amplification

Relative Quantitation of mRNA: Real-Time PCR vs. End-Point PCR

Khaldun Al-Khatib, MD and Daniel J J Carr, PhD, Department of Ophthalmology, Microbiology, and Immunology, Dean A McGee Eye Institute, University of Oklahoma Health Sciences Center, 608 Stanton L Young Blvd, Oklahoma City, OK 73104 USA

Introduction

Herpes simplex virus type 1 (HSV-1), a DNA virus and a member of the family of alpha herpesviruses, primarily infects epithelial cells, replicates at the sites of infection, and migrates to sensory ganglia where it establishes latency in neurons. In the human host, ocular HSV-1 infection can lead to permanent loss of vision due to the inflammatory response to repetitive reactivation of the latent virus, ultimately resulting in destruction of the cornea. In rare instances, HSV-1 can lead to death as a result of encephalitis. During acute infection or reactivation, viral replication takes place through the sequential expression of genes referred to as lytic genes, which are defined as immediate early (α), early (β), or late (γ) genes according to the stage at which they are activated during the replication cycle (Roizman and Sears 1996). Interference with the expression of these lytic genes would hamper the ability of HSV-1 to replicate and to elicit an inflammatory reaction by the host. Consequently, the ability to detect changes in the expression of these genes would be useful in identifying molecules or drugs that hamper the production of viral progeny.

PCR and RT-PCR are commonly used methods for detecting low-abundance species of DNA and RNA, respectively. However, the quantitative application of these methods has been more limited. Although there is ample evidence that PCR and RT-PCR can be performed in a quantitative manner, considerable debate has revolved around the conditions that must be met to validate the quantitative reliability of such an assay. Nevertheless, end-point PCR has been used by many investigators as a semi-quantitative measure of relative differences in template input when comparing samples. Real-time quantitative PCR is a single-step method that includes both amplification and analysis with no need for slab gels, radioactivity, or sample manipulation post-PCR. There are now several platforms commercially available for combining thermal cycling with fluorescence acquisition (Foy and Parkes 2001). The fluorescence of DNA dyes or probes is monitored each
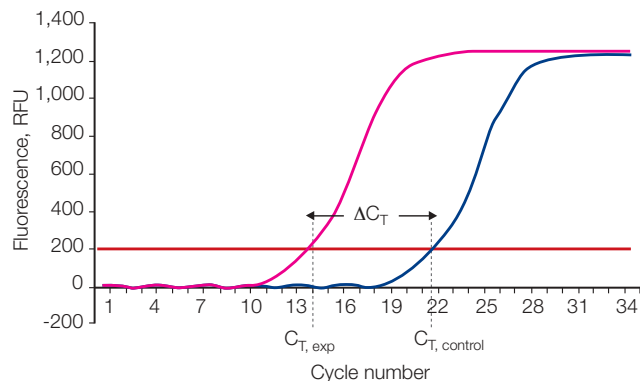

Fig. 1. Concepts of real-time PCR: calculating relative copy numbers. The threshold cycle (C_T) for a sample is the cycle in which fluorescence crosses the threshold (approximately 195 RFU on this graph). The difference in C_T between two samples (ΔC_T) reflects the difference in their relative copy numbers.

cycle during amplification. At a certain point during amplification, the targeted PCR product accumulates above the background, resulting in the detection of fluorescence (expressed in relative fluorescence units, or RFU). The point in the amplification profile where fluorescence rises significantly above the background determines the threshold cycle (C_T). By default, the threshold is 10 times the mean of the standard deviation of the fluorescence reading of each well over the first 10 cycles, excluding cycle 1. The threshold cycle correlates well with the number of copies of the targeted sequence within a sample.

Assuming equally efficient amplification of two templates, the greater the number of copies of the initial template, the earlier fluorescence appears and the lower the C_T value. The relative copy numbers of two samples (e.g., experimental and control) can be determined by the difference in their C_T values (Figure 1, ΔC_T). Because PCR is an exponential process, the relative copy number difference (n) is equal to the PCR efficiency (f) raised to the power ΔC_T:

$$n = f^{(C_{T,\ experimental} - C_{T,\ control})} = f^{(\Delta C_T)}$$

Although it may be difficult to determine the total amount of cDNA present in different samples, quantitation of test mRNA transcripts is often normalized to a reference gene presumed to be invariant. The efficiency relative to the

BIO·RAD

reference gene is then $f^{(\Delta C_{T, test})}/f^{(\Delta C_{T, reference})}$, which in an optimal reaction equals 2. Relative quantitation by real-time PCR often uses SYBR Green I as a fluorescent indicator of double-stranded DNA synthesis (Wittwer et al. 1997).

