

## *Supplementary Material*

# **Construction of Pseudorabies Virus (PRV) Variant Attenuated Vaccine: Codon Deoptimization of US3 and UL56 Gene Based on PRV gE/TK Deletion Strain**

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## Supplementary Information

**Supplementary Information S1** Nucleotide sequence of US3-S (accession numbers: KM061380), US3-S<sup>F10-CD</sup> (accession numbers: OR228539), US3-S<sup>T-CD</sup> (accession numbers: OR228540), UL56 (accession numbers: KM061380), UL56<sup>F10-CD</sup> (accession numbers: OR228541) and UL56<sup>T-CD</sup> (accession numbers: OR228542). Nucleotide changes compared to wide type are highlighted in red.

### US3-S

ATGGCCGACGCCGGAATCCCCGACGAGATCCTGTACTCGGACATCAGCGACGACGAGAT  
CATCATCGACGGCGACGGCGACAGCAGCGGGGACGAGGACACCGACGATGACGGGGGG  
CTGACGCGGCAGGCCGCGTCGCGCATCGCCACGGACCTGGGCTTCGAGGTGCTGCAGCC  
CCTGCAGTCGGGCTCGGAGGGCCGCGTCTTCGTGGCCCCGCCGGCCCCGGCGAGGCGGACA  
CGGTGGTGCTGAAGGTGGGCCAGAAGCCCTCGACGCTGATGGAGGGCATGCTGCTGAA  
GCGCCTGGCCCACGATAACGTCATGAGCCTGAAGCAGATGCTCGCCCCGGGGCCCCGGTGA  
CGTGCCCTGGTCCTGCCGCACTTTCGGTGCGATCTGTACAGCTACCTGACCATGCGGGACG  
GGCCGCTGGACATGCGCGACGCCGGCCGCGTGATCCGTTCCGTGCTCCGCGGGCTCGCC  
TACCTGCACGGGATGCGCATCATGCACCGCGACGTCAAGGCGGAGAACATCTTCCTCGA  
GGACGTGGACACGGTGTGCCTGGGGGACCTCGGGGGCCGCGCGCTGCAACGTGGCGGCG  
CCCAACTTTTACGGGCTCGCCGGGACCATCGAGACCAACGCCCCCGAGGTGCTCGCGCG  
CGACCGCTACGACACCAAGGTCGACGTCTGGGGTGCGGGGGTGGTGCTCTTCGAGACGC  
TGGCCTACCCCAAGACGATCACCGGCGGGGACGAGCCCGCGATCAACGGGGAGATGCA  
CCTGATCGACCTCATCCGCGCCCTCCGCGGGGTGCACCCCGAGGAGTTCCCGCCCCGACA  
CGCGCCTCCGGAGCGAGTTCGTCCGGTACGCCGGGACCCATCGCCAGCCGTACACGCAG  
TACGCGCGCGTGGCTCGCCTCGGGCTGCCCGAGACGGGGGCTTTCCTGATTTACAAGAT  
GTTGACGTTTGATCCCGTCCGCGCCCTTCCGCTGATGAGATACTCAACTTTGGAATGTG  
GACCGTATAA

### US3-S<sup>F10-CD</sup>

ATGGC**GGAT**TGC**GGG**TAT**ACCGGAT**G**AA**TA**ACT**GTACTCGGACATCAGCGACGACGAGAT  
CATCATCGACGGCGACGGCGACAGCAGCGGGGACGAGGACACCGACGATGACGGGGGG  
CTGACGCGGCAGGCCGCGTCGCGCATCGCCACGGACCTGGGCTTCGAGGTGCTGCAGCC  
CCTGCAGTCGGGCTCGGAGGGCCGCGTCTTCGTGGCCCCGCCGGCCCCGGCGAGGCGGACA  
CGGTGGTGCTGAAGGTGGGCCAGAAGCCCTCGACGCTGATGGAGGGCATGCTGCTGAA  
GCGCCTGGCCCACGATAACGTCATGAGCCTGAAGCAGATGCTCGCCCCGGGGCCCCGGTGA

