Cryopreservation of Endothelial Cells in Suspension

Principle

An optimized protocol for the cryopreservation of endothelial cells that has been validated for human umbilical vein endothelial cells and porcine and human corneal endothelial cells providing post-thaw viabilities above 85% (Marquez-Curtis et al. 2016, Marquez-Curtis, McGann, and Elliott 2017, Sultani et al. 2016). Endothelial cells form the inner lining of blood vessels and other tissues and provide a selectively permeable barrier. Vascular endothelial cells play pivotal roles in hemostasis, coagulation, immune responses, and angiogenesis and are often used to study dysfunctions and pathologies such as atherosclerosis and thrombosis.

Equipment and Supplies

Equipment

1) Analytical balance (Mettler-Toledo)
2) Biological safety cabinet (Western Scientific Services, Ltd)
3) Pipet-aid and Pipettors (Eppendorf)
4) Phase-contrast microscope (Labovert, Leitz)
5) Coulter counter or hemocytometer
6) Centrifuge (Eppendorf 5810R tabletop centrifuge, Hamburg, Germany)
7) Programmable methanol cooling bath (FTS Systems, Stone Ridge, NY, USA), or, alternatively, −80°C freezer (Revco Ultima II)
8) T-type thermocouple and OMB-DAQ-55 data acquisition module (OMEGA Engineering Inc, Stamford, CT, USA)
9) Water bath (VWR)
10) Timer
11) Liquid nitrogen (LN₂) Dewar
Supplies

1) 50-ml conical centrifuge tubes (Corning, Inc)
2) Pipettes and tips (200 µl, 1000 µl, Eppendorf)
3) Borosilicate glass culture tubes (6 × 50 mm, VWR)
4) O-rings and corks (VWR)
5) Styrofoam floaters
6) Plastic cryovials (Nunc, ThermoFisher Scientific, Inc)
7) Dimethylsulfoxide (DMSO; Fisher Scientific)
8) Hydroxyethyl starch (HES, Bristol-Myers Squibb), or, alternatively, Pentastarch, a 20% w/v HES solution (Preservation Solutions, Inc)
9) Endothelial cell media (LONZA)
10) Methanol (Fisher Scientific)
11) Liquid nitrogen (Praxair)
12) Long metal forceps
13) Styrofoam buckets to hold icewater and LN₂

Safety

1) Exercise caution when handling LN₂. Use only special containers designed for cryobiological use, typically double-walled pressure vessels. Always use stable four-wheeled carts when moving containers. Styrofoam boxes must be constructed with suitable wall thickness and loosely covered to allow for increased pressure. Exposure to the extremely low LN₂ temperature (–196°C) can cause frostbite and tissue damage, as well as asphyxiation because nitrogen is able to displace the oxygen in the air. Wear thick insulated loose-fitting gloves, goggles and full face shield, lab gown, full-length pants, and closed-toed shoes when dispensing LN₂ from a tank or a Dewar. Experiments using LN₂ should be carried out in a well-ventilated area. In case of a spill or leak of LN₂, isolate the area and stop the source of the leak if possible. Ventilate area or move exposed personnel to fresh air. In case of skin contact, immerse skin in circulating warm water (37.8–43.3°C) but do not use dry heat.

2) Methanol is flammable and should be kept away from open flames or other ignition sources. Keep stock in a designated flammable solvents storage cabinet. Keep the bath covered when not in use. Methanol is toxic if swallowed, absorbed through the skin, or inhaled. Basic personal protective equipment (gloves, safety goggles, lab gown, full-length pants, and closed-toed shoes) is required. Wash with copious amounts of water in case of skin contact and move the affected person to a ventilated area in case of inhalation.

3) DMSO is a combustible liquid, which may cause skin, eye, and respiratory tract irritation. It readily penetrates the skin and may carry other dissolved
materials into the body. Wear basic personal protective equipment; wash with copious amounts of water in case of skin contact.

4) Glassware can cause cuts and lacerations when it breaks. Inspect glassware for cracks before use. Always wear gloves and safety goggles. Loosen corks to relieve pressure build-up when transferring glass tubes from LN₂ to the 37°C water bath, and carry out the procedure at arm's length, away from the face.

5) Water baths can get contaminated with bacteria and/or fungi. Add antibacterial and antifungal drops during routine lab maintenance and always wear gloves.

6) Methanol bath, water bath, and thermocouple are electrical equipment, which may cause shock to the user. Exercise electrical safety precautions.

Procedure

Cell Preparation

Note: Cells can be isolated from tissues or purchased. If purchased, the cells are obtained cryopreserved, thawed, and cultured according to the manufacturer's instructions using the recommended media and other reagents. Cells isolated from tissue are also typically cultured prior to cryopreservation. Follow culture confluency and passage recommendations according to cell type.

1) Ensure that cells have reached appropriate confluency by examining the cultures under a phase-contrast microscope before trypsinization.

