Cryopreservation

RATIONALE FOR FREEZING

Cell lines in continuous culture are prone to variation due to selection in early-passage culture, senescence in finite cell lines, and genetic and phenotypic instability in continuous cell lines.

In addition, even the best-run laboratory is prone to equipment failure and contamination. Cross-contamination and misidentification also continues to occur with an alarming frequency.

There are many reasons, therefore, for freezing down a validated stock of cells; these reasons can be summarized as follows:

(1) Genotypic drift due to **genetic instability**

(2) Senescence and the resultant **extinction of the cell line**

(3) Transformation of **growth characteristics** and acquisition of malignancy-associated properties

(4) Phenotypic instability due **to selection and dedifferentiation**

(5) **Contamination** by microorganisms

(6) **Cross-contamination** by other cell lines

(7) **Incubator failure**

(8) **Saving time and materials** maintaining lines not in immediate use

(9) **Need for distribution** to other users

ACQUISITION OF CELL LINES FOR CRYOPRESERVATION

There are certain requirements that should be met before cell lines are considered for cryopreservation.

Validation.

- Cell lines should be shown to be free of contamination and authentic before cryopreservation.
- Although a few ampoules of a newly acquired cell line may be frozen before complete validation has been carried out, proper validation should be carried out before major stocks are frozen.

When to freeze.

- If it is a finite cell line, it is grown to around the fifth population doubling in order to create a sufficient number of cells for freezing.
- Continuous cell lines should be cloned, a characterized clone selected, and sufficient stocks grown for freezing. Continuous cell lines have advantages; they **survive indefinitely**, **grow more rapidly**, and can **be cloned** more easily; but they may be **less stable genetically**.
- Finite cell lines are usually diploid or close to it and are **stable** between certain passage levels, but they are **harder to clone**, **grow more slowly**, and eventually **die** out or **transform**.

PRINCIPLES OF CRYOPRESERVATION

1. Theoretical Background to Cell Freezing

Optimal freezing of cells for **maximum viable recovery** on thawing depends on minimizing intracellular ice crystal formation and reducing cryogenic damage from foci of high concentration solutes formed when intracellular water freezes.

This is achieved:

(a) by **freezing slowly** to allow water to leave the cell but not so slowly that ice crystal growth is encouraged,

(b) by using a **hydrophilic cryoprotectant** to sequester water,

(c) by storing the cells at the **lowest possible temperature** to minimize the effects of high salt concentrations on protein denaturation in micelles within the ice, and

(d) by **thawing rapidly** to minimize ice crystal growth and generation of solute gradients formed as the residual intracellular ice melts.

2. Cell Concentration

- Cells appear to survive freezing best when frozen at a **high cell concentration**.

✓ This is largely an empirical observation but probably is related partly to the reduced viability on thawing requiring a higher seeding concentration and partly to improved survival at a high cell concentration if cells are leaky because of cryogenic damage.

✓ A high concentration at freezing also allows sufficient dilution of the cryoprotectant at reseeding after thawing so that centrifugation is unnecessary (at least for most cells). The number of cells frozen should be sufficient to allow for 1:10 or 1:20 dilution on thawing to dilute out the cryoprotectant but still keep the cell concentration higher than at normal passage;

✓ for example, for cells subcultured normally at 1×10^5 /mL, 1×10^7 should be frozen in 1 mL of medium, and, after thawing the cells, the whole 1 mL should be diluted to 20 mL of medium, giving 5×10^5 cells/mL (five times the normal seeding concentration).

✓ This dilutes the cryoprotectant from **10% to 0.5%,** at which concentration it is less likely to be toxic.

✓ **Residual cryoprotectant** may be diluted out as soon as the cells start to grow (for suspension cultures) or the **medium changed** as soon as the cells have attached (for monolayers).

Freezing Medium

- The cell suspension is frozen in the presence of a **cryoprotectant** such as **glycerol** or **dimethyl sulfoxide (DMSO).**
- Of these two, DMSO appears to be the more effective, possibly **because it penetrates the cell better than glycerol.**
- Concentrations of between 5% and 15% have been used, but 7.5% or 10% is more usual.

