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Development of a Markerless Gene Deletion System for HSV-1

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Depositories
The GenBank accession number of the Human herpesvirus 1 strain17 is JN555585.1.

Abstract: ***HSV-1 is a widespread human pathogen that causes various diseases worldwide, highlighting the need for novel approaches to treatment and prevention. However, the lack of some efficient gene deletion techniques has hampered the pathological studies, pharmaceutical research and vaccines development of this virus. In this study, we developed an efficient and markerless deletion system mainly composed of two recombinant plasmids, BAC-HSV-1 and pREDI. And the homologous fragment "a-c-Kan-sacB-b-c" used in this system contained three homology regions and two selection markers (positive/ counter). Rapid deletion was achieved in three steps: (1) amplification of homologous fragment; (2) antibiotic and sucrose screening of recombinants; and (3) BAC excision. The overall deletion efficiency was greater than 83.9%. We were the first to develop a special in-vitro gene deletion system to aim to delete HSV-1 genes. And we had successfully deleted the ICP34.5, UL5, UL41, UL29, and US8 genes of HSV-1 and conducted preliminary analysis of the virus titer of mutants.***

Keywords: HSV-1, unmarked, homologous recombination, gene deletion

Highlight

1) The gene deletion system was realized in an efficient and markerless way.
2) We were the first to develop a special gene deletion system to aim to knock out HSV-1 genes.
3) The overall deletion efficiency arrived about 83.9%.
4) We had successfully deleted the ICP34.5, UL5, UL41, UL29, and US8 genes.

1. Introduction

Herpes simplex virus type 1 (HSV-1) was the first human herpes virus reported and belongs to the family Herpesviridae, subfamily Alphaherpesvirinae, and genus Simplexvirus [1]. Its structure is highly complex and is composed of a particle 170-200nm in diameter [2] and contains four components: (1) an electron-dense core containing viral DNA of at least 152 kb; (2) an icosadeltahedral capsid; (3) an amorphous, at times eccentric layer of proteins, designated tegument, which surrounds the capsid; and (4) an envelope [3]. HSV-1 is widespread in nature and causes large numbers of infection in humans from mild orofacial infections to severe keratitis or encephalitis [4, 5]. Additionally, it can establish latent infections in trigeminal neurons and become reactivated at or near the point of entry, resulting in secondary lesions [6-8]. Although the genome has been sequenced, most of pathologies are not fully understood.

Targeted gene deletions are often followed by a series of biological changes [9-12]. So, specific gene deletion techniques have been exploited as a powerful tool for evaluating gene functions, studying proteomics, or developing live attenuated vaccines. In comparison, homologous recombination is a classic and reliable method to manipulate. And scientists have done crowds of experiments through this method. The selection markers are necessary to find the clones with the desired genetic change or changes [13-16]. However, the residual exogenous markers prevent gene function and affect the analysis of gene function. To prevent selection markers from remaining after genome modification, the Flp recombinase target and the Cre/loxP-mediated site-specific recombination systems have been used for the precise excision of selection markers with the corresponding recombinase [17-21]. However, even using these site-specific recombination systems, at least one copy of the Flp recombinase target site or loxP site remains after excision [22], limiting further analyses.

Many new technologies for gene modification have been developed, such as TALENA, CRISPR-Cas9, and NgAgo-gDNA. However, these methods, mostly directly involving live virus, exist some deadly deficiency in the experiments, such as in-vivo toxicity, off-targeting, non-specific reaction and generated biological poison et al. A team of researchers have cloned the simple herpes virus type 1 whole genome into a bacterial artificial chromosome (BAC) [23]. Thus, the constructed BAC-HSV-1 become an effective tool to in-vitro operates this virus. Additionally,

another researchers have also reported plenty of unmarked methods for rapid gene knockout in bacterial cells [24-27].

In this study, combining the aforementioned two aspects, we have developed a more efficient and precise method for gene deletions of HSV-1 in vitro without other genome alterations. The construction strategy involves six steps: (1) pREDI construction, (2) homologous cassette amplification, (3) electrical transformation, (4) selection marker removal, (5) virus reconstitution and BAC excision, and (6) virus titer detection. To verify the reliability and maneuverability of this targeted deletion technique, and study some pathogenicity of HSV-1 gene, we choose to delete the ICP34.5, UL5, UL41, UL29, and US8. At last, compared to wild-type virus, infectivity was greatly reduced when ICP34.5 and UL41 were deleted. HSV-1 could not survive when UL5, UL29, and US8 were defective. Those mutants provide some important materials for HSV-1 research.

In conclusion, we constructed a markerless and efficient deletion system to modify the HSV-1 genome, which not only bypasses the traditional time-consuming and labor-intensive restriction/ligation-dependent vector construction, but also does not leave selection markers behind. This in vitro system is a powerful tool for studying HSV-1 virus.

2. Material and Methods

2.1 Reagents

PrimeSTAR® HS DNA Polymerase (#R010A), PrimeScriptTM RT Master Mix (Perfect Real Time) (Takara, #RR036A), SYBR Green qPCR Master Mix (#638320), NheI (#1241S), EcoRI (#1040S), and T4 DNA Ligase (#2011A) were purchased from Takara (Shiga, Japan). The Gel Extraction Kit (#D2500-01) and BAC/PAC DNA Isolation Maxi Kit (#D2154-01) were purchased from Omega bio-tek (Norcross, GA, USA). Lipofectamine 3000 (#3000015) and TRIzol® Reagent (#15596026) were purchased from Invitrogen (Carlsbad, CA, USA). The RNeasy Mini kit (#74106) was purchased from QIAGEN (Hilden, Germany). Fetal bovine serum (#16000044), penicillin-streptomycin mixtures (#15140163), and G418 (geneticin, #11811-023) were purchased from Gibco (Grand Island, NY, USA). The primers were designed in Primer Premier 5.0 (Premier Biosoft, Palo Alto, CA, USA) and synthesized by Invitrogen. Other reagents were purchased from TIANGEN Biotech (Beijing, China) Co., Ltd., including ampicillin (#RT501), chloramphenicol (#RT502), and kanamycin (#RT503), et al.

