Title	McEwen - Cardiac differentiation
Date Submitted	March 2012
Submitted by -	Sunita D'Souza
Adapted from -	**Human cardiovascular progenitor cells develop from a KDR+ embryonic-stem-cell-derived population.** Nature. 2008 May 22;453(7194):524-8. Epub 2008 Apr 23. AND SIRPA is a specific cell-surface marker for isolating cardiomyocytes derived from human pluripotent stem cells. 2011. Nature Biotechnology, 29, 1011–1018 (2011)
Contributors -	Yang L, Soonpaa MH, Adler ED, Roepke TK, Kattman SJ, Kennedy M, Henckaerts E, Bonham K, Abbott GW, Linden RM, Field LJ, Keller GM. AND Nicole C Dubois, April M Craft, Parveen Sharma, David A Elliott, Edouard G Stanley, Andrew G Elefanty, Anthony Gramolini & Gordon Keller
Affiliation(s) -	Mount Sinai School of Medicine, New York, NY 10029

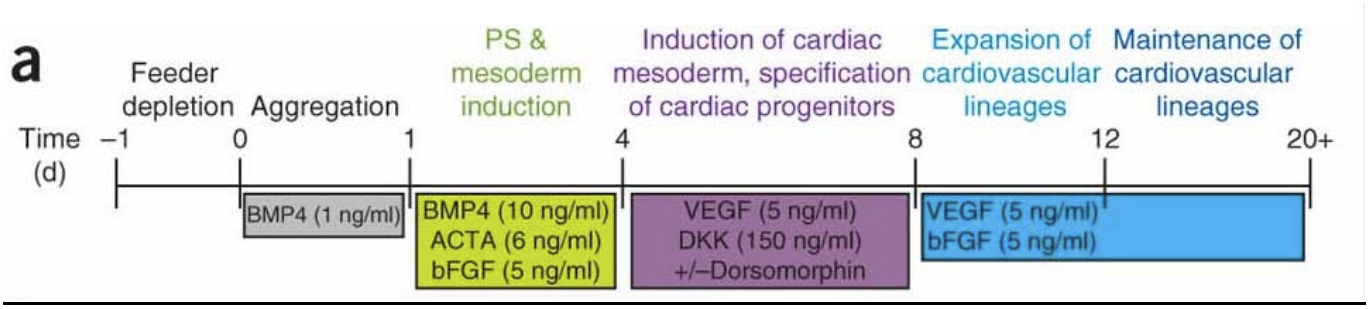
Table of Contents

Introduction

To direct the differentiation of human embryonic stem cells (ESCs) to the cardiac lineage, Lei Yang et al., showed that the combination of BMP4 and activin A will promote cardiac development in human ESC cultures[8]. However, the stage at which these pathways function in the establishment of this lineage was not defined. Using the protocol developed here, the combination of activin A and BMP4 at stage 1 induces a primitive-streak-like population and mesoderm, as demonstrated by the upregulation and transient expression of *T* (brachyury) and *WNT3A*—genes known to be expressed in these populations in the mouse. At stage 2, the WNT inhibitor DKK1 is added to specify cardiac mesoderm and VEGF is included to promote the expansion and maturation of the KDR^{+} population. bFGF is added again at day 8 of differentiation to support the continued expansion of the developing cardiovascular lineages.

Dubois et al., went on to optimize concentration of BMP4, ACTA to increase the efficiency of differentiation (Sunita, the characterization of different BMP/Activin signaling to induce primitive streak populations was described in Steve's Cell Stem Cell paper, which would be the correct reference here). She also identified a unique cell surface marker referred to as SIRPA that tracks the specification and the expansion of the cardiac progenitor.

