Monitoring Stem Cell Differentiation and Pluripotency Using the StemElite™ Gene...

Monitoring Stem Cell Differentiation and Pluripotency Using the StemElite™ Gene Expression System

ABSTRACT

In stem cell research, the need for thorough characterization of molecular markers associated with stem cell pluripotency, as well as putative induced pluripotent stem cells, is well recognized. Stem cell differentiation is routinely monitored by measuring gene expression using real-time reverse transcription PCR. The StemElite™ Gene Expression System uses validated primer sets in conjunction with qPCR technology to monitor stem cell differentiation via multiplexed amplification.

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Introduction

Real-time PCR is a quantitative method used to determine the amount of sequence-specific PCR template present in a sample. Known as quantitative PCR, or qPCR, this method detects and measures the PCR product accumulation at each cycle as amplification proceeds. Traditional endpoint-PCR assays are often ineffective at quantitating the amount of starting material in an amplification reaction; qPCR addresses this problem. Monitoring the product formation during the exponential phase of amplification makes for a highly quantitative assay, and allows for a greater degree of sensitivity(1) .

The StemElite™ Gene Expression System (Cat.# B1001) is a novel quantitative PCR system for the detection and relative quantification of RNA expression levels. This system uses fluorescent dye-labeled primers to facilitate multiplexed amplification for qPCR. Multiplex PCR simultaneously amplifies multiple targets by incorporating more than one primer pair in a single reaction. This reduces the number of reactions required for measuring multiple transcripts and improves the quality of the experimental data because all the transcripts are quantified in the same well.

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For each target, one primer contains a non-natural nucleotide (isoC) and a fluorescent label. The second primer is a standard, unmodified oligonucleotide. Thermal cycling is performed in the presence of a modified *Taq* DNA polymerase, four normal dNTPs, and a dabcyl-iso-dGTP. Dabcyl is a potent contact quencher. When the polymerase encounters the isoC nucleotide, it preferentially incorporates the dabcyl-iso-dGTP(2) (3) . The close proximity of the dabcyl and fluorescent reporter results in contact quenching. A single reaction can contain multiple primer pairs; each primer pair is specific to a different target sequence, and labeled with different fluorescent dyes. The dabcyl-iso-dGTP will quench the fluorescence of all the dyes present in the reaction, if incorporated.

