Assessing Gene Expression by Real-Time Polymerase Chain Reaction

Ngoc Dang, Paul Tran, Ben Colton, Ronald Torry
College of Pharmacy and Health Sciences, Drake University, Des Moines, IA

ABSTRACT

End-point Polymerase Chain Reaction (PCR) is a highly sensitive technique that can be used to assess gene expression in limited tissue/cell samples. However, accurately quantifying gene expression with this method is tedious and inconsistently performed properly. Real-time PCR refers to using fluorescent probes that bind double-stranded DNA to form DNA-probe hybrids during PCR. The number of cycles required for the fluorescence to reach a threshold level is inversely correlated with the initial amount of target sequence present. The present study compared end-point and real-time PCR techniques to determine whether hypoxia alters growth factor mRNA expression in cardiomyocytes. We choose to assess plasma growth factor (PIGF) expression, an important angiogenic factor with normally low level expression outside of the placenta. End-point RT-PCR showed that cardiomyocytes express PIGF and its receptor, VEGFR1, mRNA under normal culture conditions. Cells were cultured under normoxic (N2: 21% O2) or hypoxic (N2: 2% O2) conditions for 1, 3, 6, 12, or 24 hours and total RNA was isolated, reverse-transcribed, and resultant cDNA was used to assess changes in PIGF mRNA expression by end-point PCR. After optimization, the induction of PIGF mRNA expression at 12/24 hours of hypoxia were confirmed using real-time PCR: 12 hour: 3.38 ± 0.62 fold increase; 24 hour: 14.97 ± 0.83 fold increase (n = 6, p<0.01). The results demonstrate good correlation between end-time and real-time PCR.

Advantages of using real-time over end-point PCR: 1) its ability to measure DNA concentrations over a wider range, 2) higher throughput, 3) more quantitative than end-point PCR, and 4) no post PCR handling is necessary. In conclusion, real-time PCR can accommodate for a wider variance in initial gene expression between samples and allows for more depth and rapid quantification of gene expression.

RESULTS

PIGF Expression in Cultured Neonatal Rat Cardiomyocytes Using End-point PCR

1000 bp 1N 1N 3N 1N 4N 1N 12N 1N 24N 1N

PIGF mRNA Expression

- Normoxic
- Hypoxic

1 3 6 12 24 Hours

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Cells were cultured under normoxic (N2: 21% O2) or hypoxic (N2: 2% O2) conditions for 1, 3, 6, 12, or 24 hours and total RNA was isolated and subjected to changes in PIGF mRNA expression by PCR (30 cycles). Expected products 18S classic II (485bp); PIGF (224bp). Gel electrophoresis analysis showed significant increases in PIGF mRNA expression at 12 and 24 hours of hypoxia. 12 hours: 2.07 ± 0.43 fold increase (n = 6, p<0.01). 24 hours: 2.18 ± 0.27 fold increase (n = 7, p<0.01).

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END-POINT CYCLE RANGE OPTIMIZATION

Cycle Number

PIGF mRNA Expression

- End-point
- Real-time

1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20 21 22 23 24 25 26 27 28 29 30

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PIGF mRNA expression assessed using end-point PCR. Quantification of the threshold cycle (Ct) values confirmed the significant increases in PIGF mRNA expression shown by end-point PCR at 12 and 24 hours of hypoxia:

12 hours: 3.38 ± 0.62 fold increase (n = 6, p<0.01)
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METHODS

BACKGROUND

Plasma growth factor (PIGF) is an important angiogenic factor that protein placentae trophoblast from apoptosis but little is known regarding its expression and physiological effects in heart tissue. Polymerase chain reaction (PCR) is a highly sensitive technique that can be utilized to assess gene expression, even at very low levels. End-point and real-time PCR are two different variants of the PCR process. In end-point PCR, samples are run at a predetermined number of cycles and post-PCR gel electrophoresis is used to quantify the amount of DNA present. This data is only useful if the cycle number and DNA template has been optimized so that amplification is still in the exponential phase. However, amplification can only occur efficiently (the exponential phase) until a certain amount of product has accumulated and begins to plateaus (the plateau phase) with greater cycle numbers. The range of cycles when exponential amplification occurs changes depending on the initial gene expression. With real-time PCR, an amplification plot is obtained where the exponential and plateau phase can be clearly seen and no post-PCR handling is necessary. Complete electronic monitoring of the amplification process allows for higher throughput and more rapid, more quantitative analysis.

PIGF mRNA expression assessed using real-time PCR. Quantification of the threshold cycle (Ct) values confirmed the significant increases in PIGF mRNA expression shown by end-point PCR at 12 and 24 hours of hypoxia:

12 hours: 3.38 ± 0.62 fold increase (n = 6, p<0.01)
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SUMMARY AND CONCLUSION

1) PIGF mRNA expression was detected in cultured cardiomyocytes and by real-time PCR. Its expression increased ~3.76 ± 0.30 fold after 12-24 hours of hypoxia.
2) There is a high concordance between results from end-point PCR analysis and from real-time PCR analysis. However mean fold change increase was higher with real-time PCR.
3) Optimization of PCR parameters was more tedious and time-consuming for end-point PCR than for real-time PCR.
4) Real-time PCR provided more quantitative and reliable data over a wider range of initial gene expression when compared to semi-quantitative data yielded by end-point PCR.

Conclusions: Real-time and end-point PCR are highly sensitive processes that can be used to assess gene expression in limited tissue/cell samples. In our current study, both real-time and end-point PCR indicated that cardiomyocytes have a higher level of expression of PIGF, an important angiogenic growth factor, after 12-24 hours of hypoxia. We observed that real-time PCR has several advantages over real-time PCR: 1) real-time PCR automation allows for more quantitative data to be collected in a shorter amount of time, therefore increasing throughput; 2) eliminating post-PCR manipulation saves time and decreases risk of contamination and human error; 3) real-time PCR can provide reliable data over a wider range of gene expression.