Freezing Human Pluripotent Stem Cells in Feeder-Free Conditions

Purpose: This protocol describes the cryopreservation process for human pluripotent stem cells cultured on basement membrane extract such as Matrigel in hPSC Media such as mTeSR in a 6-well plate format.

Materials:

- Human pluripotent stem cells-80-90% confluent
- Cryopreservation Media: 50% hPSC media, 30% KOSR, 20% DMSO
- 30% KOSR: 70% hPSC media, 30% KOSR
- Cell dissociation reagent such as CellStripper (Corning), Versene (Gibco), or ReleSR (Stem Cell Technologies)
- DPBS(-/-)
- DMEM-F12
- Labeled Cryovials
- 15 and 50mL tubes

Procedure:

1. Mark areas of differentiation using the objective marker or pen.
2. Aspirate off the spent media and aspirate marked areas of differentiation in the well using the aspirating tip. Remember to change the tip between different cell lines to avoid cross contamination.
3. Wash each well with 1 mL DPBS.
4. Add 1 mL of room temperature dissociation regent to each well and incubate at 37°C for 4-5 minutes. Incubation times will vary based on cell density. (if using ReLeSR, aspirate immediately and incubate at RT)
5. After incubation, check the cells under a microscope. Cells should appear shiny and separated with small gaps or holes appearing inside colonies. Cells should still be attached to the surface. In some cases, colonies will have slightly curled edges and minimal amounts of floating cells/debris will be visible.
6. When cells appear as mentioned in step 6, gently aspirate CellStripper/Versene EDTA from the well without disturbing the loosely attached cells.
7. Add 1-1.5mL of 30/70 KOSR/hPSC to each well and gently pipette to remove cells from the growth surface. Adjust volumes for number of vials to generate, 0.5mL/vial. Leave cells in well unless pooling many wells.
8. Add equal volume of cryopreservation media to cell solution, gently mix well. The final DMSO concentration will be 10%.
9. Aliquot 1mL of cell/ suspension into labeled cryovials.
10. Cap cryovials and transfer to a Styrofoam freezing rack.
11. Place Styrofoam rack in -80C freezer overnight. Transfer to liquid nitrogen cryostorage the following day. Foam freezing racks and similar cell freezing containers (like Nalgene's Mr. Frosty) allow for a continuous cooling rate of -1C/min. Slow continuous cooling in combination with cyroprotectants (DMSO) prevents the formation of cell damaging ice crystals.

Troubleshooting: -80C freezers are not suitable long-term storage solutions for frozen cell stocks. Prolonged storage at -80C will result in reduced cell viability. Transfer frozen cell stocks to a liquid nitrogen cryostorage system within 1 week. Always map the box position of cell stocks when transferring to liquid nitrogen storage,