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Chemically Defined Culture and Cardiomyocyte Differentiation of Human Pluripotent Stem Cells

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Abstract

Since the first discovery that human pluripotent stem cells (hPSCs) can differentiate to cardiomyocytes, efforts have been made to optimize the conditions under which this process occurs. One of the most effective methodologies to optimize this process is reductionist simplification of the medium formula, which eliminates complex animal-derived components to help reveal the precise underlying mechanisms. Here we describe our latest cost-effective and efficient methodology for the culture of hPSCs in the pluripotent state using a modified variant of chemically defined E8 medium. We provide exact guidelines for cell handling under these conditions, including non-enzymatic EDTA passaging, which have been optimized for subsequent cardiomyocyte differentiation. We describe in depth the latest version of our monolayer chemically defined small molecule differentiation protocol, including metabolic selection-based cardiomyocyte purification and the addition of triiodothyronine to enhance cardiomyocyte maturation. Finally, we describe a method for the dissociation of hPSC-derived cardiomyocytes, cryopreservation, and thawing.

Keywords

Human induced pluripotent stem cells; differentiation; cardiac; cardiomyocyte; chemically defined; monolayer

BASIC PROTOCOL

This unit describes methods for the culture and cardiac differentiation of human pluripotent stem cells (hPSCs), which include human embryonic stem cells (hESCs) and human induced pluripotent stem cells (hiPSCs) (Burridge et al., 2014). In this approach, hiPSCs are grown in a modified version of chemically defined E8 medium on low-density (1:400) Matrigel. E8 medium is changed every day, and the cells are grown for 3–4 days, by which time they become 65–85% confluent; afterwards, cells are either passaged or differentiated. Cells are passaged non-enzymatically using EDTA, and a Rho-associated protein kinase inhibitor (10

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μM Y27632) is used for 24 hours after splitting to improve cell survival and split ratio reliability, and to reduce selective pressure. Cells are passaged at a 1:15 split ratio (equal to seeding densities of ~1.25×10^4 cells per cm^2). The timing for splitting and subsequent 3–4 days of growth are crucial to the efficiency of the protocol.

In this protocol, hiPSCs are differentiated as a monolayer, which eliminates embryoid body formation variability (Burridge et al., 2007) but requires careful control of pluripotent cell seeding density. Differentiation towards cardiomyocyte lineage is induced 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 (Burridge et al., 2014; Gonzalez et al., 2011; Lian et al., 2012). The basic culture medium used throughout cardiac differentiation and cardiomyocyte maintenance is CDM3 (Burridge et al., 2014), a chemically defined medium consisting of RPMI 1640, rice-derived recombinant human albumin, and L-ascorbic acid 2-phosphate. This protocol is designed to be very simple and cost-effective. This protocol is highly reproducible, and has been shown to generate ~80–95% $TNNT2^+$ cells in >200 hiPSC lines we have tested. We have empirically demonstrated that higher splitting ratios (lower seeding densities) result in higher $TNNT2^+$ cell yields. At day 0, the medium is changed to CDM3-C containing 6 μM CHIR99021 (GSK3B inhibitor). After 48 hours (day 2), the medium is changed to CDM3-C59 containing 2 μM Wnt-C59 (WNT inhibitor). The medium is then changed with CDM3 every other day and contracting cells will be seen from day 8 to day 9. From day 10 to day 16, CDM3 is replaced with CDM3-L (with no D-glucose but with L-lactic acid) to metabolically select and purify cardiomyocytes (Tohyama et al., 2013). From day 20 to day 30, CDM3 is replaced with CDM3-T (with triiodothyronine) to enhance cardiomyocyte maturation (Yang et al., 2014). Beating cardiomyocytes can be maintained indefinitely in CDM3 (>6 months). The time course of pluripotent growth and cardiac differentiation is shown in Figure 1.

The major optimizable factors are the seeding density (1:12 to 1:20), the number of days of pluripotent growth (3 or 4 days), and the narrow range at which the CHIR99021 is effective in this system (5–7 μM). A simple experiment is to seed the cells at densities of 1:12, 1:15, and 1:20, treat them with 5 μM, 6 μM, or 7 μM of CHIR99021 during the day 0 to day 2 window, and assess for cell survival and epithelial to mesenchymal transition. We found only a minimal influence of modification of the later steps of differentiation.

