

Guidelines for WTC Derived AICS hiPSC Lines Scale Up & Banking (up to 80 vials)

We are always working to optimize and improve our protocols. Here is a summary of changes made since Version 1.2 of this protocol:

- Penicillin/Streptomycin switched from product with catalog #15140-122 to #15070-063. The updated protocol uses a reduced concentration of 5,000U/mL.
- The catalog number of T-175 flasks switched from #155920 to #660175 and the volume of Matrigel used to coat them is refined in Table 1.
- The final protein concentration used for diluted Matrigel was added.

Note: The following protocol is for banking ~60- 80 vials of cells (1 million/vial) depending on the cell line. If a smaller bank is needed, please adjust accordingly.

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 # 85850, STEMCELL™ Technologies) with added 5 mL (1% v/v) Penicillin/Streptomycin (catalog # 15070-063, Gibco). *Refer to page 14 of the STEMCELL™ Technologies technical manual about preparation, storage and shelf life of this media.*
- Growth Factor Reduced (GFR) Matrigel®, phenol red-free (catalog # 356231, 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 (Y-27632, catalog # 72308, STEMCELL™ Technologies)
- 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-Sigma)
- DMSO (catalog # D2650, Sigma-Aldrich)
- Knock Out Serum Replacement (catalog # 10828-028, Gibco Life Technologies), referred to in this protocol as “KSR”
- CryoVials with rubber gasket and internal threading (catalog # 377267 Nalgene Nunc International)
- Mr. Frosty freezing container (catalog # 5100-0001, Thermo Fisher Scientific)
- Isopropanol (catalog # 19516-500ML, Sigma-Aldrich)

Recommended Equipment:

- All work described in this protocol should be performed in a sterile Bio Safety Cabinet (Nuair Class II Type A2, or similar) using proper sterile technique.
- Tissue culture incubator capable of maintaining an environment with 5% CO₂ 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)

Related SOP:

The following SOP is used in conjunction with this protocol:

- WTC culture v1.7: Culture and Freezing Methods for WTC Derived AICS hiPSC Lines (can be accessed at: <https://www.allencell.org/methods-for-cells-in-the-lab.html#sops>)

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% confluency for passaging and freezing, and cells should be fully recovered from the previous passage. Some dead cells in the media is normal, but this should not be more than 5%.
2. If necessary, prepare fresh mTeSR1 media (refer to manufacturer protocol for added details):
 - 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 basal media 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 or allow to come to RT.
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 24h after they are treated with Accutase to promote cell survival.
 - a. Dilute Ri at 1:1000 in 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 protocol. We recommend making 250 µL aliquots in 1.5 mL Eppendorf tubes and storing at -20°C for up to 6 months. Minimize freeze/thaw of aliquots.

Matrigel Coating Plastic Tissue Culture Vessels (Matrigel final protein concentration = 0.337mg/mL)

1. Prepare Matrigel coated vessels as needed. Per manufacturer protocol, coated vessels are only good for 14 days, Matrigel should be stored at -80°C long term or -20°C for short term, and should be thawed **only** at 4°C or on ice. **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. 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. Make sure to change pipette between uses (maximum of 2 minutes at RT) to a fresh pre-chilled pipette at -20°C.
 - b. Store aliquots at -20°C for up to 3 months.
3. To prepare Matrigel dilutions for vessel coating:
 - a. Thaw Matrigel aliquot in a 4°C fridge until frozen pellet is no longer visible (~ 1-2 h or overnight for 1 mL).
 - b. Dilute 4°C Matrigel by adding chilled (4°C) DMEM/F12 media to the 1 mL of Matrigel in a 50 mL conical tube using a pre-chilled pipette.
 - c. Ensure diluted Matrigel is homogenously mixed by carefully, but thoroughly, pipetting the full mixture up and down 3-5 times with the pre-chilled 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 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 (kept on ice) 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 to do this.
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% CO₂ and 37°C until ready to seed.
- a. 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% CO₂ and 37°C until ready to seed.

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 (cat. 353003, Corning)	5	10-12	3	3-7	7-8	10	500K-1M / 3-4 days
T-175 flask (cat. 660175, Greiner Bio-One)	10-12	25 @ d0 30 @ d1 35 @ d2 40 @ d3 & 4	10	20	20	15-25	1.5M-3M / 3-4 days

^ACan be cell line-dependent.

