Cryopreservation and Banking

Cell lines have been used by research laboratories since the 1940s (see Table 1.1). Efforts were made to collect cell lines at early passage and freeze stocks for later use. The early cell lines were frozen in glycerol, since it was shown as early as 1949 that glycerol prevents cells from dying while being frozen (Polge et al. 1949). Cells frozen in glycerol were later found to retain viability after prolonged storage in liquid nitrogen (Coriell et al. 1964; Greene et al. 1967). Using this approach, the Cell Culture Collection Committee collected an initial panel of 23 "certified cell lines" for deposit at the American Type Culture Collection (ATCC) under accession numbers CCL-1 to -25 (Coriell et al. 1964; Ledley 1964). Participating laboratories tested all cell lines for microbial contamination and species of origin. Unfortunately, methods to detect interspecies cross-contamination were limited at that time; 14 of these cell lines are now known to be misidentified (see Chapter 17). However, the overall approach was successful in preserving early passage cultures and managing the risk of contamination through specific testing. This chapter focuses on how to perform cryopreservation, which is defined as the application of low temperatures to preserve the structural and functional integrity of cells and tissues (Pegg 2007; Hunt 2019), and how to prepare seed stocks or "cell banks" for ongoing use (Hay 1988). Although cell repositories specialize in these procedures, the general principles can be applied in any laboratory to ensure that unique and irreplaceable cell lines are preserved at low passage for future use.

15.1 PRINCIPLES OF CRYOPRESERVATION

Unprotected freezing is normally lethal to cells. Freezing of water and the formation of crystalline ice results in cellular damage, primarily due to the mechanical action of the ice crystals and secondary changes in the composition of the liquid phase (Pegg 2007; Hunt 2019). To preserve cell viability, conditions and procedures must minimize intracellular ice crystal formation and reduce cryogenic damage from foci of high-concentration solutes that form when intracellular water freezes. These aims can be achieved by (i) using a hydrophilic cryoprotectant to sequester water; (ii) freezing slowly at a controlled rate to allow water to leave the cell, but not so slowly that ice crystal growth is encouraged; (iii) storing the cells at the lowest possible temperature to minimize the effects of high salt concentrations; and (iv) thawing rapidly to minimize ice crystal growth and generation of solute gradients as the residual intracellular ice melts. Alternatively, cell viability can be preserved by inducing a vitreous or glassy state which prevents ice crystal formation (vitrification; see Section 15.1.4).

15.1.1 Cryoprotectants

Cryoprotective agents (referred to throughout this book as cryoprotectants) act to reduce the amount of ice formed at any given temperature and also protect against other adverse chemical and physical phenomena during the

Freshney's Culture of Animal Cells: A Manual of Basic Technique and Specialized Applications, Eighth Edition. Amanda Capes-Davis and R. Ian Freshney.

Companion website: www.wiley.com/go/freshney/cellculture8

freezing process. For living cells there are two broad groups of cryoprotectants: penetrating and non-penetrating. Penetrating cryoprotectants work by lowering the freezing point of the cells, protecting against solute toxicity, stabilizing the cell membrane, and protecting the cytoskeleton. They must be soluble in water and able to penetrate cells with low toxicity (Pegg 2007). Non-penetrating cryoprotectants work by enhancing dehydration, increasing the glass-forming potential, and protecting the cell membranes. Examples of penetrating cryoprotectants include dimethyl sulfoxide (DMSO), glycerol, propanediol, ethanediol, and methanol. Examples of non-penetrating cryoprotectants include polyethylene glycol (PEG), sucrose, trehalose, Ficoll, serum, and albumin.

Glycerol and DMSO are the most widely used cryoprotectants and are typically added to the medium in which cells are suspended before freezing (see Section 15.3.2) (Polge et al. 1949; Lovelock and Bishop 1959). Concentrations of 5–10% are common, although a wide range of concentrations (2–20%) can be used. DMSO should be colorless; it must be stored in glass or polypropylene, as it is a powerful solvent and will leach impurities out of rubber and some plastics. DMSO is assumed to be self-sterilizing but can be filter sterilized using a nylon filter, preferably after dilution in medium. Glycerol should be not more than one year old; it may become toxic after prolonged storage, due to light-induced conversion to acrolein. It can be sterilized by autoclaving.

DMSO is typically more effective than glycerol, possibly because it diffuses more rapidly through cell membranes (Coriell et al. 1964). However, its use can cause problems for some cultures. DMSO is known to induce differentiation in hematopoietic cell lines (Collins et al. 1979; Young et al. 2004; Jiang et al. 2006). It may also be toxic at the concentrations used in freezing media (Matsumura et al. 2010). Toxicity is likely to be a cell-type-specific event, perhaps relating to intracellular penetration. Some laboratories find that DMSO is less toxic if freezing medium is used at 4 °C; once added, all subsequent steps should be performed at the same temperature until freezing commences (see Protocol P15.1) (Wewetzer and Dilmaghani 2001). There are additional concerns about its use for biological products with potential therapeutic use *in vivo*. DMSO has well-recognized side effects when administered to patients, many of which are associated with induction of histamine release (Morris et al. 2014). Laboratory personnel should also be careful when handling DMSO, which can penetrate many synthetic and natural membranes (see Section 6.2.2). Consequently, DMSO may carry hazardous substances into the circulation through skin and even through gloves.

