Humboldt-CIRM Shared Resource Lab	STANDARD OPERATING PROCEDURE

Title: iPSC Maintenance and Cryopreservation
Purpose: To describe standard procedure for thawing, maintaining, and freezing iPSCs
Version: 1
Last Updated: 20260218
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Introduction

This Standard Operating Procedure outlines the protocols for thawing, maintaining, and cryopreserving human pluripotent stem cells. The document is organized chronologically, though each protocol may be used individually as needed. In protocol we use mTeSR Plus medium for expansion and hESC-qualified Matrigel for surface coating. Instructions cover clump passaging using ReLeSR for routine maintenance and single-cell dissociation for precise seeding or cryopreservation. The cryopreservation protocol is optimized for CryoStor CS10 to ensure high cell viability during storage in liquid nitrogen.

Aliquoting, Diluting, and Coating with hESC-Qualified Matrigel

Materials and Supplies

Item	Purpose	Storage	Cat #
Matrigel (hESC-Qualified)	Basement membrane	-20°C	Corning 354277
KnockOut DMEM	Diluent for Matrigel aliquots	2-8°C	Thermo 10829018
1.5 ml Tubes	Storage of aliquots	RT	
Filter Pipette Tips	Liquid handling	RT	
Cooling Block	Maintaining low temperature	RT / -80°C	
Bucket of Ice	Prepared bed of crushed ice for handling		

Aliquoting Matrigel

This protocol is designed specifically for the standard expansion and maintenance of undifferentiated hPSCs using hESC-Qualified Matrigel. Matrigel was originally derived from the Engelbreth-Holm-Swarm (EHS) mouse sarcoma. While the tumor's ability to produce a basement membrane was first described by Orkin, Martin, and colleagues in 1977 [(1)], the matrix was later developed into the standardized laboratory tool known as Matrigel by Hynda Kleinman and her team at the NIH [(2)]. **Note:** If you will be transitioning from expansion to differentiation, consider switching to Growth Factor Reduced (GFR) Matrigel which contains reduced concentrations of growth factors (e.g., TGFβ and EGF) that otherwise might affect the differentiation [(3-5)].

1. **Thawing:** Thaw a bottle of Matrigel overnight on ice in the back of a 4°C refrigerator, in order to prevent exposure to temperature fluctuations due to opening and closing.

2. **Calculating aliquot volumes:** check the lot-specific Certificate of Analysis (CoA) for the dilution volume. This is the amount of Matrigel required for 25 mL of KnockOut (KO) DMEM. (Lot-specific dilution). **Note:** For lower usage rates, you can pipette 50% of the CoA volume per tube to be suspended in 12.5 mL of KO DMEM (e.g., if the lot-specific volume is 270 µL, pipette 135 µL per tube) and then resuspending that half-aliquot in 12.5 mL of KO DMEM.

3. **Pre-Chill Plasticware:** Place the predetermined number of sterile 1.5 ml microcentrifuge tubes in a cooling block, along with the appropriate pipette tips, the in the -80°C freezer for at least 1 hour before use.

4. **Remove chilled materials**:

    a. Transfer the pre-chilled cooling block (with tubes) onto a prepared bed of crushed ice inside the TC hood. Sanitize both the block and the ice bed with 70% EtOH, wipe dry, and then open the tube lids.

    b. Remove the Matrigel from the 4°C, spray with 70% EtOH, wipe dry. Carefully remove the metal band and stopper, then place the bottle securely into the ice bucket in a stable location.

    c. Remove chilled tip box from the -80°C, spray with 70% EtOH, place in the ice box.

5. **Aliquot:** Immediately begin to aliquot the Matrigel into the pre-chilled tubes, aiming to complete the entire process within 5 -10 minutes. Use a new pipette tip every few aliquots as they will begin to warm and can cause the Matrigel to solidify. Close the lids.

6. **Storage:** Upon completion, immediately transfer the aliquots to a -80 (preferably towards the rear) avoid repeated freeze-thaw cycles.

