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A knockdown of the herpes simplex virus type-1 gene in all-in-one CRISPR vectors

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Abstract

Introduction. Herpes simplex virus type 1 (HSV-1) is a virus that causes serious human disease and establishes a long-term latent infection. The latent form of this virus has shown to be resistant to antiviral drugs. Clustered Regularly Interspace Short Palindromic Repeats (CRISPR), is an important tool in genome engineering and composed of guide RNA (gRNA) and Cas9 nuclease that makes an RNA-protein complex to digest exclusive target sequences implementation of gRNA. Moreover, CRISPR-Cas9 system effectively suppresses HSV-1 infection by knockout of some viral genes.

Materials and methods. To survey the efficacy of Cas9 system on HSV-1 genome destruction, we designed several guide RNAs (gRNAs) that all packaged in one vector. Additionally, we performed a one-step restriction using *BamHI* and *Esp3I* enzymes.

Results. CRISPR/Cas9 system targeted against the gD gene of HSV-1 was transfected into HEK-AD cells that showed a significant reduction of HSV-1 infection by plaque assay and real-time PCR.

Conclusion. The *pCas-Guide-EF1a-GFP CRISPR* vector can create a fast and efficient method for gRNA cloning by restriction enzymes (*Esp3I* (*BsmBI*) and *BamHI*). Therefore, the CRISPR/Cas9 system may be utilized for the screening of genes critical for the HSV-1 infection and developing new strategies for targeted therapy of viral infections caused by HSV-1. *(Folia Histochemica et Cytobiologica 2020, Vol. 58, No. 3, 174–181)*

Key words: CRISPR; Cas9; gRNA; genome editing; HSV-1; HEK-AD cells

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Introduction

Herpes simplex virus type 1 (HSV-1) is a very contagious and lifetime infectious pathogen and remains an important problem worldwide [1, 2]. Herpes viruses are known to be involved in lytic infection in which viruses lyse their host cells or latent infection in which viruses remain silent within the host cells [1, 3].

In recent years, several studies have focused on DNA editing [4, 5] such as zinc-finger nucleases (ZFNs), transcription activator-like effector nucleases (TALENs) system. However, Clustered Regularly Interspace Short Palindromic Repeats (CRISPR) has been considered as an important acceptable system for genome editing [6]. CRISPR/Cas9 system which originated from a wide range of bacteria and archaea can be an adaptive bacterial immune system that mainly targets genome of bacterial invasive phages [7].

CRISPR-associated (Cas) genes are located adjacent to a CRISPR locus [8]. The CRISPR-Cas system is clustered into three major types (I, II, III) that CRISPR associated protein 9 (Cas9) is the signature gene of type II and also, the most efficient genome editing tool [9].

The Cas9 system consists of two major components including an endonuclease protein which can produce a double-strand break in DNA and two RNA components including trans-activating crRNA (tracrRNA) and mature CRISPR RNA (crRNA) which are responsible for identifying a short DNA sequence; 5'-NGG-3'; called the protospacer adjacent motif (PAM). The crRNA/tracrRNA complex associated with Cas9 creates an active ribonucleoprotein (RNP) complex [10]. In this system, crRNA can conduct the Cas9 protein as an RNA-guided endonuclease. Briefly, the crRNA 5'-end (twenty nucleotides) can interact with Guanine nucleotide of the PAM on the non-complementary DNA strand to form RNA-DNA complementarity due to the interaction between DNA backbone 5' and a phosphate-lock loop in Cas9. As mentioned above, Cas9 protein as an endonuclease protein cleaves the recognized target site to create a double-strand break (DSB) [10]. To fix this break, there have been two cellular repair mechanisms identified, including Non-Homologous End Joining (NHEJ) which joins the damaged ends of chromosomes and needs little to no DNA sequence homology.

Another mechanism is Homology Directed Repair (HDR) that has high precision to repair the DSB with the pattern of a DNA strand. For example, in humans, the NHEJ system has a higher recovery rate than the HDR [6, 11, 12].

