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A versatile dual-use RT-PCR control for use in assays for the detection of peste des petits ruminants virus

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ABSTRACT

Peste des petits ruminants (PPR) is an acute and highly contagious disease with high mortality in small ruminants and significant socioeconomic impact in developing countries. The causative agent is peste des petits ruminants virus (PPRV). The Food and Agriculture Organization of the United Nations (FAO) and the World Organization for Animal Health (OIE) have set up a goal for the global eradication of PPR by 2030. To assist in this effort, an easily produced, specific, non-pathogenic bacteriophage Qβ based real-time RT-PCR (qRT-PCR) PPRV positive control was developed. This control is compatible for use with two previously described PPRV qRT-PCR assays either as singleplex or multiplex platform. Additionally, the control can also be used for assembling proficiency testing panels for competency testing in diagnostic laboratories. Use of the Qβ phage based PPRV control as a positive control or in proficiency testing panels reduces the risk of inadvertent release of pathogenic PPRV from diagnostic laboratories, which would be especially important should PPR be eradicated in the future.

The peste des petits ruminants virus (PPRV) belongs to the genus *Morbillivirus*, in the family *Paramyxoviridae*. PPRV is closely related to rinderpest virus (RPV), measles virus (MV) and canine distemper virus (CDV) (Baron et al., 2016). The 16 kb negative-sense single stranded RNA (ssRNA) genome codes for six structural and two nonstructural proteins. The virus may be categorized into four distinct lineages based on nucleocapsid (N) and fusion protein (F) gene sequences (Bao et al., 2008). PPRV causes peste des petits ruminants (PPR), an acute and highly contagious disease with high mortality in small ruminants, with significant socioeconomic impact leading to poverty, malnutrition, and social instability in developing countries (Banyard et al., 2010). Through international consensus, a PPR Global Control and Eradication Strategy (PPR GCES) was endorsed in 2015 at the International Conference for the Control and Eradication of PPR organized by the Food and Agriculture Organization of the United Nations (FAO) and the World Organization for Animal Health (OIE). The stated goal is to eradicate PPR globally by 2030 (FAO and OIE, 2016).

One of the key components of the effort to control and eradicate PPR is early detection of the PPRV. Several real-time RT-PCR (qRT-PCR) assays have been developed, and used for the PPR diagnosis (Forsyth and Barrett, 1995; Couacy-Hymann et al., 2002; Bao et al., 2008; Kwiatek et al., 2010; Batten et al., 2011). These assays target genomic locations conserved across all strains and lineages of PPRVs while excluding other closely related species in the genus. A valid qRT-PCR result relies on using well defined positive controls, which typically involves the use of the original agent. However, in certain circumstances (e.g. eradicated disease), use of the original agent is prohibitively risky and therefore not advisable. In such circumstances, a surrogate which mimics the behavior of the original agent but which carries none of the risks can be used as a control for the assay. When a surrogate is prepared for use as a control, nucleic acid sequences of the control should include specific binding sites for the primers and probes used in the assay and in the case of Positive Extraction Control (PEC) be encapsulated within a protein coat. It has been previously reported that introduction of primer/probe binding sites into the gene of the capsid protein A of Qβ phage can be used to generate Qβ phage particles for use in desired RNA-based viral diagnostic assays (Villanova et al., 2007). Other Qβ based controls including rinderpest virus, foot and mouth disease virus, and classical swine fever virus were previously developed in our laboratory and used for proficiency testing. These controls are non-pathogenic and valuable for broad use to support surveillance and proficiency testing of those high risk animal diseases.

As genetic libraries of pathogen strains grow, finding unique primer binding sites, which are virus species specific but generic enough to be used for positive identification of the breadth of variants within the species, is increasingly challenging. Incorporation of degenerate bases,

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nesting primer sets and targeting multiple genomic locations are all approaches that have been taken to avoid false positive and negative results (Forsyth and Barrett, 1995; Couacy-Hymann et al., 2002; Bao et al., 2008; Kwiatek et al., 2010; Batten et al., 2011).

To distinguish PPRV from other *Morbillivirus* species, regions within the N gene, F gene and phosphoprotein (P) gene have been targeted for specific primer selection. (Forsyth and Barrett, 1995; Bao et al., 2008). Many primer sequences described target the N gene which contains reduced 3' sequence homology with non-PPRV *Morbillivirus* and is the most profusely transcribed mRNA during a PPRV infection (Couacy-Hymann et al., 2002; Bao et al., 2008). Two assays developed by PPRV reference labs that utilize this region include the Kwiatek et al. (2010) and the Batten et al. (2011) assays. Both were evaluated using representatives from all four lineages of PPRV and are exclusive of other *Morbillivirus* (Kwiatek et al., 2010; Batten et al., 2011).