- There are situations in which DMSO may **be toxic** or **induce cells to differentiate**, e.g., with hematopoietic cell lines such as L5178Y or HL60, and in these cases it is preferable either to use glycerol or to centrifuge the cells after thawing to remove the cryoprotectant.

- It has been claimed that cells should be kept at 4◦C after DMSO is added to the freezing medium and before freezing, but preliminary experiments by the author suggested that this did not improve survival after freezing and may even have reduced it, perhaps by inhibiting intracellular penetration.

- DMSO should be colorless, and it needs to be stored in glass or polypropylene, as it is a **powerful solvent** and will leach impurities out of rubber and some plastics.

- Glycerol should be not more than one year old, as it may become toxic after prolonged storage.

- Other cryoprotectants have been suggested, such as **polyvinylpyrrolidone (PVP), polyethylene glycol (PEG) and hydroxyethyl starch (HES)** but none has had the general acceptance of either DMSO or glycerol, although there may be some improvement with **trehalose**.
- Many laboratories also increase the serum concentration in freezing medium to 40%, 50%, or even 100%.

Cooling Rate

- Most cultured cells survive best if they are cooled at 1°C/min.

- This is probably a compromise between **fast freezing minimizing ice crystal growth** and **slow cooling encouraging the extracellular migration of water**.

- The rate of the cooling is governed by (a) the ambient temperature, (b) any insulation surrounding the cells, including the ampoule, (c) the specific heat and volume of the ampoule contents, and (d) the latent heat absorption during freezing.

- When cells are transferred to the liquid nitrogen freezer (or the end stage is reached in a programmable freezer) the temperature drops rapidly to between −180 and −196°C.

A controlled cooling rate can be achieved in several ways:

(1) Lay the ampoules on cotton wool in a polystyrene foam box with a wall thickness of ~15 mm. This box, plus the cotton wool, should provide sufficient insulation such that the ampoules will cool at 1°C/min when the box is placed at −70°C or −90°C in a regular deep freeze.

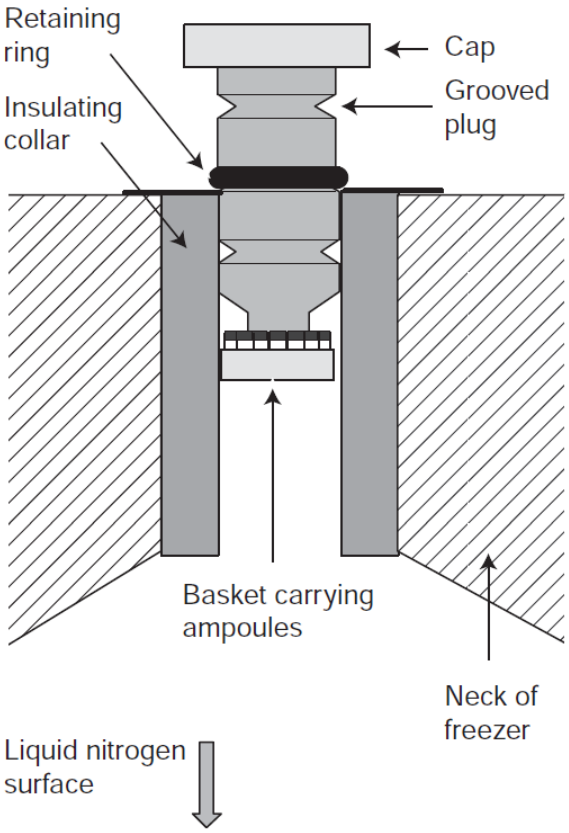
Cooling Rate

(2) Insert the canes in tubular foam pipe insulation, with a wall thickness of ~15 mm, and place the insulation at −70°C or −90°C in a regular deep freeze.