In the CRISPR system, gRNA and Cas9 are needed to knock out or in any gene. Although there are many vectors for the gRNAs, p-Cas-Guide-EF1a-GFP CRISPR vector Origene Technologies (Rockville, MD, USA) as an all-in-one vector has both Cas9 and gRNA. Additionally, the vector contains an enhanced green fluorescent protein (EGFP) gene that can detect transfected cells. The gRNA and Cas9 protein are also under the control of the U6 promoter. For the high efficiency of gRNA transcription, U6 is an excellent housekeeping promoter, which is transcribed by RNA polymerase III [13].

One of the advantages of this system is the high and continuous transcription gRNA and Cas9 by stable functional promoters in p-Cas-Guide-EF1a-GFP CRISPR Vector. To design DNA with high-efficiency expression by U6 promoter, guanine should be considered as the first transcribed nucleotide. In the previous studies, multi-vector systems were used to knock out or in any gene, which was costly and time-consuming. For solving this problem, we designed a single vector CRISPR system in the present study.

The objective of this study is to establish a novel method to clone gRNA in p-Cas-Guide-EF1a-GFP CRISPR Vector by using *Bsm*BI and *Bam*HI restriction enzymes. These enzymes have specific detection sites (*Bam*HI: 5-GGATCC-3 and *Bsm*BI: 5-CGTCTC (N1) -3, 3-GCAGAG (N5) -5) and also, gRNAs can be easily and quickly clone between two restrictions sites. Here, we used a one-step restriction digestion method using *Esp3I* and *BamHI* and the ligation reaction (defined as a construction procedure of one g-RNA expression vector). Additionally the main goal of designing this new system is to perform knockouts in the HSV-1 genome and study the function of essential and non-essential genes in the virus.

Materials and methods

Software selection. Designing an efficient and functional sgRNA which can accurately join to the target DNA is the first step in the CRISPR system. However, this proposed gRNA is restricted to avoid off-target sites. In this regard, there have been several software programs currently used, and are listed in Table 1.

CHOPCHOP is one of the most powerful tools used to design CRISPR/Cas9, CRISPR/Cpf1, and TALEN systems. This software supports many features in order to design genome editing systems and allows us to add the undefined genomes of organisms. A target can be mentioned in both the name of the gene and the sequence (FASTA format).

Table 1. Several online software for gRNA design. These software are usually designed and supported by universities and research centers

Software	CHOPCHOP	E-CRISP	Cas-OFFinder	Genetic perturbation platform web portal
PAM	Ability to choose PAM	NGG	Different PAM	NGG, NNGRR, TTTV and enAsCas12a
Cas	Cas9, Cas12 and Cas13	Cas9	Cas9, Cas12 and MAD7 nuclease	Cas9 and Cas12
Target genome	It includes a large number of creatures and the ability to add genomes that non-defined in the software	55 Organisms	It includes a large number of creatures and the ability to add genomes that non-defined in the software	Human Mouse Rat
On target scoring	Doench *et al.* 2014 [22] Doench *et al.* 2016 [23] Xu *et al.* 2015 [24] Moreno-Mateos *et al.* 2015 [25] Shen *et al.*, 2014 [26]	Xu *et al.*, 2015 [24] Doench *et al.*, 2014 [22]		Doench, Fusi *et al.* 2016 [23] Snson, *et al.* 2018 [27] Kim, *et al.* 2018 [28]
Off target scoring	Hsu *et al.*, 2013 [29]	S-score	Standalone command--line program [30]	Use on target scoring for off target and on target prediction
Scientific institute or university	Harvard	German Cancer Research Center	Seoul National University	Broad Institute
Web site	http://chopchop.cbu.uib.no	http://www.e-crisp.org/E-CRISP/ /designcrispr.html	http://www.rgenome.net/cas-designer/	https://portals.broadinstitute.org/gpp/public/analysis-tools/sgrna-design

For example, the gene name refers to the area which can be designed for the gRNA (coding region, promoter, selected exons, 3' or 5' UTR, and splice sites). Other advantages of this software include selecting the PAM sequence (for different CRISPR systems), calculating the efficiency score method, detecting off-targets in the genome, calculating the number of bases (which can be self-complementary), and choosing different gRNA backbones. Furthermore, to increase transcriptional efficiency, the promoter requires specific bases at transcription initiation. For example, for an optimal transcription by the T7 promoter, the two primary transcripts of the nucleotide should be GG and for the U6 promoter must be G. Also, this software can design the primers to amplify the area in which the indel mutation is created by the CRISPR system. This software can identify the restriction enzymes that are at one or more points within the replication region through the primers. In the current study, *Herpes simplex virus type 1 KOS strain* and human including US6 (glycoprotein D), UL39 (ribonucleotide reductase subunit 1), UL23 (Thymidine kinase), ICP34.5 and ASS1 (argininosuccinate synthase 1) were respectively selected and included into CHOPCHOP.

