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GeXPS multiplex PCR analysis of the simian varicella virus transcriptome in productively infected cells in culture and acutely infected ganglia

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Abstract

Simian varicella zoster virus (SVV) infection of non-human primates serves as a model to study varicella zoster virus (VZV) infection and pathogenesis in humans. While macroarray analysis detected all 69 predicted unique open reading frames (ORFs) in SVV-infected cells in culture, it lacked the sensitivity to detect the low-abundance transcripts expressed in latently infected monkey ganglia. Recently, a multiplex RT-PCR assay using the GenomeLab Genetic Analysis System (GeXPS) identified 10 VZV transcripts in latently-infected human ganglia. GeXPS was used to analyze the SVV transcriptome in SVV-infected monkey cells in culture as well as in acutely infected ganglia from African green monkeys. Oligonucleotide primers containing both SVV ORF- and cell-specific sequences linked to universal DNA sequences were used in RT-PCR to produce products of predetermined sizes. Amplification products were resolved by capillary gel electrophoresis and detected by fluorescence spectrophotometry. Transcripts corresponding to all 69 predicted SVV ORFs, in addition to transcripts within the leftward end region and ORF 61 antisense transcripts were detected in virus-infected cells in culture. Except for two transcripts (ORFs 14 and 35), all transcripts found in infected tissue culture cells were also found in acutely infected monkey ganglia.

Keywords

simian varicella virus transcriptome; GeXPS; PCR

1. Introduction

Varicella zoster virus is an exclusively human, neurotropic alphaherpesvirus. Primary infection causes varicella (chickenpox), after which virus establishes latency in cranial

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nerve, dorsal root and autonomic ganglia. With a decline in VZV-specific cell-mediated immunity in immunosuppressed individuals, VZV reactivates and causes herpes zoster (shingles) which can be complicated by postherpetic neuralgia as well as VZV vasculopathy, myelopathy, and multiple ocular disorders. Postmortem analysis of latently-infected human ganglia reveals limited transcription, with VZV open reading frame (ORF) 63 being the most prevalent and abundant (Cohrs et al., 2007). VZV becomes latent and produces disease only in humans.

Like VZV, simian varicella zoster virus (SVV) is a neurotropic alphaherpesvirus. Primary SVV infection causes varicella, after which virus becomes latent in ganglionic neurons along the entire neuraxis in monkeys (Kennedy et al., 2004; Messaoudi et al., 2009) and reactivates to produce zoster. The 124.5-kbp SVV genome contains 69 ORFs (Gray et al., 1992; Clarke et al., 1995); transcripts corresponding to each of these ORFs have been detected by macroarray analysis of RNA from SVV-infected Vero cells (Deitch et al., 2005). Using RT-PCR, transcripts corresponding to SVV ORFs 21, 62, 63 and 66, but not ORF 40, were detected in ganglia latently infected with SVV (Messaoudi et al., 2009), in addition to detection of antisense ORF 61 transcript (Ou et al., 2007). However, examination of the entire SVV transcriptome, including low-abundance SVV transcripts, in ganglia during latency requires a more sensitive assay. Recently, the GenomeLab Genetic Analysis System (GeXPS) was used to develop a multiplex reverse transcription (RT)-PCR assay to analyze the entire VZV transcriptome in VZV-infected cells in culture and in human ganglia latently infected with VZV (Nagel et al., 2009). This assay identified transcripts specific for 10 VZV ORFs in latently infected human trigeminal ganglia, 5 of which had not been found by earlier array analysis (Nagel et al., 2011). Herein, we used GeXPS multiplex RT-PCR to analyze the entire SVV transcriptome *in vitro* and *in vivo*.

2. Materials and methods

2.1 Virus, cells and tissue

SVV (Delta strain) was isolated from a Patas monkey (*E. patas*) as described (Allen et al. 1974) and propagated in African green monkey kidney epithelial cells (Vero) and in Rhesus macaque fibroblasts as described (Gray and Gusick, 1996). At 3 days post-infection, cells were released with trypsin and centrifuged at $450 \times g$ for 10 min. An African green monkey inoculated intratracheally with 10^4 pfu of SVV developed rash on day 13; on day 21, the monkey was euthanized and trigeminal, cervical, thoracic, lumbar and sacral ganglia were harvested and flash frozen in liquid nitrogen. Ganglia corresponding to each paravertebral region were pooled before RNA extraction.

