Generating Cardiomyocytes from Human Pluripotent Stem Cells using Stemgent MesoFate™ Differentiation Medium

Overview

Analysis of mouse and human embryonic stem cell differentiation cultures indicate the existence of a cardiovascular progenitor representing one of the earliest stages in mesoderm specification to the cardiovascular lineages. Human pluripotent stem cell derived embroid bodies (EBs) induced with combinations of Activin A, bone morphogenetic protein 4 (BMP-4), basic fibroblast growth factor (bFGF), vascular endothelial growth factor (VEGF), and dickkopf homolog 1 (Dkk-1) (or small molecule Wnt pathway antagonist such as IWP2) in serum free media generates a KDR^{low}/$c\text{-}Kit^{neg}$, $PdgfR\text{-}\alpha^{high}$ population that can give rise to cardiomyocyte, endothelial, and vascular smooth muscle cells[1,2].

The following staged protocol, developed in collaboration with the laboratory of Dr. Gordon Keller, describes the cardiovascular differentiation of human pluripotent stem cells (hPSCs) using Stemgent MesoFate Differentiation Medium in a 6-well format. While the following describes the setup for three wells of a 6-well plate, the reagent volumes can be adjusted according to the desired number of experimental samples. Cardiovascular differentiation from Days 0 - 12 is carried out under hypoxic conditions, and Days 12 - 20 are done under normoxic conditions. This protocol was developed on the human embryonic stem cell line, HES2. Additional optimization may be required for cardiovascular differentiation of other human pluripotent stem cell types as proliferation rate and differentiation efficiency will vary. It may be necessary to titrate cytokine concentrations and/or vary the timing of the various stages of differentiation. For a range of recommended cytokine concentrations to test, see Appendix A.

Required Materials

Product Description	Cat. No.	Format	Storage
Stemgent MesoFate Differentiation Medium	00-0072	500 ml	4°C
Product Components*		**Format**	**Storage**
MesoFate Basal Medium (Part No. 01-0017)		500 ml	4°C
MesoFate Supplement (Part No. 01-0018)		4×10^6 cells/vial	Liquid Nitrogen

*Product components not available for individual sale

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Additional Materials Required

Product Description	Cat. No.
DMEM/F-12 (Corning Cellgro®)	10-092-CV
Penicillin-Streptomycin (Life Technologies™)	15070-063
DNase I, Bovine Pancreas (Calbiochem®)	260913
TrypLE™ Express (1X), Phenol Red (Life Technologies)	12605-100
KnockOut™ Serum Replacement (Life Technologies)	10828-010
L-Glutamine, 200 mM Solution (Life Technologies)	25030-081
L-Ascorbic acid (Sigma-Aldrich®)	A4544
1-Thioglycerol (Sigma-Aldrich)	M6145
Y267632 (Stemgent-Asterand)	04-0012
Transferrin (Roche)	10652202
Recombinant Human BMP-4 (R&D Systems®)	314-BP
Recombinant Human FGF basic (R&D Systems)	233-FB
Recombinant Human/Mouse/Rat Activin A,CF (R&D Systems)	338-AC/CF
Recombinant Human VEGF 165 (R&D Systems)	293-VE
Recombinant Human IWP2 (R&D Systems)	3533
Phosphate Buffered Saline (PBS), (without Ca^{2+} and Mg^{2+}) pH 7.4 (Corning Cellgro)	01-031-CM
Iscove's Modified Dulbecco's Medium (IMDM) (Life Technologies)	12440-053
Fetal or Newborn Calf Serum (FCS)	
0.22 µm pore size, low protein-binding filter	
50mL polypropylene (PP) conical centrifuge tubes	
Falcon® 14mL Round Bottom Polystyrene Test Tube, with Snap Cap (Corning)	352001
Falcon 5mL Round Bottom Polystyrene Test Tube, with Cell Strainer Snap Cap (Corning)	352235
Ultra Low Attachment 6 well plate (Corning)	3471
70 µM and 100 µM cell strainer	

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Material Preparation

hPSC Wash Medium Preparation

500 mL	DMEM/F12
2.5 mL	KnockOut Serum Replacement
5 mL	Penicillin-Streptomycin, Liquid

DNase I Stock Solution Preparation

1. Dissolve 5 mg of DNase I in 5 mL of tissue grade water to achieve a final concentration of 1 mg/mL.
2. Stir gently at room temperature in the fume hood for 1 hour.
3. Filter-sterilize and pipet into 100 µL single-use aliquots.
4. Store the DNase I aliquots at -20°C.

