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Development of a Generic Real-time PCR Assay for Simultaneous Detection of Proviral DNA of Simian *Betaretrovirus* Serotypes 1, 2, 3, 4 and 5 and Secondary Uniplex Assays for Specific Serotype Identification

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Abstract

Simian betaretroviruses, (formerly Type D retroviruses; SRV) are a group of closely related retroviruses for which the natural host species are Asian monkeys of the genus *Macaca.* Five serotypes have been identified by classical neutralization assays and three additional untyped variants have been reported ($SRV_{Tsukuba}$, SRV-6, SRV-7). These viruses may be significant pathogens in macaque colonies, causing a broad spectrum of clinical disease secondary to viral-induced immune suppression. Undetected SRV infections in research macaques also represent a potential confounding variable in research protocols and a concern for human caretakers. Intensive testing efforts have been implemented to identify infected animals in established colonies. A real-time quantitative generic multiplex PCR assay was developed that is capable of simultaneous detection of proviral DNA of SRV serotypes 1, 2, 3, 4 and 5. This assay incorporates amplification of the oncostatin M (OSM) gene for confirmation of amplifiable DNA and allows quantitation of the number of proviral copies per cell analyzed in each multiplex reaction. Detection of multiple serotypes by PCR increases the efficiency and cost-effectiveness of SRV screening programs. A panel of SRV serotype-specific uniplex real-time PCR assays for discrimination among the 5 recognized serotypes is also described.

Keywords

Simian betaretrovirus; SRV; PCR; SPF

1. Introduction

The exogenous simian type D retroviruses (SRV), now classified in the genus *Betaretrovirus*, subfamily *Orthoretrovirinae* comprise a group of closely related viruses that are important pathogens in many species of Asian monkeys of the genus *Macaca*, that are capable of causing significant morbidity and mortality secondary to viral-induced

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immunosuppression (Nishida et al., 1986; Chopra, 1970; Jensen et al., 1970; Lerche, 1992; Lerche et al., 1997; Lerche, 2003; Nishida, 1992; Power et al., 1986; Sonigo et al., 1986). SRV is endemic in many populations of wild and captive macaques and to date five distinct serotypes have been identified by conventional virus neutralization assays (Hara et al., 2007; Hara et al., 2005; Lerche, 2005; Lerche et al., 1997; Lerche et al., 1986; Lerche et al., 1994; Lowenstine et al., 1986; Maul et al., 1986; Wolfe-Coote, 2005). Undetected SRV infection has also been recognized as a potential confounding variable in biomedical research protocols using macaques as research subjects (Lerche, 2003; Maul et al., 1986). As a result, SRV is one of several persistent viruses targeted for elimination to establish and maintain specific pathogen-free (SPF) breeding colonies of rhesus macaques (*Macaca mulatta*) for biomedical research. Antibody testing alone, while effective in identifying the presence of SRV at a population level, is inadequate to identify all infected individuals within a population, as infected macaques may exhibit a long interval between infection and seroconversion, and some infected macaques fail to make a detectable antibody response (Lerche, 2003; Lerche et al., 1994; Liska et al., 1997). The application of SRV testing algorithms incorporating real-time PCR together with sensitive and specific assays for SRV antibody detection have been successful in detecting and eliminating SRV infection in breeding and research colonies in which recognized serotypes are endemic (Lerche et al., 1997; Liska et al., 1997; Lowenstine et al., 1986). The existence of five distinct serotypes of exogenous SRV, as determined by conventional virus neutralization assays, recognition of significant genetic variation within specific serotypes, as well as the presence of an endogenous full-length intact betaretrovirus genome in all Old World monkeys, has complicated diagnostic and screening programs utilizing PCR (Anderson, 1999; Chung et al., 2008; Hara et al., 2005; Lerche, 1992; Lerche et al., 1994; Li et al., 2000; Marracci et al., 1995; Marx et al., 1985; Nishida, 1992). While some generalizations are possible regarding the association of the various serotypes with different macaque species, rhesus macaques *(M. mulatta)* are most commonly infected with SRV type 1, while cynomolgus *(M. fascicularis)* and pig-tailed macaques *(M. nemestrina)* are most often infected with SRV type 2 viruses (Lerche, 1992; Lerche et al., 1987; Lowenstine et al., 1986; Marx et al., 1984; Nishida, 1992), these virus-macaque species associations are not exclusive. In the research colony environment macaques may be infected with any of the five serotypes and infection with more than one serotype has been observed (Lerche, 2005; Wolfe-Coote, 2005). Sensitive and specific real-time PCR assays have been developed for detection of proviral DNA of various SRV serotypes, but to date, real-time multiplex or generic PCR assays for SRV have not been reported (Chung et al., 2008).

