Differentiation of Human Pluripotent Stem Cells in to Cardiomyocytes

Background

This protocol describes methods for the culture and cardiac differentiation of human induced pluripotent stem cells (hiPSC). In this approach, hiPSC are grown in a modified version of chemically defined E8 and passaged non-enzymatically. A Rho kinase inhibitor (10 μM Y27632) is used for 24 h after splitting to improve cell survival, split ratio reliability, and reduce selective pressure. Cells are passaged at a **1 to 12** split ratio (equal to seeding densities of ~**1.25 x 10^4** cells per cm^2), E8 is changed every day, and the cells are grown for 3-4 days, by which time they become 70-80% confluent. These cells are either passaged or differentiated. **The timing (3-4 days of growth) is crucial to the efficiency of the protocol.**

Human pluripotent stem cells can be induced to differentiate towards cardiomyocyte lineage using small molecules to modulate the WNT signaling pathway, first with a GSK3B inhibitor to potentiate WNT signaling and then 2 days later with a WNT inhibitor to attenuate WNT signaling (1-3). This protocol is designed to be as simple as possible. It is optimized to be compatible with hiPSC grown in E8 on Matrigel and passaged with EDTA. This protocol is highly reproducible, generating ~80-95% $TNNT2^+$ cells in >50 hiPSC lines tested. In this protocol, cells are differentiated as a monolayer, eliminating embryoid body formation variability but requiring careful control of pluripotent cell seeding density. We have empirically demonstrated that higher splitting ratios (lower seeding densities) result in higher $TNNT2^+$ cell yields. The protocol I sbased on the modulation of WNT pathway using small molecules. At the start of differentiation (d0), the E8 media is switched to cardiac differentiation media (RPMI+B27-insulin) supplemented with CHIR99021 – an inhibitor of GSK3b – to activate the WNT signaling pathway. After 72 h (d3) the cells are treated with IWR-1 – a WNT inhibitor. Contracting cells will be seen from d8-d9. CDM-L (with no D-glucose, with L-lactic acid) to is used to metabolically select cardiomyocytes (4). From d16 to d30, cardiomyocytes are maintained in RPMI 1640 supplemented with B27 supplement (CDM-B27 with insulin). Beating cardiomyocytes can, in theory, maintained indefinitely in CDM-B27 (>6 months) with media changed every other 2-3 days.

The two major factors are the seeding density and the narrow range at which the CHIR99021 is effective in this system. A simple optimization experiment is to seed the cells at 1:6, 1:9, and 1:12 and treat with 4 μM, 6 μM, or 8 μM of CHIR99021 during the d0 to d2 window and assess for cell survival and epithelial to mesenchymal transition.

Materials

- DMEM/F12, 1L (Corning, cat. no. 10-092-CM)
- L-ascorbic acid 2-phosphate tri sodium salt (Wako USA, cat. no. 321-44823)
- Recombinant human insulin (Life Technologies, cat. no. A11382ij)
- Recombinant human transferrin (Sigma-Aldrich, cat. no. T3705-5G)
- Heparin sodium salt (Sigma-Aldrich cat. no. H3149-250KU) make a 10 mg/mL stock solution in WFI water, store at 4 °C
- Sodium selenite (Sigma-Aldrich, cat no. S5261-10G), make a 1.4 mg/mL stock solution, (70 mg/50 mL WFI water), make 1 mL aliquots and store at -20 °C
- Recombinant human FGF2 (Peprotech, cat. no. 100-18B)