In this study, we compare the use of end-point PCR (35 cycles) and real-time PCR as methods of relative quantitation for viral gene expression. The chosen viral genes are representative of each phase of HSV-1 replication and include an α gene, encoding infected cell protein-27 (ICP27), a β gene, encoding thymidine kinase (TK), and a γ gene, encoding viral protein 16 (VP16).

Methods

Cell Lines and Viruses
The mouse fibroblast line L929 was obtained from the American Type Culture Collection (ATCC, Manassas, VA) and propagated in DMEM medium containing 0.375% HCO_3 supplemented with 10% fetal bovine serum and antibiotic/antimycotic solution (GIBCO, Gaithersburg, MD) (referred to as complete DMEM). The cell culture was maintained at 37°C, 5% CO_2, and 95% humidity. The highly virulent McKrae strain of HSV-1 was used in this study.

Reverse Transcription
L929 cells were infected with HSV-1 at a multiplicity of infection (MOI) of 1. The cells were harvested 10 hr post-infection and the RNA was extracted using Ultraspec RNA isolation reagent (Biotecx Inc., Houston, TX). First-strand cDNA was synthesized using avian myoblastosis virus reverse transcriptase (Promega, Madison, WI).

Semi-Quantitative PCR
PCR was performed in the iCycler iQ™ system for 35 cycles of 25 sec at 95°C followed by 45 sec at 60°C. PCR primers used were as follows:

GAPDH (reference gene)	Forward: 5'-GAATCTACTGGCGTCTTCACC-3' Reverse: 5'-GTCATGAGCCCTTCCACGATGC-3'
ICP27	Forward: 5'-TTCTCCAGTGCTACCTGAACC-3' Reverse: 5'-TCAACTCGCAGACACGACTCG-3'
TK	Forward: 5'-ATGGCTTCGTACCCCTGCCAT-3' Reverse: 5'-GGTATCGCGCGGCCGGGTA-3'
VP16	Forward: 5'-GGACTGTATTCCAGCTTCAC-3' Reverse: 5'-CGTCCTCGCCGTCTAAGTG-3'

A PCR supermix from Bio-Rad (iQ™ SYBR Green® supermix, catalog #170-8880) containing *Taq* DNA polymerase (iTaq™ polymerase), $MgCl_2$, dNTPs, SYBR Green I, and fluorescein was used. Primers were added to the reaction mix at a final concentration of 200 nM. For real-time PCR, the C_T was automatically determined using the accompanying iCycler iQ software by calculating the second derivative of each trace and looking for the point of maximum curvature. While the baseline cycles are determined individually for each trace, the threshold must be a global value that is the same for all traces. The threshold is calculated automatically using the iCycler iQ software.

For end-point PCR, the PCR products were electrophoresed and visualized in ethidium bromide-stained 1% agarose gels. The products were then analyzed using a Bio-Rad Gel Doc™ 1000 image documentation system.

