

CRISPR-Cas9-mediated genome editing in *Caenorhabditis elegans* using in-house purified Cas9 protein and sgRNAs

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Abstract

CRISPR-Cas9-mediated genome editing has emerged as a powerful tool for research in *Caenorhabditis elegans* (*C. elegans*). This protocol describes a comprehensive workflow for producing CRISPR-Cas9 RNPs using in-house purified Cas9 protein and in vitro-transcribed single-guide RNAs (sgRNAs), followed by microinjection into the *C. elegans* germline to generate genome-edited animals. The protocol covers Cas9 protein expression and purification, sgRNA design, synthesis and purification, repair template preparation, RNP assembly, germline microinjection, co-CRISPR screening, and genotyping of edited progeny. The complete procedure, from Cas9 protein purification to the isolation of homozygous mutant animals, can typically be completed within 2-3 weeks. The protocol enables the efficient generation of targeted knockouts, knock-ins, and point mutations while taking advantage of the immediate activity and reduced off-target effects associated with RNP delivery. It provides a reliable and cost-effective protocol for genome engineering and functional genomic studies in *C. elegans*.

Keywords

Caenorhabditis elegans (*C. elegans*), CRISPR-Cas9, Purification of Cas9 protein, sgRNA synthesis and purification, Ribonucleoprotein (RNP), Microinjection, Gene editing

Introduction

Caenorhabditis elegans (*C. elegans*) is a widely used model organism for studies of development, neuroscience, aging, and metabolism because of its well-characterized genetics, short life cycle, and optical transparency (Brenner 1974; Consortium 1998). CRISPR-Cas9-mediated genome editing has become a standard approach for generating targeted genetic modifications in *C. elegans*, including gene knockouts, precise point mutations, genomic deletions, and fluorescent protein knock-ins (Arribere *et al.* 2014; Dickinson and Goldstein 2016; Dickinson *et al.* 2013; Friedland *et al.* 2013; Paix *et al.* 2017; Wan *et al.* 2018). Microinjection into the syncytial germline enables efficient delivery of genome-editing reagents and the rapid recovery of heritable genome-edited animals.

Several strategies have been developed to deliver CRISPR-Cas9 components in *C. elegans*, including plasmid DNA, Cas9 mRNA, and Cas9 ribonucleoprotein (RNP) complexes (Chiu *et al.* 2013; Kim *et al.* 2014a; Schwartz and Sternberg 2014; Schwartz *et al.* 2021; Waaijers and Boxem 2014). Among these, RNP-mediated delivery has become widely adopted because the preassembled Cas9-sgRNA complexes are immediately active after injection, eliminating the need for endogenous transcription and translation (Paix *et al.* 2015). The transient activity of RNPs also minimizes prolonged Cas9 expression, which may reduce off-target effects and germline toxicity while providing high editing efficiency for a broad range of genome modifications (Kim *et al.* 2014b).

Here, we present a detailed and cost-effective protocol for CRISPR-Cas9 genome editing in *C. elegans* using in-house purified Cas9 protein and in vitro-transcribed sgRNAs assembled into RNP complexes. The protocol describes the complete workflow, including Cas9 protein expression and purification, sgRNA design, synthesis and quality control, repair template preparation, RNP assembly, germline microinjection, co-CRISPR screening, and genotyping of edited progeny. By providing detailed experimental procedures and practical recommendations, this protocol enables laboratories with standard molecular biology and microinjection equipment to implement efficient and economical genome editing (Table 3).

MATERIALS

1. Chemicals

HEPES (Sigma-Aldrich, V900477), HCl (Guangzhou Chemical Reagent Factory, GS0006AR), NaCl (Sangon, A100241), Imidazole (Sangon, A600277), DTT (Sigma-Aldrich, V900830), PMSF (Beyotime, ST2573), Pepstatin A (APExBIO, A2571), Glycerol (Sangon, A600232), Absolute ethanol (Guangzhou Chemical Reagent Factory, HB15), NaOH (Sigma-Aldrich, 221465), RNase-free H₂O (Takara, 9012), TRIzol (Thermo Fisher, 15596026CN), Trichloromethane (Guangzhou Chemical Reagent Factory, GD10), RNA Loading Dye (NEB, B0363S), Isopropyl alcohol (Guangzhou Chemical Reagent Factory, HC16), Agarose (Biowest, RS0481), NTP Mix (Beyotime, D7387), Tris-Base (Biofroxx, 1115KG001), Potassium dihydrogen phosphate (Sangon, A100781), Potassium phosphate, dibasic, trihydrate (Sangon, A501728), Sodium phosphate dibasic dodecahydrate (Guangzhou Chemical Reagent Factory, 10039-32-4), Ampicillin (Sangon, A610028), Chloramphenicol (Sangon, A600118), Tryptone (OXOID, LP0042B), Yeast extract (OXOID, LP0021B), IPTG (MDBio, I001), Triton X-100 (Sigma-Aldrich, T8787).

Note: All toxic and hazardous chemicals must be handled in a certified fume cupboard while wearing appropriate personal protective equipment, including a laboratory coat, safety goggles, and nitrile gloves. TRIzol contains phenol and guanidine isothiocyanate and should be handled with care to avoid inhalation and skin contact. All TRIzol-containing waste must be disposed of in accordance with institutional hazardous waste disposal guidelines. Chloroform (trichloromethane) is a suspected carcinogen and must be handled exclusively in a fume cupboard, with waste segregated and disposed of as hazardous chemical waste. Phenylmethylsulfonyl fluoride (PMSF) is a potent cholinesterase inhibitor; stock solutions should be prepared fresh immediately before use and handled with extreme caution to minimize exposure.

