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Maintenance of Undifferentiated hiPSC Cultures and Differentiation to Cardiomyocytes on Glass Surfaces

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ABSTRACT

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 glass 96-well plate format using the Allen Cell Collection, derived from the hiPSC WTC parental line released by the Conklin Laboratory[2] at the J. David Gladstone Institute.

Differentiation on glass allows high resolution imaging without replating steps. It also allows observations at the earliest transitions and cell states during differentiation.

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Related SOP's:

The following SOP's are 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/uploads/8/1/9/9/81996008/aics_wtc_culture_sop_external_v1-7_200210.pdf
- Cardiomyocyte differentiation methods v1.2 (can be accessed at: - https://www.allencell.org/uploads/8/1/9/9/81996008/sop_for_cardiomyocyte_differentiation_methods_v1.2_200210.pdf

The following video tutorials demonstrate the techniques used in this protocol:

- Allen Cell Methods: Setting up the WTC parental line and our gene-edited cells for cardiac differentiation can be accessed at: https://www.allencell.org/instructional-videos-and-tutorials-for-cell-methods.html)

References:

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.
3. Roberts, B. *et al.* Systematic gene tagging using CRISPR/Cas9 in human stem cells to illuminate cell organization. *Mol Biol Cell* 28(21), 2854-2874, doi:10.1091/mbc.E17-03-0209 (2017).

MATERIALS TEXT

Required Reagents:

- ☒ mTeSR™1 500 mL Kit **Stemcell Technologies Catalog #5850** In 4 steps , referred to in this protocol as simply "mTeSR1": 400 mL basal media with provided 100 mL 5X supplement with added 5mL (1% v/v)
- ☒ Penicillin-Streptomycin (10,000 U/mL) **Gibco - Thermo Fisher Catalog #15140122** In 5 steps . *Refer to page 20 of the STEMCELL™ Technologies technical manual about preparation, storage and shelf life of this media.*
- ☒ Growth Factor Reduced (GFR) Matrigel® **Corning Catalog #354230** In 2 steps
- ☒ Growth Factor Reduced (GFR) Matrigel® phenol red-free **Corning Catalog #356231** In 2 steps
- ☒ DMEM/F-12 Media - No Phenol Red **Gibco - Thermo Fisher Catalog #11039-021** In 2 steps
- ☒ ROCK inhibitor (Ri) Y-27632 **Stemcell Technologies Catalog #72308** In 3 steps (Ri) $[10mM]_{stock}$ reconstituted in DMSO per manufacturer's instructions.
- ☒ DPBS (no Ca, no Mg) **Thermofisher Catalog #14190144** In 4 steps
- ☒ DPBS with Ca and Mg **Gibco - Thermo Fischer Catalog #14040-133** In 2 steps
- ☒ Poly-D-Lysine solution 1.0 mg/mL **Millipore Catalog #A-003-E** In 2 steps

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rhLaminin-521

- **Biolamina Catalog #LN521** In 2 steps

  Fibronectin **Gibco - Thermo**
- **Fisher Catalog #33016-015** In 2 steps

  StemPro™ Accutase™ Cell Dissociation Reagent **Gibco - Thermo**
- **Fisher Catalog #A1110501** In 3 steps

  Falcon® 100 mm TC-treated Cell Culture
- Dish **Corning Catalog #353003** In 5 steps , or similar treated plastic dishes/plates.

  96 Well glass bottom
- plate **Cellvis Catalog #P96-1.5H-N** In 5 steps

  0.22 µM Sterile Media Filter, Stericup™ 500 m **Emd**
- **Millipore Catalog #S2GPU05RE** In 3 steps , or similar.

  Dimethyl sulfoxide (DMSO) **Sigma**
- **Aldrich Catalog #D2650** In 3 steps

  RPMI 1640 Medium **Gibco - Thermo**
- **Fisher Catalog #A1049101** In 3 steps

  B-27™ Supplement (50X), serum free **Gibco - Thermo**
- **Fisher Catalog #17504044** In 3 steps

  B-27™ Supplement, minus insulin **Gibco - Thermo**
- **Fisher Catalog #A1895601** In 3 steps

  CHIR99021 **Cayman Chemical**
- **Company Catalog #13122** In 3 steps $[25mM]_{stock}$ reconstituted in DMSO per manufacturer's instructions.

  IWP 2 **R&D**
- **Systems Catalog #3533** In 3 steps $[7.5mM]_{stock}$ reconstituted in DMSO per manufacturer's instructions.

Recommended Equipment:

- Plasma Treater (Diener ATTO, or similar).
- All work described in this protocol (with the exception of plasma treating the glass-bottom plates) 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 211xg (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).
- Electronic repeater multichannel pipette capable of pipetting 100-1000uL (Rainin E4 XLS+, or similar).