MesoFate Supplement Aliquot Preparation

For ease of use and to minimize degradation of components, the MesoFate Supplement (Stemgent-Asterand 01-0018) should be aliquotted into single-use vials and stored separately as directed.
1 mL of MesoFate Supplement is required for 50 mL of MesoFate Basal Medium.

1. Thaw the 10 mL vial of MesoFate Supplement at 4°C overnight.
2. Pipet 1 mL of supplement into ten individual sterile, low protein-binding microcentrifuge tubes.
3. Store the MesoFate Supplement aliquots at -20°C for up to 3 months.

NOTE: Once a single-use aliquot has been thawed it must be used immediately and cannot be refrozen.

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Complete MesoFate Differentiation Medium

Prepare Complete MesoFate Differentiation Medium just prior to preparing the various induction media used throughout the protocol.

50 mL	MesoFate Basal Medium
500 µL	Penicillin-Streptomycin, Liquid
1 mL	MesoFate Supplement (Stemgent 01-0018)

1. Add 500 µL of Penicillin-Streptomycin (P/S) to the 50 mL of MesoFate Basal Medium and pipet thoroughly to mix.
2. Warm the 50 mL MesoFate Basal Medium containing P/S and one 1 mL MesoFate Supplement aliquot in a 37°C waterbath for 15 to 20 minutes.
3. Mix the Supplement aliquot well with a pipet and add to the warmed Basal Medium containing P/S.
4. Warm the Complete MesoFate Medium in a 37°C waterbath for an additional 30 minutes.

L-Ascorbic Acid Aliquots Preparation

25 mg	L-Ascorbic acid
5 mL	Cold tissue grade water

1. Dissolve 25 mg of L-Ascorbic acid in 5 mL of cold tissue grade water to achieve a final concentration of 5 mg/mL.
2. Leave on ice and vortex periodically until completely dissolved.
3. Filter-sterilize and pipet into 550 µL single-use aliquots. Store L-Ascorbic Acid aliquots at -20°C.

NOTE: Once a single-use aliquot has been thawed it must be used immediately and cannot be refrozen.

1-Thioglycerol Working Stock Solution Preparation

26 µl	1-Thioglycerol
2ml	IMDM

Dilute 26 µl of concentrated 1-Thioglycerol in 2 ml of IMDM just before use. The working stock solution is always made fresh and should not be reused.

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Aggregation Medium Preparation

50 mL	Complete MesoFate Differentiation Medium
500 µL	L-Glutamine, 200 mM Solution
250 µL	Transferrin
500 µL	L-Ascorbic acid
150 µL	1-Thioglycerol working stock solution
2.5 µL	Recombinant Human BMP-4 (10 µg/mL stock concentration)
1 µM	Y27632

Prepare Aggregation Medium just prior to use as instructed within the protocol. Discard any unused Aggregation Medium after use.

Induction 1 Medium Preparation

50 mL	Complete MesoFate Differentiation Medium
500 µL	L-Glutamine, 200 mM Solution
250 µL	Transferrin
500 µL	L-Ascorbic acid
150 µL	1-Thioglycerol working stock solution
50 µL	Recombinant Human BMP-4 (10 µg/mL stock concentration)
25 µL	Recombinant Human FGF basic (10 µg/mL stock concentration)
30 µL	Recombinant Human/Mouse/Rat Activin A, CF (10 µg/mL stock concentration)

Prepare Induction 1 Medium just prior to use as instructed within the protocol. Discard any unused Induction 1 Medium after use.

