Published in final edited form as:

J Virol Methods. 2014 June ; 201: 101–111. doi:10.1016/j.jviromet.2014.02.010.

Development and evaluation of SYBR Green-I based quantitative PCR assays for herpes simplex virus type 1 whole transcriptome analysis

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Abstract

There is an emerging need for viral gene specific quantitative PCR (qPCR) assays that validate and complement whole transcriptome level technologies, including microarray and next generation sequencing. Therefore, a compilation of qPCR assays that represented the breadth of the entire Herpes simplex virus type 1 (HSV-1) genome were developed and evaluated. SYBR Green-I-based quantitation of each of the 74 HSV-1 lytic genes enabled accurate and reproducible detection of viral genes using a minimal number of reaction conditions. The amplification specificity of these assays for HSV-1 target genes was confirmed by amplicon size and purity determination on agarose gels, melt temperature dissociation curve analysis, and direct DNA sequencing of amplified products. Analysis of representative target genes demonstrated that these assays accurately and reproducibly quantified target gene expression across a wide and linear range of detection. In addition, minimal intra- and inter-assay variability was observed with significant well-to-well and plate-to-plate/assay-to-assay precision. To evaluate the utility of the developed qPCR assay system, kinetic profiles of viral gene expression were determined for an array of representative genes from all HSV-1 transcriptional gene classes. Collectively, these data demonstrate that the compiled optimized qPCR assays is a scalable and cost-effective method to assess HSV-1 gene expression with broad application potential, including investigation of pathogenesis and antiviral therapies. In addition, they can be employed to validate and complement evolving technologies for genome-wide transcriptome analysis.

Keywords

Herpes simplex virus; RT-PCR; SYBR Green-I; Transcriptome; HSV-1; Quantitative PCR

1. Introduction

The human Herpesviridae family consists of 9 large DNA viruses that encode open reading frames that specify between 74 and 164 genes. The alphaherpesvirus, Herpes simplex virus type 1 (HSV-1), specifies a 152 kilobase pair genome that encodes 77 open reading frames of which three are diploid (Pellett and Roizman, 2007; Roizman et al, 2007). These genes are expressed during the HSV lytic replicative cycle in a triphasic sequential and coordinately regulated temporal cascade of gene expression (Honess and Roizman, 1974, 1975; Roizman et al., 1975). First, immediate early or alpha (IE/a) genes are expressed immediately upon deposition of viral DNA into the nucleus (Honess and Roizman, 1974, 1975). Next, IE proteins launch the expression of early or Beta (E/ β 3) genes that initiate viral DNA replication and elicit subsequent late or gamma (L/ γ) gene expression (Honess and Roizman, 1974); Lastly, the L/ γ genes consist of proteins that are either structural in nature or function to mediate capsid assembly, virion maturation, and viral egress (Honess and Roizman, 1974). The gamma gene class has been further subdivided into γ 1 and γ 2 genes, with γ 2 gene transcription absolutely dependent on viral DNA replication. Consequently, the effects of cellular proteins, viral gene mutations/deletions, or pharmaceutical drugs on these highly ordered viral transcriptional patterns can provide insights into their functional activities. For example, HSV infected cells that are treated with the viral DNA replication inhibitors foscarnet or acyclovir exhibit an altered transcriptional pattern, whereby there is an accumulation of early β transcripts and suppression of late γ gene expression (Jones and Roizman, 1979; Holland et al, 1980; Hall et al., 1982).

Microarray and RNA sequencing (RNA-Seq) are emerging and expanding high throughput technologies that enable whole genome transcriptional analysis of the effects of various experimental conditions. In virus infected cells, these technologies enable simultaneous assessment of both host and viral transcription. However, despite these technologies generating data with immense depth, results still require confirmation by more standard and accepted approaches, such as quantitative real-time PCR (qPCR). qPCR arrays, such as the system presented in this study, can complement, corroborate, and validate results obtained from these contemporary methodologies. For most viruses with small genomes, designing qPCR assays that can assess whole genome transcriptional changes is relatively uncomplicated. However, for viruses with large complex genomes, like those of the Herpesviridae family, the enormity of the task can be challenging.

In order to assess the effects of various conditions on viral transcription, we have developed a compilation of qPCR assays within plate arrays that represent the breadth of the whole viral genome. These qPCR arrays were designed to: (1) represent the entire HSV genome across all HSV transcriptional classes; (2) utilize adaptable SYBR Green-I-based quantitation to alleviate probe design, synthesis and development that is expensive and would be required for 74 individual HSV-1 genes; (3) be specific for target viral genes without cross-reactivity to host cellular transcripts; (4) minimize the required number of amplification conditions; (5) follow, where possible, Minimal Information for Publication of Quantitative Realtime PCR Experiments (MIQE) guidelines for qPCR assay development (Bustin et al., 2009; Bustin, 2010; Huggett et al., 2013); (6) quantify levels of viral transcripts relative to a host reference gene; (7) be linear in amplification across varying

target concentrations; and (8) be reproducible with minimal intra- or inter-assay variation. Described in this study is the development, evaluation and implementation of these assays to assess relative transcriptional kinetic profiles of representative genes from each transcriptional class.

2. Materials and methods

2.1. Viruses and cells

African green monkey kidney (Vero) cells were obtained from ATCC (Manassas, VA) and were maintained in DMEM supplemented with 5% FCS. The RE strain of HSV-1 (HSV-1(RE)), a known corneal keratitis inducing strain of HSV-1 (Hendricks et al., 1989; Hendricks and Tumpey, 1990), was one of the parental wildtype strains utilized in these studies and was obtained initially from Dr. James Hill, Louisiana State University Health Sciences Lions Eye Center. HSV-1(RE) virus stocks were propagated in Vero cells and stored as infectious cell preparations at -80°C . Purified HSV-1 KOS strain (ATCC) viral genomic DNA was prepared on a sodium iodide gradient, resuspended in Tris EDTA buffer, and stored as aliquoted frozen stocks.

