Culture and freezing methods for AICS cell lines

Required Reagent List:

- Complete mTeSR1 culture media, referred to in this protocol as simply "mTeSR1": 400 mL basal media with provided 100 mL 5X supplement (catalog # 05850, Stem Cell Technologies) with added 5 mL (1% v/v) Penicillin/Streptomycin (catalog # 15140-122, Gibco) *Refer to page 16 of the Stem Cell Technologies technical manual about preparation, storage and shelf life of this media*.
- Matrigel (catalog # 354230, Corning)
- DMEM/F12 media, phenol red-free (catalog # 11039-021, Gibco Life Technologies)
- ROCK inhibitor (Ri) $[10mM]_{stock}$ reconstituted in DMSO per manufacturer's instructions (Stemolecule™ Y27632, catalog # 04-0012-10, Stemgent)
- DPBS, without Ca^{+} or Mg^{++} (catalog # 14190-144, Gibco Life Technologies)
- StemPro® Accutase® (catalog # A11105-01, Gibco Life Technologies)
- Treated plastic Tissue Culture dishes and/or plates (see Table 1 for appropriate catalog numbers)
- 0.22 µM Sterile Media Filter, Stericup™ 500 mL, or similar (catalog # SCGPU05RE, Millipore)
- DMSO (catalog # D2650, Sigma)

Additional for Freezing:

- Knock Out Serum Replacement (catalog # 10828-028, Gibco Life Technologies)
- CryoVials with rubber gasket and internal threading (catalog # 12-565-167N, Fisher Scientific)
- Mr. Frosty Freezing container (catalog # 5100-0001, Fisher Scientific)
- Isopropanol (catalog # 19516-500ML, Sigma)

Recommended Equipment:

- All work described in this protocol should be performed in a sterile Bio Safety Cabinet (Nuaire Class II Type A2, or similar) using proper sterile technique.
- Tissue culture incubator capable of maintaining an environment with 5% CO_2 and 37˚C (ThermoFisher Scientific Heracell™ VIOS 160i, or similar)
- Swinging bucket centrifuge capable of reaching speeds of 211 x g (Eppendorf 5810R, or similar). *Throughout protocol, RPM values are directly applicable to Eppendorf S-4-104 rotor (18.9 cm radius).*
- Cell Counter (Beckman Coulter® Vi-CELL™, or similar)
- Phase-contrast microscope with 4X and 10X objectives (Nikon Eclipse TS100, or similar)
- Serological pipettes (5-25 mL) and pipet-aid (Drummond Pipet-Aid, or similar)
- Standard size pipette set capable of pipetting 2-1000 µL (Rainin LTS, or similar)

Steps before starting:

1. If passaging, check that the morphology of your cells is consistent with known, good hiPSC morphology (Fig. 1). Ideally, cells should be at ~75% confluence for passaging and freezing, and cells should be fully recovered from previous passage. Some dead cells in the media is normal, but this should not be more than 1-5%.
2. If necessary, prepare fresh mTeSR1 media:
    a. Thaw 5X supplement at room temperature (RT) for ~4-6 h, or at 4˚C overnight. Do not thaw 5X supplement at 37˚C.
    b. Combine 5X supplement with 400 mL mTeSR1 and 5 mL Pen/Strep.
    c. Sterile filter media with a 0.22 µM media filter before first use.
3. Bring mTeSR1 media to RT on the bench. Do not warm mTeSR1 in a 37˚C water bath.
4. Pre-warm Accutase in a 37˚C water bath.
5. Label vessel(s) (culture dish or CryoVials, etc.) with cell line name, clone, passage number, date, and attach barcode (as applicable).
6. Prepare mTeSR1 +ROCK inhibitor (Ri) media. mTeSR1 + Ri should always be used with cells for 24 h after they are treated with Accutase to promote cell survival.
    a. Add Ri at 1000X dilution to mTeSR1 media.

b. Mix well by pipetting.
e.g., for 100 mL mTeSR1, add 100 µl Ri.
Note: Lyophilized Ri stock is reconstituted in DMSO at 10 mM, per manufacturer's instruction. We recommend making 200 µL aliquots in 1.5 mL Eppendorf tubes and storing at -20˚C for up to 6 months.