Dilution and Coating

1. **Thawing**: Remove one aliquot of Matrigel from the -80°C freezer, immediately place on ice, and into the back of a 4°C refrigerator to thaw overnight.

2. **Resuspension:** Once thawed, spray the aliquot with 70% EtOH, dry the tube and then immediately resuspend the Matrigel aliquot into an appropriate, predetermined volume of cold KnockOut DMEM.

3. **Coating:** Add 1 ml of the solution per well of a 6-well plate. It is critical to make sure that the entire surface area of the well or vessel is covered. Different volumes will be required for different culture vessels (for information about approximate volume conversions click here). Any remaining Matrigel/KO-DMEM solution can be stored for up to 2 weeks in the back of the 4°C refrigerator.

4. **Incubation:** Incubate the coated dish at 37°C for ≥ 1 hour or overnight at 4°C, before use.

Troubleshooting:

Problem	Possible Cause	Solution
Matrigel is solid	It has been allowed to warm	Order a new bottle
Tips are clogging or Matrigel is solidifying	Tips or Matrigel are warming to room temperature.	Change pipette tips every few aliquots; keep the Matrigel bottle securely in a stable ice bucket.
Well surface is not fully coated	Insufficient volume or the solution was not spread evenly.	Ensure the entire surface area of the vessel is covered. Use 1 ml of solution per well for a 6-well plate. Discard any plate not fully coated.
Poor cell attachment	Well surface not fully coated, Matrigel is old, dried out, or improperly stored.	Ensure coated plates are used within 2 weeks. Seal plates (e.g., with Parafilm) to prevent evaporation during 4°C storage.

Further Reading:

1. Orkin RW, et al. (1977). J Exp Med. 145(1): 204–220. doi: 10.1084/jem.145.1.204
2. Kleinman HK, et al. (1986). Biochemistry. 25(2): 312–318. doi: 10.1021/bi00350a005
3. Vukicevic S, et al. (1992). Exp Cell Res. 202(1): 1–8. doi: 10.1016/0014-4827(92)90397-q
4. Hughes CS, et al. (2010). Proteomics. 10(9): 1886–1890. doi: 10.1002/pmic.200900758
5. Waxman EA, et al. (2023). Curr Protoc. 3(2): e681. doi: 10.1002/cpz1.681

Thawing human pluripotent stem cells

Media and Supplies:

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

Clump passaging of hPSCs using ReLeSR

Media and Supplies

Item	Purpose	Storage	Catalog Number	Supplier
mTeSR Plus	Primary maintenance culture media	2–8°C	100-0276	VWR
ReLeSR	Clump-based, non-enzymatic dissociation reagent	RT	100-0484	VWR
Matrigel (hESC-Qualified)	Basement membrane matrix	-20°C	BD354277	VWR
DPBS (no Ca^{2+}, no Mg^{2+})	Ca2+/Mg2+ free wash buffer	RT	D8537-500ML	Sigma-Aldrich Inc
ROCK Inhibitor (Ri)	Optional: survival support if too many single cells are generated	-20°C	101763-964	VWR
Advanced DMEM/F12 (ADF)	Washing/quenching medium	2–8°C	12634010	Fisher Scientific
Penicillin-Streptomycin	Optional antibiotic for contamination prevention	-20°C	15-140-122	Fisher Scientific
70% Ethanol (EtOH)	Sanitization of surfaces and materials	RT	—	—
6-well TC Plates	Multiwell culture plates (Advanced TC treated)	RT	89131-688	VWR
Conical Tubes	Centrifugation and media preparation	Sterile	21008-936	VWR
Serological Pipettes	Sterile liquid handling	RT	76184-746	VWR
PPE (Gloves)	Nitrile Exam Gloves	RT	99452683	Fastenal
Permanent Marker	Labeling culture vessels and tubes	RT	—	—

Clump Passaging using ReLeSR

Once wells reach 70–80% confluency, they can be passaged for expansion or other downstream applications e.g., generating embryoid bodies. Clump passaging is generally recommended over single-cell dissociation, as it lowers the vulnerability to apoptosis and subsequent genetic drift [1-3] while circumventing the necessity for ROCK inhibitor supplementation. While 0.5 mM EDTA is a common alternative for clump passaging, this protocol relies on ReLeSR for its ability to selectively detach undifferentiated colonies while leaving differentiated cells behind.

