Chapter 12

Highly Multiplexed Analysis of CRISPR Genome Editing Outcomes in Mammalian Cells

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Abstract

CRISPR–Cas-based genome editing has enabled efficient genetic engineering of a range of organisms and sparked revolutions in many fields of biology. After *Streptococcus pyogenes* Cas9 was first demonstrated for mammalian genome editing, many CRISPR-associated (Cas) protein variants have been isolated from different species and adopted for genome editing. Furthermore, various effector domains have been fused to these Cas proteins to expand their genome-editing abilities. Although the number of genome-editing tools has been rapidly increasing, the throughput of cell-based characterization of new genome-editing tools remains limited. Here we describe a highly multiplexed genome editing and sequencing library preparation protocol that allows high-resolution analysis of mutation outcomes and frequencies induced by hundreds to thousands of different genome-editing reagents in mammalian cells. We have successful experiences of developing several key genome-editing tools using this protocol. The protocol also is designed to be compatible with robotic liquid handling systems for further scalability.

Key words Genome editing, CRISPR–Cas9, Base editing, High-throughput sequencing, Amplicon sequencing

1 Introduction

A number of CRISPR genome-editing systems have been developed. *Streptococcus pyogenes* Cas9 (SpCas9) was the first to be demonstrated for mammalian genome editing [1, 2] and it has been the most widely used in a range of fields, including genome-wide gene knockout screens and development of genome editing-based therapeutic approaches [3]. In CRISPR–Cas9 genome editing, a chimeric single guide RNA (gRNA) recruits Cas9 to the target genomic sequence that is adjacent to a 3′ protospacer adjacent motif (PAM) through sequence complementarity (Fig. 1). The two endonuclease domains of Cas9 then induce a double-stranded DNA break (DSB) at the target genomic region. This has been used to induce either mutations (mostly deletions) in target genes through error-prone nonhomologous end-joining

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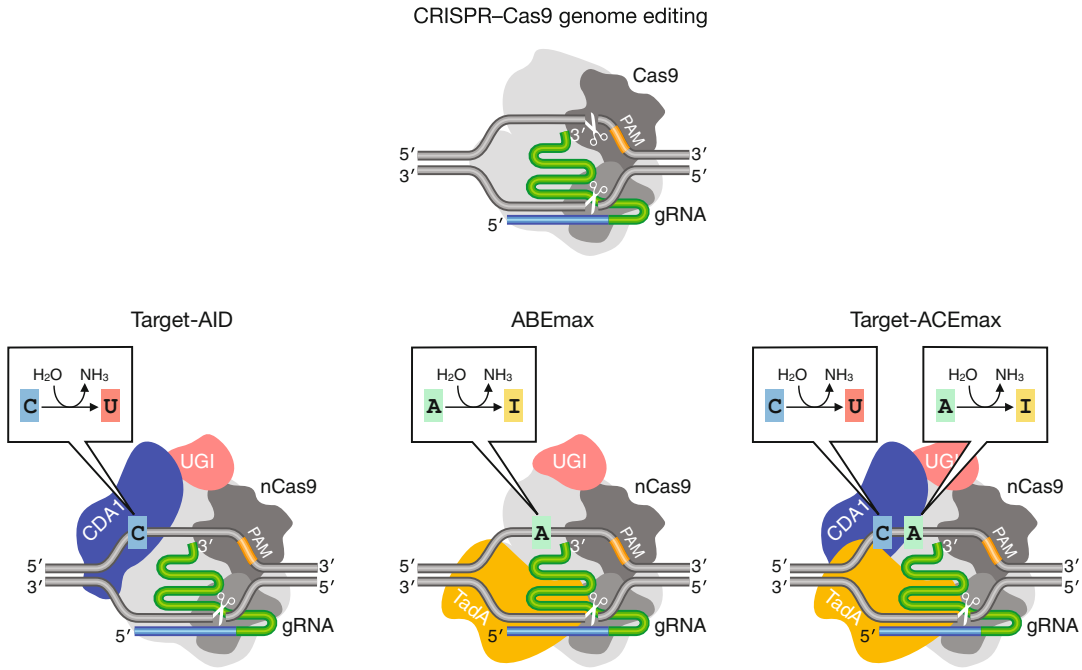

Fig. 1 CRISPR–Cas9 genome editing and base editing. Conceptual diagrams of genome editing by wild-type Cas9 and three base editors. The base editing tools, Target-AID [11], ABEmax [17], and Target-ACEmax [35], convert C·G→T·A, A·T→G·C, and simultaneously C·G→T·A and A·T→G·C, respectively

DNA repair or transgene insertions through homology-directed DNA repair in eukaryotic cells. While wild-type SpCas9 has been adopted in diverse applications, many protein engineering efforts have further enhanced its genome-editing ability. For example, recent studies have expanded the targeting scope of SpCas9 by relaxing its wild-type PAM restriction (5′-NGG-3′ or 5′-NGA-3′). Such PAM-altered Cas9 variants include SpCas9-NG [4] and xCas9-3.7 [5] for 5′-NG-3′ PAM, and near PAM-less SpG and SpRY [6]. Other efforts have focused on minimizing undesired off-target edits that usually occur through mismatch-tolerant annealing of gRNA to nontargeting genomic regions, and several high-fidelity Cas9 variants, such as SpCas9-HF1 [7], eCas9(1.1) [8], and evoCas9 [9], have been derived.

