Prime Editing-mediated Genome Modification in *Saccharomyces cerevisiae*

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ii. Abstract

Prime editing is a highly versatile genome editing technology that enables precise genetic modifications in eukaryotes. Here, we present a detailed step-by-step protocol for conducting prime editing in *S.cerevisiae*. In particular, we describe the design of prime editing guide RNAs (pegRNAs), yeast transformation with prime editor and pegRNA plasmids, and controlled expression through galactose induction. We demonstrate the protocol by targeting the *CAN1* gene and achieving editing efficiencies of 22% and 55% with two different pegRNAs. We provide protocols for genomic DNA extraction, PCR amplification, and editing quantification using both Sanger and next-generation sequencing. Given the important role of yeast in both basic research and biotechnology applications, this protocol provides valuable tools for studying gene function and precise engineering of yeast strains.

iii. Key words: *Saccharomyces cerevisiae*, genome engineering, marker-free genome editing, CRISPR-Cas9, prime editing, pegRNA design, yeast transformation, editing efficiency.

1. Introduction

Saccharomyces cerevisiae has been widely used as a eukaryotic model organism for genetic research and biotechnological applications[1, 2]. Its rapid growth rate, well-annotated and characterized genome, and genetic tractability make it an invaluable model system for studying fundamental biological processes and developing novel technologies[3]. The ability to manipulate the genome of yeasts has been crucial for both basic research and applied biotechnology, including food production, biosynthesis of pharmaceuticals and biofuels[4]. Traditionally, genetic modifications in yeast have relied on homologous recombination (HR), leveraging the yeast's highly efficient HR machinery[5, 6]. However, this approach involves transforming cells with linear DNA fragments in which a selectable marker is flanked by DNA sequences homologous to the targeted genomic locus[7]. While effective for introducing genetic modifications, these selectable markers can alter the function of nearby genes by modifying their transcription and changing the local chromatin environment. Furthermore, the requirement for selection markers constrains multiplex engineering approaches.

Recent advances in genome editing technologies, such as CRISPR-Cas9, base editing or prime editing, have expanded the toolkit for yeast genetic manipulation[8-13]. The CRISPR-Cas9 system enables marker-free genome engineering by introducing a DNA double-stranded break (DSB) at a targeted genomic site as defined by a guide RNA (gRNA)[14, 15]. In yeast, these DSBs are predominantly repaired through HR, enabling precise genetic modifications when a donor DNA template is provided[13]. However, DSB induction carries risks including unintended mutagenesis, chromosomal rearrangements, chromosome loss, large deletions, and off-target effects[16]. Base editing emerged as an alternative approach that enables direct nucleotide conversion without DSBs or DNA donors. While highly efficient for gene inactivation[17] or introducing transition mutations[12, 18], base editing is constrained to specific nucleotide changes and its editing window of 5 to 8 nucleotides can result in undesired bystander edits, complicating the introduction of precise genomic modifications.

Prime editing allows the introduction of specific mutations, including substitutions and precise small insertions or deletions, without DSB formation[10, 19]. The prime editor consists of a fusion between a Cas9 nickase (H840A) and an engineered reverse transcriptase (RT), guided by a prime editing gRNA (pegRNA). This pegRNA contains a spacer sequence directing the prime editor to the target site, a scaffold that forms the complex with nCas9, and a 3' extension comprising a primer binding site (PBS) and an RT template encoding the desired edit. The mechanism leading to successful prime editing involves several distinct steps. The prime editor first introduces a nick in the non-targeted strand, allowing the 3' extension of the pegRNA to hybridize with the exposed single-strand DNA. The RT then synthesizes a complementary strand containing the desired edit[20], creating a DNA flap that the cellular DNA repair mechanisms resolve[15, 21]. Prime editing provides an effective approach for precision genome editing without relying on DSBs or donor DNA templates.

The first demonstration of the feasibility of prime editing in eukaryotes was performed in *S. cerevisiae[10]*. Electroporation of a plasmid containing a DNA flap mimicking those generated by prime editors established that eukaryotic cells possess the necessary machinery for prime editing. Recent work combined prime editing with the OrthoRep continuous evolution system in yeast[22] to develop enhanced prime editors through directed evolution[23].

This protocol describes the conduction of prime editing in *S. cerevisiae* (**Figure 1**). We demonstrate the method by inactivating *CAN1*, which encodes an arginine permease responsible for arginine uptake, through precise small deletions. Inactivation of *CAN1* confers resistance to canavanine, a toxic arginine analog, enabling direct phenotypic selection of edited cells. The protocol covers pegRNA design and cloning, and lithium acetate-mediated co-transformation of pegRNA and prime editor plasmids. Induction of prime editing is achieved through galactose induction. Finally, the protocol includes methods for quantifying editing, using both Sanger sequencing and next-generation sequencing.

“[Fig 1 near here]”

2. Materials

2.1 Reagents and solutions

1. Carbenicillin: Dissolve 1 g in 10 mL of double distilled H_2O (ddH_2O) to prepare a 100 mg/mL stock solution. Filter-sterilize and store at -20°C.
2. Canavanine: Dissolve 0.1 g in 1 mL sterile H_2O to prepare a 100 mg/mL stock solution. Store at -20°C.
3. Polyethylene glycol (PEG, 50% w/v): Dissolve 50 g of PEG 3350 in 30 mL of ddH_2O. Stir until fully dissolved, then bring the volume to 100 mL. Warm gently if needed to aid dissolution. Transfer to a glass bottle and autoclave. Store at room temperature.
4. Lithium acetate (LiOAc, 1M): Dissolve 10.2 g of lithium acetate dihydrate in 70 mL of ddH_2O. Adjust pH to 8.9 and bring the volume to 100 mL. Autoclave and store at room temperature.
5. LiOAc (100 mM): Dilute 5 mL of 1M LioAc to 50 mL with ddH_2O.
6. Ethylenediaminetetraacetic acid (EDTA 500 mM, pH 8.0): Dissolve 186 g of EDTA in 800 mL of ddH_2O. Adjust the pH to 8.0 with NaOH, then bring the volume to 1 L. Store at room temperature.
7. Tris-HCl (1 M, pH 8.0): Dissolve 121.1 g Tris-base in 800 mL of ddH_2O. Adjust the pH to 8.0 with HCl, then bring the volume to 1 L. Store at room temperature.
8. Tris-HCl and EDTA (TE) Buffer: Combine 1 mL of 1M Tris-HCl (pH 8.0) and 200 µL of 500 mM EDTA (pH8.0) with 98.8 mL of ddH_2O. Filter-sterilize and store at room temperature.
9. Single-stranded carrier DNA (2 mg/mL): Dissolve 200 mg of salmon sperm DNA in 100 mL of sterile TE buffer. Stir at 4°C for several hours to ensure complete dissolution. Aliquot and store at –20°C.
10. TAE (50X): Dissolve 242 g Tris-base in 700 mL of ddH_2O. Add 57.1 mL of glacial acetic acid and 100 mL of 500 mM EDTA. Bring volume to 1L. Store at room temperature.

