Chapter 17

RNA-Guided Genome Editing of Mammalian Cells

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Abstract

The microbial CRISPR-Cas adaptive immune system can be harnessed to facilitate genome editing in eukaryotic cells (Cong L et al., Science 339, 819–823, 2013; Mali P et al., Science 339, 823–826, 2013). Here we describe a protocol for the use of the RNA-guided Cas9 nuclease from the *Streptococcus pyogenes* type II CRISPR system to achieve specific, scalable, and cost-efficient genome editing in mammalian cells.

Key words CRISPR-Cas Genome editing DNA cleavage Cas9 Guide RNA PAM sequence NHEJ Gene knockout

1 Introduction

The ability to introduce targeted modifications into genomes and engineer model organisms holds enormous promise for biomedical and biotechnological applications. The development of programmable nucleases [1–9] has allowed targeting of specific genomic loci to introduce double-strand breaks (DSBs) in the DNA. These DSBs are subsequently repaired through either the error-prone nonhomologous end-joining (NHEJ) pathway or the homology-directed repair (HDR) pathway, allowing formation of indels or precise editing of the genome, respectively [10]. These endonucleases can be used for studies in basic biology, biotechnology, and medicine, including the development of reporter cell lines [11], transgenic organisms [12], disease models [13], and gene therapy [14], among others. Although ZFNs and TALENs can be reprogrammed to target specific DNA sequences, these tools still require time-consuming engineering of proteins de novo for each target, and there remains a deficit for technologies that are easily customizable, multiplexable, and affordable.

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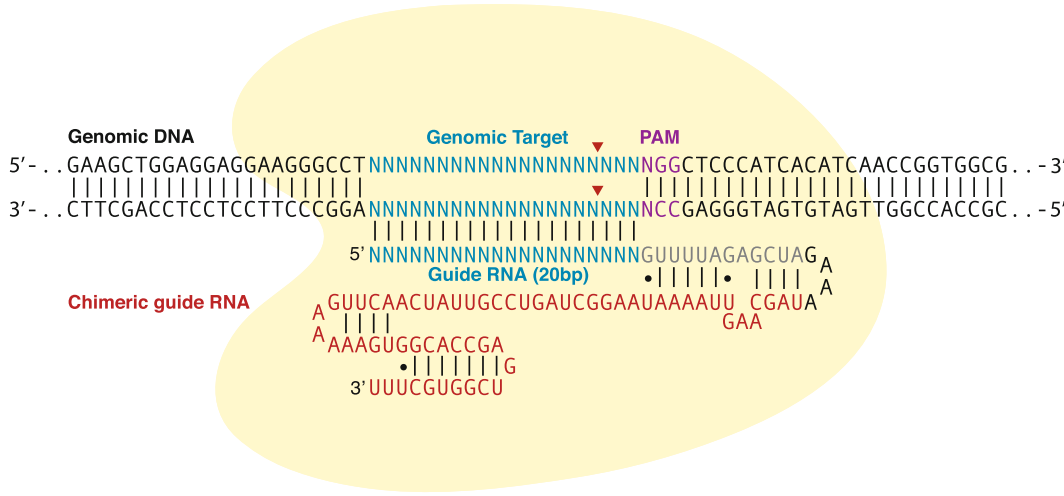

Fig. 1 Targeted DNA cleavage by SpCas9 in the human *EMX1* locus. The SpCas9 enzyme (*yellow*) interacts with its genomic target (*blue*) with the help of a guide RNA. The genomic target is directly 5′ to the PAM sequence, which is -NGG- for SpCas9. The guide RNA is composed of the guide sequence (*blue*), which anneals with the genomic target via Watson–Crick base pairing and a chimeric guide RNA scaffold consisting of a fusion between the crRNA (*gray*) and the tracrRNA (*red*)

The microbial adaptive immune system CRISPR (Clustered Regularly Interspaced Short Palindromic Repeats) consists of a set of enzymes and noncoding RNA elements [15–17]. Among the three types of CRISPR systems in bacteria and archaea [15, 16], type II requires only a single protein, Cas9 (formerly Csn1), to mediate DNA cleavage [18]. Cas9 is targeted to specific DNA sequences by a pair of noncoding RNA elements: the CRISPR RNA (crRNA), which carries the target-specifying guide sequence via Watson–Crick base pairing (Fig. 1), and the trans-activating crRNA (tracrRNA), which hybridizes with crRNA and is required for loading onto Cas9 [19, 20].

