StemXVivo™

Cardiomyocyte Differentiation Kit

Catalog Number SC032

Reagents for the directed differentiation of human pluripotent stem cells into the cardiomyocyte lineage.

This package insert must be read in its entirety before using this product.
For laboratory research use only. Not for diagnostic use.
The safety and efficacy of this product in diagnostic or
other clinical uses has not been established.

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INTRODUCTION

Pluripotent stem cells, including both embryonic and induced pluripotent stem cells, provide much promise for the generation of sufficient quantities of specialized cells for use in regenerative medicine. Additionally, these cells are an important tool for understanding developmental and disease mechanisms.

The ability of pluripotent stem cells to differentiate into functional cardiomyocytes holds tremendous promise for applications in therapeutics, pharmaceuticals, and developmental biology research. The potentially unlimited supply of cardiomyocytes generated from pluripotent cell sources can be used in high throughput platforms for drug toxicity screening, eliminating harmful drug candidates before clinical trial stages. Additionally, fully characterized differentiated cardiomyocytes may eventually be transplanted as a therapy for heart disease. Studying the process of cardiomyocyte differentiation from pluripotent cells will increase our understanding of the processes involved in embryonic development and organogenesis (1-3).

PRINCIPLE OF THE ASSAY

The StemXVivo™ Cardiomyocyte Differentiation Kit contains specially formulated media supplements and growth factors that can be used to direct differentiation of pluripotent stem cells toward a cardiomyocyte fate. An antibody is included to characterize the extent of differentiation. The quantity of each component in the kit is sufficient to differentiate two 24-well plates, or an equivalent surface area, of pluripotent stem cells.

TECHNICAL HINTS & LIMITATIONS

- FOR LABORATORY RESEARCH USE ONLY. NOT FOR DIAGNOSTIC USE.
- Do not mix or substitute reagents with those from other lots or sources.
- The safety and efficacy of this product in diagnostic or other clinical uses has not been established.
- The quality and differentiation potential of human pluripotent stem cells at the onset of the differentiation protocol are of paramount importance to the efficiency of differentiation.
- If no beating is observed during the differentiation, test the cells' ability to differentiate into mesoderm by either differentiating with the StemXVivo Mesoderm Kit (R&D Systems, Catalog # SC030) or by staining the cells with Goat Anti-Human/Mouse Brachyury Affinity-purified Polyclonal Antibody (R&D Systems, Catalog # AF2085) after the first 24 hours of differentiation (prior to the Day 1 Media Change).
- For high quality pluripotent stem cells with a low propensity to form mesoderm, supplement the Day 0 Cardiomyocyte Differentiation Media with the selective GSK-3 inhibitor CHIR 99021 (Tocris, Catalog # 4423/10). The optimal concentration will vary with each cell line.
- If beating is observed, but with low efficiency, the quality of the starting population should be verified at Day 0 of the differentiation (prior to the Day 0 Media Change). Cell quality can be determined morphologically, by staining with pluripotency markers such as Oct-4A (R&D Systems, Catalog # MAB17591), Nanog (R&D Systems, Catalog # AF1997), SSEA-4 (R&D Systems, Catalog # MAB1435), or others, or functionally by using the Human Pluripotent Stem Cell Functional Identification Kit (R&D Systems, Catalog # SC027) to determine the ability of the starting cell population to differentiate into each of the three germ layers.

PRECAUTION

The acute and chronic effects of overexposure to reagents of this kit are unknown. Safe laboratory procedures should be followed and protective clothing should be worn when handling kit reagents.

MATERIALS PROVIDED & STORAGE CONDITIONS

Store unopened kit at ≤ -70 °C in a manual defrost freezer. Do not use past kit expiration date.

