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High-Throughput Screening Assay for Embryoid Body Differentiation of Human Embryonic Stem Cells

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Abstract

Serum-free human pluripotent stem cell media offer the potential to develop reproducible clinically applicable differentiation strategies and protocols. The vast array of possible growth factor and cytokine combinations for media formulations makes differentiation protocol optimization both labor and cost-intensive. This unit describes a 96-well plate, 4-color flow cytometry-based screening assay to optimize pluripotent stem cell differentiation protocols. We provide conditions both to differentiate human embryonic stem cells (hESCs) to the three primary germ layers, ectoderm, endoderm, and mesoderm, and to utilize flow cytometry to distinguish between them. This assay exhibits low inter-well variability and can be utilized to efficiently screen a variety of media formulations, reducing cost, incubator space, and labor. Protocols can be adapted to a variety of differentiation stages and lineages.

Keywords

human embryonic stem cells; hESCs; differentiation; high-throughput; serum-free differentiation; embryoid body; suspension culture

Introduction

This unit describes a 96-well plate differentiation assay to screen and optimize human embryonic stem cell (hESC) differentiation conditions. hESCs are differentiated in suspension as embryoid bodies (EBs) in serum-free conditions and analyzed with 96-well plate flow cytometry to distinguish germ layer identity and differentiation efficiency. Conditions are provided for differentiation to all three primary germ layers. The first step involves preparing hESCs in culture for differentiation by depleting feeder cells (see Support Protocol 1). Next, in Basic Protocol, adherent hESCs are harvested and seeded as EBs in serum-free differentiation (SFD) conditions. EBs are transferred to 96-well plates and induced to differentiate to one of the three germ layers. At a selected endpoint, EBs are dissociated and cells are analyzed with 96-well plate flow cytometry for surface markers to distinguish germ layer identity between endoderm, mesoderm, and ectoderm.

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This assay is designed to allow numerous differentiation conditions to be screened in a short amount of time while consuming a minimal amount of reagents. With the provided SFD conditions, differentiation efficiencies are consistently high and inter-well variability is remarkably low. Although conditions are provided for the three germ layers, the assay allows a variety of conditions (growth factor concentrations, siRNA molecules, etc.) to be screened in order to investigate the effects upon differentiation.

A 4-color, 96-well flow cytometer is utilized for data acquisition. Four surface markers are provided to distinguish early endoderm, mesoderm, and ectoderm. Depending upon the differentiation pathway and cell types to distinguish, any variety of marker combinations corresponding to the 4 fluorescent channels may be utilized in order to optimize or investigate differentiation.

NOTE: All culture procedures should be performed under sterile conditions. hESC maintenance incubations are performed in a 37°C, 5% CO_2 incubator. hESC differentiations are performed in a 37°C, 5% O_2, 5% CO_2, 90% N_2 incubator.

NOTE: All reagents and equipment coming into contact with live cells must be sterile, and proper aseptic technique should be used accordingly.

Basic Protocol 1 – 96-well Embryoid Body Differentiation

EB differentiation of hESCs in 96-well plates is initiated by harvesting the cells from Matrigel and seeding at desired densities into the well plates. Alternatively, depending upon the most appropriate stepwise protocol, cells can be seeded in suspension into petri dishes or 6-well plates at day 0 and later transferred to 96-well plates. With the appropriately defined medium condition, hESCs can be differentiated into ectoderm, endoderm, or mesoderm (see Fig. 1). Depending upon seeding density, medium should be added/replaced at least every three days. Cells can be harvested and analyzed with 96-well flow cytometry (Basic Protocol 2) at specified time points.

Materials

Cultures of hESCs on Matrigel (approximately 70% confluent is optimal; see Support Protocol 1)

Collagenase B (see recipe)

1 mg/mL DNase I (see recipe)

0.25% Trypsin-EDTA

Serum-free differentiation (SFD) media; see recipe)

Cytokines required for SFD media (see recipe)

0.25% Trypsin-EDTA (1:4 dilution; see recipe)

Stop medium (see recipe)

Iscove’s modified Dulbecco’s medium (IMDM; Invitrogen)

Y26732 ROCK Inhibitor (see recipe)

Cell scrapers

Serological pipets

50-mL conical tubes

96-well ultra low cluster flat bottom plates (Costar cat. no. 3474)

