User Manual

Recombinant Lentivirus-CRISPR/Cas9 Knockout System

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Safe Use of Lentivirus (Lv)

1. Lentivirus (Lv) related experiments should be conducted in biosafety level 2 facilities (BL-2 level).

2. Please equip with lab coat, mask, gloves completely, and try your best to avoid exposing hand and arm.

3. Be careful of splashing virus suspension. If biosafety cabinet is contaminated with virus during operation, scrub the table-board with solution comprising 70% alcohol and 1% SDS immediately. All tips, tubes, culture plates, medium contacting virus must be soaked in chlorine-containing disinfectant before disposal.

4. If centrifuging is required, a centrifuge tube should be tightly sealed. Seal the tube with parafilm before centrifuging if condition allowed.

5. Lentivirus related animal experiments should also be conducted in BL-2 level.

6. Lentivirus associated waste materials need to be specially collected and autoclaved before disposal.

7. Wash hands with sanitizer after experiment.

Storage and Dilution of Lentivirus

Storage of Lentivirus

Virus can be stored at 4°C for a short time (less than a week) before using after reception. Since Lentiviruses are sensitive to freeze-thawing and the titer drops with repeated freeze-thawing, aliquot viral stock should be stored at -80°C freezer immediately upon arrival for long-term usage. While virus titer redetection is suggested before using if the lentiviruses have been stored for more than 12 months.

Dilution of Lentivirus

Dissolve virus in ice water if virus dilution is required. After dissolving, mix the virus with medium, sterile PBS or normal saline solution, keeping at 4°C (using within a week).

Precautions

- Avoid lentivirus exposure to environmental extremes (pH, chelating agents like EDTA, temperature, organic solvents, protein denaturants, strong detergents, etc.)
- Avoid introducing air into the lentivirus samples during vortexing, blowing bubbles or similar operations, which may result in protein denaturation.
- Avoid repeated freezing and thawing.
- Avoid exposing to “regular” plastics (especially polystyrene or hydrophobic plastics) for prolonged periods in liquid phase. Most lentivirus viruses are very sticky and loss can occur if exposed to regular plastics, including tubes, cell culture plates, pipette tips, if not frozen. It is best to store lentivirus in siliconized or low protein binding tubes. Pluronic F-68 used at 0.01%-0.1% in the formulation buffer will minimize sticking if regular plastics are used.
- Avoid diluting lentivirus into low salt solution. Some lentiviruses aggregate in low salt solution, which will be non-infectious.

Introduction of Lentivirus

Lentivirus (rLv)

Lentivirus (lente-, Latin for "slow") is a genus of retroviruses, causing chronic and deadly diseases by long incubation periods in human or other mammalian species [1]. To date, 5 serogroups have been recognized according to the vertebrate hosts they are associated with (primates, sheep and goats, horses, domestic cats, and cattle). Among them, the primate lentiviruses are distinguished by the utilization of CD4 surface protein as a receptor and the absence of dUTPase [2]. Derived from HIV-1, lentiviruses can integrate a significant amount of viral cDNA into the host genome, mediate stable and long-term transgene expression, and efficiently infect dividing cells and nondividing cells, which makes lentivirus an attractive gene delivery vehicle in most cell types [3].

Considering the key safety concerns during the use of HIV-derived lentivirus vectors, recombinant lentivirus has been designed and widely used for gene delivery in most cell types. As a research tool used to introduce a gene product into *in vitro* systems or animal models, lentiviral vector has been put into large-scale efforts to down-regulate or up-regulate gene expression in high-throughput formats, allowing researchers to examine the necessity and effects of transgenes in disease model systems, which is an indispensable for the discovery of novel transgenic drugs.

Nowadays, several generations of lentivirus packaging system are developed, in which the second-generation lentivirus vector and the third-generation lentivirus vector are the two most popular ones. The current method of the recombinant lentivirus production in Genemedi is based on three plasmids co-transfection system, involving the co-transfection of 3 plasmids (lentivirus series plasmid containing gene of interest (GOI) pLv-GOI, envelope expressingplasmid pMD2G and packaging plasmid pSPAX2) into 293T cells to generate lentivirus vectors.

CRISPR/Cas9 System

CRISPR (clustered regularly interspaced short palindromic repeats) is a family of DNA sequences derived from viruses that have previously infected the prokaryotes and has the ability to recognize invasive homologous DNA sequences from similar viruses, then directing Cas9 to specifically cleave them during subsequent infections, thus playing a key role in the antiviral defense system of prokaryotes [4]. Cas9 (CRISPR-associated 9) is an enzyme guided by CRISPR sequences to recognize and cleave specific strands of DNA which are complementary to the CRISPR sequence [5]. CRISPR sequences together with Cas9 enzymes make up the basis of a CRISPR/Cas9 technology which is a versatile genome-editing platform within organisms.