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Plasma and PDL treatment of 96-well glass bottom plates 2h

1

Plate preparation should be completed ahead of time. It is easiest if completed in two steps over two days: one day for plasma and PDL treatment, one day for rhLaminin-521/Fibronectin coating.

2 Thaw Poly-D-Lysine (PDL) in **4 °C overnight** and aliquot:

a. Aliquot 2mL units into individual 50mL conical tubes from the 20mL vial of thawed PDL

b. Use directly or store aliquots at **-20 °C for up to 18 months** .

Poly-D-Lysine solution 1.0 mg/mL **Millipore Catalog #A-003-E**

3 Plasma treat 96-well glass plate for 1 minute using a 13.56MHz system at 250mTorr pressure and 100Watts power

4 To ensure sterility, expose 96-well plates and lids to UV for 10 minutes.

5 Prepare 1:20 PDL dilutions for vessel coating:

a. Thaw PDL aliquot in a **4 °C overnight** or at **Room temperature** until frozen pellet is no longer visible.

b. Dilute PDL 1:20 by adding 38mL of **Room temperature** DPBS (without Ca^{++} and Mg^{++}) to the 2mL of PDL in a 50mL conical tube.

c. Ensure diluted PDL is homogenously mixed by carefully pipetting the full mixture up and down 3-5 times using a 25mL pipette.

DPBS (no Ca, no Mg) **Thermofisher Catalog #14190144**

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6 Using a multichannel pipette, add 100µL of PDL/DPBS solution to each well and incubate at **Room temperature** for 20-60 minutes.

7 Aspirate PDL/DPBS solution and wash wells 2 times with 200µl DPBS per well.

8 Let plates dry in the TC biosafety cabinet with lid off for a minimum of 1 hour or until plates are visibly dry.

9 Dried Plasma/PDL treated plates can be wrapped in parafilm and stored at **4 °C for up to 1 week** .

rhLaminin-521/Fibronectin coating 96-well plates

10 Prepare Laminin/Fibronectin coated 96-well plates as needed. Use coated plates within 7 days.

11 Reconstitute Fibronectin:

a. Add 5mL sterile water to the bottle containing 5mg of Fibronectin to make a 1mg/mL concentration.
b. Mix thoroughly.
c. Aliquots can be stored at **-20 °C for up to 6 months** .

Fibronectin **Gibco - Thermo Fisher Catalog #33016-015**

12 Thaw rhLaminin-521 in **4 °C for 3 hours - overnight** .

rhLaminin-521 **Biolamina Catalog #LN521**

13 On ice, prepare 1:5 dilution of rhLaminin-521 (20µg/mL) in **4 °C chilled** DPBS with Ca^{++} and Mg^{++}.

DPBS with Ca and Mg **Gibco - Thermo Fischer Catalog #14040-133**

14 On ice, prepare 1:20 dilution of Fibronectin (50µg/mL) in **4 °C chilled** DPBS with Ca^{++} and Mg^{++}.

15 Diluted rhLaminin-521 and Fibronectin should be combined at a 90/10 ratio.
e.g. for 100µL solution, add 90µL 20µg/mL rhLaminin to 10µL 5µg/mL Fibronectin.

16 Using a multichannel pipette, add 100µL of rhLaminin-521/Fibronectin per well desired.

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17 Coated 96-well plates should be wrapped in parafilm and stored at **4 °C for up to 1 week** .

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

18 Prepare Matrigel coated vessels as needed for culturing undifferentiated cells. Per manufacturer protocol, coated vessels are good for 14 days.

☒ Growth Factor Reduced (GFR) Matrigel® **Corning Catalog #354230**

☒ Growth Factor Reduced (GFR) Matrigel® phenol red-free **Corning Catalog #356231**

Matrigel should be stored at **-20 °C short term** or **-80 °C long 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 #19 below*).

We use our Matrigel at a final protein concentration = 0.337mg/mL. We lot test our Matrigel to make sure cells cultured show expected morphology and expression of stem cell markers over 3-5 passages.

19 Aliquoting Matrigel:

a. From a 10mL glass vial of thawed Matrigel, aliquot 1mL units into individual 50mL conical tubes.

1. Keep 10mL source glass vial and 50mL conical tubes on ice while working.
2. When making aliquots, we recommend using 5mL serological pipettes that have been pre-chilled at **-20 °C** to prevent Matrigel from gelling inside the pipette. Make sure to change pipettes between uses (maximum of 2 minutes at **Room temperature**) to a fresh, pre-chilled pipette.

b. Store aliquots at **-20 °C for up to 3 months** .

Video demonstrating Matrigel aliquoting, available here.

