Chapter 5: Cardiomyocyte differentiation of human pluripotent stem cells

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OVERVIEW

Human cardiomyocytes differ significantly from those of rodents, most obviously in their electrophysiological properties. For this reason, many potential biomedical applications of cardiomyocytes would benefit from using human rather than rodent cells. This is of growing interest as new opportunities arise for using induced pluripotent stem cells (iPSCs) carrying cardiac disease-associated mutations to understand the pathophysiology of heart disease and to develop new treatment modalities.

There is an obvious need for robust and efficient cardiac differentiation protocols for human pluripotent stem cells, preferably under defined, serum free conditions. Although protocols that are effective on multiple pluripotent cell lines are beginning to emerge, none has yet been shown to be universally applicable without individual optimization, and most still require a cell aggregation step to initiate differentiation. More than 10 years of research on human embryonic stem cells (hESCs) has, however, provided important clues as to which parameters are candidates for further improvement. Many of the recent methodological advances made using hESCs are proving to be transferable to human iPSCs with minor modifications. Thus, it is now possible to produce cardiomyocytes from most hESC and hiPSC lines, although the efficiencies with which individual cell lines differentiate can still be highly variable.

Most protocols mimic in some way the signals that take place during fetal heart development. Among the first was one that recapitulated signals from endoderm adjacent to the developing heart: mechanically passaged hESC colonies were co-cultured with a mouse visceral endoderm-like cell line (END2) in serum-free culture medium. This method is effective in most hESC and hiPSC lines and will yield 5–15% cardiomyocytes depending on the individual line. This technique provides a simple way to check for cardiomyocyte differentiation capacity of multiple hiPSC clones while they are still at very early (mechanical passage) stages of development. For producing larger numbers of cardiomyocytes from pluripotent cells already adapted to enzymatic passage on feeder cells, however, a more effective method is based on centrifuging the cells to force them to make aggregates, which are called “Spin embryoid bodies”. Human pluripotent cells have less inclination to form aggregates spontaneously in suspension than mouse pluripotent cells and forced aggregation makes cells attach more firmly to one another. In the cardiomyocyte differentiation protocol, the aggregates do not become true embryoid bodies, since they are not cultured long enough to induce all three germ layers.

In this chapter we outline a basic protocol for the differentiation of hESCs into cardiomyocytes. The method is based on the formation of aggregates of a standard size using a fixed number of undifferentiated cells in each aggregate. The aggregates are formed by centrifugation of the cells into embryoid body (EB)-like structures and are referred to as “Spin EBs”. Key to the procedure, and most difficult, is the prior adaption of the undifferentiated cells to a standardized single cell passaging bulk culture system in which cells are passaged enzymatically and grown on feeder layers. The method we recommend for making spin EBs after bulk culture adaption is straightforward and has worked well in our hands for multiple hESC lines and several hiPSC lines. The method requires titration of growth factor concentrations for each line to optimize efficiencies. We also discuss alternative methods and reagents that work but are not routinely used in our laboratories. In addition, we discuss aspects of the protocol that could be further optimized. The key variables that we outline in this chapter are:

- Basal culture conditions
- Adaption to bulk passage
- Generating spin “embryoid bodies” (Spin EBs)
- Growth factor titration curves
- Dissociation of EBs and replating cardiomyocytes.

While optimizing and standardizing conditions, it is important to keep in mind that changing one reagent or parameter in the system may unexpectedly impact the outcome of the entire protocol.

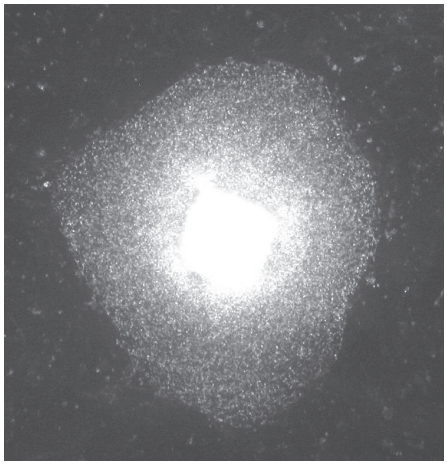

Figure: 1
Example of a healthy looking hESC colony with a small amount of differentiation in the center of the colony.

Procedures

Basal Culture Conditions

In our laboratory, many of our stock hESC lines (e.g. HES2, HES3, HES4, NL-HES1) are grown and maintained as mechanically passaged "cut and paste" colonies in KnockOut Serum Replacement-based medium on irradiated or mitomycin C-treated mouse embryonic feeder cells (MEFs). The colonies are cut into small pieces using glass needles and, using a pipette, are transferred to new feeder layers once weekly. See Chapters 1 and 2. For details see Mummery et al., 2007.

