Chapter 19

Manufacturing and Delivering Genome-Editing Proteins

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Abstract

Genome-editing technologies have revolutionized the biomedical sciences by providing researchers with the ability to quickly and efficiently modify genes. While programmable nucleases can be introduced into cells using a variety of techniques, their delivery as purified proteins is an effective approach for limiting off-target effects. Here, we describe step-by-step procedures for manufacturing and delivering genome-modifying proteins—including Cas9 ribonucleoproteins (RNPs) and TALE and zinc-finger nucleases—into mammalian cells. Protocols for combining Cas9 RNP with naturally recombinogenic adeno-associated virus (AAV) donor vectors for the seamless insertion of transgenes by homology-directed genome editing are also provided.

Key words ZFNs, TALENs, CRISPR, RNP, Protein delivery, Genome editing

1 Introduction

Programmable nucleases—including the RNA-guided Cas9 endonuclease from type II clustered regularly interspaced short palindromic repeats (CRISPR)-associated (Cas) systems, transcription activator-like effector (TALE) nucleases (TALENs), and zinc-finger nucleases (ZFNs)—have revolutionized biomedical research and biotechnology. These technologies can be configured to recognize a specific genomic site to induce a DNA double-strand break (DSB) that is processed by cellular DNA repair mechanisms, enabling a range of genomic modifications, including gene knockout via non-homologous end joining (NHEJ) [1, 2] and the site-specific insertion of transgenes by homology-directed repair (HDR) [3–5].

ZFNs and TALENs—among the first widely used tools for genome editing—are fusions of the DNA cleavage domain from the FokI restriction endonuclease and engineered Cys_2-His_2 zinc-finger [6] and TALE DNA-binding proteins [7], respectively. An individual zinc finger binds to 3–4 nucleotides (with occasional target site overlap from an adjacent zinc-finger domain) [8], whereas a TALE domain recognizes only a single base pair (bp) [9, 10], with

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the specificity of each TALE conferred almost entirely by two hypervariable amino acid residues in the center of each domain (dubbed the repeat variable diresidues). Importantly, because the FokI nuclease domain cleaves DNA as a dimer, two ZFN and TALEN monomers are required to form an activate nuclease.

Unlike ZFNs and TALENs, CRISPR-Cas systems rely on a single guide RNA (sgRNA) molecule to direct a Cas nuclease, such as Cas9 or Cas13a, to a specific DNA [11] or RNA sequence [12, 13], respectively. CRISPR-based tools can thus be harnessed for genome editing with only minimal molecular engineering [14–17]. For example, the only major requirement for targeting DNA using CRISPR-Cas9 is the presence of a conserved protospacer adjacent motif (PAM) located at the 3′-end of the Cas9 target site [11].

Nuclease-encoding gene(s) can be introduced into cells using viral and nonviral methods. Nonviral delivery, for instance, can be performed using electroporation [18] and nucleofection [19] but also with chemical methods, including cationic liposomes [20] and charged polymers [21]. These approaches are easy to use but limited to certain cell lines or associated with toxicity [22]. In addition to nonviral methods, viral vectors, including lentivirus, adeno-associated virus (AAV), and adenovirus, can be harnessed to facilitate the expression of nuclease-encoding gene(s) in more difficult-to-transfect cell types and in vivo [23]. AAV vectors, in particular, have emerged as effective tools for delivering CRISPR-Cas9 to the central nervous system [24–26], among other tissues [27].

Regardless of the delivery strategy employed, persistent transient expression of nuclease-encoding gene(s) can lead to increased off-target (OT) effects [28]. One approach for improving nuclease specificity is minimizing the amount of time that a nuclease is exposed to the cell. Crucially, when delivered directly into cells as purified proteins, genome-modifying nucleases are rapidly degraded and induce fewer OT modifications compared to methods that rely on transient expression from nucleic acids [29].

Each of the three major genome-editing platforms can be delivered into cells as proteins [30]. ZFNs—which were the first technology used for DNA-free genome editing—can naturally cross cell membranes [29] due to the intrinsic cell-penetrating capabilities of zinc-finger proteins [31, 32] (notably, their internalization efficiency can be increased following the fusion of multiple nuclear localization sequences [NLS] [33]). In addition to this mode of entry, ZFN proteins can be engineered to enter cells via receptor-mediated endocytosis [34] or packaged into retroviral [35] and lentiviral [36] protein-based platforms. Similarly, TALEN proteins can be introduced into cells via lentiviral based particles [36] or cell-penetrating peptides (CPPs), which are tethered onto the TALEN protein surface [37] or genetically fused to their termini [38]. Moreover, both Cas9 and its sgRNA can be delivered to cells as a preformed ribonucleoprotein (RNP) using nucleofection

[39, 40] or transient transfection [41], though CPPs [42], nanoparticles [43–46], and small-molecule-induced osmocytosis [47] can also facilitate RNP delivery. Additionally, RNPs can be easily complemented with highly recombinogenic single-stranded DNA oligonucleotides [48] and AAV donor vectors [48, 49] for homology-directed genome editing, which expands on the capabilities of the protein-based genome-editing toolbox.

Here, we provide detailed step-by-step procedures for manufacturing and delivering genome-modifying proteins, including RNPs, TALEN, and ZFN proteins. We also provide protocols for combining Cas9 RNP with AAV donor vectors for homology-directed repair (HDR)-mediated genome editing. These procedures are broadly applicable and can be used to modify cells within 10–14 days.

