

Cardiomyocyte Differentiation Methods

The methods described below were adapted from the small molecule protocol described in Lian et al.¹, and have been optimized for the differentiation of cardiomyocytes in a 6 well format using the Allen Cell Collection, derived from the hiPSC WTC parental line released by the Conklin Laboratory² at the J. David Gladstone Institute.

Related SOP:

The following SOP is used in conjunction with this protocol:

- WTC culture v1.5: Culture and Freezing Methods for WTC Derived AICS hiPSC Lines (can be accessed at: <http://www.allencell.org/detailed-methods.html#sops>)

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, STEMCELL™ Technologies) with added 5 mL (1% v/v) Penicillin/Streptomycin (catalog # 15140-122, Gibco). *Refer to page 16 of the STEMCELL™ Technologies technical manual about preparation, storage and shelf life of this media.*
- Growth Factor Reduced (GFR) 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 (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)
- RPMI Medium 1640 (1x) (catalog # A10491-01, Gibco Life Technologies)
- B-27™ Supplement (50x), serum free (catalog # 17504044, Gibco Life Technologies)
- B-27™ Supplement, minus insulin (catalog # A1895601, Gibco Life Technologies)
- CHIR99021 [25mM]_{stock} reconstituted in DMSO per manufacturer's instructions (catalog # 13122, Cayman Chemical Company)
- IWP 2 [5mM]_{stock} reconstituted in DMSO per manufacturer's instructions (catalog # 3533, R&D Systems)

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)

Steps before starting:

- We recommend preparing all the necessary materials and calculations needed for the differentiation before beginning.
- 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 differentiating, and cells should be fully recovered from previous passage. Some dead cells in the media is normal, but this should not be more than 5%.
- If necessary, prepare fresh mTeSR1 media for passaging hiPSCs:
 - Thaw 5X supplement at room temperature (RT) for ~4-6 h, or at 4°C overnight. Do not thaw 5X supplement at 37°C.
 - Combine 5X supplement with 400 mL mTeSR1 and 5 mL Pen/Strep.
 - Sterile filter media with a 0.22 µm media filter before first use.
- Bring mTeSR1 media to RT on the bench. Do not warm mTeSR1 in a 37°C water bath.

5. Pre-warm Accutase in a 37°C water bath.
6. Label culture dishes for both passaging and differentiation with cell line name, clone, passage number, date, and cell density to be used.
7. Prepare mTeSR1 + ROCK inhibitor (Ri) media. After treating with Accutase, cells should remain in mTeSR1 + Ri for 24 h to promote cell survival.
 - a. Dilute Ri at 1:1000 in mTeSR1 media.
e.g., for 100 mL mTeSR1, add 100 µl Ri.
 - b. Mix well by pipetting.
Note: Lyophilized Ri stock is reconstituted in DMSO at 10 mM, per manufacturer's instruction. 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.
8. Prepare RPMI Medium 1640 + B-27 Supplement (50x), referred to in this protocol as simply "RPMI/B27(+)".
 - a. Thaw B-27 Supplement (50x) overnight at 4°C or at room temperature for ~1 h.
 - b. Mix the entire volume of thawed B-27 Supplement (50x) with a 10 mL serological pipette two times.
 - c. Combine the entire volume of B-27 Supplement (50x) (10 mL) with RPMI Medium 1640 (500 mL) and 5 mL Pen/Strep in the RPMI Medium 1640 bottle. We do not sterile filter RPMI/B27(+).
Note: We recommend not using any media that is more than 2 weeks old. Smaller volumes of the media could be made with the final concentrations of 10% (v/v) B-27 Supplement and 5% Pen/Strep, if necessary.
9. Prepare RPMI Medium 1640 + B-27 Supplement minus insulin, referred to in this protocol as simply "RPMI/B27(-)".
 - a. Thaw B-27 Supplement minus insulin overnight at 4°C or at room temperature for ~1 h.
 - b. Mix the entire volume of thawed B-27 Supplement minus insulin with a 10 mL serological pipette two times.
 - c. Combine the entire volume of B-27 Supplement minus insulin (~10 mL) with RPMI Medium 1640 in the RPMI Medium 1640 bottle. We do not sterile filter RPMI/B27(-).
Note: We recommend not using any media that is more than 2 weeks old. Smaller volumes of the media could be made with the final concentration of 10% (v/v) B-27 minus insulin, if necessary. We also strongly recommend lot-testing the B-27 Supplement minus insulin, as we have found lot-to-lot variability to affect the success of differentiation.