From Mechanical Colonies to Bulk Culture: Tips for Successful Adaption Prior to Generating Spin EBs

Several of the hESC lines we use are routinely passaged enzymatically, twice weekly on MEFs using TrypLE Select. These cells can be used immediately in the spin EB protocol for cardiomyocyte differentiation. For cell lines that are not enzymatically passaged, the spin EB protocol requires that the undifferentiated colonies be adapted to single cell enzymatic passage on feeder layers (referred to as bulk culture).

It is important to select hPSC lines for adaption that have been well-maintained with timely medium refreshment and regular mechanical passaging. The colonies should look healthy with little debris and few differentiated cells around the colony edges (Figure 1).

For best results use 80–100 mechanically-passaged colonies per line to start bulk adaption of a line.

MEFs are inactivated by prior irradiation at 3000 rads in suspension culture.
Plate irradiated MEFs the day before bulk culture adaption to allow attachment and spreading.

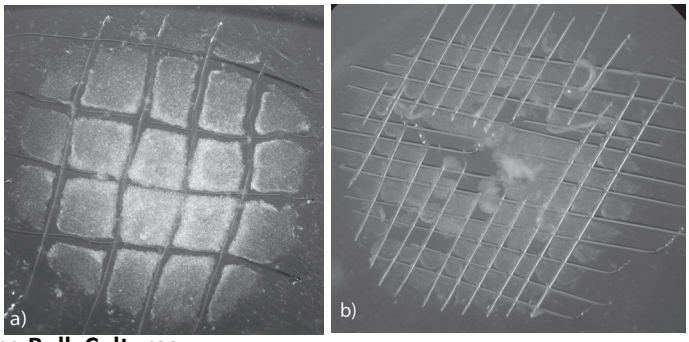

Figure: 2
(A) Example of the size of pieces for mechanical passaging (pieces approximately 400–500 µm in length). (B) Example of grid made for bulk culture initiation (pieces approximately 100–150 µm in length).

Starting Bulk Cultures

1. Cut away any residual differentiated parts of the colonies. These are usually the flat cells around the edge and the cluster at the center.
2. Cut the colonies into extremely small square pieces in a grid (cut closely spaced lines first in one direction over the whole colony then similarly spaced lines in the perpendicular direction) with standard tungsten or glass needles. Note that the squares should be much smaller than those generated during routine mechanical passaging (Figure 2).
3. Lift the cut pieces from the dish using a p1000 pipette and place in a 15 mL tube.
4. Collect all of the pieces from a total of 80–100 colonies.
5. Centrifuge for 3 minutes at 250 *g*.
6. Aspirate medium and resuspend in HESC-medium using a p1000 to pipette colony pieces up and down gently several times.
7. Plate all of the pieces in a T25 flask seeded the day before with 2-3 x 10^4 irradiated MEFs (density of MEFs depends on mouse strain used to produce MEFs and should be optimized empirically) per cm2 with the appropriate amount of HESC-medium.
8. Gently passage the cells in the flask 4 days later in a 1:1 ratio by incubation in TrypLE Select and resuspend in hESC-medium to spread the small colonies, which tend to pile up, over the surface of the flask (Figure 3).
9. The cultures should be observed and the medium should be changed daily. The cells should be passaged at a low ratio (1:2 or 1:3) on new feeder layers in HESC-medium when the islands of undifferentiated cells start to fill the flask (Figure 4).

- **Note**

This low split ratio should be continued until the cells adapt (possibly up to five or six passages). This depends somewhat on the individual cell line; some lines accept a higher split ratio after only two passages. The cells are then cultured according to the procedure below (see Maintaining Bulk Cultures).

Maintaining Bulk Cultures

Bulk cultures should be passaged approximately every 3–4 days as follows:

1. Wash once with phosphate buffered saline (PBS).
2. Add 1–1.5 mL of TrypLE Select to the T25 flask.
3. Place at 37°C for 5 minutes.
4. Tap the flask with fingers to dislodge cells.
5. Resuspend cells in 5 mL of culture medium containing 10% serum and place in a 15 mL tube.
6. Rinse the flask with an additional 3–5 mL of the same serum-containing medium and add to the same tube.
7. Centrifuge for 3 minutes at 250 *g*.

8. Gently resuspend the pellet in the appropriate amount of HESC-medium.
9. Plate down in an appropriate sized flask, plate, or dish pre-plated with
10. 2-3 x 10^4 irradiated MEFs per cm^2.

