

## **Modeling Cardiac Hypertrophy: *Endothelin-1 Induction with Flow Cytometry Analysis***

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### **Introduction**

Cardiac hypertrophy is characterized by several different cellular changes, including reactivation of the fetal gene program (1, 2). One of the genes in this group, *NPPB*, and the protein product for which it encodes, B-type natriuretic peptide (BNP), have been utilized as classical biomarkers for hypertrophy. Because cardiac hypertrophy is observed under many conditions, including myocardial infarction, ischemia, hypertension, valvular dysfunctions, and toxic side effects of small molecule/protein stimulation, there is a need for reproducible methods for its detection and quantification.

iCell® Cardiomyocytes are derived from human induced pluripotent stem cells (iPSCs) and have been shown to recapitulate the biochemical, electrophysiological, mechanical, and pathophysiological properties of native cardiac myocytes, including hypertrophic responses (3). Due to their human origin, high purity, and functional relevance, iCell Cardiomyocytes enable the interrogation of cardiac biology in basic research and drug discovery.

This Application Protocol describes how to utilize iCell Cardiomyocytes in a 5-day post-plating in vitro cellular model of cardiac hypertrophy that is induced by endothelin-1 (ET-1) and quantified via increased BNP expression using a reproducible flow cytometry-based assay readout. Longer post-plating incubation times can be implemented to better fit end-user workflows or to increase the assay response window.

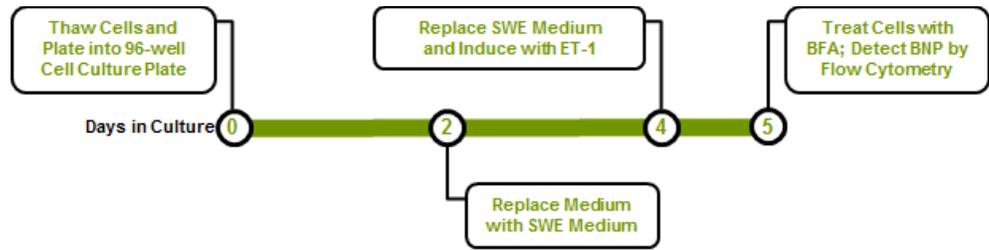
## Required Equipment and Consumables

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

Item	Vendor	Catalog Number
<b>Equipment</b>		
Flow Cytometer	Multiple Vendors	N/A
Multichannel Pipettor	Multiple Vendors	N/A
<b>Consumables</b>		
iCell Cardiomyocytes Kit	Cellular Dynamics International (CDI)	CMC-100-010-001 CMC-100-010-005
0.5% Trypsin-EDTA (10X), no Phenol Red	Life Technologies	15400-054
96-well Cell Culture Plate	Multiple Vendors	N/A
96-well V-bottom Microwell Plate	Nunc	249570
AlexaFluor 647 Donkey Anti-Mouse IgG (H+L) (Secondary Antibody)	Life Technologies	A31571
Anti-proBNP Mouse Monoclonal Antibody [15F11] (Detection Antibody)	Abcam	ab13115
Brefeldin A (BFA)	Sigma-Aldrich	B7651
Cocktail B from Hepatocyte Maintenance Supplement Pack (Supplement Pack)	Life Technologies	CM4000
Dulbecco's Phosphate Buffered Saline without Ca <sup>2+</sup> and Mg <sup>2+</sup> (D-PBS)	Invitrogen	14190
Endothelin-1 (ET-1)	Sigma	E7764
Fetal Bovine Serum (FBS)	Multiple Vendors	N/A
Fibronectin	Roche Applied Science	11051407001
Flow Cytometry Tubes	Multiple Vendors	N/A
Formaldehyde Solution (37%)	Sigma-Aldrich	F8775
HPLC-grade Methanol	Multiple Vendors	N/A
IgG2b Isotype Control (Control Antibody)	Sigma-Aldrich	M5534
Live/Dead Fixable Green Dead Cell Stain Kit (Live/Dead Dye)	Life Technologies	L-23101
Saponin from Quillaja Bark	Sigma-Aldrich	S7900
William's E Medium	Life Technologies	A12176

## Workflow

iCell Cardiomyocytes are thawed and plated into a 96-well cell culture plate previously coated with fibronectin. On day 2 post-plating, iCell Cardiomyocytes Plating Medium is replaced with supplemented William's E (SWE) medium. On day 4 post-plating, ET-1 is added in fresh SWE medium. On day 5 post-plating, cells are treated with BFA before harvesting and then analyzed for BNP expression using flow cytometry. The assay is optimized for a 5-day workflow but can be expanded to longer durations if larger signal-to-noise ratios are necessary. Contact CDI's Technical Support ([support@cellulardynamics.com](mailto:support@cellulardynamics.com); +1 (877) 320-6688 (US toll-free) or (608) 310-5100) for instructions on extending the assay workflow.



