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## Directed cardiomyocyte generation from human pluripotent stem cells using a chemically defined protocol (GiWi2)

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**We use this protocol and it's working**

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## Abstract

The GSK3 inhibitor and Wnt inhibitor, named GiWi2, are utilized for robust differentiation of human pluripotent stem cells (hPSCs) into cardiomyocytes under serum-free and growth factor-free conditions. This differentiation protocol, developed previously by Dr. Xiaojun Lian (Nat Methods. 2015 Jul; 12(7): 595–596), applies two small molecules at precise developmental stages to sequentially promote mesoderm formation and ventricular cardiomyocyte (vCM) specification. The protocol produces 88–98% cTnT+ cells with yields exceeding 1×10<sup>6</sup> cardiomyocytes/cm<sup>2</sup> across multiple human embryonic stem cell (hESC) and human induced pluripotent stem cell (hiPSC) lines. The resulting cardiomyocytes exhibit spontaneous contraction for over eight months, demonstrating their functionality and long-term viability.



## Materials

1. **Matrigel** (*life science*, cat# 354230)
2. **Y27632** (*Tocris*, cat# 1254), 5 mM Y27632 stock (1:1000)
3. **TeSR-E8** (*Stem Cell Technologies*, cat# 05990): Thaw TeSR-E8 25X Supplement at room temperature (15–25°C) or overnight at 2 – 8°C. Add 20 mL of TeSR-E8 25X Supplement to 480 mL of TeSR-E8 Basal Medium. Mix thoroughly. Store complete medium at 2 – 8°C for up to 2 weeks.
4. **Accutase** (*Innovative Cell Technology*, cat# AT104)
5. **CHIR99021** (*Selleckchem*, cat# S1263-25mg): 10 mM CHIR99021 stock
6. **IWP2** (*Tocris*, cat# 3533-10mg): 5 mM IWP2 stock
7. **Insulin** (*Sigma*, cat# 91077C-250MG): 5 mg/mL in stock (1:500)
8. **RPMI** (*Life Technologies*, cat# 11875-085)
9. **B-27 Minus Insulin** (*Gibco*, cat# A18956-01 ), 50X for RPMI-B27 [RPMI1640 (*Life Technologies*); 2% B-27 Supplement Minus Insulin (B27-Insulin)]
10. **L-Ascorbic acid 2-phosphate sesquimagnesium** (*Sigma*, cat# A8960-5G): 50 mg/mL in stock (1:250)
11. **Cardiomyocyte Maintenance Medium (CMM)**: RPMI1640 supplemented with 10 µg/mL of insulin and 200 µg/mL of L-Ascorbic acid 2-phosphate sesquimagnesium. Add 0.5 mL of insulin stock (5 mg/mL) and 1 mL of ascorbic acid (50 mg/mL) to 250 mL of RPMI1640 basal medium. Sterilize by filtration and store at 4°C.
12. **Freezing medium (2X)**: 20% DMSO, 60% defined FBS, 20% hES cell culture medium, and need to add fresh 10 µM Y27632 (ROCKi).

## Materials

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## Feeder-free culture of hPSCs

- 2 Prepare a Matrigel-coated 6-well plate and store it at 4°C, put it at room temperature at least 1 hour before use.
- 3 Remove a frozen cell vial of hPSCs, from a liquid N<sub>2</sub> tank and immerse the vial in a 37°C water bath without submerging the cap. Swirl the vial gently for 2 – 3 min until the contents are almost completely thawed.
- 4 Spray the vial with 70% (v/v) ethanol and place it in a tissue culture hood. Use a sterile 1-mL pipette to gently transfer the cells into a sterile 15-mL conical tube containing 5 mL of room temperature mTeSR1 medium. Rinse the cell tube a few times without disturbing "little" cell clusters.
- 5 Centrifuge the cells at 200 g for 5 min at room temperature. Aspirate and discard the supernatant with a sterilized electronic pipette. Resuspend the cell pellet in 2 mL of

TeSR-E8 medium(with 5  $\mu$ M ROCKi), and gently resuspend the cells without disturbing "little" cell clusters.

- 6 Aspirate the liquid from the wells of the Matrigel-coated plate.
- 7 Slowly add the cell suspension to the Matrigel-coated 6-well plate. Put the plate back into the 37°C, 5% CO<sub>2</sub> incubator.
- 8 The next day, aspirate the medium in each well and replace it with 2 mL of fresh room temperature mTeSR-E8 medium (without ROCKi). Repeat this medium replacement daily until cells are ready for passage.
- 9 Passage the cells with EDTA-method: Aspirate the medium and wash once with DPBS. Add 1 mL 0.5 mM EDTA-DPBS buffer to each well to dissociate the cells. Incubate for 3 - 5 min at 37°C and observe with microscopy. Aspirate the EDTA buffer. Add 1 mL TeSR-E8 with Rock-inhibitor (1:1000) to the cells and gently detach them by pipetting them up and down. Transfer the cells to a 15 mL tube with 5 mL TeSR-E8 (with ROCKi), and rinse the well with 1 mL medium. Add the cells to a Matrigel-coated plate (6 wells).