2. Enzymes

T7 RNA polymerase (Vazyme, DD4101-PC-00), Recombinant RNase Inhibitor (Thermo Fisher, EO0381), DNase I (Thermo Fisher, EN0525)

3. Instruments and consumables

ÄKTA pure (Cytiva), UV spectrophotometer (Thermo Fisher, NanoDrop One), Ultrasonic Disruptor (SCIENTZ, JY92-IIN), HisTrapTM HP prepacked column (Cytiva, 17524801), SuperdexTM 200 Increase 10/300 GL (Cytiva, 28990944), Dual-Stage Glass Micropipette Puller (Narishige, PC-100), Electric Microsurgery Injection System (NARISHIGE, IM 400), Acrodisc 25 mm Syringe Filter with 0.45 µm Supor Membrane (PALL, 4614), Acrodisc 25 mm Syringe Filter with 0.22 µm Supor Membrane (PALL, 4612), Amicon® Ultra-15 Centrifugal Filter Unit, 100 kDa MWCO (UFC9100), 24*50 mm Microscope cover glass (CITOTEST, 10212450C), 22*22 mm Microscope cover glass (CITOTEST, 10212222C), Standard Glass Capillaries (WPI, 1B100F-4), Microloader micro-volume loading needle (Calibre Scientific, 2229001206)

4. Buffers and culture medium

Binding buffer: 20 mM HEPES (pH 7.5), 500 mM NaCl, 30 mM imidazole, 1 mM DTT;

Lysis buffer: 20 mM HEPES (pH 7.5), 500 mM NaCl, 30 mM imidazole, 1 mM DTT, supplement with 0.4 mM PMSF and 0.5 ng/mL Pepstatin A immediately prior to use;

Wash buffer: 20 mM HEPES (pH 7.5), 500 mM NaCl, 30 mM imidazole, 1 mM DTT;

Elution buffer: 20 mM HEPES (pH 7.5), 250 mM NaCl, 300 mM imidazole, 10% (v/v) Glycerol;

SEC buffer: 20 mM HEPES (pH 7.5), 150 mM NaCl, 1 mM DTT;

Cas9 protein storage buffer: 20 mM HEPES (pH 7.5), 150 mM NaCl;

E4 buffer: 1.25 M NaCl, 100 mM Tris-HCl (pH 8.5);

Note: All buffers must be filtered through a 0.22 µm membrane and pre-cooled to 4°C prior to use.

10x Phosphate buffer: 23.1 g KH₂PO₄, 164.32 g K₂HPO₄·3H₂O, dilute to 1L and autoclave;

TB medium: 24 g Yeast extract, 12 g Tryptone, 4 mL Glycerol, dilute to 900mL, and autoclave. After autoclaving, add 100 mL of 10× phosphate buffer to 900 mL of TB medium;

M9 buffer: 3 g KH_2PO_4 , 15.14 g $\text{Na}_2\text{HPO}_4 \cdot 12\text{H}_2\text{O}$, 5 g NaCl, 0.1204 g MgSO_4 , dilute to 1L and autoclave. After autoclaving, add 10 μL of Triton X-100;

EXPERIMENTAL PROCEDURES

As shown in Fig. 1, this is the overall workflow of the protocol.

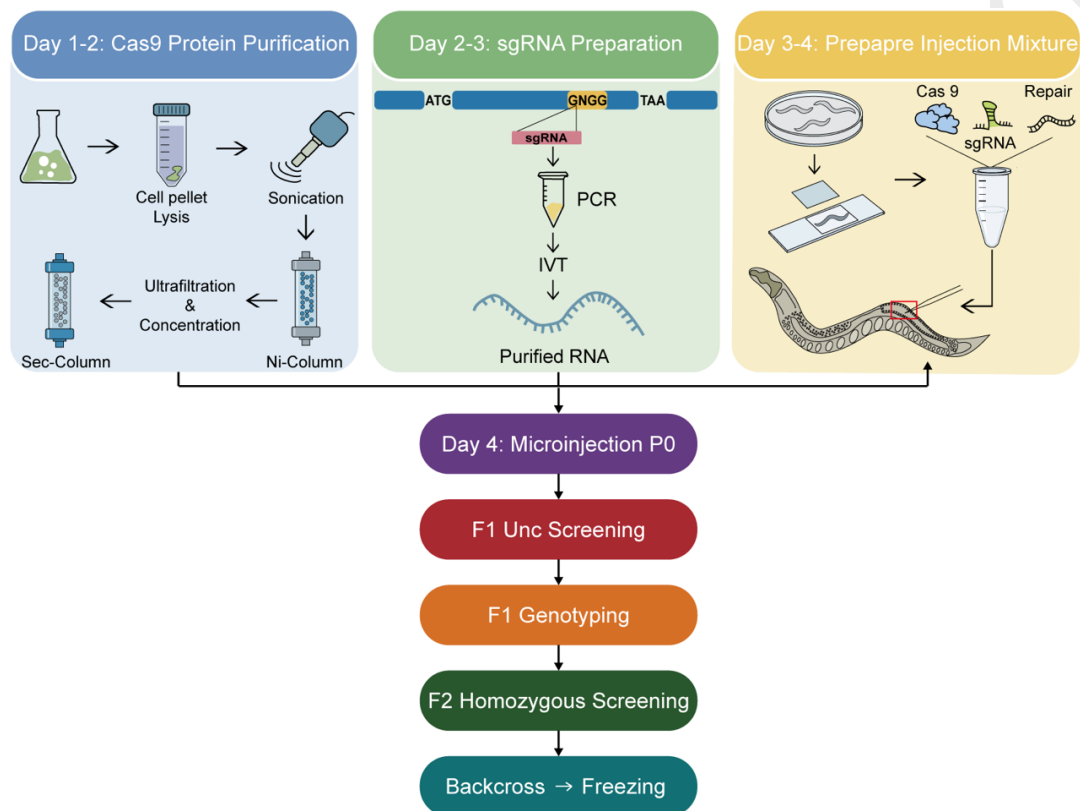


Fig. 1. Schematic diagram of the complete RNP mediated-CRISPR-Cas9 gene editing workflow for *C. elegans* in this protocol. This workflow illustrates the standard procedure from sample preparation to the generation of homozygous mutant lines.