Table 1: Vessel Format for Passaging AICS Cells

Vessel format	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 re-suspend pellet in for accurate counting	Cell Plating Density/ approx. days to 70% confluency ^A
10 cm dish (cat. 353003, Corning)	5	10-12	3	4	7	10	500K-1M / 3-4 days

^AThe appropriate densities can be cell line-dependent. Please see recommended seeding densities in the AICS catalog.

Table 2: Vessel Format for Differentiating AICS Cells to Cardiomyocytes

Vessel format	Vol. (mL/well) for Matrigel coating	Vol. (mL/well) for media	Recommended Cell Plating Density to reach 70-85% confluency in 3 days ^A		
6 well plate (cat. 657-160, Greiner Bio-One)	1.5	3	175k	225k	250k

^AThe appropriate densities can be cell line-dependent. We recommend testing a range of seeding densities (125-350k) to accommodate for variation in cell growth rate and single cell passaging technique. We have found cell density to be very important for a successful differentiation, and therefore plate cells at a range of densities when setting up a differentiation experiment.

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 should be used within 14 days of coating, 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 ensure cultured cells 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 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 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 or overnight 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 and Table 2). 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.
 - b. Storage Protocol (for use next day or up to 2 weeks later):
 - i. Wrap Matrigel-coated 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.

Passaging AICS Cells on Plastic Tissue Culture Treated Vessels for Cardiomyocyte Differentiation Setup

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% CO₂ incubator at 37°C and must be fed fresh mTeSR1 once every day. The day of passaging is denoted as Day -3 in the differentiation protocol, where Day 0 is the start of differentiation. This protocol should take no more than 1-1.5 h to minimize the time cells are handled. We recommend starting with setting up only 1 line and gradually increasing the scale of differentiation setup to stay within this time range.

1. When cells reach 70-85% confluency, passage the cells. See Figure 1 for example images.
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 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 5 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 6-10 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 5mL serological pipette to get better single cell suspension.

- 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 (as described above in step 7a) and check again.

Note: Cells can be passaged in small clumps, but cell counting may not be accurate.

8. Transfer cell suspension to 15 mL conical tube.
9. Rinse the vessel one time with DPBS for final wash (see Table 1 for volumes). Add to conical.
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. Matrigel-coated vessels cannot be re-used, so always seed cells onto fresh Matrigel coated vessels prepared with RT mTeSR1 + Ri.
 - a. To maintain the cell line for further applications, cells are seeded directly into new 10 cm dish(es) at a specific cell count (3-4 days to confluency). See Table 1 for recommended cell plating densities. 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.

 - i. 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.
 - ii. Incubate cells at 37°C and 5% CO₂.
 - iii. Observe 24 h after seeding (see Figure 1a) and change media to mTeSR1 (no Ri).
 - iv. Change media every 24 h with mTeSR1.
 - b. To prepare cells for differentiation into cardiomyocytes, cells are seeded directly into new 6 well plate(s) at three different specific cell densities (See Table 2). To seed the cells, gently mix the entire volume three times using a serological pipette. Pipette the required volume of cell suspension into each well of the vessel, using a P200 or P1000. We recommend pre-loading the plate with mTeSR1 (3 mL/well), and adding the cell suspension dropwise throughout the well.

Note: Although we recommend 6 well plates for differentiation setups, other plate formats can be used with proper seeding density optimization.

