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Patient-Specific Induced Pluripotent Stem Cell Models: Generation and Characterization of Cardiac Cells

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Abstract

The generation of human induced pluripotent stem cell (hiPSC)-derived cardiomyocytes has been of utmost interest for the study of cardiac development, cardiac disease modeling, and evaluation of cardiotoxic effects of novel candidate drugs. Several protocols have been developed to guide human stem cells toward the cardiogenic path. Pioneering work used serum to promote cardiogenesis; however, low cardiogenic throughputs, lack of chemical definition, and batch-to-batch variability of serum lots constituted a considerable impediment to the implementation of those protocols to large-scale cell biology. Further work focused on the manipulation of pathways that mouse genetics indicated to be fundamental in cardiac development to promote cardiac differentiation in stem cells. Although extremely elegant, those serum-free protocols involved the use of human recombinant cytokines that tend to be quite costly and which can also be variable between lots. The latest generation of cardiogenic protocols aimed for a more cost-effective and reproducible definition of the conditions driving cardiac differentiation, using small molecules to manipulate cardiogenic pathways overriding the need for cytokines. This chapter details methods based on currently available cardiac differentiation protocols for the generation and characterization of robust numbers of hiPSC-derived cardiomyocytes under chemically defined conditions.

Keywords

Human induced pluripotent stem cells; Cardiomyocyte; Cardiac differentiation; Cardiac assays; Small molecule; Wnt signaling pathway

1 Introduction

Since their establishment, human induced pluripotent stem cells (hiPSCs) have been considered a new gold standard for modeling human genetic-based diseases. Similar to human embryonic stem cells, hiPSCs have the potential to differentiate into virtually any cell type in the human body, offering enticing possibilities to study organ- and system-specific disorders, including diseases affecting the heart (1). Additionally, hiPSC-derived cardiomyocytes constitute a robust platform for the assessment of cardiotoxicity, a major contributor to the failure of potential novel drugs in later stages of clinical trials (2). Several genetic diseases bearing cardiac phenotypes have been modeled with hiPSCs including LEOPARD syndrome (3), long QT syndrome (4–7), Timothy syndrome (8), catecholaminergic polymorphic ventricular tachycardia (9–12), familial dilated (13) and

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hypertrophic cardiomyopathies (14, 15), arrhythmogenic right ventricular cardiomyopathy (16–18), as well as an overlapping syndrome of a cardiac Na^+ channel disease (19). As the field has evolved, several differentiation protocols have been developed for the differentiation of hiPSCs toward the cardiac lineages (1). Currently, the most popular protocols rely on small-molecule-mediated temporal modulation of the Wnt pathway (20, 21). Here we present an adapted version of a small-molecule-based protocol, which has been used successfully across many independent hiPSC control and diseased lines.