2.2 RNA extraction

Total RNA was extracted using Tri-Reagent (Molecular Research Center, Cincinnati, OH). mRNA was extracted using a μ MACS mRNA Isolation Kit (MiltenyiBiotec, Auburn, CA) and treated twice with amplification grade DNase (Invitrogen, Eugene, OR).

2.3 GeXPS primer design

Four independent multiplex primer sets (A–D) were designed to analyze the entire SVV transcriptome using GenomeLab GeXPS eXpress Profiler software (Table 1). The SVV transcriptome consists of SVV ORFs A, B, 1, 3–68, transcripts corresponding to the leftward end region (LE) and antisense ORF 61 (–61). ORF 2, unique to VZV, is absent in SVV (Gray et al., 2001). Reverse primers consisted of 20–24 nucleotides (nt) complementary to the target SVV gene and coupled to a 5′-19-nt universal reverse sequence. Forward primers consisted of 20–25 nt complementary to the target gene and coupled to a 5′-18-nt universal forward sequence. All primers were obtained from Invitrogen Life Technologies (Grand

Island, NY) and supplied at a stock concentration of 100 μM in 96-well plates. Primers contained in GeXPS reverse primer plex sets A–D were diluted 1:100 in nuclease-free water to a final concentration of 1 μM , while primers contained in forward primer plex sets A–D were diluted 1:1000 in nuclease-free water to a final concentration of 0.2 μM .

2.4 Efficiency of multiplex RT-PCR by Sybr green qPCR

To determine the efficiency of the multiplex RT-PCR primer pairs and to confirm that each primer pair amplified a unique SVV-product, serial dilutions of SVV DNA (1–5000 copies) were amplified in separate reactions for each of the 69 SVV ORFs, LE and γ -61 transcripts using the respective primer pairs using Sybr green qPCR as previously described (Nagel et al., 2011). Table 2 lists the linear ranges of DNA copies detected by each PCR primer pair, including the linear range, regression line slope, intercept, R^2 value and PCR efficiency, along with the midpoint dissociation temperature (T_m) and dissociation temperature range (lower and higher T_m) for each PCR product. All primer pairs amplified SVV DNA with a linear response between 1 to 5,000 copies of SVV DNA. The slope of the linear regression lines from each amplification reaction showed that all primer pairs amplified target SVV DNA with efficiencies of 1.0 ± 0.1 . Melting point analysis of amplified SVV DNA for all SVV ORFs revealed a single discrete peak, indicating that a single DNA product was synthesized.

2.5 GeXPS multiplex RT-PCR

For each GeXPS multiplex RT-PCR experiment, RT and subsequent PCR was performed as described (Nagel et al. 2009). Each of 3 tissue culture plates was infected with SVV, and RNA extracted from these plates was analyzed in triplicate. Similarly, RNA from pooled monkey ganglia was analyzed in triplicate. The RT reaction mix (20 μl) contained 100 ng RNA, 250 mM kanamycin-resistant (Kan^r) RNA (internal control), 1X RT master mix buffer containing 10 mM HCl, 50 mM KCl, 2.5 mM MgCl_2 , 10 mM DTT, 0.1 μM reverse primer plex mix, 1 mM each dNTP and 0.05 μM Kan^r RT reverse primer. RT enzyme, RNase inhibitor, 1X RT master mix buffer and Kan^r RNA were supplied in the GenomeLab GeXP Start Kit (Beckman Coulter, Fullerton, CA). RT was carried out at 48°C for 1 min followed by 37°C for 5 min, 42°C for 60 min and 95°C for 5 min.

PCR was performed using 9.3 μl of each RT product, 3.5 U Thermo Start *Taq* DNA polymerase (Thermo Fisher Scientific, Pittsburgh, PA), 0.02 μM forward primer plex set, 5 mM MgCl_2 , and 1X PCR master mix buffer (GenomeLab GeXP Start Kit) containing 10 mM HCl, 50 mM KCl, 0.3 mM each dNTP, 0.02 μM Kan^r PCR forward primer, 1 μM universal reverse primer, and 1 μM D4-labeled universal forward primer. PCR conditions were as follows: 95°C for 15 min, followed by 35 two-step cycles of 94°C for 30 s and 55°C for 30 s, ending with a single extension cycle of 70°C for 1 min.

2.6 GeXPS multiplex data analysis

Two μl of each PCR product from primer plex sets A–D was added to 37 μl of sample loading solution along with 1 μl of DNA size standard-400 (GenomeLab GeXP Start Kit). The GeXPS system was then used to analyze PCR products based on size by capillary gel electrophoresis. To identify each transcript, PCR product sizes along with DNA size standards were analyzed with GenomeLab GeXPS software and correlated with predicted sizes of PCR products.