11. Diluted SYBR Gold: Dilute 10 µL of SYBR Gold in 990 µL DMSO. Store at room temperature, protected from light.
12. Lysis buffer: Pierce™ RIPA Buffer (Thermo 89901).
13. Ethanol 99.5% (Molecular biology grade).
14. Phenol/chloroform/Isoamyl alcohol (25:24:1). Use in a chemical hood with appropriate personal protective equipment. Store at 4°C.

2.2 Preparation of growth media

1. Lysogeny broth (LB): Dissolve 25 g of LB powder in 1 L of ddH_2O. Autoclave at 121°C and store at room temperature.
2. LB agar plates with carbenicillin (100 µg/mL): Add 15 g/L agar to LB medium before autoclaving. Cool the autoclaved medium to 50-60°C, add carbenicillin to a final concentration of 100 µg/mL, and pour approximately 20 mL into each sterile petri dish. Allow plates to solidify at room temperature and store at 4°C.
3. Yeast Peptone Dextrose (YPD) medium: Dissolve 10 g bacto yeast extract, 20 g bacto peptone and 20 g dextrose in 700 mL ddH_2O. Once dissolved, bring the volume to 1 L. Autoclave at 121°C for 20 minutes and store at room temperature. For YPD plates, add 20 g agar before autoclaving, cool to 50-60°C, pour into petri dishes and store at 4°C.
4. Yeast Nitrogen Base (YNB, 10X): Dissolve 14.5 g YNB (without amino acids or ammonium sulfate) and 50 g ammonium sulfate in 800 mL of ddH_2O. Bring the volume to 1 L. Filter sterilize and store at room temperature.
5. Dropout amino acid solution (10X): Dissolve the components in **Table 1** in 800 mL ddH_2O. Bring the volume to 1 L. Filter sterilize and store at room temperature.
6. Glucose solution (20%): Dissolve 200 g glucose in 800 mL ddH_2O, then bring the volume to 1 L. Autoclave and store at room temperature.

7. Raffinose solution (20%): Dissolve 200 g raffinose in 800 mL ddH_2O, then bring the volume to 1 L. Filter sterilize and store at room temperature.
8. Galactose solution (20%): Dissolve 200 g galactose in 800 mL ddH_2O, then bring the volume to 1 L. Filter sterilize and store at room temperature.
9. Individual amino acid solutions (w/v):
    a) 0.1% Uracil
    b) 0.1% Adenine
    c) 1% Lysine
    d) 1% Tryptophan
    e) 2% Leucine
    f) 1% Histidine

Dissolve each amino acid separately in ddH_2O, filter sterilize, and store at room temperature.

10. Canavanine selection plates: Combine the sterile components listed in **Table 2** after autoclaving and cooling agar solution (20 g agar in 619 mL H_2O) to 50-60°C. Mix thoroughly and pour into sterile petri dishes. Store at 4°C.

11. Selection media lacking leucine and uracil (Leu^- Ura^-): Combine the sterile components listed in **Table 3** and bring to 1 L with sterile H_2O.

2.3 Commercial reagents and Kits

1. T4 Ligase
2. T4 polynucleotide Kinase
3. Competent bacteria
4. Restriction enzymes: BsaI
5. Plasmid miniprep kit

6. Gel extraction kit
7. DNA ladder

2.4 Equipment

1. Microcentrifuge tubes
2. PCR tubes
3. Petri dishes
4. Bacterial culture tubes
5. Thermocycler
6. Electrophoresis apparatus
7. Acid washed glass beads
8. Nanodrop

3. Method

All steps should be conducted at room temperature unless otherwise stated.

3.1 Design of pegRNAs and oligonucleotides (*see* **Note 3**)

Designing pegRNAs involves identifying the target sequence and determining the pegRNA components: the protospacer sequence, primer binding site (PBS), and reverse transcriptase (RT) template. The following steps describe pegRNA design using *CAN1* inactivation as an example.

1. First, identify the genomic target sequence containing the desired mutation site and locate a suitable SpCas9 PAM sequence (NGG) in proximity to the mutation on either strand. In this example, the target sequence with the deletion site (underlined) (see **Table 4**, step 1)
2. Define the 20-nucleotides protospacer sequence of the PAM sequence (see **Table 4**, step 2)

3. Determine the nick location, which occurs between positions 3 and 4 upstream of the PAM sequence (indicated by "x") (see **Table 4**, step 3)
4. Design the primer binding site (PBS) by reverse complementing 13 nucleotides upstream of the nick site: (see **Table 4**, step 4; *see* **Note 4**)
5. Generate the reverse transcriptase (RT) template by reverse complementing 13 nucleotides downstream of the nick site, while incorporating the deletion: (see **Table 4**, step 5; *see* **Note 4**)
6. Design oligonucleotides and add the appropriate handle sequences (bold) (see **Table 4**, step 6i and ii)
7. Use the scaffold oligonucleotides (see **Table 4**, step 7i and ii), which remain constant across all pegRNA designs
8. Combine the RT template and PBS into a single oligonucleotide to create the 3' extension (see **Table 4**, step 8i and ii)
9. Order all the above oligonucleotides for synthesis.

