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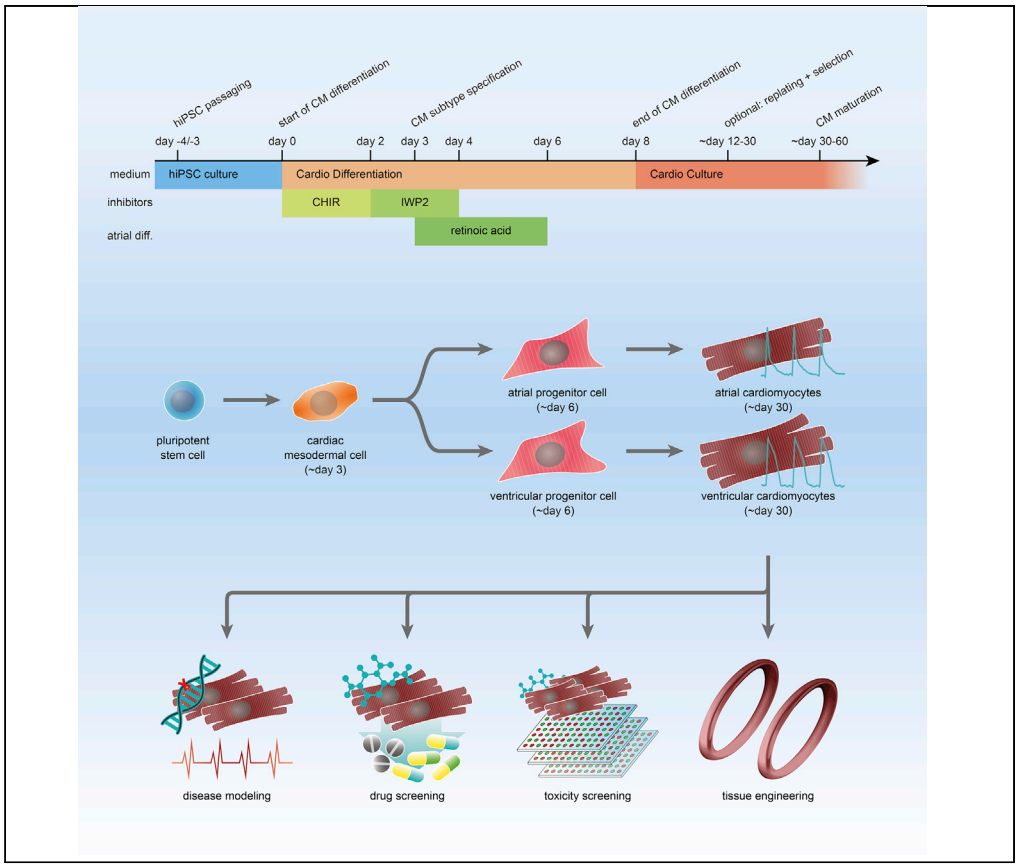
Subtype-Directed Differentiation of Human iPSCs into Atrial and Ventricular Cardiomyocytes



The generation of homogeneous populations of subtype-specific cardiomyocytes derived from human induced pluripotent stem cells (hiPSCs) is crucial in cardiovascular disease modeling as well as in drug discovery and cardiotoxicity screenings. This protocol describes a simple, robust, and efficient monolayer-based differentiation of hiPSCs into defined atrial and ventricular cardiomyocytes.

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HIGHLIGHTS

Subtype-directed differentiation of hiPSCs into atrial and ventricular cardiomyocytes

Simple, robust, and efficient monolayer-based differentiation protocol

Approx. 90%–95% of the intended cardiac subtype within cTNT^{+} cells can be obtained

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Subtype-Directed Differentiation of Human iPSCs into Atrial and Ventricular Cardiomyocytes

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SUMMARY

The generation of homogeneous populations of subtype-specific cardiomyocytes derived from human induced pluripotent stem cells (hiPSCs) is crucial in cardiovascular disease modeling as well as in drug discovery and cardiotoxicity screenings. This protocol describes a simple, robust, and efficient monolayer-based differentiation of hiPSCs into defined atrial and ventricular cardiomyocytes.

For complete details on the use and execution of this protocol, please refer to Cyganek et al., 2018.

BEFORE YOU BEGIN

Human induced pluripotent stem cells (hiPSCs) have to be obtained and used according to the respective legal and ethical guidelines. hiPSCs and derivatives thereof are maintained in a humidified incubator at 37°C and 5% CO_2. All cell culture procedures are performed in a Class II biosafety cabinet using sterile conditions.

Coating of Culture Plates

For enhanced and homogeneous attachment of cells, culture plates must be coated with extracellular matrix proteins or protein mixtures.

1. Dilute Matrigel:
   a. Thaw Matrigel (7-10 mg/ml) on ice at 4°C for 16-24 h.
   b. Distribute Matrigel in 250 µl aliquots into 50 ml centrifuge tubes using ice-cold pipette tips and tubes, and directly freeze.
   c. Store aliquots at -20°C for up to one year.
2. Coat cell culture-treated multi-well plates (e.g. 6-well plates):
   a. Resuspend a frozen Matrigel aliquot in 29.75 ml cold PBS to obtain the working dilution of 1:120.
   b. Add 1 ml of diluted Matrigel per well of a 6-well plate (approx. 6-9 µg/cm^2) and ensure that the entire well surface is covered.
   c. Incubate at 37°C for 60 min or at 4°C for 16-24 h.

Note: Coated plates can be stored at 4°C for up to 2 weeks, but should not be used if the Matrigel has dried up.

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Alternatives: Instead of Matrigel, plates can also be coated with Geltrex (approx. 17 μg/cm^2) or defined matrices such as recombinant Laminin-521 (approx. 0.9 μg/cm^2).

