Whole-Genome Transcription Profiling of Rhesus Monkey Rhadinovirus

Dirk P. Dittmer,^{1,2} Carlos M. Gonzalez,^{1,2} Wolfgang Vahrson,¹ Scott M. DeWire,^{2,3} Rebecca Hines-Boykin,¹ and Blossom Damania^{1,2,3}*

Department of Microbiology and Immunology,¹ Lineberger Comprehensive Cancer Center,² and Curriculum in Genetics and Molecular Biology,³ University of North Carolina at Chapel Hill, Chapel Hill, North Carolina 27599

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Rhesus monkey rhadinovirus (RRV) and Kaposi's sarcoma-associated herpesvirus (KSHV; also called human herpesvirus 8) belong to the gamma-2 grouping of herpesviruses. RRV and KSHV share a high degree of sequence similarity, and their genomes are organized in a similar fashion. RRV serves as an excellent animal model system to study the gamma herpesvirus life cycle both in vitro and in vivo. We have developed a high-sensitivity, high-throughput, high-specificity real-time quantitative reverse transcriptase-based PCR assay for RRV and have used this assay to profile transcription from the whole RRV genome during de novo productive infection of rhesus fibroblasts. Using this assay, we demonstrate that the genome-wide transcription profile for RRV closely parallels the genome-wide transcription profile for KSHV.

Kaposi's sarcoma (KS)-associated herpesvirus (KSHV; also called human herpesvirus 8) was identified in KS lesions from AIDS patients by using representational differential analysis (6). KS was first described as a cancer of elderly men and is also found in non-human immunodeficiency virus-infected individuals who are immunocompromised, such as transplant recipients. The classic form of KS also occurs in nonimmunocompromised hosts. The KS lesion is comprised of a mixed population of cells including endothelial, inflammatory, and spindle cells. The virus is found in the spindle cells and at times in macrophages in the lesion but not the infiltrating T lymphocytes. KSHV has also been linked to two B-cell lymphoproliferative diseases, namely, primary effusion lymphoma (PEL) and multicentric Castleman's disease (4, 51). In 1997, the New England Primate Research Center reported the isolation of a new herpesvirus, rhesus monkey rhadinovirus (RRV), showing close sequence relatedness to KSHV. Two different RRV isolates were subsequently sequenced at the New England Primate Research Center (RRV strain 26-95) and the Oregon Regional Primate Research Center (RRV strain 17577) (1, 50), and both isolates exhibit high overall sequence similarity to KSHV. The RRV genome, for the most part, is organized in a colinear fashion with KSHV. However, unlike KSHV, RRV grows to high titers in culture and currently represents the closest nonhuman primate model for KSHV and KSHV-associated malignancies.

Wong et al. (57) have reported that naive rhesus macaques that were coinfected with RRV (strain 17757) and simian immunodeficiency virus developed lymphoid hyperplasia comparable to KSHV-associated multicentric Castleman's disease. Similarly, Mansfield et al. (34) reported that RRV-negative naive macaques infected with RRV (strain 26-95) developed clinical lymphadenopathy consisting of paracortical and vascular hyperplasia, which over time evolved into marked follicular hyperplasia but ultimately resolved approximately 12 weeks postinfection (34). The phenotypes seen with RRV closely resemble the clinical presentation of KSHV-associated lymphoproliferative diseases and conform to the clinical manifestations of primary gammaherpesvirus infections in the human population. Studying RRV in its natural host overcomes two fundamental roadblocks in KSHV research. First, RRV provides an animal model system to study the relationship between simian immunodeficiency virus and RRV coinfection that can closely model human immunodeficiency virus and KSHV coinfection. Such a model does not otherwise exist for KSHV. Second, studying the lytic life cycle of KSHV is hampered by the fact that at most 20 to 30% of latently infected PEL cells can be reactivated by tetradecanoyl phorbol acetate (TPA) (43). Such TPA reactivation assays are widely used to study KSHV lytic gene expression. Recently, an Rta/ORF50inducible BCBL-1 cell line was developed to study lytic gene expression (36). Systems to study de novo infection of KSHV, however, are limited by low viral titers and the propensity for KSHV to enter latency after a few passages in tissue culture of infected cells (15, 30, 42, 47). In contrast, RRV can be grown to high titers ($\sim 10^6$ PFU/ml) in primary rhesus fibroblasts (RhFs) and can be serially propagated ad infinitum. This greatly facilitates the construction of recombinant viruses (13) and can be used, for instance, to evaluate loss-of-function phenotypes of mutant viruses after primary infection.

As with other surrogate viruses for human pathogens, the usefulness of the RRV model rests on establishing close correlations between the molecular machinery of RRV and KSHV. We have previously shown that the kinetics of key RRV transcripts after primary infection in RhFs mirror the kinetics of the homologous KSHV transcripts after reactivation in PEL cell lines (12). This can be attributed, in part, to the functional conservation between the major immediate-early transactivator of both viruses, namely, Rta/ORF50. RRV

^{*} Corresponding author. Mailing address: Lineberger Comprehensive Cancer Center, CB #7295, University of North Carolina, Chapel Hill, NC 27599. Phone: (919) 843-6011. Fax: (919) 966-9673. E-mail: damania@med.unc.edu.

open reading frame 50 (ORF50) can transactivate several KSHV promoters, albeit to a lesser extent than KSHV ORF50 (11), and KSHV ORF50 can transactivate a subset of RRV promoters tested to date (12). In order to further elucidate commonalities and differences between RRV and KSHV, we have developed a real-time reverse transcription (RT)-PCR-based array for every mRNA in the entire RRV genome. This assay is high throughput and highly sensitive, making it amenable to profiling of the viral transcription of the more than 80 RRV genes simultaneously and with multiple samples. In this report, we describe the transcription profile of RRV after lytic infection in RhFs.

Real-time QPCR array for RRV. The primary achievement of real-time quantitative PCR (QPCR) is that, for the first time, PCR (and RT-PCR) delivers reliable quantitative information without the need for dilution series or internal competitors, etc. Quantitative information can be extracted because the QPCR is monitored in real time (23) and the reaction product is quantified at every cycle using a doublestrand-specific intercalating dye (SYBR). We have recently shown that using the fluorescent dye SYBR is as sensitive as TaqMan-based detection (38) and have thus used SYBR for every primer pair in the RRV QPCR array. This removed one layer of variation, namely, the hybridization efficiency of the indicator oligonucleotide (TaqMan, Beacon, etc.), and yielded a high-throughput, low-cost approach, without compromising sensitivity or linearity (6 orders of magnitude) of the assay.

The RRV primer set is shown in Table 1. Primer design is one of the most important aspects in achieving a successful QPCR array. Based upon our prior experience (16, 19), we used the following guidelines to attain the best primer pairs possible. (i) The melting temperature (T_m) of the primers should be in the range of 59 \pm 2°C. The T_m was calculated using the Primer3 program (46) and the default setting for salt (50 mM KCl) and a 50 nM primer DNA concentration. (ii) The maximal difference between two primers within the same primer pair should be no more than 2°C. (iii) The total guanidine (G) and cytosine (C) content within any given primer should be 20 to 80%. (iv) There should not be any GC clamp designed into any of the primers. (v) Primer length should fall into the range of 9 to 40 nucleotides. (vi) Hairpins with a stem length four or more residues should not exist in the primer sequence. (vii) Fewer than four repeated N homonucleotide residues should be present within a primer. (viii) The resulting amplicon should be at least 50 nucleotides in length but no larger than 100 nucleotides. (ix) The primers should be located toward the 3' end of the ORF. (x) In cases where predicted ORFs overlap, primers should be selected outside the region of overlap. However, it is important to note that until a complete transcript map for RRV is known, one cannot exclude the possibility that some primers are located in regions in which 3' untranslated regions (UTR) or 5' UTR segments of one gene overlap the ORF of an adjacent gene. Primers were designed using the PrimeTime program (W. Vahrson and D. P. Dittmer, unpublished data), based on European Molecular Biology Open Software Suite and Eprimer3, modules. The European Molecular Biology Open Software Suite (44) is a comprehensive collection of free open-source programs for sequence analysis. Eprimer3, is a program for searching PCR

primers and is based on the Primer3 program (46) from the Whitehead Institute/MIT Center for Genome Research.

Each experimental sample was analyzed as follows. RNA from RRV-infected RhFs was isolated using RNAzol (Tel-Test Inc.) as previously described (16, 19). Poly(A) mRNA was prepared using dT-beads (QIAGEN Inc.) and reverse transcribed using Superscript II reverse transcriptase (Life Technologies Inc.) according to the manufacturer's recommendations. Five hundred nanograms of RNA was reverse transcribed in a 20-µl reaction volume with 100 U of Superscript II reverse transcriptase (Invitrogen Inc.), 2 mM deoxyribonucleoside triphosphates, 2.5 mM MgCl₂, 1 U of RNasin (Applied Biosystems Inc.), and 0.5 µg of random hexanucleotide primers (Amersham Inc.). The reaction mixture was sequentially incubated at 42°C for 45 min, 52°C for 30 min, and 70°C for 10 min. Heating to 95°C for 5 min stopped the RT reaction. Next, 0.5 U RNase H (Invitrogen Inc.) was added and the reaction mixture was incubated at 37°C for an additional 30 min. Afterwards, the cDNA pool was diluted 25-fold with diethyl pyrocarbonate-treated, distilled H₂O and stored at -80°C. The forward primer and reverse primer sets were synthesized (MWG Biotech Inc.) and pipetted on separate plates. Individual primers were stored at 100 pmol/ μ l at -80° , combined and diluted to yield enough forward and reverse primer mix for 100 reactions at a 267 nM final primer concentration. A 2.5-µl volume of primer mix was combined with 7.5 µl SYBR Green 2 \times PCR mix (Applied Biosystems Inc.) and 5 μ l cDNA and subjected to real-time QPCR on an ABI5700 or MJR Opticon2 cycler using universal cycling conditions (see reference 37 for details).

Following the criteria outlined above, we initially computed three primer pairs for each predicted ORF in the RRV genome (data not shown). To ascertain the potential for nonspecific amplification and cross-reactivity to other herpesviruses, we conducted a National Center for Biotechnology Information (NCBI) BLAST search with each primer against (i) the RRV genome, (ii) all herpesvirus sequences in the GenBank database, and (iii) the human genome. The results are depicted in Fig. 1. For each individual primer (mean primer length, 20.33 nucleotides; 95% CI, 20.17 to 20.49; n = 568) the second closest alignment in the RRV genome contained, on average, 9.73 mismatches (95% CI, 9.55 to 9.91; n = 568), making it highly unlikely that any primer would anneal anywhere other than at its cognate sequence in the RRV genome. For example, a single primer that aligned perfectly at two different positions in the RRV genome (at nucleotide positions 115092 and 115023 in the RRV 26-95 genome) was eliminated from the array. The alignments for any RRV primer on any herpesvirus DNA segment in the NCBI GenBank database averaged 6.40 mismatches (95% CI, 6.16 to 6.64; n = 568). These matches were located in RRV genes homologous to other herpesviruses. Primers which showed ≥ 15 matches to any herpesvirus genome other than RRV were eliminated from the array and thereby enabled the specific detection of only RRV in samples containing other herpesviruses. Lastly, we compared all RRV primers against the human genome. The alignments for any RRV primer against the human genome averaged 4.11 mismatches (95% CI, 3.94 to 4.28; n = 568), which is statistically expected as the universe of possible target sequences increases by orders of magnitude (from $\sim 1.2 \times 10^6$



FIG. 1. RRV primer design and characteristics. Shown on the vertical axes are the numbers of nucleotide matches for each of the 568 primers in the initial RRV array, which contained three primers per ORF. On the horizontal axes, the nucleotide positions for each primer start site on the RRV genome (26-95) are indicated. In all three panels, A, B, and C, the small black dots represent the intended primer and numbered matches, which equals the primer length. In panel A, the large gray circles represent the second closest match for a given primer on the RRV genome. In panel B, the large gray circles represent the highest match for a given primer on any herpesvirus nucleotide sequence in the NCBI GenBank database, except RRV. In panel C, the large gray circles represent the highest match for a given primer on the human genome.

for all herpesvirus sequences to $\sim 10^9$ for the human genome). Only two primers (0.4%) exhibited a perfect match to a human DNA sequence, and these were removed from the array. We have shown previously that any \geq 3-nucleotide sequence difference can be recognized by dissociation profile analysis (38) and thus nonspecific amplification products would have been identified. No nonspecific amplification products were generated using the primer set listed in Table 1 (Fig. 2). To gauge the specificity and sensitivity of our approach, we conducted the following quality control experiments. (i) The cDNA was subjected to PCR with the primer pairs shown in Table 1. We analyzed the PCR products by agarose gel electrophoresis and found that every primer pair in the RRV array yielded a single product of uniform size (Fig. 2A). (ii) We conducted melting curve analysis for every experiment (data not shown) and excluded data for primer pairs which did not yield a single peak dissociation profile from further analysis. (iii) Under the stringent real-time QPCR conditions used (60°C annealing temperature, 60-s extension phase), no primer in the RRV array yielded a signal using either KSHV- or Epstein-Barr virusinfected cell mRNA as the target sample (data not shown).

Figure 2B shows the unmanipulated cycle number (CT) signals for each primer using the following samples as targets: (i) water, as a nontemplate control (NTC; open squares), (ii) RNA from uninfected cells that was DNase I treated, reverse transcribed, and RNase H treated (gray circles), (iii) RNA from uninfected cells that was DNase I treated but prepared without reverse transcriptase in the cDNA reaction and subjected to RNase H digestion (gray squares), and (iv) RRV virion DNA (gray line). Only cDNA from RRV-infected cells or RRV virion DNA yielded a significant signal. The positive signal was, on average, 15.84 CT units (95% CI, 14.86 to 16.81) or $2^{CT} = 58,656$ -fold above the background of the mockinfected, the RT-negative, or the NTC sample. This outcome demonstrates that the primer pairs were specific for RRV mRNAs, not cellular mRNAs. This experiment verified our contention that even if an individual primer showed limited similarity to a cellular gene (Fig. 1), the chance of both 20-mer primers in a primer pair containing significant sequence identity to the same cellular gene and being located in close enough proximity ($\leq 1,000$ bp) to be amplified during the 60-s extension phase of the real-time QPCR protocol is infinitesimal. Furthermore, we confirmed that our mRNA/cDNA preparations did not contain contaminating viral DNA (Fig. 2B). In certain reactions, the NTC target yielded a higher background signal than cDNA from mock-infected cells, which is not unexpected, since even unspecific nucleic acids have a quenching effect on the PCR, thereby affecting overall PCR efficiency. Ten of the initial 83 (12%) RRV-specific primers did not amplify virion DNA or cDNA from productively infected cells (data not shown). These "primer failures" were replaced with alternative primers in subsequent experiments (Table 1 depicts only the experimentally validated primer pairs for RRV). For all primers, the virion DNA target yielded a mean CT of 24.41 (95% CI, 23.41 to 25.41; n = 72). In other words, any change in CT of ≥ 1 could not be attributed to differences in primer efficiency but was due to changes in target mRNA. Figure 2C plots the standard deviation (SD; n = 5) relative to the mean CT for each gene in the RRV array. Note that Fig. 2C, is on a log₂ scale. Five CT units represents a 32-fold change, and 10 CT units represents a 1,024-fold change in relative mRNA levels. There was no significant correlation between the magnitude of the SD and the mean CT (r = 0.31; n = 82), demonstrating that changes in RRV gene expression did not depend on the overall levels of any particular viral mRNA (Fig. 2C).