3.2 Plasmid digestion

1. Prepare the cloning vector by digesting plasmid with BsaI. Assemble the digestion reaction as shown in **Table 5**, and incubate the reaction overnight at 37°C.
2. Prepare a 1% (w/v) agarose gel by dissolving 0.5 g of agarose in 50 mL of 1X TAE buffer using a microwave, stirring periodically. Cool the solution, pour into a small electrophoresis tank and allow it to solidify at room temperature.
3. Add 4 μL of loading dye and 1 μL of diluted SYBR Gold to the 20 μL digestion reaction.
4. Load the sample alongside a 1kb DNA ladder and run the samples at 120V for 45 minutes.
5. Using a blue light transilluminator, excise the larger DNA fragment.
6. Extract the DNA using a commercial gel extraction kit following the manufacturer's instructions. Determine the DNA concentration using a nanodrop.

3.3 Phosphorylation, annealing of oligonucleotides and ligation into plasmid

1. Resuspend dried oligonucleotides in TE buffer at 100 µM.
2. Combine the components listed in **Table 6** for oligonucleotide phosphorylation and anneal as follows.
3. Incubate the reaction at 37°C for 1 hour, then gradually cool from 95°C to 25°C at 0.5°C/second.
4. Dilute 1 µL of phosphorylated and annealed oligonucleotides into 99 µL of H_2O.
5. Prepare ligation reactions as outlined in **Table 7**.
6. Aliquot 8 µL of master mix into 2 PCR tubes. Add 1 µL each of diluted protospacer and 3' extension to one tube, and 2 µL of H_2O to the second tube, as a negative control.
7. Incubate ligation reactions at 25°C for 15 minutes.

3.4 Bacterial transformation

1. Transfer 20 µL of competent bacteria to a 1.5 mL microcentrifuge tube and add 1 µL ligated product. Incubate the mixture on ice for 20 minutes.
2. Heat shock the cells at 42°C for exactly 45 seconds in a water bath, then immediately return to ice for 5 minutes.
3. Spread the entire transformation mixture onto LB agar plates containing 100 µg/mL carbenicillin. Incubate the plates overnight at 37°C.
4. Select a single colony and inoculate into 3 mL LB medium containing 100 µg/mL carbenicillin. Grow overnight at 37°C with shaking at 220 rpm.
5. Extract plasmid DNA from the overnight culture using a commercial miniprep kit according to manufacturer's protocol.
6. Verify successful cloning of the pegRNA by Sanger sequencing using the universal M13R primer (see **Table 4**, step 9) or full plasmid sequencing (*e.g.*, Plasmidsaurus, Azenta).

3.5 Yeast transformation

The following high-efficiency yeast transformation protocol is modified from Gietz et.al., 2007 [25]. (*see* **Note 6**).

1. Inoculate a single yeast colony of BY4741 (*see* **Note 7**) into 3 mL of YPD medium and grow overnight at 30°C with shaking at 220rpm.
2. Dilute the overnight culture to OD_{600} = 0.3 in fresh YPD medium (*see* **Note 8**). For three transformations (control, pegRNA1 and pegRNA2) prepare 35 ml culture, with 30 mL used for transformation and 5 mL for OD measurements.
3. Grow the diluted culture for approximately 3 hours at 30°C with shaking at 220 rpm until OD_{600} reaches 0.4-0.6.
4. Harvest cells by centrifugation at 3,000 g for 5 minutes at 25°C. Wash the cell pellet with 15 mL sterile H_2O, then centrifuge again under the same conditions.
5. Resuspend the washed cell pellet in 300 µL of 100 mM LiOAc and transfer to a microcentrifuge tube. Collect cells by centrifugation at 21,000 g for 30 seconds at 25°C.
6. Resuspend cells in 150 µL of 100 mM LiOAc, vortex for 30 seconds, and incubate at room temperature for 5 minutes. Distribute 50 µL aliquots into three microcentrifuge tubes for transformation.
7. Pellet cells in each tube at 21,000 g for 30 seconds and remove supernatant. Add transformation components in the order outlined in **Table 8**.
8. Vortex each tube vigorously for 1 minute, then incubate at 30°C for 1 hour with shaking at 220 rpm.
9. Heat shock the cells at 42°C for 20 minutes in a water bath. Collect cells by centrifugation at 21,000 g for 30 seconds and remove supernatant.
10. Gently resuspend cells in 500 µL of sterile water, pellet by centrifugation, and remove supernatant. Resuspend in 500 µL YPD medium and incubate at 30°C for 1 hour with shaking at 220 rpm.

11. Collect cells by centrifugation, wash once with 500 µL of sterile H_2O, and resuspend the pellet in 100 µL of sterile H_2O. Plate entire volume on Leu^- Ura^- selective medium(*see* **Note 2**).
12. Incubate plates at 30°C for 2-3 days until colonies appear (*see* **Note 9**).

3.6 Prime editing induction

1. Pick a single colony from the transformation plate and inoculate into 3 mL of Raf^+ Leu^- Ura^- medium. Grow overnight at 30°C with shaking at 220 rpm.
2. Dilute the overnight culture to OD_{600} = 0.1 in 3 mL of Gal^+ Leu^- Ura^- medium (*see* **Note 8**) to initiate prime editor expression. Maintain cells in galactose-containing medium for three days, with daily dilution to OD_{600} = 0.3 in 3 mL of fresh Gal^+ Leu^- Ura^- medium.
3. Following galactose induction, prepare dilutions for plating on selective media:
   a) For canavanine selection: Dilute culture 1/2,000 and plate 100 µL on CAN^+ ARG^- plates.
   b) For viability assessment: Dilute culture 1/20,000 and plate 100 µL on YPD plates.
4. Incubate plates at 30°C for 2-3 days until colonies develop (**Figure 2**).
5. Calculate prime editing efficiency by comparing colony counts between selective and non-selective conditions: Editing efficiency (%) = (Number of canavanine-resistant colonies x 2,000) / (Number of YPD colonies x 20,000) x100
6. For subsequent analysis of editing frequencies, harvest all colonies on the plates by adding 2 mL sterile H_2O to each plate and carefully collect the cells by scraping the plate.

