Tech Note
No. 14

Cryopreservation of Mammalian Cells – Protocols

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Most mammalian cells can be stored at temperatures below –130°C for many years*.
The viability of the cells after cryopreservation depends on their ability to cope with the variety of stresses imposed on them during the freezing and thawing procedures. The protocols given in this Tech Note will be suitable for most cells. Minor adjustments are always cell line dependent and therefore in each case must be found emperically.

Protocol for freezing of cells

1. Cells to be frozen must not be infected with micro-organisms (bacteria, yeast, mold, mycoplasma and in special cases virus). In addition the cells must be viable and in exponential growth.

2. Harvest the cells by spinning the cells as gently as possible (speed should not exceed 400 x g). Resuspend the cells in growth medium at room temperature to a concentration of 2 x 10^6–2 x 10^7 cells per mL.
   Count the viable cells. Cells grown in protein free (or serum free) medium cannot be expected to survive cryopreservation in their growth medium. Protein (e.g. BSA or serum) must be added to the medium.
   After thawing, the cells can then be returned to the required protein free medium.

3. To avoid damage to the cell during freezing, a cryoprotectant is added to the growth medium in which the cells are to be frozen. Glycerol or DMSO (dimethyl sulphoxide) in 10% concentration is most commonly used. Glycerol is non-toxic to the cells (and to the personnel) and can be added directly to the cells. DMSO enters the cells more rapidly than glycerol. DMSO is toxic to the cells (and to the personnel) if high concentrations are used, and if the cells are exposed to DMSO for prolonged periods (e.g. 10% DMSO for several hours at room temperature). Because heat is generated when DMSO is dissolved in aqueous solutions, it cannot be added directly to the cells. DMSO must be diluted to e.g. 20% in medium, allowed to cool to below 37°C and subsequently added to the cells to a final concentration of 10%.

4. Mix cells and cryoprotectant at room temperature to final concentration of viable cells in the range between 10^6 and 10^7 cells per mL and final concentration of cryoprotectant of 10%. Cells frozen in lower or higher cell concentration often tend to have less viability.
   Allow the cryoprotectant to enter the cells. At least 20 minutes and, for DMSO, not more than 30 minutes should pass before the cooling procedure is started.

5. If a uniform batch of cells where every aliquot is representative of the whole batch is required, all the cells must be collected at this step into one vial and properly mixed, before aliquoting is performed. Usually 1 mL aliquots are used, but other volumes can be used. As 1 mL Thermo Scientific Nunc CryoTube vials are very small, it is advisable to use sterile gloves when opening and closing the vials in order to minimize the risk of contamination during aliquoting. Care should be taken not to exceed the maximum filling volume of the Nunc™ CryoTube™ vial. CryoTube vials made of polypropylene are most commonly used for storage of the cells. When cells are to be stored in the vapour phase of liquid nitrogen or in a –140°C mechanical freezer, CryoTube vials with internal thread and silicone gasket are the safest to use. This kind of CryoTube vials has the best seal and, provided that the vial is properly closed (neither too loose nor too tight), there is no risk of contamination with micro-organisms during storage.
   When cells are to be stored in the liquid phase of liquid nitrogen, it is recommended to enclose the internally threaded

*) CryoTube vials are recommended by ECACC (European Collection of Animal Cell Cultures) for shipping and depositing cell lines (ECACC general catalogue and ECACC shipping instructions).

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CryoTube vials in heat sealable Thermo Scientific Nunc CryoFlex tube wrap. Be careful not to heat the vial excessively as this will injure the cells. If the CryoTube vials contain hazardous material – no matter how they are stored – the vials must be sealed with CryoFlex™ tube wrap to reduce the risk for the personnel handling the CryoTube vials.

6. The most common cooling rate used is 1°C per minute in the range from room temperature and down to below –50°C. Once the temperature is below –50°C, the CryoTube vials can be lowered to the final storage temperature (–140°C to –196°C). (NB only internally threaded tubes protected by correctly applied CryoFlex must be submerged in the liquid phase of nitrogen).
   Special equipment for precise controlling of the cooling rates is available on the market. Such equipment is very expensive and usually only needed for very sensitive cells.
   In most cases the cooling rate can be controlled in a less expensive manner: As a rule of thumb, a –70°C mechanical freezer cools a litre cube of water 1°C/minute.
   To achieve a similar cooling rate, the cryo vials can be placed in an insulated cube of approximately 1 litre capacity. The insulation can be paper, cotton, wool, or styrofoam.
7. In theory, cells can be stored indefinitely below –130°C, as no biological process takes place below this temperature. In practice, background ionizing radiation will in time (decades) injure the cells. Cells stored for prolonged periods above –130°C tend to be less stable.
   Be aware that cells stored in small liquid nitrogen tanks without automatic refill may not be stored permanently below –130°C if the tanks are opened frequently. The temperature at the top of the tank rises as the nitrogen evaporates from the tank. Therefore, a temperature gradient is established from the surface of the liquid nitrogen towards the top of the tank.

Protocol for thawing of cells

1. To obtain the best possible survival the thawing of the cells must be performed as quickly as possible. Once the CryoTube vial is removed from the freezer/liquid nitrogen tank, it should be placed directly into a 37°C water bath and shaken until it is completely thawed. If liquid nitrogen has entered the CryoTube vial, it will explode upon warming, because liquid nitrogen will expand 700 times during the warming process. Personnel should always wear protective clothes, glasses and gloves, while handling CryoTube vials from liquid nitrogen tanks, and should transport the vial in a covered, insulated bucket or box. An explosion may happen seconds after the vial has been removed from the liquid nitrogen.
2. To avoid transfer of micro-organisms from the freezer/liquid nitrogen tank or the water bath to the laminar flow cabinet and subsequently to the culture, the CryoTube vials are soaked in 70% ethanol before they are transferred to the laminar flow cabinet.
3. If the cryo protectant used is glycerol, the cells can be diluted 10 times directly into a TC flask or TC dish. If DMSO is used, the cells should be washed once in growth medium before being added to the TC flask in fresh growth medium. Some cells are very sensitive at this stage of the procedure, and it may be advisable to dilute the DMSO stepwise to minimize the osmotic stress imposed upon the cells when DMSO is diluted. It is advisable to take out a sample of the thawed cells for a viability test before the cells are placed in the incubator.
4. In order to avoid physical damage of cells that form a monolayer, such cultures should be left untouched in the incubator for at least 16 hours before assessing the result.

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