FLOWCHART



Caption : Analysis of mouse and human embryonic stem cell differentiation cultures indicate the existence of a cardiovascular progenitor respresenting one of the earliest stages in mesoderm specification to the cardiovascular lineages (1,2). Induction with combinations of Activin A, bone morphogenetic protein 4 (BMP4), basic fibroblast growth factor (bFGF), vascular endothelial growth factor (VEGF), and dickkopf homolog 1 (DKK1) in serum free media generates a KDR^{low}/c-Kit^{neg} population that displays cardiac, endothelial and vascular smooth muscle potential (2). The following schematic outlines a staged protocol for generating cardiovascular progenitors from hESCs.

Protocol: Step I: Reagents used

	Reagent	Company	Cat #
1.	StemPro	Invitrogen	10828-028
	Knockout Serum Replacement	Invitrogen	10828-028
2.	DMEM/F12	(Cellgro	10-092-CV
	Penicillin/Streptomycin	Invitrogen	15070-063
3.	Glutamine	Invitrogen	25030-081
4.	Ascorbic acid	Sigma	A-4544
	Collagenase B	Sigma	T-4799
5.	Monothioglycerol (MTG	Sigma	# M-6145
6.	Trypsin	Sigma	T4799
7.	EDTA 0.5 M (pH 8)	Sigma	E6635-100G
8	PBS (without Ca^{2+}, Mg^{2+})	(Cellgro	21-031-CM
9	+/- Matrigel (1:1) *	BD	354230
10	Collagenase Type 1	Sigma	C0-130
11	Fetal Calf serum	Gemini Biologicals	100106
12	DNAse I	Calbiochem	260913
13	Transferrin	Roche	4038377
14	hBMP-4	R&D Systems	314-BP
15	hbFGF	R&D Systems	233-FB
16	hVEGF	R&D Systems	293-VE
17	ActivinA	R&D Systems	338-AC
18	Dkk	R&D Systems	1096-DK
19	hBMP-4	R&D Systems	314-BP

20	Dorsomorphin	ToCris	3093
21	collagenase type II	Invitrogen	17101-015
22	Hanks solution	Invitrogen	14170
23	Taurin -10mM	Sigma	491330
24	EGTA – 0.1mM	Sigma	E0396
25	anti-SIRPA–IgG-phycoerythrin-Cy7 clone SE5A5; BioLegend; 1:500	Biolegend	323808
26	anti-cardiac isoform of cTNT (clone 13-11	NeoMarkers; 1:400	MS-295-P1
27	anti-IgG1κ - phycoerythrin-Cy7 (clone MOPC-21, 1:500	Biolegend	400129

Media preparation

L-ASCORBIC ACID (AA) *(SIGMA # A-4544)*
Prepare a stock solution of 5 mg/mL in cold TC-H_2O. Leave on ice and vortex periodically until completely dissolved. Filter sterilize, aliquot and store at -20ºC. Use once and discard

MONOTHIOGLYCEROL (MTG) *(SIGMA# M-6145)*
The amounts of MTG indicated in our protocols are recommended concentrations. However, it is important to test each new batch of MTG as there is variability between them. MTG should be aliquoted (1 mL) and stored frozen (-20ºC). When aliquots are thawed, they can be used for several experiments and then discarded. Aliquoting of MTG is strongly recommended as it minimizes the amount of oxidation due to repeated opening

TRANSFERRIN *(ROCHE# 10 652 202)*
The amounts of Transferrin indicated in our protocols are recommended concentrations. However, it is important to test each new batch of transferrin as there is variability between them. It should be aliquoted (2 mL) and stored at 4ºC.

COLLAGENASE B *(*Cat # 11 08 831 001*)*

		Final Conc.	For 1 Liter
Collagenase B	*(Sigma# T-4799)*	1mg/ml	1 g
DMEM/F12 + Glut +P/S	*(Cellgro# 10-092-CV)*	100%	1000 mL

1. Weigh 1 g of Collagenase B in the fume hood (inhalation is hazardous) and dissolve in 1000 mL of DMEM-F12 containing 2mM of Glutamine and 50ug/ml of Penicillin/Streptomycin by gently stirring. Make sure it is completely dissolved.
2. Filter sterilize (may need more than one filtration apparatus)
3. Store at -20ºC as 12 mL aliquots in 15 mL screw cap tubes
4. One cycle of freeze-thaw is acceptable
5. Note: final concentration is 0.2% or 2 mg/mL in 20% FCS/PBS

Collagenase II – Overnight solution for the dissociation of EBs: Collagenase type II (1 mg/ml; Worthington) was dissolved in Hanks solution (NaCl, 136 Mm; $NaHCO_3$, 4.16 mM;

$NaPO_4$, 0.34 mM; KCl, 5.36 mM; KH_2PO_4, 0.44 mM; dextrose, 5.55 mM; HEPES, 5 mM), filtered, aliquoted and frozen.

