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Rapid and sensitive detection of 68 unique varicella zoster virus gene transcripts in five multiplex reverse transcription-polymerase chain reactions

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Abstract

Varicella zoster virus (VZV) becomes latent in ganglionic neurons along the entire neuraxis. Although all predicted VZV open reading frames (ORFs) have been detected by macroarray and microarray analysis in virus-infected cells in culture where virus gene expression is abundant, array technology does not detect all VZV gene transcripts in latently-infected human ganglia, where the abundance of ganglionic RNA is low and VZV gene transcription is highly variable. Using reverse transcription-polymerase chain reaction (RT-PCR) and the GenomeLab Genetic Analysis System (GeXPS), transcripts mapping to all 68 predicted unique VZV ORFs were detected in VZV-infected MeWo cells. Oligonucleotide primers contained both VZV- and cell-specific sequences linked to universal DNA sequences such that PCR amplification products were of predetermined sizes. Amplification products were resolved by capillary gel electrophoresis and detected by fluorescence spectrophotometry. Serial dilutions of total RNA extracted from VZV-infected MeWo cells were analyzed in parallel by GeXPS multiplex RT-PCR and real-time RT-PCR. GeXPS technology detected as few as 20 copies of VZV gene-specific transcripts. Only five multiplex RT-PCR assays were needed to analyze the entire VZV transcriptome. This technology will allow rapid analysis of all VZV genes transcribed during latency in human ganglia.

Keywords

VZV; GeXPS multiplex PCR

1. Introduction

Varicella zoster virus (VZV) is an exclusively human neurotropic alpha-herpesvirus. Primary infection typically causes childhood chickenpox, after which virus becomes latent in ganglionic

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neurons along the entire neuraxis (Gilden et al., 2003). Decades later, virus can reactivate to produce zoster (shingles), frequently complicated by postherpetic neuralgia (Rogers and Tindall, 1971; Bowsher, 1997). Reactivation can also lead to VZV vasculopathy, myelopathy and retinal necrosis (Nagel and Gilden, 2007).

The 125,000-basepair VZV genome contains 71 identified open reading frames (ORFs) of which 3 are repeated (Davison and Scott, 1986). All predicted VZV ORFs have been detected by macroarray (Cohrs et al., 2003a) and microarray analysis (Kennedy et al., 2005) in virus-infected cells in culture where virus gene expression is abundant. Unfortunately, array technology does not detect all VZV gene transcripts in latently-infected human ganglia, where the abundance of ganglionic RNA is low. In anticipation of studying VZV transcription in latently infected human ganglia, the Beckman Coulter GenomeLab Genetic Analysis System (GeXPS) was adapted to detect transcripts mapping to all predicted VZV ORFs in virus-infected cells in tissue culture and the results were compared with those obtained using quantitative real-time PCR.

2. Materials and methods

2.1. Virus and cells

VZV (Ellen strain) was isolated from a zoster lesion and propagated by co-cultivation in human malignant melanoma MeWo cells (Grose and Brunel, 1978) as described (Cohrs et al., 2002).

2.2. RNA extraction

VZV-infected MeWo cells (2×10^7) were harvested at the peak of virus-induced cytopathic effect (3 days post-infection). Uninfected MeWo cells (2×10^7) were processed in parallel. Uninfected and VZV-infected cells were scraped into phosphate-buffered saline (20 mM phosphate pH 7.4, 150 mM NaCl), pelleted at 1000 × *g* for 5 min at 4°C, and snap-frozen in liquid nitrogen. Cell pellets were thawed in 9 ml Tri-Reagent (Molecular Research Center, Cincinnati, OH), disrupted by sonication (3 times for 30 sec at 4°C), and total RNA was extracted (Cohrs et al., 1992). Residual DNA was digested with RNase-free DNase (DNase I, amplification grade; Invitrogen, Carlsbad, CA) at room temperature for 15 min. After heat inactivation for 10 min at 65°C in 2 mM EDTA, RNA in 1.5 μl was removed for quantitation (Nanodrop Technologies, Wilmington, DE), and 5 μl was analyzed for RNA integrity by microfiltration on Bioanalyzer chips (Agilent Technologies, Quantum Analytics, Inc., Foster City, CA).

2.3. GeXPS primer design

Five multiplex primer sets were designed to analyze the VZV gene transcription from all 68 predicted unique VZV ORFs (Table 1) using GenomeLab GeXP eXpress Profiler software. Multiplex primer sets were grouped based on the abundance of each VZV gene transcript found earlier (Cohrs et al., 2003a). Multiplex primer sets A and B amplified high-abundance VZV transcripts, and multiplex primer sets C, D and E amplified low-abundance transcripts. Reverse primers consisted of 20 nucleotides complementary to the target gene coupled to a 19-nucleotide universal reverse sequence. Forward primers consisted of 20 nucleotides corresponding to the target gene coupled to an 18 oligonucleotide universal forward sequence. All primers were obtained from Integrated DNA Technologies (Coralville, IA) and supplied at a stock concentration of 100 μM in 96-well microtiter plates. GeXPS primer stocks were diluted 1:100 in nuclease-free water to a final concentration of 1 μM for reverse primer sets AE, and diluted 1:1000 to a final concentration of 0.2 μM for forward primer sets A–E (Table 1).

2.4. GeXPS multiplex RT-PCR

In each GeXPS multiplex RT-PCR experiment, reverse transcription (RT) was followed by PCR (Fig. 1). RT reactions mixtures (20 μl) contained 10–20 ng RNA, 0.1 μM reverse primer set mix, 20 U reverse transcriptase with RNase inhibitor (0.1 U/mL), 250 fM kanamycin resistant (Kan^r) RNA and 1x RT Master Mix Buffer containing 10 mM HCl, 50 mM KCl, 2.5 mM MgCl2 10 mM DTT, 1mM each dNTP and 0.05 μM Kan^r RT reverse primer (Fig. 1A). RT, RNase inhibitor, Kan^r RNA and 1x RT Master Mix Buffer were supplied in kit form (GenomeLab GeXP Start Kit; Beckman Coulter, Fullerton, CA). RT reactions were incubated at 48°C for 1 min, 37°C for 5 min, 42°C for 60 min, and 95°C for 5 min (Fig. 1A).