20 Preparing Matrigel dilutions for vessel coating:

a. Thaw Matrigel aliquot at **4 °C for 2 hours - overnight** until frozen pellet is no longer visible.

b. Dilute thawed Matrigel by adding the appropriate volume of **4 °C chilled** DMEM/F12 media to the 1mL of Matrigel in a 50mL conical tube using a pre-chilled pipette. The volume of DMEM/F12 will be determined by the final protein concentration of the Matrigel.

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.

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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 at **4 °C or on ice** while working.

DMEM/F-12 Media - No Phenol Red **Gibco - Thermo Fisher Catalog #11039-021**

21 Add enough diluted Matrigel into each well or vessel to coat bottom (*See Table 1 at step #31/32 for volumes*). If preparing 96-well plates, we recommend transferring the diluted Matrigel into a boat 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.

22 Select either the "**Fast**" or "**Storage**" Protocol below depending on your use.
Step 22 includes a Step case.
Fast protocol
Storage Protocol

step case

Fast protocol

For same day use of coated plastic vessels.

23 Incubate at **Room temperature** for at least 1 hour and up to max 6 hours.

24 Tip plate to a 45˚ angle, aspirate and discard all excess liquid. Gently add fresh **Room temperature** mTeSR1 + Ri. Work quickly so that wells do not dry out. Try to limit time between aspirating and dispensing medium to <30 seconds.

25 Seed cells within 10 minutes of adding medium. If not seeding plate within 10 minutes, store prepared plates with medium at **37 °C for up to 1 hour** in a tissue culture incubator at 5% CO2 until ready to seed.

Medium and vessel preparation

26 Prepare fresh mTeSR1 medium for passaging hiPSCs:

a. Thaw 5X supplement at **Room temperature for 4-6 hours** , or at **4 °C overnight** . Do not thaw 5X supplement at 37˚C.
b. Combine 5X supplement with 400mL mTeSR1 and 5mL Pen/Strep.
c. Sterile filter medium with a 0.22µM medium filter before first use.

mTeSR™1 500 mL Kit **Stemcell Technologies Catalog #5850**

Penicillin-Streptomycin (10,000 U/mL) **Gibco - Thermo Fisher Catalog #15140122**

0.22 µM Sterile Media Filter, Stericup™ 500 m **Emd Millipore Catalog #S2GPU05RE**

27 Prepare mTeSR1 + 10µM ROCK inhibitor (Ri). The inclusion of Ri promotes cell survival after treatment with Accutase.

a. Dilute Ri at 1:1000 in mTeSR1 medium. *e.g. for 100mL mTeSR1, add 100µl Ri.*
b. Mix well by pipetting.

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ROCK inhibitor (Ri) Y-27632 **Stemcell Technologies Catalog #72308**

Dimethyl sulfoxide (DMSO) **Sigma Aldrich Catalog #D2650**

Lyophilized Ri stock is reconstituted in DMSO at 10mM, per manufacturer's instruction. We recommend making 250µL aliquots in 1.5mL Eppendorf tubes and storing at **-20 °C for up to 6 months** . Minimize freeze/thaw of aliquots.

28 Label culture dishes for both passaging and differentiation with cell line name, clone, passage number, date, and seeding density.

Falcon® 100 mm TC-treated Cell Culture Dish **Corning Catalog #353003**

96 Well glass bottom plate **Cellvis Catalog #P96-1.5H-N**

29 Prepare RPMI Medium 1640 + B-27 Supplement (50x), referred to in this protocol as simply "RPMI/B27(+)":

a. Thaw B-27 Supplement (50x) at **4 °C overnight** or at **Room temperature for 1 hour** .

b. Mix the entire volume of thawed B-27 Supplement (50x) with a 10mL serological pipette two times.

c. Combine the entire volume of B-27 Supplement (50x) (10mL) with RPMI Medium 1640 (500mL) and 5mL Pen/Strep in the RPMI Medium 1640 bottle. We do not sterile filter RPMI/B27(+).

RPMI 1640 Medium **Gibco - Thermo Fisher Catalog #A1049101**

B-27™ Supplement (50X), serum free **Gibco - Thermo Fisher Catalog #17504044**

Penicillin-Streptomycin (10,000 U/mL) **Gibco - Thermo Fisher Catalog #15140122**

We recommend not using any medium that is more than 2 weeks old. Smaller volumes of the medium could be made with the final concentrations of [M]**2 % volume** B-27 Supplement and [M]**1 % volume** Pen/Strep, if necessary.

30 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 at **4 °C overnight** or at **Room temperature for 1 hour** .

b. Mix the entire volume of thawed B-27 Supplement minus insulin with a 10mL serological pipette two times.

c. Combine the entire volume of B-27 Supplement minus insulin (10mL) with RPMI Medium 1640 (500mL) in the RPMI Medium 1640 bottle. We do not sterile filter RPMI/B27(-).