The type II CRISPR system of *Streptococcus pyogenes* can be reconstituted in mammalian cells to mediate DNA cleavage with three minimal components: Cas9, crRNA, and tracrRNA. The latter two components can further be truncated and fused into a single chimeric guide RNA scaffold (Fig. 1) [18] for a target sequence selected from any genomic locus with its 3' end followed by a NGG trinucleotide motif [19]. This protospacer-adjacent motif (PAM) is specific to each CRISPR system [21]. Generation of specific guide RNAs for targeted genome editing only requires the purchase of two short oligos and simple cloning that can take as little as two days.

The wild-type *S. pyogenes* Cas9 (SpCas9) enzyme has multiple endonuclease domains, two of which cleave DNA in a strand-specific manner. Two catalytic residues, D10 or H840 [18], can be mutated to convert the wild-type SpCas9 into a DNA-nicking enzyme (SpCas9n) [1, 18]. Given that single-stranded nicks in the

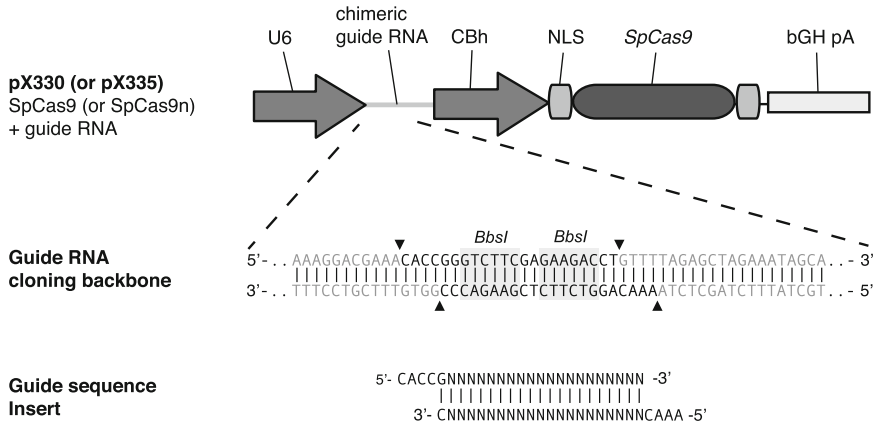

Fig. 2 Bicistronic expression vector for guide RNA and SpCas9 (or SpCas9n). A genomic target directly upstream to the PAM sequence can be cloned into the expression vector. After a target is selected, two DNA oligos can be designed based on the schematic showing the guide sequence insert. One oligo (*top* strand, written 5′–3′) contains ligation adapter sequences for cloning into the expression vector and $G(N)_{19}$, which is the selected genomic target sequence. The other oligo (*bottom* strand, written 3′–5′) also contains ligation adapter sequences for cloning into the expression vector and the complementary bases to the genomic target sequence. Once annealed and phosphorylated, the oligos can be inserted into the vector digested with *BbsI*

target DNA can also stimulate HDR, SpCas9n reduces the likelihood of error-prone repair by NHEJ. Furthermore, both catalytic domains of SpCas9 can be mutated to convert SpCas9 into a RNA-guided DNA-binding protein [18, 22]. This chapter describes a set of protocols for using the SpCas9 system for genome editing in mammalian cells.

2 Materials

2.1 Molecular Cloning Components

1. Cloning plasmids: *pX330* (CBh::SpCas9 + U6::chimeric guide RNA) (Addgene) or *pX335* (CBh::SpCas9n (D10A) + U6:: chimeric guide RNA) (Addgene) (Fig. 2).
2. Oligos for target sequence. *See* Subheading 3.1 for discussion regarding locus selection and Subheading 3.2 on oligo design (Integrated DNA Technologies).
3. Restriction enzymes and phosphatase: FastDigest *Bbs*I (Fermentas), FastAP (Fermentas), 10× FastDigest Buffer (Fermentas) (*see* **Note 1**).
4. QIAquick Gel Extraction Kit (QIAGEN).
5. Phosphorylation, annealing, and ligation reagents: 10× T4 Ligation Buffer (NEB), T4 Polynucleotide Kinase (NEB), 2× Quick Ligation Reaction Buffer (NEB), Quick Ligase (NEB).
6. Plasmid-Safe exonuclease (Epicentre Biotechnologies).
7. Competent cells and bacterial growth reagents.