A prototype generic SRV multiplex real-time PCR assay (GSRV), incorporating GAPDH as an internal control for the presence of amplifiable DNA, was developed and validated in parallel testing with uniplex end point PCR on over 1000 samples submitted for SRV diagnostic testing from a variety of primate facilities to the pathogen detection laboratory at the California National Primate Research Center (CNPRC) ((Lerche et al., 1997), N. Lerche, unpublished). While the SRV detection capabilities of this multiplex prototype were robust compared to a previously published end point assay (kappa=0.6966), frequent failure to amplify GAPDH, possibly due to competition between the GADH and SRV amplification reactions, rendered many of the assays invalid, requiring repeat testing (Lerche et al., 1994). A generic SRV multiplex real-time PCR assay (OSRV) is described that is capable of simultaneous detection of a conserved sequence region in all 5 SRV serotypes and that incorporates detection of the diploid oncostatin (OSM) gene as an internal control for the presence of amplifiable DNA that reduces competition with SRV amplification in the multiplex reaction, greatly reducing the need for repeat testing. Incorporating OSM into the assay has the added benefit of allowing quantitation of the number of SRV proviral DNA copies. (Biosystems, 2008; Bruce et al., 2005; Lerche et al., 1997; Malik et al., 1989). In addition a panel of SRV serotype-specific uniplex real-time PCR assays is described for discrimination among the 5 different serotypes.

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2. Materials and Methods

2.1 Animal Samples

SRV infection status of samples used to generate a test validation panel was determined by previous analysis for SRV-specific antibodies by multiplex microbead immunoassay (MMIA) and confirmatory Western immunoblot (WB) and for SRV proviral DNA by both end point PCR and a prototype generic multiplex real-time PCR. An SRV positive was defined as a sample with PCR amplification in 2 of 2 or 3 of 4 tests in both an endpoint PCR assay and a prototype real-time PCR assay as well as positive antibody results from the MMIA. An SRV indeterminate sample had PCR amplification in either 1 or 2 out of 4 PCR tests on the end point and prototype multiplex PCR assay as well as indeterminate antibody results from the MMIA and WB assays. An SRV negative sample was defined as having no PCR amplification in either the end point or prototype real time PCR assays as well as no antibody detection in either the MMIA or the WB assays. A panel of 44 samples comprising 15 SRV positive, 15 SRV negative, and 14 SRV indeterminate DNA samples were randomly selected from a reference DNA bank derived from >1000 heparinized whole blood samples obtained from various species of macaque and submitted to the pathogen detection laboratory for diagnostic SRV testing in 2007.

2.2 SRV variants

In development of the generic and uniplex assays both sequence data and DNA from specific isolates were used. Full sequence data was available for SRV-1, -2, and -3 serotypes while partial sequence data was used for SRV-5_{ORE} and $SRV_{Tsukuba}$. Two isolates of SRV-5, SRV-5_{Cal} and SRV-5_{Ore} (generously provided by Dr. Michael Axthelm, Oregon NPRC), $SRV_{Tsukuba}$ (generously provided by Dr. Ryozaburo Mukai, Tsukuba Primate Research Center, Japan)(Hara et al., 2005) were tested on both the generic multiplex and uniplex assays, as was the prototype SRV-4_{Cal} isolate originally identified by neutralization assays, but which has only been partially sequenced (N. Lerche, unpublished). Variants identified as “SRV-6” and “SRV-7” were not available for analysis in the generic assay (Nandi et al., 2000; Nandi et al., 2003; Nandi et al., 2006).