25-mL sterile reagent reservoirs

Multi-channel pipet

12-channel sterile reagent reservoir (Costar cat. no. 4877)

8-channel sterile reagent reservoir (Costar cat. no. 4878)

5-mL round bottom polystyrene tubes

Breathe Easy gas permeable membranes (Diversified Biotech)

Stainless steel micro plate lids (Wako/Kalypsys)

Additional reagents necessary for counting cells (Phelan, 2006; Ungrin et al, 2009)

Differentiation Initiation (day 0)

EB differentiation can be initiated in petri dishes, 6-well plates, or 96-well plates. Depending upon the differentiation strategy, it may be advantageous to seed EBs into petri dishes or 6-well plates and transfer to 96-well plates at a later stage. If such a strategy is adopted, it is necessary to dissociate EBs from a sample of cells to perform a cell count prior to 96-well seeding.

1 Thaw 1 mL collagenase B per well of hESCs on Matrigel in 6-well plates. Add DNase I to a final concentration of 10 μg/mL.

Adding DNase I at this step reduces the tendency for EBs to stick together during differentiation

2 Trypsinize 1 well and count cells (see Preparation of a single-cell suspension of viable hESCs, Ungrin et al, 2009).

3 Prepare day 0 SFD media.

4 Add 1 mL per well collagenase B with DNase I and incubate at 37°C for 20 minutes.

5 Aspirate the collagenase B. Add 1 mL per well 0.25% trypsin-EDTA (1:4 dilution) and incubate at RT for 1.5 minutes.

It is necessary to trypsinize hESCs following collagenase B treatment. The authors have obtained the best results with homemade 0.25% trypsin-EDTA diluted 1:4 in PBS. For other trypsinization procedures of hESCs or EBs within this protocol, commercially made 0.25% trypsin-EDTA is utilized.

6 Aspirate trypsin-EDTA. Add 1 mL Stop Medium.

7 Scrape cells to detach from bottom of well.

8 Add 1 mL per well IMDM to rinse.

9 Transfer cells to 50-mL conical tube and spin at 110 × *g* for 3 min at 22°C.

10 Remove the supernatant.

11 Resuspend in Day 0 SFD medium to give the desired cell concentration. Pipet the clumps up and down 2–3 times as needed to break up large clumps.

See Table 1 for germ layer-specific supplementation recipes

It is advisable to perform flow cytometry on day 0 cells to ensure expression of pluripotency markers (e.g. SSEA-3, SSEA-4). This can

either be done with a separate well designated for this purpose or with an aliquot taken at this step.

The optimal seeding density will depend upon the cell line and differentiation medium. A typical 96-well seeding density for hESCs is 2×10^4 cells per well. Thus, assuming that cells are seeded in 100 μL differentiation medium, cells should be resuspended at a density of 2×10^5 cells/mL. If cells are to be seeded at day 0 into 10 cm petri dishes, seed at 2×10^6 cells per dish in 15 mL medium.

12 Transfer cells to a 25-mL sterile reagent reservoir.

13 Using a multichannel pipet, transfer 100 μL per well medium to 96-well, ultra low-cluster plates.

EBs will rapidly settle to the bottom of the reservoir. Therefore, a serological pipet should be used frequently to gently resuspend EBs in the medium.

If conditions are being screened across the plate, cells can be seeded in 50 μL of medium. 50 μL of additional supplementation medium can then be added to the wells.

14 Incubate at 37°C in an environment of 5% CO_2, 5% O_2, and 90% N_2.

Differentiation Media Addition

To be performed at defined time points during differentiation. If media needs to be replaced, a multichannel pipet is utilized to withdraw media prior to addition. Media can be uniform across all wells or can be designed to analyze a range of conditions.

15 Prepare germ layer-specific SFD media.

16 Remove Breathe Easy membrane from 96-well plate.

17 Allow EBs to settle and carefully withdraw media as needed from wells using a multichannel pipet and discard.

This step can be omitted if media is only being added to wells.

It is safest to leave at least 100 μL in each well to avoid withdrawing any EBs.

If this step is performed at a later stage of differentiation and single cells must be preserved, the supernatant can be transferred to a V-bottom 96-well plate, centrifuged, and removed, allowing cells to be resuspended in differentiation media and transferred back to the culture plate.