Cloning of gRNA. Next step, sgRNAs should be cloned in pCas-Guide-GFP vector from now on as pCas-Guides. For cloning into this vector, several nucleotides (5-*GATCG*NNNNNNNNNNNNNNNNNNN*G*-3 and 5-*AAAAC*NNNNNNNNNNNNNNNNNNN*C*-3) are added to two sgRNA target ends complementary to the target region, and were ordered in two separate strands. When two oligo strands are annealed together, pre-cut sticky end of *BamHI* and *BsmBI* was established. Table 2 shows the protocol for annealing two proposed strands.

pCas-Guides CRISPR vector was cut by *BamHI* and *BsmBI* and followed by DNA clean up (Favorgen, Taiwan). Moreover, this tool can show the molar ratio of insert: vector that of 5:1 was recommended in the current study. Ligation reaction of gRNAs into double digested *pCas-Guides*

Table 2. Oligo annealing protocol used in the study

PCR program for oligo annealing		Add and mix the material in a PCR tube
Temperature	Time	5 μl forward oligo (10 μM) 5 μl reverse oligo (10 μM) 40 μl DNase free water
95°C	5 min.	
90°C	30 sec.	
80°C	30 sec.	
70°C	30 sec.	
60°C	30 sec.	
50°C	30 sec	

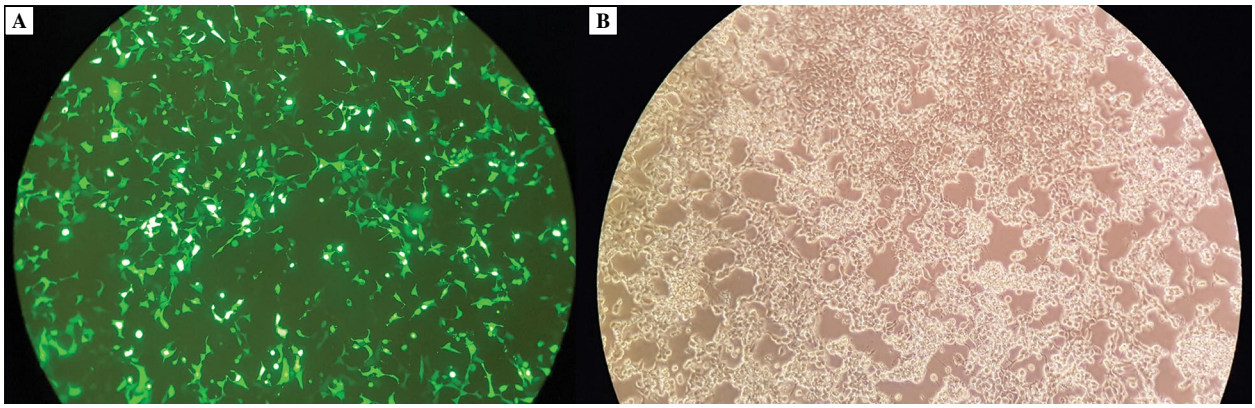

Figure 1. GFP expression of *pCas-guides* in HEK293-AD cells. **A.** Observation at 48 h post-transfected HEK293-AD cells. **B.** Control cells using an inverted fluorescent microscopy.

CRISPR vector was prepared in a 10 μl volume including 2 ng of gRNA, 150 ng of vector, 0.5 μl of T4 ligase, and 1 μl of 10× ligation buffer.
1 μL of 10× ligation buffer, 150 ng precut *pCas-Guide* vector (10 ng/μL), 5 ng annealed double-stranded oligo (diluted), 0.5 μL ligase (0.5 μL), and 6.5 μL dH_2o were mixed incubated for 2 hours at 22°C. Of this, 5 μL of the ligation mixture was added to competent cells.

