

## Automated Optimization of Murine Embryonic Stem Cell Differentiation into Cardiomyocytes

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Blood Banking  
Capillary Electrophoresis  
Centrifugation  
Flow Cytometry  
Genomics  
**Lab Automation**  
Lab Tools  
Particle Characterization

### Abstract

Pure populations of cardiomyocytes derived from embryonic stem cells offer great promise as potential cell replacement therapies, as well as for use in pharmaceutical studies. The differentiation process, however, is frequently inefficient and nonspecific. We sought to improve upon the current processes by coupling automation and Design of Experiment (DOE). DOE-generated combinations of pro-cardiomyocyte compounds were converted to dispensing volumes using Automated Assay Optimization (AAO) for BioRAPTR<sup>®</sup> software, and reagents and cells were dispensed into 384-well plates using the BioRAPTR FRD<sup>®</sup> Microfluidic Workstation. After five days of culture, the resulting embryoid bodies (EBs) were transferred to 96-well gelatin-coated plates using the Biomek FX<sup>®</sup> liquid handler. Over an additional three days, the wells were observed for spontaneously beating regions of cardiomyocytes, and the differentiated cells were further analyzed using flow cytometry to detect myosin heavy chain as a marker of cardiomyocytes. This bulletin illustrates the potential of a system that can both automate and optimize the differentiation of murine embryonic stem cells.

### Materials and Methods

#### Murine Embryonic Stem Cell (mESC) Differentiation

129 mESCs (Invitrogen) were maintained as directed and differentiated in 15% FBS medium without LIF. For differentiation experiments (Figure 1), the BioRAPTR FRD Workstation was used to dispense 500 cells/well into 384-well polypropylene plates (Nunc) in a culture volume of 40  $\mu$ L/well. These plates prevented cell adherence, and the suspension cells coalesced into a single embryoid body (EB) per well (Figure 2A). An additional 80  $\mu$ L/well of media was added, and on day 5, the EBs were transferred to 96-well gelatin-coated tissue culture plates containing 100  $\mu$ L fresh media (~200  $\mu$ L total volume), using the multi-channel pipettor of the Biomek FX<sup>®</sup> Workstation. The EBs adhered to the well surface, allowing for further differentiation. Cultures were analyzed for cardiomyocytes after 8 total days of culture (3 days post-adherence).

#### Cardiomyocyte Optimization/Experimental Design

To optimize ES cell differentiation into a cardiomyocyte lineage, combinations of pro-cardiomyocyte compounds and proteins were tested using DOE methodology.

Design-Expert (Stat-Ease) software was used to generate test conditions using a D-Optimal Factorial design and these conditions were converted to reagent volumes using AAO for BioRAPTR software. Reagents were dispensed to 384-well plates, along with cells and media using the BioRAPTR dispenser as referenced above.

#### Assays

**Beating Cells:** Cardiomyocytes are capable of contracting spontaneously, and bright field microscopy was used to detect motion in the differentiated cell cultures as an initial measure of cardiomyocyte formation (Figures 2B, 5B).

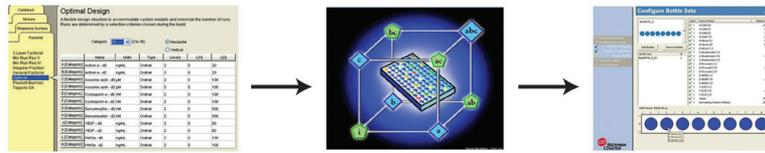
**Sarcomere Staining:** The presence of sarcomeres was used to confirm the identity of cardiomyocytes in the differentiated cells. Cells were processed using FIX & PERM Cell Fixation and Permeabilization Kit (Invitrogen) and blocked with 2% goat serum. Sarcomeres were stained with Alexa 488-conjugated anti-myosin heavy chain antibody (clone MF20, 1:500, eBioscience) (Figures 2C, 2D). Images were generated on an EVOS fluorescent microscope (AMG).

**Flow Cytometry:** The Biomek FX<sup>®</sup> multi-channel pipettor was used to release cells from the plate through ACCUMAX (Millipore) treatment coupled with robust pipetting. A Biomek NX<sup>®</sup> with Span-8 Pipettor was used to stain cells for myosin using the reagents described above (please refer to Beckman Coulter Information Bulletin IB-15496A for more details). Samples were run on a FC500 MPL cytometer and the resulting data were analyzed using Kaluza<sup>+</sup> software. Cardiomyocytes were identified as a highly positive myosin population (Figure 3). The percentage of cells in this population was used for the cardiomyocyte optimization screens (Figure 4).

