

Modeling Cardiomyocyte Differentiation: *Wnt- and Activin/TGFβ-inhibitor Induction with Flow Cytometry Analysis*

Introduction

The ability of cardiac progenitor cells to proliferate and differentiate into cardiomyocytes is fundamental during cardiac development in the embryonic and postnatal heart and contributes to the myocyte replacement in a damaged adult heart. iCell® Cardiac Progenitor Cells are human induced pluripotent stem cell-derived cardiac progenitor cells that recapitulate the physiological characteristics of native human cardiac progenitor cells. Due to their human origin, high purity, functional relevance, and ease of use, iCell Cardiac Progenitor Cells represent an optimal in vitro test system for interrogating cardiac regenerative biology in basic research and many areas of drug development. The protocol presented here has demonstrated utility in inducing differentiation of iCell Cardiac Progenitor Cells into cardiomyocytes with the Wnt inhibitor XAV939 and activin/TGFβ inhibitor SB431542 as assessed by the expression of cardiac troponin T (cTNT) using flow cytometry analysis.

Required Equipment and Consumables

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

Item	Vendor	Catalog Number
Equipment		
Flow Cytometer	Multiple Vendors	
Consumables		
96-well V-bottom Plates	Multiple Vendors	
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 Green Dead Cell Stain Kit (Live/Dead Dye)	Life Technologies	L-23101
Saponin from Quillaja Bark	Sigma	S7900
SB431542	Sigma	S4317-5MG
TrypLE	Life Technologies	12563
XAV939	Sigma	X3004-5MG

Recommended Antibodies

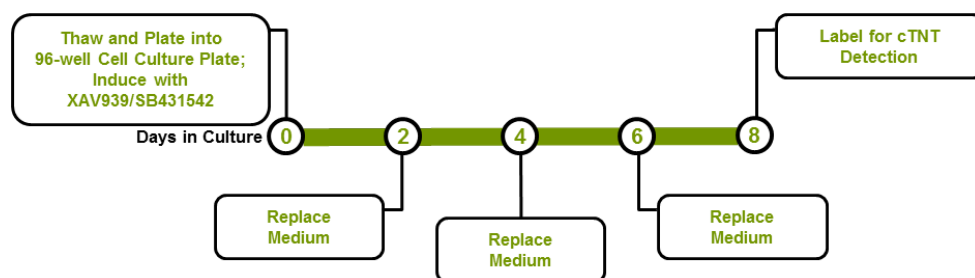
The following table of primary and secondary antibodies provides the dilution factor to use for labeling iCell Cardiac Progenitor Cells.

Item	Vendor	Catalog Number	Dilution Factor
Primary Antibodies			
Mouse Anti-cardiac Troponin T (Detection Antibody)	Thermo Fisher	MS-295-P	1:1,000
Mouse IgG1 Isotype (Control Antibody)	Sigma	M5284	1:1,000
Optional Primary Antibodies			
Mouse Anti-CD117(c-Kit)-APC (Detection Antibody)	Life Technologies	CD11705	1:1,000
Mouse Anti-KDR-PE (Detection Antibody)	R&D Systems	FAB357P	1:1,000
Mouse IgG1 Isotype-APC (Control Antibody)	R&D Systems	IC002A	1:1,000
Mouse IgG1 Isotype-PE (Control Antibody)	R&D Systems	1C002P	1:1,000
Secondary Antibody			
AlexaFluor 647 Goat Anti-mouse IgG (Secondary Antibody)	Life Technologies	A21240	1:1,000

Workflow

iCell Cardiac Progenitor Cells are thawed and plated in XAV939/SB431542 containing Maintenance Medium into a 96-well cell culture plate previously coated with fibronectin. On day 2 post-plating, spent medium is replaced with XAV939/SB431542-free Maintenance Medium, and spent medium is replaced every 2 days thereafter. From day 6 - 8 post-plating, differentiated cardiomyocytes can be labeled for cTNT detection.

Optional: On day 2 post-plating, the cardiac progenitor cell population can be labeled for KDR/cKit detection.



