Genemedi Protocol

Crispr/cas9 mediated Gene knockout in Mammalian Cells

GENEMEDI
Innovation In Gene Therapy

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Introduction of Crispr/cas9 mediated Genome editing

Gene editing(also called Genome editing) is a group of technologies to change the sequence of DNA in the genome. Several approaches to genome editing have been developed, which including Zinc Finger, TALEN, and CRISPR/Cas9. The CRISPR/Cas9 system is faster, cheaper, more accurate, and more efficient than other existing genome editing methods.Cas9, an RNA-guided endonuclease derived from the type II CRISPR-Cas bacterial adaptive immune system, has emerged as a versatile genome-editing platform.

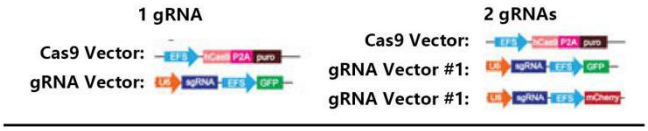
Crispr/Cas9 delivery

To knock out a gene of interest, the nuclease Cas9 and a target-specifying guide RNA must be delivered to the cell. Various CRISPR platforms are suitable for generating knockouts, including “all-in-one" or one-plasmid system (in which Cas9 and the guide RNA are encoded on the same vector), two-plasmid systems (in which Cas9 and the guide RNA are encoded on different vectors). GeneMedi has developed different kinds of the viral vectors (lentivirus, adenovirus and AAV) combining with both of these crispr/cas9 systems.

Workflows of Crispr/cas9 mediated Gene knockout in Mammalian Cells

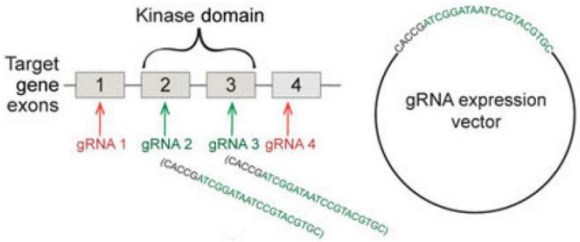
1) Choose a knockout strategy

Two-vector system:

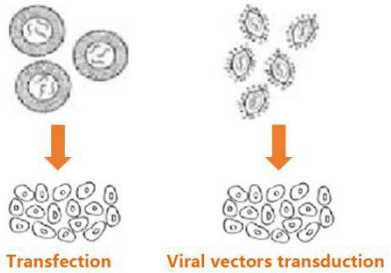


All-in-one system:

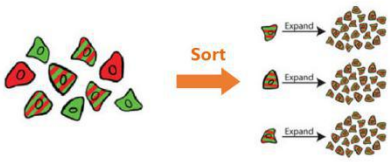
2) Select gRNAs and generate vectors



3) Delivery CRISPR/Cas9 plasmids transfection or viral vector transduction (lentivirus, adenovirus, AAV, etc.)



4) Isolate and expand single-cell clones



5) Verify clonal knockouts

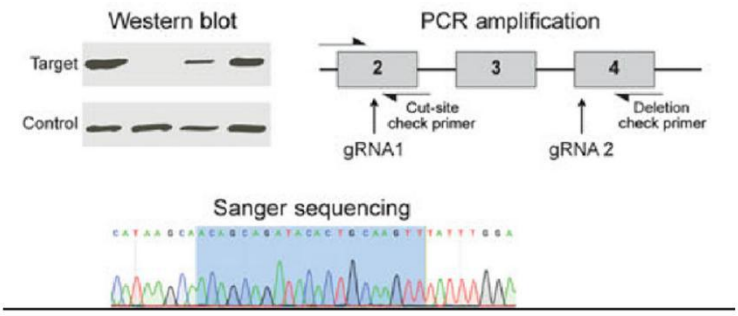


Figure 1: *A simple of workflows of Crispr/Cas9 mediated cell lines knockout process*

Single-Cell Expansion By Dilution Plating

After introduction of the gRNA(s) by transduction or transfection, single cells must be isolated in order to generate clonal lines that can be verified as complete knockouts. Two common methods for this are dilution cloning and fluorescence-activated cell sorting (FACS) of single cells (Alternate Protocol 1). Although both approaches can yield single cell-derived clones, FACS sorting is often more effective given its ability to specifically isolate double-positive cells. However, dilution cloning is cheaper and may cause less cellular stress than sorting.

Materials

·Appropriate cell culture medium

·Standard cell culture plates:

1) 10-cm plates

2) 96-well flat-bottom plates

·Microscope

·Additional reagents and equipment for cell culture and trypsinization

1) Harvest the transduced cells by trypsinization.

2) Count the cells and dilute to a concentration of 20 cells per 100 µl.

3) Using a multichannel pipette, pipette 200 pl of the diluted cells into the first row of a 96-well plate.

4) Pipette 100 µl of the appropriate culture medium into all remaining wells in the plate.