NOTE: Optimization may be required as proliferation rate and differentiation efficiency will vary between pluripotent stem cell lines.

Induction 2 Medium Preparation

50 mL	Complete MesoFate Differentiation Medium
500 µL	L-Glutamine, 200 mM Solution
250 µL	Transferrin
500 µL	L-Ascorbic acid
150 µL	1-Thioglycerol working stock solution
100 µL	Recombinant Human VEGF 165 (5 µg/mL stock concentration)
1 µM	IWP2 (range from 1-4 µM)

NOTE: For optimal cardiac differentiation of some hPSC lines, the addition of small molecules such as SB431542 (1-10 µM) and Dorsomorphin (0-2 µM) may be required.

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Induction 3 Medium Preparation

50 mL	Complete MesoFate Differentiation Medium
500 µL	L-Glutamine, 200 mM Solution
250 µL	Transferrin
500 µL	L-Ascorbic acid stock solution
150 µL	1-Thioglycerol working stock solution
100 µL	Recombinant Human VEGF 165 (5 µg/mL stock concentration)

Prepare Induction 3 Medium just prior to use as instructed within the protocol. Discard any unused Induction 3 Medium after use.

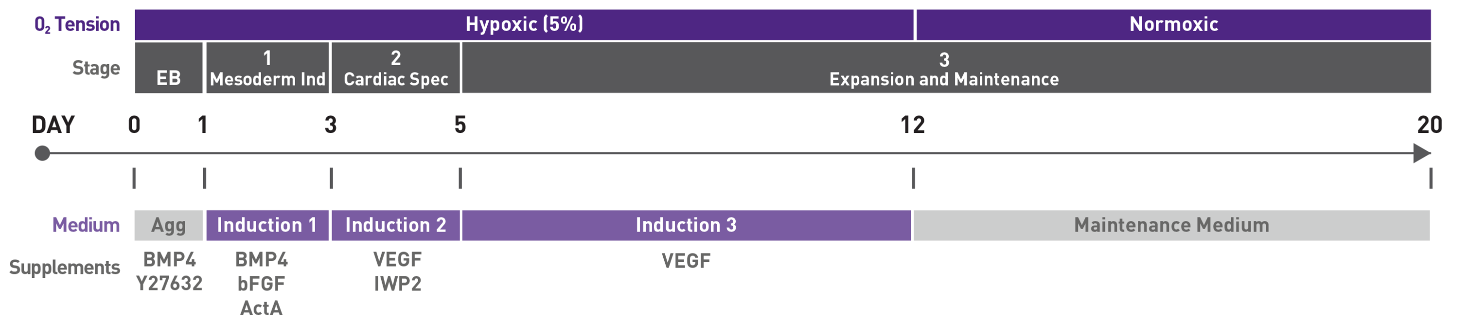
Maintenance Medium (Mesofate Backbone Medium) Preparation

50 mL	Complete MesoFate Differentiation Medium
500 µL	L-Glutamine, 200 mM Solution
250 µL	Transferrin
500 µL	L-Ascorbic acid stock solution
150 µL	1-Thioglycerol

Prepare Maintenance Medium just prior to use as instructed within the protocol. Discard any unused Maintenance Medium after use.

Specification and Differentiation Schematic

The schematic below outlines the staged protocol for generating cardiomyocytes from human pluripotent stem cells using MesoFate Differentiation Medium.



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Day 0: Stage EB

Differentiation of Human Pluripotent Stem Cells

The formation of EBs is the first important step in the differentiation of hPSCs towards cardiovascular lineages and is best achieved by culturing small aggregates of hPSCs in minimal amounts of BMP-4 for 24 hours. At this stage, BMP-4 functions to promote the survival of the hPSCs. The aggregation protocol below is based on a healthy, starting culture of hPSCs grown on mouse fibroblasts/feeders in 6-well format. Cells should be growing within a density range of 60 - 80% confluency (Figure 1B).