1. **Preparation:** Determine the optimal split ratio and coat the appropriate number of wells with Matrigel at 37°C for ≥ 1 hour (See: **Matrigel – Aliquoting, Thawing, and Coating**). Generally, a 1:6 split is recommended for standard maintenance. Calculate the total volume of mTeSR Plus required based on 2 ml per well (e.g., 12 ml for an entire 6-well plate), place this volume into a 50 ml falcon tube, and allow it to reach room temperature (do not warm it in a 37°C incubator). **Note:** the split ratio can be adjusted higher (e.g., 1:10) if the culture is very confluent or lower (e.g., 1:4) if growth is slow, depending on your desired timeline.

2. **ReLeSR Treatment:** Wash each well 2x with 1-2 ml DPBS (no Ca2+, no Mg2+). Add enough ReLeSR to entirely cover the bottom of the well (e.g., 1 ml per well of a 6-well plate) then immediately and completely aspirate the ReLeSR, leaving only residual liquid.

3. **Incubation:** Incubate at 37°C for roughly 5-7 minutes. Colonies should begin to ball up or detach entirely while differentiated cells remain. **Note:** the exact duration of incubation is less important than what is physically happening with the colonies themselves, and this will vary depending on numerous factors e.g., cell line, density, passage number, DIV, etc. Therefore, it is **critical to observe the cells** every couple of minutes to identify the time window in which healthy colonies are lifting while any potential differentiated cells remain attached.

4. **Detachment:** Add exactly 1 ml of mTeSR Plus to each individual well. Firmly tap each side of the plate for a total of 30–60 seconds to detach the colonies. Once detached, gently transfer the 1 ml suspension from each well into its own fresh well; do not mix the contents of different wells.

   Gently and uniformly dissociate the suspension by pipetting the entire volume up and down to break the colonies into clumps of approximately 15–40 cells. Maintaining a uniform clump size is critical to reduce the potential for non-

specific differentiation. Ensure you pipette the full volume of media and cells with a consistent, uniform force with each stroke to achieve maximum consistency. Monitor the aggregate size under the microscope after every 1 or 2 pipette strokes to ensure the desired clump size is obtained.

5. **Seed:** Dilute clumps in volume of mTeSR Plus, ***without Rock inhibito***r. Aspirate/remove Matrigel from fresh wells, and plate. **Note:** If there is a large proportion of single cells after detachment/dissociation, then it may be necessary to culture cells in the presence of Rock inhibitor (no longer than 24 hours). To prevent over dissociation in the future it may be necessary to incubate in ReLeSR for less time, or to break apart colonies with less force or frequency.

6. **Plating & Incubation:** Next, 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.

Troubleshooting

Problem	Possible Cause	Solution
Colonies remain attached / hard to dislodge	Insufficient incubation time with ReLeSR.	Increase incubation time by 1–2 minutes.
Aggregates are too large	Insufficient pipetting	Pipette cells up and down a few more times and with more force. Check using the microscope.
Aggregates are too small. Many single cells generated	Over-incubation or excessive pipetting.	Decrease incubation time by 1–2 minutes. Minimize manipulation after dissociation.
Differentiated cells detaching with colonies	Incubation is too long or temperature is too high.	Decrease incubation time by 1–2 minutes. Decrease incubation temperature to room temperature (15–25°C).
Non-uniform attachment	Clumps were not evenly distributed during plating.	Move the plate in brisk side-to-side and forward-backward motions (avoid circular motions) before settling.