All current reported PPRV qRT-PCRs assays use live PPRV as positive controls, which increases the risk of PPRV release from a laboratory and could compromise global PPRV eradication efforts. Moreover, use of synthetic DNA plasmids as a PCR control is not adequate in a reverse transcription PCR assay for RNA virus detection. In a post eradication future, while appropriate PPR diagnosis and surveillance capacity must be maintained in diagnostic laboratories, the ability to obtain and manipulate PPRV will likely be highly regulated and restricted. To assist in the efforts of PPR eradication and post eradication PPR diagnosis and surveillance, this report describes the development of a Qβ phage based PPRV PCR control, which is non-pathogenic, distinguishable from wild type virus, and suitable for use in routine (single and multiplex) PPRV detection and proficiency testing associated with two previously described and widely used PPRV qRT-PCR assays (Kwiatek et al., 2010; and Batten et al., 2011). Both assays were developed by PPRV reference labs, result in similar sized amplicons and contain compatible primer and probe sequences. The PPRV phage-based controls amplicon sizes and cycling conditions are similar to the two assays. The amplification efficiency obtained utilizing the Batten primer probe combination and this studies chemistry, equipment and parameters was nearly identical to that reported by Batten et al. (95.2 and 95.7 % respectively) a slight drop to 93 % amplification efficiency was observed when the same experiment was performed utilizing a redesigned probe (Table 1).

Primer and probe sequences, plasmids and competent cells used in this study are listed in Tables S1 and S2. No significant similarities were found when selected Qβ primer and probe sequences were aligned to rinderpest morbillivirus (taxid:11241), peste des petits ruminants virus (taxid:31604), measles morbillivirus (taxid:11234), and canine morbillivirus (taxid:11232) genomic sequences utilizing the National Center for Biotechnology Information's nucleotide Basic Local Alignment Search Tool suite (BLASTN 2.9.0) (Zhang et al., 2000; Morgulis et al., 2008). The original probes for both PPRV assays [Kwiatek et al. (2010) and Batten et al. (2011)] (NPPRp and PPRVPROB) utilized a 5' FAM dye; to allow for multiplexing, the probes were redesigned (Table S1). The probes (KPPRV and BPPRV) contain the original NPPRp and PPRVPROB nucleotide sequences, respectively; however, the quenchers in both probes were changed to non-fluorescent quenchers (NFQ) QSY (Applied Biosystems). In addition, the FAM dye in PPRVPROB was changed to ABY (BPPRVp). The Qβ probe contains the dye JOE and a NFQ (IDT).

The design of the synthetic 198 bp control template sequence is shown in Fig. 1. Primer and probe binding sites for both the Kwiatek et al. (2010) and the Batten et al. (2011) assays as well as recombinant Qβ phage (rQβ) specific primer and probe sequences were integrated into the template. Primer sites for both were staggered to generate similar sized amplicons, 126 bp and 128 bp, respectively.

Construction of the rQβ phage control was done following the methods of Villanova et al. (2007) and Diane Holder (personal communication, 2018) . Briefly, the control template sequence was amplified in 50.0 µL reactions containing 25.0 µL GoTaq Green 2X master mix (Promega), 22.0 µL nuclease free water, 1.0 µL / each PPRVTem_F and PPRVTem_R (20 pmol/µL) (Supplemental Table 1), and 1.0 µL template sequence (0.1 pmol/µL). Cycling conditions consisted of 94 °C for 5 min followed by 35 cycles of amplification (94 °C for 1 min, 65 °C for 1 min and 72 °C for 30 s) and a 5 min final extension of 72 °C. The final PCR product was then gel purified (Invitrogen Clonewell II) and stored at -20 °C prior to digestion. The insert was digested with NsiI-HF and AFlIII [New England Biolabs (NEB)] at 37 °C for 4 h and then allowed to cool to room temperature (~20 °C) at which point the digested template was gel purified (Invitrogen Clonewell II) and stored at -20 °C prior to ligation (Fig. 2).

pBRT7Qβ plasmid DNA (Taniguchi et al., 1978) was digested with NsiI-HF and AFlIII (NEB) and dephosphorylated with FastAP (Thermo Scientific) at 4 °C overnight followed by incubation for 3.0 h at 37 °C