2 Materials

2.1 Commercially Available Reagents.	*See* Table 1
2.2 Preparation of Extracellular Matrices (ECMs)	Add the corresponding volume of diluted ECMs to coat different tissue culture plates according to Table 2.
2.2.1 Growth Factor-Reduced Matrigel	Thaw a 10 ml vial of growth factor-reduced matrigel on ice for 1 hour (h) or overnight at 4 °C. Add 10 ml of cold DMEM/F12 medium and mix quickly and thoroughly. Quickly prepare 600 μl aliquots, to be stored at −80 °C.
2.2.2 Diluted Growth Factor-Reduced Matrigel	Thaw a 600 μl aliquot of growth factor-reduced matrigel on ice for 1 h or overnight at 4 °C. Add 50 ml of cold DMEM/F12 medium to a 50 ml falcon tube. Dilute the matrigel aliquot into DMEM/ F12 and quickly mix thoroughly by inverting the falcon tube several times. Cell culture plates are subsequently incubated with matrigel solution to coat surfaces. Coated plates may be stored at 37 °C for up to 5 days prior to cell seeding.
2.2.3 Laminin	A 1 ml vial of laminin is thawed on ice and 50 μl aliquots are prepared for storage at −80 °C. For laminin coating, a 50 μl laminin aliquot is thawed on ice and diluted into a 5 ml solution of PBS+, mixed thoroughly and incubated on tissue culture dish surfaces overnight at 37 °C. Coated dishes may be stored at 4 °C for up to 7 days prior to cell seeding.
2.3 Cell Culture Medium	hiPSCs are maintained in Essential 8 (E8) medium (22). Prepare stock solutions and make aliquots as indicated in Table 3. *See* Note 1.
2.3.1 hiPSC Culture Medium	Prepare a 500 ml bottle of E8 medium by adding supplements according to Table 4. *See* Note 2.
2.3.2 Differentiation Initiation Medium: RPMI Medium with B27 Supplement Without Insulin (RB–)	Take a 500 ml bottle of RPMI and add one 10 ml vial of B27 without insulin. Add one 5 ml aliquot of P/S. Mix well. If the medium will be used within 1–10 days, then the bottle can be kept at 4 °C and 50 ml aliquots can be generated to be warmed when needed. Otherwise, 50 ml aliquots should be prepared for storage at −20 °C.
2.3.3 Differentiation Medium: RPMI Medium with B27 Containing Insulin (RB+)	Take a 500 ml bottle of RPMI and add one 10 ml vial of B27 with insulin. Add one 5 ml aliquot of P/S. Mix well. If the medium will be used within 1–10 days, then the bottle can be kept at 4 °C and 50 ml aliquots can be generated to be warmed when needed. Otherwise, 50 ml aliquots should be prepared for storage at −20 °C.
2.3.4 Maturation Medium: DMEM/M199 Medium with B27 Containing Insulin (D/199B+)	Take a fresh bottle of DMEM and remove 140 ml of the medium. Add 125 ml of M199. Add one 10 ml vial of B27 with insulin. Add one 5 ml aliquot of P/S. Mix well. If the medium will be used within 1–10 days, then the bottle can be kept at 4 °C and 50 ml aliquots can be generated to be warmed when needed. Otherwise, 50 ml aliquots should be prepared for storage at −20 °C.
2.4 Small Molecules *2.4.1 Rock Inhibitor/ Y27632: $Rock_i$*	For a 10 mM (2,000×) solution, re-suspend 10 mg vial in 3.122 ml of DMSO. Mix well, vortex, and make 25–50 μl aliquots, to be stored at −20 °C. Aliquots may be reused up to three times.

[1]The solvent for all stock solutions is tissue culture-grade water, except for TGFβ, for which 10 mM citric acid with pH = 3 should be used. When preparing the ascorbic acid stock solution, pre-warm, pre-warm tissue culture-grade water to 37 °C before slowly dissolving with constant agitation and vortexing. Crystals may take several hours to dissolve completely. Store all aliquots of stock solutions at −20 °C.

[2]The cytokines in E8 medium are not stable at 37 °C for long periods of time. Therefore it is preferable to warm the medium to room temperature instead of 37 °C.

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2.4.2 CHIR99021: CH

For a 12 mM (2,000×) solution, re-suspend 25 mg vial in 4.152 ml of DMSO. Mix well, vortex, and make 25–50 μl aliquots, to be stored at −20 °C. Aliquots may be reused up to three times.

2.4.3 IWP-2

For a 5 mM (1,000×) solution, re-suspend 10 mg vial in 4.286 ml of pre-warmed DMSO. Mix well, vortex, and make 25–50 μl aliquots, to be stored at −20 °C. Aliquots may be reused up to three times.

2.5 Dissociation Reagents

2.5.1 hiPSC Dissociation Solution: 0.5 mM EDTA

Add 500 μl of 0.5 M EDTA to a bottle of PBS−.

2.5.2 Collagenase Type II Solution

Cardiomyocytes are dissociated in a solution containing 200 units/ ml of HBSS−. Estimate the required volume of collagenase II solution, and calculate and weigh the mass necessary to reach the target concentration in units/ml. Dissolve into HBSS− and filter sterilize. The solution can be kept at 4 °C for up to 1 week.