Subsequent PCR was done with each reaction containing 9.3 μl RT reaction, 0.02 μM forward primer set mix, 5 mM $MgCl_2$, 3.5 U Thermo Start Taq DNA polymerase (Thermo Fisher Scientific, Pittsburgh, PA) and 1x PCR Master Mix buffer (GenomeLab GeXP Start Kit; Beckman Coulter) containing 10 mM HCL, 50 mM KCl, 0.3 mMeach dNTP, 0.02 μM Kan^r PCR forward primer, 1 μM universal reverse primer, and 1 μM D4-labeled universal forward primer. Amplification conditions consisted of initial denaturation at 95°C for 15 min, followed by 35 two-step cycles of 94°C for 30 sec and 55°C for 30 sec, ending in a single extension cycle of 70°C for 1 min (Fig. 1 B and C).

2.5. GeXP multiplex data analysis

PCR products from multiplex primer sets A and B reactions were diluted 1:100 in water, and 2-μl was added to a 37.75 μl sample loading solution along with 0.25 μl DNA size standard-400 (GenomeLab GeXP Start Kit; Beckman Coulter). For multiplex primer sets C,D, and E reactions, 2-μl of the undiluted PCR product was added to a 37.75 μl sample loading solution along with 0.25 μl DNA size standard-400. The GeXPS system was used to separate PCR products based on size by capillary gel electrophoresis and to measure their dye signal strength in arbitrary units (A.U.) of optical fluorescence, defined as the fluorescent signal minus background.

PCR product sizes were determined using GenomeLab GeXPS software and were compared to the expected PCR product size to identify each transcript. A VZV ORF transcript was considered present in the initial RNA when dye signal of the VZV ORF-specific product was greater than 2,000 A.U. (signal background), and when no corresponding PCR product of the same size was present in RT-PCR reactions with uninfected MeWo cell RNA, VZV-infected MeWo cell RNA without RT, and reactions containing only Kan^r RNA.

2.6. Real-time PCR and data analysis

VZV-infected MeWo cell RNA (0.01–20 ng) was added to uninfected MeWo RNA to yield a total of 20 ng RNA in 5 μl. RNA was reverse transcribed in a total reaction volume of 20 μl using oligo-dT primers (Transcriptor First Strand cDNA Synthesis Kit; Roche, Mannheim, Germany). Taqman primer and probes for glyceraldehyde-3-phosphate dehydrogenase (GAPDH) and VZV ORFs 21, 29, 62, 63, and 66 (Cohrs and Gilden, 2007) were used to quantitate the abundance of each transcript. These five VZV ORFs were selected for study since they are expressed during latency (Cohrs et al., 1996, 2007). Real-time PCR was performed in a 20-μl volume of 1x qPCR Mastermix (VWR, West Chester, PA) containing 900 nM of each primer, 250 nM probe and 9.3 μl of the cDNA reaction using the 7500-Fast real-time PCR system (Applied Biosystems, Foster City, CA). Amplification conditions consisted of initial denaturation at 95°C for 10 min, followed by 40 two-step cycles of 95°C for 15 sec and 60°C for 1 min. Samples were analyzed in duplicate and VZV DNA ($1–10^6$ copies) was used to generate standard graphs (Cohrs and Gilden, 2007).

3. Results

3.1. Detection of VZV transcripts in VZV-infected MeWo cells by GeXPS multiplex RT-PCR

Five independent multiplex PCR reactions using multiplex primers sets A–E detected transcripts from all 68 predicted VZV ORFs in RNA from VZV-infected MeWo cells (Fig. 2, left panels), whereas no PCR products corresponding to any VZV gene transcript were detected in uninfected cells using the multiplex primer sets A-E (Fig. 2, right panels). PCR products corresponding to the internal control Kan^r RNA and to cellular GAPDH, β-actin and cyclophilin genes were detected in RNA from uninfected and VZV-infected cells (Fig. 2, all panels). PCR products corresponding to VZV and all control gene transcripts were not detected in DNase treated RNA (data not shown), indicating an absence of contaminating DNA.

3.2. Detection of low-abundance VZV gene transcripts by GeXPS multiplex RT-PCR

The number of VZV ORF 21, 29, 62, 63 and 66 cDNA copies synthesized from decreasing amounts (20-0.01 ng) of RNA from VZV-infected MeWo cells added to uninfected MeWo cell RNA for a total of 20 ng was quantitated by real-time PCR. Copy number was linear over the range of VZV-infected MeWo cell RNA tested (Fig. 3). In 0.01 ng VZV-infected MeWo cell RNA, there were 33.4 (+/− 0.8) copies of ORF 21, 54.5 (+/− 19.1) copies of ORF 29, 44.5 (+/− 16.3) copies of ORF 62, 99.0 (+/− 46.7) copies of ORF 63 and 20.0 (+/− 0.0) copies of ORF 66 (Fig. 3).

The same decreasing amounts of VZV-infected MeWo cell RNA used in real-time PCR was analyzed by GeXPS multiplex RT-PCR assay using primer sets A and D (Fig. 4, top and bottom panels, respectively). These primer sets were selected to compare realtime PCR quantitation with GeXPS multiplex RT-PCR, since multiplex primer set A contained primers specific for ORFs 63, 62 and 29, and multiplex primer set D contained primers specific for ORFs 21 and 66. At the lowest concentration of VZV-infected MeWo RNA (0.01 ng), PCR products corresponding to ORFs 21, 29, 62, 63, and 66 were detected. A plot of fluorescence intensity of each PCR product as a function of the VZV-infected MeWo RNA revealed a linear relationship between dye signal and RNA concentration when dye signal was <120,000 A.U., plateauing thereafter (Fig. 5).

4. Discussion

VZV gene transcription has been analyzed by Northern blotting (Ostrove et al., 1985), macroarrays (Cohrs et al., 2003a) and microarrays (Kennedy et al., 2005); however, none of these techniques detects low-abundance transcripts. Herein, a PCR-based assay was developed to identify transcripts corresponding to all predicted VZV ORFs simultaneously. Multiplex RT-PCR detected as few as 20 VZV transcripts in a background of 20 ng total RNA and was specific for each VZV gene transcript, since no amplification product was detected in RNA extracted from uninfected cells.