Further Reading:

1. Beers, J., Gulbranson, D.R., George, N., Siniscalchi, L.I., Jones, J., Thomson, J.A. and Chen, G., 2012. Passaging and colony expansion of human pluripotent stem cells by enzyme-free dissociation in chemically defined culture conditions. *Nature protocols*, *7*(11), pp.2029-2040.
2. Bai, Q., Ramirez, J.M., Becker, F., Pantesco, V., Lavabre-Bertrand, T., Hovatta, O., Lemaitre, J.M., Pellestor, F. and De Vos, J., 2015. Temporal analysis of genome alterations induced by single-cell passaging in human embryonic stem cells. *Stem cells and development*, *24*(5), pp.653-662.
3. Nguyen, H.T., Geens, M., Mertzanidou, A., Jacobs, K., Heirman, C., Breckpot, K. and Spits, C., 2014. Gain of 20q11. 21 in human embryonic stem cells improves cell survival by increased expression of Bcl-xL. *Molecular human reproduction*, *20*(2), pp.168-177.

Single-Cell Passaging using Accutase

Media and Supplies

Item	Purpose	Storage	Catalog Number	Supplier
mTeSR Plus	Primary maintenance culture media	2–8°C	100-0276	VWR
Accutase	Gentle single-cell dissociation enzyme	-20°C	10761-312	VWR
Matrigel (hESC-Qual)	Basement membrane matrix	-20°C	BD354277	VWR
ROCK Inhibitor (Ri)	Y-27632; prevents apoptosis in single cells	-20°C	101763-964	VWR
DPBS (no Ca^{2+}, no Mg^{2+})	wash buffer	RT	D8537-500ML	Sigma
Advanced DMEM/F12	Washing/quenching medium	2–8°C	12634010	Fisher
Pen/Strep (Optional)	Antibiotic for contamination prevention	-20°C	15-140-122	Fisher
70% Ethanol (EtOH)	Sanitization of surfaces and materials	RT	—	—
6-well TC Plates	Multiwell plates (Advanced TC treated)	RT	89131-688	VWR
Conical Tubes				
Serological Pipettes				
Pipette Controller				
Inverted Microscope				

Single-Cell Passaging using Accutase

Once wells reach 70–80% confluency, they are ready for single-cell dissociation. This method is necessary for applications requiring precise cell counts, such as cryopreservation or seeding specific densities. We prefer to use Accutase as it is very gentle [1], other options e.g., recombinant trypsin alternatives are also used and are more cost effective [2].

Preparation: coat the appropriate number of wells with Matrigel at 37°C for ≥ 1 hour. Prepare the required volume of plating medium by adding ROCK inhibitor to mTeSR Plus to a final concentration of 10 µM and warm to room temperature. Accutase should be thawed in the 4°C and applied to the at this temp as well i.e., no need to warm the Accutase (**Note:** Accutase should be stored in single-use aliquots at -20°C to avoid repeated freeze-thaw cycles which degrade enzymatic activity.

1. **Wash:** Completely aspirate the media from the wells and wash 2x with 1 ml DPBS (no Ca2+, no Mg2+).

2. **Dissociate:** Add 1 ml of Accutase per well and incubate at 37°C for 5-10 minutes (Accutase is very gentle – do not rush this step, let the enzyme do its job). Firmly tap the side of the plate to detach cells.

3. **Quench:** *Gently* pipette the suspension 5 times to singularize the cell and then transfer the cell suspension to a conical tube containing 9 ml of Advanced DMEM/F12 to rinse the wells and dilute the Accutase.

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

5. **Resuspend:** Aspirate the supernatant without disturbing the pellet. Tap the bottom of the tube firmly to flick-loosen the pellet then resuspend the cells in the required volume of mTeSR Plus + 10 µM ROCK inhibitor **Note:** ROCK inhibition is essential during single-cell dissociation to prevent apoptosis and ensure post-passaging viability and attachment [3,4].

6. **Plating:** Aspirate the Matrigel from fresh wells and **immediately** dispense **2 ml of the cell suspension** into each. Move quickly and do not allow the coated surface to dry out, as this compromises substrate integrity and cell attachment.