Materials

Matrigel coated plates (see recipe)

E8 medium (see recipe)

E8-Y medium (see recipe)

0.5 mM EDTA in DPBS (see recipe)

CDM3 medium (see recipe)

CDM3-C medium (with CHIR99021; see recipe)

CDM3-C59 medium (with Wnt-C59; see recipe)

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CDM3-L medium (without glucose; see recipe)

CDM3-T (with T3; see recipe)

WFI water (Corning, cat. no. 25-055-CV)

TrypLE Express (Life Technologies, cat. no. 12605-036)

1 N HCl (Fisher Scientific, cat. no. SA48-500)

10 N NaOH (Fisher Scientific, cat. no. SS255-1)

Y27632-HCl (Biorbyt, cat. no. orb154626), 1 mL aliquots at 10 mM in WFI water, store at −20 °C

CHIR99021-HCl (Biorbyt, cat. no. orb154612), 600 μL aliquots at 10 mM in DMSO, store at −20 °C

Wnt-C59 (Biorbyt, cat. no. orb181132), 200 μL aliquots at 10 mM in DMSO, store at −20 °C

L-lactic acid (Wako Chemicals USA, cat. no. 129-02666), make a 1 M stock solution (supplied as 10 M) in 1M HEPES (Corning, cat. no. 25-060-CI), store at 4 °C

3,3′,5-Triiodo-L-thyronine sodium salt (Sigma-Aldrich, cat. no. T6397), make a 2 μg/mL stock solution by adding 1 mL 1N NaOH to 100 μg 3,3′,5-triiodo-L-thyronine; mix and top up to 50 mL with WFI water, make 100 μL aliquots

Liberase TH, 260 U/50 mg (Roche, cat. no. 05401151001), resuspend in 10 mL WFI water and make 500 μL aliquots, store at −20°C

DNase I, 277 U/μL (Life Technologies, cat. no 18047-019)

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)

50 mL reagent reservoir, individually wrapped (E&K Scientific, cat. no. D620051)

BamBanker (Wako Chemicals USA, cat. no. 302-14681)

FBS (Life Technologies, cat. no. 10082-147)

DMSO (Fisher Scientific, cat. no. BP231-1)

Coolcell LX (Biocision, cat. no. BCS-405)

Cyrovials (Greiner, cat. no. 122261)

150 mL cell culture bottle (Corning, cat. no. 431175)

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 polystyrene conical tubes (Corning Falcon, cat. nos. 352097, 352098)

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2 mL aspiration pipettes, individually wrapped (Greiner, cat. no. 710183)

5, 10, 25 mL pipettes sterile, individually wrapped (Corning Falcon, cat. nos. 357543, 357551, 357535)

250, 500, and 1000 mL PES 0.2 µm filters (Thermo Scientific Nalgene, cat. no. 568-0020, 569-0020, 567-0020)

100 µm cell strainer (Corning Falcon, cat. no. 352360)

0.6 mL sterile microtubes (E&K Scientific, cat. no. 280060-S)

2 mL sterile microtubes (E&K Scientific, cat. no. 280200-S)

100 mL glass beaker (Fisher Scientific)

Magnetic stir bar (Fisher Scientific)

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 or 5% CO_2/5% O_2 such as Thermo Scientific Heracell VIOS

Centrifuge such as Thermo Sorvall ST8

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 before use due to concerns of the temperature stability of the FGF2 in the media (Chen et al., 2011). Bringing the media to room temperature is sufficient, and we found no noticeable effects on cell growth by using 4 °C media.

Thawing and initial plating of hiPSCs

1. Generate hiPSCs in-house or obtain from reputable sources such as WiCell (http://www.wicell.org/) or Stanford CVI Biobank (http://med.stanford.edu/scvibiobank.html) (Marx, 2015; McKernan and Watt, 2013).

2. Remove the vial from liquid nitrogen and place it in a 37 °C water bath until only a sliver of ice remains. Fill a 10 mL pipette with E8-Y medium, and use this to remove contents of vial, transfer ~5 mL to a 15 mL conical tube, wash out vial with ~1 mL of medium, and transfer the remainder to the conical tube.

   NOTE: *The addition of Y27632 ROCK inhibitor in the E8-Y medium improves cell survival after dissociation which enhances the consistency of plating.*

3. Centrifuge at 200 × *g* for 4 min. Aspirate supernatant. Resuspend in 2 mL of E8-Y and transfer to 2 wells of a Matrigel-coated 6-well plate.

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4. Change media every 24 h with E8 (without Y27632).

Passage of hiPSCs with EDTA

1. Ideally, cells should have reached 65–85% confluence in 3–4 days (adjust split ratio ~1:12 to 1:20 to achieve this, as higher split ratios result in more efficient differentiations).
2. Aspirate culture medium.
3. Add 1 mL per well of 0.5 mM EDTA, and incubate for 6 min at RT (in hood).
4. Aspirate EDTA from well.
5. With a P1000 tip, add 1 mL of E8-Y medium to the well, and blast medium against cell surface to dissociate cells. Cells should come off easily after ~5 times pipetting. Top up well to 12 mL of E8-Y. For a 1:15 split, remove 200 μL and discard; for a 1:20 split, remove 400 μL and discard.
6. Plate out cells at 1 mL per well in to two new Matrigel-coated 6-well plates and top up each well to 2 mL with E8-Y.

   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 85% confluent (i.e., 85% of the culture surface covered with cells). This prevents cells from becoming contact inhibited, which would result in a slow lag phase growth after passaging. E8 is less well buffered that other media and cells that are overgrown (i.e., >100% confluence) cell death is noted rather than spontaneous differentiation.*

Day 0: Beginning differentiation with CDM3-C medium (with CHIR99021)

1. Aspirate medium in wells.
2. Add 2 mL of CDM3-C.

   NOTE: *Significant cell death may be noted (*Fig. 1). hiPSCs that undergo epithelial to mesenchymal transition at d2 will result in higher cardiomyocyte yields than those that maintain an epithelial morphology (Fig. 1).