Seeding Flasks

Before seeding flasks, the following criteria should be met for each AICS cell line to be banked:

- **Thaw cells according to “Thawing AICS cells” section outlined in “Culture and Freezing Methods for WTC Derived AICS hiPSC Lines” (can be accessed at: <https://www.allencell.org/methods-for-cells-in-the-lab.html#sops>)**
- **24 h after thawing, image cells at 4x and 10x (compare to images in Certificate of Analysis).**
- **When cells have recovered to 70-80% confluent and are ready to be passaged, capture a second set of images at 4x and 10x (compare to images in Certificate of Analysis).**
- **Passage cells one time post-thaw using standard passaging conditions in a 10 cm plate (recovery passage). Note: seed additional 10 cm plates if needed to acquire enough cells to seed to 3-4 T-175 flasks.**
- **AICS cell line has appropriate morphology and is at a healthy density for passaging (compared to images in Certificate of Analysis).**
- **Recommended: seeding into T-175 flasks should ideally occur on a Monday or Friday to avoid weekend passaging.**

1. Proceed with “Passaging and Maintaining AICS Cells on Plastic Tissue Culture Treated Vessels” method from “Culture and Freezing Methods for WTC Derived AICS hiPSC Lines” AICS SOP to the point where cells are counted and in suspension of mTeSR1 + Ri (step 11).
2. Use a long VWR plastic aspirating pipette (or a long autoclaved glass Pasteur pipette) when aspirating Matrigel or media from a T-175 flask to avoid contamination. Always tilt the flask so that the pipette tip is aspirating from the “top-back” corner so cells are not disrupted. See Figure 2.
3. Seed T-175 flasks with approximately three times the number of cells you would seed a 10 cm plate with, this number may vary based on cell line growth dynamics and number of days growth desired. Seed 4xT-175 flasks for 3 day growth and 3xT-175 flasks for 4 day growth.

Note: On average, ~3 million cells will be 70-80% confluent on day 3, and 1.5 million cells will be 70-80% confluent on day 4. Approximately 30 million cells are obtained from each T-175 flask. Total cell counts from T-175s can vary between AICS cell lines.

4. Ensure proper distribution of cells is observed for even plating within each flask by sitting each T-175 upright and slowly rock back and forth. Carefully lay each T-175 flask flat in the incubator.
5. In order to guarantee cells are able to sit down evenly in each flask, do NOT stack flasks in the incubator until cells have been given a chance to adhere to matrix for at least 3-4 hrs.
6. Incubate flasks at 37°C and 5% CO₂, changing media daily with fresh mTeSR1. See Table 1 for recommended volume of media.

Note: Volume of media increases by 5 mL per day to provide sufficient nutrients for increasing numbers of cells.

7. When dispensing into a T-175 flask, position the serological pipette tip so that media is being dispensed on either the side or top of the flask, never directly onto cells. See Figure 2.

Banking Cell Lines from T-175 Flasks

Before starting, ensure the following criteria are met:

- **AICS cell line has appropriate morphology and is at a healthy density for passaging. Image at 4X and 10X prior to banking. (Compare images to images in certificate of analysis.)**
- **Make sure the required number of Mr. Frosty containers are available at room temperature**

1. Prepare 80 vial labels prior to banking with appropriate information. Make sure each label contains AICS cell line name, clone number, passage number, cell number per vial (1x10⁶ cells per vial) and date cells banked.
2. Prepare fresh freezing media by thoroughly mixing 30 mL of mTeSR + P/S, 15 mL of KSR and 5 mL of DMSO (total volume 50 mL, enough for freezing 80 vials with excess).

Note: Add DMSO last to avoid forming precipitate.

3. Retrieve flasks for the AICS cell line to be banked from the incubator.
Recommended: work with one T-175 flask at a time and start with second T-175 once Accutase has been on first T-175 for approximately 3-4 minutes. Once comfortable with timing, may work with two T-175 flasks at a time.
4. Aspirate and discard old medium.
5. Gently add RT DPBS to rinse the cells. Do not dispense DPBS directly onto cells, but rather introduce DPBS at the side of the vessel. Then rotate the flask, allowing it to slowly cover the vessel surface without disturbing the cells.
6. Aspirate and discard DPBS wash.
7. Add 37°C pre-warmed Accutase (see Table 1 for volumes) and incubate in 37°C incubator for 7 min (start with second set of T-175 flasks once first set of flasks have been in accutase for 3-4 minutes).
8. Check for detachment by gently tilting vessel and/or observing under the microscope. If all cells have not detached in 7 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.