- ***Safety Note. Always wear suitable personal protective equipment (PPE) when handling DMSO and check chemical resistance when selecting gloves. Nitrile gloves are suitable for brief contact but will degrade if exposed to DMSO for a few minutes, so gloves should be changed promptly after contact.***

These concerns have led to the adoption of other cryoprotectants, including PEG, polyvinylpyrrolidone (PVP), hydroxyethyl starch (HES), or trehalose (Suzuki et al. 1995; Monroy et al. 1997; Pasch et al. 2000). Trehalose is a sugar that is used by some organisms to withstand extreme cold or desiccation, making it a "natural" cryoprotectant with low toxicity compared to other choices (Crowe and Crowe 2000). Trehalose normally does not enter cultured cells, but it can be introduced when the cell membrane is more permeable, e.g. following genetic modification or the use of specific buffers (Beattie et al. 1997; Eroglu et al. 2000; Buchanan et al. 2010).

15.1.2 Cooling Rate

Most cultured cells survive best if they are cooled at −1 °C/min (Coriell et al. 1964; Leibo and Mazur 1971; Hunt 2019). This is probably a compromise between fast freezing (minimizing ice crystal growth) and slow cooling (encouraging the extracellular migration of water). However, maintaining a consistent cooling rate is not a straightforward process, as can be seen by the temperature curve that is generated during freezing (see Figure 15.1a). Formation of ice crystals is an exothermic process, resulting in a spike in temperature and associated loss of viability; this is referred to as the latent heat of fusion (see Figure 15.1b) (Morris 2007). Latent heat must be absorbed in some way, either by dispersal through the insulating material that surrounds the sample or by accelerated cooling just before ice crystals begin to form (see Figure 15.1c).

The shape of the freezing curve will vary based on a number of factors, including (i) the ambient temperature; (ii) any insulation surrounding the cells, including the cryovial; (iii) the specific heat and volume of the cryovial contents; (iv) the onset of freezing (nucleation); and (v) latent heat absorption once nucleation occurs. The actual cooling rate in Figure 15.1c is faster than the recommended −1 °C min, but this may be less important than the control around the eutectic point, which is defined as the temperature at which a homogenous mixture of substances melts or solidifies. Seeding ice crystal formation as the cell suspension reaches the eutectic point can initiate freezing and improve survival. These factors are explored elsewhere in more depth and are important for optimization of cryopreservation procedures (Mazur 1984; Morris and Acton 2013; Hubel 2017; Hunt 2019).

15.1.3 Storage Temperature

What temperature should be used for long-term storage of frozen cells? Storage at −80 °C may be sufficient for some cell types but results in loss of viability and degradation of

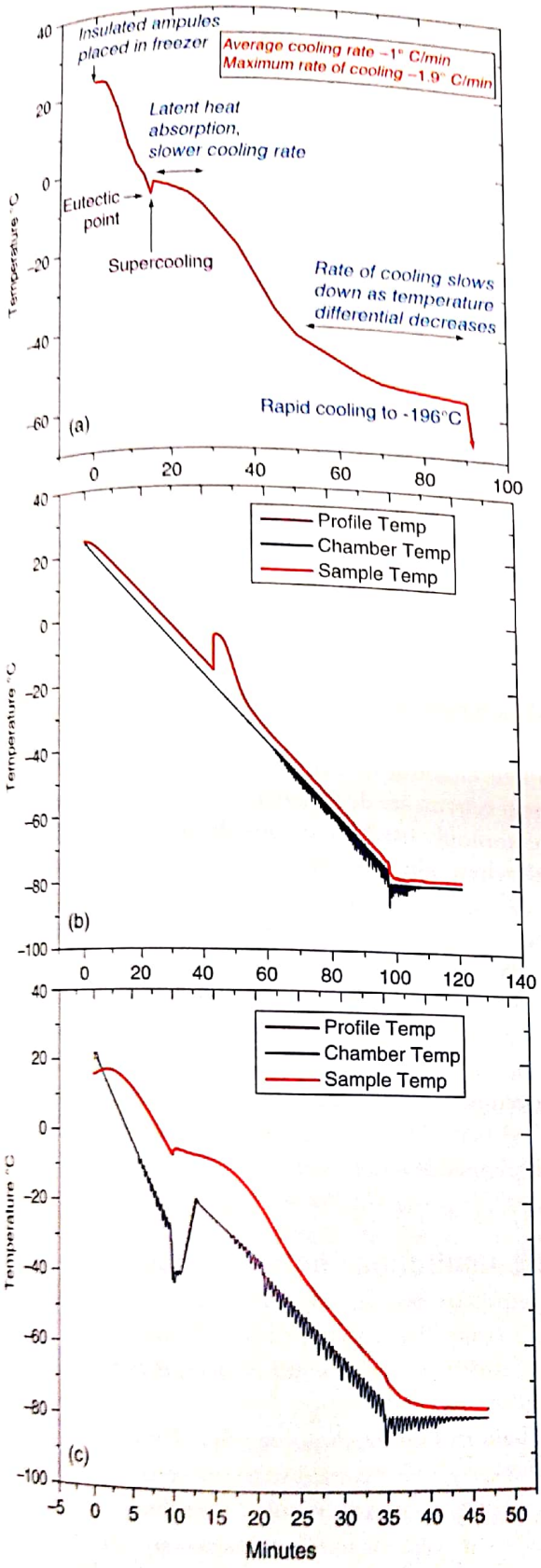

Fig. 15.1. Freezing curves. Temperature within a cryovial containing medium during freezing under different conditions. (a) Cryovial clipped with five other cryovials on an aluminum cane, enclosed in a cardboard tube, placed within a polyurea-foam tube, and placed in a freezer at −70 °C. (b) Cryovial placed in a rate-controlled freezer set to cool at −1 °C/min with no attempt to control for cooling. (c) Cryovial placed in a rate-controlled freezer with a multistep program to control cooling conditions. *Source*: (a) R. Ian Freshney; (b, c) courtesy of Elsa Moy and Amanda Capes-Davis, CellBank Australia.

phenotype for most cryopreserved cells (Hubel et al. 2014; Simione and Sharp 2017). Once frozen, cells should be stored at a temperature below the glass transition temperature (T_g), which for a solution of water and 10% DMSO is calculated to be −132.58 °C (Murthy 1998; Hubel et al. 2014). Below this temperature, the solution is highly viscous and the mobility of molecules within the sample is greatly reduced, which essentially means that metabolic processes are placed in stasis. For long-term storage, frozen cells should be kept in cryofreezers that maintain temperatures at less than −150 °C throughout the storage area (see Section 15.2.3) (Simione and Sharp 2017).