Day 2: Change to CDM3-C59 medium (with Wnt-C59)

1. Aspirate medium in wells.
2. Add 2 mL of CDM3-C59.

Days 4, 6, and 8: Change CDM3 medium

1. Aspirate medium in wells.
2. Add 2 mL of CDM3.

Days 10, 12, 14: Change CDM3-L medium (without glucose)

1. Aspirate medium in wells.

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2. Add 2 mL of CDM3-L.

   NOTE: *At least 4 days of metabolic selection is required. Some non-cardiomyocyte cell types can survive metabolic selection. Cease metabolic selection if cardiomyocyte death is noted.*

Day 15: Dissociation of cardiomyocytes

1. Aspirate medium in wells. NOTE: For cells later than day 15, add 0.5 U/mL Liberase TH and 50 U/mL DNase I to the TrypLE to break down deposited collagen.
2. Wash cells three times with DPBS to remove calcium and inhibit contraction.
3. Add 1 mL of TrypLE Express, incubate for 5 min at 37 °C.
4. Pipette up and down with a P1000 ~10 times to dislodge cells and to break up aggregates.

   Avoid forming bubbles.
5. Return cells to incubator for another 5 min at 37 °C.
6. Pipette up and down with a P1000 ~10 times to dislodge cells and to break up aggregates.

   Avoid forming bubbles.
7. Transfer cells to a 15 mL conical tube, top up with CDM3, and centrifuge at 300 × *g* for 5 min.
8. Resuspend in 1 mL CDM3 and pipette up and down with a P1000 for ~10 times to release single cells.

   NOTE: *In our experience, the addition of Y27632 did not improve cardiomyocyte cell survival after dissociation.*
9. Pass cells through a 100 μm cell strainer.
10. Count cells with an automated cell counter.
11. Dilute to 1×10^6 per mL with CDM3.
12. Seed into a Matrigel-coated 24-well plate at 750,000 cells per well, a 96-well plate at 100,000 cells per well, or a 384-well plate at 25,000 cells per well.
13. Change media every other day, cells should begin contraction after ~2–5 days.

Freezing cardiomyocytes (d15)

1. Resuspend at $>2\times10^6$ per mL in 90% FBS/10% DMSO, transfer 1 mL to a cryovial, and place in a Biocision CoolCell. Place CoolCell at –80 °C overnight and then transfer vials to liquid nitrogen.

NOTE: BamBanker cell freezing medium is not suitable for the cryopreservation of cardiomyocytes. In our experience, cell survival was superior in cells cryopreserved in FBS when compared to KSR (Knockout Serum Replacement).

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 conical tube, and add ~10 mL of CDM3 supplemented with 20% FBS dropwise.
3. Invert to mix then centrifuge at 200 × *g* for 4 min.
4. Resuspend pellet in CDM3 at a ratio of ~1 mL per million cells to be plated in a 12-well plate or equivalent.
5. After 48 hours, replace medium with CDM3 and then change medium every other day.

   NOTE: Expect ~60% survival after thawing.

Days 16 and 18: Change CDM3 medium

3 Aspirate medium in wells.

4 Add 2 mL of CDM3.

Days 20, 22, 24, 26, 28: Change medium to CDM3-T (with T3)

1. Aspirate medium in wells.
2. Add 2 mL of CDM3-T.

   NOTE: We have not validated the concentrations and timing of T_3 treatment. It is possible further optimization may improve maturation.

SUPPORT PROTOCOL 1: Characterization by flow cytometry

Cardiomycytes can be analyzed using flow cytometry (see Figure 2). Day 20 cardiomyocytes are first stained with troponin T (TNNT2) antibodies and labeled with Alexfor 488 goat anti-mouse IgG_1. Follow the manufacturer's instructions for operation of the flow cytometer.

For this procedure we use a Thermo 5/7 mL bucket with decanting aid allowing the simultaneous processing of a large number of tubes.

Materials

TNNT2 (Troponin T) primary antibody, rabbit polyclonal IgG (Abcam, cat. no. ab45932)

20% PFA (Electron Microscope Services, cat. no. 15713-S)

Triton X-100 (Sigma Aldrich, cat. no. X100)

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BSA (Sigma-Aldrich, cat. no. A3311)

AlexaFluor 488 goat anti-rabbit IgG (Life Technologies, cat. no. A11008)

Methanol (Fisher, cat. no. A412-1)

Flow cytometry tubes (Corning Falcon, cat. no. 352235)

5/7 mL tube buckets with decanting aid for TX-750 rotor (Thermo, cat. no. 75003732)

Flow cytometer capable for analyzing FITC and Texas Red such as Beckman Coulter CytoFLEX