- **Note**

If the bulk cultures were started using T25 flasks, the flask size can easily be increased for cryopreservation or scaled down to 6-well plates for general maintenance. One well of a 6-well plate contains a sufficient number of cells for the next step of the procedure and reduces the number of MEF feeder cells required at each passage.

Generating Spin EBs

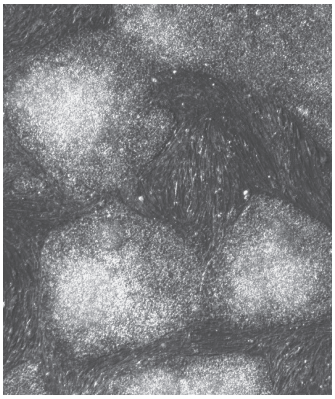

Figure 3:
Example of a bulkculture after 4 days, just before the first passaging.

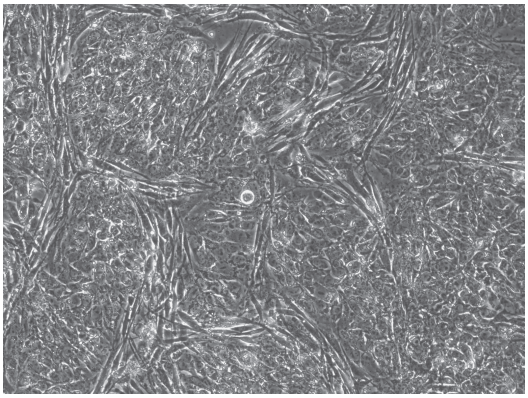

Figure 4:
Example of bulk culture that has been adapted and is ready to be passaged.

One Day Before Starting the Spin EB Differentiation Protocol

Deplete cultures of MEF feeder cells by passaging the bulk cultures and plating on to Matrigel-coated dishes or flasks (ratio about 1:2/1:3 or about 8 x 10^5 cells per well of a 6-well plate) (Figure 5). The following protocol uses one well of a 6-well plate.

Day 0

1. Early morning on the day of differentiation: refresh the medium on the bulk culture in the well of the 6-well plate to maintain culture quality and wash away dead cells.
2. Prepare the growth factor solutions prior to harvesting the cells.
3. Midday on the day of differentiation, start harvesting the cells by washing the culture once with PBS.
4. Add 1 mL of TrypLE™ Select (this is for one well of a 6-well plate; adjust as necessary to the size of culture flask, dish, or well) and incubate for 5 minutes at 37°C.
5. Add 3 mL (adjusting again as necessary to the size of culture flask, dish, or well) of serum-containing medium to collect cells.
6. Wash the flask again with 3 mL of serum-containing medium to collect remaining cells.
7. Centrifuge for 3 minutes at 250 *g*.
8. Resuspend cells in 2 mL of BPEL medium. BPEL medium is basal medium with BSA, Polyvinylalcohol (PVA), and Essential Lipids (see Recipes).
9. Centrifuge for 3 minutes at 250 *g*.
10. Resuspend cells in BPEL medium (1 mL per well of 6-well plate).
11. Count cells.

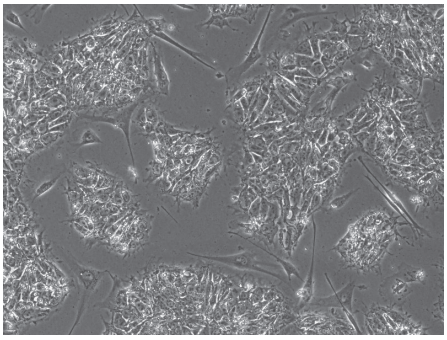

Figure 5:
Bulk cultures one day after plating on Matrigel™ for MEF feeder cell depletion.

12. Plate cells at a final density of 3000 cells in 50 µL of BPEL medium plus growth factors in each V-shaped well of a 96-well plate from a stock solution containing the required number of cells in the correct volume for all wells to be seeded.

- **Note**

For a complete plate only use the 60 inner wells and add 100 µL of PBS to the outer wells to compensate for any evaporation of culture medium.

13. Centrifuge plate for 3 minutes at 450 *g* to form aggregates (Spin EBs) (Figure 6A).
14. Place in the incubator at 37°C for 3 days.

Day 3

15. Remove the BPEL medium containing growth factors and add 100 µL of growth factor-free BPEL medium.

Day 7

16. Plate the Spin EBs in BEL medium (BPEL without PVA) on 0.1% gelatincoated 96-well plates with flat-bottomed wells (Figure 6B).

- **Note**

For practical reasons BPEL can also be used but PVA is not needed after 7 days for the continuous differentiation of the EBs.