RRV transcription upon de novo infection of fully permissive RhFs. To chart the transcription profile of a rhadinovirus upon primary infection of highly permissive cells, we infected RhFs with RRV at a multiplicity of infection (MOI) of 1, and isolated mRNA at different time points after viral infection. The mRNA pools were reverse transcribed using hexamer

TABLE 1.	Real-time	QPCR	primers	for	the	RRV	array ^a

Ai DorREN:H CACREGACIGAAAAACAAATTA (a) 198 RORE12 CCATEGACAACATACACATTCACC (b) 191 AS RORE31 CACUCACATTCACGACACTTCAC (a) 370 RORE32 CANLAAGGCACACACACACACAC (a) 373 AS RORE31 CACUCACATTCACACACACACAC (a) 370 RORE31 CACUCACATACACACACACAC (a) 370 AS RORE31 CACUCATACACACACACACAC (a) 1818 RORE32 CACUCACACACACACACAC (a) 1818 AS RORE31 CACUCATACACACACACACAC (a) 1823 RORE32 CARUCACUCACAGAGACACACACACACACACACACACACACAC	Well	Name	Forward primer sequence	T_m (°C)	Position	Name	Reverse primer sequence	T_m (°C)	Position
A2 BORES-1F ACUECACTIALGUALANTIT 60 1941 ROBES-2F TIGLALETTUCLCUCULAGUACC 60 1944 A BORES-1F CCTGACGACOCCTACT 6 987 ROBES-2F TICCAGTOCTACGTACGACAGAC 9878 A BORES-1F CCTGATACCACCACCAC 60 1938 ROBES-1F TICCAGTOCTACGTACGACAGC 1934 A BORES-1F CCTGATACGACACCAC 60 1936 ROBES-1F CCCAGACACACCACGAC 1937 A ROBES-1F CCTTGATACGACACCAC 91517 ROBER-2F CCCAACACACACCACGTACGAGAAC 1937 A ROBER-1F CCGCGCTGAACGACATTAGGAAAC 1937 ROBER-2F COTTGAAGACACACACAC 1938 A ROBER-1F GGACTCACCACTGAAGAAC 23507 ROBER-2F COTTGAAGACACACACAC 23539 B ROBER-1F GGACTCACGTACAGACAAC 23508 ROBER-1F COTTGAAGACACACCCACTAGAGACAC 2359 B ROBER-1F GGACCACTCACGTACAGACAC 23509 ROBER-1F COTTGAAGACACACCCACTGACACACACACACACACACACAC	A1	R-ORFR1-1f	GACCGCAGGAAAACACAATTA	60	1495	R-ORF1-2r	CCATGTGCTCAACATTATCCTC	59	1575
A) BORE-LI CALLAGACUCALANTAGE 0 598 ROBE-L2 CALLAGACUCALANAGACUCALANAGA 0 127 A) ROBE-L1 CALGAACUCACAACAACAA 0 958 ROBE-L2 CALLAGACUCALANAGACUCA 0 1234 A) ROBE-L1 CACCIGATACACAACACACA 0 1345 ROBE-L2 CALAGACUCATACAACAACAACAACAACAACAACAACAACAACAACAA	A2	R-ORF2-1f	ACGCGCATTATGGAGAGTTT	60	1991	R-ORF2-2r	TTGTATCTTTGCCCGGTAGC	60	1914
AS ROBF:11 CCTEDATCTAACGTACGGACCAGAG 9957 ROBF:22 TCGACTTCAGCGGTTA 9958 AS ROBF:11 CCCGACGGACAGCAGCAG 00 1518 ROBF:22 CCGAACGGACAGCGAG 00 1524 AS ROBF:11 CCCGGATTCACGTCACACATCC 90 1578 ROBF:22 CCGAACGGACACGGAGCAG 00 1524 AS ROBF:12 CCGACGTCACGTCACACATCC 90 1578 ROBF:24 CCATACGTCACGTCACACATC 00 1581 AN ROBF:14 GCGGCTGAAGACACGTCAGGAAC 00 1578 ROBF:24 CCCATCCCCACGAACACC 00 1581 AN ROBF:14 CCGCCCGAAGACTCAGGTCAGG 00 2581 ROBF:24 CCCATCCCCCAGAACACC 00 2584 B ROBF:14 CCCCACCCAGACACACCAGTCAGG 00 2584 ROBF:24 CCATCGCCGAGAACACAC 00 2584 B ROBF:14 CCCCACCCAGACACACCAGC 00 3377 ROBF:24 CATCGCAGACACACAC 00 3385 B ROBF:24 TATCGGAGAGGAGGACATACAGAC	Α3 Δ4	R-ORF4-11 R-ORF6-1f	ATATCGCCACCGCTTCCT	60 61	3070 7493	R-ORF4-2r R-ORF6-2r		60 61	3740 7576
AB RORB-SH GOGGTTAGACCAACAGCAG 00 1204 R.ORB-SH CCGAATCAGACCAGGAGGAT 00 1224 AB RORB-IL-I CATCAGGATLAGGACATAGGAG 00 1235 RORB-IL-I CGGAACACAGCACTGAGGAGT 00 1235 AB RORB-IL-I CGGAACACAGCAGTGAGGAAT 00 1237 RORB-IL-I GGGAACACAGACAGCGGGGGGGGGGGGGAGTGAGGAAA 00 1007 ROARB-IL-I GGAACACAGACAGTGGGAGGGGGGGGGGGGGGGGGGGGG	A5	R-ORF7-1f	CCTGTATCTAACGTACGAGCAAGA	60	9567	R-ORF7-2r	TGCAGGTGATGGTACAGAAGA	59	9648
AT R. BRUPF-IF ACCCGGATTACCATCAGACAG 60 1548 R. BRUPF-IF CGAAACAGCAACATGGGATT 60 AS R. BORFIL-IF CCGAACCGGCAGATAGAGAG 777 R. BORFIL-IF CGAACCGGCAGACATAGAGAGAG 60 AS R. BORFIL-IF CGAACCGGCAGACAGAGAGAGAG 778 R. BORFIL-IF CGAACCGCCAGACAACAGAGAG 7772 ALI R. BORFIL-IF GGAACCGCGCAGATGAAGAACAG 78 R. BORFIL-IF CGCAACCAGACAGACACAGAGAGAGAGAG 7872 BL R. BORFIL-IF GGAGCGCGCGAGATGAAGAGAGAG 78 25851 R. BORFIL-IF CGCAACCAGACGAGACAGAGAGAGAGAGAGAGAGAGAGA	A6	R-ORF8-1f	GCGGTTAGACGAACAGCAG	60	12043	R-ORF8-2r	CTTCGTTTTCCAGCGGTTTA	60	12124
AS ROPRIP.H CATGGTTCATCGTTCGT 61 16600 AS ROPRIP.H CCGAACCGAGATAGAGAT 00 1987 ROPRIP.H CGGAACAGAGATGGTTGT 00 1982 AII ROPRIP.H GCGACCGAGATAGAGAT 00 1987 ROPRIP.H CGGAACAGAGATGTGAA 00 1982 AII ROPRIP.H GCGACCGAGATGGAACA 00 1987 ROPRIP.H CGTGTGAAGACACGTGAACA 00 1982 B ROPRIP.H GGACGTGCAGATCGTAAG 00 2520 ROPRIP.H CGGCCCAGAGACCACG 00 2530 B ROPRIP.H CATGGCAGGGACTCGAACA 00 2577 ROPRIP.H CATGGCAGGGATGGTGATCAA 00 3107 B ROPRIP.H TATGCCAGAGAGACCCCCATCA 00 3107 ROPRIP.H CATGGCAGGGGGGACACGCACTCA 00 3108 B ROPRIP.H TCGCACGCCAATTTAAG 00 3108 ROPRIP.H TCGCACGCAATTTAAG 00 3108 B ROPRIP.H TCGCAACCCAATTTAAGCACTTCACTTAG 3108 ROPRIP.H ROPRI	A7	R-ORF9-1f	ACCCGGATTACGTCAGACAG	60	15165	R-ORF9-2r	CCGAACAGACACTGGAGGAT	60	15245
49 ROBREJLH CCGAACCCGCAGATAGAGAT 60 1764 ROBREJL CGAACCCGCTTCAGATACACAG 60 1772 AU ROBREJLI GCAACCCGCATCAAGGACAC 61 1873 ROBREJLI GCAACCCGCCGAGATCAGGAGAC 60 1835 AL2 ROBREJLI GCAACCCGCGAGACACACAC 61 2550 ROBREJLI CCACLAGCCGCAGACACACA 60 2541 B ROBREJLI GCAGACCGCGAGATCG 60 2541 ROBREJLI CCACACCGCAGATACCG 60 2541 B ROBREJLI ACTOCTGCGCAGTCTCAGG 60 2814 ROBREJLI CCACACCGCAGTACTG 60 2902 B ROBREJLI TCACACGGCAGCATTCAGG 60 2902 ROBREJLI CCACAGCCAGTGACATG 60 3935 B ROBREJLI TCACACGGCAGCCATTCAGG 60 3372 ROBREJLI CCACAGCCAATTTACGT 60 3385 B ROBREJLI TCACACGCAGGTGCTTCAGT 90 348 ROBREJLI CCACAGCCAATTTACACTT 60 43512 B ROBREJLI	A8	R-ORF10-1f	CATTAACGCGTTCACAATCC	59	16577	R-ORF10-2r	CAATGGTTCATGCGTTCGT	61	16660
AID ROBERS-T DEAGRES-T DEAGRES-T <thdeagres-t< th=""> <thdeagres< td=""><td>A9</td><td>R-ORF11-1f</td><td>CCGAACCGGGAGATAGAGAT</td><td>60</td><td>17684</td><td>R-ORF11-2r</td><td>CGAACACGAAGATGGCTTG</td><td>60</td><td>17762</td></thdeagres<></thdeagres-t<>	A9	R-ORF11-1f	CCGAACCGGGAGATAGAGAT	60	17684	R-ORF11-2r	CGAACACGAAGATGGCTTG	60	17762
A12 ROBER-1: GGTTGGAAGCATCTCATT 00 2389 B ROBER-1: GGTTGGAAGCATCTCATT 00 2389 B ROBER-1: GGCGACCCAGAGACCTGAGACA 00 2389 B ROBER-1: GGCGATCCCCCAGAGTCTCGG 00 2561 8.00 2389 B ROBER-1: IAGTGGTCCCCAGAGTCGC 00 2980 ROBER-2: GGATGGTCCCGAGAGTCG 00 2980 B ROBER-1: IAGTGGTCCCCAGAGTCACAGC 00 2980 ROBER-2: CATTGCTTCCCAATGGTT 00 300 B ROBER-1: IAGGGCAGGAGTGATGCAATG 00 3159 ROBER-2: CATTGCTTCCAAATGGTT 00 3238 B ROBER-1: ITGGGCAAGTGAATGTATAG 00 3228 ROBER-2: CAGTGGAAGTGAAGTCGAACGCAATGTAAG 04 3333 B ROBER-1: ITGGGGAAGGAGGATGTACTCAAGC 04 3228 ROBER-2: CAGTGGAAGTGAAGTCGAACGCAATGTAGAG 43383 B ROBER-1: TCGGGAAGGAGGGTGTTGTAA 04 3228 ROBER-2: <t< td=""><td>A10</td><td>R-ORFR2-II P OPE70 1f</td><td>GCGCTGAAGATTTTGAGAA</td><td>59 60</td><td>18233</td><td>R-ORFR2-2r</td><td>GAAAUGUGUIGAGAAUAAU</td><td>60 60</td><td>18151</td></t<>	A10	R-ORFR2-II P OPE70 1f	GCGCTGAAGATTTTGAGAA	59 60	18233	R-ORFR2-2r	GAAAUGUGUIGAGAAUAAU	60 60	18151
B RORFID-1 ACTAGEGGAGGACT AGGAAT 60 2524 RORFID-2 CACTAGECCCAGAGACC 60 2536 B RORFID-1 CACCAAGCAGCTCACGTTCG 60 2541	A12	R-ORFR4-1f	GGACGACGCAGTGAAGAAAC	61	20570	R-ORFR4-2r	CGTTGGAAGCATCGTCAAT	60	20495
B2 RORFIF-11 CCCCCCAGAGATCGTAATG 60 2561 RORFIP-22 GGACTGTCCCGATGTCGG 61 2778 B4 RORFIP-11 ACCGAGAGCCGAGTGTCG 62 2805 RORFIP-32 GATGGTCCCAAGAGTGGTCGAGA 60 2902 B6 RORFIP-11 CAGGTCGTTGTCACAAAG 60 2909 RORFAP CATGGTTCGCAATGGTGT 59 20012 B6 RORFAP CAGGTCGTTGTCACAATAG 60 3159 RORFAP CAAGGGGAAGTGAAGTAG 60 3373 B7 RORFAP TGGAGCGAATTATATGTCGAATAAGC 60 3238 RORFAP CCCAACTGCAAGTTATATATGTCGAATAACG 60 4238 B10 RORFAP TGGAGGAATTATATGTCGAATTAACG 60 4239 RORFAP CCCAACTGCAAGGAGTGTATTATA 60 4357 B11 RORFAP TCCCAACTGCAAGGAGTGTACTTGTA 60 4357 RORFAP CCCACTGAAGCGAAGTGAACTGAA 64 4578 B12 RORFAP TCCCAACTGAACGAAGGGGTTACTGTTTGT 60 4579 60 4579 60 4579 60 45799 <td>B1</td> <td>R-ORF16-1f</td> <td>AATGGGAGGGGACTCAGGAAT</td> <td>60</td> <td>25224</td> <td>R-ORF16-2r</td> <td>CACTAGCGCCAGAAGACCA</td> <td>60</td> <td>25309</td>	B1	R-ORF16-1f	AATGGGAGGGGACTCAGGAAT	60	25224	R-ORF16-2r	CACTAGCGCCAGAAGACCA	60	25309
Bit BORPH3-II AGEIGIGICGCGAGTGITCG 59 2710 RORPH3-2 CACAAACAGTGICCCAGTCG 60 27962 Bit RORPJ3-II CACCAACGCCACGTTGATCAAAC 60 2999 RORPJ3-2 CACCATGGTTTGGTCAAT 59 2001 Bit RORPJ3-II CACCATGGTTGTCACAAT 60 3999 RORPJ3-2 CATCGTTGCTCAACGACGATGTTGGTCAAT 60 3939 RORPJ3-2 TAGCGGGGGGTGTGGTGGAGTGTCAAT 60 3932 RORPJ3-2 TAGCGGGGGGTGTGGTGGGGGGGGGGGGGGGGGGGGGGG	B2	R-ORF17-1f	CCGCCCAAGAATCTGTAATG	60	25616	R-ORF17-2r	GGACTGTCCGCATTCTGG	60	25541
Bit R. DRF19-IF ACCAAAAGCTCACGTTGAGG 60 29017 CARTGGTTGGTACAATAG 60 29007 CARTGGTTGGTACAATAG 60 29007 CARTGGTTGGTACAATAG 60 29007 CARTGCTTTGGTACAATAGGTGGTT 60 29007 CARTGCTTTGGTACAATAGGTGGTTT 60 29007 CARTGCTTTGGTTGGACAATAGG 60 29007 CARTGCTTTGGTGTGCAATAGGTGGTT 60 29007 CARTGCTTGTGTGTGTAAAGG 60 29007 CARTGCAATGGTGGTGTGTGTGTGT 60 28308 R-ORF2-2+ CAAGGAAGCGCAATTTTCAAGG 60 41233 B10 RORF2-11 CCCCCCTGCACGTATCTGTGTGTGTA 60 4229 RORF2-2+ CAGGAACCCCAATAGCTGTA 41423 RORF2-2+ CAGGAACCCAACGTTTAAGGCAATTCCAG 41423 RORF2-2+ CAGGAACCCAACGTTAAGG 41222 CARCGAACCCAATAGCTGTA 4122 RORF2-2+ CAGGAACCCAACCATTCCAACG 4123 RORF2-2+ CAGGCACCAACCATTCAACGTTAAGGA 4123 RORF2-2+ CAGGCACCAACCATCCAACGTTGAACGTGTGTGTGTAAGA 41232 RORF2-2+ CAGCTGGTGGTGTGTGTAAGA 41232 RORF2-2+ CAGCTGGCAGACGTGCAACGTGCAACGGTGAACGTGTGTTTGAAGACGAAGAGAGAACGTGAAAACGGAAGA 412904 RORF3-2+ CAGCCAACG	B3	R-ORF18-1f	AGTGTGTCGCGATGTTCG	59	27719	R-ORF18-2r	CGCAAACAAGTAGTCCGTCTG	61	27798
