Humboldt-CIRM Shared Resource Lab	STANDARD OPERATING PROCEDURE

Title: Thawing human pluripotent stem cells
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Media and Supplies:

Item	Purpose	Storage	Catalog Number	Supplier
mTeSR Plus	Primary maintenance culture media	2–8°C	MSPP-100-0276	VWR Funding Inc
Advanced DMEM/F12 (ADF)	Washing media to minimize osmotic shock	2–8°C	12634010	Fisher Scientific
KnockOut DMEM	Diluent for Matrigel and washing	2–8°C	10829018	Fisher Scientific
ROCK Inhibitor (Ri)	Y-27632; critical for post-thaw survival	-20°C	101763-964	VWR Funding Inc
Pen/Strep (Optional)	Antibiotic for contamination prevention	-20°C	15-140-122	Fisher Scientific
70% EtOH	Sanitization of vials and surfaces	RT	—	—
Conical Tubes	Centrifugation and media preparation	Sterile	21008-936	VWR Funding Inc
Serological Pipettes	Sterile liquid handling	RT	76184-746	VWR Funding Inc
Cooling block at -80	Transfer of cells from LN2 to 37°C			

Thawing hPSCs:

This protocol describes the thawing of human pluripotent stem cells (hPSCs) from cryopreservation. Successful thawing is critical as improper handling during the transition from liquid nitrogen to culture is a major stress point that can result in increased cell loss, genetic drift, and selection for abnormal cells [1,2]. **Note:** In this protocol 6-well plates are being used. If alternative culture vessels are used, volumes of reagents will accordingly require adjustment (for information about approximate volume conversions click here.)

1. **Preparation:** Consult the **Matrigel – Aliquoting, Thawing, and Coating** protocol to prepare your culture vessels. For every vial ($1x10^6$ cells), coat two wells of a 6-well plate.

   Ensure mTeSR Plus complete medium is prepared (Pen/Strep is optional) before proceeding. While Matrigel-coating is incubating, dispense 10 ml of Advanced DMEM/F12 into one 15 ml tube and 4 ml of mTeSR Plus with ROCK Inhibitor (10 µM; 1:1000) into another. Let media reach room temperature on the benchtop. Do not use a 37°C water bath.

2. **Thaw:** Retrieve one vial of frozen cells from liquid nitrogen and place **immediately** into a pre-frozen cold block (stored at -80°C) or a bucket of dry ice. Thaw at 37°C in a water or bead bath, by gently swirling until only a small ice pellet remains **Note:** Keep the cap-tube interface above the water level to minimize contamination risk. Remove vial from the bath, spray thoroughly with 70% EtOH, wipe dry, and place in a suitable rack in the TC hood.

3. **Wash:** Uncap the lid of the cryovial and use a p1000 pipette to transfer 1 ml of the warmed Advanced DMEM/F12 to the cryovial. Next, ***very gently*** pipette the cell suspension up and down a couple of times before ***slowly*** transferring the mixture ***dropwise*** back to the tube of ADF in order to minimize osmotic shock.

4. **Centrifuge:** Pellet cells by spinning for 5 minutes at 200 x g (RCF).

5. **Plating:** Aspirate supernatant without disturbing the pellet. Next, tap the bottom of the tube several times in order to break up the cell pellet, and then **gently** resuspend in 4 ml of mTeSR Plus + 10 µM ROCK inhibitor. **Note:** The addition of ROCK inhibitor at this step is critical for post-thaw viability [3,4]. Next, aspirate the Matrigel from two prepared wells and ***immediately*** dispense 2 ml of cell suspension into each - do not allow the wells to dry, as this compromises substrate integrity and cell attachment.

6. **Incubation:** place the plate in the incubator on the shelf. While maintaining contact between the plate and the shelf, in a brisk manner, move the plate forward-backwards (3-5 times) and then side-to-side (3-5 times), in order to disperse the clumps evenly throughout the well. Leave plate undisturbed overnight before moving. **Note:** Some groups perform this entire sequence (forward-backwards and side-to-side) directly in the TC hood and then leave the plate undisturbed for roughly 30 minutes before carefully transferring to the incubator to prevent convection currents from causing uneven settling.

7. **Removing ROCK inhibitor:** The following day replace medium with fresh mTeSR Plus without ROCK inhibitor. **Note:** removal is critical because prolonged exposure can cause abnormal morphology, metabolic stress, and spontaneous differentiation [5-7].

Further Reading:

1. Baker DE, et al. (2007). Nat Biotechnol. 25(2): 207–215. doi: 10.1038/nbt1285
2. International Stem Cell Initiative. (2011). Nat Biotechnol. 29(12): 1132–1144. doi: 10.1038/nbt2051
3. Watanabe K, et al. (2007). Nat Biotechnol. 25(6): 681–686. doi: 10.1038/nbt1311
4. Ohgushi M, et al. (2010). Cell Stem Cell. 7(2): 225–239. doi: 10.1016/j.stem.2010.05.019
5. Vernardis SI, et al. (2017). Sci Rep. 7: 42138. doi: 10.1038/srep42138
6. Alasmar S, et al. (2023). Stem Cells. 41(11): 1006–1021. doi: 10.1093/stmcls/sxad059
7. Matsumoto T, et al. (2022). Bioengineering. 9(11): 613. doi: 10.3390/bioengineering9110613