Identification of the Suitable Insertion Site for Expression of Spike Gene of A Porcine Epidemic Diarrhea Virus Variant in A Pseudorabies Virus Vector

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Research Article

Keywords: pseudorabies virus, bacterial artificial chromosome, noncoding region, insertion site, spike gene, porcine epidemic diarrhea virus

Posted Date: December 16th, 2020

DOI: https://doi.org/10.21203/rs.3.rs-124699/v1

Abstract

Background: The emergence of variant porcine epidemic diarrhea virus (PEDV) strain and pseudorabies virus (PRV) in China in recent years has decreased the effectiveness of CV777 and Bartha K61 vaccines, causing significant loss to the swine industry. Previously, we generated a TK&gE-deleted PRV ($PRV^{TK\&gE\text{-}AH02}$) based on a virulent PRV AH02LA strain. It was shown to be safe for 1-day-old piglets with maternal PRV antibodies and 4–5 week-old PRV antibody negative piglets and provide rapid and complete protection in weaned pigs against lethal challenge with the PRV variant strain. $PRV^{TK\&gE\text{-}AH02}$ may be a promising live vaccine vector for construction of recombinant vaccine in pigs. PEDV spike (S) protein is mainly used in the development of PEDV vaccines. Therefore, the gene-deleted PRV (from PRV variants) vectored vaccine expressing variant PEDV S gene may be viable PEDV and PRV vaccine candidates. However, insertion site is an important factor affecting foreign gene expression and vaccine efficacy.

Results: In this study, we constructed four recombinant PRV-S bacterial artificial chromosomes (BACs) carrying the same S expression cassette in different noncoding regions (UL11-10, UL35-36, UL46-27 or US2-1) from AH02LA BAC with TK, gE and gI deletion. The successful expression of S gene (UL11-10, UL35-36 and UL46-27) in recombinant viruses was confirmed by virus rescue, PCR, RT-PCR and indirect immunofluorescence. We observed higher S gene mRNA expression level in Swine testicular cells infected with PRV-S(UL11-10)ΔTK/gE and PRV-S (UL35-36)ΔTK/gE compared to that of PRV-S(UL46-27)ΔTK/gE at 6 hour post infection ($P < 0.05$). Moreover, at 12 hour post infection, cells infected with PRV-S (UL11-10)ΔTK/gE exhibited higher S gene mRNA expression than those infected with PRV-S (UL35-36)ΔTK/gE ($P = 0.097$) and PRV-S (UL46-27)ΔTK/gE ($P < 0.05$). Recovered vectored mutant PRV-S (UL11-10, UL35-36 and UL46-27) exhibited similar growth kinetics to the parental virus ($PRV^{TK\&gE\text{-}AH02}$).

Conclusions: The identification and comparison of the insertion sites in PRV genome laids a foundation for future development of recombinant PRV vaccines.

Background

Porcine epidemic diarrhea (PED), a highly contagious disease of pigs, is caused by porcine epidemic diarrhea virus (PEDV) and has caused substantial economic losses to swine industry in many countries [1-3]. PEDV infection may cause high mortality (50%-100%) in the neonatal piglets and suckling piglets, and weight loss in older pigs. In China, large-scale outbreaks of PED caused by new emerging PEDV variants took place in many pig since 2010, and rates of morbidity and fatality approaches 100% in 1-week old piglets [4]. Previous studies have shown that attenuated, inactivated, or subunit PEDV vaccines fail to induce sufficient levels of lactogenic immunity and mucosal immune response in piglets [5, 6]. Therefore, it is critical to develop a safe and efficient vaccine to control PED. PEDV is an *Alpha-coronavirus* belonging to the family *Coronaviridae*. Its genome is a 28 kb-long, single-stranded, positive-sense RNA encoding for seven open reading frames (ORFs), including four structural proteins: spike (S), integral membrane, envelope and nucleoprotein [7]. S protein has higher antigenicity than other encoded proteins, playing an important role in attachment and entry of virus and induction of neutralizing antibodies [8, 9]. Variant PEDV strains isolated from various regions in China contain sequence mutations (base insertions and deletions) in S protein compared with the classic strains [10], which may be responsible for the increased virus transmission and the reduced protection efficiency of currently available commercial vaccines. Therefore, S gene of the variant PEDV strain is an appropriate viral gene for developing new genetically engineered vaccine.

Pseudorabies viruses (PRV) belongs to the herpesvirus group. Its genome is approximately 145 kb containing some nonessential regions that can be replaced by heterologous gene [11]. Several previous reports have shown that recombinant PRVs expressing foreign antigens are able to stimulate humoral and cell-mediated immune responses against both PRV and other viruses [12, 13]. Thus, the attenuated PRV strain might be a promising live vaccine vector for

expressing antigens of other pathogens. Insertion of the foreign gene should have a minimal effect on growth characteristics, vaccine efficacy and vector quality of parental virus. Several non-essential genes sites (gG, gI, gE and gI gene, and TK) are usually the targets of foreign gene insertions for PRV vaccine vectors [14-17]. However, insertion of foreign genes into non-essential genes may partly affect properties of the parental virus, expression of foreign antigens and efficacy of vaccine [15]. Therefore, it is important to identify other sites in PRV genome where foreign genes can be inserted and expressed stably without disrupting properties of the parental virus.

In PRV genome, several noncoding regions are located in the *downstream of two ORFs* in the *opposite* direction [18]. Insertion of a foreign gene into these noncoding regions should not disrupt any viral genes expression. Therefore, noncoding region in the *downstream of two ORFs* in the *opposite* direction may be suitable sites for insertion of foreign genes. Recently, we generated a TK&gE-deleted PRV ($PRV^{TK\&gE\text{-}AH02}$), which is safe for 1-day-old piglets with maternal PRV antibodies and 4–5 week-old PRV antibody negative piglets and can provide complete protection in weaned pigs against challenged with virulent AH02LA strain at 7 days post vaccination [19]. It suggests that $PRV^{TK\&gE\text{-}AH02}$ is a technically appropriate vector for the expression of foreign antigens of other swine diseases. In this study, we constructed recombinant PRVs expressing S gene of a variant PEDV strain in which S expression cassette was inserted in the noncoding regions (UL11-10, UL35-36, UL46-27) of $PRV^{TK\&gE\text{-}AH02}$ genome and evaluated the effect of insertion site on antigen expression and growth ability of the vector virus.

Results

Construction of PRV-S bacterial artificial chromosome (BAC)

Based on the AH02LA BAC with TK, gE and gI deletion ($BAC^{PRV\Delta TK/gE/gI}$), noncoding regions (UL11-10, UL35-36, UL46-27 or US2-1) was replaced with S^{cas}-KAN (S expression cassette with kanamycin resistance gene) through the first recombination. The kanamycin resistance gene was deleted from the S expression cassette through a second recombination, generating four PRV-S recombinant BACs ($BAC^{PRV\text{-}S(UL11\text{-}10)\Delta TK/gE/gI}$, $BAC^{PRV\text{-}S(UL35\text{-}36)\Delta TK/gE/gI}$, $BAC^{PRV\text{-}S(UL46\text{-}UL27)\Delta TK/gE/gI}$ and $BAC^{PRV\text{-}S(US2\text{-}1)\Delta TK/gE/gI}$, Fig. 1). Restriction fragment length polymorphism (RFLP) analysis of these constructs with *BamH* I digestion corresponded with the predicted pattern with minor differences (Fig. 2). S expression cassette was confirmed by PCR and sequencing (data not shown).