Day 8

17. Begin checking for beating areas in the cell aggregates under a phase contrast microscope (203 objective) (Figure 6C).

- **Note**

Beating may begin up to 20 days later, depending on the cell line. HES3 Spin EBs usually start to beat around day 8 and reach a maximum at day 12 (that is, all EBs containing cardiomyocytes are beating). Note that EBs that do not beat visibly do not usually contain cardiomyocytes if stained retrospectively with an antibody against sarcomeres.

18. After 14 days, refresh wells with BEL or BPEL medium once a week.

Growth Factor Titration Curves

Titration of Activin A and BMP4 is usually needed to determine the optimal concentrations for the induction of beating cells. Titration will be required for each new batch of growth factors (which are supplied on the basis of protein concentration and not specific activity) and each new cell line or clone:

	Final (ng/mL)	Stock (ng/µL)
BMP4	Determine using titration	30
VEGF	30	50
SCF	40	40
Activin A	Determine using titration	25

One way in which a titration can be done is to test new growth factor batches or cell lines in the middle 60 wells (6 wells by 10 wells) of a 96-well plate. Vary the concentration of Activin A (0–30 ng/mL) in one direction and BMP4 (0–30 ng/mL) in the other direction. Duplicate rows and columns so that there are multiple samples of each combination.

Constant concentrations of VEGF (30 ng/mL) and SCF (40 ng/mL) are present in the Spin-EB medium. PVA is an essential component and should not be omitted (Figure 7).

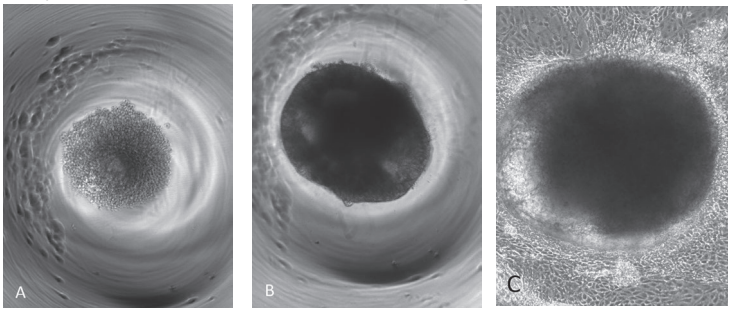

Figure 6:
(A) Example of Spin EB in 96 well V-wells at day 0 after centrifuging. (B) Example of spin EB in V-wells on day 7 (C) Example of Spin EB after plating onto gelatin on day 8.

Dissociation of Beating Spin EBs for Experimentation on Single Cardiomyocytes

1. Wash Spin EBs with PBS.
2. Add TrypLE Select for 20 min at 37°C (this depends on the size of the EBs, days of differentiation, etc.). Break up the EBs by gentle pipetting.

- **Note**
  The time needed for dissociation increases with the age of the EBs and thus the time mentioned above should be monitored.

3. Neutralize the enzyme by resuspending cells in serum-containing medium and place in a 15 mL tube.

- **Note**
  Pipette up and down vigorously (maximum three times) if clumps of EBs are still visible

4. Centrifuge for 3 minutes at 250 *g*.

- **Note**
  For use in FACS, begin standard FACS staining protocol after this step.

5. Resuspend in BEL medium containing 5 µM Rock Inhibitor.
6. Plate dissociated cells on the appropriate 0.1% gelatin-coated cell culture treated dish, plate, or flask.

- **Note**
  5000 cells per cm2 gives a nice spread of single cells although the ideal cell density depends on the subsequent experiments that will be carried out. Coating dishes, plates, or flasks with other ECM materials (e.g. Matrigel or fibronectin) may improve attachment in some cases.