B REMERSING REMERS	B4	R-ORF19-1f	ACCAAAAGCTCACGTTCAGG	60	28045	R-ORF19-2r	GATGGGTGCGAGAGGATG	60	27962
B CORRE2-1: TIGTACAGAAGGACGEATCA 60 3377 F-ORRE2-2: TAGGCAGGAGTGAAAACAG 60 3383 B R-ORRE3-1: TAGGCAGGAGTTAGG 60 3383 3893 R-ORRE3-2: TAGGCCAGGAGTTAGG 61 3393 B R-ORRE3-1: TGGAGGAATATATGTGCAATAGC 64 4123 R-ORRE3-2: TAGGGAGAGTATTACGE 64 4123 BI R-ORRE3-1: TGCCCACCTACACTATCTACCG 64 4220 R-ORRE3-1: CCCACCGGAGTACTACCG 64 4326 BI R-ORRE3-1: CTCCGGAGGGAGTCTACCG 64 4386 R-ORRE3-2: CGGACGACGCACCTACACAT 64 4570 C R-ORRE3-1: CTCCGGAGGGAGGTCACCG 64 4820 R-ORRE3-2: CGGCACGACGCACCTACACAT 64 4570 C R-ORRE3-1: CTCCATGGAGCAGGAGGTCACACG 4539 R-ORRE3-2: AGGCCAACGTACACAT 64 4570 C R-ORRE3-1: CGCAGCAGGAGCTGACATA 4539 R-ORRE3-2: AGGCCAGCAGCAGGAGGGGGGGGGGGGGAGA 45678 C R-ORRE3-1	B5 B6	R-ORF20-II R-ORF21-If	TATGGGCAGGTGATGTCAAA	60 60	29097	R-ORF20-2f	CATTGCTTTCCAAGCTGGTT	59 60	29012
BR CORF2-1:1 CAGACCTGACCATTTAAGTT 99 10008 F.ORF2-2: TACCCGGGACTGACCTAACGTCATGAGG 60 39933 BI0 RORP2-1: CCCATGAACTTACTGACG 99 41423 BI1 RORP2-1: CCCATGAACTTATCGACG 91 41423 BI1 RORP2-1: CACAGGAACGTAACGTACGG 64 4229 RORP2-2: RACAGGAACGTAACATTACAC 59 4322 CI RORP2-1: TCGAACGTATAGACTATACGC 64 4320 RORP3-2: GCGAACCTAACACTTAAGCACTACG 64 4322 CI RORP3-1: TCGAACGTAACCTAACACTTAAGCACTACG 64 4320 RORP3-2: GCGATGCAGCTACTTAG 59 4578 CI RORP3-1: TCGAACCCAACTTACTCCCACTATAAA 59 4578 CACCACTTAGGCCTTAATA 64 4502 CI RORP3-1: GCGAAGAACCCAACTTAACTCCACTAAA 59 4577 CACCACTTAGGCCTTAAGCACTAAA 64 4502 CI RORP3-1: GCGATGAGAACCCAACTAAAA 59 4577 RORP3-2: GCGCAGCACACACACACACAACAACAAAAAAAAAAAAA	B7	R-ORF22-1f	TTGTACAGAAGACACGCACTCA	60	33772	R-ORF22-2r	TAGGCCAGGATGGAAAACAG	60	33856
B9 B0 CORP2+11 COCCAACGCCAATTATATCGCAATAAGC 60 35122 B10 RORP2-11 TOGCCAACATTATATCGCAATAAGC 60 4239 RORP2-2-2 CACAGGAACCTATCATCAG 60 42380 B11 RORP2-11 CCCCACCTATGAACATATCAG 60 4239 RORP2-2-2 CACAGGAACCTATTCAGC 60 43846 RORP3-2-1 CGCAACCACTATACACCACTATCACAG 61 43564 C RORP3-12 CGCAGCACACTATACGACG 61 43564 C RORP3-11 TITAAACCACTTACCCACCATACACG 60 43846 RORP3-12 CACCCATCAGGAGCACTATACGACTA 60 4902 CORT3-22 AGGTCGTAGGGGCATTAATAG 60 4902 CORT3-22 AGGTCGTAGGGGCATTAATA 60 4902 CORT3-22 AGGTCGTAGGGCACTTAATAG 60 4902 CORT3-11 AGGTCGTAGGGCACTTAATAG 60 4902 CORT3-22 CACGTCCAACGGCCATTACATAG 60 4902 CORT3-22 CACGTCCACACTACCACACGACGCCATTACA 60 4902 CORT3-22 CACGTTCACACACTACGCACACACGACACCACGACACACAC	B8	R-ORF23-1f	GAGAGCTGGCCATTCAAGTT	59	34008	R-ORF23-2r	TACGCGGGGGACTGAGATTAG	60	33933
BIO R.ORPZ-511 TOCGACIGATIATIATIOTEGAATIAAGC 59 41439 R.ORPZ-52- TACCAGGAAGGGGTATTICGTIG 60 41230 BII R.ORPZ-51-1 CCCCACCTGGAATIGATGTA 50 4312 R.ORPZ-52- GACAGGAAGGGGTATTAGCC 54 43222 C R.ORPZ-51-11 CTCCAACCTAAGCCACTTAAGC 64 43578 RORP30-2- CGCATCGTGGTATAAGGAGAGA 54 43574 C R.ORPZ-11 TATAAGCACGAGGGTCATTCACG 64 4302 RORP30-2- CGCATCGTGGTATAAGGAGAGA 54 4570 C R.ORPZ-11 ACGCCACCTAACTTCACCTACTCACGA 59 45578 CORT32-2- CATCCATTAAGCACCTACTAA 54 4578 C R.ORPZ-11 ACGCCTATGTGTGGGCGCACTCA 64 4902 RORP3-2- CATCCATTACCCC 64 4900 C R-ORPZ-11 AGCGCTAGGGAACTGGAACGA 59 5292 CACCCTTATAGCACAAGGGGCTATTACA 60 5932 C R-ORPZ-11 AGCGCAACTATAAA 50 5304 CORT3-2- TATGAAGCACTATACA 60 5932	B9	R-ORF24-1f	CGCAACGCCAATTTTATGT	60	35238	R-ORF24-2r	CCAGTGAAGTCCCAAACGTC	61	35152
BII RORP2:11 ICCCCTAGAGACCTATCTA 60 4229 RORP2:2-27 AACAGGAACCGATACACACG 60 4222 C RORP2:11 CCCCACCTGGAATGATATA 60 4357 RORP2:2-27 GGAACCACCATACAACATCA 61 4352 C RORP2:11 TITAAACGACGTGTTGTACACG 60 4386 RORP2:2-27 GGAACCACCACTACACACAACAACGA 61 4350 C RORP3:11 TITAAACCACTTAGCCACTACACAC 64 4902 RORP3:2-27 GCGATGCAGCGCCTGTAAAGAGAA 61 4510 C RORP3:11 TITAAACCACTTAGCTACACGA 64 4902 RORP3:2-27 AGGCAGTACCGAGCGCCTGAAAA 60 4903 C RORP3:11 GGGGACGCACCACTCGTGGAGAT 64 4902 RORP3:2-27 AGGCAGTATGCCGAAAAAAGAAAGAAGAAGAAGAAGAAGAAGAAGAAGA	B10	R-ORF25-1f	TGGACGAATATATGTCGAATAAGC	59	41349	R-ORF25-2r	TCCGATCTTTAATAGTCTTTTCATAGG	60	41423
Dip RORDZY-11 COCCUCACCTACATATIONTON (CARACAC) Set al: Constraint (CARACAC)	B11	R-ORF26-1f	TGCCCTCATGACCTATCTACG	60 50	42299	R-ORF26-2r	AACAGGAAGCGGTATTCGTG	60 50	42380
C2 NORP39-17 CTAAAAGACGGAGGTCACACG 60 48966 R-ORP39-27 TCCTTOGGTTGCCATGTAGTGT 61 45102 C3 RORP3111 TTCAAACCACTTTAGCATT 64 4502 R-ORP3127 GCGATGGTGTAACAGTGTAGCACTA 59 4554 R-ORP3127 GCGATGGTGTACTGTATGG 59 4554 C4 RORP3111 GTGCAACCACTTGCGATACTGTTTG 60 49027 R-ORP3227 AGGTGGTAGCGCACTGGCACAC 61 48031 C7 R-ORP3211 AGGCGATGGTCACACAC 61 48031 R-ORP32-17 AGGACACTGCCACACC 61 48031 C8 R-ORP32-11 AGGCGATAGGACACGGACAC 50 50247 R-ORP32-27 TGGAAGCACGCACACAC 61 5031 C8 RORP32-11 AGGCACATGGTGAAGCAA 50 5020 R-ORP32-27 GCGATTGCACAAGAGGG 50 51546 C1 R-ORP32-11 AGGCACATGTGTAGAAAGAAGAACATGTGTTGTAG 50 51542 CCRCATGAGAAGGAGGT 50 51542 C1 R-ORP32-11 GGCGATGAGAAGAAGAAGAAGTTTGTGTGTGTGTAGGAAGAAG	Б12 С1	R-ORF2/-II R-ORF28-If	ATGCGGAGGGTGTGTGTGTA	59 60	43142	R-ORF2/-2f R-ORF28-2r	GCATCACCATTAAGCACTATTACAC	59 61	43222 43654
C3 P.ORFF9-17 TITTAAACCACTITTAGCCTATCGAATC 69 4592 P.ORFF3-27 CATCCATGCGTGTAAGAGGACA 61 45102 C3 R.ORFF3-11 TGCGCAACCCACTIGGTTG 69 49027 R.ORFF3-27 AGTCGATGCGGCCTTTAAT 60 49027 C6 R.ORFF3-11 GGCGAAGCACCTGAACATAAA 59 4913 R.ORFF3-27 AGTCGAAGCACCTCCAC 61 4902 C7 R-ORFF3-11 GGCGAAGCACTGGGGCAA 50 4913 R.ORFF3-27 GAGGCACGTTCCCT 60 59126 C8 R-ORFF3-11 AGCCGTGACAGCACTGGTGAAGA 50 59046 R.ORFF3-27 GGCGAATTGCTCAAGGAGA 60 51546 C11 R-ORFF3-11 GAGCAAGTGTTGCTGATGAC 59 5900 R-ORFF3-27 GGCGAATCATTGTGAAGAA 60 51546 C12 R-ORFF3-11 GAGCACTGTTGATAAACAAC 60 55807 GAGGTTGTGTGAACGAACTATTGTGGTGTGT 60 5582 C3 R-ORFF4-11 GGCGCAACTGTTAAACAAC 60 61414 R-ORFF4-27 GGTGTGATGCTGAAGTACTAGG 55899 C3	C2	R-ORF29b-1f	CTAAAAGACGGAGGTCACACG	60	43846	R-ORF29b-2r	TCGTTGGTTGCCATGTAGTG	61	43769
C4 R-ORF31-11 CCTCCATTGCCCCTTCAAAGCGTA S9 45594 R-ORF31-22 CATCCATTGCCCCTTGATAG S9 4576 C5 R-ORF33-11 GCGCAGCCACTCACTGTTGC S9 47977 R-ORF33-27 AGGTCGTACGCGCCTTGATAG S9 48181 C7 R-ORF39-27 TATTGAAGCACCTGAACCAC 61 49802 TATTGAAGCACCTGACCCAC 61 48002 C8 R-ORF39-11 ACGTCTAGTGTTCCGGCGCAC 61 49814 R-ORF39-27 GATGCCCCCACTGACCTAC 60 59322 C10 R-ORF39-11 ACGCCACACGTGGTGAACC 60 51468 R-ORF39-27 GCGTTTCCACAGGAGGAAGAAGCATTGTCTTGTAT 60 5306 R-ORF39-27 GCGCTGTAGACAAGGAAGAAGCATTGTCTTGTTA 60 5306 R-ORF49-27 CTCTGTAAGGCCCAAGAGAAGGAA 59 5890 D1 R-ORF39-11 CCCGGCACATTGTAGAAAACAAC 60 5582 R-ORF49-27 CTCTGTAGAGGACGCATTGTGAGGA 59 5898 D2 R-ORF49-11 CCGGCCACATTGTAGAAAACAAC 60 55815 COTCTAGAGGACACTCGAGAGA 59 54957 D4 <	C3	R-ORF30-1f	TTTAAACCACTTTAGCCTATCTGAATC	60	45020	R-ORF30-2r	GCGATCGTGGTATAAGAGAGACA	61	45102
CS R.ORF3-1/F AGCGGGCAGTICTACTGTTG 60 49027 R-ORF3-2r AGCGGTCGTAGCGGCCTTTAAT 60 47002 C7 R-ORF3-1/F GGCGAAGCACCCACCCCTCC 59 49813 R-ORF3-2r AGCGGTCGTACGGACACC 61 4984 R-ORF3-2r GAGGCCCTTTACAG 60 49902 C8 R-ORF3-1/F AGCTGTACGGAGAACTGGAGAT 59 50247 R-ORF3-2r GAGGCCCACATTATCA 60 50332 C10 R-ORF3-1/F AAACCACACCGACTGGTAGCA 60 5360 R-ORF3-2r GCAGTTCCCCAAGGACGAGG 60 53282 C12 R-ORF3-1/F AAACCAAC 60 53307 R-ORF4-2r TTTCCTACGGCAACGGG 60 53286 D2 R-ORF4-1/F CCTAAGGGAGCACTATGTGTTT 60 56427 R-ORF4-2r CTTTCAAGTGGCAACGGG 60 5318 D4 R-ORF4-1/F CCTAAGGCAGCACATTGTGTAAG 60 56417 R-ORF4-2r TGTCGTAAGGGAGACGGGGGGGGGGGGGGGGGGGGGGGG	C4	R-ORF31-1f	TCTCCATATACTCCACTAAAACGGTA	59	45594	R-ORF31-2r	CATCCATTGCGCTCTGATAG	59	45678
Ch RORF3-11 CIGCAACCTACTICUCIAC 59 4757 FORF3-2r ACORF3-2r ACCAVALCCTACACATAA 59 48131 CR RORF3-11 ACGICTAGTGTTCGGGCTCA 61 49834 R-ORF3-2r GAGGCACATGTCACCACAC 60 49919 C10 R-ORF3-11 ACGICTACGAGAACTGAAGCA 59 5200 R-ORF3-2r GAGGTTCCACACATGCTAA 60 5146 C11 R-ORF3-11 AGACACCACACGTGTCAAGGA 59 5200 R-ORF3-2r GAGGTTCACACACAGGGCCAAAGACACTGATCTGTTT 60 53142 D1 R-ORF3-11 CCAGGCCATAGAGAAGAACACAC 60 5306 R-ORF3-2r CACCATTGGTCACGCACATAGGTGT 60 53142 D2 R-ORF4-11 CCCAGCAGGCACATTGTGTTT 60 5522 R-ORF4-2r CCTTTGCTGGGCACATTGTAGTG 60 5542 D4 R-ORF4-11 CCCAGCACACCACCTAA 61 57418 R-ORF4-2r CCTTTGCTGGGCATAGTGTAACCCACAGAG 61 6144 R-ORF4-2r CCTTTGCTGGGCATAGTTGTAGGAGAAC 60 5142 R-ORF4-2r CGCTTGGTATTTGGGCGCACTTAACCCACAGAGG 61	C5	R-ORF32-1f	AGCGGGCAGTCTACTGTTTG	60	46927	R-ORF32-2r	AGGTCGTAGCGGCCTTTAAT	60	47002
C RORE34-II ODCONTRACTION 59 48.03 RORE32-II AGGECATGGTCAGGGCTCA 61 4981 CS RORE34-II AGGICTAGGGAGACTGGAGAAT 5024 R-ORE32-II GAGGECATAATACCA 60 3032 CI RORE34-II AGGACAGAAGAAGCATCTC 60 51546 R-ORE32-II GAGGTCCAATTTCCTTACGGAGACA 51246 CI R-ORE34-II AAACACCAACCGGTGAAGCA 60 5320 R-ORE42-II TITTATTGTTTTATTGTTTTAGGTCCAAGGAG 60 5321 CI R-ORE34-II GGAGGCACTTGATAAAACAAC 60 5320 R-ORE42-I TITTCTAGGGCAACTATGTGTG 60 53286 DR R-ORE41-II GCAGCACCTGATAAAACAAC 60 5320 R-ORE42-I TITTCTAGGGCAACTGTG 60 5495 DR R-ORE41-II GGACCATGTGTAAAAAAACAAC 60 5522 R-ORE42-II TITTCTAGGGCAACTGTGT 60 55495 DR R-ORE41-II GGACGTCGTTGGCACACACTGTAAACCAACTGTGT 60 56495 57333 57333 5748 R-ORE42-I GAGGTTGTGTGGAGGAGGAGGAG	C6 C7	R-ORF33-1f	GIGCAACCCACIICGCIAC	59	47957	R-ORF33-2r	AGCAGATGCTTTTTAAGTTCTGG	59 61	48031