2.3 DNA Isolation and Sample Preparation

DNA was isolated from heparinized whole blood on a Qiagen M48 Biorobot using the QiaAmp midi kit (cat. # 951336, Valencia, CA, USA). From each sample, 200ul of heparinized blood was removed and placed in an open microfuge tube in the Biorobot for DNA extraction according to the kit protocol. The remaining heparinized blood sample was centrifuged and plasma was removed for SRV specific antibody testing (Khan et al., 2006). Negative control samples from uninfected macaques and positive control samples obtained from SRV-1 and -2 infected macaques were processed as described above or by “bulk” processing of 10ml whole blood using the Gentra Puregene DNA Purification Kit (Qiagen cat.# 158389, Valencia, CA, USA). DNA isolated using the “bulk” method was re-suspended in (~12ml) DNA hydration solution to make a final concentration of ~25ng/ul. A negative cell culture control consisting of DNA extracted from uninfected Raji or Sup-T-1 cell lines and positive cell culture controls consisting of proviral DNA of SRV serotypes -3, -4_{Cal} -5_{Cal}, -5_{Ore} and $SRV_{Tsukuba}$ obtained from infected Raji or SupT cell cultures were prepared as described. Isolated DNA was re-suspended in 4ml of DNA hydration solution to make a final concentration of ~25ng/ul.

2.4 Primers and Probes

Probe and primer sequences (Tables 1 and 2) were selected according to parameters defined by the Primer Express software (version 2.0, Applied Biosystems, Foster City, CA) to amplify proviral DNA sequences of exogenous SRV serotypes 1, 2, 3, 4, and 5 but not endogenous

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betaretrovirus sequences. Full length sequence information from SRV serotypes 1, 2, and 3 were used in primer and probe design in addition to partial sequence data from SRV-5_{Ore} and $SRV_{Tsukuba}$. Primers and probes for the generic PCR assay were derived from highly conserved sequences within the envelope (*env*) gene. Once primers and probes were designed based on the sequence data available, testing was completed with additional unsequenced SRV isolates. Primers and probes for the uniplex type-specific PCR assays were derived from highly variable regions within the *env* gene incorporating sequences coding for the neutralization epitope region that define the specific serotypes (Anderson, 1999;Malley et al., 1991;Torres et al., 1991a;Torres et al., 1991b;Werner et al., 1991). Partial sequence data for $SRV_{Tsukuba}$ was used in development of an SRV-4 specific assay. Isolates of SRV-4_{cal} and $SRV_{Tsukuba}$ were both tested on the SRV-4 specific assay to confirm the sensitivity of the assay. In order to confirm specificity of all the uniplex assays DNA controls for all 5 serotypes were tested on each assay. (Table 4)

2.5 Calibration Plasmids

Full-length plasmid constructs for SRV-1, -2, -3 were generously provided by Dr. Paul Luciw, UC Davis. Plasmid constructs for SRV-4 and -5 were prepared commercially (BioClone, San Diego, CA, USA) to specifications based on unpublished sequence. Plasmid constructs for each of the 5 serotypes were prepared at a concentration of 10^7 copies/ul in 1×TE (Tris EDTA) buffer, pH 8.0. Serial tenfold dilutions from 10^6 copies/reaction (15ul) to 10^{-1} copies/reaction were made in a background of DNA extracted from negative macaque buffy coat at a concentration of 25ng/ul. Plasmids for SRV serotypes 1, 3 and 5 were diluted in rhesus macaque DNA while plasmids for serotypes 2 and 4 were diluted in cynomologus macaque DNA to more accurately represent species-serotype associations in test samples (Anderson, 1999; Lerche, 1992; Lerche et al., 1994; Nishida, 1992). (Table 3)