Results
cDNA from HSV-1 infected L929 cells (100 ng/μl) was serially diluted 10-fold using Mg^{2+}-free 1x buffer, and a 35-cycle PCR run was performed on each sample (the original, the 10-fold dilution, and the 100-fold dilution) in triplicate (Figures 2 and 3). Using either real-time PCR or end-point PCR, the levels of all three viral transcripts (ICP27, TK, and VP16) were inversely proportional to the dilution used. To determine whether the dependence of all three viral gene RT-PCR product yields on template abundance was significant, a more rigorous analysis was performed. Using real-time PCR, ICP27, TK, and VP16 products decreased in proportion to the logarithm of the HSV-1 cDNA concentration in the PCR reactions. The difference in relative cDNA copy number was statistically significant ($p < 0.05$) between the original sample and the first dilution, as well as between the first and second dilutions, for each viral gene tested. Within each group of replicate PCR samples, the coefficient of variation [CV; defined as 100 x (standard deviation/mean)] of product yield ranged from 0.1 to 2.0%. End-point PCR yielded significant differences among dilutions in most of the samples. However, it failed to show significant differences in the ICP27 PCR product when comparing the undiluted to the first log dilution (Figure 3 A, lanes labeled 3 and 2, respectively). The CV of end-point PCR product yield among replicates ranged from 7 to 17%. This indicates relatively high variability among the triplicates of the same dilution. For both real-time (Figure 2) and end-point PCR (Figure 3), it was possible to show a linear relationship between the response variable (log dilution) and the explanatory variable (PCR product yield) in all three viral genes using least-squares regression analysis. However, the correlation coefficients (r) were superior for all viral genes when real-time PCR was employed (0.998 for ICP27 curve, 0.999 for TK curve, and 0.998 for VP16 curve) compared to the values for end-point PCR (0.872 for ICP27 curve, 0.935 for TK, and 0.966 for VP16). It is clear from these results that real-time PCR achieves results with less variability and increased sensitivity compared to end-point PCR.
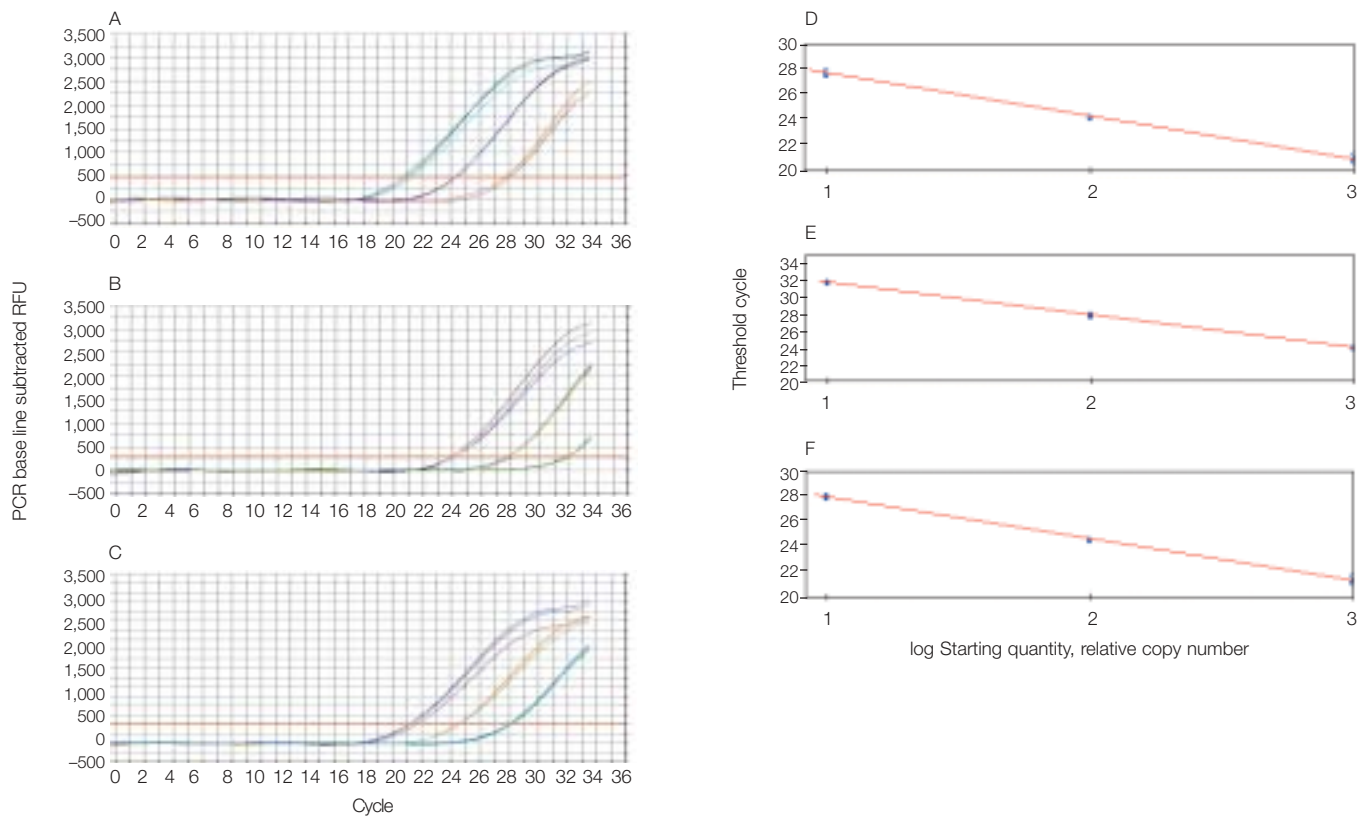
Fig. 2. Real-time PCR analysis of HSV-1 lytic gene expression. cDNA generated from RNA obtained from HSV-1 infected L929 cells was serially diluted 10-fold and used to establish standard curves for HSV-1 α (ICP27), β (TK), and γ (VP16) genes. Each dilution was performed in triplicate. A, B, and C, representative amplification profiles for ICP27, TK, and VP16, respectively. D, E, and F, standard curves for A, B, and C, with slopes of –3.4, –3.8, and –3.3, respectively.
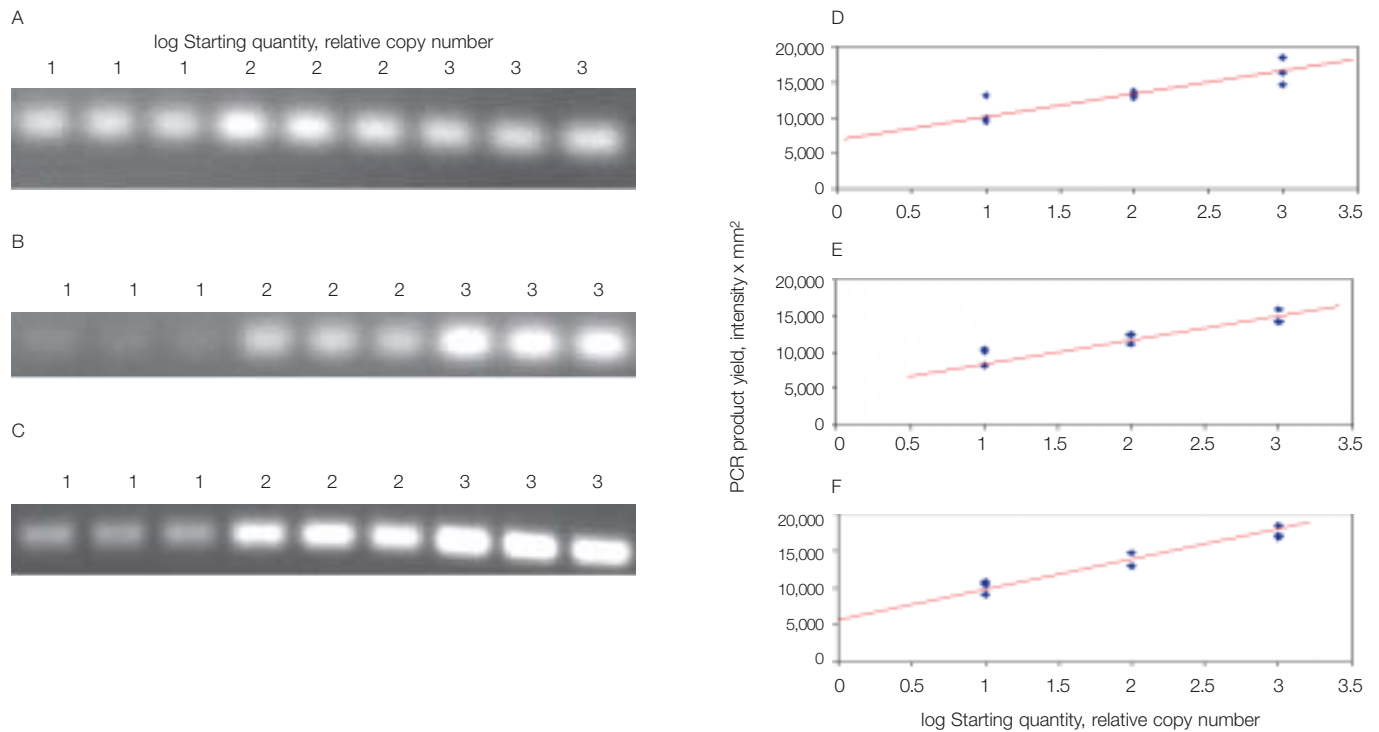