18 Transfer SFD medium to a 25-mL sterile reagent reservoir.

19 Using a multichannel pipet, transfer 200 μL medium from the reservoir to the 96-well plate.

If conditions are being screened across the plate, this method can be modified to accommodate numerous samples. Base medium can be aliquoted to appropriately sized tubes (5 mL round bottom tubes generally suffice), and corresponding supplementations can be added. Media should be transferred to multichannel reagent reservoirs (8-

channel or 12-channel reagent reservoirs) and then added to the 96-plate using a multi-channel pipet.

20 Cover with a Breathe Easy gas permeable membrane and a stainless steel micro plate lid.

21 Incubate at 37°C in an environment of 5% CO_2, 5% O_2, and 90% N_2.

Basic Protocol 2 – Flow Cytometry Analysis

After a defined length of differentiation, EBs are harvested, disassociated, and analyzed with 96-well flow cytometry. Cells can also be utilized for various other analyses, such as PCR, colony assays, etc. After EB dissociation, cells are labeled with primary conjugated fluorescent antibodies according to surface marker profiles designed to distinguish between lineages. Cells are then analyzed with flow cytometry.

Materials

0.25% Trypsin-EDTA

Stop media (see recipe)

IMDM

Cell staining buffer (CSB; Biolegend)

1 mg/mL DNase I (see recipe)

Fluorescent conjugated antibodies

96-well V-bottom plates

8-channel aspirator nozzle

Plate agitator

V-bottom 96-well plates

C6 Flow Cytometer with C-sampler (Accuri)

Multi-channel pipet

Flow cytometry analysis

1 Using a 200–300μL multichannel pipet, withdraw 100 μL media per well and discard.

If this step is performed at later differentiation time points and single cells in suspension are present, supernatant can be transferred to a 96-well V-bottom plate, centrifuged, resuspended, and later combined with disassociated EBs.

2 Transfer remaining solution containing EBs to a 96-well V-bottom plate.

3 Centrifuge at 110 × *g* for 5 minutes.

4 Aspirate supernatant using an 8-channel aspirator nozzle, ensuring not to disrupt the cell pellet.

5 Resuspend cells in 50 μL 0.25% trypsin-EDTA and pipet up and down to ensure resuspension.

6 Incubate at 37°C for 4 minutes, vortexing on plate agitator every 1 minute.

7 Add 50 μL per well stop media.

8 Add 100 μL per well DMEM and pipet up and down 4–5 times to facilitate dissociation of EBs.

9 Centrifuge at 440 × *g* for 2 minutes.

10 Discard supernatant.

11 Resuspend cells in 100 μL cell staining buffer (CSB).

This protocol focuses on labeling surface markers for lineage identification. If intracellular markers are to be labeled, a permeabilization step should be inserted here.

12 Centrifuge at 440 × *g* for 2 minutes and discard supernatant.

13 Resuspend cells in 50 μL per well primary antibody solutions containing 10 μg/mL DNase I.

DNA from disrupted cells causes cells to clump together, resulting in higher tendencies for occlusion during flow cytometry. DNase I added at this step greatly reduces the frequency of this occurrence.

Primary antibodies should be utilized that allow different lineages to be distinguished based upon the available fluorescent channels of the flow cytometer. The 96-well flow cytometer utilized in this protocol has a four-color system. For distinguishing the three primary germ layers and pluripotent stem cells, the authors have defined the following combinations of surface markers (Outten et al., 2011):

Undifferentiated hESCs: SSEA-3^{+}NCAM $^{-}$

Ectoderm: SSEA-3^{+}NCAM $^{+}$

Endoderm: CXCR4^{+}KDR $^{-}$

Mesoderm: KDR^{+}SSEA-3^{-}

Primary antibodies utilized for early stage three germ layer differentiation:

Alexa 488 conjugated anti-SSEA-3 (Biolegend cat no. 330306)

PE conjugated anti-CXCR4 (Invitrogen cat. no. MHCCXCR404)

PE-Cy7 conjugated anti-NCAM (Biolegend cat. no. 318318)

Alexa 647 conjugated anti-KDR (Biolegend cat. no. 338909)

Antibodies should be titrated to determine the optimal concentration. The four antibodies given here can be utilized at 1:200 dilutions in CSB.

14 Incubate in the dark at room temperature for 20 minutes.

15 Add 100 μL CSB, centrifuge at 440 × *g* for 2 minutes, and discard supernatant.

16 Wash once more with 100 μL CSB and resuspend in 80–150 μL per well CSB.

17 Analyze on 96-well flow cytometry unit, pipetting groups of 8–12 wells up and down before flow to ensure complete resuspension of cells.

