

Cardiac Differentiation using Wnt Modulation V1.4 “6 μ M/48 hour CHIR”

SOP Number:

Effective Date: 30Jan20

Written/Revised By: Wendy Runyon

Table of Contents:

1. Purpose	1
2. Supplies	1
3. Scope	1
4. Procedure	2
4.1. Preparing iPSCs for differentiation	2
4.2. Day 0-14: Cardiac Differentiation	3
4.3. Day 15: Cryopreservation	3

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1. PURPOSE

To describe the procedure for cardiac differentiation from induced pluripotent stem cells (iPSCs) using the Wnt modulation protocol, version 1.4 “6 μ M/48 hour CHIR”.

2. SUPPLIES

GFR (356231, Corning) or hESC Matrigel coated flasks (354277, Corning)
 Essential 8 Complete Medium (E8) (A1517001, Life Technologies)
 or mTeSR (85850, Stem Cell Technologies)
 ROCKi, 10mM stock (Ri) Y-27632 (S1049, Selleck Chemicals)
 DPBS (-/-) (14190250, Thermo Fisher)
 CHIR99021, 12mM stock (4423/50, R&D Systems)
 IWP2, 5mM stock (3533/50, R&D Systems)
 B27 Supplement (B27+) (17504044, Life Technologies)
 or N21-MAX Supplement (N21+) (AR008, R&D Systems)
 B27 Minus Insulin (B27-) (A1895601, Life Technologies)
 or N21-MAX Insulin Free Supplement (N21-) (AR010, R&D Systems)
 RPMI 1640 (11875093, Life Technologies)
 Accutase (07920, Stem Cell Technologies)
 Countess Slides/Trypan Blue
 Cryovials
 Mr. Frosty freezing container
 Trypsin
 EB20 Media
 CryoStor (07930, Stem Cell Technologies)

EB 20 Media:

500 mL KO DMEM (10829018, Life Technologies)
 120mL Hyclone FBS (SH30071.03, Hyclone/GE)
 6mL GlutaMAX-I (Gibco, 35-050-061)
 6mL MEM NEAA(Gibco, 11140050)
 4.2 μ L β -mercaptoethanol (M6250, Sigma)
 Filtered through 0.22 mesh filter.

3. SCOPE

This protocol applies to iPSC differentiation to cardiomyocytes using the V1.4, 6 μ M/48 hour, Wnt modulation method.

mTeSR may be substituted in place of E8 for some cell lines.

hESC Matrigel may be substituted in place of GRF Matrigel for some cell lines.

N21 MAX supplement may be substituted for B27 supplement for some cell lines.

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4. PROCEDURE

Note: All work must be performed in a sterile environment such as a biosafety cabinet.

Table 1.

Vessel Format	Volume of Feeding Media	Surface area (cm ² /well)
12-well plate	1mL/well (12mL/plate)	3.8
6-well plate	2mL/well (12mL/plate)	9.6
10cm dish	10mL	56.7
T25 flask	4mL	25
T75 flask	12mL	75

4.1. Preparing iPSCs for differentiation

- 4.1.1. Prepare GFR Matrigel coated flasks by placing them at 37°C for at least one hour.
- 4.1.2. Remove 70-80% confluent flasks from incubator and aspirate spent media.
- 4.1.3. Wash flask with an appropriate volume of DPBS (-/-) based on flask size.
- 4.1.4. Aspirate DPBS and add an appropriate volume Accutase.
- 4.1.5. Incubate at 37°C for 3-5 minutes. Check cells for detachment by gently tapping the side of the flask. If >90% detachment is observed, incubate for up to an additional 5 minutes.
- 4.1.6. Pipette the cell suspension up and down before transferring to an appropriately sized conical. Add an equal volume DPBS to the conical to stop the dissociation.
- 4.1.7. Centrifuge at 800 rpm for 3 minutes.
- 4.1.8. Aspirate supernatant and add an appropriate volume E8+Ri (1:1000) without resuspending the pellet.
- 4.1.9. Pipette up and down to resuspend the pellet and singularize the cells.
- 4.1.10. In a 1.5mL microcentrifuge tube, mix 10μL trypan blue with 10μL cell suspension.
- 4.1.11. Pipette 10μL from the stained cell suspension into the chamber of a Countess cell counting slide.
- 4.1.12. Insert the slide into the Countess and count cells, obtaining the number of live cells.
- 4.1.13. Determine the volume of cell suspension to seed using the following calculation:

