, when methanol is used as the cryoprotectant at the UTEX algal collection at –45 to –55°C is used as a terminal temperature ***(13)***.
16. For some algae, including members of the Prasinophyceae, incubation for 30 min at –40°C is used.
17. The chamber cools at approx –1°C/min over a temperature range of 0°C to –50°C. For some strains it may be desirable to leave the container in the –80°C freezer for only 60 min, at which time the content of the cryogenic vial reaches approx –40°C. For some strains it has been found to be advantageous to leave the Mr. Frosty for up to 4 h at –80°C. A convenient protocol suitable for many strains of microalgae is to remove the canister after the desired temperature is reached and quickly transfer the frozen vials to an ultra-cold storage vessel.

18. One can use cooling rates of up to –10°C/min for many members of the Chlorococcales. Slow cooling rates (0.5–5°C/min) are generally used for many larger and more complex microalgae ***(14–16)***.
19. A more complex cooling program successfully employed at CCMP for marine strains involves cooling the contents of cryogenic vials from ambient temperature to 4°C at –1°C/min, then holding the temperature constant for up to 5 min. This dwell time is especially effective for cold polar strains and is sometimes required for adequate penetration of the cryoprotectant. Vial contents are next cooled at –1°C/min until they reach –9°C. Seawater remains as super-cooled liquid at that temperature. The cooling chamber is then cooled rapidly to –45°C in order to quickly drive the contents of cryogenic vials down to –12°C. This induces ice nucleation and rapidly removes the latent heat of fusion. The contents of vials are then cooled at –1°C/min until they reach –45°C, which is below the eutectic point (*see* Glossary). The vials are then cooled rapidly to –90°C and finally transferred from the cooling chamber to a liquid nitrogen storage system ***(8)***.
20. Cryopreserved cultures in cryogenic vials are generally maintained for long periods of time in one of three ways: (1) submerged in liquid nitrogen (–196°C), (2) in the cold vapor phase above liquid nitrogen (approx –165°C), or (3) in an ultra-cold electrically driven freezer (–150°C).
21. Using a floating vial holder (e.g., Nalgene 5974-4015) reduces the possibility of contamination caused by contact with the water in the waterbath.
22. Liquid nitrogen leakage into the vial is potentially dangerous. On thawing, the nitrogen will evaporate and this could cause the vial to explode. Care should be taken when handling vials containing nitrogen and full-safety equipment (gloves, apron, goggles, and so on) should be used.

    Liquid nitrogen may contain low levels of viable bacteria and these, in turn, may contaminate axenic cultures if leakage occurs. Where alternative frozen specimens are available, vials containing liquid nitrogen should be discarded.
23. For cultures that do not settle rapidly, the cryogenic vial may be subjected to gentle centrifugation in order to pellet the culture. Discard the supernatant and dilute the pellet with fresh culture medium.
24. Allow the recovered culture to remain in darkness, or in subdued light (normal room light is generally acceptable, but not in close proximity to a source of artificial illumination, or a window exposed to bright outdoor light) for several hours, preferably overnight. Then place the culture under normal culture conditions and expect growth of viable cells to resume within 1–2 d. The addition of a small amount of yeast extract (0.1 gL), proteose peptone (0.1 gL), or soil extract (10 mL/L) sometimes enhances viability of axenic cultures.
25. An incubation period that allows repair of sublethal damage, but which is too short for cell division to occur, will give a more accurate index of viability when using vital staining rather than staining immediately after thawing.
26. Temperature levels of the specimen increase rapidly during fluorescence microscopy and cells will die within 1–2 min. Observations and counts must be performed rapidly to prevent an underestimate of the viability level.

27. For many algae, particularly nonmotile algae that do not survive embedded in agar, spread plates of logarithmic dilutions may be used instead of pour plates. Spread 0.1 mL logarithmic dilutions of control and frozen and thawed culture onto the surface of Petri dishes (50 mm) containing an appropriate agar (1.5% [w/v]) solidified medium. Incubate and enumerate as detailed for pour plates.
28. A sufficient number of algal units should transferred to the 50-mm plates to yield between 50 and 200 colonies per plate, because that provides statistically meaningful numbers, yet allows convenient and accurate counting.
29. Exposure time to the hot agar should be minimized; 40°C for more than a few minutes will be lethal for many nonthermophilic algae. For temperature-sensitive strains, melted agarose with a lower melting point may be mixed with the culture prior to transferring to plates.
30. For most algae, incubation at 25°C will reduce the period required to obtain discrete countable colonies.

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11

Cryopreservation of Plant Cell Suspensions

Brian W. W. Grout

Summary

The cryopreservation of dedifferentiated cells, grown in suspension culture, is one of the portfolio of techniques employed for the long-term conservation of higher plant germplasm. Suspension cultures are also important in biotechnology, particularly in transformation studies and for the production of specific metabolites, and, here, there is also a pressing need for genetically stable, long-term storage of cell lines.

Cryopreservation of suspension cell cultures can be exploited by either slow, or rapid, cooling techniques. During slow cooling the extracellular solutions are nucleated and the cells cryodehydrate during controlled cooling as a consequence of extracellular ice, to the point where their intracellular fluids will vitrify on subsequent transfer to liquid nitrogen. In the rapid cooling protocols, the cells are prepared by extreme osmotic dehydration, with cryoprotection, before plunging the samples directly into liquid nitrogen to achieve vitrification. Extensive success has been achieved with both techniques but rapid cooling is, currently, widely favored because of its simplicity.

Key Words: Higher plant; cell suspension; cryopreservation; slow cooling; vitrification; genetic conservation; long-term storage.

1. Introduction

Suspension cultures may be considered being dedifferentiated and at the simplest level of organization of higher plant material held in vitro. Typically, they are diverse in morphology and development and contain varying proportions of single cells and aggregates of differing sizes and morphologies. These include cells that range from densely cytoplasmic to massively vacuolate, with varying vacuolar volumes and cell wall properties.

Plant suspension cultures are valued for a diversity of reasons, including:

1. Their role in genetic transformation studies and the development of transformation technology (e.g., **refs. *1–5***).

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2. The production of specific metabolites, often following transformation (e.g., **refs. *6–10***).
3. Their utility as starting material in the process of embryogenesis and whole-plant recovery (e.g., **refs. *11–15***).

In many instances more than one of these attributes may be combined in a single program of research and development, particularly in biotechnology.

It is widely assumed, from a genetic conservation standpoint, that the cell population in suspension is clonal but, if not, then any genetic diversity that might exist is uniformly distributed throughout the culture, albeit that it might be restricted to particular phenotypes. Further diversity, evident in most cultures, is the differing placement of individual cells in the suspension within the mitotic cycle. Few suspension cultures can be brought to high levels of mitotic synchrony without significant effort ***(15,16)***, but the high levels of survival (90%) reported following cryopreservation of *Arabidopsis* suspension cultures ***(17)*** may indicate that such efforts are worthwhile.

It follows from the morphological and developmental diversity within most plant cell suspension cultures that it is unlikely that any particular cryopreservation protocol, however devised, will be successful for more than a limited portion of the cell population. Clearly, this understanding should influence estimates of successful post-thaw viability.

The consequences and advantages of cryoconservation and its application to plant suspension cultures are well documented ***(18–23)***. The first generation of techniques, still current and widely employed, were based on a protocol reliant on relatively slow-cooling rates, at or near 1°C/min ***(18–24)*** and a cryoprotectant mixture containing dimethyl sulfoxide (DMSO), glycerol, and sucrose. For many culture types, acceptable levels of success with this basic method depended on empirical optimization of:

1. The duration and nature of any pretreatments used to take advantage of natural cell adaptation.
2. The type, mixture, and concentration of cryoprotectants.
3. The incubation time in the protectant solution.
4. Precise cooling rate.
5. The duration of any necessary subzero-holding temperatures during the cooling protocol that increased cryodehydration.
6. The recovery conditions.

Typically, suspensions are concentrated from the growth medium at a point in the culture cycle where the proportion of small, more densely cytoplasmic cells is at a maximum, with these being viewed as the likely survivors of the freezing protocol. Dependant on the particular genotype, the suspensions may benefit from pregrowth with enhanced concentrations of an osmoticum such as

mannitol or sorbitol and proline and DMSO have also been used in pretreatment ***(17,19,25,26)***. This pregrowth takes advantage of any natural, adaptive responses that result from osmotic stress and/or activation of the synthesis of natural protectants ***(27)***. Pregrowth at low temperature may also be beneficial ***(28,29)***. Thereafter, the cells are exposed to a cryoprotectant solution containing, in the original protocol, DMSO, glycerol, and sucrose at final concentrations of 0.5, 0.5, and 1 *M*, respectively ***(19,30)***. The optimum concentrations will vary with circumstance, and empirical optimization is likely to be needed. Other protectant compounds used include proline and polyethylene glycol (*see* **Note 1**). Once pretreated, volumes of cell suspension of less than 2 mL are cooled at a rate typically between 0.5 and 2.0°C/min to –40°C, before plunging into liquid nitrogen. At these slow-cooling rates, the unfrozen suspension cells become embedded in ice following nucleation of the extracellular solution, resulting in ongoing cryodehydration ***(22,23)***. As temperature decreases, the dehydration continues and intracellular solutions become increasingly concentrated and viscous. A point is reached where the residual intracellular solutions will vitrify when the cell sample is plunged directly into liquid nitrogen. A holding period at an appropriate subzero temperature may be required to achieve a sufficient level of dehydration. This pattern of cooling, followed by the plunge to ultra-low temperature, provides the classic "two-step" freezing protocol. The expectation is that, with rapid rewarming to avoid intracellular ice crystallization, a proportion of the cells in suspension will retain organization and viability following thawing ***(19,30,31)***.

A second major step in the cryopreservation of plant cell suspensions is seen in the more recent proliferation of vitrification techniques, whereby cells can be cooled rapidly to ultra-low temperatures avoiding the phase transition from liquid water to crystalline ice and, instead, producing an amorphous glass ***(31)***. Two mechanisms are at work in the successful vitrification protocol, the first being extensive cytoplamic dehydration resulting from high concentrations of essentially nonpenetrating protectants, e.g., glycerol at 30% (w/v) in the extracellular medium ***(31)***. The second is the increase in viscosity of the cytoplasmic solution that follows from the penetration of high concentrations of protectant, such as DMSO, included in the added vitrification medium into the cells.

Following treatment with vitrification medium, both the extra- and intracellular solutions are radically modified as a consequence of osmotic dehydration and loading with high concentrations of cryoprotectants. The cells that survive are those that become sufficiently dehydrated so that their intracellular solutions form a stable glass when, with rapid cooling by direct immersion of the sample in liquid nitrogen, they pass the appropriate glass transition point. Typically, the cooling rates employed are in excess of 200°C/min. The extracellular solutions

must undergo a similar transition. For the widely used plant vitrification solution PVS2 containing high levels of glycerol, ethylene glycol, and DMSO, this occurs in the region of –115°C ***(31)***. The presence of a glass provides stability in the system to be preserved, but successful recovery depends on a thawing procedure that transforms the aqueous glass phase directly to a liquid without the intervention of crystalline material, which can only be achieved by rapid rewarming. Precise rates are not usually quoted for this part of published protocols, perhaps because of the difficulties in making appropriately cited, accurate measurements. However, immersion of 2-mL cryovials, foil packets, or straws in water at 40°C for 1–2 min is widely reported ***(29,31,32)***. On occasion, the vitrification protocol can be modified to include an encapsulation stage, whereby the cells are embedded in alginate beads prior to the vitrification procedures ***(33)***.

2. Materials

2.1. Slow-Cooling Protocols

1. A cell suspension selected at a point in the culture cycle where the proportion of smaller, more densely cytoplasmic cells are at a maximum. The culture may have been subjected to pretreatments, such as growth in osmoticum-supplemented medium (e.g., sorbitol or mannitol at 0.3–0.5 *M*) and/or at low temperatures (6–0°C). Compounds such as desferrioxamine may also be used (*see* **Note 2**).
2. An apparatus capable of providing uniform, slow cooling to temperatures between –35 and –40°C. Reliable, programmable freezers that achieve this are commercially available, but simpler constructions relying on cooled liquid baths may be used (*see* **Note 3**).
3. An appropriate storage Dewar that allows for storage in the liquid or vapor phase. For secure cryoconservation, the storage Dewar should have an effective inventory system (drawers, canes) so that the precise location of samples can be achieved without unnecessary movement of samples from the storage environment. Duplicate samples should be stored in separate Dewars, ideally in a separate facility. Level alarms for liquid nitrogen should be fitted to prevent compromise of the storage temperature.
4. Autoclaved/sterile polypropylene cryopreservation vials, typically of 2-mL volume. Alternatives will include the straws used for semen cryopreservation, with volumes up to 1 mL, and self-made foil packets made from double layers of aluminium foil.
5. Suitable filtration or low-speed centrifugation to allow cell suspensions to be concentrated before freezing.
6. Cryoprotectant solutions: a double-strength solution of 1 *M* DMSO, 1 *M* glycerol, and 2 *M* sucrose to be added to an equal volume of cells, and pregrowth medium is widely employed. Other protectants and concentrations may be more appropriate (*see* **Note 1**).
7. A thawing bath set a 40°C, containing smaller vessels holding sterile water. Thawing vials in this water reduces the incidence of post-thaw contamination.

8. Suitable post-thaw recovery media, either liquid or solidified.
9. A solution of 0.1% (w/v) fluoroscein diacetate (FDA) in acetone, to carry out a post-thaw assessment of potentially viable cells. A microscope with an ultraviolet (UV) light source, and excitation and barrier filters at 490 and 530 nm, respectively, is also needed.

2.2. Vitrification Protocols

1. The materials and requirements for pregrowth and the selection of samples at an appropriate place in the culture cycle is as for slow cooling (*see* **Subheading 2.1.**). The requirements for concentrating the cell suspension, the sample vials for cryopreservation, frozen storage, a thawing bath, and the fluorescence viability assay are also similar.
2. A "loading" or osmoprotection solution that produces a final concentration of 2 *M* glycerol and 0.4 *M* sucrose.
3. The PVS2 solution ***(34)*** comprising 30% (w/v) glycerol, 15% (w/v) ethylene glycol, and 15% (w/v) DMSO in the appropriate growth medium supplemented with 0.4 *M* sucrose.
4. An "unloading" or dilution medium of 1.2 *M* sucrose in the appropriate growth medium.
5. A suitable recovery medium.

3. Methods

3.1. Slow Cooling

1. Aliquots of the suspension culture are transferred to pregrowth medium for a period of 2–7 d, the optimum period being determined by investigation.
2. Pregrown cultures are concentrated by settling, filtering, or centrifugation and the more concentrated suspension cooled on ice for 30 min.
3. A volume of prechilled cryoprotectant equal to the volume of cell suspension, and at twice the required final concentration, is added to the suspension culture in three aliquots over a 20-min period, with mixing by gentle agitation at each addition.
4. After an incubation of longer than 1 h in the final concentration of protectant, excess solution is pipetted off to leave a 30% loosely packed cell volume.
5. An appropriate aliquot is transferred to the containers for freezing (1 mL into a 2-mL cryovial) and then transferred to the cooling apparatus. A programmable freezer should be precooled to 4°C.
6. The samples are cooled at 1°C/min to –40°C, with a holding period of up to 40 min at this temperature, or higher, to enhance cryodehydration.
7. Frozen samples are transferred into a small Dewar of liquid nitrogen as rapidly as possible, and once equilibrated (when bubbling ceases) can be transferred to the storage Dewar.
8. To thaw, samples are removed from the storage Dewar and immediately plunged into sterile water at 40°C, with continuous agitation. The samples are thawed when no more ice is visible in the sample.

9. To recover the cells, the thawed container is wiped with 70% (v/v) ethanol to minimize surface contaminants, is then opened, and the cells are returned to a recovery medium, when any dilution procedures can take place.
10. An estimate of initial, post-thaw viability can be made at this stage using 0.5 mL of cell suspension to which several drops of FDA stain is added. The sample is viewed microscopically for UV fluorescence.

3.2. Vitrification

1. Aliquots of the suspension culture are transferred to pregrowth medium for a period of 2–7 d, the optimum period being determined by investigation.
2. Pregrown cultures are concentrated by settling, filtering, or centrifugation.
3. Cells are transferred to a loading solution adjusted to give a final concentration of 2 *M* glycerol and 0.4 *M* sucrose for 20–30 min at room temperature.
4. Cells concentrated and transferred to PVS2 at 0°C for 60–90 min.
5. During the PVS2 incubation the cells are transferred to the vials/straws for freezing.
6. Direct immersion of the sample containers (vials/straws) into liquid nitrogen, with subsequent transfer to the storage dewar.
7. To thaw, samples are removed from the storage Dewar and immediately plunged into sterile water at 40°C, with continuous agitation. The samples are thawed when no more ice is visible in the sample.
8. To recover the cells, the thawed container is wiped with 70% (v/v) ethanol to minimize surface contaminants and then opened, and the cells are returned to an unloading solution of 1.2 *M* sucrose for 20–30 min at room temperature before transferring the cells returned to a recovery medium, when any further dilution procedures can take place.
9. An estimate of initial, post-thaw viability can be made at this stage using 0.5 mL of cell suspension to which several drops of FDA stain is added. The sample is viewed microscopically for UV fluorescence.

4. Notes

1. The selection of an optimally effective protectant solution is likely to require empirical investigation. Variants previously reported ***(19,35,36)*** have included:
   a. DMSO at 5–10% (v/v).
   b. DMSO and glycerol both at 5% (v/v).
   c. 4% (v/v) DMSO, 4.6% (w/v) glycerol, and 11.5% (w/v) proline.
   d. 10% (v/v) DMSO, 8% (w/v) glucose, and 10% (w/v) polyethylene glycol (PEG2000).
   e. 10% (v/v) DMSO and 18% (w/v) sucrose.
   f. 18.4% (w/v) glycerol and 14.4% (w/v) sucrose.
2. Desferrioxamine is intended to reduce oxidative stress by reducing cation availability, thereby reducing the Fenton reaction and the production of hydroxyl radicals.
3. Alternatives to commercial programmable freezers will include baths of suitably low freezing point solutions (e.g., methanol, glycol) with a dip cooler to lower the temperature and a stirring device to ensure uniform cooling ***(19,24)***. Where

laboratory freezers with temperatures below –30°C are available, suitably insulated containers (polystyrene) may allow sufficient control of the cooling rate to provide successful cryopreservation.

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12

Cryopreservation of Shoot Tips and Meristems

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Summary

Shoot-tip meristem cryopreservation methodologies are reported for the complementary cryoprotective strategies of vitrification and equilibrium freezing using traditional controlled-rate freezing and chemical additive cryoprotection. Pregrowth, pretreatment, and cold acclimation approaches for the improvement of tolerance to liquid nitrogen are also presented. The chapter concludes by reporting an analytical protocol that profiles volatile hydrocarbon stress markers (for ethylene, hydroxyl radicals, and lipid peroxidation products) during cryopreservation. This method uses noninvasive headspace sampling and gas chromatography, and it is widely applicable across cryogenic systems.

Key Words: Meristems; vitrification; cryoprotection; cryoinjury; plant genetic resources.

1. Introduction

Recent advances in shoot-tip cryopreservation have been significant, this is largely because of: (1) development of vitrification-based cryoprotection protocols, (2) refinements in tissue culture practices, (3) identification of critical points in cryopreservation technology transfer, and (4) the wider uptake and validation of cryostorage technologies in international germplasm repositories. There still remain some genotypes intractable to cryogenic storage, and fundamental research is progressively facilitating the identification of decisive factors in recalcitrance, with a view to aiding storage protocol development. With these issues in mind, this chapter will report the routine storage and investigative methodologies currently applied to shoot cryopreservation. Generic cryopreservation protocols will be described and *Ribes* (currants) are used as an exemplar, as this genus has been studied in detail with regard to critical point thermal analysis, protocol validation, and technology transfer to germplasm

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repositories. An analytical gas chromatogram (GC)-head space sampling technique will also be described. This is a noninvasive tool that has been optimized to pinpoint stress components in shoot-tip cryopreservation protocols with a view to assisting method development.

Anatomically, the shoot meristem contains the apical dome and the youngest, unexpanded primordial leaf. For cryopreservation, selection of tissues that comprise the meristem apex, subjacent tissue, and several larger leaf primordia may be also necessary. Shoot-tip size and origin (apical, lateral, axillary) influences poststorage survival and it is also possible to cryopreserve nodal stem cuttings containing an axillary meristem. Physiological status is critical and storage responses contrast between temperate and tropical species. In temperate genotypes, cold hardiness, acclimation, and dormancy responses are important factors and are used in cryoprotective treatments. Cryopreservation of cold-hardened winter buds of woody perennial species is therefore an alternative approach to in vitro cryopreservation. In contrast, tropical and warm temperate species are more sensitive to chilling and desiccation stresses, and their cryopreservation protocols have to be developed accordingly.

As described in Chapter 3, there are two main approaches to cryopreservation based on cryoprotective modality. The first is termed traditional, controlled rate, or two-step freezing and involves the application of single or combined mixtures of colligative cryoprotectants, sometimes in conjunction with osmotic additives. Controlled cooling of tissues to an intermediate freezing temperature causes a water vapor deficit to be created between the inside and outside of the cell initiated by the preferential formation (termed "seeding" or ice nucleation) of extracellular ice. Thus, intracellular water moves across the plasmalemma and cellular dehydration results; this process is called equilibrium freezing. Under optimum freezing rates this is a cryoprotective process, as the amount of water available for intracellular ice formation is reduced. However, excessive dehydration can lead to colligative damage owing to the harmful concentration of solutes. Critical cryogenic factors in controlled-rate freezing are:

1. Cryoprotectant composition, the components of which *must* include a penetrating colligative cryoprotectant.
2. Cooling rate.
3. The control of, and point at which, ice nucleation occurs.
4. A "hold step" programmed as a fixed time at a fixed terminal transfer temperature. Usually at, or around, the point of homogeneous ice formation, which is –40°C.
5. Transfer to liquid nitrogen.

The second approach to plant cryopreservation is vitrification which usually comprises different permutations of chemical additive as well as vitrification, and

encapsulation, osmotic-, evaporative-, and chemical (silica gel)-dehydration. The cryoprotective basis is the concentration of solutes to such an extent that cell viscosity becomes so high that on exposure to cryogenic temperatures, a glass, rather than ice, is formed. The process, termed vitrification, involves the creation of a metastable amorphous glassy state, characterized by the glass transition temperature, Tg, the thermal point at which a glass is formed. However, glasses can be thermally unstable and it is possible that devitrification occurs on cooling and rewarming (as this risks the formation of ice it is critical that glasses are stabilized). This can be achieved by the manipulation of dehydration and cryoprotective additives, particularly the inclusion of sugars and careful control of rewarming. Vitrification is a useful alternative to controlled-rate cooling and freezing, as it has the major advantage that tissues are directly immersed into liquid nitrogen, circumventing the need for expensive programmable freezing equipment. In addition to cryogenic factors, the development of successful cryopreservation protocols also depends on the physiological status of the tissues, and pre- and post-treatments are applied to maximize the ability of shoot tips to survive cryopreservation.

To assist the reader in accessing information as to the theory and applications of plant cryopreservation, a bibliography is recommended. For fundamental theory *see* Mazur ***(1)*** and Chapter 3 of this volume; for cryophysics and instrumentation *see* Benson et al. ***(2)***; for historical perspectives and wider areas of in vitro plant conservation *see* Benson ***(3,4)***; and for a comprehensive overview of the contemporary aspects of fundamental and applied cryobiology, *see* Fuller et al. ***(5)***.

2. Materials

Cryogenic safety protocols and protective clothing should be used throughout all liquid nitrogen handling procedures. In vitro materials should be sterile and manipulations performed in a laminar-flow cabinet. Shoot cultures should be checked before cryopreservation for health, vigor, and the presence of contaminants, particularly incipient systemic infections that are manifested as opaque white-cream haloes in the culture medium surrounding explants.

2.1. Shoot-Tip Dissection

1. Binocular, dissecting microscope (magnification ×20).
2. Two 10-mL syringes fitted with hypodermic needles.
3. Liquid growth medium (*see* **Note 1**).
4. 50-mm Petri dish lined with a sterile filter paper.
5. Scissors, scalpel, forceps, Pasteur pipets.

2.2. Common Cryopreservation Materials

1. Liquid nitrogen.
2. Small bench top Dewar with 1-L capacity.

3. Cryovials, canes, Pasteur pipets, 90- and 50-mm Petri dishes, sterile filter papers, forceps, scissors, scalpels, heated magnetic stirrer, and binocular dissecting microscope.
4. Long-term storage Dewar and inventory system.
5. Recovery medium (*see* **Note 2**).
6. Safety equipment.

2.3. Controlled-Rate Freezing

1. Two representative cryoprotectants have been selected for controlled-rate freezing, both use dimethyl sulfoxide (DMSO), one of the most penetrating colligative cryoprotectants known (*see* **Notes 3–5**). The second incorporates a mixture of additives and includes the dehydrating osmotic agent polyethylene glycol and glucose.
   a. 5 or 10% (w/v) DMSO or (v/v) corrected for the specific gravity of DMSO ***(6)***.
   b. Or, 10% (w/v) each of polyethylene glycol (MW 8000), glucose, and DMSO ***(7)***.
2. Programmable freezer.
3. Water bath at 45°C.

2.4. Cryoprotective Dehydration

1. One or more of a series of pregrowth media containing 0.5–1 *M* of a dehydrating agent selected from sucrose, mannitol, or sorbitol (*see* **Note 6**).
2. An airtight vessel (e.g., a Parafilm-sealed 9.0-cm glass Petri dish) containing a known loading (30–50 g) of sterile heat-activated silica gel.
3. An open sterile Petri dish located in sterile, horizontal laminar airflow.

2.5. Vitrification Using Cryoprotective Additives

1. 100 mL of plant vitrification solution 2 (PVS2 ***[8–11]***) made up in standard liquid growth medium to which is added 30% (w/v) glycerol, 15% (w/v) ethylene glycol, 15% (w/v) DMSO, and sucrose to a final concentration of 0.4 *M* (*see* **Notes 3–5**).
2. A dilution series range (50, 60, 70, 80% [v/v]) of PVS2 solution made in liquid medium (*see* **Note 6**).
3. Ice and ice bucket.
4. 100 mL of "unloading" solution comprising 1.2 *M* sucrose made up in liquid culture medium.
5. Water bath at 45°C.

2.6. Encapsulation and Dehydration

1. A 5-mL Gilson Inc., Middleton "Pipetteman" fitted with a tip or a 3-mL plastic Pastet (*see* **Note 7**).
2. Liquid culture medium containing 0.75 *M* sucrose (*see* **Note 6**) aliquoted (20 mL) into 100-mL conical flasks.
3. Calcium-free, liquid culture medium containing 3% (w/v) sodium alginate (SIGMA [SIGMA-ALDRICH, Gillingham, Dorset, UK] low viscosity, sodium salt derived from sea kelp) dispensed into small bottles as 20-mL aliquots (*see* **Notes 8** and **9**).

4. Liquid culture medium containing 100 m*M* $CaCl_2$ dispensed as 30-mL aliquots into conical flasks or beakers.
5. A 250–500-µm mesh sieve.
6. Sterile tissues or pieces of filter paper.
7. Reciprocal shaker.

2.7. Encapsulation and Vitrification

1. PVS2 solution (*see* **Subheading 2.5.**).
2. Calcium-free, liquid culture medium ***(12)*** containing 2 or 3% (w/v) sodium alginate (SIGMA low viscosity, sodium salt derived from sea kelp) and incorporating 0.4 *M* sucrose ***(12)*** dispensed into small bottles as 20-mL aliquots (*see* **Note 8**).
3. Liquid culture medium containing 100 m*M* $CaCl_2$ ***(12)*** dispensed as 30-mL aliquots into conical flasks or beakers.
4. Liquid culture medium containing 0.75 or 0.8 *M* sucrose (*see* **Note 6**) aliquoted (20 mL) into 100-mL conical flasks.
5. A 250–500-µm mesh sieve.
6. Sterile tissues or pieces of filter paper.

2.8. Droplet Freezing

1. Sterile pieces of aluminium foil (of 2–3 cm length × 0.5–1.0 cm width × 0.003 to 0.005 cm depth dimensions).
2. Sterile liquid culture medium.

2.9. Volatile Headspace Analysis of Cryopreservation Injury in Shoot Tips

1. GC equipped with an injector at 150°C, Porapak Q- or Haysep Q-packed column (2-m long, 1/8-in. ID stainless steel tubing, 80–100 mesh), oven temperature initially at 60°C for 3 min (increase to 160°C at 20°C/min, and hold at 160°C until all compounds have eluted from the column [*see* **Note 10**]), carrier gas of oxygen-free nitrogen set to a flow-rate of 25–30 mL/min, flame ionization detector at 225°C, supply of hydrogen (30 mL/min) and air (300 mL/min) for the flame ionization detector, and an output linked computer, integrator, or chart recorder.
2. Gas calibration standards (Supelco, Scotty I-analyzed gases, cat. no. 2-2566).
3. 4 mL autoclave-resistant glass vials with open-top caps and inert silicon/Teflon septum (Supelco, SIGMA-ALDRICH cat. no. 27209-U).
4. 1-mL glass, gas-tight syringe fitted with closable inlet valve (*see* **Note 11**).
5. 5% (v/v) DMSO in distilled H_2O (filter-sterilized).
6. Preculture and recovery medium (*see* **Note 12**).
7. 100-µL and 5-mL automatic pipets and sterile tips.

3. Methods

3.1. Pregrowth

Pregrowth of cultures, shoot nodes, and excised shoot-tips can be undertaken 24–72 h before cryopreservation (*see* **Notes 6** and **13**). The procedure involves

the application of an osmotica (sucrose, sorbitol, mannitol), DMSO, a plant growth regulator (abscisic acid), and a stress-ameliorating factor (proline) at predetermined optimized concentrations.

3.2. Acclimation

Cold acclimation may be simulated for chill-sensitive species, as well as for naturally acclimating genotypes. This can be achieved by the application of a 0.75 *M* sucrose pregrowth treatment ***(13)*** to shoot nodes supported on semisolid growth medium 3–7 d before meristem excision (*see* **Note 13**). Cold-hardened dormant buds, which have undergone natural seasonal acclimation, may also be used ***(10,11)***. Alternatively, it is possible to induce cold hardiness in rejuvenated buds or in vitro plants using temperature-controlled environments (***14***; *see* **Notes 14** and **15**). It may be necessary to optimize low temperatures (–5 to 10°C), treatment duration (days to months), and temperature cycling (diurnal alternation of growth and low-temperature treatments) to achieve enhanced shoot-tip survival after cryopreservation (*see* **Note 15**).

3.3. Shoot-Tip Dissection

1. With the aid of a dissecting microscope (×20–40) and two hypodermic needles, remove the larger expanded leaves from the shoot tips, while retaining several non- or partly expanded leaf primordia and a small amount of subjacent tissue.
2. Dissection is best achieved on a medium-moistened, sterile filter paper platform in a Petri dish placed on the microscope-viewing platform.
3. Trim the shoot tip to an approximate size of 1–5 mm (*see* **Note 15**).
4. Immediately after dissection transfer shoot tips to filter paper bridges (placed in a 50-mm Petri dish or similar) soaked with pregrowth medium (*see* **Note 16**).
5. For some species and genotypes a short recovery period after dissection may be required to overcome wounding injury. Thus, transfer dissected shoot tips from filter paper bridges to standard solid or liquid medium (*see* **Note 17**).
6. For some germplasm the incorporation of 5% (v/v) DMSO in the recovery medium can enhance survival and help ameliorate the effects of wounding.

3.4. Chemical Cryoprotection and Controlled Freezing

This method is adapted from that originally developed for potatoes ***(6)*** and *Ribes* ***(7)***.

1. Transfer 10–20 shoot tips to a cryovial containing an appropriate cryoprotective additive (*see* **Subheading 2.3.**) that can be applied all at once, gradually at ambient temperature, or chilled/ice temperatures.
2. Incubate at room temperature for 1 h (*see* **Note 6**).
3. Transfer to a programmable freezer and set a freezing cycle (*see* **Note 18**) with parameters in the following ranges:
   a. Cooling rate (–0.25 to –5°C/min).

   b. Terminal transfer temperature (0 to –40°C).
   c. Holding time at temperature of transfer (30–45 min).
4. An optional "seeding" stage can also be incorporated within a range normally between –5 and –15°C, which includes a hold for 5–15 min to allow ice nucleation. This is achieved manually by touching the cryovials with an ultracold instrument (e.g., a spatula that has been briefly held in liquid nitrogen), or by initiating the automatic seeding facility on a programmable freezer (*see* **Note 18**).
5. On completing the controlled temperature program, rapidly transfer the vials to a small liquid nitrogen-filled Dewar located by the side of the freezer (*see* **Note 19**).
6. Subsequently transfer the vials to a large storage Dewar.
7. For thawing, transfer the vials to a 45°C water bath until all the ice has melted, then place in a flow bench and wipe the outside of the vial with a tissue soaked in a sterilizing solution to remove excess and potentially contaminating water.
8. Expel the vial contents onto filter papers contained in a Petri dish; the cryoprotectant is removed by capillary action.
9. Transfer the shoot tips to recovery medium (*see* **Note 2**).

3.5. Cryoprotective Dehydration

This method is adapted from those of Niino et al. ***(8)***, Uragami ***(9)***, Bagniol and Engelmann ***(15)***, and Sherlock et al. ***(16)***.

1. Remove nodal segments or shoot tips (2–5 mm) from plantlets and detach expanded leaves using a scalpel (*see* **Note 20**).
2. Culture the prepared explants in a series of media containing 0.5–0.75 *M* of osmotic agent selected from sucrose, mannitol, or sorbitol prepared on standard solid medium for 1–5 days (*see* **Notes 13** and **17**).
3. Transfer plant tissues to standard medium in order to optimize **step 2** and select the protocol that maximizes dehydration with minimal loss of viability (*see* **Note 18**).
4. After **step 3** has been optimized, transfer the osmotically dehydrated tissues to airtight vessels containing activated silica gel and desiccate for 2–16 h. Alternatively, the germplasm may be desiccated in an evaporative airflow.
5. Place shoot tissue on recovery medium to determine which treatment is optimal for maximum dehydration and minimal loss of viability.
6. Choose the optimal pregrowth treatment and test its effectiveness in supporting postfreeze recovery after ultrarapid freezing (*see* **Notes 13** and **17**).
7. Transfer the dehydrated shoot tips to cryovials and plunge directly into liquid nitrogen.
8. Rewarm the vials at ambient temperatures.
9. Transfer tissues to recovery medium (*see* **Note 2**).

3.6. Chemical Additive Vitrification

These methods are largely based on those originally developed by Uragami ***(9)*** and Sakai ***(11)*** and has since been adapted extensively by Kim et al. ***(17)*** and Thin et al. ***(18)*** to produce different derivative methodologies. These use

different combinations of cryoprotectants applied at higher concentrations than would be the case for standard cryoprotection. They also include protocol permutations that incorporate pregrowth and dehydration treatments that assist the recovery of more sensitive genotypes ***(11)***. The basic protocols are presented with some alternative options highlighted.

3.6.1. PVS2

1. Prepare PVS2 (*see* **Subheading 2.5.**; *see* **Note 22**).
2. Prepare a range of diluted PVS2 solutions (e.g., 10–80% [v/v] PVS2 solutions in standard liquid medium).
3. Prepare a solution of standard liquid medium containing 1.2 *M* sucrose (*see* **Note 23**).

PVS3 is an alternative solution comprising 50% (w/v) sucrose and 50% (w/v) glycerol prepared in standard liquid culture medium ***(8)***.

3.6.2. Basic Vitrification Procedure

Prepare shoot tips (*see* **Subheading 3.3.**) and construct a toxicity test (*see* **Notes 23** and **24**) for the PVS2 solutions as follows:

1. Add on ice, chilled 1-mL aliquots of PVS2 within the range of 50–80% (v/v) to a specific concentration for 1–10 min and remove almost all the vitrification solution (*see* **Note 25**).
2. Replace the intermediate concentrations with a stepwise, higher concentration of PVS2 solution (*see* **Note 6**).
3. Gradually increase the concentration of PVS2 until the tissues are in the 100% solution (*see* **Note 24**).
4. Remove the 100% PVS2 and replace with "unloading" solution containing 1.2 *M* sucrose in standard liquid medium. Perform two to three washes in fresh unloading solution and maintain in this solution for up to 30 min.
5. Expel vial contents onto a filter paper; the paper will soak away the liquid excess.
6. Transfer the shoots to recovery medium and select the treatment that permits maximum survival and cryoprotection.
7. After the stepwise delivery of PVS2 has been optimized, transfer the vials directly to liquid nitrogen.
8. For rewarming, place vials in a water bath at 45°C and recover shoot tips as described in **steps 4–5** of this protocol.

3.7. Encapsulation and Dehydration

Fabre and Dereuddre ***(19)*** developed this cryoprotective approach and applied it for the first time to *Solanum phureja*. The following method has been adapted for *Ribes* spp. ***(20–22)*** and as such, has been used to assist in cryopreservation technology transfer and training programs that also incorporate

PVS2 and controlled-rate freezing methodologies. It may be applied with appropriate modification and optimization to other shoot-tip systems.

1. Transfer shoot-tip meristems to 3% (w/v) alginate solution (*see* **Subheading 2.6.**) and gently swirl the vial to assist tissue immersion, taking care not to form air bubbles.
2. Fill a 3-mL plastic pastette with 2 mL of alginate containing ca. five meristems, care is again required to ensure the alginate contains no air bubbles.
3. Hold the pastette vertical to ensure that spherical drops are formed, and deliver droplets at ca. 1-s intervals into 0.1 *M* $CaCl_2$ containing liquid culture medium to form uniform spherical beads (45–50 µL in size).
4. Allow the beads to polymerize in the calcium solution for ca. 15 min.
5. Pour the calcium solution and beads into a 90-mm sterile Petrie dish, and retrieve the beads containing shoot tips.
6. Transfer the encapsulated germplasm to a 100-mL conical flask containing ca. 25 mL 0.75 *M* sucrose liquid pretreatment medium.
7. Place flasks on an orbital shaker (e.g., a Gertomat M, 125 revolutions per min) for 16–22 h under standard culture conditions.
8. Pour the beads from the flask into a 90-mm sterile Petri dish or through a sieve, and blot dry on sterile filter paper to remove excess surface moisture.
9. Transfer the beads to the base of a sterile, empty 90-mm sterile Petri dish. Ensure the beads do not touch each other and that they are evenly distributed across the base of the dish.
10. Place the dish to the back of a horizontal air stream of a laminar flow hood for 4 h, or as optimized for the germplasm type (*see* **Notes 26–28**).
11. Record the temperature and relative humidity (RH) of the laminar air-flow cabinet at the onset and end point of the 4-h period to ensure quality control (*see* **Note 29**) between different cryopreservation experiments. RH and ambient temperature can critically influence the bead desiccation rate and final moisture content (this should be 20–25% on a fresh weight basis in order to ensure that a stable glass is form during cryopreservation).
12. Place desiccated beads in a cryovial and immerse in liquid nitrogen until required.
13. Warm at ambient temperature in the laminar flow hood for 20–30 min.
14. Wipe the exterior of the cryovials with a sterilant solution.
15. Rehydrate beads in liquid medium for 20 min to remove sucrose (*see* **Note 30**).
16. Transfer to recovery medium (*see* **Note 31**).

3.8. Encapsulation–Vitrification

This method is a combination of the encapsulation technique created by Fabre and Dereuddre ***(19)*** and the vitrification protocol developed by Sakai ***(11)***. It involves encapsulation followed by treatment with PVS2 solutions. Use the basic preparative and bead-making procedures for alginate and PVS2 (*see* **Subheadings 3.6.** and **3.7.**).

1. Suspend shoot-tips meristems following an appropriate pregrowth or hardening treatment (*see* **Subheadings 3.2.** and **3.3.**), in Ca^{2+}-free 2 or 3% (w/v) Na-alginate loaded with a final concentration of 0.4 *M* sucrose, or a mixture of 2 *M* glycerol and 0.4 *M* sucrose.
2. Dispense as droplets into 0.1 *M* $CaCl_2$ solution made up in liquid culture medium containing a final concentration of 0.4 *M* sucrose, or a mixture of 2 *M* glycerol and 0.4 *M* sucrose.
3. Incubate in this solution for 1 h at 25°C.
4. Remove the encapsulated shoot-tip meristems and place in a 50- to 100-mL flask containing 100% PVS2 solution (**Subheading 2.5.**).
5. Place on a reciprocal shaker for an optimized incubation time (e.g., 60 rpm up to 1 h).
6. Remove beads and place in a 1.8- to 2.0-mL cryovial at 5–15 beads/vial and add 1 mL of PVS2 solution.
7. Directly immerse the beads into liquid nitrogen.
8. On retrieval from storage, rapidly rewarm the vials in a water bath at 35–45°C for approx 1 min.
9. Drain the PVS2 solution away and replace with 1.2 *M* sucrose unloading solution for 10 min.
10. Blot the beads dry of excess moisture and place on recovery medium.
11. Hirai et al. ***(12)*** also report a variation on this method (*see* **Notes 26–31**), which involves an additional evaporative drying step.
    a. Prepare encapsulated shoot tips in 2% (w/v) alginate as described in **steps 1–2**.
    b. Transfer to 0.8 *M* sucrose prepared in liquid culture medium and incubate for 16–20 h on a rotary shaker at approx 60 rpm.
    c. Remove, blot dry on sterile filter papers, and transfer to sterile glass Petri dishes containing sterile heat-activated 50 g silica gel and sealed with Parafilm.
    d. Desiccate for 2–5 h optimized to a moisture content of approx 25% on a fresh weight basis.
    e. Proceed as in **steps 6–10**, but omitting the application of PVS2 and sucrose unloading stages.

3.9. Droplet Freezing

This method is based on that originally developed for cassava and potato ***(23)***; *see* **ref. *2*** for a review of droplet freezing. It uses DMSO as the cryoprotectant. More recently it has been applied in combination with vitrification solutions ***(24)***. Shoot tips may require pregrowth treatments before proceeding with the protocol (**Subheadings 3.2.** and **3.3.**).

1. Prepare 10% (v/v) DMSO in liquid medium (**Subheading 2.3.**).
2. Dispense using an analytical 2.5–20 µL Gilson Pipetteman or equivalent 2.5-µL droplets of cryoprotectant (5–10 droplets/strip dependent on loading size) onto the surface of sterile, aluminium foil strips (**Subheading 2.8.**).
3. Transfer one shoot-tip meristem to each droplet.

4. Using liquid nitrogen-tolerant sterile forceps drop the foils directly into liquid nitrogen containing a small (5–10 mL) Dewar located in a laminar flow cabinet.
5. Transfer the foils to cryovials at two foils per vial.
6. Store in liquid nitrogen storage vessels.
7. For rewarming, remove the foils from the vials and place on liquid medium at ambient room temperatures (~25°C). The shoots will dislodge on rewarming and can be retrieved and plated onto the recovery medium.

3.10. Postfreeze Recovery Assessments

It is not always possible to perform viability assays based on microscopy (e.g., vital staining) on shoot tissues that are often optically too dense. Recovery can be assessed initially on survival and then regeneration, and it is the latter that is the definitive assessment of protocol success.

1. Within 7 d of thawing, examine tissues under a binocular microscope.
2. Record the number of shoot tips, which are green and have expanded leaf primordia (*see* **Note 32**).
3. Continue to undertake weekly assessments and record a time-course of recovery events as leaf expansion, callus formation, shoot and plantlet regeneration (*see* **Note 33**).
4. Evaluate recovery as (1) normal events (plantlet formation in the absence of callogenesis and adventitious shooting), and (2) abnormal events (callus and adventitious shoot development) (*see* **Note 33**).

3.11. Volatile Headspace Analysis of Cryopreservation Injury in Shoot Tips

The survival and regeneration of shoot tips following cryopreservation often varies between genotypes and this can require lengthy optimization steps to determine the best cryopreservation protocol. This approach does not identify the underlying physical and physiological causes of cryopreservation injury in recalcitrant species, making it difficult to develop protocols that match the physiological requirements (e.g., desiccation and cold sensitivities) of each species. A complementary approach is to use analytical techniques to measure stress responses in tissues at each stage of a cryopreservation protocol so that critical points for recovery can be identified. This approach has been successfully applied to dedifferentiated cultures of *Daucus carota* ***(25)*** and algae ***(26)*** using volatile markers of oxidative stress and were used to create improved cryopreservation protocols. The method has now, and as follows, been optimized for the profiling of cryoinjury in shoot-tip meristems. The small mass of the shoot tips and the time-consuming nature of meristem excision manipulations often restricts the production of sufficient tissue mass to detect stress markers using analytical procedures based on calorimetry. The nondestructive and sensitive nature of headspace volatile analysis eliminates this problem as it reduces

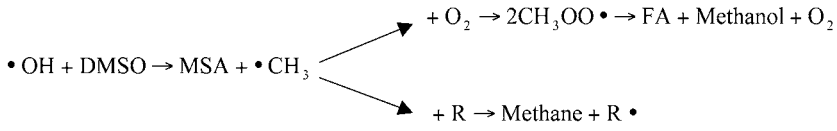

Fig. 1. Reaction between dimethylsulfoxide and the hydroxyl radical (·OH) to produce methane. $\cdot CH_{3,}$ methyl radical; FA, formaldehyde; MSA, methane sulfinic acid.

the need for large numbers of meristems to be surveyed. Moreover, the issue of compound detection from tissues with low mass can be circumvented by increasing the time shoot tips are sealed in air-tight vials, allowing more time for volatile accumulation before sampling. The nondestructive attribute of this method also allows time-course experiments to be conducted on the same batch of vials, and assuming sterile conditions are maintained, the same tissues can be assessed for recovery. This means direct comparisons can be made between volatile markers and shoot-tip recovery.

The following headspace protocol describes the detection of ethane, ethylene, pentane, and methane. Ethane, ethylene, and pentane are secondary products of lipid peroxidation, and the detection of these compounds during cryopreservation may indicate membrane damage ***(25,26)***. Ethylene and ethane are produced from the peroxidation of linolenic acid, and pentane from linoleic acid ***(27,28)***. A second, and usually more abundant, source of ethylene in plants is produced enzymatically ***(29)*** from 1-aminocyclopropanecarboxylic acid. An intact membrane is considered essential for 1-aminocyclopropanecarboxylic acid-dependant ethylene biosynthesis, and a reduction in ethylene production during in vitro plant cryopreservation intimates that membrane damage has occurred ***(25)***. The growth-regulating effects of ethylene also influence growth and development, such as inhibiting in vitro shoot growth and promoting senescence ***(29,32)***, and these could impact postcryopreservation regeneration. Ethylene may also affect the oxidative stress status of the tissue as it can induce lipid peroxidation, deplete ascorbate and α-tocopherol levels, and induce antioxidant enzyme activity ***(30,31)***.