Transfection of pCas-guide-Ef1-GFP. HEK293-AD, a derivative of the HEK293 cell line [14], was grown in a 24-well plate which contained Dulbecco's modified Eagle's medium (DMEM) (Gibco, Gaithersburg, MD, USA) supplemented with 10% fetal bovine serum (FBS) (Gibco, USA) and 100 IU/mL penicillin and 100 μg/mL streptomycin (Sigma-Aldrich, St. Louis, MO, USA) at 37°C in a humidified atmosphere containing 5% CO_2 for 48 hours. For transfection efficiency, *pCas-guides* were transfected using the *PolyFect* reagent (Qiagen, Germany). For each well, both DNA (1, 1.5, and 2 μg) and *PolyFect* (2, 4, and 6 μl) were diluted in DMEM to the final volume 100 μl. The complex solution was mixed and incubated for 15 min at room temperature, and then dropped into wells. After 5 hours, the supernatant was removed and fresh complete media was added into each well. At 24–48 hours post-transfection, expression of GFP reporter *pCas-guide* was examined by an inverted fluorescent microscope (Fig. 1) [15].

Virus production assay. HSV-1 genomic DNA in the supernatants from HSV-1 infected or transfected with gD gRNA was extracted by the DNA extraction kit according to the kit instructions (Favorgen, Taiwan). Relative quantitative real-time PCR for DNA of the virus was used and 2-ΔΔCt (CtHSV-1CtHSV-1 with gRNA treated) was applied.

The reaction contained 2 μL template, 1 μL ROX as a reference dye, 0.2 μM probe, 0.4 μM of each primer, 10 μL Qiagen Taqman Master Mix (2×), and 5 μL water. The amplification was carried out as follows: initial incubation at 95°C for 2 min, 35 cycles of 10 s at 95°C, and 15 s at 60°C. The standard curve was drawn using ten-fold serial dilutions of HSV-1 DNA for determining the reaction efficiency. Viral DNA was amplified using a QuantiNova Probe PCR Mix kit (Qiagen, Germany). The HSV-1 primers and probe (Metabion, GmbH, Germany) were designed to target the UL27 gene which encodes the glycoprotein B (gB) of the virus. For checking primer specificity, NCBI primer BLAST was used. The sequence and characteristics of the used oligonucleotides in this study are shown in Table 3.

To determine the virus titer, a plaque assay was performed. Vero cell monolayers in a six-well plate were infected with a serial ten-fold dilution of supernatants collected from HSV-1 infected or gRNA transfected HEK293-AD cells. After incubation for 1 hour at 37° C, 5% CO_2, the inoculum was replaced by 2X DMEM with agarose 1.5% in equal volume as an overlay. Nearly 48h later, cells were fixed in 4% formaldehyde and stained with 1% crystal violet (in 20% ethanol). The titer of a virus stock can be measured in plaque-forming units which is calculated by the number of plaques multiplied by dilution factor and the virus amount.

Statistical analysis. Data from the study are described as means or means ± SD. Effects were evaluated by one-way ANOVA and Turkey's *post hoc* test.

Results

In the current study, g-RNA was selected based on the updated CHOPCHOP software [16], along with top rank, high cutting efficiency, minimum self-complementary, and least off-target. Furthermore, among selected g-RNAs, the low cutting efficiency g-RNA

Table 3. The sequence and characteristics of the used oligonucleotides

Oligonucleotide	Sequence	Region in gB gene	Amplicon Size
Probe	5'-6-FAM-ATCACCACCGTCAGCACCTTCATCG-B00HQ-1-3'	702-726	96 bp
Sense primer	5'-TCCAGCATGGTGATGTTGAG-3'	680-996	
Antisense primer	5'-CGTGTACTTCGAGGAGTACG-3'	756-775	