Table 1: Vessel Formats

Vessel formats	Vol. (mL/well) for Matrigel coating	Vol. (mL/well) for media	Vol. (mL/well) for Accutase	Vol. (mL/well) of DPBS for trituration	Vol. (mL/well) of PBS for final wash	Vol. (mL/sample) of media to resuspend pellet in for accurate counting	Cell Plating Density/ approx. days to 70% confluency[A]
10 cm dish[B] (cat. 353003, Corning)	5	10-12	3	2	8	10	500K-1M / 3-4 days
6 well plate (cat. 657-160, Greiner Bio-One)	1.5	2-4	1	4	5	3-4	50-100K / 3-4 days
24 well plate (cat. 662-160, Greiner Bio-One)	0.5	1-2	0.5	1	1	n/a	20-40K / 3-4 days
96 well plate (cat. 353072, Falcon)	0.1	0.15	0.04	0.170	n/a	n/a	2K / 3-4 days
T25 flask (cat. 353014, Falcon)	4	5	2	2	4	5-7	350K /3-4 days
T175 flask (cat. 159920, Nunc)	10	25-35	3	20	20	25	1.5M-3M / 3-4 days

[A]Can be cell line-dependent. Please see recommended seeding densities in AICS catalog. [B]We recommend culturing in 10 cm dishes for most purposes, especially when more than 1 x 10^6 cells are needed for downstream applications.

Methods:

Matrigel Coating Plastic Tissue Culture Vessels (Matrigel:DMEM/F12= 1:30)

1. Prepare Matrigel coated vessels as needed. Per manufacturer's instruction, coated vessels are only good for 14 days, Matrigel should be stored at -80˚C long term, and should be thawed **only** at 4˚C overnight. **Never thaw in a water bath or at RT**. Freeze-thaws should be avoided, therefore we recommend making aliquots of Matrigel. See Step 2, below.
   Note: We lot test our Matrigel to make sure cells cultured show expected morphology and expression of stem cell markers over 3-5 passages.
2. To aliquot Matrigel:
   a. From a 10 mL glass vial of thawed 4˚C Matrigel, aliquot 1 mL units into individual 50 mL conical tubes.
      i. Keep 10 mL source glass vial and 50 mL conical tubes on ice or at 4˚C while working.
      ii. When making aliquots, we recommend using 5 or 10 mL serological pipettes that have been pre-chilled at -20˚C to prevent Matrigel from gelling inside pipette.
   b. Store aliquots at -20˚C for up to 3 months.
3. To prepare 1:30 Matrigel dilutions for vessel coating:
   a. Thaw Matrigel aliquot in a 4˚C fridge until frozen pellet is no longer visible (~ 2 h for 1 mL).
   b. Dilute 4˚C Matrigel 1:30 by adding 29 mL of ice-cold (4˚C) DMEM/F12 media to the 1 mL of Matrigel in a 50 mL conical tube.
   c. Ensure diluted Matrigel is homogenously mixed by carefully, but thoroughly, pipetting the full mixture up and down 3-5 times using a 25 mL pipette.
   d. Diluted Matrigel should be used to coat vessels immediately and should not be re-frozen. If spending more than 5 minutes coating vessels from a prepared dilution of Matrigel, keep the dilution on ice (or at 4˚C) while working.
4. Transfer enough 1:30 diluted Matrigel into each well or vessel to coat bottom (see Table 1). If preparing 96 well plates, we recommend transferring the diluted Matrigel into a boat and using a multi-channel pipette to quickly dispense Matrigel into each well. An electronic repeater multi-channel pipette is ideal for fastest dispensing. Ensure that the entire vessel surface is coated with liquid- if necessary, rock or tap vessel.
5. Choose either the "Fast" or "Storage" Protocol, below
   a. Fast Protocol (for same day use of coated plastic vessels):
      i. Incubate at least 1 h, or up to 6 h, at RT.
      ii. Tip plate to a 45˚ angle and aspirate and discard all excess liquid by aspiration. Gently add fresh RT mTeSR1 + Ri. Work quickly so that wells do not dry out. Try to limit time between aspirating and dispensing media to < 30 s.

iii. Seed cells within 10 min of adding media. If not seeding plate within 10 min, store prepared plates with media for up to 1 h in a tissue culture incubator at 5% CO_2 and 37˚C until ready to seed.

b. Storage Protocol (for use next day or up to 2 weeks later):
   i. Wrap vessels with Parafilm to prevent evaporation and store on level surface in 4˚C fridge for up to 2 weeks.
   ii. When ready to use, remove from fridge and allow plates to come to room temperature in hood or on bench top.
   iii. Tip plate to a 45˚ angle and aspirate and discard all excess liquid by aspiration. Gently add fresh RT mTeSR1 + Ri. Work quickly so that wells do not dry out. Try to limit time between aspirating and dispensing media to < 30 s.
   iv. Seed cells within 10 min of adding media. If not seeding plate within 10 min, store prepared plates with media for up to 1 h in a tissue culture incubator at 5% CO_2 and 37˚C until ready to seed.