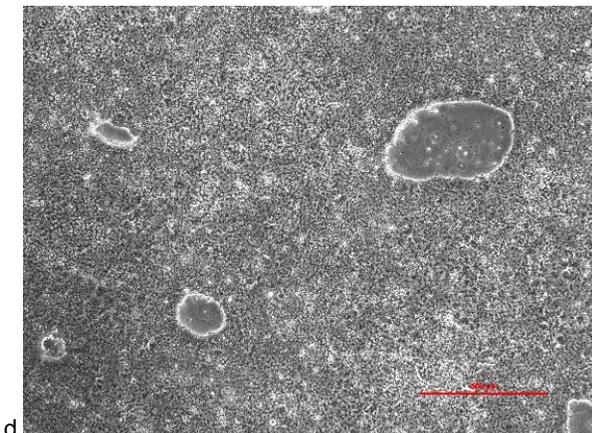
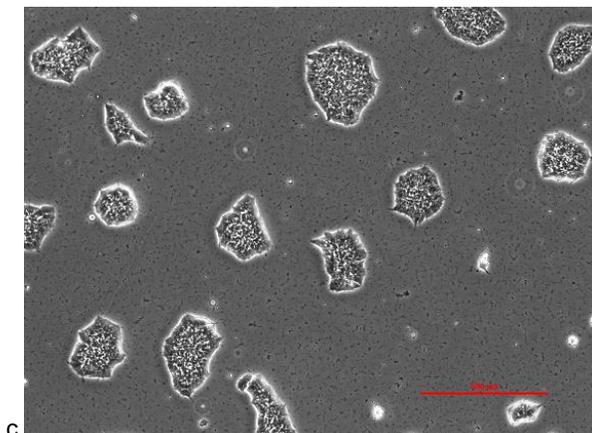
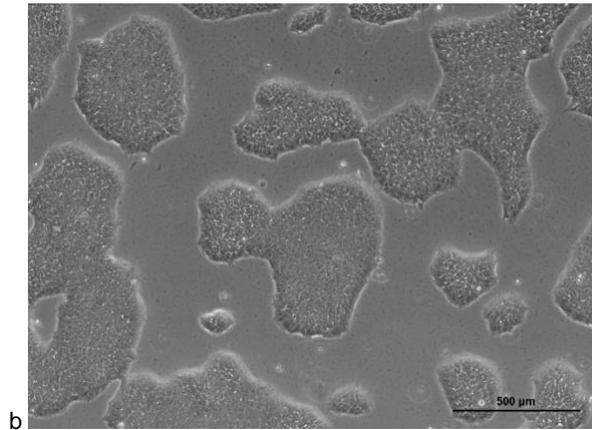
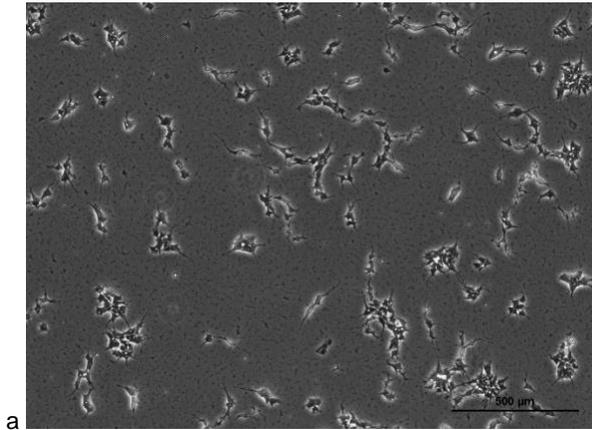
Note: Avoid incubating cells with Accutase for longer than is necessary to detach from plate surface. Most clonal lines derived from WTC will be fully detached from vessel surface in less than 10 min.

9. Add RT DPBS to dilute 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 8-10 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 3-5 more times and check again.
Note: Cells can be passaged in small clumps, but cell counting may not be accurate, and thawed vials may become confluent sooner than expected.
10. Transfer cell suspension to a 125 or 250 mL square Nalgene bottle.
11. Rinse each vessel one time with DPBS for final wash (see Table 1 for volumes). Add to Nalgene bottle.
12. When cell suspension from all flasks has been collected into one 125 or 250 mL square Nalgene bottle, aliquot the suspension into 50 mL conicals and pellet cells at 211 x g for 3 min at RT.
13. Re-suspend each pellet in 10 mL of mTeSR1 + Ri and combine all suspensions into one 50 mL conical. Bring the final volume of cell suspension to 50 mL, mixing thoroughly 3-5 times with a 50 mL pipette.

14. Prepare three samples for counting the suspension three times. *If using ViCell, take care to change pipette tips when preparing each cuvette so as to not cross-contaminate the cell suspension. Average the three counts.*
15. Prepare aliquots of maximum 19 million cells (enough cells for one extra CryoVial) in 15 mL or 50 mL conicals (depending on cell suspension concentration) using the following equation (this calculation is enough to fill one Mr. Frosty with 18 vials frozen at 1×10^6 cells per vial):
 - a. $19 \text{ million cells needed} / (\text{cells per mL in suspension}) = \text{mL of suspension per conical}$
 - b. Mix parent suspension thoroughly (full volume, 4-5 times) before aliquoting.

Note: Excess is calculated in for pipetting error.
16. Working with one aliquot at a time, pellet cells at $211 \times g$ for 3 min at RT.