Table 1
Cq values of various primer probe combinations. Cq Values for singleplex reactions (a) approximate those of the multiplex reactions (b) with the exception of KPPRVp. Heterogeneous primer probe combinations utilizing QPPRV primers and KPPRVp or BPPRVp (c) resulted in Cq values similar to those observed in both QPPRVp singleplex and multiplex reactions.

a)

Primers	Probe	Cq (σ)	Amplification Efficiency	R^2
NPPRf / NPPRr	NPPRp	21.77 (± 0.34)	96.9	0.9989
NPPRf / NPPRr	KPPRVp	21.56 (± 0.26)	97.7	0.9999
PPRVFOR / PPRVREV	PPRVPROB	22.83 (± 0.30)	95.2	0.9977
PPRVFOR / PPRVREV	BPPRVp	27.28 (± 0.61)	93.0	0.9942
QPPRVf / QPPRVr	QPPRVp	28.33 (± 0.57)	90.3	0.9995

b)

Primers	Probe	Cq (σ)
NPPRf / NPPRr	KPPRVp	26.38 (± 0.05)
PPRVFOR / PPRVREV	BPPRVp	26.81 (± 0.15)
QPPRVf / QPPRVr	QPPRVp	27.89 (± 0.05)

c)

Primers	Probe	Cq (σ)
QPPRVf / QPPRVr	KPPRVp	27.93 (± 0.04)
QPPRVf / QPPRVr	BPPRVp	27.84 (± 0.08)

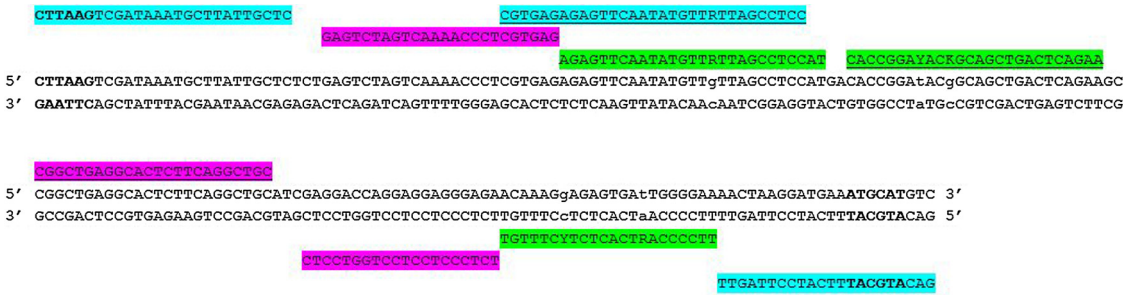

Fig. 1. Synthetic control template sequence designed for construction of PPRV_rQβ Phage. The template sequence incorporates Kwiatek et al. (2010) (red) and Batten et al. (2011) (green) primer and probe sites as well as rQβ specific primer and probe (underlined) sites (blue). Amplicon sizes are 128 bp, 126 bp, and 201 bp respectively. Lowercase bases denotes degenerate primer and probe sites. Restrictions sites AflII (CTTAAG) and NsiI (ATGCAT) are shown in bold.

and heat inactivation at 80 °C for 20 min. The PPRV control template was ligated into the 3′ end of the A1 gene of phage Qβ located on pBRT7Qβ in a 30.0 µL reaction containing 1.0 µL T4 ligase (NEB); 11.0 µL nuclease free water; 15.0 µL 2X ligation buffer (NEB); 1.0 µL digested pBRT7Qβ (29 ng) and 2.0 µL digested control insert (6.0 ng). the reaction was allowed to proceed for 45 min at room temperature and then moved to 4 °C overnight.

Transformation via electroporation was carried out using 2.0 µL of ligation reaction and 100.0 µL of *Escherichia coli* DH5α (Invitrogen) electrocompetent cells. Following electroporation, transformed cells were suspended in 900.0 µL of SOC (Invitrogen) and incubated 37 °C with orbital agitation (~200 rpm) for 2 h. Following outgrowth, the