COMPARIANT AGCTGTACCGAGAACTGGAGAT 59 50247 R.ORF35-2r GACGCTTCCAACTTTCCACCTA 60 50352 C10 R.ORF37-1r AACACCAACTGGTGAAGCA 59 50247 R.ORF37-2r GACGCTTCCAACTTTCCGACATGACTG 60 51362 C11 R-ORF37-1r AACACCAACTGGTGAAGCA 59 52900 R-ORF37-2r GCGTTTGCACAACGAGGAG 60 53122 C12 R-ORF39-1r TCTCAAGGCCATAAGGAGAAGAAGC 60 53126 R-ORF49-2r CCTCGTAATGGGCACACTTATGTGTT 60 55128 D1 R-ORF40-1r TCTTCAGGGCACACTATGTGTT 60 56127 R-ORF41-2r CCTTTCAAACTCCTAATGTGGCAGCACCA 60 57333 D3 R-ORF41 CCTAGGTATGTGGACACCA 61 57418 R-ORF43-2r GCCTTGGAGTATGTGGACGCA 60 61317 D4 R-ORF45-1r GGGCAGGAAGCAACCCCCTTAAA 60 61241 R-ORF45-2r GGCCTTGGGGATGTGTGT 60 61300 D7 R-ORF45-1r GGCAGGAGCAGCAACCACCCCCTAAA 60 62141 R-ORF45-2r GGCCTTGCAGAGCACCACCACCAAGGA 61 <td>C8</td> <td>R-ORF34-11</td> <td>ACGTCTAGTGTTCGGGCTCA</td> <td>61</td> <td>40139</td> <td>R-ORF34-2r</td> <td>GAGGCCATGTTCCCTTATCA</td> <td>60</td> <td>40002</td>	C8	R-ORF34-11	ACGTCTAGTGTTCGGGCTCA	61	40139	R-ORF34-2r	GAGGCCATGTTCCCTTATCA	60	40002
C10 R-ORF3-1f GCGTTAGAAAAGCGCATCCTC 60 51468 R-ORF3-2r GCGTTTCGCGAATGACAAA 60 51362 C11 R-ORF3-1f AAGACACCAACTGGTGTGTGTGTA 60 53066 R-ORF3-2r GCGTTGCAACACAGAGA 60 53126 D1 R-ORF3-1f TCCTCAAGGACCCTAAAAACAAC 60 53360 R-ORF3-2r CCTCGTAATGGCTACATTGTGGACAGC 53280 D2 R-ORF4-1f TCCTCAAGGAGGCACTATGTGTT 60 56422 R-ORF4-2r TTTCCTAAGGCAGCACTTTGGGGGGGG 60 5542 D4 R-ORF4-1f TGTGCAAGGCACACATTTT 60 5642 R-ORF4-2r CATTTGTGGGGCAGGCGGGGGGGGGGGGGGGGGGGGGGG	C9	R-ORF35-1f	AGCTGTACGGAGAACTGGAGAT	59	50247	R-ORF35-2r	GACGCTCCAATTTCCACCTA	60	50332
C11 R-ORF3-1/I AAACACCAACTGGTGAAGCA 59 5200 R-ORF32-g GCGTTTGCACACCACAGAGAGG 61 52982 D1 R-ORF33-1F TCCTCAAGGCCATAGATGACTTGTTTTTTAG 60 53160 R-ORF32-2F TCCACACACATTGATTGGCGCACACATTTGG 60 53287 R-ORF41-12 CCTCGTAATGGCTCACGTCA 60 55897 D2 R-ORF41-11 CCTACAGGAGGCACATTGTGTGTT 60 56422 R-ORF41-27 CTTTTCTCACGCACACTTGTTGGGC 60 56495 D4 R-ORF41-11 CCTCACAGGAGCACCACCTTTAT 60 56417 R-ORF41-27 CTTTGCAGTAGTGTGTGT 60 56495 D4 R-ORF41-11 GGCACGTAATCTAACCCAGAG 61 61471 R-ORF42-27 GCTTGGAGCAGGATGG 61 61400 D8 R-ORF41-11 GGCGTGTCTCACACGAGGG 60 61474 R-ORF42-27 CGCCTGGAGCAGGATGCCA 61 62303 D10 R-ORF41-11 ACGGGGTATTCCAGAGGGT 60 6317 R-ORF82-27 CGCACGAGAGACACACCACAGAGA 63920 D11 R-ORF83-11 ACGGCCCTCTTAACCAGGACACCCAGAGA 60	C10	R-ORF36-1f	GCGTTAGAAAACGCATCCTC	60	51468	R-ORF36-2r	GCAGTTTCCGCAATGACTAAA	60	51546
C12 R-ORF38-If AGGAGAAGAAGCATTGTCTTGTTA 60 5306 R-ORF39-2r CCTCGTAATCGCTACAGGTCACGT 60 53286 D2 R-ORF39-II CCTCAAGGCGCACACTATGTGGAAGAGA 60 5527 R-ORF49-2r CTTTCTAAGGCACCACGTGGT 60 55287 D3 R-ORF41-II CCTACAGGAGGCACAATTTTGT 60 56422 R-ORF41-2r CCTTTCTAAACTCTCTCTAATGTGGGACAGCA 61 57418 R-ORF42-2r GCTCAGTATTGGGGACAGCA 60 57335 D6 R-ORF41-II CCAAGCGACACCCCTTAAA 60 61241 R-ORF42-2r GGCCACGGATAGTTGGGTCATT 60 61318 D7 R-ORF4-1I GGGCACCTTCTCAAGCCCAGGAT 60 6317 R-ORF4-2r GGCCACGGATAGTTGGGTCATT 60 61318 D8 R-ORF4-1I TGCCCAGGCTTCAGAGGGT 60 6317 R-ORF4-2r GATCAGGCACAGCACCC 63989 D10 R-ORF4-1I AGCGCGACCTCAGAGCACT 61 6466 R-ORF4-2r GATCCAGGCAGCTTCC 60 63989 D11 R-ORF4-1I AGACACGGACACCCCAGACACAGAG 67767 R-	C11	R-ORF37-1f	AAACACCAACTGGTGAAGCA	59	52900	R-ORF37-2r	GCGTTTGCACACAGAGAGG	61	52982
D1 R-ORF-39-II ICCICAAGGCCCITAAAAGGAA 60 53520 R-ORF40-27 ICICGIAAGCCCICTCIT 60 55280 D3 R-ORF40-IF CCGGCCATGATATAAACAAC 60 55827 R-ORF40-27 ICITCCTACGGCAACTATTGTGT 60 56495 D4 R-ORF41-IF CCACACGCACCACTTTTG 60 56617 R-ORF42-27 GCTTCAGTATTGTGCCCAGC 60 57333 D6 R-ORF45-IF GGGGCTACCTTCAAACCAGAG 60 61241 R-ORF42-27 GCTCAGGATGTGTCAGGAGT 60 61318 D7 R-ORF45-IF GGGGCTACTTCAAACCAGAG 60 61241 R-ORF42-27 CGTCTGAGGAGAGATG 61 61400 D8 R-ORF45-IF GGGCACCTTCAGAGGGT 60 61241 R-ORF42-27 CGTCTCAGAGCAATTCCA 59 63241 D10 R-ORF45-IF GGGCACCTTAACCCGAGCATTAACC 60 64066 R-ORF42-27 CGTCTCGAGCAGATTCCA 59 63320 D11 R-ORF45-IF AAACGACCGACCACCAGAGA 60 74067 R-ORF52-27 CGTCCTCAAGACAGATTTCC 60 63389	C12	R-ORF38-1f	AGGAGAAGAAAGCATTGTCTTGTTA	60	53066	R-ORF38-2r	TGACAATTTATTGTTTTTATGTTTGGA	60	53142
D2 RORH941-II CCTACAGGAGGCACTATGTGTTT 60 56422 RORH941-II CCTACAGGAGGCACATTTTT 60 56422 RORH941-II CCTACAGGAGGCACATTTT 60 56422 D4 R-ORF44-II CCACCGTGATGTGGACAGCA 61 7418 R-ORF42-27 AGTITGTCGGGGAGCTGT 60 56423 D5 R-ORF44-II GGACGCGTACTTCAACCCAGAG 60 61214 R-ORF42-27 GGCTCGTGTATGGTGGGGTGT 60 61338 D7 R-ORF44-II TCTGCCAAGCTTCAACCCAGAG 69 61474 R-ORF42-27 CGCCACGGAACAGTGTGTGT 60 61406 D8 R-ORF46-1I TTCTGCCAAGCTTGGACTCA 60 63317 R-ORF42-27 CGCCACGACAAGTATCCA 59 63240 D10 R-ORF48-1I AGGCGTATCTCAAGCACATCA 60 64066 R-ORF42-27 CGTCCAAGGCACACTTCA 60 63392 D11 R-ORF50-1I AAACGACCACCAGACATTCA 60 64066 R-ORF52-27 CGTCCATCTCAAGGGCAGCATTCT 60 70389 D2 R-ORF51-1I CAAAGGAAAGCGAGGTCTCCC 60 7	DI D2	R-ORF39-If		60 60	55827	R-ORF39-2r		60 50	55800
D4 R-ORF42-If TTTATTGCGGCACAATTTT 60 56617 R-ORF42-2r AGTTTGTCTGGGGATGCTGT 60 55542 D5 R-ORF43-IF CCAGCTGTATGTGGACAGCA 61 57148 R-ORF43-2r GGTTTGTCTGGGGATGCTGT 60 57333 D6 R-ORF44-IF GGGGCTACTTCTAACCCCCTTAAA 60 61241 R-ORF44-2r GGCGTGGGTGTGTTGTGGGACGAGATG 60 61318 D7 R-ORF45-IF GGGGCTACTTCCACAGGT 60 62714 R-ORF45-2r GGCCACGAGATGTGATCA 61 62333 D9 R-ORF47-IF AGCGGTGTGCCGGACTTAACC 60 63317 R-ORF47-2r TGTCTCGAGCACAGTTCC 63 63240 D10 R-ORF49-1F AGCACCGAGCACTCA 61 64066 R-ORF49-2r CGATCAGGAGCAGCATTCC 61 65392 D11 R-ORF50-1F AAAACGACGACCCCCGAGA 60 70467 R-ORF50-2r TCCTCATGTGGTGTTTTCG 60 70389 E1 R-ORF53-1F GTAGCACCCAAGAGGT 60 7108 R-ORF53-2r TGGGTATCATAAGAGAGAGGAGGA 77214	D2 D3	R-ORF41-1f	CCTACAGGAGGCACTATGTGTTT	60	56422	R-ORF41-2r	CCTTTTCAAACTTCTCTAATTGTCG	60	56495
D5 R-ORF43-If CCACCTGATATGGGCAGCA 61 57418 R-ORF43-2r GCTTCAGTATTGGTCCATCG 60 57333 D6 R-ORF44-IF GGGCACCCCTTAAA 60 61214 R-ORF45-2r GGCCACGGATAGTTGTGTTTGGTTGGT 60 61318 D7 R-ORF45-1f TGTGCCAAGCTTCAACCCAGAG 59 61474 R-ORF46-2r CGCCTGAGGACGACGAC 59 63240 D10 R-ORF45-1f AGGGGGTATCCTGGACTCA 60 63317 R-ORF48-2r CTAAGCTCCATCCAAGCACACCACA 60 633240 D11 R-ORF49-1f AGGAGGGACCCCCGACCACA 60 64066 R-ORF49-2r CGTCAAGCAGAGCGGACGACTGCT 61 65392 D11 R-ORF50-1f AAAACGACGAACCACCACAGAG 60 70467 R-ORF50-2r TCCTCATTGTGCGAGACGACGTGT 61 70393 E3 R-ORF53-1f AGGACACACCCTCCACAGAG 60 71980 R-ORF54-2r TGGGTGCTGAGACA 61 72045 E4 R-ORF55-1f TGCACACACACCACAGAGGTCCTC 60 72026 R-ORF52-2r TGGGTACCACAAAAGAAAGGAGAGGA 60	D4	R-ORF42-1f	TTTTATTGCGGCACAATTTTT	60	56617	R-ORF42-2r	AGTTTGTCTGGGGGATGCTGT	60	56542
D6 R-ORF44-1f GGACAGCAACCCCCTTAAA 60 61241 R-ORF44-2r GAGGGTTCTGTTTGGGTTGT 60 61318 D7 R-ORF45-1f GGGCTACTTCTAACCCAGAG 59 61474 R-ORF46-2r GGCCAGGATGTGTCATACCA 61 62533 D9 R-ORF46-1f TTCTGCCAAGGCTTAACC 60 63317 R-ORF47-2r CTACGCTCCCAAGCAACATA 59 63240 D10 R-ORF49-1f AGCACGGGACTCA 61 64066 R-ORF49-2r CTACGCTCCCAAGCACACACA 60 63989 D11 R-ORF49-1f AAACGACGACACACAGAGAG 60 70467 R-ORF50-2r TCTCATATTGCCGAGCTGTTT 60 63989 D12 R-ORF53-1f AAACGACCACACAGAGAG 60 70467 R-ORF52-2r CGCCACCAGAGAGTGTG 61 70389 E2 R-ORF54-1f GACGACCCCTCCAGATA 60 7180 R-ORF52-2r TTGGGTTGGTTGGTTGGTGT 61 70389 E3 R-ORF54-1f GACGACCCCTCCAGATA 60 7180 R-ORF52-2r TTGGGTACCAGACAGATGGT 61 70389 <t< td=""><td>D5</td><td>R-ORF43-1f</td><td>CCAGCTGTATGTGGACAGCA</td><td>61</td><td>57418</td><td>R-ORF43-2r</td><td>GCTTCAGTATTTGGTCCATCG</td><td>60</td><td>57333</td></t<>	D5	R-ORF43-1f	CCAGCTGTATGTGGACAGCA	61	57418	R-ORF43-2r	GCTTCAGTATTTGGTCCATCG	60	57333
D/ R-ORF45-II GGGGG1ACT1CIAACCCAGAG 59 614/4 R-ORF45-2r GGCCACGGAIAGTIGICATIC 60 61400 D8 R-ORF46-II TCTGCCAAGGGTTACACC 60 63317 R-ORF47-2r TGTCTGAGGACGAGAATTCCA 59 63240 D10 R-ORF49-II AGCACCTGATCCGAGCACTA 60 6406 R-ORF49-2r GATTCAGGAGGGCATGTTA 60 63392 D11 R-ORF50-II AAACGACGACGACACAGCAT 59 67876 R-ORF52-2r GCTCATGGGTGTGTTG 61 67956 E1 R-ORF53-II AAAGGACACACCAGAGA 60 70467 R-ORF52-2r AGTCATGGCGTGTTTAGGGT 61 70939 E2 R-ORF53-II GAGCACACCCTCCAGATA 60 71908 R-ORF53-2r CGCACACACACAGAGTGT 61 72085 E4 R-ORF57-II GTCAACAAAGAAAGCGAGGT 60 75207 R-ORF57-2r CGGTACCATAAAATGAAAAGGAAAG 60 75286 E6 R-ORF8-3I TTGGGGGCACACACTACAA 60 75277 R-ORF9-4r AGAGGGGACAAA 60 7224 <	D6	R-ORF44-1f	GGACAGCAACCCCCTTAAA	60	61241	R-ORF44-2r	GAGGGTTGTGTGTTGGGTTGT	60	61318
Dis R-ORT-97-11 AGCTGGCCACCCTACAGGACTTACCC 60 62/14 R-ORT-97-27 CGCCCTGGACAGTACCG 60 63240 D10 R-ORF47-11 AGCGGGGACTTACCGGACTTAA 60 64066 R-ORF47-27 CTACGAGGGGGGTTTACCG 60 63982 D11 R-ORF49-11 AGCACCGACTCAAGGACACACA 61 65468 R-ORF49-27 CATCCAGGGGGGGTTTAC 60 65392 D12 R-ORF50-11 AAAGGACGACGACACACAGGA 60 70467 R-ORF50-27 TCCTCATGGGGCGGTTTACT 60 70389 E3 R-ORF51-11 AGGCGCCTCTTTATTAGGAGGAGGA 60 70467 R-ORF52-27 TCGGACACACAGAGGTG 61 70399 E3 R-ORF51-11 GGCACACACCGAGAGGT 60 71980 R-ORF54-27 TCGGTTGGACACAAAGGGGT 61 7205 E4 R-ORF55-11 GTAGAACACGAAGAGGGTCCTC 60 72206 R-ORF57-27 CCTGTGTGTTTCGGCACAAT 60 78280 E5 R-ORF87-11 ATTAAATCAAAAGGGGGCGTACCTACA 60 7727 R-ORF89-17 TATGTCGAGAGACACACAATT 60	D/	R-ORF45-If	GGGGCTACITCIAACCCAGAG	59 60	61474 62714	R-ORF45-2r	GGCCACGGAGAGAGGAGATG	60 61	61400
