Modeling Cholangiocyte Differentiation with Flow Cytometry Analysis

Introduction

iCell® Hepatoblasts represent a progenitor population upstream in differentiation from the iCell Hepatocytes 2.0 and iCell Hepatocytes products. This cell type reflects the point in differentiation from induced pluripotent stem (iPS) cells past endoderm but before being a fully specified hepatocyte. Thus, the hepatoblast cells retain the potential to differentiate into both hepatocytes and cholangiocytes. The protocol presented here has demonstrated utility in inducing differentiation of iCell Hepatoblasts into the cholangiocyte lineage.

Required Equipment and Consumables

The following equipment and consumables are required in addition to the materials specified in the iCell Hepatoblasts Prototype User's Guide.

Item	Vendor	Catalog Number
Equipment		
Flow Cytometer	Multiple Vendors	
Tabletop Centrifuge	Multiple Vendors	
Consumables		
iCell Hepatoblasts Prototype	Cellular Dynamics International (CDI)	HBC-100-020-001-PT
96-well Polystyrene V-bottom MicroWell Plate (96-well V-bottom Plate)	Nunc	249570
Biocoat Collagen I Multiwell Plates (Cell Culture Plates)	Becton Dickinson	354408 - 24-well) 354407 - 96-well)
Dulbecco's Phosphate Buffered Saline without Ca^{2+} and Mg^{2+} (D-PBS)	Multiple Vendors	
Fetal Bovine Serum (FBS)	Multiple Vendors	
Flow Cytometry Tubes	Multiple Vendors	
Formaldehyde, 37%	Multiple Vendors	
Live/Dead Fixable Red or Dead Cell Stain Kit (Live/Dead Dye)	Thermo Fisher	L-23102
Saponin, Practical Grade	MP Biomedicals	02102855
Trypsin-EDTA, 0.5%, 10X, No Phenol Red	Life Technologies	15400-054
Media Components		
Activin A	R&D Systems	338-AC
B27 Supplement	Life Technologies	17504
EGF	R&D Systems	236-EG
FGF10	R&D Systems	345-FG
Gentamicin	Life Technologies	15750

Item	Vendor	Catalog Number
HGF	R&D Systems	294-HGN
RPMI	Life Technologies	11875

Notes

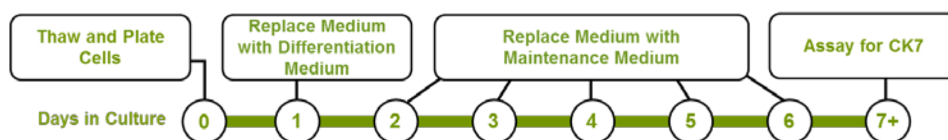
Recommended Antibodies

The following table of primary and secondary antibodies provides the dilution factor to use for labeling differentiated iCell Hepatoblasts.

Item	Vendor	Catalog Number	Dilution Factor
Primary Antibodies			
FITC Conjugated Anti-Human Alpha-1 Antitrypsin (A1AT) Antibody (FITC-A1AT)	Bethyl Laboratories	A80-122F	1:400
Mouse Anti-cytokeratin 7 (CK7) Antibody	Abcam	Ab9021	1:100
Mouse IgG1 Isotype Antibody (Control IgG1)	Sigma	M5284	1:20
Secondary Antibodies			
AlexaFluor 647 Conjugated Goat Anti-mouse IgG1 Antibody (AlexaFluor 647-IgG1)	Life Technologies	A21240	1:500
FITC Conjugated Goat IgG Isotype Antibody (Control FITC-IgG)	Bethyl Laboratories	P50-100F	1:400
Optional Live/Dead Dye (if staining for only CK7)			
Live/Dead Fixable Green Dead Cell Stain Kit (Live/Dead Dye)	Thermo Fisher	L-23101	

Workflow

iCell Hepatoblasts are thawed and plated according to the iCell Hepatoblasts Prototype User's Guide. On day 1 post-plating, spent medium is replaced with Cholangiocyte Differentiation Medium. On day 2 post-plating, spent medium is replaced with Cholangiocyte Maintenance Medium, and spent medium is replaced daily thereafter. On day 7, differentiated cholangiocytes can be labeled for CK7 and A1AT. The expression of CK7 is dependent on the concentration of activin A in the Differentiation Medium.



Methods

Thawing and Plating iCell Hepatoblasts

Thaw, plate, and maintain iCell Hepatoblasts according to the User's Guide until day 1 post-plating.