"[Fig 2 near here]"

3.7 Genomic DNA extraction

1. Collect yeast colonies from CAN^+ ARG^- and YPD plates in separate microcentrifuge tubes. Pellet cells by centrifugation at 21,000 g for 30 seconds and remove supernatant. Wash pellet with 500 µL H_2O, centrifuge at 21,000 g for 30 seconds and remove supernatant.
2. Resuspend each cell pellet in 200 µL RIPA buffer and add 200 µL of acid-washed glass beads. Add 400 µL phenol/chloroform/isoamyl alcohol (25:24:1) under a fume hood (*see* **Note 11**).
3. Lyse cells by vigorous vortexing for 2 minutes. Add 400 µL TE Buffer and briefly mix by vortexing. Separate phases by centrifugation at 21,000 g for 10 minutes at room temperature.
4. Carefully transfer 400 µL of the upper aqueous phase to a fresh microcentrifuge tube. Add 1 mL ice-cold 99.5% ethanol and mix by gentle inversion. Collect the precipitated DNA by centrifugation at 21,000 g for 5 minutes.
5. Remove supernatant and wash the DNA pellet with 500 µL of 70% ethanol. Centrifuge at 21,000 g for 5 minutes, carefully remove the supernatant and air-dry the pellet at room temperature for 5 minutes.
6. Resuspend the DNA pellet in 100 µL TE Buffer. Determine DNA concentration with a nanodrop. Store the gDNA at -20°C for short-term use or -80°C for long-term storage.

3.8 Editing evaluation by Sanger sequencing (*see* **Note 10**)

1. Design PCR primers to amplify the edited region of the *CAN1* using Primer 3 (http://primer3.ut.ee/). Input the *CAN1* sequence with square brackets flanking the target site.
2. Configure Primer3 with the following parameters:

   Mispriming library = "NONE"

   Primer size ‘‘min = 25, Opt = 27, Max = 30’’

   Primer T_m ‘‘Min = 57.0C, Opt = 60.0C, Max = 63.0C”

   Leave all other parameters as default.
3. Prepare the PCR reactions as outlined in **Table 9** (*see* **Note 12**).
4. Program the thermocycler as outlined in **Table 10** (*see* **Note 13**).

5. Analyze the PCR products on a 2% (w/v) agarose gel as described in section 3.2 steps 2-6. Excise the band of expected size and purify using a commercial gel extraction kit according to the manufacturer's instructions.
6. Submit purified PCR products for Sanger sequencing using the forward primer (*see* **Note 14)**. Evaluate chromatogram quality and analyze editing efficiency using the Inference of CRISPR Edits (ICE) web tool (https://ice.synthego.com). Upload the sequencing file (.ab1 format) and input the target sequence to determine editing efficiency based on nucleotide frequencies at the edit site (*see* **Note 15**) (**Figures 3A and 3B**).

3.9 Editing evaluation by next-generation sequencing (*see* **Note 10**)

1. Modify the PCR primers designed in section 3.8 by adding Illumina adaptor sequences:

Forward primer: 5'- ACACTCTTTCCCTACACGACGCTCTTCCGATCT[N20]-3'

Reverse primer: 5'-GACTGGAGTTCAGACGTGTGCTCTTCCGATCT[N20]-3' where [N20] represents the gene-specific primer sequence. Ensure the total amplicon length, including adaptors, remains compatible with the sequencing platform (*e.g.*, maximum 500 bp for Azenta Amplicon-EZ).

2. Amplify the target region using the modified primers following the PCR conditions described in section 3.8. Purify the amplicons on a 2% agarose gel and extract DNA using a commercial kit.

3. Measure amplicon concentration with a nanodrop, and prepare sequencing samples according to Azenta Amplicon-EZ specifications (25 µL at 20 ng/µl).

4. After receiving the sequencing data, download the FASTQ files and analyze them using CRISPResso2[24] (http://crispresso2.pinellolab.org/submission) to measure editing efficiency (*see* **Note 15**).

5. Select the 'prime editors' option. Upload the forward and reverse FASTQ files under the 'Paired-end reads' tab. Input the WT *CAN1* amplicon sequence.

6. Under optional parameters, input the protospacer sequence in the ‘pegRNA spacer sequence’. Input the 3’ extension sequence in the ‘pegRNA extension sequence’. Leave all other parameters as default (*see* **Note 16**).

7. The CRISPResso2 results give the proportion of reads containing the edit, as well as indels and byproducts (**Figures 3C and 3D**).

“[Fig 3 near here]”

4. Notes

1. For canavanine selection plates, omit arginine from the dropout amino acid mixture to maintain selective pressure.

2. For prime editor and pegRNA transformation, selective media must lack both leucine and uracil (leu^- ura^-) to maintain plasmids throughout the experiment.

3. pegRNA design can be automated using the PRIDICT webtool (https://pridict.it/about)[25]. While this tool provides efficiency predictions based on data generated from human cells, its design principles may potentially be applicable to yeast. Consider multiple pegRNA designs for optimal editing efficiency.

4. The optimal lengths of the PBS and RT template sequences depend on multiple factors including melting temperature and GC content. Refer to Mathis et al., 2023[25] for detailed optimization guidelines.

5. Use molecular biology grade reagents and nuclease-free water for all DNA manipulation steps to prevent degradation and contamination.

6. Maintain strict sterile technique throughout all procedures involving yeast cells to prevent contamination.

7. When using yeast strains other than BY4741, verify compatibility of auxotrophic markers with the selection strategy.

8. Always culture yeast cells to exponential phase before transformation or galactose induction to ensure optimal efficiency.

9. If transformation yields no colonies, verify cell viability, plasmid integrity, selection medium composition and transfection reagent quality.

10. Multiple methods are available for quantifying editing efficiency:

a) Colony counting (section 3.6 steps). Rapid and inexpensive but limited to *CAN1* inactivation.

b) Sanger sequencing (section 3.8). Reliable and widely accessible, but slow and relatively expensive.

d) Next-generation sequencing (section 3.9). Highly accurate but the most expensive.

e) One-pot DTECT (detailed protocols in[26, 27]). Quick and cost-effective method.

11. Perform phenol-chloroform extraction under a fume hood using appropriate personal protective equipment.

12. This protocol is based on the Q5 DNA polymerase, but other high-fidelity DNA polymerases may be substituted with appropriate optimization of reaction conditions.

13. Optimize PCR conditions for each primer pair by adjusting annealing temperature-based on primer Tm, modifying extension time according to amplicon length and validating amplification specificity.