1. Expression and in-house purification of Cas9 protein

(1) Culture preparation and expression induction

Transform the pET-21a_3xNLS_SpCas9 plasmid (Addgene plasmid #114365) into *E. coli* Rosetta (DE3) cells. Pick three single colonies from the transformation plate and inoculate each

into 100 mL of Terrific Broth (TB) medium supplemented with 100 µg/mL ampicillin and 34 µg/mL Chloramphenicol. Incubate the cultures at 37 °C with shaking at 220 rpm for 16 hours. To scale up the culture, inoculate 10 mL of the overnight culture into each 1 L of fresh TB medium containing 100 µg/mL ampicillin and 34 µg/mL Chloramphenicol. Grow the cultures at 37°C with shaking at 220 rpm until the OD₆₀₀ reaches approximately 1.0. Reduce the incubation temperature to 16°C while maintaining shaking at 220 rpm, then induce protein expression by adding IPTG to a final concentration of 1 mM. Continue incubation for an additional 16 hours.

(2) Cell harvesting

Transfer the induced cultures into sterile centrifuge bottles and harvest the cells by centrifugation at $2,535 \times g$ for 15 minutes at 4°C. The resulting cell pellets may be stored at -80°C until protein purification. A typical yield is approximately 5-7 g of wet cell pellet per liter of TB medium.

(3) Ultrasonic lysis and lysate preparation

Thaw the frozen cell pellets on ice. Resuspend the cells in binding buffer at a ratio of 35 mL per 7 g of cell pellet, and add PMSF and pepstatin A to final concentrations of 0.4 mM and 0.5 ng/mL, respectively. Keep the cell suspension on ice throughout the procedure.

Lyse the cells using an ultrasonic homogenizer equipped with a No. 6 probe, ensuring that the output power does not exceed 400 W. Place the centrifuge tube in an ice-water bath and position the probe slightly below the midpoint of the suspension. Sonicate the sample using 3-second pulses followed by 7-second intervals for a total sonication time of 3 minutes per cycle. Perform five cycles of sonication, allowing the sample to cool on ice for 5 minutes between cycles to prevent overheating.

Clarify the lysate by centrifugation at $19,600 \times g$ for 20 minutes at 4°C. Collect the supernatant and filter it through a 0.45 µm membrane filter. Keep the filtered lysate on ice until protein purification.

(4) Nickel affinity chromatography

Nickel affinity purification was performed at 4°C using a HisTrap HP prepacked column (Cytiva, catalog no. 17524801; 5 mL bed volume). The column back pressure was maintained below 0.5 MPa (recommended flow rate: 0.5-2.0 mL/ minute). Protein elution was monitored by measuring UV absorbance at 280 nm.

(A) System Preparation:

Rinse the chromatography system with ddH₂O and prime the fluid lines using Pump A1. Set the column pressure limit and flow rate according to the manufacturer's recommendations.

(B) Column Equilibration:

Wash the column with at least 5 column volumes (CV) of ddH₂O to remove the storage solution (20% ethanol). Equilibrate the column with 5 CV of Binding Buffer.

(C) Protein Purification:

Load the filtered lysate onto the equilibrated column. Wash the column with 10-20 CV of Wash Buffer to remove nonspecifically bound proteins. Elute Cas9 using Elution Buffer and collect fractions (500 µL per tube) corresponding to the UV absorbance peaks at 280 nm.

(D) Protein Analysis:

Analyze the expression level and purity of the eluted fractions by SDS-PAGE followed by Coomassie Brilliant Blue staining. Pool the fractions containing the purest Cas9 protein for subsequent purification.

(5) Ultrafiltration and Concentration

Concentrate the pooled Cas9 protein fractions using an Amicon® Ultra-15 centrifugal filter unit (100 kDa molecular weight cutoff (MWCO)). Perform all procedures on ice to preserve protein stability.

(A) Pre-treatment of the Ultrafiltration Unit:

Rinse the filter unit three times with deionized water and once with elution buffer. During the final rinse, centrifuge the unit at $1,356 \times g$ for 6-8 minutes at 4°C (ensuring proper rotor balance) to thoroughly remove residual contaminants and condition the membrane.

(B) Protein Concentration:

Discard the filtrate from the collection tube and load the pooled protein sample into the filter reservoir. Centrifuge at $1,356 \times g$ for 6-8 minutes at 4°C . After each centrifugation, gently pipette the retentate to ensure a homogeneous solution before continuing the concentration process. Repeat the centrifugation until the sample volume is reduced to approximately 500-800 μL . Collect the final filtrate from the collection tube and retain it for subsequent analysis.

(C) Determination of protein concentration:

Measure the protein concentration using a NanoDrop spectrophotometer by recording the absorbance at 280 nm (A_{280}), using Elution Buffer as the blank. Determine the protein concentrations of both the concentrated retentate and the filtrate to evaluate protein recovery and concentration efficiency.

(6) Size-exclusion chromatography (SEC)

To obtain highly purified 3 $\times$ NLS-Cas9 protein, perform size-exclusion chromatography (SEC) to further separate Cas9 from residual contaminants based on differences in molecular size.

Column and running conditions:

Use a SuperdexTM 200 Increase 10/300 GL column (Cytiva, catalog no. 28990944; bed volume approximately 24 mL). Maintain the column back pressure below 3.0 MPa (recommended flow rate: 0.2-0.5 mL/minute).

(A) System preparation:

Rinse the chromatography system with ddH₂O and prime the fluid lines using Pump A1. Set the column pressure limit and flow rate according to the manufacturer's recommendations.

(B) Column equilibration:

Install the column and wash it with 2 CV of ddH₂O, followed by equilibration with at least 2 CV of SEC Buffer.

(C) Sample loading and elution:

Load 400-500 μL of the protein sample using a 1 mL syringe. Configure the flow path to include the injection loop and initiate the chromatographic run. Collect eluted fractions (500 μL per tube) corresponding to the detected protein peaks.

Note: Lower flow rates generally provide improved resolution and separation during SEC.

(D) Protein analysis:

Analyze the collected fractions by SDS-PAGE to confirm the presence and purity of the target Cas9 protein.

(E) Protein storage:

The purified Cas9 protein was concentrated to 10 mg/mL using an Amicon® Ultra-15 centrifugal filter unit (100 kDa MWCO), buffer-exchanged into the storage buffer as described above, aliquoted, and stored at -80°C .