Methane is produced during DMSO scavenging of hydroxyl radicals (·OH), one of the most reactive and damaging free radicals known ***(25)***. The ·OH reacts with DMSO to produce methane sulfinic acid and a methyl radical (*see* **Fig. 1**), which in turn produces either methane or methanol and formaldehyde ***(33)***.

The following protocol has been adapted to measure methane, ethane, ethylene, and pentane in cryopreserved shoot-tips cultures. It may also detect other small-chained products of lipid peroxidation, such as alcohols and aldehydes ***(28)***, allowing further insight into the stress responses during cryopreservation.

1. Measure the empty weight of each capped vial.
2. Submerge each vial in H_2O, cap, and record the full weight.

3. Calculate the empty headspace volume of each vial by subtracting the empty weight from the full weight (normally ~5.1 mL for 4-mL vials).
4. Autoclave the vials, lids, and septa in the absence of plastic. Then immediately vent each vial in a laminar flow bench for 1–2 h to remove any contaminating volatiles.
5. To each vial add a known volume of medium using a 5-mL automatic pipet. Once the medium is solidified, ventilate the vials for a further 10 min (*see* **Note 34**).
6. Add 75 µL of 5% (v/v) DMSO to each vial, cap, and record the weight.
7. Add five alginate-encapsulated meristems or 10 nonencapsulated meristems to each vial, ensuring the bead or tissue is in contact with the medium and DMSO layer. Seal the vials, note the time, and record the weight. Also incorporate control vials (*see* **Note 35**).
8. Place vials in culture room under normal temperature and light conditions.
9. Calculate the headspace volume (**Eq. 1**; *see* **Note 36**):

$$\text{Headspace vol.} = \text{vial vol.} - (\text{medium vol.} + \text{DMSO vol.} + \text{plant tissue vol.}) \quad (1)$$

   where: vol. = volume (L) and DMSO = dimethylsulfoxide.
10. Turn on the GC, turn on the flow of carrier gas, and set the temperature for the injector, column, and detector. Leave to equilibrate overnight.
11. On the day of headspace sampling (*see* **Note 37**), turn detector on and equilibrate for 30 min.
12. Inject 1 mL of standard into the GC and calibrate the integrator. Repeat at least three times (*see* **Note 38**).
13. Flush the syringe in the flow bench, take a 1-mL sample from a vial, and record the time (*see* **Note 39**).
14. Once all the vials have been sampled, open the vials and allow them to vent for 10 min.
15. If required, reseal each vial and record the time. Repeat **steps 11–15** as needed (*see* **Note 40**).
16. Calculate volatile production (**Eq. 2**):

$$r_{\text{volatile}}\ (\text{mol.s.g}^{-1}) = \frac{(\text{s conc} \times \text{s vol}) - (\text{b conc} \times \text{b vol})}{\left(22.4 \times \frac{\text{T}}{273.15}\right) \times 10^6} \times \frac{1}{t_2 - t_1} \times \frac{1}{\text{FW}} \quad (2)$$

   where: r_{volatile} = rate of volatile production; s conc = concentration in a 1-mL sample (µL/L); b conc = blank concentration in a 1-mL sample (µL/L); s vol = headspace volume (L); b vol = blank headspace volume (L); $t_2 - t_1$ = time between sealing and removal of a 1-mL sample(s); FW = sample tissue weight (g); T = temperature during sampling (K); 22.4 = L of space occupied by 1 mol of gas at 273.15 K and 1 atm; 10^6 = conversion factor from L to µL.

4. Notes

1. Tissue culture growth optimization must precede cryopreservation applications.
2. Recovery medium composition is species specific and may require the incorporation of growth regulators for shoot regeneration.

3. Cryoprotectants and pregrowth additives must be of high purity; spectroscopically pure DMSO is recommended.
4. Cryoprotectants and vitrification solutions are usually prepared in standard liquid culture medium; it is advisable to check medium pH after incorporating the additives.
5. Where possible, cryoprotectant mixtures should be filter sterilized, this may be technically difficult for high-viscosity solutions for which autoclaving may be the only practical option.
6. Concentration, duration, and temperature (ambient or on ice) of exposure to PVS2, sugars, and other cryoprotectants is genotype specific, this range is only a guide.
7. Uniform bead size is critical for reproducible bead dehydration and desiccation so it is important to use an alginate dispenser of a constant size. Adjusting the pipet tip diameter with a hot scalpel can accommodate larger structures. The authors' use Fisherbrand (Fisher Scientific, Loughborough, UK) 5 mL plastic pastettes. However, any pastette of similar type will suffice, but it is important to standardize the pastette used, as different manufacturers products have different apertures. If undertaking technology transfer with other laboratories ensure that an identical or equivalent type of Pastette is used. Size difference in beads will lead to nonuniform desiccation profiles on air and silica gel drying.
8. Prepare calcium-free standard liquid medium (Ca^{2+} salts of vitamins accepted) and add 3% (w/v) sodium alginate (SIGMA low-viscosity kelp alginate as the sodium salt). It is very difficult to solubilize alginate and this is best achieved by: (1) adding the alginate to liquid medium on a magnetic stirrer and then heating to boiling while agitating vigorously, dispense small amounts of alginate, step by step; (2) the alginate solution may be vigorously agitated by shaking and then autoclaved. Discard flocculated solutions.
9. Knowledge of cold-hardiness status is especially important for woody perennial species and seasonal screening for freeze tolerance is recommended to determine at which time in the growth cycle buds are best able to survive cryopreservation. Laboratory-induced cold hardiness is frequently used to enhance poststorage recovery. A suggested procedure for plants normally maintained at 20–25°C in vitro is cold acclimation for 7–10 d using 8 h days at 15–22°C and 16 h nights at –1 to 4°C. If specialist growth room facilities are not available for cold hardening it is possible to improvise and use manual transfers from a refrigerator set at the lowest setting to a standard growth room.
10. A shorter isothermal (constant temperature) program can be used when measuring C1 to C3 compounds. Gradient separation of C1 to C6 compounds requires 25–30 min (**Fig. 2**), whereas isothermal separation of C1 to C3 compounds requires 2–10 min (**Fig. 3**). A temperature of 60°C is suggested for isothermal separation.
11. If a glass syringe is unavailable, a plastic syringe can be used as an alternative as long as samples are injected into the GC immediately to minimize syringe compound adsorption.

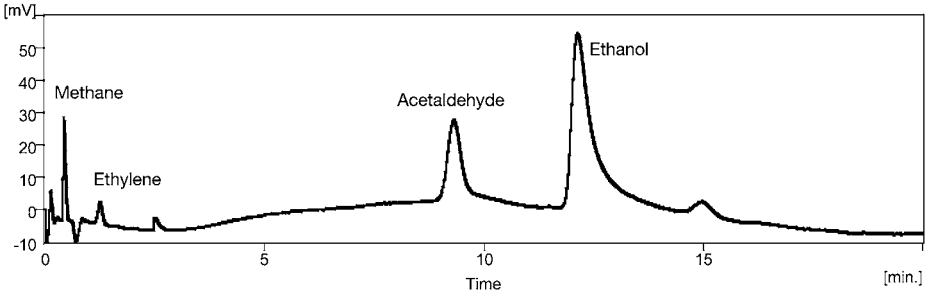

Fig. 2. Gradient separation of headspace volatiles from *Ribes sanguineum* (cv King Edward VII) shoot tips on Ribes (RIB) medium containing 0.75 *M* sucrose.

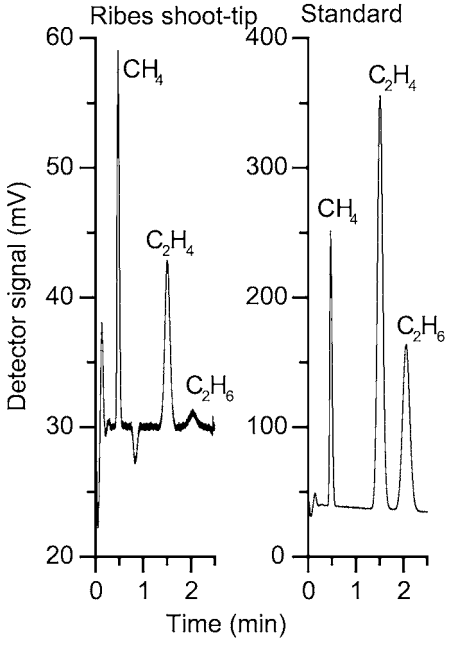

Fig. 3. Isothermal separation of headspace volatiles from *Ribes ciliatum* shoot tips and gas standard (15 µL/L for methane and ethane, 30 µL/L for ethylene).

12. To determine volatile production during each phase of cryopreservation, vials will need to contain the corresponding medium.
13. The duration of exposure and loading of sucrose and other compounds (e.g., polyols, mannitol, proline, abscisic acid) used as a pregrowth additive or to simulate cold acclimation will require genotype-specific optimization for concentration.

14. Tissues harvested *ex situ* may exhibit different natural cold-hardiness responses to freezing. For cold acclimation in vitro it will be necessary to optimize the cold-hardening regimes on a species and sometimes genotype basis.
15. It is important to standardize and optimize shoot tip and nodal cutting size.
16. Shoot tips rapidly dehydrate after dissection and they should be transferred to a suitable medium immediately after removal. Ensure that this has the appropriate osmotic balance relative to the pregrowth medium from which the shoots were originally removed.
17. Newly dissected tissues are frequently sensitive to wounding, which is exacerbated by freezing. A short period of pregrowth (e.g., 16–48 h) in the presence of chemical additives such as (at 1–5% [v/v]) DMSO can significantly enhance recovery. Dehydrating pregrowth treatments applied as osmotica do not provide adequate protection against cryogenic treatments and additional cryoprotection is required. Comparing the efficacy of several treatments leads to the development of a pregrowth strategy. Pregrowth additives are incorporated into solid media or in filter papers moistened with additive-containing liquid media. The most commonly used osmotic pregrowth additives are, sorbitol, mannitol, and sucrose.
18. Modern programmable freezers consist of a freezing chamber cooled by liquid nitrogen. With the use of temperature probes (connected to the sample and the chamber) and precise computer programming it is possible to investigate a wide range of cooling/freezing parameters. With an appropriate output device (e.g., chart recorder/PC) the temperature at which extracellular ice is formed (latent heat of crystallization) may be determined with reasonable accuracy. This event, termed nucleation, can mark the onset of intracellular dehydration and it can be an important factor in developing a controlled cooling method for shoot tips. Extracellular ice formation causes a vapor pressure deficit, which is compensated by the movement of intracellular water to the outside of the cell. The effect is "cryoprotective" as the amount of intracellular water available for ice formation is reduced. In the absence of external intervention, it is possible for extracellular ice nucleation to occur randomly; however, this may be problematic and lead to variable freezing responses. The control of nucleation is thus an important consideration in developing slow-freezing methods for shoot tips. Some programmable freezers are fitted with a device that initiates nucleation by mechanically agitating the cryogenic samples. It is also possible to induce nucleation by touching the outside of the tube with a liquid nitrogen-chilled instrument.
19. It is important that vials are plunged immediately into liquid nitrogen after they are removed from the programmable freezer. Rewarming of the samples during this transition is detrimental to the tissues. For convenience a small Dewar (e.g., of 1-L capacity) can be located near the programmable freezer and used to transport vials to the long-term storage Dewar site.
20. Nodal segment stem size may be difficult to standardize and optimization is required.
21. Determination of water loss (using controlled oven drying) aids in the development of cryoprotective–dehydration protocols. However, it is important to use an

appropriate oven temperature (e.g., 105°C) and duration time for accurate moisture-content determinations. Moisture-content status should be accurately determined on a fresh and dry weight basis as appropriate. In humid environments, transfers to and from the drying ovens should be undertaken over activated silica gel desiccators.

22. The mixture will be extremely viscous and takes some time to completely dissolve in solution. Gradual addition of the cryoprotectants during vigorous agitation on a magnetic stirrer is recommended.
23. To reduce osmotic shock, thawed tissues may be sequentially transferred to a range of media containing decreasing concentrations of a nontoxic osmoticum such as sucrose.
24. Shoot tips may not survive direct exposure to 100% PVS2 and stepwise addition of PVS2 in an increasing concentration series is necessary. Following vitrification and exposure to liquid nitrogen the shoots are initially recovered in an "unloading" solution (1.2 *M* sucrose). This prevents osmotic shock while replacing the toxic vitrification solution with a less damaging sucrose solution.
25. Gradually add increasing concentrations of PVS2 solution, e.g., in a range of 50–100% (v/v), exposure duration is short (5–30 min), and requires species optimization. Adding chilled solutions on ice may reduce the toxic effects of vitrification cocktails. It may be possible to expose tissues to higher concentrations of PVS2 if applied at 0°C.
26. This is a critical point (*see also* **Note 21**) as it is essential that the beads desiccate uniformly and reproducibly between different cryopreservation experiments. Desiccation rates can be determined by assessing the changes in moisture content of a batch of empty (do not include meristems) beads before and after 4 h of desiccation. This can be determined from bead fresh and dry weights as follows:

$$\text{Residual moisture (\% fresh weight)} = \frac{\text{fresh weight (g)} - \text{dry weight (g)}}{\text{fresh weight (g)}} \times 100$$

27. New desiccation profiles should be determined when working in different laminar flow benches and/or laboratories to ensure the bead residual water content is optimum after desiccation. Note that alginate beads become extremely hard when air-dehydrated, and are sometimes difficult to manipulate. It is important that bead dry weights are determined at the correct temperature (105°C) as lower temperatures will underestimate their water content.
28. Failure to take note of environmental quality control parameters will result in nonuniform bead desiccation.
29. Another option in high RH environments is to desiccate beads over activated silica gel.
30. Encapsulated shoot tips may be rewarmed at ambient temperatures and placed on culture medium. Beads rehydrate within 1 h of placing on solid medium and can be rehydrated by placing in liquid culture medium for ca. 30 min following rewarming and before placing on recovery medium.
31. Regenerating shoots usually grow out of the bead, although this is not the case for all species. Removal from the alginate may be necessary for regeneration to proceed.

32. This is usually the first postfreeze recovery response.
33. Shoot-tip cryopreservation is successful if whole plants are regenerated from the cryopreserved meristem. Recovery proceeds via several morphological patterns of development. Leaf expansion, callus, and root formation indicate viability but not regeneration, which should proceed via an original meristem (primary or axillary) and not an indirect adventitious route (e.g., organogenesis from a callus). This criterion is important to ensure the genetic stability of plants regenerated from cryopreserved tissues. Quantitatively acceptable levels of recovery are of obvious importance. In the first stages of method development, survival levels approaching 50% of the total number of shoot tips frozen may be considered encouraging. The application of the technique on a reproducible and routine basis demands high levels of regeneration (e.g., approx 80% of the total number of shoots frozen).
34. For 4-mL vials, a medium volume of 3.5 mL is recommended as this allows sufficient space for five alginate-encapsulated meristems; volume will need modification for meristem size. A large headspace volume will make volatile detection more difficult.
35. Control vials should be prepared with medium alone, medium + DMSO, and tissue without DMSO. This will measure background volatiles in the laboratory (medium alone), medium methane production (difference between medium ± DMSO), tissue methane production not produced by the reaction between DMSO, and the hydroxyl radical (difference between tissue and medium-only vials without DMSO). All vials should be sealed at the same time to prevent background differences in volatiles.
36. It is often difficult to accurately measure the volume of small pieces of plant tissues. For nonencapsulated meristems, determine the average mass of a meristem and use this number to estimate the collective meristem mass in each vial (assume 1 g of tissue equates to a volume of 1 mL [density of water]). For encapsulated tissues, weigh the vial before and after the addition of beads. Use this weight to estimate bead volume, again assuming 1 g = 1 mL.
37. The sealing time before headspace sampling will depend on the tissue mass. For some species, the meristems will need sealing for up to 7 d to allow the volatiles to accumulate to detectable concentrations. It is equally important not to seal the meristems for too long, as this may induce anaerobiosis (detected as ethanol and acetaldehyde peaks during gradient separation; **Fig. 2**).
38. Blocked needles and leaky syringes cause considerable error. To check the syringe is gas tight, fill it with air, block the needle outlet by pushing it gently into a rubber bung or by closing the needle valve, submerge the syringe under H_2O, and gently press the syringe plunger. If air bubbles escape from any part of the syringe other than the needle, then the syringe is no longer gas tight. A blocked needle often occurs if the GC septum is too tight. A blockage can also occur while puncturing a vial septum. A blocked needle is difficult to detect during injection if the syringe plunger does not move freely in the barrel. To check if the needle is blocked, fill the syringe with air, submerge the needle tip under water, and gently press the syringe plunger. The absence of air bubbles indicates a needle blockage.

39. If the sample volume to be withdrawn is greater than 10% of the headspace volume, a negative pressure will develop inside the vial and syringe needle, which upon withdrawal of the needle from the vial, will cause air to move uncontrollably into the syringe diluting the sample. This problem is solved by closing the valve on the syringe needle prior to withdrawal from the vial, or by adding a known volume of sterile air to the vial before sample withdrawal (added volume has to be included in headspace volume calculations). A disadvantage of adding sterile air is that the sample will be diluted, which may make peak detection more difficult. Solid phase microextraction provides another viable sampling option.
40. After a number of injections high molecular weight compounds may accumulate in the column and cause a noisy baseline. These compounds can be removed by increasing the column temperature to 150–170°C for 1–4 h. It is recommended that this column cleaning procedure is routinely undertaken before and after each experiment.

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Cryopreservation of Desiccation-Tolerant Seeds

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Summary

The cryopreservation of desiccation-tolerant seeds depends on two key steps: specimen dehydration in an environment that ensures the attainment of water contents below the high-moisture freezing limit; and transfer and maintenance at a subzero temperature that may be optimized in relation to the seed-lot moisture content and species. Temperatures about 70°C below the glass transition temperature (Tg–70) or lower are recommended for seed storage. For fully desiccation-tolerant (type I) seeds, cryopreservation success tends to be independent of cooling regime and subzero (°C) temperature treatment when the seeds are dry. However, to maximize recovery of partially desiccation-tolerant (type II) seeds there can be a requirement for slow cooling, avoidance of storage at certain subzero temperatures, and controlled imbibition following cryopreservation, particularly for endospermic seeds that are high in lipid.

Key Words: Seed storage; longevity; glassy state; freezing stress; imbibitional injury.

1. Introduction

The impetus for the *ex situ* conservation of seeds has gathered pace with growing concerns about the risk that, *inter alia*, climate change, habitat fragmentation, and overexploitation pose for the extinction of wild-plant species ***(1)***. Seed storage is arguably the most effective and efficient method for the *ex situ* preservation of plant genetic resources, combining low storage costs with ease of seed distribution and regeneration of whole plants from genetically diverse material as each seed is genetically different ***(2)***. Recommendations for conventional seed bank storage are 3–7% seed moisture content (MC) (w/w) (fresh weight [FW] basis) and –18°C in hermetically sealed containers; such conditions probably guarantee the retention of high levels of viability over many decades, possibly centuries ***(3)***. Why then consider the use of cryopreservation

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or other nonconventional subzero storage temperatures? A combination of theoretical consideration and practical observation frame the answer. Although all seeds die eventually, seed deterioration is virtually stopped at cryopreservation temperatures ***(4–6)***. An earlier prediction suggested that seed longevity at cryogenic temperature could be about 175 times longer than at conventional seed bank temperature ***(5)***. More recently, cryogenic half-lives of up to c. 3400 yr are predicted for lettuce seeds at 6.5% MC, based on experiments running for longer than 10 yr ***(6)***. Comparable estimates or lettuce seed longevity at –18°C, based on the seed viability equation (P), are approx 46–70 yr, i.e., up to 74 times less than at cryogenic temperatures.

An additional complication concerning the low-temperature dry storage of seeds are observations of a subzero temperature-specific effect on seed germinability/viability ***(7)***. Such effects can be observed in dry seeds at approx 5–10% MC after as little as 1 wk at –20°C ***(8)*** and other, but not all, subzero storage temperatures. Such seeds are now defined as type II in storage category, to reflect their differential temperature sensitivity in relation to their level of desiccation tolerance within the moisture range of zone II of the water sorption isotherm ***(7)***. Such seeds tend to readily tolerate the removal of "free, unbound" water and survive drying to MCs below the unfrozen water content ***(9)***. The level of seed-desiccation tolerance is a function of development status, which is itself dependent on climate conditions. Cumulative developmental heat sum even across continental scales determines seed storage "type" at the point of natural seed shed ***(10,11)***.

Dehydration below the high-moisture freezing limit (HMFL) ***(4,5)***, which is analogous although not identical to the unfrozen water content, is the primary consideration and first step in the development of successful cryopreservation protocols for desiccation-tolerant seeds. Methods of screening seeds for desiccation tolerance vary from full characterisation of critical MCs for survival using thousands of seeds, e.g., approx 50 tropical species ***(12)***, to an all-or-nothing-type response to drying based on 100 seeds ***(13)***. For seeds that are partially desiccation tolerant, optimum MCs for cryopreservation are often approx 10% MC, equivalent to an ambient RH of about 65–75%. Such seeds are not likely to be in the glassy state at room temperature ***(3,6,14–16)*** and subsequent cooling rate to and warming rate from liquid nitrogen temperature could be critical for survival when moisture levels are close to the HMFL. Also, "excessive" drying may result in reduced viability before cooling. In contrast, seeds that are fully desiccation tolerant and have been dried to low (<20%) relative humidity (RH) general tolerate direct transfer to liquid nitrogen, i.e., uncontrolled cooling. Exceptions could be some lipid-rich seeds that appear to benefit from slow cooling; however, the majority of such species do not require such treatment.

Finally, the rehydration method could be a major factor in the ultimate success of the cryopreservation treatment.

Interest in cryopreservation studies has increased significantly ***(17)*** since two earlier reviews on the cryopreservation of seeds ***(4,5)***. It was suggested previously that candidate species for dry-seed cryopreservation were those with inherently short-lived seeds and endangered species with critically small population sizes ***(5)***. A review of species researched from 1995 to 2005 reveals considerable interest recently in the cryopreservation of more than 60 socioeconomically important species, particularly woody and horticultural species (**Table 1**). Considerable earlier literature, stretching back more than 100 yr, also reported cryopreservation of a broad range of economically important species, mainly crops; the reader is referred to earlier reviews as an access point ***(4,5)***. Seeds of most of these species are easily preserved at cryogenic temperatures and a basic protocol is re-emphasised here. Emphasis is given here to studies that have improved the handling of relatively difficult-to-store type II seeds ***(5)***. Such studies have started to form a knowledge base against which hypotheses are being tested on the mechanisms that may limit the performance of partially desiccation-tolerant seeds when stored at subzero (°C) temperatures.

2. Materials

1. Seed lots of named species from known provenance and of high quality, e.g., more than 90% germinable.
2. Drying chambers: large-scale drying room at approx 15–20% RH, plastic box or glass jar/cabinet with tightly-fitting lid, and clear plastic bags. In all instances, the drying chamber volume will depend on the quantity of seeds to be desiccated.
3. Saturated salt solution(s), indicator-type silica gel desiccant.
4. Dissecting instruments.
5. Stainless steel or aluminium weighing dishes with lids.
6. Water activity measuring unit and chamber, e.g., Rotronic, UK.
7. Five- to seven-place balance.
8. Fan-assisted ovens set to 103 and 130°C.
9. Seed storage containers: screw-cap polypropylene ampoules with gasket, borosilicate vials with neoprene insert and crimped aluminium caps, aluminium foil laminate bags (heat sealable), and polyolifin tubing are suitable. The most appropriate container to use will depend on the volume of the seed for storage. Endeavor to limit the container's cross-sectional area to ensure relatively uniform cooling and warming rates throughout it.
10. Protective wear for cryowork: cryogloves, goggles, apron, and long forceps.
11. Storage system: small-seed samples are easily accommodated in drawer-based inventory systems; larger-seed samples suit storage in canisters.
12. Programmable freezer.
13. A water bath preset to approx 40°C.

Table 1
Examples of Species With Value Category for Which Seed Cryopreservation Research has Been Progressed During 1995–2005 (Mainstream Journals)[a]

Value category	Species	Refs.
Crop	*Apium graveolens, Brassica* sp. (*B. cretica*, *B. drepanensis*, *B. incana*, *B. macrocarpa*, *B. montana, B. napus, B. oleracea* [two cvs]), *Capsicum annuum, Eruca vesicaria, Gossypium hirsutum* (five cvs), *Helianthus annuus, Solanum melongena*	***18–22***
Horticulture and ornamental	*Onopordum acanthium*, orchids (*Anacamptis morio, Bratonia*, *Dactylorhiza fuchsii, Dactylorhiza majalis, Dendrobium anosmum, Dendrobium candidum*, *Eulophia gonychila, Paphiopedilum rothschildianum*)	***19,20–25***
Native, threatened or endemic species	*Antirrhinum microphyllum, Antirrhinum majus, Arabidopsis thaliana, Acacia bivenosa, Anigozanthos manglesii, Banksia ashbyi, Cistus osbeckiifolius*, *Coronopus navasii*, *Diplotaxis virgata, Gypsophila struthium*, *Helianthemum polygonoides, Helianthemum squamatum, Iberis pectinata, Mesomelaena tetragona, Onobrychis peduncularis, Onopordum nervosum, Ononpordum nogalesii, Plantago lanceolata, Vella pseudocytisus*	***26–30***
Woody species: timber	*Cassia siamea, Cedrela fissilis, Pinus* sp. (*P. canariensis*, *P. halepensis. P. pinaster, P. pinea, P. nigra, P. uncinata, P. sylvestris*), *Populus deltoides, Salix* (two hybrids)	***31–35***
Woody species: fruits	*Citrus* sp. (*C. aurantifolia, C. aurantium, C. deliciosa, C. grandis, C.limon, C. madurensis, C. sinensis, C. reticulata, C. suhuiensis*)	***36–39***
Woody species: medicinal, spice, beverage, or industrial	*Azadirachta indica, Coffea* sp. (*C. arabica, C. brevipes, C. canephora, C. costatifructa, C. liberica, C. stenophylla, C. eugenioides, C. pseudozanguebariae, C. racemosa, C. sessiliflora*), *Elaeis guineensis*, *Piper* sp. (*P. mullesua, P. attenuatum, P. argyrophyllum, P. trichostachyon, P. galeatum, P. nigrum*), *Warburgia salutaris*	***40–49***

[a]Most species listed have desiccation-tolerant seeds; the few desiccation-sensitive seeded species listed were used for high-moisture freezing limit or unfrozen moisture content determinations.

14. Seed rehydration: plastic jar/box containing a pool of water or glycerol solution.
15. Seed germination: filter paper/paper towels or 1% (w/v) agar-solidified water or washed sand, Petri dishes or plastic or metal trays.
16. 1% (w/v) triphenyl tetrazolium chloride (TTC) in phosphate buffer.
17. Incubator(s) with temperature and lighting control.

3. Methods

If involved directly in the collection of seeds, permission from the appropriate land-owner should be sought. The conservation authorities should also be consulted when dealing with threatened species or species that are listed under CITES (Convention on International Trade in Endangered Species). Also during fieldwork valuable data should be recorded on, *inter alia*, the harvest method, the number of individual plants sampled, and the ripeness of the seed lot.

3.1. Initial Determinations in the Laboratory

Assessments should be made as soon as practically possible after receipt of seed lot from reputable supplier or arrival of the seed from the collection site. This is particularly important if handling moist seeds.

3.1.1. Seed Moisture Content

1. Determine initial moisture content (MC_i) of cleaned seed sample by weighing seeds, in metal dishes, before and after drying in fan-assisted oven. For oily seeds, use 103°C for 17 h; for nonoily seeds use 103°C as above or 130°C for 2 h. Allow seed and containers to cool over silica gel before reweighing.
   a. For large seeds, cut seeds in half or quarters, using one seed per dish and $n \geq 25$.
   b. For small seeds, prepare at least five (preferable ≥ 10) subsamples of seeds randomly drawn from the seed lot. The quantity of seeds will ultimately depend on the total availability of seed for the experimentation; seeds could be in short supply if assessing responses of threatened or endemic species.
2. Express MC on a FW basis, as follows: %MC = ([seed FW – seed DW]/seed DW) × 100. Alternatively, express moisture as water content in units of g H_2O g DW. The water status of the seed can also be determined nondestructively in the chamber of a water activity measuring unit allowing the seeds to equilibrate for about 30 min.

*3.1.2. Seed Germination (*see ***Note 1****)*

Determine the initial germination of seeds using a prescribed test condition in relation to the provenance of the seeds. Conditions may involve constant or an alternating temperature regime. When alternated, the shift is often 10–15°C and occurs on an approx 12 h cycle. When light is required, it is applied during the warm temperature phase.

3.1.2.1. Moist Seeds

1. For moist seeds, place on top of moistened (not soaking wet) filter paper/paper towel or agar water in Petri dishes or other suitable transparent container. Alternatively, half bury in clean, washed sand.
2. Score seeds for germination regularly; the interval may vary considerably, from more than once a day to once a week, depending on the vigor of the seed lot and environmental conditions used.

3.1.2.2. Dry Seeds

1. For dry seeds (approx < 30% RH), avoid rapid rehydration by soaking in water, as seeds of some species are sensitive to imbibitional injury; rather, place seeds above water at room temperature (≥ 25°C) for approx 24 h before sowing for germination.
2. Score germination as radicle emergence and/or seedling emergence, if assessment of "normal" seedling growth is required.

*3.1.3. Seed Viability (*see ***Notes 2*** *and* ***3****)*

As an alternative to, or in combination with, germination seed viability can be assessed using tetrazolium staining.

1. Prepare 1% (w/v) TTC in phosphate buffer as follows: dissolve 3.631 g KH_2PO_4 in 400 mL distilled water, dissolve 7.126 g $Na_2HPO_4 \cdot 2H_20$ in 600 mL of distilled water, mix the two solutions; dissolve 10 g of 2,3,5-triphenyl tetrazolium chloride in 1000 mL of the buffer solution. **Note**: the solution is light sensitive and should be stored cool in the dark.
2. Rehydrate dry seeds over water or on agar water at approx 25°C for approx 24 h (step not needed if seeds are already moist).
3. Penetrate covering seed structures or surgically remove the embryo to allow permeation of the stain.
4. Soak seed/embryo individually in a small volume (approx 2 mL) of TTC for 24–48 h in the dark at approx 30°C.
5. Wash seed/embryo several times in distilled water.
6. Evaluate immediately after washing or following brief storage of stained material on moistened filter paper at 3–5°C. The intensity and topography of the red-staining pattern on the embryo tissues, i.e., embryonic axis and cotyledon. No staining of the embryo axis indicates no viability.

3.2. Seed Drying

Seeds need to be dried (*see* **Note 4**; **Table 2**) to below the HMFL (*see* **Note 5**; **Table 3**) prior to cooling and storage at subzero temperature.

1. Preweigh a small sub lot of seed and include with the main seed lot as a moisture-content monitor.

Table 2
Constant Humidity Solutions and Predicted Seed Equilibrium Moisture Contents[a]

Salt (saturated solution) or % mass glycerol (g per 100 g solution)	RH (%)	Seed moisture content (%)		
		Pisum sativum	*Helianthus annuus*	*Sesamum indicum*
$LiCl{\cdot}H_2O$	11	4.9	3.4	2.7
$LiI{\cdot}3H_2O$	18	6.3	4.3	3.4
$CaCl_2{\cdot}6H_2O$	30	8.1	5.7	4.5
88% glycerol	37	9.3	6.5	5.2
$NaI{\cdot}2H_2O$	38	9.4	6.6	5.2
$Mg(NO_3)_2{\cdot}6H_2O$	53	11.5	8.1	6.5
72% glycerol	62	12.9	9.1	7.3
KI	69	14.0	9.9	8.0
NaCl	75	15.0	10.7	8.6
52% glycerol	80	16.0	11.5	9.2
$(NH_4){\cdot}2SO_4$	81	16.2	11.6	9.3
$BaCl_2{\cdot}2H_2O$	90	18.5	13.4	10.8
20% glycerol	96	21.2	15.5	12.5

[a]Fresh weight basis at 25°C for pea (1.5% lipid, dry weight basis), sunflower (33% lipid), and sesame (47.5% lipid). For primary data sources and limitations of the predictions *see* **refs. *5*** and ***51***.

Table 3
High Moisture Freezing Limit and Unfrozen Water Contents for a Selection of Fully Desiccation-Tolerant Seeds

Species	Oil content (% dry weight)	HMFL[a] ***(5)*** or unfrozen water content ***(39,43)*** (% fresh weight)	Ref.
Sesamum indicum	c. 52	9	***5***
Citrus aurantifolia	52	8	***39***
Coffea pseudozanguebariae	c. 35	12	***43***
Coffea sessiliflora	c. 21	15	***43***
Glycine max	18	14	***5***
Coffea racemosa	c. 14	19	***43***
Daucus carota	13	22	***5***
Hordeum vulgare	2	21	***5***
Triticum aestivum	2	27	***5***

[a]Signifies the moisture content above which the seed viability would be expected to fall significantly on transfer to liquid nitrogen. The values could vary with cooling/warming rates; generally, studies have used rapid cooling and rewarming.

HMFL, high-moisture freezing limit.

2. Assume that the monitoring seed lot has the same MC as the initial seed sample. This will only be the case if two conditions are met: (1) there is little delay to the experiment after the arrival of the seed lot, and (2) the seed lot has been held at a RH very similar to that determined for the seed on receipt (*see* **Subheading 3.1.1.**).
3. Prepare saturated salt solution of desired RH. Add excess solid to a small quantity of water. The ratio will vary with each particular salt (*see* **Note 6**).
4. Put seeds to desiccate as a monolayer to enable more uniform drying. Place seed, including separate monitor, in: (1) the dry room on a slated tray, (2) in the relative humidity cabinet above saturated salts or glycerol, or (3) in a plastic bag with approximately an equal weight of indicator silica gel.
5. Regularly weigh the monitor sample and calculate the current MC of the sample in relation to the target moisture content (TMC). The TMC is estimated using the following formula:

$$\text{weight of seed(g) at TMC} = (100 - Mc_i)/(100 - TMC) \times \text{initial seed weight(g)}$$

6. Remove seed from the drying environment when MC has been reduced to the desired level; the level below the HMFL that is optimal for subsequent cryopreservation can be species specific in relation to the desiccation tolerance of the seed lot (*see* **Note 7**).
7. Reassess germination level/stainability of seed. If test is negative, seed may be sensitive to drying below a critical level that is dependent on a combination of seed lot and species characteristics ***(7,10)***. In addition to the risks of imbibition damage to dry seeds, water-uptake characteristics may have altered as a result of drying. Consequently, careful monitoring in the subsequent germination test may be needed (*see* **Note 8**).
8. Either count the number of dried seed for storage or take the total weight of sample and 100 seed weight and calculate the total number of seed in sample.

3.3. Seed Cooling and Storage

Always wear appropriate protective equipment (goggles, gloves, apron, long metal forceps) for the transfer of samples into and from liquid nitrogen. Storage in the vapor phase is more convenient if seed samples are to be removed on a frequent basis. Storage in the liquid phase does, however, reduce the possibility of inadvertent warming, but extreme care must be exercised when removing samples from the liquid; defective containers that allow the penetration of liquid may explode during rewarming as the liquid expands to become a gas within a confined space.

1. Place small quantities (i.e., grams) of seeds dried below the HMFL in appropriate containers (e.g., polypropylene cryovials, polyolifin tubing), close, and then cool by transferring to the liquid or vapor phase of liquid nitrogen. Such cooling tends not to affect germination adversely except in a limited number of species (*see* **Note 9**).

Alternatively, slower, controlled-cooling rates of approx 10°C/min to –50°C may be appropriate for some seed lots/species prior to transfer to liquid nitrogen (*see* **Note 10**).

Other subzero storage temperatures may be used, but the level of success will be dependent on the seed MC (*see* **Note 11**).

2. Store for at least 24 h (*see* **Note 11**).

3.4. Seed Warming, Rehydration, and Regrowth (see *Notes 12 and 13)*

1. To thaw, warm containers in a water bath at approx 40°C, particularly if seeds are close to their HMFL; ambient laboratory conditions can be used if the seed is drier.
2. After thawing allow seed to equilibrate to room temperature (≥25°C) before sowing for germination or transferring to the viability test (*see* **Subheadings 3.1.1.** and **3.1.2.**).
3. Assume the need to run the germination test for longer than the initial test as both drying and cooling/rewarming may have altered the properties of the seed, i.e., changed the germination or vigor of the seeds.

4. Notes

1. Conditions for germination testing are species specific in relation to the environment from which they were collected. In addition, the level of germination in seeds of a species can vary throughout the season of harvest and between harvest years. Such variability is primarily a function of the inherent level of seed dormancy in the seed lot. The dormancy can be removed and/or germination stimulated by the application of various temperature, light, and chemical stimulants, details of which are beyond the scope of this chapter. Reference should be made to a number of online information sources for science specialists and/or horticulturalists, including: Kew's Seed Information Database (http://www.kew.org/data/sid/sidsearch.html), International Seed Testing Association (http://www.seedtest.org/en/home.html), and Backyard Gardener (http://www.backyardgardener.com/tm.html).
2. TTC is a mild irritant and gloves should be worn during its preparation, which should take place under a fume hood. Store the prepared solution in the dark as it is light sensitive; ultraviolet light stimulates a slow reduction of the salt. Also store cool, but allow to warm up to room temperature before use to avoid any likelihood of chilling injury, especially in tropical seeds. The prepared solution should remain effective over a few weeks. Dissecting instruments are required to facilitate the penetration of the stain into the seed. Use Petri dishes with individual wells. Wrap the dishes in aluminium foil as the development of the staining pattern must take place under dark conditions.
3. When dealing with the cryopreservation of new species, some empirical studies may be required to develop optimum viability test conditions. The assessment of tetrazolium staining can be subjective and the determination of the correlation between this test, which reflects dehydrogenase enzyme activity, and the germination test is recommended for each species ***(50)***.

4. Manipulation of seeds to specific moisture levels can be achieved using saturated salt solutions and glycerol solutions (**Table 2**). For drying to less than 11% RH, freshly regenerated silica gel can be used. At any one temperature, the final MC achieved at each RH is a function of seed lipid content (LC). Note that the final or TMC can be interpolated from **Table 2** for seeds for which the lipid content is known but falls between the values given. After drying, the MC of the seed lot should be confirmed before freezing using the method given in **Subheading 3.1.1.** Alternatively, nondestructive determination of water status, specifically equilibrium RH or water activity, can be performed with a Rotronic water activity device (http://www.rotronic.co.uk). Accurate determinations of eRH by this method take approx 30 min. As RH is temperature specific, record the operating temperature.
5. The HMFL varies with species, being around 10% and coincides with approx 75–90% RH (approx –40 to –15 MPa) (**Table 3**). In the case of four *Citrus* sp., unfrozen water content (WC_u) is remarkably consistent with those at which seed tolerance to liquid nitrogen (LN) exposure is maximal ***(39)*** and for 13 species is inversely related to seed C; thus, HMFL = 23.1 – 0.21LC ***(5)***. Similarly, the WC_u of 23 species (including four *Citrus* and seven *Coffea* sp.) is inversely related to lipid content, thus, WC_u = 23.4 – 0.28LC ***(39)***. **Note**: in both instances, moisture/water and oil/lipid are on %FW and DW bases, respectively.
6. Gently boil the solution to ensure maximum solubility and transfer to equilibration/ drying chamber. To be effective, the saturated solution should always contain a large quantity of undissolved salt. Alternatively, dilute a pure solution of glycerol to produce the desired environmental RH. Examples of the glycerol concentrations and salts used to generate a particular RH are given in **Table 2**. The greater the differential between the MC_i and the drying environment RH, the faster the drying rate. Rates and final MCs will be also temperature and seed oil content dependent (**Table 2**). To reduce the likelihood of ice formation during cooling, seeds should be equilibrated to ≤80% RH, i.e., to MCs of <9, <12, and <16% for oily, moderately oily, and nonoily seeds, respectively (**Table 2**).
7. The optimum MC for seed cryopreservation can vary from about 7–14%, depending on species and the seed oil content (**Table 4**). In many species the optimum is around 10% MC, e.g., in *Citrus* (four species; ***37***), *Azadirachta indica **(40)***, *Piper* (six species; ***48***), and *Warburghia salutaris **(49)***. For *Piper* sp. it is suggested that this is equivalent to approx 65 % RH (prestorage). For four *Citrus* species, the preferred drying level is approx 75% RH ***(39)***. At MCs just above this optimum, i.e., close to the HMFL, small quantities of ice could form during cooling/warming. This risk seems to be higher in lipid-rich seeds; lipid thermal transitions possibly enable coalescence of small, benign ice crystals into larger pernicious ones (*see* **ref.** ***43*** for discussion). Such an effect might be observed in both type I and II seeds.
8. Drying of most type I seeds below this optimum MC for cryopreservation usually has little or no adverse effect on seed germination and vigor. Indeed, in *Dactylorhiza fuchsii* seed drying to low RH improved cryopreservation ***(31)***. However, drying *Dendrobium candidum **(25)*** and *Plantago cordata **(30)*** seeds to MCs less than 12% and approx 5%, respectively, slowed subsequent germination,

Table 4
Examples of Optimum Equilibrium Seed Water Status at 25°C[a]

Species	Oil content (% dry weight)	Optimum moisture content (% fresh weight)	RH (%)	Ref.
Arachis hypogaea (peanut)	45	c.7	c.57	***5***
Citrus aurantifolia (lime)	52	9	74	***39***
Azadirachta indica (neem)	c.40	c. 9	ND	***40***
Coffea costatifructa (wild coffee)	NA	16	ND	***45***
Glycine max (soybean)	18	c.10	58	***5***
Guizotia abyssinica (niger)	35	c.11	77	***5***
Helianthus annuum (sunflower)	33	c.9	c. 61	***5***
Piper sp. (pepper; six sp.)	NA	10-14	65	***48***
Sesamum indicum (sesame)	c.48	c.9	c. 78	***5***

[a]For subsequent safe exposure to liquid nitrogen temperatures (cooling rate of approx 200°C/min) for fully desiccation-tolerant seeds.

but this was not exacerbated by cryo. Also, some cotton seeds may become more difficult to germinate after drying to approx 5% MC ***(18)***, possibly as a result of changes in hardness of the seed coat (i.e., physical dormancy induction). As with some legumes that show this type of response, subsequent liquid nitrogen treatment of cotton seeds restores the ease of germination ***(18)***, probably by cracking the seed coat, i.e., to allow water in during the subsequent germination test. Also, in two Spanish endemics (*Cistus osbeckiifolius*, *Helianthemum polygonoides*) there can be a significant interaction between cryopreservation and dormancy breaking treatment ***(26)***. However, such physical stresses and strains can be too great in some dry seeds, in which case use slower cooling or warming rates (e.g., ~10°C/min). For type II seeds, drying to low MC can immediately reduce the germination level and have a negative effect on cryopreservation. Such variability in seed responses to drying means that it is essential to fully characterize the response when attempting seed cryopreservation on a species for the first time.

9. Plunging small containers of seed into liquid nitrogen produces a cooling rate of approx 200°C/min. Always chart the cooling (and rewarming) regimes using a copper constantan, or similarly, thermocouple and electronic thermometer. Such a cooling rate rarely affects most seeds even after repeated cooling and warming. However, *Capsicum annuum* and *Solanum melongena* seeds at approx 20% MC (probably close to their HMFL) respond negatively to repeated liquid nitrogen cycles ***(20)***. In contrast, seeds dried to approx 5% MC will be in the glassy state at room temperature ***(3,6,14–16)*** and have limited requirement for controlled cooling or rewarming. The exceptions appear to be some lipid-rich seeds, such as *Coffea* sp. ***(45,46)*** and *Onopordum* sp. ***(19)*** that benefit from the use of slow cooling rates

(*see* **Note 9**). Lipid-rich seeds of *Pinus pinea* (LN ***[33]***), *Agathis macrophylla* (–70°C ***[52]***), and *Anigozanthos manglesii* (LN ***[29]***) also show cryopreservation-related stress. The cause of such stress could relate to rate-dependent destabilization of lipid glasses ***(5)***. However, most oil seeds appear not to require slow cooling for high levels of survival ***(19,21,33,36–38)***.

10. Slower cooling rates can be achieved through the use of differing thicknesses of insulation materials (e.g., ampoules/vials inside layers of aluminium) and by altering the distance of the sample from the surface of the LN. It is also possible to improvise with various wide-neck Dewars of varying depth. Using a programmable freezer to control the cooling and warming rate is probably the best option; although such equipment can be expensive. *Coffea arabica* seeds can be cryopreserved by precooling at 1 to 2°C/min to –50°C ***(45,46)***.
11. Survival is also a function of storage time. Earlier studies on the cryopreservation of plant material tended to expose samples to LN for about 1 h only. However, there is good evidence to show that periods of 24 h or longer should be used ***(24,31)***. Cold stress in dry seeds can be observed also after various times at subzero temperatures other than those of LN. For example, at –20°C for 1 wk (*Hyphaene* palm ***[8]***, ~6 wk [*Agathis australis* ***(52)***], ~12 wk [*Carica papaya* ***(53)***, *Coffea arabica* ***(54)***, *Lannea microcarpa* ***(55)***, and oil palm ***(56)***] and 6–8 mo [*Azadirachta indica*, *Swietenia macrophylla*, *Khaya senegalensis* ***(57)***]). Similarly, cold stress is evident in seeds of three orchids (*Dactylorhiza fuchsii, D. majalis, Paphiopedilum rothschildianum*) after 1 mo at –30 and –50°C, but not at –20°C ***(20)*** and in *Carica papaya* after 1 d at –13°C ***(58)***. It is suggested that the temperature for long-term seed storage should be approx 70°C below the glass transition (Tg), i.e., Tg–70, or lower ***(7)***.
12. The rehydration method can also affect survival levels, particularly of oil seeds ***(59)***. Recovery after cryopreservation in oily *Coffea arabica* seeds is improved when either prehumidified for 24 h at a relatively high temperature (37°C) prior to transfer to germination conditions ***(42)*** or osmoconditioned for 6 wk in contact with a –1.25 MPa solution of polyethylene glycol ***(44)***. Such treatment increased the germination rate once the mild osmotic stress had been removed. Such priming treatments are often used to improve both germination speed, and uniformity in crop seeds and the subsequent application of cryopreservation to primed (and redried) *Apium graveolens* seeds had no adverse effect on germination rate ***(22)***. It is preferable not to immerse seeds in polyethylene glycol solution because of the risk of anoxia. For nine *Coffea* sp. a highly significant correlation was found between the percentage of unsaturated fatty acids and seedling recovery ***(43)***. In other words, higher levels of saturated fatty acids may hinder survival. Elevating seed moisture and using high temperatures approx 35°C prior to the germination test aids recovery in *Azadirachta indica* after –20°C storage ***(60)*** and *Coffea arabica* after cryopreservation ***(42,44)***, probably by facilitating gel-to-liquid crystalline-phase transitions in membranes.
13. There are various "other" factors that impinge on recovery level. Although few whole seeds of *Citrus suhuiensis* germinated after cryopreservation, the majority of isolated embryonic axes tolerated rapid drying and cryopreservation ***(36)***.