15.1.4 Vitrification

Vitrification refers to the solidification of a liquid into a glass, which is defined as a non-crystalline solid with the molecular structure of a liquid (strictly, an extremely viscous liquid with the mechanical properties of a solid) (Fahy and Wowk 2015). Rapid cooling of cells below the T_g induces vitrification and can be used to avoid ice crystal formation. Vitrification is particularly useful for cryopreservation of embryos. Their three-dimensional (3D) structure, although very small, is likely to limit diffusion during a slow cooling process. Vitrification is also commonly used for human pluripotent stem cells (hPSCs) (Richards et al. 2004; Kaindl et al. 2018).

During vitrification, cryoprotectants are typically added at stepwise increasing concentrations to minimize osmotic effects. Suitable agents include DMSO, glycerol, ethylene glycol, and sucrose. Embryos or cell suspensions are placed in a plastic capillary tube ("straw") or loaded onto a vitrification device before being placed directly into liquid nitrogen. Although vitrification can be very effective, it is difficult to perform in bulk and samples cannot be transported afterwards in dry ice, which (at a temperature of −78.5 °C) is above the T_g of the sample and will therefore affect its viability. Over time, techniques have been developed to allow stem cell cryopreservation at −1 °C/min, e.g. using Rho kinase (ROCK) inhibitors to promote survival. A protocol for stem cell cryopreservation is included in a later chapter (see Protocol P23.3).

15.2 APPARATUS FOR CRYOPRESERVATION

15.2.1 Cryovials

Glass ampoules or plastic cryovials can be used for cryopreservation. Cell repositories have traditionally preferred heat-sealed glass ampoules for seed stocks because the long-term storage properties of glass are well characterized and, when correctly performed, sealing is absolute. However, glass ampoules carry a risk of explosion during thawing (see Section 6.2.3). Glass ampoules have now largely been replaced by plastic cryovials, which are safer than glass (provided they are rated for cryogenic storage) and are more convenient and easier to label. Cryovials are usually made

(d) Color-coded Cryovials. Polypropylene cryovials of various sizes, ranging from 1.0 to 4.6 mL. Colored inserts can be added to the caps to help identify cryovials. Source: Courtesy of Alpha Laboratories.

Fig. 15.2. Color-coded cryovials. Polypropylene cryovials of various sizes are shown, ranging from 1.0 to 4.6 ml. Colored inserts can be added to the caps to help identify cryovials. *Source*: courtesy of Alpha Laboratories.

from polypropylene and are available in a range of sizes (e.g. 1–5 ml; see Figure 15.2).

- ***Safety Note. Inexperienced users should avoid using glass ampoules due to the risk of explosion during thawing. If glass ampoules are used, they must be perfectly and quickly sealed in a gas-oxygen flame. If sealing takes too long, the cells will heat up and die, and the air in the cryovial will expand and blow a hole in the top of the ampoule. If the ampoule is not perfectly sealed, nitrogen may enter during storage and cause explosion of the vial during thawing. If glass ampoules are used, they should be stored in vapor phase or in an isothermal freezer (see Section*** 15.2.3***).***

Plastic cryovials have a screw cap and may have an external or internal thread; externally threaded vials are designed to reduce contamination, while the internally threaded vials usually have an "O" ring to improve their seal (Ryan 2004). Both require the correct torsion for closing, as they will leak if too slack or too tight (due to distortion of the "O" ring). It is worth practicing with a new batch to make sure that they seal correctly. Cryovials can be checked for leakage by placing them in a dish of stain, e.g. 1% methylene blue in 70% alcohol, at 4 °C for 10 minutes before freezing (Ryan 2004).

Cryovials must be clearly labeled using a method that will survive prolonged periods of time in cryogenic conditions. Remember, cultures that are stored in liquid nitrogen may well outlive you! At the very least, they will outlive your stay in a particular laboratory. The label should show the cell line's name or numerical identifier and, preferably, the date, passage number, and user's initials, although the latter is not always feasible in the available space. Use a fine-tipped marker that is alcohol-resistant or a cryogenic label that is reported to withstand liquid nitrogen storage (e.g. Cryo-Babies, Sigma-Aldrich; FreezerBondz™ label, Brady; LabTAG cryogenic labels, GA International). Always test to see if the printing remains legible and the label remains firmly adherent after handling. This can be done by wiping the labeled cryovial with alcohol, storing in the cryofreezer, and thawing in a water bath.

Some tissue culture laboratories use additional measures to ensure that labeling remains intact for the life of the cryovial and its records are rapidly accessible. Such measures include adding printed barcodes to the cryovial or using vials with pre-existing labels that were added during manufacture. Commercial sample management systems are available with barcoded cryovials and compatible barcode readers, sample racks, and software (e.g. FluidX, Brooks Life Sciences). Laboratories may also develop their own customized systems using standalone electronic databases, barcode software, and printable labels. Customized systems are likely to be cheaper and specific to your needs but will take time and expertise to make the various components work together. Simple additions are also helpful; for example, colored caps or cap inserts can be used to identify stocks that are reserved for cell banking procedures (see Section 15.5).