- Recombinant human TGFB1 (Peprotech, cat. no. 100-21)
- 500 mL DMEM/F12 (Corning, cat. no. 10-092-CV)
- RPMI 1640 (Life Technologies 11875-093
- B27 Supplement (50X) (Life Technologies 17504044)
- B27 minus insulin supplement (50X) (Life Technologies A1895601)
- IWR-1 (Selleck S7086)
- Matrigel™ hESC-Qualified Matrix (Corning, cat. no. 354277)
- Gentle Dissociation Solution (Stem Cell Technologies 07174)
- DPBS without calcium and magnesium (Corning, cat. no. 21-031-CV)
- RPMI 1640 (Corning, cat. no. 10-040-CM)
- Recombinant human albumin (ScienCell, cat. no. OsrHSA-100)
- WFI water (Corning, cat. no. 25-055-CV)
- TrypLE™ Select Enzyme (10X), no phenol red (Life Technologies, cat. no. A1217701)
- 1 N HCl (Fisher, cat. no. SA48-500)
- 10 N NaOH (Fisher, cat. no. SS255-1)
- Y-27632-HCl (Biorbyt, cat. no. orb154626), 10 mM 600 µL aliquots in WFI water, store at -20 °C
- CHIR-99021-HCl (Biorbyt, cat. no. orb154612), 10 mM stock, 1 mL aliquots in DMSO, store at -20 °C
- 15 and 50 mL polystyrene conical tubes (Corning, cat. no. 352097/352098)
- Luna Automated Cell Counter (Logos Biosystems, cat. no. L20001)
- Cell counting slides and trypan blue (Logos Biosystems, cat. no. L12003/T13101)
- 96-well V-bottom plate (Thermo Scientific, cat. no. 249952)
- Reagent reservoir (Accuflow, cat. no. D520051)
- FBS (Life Technologies, cat. no. 10082-147)
- Coolcell LX (Biocision, cat. no. BCS-405)
- Cyrovials (Greiner, cat. no. 122261)
- 6, 12, 24, 96, 384-well cell culture plates (Greiner, cat. no. 657160, 665180, 662160, 655090, 781091)
- 100 and 145 mm dishes (Greiner, cat. no. 664160, 639160)
- 15 and 50 mL conical tubes (Corning Falcon, cat. nos. 352097, 352098)
- 2 mL aspiration pipettes (Corning Falcon, cat. no. 357558)
- 5, 10, 25, and 50 mL pipettes (Corning Falcon, cat. nos. 356543, 356551, 356525, 356550)
- 250, 500, and 1000 mL PES 0.2 µm filters (Thermo Scientific Nalgene, cat. no. 568-0020, 569-0020, 567-0020)
- 0.6 mL sterile microtubes (Accuflow, cat. no. 280060-S)
- 2 mL sterile microtubes (Accuflow, cat. no. 280200-S)
- Disposable collection canisters (Allied Healthcare Products, cat. no. 20-08-0002)
- 100 mL glass beaker (Fisher)
- Magnetic stir bar (Fisher)
- Magnetic stir platform such as IKA Lab Disc
- Analytical balance such as Mettler Toledo ML204
- pH meter such as Mettler Toledo SevenCompact S220
- Cell culture incubators with 5% CO_2 such as Panasonic Cell IQ

- Centrifuge such as Eppendorf 5810 or Sorvall Legend X1
- Class II, Type A2 or better biosafety cabinet such as Labconco Purifier Cell Logic+

NOTE: we have found that 5% O_2 (hypoxic) incubators are not essential for the success of this protocol

NOTE: we do not place any media in a 37 °C water bath due to concerns of the temperature stability of the FGF2 in the media (Chen et al 2012), bringing the media to room temperature is sufficient and we have not noted any effect on cell growth by using 4 °C media

Making Matrigel-Coated Plates

Thaw a bottle of Matrigel™ hESC-Qualified Matrix overnight on ice at 4 °C. Prepare aliquots based on the Lot -specific instructions, typically 270-350µl (see product insert). **Make sure to always keep matrigel on ice when thawing and handling to prevent it from gelling.**

1. Add one aliquot of Matrigel to 50 mL of 4 °C DMEM/F12 (enough for 4 plates).
2. Mix and plate at 2 mL per well of a 6-well plate (or equivalent).
3. Place plates at RT for at least 30 min. Plates may be kept at 37 °C for 1-2 weeks without risk of wells drying out and being unusable.

NOTE: we use 2 mL per well so that the plates do not dry out with extended storage at 37 °C.

E8 media

E8 media is commercially available from ThermoFisher Scientific or Stem cell Technologies. Alternatively, the E8 media can be prepared in the lab as described by ***Chen, G. et. al., Nature Methods 8, 424–429 (2011)***. Below are directions for making 100 x 1.5 mL E8 supplement aliquots. This will generate 100 L of E8.

