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A lentivirus-based system for Cas9/gRNA expression and subsequent removal by Cre-mediated recombination

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Abstract

A major concern of CRISPR and related genome engineering technologies is off-target mutagenesis from prolonged exposure to Cas9 and related editing enzymes. To help mitigate this concern we added a *loxP* site to the 3'-LTR of an HIV-based lentiviral vector capable of expressing Cas9/gRNA complexes in a wide variety of mammalian cell types. Transduction of susceptible target cells yields an integrated provirus that expresses the desired Cas9/gRNA complex. The reverse transcription process also results in duplication of the 3'-LTR such that the integrated provirus becomes flanked by *loxP* sites (floxed). Subsequent expression of Cre recombinase results in *loxP-to-loxP* site-specific recombination that deletes the Cas9/gRNA payload and effectively prevents additional Cas9-mediated mutations. This construct also expresses a gRNA with a single transcription termination sequence, which results in higher expression levels and more efficient genome engineering as evidenced by disruption of the *SAMHD1* gene. This hit-and-run CRISPR approach was validated by recreating a natural *APOBEC3B* deletion and by disrupting the mismatch repair gene *MSH2*. This hit-and-run strategy may have broad utility in many areas and especially those where cell types are difficult to engineer by transient delivery of ribonucleoprotein complexes.

Keywords

Cas9; Cre recombinase; CRISPR; gRNA; loxP; pLentiCRISPR1000

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⁷Competing interests

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1. Introduction

CRISPR technologies are rapidly eclipsing other methods to engineer the genomes of human cell lines including unassisted homologous recombination and endonuclease-assisted homologous recombination. However, a major liability of CRISPR knockout technology is its potential to cause off-target mutations (1–8). Here, we report two modifications to an existing lentivirus-based CRISPR vector called pLentiCRISPR(v1) (9). The first modification removed a premature guide (g)RNA transcription termination site, which results in greater gRNA expression and higher gene disruption frequencies. The second modification introduced a *loxP* site-specific recombination site into the 3'-LTR of the vector, which following reverse transcription and integration becomes copied into the 5'-LTR. As a result, the tandemly arranged dual LTR sequences become a substrate for Cre-mediated recombination and removal of the intervening gRNA and Cas9 sequences. The net result is a new “hit-and-run” system for transient expression of Cas9/gRNA complexes.

2. Materials and Methods

2.1 Plasmid constructions.

LentiCRISPR(v1) was a gift from Feng Zhang (Addgene plasmid #49535) (9). A gBlock (IDT) was designed to change the third T:A base pair in a run of Ts in the stem-loop region of the guide (g)RNA to a C:G base pair. This gBlock was digested with NsiI and XhoI and cloned into lentiCRISPR digested with the same enzymes. This vector was then engineered using overlap extension PCR to remove a SacII site in the puromycin resistance gene (thereby making the SacII site in the WPRE region unique). A gBlock (IDT) was designed with a *loxP* site inserted between the deltaU3 and HIV-1 3'-UTR and containing sequence from the SacII site in the WPRE of the original vector through the SnaBI site at the end of the 3'-LTR. The gBlock and vector were digested with SacII and SnaBI then ligated to make pLentiCRISPR1000. Primer sequences are listed in Table 1.

Two top-ranked gRNAs were chosen to disrupt *MSH2* (www.crispor.org) and four top-ranked gRNAs were chosen for *SAMHD1* exon 1 (crispr.mit.edu). To recapitulate the natural *A3B* deletion, a gRNA was selected manually based within a region of 100% sequence identity in the introns between *A3A* exons 4/5 and *A3B* exons 7/8, respectively. Each gRNA was cloned into pLentiCRISPR-*loxP* via Golden Gate cloning using the BsmBI sites. Restriction enzymes and T4 DNA ligase were obtained from NEB. All constructs were confirmed by Sanger DNA sequencing (GeneWiz). Primer sequences are listed in Table 1.