2) Cryopreserve cells when they are at an appropriate early passage and have the typical morphology of healthy growing cells. Overgrown and late-passage cells approach senescence and are not recommended for cryopreservation.

3) Take an aliquot of the cell suspension and perform a cell count using a Coulter counter/cell analyzer or a hemocytometer. Dilute the cells to a density of 1–2 × 10⁶ cells/ml of media. If necessary, concentrate the cells by centrifugation at 200 × g for 5 min at room temperature, remove the supernatant, add the appropriate amount of media, and resuspend the cells by gently pipetting up and down.

4) The cell suspensions may be kept on icewater, to prevent cell clumping, for 2–3 h after passaging if needed.

Preparation of Cryoprotectant Solution

Note: DMSO and HES are prepared at twice the concentration of the final solution as they are diluted two-fold upon mixing with an equal volume of the cell suspension. Because volume measurements are temperature-dependent, the amounts of components of the cryoprotectant solution are weighed.
Using Powdered HES

1) Weigh 5 ml of media in a 50-ml conical tube.
2) Add approximately 0.6 ml DMSO (approximately 0.66 g, assuming a density of 1.1 g/ml at room temperature) and record the weight.
3) Add approximately 0.75 g HES.
4) Calculate the weight percentages of DMSO (should be close to 10%) and HES (should be close to 12%).
5) Immerse the tube in a 37°C water bath and swirl occasionally to allow the HES to dissolve. Once complete dissolution is achieved, transfer the tube to icewater.

Using Pentastarch Solution

1) HES supplied by Bristol-Myers Squibb and used in our references 1 and 2 has been discontinued. As an alternative, we have validated Pentastarch (20% w/v, Preservation Solutions, Inc (PSI), Elkhorn, WI, USA). To prepare 10% DMSO and 12% Pentastarch in media, measure 2 ml media, 0.6 ml DMSO, and 3.75 ml PSI Pentastarch solution, taking note of the weight after each addition. Mix well by gently pipetting up and down. Calculate % by weight composition.

Cryoprotectant Addition

1) Weigh an equal volume of cell suspension and cryoprotectant solution. Calculate the weight percent of DMSO (should be close to 5%) and HES (should be close to 6%) in the final mixture.
2) Mix the cells and cryoprotectants by pipetting gently up and down. Leave the cell–cryoprotectant mixture in icewater for 15 min to allow the DMSO to penetrate the cells.

Freezing

Controlled-rate Freezing with a Methanol Bath

1) Set the programmable methanol cooling bath to –5°C with a mixing speed of about 70 rpm and a cooling rate of 1°C/min and begin the temperature data acquisition program to record the actual temperature in the methanol bath. Allow sufficient time for the bath to reach –5°C.
2) Aliquot 200 µl of the cell–cryoprotectant mixture to the borosilicate glass culture tubes (with O-rings to allow them to sit on a Styrofoam floater in the methanol bath) and cover with corks.
3) Place the culture tubes in the methanol bath and allow equilibration at –5°C for 2 min.
4) Induce ice nucleation by touching the tubes with LN$_2$–cooled metal forceps. Allow the release of the latent heat of fusion by keeping at −5°C for 3 min.

5) Set the methanol bath temperature to −50°C and allow controlled cooling at 1°C/min until about −35°C. (See the following for alternative procedure.)

6) Once the desired temperature is attained, transfer the tubes to LN$_2$.

**Alternative Freezing Procedure**

1) In case a programmable methanol–cooling bath or controlled-rate freezer is unavailable, we suggest as an alternative cooling 1 ml cell suspensions in the presence of 5% DMSO and 6% HES in plastic cryovials (Nunc 1.8 ml CryoTube vials, ThermoFisher Scientific, Waltham, MA, USA) placed in a polystyrene tube holder and kept in a −80°C freezer. We have validated the cooling rate to be 1.4 ± 0.3°C/min. The next day the cryovials can be transferred to LN$_2$ for long-term storage. After at least 8 months of storage we found that the post-thaw viability was similar to those of samples in glass tubes, cooled at 1°C/min in a controlled-rate methanol bath, that were plunged into LN$_2$. Commercially available cooling containers, such as Mr. Frosty (ThermoFisher Scientific) and CoolCell containers (Corning, Inc, Corning, NY, USA), in combination with a dry ice locker, can also provide a cooling rate of approximately 1°C/min.

**Thawing**

1) Rapidly thaw the cells in a 37°C water bath until only a sliver of ice remains.

**Expected Results**

Post-thaw membrane integrity for human umbilical vein endothelial cells (HUVECs) should be 87.7 ± 0.8%, assessed by flow cytometric analysis, which was equivalent to 94.0% when normalized against fresh, unfrozen control cells. The HUVECs should also exhibit tube-forming ability, an *in vitro* assay for angiogenesis. The post-thaw membrane integrity for porcine corneal endothelial cells should be near 85%.

**References**