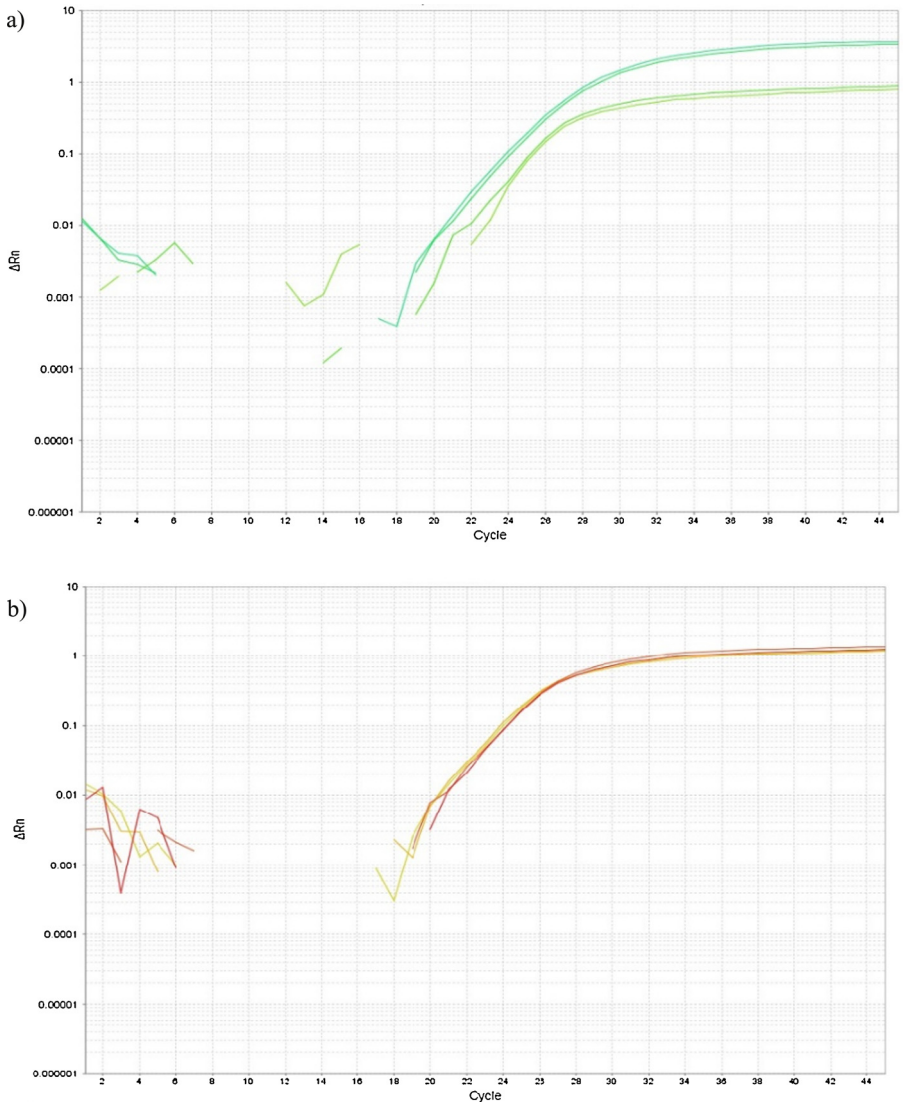

Fig. 2. Singleplex amplification plots of RT-PCR reactions. Singleplex amplification plots of RT-PCR reactions comparing either BPPRVp and PPRVPROB (a) or NPPRp and KPPRVp (b). The dye and quencher in PPRVPROB (light green) was modified to ABY and a QSY respectively creating BPPRVp (dark green). Replacement of the TAMRA quencher by a NFQ resulted in higher fluorescent intensity. The quencher of NPPRp (red) was changed to QSY creating KPPRVp (yellow); NPPRp and KPPRVp profiles are virtually indistinguishable.

culture was plated on Lysogeny broth Miller formulation (LB) plates (Teknova) containing 100 μg/mL carbenicillin and incubated at 37 °C overnight. Colony PCR (cPCR) was performed to screen for positive colonies. Briefly, potentially positive colonies were suspended in 8.0 μL of nuclease free water, 1.0 μL of which was added to 50.0 μL PCR reactions containing 25.0 μL GoTaq Green 2X master mix (Promega); 22.0 μL nuclease free water; 1.0 μL/each KPPRV_F and KPPRV_R (20 pmol/μL). Cycling conditions consisted of 94 °C for 5 min followed by 30 cycles of amplification (94 °C for 30 s, 60 °C for 30 s and 72 °C for 30 s) and a 5 min final extension at 72 °C. PCR products were visualized on a 2 % agarose gel (Invitrogen E-gel). A colony containing the expected 128 bp amplicon was cultured overnight at 37 °C with orbital agitation (200 rpm) in 2.0 mL LB broth after which a plasmid purification was performed (Qiagen). The purified pPPRV_rQβ plasmid was screened by PCR utilizing NPPR, PPRV and QPPRV primer sets with identical reaction and cycling conditions as the cPCR. Expected amplicons for each primer set (128 bp, 126 bp, and 201 bp respectively) were visualized on a 2 % agarose gel (Invitrogen E-gel). A three hour diagnostic digest was then performed on the vector utilizing identical reaction conditions as described above. The digest was run on a 2 % agarose E-gel SizeSelect II (Invitrogen). The plasmid was linearized as expected and a 200 bp band was visualized on the gel. Purified plasmid pPPRV_rQβ was then used to transform electrocompetent *E. coli* BL21 (DE3) pLysS cells (Promega).

