CReM
CENTER FOR REGENERATIVE MEDICINE
BOSTON UNIVERSITY
BOSTON MEDICAL
670 ALBANY STREET, 2ND FLOOR
BOSTON, MA 02118
TEL: 617-414-2969
HTTP://WWW.CREM.BU.EDU

Human ESC/iPSC Derived Hepatic Cell Directed Differentiation Protocol from Feeder Free Conditions – Wilson Laboratory

INTRO

This protocol describes the methods to derive iPSC Hepatic cells from human iPSCs/ESCs. As published in: Wilson AA et al. Stem Cell Reports. 2015, Kaserman JE et al. Stem Cell Reports 2020, and Kaserman JE et al. Cell Reports 2022.

REAGENTS

A) cSFDM (complete serum free differentiation media):

Volume for 500 ml	Final concentration	Reference
375 ml IMDM	75%	Thermo Fisher (Gibco) 12440053
125 ml Ham's F12	25%	Thermo Fisher (Corning) 10080CV
5 ml B-27 (minus vitamin A) supplement	1%	Thermo Fisher (Invitrogen) 12587010
2.5 ml N-2 supplement	0.5%	Thermo Fisher (Invitrogen) 17502048
3.3 ml BSA (7.5% stock)	0.05%	Thermo Fisher (Invitrogen) 15260037
1 ml Primocin (50 mg/ml stock)	100 ug/ml	Thermo Fisher (Invivogen) ANTPM2
5 ml Glutamax 100X	1X	Thermo Fisher (Gibco) 35050061
500 µl Ascorbic Acid (50 mg/ml stock)	50 µg/ml	Millipore Sigma A4544
1.5 ml dilute 1-Thioglycerol (MTG) (26 µl MTG in 2 ml IMDM)	$4.5x10^{-4}M$	Millipore Sigma M6145

B) STEMdiff Definitive Endoderm Kit, StemCell Technologies 05110

C) <u>Days 5-6 Media</u>

cSFDM base media supplemented with following growth factors (final concentrations)
50ng/ml Activin A (R&D 338-AC)
10ng/ml BMP4 (R&D 314-BP)
10ng/ml FGF2 (R&D 233-FB)
10ng/ml VEGF (R&D 293VE)

D) <u>Days 7-12 Media</u>

cSFDM base media supplemented with following growth factors (final concentrations)
50ng/ml BMP4
10ng/ml FGF2
10ng/ml VEGF
10ng/ml EGF (R&D 293-EG)
20ng/ml TGFa (R&D 239-A)
100ng/ml HGF (R&D 294-HG)
100nM Dexamethasone (Millipore Sigma D4902)

E) <u>Days 13-18 Media</u>

cSFDM base media supplemented with following growth factors (final concentrations)
10ng/ml FGF2
10ng/ml VEGF
10ng/ml EGF
100ng/ml HGF
20ng/ml Oncostatin M (Thermo Fischer (Gibco) PHC5015)

6ug/ml Vitamin K (Millipore Sigma 47773)
100nM Dexamethasone
1.5uM γ-Secretase Inhibitor X (Millipore Sigma 565771)
1% DMSO (Millipore Sigma D2650)

E) Days 19-25+ Media

cSFDM base media supplemented with following growth factors (final concentrations)
100ng/ml HGF
20ng/ml Oncostatin M
6ug/ml Vitamin K
0.1uM Dexamethasone

F) Other Reagents

Y-27632 Rho-associated kinase inhibitor (Tocris 1254)
DMEM/F12 (Thermo Fisher (Gibco) 11330032)
Gentle Cell Dissociation Reagent (StemCell Technologies 100-0485)
Corning Matrigel hESC-Qualified Matrix (Thermo Fisher (Corning) 354277)

PROTOCOL

A) Definitive Endoderm Induction (STEMdiff Definitive Endoderm kit: Days 0 to 4)

