Version 1.8 2024

Culture and Freezing Methods for WTC Derived AICS hiPSC Lines

We are always working to optimize and improve our protocols. Here is a summary of changes made since Version 1.6 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 suggested densities for seeding stem cells into a 6-well plate has been updated.**
- **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.**
- **The number of triturations during passaging has been refined.**
- **During passaging of AICS cells, an alternative to washing the plate with DPBS after collecting cells has been added.**

New changes since Version 1.7 of this protocol

- **Added a new section on thawing AICS cells. Part A – Standard Cryovial Format and Part B – Newest Cryovial Format with 2D barcodes on bottom of the vial. Please refer to thaw videos on allencell.org and Coriell's website for further details.**

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, 5,000U/mL) *Refer to page 16 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)
- DMSO (catalog # D2650, Sigma)

Additional for Freezing:

- 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 (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% 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.

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	3-7	7-8	10	500K-1M / 3-4 days
6 well plate (cat. 657-160, Greiner Bio-One)	1.5	2-4	1	1-4	2-3	3-4	80K-175KK / 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- 0.2	0.04	0.170	n/a	n/a	2.5K-4K / 3-4 days

T25 flask (cat. 353014, Falcon)	**4**	**5**	**2**	**2**	**4**	**5-7**	**350K /3-4 days**
T175 flask (cat. 660175, Greiner Bio- One)	**10-12**	**25- 40**	**10**	**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 final protein concentration = 0.337mg/mL)

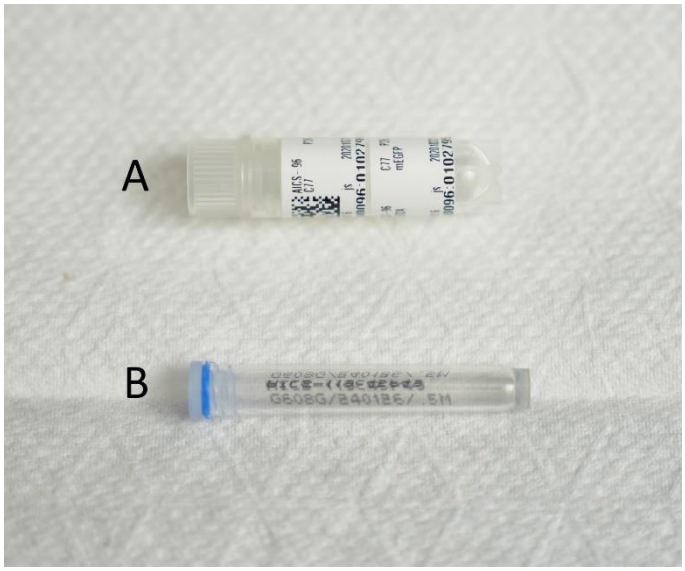
1. Prepare Matrigel coated vessels as needed. Per manufacturer protocol, coated vessels are only good for 14 days, Matrigel should be stored at -80˚C long term or -20 ˚C for short term, and should be thawed **only** at 4˚C or on ice. **Never thaw in a water bath or at RT**. Freeze-thaws should be avoided, 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% CO_2 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% CO_2 and 37˚C until ready to seed.

Thawing AICS Cells

STOP! If receive Vial B **DO NOT store these in LIQUID nitrogen dewars. Can store these ONLY in dewars that are vapor phase.** If you do not have a vapor phase dewar for cell storage, make sure to thaw and bank the cells immediately.



A (Follow Steps 1-6 below if receive vial "A" pictured above).

1. Prepare a 15 mL conical tube with 5 mL of RT mTeSR1 + Ri (Dilute Ri at 1:1000 in mTeSR1 media).
2. Carefully remove vials from Liquid Nitrogen (LN_2) storage, allow any LN_2 trapped inside to vent.
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 (containing DMSO) at RT to less than 8 minutes upon thaw.
4. Fully rinse outside of closed vial with 70% EtOH and dry with Kimwipe before transferring vial to sterile hood.
5. Using a **5 mL serological pipette**, slowly add 1 mL of RT mTeSR1 + Ri to the vial with the cells. Do not mix. Using the same pipette, slowly aspirate the now ~ 1.5 mL volume of diluted cells from the vial. Work carefully to remove all the liquid without creating extensive bubbles. Transfer the diluted cells from the vial into the prepared 15 mL conical tube from step 1 for a total of 5.5 mL**. [Do not mix; avoid disrupting the cells].** 6. Continue with steps 14-20.