Fig. 3. End-point PCR analysis of HSV-1 lytic gene expression. Amplified products from Figure 2 were run on agarose gels and quantitated on a Gel Doc™ 1000 image documentation system. A, B, and C, agarose gel images of ICP27, TK, and VP16 products, respectively. D, E, and F, standard curves for A, B, and C, with slopes of 4.95, 5.45, and 6.89, respectively.

Discussion

The use of PCR to detect the presence or absence of specific mRNA transcripts has been widely used over the last two decades. This is generally considered a qualitative assay. However, the use of this tool as a quantitative assay has been tempting, and even become more powerful since the advent of real-time PCR. End-point PCR has been used by many researchers as a quantitative method if a standard curve can be generated for a selected set of primers in a competitive PCR approach. However, it is still unclear how PCR can enter the plateau phase before maximal amplification of specific products. Loss of polymerase activity and accumulation of pyrophosphates are commonly cited as significant inhibitory factors during the amplification process (Gause and Adamovicz 1994). In addition, nonspecific amplification of primer-dimers also affects amplification (Halford et al. 1999). These factors affect the reliability of end-point PCR as a quantitative measure. On the other hand, the above study has demonstrated real-time PCR to be superior to end-point PCR for relative quantitation of mRNA input using three targeted viral lytic genes during viral replication. Specifically, the reproducibility of real-time PCR was found to be superior to end-point PCR as shown by measuring the CV for each group of replicates. The CV for end-point PCR products was found to be much higher than the CV calculated for real-time PCR products (7–17% vs. <2%, respectively). The variability was independent of the gene of interest and seems to be inherent in the nature of the procedures themselves. In addition, the level of confidence suggested by the r values strongly favors real-time PCR as an accurate method to determine unknown sample values. The r values for end-point PCR varied to a greater degree among the three viral genes analyzed and were significantly lower in comparison to the real-time PCR approach. When end-point RT-PCR is performed using a cDNA standard curve in parallel with unknown samples, the variability of the PCR is reflected in the standard curve. Thus, product yields obtained from the standard curve can be used to define the quantitative reliability and range over which end-point RT-PCR product yields provide a useful measure of target cDNA abundance. The slope of the standard curve in end-point PCR varies depending on the fidelity of the primer sets used. Moreover, the intensity of the PCR product varies even for the same primer sets if run at different times (the intensity of the PCR product measured by the imaging system varies depending on the efficiency of the PCR reaction for that run and also on the image setting). Therefore, a standard curve should be generated when new samples are to be evaluated, whether they are run simultaneously or at different time points. However, in real-time PCR, since the slope of the regression curve approaches 3.3 for any log dilution and for any suitably chosen primer set, there is little need to run a standard curve each time a comparison of cDNA input for a specific gene from different samples is undertaken, providing that the samples are run simultaneously. Because the y-intercept varies between runs (it depends on the efficiency of the PCR amplification, which can vary between different runs), a standard curve made from the stock cDNA and its dilutions should be included if the samples are evaluated in different runs. Collectively, in assessing the data generated in this report using representative viral genes as templates, real-time PCR is more accurate and reproducible than end-point PCR. In addition, real-time PCR is more efficient than end-point PCR based on the ease of determining relative gene expression through the software provided, the single-step method of amplification and analysis, the reproducibility of the results, and the time required for analysis of targeted gene quantitation. Specifically, a typical end-point PCR run and assessment of gene products by gel electrophoresis can take 5 hr to complete. In contrast, real-time PCR runs typically take 60–90 min to complete with relative values for each targeted gene being measured.

References

Foy CA and Parkes HC, Emerging homogeneous DNA-based technologies in the clinical laboratory, Clin Chem 47, 990–1000 (2001)

Gause WC and Adamovicz J, The use of the PCR to quantitate gene expression, PCR Methods Appl 3, S123–S135 (1994)

Halford WP et al., The inherent quantitative capacity of the reverse transcription-polymerase chain reaction, Anal Biochem 266, 181–191 (1999)

Roizman B and Sears AE, Herpes simplex viruses and their replication, pp. 2231–2295 in (Fields BN et al., eds) Fields Virology, 3rd ed., Lippincott-Raven Publishers, Philadelphia (1996)

Wittwer CT et al., Continuous fluorescence monitoring of rapid cycle DNA amplification, Biotechniques 22, 130–131, 134–138 (1997)