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The Use of Competitive PCR for Quantitation of HSV-1 DNA

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Purpose: Polymerase chain reaction (PCR) detects the genomic materials of etiological agents with high specificity and sensitivity. However, in herpes simplex virus type 1 (HSV-1) infection, the clinical significance of the results often poses controversy because of the subclinical viral shedding during latent infection. Quantitative PCR might provide additional information to help clinical evaluation of the results.

Methods: Virus DNA was extracted from high titer stock of human HSV-1 (FK 25) by phenol/chloroform treatment. Construct (p/HSV-1) was made by inserting the glycoprotein D gene obtained from virus DNA into p(GEM-T) vector. Competitor (p/ΔHSV-1) was made by deleting the inner 40 bp of construct (p/HSV-1) with restriction enzyme. Competitive PCR was performed using primers that amplify the glycoprotein D gene, and a template made of a 1:1 molar mixture of HSV-1 DNA and the competitor.

Results: The PCR product reflected the initial template dose from 20 to 30 cycles. Minimum detection level of HSV-1 DNA was 0.01 ng.

Conclusion: Competitive PCR can quantitate HSV-1 DNA. **Jpn J Ophthalmol 2003;47:240–245**

Key Words: Competitive PCR, cornea, glycoprotein D, herpes simplex virus type-1, human.

Introduction

Herpes simplex virus type 1 (HSV-1) causes three types of corneal disease: dendritic or geographic ulcer of the epithelium, disciform or necrotizing keratitis in the stroma,[1] and endothelitis.[2] Positive immunofluorescence of HSV antigen and virus isolation are diagnostic criteria for HSV-1 infection,[3] however, diagnosis is often hampered by a nonspecific reaction, low sensitivity, and time-consuming, meticulous laboratory work.[4,5]

Polymerase chain reaction (PCR) was initially devised as a technique to make a large quantity of a specified DNA fragment.[6] It has become a powerful tool for the diagnosis of infectious diseases, with extremely high sensitivity and specificity.[7] However, its qualitative nature poses a controversial difficulty in the clinical diagnosis of herpes virus infections, because of the subclinical shedding of the virus or the presence of the latent virus-harboring cells. Quantitative PCR offers additional information to establish a more solid clinical diagnosis and evaluation of these virus infections. Previously, Piatak et al demonstrated quantitative analysis of human immunodeficiency virus type 1 (HIV-1) RNA by competitive PCR and reported that it correlated with the amounts of HIV-1 RNAs and CD4+ T cells in the blood.[8] Furthermore, an upgraded variety of assays to quantitate HIV DNA and RNA levels were reported.[9–11] In addition to HIV, PCR was applied to quantitate hepatic hepatitis C virus (HCV) in patient blood.[12–14]

About herpes, it was reported that virus DNA can be quantitated in genital tract secretion, trigeminal ganglia, and cerebrospinal fluid by competitive PCR.[15–18] During this experiment, we tried to develop a new application of quantitation for HSV-1 DNA by competitive PCR, to quantitate virus DNA levels in human tear film.

Received: January 28, 2002
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Published by Elsevier Science Inc.

0021-5155/03/$–see front matter
doi:10.1016/S0021-5155(03)00010-8

Materials and Methods

Constructing Vectors Containing Glycoprotein D (gD) and Truncated gD Gene of HSV-1

DNA was extracted from stock of HSV-1 (2.5×10^9 pfu/mL) (strain F25) by phenol/chloroform treatment. In brief, 865 μL of the virus was mixed with 20 μL of 5 N NaCl, 5 μL of 2 M Tris-HCl (pH 8.0), 50 μL of 0.5 M ethylenediaminetetraacetic acid (EDTA, pH 8.0), 50 μL of 10% sodium dodecyl sulfate and 10 μL of 10 mg/mL Proteinase K solution (Merck, Darmstadt, Germany) to give a final concentration of 100 μL/mL. It was incubated at 55ºC for 18 hours. Viral DNA was extracted with an equal volume of 25:24:1 phenol/chloroform/isoamyl alcohol saturated with Tris-EDTA buffer. After centrifugation at $20{,}879 \times g$ for 3 minutes, the pellet was washed in ice-cold ethanol and then resuspended in a minimal volume of Tris-EDTA (0.1–0.5 mL/g of starting material). The HSV gD gene was amplified by PCR, using primer sequences delineating 570 bp (sense, 5-TGTGACACTA TCGTCCATAC-3; antisense, 5-AAGCGATGGTCAGG TTGTAC-3). The 100-μL reaction mixture containing 50 mM KCl, 10 mM Tris-HCl (pH 9.0), 1.5 mM $MgCl_2$ 0.1% (w/v) Triton X-100, 200 M of dNTP, 0.5 M of each primer, 2.5 units of *Taq* DNA Polymerase (Promega, Madison, WI, USA). The amplification steps were as follows: initial denaturation step 94ºC for 2 minutes; 30 cycles at 94ºC for 30 seconds, 55ºC for 30 seconds, 72ºC for 30 seconds; additional extension step 72ºC for 7 minutes on a GeneAmp PCR System 9600 thermocycler (Perkin Elmer, Foster City, CA, USA).