PART	PART #	DESCRIPTION	STORAGE OF OPENED/ RECONSTITUTED MATERIAL
Stem Cell Qualified RGF BME, Pathclear®, 1.5 mL	894860	1.5 mL of frozen reduced growth factor basement membrane extract.	Store at 2-8 °C on ice for up to 1 week or aliquot and store at ≤ -70 °C for up to 3 months.* Avoid repeated freeze-thaw cycles.
Cardiomyocyte Differentiation Base Media Supplement I	390537	2.8 mL of a 50X concentrated solution.	Store at 2-8 °C for up to 2 weeks or aliquot and store at ≤ -20 °C in a manual defrost freezer for up to 6 months.* Avoid repeated freeze-thaw cycles.
Cardiomyocyte Differentiation Base Media Supplement II	390538	10 mL of a 50X concentrated solution.	
Cardiomyocyte Differentiation Cocktail I	967589	1 vial of lyophilized growth factors; enough to make 50 µL of a 500X stock.	Store under sterile conditions at 2-8 °C for up to 1 month or aliquot and store at ≤ -20 °C in a manual defrost freezer for up to 6 months.* Avoid repeated freeze-thaw cycles.
Cardiomyocyte Differentiation Cocktail II	895865	1 vial of lyophilized growth factors; enough to make 100 µL of a 500X stock.	
Cardiomyocyte Differentiation Cocktail III	967590	1 vial of lyophilized growth factors; enough to make 100 µL of a 500X stock.	
Anti-Human Cardiac Troponin T	893409	25 µg of lyophilized mouse anti-human Troponin T monoclonal antibody. Enough to make 2.5 mL of staining solution when used at the suggested concentration of 10 µg/mL.	

*Provided this is within the expiration of the kit.

OTHER SUPPLIES REQUIRED

Materials

- Human pluripotent stem cells
- 24-well culture plates (or other, as needed)
- 60 mm culture plates
- 12 mm coverslips (optional)
- 15 mL and 50 mL centrifuge tubes
- 0.2 µm syringe filter
- 10 mL syringe
- Pipettes and pipette tips
- Serological pipettes
- Glass slides
- Fine pointed curved forceps
- FACS tubes

Reagents

- MEF Conditioned Media (R&D Systems, Catalog # AR005)
- RPMI 1640
- BSA, very low endotoxin
- DMEM/F-12 (1X)
- GlutaMAX™ (Invitrogen, Catalog # 35050-079 or equivalent)
- Penicillin-Streptomycin (optional)
- Phosphate Buffered Saline (PBS)
- Accutase®
- 95% Ethanol
- 4% Paraformaldehyde
- 1% BSA in PBS
- 0.3% Triton X-100, 1% BSA, 10% normal donkey serum in PBS
- Mounting medium (R&D Systems, Catalog # CTS011)
- Secondary antibody for immunocytochemistry (R&D Systems, Catalog # NL007)
- Secondary antibody for flow cytometry (R&D Systems, Catalog # F0102B)
- Isotype control for flow cytometry (R&D Systems, Catalog # MAB003)
- Deionized or distilled water
- Flow Cytometry Fixation/Permeabilization Buffer I (R&D Systems, Catalog # FC007) supplemented with 0.1% Triton™ X-100
- Flow Cytometry Permeabilization/Wash Buffer I (R&D Systems, Catalog # FC005)

Equipment

- 37 °C and 5% CO_2 incubator
- 37 °C water bath
- Centrifuge
- Inverted microscope
- Fluorescence microscope
- Hemocytometer

REAGENT & MATERIAL PREPARATION

Stem Cell Qualified RGF BME, Pathclear - Store tubes of RGF BME at ≤ -70 °C until ready to use. As needed, thaw RGF BME overnight on ice at 2-8 °C. Dilute 1:40 in ice cold stem cell growth media for coating the tissue culture dish prior to seeding cells for differentiation. Dilute 1:60 in ice cold stem cell growth media for overlay procedure on Day (-1). Dilute 1:60 in Cardiomyocyte Differentiation Base Media I to initiate differentiation on Day 0.

0.1% BSA in PBS - Dissolve 10 mg of BSA in 10 mL of PBS. Sterile filter the solution by syringe filter and store at 2-8 °C for up to 3 months.

Cardiomyocyte Differentiation Cocktail I (500X) - Reconstitute with 50 µL of sterile 0.1% BSA in PBS. Mix gently.

Cardiomyocyte Differentiation Cocktail II (500X) - Reconstitute with 100 µL of sterile 0.1% BSA in PBS. Mix gently.

Cardiomyocyte Differentiation Cocktail III (500X) - Reconstitute with 100 µL of sterile 0.1% BSA in PBS. Mix gently.

Cardiomyocyte Differentiation Base Media I - Dilute Cardiomyocyte Differentiation Base Media Supplement I 1:50 with RPMI 1640 containing 1X Penicillin/Streptomycin (optional) and 1X GlutaMAX. Prepare fresh as needed.