Thawing WTC Cells

1. Prepare a 15 mL conical tube with 5 mL of RT mTeSR1 + Ri.
2. Carefully remove vials from LN_2 storage, being sure to vent cap to relieve any LN_2 trapped inside, then re-tighten cap.
3. Quickly thaw the frozen vials using 37˚C water bath until only a small ice pellet is visible. Limit the amount of time cells are in Freezing Media at RT to 10 min or less.
4. Fully rinse outside of closed vial with 70% EtOH and dry with Kimwipe before transferring vial to sterile hood.
5. Remove cell suspension from thawed vial using a 1000 µL pipette tip to transfer the cells to the 15 mL conical tube containing 5 mL mTeSR1 + Ri prepared in step 1. Pipette slowly and do not mix. Avoid creating bubbles or disturbing the cells.
6. Spin cells at 1000 rpm (RCF= 211 x g) for 3 min at RT in a swinging bucket centrifuge.
7. Aspirate and discard supernatant, then re-suspend pellet in 5 mL RT mTeSR1 + Ri.
8. Seed cells in Matrigel-coated vessels; see chart for plating densities. Gently rock vessel and then keep level for even cell attachment.
   Note: Cells settle and attach to Matrigel VERY quickly (<2-3 min), so it's important to place plates on a level surface as quickly as possible.
9. Incubate cells at 37˚C and 5% CO_2.
10. Change media to mTeSR1 (no Ri) at 24 h after seeding.
11. Change media every 24 h with mTeSR1.

Passaging and Maintaining WTC Cells on Plastic Tissue Culture Treated Vessels

Accutase can be warmed to RT in a water bath at 37˚C if necessary, while mTeSR1 must only be warmed to RT on the bench. WTC are grown in a standard 5% CO_2 incubator at 37˚C and must be fed fresh mTeSR1 once every day.

12. When cells reach 70-85% confluency, passage the cells. See Figure 1.
13. Aspirate and discard old medium.
14. Gently add RT DPBS. Do not dispense DPBS directly onto cells, but rather introduce DPBS at the side of the vessel, allowing it to slowly cover the vessel surface without disturbing the cells.
15. Aspirate and discard DPBS wash.
16. Add pre-warmed Accutase (see Table 1 for volumes) and incubate in 37˚C incubator for 3-5 min.
17. Check for detachment by gently tilting vessel and/or observing under the microscope. If all cells have not detached in 3-5 minutes, incubate an additional 2 min and check again. Continue to incubate and check as necessary, only until cells are no longer attached to the plate surface, although cells may remain in large clumps.
    Note: Avoid incubating cells with Accutase for longer than is necessary to detach from plate surface. Most clonal lines derived from WTC will be fully detached from vessel surface in less than 8 min.
18. Add RT DPBS to dilute out the Accutase in the vessel as follows (see Table 1 for volumes):
    a. Add DPBS to vessel and very gently triturate cell suspension by aspirating and then gently rinsing the cells across the vessel surface 5-8 times to the point where all cells have been released from the vessel surface and the suspension is homogenous- all without creating bubbles.
    b. Check that single cell suspension has been achieved in >90% of the suspension using microscope. If single cell suspension is not achieved, triturate 4-5 more times and check again.
    Note: Cells can be passaged in small clumps, but cell counting may not be accurate. Single cell suspension is especially important for certain downstream applications such as transfection, cell sorting by FACS, or plating at low density for clone picking.