7. **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 (3-5x) and then side-to-side (3-5x) to disperse the cells evenly. Leave the plate undisturbed overnight. **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 20 minutes before carefully transferring to the incubator to prevent convection currents from causing uneven settling.

8. **Recovery:** The following day replace the medium with **2 ml of fresh mTeSR Plus** (without ROCK inhibitor). **Note:** Removal of ROCK inhibitor is critical because prolonged exposure can cause abnormal morphology, metabolic stress, and spontaneous differentiation, compromising iPSC pluripotency [5-7].

Troubleshooting

Problem	Possible Cause	Solution
Cells remain attached after 10 min	Insufficient incubation, insufficient rinsing, and/or degraded Accutase.	Increase incubation time up to 30 min. Remove Accutase from well, rinse 2x with DPBS (no Ca2+, no Mg2+) add a new ml of Accutase. Use new bottle or thaw a new aliquot.
High cell death / low viability	Mechanical stress due to over pipetting or pipetting before the cells have detached	Pipette gently and only after cells detach with gentle tapping.
Cell suspension is sticky or stringy	Lysed cells and leaked genomic DNA	Add 1-2 drops of a 1mg/ml stock solution of DNase I to the 10 ml cell suspension (Cells/Accutase/ADF). Incubate at 37°C for 5 – 10 min. Do not vortex.

Further Reading:

1. **Bajpai, R.** et al. (2008) 'Efficient propagation of single cells accutase-dissociated human embryonic stem cells', *Molecular Reproduction and Development*, 75(5), pp. 818–827. doi: 10.1002/mrd.20819.
2. **Ellerström, C.** et al. (2007) 'Facilitated expansion of human embryonic stem cells by single-cell enzymatic dissociation', *Stem Cells*, 25(7), pp. 1690–1696. doi: 10.1634/stemcells.2006-0590.
3. **Watanabe, K. et al.** (2007) 'A ROCK inhibitor permits survival of dissociated human embryonic stem cells', *Nature Biotechnology*, 25(6), pp. 681–686. doi: 10.1038/nbt1311.
4. **Ohgushi, M. et al.** (2010) 'Molecular Pathway and Cell State Responsible for Dissociation-Induced Apoptosis of Human Pluripotent Stem Cells', *Cell Stem Cell*, 7(2), pp. 225–239. doi: 10.1016/j.stem.2010.05.019.
5. **Vernardis, S. I. et al.** (2017) 'Mass spectrometry-based proteomics reveals that cell-cell adhesion is a key determinant of human pluripotent stem cell survival', *Scientific Reports*, 7, p. 42138. doi: 10.1038/srep42138.
6. **Alasmar, S. et al.** (2023) 'Human pluripotent stem cell-derived limbal epithelial stem cells: a review of current methods and future perspectives', *Stem Cells*, 41(11), pp. 1006–1021. doi: 10.1093/stmcls/sxad059.
7. **Matsumoto, T. et al.** (2022) 'Advanced Culture Systems for Mammalian Cells', *Bioengineering*, 9(11), p. 613. doi: 10.3390/bioengineering9110613.

hPSC Maintenance

Media and Supplies

Item	Purpose	Storage	Catalog Number	Supplier
mTeSR Plus	Primary maintenance culture media	2–8°C	1000276	STEMCELL
70% Ethanol (EtOH)	Sanitization of surfaces and materials	Room Temp		
Pen-Strep	Optional antibiotic for contamination prevention.	-20°C.	15-140-122.	Fisher
Conical Tubes	Aliquoting and warming media	Sterile	21008-936	VWR
6-well TC Plates	Advanced TC treated culture vessels.	Room temperature.	89131-688 (CS).	VWR
Serological Pipettes	Sterile liquid handling (10 ml)	Room Temp	76184-746	VWR
Inverted Microscope	Culture assessment (4x–20x)	Room Temp	LMI3PH2	Life Technologies
PPE (Gloves)	Nitrile Exam Gloves	Room Temp	99452683	Fastenal
Permanent Marker	Labeling culture vessels			

Maintenance of Cells

This protocol uses mTeSR Plus for the standard expansion and maintenance of undifferentiated hPSCs. This medium was selected as it is the most widely used in the field, it is easy to use and highly robust, and it contains stabilized FGF2 that allows for weekend-free feeding schedule. While alternative media may be substituted, these may require further optimization to ensure comparable results. Before beginning, ensure the mTeSR™ Plus medium has been previously prepared with supplement according to the manufacturer's instructions. Official Technical Manual: Maintenance of Human Pluripotent Stem Cells in mTeSR™ Plus (See Section 6.0 for flexible feeding and weekend-free schedules).