Similarly, embryo viability after seed cryopreservation was much higher compared with seed germination in *Coffea arabica*, indicating a negative influence of the presence of the endosperm ***(41,46)***. Using *Citrus aurantifolia* without testas (coats) approximately doubled recovery after desiccation to approx 5% MC and cryopreservation ***(38)***. The beneficial effects of these treatments and of osmoconditioning/priming (*see* **Note 11**) suggest treatments that enable easier growth of the embryo can be critical to the final level of recovery. It follows that germination testing periods after both drying and cryopreservation may need to be extended to give partially stressed (reduced vigor) seed the opportunity to regrow, e.g., 4 mo for *Coffea arabica* ***(46)***. Cryopreservation may not only reduce seed vigor but induce morphological abnormalities, e.g., for nine *Coffea* sp. total germination after cryopreservation was higher than the normal seedling development because of damage to the shoot apex ***(45)***. Finally, do not expect all seed lots of the same species to respond identically to cryopreservation; inter seed lot variability in response has been seen in *Coffea arabica* ***(41)*** and *Anigozanthos manglesii* ***(29)***.

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14

Cryopreservation of Fish Sperm

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Summary

This chapter provides detailed descriptions of the protocol used for fish sperm cryopreservation. The principles described can be applied to any species, but details are also given for individual fish species. Successful cryopreservation of fish spermatozoa depends on a range of factors including the collection of high quality sperm, equilibration conditions, choice of cryoprotectant medium, cooling/thawing regimes, and conditions for fertilization. Even though some general rules can be applied to any fish species, optimization of the protocol is needed for each individual species. Furthermore, because sperm derived from marine and freshwater fish differs markedly, the described protocol is mainly focused on the cryopreservation of sperm for freshwater fish because this poses the greater technical challenge.

Key Words: Fish sperm cryopreservation; freshwater species; carps; salmonids; sturgeon.

1. Introduction

The development of routine use of "artificial" fertilization in aquaculture has raised the requirement for storage of reproductive materials. The short period of time for which fish gametes remain in good condition after collection became a significant obstacle to hybridization between species of fish inhabiting different geographical locations, or having different spawning times. The asynchronous maturation of breeders invariably causes problems in aquaculture and has stimulated research to develop a method suitable for the prolonged storage of fish sperm. Several different approaches were initially tested including storage of fish sperm in medium saturated with different gases ***(1)***, preservation of sperm at temperatures above zero ***(2)***, as well as in the frozen state ***(3)*** and drying ***(4)***. However, to date, low-temperature preservation has proven to be the most effective approach, with the first successful cryopreservation of fish sperm reported by Blaxter in 1953 ***(3)***. The method has subsequently been applied

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widely and has become not only a routine tool in aquaculture for fish hybridization and selective breeding, but also an important tool in programs on biodiversity and the preservation of endangered species. Gamete banks of rare or almost extinct species are currently being created ***(5)*** with the objective of protecting endangered species. The technique has also found applications in research programs for maintaining laboratory animals and, in recent years, sperm of more than 200 species of fish have been successfully cryopreserved ***(6)***. However, despite the extensive number of studies that have been undertaken there is still ambiguity in the data reported in the literature, primarily because of poor standardization of methodology and data analysis.

Male reproductive cells derived from fish are significantly different than mammals', not least because the gametes of the vast majority of fish are fertilized externally, their spermatozoa need activation (fish spermatozoa remain immotile until they are expelled into water), and have a relatively short duration of motility. Fish inhabit almost all surface waters on Earth ranging from freshwater to hypersaline lakes and cold Arctic water to hot waters of the California desert ***(7)***. Because fish have evolved to live in these diverse environments, there are substantial differences in fish morphofunctional characteristics. Fish have had to develop adaptation mechanisms to survive in these diverse environmental conditions, and as a result spermatozoa from fish species demonstrate significant differences in their reactions to cryopreservation protocols. For example, there is a striking difference in post-thaw survival of reproductive cells of marine and freshwater species. Sperm of marine species were successfully cryopreserved ***(3)*** soon after the discovery of the first cryoprotectant, whereas the cryopreservation of freshwater fish gametes was more challenging and took longer to achieve ***(8–10)***.

Although there are many protocols available for low-temperature storage of sperm of freshwater fish ***(11,12)*** there is much work still to be done to improve this technology. In general, approx 40–90% of spermatozoa from freshwater species are usually damaged after cryopreservation, whereas only 10–20% of spermatozoa are damaged in marine species ***(11)***. The difference between species is presumably a result of evolution in their cellular properties developed under the pressure of their niche environment. In the author's experience, post-thaw survival of fish sperm is strongly predetermined by their sensitivity to osmotic changes in extracellular media. Freshwater species inhabit an environment of 0–50 mOsm, whereas marine fish live in a range of 600–1000 mOsm. Consequently, the osmolality of the seminal plasma differs between species, for example, Siberian sturgeon (*Acipenser baeri*) having semen of 20 to 60 mOsm, whereas most of freshwater or seawater teleost has semen osmolarity between 230 and 320 mOsm ***(13)***. The activation of freshwater sperm is because of a

decrease in extracellular osmolarity, and sperm originating from saltwater species is activated by an increase in external osmolarity ***(14)***. The sperm of marine species is not damaged by an environment with an increased osmolarity and it can easily tolerate the increased osmolarity it experiences during cryoprotectant treatment and cryopreservation. Sperm from freshwater fish are not naturally accustomed to an increased osmolarity, and thus are subjected to significant stress during equilibration with cryoprotectants and subsequent cryopreservation.

High variability in cryoresistance is reported not only between species of fish, but also between individual males ***(15)***. In this chapter, it is our intention to summarize the factors that, in our opinion, are important in achieving a successful protocol. It is unfortunately impossible to offer a single universal protocol for such a diverse kingdom as fish. Instead, examples of successful protocols used for cryopreservation of the most widely studied species of freshwater fish are given. To develop reliable protocols of cryopreservation for fish spermatozoa, individual fish and species-specific properties have to be taken into consideration. Important factors that can undermine the final quality are outlined and these should be taken into consideration when a new protocol is being developed for a previously unstudied species.

2. Materials

1. Gamete materials: sperm and a suspension of sperm cells prepared from testes. Fresh eggs collected immediately before fertilization.
2. Solutions.
   a. Extenders (solutions without cryoprotectant): the choice of appropriate extender depends on the species to be investigated (*see* **Note 1**).
   b. Freshly prepared cryoprotectant solutions. The most commonly used cryoprotectants for fish sperm cryopreservation are dimethyl sulfoxide (DMSO), ethylene glycol, methanol, ethanol, glycerol, and dimethylacetamide. The concentration usually varies between 5 and 12% (w/v). Sucrose, polyethyleneglycol, albumin, and hen egg yolk have also been used (*see* **Note 1**).
   c. Activating medium for fertilization: 25 m*M* Tris and 150 m*M* NaCl medium can be used for sturgeons and carp ***(12)***; 60 m*M* $NaHCO_3$, 20 m*M* glycine, 5 m*M* theophylline, 50 m*M* Tris-HCl (pH 9.0) is used for Salmonid fishes ***(16)***.
3. Containers for milt collection: vials, flasks, tissue culture flasks. The type and size of container depends on species and volume of collected sperm (*see* **Note 2**).
4. Container with ice.
5. Cryocontainers: cryovials and straws.
6. Cryostorage equipment.
7. Water bath.
8. Refrigeration systems:

a. Programmable variable-rate cooler.
b. Wide-necked Dewar (10–15 L) with liquid nitrogen. The neck diameter of 10–15 cm.
c. Refrigerated alcohol cold baths (for –20 and –40°C).
d. Polystyrene box with dry-ice.

9. Thermocouple and device for monitoring temperature changes.
10. Storage system: crystorage containers with appropriate storage racks and inventory system suitable for holding cryovials.
11. Microscope with a computer-assisted semen analysis system.
12. Petri dishes for fertilization.
13. Low temperature-resistant markers for glass.
14. Hemocytometer.

3. Methods

1. Select the appropriate breeders and prepare them, if necessary, for spawning (*see* **Note 3**).
2. Stimulation of maturation. Unless naturally spawning fish are used, artificial stimulation is required. In order to obtain good quality sperm, keep fish under optimal conditions and avoid fluctuations and/or changes of temperature after stimulation and during spermiation. Inject chorionic gonadotropin or solution of the pituitary gland. The dose depends on species, fish weight, degree of maturation, and the quality and type of injected hormones. For examples of dosage refer to **Table 1** (*see* **Note 4**). The injections are made intramuscularly in the cranial part of the body, or under the one of pectoral fins (*see* **Note 5**).
3. Collection of milt.
   a. Collect sperm from mature males into clean and dry flasks applying gentle abdominal massage. For small males it can be useful to sedate the fish (with 25 mg/L tricaine methanesulfate [MS-222]) and use a capillary tube. The collection of sperm from large fish such as sturgeon is performed on a specially designed table. The table has two surfaces under a 90° angle and a 10° slope with soft cover. A tube with flowing water is inserted into the fish's mouth, while a transparent polyethylene tube is placed into its genital orifice. This method of sperm collection helps to prevent sperm contamination and traumatization of the fish (*see* **Note 6**).
   b. Collection of sperm by sacrificing the fish. This approach is usually only necessary in the case of small aquarium fish or in some species where the collection of liquid sperm is not possible without extraction of testes (for example, Weather Loach *Misgurnus fossilis*). Anesthetize fish by placing them into a solution of 100 mg/L tricaine methanesulfate or 25–50 mg/L benzocaine. Extract testes from abdominal cavity and transfer them to a dry, clean dish.
4. Maintain the milt samples obtained at 4–5°C, or on ice in well-aerated flasks in a thin layer of sperm sample. It is preferably not to store sperm samples after collection, one should proceed immediately with cryopreservation (*see* **Notes 7** and **8**).

Table 1
Examples of Hormone Doses Used for Stimulation of Fish Spermiation

Species	Injected material	Dose
Sturgeon (*Acipenser baeri*)	Powder of sturgeon pituitary gland dissolved in water	2 mg/kg
Carp (*Cyprinus Carpio*)	Powder of carp pituitary gland dissolved in water	4 mg/kg
Loach (*Misgurnus fossilis*)	Chorionic hormone	100 U/fish

5. Inspection of cell quality under the microscope (*see* **Note 9**). Activate cell movement by addition of the appropriate activating medium. Fresh sperm can be activated by water from the aquarium tank after dilution not less than in 40 times followed by good, rapid mixing. Sperm with low motility rate and with low cell density should not be used for cryopreservation. For activation after cryopreservation, *see* **step 15** (*see* **Note 10**). If necessary, cell density can be established with the help of a hematocytometer.
6. Preparation of milt suspension if **step 2** was used. Prepare sperm suspension from extracted testes. Care must be taken to avoid milt contact with water, urine, blood, and feces to prevent activation and contamination of sperm. Testes are cut into small pieces and homogenized with the extender.
7. Dilute sperm with appropriate cryoprotectant (*see* **Notes 11** and **12**). Establish the appropriate ratio of dilution for each species (*see* **Note 13**). Cryoprotectant solutions that causes more than a 50% decline in sperm motility rate must not be used. Cryoprotectants should be added very slowly and gradually (*see* **Note 14**) with a constant mixing of sperm with the added solution. The concentration of cryoprotectant usually varies in the range between 5 and 12% (v/v).
8. Equilibration. Immediately after sperm collection, it is cooled down to 5°C and diluted with isothermal cryoprotectant medium. The duration of exposure to the cryoprotectant solution at 5°C is generally in the range of 20 to 60 min. However, for some species equilibration might not be necessary (*see* **Note 15**).
9. Load samples in 1–2 mL vials. The volume of the cryopreserved sample should not usually exceed 2 mL (*see* **Note 2**). The smaller the volume that is used, the easier it is to achieve the desired freezing regime within the sample. Leave at least one-fourth of ampoule volume free of sample (*see* **Note 16**).
10. Freezing.
    a. Freezing in vapor-phase liquid nitrogen (*see* **Note 17**). Place vials or straws above the liquid nitrogen horizontally on the rack at a predetermined height. The height of sample placement and the time of exposure at this height depend on sample volume, type of container, type of the Dewar, and temperature at that height. For example, samples of sturgeon sperm in 12% (v/v) DMSO placed in vials in 0.7- or 1.5-mL volumes are kept at 20-cm above the surface

of liquid nitrogen for 20 min ***(17)***. During this time sperm is cooled down to –20°C. After that samples are moved at 2-cm above the liquid nitrogen level for 5 min followed by plunging into liquid nitrogen.

Sperm of salmonid fish is cryopreserved in straws with volumes of 0.5 or 1.2 mL ***(16)***. Straws containing 0.5 and 1.2 mL are frozen at 1.5- and 2.5-cm above the level of liquid nitrogen (LN) respectively, with a holding time of 10 min ***(16)***.

b. Freezing in alcohol baths. Similar results can be obtained by freezing sperm in cold baths that are capable of maintaining a set temperature. Prepare two cold baths precooled to –20 and –40°C. Vials (0.7 or 1.5 mL) are placed in the bath at –20°C for 2.5 min and then are transferred to a bath at –40°C for 2.5 min. The samples are then plunged into liquid nitrogen.
c. Freezing in dry-ice. Place small aliquots (100–250 µL) of sperm diluted with cryoprotectant directly onto dry ice and allow them to cool for 4–5 min. Then transfer to cryovials and store in liquid nitrogen.
d. Controlled-rate cooling. The following protocol can be applied for sperm of different species. The first step from 0 to –15°C involves cooling at 1–5°C/min (1–2°C/min for Carp); second step from –15 to –70°C cooling at 15–20°C/min, and the third step plunging into liquid nitrogen (*see* **Note 18**).

11. Store in liquid nitrogen (*see* **Note 19**).
12. Thawing. Transfer the vials or straws from liquid nitrogen directly into a water bath at 40°C (*see* **Note 20**). Rotate the vials while they are immersed in a water bath until the appearance of the liquid phase. Then shake the vials in the air until no ice remains.

    For samples frozen as pellets, add them directly to eggs along with the activator, the temperature of which would be the same as river (or tank) water from which the fish originated. In the case of carp, the thawed sperm samples could be stored on ice and the fertility rate could be improved after some additional treatment (*see* **Note 21**).
13. "Rehabilitation" of sperm. Aerate sperm from carp for 5–15 min after thawing before fertilization (*see* **Note 21**).
14. Fertilization. Ensure that the eggs are ready for fertilization. Place sperm samples directly into a container with eggs together with activating medium. Use 42 m*M* $NaHCO_3$ or NaCl as an activating media (*see* **Note 10**). If available use a goose feather for mixing eggs if a large quantity is to be used.
15. Assessment of sperm quality after cryopreservation. Use microscope assessment as outlined in **step 5**, plus fertilization and hatching rate for assessment of sperm quality.
16. Keep detailed comprehensive records of the cryopreserved materials and protocol.

4. Notes

1. The choice of extender depends on the species from which the sperm is obtained (*see* **Table 2**).
2. Good results are usually obtained when fish sperm is frozen in granules, straws, and/or plastic vials of 0.05–2 mL volume. Larger volumes create difficulties in achieving uniformed freezing of the sample. Containers larger than 5 mL are not normally recommended for the cryopreservation of fish sperm.

Table 2
Example of Cryoprotectant Media for Some Species

Fish species	Medium ingredients	Quantity
Carp (*Cuprinus Carpio*) ***(18)***	NaCl	42 mg
	KCl	6 mg
	$CaCl_2 \cdot 6H_2O$	18 mg
	$MgSO_4 \cdot 7H_2O$	62 mg
	$NaHCO_3$	280 mg
	Sucrose	137 mg
	D-Mannitol	1.5 g
	Tris-oxymethyl-aminomethane basis	1.697 g
	Glutathione red	56 mg
	Polyvinyl alcohol	5 mg
	Hen egg yolk	12.0 mL
	HCl	Adjust pH 8.1
	H_2O	up to 100 mL
	Ethylene glycol	19.6 mL
Sturgeon fish ***(17)***	Tris-HCl buffer	0.05 *M*
	Egg yolk	20%
	DMSO	25% (after dilution 1:1 final concentration 12.5%)
Salmonid fish ***(16)***	NaCl	600 mg
	KCl	315 mg
	$CaCl_2 \cdot 2H_2O$	15 mg
	$MgSO_4 \cdot 7H_2O$	20 mg
	HEPES	470 mg
	H_2O	Up to 100 mL
	Methanol	10 mL
	Bovine serum albumin	1.5 g
	Sucrose	0.5 g
	Hen egg yolk	7 mL

3. The success of cryopreservation strongly depends on the initial quality of the sperm. Higher quality material can be obtained from matured breeders in the middle of a breeding season ***(19,20)***. The quality of sperm drops substantially by the end of the spawning period in trout, herring, and others species ***(21)***. The recommended motility rate of sperm that can be used for cryopreservation should ideally not be lower than 80%. For example, the sperm of sturgeon with a motility rate lower than 40% should not be used for cryopreservation. Sturgeon sperm with low motility and cell concentration is not recommended to be used for fertilization, even without cryopreservation ***(2)***. Better quality sperm can

usually be obtained in the morning, when natural spawning takes place for the majority of species.

4. A variety of hormones and doses may be employed (*see* **Table 1**).
5. The injecting is performed slowly and gentle rubbing to the site of injection is applied afterward to avoid injected hormone leaking out because of muscle constriction. It is optimal to inject the fish in the morning. After injection place the fish back into water at their optimal light and temperature regime. Spermiation normally occurs 12–43 h after injection, depending on species and spawning temperature.
6. It is of paramount importance to avoid sperm contamination with water, urine, and feces during fish stripping. As it was demonstrated in salmon (*Salmo salar*) ***(22)***, the collection of sperm by abdominal pressure causes urine contamination that can dilute milt by as much as 80% (v/v). This causes substantial variability of sperm osmolarity, a decrease of K^+ concentration, and motility. The contamination of sperm with up to 25% (v/v) urine causes the one-third decrease in motility after cryopreservation in comparison with uncontaminated samples. Other authors ***(23)*** also reported the decrease of survival in cryopreserved sperm of Atlantic salmon (*Salmo salar*) after mixing it with different amounts of urine. Therefore, it is recommended ***(24)*** that one wipes the abdominal part of a male thoroughly before collecting a sperm sample to clear any water and to expel urine by gentle abdominal pressure. It is more reliable to use catheters for sperm collection, this minimizes contamination. Feces in sperm also affects the motility rate. Clean, uncontaminated, sperm samples from the Walleye (*Stizostedion vitreum*) could be stored almost six times longer (11.7 d) than sperm contaminated with feces (2 d) ***(25)***. Contamination of sperm with blood also decreases the duration of possible storage, as well as the motility rate. Bacterial contamination has also been demonstrated, in our laboratories, to have a negative impact on the functional stability of cells. Antibiotic treatment helps significantly to extend the duration of hypothermal storage in Stellate sturgeon (*Acipenser stellatus*) ***(26)***. The risk of sperm contamination can be one of the reasons why cryopreservation is recommended straight after sample collection without any preliminary storage.
7. The container for holding sperm samples can also affect the quality of sperm. Tall and narrow containers must be avoided to minimize the possibility of hypoxic conditions developing in the holding container. The conditions for short-term storage/holding of sperm samples need to provide the best possible conditions for appropriate access of oxygen, and the use of wide-neck containers is optimal. Depending on sperm concentration the depth of sperm sample should be in a range of 5 to 15 mm. For example, sturgeon sperm placed into a fridge in closed vials, without access of oxygen, loses motility in 3 h. Samples kept in refrigerators in open humidifying containers, with a depth of less than 15 mm, preserve the sperm motility for as long as several days ***(26)***. Experiments with carp sperm also indicates that decrease of storage temperature and thickness of samples increase sperm survival (Kopeika, unpublished data). The layer of air above the sperm sample should be 7–10 times thicker than the layer of sperm. These conditions

are especially important when sperm is not going to be used immediately for cryopreservation.

8. In order to decrease the energy lost of spermatozoa during maintenance, the temperature of temporary storage needs to be approx 0–5°C. If ice is used for temporary storage, direct contact between ice and sample needs to be avoided. Sperm of sturgeon (*Acipenser guldenstadti*) stored at 1–4°C maintained fertilizing ability for 5–6 d ***(27)***, whereas when stored in ice fertilizing ability was maintained for up to 8 d ***(2)***. Prolonged storage at 1–4°C was also shown to be effective for many other species of fish ***(28)***. However, storage at temperatures of 1–4°C is not adequate for the long-term preservation of sperm.
9. Motility assessment. The percentage of motile sperm can be assessed by light microscopy. However, the operator requires experience to assess the motility rate accurately. Therefore, where available, a computer-assisted semen analysis system should be used wherever possible to standardize measurement of motility. This system not only allows the accurate estimation of sperm motility, but also a range of other valuable measurements for the assessment of sperm quality. Adequate assessment of sperm quality also depends on their appropriate dilution. It has been demonstrated for sperm of trout, carp ***(29)***, and Siberian sturgeon ***(30)*** that after low levels of dilution not all sperm were activated simultaneously and the process of activation can last from 15 s to 3 min after dilution. It is advised that in some cases two-step dilution should be used; first dilute sperm with nonactivating medium and then dilute sperm with activating medium. The degree of dilution needs be optimized for each species. As a general approach we recommend that 5 µL of sperm should be activated by 2 mL H_2O for "native" sperm, or activating medium for freeze–thawed sperm. If larger volumes of sperm need to be assessed, then two-step dilution is preferable ***(30)***. First, dilution with a 1:20 ratio in nonactivation solution of 400 m*M* sucrose, 20 m*M* Tris (pH 8.0) (sperm remains immotile); second, activation of sperm using 30 m*M* Tris (pH 8.0).
10. By the time sperm is thawed and ready to be used for fertilization they have gone through a range of stresses. Further treatments, such as activation of motility in pure water, can also affect functional activity of weak sperm cells post-thaw. Therefore, care has to be taken during handling of sperm after thawing and pure water should not be used as an activator for cryopreserved-thawed sperm during fertilization. Better activation will be attained in activation media that have higher osmolarity than pure water. However, the increase in osmolarity in the activating medium has to be within the range that is safe for the eggs. There are different media used for sperm activation (40 m*M* NaHCO3, or 60 m*M* NaCl + 50 m*M* Tris-HCl ***(12)***, Tris/NaCl/$CaCl_2$ 10/20/2 mmol/L, pH 8.5) ***(31)***. There is also some evidence that the addition of certain chemicals during, or immediately after, fertilization ***(32)*** with cryopreserved fish sperm can enhance the hatching rate.
11. Choice of extender. The osmolarity of the extender solution appears to be one of the most important factors in preparation of an appropriate extender. Because large interindividual differences have been demonstrated in fish semen osmotic responses, in order to maximize the outcome of cryopreservation the osmolarity

of extender should be adjusted on the individual bases if possible. Increased concentration of buffer or K^+ may be used to inhibit sperm motility in extenders for some species such as sturgeon.

12. Both permeable (glycerol, DMSO, ethylene glycol, dimethylacetamide, methanol) and nonpermeable (sucrose) cryoprotectants may be used in protocols to cryopreserve fish sperm. The absence of an ideal cryoprotectant, as well as the lack of a full understanding of their mechanisms in cell protection, make selection of a single cryoprotectant difficult for different species. However, the optimal cryoprotectant can be determined empirically. The concentration usually varies in the range between 5 and 12% (v/v). Better cell protection can be achieved on employing higher concentrations, but this has to be balanced with toxicity effects of the cryoprotectant. Addition of nonpenetrating agents, such as sucrose, is generally considered to be beneficial; however, direct mixing of fish sperm with cryoprotectants inevitably leads to the death of all cells ***(33)***. Clearly, extenders play an important role in sperm protection. It has also been demonstrated for salmon sperm that multicomponent salt media are much more effective for cryopreservation ***(16)***. For marine species, the first successful protocol described by Blaxter ***(3)***, utilized a mixture of seawater and distilled water with 12.5% (v/v) glycerol as a cryoprotectant. The authors used the carp medium detailed in **Table 1** with 8% (v/v) DMSO (final concentration) for freezing marine species.
13. The level of dilution of the cryoprotectant medium is equally important and it is species sensitive ***(16)***. High-density semen needs a greater level of dilution. It was found for sturgeon sperm that the optimal ratio was 1:1 sperm:medium (0.1 *M* Tris-HCl buffer, 10% [v/v] egg yolk, 25% [v/v] DMSO] ***(17)***; however, the ratio can vary between species and even individuals. It has also been demonstrated that the concentration of sperm cells in beluga (*Huso huso*) semen may vary between males in the range of 0.58 to 6.4 billion of cells/mL and in stellate (*Acipenser stellatus*) from 0.9 to 10.4 billion of cells/mL ***(2)***. Studies on the effect of cell concentration showed that increasing carp sperm concentration from 0.5–0.8 billion/mL to 5–8 billion/mL, or 11–16 billion/mL in freezing medium resulted in a significant reduction of the temperature of ice nucleation from -4.73 ± 0.23°C to -5.75 ± 0.56°C, and -7.16 ± 2.04°C, respectively ***(34)***. We believe that a standard dilution level cannot be applied for such a broad range of sperm concentrations. For salmonids, sturgeon, carp and tilapia, milt is generally diluted between 1- and 20-fold.
14. The osmotic potential of media is inevitably substantially increased by the addition of cryoprotectant. One option that may be employed to avoid dramatic osmotic shock is the addition of egg yolk, which we have found to improve sperm survival ***(17)***. An additional factor that may be manipulated to minimize osmotic shock is the way dilutions are performed. We have observed that rapid mixing of sperm with cryoprotectant medium can damage substantive amount of cells or affect their motility. For that reason slow addition of cryoprotectant is essential for obtaining optimal results. The best sperm survival is obtained when cooled cryoprotectant medium is slowly added to sperm of equal temperature by pouring it to the wall of a slowly swirled container.

15. Equilibration time period is another important factor. Spermatozoa of salmon are relatively small and readily permeable to cryoprotectants ***(16)***. Therefore, the exposure time of sperm to cryoprotectants can be short to avoid any potential toxic effect. However, this is not the case for all species. When the motility level of freeze–thawed sturgeon sperm was compared following different equilibrating durations, it was observed that the samples that had a 40-min exposure to the cryoprotectant medium at 5°C had a significantly higher motility rate than the ones that were frozen almost immediately after placing them in cryoprotectants ***(12)***.
16. During freezing the volume of the sperm suspension expands and may consequently increase the pressure in the cryovials, potentially causing cryodamage. The increased pressure can also disrupt the hermetically sealed ampoules and thus lead to contamination of the samples. Therefore, it is important not to completely fill the vials leaving enough space for sample expansion.
17. In order to minimize the fluctuations in cooling rate when freezing in liquid nitrogen vapor, it is important to make sure that conditions of freezing are controlled appropriately. To estimate the optimal height above liquid nitrogen, where the samples are going to be held, several sperm samples should be placed at different levels above liquid nitrogen surface. The higher the sample is above the surface, the longer the freezing time should be. All samples should then be moved to approx 1 cm above liquid nitrogen for a further 10–15 min before plunging into liquid nitrogen. Assessment of motility rates in the thawed samples will indicate which holding height is optimal, and this should be used for routine cryopreservation. The cooling rate in vapor can be estimated using a thermocouple, one end of which is placed in the sample and the other in ice.
18. The cooling rate can be finely controlled by employing a controlled-rate freezer. However, it is not always practically possible to have it on the site of sperm collection. Fish sperm freezing is often performed under field conditions, where other methods of freezing, such as freezing in liquid nitrogen vapor or on dry ice, are more practical. The majority of modern controlled-rate freezers provide linear cooling rates and this may not always be the optimal cooling regime as some workers have found exponential cooling to be more effective ***(34)***. The closest easily achieved approximation to an exponential cooling regime can be achieved by using liquid nitrogen vapor-phase freezing or dry ice.
19. The authors have noted that storage of cryopreserved sperm in liquid nitrogen is possible for a long period of time without any detectable decline in sperm quality.
20. Studies on optimization of the thawing regime have demonstrated that the best thawing regime for 1–2 mL vials is using a 40°C water bath**.** As soon as sperm samples of sturgeon or salmon are thawed they should be immediately used for fertilization. Granules (*see* **Fig. 1**) can be thawed in a different way; granules are placed directly into eggs along with activating medium, the temperature of which should be the same as river water. In the case of carp, the thawed sperm samples could be stored on ice and the fertility rate could be improved after some additional treatment as described in **Note 21**.

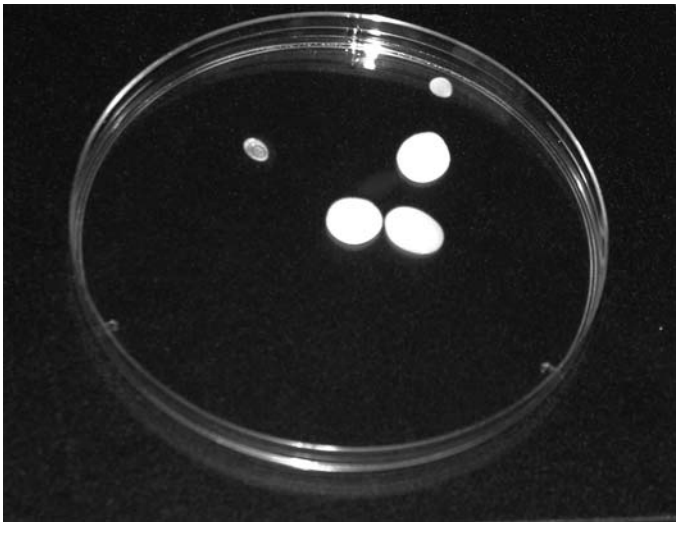

Fig.1. Sperm granules after cryopreservation.

21. Ice nucleation/crystallisation results in the removal of gases from the suspension of frozen cells. Saturation of sperm with oxygen has been reported to improve the quality of spermatozoa for cryopreserved-thawed sperm of carp ***(35)***. Significant increases in fertility were observed in all sperm samples that were aerated over 5 and 15 min at 0°C. To explain this effect the dynamics of energetic components of sperm cells was studied in fresh, sperm cryoprotectants treated and cryopreserved samples ***(36,37)***. It was found that exposure to cryoprotectants, as well as cryopreservation, affects almost all elements of the energetic system of sperm including a decrease of ATP and an increase of creatine phosphate levels ***(36,37)***.

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15

Cryopreservation of Avian Spermatozoa

Graham J. Wishart

Summary

This chapter describes a simple system for cryopreservation of avian spermatozoa as pellets, formed by dropping volumes of sperm suspension directly into liquid nitrogen with dimethylacetamide as cryoprotectant. The method originates from the group at the Research Institute of Farm Animal Breeding and Genetics at St. Petersburg–Pushkin and is described here for chicken spermatozoa, but has also been adapted successfully for other species, such as Houbara bustards and pheasants.

Key Words: Spermatozoa; avian; poultry; spermatozoa; cryopreservation; dimethylacetamide; pellet; liquid nitrogen.

1. Introduction

Since Polge and colleagues first produced live chickens from glycerolated spermatozoa frozen to –79°C ***(1)***, there have been several alternative methods developed for avian sperm cryopreservation (*see* **Note 1**).

In the previous edition of this volume, a method for cryopreserving chicken spermatozoa in glycerol using a programmable freezer to produce a freezing rate of –1 to –7°C was described as the then optimum system for maintenance of fertilizing ability in that species ***(2)***. The "downside" of this method is that the equipment is expensive and not easily adapted to "field" conditions, and that glycerol is a contraceptive and must be removed from the semen before insemination (*see* **Note 2**).

Since then, in at least one publication ***(3)***, a simpler method of chicken sperm cryopreservation using dimethylacetamide as cryoprotectant and freezing samples as pellets produced directly in liquid nitrogen, has been shown to preserve fertilizing ability more efficiently that the typical glycerol/slow-freeze method

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outlined previously. Furthermore, because the "pellet" method has also used successfully to produce offspring from other species, such as Houbara bustards ***(4)*** and pheasants ***(5)***, it seemed appropriate to describe that method in this chapter. However, it should be noted that this method has not been successful for spermatozoa from all avian species, for example those of turkeys, cranes ***(6)***, and eagles ***(7)*** and that different diluents, cryopreservatives, freezing systems, and thawing systems, may need to be considered for any "new" species (***[6,8]***; *see* **Note 1**). But, it is certainly better to start with the simplest method.

The method presented here originates from the group at the Research Institute of Farm Animal Breeding and Genetics at St. Petersburg–Pushkin led by Kurbatov ***(9)*** and published in English by Tselutin et al. ***(10)***. The system is described for chicken spermatozoa, but can be adapted for other species by altering volumes and insemination doses.

A word of caution: avian sperm cryopreservation technology has been best described for, and probably most successfully applied to, chicken breeding; however, quantitative studies have shown that, following intravaginal insemination, optimally cryopreserved chicken sperm retain only 2% of their prefreeze fertilizing ability ***(8)***. For any strain or group of chickens, which may vary in their integral fecundity, it is therefore important to establish that intravaginal artificial insemination is efficient enough to withstand this reduction in sperm quality (*see* **Note 3**) or otherwise consider alternative methods of insemination (*see* **Note 4**).

Despite the inefficiency of the system as previously described, progeny can be derived and avian sperm cryopreservation is recommended as a conservation measure for domestic and nondomestic birds ***(11,12)***. For intensively reared domestic species, this is particularly pertinent with the current threat of avian influenza.

2. Materials

2.1. Semen

Semen collection techniques for poultry are well documented ***(13)*** and information on these and the many and varied systems that have been employed for other species have been reviewed recently by Gee et al. ***(6)***. Whichever system is employed, maximum efficiency will require optimization of housing and husbandry conditions and conditioning of the birds to a routine that is as stress-free as possible. It is most desirable that the semen is free of contamination and is diluted and cooled as soon after collection as possible.

2.2. Diluent

The diluent used is that described by Lake and Ravie ***(14)***. It should be prepared using distilled or deionized water and can be kept frozen in aliquots, or otherwise stored after sterilization by filtration (*see* **Table 1**).

Table 1
Cryopreservative Solution

Constituent	Molarity (*M*)	g/100 mL
Sodium glutamate (monohydrate)	0.103	1.92
Magnesium acetate (tetrahydrate)	0.004	0.08
Potassium acetate (anhydrous)	0.051	0.50
Polyvinyl pyrrolidine (MW 10,000)	0.0003	0.30
Glucose	0.044	0.80

Dimethylacetamide (liquid; 99%+) is added to the sperm suspension in diluent to a concentration of 6% (v/v); 0.645 *M*), just before pellet formation.

2.3. Equipment

1. A polystyrene box for cooling semen samples, ideally with an inner well of around 20 cm^3, walls 5-cm thick, and a fitted lid. The boxes used by chemical companies to transport reagents in dry ice are ideal. Fill the box with ice to a depth of 8–10 cm and cover this with a few sheets of tissue (*see* **Note 5**).
2. A similarly sized polystyrene box for liquid nitrogen. It is helpful to line the base with black card against which the white frozen pellets can be seen.
3. Liquid nitrogen source.
4. Liquid nitrogen storage container, for example, with cylinder storage for aluminum canes.
5. 20–30 mL volume flat-bottomed polystyrene vials.
6. 2-mL cyovials. We use cryovials that can withstand immersion in liquid nitrogen, and have screw caps in which we puncture a small (1-mm diameter) hole (*see* **Note 6**).
7. Automatic pipets: volumes as required, including a 1-mL pipet (blue disposable tip) for dropping semen into liquid nitrogen.
8. Tongs or large forceps.
9. Beaker of water at 60°C (*see* **Note 7**).

3. Methods

3.1. Freezing Protocol

1. Collect semen (1–2 mL) into a polystyrene vial and place this in the ice box for 10 min to cool, then mix with the same volume of Lake's solution at around 5°C and equilibrate in the same container for 20–30 min.
2. Add liquid nitrogen to the other polystyrene box to a depth of around 10 cm and let stand for at least 10 min before use.
3. With constant agitation, add dimethylacetamide, drop by drop to the diluted semen to give a final concentration of 6% (v/v). Equilibrate for 1 min.
4. Using the 1-mL automatic pipet, take up around 0.5 mL and allow individual droplets to fall into the liquid nitrogen. The droplets, of around 0.05 mL, will form

pellets that move around on the surface of the liquid nitrogen before sinking to the base of the container. Make sure that the pellets on the surface do not fuse with each other before sinking.
5. Using the tongs, immerse cryovials into the liquid nitrogen so that they are filled with liquid and place in indentations made in the wall of the polystyrene container.
6. Using the tongs, transfer six pellets into each cryovial, place the cap on the cryovial, clip onto an aluminium cane or other suitable storage system, and transfer these to the liquid nitrogen storage container.

3.2. Thawing Protocol

1. Remove cryovials from the canes and place in the indentations in the wall of the polystyrene container. Remove the caps.
2. Before the nitrogen completely vaporizes, transfer to a polystyrene vial and hold this with gentle, but constant, agitation in the 60°C "bath" until the pellets liquefy. When this happens, remove them quickly from the water bath (*see* **Note 6**).

3.3. Insemination (see Note 7)

1. A volume of around 0.3 mL should be obtained from six pellets sufficient for two to three insemination doses of approx 300 million spermatozoa.
2. Inseminate 0.1–0.15 mL of samples 3–4 cm into the vagina of each hen as quickly as possible (within a minute) of thawing.
3. Repeat inseminations three to four times at 2- to 3-d intervals to maximize chances of fertilization.
4. Collect and incubate eggs. Test for fertility after about 7 d by "candling," Open any "clear" eggs to check for signs of early embryonic death.

4. Notes

1. Reviews that describe the use of programmable freezers, customized systems for freezing in nitrogen vapor or alcohol baths, formation of pellets on CO_2 and in liquid nitrogen for freezing spermatozoa from a range of avian species, using a range of diluents and cryoprotectants are cited in the reference list ***(2,6,8,9,15–17)***. The choice of method may often be unclear because comparisons of only limited permutations of different systems have been made in single reports. Furthermore, even for any one species, the innate fecundity of the birds being used in different reports will vary, as well as the systems used for artificial insemination, so comparisons of resultant fertility between reports are unreliable. For any new species, the choice of system is pretty much empirical and no rules can be offered. The justification for describing the pelleting system is that it is inexpensive and simple and so a good place to start. For a definitive study for optimizing conditions for freezing spermatozoa of any new species, use of a programmable freezer and careful analysis of sperm function after cryopreservation is recommended. However, it must be remembered that in vitro properties of cryopreserved spermatozoa have been shown to be poor predictors of their fertilizing ability.

2. Glycerol has been shown to be less toxic to spermatozoa than other cryopreservatives, but the need to remove glycerol from spermatozoa before intravaginal insemination really undermines the use of glycerol for "field" use or where only very small volumes of spermatozoa are available. If these restrictions are not a problem, then glycerol can be removed by centrifugation and reconstitution ***(2)*** or by dialysis ***(18)***.
3. The "innate fecundity" depends on sperm quality and quantity, on artificial insemination efficiency, and on sperm sequestration and storage in the female tract. This is liable to vary between birds, strains, and species.

   The whole process can be checked with nonfrozen spermatozoa by examination of laid eggs for the presence of spermatozoa, or the holes that they digest in the inner perivitelline layer (IPVL-holes) around the germinal disc ***(19)***. In chickens, the probability of an egg being fertile approaches 100% with at least six "IPVL-holes" ***(19)***. So, on the basis that 98% of chicken spermatozoa lose their fertilizing ability on cryopreservation ***(8)***, only combinations of (fresh) semen donors and recipients that result in eggs with 300 or more IPVL-holes (likely to result in six IPVL-holes with cryopreserved spermatozoa) should be considered for a sperm cryopreservation program.

   This is certainly true for chickens and maybe for other poultry, where artificial insemination is carried out by mechanically "everting" the vagina ***(13)***, but the process may be more efficient in other species, for example in "imprinted" birds, such as raptors, that are behaviorally primed for copulation and will voluntarily "present" a cloaca with exposed vaginal opening to the handler ***(20)***.

   Variations in "innate fecundity" following artificial insemination (AI) with unfrozen spermatozoa will be compounded by variation in the response of spermatozoa to cryopreservation, which has been the basis of genetic selection ***(21)***.
4. The normal route for inseminating birds is into the vagina, which may be mechanically everted by pressure on the abdomen in chickens and turkeys and some other species ***(13)***, or may be visualized by opening the cloaca with retractors (e.g., **ref. *22***) or may be voluntarily presented by imprinted birds ***(20)***.

   Alternative insemination routes into the uterus, magnum, or infundibulum provide greater access of spermatozoa to the upper female tract and enable fertilization in situations where intravaginal inseminations are ineffective. However, there are problems associated with these alternative methods ***(2)***; for example, the latter two require surgical procedures. These methods have been used to obtain fertility with glycerolated spermatozoa ***(15)*** and have been used to obtain fertility with poor quality cryopreserved avian spermatozoa ***(2)***. Recently a nonsurgical intramagnal insemination technique has been described for poor-quality eagle spermatozoa ***(23)***.
5. A liquid cool bath at 5°C or a refrigerator are alternatives.
6. Clearly these punctured cryovials will flood with liquid nitrogen during storage, as will the vials plugged with cotton wool, described in the original method ***(10)***. Thus cross-contamination between samples will, theoretically, be possible. For this reason, such containers have not been recommended for gene banks where different samples are held in the same liquid nitrogen container ***(12)***.

7. The original method utilized a funnel, 10 cm in length and maintained at 60°C by a water jacket ***(10)*** or a thermoregulated conical hotplate ***(3)***, into which pellets were dropped, and the thawed semen retrieved through the funnel in 3–5 s. This is clearly more convenient for larger volumes. In the method described here, heat transfer and thawing would be faster if pellets were transferred to a glass vial before holding at 60°C.

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16

Cryopreservation of Animal and Human Cell Lines

Christopher B. Morris

Summary

The long-term storage of human and animal cell lines is a relatively new branch of the science of cryopreservation of living organisms. The need to maintain the increasing numbers of cell lines, as they started to emerge from research laboratories from the mid-1900s, necessitated a radical approach to the problem. The realization that they could survive cryopreservation, and that a slow rate of cooling is essential for this survival, led to the eventual discovery of cryoprotectants. Subsequent development of mechanical freezers, which can accurately control the rate of cooling, now allows cells to be cryopreserved at their maximum viability. This chapter outlines the essentials steps for the successful preparation, freezing, and storage of cell lines.

Key Words: Cryopreservation; human and animal cell lines; cryoprotectant; cryovials; freeze-medium; programmable freezer; liquid nitrogen; cross-contamination; viable cells.

1. Introduction

The emergence of mammalian cell culture has its origin in the first attempts to culture tissue explants in vitro at the turn of the twentieth century ***(1–3)***. The subsequent development of complex culture medium formulations in the 1950s ***(4,5)*** enabled the establishment of a wide range of cell lines and provided a valuable research tool for the study of growth mechanisms and disease. Today, the availability of thousands of animal cell lines offers a reproducible source of material for all aspects of medical and agricultural research.

Continuous maintenance of cell lines in culture is impractical for reasons of:

1. Cost, e.g., serum containing media is currently about 50–300 GBP/L.
2. Risk of exposure to microbial contamination.
3. The possibility of culture cross-contamination.
4. Genetic drift with the possible consequence of phenotypical changes and the loss of the cell's original characteristics.

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Concomitant research into subzero storage methods at the time when cell culture methodology was being developed led to the discovery that addition of glycerol to fowl semen enhanced survival of spermatozoa after storage at –79°C ***(6)***. The use of nitrogen in the gaseous (below –130°C) and liquid phase (–196°C) now allows indefinite storage of most mammalian cell lines after cryopreservation. The precise mechanism for optimal cryopreservation has been established by studying the effects of freezing and thawing at various rates from which a hypothesis of freezing injury to cells was proposed ***(7)***.

The majority of cell lines must be cooled slowly, i.e., –1 to –5°C/min, and then thawed rapidly to achieve maximum viability. The rate of cooling is optimized to allow time for intracellular water to escape, and subsequently reduces the amount of intracellular ice formed (*see* Chapter 3). The presence of intracellular ice during cooling and thawing, i.e., post-freezing resuscitation, may cause lethal damage to intracellular membranes ***(8)***. Addition of cryoprotectant to the cells depresses the temperature at which intracellular ice is formed and allows cooling rates to be reduced for more efficient water loss ***(9)***.

The development of programmable controlled-rate freezers now allows individual cooling profiles to be designed that give maximum cell viability during cryopreservation, i.e., identical pre- and postfreezing viabilities. Culture collections such as the European Collection of Animal Cell Cultures (ECACC) (http://www.ecacc.org.uk/), supply many thousands of cell lines and many new cell lines are added annually. A systematic approach to the quality control of cell lines and their cell banks is therefore essential to ensure future supplies of authentic material ***(10,11)***.

