Standard Operating Procedures (SOP)
Human iPSC Culture and Cardiac Differentiation
Biobank and NHLBI investigators

Culturing hiPSCs/hESCs.

Human induced pluripotent stem cells (iPSCs) or human embryonic stem cells (hESCs) are cultured in E8 medium (*1*) on a matrigel layer.
Sterile culture conditions at 37°C, 5 % CO_2 are required. Hypoxic culture conditions (5 % O_2) are optional. Avoid keeping iPSCs/hESCs at room temperature for prolonged periods of time. Culture medium needs to be changed daily. Note: For medium aspiration, change to a new 2 ml aspirator pipette for every culture dish.

Preparation of Matrigel-coated dishes.

1) Thaw the full-strength matrigel stock solution on ice. To aliquot, pre-cool 1.5 ml protein low retention tubes on ice. Keep the Matrigel full-strength stock solution on ice and quickly aliquot into the pre-cooled 1.5 ml tubes on ice. Store aliquots at -20 °C.
2) To prepare a working solution of matrigel, thaw an aliquot of the full-strength matrigel stock on ice or at 4 °C.
3) Dilute matrigel stock in ice-cold DMEM-F12 medium at 1:500 without delay. Note: This dilution of the full-strength matrigel stock is more than sufficient.
4) Quickly distribute into 6 well plates or desired plate format, respectively, e.g. at 1.5 ml per well of a 6 well plate.
5) Transfer coated culture dishes to a 37 °C incubator. Incubate at least 30 min before use. Ideally, matrigel-coated plates should be prepared freshly before passage, but may be stored for several days at 37°C, e.g. until reaching the next passaging date.

Splitting iPSCs/hESCs.

Passage iPSCs/hESCs every 4 days for maintenance at a 1:12 ratio ($1.2x10^5$ cells per one well of a 6 well plate) and for cardiac differentiation at a 1:6 ratio ($2x10^5$ cells per one well of a 6 well plate). To determine live cell numbers, employ Trypan Blue staining using an Automated Cell Counter, as described under "Freezing iPSC-CMs/hESC-CMs".

1) Prepare matrigel-coated culture dishes. Aspirate matrigel, taking care to completely remove all residual liquid, and quickly replace with 1.5 ml E8 medium plus 10 µM

ROCK inhibitor (10 mM stock in sterile ddH_2O) per each well of a 6 well plate. Critical: Do not let plates dry out.

2) Wash iPSCs/hESCs with 1 ml PBS per each well of a 6 well plate.
3) Remove PBS and replace with 500 µl Accutase per well of a 6 well plate to detach cells. Incubate for 3 min at 37°C and subsequently quench with an equal volume of E8 medium. Pellet cells at 200g for 3min at room temperature (RT). (*)
4) Aspirate medium and gently resuspend iPSC/hESC pellet in E8 medium plus 10 µM ROCK inhibitor in a volume corresponding to 0.5 ml per each well of a 6 well plate.
5) Distribute cell suspension evenly into the desired plate format. Place plates into a 37 °C sterile incubator and allow iPSCs/hESCs to attach overnight or for at least 12 h.

(*) An alternative option is to passage iPSCs/hESCs using EDTA (0.5 mM in PBS) (*1*) by incubating for 10 min at 37°C. Check cells under a brightfield microscope:
If cells are rounded, but still attached, carefully remove EDTA and gently rinse cells off with a p1000 µl pipette, using E8 medium plus 10 µM ROCK inhibitor at a volume corresponding to 0.5 ml per each well of a 6 well plate. Proceed with step 5).
If cells are floating, quench with an equal volume of E8 medium plus 10 µM ROCK inhibitor and pellet iPSCs/hESCs at 200g for 3 min (RT). Proceed with step 4).

Freezing iPSCs/hESCs.

Freeze a ***minimum*** of 3 million cells per 1ml freezing medium in each cryo vial. To determine live cell numbers, employ Trypan Blue staining using an Automated Cell Counter, as described under "Freezing iPSC-CMs/hESC-CMs".

1) Detach iPSCs/hESCs as described above.
2) Pellet cells at 200g for 3min (RT).
3) Aspirate medium and gently resuspend cell pellet in freezing medium (Bambanker or alternatively, KSR +10 % DMSO, sterile filtered 0.22 µm). Quickly transfer cell suspension into cryo vials at a volume of 1 ml per cryo vial.
4) Immediately place cryo vials into a freezing container. Store at -80 °C for at least 24h.
5) Transfer cryo vials into the vapor phase of a liquid nitrogen freezing tank for long-term storage.

Freezing iPSC-CMs/hESC-CMs.