2.2 Cells, bacteria, and plasmids

African green monkey kidney cells (Vero) were obtained from the American Type Culture Collection (ATCC, Manassas, VA, USA), maintained in DMEM medium supplemented with 10% fetal bovine serum, 250 U of penicillin ml^{-1}, and 250 g of streptomycin ml^{-1} at 5% CO_2 and 37°C. Vero cell lines steadily expressing Cre-recombinase were constructed as follows. *Cre* gene was ligated to pEGFP-N1 at the NheI and EcoRI sits, and then transfected into Vero by Lipofectamine 3000. Twenty-four hours later, the cells were washed 3 times with PBS and then, new medium containing 1000 μg ml^{-1} G418 was added. Cultivation was continued for another 2 days, during which time some cells died, while successfully transfected colonies were G418-resistant. Ten colonies were subjected to limiting dilution. Pure subclones steadily expressing Cre-recombinase were harvested. The *Cre*-Vero cells were cultured in the same manner as Vero except 200 g.ml-1 G418 was included in the mediums. Escherichia coli strain DH5α was cultured in LB medium. BAC-HSV-1 17 was a kind gift from David A. Leib. Plasmid pEPKan-s, pKD46, Bacillus subtilis and MG1655 were purchased from National Type Culture Collection (Biovector, NTCC), the plasmid profiles or genomes were available in the website (http://www.biovector.net/). And pBAD-I-sceI was obtained from Addgene, this plasmid allows an arabinose-inducible expression of the I-SceI homing endonuclease- for the en passant (two-step Red-mediated) mutagenesis. This plasmid profiles was available in http://www.biovector.net/.

2.3 pREDI construction

The construction process was as follows. (1) a 2.0-kb DNA fragment containing the *rhaRS* regulator and *PrhaB* gene from the MG1655 genome was amplified by PCR using the following forward (Prha-F) and reverse (Prha-R) primers: Prha-F, 5′-CAT GCC ATG GGG CAT GGC GAA TTA ATC TTT CTG CG-3′, and Prha-R, 5′-ATT ACC TGG TTT TTT TGA TGC ATT ATG TGA TCC TG-3′. The resulting fragment contained an NcoI restriction site at its 5′-end. (2) a 0.7-kb DNA fragment containing the *I-SceI* endonuclease gene from the pBAD-I-sceI genome was amplified by PCR using the following forward (I-SceI-F) and reverse (I-SceI-R) primers: I-SceI-F, 5′- TTA GAC TGG TCG TAA TGA AAT TCA GCA GGA TCA CAT CTG GGT C -3′, and I-SceI-R, 5′- CAT GCC ATG GGT CGA CTT ATT ATT TCA GGA AAG TTT CGG AGG AGA TAG TG -3′. This resulting fragment contained a 50-bp flanking sequence at its 5′-end, which overlapped with the 3′-end of the 2.0-kb *rhaRS-PrhaB* and had a NcoI restriction site at its 3′-end. (3) The 2.0- and 0.7-kb fragments were connected by the Prha-F and I-SceI-R primers to produce a 2.7-kb DNA cassette (*PrhaB-I-SecI*). (4) *PrhaB-I-SecI* was digested with NcoI endonuclease and cloned into the pKD46 plasmid. Finally, the positive recombinant clones were selected and further evaluated.

2.4 Primer design and homologous fragment amplification

The final "a-c-*Kan-sacB*-b-c" cassettes contained two especial sequences, *kanamycin* (*Kan*) and *SacB*, serving as positive and counter selection markers respectively. The construction process included a series of PCRs as follows: (1) a 1.0-kb *Kan* fragment from the pEPKan-s genome was amplified by PCR with the Kan-F and Kan-I-SecI-R primers: Kan-F, 5′- CGA TTT ATT CAA CAA AGC CAC GTT GTG TCT CAA AAT CTC TGA TGT TAC AT -3′, and

Kan-I-SecI-R, 5′- TAC GGG CCT AGG GAT AAC AGG GTA ATT GCC AGT GTT ACA ACC AAT TAA CC -3′. (2) a *SacB* fragment from the Bacillus subtilis genome was amplified with the I-SecI-sacB-F and sacB-R primers: I-SecI-sacB-F, 5′- CAC TGG CAA TTA CCC TGT TAT CCC TAG GCC CGT AGT CTG CAA ATC CTT TT -3′, and sacB-R, 5′- GCA TCT TGC AAG AAT GGC GCT CGT TTA ATA GTT GTC TTC TTT TTT CTT GA -3′. (3) *Kan* and *SacB* were ligated by the Kan-F and SacB-R primers to construct the *Kan-SacB* intact cassette. (4) ligating an approximately 50-bp flanking sequence in *Kan-sacB* 5′-end and 3′-end, which overlapped the downstream sequence of the target deletion gene, composing a c-*Kan-sacB*-b-c cassette. (5) Finally, using c-*Kan-sacB*-b-c fragments as templates, ligation with another 50-bp flanking sequence at its 5′-end, which overlapping the upstream sequence of the target deletion gene, was used to obtain a a-c-*Kan-sacB*-b-c fragment.

Specifically, because the *ICP34.5* gene included two ORFs, we named them as *ICP34.51* and *ICP34.52* in accordance with their order in the gene sequences. In order to knockout *ICP34.51*, *ICP34.52*, *UL5*, *UL41*, *UL29*, and *US8*, the c-Kan-sacB-b-c and a-c-Kan-sacB-b-c amplification primers were used (Table 1).

2.5 Preparation electro-competent cells

Bacterial strains were grown on LB cultures at 37°C and then harvested in early log phase (OD600 = 0.4) by centrifugation at 11,000g for 30 s in 4°C. The supernatant was removed immediately, and the cells were washed 3 times with pre-chilled 10% glycerol. The washed cells were resuspended in 10% glycerol, divided into eppendorf tube, and stored at -80°C.

2.6 Electroporation and identification

First, the recombinant plasmids pREDI and BAC-HSV-1 17 were co-transformed (1:1) into DH5a, to construct the gene knockout system. Next, the BAC-HSV-1 17-pREDI strains were made into electro-competent cells and transformed with appropriate homologous cassettes (10 µl, 200–400 ng) per 100 µl at 1500 V for 40 µs. Next, the cells were incubated in 500 µl SOC medium containing 10 mM arabinose at 30°C for 40 min and spread onto LB plates containing 10 mM arabinose, ampicillin, chloramphenicol, and kanamycin. These cells containing the homologous deletion cassettes were Kan-resistant. The targeted deletion genes were further tested by PCR with the primers shown in Table 1.

2.7 Removal of selection marker

The recombinant mutants were grown to OD_{600} = 0.4 at 30°C in 5 ml LB liquid medium, which contained ampicillin, chloramphenicol, kanamycin and 10 mM arabinose. Next, the bacterial suspension was diluted by 10-fold into another 5 ml LB medium, which contained ampicillin, chloramphenicol, 10 mM arabinose, and 5 % sucrose. Culture was continued till OD_{600} = 0.4. After diluting three times, the cells were plated on LB plates, which also contained 10 mM arabinose and 5% sucrose. Sucrose-resistant but Kan-sensitive clones were selected and verified by PCR further.

2.8 Virus reconstitution and BAC-excision

The deleted BAC-HSV-1 17 DNA was extracted and then transfected into *Cre*-Vero cells using Lipofectamine 3000 to excise the BAC backbone and reconstitute the virus. Following limited dilution, the virus was plaque-purified three times on *Cre*-Vero cells to ensure the harvest of a pure population with the BAC cassette excised.