14. High quality Sanger sequencing results should have clear, single-color peaks. If quality is poor, sequence both strand and consider PCR optimization if problem persist.

15. When prime editing efficiency is low, redesign pegRNA with modified PBS and RT template lengths.

16. For CRISPResso2 analysis, if you editing events are not properly detected or quantified, you may need to adjust the quantification window parameters to ensure proposer detection of intended edits.

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Figure Captions

Figure 1: Workflow for prime editing in *S. cerevisiae*. (A) Design and composition of a pegRNA targeting *CAN1*. The genomic target sequence contains the protospacer (orange), PAM sequence (purple), and nick site (purple arrow). The pegRNA components include the PBS (red) and RT template (green) encoding the GG deletion. **(B)** Transformation of yeast cells with prime editing components. The prime editor plasmid contains a galactose-inducible Cas9(H840A)-reverse transcriptase fusion, while the pegRNA plasmid carries the protospacer, scaffold, and 3' extension sequences as indicated in Fig. 1A. Following transformation, cells are selected on leucine and uracil dropout media. **(C)** Prime editing induction and phenotypic selection. Single colonies from transformation plates are grown in raffinose-containing medium, followed by galactose induction to express the prime editor. Edited cells are

identified by parallel plating on YPD (viability) and canavanine selection plates (CAN+ ARG-). **(D)** Molecular characterization of editing outcomes. Genomic DNA is extracted from both YPD and CAN^+ ARG^- plates, followed by PCR amplification of the target region. Editing efficiency is determined by Sanger sequencing and next-generation sequencing analysis.

Figure 2: Prime editing enables efficient *CAN1* inactivation in yeast. Schematic representation of the *CAN1* locus showing the target sites for pegRNA1 and pegRNA2. The protospacer sequences are represented in blue. Representative images of yeast colonies on YPD plates (top row, 1:20,000 dilution) and canavanine selection plates (CAN^+ ARG^-, bottom row, 1:2,000 dilution) after prime editing induction. Control cells (left) lacking pegRNA show no growth on canavanine plates, while cells expressing either pegRNA1 (middle) or pegRNA2 (right) form colonies, indicating successful *CAN1* inactivation. Colony growth on YPD plates demonstrates comparable cell viability across all conditions.

Figure 3: Molecular characterization of prime editing outcomes at the *CAN1* locus. (A) Sanger sequencing analysis of pegRNA1-targeted region. Chromatograms show the unedited WT sequence (left), mixed sequence from pooled cells on YPD plates indicating partial editing (top right), and clean edited sequence from canavanine-resistant colonies showing complete deletion (bottom right). **(B)** Sanger sequencing analysis of pegRNA2-targeted region, displaying similar patterns of unedited, mixed population, and successfully edited sequences as observed for pegRNA1. **(C)** Next-generation sequencing quantification of editing outcomes for pegRNA1. The bar graph displays five distinct sequence categories: unmodified sequences matching WT (76.15%), sequences with unintended modifications (1.44%), sequences containing the desired prime edit (22.22%), sequences with both the prime edit and additional modifications (0.19%) and ambiguous (0.00%). Values in parentheses indicate total read counts for each category. **(D)** Next-generation sequencing analysis for pegRNA2 editing outcomes. The

histogram shows the distribution across the same sequence categories: unmodified WT sequences, sequences with unintended modifications, sequences with successful prime edits (55.09%), and sequences containing both prime edits and additional modifications. Read counts are provided in parentheses for each category.

Tables

Table 1. Dropout amino acid solution (10X)

Methionine	0.2 g
Tyrosine	0.6 g
Isoleucine	0.8 g
Phenylalanine	0.5 g
Glutamic acid	1 g
Threonine	2 g
Aspartic acid	1 g
Valine	1.5 g
Serine	4 g
Arginine (*see* **Note 1**)	0.2 g

Table 2. Media components for canavanine plates

Glucose (20%)	100 mL
YNB (10X)	100 mL
Dropout amino acid solution (10X)	100 mL
Uracil (0.1%)	35 mL

Adenine (0.1%)	20 mL
Lysine (1%)	12 mL
Tryptophan (1%)	8 mL
Leucine (2%)	4 mL
Histidine (1%)	2 mL
Canavanine (100 mg/mL)	0.5 mL

Table 3. Media without leucine and uracil

Raffinose (20%) or Galactose (20%)	100 mL
YNB (10X)	100 mL
Dropout amino acid solution (10X)	100 mL
Adenine (0.1%)	20 mL
Lysine (1%)	12 mL
Tryptophan (1%)	8 mL
Histidine (1%)	2 mL

Table 4. Designing pegRNA for prime editing

Step	Description	Sequence
1.	Target sequence with deletion site	5’-GAAGCATATGTACAATGAGC(CGG)TCACACCCTCTTT-3’
2.	Protospacer sequence	5’-GAAGCATATGTACAATGAGC-3’
3.	Nick location (x)	5’-GAAGCATATGTACAATGxAGC(CGG)TCACAACCCTCTTT-3’
4.	Primer Binding site	5’-CATTGTACATATG-3’

5.	Reverse transcriptase template	5'-TGTGACCGGCT-3'.
6i.	Oligonucleotides with handles (forward)	5'-**GATC**GAAGCATATGTACAATGAGC**GTTTT**-3'
6ii.	Oligonucleotides with handles (reverse)	5'-**CTCTAAAA**CGCTCATTGTACATATGCTTC-3'
7i.	Scaffold oligonucleotide	5'-AGAGCTAGAAATAGCAAGTTAAAATAAGGCTAGTCCGTTATCAACTTGAAAAAGTGGCACCGAGTCG-3'
7ii.	Scaffold oligonucleo	5'-GCACCGACTCGGTGCCACTTTTTCAAGTTGATAACGGACTAGCCTTATTTTAACTTGCTATTTCTAG-3'
8i.	3' extension (forward)	5'-GTGCGTTGTGAGGCTCATTGTACATATG-3'
8ii.	3' extension (reverse)	5'-TCAACATATGTACAATGAGCCTCACAAC-3'.
9.	M13 primer	(5'-CAGGAAACAGCTATGAC-3')