Generation of recombinant PRVs expressing S gene of PEDV from cloned DNA

To generate recombinant PRVs expressing S gene of PEDV, co-transfection of DNA of $BAC^{PRV\text{-}S(UL11\text{-}10)\Delta TK/gE/gI}$, $BAC^{PRV\text{-}S(UL35\text{-}36)\Delta TK/gE/gI}$, $BAC^{PRV\text{-}S(UL46\text{-}UL27)\Delta TK/gE/gI}$ or $BAC^{PRV\text{-}S(US2\text{-}1)\Delta TK/gE/gI}$ and H1-H2-gI-ΔgE, non-fluorescent plaques of PRV-S(UL11-10)ΔTK/gE, PRV-S(UL35-36)ΔTK/gE and PRV-S(UL46-27)ΔTK/gE were observed under UV light (488nm) (Fig. 3). $BAC^{PRV\text{-}S(US2\text{-}1)\Delta TK/gE/gI}$ failed to rescue the virus, indicating that US2-1 region may be crucial for PRV replication. To obtain a homogeneous population, one plaque was isolated after 5 rounds of plaque purification. The correct sequences of the inserted S expression cassette and H1-H2-gI-ΔgE of PRV-S (UL11-10)ΔTK/gE, PRV-S (UL35-36)ΔTK/gE and PRV-S (UL46-27)ΔTK/gE were confirmed by PCR and sequencing (Fig. 4). To investigate the genetic stability of S expression cassette, the recombinant viruses were passaged 20 times on Swine testicular (ST) cells. The viral DNAs were extracted, and S expression cassette was detected by PCR. The correct sequences of the inserted S expression cassette were confirmed by sequencing (data not shown).

Comparative analysis of S expression capacity

S and PRV protein expression in recombinant PRVs were confirmed by using indirect immunofluorescence assay (IFA) in infected ST cells (Fig. 5). Cells infected with PRV-S(UL11-10)ΔTK/gE, PRV-S(UL35-36)ΔTK/gE and PRV-S(UL46-

27)ΔTK/gE reacted with anti-S and anti-PRV antibody, emitting a green fluorescent signal under UV light (488 nm). However, no fluorescence for S protein was detectable in cells infected with $PRV^{TK\&gE\text{-}AH02}$ or ST cells (Fig. 5). S gene mRNA expression of recombinant PRVs in ST cells was also confirmed by RT-PCR (Fig. 6). S gene mRNA expression in three recombinant viruses differed at 6 and 12 hours post infection, and cells infected with PRV-S (UL11-10)ΔTK/gE and PRV-S (UL35-36)ΔTK/gE exhibited higher S gene mRNA expression than that infected with PRV-S (UL46-27)ΔTK/gE at 6 hour post infection ($P < 0.05$). At 12 hour post infection, S gene mRNA expression from PRV-S (UL11-10)ΔTK/gE was higher than those from PRV-S (UL35-36)ΔTK/gE ($P = 0.097$) and PRV-S (UL46-27)ΔTK/gE ($P < 0.05$).

Growth kinetics of recombinant PRVs expressing S gene of PEDV

The growth kinetics of the PRV AH02LA, $PRV^{\Delta TK\&gE\text{-}AH02}$, PRV-S(UL11-10)ΔTK/gE, PRV-S(UL35-36)ΔTK/gE and PRV-S(UL46-27)ΔTK/gE on ST cells were shown in Fig. 7. The growth kinetics of PRV-S(UL11-10)ΔTK/gE, PRV-S(UL35-36)ΔTK/gE and PRV-S(UL46-27)ΔTK/gE were similar to those of $PRV^{\Delta TK\&gE\text{-}AH02}$, indicating that S expression cassette insertion did not affect the replication of parental virus. However, at 6, 36, 48 and 60 hours post infection, the titers of $PRV^{\Delta TK\&gE\text{-}AH02}$, PRV-S(UL11-10)ΔTK/gE, PRV-S(UL35-36)ΔTK/gE and PRV-S(UL46-27)ΔTK/gE were lower than PRV AH02LA, which may due to the deletion of TK. Peak titers for PRV-S(UL11-10)ΔTK/gE, PRV-S(UL35-36)ΔTK/gE and PRV-S(UL46-27)ΔTK/gE were $10^{7.87}$, $10^{8.27}$ and $10^{8.05}$ $TCID_{50}$/mL, respectively (Fig. 7).

Discussion

It has been reported that attenuated PRV strain may be developed as virus vectors to express foreign genes [11]. A TK&gE-deleted ($PRV^{TK\&gE\text{-}AH02}$) previously constructed in our lab may be an appropriate vector for the expression of foreign antigens. The S protein of PEDV is the main target for neutralizing antibodies, and can induce immune protection [8]. Therefore, recombinant $PRV^{TK\&gE\text{-}AH02}$ expressing S protein may be a novel multivalent vaccine candidate against PRV and PEDV. However, insertion site is the main factor influencing foreign gene expression and vaccine efficacy. In PRV genome, noncoding region in the *downstream of two ORFs* with *opposite* direction may be suitable sites for insertion of foreign genes. In this study, we constructed four recombinant PRV-S BACs based on AH02LA BAC with TK, gE and gI deletion in which S expression cassette was inserted in the different noncoding regions (UL11-10, UL35-36, UL46-27 or US2-1). Based on virus rescue, PCR and IFA analysis, S gene inserted in the UL11-10, UL35-36 and UL46-27 region of PRV were successfully expressed. However, $BAC^{PRV\text{-}S(US2\text{-}1)\Delta TK/gE/gI}$ in which US2-1 region was replaced with S expression cassette failed to rescue the recombinant PRV. S gene mRNA expression of PRV-S(UL11-10)ΔTK/gE and PRV-S(UL35-36)ΔTK/gE were significantly higher than that of PRV-S(UL46-27)ΔTK/gE at 6 hour post infection. At 12 hour post infection, PRV-S (UL11-10)ΔTK/gE exhibited highest S gene mRNA expression among the tested three recombinant PRVs. The study first reported noncoding region of PRV as foreign gene insertion site, which provide new appropriate sites for construction of PRV-based vaccines.

Compared with traditional homologous recombination, BAC-based genetic manipulation platform for PRV facilitates the generation of recombinant PRVs with gene deletions or insertions. A few of PRV genomes have been maintained in BACs as infectious clones [20, 21]. $BAC^{PRV\Delta TK/gE/gI}$ was previously constructed in our lab, and used to generate PRV-S BAC by *En Passant* protocol in this study. It is surprising for us to find out that a recombinant PRV BAC in which US2-1 region was replaced with S expression cassette was not successfully rescued, which indicates that US2-1 may be crucial for PRV replication.

To develop a PRV vector-based vaccine, it is important that the recombinant virus still retains the growth ability of the vector virus. In this study, there was no difference in virus titers between three recombinant PRVs expressing S gene and the parent virus $PRV^{TK\&gE\text{-}AH02}$, indicating that the insertion of the S gene in the UL11-10, UL35-36 or UL46-27 did not influence the growth of the vector virus. Since three sites used in this study for insertion of S expression cassette were

noncoding region in the *downstream of two ORFs* with *opposite* direction, the insertion of S expression cassette at the above three sites might not influence the PRV replication on ST cells.