7. Refresh medium, removing the Rock Inhibitor 24 hours later.

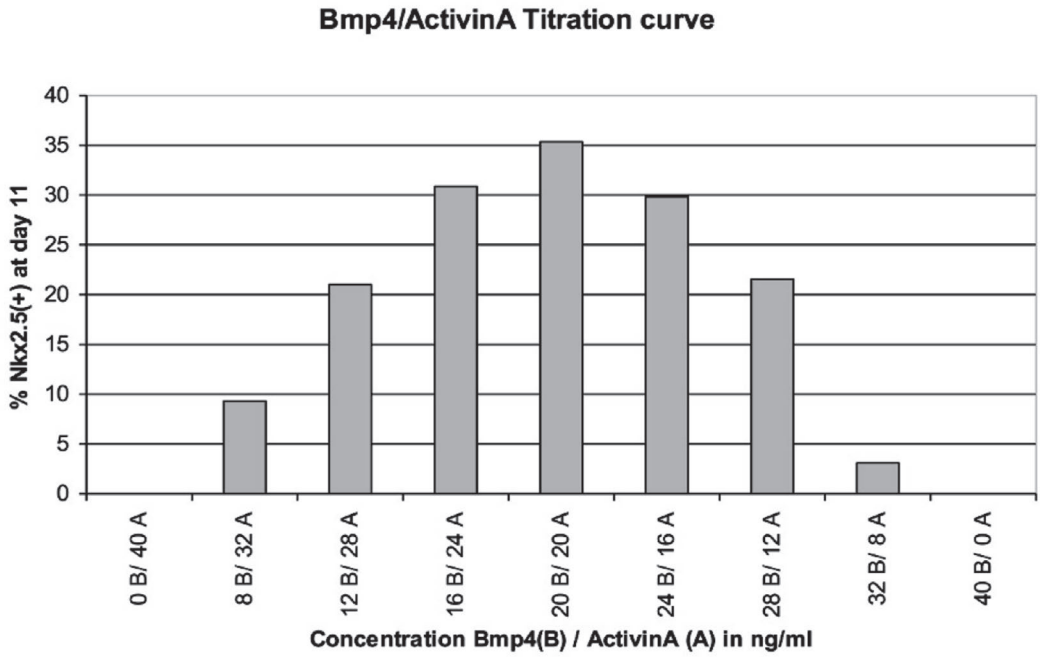

FIGURE 7
The outcome of a typical titration curve of BMP4 and Activin A, using HES3 cells, showing optimal BMP4/ Activin A concentrations of 20 ng/mL for each. Higher Activin A concentrations in HES3 lead to endoderm formation. Lower Activin A and higher BMP4 concentrations lead to other mesoderm derivatives like blood. Here, the number of Nkx2.5 1 cells present was determined by FACS analysis of GFP (NKX2.5-GFP-Hes3 line [Elliot et al., 2011], but it may also be done using an Nkx2.5 antibody) in a single cell suspension (dissociated beating Spin EBs, see protocol below). For other cell lines, similar titration experiments showed concentrations as low as 5 ng/mL for these factors to be optimal for inducing cardiomyocyte differentiation.

ALTERNATIVE PROCEDURES

Use of APEL Instead of BPEL

The use of Bovine Serum Albumin (BSA) in the BPEL medium has some drawbacks, including batch-to-batch variation and the fact that it is an animal product. Replacing BSA with human recombinant albumin may decrease the batch-to-batch variation observed with BSA. Human recombinant albumin may also produce higher efficiencies of differentiation. However, human recombinant albumin is much more expensive than BSA. Stem Cell Technologies™ also supplies pretested APEL medium on a commercial basis.

PITFALLS AND ADVICE

Defined Media for hESC Cultures

The use of defined media for culture of hESCs is ideal for reducing the dependence on MEFs, eliminating the unknown components they produce and reducing variability in cultures over time. There are two commercial media, mTeSR and Nutristem, which we have used to grow hESC cultures under defined, feeder-free conditions for up to 20 or so passages. Long-term passage in either media may be more likely to cause earlier karyotypic abnormalities compared to MEF-based mechanically passaged cultures because it is thought that the total number of cell divisions in a given time period may be higher in defined media. In addition, the potential for subsequent differentiation using other protocols can vary depending on the media used, possibly due to the differences in media components. For example, higher bFGF levels in mTeSR have been found by some to impact the efficiency of differentiation to cardiomyocytes and endoderm derivatives.

Other Factors Influencing Differentiation Efficiency

While we have optimized the Spin EB method using specific growth factors, there are a number of factors which could still be added to help improve efficiency further. These include the addition of ascorbic acid or small molecular inhibitors of transforming growth factor β receptors. The presence of Wnts or Wnt inhibitors has also been reported to enhance cardiomyocyte differentiation if present during the earliest phases of mesoderm formation. Efficient nascent mesoderm formation is a prerequisite for subsequent patterning to cardiogenic mesoderm.