2.2. Primer design and synthesis

The published genomic sequence of human herpesvirus 1, strain 17 (Genbank Accession no. NC_001806.1) was used as template to identify comprehensive, genome-wide coding sequences for each HSV-1 transcript (McGeoch et al., 1988a, b). In order to specifically amplify regions within each coding sequence, unique primers were designed to avoid overlapping genes and assessed subsequently for potential hairpin formations, 3' complementarities, and self-annealing sites using OligoCalc (Kibbe, 2007). Primer pairs were selected to generate amplicons of 90–200 base pairs (Tables 1–5). All primer sets were designed initially with uniform annealing temperatures to facilitate assessing multiple gene targets within the same PCR run. Prospective primers and their generated amplicons were analyzed in silico for cross-reactivity and recognition of other HSV-1 strains and verified to have no significant homology to *Homo sapiens* host genes using the genomic database for human and human transcripts and BLASTn (NCBI). All designed iterations of primer pairs were tested empirically for amplification of target genes from HSV-1 KOS genomic viral DNA and cDNA isolated from HSV-1 RE infected cells. Any primer pairs that exhibited any amplification product at or before 30 cycles from cDNA prepared from non-infected cell lysates were excluded from further analysis. The specificity of each selected primer pair was evaluated by melt curve analysis, agarose gel electrophoresis, and DNA sequencing and confirmed to be a single uniform amplification product of appropriate apparent molecular size and sequence. Final optimized primer sequences, associated gene references, specific assay conditions, and amplicon characteristics are described in Tables 1–5. In addition, cellular 18s ribosomal RNA (rRNA) was utilized to normalize qPCR reactions (FOR: 5'CCAGTAAGTGCGGGTCATAAGC3'; REV: 5'GCCTCACTAAACCATCCAATCGG3') (Mbong et al., 2012). All primers were synthesized by Integrated DNA Technologies (IDT-DNA, Coralville, IA, USA).

2.3. Total RNA extraction, isolation, and purification

To initiate synchronous infection for kinetics analysis, HSV-1 RE strain (MOI = 5) was adsorbed for 1 h on subconfluent Vero cells in 12-well plates that were pre-chilled (4°C) in 500 µl DMEM, 1.5% heat-inactivated FCS, as we have described previously (Foster et al., 1998, 1999; Kadeppagari et al., 2012; Sanchez et al., 2012). Following the 1 h incubation, all media was removed, replaced with 1 ml of pre-warmed (37 °C) media and incubated at 37 °C for 0, 1, 2, 4, 8, 12, 18, or 24 h. At the respective time points, culture supernatants were removed and cells were lysed in 350 µl RLT Buffer (Qiagen RNeasy Mini Kit) with gentle scraping of each well to harvest all cell lysates. Cell lysates were stored at –80 °C until RNA purification. Within 24 h of harvest, cell lysates were thawed on ice and total RNA was purified using the RNeasy Mini Kit (Qiagen, Valencia, CA, USA) according to the manufacturer's protocol with the exception that two optional DNase I digestion steps were employed to ensure the absence of contaminating viral genomic DNA. First, DNaseI was added to cell lysates prior to RNA purification and then contaminating DNA was further digested via an on-column digestion using RNase-free DNase I in Buffer RDD for 15 min at room temperature. All samples were eluted in 50 µl of RNase-free water and quantified by spectrophotometry.

2.4. cDNA synthesis

cDNA was synthesized immediately after RNA purification using the iScript™ cDNA Synthesis Kit (Bio-Rad Laboratories, Hercules, CA, USA). Each 100 µl cDNA reaction consisted of 20 µl 5 × iScript reaction mix, 5 µl iScript reverse transcriptase, and 4 µg RNA template. Thermal cycling was conducted as follows: 5 min at 25 °C, 30 min at 42 °C, followed by 5 min at 85 °C. Parallel cDNA reactions lacking reverse transcriptase enzyme were included for each experiment to evaluate the presence of contaminating genomic DNA.

2.5. SYBR Green-I qPCR conditions

SYBR Green-I based qPCR was performed using the indicated primer pairs for each target gene (Tables 1–5). Real-time qPCR was carried out using iQ™ SYBR® Green Supermix (Bio-Rad Laboratories; 40 mM Tris-HCl pH 8.4, 100 mM KCl, 1.6 mM dNTPs, 0.625U antibody-mediated hot-start iTaq DNA polymerase, 6 mM MgCl₂, SYBR Green-I, 20 nM fluorescein, and stabilizers). Each 25 µl total PCR reaction contained 3 µl of 1:4 diluted cDNA template, 12.5 µl iQ™ SYBR® Green Supermix, and 200 nM of each primer. All realtime qPCR reactions were carried out using a Bio-Rad CFX96™ Real-Time System and CFX Manager 2.0 (Bio-Rad Laboratories, Hercules, CA, USA) in white Multiplate™ 96-well plates covered with Microseal 'B' adhesive seals. Three-step cycling parameters were used for a majority of PCR reactions and were as follows: initial denaturation at 95 °C for 3 min followed by 30 cycles of 95 °C for 15 s, annealing at 55 °C for 30 s, followed by a 30 s extension at 72 °C. To minimize cross-reactivity issues and increase specificity, a two-step cycling parameter was used for PCR reactions against UL30 and UL42 and was as follows: initial denaturation at 95 °C for 3 min followed by 30 cycles of 95 °C for 15 s and annealing at 65 °C for 30 s. Following amplification, amplicon specificity and purity for each PCR run was confirmed by melt curve analysis.

2.6. Agarose gel electrophoresis, PCR purification, and DNA sequencing

7 µl aliquots of all HSV target gene PCR products were analyzed for purity and specificity on a 1.8% agarose gel run in $0.5 \times$ Tris Borate EDTA (TBE) buffer. The remaining amplified DNA was purified using DNA Clean and Concentrator-5 columns and reagents (Zymo Research) according to the manufacturer's directions. Purified PCR products were sequenced directly (ACGT, Inc) using gene specific primers. Sequences were compared to the HSV-1 genome and the NCBI database (BLASTn) to confirm specificity of each HSV target gene amplified.

2.7. Assay precision and reproducibility

Assay precision was determined for representative HSV target genes. Amplification of target genes across a 2-fold dilution series of HSV-1 KOS strain genomic DNA (from 42.2 pg to 1350 pg) was assessed and the Cq values were plotted relative to genomic DNA. The linearity of amplification for each gene was determined by the R^2 values of each dilution series. Intra-assay (within the day/across the plate) and inter-assay (independent assessment over 4 days/between plate) variability was determined according to MIQE (Minimal Information for Publication of Quantitative Realtime PCR Experiments) guidelines (McGowin et al., 2009; Bustin et al., 2009; Bustin, 2010; Huggett et al., 2013). The mean, standard deviation (S.D.), coefficient of variation (CV) of the cycle of quantification (Cq) were calculated separately for amplification of representative target genes from cDNA prepared at 4 and 12 h post infection (h p.i.) or genomic viral DNA. The intra- and inter-assay variability assessed by the CV was determined by the S.D./mean multiplied by 100 to express as a percentage CV, as described previously (Santhosh et al., 2007; Bustin et al., 2009; Amer and Almajhdi, 2011).

