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# TPP Cryo Tube
# Cryopreservation of Adherent Cells

The establishment of a cell bank through cryopreservation is essential to maintain cell quality. Continuous cultivation can lead to genetic changes, reduction of proliferation rate, transformation and altered expression patterns. Cryopreservation allows cell lines to be stored almost indefinitely without loss of quality.

**Note**: This protocol describes the basic steps for freezing adherent cells. It may not be appropriate for every cell line. Please refer to the specific protocols of the respective cell banks or sources

## Handling and Safety

Handling of biological materials shall be performed in full compliance with all applicable national and international regulations.

Activities must conform to the laboratory's assigned biological safety level, the relevant Safety Data Sheets (SDS), and the manufacturer's Instructions for Use (IFU).

Appropriate personal protective equipment (PPE) should be always worn during handling.

## General Information

Before freezing, examine cells morphologically and check for contamination. Cells should be in late log phase. For adherent cells, harvesting at 70 – 80% confluence is recommended. Perform a media change 24 hours before freezing. Pre-cool serological pipettes, freezing media, freezing containers and cryovials in the refrigerator before use.

## Freezing Media Options

The presence of cryoprotective agents, together with the high protein content, plays a critical role in protecting cells from damage caused by ice crystal formation. These components are particularly important during both the freezing and thawing processes, where they help preserve cellular integrity and viability. In serum-free media, the intrinsic protective effect provided by serum is absent; however, this loss can be effectively compensated for by the addition of cryoprotective agents such as hydroxyethyl starch (HES), Pluronic F68™, and methylcellulose.

The following media are suitable as freezing media

| Media Type | Composition |
|---|---|
| **Standard** | 90 % culture media +10 % DMSO |
| **Conditioned** | 45 % sterile filtered conditioned media / 45 % fresh media + 10 % DMSO |
| **Serum-Free** | 89% chemically defined, serum-free media, 10% DMSO, 1% Pluronic F68™ [5] |
| | Commercially available serum-free freezing media |

**Freezing Procedure**

- Remove all the culture media from the flask and wash the cells twice with warm PBS (w/o $Ca^{2+}$ and $Mg^{2+}$) at 0.1 mL/cm².
- Detach the cells with a suitable dissociation reagent (e.g., Trypsin, Trypsin-EDTA). Follow the protocol established in your laboratory. To prevent cell damage, avoid over-trypsinization, insufficient neutralization, and harsh mechanical handling during the detachment process.
- After addition of fresh media containing a dissociation inhibiting component, transfer the cells into a centrifuge tube.
- Resuspend the cells with fresh medium and determine the total cell count and viable cell count (viability).
- Calculate the required volume of freezing medium to achieve a final live cell count of 1 x $10^6$ - 1 x $10^7$ viable cells/mL
- Centrifuge the cells at 200 x g for 5 min.
- Remove the supernatant and gently resuspend the cells with the calculated volume of cold freezing medium.
- Aliquot the cell suspension into the appropriately labeled cryotubes.
- Freeze the cells at a controlled rate of -1 °C/min using programmable freezers or a freezing container.
- Store the freezing container overnight in an -80 °C freezer.
- The next day, transfer to a -152 °C freezer or the gas phase of liquid nitrogen ($LN_2$). Storage in the gas phase is possible and recommended for safety reasons [8]. Storage below **–130 °C** effectively stops all biological activity and is recommended for long-term preservation.
- Check the quality of the cryopreservation 24 hours after freezing by performing the first thawing control (vitality and sterility).

**Thawing of the Cells:**

- Fill a T-75 cm² flask with 14 mL of warm culture medium without antibiotics and prepare a centrifuge tube containing 10 mL warm medium.
- Remove a cryo tube from the $LN_2$ cry storage unit and immediately place it a 37°C warm water bath equilibrated to the culture temperature of the respective cell line.
- When ice crystals are no longer visible, transfer the contents of the cryo tube to the centrifuge tube using a sterile pipette.
  Centrifuge the cells at 200 x g for 5 minutes. Remove the supernatant to eliminate residual DMSO. Gently resuspend the cells in 1 mL fresh medium. Avoid foaming during resuspension, as this may result in poor and uneven cell attachment. Foaming can be minimized by maintaining a steady, gentle pipetting rhythm without rapid or abrupt movements.
- Transfer cells to the T-75 cm² flask and incubate for 24 hours.
- After 24 hours, check vitality, identity, and sterility. Perform periodic thawing controls to ensure bank stability.

**Literature**

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