8. QIAGEN Plasmid Midi Kit (QIAGEN).
9. Standard gel electrophoresis reagents.

2.2 Tissue Culture, Transfection, and DNA Extraction Components

1. Cell line: For validation, human embryonic kidney (HEK) cell line 293FT (Life Technologies). For additional discussions on working with other cell lines, *see* **Note 2**.
2. Cell culture reagents for maintenance of 293FT cells: Dulbecco's Modified Eagle's Medium (DMEM) (Life Technologies), 10 % fetal bovine serum (HyClone), 2 mM GlutaMAX (Life Technologies), 100 U/mL penicillin, and 100 μg/mL streptomycin.
3. Dissociation reagent: TrypLE™ (Life Technologies).
4. Transfection reagent: Lipofectamine 2000 (Life Technologies) for HEK293FT or Neuro-2a cells (Sigma Aldrich) (*see* **Note 3**).
5. 24-well tissue culture plates (Corning).
6. Transfection Control Plasmid: pMaxGFP (Lonza).
7. QuickExtract™ DNA extraction kit (Epicentre Biotechnologies).

2.3 Components for the Analysis of Genome Modification

1. SURVEYOR Mutation Detection Kit (Transgenomic).
2. 4–20 % Novex TBE polyacrylamide gels (Life Technologies).
3. Amplification primers specific to the targeted locus (Integrated DNA Technologies).
4. Herculase II High Fidelity Polymerase (Agilent).

3 Methods

3.1 Target Selection

For use with the SpCas9 system, target sites must be followed by a NGG trinucleotide motif on the 3′ end (*see* **Notes 4** and **5**).

3.2 Construct Design

We designed cloning vectors (pX330 for SpCas9 or pX335 for SpCas9n, a D10A nickase) to aid co-expression of SpCas9 and guide RNA in mammalian cells (Fig. 2). In this vector, SpCas9 is driven by the CBh promoter [23], and the guide RNA is driven by the human PolIII promoter U6. Phosphorylated and annealed oligos (design indicated in Fig. 2) can be cloned into the *BbsI* digested plasmid containing the entire guide RNA scaffold. The oligos are designed based on the target site sequence (20 bp sequence corresponding to the target site). The $G(N)_{19}$ refers to the sequence selected upstream of the PAM sequence in the genomic DNA (*see* **Note 6**). Create oligos using the schematic in Fig. 2.

3.3 Molecular Cloning: Oligo Annealing and Cloning into Backbone Vectors

1. Digest 1 μg of pX330 or pX335 with *Bbs*I for 30 min at 37 °C:

1 μg	pX330 or pX335
1 μL	FastDigest *Bbs*I (Fermentas)
1 μL	FastAP (Fermentas)
2 μL	10× FastDigest Buffer
*X*μL	ddH_2O
20 μL	Total

2. Gel purify digested pX330 or pX335 using QIAquick Gel Extraction Kit and elute in EB.
3. Phosphorylate and anneal each pair of oligos for the insert piece:

1 μL	Oligo 1 (100 mM)
1 μL	Oligo 2 (100 mM)
1 μL	10× T4 Ligation Buffer (NEB)
6.5 μL	ddH_2O
0.5 μL	T4 PNK (NEB)
10 μL	Total

Anneal in a thermocycler using the following parameters:

37 °C	30 min (for addition of 5′ phosphates)
95 °C	5 min and then ramp down to 25 °C at 5 °C/min

4. Set up ligation reaction and the negative control. Incubate at room temperature for 10 min:

*X*μL	*Bbs*I digested pX330 or pX335 from **step 2** (50 ng)
1 μL	Phosphorylated and annealed oligo duplex from **step 3** (1:200 dilution)
5 μL	2× Quick Ligation Buffer (NEB)
*X*μL	ddH_2O
10 μL	Subtotal
1 μL	Quick Ligase (NEB)
11 μL	Total

5. (Optional but highly recommended) Treat ligation reaction with Plasmid-Safe exonuclease (*see* **Note 7**):

11 μL	Ligation reaction from **step 4**
1.5 μL	10× Plasmid-Safe buffer
1.5 μL	10 mM ATP
0.7 μL	ddH_2O
0.3 μL	Plasmid-Safe DNAse
15 μL	Total

Incubate reaction at 37 °C for 30 min.

6. Transform 2 μL of reaction from **step 5** into competent cells and plate on ampicillin selection plates.
7. Pick two colonies the following day and analyze for correct insertion of the target sequence oligos.

3.4 Cell Culture and Transfection

The CRISPR-Cas DNA cleavage system has been validated for use in a variety of mammalian cell lines [1, 2, 24] (*see* **Note 2**). The protocol below is for HEK239FT cells.