CGTGCCTGGTCCTGCCGCACTTTCGGTGCGATCTGTACAGCTACCTGACCATGCGGGACG  
GGCCGCTGGACATGCGCGACGCCGGCCGCGTGATCCGTTCCGTGCTCCGCGGGCTCGCC  
TACCTGCACGGGATGCGCATCATGCACCGCGACGTCAAGGCGGAGAACATCTTCCTCGA  
GGACGTGGACACGGTGTGCCCTGGGGGACCTCGGGGCCGCGCGCTGCAACGTGGCGGCG  
CCCAACTTTTACGGGCTCGCCGGGACCATCGAGACCAACGCCCCCGAGGTGCTCGCGCG  
CGACCGCTACGACACCAAGGTCGACGTCTGGGGTGCGGGGGTGGTGCTCTTCGAGACGC  
TGGCCTACCCCAAGACGATCACCGGCGGGGACGAGCCCGCGATCAACGGGGAGATGCA  
CCTGATCGACCTCATCCGCGCCCTCCGCGGGGTGCACCCCGAGGAGTTCCCGCCCGACA  
CGCGCCTCCGGAGCGAGTTCGTCCGGTACGCCGGGACCCATCGCCAGCCGTACACGCAG  
TACGCGCGCGTGGCTCGCCTCGGGCTGCCCAGACGGGGGCTTTCCTGATTTACAAGAT  
GTTGACGTTTGATCCCGTCCGCCGCCCTTCCGCTGATGAGATACTCAACTTTGGAATGTG  
GACCGTATAA

### US3-S<sup>T</sup>-CD

ATGGCGGATGCGGGTATACCGGATGAAATACTATATTCGGATATAAGTGATGATGAAAT  
AATAATAGATGGTGATGGTGATAGTAGTGGTGATGAAGATACGGATGATGATGGTGGTC  
TAACGCGTCAAGCGGCGTCGCGTATAGCGACGGATCTAGGTTTTGAAGTACTACAACCC  
GTGCAATCGGGTTCGGAAGGTCGTGTATTTGTAGCGCGTCGTCCGGGTGAAGCGGATAC  
GGTAGTACTAAAAGTAGGTCAAAAACCGTCGTTCGCTAATGGAAGGTATGCTACTAAAC  
GTCTAGCGCATGTAAATGTAAATGAGTCTAAAACAATGCTAGCGCGTGGTCCGGTAACG  
TGTTCTAGTACTACCGCATTTTCGTTGTGATCTATATAGTTATCTAACGATGCGTGATGGT  
CCGCTAGATATGCGTGATGCGGGTCGTGTAATACGTTCCGGTACTACGTGGTCTAGCGTAT  
CTACATGGTATGCGTATAATGCATTCGTGATGTAAAAGCGGAAAATATATTTCTAGAAGA  
TGTAGATACGGTATGTCTAGGTGATCTAGGTGCGGGCGCGTTGTAATGTAGCGGCGCCGA  
ATTTTTATGGTCTAGCGGGTACGATAGA AACGAATGCGCCGGAAGTACTAGCGCGTGAT  
CGTTATGATACGAAAGTAGATGTATGGGGTGCGGGTGTAGTACTATTTGAAACGCTAGC  
GTATCCGAAAACGATAACGGGTGGTGATGAACCGGCGATAAATGGTGAAATGCATCTA  
ATAGATCTAATACGTGCGCTACGTGGTGTACATCCGGAAGAATTCCGCCCGGATACGCG  
TCTACGTAGTGAAATTTGTACGTTATGCGGGTACGCATCGTCAACCGTATACGCAATATGC  
GCGTGTAGCGCGTCTAGGTCTACCGGAACGGGTGCGTTTCTAATATATAAAATGTTAA  
CGTTTGATCCGGTACGTTCGTCCGTGCGCGGATGAATACTAAATTTTGGTATGTGGACGG  
TATAA