The DSB-based genome editing by wild-type SpCas9 is particularly effective in gene knockout, but has a drawback in precision genome editing, where mutation outcomes are not always predictable. Induction of a single-base substitution has been proposed with the transgene insertion approach to replace target sequence with a donor template sequence by homology-directed DNA repair, but its efficiency is limited. Furthermore, Cas9-derived DSBs are cytotoxic in cells [10, 11]. Harnessing a nucleoside deaminase domain to catalytically inactive Cas9 (dCas9) or nickase Cas9 (nCas9), base editing technologies have enabled targeted

nucleotide substitutions to be induced without DSB [12]. The current base editors are categorized as cytosine base editors (CBEs), for targeted C·G→T·A substitution, and adenine base editors (ABEs), for targeted A·T→G·C substitution (Fig. 1). The CBEs use a cytidine deaminase such as rAPOBEC1 or PmCDA1 to convert cytidines into uridines in the nontargeting DNA strand of gRNA and a uracil glycosylase inhibitor to prevent base excision repair of uracil bases [11, 13]. Following DNA replication, uracils are converted into thymines. The ABEs instead use an evolved tRNA adenosine deaminase (TadA) that converts adenosines to inosines on single-stranded DNA (ssDNA). Although none of the natural adenosine deaminases have been found to have catalytic activity for ssDNA, a directed protein evolution approach derived the evolved TadA heterodimer for A·T→G·C base editing [14]. The CBEs and ABEs both have narrow base editing windows within the gRNA target site and are suited for targeted sequence alternation. The idea of base editing has been rapidly applied to the engineered SpCas9 variants to expand their targeting scopes [15] and enhance their targeting fidelities [16]. Furthermore, several studies have reported that codon optimization, linker optimization, and other accessory domains, such as nuclear localization signals and ssDNA-binding domains, can be used to derive more efficient base editors if they are fused in an optimal order [17–20]. Non-specific C→U and A→I edits of the cellular transcriptome have been a concern for CBEs using rAPOBEC1 and ABEs using TadA, the original wild-types of which have catalytic activity for RNA (this has been reported not to be the case for PmCDA1-based CBEs). However, protein engineering approaches also have derived deaminase variants that minimize such nonspecific RNA off-target activities [21–24].

Although the delivery efficiency of genome-editing reagents to cells and nuclei is important, SpCas9 is 1358 aa long, which limits the delivery efficiency of SpCas9 to cells by, for example, adeno-associated viral vectors. To resolve this issue, other compact Cas9 orthologues have been explored and reported for genome editing, such as SaCas9 from *Staphylococcus aureus* (1053 aa) [25] and CjCas9 from *Campylobacter jejuni* (984 aa) [26]. These Cas9 orthologues also have been fused to deaminase domains to obtain compact base editing tools [27, 28]. Furthermore, other CRISPR–Cas systems from outside the CRISPR–Cas9 family have been characterized for efficient genome editing [29]. For example, LbCas12a from *Lachnospiraceae* bacterium (formally characterized as LbCpf1) catalyzes site-specific cleavage of DNA with T-rich 5′-TTTV-3′ PAM adjacent to the 5′-end of the gRNA-targeting sequence [30]. rAPOBEC1 and TadA also have been fused to catalytically inactive LbCas12a for base editing [31].

Accordingly, a range of Cas proteins, mutations, and different effector and linker domains have been reported for genome editing and expanding the design space to develop new genome-editing tools. However, the number of possible design combinations to derive a new tool has encountered a combinatorial explosion, making it difficult to derive an optimal-best tool for a specific target of application because the editing outcome patterns of each tool need to be thoroughly evaluated for a range of genomic on-target and predicted off-target sites in cells. Recently, along with three other similar studies [32–34], we have developed a novel dual-function base editor Target-ACEmax for the simultaneous introduction of C·G→T·A and A·T→G·C substitutions by fusing both cytidine and adenosine deaminases to nCas9 [35]. We tested genome-editing outcome patterns of 47 endogenous target sites for three dual-function base editor candidates, six single-function base editors, and four single-function base editor mixes, as well as a non-functional enzyme control in triplicates by amplicon sequencing (a total of 1833 assays). In this methodology article, we describe a modified protocol that enables genome-editing outcome assays of 48 different gRNA target sites in human embryonic kidney 293 T (HEK293T) cells for genome-editing tools as well as a control enzyme in triplicate (Fig. 2). The protocol has five sections: (1) screening of gRNA target sites and target amplification primers, (2) preparation of a gRNA expression plasmid library, (3) culturing of cells, (4) massively parallel transfection of genome-editing reagents, (5) template genomic DNA preparation, and (6) generation of a multiplexed amplicon sequencing library. This protocol produces a single multiplexed amplicon sequencing library for hundreds to thousands of genome-editing assay samples and enables analysis of their genome-editing outcomes by one-shot massively parallel sequencing (Fig. 3). This basic protocol can easily be scaled up with increased numbers of gRNA on-target and predicted off-target sites. It can also be modified for different cell lines with optimal cell culture and transfection protocols. (We have successful experiences of performing similar genome-editing assays for HeLa, HCC827, and mouse embryonic stem cells with similar library-scale protocols.) Furthermore, we designed many of the process modules in the protocol to be compatible with robotic liquid handling systems for more systematic characterization of a large number of genome-editing tools. We believe that this step-by-step protocol will be of great use for researchers to facilitate the rapid development of new genome-editing tools in mammalian cells.