The GeXPS system measures the dye signal strength in arbitrary units (A.U.) of optical fluorescence, defined as the fluorescent signal minus background. An SVV transcript was considered to be present when the dye signal of the SVV ORF-specific product exceeded

5000 A.U. (signal background) and when no PCR product of the same predicted size was present in RT-PCR reactions of uninfected Vero cells or Rhesus fibroblasts.

2.7 Standard protocol approvals

All procedures were performed following guidelines and protocols approved by the Institutional Animal Care and Use Committee of the Tulane National Primate Research Center or each GeXPS.

3. Results

3.1 Detection of SVV transcripts in SVV-infected Vero (African green monkey kidney) cells and in Rhesus fibroblasts by GeXPS multiplex RT-PCR

Use of four independent PCR reactions with multiplex primer sets A–D revealed SVV ORFs A, B, 1, 3–68, and LE and γ -61 ORF 61 transcripts, in cDNA synthesized from RNA extracted from SVV-infected Vero cells (Fig. 1, left panels) and Rhesus fibroblasts (Fig. 2, left panels). No PCR products corresponding to any SVV transcript were detected in uninfected Vero cells or Rhesus fibroblasts (Figs. 1 and 2, right panels, respectively). PCR products corresponding to control kanamycin RNA (325-nt peak) and cellular GAPdH (344-nt peak) were detected in all reactions. PCR products corresponding to SVV and control gene transcripts were not detected in DNase-treated RNA (data not shown), indicating absence of contaminating DNA.

3.2 GeXPS multiplex RT-PCR analysis of SVV transcripts in pooled trigeminal, cervical, thoracic, lumbar and sacral ganglia from an acutely infected African green monkey and uninfected ganglia from a different African green monkey

Twenty-one days after intratracheal inoculation of an African green monkey with 10^4 pfu SVV (13 days after onset of rash), poly (A⁺) RNA was extracted from pooled trigeminal cervical, thoracic, sacral and lumbar ganglia and analyzed by GeXPS multiplex plex sets A–D. All SVV ORFs, LE and γ -61 transcripts were detected (Fig. 3) with the exception of ORF 14, with an expected peak size of 207 nt (Fig. 3B), and ORF 35, with an expected peak size of 256 nt (Fig. 3C). Transcripts specific for ORFs 41 and 13 (A), ORF 48 (B), ORF 51 (C) and ORFs 64 and 4 (D) were detected at the expected sizes but at low abundance, with peak sizes below the background of dye signal (5000 A.U.). Subsequent efforts to verify individual sequences of the 2 absent ORFs by nested PCR (Nagel et al., 2011) were unsuccessful, most likely reflecting the low abundance of each transcript in the original samples.

Similar analysis of uninfected monkey ganglia revealed no peaks corresponding to any SVV ORFs, but did reveal multiple nonspecific peaks (Fig. 4).

All multiplex RT-PCR analyses of acutely infected and uninfected ganglia from African green monkeys revealed the presence of control kanamycin RNA and cellular GAPdH. No peaks were seen in DNase-treated RNA (data not shown), indicating absence of contaminating DNA.

4. Discussion

We describe here the development of an RT-PCR-based assay that successfully identified transcripts corresponding to all predicted SVV ORFs in Vero cells and Rhesus fibroblasts. In pooled trigeminal, cervical, thoracic and lumbar ganglia from an acutely infected African green monkey, our assay identified all predicted SVV ORFs A, B, 1, 3–68, LE and γ -61 transcripts with the exception of ORF 14 and ORF 35. The latter transcripts are expressed at

high levels (125,000 A.U. for ORF 14 and 150,000 A.U. for ORF 35) during productive infection *in vitro* (Figs. 1 and 2), and their lack of detection *in vivo* may reflect basic differences in the biology of productive infection in ganglia compared to that in tissue culture, resulting in an abundance below the level detectable by GeXPS analysis (500 copies for ORF 14 and 10 DNA copies for ORF 35; Table 2). In VZV-infected cells in tissue culture, transcripts corresponding to ORFs 14 and 35 were found (Nagel et al., 2009), similar to their detection in SVV-infected tissue culture cells herein. ORFs 14 and 35 transcription was not detected in human TG latently-infected with VZV (Nagel et al., 2011). In addition, transcripts encoding ORFs 4, 13, 41, 48, 51 and 64 were detected at the expected sizes but at low abundance, with peak sizes below the arbitrarily set background. The abundance of these transcripts may be near the lower limit of detection (5 DNA copies for ORF 4; 1 copy for ORF 13, 41 and 48; 100 copies for ORF 51; and 500 copies for ORF 64), explaining their low dye signal. Analysis of uninfected monkey ganglia yielded no peaks corresponding to any predicted SVV ORFs. Overall, this assay provides a sensitive method for analyzing virus gene transcription in monkey tissue not only during primary infection, but also during latency and reactivation. GeXPS can also readily be applied for rapid transcriptional analyses of other herpesviruses.