Over the past decade, characterization of VZV gene transcripts in latently-infected human ganglia has been hindered by: (1) the limited amount of RNA present in human ganglia (~250 μg total RNA or 5 μg mRNA in human trigeminal ganglia); (2) the low abundance of VZV transcripts present in ganglionic neurons (64 to 3,710 transcripts per μg mRNA (Cohrs and Gilden, 2007); and (3) the lack of sensitivity of macroarray and microarray assays (10,000 VZV mRNA copies; data not shown). Consequently, only 12 of the 68 ORFs in the VZV genome have been analyzed for latent VZV gene transcription (Kennedy et al., 1999, 2000; Cohrs et al., 1996, 2003b), and quantitative analysis has been limited to only 5 of these transcripts (Cohrs and Gilden, 2007). Use of our GeXPS multiplex RT-PCR assay system allows detection of low-abundance VZV gene transcripts mapping to all predicted virus ORFs.

Additionally, since GeXPS analysis can be performed with small amounts of RNA (100 ng total RNA per reaction), all VZV gene transcripts can be assayed with 500 ng RNA. Thus, it will now be possible to analyze latently-infected human ganglia for the entire VZV transcriptome, a goal previously unattainable. In addition, application of our multiplex RT-PCR technology to animal models of varicella latency (White et al., 2001) will permit analysis not only of virus transcription in latently-infected ganglia, but also after experimentally-induced reactivation.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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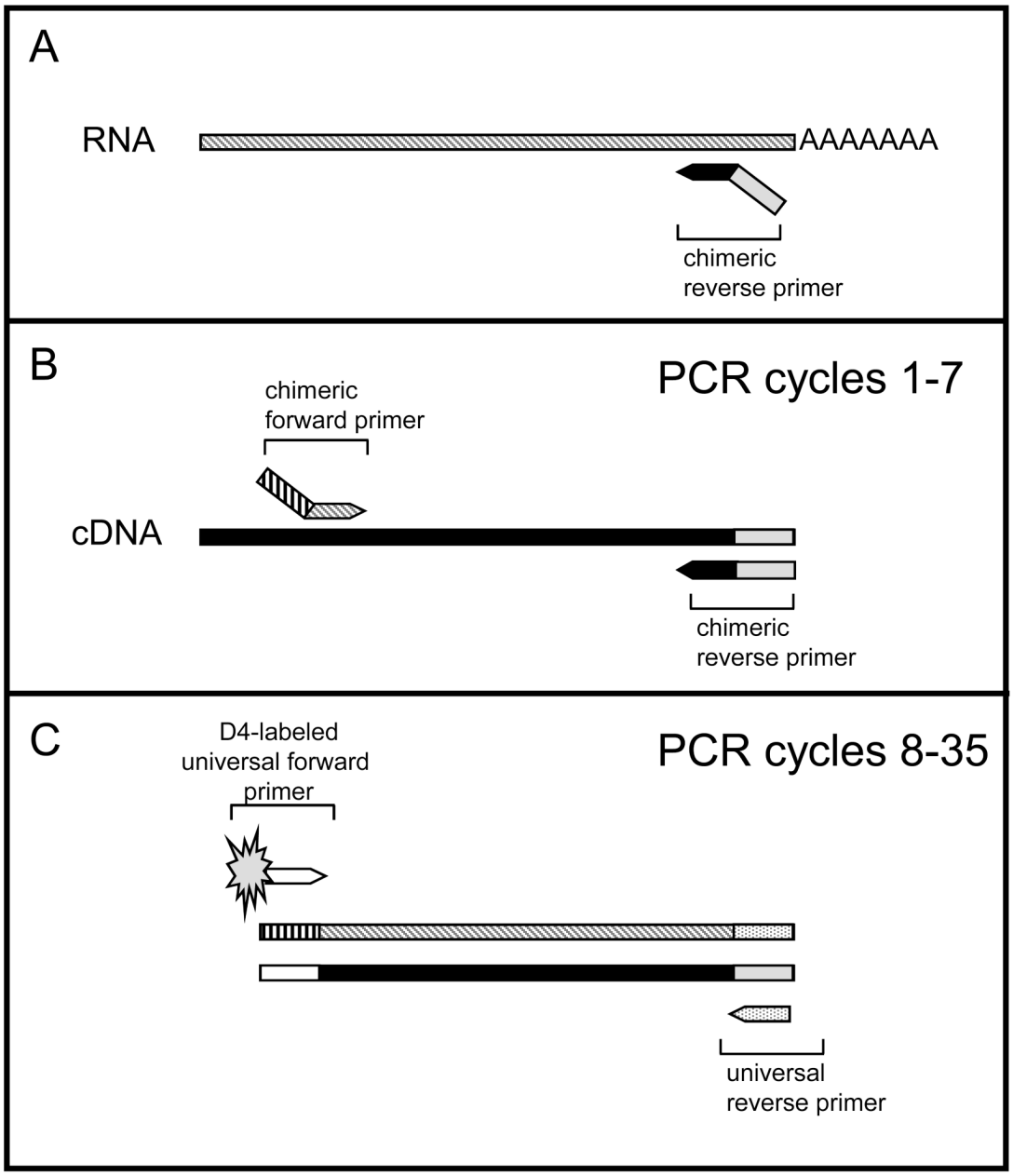

Fig. 1.

GenomeLab eXpress (GeXPS) multiplex RT-PCR strategy. A. Total RNA extracted from 2 × 10^7 uninfected or VZV-infected MeWo cells was reverse transcribed using 19–22 chimeric reverse primers each containing 20 nucleotides complementary to the target gene (solid black) coupled with a 19-nucleotide universal reverse sequence (solid gray). B. The cDNA was PCR-amplified in cycles 1–7 using the chimeric primers to synthesize the 19–22 gene-amplification products, each of which contained universal tags at both termini. C. Subsequent PCR amplifications (cycles 7–35) using universal forward and reverse primers to yield D4-fluorescent-labeled amplification products corresponding to each of the 19–22 specific genes tested.