NOTE: One well of hPSCs at 60 - 80% confluency should yield – 1-2 x 10^6 cells, and 1 x 10^6 hPSCs will typically yield 1.5 x 10^6 - 2 x 10^6 cardiovascular progenitors at Day 4, and 3 x 10^6 – 3.5 x 10^6 cardiomyocytes at Day 20 in an optimized protocol.

Refer to **Figure 1** for an overview of the experimental set-up and examples of proper density, health, and confluency of starting hPSCs cultures. The following instructions should be done under Hypoxic conditions.

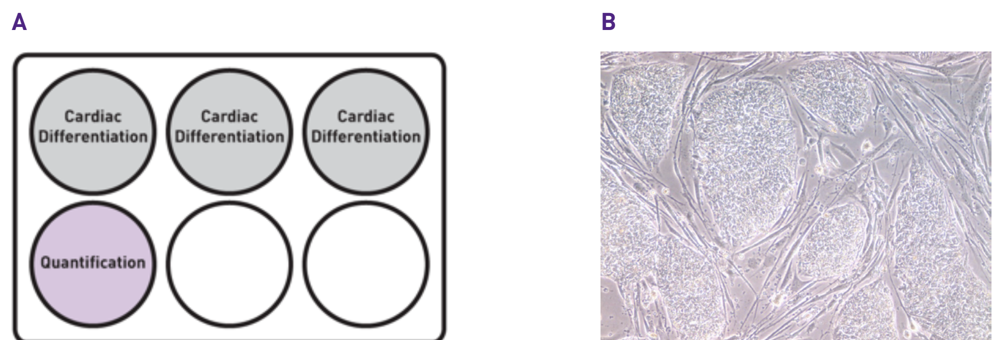

Figure 1. Plating and starting culture of hPSCs. (A) Experimental set up in tissue culture plate. Three wells containing hPSCs will be used for cardiovascular differentiation while the fourth well will be used to estimate total cell number. **[B]** This image represents a healthy, feeder-free starting culture of hPSCs grown on mouse fibroblasts/feeders at approximately 80% confluency.

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1. Aspirate the culture medium from the well of hPSCs designated for quantification of starting cell number and add 2 mL of PBS to wash.
2. Aspirate the PBS and add 1 mL of TrypLE.
3. Incubate the cells for 3 to 5 minutes at 37°C.
4. Aspirate the TrypLE and add 1 mL per well of hPSC Wash Medium containing 10 µg/mL of DNase I.
5. Resuspend cells into single cells with a P1000 pipette. Determine amount of cells in a single well in order to prepare an appropriate amount of aggregation medium.
6. Add an additional 1 mL per well of hPSC Wash Medium containing 10 µg/mL of DNase I.
7. Combine the cell suspensions from the three wells and filter the cells using a 70 µM filter into a 50 mL conical tube.
8. Add an additional 4 mL of hPSC Wash Medium containing 10 µg/mL of DNase I, to bring the total volume to 10 mL.
9. Centrifuge the cell suspension for 3 minutes at 1200 rpm.
10. Aspirate the supernatant and wash cells with 6mLs of DMEM/F12.
11. Centrifuge the cell suspension for 3 minutes at 1200 rpm.
12. Aspirate the supernatant and add an appropriate amount of Aggregation Medium to the cells to bring the cell density to 2.5×10^5 - 5×10^5 cells/mL with preference to the upper limit. Pipet up and down gently to resuspend the clusters.
13. Distribute 2 mL per well of the cell suspension into three individual wells of an Ultra Low Attachment 6-well plate. This should result in a final density of 5×10^5 - 1×10^6 cells per well.
14. Incubate the cells for 24 hours at 37°C in 5% CO_2, 5% O_2 on an orbital shaker set between 60-70 rpm.

Day 1-3: Stage 1

Before starting, prepare Induction 1 Medium, enough for 2 mL for each well harvested. It is co mon to observe some debris after 24 hours in culture from a small percentage of cell death during the aggregation process. It is best to remove this debris prior to induction of differentiation. The efficiency of mesoderm induction and cardiovascular progenitor specification in the EBs can be assessed starting at Day 3. This can be monitored by flow cytometric (FC) analysis, evaluating the cells for expression of CD56 and PdgfR-α (see Appendix B for Day 3 FC analysis instructions).

