Cellular Dynamics international

iCell® Cardiomyocytes
Application Protocol

Modeling Cardiac Hypertrophy:

Endothelin-1 Induction with qRT-PCR 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 *NPPB* expression using quantitative real-time PCR (qRT-PCR). This protocol should serve as a guide for detecting the regulated expression of other fetal genes (e.g. NPPA) in iCell Cardiomyocytes. 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

Notes

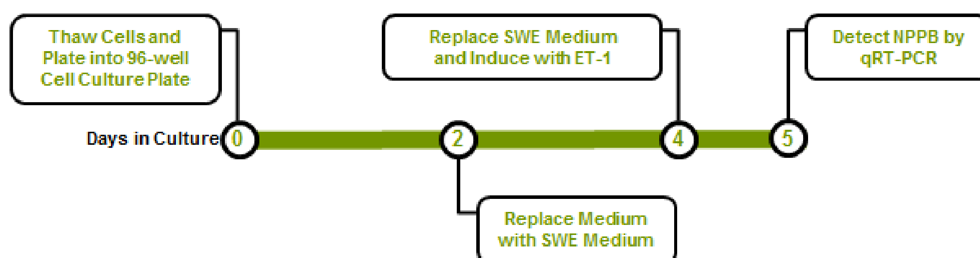
The following equipment and consumables are required in addition to the materials specified in the iCell Cardiomyocytes User's Guide.

Item	Vendor	Catalog Number
Equipment		
Multichannel Pipettor	Multiple Vendors	N/A
Quantitative Real-time PCR (qRT-PCR) Thermal Cycler	Multiple Vendors	N/A
Consumables		
iCell Cardiomyocytes Kit	Cellular Dynamics International (CDI)	CMC-100-010-001 CMC-100-010-005
384-well Nuclease-free PCR Multiwell Plate	Multiple Vendors	N/A
96-well Cell Culture Plate	Multiple Vendors	N/A
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
Nuclease-free Microcentrifuge Tubes	Multiple Vendors	N/A
Nuclease-free Water	Multiple Vendors	N/A
TaqMan Gene Expression Assay for B2M detection (Control Gene TaqMan Assay)*	Life Technologies	Hs000984230_m1
TaqMan Gene Expression Assay for NPPB detection (NPPB TaqMan Assay)	Life Technologies	Hs00173590_m1
TaqMan Gene Expression Cells-to-C_T Kit	Life Technologies	AM1728
William's E Medium	Life Technologies	A12176

* Other control genes that can be used include ACTB or GAPDH.

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 analyzed for NPPB expression using qRT-PCR. 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; +1 (877) 320-6688 (US toll-free) or (608) 310-5100) for instructions on extending the assay workflow.



Notes

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.

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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.*

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 NPPB 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.

Data Acquisition and Analysis

The following sections detail how to prepare cell lysates, perform reverse transcription, and run qRT-PCR for data acquisition and then how to perform data analysis.

Preparing Cell Lysates from iCell Cardiomyocytes

The optimal time to detect ET-1-induced NPPB expression in iCell Cardiomyocytes is 18 hours post-treatment. This procedure is modified from the TaqMan Gene Expression Cells-to-C_T Kit User Guide.

1. Remove the 96-well cell culture plate containing ET-1-stimulated iCell Cardiomyocytes from the incubator.
2. Aspirate the spent medium. Wash with 100 µl/well of cold D-PBS.
3. Add 50 µl/well of Lysis Solution. Triturate the cells 5 times using a multichannel pipettor set at 35 µl to avoid introduction of air bubbles.
4. Incubate at room temperature for 5 minutes.

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5. Add 5 µl/well of Stop Solution. Mix the solution 5 times.
6. Incubate at room temperature for 5 minutes.

   Note: *If necessary, store cell lysates on ice for up to 2 hours or at -20°C for up to 5 months.*

Performing the Reverse Transcription

This procedure is modified from the TaqMan Gene Expression Cells-to-C_T Kit User Guide.