Methods

Thawing iCell Cardiac Progenitor Cells

1. Thaw iCell Cardiac Progenitor Cells according to the iCell Cardiac Progenitor Cells Prototype User's Guide.

Notes

2. Remove a sample of cells to perform a cell count using a hemocytometer (using trypan blue exclusion to identify viable cells) or an automated cell counter.
3. Dilute the cell suspension in Maintenance Medium to 5.6×10^5 viable cells/ml.

Plating iCell Cardiac Progenitor Cells

The following procedure details plating iCell Cardiac Progenitor Cells in a 96-well cell culture plate. Scale volumes appropriately for other vessel formats.

1. Reconstitute XAV939 in DMSO at 10 mM and SB431542 at 2.5 mM according to the manufacturer's instructions.

Note: *If necessary, aliquot and store reconstituted XAV939 and SB431542 individually at -20°C.*

2. Dilute 10 mM XAV939 and 2.5 mM SB431542 to a 20X concentration of 200 μ M and 50 μ M in Maintenance Medium, respectively. See the iCell Cardiac Progenitor Cells Prototype User's Guide for medium preparation.
3. Aspirate the fibronectin solution from a pre-coated 96-well cell culture plate.
4. Invert the cell suspension 6 times. Immediately dispense 90 μ l/well of cell suspension (~50,000 viable cells/well).
5. Immediately add 5 μ l/well of 200 μ M XAV939 and 5 μ l/well of 50 μ M SB431542 into experimental wells containing 90 μ l/well cell suspension. Mix gently by pipetting. Do not add XAV939 or SB431542 in control wells.
6. Culture iCell Cardiac Progenitor Cells in a cell culture incubator at 37°C, 7% CO₂ for 6 - 8 days, replacing the spent medium with 37°C XAV939/SB431542-free Maintenance Medium every other day.

Collecting Differentiated Cardiomyocytes from a 96-well Cell Culture Plate

1. Aspirate the Maintenance Medium from the 96-well cell culture plate containing differentiated cardiomyocytes.
2. Add 50 μ l/well of TrypLE. Incubate in a cell culture incubator at 37°C for 5 minutes.
3. Add 50 μ l/well of FBS to a clean 96-well V-bottom plate.
4. Triturate the cells 4 times using a multichannel pipettor.
5. Transfer the differentiated cardiomyocytes cell suspension to the corresponding wells of the 96-well V-bottom plate containing FBS.
6. Cover the plate with a lid and centrifuge 400 x g for 3 minutes.
7. Aspirate or quickly decant the supernatant. Resuspend the cells in 100 μ l/well of D-PBS.
8. Cover the plate with a lid and centrifuge at 400 x g for 3 minutes.

Staining Live/Dead Differentiated Cardiomyocytes

Stain differentiated cardiomyocytes to distinguish live and dead populations before fixation for intracellular labeling for cTNT expression.

1. Dilute 10 μ l of live/dead dye solution to 1:1,000 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 the cells in 100 μ l/well of diluted live/dead dye.
3. Incubate the cells at room temperature for 10 minutes.
4. Cover the plate with a lid and centrifuge at 400 x g for 3 minutes.
5. Aspirate or quickly decant the D-PBS. Resuspend the cells in 200 μ l/well of D-PBS.
6. Cover the plate with a lid and centrifuge at 400 x g for 3 minutes.
7. Prepare 100 ml of wash buffer by diluting 2 ml of FBS in 98 ml of D-PBS to achieve a final FBS concentration of 2% (v/v).
8. Aspirate or quickly decant the D-PBS. Resuspend the cells in 200 μ l/well of wash buffer.