Ampoules on Cane. Plastic ampoules are clipped onto an aluminum cane (bottom), enclosed in a cardboard tube (middle), and placed inside an insulating foam tube (top). The insulating tube is plugged at either end with cotton or another suitable insulating material.

Cooling Rate

(3) Place the ampoules in a **Taylor Wharton freezer neck plug** and insert the plug into the neck of the nitrogen freezer.



Neck Plug Cooler. Modified neck plug for narrow necked freezers, allowing controlled cooling at different rates (Taylor Wharton). Shown is the section of the freezer neck with the modified neck plug in place. The ‘‘O’’ ring is used to set the height of the ampoules within the neck of the freezer. The lower the height, the faster the cooling

Cooling Rate

(4) Place the ampoules in a Nalgene Nunc freezing container and place at −70°C or −80°C.

Nalgene Nunc Cooler. Plastic holder with fluid-filled base. The specific heat of the coolant in the base insulates the container and gives a cooling rate of ~1°C/min in the ampoules.

Cooling Rate

(5) Use a controlled-rate freezer programmed to freeze at 1˚C/min.

Programmable Freezer.

Ampoules are placed in an insulated chamber, and the cooling rate is regulated by injecting liquid nitrogen into the chamber at a rate determined by a **sensor** on the rack with the ampoules and a preset program in the console unit (Planer Biomed).

(a) Control unit and freezing chamber (lid open).

(b) Close-up of a freezing chamber with four ampoules, one with a probe in it.

Cooling Rate

- If recovery is low, it is possible to change the average cooling rate (i.e., by use of more or less insulation).

- Use of a programmable freezer with a probe, which **senses the temperature of the ampoule and adds liquid nitrogen to the freezing chamber at the correct rate to achieve a preprogrammed cooling rate**, minimizes the stress of supercooling and can achieve a linear cooling rate throughout the range.

- Programmable coolers are, relatively expensive, compared to the simple devices described in (1)– (4) above, and have few advantages unless you wish to vary the cooling rate.

- With the insulated container methods, the cooling rate is proportional to the difference in temperature between the ampoules and the ambient air. If the ampoules are placed in a freezer at −70°C, they will cool rapidly to around −50°C, but the cooling rate falls off significantly after that. Hence, the time that the ampoules spend in the −70°C freezer needs to be longer than the amount of time projected by a 1°C/min cooling rate.

- It is safer to leave the ampoules at −70°C overnight before transferring them to liquid nitrogen.

- Furthermore, when removed from the freezing device, they will heat up at a rate of ∼10°C/min. It is critical that they do not warm up above −50°C, as they will start to deteriorate, so the transfer to the liquid nitrogen freezer must take significantly less than two minutes.

Cryofreezers

- Storage in a liquid nitrogen freezer is currently the most satisfactory method of preserving cultured cells.

- The frozen cells are transferred rapidly to the cryofreezer when they are at or below −70°C.

- Cryofreezers differ in design depending on **size of the access neck**, **storage system employed**, and **location of liquid nitrogen**.

Neck size.

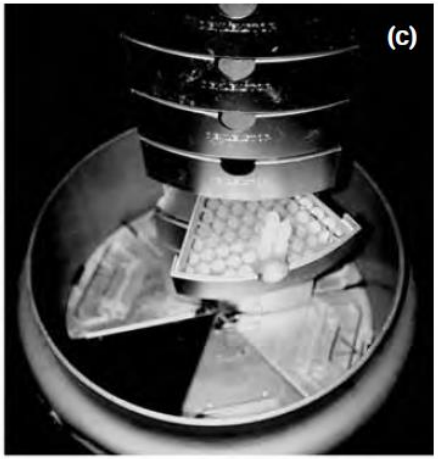
✓ Canister storage systems tend to have narrow necks (Figs. 20.6a,b, 20.7a), which **reduces the rate of evaporation of the liquid nitrogen but makes access a little awkward**.