2 Materials

2.1 Molecular Cloning

1. Centrifuge.
2. DNA imaging system.
3. UV-Vis spectrophotometer.
4. PCR thermocycler.
5. Sterile 1.5 mL microcentrifuge tubes.
6. 5 mL Polystyrene tubes.
7. Sterile 15 mL conical tubes.
8. Sterile 50 mL conical tubes.
9. 0.2 mL Flat-cap PCR tubes.
10. Bacterial (e.g., pET-28B; EMD Biosciences) and mammalian expression vectors (e.g., pcDNA 3.1; Life Technologies): Note that the expression vectors encoding Cas9 and the TALENs and ZFNs used here can be obtained from J.L.
11. Deionized water (dH_2O).
12. Nuclease-free water.
13. High-fidelity PCR DNA amplification system.
14. *Nco*I restriction enzyme.
15. *Xho*I restriction enzyme.
16. *Xba*I restriction enzyme.
17. Master buffer for restriction enzymes.
18. T4 DNA ligase with buffer.
19. Plasmid purification kit.
20. PCR amplicon purification kit.
21. DNA gel extraction kit.

22. T7 RNA polymerase in vitro transcription kit.
23. Phenol:chloroform:isoamyl alcohol solution (25:24:1, v/v/v) (Sigma-Aldrich).
24. 3 M Sodium acetate stock solution (dissolve 24.6 g of anhydrous sodium acetate powder in 100 mL of dH_2O): Adjust pH to 5.2 and then autoclave. Can be stored at room temperature (RT) for up to 1 year.
25. 1 kilobase (kb) DNA ladder.
26. Agarose.
27. 10× TAE running buffer.
28. 6× DNA-loading dye.
29. Ethidium bromide (*see* **Note 1**).

2.2 Bacterial Culture

1. Kanamycin.
2. LB medium (Gibco).
3. Bacto-agar.
4. LB agar plates (1 L): Add 15 g of bacto-agar to 1 L of LB medium. Sterilize by autoclave and pipet 22 mL of LB agar to a sterile petri dish. Can be stored at 4 °C for up to 1 month.
5. Chemically competent TOP10 cells.
6. Chemically competent BL21 (DE3) cells.

2.3 Protein Purification

1. Sonicator.
2. 0.22 μm Low-protein-binding filter.
3. 0.45 μm Low-protein-binding filter.
4. Polypropylene gravity-flow purification column.
5. Protein concentrator: 10 kDa MWCO.
6. Protein concentrator: 30 kDa MWCO.
7. HEPES stock solution: 200 mM, pH 8.0. Dissolve 52.1 g HEPES sodium salt in dH_2O. Adjust volume to 1 L and pH to 8.0 and autoclave. Store at 4 °C for up to 6 months.
8. Tris–HCl stock solution: 1 M. Dissolve 12.1 g Tris base in 100 mL dH_2O and autoclave. Store at room temperature for up to 1 year.
9. NaCl stock solution: 5 M. Dissolve 29.2 g NaCl in 1 L dH_2O and autoclave. Store at room temperature for up to 1 year.
10. $MgCl_2$ stock solution: 1 M. Dissolve 20.3 g $MgCl_2$-$6H_2O$ in 1 L dH_2O and autoclave. Store at room temperature for up to 1 year.
11. $ZnCl_2$ stock solution: 9 mM. Dissolve 1.23 g $ZnCl_2$ in 1 L dH_2O and autoclave. Store at room temperature for up to 1 year.
12. Glucose.

13. β-Mercaptoethanol (β-ME): 55 mM (*see* **Note 2**).
14. Glycerol stock solution: 50%, v/v. Mix glycerol with dH_2O and autoclave. Store at room temperature for up to 6 months.
15. Protease inhibitor cocktail: 25× Stock solution. Dissolve one protease inhibitor cocktail tablet in 2 mL dH_2O and sterilize using a 0.22 μm filter. Store at −20 °C for up to 1 week.
16. Imidazole stock solution: 2 M. Dissolve 13.6 g imidazole in 100 mL dH_2O and autoclave. Store at room temperature for up to 1 year.
17. D, L-Dithiothreitol (DTT) stock solution: 1 M. Dissolve 1.54 g in 10 mL dH_2O. Prepare 1 mL aliquots. Sterilize using 0.22 μm filter and store at −20 °C for up to 6 months.
18. L-Arg stock solution: 1 M. Dissolve 17.4 g in 100 mL dH_2O. Adjust pH to 7.4 and sterilize using 0.22 μm filter (*see* **Note 3**). Store at room temperature for up to 6 months.
19. Isopropyl-β-D-1-thiogalactopyranoside (IPTG).
20. Nickel agarose resins for purification of His-tagged proteins.
21. Protein-binding buffer (1 L): Add 100 mL of 200 mM HEPES stock solution, 1 mL of 1 M $MgCl_2$ stock solution, 29.2 g NaCl, and 100 mL glycerol into 799 mL dH_2O. For ZFN-binding buffer, add 10 mL of 9 mM $ZnCl_2$ stock solution and an additional 87.7 g NaCl. Adjust volume to 1 L and autoclave. Store at 4 °C for up to 6 months.
22. Lysis buffer: Add 1 mM β-ME and 1× protease inhibitor cocktail to binding buffer before use (*see* **Note 4**). Store at 4 °C for up to 24 h.
23. Wash buffer A: Add 2.5 mL of 2 M imidazole to 1 L of binding buffer. Store at 4 °C for up to 6 months.
24. Wash buffer B: Add 17.5 mL of 2 M imidazole to 1 L of binding buffer. Store at 4 °C for up to 6 months.
25. Elution buffer: Dissolve 20.4 g imidazole into 1 L of binding buffer. Store at 4 °C for up to 6 months.
26. Storage buffer: Add 100 mL of 200 mM HEPES stock solution, 1 mL of 1 M $MgCl_2$ stock solution, 29.2 g NaCl, and 100 mL glycerol into 799 mL dH_2O. For ZFN storage buffer, add 100 mL of 1 M L-Arg. For Cas9 protein storage buffer, add 1 mL of 1 M DTT stock solution. Store at 4 °C for up to 2 weeks.
27. 4–20% Tris-glycine SDS-PAGE gel: Purchased from commercial vendor.
28. 2× Protein-loading dye.
29. Protein marker.
30. Bovine serum albumin (BSA): 10 mg/mL. Dissolve 10 mg BSA powder in 1 mL dH_2O.