1. Dissociate cells as above, transfer 1×10^6 cells to a flow cytometry tube and centrifuge at $300 \times g$ for 4 min. Decant supernatant.
2. Add 1 mL of 1% PFA in PBS, vortex, incubate for 20 min at RT, centrifuge, and decant supernatant.
3. Add 1 mL of cold 90% methanol, incubate for 15 min at 4 °C, centrifuge, decant supernatant.
4. Wash with 2 mL 0.5% BSA in DPBS, centrifuge, decant supernatant.
5. Repeat step 4.
6. Re-suspend in 100 uL of 0.5% BSA, 0.1% Triton X-1 in DPBS with 1:200 dilution of TNNT2 mouse monoclonal (13-11) primary antibody, vortex, incubate for 1h at RT, centrifuge, decant supernatant.
7. Wash with 2 mL 0.5% BSA, 0.1% Triton X-100 in DPBS, centrifuge, decant supernatant.
8. Re-suspend in 100 µL of 0.5% BSA, 0.1% Triton X-1 in DPBS with 1:1000 dilution of Alexflor 488 goat anti-mouse IgG_1, vortex, incubate for 30 min at RT, centrifuge, decant supernatant.
9. Wash with 2 mL 0.5% BSA, 0.1% Triton X-100 in DPBS, centrifuge, decant supernatant.
10. Repeat step 9.
11. Resuspend 300 µL 0.5% BSA in DPBS.
12. Analyze with flow cytometer such as Beckman Coulter CytoFLEX, following instrument manufacturer's instructions.

SUPPORT PROTOCOL: Characterization by immunofluorescent staining

Day 15 cardiomyocytes can be evaluated by immunofluorescent staining using antibodies against troponin T (TNNT2) and α-actinin (ACTN2), as shown in Figure 2.

4% PFA in DPBS (20% PFA (Electron Microscope Services, cat. no. 15713-S)

0.5% Triton X-100 in DPBS (Triton X-100 (Sigma Aldrich, cat. no. X100)

3% BSA in DPBS (BSA (Sigma-Aldrich, cat. no. A3311)

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3% BSA in PBS

DPBS

Coverslips

TNNT2 (Troponin T) primary antibody, rabbit polyclonal IgG (Abcam, cat. no. ab45932)

ACTN2 (α-actinin) primary antibody, mouse monoclonal IgG_1, clone EA-53 (Sigma-Aldrich, cat. no. A7811)

8-well Lab-Tek II chamber slides (Thermo Nunc, cat.no. 154534)

12-well Matek glass No. 1.5 plates (Matek, cat no. P12G-1.5-14-F)

AlexaFluor 488 goat anti-rabbit IgG (Life Technologies, cat. no. A11008)

AlexaFluor 594 goat anti-mouse IgG1 (Life Technologies, cat. no. A21125)

Prolong Diamond with DAPI (Life Technologies, cat. no. P36962)

1. Plate cells on to a Matrigel-coated 8-well chamber slides or 24-well plate glass-bottom Matek plate and allow cells to grow for >2 days. Remove media from cells.
2. Fix cells by adding 4% PFA in DPBS, and incubating 15 min at RT.
3. Permeabilize with 0.5% Triton X-100 in DPBS. Incubate for 15 min at RT.
4. Block with 3% BSA in DPBS, and incubate for 30–60 min at RT.
5. Stain cells with TNNT2 and ACTN2 antibodies at 1:200 and 1:500 dilution, respectively, in 3% BSA in PBS. Incubate for 1–3 h at RT or overnight at 4 °C.
6. Wash 3 times with DPBS, 2–3 min each.
7. Stain with secondary antibodies at 1:1000 dilution in 3% BSA in PBS for 30–60 min RT in dark.
8. Wash 3 times with DPBS, 2–3 min each.
9. Adhere coverslip with 1–2 drops of Prolong Diamond and evaluate by fluorescence microscopy.

REAGENTS AND SOLUTIONS

Matrigel-coated plates

Growth factor-reduced Matrigel (Corning, cat. no. 356230)

DMEM/F12

6-well plates

- Thaw a bottle of growth factor-reduced Matrigel overnight at 4 °C, then store it at 4 °C.

  There is no need to aliquot or leave on ice. The bottle of Matrigel will be stable at 4 °C for > 3 months.

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- Add 1.25 mL of Matrigel to 500 mL of 4 °C DMEM/F12 (enough for 42 plates). Return the Matrigel bottle to 4 °C quickly to prevent gelling.
- Mix the bottle by inversion and plate at 2 mL per well of a 6-well plate (or equivalent).
- Place plates at 37 °C for at least 30 min. Plates may be kept here at 37 °C for >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 during extended storage at 37 °C.

NOTE: We use Matrigel at a 1:400 dilution. Matrigel is supplied at a 10–12 mg/mL stock concentration (see product insert). At a 1:400 dilution and using 2 mL per well, this equates to ~5 μg/cm^2, which is well within the suitable range (>3 μg/cm^2) as previously described (Burridge et al., 2011; Miyazaki et al., 2012).

