User Manual

Recombinant Adenovirus-CRISPR/Cas9 Knockout System

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Safe Use of Adenovirus (AdV)

1. Adenovirus 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. Adenovirus related animal experiments should also be conducted in BL-2 level.

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

7. Wash hands with sanitizer after experiment.

Storage and Dilution of Adenovirus

Storage of Adenovirus

Virus can be stored at 4°C for a short time (less than a week) before using after reception. Since AdV viruses 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 AdV viruses have been stored for more than 12 months.

Note: 1. Repeated freeze-thaw cycles must be avoided in case of a downside effect on virus titer (for each freeze-thaw cycle, there would be a 10%-50% decrease). Genemedi will provide rAdV products in small aliquots (200 μl/tube) that can be directly stored in -80 centigrade for multiple usage.
2. For viruses stored more than 6 months, it is suggestive to re-analyze virus titer before use.

Dilution of Adenovirus

To properly thaw rAdV frozen aliquots, transfer viruses from -80 ℃ freezer to an ice-water bath till completely melted. When melted, add proper amount of sterile PBS or serum-free culture medium, and keep in 4℃ for no more than a week.

Precautions

- Avoid Adenovirus exposure to environmental extremes (pH, chelating agents like EDTA, temperature, organic solvents, protein denaturants, strong detergents, etc.)

- Avoid introducing air into the Adenovirus 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 AdV 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 Adenovirus 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 Adenovirus into low salt solution. Some viruses aggregate in low salt solution, which will be non-infectious.

Introduction

Recombinant Adenovirus (rAdV)

Recombinant adenovirus (rAdV) is a replication-defective adenoviral vector, which is widely used for a variety of purposes including gene transfer and engineering, vaccination and gene therapy1,2. There are several advantages of using rAdV as a gene transfer mediator. Firstly, it can deliver as large as 8 kilo-base (kb) gene sequences into cells and tissues without insertion of exogenous fragment in the genome. Secondly, almost all the dividing and non-dividing cells, primary cells and organ tissues can be transduced by rAdV. Moreover, rAdV is easy to operate and expand into large-scale, and the efficiency can reach up to 100%. Thus, rAdV plays an important role in gene engineering research and potential therapeutic treatment of diseases.

The most commonly used adenovirus is serotype 5 (Ad5) of Homo Sapiens consisting of a double-stranded linear DNA molecule at about 36 kb in size1. The cytoplasmic membrane receptors and fibers facilitate endocytosis of adenovirus into cell cytoplasm, where virus particles further migrate into cell nucleus for self-replication using replication machinery of the host3. Once replicated, the virus genome is assembled into its protein shell and released from cells, causing cell lysis3.

Nowadays, several packaging systems of rAdV are developed, in which AdEasy1 and AdMAX2 are the two most popular ones, sharing a common strategy that target gene sequence is cloned into a shuttle vector, then recombined into a viral backbone vector. Early viral transcription units, E1 and E3, are defected in both of these two systems, while E3 gene is not necessary for virus replication1. Thus, packaging of rAdV is usually conducted in cell lines expressing E1 gene, such as HEK-293, HEK-293A etc.2.

In comparison with AdEasy, AdMAX system is relatively easy to handle and can achieve higher virus titer during virus production. This rAdV protocol is developed according to AdMAX system, using a two-vector system composed of a pAd shuttle vector and a rAdV backbone vector pBHGlox(delta) E1-3cre.

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].

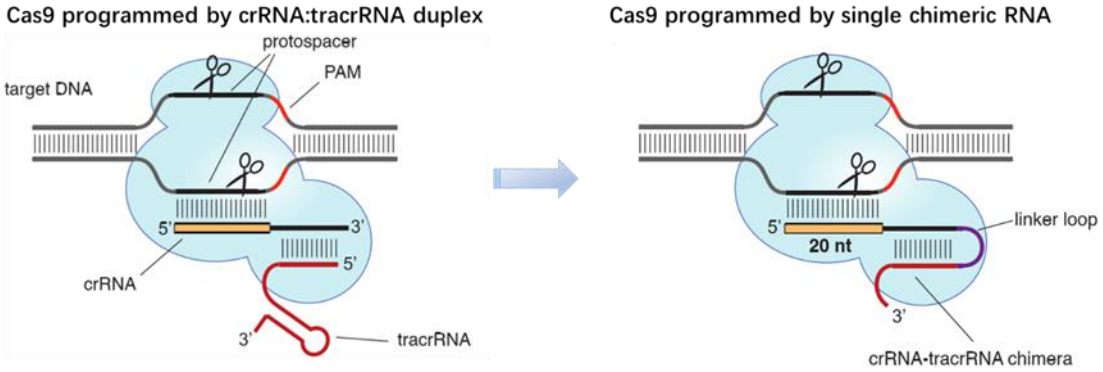

Figure 1. Cas9 can be programmed using a single engineered RNA molecule covering tracrRNA and crRNA features. Left, Cas9 is directed by a dual RNA structure formed by activating tracrRNA and targeting crRNA to cleave site-specifically–targeted dsDNA in type II CRISPR/Cas systems. Right, a chimeric RNA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

rAdV-CRISPR/Cas9 Knockout System

By combining recombinant adenovirus packaging system with Cas9 genome-editing platform, Genemedi has further developed Adenovirus-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 pAdV-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'

pAdV-Cas9-gRNA Vector Construction

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

Overall Experiment Procedure of Adenovirus Production

The Adenovirus-CRISPR/Cas9 knockout system virus can be packaged using 293A cells, purified with CsCl density gradient centrifugation method, and titer is detected by plaque assay. The detailed protocol can be consulted in the Adenovirus Protocol.