2. Materials

1. Class II or III microbiological safety cabinets in either containment level 2 or 3 laboratory facilities ***(12)***. The handling requirements of any cell line should be assessed using the current guidelines issued by national regulatory bodies in consultation with your safety officer, prior to their introduction into the laboratory ***(13)***.
2. Cell cultures: these should be in active growth, i.e., log phase, usually 2–4 d after subculture for most mammalian cells, although slower growing cells may need a longer period to achieve sufficient numbers to freeze. Cells that have entered the stationary phase should not be used (*see* **Note 1**).
3. Freeze medium: this will be either the growth medium supplemented with 20–25% (v/v) serum and 10% (v/v) cryoprotectant, or whole serum and 10% (v/v) cryoprotectant. The choice of cryoprotectant will be determined by the cell type, but for the majority of cell lines dimethyl sulfoxide (DMSO) or glycerol can be used. An alternative is polyvinyl pyrrolidone, a high-molecular weight polymer (*see* **Note 2**).

4. Cryovials or ampoules: these are obtained from tissue culture plasticware supplies, e.g., Nunc, Becton-Dickinson, Corning as presterilized (irradiated) screw cap 1.0- or 1.8-mL vials (*see* **Note 3**). Some manufacturers also supply special racks for holding the vials during filling.
5. Freezing system: the best and most reproducible method is the programmable freezer, e.g., Planer Products (*see* **Notes 4** and **5**).
6. Storage system: liquid nitrogen storage vessels with inventory systems suitable for cryovials. Most vessels can be arranged to store vials in the vapor phase, liquid phase, or a combination of both.

   Automatic filling (top-up) and alarm systems are advisable to prevent accidental loss of stored material. A recent development is the isothermic vessel that provides storage in the vapor phase, but is designed to maintain an even temperature throughout the vessel (*see* **Note 6**).
7. Improved Neubauer hemocytometer and 0.4% (w/v) Trypan blue in phosphate-buffered solution for calculating both total and viable cell numbers.
8. A small liquid nitrogen vessel for transporting ampoules or preferably a "dry shipper," i.e., a unit designed to transport vials in the vapor phase instead of in the liquid phase.
9. Protective full face mask, cryogenic (insulated) gloves, waterproof apron, long forceps, and clamping scissors.

3. Methods

1. Cell lines must be handled in appropriate laboratory conditions providing adequate protection to the operator. Only one cell line should be handled at a time, with separate reagents used for each cell line to avoid the risks of microbial and cell cross-contamination.
2. Microscopically examine all cultures to be frozen for cell morphology, density, and microbial contamination using a good quality inverted-phase microscope fitted with at least a ×10 and ×20 objective, i.e., ×100 and ×200 final magnification. Reject any suspect cultures.

   Normally the cell density should not exceed 90% of its maximum growth density, and they should have been "passaged" at least twice in the absence of all antibiotics prior to freezing (*see* **Note 7**).
3. Count the cells after staining with Trypan blue to estimate the percentage of viable cells in the culture. Suspension cells can be counted directly by diluting 100 µL between 2-fold (1:1) and 10-fold (1:9) with the Trypan blue solution.

   Adherent cells will normally require a proteolytic enzyme, e.g., trypsin or trypsin + EDTA to remove the cell sheet. Some lines will detach in the presence of EDTA only. Cells should be prepared as for routine subculture, remembering to neutralize the enzyme by addition of serum containing medium or soya bean inhibitor in the case of serum-free cultures. Dilute an aliquot of cells in Trypan blue.
4. Load a prepared hemocytometer with the diluted cells using a fine-tip Pasteur or micropipet. Allow the mixture to be drawn under the cover slip by capillary, rather than active, pipetting. Fill the chamber completely.

5. Count the cells over one of the nine 1-mm^2 squares, (bright, refractile cells are viable, dark-blue cells are dead), using a phase microscope (an inverted type is the most suitable).

   Repeat the process over three more squares. Usually the corner squares are used. For statistically accurate counts a range of 30–100 cells/mm^2 should be counted per 1-mm^2 square. Prepare another sample if the counts are outside this range.
6. Estimate the total and viable cell count as follows:

$$\text{cells/mL} = \frac{\text{No of cells counted } (\times \text{ dilution} \times 10^4)}{\text{No of 1-mm}^2 \text{ squares counted}}$$

   The percentage of viable cells is:

$$\frac{\text{Total viable cells } (\times 100)}{\text{Total cell count (viable + dead)}}$$

   Healthy cultures should exceed 90% viability. Low viabilities or the presence of large quantities of cell debris are an indication of suboptimal culture conditions or exhaustion of the nutrient supply. These cultures would normally be rejected for cryopreservation.
7. Ampoules (cryovials) are normally filled with 1-mL aliquots of cells in freeze medium. The number of viable cells per ampoule would typically be in the range $3 - 10 \times 10^6$ cells in order to rapidly establish a new culture after resuscitation. Calculate the number of cells required to fill the ampoules, e.g., 10 ampoules at 5×10^6 cells/ampoule will need a total of 50×10^6 cells. Dispense the required number of cells into centrifuge tubes.
8. Centrifuge the cells using the minimum g-force necessary to sediment them, e.g., 100*g* for 5 min with small volumes per centrifuge tube, i.e., 10–50 mL.
9. Decant the medium and resuspend the cell pellet(s) in freeze medium to the required cell density (*see* **Note 8**). To aid resuspension, agitate the pellet gently with a finger after decanting the medium, and before adding freeze medium.
10. Dispense the cells into premarked ampoules in 1-mL aliquots. Ampoules must be clearly marked with the cell designation, e.g., passage number, freeze batch number, and date of freezing (*see* **Note 9**).
11. Keeping the ampoules vertical to avoid spillage into the cap, transfer them to the freezer (*see* **Subheading 2.**, **item 5**; **Notes 4** and **5**).
12. Transfer the ampoules to nitrogen storage once frozen to at least –130°C or below. Wear a face mask and full-protective clothing to prevent injury from exploding ampoules. Record the ampoule location. Graphical databases specifically designed for use with cryogenic systems are available from commercial sources.
13. Check at least one ampoule from each frozen cell bank for viability and growth potential. Always allow at least 24 h storage in their final location before starting quality control tests.
14. Transfer an ampoule to a tray in the top of the storage vessel using long forceps or clamping scissors. If the ampoule has been stored in liquid nitrogen, allow it to

equilibrate for at least 2 min in the vapor phase to reduce the risk of explosion from liquid trapped inside the ampoule rapidly expanding. Place the ampoule(s) in an aluminium screw-top canister to enable decontamination in the event of leakage or explosion during transit. Full-protective clothing must be worn when removing ampoules from storage.

15. Transport the ampoule to a water bath by covering the canister in solid carbon dioxide pellets (dry ice) placed in a loose-lid insulated container.
16. Thaw ampoules in a water bath set at the cell's normal growth temperature (e.g., 37°C for mammalian cells, 25°C for amphibian cells). Rapid and complete thawing is vital to retain viability. Float ampoules to half their height in racks or polystyrene, i.e., do not submerge to avoid water entering/potentially contaminating the screw cap.
17. Transfer ampoules to a microbiological safety cabinet and thoroughly wipe the ampoule surface with 70% (v/v) ethanol. Remove the contents using a sterile Pasteur or 1-mL pipet, and transfer to a 15-mL screw-cap centrifuge tube. Add dropwise 2 mL of antibiotic-free growth medium, mixing gently by swirling. An additional 2 mL of medium is added at normal speed. Remove 100 µL for total and viable cell counts (*see* **step 3–6**). To establish a rapid culture, set up new cultures at between 30–50% of their maximum cell density (*see* **Note 10**).
18. Maintain the culture for at least 5 d to monitor cell growth and absence of microbial contamination. Master cell banks should be fully quality controlled to ensure their authenticity (*see* **Note 11**).

4. Notes

1. Cells harvested for cryopreservation should be at their optimum viability level to ensure maximum survival during freezing and after thawing. This is especially relevant when the method of cryopreservation reduces the number of viable cells and increases the chances of selecting freeze-tolerant populations that may have different characteristics from the original population.
2. DMSO is sterilized by filtration using a 0.2-µm pore size, DMSO-resistant filters. Glycerol can be sterilized by autoclaving. DMSO is toxic if left in contact with cells for more than a short period of time. Polyvinyl pyrrolidone can be used as an alternative to DMSO when it is important to maintain the structural integrity of cells, e.g., pancreatic islet cells. Once the cells have been prepared for freezing, 1-mL aliquots are pipetted into ampoules and frozen as soon as possible. Addition of DMSO to culture medium normally increases the pH, especially if it contains sodium bicarbonate. This increase will further reduce cell viability at values above pH 8.0. It may then be necessary to gas the medium with 5–10% (v/v) CO_2 in air.

   An alternative freeze medium is whole serum, i.e., fetal bovine serum or newborn calf serum, to which the cryoprotectant is added. This has the double advantage of greater pH control and protection against freeze damage because of the increased levels of albumins.

   A further consideration, often overlooked, is the country of origin of the serum used to grow and freeze the cells. If cells are to be used in commercial processes or

shipped to other countries, e.g., United States, it is necessary to prove that the serum has been obtained from a country know to be free of certain contaminants and adventitious agents before an import permit is issued. Using serum from countries of origin other than those designated Zone one, i.e., United States, Australia, and New Zealand, may lead to difficulties in importation. Consult your supplier for the current situation.

When cells have been grown in serum-free medium it may be desirable to omit serum from the freeze medium. This can reduce the viability of the cells during freezing, owing to the protective nature of serum on surface membrane components. Addition of 0.1% (w/v) methyl cellulose in the freeze medium has been found to reduce cell death ***(14)***.

Cell lines requiring complex media or addition of growth factors should initially be frozen with these additives included in the freeze medium, e.g., interleukin-2 for the mouse T-cell CTLL. Their inclusion may help to stabilize surface proteins acting as receptors for the growth factors.

If zwitterion buffers, e.g., HEPES, tricine are usually included in the growth medium, they must be excluded from the freeze medium to avoid hypertonic stress during cooling. Antibiotics should never be included (*see* **Note 7**).

3. Cryovials can be obtained with either an internal or external thread. In long-term storage both types can allow entry of nitrogen liquid or gas. On removal from storage, extreme caution must be exercised to prevent explosion of the cryovial because of sudden expansion of the trapped nitrogen. Cryovials that have been stored in liquid should be allowed to equilibrate to the temperature at the top of the storage vessel, i.e., gas phase, before transferring to a water bath or laboratory. It is advisable to place ampoules during this period in a screw-top aluminium canister in case there is the need for decontamination because of leakage or explosion during transit (*see* **Note 5**).
4. To retain maximum viability during cryopreservation, cells must be cooled at a constant slow rate, –1 to –5°C/min ***(15)***. Programmable freezers are therefore the only means of achieving total viability, i.e., recovery of the same number of viable cells after freezing as before freezing. This is because they can increase the cooling rate at the most critical point in the program, the eutectic point (*see* Glossary), when the cells freeze and release energy from the latent heat of fusion, usually between –4 to –10°C, when water (liquid) changes to ice (solid). If rapid cooling does not occur at this point to compensate for the increase in temperature, the cells warm up with subsequent cell injury.
5. A less expensive alternative is the two-stage freezer. Ampoules are placed in the neck of a nitrogen Dewar, which contains a low level of liquid nitrogen, exposing them to the gaseous phase at a point where the temperature is about –25 to –30°C (prolonged incubation will result in the samples being cooled to –130°C). After 20–30 min the ampoules should have frozen and are then plunged into the liquid prior to transfer to their final storage location.

   The least expensive and reliable method is to place the ampoules in a heavily insulated box, e.g., polystyrene, either directly into precut holes or wrapped in paper towel and place the box at –80°C for 24 h. The ampoules are then transferred to their final storage location.

A better alternative to polystyrene is a cooling box (Mr. Frosty) produced by Nalgene (cat. no. 5100/001) which on following the manufacturers instructions cools at –1°C/min when placed at –80°C. However, none of the previously mentioned systems can compensate for the latent heat released when the cells start to freeze below –4°C.

6. To safeguard against loss of cryopreserved material from sudden vacuum loss or staff forgetting to fill vessels, it is essential to install autofill and alarm systems on storage vessels. Many large volume vessels already include these facilities. Storage vessels can be automatically supplied from 100- to 200-L self-pressurising reservoirs placed in the same room. Larger reservoirs will require special housing arrangements. Local alarms provide audible warning of problems, but to fully cover all emergencies they need to be connected to a telemetric system that will signal a radio pager. An additional precaution is to divide up the cell banks and store them in several vessels. It is essential that storage vessels are located in a ventilated room, which will be checked regularly. When large numbers of vessels or large volumes of nitrogen are used, an oxygen monitor must be fitted in the room, i.e., under the guidance of your safety officer and nitrogen supplier. At The European Collection of Animal Cell Cultures the monitor has been connected to an automatic ventilation system that operates when the oxygen level falls below 18.0% (v/v).
7. Routine addition of antibiotics to cell cultures must be avoided at all times, especially in stock cultures. As most antibiotics will only suppress persistent infections, which in their absence could be easily identified and eliminated, it is essential that cells to be cryopreserved are known to be free of infection. To further reduce the risk of both microbial and cell culture cross-contamination, a segregation policy should be implemented, i.e., separate reagents for each line and only one line handled at a time in the cabinet.
8. If whole serum with cryoprotectant is used this can be prepared in advance and checked for microbial contamination by removing a 5–10% (v/v) sample and incubating with thioglycollate and tryptone soya broths for 7 d. Aliquot the freeze medium into appropriate volumes, and store at –20°C or below. However, preparation of freeze media containing cell culture medium, e.g., MEM or RPMI 1640 is necessary just prior to use to avoid pH changes on standing. To minimize the risk of contamination all components should be pretested. Resuspending cells for freezing in precooled freeze medium, 0–4°C, may improve their survival prior to freezing. When this approach is used, it is important to maintain the cells at a constant temperature during all subsequent handling procedures by placing ampoules in ice until they are frozen.
9. The use of printed labels designed to withstand liquid nitrogen is recommended, as these can be produced with a barcode for easy identification with a barcode reader. Also microchips placed in the ampoule lid can store this information.
10. After thawing cells it is necessary to slowly dilute the cryoprotectant to prevent osmotic shock. The requirement to remove the cryoprotectant will depend on how much the cells are diluted. Above 10 mL is usually sufficient to overcome toxic effects. DMSO will also evaporate from the medium at 37°C.

When it is necessary to centrifuge the cells, use the minimum g-force to sediment them to prevent shearing damage, i.e., 70–100*g*. To initiate rapid growth it is advisable to inoculate new cultures at a higher density than for routine subculture, e.g., between 3–5 × 10^5 viable cells/mL for most suspension cells, and 3–5 × 10^4 viable cells/cm^2 for adherent cells. Monitor their growth and subculture once they reach a maximum density.

At the ECACC cell banks are frozen in a programmable freezer. The majority of post-thaw viabilities exceed 85% and are normally only a few percent lower than the prefreezing viability. Those with post-thaw viabilities below 75% are rejected. Viabilities of cells frozen by other methods tend to be much lower, and consequently resuscitated cultures contain a high level of debris and dead cells, which may have an inhibitory effect on the remaining viable cells. Debris can be removed from adherent cells by allowing the culture to grow for 24 h and then changing the medium, washing the attached cells with medium first if necessary. Suspended cells present a greater problem, as the debris may settle at the same rate as the viable cells, and may require a sedimentation stage to separate viable and dead cells.

11. The minimum number of tests that should be carried out on Master cell banks are total and viable cell counts, growth potential, and screening for bacteria and fungi. Quality control and the principles of cell banking should be an established part of the laboratory procedures if you are to establish problem-free cell stocks.

    Other tests which therefore became essential to comply with these criteria are:

    a. Screening for mycoplasma: these are very small microorganisms in a size range below most bacteria. Therefore, although they may be present at concentrations between 10^6 and 10^8 organisms/mL, they do not usually cause turbidity in cultures and often remain undetected. Mycoplasmas constitute one of the greatest problems in cell culture, and cultures should be routinely checked for their presence ***(16)***. One of the more recent developments has been the use of polymerase chain reaction to detect mycoplasma. This has the advantage of giving results with in a few hours of testing.
    b. Cell line authenticity: cross-contamination of cell cultures has been, and still is to some extent, a major problem ***(17,18)***. The species of origin of a cell line can be identified using isoenzyme analysis ***(19)***. A kit is available that is suitable for use in any laboratory (Authentikit from Innovative Chemistry Inc., Marshfield, MA). Cytogenetic analysis is used to identify normal and abnormal karyotypes as well as species but requires considerable expertise to interpret ***(20)***. More recent techniques are DNA fingerprinting ***(21)*** and short tandem repeat profiling ***(22)*** is used at ECACC for species verification of cell lines and master cell banks.

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17

Cryopreservation of Hematopoietic Stem/Progenitor Cells for Therapeutic Use

Suzanne M. Watt, Eric Austin, and Sue Armitage

Summary

To date, more than 25,000 hematopoietic transplants have been carried out across Europe for hematological disorders, the majority being for hematological malignancies. At least 70% of these are autologous transplants, the remaining 30% being allogeneic, which are sourced from related (70% of the allogeneic) or unrelated donors. Peripheral blood mobilized with granulocyte colony stimulating factor is the major source of stem cells for transplantation, being used in approx 95% of autologous transplants and in approx 65% of allogeneic transplants. Other cell sources used for transplantation are bone marrow and umbilical cord blood. One crucial advance in the treatment of these disorders has been the development of the ability to cryopreserve hematopoietic stem cells for future transplantation. For bone marrow and mobilized peripheral blood, the majority of cryopreserved harvests come from autologous collections that are stored prior to a planned infusion following further treatment of the patient or at the time of a subsequent relapse. Other autologous harvests are stored as backup or "rainy day" harvests, the former specifically being intended to rescue patients who develop graft failure following an allogeneic transplant or who may require this transplant at a later date. Allogeneic bone marrow and mobilized peripheral blood are less often cryopreserved than autologous harvests. This is in contrast to umbilical cord blood that may be banked for directed or sibling (related) hematopoietic stem cell transplants, for allogeneic unrelated donations, and for autologous donations. Allogeneic unrelated donations are of particular use for providing a source of hematopoietic stem cells for ethnic minorities, patients with rare human leukocyte antigen types, or where the patient urgently requires a transplant and cannot wait for the weeks to months required to prepare a bone marrow donor. There are currently more than 200,000 banked umbilical cord blood units registered with the Bone Marrow Donors Worldwide registry. In this chapter, we describe several protocols that we have used to cryopreserve these different sources of hematopoietic stem/progenitor cells, keeping in mind that the protocols may vary among transplant processing centers.

Key Words: Hematopoietic stem cells; hematopoietic progenitor cells; HPC-A; HPC-M; cord blood; cryopreservation; cryoprotectant; controlled-rate freezer; DMSO.

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1. Introduction

Cryopreservation of hematopoietic stem/progenitor cells (HSC/HPC) for therapeutic use has been carried out for more than 30 yr. Three types of HSC/HPC harvests are now routinely used for transplantation for hematological disorders and each may be cryopreserved with, or without, preprocessing. These are bone marrow (BM), mobilized peripheral blood (MPB; most often following granulocyte colony stimulating factor [G-CSF] administration), and umbilical cord blood (UCB). The latter is sourced at birth and may be collected *in utero* or *ex utero* ***(1,2)***. The HSC/HPC may be autologous (cells to be transplanted back in the donor) or allogeneic (cells to be transplanted in recipients other than the donor) donations, preference being given to autologous transplants where these are deemed possible.

Over the past decade, there have been considerable changes in the indications for usage of these different cell sources for transplantation ***(3,4)***. In Europe, there has been a 600% increase in BM and MPB HSC/HPC transplants from just over 4200 in 1990 to 25,207 in 2003. Most transplants (95%) were for malignant diseases. In 1990, most HSC/HPC transplants used autologous or allogeneic BM donations. This changed first to MPB for autologous transplants, and by 2003, 97% of autologous HSC transplants were MPB derived. For allogeneic HSC transplants the pace of change was slower, but by 2003 65% of all allogeneic HSC transplants were MPB derived, with BM remaining a significant source of HSC/HPC in some European and Asian countries. UCB, a rich source of HSC/HPC is an established alternative for bone marrow (particularly in children), when a matched unrelated allogeneic bone marrow donor is not available. The sourcing of these stem cells for patients from ethnic minorities or with rare tissue (human leukocyte antigen [HLA]) types is a particular challenge, and it is for these patients that UCB may provide a unique source of matched stem cells. Banked UCB units are also important for patients needing immediate unrelated allogeneic transplantation. Currently, more than 200,000 banked UCB units are available for transplantation via the Bone Marrow Donors Worldwide registry (www.bmdw.org) or Netcord (www.NET CORD.org). There has recently been renewed interest in the use of UCB for HSC transplantation with the development of: (1) multiple UCB transplants into a single recipient, (2) nonmyeloablative transplants, (3) improvements in hematological reconstitution by transplantation of UCB with other sources of CD34+ HSCs, T-cell subsets, ex vivo expanded UCB cells, or mesenchymal stem cells, and (4) genetic selection of UCB units ***(1,2)***. Thus, cryopreservation of hematopoietic stem cells for therapeutic use takes place mainly for autologous MPB and for banked UCB units.

Optimal cryopreservation and recovery of viable HSC/HPC (as determined by engraftment potential) from each of the sources described depends on a

number of parameters, including (1) transit times and storage temperatures following harvest, (2) preprocessing prior to cryostorage, (3) the selected cryoprotectant, (4) cooling and thawing rates, temperatures, and protocols, and (5) longer term storage temperatures. As well as optimizing HSC/HPC viability and function, the aim is to reduce cryoprotectant toxicity and osmotic stress. In 1959, dimethyl sulfoxide (DMSO) was introduced as a cryoprotectant for HSC/PPC ***(5)*** and is the cryoprotectant used in the protocols. For therapeutic use of HSC/HPC, cryopreservation is performed as soon after harvesting as possible ***(6)*** with DMSO concentrations of 1–2 *M*, a controlled-rate freezer (CRF) cooling rate of 1°C/min, storage and/or transport below –135°C, preferably between –150 and –196°C, and rapid thawing at 37°C with slow dilution of DMSO ***(7–11)***. The theory and experimental evidence and other practices that have been developed for optimizing cryostorage and thawing of these cells are detailed in a number of publications ***(7–25)*** and elsewhere. An established protocol for cryostorage of MPB HSC/HPC collected by apheresis (termed HPC-A in JACIE guidelines) and BM HSC/HPC (termed HPC-M in Joint Accreditation Committee of ISCT Europe and EBMT (JACIE) guidelines) donations is described first, and this is followed by a description of the cryostorage of UCB from related (directed donations) or unrelated donors (*see* **Notes 1–21**).

2. Materials

2.1. Cryopreservation of HSC/HPC From Mobilized Peripheral Blood and Bone Marrow

1. Cryocyte freezing bags of varying capacities (Miltenyi Biotec., cat. no. Baxter R995).
2. Cryosure DMSO (Wak-Chemie Medical GmbH).
3. 4.5% (w/v) human albumin solution (HAS) if autologous plasma is not available.
4. Transfer set with luer adaptor (Baxter Biotec., cat. no. EMC2240).
5. Sample site coupler (Baxter Biotec., cat. no. EMC1401).
6. Plasma transfer set with two couplers (Baxter Biotec., cat. no. EMC2243).
7. Air darts (Baxter Biotec., cat. no. EMC0413).
8. Syringes (2.5, 5, 20, and 50 mL).
9. Needles (23 and 19 gage).
10. Cryovials (NalgeNunc Int.).
11. Aerobic/anaerobicBactAlert bottles (BioMerieux Corp.).
12. Overbags for double bagging (Synpac Ltd.).
13. Laminar air flow (LAF) cabinet.
14. Sterile connecting device (SCD) (Terumo Medical Corp.).
15. Dielectric line sealer Baxter Biotec.
16. Vacuum packer/sealer (Multivac).
17. Melting ice packs-sealed into plastic bags.

18. Programmable liquid nitrogen controlled rate freezer (CRF) (e.g., Kryo 10) (Planer plc, UK).
19. Freezing cassettes (Planer plc).
20. Ice bucket to hold melting ice packs.
21. Environmental monitoring plates (Biomerieux Corp.).
22. Yellow bin bag or plastic box for clinical waste.
23. Sharps bin.
24. Laminated harvest calculation sheet and processing sheets.
25. Labels (*see* **Note 3**).
26. Automated cell counter.
27. Top pan balance (accuracy to 0.1 g).
28. Scissors.
29. Particle counter.
30. Isopropyl alcohol (IPA) wipes.
31. Sterile biocide (e.g., Klercide, Shield Medicare Ltd.).
32. Cryovat liquid nitrogen vapor phase storage vessel.

2.2. Umbilical Cord Blood Cryopreservation

1. Harvest bag.
2. Cord blood collection: either whole blood or red cell and plasma depleted product.
3. Yellow bin bag for clinical waste.
4. Sharps bin.
5. Syringes, luer-lock (1, 5, 10, 20, and 50 mL).
6. Cryovials (2 and 4.5 mL).
7. Needles (19 gage).
8. "2991" coupler (Gambro BCT).
9. Spike-to-luer transfer set (Gambro BCT).
10. Luer/spike interconnector (Gambro BCT).
11. Three-way stopcock (Discofix).
12. 600-mL transfer bag.
13. Sample site coupler.
14. Preinjection swabs for disinfection of sampling sites.
15. 10% Dextran (Gentran 40, Baxter Corp.).
16. Double-bagged ice block.
17. DMSO.
18. 50% (w/v) DMSO diluted in Dextran-40 (Pall Corp. Europe).
19. Cryocyte or appropriate freezing bags.
20. Labels for freezing bags (*see* **Note 3**).
21. 1 tube cane (Planer plc).
22. Programmable CRF (e.g., Kryo 10, Planer plc, Sunbury, UK).
23. Liquid nitrogen storage vessel (Planer plc).
24. BioArchive automated, controlled rate, liquid nitrogen freezer (intended for cryopreservation and storage of HPC) (ThermoGenesis Corp.).
25. BioArchive controlled rate freezing module (ThermoGenesis Corp.).

26. Syringe pump (Invac.)
27. Dielectric hand held line sealer (RFS Services).
28. Overwrap bags for double bagging (Thermo Genesis Corp./Synpac Ltd.).
29. SCD (Terumo Medical Corp.).
30. Mixer.
31. Syringe pump (Invac).
32. Vacuum sealer (Fuji/Multivac).
33. Freezing cassettes for BioArchive (Thermo Genesis Corp.).
34. Jig for PALL freezing bag (Thermo Genesis Corp.).
35. PALL freezing bags compatible with BioArchive (Pall Corp. Europe).
36. Environmental monitoring plates for contact, settle, and finger dabs (Biomerieux Corp.).
37. Aerobic/anaerobic culture bottles, e.g., BacTAlert (Biomerieux Corp.).

3. Methods

Details are given of protocols for the cryopreservation of HSC/HPC from MPB and BM (*see* **Subheading 3.1.**) and UCB cryopreservation (*see* **Subheading 3.2.**).

3.1. Cryopreservation of HSC/HPC From Mobilized Peripheral Blood and Bone Marrow

Bone marrow (HPC-M) or mobilized peripheral blood (HPC-A) HSC/HPC are harvested from patients prior to autologous transplant for backup or "rainy day" purposes (specifically being intended to rescue patients who develop graft failure following an allogeneic transplant or who may require this transplant at a later date). Less frequently, cells for allogeneic use are cryopreserved prior to transplantation. The following protocol describes the cryopreservation of these harvests using a CRF, with DMSO (in autologous plasma or HAS if no plasma is available) as a cryoprotectant. Donor T lymphocytes or therapeutic T cells (termed TC-T in JACIE guidelines; *see* **Note 4**) may also be harvested from the blood and given to the patient as part of a low-intensity conditioning transplant procedure or to induce a graft vs tumor reaction. The TC-T are cryopreserved in a similar manner (*see* **Notes 1**, **2**, and **6**). Harvests are then stored longer term in liquid nitrogen (vapor phase) storage facilities at a temperature of less than –135°C. Sterile techniques are required for all procedures. In the United Kingdom, this process is carried out in a good manufacturing practice (GMP)-grade clean room environment as an open process, although closed processes are under investigation. The clean rooms are environmentally monitored for bacterial and fungal contamination. All BM and all blood products are treated as potentially infectious (*see* **Note 13**). Samples for microbiology testing are taken to check contamination of the product during processing. Because HSC/HPC occur in the CD34 subset, samples are also taken for both nucleated and CD34 cell counts and viability (as determined by dye exclusion

using 7-amino-actinomycin. D. (7-AAD) and flow cytometry, or using the colony forming unit (CFU) assay ***(22,26)***. This allows the efficiency of cell manipulations and the robustness of the cryopreservation to be evaluated, while at the same time determining the cell or CD34+ cell dose per kg of patient or recipient body weight ***(26,27)***. Cells are best processed the same day and as soon after harvesting as possible ***(6),*** but when unavoidable may be stored overnight at 4°C (*see* **Note 12**). The BM or MPB harvests may be volume reduced or red cell depleted ***(6)***, prior to cryostorage in order to reduce the numbers of cryocyte bags stored or the volume of incompatible (i.e., Rh and ABO mismatched) red cells in the graft. In such cases, the buffy coat, reduced from over 1 L to a volume of 100–150 mL, is then cryopreserved using similar techniques to those described next.

3.1.1. Preparations Prior to Processing Samples

1. Relevant forms and labels are used and completed to record and track products for processing, testing, and cryostorage. The harvest should be correctly labeled according to JACIE or other accreditation guidelines (*see* **Notes 3–8** and **11**). A unique donation barcode number (ISBT128 standards) is assigned to the harvest and is subsequently attached to the relevant harvest record form and cryobag labels. The time of collection and receipt into the processing facility should be noted. The temperature on arrival is taken and should be below 8°C if the time from collection is greater than 1 h.
2. The total nucleated cell count is obtained using a hematology analyser. The sample for this can be taken from the harvest bag by either:
   a. Removing a section of the bag line. The line is first "stripped" into the bag a minimum of five times. A double heat seal is then made in the line isolating approx 0.5 mL of cell suspension. The seal furthest away from the bag is then cut removing the section of line containing the cell sample.
   b. Taking the cell collection into the clean room, inserting a sample site coupler into the collection bag through one of the sample ports, and removing approx 0.5 mL of sample using a syringe and 23-gage needle. This operation is carried out in the laminar air flow (LAF) cabinet.
3. Prior to analysis on the hematology analyser, the cell sample is diluted 1 in 5 and 1 in 10 using phosphate buffered saline either alone or supplemented with 0.5% (w/v) HAS (*see* **Notes 12** and **13**).
4. Processing of harvests and their cryopreservation are currently carried out in the clean room as open processes, although closed processes may be developed. The number of cryocyte bags required for freezing is determined prior to entering the clean room. Each "50 cryocyte bag" can hold a maximum of 20 mL, a "250 cryocyte bag" can hold a minimum of 30 mL and a maximum of 70 mL, a "500 cryocyte bag" can hold a minimum of 70 mL and maximum of 100 mL, and a "750 cryocyte bag" can hold a minimum of 100 mL and maximum of 130 mL. The "50 and 250 cryocyte bags" are usually used for TC-T preparations only.

5. Prime the CRF liquid nitrogen Dewar prior to entering the clean room.
6. Prior to entering the clean room and sterile area preparation, all items for transfer into the clean rooms are wiped down with IPA wipes.
7. Staff should proceed through clean room lobbies, where they gown in clean room garments. On entering change areas, a particle counter, a hand sealer, and charged battery pack are collected, wiped with an IPA impregnated wipe, and carried in to the clean room. All of the cell processing is performed in a LAF hood producing a Grade A environment and within a clean room giving a Grade B background (*see* **Notes 3**, **5**, and **6**).
8. The LAF hood is wiped down with Klercide (or an equivalent), left for 2–5 min, and then wiped down again with sterile 70% (v/v) IPA. Two settle agar plates are placed to the left- and right-hand sides of the workspace within the hood for environmental monitoring.
9. The harvest and plasma bag details are checked in order to match the paperwork details and the clean room processing sheet is signed to verify this. All bags are weighed using a top pan balance to confirm volumes (assuming approx 1 g/mL).
10. The paperwork is prepared on the bench away from the hood. By the end of the processing, the clean room operation record sheet will be filled in with the details of the procedure being performed, batch numbers of consumables used, operator details, cleaning details, and environmental monitoring details, whereas the stem cell processing sheet will be completed with details of the processing volumes, total cell and CD34 positive cell counts, and other relevant information.

3.1.2. Preparation and Addition of Diluent

The required amount of diluent, either as plasma (*see* **Subheading 3.1.2.1.**) or HAS (*see* **Subheading 3.1.2.2.**) is added in the clean room within the LAF hood as described next.

3.1.2.1. Autologous Plasma

1. Sterile connect (*see* **Note 14**) together the plasma and cell collection bags using either a sterile connecting device (use a pair of "Spencer wells" to clamp the line prior to opening the sealed join) or by a plasma transfer set with two couplers, one coupler inserted into each bag. It is important to ensure the wheel clamp is closed when inserting the spike couplers.
2. Place the bag of cells onto a balance and tare its weight to zero.
3. Open the wheel clamp or unlock the Spencer wells.
4. Allow the diluent to flow into the bag of cells until the required volume has been added and then reclamp the line.
5. Heat seal the tubing in three places and break the middle seal to separate the two bags.
6. Thoroughly mix the cells and diluent by gentle hand agitation.

3.1.2.2. 4.5% (w/v) HAS

1. Remove the metal cap from a bottle of 4.5% (w/v) HAS, disinfect with an IPA wipe, and place bottle inside LAF.
2. Insert an air dart into the bottle.
3. Remove a volume of 4.5% (w/v) HAS using a 50-mL syringe with 23-gage needle.
4. Insert sample site coupler into cell bag port.
5. Wipe injection site of sample site coupler with sterile alcohol wipe.
6. Inject required volume into cell bag.
7. Thoroughly mix the cells and diluent.

3.1.3. Preparation of Cryoprotectant

The cryoprotectant used here is 20% (v/v) DMSO diluted in either autologous plasma or 4.5% (w/v) HAS. The 20% (v/v) DMSO solution allows equal volumes to be added to the cell suspension resulting in a final concentration of 10% (v/v) DMSO (*see* **Notes 15–17**).

3.1.3.1. With Autologous Plasma

1. Prior to adding DMSO, the plasma must be cooled to below 8°C. This is achieved by placing the plasma bag between two bags of melting ice.
2. Sterile connect the plasma bag to a cryocyte bag either using a sterile connecting device or with a plasma transfer set with two couplers.
3. Place the cryocyte bag on a balance and tare its weight to zero.
4. Open the connecting device and transfer the required volume of plasma to the cryocyte bag (assuming 1 g/mL).
5. Heat seal the tubing in three places and break the middle seal to separate the two bags.
6. Place the cryocyte bag containing plasma on an ice bag prior to adding DMSO.

3.1.3.2. With 4.5% (w/v) HAS

1. Disinfect rubber septum on bottle of 4.5% (w/v) HAS and place inside LAF hood as in **Subheading 3.1.2.2.**, **step 1**.
2. Insert an air dart into the bottle.
3. Remove a volume of 4.5% (w/v) HAS using a 50-mL syringe with 23-gage needle.
4. Wipe injection site of sample site with sterile IPA wipe.
5. Inject 4.5% (w/v) HAS into the cryocyte bag.
6. Repeat **steps 3–5** until the required volume of 4.5% (w/v) HAS is achieved.
7. Place the cryocyte bag containing 4.5% (w/v) HAS on an ice bag prior to adding DMSO.

3.1.3.3. Adding DMSO

1. Calculate the required volume of DMSO (1 vol DMSO to 4 vol HAS or plasma).
2. Remove the metal cap from the bottle of DMSO, disinfect the rubber septum with an IPA wipe, and place the bottle inside the LAF.

3. Insert an air dart into the bottle of DMSO.
4. Remove a volume of DMSO using a 20- or 50-mL syringe with a 23- or 19-gage needle together with 3 mL of air.
5. Inject DMSO very gradually into a cryocyte bag containing plasma or HAS (on bag of melting ice), ensuring the diluent is gently agitated (*see* **Note 18**).
6. Repeat **steps 3** and **4** until required the volume of DMSO has been added.
7. Use the air in the syringe to empty the coupler tubing of DMSO.
8. Record the start and finish times for the addition of the DMSO on the calculation sheet.
9. Leave the cryocyte bag containing cryoprotectant on a bag of melting ice for at least 10 min prior to adding to cells.

3.1.4. Adding Cryoprotectant to Cells

Do not add leukocytes to the cryoprotectant. Aim to keep the time from adding the cryoprotectant to putting the cryocyte bags on to freeze to less than 20 min. Monitor the weight of the cryoprotectant added using the top pan balance.

It is important to maintain the cells at around 4–8°C during the addition of the cryoprotectant to prevent an increase in temperature owing to the DMSO that will result in toxicity (*see* **Notes 15–18**).

The procedure is as follows:

1. Sterile connect together the cell bag and bag of cryoprotectant (*see* **Note 14**).
2. Place the cell bag on a balance and tare the measured weight to zero.
3. Open the clamp between the two bags and allow the required weight of preservative to be added to the cell bag. The mixing rate should be about 10 mL/min with constant mixing.
4. When all the cryoprotectant has been added seal the tubing in three places and break the middle seal.
5. Place the bag of cells with cryoprotectant between two ice bags for 10 min prior to distributing into cryocyte bags.
6. Prepare cryocyte bags by sealing off and removing one dark blue and one light blue luer port (*see* **Notes 18** and **19**).
7. Close the clamp on the remaining port line.
8. Insert a transfer set with spike coupler and luer adapter into a port of the bag of cells.
9. Connect the bag of cells and cryocyte bag via luer connectors.
10. Place the cryocyte bag on a balance and tare as before.
11. Open the line clamp and transfer the required volume of cells into the cryocyte bag.
12. Express excess air in the cryocyte bag back into the cell bag.
13. Seal off the line as close as possible to the cryocyte bag.
14. Close off the line clamp and remove luer connector and remaining line from the cell bag.
15. Repeat **steps 9–14** for each cryocyte bag until all bags have been filled.
16. Take samples from the final cryocyte bag for archiving (into cryovials) and microbiology testing (Bact Alert bottles or equivalent) before heat sealing the line.

17. A control cryocyte bag for the CRF (*see* **Note 20**) is set up as in **steps 9–16, Subheading 3.1.3.1., steps 1–6, Subheading 3.1.3.2., steps 1–7,** and **Subheading 3.1.3.3., steps 1–8** but by adding equal volumes of plasma or 4.5 % (w/v) HAS and cryoprotectant to a cryocyte bag.
18. The cryocyte bags are labeled and placed into an outer bag and vacuum sealed using the Multivac vacuum sealer at 225 mBar. The bags are kept on ice until frozen in the CRF.
19. The laminar hood surface is monitored after the procedure on left- and right-hand sides of the work area by contact plates (10 s contact per plate). Left and right glove prints of the operator are made by dabbing the surface of an agar settle plate with the fingers of each hand. The clean room is decontaminated by thoroughly spraying with Klercide and wiping down/mopping the floor with a dedicated mop, the head of which is changed daily. The empty collection bags are retained for 24 h in the fridge.

3.1.5. Freezing Cells

This operation should ideally be carried out by another member of staff, who can receive the cryocyte bags through the hatch immediately after they are filled, so that the time prior to freezing is minimized.

The bags of cells are frozen in a CRF (*see* **steps 1–10** and **Note 20**) to ensure that the optimum freezing parameters resulting in maximum cell viability are achieved. One such cell freezer is the Kryo 10 produced by Planer plc. This freezer can be programmed manually within the MR3 controller or from a computer linked to the controller using the KSK10 software. The software allows a full audit trail for each individual freezing procedure including details of the products frozen together with a graph of the freezing run itself. The Kryo 10 freezer has two temperature probes, one for measuring the temperature within the freezer chamber and the second that is inserted into a control bag monitoring the temperature of a simulated product throughout the freezing run.

1. Fill the liquid nitrogen Dewar to 12-cm depth with liquid nitrogen.
2. Attach the heater and vapor withdrawal tube to the Dewar ensuring all clips are in place.
3. Turn on the CRF and press rocker switch on heater control box. The heater will stay on until a pressure of approx 5 lb/sqin is attained.
4. Select program to be used either within MR3 control box or download from computer software. An example program is shown in **Table 1**.
5. Place cryocyte bags into precooled stainless steel cassettes and keep on ice until freezer is ready for loading.
6. When freezer indicates the loading temperature has been reached, place freezing cassettes into the rack inside the chamber ensuring the cassettes are parallel to the direction of the fan.
7. Insert bag probe into dummy bag and place lid onto freezing chamber.
8. Press run to start the freezing cycle.

Table 1
Example of Freezing Profile Used on the KRYO10 Controlled Rate Freezer

Start temperature	6°C
Ramp 1	Hold for 12 min
Ramp 2	Rate –2°C per min to –5°C (Chamber)
Ramp 3	Rate –1°C per min to –40°C (Chamber)
Ramp 4	Rate –5°C per min to –160°C (Chamber)
No seeding	Trigger on temperature

Validation was carried out in-house.

9. Once the freezing run has completed remove cassettes and open carefully and place frozen bags into a liquid nitrogen vapor-phase storage vessel at –180°C.
10. Archive sample cryovials must be frozen within the same run as the bags and preferably stored with the frozen harvests.

3.1.6. Environmental and Product Monitoring/Testing

1. To complete the process, the agar plates (including those with a set of glove prints) used for environmental monitoring are incubated at 35–37°C and 22°C for 3 and 7 d, respectively, to check for bacterial and fungal/yeast growth. Any positive plates and Bactalert bottles inoculated with the final processed product are sent for identification to the relevant microbiology laboratories.
2. As part of quality monitoring, CD34 with viability (using 7-AAD, flow cytometry, and CFU) assays are routinely set up on each harvest before, 1 wk after cryopreservation, and at the time of issue.

3.1.7. Retention of Records

It is important to ensure that all records relating to the harvest and cryopreservation are completed fully. The record forms must be filled in at all relevant stages to ensure that all actions and instructions are traceable. Records must be stored for 30 yr according to EU legislation (*see* **Note 10**).

3.2. UCB Cryopreservation

This section describes two procedures for cryopreserving donated UCB. The first is a method used for an intended recipient or is a directed UCB donation. These directed UCB units are usually collected from mothers who have a child with a condition that may be treated with an HSC/HPC transplant or where there is a family history of a genetic disease that may benefit from HSC/HPC transplantation. This procedure is compared with one for cryopreserving altruistic unrelated allogeneic UCB donations for an unrelated UCB bank (*see* **Notes 9** and **10**).

There are two techniques for collecting UCB, (1) *in utero*—following the birth of the baby, prior to delivery of the placenta, and (2) *ex utero*—following delivery of the placenta. Generally *in utero* collections are performed by the midwife and this is the technique favored for directed collections. *Ex utero* techniques are predominantly performed by trained cord blood bank staff collecting for unrelated UCB banks (*see* **Note 21**). At the time of collection, the midwives would have been fully informed and supplied with a collection kit from the stem cell processing laboratory. Following stringent cleaning of the umbilical cord, a needle is inserted into the umbilical vein and the blood drains out of the placenta under gravity into a purpose designed collection bag containing an adequate volume of anticoagulant to prevent the blood clotting. A 2- to 3-cm cord biopsy is collected as an alternative source for DNA extraction that can be used for testing and HLA typing. Directed UCB units are stored without volume reduction in the vapor phase of liquid nitrogen in cryovats. Where the cord blood banks are planning to bank thousands of donations, storage space is crucial. Therefore, it is often the practice for collections for the unrelated bank to be red cell and plasma depleted using such systems as the Sepax system (Biosafe SA www.biosafe.ch) to reduce the UCB collection to a standard volume. This reduces storage requirements of the bank and provides a standard product for cryopreservation. The UCB donations are cryopreserved and stored in either liquid or vapor-phase nitrogen. Many cord blood banks use a BioArchive system that provides automatic controlled rate freezing, liquid nitrogen storage, and retrieval facilities using robotics in a single machine. Details of these preprocessing procedures are not described here but may be found elsewhere ***(1,28)***. The directed UCB unit and cord biopsy once collected are cooled and delivered by courier immediately with appropriate paperwork to the processing center for cryopreservation. All collections for the unrelated UCB Bank are stored at 22 ± 2°C and processed within 24 h of collection.

3.2.1. Receipt of Harvest and Preprocessing Requirements

The objectives of the protocols below are to ensure that the UCB units are processed and cryopreserved in a uniform and aseptic manner, tested for microbiological contamination, and assessed for nucleated cell and CD34 content and viability prior to and following cryostorage. The cryostorage of UCB units described here is carried out in a LAF cabinet in a clean room as in **Subheading 3.1.** pending the introduction of closed systems where this may not be required ***(29)***. For directed UCB units, procedures are similar to those for BM and MPB as detailed in **Subheading 3.1.** Specific modifications to these processes for archiving unrelated allogeneic reduced volume UCB units in a BioArchive are also described.

UCB should be treated as potentially infectious. In handling it, the same precautions should be observed as with MPB and BM donations. The virology status of the mother donating the UCB unit must be confirmed. Although collections and storage are usually restricted to mothers who have had mandatory virology negative clearance, occasionally a request may be granted for the collection and storage of a directed UCB unit from a mother who is virology positive. Potentially, virology positive UCB harvests are quarantined in a designated cryovat until the status is confirmed. Unrelated collections are accepted only from mothers who have consented to be tested for HIV and hepatitis C virus (HCV) during their pregnancy.

1. Appropriate forms and labels are completed to record and track the donations from collection, processing, testing, freezing, storage, and issue. All UCB donations, associated samples, products, and records must be labeled according to appropriate standards (i.e., FACT-Netcord; *see* **Note 9**). All collections are assigned a unique barcode number (ISBT 128 standard) that is subsequently used to identify all related samples, paperwork, and the final cryopreserved product. Labels for directed donations include the mother's name and date of birth and clearly identify the donation as restricted for either a named person or a family. All unrelated donations are identified with their unique ISBT 128 donation number.
2. For the directed donations, the Kryo 10 CRF Dewar should be prepared ready for use before entering the clean room and set up using the appropriate freezing program as described in **Subheading 3.1.** (*see* **Notes 5**, **6**, **8**, **9**, and **11**). Following the automatic nightly maintenance period including liquid nitrogen filling of the system, the Bio Archive defaults to a ready status for freezing.
3. Any part of the processing or cryopreservation that is not a closed process must be carried out in a clean room. Procedures for entering and preparing the clean rooms are as described in **Subheading 3.1.1.**

3.2.2. Cell Analyses

A sample of UCB taken from the collection or from the bleed-line (as described in **Subheading 3.1.1.**) is used to perform a full blood count using a hematology analyser. The nucleated cell dose of a donation is used to assess the suitability for transplant. For directed UCB units, CD34 and viability assays (7-AAD, flow cytometry, and CFU) are performed on the starting material and on aliquots of the cryopreserved harvest stored in cryovials either 1 wk after cryopreservation or at the time of issue. The unrelated UCB units are assessed for nucleated cell content pre- and postvolume reduction, and the process product is assayed for CD34+ cell content and dye exclusion viability with 7-AAD. At the time of issue for transplant an integral sample attached to the frozen unit is removed and assayed for functional and dye exclusion viability (CFU and 7-AAD) and nucleated and CD34+ cell content as an indicator of the state of the cryopreserved cells at the time of transplant. The UCB samples are tested for ABO

and Rh blood group antigens, for specific viruses and bacterial and fungal contaminants, HLA typed, and a hemoglobinopathy screen is performed ***(1,28)***. Short tandem repeat analysis on a sample removed from the bleed-line of the freezing bag is used to confirm identity of the donation at issue for transplantation ***(30,31)***.