By 2010, three CRISPR/Cas9 systems have been identified in bacteria: Type I, II and III. Due to the relative simplicity, Type II CRISPR system from Streptococcus pyogenes have been eventually adapted for genome editing in mammalian cells [6]. This simpler CRISPR system is made up of four components, that is, the Cas9 endonuclease, trans-activating CRISPR RNA (tracrRNA) and two small RNA molecules named CRISPR RNA (crRNA) [7]. In this system, the mature crRNA base-paired to trans-activating crRNA (tracrRNA) forms a two-RNA structure, guiding Cas9 protein to induce double-stranded breaks in target DNA. At sites complementary to the crRNA-guide sequence, the Cas9 HNH nuclease domain cleaves the complementary strand, whereas the Cas9 RuvC-like domain cleaves the noncomplementary strand. To further apply this system to mammalian cells, Jennifer Doudna et.al. re-engineered the CRISPR/Cas9 system into a more manageable two-component system by fusing dual-tracrRNA and crRNA molecules into a "single-guide RNA (sgRNA)", in which Cas9 endonuclease could search and cleave the target DNA specified by the sgRNA. By manipulating the nucleotide sequence of the sgRNA, the artificial Cas9 system could be programmed to target any DNA sequence for cleavage [8].

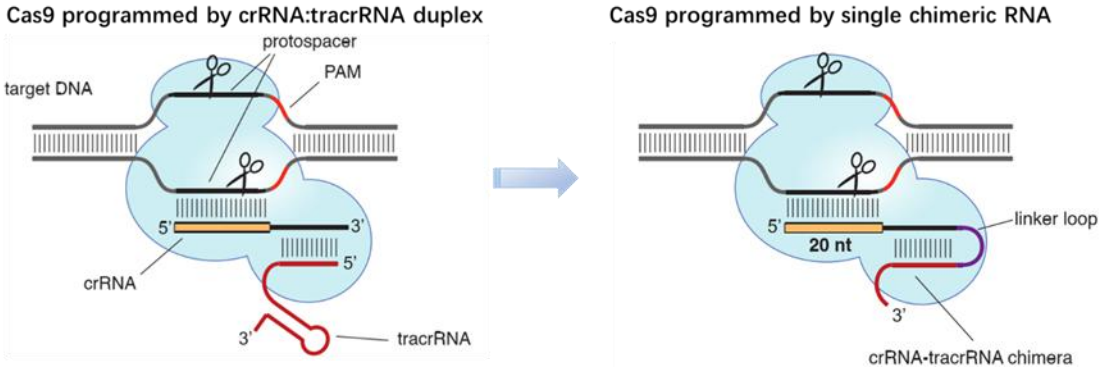

Figure 1. Cas9 can be programmed using a single engineered RNA molecule covering tracrRNA and crRNA features. Left, Cas9 is directed by a dual RNA structure formed by activating tracrRNA and targeting crRNA to cleave site-specifically–targeted dsDNA in type II CRISPR/Cas systems. Right, a chimeric RNA generated through fusion of crRNA to tracrRNA [8].

Compared to other gene editing technology, there exists many advantages in CRISPR/Cas9 system: 1) Remarkable versatility; 2) The artificial CRISPR/Cas9 system can be programmed to target any DNA sequence for cleavage by manipulating the nucleotide sequence of the sgRNA and displays remarkable versatility from cells *in vitro* to animals or plants *in vivo*; 3) Simple to design and easy to operate; 4) Compared to the previous genome editing tools, such as Zinc Finger Nucleases (ZFN) and Transcription activator-like effector nucleases (TALEN), the CRISPR/Cas9 system doesn't need to rely on protein design, just requires a few simple DNA constructs to encode Cas9 protein and sgRNA, which is much more simple and easier; 5) Low cost; 6) Compared to genome editing tools, such as ZFN and TALEN, the CRISPR/Cas9 system only requires less time and resources.

rLv-CRISPR/Cas9 Knockout System

By combining recombinant lentivirus packaging system with Cas9 genome-editing platform, Genemedi has further developed Lentivirus-Cas9 system to achieve gene knockout with high efficiency, which is one of the top advanced gene knockout technology in the world.

sgRNA Design and pLv-Cas9-gRNA Vector Construction

sgRNA Design

Cas9-gRNA design principles:

1） The PAM sequence that Cas9 recognizes is NGG (AGG, TGG, CGG, GGG), for sgRNA, the length is about 21 or 22 nucleotides.

2) For sgRNA, the GC content in 40%~60% is better.

3) If the sgRNA is driven by U6 or T7 promoter, the 5' end of sgRNA can be designed as G or GG to improve transcription efficiency, which should be considered.

4) The binding site of sgRNA should be as close to the coding region in the downstream of ATG as possible to induce frameshift mutation, the first or second exons is better.