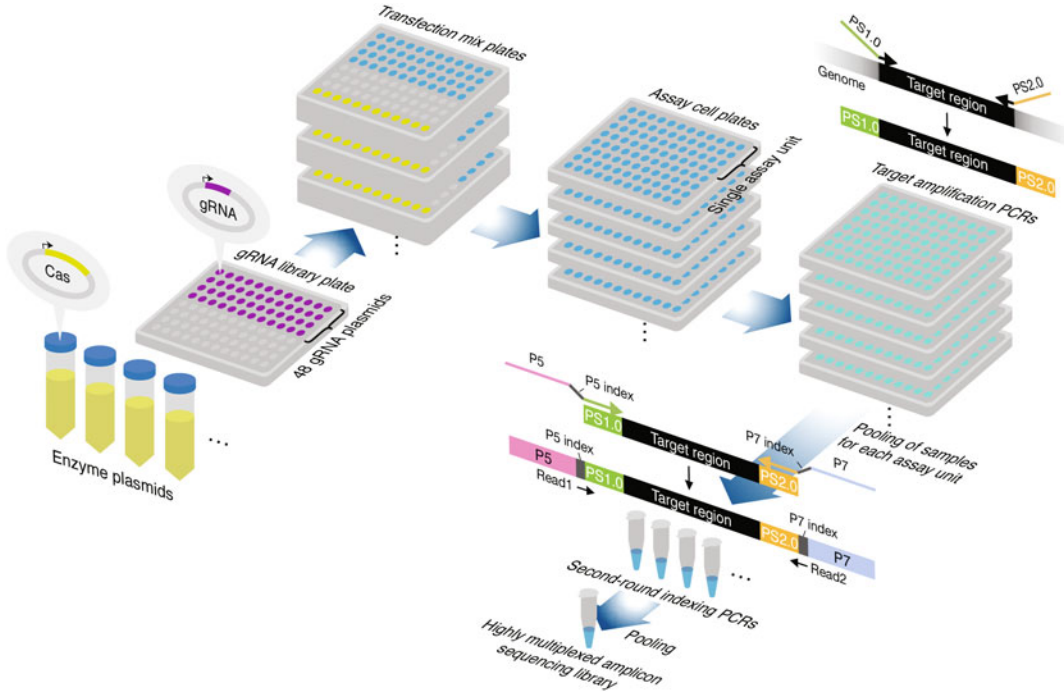

Fig. 2 Overview of the highly multiplexed amplicon sequencing protocol to analyze CRISPR genome editing outcomes and frequencies. First, the genome-editing enzyme plasmids and gRNA plasmids are assembled to produce all possible combinatorial transfection reagents. Then, highly parallel genome-editing assays are performed in triplicates by cell transfection. Next, cell samples are lysed and direct PCR is used to amplify the gRNA target regions with primers that have common adapter sequences (PS1.0 and PS2.0). The first-round PCR products are then pooled for each genome-editing enzyme of each replicate and the second-round PCR is performed to attach custom Illumina index adapters. After sample normalization, the amplicon sequencing libraries are pooled into a single multiplexed library and subjected to an Illumina sequencing

2 Materials

2.1 General Considerations

All the experimental steps described here use PCR-grade ultrapure distilled water (not autoclaved distilled water) to minimize the potential risks of nucleic acid and DNase/RNase contaminations that may affect the genome-editing assays and the quality of the sequencing libraries. We also recommend performing all the experiments using filtered tips on a clean bench. In the following sections, we provide a basic protocol to measure mutation outcomes of given genome-editing tools and a nonfunctional control enzyme (EGFP) condition for 48 different endogenous gRNA targeting sites of HEK293T cells in triplicates (hundreds to thousands of genome-editing assays). However, this protocol can be easily scaled up for an increased number of gRNAs and target sites, and the cell line also can be replaced with others with their own cell culture and transfection protocols.

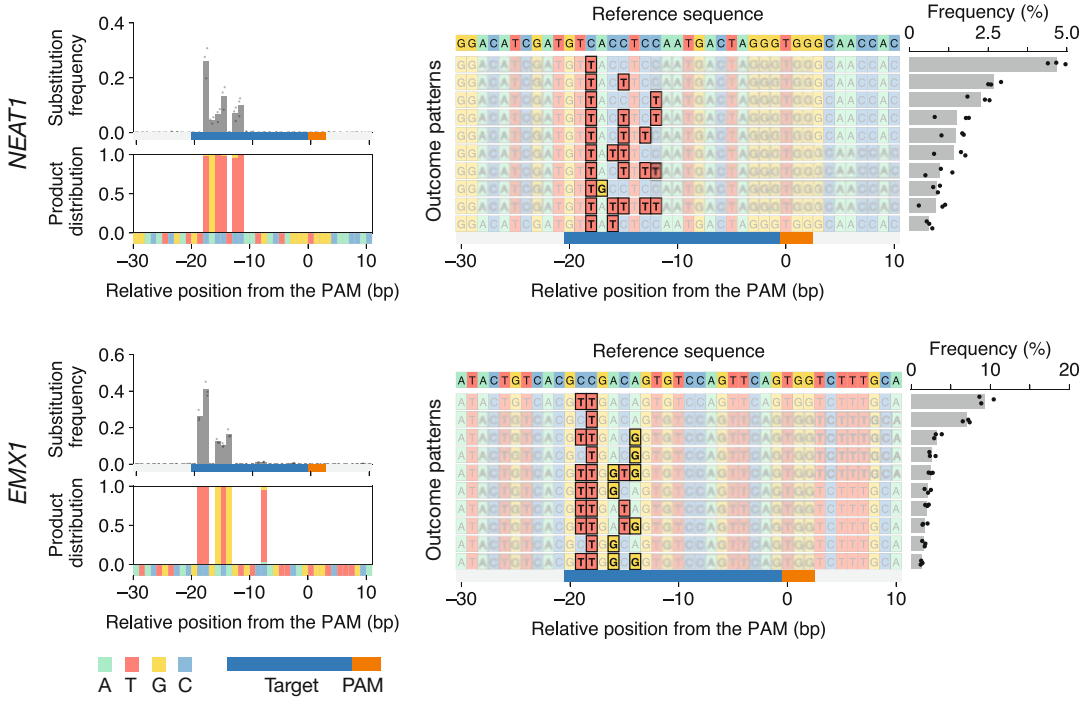

Fig. 3 Data analyses of two arbitrary selected genome-editing assays in triplicates multiplexed with a total of nearly two thousand assays. Base editing of *NEAT1* and *EMX1* encoding regions by Target-ACEmax