2.6 Multiplex Detection and Quantitation of SRV Proviral DNA

The procedure for PCR amplification of OSM gene sequences as described previously (Bruce et al., 2005) was modified slightly for use on the Applied Biosystems platform and was multiplexed with the SRV reaction. The reaction conditions have been optimized for the multiplex detection of OSM and all five SRV serotypes. The multiplex reaction mixture consisted of 50ul containing 1ul of 5uM OSM probe, 0.25ul of each 10uM OSM primers, 0.25ul of 20uM SRV probes (SRVa/SRVb) and 0.5ul of each 20uM SRV primers (SRVFor/ SRVRev), 25ul of TaqMan Universal master mix (cat # 4304437, Applied Biosystems, Foster City, CA), 7ul 1×TE buffer and 15ul of template DNA. Cycling conditions were 2min at 50°C, 10min at 95°C; 55 cycles of amplifications for 15sec at 95°C and 1min at 62°C. All reactions were carried out in a 7900 ABI PRISM Sequence Detector from Applied Biosystems. Positive controls consisted of proviral DNA of SRV-1 -2, -3, -4, and -5 prepared as described above.

2.7 Detection of Specific SRV Serotypes

To further identify specific serotypes in samples testing positive (or indeterminate) in the generic SRV multiplex PCR assay, 5 additional serotype-specific uniplex real-time PCR assays are performed. Probe and primer sequences (Table 2) were selected according to parameters defined by the Primer Express software (version 2.0, Applied Biosystems, Foster City, CA) for serotype specificity and to not amplify the endogenous betaretrovirus sequences of Old World monkeys. Reaction conditions for the individual uniplex assays are summarized in table 5.

Cycling conditions for each assay consisted of 2min at 50°C, 10min at 95°C; 55 cycles of amplifications for 15sec at 95°C and 1min at 60°C. All reactions were carried out in an ABI 7900 ABI PRISM Sequence Detector (Applied Biosystems). The type-specific plasmid constructs for all 5 SRV serotypes and cell culture controls described above were tested on

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each individual typing assay in order to determine both the lower limit of detection and for confirmation of serotype specificity.

2.8 Interpretative Criteria

The cycle threshold (CT) value reflects the linear phase in the amplification curve that can be used for quantitation of the template input (Biosystems, 2008). Samples were initially amplified in duplicate on separate reaction plates. Samples were considered SRV negative if no amplification was present in the initial duplicate samples but amplification was observed in the control gene OSM. Samples giving discrepant results on initial testing were repeated in duplicate. Samples were considered SRV positive if amplification occurred in both the initial duplicate tests, or in 3 out of 4 replicates. Samples that showed amplification in 1 or 2 out of 4 replicates were considered “indeterminate”.

2.9 Statistical Analysis

Kappa statistic, a proportion of the amount of agreement observed due to chance alone compared to the maximum amount of agreement possible (Landis, 1977), was calculated to compare the agreement between a previously published end point PCR assay and a previously unpublished generic multiplex real-time PCR (GSRV). A second *kappa* analysis was performed to compare the infection status (determined by combining GSRV and antibody testing) to the newly described OSRV generic multiplex real-time PCR assay (Table 6). *Kappa* calculations were carried out using the STATA software package (STATA Statistics/ Data Analysis ver. 9.2, Statacorp, College Station, Texas, USA).

3. Results

3.1 Multiplex Detection of SRV Proviral DNA

Proviral DNA of all 5 known serotypes isolated from the blood of infected macaques or infected cell lines in tissue culture was consistently amplified in the multiplex real-time PCR assay (Table 4). Two different isolates of SRV-5 (SRV-5_{Ore} and SRV-5_{Cal}) were detected in the generic PCR, as was $SRV_{Tsukuba}$, an isolate of undetermined serotype. Results of the panel of 44 DNA test samples by the OSRV assay and the GSRV prototype showed very good agreement between the two assays, with only 2 discrepant samples detected among the “indeterminates” (Table 6, *Kappa* = 0.8977).

The standard curves generated to determine the detection limits of the multiplex assay for SRV serotypes 1 through 5, and for OSM are presented in Figure 1 A-F. The lower limit of detection in the multiplex assay for SRV serotypes 1 through 3 is from 10-100 copies per reaction, while the limit of detection for serotype 2 in this assay is from 1-10 copies per reaction. The limit of detection for serotypes 4 and 5 are not as sensitive (SRV-4 100-1000, SRV-5 1000-10,000 per reaction) but these standard curves are based on limited sequence data from the CNPRC isolates. The sensitivity of the multiplex PCR assay for each of the 5 serotypes is summarized in Table 3.