Notes

Preparing Medium

Prepare and store the Cholangiocyte Differentiation Medium and Cholangiocyte Maintenance Medium as follows:

Cholangiocyte Differentiation Medium[1,2,3]		
Component	Amount (ml)	Final Concentration
RPMI	96	98%
Activin A, 25 µg/ml	0 - 0.2	0 - 50 ng/ml
B27 Supplement, 50X	2	1X
Gentamicin, 50 mg/ml	0.05	25 µg/ml

1 Prepare the Cholangiocyte Differentiation Medium immediately before use.
2 Filter the medium using a 0.2 µm PES filter unit.
3 Store the Cholangiocyte Differentiation Medium at 4°C for up to 1 week. Do not store at -20°C.

Cholangiocyte Maintenance Medium[1,2,3]		
Component	Amount (ml)	Final Concentration
RPMI	96	98%
B27 Supplement, 50X	2	1X
EGF, 500 µg/ml	0.004	20 ng/ml
FGF10, 500 µg/ml	0.01	50 ng/ml
HGF, 25 µg/ml	0.001	20 ng/ml
Gentamicin, 50 mg/ml	0.05	25 µg/ml

1 Prepare the Cholangiocyte Maintenance Medium immediately before use.
2 Filter the medium using a 0.2 µm PES filter unit.
3 Store the Cholangiocyte Maintenance Medium at 4°C for up to 1 week. Do not store at -20°C.

Differentiating iCell Hepatoblasts into Cholangiocytes

The following protocol details the procedures for inducing differentiation of iCell Hepatoblasts into cholangiocytes in a 96-well or 24-well cell culture plate. Scale volumes appropriately for other vessel formats.

1. Equilibrate Cholangiocyte Differentiation Medium at room temperature before use.
2. Approximately 24 hours post-plating iCell Hepatoblasts, aspirate the spent medium and replace (100%) with the appropriate volume of Cholangiocyte Differentiation Medium. Recommended volumes are as follows:
   - **24-well cell culture plate:** 0.5 ml/well
   - **96-well cell culture plate:** 100 µl/well
3. Return plates to a cell culture incubator at 37°C, 5% CO_2.
4. Approximately 24 hours post-differentiation induction, aspirate the spent medium and replace (100%) with the appropriate volume of Cholangiocyte Maintenance Medium.
5. Replace 100% of the spent Cholangiocyte Maintenance Medium daily until day 7 post-plating.

Collecting Differentiated Hepatoblasts from a 96-well Cell Culture Plate

The following protocol details the procedures for collecting differentiated hepatoblasts from a 96-well cell culture plate. Scale volumes according to the surface area of other vessel formats.

1. Aspirate the Maintenance Medium from the 96-well cell culture plate containing differentiated hepatoblasts.
2. Wash cells with 200 µl/well of D-PBS using a multichannel pipettor.
3. Add 50 µl/well of 0.5% trypsin-EDTA. Incubate in a cell culture incubator at 37°C for 3 minutes.
4. Add 50 µl/well of FBS to a clean 96-well V-bottom plate.
5. Triturate cells 4 times using a multichannel pipettor.
6. Transfer the cell suspension to the corresponding wells of the 96-well V-bottom plate containing FBS.
7. Cover the plate with a lid and centrifuge 250 x g for 4 minutes.
8. Aspirate or quickly decant the supernatant. Resuspend cells in 100 µl/well of D-PBS.
9. Cover the plate with a lid and centrifuge at 250 x g for 4 minutes.

Staining Live/Dead Differentiated Hepatoblasts

Stain differentiated hepatoblasts to distinguish live and dead populations before fixation for labeling for CK7 and A1AT expression.

1. Dilute 10 µl of live/dead dye solution to 1:1000 in 9.99 ml of D-PBS immediately before use.

   Note: *Reconstitute live/dead dye according to the manufacturer's instructions.*
2. Aspirate or quickly decant the D-PBS from the 96-well V-bottom plate. Resuspend cells in 100 µl/well of diluted live/dead dye.
3. Incubate at room temperature for 15 minutes.
4. Cover the plate with a lid and centrifuge at 250 x g for 4 minutes.
5. Aspirate or quickly decant the supernatant. Resuspend cells in 200 µl/well of D-PBS.
6. Repeat steps 4 and 5 three times to complete the wash.

Labeling Differentiated Hepatoblasts: Fixation, Permeabilization, and Antibody Incubation

1. Prepare the fixative solution by diluting a stock solution of formaldehyde solution to 4% (v/v) in D-PBS.
2. Cover the plate with a lid and centrifuge at 250 x g for 4 minutes.
3. Aspirate or quickly decant the D-PBS wash buffer from the plate. Resuspend cells in 100 µl/well of fixative solution.
4. Incubate at room temperature for 15 minutes.
5. Prepare 100 ml of FACS buffer by diluting 2 ml of FBS in 98 ml of D-PBS to achieve a final FBS concentration of 2% (v/v).
6. Cover the plate with a lid and centrifuge at 250 x g for 4 minutes.
7. Aspirate or quickly decant the supernatant. Resuspend cells in 100 µl/well of FACS buffer.