1. HEK293FT cells are maintained in DMEM supplemented with 10 % fetal bovine serum and passaged before reaching 70 % confluency. Cells are maintained in an incubator set at 37 °C supplemented with 5 % CO_2.
2. HEK293FT cells can be transfected using Lipofectamine 2000 according to the manufacturer's protocol.
3. For each well of a 24-well plate, a total of 500 ng of plasmid is transfected. One well should be a control to see the relative transfection efficiency using a plasmid such as pmaxGFP.
4. After 12 h of transfection, replace the medium with pre-warmed maintenance medium. After 72 h, genomic DNA can be isolated using the QuickExtract DNA extraction kit following the manufacturer's protocol. Briefly, cells are resuspended in QuickExtract solution (50 μL per 24 well) and incubated at 65 °C for 15 min followed by 98 °C for 10 min.

3.5 Analysis of Genomic Modification: SURVEYOR and Sequencing

1. The efficiency of cleavage can be detected by assessing the percentage of cells containing indels in the target region (*see* **Note 8**). In order to detect indels in the DNA, follow the instructions provided in the SURVEYOR Mutation Detection Kit manual (*see* **Note 9**).
2. It is recommended that the SURVEYOR Nuclease digestion products are analyzed on a PAGE gel.

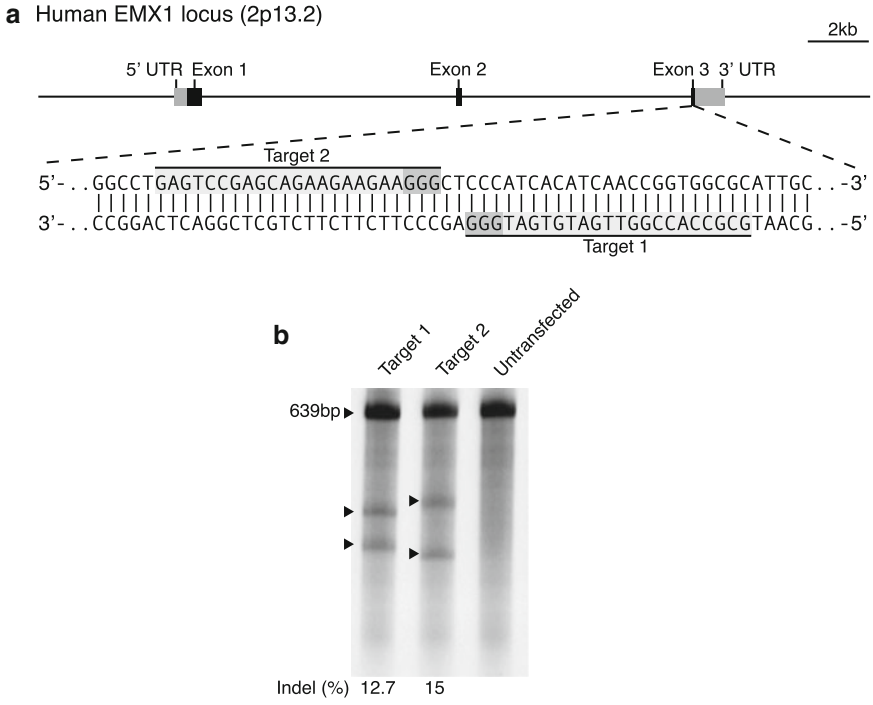

Fig. 3 SURVEYOR assay comparing SpCas9-mediated DNA cleavage at two different targets in the same gene. (**a**) The third exon of the human *EMX1* locus was targeted using guide RNAs at two unique sites. (**b**) A representative SURVEYOR assay gel image comparing the targeted cleavage efficiency by SpCas9 at the two targets in the human *EMX1* locus

3. To calculate the percent cutting efficiency of a CRISPR locus, use the following formula: $\%indel = \left(1 - \sqrt{1 - \frac{(a+b)}{(a+b+c)}}\right)100$ where a and b refer to the relative concentrations of the cut bands and c equals the relative concentration of the full-length PCR template. A representative SURVEYOR gel image and quantitation is shown in Fig. 3.

4 Notes

1. Conventional restriction enzymes can be substituted for FastDigest restriction enzymes. In this case, adjust digestion reagents and digestion times according to manufacturer's protocol.
2. Experimental conditions may need to be optimized for each cell line.