### UL56

ATGCCTCCACAACGAGCCCGCGGGGCTCCGCCGCGCCGTGCGGGCAGCGACCCGCCCGA  
TCCAGGCAGCCTCGCCGGGCGGGCCCTCGCCCGGGGGGAGAGGAGGCGGAGGGCAGCGC  
CGCCCCCTCTCGCGCAGCAGCTCGCTCACGTCCGTGCGCTCGGCGCCCGTGGAGACGCC  
CGTCGTGCGGAGGCTCCGGGGCTCGGCGCCCCCGGCTCCAGGCCGCCCTCCTACGGGG  
ACGTGTCGCGGTGCGGGCCGCGCCCGCACCGATCGCCGGACACGCCGCTGTTTGCCCGG  
GGCCCGCCCCCGTCTACTCGGAGACGCTCCTGTTGACCCGCCCGCGTACGCGGTGAC

CATCCCGGACCCGCCGGCGTACGAGCCCACCGTCATCGGGCCGCACCCGCCGCGCCCC  
GCGACTGGATCTCCTCGCCCTCGGTGGTGCAGCCGTCGCTGCTGGGCCCCCTTCAGCCAGT  
GCCTCCCGCGGGTGACCTGCCCCGACTGCCGCTACCCCGAAGACCGCCCGATGGTGCTC  
GTGGGCTTCCTCTGGGGGGGACTGCTCCTGCTGGTGGGCCTCGTGTTTCTGATCCTGCTC  
CCGGTGCTCCGGGAGTCCGTCGTGTTTCCCTGA

#### UL56<sup>F10-CD</sup>

ATGCCGCCGCAACGTGCTCGTGGTGC<sup>G</sup>CCGCCGCGCCGTCGCGGCAGCGACCCGCCCGA  
TCCAGGCAGCCTCGCCGGGCGGCCCTCGCCCGGGGGAGAGGAGGCGGAGGGCAGCGC  
CGCCCCCTCTCGCGCAGCAGCTCGCTCACGTCCGTCGCCTCGGCGCCCGTGGAGACGCC  
CGTCGTCGCGGAGGCTCCGGGGCTCGGCGCCCCCGGCTCCAGGCCGCCCTCCTACGGGG  
ACGTCGTCCGCGTCGGGCCGCGCCCGCACCGATCGCCGGACACGCCGCTGTTTGCCCGG  
GGCCCGCCCCCGTCTACTCGGAGACGCTCCTGTTCGACCCGCCCGCGTACGCGGTGAC  
CATCCCGGACCCGCCGGCGTACGAGCCCACCGTCATCGGGCCGCACCCGCCGCGCCCC  
GCGACTGGATCTCCTCGCCCTCGGTGGTGCAGCCGTCGCTGCTGGGCCCCCTTCAGCCAGT  
GCCTCCCGCGGGTGACCTGCCCCGACTGCCGCTACCCCGAAGACCGCCCGATGGTGCTC  
GTGGGCTTCCTCTGGGGGGGACTGCTCCTGCTGGTGGGCCTCGTGTTTCTGATCCTGCTC  
CCGGTGCTCCGGGAGTCCGTCGTGTTTCCCTGA

#### UL56<sup>T-CD</sup>

ATGCCGCCGCAACGTGCTCGTGGTGC<sup>G</sup>CCGCCGCGT<sup>G</sup>CGTCG<sup>T</sup>GGTAG<sup>T</sup>GATCCGCC<sup>G</sup>GA  
TCC<sup>G</sup>GGTAG<sup>T</sup>TCTAGC<sup>G</sup>GGTCG<sup>T</sup>CCGTCGCC<sup>G</sup>GGTGGTAGAGGTGG<sup>T</sup>GGTGGTCAACGTC  
GTCC<sup>G</sup>GCTATCGCGTAG<sup>T</sup>AGTTTCGCTAACGTC<sup>G</sup>GTAGC<sup>G</sup>TCGGCGCC<sup>G</sup>GTA<sup>G</sup>AAACGCCG  
GTAGTAGCGGAAGC<sup>G</sup>CCGGGTCTAGGTGCGCC<sup>G</sup>GGTTCGAGACCGCCGTCGTATGGTGA  
TGTAGTACGTGTAGGTCCGCGTCCGCATCG<sup>T</sup>TCGCCGGATACGCCGCTATTTGCGCGTGG  
TCCGCC<sup>G</sup>CCGTCG<sup>T</sup>ATTTCGGA<sup>A</sup>ACGCTACTATTTGATCCGCC<sup>G</sup>GCGTATGCGGTAA<sup>G</sup>GAT  
ACCGGATCCGCCGGCGTATGAACCGACGGTAATAGGTCCGCATCCGCCGCGTCCGCGTG  
ATTGGATATC<sup>G</sup>TCGCCGTCGGTAGTACAACCGTCGCTACTAGGTCCGTTAGTCAATGTC  
TACCGCGTGTAA<sup>G</sup>CGTGTCCGGA<sup>T</sup>TGTCGTTATCCGGAAGATCG<sup>T</sup>CCGATGGTACTAGTAG  
GTTT<sup>T</sup>TCTATGGGGTGGTCTACTACTACTAGTAGGTCTAGTATTTCTAATACTACTACCGG  
TACTACGTGAATC<sup>G</sup>GTAGTATTTCCG<sup>T</sup>TGA