15. Following the 24-hour incubation, separate the EBs from the debris by transferring the cell suspensions from the three wells into a single 14 mL round bottom tube. (Large clumps of cells can be removed by filtering the EB suspension through a 100 µM cell strainer).
16. Incubate for 20 minutes at 37°C in 5% CO_2, 5% O_2 to allow the EBs to settle.
17. Following the 20 minute incubation, aspirate the supernatant and add 6 mL of the freshly prepared Induction 1 Medium. Pipet gently to resuspend the EBs.
18. Distribute 2 mL per well of the cell suspension to three individual wells of a new Ultra Low Attachment 6-well plate.
19. Incubate the EBs for 2 days at 37°C in 5% CO_2, 5% O_2 without agitation.

NOTE: No media changes are necessary during this 3 day incubation period.
(Optimal duration of induction will need to be determined by the end user.)

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Day 3-5: Stage 2

Before starting, prepare Induction 2 Medium containing VEGF andIWP2, enough for 2 mL for each well harvested. The efficiency of mesoderm induction and cardiovascular progenitor specific tion in the EBs is again assessed at Days 3. This can be monitored by flow cytometric (FC) analysis, evaluating the cells first for expression of CD56 and PdgfR-α followed by KDR and PdgfR-α one day later (see Appendix B for Day 3/4 FC analysis instructions).

20. Following the 2-day incubation, separate the EBs from the debris by transferring the cell suspensions from the three wells into a single 14 mL round bottom tube.
21. Incubate for 20 minutes at 37°C in 5% CO_2, 5% O_2 to allow the EBs to settle.
22. Following the 20-minute incubation, aspirate the supernatant and add 10 mL of IMDM to the EBs to wash out residual inductive cytokines.

NOTE: Activin A is a potent signaling molecule even at very low concentrations. Its complete removal is therefore necessary for efficient differentiation to the next stage.

23. Centrifuge the EB suspension for 5 minutes at 800 rpm.
24. Aspirate the supernatant and add 6 mL of the freshly prepared Induction 2 Medium. Pipet gently to resuspend the EBs.
25. Distribute 2 mL per well of the cell suspension to three individual wells of a new Ultra Low Attachment 6-well plate.
26. Incubate the EBs for 2 days, until Day 5, at 37°C in 5% CO_2, 5% O_2.

Day 5-12: Stage 3

Before starting, prepare Induction 3 Medium containing VEGF, enough for 2 mL for each well harvested.

NOTE: The addition of small molecules are no longer necessary in the media at this stage.

27. Following the 2 day incubation, separate the EBs from the debris by transferring the cell suspensions from the three wells into a single 14 mL round bottom tube.
28. Centrifuge the EB suspension for 5 minutes at 800 rpm.
29. Aspirate the supernatant and add 6 mL of the freshly prepared Induction 3 Medium. Pipet gently to resuspend the EBs.
30. Distribute 2 mL per well of the cell suspension to three individual wells of a new Ultra Low Attachment 6-well plate.
31. Culture the EBs in Induction media 3 containing VEGF until Day 12 at 37°C in 5% CO_2, 5% O_2, changing the media every 2-3 days as required.

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Day 12-20: Maintenance

Before starting, prepare Maintenance Medium, enough for 2 mL for each well harvested.

NOTE: The following instructions should be done under normoxic conditions.