3.2.3. Preparation and Addition of the Cryoprotectant

The cryoprotectant stock used is 50% (w/v) DMSO in Dextran-40 (purchased ready prepared) or 20% (v/v) DMSO made up in 10% (w/v) Dextran-40 in 0.9% (w/v) saline. The final concentration of DMSO after addition to the harvest is 10% (v/v) DMSO (*see* **Notes 15–17**). Fifty percent (v/v) DMSO solution is generally used for cryopreserving cord blood units for the unrelated bank, a strategy implemented to minimize liquid nitrogen storage requirements.

For the directed UCB unit:

1. Insert a sample site coupler into a port on the harvest bag (*see* **Note 18**).
2. Remove a 4-mL sample using a 5-mL syringe and place into a labeled 4.5-mL cryotube to be used for the test **(Subheading 3.2.2.)**.
3. Place the collection bag on the flat bed mixer to mix thoroughly. Ensure collection details, process volume, and batch details of reagents are recorded before proceeding.
4. Pre-cool the cryoprotectant and diluent on ice and add the DMSO slowly as in **Subheading 3.1.** and as follows:
   a. Calculate the volume of cryoprotectant needed to freeze the UCB volume. The volume of DMSO to be added to the Dextran will give a concentration of 20% (v/v) DMSO in 8% (w/v) Dextran-40 in saline.
   b. Insert a "2991" coupler into the port of a 600-mL transfer bag.
   c. Withdraw the required volume of 10% (w/v) Dextran-40 in 0.9% (w/v) saline into 50-mL syringes and transfer into the 600-mL bag via the coupler. Place on the cold block.
   d. Remove the required volume of DMSO using a 50-mL syringe with a 19G needle together with 3 mL of air.
   e. Remove the needle and connect to the coupler on the Dextran-40 bag. Add the DMSO at a rate of 2.5 mL per 15 s (i.e., 10 mL/min) to the transfer bag containing Dextran-40 (on a cold block) ensuring the bag is gently agitated.
   f. Use the air in the syringe to empty the coupler tubing of DMSO.
   g. Record the start and finish times for the addition of the DMSO on the calculation sheet.
   h. Once all the DMSO is added, close the coupler and seal the coupler tubing with three seals.

      Allow the solution to cool on the cold block for a further 5 min. If necessary, the cryoprotectant solution can be stored between 2 and 8°C for up to 24 h. Ensure that the bag is in a secondary container and placed in a controlled and monitored cold room or refrigerator.

3.2.4. Cryopreservation Preparation

For open processes in the clean room, all items are assembled in the Class 2 LAF safety cabinet after wiping with IPA as in **Subheading 3.1.** Once the procedure has started it must be completed without interruption. As with BM and MPB, it is recommended that only one donation be processed at a time in an individual Class 2 cabinet. Record all data during processing and cryostorage. Environmental and product monitoring are essentially as described in **Subheading 3.1.**

For the directed donations:

1. Calculate the number of cryocyte bags required as in **Subheading 3.1.**
2. Prepared cryocyte bag labels (with time, bag volume, and number of bags) are completed and placed into the label pocket of each bag. Using the line sealer, place three spot seals across the edge of each pocket.
3. Check that all roller clamps are closed and the freezing bag are joined using the light blue and dark blue luer connectors.
4. Unused lines are clamped or sealed and removed.
5. Four cryovials are labeled with the baby's surname and cryopreservation date.

For the unrelated donations one freezing bag is required:

1. Prepare printed labels with the unique barcoded and eye readable donation number, proper name of product, i.e., red cell and plasma-depleted cryopreserved cord blood, name, and additives including anticoagulant and cryoprotectant, recommended storage temperature, and name of cord blood bank. Affix to the freezing bag.
2. Prepare the freezing canister with the barcoded donation label inside and affix barcoded label on the allocated area on the outside, which is scanned by the BioArchive machine in order to function.

3.2.5. Adding the Cryoprotectant to the Cells

3.2.5.1. Procedure for Directed UCB Donation

1. Place the flat bed mixer in the LAF cabinet.
2. Prepare the following transfer assembly for addition of cryoprotectant to the harvest, taking the red port on the three-way stopcock (*see* **Note 18**) nominally as NORTH Attach:
   a. A 50-mL syringe to the SOUTH port.
   b. A transfer set to the EAST port.
   c. A luer/spike interconnector to the NORTH (**Fig. 1**).
   d. Ensure the stopcock is shut.
3. Insert the spike (EAST) on the transfer assembly into the spare port on the cryoprotectant bag. Insert the interconnector (NORTH) into the harvest bag and leave on the mixer to rotate. Leave the cryoprotectant bag on the cold block.
4. Add volume of diluted cryoprotectant equal to that of the cells to the harvest bag via the 50-mL syringe.

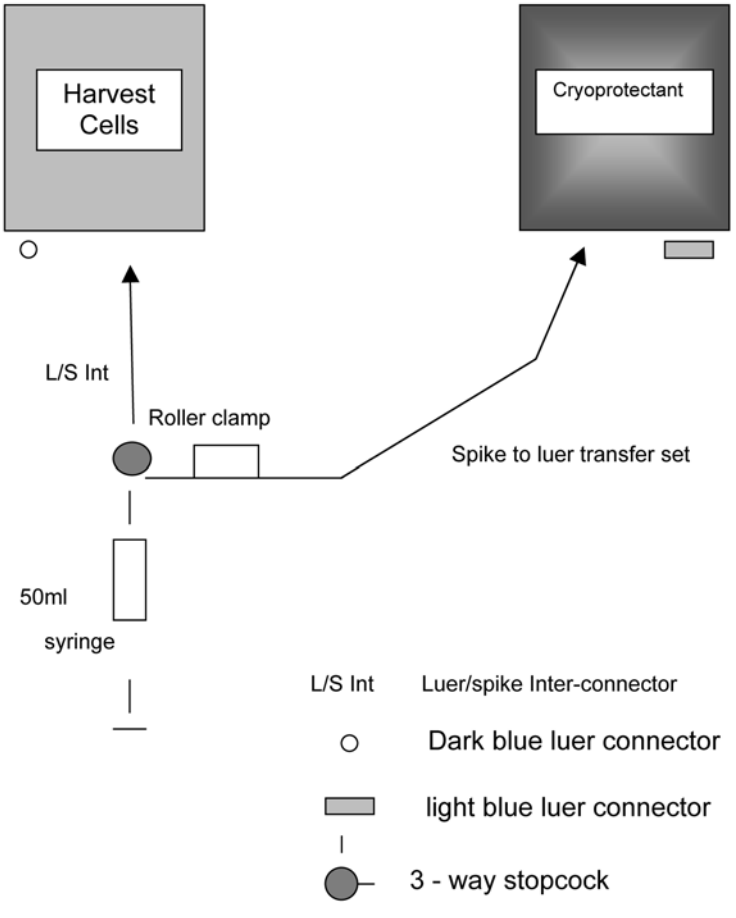

Fig. 1. Addition of cryoprotectant to harvest cells. The protocol is described in **Subheading 3.2.5.1.** and allows optimal transfer of the cryoprotectant to the harvested cells.

a. Adjust the stopcock to allow the cryoprotectant to be pulled into the syringe.
b. Ensure all air is pushed back into the cryoprotectant bag before readjusting the stopcock and adding the contents of the syringe through the interconnector into the harvest bag.
c. The cryoprotectant must be added to the harvest cells slowly (5 mL every 30 s, i.e., 10 mL/min).
d. Record the start and finish times on the calculation sheet.

5. On completion of the cryoprotectant transfer, close the stopcock and move the roller clamp on the transfer setup to the stopcock and close. Carefully detach the transfer set from the EAST port of the stopcock and attach the cryocyte bag to

the spare male luer connector. Discard the cryoprotectant bag and attached transfer set into a sharps bin.
6. Place the cell and cryoprotectant solution on the mixer on the cold block.
7. With a 50-mL syringe/tap assembly, opening roller clamps, and using Spencer wells as necessary, transfer the appropriate amount of cells/cryoprotectant into the first bag. Remove any residual air from the cryocyte bag and clamp.
8. Fill the remaining bags in the same manner, retaining 4 mL in the syringe for the cryotubes and blood culture bottles.
9. Close the stopcock and seal off the cryocyte bags with three seals close to the ports. Remove the bags at the third seal. Detach the syringe from the assembly and attach a 19-gage needle.
10. Dispense 0.5-mL aliquots into the four prelabeled cryotubes. Inoculate two BactAlert culture bottles.
11. Check the cryotubes are correctly labeled and put them into the storage cane.
12. Perform right and left glove prints as in **Subheading 3.1.4.**
13. Place each cryocyte bag into an individual vacuum-pack bag and seal in the Multivac C100 vacuum packer at 250 mbar for 1.8 s. Seal the bags so that they fit the cryocyte bags firmly.
14. Keep the cryocyte bags and cryotube cane on the cold pack, and freeze without delay.

3.2.5.2. Concentrated UCB Samples

The following describes the procedure for UCB that has been reduced to a standard volume of 21 mL and have been cooled to 4°C. The concentrated cells are automatically transferred to the freezing bag on completion of the volume–reduction process when a system such as the Sepax process and the Pall cryobags specified are used.

1. Place a cold pack with the cooled UCB cell concentrate secured on top to a mixer. Attach a luer connector to the freezing bag bleed-line. Switch on the mixer.
2. Attach a syringe filled with 5.5 mL 50% (w/v) DMSO diluted in Dextran-40 to the luer connector. Push 0.5 mL of DMSO into the line and break the seal (*see* **Notes 15–17**).
3. Install the syringe on a syringe pump. Switch on the pump, confirm the syringe size, and select an infusion rate of 20 mL/h allowing the DMSO to be added over 15 min. Press start to commence infusion. During infusion the edges of the pack must be manually manipulated frequently to ensure thorough mixing.
4. A warning alarm on the pump sounds when 4% of the DMSO volume in the syringe remains, and the pump continues infusing. On completion the pump automatically stops and sounds an alarm.
5. Remove the syringe from the pump and slowly transfer the remainder of the DMSO to the freezing bag, continuing to mix.
6. Remove air from the freezing bag using the syringe, ensuring all cells and DMSO are returned to the freezing bag; heat-seal the bleed-line just above the injection port.

7. Remove samples from the freezing bag for archiving (into cryovials) and bacterial and fungal screening (BacTAlert bottles or equivalent).
8. Place the freezing bag in the jig for the Pall cryobags (this ensures that the bag is not over-filled) and heat-seal as follows: one immediately below the injection port, one in the middle of the bleed-line segment, one on the bleed-line as close as possible to the freezing bag, and one on each of the links between the major and minor bag compartments.
9. Visually check all heat-seals and pressure test to ensure the integrity of the seals.
10. Weigh the final freezing bag and place in an overwrap bag and vacuum seal as close to the freezing bag as possible, check the seal.

3.2.6. Freezing Cells, Testing, and Records

Directed donations are frozen using the CRF as detailed in **Subheading 3.1.** Once the freezer program is completed the frozen bags and sample tubes are transferred to the storage vessel. Any material of unknown virology status must be stored in a quarantine tank until the virology status is negative. The cryovials are stored in the same vessel as the cryobags until such time as all the harvest has been reinfused or discarded. All records relating to the collection and cryopreservation, including a copy of the freezing profile printout, must be completed, ensuring all actions and procedures are traceable. Reports and records must be stored securely for 30 yr according to EU legislation (*see* **Note 8**).

For the volume reduced unrelated UCB units to be banked and stored in the BioArchive, the following procedure is followed:

1. Place each freezing bag in the appropriately labeled BioArchive metal freezing canister.
2. Transfer to the CRF module of the BioArchive system and initiate freezing. Select the port to be used and insert the CRF module. Once the donation number has been read from the canister, verify the port, select the freezing profile, and scan the barcode of the unit from the paperwork. Following barcode verification of the unit with the sample barcode "wanded" by the operator, the freeze program is initiated (*see* **Table 2** for an example).
3. On completion of the freeze process, the BioArchive system automatically moves the canister containing the frozen unit to a permanent storage location in the Dewar. The location is recorded in the BioArchive system database and on the freeze profile printout. Status information throughout the process is displayed on the computer monitor.
4. Cryovials are frozen separately in this procedure and are placed in the next available position in a separate Dewar.
5. Environmental monitoring is carried out as in **Subheading 3.1.**
6. Records must be fully completed and stored for 30 yr (*see* **Notes 8** and **21** for additional information on UCB bank programs).

Table 2
Example of a Freezing Profile Used for Unrelated UCB Volume Reduced Units

Prefreeze rate	10°C/min
Freeze start temp	–1°C
Freeze power	100%
Freeze exit temp	–11°C
Postfreeze rate	–2°C/min
Target temp	–50°C

Validation was carried out in-house.

4. Notes

1. There have been a number of excellent review papers mainly in the journal *Cryobiology* published by Academic Press (ISSN 0011-2240).
2. Websites of interest include the Society for Low Temperature Biology (www.sltb.info) and the Society for Cryobiology (www.societyforcryobiology.org).
3. Relevant national and international standards/guidelines are found (where not provided here in detail) in the most recent versions of or on relevant websites as in **Notes 4–10**.
4. JACIE guidelines are standards for blood and marrow progenitor cell processing, collection, and transplantation. From the Joint Accreditation Committee of ISCT-Europe and EBMT (JACIE) (www.jacie.org; www.ebmt.org).
5. Department of Health. A code of practice for tissue banks providing tissues of human origin for therapeutic purposes (http://www.dh.gov.uk; www.mhra.gov.uk; www.hta.gov.uk).
6. UKBTS/NIBSC (Red Book): guidelines for the blood transfusion services in the United Kingdom.
7. Department of Health. Guidance on the microbiological safety of human organs, tissues, and cells used in transplantation. Advisory committee on the microbiological safety of blood and tissues for transplantation (MSBT) (http://www.dh.gov.uk).
8. The EU directive for tissues and cells (http://www.hfea.gov.uk; www.dh.gov.uk). In the United Kingdom, mandatory licensing of cell processing and storage facilities for clinical transplantation became the remit of the Human Tissue Authority in April 2006 (www.hta.gov.uk).
9. FACT-Netcord international standards for cord blood collection, processing, testing, banking, selection, and release (https://office.de.netcord.org; www.unmc.edu; www.ebmt.org).
10. UKBTS/NIBSC cord blood donor selection guidelines (http://www.transfusionguidelines.org.uk).
11. Rules and guidance for pharmaceutical manufacturers and distributors (The "Orange Guide") Author: Medicines and Health Products Regulatory Agency. Published by The Stationary Office, London UK.

12. If the apheresis collection has a total nucleated cell count greater than 200×10^9/L, it requires diluting back to this cell concentration. If the collection is to be processed immediately the dilution can be done prior to adding the cryoprotectant, however, if the cell collection is to be stored overnight at 4°C, the collection will need diluting prior to storage. The diluent can be autologous plasma collected during the leukapheresis procedure or 4.5% (w/v) HAS.
13. It is important to ensure that the predonation virology screening (maximum 30 d prior to donation) has been done and a hardcopy of the results has been obtained. Untested donations may be processed, but must be stored in quarantine tanks until product testing is completed. In cases of clinical need, known mandatory marker positive donations may be processed while ensuring their complete separation from other donations. All waste materials used in processing viral marker positive donations must be autoclaved prior to disposal. Staff handling hepatitis B virus positive donations must have up-to-date immunization against hepatitis B virus.
14. A problem with using a sterile connecting device within a clean room is that of the excessively high particle numbers that are generated. To obviate this we place the sterile connecting device in a small cabinet (Powdercap™) that houses a Hepa filter allowing total removal of the particles.
15. Neat DMSO can dissolve various types of plastic, therefore it is important to ensure that any plastic material coming into contact with neat DMSO is resistant. At the time of writing, there are no commercially available sample site couplers fully validated that are resistant to DMSO, although closed systems are under development. Therefore, we have found that it is best to use a cryocyte bag to make up the cryoprotectant. The volume of cryoprotectant required is equal to the volume of cell suspension plus 10 mL with an additional 40 mL for a control bag.
16. The addition of DMSO to water causes an exothermic reaction. The heat release is such that plasma proteins may be denatured. This is avoided by precooling the diluent and adding the DMSO to the diluent slowly.
17. Although the method described here uses 10% (v/v) DMSO as the cryoprotectant other methods use 5% (v/v) DMSO ***(13,15,24,32,33)*** either alone or in combination with 6% (w/v) hydroxyethyl starch ***(34,35)***. In a randomized phase III trial, Rowley et al. ***(21)*** compared 10% (v/v) DMSO vs 5% (v/v) DMSO in combination with 6% (w/v) hydroxymethyl starch that indicated that engraftment times for patients receiving cells frozen using the combination cryoprotectant was 1 d faster than for those frozen with DMSO alone. An advantage of using the lower concentration of DMSO is that it reduces the exposure to the patient. The exposure to DMSO can also be lowered by freezing the cells at a higher concentration. This has been shown to have little effect on engraftment ***(36)***.
18. Some methods use syringes and three-way taps to prepare cryoprotectant, add cryoprotectant to cells, and aliquot into freezing bags. A useful tap system, Discofix, is produced by Braun with up to five three-way taps linked together in series.
19. When adding cells to cryocyte bags it is important that the volume of cells put into each bag does not exceed the manufacturer's limits both maximum and minimum

(*see* **Subheading 3.1.1., step 4**). If these values are not adhered to, the viability of the cells may be compromised during the freezing procedure.

20. It is not essential to use a CRF. Some groups freeze cells by placing directly into a –80°C mechanical freezer ***(32–34)***.
21. **Reference 37** describes requirements for an effective national cord blood stem cell bank program being developed in the United States and contains additional information on UCB banking.

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18

Cryopreservation of Human Embryonic Stem Cell Lines

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Summary

Two different approaches have been adopted for the cryopreservation of human embryonic stem cells (hESCs): vitrification and conventional slow cooling/rapid warming. The vitrification method described here is designed for hESCs that grow as discrete colonies on a feeder cell monolayer, and are subcultured by manual subdivision of the colonies into multicellular clumps. hESCs that are subcultured by enzymatic dissociation can more conveniently be cryopreserved by conventional slow cooling/rapid warming methods. Although both methods are suitable for use in a research context, neither is suitable for cryopreservation of embryonic stem cells destined for clinical diagnostic or therapeutic uses without modification.

Key Words: Human embryonic stem cells; hESCs; vitrification; cryopreservation; controlled-rate cooling.

1. Introduction

In 1998, the successful derivation of a human embryonic stem cell (hESC) line was first reported by Thomson et al. ***(1)***. Since then, the derivation of hESC lines from donated in vitro fertilized embryos has been reported by an increasing number of groups worldwide (*see* **ref. *2*** for review). Two different approaches to cryopreserving hESCs have been adopted: conventional slow cooling/rapid warming and vitrification. The former is based largely on protocols developed for murine embryonic stem cells (mESCs), while the latter is based on vitrification methods developed for embryos. To date, no systematic studies on optimizing cryopreservation protocols for hESCs have been conducted and the few empirical studies that have been undertaken have yielded inconsistent results; not only in terms of "viability" of the cell lines examined, but also in a clear choice of a preferred cryopreservation protocol.

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Methods for the cryopreservation and storage of mESCs are well established ***(3)***. They generally employ a conventional cryoprotectant solution, which consists of 10% (v/v) dimethyl sulfoxide (DMSO) in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10–90% (v/v) fetal bovine serum (FBS). Slow cooling in cryovials, at or around 1°C/min, and rapid thawing within a 37°C water bath complete the process. Apart from minor variations, such as preservation on microplates ***(4,5)***, the methods adopted for mESCs are similar to standard protocols routinely used for established cell lines and primary cultures.

Adapting the mouse protocols for hESCs has, in some hands, proved problematic (*see* **ref. *6*** for review). This has been attributed to the highly "cooperative" nature of hESCs (as compared with mESCs), which appear to require intimate physical contact between the cells of the colony (to permit cell–cell signaling) and an optimum clump size of approx 100 cells during serial passage ***(7,8)***. A number of studies of hESCs, comparing vitrification and slow cooling, have reported extremely low viability following the latter ***(9–11)***. In these studies, survival rates of between 6 and 30% (as determined by the ability of colony fragments to reattach, multiply, and retain their undifferentiated state) were reported, with the conclusion that vitrification was the preferred option.

However, a number of stem cell lines have been reported to survive conventional slow cooling/rapid thawing. Whole cell colonies, cryopreserved while still adhering to the culture dish, or embedded in a Matrigel matrix showed an increased level of survival (as measured by Trypan blue dye exclusion or the metabolic assays MTT and Alamar blue) when compared with colony fragments cryopreserved in suspension ***(12)***. Colony fragments, when frozen in suspension, can also show high levels of survival and growth. The HuES cell lines may be cryopreserved in this way, using 10% DMSO and FBS or DMEM ***(13)***. A study using the hESC line H1 also showed that survival rates (as measured by colony number and size) of up to 80% could be achieved using a DMSO-FBS-DMEM cryoprotectant solution. The factors critical for optimizing colony (and cell) survival were a cooling rate of between 1 and 1.8°C/min, and seeding samples at –7 to –10°C ***(14)***. A similar study, again using a DMSO-FBS-DMEM cryoprotectant solution, has reported high levels of survival (>80%) with a cooling rate of 0.5°C/min and seeding at –10°C ***(15)***. The use of a controlled-rate cooling device for slow cooling, although desirable from a quality system and regulatory perspective, is not a requirement as passive cooling devices were no less effective than controlled-rate cooling in a number of studies ***(14,16)***. The protocol used by Ware et al. is published in their paper ***(14)***, whereas that for the HuES cell lines can be found in the technical appendix published separately to the paper ***(13)***. However, details of the cooling rate used have been omitted from the supplement.

Nevertheless, the majority of groups deriving stem cell lines have adopted vitrification as the method of choice despite the practical difficulties associated with this methodology. This choice is based largely on the comparative studies previously referred to ***(9–11)***, all of which indicated that vitrification led to increased post-thaw survival of colony fragments when compared with conventional slow cooling. Survival rates of more than 75% were reported in all three studies.

The vitrification protocols reported in all three studies are very similar, based as they are on one previously developed for the cryopreservation of bovine ova and embryos ***(17)*** and applied to hESCs with some modification by Reubinoff et al. ***(9)***. The essence of this protocol has since been described in a manual on hESC culture produced by ESI International ***(18)*** and available via their website. The protocol requires stepwise exposure of colony fragments to two vitrification solutions of increasing cryoprotectant concentration. The common components of which are DMSO and ethylene glycol (EG). The composition of the vehicle solution varies, with differences in sucrose concentration and the presence or absence of FBS, human serum albumin, and buffer (HEPES).

Extremely rapid cooling rates are required to achieve vitrification using this two-component system. This is accomplished by direct immersion into liquid nitrogen (LN_2) of open-pulled straws (OPS) containing small droplets (typically 1–20 μL) of vitrification solution within which were held the colony fragments. Warming, too, has to be sufficiently rapid to avoid devitrification and ice crystallization. This is accomplished by direct immersion of the vitrified samples into a prewarmed, sucrose-based thawing solution followed by stepwise elution of the cryoprotectants using sucrose as an osmotic buffer.

The requirement for rapid cooling and warming has precluded the use of conventional cryovials in the vitrification protocols so far developed for hESCs. The preferred option has been for OPS, though a closed-straw alternative has been shown to provide equally high survival ***(10)***. An alternative method, using intact adherent colonies and a specially designed culture dish and cryovial, has been proposed ***(19)*** but no trials using this method have so far been published.

The vitrification methods so far developed, although suitable for research applications where only small stocks of cells are required, nevertheless impose a number of constraints on large-scale cell banking and storage of embryonic stem cells for use in regenerative medicine or pharmacological and cytotoxicity studies. These applications will require large banks of cells that will have been produced under regulatory frameworks requiring a sterile product prepared in media that is free, as far as possible, from xeno-derived components. Some attempt has been made to address these problems. Substituting closed

straws for open straws, human alternatives such as human serum albumin for animal components such as FBS, and storage in the vapor phase above LN_2 rather than in liquid phase have all been shown to have little or no detrimental effect on colony survival or the level of cell differentiation ***(10)***. However, many of the regulatory requirements, particularly those relating to contamination, and their impact on the cryopreservation and storage of hESCs, have yet to be addressed.

Although the method detailed in this chapter has been shown to be effective for a range of hESC lines under laboratory conditions, it is extremely labor intensive, requiring time-consuming manual dissection and manipulation of the colonies through a series of solutions under a low-magnification stereomicroscope. Transfer of the colony fragments into and out of the various vitrification solutions is time critical, as is the process of transfer to and from liquid nitrogen. Moreover, the use of straws (sealed or otherwise) is incompatible with efficient, routine preparation of large banks of cells. All these procedures require a high level of acquired skills and do not lend themselves to the preparation of the bulk quantities of hESCs required for therapeutic and diagnostic applications.

2. Materials

Appropriate facilities for following good cell culture practice are required (*see* **Note 1**).

2.1. Equipment

1. Laminar flow hood.
2. Cell culture incubator.
3. Binocular stereo-zoom microscope with transmitted light base and tilting substage mirror or darkfield illumination system (*see* **Note 2**).
4. 37°C warm plate or heated microscope stage (optional) (*see* **Note 3**).
5. Appropriate protective clothing and safety equipment for handling LN_2.
6. Small (500 mL or 1.0 L) wide-neck vacuum flask/Dewar.
7. LN_2 vessel for precooling cryovials.
8. LN_2.
9. Pipettors and sterile tips for 1- to 1000-µL volume range.
10. Dual-function countdown timer.
11. Colony cutting tool (*see* **Note 4**).
12. 4- or 5-well cell culture plate or small culture dish (*see* **Note 5**).
13. 6-cm bacteriological Petri dishes (*see* **Note 6**).
14. 5-mL screw-cap cryovials.
15. Aluminium cryovial storage canes (*see* **Note 7**).
16. 21-gage × 1 in. needles.
17. Sterile OPS (*see* **Note 8**).
18. Long (20 cm) and short (12 cm) forceps.
19. Test tube rack to hold cryovials and solutions.

2.2. Cells

hESC colonies of 1–3-mm diameter, growing on a feeder cell layer in a single or multiwell cell culture dish.

2.3. Solutions

1. Growth medium (GM): use appropriate GM recommended for your particular hESC line.
2. Base medium (BM) for vitrification and thawing: 89% (v/v) 25 m*M* HEPES-buffered DMEM (Invitrogen, cat. no. 42430-025, Paisley, Scotland, UK), 10% (v/v) FBS, and 1% (v/v) nonessential amino acids (Invitrogen, cat. no. 111430-035, 10 m*M* solution). Store for up to 1 wk at 4°C.
3. Base medium with 1 *M* sucrose (BMS): for a 10-mL final volume, add 3.42 g sucrose to 6 mL 25 m*M* HEPES-buffered DMEM and warm to 37°C to dissolve. Add 2 mL FBS, plus enough DMEM to bring the final volume to 10 mL. Sterilize by passing through a 0.2-µm syringe filter. Store for up to 1 wk at 4°C.
4. Vitrification solution 1 (V1): 80% (v/v) BM, 10% (v/v) DMSO, 10% (v/v) EG. Filter-sterilize as above can be aliquotted and stored at –80°C.
5. Vitrification solution 2 (V2): 50% (v/v) BMS, 10% (v/v) BM, 20% (v/v) DMSO, 20% (v/v) EG (*see* **Note 9**). Filter-sterilize as above can be aliquotted and stored at –80°C.
6. Thawing solution (T1): 80% (v/v) BM and 20% (v/v) BMS. This solution is only required for the alternative thawing method (*see* **Note 10**).
7. Thawing solution with 0.1 *M* sucrose (T2): 50% (v/v) T1 and 50% (v/v) BM. This solution is only required for the alternative thawing method (*see* **Note 10**).

3. Methods

All culture manipulations should be carried out following good cell culture practice/aseptic technique in a laminar flow hood if possible (*see* **Note 1**).

3.1. Vitrification

1. Prepare all solutions in advance. Fill the wide-neck vacuum flask/Dewar with LN_2 using the appropriate safety equipment.
2. Set out equipment in the work area (laminar flow hood) including the flask/Dewar containing LN_2.
3. Prepare a well or dish containing 1 mL of fresh GM to hold the colony fragments after dissection.
4. Prepare a vitrification plate. This can be the base or lid of a 6-cm Petri dish (*see* **Note 6**). Pipet one 200-µL drop of V1 and two 100-µL drops of V2 spacing the drops well apart on the plate and avoiding air bubbles.
5. Program one timer to count down 1 min, and the other to count down 25 s.
6. Place the culture dish containing hESC colonies on the microscope stage and cut one or more colonies into appropriate-sized fragments (*see* **Notes 11–13**).
7. Using a pipettor set on 5–10 µL, prewet the tip with GM from the culture dish and transfer the colony fragments to the holding well containing fresh GM (*see* **Notes 14** and **15**). Return the culture dish to the incubator.

8. Place the vitrification plate alongside the holding plate on the microscope stage or nearby.
9. Set the pipettor to 5 µL, prewet a tip with GM and take up six fragments from the holding well (*see* **Note 13**). Focus the microscope on the 200-µL drop of V1 solution on the vitrification plate. Submerge the open end of the pipet tip in the center of the V1 drop and gently expel the colony fragments into the drop avoiding the meniscus.
10. Start the timer (set for 1 min). The fragments will gradually sink to the bottom of the V1 solution.
11. While the timer is counting down, place a new tip on the pipettor and prewet it in the V1 drop. At the end of the 1-min period collect the colony fragments in the pipet tip and transfer them into the center of the first 100-µL drop of V2 solution avoiding the meniscus.
12. Start the other timer (set to 25 s). The fragments will float in the V2 solution.
13. While the timer is counting down, change the pipet tip and prewet it in the first V2 drop. At the end of the 25-s period transfer the fragments to the second 100-µL drop of V2 solution. Proceed immediately with the next step.
14. Change the tip and prewet it in the second drop of V2 solution. Collect the colony fragments in 3–5 µL and pipet this onto a clean area of the vitrification plate to form a small drop. It is important to avoid introducing an air bubble into this drop. Make sure that the tip is prewetted and take a small volume of V2 solution into the tip ahead of the fragments so that they can be deposited onto the plate without depressing the plunger to its full extent, leaving a small residue of liquid in the pipet tip.
15. Set the pipettor aside and pick up a pulled straw (*see* **Note 16**). Holding the straw at a slight angle to the vertical, touch the narrow open end of the straw against the surface of the drop containing the fragments. The liquid and fragments should enter the straw by capillarity (*see* **Note 17**).
16. Vitrify the fragments by holding the straw vertically (narrow end down) and immersing the lower end of the straw immediately in LN_2. Rest the straw against the edge of the Dewar, allowing approx 1 cm of the straw to protrude above the surface of the LN_2. Multiple straws can be lined up around the edge of the Dewar and held in this way until transferred to cryovials. Take care that the vitrified samples always remain submerged in LN_2.
17. Repeat the procedure (**steps 5–16**) with further batches of fragments using the drops of vitrification solutions V1 and V2 on the vitrification plate up to three times. For further repetitions set up a new vitrification plate with fresh drops of vitrification solution (*see* **step 4**).
18. When the required number of straws has been processed, label an appropriate number of 5-mL cryovials and puncture each vial several times with a 21-gage needle. Remove the lids, attach each vial to the bottom of an aluminium cane, and precool in vapor phase or LN_2 (*see* **Note 7**).
19. Remove one precooled cane at a time and transfer it to the Dewar containing the straws, taking care not to disturb them. Use sterile forceps to transfer the appropriate number of straws to the cryovial (*see* **Note 18**).
20. Replace the cap and transfer to cryostorage below –160°C (*see* **Note 19**).

3.2. Thawing

The choice of method for thawing vitrified hESCs will depend on the particular embryonic stem cell line. The method described next is relatively quick and simple (for an alternative method, *see* **Note 10**).

1. Prepare a culture dish or a well containing the appropriate feeder cell layer incubated in advance in GM.
2. Retrieve a cryovial containing vitrified straws from cryostorage. Transport the vial to the work area in a container capable of maintaining the temperature below –160°C (*see* **Note 19**).
3. Fill the wide-neck vacuum flask/Dewar with LN_2 using the appropriate safety equipment.
4. Prepare a holding dish or well containing 1 mL of GM, warmed to 37°C in a cell culture incubator.
5. Remove the cryovial from the transport container, attach it to the bottom of an aluminium cane, remove the cap, and submerge the vial in the small dewar of LN_2.
6. Set a pipettor to 20 µL and attach a tip that will fit snugly into the wide end of a straw.
7. Place the holding dish/well under the microscope and remove the lid.
8. Raise the cryovial to the surface of the LN_2 and remove one straw with sterile forceps.
9. Take the straw between the thumb and middle finger and submerge the narrow end in the well of GM. As soon as the liquid inside the straw melts, place a finger over the upper end of the straw to help force the vitrification solution and colony fragments into the well. Take care not to damage the fragments with the end of the straw as they fall into the well. It is important to minimize any mechanical disturbance or mixing of the medium at this stage.
10. If the contents of the straw do not empty readily into the holding well, insert the tip of the prepared pipettor into the upper (wide) end of the straw, twisting to obtain a seal, and depress the plunger gently. If necessary, rinse out the end of the straw by gently drawing up and expelling some of the GM.
11. Carefully place the holding well in the cell culture incubator and leave to equilibrate for 5 min.
12. Bring the holding well and culture dish containing prepared feeder cells to the microscope. Using a fresh, prewetted tip gently transfer the colony fragments from the holding well to the culture dish, spacing them out evenly on the feeder layer.
13. Allow the fragments to sink onto the surface of the feeder layer before carefully returning the culture dish to the cell culture incubator.
14. Viable colony fragments will generally attach to the feeder layer within 24–72 h after thawing.

4. Notes

1. The use of appropriate facilities, good cell culture practice, and good aseptic technique should prevent contamination and provide viable colony fragments.

However, this cryopreservation method is not intended *per se* to provide cell material for clinical, diagnostic, or therapeutic use. Such material will be required to comply with regulatory requirements, which encompass the derivation, processing, storage, and distribution of the cell line, as well as cryopreservation when destined for such uses.

2. It is important that the microscope has an objective lens with a wide enough field of view to include the whole hESC colony and its environs, with sufficient working distance to allow the use of the pipettor and cutting knife.
3. Using the method described here, we have not found it necessary to warm the holding well or processing plate during vitrification or thawing. However, it may be advantageous to use a warming plate during the alternative (longer) thawing method (*see* **Note 10**) to obviate moving the thawing plate repeatedly to and from the incubator during the washing steps.
4. A number of options are available as cutting tools, including pulled glass Pasteur pipets, plastic pipet tips, sterile manufactured stem cell knives, or glass holding-pipets.
5. Multiwell cell culture plates (e.g., Medical Technology Ventriebs-GMbH Altdorf, Germany, cat. no. 19021/0005) or in vitro fertilized/organ culture dishes (e.g., Corning/Costar, Corning, NY, cat. no. 3260MTG) are required for holding the colony fragments after dissection. Shallow wells with sloping sides are preferable for ease of pipetting under the microscope.
6. Dishes that have not been coated or treated for cell attachment are preferred so that solutions can be pipetted onto the surface in discrete drops that will not spread over the plastic.
7. For easier handling of 5-mL cryovials, use canes designed to hold 2-mL vials so that the 5-mL vial will stand out at an angle from the cane affording better access when adding or removing the cap or contents.
8. OPSs are available from LEC Instruments (Victoria, Australia; www.lecinstruments.com).
9. The vitrification solution V2 used in this protocol contains 0.5 *M* sucrose. Other protocols use different sucrose concentrations, e.g., 0.33 *M*, *see* protocols at www.escellinternational.com.
10. An alternative thawing method utilizing a stepwise elution protocol involves thawing the vitrified fragments and incubating for 1 min in thawing solution (T1), followed by sequential 5-min incubations in T2, and two aliquots of BM before being plated onto the appropriate feeder cell layer. Details can be found in the ESI Methodology Manual Embryonic Stem Cell Culture, July 2005, available at www.escellinternational.com.
11. Only choose undifferentiated colonies for vitrification, and preferably those that appear relatively thick. Colonies with a flatter, thinner morphology (typically those growing on sparser areas of the feeder layer) tend to be more difficult to cut cleanly.
12. The fragments should be the largest size that can be taken up into the pipet tip (in 3–5 μL of liquid). The fragments will shrink significantly as they are passed through the vitrification solutions.

13. Cut fragments in multiples of six in order to vitrify six fragments per straw. Until practiced, it is recommended to process just six fragments at a time. With experience it is possible to process enough fragments for several straws in one operation, the limiting factor being the number of fragments that can be taken up in 5 µL of liquid. Increasing this volume is not advisable, as this may lead to excessive dilution of the vitrification solutions.
14. When transferring colony fragments from one solution to another, or whenever you use a new pipet tip, it is important to prewet the tip by drawing up and expelling some of the appropriate solution before picking up the fragments. This will help to prevent the fragments from sticking to the tip and avoid air bubbles.
15. Always minimize the pipetting of fragments. Pipet slowly, using the smallest volume pipettor (5- or 10-µL size), and set it to the minimum volume necessary to transfer the fragments. With practice it is possible to pipet 12 or more fragments in 3 µL.
16. As there may be some variability in the length of the OPSs within a packet, check that your straws will fit inside your 5-mL cryovials before picking up the processed fragments.
17. Taking up the fragments into the straw can take several seconds, and may be helped by resting the tip of the straw on the plate and slowly tilting it into a vertical orientation. Because of the short incubation time in V2, the fragments do not become fully equilibrated and will tend to float toward the top of the column of liquid as it enters the straw. If all of the liquid from the small drop is not taken up, leaving the fragments close to the open end of the straw, it is often possible to move them further into the straw by dipping the end of the straw into one of the larger drops of V2.
18. In terms of safe storage and stock inventory, it is advisable to place only one or two straws per cryovial. This enables the minimum number of straws to be removed from cryostorage when cells are required for culture, and obviates the need for relabeling or replacing vials when part of their contents have been removed.
19. Vitrified cells must always be maintained below –160°C to avoid devitrification. Straws must not be stored or transported on dry ice.

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19

Cryopreservation of Primary Animal Cell Cultures

Glyn N. Stacey and Stuart Dowall

Summary

Cells isolated directly from tissues (primary cultures) have many applications in research and applications in the fields of toxicology, pharmacology, and virology. Where such preparations remain the only scientific option to achieve necessary results it is still possible to refine and reduce the use of animal and human sources of the tissue by cryopreservation of the primary cells for later use, this reduces the need for fresh tissues and enables improvements in standardization through the ability to provide the same cell preparation at different times and to different laboratories. The methods described provide options for adherent cultures as monolayers, harvested cell suspensions, and also lymphocytes isolated from peripheral blood.

Key Words: Primary cells; cryopreservation; primate kidney cells; mononuclear cells; adherent cultures; animal cells.

1. Introduction

Historically the fields of toxicology, pharmacology, and virology have relied on the use of animals to provide vital research data for the development of new products and the isolation and investigation of infectious diseases. However, over a number of decades there has been a strong movement toward adopting the three Rs principle to refine, reduce, and ultimately replace the use of animals for research and testing ***(1)***. This is now supported at national and international levels ***(2)*** and much interest has been focused on the development of alternative in vitro techniques. The requirement for functional cell-based assays and virus isolation from infected material has maintained the need for tissue preparations in the form of primary cell cultures. Where such preparations remain the only scientific option to achieve necessary results it is still possible to refine and reduce the use of animal and human sources of the

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tissue by cryopreservation of primary cells for later use. Cryopreservation techniques provide an opportunity to reduce the need for regular fresh animal tissue and can enhance standardization of laboratory work by enabling the use of the same source of cells over a period of an experimental program and in different laboratories.

It may be tempting to reserve the use of cryopreservation methods for spare or suboptimal cultures left over from formal experiments to provide a perceived "backup" for emergencies in future experiments. However, attempts to use cryopreserved spare cultures will not enable the full benefits of the prospective establishment and qualification of cryopreserved cell stocks to be realized. It is also valuable to try to assure the quality of the primary cell obtained for preservation and the establishment of a protocol agreed with clinicians or other staff providing the cells or tissues ***(3)***. This protocol should also ensure that all legal and ethical requirements have been addressed; this is especially important for human cells and tissues where those responsible for the use of the cells should be able to demonstrate that the materials were obtained with fully informed consent ***(4)***.

Once a reliable archive of preserved cells has been established it can be quality controlled and qualified for particular purposes, and if necessary, tested for infectious agents. This provides a valuable reference stock of cells that may be considered safer for laboratory use than unqualified supplies of fresh tissue. Primary cell preparations are often very complex mixtures of cells with a very broad range of functions and morphologies. These various populations are likely to vary in their amenability to preservation. Loss of critical cell populations could therefore affect the utility of preserved cultures so it is particularly important to confirm the retention of key functional characteristics (or to identify levels of certain key cell types) in cultures recovered from a cryopreserved "bank" of cells.

Such qualified banks of stored material can provide a reliable and convenient source of fresh cells for routine experimentation. This is invaluable where primary feeder cells are required for the isolation, maintenance, and expansion of complex and potentially unstable cultures that are difficult to grow. An important example is the case of primary mouse embryonic fibroblasts used for the culture of human embryonic stem cells ***(5)***.

When carrying out the preparation of a stock of cryopreserved cells it is important to select the cultures to be used to eliminate any that do not meet certain criteria that include:

1. Cultures should be subconfluent to avoid the use of cells in the plateau phase of growth that may not survive cryopreservation (e.g., **ref. *6***).
2. Under the microscope cultures should exhibit typical, healthy morphology without cytopathic effects such as high levels of vacuolation or cell death.

3. There should be no evidence of microbial contamination, which may include observation as rafts of organisms seen under the microscope (i.e., fungi and bacteria) or gross turbidity in the growth medium, positive results in sterility tests (*see* **Note 1**), evidence of viral contamination such as "plaques" (areas of cell death), or cell fusion or enlargement.

At the point of preparing the selected cultures for preservation it is important to eliminate any materials that might lead to loss of viability during preservation (*see* **Note 2**) and to ensure homogeneity of the cell bank being prepared, i.e., does vial 1 of the stock provide the same representative culture as vial 100 (*see* **Note 3**).

The methods described here provide options for adherent cultures as monolayers and harvested cell suspensions, and also lymphocytes isolated from peripheral blood based on the original principle of Boyum *(7)* as general examples of some of the more common methods used for preservation of primary cells.

2. Materials

2.1. Equipment

1. Bench centrifuge with sealable biohazard-containment centrifuge buckets.
2. Inverted microscope with ×20 and ×40 objectives.
3. Class II biological safety cabinet compliant with the British Standard 5726 (2005).
4. 1 and 10 mL plastic sterile pipets and a pipet pump.
5. Trypan blue (0.4% [w/v] in PBS-A).
6. Hemocytometer (Neubauer improved).
7. Programmable freezer or alternative slow cooling system (*see* **Note 4**).
8. Liquid nitrogen storage system and supply of liquid nitrogen: cryostorage containers with appropriate storage racks and an inventory system suitable for holding cryovials or other vessels described in **Subheadings 3.1.–3.3**.
9. Low-temperature-resistant marker pens or labeling machine.
10. Biohazard waste bags and waste bag containers for contaminated plastics (all waste must be disposed of according to the local safety guidance).

2.2. Cryopreservation of Adherent Primary Cells

1. Near confluent monolayer cultures of primary cells in T75-cm^2 tissues culture flasks or multiwell (6 or 24 well) trays (γ irradiated and cell culture tested).
2. Growth medium: 1X Eagle's Minimal Essential Medium basal medium, 10% (v/v) fetal calf serum, 2 m*M* glutamine, and 1% (w/v) nonessential amino acids.
3. 0.5 g/L trypsin/0.2 g/L EDTA in Puck saline A (0.4 g/L potassium chloride, 8 g/L sodium chloride, 0.35 g/L sodium hydrogen carbonate, 1 g/L D-glucose, and 0.005 g/L phenol red).
4. Phosphate buffered saline (PBS): 0.2 g/L potassium dihydrogen phosphate, 1.15 g/L anhydrous disodium hydrogen phosphate, 0.2 g/L potassium chloride, and 8.0 g/L sodium chloride dissolved in deionized water with a final pH of 7.4 ± 0.2 pH units).

5. Humidified carbon dioxide/air (5/95% [v/v]) incubator.
6. Water bath (37–40°C).
7. Cryoprotectant solution: 90% (v/v) fetal calf serum and 10% (v/v) dimethyl sulfoxide (DMSO; spectroscopic grade) (*see* **Note 5**).
8. Adhesive tape.
9. Cryostorage ampoules (e.g., Cryovials from Nunc/Invitrogen).