1. Add 50 mL of room temperature WFI water to 250 mL cell culture bottle, slowly add 6.4 g of L-ascorbic acid 2-phosphate, inverting intermittently. Mix until clear.
2. Place 46 mL of room temperature WFI water in a sterile 100 mL glass beaker with a stir bar on a stir plate, add 2 g of insulin, pH to 3 with 1 N HCl to dissolve (~1.4 mL), pH to 7.4 with 10 N NaOH (~200 µL), add 500 mg of transferrin, 1 mL of 10 mg/mL heparin sodium salt and 1 mL of 1.4 mg/mL sodium selenite. Make up to 50 mL and add to the ascorbic acid solution.

NOTE: Insulin will go back through its isoelectric point and transiently come out of solution as it progresses from pH 3 through to pH 8.4. As it passes pH 7, it will go back in to solution. If the mixture does not stay in solution, then increase the pH with additional 10 N NaOH (~200 µL).

3. Add 48 mL of WFI water to a 50 mL Falcon tube, use this to resuspend the contents of 10 x 1 mg vials of FGF2.
4. Add 2 mL of WFI water to 2 x 100 µg vials of TGFB1
5. Add the growth factors to the 250 mL cell culture bottle, mix well and filter sterilize. Make 1.5 mL aliquots in 2 mL microfuge tubes, label and store at -20 °C.

E8

DMEM/F12 with L-glutamine and	1000 mL	Corning 10-092-CM
64 µg/mL L-ascorbic acid 2-phosphate	1.5 mL	Wako 321-44823
20 µg/mL insulin		Life Technologies A11382
5 µg/mL transferrin		Sigma-Aldrich T3705-1G
14 ng/mL sodium selenite		Sigma-Aldrich S5261-10G
100 ng/mL FGF2		Peprotech 100-18B
2 ng/mL TGFβ1		Peprotech 100-21
100 ng/mL heparin sodium salt		Sigma H3149-250KU

Add one thawed aliquot to a bottle of DMEM/F12, filter sterilize, media is stable at 4 °C for >4 weeks.

E8Y: E8 suplemented with Rho-kinase inhibitor -Y27632

E8	500 mL	
10 µM Y27632 (10 mM)	0.2 mL	Biorbyt orb154626

Filter sterilize, E8Y is stable in E8 at 4 °C for >4 weeks.

Thawing hiPSC

1. hiPSC should be either generated in house or can be obtained from available depositories e.g Wicell, Coriell or the Stanford CVI iPSC Biobank.
2. Remove vial from liquid nitrogen, place in 37 °C water bath until only a sliver of ice remains. Transfer the vial content dropwise (~1 mL) to a 15 mL conical tube filled with 4 ml of pre-warmed E8Y.
3. Centrifuge at 200 × *g* for 4 min. Carefully aspirate the supernatant. Re-suspend the cell pellet in 2 mL of E8Y and transfer to 1 well of a Matrigel-coated 6-well plate
4. Change media every 24 h with fresh E8 (without Y27632). Cells should be 70-80% confluent in 3-4 days, but could take longer depending on the viability of the iPSCs.

Daily E8 medium change

1. Aspirate medium in wells. Be careful not to use the same aspiration tip on different cell lines
2. Add 2 mL of E8 per well. Repeat for 3-4 days total.

Passage of hiPSC with Gentle Dissociation Solution

1. Ideally cells should have reached 60-80% confluence in 3-4 days (adjust split ratio accordingly, typically 1/6-1/12.
2. Aspirate the E8 culture medium.
3. Add 2 mL per well of Gentle dissociation Solution, incubate for 6-8 min at RT (in hood)
4. Whilst waiting, aspirate medium from Matrigel-coated plates and replace with 1 mL of **E8Y.**
5. Aspirate Gentle Dissociation Solution from each well.
6. Add 1 mL of **E8Y** medium to the well. Gently detach the colonies by scraping with a serological pipette or a cell scraper. Add 6 mL of **E8Y** for a 1:6 split.
7. Mix gently and transfer 1 mL in each well of the 6-well plate (2ml per well total volume).

NOTE: In this protocol we aim to keep the pluripotent cells in the logarithmic growth phase. Cells should not be allowed to become more than 90% confluent (i.e. 90% of the culture surface covered with cells).