## Differentiate hPSCs into Cardiomyocytes

### 10 (Day -2) Passaging hPSCs using Accutane:

- 10.1 Take hPSCs cultured on Matrigel-coated 6-wells in mTeSR1 medium at 80-90% confluence. Aspirate medium and wash once with DPBS. Add 1 mL of room-temp Accutase to each 6-well. Place plate in 37°C, 5% CO<sub>2</sub> incubator for ~5 min.
- 10.2 Add 0.5 mL mTeSR1 to each well and gently detach the cells by pipetting the cells up and down and pool the cells in a 15-mL conical tube. Count total cells and centrifuge at 200 g for 5 minutes at room temp.
- 10.3 Aspirate supernatant, resuspend cells in TeSR-E8 + 5  $\mu$ M Y27632, and plate 200 k cells/cm<sup>2</sup> (i.e. ~700 k cells/3.5 cm<sup>2</sup> for 12-well) onto Matrigel-coated plate. Add TeSR-E8 + 5  $\mu$ M Y27632 medium to each well to obtain a final volume of 1 mL in each well of the 12-well plate. Incubate the cells. Move the plate in quick, short, back-and-forth, and side-to-side motions to disperse the cells across the surface of the wells.

#### Note

- 1) This time point corresponds to Day -2 of differentiation.
- 2) Seeding density is crucial: recommended cell density by day 0 should be 250-400 k cells/cm<sup>2</sup>.

- 11 **Day -1 Maintenance:** On day -1 of differentiation, aspirate the medium and replace it with 2 mL of room temperature TeSR-E8 per well of the 12-well plate.
- 12 **Day 0 Add GSK3 inhibitor CHIR99021:** On day 0 of differentiation, prepare 2 mL of 6  $\mu$ M CHIR99021 in RPMI-B27 medium for each well of 12-well. Aspirate the old medium and add 2 mL of this mixture to each well. **Record the time, as the medium needs to be changed exactly 24 hours later.**
- 13 **Day 1 Remove GSK3 inhibitor CHIR99021.** After 24 h, aspirate medium from each well and replace with 2 mL of room-temp RPMI-B27. Put the plate back into 37°C, 5% CO<sub>2</sub> incubator.

**Note**

If a lot of cell death/detachment is observed, which might be less than the optimal initial seeding density or too much CHIR (concentration vs time).
- 14 **Day 3 add Wnt inhibitor IWP2:** On day 3 of differentiation, prepare the combined medium by using a 5 mL pipette to collect 1 mL of medium from the 12-well plate; mix it with 1mL of fresh RPMI-B27 medium in a 15 mL conical. Add 1  $\mu$ L of 5 mM IWP2 (final conc. is 2.5  $\mu$ M) into the 2 mL of combined medium. Before aspirating 1 mL of the remaining medium in the well of the 12-well plate, gently rock the plate back and forth to get cell debris into suspension, ensuring that the cell debris will be discarded via aspiration. Aspirate the remaining 1 mL of medium from each well of the 12-well plate, and then add 2 mL per well of the combined medium containing IWP2 to each well and incubate the cells.
- 15 **Day 5 Remove Wnt inhibitor IWP2:** On day 5 of differentiation, aspirate the medium from each well of the 12-well plate and add room-temperature RPMI-B27 medium at a volume of 2 mL per well. Put the plate back into the 37°C, 5% CO<sub>2</sub> incubator.
- 16 **Day 7 Change medium with CMM:** On day 7 of differentiation and every 2 days thereafter, aspirate the medium from each well of the 12-well plate and add room-temperature RPMI medium supplemented/filtered together with B27(with insulin) and 10  $\mu$ g/mL insulin and 200  $\mu$ g/mL L-Ascorbic acid 2-phosphate sesquimagnesium salt hydrate at a volume of 2 mL per well. Put the plate back into the 37 C, 5% CO<sub>2</sub> incubator.
- 17 Robust spontaneous contraction should occur by day 12. The cells can be maintained with this spontaneously beating phenotype for more than 6 months.

## References

- 18 1. Lian X, Zhang J, Azarin SM, Zhu K, Hazeltine LB, Bao X, Hsiao C, Kamp TJ, Palecek SP. Directed cardiomyocyte differentiation from human pluripotent stem cells by modulating Wnt/ $\beta$ -catenin signaling under fully defined conditions. *Nat Protoc*. 2013 Jan;8(1):162-75. doi: 10.1038/nprot.2012.150. Epub 2012 Dec 20. PMID: 23257984; PMCID: PMC3612968.
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1. Lian X, Zhang J, Azarin SM, Zhu K, Hazeltine LB, Bao X, Hsiao C, Kamp TJ, Palecek SP. Directed cardiomyocyte differentiation from human pluripotent stem cells by modulating Wnt/ $\beta$ -catenin signaling under fully defined conditions. *Nat Protoc*. 2013 Jan;8(1):162-75. doi: 10.1038/nprot.2012.150. Epub 2012 Dec 20. PMID: 23257984; PMCID: PMC3612968.
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