Table 5. Plasmid digestion reaction mixture

Reagent	**Amount**
rCutsmart buffer (10X)	2 µL
Plasmid DNA	1 µg
BsaI-HF v2 (20,000 units/mL)	0.5 µL
H_2O (*see* **Note 5**)	Up to 20 µL

Table 6. Oligonucleotide phosphorylation reaction mixture

Reagent	**Volume (µL)**
H_2O	5.5
5X ligase buffer (5X)	2
T4 PNK (10,000 units/mL)	0.5

Forward oligonucleotide (100 μM)	1
Reverse oligonucleotide (100 μM)	1

Table 7. Ligation reaction mixture

Reagent	**Amount (1X)**	**Master Mix (2.5X)**
H_2O	Up to 8 μL	Up to 20 μL
5X Ligase buffer (5X)	2 μL	5 μL
Digested plasmid	10-20 ng	25-50 ng
T4 ligase (400,000 units/mL)	0.5 μL	1.25 μL
Diluted scaffold	1 μL	2.5 μL

Table 8. Yeast transformation mixture

PEG (50%)	240 μL
LiOAc (1M)	36 μL
ssDNA (2 mg/mL)	25 μL
plasmid (2 μg of prime editor and 2 μg of pegRNA) with sterile water	50 μL

Table 9. PCR reaction mixture

Reagent	**Amount per reaction (µL)**	**Amount per 2.5 reactions (µL)**
Q5 Reaction buffer (5X)	5	12.5
dNTP mix (10mM each)	0.25	0.625
Forward primer (100 µM)	0.25	0.625
Reverse primer (100 µM)	0.25	0.625
Q5 High-Fidelity DNA polymerase (2 units/µL)	0.2	0.5
H_2O	18.05	32.625
gDNA (200 ng/µL)	1	X

Table 10. PCR reaction conditions

Steps	Temperature (°C)	Time (sec)	Cycles
Initial Denaturation	95	30	1
Denaturation	95	10	40
Annealing	62	10	
Extension	72	30	
Final extension	72	60	1
Hold	4	infinite	

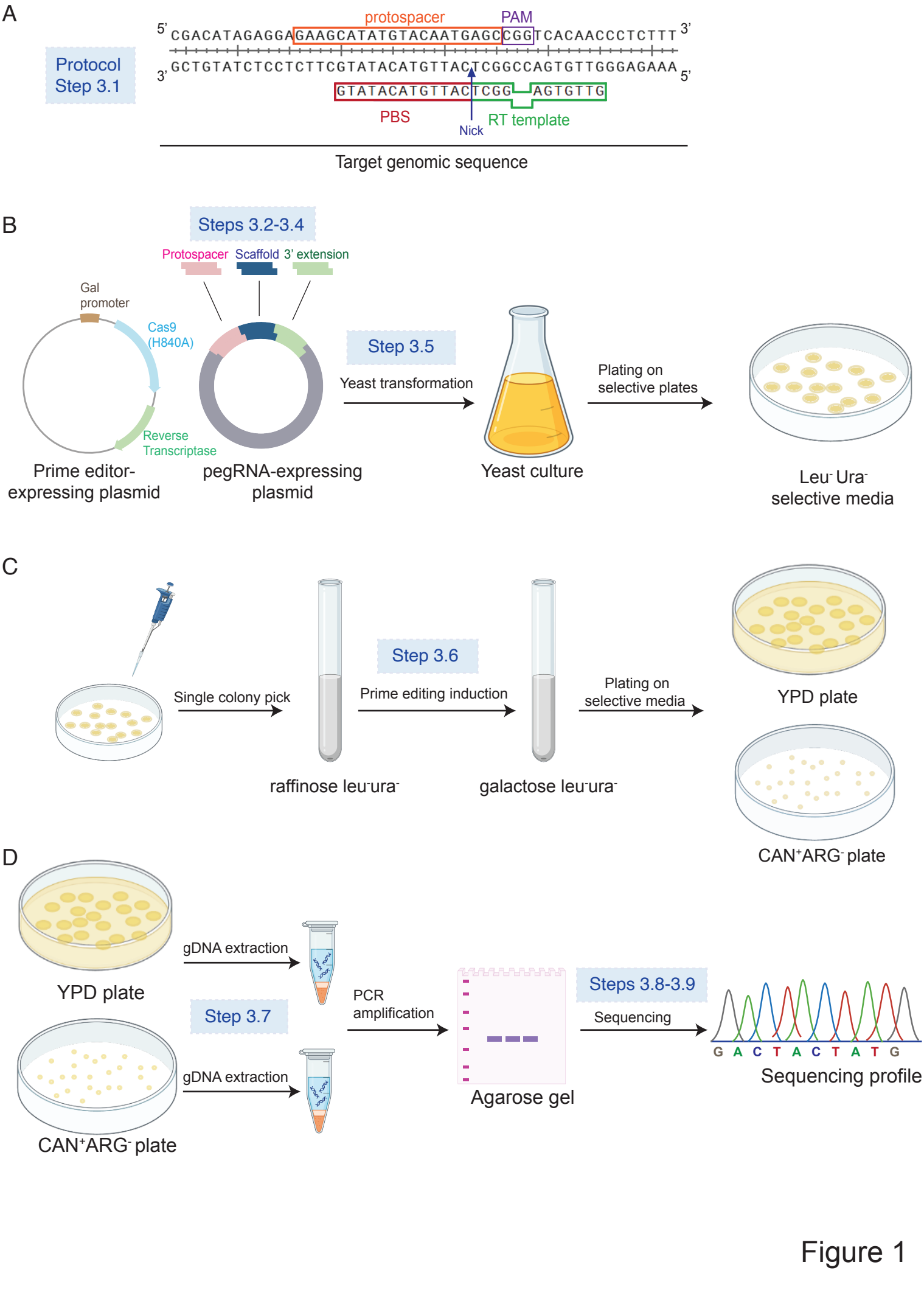

A
Protocol Step 3.1
protospacer
PAM
5' CGACATAGAGGAGAAGCATATGTACAATGAGCCGGTCACAACCCTCTTT 3'
3' GCTGTATCTCCTCTTCGTATACATGTTACTCGGCCAGTGTTGGGAGAAA 5'
GTATACATGTTACTCGG
AGTGTTG
PBS
Nick
RT template
Target genomic sequence
B
Steps 3.2-3.4
Protospacer
Scaffold
3' extension
Gal promoter
Cas9 (H840A)
Reverse Transcriptase
Prime editor-expressing plasmid
pegRNA-expressing plasmid
Step 3.5
Yeast transformation
Yeast culture
Plating on selective plates
Leu- Ura- selective media
C
Single colony pick
raffinose leu-ura-
Step 3.6
Prime editing induction
galactose leu-ura-
Plating on selective media
YPD plate
CAN+ARG- plate
D
YPD plate
gDNA extraction
Step 3.7
CAN+ARG- plate
gDNA extraction
PCR amplification
Agarose gel
Steps 3.8-3.9
Sequencing
G A C T A C T A T G
Sequencing profile