2.5.3 Cardiomyocyte Dissociation Solution

Prepare cardiomyocyte dissociation solution immediately before use. For 1 ml cardiomyocyte dissociation solution, prepare the stock solutions in tissue culture-grade water as described in Table 5.

2.6 Buffers

2.6.1 Immunofluorescence Permeabilization Buffer

In order to prepare 50 ml of immunofluorescence permeabilization buffer, dilute 100 μl of Triton-X in 50 ml of PBS+, for a final concentration of 0.2 % Triton-X.

2.6.2 Immunofluorescence Dilution Buffer

In order to prepare 50 ml of immunofluorescence buffer, prepare the stock solutions listed in Table 6 and add the appropriate volumes for the final desired concentrations.

2.6.3 Immunofluorescence Blocking Buffer

In order to prepare 10 ml of immunofluorescence blocking buffer, take 9.5 ml of immunofluorescence dilution buffer and add 500 μl of donkey serum for a final concentration of 5 %.

2.6.4 FACS Dilution Buffer

BD Perm/Wash buffer is sold as a 10× concentrate that must be diluted in PBS− before use. For 10 ml of 1× ready-to-use BD Perm/Wash buffer, add 1 ml of 10× concentrate BD Perm/Wash buffer to 9 ml of PBS−.

2.6.5 FACS Blocking Buffer

For 10 ml of FACS blocking buffer, take 9.5 ml of 1× ready-to-use BD Perm/Wash buffer and add 500 μl of donkey serum for a final concentration of 5 %.

3 Methods

3.1 hiPSC Culture

hiPSCs are grown as confluent monolayers on growth factor-reduced matrigel-coated 6 cm^2 plates. Passaging is performed at a ratio of 1:4–1:10 every 4–6 days and achieved as outlined below:

1. Wash cells once with warm PBS−.
2. Add 2 ml of warm 0.5 mM EDTA in PBS−.
3. Incubate for 5 min at 37 °C.
4. Aspirate the 0.5 mM EDTA and re-suspend cells in 4 ml of E8 medium. If cells do not detach completely, use a cell lifter to gently scrap them and break larger clusters by pipetting 6–10 times.
5. Split cells as required and add $Rock_i$ to a final concentration of 5 μM.

3.2 hiPSC Seeding for Cardiac Differentiation

Cardiac differentiation is typically performed in a 12-well plate format, although other formats can be used with the appropriate adjustments to cell growth surfaces. Prior to seeding, coat a 12-well plate with diluted growth factor-reduced matrigel as described in Sections 2.2 and 2.2.1.

1. When hiPSCs (6 cm plate) reach 80–90 % confluence, aspirate E8 medium and wash cells once with 4 ml of warm PBS−.
2. Add 1.5 ml of warm Accutase, and move the plate in perpendicular directions to spread the enzyme evenly. Remove 1 ml of the Accutase and move the plate in perpendicular directions again.
3. Incubate hiPSC for 3–5 min at room temperature, until cells are singularized. *See* Note 3.

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4. Remove Accutase quickly and add 4 ml of maintenance medium. Pipette up and down 6–10 times to ensure that cells are singularized or reduced to very small clusters. Transfer cells to a 15 ml Falcon tube. Add 5 μM of $Rock_i$ (2.5 μl of a 10 mM aliquot).
5. Count cells in the suspension.
6. Prepare 12.5 ml of a second suspension containing 5 μM $Rock_i$ in which the cell concentration is adequate for the desired number of cells to be plated in each well. *See* Note 4.
7. Plate 1 ml of the cell suspension prepared in step 4 in each well of the 12-well plate. Move the plate vigorously in perpendicular directions to ensure that cells are evenly distributed.