3.2 Serotype Determination by Uniplex Real-time PCR

Samples positive in the multiplex real-time PCR assay may be further characterized in uniplex typing assays. Specificity of the uniplex PCR reactions is summarized in Table 4. Uniplex PCR amplification was specific only for the matched SRV serotype. Primers and probe generated to amplify sequences of $SRV_{Tsukuba}$, also amplified SRV-4 (for which no sequences have been published), suggesting that $SRV_{Tsukuba}$ is a type 4-like variant. No amplification was observed in genomic DNA isolated from SRV negative macaques, demonstrating that none of the type-specific primers amplified endogenous betaretrovirus sequences.

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3.3 Quantitation of Proviral DNA copy number

Once the specific serotype has been determined, the equations generated by the standard curves shown in Figure 1 A-F allow for the calculation of the number of proviral DNA sequences detected in the multiplex assay in relation to the number of cells analyzed (Bruce et al., 2005). Once the specific serotype in a given sample is determined by previous information or uniplex PCR assays, the results from the multiplex reaction can be used to perform relative quantitation of proviral load for the specific SRV serotype per number of cells tested in the same reaction well. Using the appropriate SRV standard curve for whichever serotype is present in a sample and inserting the CT value will give the number of proviral copies detected (numerator). Using the equation generated from the OSM standard curve from the multiplex reaction the number of cellular genomes can be calculated (denominator). This allows for a relative quantitation of the number of proviral copies of a specific SRV serotype detected in a sample compared to the number of cellular genomes detected in the same reaction well.

4. Discussion

The OSRV generic multiplex real-time PCR reaction is a sensitive and specific test for routine SRV surveillance that is capable of detecting all 5 SRV serotypes in one PCR reaction. Efficient, accurate and cost-effective testing for SRV in macaque populations is important for maintaining the health of animals maintained in research colonies, as well as the integrity of research data collected utilizing macaques. Incorporation of OSRV generic real-time multiplex PCR into surveillance testing algorithms can significantly increase the efficiency of such programs for detecting the presence or absence of generic SRV in macaque populations. If desired, secondary specific SRV type determinations and quantitation of proviral DNA copy number can be achieved using a second tier of uniplex assays and associated standard curves.

There is also potential for use of the generic SRV multiplex PCR in combination with the specific typing PCR assays as a tool for detection of novel SRV variants. Amplification of proviral DNA sequences in the multiplex assay, but not in any of the 5 type-specific assays could indicate the presence of a previously unrecognized variant of SRV. Recently reported “SRV-6” and “SRV-7” of Indian langurs and rhesus macaques respectively (Nandi et al., 2000; Nandi et al., 2003; Nandi et al., 2006), have not been typed by conventional serum neutralization assays and were not available for testing in the multiplex assay. However, recent detection of SRV by the OSRV multiplex assay in samples obtained from langurs and rhesus macaques in Bangladesh which do not amplify in any of the type-specific uniplex assays (data not shown), suggests the existence of additional novel variants requiring further characterization.

5. Conclusions

A multiplex real-time PCR was developed for the detection of all 5 SRV serotypes. Separate serotype-specific uniplex real-time PCR assays were also developed allowing for differentiation between the different serotypes. These assays allow for accurate, efficient and less expensive screening of macaque colonies.

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Abbreviations

SRV, Simian betaretrovirus; OSM, oncostatin M gene; GSRV, GAPDH SRV multiplex PCR reaction; OSRV, oncostatin M gene and SRV multiplex PCR; env, SRV envelope gene; MMIA, multiplex microbead immunoassay; WB, western blot.