2.8. Analysis of HSV transcriptional profiles

qPCR was performed on arrays of representative genes from each HSV-1 transcriptional gene class to quantify viral transcript abundance at 0, 1, 2, 4, 8, 12, 18, and 24 h p.i. Viral gene transcripts were normalized to the mean Cq values ($\text{meanCq}_{\text{of18s@timeX}}$) of cellular 18s rRNA expression, which was determined as the mean Cq of in triplicate parallel reactions for each time point (Chey et al., 2010). Normalized viral transcript expression levels relative to the internal cellular 18s rRNA reference gene were calculated by the Cq at time X using the equation: $\text{Cq}_{\text{timeX}} = (\text{Cq}_{\text{ofviraltranscript@timeX}} - \text{meanCq}_{\text{of18s@timeX}})$. Relative fold change in gene expression for each specific viral gene was calculated by the $2^{-\text{Cq}}$ method (Livak and Schmittgen, 2001). For each time point of each gene analyzed, the Cq was determined relative to that gene's Cq value at a time point for that gene's kinetic class where gene expression was first detected. That was for α genes at 1 h p.i., for β genes at 2 h p.i. and for γ genes at 4 h p.i. Therefore, the $\text{q} = (\text{Cq}_{\text{of viral transcript@timeX}} - \text{Cq}_{\text{ofviraltranscript@timeY}})$; where $Y = (\alpha \text{ genes:1 h p.i.; } \beta \text{ genes:2 h p.i.; } \gamma \text{ genes:4 h p.i.})$. Relative fold change for each time point was then expressed by the equation $2^{-\text{Cq}}$.

2.9. Heat map visualization of HSV transcriptional profiles

Heat maps were generated within Microsoft Excel to assist in visualization and comparative analysis of gene expression patterns. The fold change values across each individual gene, as

calculated by the 2^{-C_q} method (Livak and Schmittgen, 2001), were converted to colorized values using Excel's conditional formatting color-scale function and a three defined point color scale gradient: (1) no expression was assigned a bright green color; (2) the highest value in the kinetic series was assigned a bright red color; (3) the 50th percentile within the kinetic series was assigned a bright yellow color; (4) values that fell within these three specific color identifiers were assigned within the gradient of colors between these values. Assigned color values and genes were segregated by gene transcriptional class and figures were compiled in Adobe Photoshop.

3. Results

3.1. Design and optimization of SYBR Green-I-based quantitative PCR assays for the whole transcriptome evaluation of HSV-1 gene expression

In order to enable whole HSV-1 transcriptome level validation of microarray and next generation sequencing (NGS) methodologies, we developed a series of primer sets that target each lytic gene of the HSV-1 genome. After several design and testing iterations, final optimized primer sets were selected to not be affected by nucleotide variations amongst HSV-1 strains, to not be cross-reactive with cellular genes, and to not form primer-dimers. The detailed sequences, gene identification numbers, and amplicon size of these primers are segregated by their respective documented or suggested gene transcriptional class (Pellett and Roizman, 2007; Roizman et al, 2007) and are shown in Tables 1–5. Criteria for primer set acceptability included: (1) number of cycles required to develop a detectable signal (C_q) from HSV-1 genomic DNA and infected cell cDNA; (2) complete absence of amplification in control samples; (3) uniform primer concentrations for all gene targets; (4) simultaneous arrayed analysis of most HSV-1 genes by minimizing the number of annealing temperatures required; (5) linearity and accuracy of gene amplifications; (6) reproducibility and precision of amplification reactions; and (7) ability to detect target genes from several HSV-1 strains, including HSV-1 syn17+, KOS, and RE from genomic and cDNA templates.

Optimal quantitative SYBR Green-I PCR assay conditions were established for these primer sets that resulted in robust linear amplification of HSV-1 target gene transcripts with no amplification signals from uninfected cDNA controls. This required two different reaction conditions. For the vast majority of HSV genes (72 of 74 genes) and cellular 18s rRNA reference gene, reaction conditions with a 55 °C annealing temperature was utilized (Tables 1–5; Fig. 1C; Supplemental Figs. 1C, 2C, and 3C). However, two beta genes, UL30 and UL42, required altered reaction conditions with an annealing temperature of 65 °C in order to fulfill all of our specificity requirements (Table 2; Supplemental Fig. 2B and C). Specific reaction conditions are provided in Section 2.5. All individual qPCR assays exhibited a single linear amplification phase within the logarithmic curve for both genomic viral DNA and cDNA of viral transcripts from infected cells. However, the HSV-1 genomic DNA and infected cell cDNA amplification curves differed due to variances in the levels of the target gene at the specific time point assessed. The method of calculating C_q values from where they cross the indicated threshold are provided within the Section 2. Since HSV-1 has a limited number of spliced genes, primers could not be designed across introns. Therefore, the most critical assay optimization was ensuring that viral genomic DNA was removed

completely prior to quantitative analysis. This was verified by the absence of detectable amplification within the no reverse transcriptase (NRT) controls. The complete degradation of genomic viral DNA was ensured by extracting, processing, and purifying RNA with the protocols in Section 2.3.

Supplementary material related to this article can be found, in the online version, at <http://dx.doi.org/10.1016/j.jviromet.2014.02.010>.

3.2. Melting curve analysis of amplified gene products

Following real-time gene amplification, a dissociation curve analysis was performed to establish each gene product's specific melting temperature (T_m) and that only a single T_m , which represented a single amplification product, was present. T_m 's for individual HSV-1 genes ranged from 83.5 °C to 91.5 °C and are presented for each gene in Tables 1–5. All reactions where potential HSV-1 target genes were present (HSV-1 infected cell cDNA and HSV-1 KOS genomic DNA) exhibited a single dissociation peak (Fig. 1B, red or blue lines; Supplemental Figs. 1B, 2B and 3B, red or blue lines). No dissociation curve/ T_m was reported for any reactions from cDNA made from uninfected cells due to lack of PCR amplification (Fig. 1B, green lines; Supplemental Figs. 1B, 2B and 3B, green lines). For two genes, UL23/thymidine kinase (TK) and UL41/virion host shutoff (VHS), there was a 0.5 °C difference between amplification products from HSV-1 genomic DNA from the KOS strain versus cDNA from the RE strain (Tables 2 and 4; Fig. 1 :B2, red versus blue lines). In silico and sequencing analysis of these amplification products revealed a one nucleotide difference within the PCR products of these two strains that could account for the observed differences in T_m . The T_m 's reported in Tables 1–5 can be utilized as a reference to confirm specificity and proper gene amplification in future experiments.