Assessment (Daily): Evaluate culture health and confluency daily under a microscope (4x–20x magnification). Look for healthy, undifferentiated colonies characterized by highly compressed cells with a high nuclear-to-cytoplasmic ratio and clearly defined, smooth colony borders. Cultures require feeding if <70% confluent and are ready for passaging once they reach 70–80% confluency.

Preparation (Feeding/Passage Days Only): On scheduled maintenance days (Mon/Wed/Fri), calculate the total volume required for your specific vessel and aliquot this amount into a sterile conical tube (e.g., a 15 mL or 50 mL tube). Allow the medium to warm to room temperature.

Media Exchange and Incubation: When performing media changes, leave a small amount (100–200 µL) of conditioned medium in the well. This prevents the cell surface from drying out and maintains a baseline level of paracrine signaling factors. Add fresh, room-temperature mTeSR Plus and return the plate to a 37°C incubator.

Weekly Maintenance Schedule (for weekend-free culture):
To maintain healthy, undifferentiated cultures, follow the routine below using volumes specified for a 6-well plate (refer to Link for other vessels). **Note**: cell cultures are dynamic - if a weekend-free schedule becomes impractical e.g., due to specific experimental needs, rapid growth rates, or cell death, resume a standard every-other-day feeding regimen using 2.0 ml of medium.

1. **Monday Morning**: Perform a full media change (2.0 mL per well) if cells were passaged the previous Friday.
2. **Wednesday**: Perform a full media change (2.0 mL per well).

3. **Friday Late Afternoon Assessment:** Evaluate culture confluency. If confluency is low < 50%, you may proceed with feeding only (Option A). If cells are ≥ 50% confluent or rapidly growing, you must passage (Option B).

   Option A: Feeding Only. To skip two consecutive days (Saturday/Sunday), perform full media change, however, replace spent media with double the standard media volume (4.0 ml per well of a 6-well).

   Option B: Passaging. Perform clump passaging with ReLeSR and seed aggregates at a low-density (e.g., 1:10 split) into 4.0 ml of room temperature mTeSR Plus (no ROCK inhibitor). The double media volume and low-density split allows the culture to remain unattended until Monday.

   Note: This weekend-free schedule applies only to clump passaging. Single-cell dissociation requires a Saturday media change to remove ROCK inhibitor. Do not attempt a weekend skip with single-cell cultures.

Standard Maintenance Schedule (feeding on alternate days): Use this regimen if the weekend-free protocol is not utilized or if rapid growth prevents a two-day skip. Replace the medium with 2 ml of fresh mTeSR Plus every other day until the culture reaches 70–80% confluency. While cells can be fed at intervals shorter than 48 hours, they must never exceed the 48-hour window. For routine maintenance and expansion, use the clump passaging method with ReLeSR.