Preparing Cardiomyocyte Differentiation Media: CDM, CDM-C, CDM-IWR, CDM-B27, and CDM-L media

CDM medium

1. Thaw a vial of B27 minus insulin supplement overnight at 4°C.
2. Add 10ml of the supplement to 0.5L of RPMI media.

RPMI 1640	0.5 L	Life Technologies 11875-093
B27 minus insulin supplement (50X)	10ml	Life Technologies A1895601

Day 0: Beginning differentiation with CDM-C medium

CDM-C medium

CDM	50 mL	
6 µM CHIR99021-HCl (10 mM stock)	30 µL	Biorbyt orb154612

CDM-C is stable at 4 °C for ~2 weeks.

1. Aspirate E8 medium in all wells
2. Add 3 mL of CDM-C per well
3. Return to the incubator – incubate for 48h

NOTE: Some cell death will be noted; hPSC that undergo epithelial to mesenchymal transition will result in higher cardiomyocyte yield than those that maintain an epithelial morphology.

Day 2: Change to CDM medium (without CHIR99021)

1. Aspirate medium in all wells
2. Add 3 mL of CDM per well

Day 3: Change to CDM-IWR medium

1. Aspirate medium in all wells
2. Add 3 mL of CDM-IWR per well

CDM-IWR medium

CDM	50 mL	
3 µM IWR-1 (10 mM stock)	15 µL	Selleck S7086

CDM-IWR is stable at 4 °C for ~2 weeks.

Day 5 and 7: Change to CDM medium

1. Aspirate medium in all wells
2. Add 3 mL of CDM

Day 9 and 11: change media to CDM-B27

1. Aspirate medium in all wells

2. Add 3 mL of CDM-B27

CDM-B27 medium

RPMI 1640	0.5 L	Life Technologies 11875-093
B27 Supplement (50X)	10 mL	Life Technologies 17504044

There is no need to filter sterilize, CDM-B27 is stable at 4 °C for ~4 weeks.

Day 13: Change CDM-L medium

1. Aspirate medium in all wells
2. Add 3 mL of CDM-L

CDM-L medium:

RPMI 1640, no Glucose	0.5 L	**11879-020**
B27 minus insulin supplement (50X)	10 mL	Life Technologies A1895601
Sodium DL-lactate solution (60% w/v)	0.5 mL	Sigma L7900

CDM-L is stable at 4 °C for ~2 weeks.

Day 16: Change media to CDM-B27

1. Aspirate medium in all wells
2. Add 3 mL of CDM-B27

NOTE: At least 3 days of metabolic selection is required. Some non-cardiomyocyte cell types can survive metabolic selection. Selection can be extended up to 6 days.

Day 18-30: maintain cardiomyocytes in CDM-B27

1. Aspirate medium in wells
2. Add 3 mL of CDM-B27
3. Change media every 2 days

Dissociation of cardiomyocytes and re-plating

1. On Day-30 aspirate medium in wells.
2. Wash cells with 2 mL D-PBS.
3. Aspirate. Add 1.5 mL of TrypLE (10x), incubate for 10-15 min at 37 °C
4. Gently pipette up and down with a P1000 ~5-6 x to dislodge cells and to break up aggregates. Avoid forming bubbles.
5. Transfer cells to a 15 mL Falcon tube, and centrifuge at 300 *g* for 4 min.
6. Decant (do not aspirate). Resuspend the cell pellet in 5 mL CDM-B27 and pipette up and down a few times
7. Take a small aliquot and count cells with automated cell counter.
8. Centrifuge at 300 *g* for 4 min.
9. Decant the media.
10. Dilute cells to 1 x 10^6 per mL with **CDM-B27 (+10%KSR).** Filter through a 100um Nylon mesh filter.

11. Seed into Matrigel-coated plates (6-well @ 1.5 x 10^6 cell per well; 12-well @ 7 x 10^5 cells per well ; 24-well plate @ 350,000 cells per well, 96-well plate at 80,000 cells per well, or 384-well plate at 20,000 cells per well
12. Change media every other day, cell should resume contraction after ~1-2 days

NOTE: We have found a significant improvement in survival by adding 10% Knockout Serum replacement in the CDM-B27 for 48-72h after dissociation.
NOTE: The addition of Y27632 or Thiazovivin does not improve cardiomyocyte cell survival after dissociation.