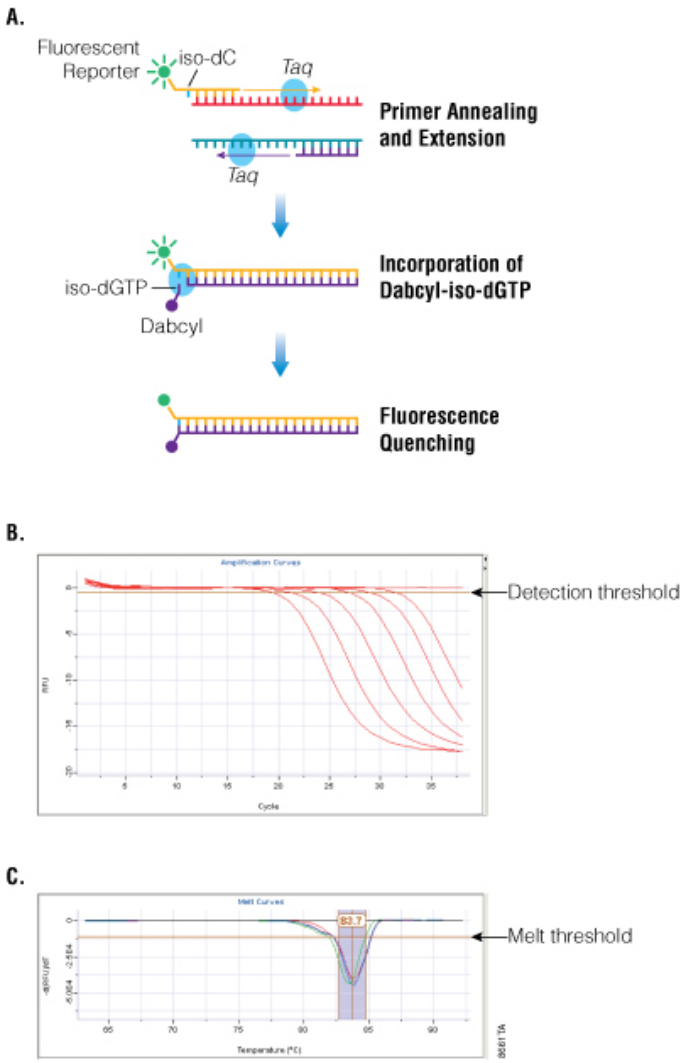

Figure 1. Real-time PCR technology. Panel A. An overview of the qPCR chemistry. One primer contains a non-natural nucleotide (isoC) and a fluorescent label. The second primer is a standard, unmodified oligonucleotide. Thermal cycling is performed in the presence of a modified *Taq* DNA polymerase, four normal dNTPs, and a dabcyl-iso-dGTP. Dabcyl is a potent contact quencher. When the polymerase encounters the isoC nucleotide, it preferentially incorporates the dabcyl-iso-dGTP. The close proximity of the dabcyl and fluorescent reporter results in contact quenching. A single reaction can contain multiple primer pairs; each primer pair is specific to a different target sequence, and labeled with different fluorescent dyes. The dabcyl-iso-dGTP will quench the fluorescence of all the dyes present in the reaction, if incorporated. **Panel B.** The qPCR amplification curves show that fluorescent dyes are quenched as PCR proceeds. This results in a decrease in fluorescence during amplification. The point where the decrease in fluorescence crosses the d etection threshold is the quantification cycle, or C_q. Samples are compared using this number. **Panel C.** qPCR thermal melt analysis occurs after amplification is complete when the amplified product is denatured during a "melt" step. When the product denatures, the quencher molecule is no longer in close proximity to the

fluorescent dye. The denaturation of the product is detected by a sudden increase in fluorescence; this temperature is the T_m. Examining the melt profile enables the user to confirm reaction specificity.

Methods and Results

RNA was extracted from various cell samples using the SV Total RNA Isolation System (Cat.# Z3100). The extraction protocol was modified to add an extra DNase digestion step following RNA purification using RQ1 RNase-Free DNase (Cat.# M6101). To avoid potential amplification of contaminating genomic DNA, the RNA templates should be DNase-treated prior to cDNA synthesis. This was particularly important when using amplification assays that are designed within a single exon (for example, the SOX2 transcript contains no introns).

In preparation for qPCR, first-strand cDNA was synthesized from 100ng of each RNA template using the GoScript™ Reverse Transcription System (Cat.# A5000). Following heat inactivation of the reverse transcriptase, the cDNA was used as the template in StemElite™ Gene Expression System amplification reactions.

MONITORING CELL DIFFERENTIATION

Human embryonic stem cells (H9 line) were treated with various growth factors in three separate experiments to stimulate neural, cardiomyocyte and pancreatic differentiation(4) (5) (6) . Neural cells were collected after differentiating for 10 and 30 days. Cardiac cells were collected after differentiating for 40 days; beating cardiomyocytes were observed. Pancreatic cells were collected after differentiating for 1, 3, 6, 9 and 12 days. The StemElite™ Gene Expression System was used to assess the expression level of pluripotency-associated transcripts for all cell samples. In all cases, expression levels of pluripotency-associated transcripts were observed to decrease as the cells differentiated.