EQUIPMENT

- Tissue culture hood: Class II A/B3
- Tissue culture incubator, 37°C, 5% CO2, in humidified air
- Inverted phase/contrast microscope with 43, 103 and 203 objectives
- Centrifuge, 250 to 450 *g*
- Water bath, 37°C
- Pipettes, such as Eppendorf p2, p20, p200, p1000, multichannel
- Pipette Aid, automatic pipettor for use in measuring and dispensing media
- Aspirator in the hood, with flask
- Eight-channel plastic aspirator
- Roller bank
- Refrigerator, 4°C
- Freezers:-20°C, -80°C

REAGENTS AND SUPPLIES

Supplies

- 5 mL, 10 mL, 25 mL sterile disposable pipettes
- 6-well culture dishes
- T25 culture flask
- 96-well Greiner bioone V-shaped bottom low attachment tissue culture plates
- 96-well flat-bottomed tissue culture plates
- 15 mL sterile conical tubes
- 50 mL sterile conical tubes
- Sterile 9” Pasteur pipettes (autoclaved)
- Pipette tips for Eppendorf or similar pipette (use blue and yellow low attachment polypropylene pipette tips from Corning)
- Hemocytometer
- 0.22 µm Stericup filtration unit
- Other filters and syringes
- Reagent reservoir, optional

Recommended Reagents

Item	Supplier	Catalog #
Basal medium		
DMEM/high glucose (Dulbecco's Modified Eagle's Medium)	Life Technologies	11960-044
DMEM/F12 1:1 (GlutaMAX, no HEPES)	Life Technologies	31331-028
IMDM, L-glutamine, 25 mM HEPES, no phenol red (Iscove's Modified Dulbecco's Medium)	Life Technologies	21056-023
F12 Nutrient Mixture (Ham), GlutaMAX	Life Technologies	31765-027
Serum components		
Knockout Serum Replacement (contains bovine products)	Life Technologies	10828-028
Fetal Bovine Serum (FBS) (batch selected)	Sigma-Aldrich	F7524
L-Glutamine 200 mM	Life Technologies	25030-024
GlutaMAX™-I Supplement 200 mM	Life Technologies	35050-038
MEM-Non-essential amino acids 100x	Life Technologies	11140-035
2-Mercaptoethanol 50 mM (1000x)	Life Technologies	31350-010
D-PBS (Dulbecco's Phosphate- Buffered Saline without Calcium and Magnesium)	Life Technologies	15230-089
Distilled H_2O	Invitrogen	15230-089
Basic FGF (FGF-2) Recombinant human	PeproTech	100-18B
Albumin from bovine serum, powder	Sigma	A3311
Resin Beads	Bio-Rad	142-6425
Poly(vinyl alcohol) (PVA)	Sigma-Aldrich	P8136
Recombinant Human VEGF 165	R&D systems	293-VE
Recombinant Human SCF	PeproTech	300-07
Recombinant Human/Mouse/Rat Activin A	R&D systems	338-AC
Recombinant Human BMP-4, CF	R&D systems	314-BP
Fasudil, Monohydrochloride Salt, >99%	LC Laboratories	F-4660
BD Matrigel Matrix Growth Factor Reduced (GFR)	BD Biosciences	354230
Chemically Defined Lipid Concentrate	Invitrogen	11905-031
TrypLE Select (1x)	Invitrogen	12563-029

1-Thioglycerol	Sigma-Aldrich	M6145
Insulin-Transferrin-Selenium-X Supplement (100x)	Invitrogen	51500-056
Gelatin from porcine skin	Sigma-Aldrich	G1890
PFHM II: Protein free Hybridoma medium 1x	Invitrogen	12040-051
L-Ascorbic acid 2-phosphate	Sigma-Aldrich	A8960
Pen/Strep (100x)	Life Technologies	15070-063

RECIPES

- **Note**

Glassware should be dedicated to tissue culture use only. If glassware is to be used instead of pre-sterilized plasticware, do not expose bottles to detergent.

Stock Solution Human Basic FGF (bFGF) 100 µL

Component	Amount	Stock Concentration
Human bFGF	10 µg	100 ng/µL

1. Dissolve 10 µg of human basic FGF in 10 µL of 5 mM Tris (according the product sheet).
2. Add 90 µL of PBS +0.1% BSA.
3. Aliquot in 5, 10 and 25 µL samples.
4. Store thawed aliquots at 4°C for up to 2 weeks.
5. Store frozen aliquots at -80°C (stable for up to 12 months).

Stock Solution 10% Deionized BSA 45 mL

Component	Amount	Stock Concentration
10% Deionized BSA	4.5 g	10% w/v in IMDM

1. Dissolve 4.5 *g* in 45 mL of IMDM at 37°C in a 50 ml tube.
2. Vortex to help mix.
3. After completely dissolved, add Bio-Rad Resin Beads in excess (.2 grams).
4. Set rolling at 4°C on a roller bank until beads turn yellow (beads aresaturated).
5. Remove the supernatant and place in a new Falcon tube.
6. Add new beads and set rolling on a roller bank at 4°C.
7. Repeat until beads remain blue.