2.3. Cryopreservation of Peripheral Blood Mononuclear Cells

1. Primary cells: 10–20 mL of whole blood taken in heparin-containing tubes.
2. Culture medium: RPMI-1640, 2 m*M* glutamine, and 10% (v/v) fetal calf serum.
3. Cryoprotectant medium: culture medium with 20% fetal calf serum and 10% (v/v) DMSO without antibiotics.
4. 15 mL sterile "PVC" sterile conical centrifuge tubes.
5. Separation medium: ACCUPSPIN™ System-HISTOPAQUE®-1077 (Sigma Aldrich-UK) (www.sigmaaldrich.com) tubes (one tube required for maximum volume of 15 mL blood). HISTOPAQUE-1077 consists of an aqueous solution of a high molecular weight polysaccharide and an aggregating agent sodium diatrizoate adjusted to a density of 1.077 ± 0.001.
6. PBS-A: 10 g/L sodium chloride, 0.25 g/L potassium chloride, 1.44 g/L disodium hydrogen orthophosphate-anhydrous (Na_2HPO_4), 0.25 g/L potassium dihydrogen orthophosphate (KH_2PO_4) at pH 7.3–7.5.
7. Benchtop centrifuge. Values for centrifugation given for use of an Eppendorf 5810 centrifuge.
8. Class II biological safety cabinet compliant with the British Standard 5726 (2005).
9. Plastic gown (to be worn over the laboratory coat while handling blood) and gloves.

3. Methods

3.1. Cryopreservation of Monolayer Primary Cell Cultures as Single Cell Suspensions

This method has been used by the author for preservation of a variety of primary cells including those from overnight cold trypsinization of tissues ***(8)*** and perfusion of organs (liver, kidney, and small intestine) with prewarmed collagenase or trypsin (Dowall, S., Health Protection Agency ***[9]***).

1. Inspect and select cultures that appear morphologically normal, show no signs of microbial contamination (*see* **Note 1**), and are subconfluent (considered to be more amenable to cryopreservation, *see* **ref. *6***.
2. Aspirate the growth medium from the monolayer and wash twice in approx 10 mL PBS (i.e., approx 0.1 mL/cm^2) using sterile pipets.
3. Use a 5-mL pipet to add 2–4 mL (i.e., 0.5 mL/25cm^2) prewarmed (approx 37°C) trypsin solution to cover the cell monolayer, recap the flask, and incubate at approx 37°C for 5 min.

4. Examine the monolayer and tap the flask to dislodge cells. If the large majority cells are not dislodged, incubate for up to a further 10 min.
5. Recover cells in trypsin in a sterile pipet and add to approx 10 mL PBS in a conical-based centrifuge tube and take a 1-mL sample to determine the total number of viable cells by Trypan blue dye exclusion (***[10,11]***; and the Cell Biology and Imaging Laboratory Manual of Basic Techniques [http://www.nibsc.ac.uk/aboutus/cell_techniques.html]) or an appropriate alternative method ***(12)***.
6. Meanwhile centrifuge the remaining suspension at approx 80–100*g* for 5 min.
7. Aspirate the supernatant and resuspend the cell pellet in 1 mL of culture medium.
8. Add cryoprotectant medium to the cells to give a final cell concentration of approx 5×10^6–10^7 cells/mL.
9. Mix the cell suspension gently to provide a homogenous suspension and carefully aliquot the cell/cryoprotectant mixture into 1- to 2-mL aliquots in sterile cryovials, sealing each tube as it is filled.
10. Transfer all sealed vials to a programmable cooler. One vial should be taken to determine postcryoprotection viability (*see* **Notes 3** and **6**).
11. Prepare a reference vial containing the cryoprotectant solution used for the cells and a thermocouple, and set the machine to cool as follows:
    a. Step 1: hold for 4°C for 10 min.
    b. Step 2: –3°C/min to –10°C.
    c. Step 3: –15°C/min to –30°C.
    d. Step 4: –3°C/min to –60°C.
    e. Step 5: –10°C/min to –140°C and hold at –140°C.
12. Using an appropriate cryoprotective mask, gloves, and apron, the cooling machine is opened, according to the manufacturer's instructions, and vials are transferred to a temporary storage rack in the vapor phase of liquid nitrogen prior to archiving frozen vials in the vapor phase of liquid nitrogen.
13. Vials of cells for quality control should be recovered for by rapid thawing at 37°C (e.g., in a water bath at 37–40°C) followed by gradual dilution of thawed cells by dropwise addition of prewarmed growth medium.
14. A sample ampoule should be removed from the liquid nitrogen storage after overnight equilibration and a viability assay again performed and compared with pre- and postcryoprotection treatment results to determine the overall success of the cryopreservation process (*see* **Note 3**). The remainder of this sample should also be recovered in antibiotic-free medium to demonstrate the appropriate growth and morphology of the cells, as well as the absence of contamination (*see* **Note 1**).

3.2. Cryopreservation of Adherent Monolayers of Primary Cells

This method has been used for hepatocyte and kidney epithelial cell cultures.

1. Seed fresh primary cells into culture vessels (e.g., 2 mL of growth medium at 2×104 cells/mL in each well of a 24-well plate) and incubate the cultures in a humidified atmosphere of 95% air/5% carbon dioxide for several days until the culture has almost formed a confluent monolayer.

2. Inspect and select primary cultures that appear morphologically typical, show no signs of microbial contamination, and are subconfluent.
3. Hold the cultures at room temperature (18–24°C) for 15 min.
4. Aspirate the growth medium supernatant, replace with cryoprotectant medium, and incubate for 15 min (*see* **Note 7**).
5. Aspirate almost all of the cryoprotectant medium, leaving the cell monolayer with sufficient medium such that its meniscus against the side walls of the wells is still visible.
6. Secure the lid of each 24-well plate with adhesive tape to provide a seal that will protect against ingress of nitrogen vapor during storage.
7. Check that each culture tray is labeled with the date, growth medium, cell type, and any other key information.
8. Transfer the plates to a programmable freezer set to cool its chamber as follows (*see* **Note 8**):
   a. Step 1: initial hold at 4°C for 15 min to allow chamber and vessels to equilibrate.
   b. Step 2: cool to –5°C at –1°C/min.
   c. Step 3: cool to –12°C at –3°C/min.
   d. Step 4: cool to between –10 and –14°C at –5°C/min (*see* **Note 9**).
   e. Step 5: cool to –20°C at –7.5°C/min.
   f. Step 6: cool to –25°C at –6.5°C/min and hold at –25°C for 2 min.
   g. Step 7: raise temperature to –20°C at 3°C/min (*see* **Note 9**).
   h. Step 8: cool to –50°C at –1°C/min.
   i. Step 9: cool to –130°C at –10°C/min and hold at this temperature.
9. Transfer the cryopreserved cells to appropriate and secure storage containers in the vapor phase of liquid nitrogen being careful to avoid cell rewarmth.

3.3. Cryopreservation of Peripheral Mononuclear Cells

1. Equilibrate blood sample(s) and the required number of mononuclear cell separation tubes to room temperature. If necessary, protect tubes from excessive light (in exceptional cases of exposure to strong sunlight, wrap in aluminium foil while on the open bench).
2. Centrifuge tubes at 1000 rcf (~2230 rpm Eppendorf 5810 centrifuge) for 30 s at room temperature to insure all HISTOPAQUE-1077 is above the "frit" prior to use (*see* **Fig.1**; **Note 10**).
3. Working in a class II safety cabinet, pour 5–15 mL of whole blood (previously mixed with an anticoagulant such as heparin) into the upper chamber of each prefilled ACCUPSPIN tube.
4. Dilute blood directly in the upper chamber of the ACCUPSPIN System-HISTOPAQUE-1077 tube with an equal volume of PBS-A (maximum final volume of 30 mL per tube).
5. Centrifuge at 800 rcf (~2000 rpm Eppendorf 5810 centrifuge) for 15 min. Set centrifuge to low-brake deceleration (setting 0 on the Eppendorf 5810) (*see* **Note 11**).
6. After centrifugation, carefully aspirate and discard the plasma layer with a Pasteur pipet to within 0.5 cm of the opaque interface containing the mononuclear cells (*see* **Fig. 1**).

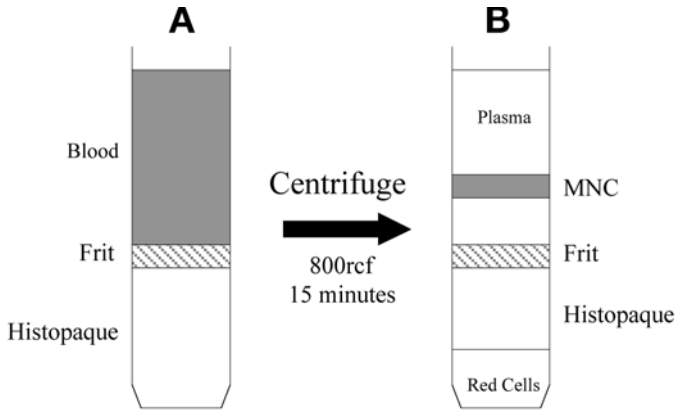

Fig. 1. Separation of mononuclear cells from whole blood using the ACCUSPINTM system. **(A)** Diluted blood prior to centrifugation. **(B)** Appearance of cell layers after centrifugation. MNC, mononuclear cells; red cells, erythrocyte pellet. (Figure kindly provided by Byrne, E., National Institute for Biological Standards and Control [NIBSC].)

7. Carefully transfer the mononuclear cell band using a sterile Pasteur pipet into a sterile, 15-mL centrifuge tube.
8. Add PBS-A to a total volume of 15 mL and gently mix by inversion.
9. Centrifuge at 290 rcf (1200 rpm; Eppendorf Centrifuge 5810) for 10 min.
10. Check to see that a cell pellet has formed before discarding the supernatant (*see* **Note 11**).
11. Wash the mononuclear cells by adding 10 mL PBS-A and resuspend cells by gentle aspiration with a sterile Pasteur pipet.
12. Centrifuge at 1200 rpm (290 rcf; Eppendorf Centrifuge 5810) for up to 5 min and discard the PBS-A supernatant.
13. Resuspend the pellet in 5 mL PBS-A, take a sample (0.2–0.5 mL) to perform a total and viable cell count (***[10,11]***; or go to www.nibsc.ac.uk/aboutus/research.html and click on Cell Biology and Imaging Laboratory Manual of Basic Techniques), and repeat **step 9**.
14. Resuspend the mononuclear cell pellet in freeze medium at approx 1×10^6 cells/mL (approx $5–10 \times 10^6$ lymphocytes can be expected from a fresh 10-mL blood sample).
15. Mix and aliquot the cell/cryoprotectant mix to prelabeled cryovials.
16. Transfer to a programmable freezer and set to cool according to the program given in **Subheading 3.1., step 11** or alternatively by a passive preservation method as follows (**steps 17–19**).
17. Wrap the vials of cells in insulated paper towels inside a polystyrene box (e.g., protective boxes used by manufacturers to ship individual 500-mL bottles of cell culture medium) and tape the box lid in place.
18. Place the box in a freezer (at or below –70°C) overnight (minimum of 16 h).
19. Recover the box, place it in the vapor phase of liquid nitrogen, and transfer all vials (taking care to avoid rewarming) to a storage location in the vapor phase of

liquid nitrogen (*see* **Note 12**). An atypical cooling profile obtained by this method is given in **ref. *13***.

4. Notes

1. Sterility tests for fungi and bacterial contaminants can prove highly useful to give confidence to workers in the quality of their aseptic technique, and are an important element in the quality control of stocks of primary cells. There are various methods for carrying out sterility tests, including standard Pharmacopeial tests. A typical method is given in Stacey and Stacey ***(14)*** and there are also industrial standards published in Pharmacopeia ***(15)***. Broader microbiological issues and other biosafety matters in cell culture work are covered in Coecke et al. ***(4)*** and Doblhoff-dier and Stacey ***(16)***.
2. A variety of chemicals in cell culture media are highly toxic at high concentrations, including antibiotics and HEPES. As ice forms in a cryopreserved suspension the cells become surrounded by the residual unfrozen water with an ever increasing concentration of solutes. Thus, it may be necessary to wash the cells in a basal medium without any potentially toxic additives before carrying out cryopreservation and to avoid incorporation of such toxic chemicals in cryoprotectant solutions.
3. In order to ensure that each frozen ampoule in a particular bank will give the same representative culture on thawing it is important to pool harvested cells and mix them well prior to processing for cryopreservation. It will be important to check the homogeneity of cultures recovered from cryopreserved stocks; this will be especially important for very large frozen stocks of cells where cryoprotection and aliquotting may have taken a considerable period of time, and may affect the viability and the quality of the frozen cells. Accordingly, initial experiments on preservation of a new type of primary culture reproducibility within and between cryopreserved stocks should be addressed. For larger banks of cells, testing should be performed on early-, mid-, and late-vials to ensure intrabank homogeneity. It should also be born in mind that a simple viability test, such as trypan blue dye exclusion, does not indicate the functionality of the cells, and cryopreserved banks of cells should be qualified in appropriate tests and characterisation to ensure that they retain the critical features and functions for their intended use.
4. A range of machines are available for the slow rate-controlled cooling of cells. One company that provides a number of commonly used models, such as the Kryo 10, is Planer Ltd, UK (www.planer.co.uk).
5. DMSO at a final concentration of between 5 and 10% (w/v) has become the cryoprotectant of choice for most mammalian cell preservation methods based on slow cooling rates (−1°/m). However, it has broad range of potentially toxic biological effects. If it is suspected that the use of DMSO is affecting the quality of recovered cells then it may be necessary to carry out cryoprotectant toxicity experiments to explore the quality of cells after different time/concentration treatments, and also to consider the use of alternative cryoprotectants such as glycerol. There is a significant amount of literature on the development of cryopreservation

methodologies in journals such as the *Journal of Cryobiology* (http://www.sciencedirect.com/science/journal/00112240) and *CryoLetters* (www.cryoletters.org).

6. A range of alternative methods have been developed to approximate the cooling rates required to reserve cells (generally –1°C/min). One method of passive cooling is given in **Subheading 3.3.** and this has proven effective for a range of cell lines as well as primary cells. In addition, passive cooling devices are commercially available such as the Handi-Freeze™ (Taylor Wharton, UK) and Mr. Fosty™ (Nalgene/Invitrogen).
7. In unpublished work by Dowall, S. (Health Protection Agency, Salisbury) ***(9)*** it appears that this method is also effective using glycerol (10% [v/v]) in culture medium as the cryoprectective solution.
8. Kidney primary epithelial cells may take 5–6 d to form semiconfluent cultures, whereas primary hepatocyte cultures will have already begun to lose key biochemical functions in this time and will need to be cryopreserved when freshly isolated or after a shorter period of culture. The optimal culture harvest point for cryopreservation will vary depending on a range of factors including cell type and species, culture medium, vessel type, gaseous atmosphere, and culture medium volume.
9. The profile given was developed at the University of Sheffield, Sheffield, UK originally for preservation of insect embryos. It shows a sharp drop in temperature over the range –50 to –25°C with a brief rewarming to –20°C. This eliminated the sharp temperature peak that occurs from the formation of the first ice, which is an exothermic event. In order to use the type of profile given in **Subheading 3.2.** it is important to characterize the nature (timing and degree) of the exothermic point for a particular cell/cryoprotectant combination.
10. Other media (e.g., Ficoll-Paque [GE Healthcare], Percoll [GE Healthcare]) are available to provide the discontinuous density gradient for separation of cells by buoyant density. The preparation of lymphocytes from the blood of different species may require specialized discontinuous density gradients and two examples of gradient compositions using Percoll are given here that have been used for isolation of primate and mouse mononuclear cells:

a. Primate:	Percoll (1.13 g/mL stock)	15 mL
	PBSA (*see* **Subheading 2.2.**)	10.3 mL
	10X PBS (divalent cation free)	1.6 mL
	1 *M* sulphuric acid	0.1 mL
b. Mouse:	Percoll (1.23 g/mL stock)	57 mL
	5 *M* NaCl in water	10 mL
	Distilled water	33 mL

11. The tubes can be centrifuged for a further 10 min if a good separation has not been obtained. This is often the case with blood samples separated 48 h after collection (Byrne, E., unpublished data, National Institute for Biological Standards and Control).
12. Usually only sufficient cells are recovered from individual whole blood samples for about one to three vials and, thus, it is not usual to recover a vial to check viability

but to try to ensure samples are obtained as soon as possible after blood donation and that the separation and cryopreservation process is carried out in a reproducible way. Great care should be taken with small samples as only a small proportion of the mononuclear cells received may be of value (e.g., studies of minority lymphocyte populations, Epstein–Barr virus transformation). If a cell pellet is not visible following the first PBS-A wash after Histopaque separation, repeat the centrifugation in **step 7** (Byrne, E., unpublished data, National Institute for Biological Standards and Control). If the blood has a very low lymphocyte count then the pellet may only just be visible to the naked eye. Very small samples of whole blood may provide a useable source of mononuclear cells by scaling down the use of Histopaque or Ficoll separation to a total volume of 1.5 mL in a microtube and using a microfuge.

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Cryopreservation of Red Blood Cells and Platelets

Andreas Sputtek

Summary

Blood cells can be regarded as a classical field of application of low-temperature biology. Cryopreservation methods have been developed for different categories of blood cells namely red blood cells (RBCs) (erythrocytes), platelets (thrombocytes), mononuclear cells (i.e., lymphocytes, monocytes), and hematopoietic progenitor cells. This chapter outlines the four most commonly applied techniques for RBCs and two for platelets.

Key Words: Cryopreservation; red blood cells; platelets; dimethyl sulfoxide; hydroxyethyl starch.

1. Introduction

Frozen red erythrocytes, thrombocytes, mononuclear cells (i.e., lymphocytes, monocytes), and hematopoietic progenitor cells (from peripheral blood as well as from bone marrow) are being used for various diagnostic and therapeutical purposes (to *see* reviews, e.g., **refs. *1*** and ***2***). A variety of cell-specific cryopreservation protocols have evolved so far. The methods differ with regard to (1) cell concentrations, (2) protective solutions used (cryoprotectants and their concentrations), (3) temperature–time histories during cooling and rewarming, and (4) storage temperature. Additionally, some of the cryoprotectants are not well tolerated in the concentrations required (e.g., dimethyl sulfoxide [DMSO] for platelets) or lead to an osmotically induced lysis of the cryoprotectant-loaded cells when transfused into an isotonic organism (e.g., glycerol for red blood cells [RBCs]). In these cases, a washing procedure is required after thawing prior to the application/transfusion.

The four techniques for RBCs and two for platelets (PLT) described in detail in the following are:

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No.	Cell	Method	Author
1.	RBC:	High glycerol–slow cooling technique	(Meryman ***[3,4]***)
2.	RBC:	Low glycerol–rapid cooling technique	(Rowe ***[5,6]***)
3.	RBC:	Hydroxyethyl starch–rapid cooling technique	(Sputtek ***[7,8]***)
4.	RBC:	Hydroxyethyl starch–rapid cooling technique	(Thomas ***[9,10]***)
5.	PLT:	Dimethyl sulphoxide–intermediate cooling technique	(Schiffer ***[11,12]***)
6.	PLT:	Hydroxyethyl starch–intermediate cooling technique	(Choudhury ***[13]***)

Please note that (1) methods 1, 2, 3, 4, and 6 as described next are "adjusted" to whole blood donations (approx 450–500 mL), whereas 5 deals with platelets obtained by apheresis. This is usually equivalent with what can be obtained from four to five whole blood donations. (2) Deviations regarding the volume and sample geometry (e.g., by changing from bags to vials for laboratory purposes) may lead to different temperature–time histories ("rates") during cooling and rewarming; this can be very critical in the case of the rapid cooling techniques and may result in less favorable results, and (3) some of the methods have been developed at a time when sterile tube-docking devices were not available. In this case, the use of laminar air-flow cabinets are highly recommended, and as nowadays some of the "old" cryogenic bags are not available, they have been replaced by what the author thinks to be suitable as well. It is assumed for all methods that standard blood bank equipment is available, e.g., (refrigerated) bag centrifuge, scales, freezer, plasma extractor, tube stripper, clamps, and so on. A list of companies selling such devices (as cleared by the FDA's Center for Biologics Evaluation and Research) can be found at http://www.fda.gov/cber/dap/510kman.htm. Isotonic saline, ACD-A, ACD-B, CPD*, CPD-A1*, SAG-M*, PAGGS-M*, AS-1*, AS-3* are "standard solutions" that contain the "ingredients" at prescribed concentrations and can be obtained from many companies supplying blood banks. The solutions marked with an asterisk are usually contained in the blood donation/processing/storage bag system.

1.1. RBCs

Cryopreserved RBCs for transfusion are of advantage in the case of patients with rare blood groups, adverse antibody problems, and civil as well as military disasters. Additionally, they can be used for blood typing, antibody screening, and compatibility testing. In principle, three different methods have been established for clinical use: (1) the Huggins ***(14)*** technique, using glycerol in a nonionic suspension and removal of the cryoprotectant by

reversible agglomeration of the RBC, (2) the "high glycerol–slow cooling technique" according to Meryman and Hornblower ***(3,4)***, which is the dominant method in the United States. (3) The "low glycerol–rapid cooling technique" according to Rowe ***(5,6)*** and Krijnen ***(15)*** is the dominant method for the cryopreservation of RBCs in Europe. The utilization of macromolecular cryoprotectants goes back to Rinfret ***(16)*** and coworkers, and water-soluble, cryoprotective macromolecules such as albumin, dextrans, modified gelatin, polyvinylpyrrolidone (PVP), polyethylene oxide, polyethylene glycol, and hydroxyethyl starches (HES) exhibit the principal advantage of not entering into the cells. This property significantly facilitates their removal after thawing. In the case of emergencies, this step could be omitted if the additive, e.g., albumin, dextrans, modified gelatin, and HES are biodegradable and tolerated by the human organism. In 1967, Knorpp et al. ***(17)*** described for the first time the successful cryopreservation of human RBCs using HES and liquid nitrogen (LN_2), comparing the efficacy of HES to that of PVP. They preferred the colloid HES to PVP as the latter is retained to a considerable extent in the recipient (as are polyethylene oxide and polyethylene glycol). Moreover, in the case of hypovolemia, albumin, dextrans, modified gelatins, and HES serve as blood volume substitutes. We have carried out several in vitro investigations and optimizations of the HES procedure, and after in vivo experiments in dogs a successful in vivo study including seven healthy volunteers has been carried out ***(8)***. Finally, we have performed a systematic clinical trial in patients ***(18)***. Based on the work published by Robson ***(19)***, Thomas et al. ***(9,10)*** have developed another procedure for the freezing of RBCs using HES. The major differences compared to our procedure are (1) no prefreeze washing, (2) different HES modification, (3) lower HES concentration, (4) higher electrolyte concentration, (5) higher hematocrit (HCT), (6) larger freezing bag, (7) smaller sample thickness, (8) smaller volume, (9) higher viscosity, (10) different freezing container, and (11) uncontrolled thawing. For an overview regarding the nomenclature of different HES modifications and specific aspects of their usefulness for the cryopreservation of human cells, *see* http://www.sputtek.de/Info/HES.pdf.

1.2. Platelets (see *Note 1)*

Since the first reported attempt in 1956 to stop thrombocytopenic bleeding by the infusion of previously frozen platelets by Klein ***(20)***, a broad variety of in vitro and in vivo studies on cryopreserved platelets have been published. The most widely used method for the cryopreservation of platelets is a "10% DMSO–slow cooling" method by Schiffer ***(11,12)***, another one is a "low glycerol/glucose–intermediate cooling" method described by Dayian and Rowe in 1976 ***(21)***. HES-cryopreserved

platelets (frozen in the presence of 4% [w/v] HES at 1°C/min), turned out to be hemostatically effective when using this 4% HES method ***(13)***.

1.3. Mononuclear Cells (see *Note 2)*

The use of cryopreserved mononuclear cells (i.e., lymphocytes and monocytes) is well established and a routine procedure for clinical laboratory testing. Most recently, there is a growing clinical interest in cryopreserved lymphocytes for the supplemental treatment of patients after blood stem cell transplantation. Usually, they are frozen according to methods that are more or less modifications of a technique that was first described for bone marrow by Ashwood-Smith ***(22)*** in 1961 using 10% DMSO. During cooling, the heat is removed either by computer-controlled and LN_2-operated machines or in mechanical (–80°C) refrigerators. Stiff et al. ***(23)*** have demonstrated that the addition of 6% HES reduced the "original" concentration of DMSO (10%) by one-half. Cryopreserved autologous and homologous blood stem cells (in combination with high-dose chemotherapy and/or irradiation) have become a "standard" blood component for the treatment of several malignant diseases. Frozen cryopreserved mononuclear cells are used for various diagnostic purposes, e.g., human leukocyte antigen typing, detection of human leukocyte antigen antibodies in patients on waiting lists for organ/bone marrow transplantations, and mixed lymphocyte reactions/cultures. They are also of interest with respect to look-back procedures in transfusion medicine or diagnosis in patients. The methods for freezing mononuclear cells reported in the literature vary from one author to another ***(1,2)***.

1.4. Granulocytes

There have been publications in the past that claim the successful cryopreservation of granulocytes (*see* reviews, e.g., **refs.** ***24,25***). Despite reports appearing now and then in the newer literature (mostly as abstracts), it is our opinion that no clinically suitable method for the preservation of granulocytes has been found. The huge variation of the in vitro results shows how cumbersome the viability assays are, and how unsuitable they will be to predict anything that is going to happen in vivo. Membrane integrity tests (i.e., staining tests often referred to as "viability tests") measure only a *conditio sine qua non* (i.e., an intact cell membrane). However, what is the meaning of these results if tests measuring typical granulocytic functions (e.g., chemotaxis, bactericidal activity) fail to detect any significant activity? Takahashi et al. ***(26)*** have proposed some explanations why granulocytes are so unrewarding regarding their cryopreservation. Already at temperatures below –5°C without the formation of ice, a significant loss of function can be observed. This could be prevented by the addition of DMSO, whereas glycerol failed to show this effect. Because of their limited osmotic tolerance (already a twofold increase compared to isotonicity caused a

significant loss of function), they are highly susceptible to the electrolyte enrichment taking place during ice formation. Granulocytes also showed a limited tolerance to hypotonic stress, which may occur upon thawing.

2. Materials

2.1. High Glycerol–Slow Cooling Technique

1. Plasma transfer sets (e.g., Terumo 1TC*00505, Baxter 4C2298).
2. Polyvinyl chloride storage containers.
3. 400 mL 6.2 *M* glycerol solution containing 0.14 *M* sodium lactate, 5 m*M* potassium chloride, and 5 m*M* sodium phosphate (pH 6.8 after autoclaving).
4. Cryogenic freezing containers made of polyolefin, polytetrafluorethylene (i.e., Teflon®, polyimide [Kapton®]/Teflon), or ethylenevinylacetate (EVA).
5. Cardboard or metal canister for freezing.
6. Freezer (–65°C or below).
7. 37°C water bath or 37°C dry warmer.
8. 12% (w/v) hypertonic sodium chloride solution.
9. 1.6% (w/v) hypertonic sodium chloride solution.
10. 0.9% (w/v) isotonic sodium chloride with 200 mg/dL glucose.

2.2. Low Glycerol–Rapid Cooling Technique

1. Plasma transfer sets (e.g., Terumo 1TC*00505, Baxter 4C2298).
2. Polyvinyl chloride storage containers.
3. Cryoprotective solution (CPS): 35 g glycerol, 3 g mannitol, and 0.65 g sodium chloride per 100 mL.
4. Cryogenic freezing containers made of polyolefin, polytetrafluorethylene (Teflon), or polyimide (Kapton)/Teflon.
5. Heat sealer.
6. Freezing bag holder, retainer, gloves, and goggles.
7. Open LN_2 Dewar.
8. LN_2 storage tank.
9. 45°C water bath.
10. 3.5% hypertonic sodium chloride solution.
11. 0.9% isotonic sodium chloride with 200 mg/dL glucose.

2.3. HES–Rapid Cooling Technique (7,8)

1. Plasma transfer sets (e.g., Terumo 1TC*00505, Baxter 4C2298).
2. 600 mL transfer packs with couplers (e.g., Terumo Teraflex B600, Baxter 4R2027).
3. Sterile tube-connecting device.
4. CPS: 220 mL of 23% (w/w) (i.e., 25% [w/v]) HES 200/0.5 in 60 mmol/sodium chloride solution (*see* **Note 3**).
5. Platform rocking device.
6. Cryogenic freezing containers made of polyimide (Kapton)/polytetrafluorethylene (Teflon), e.g., Fresenius Gambro Hemofreeze Bag DF 1200.

7. Heat sealer.
8. Patented freezing container (*see* **Note 4**). The container can be obtained by contacting the patent holders.
9. Open LN_2 Dewar.
10. LN_2 storage tank.
11. Hand tongs, gloves, and goggles.
12. 48°C shaking water bath with pouch.

2.4. HES–Rapid Cooling Technique (9,10)

1. Plasma transfer sets (e.g., Terumo 1TC*00505, Baxter 4C2298).
2. Polyvinyl chloride storage containers.
3. CPS: 40 mL of 40% (w/v) HES 200/0.5 in isotonic saline (*see* **Note 3**).
4. Cryogenic freezing containers made of polyimide (Kapton)/tetrafluoretylene (Teflon).
5. Sterile tube connecting-device.
6. Heat sealer.
7. Aluminium freezing frame and metal clips.
8. Open LN_2 Dewar.
9. LN_2 storage tank.
10. Hand tongs, gloves, and goggles.
11. 43.5°C controlled circulating water bath.

2.5. DMSO–Intermediate Cooling Technique

1. Sampling site couplers (e.g., Terumo TC*MP1, Baxter 4C2405).
2. 300 and 600 mL transfer packs with couplers (e.g., Terumo Teruflex B-600, Baxter [300 mL] 4R2014A, [600 mL] 4R2027).
3. Heat sealer.
4. 3-, 10-, and 60-mL sterile disposable plastic syringes.
5. 18-gage needles.
6. Cryogenic freezing bag: polyolefin, polytetrafluorethylene (Teflon), polyimide (= Kapton)/Teflon, or EVA.
7. 10-mL vials of sterile DMSO.
8. 16-gage butterfly needle with 75-cm tubing.
9. Infusion pump for 60-mL syringe.
10. Platform rocking device.
11. Metal plates with clamps.
12. Ultralow temperature freezer with temperature range down to –135°C.
13. Protective bags.
14. 37°C water bath.
15. Infusion set.

2.6. HES–Intermediate Cooling Technique

1. CPS: 8% (w/w) HES 70/0.5 or HES 200/0.5, sodium chloride concentration 0.6% (*see* **Note 3**).
2. Sterile, disposable, 2-, 20-, and 50-mL plastic syringes with 18-gage needles.

3. Cryogenic freezing bags made of polyolefin, polytetrafluorethylene (Teflon), polyimide (Kapton)/Teflon, (e.g., Fresenius Hemofreeze Bag DF 200), or EVA.
4. Heat sealer.
5. Platform rocking device.
6. Closed aluminium container with 3-mm inner thickness.
7. Controlled-rate LN_2 operated freezer.
8. Hand tongs, gloves, and goggles.
9. LN_2 storage tank.
10. Punched aluminium thawing container.
11. 37°C shaking water bath.
12. 250-mL transfer pack.

3. Methods

3.1. High Glycerol–Slow Cooling Technique (3,4)

1. Prepare a RBC concentrate from whole blood using standard blood bank techniques (e.g., **ref.** *27*). RBC preserved in CPD (combined citrate/phosphate buffer and glucose containing whole blood/RBC anticoagulant/storage solution) or CPD-A1 (also contains adenine) may be stored at 1–6°C for up to 6 d before freezing. RBC preserved in AS-1 (Adsol®) and AS-3 (Nutricel®) (both are glucose and adenine containing RBC storage solutions, the latter also contains a combined citrate/phosphate buffer) may be stored at 1–6°C for up to 42 d before freezing. RBC that have undergone a rejuvenation procedure may be processed for freezing up to 3 d after their original expiration. RBC in any preservative solution that have been entered for processing must be frozen within 24 h after opening the system.
2. The combined mass of the cells and the collection bag should be between 260 and 400 g.
3. Underweight units can be adjusted to approx 300 g either by adding isotonic saline or by the removal of less plasma than usual.
4. Warm the RBC and the 6.2 *M* glycerol solution to at least 25°C by placing them in a dry warming chamber for 10–15 min or by allowing them to remain at room temperature for 1–2 h. The temperature must not exceed 42°C.
5. Place the container of RBC on a shaker and add approx 100 mL of the glycerol solution as the red cells are gently agitated.
6. Turn off the shaker and allow the cells to equilibrate, without agitation, for 5–30 min.
7. Allow the partially glycerolized cells to flow by gravity into the freezing bag.
8. Add the remaining 300 mL of the glycerol solution in a stepwise fashion, with gentle mixing. Add smaller volumes of the glycerol solution for smaller volumes of red cells. The final glycerol concentration is 40% (w/v).
9. Maintain the glycerolized cells at temperatures between 25 and 32°C until freezing. The recommended interval between removing the RBC unit from refrigeration and placing the glycerolized cells in the freezer should not exceed 12 h.
10. Place the glycerolized unit in a cardboard or metal canister and place in a freezer at –65°C or below. The freezing rate should not be less than 10°C/min.
11. Do not "bump" or handle the frozen cells roughly.

12. Storage of the frozen RBC at –65°C or colder is possible but not recommended for up to 10 yr (and more).
13. For thawing place the protective canister containing the frozen RBC in either a 37°C water bath, or 37°C dry warmer.
14. Agitate gently to speed up thawing. The thawing process takes at least 10 min, and the thawed cells should be at 37°C.
15. After the RBC are thawed, you may either use a commercial instrument (e.g., centrifuge) for batch or a continuous-flow washing device to deglycerolize cells. Follow the manufacturer's instructions precisely, especially when using a special device (e.g., Cobe Cell Processor 2991).
16. For batch washing, dilute the unit with a quantity of hypertonic (12%) sodium chloride solution appropriate for the size of the unit. Allow to equilibrate for approx 5 min.
17. Wash again with 1.6% sodium chloride until deglycerolization is complete. Approximately 2 L of wash solution per unit are required.
18. Suspend the deglycerolized RBC in isotonic saline (0.9%) with 0.2% glucose.
19. If you have opened the system for the processing, deglycerolized RBCs must be stored at 1–6°C for no longer than 24 h (in the case of a transfusion).

3.2. Low Glycerol–Rapid Cooling Technique (5,6)

1. After collection of a unit of whole blood in ACD (citrate buffer and glucose containing whole blood/RBC/platelet anticoagulant/storage solution; formulations ACD-A and ACD-B vary with regard to the concentration of the solutes) or CPD anticoagulant, the plasma is removed from the cells after centrifugation. The RBCs should be frozen as soon after the collection as possible, but preferably before they are 5 d old.
2. The remaining packed RBCs are weighed, and an equal volume by weight of the glycerol freezing solution is added at room temperature to achieve a final concentration of 14% (v/v). The freezing solution contains 28% (v/v)–35% (w/v) glycerol, 3% mannitol, and 0.65% sodium chloride.
3. After 14–30 min equilibration at room temperature (22°C), the RBC suspension is transferred into a suitable (e.g., polyolefin) freezing bag. Conventional PVC plastic bags cannot be used, as they become brittle and crack upon freezing in LN_2.
4. The bag is placed between two metal plates (holder). These are used to keep the bag in a flat configuration. The top and bottom of the bag are tucked under to allow the holder plates to close without pinching the bag.
5. Cooling is performed by complete immersion of the container in the open LN_2 filled without agitation. A freezer retainer should be used to prevent excess bulging of the container during freezing. Freezing is complete in 2–3 min when the LN_2 stops boiling.
6. The unit is stored in its metal plate holder in a LN_2 storage tank either in the vapor or in the liquid phase.
7. Thawing: on retrieval from the LN_2 storage, the unit is immediately thawed by immersing the complete unit (bag and holders) into a 40–45°C warm water bath under gentle agitation (approx 60 cycles/min) for about 2.5 min.

8. The bag is removed from the metal holding plates and it is checked that all ice has disappeared. If not, immediately reimmerse the bag into the thaw bath and knead the bag under the warm water until the ice has completely melted.
9. Following centrifugation and removal of the supernatant containing free hemoglobin, glycerol, and debris, the RBCs are washed three times using a bag centrifuge. The first wash is with 300–500 mL 3.5% sodium chloride at 4°C, the last two are with 1000–2000 mL isotonic saline (or preferably with 0.8 % NaCl containing 200 mg/dL glucose). All washes must be added slowly to the cells at room temperature with gently mixing.
10. For resuspension of the RBC after the deglycerolization, the glucose-supplemented sodium chloride solution may be used as well.

3.3. HES–Rapid Cooling Technique (7,8)

For an overview of this method, *see* http://www.sputtek.de/Info/V140.pdf.

1. 450–500 mL whole blood are collected in blood bank packs containing CPD-A as anticoagulant.
2. Plasma and buffy coat are removed using standard blood bank techniques ***(28)*** and the RBCs are leukodepleted by filtration and stored in an additive solution (SAG-M [adenine-, glucose-, and mannitol-containing saline solution]), PAGGS-S (phosphate buffer, adenine, glucose, guanosine-containing saline solution, AS 1, AS 3, and so on) (*see* **Note 5**).
3. The leukodepleted RBCs can be kept at 4 ± 4°C for a maximum of 3 d prior to freezing.
4. The first centrifugation takes place at 4000*g* for 10 min at 4°C.
5. The supernatant additive solution is removed by means of a plasma extractor.
6. The RBC concentrate is resuspended in 333 mL isotonic saline solution.
7. The suspension is then centrifuged again. The centrifugation step is repeated three times to make sure that all of the additive solution and plasma have been removed and that the "contamination" of the RBC concentrates by leukocytes and platelets is minimal.
8. After the removal of the last supernatant-purified RBC concentrates, a volume of approx 220 mL at a HCT of 85% ± 5.0% should be obtained.
9. An equal part of the CPS containing 23% (w/w) HES and 60 mmol/L sodium chloride is added to the purified RBC concentrate while mixing continuously.
10. Two aliquots of 220 mL of this suspension are transferred in two freezing bags.
11. The bags must be carefully deaerated and heat sealed below the inlet port. As the densities of the CPS and the RBC concentrates are quite similar (ca. 1.08 g/mL), the final HES concentration in the suspension to be frozen is 11.5% (w/w).
12. The two bags are then placed in two aluminium containers (*see* **Note 6**).
13. Cooling at the required cooling rate of about 240°C/min is achieved by complete vertical immersion of the containers into the open LN_2-filled Dewar. Use hand tongs, gloves, and goggles. Cooling is complete within 3 min. The total time is not critical as long as the containers are immersed for at least 3 min.

14. Lift the reusable container from the LN_2. Open it quickly and remove the freezing bag. Transfer the freezing bag to the vapor phase of a LN_2 storage tank within 30 s to avoid the risk of premature thawing. The bags must be stored in vapor phase over the LN_2 though not actually in the LN_2. Storage in the vapor phase over LN_2 below –130°C results in no time-dependent degradation.
15. Thawing is achieved by means of a shaking water bath. To guarantee thermally defined and reproducible conditions during rewarming, the bags have to be transferred from the LN_2 vapor phase into the pouch of the shaking water bath. This pouch maintains a well-defined flat geometry, and effectively transduces the shaking frequency (300 cycles/min) and amplitude (2 cm) of shaking in the water bath (48°C) to the frozen unit. After 75 s, the temperature in the bag is about 20°C and they are immediately removed from the bath and the thawing container.
16. Repeat this with the second bag prepared from this donation if needed.
17. If desired, free hemoglobin, HES, and debris can easily be removed by washing the RBC once with 300–500 mL of isotonic saline (or preferably with 0.8% NaCl containing 200 mg/dL glucose). Centrifuge refrigerated (4°C) at 4000*g* for 10 min, and remove the supernatant by means of a plasma extractor.
18. For resuspension of the washed RBC, the glucose-supplemented sodium chloride solution can be used. Standard RBC additive solutions (SAG-M, PAGGS-S, AS-1, and AS-3) may be used as well.

    The percentage recovery of intact cells, immediately post-thaw, is not used as a quality control procedure because it is only a crude indicator of quality (*see* **Note 7**).

3.4. Hydroxyethyl Starch–Rapid Cooling Technique (9,10)

1. This patented method (UK patent PCT/GB 90/0140, priority 08.02.1989) starts with a standard 450–500 mL whole donation collected in a suitable anticoagulant (e.g., CPD). In this case, the whole blood has to be filtered first, e.g., using standard blood bank techniques ***(27)*** (*see* **Note 8**).
2. Place the filtrated whole blood unit in a centrifuge and spin at 3000*g* for 20 min. Express the plasma from this bag into an attached satellite bag. It is important that all visible plasma is expelled from the filtrate bag to ensure that a packed cell volume (HCT) of at least 90% is achieved. Detach the plasma bag (*see* **Note 9**).
3. Connect the filtrate bag to the tubing of the freezing bag. Invert the bag and suspend it 2 m above ground level. Allow the packed red cells to flow into the freezing bag.
4. Express any air, which has collected in the freezing bag, back up the tubing into the previous bag together with a small volume of red cells.
5. Aseptically connect the freezing bag to the bag containing the CPS. Suspend the inverted bag 2 m above ground level and allow the HES solution to flow into the freezing bag, displacing all red cells from the tubing into the freezing bag. Roll up the nearly empty bag that has contained the cryoprotective solution to ensure that it is completely emptied.
6. Seal off the freezing bag, leaving as great a length of tubing as possible attached to it.
7. Mix the contents of the freezing bag thoroughly but carefully by repeated manual rotation and inversion of the bag for 4 min.

8. Freezing the HES/red cell mixture. Place the freezing bag into the base of the freezing frame. Ensure that the bag is lying smoothly and centrally and that the tubing/insert area is positioned within the frame recess.
9. Place the top of the freezing frame in position and secure this with six metal clips placed around the edges of the frame.
10. Carefully drop the frame vertically into the LN_2-filled Dewar so as to totally immerse the frame. Use hand tongs, gloves, and goggles. The nitrogen will boil vigorously. Bubbling should cease in approx 30 s. Leave the frame totally immersed for a further 30 s.
11. Lift the frame from the LN_2. Quickly place the frame on a steel table, unclip the frame, and remove the freezing bag. Transfer the freezing bag to the LN_2 storage tank. This stage is to be completed within 30 s to avoid the risk of premature thawing.
12. Storage of HES-cryopreserved red cells. Bags are to be stored flat. The bags must be stored in vapor phase over the LN_2n, not actually in the LN_2 (*see* **Note 10**).
13. Thawing HES-cryopreserved red cells. Check that the temperature of the water in the circulating water bath is within one degree of 43.5°C.
14. Remove the required frozen unit of cryopreserved red cells from the storage tank.
15. Place the unit in the water bath within 30 s of removing it from the storage tank, ensuring that the unit is totally immersed.
16. Holding the bag horizontally move it gently to and fro during the first minute of thawing. Rapid freezing is essential.
17. Leave the bag in the water bath for a further 9 min to allow the temperature to equilibrate and then remove the thawed unit from the bath. Blot it dry with paper towels. After thawing, the pack of HES/RBC can be stored at 4°C for 10 d without deterioration. It still contains the original amount of HES if no washing step is performed. If mixed with a diluent or optimal additive solution, storage times at 4°C are not improved over those of the mixture stored in the undiluted state.
18. Viability testing. The saline stability test may be performed 2 h after resuspension for the determination of the "2 h saline-stability value" as well. "Plasma stability" can be measured by using red cell-free nonlipemic plasma instead of the saline solution. In this case, the plasma should also be used for the determination of the blank (*see* **Note 11**).

3.5. DMSO–Intermediate Cooling Technique (11,12)

1. Platelet concentrates (PC) can be obtained from whole blood donations using standard blood bank techniques ***(28)*** or by apheresis.
2. Record the net weight of the PC.
3. Using a sampling site coupler, add 4.5 mL of additional ACD-A per 100 mL PC if the platelets have been obtained by apheresis. (This additional ACD-A is necessary to prevent platelet clumping following centrifugation because anticoagulant ratios used in automated procedures are lower than when platelets are manually collected from individual whole blood units.)
4. Allow the PC to stand undisturbed at room temperature for 1–2 h to permit disaggregation of any platelet clumps.

5. Transfer the PC to a 600-mL transfer pack and heat seal the tubing approx 20 cm from the bag. Discard extra tubing.
6. Strip the tubing several times, mixing the PC thoroughly (but gently) in between.
7. Heat seal the tubing 2–5 cm from the bag. Remove the segment.
8. Perform a platelet count on the segment and weigh the PC; record the information.
9. If gross RBC contamination of the PC is obvious, centrifuge the PC at 65*g* for 12 min.
10. Express the platelet-rich plasma (PRP) supernatant (minus the RBC button) into another 600-mL transfer pack. Obtain a specimen segment as in **steps 5–7**. Perform a platelet count on the segment, weigh the concentrate, and record the net weight and platelet count on a data sheet.
11. If the initial PC has few or no RBC, skip **steps 9** and **10** and proceed with **step 12**.
12. Calculate the platelet yield. If it is greater than 4.4×10^{11}, split the material prepared for cryopreservation equally such that each bag contains less than 4.4×10^{11} platelets. (Example: if the platelet yield = 10.5×10^{11}, split the freeze into three bags, each containing 3.5×10^{11} platelets.) Prepare a record for each bag of platelets frozen.
13. Centrifuge the PC at 1250*g* for 15 min.
14. Express the platelet-poor plasma (PPP) into another 600-mL transfer pack, leaving behind the undisturbed platelet button and approx 10 mL of plasma. Clamp the tubing between the two transfer pack; do not seal it.
15. Gently mix the platelet button with the 10 mL of plasma.
16. Using a wet folded paper towel, gently rub the platelet button until the suspension is homogeneous.
17. If the platelet button is still clumped, stop. Allow it to stand undisturbed at room temperature until the clumping has disappeared, usually for 1–2 h.
18. Using a sampling site coupler, a 60-mL syringe, and an 18-gage needle, withdraw the 10 mL of PC.
19. Unclamp the tubing between the two transfer packs and run 10–15 mL of the PPP into the now empty platelet bag. Reclamp the tubing.
20. Swirl the plasma around inside the platelet bag in order to mix any residual platelets with the plasma.
21. Using the same 60-mL syringe as in **step 18**, withdraw the residual platelet mixture.
22. Repeat **steps 19–21** until all of the platelet mixture is withdrawn and the syringe contains a final volume of 45 mL.
23. Insert a sampling site coupler into one of the ports of a platelet freezing bag and inject the 45 mL of PC (*see* **Note 12**).
24. Using the same syringe, withdraw 40.5 mL of plasma from the PPP-bag. Draw up to 4.5 mL DMSO (final syringe volume will now equal 45 mL); gently invert the syringe several times to mix the contents.
25. Disconnect the 18-gage needle from the syringe, and connect a butterfly needle with approx 75-cm tubing. Insert the 16-gage needle into the sampling site coupler already in place in the freezing bag, taking care not to puncture the bag.
26. Place the syringe in an infusion pump set to deliver 3.0 mL/min.