Foreign gene expression level is correlated with live vector vaccine efficacy [22]. Therefore, we investigated S expression of three recombinant PRVs by IFA and RT-PCR. The results revealed that S protein inserted in the UL11-10, UL35-36 and UL46-27 region of PRV were successfully expressed. ST cells infected with PRV-S(UL11-10)ΔTK/gE and PRV-S(UL35-36)ΔTK/gE showed significantly higher S gene mRNA expression level than those infected with PRV-S(UL46-27)ΔTK/gE at 6 hour post infection. At 12 hour post infection, S gene mRNA expression level on ST cells infected with PRV-S(UL11-10)ΔTK/gE was higher than those of PRV-S (UL35-36)ΔTK/gE and PRV-S (UL46-27)ΔTK/gE. Considering S gene expression and viral replication rate observed in this study, we propose that UL11-10 may be an optimal insertion site among three noncoding regions for foreign gene expression.

Conclusion

We constructed three recombinant PRVs in which S expression cassette was inserted in the UL11-10, UL35-36 or UL46-27 region of the $PRV^{TK\&gE\text{-}AH02}$ genome through BAC technology using homologous recombination. S gene inserted in three noncoding regions of PRV were expressed. High S gene mRNA expression level was discovered in ST cells infected with PRV-S (UL11-10)ΔTK/gE at 6 and 12 hours post infection among the tested recombinant PRVs. Furthermore, S expression cassette inserted in the UL11-10, UL35-36 or UL46-27 region did not affect the replication of parental virus. The identification and comparison of the insertion sites in PRV genome in this study will be useful for the further development of recombinant PRV vaccines. Future studies involving animal experiments are necessary to evaluate safety and efficacy of three recombinant PRVs.

Methods

Viruses, cells and plasmids

The PRV AH02LA strain (a PRV variant) was isolated and identified in our lab [23]. A gE/TK-deleted PRV ($PRV^{TK\&gE\text{-}AH02}$) based on the PRV AH02LA strain were constructed in our lab [19]. ST cells from CVCC was cultured in Dulbecco's Modified Eagle's Medium (Gibco, USA) supplemented with penicillin (100U/mL), streptomycin (100μg/mL) and 2–10% fetal calf serum in a humidified incubator with 37°C and 5% CO_2. The S gene was amplified by reverse transcription PCR with a pair of primers (S-F and S-R) from jejunal tissue sample of piglets that displayed typical PED clinical symptoms (acute watery diarrhea and vomiting) as previously described [24]. The amplified fragment was cloned to downstream of a pMCMV promoter. The resulting S gene expression cassette was inserted pMD19 (Takara), and named pS-T. Furthermore, to construct plasmid pS-KAN-T, a kanamycin resistance gene was cloned into the *EcoR* I site in pS-T.

Bacterial manipulations, PCR and sequencing

AH02LA BAC with TK, gE and gI deletion ($BAC^{PRV\Delta TK/gE/gI}$) constructed previously in our lab [19], was used to generate PRV-S BACs. Electroporation was carried out as described earlier [25]. Plasmid and PRV-S BAC DNA were performed with commercial kits (QIAGEN) according to manufacturer's instructions. PRV-S BAC was confirmed by RFLP with *BamH* I.

Primers KAN ins S F/KAN ins S R (Table 1) with two *EcoR* I restriction sites in both terminals for cutting and ligation were used to insert a kanamycin resistance gene into plasmid S-T. Primers (PRV ins S cas UL11-10 F/PRV ins S cas UL11-10 R, PRV ins S cas UL35-36 F/PRV ins S cas UL35-36 R, PRV ins S cas UL46-27 F/ PRV ins S cas UL46-27 R or PRV ins S cas US2-1 F/PRV ins S cas US2-1 R; Table 1) were used to insert the S^{cas}-KAN into the UL11-10, UL35-36, UL46-27 or US2-1 of $BAC^{PRV\Delta TK/gE/gI}$ through the *En Passant* protocol [26]. Specific primers (S cas check F/S cas check R, Table 1) were used to verify the sequence of the inserted S gene expression cassette. A pair of primers (PRV BAC H1 F and PRV BAC H2 R)

were used to amplify a DNA fragment (H1-H2-gI-ΔgE) including the gI gene, part of gE with the deletion of the 1286bp fragment (position 13 to 1298), and upstream and downstream homologous sequences using PRV LA-A^B strain DNA as template. The primers PRV ΔgE check F and PRV ΔgE check R were used for sequencing gI and ΔgE gene. S expression cassette were confirmed by sequencing (S cas check F and S cas check R).

Generation of recombinant PRVs expressing S gene of a PEDV variant

S expression cassette was inserted into the noncoding area of $BAC^{PRV\Delta TK/gE/gI}$ to replace nucleotide fragments of UL11-10, UL35-36, UL46-27 or US2-1 through the *En Passant* method [26]. Briefly, S^{cas}-KAN with 40-bp homologous sequences of PRV in both terminals were amplified. After digestion with *Dpn* I, four PCR products was electroporated into GS1783 with $BAC^{PRV\Delta TK/gE/gI}$ to achieve the first recombination at the UL11-10, UL35-36, UL46-27 or US2-1 sites. Four target recombinant clones were generated by deletion of the kanamycin resistance gene through the second recombination (Fig. 1). Selected clones were confirmed by RFLP after digestion with *BamH* I, and named $BAC^{PRV\text{-}S(UL11\text{-}10)\Delta TK/gE/gI}$, $BAC^{PRV\text{-}S(UL35\text{-}36)\Delta TK/gE/gI}$, $BAC^{PRV\text{-}S(UL46\text{-}UL27)\Delta TK/gE/gI}$ and $BAC^{PRV\text{-}S(US2\text{-}1)\Delta TK/gE/gI}$. Moreover, S gene expression cassette was confirmed using PCR and sequencing. To obtain recombinant PRVs expressing S gene of PEDV, ST cells were transfected with approximately 1μg $BAC^{PRV\text{-}S(UL11\text{-}10)\Delta TK/gE/gI}$, $BAC^{PRV\text{-}S(UL35\text{-}36)\Delta TK/gE/gI}$, $BAC^{PRV\text{-}S(UL46\text{-}UL27)\Delta TK/gE/gI}$ or $BAC^{PRV\text{-}S(US2\text{-}1)\Delta TK/gE/gI}$ and 1μg H1-H2-gI-ΔgE. One to two days after transfection, non-fluorescent plaques were selected and purified to obtain homogeneous population of recombinant PRVs expressing S gene of PEDV under UV light (488nm). The S expression cassette and H1-H2-gI-ΔgE were identified with PCR and sequencing. To evaluate the genetic stability of S gene, the recombinant viruses were passaged 20 times on ST cells, and S expression cassette was verified using PCR and sequencing.