**Quantitative PCR:** Optimal differentiation conditions were validated by measuring the induction of cardiomyocyte-specific gene expression (Figure 5A). Differentiated cells from 8 wells per condition were combined, and total RNA was extracted using the SV Total RNA Isolation System (Promega). RNA was reverse transcribed using the High Capacity cDNA Reverse Transcription Kit (Applied Biosystems) and gene expression was analyzed using TaqMan probes and a 7900 Real-Time PCR System (Applied Biosystems). Relative gene expression was determined using the  $\Delta\Delta$ Ct method with GAPDH as a reference gene.

# Automated Optimization of mESC Differentiation

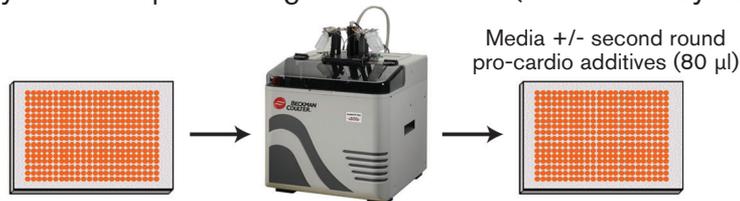
Design experiment (Design-Expert + AAO for BioRAPTR Software)



Day 0 – Dispense reagents and mES cells (BioRAPTR System)



Day 1-4 – Dispense reagents and media (BioRAPTR System)



Day 5 – Transfer EBs to 96-well gelatin-coated plates (Biomek MC Workstation)

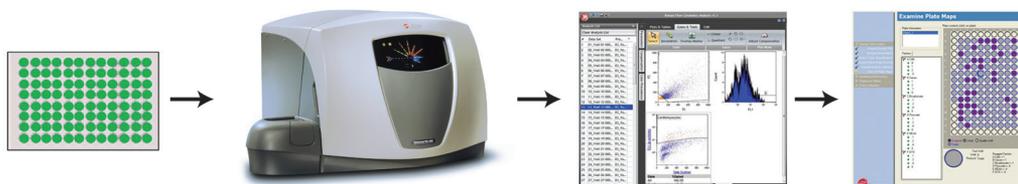


Day 8 – Prep cells for flow cytometry analysis (Biomek Span-8 Workstation)

For more information on this process, see Beckman Coulter Information Bulletin IB-15496A