Note: This step is essential to minimize the amount of time cells are in freezing media; limit time in freeze media at RT to 10 min or less.
17. Aspirate supernatant and thoroughly but gently resuspend pellet in appropriate volume of fresh freezing media prepared in step 2 (9.5 mL for tubes containing 19 million cells).
18. Use a 5 mL serological pipette to dispense 0.5 mL aliquots of cell suspension into each of 18 prepared CryoVials. Work quickly so cells don't settle toward bottom of pipette.
19. Tightly seal CryoVial caps and quickly transfer to a room temperature Mr. Frosty. After securing the lid, quickly transfer Mr. Frosty to -80°C freezer.
20. Repeat steps 16-19 for remaining aliquots.
21. After **24 h**, transfer CryoVials to liquid nitrogen storage. Do not leave cells in Mr. Frosty containers in the -80°C freezer for longer than 72 h.
 - a. Use a Styrofoam box or appropriate container filled with about 1" of liquid nitrogen to place the destination box into so that cells stay frozen during the transfer process.
 - b. Retrieve Mr. Frostys from the -80°C freezer and transfer CryoVials into the destination box for liquid nitrogen storage.



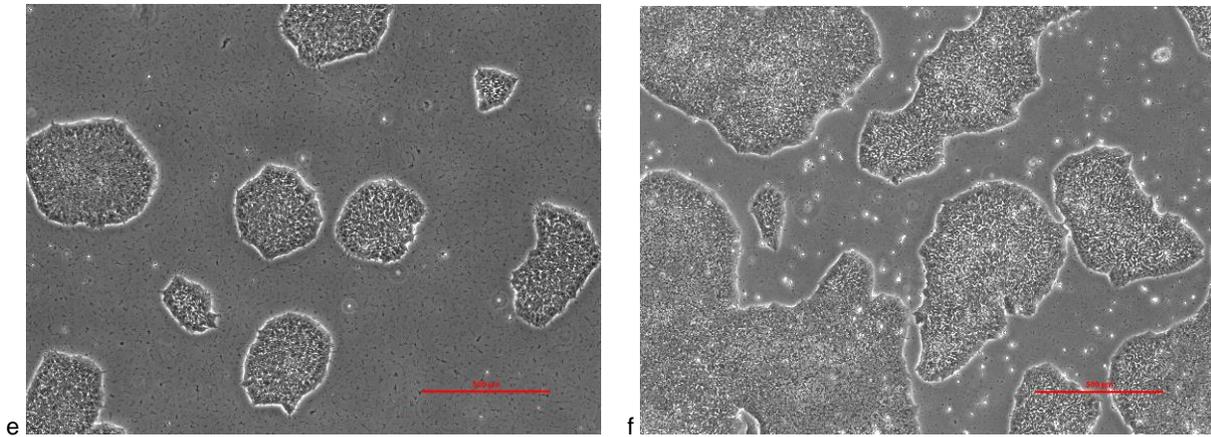


Figure 1. Examples of Confluency: (a) 1×10^6 cells plated in 10 cm dish, 24 h after seeding. Cells maintain a “spiky” morphology due to RI treatment. Cells should be allowed to grow 3-4 days before subsequent passaging. (b) Same cells from (a) after 3 days of growth. Cells have good mature stem cell morphology and are at an ideal density to be passaged again. (c) Immature colonies have a slightly spiky 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. Scale bars, 500µm.

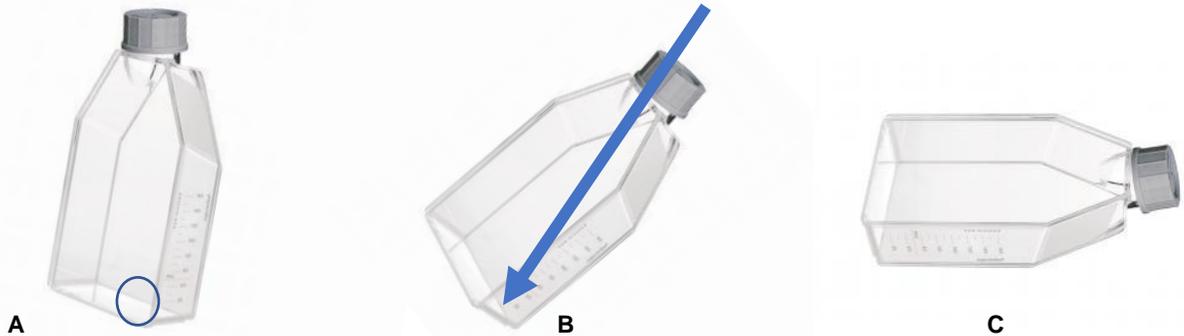


Figure 2. Flask Orientations. A. Location to place pipette tip when aspirating from a tilted flask. B. Dispense liquid onto the side of the flask(s) to avoid disrupting attached cells. C. When cells are seeded, lay flask(s) flat with media evenly covering the bottom surface.