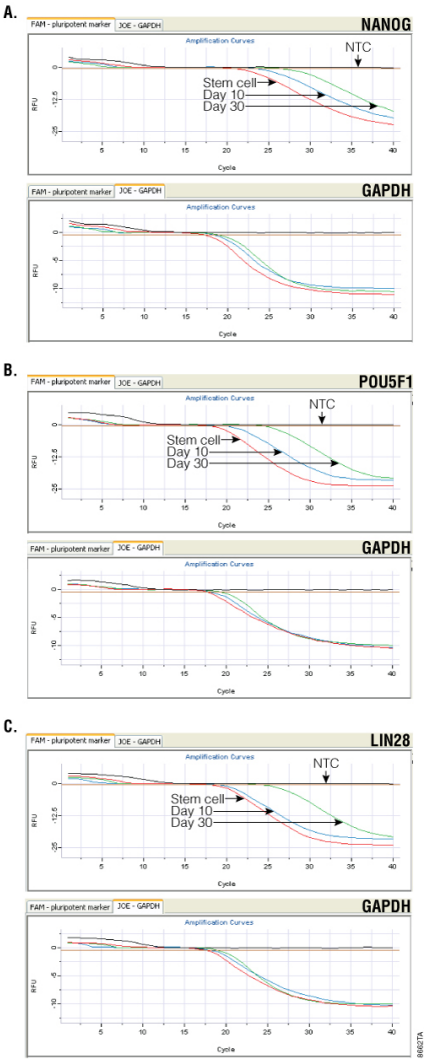

Figure 2. Monitoring neural cell differentiation. In the differentiating neural cell samples, NANOG, POU5F1 and LIN28 were each amplified in a duplex reaction with GAPDH. Gene expression in the differentiated cells was compared to undifferentiated human embryonic stem (hES) cells. **Panel A.** Amplification curves for NANOG and GAPDH duplex reactions. **Panel B.** Amplification curves for POU5F1 and GAPDH duplex reactions. **Panel C.** Amplification curves for LIN28 and GAPDH duplex reactions.

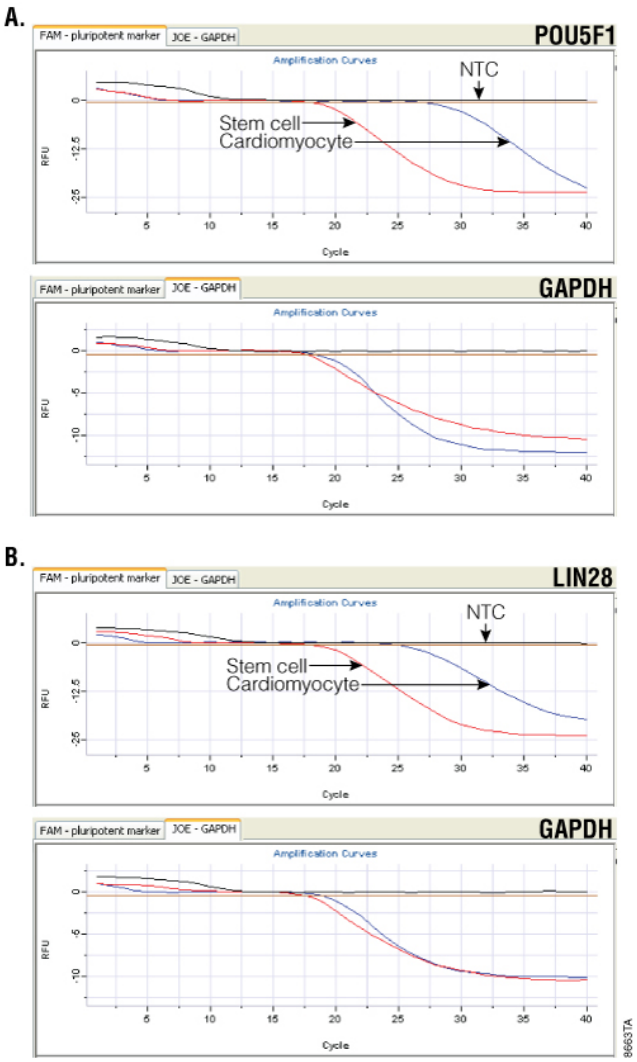

Figure 3. Monitoring cardiomyocyte cell differentiation. In the differentiating cardiac cell samples, POU5F1 and LIN28 were each amplified in a duplex reaction with GAPDH. Gene expression in the differentiated cells was compared to undifferentiated hES cells. **Panel A.** Amplification curves for POU5F1 and GAPDH duplex reaction. **Panel B.** Amplification curves for LIN28 and GAPDH duplex reaction.