Freeze a ***minimum*** of 3 million ***live*** iPSC-CMs/hESC-CMs per 1ml volume of freezing medium (FBS+10% DMSO, sterile filtered 0.22 µm). Determine live cell number by Trypan Blue staining using an Automated Cell Counter (e.g. Countess/Life Technologies). Generally, freeze iPSC-CMs/hESC-CMs batches in aliquots of 5 and 10 million. Adding ROCK inhibitor does not improve cell survival of iPSC-CMs/hESC-CMs.

1) Wash iPSC-CMs/hESC-CMs with 1 ml PBS per well of a 6 well plate.

2) Remove PBS and replace with 1 ml Accutase per well of a 6 well plate to detach cells. Incubate for 10 min at 37°C. (**)
3) Check for cell detachment using a brightfield microscope. Note: If the cell layer does not detach after a 10 min incubation period, incubate for an additional 5 min.
4) Quench by adding an equal volume of culture medium (RPMI plus B27 supplement with insulin) and gently resuspend cell suspension.
5) Count cells by mixing 10 ul cell suspension with 10 ul of Trypan blue solution. Use a p200 ul pipette to transfer mixture into a Cell Counting Chamber Slide. Count cells using an Automated Cell Counter.
6) Pellet cells at 200g for 3min (RT).
7) Aspirate medium and resuspend cell pellet in freezing medium (FBS+10% DMSO, sterile filtered 0.22 µm) and transfer into cryo vials at 1 ml per vial.
8) Immediately transfer cryo vials into a freezing container. Store at -80 °C for at least 24h.
9) Transfer cryo vials into the vapor phase of a liquid nitrogen freezing tank for long-term storage.

(**) An alternative option is to detach iPSC-CMs/hESC-CMs with TrypLE Express for 10 min at 37 °C.

Thawing iPSC-CMs/hESC-CMs.

Proceed quickly with each step. Adding ROCK inhibitor does not improve cell survival of iPSC-CMs/hESC-CMs.

1) Prepare a 15 ml sterile falcon with 8 ml of room-temperature culture medium (RPMI with B27 supplement) plus 10 % FBS. Prepare matrigel-coated plates of desired format, e.g. 6 well plates. Aspirate matrigel and replace with 3 ml of culture medium (RPMI with B27 supplement) plus 5 % FBS per each well of a 6 well plate.
2) Take out cryo vial with frozen iPSC-CMs/hESC-CMs from -80 °C or liquid nitrogen storage, and incubate in a 37°C sterile water bath, until the frozen cell clump has dissolved, but maximal for 1-2 min. Do not incubate longer.
3) Quickly transfer iPSC-CM/hESC-CMs: Gently deliver the cell suspension with a p1000 µl pipette into the prepared 15 ml falcon with medium.
4) Pellet iPSC-CMs/hESC-CMs at 200 g for 3 min.
5) Aspirate medium and resuspend pellet in culture medium (RPMI with B27 supplement). Determine percent viability of iPSC-CMs/hESC-CMs via Trypan Blue exclusion: Mix 10 ul cell suspension with 10 ul of Trypan blue solution. Use a p200 ul pipette to transfer mixture into a Cell Counting Chamber Slide, and determine percent viable cells using an Automated Cell Counter.
6) Plate iPSC-CMs/hESC-CMs at a minimum density of 1.5 million per each well of a 6 well plate. Distribute cells evenly and immediately transfer dishes to a 37°C, 5 % CO_2 incubator.

7) Allow iPSC-CMs/hESC-CMs to attach for 24 h. Generally, iPSC-CMs/hESC-CMs will have resumed contraction at 24 h post-thaw. For some individual iPSCs/hESCs lines, iPSC-CMs/hESC-CMs may resume beating as late as 48 – 72h post-thaw.

Important:

For each batch of iPSC-CM/hESC-CMs, set aside ***at least 2 vials*** of 5 million iPSC-CM/hESC-CMs per vial from each batch. Use these for the following minimal ***quality control tests***:

1) 24 h post-freezing or later, thaw 1 vial as described above to assess the quality of the cells.

2) Determine when cells resume beating, e.g. after 24 h, 48 h, or later. Take images and videos of beating cells at the indicated time points.
   Generally, if availability of time and staff allows, use the same cells for immunohistochemistry (cardiac Troponin T) and acquire images using a brightfield microscope. Perform FACS analysis for % of cardiac Troponin T positive cells.

3) Keep the 2nd frozen vial of 5 million CMs from the same batch as minimal backup in case of specific requests regarding a particular batch; this allows for further analysis if requested by a collaborating NHLBI investigator.