## Supplementary Tables

**Supplementary Table S1** Primers for PCR, sequencing or RT-PCR

| Primer                               | Sequence (5'- 3')                                                               |
|--------------------------------------|---------------------------------------------------------------------------------|
| US3-S ins mKate2 F                   | AGATCTCGAGCTCAAGCTTCGAATTCATGGCCGACGCC<br>GGAATCCC                              |
| US3-S ins mKate2 R                   | CCGCGGTACCGTCGACTGCAGAATTCTACGGTCCACAT<br>TCCAA                                 |
| US3-S <sup>F10-CD</sup> ins mKate2 F | AGATCTCGAGCTCAAGCTTCGAATTCATGGC GGATGCG<br>GGTATACC                             |
| US3-S <sup>F10-CD</sup> ins mKate2 R | CCGCGGTACCGTCGACTGCAGAATTCTACGGTCCACAT<br>TCCAA                                 |
| US3-S <sup>T-CD</sup> ins mKate2 F   | AGATCTCGAGCTCAAGCTTCGAATTCATGGC GGATGCG<br>GGTATACC                             |
| US3-S <sup>T-CD</sup> ins mKate2 R   | CCGCGGTACCGTCGACTGCAGAATTCTACCGTCCACAT<br>ACCAA                                 |
| UL56 ins mKate2 F                    | AGATCTCGAGCTCAAGCTTCGAATTCATGCCTCCACAA<br>CGAGCCCCG                             |
| UL56 ins mKate2 R                    | CCGCGGTACCGTCGACTGCAGAATTCGGGAAACACGAC<br>GGACT                                 |
| UL56 <sup>F10-CD</sup> ins mKate2 F  | AGATCTCGAGCTCAAGCTTCGAATTCATGCCGCCGCAA<br>CGTGCTCG                              |
| UL56 <sup>F10-CD</sup> ins mKate2 R  | CCGCGGTACCGTCGACTGCAGAATTCGGGAAACACGAC<br>GGACT                                 |
| UL56 <sup>T-CD</sup> ins mKate2 F    | AGATCTCGAGCTCAAGCTTCGAATTCATGCCGCCGCAA<br>CGTGCTCG                              |
| UL56 <sup>T-CD</sup> ins mKate2 R    | CCGCGGTACCGTCGACTGCAGAATTCGGGAAATACTAC<br>CGATT                                 |
| KAN ins US3-S <sup>F10-CD</sup> F    | CCCCCGGGGCCCCGGTGACGTGCCTGGTCCTGCCGCACT<br>TTCGGTGCAGGATGACGACGATAAGTAGGGATAAC  |
| KAN ins US3-S <sup>F10-CD</sup> R    | CCCCCGGGGGGTAATGCCAGTGTTACAACCA                                                 |
| KAN ins US3-S <sup>T-CD</sup> F      | TGCTCTAGAAGATGTAGATACGGTATGTCTAGGTGATC<br>TAGGTGCGGCGGGATGACGACGATAAGTAGGGATAAC |
| KAN ins US3-S <sup>T-CD</sup> R      | TGCTCTAGAGGGTAATGCCAGTGTTACAACCA                                                |
| KAN ins UL56 <sup>F10-CD</sup> F     | GGGCCGGCGTACGAGCCACCGTCATCGGGCCGCACCC<br>GCCGCGCCCCGGATGACGACGATAAGTAGGGATAAC   |
| KAN ins UL56 <sup>F10-CD</sup> R     | GGGCCGGCGGGTAATGCCAGTGTTACAACCA                                                 |
| KAN ins UL56 <sup>T-CD</sup> F       | CCGGATATCGTCGCCGTCGGTAGTACAACCGTCGCTAC<br>TAGGTCCGTTTGGATGACGACGATAAGTAGGGATAAC |
| KAN ins UL56 <sup>T-CD</sup> R       | CCGGATATCGGGTAATGCCAGTGTTACAACCA                                                |
| US3-S <sup>F10-CD</sup> En pa F      | GTTGTCGCGCGTCCACGCCAGCGCTCGCACGCAGCAA<br>CAATGGCGGATGCGGGTATACC                 |