2.2 Cell culture, virus production, and transduction.

293T and 293-TREx cells were maintained in RPMI with 10% FBS. MCF-7L was maintained in modified IMEM with 5% FBS and 11.25 nM insulin. Cells were transfected with TransIT-LT1 (Mirus Bio) at 60-70% confluence. pLentiCRISPR or pLentiCRISPR1000 and GagPol and VSV-G plasmids were transfected in a 3:2:1 ratio with 20 µg total per 15 cm plate. Three days post-transfection, the culture medium was filtered through 0.45-micron filters to remove cells and debris, and supernatants were spun at 20,000 g for 2 hr to pellet viruses. Viruses were then added to cell culture media. 3 days post-infection, infected cells

were selected with 1 µg/mL puromycin and the surviving pools were screened for knockout by PCR. Single cell subclones were generated by limiting dilution and then screened again for knockout by PCR, immunoblotting, and/or loss of protein function assays. Cre treatment was performed on selected clones by infecting cells with Adenovirus-Cre followed by subcloning. Excision of the integrated virus was determined by loss of puromycin resistance in colony formation assays in the presence versus absence of puromycin.

2.3 Colony Formation Assays.

293-TREx-A3Bi-GFP cells were seeded into 6-well plates (500 cells per well). 24 hrs after seeding, the semi-confluent cells were treated with DMSO (vehicle) or 6-thioguanine (6-TG) (Abcam). Once colonies of greater than 50 cells were formed in the vehicle-treated wells (7-9 days), the drug-treated plates were washed with PBS, stained with a solution of 50% methanol and 0.5% crystal violet, and colonies were counted.

2.4 Immunoblots.

Cells were collected, washed with PBS, and re-suspended in 100 µL reducing sample buffer per 1E6 cells (0.5 M Tris-HCl pH 6.8, 1% 2-mercaptoethanol, 10% sodium dodecyl sulfate, 50% glycerol). Proteins were denatured by boiling samples for 20 min and resolved by SDS-polyacrylamide gel electrophoresis. Proteins were transferred to PVDF-FL membranes (Millipore), and blocked in 5% milk in phosphate-buffered saline with 0.1% Tween 20 (PBST). Primary antibodies were incubated in PBST, and secondary antibodies were incubated in PBST + 0.2% SDS. Membranes were imaged using a LI-COR Odyssey (LI-COR Biosciences, Lincoln, NE, USA) for fluorescent antibodies and with film (Denville Scientific) for HRP. The primary antibody for MSH2 detection was a mouse monoclonal anti-MSH2 antibody (Abeam ab52266, 1:2,000 dilution) and the primary antibody for β-ACTIN was a rabbit monoclonal antibody (Cell Signaling Technologies 13E5, 1:2,500 dilution). The secondary antibodies used were Alexa Fluor 680 goat anti-mouse antibody (Molecular Probes A28183, 1:10,000 dilution) and IRDye 800CW goat anti-rabbit antibody (LI-COR Biosciences, 1:10,000 dilution), respectively.

3. Results

3.1 Construction of pLentiCRISPR-loxP.

One of the plasmids that helped ignite the CRISPR field is pLentiCRISPR (9). This vector expresses a synthetic guide (g)RNA from a hU6 Pol III promoter and Cas9 from a short eEF1α Pol II promoter and thereby enables target gene disruption following transfection or transduction of mammalian cells. A drawback of lentivirus-mediated transduction is permanent expression of the Cas9/gRNA complex and potential off-target mutations (1–8). We reasoned that adding a *loxP* site to the 5′- and 3′-LTRs of this lentivirus-based vector would enable efficient Cre recombinase-mediated removal of the gRNA and Cas9 coding sequences following each desired on-target genome engineering event. Accordingly, such reverse engineering to leave only an inert solo LTR would help to minimize potentially confounding off-target events.

To achieve this goal, as detailed in the Materials and Methods, we created a derivative of pLentiCRISPR by inserting a single 34 bp *loxP* site between the U3 and R elements within the 3'-LTR region (Figure 1). Based on the established reverse transcription mechanism of retrovirus replication (10), the genomic RNA will be copied into a full-length double-stranded proviral DNA capable of integration into cellular genomic DNA. During the strand-transfer stage of this process, the 3' U3 and R regions anneal to the 5'-end of the nascent proviral genome and become duplicated. Additional DNA synthesis leads to the production of a full-length proviral genome with *loxP* sites on both the 5' and 3'-ends. Upon integration into the genomic DNA of a susceptible target cell, the full genetic payload will be expressed constitutively which will mediate target gene disruption. Subsequent expression of Cre recombinase (below by virus-mediated transduction) will lead to site-specific recombination between 5'- and 3'-*loxP* sites and removal of the entire intervening sequence. The only element remaining will be a single LTR element, which has minimal drawbacks that can be easily mitigated by characterizing multiple clones with each desired genetic engineering event.

