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Preservation Strategies Across the Biorepository Spectrum: Ensuring Longevity and Integrity of Biological Specimens

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Abstract

Biorepositories are essential for preserving biological specimens for scientific research and diagnostic purposes. Effective preservation methods for viruses, bacteria, and serum/plasma are crucial for maintaining their viability and integrity over time. Viruses, characterized by their simple structure, are preserved through techniques such as freeze-drying, cryopreservation, and storage in liquid nitrogen, emphasizing the importance of proteins, small volumes, and high-titre preparations. Cryopreservation ensures virus infectivity at temperatures below 60°C, with cryoprotectants playing a vital role in preventing cellular damage during freezing. Lyophilization emerges as a valuable technique, involving freezing, primary drying, and secondary drying stages, although it is time and energy-intensive. Bacterial preservation relies on cryopreservation and freeze-drying, with -80°C being suitable for most isolates, while serum/plasma preservation is crucial for nucleic acid extraction from blood-borne viruses or bacteria, with -20°C generally sufficient for immunoglobulin stability. These preservation methods contribute to the longevity and accessibility of biological specimens, facilitating scientific investigation and diagnostic advancements.

Biorepository of Viruses

Viruses, being non-cellular entities, are notably smaller and less complex biochemically compared to even the simplest unicellular organisms. They typically comprise RNA or DNA, often enclosed by protective proteins. These proteins shield the nucleic acid, aid in its delivery to host cells, facilitate transcription, and assist in exposing it to the host's biochemical machinery. This relative simplicity has contributed to viruses' widespread coexistence with various life forms (Gould, 1999).

While DNA viruses generally exhibit greater stability than RNA viruses, both types can be preserved relatively easily. Many viruses endure for months at refrigeration temperatures and remain viable for years at very low temperatures without requiring specialized preservatives or meticulously controlled freezing methods. This resilience is largely attributed to their uncomplicated structure, diminutive size, and the absence of free water. Although viruses with lipid envelopes may be less stable at ambient temperatures, they fare well at ultra-low temperatures or when subjected to freeze-drying (Gould, 1999).

Some General Rules for Preservation Apply to Most Viruses (Forrest *et.al.*, 2022).

1. **Freeze dried preparations of virus can be maintained for decades at 4°C in the dark and lower temperatures increase the storage time**. Although this principle has not been tested exhaustively for every known virus, it has been demonstrated with very many different viruses.

2. **Virus infectivity is retained for very long periods in liquid nitrogen.** While not the most cost-effective method for storing viruses, liquid nitrogen proves highly effective, allowing viruses to endure almost indefinitely. To enhance safety, it's crucial to encase individual cryotubes in heat-shrinkable tubing like Nunc CryoFlex. This prevents liquid nitrogen penetration, minimizing the risk of virus cross-contamination, reducing the potential for skin burns during cryotube exposure, and avoiding exposure to virus aerosols.
3. **Proteins are effective protectants for virus cryopreservation**. For many viruses, the preferred suspending medium is tissue culture medium supplemented with serum or proteins, typically at concentrations of 10% or higher. These proteins are thought to safeguard virus infectivity during freezing or freeze-drying, although the exact mechanisms remain unclear. They may offer buffering against pH fluctuations, aid in dispersing virus particles, and hinder processes that harm nucleic acids. Viruses from human or animal specimens, when contained in serum or tissues, can be stored at ultra-low temperatures without additional processing. (Hubalek, 2003).
4. **It is good practice to preserve small volumes of virus suspension**. Preserving virus samples in small volumes proves more effective in maintaining infectivity, as it allows for quicker processes of freezing, thawing, freeze-drying, and reconstitution. Rapid freezing, thawing, or reconstitution is less detrimental to the virus compared to slower methods. Dispensing small aliquots enables storage of numerous samples of the same preparation in a freezer, ensuring each is readily available to replicate consistent performance (Tedeschi and Paoli, 2011).
5. **High titre virus preparations are preferable for long-term storage**. Since the half-life of a virus held either at low temperature or freeze dried is unaltered by the quantity of infectious virus in the preparation, it follows that a high titre virus preparation will retain viability for longer than a low titre preparation (Jiang *et.al*., 2015).
6. **Dry ice should only be used to preserve viruses in totally sealed containers.** The ideal pH range for storing viruses is between 7.0 and 8.0, as they are less stable at pH levels of 6.0 or below. Storing virus preparations in unsealed containers on dry ice is discouraged because released carbon dioxide can be absorbed through the joint between the cap and the cryotube, lowering the pH of the preserved virus suspension (Best, 1961).
7. **Viruses can be preserved for long periods as nucleic acid**. The purified nucleic acid of positive-stranded RNA viruses and certain DNA viruses, lacking essential enzymes in their structure, retains infectivity. This principle enables the long-term preservation of these viruses. Ethanol-precipitated RNA and DNA can be stored nearly indefinitely at 4°C or lower temperatures under ethanol. Ethanol is crucial for RNA storage, inhibiting RNA-degrading enzymes. DNA can be stored under ethanol or as dried DNA. Although highly effective, this method of virus preservation is not widely adopted. Viruses frozen as nucleic acid likely remain viable indefinitely and can be stored in minimal volumes, reducing the need for extensive storage capacity (Gould, 1999)