4) Attempt to freeze more than the originally requested number of iPSC-CMs for each NHLBI investigator request. This allows the Biobank to provide additional cells from the same batch.

Cardiac differentiation

***Day 0* - Induction of mesoderm differentiation**.
Induce cardiac differentiation of iPSCs/ESCs at 80 % confluency, usually day 3 after passage. Remove E8 medium. Add RPMI medium with B27 supplement ***minus*** insulin and the Glycogen synthase kinase 3 (GSK3) inhibitor CHIR-99021 (*2*). The standard CHIR concentration is 6 µM (10 mM stock), but may be adapted as needed for individual iPSCs/ESCs lines, varying between 4-10 µM. Volume: 2 ml per each well of a 6 well plate.

***Day 2* – Recovery**.
Replace medium with 2 ml RPMI with B27 supplement ***minus*** insulin.

Day 3 - **Cardiac mesoderm specification**.
Remove medium and replace with 2ml RPMI with B27 supplement ***minus*** insulin with the addition of a canonical Wnt-inhibitor (*2*), IWR1 (10 mM stock in DMSO), at 5 µM. Note: The choice of Wnt inhibitor is not critical; IWR1 may be substituted with other small molecule Wnt inhibitors, e.g. IWP2 or IWP4 (*2*).

***Day 5* – Recovery**.
Replace medium with 2ml of RPMI with B27 supplement ***minus*** insulin.

***Day 7* – Completion of differentiation**.
Replace medium with 2ml of RPMI with B27 supplement ***plus*** insulin. Contracting cells may be observed as early as day 6 (normally between day 7-9). For some particular iPSC/ESC lines beating cells may become evident as late as day 11.

Cardiac purification

***Day 7-11* - Propagation**.
Replace medium on day 9 and 11 with ***3*** ml fresh RPMI and B27 supplement ***plus*** insulin.

***Day 12* - Purification I**.
(Day 12-16)
Change medium to ***3*** ml RPMI ***minus* Glucose** with B27 supplement ***plus*** insulin.

***Day 14* - Purification I**.
Change medium on day 12 to ***3*** ml RPMI ***minus* Glucose** with B27 supplement ***plus*** insulin (Life Technologies).

***Day 16* – Recovery**.
(Day 16-20).
Replace medium with ***3*** ml RPMI and B27 supplement ***plus*** insulin.

***Day 18* – Recovery**.
Replace medium with ***3*** ml RPMI and B27 supplement ***plus*** insulin.

***Day 20* – Purification II**.
(Day 20-24).
Change medium to ***3*** ml RPMI ***minus* Glucose** with B27 supplement **plus** insulin.

***Day 22* – Purification II**.
Replace medium with ***3*** ml RPMI and B27 supplement ***plus*** insulin.

***Day 24* – Recovery**.
(Day 24-30).
Replace medium with ***3*** ml RPMI and B27 supplement ***plus*** insulin on days 24, 26, and 28.

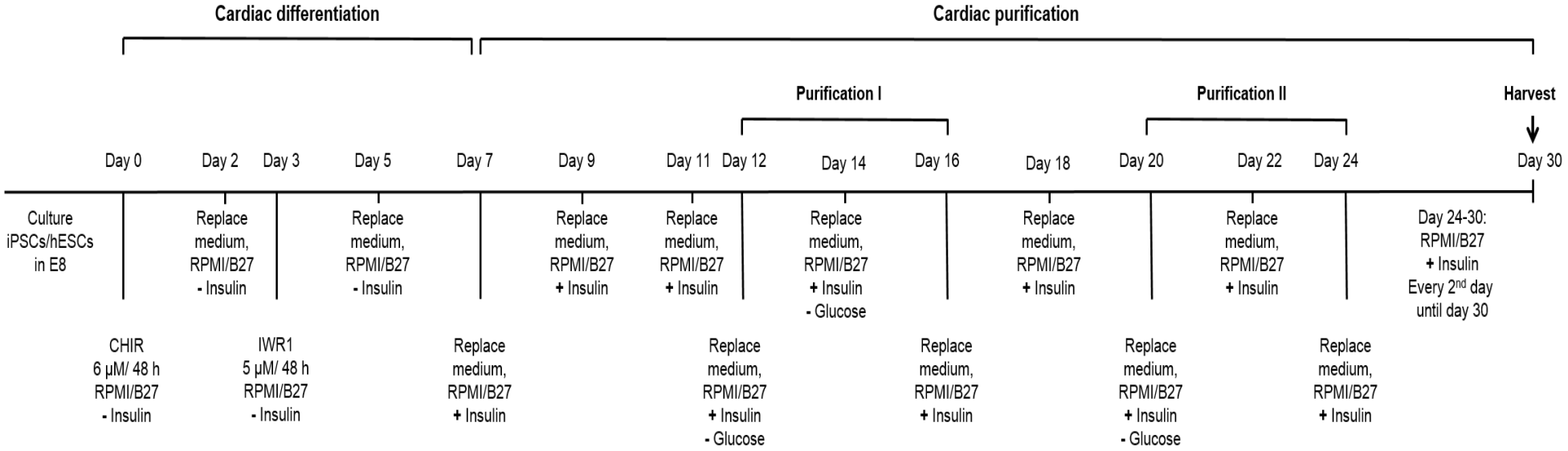
***Day 30* – Harvest**.
Detach and freeze iPSC-CMs/hESC-CMs as described above.