3.2 Optimization of gRNA expression.

During our initial studies with pLentiCRISPR, we noticed that the gRNA stem-loop region contained a putative Pol III transcription termination site (poly-U in the gRNA), which would result in production of a truncated and therefore defective gRNA (Figure 2A). We also observed rare target site cleavage activity at a variety of different target genes. To systematically test this idea, we made two single base substitutions in the gRNA portion of pLentiCRISPR that changed the likely premature termination site 5'-UUUU to 5'-UUCU and simultaneously maintained the perfect complementarity of the stem region of the gRNA (Figure 2A). This "new" construct – pLentiCRISPR1000 – was tested in parallel with otherwise identical "old" constructs using *SAMHD1* as a target gene in 293T cells. In each instance, simultaneous Cas9 cleavage of exon 1 and intron 12 is predicted to lead to deletion of the 47 kbp intervening sequence and creation of a *SAMHD1* null allele (Figure 2B). In each instance, PCR products spanning the deletion interval were only obtained using the new constructs with the premature transcription stop sequence removed (Figure 2C). Sanger DNA sequencing confirmed junctions comprised on the 5'-end by exon 1 sequence and on the 3'-end by intron 12 sequence. After we completed this work, we learned that another group had previously disrupted this premature termination sequence and demonstrated an increase in CRISPR-mediated gene binding efficiency (11).

3.3 Validation of the floxed "hit-and-run" system.

We next undertook a series of studies to validate the efficiency of our new transcriptionally optimized, *loxP*-flanked construct. The first target gene was *APOBEC3B* (*A3B*), which encodes an antiviral single-stranded DNA cytosine deaminase that is also overexpressed in a variety of different cancers (reviewed by (12–14)). In addition, rather than simple gene disruption, we sought to re-create a naturally occurring 29.5 kbp *A3B* deletion allele that lacks the entire *A3B* coding sequence due to fusion of 3' untranslated regions (UTRs) of *A3A* and *A3B* (15) (Figure 3A). A gRNA was designed to a region of 100% sequence identity between intron 4 of *A3A* and intron 7 of *A3B*. This construct was used to stably transduce the estrogen receptor-positive breast cancer cell line MCF-7L and, following

puromycin selection, the presence or absence of the intended deletion allele was assessed by locus-specific PCR. In all instances, the parental cell line and the knockout (KO) pool contained a 500 bp wild-type PCR product spanning the Cas9 cleavage site in *A3A* (or *A3B*) (Figure 3B). In comparison, only the KO pool and derivative clones such as H24 yielded the predicted 700 bp *A3A/B* fusion PCR product (Figure 3B). The H24 null clone was homozygous for this deletion allele as evidenced by no wild-type PCR product and Sanger sequence analysis of the fusion PCR products. Moreover, RTqPCR analyses of MCF-7L and the H24 clone showed no *A3B* mRNA above background in the clone and normal expression levels of flanking *APOBEC3* genes, most importantly *A3A* on the 5' side and *A3C* on the 3' side (Figure 3C). Finally, the H24 clone was transduced with a Cre-expressing adenovirus, and complete removal of the gRNA/Cas9 portion of the LentiCRISPR construct was evidenced by a lack of RTqPCR product above background for *Cas9* mRNA and a fully restored sensitivity to puromycin (Figure 3D-E).

As a second and simpler example (similar to almost any non-essential gene), we chose to disrupt the *MSH2* gene in a 293-derived cell line (16–18). *MSH2* encodes the lesion recognition protein of the mismatch repair system (19, 20). A gRNA was designed to exon 1 of the *MSH2* gene, which following Cas9 cleavage and error-prone nonhomologous end-joining was predicted to create a null allele (Figure 4A). As above, cells were transduced with the new pLentiCRISPR1000 construct, subjected to selection by puromycin, and screened for gene disruption events. In this case, the MSH2 protein was no longer detected in the pool indicating near 100% engineering efficiency (data not shown). Therefore, pools were transduced with Cre-expressing adenovirus to gut the LentiCRISPR vector, and then limiting dilutions were done to derive single cell subclones for subsequent experimentation. As predicted from analysis of the engineered pool, immunoblotting showed that 4/4 single cell subclones completely lacked the MSH2 protein and became resistant to the drug 6-thioguanine (a hallmark of mismatch repair defective cells (21, 22)) (Figure 4B-C). Lastly, the clones were screened for resistance to puromycin, and 3/4 proved to be as sensitive as the original wild-type parental cell population. One clone was still resistant consistent with prior lab experiences and literature on Cre recombinase showing that it is highly effective but not 100% efficient (23–25).