3. For other cell lines, we suggest doing an initial comparison of different transfection reagents (e.g., FuGENE HD, nucleofection, and TransIT).
4. A free computational resource maintained by the Zhang lab (http://www.genome-engineering.org) contains the most up-to-date information relevant for Cas9 systems.
5. It is ideal for these targets to be unique within the genome. We also recommend testing multiple target sites for each gene and selecting the most effective target.
6. Selecting a target site with a 5′ G allows for efficient transcription of the guide RNA from the U6 promoter.
7. Plasmid-Safe treatment is recommended because it degrades linear dsDNA, helping to prevent unwanted recombination products.
8. SpCas9-induced double-strand breaks in the target DNA are usually repaired through the error-prone NHEJ process in HEK293FT cells.
9. It is important to make sure that the genomic PCR primers yield a single amplicon for reliable quantification of the percent cutting efficiency. In the case that primers do not yield a single amplicon, the PCR product needs to be gel purified or new primers should be designed.

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References

1. Cong L et al (2013) Multiplex genome engineering using CRISPR/Cas systems. Science 339:819–823
2. Mali P et al (2013) RNA-guided human genome engineering via Cas9. Science 339:823–826
3. Urnov FD et al (2005) Highly efficient endogenous human gene correction using designed zinc-finger nucleases. Nature 435:646–651
4. Porteus MH, Baltimore D (2003) Chimeric nucleases stimulate gene targeting in human cells. Science 300:763

5. Miller JC et al (2007) An improved zinc-finger nuclease architecture for highly specific genome editing. Nat Biotechnol 25:778–785
6. Christian M et al (2010) Targeting DNA double-strand breaks with TAL effector nucleases. Genetics 186:757–761
7. Miller JC et al (2011) A TALE nuclease architecture for efficient genome editing. Nat Biotechnol 29:143–148
8. Sanjana NE et al (2012) A transcription activator-like effector toolbox for genome engineering. Nat Protoc 7:171–192
9. Stoddard BL (2005) Homing endonuclease structure and function. Q Rev Biophys 38: 49–95
10. Rouet P, Smih F, Jasin M (1994) Expression of a site-specific endonuclease stimulates homologous recombination in mammalian cells. Proc Natl Acad Sci U S A 91:6064–6068
11. Hockemeyer D et al (2011) Genetic engineering of human pluripotent cells using TALE nucleases. Nat Biotechnol 29:731–734
12. Cui X et al (2011) Targeted integration in rat and mouse embryos with zinc-finger nucleases. Nat Biotechnol 29:64–67
13. Ding Q et al (2013) A TALEN genome-editing system for generating human stem cell-based disease models. Cell Stem Cell 12:238–251
14. Maier D et al (2013) Efficient clinical scale gene modification via zinc finger nuclease targeted disruption of the HIV co-receptor CCR5. Hum Gene Ther 24:245–258
15. Deveau H, Garneau JE, Moineau S (2010) CRISPR/Cas system and its role in phage-bacteria interactions. Annu Rev Microbiol 64:475–493
16. Garneau JE et al (2010) The CRISPR/Cas bacterial immune system cleaves bacteriophage and plasmid DNA. Nature 468:67–71
17. Horvath P, Barrangou R (2010) CRISPR/Cas, the immune system of bacteria and archaea. Science 327:167–170
18. Jinek M et al (2012) A programmable dual-RNA-guided DNA endonuclease in adaptive bacterial immunity. Science 337:816–821
19. Deltcheva E et al (2011) CRISPR RNA maturation by trans-encoded small RNA and host factor RNase III. Nature 471:602–607
20. Jore MM et al (2011) Structural basis for CRISPR RNA-guided DNA recognition by Cascade. Nat Struct Mol Biol 18:529–536
21. Mojica FJ, Diez-Villasenor C, Garcia-Martinez J, Almendros C (2009) Short motif sequences determine the targets of the prokaryotic CRISPR defence system. Microbiology 155: 733–740
22. Qi LS et al (2013) Repurposing CRISPR as an RNA-guided platform for sequence-specific control of gene expression. Cell 152:1173–1183
23. Gray SJ et al (2011) Optimizing promoters for recombinant adeno-associated virus-mediated gene expression in the peripheral and central nervous system using self-complementary vectors. Hum Gene Ther 22:1143–1153
24. Cho SW, Kim S, Kim JM, Kim JS (2013) Targeted genome engineering in human cells with the Cas9 RNA-guided endonuclease. Nat Biotechnol 31:230–232