Note: For initial differentiations, test 4 iPSC seeding densities for optimization. Standard optimization densities are 6.125e3, 1.25e4, 2.5e4, and 5e4 cells/cm².

$$\text{Seeding density} \times \text{surface area} \div C_{\text{live cells/mL}} = \frac{V_{\text{cells}}(\text{mL})}{\text{well}}$$
- 4.1.14. Aspirate Matrigel from flasks and pre-fill with an appropriate volume E8+Ri according to Table 1 minus the volume calculated above.
- 4.1.15. Add the volume of cell suspension calculated above.
- 4.1.16. Place in a 37°C incubator, shaking flasks left/right and up/down to ensure even seeding.

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4.1.17. Replace media daily with E8 until flasks reach 70-90% confluence. (Generally 2-3 days. Optimal confluence is cell line dependent.)

4.2. Day 0-14: Cardiac Differentiation

4.2.1. **Day 0:** When flasks reach 70-90% confluence, replace media with RPMI/B27(-) (1X) +6uM CHIR (1:2000).

4.2.2. **Day 1:** [Optional] Replace media with RPMI/B27(-) (1X) +6uM CHIR (1:2000) 23-25 hours after D0 media change.

4.2.3. **Day 2:** Replace media with RPMI/B27(-)(1X) +5uM IWP2 (1:1000) 47-49 hours after D0 media change.

4.2.4. **Day 3:** [Optional] Replace media with RPMI/B27(-) (1X) +5uM IWP2 (1:1000) 23-25 hours after D2 media change.

4.2.5. **Day 4:** Replace media with RPMI/B27(-) (1x) 47-49 hours after D2 media change.

4.2.6. **Day 5-9:** Replace media daily with RPMI/B27(-) (1X). Ideally, beating should first be observed between days 7-9.

4.2.7. **Day 10:** Replace media with RPMI/B27(+) (1X).

4.2.8. **Day 13:** Replace media with RPMI/B27(+) (1X).

4.3. Day 15: Cryopreservation

4.3.1. Prepare cryovials by labeling with cell line, passage number, number of cells per vial, date, and operator initials.

4.3.2. Remove D15, visibly beating flasks from incubator and aspirate spent media.

4.3.3. Wash flask with an appropriate volume of DPBS (-/-) based on flask size.

4.3.4. Aspirate DPBS and add an appropriate volume Trypsin. (Typically half the volume of feeding media used.)

4.3.5. Incubate at 37°C for approximately 15 minutes. Check cells for detachment by gently tapping the side of the flask. If >90% detachment is observed, incubate for up to an additional 5 minutes.

4.3.6. Pipette the cell suspension up and down before transferring to an appropriately sized conical. Add twice the volume EB20 to the conical to stop the dissociation.

4.3.7. Pipette the cell suspension up and down in the conical to singularize the cells.

4.3.8. Centrifuge at 300 rpm for 5 minutes.

4.3.9. Aspirate supernatant and add an appropriate volume RPMI/B27(+) (1X) +Ri (1:1000) without resuspending the pellet.

4.3.10. Leave the pellet undisturbed for 10-15 at room temperature.

4.3.11. Pipette up and down to resuspend the pellet and singularize the cells.

4.3.12. In a 1.5mL microcentrifuge tube, mix 10μL trypan blue with 10μL cell suspension.

4.3.13. Pipette 10μL from the stained cell suspension into the chamber of a Countess cell counting slide.

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- 4.3.14.** Insert the slide into the Countess and count cells, obtaining the number of live cells.
- 4.3.15.** Based on cell counts, remove 25e5 cells for FACS analysis.
- 4.3.16.** Centrifuge the remaining cells at 300 rpm for 5 minutes.
- 4.3.17.** Aspirate supernatant and resuspend in the volume of CryoStor calculated below.
- $$Total\ Cells\ (V_{Total} \times C_{live\ cells/ml}) \div Cells/vial = V_{CryoStor}(mL)$$
- 4.3.18.** Dispense 1mL cell suspension into pre-labeled cryovials and place in a Mr. Frosty. If there is a large number of vials to be filled, place cell suspension on ice while dispensing.
- 4.3.19.** Store the Mr. Frosty at -80°C overnight and transfer to the liquid nitrogen tank the following day. Keep vials on dry ice while transporting to the liquid nitrogen and transfer vials as quickly as possible without warming.