4. Discussion

Many different areas of biology and biomedical research, including epitranscriptomics, are undergoing a revolution enabled by advances in genome engineering. CRISPR technologies have been particularly innovative due to high efficiencies of on-target engineering events and relatively low technical barriers to success (26, 27). For instance, most labs with basic skills in molecular biology are able to use CRISPR to knockout a gene-of-interest in a relevant cell line in a relatively short period of time (days to weeks).

However, one of the well-documented liabilities of CRISPR technologies is its potential to cause off-target mutations (1–8). These can occur, for instance, when the Cas9/gRNA complexes bind to imperfect (mismatched) target sequences and cause double-stranded DNA breaks, which are then repaired by error-prone processes. This potential problem can be minimized by selecting unique gRNA sequences and by using Cas9 variants with improved

fidelity (28–31). It can also be diminished by delivering preassembled Cas9/gRNA ribonucleoprotein complexes or chemically modified RNAs encoding both Cas9 and gRNA directly to cells (32–36), which will be relatively short-lived and may last long enough to mediate the desired on-target genome engineering event(s).

As an alternative, we report refinements of an existing lentivirus-based vector, which enable higher gRNA expression levels, improved rates of gene disruption, and importantly, an option to permanently excise the Cas9/gRNA coding sequences before off-target lesions can accumulate. We demonstrate this technology by disrupting three separate human genes in three different human cell lines. We anticipate that this new genetic engineering system, called pLentiCRISPR1000, may be useful for modifying many different mammalian cell lines and particularly those that are difficult to engineer by non-viral methods such as transfection.

5. Conclusions

We report two refinements to the original pLentiCRISPR system (9) that we have renamed pLentiCRISPR1000. This system yields higher gRNA expression levels, which are directly reflected in higher rates of Cas9-mediated gene disruption. The pLentiCRISPR1000 system also includes a *loxP* site in the 3'-LTR that becomes duplicated upon replication and integration into a target cell genome. Thus, expression of Cre recombinase can be used to excise the gRNA/Cas9 core of the vector and thus help to avoid accumulating undesirable off-target mutations due to constitutive Cas9 nuclease activity. This system may have broad utility in many areas of genome engineering, and particularly those where transfection efficiencies are low.

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Abbreviations:

A3, APOBEC3	Apolipoprotein B mRNA editing enzyme, catalytic polypeptide-like 3
indel	insertion/deletion mutation
HIV	human immunodeficiency virus
LTR	long-terminal repeat
SNP	single nucleotide polymorphism
ssDNA	single-stranded DNA

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Highlights

- Construction of a *loxP*-flanked lentivirus-based gene expression system
- Utility demonstrated for genome engineering using CRISPR
- Cre-mediated removal of viral payload may help to minimize off-target effects

Figure 1.

Schematic of the replication cycle of pLentiCRISPR1000. The single *loxP* site (red triangle) is duplicated through reverse transcription resulting in the integration of a provirus containing flanking *loxP* sites in the same orientation. This enables Cre-mediated removal of the gRNA and Cas9 expression cassette, which leaves only one LTR and a single *loxP* site. Newly copied DNA is depicted in dark blue behind key viral features.

Figure 2.

Guide (g)RNA optimization for RNA Polymerase III transcription. (A) Schematic of the pLentiCRISPR1000 gRNA bound to a representative target region. The stem-loop base substitution mutations that removed the premature transcription termination site are highlighted. (B) *SAMHD1* gene schematic showing exon/intron structure, Cas9 cleavage sites (scissors), and primers used for genotyping (a, b, c). (C) An agarose gel depicting the results of simultaneous attempts to disrupt the *SAMHD1* gene in 293T cells with pLentiCRISPR1000 (new) versus pLentiCRISPR (old) constructs. PCR products using primers a and c represent the 47 kbp deletion, and those with primers a and b represent the wild-type locus.