E8 media aliquots

DMEM/F12, 1 L (Corning, cat. no. 10-092-CM)

L-ascorbic acid 2-phosphate tri sodium salt (Wako Chemicals 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)

- Shown below are directions for making 100 × 1.5 mL E8 supplement aliquots. This will generate 100 L of E8.
- Add 50 mL of room temperature WFI water to 150 mL cell culture bottle, then slowly add 6.4 g of L-ascorbic acid 2-phosphate, inverting intermittently. Mix until clear.
- 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), and pH to 7.4 with 10 N NaOH (~200 μL), then 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 come back out of solution as it progresses from pH 3 through to pH 7.4. 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).

- Add 48 mL of WFI water to a 50 mL conical tube, and use this to resuspend the contents of 10 × 1 mg vials of FGF2.
- Add 2 mL of WFI water to 2 × 100 μg vials of TGFB1.
- Add the growth factors to the 150 mL cell culture bottle, mix well and filter sterilize. Make 1.5 mL aliquots in 2 mL microfuge tubes and store at −20 °C.

E8		
DMEM/F12 with L-glutamine and HEPES	1000 mL	Corning 10-092-CM
64 μg/mL L-ascorbic acid 2-phosphate	1.5 mL	Wako Chemicals USA 321-44823
20 μg/mL insulin		Life Technologies A11382ij
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; there is no need to filter sterilize. The medium is stable at 4 °C for >4 weeks.

E8-Y		
E8 as above	1000 mL	
10 μM Y27632 (10 mM)	1 mL	Biorbyt orb154626

There is no need to filter sterilize. E8-Y is stable in E8 at 4 °C for >4 weeks.

0.5 mM EDTA		
DPBS without calcium and magnesium	500 mL	Corning 21-031-CV
0.5 mM EDTA (0.5 M)	500 μL	Corning 46-034-CI

Store at RT

CDM3 Media Aliquots

- Slowly add 10.56 g of L-ascorbic acid 2-phosphate to a 500 mL bottle of WFI water, inverting intermittently. Mix until clear.
- Add 25 g of recombinant human albumin, inverting intermittently, mix until dissolved.
- Filter sterilize. Make 10 mL aliquots in 15 mL conical tubes and store at −20 °C.

CDM3-C		
RPMI 1640	1 L	Corning 10-040-CM
CDM3 supplement	10 mL	As above

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6 μM CHIR99021-HCl (10 mM)	600 μL	Biorbyt orb154612

There is no need to filter sterilize. CDM3-C is stable at 4 °C for >4 weeks.

CDM3-C59		
RPMI 1640	1 L	Corning 10-040-CM
CDM3 supplement	10 mL	As above
2 μM Wnt-C59 (10 mM)	200 μL	Biorbyt orb181132

There is no need to filter sterilize. CDM3-C59 is stable at 4 °C for >4 weeks.

CDM3		
RPMI 1640	1 L	Corning 10-040-CM
CDM3 supplement	10 mL	As above

There is no need to filter sterilize, CDM3 is stable at 4 °C for >4 weeks.

CDM3-L		
RPMI 1640 no glucose	1000 mL	Invitrogen 11879-020
CDM3 supplement	10 mL	As above
4 mM L-lactic acid (1 M, from 10 M stock)	1 mL	Wako Chemicals USA 129-02666

First dilute L-lactic acid in 1M HEPES (Corning). Filter sterilize. CDM3-L is stable at 4 °C for >4 weeks.

CDM3-T		
RPMI 1640	1 L	Corning 10-040-CM
CDM3 supplement	10 mL	As above
20 ng/mL triiodo-L-thyronine (2 μg/mL)	100 μL	Sigma T6397-100MG

There is no need to filter sterilize. CDM3-L is stable at 4 °C for >4 weeks.

COMMENTARY

Background information

Numerous techniques now exist for the cardiac differentiation of hiPSCs, yet only minimal differences in the cardiomyocytes produced have been demonstrated (Burridge et al., 2014). The chemically defined methodology described here was specifically designed to provide improved control and understanding of the constituents required for cardiomyocyte differentiation while simultaneously providing an 'inert' platform for subsequent drug testing assays and manipulation of factors controlling maturation and subtype specification. It has yet to be demonstrated what effect differentiation in CDM3 has on the cardiomyocytes it produces in comparison to RPMI+B27 and various other media such as StemPro-34 (Life Technologies) or APEL (Stemcell Technologies), each of which has a different basal medium. It is likely that differences in metabolism exist due to the single energy source (glucose) in CDM3 and the lack of pyruvate, galactose, and fatty acids (such as linoleic, linolenic, or oleic acid), resulting in cardiomyocytes relying on glycolysis rather than pyruvate decarboxylation, fatty acid oxidation, the TCA cycle, and/or oxidative

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phosphorylation. The lack of lipids in the medium may impact cell membrane structures, which may affect successful patch clamp experiments. In addition, the lack of retinoic acid, which is a component of B27, may influence subtype specification, although we have not noted this to date (Burridge et al., 2014). It has been demonstrated that the addition of creatine, carnitine, taurine, and insulin may be useful for long-term cardiomyocytes culture (Xu et al., 2006), and this combination is commonly used for rat neonatal ventricular myocytes (RNVMs). We have previously shown that hiPSC-CM can adhere to a variety of surfaces such as Matrigel, fibronectin, laminin, and collagen, and what role these surfaces have on maturation and subtype specification is still to be established. CDM3 provides suitable platform for the analysis of each of these variables and high level of control over the cell environment during differentiation. Finally, cost-effectiveness of this protocol makes it highly suitable for large scale differentiation techniques, both adherent- and suspension-based.