Indirect immunofluorescence assay

The ST cells were infected with $PRV^{TK\&gE\text{-}AH02}$ or recombinant PRVs expressing S gene of PEDV. At 2 day post inoculation, cells were washed 3 times with phosphate buffered saline (PBS) and fixed with cold fixing solution (96% ethanol: acetone =3:1) for 20 min. Cells were then washed three times with PBS and blocked with PBS+10% bovine serum albumin for 1 h. After this blocking reaction, the cells were incubated with anti-S or anti-PRV pig serum produced in our lab at 37°C for 1 h. After washing three times with PBS, the cells were incubated with FITC-labeled goat anti-pigs IgG (Solarbio, diluted 1:100) at 37°C for 1 h. Cells were then washed as above and analyzed by inversion fluorescence microscope.

Real-time quantitative PCR

To evaluate S gene mRNA expression on the cell surface, ST cells in the six-well plates were infected with $PRV^{\Delta TK\&gE\text{-}AH02}$ or recombinant PRVs expressing S gene of PEDV at a multiplicity of infection (MOI) of 10. At 6 and 12 hours post infection, infected-cells were separately harvested. Total RNA of infected-cells was extracted using TRIzol reagent [27]. A total of 1μg total RNA from different treatments was reverse transcribed using a PrimeScript® RT Reagent Kit with gDNA Eraser (Takara Bio). Real-time quantitative PCR with a pair of primers for S gene (S exp F/ S exp R, Table 1) was carried out on Roche Light Cycler® 480 system (Roche Diagnostics, Burgess Hill, UK) using SYBR Premix Ex Taq dye (Takara) [28]. Each cDNA was analyzed in triplicate, and sample data were normalized to Beta actin expression using the $2^{-\Delta\Delta Ct}$ method.

Multi-step growth kinetics

Multi-step growth kinetics of PRV AH02LA, $PRV^{\Delta TK\&gE\text{-}AH02}$ and recombinant PRVs expressing S gene of PEDV were tested on ST cells with a MOI of 0.01 as described previously [21], the culture cells were harvested at 6, 12, 24, 36, 48 and

60 hours post infection, and then were titrated in cell monolayers. Experiments were performed in triplicate.

Abbreviations

BAC: Bacterial artificial chromosome; DNA: Deoxyribonucleic acid; gE: glycoprotein E; gG: glycoprotein G; gI: glycoprotein I; IFA: Indirect immunofluorescence assay; MOI: Multiplicity of infection; PCR: Polymerase chain reaction; PED: Porcine epidemic diarrhea; PEDV: Porcine epidemic diarrhea virus; ORFs: Open reading frames; PRV: Pseudorabies virus; RFLP: Restriction fragment length polymorphism; S: Spike; ST cells: Swine testicular cells; TCID50: 50% tissue culture infectious dose; TK: Thymidine kinase; UL: Unique long; US: Unique short

Declarations

Ethics approval and consent to participate

Not applicable

Consent to publish

Not applicable

Availability of data and materials

The datasets used and analysed during the current study are available from the corresponding author on reasonable request.

Competing interests

The authors declare that they have no competing interests.

Funding

This study was supported by the Natural Science Foundation of Jiangsu Province (BK20181243).

Authors' contributions

CJZ and JCW were in charge of the whole trial; CJZ and JCW wrote the manuscript; CJZ, SQG, RLG, SSC, YTZ and MWX constructed PRV-S BAC and recombinant PRVs. SQG, ZSW and YML conducted laboratory analyses; All authors read and approved the final manuscript.

Acknowledgements

The authors thank professor Nikolaus Osterrieder for providing *E.coli* GS1783.

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Tables

Table 1 Primers for PCR, sequencing or RT-PCR

Primer	Sequence (5’- 3’)
S-F	CATGAATTCGCGTCGACGATGCTGCTCGG
S-R	TCACTGCACGTGGACCTTTTG
Kan ins S F	CCGGAATTCAAACGCCATGTACTTTCCCACCATTGACGTCAATGGGCTAGGATGACGACGATAAGTAGGGATAAC
Kan ins S R	GGCGAATTCGGGTAATGCCAGTGTTACAACCA
PRV ins S cas UL11-10 F	TCGCGGGCGTACTGACTGCAATAAACCCGTTTGTCATACTCTAGTGGATCCCCCAACTCC
PRV ins S cas UL11-10 R	CGGCGACGAGGTCGTGTACGAGAACCTCGGCTTTGAATAATTGTCGACTCTAGAGGATCCG
PRV ins S cas UL35-36 F	TGCCCTCGCCCCTCGCCCTAGCCCCGCGCGATCAATAAAGCTAGTGGATCCCCCAACTCC
PRV ins S cas UL35-36 R	GTGGACCTATTTCAGGTCCGCCTGATTCTTGGTTAATAAATTGTCGACTCTAGAGGATCCG
PRV ins S cas UL46-27 F	TGCCCCCCTGTGTGGAAATAAAGTTTTTTTCTAATTCTGTCTAGTGGATCCCCCAACTCC
PRV ins S cas UL46-27 R	CTACCAGCGCCTCGAGAACGAGGACCCCGACGCCCCCTAGTTGTCGACTCTAGAGGATCCG
PRV ins S cas US2-1 F	CAACGGACGCGAGCGCGCCCCGCGATGTACCATCTCCTAGCTAGTGGATCCCCCAACTCC
PRV ins S cas US2-1 R	CTCTGTTGTGCCCTCAATAAACACGGCGGCCCGCCGCTCGTTGTCGACTCTAGAGGATCCG
PRV BAC H1 F	GTACCCGTACACCGAGTCGT
PRV BAC	TTGTGGACCCGCGCGAACAT

H2 R	
S cas check F	gcgtcgacgatgctgctcgg
S cas check R	CCGTGAACAACATGCTGCTG
PRV ΔgE check F	AGCCCCGGGAAGATAGCCAT
PRV ΔgE check R	ATCGCGGAACCAGACGTCGAAG
S exp F	CGGTAACACTAATGCTACTGCGCG
S exp R	CGATCATTATCCCATGTTATGCCG
Beta actin F	AGAGCGCAAGTACTCCGTGT
Beta actin R	ACATCTGCTGGAAGGTGGAC