## Methods

### Thawing and Culturing iCell Cardiomyocytes

1. Dilute 1 mg/ml fibronectin solution in sterile D-PBS to a final concentration of 5 µg/ml immediately before use. Approximately 10 ml are required to coat a 96-well cell culture plate.

**Note:** Reconstitute the fibronectin in sterile water at 1 mg/ml according to the manufacturer's instructions. Aliquot and store at -20°C.

2. Add 100 µl/well of 5 µg/ml fibronectin solution to a 96-well cell culture plate.

**Note:** If necessary, store fibronectin-coated plates at 4°C for up to 1 week.

3. Incubate at 37°C for 1 hour.

4. Thaw iCell Cardiomyocytes according to the iCell Cardiomyocytes User's Guide.

5. Dilute the cell suspension in iCell Cardiomyocytes Plating Medium (Plating Medium) to 200,000 plated cardiomyocytes/ml. Refer to the iCell Cardiomyocytes User's Guide for instructions to calculate the *Target Plating Density* based on *Plating Efficiency*.

6. Aspirate the fibronectin solution. Immediately add 100 µl/well of the cell suspension (20,000 plated cardiomyocytes/well).

7. Incubate in a cell culture incubator at 37°C, 7% CO<sub>2</sub> for 48 hours.

**Note:** CDI recommends leaving the cells undisturbed for maximum attachment.

8. Prepare the supplemented William's E (SWE) medium by diluting Cocktail B to 1:25 in William's E Medium immediately before use. Invert to mix. Do not filter.

**Note:** Cocktail B is provided in the Supplement Pack and supplies the cardiomyocytes with a source of energy in the serum-free SWE medium. Also provided in the Supplement Pack is dexamethasone, which is not used for this protocol.

**Note:** Stored separately, William's E Medium and Cocktail B are stable at 4°C for 1 year according to the manufacturer. CDI recommends preparing fresh 50 ml aliquots of SWE medium before performing this protocol.

9. Aspirate the Plating Medium from the 96-well cell culture plate. Immediately add 100  $\mu$ l/well of SWE medium.

**Note:** CDI recommends replacing the Plating Medium with SWE medium in the morning on day 2 post-plating to maximize the time that the cardiomyocytes are maintained in culture in serum-free medium before inducing a hypertrophic response.

10. Gently wash off the non-adherent cells and debris using a multichannel pipettor by repeatedly aspirating and dispensing the SWE medium over the plate surface. Be mindful as to not disrupt the cell monolayer.
11. Aspirate the SWE medium. Immediately add 100  $\mu$ l/well of fresh SWE medium.
12. Incubate in a cell culture incubator at 37°C, 7% CO<sub>2</sub> for 48 hours.

**Note:** Contact CDI's Technical Support ([support@cellulardynamics.com](mailto:support@cellulardynamics.com); +1 (877) 320-6688 (US toll-free) or (608) 310-5100) for instructions on extending the assay workflow beyond 5 days.

### Inducing a Hypertrophic Response in iCell Cardiomyocytes

iCell Cardiomyocytes are suitable for inducing a hypertrophic response with ET-1 on day 4 post-plating when cultured according to this protocol. CDI recommends inducing a hypertrophic response with ET-1 in iCell Cardiomyocytes in the afternoon on day 4 post-plating. This timing allows for data acquisition and analysis in the morning on day 5 post-plating at 15 hours post-treatment.

1. Reconstitute the ET-1 in sterile water to a stock concentration of 10  $\mu$ M.
2. Prepare a serial dilution of ET-1 in SWE medium at 5X the final concentrations to allow for assessment of a 10-point dose-response curve of induced BNP expression during data analysis.

**Note:** CDI recommends preparing a 4-fold titration with the top concentration of 10 nM. EC<sub>50</sub> values in the low pM range have been observed for ET-1.

3. Remove the 96-well cell culture plate containing iCell Cardiomyocytes from the incubator.
4. Aspirate the spent SWE medium. Immediately add 80  $\mu$ l/well of fresh SWE medium.
5. Add 20  $\mu$ l/well of serially diluted ET-1.
6. Incubate in a cell culture incubator at 37°C, 7% CO<sub>2</sub> for 15 hours.