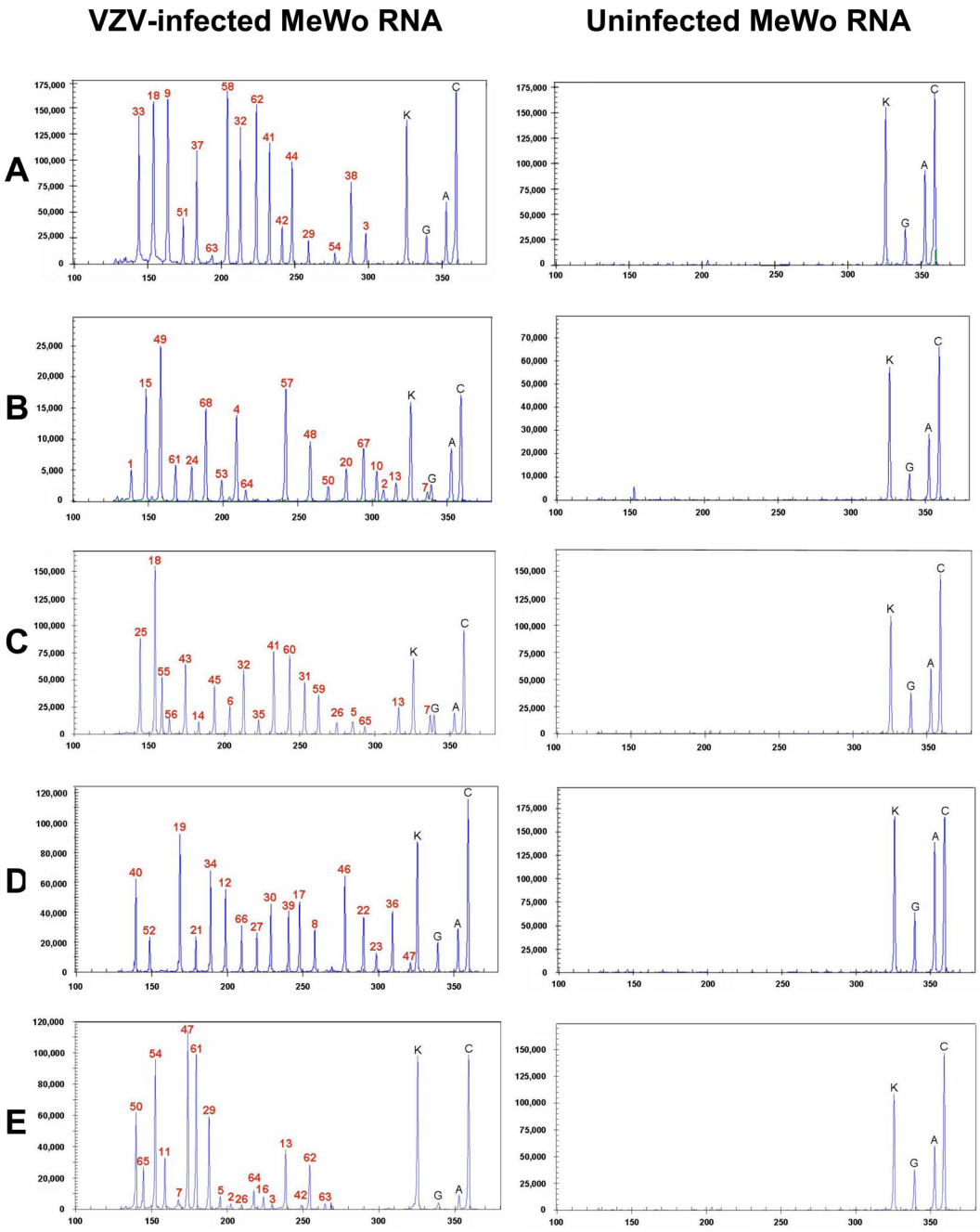

Fig. 2.

Electropherogram of D4-labeled PCR products synthesized with multiplex primer sets A–E. D4-labeled PCR products amplified from uninfected MeWo cells (right panels) and VZV-infected MeWo cells (left panels) with multiplex primer sets A–E (left) were separated by capillary electrophoresis and detected by fluorescence spectrophotometry given as dye signal in arbitrary units on the y-axis. Each peak was identified by comparing expected to actual PCR product size (x-axis). Uninfected cells showed peaks corresponding only to the internal control kanamycin-resistance gene (K) and cell control genes GAPDH (G), beta-actin (A) and cyclophilin (C). In VZV-infected MeWo cells, peaks corresponding to amplification products from VZV ORFs incorporated in multiplex primer sets A–E.

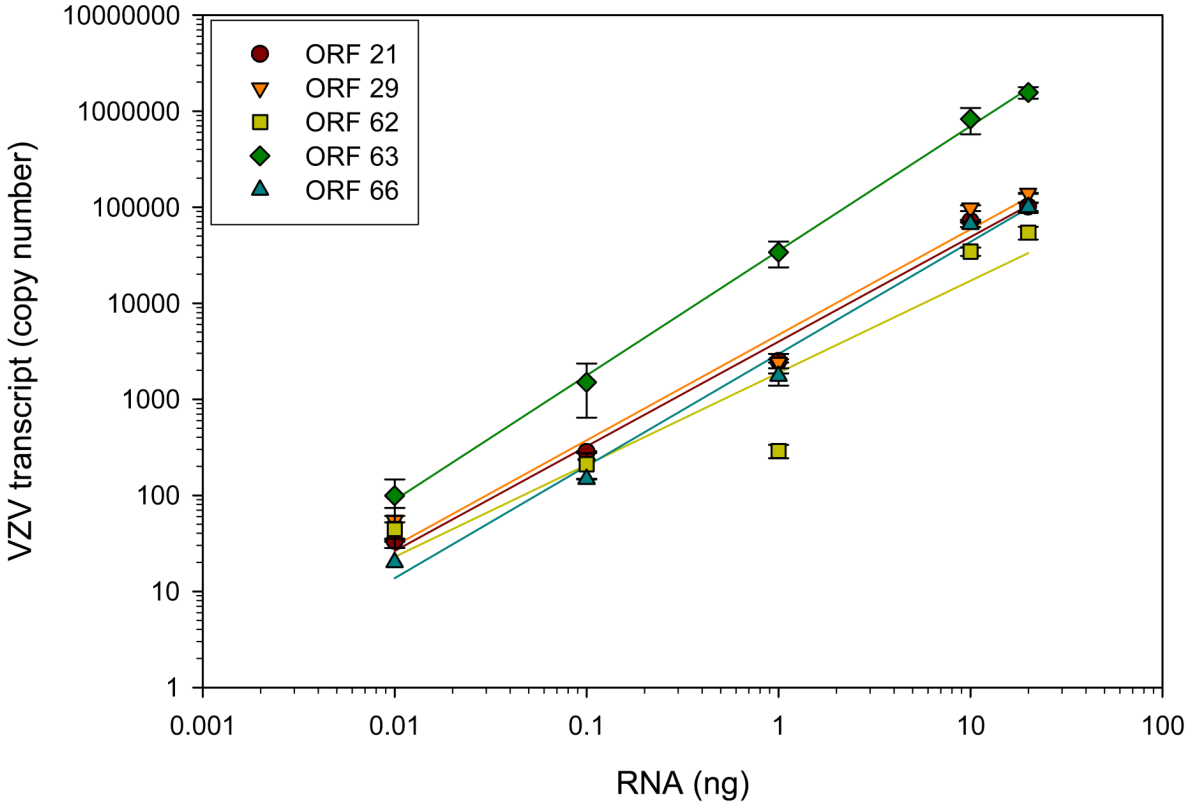

Fig. 3.