Dip RORF#8-1f ACGAGGGTATCCTGGACTCA 60 60466 R-ORF48-2r CTAAGCTCCTCAAAGACAATTTCC 60 65398 D11 R-ORF48-1f AGCACCTGATCCTGGACTCA 61 65468 R-ORF49-2r GATTCAGGAGGGGGTGTTTA 60 65392 D11 R-ORF50-1f AAACGACGACGACATGCTA 59 67876 R-ORF50-2r TCCTCATTGTCCGGGTTGCT 61 67956 E1 R-ORF51 AATGGACGAGACACCCACAGAG 60 70467 R-ORF52-2r AGTCCGCGTGTTATTTCTG 60 70389 E2 R-ORF54-1f GGCACCACCCGAAGAGGTCCTC 60 71980 R-ORF54-2r TTGGGTTGGGCAGGAG 60 72296 R-ORF55-2r CTGTATTATTTTGTCGGCAGA 59 7214 E5 R-ORF57-1f CTAAAATCAATAGTGGTGAGAG 60 75207 R-ORF57-2r CGTGTGTTTCGCGAGAT 60 78280 E7 R-ORF8-21 AGAGGGGACACACCGAATTGC 60 78772 R-ORF8-3r ACCATTCACCGGAGACA 60 78772 E8 R-ORF8-3r TATGGGTGGGACAA 60 8133 <t< td=""><td>D0 D9</td><td>R-ORF47-1f</td><td>AGCTGTGCCGGACTTAACC</td><td>60</td><td>63317</td><td>R-ORF47-2r</td><td>TGTCTCGAGCCAAGTATCCA</td><td>59</td><td>63240</td></t<>	D0 D9	R-ORF47-1f	AGCTGTGCCGGACTTAACC	60	63317	R-ORF47-2r	TGTCTCGAGCCAAGTATCCA	59	63240
D11 R-ORF49-1f AGCACCTGATCCGAGCACTA 61 65468 R-ORF49-2r GATTCAGGAGGGCGTGTTTA 60 65392 D12 R-ORF50-1f AAAACGACGACGACACAGGC 59 67876 R-ORF50-2r TCCTCATTGTCCCGAGTGCT 61 67936 E1 R-ORF51-1f AATGGACGACCACCAGAG 60 70467 R-ORF52-2r AGTCCGCGCTGTTTTATTTCG 60 70398 E2 R-ORF54-1f GGCAGCACCCTCCAGATA 60 71980 R-ORF53-2r CGGGACCAGAAAGAAAGCAGGT 61 70939 E3 R-ORF55-1f GTCACACAAAGAAAGCAGGT 60 71296 R-ORF56-2r TGGGTACCATAAAAGAAAAGCAAGGT 60 75207 R-ORF56-1r TCCAAAAAGAAAGCAAGCAGGT 60 75207 R-ORF8-1r TATTTCAGCGGGAGAAA 60 75208 E6 R-ORF8-1f TCCCACAAAAGAAATGTGG 61 77348 R-ORF8-1r TATTACGCGGGGACAAA 60 78296 E7 R-ORF8-31 ATCCGTTGCAGATGTACTAC 60 80335 R-ORF8-1r TATTACGCGGGGACAAA 60 78290 E8	D10	R-ORF48-1f	ACGAGGGTATCCTGGACTCA	60	64066	R-ORF48-2r	CTAAGCTCCTCAAAGACAATTTCC	60	63989
D12 R-ORF50-If AAAGGACGACGACAGCAGGAC 59 678/6 R-ORF50-2r TCCTCATTGTCCGAGTTGCCT 61 67956 E1 R-ORF52-If AATGGACGAGACCACAGAG 60 70467 R-ORF52-2r AGTCCGCGTGTATTTCTG 60 70899 E3 R-ORF53-If GGCACCCTCCTTTTTATGACTATGA 60 71980 R-ORF54-2r TCCGGTTGGCTTGAGAGGT 61 72065 E4 R-ORF56-If GTAGACACCGAAGCAGCAGGT 60 7220 R-ORF56-2r CTGTATTATTTGTCTGGCACAGA 59 72214 E5 R-ORF56-1f CCACAAAGAAAGCAGAGCA 60 7672 R-ORF57-2r CTTGTGTTTGCTTGGCAGAAA 60 72216 E6 R-ORF8-1f ATCCATTTCAAATAGTGGTGGAGCA 60 76772 R-ORF87-1r TATTTCACCGCTGGTGGGACAA 60 7820 E7 R-ORF8-1f ATCCGTTTGCACAAGATTGCT 60 7837 R-ORF8-1r TATTCACCGCGGGACAAA 60 7820 E8 R-ORF8-3f TTGGGGGCGAACACTGTATCC 60 80335 R-ORF8-3r AACATTAGCGGCGGAAAA 60 80248<	D11	R-ORF49-1f	AGCACCTGATCCGAGCACTA	61	65468	R-ORF49-2r	GATTCAGGAGGGCGTGTTTA	60	65392
E1 R-ORF52-1f AATGGACGACACCACAGAG 60 70447 R-ORF52-2r AGTCCGCGTCGTTATTCTG 60 70389 E2 R-ORF53-1f ACGCCCTCTTTATGACTATGA 59 71018 R-ORF53-2r CGCGACACAGAGAGTG 61 70939 E3 R-ORF54-1f GCAGCACCCTCCAGATA 60 72296 R-ORF54-2r TTCGGTTTGACTTATTTGCGGACAGA 59 72214 E5 R-ORF56-1f TCCACAAAAGAAAGCGAGGTCTC 60 72296 R-ORF56-2r CGTGATATATTGCGCAGAA 60 73286 E6 R-ORF51-1f CTCAAATCAATAGTGGTGGAGCA 60 76772 R-ORF56-2r CCTTGTTTTTCACCAGATGTG 60 78286 E7 R-ORF89-1f ATCCGTTTGCATAGTGGTGGAGCA 60 76772 R-ORF89-1r TATTTCACCGCTGCTGTC 60 78280 E8 R-ORF89-3f ATGGGGGCAACACACTGTATCTC 60 78772 R-ORF89-4r ACACTAGCGCGCCAAA 60 80248 E10 R-ORF89-4f ACGGGGCAACACACTGTATCTC 60 81582 R-ORF89-4r GACATCACCACGACTC 60 81501	D12	R-ORF50-1f	AAAACGACGACGACATGCTA	59	67876	R-ORF50-2r	TCCTCATTGTCCGAGTTGCT	61	67956
E2R-ORF3-11ACGCCCTCTTITAGTATGA5971018R-ORF52-2rCGCGACCAGAAACAGAGTIG6170939E3R-ORF54-11GGCAGCACCCTCCAGATA6071980R-ORF55-2rTTGGTTTGTTTGGCACGA5972214E5R-ORF56-11TCCACAAAAGAAAGCGAGGT6072296R-ORF56-2rTGGGTACCATAAATGAAAAATGTG6075286E6R-ORF57-11CTTAAATGCAATAGTGGTGGAGCA6076772R-ORF56-2rTGGTGTGTTTCGCCAGAT6076850E7R-ORF89-11ATCCGTTTGCACAGATTTGG6177348R-ORF89-1rTATTTCACGCTGGTGGACAA6078702E8R-ORF89-2fAGAGGGGAGGCAACACTGACA6078772R-ORF89-3rAACATTAGCGGCGGACAA6080248E10R-ORF89-3fTTTGGGGGCGTACCTACA6081582R-ORF89-3rAACATTAGCGGCGGACAA6080248E11R-ORF89-5fAACTCTGGGTTTCGGGTTGT6183120R-ORF89-5rTCAAAGTGCCCACAGCTC6083040E12R-ORF89-5fAACTCTGGGTTACGCAAA5984455R-ORF89-5rTCAAAGTGACCAAGAGG6085920F2R-ORF89-5fGCGAACTCGCCAAGAATAGT598455R-ORF89-5rGCACACACGAGCACACC598493F3R-ORF89-1fGACGCGGACCCAAGAATAGT5988529R-ORF89-5rGCAACATGAGACCAGAGC6085920F2R-ORF89-1fGAACCCCGGACCCAGAGATAGT5988529R-ORF89-2rGCAGACCACAGAGCACACC608948F4R-ORF89-1fGAACCCCTGACCTGGCTTAGAT<	E1	R-ORF52-1f	AATGGACGAGACCACCAGAG	60	70467	R-ORF52-2r	AGTCCGCGTCGTTATTTCTG	60	70389
LDNORFST-IIGUAGACACCECACAGCITC607200RORFST-1ICITATATTITITGTCTGGCACGA6172014E5R-ORF55-1fTCCACAAAAGAAAGCGAGGT6075207R-ORF55-2rCTGTATTATTTTGTCTGGCACGA6075286E6R-ORFS7-1fCTTAAATTCAATAGTGGTGGAGCA6076772R-ORF57-2rCCTIGTGTTTTCCCGGACGA6076850E7R-ORFR9-1fATCCGTTTGCACAGATTGG6177348R-ORFR9-1rTATTTCACGCGGGGACAA6077270E8R-ORFR9-3fTTTGGGGGGCGACCAATC6078772R-ORFR9-2rACCATTACCGCTGCTTC6080248E10R-ORFR9-4fACGGGGCAACACTGTATCT6081582R-ORFR9-3rAACATTAGCGGCGGACAA6080248E10R-ORF89-4fACGGGGCAACACTGTATGTC6081582R-ORFR9-5rTCAAAGTGCCTACAGATTCTGTTG6081501E11R-ORF89-4fACGGGGCAACACTGTATGTCAGCAA5984455R-ORFR9-5rTCAAAGTGCTTACAGAGG6080248E12R-ORF89-7fGATTTCCAGCCGCCTATG5985998R-ORF89-7rGCCAAATTTGAATGGACAGG6085920F2R-ORF89-8fGCCAGTCACTCTTTACCTTGG6087213R-ORF89-7rGCCAAACATGGAATAGT5984455F3R-ORF89-8fGCCAGTCCCTGGACATAA6087213R-ORF89-7rGCCAAACCATGAGGAAACAC6089248F4R-ORF59-1fGAACGCCTGACCAAGAATAGT5988529R-ORF59-2rGCTCGTCGCAAGGCAAAC6089448F5R-ORF60-1fCAAACGCGT	E2 E3	R-ORF53-If R-ORF54-If	ACGCCCTCTTTTTATGACTATGA	59 60	71018	R-ORF53-2r R-ORF54-2r	TTCGGTTTGGCTTTGAGTGT	61 61	70939
E5R-ORF56-1fTCCACAAAAGAAAGCGAGGT6075207R-ORF56-2rTGGGTACCATAAATGAAATGAAAATGTG6075286E6R-ORF57-1fCTTAAATTCAATAGTGGTGGAGCA6076772R-ORF57-2rCCTTGTTGTTTTCGCCAGAT6076850E7R-ORF89-1fATCCGTTTGCACAGATTGG6177348R-ORF89-1rTATTTCACGCGGGGGACAA6077270E8R-ORF89-2fAGAGGGGCGACCAATC6078772R-ORFR9-2rATCATCTTCACCGCTGGTC6078690E9R-ORF89-3fTTTGGGGGCGAACACTGTATCTC6080138R-ORFR9-3rAACATTAGCGGCGGACAA6080248E10R-ORF89-4fACGGGGGCAACACTGTATCTC6081582R-ORF89-4rGTGTCCCTGCACCAGGCC6081501E11R-ORF89-5fAACTCTGGGTTGTGAGTGTCAGCAA5984455R-ORF89-5rTCAAAGTGCCTATAGATTTCTGTTTG6083040E12R-ORF89-8fCGCAGTCACTCTTTACCTTGG6087213R-ORF89-8rGTCACCATGAGTCAATCATC5985920F0R-ORF89-1fGAACGCCTGACCTGGACTGACATTA6089723R-ORF89-2rTAAAGGGCACCAAGCTCAAC6089648F4R-ORF59-1fGAACGCCTGACCTGGACTTA6089723R-ORF69-2rCAGATCATTATGAAGGGATGTGA6090856F6R-ORF61-1fATGCCCTGGACTGGACTTA6091734R-ORF62-2rCTGCGCAAGAGCTCACC6091794F5R-ORF61-1fGACGCCTGACTGGACTAGAT6091758R-ORF62-2rCTGCGCAATAGCCTGGACTAG6091794F6R-	E4	R-ORF55-1f	GTAGACACCGAAGCGTCCTC	60	72296	R-ORF55-2r	CTGTATTATTTTGTCTGGCACGA	59	72214
E6R-ORF57-1fCTTAAATTCAATAGTGGTGGAGCA6076772R-ORF87-2rCCTTGTTGTTTTCGCCAGAT6076850E7R-ORFR9-1fATCCGTTTGCACAGATTTGG6177348R-ORFR9-1rTATTCACCGCTGGGAGAAA6077270E8R-ORFR9-2fAGAGGGGACGACCAATC6078772R-ORFR9-2rATCATCTTCACCGCTCGTTC6078690E9R-ORFR9-4fACGGGGCAACCTGATCAA6080335R-ORFR9-3rAACATTAGCGGCGGACAA608024E10R-ORFR9-4fACGGGGCAACACTGTATCTC6081582R-ORFR9-4rGTGTCCCTGCACCAGCTC6081501E11R-ORF89-5fAACTCTGGGTTTCGGGTTGT6183120R-ORFR9-5rTCAAAGTGCCTATAGATTTCTGTTTG6083040E12R-ORF89-6fATGTGTCAGATGATGTCAGCAA5984455R-ORF89-6rAGATGTGCTCACTAGATGATGTCAGCAA6083204F1R-ORF89-7fGATTTCCAGCCGCCTATG598598R-ORF89-7rGCCAAATTGAATGGACAGG6085920F2R-ORF89-81GCAGCACACTCGCCAAGAATAGT5984529R-ORF58-2rTAAAGGGCACCAAGCCAAGC6088448F4R-ORF59-1fGAACGCCTGACCTGGACTTA6089723R-ORF59-2rGCTCGTCCGCAGTGATGTGA6090856F6R-ORF61-1fCTTCCGGTCACAACCATCT609173R-ORF60-2rCAGATCATTATGAAGGAGTGGA6090856F6R-ORF61-1fGTGCCCGAGATTAGAAT6091878R-ORF61-2rACTACCTGGGATTAGTGG6091794F8R-ORF61-1fAG	E5	R-ORF56-1f	TCCACAAAAGAAAGCGAGGT	60	75207	R-ORF56-2r	TGGGTACCATAAATGAAAAATGTG	60	75286
E7R-ORFR9-1fATCCGTTTGCACAGATTTGG6177348R-ORFR9-1rTATTTCACGCTGGTGGACAA6077270E8R-ORFR9-2fAGAGGGGACGAGCCAATC6078772R-ORFR9-2rATCATCTTCACCGCTGGTTC6078690E9R-ORFR9-3fTTTGGGGGGGACAACACTGTACA6080335R-ORFR9-3rAACATTAGCGGGCGACAAA6080248E10R-ORFR9-4fACGGGGACAACACTGTATCTC6081582R-ORFR9-4rGTGTCCTGCACACAGGTTC6081501E11R-ORFR9-4fACGGGGCAACACTGTAGCAA5984455R-ORFR9-5rTCAAAGTGCCTATAGATTTCTGTTTG6083040E12R-ORFR9-7fGATTTCCAGCCGCCTATG5985998R-ORFR9-6rAGATGTGCTTCCCGTTGTTC6084373F1R-ORFR9-7fGATTTCCAGCCGCCAAGAATAGT5985998R-ORFR9-7rGCCAAATTTGAATGGAACAGG6085920F2R-ORF89-8fCGCAGTCACTCTTTACCTTGG6087213R-ORF59-2rGTCGCCAAGGTCAATTCATCC5987134F3R-ORF50-1fGAACGCCTGACCTGGACTTA6089723R-ORF50-2rTAAAGGGCACCAAGCTCAAC6089648F4R-ORF61-1fATGCCCTGCAGTGCTTAGAT6091878R-ORF61-2rACATACCTTGGTCAGCAC6091794F7R-ORF61-1fAGCCCTGAGTGCACAACCATCT6091878R-ORF61-2rCTGCAAACATGCGATTAGGG6091794F7R-ORF63-1fTGTCCCTAGATTGGAGCACAA6097852R-ORF61-2rCTGCAAACATGCGATTAGGG6091794F7R-ORF65-1f <td>E6</td> <td>R-ORF57-1f</td> <td>CTTAAATTCAATAGTGGTGGAGCA</td> <td>60</td> <td>76772</td> <td>R-ORF57-2r</td> <td>CCTTGTTGTTTTCGCCAGAT</td> <td>60</td> <td>76850</td>	E6	R-ORF57-1f	CTTAAATTCAATAGTGGTGGAGCA	60	76772	R-ORF57-2r	CCTTGTTGTTTTCGCCAGAT	60	76850
E8R-ORFR9-21AGAGGGGGACGAGCCAATC6078772R-ORFR9-2rATCATCTCTCACCGCTCGTTC6078690E9R-ORFR9-3fTTTGGGGGGCGACCAACAC6080335R-ORFR9-3rAACATTAGCGGGCGACAA6080248E10R-ORFR9-4fACGGGGCAACACTGTATCTC6081582R-ORFR9-4rGTGTCCTGCACACAGGTC6081501E11R-ORFR9-4fAACTTCGGGTTCGGGTTGT6181120R-ORFR9-5rTCAAAGTGCCTATAGATTTCTGTTTG6083040E12R-ORFR9-6fATGTGTCAGAATGATGTCAGCAA5984455R-ORFR9-6rAGATGTGCTTCCCGTTGTTC6084373F1R-ORFR9-7fGATTTCCAGCCGCCTATG5985998R-ORFR9-7rGCCAAATTGAATGGACAGG6085920F2R-ORF89-8tCGCAGTCACTCTTTACCTTGG6087213R-ORF89-8rGTCACCATGAGTCAATTCATCC5987134F3R-ORF59-1fGAACGCCTGACCTGGACTTA6089723R-ORF50-2rGCTGGTCCGCAAGGTGAAC6089648F4R-ORF61-1fAACGCCTGCAGTGCTTAGAT6091878R-ORF61-2rCAGATCACGAGTGTAG6090856F6R-ORF61-1fATGCCCTGCAGTGCTTAGAT6091878R-ORF61-2rCTGCAAACATGCGATTAGGGC6091794F7R-ORF63-1fTGTCCCTAGATTGGAGCACA6097852R-ORF61-2rCTTCCAAGACCCGGATT6091794F7R-ORF65-1fACGGGCCCCAATGGAGCAAA6097852R-ORF61-2rCTGCCAAACATGCGATTAGGG6010575F9R-ORF65-1fAGGGCCC	E7	R-ORFR9-1f	ATCCGTTTGCACAGATTTGG	61	77348	R-ORFR9-1r	TATTTCACGCTGGTGGACAA	60	77270