Collagenase II –dissociation solution. Collagenase type II was dissolved in Hanks solution: taurin, 10 mM, EGTA 0.1 mM, BSA 1 mg/ml, filtered, aliquoted and frozen

(A) COLLAGENASE TYPE 1 *(SIGMA# C-0130)*

		Final Conc.	For 500ml
Collagenase Type 1	*(Sigma# # C-0130)*	2mg/ml	1 g
PBS1X Ca/Mg	*(CellGro # 21-030-CM)*	100%	400 mL
FCS	*Gemini Biologicals # 100106*	20%	100ml

Collagenase Type 1 is mainly used for dissociation of the embryoid bodies.

1. Weigh 1 g of Collagenase Type 1 in the fume hood (inhalation is hazardous) and dissolve in 400 mL of PBS by gently stirring. Make sure it is completely dissolved.
2. Add 100 mL FCS
3. Filter sterilize (may need more than one filtration apparatus)
4. Store at -20ºC as 12 mL aliquots in 15 mL screw cap tubes
5. One cycle of freeze-thaw is acceptable
6. Note: final concentration is 0.2% or 2 mg/mL in 20% FCS/PBS

(B) DNASE I (VWR, Cat # 80510-412, 10MU)

1. Want final concentration to be 1mg/ml

2. $10MU \times \frac{1X10^6\ U}{1\ MU} \times \frac{1mg}{65150\ U} = 153mg$

3. In the hood transfer powder to a 125 ml bottle
4. Bring the volume up to 153 ml with ice cold sterile water
5. Let dissolve on ice for 1-2 hours
6. Filter and aliquot 1ml/eppendorf
7. Store at –20.
8. Filter sterilize, aliquot in 1 mL amounts and store frozen at -20ºC
9. Use aliquots once and discard excess

STEMPRO 34 *(Invitrogen# 10639-011)*

Stempro 34 is sold as a kit with 2 components. The supplement is kept at -20°C and the liquid media at 4°C. When combined, the media is unstable, therefore, we use it for a maximum of 2 weeks. If not used right away, we store the medium as 50mL aliquots and supplement them as needed. The supplement is frozen as 1.3mL aliquots which is the amount required for 50mL. of medium

	Final Conc.	For 500mL
STEMPRO 34 Kit		500 mL
Penicillin/Streptomycin P/S	1%	5 mL

1. Warm the media, P/S and frozen supplement in a 37°C waterbath for 15 to 20 minutes
2. Mix the supplement well with a pipette and add to the warm media along with the P/S.
3. Warm the mixture for another 30 minutes and then aliquot into 50mL. (Label the tube that supplement has been added, to avoid confusion.)
4. Store for a maximum of 2 weeks

MATRIGEL (REDUCED FACTOR) *(BD# 354230)*

Each batch of matrigel has its own unique levels of endotoxin and protein concentrations. We find that the endotoxin levels should not be higher than 2 endotoxin units/mL and the protein levels should range between 7 to 10 mg/mL. If the protein levels are higher than this you may need to dilute the matrigel more than 1:1. This is determined by observing the hESC colony morphology and the ability of the hESCs to differentiate into the lineage required of them.
Caution: When working with matrigel, all tubes, plates and pipettes should be pre-chilled, as matrigel solidifies at room temperature.