Figures

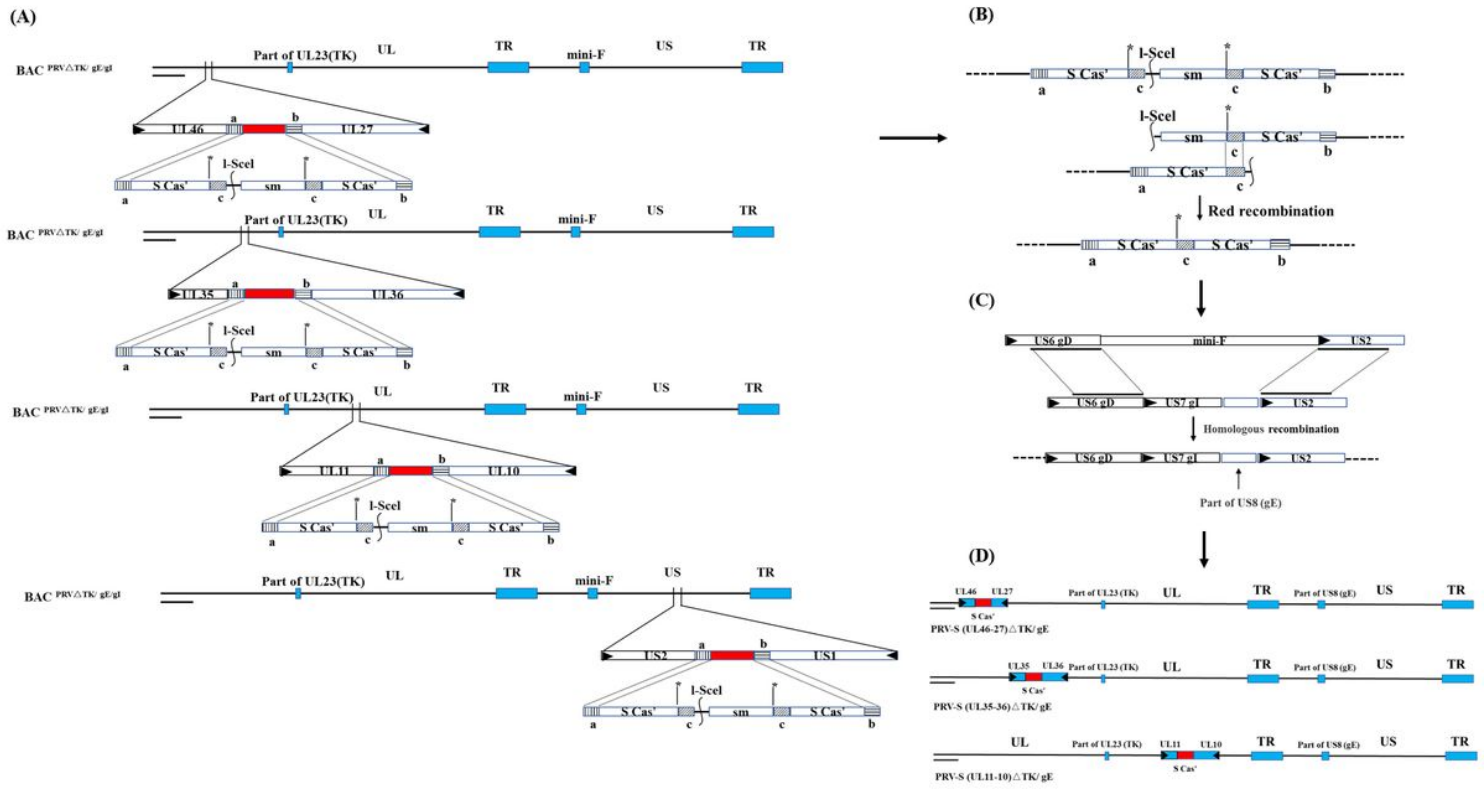

Figure 1

Construction of PRV-S(UL11-10)ΔTK/gE, PRV-S(UL35-36)ΔTK/gE and PRV-S(UL46-UL27)ΔTK/gE. A: The S expression cassette with a kanamycin resistance gene was inserted into the noncoding area (UL11-10, UL35-36, UL46-UL27 or US2-1) through the first recombination to generate four recombinant BAC clones (BACPRV-S-KAN(UL11-10)ΔTK/gE/gI, BACPRV-S-KAN(UL35-36)ΔTK/gE/gI, BACPRV-S-KAN(UL46-UL27)ΔTK/gE/gI and BACPRV-S-KAN(US2-1)ΔTK/ gE/gI). B: The second recombination was performed to delete the kanamycin resistance gene and generate the final recombinants (BACPRV-S(UL11-10)ΔTK/gE/gI, BACPRV-S(UL35-36)ΔTK/gE/gI, BACPRV-S(UL46-UL27)ΔTK/gE/gI and BACPRV-S(US2-1)ΔTK/gE/gI). C: Homologous recombination was performed to recover the intact gI gene and part of gE gene (1299bp to 1735bp of gE open reading frame). D: Schematic presentation of the PRV-S(UL11-10)ΔTK/gE, PRV-S(UL35-36)ΔTK/gE and PRV-S(UL46-27)ΔTK/gE were shown.

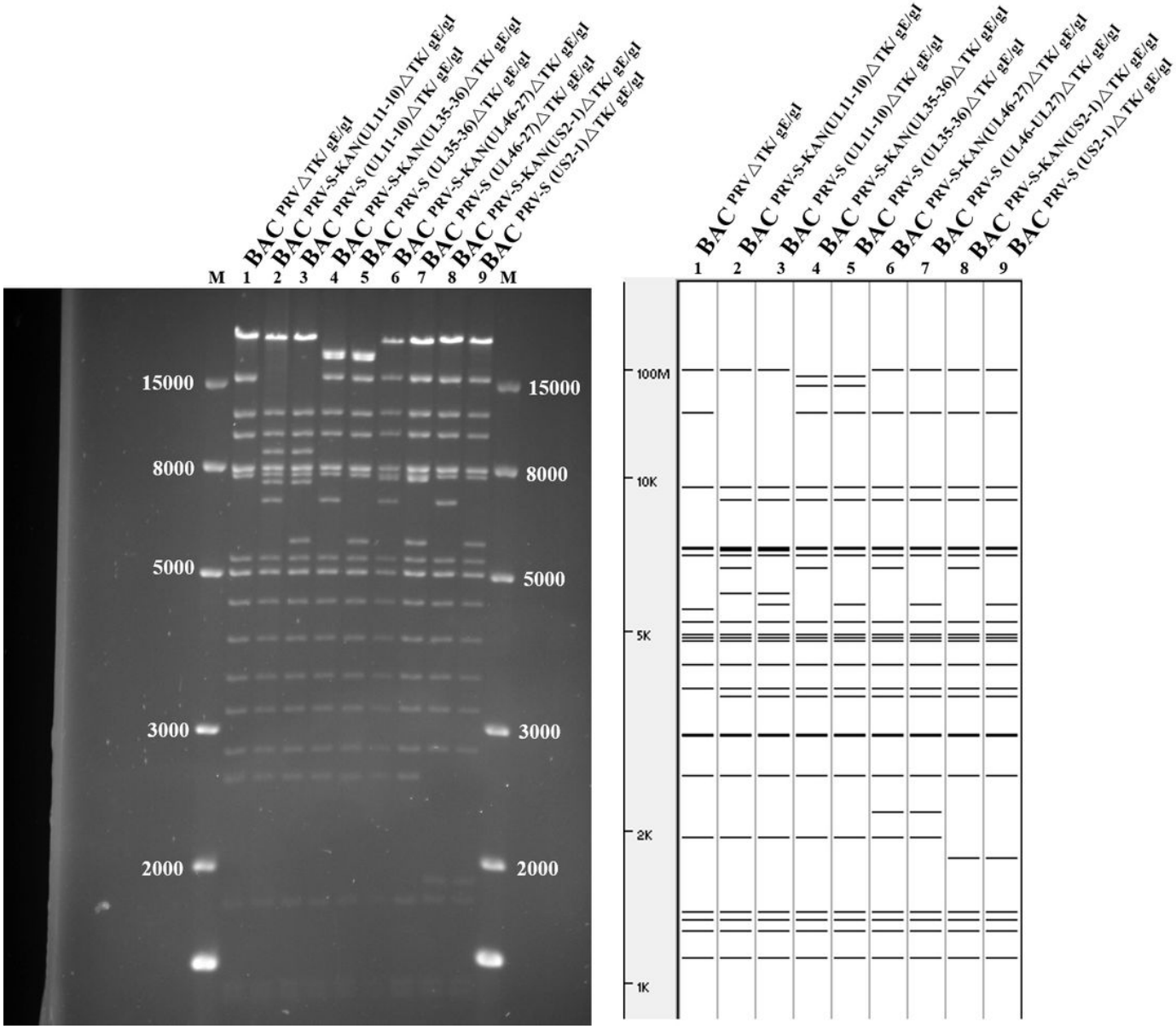

Figure 2

Restriction fragment length polymorphism (RFLP) of BACPRVΔTK/gE/gI, BACPRV-S-KAN(UL11-10)ΔTK/gE/gI, BACPRV-S(UL11-10)ΔTK/gE/gI, BACPRV-S-KAN(UL35-36)ΔTK/gE/gI, BACPRV-S(UL35-36)ΔTK/gE/gI, BACPRV-S-KAN(UL46-