KEY RESOURCES TABLE

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Antibodies		
α-actinin monoclonal mouse IgG1	Sigma-Aldrich	Cat# A7811, RRID:AB_476766
CTNT monoclonal mouse IgG1	Thermo Fisher Scientific	Cat# MA5-12960, RRID:AB_11000742
RYR2 polyclonal rabbit IgG	Sigma-Aldrich	Cat# HPA020028, RRID:AB_1856528
MLC2A monoclonal mouse IgG2a	Synaptic Systems	Cat# 311 011, RRID:AB_887737
MLC2V polyclonal rabbit IgG	Proteintech	Cat# 10906-1-AP, RID:AB_2147453
Alexa Fluor 488 donkey anti-rabbit	Thermo Fisher Scientific	Cat#A21206, RRID:AB_2535792
Alexa Fluor 555 donkey anti-mouse	Thermo Fisher Scientific	Cat#A31570, RRID:AB_2536180
Chemicals, Peptides, and Recombinant Proteins		
StemPro Accutase	Thermo Fisher Scientific	Cat#A1110501
Albumin, Human Recombinant	Sigma-Aldrich	Cat#A9731
B-27 Supplement	Thermo Fisher Scientific	Cat#17504044
B-27 Supplement minus Insulin	Thermo Fisher Scientific	Cat#A18956-01
Bovine Serum Albumin (BSA)	Sigma-Aldrich	Cat#A2153
CHIR99021	Merck Millipore	Cat#361559
Cryostor CS10	STEMCELL Technologies	Cat#07930
DAPI (4′,6-Diamidino-2-Phenylindole, Dilactate)	Thermo Fisher Scientific	Cat#D3571
DMSO	Sigma-Aldrich	Cat#D2650
DPBS 1×	Thermo Fisher Scientific	Cat#14190094
E8 Medium	Thermo Fisher Scientific	Cat#A1516901
EDTA	Carl Roth	Cat#8040
Fetal Bovine Serum (FBS)	Thermo Fisher Scientific	Cat#10270-106
Fluoromount-G	Thermo Fisher Scientific	Cat#00-4958-02
Geltrex (Growth Factor Reduced)	Thermo Fisher Scientific	Cat#A1413302
HEPES Buffer (pH 7.0-7.6)	Sigma-Aldrich	Cat#H3662
Hoechst 33342, Trihydrochloride, Trihydrate	Thermo Fisher Scientific	Cat#H3570
ROTI Histofix 4%	Carl Roth	Cat#P087.5
IWP2	Merck Millipore	Cat#681671
IWP4	Reprocell Europe	Cat#04-0036C
L-Ascorbic Acid 2-Phosphate	Sigma-Aldrich	Cat#A8960
Laminin 521	Biolamina	Cat#LN521-05
Matrigel (Growth Factor Reduced)	BD	Cat#354230
Penicillin/Streptomycin	Thermo Fisher Scientific	Cat#15140-122
Pro Survival Factor DDD00033325	Merck Millipore	Cat#529659
Retinoic Acid	Sigma-Aldrich	Cat#R2625
ROCK Inhibitor Y27632	Merck Millipore	Cat#688000

(*Continued on next page*)

Continued

REAGENT or RESOURCE	SOURCE	IDENTIFIER
RPMI 1640 with GlutaMax and HEPES	Thermo Fisher Scientific	Cat#72400021
RPMI 1640 (without glucose and HEPES)	Thermo Fisher Scientific	Cat#11879020
Sodium Chloride	Carl Roth	Cat#9265.1
Sodium DL-Lactate Solution	Sigma-Aldrich	Cat#L4263
Stem MACS iPS Brew XF Medium	Miltenyi Biotec	Cat#130-104-368
StemPro Accutase Cell Dissociation Reagent	Thermo Fisher Scientific	Cat#A1110501
StemFlex Medium	Thermo Fisher Scientific	Cat#A3349401
Thiazovivin	Merck Millipore	Cat#420220
Triton X-100	Sigma-Aldrich	Cat#T8787
10× TrypLE Select	Thermo Fisher Scientific	Cat#A1217701
0.05% Trypsin-EDTA	Thermo Fisher Scientific	Cat#25300-054
0.25% Trypsin-EDTA	Thermo Fisher Scientific	Cat#25200056
Versene Solution (0.48 mM EDTA)	Thermo Fisher Scientific	Cat#15040033
Wnt-C59	Peprotech	Cat#1248913

MATERIALS AND EQUIPMENT

hiPSC Culture Medium

Complement Stem MACS iPS-Brew XF Basal medium with Stem MACS iPS-Brew XF 50× supplement. Store at 4°C for up to two weeks.

Alternatives: Instead of StemMACS iPS-Brew XF, E8 medium or StemFlex medium can be used for the maintenance culture of hiPSCs.

EDTA Dissociation Buffer 0.5 mM

Add 1 µl/ml EDTA (from a stock solution of 0.5 M in ddH_2O and pH 8.0) to PBS (Ca^{2+}/Mg^{2+}-free). Add 1.8 mg/ml sodium chloride to adjust the osmolarity to ~340 mOsm. Sterilize by filtration. Store at 4°C for up to 6 months.

Alternatives: Instead of preparing EDTA Dissociation Buffer, commercially available EDTA solutions, such as Versene, can be used.

Thiazovivin 2 mM

Dissolve 0.62 mg/ml Thiazovivin in DMSO to obtain a 1,000× stock solution of 2 mM. Aliquot and store at -20°C for up to one year.

Alternatives: Instead of Thiazovivin, ROCK Inhibitor Y27632 or Pro Survival Factor DDD00033325 can be used to minimize cell death during splitting/digestion. Prepare a 5 mM stock solution (1,000×), by dissolving 1.60 mg/ml Y27632 in DMSO or 1.47 mg/ml of DDD00033325 in DMSO. Apply either of these at a final concentration of 5 µM.

hiPSC Cryopreservation Medium 2×

Supplement hiPSC Culture Medium with 20% DMSO and a final concentration of 4 µM Thiazovivin (1:500 from 2 mM stock). Store at 4°C for up to one week.