Always check that your cryovials are compatible with the cryofreezer and its storage system (see Section 15.2.3). Most current systems are designed to accept 1.2-ml cryovials, which are commonly used for storage. It may be difficult to fit cryovials where additional material has been added, such as Cryoflex™ (ThermoFisher Scientific). Cryoflex is recommended when cryovials are stored in liquid phase to minimize the risks of explosion or contamination (see Section 15.2.3). If Cryoflex is used, it is heat sealed in position over the vial before freezing, with the cryovials placed on ice to prevent overheating (Wewetzer and Dilmaghani 2001). Cryoflex must be removed before thawing; if left in position, it will slow thawing and may allow contaminated water from the water bath to be trapped between the Cryoflex and the vial.

15.2.2 Controlled Cooling Devices

A controlled cooling rate (see Figure 15.1) can be achieved using a controlled cooling device. Tissue culture laboratories use an assortment of cooling devices, including:

(1) ***Foam insulation.*** A polystyrene foam box (~ 15 mm wall thickness) can be used with the cryovials placed in a rack or lying on cotton wool. Alternatively, a foam rack can be used with a second rack taped over the top (e.g. racks supplied with Falcon 15-ml conical tubes #C352099, Corning). The insulation should be sufficient for cryovials to cool at −1 °C/min when placed at −70 to −90 °C but also absorb the latent heat of fusion. Foam boxes can be surprisingly effective, but this effectiveness may vary between boxes and between different locations inside the box.

- ***A commercial freezing container*** (see Figure 15.3). Various formats are available to suit different cryovial sizes and sample numbers. Freezing containers are placed in a freezer at −70 °C or −90 °C; devices may need isopropanol (e.g. Mr. Frosty™, ThermoFisher Scientific) or may be alcohol-free (e.g. CoolCell®, Corning). Commercial freezing containers usually produce consistent freezing profiles but have some limitations, e.g. space is limited to a set number of cryovials.
- ***A programmable rate-controlled freezer*** (see Figure 15.4). This allows increased reproducibility, active control of the freezing process, and handling of large volumes, e.g. >100 cryovials or cryobags containing > 100 ml (Massie et al. 2014). Users can monitor temperature data using a sample probe and adjust the freezing curve by editing the freezing program (see Figure 15.1c). Rate-controlled freezers are available from several suppliers (e.g. Custom Biogenic Systems, Planer PLC, or ThermoFisher Scientific). These devices typically work by injecting liquid nitrogen into the sample chamber at variable rates, in response to a preset freezing program. Liquid nitrogen-free devices are also available that utilize Stirling closed-cycle, regenerative heat engines as a completely sealed heat transfer system (e.g. via Freeze™ controlled-rate freezers, GE Healthcare Life Sciences) (Walker 1983).

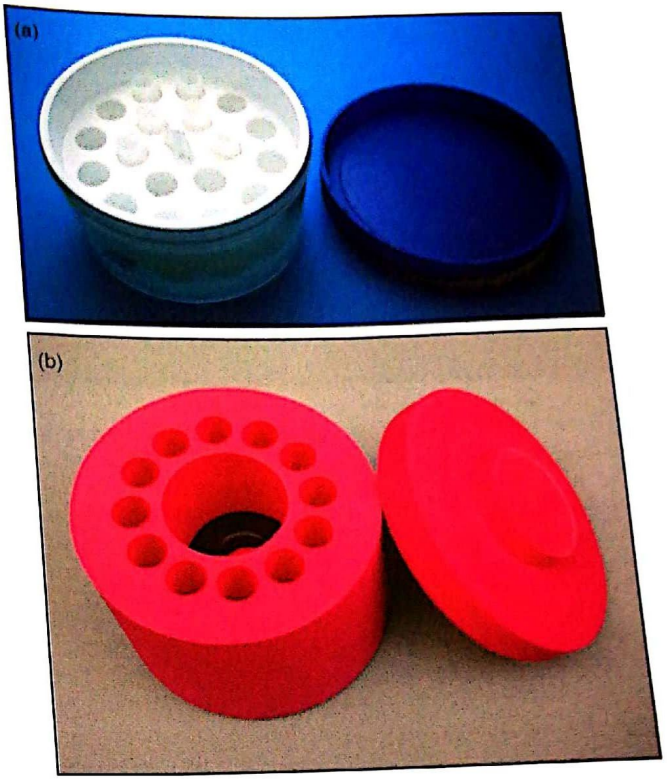

Fig. 15.3. Freezing containers. Insulated blocks with spaces for cryovials. (a) Mr. Frosty (ThermoFisher Scientific), which relies on the specific heat of the coolant (isopropanol) added to the base to insulate the container and give a cooling rate of approximately −1 °C/min. (b) CoolCell (Corning), which relies on an insulating outer housing and thermoconductive solid core. *Source*: R. Ian Freshney.

Always perform viability testing to ensure that the device is satisfactory, particularly when freezing a new cell type or a new cell line (see Section 15.4.4). Poor viability is addressed by looking at the entire cryopreservation procedure (see Section 15.4). Even small details are important, such as the length of time spent in the freezing container. If cryovials are placed in a freezing container at −70 °C, they will cool rapidly to around −50 °C, but the cooling rate falls off significantly after that (see Figure 15.1a). Hence, the time that they spend in the −70 °C freezer needs to be longer than the amount of time projected by a − 1 °C/min cooling rate, as the bottom of the curve is asymptotic. Freezing containers should be kept in the freezer overnight and cryovials transferred to the cryofreezer on the following day. Cryovials will heat up at a rate of −10 °C/min when removed from the freezing device. It is critical that they do not warm up above −50 °C, as they will start to deteriorate, so the transfer to the cryofreezer must take significantly less than two minutes.