27)ΔTK/gE/gI, BACPRV-S(UL46-27)ΔTK/gE/gI, BACPRV-S-KAN(US2-1)ΔTK/gE/gI and BACPRV-S(US2-1)ΔTK/gE/gI. DNA of BACPRVΔTK/gE/gI, BACPRV-S-KAN(UL11-10)ΔTK/gE/gI, BACPRV-S(UL11-10)ΔTK/gE/gI, BACPRV-S-KAN(UL35-36)ΔTK/gE/gI, BACPRV-S(UL35-36)ΔTK/gE/gI, BACPRV-S-KAN(UL46-U27)ΔTK/gE/gI, BACPRV-S(UL46-27)ΔTK/gE/gI, BACPRV-S-KAN(US2-1)ΔTK/gE/gI and BACPRV-S(US2-1)ΔTK/gE/gI were digested with BamH I. Predicted RFLP pattern with BamH I was performed using PRV ZJ01 strain (GenBank KM061380.1) as a reference

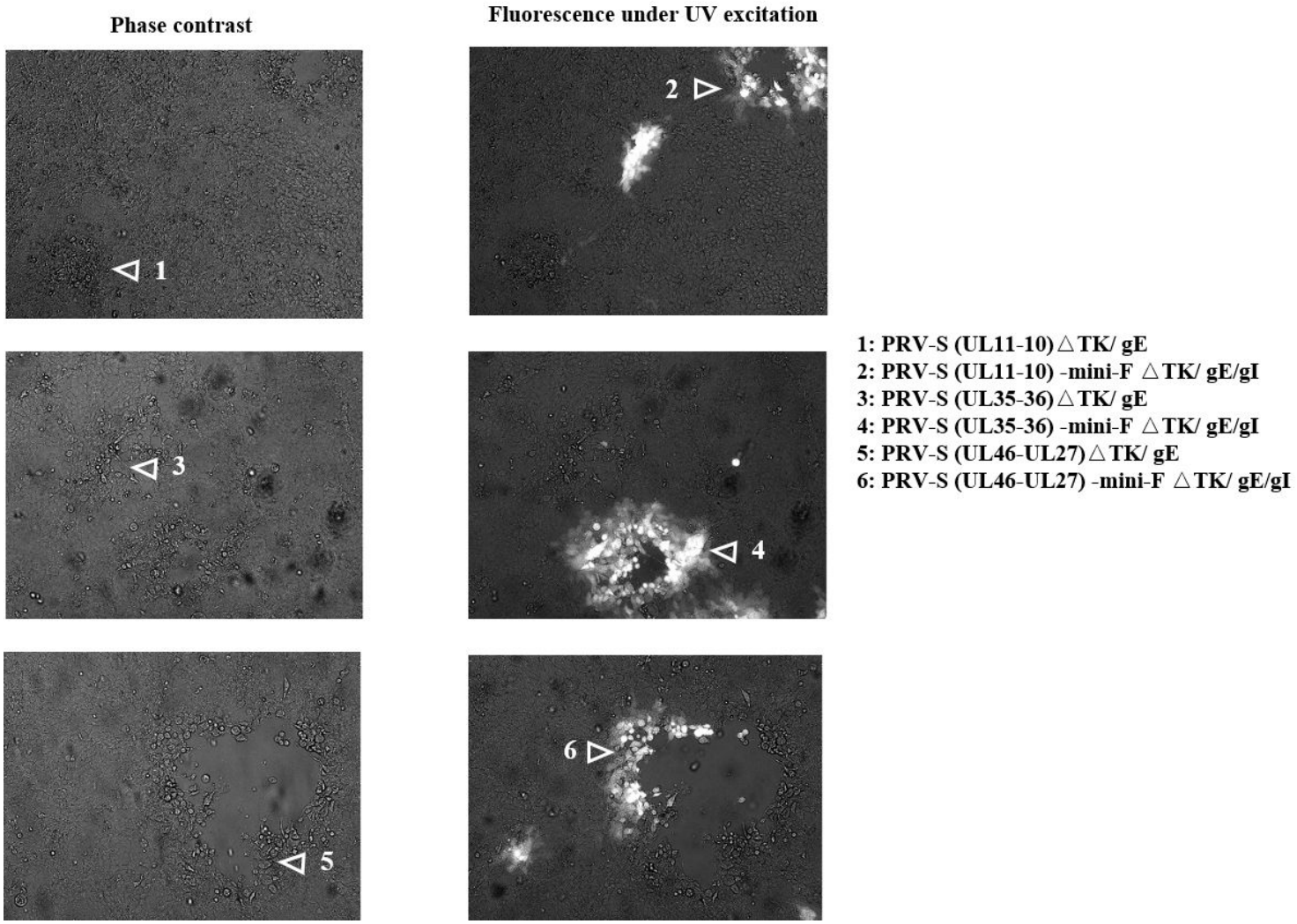

Figure 3

A Images of PRV-S(UL11-10)ΔTK/gE, PRV-S(UL11-10)-mini-FΔTK/gE/gI, PRV-S(UL35-36)ΔTK/gE, PRV-S(UL35-36)-mini-FΔTK/gE/gI, PRV-S(UL46-UL27)ΔTK/gE and PRV-S(UL46-UL27)-mini-FΔTK/gE/gI under UV excitation and phase contrast are shown. Each panel represents a view of 200 × 200 μm in size.

