V1.4 Cardiac Differentiation Protocol

V1.4 is modified from V1.0 (standard protocol). The main change is the amount of, and duration of CHIR99021 treatment.

- For new iPS cell (iPSC) lines, start with this protocol.
- *For initial CM differentiations, test 4 iPSC seeding densities (e.g. 6.125E3, 1.25E4, 2.5E4, 5E4).*
- *If a poor differentiation is obtained, try V1.2 (Day5 replating method), V1.0 (CHIR treatment modification)*

Table 1. Volume for each vessel format

Vessel formats	Vendor	Cat #	Vol. (ml/well) for Matrigel coating	Vol. (ml/well) for media exchange	Surface area (cm^2/well)
15 cm dish	Corning	430599	15 mL	25 ml	148
10 cm dish	Nunc	174902	5-7 mL	10 ml	56.7
6 well plate	BD Science	351146	1.5 mL	2 ml	9.6
12 well plate	BD Science	351143	1 mL	1 ml	3.8
24 well plate	BD Science	351147	0.5 mL	0.5 ml	2
48 well plate	BD Science	351178	0.25 mL	0.25 ml	0.75
96 well plate	BD Science	351172	0.125 mL	0.125 ml	0.32
T25 flask			4 mL	5 ml	25
T75 flask			12 mL	15ml	75

Reagents:

GFR Matrigel Phenol Red Free (BD Bioscience)
Knockout DMEM #10829-018 (Life Technologies)
Essential 8™ Medium (prototype), #A14666SA, (Life Technologies)
Y-27632 ROCK inhibitor
CHIR99021 10mg (R&D system), or 4953 (Tocris)
IWP2, #3533, (Tocris)
B27® Serum-Free Supplement (50X), liquid, #17504-044 (Life Technologies)
B27® minus (-) insulin (50X), liquid, #0050129SA (Life Technologies)
RPMI1640 #11875-093 (Life Technologies)
0.25% Trypsin with EDTA in Saline A (UCSF Cell Culture Facility)
Accutase (Stemcell Technologies)

80 ug/mL GFR Matrigel/KO DMEM media components (MG medium):
500 uL of 4°C cold 8 mg/mL GFR Matrigel Phenol Red Free (BD Bioscience)
50 mL of 4°C cold Knockout (KO) DMEM #10829-018 (Life Technologies)

EB20 media components:
Knockout DMEM #10829-018 (Life Technologies)
120 ml of Hyclone Fetal Bovine Serum Characterized # SH30396.03 (Fisher)
6 ml of GlutaMAX-I, 100X, #35050-061 (Life Technologies)
6 ml of MEM NEAA 10mM (100X), #11140-050 (Life Technologies)
4.2 ul of β-mercaptoethanol
Filtered the media through 0.22 µm mesh screen.

iPS and CM Freezing media:
10% DMSO in 90% FBS (+) Ri (10 µM Ri final concentration)

A. iPSC passaging before CM differentiation

- *For newly received cell lines, passage <u>at least 3 times</u> before starting a cardiac diff.*

1. Coat a T25 flask with 4 mL of MG medium overnight in the 37°C incubator (1 day prior to cell seeding).
2. Split the cells at 70-80% confluency.
3. Wash the iPS cells 1X with PBS.
4. Add 1.5 mL of Accutase to the cells.
5. Incubate cells in 37°C incubator for 3 – 5 min.
6. Check the cells for detachment by knocking the flask against your palm.
7. Add 10 mL of PBS to the cells to dilute down the Accutase.
8. Pipette up and down a few time for dissociation.
9. Centrifuge the cells at 800 rpm for 3 min and remove the supernatant.
10. Add 3ml of E8 (+) Ri medium to the pellet without resuspending the pellet.
11. Leave the pellet in the medium at room temperature for 10 - 15 min in the hood.
12. Pipette up and down to singularize the cells.
13. Prepare Countess slide for counting: add 10 µL of singularized cell suspension and 10 µL of Trypan blue to an eppendorf tube.
15. Pipette up and down a few times for further dissociation.
16. Take a 10 µL aliquot of Trypaned cells and inject into a chamber of the Countess for counting. Use iPS cells program to count live cells: make sure there are less than 5 cells excluded, marked by black rings by pressing the Zoom In button. *If there are many black rings shown, go back to the original cell suspension, pipette up and down for further dissociation, and prepare a new aliquot for re-counting.*
19. Aspirate the excess Matrigel from the coated flask.
20. Add 5 mL of E8/Ri medium in the flask.
21. For eWTc11, transfer 2E5 cells to the flask. *For other iPS cell lines, try 2E5 to 4E5 for a T25 flask.*
22. Rock the flask in an East/South/West/North direction and incubate the cells at 37°C.
24. The cells should be at 70-80% confluency on day 4.