Freezing cardiomyocytes

1. After dissociation, follow the protocol up to step 9 (see above).
2. Resuspend at 3-4 x10^6 per mL in Cryostor 10 and transfer 1 mL to a cryovial and place in a Biocision CoolCell. Typically, 1 well of a 6-well plate is frozen in 1 vial (1ml)
3. Place CoolCell at -80 °C overnight and then transfer vials to liquid nitrogen. Expect 60-70% survival after thawing.

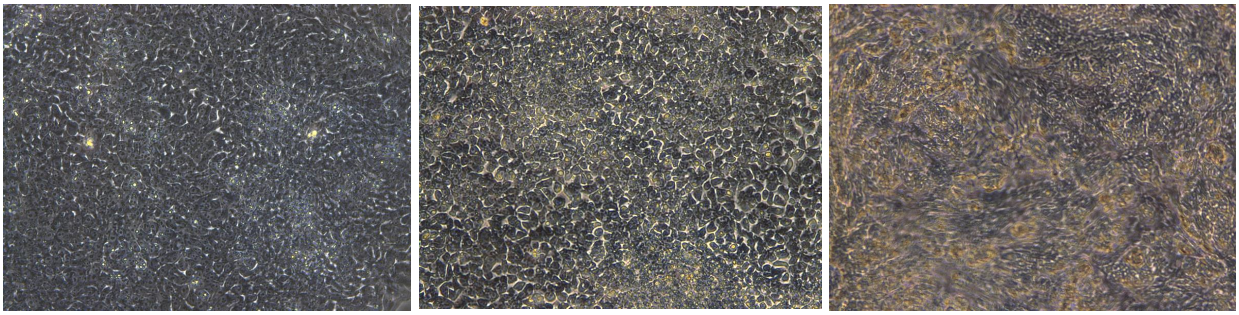
NOTE: We have also tested BamBamker for the cryopreservation of cardiomyocytes with good recovery.

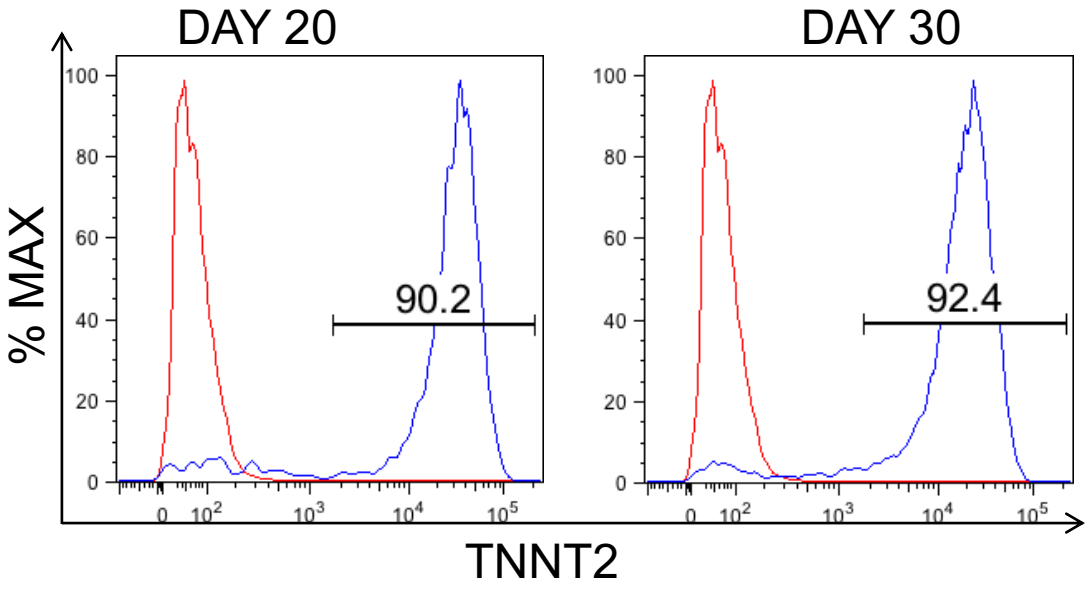
Thawing cardiomyocytes

1. Remove vial from liquid nitrogen and place in a 37 °C water bath for approximately 1 min until there is just a sliver of ice left.
2. Transfer vial contents to a 15 mL Falcon tube, add ~10 mL of CDM-27 supplemented with 10% KSR dropwise.
3. Invert to mix then centrifuge at 300 g for 4 min.
4. Resuspend pellet in CDM-B27 + 10% KSR at a ratio of ~1 mL per million cells to be plated in a 12-well plate or equivalent.
5. After 48 h replace media with CDM-B27 then change media every other day.