Quantitation of VZV transcripts in VZV-infected MeWo cells. Decreasing amounts of RNA (20-0.01 ng) extracted from VZV-infected MeWo cells was added to uninfected MeWo cell RNA to yield a total of 20 ng. cDNA was synthesized and the abundance of VZV ORFs 21, 29, 62, 63 and 66 transcripts was quantitated by real-time PCR. For all five VZV ORFs, there were 20–99 copies of VZV transcripts in 0.01 ng total VZV-infected MeWo RNA.

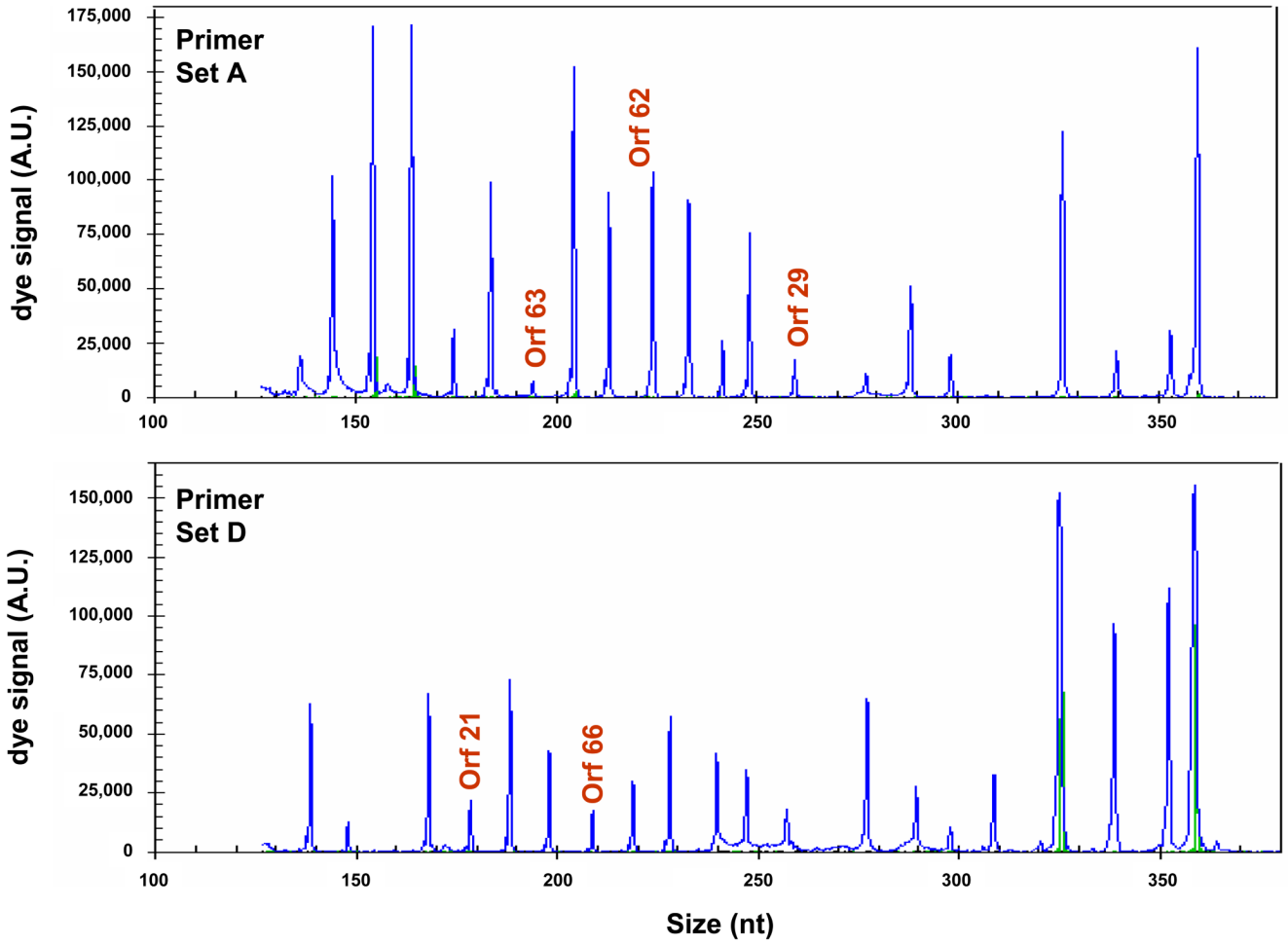

Fig. 4.

GeXP multiplex technology detection of low-abundance VZV transcripts. PCR products were synthesized using multiplex primer set A (top panel) and D (bottom panel) from 0.01 ng total VZV-infected MeWo cell RNA (shown in Fig. 3) and resolved by capillary gel electrophoresis. Peaks corresponding to VZV ORFs 63, 62, 29, 21 and 66 were detected.