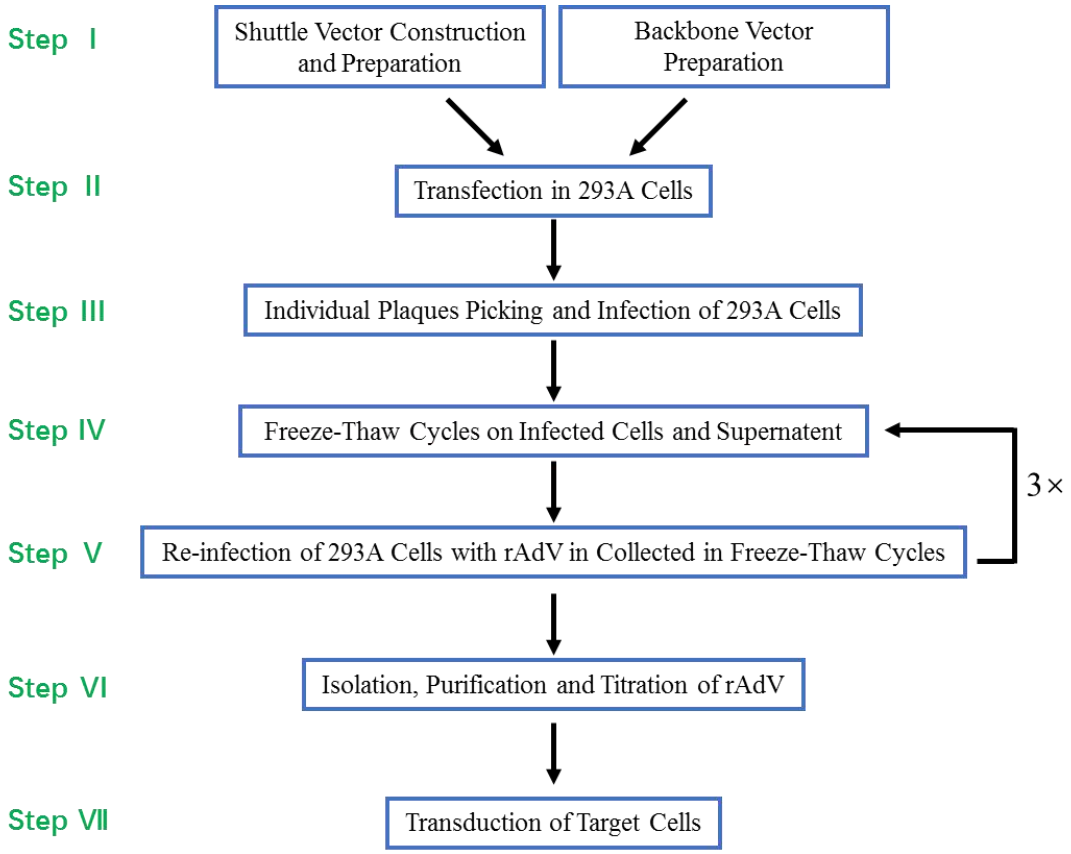

Figure 2. Adenovirus packaging experiment flow chart.

Target Validation of Adenovirus-Cas9 *in vitro*

After Adenovirus-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 Adenovirus Protocol. Infect 293T cells with Adenovirus-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.

Target Validation of Adenovirus-Cas9 *in vivo*

Caution: Purification of rAdV is required for animal injection.

The successful validated Adenovirus-Cas9-gRNA3 can be delivered *in vivo*. Genemedi has systematically organized the corresponding gene delivery method and injection volume for mouse and rat tissue infection, please consult our technical support.

About 3 weeks post infection, get the target tissues or organs, extract the genome and perform PCR and Sanger sequencing to confirm the knockout effects, which are the same as target validation of Adenovirus-Cas9 *in vitro*.

You can also extract protein from target tissues or organs 3 weeks post infection, and carry out Western blot to make sure the target protein is knocked out.

References

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2. He T. C. et al. A simplified system for generating recombinant adenoviruses. PNAS. 95, 2509-2514 (1998).

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7. Barrangou R. Diversity of CRISPR-Cas immune systems and molecular machines. Genome Biol 16:247. (2015).

8. Jinek M, K Chylinski, I Fonfara, M Hauer, JA Doudna and E Charpentier. A programmable dual-RNA-guided DNA endonuclease in adaptive bacterial immunity. Science 337:816-21. (2012).

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 Lentivirus-CRISPR/Cas9 Knockout System - User Manual
https://www.genemedi.net/pdf/Genemedi-Lentivirus-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

- Adenovirus Production Protocol:
https://www.genemedi.net/pdf/Adenovirus%20production%20protocol-packaging%20concentration%20and%20purification-GeneMedi.pdf

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