Further optimization of the cooling process is discussed elsewhere (Hubel 2017). A programmable rate-controlled freezer is useful, if available, to analyze temperature data and adjust the freezing program (see Figure 15.1). Freezing programs typically consist of a series of steps, including (i) a holding period to allow the samples and chamber to equilibrate; (ii) slow cooling until the sample temperature is close to the eutectic point; (iii) a brief period of rapid cooling to induce nucleation in the sample and absorb the latent heat of fusion; (iv) a brief period of rewarming to −20 °C to correct for supercooling in the chamber; and (v) a return to slow cooling until the sample reaches −60 °C. Each step can be edited to optimize cryopreservation for various cell types.

15.2.3 Cryofreezers

Mechanical freezers can be used for storage at ultralow temperatures, but access to a mechanical freezer may result in temperature fluctuations as high as −30 °C (Simione and Sharp 2017). Liquid nitrogen Dewars or freezers (referred to here as "cryofreezers") are preferred for storage of cells and tissue, ensuring that correct temperature conditions are maintained with maximum stability and security (Coecke et al. 2005; Simione and Sharp 2017). Various cryofreezer designs may be used (see Figures 15.5, 15.6). Tissue culture laboratories often have more than one design; a new cryofreezer may be purchased to add to existing storage capacity rather than replace the previous model (see Figure 15.6e). The various designs affect how samples are stored and how cryofreezers are maintained.

Many larger laboratories, biobanks, and cell repositories have converted to using a consensus format developed by the Society for Biomedical Screening (SBS) – now the Society for Laboratory Automation and Screening (SLAS) – for cryovial boxes, racks, and racking systems (SLAS 2019). This format will typically hold 96 cryovials in an SBS microtiter format box and has the advantage of allowing more samples to be stored in a given space. It is also compatible with most of the robotic liquid handling platforms that are used in larger tissue culture laboratories and facilities (see Sections 5.1.3, 28.7.1, 28.7.2). These cryostorage boxes and racks are often used with cryovials that have permanent unique barcodes on their base and sides, which can be read by many automated systems. SBS format cryovials and boxes are available from several specialist suppliers (e.g. FluidX, Greiner Bio-One, Micronic).

Cryofreezer maintenance. The investment in the contents of a cryofreezer may be considerable and must be protected by ongoing maintenance and automated monitoring and alarm systems. Maintenance should include regular removal of ice crystals on the lid and other accessible locations. Ice is the enemy of all frozen storage and will increase the risk of equipment breakdown and contamination over time. Always report cryofreezer faults promptly for repair by trained and competent personnel. Never adjust liquid nitrogen connections or carry out repairs yourself unless you are trained and competent to do so safely. Otherwise, "repairs" can lead to catastrophic accidents (Lowe 2006).

Monitoring and alarms are best carried out using an equipment alarm system with independent temperature sensors (see Section 4.3.2). At least one sensor should be positioned high in the chamber to act as an early warning system for rising chamber temperatures. Temperature and alarm information should be accessible remotely so that laboratory members are alerted to problems at an early stage, e.g. if temperatures rise above −150 °C. Cryofreezers that are currently on the market typically include monitoring of temperature and liquid nitrogen levels; these systems are designed to minimize the risk of faults or failures and may have auto-fill cycles that are triggered if liquid nitrogen levels reach the set low point. However, monitoring systems may still fail, and failures can be difficult to detect if cryofreezers are infrequently accessed or positioned away from the main laboratory. Contingency planning should always cover loss of sample integrity due to cryofreezer failure, e.g. loss of liquid nitrogen supply. Simple measures can be adopted to manage the associated risks, including storing samples across multiple cryofreezers and arranging offsite storage (see Section 4.3.1).

15.3 REQUIREMENTS FOR CRYOPRESERVATION

15.3.1 When to Freeze

There are certain requirements that should be met before cell lines are considered for cryopreservation (see Table 15.1) (Stacey and Dowall 2007; Parker 2011). The most important considerations relate to the culture itself. Cells should appear healthy on routine observation (see Table 14.3). The culture should be in an exponential "log" phase of growth (see Figure 14.1) (Terasima and Yasukawa 1977). Do not allow the culture to become confluent and enter its plateau phase of growth, where viability is likely to be impaired (Stacey and Dowall 2007). The cell yield should be sufficient for a high cell concentration in each cryovial (see Section 15.3.3).

How soon should a culture be frozen after its inception? In theory, you should optimize the culture conditions and wait for cells to proliferate before you start to freeze down samples. Finite cell lines are grown to around the fifth population doubling (see Section 14.2.2) in order to generate sufficient cells for freezing. However, in practice, you may have too many cells to handle in the time available or you may be concerned that the culture or its properties will be lost with further handling. Many tissue culture personnel choose to freeze down "token" vials whenever additional cells become available at subculture, e.g. plating half of the culture in a new flask and freezing the other half in one or two cryovials. Token vials are particularly useful if you discover a problem with the culture, e.g. if later authentication testing shows that it is misidentified. Preserving early passage material gives the best possible opportunity to understand the cause of the problem and hopefully retrieve authentic material.

Continuous cell lines usually grow rapidly, giving no shortage of cells available for freezing. It may be tempting to reserve cryopreservation for "backup" of the cell line after experimental work is complete. However, experienced practitioners have found that it is far better to freeze cells first and perform validation testing, resulting in a reliable source of cells for experimental work (Stacey and Dowall 2007). Cell banking procedures are discussed later in this chapter (see Section 15.5).