(7) Cas9 protein purity assessment

Assess the purity of the purified Cas9 protein by SDS-PAGE followed by Coomassie Brilliant Blue staining. The purified Cas9 protein should appear as a single predominant band at the expected molecular weight of approximately 166.1 kDa, with a purity of $>90\%$ (Fig. 2). For downstream microinjection applications, Cas9 protein with a purity of $>90\%$ is recommended.

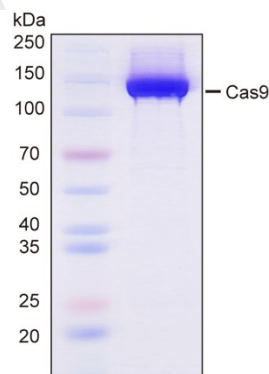


Fig. 2. SDS-PAGE analysis of purified Cas9 protein followed by Coomassie Brilliant Blue staining to assess protein purity.

2. sgRNA design, synthesis, and purification

(1) sgRNA design

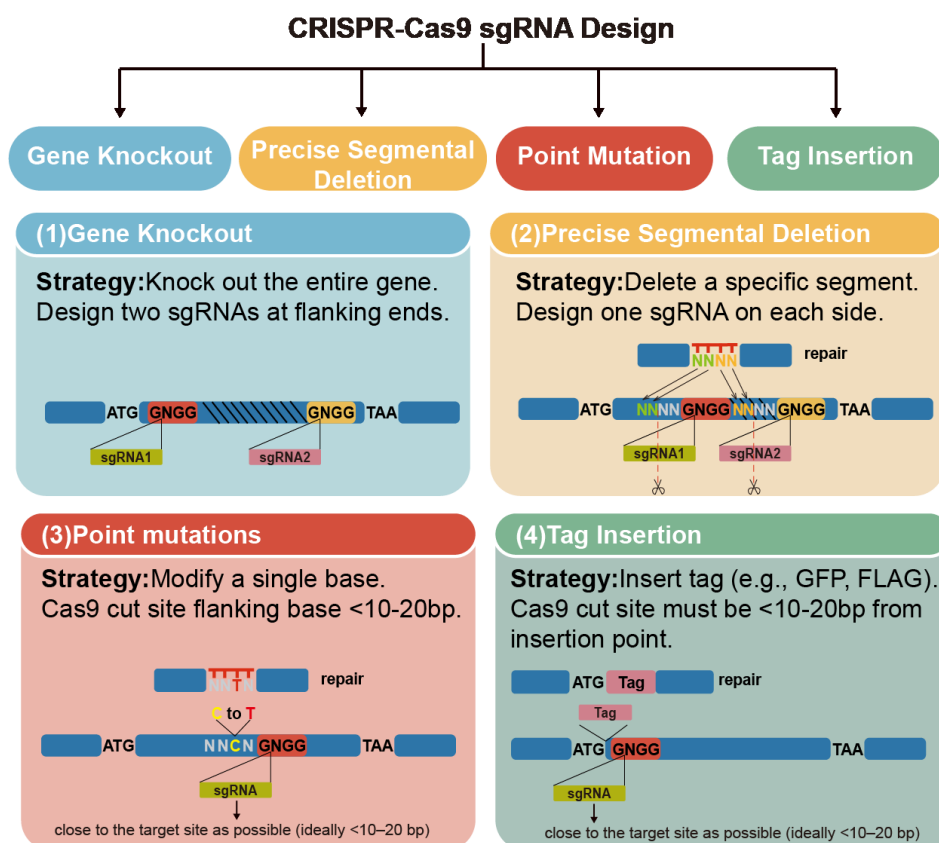


Fig. 3. Schematic illustration of sgRNA target site selection for four CRISPR genome editing strategies

(A) Gene Knockout:

To disrupt the target gene, design two sgRNAs targeting the coding regions near the start and stop codons, respectively. Use SnapGene to identify candidate sgRNAs containing the GNGG/NGG protospacer adjacent motif (PAM), with preference given to sgRNAs containing the GNGG PAM sequence. Evaluate the predicted editing efficiency of selected sgRNAs using online tools such as CRISPOR (<https://crispor.gi.ucsc.edu>) (Concordet and Haeussler 2018).

Note: Ensure that the 20 bp sgRNA guide sequence upstream of the NGG PAM has a GC content between 40% and 60%. Avoid sgRNA sequences containing poly-nucleotide stretches (e.g., AAAA or TTTT). If the first nucleotide of the sgRNA sequence is not a G, manually add a G nucleotide to satisfy the transcriptional requirement of the T7 promoter. Whenever possible, prioritize designing sgRNAs on the sense strand, as sgRNAs targeting the antisense strand often exhibit lower efficiency.

(B) Precise Segmental Deletion:

Design two sgRNAs targeting the 5' and 3' boundaries of the genomic segment to be deleted, respectively.

(C) Point mutations:

Design one sgRNA targeting the coding region near the desired mutation site.

Note: The sgRNA cleavage site should be positioned as close as possible to the desired mutation site (ideally within 10-20 bp). Greater distances substantially increase the risk of proximal repairing, in which the sgRNA recognition sequence is disrupted by Cas9 cleavage and subsequently repaired using only part of the repair template, without incorporating the desired point mutation.

(D) Tag Insertion:

To generate fusion proteins, design one or two sgRNAs near the start codon or stop codon. The distance between the Cas9 cleavage site and the insertion site is critical; ideally, the cleavage site should be located within 0-30 bp of the intended insertion site.

(2) sgRNA amplification and *in vitro* transcription (IVT)

(A) Template Preparation:

Using U6>sgRNA(F+E) (Addgene, 59986) as the template, amplify the DNA fragment containing the T7 promoter, the target-specific sgRNA sequence, and the sgRNA(F+E) tracrRNA scaffold by PCR(Chen *et al.* 2013). Purify the amplified fragment using gel extraction.

