Protocol for Differentiating KOLF2.1J hiPSCs into Cardiomyocytes

hiPSC Handling and Maintenance

Matrigel Aliquot Preparation & Plate Coating

Reagents Required

- DMEM: Gibco; Thermo Fisher Scientific; catalogue #11966025
- Matrigel: Corning; catalogue # 354230
- mTeSR1: Stem Cell technology; catalogue #85851
- ROCK inhibitor (Y27632 dihydrochloride): Tocris; catalogue #1254
- Accutase: EMD Millipore; catalogue # SCR005

Preparation of Matrigel Aliquots

Store the Matrigel bottle in an ice bucket and a new 200 µL tip box at 4°C overnight.

On the following day, transport the ice bucket containing Matrigel and the new 200 µL tip box into a sterile hood. Open a new bag of 1.5 mL Eppendorf tubes and arrange them on the ice. Place the Matrigel bottle in another small ice box and carefully open the bottle. Quickly prepare 150 µL Matrigel aliquots in 1.5 mL tubes placed on the ice. Transfer these aliquots into a 96-tube sample box and store at -20°C until use.

Coating Matrigel Plates for hiPSC Culture

1. Aliquot 12 mL of cold DMEM into a 15 mL Falcon tube.
2. Remove one aliquot of Matrigel from -20°C and quickly thaw it by pipetting up and down with ~1 mL of DMEM from the 12 mL aliquot.
3. Add 1 mL of the Matrigel-DMEM mixture to each well in a 6-well plate, ensuring all wells are covered, and incubate the plate at 37°C with 5% CO2 for at least 1 hour.
   (For a 12-well plate, add 500 µL of Matrigel-DMEM mixture per well).

iPSC Passaging and Maintenance

1. Before splitting cells, prepare a mixture of mTeSR1 containing 1:2000 ROCK inhibitor from a 10 mM stock concentration. Make sure to prepare enough mTeSR1-ROCKi for the upcoming split plus an extra 1 mL.
2. Take out a plate with hiPSCs, aspirate the mTeSR1 medium from the split well, and wash with 1 mL PBS per well.
3. Add 500-600 µL Accutase to the split well and incubate at 37°C, 5% CO2 for 5 minutes. While waiting, prepare inactivation tubes by adding 2 mL fresh mTeSR1 to a 15mL Falcon tube.
4. After 5 minutes, remove the plate from the incubator and pipette out the Accutase/cell solution to dislodge remaining cells. Spin hiPSCs at 200 g for 3 minutes.
5. Aspirate the supernatant, then resuspend cell pellet in 1 mL of the mTeSR1-ROCKi solution.
6. Seed cells at a density of 500,000 per well (count using a hemocytometer).
7. Place the plate in the incubator at 37°C, 5% CO2 for 24 hours.
8. For the next 2-3 days, add 2 mL fresh mTESR1 medium daily until cells reach >90% confluence.

hiPSC to Cardiomyocyte Differentiation

Reagents Required

- RPMI 1640 medium: Gibco; Thermo Fisher Scientific; catalogue # 11875093
- B-27 minus supplement: Gibco; Thermo Fisher Scientific; catalogue # A1895602
- CHIR 99021: Tocris; catalogue #4423
- IWP4: Tocris; catalogue #5214
- B-27 Supplement: Gibco; Thermo Fisher Scientific; catalogue #17504001
- RPMI 1640 medium no glucose: Gibco; Thermo Fisher; catalogue # 11879020
- Nalgene Rapid-Flow™ Sterile Disposable Filter units 0.1µM: Thermo Fisher Scientific; catalogue #566-0010
- Glutamax: Gibco; Thermo Fisher Scientific; catalogue # 35050061
- PenStrep: Gibco; Thermo Fisher Scientific; catalogue #15140122

Cell Culture Media

RPMI without insulin: RPMI 1640 medium + 2% B-27 minus insulin + 1% penicillin/streptomycin. In a sterile environment, combine these components in the RPMI 1640 medium bottle and filter using Nalgene Rapid-Flow sterile disposable filter units.
RPMI with insulin: RPMI 1640 medium + 2% B-27 supplement + 1% penicillin/streptomycin. In a sterile environment, combine these components in the RPMI bottle and filter using Nalgene Rapid-Flow sterile disposable filter units.

RPMI without glucose: RPMI 1640 without glucose medium + 2% B-27 supplement + 1% penicillin/streptomycin. In a sterile environment, combine these components together in the RPMI bottle and filter using Nalgene Rapid-Flow sterile disposable filter units.

Day 0: When hiPSCs are >90% confluent, aspirate the medium and wash each well with PBS. Add RPMI-B27 without insulin mixed with CHIR 99021 at a final concentration of 4 µM (from a 12 mM stock). Incubate at 37°C, 5% CO2 for 48 hours.

Note: KOLF2.1J hiPSCs respond differently to varying CHIR concentrations, so optimization is required. We have optimized KOLF2.1J cardiomyocyte differentiations at:

- *4 µM (RPMI without insulin for 2 days)*
- *9 µM (RPMI with insulin for 1 day)*
- *10 µM (RPMI with insulin for 1 day)*

Day 2: After 48 hours, aspirate the medium, wash each well with PBS, and add 2 mL of RPMI-B27 without insulin. Incubate at 37°C, 5% CO2 for 24 hours.

Day 3: Aspirate the medium and add RPMI-B27 without insulin mixed with IWP4 at a 2:1000 dilution (from a 2.5 mM stock). Incubate at 37°C, 5% CO2 for 48 hours.