5) Take 100 µl from the first row of the plate containing cells and mix it with the 100 µl of appropriate medium in the row below. This will result in a two-fold dilution of the cell concentration for the second row.

6) Repeat this process down the rows of the plate, resulting in a series of two-fold dilution down the rows. This will result in some proportion of the wells containing a single cell.

7) Check by microscopy for wells that contain a single cell that are single or double positive for the gRNA marker(s). This can be done immediately following dilution or 12-24 hr later, after the cells have settled. Circle these wells.

8) Wells with single cells should be expanded: allow the cells to grow to form microcolonies (∽50-100 cells). Split the microcolony into a fresh well of a 96-well plate. Once that well is near confluence, split to a well of a 48-well plate. Continue expanding the line until a sufficient number of cells can be harvested for the validation assays described below.

Single-Cell Isolation And Expansion By Facs

FACS can be used in multiple ways to isolate single cells. When using the two-gRNA approach, FACS can be used to isolate cells that express both gRNAs by sorting for both fluorescent markers. Depending on your choice of system and the setup in your FACS facility, there are several approaches for this, listed below.

1. Double-positive single cells can be sorted directly by FACS and plated into 96-well plates.

2. Bulk populations of double-positive cells can be isolated by FACS and plated into 96-well plates by dilution

plating, as described in Basic Protocol 6.

3. Bulk populations of double-positive cells can be isolated by FACS and plated on 10-cm plates at a low concentration to allow single cells to form colonies:

1) Plate 100-200 cells from the FACS-sorted sample of double-positive cells onto a 10-cm plate.

2) Use a microscope to identify isolated microcolonies that begin to grow.

3) Use cloning cylinders (Chemglass; cat. no. CLS-1777-02) to isolate and trypsinize the individual microcolonies, and then move them to a 96-well or 48-well plate for clonal expansion.

4) Clones can be expanded by plating to progressively larger plates until there are enough cells to freeze and take DNA and protein lysates for knockout verification.

Knockout Clones Screening And Validation

The cornerstone of any strategy to verify that a protein of interest has been ablated is demonstrating its absence by western blot analysis. However, single western blots may be misleading, and characterizing the precise alterations caused by CRISPR at the DNA level may provide additional useful information. The options for knockout validation also differ slightly depending on whether the one-guide or two-guide approach was used. In particular, if a gene was targeted by two independent guide RNAs, a PCR-based screening strategy can be applied to rapidly assess the knockout status of dozens of potential clones.

Alternatively, the presence of indel mutations can be verified at each cut site, though this becomes more challenging if the resultant mutations are heterozygous. No single technique is perfect, and we recommend that researchers apply a combination of the methods described below to characterize the CRISPR-induced lesions and identify valid knockout clones.

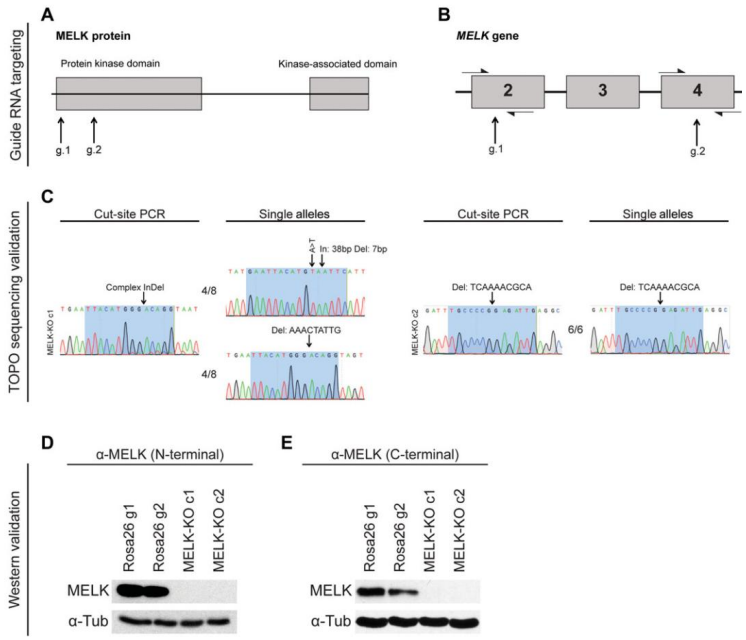


Figure 2: *A simple of workflows of knockout (KO)validation by Crispr/Cas9 system*

Genomic Dna Extraction From Potential Knockout Cells

To confirm gene knockout by PCR or mutation sequencing, genomic DNA must first be prepared from control and potential knockout cell lines.

Materials

Potential knockout cells
DNeasy Blood & Tissue Kit (Qiagen; cat. no. 69506)
Cell culture materials

6-well tissue culture plates
15-ml tubes
1.5-ml microcentrifuge tube
NanoDrop spectrophotometer or equivalent

1. Plate ∽300,000 cells in one well of a 6-well plate for each putative knockout clone.
2. The next day, follow the manufacturer's protocol from the DNeasy Blood & Tissue Kit to isolate the genomic DNA.
3. Quantify the concentration and purity of the DNA with a NanoDrop spectrophotometer. A DNA yield of ∽1 µg is ideal.