E9R-ORFR9-31THEOGOGOGOGIACCTACA6080248E10R-ORFR9-4fACGGGGCAACACTGTATCTC6081582R-ORFR9-4rGTGTCCTGCACCACGTC6081501E11R-ORFR9-4fACGGGGCAACACTGTATCGGGTTGT6183120R-ORFR9-5rTCAAAGTGCCTATAGATTTCTGTTTG6083040E12R-ORFR9-6fATGTGTCAGAATGATGTCAGCAA5984455R-ORFR9-6rAGATGTGCTTCCGGTTGTC6084373F1R-ORFR9-7fGATTTCCAGCCGCCTATG5985998R-ORFR9-7rGCCAAATTGAATGGACAGG6085920F2R-ORF89-8tCGCAGTCACTCTTTACCTTGG6087213R-ORF89-8rGTCACCATGAGTCAATTCATCC5987134F3R-ORF59-1fGAACGCCTGACCTGGACTTA6089723R-ORF59-2rGCTCGTCGCAAGTGATTT6089648F4R-ORF61-1fCAAACGCGTGTCCCTTGT6190933R-ORF60-2rCAGATCATATAGAACGGATGTGA6090856F6R-ORF61-1fATGCCCTGCAGTGCTTAGAT6091878R-ORF61-2rACAAACACCGGATCTAGTGC6091794F7R-ORF62-1fCTTCCGGTTCACAACCATCT6094241R-ORF62-2rCTTCCAAGACCCGGATTAGGC5997935F9R-ORF64-1fAAGAAATCACACGAGCCAAGA60105490R-ORF63-2rCGCAAACATGCGATCAGGG60105570F10R-ORF65-1fGGGCCCCATTCATTGGTAG60105579R-ORF66-2rATAAAACAGCTCGGCAACA60105570F11R-ORF66-1fACGGGAACTCCCTGGTAGA60105590R-ORF66-2r<	E8	R-ORFR9-2f	AGAGGGGACGAGCCAATC	60	78772	R-ORFR9-2r	ATCATCITCACCGCICGTTC	60	78690
$ \begin{array}{cccccccccccccccccccccccccccccccccccc$	E9 E10	R-ORFR9-31		60	81582	R-ORFR9-31	GTGTCCCTGCACCAGCTC	60	81501
E12R-ORFR9-6fATGTGTCAGAATGATGTCAGCAA5984455R-ORFR9-6rAGATGTGCTTCCCGTTGTTC6084373F1R-ORFR9-7fGATTTCCAGCCGCCTATG5985998R-ORFR9-7rGCCAAATTTGAATGGACAGG6085920F2R-ORFR9-8tCGCAGTCACTCTTTACCTTGG6087213R-ORFR9-8rGTCACCATGAGTCAATTCATCC5987134F3R-ORF58-1fGCGAACTCGCCAAGAATAGT5988529R-ORF58-2rTAAAGGGCACCAAGCTCAAC6088448F4R-ORF59-1fGAACGCCTGACCTGGACTTA6089723R-ORF59-2rGCTCGTCGCAGTGATTT6089648F5R-ORF60-1fCAAACGCGTGTCCCTTGT6190933R-ORF60-2rCAGATCATTATGAACGATGTGA6090856F6R-ORF61-1fATGCCCTGCAGTGCTTAGAT6091878R-ORF61-2rACATACCTTGGTCCGCTCAC6091794F7R-ORF62-1fCTTCCGGTTCACAACCATCT6094241R-ORF62-2rCTTCCAAGACCCGGATT6094160F8R-ORF63-1fTGTCCGTAGATTGGAGCACA6097852R-ORF63-2rGCAAACATGCGATCTAGTGC5997935F9R-ORF64-1fAAGAAATCACACGAGCCAAGA60105490R-ORF63-2rCGCAAGATGCGAAAGTGG60105570F10R-ORF65-1fGGCCCCATTCATTGATAG60105579R-ORF66-2rATAAAACAGCTCGGCCAACA60105575F11R-ORF65-1fGACCGGTTTTGCCCTGGATAG60105579R-ORF66-2rATAAACAGCTCGGCCAACA60105758F12R-ORF67-1fGA	E10	R-ORFR9-5f	AACTCTGGGTTTCGGGTTGT	61	83120	R-ORFR9-5r	TCAAAGTGCCTATAGATTTCTGTTTG	60	83040
F1R-ORFR9-7fGATTTCCAGCCGCCTATG5985998R-ORFR9-7rGCCAAATTTGAATGGACAGG6085920F2R-ORF89-8fCGCAGTCACTCTTTACCTTGG6087213R-ORFR9-8rGTCACCATGAGTCAATTCATCC5987134F3R-ORF58-1fGCGAACTCGCCAAGAATAGT5988529R-ORF58-2rTAAAGGGCACCAAGCTCAAC6088448F4R-ORF59-1fGAACGCCTGACCTGGACTTA6089723R-ORF59-2rGCTCGTCGCAGTGATTT6089648F5R-ORF61-1fATGCCCTGCAGTGCTCTGT6190933R-ORF60-2rCAGATCATTATGAACGATGTGA6090856F6R-ORF61-1fATGCCCTGCAGTGCTTAGAT6091878R-ORF61-2rACATACCTTGGTCCGCTCAC6091794F7R-ORF62-1fCTTCCGGTTCACAACCATCT6094241R-ORF62-2rCTTCCAAGACCCGGATT6094160F8R-ORF63-1fTGTCCGTAGATTGGAGCACA6097852R-ORF63-2rGCAAACATGCGATCTAGTGC5997935F9R-ORF64-1fAAGAAATCACACGAGCCAAGA60105490R-ORF63-2rCTGCAAAGATGCGAATAG60105570F10R-ORF65-1fGGCCCCATTCATTGATAG60105579R-ORF66-2rATAAAACAGCTCGGCCAACA60105670F11R-ORF67-1fGAACGGTTTTGCCCTGGATAG60105759R-ORF66-2rATAAAACAGCTCGGCCAACA60106188F12R-ORF67-1fGAACGGTTTTGCCCTGGAC60107538R-ORF67-2rCGCCAATATCCTTCAACTC60106188	E12	R-ORFR9-6f	ATGTGTCAGATGATGTCAGCAA	59	84455	R-ORFR9-6r	AGATGTGCTTCCCGTTGTTC	60	84373
F2R-ORFR9-8fCGCAGTCACTCTTTACCTTGG6087213R-ORFR9-8rGTCACCATGAGTCAATTCATCC5987134F3R-ORF58-1fGCGAACTCGCCAAGAATAGT5988529R-ORF58-2rTAAAGGGCACCAAGCTCAAC6088448F4R-ORF59-1fGAACGCCTGACCTGGCATTA6089723R-ORF59-2rGCTCGTCGCAGTGATTT6089648F5R-ORF61-1fCAAACGCGTGTCCCTTGT6190933R-ORF60-2rCAGATCATTATGAACGATGTGA6090856F6R-ORF61-1fATGCCCTGCAGTGCTTAGAT6091878R-ORF61-2rACATACCTTGGTCCGCTCAC6091794F7R-ORF62-1fCTTCCGGTTCACAACCATCT6094241R-ORF62-2rCTTCCAAGACCCGGATT6094160F8R-ORF63-1fTGTCCGTAGATTGGAGCACA6097852R-ORF63-2rGCAAACATGCGATCTAGTGC5997935F9R-ORF64-1fAAGAAATCACACGAGCCAAGA60105490R-ORF63-2rCGCAAACATGCGATAG60105570F10R-ORF65-1fGGGCCCCATTCATTGATAG60105759R-ORF66-2rATAAAACAGCTCGGCCAACA60105670F11R-ORF67-1fGAACGGTTTTGCCCTGGATAG60106267R-ORF66-2rATAAACAGCTCGGCCACAC60106188F12R-ORF67-1fGAACGGTTTTGCCCTGAC60107623R-ORF67-2rCGCCCAATATCCTTCAACTC60107538	F1	R-ORFR9-7f	GATTTCCAGCCGCCTATG	59	85998	R-ORFR9-7r	GCCAAATTTGAATGGACAGG	60	85920
F3R-ORF58-1fGCGAACICGCCAAGAATAGT5988529R-ORF58-2rTAAAGGGCACCAAGCICAAC6088448F4R-ORF59-1fGAACGCCTGACCTGGCATTA60 89723 R-ORF59-2rGCTCGTCGCAGTGATTT6089648F5R-ORF61-1fCAAACGCGTGTCCCCTTGT61 90933 R-ORF60-2rCAGATCATTATGAACGATGTGA6090856F6R-ORF61-1fATGCCCTGCAGTGCTTAGAT6091878R-ORF61-2rACATACCTTGGTCCGCTCAC6091794F7R-ORF62-1fCTTCCGGTTCACAACCATCT6094241R-ORF62-2rCTTCCAAGACCCCGGATT6094160F8R-ORF63-1fTGTCCGTAGATTGGAGCACA6097852R-ORF63-2rGCAAACATGCGATCTAGTGC5997935F9R-ORF64-1fAAGAAATCACACGAGCCAAGA60105490R-ORF63-2rCTGGCAATAGCCTCGGATAG60105570F10R-ORF65-1fGGGCCCCATTCATTGATAG60105759R-ORF66-2rCGCAAGGTGATAACGAAGTGG6010570F11R-ORF66-1fACGCGAACTCCCTGGTTAG60106267R-ORF66-2rATAAAACAGCTCGGCCAACA60106188F12R-ORF67-1fGAACGGTTTTGCCCTGAC60107623R-ORF67-2rCGCCCAATATCCTTCAACTC60107538	F2	R-ORFR9-8f	CGCAGTCACTCTTTACCTTGG	60	87213	R-ORFR9-8r	GTCACCATGAGTCAATTCATCC	59	87134
F4R-ORF9-11GAACGCCTGACCTGACTTA6059/23R-ORF9-21GCTCGTCCGTAGTGATTA6059/43F5R-ORF60-1fCAAACGCGTGTCCCTTGT6190933R-ORF60-2rCAGATCATTATGAACGGATGTGA6090856F6R-ORF61-1fATGCCCTGCAGTGCTTAGAT6091878R-ORF61-2rACATACCTTGGTCCGCTCAC6091794F7R-ORF62-1fCTTCCGGTTCACAACCATCT6094241R-ORF62-2rCTTCCAAGACCCCGGATT6094160F8R-ORF63-1fTGTCCGTAGATTGGAGCACA6097852R-ORF63-2rGCAAACATGCGATCTAGTGC5997935F9R-ORF64-1fAAGAAATCACACGAGCCAAGA60105490R-ORF63-2rCTGGCAATAGCCTCGGATAG60105570F10R-ORF65-1fGGGCCCCATTCATTGATAG60105759R-ORF66-2rCGCTAGGTGATAACGAAGTGG6010576F11R-ORF66-1fACGGGAACTCCCTGGTTAG60106267R-ORF66-2rATAAAACAGCTCGGCCACAC60106188F12R-ORF67-1fGAACGGTTTTGCCCTGAC60107623R-ORF67-2rCGCCCAATATCCTTCAACTC60107538	F3 E4	R-ORF58-1f	GCGAACTCGCCAAGAATAGT	59	88529	R-ORF58-2r	TAAAGGGCACCAAGCTCAAC	60	88448
F6R-ORF61-1fATGCCCTGCAGTGCTTAGAT6090878R-ORF61-2rACATACCTTGGTCCGCTCAC6091878F7R-ORF62-1fCTTCCGGTTCACAACCATCT6091878R-ORF62-2rCTTCCAAGACCCCGGATT6094160F8R-ORF63-1fTGTCCGTAGATTGGAGCACA6097852R-ORF63-2rGCAAACATGCGATCTAGTGC5997935F9R-ORF64-1fAAGAAATCACACGAGCCAAGA60105490R-ORF64-2rCTGGCAATAGCCTGGATAG60105570F10R-ORF65-1fGGGCCCCATTCATTGATAG60105759R-ORF65-2rCGCTAGGTGATAACGAAGTGG60105675F11R-ORF66-1fACGCGAACTCCCTGGTTAG60106267R-ORF67-2rCGCCAATAGCCGGCCAACA60106188F12R-ORF67-1fGAACGGTTTTGCCCTGAC60107623R-ORF67-2rCGCCCAATATCCTTCAACTC60107538	гч F5	R-ORF60-11	CAACGCGTGTCCCTTGT	61	07/23 90933	R-ORF60_2r	CAGATCATTATGAACGGATGTGA	60 60	09048 90856
F7R-ORF62-1fCTTCCGGTTCACAACCATCT6094241R-ORF62-2rCTTCCAAGACCCCGGATT6094160F8R-ORF63-1fTGTCCGTAGATTGGAGCACA6097852R-ORF63-2rGCAAACATGCGATCTAGTGC5997935F9R-ORF64-1fAAGAAATCACACGAGCCAAGA60105490R-ORF64-2rCTGGCAATAGCCTCGGATAG60105570F10R-ORF65-1fGGGCCCCATTCATTGATAG60105759R-ORF65-2rCGCTAGGTGATAACGAAGTGG60105570F11R-ORF66-1fACGCGAACTCCCTGGTTAG60106267R-ORF66-2rATAAACAGCTCGGCCACAC60106188F12R-ORF67-1fGAACGGTTTTGCCCTGAC60107623R-ORF67-2rCGCCAATATCCTTCAACTC60107538	F6	R-ORF61-1f	ATGCCCTGCAGTGCTTAGAT	60	91878	R-ORF61-2r	ACATACCTTGGTCCGCTCAC	60	91794
F8 R-ORF63-1f TGTCCGTAGATTGGAGCACA 60 97852 R-ORF63-2r GCAAACATGCGATCTAGTGC 59 97935 F9 R-ORF64-1f AAGAAATCACACGAGCCAAGA 60 105490 R-ORF64-2r CTGGCAATAGCCTCGGATAG 60 105570 F10 R-ORF65-1f GGGCCCCATTCATTGATAG 60 10579 R-ORF65-2r CGGCAAGGCCAAGA 60 105570 F11 R-ORF66-1f ACGCGAACTCCCTGGTTAG 60 10579 R-ORF66-2r CGCTAAGTGGATCACGAGCCAACC 60 105675 F12 R-ORF67-1f GAACGGTTTTGCCCTGAC 60 107623 R-ORF67-2r CGCCAATATCCTTCAACTC 60 107538	F7	R-ORF62-1f	CTTCCGGTTCACAACCATCT	60	94241	R-ORF62-2r	CTTCCAAGACCCCGGATT	60	94160
F9 R-ORF64-1f AAGAAATCACACGAGCCAAGA 60 105490 R-ORF64-2r CTGGCAATAGCCTCGGATAG 60 105570 F10 R-ORF65-1f GGGCCCCATTCATTGATAG 60 105759 R-ORF65-2r CGCTAGGTGATAACGAAGTGG 60 105675 F11 R-ORF66-1f ACGCGAACTCCCCTGGTTAG 60 106267 R-ORF66-2r ATAAAACAGCTCGGCCACAC 60 106188 F12 R-ORF67-1f GAACGGTTTTTGCCCTGAC 60 107623 R-ORF67-2r CGCCCAATATCCTTCAACTC 60 107538	F8	R-ORF63-1f	TGTCCGTAGATTGGAGCACA	60	97852	R-ORF63-2r	GCAAACATGCGATCTAGTGC	59	97935
F10R-ORF65-1fGGGCCCCATTCATTGATAG60105/59R-ORF65-2rCGCTAGGTGATAACGAAGTGG60105675F11R-ORF66-1fACGCGAACTCCCTGGTTAG60106267R-ORF66-2rATAAAACAGCTCGGCCACAC60106188F12R-ORF67-1fGAACGGTTTTTGCCCTGAC60107623R-ORF67-2rCGCCCAATATCCTTCAACTC60107538	F9	R-ORF64-1f	AAGAAATCACACGAGCCAAGA	60	105490	R-ORF64-2r	CTGGCAATAGCCTCGGATAG	60	105570
F12 R-ORF67-1f GAACGGTTTTTGCCCTGAC 60 107623 R-ORF67-2r CGCCCAATATCCTTCAACTC 60 107538	F10 F11	K-UKF65-If	GGGCGAACTCCCTGGTTAG	60 60	105759	K-ORF65-2r R-ORF66-2r		60 60	105675
	F12	R-ORF67-1f	GAACGGTTTTTGCCCTGAC	60	107623	R-ORF67-2r	CGCCCAATATCCTTCAACTC	60	107538