31. PCR amplicons carrying designated nuclease-targeting sequence.
32. In vitro cleavage reaction buffer (10×): 500 mM NaCl, 10 mM Tris–HCl, 10 mM $MgCl_2$, 1 mM DTT, pH 7.9. Mix 1 mL of 5 M NaCl solution, 0.1 mL of 1 M Tris–HCl solution, 0.1 mL of 1 M $MgCl_2$ solution, and 0.1 mL of 1 M DTT solution with water up to 10 mL. Adjust pH to 7.9 and autoclave. Store at −20 °C for up to 2 months.

2.4 Mammalian Cell Culture

1. Tissue culture hood.
2. Tissue culture incubator.
3. Hemocytometer.
4. Leica microscope.
5. Sterile serological pipets: 5, 10, and 25 mL.
6. 10 cm Petri dish.
7. Tissue culture plate: 24-well, 12-well, and 6-well plates.
8. Ethanol: 75%. Mix 25 mL dH_2O with 75 mL ethanol. Store at room temperature (25 °C) for up to 1 month (*see* **Note 5**).
9. Complete DMEM medium: Add 50 mL of fetal bovine serum (FBS) and 5 mL of 100 U/mL of penicillin-streptomycin to 500 mL DMEM medium. Store solution at 4 °C for 3 months. Pre-warm the medium to 37 °C before use.
10. Complete RMPI 1640 medium: Add 50 mL of FBS and 5 mL of 100 U/mL of penicillin-streptomycin to 500 mL RPMI 1640 medium. Store solution at 4 °C for 3 months. Pre-warm the medium to 37 °C before use.
11. Trypsin-EDTA, 0.05% (v/v).
12. Dulbecco's phosphate-buffered saline, no calcium, no magnesium (DPBS).
13. CD4+ primary T cells: Purchased from commercial vendor.
14. CD3/CD28 human T-cell activation beads.
15. Recombinant interleukin-2 (rIL-2).
16. Poly-L-lysine solution.
17. Poly-lysine-coated culture plates: Add 250 mL poly-lysine solution to each well of a 24-well culture plate and incubate at 37 °C for 1 h. Remove solution by aspiration and wash twice with 0.5 mL of DPBS. Dry poly-lysine-treated plates at room temperature for 2 h. Store coated plates at 4 °C for up to 1 month (*see* **Note 6**).

2.5 Transfection Reagents

1. 4D-Nucleofector System (Lonza; core unit, cat. no. AAF-1002B; X unit, cat. no. AAF-1002X).
2. P3 Primary Cell 4D-Nucleofector kit (Lonza, cat. no. V4XP-3032).

3. EGFP reporter cell line: House-made HEK293-derived cells (*see* **Note 7**).
4. Sodium phosphate buffer stock solution: 200 mM, pH 5.5. Dissolve 23.4 g sodium phosphate monobasic in 1 L dH_2O. Adjust pH to 5.5 and autoclave. Store at 4 °C for up to 6 months.
5. Sodium hydroxide: 1 M. Dissolve 4 g NaOH in 100 mL dH_2O and autoclave. Store solution at room temperature for up to 6 months.
6. Peptide stock solution of Cys-Npys-modified peptides: 2.3 mM. Dissolve 1 mg peptides in 250 μL dH_2O. Sterilize using a 0.22 μm filter and store at −20 °C for up to 6 months.
7. Genomic DNA extraction kit.
8. T7E1 endonuclease and buffer.
9. PCR TA cloning kit.
10. Flow cytometry buffer: Supplement DPBS with 2% FBS.

3 Methods

3.1 Expression Vector Construction

1. PCR amplify the genes encoding the "left" and "right" ZFN and TALEN monomers or the gene encoding the Cas9 nuclease from *Streptococcus pyogenes* (SpCas9) using primers encoding 5′ *Nco*I and 3′ *Xho*I restriction sites (*see* Table 1). Carry out the PCR reaction using 5 ng of template DNA, 5 μL of 10× PCR buffer, 0.2 μM of each primer, 0.2 mM of dNTPs, and 2 U of high-fidelity DNA polymerase with water to 50 μL. Use the following cycle: initial denaturation at 95 °C for 5 min; 30 cycles of 95 °C for 30 s; 55 °C for 30 s; and 72 °C for 2 min (for ZFNs) or 4 min (for TALENs) or 5 min (for Cas9); final extension at 72 °C for 10 min (*see* **Note 8**).
2. Purify the PCR products from Subheading 3.1, **step 1**, using a PCR purification kit.
3. Separately incubate pET-28b and the nuclease-encoding genes from Subheading 3.1, **step 2**, with restriction enzymes in separate 50 μL reactions containing 2 μg of DNA; 5 μL of 10× restriction enzyme buffer; and 20 U each of *Nco*I and *Xho*I. Incubate at 37 °C for 3 h.
4. Mix the restriction digests from Subheading 3.1, **step 3**, with 10 μL of 6X DNA-loading dye. Resolve the DNA by gel electrophoresis using a 1% agarose gel. Excise the desired band from the agarose gel, and isolate the DNA using a gel extraction kit, following the manufacturer's instructions (*see* **Note 9**). Determine DNA concentration using a spectrophotometer by measuring $Abs_{260} \times 50$ μg/mL.