3.3 Cardiac Differentiation

The protocol described herein is an adaptation of a previously described small-molecule-based method (20, 23).
The key differences are the following:

1. Washing hiPSCs before cardiac differentiation/induction. The purpose of this step is to remove traces of FGF at the beginning of differentiation.
2. Use of insulin during CH treatment. We have observed that cell death can significantly impact cardiac differentiation. Insulin, acting as a pro-survival cytokine, facilitates survival during the first 48 h of the cardiac differentiation protocol.
3. Washing hiPSCs after cardiac differentiation/induction. Insulin has been proposed to have a detrimental effect on cardiac differentiation after 72–96 h of induction (24). Therefore, cultures are washed with RPMI before the addition of RB−.
4. Switching to D/199B+ after day 30 of cardiac differentiation. After cardiomyocytes are differentiated, it is recommended that they are maintained in culture for an additional time to allow for further maturation. The nutrient composition of RPMI medium is relatively conservative; thus, cardiomyocyte death can occur also at later stages of differentiation/maturation due to stringent culture conditions. Thus, an adaptation of the medium used for neonate mouse cardiomyocyte cultures has been incorporated in this protocol to promote an environment richer in nutrients, allowing for enhanced cardiomyocyte health and survival during maturation.

3.3.1 Day 0: Induction

1. When hiPSCs plated on the 12-well plate reach 100 % confluence (within 2–5 days of plating), warm an aliquot of RPMI and one of RB+ medium.
2. Take 24.5 ml of RB+ and add CH to a final concentration of 6 μM. Mix well. *See* Note 5.
3. Wash cells once with warm RPMI medium (1 ml per well).
4. Add 2 ml of RB+ with CH to each well. Record the time at which CH is added.

3.3.2 Day 2: Wash and Switch to RB−

After CH treatment, mild to considerable cell death is expected. Proceed with the protocol as outlined below:

1. Warm an aliquot of RPMI and RB− medium.
2. 48 h after adding CH to the cells:

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[3]Since Accutase is an aggressive enzyme, cell dissociation should be monitored frequently as some cell lines will singularize and even detach at a shorter time. If it is noted that cells start to peel off, remove the enzyme immediately. If all cells become detached, add 3 ml of maintenance medium, pipette up and down to collect all cells, and transfer to a 15 ml Falcon tube. Add 5 μM of $Rock_i$ (2.5 μl of a 10 mM aliquot) and spin at 800 rpm for 5 min. Re-suspend cells in 4 ml of maintenance medium, add 5 μM of $Rock_i$, and proceed to next step (4).

[4]Seed 1×10^5–5×10^5 cells per well in matrigel-coated 12-well plates. Cell density should be optimized for each cell line, so confluence is reached within 2–5 days after plating. Ideally, cells should reach confluency within wells at the same approximate time frame that it took to reach confluency in 6 cm plates.

[5]CH aliquots are prepared at 12 mM (2,000×), so for 24.5 ml of RB+, 12.25 μl of a CH aliquot is required. Also note that 6 μM CH for 48 h seems to be optimal for most cell lines; however, specific lines may have different requirements. It is recommended that a range of 6–14 μM is tested at treatment durations of 24 h (12–14 μM) and 48 h (6–8 μM).

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a. Wash cells once with warm RPMI medium (2 ml per well).

b. Add 2 ml of RB− to each well.

3.3.3 Day 4: IWP-2 in Conditioned Medium

1. Warm an aliquot of RB− medium.
2. Collect 1 ml of conditioned medium from each well of the differentiation plate at 96 h after adding CH, plus an excess of 0.5 ml, and transfer to a 50 ml Falcon tube. The total volume of conditioned medium collected must be 12.5 ml.
3. Add equal volumes (12.5 ml) of RB−. Add IWP-2 to the conditioned medium to a final concentration of 5 μM. Mix well. *See* Note 6.
4. Aspirate the remaining conditioned medium from all wells of the differentiation plate.
5. Add 2 ml of combined medium with IWP-2 to each well.

3.3.4 Day 6: Medium Change

See Note 7.