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## Data Acquisition and Analysis

The following sections detail how to prevent BNP secretion, how to collect and stain cells for data acquisition, and then how to perform data analysis.

### Preventing Secretion of the BNP Protein

BNP is a secreted protein. Incubation of cardiomyocytes in BFA prevents BNP secretion, increasing cellular BNP levels and thus experimental signal. The optimal time to prevent BNP secretion is 15 hours post-ET-1 treatment.

## Notes

1. Dilute BFA solution in SWE medium to a 10X concentration of 100 µg/ml immediately before use.

**Note:** Reconstitute BFA in HPLC-grade methanol to prepare a 10 mg/ml stock solution. Aliquot and store at -20°C.

2. Remove the 96-well cell culture plate containing ET-1-stimulated iCell Cardiomyocytes from the incubator.
3. Add 10 µl/well of the 10X BFA solution without removing the spent medium to achieve a final concentration of 10 µg/ml.
4. Incubate at 37°C, 7% CO<sub>2</sub> for 3 hours.

### Collecting iCell Cardiomyocytes from the 96-well Cell Culture Plate

The optimal time to detect ET-1-induced BNP expression in iCell Cardiomyocytes is 18 hours post-ET-1 treatment.

1. Remove the 96-well cell culture plate containing ET-1-stimulated and BFA treated iCell Cardiomyocytes from the incubator.
2. Aspirate or quickly decant the spent medium. Wash the cells with 200 µl/well of D-PBS.
3. Add 100 µl/well of 0.5% Trypsin-EDTA.
4. Incubate at 37°C for 5 minutes.
5. Add 50 µl/well of FBS to a fresh 96-well V-bottom microwell plate.
6. Remove the 96-well cell culture plate containing ET-1-stimulated and BFA treated iCell Cardiomyocytes from the incubator.
7. Triturate the cells 4 times using a multichannel pipettor.
8. Transfer the cell suspension into the corresponding wells of the 96-well V-bottom microwell plate containing FBS.
9. Cover the plate with a lid and centrifuge at 400 x g for 3 minutes.
10. Aspirate or quickly decant the supernatant. Resuspend the cells in 100 µl/well of D-PBS.
11. Cover the plate with a lid and centrifuge at 400 x g for 3 minutes.

### Staining Live/Dead iCell Cardiomyocytes

Stain iCell Cardiomyocytes to distinguish live and dead populations before fixation for intracellular labeling for BNP expression.

1. Dilute 10 µl of live/dead dye solution in 9.99 ml of D-PBS to make a 1:1,000 dilution 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 microwell plate. Resuspend the cells in 100 µl/well of diluted live/dead dye.
3. Incubate 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 µl/well of D-PBS.

6. Cover the plate with a lid and centrifuge at 400 x g for 3 minutes.
7. Prepare FACS wash buffer by diluting FBS to 2% (v/v) in D-PBS.
8. Aspirate or quickly decant the D-PBS. Resuspend the cells in 200 µl/well of FACS wash buffer.

#### Labeling iCell Cardiomyocytes: Fixation, Permeabilization, and Antibody Incubation

1. Prepare the fixative solution by diluting a stock solution of formaldehyde to 4% (v/v) in D-PBS.
2. Aspirate or quickly decant the FACS wash buffer from the 96-well V-bottom microwell plate. Resuspend the cells in 100 µl/well of fixative solution.
3. Incubate at room temperature for 15 minutes.
4. Add 100 µl/well of FACS wash buffer and mix.
5. Cover the plate with a lid and centrifuge at 400 x g for 3 minutes.
6. Aspirate or quickly decant the D-PBS. Resuspend the cells in 100 µl/well of FACS wash buffer.
7. Repeat steps 5 and 6 three times to complete the wash.

**Note:** *If necessary, store fixed cells at 4°C overnight in FACS wash buffer.*

8. Prepare the permeabilization buffer by diluting FBS to 2% (v/v) and Saponin to 0.1% (w/v) in D-PBS.
9. Prepare the primary antibody solution by diluting the detection antibody to 1:1000 in permeabilization buffer.
10. Prepare the isotype control solution by diluting the control antibody to 1:1000 in permeabilization buffer.
11. Centrifuge the 96-well V-bottom microwell plate at 400 x g for 3 minutes.
12. Aspirate or quickly decant the FACS wash buffer. Resuspend the cells in 100 µl/well of permeabilization buffer.
13. Cover the plate with a lid and centrifuge at 400 x g for 3 minutes.
14. Aspirate or quickly decant the permeabilization buffer. Resuspend the cells in 50 µl/well of primary antibody (or isotype control) solution.
15. Cover the plate and incubate at room temperature for 1 hour, protected from light.