B. CM differentiation Protocol

1. **Day -4,** coat a 12WP (1 ml/well) with 80 µg/ml GFR Matrigel overnight in 37°C incubator (1 day prior to cell seeding).
2. **Day -3,** wash the iPS cells in a T25 flask with PBS (-) Ca^{2+}/Mg^{2+}.
3. Add 1.5 ml of Accutase to the cells.
4. Incubate at 37°C for 3 - 5 min.
5. Check the cells for detachment by rocking the flask or knocking it against your palm.
6. Quench the Accutase with 10 ml of PBS.
7. Pipette up and down a few times to dissociate the cells.
8. Centrifuge the cells at 800 rpm for 3 min and remove the supernatant.
9. Add 3 ml of E8 (+) 10 µM Ri medium to the pellet without resuspending the pellet.
10. Leave the pellet in the medium in the hood for 10 - 15 min without disturbance.
11. Pipette up and down to singularize the cells.
12. For counting cells, prepare Countess slide: in an eppendorf tube, add 10 µl of singularized cell suspension and 10 µl of Trypan blue.
13. Pipette up and down a few times for further dissociation.
14. Take a 10 µl aliquot of “trypaned” cells and inject into one of the Countess chambers.
15. Use’ iPS cells’ program to count cells: Obtain the # of live cells. Make sure that less than 5 cells are excluded, marked by black ring by pressing the “Zoom In” button.
    **If there are many cells circled with black rings shown, go back to the original cell suspension, pipette up and down for further dissociation, and prepare a new aliquot for re-counting.*

16. Seed cells in E8 (+) 10 μM Ri medium. Use a reservoir and a multi-channel pipette to seed the cells at 4 densities in the Matrigel-coated 12WP. N.B. Pipette up and down to mix the cell suspension before transferring the cells each time.
17. Rock the plate in East/South/West/North direction to distribute the cells evenly.
18. Incubate the cells at 37°C.
19. **Day -2 and Day -1**, replace the medium with E8 (-) Ri.
20. **Day 0**, incubate the cells with 6 μM CHIR in RPMI/B27 (-) insulin.
21. **Day 1,** no media change.
22. **Day 2**, exactly 48h after adding CHIR, replace media with 5 uM IWP2 in RPMI/B27 (-) insulin.
23. **Day 3**, no media change.
24. **Day 4**, change media to incubate cells with RPMI/B27 (-) insulin.
25. **Day 5**, no media change.
26. **Day 6**, feed the cells with RPMI/B27 (+) insulin.
27. For **subsequent days** after, change media every 3 to 4 days (2 times per week).
28. **Day 15**, check qualitatively the amount of beating cells. If there are beating iPS-CM (>20%), continue to lactate purification protocol (V3.1). If no beating cells are observed, do not continue.

3. iPS-CM freezing and storage for non-lactate-treated iPS-CMs

1. Wash the cells with 1X PBS.
2. For a 10cmD diff, trypsinize the cells in 8 ml of 0.25% Trypsin for ~15 min.
3. Check the cells for detachment and titurate the cells to dissociate the cells.
4. Quench the trypsin with 16 ml of EB20 media and pipette up and down for further dissociation.
5. Centrifuge the cell suspension at 300 rcf for 5 min.
6. Remove the supernatant.
7. Add 20 ml of RPMI/B27 (+) insulin/(+) Ri media to the pellet without resuspending the pellet.
8. Leave the pellet in the medium for 10 - 15 min at room temperature.
9. Pipette up and down the cell suspension for further dissociation.
10. Count the iPSC-CMs using hemocytometer: in an eppendorf tube, add 40 μl of Trypan blue; 50 μl of PBS; 10 μl of iPSC-CM suspension.
    # of cells/ml = (# of cells counted in 4 squares/4) * 10 * 10^4
11. Take an aliquot of 2E5 of cells for FACS analysis.
12. Centrifuge the remaining cells at 300 rcf for 5 min, then remove the supernatant.
13. Resuspend the pellet in freezing media, 500 μl per vial
14. Freeze down cells at a) 2E5 or b) 2E6 cells per cryovial by placing vials in a room temperature Mr Frosty and then transferring the Mr Frosty to the -80°C for cooling at close to -1°C/minute, the optimal rate for cell preservation.
15. Store at -80°C for at least 2 days, before transferring to liquid N2 tank.