 - i. As quickly as possible 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.
 - ii. Incubate cells at 37°C and 5% CO₂.

Note: The day of seeding for differentiation is denoted as Day -3.

Cardiomyocyte Differentiation of AICS Cells on Plastic Tissue Culture Treated Vessels

These methods are a continuation of the steps involved to passage AICS cells onto a 6 well plate, as described in Passaging AICS Cells on Plastic Tissue Culture Treated Vessels for Cardiomyocyte Differentiation Setup. We denote the day of passaging and seeding of cells as Day -3 in the differentiation protocol. AICS cells are grown in a standard 5% CO₂ incubator at 37°C. Our standard protocol is modified from Lian et al.¹ and the Conklin Lab².

13. Day -3: Passage and Seed Cells (as detailed in step 12 of Section *Passaging AICS Cells on Plastic Tissue Culture Treated Vessels for Cardiomyocyte Differentiation Setup*)
14. Day -2: mTeSR1 Media Change
 - a. Change media with 3 mL mTeSR1 per well.
 - b. Observe 24 h after seeding (see Figure 1a).
15. Day -1: mTeSR1 Media Change
 - a. Change media with 3 mL mTeSR1 per well.
16. Day 0: Start of Differentiation, CHIR Addition
 - a. Check to ensure cells look healthy and are at 70-85% confluency.
 - b. Prepare and warm 7.5 µM CHIR in RPMI/B27(-) in a water bath at 37°C. Prepare 20 mL per 6 well plate (3 mL/well with an additional 2 mL to accommodate for pipetting excess).
 - i. Dilute CHIR at 1:3333 in RPMI/B27(-). Make sure to completely thaw and mix CHIR before use.

e.g., for 20 mL RPMI/B27(-), add 6 µL CHIR
 - ii. Mix well by pipetting.

Note: Lyophilized CHIR stock is reconstituted in DMSO at 25mM, per manufacturer's instruction. We recommend making 50 µL aliquots in 1.5mL Eppendorf tubes and storing at -20°C for up to 6 months. Minimize freeze/thaw of aliquots.
 - c. Aspirate and discard old medium.
 - d. Gently add 3 mL 7.5 µM CHIR RPMI/B27(-) to each well. Do not dispense media directly onto cells, but rather introduce media at the side of the vessel, allowing it to slowly cover the vessel surface without disturbing the cells.

Note: Normally, this step takes around 1 min per 6 well plate.
 - e. Return the cells to the incubator as quickly as possible.

Note: Vessels should be out of the incubator for a maximum of 5 minutes during any media change. If necessary, remove one plate at a time during feeding, as cells are sensitive during the early part of the differentiation protocol.
 - f. Incubate cells at 37°C and 5% CO₂ for 48 h.
 - g. Start a timer or record the time at which the cells were treated with CHIR, as the addition of IWP 2 is time sensitive.

17. Day 2: IWP 2 Addition- TIME SENSITIVE

- a. Prepare and warm 7.5 μ M IWP 2 in RPMI/B27(-) in a water bath at 37°C. Prepare 20 mL per a 6 well plate (3 mL/well with an additional 2 mL to accommodate for pipetting excess).
 - i. Make sure to completely thaw and mix IWP 2 before use. Dilute IWP 2 at 1:667 in RPMI/B27(-). Excess IWP 2 should not be refrozen.
e.g., for 20 mL RPMI/B27(-), add 30 μ L IWP 2
 - ii. Mix well by pipetting.
Note: Lyophilized IWP 2 stock is reconstituted in DMSO at 5mM, per manufacturer's instruction. We recommend making 150 μ L aliquots in 1.5mL Eppendorf tubes and storing at -20°C for up to 6 months. We do not recommend refreezing thawed aliquots.
- b. Ensure 48 h have elapsed since step 16. It is important to change the media to IWP 2 as close as possible to the 48 h mark. We keep media changes within +/- 5 min of 48 h.
- c. Aspirate and discard old medium.
- d. Gently add 3 mL 7.5 μ M IWP 2 RPMI/B27(-) to each well. Do not dispense media directly onto cells, but rather introduce media at the side of the vessel, allowing it to slowly cover the vessel surface without disturbing the cells.
Note: Normally, this step takes around 1 min per 6 well plate.
- e. Return the cells to the incubator as quickly as possible.
- f. Incubate cells at 37°C and 5% CO₂ for 48 h.
- g. Stop timer or record time at which media was replaced with IWP 2 RPMI/B27(-).