Support Protocol

Feeder Depletion of Human Embryonic Stem Cells

This step is performed 1–2 days prior to the onset of differentiation in order to deplete hESCs from the MEF feeder cells. Cells should be 70–80% confluent on MEF feeder cells. Higher confluency tends to result in lower viability after seeding.

In the authors' experience, mesoderm differentiation is more sensitive to Matrigel inconsistencies than endoderm. Matrigel should be freshly thawed and kept at 4°C for mesoderm differentiation, whereas endoderm differentiation is more robust to Matrigel that has been allowed to warm to room temperature. To minimize any potentially confounding influence of Matrigel quality, it is best to use freshly thawed Matrigel regardless of differentiation protocol.

The target confluency to harvest cells from Matrigel is 70%. Although viability varies between cell lines, typically, a 1:3 split onto Matrigel results in cells approximately 70% confluent in 1 day. A 1:6 split results in cells ready to harvest in 2 days. It isn't recommended to split at ratios higher than 1:3 due to the resulting high confluency, leading to lower viability upon harvest, after 1 day. Viability is significantly enhanced with the inclusion of Y26732 in the seeding media.

Materials

Cultures of hESC cells on MEF feeder cells (70–80% confluent is optimal)

Matrigel (frozen; diluted 1:3 v/v with IMDM)

hESC maintenance medium (see recipe)

TrypLE Express cell dissociation enzyme (Invitrogen)

IMDM

Y26732 ROCK Inhibitor (see recipe)

6-well tissue culture plates

Cell scrapers

Serological pipets

50-mL conical tubes

Prepare matrigel-coated plates

1. Thaw 1:3 matrigel solution at 4°C overnight (see recipe)
2. Chill 5-mL serological pipet and 6-well plates at −20°C for approximately 10 minutes.
3. Place 6-well plates on ice in culture hood.
4. Pipet 3 mL 1:3 matrigel into the first well of a 6-well plate using the chilled serological pipet, tilting to ensure uniform coverage.
5. Transfer excess matrigel to next well and repeat process.
6. Aspirate any additional excess Matrigel from wells.
7. Incubate plates at 37°C for at least 30 minutes.

Seeding hESCs onto matrigel

8 Dilute Y26732 in hESC medium to a final concentration of 10 μM.

Cells will be seeded in 2 mL per well hESC medium.

9 Aspirate media from hESC plates.

See Costa et al 2009 for expanding hESCs on MEFs.

10 Add 1 mL per well TrypLE Express and incubate at room temperature for 4 minutes.

11 Aspirate TrypLE and wash twice with 1 mL per well IMDM. Aspirate.

12 Add 1 mL per well hESC medium containing Y26732.

13 Using a cell scraper, scrape the cells off of the entire well, ensuring complete removal.

14 Add 1 mL per well hESC medium containing Y26732 to rinse well.

15 Using a 5 mL serological pipet, transfer detached cell clumps to a 50 mL conical tube.

16 Add hESC media containing Y26732 to give 2 mL per well to be seeded. Using a 5-mL serological pipet, pipet the clumps up and down as needed to break up large clumps.

17 Seed cells at 2 mL cell solution per well onto Matrigel coated plates.

18 Incubate cells at 37°C at 5% CO_2 and replace media daily.

Reagents and Solutions

Antibodies

Alexa 488 conjugated anti-SSEA-3 (Biolegend cat. no. 330306)

Alexa 647 conjugated anti-KDR (Biolegend cat. no. 338909)

PE conjugated anti-CXCR4 (Invitrogen cat. no. MHCCXCR404)

PE-Cy7 conjugated anti-NCAM (Biolegend cat. no. 318318)

Antibodies were utilized at 1:200 dilutions in Cell Staining Buffer. Dilutions should be prepared the day of staining. Store antibodies at 4oC for the manufacturer specified durations.

Collagenase B

1L Dulbecco's modified Eagle's medium (DMEM)/F12

1 g Collagenase B (Roche)

Mix to dissolve collagenase B in DMEM/F12 and filter sterilize using a 0.22 μM filtration unit. Store aliquots at −20°C. Thawed solution can be stored for 2 weeks at 4°C.