Cryopreservation of hPSCs using CryoStor CS10

Media and Supplies

Item	Purpose	Storage	Catalog Number	Supplier
CryoStor CS10	Specialized protein-free cryopreservation medium	2–8°C	07930	STEMCELL
Accutase	Gentle enzymatic dissociation into single cells	-20°C (Aliquots)	10761-312	VWR
mTeSR Plus	Primary culture medium for preparation and recovery	2–8°C	100-0276	STEMCELL
DPBS (-/-)	Ca2+/Mg2+ free wash buffer	RT	D8537-500ml	Sigma
Advanced DMEM/F12	Washing and quenching dissociation enzymes	2–8°C	12634010	Fisher
Penicillin-Streptomycin	Optional antibiotic	-20°C	15-140-122	Fisher
Trypan Blue (0.4%)	Cell viability counting	RT	15250061	Thermo
Cryovials	2 ml sterile vials for long-term storage	RT	66021-987	VWR
15 ml Conical Tubes	Centrifugation and cell collection	Sterile	21008-936	VWR
50 ml Conical Tubes	Pooling large volumes of cell suspension	Sterile	21008-940	VWR
Serological Pipettes	Sterile liquid handling (5 ml or 10 ml)	RT	76184-746	VWR
Pipette Controller	For use with serological pipettes	RT	13-681-161	Fisher
Controlled Rate Freezer	Isopropanol container (e.g., "Mr. Frosty")	RT	5100-0001	Thermo
Countess Cell Counter	Automated viable cell counting	RT	A50298	Fisher
Inverted Microscope	Culture assessment before harvest (EVOS)	RT	LMI3PH2	Life Tech
Nitrile Exam Gloves	Personal safety	RT	99452683	Fastenal
Permanent Marker	Labeling cryovials with cell line and date.	RT		

Cryopreservation

This protocol is optimized for use with CryoStor CS10, a specialized, protein-free cryopreservation medium designed to maintain high viability during the freezing and thawing process. While CryoStor CS10 is the primary recommendation for this workflow, other options such as STEM-CELLBANKER or a combination of ES-cell qualified FBS with culture medium (e.g., mTeSR Plus) may be suitable alternatives depending on your specific experimental requirements or laboratory situation.

Preparation: Prior to harvesting, confirm that iPSC cultures have reached 70 - 80% confluency and exhibit minimal to no differentiation. Keep CryoStor CS10 in the 4°C refrigerator until just prior to use. Ensure the controlled-rate freezing container is at room temperature and the isopropanol is fresh. Label cryovials with cell line name, passage, date, concentration, and initials. **Note:** If differentiated cells are present, consider delaying cryopreservation and instead perform a "cleanup" passage using a conservative application of ReLeSR (minimal incubation) to selectively detach healthy undifferentiated colonies while leaving differentiated cells behind, to ensure that an optimal population of pluripotent cells are advanced to the cryopreservation stage.

Single cell dissociation: Refer to the Single-Cell Passaging (using Accutase) protocol for technical details. **Briefly:** For one well of a 6-well plate, completely aspirate media from cells, wash 2x with 1 ml DPBS (no Ca2+, no Mg2+). Add 1 ml pre-warmed Accutase and incubate at 37°C for 5–10 minutes until cells round. Tap plate firmly on each side, to detach cells. Gently pipette up and down 5x to singularize, transfer to a conical tube with 9 ml Advanced DMEM/F12 to dilute the enzyme out. **Note:** If scaling up e.g., freezing down an entire plate, you can pool the 6 ml of cell suspension into a 50 ml falcon containing roughly 44 ml of Advanced DMEM/F12.

Counting and Calculation: Ensure the cell suspension is thoroughly mixed in the quench media. Remove 100 µl of the suspension to a small tube containing 100 µl of Trypan Blue, gently pipette up and down a few times to mix, and load 10 µl of

the mixture onto a Countess slide. Using the Countess cell counting SOP, determine the total viable cell count. Finally, calculate the volume of chilled CryoStor required to reach a final concentration of 1.0 x 10^6 cells/ml.

Pellet and Resuspend: Centrifuge the suspension for 5 minutes at 200 x g (RCF). Aspirate the supernatant completely, taking care not to disturb the cell pellet. Tap the bottom of the tube firmly to flick-loosen the pellet before adding the predetermined volume of CryoStor. Gently pipette up and down several times to ensure the cells are nicely mixed and then distribute 1 ml (1 x 10^6 cells) to each cryotube. Ensure lids are screwed on tightly, place cryotubes in a controlled rate freezer, and then place the controlled rate freezer and cells in -80°C overnight. The following day, transfer the vials to LN2 for long-term storage.