For further references, see (*3-5*)

Materials

DMEM-F12	Life Technologies 11330-057
Essential 8 (E8) medium (***)	Gibco/Life Technologies A1517001
Matrigel	Corning/Fisher Scientific CB40230C
PBS	Gibco/Life Technologies 14190-250
Accutase	Sigma A6964-100ML
EDTA (****)	0.5 M stock, Gibco/Life Technologies 15575-020
ROCK inhibitor	Selleck Chemicals Y27632
BamBanker	Fisher Scientific NC9582225
KnockOut Serum Replacement (KSR)	Life Technologies 10828-028
RPMI 1640	Life Technologies 11875-119
RPMI 1640 Medium, no glucose	Life Technologies 11879-020
B27 supplement minus insulin	Gibco/Life Technologies A1895601
B27 supplement plus insulin, serum-free	Gibco/Life Technologies 17504-044
CHIR-99021	Selleckchem S2924
IWR-1	Selleckchem S7086
Matrigel	Corning/Fisher Scientific CB40230C
TrypLE Express	Gibco/ Life Technologies 12605-010
Trypan Blue Stain (0.4%)	Life Technologies T10282
Fetal Bovine Serum (FBS)	Life Technologies 26140-079
Freezing container	Thermo Scientific 5100-0001
Countess Cell Counting Chamber Slides	Invitrogen Life Technologies C10228
Protein low retention tubes	Fisher Scientific (Protein LoBind, 13-698-794)

(***) Alternatively, prepare from E8 medium from individual components (*1*).
(****) Alternatively, prepare a 0.5 mM EDTA working solution in D-PBS. Sterile filter before use.


Cardiac differentiation
Cardiac purification
Purification I
Purification II
Harvest
Day 0
Day 2
Day 3
Day 5
Day 7
Day 9
Day 11
Day 12
Day 14
Day 16
Day 18
Day 20
Day 22
Day 24
Day 30
Culture iPSCs/hESCs in E8
Replace medium, RPMI/B27 - Insulin
Replace medium, RPMI/B27 - Insulin
Replace medium, RPMI/B27 + Insulin
Replace medium, RPMI/B27 + Insulin
Replace medium, RPMI/B27 + Insulin - Glucose
Replace medium, RPMI/B27 + Insulin
Replace medium, RPMI/B27 + Insulin
Replace medium, RPMI/B27 + Insulin
Day 24-30: RPMI/B27 + Insulin Every 2nd day until day 30
CHIR 6 µM/ 48 h RPMI/B27 - Insulin
IWR1 5 µM/ 48 h RPMI/B27 - Insulin
Replace medium, RPMI/B27 + Insulin
Replace medium, RPMI/B27 + Insulin - Glucose
Replace medium, RPMI/B27 + Insulin
Replace medium, RPMI/B27 + Insulin - Glucose
Replace medium, RPMI/B27 + Insulin

Literature

1. G. Chen *et al.*, Chemically defined conditions for human iPSC derivation and culture. *Nat Methods* **8**, 424-429 (2011).
2. X. Lian *et al.*, Robust cardiomyocyte differentiation from human pluripotent stem cells via temporal modulation of canonical Wnt signaling. *Proc Natl Acad Sci U S A* **109**, E1848-1857 (2012).
3. X. Lian *et al.*, Directed cardiomyocyte differentiation from human pluripotent stem cells by modulating Wnt/beta-catenin signaling under fully defined conditions. *Nat Protoc* **8**, 162-175 (2013).
4. J. Zhang *et al.*, Extracellular matrix promotes highly efficient cardiac differentiation of human pluripotent stem cells: the matrix sandwich method. *Circ Res* **111**, 1125-1136 (2012).
5. S. J. Kattman *et al.*, Stage-specific optimization of activin/nodal and BMP signaling promotes cardiac differentiation of mouse and human pluripotent stem cell lines. *Cell Stem Cell* **8**, 228-240 (2011).