BAC were placed between the *UL37* and *UL38* genes of HSV-1 in the BAC-HSV-1 plasmid (1). Whether BAC was excised was determined by DNA sequencing using the following primer, UL37F: 5′- CGG GGG CCT CGG ACG GGA GAC CG -3′. If the BAC was excised, the downstream sequence of *UL37* would be the *UL38* gene; if not, the BAC vector sequence would be present.

2.9 Virus titration

To compare the virus titer of the deleted and intact HSV-1, tissue culture infectious dose (TCID50, 50% tissue culture infective dose) was measured. Equivalent amounts of deleted and intact HSV-1 17 plasmids were transfected into *Cre*-Vero cells. Three to four days later, the cultures were lysed by three freeze-thaw cycles. Ten-fold dilutions were utilized to inoculate Vero cells that had been seeded in 96-well plates the previous day, and then maintained for another 4 days. $TCID_{50}$ values were calculated using the Reed-Muench method.

2.10 Real-time quantitative PCR

At 3–4 days post-transfection, RNA was extracted from the cells to detect gene expression. Detailedly, the medium was removed from the dishes, and the monolayers were washed twice with PBS. Next, total RNA was isolated and purified using the RNeasy Mini kit, and the concentration was measured using a NanoDrop (Thermo Scientific, Waltham, MA, USA). High-quality RNA (A260/A280 > 1.9) was converted into cDNAs. Gene expression was detected by RT-PCR. Detected genes included: *UL27* (IE gene), *UL30* (E gene), *UL18* (L gene), and latency-associated transcript gene (*LAT*). UL27: UL27-F, 5′- GCC TTC TTC GCC TTT CGC -3′, and UL27-R, 5′-CGC TCG TGC CCT TCT TCT T-3′. UL30: UL30-F, 5′-GGC GTG CGT GAC ATT CAA-3′, and UL30-R, 5′-GCA ACA TTC GAC GAG TTT CCT-3′. UL18: UL18-F, 5′-TGG CGG ACA TTA AGG ACA TTG-3′, and UL18-R, 5′- TGG CCG TCA ACT CGC AGA -3′. LAT: LAT-F, 5′-TGG CGG ACA TTA AGG ACA TTG-3′, and LAT-R, 5′-CCA TCG CCT TTC CTG TTC TCG-3′.

2.11 Statistical analysis

Data were evaluated using the student *t*-test. Each

experiment was performed at least three times. The results were considered significant when $P < 0.05$(*) or $P < 0.01$(**).

3. Results

3.1 Construction of pREDI

The knockout system was mainly composed of two recombinant plasmids, pREDI and BAC-HSV-1 (Fig. 1a). pREDI included *λ-red* recombinase and *I-SecI* endonuclease genes under control of the arabinose-inducible promoter *ParaB* and rhamnose-inducible promoter *PrhaB*, respectively. Thus, efficient and markerless gene knockout in the BAC-HSV-1 virus was achieved by adding different carbohydrates to the medium to induce the expression of specific genes.

First, the *I-SceI* was obtained from the pBAD-I-sceI genome (Fig. 1b); *rhaRS* regulator genes and *PrhaB* were obtained from the MG1655 genome (Fig. 1c). Next, the two cassettes were connected by recombinant PCR to acquire *PrhaB-I-SecI* (Fig. 1d). *PrhaB-I-SecI* was digested with NcoI and ligated into the pKD46 plasmid, followed by transformation into DH5α. Sequencing analysis (data not shown) and enzyme digestion further confirmed that the plasmid was constructed as expected (Fig. 1e).

3.2 Amplification of homologous fragments

In order to verify the effectiveness of this knockout system, as well as study HSV-1 pathogenicity, we deleted the *ICP34.51*, *ICP34.52*, *UL5*, *UL41*, *UL29*, and *US8* genes. Approximately four PCRs were required to amplify homologous fragments containing three homology regions (boxes a, b, and c) (Fig. 2a). First, *Kan* and *I-SecI-SacB* were obtained (Fig. 2b, 2c). Next, we ligated the two fragments by recombinant PCR (Fig. 2d). Next, using the *Kan-SacB* fragment as a universal template, we amplified six different c-*Kan-SacB*-b-c cassettes according to their varied downstream regions (boxes b and c) of the targeted genes (Fig. 3a). Using c-*Kan-SacB*-b-c as a template, the fragment was ligated with another 50-bp flanking sequence at the 5′-end (Fig. 3b). We obtained homologous fragments that overlapped with the up- and down-stream sequence of the target gene.

3.3 Rapid knockout of HSV-1

The a-c-*Kan-SacB*-b-c fragments were transformed into BAC-HSV-1 17-pREDI electro-competent cells and screened on selective plates. Kan-resistant colonies were further identified by PCR. More than 89.5% of Kan-resistant clones were the correct replacement strains (Table 4). In the deleted genomes, *Kan-SacB* was present, but not the target gene (Fig. 4).

3.4 Removal of selection markers

SacB proteins were lethal to *E. coli*. Thus, Kan-resistant but sucrose-sensitive cells represented marked mutants (Fig. 5a). When the *I-SceI* site between *Kan* and *sacB* be digested with the I-SceI endonuclease expressed from pREDI, the subsequent double-strand break-mediated intramolecular recombination between the two homology arms (box c) resulted in the removal of the inserted selection markers (*Kan-SacB*), producing a clean, markerless deletion. After three induction steps in rhamnose selective mediums, more than 80.0% of clones were sucrose-resistant, indicating that the efficiency of rhamnose-induction and DSB-repair was very high (Table 4). Sucrose-resistant but Kan-sensitive cells were considered unmarked mutants (Fig. 5b). PCR further demonstrated that both *Kan-SacB* (Fig. 5c) and the target gene (dates not show) were successfully excluded.

We successfully deleted the HSV-1 *ICP34.5*, *UL29*, *UL5*, *UL41*, and *US8* genes *in vitro*.

3.5 Virus reconstitution and BAC-excision

In order to reconstitute the mutant virus from deleted BAC-HSV-1 17 genomes and to remove the BAC backbone, *Cre*-Vero was transfected with the plasmids. Three days later, lesions were observed in HSV-1 17, Δ*ICP34.51*, and Δ*ICP34.52*. Lesions were also present in ΔUL41 after another three days (Fig. 6a). The virus was harvested and plaque-purified three times on *Cre*-Vero to ensure that a pure population of virus with BAC excised was obtained. The DNA sequencing results showed that the region downstream of *UL37* was close to *UL38* but not to the BAC (data not shown). This indicates that the BAC backbone of the mutants was removed. However, lesions were not observed in Δ*UL5*, Δ*UL29*, and Δ*US8* until 14 days after transfection (Fig. 6b). These results suggested that *UL5*, *UL29*, and *US8* were essential for the propagation of HSV-1.