The sgRNA(F+E) template sequence is as follows:

5'-

GNNNNNNNNNNNNNNNNNNNGTTTAAGAGCTATGCTGGAAACAGCATAGCAAG
TTTAAATAAGGCTAGTCCGTTATCAACTTGAAAAAGTGGCACCGAGTCGGTGCTTT
TTTT-3'

Use the following primers to amplify U6>sgRNA(F+E). Replace 20N or G20N with the sequence of the sgRNA of interest.

Forward primer: 5' TTCTAATACGACTCACTATA (G)

NNNNNNNNNNNNNNNNNNNNNGTTTAAGAGCTATGCTGG 3'

Reverse primer: 5' AAAAAAAGCACCGACTCGGTGCCACTTTTTCAAG 3'

(B) *In vitro* transcription:

As shown in Table 1, prepare a 20 μ L *in vitro* transcription reaction mixture in a 0.2 mL RNase-free tube on ice.

Table 1. *In vitro* transcription system.

Components (Stock concentration)	Final concentration	Mass / Volume
10X T7 Transcription Buffer	1 X	2 μ L
NTP Mix (25 mM each)	1 mM	0.8 μ L
T7 RNA Polymerase (50 U/ μ L)	2.5 U/ μ L	1 μ L
Recombinant RNase Inhibitor (40 U/ μ L)	1 U/ μ L	0.5 μ L
DNA template	14 ng/ μ L	280 ng
RNase-free H ₂ O	To 20 μ L	To 20 μ L

Incubate the reaction mixture in a thermocycler at 37°C for 4-12 hours. Following transcription, assess the specificity and yield of the synthesized RNA by 2% agarose gel electrophoresis.

(C) DNA template digestion:

Add 10 μ L of 10 $\times$ DNase I buffer, 2 μ L of DNase I, and 68 μ L of RNase-free H₂O directly to the IVT reaction mixture. Mix gently by inversion and incubate at 37°C for 30 minutes to digest and remove the DNA template.

(3) sgRNA extraction and purification

Note: From this step onward, strictly maintain RNase-free conditions throughout the procedure.

(A) TRIzol treatment:

Transfer the DNase I-treated reaction mixture to a 1.5 mL RNase-free tube. Add 500 μ L of TRIzol and mix thoroughly. Add 100 μ L of chloroform, shake vigorously for 30 seconds, and incubate at room temperature for 5 minutes. Centrifuge at 18,000 $\times g$ for 10 minutes at 4°C.

(B) RNA precipitation:

Transfer the aqueous phase to a new RNase-free tube and add 2.5 volumes of pre-chilled absolute ethanol. Mix gently by inversion and incubate at -80°C for 2 hours to overnight to precipitate the RNA.

(C) Washing:

Centrifuge the sample at $18,000 \times g$ for 10 minutes at 4°C and carefully remove the supernatant. Wash the RNA pellet with 1 mL of 75% ethanol, centrifuge at $6,000 \times g$ for 5 minutes at 4°C , and remove the supernatant. Air-dry the pellet for 10 minutes.

(D) Resuspension and storage:

Dissolve the RNA pellet in 12 μL of RNase-free H_2O . Measure RNA concentration and purity using a spectrophotometer. Evaluate sgRNA integrity by electrophoresis (mix 1 μL of RNA with 1 μL of $2\times$ RNA loading buffer). Store the purified sgRNA at -80°C until further use.

(4) sgRNA quality assessment

(A) Expected yield:

IVT using a 20 μL reaction volume typically produces 6-20 μg of sgRNA. A yield below 6 μg may indicate issues with the DNA template quality, T7 RNA polymerase activity, transcription efficiency, or RNA stability.

(B) Purity assessment:

RNA purity was evaluated by measuring the A260/A280 and A260/A230 ratios using a NanoDrop spectrophotometer. High-quality purified RNA should exhibit an A260/A280 ratio of 2.0-2.1 and an A260/A230 ratio of >2.0 .

(C) Integrity assessment:

Analyze 1 μL of purified sgRNA by 2% agarose gel electrophoresis alongside an RNA size marker. A successful preparation should appear as a single, sharp band at the expected size (~ 100 nt). Smearing or the presence of multiple bands may indicate RNA degradation, incomplete transcription, or nonspecific transcription products. Only sgRNA preparations that meet *all* three

quality criteria (yield >6 µg, A260/A280 ratio is 2.0-2.1, and a single sharp band on gel electrophoresis) should be used for microinjection.

3. Construction and purification of repair templates

(1) Construction of repair templates

(A) For knockout of the target gene:

No repair template is required.

(B) For deletion of a specific fragment of the target gene:

A 100-500 bp sequence immediately upstream of the target region is selected as the left homology arm, and a 100-500 bp sequence immediately downstream of the target region is selected as the right homology arm. The left and right homology arms are amplified by PCR and cloned into a linearized pGEM-7zf(+) vector (Promega, TB069) digested with EcoRI and HindIII to generate the recombinant plasmid. After sequence verification, the fragment containing the homology arms (i.e., the repair template) is amplified by PCR and purified by gel extraction.

(C) For site-specific base substitutions:

Left and right homology arms (~100 bp each) flanking the sgRNA target site are designed to introduce the desired nucleotide substitutions. Following construction and sequence confirmation of the recombinant plasmid, the repair template is amplified by PCR and purified as described above.

(D) For knock-in of a tag coding sequence into the target gene:

Left and right homology arms (~500 bp each) flanking the tag-coding sequence are designed on both sides of the sgRNA target site. Following construction and sequence confirmation of the recombinant plasmid, the repair template is amplified by PCR and purified as described above.

Note: For all repair templates, introduce silent mutations into the PAM sequence or the protospacer sequence adjacent to the PAM site (typically 3-4 nucleotide substitutions near the PAM region) to prevent Cas9-mediated re-cleavage after successful genome editing. For site-

specific base substitutions, introduce additional silent mutations that either create a novel restriction enzyme recognition site or disrupt an existing restriction site to facilitate genotyping.

