Protocol for examining and eliminating base editor-induced genome-wide and transcriptome-wide off-target mutations

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Method Article

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Abstract

Fusion of CRISPR-Cas9 with cytidine deaminases leads to base editors (BEs) for programmable C-to-T editing, which holds potentials in clinical applications but suffers from off-target (OT) mutations. Here, we applied a cleavable deoxycytidine deaminase inhibitor (dCDI) domain to construct a transformer BE (tBE) system that induces efficient editing with only background levels of genome-wide and transcriptome-wide OT mutations. This step-by-step protocol describes the plasmid construction of tBE system, determination of genome/transcriptome-wide OT mutations and tBE-mediated base editing *in vivo*.

Introduction

Reagents

PrimeSTAR® HS DNA Polymerase, TaKara, R010B

Agarose, ThermoFisher, 75510019

T4 DNA Ligase, NEB, M0202M

BsmBI, NEB, R0580S

Trans5α Chemically Competent Cell, TransGen, CD201-01

NucleoBond Xtra Midi, MACHEREY-NAGEL, 740410.50

Lipofectamine LTX Reagent, ThermoFisher, 15338100

FastPure® DNA isolation Kit, Vazyme, DC102-01

TransZol® Up Plus RNA Kit, TransGen, ER501-01

Puromycin, InvivoGen, ant-pr-1

Mouse PCSK9 ELISA Kit, R&D Systems, MPC-900

Total Cholesterol Kit, Shanghai ShenSuo UNF Medical Diagnostic Articles, 1040280

E.Z.N.A.® Tissue DNA Kit, Omega Bio-tek, D3396-01

HiPure Total RNA Plus Mini Kit, Magen, R4121-02

Equipment

Procedure

Plasmid construction of tBE system:

1, Amplification of DNA fragments containing gRNA sequences.

a) PCR is performed to amplify the gRNA sequences.

The reaction mixture:

Oligonucleotides (pUC57-sgRNA-MS2-U6), 50 ng

10 μM F primer, 1.5 μl

10 μM R primer, 1.5 μl

PrimeSTAR Buffer (5×), 10 μl

dNTP Mix (2.5 mM), 4 μl

PrimeSTAR® HS DNA Polymerase, 1 μl

H_2O, up to 50 μl

PCR program:

94 ℃ for 5 minutes (min)

35 cycles of:

98 ℃ for 15 seconds (s)

68 ℃ for 15 s

72 ℃ for 30 s

Then:

72 ℃ for 3 min

b) Purify amplified DNA fragments with 2% agarose gel.

c) Measure the concentration of DNA fragments (containing gRNA sequences) by Nanodrop.

2, Linearization of gRNA-expressing backbone vector of tBE system and insertion of the DNA fragments containing gRNA sequences.

a) Digest the gRNA-tBE-expressing backbone vector by BsmBI, such as U6-ccdB-boxB-tBE-V5-mA3 and insert the DNA fragments containing gRNA sequences by T4 DNA Ligase into the BsmBI digested backbone, simultaneously.

Restriction enzyme (BsmBI) digest and T4 DNA Ligase mixture:

gRNA-tBE-expressing backbone vector (U6-ccdB-boxB-tBE-V5-mA3), 50 ng

DNA fragments containing gRNA sequences, 75 ng

BsmBI (NEB), 0.5 μl

T4 DNA Ligase, 0.5 μl

T4 DNA Ligase Reaction Buffer (10×), 1 μl

H_2O, up to 10 μl

Incubate reaction program:

37 ℃ for 5 minutes (min)

10 cycles of:

16 ℃ for 10 min

37 ℃ for 15 min

Then:

80 ℃ for 15 min

3, Transform the tBE assembled gRNA vectors into competent cells.

4, Plate the transformation mixture on the petri dish. Invert plates and culture at 37 ℃ for 14 hrs.

5, Scrape colonies into a 50 ml tube with 20 ml LB to harvest colonies.

6, Use maxi plasmid prep columns to purify plasmids from transformation colonies according to the manufacturer's protocol.

Determining genome-wide off-target mutations

1, Establish APOBEC3-knockout cell lines.

a) Seed the 293FT cells containing *Pgm3*-T248C into a 60-mm plate at a density of 4×10^5 per well and culture for 24 hrs.

b) Transfect the cells with plasmids expressing Cas9 and two APOBEC-targeting sgRNAs (sghA3A and sghA3H) by the Lipofectamine LTX Reagent, according to the manufacturer's instruction.

c) After 48 hrs, add 10 μg/ml puromycin into the media in the following two weeks, and then single cell colonies are picked up.

2, Plasmid transfection.

a) The APOBEC3-knockout cell lines are seeded in a 24-well plate at a density of 1×10^5 per well and transfect with the plasmid of tBE or other base editor system by LIPOFECTAMINE LTX Reagent.

b) After 24 hrs, puromycin is added to the medium at the final concentration of 4 μg/ml.

c) After another 48 hrs, the single cell colonies are picked up. The Genomic DNA of edited cells expand from single colonies are extracted with FastPure® DNA isolation Kit.

3, Whole-genome sequencing is conducted by Illumina HiSeq X Ten (2×150) at CAS-MPG Partner Institute for Computational Biology Omics Core, Shanghai, China.

Determining transcriptome-wide off-target mutations

1, Plasmid transfection.

a) The $293FT^{PGM3\text{-}T248C}$ cells are seeded in a 24-well plate at a density of 1×10^5 per well and transfect with the plasmid of tBE or other base editor system by LIPOFECTAMINE LTX Reagent.

b) After 40 hrs, cells in the first 15% of the fluorescence intensity are sorted by FACSAriaIII. Total RNAs of sorted cells are extracted by using the TransZol® Up Plus RNA Kit.

2, Indexed RNA libraries are prepared by using the TruSeq Stranded Total RNA with Ribo-Zero Globin. RNA-seq libraries are sequenced on an Illumina HiSeq X Ten (2×150) at CAS-MPG Partner Institute for Computational Biology Omics Core, Shanghai, China.

tBE-mediated base editing *in vivo*

1, Tail vein injection of AAV vectors

a) All dosages of AAV are adjusted to 200 μl with sterile PBS before injection. The total doses of AAV are 1.6×10^{11}, 8×10^{11}, or 4×10^{12} vg per mouse.

b) AAV vectors are delivered to 8-week-old female C57BL/6 mice intravenously via lateral tail vein injection.

2, Two or four weeks after injection, mice are euthanized by carbon dioxide.

3, The blood is collected and the serum is separated by centrifugation. Serum levels of PCSK9 and total cholesterol are measured using the Mouse PCSK9 ELISA Kit and Total Cholesterol Kit, respectively.

4, The tissues are cryogenically grounded into powder. The genomic DNA and total RNA are isolated using E.Z.N.A.® Tissue DNA Kit and HiPure Total RNA Plus Mini Kit, respectively.