2.2 Screening of gRNA Target Sites and Target Amplification Primers

1. DNase/RNase-free ultrapure distilled water.
2. 8-strip individually capped PCR tubes.
3. Hard-Shell® 96-Well PCR Plates, low profile, thin wall, skirted, white/clear (Bio-Rad HSP9601).
4. Microseal® 'C' PCR Plate Sealing Film (Bio-Rad MSC1001).
5. 5× Phusion HF Buffer (NEB B0518S).
6. Phusion DNA Polymerase (NEB M0530S).
7. dNTPs (25 mM each).
8. 1/100 diluted SYBR Green: mix 10 μL of SYBR™ Green I Nucleic Acid Gel Stain (Invitrogen S7563) and 990 μL of 100% dimethyl sulfoxide. Wrap with aluminum foil to protect from light and store at 4 °C.
9. 6× loading dye.
10. Target amplification of forward primers with PS1.0 adapter sequence: 5′-TAACTTACGGAGTCGCTCTACG XXXXXXX XXXXXXXXXXXX -3′ (Xs: annealing sequence of forward primer for target amplification).
11. Target amplification of reverse primers with PS2.0 adapter sequence: 5′-GGATGGGATTCTTTAGGTCCTG XXXXXXX XXXXXXXXXXXX -3′ (Xs: annealing sequence of reverse primer for target amplification).

2.3 Preparation of gRNA Expression Plasmid Library

1. DNase/RNase-free ultrapure distilled water.
2. DH5-alpha Competent *E. coli*.
3. Super Optimal broth with Catabolite repression (SOC) outgrowth medium.
4. T4 DNA Ligase (NEB).
5. 10× T4 Ligase Buffer with 10 mM ATP (NEB).
6. T4 Polynucleotide Kinase (NEB).
7. BsmBI (NEB R0580S).
8. 1 mg/mL BSA: mix 10 μL of 10 mg/mL BSA and 90 μL of ultrapure distilled water.
   Store at −20 °C.
9. gRNA cloning backbone plasmid (pSI-356 v2; available at Addgene https://www.addgene.org/158431/) (*see* **Note 1**).
10. DNA miniprep kit.
11. 100-mL disposable DNase/RNase-free reagent reservoirs.
12. 96-well DNase/RNase-free 2-mL round bottom deep-well plates.
13. 96-well DNase/RNase-free PCR plates.
14. Microseal® 'C' PCR Plate Sealing Films (Bio-Rad MSC1001) or any alternative that can perfectly seal up the wells.
15. Lysogeny broth (LB) medium: add 25 g of LB powder to distilled water, top up to 1 L, autoclave and store at 4 °C.
16. LB-Ampicillin medium: add 100 μL of 100 mg/mL ampicillin to 100 mL of LB medium.
17. LB-Ampicillin agar plate: add 15 g of agar, and 25 g of LB powder to distilled water in a glass bottle, top up to 1 L, autoclave, cool down to 55 °C, then add 1000 μL of 100 mg/mL ampicillin, and pour to bacterial petri dishes.
18. 15-mL bacterial culture tubes.
19. Sanger sequencing primer to validate gRNA spacer sequences.
    Use 5′- TTTCCCATGATTCCTTCATATTT -3′ for the gRNA cloning backbone plasmid above (compatible with any human U6 promoter-gRNA expression plasmid).

2.4 Culturing of Cells

1. Human embryonic kidney cells 293T (HEK293T cells).
2. 0.05 w/v% Trypsin-0.53 mmol/L EDTA-4Na Solution with Phenol Red.
3. Heat-inactivated fetal bovine serum (FBS): thaw FBS (Invitrogen 10437-028) at 4 °C overnight, incubate for 30 min in 56 °C water bath, and aliquot to 50-mL tubes. Store at −20 °C.

4. 1× Phosphate-Buffered Saline (PBS): mix 100 mL of 10× D-PBS(−) and 900 mL of DNase/RNase-free ultrapure distilled water in a 1 L glass bottle. Store at 4 °C.
5. 100× penicillin–streptomycin.
6. Cell culture medium: mix 500 mL of DMEM, 50 mL of heat-inactivated FBS, and 5 mL of 100× penicillin–streptomycin. Store at 4 °C.
7. 5 mM Acetic Acid (AcOH): add 500 μL of 1 M AcOH to 99.50 mL of DNase/RNase-free ultrapure distilled water in 200 mL glass bottle and autoclave. Store at 4 °C.
8. Collagen-I solution: mix 40 μL of Collagen Type I and 11.96 mL of 5 mM AcOH in a 15-mL tube. Store at 4 °C.
9. 96-well cell culture plates.
10. 10-cm cell culture dishes.