Critical Parameters

The chemically defined pluripotent culture medium E8 (Chen et al., 2011) is used to provide a more reproducible environment for pluripotent growth. In our experience, suitable pluripotent growth is the most important variable in achieving efficient subsequent cardiac differentiation. We have made small modifications to the E8 formula. These include (1) replacing human transferrin with a recombinant version and reducing the concentration; (2) selecting a DMEM/F12 with higher levels of sodium bicarbonate so that it does not have to be supplemented (this DMEM/F12 is also supplied in 1 L bottles, thus eliminating the need for transferring to a larger bottle and filtration); (3) and adding heparin sulfate, which has been demonstrated to stabilize FGF2 at 37 °C, preventing the transient decrease in FGF2 levels over 24 hours as previously noted (Chen et al., 2012). Our experience indicates that cells grow exceptionally well in this medium, and by negotiating with vendors, we have reduced the cost of E8 by 75% compared to commercial media such as Essential 8™ (Life Technologies) or TeSR™ -E8™ (Stemcell Technologies).

Passaging hPSCs as single cells and growing them as monolayers is well established (Denning et al., 2006). The use of EDTA simplifies this process by eliminating the need for centrifugation and dissociating cells to small clumps to improve cell survival (Beers et al., 2012; Yu et al., 2011). We add 10 μM Y27632 for the first 24 h after passaging to improve consistency of plating and minimize selective pressure. A number of Rho kinase inhibitors have been shown to improve survival of dissociated hiPSCs, although Y27632 has been demonstrated to be more effective than others such as thiazovivin (Chen et al., 2014). We use either atmospheric (~20%) O_2 or physiological (5%) O_2 for pluripotent culture and have not detected a significant impact on subsequent differentiation efficiency. Physiologic 5% O_2 has been demonstrated to improve reprogramming efficiency (Yoshida et al., 2009), enhance expression of pluripotency genes (Forristal et al., 2010; Narva et al., 2013), and reduce spontaneous differentiation (Ezashi et al., 2005), and is optimal when used with mTeSR1™ (Stem Cell Technologies) medium (Ludwig et al., 2006) and E8 (Chen et al., 2011). Low O_2 also pushes hPSCs to anaerobic glycolysis, resulting in the production of less reactive oxygen species (ROS) and DNA damage, and improved genetic stability, and is therefore recommended where available. In particular, even when used for just the first two

days of the differentiation time-course, 5% O_2 can result in inhibition of cardiac differentiation. In our original publication, we demonstrated that a variety of matrices were suitable for cardiac differentiation, although adhesion of cardiomyocytes after day 12 became problematic with all except Matrigel and laminin 511 or 521 (Burridge et al., 2014). We commonly use Matrigel at a 1:400 dilution and have not found a more cost-effective alternative. We have found no added benefit in using hESC-qualified Matrigel.

For cardiac differentiation, we have now used the protocol described here for >200 hiPSC lines. The protocol is not 100% reproducible and we have found that some lines go through periods of very successful differentiation followed by periods when they become refractory as they progress from passage 25 to beyond passage 90. Nevertheless, we have not found a hiPSC line that is completely recalcitrant to differentiation. In 6-well plates, we have also commonly observed some wells differentiate acceptably, and others not differentiating at all or are subject to total cell death. Taken together, these observations suggest that a controlling factor in differentiation efficiency is a combination of passage number, cell density, proliferation rate, and the microenvironment established in response to CHIR99021 and Wnt-C59 treatment.

During the continued development of our protocol, we have used numerous suppliers of recombinant human albumin and currently use one from ScienCell based on cost. We did not notice any significant differences in differentiation efficiency among the various alterative manufacturers. Similarly, we selected an L-ascorbic acid 2-phosphate that can be purchased in large volumes. Despite continued assessment of CHIR99021 and Wnt-C59 dosing alternatives and timing in a large number of lines, we have not discovered a clearly superior regimen. The differentiation protocol described above was simply one that most commonly worked with the largest number of the lines.

Metabolic purification is the process of replacing glucose in the media with lactate, based on the premise that only cardiomyocytes in the culture are able to use the TCA cycle to produce ATP (Tohyama et al., 2013). As first demonstrated, this method used αMEM and FBS and showed that cardiomyocytes could survive long term without glucose. The method has not proven to be as straightforward in CDM3-based media, and we have found high levels of variability in survival among lines and differentiations during the metabolic selection. To counteract this, we have reduced the length of time the cells are treated to 4–6 days so that we could observe cells to identify early signs of cardiomyocyte death. Additionally, we noted that some differentiation runs contain contaminating non-cardiomyocyte cell types which cannot be removed by metabolic selection. In our original publication (Burridge et al., 2014), we used sodium DL-lactate to overcome the lack of membrane permeability of L-lactic acid as previously described (Tohyama et al., 2013), but we have found that cardiomyocytes cannot be maintained long-term using either approach.