1. Warm an aliquot of RB− medium.
2. 144 h after adding CH to the cells: remove medium and add 2 ml of fresh RB− to each well.

3.3.5 Day 8: Medium Change

1. Warm an aliquot of RB− medium.
2. 192 h after adding CH to the cells: remove medium and add 2 ml of fresh RB− to each well.

3.3.6 Day 10: Medium Change (Switch to RB+)

See Note 8.

1. Warm an aliquot of RB+ medium.
2. 240 h after adding CH to the cells: remove medium and add 2 ml of fresh RB+ to each well.

3.3.7 Day 11–30: Differentiation and Maintenance

Change medium every 2–3 days with RB+ until day 30. *See* Note 9.

3.3.8 Day 31–45+: Maturation

Switch medium to D/199B+:

1. Warm an aliquot of D/199B+ medium.
2. Remove medium from cells and add 2 ml of fresh D/199B+ to each well.
3. Change medium every 2–3 days with D/199B+ until cardiomyocytes have achieved desired time points.

   See Note 10.

3.4 Characterization of hiPSC-Derived Cardiomyocytes

Bona fide hiPSC-derived cardiomyocytes express appreciable levels of the contractile protein sarcomeric α-actinin and the transcription factor NKX2.5. Co-staining of cells with this

[6]IWP-2 aliquots are prepared at 5 mM (1,000×), so if a whole 12-well plate is used, add 25 μl of IWP to the 25 ml of combined medium prepared in step 3.

[7]From this time point onward, cells may display a type of growth pattern that resembles epithelial-to-mesenchymal transition (EMT). As a result, instead of growing flat and spreading out horizontally, they may grow vertically and pile up in the form of foci in the well. This type of growth is indicative of successful mesoderm and precardiac mesoderm formation.

[8]If medium is changed every 2 days, add 2 ml of medium per well. If medium is changed every 3 days, add 3 ml of medium per well.

[9]At this time point, there should be sheets and/or clusters of beating cells that can be readily visualized by light microscopy. Beating is first observed between days 7 and 11 for most of cell lines.

[10]If medium is changed every 2 days, add 2 ml of medium per well. If medium is changed every 3 days, add 3 ml of medium per well.

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combination of cardiac markers robustly identifies hiPSC-derived cardiomyocytes.

3.4.1 Cardiomyocyte Dissociation

Beating clusters or sheets of hiPSC-derived cardiomyocytes can be microdissected, dissociated, and replated for specific downstream applications. *See* Note 11.

1. Wash cells once with warm HBSS−.
2. Add 1 ml of collagenase II solution. Add 10 μM $Rock_i$ and incubate for 30 min at 37 °C, swirling the plate or vortexing the microdissected cells every 5 min.
3. Pipette cells repeatedly 5–10 times to break larger clusters and transfer cells to a 2 ml Eppendorf tube.
4. Add 1 ml of dissociation solution dropwise. Mix gently.
5. Pass cells through a 20 G syringe needle 3–6 times until larger clumps can no longer be observed by the eye.
6. Spin cells for 5 min at 130 × *g* and re-suspend cells in 1 ml of D/199B+. Add 10 μM $Rock_i$.
7. Count cells and replate at an appropriate density. For a 3.5 cm plate, a minimum of 2×10^5 cells/plate should be added. Add 10 μM $Rock_i$.

   See Note 12.

3.4.2 Flow Cytometry

Flow cytometry allows for robust, quantitative, and rapid estimation of cardiomyocyte throughputs in a given differentiation experiment. The staining protocol outlined below relies on co-staining of undifferentiated hiPSCs (negative control) and hiPSC-derived cardiomyocytes resulting from a differentiation experiment with sarcomeric α-actinin and NKX2.5. *See* Note 13.