2.5 Massively Parallel Transfection of Genome-Editing Reagents

1. Genome-editing enzyme expression plasmids. For example, PpX165 is a wild-type SpCas9 nuclease expression plasmid for mammalian genome editing (Addgene 48137). A range of base editor plasmids also can be obtained from Addgene (https://www.addgene.org/crispr/base-edit/).
2. EGFP expression plasmid as non-functional enzyme control (pLV-eGFP, Addgene 36083, or any alternative).
3. Plasmid DNA midiprep Kit.
4. Plasmid DNA miniprep Kit.
5. 96-well DNase/RNase-free 1.1 mL round-bottom deep well plates.
6. Microseal® 'C' PCR Plate Sealing Film (Bio-Rad MSC1001).
7. 1 mg/mL Polyethylenimine (PEI) MAX solution: dissolve 10 mg PEI MAX (Polysciences 49553-93-7) in 10 mL of ultrapure distilled water, filter through a 0.22-μm filter, and aliquot 1000 μL into each 1.5-mL microcentrifuge tube with screw cap. Store at −20 °C for up to 12 months.

2.6 Template Genomic DNA Preparation

1. 50 mM NaOH: add 500 μL of 5 N NaOH to 49.5 mL of DNase/RNase-free ultrapure distilled water in a 50-mL tube. Store at 4 °C (*see* **Note 2**).
2. 1 M Tris–HCI, pH 8.0.
3. Hard-Shell® 96-Well PCR Plates, low profile, thin wall, skirted, white/clear (Bio-Rad HSP9601).
4. Microseal® 'C' PCR Plate Sealing Film (Bio-Rad MSC1001).

2.7 Generation of a Multiplexed Amplicon Sequencing Library and Sequencing

1. DNase/RNase-free ultrapure distilled water.
2. 8-strip individually capped PCR tubes.
3. Hard-Shell® 96-Well PCR Plates, low profile, thin wall, skirted, white/clear (Bio-Rad HSP9601).
4. Microseal® 'C' PCR Plate Sealing Film (Bio-Rad MSC1001).
5. 5× Phusion HF Buffer (NEB B0518S).
6. Phusion DNA Polymerase (NEB M0530S).
7. dNTPs (25 mM each).
8. 1/100 diluted SYBR Green: mix 10 μL of SYBR™ Green I Nucleic Acid Gel Stain (Invitrogen S7563) and 990 μL of 100% dimethyl sulfoxide. Wrap with aluminum foil to protect from light and store at 4 °C.
9. 6× loading dye.
10. Agencourt AMPure XP (Beckman Coulter A63881).
11. 1.5 mL DNA LoBind tubes (Eppendorf 0030122348).
12. 70% Ethanol: add 30 mL of 100% Ethanol to 70 mL of ultrapure distilled water. Store at 4 °C.
13. 1× Tris-EDTA (TE) buffer.
14. KAPA Library Quantification Kits (Illumina) (KAPA Biosystems KK4824, or any alternative).
15. Illumina MiSeq v3 600 cycles kit (Illumina MS-102-3003) or Illumina MiSeq v2 Micro 300 cycles kit (Illumina MS-103-1002).
16. Illumina PhiX control v3 (Illumina FC-110-3001).
17. Target amplification forward primers with PS1.0 adapter sequence: 5′-TAACTTACGGAGTCGCTCTACG XXXXXXX XXXXXXXXXXXXX-3′ (Xs: annealing sequence of forward primer for target amplification).
18. Target amplification reverse primers with PS2.0 adapter sequence: 5′-GGATGGGATTCTTTAGGTCCTG XXXXXXX XXXXXXXXXXXXX-3′ (Xs: annealing sequence of reverse primer for target amplification).
19. Custom Illumina P5 index primer: 5′-AATGATACGGCGAC CACCGAGATCTACACTCTTTCCCTACAC GACGCTCTTCCGATCT NNNNN YYYYYYYYY T AACTTACGGAGTCGCTCTACG-3′ (Ns: random sequence for better flow cell clustering; Ys: 9-bp custom index).
20. Custom Illumina P7 index primer: 5′-CAAGCAGAAGACGG CATACGAGATCGGTCTCGGCATTCCTGCT GAACCGCTCTTCCGATCT NNNNN YYYYYYYYY G GATGGGATTCTTTAGGTCCTG-3′ (Ns: random sequence for better flow cell clustering; Ys: 9-bp custom index).

2.8 List of Equipment

1. Micropipettes (0.2–2 μL, 2–20 μL, 20–200 μL, and 100–1000 μL).
2. 12-channel micropipettes (0.2–2 μL, 2–20 μL, and 20–200 μL).
3. 5–50-mL pipete.
4. Bench-top centrifuge.
5. Centrifuge with 96-well plate and 50-mL tube rotors.
6. Heat block.
7. 96-well aluminum block.
8. 1.5-mL tube aluminum block.
9. Thermal cycler machine.
10. Agarose gel imager.
11. Agarose gel electrophoresis bath.
12. Quantitative PCR machine.
13. Vortex mixer.
14. Bacterial incubator.
15. Shaking incubator.
16. Human cell culture incubator (including CO_2 controller and aspirator).
17. Cell culture hoods (bacterial and human cell culture).
18. Automated cell counter.
19. Water baths (37 °C for human cells and 42 °C for bacterial transformation).
20. Plate seal roller.
21. Spectrophotometer.
22. Magnet stand (1.5-mL tube or 96-well plate format).
23. Illumina sequencer.