Labeling Differentiated Cardiomyocytes: Fixation, Permeabilization, and Antibody Incubation

1. Prepare 10 ml of fixative solution by diluting 1.08 ml of 37% formaldehyde in 8.92 ml of D-PBS to achieve a final concentration of 4% formaldehyde (v/v).
2. Aspirate or quickly decant the wash buffer from the 96-well V-bottom plate. Incubate the cells with 100 μ l/well of fixative solution at room temperature for 15 minutes.
3. Add 100 μ l/well of wash buffer and mix.
4. Cover the plate with a lid and centrifuge at 400 x g for 3 minutes.
5. Aspirate or quickly decant the wash buffer. Resuspend the cells in 100 μ l/well of wash buffer.
6. Repeat steps 4 and 5 three times to complete the wash.
Note: If necessary, store fixed cells in wash buffer at 4°C overnight.
7. Prepare the permeabilization buffer by diluting FBS to 2% (v/v) and saponin to 0.1% (w/v) in D-PBS.
8. Prepare the primary antibody solution by diluting the detection antibody in permeabilization buffer to 1:1,000.
9. Prepare the isotype control solution by diluting the control antibody in permeabilization buffer to 1:1,000.
10. Centrifuge the 96-well V-bottom plate at 400 x g for 3 minutes.
11. Aspirate or quickly decant the wash buffer. Resuspend the cell in 100 μ l/well of permeabilization buffer.

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12. Cover the plate with a lid and centrifuge at 400 x g for 3 minutes.
13. Aspirate or quickly decant the permeabilization buffer. Resuspend the cells in 50 µl/well of primary antibody (or isotype control) solution.
14. Cover the plate and incubate at room temperature for 1 hour, protected from light.
Note: *Alternatively, incubate the plate at 4°C overnight.*
15. Prepare the secondary antibody solution by diluting the secondary antibody in permeabilization buffer to 1:1,000.
16. Centrifuge the 96-well V-bottom plate at 400 x g for 3 minutes.
17. Aspirate or quickly decant the primary antibody (or isotype control) solution. Resuspend the cells in 100 µl/well permeabilization buffer.
18. Cover the plate with a lid and centrifuge at 400 x g for 3 minutes.
19. Repeat steps 17 and 18 two times to complete the wash.
20. Aspirate or quickly decant the permeabilization buffer. Resuspend the cells in 50 µl/well of secondary antibody solution.
21. Cover the plate and incubate at room temperature for 1 hour, protected from light.
22. Centrifuge the plate at 400 x g for 3 minutes.
23. Aspirate or quickly decant the secondary antibody solution. Resuspend the cells in 100 µl/well of permeabilization buffer.
24. Cover the plate with a lid and centrifuge at 400 x g for 3 minutes.
25. Repeat steps 23 and 24 two times to complete the wash.
26. Aspirate or quickly decant the permeabilization buffer. Resuspend the cells in 100 µl/well of wash buffer.
27. Transfer the cells to flow cytometry tubes for flow cytometry 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 (green) and cTNT signal (red) to calculate cTNT expression.
3. Calculate the percent of cTNT expression for each sample.
4. Plot the percent of cTNT⁺ cells (Y-axis) against the treatment condition (X-axis) (Figure 1).

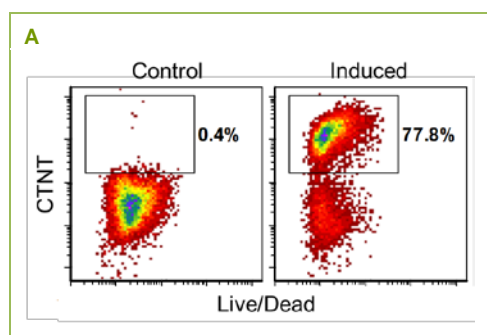



Figure 1: Flow Cytometry Detection of cTNT Expression in Cardiomyocytes Differentiated from XAV939/SB431542-induced iCell Cardiac Progenitor Cells

In this representative experiment, iCell Cardiac Progenitor Cells differentiated into cardiomyocytes as indicated by the increase in cTNT expression in the induced condition. Acquisition and analysis were performed using a BD Accuri C6 Flow Cytometer (BD Biosciences). iCell Cardiac Progenitor Cells were plated in the presence of XAV939/SB431542 for 2 days and assayed on day 8 post-plating.

Summary

iCell Cardiac Progenitor Cells are derived from human iPSCs and provide an in vitro cellular system for modeling cardiac development. The methods and data presented here highlight a reproducible cell culturing protocol for inducing cardiac differentiation in the presence of the Wnt inhibitor XAV939 and activin/TGF β inhibitor SB431542 monitored by the expression of cTNT by flow cytometry.

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