Table 1
Primer sequences for nuclease cloning

Primer name	Sequence (5′–3′)
*Nco*I ZFN Fwd	aaaCCATGGatgggtcatcatcatcatcacggtggcagcccgaaaaaaaaacgcaaa
*Xho*I ZFN Rev	aaaCTCGAGttaaaagtttatctcgccgtt
*Nco*I TALEN Fwd	aaaCCATGGatgatgggtcatcatcatcatcacggtggcagcgactacaaagaccatgacggt
*Xho*I TALEN Rev	aaaCTCGAGttaaaagtttatctcgccgttatt
*Nco*I SpCas9 Fwd	aaaCCATGGatgggcagcagccccaagaagaagaggaaggtgggcggctccatggataagaaatactca
*Xho*I SpCas9 Rev	aaaCTCGAGttaatgatgatgatgatgggagccgcccactttgcgtttcttttttcggggagccgcc

Note: Restriction sites are capitalized

5. Ligate the purified nuclease-encoding gene(s) from Subheading 3.1, **step 4**, with the digested pET-28 vector from Subheading 3.1, **step 4**, using an insert-to-vector molar ratio of 6-to-1 in a 10 μL reaction containing ~50 ng of digested DNA, 400 U of T4 DNA ligase, and 1 μL of 10× T4 ligase buffer. Incubate at RT for 1 h.
6. Thaw 100 μL of chemically competent TOP10 cells on ice and mix gently with 10 μL of the ligation reaction from Subheading 3.1, **step 5**. Keep cells on ice for 30 min. Incubate the mixture at 42 °C for 60 s using a water batch, and then put the cells on ice for 30 s. Transfer the cells to a culture tube containing 1 mL of LB medium and incubate the cells for 1 h at 37 °C with shaking at 250 rpm. Spread 100 μL of the bacterial cell culture on a LB agar plate with 50 μg/mL kanamycin and incubate overnight at 37 °C.
7. The following day, inoculate 4 mL of LB medium containing 50 μg/mL kanamycin with one colony from the LB agar plate and culture overnight at 37 °C with shaking at 250 rpm.
8. Purify the nuclease expression vectors using a plasmid miniprep kit per the manufacturer's instructions and confirm the identity of the nuclease-encoding gene(s) by DNA sequencing (*see* **Note 10**).

3.2 In Vitro Transcription of the sgRNA

Note that the following procedures must be carried out in RNase-free environment.

1. PCR amplify the sgRNA. Carry out the PCR reaction using 5 ng of template DNA (e.g., an sgRNA-encoding mammalian expression vector); 5 μL of 10× PCR buffer with $MgCl_2$; 0.2 μM each of forward primer (e.g., 5′-GAAATTAATACGACTCACTATAGGNNNNNNNNNNNNNNNNNNGTTTTAGAGCTAGAAATA-3′, T7 promoter is under-

lined; 20 bp targeting sequence donated by N) and reverse primer (5′-AGCACCGACTCGGTGCCA-3′); 0.2 mM dNTP; 2 U of high-fidelity DNA polymerase; and water up to 50 μL. Use the following cycling program: initial denaturation at 95 °C for 5 min; 30 cycles of 95 °C for 30 s; 55 °C for 30 s; and 72 °C for 40 s; final extension at 72 °C for 10 min.

2. Purify the PCR products by gel extraction and determine DNA concentration using a spectrophotometer by measuring $Abs_{260} \times 50$ μg/mL (*see* **Note 11**).
3. Transcribe the sgRNA by a "run-off" reaction using a T7 in vitro transcription kit. Carry out a 20 μL reaction containing 1 μg of purified PCR product; 2 μL of 10× reaction buffer; 7.5 mM of each dNTP; and 1.5 μL of T7 RNA polymerase mix. Incubate at 37 °C for 16 h (*see* **Note 12**).
4. Upon completion of the reaction, add 70 μL of nuclease-free water, 10 μL of 10× DNase I buffer, and 2 μL of DNase I (RNase free). Mix well and incubate for 15 min at 37 °C.
5. Add 80 μL of nuclease-free water and 20 μL of 3 M sodium acetate to the DNase I-treated transcription reaction, followed by 200 μL of phenol:chloroform:isoamyl alcohol solution and vortex.
6. Centrifuge the mixture at 12,000 × *g* for 5 min, and transfer the upper aqueous phase to a new microcentrifuge tube.
7. Add 200 μL of chloroform and centrifuge at 12,000 × *g* for 5 min to remove residual phenol. Transfer the supernatant to a new microcentrifuge tube.
8. Repeat chloroform extraction procedure once more as described in Subheading 3.3, **step 7**.
9. Add 2.5 volumes of ethanol to the chloroform-extracted sgRNA. Store at −20 °C for 12 h.
10. Centrifuge at 12,000 × *g* for 30 min at 4 °C. Remove the supernatant and then add 500 μL of ice-cold ethanol (75%) to remove residual salt.
11. Centrifuge at 12,000 × *g* for 5 min at 4 °C and air-dry the pellet.
12. Dissolve the pellet in 20 μL of nuclease-free water (*see* **Note 13**). Determine sgRNA concentration using a spectrophotometer by measuring $Abs_{260} \times 40$ μg/mL (*see* **Note 14**). Store sgRNA at −80 °C for up to 1 year.

3.3 Nuclease Protein Manufacturing and Quality Control

1. Thaw 50 μL of chemically competent BL21(DE3) cells on ice and mix gently with 100 ng of sequence-verified nuclease-encoding expression vectors from Subheading 3.1, **step 7**. Transform as described in Subheading 3.1, **step 6**.