MATRIGEL1:1 PREPARATION

1. Thaw frozen bottles of matrigel on ice overnight in the cold room. We normally thaw 6X5-mL bottles per batch.
2. The next day, make a 50% working stock by adding an equal volume of IMDM+P/S to each bottle. Resuspend gently with a pre-chilled 5 mL pipette.
3. Leave the bottles on ice all day to allow the matrigel to completely equilibrate with IMDM.
4. Pool 3 bottles of 1:1 matrigel (30 mL) into a pre-chilled 50 mL tube. Gently mix with a chilled 10 mL pipette and aliquot.
5. Transfer 2.5 mL into pre-chilled and pre-labelled 4-mL snap cap tubes
6. Store aliquots at -20ºC

(C) TRYPSIN-EDTA

	Catalogue #	Final Conc.	For 1 Liter
Trypsin	Sigma T4799	0.25%	2.5 g
EDTA 0.5 M (pH 8)	Sigma E6635-100G	1 mM	2 mL
PBS (without Ca^{2+}, Mg^{2+})	Invitrogen 14190-136	100%	1000 mL

1. Warm to dissolve (15 min, 37ºC), filter sterilize, aliquot and store at -20ºC
2. For aggregate formation we use a 25% dilution of the above (ie: 10mL of *TRYPSIN-EDTA* in 30mL of *PBS(without Ca^{2+}, Mg^{2+})*

(D) STOP MEDIUM

	Catalogue #	Final Conc.	For 40 mL
hESC WASH Medium	See (E) below	50%	20 mL
FCS	Gemini 100106	50%	20 mL
+/- Matrigel (1:1) *	See Matrigel Prep	1:800	100 uL

(E) hESC WASH MEDIUM

	Catalogue #	Final Conc.	For 500 mL
Supplemented DMEM/F12	See Supplemented DMEM /F12 media below	95%	475 mL
Knockout™ Serum Replacement	Invitrogen 10828-028	5%	25 mL

SUPPLEMENTED DMEM/F12

	Final Conc.	For 500mL
DMEM/F12		500 mL
Penicillin/Streptomycin	1%	5 mL
Glutamine	1%	5 mL

1. Make 40 mL aliquots of the hESC WASH MEDIUM
2. You can add 100 uL of Matrigel 1:1 to each aliquot when needed

(F) AGGREGATION MEDIUM

	Stock Conc.	Final Conc	per mL	For 50 mL
STEMPRO 34				50mL
Glutamine	100x	1%	10uL	500ul
Transferrin	30mg/mL	150ug/mL	5uL	250ul
Ascorbic Acid	5mg/mL	50ug/mL	10uL	500ul
MTG	26λ/2mLs		3uL	150ul
BMP4*	10ug/mL	1ng/mL	0.1uL	5ul

*****Bmp4 concentration may vary according to lot# or the hES cell line used

(G) INDUCTION 1 MEDIUM

	Stock Conc.	Final Conc	per mL	For 50 mL
STEMPRO 34				50mL
Glutamine	100x	1%	10uL	500ul
Transferrin	30mg/mL	150ug/mL	5uL	250ul
Ascorbic Acid	5mg/mL	50ug/mL	10uL	500ul
MTG	26λ/2mL		3uL	150ul
BMP4*	10ug/mL	10ng/mL	1uL	50ul
bFGF	10ug/mL	2.5ng/mL	0.25uL	12.5ul
Activin A	10ug/mL	6ng/mL	0.6 uL	30 uL

(I) INDUCTION 2 MEDIUM

	Stock Conc.	Final Conc	per mL	For 50 mL
STEMPRO 34				50mL
Glutamine	100x	1%	10uL	500ul
Transferrin	30mg/mL	150ug/mL	5uL	250ul
Ascorbic Acid	5mg/mL	50ug/mL	10uL	500ul
MTG	26λ/2mLs		3uL	150ul
VEGF	5ug/mL	5ng/mL	1uL	50ul
Dkk	50ug/mL	150ng/mL	3uL	150ul