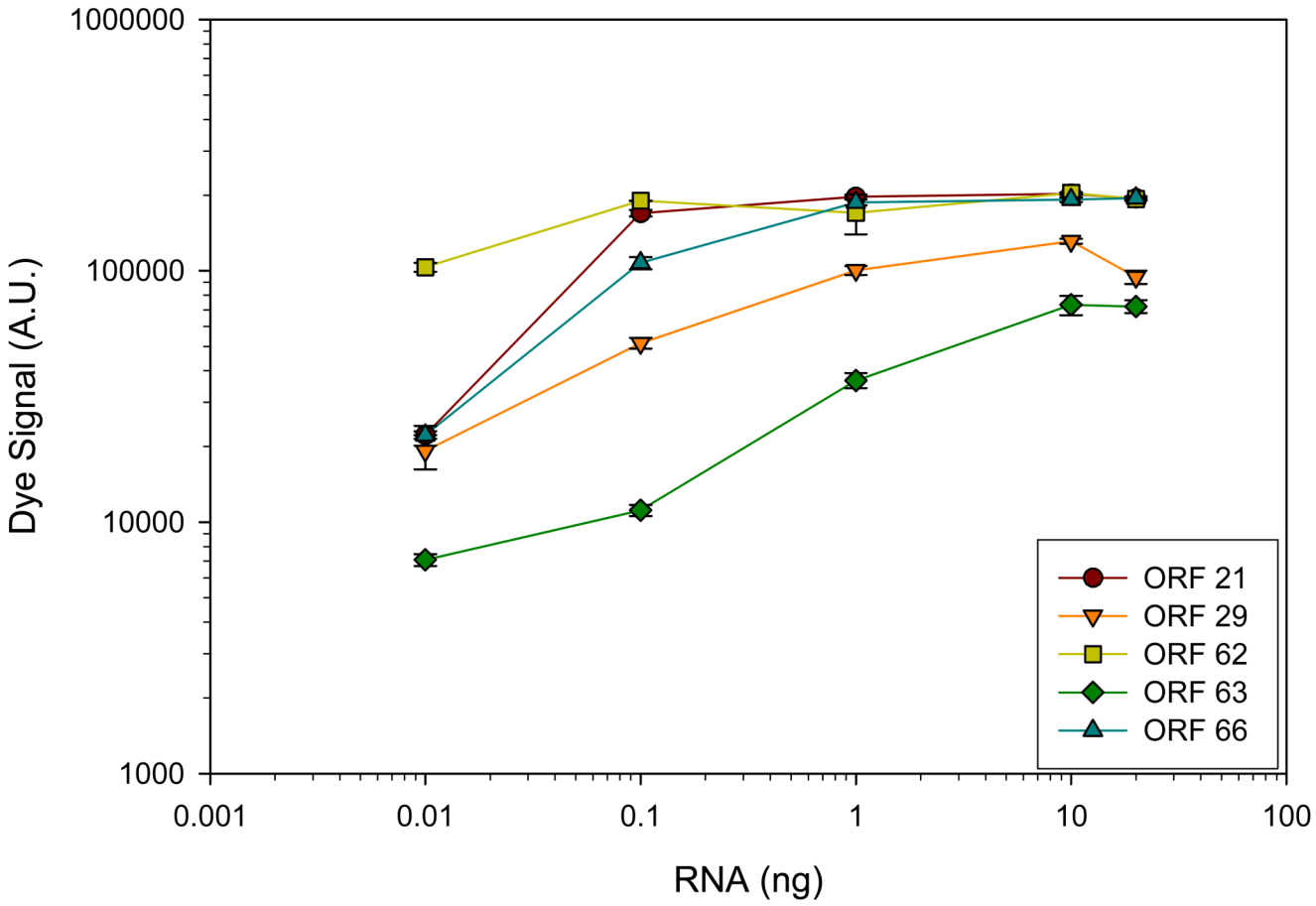

Fig. 5.

GeXP multiplex detection of VZV ORFs in serial dilutions of VZV-infected MeWo cells. Decreasing amounts of RNA (20-0.01 ng) extracted from VZV-infected MeWo cells were added to uninfected MeWo cell RNA to yield a total of 20 ng. PCR products were synthesized using multiplex primer set A and D and resolved by capillary gel electrophoresis. Peaks corresponding to VZV ORFs 63, 62, 29, 21 and 66 at various RNA dilutions were plotted.

Table 1

Oligonucleotide primer sequences and expected PCR product size.