Cytokines/Growth Factors

Recombinant human basic fibroblast growth factor (bFGF; R&D Systems cat. no. 233-FB)

Reconstitute at 10 to 100 μg/mL in sterile PBS containing at least 0.1% human or bovine serum albumin.

Recombinant human bone morphogenetic protein-4 (BMP4; R&D Systems cat. no.314-BP)

Reconstitute at 10 to 100 μg/mL in sterile 4 mM HCL containing at least 0.1% human or bovine serum albumin.

Recombinant human Activin A (R&D Systems cat. no. 338-AC)

Reconstitute at 10 to 100 μg/mL in sterile PBS containing at least 0.1% human or bovine serum albumin.

Recombinant human vascular endothelial growth factor 165 (VEGF; R&D Systems cat. no. 293-VE)

Reconstitute at 10 to 100 μg/mL in sterile PBS containing at least 0.1% human or bovine serum albumin.

Store reconstituted growth factors at −80°C. Once thawed, store for 1 month at 4°C.

DNase I

DNase I lyophilized powder (Calbiochem)

Reconstitute DNase I in sterile H_2O to a concentration of 1 mg/mL (60,000 Dornase units/mL). Aliquot to 500 μL volumes and store at −20°C for up to 9 months. Thawed aliquots can be stored up to 6 weeks at 4°C.

hESC maintenance media

DMEM/F12 (Mediatech) containing:

15–20% (v/v) Knockout serum replacement (Invitrogen)

100 μM nonessential amino acids (Invitrogen)

10^{-4} M β-mercaptoethanol (Invitrogen)

50 U/mL penicillin (Invitrogen)

50 μg/mL streptomycin (Invitrogen)

2 mM L-glutamine (Invitrogen)

Filter sterilize with a 0.22 μM filtration unit.

10 ng/mL bFGF (R&D Systems)

Store for 2 weeks at 4°C.

0.25% Trypsin-EDTA (1:4 dilution)

To prepare 500 mL, combine

500 mL PBS

1.25 g trypsin from porcine pancreas (Sigma)

1.1 mL 0.5M EDTA (Sigma)

Stir gently, then warm in a 37°C water bath, stirring occasionally, until solution is clear. Filter sterilize using a 0.22 μM filtration unit, aliquot into 125 mL units, and store at −20°C. Upon thawing, dilute 1:4 with PBS.

Inhibitors

Recombinant mouse bone morphogenetic protein receptor 1A (BMPR1A; R&D Systems, cat. no. 437-MR/CF)

Reconstitute to 250 ug/mL in sterile PBS containing at least 0.1% human or bovine serum albumin and store as aliquots at −80°C. Once thawed, store at 4°C for 1 month.

SB431542 (Tocris, cat. no. 1614)

Reconstitute to 40 mM in DMSO and store as aliquots at −80°C.

Y26732 ROCK Inhibitor (Cayman Chemicals, cat. no. 10005583)

Reconstitute to 50 mM in DMSO and store as aliquots at −20°.

Matrigel (1:3 dilution)

Matrigel Basement Membrane Matrix, Growth Factor Reduced (BD Biosciences)

Thaw Matrigel at 4°C, dilute 1:3 (v/v) with IMDM, aliquot into 3 mL volumes, and store at −20°C. Thaw overnight at 4°C prior to plating. Thawed matrigel is good for up to one week when stored at 4°C.

Matrigel formulations vary considerably between lots. Lot specifications should be examined to verify low protein concentration (<10 mg/mL) and low endotoxin (<2 EU/mL).

Serum-free differentiation (SFD) medium

IMDM (Invitrogen) containing:

25% (v/v) F12 (Mediatech)

2 mM L-glutamine

50 U/mL penicillin (Invitrogen)

50 μg/mL streptomycin (Invitrogen)

0.5X N-2 supplement (Invitrogen)

0.5X B27 without retinoic acid (Invitrogen)

0.05% (w/v) BSA (Sigma)

Make in bulk volumes and store up to 2 weeks at 4°C.

On the day of media addition to differentiation culture, prepare germ layer-specific SFD medium as follows:

SFD base medium containing:

50 μg/mL ascorbic acid (Sigma)

4×10^{-4} M monothioglycerol (Sigma)

Filter sterilize using a 0.22 μM filtration unit.

Add cytokines and/or inhibitors as needed (see Table 1)

Stop Medium

Homemade IMDM containing:

50% (v/v) fetal bovine serum (Gemcell)

Store up to 4 weeks at 4°C.