|                                 |                                                                    |
|---------------------------------|--------------------------------------------------------------------|
| US3-S <sup>F10-CD</sup> En pa R | GCAAAGGTGTGTGTGTGTCCTACCGCTCGGAGCCGGGCCG<br>TTTTATACGGTCCACATTCCAA |
| US3-S <sup>T-CD</sup> En pa F   | GTTGTGCGCGCTCCACGCCAGCGCTCGCACGCAGCAA<br>CAATGGCGGATGCGGGTATACC    |
| US3-S <sup>T-CD</sup> En pa R   | GCAAAGGTGTGTGTGTGTCCTACCGCTCGGAGCCGGGCCG<br>TTTTATACCGTCCACATACCAA |
| UL56 <sup>F10-CD</sup> En pa F  | GGGCACCGCCGTCCGCCGCCAGCCGCCGTGGGAGGCAG<br>ACATGCCGCCGCAACGTGCTCG   |
| UL56 <sup>F10-CD</sup> En pa R  | GACGCGAATATCGATGGGGCGGGCGAGCCGAGTTTATT<br>GATCAGGGAAACACGACGGA     |
| UL56 <sup>T-CD</sup> En pa F    | GGGCACCGCCGTCCGCCGCCAGCCGCCGTGGGAGGCAG<br>ACATGCCGCCGCAACGTGCTCG   |
| UL56 <sup>T-CD</sup> En pa R    | GACGCGAATATCGATGGGGCGGGCGAGCCGAGTTTATT<br>GATCACGGAAATACTACCGATT   |
| US3-S check F                   | GGAGATGGGTCACCAAGAGG                                               |
| US3-S check R                   | AGATGCGCAAAGGTGTGTGT                                               |
| UL56 check F                    | CCGCCACCCTGGGTATTAAC                                               |
| UL56 check R                    | GCCGAGTTTATTGATCAGGG                                               |
| H1-H2-gI-ΔgE F                  | GTACCCGTACACCGAGTCGT                                               |
| H1-H2-gI-ΔgE R                  | TTGTGGACCCGCGCGAACAT                                               |
| PRV ΔgE check F                 | AGCCCCGGGAAGATAGCCAT                                               |
| PRV ΔgE check R                 | ATCGCGGAACCAGACGTCGAAG                                             |
| US3-S mRNA F                    | GTCGGGCTCGGAGGGCCGCG                                               |
| US3-S mRNA R                    | CGGGCCCCGGGCGAGCATCT                                               |
| US3-S <sup>T-CD</sup> mRNA F    | ATCGGGTTCGGAAGGTTCGTG                                              |
| US3-S <sup>T-CD</sup> mRNA R    | CGGACCACGCGCTAGCATTT                                               |
| UL24 mRNA F                     | CTTCCCCCGGAACCTCAACA                                               |
| UL24 mRNA R                     | TGGGCCACAAACACCAGCAG                                               |
| UL40 mRNA F                     | AGCGCCATCCAGCTGATGCT                                               |
| UL40 mRNA R                     | GCGAACGAGGAGGCGAAGAA                                               |
| UL44 mRNA F                     | CGCCTTCGTGACCAACAGCA                                               |
| UL44 mRNA R                     | GTACCACACGGCCTCGCAGC                                               |
| UL52 mRNA F                     | TTGAAGTGC GCGTCGAGGTG                                              |
| UL52 mRNA R                     | AGCTTCTTCGAGCGCAAGGC                                               |
| UL56 mRNA F                     | GCAGCGCCGCCCCCTCTCGC                                               |
| UL56 mRNA R                     | CGGGCGCGGCCCGACGCGGA                                               |
| UL56 <sup>T-CD</sup> mRNA F     | TCAACGTCGTCCGCTATCGC                                               |
| UL56 <sup>T-CD</sup> mRNA R     | CGGACGCGGACCTACACGTA                                               |
| gB mRNA F                       | GTCCGTGAAGCGGTTTCGTGAT                                             |
| gB mRNA R                       | ACAAGTTCAAGGCCACATCTAC                                             |

