Modeling Cardiac Hypertrophy:
Endothelin-1 Induction with ELISA Analysis

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 expression of secreted BNP using a reproducible ELISA technique. 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
Equipment		
Microtiter Plate Reader	Multiple Vendors	N/A
Multichannel Pipettor	Multiple Vendors	N/A
Consumables		
iCell Cardiomyocytes Kit	Cellular Dynamics International (CDI)	CMC-100-010-001 CMC-100-010-005
96-well Cell Culture Plate	Multiple Vendors	
96-well MaxiSorp Immuno-plate (ELISA Plate)	Nunc	446612
Anti-proBNP Mouse Monoclonal Antibody [16F3], HRP-conjugate (Detection Antibody)	Abcam	ab13124
Anti-proBNP Mouse Monoclonal Antibody [5B6] (Capture Antibody)	Abcam	ab13111
Cocktail B from Hepatocyte Maintenance Supplement Pack (Supplement Pack)	Life Technologies	CM4000
Dulbecco's Phosphate Buffered Saline without Ca^{2+} and Mg^{2+} (D-PBS)	Invitrogen	14190
Endothelin-1 (ET-1)	Sigma	E7764
Fibronectin	Roche Applied Science	11051407001
Nonfat Dry Milk	Multiple Vendors	N/A
N-term proBNP (1-76) Peptide, Human	Phoenix Pharmaceuticals	011-42
TMB 2-component Microwell Peroxidase Substrate Kit	KPL	50-76-00
TMB Stop Solution	KPL	50-85-00
Tween-20	Multiple Vendors	N/A
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, the ELISA plate is prepared, and ET-1 is added in fresh SWE medium. On day 5 post-plating, supernatants are analyzed for BNP expression using ELISA. The assay is optimized for a 5-day workflow but can be extended if larger signal-to-noise ratios are necessary. Contact CDI's Technical Support (support@cellulardynamics.com; +1 (877) 320-6688 (US toll-free) or (608) 310-5100) for instructions on extending the assay workflow.

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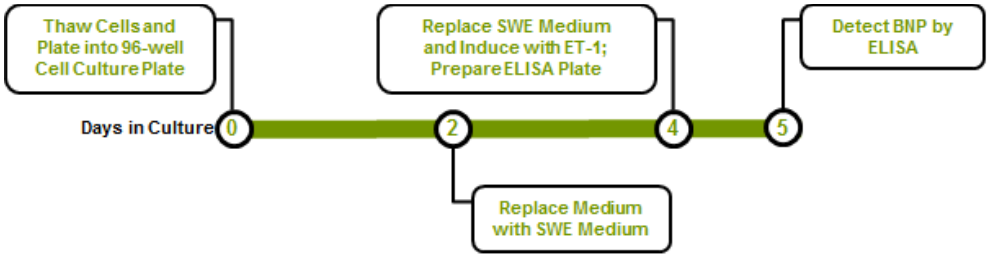

Methods

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_2 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 µl/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 µl/well of fresh SWE medium.

12. Incubate in a cell culture incubator at 37°C, 7% CO_2 for 48 hours.

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

Preparing the ELISA Plate

Prepare the ELISA plate on day 4 post-plating to ensure that it is ready for the assay on the day after iCell Cardiomyocytes are stimulated with ET-1.

1. Dilute the capture antibody in 10 ml of D-PBS to a final concentration of 2 µg/ml.

2. Add 100 µl/well of capture antibody solution to the ELISA plate.

3. Incubate at 4°C overnight to allow coating of the plate with the capture antibody solution.

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 18 hours post-treatment.

1. Reconstitute the ET-1 in sterile water to a stock concentration of 10 µM.

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_{50} 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 µl/well of fresh SWE medium.

5. Add 20 µl/well of serially diluted ET-1.

6. Incubate in a cell culture incubator at 37°C, 7% CO_2 for 18 hours.

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Data Acquisition and Analysis

The following sections detail how to prepare the ELISA BNP standard titration and perform the ELISA for data acquisition and then how to perform data analysis.

Preparing the ELISA BNP Standard Titration

1. Prepare a stock solution of the ELISA BNP standard by dissolving N-term proBNP (1-76) peptide in D-PBS at a final concentration of 2 µM.
2. Prepare the wash buffer by diluting 0.05% Tween-20 in D-PBS.
3. Prepare the blocking buffer by dissolving 1% nonfat dry milk in wash buffer.
4. Prepare a 2-fold serial dilution of the ELISA BNP standard in blocking buffer to generate a standard curve:

Sample ID	Concentration	ELISA BNP Standard	Blocking Buffer
STD1	10,000 fmol/ml	12 µl of stock solution	2.4 ml
STD2	5,000 fmol/ml	700 µl of STD1	700 µl
STD3	2,500 fmol/ml	700 µl of STD2	700 µl
STD4	1,250 fmol/ml	700 µl of STD3	700 µl
STD5	625 fmol/ml	700 µl of STD4	700 µl
STD6	312 fmol/ml	700 µl of STD5	700 µl
STD7	156 fmol/ml	700 µl of STD6	700 µl
STD8	78 fmol/ml	700 µl of STD7	700 µl
STD9	39 fmol/ml	700 µl of STD8	700 µl
STD10	19 fmol/ml	700 µl of STD9	700 µl