We obtained ΔICP34.51, Δ34.52, ΔUL41, ΔUL5, ΔUL29, and ΔUS8 mutants with our gene knockout system. Those mutants would become powerful tools for investigating gene functions, studying pathogenesis, or developing vaccines.

3.6 Virus titer detection

To compare the different pathogenicity of those mutants, viral titers were calculated firstly. The deleted and HSV-1 17 plasmids were transfected into *Cre*-Vero cells. After continuous cultivation for seven days, we calculated the TCID50 using the Reed-Muench method and detected gene expression by real-time PCR. Compared to wild-type HSV-1 17, the TCID50 values of the mutants were significantly reduced ($P < 0.01$) (Fig. 6c), indicating that those deletants reduced the toxicity of HSV-1. Moreover, the IE (*UL27*), E (*UL30*), and L (*UL18*) gene expression levels decreased (Fig. 6d), suggesting that these gene deletion mutants inhibited the production of (lytic) infection to different extents. The latency-associated transcript gene (*LAT*) was not apparently influenced in ΔICP34.51, Δ34.52, ΔUL41, and ΔUL5, suggesting that the lack of these four genes is not or only minimally related to virus latency. However, *LAT* expression was down-regulated in ΔUL29 and ΔUS8, indicating a role

in inhibiting latent progress of the virus. The detailed effects of these mutants must be further examined. Anyhow, our data provides an important tool for further studies.

4. Discussions

HSV-1 is an important human pathogen [28, 29]. Infection mainly occurs through direct contact, but also through virus-contaminated objects or indirect infection. Some surveys have shown that more than 90% of people might suffer with herpes virus during their lifetimes, and herpes simplex virus infection has become the world's fourth largest infectious disease [30, 31]. Thus, studying this virus and developing treatment for infections by the virus are urgently necessary.

Therefore, we developed an efficient and precise gene deletion system to targeted delete the regions of HSV-1 genomic. And the following pathogenicity changes of mutants could be used as a powerful tool to study the virus. Specially, this knockout system involved two recombinant plasmids, BAC-HSV-1 and pREDI. BAC-HSV-1 ligated to the full-length genome of HSV-1 on BAC, which has the potential to form intact infectious virus. pREDI expresses two specific enzymes: (1) λ-red recombinase, under the induction of arabinose, promoting replacement of the target genomic region with exogenous homologous fragments [32-34], and (2) I-SceI endonuclease, under control of the rhamnose-inducible promoter *PrhaB*, which causes double-strand breakage and further promotes deletion of the introduced selection marker by mediating intramolecular recombination [35, 36]. The "a-c-*Kan*-*sacB*-b-c" homologous fragments we amplified contained *kanamycin* and *SacB*, acting as positive/counter selection markers respectively. The interior *I-SceI* endonuclease site can be recognized and digested by pREDI, promoting the removal of the two selection markers.

The basal expression level of the *PrhaB* promoter is 10-fold lower than that of *ParaB* in the absence of the corresponding inducers (rhamnose) [37]. This has made good on its strategy to stepwise delete a target gene without leaving a selection marker behind in our knockout work, simply by changing the different carbohydrate in the media from arabinose to rhamnose. In other words, the initial homologous exchange can be promoted by λ-red recombinase, but is unaffected by endonuclease. The subsequent removal of selection marker can be tightly regulated by additional adding rhamnose, thus minimizing unwanted cleavage of the homologous fragment and increasing recombination efficiency. Furthermore, *Cre*-Vero cells specially express of *cre*-recombinase, which targeted identifies and shears the site between HSV-1 genome and BAC-backbone. The following homologous recombination in HSV-1 genome could repair this site and remove the BAC.

Compared with existing knockout systems, our method is well adapted to genomic manipulation *in vitro* for the following four advantages. First, using *SacB* as a counter selection marker makes it unnecessary for specific prerequisite strain. Second, the fragment-insertion/marker-excision cycle introduces a finally clean and unmarked mutation. Third, the whole work is timesaving and labor-extensive. Once this system is established, we can utilize it to knockout different genes, as long as changes the arms (boxes a, b or c) of a-c-*Kan*-*sacB*-b-c cassette. Fourth, the entire deletion process only involves in HSV-1 genome rather than viruses, ensuring safety of the experiment. In conclusion, we were the first to develop a special gene deletion system to aim to knock out HSV-1 genes, and we had knocked out *ICP34.5*, *UL5*, *UL41*, *UL29*, and *US8* genes of HSV-1 virus and conducted preliminary studies on the effects of these genes on virus titer.

5. Acknowledgments

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Figures:

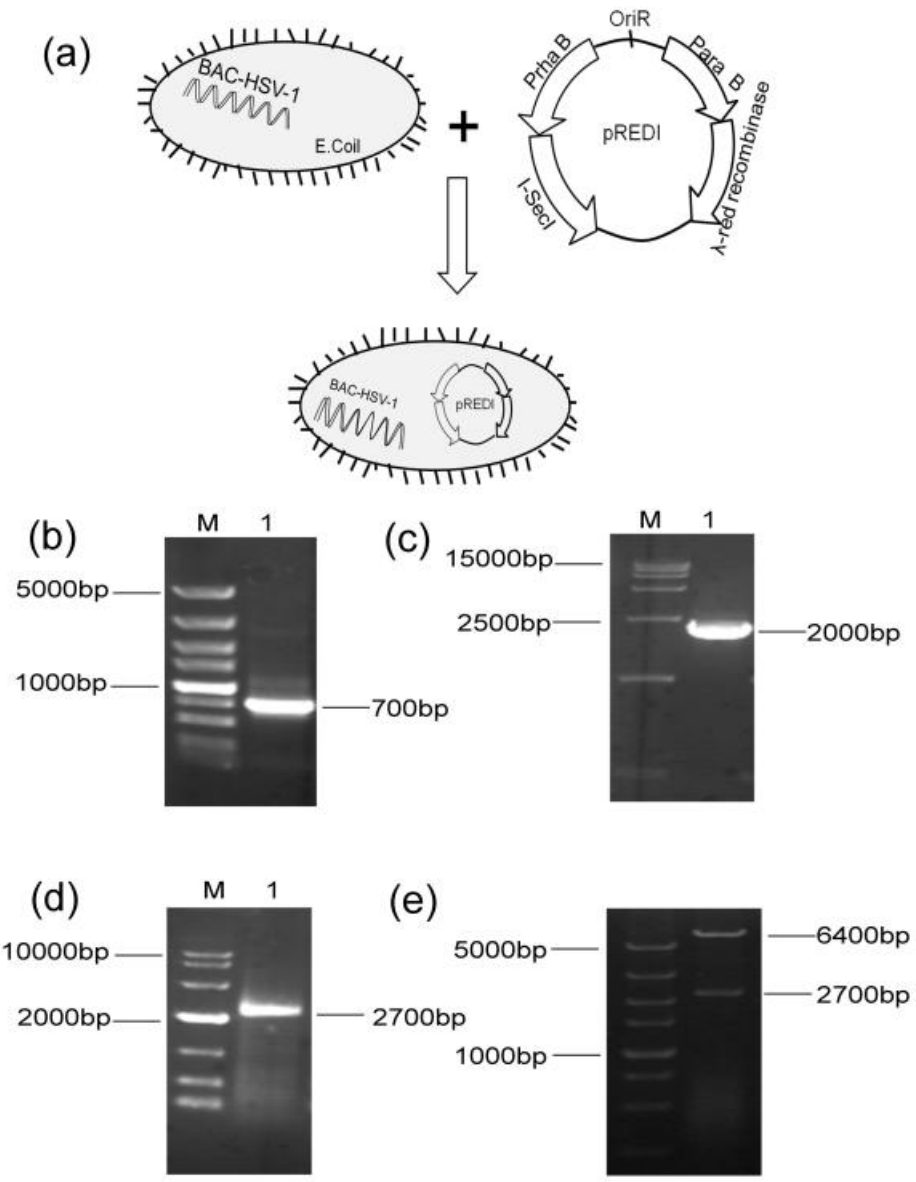

Figure 1: Construction of HSV-1 gene knockout system. (a) BAC-HSV-1 17.37 and pREDI were electroporated into DH5α cells to develop the integrated system. (b) PCR product of *I-SceI*. M: DNA marker DL5000, line 1: *I-SceI* fragment, product size 0.7 kb. (c) PCR product of *rhaRS* regulator gene and *PrhaB*. M: DNA marker DL15000, line 1: sequence of interest, product size 2.0 kb. (d) Complete sequence of *I-SceI* and *PrhaB* produced by overlapping PCR, M: DNA marker DL15000, line 1: *PrhaB-I-SceI*, product size 2.7 kb. (e) NcoI endonuclease identification of pREDI. The cleaved product, *PrhaB-I-SceI*, was harvested.

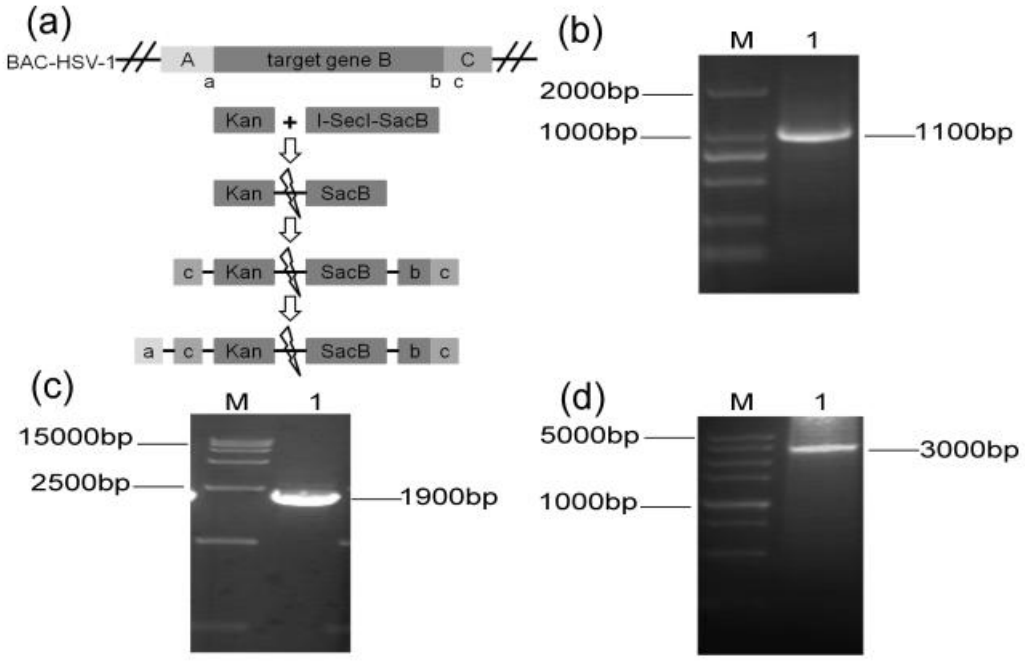

Figure 2: Schematic representation of homologous fragments. (a) Amplification strategy of homologous fragments. Four PCRs were required to amplify homologous cassettes; the final product contained a positive selective marker (*Kan*), counter-selective marker (*SacB*), and three homology regions (boxes a, b, c). (b) PCR product of *Kan*, M: DNA marker DL2000, line 1: *Kan* fragment, product size 2.1 kb. (c) PCR product of *SacB*, M: DNA marker DL15000, line 1: *SacB* fragment, product size 1.9 kb. (d) Complete sequence of *Kan* and *SacB*, M: DNA marker DL5000, line 1: *Kan-SacB*, product size 3.0 kb.