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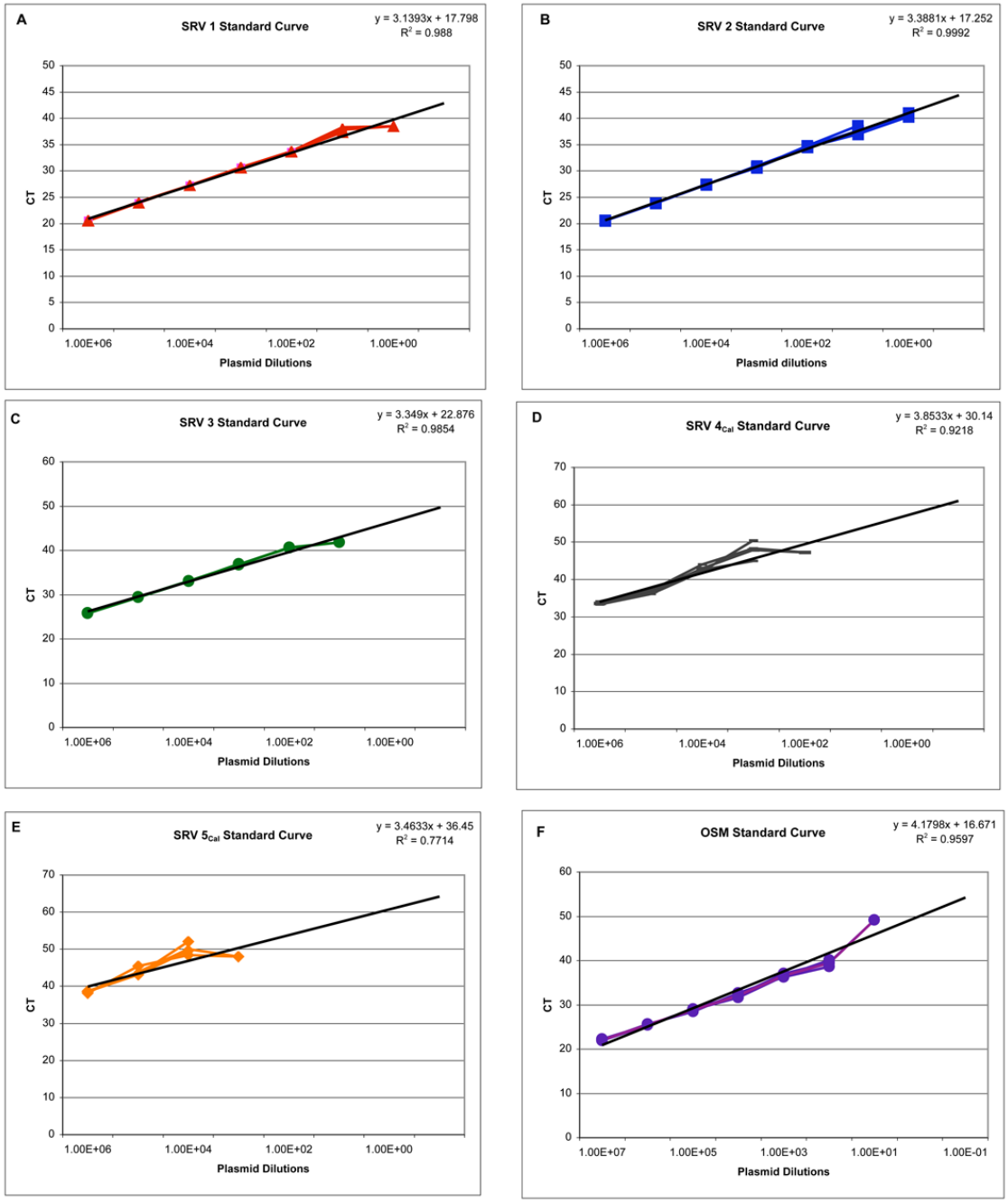

Figure 1.

Serial dilutions of SRV plasmids in the generic multiplex real-time PCR assay. Three replicates of each dilution for each plasmid are tested to generate a standard curve for each viral serotype. Dilutions of each plasmid shown on the x-axis of each figure with the corresponding cycle threshold (CT) value displayed on the Y-axis. The CT value reflects the linear phase in the amplification curve that can be used for quantitation of the template input (Biosystems, 2008). Each of the specific standard curves for SRV 1-5 are shown in A-E respectively along with the R^2 values for each dilution series. The standard curve for OSM is shown in F.