(J) INDUCTION 3 MEDIUM*

	Stock Conc.	Final Conc	per mL	For 50 mL
STEMPRO 34				50mL
Glutamine	100x	1%	10uL	500ul
Transferrin	30mg/mL	150ug/mL	5uL	250ul
Ascorbic Acid	5mg/mL	50ug/mL	10uL	500ul
MTG	26λ/2mLs	-	3uL	150ul
VEGF	5ug/mL	520ng/mL	1uL	50ul
bFGF	10 ug/mL	10ng/mL	1uL	50ul

***NB: Dkk not required beyond Day 8**

Step : III METHOD

The formation of EBs is the first important step in the differentiation of hESC. This is best achieved by culturing small aggregates of hESCs in miminal amounts of BMP-4 for 24 hours. At this stage, BMP-4 functions to promote the survival of the hESCs.

T0-T1: Generation of embryoid bodies (EBs)

1. Remove the medium from hESCs that have been feeder depleted on matrigel coated plates for 24-48 hours (see hESC cell maintainance protocol)
2. To each well, add 1mL of *COLLAGENASE Type 1* containing *10uL/mL DNase* ***(A,B)***, for 20 min. and then aspirate. The cells should still be attached.
3. During this time, completely trypsinize one well of the matrigel hESCs to single cells, to get an accurate cell count for the experiment. The average cell count per starting matrigel well using this protocol is $5x10^5$ to $1x10^6$ cells/well. To calculate the amount of *AGGREGATION MEDIUM* required for the experiment you will need 2mLs for every $5x10^5$ to $1x10^6$ cells harvested. Adjust the final volume of each tube appropriately.
4. Add 1mL of 0.*25% TRYPSIN-EDTA* ***(C)*** **diluted 1:4 in PBS** to the wells and observe carefully for 1-3 min. The cells will separate from each other, but should not lift from the plate. (Note: continuously monitor the cells since there is variation between cell lines and batches of trypsin; each cell line has a different time requirement)
5. Remove the trypsin from the well and stop the reaction with 1mL of *STOP MEDIUM +MATRIGEL****(D)*** containing *10uL/mL DNase***.** Scrape gently with a cell scraper. The hESC cells should lift as small clusters into the medium.
6. Add 1mL of *hESC WASH MEDIUM +MATRIGEL****(E)*** to each well and resuspend with a 5mL pipette 3-5x, until the clusters are **10-20** cells in size. Transfer 3 wells of a 6 well plate to a 14 mL tube containing 4mL of *hESC WASH MEDIUM +MATRIGEL*.
7. Spin at 800 rpm, aspirate and resuspend each tube with 6mL of *AGGREGATION MEDIUM* ***(F)*** using a 5mL pipette (resuspend gently 2-3 times).
8. With a 5mL pipette, evenly distribute 2 mL of aggregates into each well of a 6 well Locluster plate *(Costar#3071)*. Incubate for 24 hours at 37°C in a 5% CO_2, 5% O_2 incubator. For large scale production, plate 4 mL of a cells in a 6 cm petri dish (non adherent EO-sterilized). Optimal concentration of cells for aggregration is $2.5x10^5$-$5x10^5$ cells/mL.

T1-4: Induction 1 (Stage 1)

1. There will be some debris in the cultures after 24 hours. We separate the aggregates from the debris by harvesting 3 wells of a 6 well plate into one 14mL round bottomed tube, allowing them to settle for 30 min in a 37°C, 5%CO_2, 5%O_2 incubator.
2. While the aggregates are settling, make *INDUCTION 1 MEDIUM* ***(G)*** so that there will be 2mL for each well harvested.
3. After the aggregates have settled, aspirate the *AGGREGATION MEDIUM* and add the same volume of the *INDUCTION 1 MEDIUM*.
4. Dispense the aggregates into a new 6 well locluster plate at 2 mL per well (i.e 6mL for 3 wells) after gently resuspending the cells in the 14 mL round bottom tube.
5. Incubate as above until T4

*** For some cell lines start Stage II from Day3**

T4-T8 (OR Day D3 – D5) : Induction 2 (Stage II)