Day 5: After 48 hours, aspirate the medium, wash each well with PBS, and add 2 mL of RPMI-B27 without insulin. Incubate at 37°C, 5% CO2 for 48 hours.

Day 7: After 48 hours, aspirate the medium and add 2 mL of RPMI-B27 without insulin. Incubate at 37°C, 5% CO2 for 48 hours.

Day 9 and 11: Aspirate the medium and add 2 mL of RPMI-B27 with insulin. Incubate at 37°C, 5% CO2 for 48 hours.

Day 13: After 48 hours, aspirate the medium, wash each well with PBS, and add RPMI without glucose. Incubate at 37°C, 5% CO2 for 24 hours.

Day 14: After 24 hours, add RPMI without glucose. Incubate at 37°C, 5% CO2 for 24 hours.

Day 15: Aspirate the medium and add 2 mL of RPMI-B27 with insulin.

Day 16: Select high-quality hiPSC-CM wells and proceed with replating.

hiPSC-Derived Cardiomyocyte (hiPSC-CMs) Replating, Expansion and Splitting

Reagents Required

- TrypLE select enzyme (10x): Gibco; Thermo fisher catalogue # A1217701
- RPMI1640 medium: Gibco; Thermo fisher catalogue # 11875093
- CHIR 99021: Tocris; catalogue #4423
- ROCK-inhibitor (Y27632 dihydrochloride): Tocris; catalogue #1254
- B-27 Supplement: Gibco, Thermo Fisher Scientific; catalogue #17504001
- KnockOut serum replacement: Gibco; Thermo Fisher catalogue #10828028
- STEMdiff Cardiomyocyte Freezing Medium: Stem Cell technology; catalogue # 05030

Cell Culture Media

CM culture medium (CM): In a sterile environment, mix RPMI 1640 medium with 2% B-27 supplement (1:50) and 1% penicillin/streptomycin (1:100).

CM replating medium (RM): In a sterile environment, add ROCKi (0.1% Y-27632; 1:1,000 dilution from a 10 mM stock) and 10% KO serum replacement (1:10) to the CM culture medium (RPMI 1640 medium + B-27 with Insulin + 1% PS).

CM expansion medium: In a sterile environment, add 2µM CHIR in CM culture medium (RPMI 1640 medium + B27 + 1% PS).

CM splitting medium (with 2 µM CHIR): Add 2 µM CHIR, 0.1% Y-27632 (1:1,000 dilution from a 10 mM stock), and 10% KO serum replacement (1:10) to the CM culture medium.

1. Coat a 10 cm Corning plate with Matrigel as described in the universal hiPSC handling section.
2. Select 1-6 wells from an hiPSC-CM 6-well plate (=P2) containing 70-80 % beating/high-quality cardiomyocytes.
3. Aspirate the medium, wash the cells with PBS, and incubate hiPSC-CMs with pure TrypLE Select (10x) for 10-15 min at 37°C, 5% CO2.

   Note: Use pure TrypLE Select (10x) for efficient dissociation of hiPSC-CMs. Other reagents such as TrypLE Express or Trypsin may result in inefficient dissociation and low cell viability.
4. Gently shake to detach hiPSC-CMs. When cells come off the culture plate, add CM culture medium and gently mix 2-5 times using a 5 mL pipette.
5. Transfer the cell suspension to a 15 mL tube containing CM replating medium and centrifuge at 200 g for 3 minutes.
6. Aspirate the supernatant and resuspend the cell pellet in 1 mL CM replating medium.
7. Wash the 10 cm Matrigel-coated plate with PBS and add 9 mL CM replating medium.
8. Replate the CM cell suspension (from step #6) onto the 10 cm Corning plate and incubate at 37°C, 5% CO2.
9. After 24 hours, replace the replating medium with 37°C preheated cardiac expansion medium every other day or once every three days. For most cell lines, 2 µM CHIR induces efficient expansion of hiPSC-CMs.
10. After 3–5 days, when hiPSC-CMs reach 70%–80% confluency, passage them to prevent excessive cell-cell contact. Aspirate the medium, wash with PBS, add 3 mL of warm TrypLE (37°C), and incubate for 10–15 min at 37°C 5%, CO2.
11. After incubation (~15 minutes), detach hiPSC-CMs using a 5 or 10 mL pipette filled with 6 mL of 37°C preheated RPMI-1640, dispensing over the plate surface until all the cells are

detached. Mix gently 2–5 times using the 5 mL pipette.
12. Transfer the cell suspension to a new 15 mL tube and centrifuge at 200 g for 3 minutes.
13. Aspirate the supernatant and resuspend the cell pellet in 1 mL of 37°C preheated CM splitting medium supplemented with optimal CHIR concentration.
14. Add 37°C preheated CM splitting medium to the hiPSC-CMs.
15. Remove the Matrigel solution from the 15 cm Corning plate, wash with PBS, and add CM splitting medium along with the CM cell suspension to the prepared 15 cm plate.

Freezing of hiPSC-Derived CMs

1. Dissociate hiPSC-CMs as described in the "Replating of Differentiated hiPSC-CMs" section above. Transfer the desired cell suspension for freezing into a 15 mL tube.
2. Centrifuge the cell suspension at 200 g for 3 minutes and keep the tube on ice.
3. Remove the supernatant and resuspend the CMs in STEMdiff Cardiomyocyte Freezing Medium.
4. Freeze the vials at –80°C for a minimum of 4 hours and a maximum of 12 hours in a 2-propanol-filled freezing container.
5. Transfer the vials to liquid nitrogen for long-term storage.