27. Place the platelets (in the freezing bag) on a rocker so they will be gently mixed while the DMSO mixture is being added. Turn on the rocker and the infusion pump.
28. When all of the DMSO/plasma mixture has been injected into the freezing bag (at a setting of 3.0 mL/min), the total running time should be approx 16 min), turn off the infusion pump and remove the syringe from the pump.
29. Turn off the rocker and remove the platelet freezing bag.
30. Withdraw any air in the freezing bag into the now empty 60-mL syringe. Remove the 16-gage needle from the site coupler in the freezing bag.
31. Clamp the freezing bag port below the sampling site coupler between the bag and the coupler.
32. Remove the sampling site coupler and heat seal the port.
33. Place the freezing bag flat and gently press it to check for leaks. If there is a leak, reseal the port.
34. Place the bag between two metal plates (to produce a final thickness of approx 0.5 cm). Clamp the plates together and place them horizontally in the –135°C freezer.
35. For each bag of frozen platelets, prepare one transfer pack containing 100 mL of autologous donor plasma and one transfer pack containing 120 mL autologous donor plasma.
36. Place the bags of plasma in a conventional freezer below –18°C.
37. After 24 h, the platelets may be removed from the metal plates and filed in the freezer.
38. For thawing remove one 100-mL bag and one 120-mL bag of autologous plasma from the freezer. (If autologous plasma is not available, use ABO type-specific plasma.)
39. Remove one 10-mL vial of ACD-A from the same freezer.
40. Seal each bag of plasma in an additional protective bag (in case of breakage) and thaw the bags in a 37°C water bath without agitation.
41. Thaw the vial of ACD-A in a rack in the water bath. Make sure the water level does not reach the cap of the vial so that contamination of the ACD-A is prevented.
42. As soon as the ACD-A, and the plasma have thawed, remove them from the water bath.
43. Remove the bag of frozen platelets from the –135°C freezer.
44. Seal each bag of plasma in an additional protective bag and thaw it in the 37°C water bath without agitation.
45. Remove the platelets from the bath as soon as thawing is complete.
46. Using a 10-mL syringe and an 18-gage needle, aseptically withdraw the 10 mL of ACD-A from the vial.
47. Insert a sampling site coupler into one port of the 100-mL bag of autologous plasma.
48. Wipe the coupler with an alcohol pad and inject the ACD-A through the coupler into the plasma and gently mix it.
49. Insert one end of a blood transfusion set into the remaining port of the 100-mL bag of plasma.
50. Insert the other end of the blood transfusion set into the port of the bag of thawed platelets.
51. Hang the plasma ACD-A mixture above the platelets.

52. Place the bag of platelets on a rocker (to ensure complete mixing) and adjust the roller clamp on the infusion set such that the plasma/ACD-A mixture is added to the platelets at a rate of 10 mL/min (total running time will be 10–15 min).
53. When all of the plasma mixture has been added to the platelets, remove the platelets from the rocker.
54. Carefully disconnect the infusion set from the bag of platelets and transfer the platelet/plasma/ACD-A mixture to a 300-mL transfer pack.
55. When all of the mixture has run into the transfer pack, heat seal the tubing approx 2 cm from the transfer pack. Discard the tubing and the solution administration set.
56. Discard the empty platelet freezing bag.
57. Centrifuge the thawed platelet mixture at 1250*g* for 15 min.
58. Carefully remove the platelets from the centrifuge cup and place the bag in the plasma extractor.
59. Express the DMSO-containing supernatant plasma into another 300-mL transfer pack, leaving behind the undisturbed platelet button.
60. Heat seal the tubing between the two transfer packs and discard the bag containing the supernatant.
61. Insert one end of a double-coupler plasma transfer set into one port of the 120-mL bag of autologous plasma; insert the other end into the transfer pack containing the platelets.
62. Open the plasma transfer set roller clamp and allow a small amount (10–15 mL) of autologous plasma to run in with the platelets.
63. Using a wet folded paper towel, gently rub the platelet button until the mixture is homogeneous.
64. While gently agitating the bag of platelets, open the roller clamp on the plasma transfer set, and allow the remainder of the plasma to run in with the platelets.
65. Remove the plasma transfer set from the port of the bag of resuspended platelets; discard the set, and the empty plasma bag.
66. Insert a sampling site coupler into the open port of the bag containing the platelets.
67. To obtain a sample of the resuspended platelets, wipe the coupler with an alcohol pad and insert a 3-mL syringe and an 18-gage needle.
68. Withdraw 1–2 mL of platelets into the syringe, but do not remove the syringe from the sampling site coupler. Invert the bag several times so the platelet suspension is thoroughly mixed, then inject the sample in the syringe back into the bag.
69. Repeat **step 67** two or three times (so the specimen will be a representative sample) and remove the syringe from the sampling site coupler.
70. Perform a platelet count on the sample and obtain the net weight of the final bag of re-suspended platelets. Calculate the platelet yield and the percent recovery.

3.6. HES–Intermediate Cooling Technique

Please *see* **ref.** ***13***, which was modified according to **ref.** ***29***.

1. 450–500 mL whole blood are collected in standard blood bank packs containing CPD-A as anticoagulant.

2. The blood is kept at room temperature for at least 3 h and a maximum of 1 d before the pack is centrifuged for the first time at 180*g* for 30 min at room temperature.
3. After removal of about 120–200 mL of the supernatant platelet-rich plasma (PRP) by means of a plasma extractor, a 10% volume ACD-A is added to the PRP.
4. The PC is obtained from the PRP after a second centrifugation at 180*g* for 60 min.
5. The supernatant after this centrifugation step is removed as complete as possible into an empty bag. The PC (optimum volume 20 ± 2 mL, can be adjusted by adding previously removed supernatant plasma) is resuspended within 10 min by careful kneading of the bag.
6. 20 mL of the cryoprotective solution and 2 mL ACD-A are added by means of a syringe.
7. After removing the air by means of an empty syringe the bag is heat-sealed.
8. The resulting suspension of approx 40 ± 2 mL contains approx 4 g HES/100 mL and 700,000–1,000,000 platelets per microliter. It is transferred into the freezing bag and incubated for 15 min at room temperature under agitation.
9. The bag is then placed into an aluminium container of 3-mm inner thickness (wall thickness 1 mm) and the cooling process is started using a controlled-rate freezer. The cooling rate in the temperature region between –5 and –70°C should be approx 15 ± 5°C/min.
10. When a chamber temperature of –70°C (or lower) has been reached, the freezing chamber is opened, and the bags are removed from the freezing containers and stored in the vapor phase over LN_2. Use hand tongs, gloves, and goggles.
11. For thawing, they are transferred from the LN_2 storage tank into a punched thawing container within 30 s.
12. Thawing is performed in a shaking water bath (37°C, shaking frequency approx 60 cycles/min, shaking amplitude 2 cm) within 120 s, the temperature of the thawed suspension should be 22 ± 2°C.
13. For the removal of the HES (if desired), transfer the thawed product into a 250-mL transfer pack and add approx 200 mL of isotonic saline (or autologous or AB0-identical plasma) supplemented with 10% ACD-A by means of a 50-mL syringe. Centrifuge at 580*g* for 20 min at room temperature. Discard the supernatant and resuspend the platelets in plasma.

4. Notes

1. Optimum results can only be achieved when increasing the cooling rate from 1°C/min as described in Choudhury's method ***(13)*** to cooling rates of 15 ± 5°C/min and reducing the sodium chloride concentration in the cryoprotective solution from isotonic to 120 mmol/L ***(29)***. When comparing the optimized HES method to Schiffer's technique ***(11)***, we found that both protocols are highly effective regarding the post-thaw numerical platelet recovery (approx 90%). However, functional in vitro parameters showed that the DMSO-protected frozen platelets were inferior to fresh controls but superior compared with the HES protected ones ***(30)***.

2. Generally, the cell concentrations of mononuclear cells range from 0.5×10^6 to 50×10^6/mL. The most frequently used medium is RPMI 1640 supplemented with human or fetal calf serum or plasma, and the cryoprotectant of choice is 5–10% DMSO. Cooling is performed in 1- or 2-mL vials at 1–2°C/min down to a temperature of –30°C or less by means of a programmable LN_2-operated freezer, whereas thawing is usually performed in a water bath of 37°C. Numerical recoveries reported vary from 60 to 90%. We believe that cooling rates at temperatures below –40°C are not as critical as in the upper temperature region (i.e., above –40°C) and can be increased up to 10°C/min to save time. Additionally, we do not think that a programmable LN_2-operated freezer is always required to generate the appropriate cooling rate: –80°C refrigerators may be suitable as long as provision is taken (e.g., by using card board insulations) that the cooling rates in the upper temperature region does not exceed 5°C/min. For long-term storage (e.g., months or years), however, we recommend temperatures below –123°C, which is the glass transition temperature of DMSO ***(31)***. For days or weeks and up to months, –80°C freezers may also be acceptable.
3. The solution is commercially not available at present. It can be prepared from dry HES powder or commercially available HES solutions after dialysis and freeze-drying ***(32)*** by dissolving the dialyzed and freeze-dried HES and the appropriate amount of sodium chloride in distilled water. Suppliers of the dry HES powder are, e.g., Ajinomoto, Fresenius Kabi. Suppliers of HES solutions for infusion are, e.g., Baxter, B. Braun , Fresenius Kabi, Serag-Wiesner, and Serum Werk Bernburg.
4. Sputtek, A., Mingers, B. Freezing container, European Patent 0 786 981 (Germany, Great Britain, France, Spain, Switzerland, Italy, Netherlands), Priority 17/10/1994; German Patent P 44 37 091 C2; US Patent 5,935,848; Canadian Patent 2,203,035; Japanese Patent 8-512944.
5. In the case of not leukodepleted RBCs, five washing steps have to be performed to guarantee the same post-thaw saline stability as can be obtained with leukodepleted RBCs after three washing steps.
6. The containers have a wall thickness of 2 mm. The exterior is pasted with a microporous textile tape to improve the heat transfer during the cooling process in boiling LN_2. Additionally, the closed containers produce a well-defined flat geometry of the bags and a homogenous sample thickness (approx 5–6 mm). LN_2 is not allowed to come into contact with the samples during the initial cooling process. Please note that you will not be able reproduce our results when not using the patented freezing container.
7. HES coats the surface of the red cells and may provide a scaffolding for damaged membranes so that some cells appear intact, though they will rupture if diluted with isotonic saline. Viability in terms of "saline stability" can be determined as follows: 250 µL of the RBC suspension is diluted 40-fold in a buffered isotonic saline solution. After 30 min the suspension is separated into a supernatant (destroyed RBC) and sediment (intact RBC) by centrifugation. Saline stability is then calculated using:

$$\text{Saline stability (\%)} = (1 - Hb_S/Hb_T) \times 100,$$

where Hb_T corresponds to the total hemoglobin and Hb_S to the hemoglobin in the supernatant. The determination of the two hemoglobin concentrations can be performed spectrophotometrically at 546 nm using Drabkin's solution. A correction for the HCT is not required, as the volume fraction of erythrocytes after a 40-fold dilution is less than 2%.

8. It is essential to remove white cells and platelets. Thomas et al. ***(10)*** have speculated that the contents of the white cells and platelets are highly thromboplastic, and as these cells are destroyed on freezing, thereby liberating their contents into the HES/RBC mixture, failure to filter could cause a disseminated intravascular coagulopathy when the RBCs are subsequently thawed and transfused without post-thaw washing. Modification of the protocol is required for cryopreservation of sheep red cells; CPD or CPD-A cause slight damage to sheep cells so heparin is the anticoagulant of choice, but it is vital that special care is taken when mixing during donation.
9. For cryopreservation of pig cells, which are a similar size to human red cells, Thomas et al. ***(10)*** have reported that the main requirement is to remove all the plasma by saline rinsing prior to the addition of HES. If all the plasma is not removed, progressive hemolysis occurs, possibly from complement activation. Sheep cells are spherical and only about one-third of the volume of human red cells, so the plasma is more difficult to separate. It is therefore necessary to centrifuge for about 25 min to pack the sheep red cells to 90% HCT.
10. Both human and sheep red cells have been stored in the LN_2 vapor phase, without deterioration, for up to 12 yr, as demonstrated by in vitro testing. Storage in mechanical freezers has been carried out at the following temperatures, without deterioration of the red cells as assessed by in vitro quality control tests, for the undermentioned periods: –140°C for 4 mo; –120°C for 4 mo; –100°C for 4 mo; –90°C for 5 wk (*see* **Note 7**).
11. Plasma stabilities may be slightly higher than saline stabilities because plasma factors allow a few very slightly damaged cells to recover. The percentage recovery of intact cells, immediately post-thaw, is not used as a quality control procedure because it is only a crude indicator of quality. HES coats the surface of the red cells and may provide a scaffolding for damaged membranes so that some cells appear intact, though they will rupture if diluted with isotonic saline.
12. If performing a split-freeze, repeat **Subheading 3.5., steps 19–23** until the volume in the platelet freezing bag equals 45 times the number of bags to be frozen. (Example: if it was determined in **Subheading 3.5., step 12** that the freeze is to be split into three bags, repeat **Subheading 3.5., steps 19–23** until the volume in the first platelet freezing bag equals $45 \times 3 = 135$ mL.) Mix well. Using the same syringe, withdraw 45 mL of platelet concentrate from the first platelet freezing bag and inject it into a second platelet freezing bag. Repeat until each freezing bag contains 45 mL of platelet concentrate. (Example: there will be three freezing bags, each containing 45 mL of platelet concentrate.)

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21

Cryopreservation of Mammalian Semen

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Summary

Mammalian spermatozoa were among the very first cells to be successfully cryopreserved and over the last five decades the use of frozen-thawed semen for artificial insemination has come to play an important role in domestic livestock production. More recently, semen freezing has increasingly been utilized in the establishment of genetic resource banks for endangered species. Semen is collected, most commonly either by use of an artificial vagina or by electroejaculation of an anaesthetized animal, and basic sperm parameters assessed. Semen is extended using a TEST-egg yolk-glycerol diluent, packaged in 0.25-mL plastic straws and slowly cooled to 5°C over a period of 1–2 h. Cooled straws are frozen by suspending within liquid nitrogen vapor above the liquid nitrogen surface before plunging into the liquid phase. Straws are thawed briefly in air before immersing in a 35°C water bath for 15 s, and often are used directly for insemination without any further processing.

Key Words: Spermatozoa; mammalian; cryopreservation; glycerol; genetic resource bank.

1. Introduction

Although the first observations concerning low-temperature preservation of spermatozoa date as far back as 1776, when the Italian physiologist Spallanzani noted that spermatozoa cooled in snow became inactive but could be revived on warming; successful cryopreservation protocols truly date only from the 1950s ***(1)***. In 1949, the single most important development came with the discovery by Polge, Smith, and Parkes ***(2)*** that glycerol could act as a cryoprotectant for spermatozoa. The initial experiments with glycerol were performed using fowl sperm but after much effort, these were quickly followed by the successful preservation of bull sperm ***(3)*** with the first calf conceived by artificial insemination (AI) using frozen-thawed sperm reported in 1951 ***(4)***. Over the subsequent 50 yr

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semen cryopreservation has established a central role in livestock production with the use of frozen-thawed semen for AI becoming the norm for the cattle-breeding industry. The use of frozen semen is less dominant in other areas of livestock production, but protocols have been established and are increasingly used for all the major domestic species ***(5)***. Sperm cryopreservation has also played an increasingly important conservation role with the establishment of genetic resource banks for endangered domestic species and more recently for many at-risk wild species ***(6)***.

There are marked species differences between spermatozoa, obvious differences in size and morphology, but also more subtle differences in, for example, membrane phospholipids and metabolism. These differences reflect the high level of variation between species, and in some cases between males of the same species, in sensitivity to the freeze–thaw process. However, despite species variation there are common stages to any sperm freezing protocol. All protocols involve semen collection and extension, addition of cryoprotectant and cooling above 0°C, cooling below 0°C, storage, thawing, and insemination.

Viable semen must first be collected. With domestic species this can be ejaculated semen either by training animals to the use of an artificial vagina (e.g., bulls, rams, stallions) or by manipulation (e.g., boars, dogs). Where animals are unresponsive to training, electroejaculation by means of an anal probe may be used; this can be an effective method but has, in some instances, attracted adverse comment on welfare grounds ***(7)***. For wild species where animals may be, at best, stressed by handling, and at worst dangerous, electroejaculation under general anaesthesia with the attendant problems and risks is the only real option for obtaining ejaculated semen ***(8)***. Epididymal sperm have been successfully recovered and frozen, either from animals under anaesthetic or postmortem, when sperm may remain viable for surprisingly long periods of time making this a possible option even under some field conditions. Following collection, semen is routinely extended with an appropriate diluent to adjust concentration, usually to produce a suitable insemination dose within a fixed volume, and second to alleviate what can be pronounced adverse effects of seminal plasma constituents on sperm survival.

Sperm freezing protocols commonly use an initial slow cooling rate to take samples from body temperature or ambient collection temperature down to 5°C. This slow cooling is important as a number of mammalian species demonstrate a temperature sensitivity termed cold shock ***(9)***. Cold-shock sensitivity varies between species and between individuals within species; it is most evident in the temperature range between 0 and 20°C and is thought to be linked to temperature-dependent phase changes in the membrane lipids. The presence of

egg yolk in the diluent (5–20% [v/v]) has been shown to be protective against the effects of cold shock. Although the mechanism of this protection is not well understood it appears to be associated with the low-density lipoprotein fraction of egg yolk ***(9)***. Egg yolk-protective effects may be enhanced by the addition of the surfactant sodium triethanolamine lauryl sulphate (Orvus paste) to freezing diluents ***(10)***.

The addition of some cryoprotective agent (CPA) is necessary for sperm survival. Polge et al.'s original successes with fowl and bull semen ***(2,3)*** were dependent on glycerol and although probably hundreds of potential CPAs and combinations of CPAs have been examined, glycerol remains the cryoprotectant of choice for spermatozoa from almost all species. However, glycerol's cryoprotective actions must be balanced against a higher level of toxicity for sperm than is seen with some other cell types. Most protocols use a concentration range of between 0.5–1.5 *M* (approx 4–10% [v/v]), although some species show a greater sensitivity and will tolerate levels of only 2% or less (e.g., boar). One exception to the use of glycerol is with rabbit spermatozoa where dimethyl sulfoxide is the cryoprotectant of choice ***(11)***. CPA can be added at ambient temperature prior to the slow cooling phase or can be added at 5°C after slow cooling. Additional after cooling may minimize toxicity effects and means that sperm are not in contact with CPA throughout the prolonged slow cooling period. Some protocols have held sperm at 5°C for a so-called "equilibration" period amounting to several hours. This period may allow time for beneficial membrane phospholipid alterations to take place; however, in many species this holding period following addition of the CPA appears to confer little benefit and may even, in some cases, be detrimental to survival rates.

Compared with many other cell types, spermatozoa appear to be relatively insensitive to cooling rates below the freezing point. Sperm do show the characteristic inverted "u"-shaped curve with cryosurvival against cooling rate predicted by Mazur's two-factor hypothesis ***(12)***, but it is much more difficult to identify a clearly optimal cooling rate than it is for many other cell types, and a wide range of optimal rates from less than 10°C/min up to greater than 150°C/min have been quoted for different species under different freezing conditions ***(7)***. Linear cooling rates may be provided by controlled-rate freezers, however, rates are often determined by suspending samples at a fixed height above the liquid nitrogen surface in the vapor phase. Under these circumstances cooling will be affected by the size and surface area of the semen packaging. Vapor-phase cooling does not produce a linear cooling rate and there is some evidence that this nonlinear cooling actually increases the functional survival of spermatozoa ***(13)***.

The problems encountered in optimizing protocols are compounded by difficulties in assessing true success rates for sperm cryopreservation. Morphologically intact and apparently normal motile cells post-thaw can prove on insemination to be functionally damaged and consequently infertile, therefore, a range of parameters should be examined when assessing sperm cryosurvival where possible, including some kind of functional test. Variation occurs between species in the fertility of frozen semen used for AI compared with fresh semen, although this may be explained in part by differences in the number of spermatozoa used, in the site of deposition, and in the degree of technical difficulty involved in the insemination procedure. The freeze–thaw process also induces a "capacitation-like" state in spermatozoa and curtails their viable lifespan within the female reproductive tract ***(5)***. Consequently, insemination times relative to ovulation may need to be adjusted when using frozen semen compared with fresh semen.

Attempts to improve survival rates and to overcome species variation have produced an extensive literature detailing the largely empirical optimization of freezing protocols that has taken place over the last 50 yr ***(7)***. The effects of varying diluent composition, freezing and thawing rates, and freezing methodology together with the various interactions that exist between these factors have been reported for a wide range of species ***(7)***. However, sperm survival has generally remained around the level of 50–60%, a figure that has changed very little throughout the history of semen cryopreservation. Survival rates of approx 50% have been adequate to make practical use of cryopreserved sperm for insemination over many years; however, new developments have increasingly highlighted the need for improved survival rates. As the commercial value of livestock semen has grown, a wastage rate of between 30 and 50% has become unacceptable and the introduction of new technologies, such as sperm sex selection, has made it more important to increase survival rates. Because sperm samples may be difficult to come by and of low quality in the conservation field, it becomes important to be able to preserve the maximum number of cells possible from any given sample.

More recently there have been attempts to model sperm behavior during the freeze–thaw cycle using a number of biophysical membrane parameters with the aim of producing theoretically optimal protocols that can be tested in practice ***(14,15)***. This work has led to some new insights into sperm behavior during the freeze–thaw cycle and a greater understanding of some of the causes of sperm cryoinjury. The protocol given in this chapter, although based on established livestock methodologies, is not given as being optimal for any particular species, but as one which should result in a degree of survival over a range of species using a simple and straightforward method. The notes section gives some further information concerning the specific requirements of different species.

2. Materials

1. TEST buffer: 48.6 g/L *N*-tris(hydroxymethyl)methyl-2-aminoethanesulfonic acid (TES) and 11.0 g/L tris(hydroxymethyl)aminomethane (Tris). Dissolve TES and TRIS in 2X distilled water and adjust pH to 7.1. Filter through a 0.2-µm filter and store at 4°C.

   Although TEST buffer may be stored at 4°C prior to use, the complete TEST-egg yolk-glycerol freezing diluent is best prepared fresh on the day of use. All reagents should be ANALAR grade.
2. TEST-egg yolk-glycerol (TEST-EY-G): to TEST buffer solution add 9 g/L glucose, 500 iu/mL penicillin, and 0.5 mg/mL streptomycin sulfate. Adjust osmolarity to 350 mOsm. Add 15% (v/v) egg yolk. Egg yolk must be completely separated from the albumen and dried by rolling on filter paper. Centrifuge at 2000*g* for 20 min and decant supernatant for use discarding the pellet, then add 5% (v/v) glycerol.
3. 0.25-mL cryostraws for semen packaging.
4. Dewar containing liquid nitrogen suitable for long-term storage of the frozen samples.
5. Safety equipment for liquid nitrogen handling.

3. Methods

1. Collect sample into a clean and dry container with sufficient thermal insulation to maintain sample above 20°C (*see* **Note 1**).
2. Assess the sample for concentration, motility, and normal morphology (*see* **Note 2**).
3. Dilute in TEST-EY-G to a final concentration of approx 250–500 $\times$ 10^6/mL; if this involves a dilution of less than 5:1 TEST-EY-G to semen wash the semen once in TEST-EY and resuspend to the final concentration in TEST-EY-G (*see* **Notes 3–6**).
4. Load the diluted semen into 0.25-mL plastic straws and seal with polyvinyl acetate (PVA) powder (*see* **Note 7**).
5. Cooling to 5°C: place the straws in a 500-mL beaker of water at ambient temperature and place in a refrigerator for 1–2 h to allow slow cooling to 5°C (*see* **Note 8**).
6. Suspend the straws horizontally 5 cm above the surface of the liquid nitrogen for 7 min and then plunge rapidly into the liquid nitrogen (*see* **Note 9**).
7. Long-term storage: keep the straws submerged in liquid nitrogen. Once at –196°C, sperm may be stored for practical purposes indefinitely without any further loss of viability; however, any partial thawing and refreezing may result in degradation of the sample.
8. Thawing: remove straws from the liquid nitrogen and thaw in air for 2–3 s then plunge into a water bath at 35°C for 15 s (*see* **Note 10**).
9. Insemination: many insemination protocols use freshly thawed sperm straight from the straw without any further processing; however, a number of straws may be combined and samples can be concentrated by centrifugation where necessary to increase the insemination dose (*see* **Note 11**).

4. Notes

1. In some species males may exhibit a seasonal effect on spermatogenesis generally dependent on photoperiod. Seasonality can range from testicular regression with almost total spermatogenic quiescence through a reduction in sperm numbers outside the breeding season, to year-round semen production, depending on species and circumstances.
2. Semen assessment. Semen of poor quality is generally found to have poorer survival after freezing and thawing. A routine assessment should be performed to ensure samples conform to minimum standards for concentration, motility, and normal morphology.
3. A wide range of diluents of varying composition have been used in successful freezing protocols but the basic requirements are the same in all cases. The diluent must maintain osmolarity, pH, and ionic strength, provide an energy substrate, contain a CPA, and usually contain antimicrobial agents. The majority of diluents are slightly hyperosmotic relative to semen, although isosmotic diluents have been successfully used. Hyperosmotic diluents probably result in a degree of dehydration that is advantageous during freezing. A number of preprepared diluents are now commercially available for the major livestock species. The optimum pH for mammalian spermatozoa is close to neutrality, as most diluents buffer to pH 6.9–7.1. TES-TRIS buffer as used previously acts as a major component in the diluent serving to control pH and osmolarity. Citrate, one of the earliest and most extensively used buffers, also acts as a major component; other buffers such as MES or HEPES may be used as minor components only controlling pH. Proteins present in either fresh or skim dried milk diluents are adequate to buffer pH, and milk diluents have been extensively used for a range of species. Egg yolk proteins also have a minor buffering effect. Ionic strength appears to be of minor importance. Sperm have been successfully frozen at widely differing ionic strengths provided osmolarity is maintained by nonionic constituents. Some species, e.g., boar, do show optimal survival at relatively low ionic strengths. An energy substrate is provided by sugars such as glucose, fructose, or mannose, the only sugars glycolysable by sperm ***(1)***. Species differences in the rate at which these sugars are metabolized may govern which is optimal for any given species. Sugars also have other functions within the diluent contributing to the osmotic strength and, in some cases, having a cryoprotective effect. However, which combinations of sugars may prove to be most beneficial under any given set of conditions remains unclear.
4. The concentration of glycerol in the diluent for optimal cryosurvival varies for different species and in some species high concentrations may preserve motility or morphology but impair fertility. For example, boar spermatozoa show good postthaw motility when frozen with 7% (v/v) glycerol but fertility is only maintained with less than 3% (v/v) ***(16)***. Optimal glycerol concentrations for other domestic species are bull 6–9% (v/v) ***(17)***, ram 3–4% (v/v) ***(18)***, and stallion 4% (v/v) ***(19)***. A stepwise dilution process for the addition of glycerol is beneficial in some cases as a way of minimizing the osmotic stresses associated with the addition of permeating cryoprotectants ***(20)***.

5. Seminal fluid is normally biologically sterile; however, contamination is generally unavoidable during semen collection. The presence of microorganisms can have a range of adverse effects on fertility and the inclusion of antimicrobial agents has been variably reported as having a beneficial effect on the outcome of insemination, probably reflecting variation in initial contamination levels. A broad spectrum antibiotic as a safeguard against microorganism proliferation is included in most diluents. Penicillin (500–1000 iu/mL) and streptomycin (0.5–1.0 mg/mL) are the most commonly used and at these levels are nontoxic to spermatozoa ***(21)***. A number of other antibiotics have been used, although some have been found to have adverse effects on sperm metabolism, e.g., sulphanilamide, oxytetracycline, or gentamicin ***(22,23)***.
6. The dilution stage has three purposes, to minimize any potential toxic effects of seminal plasma, to suspend the spermatozoa in the TEST-EY-G freezing diluent, and to extend the semen to allow maximum usage. Overdilution may lead to adverse so-called dilution effects and with very low dilution rates, care must be taken that the spermatozoa are exposed to the correct levels of egg yolk and glycerol in the freezing diluent. Dilution rate is usually governed in practice by the number of spermatozoa required for artificial insemination. The relatively high post-thaw fertility of bull spermatozoa means comparatively high dilution rates are possible as a smaller number of spermatozoa are required in the inseminate (approx 2×10^7), thus maximizing the number of inseminations per ejaculate. Much greater sperm numbers are required for insemination with the boar (approx 8×10^9), the ram (approx 3×10^8), and the stallion (approx 2.5×10^8), reflecting the lower fertility of the frozen-thawed semen and consequently the lower dilution rates in these species.
7. Plastic straws are available in a range of sizes from two major suppliers IMV Technologies (L'Aigle, France) and Minitub (Landshut, Germany). Different coloured straws and seals enable samples to be colour coded for ease of identification. Automated systems are available for filling, sealing and labeling large numbers of straws (IMV Technologies, Minitub).
8. Slow cooling can be done using a controlled-rate cooling bath with a cooling rate of approx 0.25°C/min. Cold-shock sensitivity of different species will determine the maximum cooling rate that sperm will tolerate.
9. Optimal cooling rates for semen of domestic species are generally considered to be in the range 10–100°C/min. The protocol given here gives a rate of approx 90°C/min, but this is not a linear cooling rate. Spontaneous ice nucleation occurs at approx –15 to –20°C and the latent heat released causes a temperature rise of around 7 or 8°C thereafter. The cooling curve is sigmoidal. With the use of controlled-rate freezers and linear cooling programs, controlled ice nucleation can be induced at a temperature of –5°C by seeding straws (touch straws with forceps cooled in liquid nitrogen to induce local ice crystallization, which will then rapidly propagate throughout the straw). A short hold phase in the cooling program at –5°C will allow the dispersion of latent heat before resumption of linear cooling. Such seeding techniques are common in many cell-freezing protocols but have not been widely used for spermatozoa. Seeding may be beneficial for functional sperm cryosurvival but this is yet to be clearly demonstrated. The final temperature reached before plunging

into the liquid nitrogen is approx –120°C. Actual cooling rates are dependent on the conditions of the vapor column and the number of straws frozen.
Slower cooling rates to higher temperatures may be achieved by suspending the straws further from the liquid nitrogen surface. However, the straws should be cooled to at least –50°C before plunging.

10. Faster thawing rates may be achieved by using higher temperatures for shorter periods of time, e.g., 65°C for 5 s. Faster thawing is generally considered to be the most effective, although the evidence for enhanced fertility is not good. At faster thawing rates care must be taken not to allow any overheating of the sperm.
11. A number of up-to-date reviews exist that give an introduction to the very extensive literature concerning semen cryopreservation. Reviews are available concerning semen preservation and artificial insemination in general ***(7)***, as well as recent cryotheory with respect to cryopreservation of spermatozoa ***(24,25)***. Two recent review volumes on genetic resource banking "Cryobanking the Genetic Resource" ***(26)*** and "Reproductive Tissue Banking" ***(27)*** contain extensive reviews of the status of sperm freezing over a wide range of species.

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22

Cryopreservation of Mammalian Oocytes

Sharon J. Paynter and Barry J. Fuller

Summary

Two methods for the cryopreservation of mammalian oocytes are described. One method uses a relatively low concentration of the cryoprotectant propanediol plus sucrose and requires controlled-rate cooling equipment to achieve a slow cooling rate. Such a method has produced live births from cryopreserved human oocytes. The second method described employs a high concentration of the cryoprotectant dimethyl sulfoxide plus a low concentration of polyethylene glycol. This method involves cooling by plunging standard straws into liquid nitrogen vapor, hence avoiding the need for specialized equipment, but requires technical ability to manipulate the oocytes quickly in the highly concentrated cryoprotectant solutions. Murine oocytes vitrified, using this technique, have resulted in live births.

Key Words: Egg; human oocyte; oocyte; slow-cool; vitrification.

1. Introduction

Storage of unfertilized oocytes has numerous applications. In the treatment of human infertility these applications include storage of oocytes collected prior to receipt of cancer treatments or premature ovarian failure, banking of excess oocytes produced as a result of in vitro fertilization treatments, and donation of oocytes to other people (storage giving the added advantage of allowing time to screen donors for disease and in some countries, e.g., Taiwan, donation of oocytes but not embryos is authorized). Oocyte cryopreservation could also serve as a means of delaying child bearing to an age when natural fertility and/or oocyte quality has declined. Applications in animal management include the preservation of precious strains, the ability to restock following outbreaks of diseases such as foot and mouth, the preservation of genetically modified strains, thereby reducing the cost of continuous breeding and avoiding problems of genetic drift, and the preservation of endangered species. Oocyte preservation

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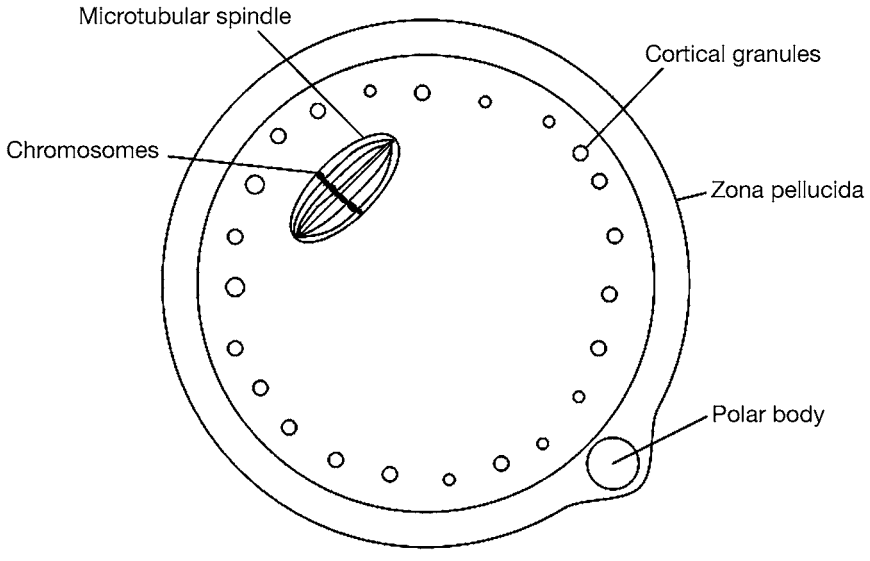

Fig. 1. Schematic representation of a mature mammalian oocyte.

is considered preferable to embryo preservation in humans because ethical concerns are fewer for unfertilized gametes. Furthermore, in some countries cryopreservation of human embryos is banned or is strictly limited. In animal management, oocyte cryopreservation gives greater flexibility in breeding programs than embryo cryopreservation.

In 1977, the first live births from cryopreserved ovulated mammalian oocytes were reported in mice ***(1)***. Since then births resulting from cryopreserved oocytes have been reported in a number of species including rabbits ***(2)***, cows ***(3)***, horses ***(4)***, and humans ***(5)***. However, in most species success rates are markedly poorer than for cryopreserved embryos.

Oocytes are proving to be very difficult cells to cryopreserve for a number of reasons. The oocyte is a large single cell with low permeability to water. This means that it has a tendency to retain water when cooled and, if this forms intracellular ice, damage to the cell results. Permeability of oocytes varies between species, strains, and maturational status of the oocyte. The oocyte is also a short-lived cell that must undergo fertilization in order for it to continue to survive and develop. For fertilization to occur naturally, the oocyte must retain integrity of a number of its unique structural features. These include the zona pellucida, the cortical granules, and the microtubular spindle (*see* **Fig. 1**). The zona pellucida is a glycoprotein coat surrounding the oocyte. Changes to this layer, triggered by the action of a single sperm binding to its receptors, induce the cortical granules to release their contents. The enzymes released from the

cortical granules act to crosslink the glycoproteins of the *zona pellucida*, thus rendering it impenetrable to further sperm ***(6)***.

Cryopreservation has been shown to result in the premature release of the contents of cortical granules, thus creating a block to sperm penetration ***(7)***. Conversely, cryopreservation can lead to damage to the *zona pellucida* resulting in multiple sperm entry. Both of these problems can be overcome by application of the intracytoplasmic sperm injection (ICSI) technique, whereby a single sperm is injected into the oocyte to achieve fertilization. The microtubular spindle is the structure on which the condensed chromosomes are aligned in mature oocytes and is responsible for the movement of chromosomes during cell division. Damage to this structure can lead to aneuploidy. During cryopreservation the microtubular spindle has been shown to disassemble, although there is growing evidence that the spindle is capable of repair on warming ***(8–10)***. Whether such repair has adverse consequences during development of the resulting embryo is still uncertain. One way of avoiding the potential for damage to the microtubular spindle is to cryopreserve the oocyte before the spindle is formed, when the chromosomes are contained within the germinal vesicle and the oocyte is termed immature. However, success following cryopreservation of immature oocytes is less than that following cryopreservation of mature oocytes. If protocols for hormonal stimulation of ovaries to induce release of numerous mature oocytes are not available or not easily applicable, then only immature oocytes, contained within the ovaries, may be available for storage. The major problem with cryopreserving immature oocytes is that they must be matured in vitro in order to become fertilizable. This maturation involves communication between the oocyte and the cumulus cells that surround it ***(11)***. The cumulus cells are much smaller than the oocyte and are connected with it by numerous gap junctions. Development of cryopreservation protocols that allow survival of the oocyte, the cumulus cells, and the connections between the two is difficult ***(12)***. Also, techniques for maturation in vitro require further refinement in most species. Maturation of oocytes in vitro prior to cryopreservation may produce poor-quality oocytes that are more prone to damage during cryopreservation. As well as these general problems, the oocytes of some species have further characteristics that make them still more difficult to freeze; for example, porcine oocytes have a high lipid content and are sensitive to chilling to temperatures at or below 15°C ***(13)***.

The protocols used for the cryopreservation of unfertilized oocytes have largely been adopted from embryo cryopreservation techniques. As with embryo cryopreservation, both slow controlled-rate cooling and vitrification techniques have been successfully applied. The technique most commonly applied to the cryopreservation of human oocytes is that of slow cooling in the presence of propanediol and sucrose. This technique, combined with

ICSI, first yielded a live birth from cryopreserved human oocytes in 1997 ***(14)***. Since then, the number of human live births has been increasing steadily, with approx 100 children having been born from cryopreserved human oocytes, fertilized using the ICSI technique, by 2004 ***(9)***. Vitrification has also produced a very small number of human births ***(15,16)***. Because of differences between species, no single cryopreservation method is suitable for all species and all developmental stages of oocyte. Despite success, the proportion of oocytes surviving and going on to produce live births is still rather disappointing. Following oocyte cryopreservation for human oocytes only 1–5% of oocytes frozen result in live births. The quality of the oocytes to be preserved is a crucial factor in survival post cryopreservation ***(17)***. Counseling regarding expected outcome should be provided to patients prior to oocyte cryopreservation being offered as a treatment option.

2. Materials

2.1. Slow Controlled-Rate Cooling

1. A controlled-rate freezing machine (available from several sources such as Planer plc, Middlesex, UK or Asymptote, Cambridge, UK) set to hold at 20°C, then cool at –2°C/min to –7°C, hold at –7°C for 10 min prior to seeding (*see* **Note 1**), hold at –7°C for 10 min after seeding, then cool at 0.3°C/min to –30°C, then at –50 to –150°C and finally hold at –150°C for 10 min (*see* **Note 2**).
2. Plastic straws (*see* **Note 3**).
3. Plugs for straws (IMV, L'Aigle, France), sealing powder or heat sealer (*see* **Note 4**).
4. Pulled glass pipets or automated pipettor for transferral of oocytes.
5. Tissue culture dishes (Falcon, Becton and Dickinson Co.).
6. Dissecting microscope (×40 magnification).
7. Hotplate set at 37°C.
8. Forceps cooled in liquid nitrogen (optional; *see* **Note 1**).
9. Liquid nitrogen and liquid nitrogen Dewars, preferably at least two storage Dewars (*see* **Note 5**) and one for transporting samples to storage Dewar and for cooling of forceps, if used.
10. Sterile scissors.
11. Syringes and needle (optional; *see* **Notes 6** and **7**).
12. Heated water bath set at 30°C.
13. Safety equipment, e.g., cryogloves, face shield, and oxygen depletion monitor.
14. Heated gassed incubator (5% [v/v] CO_2, humidified atmosphere).
15. Oocyte culture medium, e.g., for human oocytes fertilization medium (Cook IVF, Brisbane, Australia), and for murine oocytes Tyrode's medium (Invitrogen, Paisley, UK).
16. Dulbecco's phosphate-buffered solution (PBS; Invitrogen) for human oocytes supplemented with 30% (w/v) protein supplement, e.g., plasma protein supplement (Baxter AG, Vienna, Austria) or serum protein supplement (Pacific Andrology,

CGA/Diasint, Florence, Italy) for mouse oocytes supplemented with 5% (v/v) heat-inactivated fetal bovine serum (Invitrogen).
17. Freezing solutions: 1.5 *M* 1,2-propanediol (PrOH) and 1.5 *M* PrOH plus 0.2 *M* sucrose, both made up in PBS with protein supplement.
18. Thawing solutions: 1.0 *M* PrOH plus 0.2 *M* sucrose, 0.5 *M* PrOH plus 0.2 *M* sucrose, and 0.2 *M* sucrose, all made up in PBS with protein supplement.
19. Hyaluronidase (optional; *see* **Note 8**).

2.2. Vitrification

1. Plastic straws (*see* **Notes 3** and **9**).
2. Plugs for straws (IMV), sealing powder, or heat sealer (*see* **Note 4**).
3. Pulled glass pipets or automated pipettor for transferral of oocytes.
4. Tissue culture dishes (Falcon, Becton and Dickinson Co.).
5. Dissecting microscope (×40 magnification).
6. Thermocouple.
7. Device to hold straw horizontally above liquid nitrogen vapor without covering area of straw containing oocytes (*see* **Note 10**).
8. Hotplate set at 37°C.
9. Liquid nitrogen and liquid nitrogen Dewars, preferably at least two storage Dewars (*see* **Note 5**), plus a container capable of holding liquid nitrogen and accommodating a straw held horizontally.
10. Sterile scissors.
11. 3X 1-mL syringe and 2X needle.
12. Heated water bath set at 20°C.
13. Safety equipment, e.g., cryogloves, face shield, and oxygen depletion monitor.
14. Heated gassed incubator (5% CO_2, humidified atmosphere).
15. Oocyte culture medium, e.g., for human oocytes fertilization medium (Cook IVF), for murine oocytes Tyrode's medium (Invitrogen).
16. PBS for human oocytes supplemented with 30% (w/v) protein supplement, e.g., plasma protein supplement (Baxter AG) or serum protein supplement (Pacific Andrology, CGA/Diasint) for mouse oocytes supplemented with 5% (v/v) heat-inactivated fetal bovine serum (Invitrogen).
17. Vitrification solutions: 6 *M* dimethyl sulfoxide plus 1 mg/mL polyethylene glycol (MW 8000) added to 4X strength PBS medium without $CaCl_2$ (*see* **Note 11**). The solution should be made up by adding 1% (v/v) distilled water to 4X PBS prior to adding the dimethyl sulfoxide and polyethylene glycol. Then add the protein supplement and make up almost to volume with water prior to adding $CaCl_2$. Finally, make up to volume. Keep at room temperature until required. This solution will be referred to as VSDP (*see* **Note 12**). Make up dilutions of 25 and 65% (v/v) VSDP using PBS containing protein supplement.
18. Dilution solution consisting of 1 *M* sucrose made up in PBS containing the protein supplement.
19. Hyaluronidase (optional; *see* **Note 8**).

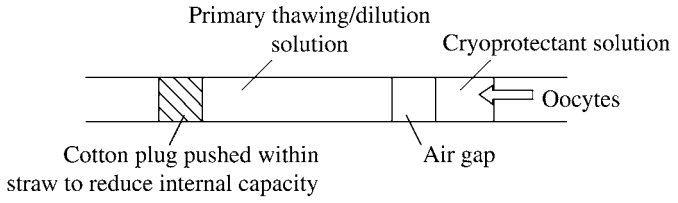

Fig. 2. Schematic diagram of straw prepared for cryopreservation of oocytes.

3. Methods

3.1. Slow Controlled-Rate Cooling

The slow controlled-rate cooling method described uses a mixture of the permeating cryoprotectant PrOH and nonpermeating sucrose. Recent studies suggest that 0.3 *M* sucrose yields better survival than 0.2 *M* sucrose in human oocytes ***(18)***. This is thought to be largely from greater dehydration of the cells prior to freezing. Optimal exposure time to PrOH plus 0.2 *M* sucrose is 5–10 min, whereas optimal exposure time to PrOH plus 0.3 *M* sucrose is yet to be determined. However, an exposure time of 2 min will give a level of dehydration equivalent to that achieved with a 5-min exposure to PrOH plus 0.2 *M* sucrose ***(19)***. A further potential modification to the method described next is the choice of medium in which to dilute the cryoprotectants. Recent studies have shown a medium in which some of the sodium has been replaced by choline to be preferable to Dulbecco's PBS medium ***(20–22)***.

1. Straws should be loaded with a 1-cm column of 1.5 *M* PrOH plus 0.2 *M* sucrose and left at room temperature (20 ± 2°C) until required (*see* **Fig. 2**; **Note 6**).
2. Place one 0.5-mL droplet each of PBS plus protein supplement, 1.5 *M* PrOH, and 1.5 *M* PrOH plus 0.2 *M* sucrose in a tissue culture dish. Label each droplet.
3. No more than five oocytes (*see* **Note 13**) should be placed in the droplet of PBS (cumulus cells may be removed, *see* **Note 8**) and then placed in the droplet of 1.5 *M* PrOH for 10 min at room temperature. The oocytes are then moved to the droplet of 1.5 *M* PrOH plus 0.2 *M* sucrose for 5 min at room temperature.
4. The oocytes are then loaded into the prepared straws within the column of 1.5 *M* PrOH plus 0.2 *M* sucrose and the straw is sealed (*see* **Note 4**).
5. The outside of the straws should be decontaminated using 70% ethanol in distilled water, an alcohol wipe, or, preferably, a less volatile disinfecting agent e.g., hypochlorite ***(23)*** and the straws are then placed immediately within a freezing machine set at 20°C. The straws should not be placed in bundles as this will affect the rate of cooling. The machine should then be cooled to –7°C at –2°C/min. Having been held at this temperature for 10 min, ice nucleation should be initiated in the solution containing the oocytes (*see* **Note 1**). The cooling regime is then resumed with the temperature being held at –7°C for 10 min to allow dissipation of the heat of crystallization. Once the cooling protocol has been completed and

the samples are held at –150°C, the straws should be decontaminated, as above, and, wearing appropriate protective equipment, placed in a liquid nitrogen storage Dewar (*see* **Note 14**).