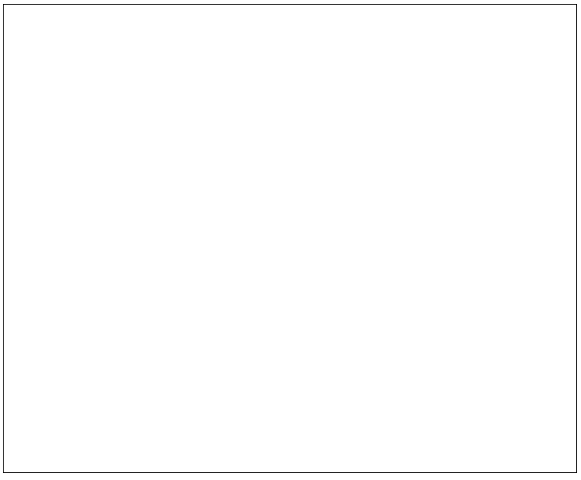
1. Harvest EBs with a 5 mL pipette pooling 3 wells of a 6 well locluster plate per 14 mL snap cap tube
2. Allow EBs to settle for 20 minutes in hypoxic conditions. It is important to allow the EBs to settle for at least 20 minutes to ensure that all EBs separate from the single cells and cellular debris.
3. Aspirate supernatant carefully and wash EBs with 10 mL IMDM (+P/S) to wash out residual inductive cytokines (especially Activin A which is potent signaling molecule even at very low concentrations)
4. Centrifuge the EBs at 800 rpm for 5 minutes and aspirate supernatant
5. Resuspend EB-pellet in 2 mL/well of *INDUCTION 2 MEDIA* ***(I)*** and re-plate into new 6 well locluster plates.
6. Incubate overnight in hypoxic conditions until T8 (OR UNTIL Day 5).

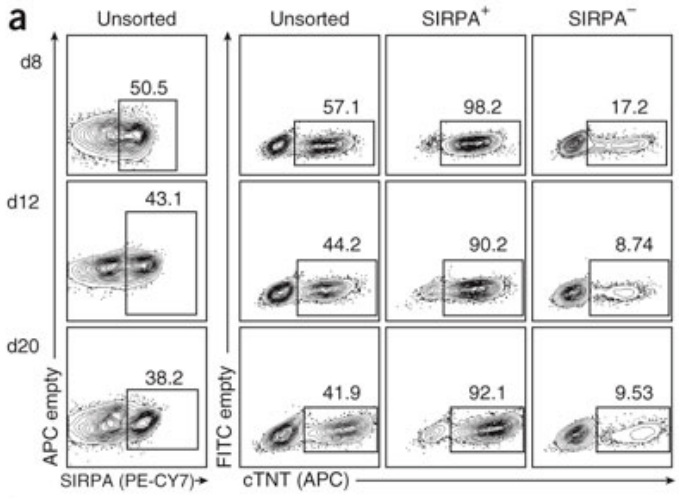
*** For some lines addition of 100ng/ml of Noggin and 0.6uM of SB431542 from D4-D5 helps to increase the specification of cardiac progenitors. In some cases the addition of 0.5uM –1uM of Dorsomorphin does the same.**

Notes

Mesoderm induction and cardiovascular progenitor specification in the EBs should be evaluated by flow cytometric analysis, monitoring the cells for expression of KDR and CD117 (c-kit). As each hESC line has its own unique kinetics, it is best to define the CV progenitor stage based on the profile seen below, rather than by time in culture. The CV progenitor stage is defined by the appearance of a population that expresses an intermediate level of KDR in the absence of CD117 ($KDR^{low}/CD117^{neg}$). EBs at this stage also contain a $KDR^{neg}/CD117^{pos}$ and a $KDR^{high}/CD117^{pos}$. The $KDR^{low}/CD117^{neg}$ profile is typically detected in hESC-derived EBs at day 5, however the kinetics of this induction vary slightly from lab to lab (and cell line to cell line…) and should be determined. Alternatively as shown in the second figure below, the first cells specified to the cardiomyocyte lineage can also be tracked by SIRPA expression (Biolegend 323808). SIPRA tracks with NKX2.5 and starts being expressed between days 7-10, depending on the efficiency of differentiation and the cell line used. and over 90% of the SIRPA+ progenitors express cardiac Troponin (this number may even be higher and the cTnT-negative cells in the SIRPA+ fraction may be sorting contaminations to some extent, however, this is difficult to prove…).