Performing the ELISA

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

1. Remove the coated ELISA plate from 4°C.
2. Aspirate the capture antibody. Wash 3 times with 200 µl/well of wash buffer.
3. Add 200 µl/well of blocking buffer.
4. Incubate at room temperature for 30 minutes.
5. Add 114 µl/well of blocking buffer to a fresh 96-well cell culture plate.
6. Remove the 96-well cell culture plate containing ET-1-stimulated iCell Cardiomyocytes from the incubator.
7. Transfer 6 µl/well of spent medium to the 96-well cell culture plate containing 114 µl of blocking buffer to make 1:20 dilutions. These dilutions are the test samples.
8. Aspirate the blocking buffer from the ELISA plate. Leave at least 3 negative control wells containing only blocking buffer.

   Note: *It is not necessary to wash the ELISA plate after the incubation with blocking buffer.*
9. Transfer 100 µl/well of the test samples to the appropriate wells of the ELISA plate.

10. Transfer 100 µl/well of the ELISA standard titration (in duplicate) to the appropriate wells of the ELISA plate.
11. Incubate the ELISA plate containing test samples, ELISA standard titration, and negative control wells at room temperature for 2 hours, protected from light.

    Note: *Alternatively, incubate the ELISA plate at 4°C overnight.*
12. Aspirate solutions from all wells of the ELISA plate. Wash 3 times with 200 µl/well of wash buffer. Remove any remaining buffer by blotting the ELISA plate upside down against a paper towel after the third wash.
13. Dilute the detection antibody in 10 ml of D-PBS to a final concentration of 1 µg/ml.
14. Add 100 µl/well of diluted detection antibody to the ELISA plate.
15. Incubate at room temperature for 1 hour, protected from light.
16. Aspirate the diluted detection antibody. Wash 3 times with 200 µl/well of wash buffer. Remove any remaining buffer by blotting the ELISA plate upside down against a paper towel after the third wash.
17. Perform one final wash with 200 µl/well of D-PBS. Remove any remaining buffer by blotting the ELISA plate upside down against a paper towel.
18. Prepare the substrate solution by mixing 5 ml of TMB Peroxidase Substrate and 5 ml of Peroxidase Substrate Solution B in a clean container.
19. Add 100 µl/well of substrate solution. Gently tap the plate to mix.
20. Incubate the ELISA plate at room temperature for at least 20 minutes.

    Note: *A blue color should develop over time indicating the presence of BNP.*
21. Stop the reaction by adding 100 µl/well of TMB Stop Solution.

    Note: *The addition of TMB Stop Solution halts the blue color development and turns the TMB Peroxidase Substrate yellow.*
22. Read the ELISA plate within 30 minutes using a microtiter plate reader to measure the absorbance at a wavelength of 450 nm.

Data Analysis

1. Calculate a standard curve by averaging the raw absorbance values from each of the ELISA BNP standard concentrations.
2. Generate a plot with the absorbance values on the Y-axis and the ELISA BNP standard concentrations on the X-axis (logarithmic scale).
3. Fit the standard curve using a 4 parameter logistics (4PL) nonlinear regression model.
4. Solve the resulting curve fitting equation for “X” and enter the test sample absorbance values as “Y.” This calculation yields the concentration of BNP detected in that well.

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5. Generate a dose-response curve for the hypertrophic response of iCell Cardiomyocytes induced by ET-1 by plotting the concentration of secreted BNP in fmol/ml (Y-axis) against the log concentration of ET-1 treatment (X-axis).
6. Analyze the data further by fitting the curve to generate an EC_{50} value from the ET-1 dose-response (Figure 1).

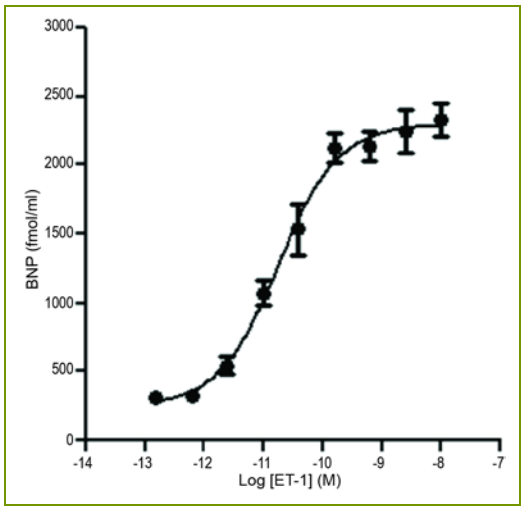

Figure 1: ELISA Detection of BNP Secretion from iCell Cardiomyocytes after ET-1 Addition and Incubation
In this representative experiment, a dose-dependent increase in secreted BNP occurred after stimulation with the indicated concentrations of ET-1 as detected by ELISA. iCell Cardiomyocytes were assayed on day 5 post-plating. The EC_{50} value for ET-1 was 18 pM (mean ± SEM; n = 3 wells 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 secretion of BNP by ELISA 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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Version: January 2014
AP-CMHYPELI140105