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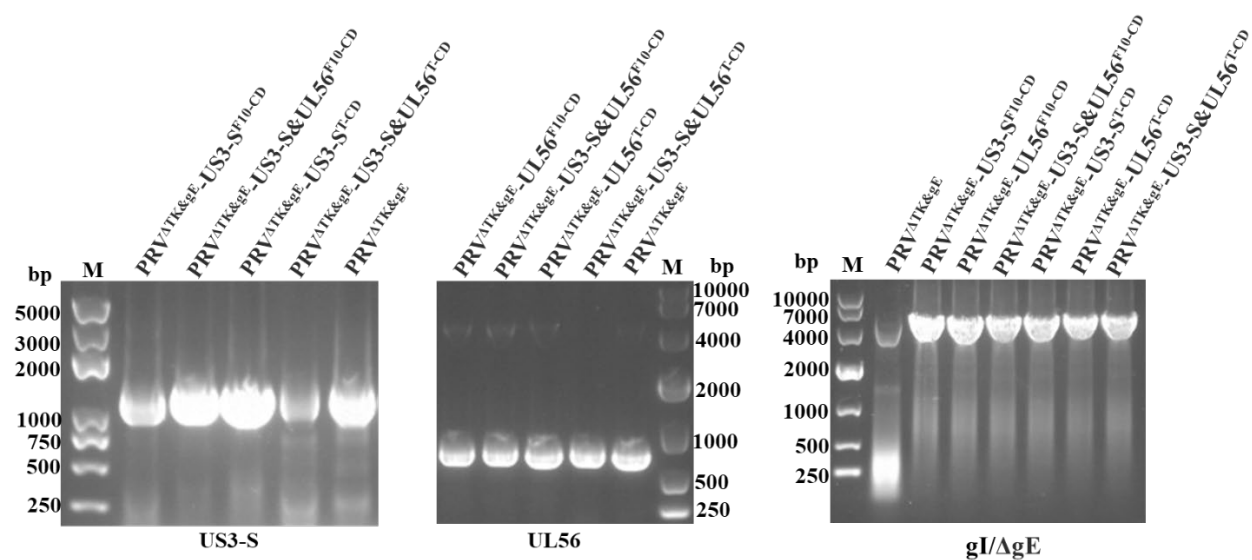
**Supplementary Table S2** The titer of recombinant viruses with US3-S and UL56 codon deoptimization at each time point post-infection