Construct (p/HSV-1) was made by inserting the PCR-amplified gD gene into pGEM-T vector (Promega) (Figure 1).

Competitor (p/ΔHSV-1) was made by deleting the inner 40 bp of the construct with restriction enzymes, *Hind* III and *Afl* II. Both p/HSV-1 and p/ΔHSV-1 were isolated from cultures of transfected *Escherichia coli* using the Flex Prep Kit (Pharmacia Biotech, Uppsala, Sweden). After purification with a PCR purification kit (Qiagen, Dusseldorf, Germany), the two constructs were sequenced with an automatic fluorescent DNA sequencer (ABI Prism 373; Applied Biosystems, Foster City, CA, USA) and a dye terminator cycle sequencing kit (Perkin Elmer).

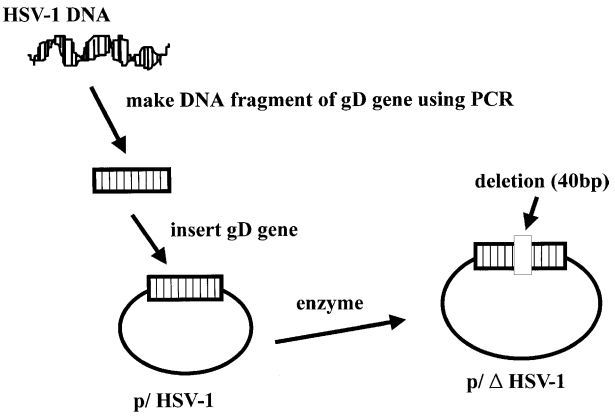

Figure 1. The competitor was made by inserting glycoprotein D gene obtained from virus DNA into the pGEM-T vector.

Quantitation of HSV-1 DNA by Competitive PCR

We performed PCR for 15 to 35 cycles at 2-cycle intervals. After the regular PCR, 10 μL of the product was electrophoresed on a 3% agarose gel, stained with SYBR green I (Molecular Probes, Eugene, OR, USA) and scanned with a laser-scanning image analyzer (Fluor Imager 595, Molecular Dynamics, Sunnyvale, CA, USA). For the quantitative PCR, new primers that amplify 350 bp located inside the constructed gD genome were used. Primer sequences were as follows: sense, 5-AGGGAGT TGTTCGGTCATAAGCT-3; antisense, 5-GCGTAGTA AACCGTGAT CGGGA-3. The amplification steps were modified as follows: initial denaturation step at 94ºC for 2 minutes; 30 cycles at 94ºC for 30 seconds, 55ºC for 30 seconds, 72ºC for 30 seconds; extension step 72ºC for 7 minutes. Competitive PCR was performed, using a template made of a 1:1 molar mixture of HSV-1 DNA and the competitor. After the defined cycles of PCR, the product was electrophoresed on a 3% agarose gel and stained with SYBR Green I.

Results

Competitive PCR Quantitates HSV-1 DNA

Initially, PCR amplifies DNA exponentially; however, as the amount of PCR product increases, the speed of amplification diminishes, and the amount of the product levels off (Figure 2A). Therefore, if PCR is allowed to go on past this point, the amount of the PCR product does not reflect the initial amount of the template. Therefore, PCR was performed using varied amounts of genome and competitor to determine an appropriate cycle number for the reaction measuring the fluorescence intensity of the product (Figure 2B). At the plateau, fluorescence intensities of the genome and competitor were the same. However, the higher the initial amount of the template, the lower the number of PCR cycles required to reach a plateau. These results indicated that it is possible to set an appropriate cycle number to quantify DNA by competitive PCR, depending upon the initial amount of the template. In our method, a target DNA and a competitor are amplified by the same primer. Therefore, the ratio