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B-27™ Supplement, minus insulin **Gibco - Thermo Fisher Catalog #A1895601**

We recommend not using any medium that is more than 2 weeks old. Smaller volumes of the medium could be made with the final concentrations of [M]**2 % volume** B-27 Supplement minus insulin.

We also strongly recommend lot-testing the B-27 Supplement minus insulin, as we have found lot-to-lot variability can affect the success of differentiation.

31 **Recommended volumes and plating densities:**

Table 1: Vessel Format for Passaging AICS Cells

A	B	C	D	E	F	G	H
Vessel format	Vol. (mL/well) for Matrigel coating	Vol. (mL/well) for medium	Vol. (mL/well) for Accutase	Vol. (mL/well) of DPBS for trituration	Vol. (mL/well) of DPBS for final wash	Vol. (mL/sample) of medium to re-suspend pellet in for accurate counting	Cell Plating Density/ approx. days to 70% confluency*
10 cm dish (cat. 353003, Corning)	5	10-12	3	4	7	10	500K-1M / 3-4 days

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

Falcon® 100 mm TC-treated Cell Culture Dish **Corning Catalog #353003**

Table 2: Vessel Format for Differentiating AICS Cells to Cardiomyocytes

A	B	C	D	E	F	G	H	I	J
Vessel Format	Vol. (µL/well) for PDL treatment	Vol. (µL/well) of DPBS wash	Vol. (µL/well) for rhLaminin-521/Fibronectin coating	Vol. (µL/well) for cell plating	Vol. (µL/well) for medium	Recommended cell plating density to reach 70-85% confluency in 3 days*			
96 well plate (cat. P96-1.5H-N, Cellvis)	100	400	100	100	200	8k	10k	12k	14k

*The appropriate densities can be cell line-dependent. We recommend testing a range of seeding densities (5K-20K) to accommodate for variation in cell growth rate and singe cell passaging technique. As appropriate cell confluency is crucial to differentiation success, we plate cells at a range of densities when setting up a differentiation experiment.

96 Well glass bottom plate **Cellvis Catalog #P96-1.5H-N**

Passaging AICS Cells for Stem Cell Maintenance and onto Glass 96-well plates for Cardiomyocyte Differentiation Setup

32

AICS cells are grown in a standard 5% CO2 incubator at **37 °C** and must be fed fresh mTeSR1 every day. The day of plating is denoted as Day -3 in the differentiation protocol, where Day 0 indicates the start of differentiation. This protocol should take no more than 1-1.5 hours to minimize the time cells are handled. We recommend setting up only 1 line at a time when first using this protocol, gradually increasing the scale of differentiation setup to stay

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within this time range.

Warm Accutase in a water bath at **37 °C** .

Warm mTeSR1 **Room temperature** on the bench.

StemPro™ Accutase™ Cell Dissociation Reagent **Gibco - Thermo Fisher Catalog #A1110501**

mTeSR™1 500 mL Kit **Stemcell Technologies Catalog #5850**

33 Check that the morphology of your cells is consistent with known, good hiPSC morphology (*See Figure 1 in step #56 for example images and note below for WTC culture guidelines*). Ideally, cells should be at ~70-85%% confluency for passaging and differentiating. Some cell death is normal, but should not exceed 5% of total cells.

WTC culture v1.7: Culture and Freezing Methods for WTC Derived AICS hiPSC Lines can be accessed here.

34 Aspirate and discard old medium.

35 Gently add **Room temperature** 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.

DPBS (no Ca, no Mg) **Thermofisher Catalog #14190144**

36 Aspirate and discard DPBS wash.

37 Add **37 °C pre-warmed** Accutase (*See Table 1 in step#31 for volumes*) and incubate in **37 °C** incubator for 3-5 minutes.

38 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 minutes and check again. Continue to incubate and check until cells are no longer attached to the plate surface. Cells may remain in large clumps

Avoid incubating cells with Accutase for longer than is necessary to detach from plate surface. Most AICS clonal lines derived from WTC-11 parental cell line will be fully detached from vessel surface in less than 5 minutes.

39 Add **Room temperature** DPBS to dilute out the Accutase in the vessel as follows (*See Table 1 in step #31 for volumes*):

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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, *e.g. 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. Use adequate but not excessive force, and avoid creating bubbles at all steps.

It is helpful to use a 5mL serological pipette to get better single cell suspension when using this technique.

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 (*See step a as described*) and check again.

40 Transfer cell suspension to 15mL conical tube.

41 Rinse the vessel one time with **Room temperature** DPBS for final wash (*See Table 1 in step #31 for volumes*). Add to conical.

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.