Alternatives: If a hiPSC Culture Medium other than Stem MACS iPS-Brew XF is used, the respective medium should also be used as basis for the Cryopreservation Medium. Alternatively, a commercial cryopreservation medium such as Cryostor CS10 may be used.

Cardio Differentiation Medium

Supplement RPMI 1640 (with GlutaMAX and HEPES) with 0.2 mg/ml L-ascorbic acid 2-phosphate and 0.5 mg/ml human recombinant albumin. Mix until dissolved and filter to sterilize. Store at 4°C for up to one month.

Optional: Add antibiotics, such as 1× penicillin/streptomycin (from 100×).

Alternatives: If no satisfactory differentiation can be achieved with a cell line using the above medium, a different medium can be applied instead: for this, supplement RPMI 1640 (with GlutaMAX and HEPES) with a final concentration of 1× B-27 Supplement minus insulin (from 50×). Store at 4°C for up to 1 month.

Cardio Culture Medium

Supplement RPMI 1640 (with GlutaMAX and HEPES) with the final concentration of 1× B-27 Supplement (from 50×). Store at 4°C for up to one month.

Optional: Add antibiotics, such as 1× penicillin/streptomycin (from 100×).

Cardio Recovery Medium

Supplement Cardio Culture Medium with 20% Fetal Bovine Serum and a final concentration of 2 μM Thiazovivin. Store at 4°C for up to two weeks.

Optional: Add antibiotics, such as 1× penicillin/streptomycin (from 100×).

Alternatives: Cardio Culture Medium with 2 μM Thiazovivin only can be used. However, the addition of serum might be beneficial for cell survival.

Cardio Selection Medium

Supplement RPMI 1640 (without glucose and HEPES) with 0.2 mg/ml L-ascorbic acid 2-phosphate and 0.5 mg/ml human recombinant albumin, as well as a final concentration of 4 mM lactate/HEPES (1:250 from 1 M stock). Store at 4°C for up to one month.

Optional: Add antibiotics, such as 1× penicillin/streptomycin (from 100×).

Cardio Cryopreservation Medium 5×

Mix 50% DMSO and 50% Fetal Bovine Serum. Add a final concentration of 10 μM Thiazovivin (1:200 from 2 mM stock). Store at 4°C for up to one week.

Alternatives: Commercially available defined cryopreservation media such as Cryostor CS10 can be used.

CHIR99021 10 mM

Dissolve 4.66 mg/ml CHIR99021 in DMSO to obtain a stock solution of 10 mM. In case of precipitations, warm the solution to 37°C for 2–5 min. Aliquot and store at -20°C for up to six months.

IWP2 5 mM

Dissolve 2.34 mg/ml IWP2 in DMSO to obtain a stock solution of 5 mM. Incubate at 37°C for 10-20 min, then aliquot and store at -20°C for up to one year.

Alternatives: Other Wnt signaling inhibitors such as IWP4 or Wnt-C59 can be applied: Dissolve 2.48 mg/ml IWP4 in DMSO or 0.76 mg/ml Wnt-C59 in DMSO to obtain a stock solution of 5 mM (1,000×) or 2 mM (1,000×), respectively. Incubate at 37°C for 10-20 min, then aliquot and store at -20°C for up to one year.

Retinoic Acid 1 mM

For a 100 mM master stock, dissolve 29.94 mg/ml retinoic acid in DMSO, vortex, aliquot and store at -80°C. For the 1 mM working stock, dilute the 100 mM master stock 1:100 in pure ethanol (95-100%) and store at -20°C for up to two weeks.

Note: Protect retinoic acid from UV light, air and oxidizing agents.

Lactate/HEPES Solution 1 M

Prepare a 1 M lactate/HEPES solution by mixing 14.3% of sodium DL-lactate solution (60% [w/w] in H_2O equals 7 M) with 85.7% of 1 M HEPES solution. Filter to sterilize. Aliquot and store at -20°C for up to one year.

PBS with 1% BSA

Dissolve 10 mg/ml of Bovine serum albumin (BSA) in PBS. Aliquot and store at -20°C for up to six months or at 4°C for up to two weeks.

PBS with 1% BSA and 0.1% Triton X-100

Add 1 µl/ml Triton X-100 to PBS with 1% BSA. Use immediately.

STEP-BY-STEP METHOD DETAILS

hiPSC Passaging and Maintenance

TIMING: 10–20 min

hiPSCs are maintained under feeder-free conditions with daily medium changes and kept at 37°C and 5% CO_2. When the cells reach 70-95% confluency, the cells can be passaged (approx. every 3-5 days).

1. Prewarm Matrigel-coated plates, hiPSC Culture Medium and EDTA Dissociation Buffer to 20-25°C.
2. Aspirate Matrigel from the precoated culture plates and add hiPSC Culture Medium (2 ml per well of a 6-well plate).
3. Aspirate medium of hiPSCs. Cover the cells carefully with EDTA Dissociation Buffer (e.g. 1 ml per well of a 6-well plate) and directly aspirate again.
4. Cover the cells carefully with EDTA Dissociation Buffer (e.g. 1 ml per well of a 6-well plate) and incubate with minimal movement for 3-5 min.

   Note: The longer the incubation, the smaller the cell clumps will be. The optimal incubation time should be determined for each cell line; however, 3 min are sufficient for most cell lines.

5. Carefully aspirate solution and rapidly wash the cells off the well with 1 ml hiPSC Culture Medium (for 6-well), but avoid excessive pipetting to keep small clumps intact. The colonies will reattach quickly when not taken off the plate.

   Note: If overtreated with EDTA and already detached from the plate, the cells could be collected, diluted in medium (e.g. 1:1), centrifuged at 200 × *g* for 5 min, and resuspended in fresh medium.