3.3. Specificity of quantitative PCR assays

The ability of each reaction to amplify a single PCR product representative of its specific gene target was further confirmed by agarose gel electrophoresis and DNA sequencing. Following amplification reactions, all HSV PCR products from each of the 3 conditions were separated on a 1.8% TBE agarose gel. All amplification reactions where potential HSV-1 target genes were present (HSV-1 infected cell cDNA and HSV-1 KOS genomic DNA) exhibited a single amplification product (Fig. 1A; Supplemental Figs. 1A, 2A and 3A) that had an apparent molecular weight that corresponded to the calculated molecular weights of the specific amplicons (Tables 1–5). As expected, no PCR products were evident in amplification reactions of cDNA from uninfected cells. Amplified gene products were sent for direct sequencing and determined to be specific for each HSV-1 target gene. Collectively, these analyses indicated that the observed amplification plots for each target gene were specific for the HSV-1 target that they were designed to detect.

3.4. Assessment of assay linearity and precision

The linearity and precision of the developed quantitative PCR assays for representative genes from the three HSV-1 transcriptional gene classes was evaluated across a 2 fold dilution series of HSV-1 genomic DNA. The resulting amplification plots and analysis of linearity are shown (Fig. 2B and A, respectively). In these reactions, amplification of each of

these representative genes across serial 2 fold dilutions of HSV-1 genomic DNA exhibited notable linearity with R^2 values ranging from 0.9905 to 0.9988.

To further assess the precision of the developed assays, intra- and inter-assay variability was determined for representative genes from each of HSV's transcriptional gene classes in accordance with MIQE guidelines (Bustin et al, 2009; Bustin, 2010; Huggett et al., 2013). For each representative gene, precision and reproducibility of each assay was established based on the Cq values obtained from either three separate reactions within a single run (intra-assay or well-to-well variability) or in three consecutive runs over 4 days (inter-assay or plate-to-plate/run-to-run variability) (Table 6) (Santhosh et al., 2007; Amer and Almajhdi, 2011). The calculated S.D. and CV for each intra-assay assessment were low, ranging from 0.02 to 0.12 and from 0.10% to 0.65%, respectively. The inter-assay variability was also exceptionally low with a calculated S.D. range from 0.05 to 0.36 and a CV range from 0.28% to 1.66%.

3.5. Evaluation of assay performance for kinetic analysis of HSV-transcriptional gene class expression

Each of the HSV-1 transcriptional gene classes follow well described coordinated temporal patterns of expression that are expressed sequentially. Regulation of these patterns of gene expression is paramount in orchestrating efficient HSV genomic replication and virion assembly. The suitability of these developed PCR assays to assess quantitatively HSV-1 gene transcription was evaluated at various times post infection across a 24 h kinetic course. Relative fold change in expression of each specific gene was determined using the 2^{-Cq} method (Livak and Schmittgen, 2001) as described in the methods section. During the course of infection, representative genes exhibited patterns of gene expression that reflected the prescribed expression patterns of each gene class (Fig. 3A).

A heat map approach was utilized to assist in visualization and comparative kinetic analysis of gene expression patterns. Segregation and alignment of these heat maps by HSV-1 gene transcriptional class revealed differences in patterns of gene expression that were specific to each class (Fig. 3B). Whereas, most genes in the beta and gamma classes exhibited one pattern of gene expression, genes in the alpha class exhibited two distinct patterns of gene expression. Interestingly, one beta gene, UL2, did not appear similar to the other beta genes, but had a pattern that was reminiscent of the gamma gene class (Fig. 3B).

4. Discussion

Emerging technologies that enable whole transcriptome analysis of viral and cellular gene expression are becoming rapidly more commonplace. Since Dr. Edward Wagner's laboratory developed an oligonucleotide-based DNA microarray approach to study global changes in HSV-1 gene transcript abundance in the late 1990s, many techniques have been developed or utilized for analyzing levels of alpha-herpesvirus gene expression to facilitate recognition of broad effects of various treatments or cellular factors on viral gene expression (Stingley et al., 2000; Wagner et al., 2002; Yang et al., 2002; Cohrs et al., 2003, 2006; Aguilar et al, 2005, 2006; Deitch et al, 2005; Kennedy et al., 2005; Kamakura et al., 2008). However, these techniques can be expensive, generate an immense dataset that requires extensive and

complicated analysis, and often still require validation or replication for statistical evaluation by secondary methods, such as qPCR. These factors limit their utility in examining the effects of various treatments at multiple kinetic points, as each new condition or time point increases exponentially the parameters required to be assessed. This study describes the development and evaluation of individual qPCR assays that represent collectively the breadth of the entire HSV-1 transcriptome in a format that would reduce overall cost and simplify assessment of gene expression. Although there are other reports of qPCR primers and conditions that represent individual or subsets of HSV-1 (Chen et al., 1997; Hukkanen et al., 2000; Broberg et al, 2003; Carr and Al-Khatib, 2003; Wang et al, 2005), this work represents a compilation of optimized primer sets and validated reaction conditions that were developed specifically to analyze the entire HSV-1 lytic transcriptome.

Each individual qPCR assay was designed to work in conjunction with other assays and therefore enabled their compilation into a plate arrays that could analyze the majority of HSV-1 genes within a single PCR run. Amplification reactions for HSV-1 target genes were designed to be quantified using SYBR Green-I-based detection, such as to not require expensive probe generation and to permit assay adaptability with additional cellular or viral genes. Quantitative analysis of gene expression via SYBR Green-I detection had several advantages for whole HSV genome level transcriptome assay design and expression analysis: (1) SYBR Green-I could be applied directly to PCR assays without the need to design, synthesize, and optimize fluorescent labeled target-specific probes. (2) SYBR Green-I detection enabled rapid and adaptable assay design, which was critical given the number of genes required to be analyzed. (3) The use of SYBR Green-I is less expensive than fluorogenic detection probes. (4) Once optimized and confirmed to be specific, SYBR Green-I detection utilizes conventional PCR protocols and primer sets that can be arrayed easily in plates for subsequent analysis. Overall, SYBR Green-I-based detection represented the most adaptable, economical approach for large-scale whole transcriptome analysis of HSV-1 gene expression.