B (Follow Steps 7-13 below if receive vial "B" pictured above).

7. Upon arrival, carefully remove vials from dry ice shipping container. **DO NOT store these in LIQUID nitrogen dewars. Can store these ONLY in dewars that are vapor phase. If you do not have a vapor phase dewar for cell storage, make sure to thaw and bank the cells immediately.**
8. Prepare a 15 mL conical tube with 5 mL of RT mTeSR1 + Ri (Dilute Ri at 1:1000 in mTeSR1 media).
9. Quickly thaw the frozen vials using a **37˚C dry bath or incubator** until only a small ice pellet is visible. Limit the amount of time cells are in Freezing Media (containing DMSO) at RT to less than 8 minutes upon thaw. **[Note:**

If thawing in the incubator be sure to sit vial in a rack to position upright and fully spray with 70% ethanol.] 10. Fully rinse the outside of closed vial with 70% EtOH and dry with Kimwipe before transferring vial to the sterile hood.

11. Be sure to spray your gloves with 70% EtOH and carefully use fingers to unscrew blue cap without touching the threads to reduce risk of contamination.

12. Using a **1 mL serological pipette**, slowly aspirate 0.5 mL of cells from the vial. Work carefully to remove all the liquid without creating extensive bubbles. Slowly transfer the cells from the vial into the prepared 15 mL conical tube from step 7 for a total of 5.5 mL. **[Do not mix; avoid disrupting the cells].** 13. Continue with steps 14-20.

Continue with steps 14-20 for either vial type.

14. Spin cells at 1000 rpm (RCF= 211 x g) for 3 min at RT in a swinging bucket centrifuge.
15. Aspirate and discard supernatant, then resuspend pellet in 3-5 mL RT mTeSR1 + Ri.
16. Seed cells in Matrigel-coated vessels containing mTESR + RI; reference Table1 for media volumes. Generally, a vial of 1 x 10^6 cells or 0.5 x 10^6 cells (refer to Certificate of Analysis to confirm number of cells in frozen vial) thawed into a 10 cm plate will be ready for passaging 3-5 days later.
17. After seeding, slide vessel front-to-back and side-to-side at least 2-4 times and keep level for even cell attachment to ensure even cell distribution across vessel surface.

    Note: Cells settle and attach to Matrigel VERY quickly (<2-3 min), so it is important to place plates on a level surface as quickly as possible.
18. Incubate cells at 37˚C and 5% CO_2.
19. Observe cells 24 h after seeding (see Figure 1a) and change media to mTeSR1 (no Ri). 20. Change media every 24 h with mTeSR1 (no Ri).

Passaging and Maintaining AICS Cells on Plastic Tissue Culture Treated Vessels

Warm accutase in a water bath at 37˚C, while mTeSR1 must only be warmed to RT on the bench. AICS cells are grown in a standard 5% CO_2 incubator at 37˚C and must be fed fresh mTeSR1 once every day.

1. When cells reach 70-85% confluency, passage the cells. See Figure 1.
2. Aspirate and discard old medium.
3. 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.
4. Aspirate and discard DPBS wash.
5. Add pre-warmed Accutase (see Table 1 for volumes) and incubate in 37˚C incubator for 3-5 min.
6. 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 1-2 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.