Table 4. The gRNA sequences used in the current study

g-RNA	g-RNA Forward (5'-3')	g-RNA Reverse (5'-3')
UL39.1	GATCG***GTTGTTCCTGTCGCGACACA***G	AAAAC***TGTGTCGCGACAGGAACAAC***C
TK.1	GATCG***GGCTGCTTGCCAATACGGTG***G	AAAAC***CACCGTATTGGCAAGCAGCC***C
ICP34.5	GATCG***GTCGTCGTCGGACGCGGACT***G	AAAAC***AGTCCGCGTCCGACGACGAC***C
ASS1.1	GATCG***ACGGCGCCACAGGAAAGGTG***G	AAAAC***CACCTTTCCTGTGGCGCCGT***C
gD	GATCG***GCTCCTAAACGCACCGTCGG***G	AAAAC***CCGACGGTGCGTTTAGGAGC***C

(< 30) with each rank and off-target was eliminated. Two or three gRNAs were selected over a span of 100 to 200 nucleotides of a target gene and primers were designed up and downstream of the target gRNA position to identify any deletion or insertion. One candidate gRNA is represented in Table 4.

The used methods in this protocol were based on the creation of an single gRNA expression vectors. In fact, these vectors can be created by ligation of pre-digesting the vector backbone (Fig. 2) and annealing short oligonucleotides in a single reaction. Successful cloning using these protocols resulted in significantly more number of clones with an appropriate insert DNA as compared to no-insert control.

Clones were screened by colony PCR and confirmed by Sanger sequencing. To perform colony PCR, the forward primer was designed based on upstream of g-RNA site, and reverse g-RNA was used as a reverse primer. The forward primer is used as a common primer. Finally, the sequencing of the clones was performed to eliminate of false-positive PCR results (Fig. 3).

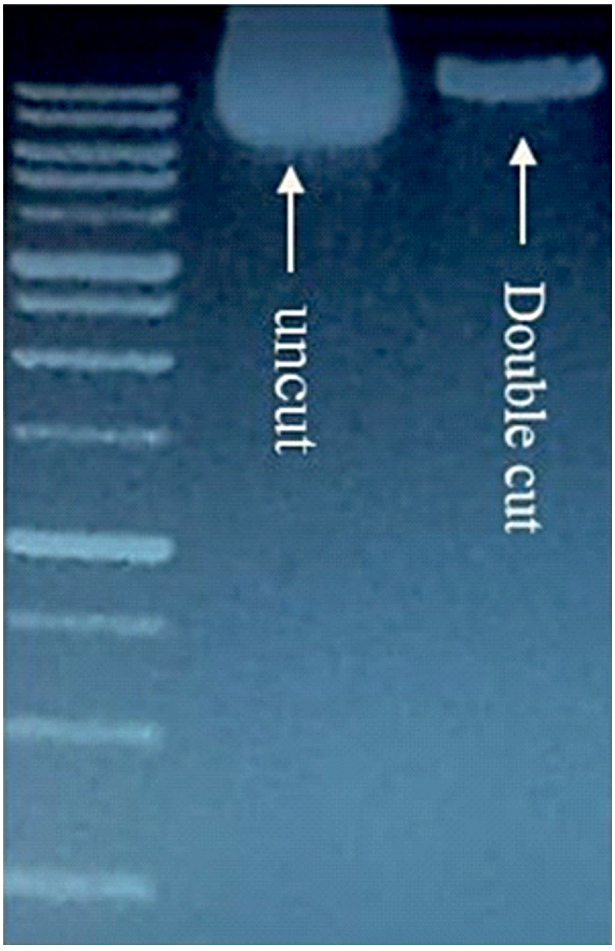

Figure 2. The digestion of a pCas-guides expression vector. 1% agarose gel was used. Lane 1: uncut pCas-guide EF1-GFP. Lane 2: pCas-guide cut with *Bam*HI and *Bsm*BI.

