V3.1 Lactate purification of iPS-CM differentiation


<table>
<thead>
<tr>
<th>Vessel formats</th>
<th>Vendor</th>
<th>Cat #</th>
<th>Vol. (ml/well) for Matrigel coating</th>
<th>Vol. (ml/well) for media exchange</th>
<th>Surface area (cm²/well)</th>
</tr>
</thead>
<tbody>
<tr>
<td>15 cm dish</td>
<td>Corning</td>
<td>430599</td>
<td>15 mL</td>
<td>25 ml</td>
<td>148</td>
</tr>
<tr>
<td>10 cm dish</td>
<td>Nunc</td>
<td>174902</td>
<td>5-7 mL</td>
<td>10 ml</td>
<td>56.7</td>
</tr>
<tr>
<td>6 well plate</td>
<td>BD Science</td>
<td>351146</td>
<td>1.5 mL</td>
<td>2 ml</td>
<td>9.6</td>
</tr>
<tr>
<td>12 well plate</td>
<td>BD Science</td>
<td>351143</td>
<td>1 mL</td>
<td>1 ml</td>
<td>3.8</td>
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<tr>
<td>24 well plate</td>
<td>BD Science</td>
<td>351147</td>
<td>0.5 mL</td>
<td>0.5 ml</td>
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<tr>
<td>48 well plate</td>
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<td>351178</td>
<td>0.25 mL</td>
<td>0.25 ml</td>
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<td>96 well plate</td>
<td>BD Science</td>
<td>351172</td>
<td>0.125 mL</td>
<td>0.125 ml</td>
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<tr>
<td>T25 flask</td>
<td></td>
<td></td>
<td>4 mL</td>
<td>5 ml</td>
<td>25</td>
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<tr>
<td>T75 flask</td>
<td></td>
<td></td>
<td>12 mL</td>
<td>15 ml</td>
<td>75</td>
</tr>
</tbody>
</table>

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 (-) Ca²⁺/Mg²⁺
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. Protocol: Lactate Purification of Day 15 iPS-CMs**

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.
   For example:
   - 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.
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.