32. Separate the EBs from the debris by transferring the cell suspensions from the three wells into a single 14 mL round bottom tube.
33. Centrifuge the EB suspension for 5 minutes at 800 rpm.
34. Aspirate the supernatant and add 6 mL of the freshly prepared Maintenance Medium. Pipet gently to resuspend the EBs.
35. Distribute 2 mL per well of the cell suspension into three individual wells of a new Ultra Low Attachment 6-well plate and incubate the EBs suspension at 37°C in 5% CO_2, ambient/normoxic O_2.
36. Monitor the EBs suspension for spontaneous contraction periodically, changing media every 2-3 days as needed until Day 20 at 37°C in 5% CO_2, ambient/normoxic O_2.
37. Prepare a single cell suspension and stain for cardiac specific markers such as CD172 α (Sirp-α) and cardiac-TroponinT (see Appendix C for Day 20 FC analysis instructions).[3]

Appendix A

Range of recommended cytokine concentrations	
Cytokine	**Concentration**
Recombinant Human BMP-4	1 – 30 ng/mL
Recombinant Human FGF basic	1 – 10 ng/mL
Recombinant Human Activin A	1 – 10 ng/mL
Recombinant Human VEGF 165	1 – 20 ng/mL
Recombinant Human IWP2	1-4 μM

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Appendix B

Day 3 and 4

Harvest Cells for Flow Cytometric Analysis ofCD56/PdgfR-α and KDR/PdgfR-α Expression

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As each human pluripotent stem cell line has its own unique induction kinetics, the cardiovascular progenitor stage cannot be defined by time in culture alone. Therefore mesoderm induction and cardiovascular progenitor specification in the EBs during Days 3 to 4 should be monitored by flow cytometric analysis. The cardiovascular progenitor stage is defined by the appearance of a CD56/ PdgfR-α double positive population at day 3 and a KDR/ PdgfR-α double positive at day 4. EBs at this stage also contain a KDR^{low}/PdgfR-α^{low} and a KDR^{high}/PdgfR-α^{low} profile. This KDR/PdgfR-α profile is typically detected in hPSC-derived EBs at Day 3-4, however the kinetics of this induction can vary slightly depending on cell line and should be determined by the user. Refer to **Figure 2** for an example of appropriate CD56/PdgfR-α and KDR/PdgfR-α profiles.

1. Separate the EBs from the debris by transferring the cell suspension from one well designated for FC analysis into a single 14 mL round bottom tube.
2. Pellet the EBs at 800 rpm for 5 minutes.
3. Aspirate the supernatant and add 1 mL of TrypLE Express (1X).
4. Incubate EBs for 5 minutes at 37°C in 5% CO_2, 5% O_2.
5. Following the 3 minute incubation, gently vortex the sample and monitor EB dissociation.
6. Incubate EBs for an additional 2 minutes at 37°C in 5% CO_2, 5% O_2.
7. Gently pipet the sample with a p1000 tip to break up any remaining EBs and add 8 mL of IMDM to the suspension.
8. Centrifuge the EB suspension for 5 minutes at 1000 rpm.
9. Aspirate the supernatant and resuspend the EBs in 4 mL PBS (without Ca^{2+} and Mg^{2+}) containing 5% FCS and DNAse I (10 ng/mL).
10. Pass the EBs through a 4 mL round bottom tube with cell strainer cap.
11. Stain $1x10^5$ cells from the EB-derived single cell suspension with CD56, KDR and PdgfR-α antibodies according to supplier's recommendations and perform flow cytometric analysis.

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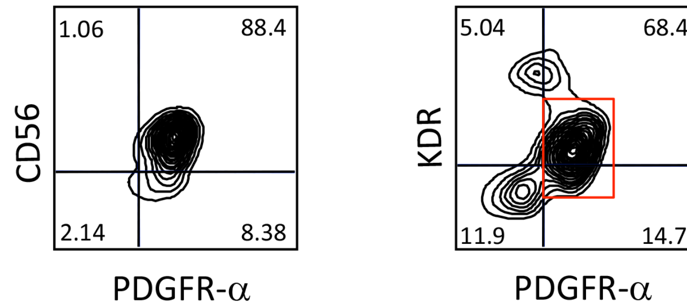

Figure 2. Flow cytometric analysis of CD56 vs. PdgfR-α and KDR vs. PdgfR-α expression on Day 3 and 4. EB-derived hPSCs were stained with CD56 and PdgfR-α at Day 3 and KDR and PdgfR-α at Day 4 of differentiation. Red box indicates cardiovascular progenitor population expressing a KDR^{low}/PdgfR-α^{high} profile.