6. Plate the desired amount onto the prepared Matrigel-coated plates with medium (e.g. 1:6-1:10 or count and plate defined cell number). Add 1 µl/ml of 2 mM Thiazovivin (final concentration 2 µM) for the first 24 h.
7. Shake the plate carefully to distribute the cells evenly and incubate at 37°C and 5% CO_2.
8. Change the medium every day to 2 ml hiPSC Culture Medium per well of a 6-well plate.

Note: On one day per week (when the cells are less than 50% confluent), the medium change can be skipped. For this, add 3 ml of hiPSC Culture Medium per well of a 6-well plate the day before and then return to daily medium changes after 48 h.

PAUSE POINT: For differentiation of hiPSCs into cardiomyocytes, continue with step 22 (see 'Subtype-Directed Differentiation of hiPSCs into Atrial and Ventricular Cardiomyocytes'). If hiPSCs are not intended to be differentiated shortly, they can be cryopreserved when they have reached a confluency of 70-95% (see 'hiPSC Cryopreservation', steps 9-14) and recovered for culture when needed (see 'hiPSC Recovery', steps 15-21).

hiPSC Cryopreservation

TIMING: 10–20 min

For cryopreservation, hiPSCs can be released from the culture plates when they reach 70-95% confluency.

9. Prewarm hiPSC Culture Medium, hiPSC Cryopreservation Medium and EDTA Dissociation Buffer to 20-25°C.
10. Aspirate medium of hiPSCs. Cover the cells carefully with EDTA Dissociation Buffer (e.g. 1 ml per well of a 6-well plate) and directly aspirate again.
11. Cover the cells carefully with EDTA Dissociation Buffer (e.g. 1 ml per well of a 6-well plate) and incubate with minimal movement for 3-5 min.

Note: The longer the incubation, the smaller the cell clumps will be. The optimal incubation time should be determined for each cell line; however, 3 min are sufficient for most cell lines.

12. Carefully aspirate solution and rapidly wash the cells off the well with 0.75 ml hiPSC Culture Medium (for 6-well), but avoid excessive pipetting to keep small clumps intact. The colonies will reattach quickly when not taken off the plate.

Note: If overtreated with EDTA and already detached from the plate, the cells could be collected, diluted in medium (e.g. 1:1), centrifuged at 200 × *g* for 5 min, and resuspended in fresh medium.

13. In a dropwise manner, add 0.75 ml of Cryopreservation Medium to the well (for 6-well) and carefully mix.
14. Transfer the cells into cryogenic vials, freeze at -80°C in an isopropanol freezing container for at least 2 h, then store in the vapor phase of a liquid nitrogen freezer.

Note: Long-term storage in the vapor phase of liquid nitrogen (at a temperature between -140°C and -180°C) for more than 5 years was tested without loss of pluripotency or loss of differentiation capacity.

hiPSC Recovery

TIMING: 10–20 min

Cryopreserved hiPSCs can be thawed as described here. Prior to starting the cardiac differentiation, thawed hiPSCs should be allowed to recover for 2-3 passages.

Note: Cryogenic vials containing hiPSCs harvested from one well of a 6-well plate can be plated to 2-3 new wells and usually reach confluency after 2-3 days.

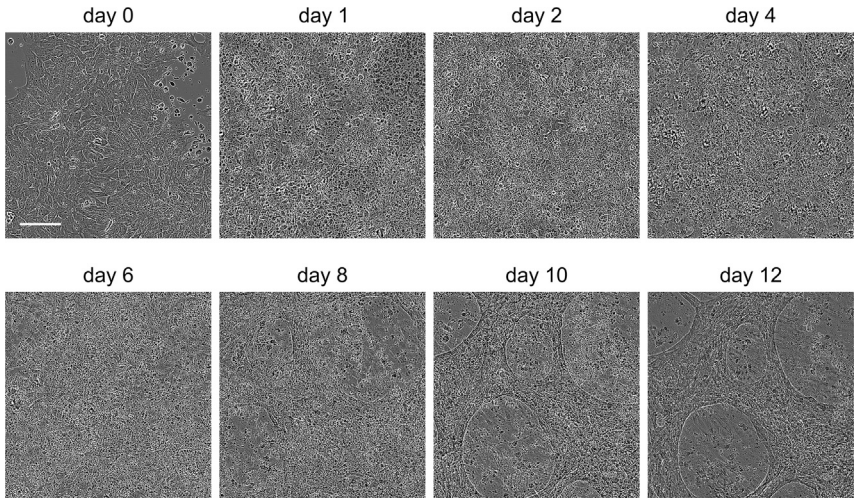

Figure 1. Subtype-Directed Differentiation of hiPSCs into Atrial and Ventricular Cardiomyocytes

hiPSCs undergoing ventricular cardiac differentiation. By day 8-10, the cells typically start to beat and develop a characteristic 'net-like' morphology. Atrial cells exhibit similar morphological changes, but can be distinguished from ventricular cells by their higher beating frequency (see Videos S1 and S2). Consistent field of view over time is shown. Scale bar represents 100 μm.

15. Prewarm Matrigel-coated plates and hiPSC Culture Medium to 20-25°C.
16. Aspirate Matrigel from the precoated culture plates and add hiPSC Culture Medium (2 ml per well of a 6-well plate).
17. Add 9 ml of hiPSC Culture Medium to a 15 ml centrifuge tube.
18. Directly transfer the cryogenic vial to a 37°C water bath and observe the thawing process. When most the content of the tube is thawed and only a small chunk of ice remains, quickly take it out and clean thoroughly with 70% ethanol.
19. Carefully transfer the cells in a dropwise manner to the prepared 15 ml centrifuge tube with medium. Centrifuge at 200 × *g* for 5 min.
20. Carefully aspirate the supernatant. Resuspend the pellet in hiPSC Culture Medium (e.g. 1 ml) and plate on the prepared Matrigel-coated plates with medium. Add 1 μl/ml of 2 mM Thiazovivin (final concentration 2 μM) for the first 24 h.
21. If the cells are well attached after 24 h, perform medium change with hiPSC Culture Medium. In case of low attachment, add 1 μl/ml of 2 mM Thiazovivin (final concentration 2 μM) for another 24 h. From the next day onwards, change the medium daily to 2 ml of hiPSC Culture Medium per well (6-well). Continue with 'hiPSC passaging and maintenance', steps 1-8.