18. Day 4: Media Change to RPMI/B27(-)

- a. We recommend changing the media at approximately 48 h.
- b. Warm prepared RPMI/B27(-) in a water bath at 37°C (see Steps Before Starting).
- c. Aspirate and discard old medium.
- d. Gently add 3 mL RPMI/B27(-) to each well. Do not dispense media directly onto cells, but rather introduce media at the side of the vessel, allowing it to slowly cover the vessel surface without disturbing the cells.
- e. Return the cells to the incubator as quickly as possible.
- f. Incubate cells at 37°C and 5% CO₂ for 48 h.

19. Day 6: Media Change to RPMI/B27(+)

- a. We recommend changing the media at approximately 48 h.
- b. Warm prepared RPMI/B27(+) in a water bath at 37°C (see Steps Before Starting).
- c. Aspirate and discard old medium.
- d. Gently add 3 mL RPMI/B27(+) to each well. Do not dispense media directly onto cells, but rather introduce media at the side of the vessel, allowing it to slowly cover the vessel surface without disturbing the cells.
- e. Incubate cells at 37°C and 5% CO₂ for 48 h.

20. Day 7: Qualitative Cell Morphology and Beating Check

- a. Check cultures briefly under a microscope for the occurrence of beating cells. Early beating can be observed between Day 6-14 but may be difficult to see, especially at the early timepoints. We suggest scanning 1-3 fields of view at 10x or 20x magnification to check for beating. It is important for the continued success of the differentiation to ensure that the cells do not remain outside of the incubator for more than 5 minutes.
- b. Record first day of observed beating, if applicable, and notes about morphology.

21. Days 8-14: Media Changes and Qualitative Checks

- a. Replace media with 3 mL of RPMI/B27(+) every 48 h. Do not dispense media directly onto cells, but rather introduce media at the side of the vessel, allowing it to slowly cover the vessel surface without disturbing the cells.
- b. Qualitatively check for beating cells every 24 h, as described in step 20.
Note: If the differentiation experiment was successful, you should expect to see beating between Day 6-14. This should be observed across the entire well with an even morphology, in contrast to isolated areas of beating.

22. Day 15 and onwards: Maintaining Cardiomyocytes after Day 15

- a. Replace media twice a week with 5 mL of RPMI/B27(+). Do not dispense media directly onto cells, but rather introduce media at the side of the vessel, allowing it to slowly cover the vessel surface without disturbing the cells.
e.g., 5mL/well on Tuesday and Friday
Note: Media dispensed directly on the cells risks dislodging them from the plate, particularly as the cardiomyocytes get older.