Many different methods are employed for the long-term storage of viruses in repositories out of which, cryopreservation and freeze drying (Lyophilization) are the commonest.

Cryopreservation

Virus infectivity is retained well at temperatures below -60°C. In many virology laboratories, -70°C (or more recently -80°C) is the favoured temperature, partly because viruses are known to survive for decades at -70°C and partly because modern freezers do not have to work at their maximum capacity to maintain this temperature thereby increasing their reliability. Viruses should be frozen rapidly and this is most readily accomplished by storing only small volumes (0.1 to 0.5 ml) of virus suspension.

Cooling and storage of live material at low temperature, that all biological processes are suspended (-196°C) and material does not decompose

- Maintains viability
- Permits high rates of survivability

It allows a virtually indefinite storage of biological material without any deterioration over many years. Techniques are available for the preservation of microorganisms, isolated tissue cells, small multicellular organisms or more complex organisms such as embroys (Wang *et.al.,* 2018).

Cryoprotectants

- Chemical compounds that prevent cells or tissues from damage due to freezing.
- These are macromolecules added to the freezing medium to protect the cells from the detrimental effects of **intracellular ice crystals formation** from the solution effects, during the process of freezing and thawing.
- Examples: Glycerol, DMSO, Ethylene glycol, Sucrose etc.
- Types of cryoprotectants: Permeating and Non-permeating.

Permeating cryoprotectants: Lowers the freezing point of the solution**,** increases the viscosity and thus reduce diffusion in solutions. Replace intracellular water to prevent the formation of large ice crystals. Examples: Glycerol, DMSO, Propylene glycol and Ethylene glycol.

Permeating cryoprotectants (extracellular): Changes the water balance of cells by shrinking cells before freezing. Examples: sucrose, maltose, trehalose, sorbitol and acetamide and BSA (Fuller, 2004).

Cryopreservation using Liquid Nitrogen: Generally, it is recommended to use only small volume cryotubes for storage of virus in liquid nitrogen and as noted earlier, each cryotube must be sealed in special tubing (Nunc Cryoflex - or equivalent) to avoid the risk of cross contamination of viruses and also exposure of the operator to virus-containing aerosols when cryotubes are removed from the nitrogen. It is also important to remember that liquid nitrogen storage tanks have to be checked and replenished with liquid nitrogen regularly (Hawkins *et al*., 1996).

Materials

i. Heat shrink cryotubing (Nunc Cryoflex or equivalent)
ii. Screw capped cryotubes - preferably small volume (1 ml or 2 ml).
iii. Liquid nitrogen storage tanks.
iv. Protective gloves and face mask.
v. Indelible marker pen.
vi. Bunsen burner.
vii. Thermos flask containing liquid nitrogen.

Procedure

1. Cut tubing to extend 2 cm beyond each end of the Cryotube.
2. Insert the labeled cryotube with virus into the center of the tubing.
3. Gently heat tubing with a Bunsen burner or heat gun to shrink around the Cryotube.
4. Reheat ends of tubing and seal with forceps to ensure closure.
5. Snap freeze sealed cryotubes in a small volume of liquid nitrogen.
6. Place frozen cryotubes into appropriate compartments of a liquid nitrogen tank and record details.
7. To use frozen virus, thaw cryotube at 37°C for minimal time, then cut the tubing at the cap's silicone gasket and unscrew the cap (Hawkins et al., 1996).