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Table 1

SRV-specific *env* primers and probes used in the generic multiplex real-time PCR assay.

Primer	Position	Virus/Sequence (5′ to 3′)	Sequence position (bp)
OSRV PCR:			
SRVFor	Sense	CTG GWC AGC CAA TGA CGG G	SRV-2,7585-7603
SRVRev	Antisense	CGC CTG TCT TAG GTT GGA GTG	SRV-2,7715-7695
SRVa	Probe	6FAM - TCA CTA ACC TAA GAC AGG AGG GTC GTC A – TAMRA	SRV-2,7621-7648
SRVb	Probe	6FAM-TCC TAA ACC TAA GAC AGG AGG GCT GTC A-TAMRA	SRV-2,7626-7642
OSMFor	Sense	CCT CGG GCT CAG GAA CAA C	(Bruce et al., 2005)
OSMRev	Antisense	GGC CTT CGT GGG CTC AG	
OSM	Probe	VIC- TAC TGC ATG GCC CAG CTG CTG GAC AA-MGBNFQ	

SRV 1 Genbank accession no. M11841, SRV 2 Genbank accession no. M16605 and Mason Pfizer monkey virus/SRV 3 (MPMV) Genbank accession no. AF033815 were used in PCR design. (Bruce et al., 2005; Hara et al., 2005)

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Table 2

SRV serotype-specific *env* primers and probes for uniplex real-time PCR

Primer	Position	Virus/Sequence (5′ to 3′)	Sequence position (bp)
SRV-1 only[a]**:**			
SRV1	Probe	FAM-TGC TGG CTG TGC TTG CGG TCA-TAMRA	SRV-1, 6599-6619
SRV1 For	Sense	CCT CCC AAC GCC AAT TAG C	SRV-1, 6573-6591
SRV 1 Rev	Antisense	GCG AGA GGA ACG GGA TCA C	SRV-1, 6639-6621
SRV-2 only[a]**:**			
SRV2	Probe	VIC-TAC ATA GAG CCA GGA GAG C-MGBNFQ	SRV-2, 6967-6985
SRV2 For	Sense	CCT GGT GAT GCA CCT GTA CCT	SRV-2, 6924-6944
SRV2 Rev	Antisense	GAC CAG CAG TCC CAG TTG AGA	SRV-2, 7053-7033
SRV-3 only:			
SRV3	Probe	FAM-CCT TTG CTT TGC TTA TTG-TAMRA	MPMV, 7214-7230
SRV3 For	Sense	CAT TTT GGA CCA GTT TTC ATG GA	MPMV, 7161-7183
SRV3 Rev	Antisense	AGG TCA AAT ATG AGC CAC CAC TGT	MPMV, 7373-7350
SRV-4 only[b]**:**			
SRV4	Probe	FAM-CCC CCT TTT TAG TGC AAC-MGBNFQ	SRV-TSUKUBA 6699-6716(Hara et al., 2005)
SRV4 For	Sense	AAA TTG TTC TTG CCC CAT TGT T	SRV-TSUKUBA 6676-6687
SRV4 Rev	Antisense	GGC AGA CAG ATT CTG TGA AAT TAA AA	SRV-TSUKUBA 6743-6718
SRV-5 only:			
SRV5	Probe	FAM- CCT TTT TTA CAG AAA TGA AC-MGBNFQ	NOT PUBLISHED
SRV5 For	Sense	TCC CTG AGA CTG CCC TTT T	
SRV5 Rev	Antisense	CAA GGC CCC AAA TGA GGA T	

SRV 1 Genbank accession no. **M11841**, SRV 2 Genbank accession no. **M16605** and Mason Pfizer monkey virus/SRV 3 (MPMV) Genbank accession no. **AF033815** were used in PCR design. (Hara et al., 2005)

[a]Primers designed by Jason Michaels, Sierra Biomedical, Charles River Laboratories.

[b]Modified from Hara, et. al. 2005.

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Table 3

Sensitivity of the cycle threshold (CT) values for 10-fold plasmid dilutions in the generic multiplex real-time PCR performed in triplicate. CT values are displayed where samples are amplified and undetermined (und) is displayed where samples were not amplified.