The success and adaptability of a qPCR assay for HSV-1 gene expression is highly dependent on several factors. First, it is imperative that the qPCR system has the ability to recognize multiple strains of HSV-1 with a high level of specificity for each target gene. In addition, with multiple gene targets on one plate, optimized performance with a single thermal cycling protocol and reaction condition are necessary for utilizing the system as a qPCR array. Lastly, it is essential for the qPCR system to quantify RNA levels accurately across a wide linear range of template concentrations with minimal intra- and inter-assay variability. Critical to these parameters is the initial design and evaluation of primer sets, which were considered carefully for the assays presented in this study. In turn, the final optimized primer sets were selected to be highly specific for their target gene and recognized multiple HSV-1 strains. The absence of significant intra- or inter-assay variation enables plate-to-plate comparisons between results obtained or to provide statistical significance when examining replicate data sets. In short, these assays can be utilized to measure relative changes in HSV-1 gene expression reproducibly and accurately, either as a primary tool for expression analysis or a confirmatory method following microarray or NGS. One intriguing finding was the ability of melting temperature dissociation curve analysis to detect single nucleotide differences within the amplified products of different

strains, especially within the TK gene where mutations can result in drug resistance (Coen and Schaffer, 1980; Furman et al., 1981; Coen et al., 1982; Frobert et al., 2005; Wang et al., 2007).

The applicability of the designed assays was demonstrated by determining the profiles of HSV-1 gene expression kinetics for an array of representative genes from all HSV-1 transcriptional gene classes. This analysis permitted determination of relative fold changes in each gene across a replicative time course. Since only two reaction conditions were utilized for all designed primer sets, the effects of various conditions can be analyzed on an individual plate that specifies an array of primer sets to most HSV genes, as well as necessary cellular reference genes. Visualization of expression values on a heat map facilitated direct comparison of transcriptional class specific expression profiles. As expected, each transcriptional class exhibited patterns of gene expression that were indicative of their known timing of expression and their roles in HSV-1 replication. Interestingly, the UL2 gene expression pattern did not match other beta genes, but rather exhibited a profile that was reminiscent of the gamma gene class (Fig. 3B). Re-examination of literature related to assignment of transcriptional class categories for the UL2 gene indicated that this gene could fall within either the beta or gamma gene classes (Pellett and Roizman, 2007; Roizman et al., 2007). These data highlight the utility of the developed qPCR array to examine genome-wide expression patterns during experimental HSV-1 infection. Foremost, since these compiled primer sets represent the entire HSV-1 lytic transcriptome, these arrays can facilitate experiments that can complement, corroborate, and validate results obtained from contemporary microarray and NGS technologies.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

This work is in memory of Dr. James M. Hill, a cherished colleague, friend, and mentor. Without his contributions and continuous support this research would not have been possible. This work was supported by a Louisiana Board of Regents Research Competitiveness Award LEQSF-RD-A-13, by a Research Commercialization grant from the Louisiana Board of Regents through the Louisiana Vaccine Center, and by the National Institutes of Health from the National Center for Research Resources (P20RR021970) and the National Institute of General Medical Sciences (P20GM103501). In addition, this work was supported in part by R01AI112402 from the National Institute of Allergy and Infectious Diseases and U54GM104940 from the National Institute of General Medical Sciences through the Louisiana Clinical and Translational Science Center. The content is solely the responsibility of the authors and does not represent necessarily the official views of the National Center for Research Resources, the National Institute of General Medical Sciences, the National Institute of Allergy and Infectious Diseases or the National Institutes of Health.

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Fig. 1.

Evaluation of amplification and assay specificity of representative genes from each transcriptional class: RL2/ICP0 (α gene: A1, B1, C1); UL23/Thymidine Kinase β gene: A2, B2, C2); UL44/gC(γ 2 gene: A3, B3, C3). Specific amplification of the indicated target genes was assessed by size and purity determination of amplified products on 1.8% TBE agarose gels (A), comparative melt curves of amplified products (B), and relative amplification curves (C). Target specificity and amplification was determined using HSV-1 KOS genomic DNA (HSV-1 genomes; blue lines), cDNA from uninfected cells (uninfected cDNA; green lines), or cDNA from HSV-1 RE infected cells (HSV-1 cDNA; red lines). (For interpretation of the references to color in this figure legend, the reader is referred to the web version of the article.)

Fig. 2.

Determination of the linearity of amplification of representative genes from each transcriptional class: RL2/ICP0 (α gene); UL23/Thymidine Kinase (β gene); UL44/gC (γ 2 gene). The Cq values for amplification of serial 2 fold dilutions of HSV-1 KOS genomic DNA were determined (B) and plotted relative to the quantity of viral genomic DNA (A). Red and light blue dotted lines (A) correspond to the 168.8 pg (red) and 1350 pg (light blue) amplification curves (B). The R² value for each representative gene's dilution series is

shown as an assessment of assay linearity. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of the article.)

Fig. 3.

Kinetic analysis of relative HSV gene expression for representative genes from each transcriptional gene class. Relative gene expression was determined for the indicated gene transcript at 0, 1, 2, 4, 8, 12, 18, and 24 h post infection. A1: Kinetic analysis of representative HSV genes that exemplify the three observed expression patterns for the alpha gene transcriptional class. A2: Kinetic analysis of representative HSV genes that exemplify the two observed expression patterns for the beta gene transcriptional class. A3: Kinetic analysis of representative Late/gamma HSV genes. B: Heat map comparison of the relative

expression levels within each transcriptional gene class at the assessed time points post infection. Expression levels of a particular gene correlate on the heat map with a three point defined gradient range from green (no expression) to yellow (50% of maximum for each gene) and to red (highest expression level for that gene). (For interpretation of the references to color in this figure legend, the reader is referred to the web version of the article.)

HSV-1 alpha (α) genes: oligonucleotide primer sequences, PCR annealing temperatures, expected PCR product size, and observed melt temperatures.