FACS Profiles





T5 Harvest for Flow Cytometry

(Perform flow cytometric analysis from Day 4-7 to determine the optimal cardiac mesoderm induction kinetics)

1. Settle the EBs in a round bottomed 15ml tube (1-3 wells per tube) for 15minutes. Aspirate the medium and add 2mls of *TRYPSIN-EDTA*. Incubate at 37^{0}C in a waterbath for 5 minutes and then stop the reaction with 1ml of *STOP MEDIUM+dnase* .
2. Make to single cells by passing the EBs 4-6x through a syringe bearing a 20 Guage needle and wash with *IMDM+10%FCS* ***(H).***
3. Spin for 5min at 1000 RPM, aspirate and resuspend in *IMDM+FCS* (usually 500ul per well harvested). Pass the cells through a 70micron filter to remove any clumps that are still remaining. You should recover 5x10^5-1x10^6 cells per well of EBs harvested.
4. Stain with the desired antibodies (KDR, c-Kit / SIRPA-PeCy7 / isotype anti-IgG1κ - phycoerythrin-Cy7) according to product datasheets and perform flow cytometric analysis

If Induction Media II (containing DKK) is added to the cultures at Day 3, followed by Noggin/SB at day 4, change media at Day 5 and replace it with Induction Media II (Day 4-day8 media).

T8-T12: Induction 3 (Stage III)

1. Harvest EBS gently using 5 mL pipette and transfer to 14 mL snap cap tube pooling up to 3 wells per tube
2. Allow 10 minutes to settle or centrifuge at 800 rpm for 5 minutes
3. Aspirate supernatant, resuspend EB-pellet in 2mL/well Stage III Induction media
4. Re-plate EBs into a 6 well low cluster dish (2mL/well)
5. Incubate in hypoxic conditions for an additional 4 days

To further increase efficiency SIRPA+ progenitors can be FACS sorted or separated using magnetic beads (Miltenyl). The sorted cells are cultured on gelatin coated dishes at a concentration ~~of …….~~(200'000 SIRPA+ cells per 96 well) in the appropriate stage media depending on the stage of development they were sorted at. For eg. EBs sorted at Day 8 will be cultured in Induction Media III.

Dissociation procedure for day 13 and older EBs. EBs generated from hPSC differentiation cultures were incubated in collagenase type II (1 mg/ml; Worthington) in Hanks solution (NaCl, 136 Mm; $NaHCO_3$, 4.16 mM; $NaPO_4$, 0.34 mM; KCl, 5.36 mM; KH_2PO_4, 0.44 mM; dextrose, 5.55 mM; HEPES, 5 mM) overnight at 25 °C with gentle shaking. On the following day, the equivalent amount of dissociation solution (in Hanks solution: taurin, 10 mM, EGTA 0.1 mM, BSA 1 mg/ml, collagenase type II 1 mg/ml) was added to the cell suspension and the EBs were pipetted gently to dissociate the cells. After dissociation, cells were centrifuged (1,000 r.p.m., 5 min), filtered and used for analysis. For EBs older than 40 d, additional treatment with 0.25% trypsin/EDTA is often required to obtain complete dissociation to single-cell suspensions.