2. The following day, inoculate 20 mL of LB medium containing 50 μg/mL kanamycin with one colony from the LB agar plate from Subheading 3.3, **step 1**, and culture overnight at 37 °C.
3. The following day, transfer 20 mL of the overnight starter culture from Subheading 3.2, **step 2**, into 1 L of LB medium containing 50 μg/mL kanamycin, 200 mM NaCl, and 0.2% glucose (*see* **Notes 15** and **16**).
4. Induce protein expression once the culture reaches an OD_{600} of 0.8 with 0.1 mM of IPTG (*see* **Note 17**). After induction, culture ZFN- and TALEN-expressing bacterial cultures at RT for an additional 4 h, and Cas9-expressing culture at 18 °C for an additional 12 h (*see* **Note 18**).
5. Harvest cells by centrifugation at 5000 × *g* for 10 min at 4 °C. Discard the supernatant. Do not dry cell pellets (*see* **Note 19**).
6. Resuspend the cell pellets from Subheading 3.2, **step 5**, with 20 mL of lysis buffer (*see* **Notes 20** and **21**). Transfer the suspension to a fresh collection tube.
7. Lyse the cells by sonication using the following settings: 50% power output and 2-min process time with 5-s on and 10-s off intervals (*see* **Note 22**).
8. Centrifuge the cell lysate at 25,000 × *g* for 30 min at 4 °C and transfer the supernatant into a fresh collection tube. Filter the supernatant through a 0.45 μm low-protein-binding filter (*see* **Note 23**).
9. Add 1 mL of nickel agarose resins (50% slurry in 30% ethanol) to the filtered lysate and incubate on a rotisserie or shaking platform for 30 min at 4 °C.
10. Rinse an empty polypropylene gravity-flow purification column with 5 mL of lysis buffer and then transfer the protein-bound slurry into the column with the bottom cap attached.
11. Remove the bottom cap and discard the flow-through. Wash the column with 20 mL of wash buffer A and 5 mL of wash buffer B.
12. Elute the nuclease protein with ten fractions of 0.5 mL elution buffer (*see* **Note 24**).
13. Analyze fractions by SDS-PAGE by mixing 5 μL of each elution with 5 μL of 2× protein-loading dye. Boil samples at 95 °C for 10 min and resolve on a 4–20% Tris-glycine gel. Combine the fractions with the highest purity.
14. Buffer exchange the combined fractions with storage buffer and concentrate the proteins to 400–800 μL using an Amicon Ultra-15 Centrifugal Filter Unit (note: use 10 kDa MWCO for

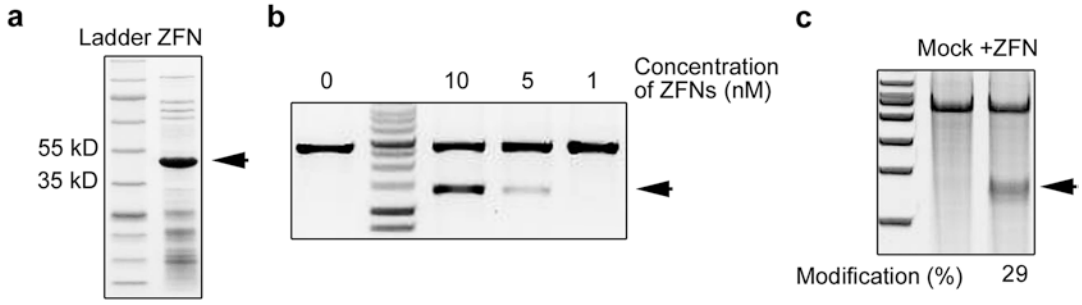

Fig. 1 Genome editing in primary CD4+ T cells using *CCR5*-targeting ZFN proteins. (**a**) SDS-PAGE of purified ZFN protein. Arrowhead indicates the predicted molecular weight of the ZFN protein. (**b**) In vitro cleavage assay assessing ZFN protein activity. (**c**) Frequency of indel formation in primary CD4+ T cells following ZFN protein delivery. (**b**, **c**) Arrowheads indicate expected cleavage product

ZFN proteins, and 30 kDa MWCO for Cas9 and TALEN proteins). Clarify the proteins through 0.22 μm low-protein-binding filters.

15. Visualize purified nuclease proteins and a BSA standard by SDS-PAGE. Determine protein concentration by densitometry using the BSA standard curve (*see* **Note 25**). Sample SDS-PAGE displaying purified nuclease proteins is shown in Fig. 1.
16. Aliquot 250 μL of each concentrated proteins to 1.5 mL microcentrifuge tubes and flash freeze samples using liquid nitrogen and store at −80 °C for up to 1 year (*see* **Note 26**).
17. Determine nuclease protein activity using an in vitro cleavage assay. Carry out the reaction with 1 μL of 10× in vitro assay buffer (*see* **Note 27**), 200 ng of PCR amplicon encoding the nuclease target site, 1 μL of 10 mg/mL BSA, and increasing concentrations (100 nM to 1 nM) of ZFN, TALEN, and RNP (*see* **Note 28**) with dH_2O up to 20 μL. A representative in vitro cleavage assay is shown in Fig. 2.

3.4 Nuclease Protein Delivery

In this section, we describe protocols for delivering nuclease proteins into cells for genome editing. Importantly, owing to their distinct characteristics, ZFNs, TALENs, and RNPs are introduced into cells through different procedures, which are described here. We also provide a protocol for site-specific gene insertion via HDR-mediated genome editing using RNP and AAV donor delivery.

3.4.1 ZFN Protein Delivery to Primary CD4+ T Cells

1. Seed 1 × 10^6 CD4+ T cells into one well of a 24-well plate containing 1 mL of complete RPMI 1640 medium with 25 μL CD3/CD28 human T-cell activation beads and 50 U rIL-2 for stimulation and expansion (*see* **Note 29**). Incubate at 37 °C with 5% CO_2 and full humidity.