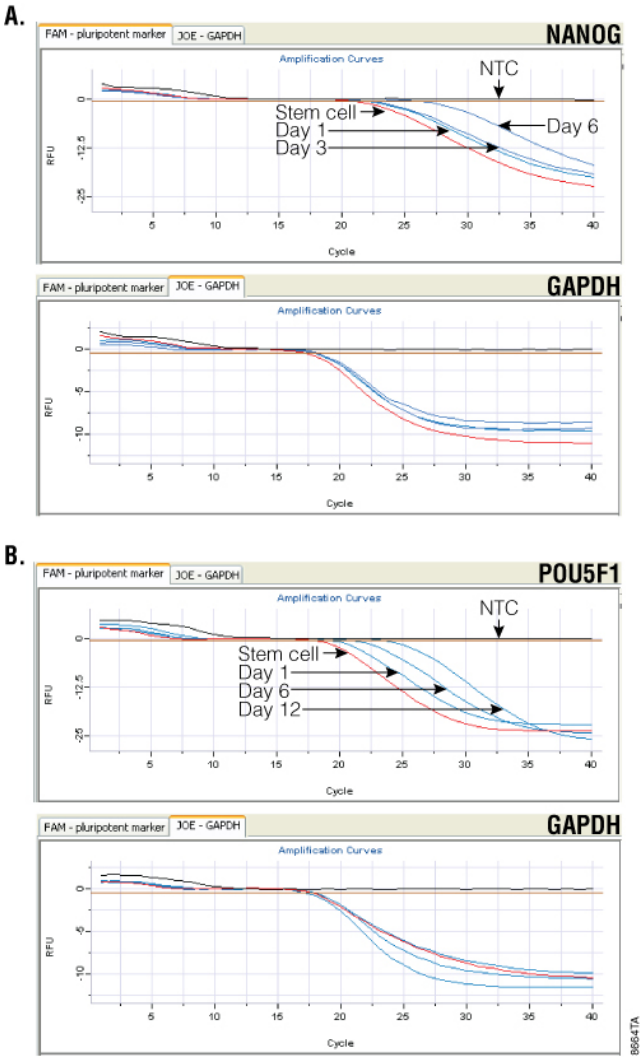

Figure 4. Monitoring pancreatic cell differentiation. In the differentiating pancreatic cell samples, NANOG and POU5F1 were each amplified in a duplex reaction with GAPDH. Gene expression in the differentiated cells was compared to undifferentiated hES cells. **Panel A.** Amplification curves for NANOG and GAPDH duplex reaction. **Panel B.** Amplification curves for POU5F1 and GAPDH duplex reaction.

MONITORING GENE EXPRESSION IN iPS CELLS

Induced pluripotent stem (iPS) cells are pluripotent cells artificially derived from an adult somatic cell. iPS technology has the potential to generate patient-specific pluripotent cells as well as eliminate the need for use of embryos to generate stem cells. Molecular markers are important to determine lineage and differentiation of iPS cells(7) .

Induced pluripotent stem cells were derived from human fibroblasts by inducing the expression of pluripotency-associated transcripts(8) . Expression was induced via lentiviral infection. The StemElite™ Gene Expression System was used to assess the expression level of pluripotency-associated transcripts; NANOG, SOX2, POU5F1, and LIN28 were each amplified in a duplex reaction with GAPDH. The iPS cells show increased expression levels of pluripotency-associated transcripts as compared to the uninduced fibroblast control; expression levels in the iPS cells are similar to the expression levels observed in undifferentiated H9 stem cells. Expression of SOX2 and LIN28 was not detected in the fibroblast controls.