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Highlights

- We have developed a multiplex PCR assay to analyze the complete SVV transcriptome.
- All virus genes were detected in SVV- infected cells in culture.
- All but two of the virus genes were found in acutely infected monkey ganglia.

Fig. 1.

Electropherogram of D4-labeled RT-PCR products from RNA isolated from SVV-infected (left panels) and uninfected (right panels) Vero cells and amplified using multiplex primer sets A–D. PCR-amplified products were separated by capillary electrophoresis (base pair; x-axis) and detected by fluorescence spectrophotometry shown as dye signal in arbitrary units (A.U.) (y-axis). Each peak was identified by comparing predicted to actual size of PCR product. Both SVV-infected and uninfected Vero cells showed peaks corresponding to the kanamycin (K) and GAPdH (G) controls. SVV-infected cells contained peaks corresponding to amplification products from all SVV ORFs analyzed.

Fig. 2.
Electropherogram of D4-labeled RT-PCR products from RNA extracted from SVV-infected (left panels) and uninfected (right panels) Rhesus macaque fibroblasts with multiplex primer sets A–D. Amplified products were separated by capillary electrophoresis (base pair; x-axis) and detected by fluorescence spectrophotometry shown as dye signal in arbitrary units (A.U.) (y-axis). Each peak was identified by comparing predicted to actual size of PCR products (x-axis). Both SVV-infected and uninfected fibroblasts showed peaks corresponding to kanamycin (K) and cell gene GAPdH (G) controls. SVV-infected cells contained peaks corresponding to amplification products from all SVV ORFs analyzed.

monkey ganglia 8 days after varicella

Fig. 3.

Multiplex RT-PCR analysis of the SVV transcriptome in ganglia from an acutely-infected African green monkey. Twenty-one days after intratracheal inoculation of an African green monkey with SVV (13 days after onset of rash), poly (A⁺) RNA prepared from pooled cervical, thoracic, lumbar and sacral ganglia was analyzed by multiplex RT-PCR using primer sets A–D. All SVV transcripts were detected with the exception of ORFs 14 (B) and 35 (C). The following transcripts were detected with specific peaks below the arbitrarily set background of 5K dye signal: ORFs 41 and 13 (A), ORF 48 (B), ORF 51 (C) and ORFs 64 and 4 (D). Peaks corresponding to internal control kanamycin (K) and cell control gene GAPdH (G) were found. Occasional peaks seen above background that did not correspond to the size of any predicted SVV amplification product are indicated as nonspecific peaks (NSP).

uninfected monkey ganglia

Fig. 4. Multiplex RT-PCR analysis of the SVV transcriptome in uninfected monkey ganglia. Poly (A⁺) RNA from pooled uninfected trigeminal, cervical, thoracic, lumbar and sacral ganglia was extracted and analyzed by multiplex RT-PCR using primer sets A–D. The electropherogram shows nonspecific peaks that do not correspond to sizes of expected SVV ORF peaks in Figs. 1–3. Peaks corresponding to internal control kanamycin (K) and cell control gene GAPDH (G) are indicated.