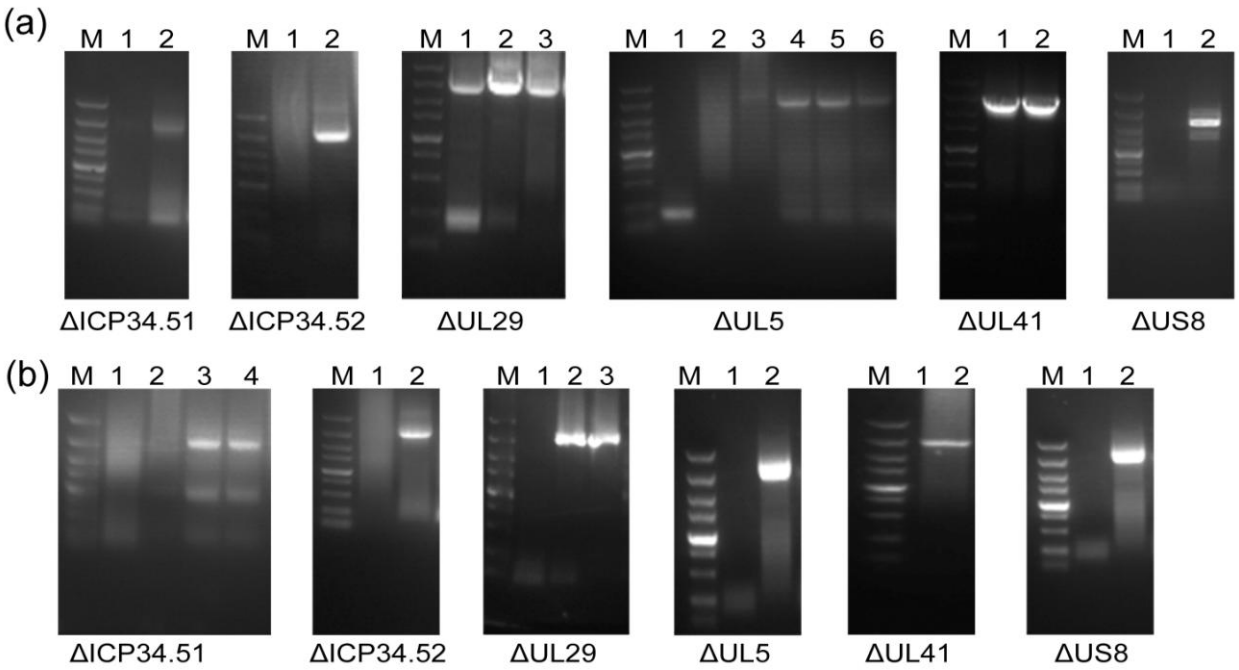

Figure 3: Amplification of homologous fragments. (a) Six different target genes of c-*Kan-SacB*-b-c cassettes. Using *Kan-SacB* fragments as a universal template, different boxes "c" and "b–c" were linked on their two terminals. (b) Six different a-c-*Kan-SacB*-b-c cassettes. Using c-*Kan-SacB*-b-c fragments as templates, boxes "a" were linked on their 5′ terminals. M: DNA marker DL5000.

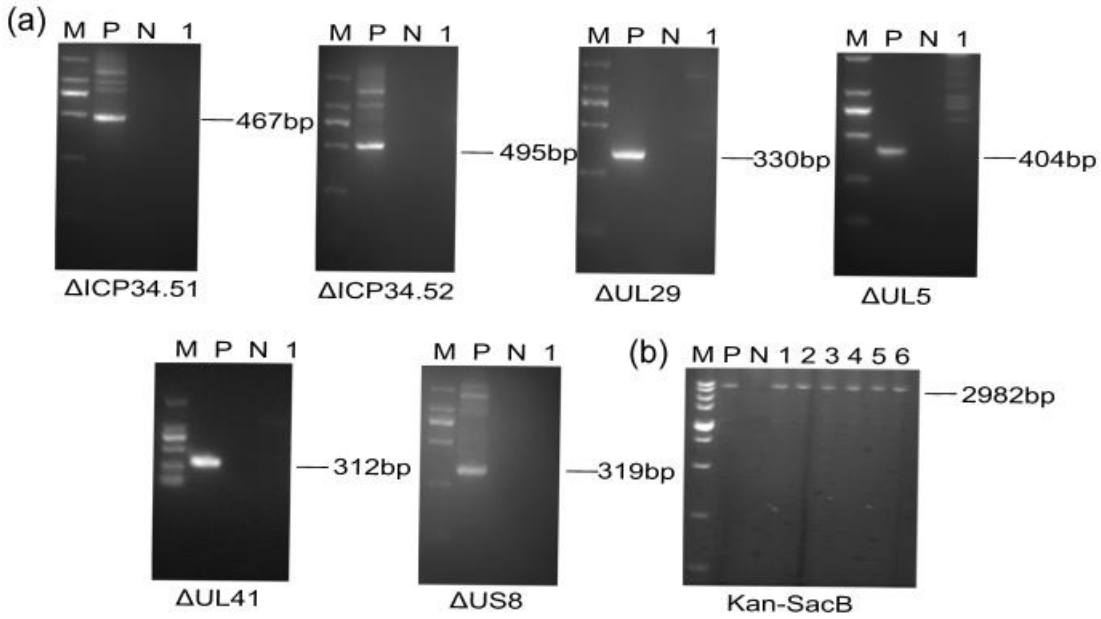

Figure 4: Rapid knockout of HSV-1. Homologous fragments a-c-*Kan-SacB*-b-c were electrically transformed into the knockout system, where the cassettes could recognize and replace their target genomic segments with the help of λ-red recombinase. (a) PCR verification of target genes *ICP34.51*, *ICP34.52*, *UL29*, *UL5*, *UL41*, and *US8*, in order. M: DNA marker DL2000, P: positive control, N: negative control, line 1: target gene, product sizes of 467, 495, 330, 404, 312, and 319 bp, respectively. The deleted mutants could not amplify the corresponding target genes. (b) PCR verification of *Kan-SacB*. M: DNA marker DL5000, P: positive control, N: negative control, line 1–6: *Kan-SacB* cassettes of six mutants were identified, product size of 2982 bp. To summarize the validation experiments above, we deleted the *ICP34.51*, *ICP34.52*, *UL29*, *UL5*, *UL41*, and *US8* genes.

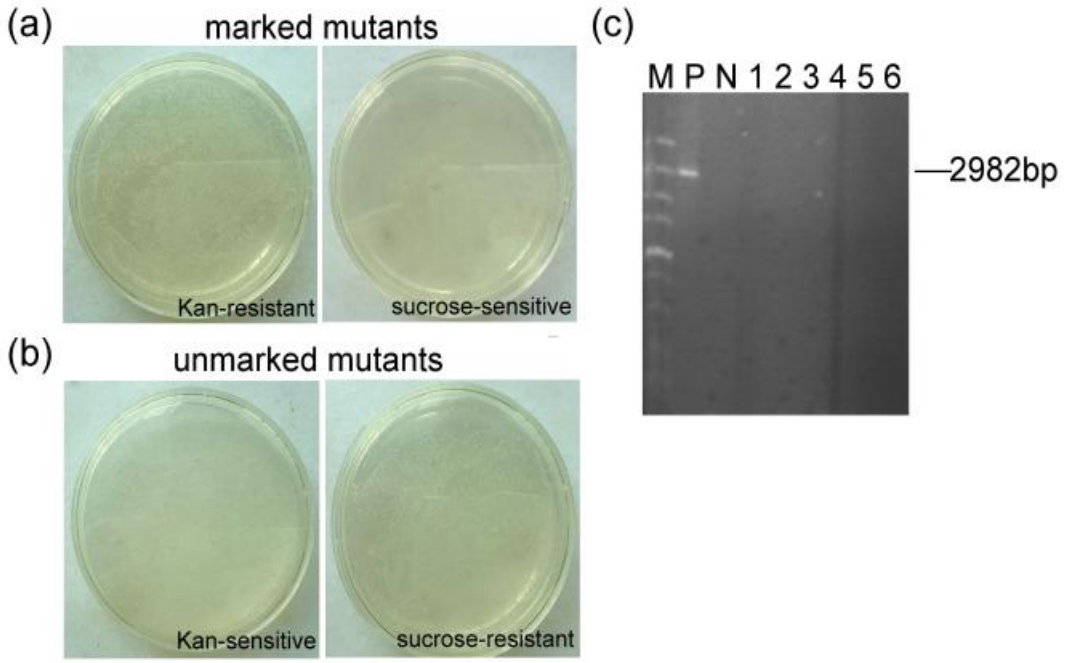

Figure 5: Removal of selection markers. (a) Plates identification of antibiotic and sucrose. The selection markers included mutants that were Kan-resistant (left), but sucrose-sensitive (right). (b) The selection markers excluded cells that were Kan-sensitive (left), but sucrose-resistant (right). (c) PCR verification of *Kan-SacB*. M: DNA marker DL5000, P: positive control, N: negative control, line 1–6: the markerless deletion of six genomes did not leave the *Kan-SacB* fragment behind.