Note: *On Day 0, the media should be prepared on ice immediately prior to use.*

Cardiomyocyte Differentiation Base Media II - Dilute Cardiomyocyte Differentiation Base Media Supplement II 1:50 with RPMI 1640 containing 1X Penicillin/Streptomycin (optional) and 1X GlutaMAX. Prepare fresh as needed.

Anti-Human Cardiac Troponin T - Reconstitute with 250 µL of PBS to obtain a 100 µg/mL stock solution. Mix gently.

PROCEDURE OUTLINE

Coat wells with Stem Cell Qualified PathClear RGF BME (RGF BME).
Incubate at room temperature for 1-2 hours.

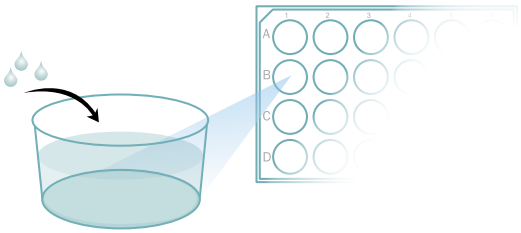
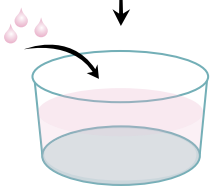
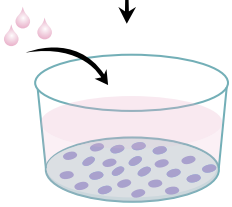


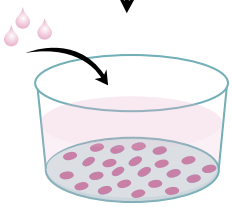
Plate human pluripotent stem cells onto the coated plates at 8-9 x10^4 cells/cm^2 in MEF Conditioned Media containing FGF basic.
Culture cells to 80-90% confluency.



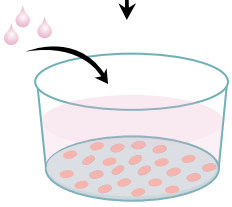
Day (-1) of Differentiation
Replace the stem cell culture media with ice cold stem cell culture media containing RGF BME diluted 1:60.
Incubate at 37 ˚C and 5% CO_2 for 18-24 hours.



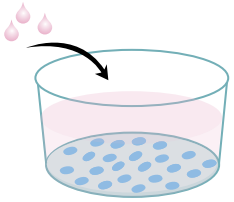
Day 0 of Differentiation
Replace the media with ice cold Day 0 Differentiation Media containing RGF BME diluted 1:60.
Incubate at 37 ˚C and 5% CO_2 for 24 hours.



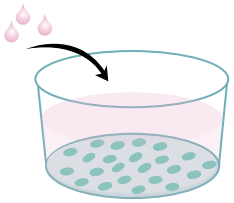
Day 1 of Differentiation
Replace the media with Day 1 Differentiation Media.
Incubate at 37 ˚C and 5% CO_2 for 4 DAYS without media exchange.



Day 5 of Differentiation
Replace the media with Day 5 Differentiation Media.
Incubate at 37 ˚C and 5% CO_2 for 2 DAYS without media exchange.



Day 7 of Differentiation and Beyond
Replace the media with Cardiomyocyte Differentiation Base Media II.
Incubate at 37 ˚C and 5% CO_2.
Replace media every 1-2 days as needed.



UNDIFFERENTIATED CELL PREPARATION

Note: *This protocol is designed for BG01V human embryonic stem (hES) cells grown in MEF Conditioned Media (Catalog # AR005) and differentiated in 24-well culture dishes on coverslips. If using different cell lines or growth media, the protocol below may need to be modified. If using different culture vessels, additional optimization may be required to determine appropriate volumes of media.*

The quality of the human pluripotent cells used in the differentiation is imperative. Use of suboptimal quality or very high passage pluripotent cells can result in decreased differentiation efficiency and/or increased cell death.

COATING PLATES WITH RGF BME

1. Thaw Stem Cell Qualified PathClear RGF BME (RGF BME) on ice at 2-8 °C overnight.
2. Dilute the RGF BME 1:40 in ice cold DMEM/F-12. This can be stored at 2-8 °C for up to 2 weeks.
3. If desired, place a sterile coverslip (sterilized with 95% ethanol and flamed) in each well of a 24-well plate for downstream immunocytochemical analysis.
4. Coat the desired number of wells or plates with diluted RGF BME (0.5 mL/well for a 24-well plate) and incubate for 1-2 hours at room temperature.