Commentary

Background Information

In an effort to develop clinically applicable therapeutic protocols, studies have begun focusing on ascertaining requisite growth factors required for serum-free differentiation of hESCs (Chase and Firpo, 2007; Kennedy et al., 2007; Vallier et al., 2009). Due to the vast array and high cost of cytokines and inhibitors, reliable techniques that minimize the usage of these consumables are practically advantageous. This system utilizes approximately 8% of the reagents and 6% of the incubator space as standard 6-well cultures. Additionally, several groups have developed 96-well plate differentiation systems in an effort to screen a variety of conditions relatively efficiently (Koike et al., 2005; Ng et al., 2005). This system uniquely allows for 96-well flow cytometry to be performed directly upon samples. The assay, typically providing $0.3–1.0\times10^4$ cells per well for flow cytometry, allows for accurate determination of marker distributions. Additionally, by differentiating numerous EBs per well, paracrine effects may be more similar to the scaled up cultures that will be required to produce differentiated cells in large quantities.

The SFD media defined here for the three germ layers is based upon pathway kinetics identified in prior studies and empirical data. Activin A and bFGF induce endoderm (D'Amour et al., 2006), BMP4 and bFGF induce mesoderm (Yang et al., 2008), and activin/nodal (SB431542) and BMP4 (soluble BMPR1A) inhibition with bFGF induce ectoderm (Chambers et al., 2009).

Any combination of biomarkers with appropriately conjugated fluorochromes can be utilized to assess differentiation outcome in this system. The protocol defined here utilizes combinations of four surface markers to distinguish between SSEA-3^+NCAM$^+$ ectoderm (Park et al., 2008; Pruszak et al., 2007), CXCR4^+KDR$^-$ endoderm (McGrath et al., 1999; Nostro et al., 2008; Yasunaga et al., 2005), KDR$^+$SSEA-3^- mesoderm (Nostro et al., 2008; Sakurai et al., 2006; Yang et al., 2008), and SSEA-3^+NCAM$^-$ undifferentiated pluripotent cells (Enver et al., 2005; Pruszak et al., 2007).

Critical Parameters and Troubleshooting

Differentiation—Post-EB seeding viability is highly dependent upon confluency of hESCs on Matrigel. The authors have obtained best results for confluencies of approximately 75%. Cells become stressed and begin to differentiate, as evidenced by lower expression of pluripotency surface markers, as the confluency approaches 100%. Higher confluencies result in lower viability at day 1.

This protocol includes an alternate day 0 step of seeding cells into a petri dish for 24 hours. Transient BMP4 supplementation has been shown to facilitate the induction of endoderm (Green et al., 2011). Additionally, bFGF helps ESCs to maintain pluripotency (Eiselleova et al., 2009). Initially culturing ESCs for 24 hours in BMP4 with no bFGF increases endoderm and mesoderm differentiation yields. Thus, the overall protocol is more efficiently performed by seeding hESCs into day 0 medium in petri dishes and transferring to 96-well plates on day 1. Alternatively, day 0 cells can be immediately seeded into 96-well plates depending on the desired differentiation strategy.

After harvesting hESCs from Matrigel, EB size is primarily dependent upon the amount of trituration upon resuspension in day 0 medium. Target EBs should be 100–200 cells large.

The authors have found that gentle pipetting 2–3 times upon resuspension with a serological pipet produces reliable EBs. EBs should be clearly visible in the resuspended day 0 medium, but not large. EBs will be disrupted even further during the transfer to 96-well plate, so it is vital to not disrupt EBs too much at this step. After 96-well plate seeding, if wells contain many single cells and EBs are sparse, it is likely due to over-trituration.

One disadvantage to seeding EBs into 96-well plates is the inevitability of having variable numbers of cells per well due to inconsistencies in EB. Profiles for the four surface markers discussed here have been shown to be relatively constant for reasonable variations in cell number (Outten et al., 2011). Seeding density optimization should be performed for different cell lines and for various differentiation media due to the differences in viability.

Surface markers are transiently expressed and frequently common to multiple cell types. Thus, the accuracy of any set of markers utilized to distinguish the three primary germ layers and pluripotent cells is subject to the efficiency and elapsed time of differentiation. The markers defined here have been utilized in prior studies and have been shown to reliably distinguish pluripotent stem cells, ectoderm, endoderm, and mesoderm in hESCs after 6 days of differentiation (Outten et al., 2011). Different cell lines differentiate at different efficiencies (Vallier and Pedersen 2009), and thus it may be necessary to validate potential marker combinations to verify their validity.