Table 1

Gene name	Gene ID no.	Primer sequence (5'-3')	Annealing	Size (bp)	Melt temp.
RS1: ICP4	2703391	FOR: 5'-CGGTGATGAAGGAGCTGCTGTTC-3'	55	145	91
		REV: 5'-CTGATCACGCGGCTGCTGTACA-3'			
US1: ICP22	2703435	FOR: 5'-ATGCAATGCTACGGCGCTCGGT-3'	55	99	88
		REV: 5'-ACAGCTGATTGATACACTGGCGC-3'			
US12: ICP47	2703441	FOR: 5'-TCGTGCACGGGGTTCTG-3'	55	110	86.5
		REV: 5'-ACCTTCTTGGACACCATGCG-3'			
RL2: ICP0	2703389	FOR: 5'-GTCGCCCTTACGTGAACAAGAC-3'	55	114	85.5
		REV: 5'-GTCGCCATGTTTCCCGTCTG-3'			
UL54: ICP27	2703426	FOR: 5'-AGACGCCCTCGTCCGACGGA-3'	55	74	87
		REV: 5'-GAGCGCGGACCAACACTGT-3'			

Table 2

HSV-1 beta (β) genes; oligonucleotide primer sequences, PCR annealing temperatures, expected PCR product size, and observed melt temperatures.

Gene name	Gene ID no.	Primer sequence (5'-3')	Annealing	Size (bp)	Melt temp.
UL2: Uracil-DNA Glycosylase	2703370	FOR: 5'-GACTTGCGTTTAGCGTGCGC-3' REV: 5'-CAACCGTGGCCGCTCATC-3'	55	109	87
UL5: Helicase-Primase-Helicase Subunit	2703420	FOR: 5'-CTCCCGGAGCGGATTTAAAG-3' REV: 5'-GGACAAGGTCGCCATCTG-3'	55	118	86
UL8: Helicase-Primase Subunit	2703432	FOR: 5'-GGTGATGAGCGCAGTCC-3' REV: 5'-GTCTGCGTGTCTGTCTC-3'	55	100	88
UL9: Ori Binding Helicase	2703434	FOR: 5'-CATGTAGCGGTCTAGTGG-3' REV: 5'-GGGAGCTGTGTGCTGT-3'	55	131	88.5
UL12: Deoxyribonuclease	2703382	FOR: 5'-TGGCGTTGTGCTTCAGCCA-3' REV: 5'-TGCCAGCTATGGGCCCTG-3'	55	106	88.5
UL23: Thymidine Kinase	2703374	FOR: 5'-TCGATGTCTGTCTCTCCG-3' REV: 5'-ATCCCATCGCCGCCCTC-3'	55	154	89 (89, 5 ^d)
UL29: ICP8	2703458	FOR: 5'-CATCAGCTGCTCCACCTCGCG-3' REV: 5'-GCAGTACGTGGACACAGGCGGT-3'	55	130	88
UL30: DNA Polymerase Catalytic Subunit	2703462	FOR: 5'-CATCACCGACCCGGAGAGGAC-3' REV: 5'-GGGCCAGGCGCTTGTGTGTA-3'	65	92	86
UL39: Ribonucleotide Reductase Subunit 1	2703361	FOR: 5'-GACTTTCACGGGGAGGAGTT-3' REV: 5'TAGATGTAGTGGCGGTTTACC-3'	55	167	87
UL40: Ribonucleotide Reductase Subunit 2	2703364	FOR: 5'-GTCCCGACATTAAACACCTG-3' REV: 5'-AAGCTGAGCTCGCCCTCG-3'	55	118	85
UL42: Polymerase Processivity	2703407	FOR: 5'ACGTCCGACGGCGAGG-3' REV: 5'-CAGGCGCAACTGAACGTC-3'	65	111	87
UL50: dUTPase	2703421	FOR: 5'-GTGACTACGGCCCTACCG-3' REV: 5'GAAACCCGGCATCTCTCTCG-3'	55	141	88.5
UL52: Helicase-Primase-Primase Subunit	2703423	FOR: 5'TGTCCGACCGTGAATTCATTAC-3' REV: 5'TTGGGGTCTCTGGGTGTC-3'	55	106	85

^dThe melt temperature for the HSV-1 KOS genomic DNA was 0.5° greater than for the HSV-1 RE cDNA).

HSV-1 gamma (γ) genes: oligonucleotide primer sequences, PCR annealing temperatures, expected PCR product size, and observed melt temperatures.

Table 3

Gene name	Gene ID no.	Primer sequence (5'-3')	Annealing	Size (bp)	Melt temp.
US5: Glycoprotein I (gI)	2703406	FOR: 5'-CACCCATCGGGACCTGC-3' REV: 5'-CAGGACTACGGCGCACAGC-3'	55	127	88.5
US9: Type 2 Membrane Protein	2703452	FOR: 5'-CGATCCGACATGTCCTGC-3' REV: 5'-ATGCGTACGAGGAAGTCGTTG-3'	55	107	85.5
UL1: Glycoprotein L (gL)	2703393	FOR: 5'-CAACCGAATATGTTATTCGGAGTCGG-3' REV: 5'-CTCTGTAACGCCCAATCAAGATCGTC-3'	55	104	82.5
UL6: Capsid Vertex	2703429	FOR: 5'-GCGTGGCGGACTACGACCG-3' REV: 5'-TCGGGAAACAGCCGCTGGA-3'	55	169	89
UL11: Envelope-Associated	2703380	FOR: 5'-GGCACGTAGAAATTACCTTCCT-3' REV: 5'-TGCTGCCGAAACAACGT-3'	55	113	85
UL13: Ser/Thr Kinase	2703383	FOR: 5'-ACGTCATAGCCAGGCCGT-3' REV: 5'-CAGCTGTCCCGGACTTCG-3'	55	110	88
UL15: DNA Packaging Terminase	2703385	FOR: 5'-GCCGTCGCCATCGCCAC-3' REV: 5'-GTACAGCACCGCGCTCCC-3'	55	129	89.5
UL17: DNA Packaging	2703388	FOR: 5'-CCGCTGACGGCCTGGTGAA-3' REV: 5'-CTCGCGTTGGTTCAAGCGGG-3'	55	131	91.5
UL19: VP5 Major Capsid Protein	2703368	FOR: 5'-TGGATGGTATGGTCCAGATGC-3' REV: 5'-GCACAACGGCGCTGCTCT-3'	55	139	90
UL21: Virion Morphogenesis Protein	2703372	FOR: 5'-CTGGTCGTTTCGAGGACAG-3' REV: 5'-ACACCTCGTCTCTCCCAATG-3'	55	112	87
UL22: Glycoprotein H (gH)	2703373	FOR: 5'-CTGCCGGAGGACAGCCGA-3' REV: 5'-GCAGCCCATCTCATCGACG-3'	55	108	87
UL26: Serine Protease Scaffold Protein	2703453	FOR: 5'-GGACTCGGGCGAGTTGGCA-3' REV: 5'-CTGCACGCGAGCGATCAG-3'	55	172	90
UL26.5: Scaffold Protein	2703454	FOR: 5'-CTCCCATTACAACAGCTCGT-3' REV: 5'-CGCACCGTGAGGCCCATAG-3'	55	112	90.5
UL28: DNA Packaging Terminase Subunit 2	2703457	FOR: 5'-GATGAGGTGGCAGTAGTTAGC-3' REV: 5'-CCGACCCCTGTCCGTATCA-3'	55	124	83.5