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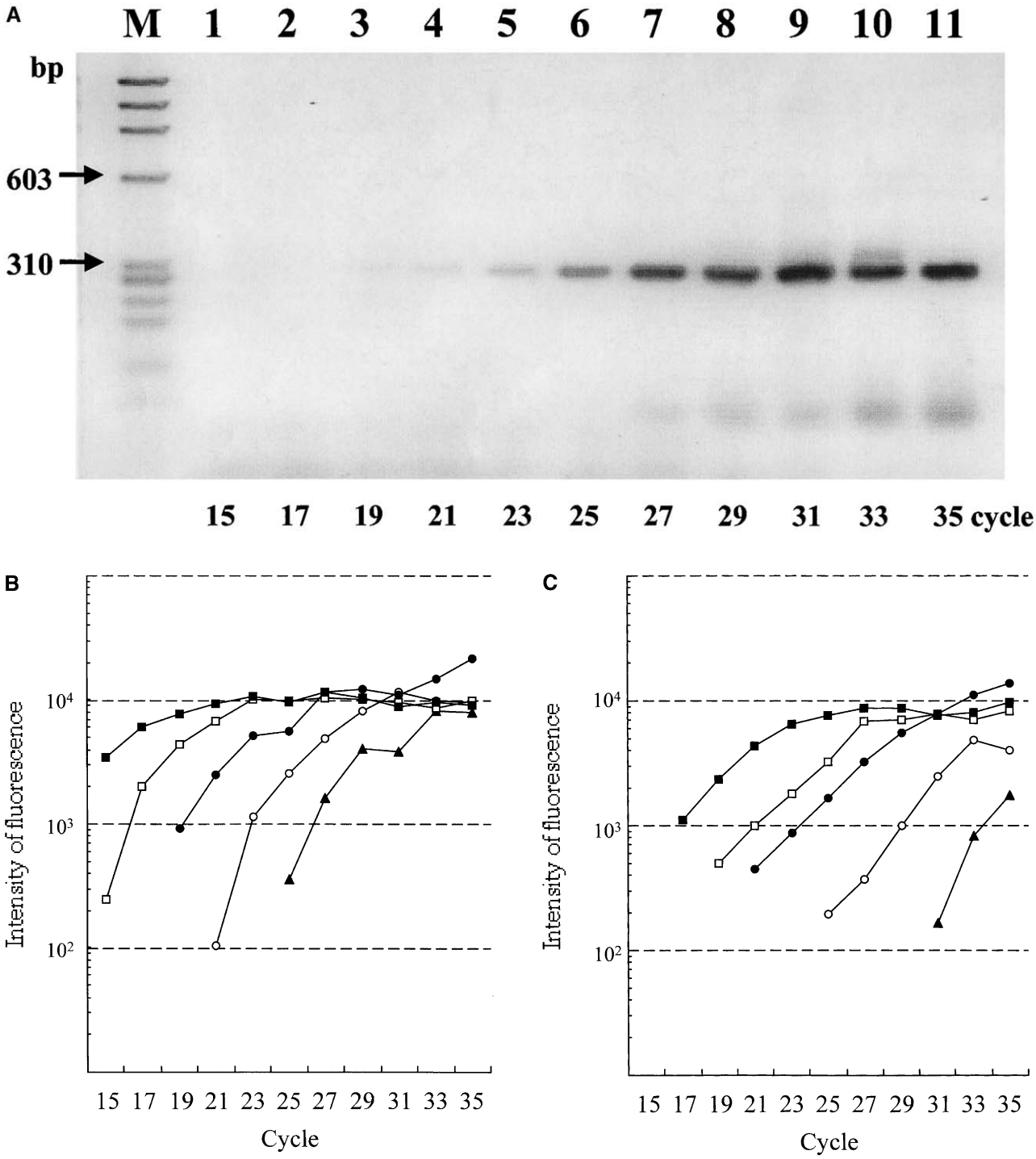

Figure 2. Number of cycles and increase of polymerase chain reaction (PCR) product measured by Fluor Imager 595. (**A**) Agarose gel stained with the SYBR Green I of competitor. Lanes are as follows: M, *Hae* III Digest of Ø × 174 as size markers. Lanes 1 to 11, 30 pg of competitor added. (**B**) Kinetics of PCR amplification of competitors. Curves represent 10^4, 10^5, 10^6, 10^7 and 10^8 copies of competitor from right to left. (**C**) Kinetics of PCR amplification of herpes simplex virus type 1 (HSV-1) genomes. Curves represent 1 pg, 10 pg, 100 pg, 1 ng, and 10 ng of HSV-1 genome from right to left.