19. Transfer cell suspension to 15 mL conical tube.
20. Rinse the vessel one time with DPBS for final wash (see Table 1 for volumes). Add to conical.
21. Spin down cells at 1000 rpm (RCF= 211 x g) for 3 min at RT.
22. Carefully aspirate and discard DPBS/Accutase supernatant from cell pellet and re-suspend in desired volume of mTeSR1 + Ri for seeding, plating and/or counting. See Table 1 for recommended re-suspension volumes for most accurate counting densities when using ViCell cell counter.
23. Cells can be seeded directly into new vessels at a ratio of 1:10 (3-4 days to confluency) to 1:20 (4-5 days to confluency) depending on day needed. See Table 1 for recommended number of cells per vessel when counting. We recommend counting cells and plating specific numbers for most reproducible culturing conditions.

    Note: Cells in suspension settle quickly. After counting, we recommend gently re-suspending cell suspension before seeding by triturating the entire volume two times using a serological pipette. Cells should be mixed approximately every 2-3 min when seeding multiple vessels.
24. Matrigel-coated vessels cannot be re-used, so always seed cells onto fresh Matrigel coated vessels prepared with RT mTeSR1 + Ri.
25. After seeding, gently rock vessels front-to-back and side-to-side to ensure even cell distribution across vessel surface.

    Note: Cells settle and attach to Matrigel VERY quickly (<2-3 min), so it's important to place plates on a level surface as quickly as possible.
26. Always record the correct date, passage number and identifier on vessel(s).
27. Incubate cells at 37˚C and 5% CO_2.
28. Change media to mTeSR1 (no Ri) at 24 h after seeding.
29. Change media every 24 h with mTeSR1.

WTC Cell Freezing (Cryo-preserving)

1. Prepare fresh Freezing Media. Freezing media should be made 10 min-6 h in advance of use and discarded thereafter.
    a. mTeSR1 with 30% KSR, and 10% DMSO
    e.g. for a 10 mL volume: 6 mL mTeSR1 + 3 mL KSR + 1 mL DMSO
    Note: No Ri is used in the Freezing Media.
2. Prepare Mr. Frosty freezing containers by adding room temperature isopropanol per manufacturer's instructions.
3. Label CryoVials as appropriate.
4. When cells are 70-80% confluent, detach and pellet using Accutase per Passaging and Maintenance protocol, and re-suspend final pellet in mTeSR1 + Ri (see Table 1 for recommended volumes based on source well size).
5. Count cells using the counting protocol, or similar method.
6. Calculate the mL of cell suspension needed using the following equation:
$$mL\ cell\ suspension\ to\ spin\ down = \frac{\#\ vials\ desired\ x\ 1x10^6}{\#\frac{cells}{mL}}$$
7. Pellet cells to be frozen in a 15 mL conical by spinning at 1000 rpm (RCF= 211 x g) for 3 min at RT.
8. Re-suspend cells in appropriate volume of fresh freezing media (0.5 mL Freezing Media per vial of $1x10^6$ cells) and aliquot 0.5 mL volumes of cells in freezing media into pre-labeled and prepared CryoVials.
9. Tightly close vial caps.
10. Quickly transfer CryoVials to room temperature Mr. Frosty containers and place containers in -80˚C freezer. Limit the amount of time cells are in Freezing Media at RT to 10 min or less.
11. After 24 h, transfer CryoVials to LN_2 storage. Do not leave cells in Mr. Frosty containers in the -80˚C freezer for longer than 96 h.