6. Prior to warming the straws, prepare dishes containing a 0.5–1 mL droplet each of 1.0 *M* PrOH plus 0.2 *M* sucrose, 0.5 *M* PrOH plus 0.2 *M* sucrose, and 0.2 *M* sucrose and PBS with protein supplement. Label each droplet.
7. Wearing suitable protective equipment, warm the straws by holding them in air for 30 s, holding either end of the straw *not* the area containing the oocytes. Then place the straws in the water bath set at 30°C for 30 s or until the ice has just melted.
8. Decontaminate the straw. The plug, if used, should be removed from the straw or the ends of the straw should be cut with sterile scissors and the contents then expelled (*see* **Note 7**) into the dish containing 1 *M* PrOH plus 0.2 *M* sucrose.
9. The oocytes should remain in this solution for 5 min at room temperature before being moved into the droplet of 0.5 *M* PrOH plus 0.2 *M* sucrose again for 5 min at room temperature.
10. The oocytes are moved into 0.2 *M* sucrose for 10 min at room temperature.
11. The oocytes are then placed in the droplet of PBS for 20 min, 10 min at room temperature, and 10 min at 37°C on a hotplate.
12. The oocytes should then be cultured (2–3 h human, 30 min mouse) within an incubator at 37°C (5% CO_2) in a suitable culture medium to allow recovery prior to attempted fertilization (*see* **Note 15**).

3.2. Vitrification

Vitrification is advantageous over slow controlled-rate cooling in that expensive cooling machines are not required and the technique can be performed in the field. However, the need for high concentrations of cryoprotectant means that the times stated for cryoprotectant exposure must be strictly adhered to and manipulation of oocytes in these highly viscous solutions is technically demanding. The vitrification method described has resulted in high blastocyst formation ***(24)*** and live births ***(25)*** in mice, although the technique is prone to variability ***(26)***. The cryoprotectant mixture and cryopreservation vessel could be modified but the basic techniques described are applicable to all vitrification protocols.

1. Straws should be loaded with an approx 0.5-cm column of 100% VSDP (*see* **Fig. 2**; **Note 6**). Take care not to wet the sides of the straw with sucrose (*see* **Note 16**). Leave prepared straw at room temperature until required.
2. Place one 50-µL droplet each of 25, 65, and 100% (v/v) VSDP in a tissue culture dish. Label each droplet.
3. Place liquid nitrogen to a level equivalent to at least the length of the straw into the vessel capable of accommodating the straw horizontally. Use the thermocouple to determine the point above the liquid nitrogen at which the temperature is –140°C and temporarily affix the thermocouple at this level.

4. Pipet a maximum of five oocytes (*see* **Note 13**) into a 25% VSDP droplet and leave at room temperature for 3–5 min.
5. Transferring as little of the solution as possible (*see* **Note 17**), pipet the oocytes from the 25% (v/v) VSDP into a 65% (v/v) VSDP droplet.
6. As quickly as possible, move the oocytes into a droplet of 100% VSDP.
7. Immediately draw up a small amount of 100% VSDP into a glass pipet and collect the oocytes. Transfer the oocytes into the column of 100% VSDP contained within the prepared straw.
8. Seal the open end of the straw (*see* **Note 4**) and decontaminate its surface with 70% (v/v) ethanol, an alcohol wipe or, preferably, a less volatile disinfecting agent (e.g., hypochlorite) ***(23)***. Hold the straw using a holder and, wearing suitable protective equipment, immediately position the straw horizontally at the position above the liquid nitrogen at which the temperature is –140°C (*see* **Note 18**). Keep the straw in this position for 3 min and then plunge the straw into liquid nitrogen.
9. Transfer the straw to a liquid nitrogen storage vessel (*see* **Note 14**).
10. Prior to warming the straw, place 1 mL of 1 *M* sucrose solution in a culture dish, half fill a 1-mL syringe with 1 *M* sucrose, and place two 50-µL droplets of sucrose and two 50-µL droplets of PBS with protein supplement in a culture dish. Label the droplets.
11. Wearing suitable protective equipment, remove the straw from liquid nitrogen storage. Hold the straw in air for 10 s, holding either end of the straw and *not* the area containing the oocytes, then plunge the straw into the water bath at 20°C for 10 s.
12. Decontaminate the straw, as in **step 8**, and cut through it using sterile scissors in the area containing the sucrose. Remove the plug from the other end, if used, or cut with sterile scissors. Attach the syringe containing sucrose to one end of the straw and hold the other end over the dish containing 1 mL sucrose. Flush the contents of the straw and syringe into the dish and ensure good mixing.
13. Immediately begin to look for the oocytes using the dissecting microscope (*see* **Note 19**) maintaining the oocytes at room temperature throughout. As soon as the oocytes are located place them in one of the droplets of sucrose.
14. Immediately move the oocytes into the second droplet of sucrose at room temperature. When the oocytes have been in contact with 1 *M* sucrose for a total of 5 min, transfer the oocytes into a droplet of PBS and leave for 10 min at room temperature. Move the oocytes into the second droplet of PBS and leave them for 10 min on the hotplate.
15. The oocytes should then be placed in oocyte culture medium, within an incubator (2–3 h human, 30 min mouse), prior to attempted fertilization (*see* **Note 15**).

4. Notes

1. Seeding is the initiation of ice nucleation. This can be achieved automatically in some freezing machines, or can be initiated manually by removing the straws from the machine and touching the solution with forceps that have been precooled in liquid nitrogen.

2. Samples should be cooled below the glass transition temperature (–130°C) within the cooling machine. Warming above this temperature during transfer to storage and during storage should be avoided because only below this temperature will all biological activity cease and the samples be safe from deterioration.
3. Oocytes can be slow cooled within cryovials or straws. The poor thermal conductivity properties and possibility of vial leakage makes straws the preferred option. Cryovials are not suitable for vitrification. Straws are available from IMV, Minitub, (Tiefenbach, Germany), and CryoBioSystem (France). The use of high-security straws is recommended because they are heat sealable, guaranteed leak-proof to given pressures, shatterproof, bacteria/virus proof, and tested extensively to verify nontoxicity ***(27)***. Also, straws are available with two compartments, allowing identification information to be stored within the straw itself thereby reducing the risk of tampering.
4. Straws can be sealed by heating the ends, although care must be taken not to heat the solution containing the oocytes, or to damage the straw by creating stress fractures that can crack on cooling and allow contamination of the sample or liquid nitrogen entry that risks explosion on warming. Alternatively, straws can be sealed by insertion of plugs or sealing powder, both of which should be wet in order to ensure a good seal.
5. Ideally, oocytes should be stored individually within straws and the straws kept in at least two different locations so that should an accident occur with one straw then all is not lost. In areas of high risk, storage on two sites may be advisable. Samples from patients who have been screened, those who are unscreened, and those with known pathogens should be stored in separate storage tanks. Accurate records should be kept of the location of the straws within each storage vessel.
6. The straw can be filled using a small syringe and needle. Pushing the cotton plug along the straw will reduce the capacity of the straw (*see* **Fig. 2**). The plug should be wetted as this will help prevent liquid nitrogen entry. If the plug is wetted by insertion of a sizeable column of the primary thawing/dilution solution, then expulsion of this solution along with the oocytes following thawing will aid dilution of the cryoprotectant. An air gap should be left between this section and the area to contain the oocytes—a column of cryoprotectant inserted using a fresh syringe and needle. Straws should be clearly labeled, with an appropriate marker, i.e., one that will not smudge or be erased during storage.
7. The straw contents can be expelled by inserting a 1-mL syringe containing at least 0.5 mL of primary thawing/dilution solution and expelling the contents of both the straw and syringe into a dish.
8. Studies have been performed with oocytes that have been denuded of cumulus cells (by means of gentle pipetting and/or treatment with hyaluronidase) prior to freezing or frozen with the cumulus intact. No clear advantage either way is evident.
9. Faster cooling/warming rates can be achieved and the risk of ice crystal formation reduced by using straws that have been pulled to reduce the wall thickness, or by use of such devices as nylon loops or microscope grids.

10. A piece of plastic that can be inserted into a straw and is approximately twice the length of a straw is ideal. The plastic is inserted into the end of the straw and then bent through 90°. The straw can then be placed close to the nitrogen in the horizontal position and the holder can be held by hand at a safe distance from the nitrogen.
11. Addition of the high concentrations of cryoprotectant used in vitrification solutions directly to the PBS medium will result in reduction of the salt concentrations within that medium. Using a concentrated PBS medium allows the cryoprotectant to be added prior to the addition of water to give the required volume of single-strength PBS medium.
12. The vitrification solution could be replaced with other combinations of cryoprotectant, for example, ethylene glycol together with sucrose, often in combination with a macromolecule such as Ficoll, which has been used successfully for the preservation of bovine and human oocytes. Exposure to this cryoprotectant mixture can be performed at 37°C, thereby avoiding any cooling-related damage.
13. In order to adhere to the timing of each step of the procedure, oocytes should be processed in small batches.
14. It should be noted that straws allow rapid heat transfer and hence are susceptible to temperature change during handling, for example on transfer from the cooling machine to liquid nitrogen for storage and during handling for identification purposes prior to thawing. Auditing of cryopreserved samples should only be performed after careful consideration of the risks of warming the samples. Both liquid nitrogen and liquid nitrogen vapor should be considered to contain pathogens. Straws that are not intact should be discarded. Care should be taken to prevent the bending of straws, which may lead to cracks and entry of contaminants from the liquid nitrogen, as well as a risk of explosion on warming as liquid nitrogen within the vessel expands. If storing in the vapor phase, the level of liquid nitrogen should be carefully monitored to ensure stability of storage temperature. Alarms and automatic filling systems are available, but it is recommended that the level of liquid nitrogen still be checked manually at regular intervals.
15. There is evidence to suggest that the microtubular spindle is capable of repair during a period of culture post-thaw.
16. The presence of the sucrose solution on the sides of the straw would dilute the concentration of cryoprotectant in the VSDP column and, more importantly, may allow the propagation of ice crystals from the sucrose solution into the area within which the oocytes are contained.
17. Care must be taken not to dilute the cryoprotectant concentration in these small droplets, as the use of small droplets aids location of the oocytes in such viscous solutions.
18. The straw is held at –140°C just below the glass transition temperature, rather than plunging directly into liquid nitrogen in order to reduce the occurrence of cracks in the vitrified glass which, on warming, may form sites of ice nucleation. It is important to measure the temperature, as extreme fluctuations in temperature are present across a few centimetres in liquid nitrogen vapor.

19. Oocytes are particularly difficult to locate in this solution. The oocytes must spend no longer than 5 min in sucrose at this point and this time can pass quickly.

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23

Cryopreservation of Mammalian Embryos

Barry J. Fuller and Sharon J. Paynter

Summary

The cryopreservation of mammalian embryos has expanded over the past 20 yr by encompassing a range of sophisticated methods to deal with different developmental stages and different sensitivities to low-temperature exposure. We have described a method for slow, controlled-rate freezing of early stage embryos based on exposure to 1,2-propanediol and sucrose, while the method for late-stage (blastocyst) embryos employs mixtures of glycerol and sucrose. Both methods have been used for animal and human embryos. A third rapid cooling or "vitrification" technique is described, which depends on brief but controlled exposure of multicellular embryos to mixtures of glycerol and 1,2-propanediol at high concentrations. This technique is used for successful animal embryo cryopreservation but is not yet widely applied in the clinic.

Key Words: Embryo; cryopreservation; slow cooling; vitrification.

1. Introduction

The development of cryopreservation techniques for preimplantation-stage mammalian embryos has progressed hand in hand with, and largely driven by, advances in their in vitro manipulation and culture. Important early work included the in vitro culture of eight-cell mouse embryos to the blastocyst stage ***(1)***, the demonstration that mouse embryos cultured in vitro could be transferred to host mothers and yield live offspring ***(2)***, the growth of human embryos to the blastocyst stage in culture ***(3)***, and the birth of a calf after in vitro fertilization and embryo culture ***(4)***.

Throughout this period, it was becoming obvious that embryo cryopreservation would too become centrally important in reproductive technologies by allowing optimal utilization of resources in the development of breeding programs in

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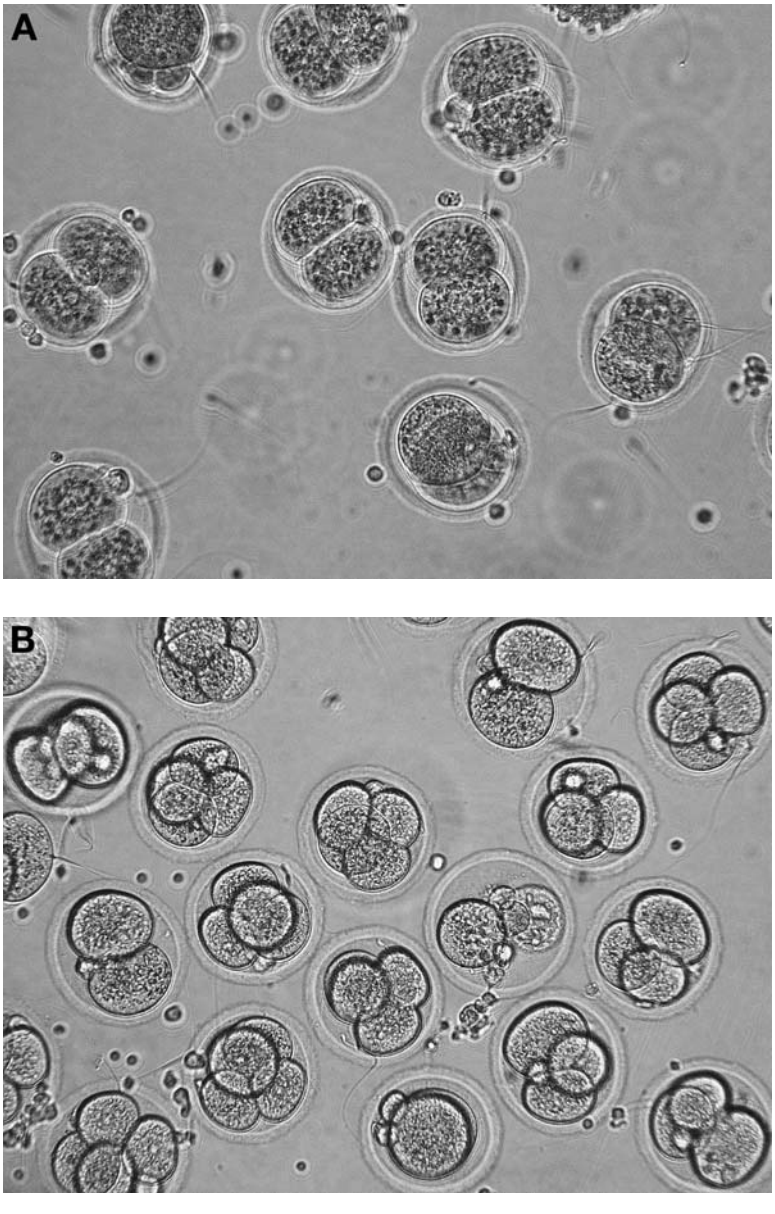

Fig. 1. *(Continued)*

animals ***(5)*** by permitting genetic banking (for example, of rare or economically important mouse strains ***[6]***), and by enhancing the clinical approach to infertility treatment, where often more embryos are produced in vitro than can be safely implanted in the mother ***(7)***.

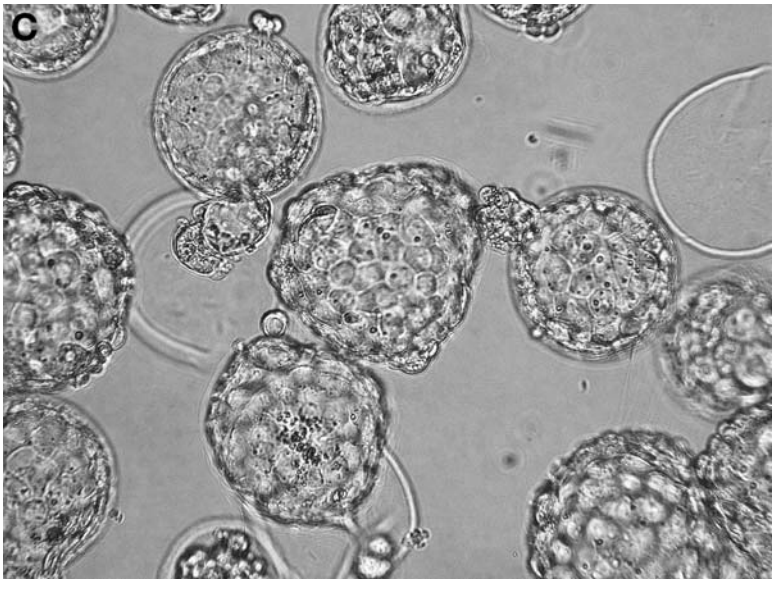

Fig. 1. Micrographs of mouse embryos of different developmental stages (magnification ×40).**(A)** Two-cell embryos at day 1 after fertilization. A group of two-cell embryos; for each, the acellular glycoprotein coating *zona pellucida*—surrounds the two equally sized blastomeres produced at the first cleavage stage of each fertilized oocyte. **(B)** Four- to eight-cell embryos at day 2 after fertilization. Each embryo is still surrounded by its' *zona pellucida*; some embryos have cleaved at a slightly different rate. **(C)** Blastocysts at day 5 after fertilization. Multiple cell divisions have taken place yielding many small blastomeres (more than 60). In several cases, the blastocysts have "hatched" or broken free from the *zona pellucida* leaving an empty, fractured zona in a step that would be a preparation for implantation in vivo.

The first mammalian species to have embryos successfully cryopreserved was the mouse in the early 1970s ***(8,9)***, and over the next three decades this has been extended to embryos of most species, including rat, rabbit, cow, goat, sheep, pig, horse, canine and feline species, primates, and man. Across all these different species, the protocols applied to embryo cryopreservation tend to fall into one of two broad categories, (1) slow controlled-rate cooling and (2) ultra-rapid cooling or vitrification (*see* Chapter 3). The choice of protocol is most often dictated by logistical or regulatory concerns, and may be affected by additional factors such as species peculiarities and the stage of embryonic development. These will be highlighted next, but it is worth discussing briefly the origins and concepts behind the two seemingly different approaches. It is also important to recognize that the term "preimplantation embryo" covers a range of developmental stages with specific morphologies (**Fig. 1**), which can dictate how these cells are manipulated in vitro or cryopreserved.

The earliest successes with mouse embryo cryopreservation were achieved with eight-cell mouse embryos employing prior exposure to a cryoprotective agent (CPA) and cooling slowly, at a constant rate, to deep subzero temperatures ***(8)***. In this case, the CPA was dimethyl sulfoxide at a concentration of 1.5 *M*. The contemporary work of the period highlighted the sensitivity of embryos to the cooling rate, and from this fact was the need to cool slowly dictated (at <–1°C/min). Cryomicroscope observations of embryos during this cooling procedure demonstrated the extreme dehydration encountered during the process when ice formed from pure water as extracellular ice formed around the cells, leaving the embryos in the small, residual hypertonic unfrozen fraction ***(10)***. Conditions that permitted intracellular ice to form (by cooling too fast ***[11]*** and leaving water molecules capable of nucleating ice crystal growth) were universally found to be damaging. It was also argued that slow warming was necessary to avoid cell volume stress, which might be encountered if the dehydrated embryos were warmed rapidly driving near-instantaneous redistribution of cell water content. The target temperature of the slow cooling was selected as –80°C, known from other cell types to be sufficiently low to inhibit (at a logistically practical level) further ice crystal growth, before transferring the embryos to liquid nitrogen temperature for true long-term storage. In reality, later work suggested that by using this approach, ice crystal growth *inside* the individual blastomeres of the embryos was avoided by the extreme dehydration, leaving the cell contents in a "glassy," amorphous state at very low temperatures ***(12)***.

In the intervening time, further investigations demonstrated that this low-temperature glass (and successful embryo cryopreservation) could be achieved if the embryos were slowly cooled only to a relatively high subzero temperature, and then transferred directly to –196°C. In this case, warming needed to be rapid to achieve high viability (by direct immersion of the tubes into a warm water bath between 30 and 37°C). It was here argued that this interrupted slow cool had not resulted in conversion of all water potentially available for extracellular ice formation into ice crystals, leaving a possibility for harmful intracellular ice to nucleate and form during warming. Rapid warming was needed to "out-race" the intracellular ice formation. At a prosaic level, this reduced time of cooling and rapid warming was technically attractive, making the protocols easier to handle. In general, the interrupted slow cool has become the favored approach for most groups using slow cooling in embryo cryopreservation.

The second approach (vitrification) has its' roots in the observations made by Luyet in the 1930s, that it might be possible to recover living cells from very low temperatures if they were cooled in a way that inhibited ice crystal growth all together, achieving a glass (or vitreous state) throughout the whole sample ***(13)***. In retrospect, it has become recognized that this is another facet of the

requirement to produce an "intracellular" glass either by slow or rapid cooling, discussed previously. At first, this seemed an unattainable goal in real biological samples because the rate of cooling would need to be extremely high (in excess of thousands of degrees per min), but the pioneering work of Rall and Fahy ***(14)*** showed that by careful manipulation of high concentrations of CPA and application of small sample sizes, a "quasi-vitreous" state could be achieved in mouse embryos. As long as warming was again sufficiently rapid, high rates of survival in eight-cell mouse embryos were achieved. This work had led to growing interest in the technique and its' application to different embryonic stages across a range of species, including human embryos. Much of the effort has been directed toward developing small sample holders (such as the tiny nylon loops ***[15]*** pioneered by Lane et al.) that allow embryos to be vitrified in volumes of a few microliters. Such small volumes are necessary to permit sufficient rapid heat transfer to enable very rapid cooling, and especially very rapid warming (to "outrun" the potential growth of ice crystals during rewarming). The other main area of investigation has been the concerned selection of less toxic mixtures of CPA. These are often needed in the range of 50% (w/v) to achieve the glassy state.

More extensive information concerning the underlying principles in mammalian embryo cryopreservation across a range of species can be found in a number of reviews (***[16–21]***; Chapter 3). There are interspecies differences that have dictated survival from cryopreservation. The well-documented sensitivity of porcine embryos to chilling and cryopreservation, often related to the high-lipid content of the blastomeres, has long been a major hurdle to widespread application of the technology, but vitrification may offer a hope of improvements ***(21)***. In in vitro-cultured bovine embryos, a similar problem of blastomere lipid inclusions has been encountered, and specific approaches, such as cell centrifugation, have been applied to enhance recovery from cryopreservation ***(18)***.

Beyond the descriptions of cryopreservation technologies, there are many other practical issues to consider when establishing embryos banks that are beyond the scope of the current chapter, but include clear and unambiguous methods for marking stored material (in cryovials, straws, or other containers), and a well-organized cryostorage facility with detailed and fail-safe records and inventory system for tracing banked embryos which may be held in the repository for many years. Nevertheless, the importance of cryopreserved embryo banks, both from a scientific and commercial standpoint in areas such as transgenic animal technology, is clearly established ***(22)***.

2. Materials

These are essentially the same as those needed for oocyte cryopreservation (*see* Chapter 22) but will be repeated for clarity. All chemicals, reagents,

and water should be of the highest grade (analytical reagent or tissue culture grades).

1. A source of liquid nitrogen, liquid nitrogen storage tanks, and Dewars for handling liquid nitrogen, as well as oxygen monitors for rooms where large quantities of the cryogen are stored (*see* **Notes 1** and **2**).
2. A controlled-rate freezing machine (available from several sources such as Planer plc, Middlesex, UK or Asymptote, Cambridge, UK).
3. Safety equipment, e.g., cryogloves, face shield, and an oxygen depletion monitor.
4. 37°C gassed incubators for culture.
5. Equipment for handling and manipulating embryos including an inverted microscope for visualising embryo transfer (for example, available from Nikon.co.uk), pulled glass pipets, or an automated pipet.
6. Heated water bath.
7. Embryo culture medium, such as human tubule fluid (HTF) medium, (available from several sources, including Cook IVF, Brisbane, Australia and CGA/Diasint, Florence, Italy).
8. Petri dishes (Falcon, BD Biosciences, Oxford, UK).
9. Dulbecco's phosphate-buffered solution (PBS; Gibco Life Technologies, Paisley, UK) supplemented when required with 30% (w/v) protein supplement, e.g., plasma protein supplement (Baxter AG, Vienna, Austria) or serum protein supplement (Pacific Andrology, CGA/Diasint). For animal embryos, heat-inactivated fetal bovine serum may be used (Invitrogen, Paisley, UK). These are collectively called serum-supplemented PBS (PBSs).
10. Plastic straws (CryoBiosystem, L'Aigle, France) or plastic cryovials (Nunc, Roskilde, Denmark) are commonly used to contain embryos during cryopreservation. There are particular points of consideration when using straws, such as their heat transfer capacity that dictate care in handling frozen specimens, and their safe storage (*see* **Notes 3** and **4**). There are also tried and tested methods for filling straws with the various cryoprotectant solutions and Diluents to enhance the cryopreservation protocol (*see* **Note 5**), and these should be practiced before embarking on a full cryopreservation run.
11. Microscope (×40 magnification).
12. Freezing solutions: 1.5 *M* 1,2-propanediol (PrOH) and 1.5 *M* PrOH plus 0.2 *M* sucrose, both made up in PBSs.
13. Thawing solutions: 1.0 *M* PrOH plus 0.2 *M* sucrose. 0.5 *M* PrOH plus 0.2 *M* sucrose, and 0.2 *M* sucrose, all made up in PBSs.
14. Vitrification solutions: 10% (w/v) glycerol + 20% (w/v) 1,2 propanediol in PBSs, and 25% (w/v) glycerol + 25% (w/v) 1,2-propanediol.

    1. Thawing solutions for vitrification: 1 *M* sucrose in PBSs.
    2. For vitrification (optional): small nylon loops, as described by Lane and colleagues ***(15)***, or open-pulled straws (MINITÜB, Abfüll - und Labortechnik GmbH and Co. Tiefenbach, Germany). This company also supplies equipment sets for embryo vitrification). Another source of open-pulled straws is SZIGTA, Clayton, Australia.

3. Methods

Three different methods are described here that cover the range of cryopreservation protocols for embryos of different developmental stages, applying either slow, controlled-rate cooling or vitrification (*see* **Note 6**). This obviously is not a comprehensive list of protocols in current use, but they do represent methods that are relatively straightforward, have been confirmed by several groups, and can be seen as basic expertise, which, once mastered, can be applied to other cryopreservation strategies if desired.

There are many other factors which, on a day-to-day basis, will affect the success of embryo cryopreservation procedures (*see* **Notes 7** and **8**). These need to be kept in mind when reviewing the success of embryo cryopreservation within an individual unit.

3.1. Slow Controlled-Rate Cooling of Early-Stage Embryos

The use of slow, controlled-rate cooling using a purpose-built freezing machine for embryo cryopreservation is currently the most common technique in both clinical and veterinary embryo transfer practices. One reason is that records of cooling profiles can be recorded and documented, so that the best practice can be monitored. The method described here for slow cooling of early-stage preimplantation embryos has found application in a variety of species, and is based on the method described by several authors, including Byrd ***(23)***. (For embryos from one-cell to four- to eight-cells.)

1. Straws should be loaded with a column of 1.5 *M* PrOH plus 0.2 *M* sucrose and held at room temperature (20 ± 2°C) (*see* **Note 8**; **Fig. 1**; Chapter 22).
   Prepare droplets (0.5 mL each) of PBS plus protein supplement, for example, 20% (v/v) serum, (PBSs), 0.5 *M* PrOH in PBSs, 1.0 *M* PrOH in PBSs, and 1.5 *M* PrOH with 0.2 *M* sucrose in PBSs. The droplets are placed in a tissue culture dish at room temperature. Label each droplet area.
2. Place the cohort of embryos (usually not more than six per droplet) in the PBSs for an initial wash, and then transfer to 0.5 *M* PrOH for 5 min at room temperature.
3. Transfer the embryos into the 1.0 *M* PrOH for another 5 min, and then into the 1.5 *M* PrOH plus 0.2 *M* sucrose for a final 5 min. (Total cryoprotectant exposure time of 15 min.)
4. Transfer the embryos into the prepared straws (usually two to three embryos per straw) into the column of 1.5 *M* PrOH plus 0.2 *M* sucrose. Plug the straws and place them into the cooling machine set at 20°C. Initiate cooling at a rate of –1°C/min to a holding temperature of –7°C.
5. On reaching –7°C, ice nucleation or "seeding" is carried out in the solution containing the embryos (*see* **Note 9**). The straws are returned to the cooling chamber and held at this same temperature for 10 min to allow dissipation of the latent heat of ice formation.

6. The next cooling stage is then initiated at a rate of –0.3°C/min to –35°C, and then followed by a rapid cooling phase at –50 to –150°C. The straws may then be transferred into a flask of liquid nitrogen, and finally into the liquid nitrogen storage facility. Appropriate protective equipment should be worn at all stages when handling cryogens. There are important issues concerning management of storage facilities and decontaminating outer surfaces of straws or cryovials as part of cryopreservation protocols in general (*see* **Note 10** and Chapter 1).
7. When rewarming is required, first prepare dishes containing 1-mL droplets of dilution media: PBSs + 0.2 *M* sucrose, PBSs + 0.1 *M* sucrose, and culture medium + serum supplement. Prepare a water bath at 30°C.
8. Using protective equipment, transfer the chosen straws into a flask of liquid nitrogen from the storage facility. In the laboratory, warm each straw by holding in air for 30 s then transfer the straw into the water bath at 30°C until all visible ice melts.
9. Expel the embryos plus freezing medium into the droplet of PBSs + 0.2 *M* sucrose (*see* **Note 11**), and hold them at room temperature for 5 min. Then move the embryos into the droplet of PBSs + 0.1 *M* sucrose, and again hold at room temperature for 5 min.
10. Move the embryos into tissue culture medium + serum at room temperature and hold for a further 10 min. Finally, the embryos can be transferred into the chosen tissue culture medium at 37°C for subsequent culture.

3.2. Cryopreservation of Blastocysts by Controlled-Rate Cooling

In centers where culture of embryos to the blastocyst stage is the method of choice, cryopreservation can be achieved based on a slow cooling method using glycerol as cryoprotectant. The described method is based on those previously reported by Menezo ***(19)*** and Tucker ***(24)*** using day 5/6 embryos at blastocyst or hatching blastocyst stages. Cryovials are often used in the process, but straws can also be used.

1. Prepare droplets of medium (usually modified HTF medium plus 10% [v/v] human serum albumin [HAS]: HTF-HSA, HTF-HSA + 5% (w/v) glycerol, and HTF + HSA + 10% (w/v) glycerol + 0.2 *M* sucrose. Fill a Dewar flask with some liquid nitrogen.
2. Transfer the blastocysts into HTF-HSA for 2 min at room temperature, then move them into the droplet of HTF-HSA + 5% (w/v) glycerol, and hold for 10 min at room temperature.
3. Move the blastocysts into the droplet of HTF-HSA +10% (w/v) glycerol + 0.2 *M* sucrose at room temperature for a further 10 min.
4. Transfer embryos into 0.3 mL of HTF-HSA +10% glycerol + 0.2 *M* sucrose in standard (1.2 mL) cryovials at room temperature.
5. Transfer the vials to the cooling machine and cool at –2°C/min to –7°C, and hold for 5 min.
6. Initiate ice formation or "seeding" by clamping the sides of the cryovial with forceps cooled in liquid nitrogen, then return the cryovials to the chamber of the machine at –7°C and hold for 10 min.

7. Initiate the slow cooling phase at a rate of –0.3°C/min to –38°C. Using suitable protective equipment, transfer the cryovials into the flask of liquid nitrogen. Take the vials to the liquid nitrogen storage facility for long-term banking.
8. When the embryos are required, set a water bath at 30°C in the laboratory and prepare dishes with 1-mL droplets of dilution media at room temperature: HTF-HSA + 10% (w/v) glycerol + 0.4 *M* sucrose, HTF-HSA + 5% (w/v) glycerol + 0.4 *M* sucrose, HTF-HSA + 2.5% (w/v) glycerol + 0.4 *M* sucrose, HTF-HSA + 0.4 *M* sucrose, HTF-HSA + 0.2 *M* sucrose, HTF-HSA + 0.1 *M* sucrose, and HTF-HSA. Set the warming stage to 37°C.
9. Remove the cryovials from the storage facility wearing protective equipment, and place in a flask of liquid nitrogen for transport to the laboratory.
10. Hold cryovials in air for 1 min, then transfer to a water bath until all visible ice melts.
11. Expel embryos into the droplet of HTF-HSA + 10% (w/v) glycerol + 0.4 *M* sucrose at room temperature and locate them (about 30 s).
12. Transfer the embryos into HTF-HSA + 5% (w/v) glycerol + 0.4 *M* sucrose at room temperature and hold them for 3 min.
13. Transfer to HTF-HSA + 2.5% (w/v) glycerol + 0.4 *M* sucrose at room temperature for 3 min.
14. Transfer to HTF-HSA + 0.4 *M* sucrose at room temperature for 2 min.
15. Transfer to HTF-HSA + 0.2 *M* sucrose at room temperature for 2 min.
16. Transfer to HTF-HSA + 0.1 *M* sucrose at room temperature, and move to the warm stage (37°C).
17. Wash in standard tissue culture medium alone (three washes at 37°C), then move the embryos into standard culture conditions before embryo transfer.
18. In cases where large numbers of animal embryos have been cryopreserved, it is possible to simplify the washing steps (accepting a possible slight reduction in overall recovery as a balance for convenience when handling more embryos) as:
    a. Expel thawed embryos into medium (serum supplemented) + 0.5 *M* sucrose for 10 min at room temperature.
    b. Transfer into medium (serum supplemented) + 0.2 *M* sucrose at room temperature for 10 min.
    c. Transfer into medium alone (serum supplemented), and move to the warm stage. Wash (three times in medium at 37°C) and move to standard culture conditions.

3.3. Vitrification of Murine Morulae and Early Blastocysts

There has been a rapid expansion of different methods for embryo vitrification over the past few years employing a variety of CPA mixtures and receptacles to achieve very rapid cooling rates. Many of these approaches require considerable operator skill and experience. We describe here one of the earliest methods for embryo vitrification described by Scheffen et al. ***(25)***. This method has the advantages that the CPA mixture is relatively nontoxic (mouse morulae and early blastocysts can tolerate vitrifiable concentrations for 10 min at room

temperature), and cooling can be achieved in traditional 0.25-mL straws. The sample loading can be achieved in a simple way to facilitate rapid dilution upon thawing (*see* **Note 12**). In the recent past, several other new container systems have been developed for embryo vitrification (*see* **Note 2**). The Scheffen method has also been reproduced in other laboratories (***[26]***; *see* **Note 13**). It should be seen as one method to train new users in embryo vitrification who could then go on to tackle more exacting techniques.

1. Mouse embryos recovered on day 4 of pregnancy (d 1 = presence of vaginal plug) and classified as (1) compacted morulae, (2) early blastocyst, and (3) expanded blastocyst. The method is most successful for categories 1 and 2.
2. Prepare culture dishes with droplets of PBSs Massip equilibration mixture (MEM 500-µL volume): also prepare 10% (w/v) glycerol + 20% (w/v) 1,2-propanediol in PBSs at room temperature, and a batch of PBSs + 1 *M* sucrose as Diluent to load straws, and another dish on ice at 4°C with droplets of Massip vitrification medium (MVM) (25% [w/v] glycerol + 25% [w/v] 1,2-propanediol in PBSs).
3. Rinsed 0.25-mL straws with MVM at 4°C then loaded columns with 100 µL 1 *M* sucrose in PBSs (Diluent) solution as described in the original method by syringe at this stage: sealing powder to seal one end, followed by (*see* **Subheading 3.1. step 6**) and held at 4°C. Fill a Dewar flask with liquid nitrogen (wearing appropriate safety equipment).
4. Embryos are washed in the droplet of PBSs and then transferred to the 500-µL droplet of MEM and held for 10 min at room temperature.
5. Embryos are next transferred to the droplet of MVM at 4°C for 30 s then loaded into a 20-µL droplet of MVM in straw. This is done (as described in the original method) by loading (1) 100 µL 1 *M* sucrose in PBSs (Diluent; already loaded), (2) an air bubble, (3) the embryos in 20 µL of MVM, (4) an air bubble, and (5) Diluent to the plug. The Diluent columns do not vitrify (*see* **Note 14**).
6. The straws are sealed with the plug (*see* **Note 5**), held for 30 s in the vapor just above the surface of liquid nitrogen (wearing appropriate safety equipment) in the Dewar flask (to prevent straw fracture from extremely fast cooling), and then plunged into liquid nitrogen. A simple method for holding in the vapor can be devised from a plastic "hook" (*see* **Note 15**). Transfer to the liquid nitrogen storage facility.
7. When required after storage, wearing appropriate safety equipment, the straws are transferred to a flask of liquid nitrogen and taken to the laboratory. Prepare a water bath at 20°C. Prepare dishes with droplets (1 mL) of Diluent and fresh PBSs at room temperature. The straws are removed from liquid nitrogen, held in air for 10 s, and then warmed in a 20°C water bath until the ice melts (approx 20 s). The contents of the straws are then expelled into Diluent (*see* **Note 12**; *see also* Chapter 22, **Subheading 3.2.**) and held at room temperature for 5 min.
8. The embryos are then washed three times in droplets of fresh PBSs at room temperature and transferred to the chosen culture medium at 37°C.

4. Notes

Some of the information in this section has been repeated from Chapter 22 for the reader's convenience.

1. Staff should familiarize themselves with the safety equipment and the procedures for handling liquid nitrogen. The cryogen is generally safe and easy to handle, but avoid direct handling of metal objects cooled in liquid nitrogen (e.g., metal lids of storage containers) or freeze-burns can be experienced. Treat Dewar flasks with care (they are most often constructed of mirrored glass inner sections, evacuated by vacuum, and dropping heavy objects into the chamber or the knocking the flasks themselves, can crack the glass and cause an explosion as the vacuum is lost).
2. Vitrification using some of the small sample holders (such as open-pulled straws) can result in direct exposure to liquid nitrogen. In addition to problems of security for the cryopreserved samples inside the pulled straws, it is important to remember in clinical practice that interpatient infection of equipment such as cooling tanks may be possible, and to consider equipment sterilization between usage. Some commercially available vitrification chambers have been designed with this in mind (e.g., vitset from www.minitube.de). New techniques for sealing open-pulled straws are under development ***(27)***, but will need validation before general acceptance.
3. It should be noted that straws allow rapid heat transfer and hence are susceptible to temperature change during handling, for example on transfer from the cooling machine to liquid nitrogen for storage and during handling for identification purposes prior to thawing. Auditing of cryopreserved samples should only be performed after careful consideration of the risks of warming the samples. Both liquid nitrogen and liquid nitrogen vapor should be considered to contain pathogens. Straws that are not intact should be discarded. Care should be taken to prevent the bending of straws, which may lead to cracks and entry of contaminants from the liquid nitrogen, as well as a risk of explosion on warming as liquid nitrogen within the vessel expands. If storing in the vapor phase, the level of liquid nitrogen should be carefully monitored to ensure stability of storage temperature. Alarms and automatic filling systems are available, but it is recommended that the level of liquid nitrogen still be checked manually at regular intervals.
4. Straws are convenient receptacles for use during embryo cryopreservation and have good heat transfer properties. They do, however, need consideration in handling and storage (e.g., stored away from extreme heat or light sources) before use, as the plastic can "age" and increase the incidence of cracking on exposure to extreme temperature change in the cryopreservation protocol. The practice of allowing a short period of rewarming in air before transfer to warm water helps prevent fracture of the straws from temperature stress. Cryotubes are more resilient but their size and volume restrict the rate of heat transfer, especially if very rapid heat transfer is required.

5. The straw can be filled using a small syringe and needle. Pushing the cotton plug along the straw will reduce the capacity of the straw (*see* Chapter 22; **Fig. 2**). The plug should be wetted as this will help prevent liquid nitrogen entry. If the plug is wetted by insertion of a sizeable column of the primary thawing/dilution solution, then expulsion of this solution along with the oocytes following thawing will aid dilution of the cryoprotectant. An air gap should be left between this section and the column containing the embryos—a 1-cm column of 1.5 *M* PrOH plus 0.2 *M* sucrose. Straws should be clearly labeled, with an appropriate marker, i.e., one that will not smudge or be erased during storage. Straws can also be closed by heat sealing. In this case, having a "sandwich" arrangement of: Diluent–air gap–CPA medium–air gap–CPA medium plus embryos–air gap–CPA medium can be advantageous as it protects the embryo compartment from excessive heating during sealing (*see* Chapter 22; **Fig. 2**).
6. Cryopreservation by slow cooling with freezing machines permits the recording of events such as cooling rate to be recorded directly, which may be important for clinical practice where record keeping in infertility treatment is important to ensure validation of practices. Vitrification protocols may appear more simple but are difficult to oversee on a day-to-day basis, and good continuous supervision is necessary to eliminate small changes introduced unwittingly into the multiple embryo handling steps by individual technologists.
7. In clinical practice (and in some veterinary or biotechnological applications), the optimally developed cohort of embryos from the initial embryo collection or in vitro fertilization procedure may be selected for immediate transfer to recipients. This leaves a pool of lower grade embryos available for banking by cryopreservation. Inevitably, recovery rates from this second-grade pool of embryos may be lower than would be expected if all the starting embryos were of high grade, and this needs to be recognized as a limiting factor on the success of embryo cryobanking.
8. Some studies have claimed that embryos at the exponential stage of development (e.g., 2, 4, 8 cells) may be more resistant to cryodamage during low-temperature banking than embryos cooled when at intermediate developmental stages (3, 5, 7 cells). This probably results from all the blastomeres being in interphase with chromosome completely confined within the nuclear membrane, thus avoiding potential abnormalities.
9. Seeding is in the initiation of ice nucleation. This can be achieved automatically in some freezing machines, or can be initiated manually by removing the straws from the machine and touching the solution with forceps that have been precooled in liquid nitrogen.
10. It is beyond the scope of the current chapter, but in all cryopreservation protocols, good management of the storage banks requires clear and unequivocal labeling and tracking of repositories, as well as safe methods for filling and sealing straws. This is particularly important in the clinical setting. Comprehensive ranges of technology to achieve these ends are commercially available (e.g., *see* www.cryobiosystem.com and www.planer.co.uk). Different methods for labeling straws, such as bar coding, colored insertable plugs, or colored sleeves with imprinted

numerical ID can be selected. Another point of growing significance is the use of heat sealing of ionomeric resin straws to ensure security of samples against contamination from liquid nitrogen, and reliable filling and sealing equipment is now produced (www.cryobiosystem.com). To further ensure security, use of an outer, slightly larger, security straw can be used and heat sealed in a similar fashion. The presence of the second straw does not alter cooling profiles significantly during slow cooling techniques. This will also be of importance when considering rapid cooling or vitrification techniques (*see* **Note 2**) and has been termed the "straw-in-straw" technique ***(28)***. Storage in the vapor phase above liquid nitrogen can reduce the risk of any cross-infection and is the method of choice in many clinical centers. A new generation of liquid nitrogen tanks is being developed to facilitate the best practice for vapor storage, and automatic filling systems may be provided for large-scale operations (*see* www.thamescryogenic.com). It is also recommended that the outer surfaces of straws and vials be decontaminated by wiping with active agents after filling (before cooling), and after warming (especially using a water bath) before expelling the embryos (***[29]***; *see also* Chapter 22), not only in clinical laboratories but as good practice when cryopreserving any embryos.

11. The straw contents can be expelled by inserting a 1-mL syringe containing at least 0.5 mL of primary thawing/dilution solution and expelling the contents of both the straw and syringe into a dish.
12. In vitrification using the Massip method, a straightforward way to load and expel embryos after thawing is to make the following steps. The 0.25-mL straws are plugged with the cotton/polyvinyl alcohol plug, which is sealed on wetting by carefully injecting a 4-cm long column with 1 *M* sucrose. Diluent into the straw from a 1-mL syringe (Kendall Tyco, Gosport, UK) with a 0.5 × 16-mm needle. Care is taken to avoid wetting the sides of the straw. An air gap of 0.5 cm is left, and then 0.5 mL of MVM is injected into the column and the straws are placed on ice. The equilibrated embryos are injected into the MVM column in the straw, and the straw is sealed with a wetted plastic plug. After the thawing step, the plastic plug is removed and the straw cut through in the mid region of the sucrose column, followed by flushing out the embryos in MVM using fresh 1 *M* sucrose (1 mL) Diluent from a syringe into the centre well of an organ culture dish. The embryos are then held at room temperature for 5 min before washing in PBSs as described previously (*see* Chapter 22, **Subheading 3.2.**, **step 6**).
13. Survival rates of compacted murine morulae using the Massip vitrification method are typically between 60 and 80% ***(25,26)*** and normal offspring have been born after embryo transfer ***(25)***.
14. It is worth noting that the PBSs + 1 *M* sucrose Diluent does not vitrify even on rapid cooling, but rather freezes, giving a "milky" visual appearance to the straw during cooling. It is possible to omit the Diluent from the straw, place only a column of MVM holding the embryos in the straw, and then expel the thawed embryos into a large droplet of fresh Diluent in a dish.
15. A piece of plastic that can be inserted into a straw and is approximately twice the length of a straw is ideal. The plastic is inserted into the end of the straw and then

bent through 90°. The straw can then be placed close to the nitrogen in the horizontal position and the holder can be held by hand at a safe distance from the nitrogen.

16. Vitrification using some of the small sample holders (such as open pulled straws) can result in direct exposure to liquid nitrogen. In addition to problems of security for the cryopreserved samples inside the pulled straws, it is important to remember in clinical practice that interpatient infection of equipment such as cooling tanks may be possible, and to consider equipment sterilization between usage. Some commercially available vitrification chambers have been designed with this in mind (e.g,. vitset from www.minitube.de). New techniques for sealing open pulled straws are under development ***(29)***, but will need validation before general acceptance.

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