of the two templates should remain constant during their exponential amplification phase. Actually, with 780 pg of target DNA, PCR should be performed for 21 to 27 cycles (Figure 2C). Consequently, competitive PCR was performed for 25 cycles (Figure 3A). The fluorescence intensity was measured using Fluor Image 595, and the results showed a linear function with an extremely high degree of correlation ($y = -2.924 + 0.421x$, $r^2 = 0.994$, $P < .0001$) (Figure 3B). Subsequently, the copy number of target DNA was estimated based on the calculation where the fluorescence intensities of competitor and target DNA reached the same point ($\log_{10} = 0$). Thus we estimated that there were 17.64×10^6 copies/mL of HSV-1 in 780 pg of target DNA because there were 8.82×10^6 copies in 10 to 20 μL of the reaction solution.

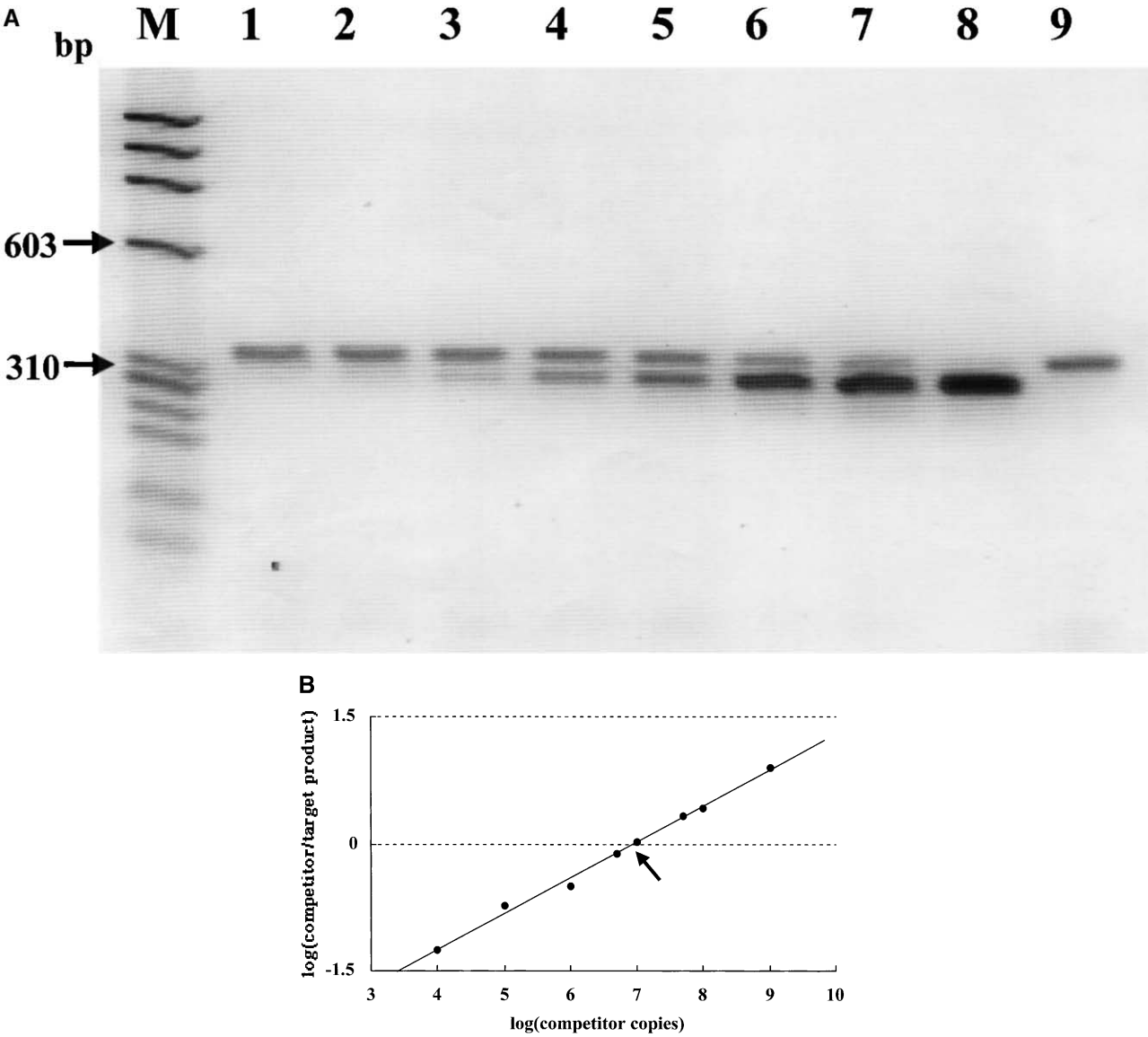

Figure 3. Competitive polymerase chain reaction (PCR) quantitates herpes simplex virus type 1 (HSV-1) DNA. (**A**) Agarose gel stained with the SYBR Green I. PCR products derived from HSV-1 genome (upper band, 350 bp) and competitor (lower band, 310 bp). Lanes are as follows: M, *Hae* III Digest of Ø × 174 as size markers. Lanes 1 to 8, 10^4, 10^5, 10^6, 5×10^6, 10^7, 5×10^7, 10^8, and 10^9 copies of competitor and 780 pg of HSV-1 genome. Lane 9; no competitor added. (**B**) Copy number of HSV-1 DNA (780 pg) is plotted on the graph. Arrow indicates a cross point of the y and amount of competitor and target product is equal: Ordinate 0. Copy number of target is obtainable at a point where a vertical line from the arrow crosses on the abscissa (Target copies/reaction = 8.82×10^6 copies, $y = -2.924 + 0.421x$, $r^2 = 0.994$).

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Discussion

Quantitation of target DNA by competitive PCR assay is based on coamplification of target and competitor where a known amount of competitor DNA is present in the same reaction mixture. The latter is genetically engineered so that it differs slightly in length or in restriction enzyme site from the target.[19,20] Quantitative PCR can be performed by using either one or two sets of primers. However, when two sets of primers are used, even if the amplification of one DNA reaches a plateau, the amplification of the other DNA will not necessarily stop. On the other hand, if one set of primers is used, primers should theoretically be depleted at the same reaction step. Therefore, the amount of the two primers should retain their initial quantities when the amplification reaches a plateau. We have chosen to employ the one set of primer method.[21]

