(Ref: Annie Truong, Po-Lin So)

V3.1 Lactate purification of iPS-CM differentiation

This protocol is modified from:
Distinct metabolic flow enables large-scale purification of mouse and human pluripotent stem cell-derived cardiomyocytes. (2013) Tohyama S, Hattori F, Sano M, Hishiki T, Nagahata Y, Matsuura T, Hashimoto H, Suzuki T, Yamashita H, Satoh Y, Egashira T, Seki T, Muraoka N, Yamakawa H, Ohgino Y, Tanaka T, Yoichi M, Yuasa S, Murata M, Suematsu M, Fukuda K. Cell Stem Cell. Jan 3;12(1):127-37. PMID:23168164

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:
Knockout (KO) DMEM #10829-018 (Life Technologies)
80 µg/mL Matrigel medium
EB20 medium
10 mM Y-27632 ROCK inhibitor (Ri)
B27® Serum-Free Supplement (50X), liquid, #17504-044 (Life Technologies), [B27 (+) insulin]
RPMI1640 #11875-093 (Life Technologies)
0.25% Trypsin with EDTA in Saline A (UCSF Cell Culture Facility)
1X PBS (-) Ca^{2+}/Mg^{2+}
Lactate powder (Sodium L-lactate #L7022-10G, Lot#: BCBJ6835V, Sigma)
1 M HEPES
100X Glutamax
100X Non Essential Amino Acids
500 mL DMEM (w/o glucose and sodium pyruvate) #11966-025 (Life Technologies)

Media preparation:

EB20 medium:
Knockout (KO) 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
-> Filter-sterilize through 0.22 µm mesh

10 µM Ri/RPMI/B27 (+) insulin medium, or RPMI/B27 (-) insulin/ (+) Ri medium:
50 µL of 10 mM Ri (10 µM final concentration)
50 mL RPMI/B27 (+) insulin medium, or RPMI/B27 (-) insulin medium

Lactate Medium:
Stock solution – 20 mL 1M lactate in HEPES solution

In a 50 mL tube, add 2.2412 g of lactate to 18 mL of 1 M HEPES solution in sterile conditions.
Invert the tube up and down to dissolve the lactate.
Allow solution to settle for a couple of minutes at room temperature. Dissolve all clumps.
After the lactate powder is completely dissolved, store as 5ml aliquots of 1 M lactate solution in 15 mL tubes at -20°C.

Working medium - 500 mL of 4 mM lactate in DMEM medium

500 mL DMEM (w/o glucose and sodium pyruvate)
5 mL 100X Non Essential Amino Acids
5 mL 100X Glutamax
2 mL of 1 M Lactate solution (final: 4 mM)

Filter the medium through a 0.22 μm filter.