1. Dissociate cardiomyocytes, and following resuspension, strain cells through a 100 μM nylon mesh.
2. Count cells and prepare a suspension of 1×10^6 cells/ml.
3. Take 2×10^5 cells (200 μl of suspension at 1×10^6 cells/ml) and transfer to an Eppendorf tube.
4. Centrifuge the cell suspension at 200 × *g* in for 2 min. Discard supernatant by carefully aspirating with a micropipette to avoid losing cells.
5. Re-suspend cells in 100 μl of 1× BD Perm/Wash buffer and incubate for 10 min at room temperature (RT).
6. Centrifuge the cell suspension at 200 × *g* for 2 min. Discard supernatant.
7. Re-suspend cells in 100 μl of FACS blocking solution and incubate for 15 min at RT.
8. Centrifuge the cell suspension at 200 × *g* for 2 min. Discard supernatant.
9. Re-suspend cells in 100 μl of primary antibody mastermix that contains mouse anti-sarcomeric α-actinin (1:100) and rabbit anti-NKX2.5

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[11] 24 h before dissociation, tissue culture dishes should be coated with 10 μg/ml laminin in PBS+, overnight at 37 °C.

[12] Dissociated cardiomyocytes may take up to 3–5 days to start beating again. Change medium every 2–3 days.

[13] Cardiomyocytes can display considerable autofluorescence within the 488 nm wavelengths; thus, secondary antibodies with emissions within the yellow and far-red regions of the light spectrum are preferable.

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(1:100) in blocking solution. Incubate for 30 min at RT.

10. Centrifuge the cell suspension at 200 × *g* for 2 min. Discard supernatant.
11. Wash once with 100 μl of 1× BD Perm/Wash buffer. Spin at 800 rpm for 2 min. Discard supernatant.
12. Re-suspend in 100 μl of secondary antibody mastermix that contains phycoerythrin-conjugated donkey anti-mouse (1:100) and PerCP-conjugated donkey anti-rabbit (1:100) in FACS blocking solution. Incubate for 30 min at RT in the dark.
13. Centrifuge the cell suspension at 200 × *g* for 2 min. Discard supernatant.
14. Wash once with 100 μl of 1× BD Perm/Wash buffer.
15. Centrifuge the cell suspension at 200 × *g* for 2 min. Discard supernatant.
16. Re-suspend cells in 200 μl of PBS−. Keep cells on ice in the dark until ready to analyze.

Expected Results

Figure 1 exemplifies FACS analysis of NKX2.5 and sarcomeric α-actinin results from hiPSC before and after cardiac differentiation. Cellular debris and cell doublets were eliminated, as well as individual signals for both antibodies were analyzed through gates not shown here. Gate P4 was set after analysis of stained undifferentiated hiPSC and adjustment of the negative control signals to the first three decades of each axis.

3.4.3 Immunofluorescence Microscopy

Cardiomyocytes may be dissociated and replated on laminin-coated dishes that are appropriate for cell imaging such as chamber slides, glass coverslips, and black-walled optic plates for immunofluorescence-based microscopy analysis.

1. Wash cells once with PBS+ solution.
2. Fix cells with 4 % PFA for 30 min at RT.
3. Wash cells once with PBS+ solution.
4. Permeabilize cells with immunofluorescence permeabilization buffer for 10 min at RT.
5. Block cells with immunofluorescence blocking buffer for 30 min at room temperature.
6. Prepare primary antibodies in immunofluorescence blocking solution:

   Mouse anti-sarcomeric α-actinin (1:100).

   Rabbit anti-NKX 2.5 (1:100).

   Aspirate blocking solution and add primary antibody mixture. Incubate overnight at 4 °C in a humidified chamber.
7. Wash cells three times for 5 min at RT with PBS +.
8. Prepare secondary antibodies and DNA stain in immunofluorescence blocking solution:

   DyLight-488-conjugated donkey anti-mouse (1:100).

   DyLight-549-conjugated donkey anti-rabbit (1:100).

   Hoechst 33342: 1 μg/ml.

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Aspirate PBS+ and add secondary antibody mixture. Incubate for 2 h in the dark at RT in a humidified chamber.