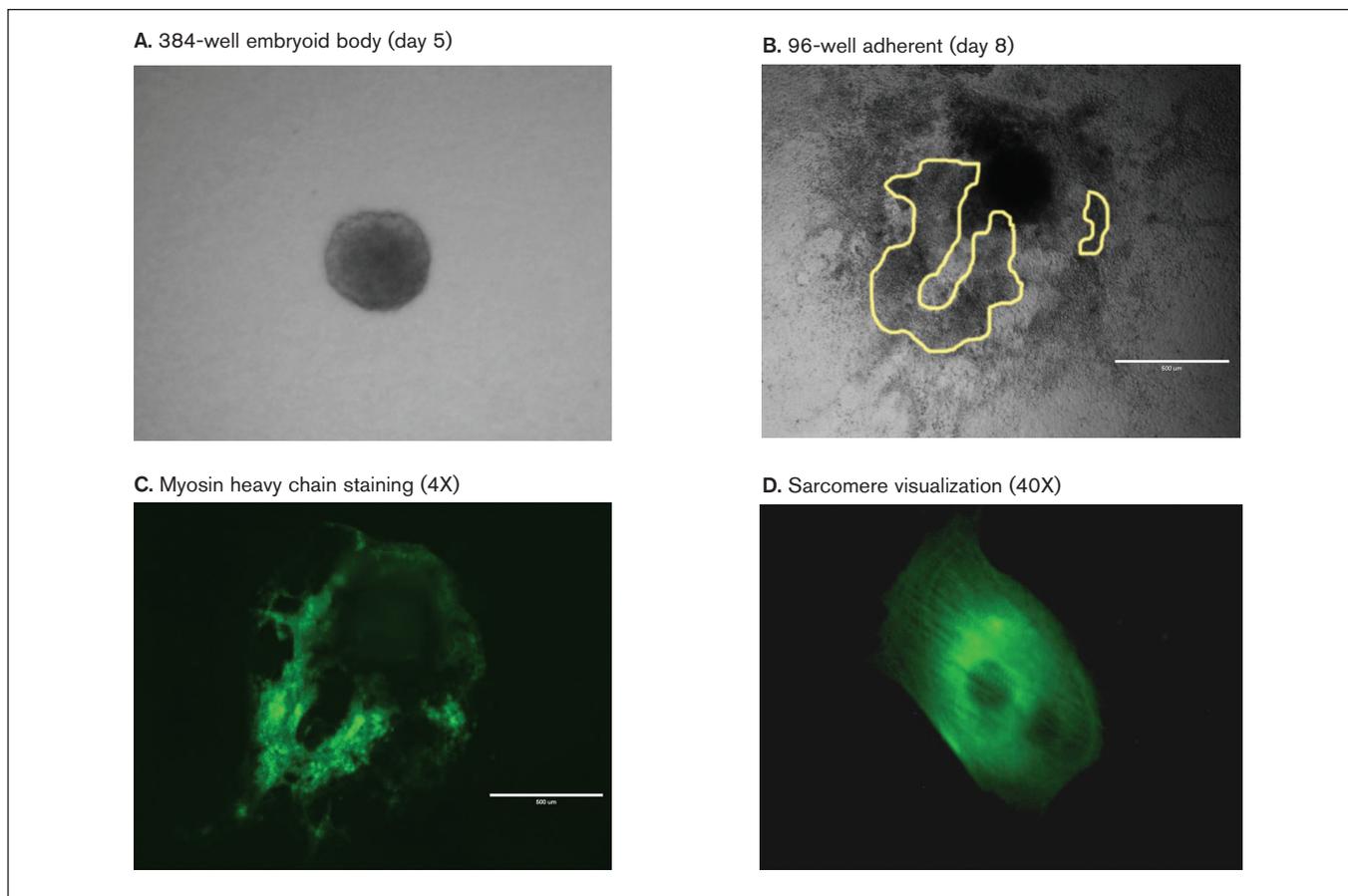


Day 8 – Assay cells on plate-based flow cytometer (FC500 MPL), analyze data (Kaluza software) and deconvolute screen results (AAO for BioRAPTR software)



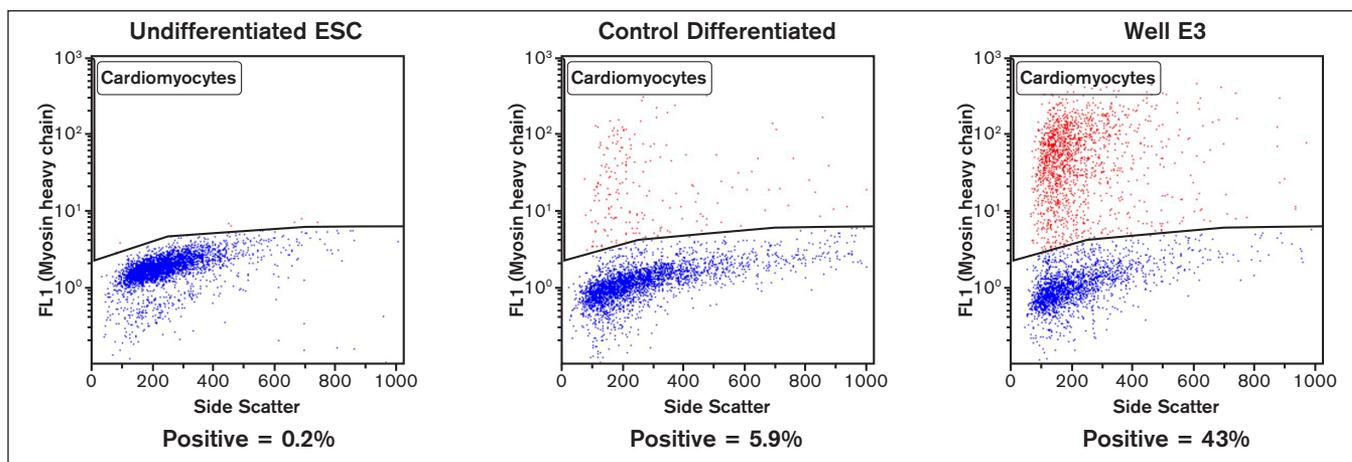
**Figure 1.** Workflow for the automation and optimization of mESC differentiation into cardiomyocytes.