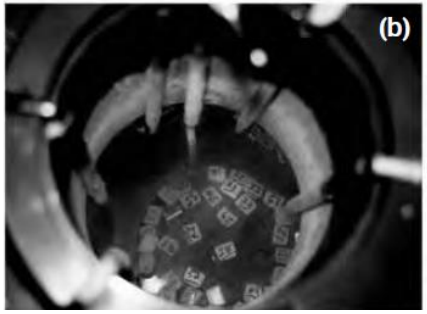
✓ Wide-necked freezers are chosen **for ease of access and maximum capacity**, usually with storage in sections within drawers, **but tend to have a faster evaporation rate**.

✓ However, it is possible to select a relatively narrow-necked freezer while still using a tray system for storage.

✓ Freezers are available with inventory control based on square-array storage trays; these trays are mounted on racks that are accessed by the same system as the cane and canister of conventional narrow-necked freezers (Figs. 20.6d, 20.7b).


(a)

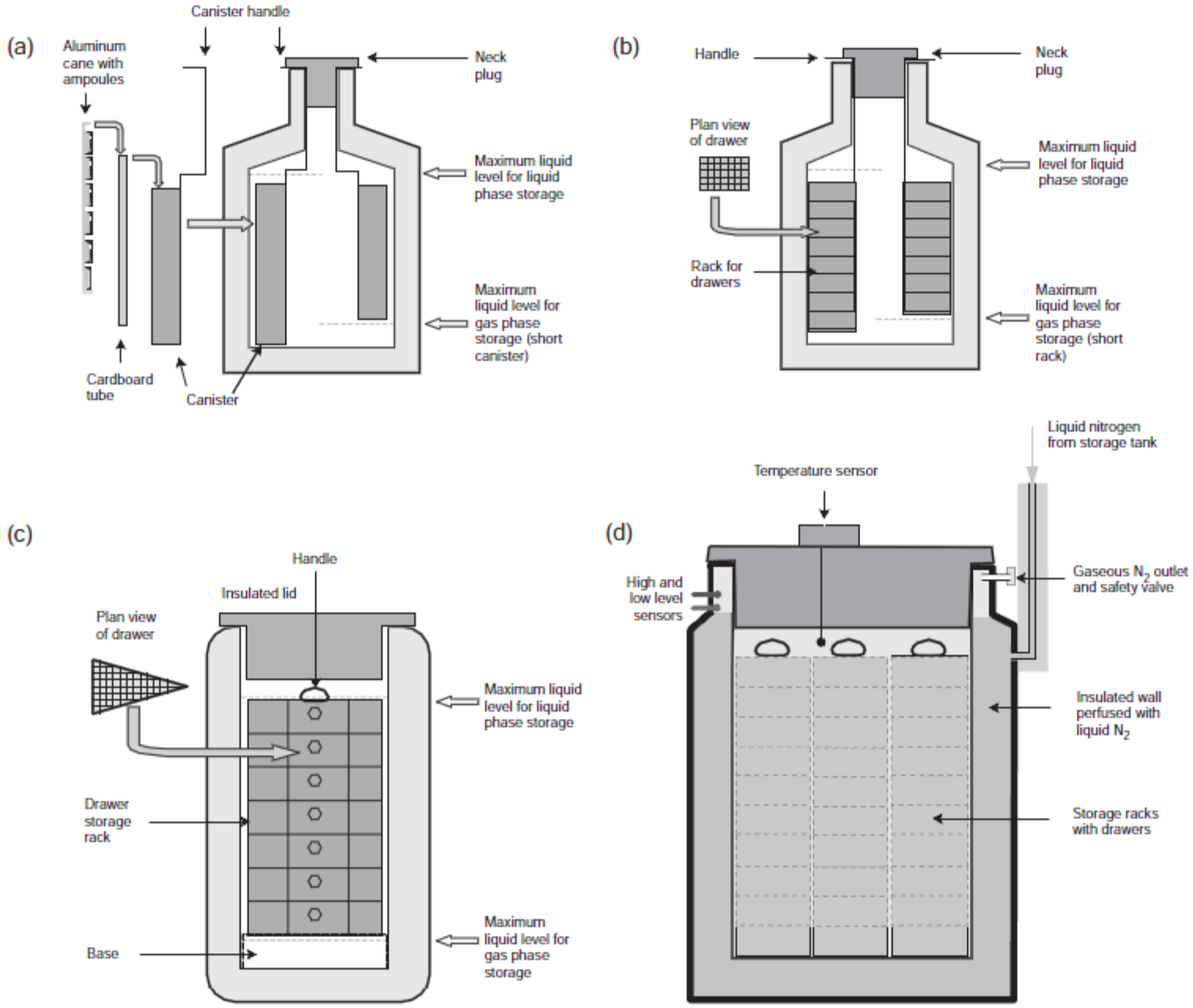

(c)


(b)


(d)


(e)


(f)

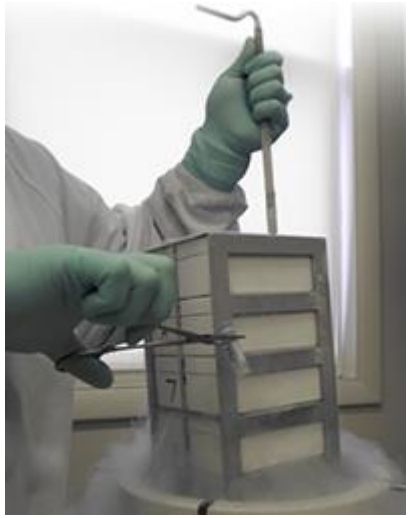

(a)
Canister handle
Aluminum cane with ampoules
Neck plug
Maximum liquid level for liquid phase storage
Maximum liquid level for gas phase storage (short canister)
Cardboard tube
Canister
(b)
Handle
Neck plug
Plan view of drawer
Maximum liquid level for liquid phase storage
Rack for drawers
Maximum liquid level for gas phase storage (short rack)
(c)
Handle
Insulated lid
Plan view of drawer
Maximum liquid level for liquid phase storage
Drawer storage rack
Base
Maximum liquid level for gas phase storage
(d)
Liquid nitrogen from storage tank
Temperature sensor
Gaseous N_2 outlet and safety valve
High and low level sensors
Insulated wall perfused with liquid N_2
Storage racks with drawers

Storage system.
There are two mains types of storage used for 1-mL ampoules for cell culture work:

1. The **cane system**, based on the storage of sperm in straws, uses ampoules clipped on to an aluminum cane, inserted into a cardboard tube, and placed within cylindrical canisters in the freezer. It has the advantage that ampoules can be handled in multiples of six at a time, with all the ampoules on one cane being from the same cell line, making the transfer from the cooling device to the freezer easier and quicker. The canes can be colored and numbered, making location fairly easy, and ampoules can be withdrawn without exposing all the other ampoules to the warm atmosphere.

Storage system.

2. Storage in **rectangular drawers** is preferred by some users, who feel that retrieval is easier and individual ampoules can be identified by the drawer number and the coordinates within the drawer. It does mean, however, that the total contents of the drawer, which can be from 20 to 100 ampoules, are exposed at one time when an ampoule is retrieved, and the whole stack must be lifted out if you are accessing one of the lower drawers. Also, loading a large number of ampoules into the drawer must be done one ampoule at a time, risking delay and overheating.



Location of liquid nitrogen.

- If the liquid nitrogen is located in the main body of the freezer there is a **choice of filling the freezer and submerging the ampoules**, or **only partfilling and storing the ampoules in the vapor phase**.

- Storage in the liquid phase means that the container can be filled and the liquid nitrogen will therefore last longer, but **risks uptake of nitrogen by leaky ampoules**, which will then explode violently on thawing.
- There is also a greater **likelihood of transfer of contamination between ampoules** and the **buildup of contamination from outside**, carried in when material is introduced and concentrated by the constant evaporation of the liquid nitrogen in the tank.