7. 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 8-10 times (ex. in a continuous zig zag or windshield wiper motion) to the point where all cells have been released from the vessel surface and the suspension is homogenous- all without creating bubbles. Do this using adequate but not excessive force. **Note: using this technique, it is helpful to use a 5 or 10 mL serological pipette to get better single cell suspension.**
    b. Check that single cell suspension has been achieved in >90% of the suspension using a microscope. If single cell suspension is not achieved, triturate 2-3 more times (as described above in step 7a) 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.
8. Transfer cell suspension to a 15 mL conical tube.
9. Rinse the vessel one time with DPBS for final wash (see Table 1 for volumes). Add to conical.
    a. Alternatively, the wash step can be skipped if the conical tube used to collect cells is pre-loaded with the volume of DPBS that would be used for the wash. It is important that the Accutase solution is diluted in the full volume of DPBS.
10. Spin down cells at 1000 rpm (RCF= 211 x g) for 3 min at RT.
11. Carefully aspirate and discard DPBS/Accutase supernatant from cell pellet and re-suspend in desired volume of mTeSR1 + Ri. See Table 1 for recommended re-suspension volumes for most accurate counts based on source vessel size.
12. Cells can be seeded directly into new vessels at a specific cell count (3-4 days to confluency) depending on day needed. See Table 1 for recommended cell plating densities based on vessel size. 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 three times using a serological pipette. Cells should be mixed approximately every 2-3 min when seeding multiple vessels.
13. Matrigel-coated vessels cannot be re-used, so always seed cells onto fresh Matrigel coated vessels prepared with RT mTeSR1 + Ri.
14. After seeding, slide vessels front-to-back and side-to-side to ensure even cell distribution across vessel surface at least 2-4 times and 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.
15. Always record the correct date, passage number and identifier on vessel(s).
16. Incubate cells at 37˚C and 5% CO_2.
17. Observe 24 h after seeding (see Figure 1a) and change media to mTeSR1 (no Ri).
18. Change media every 24 h with mTeSR1.

AICS Cell Freezing (Cryo-preserving)

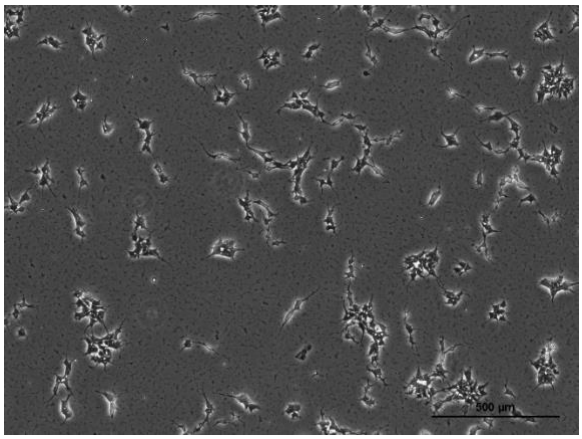
1. Prepare fresh Freezing Media. Freezing media should be made 10 min-6 h in advance of use and discarded thereafter.
    a. mTeSR1 with 30% KSR, and 10% DMSO
    e.g. for a 10 mL volume: 6 mL mTeSR1 + 3 mL KSR + 1 mL DMSO **Note:**
    No Ri is used in the Freezing Media.
    Per manufacturer's instructions, KSR is stable for up to 4 weeks at 2°C to 8°C, protected from light. Working volumes can be aliquoted and stored at –20°C to –5°C. Thaw aliquots as needed. Avoid additional freeze-thaw cycles.
2. Prepare Mr. Frosty freezing containers by adding room temperature isopropanol per manufacturer's instructions.

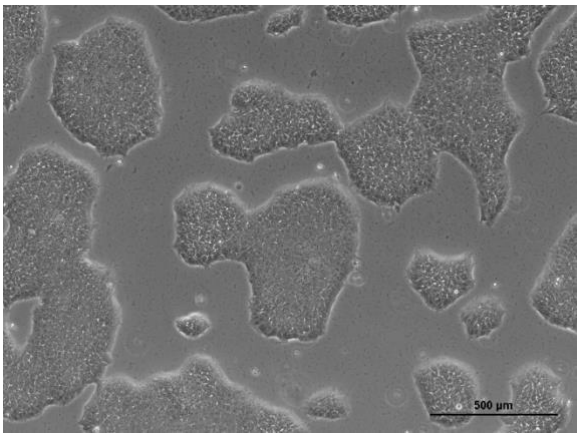
3. Label CryoVials as appropriate.
4. When cells are 70-80% confluent, detach and pellet using Accutase per Passaging and Maintenance protocol, and re-suspend final pellet in mTeSR1 + Ri (see Table 1 for recommended re-suspension volumes for most accurate counts based on source vessel size).
5. Count cells.
6. Calculate the mL of cell suspension needed using the following equation:

$$mL\ cell\ suspension\ to\ spin\ down = \frac{\#\ vials\ desired\ x\ 1x10^{6}}{\frac{\#\ cells}{mL}}$$

Note: calculate enough cells for at least 1 extra CryoVial for pipetting error.