(2) Purification of the repair template

The gel-extracted repair template is further purified using E4 buffer. Add 900 μ L of pre-chilled E4 buffer to the microcentrifuge tube containing the repair template solution, followed by 660 μ L of isopropanol. Mix gently by inversion and centrifuge at $18,000 \times g$ for 30 minutes at 4°C. Carefully remove the supernatant and add 1 mL of 70% ethanol to wash the pellet. Mix gently by inversion and centrifuge at $18,000 \times g$ for 5 minutes at 4°C. Remove the supernatant and leave the tube open at room temperature for 10 minutes to allow residual organic solvents to evaporate (do not extend the drying time beyond 10 minutes). Resuspend the pellet in 6-10 μ L of RNase-free H₂O. Use 2 μ L of the purified repair template for nucleic acid concentration measurement and store the remaining sample at -20°C until further use.

Note: All procedures after the final resuspension step should be performed under RNase-free conditions.

4. Microinjection

(1) Preparation of *C. elegans* for injection

One day before microinjection, select 20-50 healthy L4-stage larvae and transfer them to fresh NGM plates for further cultivation.

Note: If worms are obtained from an egg preparation, microinjection should preferably be performed in the late afternoon or evening to allow sufficient development of the germline. Selecting L4-stage larvae one day before injection is an effective strategy to ensure optimal germline development and size for microinjection.

(2) Preparation of Agarose Pads

Prepare a 2.5% agarose solution by dissolving 1.25 g of agarose in 50 mL of ddH₂O and heating until the agarose is completely dissolved. Place a 50 μ L drop of molten agarose onto the center

of a 24 × 50 mm coverslip. Immediately place a second coverslip perpendicular to the first one over the agarose drop to spread the agarose into a thin and uniform pad. Allow the agarose to solidify for 2 minutes, then carefully remove the upper coverslip, leaving a circular agarose pad approximately 2 cm in diameter. Air-dry the agarose pads at room temperature for at least 6 hours or overnight before use.

(3) Assemble the microinjection mixture

Prepare the injection mixture on ice. In a 0.2 mL RNase-free tube, combine the components listed in Table 2: 1.8 µg of total sgRNA (comprising equal amounts of the *unc-58* co-injection marker sgRNA and target gene-specific sgRNA(s)), 5 µg of purified 3×NLS-Cas9 protein, and RNase-free H₂O to the desired final volume.

Note: The total sgRNA amount should be maintained at 1.8 µg, including both the *unc-58* co-injection marker sgRNA and all target-specific sgRNAs. For experiments using a single target sgRNA, use 0.9 µg of *unc-58* sgRNA and 0.9 µg of target sgRNA. For experiments using two target sgRNAs, use 0.6 µg each of the *unc-58* sgRNA and the two target sgRNAs.

Incubate the mixture at 37°C for 15 minutes to allow formation of the Cas9-sgRNA RNP complexes. Subsequently, add 240 ng of the *unc-58* repair template (AF-JA-76) and, when applicable, 750 ng of the gene-specific repair template.

Note: For double-stranded DNA repair templates, perform a denaturation-reannealing step prior to addition to the injection mixture to improve genome-editing efficiency. Use the following thermocycler program: 95°C for 2 minutes; 85°C, 75°C, and 65°C for 10 seconds each; 55°C for 1 minute; and 45°C, 35°C, and 25°C for 10 seconds each. Ramp down at 1 °C/ second at every step. Immediately place the repair template on ice after completion of the program (Ghanta and Mello 2020; Lu *et al.* 2025; Zhao *et al.* 2024).

Table 2. CRISPR/Cas9 microinjection mixture (10 μ L).

Components	Final concentration	Mass / Volume
sgRNA (<i>unc-58</i>)	0.06 μ g/ μ L	Multiple sgRNAs targeting the gene of interest were pooled in equal amounts to a total of 1.8 μ g
sgRNA1 (Gene of interest)	0.06 μ g/ μ L	
sgRNA2 (Gene of interest)	0.06 μ g/ μ L	
Cas9 protein	0.5 μ g/ μ L	5 μ g
RNase-free H ₂ O		10 μ L- the volumes of all additional components, including Cas9, sgRNA, and repair template

Following incubation at 37°C for 15 minutes, the mixture was subsequently supplemented with the following components:

AF-JA-76	0.024 μ g/ μ L	240 ng
Repair template (if applicable, otherwise skip this step).	0.075 μ g/ μ L	750 ng

Centrifuge the complete injection mixture at $18,000 \times g$ for 2 minutes at 4°C to remove any aggregates that may clog the injection needle. Carefully transfer 7.5 μ L of the supernatant into a pre-chilled RNase-free tube and keep on ice until use.

Note:

The nucleotide sequence of sgRNA for *unc-58* is:

TCCACGCACATGGTCACTA

The nucleotide sequence of **AF-JA-76**(Arribere *et al.* 2014) is as follows:

5'-ATTTTGTGGTATAAAATAGCCGAGTTAGGAAACAAATTTTCTTTCAGG

TTTCTCAGTAGTGACCATGTGCGTGGATCTTGCGTCCACACATCTCAAGGCGTACT

T-3'

(4) Preparation of microinjection needles

Mount a standard glass capillary onto a dual-stage glass micropipette puller. Operate the puller according to the manufacturer's instructions to generate fine-tipped microinjection needles. Load 2.5 μ L of the chilled microinjection mixture into the needle. Hold the needle vertically with the tip facing downward to allow the solution to fill the tip by gravity, while avoiding the

introduction of air bubbles. Mount the loaded needle onto the microinjector connected to the injection arm and proceed with microinjection.

(5) Microinjection into the *C. elegans* germline

Transfer adult hermaphrodites to an NGM plate without OP50 and allow them to crawl briefly to remove bacteria attached to the body surface. Transfer one to six animals onto an agarose pad containing a drop of injection oil and immobilize them. Place the agarose pad onto the microscope stage. Under a 40× objective, adjust the position of the injection needle until the needle tip and the central region of the gonad are in the same focal plane. Using the fine-focus control, gently insert the needle into the gonad, deliver the injection mixture, and carefully withdraw the needle. Raise the injection arm and repeat the procedure for the remaining animals. After injection, remove the agarose pad and recover the injected animals by transferring them to fresh NGM plates containing 20 µL of M9 buffer.