Virus production assay

At first, we selected the HSV-1 KOS strain for evaluating whether the HSV-1 genome could be effectively cleaved via the CRISPR/Cas9 system. Among designed gRNAs, we selected gD gRNA to survey effect of the CRISPR-Cas9 system on the HSV-1 replication. Twenty-four hours after transfection, HEK-AD cells were infected with HSV-1 at different multiplicities of infections (MOIs), and when cyto-

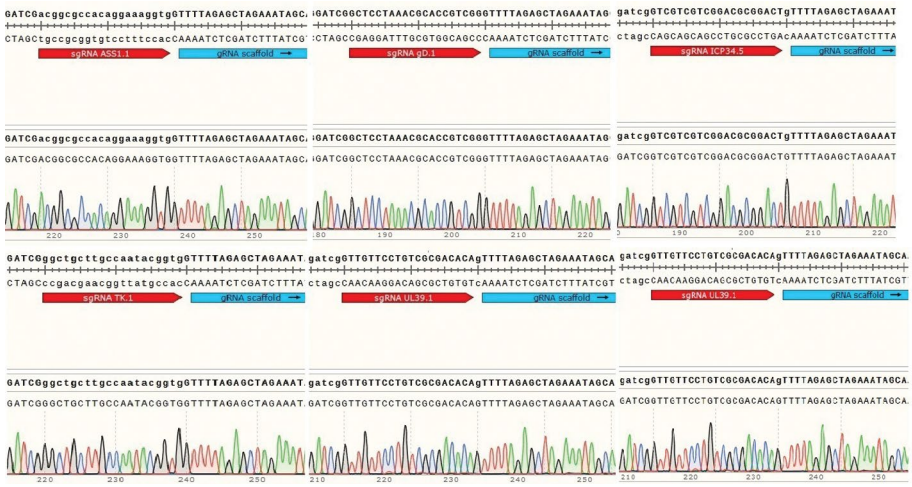

Figure 3. Sequencing of a pCas-guides g-RNA expression vector. Chromatograms corresponding to cloned g-RNA.

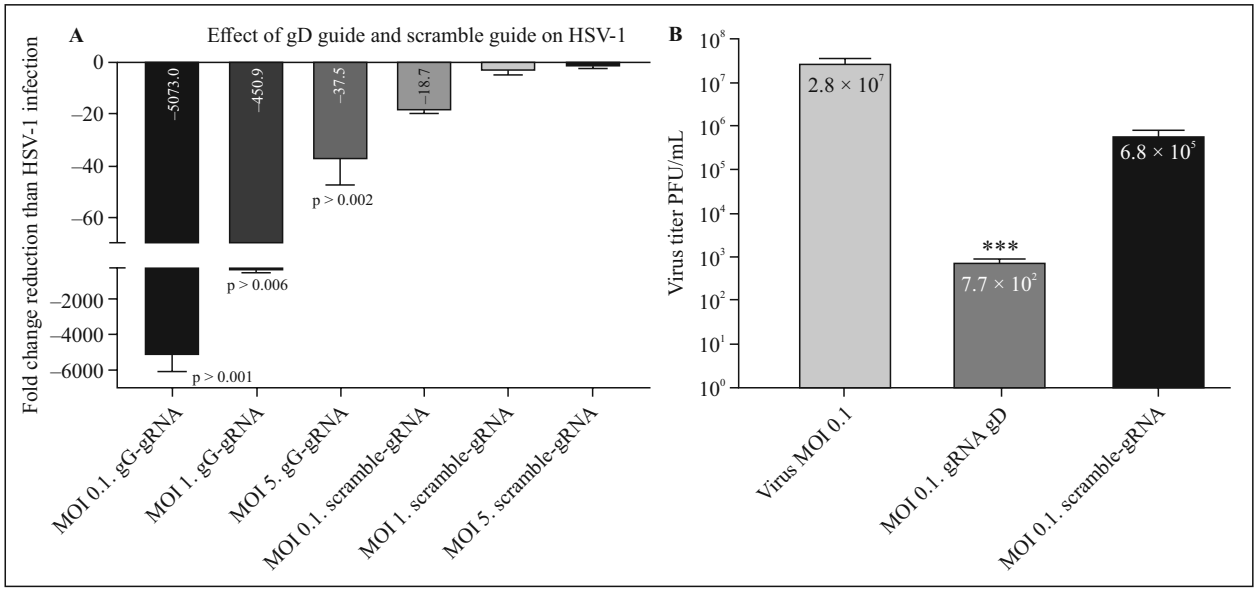

Figure. 4. The effect of gD guide on HSV-1 replication. **A**: by plaque assay. **B**: by Real Time PCR test (PFU: plaque-forming unit).