3 Methods

3.1 Screening of gRNA Target Sites and Amplification Primers

Because target genomic regions need to be stably amplified by PCR and the following genome editing assay procedures need to align with such target regions, we first screen two or three primer pairs for each candidate gRNA target region (Fig. 4), and discard candidate gRNAs with no stable PCR amplification with any of the tested primer pairs. To secure a certain number of gRNAs to be used in the genome editing assays, primer pairs for an excess number of gRNA target sites can be screened in this step. The PCR efficiencies and expected band sizes of primer pairs are evaluated by qPCR and agarose gel electrophoresis. This section describes screening of three primer pairs for 48 genomic gRNA target sites (a total of 144 primer pairs).

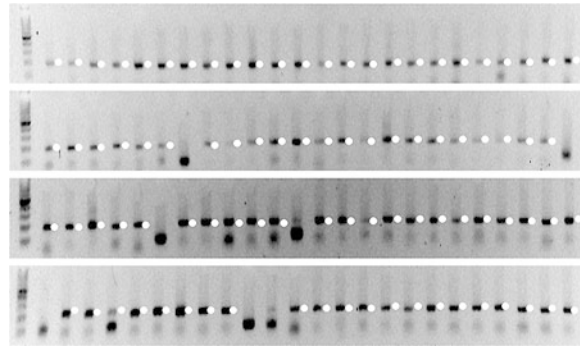

Fig. 4 Example of PCR primer screening for stable amplification of gRNA target regions. PCR products with the expected band sizes are marked with white dots

3.1.1 Primer Preparation

1. Design three PCR primer pairs for each of 48 selected gRNA targeting regions and order DNA oligos (144 forward and 144 reverse primer oligos). *See* Subheading 2.2 for the adapter sequences added to the target amplification primers. Using Primer3 or any alternative, design primer pairs each to have a melting temperature of 55–65 °C without homopolymer or palindrome sequences and to produce a PCR product of 150–230 bp for the pooled library preparation (*see* Subheading 3.6.1 for details).
2. Mix 5 μL of 100 μM forward primer and 5 μL of 100 μM reverse primer to prepare a 50 μM primer mix in each well of fresh 96-well plates using a 12-channel pipette.
3. Prepare 10 mL ultrapure distilled water in a disposable regent reservoir.
4. Transfer 40 μL of ultrapure distilled water from the reservoir to each sample well of the primer mix plates using a 12-channel pipette to obtain 10 μM primer mixes.
5. Seal the primer mix plates with optically clear adhesive PCR films (we recommend the Bio-Rad films described in Subheading 3 throughout the study for good sealability). The primer mix plates can be stored at −20 °C.

3.1.2 Template Genomic DNA Preparation

1. Place a DMEM bottle in a 37 °C water bath to warm up.
2. Thaw one vial of HEK293T cells in a 37 °C water bath and transfer the cells into 5 mL of the prewarmed DMEM in a 15-mL sample tube and centrifuge the cells at 200 × *g* for 5 min.
3. Aspirate and remove the supernatant.
4. Add 10 mL of the prewarmed DMEM into the sample tube.
5. Gently mix the cells by pipetting several times.

6. Transfer the cells into a 10-cm cell culture dish.
7. Incubate the cell culture dish for 2–3 days in a cell culture incubator at 37 °C and 0.05% CO_2 to allow cell proliferation to 80–90% confluency (*see* **Note 3**).
8. Remove the media using an aspirator and apply 1000 μL of 1× PBS to the cell culture dish.
9. Remove the reagent using an aspirator and apply 1000 μL of 0.05% trypsin-EDTA to the cell culture dish.
10. Incubate the cell culture dish for 4 min in a cell culture incubator at 37 °C and 0.05% CO_2.
11. Remove the reagent using an aspirator and add 1000 μL of 50 mM NaOH to the cell culture dish for cell lysis.
12. Transfer the sample to a 2-mL tube and incubate at 95 °C for 15 min on a heat block, place on ice for 5 min, and neutralize the sample with 100 μL of 1 M Tris–HCl (pH 8.0). This cell lysate sample can be stored at −20 °C.

3.1.3 Primer Screening by qPCR

1. To perform the following quantitative PCR (qPCR) for each of the 144 primer pairs, prepare 180× qPCR master mix in a 5 mL sample tube (*see* **Note 4**):

1× reaction unit

Component	Final concentration	Volume (μL)
5× Phusion HF buffer	1×	4
2 mM dNTPs	200 μM	2
10 μM primer mix	500 nM each	2
DNA template (lysed cell sample)		2
Phusion DNA polymerase		0.2
1/100 diluted SYBR green		0.06
Ultrapure distilled water		9.74
Total		20

180× qPCR master mix

Component	Volume (μL)
5× Phusion HF buffer	720
2 mM dNTPs	360
DNA template (lysed cell sample)	360
Phusion DNA polymerase	36

(continued)

Component	Volume (μL)
1/100 diluted SYBR green	10.8
UltraPure distilled water	1753.2
Total	3240

2. Transfer the qPCR master mix into a disposable reagent reservoir placed on ice.
3. Using a 12-channel pipette, transfer 18 μL of the qPCR master mix from the reservoir to each well of fresh 96-well PCR plates.
4. Using a 12-channel pipette, add candidate primer pair mixes, 2 μL each, to the qPCR reaction plates.
5. Seal the qPCR reaction plates with optically clear adhesive PCR films.
6. Centrifuge the qPCR reaction plates at 1000 × *g* for 2 min to spin down and remove air bubbles.
7. Perform qPCR with the following thermal cycle conditions:

Step	Temperature	Time
1	98 °C	0'30
2	98 °C	0'10
3	60 °C	0'10
4	72 °C	1'00
Go to 2		(29 times)
6	72 °C	5'00
7	4 °C	∞

8. Save the qPCR results with Ct values for the primer pairs.
9. Centrifuge the qPCR reaction plates at 1000 × *g* for 2 min and gently remove the plate seals.
10. Prepare 6× loading dye in a disposable reagent reservoir and transfer 4 μL to each sample well of the qPCR reaction plate using a 12-channel pipette and mix well.
11. Run a gel for the PCR products, 5 μL each, using 2% agarose gel for 20 min with 135 V.
12. Take gel images and evaluate PCR band sizes and amplification efficiencies with C_t values.
13. Select the best primer pair for each of the gRNA target sites and rearray the curated 10 μM primer pair mixes into a fresh 96-well PCR plate.