42 Spin down cells **200 x g, Room temperature , for 3 minutes** .

43 Carefully aspirate and discard DPBS/Accutase supernatant from cell pellet and re-suspend in desired volume of mTeSR1 + Ri. (*See Table 1 in step #31 for recommended re-suspension volumes for most accurate counts based on source vessel size*).

44 To maintain the cell line for further applications, cells are seeded directly into new 10cm dish(es) at a specific cell density for 3-4 days to confluency (*See Table 1 in step #31 for recommended cell plating densities*). We recommend counting cells and plating specific numbers for the most reproducible culturing conditions. Matrigel coated vessels cannot be re-used, so always seed cells onto fresh Matrigel coated vessels prepared with RT mTeSR1 + Ri.

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 minutes when seeding multiple vessels.

a. After seeding, place vessels in incubator and slide front-to-back and side-to-side at least 2-4 times to ensure even cell distribution across vessel surfaces. Keep vessels level for even cell attachment.

Cells settle and attach to Matrigel very quickly (<2-3 min), so it's important to place plates on a level surface in the incubator as quickly as possible.

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b. Incubate cells in a standard 5% CO2 incubator at **37 °C** .

c. Observe 24 hours after seeding (*See Figure 1 in step #56*) and change medium to mTeSR1 (no Ri).

d. Change medium every 24 hours with mTeSR1.

45 Prepare cells for differentiation into cardiomyocytes by seeding directly into new 96-well glass plate(s) at 3-5 different cell densities (*See Table 2 in step #31 for recommended cell plating densities*).

a. Pre-allocate a trough for each desired density to be seeded and calculate the required cell suspension volume.

b. Add volume of mTeSR1 + Ri to each trough to account for total number of desired wells (100µL per well), minus the cell suspension volume calculated.

c. Gently mix the entire volume of the cell suspension in the 15mL tube three times using a serological pipette.

d. For each desired density, pipette the required volume of cell suspension into the pre filled (mTeSR1 + Ri) trough.

e. Using a 5mL pipette for larger volumes or P1000/P1200 for smaller volumes, thoroughly mix each trough containing the cell suspension + medium four times.

f. Working quickly but carefully, use a multichannel aspirator to aspirate the rhLaminin-521/Fibronectin coating from all the wells. We recommend aspirating in a single up-down motion, with the pipette tip nearly perpendicular to the plate. Do not tilt the plate.

g. Starting with the lowest density trough and using a P200 multichannel pipette, dispense 100µL of the cell suspension + medium into the corresponding wells. We recommend loading the plate such that the pipette tip is nearly perpendicular to the plate, but not touching the bottom of the plate. Do not tilt the plate.

h. Repeat for all desired densities, working from the lowest density to the highest.

i. After seeding, allow the vessels to sit and keep level in the hood undisturbed for 3-4 minutes. Carefully move to incubator.

j. Incubate cells in a standard 5% CO_2 incubator at **37 °C** .

Although we recommend using 96-well plates for on-glass differentiation setups, other plate formats can be used with proper seeding density optimization. We denote the day of passaging and seeding of cells as Day -3 in the differentiation protocol.

Cardiomyocyte Differentiation of AICS Cells on Glass 96-well plates using small molecules

46 Day -3: Passage and Seed Cells (*See steps #32-45)*.

These methods are a continuation of the steps involved to passage AICS cells onto a 96-well plate, as described in the previous section, Passaging AICS Cells onto Glass 96-well Plates 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% CO_2 incubator at **37 °C** . Our standard protocol is modified from Lian et al.[1] and the Conklin Lab[2].

We recommend determining the appropriate concentration of CHIR99021 and IWP 2 for cardiac differentiation each time a new lot of these reagents are obtained. We suggest testing concentrations of 6µM, 7.5µM, and 9µM for each small molecule and further refining as necessary. The concentrations listed below have been optimized for the AICS Cell Collection and the particular small molecule lot we were using.

Citation: Angelique Nelson, Stephanie Q Dinh, Kaytlyn Gerbin, Melissa Hendershott, Caroline Hookway, Ruwanthi N Gunawardane (03/31/2021). Maintenance of Undifferentiated hiPSC Cultures and Differentiation to Cardiomyocytes on Glass Surfaces. https://dx.doi.org/10.17504/protocols.io.wjmfck6

47 Day -2: mTesR1 Medium Change.

a. Replace medium with 200µL **Room temperature** mTeSR1 medium per well.

48 Day -1: mTesR1 Medium Change.

a. Replace medium with 200µL **Room temperature** mTeSR1 medium per well.

49 Day 0: Start of Differentiation, CHIR99021 Addition.

CHIR99021 lot variability can greatly affect the success of cardiac differentiation. **We recommend determining the appropriate concentration of CHIR99021 for cardiac differentiation each time a new lot of these reagents are obtained.** The concentrations listed below have been optimized for the Allen Cell Collection and the particular small molecule lot we were using.