Table 1

Oligonucleotide primer sequences and expected PCR product size

Multiplex primer set	SVV ORF	Forward primer (5'-3') ^a	Reverse primer (5'-3') ^b	PCR product size
A	37	AGGTGACACTATAGAAATAGAACAAATTCCTGGGC	GTACGACTCCTACTATAGGGGAaatactgatactgcaaaagggc	144
A	61	AGGTGACACTATAGAAATaactgcgaacacctctggt	GTACGACTCCTACTATAGGGGAatgacgggctgaggggtgtaac	151
A	50	AGGTGACACTATAGAAATaaggaagctccaaagtcggt	GTACGACTCCTACTATAGGGGAagccttcagtaagccaatc	158
A	44	AGGTGACACTATAGAAATAcaccagttcaaatgatgcg	GTACGACTCCTACTATAGGGGAaatecttctctcgtccg	165
A	32	AGGTGACACTATAGAAATAgatgtccaaagcgggaagacat	GTACGACTCCTACTATAGGGGAgttcgctgatactggggattatg	172
A	57	AGGTGACACTATAGAAATAgcagagctttattgtctacaccacg	GTACGACTCCTACTATAGGGGAatgtaagcgtgctggtgtgtt	175
A	11	AGGTGACACTATAGAAATAcacaggggagtgatgtt	GTACGACTCCTACTATAGGGGAggggtaagcccaagaggtag	186
A	49	AGGTGACACTATAGAAATaactactggccgagggagaa	GTACGACTCCTACTATAGGGGAatttaggaaaatccggggcg	193
A	58	AGGTGACACTATAGAAATaactgcgaacacctctggt	GTACGACTCCTACTATAGGGGAaccctctgtttgtgtacc	200
A	68	AGGTGACACTATAGAAATaactcaaatgggtgggtcagc	GTACGACTCCTACTATAGGGGActggaggggaaacatgttc	207
A	8	AGGTGACACTATAGAAATAgagcctatgtgtgctgggttaa	GTACGACTCCTACTATAGGGGAgtggggcacacatcatatc	235
A	15	AGGTGACACTATAGAAATatttggaacaacttgggggg	GTACGACTCCTACTATAGGGGAcaaaaccctctctccgatac	242
A	33A	AGGTGACACTATAGAAATaacaacaacacatcccggt	GTACGACTCCTACTATAGGGGAgttaggtcacttggatcgt	249
A	24	AGGTGACACTATAGAAATaagccattaaacgtgggtgt	GTACGACTCCTACTATAGGGGAgtttacgccatttcacacga	256
A	9	AGGTGACACTATAGAAATaactgtgaaaccgagtcgc	GTACGACTCCTACTATAGGGGAatcatctgaggtgtgtgcat	263
A	23	AGGTGACACTATAGAAATAgagcgcattttgatccact	GTACGACTCCTACTATAGGGGAatctgcaaacatcgcagttc	270
A	41	AGGTGACACTATAGAAATattcggtccggtgtgtgtat	GTACGACTCCTACTATAGGGGAatttacggcgtgtggcattac	277
A	13	AGGTGACACTATAGAAATaaaacgaacccaacgacag	GTACGACTCCTACTATAGGGGAagcctctctctatgtgtgtgac	297
A	63	AGGTGACACTATAGAAATAcgtgggagcccgattacaga	GTACGACTCCTACTATAGGGGActgcccctcaatacttcgt	307
B	1	AGGTGACACTATAGAAATAgaaagaatcggatgggagacga	GTACGACTCCTACTATAGGGGAcagtttctgtttgggaaacag	137
B	34	AGGTGACACTATAGAAATAgagcagatggcgacattat	GTACGACTCCTACTATAGGGGAgtatcatattgcaacggggc	144
B	61	AGGTGACACTATAGAAATaactgcgaacacctctggt	GTACGACTCCTACTATAGGGGAatgacgggctgaggtgtgtaac	151
B	46	AGGTGACACTATAGAAATaattgttacaacatgccctc	GTACGACTCCTACTATAGGGGAatcagctgggttttttatacgc	158
B	47	AGGTGACACTATAGAAATAccacaacccaacaaactct	GTACGACTCCTACTATAGGGGAatccccctgtgtgtgagcgcac	165
B	33-A	AGGTGACACTATAGAAATaacaacaacacatcccggt	GTACGACTCCTACTATAGGGGAatctgttccgcagctgcat	172
B	59	AGGTGACACTATAGAAATatttgagaatglatccccac	GTACGACTCCTACTATAGGGGAaatttagcatgtcccccca	186
B	24	AGGTGACACTATAGAAATaactggcgttttcagtgctctt	GTACGACTCCTACTATAGGGGAaccaccacacgttttaatgg	193