15.3.2 Freezing Medium

Freezing medium refers to the solution containing cryoprotectant (see Section 15.1.1) in which cells are suspended during cryopreservation. The choice of an appropriate freezing medium will depend on whether the cells require serum (including many continuous mammalian cell lines) or can be grown serum-free (including normal/cancer stem cell cultures, certain types of suspension cultures, and cells used in human *in vivo* applications). If serum can be used, freezing medium will typically contain cryoprotectant, basal medium, and serum at an increased concentration (20–50%). The addition of DMSO to basal medium normally increases the pH; it may be necessary to gas the medium with CO_2 or adjust the pH prior to use if basal medium is present (Morris 2007). Alternatively, 90% serum can be used with cryoprotectant and no basal medium. Serum provides better protection and pH control, and serum-based freezing

TABLE 15.1. Requirements before freezing.

Status	Criterion	Action indicated
Culture	Primary culture	Freeze excess for later use but viability may be poor
	Finite cell line	Freeze token number of cryovials as early as possible; increase number as cells proliferate
	Continuous cell line	Use cell banking approach; repeat with derivative cell lines, e.g. clonal populations
Standardization	Protocol	Develop Standardized Operating Procedure (SOP) for cryopreservation and optimize for viability
	Freezing medium	Select optimal freezing medium (see Section 15.3.2)
	Medium	Select optimal medium and adhere to this selection
	Serum (if used)	Select a batch for ongoing use (see Section 9.7.2)
	Substrate	Select one type and supplier, although not necessarily one size or configuration
Validation	Provenance	Keep records on cell line, cryopreservation, and thawing (see Sections 7.3.1, 15.7)
	Viability testing	Test viability during thawing and/or 24 hours after plating (see Section 15.4.4)
	Other	Test for authenticity and microbial contamination, e.g. mycoplasma, sterility testing (see Sections 11.8.1, 16.2, 17.2.3)
Characterization	Transformation	Determine transformed status (see Table 3.2)
	Other	Test for other characteristics depending on the cell type and intended use

medium can be dispensed into aliquots and stored at −20 °C for later use.

Selection of freezing medium becomes more challenging if cells are grown in serum-free media or cannot be individualized to form a single-cell suspension. For example, hPSC aggregates are more sensitive to supercooling and vary in their permeability to cryoprotectant, making cells more vulnerable to damage (Li et al. 2018). Various reagents may be added to improve hPSC viability, including ROCK inhibitors and trehalose (Claassen et al. 2009; Hanna and Hubel 2009). Serum may be substituted with various reagents, including human serum albumin (5%), methylcellulose (0.1%), Pluronic F68 (1–5%), and PVP (3%) (Merten et al. 1995; Gonzalez Hernandez and Fischer 2007; Hanna and Hubel 2009). Optimization of the freezing process will be required and should extend to the freezing curve and the subculture conditions used (Liu and Chen 2014; Li et al. 2018). Adding 50–90% conditioned medium (serum-free medium in which the cells were grown) to both the freezing and recovery medium may improve the post freezing recovery and survival.

15.3.3 Cell Concentration

Cells appear to survive freezing best when cell suspensions are frozen at a high concentration. This is largely an empirical observation but may relate to improved survival if cells are leaky because of cryogenic damage. A high concentration at freezing also allows for loss of some cells without impairing cloning efficiency (which tends to decline at lower density) and increases dilution of the cryoprotectant when medium is added after thawing (which reduces toxicity and may render centrifugation unnecessary). The number of cells frozen per cryovial should be sufficient to allow for 1 : 20 dilution on thawing to dilute out the cryoprotectant. It is also important to keep the cell concentration higher than at normal passage (Morris 2007). For example, for cells that are normally passaged at 1×10^5/ml, 1×10^7 cells may be frozen in 1 ml of freezing medium and the cells diluted to 20 ml after thawing. This would give 5×10^5 cells/ml (five times the normal seeding concentration) and dilutes the cryoprotectant from 10% to 0.5%, at which concentration it is less likely to be toxic. Cells normally seeded at 2×10^4/ml can be diluted 1 : 100 to give 1×10^5/ml and a cryoprotectant concentration of 0.1%.

15.4 CRYOPRESERVATION PROCEDURES

15.4.1 Cryopreservation in Cryovials

Protocol 15.1 is suitable for most continuous cell lines, but some cell types will need optimization of the freezing curve (see Figure 15.1) or other conditions. Most cells are frozen in DMSO but there are some cells for which glycerol is preferred (see Section 15.1.1). Protocol P15.1 is suitable for either cryoprotectant and may be adapted for use in training (see Supp. S30.13).