**Note:** *Alternatively, incubate the plate at 4°C overnight.*

16. Prepare the secondary antibody solution by diluting the fluorescently-labeled secondary antibody to 1:1000 in permeabilization buffer.
17. Centrifuge the 96-well V-bottom microwell plate at 400 x g for 3 minutes.
18. Aspirate or quickly decant the primary antibody (or isotype control) solution. Resuspend the cells in 100 µl/well of permeabilization buffer.
19. Cover the plate with a lid and centrifuge at 400 x g for 3 minutes.

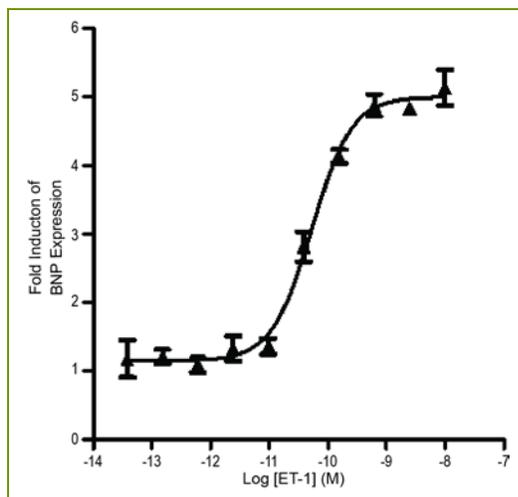
## Notes

20. Repeat steps 18 and 19 two times to complete the wash.
21. Aspirate or quickly decant the permeabilization buffer. Resuspend the cells in 50  $\mu$ l/well of secondary antibody solution.
22. Cover the plate and incubate at room temperature for 1 hour, protected from light.
23. Centrifuge the plate at 400 x g for 3 minutes.
24. Aspirate or quickly decant the secondary antibody solution. Resuspend the cells in 100  $\mu$ l/well of permeabilization buffer.
25. Cover the plate with a lid and centrifuge at 400 x g for 3 minutes.
26. Repeat steps 24 and 25 two times to complete the wash.
27. Aspirate or quickly decant the permeabilization buffer. Resuspend the cells in 100  $\mu$ l/well of FACS wash buffer.
28. Transfer the cells to flow cytometry tubes for analysis.

### Data Analysis

Refer to 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 BNP signal (red) to calculate BNP expression.
3. Calculate the median fluorescence intensity (MFI) for BNP expression for each sample.
4. Generate a dose-response curve for the hypertrophic response of iCell Cardiomyocytes induced by ET-1 by plotting the MFI values (Y-axis) against the log concentration of ET-1 (X-axis).
5. Alternatively, normalize the MFI data to unstimulated cells and generate a dose-response curve by plotting the fold induction of the ET-1-induced BNP expression (Y-axis) against the log concentration of ET-1 (X-axis) (Figure 1).



**Figure 1: Flow Cytometry Detection of BNP Expression in iCell Cardiomyocytes after ET-1 Addition and Incubation**

*In this representative experiment, a dose-dependent increase in BNP expression occurred after stimulation with the indicated concentrations of ET-1 as detected by flow cytometry. Acquisition and analysis were performed using a BD Accuri C6 Flow Cytometer (BD Biosciences). iCell Cardiomyocytes were assayed on day 5 post-plating. The  $EC_{50}$  value for ET-1 was 24 pM (mean  $\pm$  SEM;  $n = 3$  for each point on the curve).*

## Summary

iCell Cardiomyocytes are derived from human iPSCs and provide an in vitro cellular system for modeling cardiac hypertrophy. The methods and data presented here highlight a reproducible cell culturing protocol for monitoring the expression of BNP by flow cytometry after stimulation of a hypertrophic response with ET-1.

## References

1. Swynghedauw B. (1999) Molecular Mechanisms of Myocardial Remodeling. *Physiol Rev* 79.
2. Chien KR, Knowlton KU, Zhu H, Chien S. (1991) Regulation of Cardiac Gene Expression during Myocardial Growth and Hypertrophy: Molecular Studies of an Adaptive Physiologic Response. *FASEB J* 15:3037.
3. Zhi D, Irvin MR, et al. (2012) Whole-exome Sequencing and an iPSC-derived Cardiomyocyte Model Provides a Powerful Platform for Gene Discovery in Left Ventricular Hypertrophy. *Front Genet* 3:92.

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