Subtype-Directed Differentiation of hiPSCs into Atrial and Ventricular Cardiomyocytes

TIMING: 10–12 days

The cardiac differentiation of hiPSCs can be conducted along the atrial or the ventricular lineage. Both protocols comprise a series of medium changes as illustrated in the graphical abstract, in between which the cells are kept at 37°C and 5% CO_2. Although atrial and ventricular hiPSC-cardiomyocytes exhibit similar changes in morphology during differentiation (Figure 1), cultures of atrial cardiomyocytes typically contract significantly faster than ventricular cardiomyocyte cultures (see Videos S1 and S2).

Note: It is recommended to use hiPSCs between passages 15 and 50 for effective differentiation. The hiPSC line should express pluripotency markers and exhibit a normal karyotype (see Troubleshooting Problem 1).

22. Days -4 to -1: for both atrial and ventricular differentiation:
    a. Passage hiPSCs as described in 'hiPSC passaging and maintenance' (steps 1-5). After releasing the cells, count them using an automated cell counter or a hemocytometer, and plate defined cell numbers into a final volume of 2 ml per well (6-well).

    Note: The optimal seeding density for plating is cell line-dependent; it can range between 15,000-26,000 cells/cm^2, i.e. 150,000-260,000 cells per well (6-well).

    Note: Each confluent well of a 6-well maintenance plate will yield 1-3×10^6 cells for plating.

    b. Change the hiPSC Culture Medium daily with 2 ml per well (6-well).

    Note: Time for expansion to 80%-95% confluence can range between 2-4 days.

23. Day 0: for both atrial and ventricular differentiation:
    a. When the hiPSCs reach a confluency of 80-95%, start the differentiation (= d0).

    ⚠ CRITICAL: The optimal cell density at the start of differentiation is crucial and should be determined empirically for each cell line.

    b. Freshly prepare Cardio Differentiation Medium with 4-5 μM CHIR99021 (0.4-0.5 μl/ml of 10 mM stock solution).

    ⚠ CRITICAL: The optimal concentration of CHIR99021 can differ between cell lines and should be determined empirically. We recommend 4 μM as a starting point (see Trouble-shooting Problem 2).

    c. Aspirate the spent hiPSC Culture Medium and add 3 ml per well (6-well) of Cardio Differentiation Medium with CHIR99021.
24. Day 2: for both atrial and ventricular differentiation:
    a. Freshly prepare Cardio Differentiation Medium with 5 μM IWP2 (1 μl/ml of 5 mM stock solution). Aspirate the spent medium and carefully add 3 ml per well (6-well) of Cardio Differentiation Medium with IWP2.
25. Day 3: for atrial differentiation:
    a. Add 1 μl/ml of 1 mM retinoic acid (final concentration 1 μM) to the cells without aspiration of the spent medium.

    Note: For ventricular differentiation, no action is needed on day 3 of differentiation.

26. Day 4: for atrial differentiation:
    a. Freshly prepare Cardio Differentiation Medium with 1 μM retinoic acid (1 μl/ml of 1 mM stock solution). Aspirate the spent medium and carefully add 3 ml per well (6-well) of Cardio Differentiation Medium with retinoic acid.
27. Day 4: for ventricular differentiation:
    a. Aspirate the spent medium and carefully add 3 ml per well (6-well) of fresh Cardio Differentiation medium.
28. Day 6: for both atrial and ventricular differentiation:
    a. Aspirate the spent medium and carefully add 3 ml per well (6-well) of fresh Cardio Differentiation medium.
29. From day 8: for both atrial and ventricular differentiation:
    a. From day 8 onwards, change the medium to 2 or 3 ml of Cardio Culture Medium per well (6-well) every 2 or 3 days, respectively (e.g. 2 ml on Monday and Wednesday, 3 ml on Friday).

30. Optionally, replate the cells in a lower density (see 'Digestion of hiPSC-Derived Cardiomyocytes' steps 31-38) and perform cardiomyocyte selection (see 'Metabolic Selection of hiPSC-Derived Cardiomyocytes' steps 39-43), ideally between days 12-25 of differentiation.

Note: First spontaneous contractions of cultures can be observed after 8 days of differentiation, robust spontaneous contractions after 10-12 days. If, after day 15, no beating cells have been present on several consecutive days of observation, the differentiation has likely failed. Please note that the spontaneous contraction is affected by several factors including medium contents and environment temperature, so that, if necessary, additional characteristics should be examined such as expression of cardiac markers via flow cytometry or immunocytochemistry (see 'Immunostaining of hiPSC-Derived Cardiomyocytes', steps 60-71).

Note: hiPSC-derived atrial and ventricular cardiomyocytes can be maintained in culture for 2 months and longer (see Troubleshooting Problem 3).

PAUSE POINT: If differentiated atrial/ventricular cardiomyocytes are not required for immediate use, they can be cryopreserved from day 15 after the start of differentiation onwards (see 'Cryopreservation of hiPSC-Derived Cardiomyocytes' steps 44-51) and recovered for culture when needed (see 'Recovery of hiPSC-Derived Cardiomyocytes', steps 52-59).

Digestion of hiPSC-Derived Cardiomyocytes

TIMING: 30–45 min

Digestion of hiPSC-derived atrial and ventricular cardiomyocytes can be performed in order to plate cells into desired culture vessel formats at defined cell numbers if required (e.g. in lower cell density prior to metabolic selection).