Passaging of cardiomyocytes is relatively simple at day 15, but we have found that the cells lay down a layer of collagen over time in culture that must be broken down before cells can be isolated, and therefore we recommend the use of Liberase TH and DNase I. By analyzing cryopreservation techniques, we found that cells that are more effectively broken up to single cells have better survival upon thawing, and that RPMI+10% DMSO+20% FBS

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is an effective cryopreservation solution, with higher percentages of FBS (up to 40%) being more successful. Other cryopreservation media such as CryoStor10 have proven effective for the cryopreservation of hiPSC-CMs, but they are not cost-efficient. We found, that the addition of Y27632 for 24 hours after thawing had a negative effect on cell survival and therefore is not included.

Troubleshooting

- Interline variability and passage number variability in differentiation efficiency: Pluripotent cell must be undifferentiated and growing at a fast rate, ideally achieving 75–85% confluence in 3–4 days. We have found that lines over passage 25 have a higher differentiation success rate.
- Cell death after CHIR99021 treatment is commonly due to too high starting density. For some early passage lines (<p25), a lower level of CHIR99021 (e.g., 5 μM) may be more suitable.
- No signs of epithelial to mesenchymal transition at day 2 were found. Clear signs of EMT are indicative of efficient mesodermal differentiation, and the lack of EMT is likely due to overconfluence of starting cells.
- Cell death during late differentiation (day 6–8) can be observed in low passage (<p25) lines, and therefore we recommend repeating differentiation.
- No beating cells: If cells are not contracting by day 10, discard the plate and repeat differentiation. If this occurs repeatedly, try higher (1:20) or lower split ratio (1:12) or varying the level of CHIR99021 (5–7 μM).
- Total cell death during metabolic selection: There is variability in how long cells can survive the metabolic (L-lactic acid) selection, and we recommend reducing the number of days in CDM3-L

Anticipated Results

Differentiation should produce 1–2 million cardiomyocytes per well for a 6-well plate. Cardiomyocyte purity after metabolic selection will be >90% based on TNNT2 flow cytometry. Cardiomyocytes will stain positive for cardiac markers such as TNNT2 and ACTN2. Cells will beat at ~50 beats per minute, slower at lower temperatures, and faster at higher temperatures. Cardiomyocytes will demonstrate chronotropic responses to drugs such as norepinephrine. Toxicity to known cardiotoxic drugs will increase as the cells age (e.g., from day 30 to day 90). After cryopreservation, cell survival will be ~60% and cells will regain contraction in 2–5 days.

Time Considerations

This full protocol takes 19 days from passage of hiPSC through to dissociation/ cryopreservation of cardiomyocytes. After thawing cells it can take two weeks for proliferation to reach a suitable rate for successful differentiation. Media should be changed daily for pluripotent cells; this protocol is not compatible with skipping days or ‘weekend-free’ schedules. Contracting cells will be noted at approximately day 9 of differentiation.