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Multiplex primer set	SVV ORF	Forward primer (5'-3') ^a	Reverse primer (5'-3') ^b	PCR product size
C	28	AGGTGACACTATAGAAATAagttgacagcagcggaggtgttc	GTACGACTC ACTATATAGGGATttgttaaacgtcccccgtcga	285
C	38	AGGTGACACTATAGAAATAcaggatagcctttgtgtgt	GTACGACTC ACTATATAGGGAcgaggatcccatatgttaat	294
C	27	AGGTGACACTATAGAAATAtatcgttatccggaaatgggga	GTACGACTC ACTATATAGGGAccttcacgcgaacacatgggta	311
D	60	AGGTGACACTATAGAAATAatgctgtgtgtgtttgtga	GTACGACTC ACTATATAGGGAtttgtgcaccgaaacacat	137
D	21	AGGTGACACTATAGAAATAcgcacaaaactgtgtctct	GTACGACTC ACTATATAGGGATcccgtaagagcgtattcaac	144
D	25	AGGTGACACTATAGAAATAtcaacgcgttagaagcaaaa	GTACGACTC ACTATATAGGGAcctgtgaatcgggtgtgtggaa	151
D	36	AGGTGACACTATAGAAATAaatctccgataaacgacac	GTACGACTC ACTATATAGGGAcgccaattgtttggatttt	158
D	22	AGGTGACACTATAGAAATAtttgcaggtacggggaaatc	GTACGACTC ACTATATAGGGAtttcagacgacaaagtcgggg	172
D	9A	AGGTGACACTATAGAAATAtttctggcctatgtagctgttc	GTACGACTC ACTATATAGGGAaggcacaacagacacccaat	187
D	LE	AGGTGACACTATAGAAATAgcctgtgtgtctctgtcaaa	GTACGACTC ACTATATAGGGAgatgtgtgacgggtgtgtgaac	193
D	12	AGGTGACACTATAGAAATAaggagagagatggggagcagtt	GTACGACTC ACTATATAGGGAacccgcaagtcacagcgtatc	200
D	65	AGGTGACACTATAGAAATAaaagcggatagtgaggccgta	GTACGACTC ACTATATAGGGAttgggtgttttctatttcagcg	207
D	26	AGGTGACACTATAGAAATAtgagaaacgtcatttcgttg	GTACGACTC ACTATATAGGGAcgttaatacagccaccattct	222
D	16	AGGTGACACTATAGAAATAactactgtagtggggagagctc	GTACGACTC ACTATATAGGGAgcaagccaactcacatttca	229
D	64	AGGTGACACTATAGAAATAtgccggagccgttgggaagctcc	GTACGACTC ACTATATAGGGGtgtaggtctgcgtgtgcgggg	242
D	30	AGGTGACACTATAGAAATAggcaaaattttatgggagctcg	GTACGACTC ACTATATAGGGAaggtgtgtgtgtcgaacggctc	250
D	B	AGGTGACACTATAGAAATAtaccgacgtacgaagaaagcc	GTACGACTC ACTATATAGGGAataatacaacatggccgggg	260
D	62	AGGTGACACTATAGAAATAagcggcaagttctcaaacccggcc	GTACGACTC ACTATATAGGGAaacggggaggtgtgtcagagcggc	271
D	-61	AGGTGACACTATAGAAATAgtttgaaaccgccatccggcgga	GTACGACTC ACTATATAGGGAcgtgtgtgtgtgtgtgtgtgtgt	285
D	39	AGGTGACACTATAGAAATAccacttcaacattgggaacgtgtcat	GTACGACTC ACTATATAGGGAagcctgtgtgtgtgtgtgtgtgtgt	290
D	4	AGGTGACACTATAGAAATAtgggaatctgcagacgnaactg	GTACGACTC ACTATATAGGGAgataactctcgtcccatca	301
D	54	AGGTGACACTATAGAAATAtgcgtcatgtcgtgtatatt	GTACGACTC ACTATATAGGGAttttaaaagcgtctgtgcatt	312
D	17	AGGTGACACTATAGAAATAtctgttttactcgtgaactgtcggtct	GTACGACTC ACTATATAGGGAgctgcacgactcaaatggcct	333
A,B,C,D	Neurofilament	AGGTGACACTATAGAAATAaagtcaaaagagcccccaag	GTACGACTC ACTATATAGGGGacttggcactatcgtgttctct	348
A,B,C,D	GAPDH	AGGTGACACTATAGAAATAtttctctgcatcggccagcgtcat	GTACGACTC ACTATATAGGGGcagcgtcgtcccaactgtatt	344