Figure 1

A

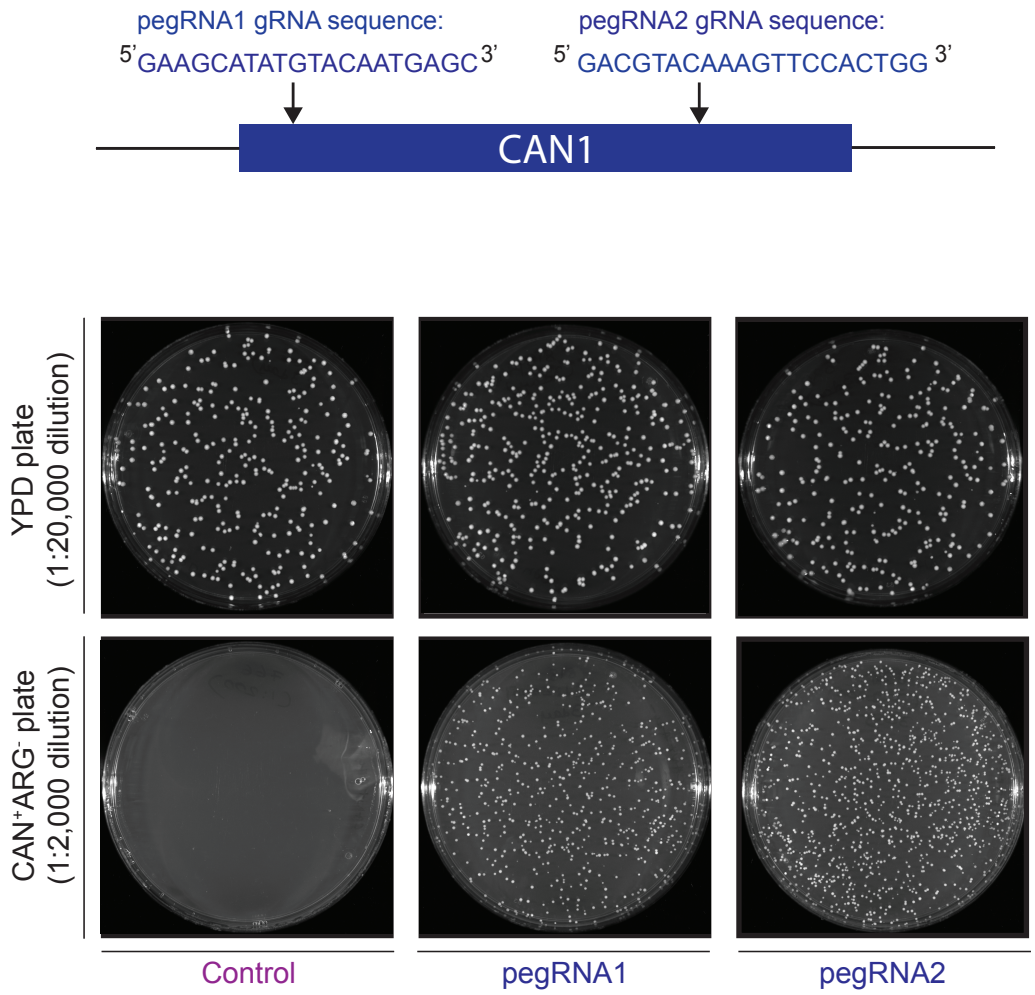
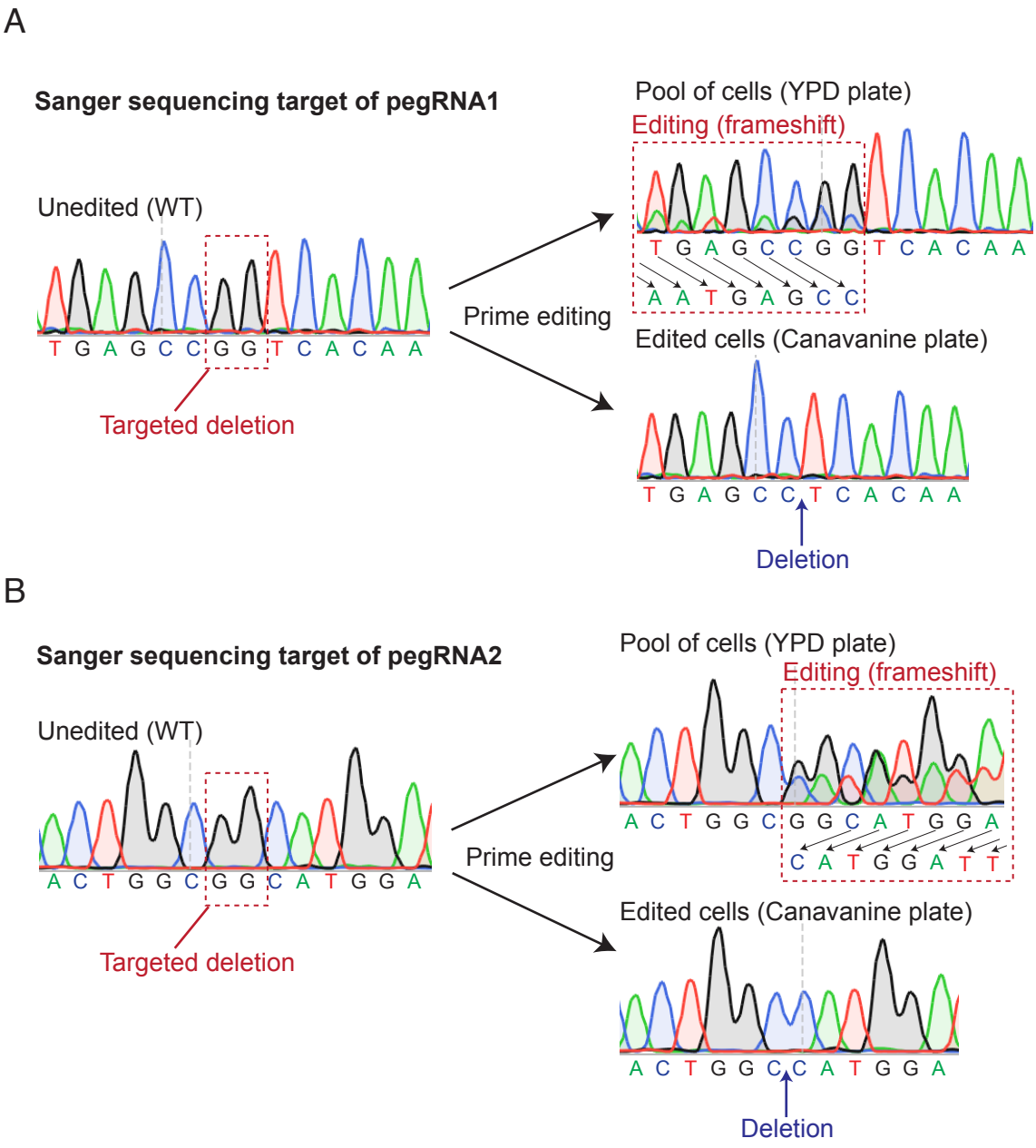
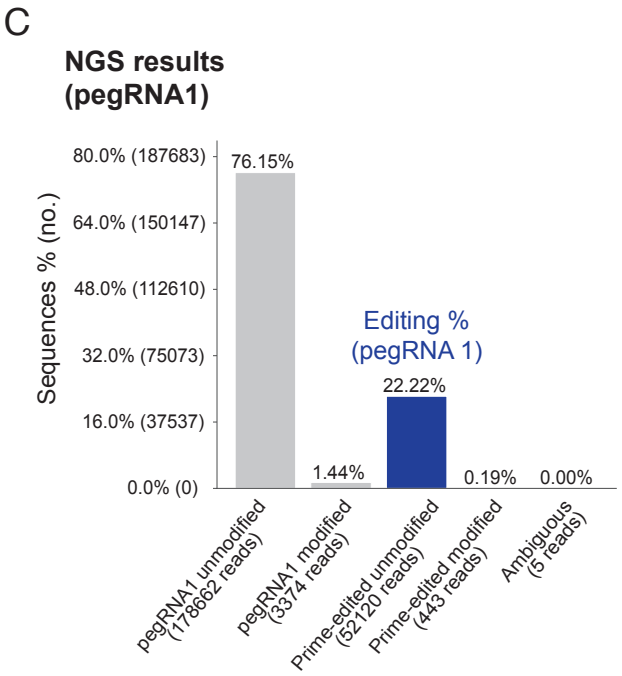