pathic effects were seen, the cells were harvested. Cytopathic effects were identified based on changes in the morphology of virus-infected HEK-AD cells after 24–48 hours. After performing a plaque assay, the CRISPR/Cas9 mediated reduction in HSV-1 infection was observed at MOI 0.1. The reduction of viral titer in this assay was computed 7.7×10^2 PFU/mL (plaque-forming unit/mL) that there was a statistically significant difference. Next, we checked the efficiency of the CRISPR/Cas9 system on the effect of viral infectious dose using real-time PCR. We found an increase of CRISPR-Cas9 (gD-gRNA) efficiency with decreasing viral infectious dose from MOI 5, 1, and 0.1 PFU/cell (Fig. 4A). In this method as in plaque assay, the decrease of HSV-1 infection was detected that there was a statistically significant difference.

The results showed that the gD gene was successfully knocked out at the infected HSV-1 into HEK-AD cells (Fig. 4B). Overall, our results showed that the CRISPR/Cas9-mediated cleavage was efficient.

Discussion

Recently, CRISPR/Cas9 technology has been used as a powerful tool in gene therapy, genetics, and genom-

ics. In this study, we used the *pCas-Guide-EF1a-GFP CRISPR* vector to create a fast and efficient method for gD gRNA cloning by restriction enzymes (*Esp3I* (*BsmBI*) and *BamHI*). We demonstrated that the CRISPR/Cas9 system could efficiently knockdown the gD locus, resulting in different sequence mutations and disrupted gD expression.

Karpof *et al.* determined that the CRISPR/Cas9 system targeted against UL52 and UL29 genes efficiently suppressed HSV-1 reproduction in Vero cells [17]. In recent years, the CRISPR/Cas9 technology has attracted much attention due to the simplicity and cost-effectiveness of gRNA design. Nevertheless, high-throughput genomic research by this method has increased the target of many viral and cellular genes with a single vector [18].

The gD is an HSV-1 envelope glycoprotein that binds to several cell receptor including HVEM, NECTIN1, and 3-O-sulfated heparan sulfate [17–19]. Mutations in gD can reduce physical interactions with some of the HSV entry receptors and also prevent cell fusion with its receptors. gD acts to block the fusion of lysosomes with the endocytic vesicles [20]. Nevertheless, it has been described that the deletion of gD produces a marked reduction in HSV-1 secondary envelopment [21].

The expression of one g-RNA is gained using oligonucleotides comprising of the forward and reverse g-RNA target sequences (each of them has 20 nt) contain extra bases (gatcg) at the 5' end of the forward sequence as a *Bam*HI overhang and 'G' at its 3' end.

The pCas-guide contains promoter RNA pol III, including U6 and H1 which have been used to express these small RNAs as gRNAs. It is believed that U6 promoter transcription starts at the +1 position, with G as the preferred initiation nucleotide [19]. To increase the efficiency of transcription site, 'aaaac' was added to the 5' end of reverse g-RNA as an *Esp3*I (*Bsm*BI) cleavage site and 'c' to its 3'end as a complementary of G in forward g-RNA.

*Esp*3I is one of the Class-IIS restriction enzymes which has an asymmetric recognition site and cleavage site separately [20]. The "aaaac" sequence also as a part of g-RNA scaffold ensures the compatibility of oligo for cloning into the *Esp*3I (*Bsm*BI)-digested pCas-guide vector. The gD gRNA among designed gRNAs was selected and the effect of the CRISPR-Cas9 system was surveyed on HSV-1 replication.

To survey results of the CRISPR-Cas9 system, plaque assay and real-time PCR were performed and the outcomes of these methods confirmed the decrease in HSV-1 infection as if they had a statistically significant difference. Nevertheless, it has been revealed that insert false-positive results were obtained when the derived primers from the insert alone or span on vector in colony PCR. The probable reason can be the amplification of the unligated insert DNA or untransformed ligated present in the bacterial plate [21]. To summarize, sequencing of the p-Cas-guide vector cloned with positive colony PCR is unavoidable. These findings provide us insights for future research, and more studies are needed to better understand the CRISPR technology.

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Ethical Guidelines

This article does not contain any studies with human participants or animals performed by any of the authors.

Conflict of interest

The authors declare that they have no conflict of interest.

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