Multiplex Primer Set	VZV ORF	Forward PCR primer (5' - 3')[a]	Reverse PCR primer (5' - 3')[b]	PCR Product Size (bp)
A	33	AGGTGACACTATAGAATAtgctgaagctgacgaagaga	GTACGACTCACTATAGGGAcgcacaatctctggggtaat	142
A	18	AGGTGACACTATAGAATAtttacatttttatcggcggc	GTACGACTCACTATAGGGAcctcgatgcactcttgttca	152
A	9	AGGTGACACTATAGAATAgcccgtttgagacatgaact	GTACGACTCACTATAGGGAcgcttcggtgaatcctctaa	162
A	51	AGGTGACACTATAGAATAacagctgcttgaccgatttt	GTACGACTCACTATAGGGActattggacatgcaagggct	172
A	37	AGGTGACACTATAGAATAtgggttcccttcgtacagac	GTACGACTCACTATAGGGAatcatggttttaaatgccgc	182
A	63	AGGTGACACTATAGAATAtcagaggagagcaccgattc	GTACGACTCACTATAGGGActttcgcggctgtatattcc	191
A	58	AGGTGACACTATAGAATAgcgttgatgcaaatgaaaaa	GTACGACTCACTATAGGGAaggcgttcataaccaagctg	202
A	32	AGGTGACACTATAGAATAcatcatccgtccaaacagtg	GTACGACTCACTATAGGGAaaggtttcgttgcacatgct	212
A	62	AGGTGACACTATAGAATAgacagcccagtggagaaaaa	GTACGACTCACTATAGGGAgaatcatccgaatttgacgg	222
A	41	AGGTGACACTATAGAATAcccagaccttaatgcgagaa	GTACGACTCACTATAGGGAaaaccaatgtgcgaatagcc	232
A	42	AGGTGACACTATAGAATAaccgttgcgaattgtgctat	GTACGACTCACTATAGGGAgtaacgcaacgccctatctc	237
A	44	AGGTGACACTATAGAATAgagtgtggtggtcagacgaa	GTACGACTCACTATAGGGAttccgcatgatttgtgacat	246
A	29	AGGTGACACTATAGAATAgtttataggcatggagggca	GTACGACTCACTATAGGGAggattcgcagctaaatgagg	256
A	54	AGGTGACACTATAGAATAgatcggtggacacatcacaa	GTACGACTCACTATAGGGAccgtaaccgtttcagctctc	276
A	38	AGGTGACACTATAGAATAtgttcttaccactcccccag	GTACGACTCACTATAGGGAcaatgggaattgatgatccc	286
A	3	AGGTGACACTATAGAATAgttggcgtcgtttacacaag	GTACGACTCACTATAGGGAcggtaacagtcgttgggaat	296
A	28	AGGTGACACTATAGAATAaagctttcgcgttgaggtta	GTACGACTCACTATAGGGAtccgacatgcagtcaatttc	343
B	1	AGGTGACACTATAGAATAgttcgggaatctgatagcga	GTACGACTCACTATAGGGAgtggtcttgtttgaacgacg	137
B	15	AGGTGACACTATAGAATAgtccatgatgaaaacctggg	GTACGACTCACTATAGGGAgctgaggttattgcacacga	147
B	49	AGGTGACACTATAGAATAatctgtggattgtgcaaacg	GTACGACTCACTATAGGGAatcggcgtcctctgttacat	157
B	61	AGGTGACACTATAGAATAgaaagggatacccgattggt	GTACGACTCACTATAGGGAatcgtttaacgtctctgccg	167
B	24	AGGTGACACTATAGAATAgcccgttgtgtttttcagtt	GTACGACTCACTATAGGGAacagacacccccgtattttg	177
B	68	AGGTGACACTATAGAATAccgagacttggagctttttg	GTACGACTCACTATAGGGAcggccaactgatcctcttta	187
B	53	AGGTGACACTATAGAATAttactgaaccctcagcctgg	GTACGACTCACTATAGGGAccgaatctcgtaaaggtcca	197
B	4	AGGTGACACTATAGAATAtccggggataaaacaaaaca	GTACGACTCACTATAGGGAacagttcatccgcagactcc	207
B	64	AGGTGACACTATAGAATAcgctatgcgccatatctacc	GTACGACTCACTATAGGGAtaacgataacaccgctctgc	216
B	57	AGGTGACACTATAGAATAcgagaacgtaatgtgtttggaa	GTACGACTCACTATAGGGAacgttgatgagccttgcag	241
B	48	AGGTGACACTATAGAATAtcggaacgtatgaatgtgga	GTACGACTCACTATAGGGAttaacgtggtgtacgtccga	256
B	50	AGGTGACACTATAGAATAccacggttaattcccacaac	GTACGACTCACTATAGGGAacgtagccgtagccttgtga	270
B	20	AGGTGACACTATAGAATAcgtggtattggcttcctgtt	GTACGACTCACTATAGGGAtgcaggataattgtgtggga	281
B	67	AGGTGACACTATAGAATAatacggtggcgttttgtttc	GTACGACTCACTATAGGGAaccaagaatgaaaccatcgg	291
B	10	AGGTGACACTATAGAATAgttcacgtgtgcgtttcatc	GTACGACTCACTATAGGGAtgaggatgtttgaccgcata	301
B	2	AGGTGACACTATAGAATAtgggtatgaatgtggtacgg	GTACGACTCACTATAGGGAtgttagggttgggagagtgg	305
B	13	AGGTGACACTATAGAATAtgggagacttgtcatgttgg	GTACGACTCACTATAGGGActttagcggcgagttctttg	313
B	7	AGGTGACACTATAGAATAacttacgctgcatgttctcg	GTACGACTCACTATAGGGAtcaaacgatgcctgaggttt	333
C	25	AGGTGACACTATAGAATAgtacgaatcggaaaatgcgt	GTACGACTCACTATAGGGAcgggtagaatcagaagaaccc	142
C	18	AGGTGACACTATAGAATAtttacatttttatcggcggc	GTACGACTCACTATAGGGAcctcgatgcactcttgttca	152
C	55	AGGTGACACTATAGAATAacaacccggactttctgttg	GTACGACTCACTATAGGGAtcgcgatttgaatgtgtttc	157