## Results – Optimization Of Cardiomyocyte Differentiation Microplate Format for ESC Differentiation



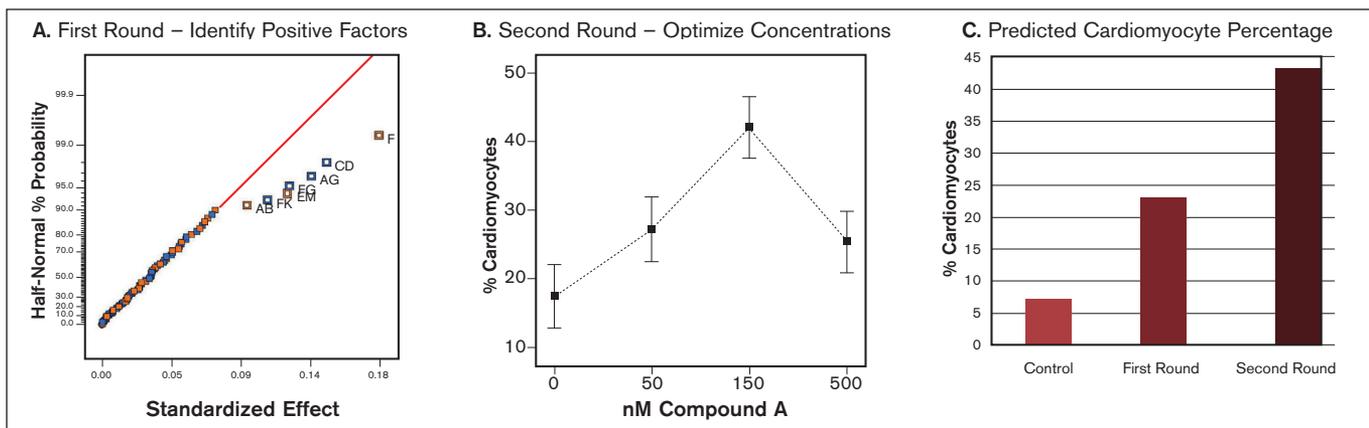
**Figure 2.** Images of mESCs during microtiter plate-based cardiomyocyte differentiation. **A)** After 5 days of suspension culture in 384-well polypropylene plates, cells have coalesced into a round ball known as an embryoid body (EB). The EBs are transferred to gelatin-coated 96-well tissue culture plates, where they adhere, spread, and differentiate further for 3 days (**B**). Cells that differentiate into cardiomyocytes can beat spontaneously, and these regions are visible by microscopy (yellow outlines). **C)** Additional identification of cardiomyocytes was achieved by staining the cells for myosin heavy chain (MF20 antibody). Positively stained cells most frequently corresponded to beating regions. **D)** Colonies were also dissociated, re-adhered, and stained with MF20 to illustrate sarcomeres, thereby further confirming the specific staining of cardiomyocytes.