CELL DISSOCIATION

1. Warm the MEF Conditioned Media to 37 °C.
2. Remove the existing media from the cells. Add 1 mL of Accutase solution to each 60 mm plate or 3 mL to each 100 mm plate. Incubate at room temperature for 2-5 minutes or until the cells begin to slough off the plate. If using cells from several plates, work in small batches (1-2 plates at a time) so the cells are not exposed to the Accutase beyond the time it takes the cells to slough off the plate.
3. Pipette gently over the plate until the cells become detached.
4. Gently pipette the cell suspension up and down to break up large cell clumps.
5. Transfer the cell suspension to a 15 mL centrifuge tube containing 5 mL of MEF Conditioned Media (or 12 mL if using a 100 mm plate) and spin at 200 x g for 4 minutes.

CELL PLATING

1. Resuspend the pellet in MEF Conditioned Media containing 4 ng/mL of FGF basic, and count the viable cells using Trypan Blue and a hemocytometer.
2. Plate the cells onto prepared RGF BME-coated plates at a concentration of 8-9 x 10^4 cells/cm^2, or an appropriate seeding density to reach 80-90% confluence within 2-3 days.

   Note: *If using cells cultured on a fibroblast feeder layer, it may be necessary to include feeder depletion prior to seeding.*
3. Allow the cells to attach and grow at 37 °C and 5% CO_2 with daily media exchanges until the monolayer reaches approximately 80% confluence.

CARDIOMYOCYTE DIFFERENTIATION PROCEDURE

RGF BME Overlay: Day (-1) through Day 0

1. Pluripotent cells should be grown to approximately 80% confluence on RGF BME prior to the initiation of differentiation.
2. For this step, MEF Conditioned Media should be kept on ice and used ice cold.
3. One tube of RGF BME should be thawed overnight and ready for dilution (see Reagent and Material Preparation on Page 5).
4. Quickly dilute an appropriate amount of RGF BME 1:60 in ice cold stem cell growth media (MEF Conditioned Media with 4 ng/mL FGF basic). For 24-well plates, use 0.5 mL/well.
5. Quickly remove and discard spent media and add the RGF BME-containing media.

   Note: *Steps 4 and 5 should be completed as quickly as possible, without sacrificing sterile technique, to prevent the RGF BME from starting to gel into solid clumps in the media.*
6. Culture the cells overnight at 37 °C and 5% CO_2.
7. RGF BME can be kept on ice at 2-8 °C for up to 1 week. Avoid repeated freeze/thaw cycles.

RGF BME Overlay and Differentiation Initiation: Day 0 through Day 1

1. Prepare Day 0 Cardiomyocyte Differentiation Media as follows:
   a. Determine beforehand the appropriate total volume of media needed. For 24-well plates, use 0.5 mL/well.
   b. Pipette the needed volume of ice cold Cardiomyocyte Differentiation Base Media I into a sterile centrifuge tube.
   c. Quickly dilute RGF BME 1:60 into the ice cold Media.
   d. Quickly dilute the Cardiomyocyte Differentiation Cocktail I 1:500 into the ice cold Media.
2. Remove and discard the spent media and add the Day 0 Cardiomyocyte Differentiation Media to each well.

   Note: *Steps 1 and 2 should be completed as quickly as possible, without sacrificing sterile technique, to prevent the RGF BME from starting to gel into solid clumps in the media.*
3. Culture the cells overnight at 37 °C and 5% CO_2.

CARDIOMYOCYTE DIFFERENTIATION PROCEDURE *CONTINUED*

Differentiation Days 1 through 5

1. Warm the Cardiomyocyte Differentiation Base Media I to 37 °C.
2. Dilute the Cardiomyocyte Differentiation Cocktail II 1:500 in the appropriate volume of Cardiomyocyte Differentiation Base Media I. For 24-well plates, use 1 mL of media/well.
3. Remove and discard the spent media and add the Day 1 Cardiomyocyte Differentiation Media to each well.
4. Culture the cells for four days (Day 1-5) at 37 °C and 5% CO_2 without media exchange.