There are highly conservative, species-specific, and/or highly variable areas in any genome. For this method, primers must be designed to target highly conserved areas. In the present study, the primers were synthesized to target the gD gene, a highly conserved area in the HSV genome.[22] Through the use of these primers, 350-bp PCR product was obtained, which extended from 100 bp upstream to 100 bp downstream in the gD gene. It was inserted in pGEM-T vector (p/HSV-1). The competitor was constructed using two restriction enzymes, *Hind* III and *Afl* II, which deleted the 40-bp fragment from p/HSV-1 (p/ΔHSV-1). As a result, the PCR product of p/ΔHSV-1 became 310 bp and was easily differentiated on an agarose gel. A sequencer was used to confirm that there were no mutations in the nucleic acid sequence of the inserted DNA in both constructs.

We used SYBR Green I staining, because its sensitivity is 25 times greater than that of ethidium bromide.[23] The sensitivity of our method was sufficient to detect 10 pg of HSV-1 DNA genome or 10^2 copies of the competitor. During this experiment, we found that in patients with untreated dendritic keratitis, the virus can be isolated from the tear film and positive immunofluorescence can be obtained using these fluid samples. However, once an antiviral agent was used, the immunofluorescence became negative quickly. Actually, we quantitated the tear films of patients with untreated dendritic keratitis using our method, resulting in 10^7 to 10^9 copies/μL, which became negative immediately after antiviral treatment (data not shown). This phenomenon suggests that the level of HSV-1 DNA had decreased to less than 10^2 copies after patients had received acyclovir ointment treatment.

Clinically, it is very important to know what dose of an antiviral agent should be given and how long. Quantitative analysis of the virus in the lesion by competitive PCR can provide useful information on these issues. However, technically speaking, competitive PCR is too time-consuming to be realistically feasible in a clinical setting. Recently, the development of real-time PCR has solved this problem by allowing for real-time monitoring of the amplification process, thereby circumventing the technically laborious steps necessary with competitive PCR.[23–25] Additionally, some researchers have reported quantitation of virus RNA levels using competitive PCR technique and real-time PCR.[11,14]

Using our method it is possible to quantify the virus DNA; however, quantitation of mRNA reflects only herpes virus replication. Piatak et al and Kaneko et al established quantitation of mRNA of HIV and HCV by reverse transcriptase-PCR (RT-PCR).[8,12] Therefore, we must not only further improve the sensitivity of our present quantification method, but in the future we must also develop quantitative RT-PCR using real-time PCR to obtain a more detailed analysis of the virus-host relationship in HSV-1 infection.

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