14. Seal the curated primer mix plate with optically clear adhesive PCR films.
15. Centrifuge the curated primer mix plate at 1000 × *g* for 2 min and store at −20 °C.

3.2 Preparation of gRNA Expression Plasmid Library

After determining the gRNA target sites and their corresponding primers, gRNA expression plasmids are prepared according to the following procedure. Two ssDNA oligos encoding the gRNA spacer region are prepared for each target site, annealed, and phosphorylated (Fig. 5a). The dsDNA insert is then ligated into a digested plasmid backbone. The assembled DNA is transformed into bacterial cells, and the plasmid clones are obtained by colony isolation (Fig. 5b), followed by miniprep and validated by Sanger sequencing. This gRNA library preparation protocol allows fast preparation of 48–96 gRNA expression plasmids within a week. gRNA libraries can be prepared for other CRISPR–Cas systems by changing the dsDNA insert design and plasmid backbone.

3.2.1 Preparation of gRNA Spacer Inserts

1. Spin down forward and reverse ssDNA oligo plates (100 μM each) (*see* **Note 5**). The ssDNA pairs need to be designed to produce sticky 5′ (CACC), and 3′ (AAAC) ends after annealing.
2. Add 10 μL of forward oligo and 10 μL of reverse oligo into a fresh 96-well PCR plate using a 12-channel pipette. The ssDNA mix plate can be stored at −20 °C.
3. To perform the following annealing and phosphorylation reactions for 48 samples, prepare 50× reaction master mix.

1× reaction unit

Component	Volume (μL)
ssDNA oligo mix (50 μM each)	2.0
10× T4 ligase buffer with 10 mM ATP	0.5
T4 polynucleotide kinase	0.25
Ultrapure distilled water	2.25
Total	5.0

50× reaction mix

Component	Volume (μL)
10× T4 ligase buffer with 10 mM ATP	25
T4 polynucleotide kinase	12.5
Ultrapure distilled water	112.5
Total	150

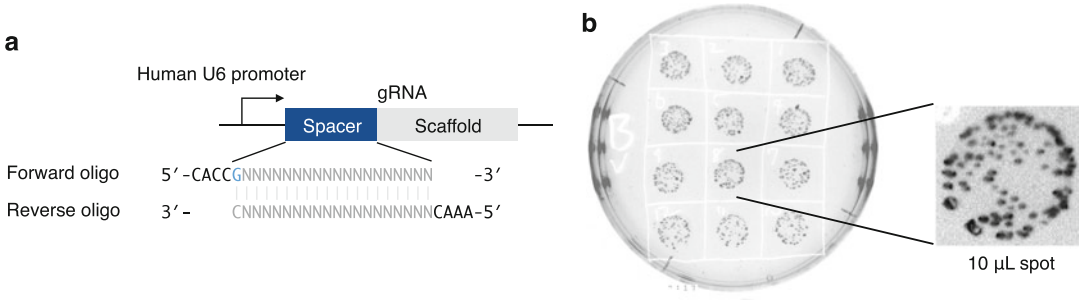

Fig. 5 Cloning of gRNA expression plasmids. (**a**) A dsDNA insert encoding a gRNA spacer is prepared by annealing of ssDNA oligos and cloned to a plasmid backbone. (**b**) Colony isolation to obtain clones of the assembled gRNA products

4. Aliquot 3 µL of the reaction mix into each well of a fresh 96-well PCR plate.
5. Add the ssDNA mixes, 2 µL each, to the reaction plate and assemble with the reaction mix.
6. Seal the reaction plate with an optically clear adhesive PCR film.
7. Centrifuge the reaction plates at 1000 × *g* for 30 s.
8. Incubate the reaction plate in a thermal cycler with the following conditions.

Step	Temperature	Time
1	37 °C	30'00
2	95 °C	5'00
3	95 °C, −1 °C/cycle	0'12
Go to 3		(69 times)
4	25 °C	∞

9. Prepare 10 mL ultrapure distilled water in a disposable reagent reservoir.
10. Remove the optically clear adhesive PCR films.
11. Add 95 µL of ultrapure distilled water to each reaction well using a 12-channel pipette and mix well.
12. Seal the plate with an optically clear adhesive PCR film.
13. Proceed to the next step, or store the dsDNA insert plate at −20 °C.