EB Harvesting/Flow Cytometry—Many 96-well differentiation protocols will stipulate end point well volumes of 300 μL. Prior to dissociation, EBs should be transferred to 96-well V-bottom plates in order to be centrifuged. Due to volume differences, it may be necessary to discard supernatant prior to EB transfer. This can be performed with a multichannel pipet. Care should be taken to ensure EBs are not removed during this process. It is generally sufficient to leave 150 μL medium in the well to be transferred with the EBs. If single cells in suspension must be preserved for analysis, the supernatant can be transferred to a V-bottom plate, and single cells can be analyzed separately or re-combined with the dissociated EBs following trypsin treatment.

Trypsin-EDTA can cleave certain surface antigens, resulting in inaccurately low or false negative flow cytometry readings. The antibodies given here are robust to trypsin treatment. However, unknown antibodies should be tested to determine sensitivity to trypsin. As an alternative, TrypLE Select (Ng et al 2009), a milder trypsin alternative, or collagenase B (Cerdan et al 2009) can be used to dissociate EBs, although the authors have observed signal-reducing effects associated with the latter.

Inadequate EB dissociation can result in occluding the flow cytometer and inaccurate flow cytometry measurements. The plate should be vigorously agitated every minute during trypsinization to assist dissociation. Additionally, samples should be triturated 4–5 times with a multichannel pipet following the neutralization of the trypsin. EB dissociation techniques have frequently employed syringe-based trituration and passing samples through a cell strainer prior to FACS analysis (Cerdan et al 2009, Ng et al 2009). These techniques are extremely inefficient for 96-well plate based analyses. The authors have obtained highly satisfactory results using the aforementioned methods and supplementing the primary antibody solutions with DNase I.

An inadequate number of cells during flow cytometry would indicate either too low of a seeding density or a differentiation medium that did not promote sufficient viability. Frequent occlusions in the flow cytometer indicate insufficiently dissociated EBs, most often due to insufficient trypsinization, insufficient trituration, or lack of DNase I.

Anticipated Results

Differentiation yields are dependent upon cell type, utilized media, and length of differentiation. Utilizing the media defined here, this system has generated high yields of approximately 80% for endoderm, 80% for mesoderm and over 40% for ectoderm (Outten et al., 2011). A representative mesoderm flow cytometry profile for directed differentiation of H9 cells is given in Figure 2. Target differentiation conditions identified with this assay can be further assessed with more exploratory analyses to verify any potential conclusions suggested by biomarker expression.

Time Considerations

Experiments should be designed to take into account time required for endpoint plate processing and cellular staining and flow cytometry. Processing and staining generally requires 1.5–2.5 hours. 96-well flow cytometry generally requires 1.5–2 hours for an entire 96-well plate, depending upon the cell density, number of desired cells, and flow rate. Low seeding densities and differentiation medium that do not promote sufficient viability result in samples that require excessive amounts of time to analyze.

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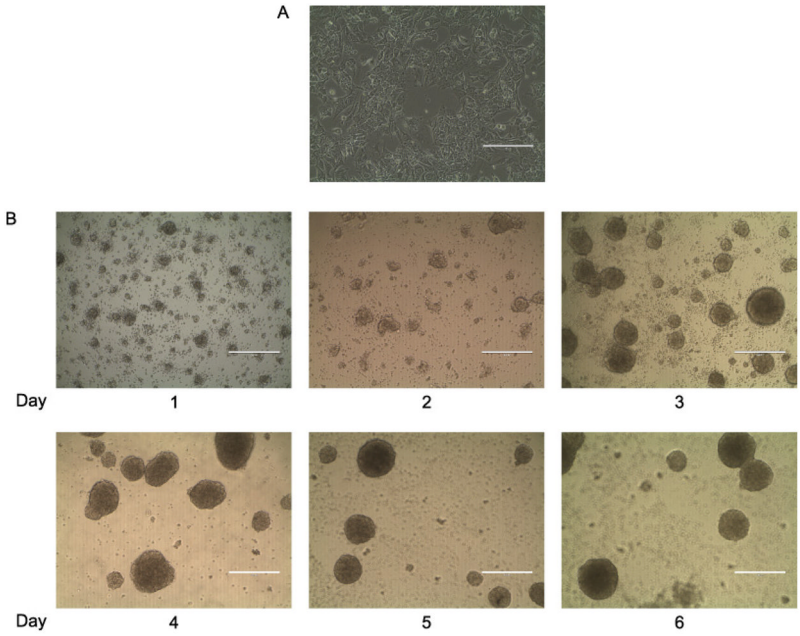