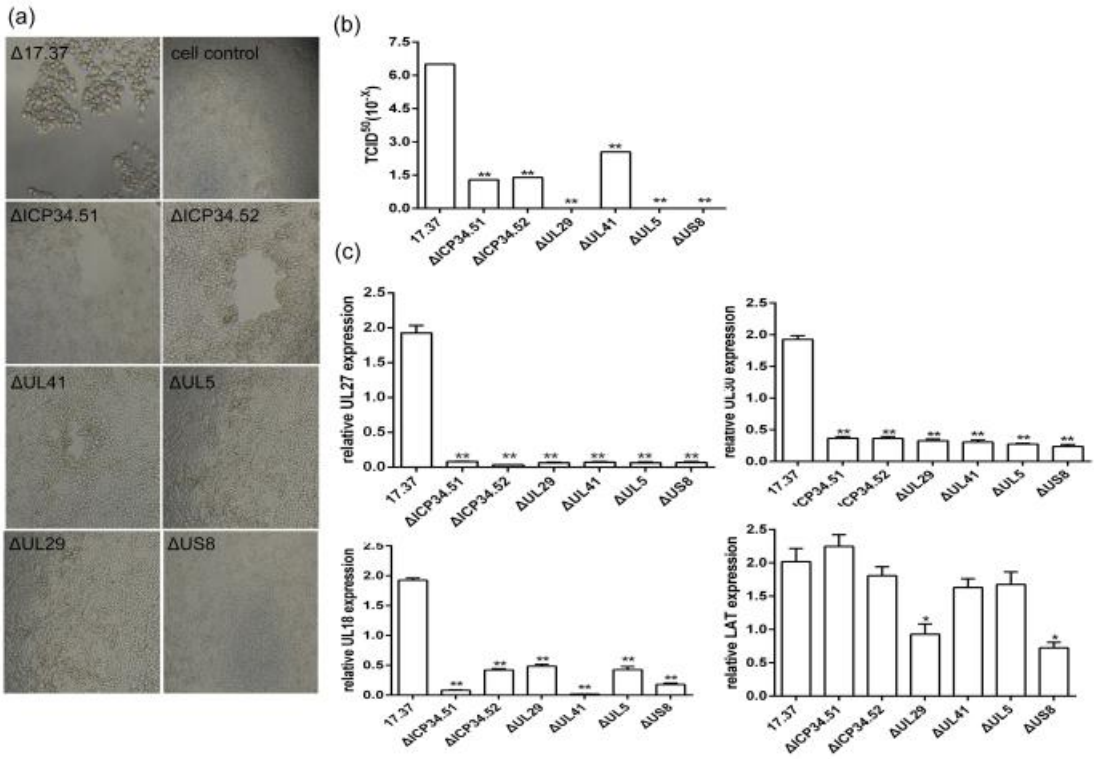

Figure 6: Pathogenic analysis of deletion mutants. (a) Lesion observation. Cells were transfected with wild-type *17.37* and six deletion plasmids. Lesions only occurred in HSV-1 *17.37*, Δ*ICP34.51*, Δ*ICP34.52*, and Δ*UL41*. (b) Virus titration of deletion mutants. Seven days after transfection, the $TCID_{50}$ of *17.37* was $10^{-6.50}$, while that of Δ*ICP34.51*, Δ*ICP34.52*, and Δ*UL41* was only $10^{-1.29}$, $10^{-1.40}$, and $10^{-2.55}$ (c), respectively, using RT-PCR to detect gene expression. IE (*UL27*), E (*UL30*), and L (*UL18*) gene expression decreased to different degrees. However, LAT levels differed from each other.

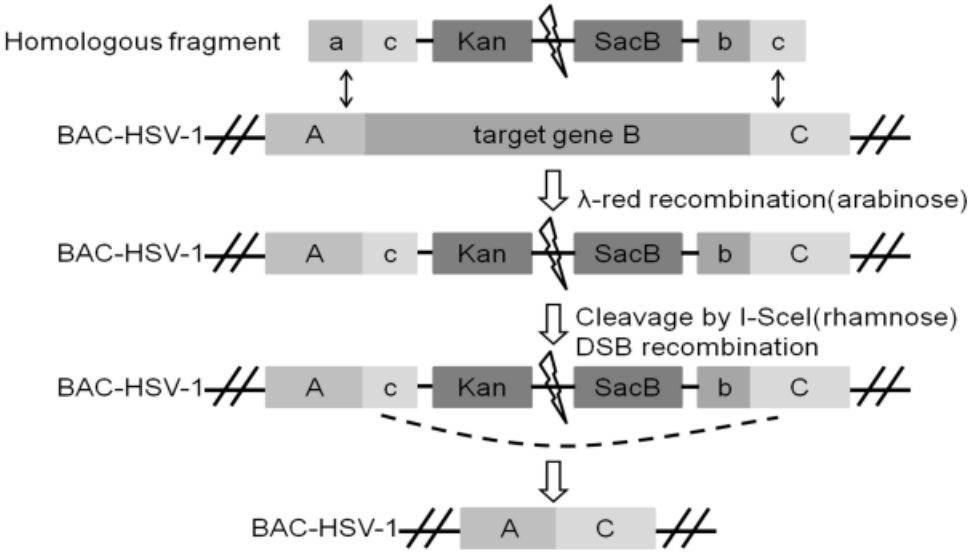

Figure 7: Flowchart of whole gene knockout. The deletion studies were conducted in BAC-HSV-1 *17.37* plasmids and regulated by pREDI. To delete the HSV-1 targeted chromosomal region between gene A and C, a homology fragment, a-c-*Kan*-*SacB*-b-c, which contained a positive selective marker (Kan), negative selective marker (SacB), and three homology (boxes a, b, and c), was amplified. Next, a-c-*Kan*-*SacB*-b-c were electroporated into the knockout system, which replaced the target genomic segment under the promotion of λ-red recombinase. Next, we induced the expression of I-SceI endonuclease (between Kan and sacB) by adding 10 mM rhamnose. The cleavage of DNA induced the double-strand break repair function to promote intramolecular recombination between the two homology arms (box c), resulting in removal of the inserted deletion cassette to produce a clean, markerless deletion.

