Cryopreservation of hPSCs by Vitrification
Brigitte Arduini, version 1, 2013-Feb-14
Modified from Scott Noggle, 2007-Jan-30

Optimal methods for cryopreservation may vary from cell line to cell line. Traditional methods (such as growth medium with serum and DMSO) do not work well for RUES hESC lines, with survival typically at less than 1%. The following procedure is optimized from a protocol for vitrification (Richards et al., 2004), and uses cryovials instead of straws for faster processing times. Recovery using this method is typically 40 – 50%. We have found that other hPSC lines, including WiCell and iPSC lines, can also be successfully preserved using this method.

Materials:

<table>
<thead>
<tr>
<th>Item</th>
<th>Vendor</th>
<th>Catalog No.</th>
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<tbody>
<tr>
<td>Growth Medium</td>
<td></td>
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<tr>
<td>Ethylene glycol</td>
<td>Sigma</td>
<td>102466</td>
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<tr>
<td>DMSO</td>
<td>Sigma</td>
<td>D2650</td>
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<tr>
<td>Sucrose</td>
<td>Sigma</td>
<td>S7903</td>
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<tr>
<td>1M HEPES solution</td>
<td>Life Technologies</td>
<td>15630</td>
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<tr>
<td>Cryovials, internal threads</td>
<td>VWR (Nunc)</td>
<td>66021-986</td>
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<td>Cryo storage box</td>
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<tr>
<td>Liquid Nitrogen in an ice bucket</td>
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FREEZING CELLS

Prepare Media
Prepare all media fresh, sterile filter, and maintain on ice while working.

Note: ethylene glycol and DMSO become very viscous when combined with sucrose solution. It is easier to combine these two reagents (1:1) and filter before adding other components. DO NOT filter these reagents individually.

HM (Growth Medium + 20mM HEPES)
80% Growth Medium (ex: MEF-CM or Essential 8)
20mM HEPES

HM+Sucrose
3.42g sucrose in 10ml HM
VS2
30% HM
30% HM+Sucrose
20% ethylene glycol
20% DMSO

VS3
40% HM+Sucrose
30% ethylene glycol
30% DMSO

Freezing Protocol

Work quickly. hPSCs cannot be exposed to the cryoprotectants for very long or they will die/differentiate upon thawing. Steps 5-7 must be timed accurately.

1. Place a cryo storage box in 1-2 inches of LN₂ in a large rectangular ice bucket, within easy reach of the biosafety cabinet.
2. Harvest hPSCs in clumps by manual dissection or collagenase/dispase treatment.
3. Centrifuge at 200 rcf, remove supernatant, and resuspend clumps in HM. Cells can be kept at room temperature in HM for up to 20 minutes. Prolonged incubation will result in clumping and reduced attachment after thawing.
4. Transfer 40µl of cells per sterile cryovial at room temperature.

**Steps 5 – 7 must be completed in no more than 1 minute. Handle only as many tubes as can be processed in this amount of time (typically two vials). Remember that handling and capping tubes will require some time.

5. Processing 2 cryovials at a time, add 40µl of VS2 and mix by gentle pipetting.
6. Add 160µl VS3 and mix by gentle pipetting.
7. Submerge the tubes quickly in LN₂ and swirl while freezing. The frozen solution should have a pink glass-like appearance, while a thin layer on the top might be opaque. Be sure caps are tightened and transfer vials to LN₂ storage boxes.
THAWING CELLS

Prepare Media & Plates

WS3
Growth medium + 1M sucrose
Prepare at room temperature and vortex periodically to completely dissolve sucrose. Filter sterilize and store on ice during the procedure.

Growth medium
Pre-warm desired growth medium to room temperature: 12ml per cell line to be thawed.

35mm dish per cell line to be thawed (with MEFs, Matrigel or other desired substrate)

Thawing Protocol

Steps 1-3 must be performed quickly so that cells are not exposed to high concentrations of cryovials for too long. The incubation times in steps 4-7 remove sucrose slowly and prevent osmotic shock and lysis of the cells.

1. Remove a tube from LN2 and quickly submerge bottom of tube in warm sterile water.
2. Quickly wipe with 70% EtOH-soaked kimwipe.
3. Immediately add 800µl of cold WS3, mix gently by stirring with the pipette tip, and let sit for 30 seconds.
4. Add 1ml of growth medium, mix as above, let sit for 2 minutes.
5. Transfer to 15ml conical tube.
6. Rinse cryotube two times with 1ml each of growth medium and add to 15ml tube, mixing gently. Let sit for 1 minute.
7. Add 6ml growth medium, slowly dropwise to cells over about 2 minutes.
8. Spin at 200 rcf for 4 minutes.
9. Resuspend gently in 1 ml of growth medium with a p1000.
10. Using a p1000, transfer to a 35mm plate containing 1ml of growth medium. Colonies should recover and show signs of growth within one week, and be ready for passage between 7 and 14 days. Change medium daily.