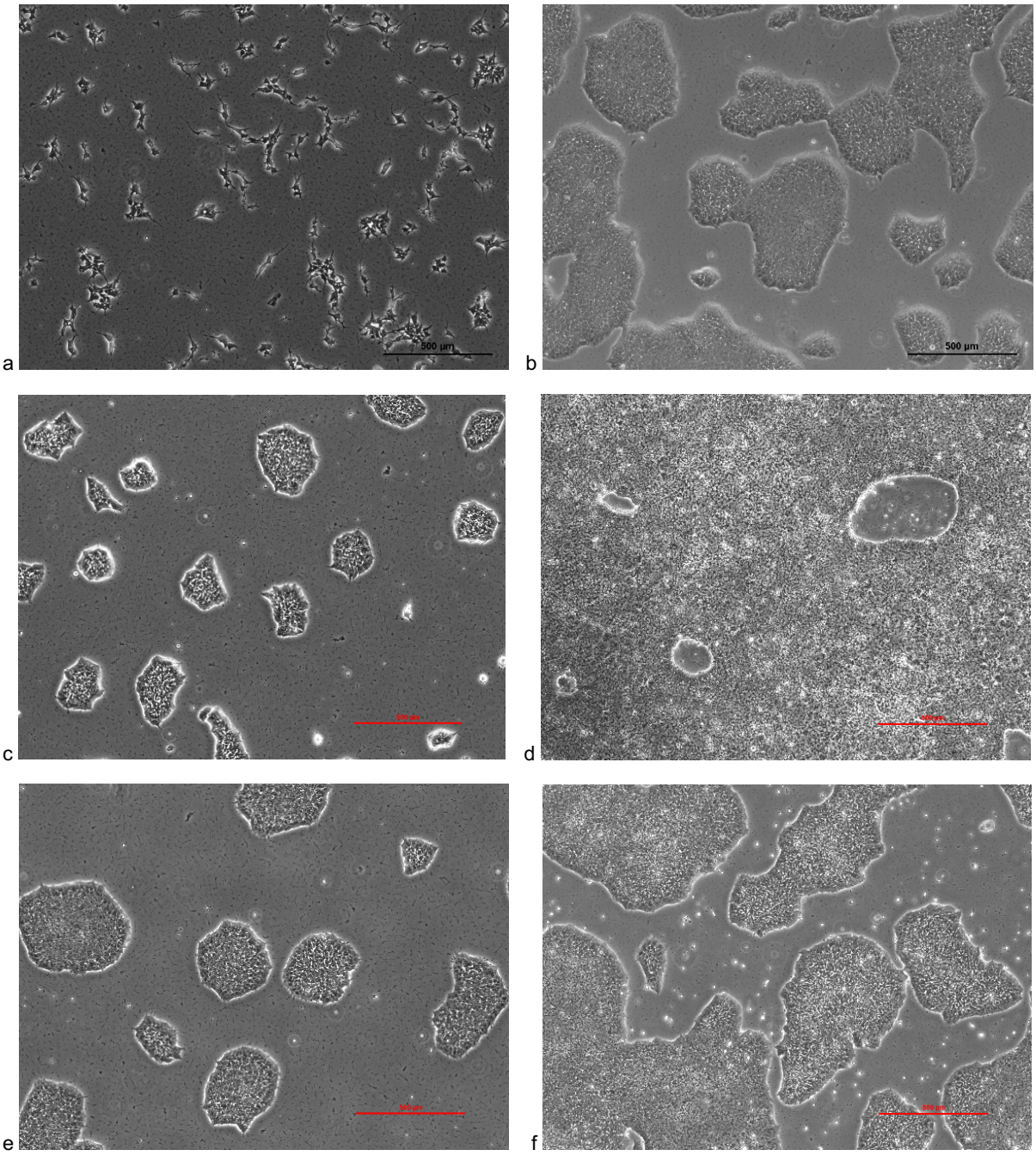

Figure 1. Examples of Confluency: (a) 1 x 10^6 cells plated in 10 cm dish, 24 h after seeding. Cells maintain a "spikey" morphology due to RI treatment. Cells should be allowed to grow 3-4 days before subsequent passaging. **(b)** Same cells from (a) after 3 days of growth. Cells have good mature stem cell morphology and are at an ideal density to be passaged again. **(c)** Immature colonies have a slightly spikey edge and are loosely packed in colony interior. Passaging immature cells should be avoided. **(d)** Overgrown culture that is too confluent to continue to use. Future genomic integrity and/or morphology may be compromised. **(e)** Mature colonies at low density, can be picked as individual colonies or passaged. **(f)** Similar to (b), another example of a culture that has good mature stem cell morphology and is at an ideal density to be passaged again.

Note on how to image the tagged structures in these cells with fluorescence microscopy:
The GFP-tagged proteins are expressed endogenously and therefore may not appear as bright as when they are overexpressed. For imaging we plate cells onto matrigel-coated high-quality glass bottom coverslips (Cellvis) and image cells in phenol-free mTeSR media (StemCell Technologies). Our most common microscope configuration are a Zeiss spinning disk fluoescence microscope with a Yokogawa CSUX1 head, Hamamatsu CMOS camera, and a 488 laser (GFP). Cells are imaged most often either with a 20x 0.8NA objective for lower magnification and 100x 1.25NA water immersion objective for higher magnification and at 37 degrees C and 5% CO2 in a temperature-controlled chamber. The approximate laser power measured at the sample for our standard 100x images is ~2.5 mW. A more detailed SOP for imaging will be available soon.