1. Calculate the number of reverse transcription (RT) reactions required to complete the experiment.
2. Prepare the RT master mix (including ~10% overage) in a nuclease-free microcentrifuge tube:

Component	Volume per Reaction
2X RT Buffer	25 µl
20X RT Enzyme Mix	2.5 µl
Nuclease-free Water	12.5 µl
Total Volume	40 µl

3. Gently (but thoroughly) mix by aspirating and dispensing the RT master mix 2 - 3 times. Briefly centrifuge to collect the contents at the bottom of the tubes. Place the RT master mix on ice.
4. Add 40 µl/well of RT master mix to a nuclease-free PCR multiwell plate.
5. Add 10 µl/well of cell lysate to the appropriate wells containing the RT master mix for a final reaction volume of 50 µl.
6. Gently mix by aspirating and dispensing the reaction 2 - 3 times. Briefly centrifuge to collect the contents at the bottom of the wells.
7. Incubate in a qRT-PCR thermal cycler at 37°C for 60 minutes to produce cDNA.
8. Incubate in a qRT-PCR thermal cycler at 95°C for 5 minutes to inactivate the RT enzyme.

   Note: *If necessary, store cDNA at -20°C.*

Running the qRT-PCR

1. Calculate the number of PCR reactions required to complete the experiment.
2. Prepare the PCR master mix (including ~10% overage) in a nuclease-free microcentrifuge tube:

Component	Volume per Reaction
TaqMan Gene Expression Master Mix (2X)	5 µl
NPPB TaqMan Assay (20X) -or- Control Gene TaqMan Assay (20X)	0.5 µl
Nuclease-free Water	2.5 µl
Total Volume	8 µl

Notes

3. Add 8 µl/well of PCR master mix to a 384-well nuclease-free PCR multiwell plate at room temperature.
4. Add 2 µl/well of cDNA to the appropriate wells containing the PCR master mix for a final reaction volume of 10 µl.
5. Gently mix by aspirating and dispensing the reaction 2 - 3 times. Briefly centrifuge to collect the contents at the bottom of the wells.
6. Program the qRT-PCR thermal cycler:

Step	Stage	Reps	Temp	Time
UDG Incubation (hold)	1	1	50°C	2 min
Enzyme Activation (hold)	2	1	95°C	10 min
PCR (cycle)	3	40	95°C	15 sec
			60°C	1 min

7. Run reactions in the qRT-PCR thermal cycler.

Data Analysis

Refer to the guide for the qRT-PCR thermal cycler for data analysis instructions.

1. Average the resulting threshold cycle (C_T) values for each reaction.
2. Determine the NPPB expression relative to the control gene (B2M) expression.
3. Normalize the NPPB expression levels to unstimulated cells and generate a dose-response curve by plotting the fold induction of the ET-1-induced NPPB expression (Y-axis) against the log concentration of ET-1 (X-axis) (Figure 1).

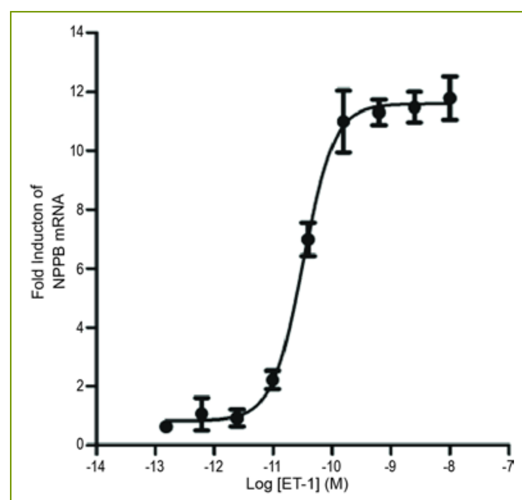

Figure 1: qRT-PCR Detection of NPPB Expression in iCell Cardiomyocytes after ET-1 Addition and Incubation
In this representative experiment, a dose-dependent increase in NPPB expression occurred after stimulation with the indicated concentrations of ET-1 as detected by qRT-PCR. Acquisition and analysis were performed using a LightCycler 480 System (Roche Applied Science). iCell Cardiomyocytes were assayed on day 5 post-plating. The EC_{50} value for ET-1 was 32 pM (mean ± SEM; n = 3 for each point on the curve).

Notes

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 the NPPB gene by qRT-PCR after stimulation of a hypertrophic response with ET-1.

References

1. Swynghedauw B. (1999) Molecular Mechanisms of Myocardial Remodeling. Physiol Rev 79.
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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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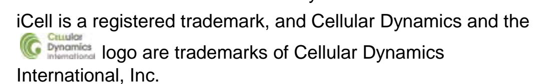

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