9 Wash cells three times for 5 min at RT with PBS +.

10 Mount cells and maintain at 4 °C in the dark until ready to image by using confocal microscopy.

Expected Results

Figure 2 exemplifies results obtained from immunofluorescence staining analysis of sarcomeric α-actinin and NKX2.5 in hiPSC following cardiac differentiation. Cells are considered positive for sarcomeric α-actinin when a clear striated pattern can be visualized. Cardiomyocytes are also defined by expression of NKX2.5, which is found confined to the cell nucleus.

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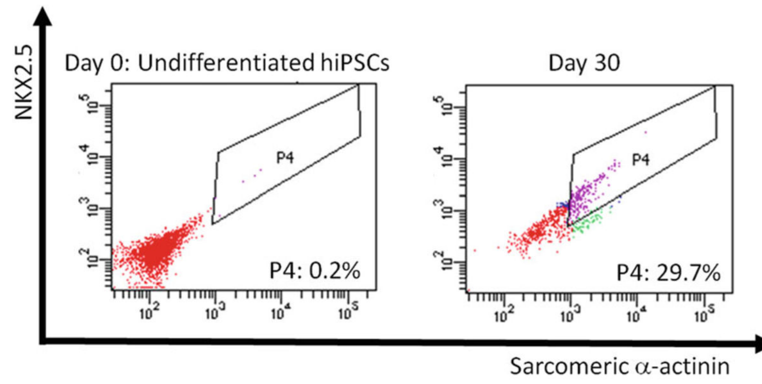

Fig. 1.

An example of FACS analysis of sarcomeric α-actinin and NKX2.5 coexpression as an indicator of cardiomyocyte throughput. Gate P4 highlights double-positive cells in undifferentiated cells on day 0 and hiPSC-derived cardiomyocytes on day 30

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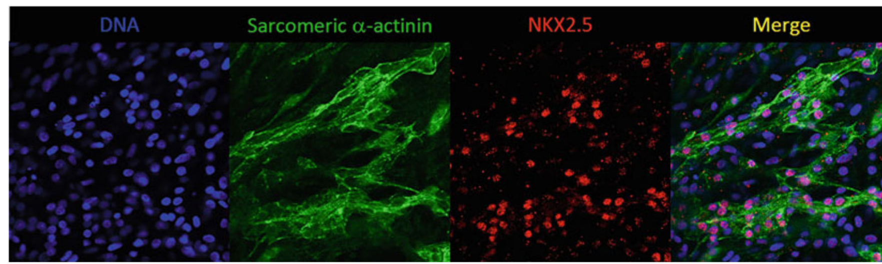

Fig. 2.

An example of immunofluorescence analysis of sarcomeric α-actinin and NKX2.5 coexpression on day 30 of differentiation as an indicator of cardiomyocyte throughput

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Table 1

Commercially available reagents

Description	Manufacturer	Catalog number
Cell growth matrices		
Growth factor-reduced matrigel	Corning	354230
Laminin	Life Technologies	23017-015
Cell culture media		
DMEM/F12 with glutamine and HEPES	Life Technologies	11330-032
RPMI 1640 with L-glutamine	Life Technologies	11875093
DMEM with L-glutamine and without sodium pyruvate	Corning	10-017
M199 with L-glutamine	Corning	10-060
Medium supplements		
L-Ascorbic acid 2-phosphate	Sigma-Aldrich	A8960
Insulin	Life Technologies	12585-014
Transferrin	Sigma-Aldrich	T3705
Sodium selenite	Sigma-Aldrich	S5261
B-27 with insulin	Life Technologies	17504044
B-27 without insulin	Life Technologies	A1895601
Penicillin/streptomycin antibiotics (P/S)	Corning	30-002
Cytokines		
FGF2	PeproTech	100-18B
TGFβ	PeproTech	100-21
Buffers		
PBS without Ca^{2+} and Mg^{2+} (PBS−)	Corning	21-031
PBS with Ca^{2+} and Mg^{2+} (PBS+)	Corning	21-030
HBSS without Ca^{2+} and Mg^{2+} (HBSS−)	Corning	21-022
7.5 % sodium bicarbonate	Life Technologies	25080-094
0.5 M EDTA	Life Technologies	15575-020
BD Perm/Wash buffer	BD Biosciences	554723
Cell dissociation reagents		
Accutase	Innovative Cell Technologies	AT 104
Collagenase type II	Worthington	LS004174
Taurine	Sigma-Aldrich	T8691-100G
EGTA	Sigma-Aldrich	E-4378-25G
25 % BSA	Life Technologies	A10008-01
Small molecules		
Y27632/Rock inhibitor ($Rock_i$)	Selleckchem	S1049
CHIR99021 (CH)	Selleckchem	S1263
IWP-2	Cayman Chemical	13951