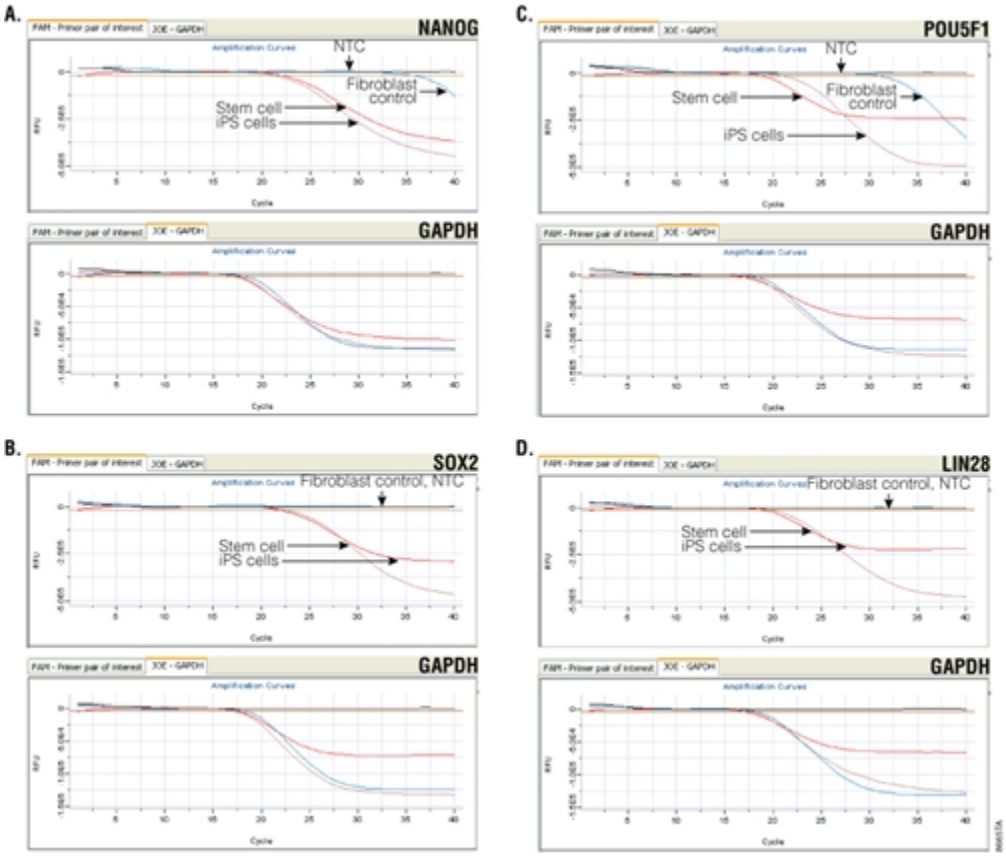

Figure 5. Monitoring gene expression in iPS cells. Panel A. Amplification curves for NANOG and GAPDH duplex reaction. **Panel B.** Amplification curves for SOX2 and GAPDH duplex reaction. **Panel C.** Amplification curves for POU5F1 and GAPDH duplex reaction. **Panel D.** Amplification curves for LIN28 and GAPDH duplex reaction.

Summary

The StemElite™ Gene Expression System uses multiplex PCR to monitor stem cell differentiation. The StemElite™ Gene Expression Primers are optimized to quantitatively amplify a two-color duplex PCR, allowing the user to amplify a transcript of interest as well as a reference transcript in a single reaction. Initial primer offerings include human-specific primers that amplify transcripts associated with stem cell pluripotency. Future sets of optimized primer pairs will include primers which target transcripts associated with lineage-specific cell differentiation, as well as mouse pluripotency-associated transcripts.

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