pegRNA1 gRNA sequence:
5' GAAGCATATGTACAATGAGC 3'
pegRNA2 gRNA sequence:
5' GACGTACAAAGTTCCACTGG 3'
CAN1
YPD plate
(1:20,000 dilution)
CAN+ARG- plate
(1:2,000 dilution)
Control
pegRNA1
pegRNA2

Figure 2


A
Sanger sequencing target of pegRNA1
Unedited (WT)
T G A G C C G G T C A C A A
Targeted deletion
Prime editing
Pool of cells (YPD plate)
Editing (frameshift)
T G A G C C G G T C A C A A
A A T G A G C C
Edited cells (Canavanine plate)
T G A G C C T C A C A A
Deletion
B
Sanger sequencing target of pegRNA2
Unedited (WT)
A C T G G C G G C A T G G A
Targeted deletion
Prime editing
Pool of cells (YPD plate)
Editing (frameshift)
A C T G G C G G C A T G G A
C A T G G A T T
Edited cells (Canavanine plate)
A C T G G C C A T G G A
Deletion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NGS results
(pegRNA1)
Sequences % (no.)
80.0% (187683)
64.0% (150147)
48.0% (112610)
32.0% (75073)
16.0% (37537)
0.0% (0)
76.15%
1.44%
Editing %
(pegRNA 1)
22.22%
0.19%
0.00%
pegRNA1 unmodified (178662 reads)
pegRNA1 modified (3374 reads)
Prime-edited unmodified (52120 reads)
Prime-edited modified (443 reads)
Ambiguous (5 reads)

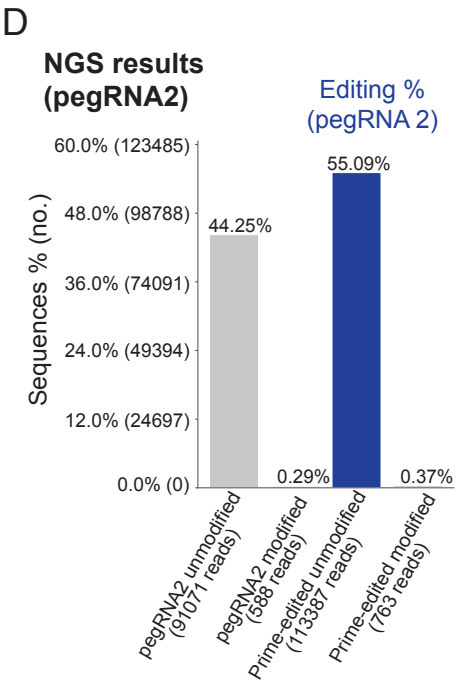
D
NGS results
(pegRNA2)
Editing %
(pegRNA 2)
Sequences % (no.)
60.0% (123485)
48.0% (98788)
36.0% (74091)
24.0% (49394)
12.0% (24697)
0.0% (0)
44.25%
0.29%
55.09%
0.37%
pegRNA2 unmodified (91071 reads)
pegRNA2 modified (588 reads)
Prime-edited unmodified (113387 reads)
Prime-edited modified (763 reads)

Figure 3