   Note: *The media will turn very yellow as the pH increases, but it is imperative to not remove the media.*

Differentiation Days 5 though 7

1. Warm the Cardiomyocyte Differentiation Base Media I to 37 °C.
2. Dilute the Cardiomyocyte Differentiation Cocktail III 1:500 in the appropriate volume of Cardiomyocyte Differentiation Base Media I. For 24-well plates, use 1 mL of media/well.
3. Remove and discard the spent media and add the Day 5 Cardiomyocyte Differentiation Media to each well.
4. Culture the cells for two days (Day 5-7) at 37 °C and 5% CO_2 without media exchange.

Differentiation Day 7 and beyond

1. Warm the Cardiomyocyte Differentiation Base Media II to 37 °C.
2. Remove and discard the spent media and add the Cardiomyocyte Differentiation Base Media II to each well. For 24-well plates, use 1 mL/well.
3. Culture the cells at 37 °C and 5% CO_2. Exchange the media every one to two days.

   Note: *Initially, media exchange every second day is standard. As beating cells begin to emerge, it may become necessary to exchange the media every day.*

4. This kit is designed to direct pluripotent stem cell differentiation toward a cardiomyocyte cell fate. For extended maintenance of these differentiated cells, StemXVivo Cardiomyocyte Maintenance Media (50X) (R&D Systems, Catalog # AR011) can be used.

FIXING & STAINING PROCEDURES

Immunocytochemistry

1. Wash the cells twice with PBS (1 mL/well for a 24-well plate).
2. Fix the cells with 4% paraformaldehyde in PBS for 20 minutes at room temperature.
3. Wash the cells 3 times with 1% BSA in PBS for 5 minutes (0.5 mL/well for a 24-well plate).
4. Permeabilize and block the cells with 0.3% Triton X-100, 1% BSA, and 10% normal donkey serum in PBS at room temperature for 45 minutes (0.5 mL/well for a 24-well plate).
5. During the blocking, dilute the reconstituted Anti-Human Cardiac Troponin T Primary Antibody to a final concentration of 10 ug/mL in PBS containing 0.3% Triton X-100, 1% BSA, and 10% normal donkey serum.

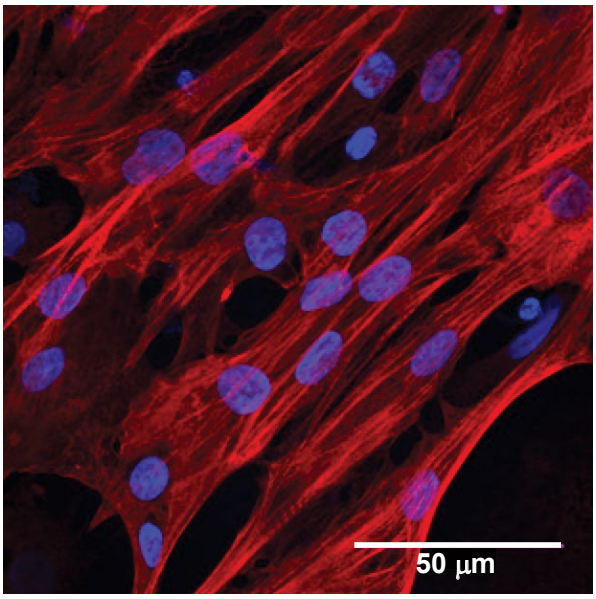
   Note: *A negative control should be performed using PBS containing 0.3% Triton X-100, 1% BSA, and 10% normal donkey serum in the absence of a primary antibody.*
6. After blocking, incubate the cells with diluted Anti-Human Cardiac Troponin T Primary Antibody (300 µL/well of a 24-well plate) for 3 hours at room temperature or overnight at 2-8 °C.
7. Wash the cells 3 times with 1% BSA in PBS for 5 minutes (0.5 mL/well of a 24-well plate).
8. Dilute the secondary antibody [*e.g.*, NL557-conjugated Donkey Anti-Mouse Secondary Antibody (R&D Systems, Catalog #NL007)] at 1:200 in PBS containing 1% BSA.
9. Incubate the cells with diluted secondary antibody in the dark for 60 minutes at room temperature (300 µL/well of a 24-well plate).
10. Wash the cells 3 times with 1% BSA in PBS for 5 minutes (0.5 mL/well of a 24-well plate).
11. Cover the cells with PBS (1 mL/well of a 24-well plate) and visualize with a fluorescence microscope. Alternatively, aspirate the PBS and add distilled or deionized water (0.5 mL/well of a 24-well plate). Carefully remove each coverslip with forceps and mount cell-side down onto a drop of mounting medium on a glass slide.
12. Slides are ready for microscopic observation.