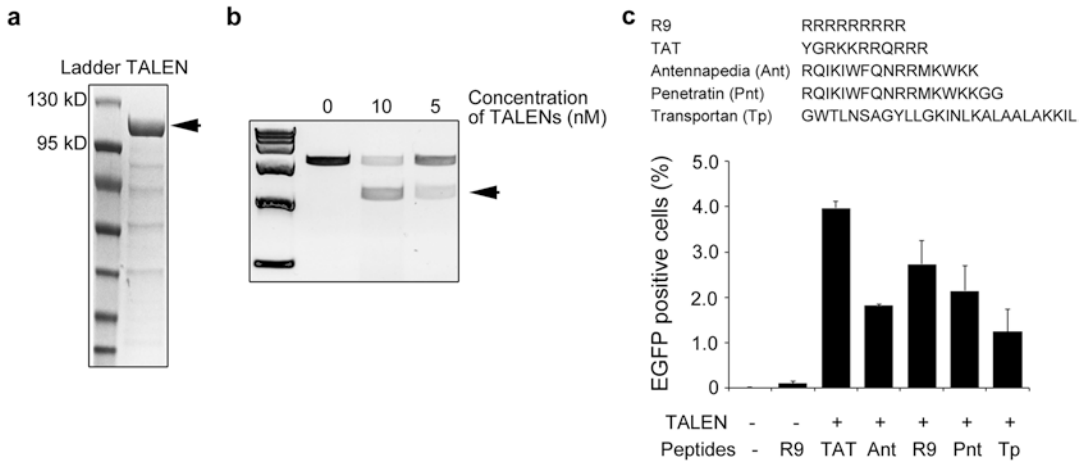

Fig. 2 Genome editing in HEK293T reporter cells using purified TALEN proteins. (**a**) SDS-PAGE of purified TALEN protein. Arrowhead indicates predicted molecular weight of the TALEN protein. (**b**) In vitro cleavage assay assessing TALEN protein activity. (**c**) Flow cytometry analysis of genome editing in HEK293T reporter cells following the delivery of TALEN proteins conjugated with various cell-penetrating peptide (CPP) domains. (**b**, **c**) Arrowheads indicate expected cleavage product

2. At 48 h after activation, harvest cells by centrifugation at 300 × *g* for 10 min, discard supernatant, and wash the cells once with serum-free DMEM medium (*see* **Note 30**).
3. Resuspend 2×10^5 cells in 250 μL serum-free DMEM containing 2 μM each of "left" and "right" ZFN proteins (*see* **Note 31**) supplemented with 90 μM $ZnCl_2$ and 100 mM L-Arg (*see* **Note 32**). Incubate cells at 37 °C for 1 h.
4. Harvest cells by centrifugation at 300 × *g* for 10 min and discard supernatant. Resuspend cells with 500 μL of complete RPMI 1640 medium containing 50 U/mL rIL-2.
5. Incubate cells at 30 °C for 24 h (*see* **Note 33**) and then 37 °C for an additional 24 h.
6. Upon completion of the treatment, centrifuge cells at 300 × *g* for 10 min, discard supernatant, and extract the genomic DNA of ZFN-treated cells using a genomic DNA extraction kit according to the manufacturer's instruction. Store genomic DNA at −80 °C for further analysis.

3.4.2 TALEN Protein Delivery to HEK293 Reporter Cells

1. Seed HEK293 cells onto a 24-well plate at a density of 1 x 10^5 cells per well and incubate at 37 °C with 5% CO_2 and full humidity.
2. At 24 h after seeding, conjugate cell-penetrating peptides (CPP) to the surface of the TALEN proteins by incubating 3.3 μM each "left" and "right" TALEN proteins with 100 μM

of Npys-modified CPPs and 1× complete protease inhibitor cocktail in 100 mM sodium phosphate buffer, pH 5.5, in a 75 μL reaction. Incubate for 2 h at RT (*see* **Note 34**).

3. Adjust the pH of the reaction by adding 7.5 μL of 1 M sodium hydroxide and mix the solution well with 175 μL of serum-free DMEM medium (*see* **Note 35**).
4. Remove medium from each well of cultured cells and wash cells once with serum-free medium (SFM). Gently remove SFM and apply the entire TALEN protein solution onto cells. Incubate cells at 37 °C for 2 h (*see* **Note 36**).
5. Replace medium with complete DMEM and incubate cells at 30 °C for 24 h, and then at 37 °C for additional 24 h for flow cytometry analysis.

3.4.3 RNP Delivery via Electroporation

1. Seed Hepa 1-6 cells onto a 24-well plate at a density of 1×10^5 cells per well. Incubate at 37 °C with 5% CO_2 and full humidity.
2. At 24 h after seeding, form the RNP complex by mixing 16 μg of Cas9 protein with 4 μg of sgRNA (molar ratio of 1:1) in a maximum volume of 2 μL. Incubate at RT for 10 min to allow the complex to form.
3. Remove the medium from the cultured cells, and remove the cells from the plate using trypsin-EDTA (0.05%; v/v). Neutralize the trypsin-EDTA by adding four volumes of complete DMEM medium, and then centrifuge the cells at 200 × *g* for 5 min. Resuspend 2×10^5 cells in 20 μL nucleofection solution containing 16.4 μL of SF Cell Line Solution and 3.6 μL of Supplement 1. Mix the cell suspension with 2 μL RNP complex (*see* **Note 37**).
4. Transfer the cells to a 16-well nucleocuvette strip and electroporate using a 4D-Nucleofector System with the manufacturer's program EH-100.
5. Immediately after nucleofection, add 100 μL of complete DMEM medium to each well and transfer the cells into a 24-well plate containing 0.5 mL of DMEM medium. Incubate at 37 °C with 5% CO_2 and full humidity.
6. At 48 h after electroporation, centrifuge cells at 200 × *g* for 10 min, discard supernatant, and extract the genomic DNA using genomic DNA extraction kit. Store DNA samples at −80 °C for further analysis.

3.4.4 AAV Donor Delivery for HDR-Mediated Genome Editing

1. Clone the AAV donor vector. Step-by-step cloning procedures to generate AAV donor templates have been described by our laboratory [50].
2. Package and purify the AAV donor vector. Step-by-step protocols for manufacturing AAV vectors have also been described by our laboratory [51].

3. Seed cells onto a 96-well plate at a density of 2×10^5 cells per well in serum-containing media with 200 ng/mL of nocodazole (*see* **Note 38**).
4. After 30 min, add AAV donor vector onto cells in the presence of 200 ng/mL of nocodazole (*see* **Notes 39** and **40**).
5. After 16 h, dissociate cells with trypsin and centrifuge at $400 \times g$ for 3 min.
6. Wash cells once with PBS and resuspend in 20 μL of Nucleofector Solution SF with 10 μL of RNP.
7. Immediately after nucleofection, add 100 μL of serum-containing medium to nucleofected wells and transfer cells into a fresh 96-well plate for further analysis.