T12-T20 <u>Induction 3 (Stage III continued)</u>

1. Harvest and re-plate EBs in *INDUCTION MEDIA 3* ***(J)*** as described above
2. Incubate cells at ambient oxygen levels for remainder of the experiment (37 °C, 5% CO_2)
3. Harvest and re-feed EBs every 4 days or as needed, until T20
4. Perform cardiac Troponin T staining at Day 20 (see protocol below)

Intracellular Troponin T Staining Protocol

A. Harvest EBs:

1. Harvest EBs from a single well of a 6 well low cluster plate into 14 ml snap cap tube
2. Pellet at 800 rpm for 3 min
3. Add 1 ml Collagenase Type I (containing 10 ug/ml DNAse (10 ul stock DNAse/ml)) and incubate at 37 °C for 1 hr
4. After collagenase treatment, wash EBs with 9 ml of IMDM containing antibiotics
5. Pellet EBs 800 rpm for 5 min
6. Aspirate supernatant and incubate EBs in 2 ml trypsin at 37 °C for 5 minutes
7. Quench with 1 ml STOP solution (1:1 ratio of FCS and IMDM) containing 30 ug/ml DNAse (30 ul stock DNAse/ml)
8. Dissoicate EBs by drawing the EB suspension into a 20 gauge syringe 3-6 times
9. Wash the cell suspension with 7 ml IMDM
10. Pellet the cells at 1500 rpm for 5 minutes
11. Resuspend cell pellet in 1 ml IMDM+3%FCS
12. Count cells and monitor cell suspension to ensure that the EBs have been adequately dissociated into single cells

<u>Staining for intracellular TroponinT (use between 100,000-300,000 cells)</u>

1. Transfer 300,000-500,000 cells to 96 well plate (include extra samples for proper controls)
2. Pellet at 2000 rpm for 2 minutes in a swinging bucket centrifuge
3. Wash cells in 200ul IMDM+3%FCS. Pellet as above
4. Fix cells in 200 ul of 4%PFA (diluted in PBS containing Ca and Mg) and for 30 minutes at room temperature
5. Pellet fixed cells at 2000 rpm for 2 minutes
6. Carefully aspirate supernatant and dispose of in a PFA waste container
7. Wash the fixed cells in 200 ul Wash Solution (PBS + 5% FCS)
8. Pellet cells as above
9. Permeabilize the cells using 100ul Permeablization/Staining Buffer (0.5% saponin in PBS+5%FCS) for 10 minutes at room temperature
10. Stain the cells accordingly to suppliers recommendations (30 minutes on ice)

a. Anti-cTnT: clone 13-11 LabVision Neomarkers MS-295 at 1:100
b. Anti-IgG1 isotype control at 1:100

11. Pellet cells at 2000 rpm for 2 minutes, wash cells pellet once with 200 ul Permeabilization buffer to remove excess unbound primary antibody
12. Pellet as above, resuspend cells in 100 ul Permeabilizaton/Staining Buffer
13. Stain cells with secondary antibody according to suppliers recommendations for 30 minutes at room temperature in the dark
14. Wash 1X in Permeablization/Staining buffer and 1X in FACS buffer (PBS+5%FCS).

Troubleshooting:

References:

Yang L, Soonpaa MH, Adler ED, Roepke TK, Kattman SJ, Kennedy M, Henckaerts E, Bonham K, Abbott GW, Linden RM, Field LJ, Keller GM. Human cardiovascular progenitor cells develop from a KDR+ embryonic-stem-cell-derived population. Nature. 2008 May 22;453(7194):524-8.

Kattman SJ, Witty AD, Gagliardi M, Dubois NC, Niapour M, Hotta A, Ellis J, Keller G. 2011. Stage-specific optimization of activin/nodal and BMP signaling promotes cardiac differentiation of mouse and human pluripotent stem cell lines. Cell Stem Cell. Feb 4;8(2):228-40.

Nicole C Dubois, April M Craft, Parveen Sharma, David A Elliott, Edouard G Stanley, Andrew G Elefanty, Anthony Gramolini & Gordon KellerSIRPA is a specific cell-surface marker for isolating cardiomyocytes derived from human pluripotent stem cells. 2011. Nature Biotechnology, 29, 1011–1018 (2011)

Acknowledgements:
Special thanks to Marion Kennedy for putting this protocol together.