(6) *C. elegans* cultivation, screening, and genotyping

After injecting approximately 10 P0 animals, distribute the injected worms evenly among five fresh NGM plates (two animals per plate), label the plates, and incubate them at 20°C. For knockout experiments, after 3 days, identify 24-48 F1 progeny exhibiting an uncoordinated (Unc) phenotype, indicating successful *unc-58* editing. Transfer each selected F1 animal individually to a fresh NGM plate. For knock-in experiments, select 96 F1 progeny displaying the Unc phenotype and transfer them individually to fresh plates.

Incubate the F1 plates for an additional 2-3 days until F2 progeny begin to hatch, then perform genotyping of each F1 animal. The principles underlying primer design for genotyping are illustrated in Figure 4.

Note: To reduce the likelihood of false-positive results, include a mixture of adult worms, larvae, and embryos whenever possible during genotyping. At this stage, plates typically contain abundant embryos; therefore, genotyping can generally be initiated 2-3 days after selecting Unc animals.

For plates containing F1 animals confirmed to be heterozygous, select 12 F2 animals with normal movement and transfer each animal individually to a fresh culture plate. Continue incubation for 3 days until F3 progeny hatch, and then genotype the F2 animals. Lines confirmed to be homozygous for the desired edit can be maintained as the final genome-edited strains. To minimize potential off-target effects associated with CRISPR/Cas9 editing, outcross the edited strains with wild-type N2 animals at least twice before performing downstream experiments. Subsequently, freeze and store the validated strains for long-term preservation.

Note: For both deletion and insertion editing strategies, establish at least two independent alleles, outcross them with N2 animals to remove background mutations, and freeze the final edited strains.

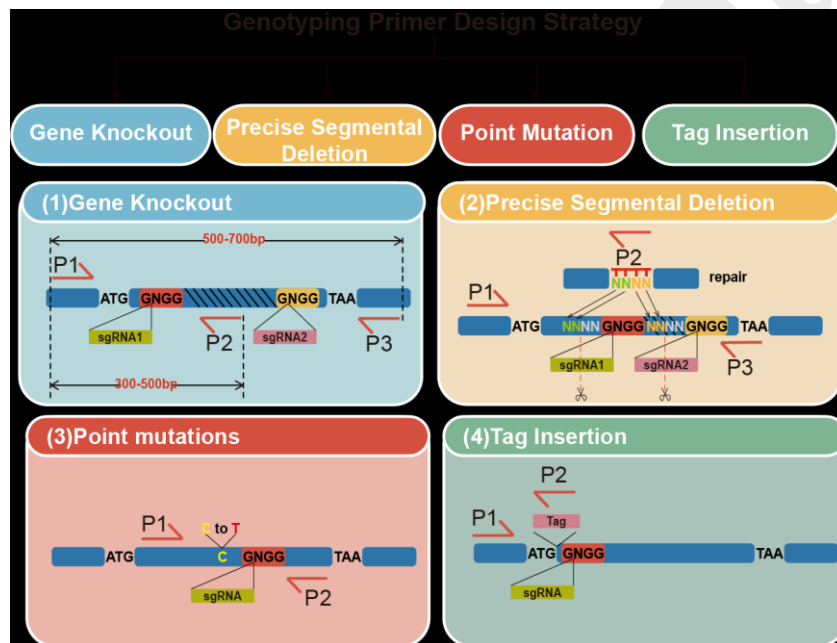


Fig. 4. Genotyping primer design strategies for different CRISPR/Cas9-mediated genome editing outcomes.

The deleted region is indicated by a black diagonal line. As illustrated in Fig. 4, the primer design strategies for genotyping (P1, P2, and P3) vary depending on the specific CRISPR/Cas9-mediated genome editing Gene.

(1) Gene knockout: A three-primer PCR assay is used, consisting of P1 (forward flanking primer), P2 (internal reverse primer), and P3 (reverse flanking primer). Due to the preferential

amplification of shorter DNA fragments, successful knockout events are identified by efficient amplification of the truncated P1+P3 amplicon (500-700 bp). In contrast, in wild-type animals, the genomic distance between P1 and P3 is typically too large to allow efficient amplification under standard PCR conditions. Therefore, the presence of the WT allele is confirmed by amplification of the shorter P1+P2 amplicon (300-500 bp).

(2) Precise fragment deletion: For precise deletion events, the 3' end of primer P2 is designed to contain sequences spanning the deletion junction, whereas P1 and P3 are located within the WT genomic regions upstream and downstream of the deletion site, respectively. Amplification of the expected P1+P2 product confirms successful introduction and genomic integration of the repair template.

(3) Point mutations: Point mutations can be introduced into the repair template to create or eliminate a restriction enzyme recognition site. Two primers are designed, with P1 located upstream and P2 located downstream of the mutation site. Successful introduction of the desired mutation is assessed by restriction enzyme digestion of the PCR-amplified product.

(4) Tag insertion: This strategy enables precise integration of epitope or fluorescent tags (e.g., GFP or FLAG) at specific genomic loci (e.g., adjacent to the ATG start codon). Genotyping is performed using a tag-specific primer (P2) paired with a genomic primer (P1). A positive PCR product is generated only when the tag sequence has been correctly integrated into the target genomic locus.

Timing

The complete CRISPR/Cas9 genome-editing workflow described in this protocol typically requires approximately 4-5 weeks, depending on the editing strategy and downstream validation requirements.

Days 1-4 | Cas9 protein expression: Transform the Cas9 expression plasmid into *E. coli* Rosetta (DE3), prepare starter cultures, inoculate large-scale cultures, and induce Cas9 protein expression with IPTG at 16°C overnight.

Days 4-5 | Cas9 protein purification: Harvest bacterial cells, perform cell lysis, and purify Cas9 protein using Ni-NTA affinity chromatography, followed by ultrafiltration, size-exclusion chromatography, and final protein concentration.

Day 1 | sgRNA template preparation:

Design sgRNA primers, generate sgRNA templates by PCR amplification, and purify the templates by gel extraction.