Multiplex Primer Set	VZV ORF	Forward PCR primer (5' - 3')[a]	Reverse PCR primer (5' - 3')[b]	PCR Product Size (bp)
C	56	AGGTGACACTATAGAATAcggttctgacccaacatctc	GTACGACTCACTATAGGGAcgtcaggtctgtttacccgt	162
C	43	AGGTGACACTATAGAATAcccgataacgccttaacaaa	GTACGACTCACTATAGGGAatcaaagaataaaccggggc	172
C	14	AGGTGACACTATAGAATAcatgccttgttacatgcgtc	GTACGACTCACTATAGGGAttcaatatgaagatcggggc	182
C	45	AGGTGACACTATAGAATAatgtttggcgcgtttctatc	GTACGACTCACTATAGGGAgctgcttcttccggtgaata	192
C	6	AGGTGACACTATAGAATAgcgtgatcgcgaatatacct	GTACGACTCACTATAGGGAtgatccactcgcagagacac	202
C	32	AGGTGACACTATAGAATAcatcatccgtccaaacagtg	GTACGACTCACTATAGGGAaaggtttcgttgcacatgct	212
C	35	AGGTGACACTATAGAATAttatgctagcgtcgcatttg	GTACGACTCACTATAGGGAtaaatccaccccacaagctg	222
C	41	AGGTGACACTATAGAATAcccagaccttaatgcgagaa	GTACGACTCACTATAGGGAaaaccaatgtgcgaatagcc	232
C	60	AGGTGACACTATAGAATAtcatcggtatatgaggcgtg	GTACGACTCACTATAGGGAccccgcttttatgttcttca	242
C	31	AGGTGACACTATAGAATAccgtgggattattggttttg	GTACGACTCACTATAGGGAcgacggttcagtgttttgtg	252
C	59	AGGTGACACTATAGAATAtgctgaataccacgcttacg	GTACGACTCACTATAGGGAgaaatggaacacgggacaat	262
C	26	AGGTGACACTATAGAATAtaccgatggagggttaccag	GTACGACTCACTATAGGGAcgcaatctttccaattagcc	273
C	5	AGGTGACACTATAGAATAactcaacgtttttggatggc	GTACGACTCACTATAGGGAgaatggcccaaacatacgac	282
C	65	AGGTGACACTATAGAATAaaaacaccatggagggtgag	GTACGACTCACTATAGGGAgctcccgtcatagcaaataca	293
C	13	AGGTGACACTATAGAATAtgggagacttgtcatgttgg	GTACGACTCACTATAGGGActttagcggcgagttctttg	313
C	7	AGGTGACACTATAGAATAacttacgctgcatgttctcg	GTACGACTCACTATAGGGAtcaaacgatgcctgaggttt	333
D	40	AGGTGACACTATAGAATAaaccctctgtttgcatgtcc	GTACGACTCACTATAGGGAgaaaaggagggattggtggt	137
D	52	AGGTGACACTATAGAATAaacaacggattccaccgtta	GTACGACTCACTATAGGGAaatacgcaaagattggacgg	147
D	19	AGGTGACACTATAGAATAgagccgatatccttcgaaca	GTACGACTCACTATAGGGAgcagcactgcgtatgattgt	167
D	21	AGGTGACACTATAGAATAatgcagatggatatcgcaca	GTACGACTCACTATAGGGAgccaacagtccgatgaagtt	177
D	34	AGGTGACACTATAGAATAcaatatgaacggggacttgg	GTACGACTCACTATAGGGAggaggcaaactgaggaatga	187
D	12	AGGTGACACTATAGAATAtaccggggaatatgtcagga	GTACGACTCACTATAGGGAataacatccacccgtagcca	197
D	66	AGGTGACACTATAGAATAtttggcaaaacggtcttctc	GTACGACTCACTATAGGGAcgggaagagtttggaaaaca	207
D	27	AGGTGACACTATAGAATAatccattttggcgctatcag	GTACGACTCACTATAGGGAtacatcataaaccctccggc	217
D	30	AGGTGACACTATAGAATActgttttccattgtggggag	GTACGACTCACTATAGGGAttctggtcgatccctgtttc	227
D	39	AGGTGACACTATAGAATAcgatcagacctgctacccctt	GTACGACTCACTATAGGGAccccacgacacaggaagtat	237
D	17	AGGTGACACTATAGAATAgttcgctgtcagaccgattt	GTACGACTCACTATAGGGAtgcatatttggaaacggaca	247
D	8	AGGTGACACTATAGAATAgaagatcatcgaaaagccga	GTACGACTCACTATAGGGAttaccgcggatagcataagg	257
D	46	AGGTGACACTATAGAATAaaagaacggaccctcgatct	GTACGACTCACTATAGGGAgcgaataaaatcgggtgcta	277
D	22	AGGTGACACTATAGAATAttgggggttacgtttactgc	GTACGACTCACTATAGGGAggagtcgatgtacggcaaat	287
D	23	AGGTGACACTATAGAATAcgtacgtttaacccgcgtat	GTACGACTCACTATAGGGAagtgcttgactgtgctccct	298
D	36	AGGTGACACTATAGAATAggcgtatggaattggaaaaa	GTACGACTCACTATAGGGAgtccatcaatgccgagattt	308
E	50	AGGTGACACTATAGAATAtcctggctacgttaattgcc	GTACGACTCACTATAGGGAccatctagggtggcgttaaa	137
E	65	AGGTGACACTATAGAATAgcgagcgcaaccaaataata	GTACGACTCACTATAGGGAgcaaaaattcatctgccgtt	144
E	54	AGGTGACACTATAGAATAatgggcgagaacgatacaac	GTACGACTCACTATAGGGAggaacgtacggtttcggtaa	151
E	11	AGGTGACACTATAGAATAgcagcaagtcctctttacgc	GTACGACTCACTATAGGGAtcctgtcttgctctttgggt	158
E	7	AGGTGACACTATAGAATAactcaaaacggcttgctgat	GTACGACTCACTATAGGGAcacttcactttcagcggtca	165
E	47	AGGTGACACTATAGAATAggacaactgcccatttcagt	GTACGACTCACTATAGGGAggggatgtcagatgggagta	172
E	61	AGGTGACACTATAGAATAcaaacctggacctggaaaga	GTACGACTCACTATAGGGAaaagcctgacttttggggt	179
E	29	AGGTGACACTATAGAATAgcccaaattctgcgttgtat	GTACGACTCACTATAGGGAgtggccatctcgatctgttt	186
E	5	AGGTGACACTATAGAATAttttggatggcctatctgg	GTACGACTCACTATAGGGActgcacgcaccaaaaataag	193

Multiplex Primer Set	VZV ORF	Forward PCR primer (5' - 3')[a]	Reverse PCR primer (5' - 3')[b]	PCR Product Size (bp)
E	2	AGGTGACACTATAGAATActggaggtgcaaggacattt	GTACGACTCACTATAGGGAttaccccgccataaacagag	200
E	26	AGGTGACACTATAGAATAggagacgttttggcgttaga	GTACGACTCACTATAGGGAaattcaaaggcagcgctaaa	207
E	64	AGGTGACACTATAGAATAcggctgatattgatggcata	GTACGACTCACTATAGGGAcgtaggttcttgggactcca	215
E	16	AGGTGACACTATAGAATAttaacacgggatgagacgtg	GTACGACTCACTATAGGGAttcacagattccatgttcgc	222
E	3	AGGTGACACTATAGAATAagtcaacccatccgagtgaa	GTACGACTCACTATAGGGAagcgtgtaaatcatcagccc	229
E	13	AGGTGACACTATAGAATAggtcaaccgattccaaagaa	GTACGACTCACTATAGGGAgctgatcgattccttgctgt	238
E	42	AGGTGACACTATAGAATAttttatatcggccgtcaacc	GTACGACTCACTATAGGGAcgccctatctcactggaaga	245
E	62	AGGTGACACTATAGAATAacccagaacggaagatgttg	GTACGACTCACTATAGGGAgttcatcggtggttttggag	254
E	63	AGGTGACACTATAGAATAacgcgagattcacgaagatt	GTACGACTCACTATAGGGAacattccagtgcgctcctat	265

Multiplex Set	Cell Gene (accession number)	Forward PCR primer (5' - 3')[c]	Reverse PCR primer (5' - 3')[d]	PCR Product Size (bp)
A, B, C, D, E	GAPdH (NM_002046)	AGGTGACACTATAGAATAatcactgccacccagaagac	GTACGACTCACTATAGGGAacctggtgctcagtgtagcc	339
A, B, C, D, E	Beta-actin (NM_001101	AGGTGACACTATAGAATAgctatccctgtacgcctctg	GTACGACTCACTATAGGGAgtcaggcagctcgtagctct	353
A, B, C, D, E	Cyclophilin (BC000689)	AGGTGACACTATAGAATAcctaaagcatacgggtcctg	GTACGACTCACTATAGGGAaggatactgcgagcaaatgg	357

[a] forward universal primer sequence (upper case) and VZV gene-specific primer sequence (lower case) in multiplex primer sets A–E

[b] reverse universal primer sequence (upper case) and VZV gene-specific primer sequence (lower case) in multiplex primer sets A–E

[c] forward universal primer sequence (upper case) and cell gene-specific primer sequence (lower case) in multiplex primer sets A–E

[d] reverse universal primer sequence (upper case) and cell gene-specific primer sequence (lower case) in multiplex primer sets A–E