A. <u>Protocol: Lactate Purification of Day 15 iPS-CMs</u>

** CM diffs should be split in a 1 to 2 ratio for lactate treatment *.*

1. Wash the CM diff 1X with PBS for trypsinization:
   *For a 12WP diff, trypsinize the cells in 0.5 mL Trypsin for ~ 10 min.
   * For a 6WP diff, trypsinize the cells in 1 mL Trypsin for ~ 10 min.
   * For a 24WP diff, trypsinize the cells in 0.25 mL Trypsin for ~ 10 min.
   * For a 10cm dish diff, trypsinize the cells in 5 mL Trypsin for ~ 10 min.
2. Check the cells for detachment and titurate to dissociate the cells.
3. Quench the trypsin with 2 volumes of EB20 media.
   (Optional: combine all similar CM diffs in one pool if required).
4. Pipette up and down a few times.
5. Centrifuge the cell suspension at 300 rcf for 5 min.
6. Remove the supernatant.
7. Add RPMI/B27 (+) insulin/ (+) Ri medium in a volume equivalent to 1 to 2 split ratio onto the pellet without resuspending the pellet.
   <u>For example:</u>
   - 6-9 wells of 12WP pooled together to replate into 1 of 10cmD.
   - 3-4 wells of 12WP pooled together and replate into 3-4 of 6WP.
   - 3 wells of 6WP pooled together to replate into 1 of 10cmD.
   - One 10cm dish diff replated into two 10cm dishes.
8. Leave the pellet in the medium for 10 - 15 min. at room temperature.
9. Pipette up and down the cell suspension to singularize the cells.
10. Count cells using hemocytometer: In an eppendorf tube, take 10 μL of cell suspension and add 10 μL of Trypan blue.
    # of cells/mL = (# of cells counted in 4 squares / 4) * 2 (dilution factor) * 10^4
11. Take an aliquot of 2E5 of cells for FACS analysis if needed.
    # of mL = 2E5 / # of cells/mL
12. Replate an appropriate volume of cell suspension in an appropriate freshly overnight-MG-coated plate/dish to get 1 to 2 split ratio (refer to step 7 above).
13. **Day 16**, change the media to RPMI/B27 (+) insulin only.
14. Day 17,18,19, no media change.
15. **Day 20**, lactate #1: remove the old media. Flush the cells 1X with PBS. Add lactate medium with the appropriate volume based on vessel type (Table 1, page 1).
16. Day 21, no media change.
17. **Day 22,** lactate #2: add new lactate medium.
18. Day 23, no media change.
    * The cells can be left in lactate for additional day if there is weekend encountered.

Cells are treated twice with lactate media in a course of 4-5 days. (see *Optional*, below).
** The cells should be beating during lactate treatment.*

19. **Day 24,** wash the cells 1X with PBS. Add RPMI/B27 (+) insulin medium. Allow recovery for the next 2-3 days without media change.
20. **Day 26**, harvest the cells for freezing.

Optional: 3x lactate treatment
If the differentiation efficiency is lower than 50% beating cells (by visualization), add a 3rd lactate treatment after the 2nd lactate.

Day 24’, *add lactate #3*
Day 26’, *wash the cells 1X with PBS. Add RPMI/B27 (+) insulin medium. Allow recovery for the next 2 days without media change.*
Day 28’, *harvest the cells*

B. iPS-CM harvest for freezing and storage

Reagents:
EB 20 medium
10 mM Y-27632 ROCK inhibitor
B-27® Serum-Free Supplement (50X), liquid, #17504-044 (Life Technologies), [B27 (+) insulin]
RPMI 1640 #11875-093 (Life Technologies)
0.25% Trypsin with EDTA in Saline A (CCF facility)
1X PBS (-) Ca^{2+}/Mg^{2+}

Freezing media:
45 mL Hyclone Fetal Bovine Serum Characterized # SH30396.03 (Fisher)
5 mL DMSO
Filtered the media through 0.22 µm mesh
50 µL 10mM Rock Inhibitor

1. Wash the cells 1X with PBS.
2. Trypsinize the cells
   - Use 5 mL of 0.25% Trypsin for a 10cmD.
   - Use 0.5 mL of 0.25% Trypsin per well of 6WP.
3. Check the cells for detachment and titurate to dissociate the cells during trypsinization.
4. Quench the Trypsin with 2 volumes of EB20 medium.
5. Collect and combine the cells with the same condition, into one pool if needed.
6. Pipette up and down a few times.
7. Centrifuge the cell suspension at 300 rcf for 5 min.
8. Remove the supernatant.
9. Resuspend the cells in RPMI/B27 (+) insulin/Ri medium.
   - Use 1-7 mL of medium for one original differentiation plate.
10. To count cells, in an eppendorf tube, remove 10 µL cell suspension and add 10 µL of Trypan blue. Count the cells using hemocytometer.
    # of cells/mL = (# of cells counted in 4 squares / 4) * 2 * 10^4
11. Take 2E5 cells for FACs analysis if needed.
    # of mL = 2E5 / # of cells/mL
12. Centrifuge the cells at 300 rcf for 5 min. Remove supernatant.
13. Resuspend the pellet in freezing media.
14. Aliquot cells at a maximum of 2E6 cells in 500 µL of freezing medium per cryovial.

15. Store at -80°C before transferring to nitrogen tank.