3.5 Genome-Editing Quantification

In this section, we describe protocols for quantifying editing with the T7E1 endonuclease assay, DNA sequencing, and flow cytometry.

3.5.1 T7E1 Endonuclease Assay

1. Amplify the genome site targeted by the nuclease protein using nested PCR (*see* **Note 41**). First, carry out an external PCR reaction using 1 μg of genomic DNA, 5 μL of 10× PCR buffer, 0.4 μM each of the forward and reverse primers, 0.2 mM dNTP, 5% DMSO, and 2 U of high-fidelity DNA polymerase with water to 50 μL. Use the following cycling program: initial denaturation at 95 °C for 5 min; 20 cycles of 95 °C for 30 s, 55 °C for 30 s, and 72 °C for 90 s; and final extension at 72 °C for 5 min. Next, carry out an internal PCR reaction using 2 μL of the PCR product from the external PCR reaction as the template, 5 μL of 10× PCR buffer, 0.2 μM each of the forward and reverse primers, 0.2 mM dNTP, and 2 U of high-fidelity DNA polymerase with water to 50 μL. Use the following cycling program: initial denaturation at 95 °C for 5 min; 30 cycles of 95 °C for 30 s, 55 °C for 30 s, and 72 °C for 40 s; and final extension at 72 °C for 5 min (*see* **Note 38**). Store the PCR product at −20 °C for up to 6 months.
2. Verify amplification by resolving 5 μL of the PCR reaction on a 1% agarose gel as described in Subheading 3.1, **step 4** (*see* **Note 39**).
3. Generate mismatched duplex DNA for the T7E1 assay by denaturing and reannealing 30 μL of the PCR amplicon using the following cycle: 95 °C for 10 min; descend from 95 to 85 °C (at a rate of −2 °C/s); 1 min at 85 °C; 85 to 75 °C (−0.3 °C/s); 1 min at 75 °C; 75 to 65 °C (−0.3 °C/s); 1 min at 65 °C; 65 to 55 °C (−0.3 °C/s); 1 min at 55 °C; 55 to 45 °C (−0.3 °C/s); 1 min at 45 °C; 45 to 35 °C (−0.3 °C/s); 1 min at 35 °C; 35 to 25 °C (−0.3 °C/s); 1 min at 25 °C; hold at 4 °C (*see* **Note 42**).

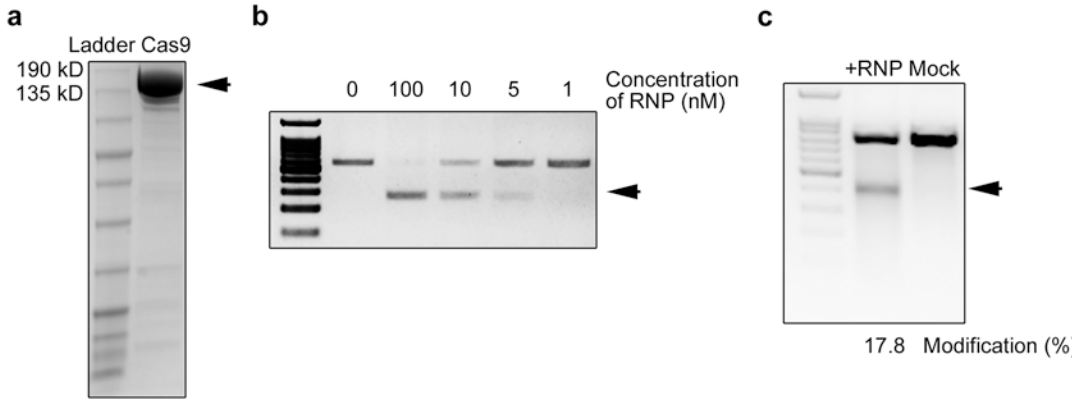

Fig. 3 Cas9-mediated modification of the mouse dystrophin gene in Hepa 1-6 cells. (**a**) SDS-PAGE of purified Cas9 protein. Arrowhead indicates predicated molecular weight of Cas9 protein. (**b**) Assessment of Cas9 RNP activity using in vitro cleavage. Arrow, cleavage product. (**c**) Frequency of indel formation in the mouse dystrophin gene in Hepa 1-6 cells following RNP delivery. (**b**, **c**) Arrowheads indicate expected cleavage product

4. Mix 10 μL of heteroduplex DNA with 5 U of T7E1 endonuclease and 1× cleavage buffer in 20 μL. Incubate the reaction at 37 °C for 30 min.
5. Resolve the T7E1 reaction product on a 1% agarose gel as described in Subheading 3.1, **step 4**.
6. Visualize the agarose gel using an agarose gel-imaging system and measure the intensity of each band. The percent gene modification can be determined by measuring the fraction of parental band cleaved at the anticipated location, as described [52]. An example of a T7E1 analysis is shown in Fig. 3.

3.5.2 DNA Sequence Analysis

1. Ligate the PCR products from Subheading 3.5.1, **step 1**, into TA cloning vector using a PCR TA cloning kit per the manufacturer's instructions. Incubate the reaction at RT for 30 min.
2. Transform the ligation reaction into chemically competent TOP10 cells as described in Subheading 3.1, **step 6**.
3. Pick individual clones from the LB agar plate, and culture them overnight in LB medium. Purify the plasmid by miniprep and submit it for Sanger sequencing using the primer recommended by the PCR TA cloning kit. Alternatively, submit the LB agar plate to a qualified service for direct colony sequencing. An example of Sanger sequencing results confirming genome editing is shown in Fig. 3.

3.5.3 Quantifying Genome Editing in Reporter Cells Using Flow Cytometry

1. Detach cells from Subheading 3.4.2, **step 5**. First, remove the culture medium and wash cells once with DPBS. Next, add 200 μL of trypsin-EDTA to each well, and incubate at 37 °C for 2 min.