31. Prewarm Cardio Recovery Medium, PBS or EDTA Dissociation Buffer, Matrigel-coated plates, 0.25% trypsin-EDTA to 20-25°C.
32. Aspirate Matrigel from the precoated culture plates and add Cardio Recovery Medium per well (2 ml per well of a 6-well plate).
33. Aspirate medium of the hiPSC-derived cardiomyocytes. Wash the cells once with PBS or EDTA Dissociation Buffer (e.g. 1 ml per well of a 6-well plate) and directly aspirate again.
34. Add 1 ml of 0.25% trypsin-EDTA per well (6-well) to the cells and incubate at 37°C for 8-12 min. Continue when the cells become round and start to detach from the plate.
35. Carefully wash all cells from the plate and transfer them into a 15 ml centrifuge tube. Add the same volume of Cardio Recovery Medium and centrifuge at 100 × *g* for 10 min.

⚠ CRITICAL: Because the cells are very sensitive, handle them carefully and avoid extensive pipetting.

36. Carefully aspirate the supernatant and resuspend the cells in a small volume (1-2 ml) of Cardio Recovery Medium.
37. Plate the desired cell number (after counting or at a particular ratio) on prepared coated plates.

Note: The cell viability should be greater than 55%.

38. After 2 days, change the medium to 2 ml of Cardio Culture Medium and change this medium every 2 or 3 days thereafter (e.g. 2 ml on Monday and Wednesday, 3 ml on Friday).

Note: Before performing analysis of or further experiments with digested cardiomyocytes, let them recover on the cell culture plate for 4-7 days (see Figure 2).

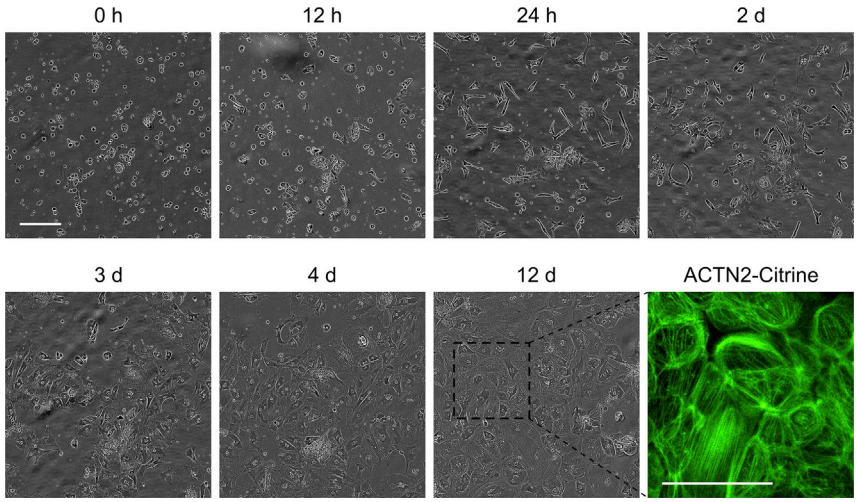

Figure 2. Recovery of hiPSC-Derived Cardiomyocytes after Replating/Thawing

A typical hiPSC-cardiomyocyte morphology can be observed from day 4 after replating or thawing of the cells displayed for the ventricular subtype (atrial cells show similar morphological changes). A CRISPR-engineered hiPSC line with endogenous tagging of ACTN2 with a fluorescence marker illustrates the formation of sarcomeric structures at 12 days after replating. Consistent field of view over time is shown. Scale bars represent 100 μm.

Note: If digestion with 0.25% trypsin-EDTA seems too harsh for any particular cell line, attempt to use a 0.05% dilution instead.

Alternatives: Instead of 0.25% trypsin-EDTA, you can use 10× TrypLE Select or Accutase.

Metabolic Selection of hiPSC-Derived Cardiomyocytes

TIMING: 3–7 days

Metabolic selection can be performed in order to purify hiPSC-derived atrial and ventricular cardiomyocytes after the differentiation. Provision of lactate as the only energy source is permissive of the survival of cells with cardiac-like metabolism only. To enhance selection efficiency and prevent detachment of cardiomyocytes, replate the cells at a lower density, ideally around day 15. The optimal time point for selection is between days 15-30, due to the cells' high sensitivity to lactate associated with their proliferative state at this age; however, selection is possible until day 60.

39. Digest the differentiated cells as described in 'Digestion of hiPSC-derived cardiomyocytes' (steps 31-36). After resuspending the cells, count them using an automated cell counter or a hemocytometer, and plate in a lower density, e.g. at a ratio of 1:3 or at a defined cell number of 1-2×10^6 cells per well (6-well).
40. Allow cells to regenerate in Cardio Culture Medium for 3-5 days before starting the selection. Change the medium every 2-3 days.
41. Start the selection process by aspirating the spent medium and adding 2 ml per well (6-well) of Cardio Selection Medium.
42. Change the medium to 2 or 3 ml Cardio Selection Medium every 2 or 3 days, respectively, for the duration of the selection process (e.g. 2 ml on Monday and Wednesday, 3 ml on Friday).
43. To stop the selection, switch to Cardio Culture Medium. Change the medium to 2 ml of Cardio Culture Medium and change this medium every 2 or 3 days thereafter (e.g. 2 ml on Monday and Wednesday, 3 ml on Friday).

Note: The incubation time in Cardio Selection Medium is dependent on the age of the cardiomyocytes: 3-5 days before day 30, and 5-7 days for cells between days 30 and 60 (see Troubleshooting Problem 4).

Cryopreservation of hiPSC-Derived Cardiomyocytes

TIMING: 3 h

hiPSC-derived atrial and ventricular cardiomyocytes can be cryopreserved for long-term storage. To ensure pure populations, the cells should ideally be frozen after having passed through a digestion and selection process. Cryopreservation can be performed from day 15 after the start of differentiation onwards.

44. Prewarm FBS, PBS or EDTA Dissociation Buffer, 0.25% trypsin-EDTA to 20-25°C.
45. Aspirate medium of hiPSC-derived cardiomyocytes. Wash the cells once with PBS or EDTA Dissociation Buffer (e.g. 1 ml per well of a 6-well plate) and directly aspirate again.
46. Add 1 ml of 0.25% trypsin-EDTA per well (6-well) to the cells and incubate at 37°C for 8-12 min. Continue when the cells become round and start to detach from the plate.
47. Carefully wash all cells from the plate and transfer them into a 15 ml centrifuge tube. Add the same volume of FBS and centrifuge at 100 × *g* for 10 min.