CHIR99021 is diluted in RPMI Medium 1640 + B-27 Supplement minus insulin, referred to in this protocol as simply "RPMI/B27(-)

Lyophilized CHIR99021 stock is reconstituted in DMSO at 25mM, per manufacturer's instruction. We recommend making 50µL aliquots in 1.5mL Eppendorf tubes and storing at **-20 °C for up to 6 months** . Minimize freeze/thaw of aliquots.

a. Prepare 5µM CHIR99021 in RPMI/B27(-) +Matrigel solution:

1. Thaw 1mL Matrigel aliquot at **4 °C for 2 hours - overnight** until frozen pellet is no longer visible.
2. Prepare 1:1 dilution of Matrigel:DMEM/F12 media, phenol red-free.
3. Dilute the solution of Matrigel:DMEM/F12 prepared in step 2 1:30 in RPMI/B27(-).
4. Dilute CHIR99021 at 1:5000 in the RPMI/B27(-)+Matrigel:DMEM/F12 solution prepared in step 3. Make sure to completely thaw and mix CHIR99021 before use.
5. Mix well by pipetting fully 3-4 times.
6. Warm in **37 °C water bath** :

e.g. for 20mL of of 5µM CHIR99021 RPMI/B27(-), add 4µL CHIR99021 and 0.667mL 1:1 MG:DMEM/F12 to 19.333mL of RPMI/B27(-).

CHIR99021 **Cayman Chemical Company Catalog #13122**

b. Gently aspirate and discard old medium without disturbing the bottom of the well.
c. Gently add 200µL 5µM CHIR99021 RPMI/B27(-) to each well. Do not dispense medium directly onto cells, but rather introduce medium at the side of the vessel, allowing it to slowly cover the vessel surface without disturbing the cells.

This step should take around 2 minutes per 96-well plate.

d. Return the cells to the incubator as quickly as possible.

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Vessels should be out of the incubator for a maximum of 5 minutes during any medium change. If necessary, remove one plate at a time during feeding, as cells are sensitive during the early steps of the differentiation protocol.

e. Incubate cells in a standard 5% CO_2 incubator at **37 °C** for 48 hours.

f. Start a timer or record the time at which the cells were treated with CHIR99021, as the addition of IWP 2 is time sensitive.

50 Day 2: IWP 2 Addition **TIME SENSITIVE**

IWP 2 lot variability can greatly affect the success of cardiac differentiation. **We recommend determining the appropriate concentration of IWP 2 for cardiac differentiation each time a new lot of these reagents are obtained.** The concentrations listed below have been optimized for the AICS Cell Collection and the particular small molecule lot we were using.

IWP 2 is diluted in RPMI Medium 1640 + B-27 Supplement minus insulin, referred to in this protocol as simply "RPMI/B27(-)

Lyophilized IWP 2 stock is reconstituted in DMSO at 7.5mM, per manufacturer's instructions. We recommend making 50-100µL aliquots in 1.5mL Eppendorf tubes and storing at **-20 °C for up to 6 months** . We do not recommend re-freezing thawed aliquots.

a. Prepare and warm 7.5µM IWP 2 in RPMI/B27(-) medium in **37 °C water bath** :

1. Completely thaw and mix IWP 2 before use. Dilute IWP 2 at 1:1000 in RPMI/B27(-). Excess IWP 2 should **not** be refrozen.
2. Mix well by pipetting.

e.g. for 20mL RPMI/B27(-), add 20µl IWP 2.

IWP 2 **R&D Systems Catalog #3533**

b. Ensure 48 hours have elapsed since step #49. It is important to change the medium to IWP 2 as close as possible to the 48 hour mark +/- 5 minutes.

c. Gently aspirate and discard old medium without disturbing the bottom of the well. Gently add 200µL 7.5µM IWP 2 RPMI/B27(-) to each well. Do not dispense medium directly onto cells, but rather introduce medium at the side of the vessel, allowing it to slowly cover the vessel surface without disturbing the cells.

This step should take around 2 minutes per 96-well plate.

d. Return the cells to the incubator as quickly as possible.

e. Incubate cells in a standard 5% CO_2 incubator at **37 °C** for 48 hours.

f. Stop timer or record time at which medium was replaced with IWP 2 RPMI/B27(-).

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51 Day 4: Medium Change to RPMI/B27(-). (RPMI Medium 1640 + B-27 Supplement minus insulin)

a. We recommend changing the medium at approximately 48 hours +/- 1 hour following IWP 2 change.
b. Warm prepared RPMI/B27(-) in **37 °C water bath** (*See step #30*).
c. Aspirate and discard old medium.
d. Gently add 200µL RPMI/B27(-) to each well. Do not dispense medium directly onto cells, but rather introduce medium 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 in a standard 5% CO_2 incubator at **37 °C** for 48 hours.