Gene name	Gene ID no.	Primer sequence (5'-3')	Annealing	Size (bp)	Melt temp.
UL31: Nuclear Egress Protein	2703350	FOR: 5'-CTCTGCGTTGCGCCGGAC-3' REV: 5'-CTGTACGTCACTTTCCCGGC-3'	55	141	88.5
UL43: Type 3 Membrane Protein	2703408	FOR: 5'-CGCGGATATACGCCCTGGCT-3' REV: 5'-CGCATAGGCGGCCCCAG-3'	55	140	91.5
UL48: VP16 Major Transactivator IE Genes	2703416	FOR: 5'-CATATCCAGAGCGCCGTAG-3' REV: 5'-CACTTAGACGGCGAGGAC-3'	55	138	88
UL49: VP22 Major Tegument Protein	2703417	FOR: 5'-GGGACGCCCGAGAACGC-3' REV: 5'-ACGAACTCCTTGGCATCACC-3'	55	144	88.5

HSV-1 gamma1 ($\gamma 1$) genes: oligonucleotide primer sequences, PCR annealing temperatures, expected PCR product size, and observed melt temperatures.

Table 4

Gene name	Gene ID no.	Primer sequence (5'-3')	Annealing	Size (bp)	Melt temp.
US3: Kinase	2703401	FOR: 5'-GTCACGTGTCTGGTCTCC-3' REV: 5'-GGAGACCGTGGGATCTG-3'	55	99	84.5
US4: Glycoprotein G (gG)	2703404	FOR: 5'-GGTCTTGTGCGTGTATCGG-3' REV: 5'-CGGTCTGGGTGTCATGTTGG-3'	55	110	85.5
US6: Glycoprotein D (gD)	2703444	FOR: 5'-CTATGACAGCTTCAGGCCGTCAG-3' REV: 5'-CGTCCAGTCGTTTATCTTCACGAGC-3'	55	112	86.5
US7: Glycoprotein I (gI)	2703446	FOR: 5'-GACGGCGTAGTGGAGGAA-3' REV: 5'-GGTAGTGCCACAGCTCT-3'	55	110	84
US8: Glycoprotein E (gE)	2703448	FOR: 5'-CGAGGACGTTTCGTTGCTTCC-3' REV: 5'-GAGACCCACGACGGGTGTA-3'	55	117	87.5
US8A: Type 2 Membrane Protein	2703450	FOR: 5'-GGCATCAATCTGCCGCCA-3' REV: 5'-GTTTCGGCGACCGACGTGG-3'	55	163	87.5
US10: Unknown Function	2703436	FOR: 5'-ACAGGTCGTGCATGTGTC-3' REV: 5'-CGGGCCAAACCAAGAGAC-3'	55	109	87
UL7: Tegument Protein	2703431	FOR: 5'-GGTTCGGTTCGTTCTGGAC-3' REV: 5'-CTCGTTGGCCGTCAGGACC-3'	55	125	86.5
UL16: Tegument Protein	2703386	FOR: 5'-CAGGTCGCTGTCCATGGCC-3' REV: 5'-AGCTGGA TGGCGCGTATCTG-3'	55	131	90.5
UL18: Capsid Triplex Subunit 1	2703366	FOR: 5'-GACCAGCGATCGCAGCTCG-3' REV: 5'-GGGTGCCAGACCTCAACG-3'	55	105	86.5
UL20: Virus Egress Membrane Protein	2703371	FOR: 5'-CGTCCGGACTGAAGGTCTC-3' REV: 5'-GCTCTCTCTGTACGTTAACATC-3'	55	137	87
UL24: Nuclear Protein	2703375	FOR: 5'-GCCCGGAGAACCGGCAG-3' REV: 5'-CCTCGAATACCGAGCGACC-3'	55	178	87.5
UL27: Glycoprotein B (gB)	2703455	FOR: 5'-TACTGCGGCTGGCCCACTTG-3' REV: 5'-GCTCTCGCGGTGGACCTG-3'	55	114	88.5
UL34: Type 2 Membrane Nuclear Egress Protein	2703355	FOR: 5'-CAGAAACCCGCCGTGTCGG-3' REV: 5'-CTTGA TGGTGTCCAGGTCGC-3'	55	150	88

Gene name	Gene ID no.	Primer sequence (5'-3')	Annealing	Size (bp)	Melt temp.
UL37: Tegument Protein	2703358	FOR: 5'-GCAGTACTGACTCACC GCGT-3' REV: 5'-CGCAGACCCCTGATTCGC-3'	55	193	91
UL41: RNAse Virion Host Shutoff (VHS)	2703365	FOR: 5'-GAAATTTTCGTCCACGAGACGCG-3' REV: 5'-GGATGTGCTGGCGGAAATGTCACT-3'	55	158	91 (91.5 ^a)
UL46: Modulator of VP16	2703413	FOR: 5'-GTTTTCGTAGACCCGCAATCC-3' REV: 5'-ATGGAAGCCACGTATCTGACG-3'	55	183	90.5
UL47: Modulator of VP16	2703415	FOR: 5'-CTCCAGAACCTCCACCTTGGG-3' REV: 5'-CGTCGCCGCTGTTCTCTAGA-3'	55	113	88.5
UL51: Tegument Protein	2703422	FOR: 5'-AGCACATCCGCGGACTTGTGTC-3' REV: 5'-ACCAACCCGGGTTTCGAGG-3'	55	145	88.5

^aThe melt temperature for the HSV-1 KOS genomic DNA was 0.5° less than for the HSV-1 RE cDNA).