PPRV_rQβ phage particles, containing the synthetic control sequence packaged as ssRNA, was prepared from a 1:100 dilution of an overnight culture of *E. coli* BL21 (DE3) pLysS cells transformed with pPPRV_rQβ in LB broth that was incubated at 37 °C with orbital agitation. After 3 h the 100.0 mL culture was induced with 1.0 mL of 100 mM IPTG (Teknova) and returned to incubate overnight. The overnight culture was then pelleted (10,000 x g, 15 min, 16 °C), the cell free supernatant was transferred to fresh tubes and pelleted again as previously described. The supernatant was transferred again to fresh tubes and treated with 5.0 μL Benzonase endonuclease (Sigma) to digest any contaminating DNA or unpackaged RNA. The digest was allowed to proceed overnight at 4 °C. After digestion the phage particles were precipitated by a 25.0 mL addition of 2.5 M NaCl / 20 % PEG 6000 (Teknova) which was then incubated at 4 °C overnight. The phage precipitation was then pelleted 10,000 x g, for 4 h at 4 °C. Once pelleted, the supernatant was removed and the phage pellets suspended in SM buffer (NaCl (Sigma) 0.1 M, MgSO4*7H20 (Fluka) 8.0 mM, Tris HCl (IBI Scientific) 50 mM, gelatin (BD) 2 %, ddH2O 500 mL).

To determine the concentration of phage isolate that would produce quantification cycle (Cq) values between 23 and 29, A tenfold dilution series of the isolate was created in nucleic acid dilution solution (AB). Each dilution of phage was extracted using a MagMax Pathogen RNA/DNA kit (Applied Biosystems) and a KingFisher 96 magnetic particle processor (Thermo Scientific). Purity of the RNA was assessed utilizing the ratio of absorbance at 260 nm and 280 nm with a NanoDrop spectrophotometer (Thermo Fisher). The average $A_{260/280}$ ratio of the extracted RNA was 1.97. Primer probe working stocks for each assay were created containing 20 pmol/ μL of each primer (IDT) and probe (IDT or Applied Biosystems). RT-PCR reactions were prepared in triplicate for each assay (6.25 μL TaqMan™ Fast Virus One-Step Master Mix, 1.25 μL primer probe mix, 15.0 μL nuclease free water and 2.5 μL of extracted phage dilutions) on a 96 well plate (MicroAmp Fast Optical, Applied Biosystems) and cycled on an ABI 7500 (Applied Biosystems) for 45 cycles of 95 °C for 3 s and 60 °C for 30 s following an initial 50 °C 5 min reverse transcription and 95 °C 20 s inactivation step. An internal reference dye (ROX) was used to normalize fluorescence. All PCR reactions contained a no-template control (NTC) containing nuclease free water (Ambion) in lieu of RNA.

The appropriate dilution that provided the ideal Cq range was selected and used to generate PECs in tris-buffered tryptose broth (TBTB) (26.0 g/L tryptose broth (BD), 1.21 g/L Trizma Base (Sigma), 18.0 mg/L phenol red (Sigma), 1000 mL H_2O, 500 mg gentamicin sulfate (Sparhawk), 2.5 mg Amphotericin B (Sigma). The inoculated TBTB was mixed with slow orbital agitation overnight at 4 °C to aid homogenization. Following the overnight agitation the inoculated TBTB was aliquoted (500 μL) to individual cryogenic tubes. To ensure that the aliquots were homogeneous, ten tubes were selected at random and 200 μL from each PEC was extracted in duplicate as described previously. An aliquot of 2.5 μL of each extraction was then used for qRT-PCR reactions performed in triplicate utilizing the NPPRp primer probe mix. Amplification resulted in a mean Cq value of 20.5 ± 0.12. The remaining extracts were pooled, diluted 1:10 in 1X TE buffer pH 8 (IDT) and aliquoted (50.0 μL) to individual cryogenic tubes to create positive amplification control (PAC) stocks. Ten tubes of PAC were selected at random and tested for homogeneity as described previously.