Flow Cytometry

1. Rinse the cells with PBS (for 24-well plates, use 1 mL/well).
2. Harvest differentiated (beating) cells from the desired number of wells into single-cell suspension using dissociation solution (*e.g.*, Trypsin or Accutase) and centrifuge at 300 x g for 5 minutes. Wash the cell pellet 2 times with PBS, and then decant the buffer.
3. Resuspend $5x10^5$ cells in 0.5 mL of Fixation/Permeabilization Buffer I supplemented with 0.1% Triton X-100 and incubate at room temperature for 30 minutes. Vortex the cells intermittently to maintain a single cell suspension.
4. Centrifuge the cells at 300 x g for 5 minutes and resuspend in Permeabilization/Wash Buffer I at $1x10^6$ cells per 100 mL. Transfer 100 µL of the cell suspension into separate FACS tubes.
5. Add 10 µL of the reconstituted Anti-Human Cardiac Troponin T Primary Antibody to the cells for a final concentration of 10 µg/mL. Pipette gently or vortex briefly to mix. Incubate the cells for 30 minutes at room temperature.

   Note: *As a control for analysis, cells in a separate tube should be treated with a flow cytometry isotype control (R&D Systems, Catalog # MAB003).*
6. Following incubation, wash the samples twice with Flow Cytometry Permeabilization/Wash Buffer I as in step 3.
7. Resuspend the cells in 100 µL of Permeabilization/Wash Buffer I and add an appropriate secondary developing reagent such as Goat Anti-Mouse IgG Phycoerythrin (R&D Systems, Catalog # F0102B), and incubate in the dark for 30 minutes at room temperature.
8. Following incubation, wash the samples twice with Flow Cytometry Permeabilization/Wash Buffer I as in step 3.
9. Resuspend the cells in 200-400 µL of PBS for flow cytometric analysis.

DATA EXAMPLES



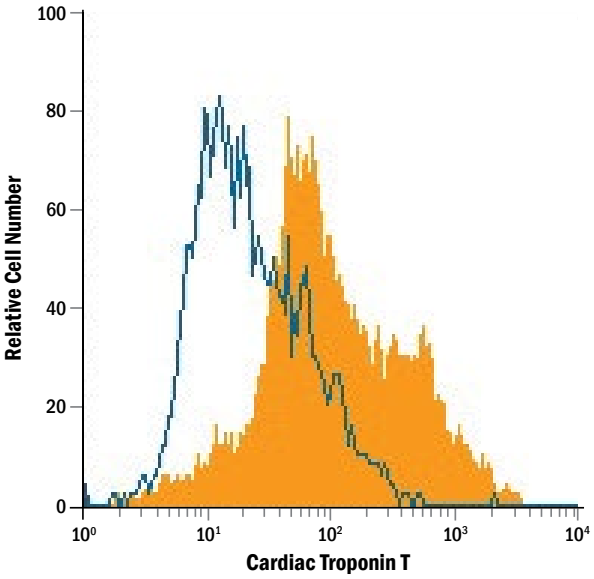


Figure 1: Differentiation of Pluripotent Stem Cells into Cardiomyocytes. BG01V human embryonic stem cells were differentiated into cardiomyocytes using the media supplements included in this kit. Aside from visually observing contracting cells, commitment to the cardiomyocyte cell fate was evaluated by labeling with the Anti-Human Cardiac Troponin T antibody included. For immunocytochemistry (left panel), the cells were stained using NorthernLights™ 557-conjugated Donkey anti-Mouse secondary antibody (R&D Systems, Catalog # NL007; red), and the nuclei were counterstained with DAPI (blue). For flow cytometry (right panel), the cells were stained using Goat Anti-Mouse IgG PE-Conjugated secondary antibody (R&D Systems, Catalog # F0102B; filled histogram) and compared to matched isotype control (open histogram).

REFERENCES

1. Dambrot, C. *et al.* (2011) Biochem. J. **434**: 25.
2. Laflamme, M.A. *et al.* (2007) Nat. Biotech. **25**: 1015.
3. Xu C. *et al.* (2002) Circ. Res. **91**: 501.

NOTES

NOTES

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