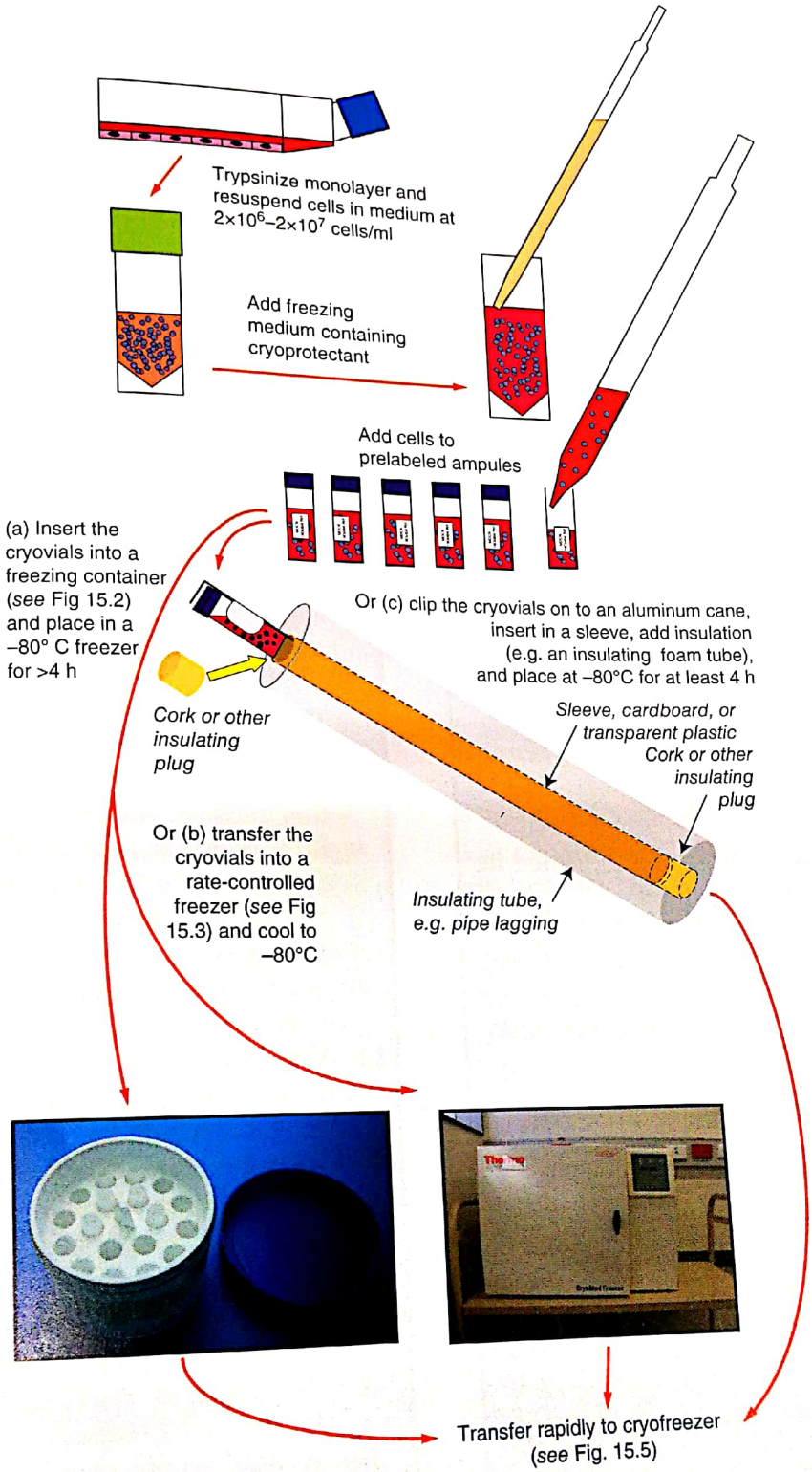

Fig. 15.7. Freezing cells. Cells are trypsinized, freezing medium is added, and the cells are aliquoted into cryovials and frozen using a controlled cooling device. Cells may be slowly cooled: (a) in a freezing container; (b) in a rate-controlled freezer; or (c) using foam insulation (e.g. pipe lagging surrounding a cane to which samples are clipped). Once cells have completed the freezing process (see Figure 15.1), cryovials should be promptly transferred to the cryofreezer for long-term storage.

PROTOCOL P15.1. FREEZING CELLS IN CRYOVIALS

Background

Cells are frozen at subculture, where they are suspended in freezing medium instead of plating into new culture vessels. Otherwise, subculture should be performed as per usual for that cell type (see Protocols P14.2,14.3). The passage number will increase by one to indicate that subculture is performed, but stays the same when cells are thawed (Parker 2011).

Outline

Grow the culture to late log phase, prepare a high-concentration cell suspension in medium with a cryoprotectant, aliquot into cryovials, and freeze slowly (see Figure 15.7).

❖ ***Safety Note. All personnel working with liquid nitrogen should be trained to do so safely and wear suitable PPE. Wear a closed lab coat, face shield, and insulated gloves when handling frozen cryovials (see Sections 6.2.3,6.2.4). Wear chemically resistant gloves when handling DMSO (see Section 6.2.2).***

Materials (sterile or aseptically prepared)

- ☐ Culture to be frozen
- ☐ Freezing medium (see Section 15.3.2), stored in aliquots at −20 °C or made up fresh
- ☐ Universal containers or centrifuge tubes
- ☐ Pipettes in an assortment of sizes, usually 1, 5, 10, and 25 ml
- ☐ Syringe, 1–5 ml, for dispensing glycerol if used (because it is viscous)
- ☐ Cryovials, 1.2 ml

Materials (non-sterile)

- ☐ PPE (see Safety Note)
- ☐ Biological safety cabinet (BSC) and associated consumables (see Protocol P12.1)
- ☐ Cell counting equipment (hemocytometer or automated counter; see Section 19.1)
- ☐ Controlled cooling device (see Section 15.2.2)
- ☐ Forceps (×1), for moving small items in the cryofreezer
- ☐ Dry ice
- ☐ Cryofreezer database (see Section 15.7)

Procedure

A. Before Cryopreservation

1. Examine the culture for cell morphology, confluence, and freedom from contamination. Cells should be healthy and subconfluent (at late log phase before plateau; see Figure 14.1). Proceed if these and other requirements for cryopreservation are met (see Table 15.1).
2. Prewarm reagents for subculture and thaw freezing medium (if stored at −20 °C).
3. Prepare a work space for aseptic technique using a BSC.
4. Perform subculture as per usual for that cell type to give a cell suspension.
5. Count the cells with a hemocytometer or an automated cell counter. If cells have been subcultured from multiple flasks, combine them at this point to give a total cell count that will be used to generate a consistent batch of cryovials.

