V1.2: "Day 5 Replating" Cardiac Differentiation Protocol

Our protocol is further modified from Conklin Lab Protocol V1.0, which is based on the Wnt Modulation protocol from Lian et al (2012) PNAS.

- *For every initial CM differentiation, test two optimal densities based on the result from V1.0 (e.g. 1.25E4, 2.5E4 cells/cm^2)*

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 #688000 (EMD)
CHIR99021 10mg (R&D system), or 4953 (Tocris)
IWP2, #3533, (Tocris)
B27® Serum-Free Supplement (50X), liquid, #17504-044 (Life Technologies)
B27® (-) 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 µg/mL GFR Matrigel/KO DMEM media components (MG medium):
500 µL 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 µl 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 final concentration)

A. iPS cell 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 3 ml of E8 (+) 10 µM 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 (with Day5 replating)

1. **Day -4,** coat a 6WP (2 ml/well) with 80 µg/ml GFR Matrigel overnight in 37°C incubator.
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 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. Prepare Countess slide for counting: add 10 µl of singularized cell suspension and 10 µl of Trypan blue in an eppendorf tube.
13. Pipette up and down a few times for further dissociation.
14. Take a 10 µl aliquot of “trypaned” cells and inject into a chamber of the Countess for counting.
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 2 optimal densities in the overnight Matrigel coated 6WP. 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**, feed the cells with 12 μM CHIR in RPMI/B27 (-) insulin.
21. **Day 1,** exactly 24h after adding CHIR, change media to RPMI/B27 (-) insulin.
22. Day 2, no media change.
23. **Day 3**, feed the cells with 5 μM IWP2 in RPMI/B27 (-) insulin.
24. Day 4, no media change.
25. **Day 5**, replate the cells into a fresh well/6WP (use overnight 37°C coated 80 μg/ml GFR Matrigel 6WP):
    - Wash the differentiating cells 1x with PBS (-) Ca^{2+}/Mg^{2}.
    - Add 1 ml of Accutase per well/6WP to dissociate the cells.
    - Incubate in 37°C incubator for 3-5 min.
    - During Accutase incubation, titurate the cells once or twice for further dissociation.
    - Quench the Accutase with 1 ml EB20 media. Pipette up and down to dissociate the cells.
    - Centrifuge the cells at 800 rpm for 3 min and remove the supernatant.
    - Resuspend the cell pellet into 2 ml of RPMI/B27/ (-) insulin/ (+) 10 μM Ri.
    - Pipette up and down the cell suspension to singularize the cells.
    - Transfer the 2 ml cell suspension to one MG-coated well of the 6WP.
    - Incubate the replated cells in the 37°C incubator.
26. **Day 6**, replace with RPMI/B27 (-) insulin to remove Ri.
27. **Day 7**, no media change.
28. **Day 8,** feed the cells with RPMI/B27 (-) insulin.
29. **Day 9**, no media change.
30. **Day 10,** feed the cells with RPMI/B27 (+) insulin.
31. For subsequent days after, change media every 3 days (2 times per week).
32. **Day 15**, check qualitatively the amount of beating cells. If there are beating iPS-CM (>50%), prepare for freezing (non-lactate). If less than >50% consider continuing to lactate purification. If no beating cells are observed, do not continue.

C. 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 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 cell suspension 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.
15. Store at -80°C for 24 h before transferring to liquid N2 tank.