Stock Solution 5% PVA 50 mL

Component	Amount	Stock Concentration
PVA	2.5 g	5% PVA in dH2O

1. Dissolve 2.5 *g* in 25 mL of dH2O (tissue culture qualified).

- **Note**

Add the powder to the dH2O and move the tube while adding the PVA powder).

2. Mix slightly.
3. Set rolling at 4°C on a roller bank until completely dissolved and adddH2O up to 50 ml (can take up to 2 days).

Stock Solution 1-Thioglycerol 10 mL

Component	Amount	Stock Concentration
1-Thioglycerol	130 µL	0.15 M

1. Add 130 µL of 1-Thioglycerol to 10 mL IMDM (making these larger volumes of 130 µL is more accurate than smaller volumes because the Thioglycerol is highly viscous).

Stock Solution BD Matrigel Matrix Growth Factor Reduced (GFR) 10 mL

Component	Amount	Stock Concentration
BD Matrigel Matrix Growth Factor Reduced (GFR)	10 mL	Undiluted

1. Thaw 10 mL stock overnight on ice and place in icebox at 4°C. Place Eppendorf tubes and pipette tips at -20°C.
2. Aliquot stock into 100, 200, and 500 µL samples on ice in ice-cold Eppendorf tubes and store at -20°C for up to 3 months.

For use in coating plates:

3. Thaw 100 µL aliquot on ice.
4. Using cold pipettes and medium add 100 µL of Matrigel to 10 mL of DMEM/F12 basal medium.
5. Using a cold pipette, add 2 mL of medium to each well of a 6-well plate.
6. Leave at room temperature for at least 45 minutes.
7. Plates can be used immediately or stored for 2 weeks at 4°C.

- **Note**

Stored plates should be left out at room temperature for 45 minutes before use to allow them to warm up.

Stock Solution Recombinant Human VEGF 165 200 µL

Component	Amount	Stock Concentration
Recombinant Human VEGF 165	10 µg	50 ng/µL

1. Centrifuge prior to opening.
2. Dissolve in 200 µL of dH2O (according to the product sheet).
3. Aliquot in 5 and 10 µL samples.
4. Store thawed aliquots at 4°C for up to 1 week.
5. Store frozen aliquots at 280°C (stable for up to 12 months).

Stock Solution Recombinant Human SCF 250 µL

Component	Amount	Stock Concentration
Recombinant Human SCF	10 µg	40 ng/µL

1. Centrifuge prior to opening.
2. Dissolve in 250 µL of dH2O (according to the product sheet).
3. Aliquot in 5, 10, and 20 µL samples.
4. Store thawed aliquots at 4°C for up to 1 week.
5. Store frozen aliquots at -80°C (stable for up to 12 months).

Stock Solution Recombinant Human/Mouse/Rat Activin A 400 µL

Component	Amount	Stock Concentration
Recombinant Human/Mouse/Rat Activin A	10 µg	25 ng/µL

1. Centrifuge prior to opening.
2. Dissolve in 400 µL of PBS +0.1% BSA (according to the productsheet).
3. Aliquot in 5, 10, and 20 µL samples.
4. Store thawed aliquots at 4°C for up to 1 week.
5. Store frozen aliquots at -80°C (stable for up to 12 months).

Stock Solution Recombinant Human BMP4 330 µL

Component	Amount	Stock Concentration
Recombinant Human BMP-4, CF	10 µg	30 ng/µL

1. Centrifuge prior to opening.
2. Dissolve in 330 µL of PBS10.1% BSA (according to the productsheet).
3. Aliquot in 5 and 10 µL samples.
4. Store thawed aliquots at 4°C for up to 1 week.
5. Store frozen aliquots at -80°C (stable for up to 12 months).

Stock Solution Fasudil, Monohydrochloride Salt, >99% 5 mL, ROCK Inhibitor

Component	Amount	Stock Concentration
Fasudil, Monohydrochloride Salt, >99%	8.15 mg	5 mM

1. Add 8.15 mg to 5 mL of dH2O.
2. Mix until dissolved.
3. Filter using 0.22 µm.
4. Aliquot in 5 and 10 µL samples.
5. Store at -20°C (stable up to 3 months).

Stock Solution "Knockout Serum Replacement" (Invitrogen 10828-028)

This product can be kept (after thawing) at 4°C for 1 week.

1. After 1 week freeze aliquots into appropriate volumes in 50 mL tubes andstore at 220°C.
2. Thaw at 37°C just prior to use.

Stock Solution L-Glutamine (200 mM) (Invitrogen 25030-081)

L-glutamine is unstable and must be stored frozen.