Representative Images

d0 d2 d9





FACS Analysis of Cardiomyocytes Produced from hiPSC

Background

Although no good surface marker for cardiomyocytes exists, the intracellular expression of Troponin T is a well-established surrogate for cardiac differentiation efficiency. We will use fluorescence activated cell sorting (FACS) to analyze the efficiency of cardiomyocyte differentiation among our differentiating hiPSC. This method is not suitable for the isolation of cardiomyocytes as fixation and permeabilization is required. The procedure consists of dissociating the cells, permeabilzing with methanol, applying antibodies, and then analyzing by FACS.

Materials

1. Day 15+ hiPSC-derived cardiomyocytes	
2. TrypLE 10x	Life Technologies A1217701
3. Cell scraper	GeneMate T-2443-4
4. D-PBS w/o Ca^{2+} and Mg^{2+})	Life Technologies 14190144
5. BSA	Sigma A1470-100G
6. FBS	Life Technologies A3160401
7. PFA (20%)	Electron Microscope 15713S
8. Isotype negative control (mouse IgG1)	Thermo MA5-14453
9. Anti-Troponin T antibody	Thermo MS-295-P
10. Falcon FACS Tubes	BD Falcon 352235
11. Triton X-100	Sigma X100-100ML
12. Zenon® Alexa-488 Mouse IgG1 Labeling Kit	Life Technologies Z25002

Reagents

- Fixation Buffer: 8% PFA in D-PBS. Make fresh.
- Blocking buffer: 4% BSA / 4% FBS in D-PBS. Filter sterilize store for up to 1 month at 4°C.
- Permabilization Buffer: 0.1% Triton-X in D-PBS. Store for up to 2 months at RT.

Protocol

Dissociation of cardiomyocyte monolayers

1. On Day-30 aspirate medium in wells
2. Wash cells with 2 mL D-PBS
3. Aspirate. Add 1.5 mL of pre-warmed TrypLE 10X, incubate for 10-15 min at 37 °C
4. Gently pipette up and down with a P1000 to dislodge cells and to break up aggregates. Avoid forming bubbles.
5. Transfer cells to a 15 mL Falcon tube, and centrifuge at 300 *g* for 4 min.
6. Re-suspend the pellet in 2 mL D-PBS (w/o Ca^{2+} and Mg^{2+}) and pipette up and down 10 times.

Fixation and permeabilization

7. Add an equal volume (2 mL) of 8% PFA, vortex, incubate for 15 min at RT.
8. Centrifuge at 300 *g* for 4 min. Re-suspend pellet in 1 mL of Permabilization Buffer , incubate for 30 min at RT.
9. Add 1 mL of blocking buffer, and incubate at RT for 30 min. Mix gently every 10 min.
10. Centrifuge at 300 *g* for 5 min. Re-suspend pellet in 0.6 mL blocking buffer.
11. Split sample into two 1.5ml eppendorf tubes (300ul each).

Primary antibody staining

12. Label the anti-Troponin T Antibody (and Isotype IgG1) with the Zenon kit. Follow Zenon protocol. Mix 5 µL of Troponin antibody with 5 µL of Zenon reagent, mix and incubate for 5 min at RT. Add 5 µL of blocking reagent (provided in the kit), mix and incubate another 5 min at RT.
13. Add 4 µL of cardiac Troponin T –Zenon conjugated Ab to one tube (1:150 dilution) and 4µl of Isotype IgG1 control to the other, mix and incubate for 60 min at RT.
14. Centrifuge at 300 *g* for 5 min. Invert to remove the supernatant.
15. Re-suspend in 300 µL D-PBS.
16. Centrifuge at 300 *g* for 5 min. Carefully invert the tube to remove the supernatant.
17. Re-suspend in 500 µL D-PBS and transfer to the FACS tube (pass through the filter).
18. Proceed to FACS