Continued on following page

Well	Name	Forward primer sequence	T _m (°C)	Position	Name	Reverse primer sequence	T _m (°C)	Position
G1	R-ORF67-5f	CACTGTGAGCACTGCATGG	60	108293	R-ORF67-2r	CGTGGCTCCCGTAAAAAC	59	108213
G2	R-ORF68-1f	GGTCTCAACTGGCCAAAATC	60	109845	R-ORF68-2r	GGGTGGGTGATTTGAATGTT	60	109922
G3	R-ORF69-1f	TAACGGTGGACTGCATCAAG	60	110700	R-ORF69-2r	CCTCGCAAATGCTGTTGAC	60	110780
G4	R-ORF71-1f	CAACCAGTCACCCACCTTTT	60	117122	R-ORF71-2r	TGCAGCAGGTCACTTAAAACC	60	117046
G5	R-ORF72-1f	CCAGGTGGTGGAGTCTGTTC	61	117798	R-ORF72-2r	GCACCGAGGCTAAACAGC	60	117716
G6	R-ORF73-1f	TCACGGTGTTCTGTCAAAGC	60	118766	R-ORF73-2r	CTATGCTGGCCTGGAAGTG	59	118681
G7	R-ORFR14-1f	CACGTGCCTGGTCACTCATA	61	121242	R-ORFR14-2r	CACCACGTAGTGGCTCGTC	60	121320
G8	R-ORF74-1f	AGCATGTATAGCGCGTTCGT	61	122616	R-ORF74-2r	AAAACACCTAAACACGGACCA	59	122691
G9	R-ORF75-1f	CCATCTCAACCAGCAGCAG	61	123101	R-ORF75-2r	GAGTTGGCAGACGGGTTG	60	123018
G10	R-R8-1f	AAACGCAACACTCGGACAC	60	68932	R-R8-2r	TGTTTTACTTTCCAGCTCCTGTT	59	69163
G11	R-R15-1f	TTGCTGCAATGTGTATGGCC	63	128129	R-R15-2r	TTGCAACATAACAAACAAGCATGT	62	128072
G12	No primer							
H1	hu-gapdhf	GAAGGTGAAGGTCGGAGTC	57	NA	hu-gapdh-f	GAAGATGGTGATGGGATTTC	57	NA
H2	hu-actinf	TCACCCACACTGTGCCCATCTACGA	72	NA	hu-actin-f	CAGCGGAACCGCTCATTGCCAATGG	77	NA
H3	mu-apoBf	TCACCAGTCATTTCTGCCTTTG	63	NA	mu-apoBr	CACGTGGGCTCCAGCATT	63	NA
H4	rh-tub P2f	CCCTTCCCACGCCTCC	63	NA	rh-tub P2r	GGCTTCCACGGCTGGTG	64	NA
H5	KSHVLAT273f	ACTGAACACACGGACAACGG	62	NA	KSHVlat335r	CAGGTTCTCCCATCGACGA	62	NA
H6	KSHVorf72f1	CATTGCCCGCCTCTATTATCA	62	NA	KSHVorf72r1	ATGACGTTGGCAGGAACCA	63	NA
H7	KSHVorf50f	CACAAAAATGGCGCAAGATGA	64	NA	KSHVorf50r	TGGTAGAGTTGGGCCTTCAGTT	62	NA
H8	KSHVorf57f	TGGACATTATGAAGGGCATCCTA	63	NA	KSHVorf57r	CGGGTTCGGACAATTGCT	62	NA
H9	KSHVK1-f	AAACAACGTGACTCAAACAAAACA	61	NA	KSHVK1r	TCTTCCGTGCACAAATCGTG	63	NA
H10	KSHVvGPCRf	TGGCCCAAACGGAGGATCCTAG	68	NA	KSHVvGPCRr	AGTTTCATTCCAGGATTCATCATC	61	NA
H11	KSHVLANA2454f	TGGCCCATCTCGCGAATA	64	NA	KSHVLANA2524R	GCCTCATACGAACTCCAGGTCT	62	NA
H12	Rh-tubP2f	CCCTTCCCACGCCTCC	63	NA	rh-tubP2r	GGCTTCCACGGCTGGTG	63	NA

TABLE 1—Continued

^a Listed are the forward and reverse primer sequences, primer name, position on the RRV genome, and predicted T_m for each well in the 96-primer RRV array. NA, not applicable.

primers and subjected to real-time QPCR using the RRV array. During real-time QPCR, the amount of product at each cycle is quantified (23) and the CT at which the product signal crossed a user-defined threshold is recorded, which was set here at five times the SD of the nontemplate control reaction.

The RRV array recorded duplicate measurements for the rhesus tubulin mRNA-specific primers for each time point. The levels of rhesus tubulin exhibited a SD of ≤ 1.7 -fold with an associated standard error of the mean (SEM) of $\pm 4\%$ for all time points (n = 5). Replicate measurements of the rhesus tubulin mRNA for any one time point on the same array also exhibited an SEM of $\pm 4\%$ in raw CT values, which was expected based on the pipetting accuracy of the robot and the instrument variation of the real-time QPCR machine (38). By contrast, viral mRNAs increased, on average, 5,379-fold (95% CI, 3,154-fold to 7,604-fold; n = 83) based upon a conservative estimate of PCR efficiency of 1.8, rather than the ideal 2.0. Hence, we concluded that for any target in the array, the biological variation was orders of magnitude above the experimental error.

All samples were highly correlated, with an average correlation coefficient $r = 0.961 \pm 0.021$ (mean \pm SD) for all possible sample correlations (Fig. 2D). Any two consecutive time points (e.g., 12 and 24 h or 24 and 48 h) were more closely correlated than unrelated time points (e.g., 12 and 48 h), indicating a progressive, gradual change in overall viral transcription. This substantiated the existing model of an ordered cascade of herpesvirus gene expression after a high MOI of fully permissive cells. It represents the first and only such demonstration for primate gammaherpesviruses, since neither KSHV nor Epstein-Barr virus currently has a highly efficient, fully permissive lytic replication system, without the use of chemicals like TPA. Kinetics of gene transcription for MHV-68, a murine gammaherpesvirus, has been determined (45). For KSHV, we and others have reported the whole-genome transcription patterns upon reactivation in lymphoma cell lines (19, 25, 39, 48, 59), with estimated reactivation frequencies of 5 to 30%, depending on the particular virus and cell line used. Krishnan et al. (28) have recently reported the induction of a limited set of lytic and latent viral genes immediately following KSHV infection of endothelial cells and fibroblasts. However, the full lytic program was only observed after TPA addition to the infected endothelial cells 48 h postinfection (28), demonstrating the predilection for KSHV to enter the latent phase of its viral life cycle in current tissue culture systems. Hence, the ability of RRV to fully replicate in RhFs and exhibit a progressive, gradual, and ordered change in viral transcription (in the absence of TPA, which might activate multiple viral promoters and hence skew the transcription profile) is important to demonstrate the ordered kinetics of gammaherpesvirus gene expression.

Microarray studies hinge upon the correct method of analysis. Therefore, we will briefly justify the approach we used for our analysis before presenting the experimental outcome. We employed several different means of statistical analysis, all of which yielded astonishingly congruent rank orders. To determine coregulated clusters of mRNAs purely upon their pattern of induction, the raw CT values were subjected to hierarchical clustering using euclidian standard correlation or a Pearson correlation-based metric. Euclidian clustering calculates distances between two datum points based on the sum of square differences (Fig. 3A). The scale encompasses the lowest level of the mRNA of overall lowest abundance (black) in the entire array to the highest level of the mRNA of overall highest abundance (red). Hence, information about overall mRNA levels strongly impacts the rank order, and even background levels exert considerable influence (This is the reason for the weak signal at t = 0 in Fig. 3A.) If the array comprises a range



FIG. 2. Quality control of the RRV RT-PCR array. (A) Agarose gel of a subset of PCR products after amplification of RRV virion DNA with primers in the RRV array. A 100-bp molecular weight marker is shown on the right. (B) Raw CT values after real-time QPCR using the following input samples: (i) water as an NTC (open squares); (ii) RNA from uninfected cells that was DNase I treated, reverse transcribed, and RNase H treated (gray circles); (iii) RNA from uninfected cells that was DNase I treated but prepared without reverse transcriptase in the cDNA reaction and subjected to RNase H digestion (gray squares); and (iv) RRV virion DNA (gray line). (C) RhFs were infected with RRV at five different time points (n = 5), 12, 24, 48, 72, and 96 h. mRNAs were harvested and subjected to real-time RT-PCR. The dCT values of RRV mRNAs were normalized to that of rhesus tubulin. Panel C is a graph representing the SD of the dCT values (vertical axis) versus the mean dCT values (horizontal axis). Lower dCT values correspond to higher levels of mRNA on a log₂ scale (dCT). (D) Scatter plot matrix of raw CT datu for each time point after productive infection of RhFs with RRV depicted as a diagonal line. Also shown is the correlation coefficient for each pair of datum sets.



of RNAs of very different abundances, such as the housekeeping genes for glyceraldehyde-3-phosphate dehydrogenase and actin and rRNAs in addition to low-abundance viral RNAs, changes in the low-abundance mRNAs will contribute to clustering but will not be visible in the color scheme. A standardcorrelation metric based clustering calculates the distance as the arc cosine of the scalar product with a maximal range of ± 1 . By definition, genes with all measurements of zero (i.e., the gene for rhesus tubulin, the normalizing gene) are excluded. This metric compresses the range to yield a unit length normalization but maintains a more realistic representation of mRNA levels among different transcripts. Finally, Pearson correlation-based clustering rescales and median centers the data such that for each gene the time point with the highest-abundance mRNA is set to 1 and the lowest to -1, regardless of overall levels for individual mRNAs. This approach to data analysis yields a relative rank ordering of mRNAs based solely upon their pattern of changes. Two genes with widely different absolute mRNA levels will group together if their transcription patterns change in a similar fashion. This metric is directly comparable to information that can be gathered from Cy3/Cy5 comparative hybridization-based microarrays (18).

Real-time QPCR-based analysis also allowed us to exclude the variation in total RNA levels and reverse transcriptase efficiency of a particular sample by calculating the abundance of a given RRV mRNA relative to the level of a cellular gene. Here we used rhesus tubulin (RhTub) as $dCT = CT_{gene}$ – CT_{RhTub} , to normalize for viral gene expression as reported in previous publications (16, 19, 32, 40, 41, 54). These dCT values were then subjected to cluster analysis. We applied hierarchical clustering as previously described (18) using ArrayMiner software (OptimaDesign Inc.) under Macintosh OsX10.3.4 (Apple Inc.). ArrayMiner uses Gaussian clustering (a genetic algorithm) as an alternative to self-organizing maps or k means clustering. Both methods yield concurrent results for highly correlated genes that change in a specific pattern, but Gaussian clustering allowed us to identify outliers, namely, genes with no recognizable pattern of transcription. By contrast, distancebased methods always force all signals into an apparent rank order, even if there is no correlation between adjacent entries. Additional calculations were performed using Excel (Microsoft Inc., Redwood, WA) and SPSS v11.0 (SPSS Science Inc., Chicago, IL). Note that the dCT values are still log₂ derivatives of the underlying mRNA levels and that clustering using a correlation metric is insensitive to differences in individual primer efficiency (see references 17 and 37 for discussions).

Taking into account the level and pattern of transcription, we obtained distinct clusters of genes after RRV infection of fully permissive RhFs. Five different time points were employed: 12, 24, 48, 72, and 96 h postinfection. None of the RRV

FIG. 3. Whole-genome profiling of RRV transcripts following de novo infection of RhFs. (A) Shown is a heat map representation of real-time QPCR data normalized to rhesus tubulin (dCT) at 0, 12, 24, 48, 72, and 96 h postinfection of permissive RhFs. Black indicates low, yellow represents intermediate, and red represents the highest level of viral mRNA detected. Panel A shows the result of rank ordering using a euclidian matrix. (B) Result of rank ordering using the scalar product of mRNA levels normalized to rhesus tubulin.

mRNA levels decreased at late time points (40, 41). Between 72 h and 96 h after RRV infection, cellular mRNAs (tubulin, actin) decreased \geq 10-fold since many cells in the population start to die and only cells that were intact were used for analysis. By definition, these cells would not have completed the viral life cycle, which destroys the host cell. Individual RRV mRNAs differed based upon how early significant levels (black-to-yellow transition) could be detected. During the course of the infection, the levels of the RRV mRNAs reached the level of tubulin mRNA in the cell (mean, 1.01-fold; 95% CI, 0.72-fold to 1.3-fold; n = 415). Thus, RRV mRNAs were easily detectable and yielded a very robust signal in the middle of the linear range of the real-time QPCR assay.

Figure 3 shows the relative abundance and change in transcription of each RRV mRNA based upon a euclidian (panel A) or a correlation-based (panel B) metric. ORFs 50, R8, 66, 8, 17, 18, 35, 47, 53, 61, 68, 71, and 74 were transcribed at the earliest time point (12 h) after infection and accumulated to the highest levels. For this group, changes in RRV transcription averaged 1,417-fold (95% CI, 983-fold to 1,850-fold; n =15) between 12 and 96 h postinfection and RRV mRNA levels climbed from 0.05-fold over tubulin mRNA levels (95% CI, 0.03-fold to 0.09-fold; n = 15) at 12 h postinfection to 65-fold over tubulin mRNA levels (95% CI, 53-fold to 77-fold; n = 15) at 96 h postinfection. This occurred exponentially (fold = $0.0048 \times e^{0.098 \times time}$). Of note, this clustering cannot be attributed to primer efficiency since primers for these mRNAs do not group together if viral DNA is used as a target (Fig. 1B). The early group of viral genes expressed within 12 to 24 h postinfection included ORF50, the RRV immediate-early transactivator (11, 12), as well as RRV R8, another early gene in RRV (12) (Fig. 3B). In addition, RRV genes ORF35, -61, and -74 were also induced early (Fig. 3A and B). A comparison with the transcription patterns for KSHV genes shows that the homologs of these RRV genes in KSHV (ORF50/Rta, K8/bZip, ORF35, ORF61/ribonucleotide reductase, ORF74/vGPCR) were also significantly induced as early as 10 h after reactivation (19, 25, 39). For these genes, the amino acid sequence and transcriptional regulation are conserved between KSHV and RRV, even though they encompass a wide range of differing biological functions. Other early RRV genes in this cluster included ORF17, -18, -53, and -66 (Fig. 3A and B), which vielded a strong signal at 24 h after infection and whose KSHV homologs were also significantly induced at 24 h after KSHV reactivation and hence represent the first wave of transcripts for both viruses (19, 25, 39).

The mRNA for ORF71/vFLIP is differently regulated between RRV and KSHV. In RRV, primary infection of RhFs resulted in early expression of ORF71/vFLIP, whose expression increases 1,249-fold from 0 to 96 h (Fig. 3A and B). By contrast, KSHV ORF71/vFLIP mRNA levels do not significantly change upon viral reactivation (16, 19). The RRV ORF72/ vCyclin and ORF73/LANA mRNAs were also induced during the course of RRV de novo infection and increased 2,588-fold and 3,217-fold by 96 h postinfection, respectively. The RRV ORF71, -72, and -73 gene expression profiles also grouped with mRNA transcripts for RRV genes including ORFs 2, 6, 11, 20, 21, 24, 37, 39, 41, 43, 44, 45, 46 48, 49, 54, 55, 57,58, 59, 60, 63, R9-5, and R9-4 (Fig. 3A and B). These transcripts appeared at 24 h postinfection but increased most drastically



FIG. 4. Impact of cycloheximide on RRV transcription. RhFs were pretreated with 50 μ g/ml cycloheximide for 1 h, infected with RRV at an MOI of 1, and maintained in cycloheximide until the end of the experiment. Plotted are the CT values for all RRV mRNAs in the array at 6 h after infection in the presence (vertical axis) or absence (horizontal axis) of cycloheximide. Black circles represent mRNAs that are immediate-early genes in KSHV, gray circles represent genes that are known transcriptional targets of ORF50/Rta, and open circles represent all other RRV mRNAs.

between 48 and 72 h and therefore were grouped separately (fold = $0.0004 \times e^{0.113 \times time}$). The transcription pattern for this group of genes also paralleled the temporal regulation of their KSHV counterparts (19, 25, 39). An exception is RRV ORF4/complement binding protein, which is significantly induced at 24 h after reactivation in KSHV but could not be detected until 72 to 96 h after de novo infection of RhFs with RRV. The mRNAs for ORFs 23, 31, 36, 52 R9-3, and R9-6 also clustered together and did not accumulate to significant levels until 96 h postinfection and were thus considered late genes.