1. Thaw the bottle completely and aliquot in 10 mL tubes. Store at -20°C.
2. Thaw a tube just prior to use.
3. Do not refreeze tube, store at 4°C and discard unused glutamine after 2 weeks.

HESC Medium 100 mL

Component	Amount	Stock Concentration
DMEM/F12 1:1 (GlutaMAX, no HEPES)	78 mL	
KnockOut Serum Replacement	20 mL	20%
100x MEM-Non-Essential amino acids	1 mL	10 mM
50 mM 2-Mercaptoethanol	200 µL	0.1 mM
Human bFGF (100 ng/µL)	10 µL	10 ng/mL
Pen/Strep (100x)	0.5 mL	1:200

1. Prepare all media in the tissue culture hood using aseptic techniques.
2. Combine the basal medium and all the components except bFGF in the filter top. Filter sterilize using a 0.22 µm Stericup filtration unit.
   Add 10 ng/mL bFGF after filtering.
3. Store at 4°C for up to 2 weeks.

BPEL Medium: Bovine Serum Albumin (BSA) Polyvinylalchohol Essential Lipids 100 mL

Component	Amount	Stock Concentration
IMDM, L-glutamine, 25 mM HEPES, no phenol red (Iscove's Modified Dulbecco's Medium)	42.6 mL	
F12 Nutrient Mixture (Ham), GlutaMAX	42.6 mL	
PFHM II: Protein free Hybridoma medium 1x	5 mL	1:20
10% Deionized BSA in IMDM	2.5 mL	0.25% w/v
5% PVA in dH2O	2.5 mL	0.125% w/v
Chemically Defined Lipid Concentrate	1 mL	1:100
Insulin-Transferrin-Selenium-X Supplement (100x)	100 µL	1:1000
0.15 M 1-Thioglycerol	300 µL	450 µM
5 mg/mL L-Ascorbic acid 2-phosphate	1 mL	0.05 mg/mL
200 mM GlutaMAX-I Supplement	1 mL	2 mM
Pen/Strep (100x)	0.5 mL	1:200

1. Prepare all media in the tissue culture hood using aseptic techniques.
2. Combine all components and filter sterilize using 0.22 µm filter.
3. Store at 4°C for up to 2 weeks.

BEL medium: Bovine Serum Albumin (BSA), Essential Lipids 100 mL

Component	Amount	Stock Concentration
IMDM, L-glutamine, 25 mM HEPES, no phenol red (Iscove's Modified Dulbecco's Medium)	42.6 mL	
F12 Nutrient Mixture (Ham), GlutaMAX	42.6 mL	
PFHM II: Protein free Hybridoma medium 1x	5 mL	5%
10% Deionized BSA in IMDM	5 mL	0.5% w/v
Chemically Defined Lipid Concentrate	1 mL	1:100
Insulin-Transferrin-Selenium-X Supplement (100x)	1 mL	1:100
0.15 M 1-Thioglycerol	300 µL	450 µM
5 mg/mL L-Ascorbic acid 2-phosphate	1 mL	0.05 mg/mL
200 mM GlutaMAX-I Supplement	1 mL	2 mM
Pen/Strep (100x)	0.5 mL	1:200

1. Prepare all media in the tissue culture hood using aseptic techniques.
2. Combine all components and filter sterilize using 0.22 µm filter.
3. Store at 4°C up to 2 weeks.

Serum-Containing Medium 100 mL

Component	Amount	Stock Concentration
DMEM/high glucose (Dulbecco's Modified Eagle's Medium)	87.5 mL	
FBS	10 mL	10%
100 x MEM-Non-essential amino acids	1 mL	10 mM
200 mM L-Glutamine	1 mL	2 mM
Pen/Strep (100 x)	0.5 mL	1:200

1. Prepare all media in the tissue culture hood using aseptic techniques.
2. Store at 4°C up to 4 weeks

QUALITY CONTROL METHODS

Lot-to-Lot Variability of Reagents

Growth factors are sold on the basis of weight/volume, not specific activity. Effective concentrations of growth factors required for growth and differentiation will then depend on how and how long the reagent has been stored. Thus, be careful to test each lot and record lot numbers.

READING LIST

Co-Culture

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Activin/BMP/Wnt signaling

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Ren, Y., Lee, M.Y., Schliffke, S., Paavola, J., Amos, P.J., Ge, X., et al., 2011. Small molecule Wnt inhibitors enhance the efficiency of BMP-4-directed cardiac differentiation of human pluripotent stem cells. J. Mol. Cell. Cardiol. 51 (3), 280–287.