Table 5

HSV-1 gamma2 (γ 2) genes: oligonucleotide primer sequences, PCR annealing temperatures, expected PCR product size, and observed melt temperatures.

Gene name	Gene ID no.	Primer sequence (5'-3')	Annealing	Size (bp)	Melt temp.
US2: Membrane-Associated Ubiquitin Interacting	2703399	FOR: 5'-GCACCGATGGGACGTCGT-3' REV: 5'-CGGACGGGAGCGGATTGT-3'	55	101	85.5
US11: dsRNA Binding Protein	2703439	FOR: 5'-TCTCTGGGAGCTCGCGGTA-3' REV: 5'-GGTGGGACACTACGATCT-3'	55	143	87.5
RL1: ICP34.5	2703395	FOR: 5'-CGCCTTCTTGTTCGTGCT-3' REV: 5'-CCAGTCGTCGTCATCGTCGT-3'	55	80	86
UL3: Nucleocytoplasmic Shuffling Protein	2703461	FOR: 5'-GGAGTTCCTCTGCGCTT-3' REV: 5'-CGAATGGAATCTGGGGAGT-3'	55	107	84.5
UL4: Nuclear Protein Associated with ICP22	2703362	FOR: 5'-TGTTGCA GTGATGTAATGGTG-3' REV: 5'-TGCCTACCGCTTTATAGCCG-3'	55	182	89
UL10: Glycoprotein M (gM)	2703379	FOR: 5'-GACGTATCTGCGTCAGGTGC-3' REV: 5'-CCGTGCA CAGGAAGACGC-3'	55	110	89
UL14: Tegument Protein	2703384	FOR: 5'-GTTAGCTGCCACATGACCAAC-3' REV: 5'-TGGACCGCGTGGAGGATCG-3'	55	145	87
UL25: Capsid Vertices Protein	2703377	FOR: 5'-GCCGCTACGATACGACAC-3' REV: 5'-CAGGTCCCGAACACAC-3'	55	99	86
UL32: DNA Packaging Protein	2703352	FOR: 5'-CACGGAGGCCCGGAGTC-3' REV: 5'-CGATGCCCGAGCCGACC-3'	55	154	90
UL33: Interacts with DNA Packaging Terminase	2703353	FOR: 5'-CGTCTGTTTCGAGGATATGAC-3' REV: 5'-AGGCCCGCGTACGTCAG-3'	55	121	87.5
UL35: Capsid Hexons	2703356	FOR: 5'-TTTCTCCGCGTCAAGCG-3' REV: 5'-TCGCCCGCGAACATAGGC-3'	55	89	87
UL36: Ubiquitin Specific Protease	2703357	FOR: 5'-ACTGTCATACTCTGTGTAGTCG-3' REV: 5'-CGCGCTGGGAAAGCTGAGT-3'	55	133	89
UL38: Interacts with Capsid Triplex Subunit 2	2703359	FOR: 5'-CGTGATCAAGAGCCAGCTCC-3' REV: 5'-ACCTGGCCAGCCGAGCAAC-3'	55	192	91
UL44: Glycoprotein C (gC)	2703410	FOR: 5'-GAGGAGTCTGTACGAACATCACCC-3' REV: 5'-CCGGTGACAGAAATACACGGAGG-3'	55	140	90.5

Gene name	Gene ID no.	Primer sequence (5'-3')	Annealing	Size (bp)	Melt temp.
UL45: Type 2 Membrane Protein	2703412	FOR: 5'-GTGATCATTTGCCGCGTTGGT-3' REV: 5'-TTGCCGACTGGACGCGTGC-3'	55	217	91
UL49A: Glycoprotein N (gN)	2703419	FOR: 5'-TGGGTCTCGCACCCGCGC-3' REV: 5'-CCTTAGCGCGGGGAGACG-3'	55	91	91.5
UL53: Glycoprotein K (gK)	2703425	FOR: 5'-CTTGAACTACGCAGGCCGCATC-3' REV: 5'- CGGTCCGCTCAAACTGAAC-3'	55	127	85.5
UL55: Nuclear Protein	2703427	FOR: 5'-GGCCGTGCGGAGTTACCTG-3' REV: 5'-GGTGGTTAACCCAGGTCCGC-3'	55	143	85
UL56: Type 2 Membrane Protein	2703428	FOR: 5'-CCTGTACAGCTCGCGCGAAT-3' REV: 5'-CCCAACCTATGCCACTGTGC-3'	55	136	89

Table 6

Representative analysis of assay reproducibility in accordance with MIQE guidelines.

Variation	Gene analyzed	Template	Cycle of quantification		
			Mean	S.D.	% CV
Intra-assay	RL2: ICP0	4 h p.i. cDNA	20.67	0.04	0.19
	RL2: ICP0	12 h p.i. cDNA	18.04	0.02	0.10
	RL2: ICP0	HSV-1 genomic DNA	20.53	0.04	0.19
	UL23:TK	4 h p.i. cDNA	17.52	0.03	0.15
	UL23:TK	12 h p.i. cDNA	17.66	0.08	0.46
	UL23:TK	HSV-1 genomic DNA	21.28	0.06	0.27
	UL44: gC	4 h p.i. cDNA	24.37	0.07	0.29
	UL44: gC	12 h p.i. cDNA	18.85	0.12	0.65
	UL44: gC	HSV-1 genomic DNA	23.04	0.04	0.19
	RL2: ICP0	4 h p.i. cDNA	20.72	0.08	0.36
Inter-assay	RL2: ICP0	12 h p.i. cDNA	18.11	0.05	0.30
	RL2: ICP0	HSV-1 genomic DNA	20.48	0.06	0.28
	UL23:TK	4 h p.i. cDNA	17.67	0.14	0.77
	UL23:TK	12 h p.i. cDNA	17.77	0.10	0.55
	UL23:TK	HSV-1 genomic DNA	21.59	0.36	1.66
	UL44: gC	4 h p.i. cDNA	24.49	0.15	0.63
	UL44: gC	12 h p.i. cDNA	18.91	0.12	0.63
	UL44: gC	HSV-1 genomic DNA	23.22	0.33	1.40