Days 2-3 | sgRNA synthesis and purification: Perform IVT at 37°C, followed by DNase I treatment, TRIzol extraction, ethanol precipitation, and quality control analysis.

Day 6 | Microinjection: Prepare injection pads (requiring at least 6 hours of drying), assemble Cas9 RNP complexes, prepare injection needles, and inject approximately 10-20 P0 animals.

Days 7-8 | P0 recovery and F1 generation: Maintain injected animals at 20°C for 48-72 hours until F1 progeny are produced.

Days 9-10 | F1 selection: Select 24-96 Unc F1 animals and transfer each animal individually to separate plates.

Days 12-13 | F1 genotyping:

Allow F1 animals to produce F2 progeny (approximately 2-3 days), then perform PCR-based genotyping to identify correctly edited heterozygous animals.

Day 14 | F2 isolation: From plates containing confirmed heterozygous F1 animals, transfer approximately 12 F2 progeny per plate for subsequent screening.

Days 16-17 | F2 genotyping: Allow F2 animals to produce F3 progeny and perform genotyping to identify homozygous edited lines.

Weeks 3-4 | Outcrossing: Outcross confirmed homozygous edited strains with wild-type N2 animals at least twice to remove potential background mutations.

Week 5 | Strain freezing: Expand confirmed edited strains and prepare frozen stocks for long-term storage.

Cost-Effectiveness of in-house purified Cas9 protein and sgRNAs

To evaluate the cost-effectiveness of our in-house CRISPR-Cas9 system, we compared the costs of commercially available CRISPR-Cas9 reagents with those of the corresponding in-house produced components described in this protocol (Table 3).

Table 3. Cost comparison of commercially available and in-house produced CRISPR-Cas9 reagents

Item	Commercial	In-house (this protocol)
Cas9 protein	Cas9 (Sigma-Aldrich, CAS9PROT-50UG), 50 µg, ¥1,085.91, ¥108.59/per injection	~2-7 mg from 2 L culture; Yeast extract (OXOID, LP0021), 2,500 g, ¥790, 48 g/per 2L, ¥15.17/per 2L; Tryptone (OXOID, LP0042), 4,000 g, ¥2,640, 24 g/per 2L, ¥15.84/per 2L; Glycerol (Sangon, A600232), 1,500 mL, ¥395, 8 mL/per 2L, ¥2.11/per 2L; Ampicillin (Sangon, A610028), 25 g, ¥240, 0.2 g/per 2L, ¥1.92/per 2L; Chloramphenicol (Sangon, A600118), 50 g, ¥136.8, 0.68 g/per 2L, ¥1.86/per 2L; IPTG (MDbio, I001), 100 g, ¥1,500, 0.476 g/per 2L, ¥7.14/per 2L; Potassium dihydrogen phosphate (Sangon, A100781), 500 g, ¥195, 4.62 g/per 2L, ¥1.8/per 2L; Potassium phosphate, dibasic, trihydrate (Sangon, A501728), 500 g, ¥61, 32.864 g/per 2L, ¥4/per 2L; Total ¥49.84/per 2L Total ¥0.1246-0.0356/per injection
sgRNA source	sgRNA (Hippobiotec), 2 OD, ¥899/per sgRNA, ¥1,798/per injection (For 2 sgRNAs)	T7 RNA polymerase (Vazyme, DD4101), 10000 U, ¥904, 50 U/per sgRNA, ¥9.04/per injection; NTP mix (Beyotime D7387), 1 mL, ¥235, 0.8 µL/per sgRNA, ¥0.38/per injection; Recombinant RNase Inhibitor (Thermo Fisher, EO0381), 5000 U, ¥558, 20 U/per sgRNA, ¥4.46/per injection; Primer (RuiBiotech), ¥75.5/per injection; RNase-free H ₂ O (Takara, 9012), 20mL, ¥150,

		20 μ L/per sgRNA, ¥0.3/per injection; TRIzol (Thermo Fisher, 15596026), 100mL, ¥750, 500 μ L/per sgRNA, ¥7.5/per injection; Total ¥97.18/per injection
Cas9 cost per injection	¥108.59	¥0.0356-0.1246
sgRNA cost per injection	¥1,798	¥97.18
Cost per injections	¥1,906.59	¥97.22-97.30
Relative cost	~20×	1× (baseline)

Troubleshooting

We have compiled troubleshooting guidelines for common issues encountered throughout the experimental workflow (Table 4).

Table 4. Troubleshooting

Problem	Cause	Solution
Low sgRNA yield after IVT	Insufficient DNA template quantity; RNase contamination.	Use at least 280 ng of purified DNA template; Extend the IVT incubation time to 12 hours, if necessary; Maintain strict RNase-free conditions throughout the procedure.
sgRNA degradation visible on gel	RNase contamination during purification or storage.	Use fresh RNase-free reagents and filter tips; Store sgRNA at -80°C in small aliquots; Perform IVT with <i>unc-58</i> sgRNA and sgRNAs targeting genes of interest at the same time for quality control.
Microinjection needles clog frequently	Aggregates present in the injection mixture.	Centrifuge the injection mixture at 18,000 $\times$ g for 2 minutes before loading.
Low editing efficiency	Poor selection of the sgRNA target site.	Run a fresh gel before each injection to verify RNA integrity; use two or three sgRNAs for each genome-editing experiment.
High false-positive rate in genotyping	Genomic DNA samples are not representative of all developmental stages; PCR contamination.	Use worm lysates containing a mixture of adults, larvae, and embryos; Include no-template and wild-type controls; prepare fresh PCR master mix for each genotyping experiment and avoid storing premixed PCR reactions.

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Author contributions G.W. designed the outline of the manuscript. H.M. and G.W. wrote the manuscript together. H.M. collected and organized the related data. G.W. revised the content of the paper. All authors discussed and edited the manuscript.

Compliance with Ethics Guidelines

Conflict of interest Haiqian Ma and Gang Wan declare that they have no conflict of interest.

Human and animal rights and informed consent This article does not contain any studies with human or animal subjects performed by any of the authors.

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