B. Cryopreservation

6. Calculate the cell yield and the volume of freezing medium that should be added for the required cell concentration, usually between 2×10^6 and 2×10^7 cells/ml. This volume will determine the number of cryovials that can be frozen, e.g. at 1 ml per vial.
7. Label the cryovials with the cell line's designation, passage number, cell count, date, etc.
8. Bring premade aliquots of freezing medium to the BSC or make up fresh, using either DMSO or glycerol (usually at 10%) with medium and/or serum (see Section 15.3.2).
9. Add freezing medium to the cells and mix gently to resuspend, e.g. by inverting the tube.
10. Aliquot 1 ml cell suspension into each cryovial. Cap each cryovial with sufficient torsion to seal the cryovial without distorting the gasket.
11. Once the cell suspension has been fully transferred or you have reached the required number of cryovials, transfer all cryovials to the controlled cooling device for freezing.
12. If you have any remaining cells, consider how these may be used, e.g. for validation testing.

C. Transfer to Cryofreezer

13. Check the cryofreezer database or other records to look for available spaces.
14. Remove cryovials from the controlled cooling device and place on dry ice. Bring to the cryofreezer. Put on PPE for working with liquid nitrogen.
15. Open the cryofreezer and navigate to the empty spaces that were previously identified. Place the cryovials in the correct locations and record the coordinates of each cryovial.
16. Return the box, drawer, or cane to its correct location. If you are working with racks that use a pin to keep boxes or drawers in place, do not forget to return the pin!
17. Update the records in the cryofreezer database (see Section 15.7).

Notes

Step 5: Some tissue culture personnel do not count cells at each passage. If you do not, always record the size of vessel that was used; if you freeze cells from a 25-cm^2 flask, the next person is likely to get good results from thawing cells into the same sized vessel (Parker 2011).

Step 9: If freezing medium is added at 4 °C (see Section 15.1.1), place cells on ice from this point onwards. Otherwise, you do not normally need to place cryovials on ice. A delay of up to 30 minutes at room temperature is not harmful when using DMSO and is beneficial when using glycerol.

Step 10: The volume suggested here is for 1.2-ml cryovials and will vary with the vial size. If multiple cryovials are used, take care to keep the cell suspension consistent, e.g. by gently mixing at regular intervals during aliquoting. Do not use a large pipette, as the cells will fall with gravity and later cryovials will have fewer cells.

Step 11: If a foam box or commercial freezing container is used, leave it overnight in a freezer between −70 and −90 °C and transfer cryovials to liquid nitrogen on the following day.

Step 12: Leftover cells are representative of that batch of cryovials and would indicate if any microbial contaminants have been introduced, e.g. during aliquoting. These cells are particularly useful for sterility and mycoplasma testing (see Sections 11.8.1, 16.4.1).

Step 15: This transfer must be done quickly (< 2 minutes), as the cryovials will reheat at ~10 °C/min, and the cells will deteriorate rapidly if the temperature rises above −50 °C. When transferring multiple samples, it is helpful to have two people at work — one to handle the cryovials and the other to record their coordinates.

15.4.2 Cryopreservation in Other Vessels

Cryopreservation may be performed in other vessels, provided they can be used safely for frozen storage (some vessels may crack or explode at low temperatures). Flasks have been frozen by growing the cells to late log phase, adding 5–10% DMSO to the smallest volume of medium that will effectively cover the monolayer, and putting the flask in a polystyrene container of 15-mm wall thickness (Ohno et al. 1994). The insulated container is placed in a freezer at −70 to −90 °C and will cool at approximately −1 °C/min. Cells may survive for several months if the flask in its container is not removed from the freezer. Multiwell plates may also be frozen in the same manner, with about 150 µl freezing medium per well for a 24-well plate (Ure et al. 1992). This approach can be used to store large numbers of clones during evaluation procedures.

15.4.3 Thawing Stored Cryovials

The procedure used for thawing is an important part of preserving viability. The cryovial should be thawed rapidly at the cell line's normal growth temperature (typically 37 °C), to minimize intracellular ice crystal growth during the warming process. This can be done using a water bath or a dry heat block, provided heat is rapidly transferred from the dry block to the cryovial. After thawing, the cell suspension should be diluted slowly to avoid osmotic damage that may reduce viability. Some tissue culture personnel centrifuge the cell suspension after dilution, particularly if cells are sensitive to cryoprotectant (e.g. some suspension-growing cells) or if toxicity is a concern for later applications (see Section 15.1.1). However, if the cell concentration has been selected to incorporate a suitable dilution factor (see Section 15.3.3), centrifugation can be omitted and the medium replaced once cells have attached. Cells should be reseeded at a relatively high concentration to optimize recovery.

PROTOCOL P15.2. THAWING FROZEN CRYOVIALS

Outline

Thaw the cells in the cryovial rapidly, dilute them slowly, and reseed at a high cell concentration (see Figure 15.8).

❖ ***Safety Note. All personnel working with liquid nitrogen should be trained to do so safely and wear suitable PPE. Wear a closed lab coat, face shield, and insulated gloves when handling frozen cryovials (see Sections 6.2.3, 6.2.4).***

Materials (sterile or aseptically prepared)

- ☐ Frozen cryovial
- ☐ Complete culture medium
- ☐ Centrifuge tubes, 15 ml (if centrifugation is required)
- ☐ Pipettes in an assortment of sizes, usually 1, 5, 10, and 25 ml