Freeze Drying / Lyophilization

Lyophilization is one of the greatest innovations in pharmaceutical industry for enhancing the long-term safety of drug products and simplifying the shipping and handling of drugs. It is a 3-stage process involving freezing, primary drying (ice sublimation), and secondary drying (unfrozen desorption of the water). It is a time-and energy-intensive process that can take days to complete (Rexroad, 2002).

Principle: The core principle of lyophilization is sublimation, where water transitions directly from solid (ice) to vapor without passing through a liquid phase. This process occurs at pressures and temperatures below the water's triple point, specifically at 4,579 mm Hg and 0.0099 degrees Celsius. The material to be dried is first frozen, then heated under high vacuum, leaving behind solid, dried components from the original liquid. The driving force for water removal during lyophilization is the water vapor concentration gradient between the drying front and the condenser (Rexroad, 2002).

The fundamental process steps:

Freezing: This provides the necessary condition for drying at low temperatures.

Vacuum: The substance is then put under vacuum after freezing. It helps the freezing substance to vaporize without going through the liquid phase, which is a process called sublimation.

Heat: Heat is added to frozen product in order to speed up sublimation.

Condensation: The vaporized liquid is removed by low-temperature condenser plates from the vacuum chamber by transforming it back into a solid. It completes process of separation (Hansen *et.al.,* 2015)

Lyophilization process architecture has historically been broken down into four parts

1. **Pre-treatment:** It includes any method of treating the product prior to freezing. This may include concentrating the product, formulation revision (i.e., addition of components to increase stability and/or improve processing), decreasing a high vapor pressure solvent or increasing the surface area.
2. **Freezing phase:** The liquid composition is cooled until the ice starts to nucleate and ice growth follows. It results in the removal of most of the water from a mixture of glassy and/or crystalline solutes into ice crystals.
3. **Primary drying:** Sublimation eliminates the crystalline ice formed during freezing. To achieve this, the chamber pressure is lowered below the vapor pressure of the shelf temperature, increasing the ice's surface area to provide the heat required for sublimation. Even after primary drying, approximately 15-20% of the product may remain unfrozen, typically at high temperatures and low pressures, to achieve the desired low humidity content. Lyophilization is typically a highly time and energy-intensive drying process.
4. **Secondary drying:** Secondary drying, compared to primary drying, is relatively short, typically occurring every hour as opposed to every day. Consequently, the emphasis in developing the Lyophilization process has often been on optimizing the primary drying stage. This optimization involves shortening the primary drying time by adjusting the shelf temperature and/or chamber pressure, all while ensuring product quality remains unaffected. Despite the critical importance of freezing during Lyophilization, historically, the significance of the freezing process has been somewhat overlooked (Rexroad, 2002).

Biorepository of Bacteria

- Bacteria are isolated from samples through incubation in standard isolation media, then suspended in liquid media for storage.
- The majority of bacterial isolates can be stored at -80°C, but factors like supporting material, initial inoculum concentration, and cryopreservative type affect survival and regrowth.

- Small beads can be utilized before freezing, allowing bacteria to coat the beads for individual retrieval without thawing the entire sample.
- Bacteria can be stored at -20°C for 1-3 years, at -70°C for 1-10 years, while storage in liquid nitrogen preserves bacteria for up to 30 years. However, without preservatives, the viability of common pathogenic bacterial species may decrease to 80-90% after 12-18 months (Tedeschi and Paoli, 2011).

Biorepository of Serum/Plasma

- Serum/plasma is used to extract nucleic acids from blood-borne viruses or bacteria during bacteremic diseases, especially when a cellular source is not readily available. They are also suitable for extracting small quantities of DNA from human or animal origin.
- Quality control programs have demonstrated that due to the stable nature of immunoglobulins, serum/plasma specimens for antibody assays can be stored for years at -20°C.
- According to Kenis et al. (2002), serum samples for Interleukin-6 and cytokine inhibitors determination can be stored at -20°C for several years, while for Interleukin-10 determinations, storage at -70°C is recommended.

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