Table 2

Multiplex RT-PCR prime efficiencies

ORF	No. of SVV DNA copies ^a			Slope ^b	Intercept ^b	$(R^2)^c$	Efficiency of qPCR	T_m^d	Lower T_m limit	Higher T_m limit
	Lower limit	Upper limit								
1	10	5000		-3.17	33.8	0.99	1.07	76.5	76.3	76.6
3	5	5000		-3.25	34.3	0.99	1.03	79.5	79.2	79.8
4	5	5000		-3.11	35.6	0.99	1.10	85.1	84.7	85.4
5	500	5000		-3.36	35.1	0.99	0.98	75.4	75.2	75.6
6	100	5000		-3.39	34.8	0.99	0.97	76.3	76.1	76.4
7	500	5000		-3.50	35.4	0.99	0.93	78.8	78.4	79.1
8	1	5000		-3.41	35.4	0.99	0.96	75.2	74.8	75.5
9	1	5000		-3.15	35.7	0.97	1.08	79.3	78.0	80.5
9A	1	5000		-3.36	34.5	0.99	0.98	78.8	78.6	78.9
10	100	5000		-3.35	34.9	0.99	0.99	77.4	77.0	77.7
11	1	5000		-3.14	34.8	0.99	1.08	79.1	78.9	79.2
12 ^e	500	5000		-3.31	34.2	0.99	1.01	77.9	77.7	78
12 ^e	1	5000		-3.27	34.8	0.99	1.02	76.6	76.4	76.7
13	1	5000		-3.39	35.6	0.99	0.97	78.8	78.6	78.9
14	500	5000		-3.59	35.6	0.99	0.90	77.0	76.8	77.2
15	1	5000		-3.51	35.1	0.99	0.93	74.3	74.0	74.6
16	1	5000		-3.16	34.2	0.98	1.07	78.6	78.3	78.9
17	1	5000		-3.29	36.0	0.99	1.01	77.5	77.1	77.8
18	500	5000		-3.41	35.1	0.99	0.96	75.5	75.3	75.6
19	100	5000		-3.41	35.1	0.99	0.96	76.3	76.1	76.4
20	5	5000		-3.20	34.4	0.99	1.05	79.2	78.9	79.5
21	1	5000		-3.20	34.9	0.99	1.05	77.2	76.8	77.5
22	5	5000		-3.25	34.9	0.99	1.03	75.6	75.2	75.9
23	1	5000		-3.40	34.8	0.99	0.97	79.4	79.1	79.7
24 ^f	5	5000		-3.17	34.2	0.99	1.07	78.3	78.1	78.4
24 ^f	500	5000		-3.43	35.1	0.99	0.96	78.3	78.1	78.4

ORF	No. of SVV DNA copies ^a		Slope ^b	Intercept ^b	(R ²) ^c	Efficiency of qPCR	T _m ^d	Lower T _m limit	Higher T _m limit
	Lower limit	Upper limit							
25	5	5000	-3.10	34.4	0.99	1.10	75.3	74.9	75.6
26	5	5000	-3.26	34.3	0.99	1.03	77.5	77.1	77.8
27	100	5000	-3.39	35.3	0.99	0.97	80.0	79.8	80.1
28	500	5000	-3.23	34.8	0.99	1.04	79.8	79.8	79.8
29	1	5000	-3.38	34.6	0.99	0.98	76.9	76.6	77.2
30 ^g	5	5000	-3.29	34.4	0.99	1.01	77.9	77.5	78.2
30 ^g	1	5000	-3.17	33.9	0.98	1.07	80.1	79.9	80.3
31	500	5000	-3.40	32.3	0.99	0.97	79.9	79.7	80.0
32	1	5000	-3.38	36.6	0.99	0.98	79.3	79.1	79.4
33A ^h	1	5000	-3.22	34.0	0.99	1.04	79.9	79.7	80.0
33A ^h	500	5000	-3.15	34.4	0.96	1.08	79.2	78.9	79.5
34 ⁱ	5	5000	-3.26	34.6	0.99	1.03	78.1	77.9	78.2
34 ⁱ	1	5000	-3.34	34.8	0.99	0.99	78.1	77.9	78.2
35	10	5000	-3.11	33.9	0.99	1.10	79.0	78.8	79.1
36	1	5000	-3.23	31.3	0.98	1.04	76.7	76.5	76.8
37	5	5000	-3.16	34.2	0.99	1.07	77.0	76.8	77.2
38 ^j	5	5000	-3.22	34.2	0.99	1.04	79.5	79.2	79.8
38 ^j	5	5000	-3.33	35.1	0.99	1.00	77.6	77.2	77.9
39 ^k	1	5000	-3.45	37.0	0.99	0.95	75.1	74.8	75.4
39 ^k	1	5000	-3.20	34.1	0.98	1.05	73.7	72.7	74.6
40	1	5000	-3.16	34.9	0.99	1.07	76.9	76.6	77.2
41	1	5000	-3.52	34.1	0.99	0.92	78.0	77.8	78.1
42-45	5	5000	-3.31	34.5	0.99	1.01	77.2	76.9	77.5
43	100	5000	-3.30	34.7	0.99	1.01	75.7	75.4	76.0
44	5	5000	-3.12	32.3	0.99	1.09	76.8	76.5	77.1
46	500	5000	-3.43	35.1	0.99	0.96	78.1	77.9	78.2
47	1	5000	-3.19	34.4	0.99	1.06	76.5	76.3	76.7
48	1	5000	-3.10	37.3	0.98	1.10	77.0	76.6	77.3