Plasmid Concentration	SRV-1 Plasmid	SRV-2 Plasmid	SRV-3 Plasmid	SRV-4 Plasmid	SRV-5 Plasmid
10^6copies/15ul	22.4, 22.7, 22.7	21.7, 21.8, 21.2	23.8, 24.4, 24.3	34.1, 33.3, 33.3	38.7, 38.6, 38.2
10^5copies/15ul	25.7, 26.0, 25.9	24.9, 25.3, 24.9	28.0, 28.0, 27.6	37.2, 36.2, 37.1	43.1, 43.2, 45.4
10^4copies/15ul	29.1, 29.4, 29.1	28.3, 30.1, 28.3	31.5, 31.7, 31.6	42.5, 42.1, 44.1	49.6, 52.1, 48.4
10^3copies/15ul	32.7, 32.7, 32.5	32.0, 32.3, 31.6	34.9, 35.1, 34.9	45.0, 50.4, 48.3	und, und, 48.0
10^2copies/15ul	36.9, 37.3, 36.2	35.0, 36.3, 35.5	38.2, 37.4, 40.6	und, und, 47.3	und, und, und
10^1copies/15ul	37.5, 39.6, und	38.1, 40.0, und	40.9, 42.0, 40.6	und, und, und	und, und, und
10^0copies/15ul	und, und, und	und, 41.3, 40.0	und, und, und	und, und, und	und, und, und
10^{-1}copies/15ul	und, 41.1, und	und, und, 40.1	und, und, und	und, und, und	und, und, und

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Table 4

SRV controls amplified in the multiplex and each of the uniplex real-time PCR assays and the generic multiplex OSRV assay.

		Sample type								
		negative whole blood control	**SRV 1 whole blood control**	**SRV 2 whole blood control**	**negative Raji cell culture control**	**SRV 3 Raji cell culture control**	**SRV 4 Raji cell culture control**	**SRV 5 CA SupT cell culture control**	**SRV 5 OR SupT cell culture control**	**Tsukuba SupT cell culture control**
PCR assay	SRV 1 only	und	**32.1**	und	und	und	und	und		und
	SRV 2 only	und	und	**29.3**	und	und	und	und	und	und
	SRV 3 only	und	und	und	und	**23.2**	und	und	und	und
	SRV 4 only	und	und	und	und	und	**26.5**	und	und	**26**
	SRV 5 only	und	und	und	und	und	und	**22.8**	**33.9**	und
	OSRV Generic	und	**30.6**	**23**	und	**22.2**	**27**	**22.7**	**34.8**	**29.5**

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Table 5

Different uniplex reaction mixtures for each of the five serotypes.

Reaction Contents:		SRV-1	SRV-2	SRV-3	SRV-4	SRV-5
	Forward Primer	0.625ul of 12uM	0.625ul of 12uM	0.5ul of 20uM	0.5ul of 20uM	1ul of 20uM
	Reverse Primer	0.625 of 12uM	0.625 of 12uM	0.5ul of 20uM	0.5ul of 20uM	1ul of 20uM
	Probe	0.625ul of 10uM	0.625ul of 10uM	0.5ul of 20uM	0.5ul of 20uM	0.5ul of 20uM
	TaqMan Universal master mix[c]	12.5ul	12.5ul	12.5ul	12.5ul	12.5ul
	1 × TE buffer	5.625ul	5.625ul	1ul	1ul	none
	DNA	5ul	5ul	10ul	10ul	10ul
total		25ul	25ul	25ul	25ul	25ul

[c] cat# 4304437, Applied Biosystems, Foster City, CA, USA

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Table 6

Results of generic multiplex real-time PCR assay for a panel of 44 DNA samples from macaques with predetermined SRV infection status. (*Kappa* = 0.8977).

		OSRV Multiplex PCR assay		
		Negative	**Indeterminate**	**Positive**
Infection status[d]	Negative	15	0	0
	Indeterminate	1	12	0
	Positive	0	2	14
	Total	16	14	14

[d] Infection status was determined by MMIA, WB, end point PCR and GSRV PCR.