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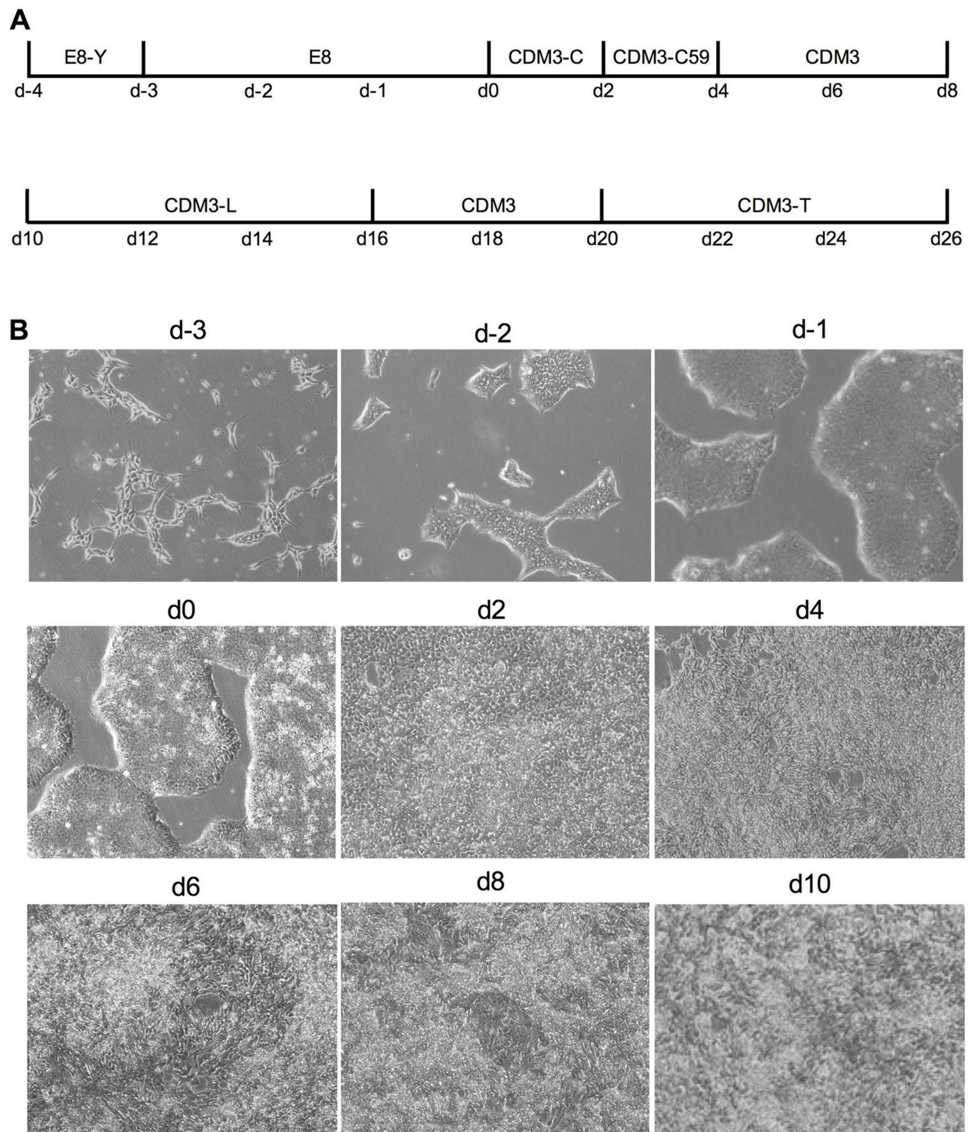

Figure 1. Human pluripotent growth and cardiac differentition

A, Time-course of pluripotent growth and subsequent cardiac differentiation showing the medium and small molcules used in each day (d) of differentiation. **B,** Representative images of hiPSCs seeded at a 1:15 split ratio in E8, followed by differentiation using the CDM3 protocol.

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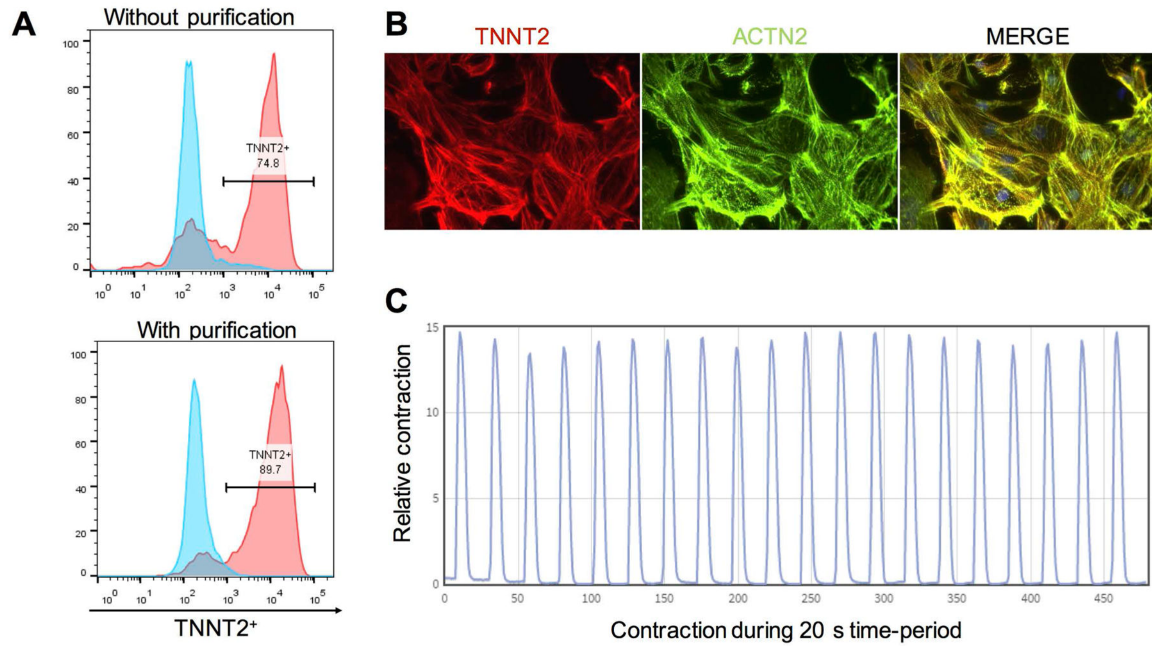

Figure 2. Characterization of cardiomycytes produced using CDM3

A, Flow cytometry of day 20 cardiomyocytes stained with troponin T (TNNT2) with or without metabolic purification. **B,** Immunofluorescent staining of day 15 cardiomyocytes for troponin T (TNNT2) and α-actinin (ACTN2). **C,** Measurement of contraction by video capture using Cellogy Pulse System.