Amplification efficiency (AE) was analyzed via singleplex PCR reactions performed in triplicate utilizing each primer probe set, 1.0 μL of a twofold PPRV_rQβ PAC template serial dilution and identical cycling conditions as previously described. Mean AE was calculated by linear regression analysis of dilution Cq values using the following equation; efficiency = $-1 + 10^{(-1/\bar{x}\text{slope})}$. Resulting Cq values ranged from 21.56 ± 0.26–28.33 ± 0.57 while efficiency values ranged from 90.3%–97.7% (Table 1a). Additionally, in all instances R^2 values were > 0.99 with the exception of reactions performed with PPRVPROB (> 0.97) (Fig. S1).

Fluorescent intensity of the BPPRVp reaction curve is greater than that of the PPRVPROB curve (Fig. 1a). Conversely, amplification plots of reactions NPPRp and KPPRVp are virtually identical (Fig. 1b). Multiplex assays were performed in triplicate under identical cycling conditions (Table 1b). A Primer probe multiplex working stock was created from equal volumes of QPPRVp, BPPRVp and KPPRVp primer / probe working stocks. Multiplex Cq values resembled those obtained from singleplex amplifications with the exception of KPPRVp whose Cq values increased. RT-PCR experiments were also carried out on extracted PPRV_rQβ utilizing QPPRV forward and reverse primers and either KPPRVp or BPPRVp which generated Cq values of 27.93 ± 0.04 and 27.84 ± 0.08 respectively (Table 1C).

Elimination of PPRV will also rely in part on effective PCR screening measures. As more PPRV strains are discovered and sequenced, assays must be continuously evaluated to ensure ongoing efficacy towards all lineages of the virus while maintaining specificity to PPRV. Minor future modifications of existing primer and or probe sequences may be deemed necessary to enhance hybridization. Decreased probe sensitivity towards lineage III due to genetic divergence has already been described by Kwiatek et al. (2010). Modifications to overcome such divergence could result in incorporation of degenerate primer and/or probe bases, or incorporation of more genetic sites. In either event, modification of our control template sequence to accommodate such eventualities can be easily achieved.

In the RPV post eradication era, all laboratories around the world that had RPV containing materials were required by FAO and OIE to either destroy their RPV-containing materials or send the materials to a handful of FAO-OIE designated high containment RPV holding facilities. Manipulation of RPV without appropriate authorization is prohibited. Reducing and eliminating manipulation of PPRV is a critical part of the efforts in PPR eradication and PPR freedom maintenance of post-eradication. The PPR freedom maintenance is likely to mimic those of the eradicated rinderpest. Manipulating live virus for purposes of research, proficiency testing or diagnostic controls will be, in most if not all of cases, prohibited. Never-the-less, diagnostic capacity and proficiency of laboratory staff must be maintained.

Qβ phage-based non-pathogenic PEC and PAC controls for the real-time PCRs of foot and mouth disease virus (FMDV), classical swine fever virus (CSFV), and RPV were developed in our lab several years ago, and provided to many domestic and some international laboratories for the surveillance and diagnosis of transboundary animal diseases. In addition to the Qβ phage-based PEC and PAC controls, proficiency test panels are also developed, and supplied to those laboratories. These blinded panels consist of several PECs of differing

dilutions and negative materials.

Successful generation of this Qβ phage based PPRV qRT-PCR control provides a proof of concept for a single control to be utilized in multiple assays both as a positive extraction control as well as a positive RT and amplification control. This non-pathogenic, non-infectious control is cost effective to produce, is easily distinguishable from the target virus, and is easily modifiable towards new target sequences. Additionally, this control can be used to generate proficiency testing panels for use with either described assay eliminating the need to manipulate live virus. Future use of this control may be expanded for the diagnosis of multiple different disease through careful design of the template sequence.

Author contributions statement

W. J. conceived the study, supervised the project, and revised the manuscript. J.L. designed the PCR template and built the constructs. D.H. gave technical support and conceptual advice. J.L. designed, administered and performed the experiments. J.L. collected, analyzed and interpreted the data and drafted the manuscript. All three authors discussed the results. K. D. reviewed and edited the manuscript.

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Declaration of Competing Interest

The authors declare that they have no competing interests.

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Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at doi:https://doi.org/10.1016/j.jviromet.2019.113799.

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