1. Culture undifferentiated PSCs in mTeSR-1 on Matrigel for at least 1 week prior to starting differentiation. Grow cells until confluent and ready for passage
2. Coat required number of wells of a 6 well plate with dilute matrigel (dilute according to Corning manufacturer's instruction and let plates sit for 1 hour at room temperature or 30 minutes at 37ºC)
3. Day 0: Dissociate PSCs in Gentle Cell Dissociation Reagent (GCDR) per manufacturer's instructions and plate $1x10^6$ (acceptable range 0.7-1.5 $x10^6$) cells onto 1 well of a matrigel-coated 6 well plate in mTeSR-1 supplemented with 10uM Y-27632.
    a. At this point cells should be placed into hypoxic conditions (5% O_2, 5% CO_2, 90%N_2).
4. Day 1: Wash cells with DMEM/F12 and change media to STEMdiff Endoderm kit Base media with supplement MR and supplement CJ. For one mL of Day1 media use 980uL of the base media and 10uL of each supplement.
5. Days 2 to 4: After 24 hours change media to STEMdiff Endoderm kit Base media and supplement CJ only and refeed every 24 hours for a total of 72 hours. For one mL of Day 2-4 media use 990uL of the base media and 10uL of supplement CJ.

    Note: You can check definitive endoderm efficiency at this point by performing FACS on a fraction of the cells using markers such as C-KIT and CXCR4. The majority of cells (>90%) are routinely positive for both markers.

B) Hepatic Specification (Days 5-6 media and Days 7-12 Media: Days 5 to 12)

1. At Day 5 cells can routinely be passaged to expand cell numbers and increase the wells available for each experiment. Coat approximately 3-5 (6 well size), 6-10 (12 well size), or 12-20 (24 well size) wells per well of endoderm with dilute matrigel*.
2. Add 1ml GCDR to each well of Day 5 endoderm, incubate for 2 minutes
3. Aspirate GCDR and add 1ml Days 5-6 media supplemented with 10uM Y-27632 to each endoderm well. Gently pipet up and down 2-6 times until all cells have lifted off the plate and are in small multicellular groups.
    a. *Gentle pipetting while replating tends to promote cell survival and achieving 100% single cell suspensions are unnecessary and will likely promote excessive cell death.*
    b. *Until comfortable with passaging it may be beneficial to evaluate cells after pipetting twice under the microscope before each subsequent pipette to ensure cells have been adequately disassociated but have not been over pipetted and show increased cell shearing/death.*
4. Passage each well 1:3-1:5 into previously coated plates in Days 5-6 media supplemented with 10uM Y-27632
    a. *The optimal cell density varies from line to line and based upon operator passaging technique*
5. 24 hours later, change media to Days 5-6 *without* 10uM Y-27632

6. Leave plates in Days 5-6 media for 24 hours
7. On Day 7, change to Days 7-12 media. At this point cells can be refed every 48 hrs.

**Note at 6-24 well sizes we reproducibly observe minimal well to well variability in the hepatic specification efficiency. Smaller well sizes may be used, however, this may cause increased well to well variability in differentiation efficiency.*

C) Hepatic Maturation (Days 13-18 media: Days 13 to 18)

1. After Hepatic specification change media to Days 13-18 media
2. Refeed every 48 hours

D) Hepatic Maintenance media (Days 19-25+ media: Day 19 to 25+)

1. After Hepatic maturation change media to Days 19-25+ media
2. Refeed every 48 hours
3. Cells may be collected for analysis at any point after day 19 through day 25+.

Note iPSC-derived hepatocyte cultures will remain stable after day 25 until day 30, however, we would not recommend continuing beyond day 30 as cell viability begins to drop at this point.

VERSION HISTORY

2017-11-29	Drafted by Prithvi Akepati.	PA
2022-1-19	Primocin final concentration 100 ug/ml	CB
2022-3-25	Updated Methods	JK