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. Resuspend 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. **Note: use a 5 mL serological pipette to dispense 0.5 mL aliquots into each CryoVial. Work quickly so cells don't settle toward bottom of pipette.**
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.

a 



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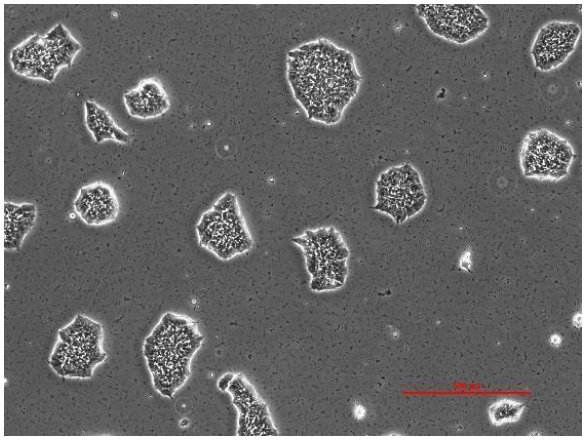
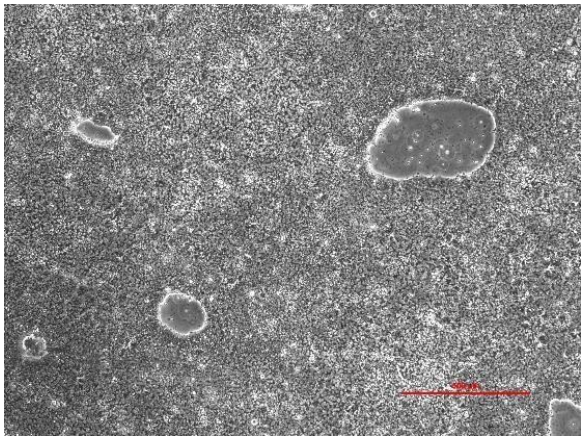
c 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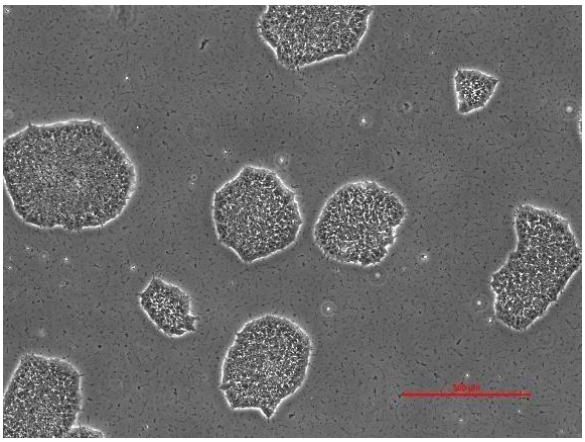
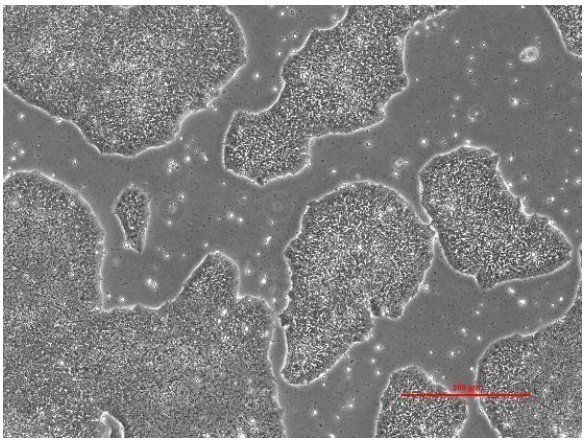
e   f

Figure 1. Examples of Confluency: (a) 1 x 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.