Characterizing Crispr-Induced Mutations By Sequencing

If the researcher chooses the single-guide knockout approach, then demonstrating the presence of an indel mutation at the guide RNA cut site is strong evidence that the targeted gene has been knocked out. (Note that this approach is also appropriate if two gRNAs are used but no full deletions are detected.) Cut-site analysis can be performed by PCR-amplifying the region targeted by the guide RNA and then subjecting the amplicon to Sanger sequencing. In the event that the lesion is found to be heterozygous, then TOPO cloning can be applied to sequence individual alleles found within the clone.

For this approach, first isolate genomic DNA from each putative knockout clone and design PCR primers to amplify a ∽200- to 400-bp region around the cut site, as described above. Then, perform PCR and sequence the amplicon:

Materials

Genomic DNA
Primers
2x Taq Master Mix (NEB; cat. no. M0270)
NucleoSpin® Gel and PCR Clean-up (Macherey-Nagel; cat. no. 740609.50) Nuclease-free water

Gel electrophoresis equipment
Thermocycler
0.2-ml thin-walled PCR tubes
1.5-ml microcentrifuge tube

Additional reagents and equipment for agarose gel electrophoresis (Current Protocols article: Voytas, 2001)

1) To set up a PCR reaction, follow the manufacturer's protocol for PCR guidelines and thermocycler settings for the 2x Taq master mix. As a control, set up a PCR reaction using the genomic DNA from a wild-type population.

1) Run the PCR product on a 2% gel. Verify that a band of the correct size is present.

2) Using the NucleoSpin® gel and PCR clean-up, isolate the PCR fragments from the mixture.

3) Set up Sanger sequencing reactions according to the sequencing facility's guidelines. The forward and reverse primers used for PCR can also be applied for sequencing.

4) Compare sequences between the wild-type population and each clone using standard chromatogram analysis software. Indels should be observed at or near the nuclease- targeted site.

Characterizing Crispr-Induced Mutations By Western Blot

Just to determine the expression of your gene by western blot.

T7E1 assay for knockout clones

T7 Endonuclease I recognizes and cleaves non-perfectly matched DNA. This protocol describes how to determine genome targeting efficiency by digesting annealed PCR products with T7 Endonuclease I. In the first step PCR products are produced from the genomic DNA of cells whose genomes were targeted using Cas9, TALEN, ZFN etc. In the second step, the PCR products are annealed and digested with T7 Endonuclease I. Fragments are analyzed to determine the efficiency of genome targeting.

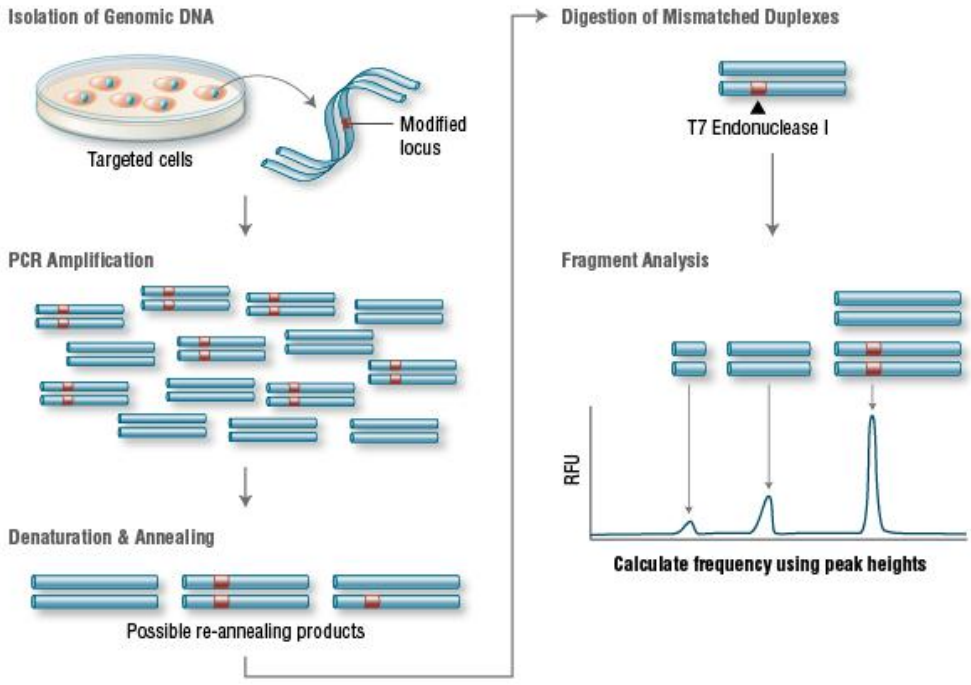


Figure 3: *Principle of T7E1 assay for knockout clones*

Note: *Find out the T7E1 assay protocol from NEB's website.*

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 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

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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