| Hours post infection | Virus titer <sup>1</sup> (lgTCID <sub>50</sub> /mL)         |                                                            |                                                                      |                                                           |                                                          |                                                                    |                                           | <i>P</i> -value |
|----------------------|-------------------------------------------------------------|------------------------------------------------------------|----------------------------------------------------------------------|-----------------------------------------------------------|----------------------------------------------------------|--------------------------------------------------------------------|-------------------------------------------|-----------------|
|                      | PRV <sup>ΔTK&amp;gE</sup> <sub>US3-S<sup>F10</sup>-CD</sub> | PRV <sup>ΔTK&amp;gE</sup> <sub>UL56<sup>F10</sup>-CD</sub> | PRV <sup>ΔTK&amp;gE</sup> <sub>US3-S&amp;UL56<sup>F10</sup>-CD</sub> | PRV <sup>ΔTK&amp;gE</sup> <sub>US3-S<sup>T</sup>-CD</sub> | PRV <sup>ΔTK&amp;gE</sup> <sub>UL56<sup>T</sup>-CD</sub> | PRV <sup>ΔTK&amp;gE</sup> <sub>US3-S&amp;UL56<sup>T</sup>-CD</sub> | PRV <sup>ΔTK&amp;gE</sup> <sub>AH02</sub> |                 |
| 6                    | 1.65 ± 0.05 <sup>a</sup>                                    | 0.00 ± 0.00 <sup>b</sup>                                   | 0.00 ± 0.00 <sup>b</sup>                                             | 0.00 ± 0.00 <sup>b</sup>                                  | 1.07 ± 0.53 <sup>ab</sup>                                | 0.53 ± 0.53 <sup>b</sup>                                           | 0.00 ± 0.00 <sup>b</sup>                  | 0.005           |
| 12                   | 2.55 ± 0.10 <sup>b</sup>                                    | 1.65 ± 0.05 <sup>c</sup>                                   | 1.78 ± 0.12 <sup>c</sup>                                             | 0.00 ± 0.00 <sup>d</sup>                                  | 2.33 ± 0.08 <sup>b</sup>                                 | 2.38 ± 0.07 <sup>b</sup>                                           | 3.13 ± 0.20 <sup>a</sup>                  | < 0.001         |
| 24                   | 4.38 ± 0.62 <sup>b</sup>                                    | 5.20 ± 0.33 <sup>ab</sup>                                  | 5.20 ± 0.60 <sup>ab</sup>                                            | 4.13 ± 0.31 <sup>b</sup>                                  | 4.20 ± 0.25 <sup>b</sup>                                 | 4.62 ± 0.22 <sup>b</sup>                                           | 6.17 ± 0.08 <sup>a</sup>                  | 0.026           |
| 36                   | 6.32 ± 0.04 <sup>bc</sup>                                   | 7.08 ± 0.30 <sup>a</sup>                                   | 6.78 ± 0.12 <sup>ab</sup>                                            | 5.70 ± 0.05 <sup>d</sup>                                  | 6.17 ± 0.10 <sup>bc</sup>                                | 6.55 ± 0.10 <sup>bc</sup>                                          | 6.81 ± 0.12 <sup>ab</sup>                 | 0.006           |
| 48                   | 6.69 ± 0.08 <sup>b</sup>                                    | 7.41 ± 0.05 <sup>a</sup>                                   | 6.77 ± 0.27 <sup>b</sup>                                             | 7.00 ± 0.14 <sup>ab</sup>                                 | 6.86 ± 0.19 <sup>b</sup>                                 | 7.00 ± 0.14 <sup>ab</sup>                                          | 7.43 ± 0.03 <sup>a</sup>                  | 0.019           |
| 60                   | 7.00 ± 0.14 <sup>b</sup>                                    | 6.86 ± 0.22 <sup>b</sup>                                   | 7.58 ± 0.08 <sup>a</sup>                                             | 6.35 ± 0.08 <sup>c</sup>                                  | 6.08 ± 0.22 <sup>c</sup>                                 | 6.58 ± 0.04 <sup>bc</sup>                                          | 7.08 ± 0.17 <sup>b</sup>                  | < 0.001         |
| 72                   | 6.05 ± 0.13 <sup>bc</sup>                                   | 6.45 ± 0.23 <sup>b</sup>                                   | 6.92 ± 0.17 <sup>a</sup>                                             | 5.92 ± 0.08 <sup>c</sup>                                  | 5.70 ± 0.15 <sup>cd</sup>                                | 6.38 ± 0.22 <sup>b</sup>                                           | 6.99 ± 0.20 <sup>a</sup>                  | < 0.001         |

<sup>1</sup> ST cells are infected with PRV<sup>ΔTK&gE</sup><sub>AH02</sub> and its six mutants at an MOI of 0.01. At 6, 12, 24, 36, 48, 60, and 72 h post-infection, the culture cells are harvested and titrated in ST cells.

<sup>a, b, c, d</sup> indicates statistical significance ( $P < 0.05$ ) of virus titer among the 7 viruses.

## Supplementary Figure



**Supplementary Figure S1** Stability of recombinant PRVs with gI-ΔgE and the recoded US3-S and UL56 genes. The recombinant viruses were passaged 20 times on ST cells, gI-ΔgE and recoded US3-S and UL56 were detected by PCR.