Tables

Table 1: Primers sequences of gene knockout

Primers	Sequences	Amplications
CB-ICP34.51	5'- TAA CGT TAC ACC CGA GGC GGC CTG GGT CTT CCG CGG AGC TCC CGG GAG CTC GAT TTA TTC AAC AAA GCC A -3'	amplificate c-*Km-sacB*-b-c cassette of *ICP34.51*
ICP34.51-ab2	5'- CGG GCC AGG GCC CGG GCA CGG GCC TCG GGC CCC AGG CAC GGC CCG ATG AGC ATC TTG CAA GA ATG GCG CT -3'	
CB-ICP34.52	5'- CGT TAC ATG GAG ACA GAG AGC GTG CCG GGG TGG TAG AGT TTG ACA GGC AAG CAT GCG ATT TAT TCA ACA A -3'	amplificate c-*Km-sacB*-b-c cassette of *ICP34.52*
ICP34.52-ab2	5'- AAC CGC ACA GTC CCA GGT AAC CTC CAC GCC CAA CTC GGA ACC CGC GGT CAG CAT CTT GCA AGA ATG GCG C -3'	
CB-UL5	5'- CAT GAC CGC ACC ACG CTC GCG GGC CCC CAC TAC GCG TGC GCG GGG GGA CAC GAT TTA TTC AAC AAA GCC A -3'	amplificate c-*Km-sacB*-b-c cassette of *UL5*
UL5-ab2	5'- GGC CGA GGC CTT TTT AAA TTT TAC GTC TAT GCA CGG GGT GCA GCC AAT CCG CAT CTT GCA AGA ATG GC GC -3'	
CB-UL41	5'- CTA CAT GTC AGG TCA ATT GTA ACT GCG GAT CGG CCT AAC TAA GGC GTG GTT CGA TTT ATT CAA CAA AGC C -3'	amplificate c-*Km-sacB*-b-c cassette of *UL41*
UL41-ab2	5'- CGT CAT GTA CAC GTT GGT GGT CAA ATA TCA GCG CCG ATA CCC CAG TTA CGG CAT CTT GCA AGA ATG GCG C -3'	
CB-UL29	5'- CAT GTC CTT TTG TCA ATC GGT CCG CGA ACG GAG GTA ATC CCG GCA CGA CGC GAT TTAT TCA ACA AAG CCA -3'	amplificate c-*Km-sacB*-b-c cassette of *UL29*
UL29-ab2	5'- CCT GGG ATA CGT GTA CGC TCG CGC GTG TCC GTC CGA AGG CAT CGA GCT TCG CAT CTT GCA AGA ATG GCG C -3'	
CB-US8	5'- TAA GGC GCC CCA TCC CGA GGC CCC ACG TCG GTC GCC GAA CTG GGC GAC CGC GAT TTA TTC AAC AAA GCC A -3'	amplificate c-*Km-sacB*-b-c cassette of *US8*
US8-ab2	5'- ACG GAG CCG TTG GTG ATA AGA TCT CAA AGC CGG ATC CAT TGG TGG AGG GAG CAT CTT GCA AGA ATG GCG C -3'	
ICP34.51-ab1	5'- CAT GCT TGC CTG TCA AAC TCT ACC ACC CCG GCA CGC TCT CTG TCT CCA TGT AAC GTT ACA CCC GAG GCG G -3'	together with ICP34.51-ab_2, amplificate a-c-Km-sacB-b-c cassette of *ICP34.51*
ICP34.52-ab1	5'- AGC TCC CGG GAG CTC CGC GGA AGA CCC AGG CCG CCT CGG GTG TAA CGT TAC ATG GAG ACA GAG AGC GTG C -3'	together with ICP34.52-ab_2, amplificate a-c-*Km-sacB*-b-c cassette of *ICP34.52*
UL5-ab1	5'- GTG AGT CCC CCG GGC CGG GTT CGG TGG AAC TGT AAG GGG ACG GCG GGT TAC ATG ACC GCA CCA CGC TCG C -3'	together with *UL5*-ab_2, amplificate a-c-*Km-sacB*-b-c cassette of *UL5*
UL41-ab1	5'- TGG TGG GTC GTT TGT TCG GGG ACA AGC GCG CTC GTC TGA CGT TTG GGC TAC ATG TCA GGT CAA TTG TAA C -3'	together with UL41-ab_2, amplificate a-c-*Km-sacB*-b-c cassette of *UL41*
UL29-ab1	5'- TAC ACA CAT TCC CCG CCC CGC CCT AGG TTC CCC CAC CCC CCA ACC CCT CAC ATG TCC TTT TGT CAA TCG G -3'	together with UL29-ab_2, amplificate a-c-*Km-sacB*-b-c cassette of *UL29*
US8-ab1	5'- TGT TGG GCT CCC ATT TTA CCC GAA GAT CGG CTG CTA TCC CCG GGA CAT GTA AGG CGC CCC ATC CCG AGG C -3'	together with US8-ab_2, amplificate a-c-*Km-sacB*-b-c cassette of *US8*
ICP34.51-t1	5'- TGC CTG TCA AAC TCT ACC ACC C -3'	test for *ICP34.51*
ICP34.51-t2	5'- TTC CGC CAA AAA AGC AAT TAG -3'	
ICP34.52-t1	5'- CTG GCT CCA AGC GTA TAT ATG CG -3'	test for *ICP34.52*
ICP34.52-t2	5'-CGG CCG AGA CTA GCG AGT TAG AC -3'	
UL5-t1	5'- CGA TGG TCG TCT GTG GAT TGG -3'	test for *UL5*
UL5-t2	5'-GGT AAG TGG TGG CGT TGA GGA -3'	
UL41-t1	5'- TGG GTC TCA TTG TGA TTC TTG TCA -3'	test for *UL41*
UL41-t2	5'-CCC TAA CGG ATT ATT GTC CTC TTG -3'	
UL29-t1	5'- AAA CAA GCA CGG ATG TCG TAG CA -3'	test for *UL29*
UL29-t2	5'-CGG AGC ACC TGA CCG TAA GCA -3'	
US8-t1	5'- TTT GCC TCT TCT GGC GGG TTG -3'	test for *US8*
US8-t_2	5'-CGG TCG CTG TGA CTC ACG ATT TT -3'	

Table 2: Efficiency of knockout

Target genes	Positive colonies/Tested colonies (%)		Overall efficiency (%)
	Positive selection marker replacement#	selection marker excision*	
ICP34.51	12/12 (100)	17/20 (85.0)	85.0
ICP34.52	12/12 (100)	17/20 (85.0)	85.0
UL5	17/19 (89.5)	20/20 (100)	89.5
UL41	14/15 (93.3)	18/20 (90.0)	83.9
UL29	15/15 (100)	18/20 (90.0)	90.0
US8	10/10 (100)	16/20 (80.0)	80.0

PCR verifying of Kan-resistant colonies after a-c-Kan-sacB-b-c cassettes transformed.
* sucrose-resistant colonies/ total rhamnose-induced colonies.

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