Appendix C

Day 20

Harvest Cells for Flow Cytometric Analysis of Cardiomyocyte Specific Protein Expression

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Refer to **Figure 3** for an example of an appropriate Sirpα and cTnT profile.
Separate the EBs from the debris by transferring the cell suspension from one well designated for FC analysis into a single 14 mL round bottom tube.

1. Centrifuge the cell suspension for 3 minutes at 800 rpm.
2. Aspirate the supernatant and add 1 mL of Collagenase B containing 10 µL DNase I Stock Solution.
3. Incubate the EBs for 1-2 hour at 37°C in 5% CO_2, 20% O_2 with periodic mixing to aid in the dissociation of the EBs.
4. Following the incubation, wash the EBs with 9 mL of PBS without Ca^{2+} and Mg^{2+}.
5. Centrifuge the EB suspension for 5 minutes at 800 rpm.
6. Aspirate the supernatant and add 1 mL of TrypLE Express (1X).
7. Incubate EBs for 5 minutes at 37°C in 5% CO_2, 20% O_2. After 3 minutes of incubation, gently vortex the sample and monitor the cell dissociation.
8. Once turbid, add 3 mL PBS (without Ca^{2+} and Mg^{2+}) containing 5% FCS and DNAse I (10 ng/mL) to the EB suspension and gently pipet to dissociate any remaining cells.
9. Filter the cell suspension through a 4 mL round bottom tube with cell strainer cap to remove any large clumps of cells.
10. Pellet at 1200 rpm for 5 minutes, and wash twice with an additional 4 mL PBS (without Ca^{2+} and Mg^{2+}) containing 5% FCS.
11. After the second wash, resuspend the single cell suspension in 1 mL of PBS (without Ca^{2+} and Mg^{2+}) containing 5% FCS.
12. Determine cell number and stain 2.5×10^5 live cells with CD90 and CD172a/Sirpα (BioLegend® 323808) according to the supplier's recommendations.

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13. For cTnT intracellular staining, fix cells in 200 µL of 4% PFA (diluted in PBS without Ca^{2+} and Mg^{2+}) and for 20 minutes at room temperature.
14. Pellet fixed cells at 1500 rpm for 2 minutes.
15. Carefully aspirate supernatant and dispose of in a PFA waste container.
16. Wash the fixed cells twice in 200 µL PBS (without Ca^{2+} and Mg^{2+}) containing 5% FCS (Fixed cells can be stored overnight at 4°C after washes)
17. Add 100 µL Permeablization Buffer (0.5% saponin (Sigma S4521) in PBS+5%FCS) for 10 minutes at room temperature.
18. Stain the cells for 30 minutes on ice with anti-cTnT (Fisher Scientific MS295P) at pre determined optimal concentration and an IgG-isotype control.
19. Pellet cells at 1500 rpm for 2 minutes and wash twice with 200 µL Permeabilization buffer.
20. Resuspend cells in 100 µL Permeabilization Buffer
21. Stain cells with goat anti-mouse-APC secondary antibody according to suppliers recommendations for 30 minutes at room temperature (in the dark).
22. Wash twice in Permeablization Buffer and once in Flow Staining buffer.
23. Analyze by flow cytometry.

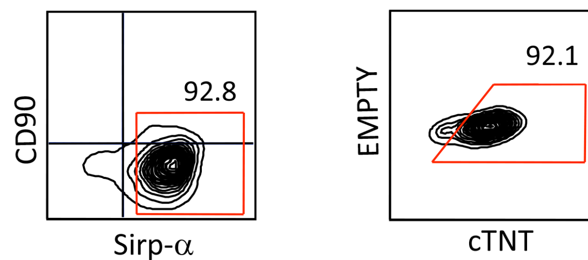

Figure 3. Flow cytometric analysis for Sirpα+ and cTnT+ expression on Day 20. EB-derived hPSCs were stained with CD90 and Sirpα or cTnT at Day 20 of differentiation. Red boxes indicate cardiovascular cell population expressing Sirpα+ and cTnT+ profiles.

References

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