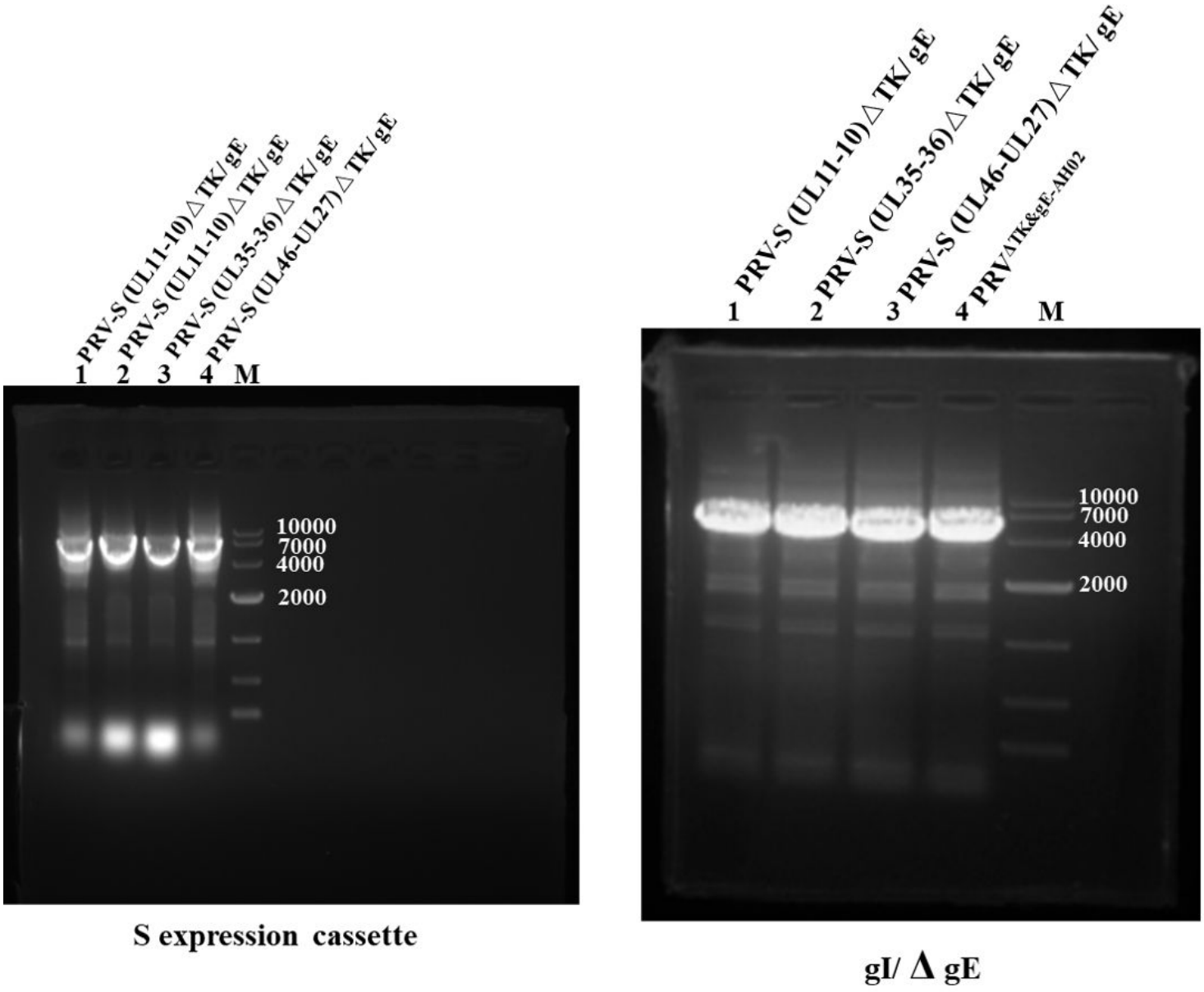

Figure 4

PCR identification of S expression cassette and gI/ΔgE. S expression cassette of PRV-S(UL11-10)ΔTK/gE, PRV-S(UL35-36)ΔTK/gE and PRV-S(UL46-UL27)ΔTK/gE was identified by PCR with primers (S cas check F/S cas check R), and gI/ΔgE was identified by PCR with primers (PRV ΔgE check F/ PRV ΔgE check R).

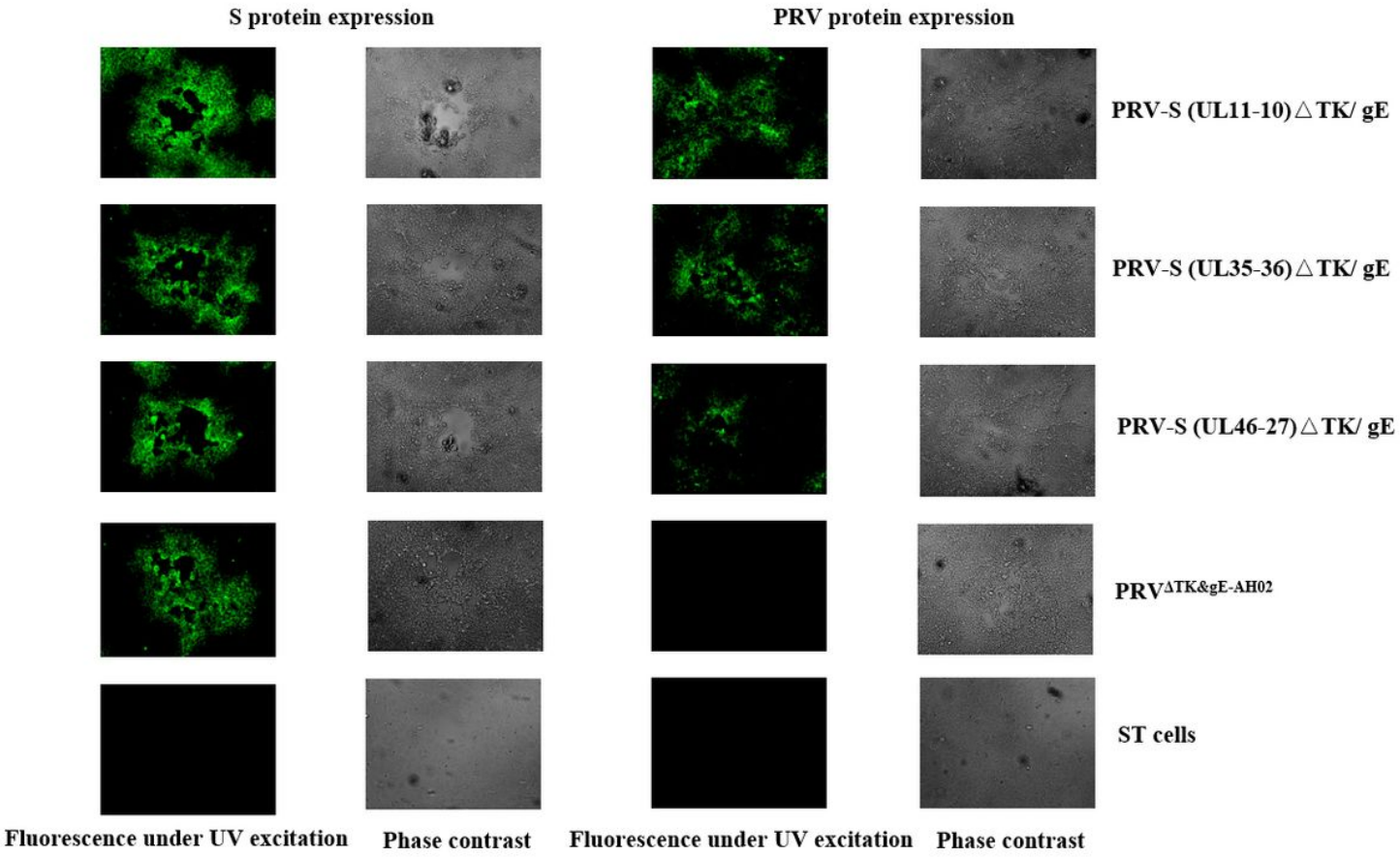

Figure 5

Confirmation of S and PRV protein expression by the recombinant PRV using indirect immunofluorescence assay (IFA). Pig anti-sera against PEDV or PRV in conjunction with FITC labelled anti-pig secondary antibodies were employed to verify S and PRV protein expression. Each panel represents a view of 200 × 200 μm in size.