5) SNPs should be checked in the binding site of sgRNA.

6) The distance between paired sgRNA should be taken into consideration before designing paired-gRNAs, if using Cas9 single nickase.

7) Whole genome off-target effect analysis is suggested. At least 5 bases can be allowed for the base mismatch and whether the off target is located in the gene encoding regions need to be confirmed. What's more, base insertion or deletion in off targets should be detected.

Cas9-gRNA design

Based on the above principles, Cas9-gRNA can be designed on the following website: https://portals.broadinstitute.org/gpp/public/analysis-tools/sgrna-design. Human TRF3 gene was taken as an example for illustration (Gray, the first exon; Yellow, the second exon).

ATGGCCTCTGCGCCCTGGCCGGAGCGGGTTCCGAGGCTGCTCGCTCCGCGCTTACCCTCTTACCCGCCCCCACCCCCAACAGTGGGATTACGGTC
CATGGAGCAGGAGGAGACCTACCTGGAGCTCTACCTGGACCAGTGCGCCGCTCAGGATGGCCTTGCCCCACCCAGGTCTCCCCTGTTCAGCCCA
GTTGTACCTTATGATATGTACATACTGAATGCATCCAATCCGGATACTGCATTTAATTCGAACCCTGAAGTCAAAGAAACATCTGGTGATTTCTC
ATCTGTGGATCTTAGCTTCCTACCAGATGAAGTTACCCAGGAAAATAAAGACCAGCCTGTCATTAGCAAACACGAAACTGAAGAAAATTCTGAA
AGCCAAAGTCCACAAAGTAGGTTGCCATCACCCAGCGAACAGGACGTTGGGCTGGGCTTAAACAGCAGCAGTTTGTCAAATTCCCATTCACAGC
TGCACCCTGGTGATACTGACTCAGTCCAGCCCTCTCCTGAGAAACCAAACTCCGACTCCTTGTCTCTGGCATCCATAACTCCCATGACACCAATG
ACCCCTATTTCAGAATGTTGTGGAATTGTACCTCAACTACAGAATATAGTTTCCACTGTAAACCTGGCCTGTAAGTTGGATCTGAAGAAAATAGC
TTTGCATGCAAAAAATGCAGAATATAACCCAAAGAGGTTTGCTGCTGTCATAATGAGGATCCGAGAGCCCAGGACAACAGCCCTTATATTTAGC
TCTGGGAAGATGGTCTGCACGGGAGCCAAAAGTGAAGAGCAGTCTCGACTTGCAGCAAGAAAATATGCTCGTGTGGTGCAGAAGCTTGGGTTC
CCTGCCAGATTCCTCGATTTTAAAATTCAGAACATGGTTGGAAGCTGTGATGTGAGATTTCCCATCAGGCTGGAAGGTTTGGTGCTAACCCATCA
GCAGTTCAGTAGTTACGAGCCTGAACTGTTTCCTGGTCTTATTTATAGAATGGTAAAACCACGAATTGTGTTGCTTATCTTTGTATCTGGAAAAG
TTGTGTTGACAGGTGCCAAAGAACGTTCTGAGATCTATGAAGCATTTGAAAACATCTATCCTATTCTAAAAGGTTTTAAAAAAGCCTGA

The confirmed sgRNAs are as follows.

Cas9-gRNA1T: 5' CACCGCTGGACCAGTGCGCCGCTC 3' (Sense strand)
Cas9-gRNA1B: 5' AAACGAGCGGCGCACTGGTCCAGC 3'

Cas9-gRNA2T: 5' CACCGACAGTGGGATTACGGTCCA 3' (sense strand)
Cas9-gRNA2B: 5' AAACTGGACCGTAATCCCACTGTC 3'

Cas9-gRNA 3T: 5' CACCGTCGGAACCCGCTCCGGCCA 3' (antisense strand)
Cas9-gRNA3B: 5' AAACTGGCCGGAGCGGGTTCCGAC 3'

pLv-Cas9-gRNA Vector Construction

sgRNA target sequences are synthesized, annealing into a double chain and cloned into pLv-Cas9 vector, which is confirmed by sequencing.

Overall Experiment Procedure of Lentivirus Production

The Lentivirus-CRISPR/Cas9 knockout system virus can be packaged using 293T cells, purified with ultracentrifugation method, and titer is detected with fluorescent microscopy. The detailed protocol can be consulted in the Lentivirus Protocol.