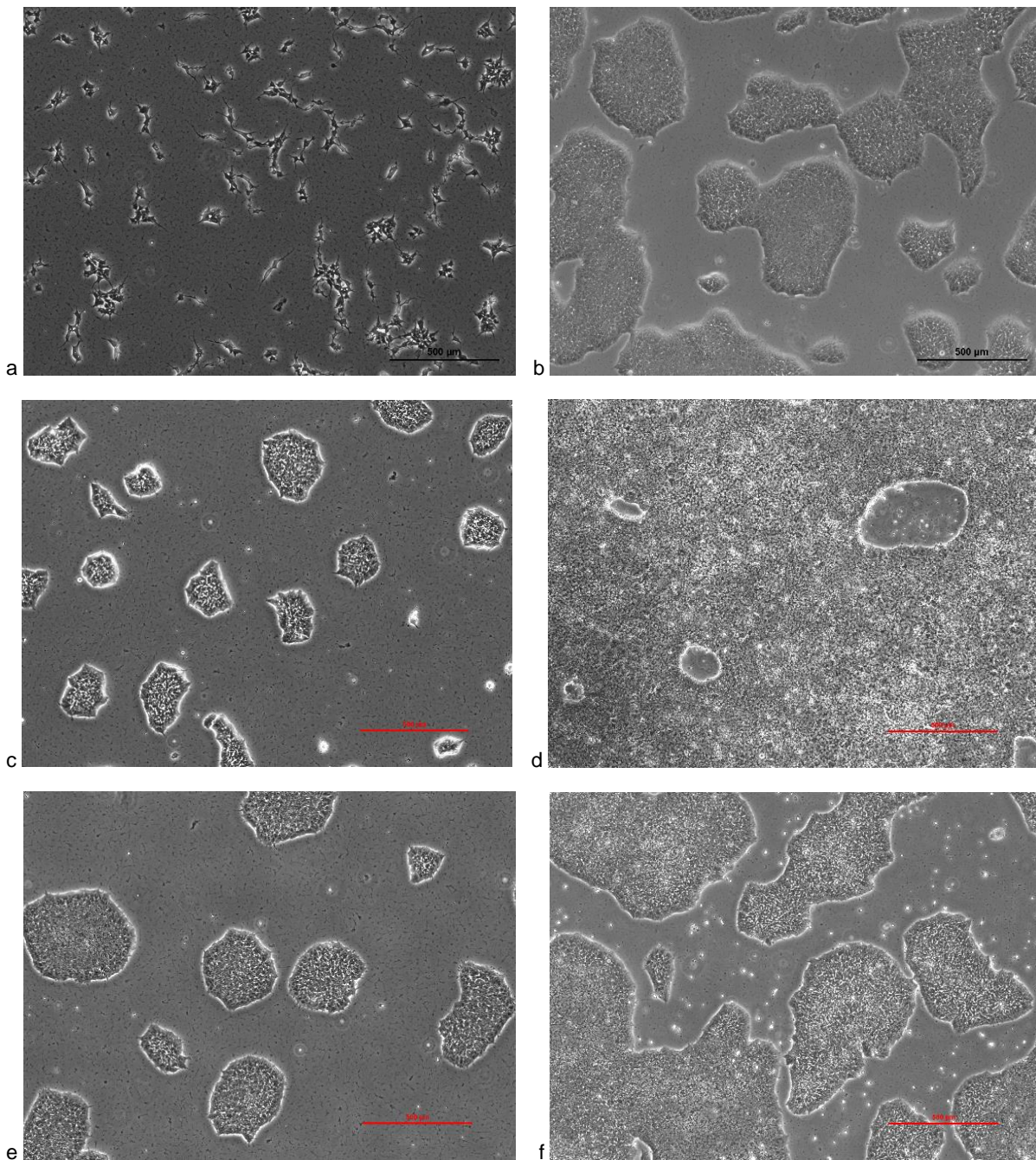


Figure 1. Examples of Confluency in hiPSC Cultures (the source cells for the differentiation): (a) 1×10^6 cells plated in 10 cm dish, 24 h after seeding. Cells maintain a “spikey” morphology due to RI treatment. Cells should be allowed to grow 3-4 days before subsequent passaging. (b) Same cells from (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.

- 1 Lian, X. *et al.* Directed cardiomyocyte differentiation from human pluripotent stem cells by modulating Wnt/beta-catenin signaling under fully defined conditions. *Nature protocols* **8**, 162-175, doi:10.1038/nprot.2012.150 (2013).
- 2 Troung, A. and So P-L. V1.4 Cardiac Differentiation Protocol. *Conklin Lab Website*, 17 June 2015, labs.gladstone.org/conklin/pages/protocols.