ORF	No. of SVV DNA copies ^a				Slope ^b	Intercept ^b	(R ²) ^c	Efficiency of qPCR	T _m ^d	Lower T _m limit	Higher T _m limit
	Lower limit	Upper limit									
49	100	5000			-3.27	32.8	0.99	1.02	78.9	78.7	79.0
50	1	5000			-3.41	33.7	0.99	0.96	77.6	77.4	77.8
51	100	5000			-3.25	35.0	0.99	1.03	78.7	78.4	79.0
52 ^f	500	5000			-3.28	34.9	0.99	1.02	78.2	77.9	78.5
52 ^f	500	5000			-3.38	35.0	0.99	0.98	76.8	76.6	76.9
53	10	5000			-3.12	34.1	0.99	1.09	76.2	76.0	76.3
54	10	5000			-3.20	33.4	0.99	1.05	77.2	76.7	77.7
55	1	5000			-3.28	34.3	0.99	1.02	78.6	78.4	78.7
56	5	5000			-3.20	34.0	0.99	1.05	76.1	75.7	76.4
57	5	5000			-3.18	34.1	0.99	1.06	78.3	77.8	78.2
58	5	5000			-3.22	32.7	0.99	1.04	76.7	76.5	76.8
59	100	5000			-3.24	34.5	0.99	1.04	78.7	78.5	78.8
60	500	5000			-3.42	35.5	0.97	0.96	78.0	77.7	78.3
61 ^m	100	5000			-3.42	34.2	0.99	0.96	81.4	81.0	81.7
61 ^m	500	5000			-3.28	34.7	0.99	1.02	80.9	80.7	81.1
61 ⁿ	1	5000			-3.52	36.2	0.96	0.92	78.4	78.1	78.7
62	500	5000			-3.39	33.9	0.98	0.97	87.4	87.3	87.6
63	1	5000			-3.33	32.6	0.99	1.00	79.8	79.4	80.1
64	500	5000			-3.21	34.7	0.94	1.05	84.3	84.1	84.5
65	5	5000			-3.19	33.9	0.98	1.06	77.3	77.0	77.6
66	5	5000			-3.15	33.9	0.99	1.08	77.4	77.0	77.7
67	1	5000			-3.15	34.3	0.99	1.08	76.0	75.7	76.3
68	1	5000			-3.31	33.5	0.99	1.01	78.1	77.9	78.2
A	100	5000			-3.24	34.4	0.99	1.04	81.1	80.8	81.4
B	1	5000			-3.33	34.3	0.99	1.00	79.2	79	79.3
LE ^o	1	5000			-3.28	34.6	0.99	1.02	81.0	80.7	81.3

^aLower limit of SVV DNA copies detected by qPCR and upper limit of SVV DNA copies added to qPCR samples detected.

^bSlope and intercept of the linear regression line calculated from the graph of SVV DNA copies and C_T values.

^c Coefficient of determination obtained from the linear regression line calculated from the graph of SVV DNA copies and C_T values.

^d Melting temperature of PCR product.

^e Primers for ORF 12 are found in primer set C and D, respectively.

^f Primers for ORF 24 are found in primer set A and B, respectively.

^g Primers for ORF 30 are found in primer set C and D, respectively.

^h Primers for ORF 33A are found in primer set A and B, respectively.

ⁱ Primers for ORF 34 are found in primer set B and C, respectively.

^j Primers for ORF 38 are found in primer set B and C, respectively.

^k Primers for ORF 39 are found in primer set B and D, respectively.

^l Primers for ORF 52 are found in primer set B and C, respectively.

^m Primers for ORF 61 are found in primer set A and B, respectively.

ⁿ Primers for antisense transcript for ORF 61

^o Left end region