In order to distinguish the immediate-early genes from the early and late transcripts, we performed RRV infections of RhFs in the presence of cycloheximide. RhFs were pretreated with cycloheximide at 50 µg/ml for 1 h, infected with RRV in the presence of drug, and kept in cycloheximide until the time of harvest. Cells were harvested at 6 and 12 h postinfection (in the presence or absence of drug), and total RNA was isolated. The RNA was subjected to array analysis as described above. Due to the facts that different cell lines exhibit different sensitivities to cycloheximide and most die by 24 h (58), we report our data as raw CT values in a two-dimensional correlation analysis (Fig. 4). We were able to identify groups of RRV transcripts that were differentially regulated by cycloheximide (data not shown). (i) Late genes were not transcribed in the presence or absence of drug at 6 and 12 h. These are ORFs 4, 25, 28, 29b, 32, 36, 38, 53, R9-3, 65, 67, 67.5, 69, 75, R9-6, 23, and 9. For these, the CT cycle numbers were \geq 38 (i.e., \leq 4-fold above background) at three of four samples points. (ii) Early genes were significantly transcribed at 6 h p.i. and strongly

inhibited by cycloheximide. Hence, they appear shifted upward of the 45° line in Fig. 4. These are ORFs R1, 70, 2, 43, 6, 29a, 17, R9-1, 27, 24, 45, 55, R8, 74, 8, and 49. Interestingly, this set includes many of the genes that are known to be regulated by ORF50/Rta (11) in KSHV, namely, ssDBP/ORF6, R1, DNApol/ORF9, gB/ORF8, vGPCR/orf74, and ORF45 (Fig. 4, gray circles). RRV TK/ORF21 was transcribed at low levels at 6 h and inhibited by cycloheximide at 12 h (data not shown). (iii) Based on our analysis, we classify RRV Rta/ORF50 and vIL6/ORF2 as the most highly induced and cycloheximideresistant immediate-early genes in RRV. At 6 h, these two genes were transcribed at approximately 8- to 10-fold higher levels than Mta/ORF57, which also was resistant to cycloheximide treatment. The RRV R8 gene was slightly sensitive to cycloheximide, suggesting that it is dependent on Rta/ORF50 to a slightly higher degree that KSHV Zta/R8, which has been reported as an immediate-early gene (60) but also as a delayedearly and Rta/ORF50-responsive gene (56). (iv) Finally, we identified a number of RNAs that are transcribed at 6 h postinfection, but to a lesser degree than Rta/ORF50, and show intermediate inhibition (fivefold or less) to cycloheximide (data not shown). These can be classified as early genes on the basis of timing, but their promoters are less stringently dependent on viral immediate-early transactivators. Whether this is biologically relevant or whether the promoters are simply leaky in the context of drug treatment remains to be determined.

We have previously reported that RRV replication is sensitive to phosphonoacetic acid (PAA) (12). RhFs were infected at an MOI of 1 in either the presence or the absence of 50 µM PAA, and viral transcription profiles were determined at 12, 24, and 48 h postinfection (Fig. 5). To exclude possible drug effects on the cellular genes, we plotted the raw CT values (higher values correspond to lower abundances). At 12 h, no effect of PAA was discernible and the RRV mRNA levels in the absence of PAA were identical to the RRV mRNA levels in the presence of PAA, as demonstrated by linear regression analysis with $r^2 = 0.9262$ and m = 0.9959, i.e., 45° (panel A). This outcome was consistent with the mechanism of action of PAA, which inhibits the viral polymerase but does not interfere with immediate-early and early transcription (53, 61). At 24 h, none of the mRNA levels were inhibited more than threefold in response to PAA. At 24 h, PAA inhibited RRV transcripts under both high-MOI and low-MOI conditions (Fig. 5B and F), specifically, ORFs7, 9, 19, 28, 29b, 32, 34, R9.2, 59, and 67. At 48 h, the effect was more pronounced (Fig. 5C and G) and in particular ORFs 7, 9, 19, 21, 32, 33, 38, 42, 48, 57, R9-3, 59, 60, 67, 69, 75, and R15 were significantly (\geq 40-fold) inhibited. This was consistent with our previously published Northern blot analysis of selected RRV latent and lytic mRNAs (12). ORFs 19, 21, 32, 29, 32, 33, 34, 38, 75, and K15 have been classified as tertiary lytic genes in KSHV (25) based upon the temporal order of transcription. A directly comparable genome-wide transcriptional profiling in response to PAA has yet to be published. The drug treatment did not affect primer specificity, as evidenced by identical melting temperatures in the presence and absence of the drug (Fig. 5E). Cluster analysis across three time points (12, 24, and 48 h) verified the individual comparisons (Fig. 5I). At 72 h postinfection, the mRNAs in the PAA-treated cultures accumulated to higher levels compared to untreated cultures because RRV egress

in the untreated cultures destroyed the cells, whereas PAAtreated cultures were arrested prior to viral DNA replication (Fig. 5D). At 96 h, the drug had lost its effect in this high-MOI system (Fig. 5H). This highlights an important limitation of all transcriptional profiling, which reflects the integration of all factors including the onset of viral transcription, mRNA stability, total mRNA level, effects of the drug (PAA), and the overall effect of the virus on cell viability and nucleotide metabolism.

As an alternative approach to classify the temporal order of RRV transcription and to test the sensitivity of our assay, we infected RhFs with RRV at different MOIs including MOIs of 5, 1, 0.1, 0.01, and prepared mRNA at a single time point, 48 h postinfection (Fig. 6A). We hypothesized that our highly sensitive real-time QPCR array would be able to detect transcripts even at the lowest MOI of 0.01. At an MOI of 5, we could detect expression of 66 RRV mRNAs. At an MOI of 1, these mRNAs could be divided into two groups: a highly abundant class (26 mRNAs), which was composed of the RRV immediate-early and early transcripts as identified by time series analysis (Fig. 6A), an intermediate class (five mRNA transcripts), and a less abundant class (35 mRNAs), which included the mRNA transcripts classified as late genes by time series analysis. Importantly, we could detect and cluster RRV mRNAs even if fewer than 1 in 100 cells were infected (MOI of 0.01). Under these conditions, the relative transcription profile of RRV mRNA transcripts was highly correlated ($r = 0.791 \pm$ 0.167; n = 6) and independent of the MOI. For example, the same group of mRNAs that were highly transcribed at 48 h after RRV infection with an MOI of 0.1 was highly transcribed at 48 h after RRV infections with an MOI of 5. Thus, once RRV enters an RhF cell, lytic gene expression commences in that cell at a fixed pace regardless of whether the neighboring cells are infected or not.

Gene profiling of a 293-RRV-green fluorescent protein latent cell line. We previously reported that RRV can infect HEK293 cells and that the virus establishes a predominantly latent infection in this model (14). This was confirmed by transcriptional array analysis. HEK293 cells were infected with RRV as previously described (14) and subjected to mRNA profiling (Fig. 6B and C). Viral mRNAs were barely detectable, but the relative comparison using the uncentered euclidian metric of rhesus tubulin-normalized data (dCT) showed that most mRNAs were transcribed to various degrees. We did not find strong transcriptional silencing of the entire genome, and just like KSHV-infected HEK293 cells (20) and KSHVinfected BCBL-1s (5), RRV-infected HEK293 cells exhibited some degree of lytic reactivation. In this culture system, TPA can induce complete lytic reactivation and at 48 h after TPA treatment all RRV mRNAs were induced (Fig. 6B). By clustering using a correlation metric on mean-centered data (Fig. 6C), we identified a set of mRNAs that were drastically upregulated by TPA and that was composed of ORF50/Rta, ORF57/Mta, ORFR8/Zta, and ORFs R9-1, 2, 4, R4, 27, 39, 41, 44, 45, 46, 47, 55, 56, 60, 61, 62, 63, 68, 71, 72, 74, and 26.

Discussion. This study set out to map the temporal order of RRV transcription and to provide a roadmap for future investigations. To achieve this goal, a technology platform was developed that was rapid and quantitative and could be used to measure RRV transcripts even if only 1 in 100 cells was in-



FIG. 5. Impact of cycloheximide and PAA on RRV transcription. RhFs were infected with RRV at an MOI of 1 (panels A to D and H) or an MOI of 0.1 (panels F and G) and either not treated or treated with 50 μM PAA. Plotted are the CT values for all RRV mRNAs in infected RhFs at 12, 24, 48, 72, and 96 h after infection. Data from RRV-infected, mock-treated cells are on the horizontal axis and data from RRV-infected, PAA-treated cells are plotted on the vertical axis. Panel E plots the melting curves of the reaction products for all RRV primers at 24 h after infection for RRV-infected, PAA-treated (vertical) and RRV-infected, untreated cells. Panel I shows standard correlation-based, hierarchical clustering of time points 12, 24, and 48 h in the presence or absence of PAA. Black indicates low and gray/white high relative levels of the corresponding viral mRNAs. pos, positive; neg, negative.



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fected (Fig. 6A). To date, this represents the only means to quantify viral transcription under conditions of a natural, lowmultiplicity infection or in mixtures of permissive and nonpermissive cells. We based the RRV array on real-time QPCR technology because PCR and RT-PCR deliver quantitative information without the need for dilution series, internal competitors, etc. We have recently shown that the SYBR-based method of detection is as sensitive as the TaqMan-based method of detection (37, 38), which reduces the cost of real-time QPCR to the level of traditional PCR without a loss in sensitivity, specificity, or dynamic range. Extensive in silico primer validation eliminated nonspecific signals (Fig. 1 and 2). Comparative array analysis of real-time QPCR data employs the same analytical methods as hybridization-based arrays (16, 19). We previously used this method to analyze KSHV transcription (19) in PEL cells and established a rank order of KSHV transcription after TPA induction, which was largely identical to that obtained in studies that measured mRNA levels by competitive hybridization to spotted viral cDNA arrays (25, 39). A direct comparison of the sensitivity of real-time QPCR arrays to hybridization-based membrane arrays, for which we spotted the real-time QPCR products onto membrane, showed that real-time QPCR-based detection of messages was more sensitive (Vahrson and Dittmer, unpublished). Hence, we were able to measure KSHV transcription in primary 2-by-2-mm punch biopsies, which hitherto was not possible using conventional arrays (16). Hybridization-based arrays profile changes in a nonlinear fashion and tend to overemphasize large changes and compress smaller less-than-twofold variations, whereas real-time QPCR has a linear range of ≥ 6 orders of magnitude (23). This offers an advantage for the analysis of viral mRNAs, which traverse a much larger dynamic range than most cellular transcripts. Cellular microarray measurements are generally verified by real-time QPCR or Northern blot analysis (27). One caveat of microarray-based profiling is of particular concern for studies in herpesvirus transcription: Neither cDNA-based, nor random-primed real-time QPCRbased arrays can distinguish between overlapping transcripts or transcripts that originate on the opposite strand. Using splice site-specific primers, we could distinguish between overlapping, spliced mRNAs in the KSHV LANA, cyclin, vFLIP locus, as well as vIRF-1 and several alpha mRNAs (26). We are currently working on a similar design strategy for known spliced mRNAs in RRV but have noticed that introducing this additional constraint into the design yields greater variation in the PCR efficiency of the individual primer pairs (Vahrson and Dittmer, unpublished). Using strand-specific oligonucleotides as used in the Affymetrix platform, one should be able to distinguish between sense and antisense transcripts, but this approach lacks sensitivity. A similar strategy using strand-specific, gene-specific RRV primers to prime the individual RT reactions can be used to identify antisense mRNAs using PCR-

FIG. 6. Applications of the RRV real-time QPCR array. (A) RhFs were infected with RRV at different MOIs (5, 1, 0.1, and 0.01). The real-time QPCR data were normalized to rhesus tubulin (dCT) at 48 h postinfection and are depicted as a heat map. Black indicates low, gray indicates intermediate, and white indicates the highest level of viral mRNA transcripts detected at the different MOIs. (B) Profiles of RRV-infected latent HEK293 cells with and without TPA induction.

Cells were treated with TPA or dimethyl sulfoxide for 48 h. Total RNA was isolated and subjected to real-time PCR. The data were normalized to tubulin (dCT). Data are depicted as a heat map using euclidian (B) or standard correlation (C) clustering. Black indicates low, yellow indicates intermediate, and red indicates the highest relative level of viral mRNA transcripts.



FIG. 7. Locations of RRV primers relative to predicted mRNA termination signals in the RRV genome. Depicted is the location of the forward primer for each RRV ORF (squares) on the RRV genome. Primers for rightward ORFs are indicated as boxes above the horizontal line, and primers for leftward ORFs are indicated below the genome axis. The sequential ORFs (either rightward or leftward) amplified by these primers are connected by vertical lines. The predicted termination signals are indicated with dots.

based arrays. Using the gene-specific reverse primer to specifically reverse transcribe only the sense transcript, we verified that all our primers will detect the mRNA that comprises the coding region for the corresponding protein (data not shown). Based upon the location and orientation of the RRV primers and predicted poly(A) sites (Fig. 7), we can identify regions of co-oriented genes (n = >3) for which it is less likely that antisense mRNAs with coding potential exist (e.g., ORFs 4 to 11, 25 to 28, 30 to 33, 34 to 38, 45 to 49, R9.1-8, 58 to 62, 65 to 67, and 71 to 73) and which can be coterminal, such as ORFs 73 to 71 (12). For all other ORFs, a complete transcription map of the RRV genome is required to resolve issues of splicing, antisense, and overlapping transcripts.

The real-time QPCR-based results corroborated our prior Northern blot analysis on a subset of RRV mRNAs (12) and supports the model of a temporal wave of viral gene expression during lytic replication. The majority of mRNAs were induced between 24 and 48 h after infection, while a few mRNAs were not detectable until 96 h postinfection. Overall, there was a good correlation between the transcriptional profiles of RRV de novo infection and KSHV reactivation. One exception to this rule was the latent transcripts for ORF71/vFLIP, 72/vCyc, and 73/LANA, which were dramatically induced upon RRV de novo infection. By contrast, LANA mRNA levels do not significantly increase after KSHV reactivation of PELs (19) and in fact appear to decrease after infection of semipermissive cells (28). The role for ORF73/LANA in RRV (14), HVS (3, 8, 55), and KSHV (2, 9, 10, 21, 22, 24) is similar for all three viruses, since LANA ensures the maintenance of the viral episome by physically tethering viral DNA to cellular chromosomes through its interaction with cellular factors (29). In addition, LANA has been shown to inhibit lytic viral replication and reactivation in HVS, RRV, and KSHV (14, 31, 49).

RRV transcription profiling during de novo infection of RhFs revealed that the temporal order of viral transcription was conserved among the gamma herpesviruses and supports the hypothesis that RRV Rta/ORF50, the master regulator of lytic transcription (33, 52), is one of the first genes to be expressed. Rta/ORF50 is highly conserved in sequence and function among the rhadinoviruses (11). Once Rta/ORF50 is expressed, viral transcription proceeds in a fixed pattern until complete lytic replication is achieved. RRV transcription in an infected cell is independent of the RRV status of the neighboring cells (Fig. 5). Interestingly, the expression of the mRNAs for the R9-1 through R9-5 genes, which represent the RRV viral interferon (vIRF) genes, was not coregulated since the mRNAs for R9-4 and R9-5 were induced early, while the other R9 mRNAs were induced late. This suggests that the multiply duplicated vIRF genes are expressed at different times in the viral life cycle and parallel the situation of the multiple KSHV vIRFs, which are differentially expressed at different phases of the viral life cycle (7, 16, 35). In conclusion,

we have developed a high-throughput RRV QPCR array that is capable of profiling gene transcription from the entire RRV genome simultaneously. An added benefit of this system is the sensitivity of this QPCR array, which will allow us to determine the RRV transcription profile in vivo, in the experimentally infected rhesus macaque.

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