- With the introduction of improved insulation and reduced evaporation, **vapor-phase** storage is probably preferable.

- It also eliminates the **risk of splashing** when the liquid nitrogen boils when something is inserted and **reduces evaporation of nitrogen into the room air**.

- There is, however, **a gradient in the temperature** from the surface of the liquid nitrogen up to the neck of approximately 80°C, from −190°C to around −110°C in gas phase storage, although the **design and composition of the racking system** may help to eliminate this gradient.

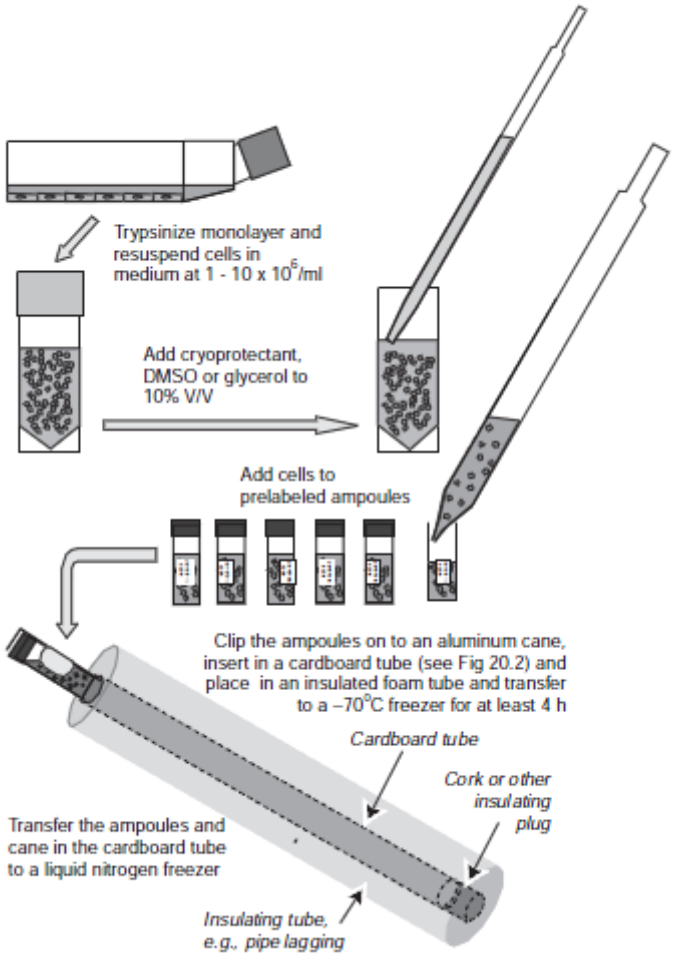
Location of liquid nitrogen.

- Some freezers have the liquid nitrogen located within the **wall of the freezer** and not in the storage compartment. It is replenished by an automatic feed with high and low level controls (Fig. 20.7d), and evaporated nitrogen is released via a relief valve.

- This has the advantages of **gas-phase storage**, with the added advantage of **a lower consumption of liquid nitrogen** and **elimination of the temperature gradient**.

- However, **the nitrogen level is not visible** and cannot be measured by dipstick, so complete reliance has to be made on electronic monitoring.

- In addition, **any blockage of nitrogen flow within the freezer wall** can be very difficult or even impossible to eliminate, so it is essential that the liquid nitrogen be filtered and that steps are taken to ensure that no water, or water vapor, enters the system, as ice can also block nitrogen flow.


Trypsinize monolayer and resuspend cells in medium at 1 - 10 x 10^6/ml
Add cryoprotectant, DMSO or glycerol to 10% V/V
Add cells to prelabeled ampoules
Clip the ampoules on to an aluminum cane, insert in a cardboard tube (see Fig 20.2) and place in an insulated foam tube and transfer to a –70°C freezer for at least 4 h
Cardboard tube
Cork or other insulating plug
Transfer the ampoules and cane in the cardboard tube to a liquid nitrogen freezer
Insulating tube, e.g., pipe lagging