### Flow Cytometry Analysis of Differentiated Cells



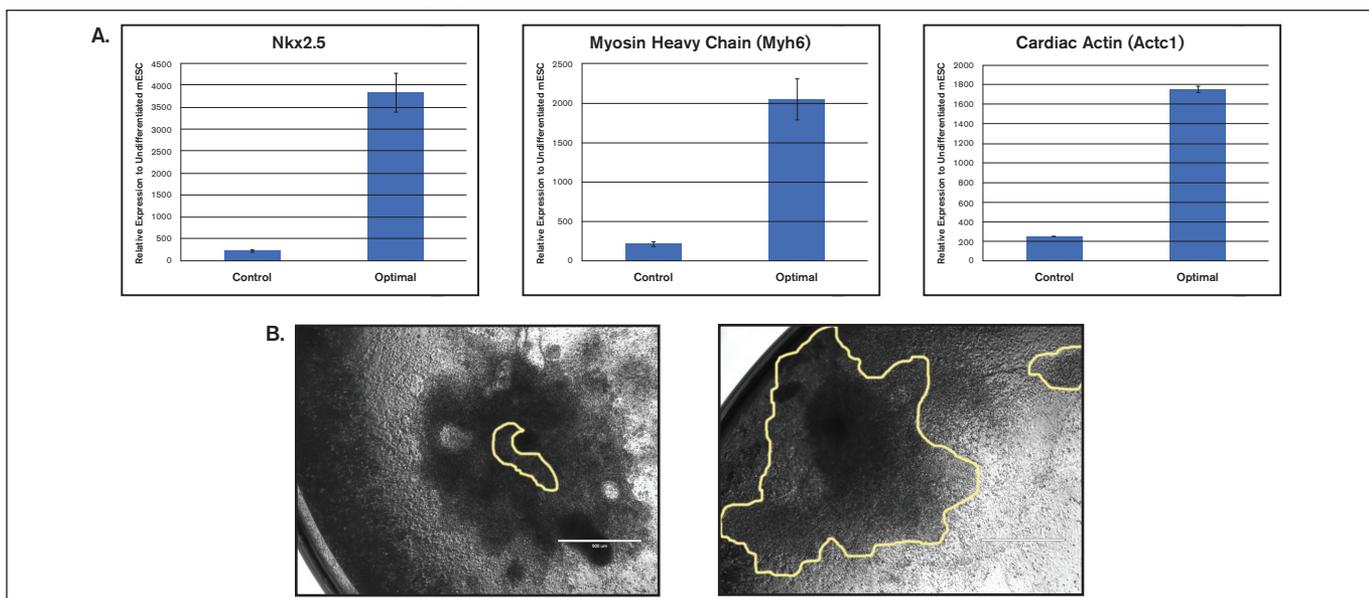
**Figure 3.** Scatter plots illustrating flow cytometry analysis of myosin heavy chain (MF20) staining of differentiated mESCs. The percentage of cardiomyocytes in each sample was quantified by gating on highly positive cell populations (“Cardiomyocytes” gate). Undifferentiated ESCs were negative for myosin staining (Left panel). Under control conditions, roughly 6% of the differentiated cells were positive (Center panel). This analysis method was utilized to screen combinations of various compounds, some of which yielded greater numbers of cardiomyocytes (Right panel - Well E3 from second screen, 43% positive).

## Cardiomyocyte Optimization Screen Results



**Figure 4.** Statistical analysis of screen results using Design-Expert software. **A)** For the initial screen, combinations of 6 compounds were tested to determine which factors promote cardiomyocyte differentiation. A half-normal plot was used to identify the treatments that significantly affect the differentiation of mESCs into cardiomyocytes (open squares), based upon their distance from the normal effect line. **B)** The positive effectors were further optimized in a second round of screening by testing combinations of different concentrations. The effect of varying Compound A concentrations on cardiomyocyte percentage is shown. **C)** The predicted cardiomyocyte percentage from optimal differentiation conditions. After two rounds of optimization, we achieved a six-fold increase in cardiomyocytes, resulting in cardiomyocytes accounting for 43% of cells. (control = average of both rounds).

## Validation of Optimized Cardiomyocyte Differentiation Conditions



**Figure 5.** Further characterization of optimal differentiation conditions. **A)** Quantitative PCR (qPCR) was used to measure expression of cardiomyocyte-specific genes (Nkx2.5, Myh6, Actc1) in cells differentiated under control and optimal conditions. All markers were increased more than two hundred-fold with differentiation (compared to undifferentiated ESCs), validating the microtiter plate differentiation method. Using the optimized differentiation conditions further increased expression of these genes seven- to seventeen-fold, illustrating a dramatic effect of optimization on cardiomyocyte gene expression. **B)** Bright field microscopy images illustrate a larger beating region with the optimal differentiation conditions, further supporting an increase in the number of cardiomyocytes.

## Summary

We have shown that the differentiation of murine embryonic stem cells can be both automated and optimized using a variety of Beckman Coulter products. Diverse experimental conditions were quickly designed and plated using AAO for BioRAPTR software and the BioRAPTR FRD. The Biomek platforms were ideal for both transferring embryoid bodies to adherence plates and for flow cytometry sample preparation. Analysis of these samples using the FC500 MPL flow cytometer and Kaluza software generated the results necessary to identify differentiation conditions that increased the percentage of cardiomyocytes in the cultures. Additional iterations based on these optimization results could be used to further refine and improve the differentiation process.