Figure 3. A recapitulation of a naturally occurring *A3B* deletion using CRISPR technology. (A) A schematic of the *A3A* and *A3B* genes (above) and the homologous intronic region targeted by Cas9 (below). Gray-shaded regions are 100% identical. (B) An agarose gel image of PCR products representing the wild-type (500 bp) and knockout (700 bp) *A3B* alleles in the indicated cell populations. (C) RTqPCR analysis of expressed *APOBEC3* genes in MCF-7L and the *A3B* KO clone H24. The black dashed line depicts the detection threshold. (D) Quantitative PCR analysis of Cas9 before and after vector collapse by Adenovirus-Cre treatment (pool vs representative subclones using primers in Table 1 and Roche UPL probe #3; hatched region is the range of 3 no-RT control reactions). (E) Representative images of crystal violet-stained colonies derived from the indicated cells treated with Adenovirus-Cre to remove the gRNA/Cas9-puro cassette (H24 mock versus H24 subclone 17 treated with Ad-Cre).

Figure 4.

MSH2 knockout construction. (A) *MSH2* gene schematic indicating the Cas9 cleavage site in exon 1. (B) Anti-*MSH2* and anti-β-ACTIN immunoblots of lysates from 293-TREx-A3Bi-GFP cells or representative *MSH2*-null derivatives following transduction with pLentiCRISPR1000. (C) Survival assays showing sensitivity (wild-type) and resistance (*MSH2*-KO) to 6-thioguanine. (D) Representative images of crystal violet-stained colonies derived from the indicated cells treated with no drug or with puromycin.

Table 1.

DNA primers.

Primer	Purpose	5'-to-3' sequence
a	SAMHD1 PCR	CGCCAGGTGTAGCCATGCAGCG
b	SAMHD1 PCR	GATGTTCTTCAGCAGCACCGGCT
c	SAMHD1 PCR	CTGGAAAACACAAGACTCCCCATG
d	A3A/B PCR	TAGGTGCCACCCCGAT
e	A3A/B PCR	TTGAGCATAATCTTACTCTGTAC
f	A3B PCR	TTGGTGCTGCCCCCTC
g	A3B PCR	TAGAGACTGAGGCCCAT
	SacII OE PCR	GCAAAGAAAGCTAGCGGCAG
	SacII OE PCR	GACATGAACTACTATACGTACTGC
	SacII OE PCR	GACGACGGCGCGGCCGTGGCGGTCTGG
	SacII OE PCR	CCAGACCGCCACGCGCGCCGTCGTC
Exon 1	SAMHD1 gRNA 1	CACCGGTCATCGCAACGGGGACGCT
Exon 1	SAMHD1 gRNA 1	GAACAGCGTCCCCGTTCGATGACC
Exon 1	SAMHD1 gRNA 2	CACCGCTCGGGCTGTCATCGCAACG
Exon 1	SAMHD1 gRNA 2	GAACCGTTGCGATGACAGCCCGAGC
Exon 1	SAMHD1 gRNA 3	CACCGTCTCGGGCTGTCATCGCAAC
Exon 1	SAMHD1 gRNA 3	GAACGTTGCGATGACAGCCCGAGAC
Intron 12	SAMHD1 gRNA	CACCGGAGACCCACTCTCACTTCC
Intron 12 old	SAMHD1 gRNA	AAAACGGAAGTGAGAGTGGGTCTCCG
Intron 12 new	SAMHD1 gRNA	GAACGGAAGTGAGAGTGGGTCTCC
Intron 4/7	A3B gRNA	CACCAGCAGGGGGTTGAATGGAGC
	A3B gRNA	GAACGCTCCATTCAACCCCTGCT
Exon 1	MSH2 gRNA 1	CACCGCGCCGTATAGAAGTCGCCC
Exon 1	MSH2 gRNA 1	GAACGGGCGACTTCTATACGGCGC
Exon 1	MSH2 gRNA 2	CACCGCCGGTCGAAAAGGCGCACTG
Exon 1	MSH2 gRNA 2	GAACCAGTGCGCCTTTTCGACCGGC
	Cas9 qPCR	AAGAAGCGGAAGGTCGGTAT
	Cas9 qPCR	GCCGATGCTGTACTTCTTGTC