52 Day 6: Medium Change to RPMI/B27(+). (RPMI Medium 1640 + B-27 Supplement plus insulin)

a. We recommend changing the medium at approximately 48 hours +/- 1 hour post RPMI/B27(-) change.
b. Warm prepared RPMI/B27(+) in **37 °C water bath** (*See step #29*).
c. Aspirate and discard old medium.
d. Gently add 200µL RPMI/B27(+) to each well. Do not dispense medium directly onto cells, but rather introduce medium at the side of the vessel, allowing it to slowly cover the vessel surface without disturbing the cells.
e. Incubate cells in a standard 5% CO_2 incubator at **37 °C** for 48 hours.

53 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 Days 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 (*See Figure 2 in step #57 for example images; See Figure 3 in step #58 for an example video*).

54 Days 8-14: Medium Changes and Qualitative Checks.

a. Replace medium with 200µL of RPMI/B27(+) every 48 hours. Do not dispense medium directly onto cells, but rather introduce medium 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 24-48 hours (*See Figure 2 in step #57 for example images; See Figure 3 in step #58 for an example video*).
c. For quantitative analysis, we recommend evaluating cardiac troponin T expression by flow cytometry. We perform this analysis at or around Day 12 after differentiation.

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.

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

a. Replace medium twice a week with 250µL of RPMI/B27(+). Do not dispense medium directly onto cells, but rather introduce medium at the side of the vessel, allowing it to slowly cover the vessel surface without disturbing the cells.

e.g. 250µL/well on Tuesday and Friday.

Medium dispensed directly on the cells risks dislodging them from the plate, particularly as the cardiomyocytes get older.

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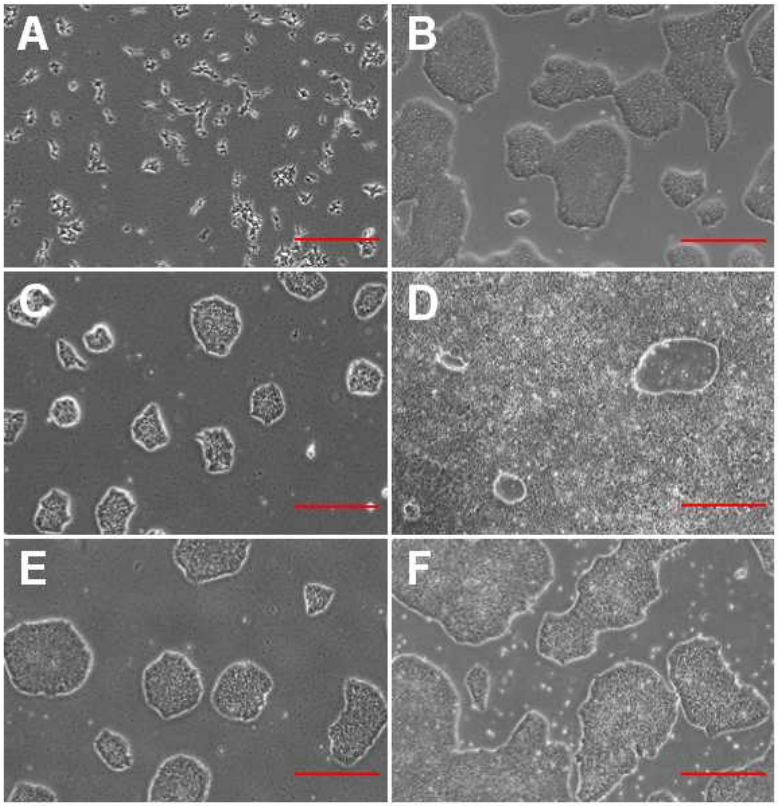

Figure 1: Examples of hiPSC confluency: **(A)** 1 x 10^6 cells plated in 10cm dish, 24 hours after seeding. Cells maintain a spikey morphology due to Ri treatment. Cells should be allowed to grow 3-4 days before subsequent passaging. **(B)** Same cells from after 3 days of growth. Cells have good mature 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)** Another example of a culture that has good mature stem cell morphology and is at an ideal density to be passaged. Scale bar, 500 microns.