⚠ CRITICAL: Because the cells are very sensitive, handle them carefully and avoid extensive pipetting.

48. Carefully aspirate the supernatant and resuspend the cells in a small volume (1-2 ml, depending on the size of the cell pellet) of FBS, and count the cells using an automated cell counter or a hemocytometer.
49. According to the desired volume and cell number per cryogenic vial and the consequent total number of cryogenic vials needed, fill up the cell suspension with FBS to 80% of the total required volume. Add a final concentration of 1× Cardio Cryopreservation Medium (from 5×), mix carefully and dispense the cell suspension into the cryogenic vials.

Note: We suggest a cell density of 2-5×10^6 cells in 1.5 ml of cryopreservation medium per vial.

50. Transfer the tubes to a controlled rate freezing device (e.g. Cryotherm Planer) and freeze the cells according to the protocol in Table 1.

Alternatives: If no automated controlled rate freezer is available, freeze the cells in an isopropanol freezing container at -80°C for at least 2 h.

51. After a minimum of 10 min at -90°C, store the tubes in the vapor phase of a liquid nitrogen freezer.

Table 1. Protocol for Controlled Rate Freezing of hiPSC-Derived Cardiomyocytes

Step	Rate (°C/min)	End Temperature (°C)
0	Starting temperature	+4
1	-1	-4
2	-25	-40
3	+10	-12
4	-1	-40
5	-10	-90
6	Hold	-90

Note: Long-term storage in the vapor phase of liquid nitrogen (at a temperature between -140°C and -180°C) for more than 2 years was tested without obvious effects on cell quality.

Note: If digestion with 0.25% trypsin-EDTA seems too harsh for any particular cell line, attempt to use a 0.05% dilution instead.

Alternatives: Instead of 0.25% trypsin-EDTA, you can use 10× TrypLE Select or Accutase.

Recovery of hiPSC-Derived Cardiomyocytes

TIMING: 10–20 min

Cryopreserved hiPSC-derived atrial and ventricular cardiomyocytes can be recovered into culture as described here.

Note: Recovery rates of cryopreserved hiPSC-cardiomyocytes might vary between cell lines. Typically, plating efficiencies of 50%–-90% can be expected.

52. Prewarm Matrigel-coated plates and Cardio Recovery Medium to 20-25°C.
53. Aspirate Matrigel from the precoated culture plates and add Cardio Recovery Medium (2 ml per well of a 6-well plate).
54. Prepare a 15 ml centrifuge tube with 9 ml of Cardio Recovery Medium.
55. Transfer the cryogenic vial containing the cells from the liquid nitrogen storage directly to a 37°C water bath and observe the thawing process. When most of the content of the tube is thawed and only a small chunk of ice remains, quickly take it out and clean thoroughly with 70% ethanol.
56. Carefully add the cells dropwise into the prepared 15 ml centrifuge tube. Gently mix and centrifuge at 100 × *g* for 10 min.
57. Aspirate the supernatant and carefully resuspend the cells in 1 ml of Cardio Recovery Medium.

⚠ CRITICAL: Because the cells are very sensitive, handle them carefully and avoid extensive pipetting.

58. Plate the desired cell number on the coated plates and incubate at 37°C and 5% CO_2.

Note: Upon thawing, we recommend an initial plating of 1-3×10^6 cells per well of a 6-well plate to ensure optimal recovery of cells.

Optional: Recount the cells before plating (note that the recovery rate might not be accurate at this point, so that the number of plated and attached cells might be lower than the total number of living cells counted).

59. Change the medium to Cardio Culture Medium (2 ml per well of a 6-well plate) after a recovery period of 2-3 days. Continue changing the medium regularly every 2 or 3 days thereafter (e.g. 2 ml on Monday and Wednesday, 3 ml on Friday).

Note: Before performing analysis of or further experiments with thawed cardiomyocytes, let them recover on the cell culture plate for 4-7 days (see Figure 2).

Immunostaining of hiPSC-Derived Cardiomyocytes

TIMING: 2 days

For the characterization and quality control of hiPSC-derived atrial and ventricular cardiomyocytes, they can be fixed and immunocytochemically stained for the expression of cardiac markers, such as α-actinin, cTNT, RYR2, MLC2a, and MLC2v.

60. Carefully place sterile glass cover slips into a multi-well plate (e.g. cover slips 20 mm in diameter into 12-well plates). Coat the cover slips with Matrigel as described in 'Before you begin' (e.g. with 500 µl per 20 mm cover slip).

    Alternatives: Chamber slides with detachable rims can be coated with Matrigel, or – if the microscopic setup allows and the stained cells do not need to be kept on mounted microscope slides – the cells can also be stained directly in the cell culture plates.

61. Digest the differentiated cells as described in 'Digestion of hiPSC-derived cardiomyocytes' (steps 31-36). After resuspending the cells, count them using an automated cell counter or a hemocytometer, and plate at the desired cell density, e.g. 70,000-150,000 cells per 20 mm cover slip.
62. Allow the cells to regenerate in Cardio Culture Medium for 7-10 days before fixation. Change the medium every 2-3 days.
63. Aspirate the medium of the cardiomyocytes on glass cover slips. Wash the cells three times with PBS.
64. Incubate the cells in 4% Histofix (or other fixative solution) at 20-25°C for 20 min. Wash them three times with PBS.

    ⏸ PAUSE POINT: Fixed cardiomyocytes can be stored in PBS with 1% BSA at 4°C for up to 1 month.