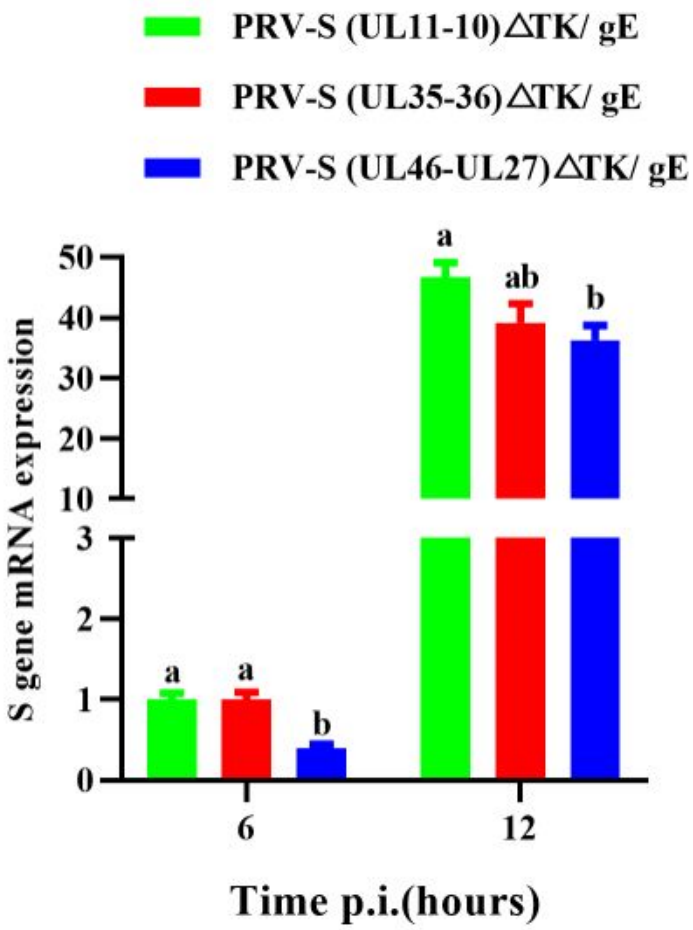

Figure 6

S gene mRNA expression on ST cells infected with PRV-S(UL11-10)ΔTK/gE, PRV-S(UL35-36)ΔTK/gE and PRV-S(UL46-UL27)ΔTK/gE at an MOI of 10 at 6 and 12 hours post infection. Data are expressed in relative arbitrary units, in comparison with the values measured in ST cells infected with PRV-S(UL11-10)ΔTK/gE at 6 hour post infection and taken as 1.00. Data were presented as mean ± SEM, and analyzed using one-way ANOVA with a Tukey's post–hoc test (SPSS Inc., Chicago, IL, USA).

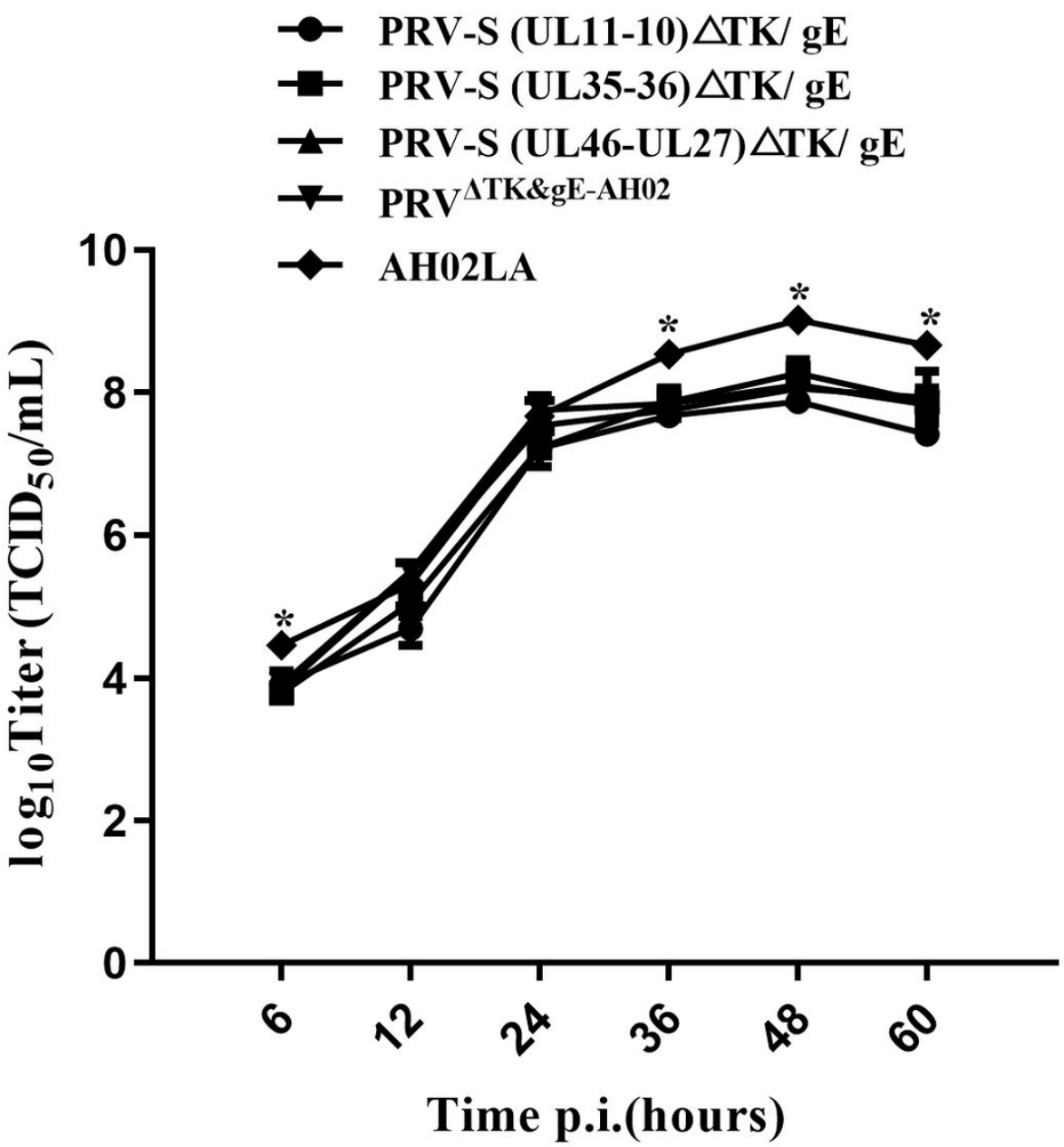

Figure 7

Multi-step growth curves of AH02LA, PRVΔTK&gE-AH02, PRV-S (UL11-10)ΔTK/gE, PRV-S(UL35-36)ΔTK/gE and PRV-S(UL46-27)ΔTK/gE. ST cells were infected with AH02LA and the four mutants at an MOI of 0.01. At 6, 12, 24, 36, 48 and 60 hours post infection, the culture cells were harvested and then were titrated in ST cells. Asterisks indicate statistical significance between AH02LA and the four mutants (*$p<0.05$). Data were presented as mean ± SEM, and analyzed using one-way ANOVA with a Tukey's post–hoc test (SPSS Inc., Chicago, IL, USA).