Notes

Notes

8. Repeat steps 6 and 7 three times to complete the wash.

   Note: *Fixed cells can be stored in FACS buffer at 4°C for up to 1 week.*

9. Prepare the permeabilization buffer by diluting FBS to 2% (v/v) and saponin to 0.1% (w/v) in D-PBS. Filter using a 0.2 µm PES filter unit.
10. Cover the plate with a lid and centrifuge at 250 x g for 4 minutes.
11. Aspirate or quickly decant the supernatant. Resuspend cells in 100 µl/well of permeabilization buffer.
12. Repeat steps 10 and 11 three times to complete the wash.
13. Prepare the primary antibody solution by diluting the CK7 antibody 1:100 in permeabilization buffer.
14. Prepare the isotype control solution by diluting the control IgG1 antibody 1:20 in permeabilization buffer.
15. Cover the plate with a lid and centrifuge at 250 x g for 4 minutes.
16. Aspirate or quickly decant the permeabilization buffer. Resuspend cells in 100 µl/well of primary antibody (or isotype control) solution.
17. Cover the plate and incubate at room temperature for 1 hour, protected from light.
18. Cover the plate with a lid and centrifuge at 250 x g for 4 minutes.
19. Aspirate or quickly decant the supernatant. Resuspend cells in 100 µl/well of permeabilization buffer.
20. Repeat steps 18 and 19 three times to complete the wash.
21. Prepare the secondary antibody solution by diluting the FITC-A1AT antibody 1:400 and AlexaFluor 647-IgG1 1:500 in permeabilization buffer.
22. Prepare the secondary isotype control solution by diluting the control FITC-IgG antibody 1:400 and AlexaFluor 647-IgG1 1:500 in permeabilization buffer.
23. Cover the plate with a lid and centrifuge at 250 x g for 4 minutes.
24. Aspirate or quickly decant the supernatant. Resuspend cells in 100 µl/well of secondary antibody (or isotype control) solution.
25. Cover the plate and incubate at room temperature for 1 hour, protected from light.
26. Cover the plate with a lid and centrifuge at 250 x g for 4 minutes.
27. Aspirate or quickly decant the supernatant. Resuspend cells in 100 µl/well of permeabilization buffer.
28. Repeat steps 26 and 27 three times to complete the wash. After the last centrifugation, resuspend cells in FACS buffer.
29. Transfer cells to flow cytometry tubes for analysis.

Data Analysis

See the guide for the flow cytometry system for data analysis instructions.

1. Use the isotype control sample to set the negative population gates.

2. Use the live/dead signal (red), CK7 (far-red), and A1AT signal (green) to evaluate hepatoblast differentiation into cholangiocytes (Figure 1).

Note: *The population of CK7+ cells increases with increasing concentration of activin A in the Cholangiocyte Differentiation Medium.*

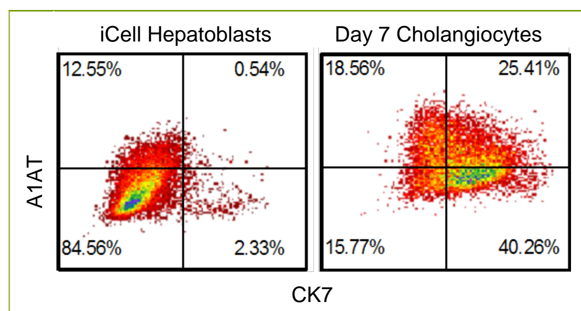
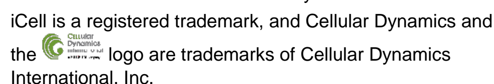


Figure 1: Flow Cytometry Analysis of A1AT and CK7 Expression in Cholangiocytes Differentiated from iCell Hepatoblasts
In this representative experiment, iCell Hepatoblasts were differentiated into cholangiocytes as indicated by the increase in CK7 expression in the induced condition (with 20 ng/ml activin A). Acquisition and analysis were performed using a BD Accuri C6 Flow Cytometer (BD Biosciences).

Notes

Summary

iCell Hepatoblasts are derived from human iPS cells and provide an in vitro cellular system for modeling hepatic development. The methods and data presented here highlight a reproducible cell culturing protocol for inducing cholangiocyte differentiation monitored by the expression of CK7 by flow cytometry.

© 2016 Cellular Dynamics International, Inc. All rights reserved.
For Life Science Research Use Only.
iCell is a registered trademark, and Cellular Dynamics and the  logo are trademarks of Cellular Dynamics International, Inc.
All other brands, product names, company names, trademarks, and service marks are the properties of their respective owners.

Version: August 2016
AP-HBCCHO160805