65. Dilute the primary antibody in PBS with 1% BSA with or without 0.1% Triton X-100 depending on the antibody (see Table 2).
66. Aspirate the supernatant of the fixed cells and add 200 µl primary antibody dilution. Incubate at 4°C for 16-24 h.
67. Dilute the secondary antibody in PBS with 1% BSA with or without 0.1 % Triton X-100 depending on the antibody (see Table 2).
68. Aspirate the primary antibody dilution and wash three times with PBS. Add the secondary antibody dilution and incubate at 20-25°C in the dark for 1 h.
69. Aspirate the secondary antibody dilution and wash once with PBS. Add diluted DAPI/Hoechst nuclear stain (1:2,000-1:3,000 in ddH_2O) and incubate at 20-25°C in the dark for 10 min.
70. Aspirate the solution and wash three times with PBS, followed by one time with ddH_2O. Mount the cover slips upside down onto clean glass slides with a few drops of an aqueous mounting medium (e.g. Fluoromount/Vectashield). Be careful not to create bubbles when lowering the cover slips onto the slides.

Table 2. Antibodies Used for the Immunocytochemical Staining of hiPSC-derived Cardiomyocytes

Antibody	Dilution	Supplier
Primary antibody		
α-actinin monoclonal mouse IgG1	1:1,000	Sigma-Aldrich
CTNT monoclonal mouse IgG1	1:200	Thermo Fisher Scientific
RYR2 polyclonal rabbit IgG	1:500	Sigma-Aldrich
MLC2A monoclonal mouse IgG2a	1:200	Synaptic Systems
MLC2V polyclonal rabbit IgG	1:200	Protein tech
Secondary antibody		
Alexa Fluor 488 donkey anti-rabbit	1:1,000	Thermo Fisher Scientific
Alexa Fluor 555 donkey anti-mouse	1:1,000	Thermo Fisher Scientific

71. Let the mounting medium dry for 1 h and seal the edges of the coverslip with nail varnish. Store the slides at 4°C in the dark.

EXPECTED OUTCOMES

The protocol for the differentiation of hiPSCs towards atrial or ventricular cardiomyocytes described here has been adapted from and modified based on previously published protocols (Zhang et al., 2011; Lian et al., 2013; Tohyama et al., 2013; Burridge et al., 2014). It has been applied successfully to more than 50 hiPSC lines in our hands, and robustly yields 80-90% of $cTNT^+$ cardiomyocytes without significant differences between atrial and ventricular specification. Upon metabolic selection, the purity can be further increased. Within the cardiomyocyte population, this protocol generates approximately 90-95% of the desired atrial or ventricular subtype. At day 30 after the start of differentiation, approximately $1\text{-}3\times10^6$ atrial or ventricular cardiomyocytes can be obtained per well of a 6-well plate, into which 150,000-260,000 iPS cells have initially been plated 2-4 days before the start of differentiation, i.e. for every iPS cell plated, approximately 10 cardiomyocytes can be generated.

At ages of 20-90 days after start of differentiation (or even older), the cells can be applied in experiments or analyzed for cardiac characteristics, such as the expression of general cardiac markers (e.g. α-actinin, cTNT, RYR2) or subtype-specific markers (e.g. MLC2v and MLC2a for ventricular and atrial cardiomyocytes, respectively). However, regarding the choice of experimentation time point it should be noted that the maturity of the cells will likely improve with prolonged culture time. The assembly of hiPSC-derived atrial or ventricular cardiomyocytes and fibroblasts into engineered heart muscle will further mature the cardiac cells and enables their investigation in a three-dimensional context (Cyganek et al., 2018).

LIMITATIONS

Our subtype-directed differentiation protocol provides relatively homogeneous cell populations for most hiPSC lines tested in our hands. However, differentiation efficiencies can vary significantly between cell lines. Although the protocol yields atrial/ventricular cardiomyocytes with purities of approximately 80-90%, a minimum of 10% cardiomyocytes of different lineages as well as non-cardiomyocytes can be found in the samples.

The hiPSC-derived cardiomyocytes generated using this protocol display embryonic- or fetal-like properties when compared to adult cardiac tissue, as evaluated by functional, transcriptomic and proteomic phenotyping (Cyganek et al., 2018). However, the generation of three-dimensional tissue has been shown to increase the maturity of the two-dimensional cell populations and already more closely reflects the *in vivo* situation (Tiburcy et al., 2017).

TROUBLESHOOTING

Problem 1

The hiPSCs differentiate on their own already in maintenance culture OR the hiPSCs do not differentiate well into cardiac cells upon application of the directed differentiation protocol.

Potential Solution

Check for pluripotency of the starting hiPSC population, e.g. by assessing marker expression and trilineage differentiation potential. Verify that the hiPSC line displays a normal karyotype.

Problem 2

Excessive cell death and detachment happens during/after CHIR99021 treatment.

Potential Solution

A substantial loss of cells is expected at this point. However, if most of the cells are lost, the differentiation will fail. Try to optimize the CHIR99021 concentration applied on day 0 of differentiation between 3-8 μM – this can vary between cell lines. Alternatively, try to optimize the starting cell density for the differentiation.

Problem 3

During long-term culture of hiPSC-derived cardiomyocytes, clusters of beating cells start to detach.

Potential Solution

The differentiated cells can be digested and replated in a lower cell density (if they were too dense) onto new coated plates.

Problem 4

Cardiac cells detach in whole clusters during metabolic selection.

Potential Solution

In order to eliminate dead cells from the cell cultures during the selection process and therefore save the cardiac cells, carefully collect the medium (including the clusters in suspension) and flush it through a 40 μm mesh. Then turn the filter and carefully wash off the collected clusters into fresh Cardio Selection Medium. Transfer the cell suspension into a new coated well.

SUPPLEMENTAL INFORMATION

Supplemental Information can be found online at https://doi.org/10.1016/j.xpro.2020.100026.

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AUTHOR CONTRIBUTIONS

L.C. conceived the protocol and edited the manuscript. M.K. drafted the manuscript.

DECLARATION OF INTERESTS

The authors declare no competing interests.

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