Description	Manufacturer	Catalog number
Antibodies and staining reagents		
Donkey serum	Jackson ImmunoResearch	017-000-121
Mouse anti-sarcomeric alpha actinin	Sigma-Aldrich	A7811
Rabbit anti-NKX2.5	Santa Cruz	sc-14033
DyLight-488-conjugated donkey anti-mouse	Jackson ImmunoResearch	715-485-150
DyLight-549-conjugated donkey anti-rabbit	Jackson ImmunoResearch	711-505-152
R-phycoerythrin-conjugated donkey anti-mouse	Jackson ImmunoResearch	715-116-150
PerCP-conjugated donkey anti-rabbit	Jackson ImmunoResearch	711-126-152
Hoechst 33342 nuclear DNA stain	Life Technologies	H1399

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Table 2

Tissue culture plate formats and coating volumes

Tissue culture plate	ECM coating volume
6 cm^2 plate	3 ml
3.5 cm^2 plate	1.5 ml
12-well plate	0.5 ml/well
96-well plate	50 μl/well

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Table 3

Stock solutions and aliquot volumes

Stock solution	Desired concentration	MW	Quantity needed	Resuspension volume	Aliquot volume (μl)
L-Ascorbic acid 2-phosphate	**64 mg/ml**	**289.54**	**5 g**	**78.125 ml**	**500**
Transferrin	53.5 mg/ml	N/A	1 g	18.692 ml	100
Sodium selenite	700 μg/ml	172.94	35 mg	50 ml	10
FGF2	100 μg/ml	N/A	1 mg	10 ml	500
TGFβ1	100 μg/ml	N/A	100 μg	1 ml	10

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Table 4

Preparation of E8 medium

Component	Stock solution	Take from stock	Final concentration
DMEM/F12 with glutamine and HEPES	1×	500 ml	1×
Sodium bicarbonate	75 mg/ml	3.62 ml	543 μg/ml
L-Ascorbic acid 2-phosphate	64 mg/ml	500 μl	64 μg/ml
Insulin	4 mg/ml	2.5 μl	20 μg/ml
Transferrin	53.5 mg/ml	100 μl	10.7 μg/ml
Sodium selenite	700 μg/ml	10 μl	14 ng/ml
FGF2	100 μg/ml	500 μl	100 ng/ml
TGFβ	100 μg/ml	10 μl	2 ng/ml
Pen/strep	100×	5 ml	1×

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Table 5

Preparation of cardiomyocyte dissociation solution

Component	Stock solution	Take from stock (μl)	Final concentration
Collagenase II solution	200 units/ml	880	200 units/ml
Taurine	200 mM	100	1 mM
EGTA	0.2 mM	10	0.1 mM
BSA	250 mg/ml	8	1 mg/ml

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Table 6

Preparation of immunofluorescence dilution buffer

Component	Stock solution	Take from stock	Final concentration
Milli-Q water	N/A	42.13 ml	N/A
Tris base, pH 7.5	1 M	1 ml	20 mM
NaCl	2 M	3.87 ml	155 mM
EGTA	50 mM	2 ml	2 mM
$MgCl_2$	100 mM	1 ml	100 mM