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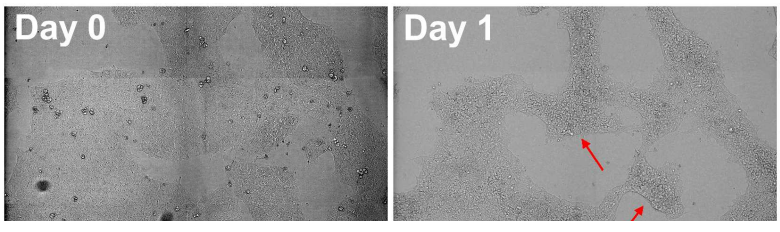

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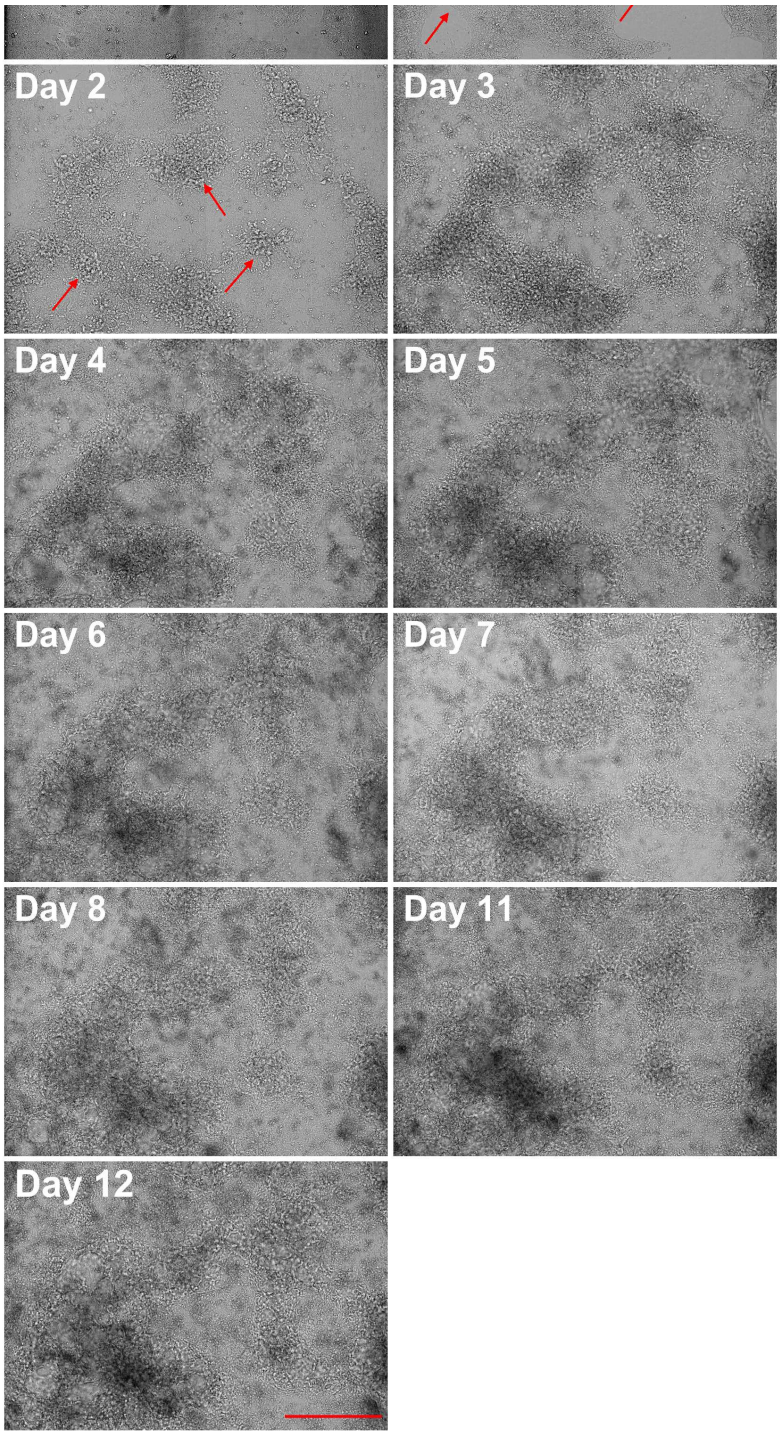

Figure 2: Day 0: hiPSC imaged 3 days after plating immediately before the addition of CHIR99021 and start of directed differentiation. **Day 1:** Contraction of differentiating colonies is apparent, indicated by the arrows. **Day 2:** Initial appearance of a multi-layered differentiating culture, indicated by arrows. **Day 3-12:** Continued development of the multi-layered tissue. From **Day 7** onwards, this tissue contains beating cardiomyocytes. Each panel shows single plane images tiled across the same region, using a 3D spinning disk confocal microscope. Scale bar, 500 microns.

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Figure 3: **(A)** Time-lapse images of a beating cardiomyocyte culture at day 11. Large region of well shown for context. Smaller region shown in transmitted light **(B)** and fluorescent **(C)** channels. Fluorescent channel shows the localization of the mEGFP-tagged gene TNNI1. Movies play at real time. Scale bars, 100 microns.

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