Figure 1.
Endoderm EB differentiation. **(A)** H9 hESCs on matrigel prior to harvesting for day 0 suspension seeding. Scale bar = 400 μm. **(B)** Typical EBs over a 6 day differentiation to endoderm. Scale bar = 200 μm.

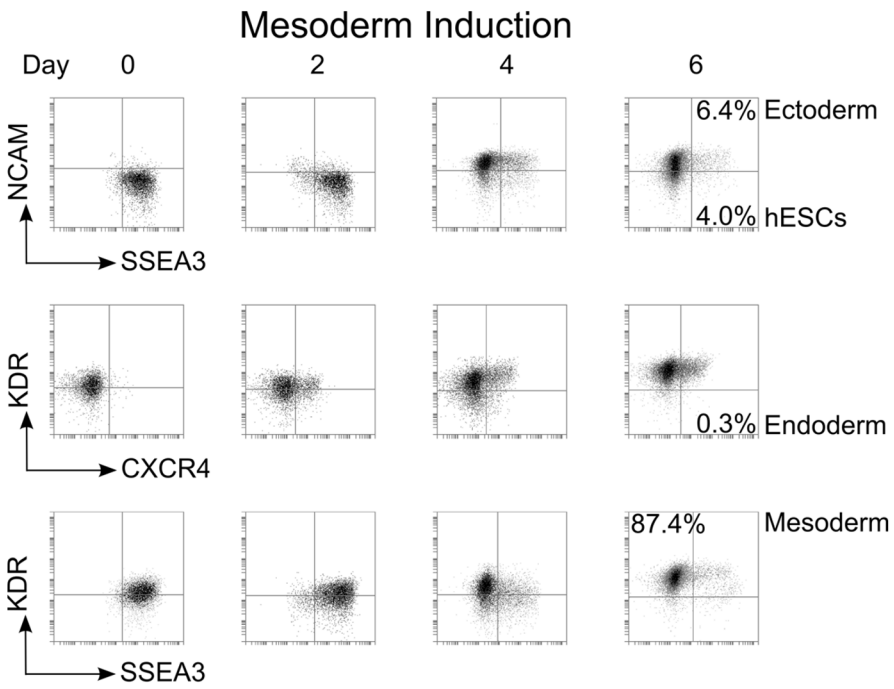

Figure 2.

Representative marker profiles for directed mesoderm differentiation of H9 cells over 6 days. EBs at each time point were dissociated and cells were stained with antibodies corresponding to the indicated surface markers (SSEA-3, CXCR4, NCAM, KDR). Plots show the transition of SSEA-3^{+}NCAM^{-} pluripotent cells to KDR^{+}SSEA-3^{-}mesoderm cells. Ectoderm (SSEA-3^{+}NCAM^{+}) and endoderm (CXCR4^{+}KDR^{-}) percentages are given at day 6 as well (Outten et al., 2011). Stem Cell Research by Elsevier. Reproduced with permission of Elsevier in the format Journal via Copyright Clearance Center.

Table 1

Cytokine/growth factor and inhibitor supplementations for germ layer differentiation media

	Day 0	Day 1	Day 4
Endoderm	• 10 ng/mL BMP4	• 0.5 ng/mL BMP4 • 100 ng/mL Activin A • 2.5 ng/mL bFGF	• 0.5 ng/mL BMP4 • 100 ng/mL Activin A • 5 ng/mL bFGF
Mesoderm	*Identical to Day 0 endoderm*	• 20 ng/mL BMP4 • 0.5 ng/mL Activin A • 2.5 ng/mL bFGF	• 20 ng/mL BMP4 • 0.5 ng/mL Activin A • 5 ng/mL bFGF
Ectoderm	• 500 ng/mL BMPR1A • 10 μM SB431542 • 5 ng/mL bFGF	*Identical to Day 0 ectoderm*	• 500 ng/mL BMPR1A • 10 μM SB431542 • 20 ng/mL bFGF