2. Resuspend cells in flow cytometry buffer and gently mix by pipetting.
3. Use untreated reporter cells to draw a gate for flow cytometry. Adjust the parameters (i.e., FSC and SSC) according to instrument manual. Specifically, for EGFP reporter cells, use the FITC channel to analyze signal.
4. Perform the analysis on nuclease-treated cells, and quantify the percentage of fluorescent cells. Note that using our previously described EGFP-293 reporter cell line, approximately one-third of nuclease-induced indels restores EGFP fluorescence. An example of this analysis is shown in Fig. 3.

4 Notes

1. Ethidium bromide is potentially carcinogenic and should be handled and deposited of in accordance with institutional guidelines and regulations.
2. βME is hazardous if swallowed or inhaled.
3. If L-Arg is difficult to dissolve, adjust the pH to 7.4 while stirring the solution over gentle heat.
4. Both β-ME and protease inhibitor cocktail should be freshly prepared and added to lysis buffer immediately before use.
5. Ethanol is flammable and should be stored and handled under appropriate conditions.
6. To ensure efficient cell attachment, allow plates to dry for 2 h prior to use.
7. The HEK293 reporter cell line used in our protocol harbors a frame-shift-disabled EGFP gene containing a strategically placed TALEN target site. Following TALEN-induced DNA cleavage, approximately one-third of indels are expected to restore the EGFP reading frame and fluorescence.
8. We recommend optimizing the PCR conditions if nonspecific amplification occurs. Alternatively, nuclease-encoding gene(s) can be sub-cloned from a separate expression vector.
9. To ensure that agarose gel slices are completely dissolved in solubilization buffer, invert the tube every 5 min. If the solution becomes pink or red after the agarose gel slice is dissolved, add 3 M sodium acetate dropwise until the solution becomes yellow.
10. We recommend confirming the full sequence of each cloned nuclease-encoding gene(s). Mutations can dramatically affect the activity of a nuclease.

11. Isolating high-quality DNA template for RNA transcription is essential for in vitro transcription.
12. Short RNA transcripts require a 16-h incubation period for maximum transcription.
13. Resuspending the pellet in nuclease-free water is critical for preventing sgRNA degradation.
14. In our experience, sgRNA concentrations >15 μg/μL are optimal for genome editing.
15. When expressing ZFN proteins, supplement the medium with 90 μM $ZnCl_2$.
16. Addition of 0.2% glucose and 200 mM NaCl to bacterial culture media can prevent leaky expression and inhibit nuclease activity, respectively. Both factors can affect cell growth.
17. Protein yield and purity are dependent on the induction conditions. We recommend monitoring cultures every 30 min until an OD_{600} of 0.8 is reached.
18. Cas9 protein yield can be improved by culturing bacteria at temperatures less than 22 °C after induction.
19. Cell pellets can be stored for up to 1 week at −20 °C or 1 month at −80 °C with no loss of nuclease activity.
20. When purifying ZFN proteins, add 90 μM $ZnCl_2$ to the lysis buffer.
21. Insufficient resuspension of the cell pellet can negatively affect cell lysis by sonication.
22. Prevent overheating by sonicating the cells on ice. Multiple sonication cycles may be necessary to completely lyse cells.
23. If the supernatant goes through the filter too slowly, consider a second centrifugation to remove remaining cell debris in the supernatant.
24. For ZFN proteins only, add 50 μL of 1 M L-Arg to each fraction (final L-Arg concentration: 100 mM) immediately after elution. Failure to add L-Arg can lead to ZFN protein precipitation.
25. To ensure maximum dosage into cells, ZFN and TALEN proteins should be concentrated to ~40 μM and Cas9 protein to ~90 μM (15 μg/μL).
26. Avoid repeating freeze-thawing to prevent damaging proteins.
27. For ZFNs, supplement the reactions with 90 μM $ZnCl_2$ and 100 mM L-Arg.
28. Prepare RNP complex by incubating Cas9 protein and sgRNA at room temperature for 10 min.

29. Activation of $CD4^+$ T cells is required to achieve maximum ZFN-mediated gene modification rates. Proper cell-to-bead ratio is critical for cell activation.
30. Activate T cells for no more than 72 h. Prolonged activation can decrease the efficiency of genome editing.
31. Maximum modification is observed using between 0.5 and 4 μM ZFN proteins. Depending on the purity of ZFN proteins, low levels of cytotoxicity could be observed at high protein concentrations.
32. Lack of L-Arg will dramatically decrease genome-editing efficiency.
33. Transient incubation at 30 °C is necessary for ZFN proteins to achieve optimal rates of gene editing.
34. The molar ratio of Npys-modified CPP to TALEN protein must be between 8-to-1 and 15-to-1 for efficient cellular internalization and genome-editing activity.
35. Depending on the purity of the TALEN proteins and the quality of the peptide, precipitation may be observed after the conjugation reaction. Precipitates can be removed by centrifuging the reaction solution at 5000 × *g* for 2 min.
36. In contrast to ZFN proteins, TALEN proteins should be incubated with cells for ~2 h for efficient internalization.
37. The total volume of the RNP should not exceed 2 μL since a protein solution that exceeds 10% of the nucleofection volume (20 μL) can reduce transfection efficiency.
38. Nocodazole enhances HDR-mediated genome editing by arresting cells at G2/M phase.
39. The transduction efficiency of AAV can vary depending on the target cell type and the AAV serotype being used. It may be necessary to screen for the optimal AAV variant.
40. High doses of AAV (multicity of infection >100,000) are necessary for efficient transduction in vitro.
41. Nested PCR was designed to yield ~1 kb and ~300–500 bp amplicons for the external and internal reactions, respectively.
42. The absence of PCR side products is critical for preventing off-target heteroduplexation of DNA.

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