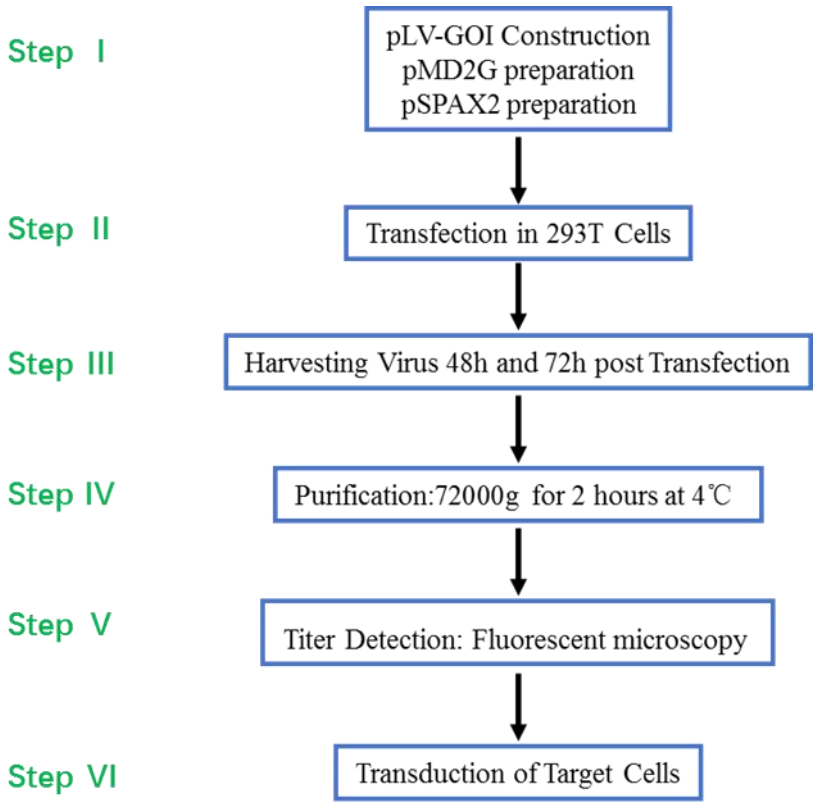

Figure 2. Lentivirus packaging experiment flow chart.

Target Validation and construction of stable cell line of Lentivirus-Cas9 *in vitro*

After Lentivirus-Cas9 titer detection, the cleavage effects of Cas9 can be validated in 293T. The detailed recommended protocol for *in vitro* cell transduction can be consulted from Lentivirus Protocol. Infect 293T cells with Lentivirus-Cas9 virus at confluency about 40%-60%. 24h post infection, change the medium. 96h post infection, harvest cells and extract the genome. Perform PCR using primers flanking target sequence, and sequence to confirm whether knockout effects exist by the appearance of emerged peaks.

Notes for infection of special cell lines.

1. **Suspension cells**

We recommend using flat fillet centrifuging transfection to infect suspension cells or semi-suspension cells. Add virus suspension into cell culture dish, sealing tightly, and centrifuge at low speed of 200g for 1 hour in the flat fillet

centrifuge. Place cells in cell culture incubator after centrifuging transfection. If the flat fillet centrifuge is inaccessible, you can suspend the cells and transfer cells into centrifuge tubes, followed by low-speed centrifuge, and discard the most of supernatant. Add virus suspension into the tubes, resuspending cells, place it at room temperature for 15 min (no more than 30 min), and transfer the cells and virus suspension into plate to culture. Replace with fresh culture medium the next day.

2. Cells difficult to infect

For cells difficult to infect, like DC cells, we recommend repeated infections. Replace with fresh virus suspension 24 hours after the first infection. Repeated infections can increase the infection efficiency markedly.

3. Non-dividing primary cells

We recommend high-titer adenovirus to infect these cells like BMSC.

References

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Virus CRISPR/Cas9 Service

- For AAV CRISPR/Cas9 service, please visit:
https://www.genemedi.net/i/aav-sacas9-packaging
- For lentivirus CRISPR/Cas9 service, please visit:
https://www.genemedi.net/i/crispr-cas9-knockout-lentivirus-production-service
- For Adenovirus CRISPR/Cas9 service, please visit:
https://www.genemedi.net/i/adenovirus-cas9-packaging

Other protocols

- Crispr/Cas9 mediated Gene knockout in Mammalian Cells - Protocol
https://www.genemedi.net/pdf/Genemedi-Crispr-cas9%20Protocol.pdf
- CRISPR/Cas9 AAV Production-User Manual
https://www.genemedi.net/pdf/Genemedi-AAV-SaCas9%20User%20Manual.pdf
- Recombinant Adenovirus-CRISPR/Cas9 Knockout System-User Manual
https://www.genemedi.net/pdf/Genemedi-Adenovirus-crispr%20User%20Manual.pdf
- Adeno-associated Virus (AAV) Production Protocol:
https://www.genemedi.net/pdf/AAV%20production%20protocol-packaging%20concentration%20and%20purification-GeneMedi.pdf
- Lentivirus Production Protocol:
https://www.genemedi.net/pdf/lentivirus%20production%20protocol-packaging%20concentration%20and%20purification-GeneMedi.pdf
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