3.2.2 Ligation Assembly

1. To perform the ligation assembly for each dsDNA insert, prepare 50× reaction mix.

1× reaction unit

Component	Volume (μL)
1/10 diluted annealed oligo	2.5
10× T4 ligase buffer with 10 mM ATP	0.625
1 mg/mL BSA	0.31
T4 DNA ligase	0.2
BsmBI	0.2
25 ng/μL backbone plasmid (pSI-356 v2)	0.25
Ultrapure distilled water	2.165
Total	6.25

50× reaction mix

Component	Volume (μL)
10× T4 ligase buffer with 10 mM ATP	31.25
1 mg/mL BSA	15.5
T4 DNA ligase	10.0
BsmBI	10.0
25 ng/μL backbone plasmid (pSI-356 v2)	12.5
Ultrapure distilled water	108.25
Total	187.5

2. Add 2.5 μL of insert dsDNA each into a fresh 96-well PCR plate.
3. Add 3.75 μL of the ligation assembly mix into each well and slowly mix with the insert dsDNA by pipetting up and down.
4. Seal the reaction plate with an optically clear adhesive PCR film.
5. Spin down the reaction plate.
6. Incubate the reaction plate in a thermal cycler with the following conditions.

Step	Temperature	Time
1	37 °C	5'00
2	20 °C	5'00
Go to 1		(14 times)
3	55 °C	30'00
4	4 °C	∞

7. Proceed to the next step, or store the reaction plate −20 °C.

3.2.3 Bacterial Transformation

1. Thaw chemically competent *E. coli* cells on ice (a total volume of 480 μL for 48 reactions).
2. Aliquot 10 μL of the competent cells into a fresh 96-well PCR plate.
3. Put a 96-well aluminum block on ice to cool down.
4. Transfer 2 μL of the ligation assembly product into each sample well of the transformation reaction plate using a 12-channel pipette.
5. Seal the transformation reaction plate with an optically clear adhesive PCR film.
6. Place the transformation reaction plate on the ice-chilled 96-well aluminum block and incubate on ice for 30 min.
7. Heat-shock the transformation samples at 42 °C for 30 s by floating the reaction plate on a preheated water bath.
8. Immediately place the transformation reaction plate on the ice-chilled aluminum block and incubate for 2 min.
9. Remove the optically clear adhesive PCR film.
10. Add 50 μL SOC outgrowth medium into each sample well of the reaction plate using a 12-channel pipette.
11. Seal the reaction plate with an optically clear adhesive PCR film.
12. Place the reaction plate in a 37 °C bacterial incubator and incubate for 10 min.
13. During the incubation, airdry 5–6 LB+Amp plates on a clean bench.
14. Remove the optically clear adhesive PCR film and briefly mix the samples by pipetting up and down several times using a 12-channel pipette.
15. Spot 10 μL of the transformation sample each on an LB+Amp agar plate (we usually spot 12 samples per 10-cm petri dish).
16. Incubate the LB+Amp sample plates overnight in a 37 °C bacterial incubator.
17. The bacterial plates should be stored at 4 °C until the obtained clones are validated in the following quality check procedure.

3.2.4 Plasmid Purification and Sanger Sequencing

1. Isolate two to three colonies for each gRNA and inoculate each to 5 mL LB+Amp liquid media in a 15-mL culture tube (*see* **Note 6**).
2. Incubate the sample tubes overnight at 37 °C using a tube rotator or shaker.
3. Purify plasmid DNAs by miniprep.

4. Validate the inserts by Sanger sequencing. The sequencing primer sequence is provided in Subheading 2.3.
5. Store the validated plasmid DNAs at −20 °C.

3.3 Culturing of Cells

Here we describe the culturing of HEK293T cells that are used for the genome-editing assay (described in Subheading 3.4). HEK293T cells are expanded and seeded into the wells of 96-well cell culture plates. One and a half 96-well cell culture plates are required for each genome-editing enzyme (or control enzyme) to perform 48 genome-editing assays with unique gRNAs in triplicates.

3.3.1 Collagen Coating of Cell Culture Plates

1. Prepare a 20-mL collagen-I coating solution in a disposable reagent reservoir.
2. Apply 50 μL of collagen-I coating solution into each well of fresh 96-well cell culture plates using a 12-channel pipette.
3. Incubate 30 min in a cell culture incubator at 37 °C and 0.05% CO_2.
4. Remove the solution from the cell culture plates using a 12-channel pipette.
5. Add 50 μL 1× PBS to each well of the cell culture plates to wash the collagen-I coating solution and remove the reagent using a 12-channel pipette.
6. Proceed to the next step, or the collagen-coated 96-well cell culture plates can be stored for at least 1 week at room temperature, wrapped with aluminum foil.

3.3.2 Seeding Cells into 96-Well Collagen-Coated Cell Culture Plates

1. Place a DMEM bottle in a 37 °C water bath to warm up.
2. Thaw 2-mL HEK293T cell stock vials in a 37 °C water bath. Make sure to have enough cells for a target screening space.
3. Transfer the cells from each stock vial to 5 mL of the prewarmed DMEM in a 15-mL sample tube and centrifuge at 200 × *g* for 5 min.
4. Aspirate and remove the supernatant.
5. Add the prewarmed DMEM, 10 mL each, into the sample tubes.
6. Gently mix the cells by pipetting several times.
7. Transfer the cells from each sample to a 10-cm cell culture dish.
8. Incubate the cell culture dishes for 2–3 days in a cell culture incubator at 37 °C and 0.05% CO_2 to allow cell proliferation to 80–90% confluency (*see* **Note 3**).
9. Remove the medium using an aspirator.

10. Apply 1× PBS, 1000 μL each, to the cell culture dishes.
11. Remove the reagent from the cell culture dishes using an aspirator.
12. Apply 0.05% trypsin-EDTA, 1000 μL, each to the cell culture dishes.
13. Incubate the cell culture dishes for 4 min in a cell culture incubator at 37 °C and 0.05% CO_2.
14. Apply DMEM, 1000 μL each, to the cell culture dishes and slowly mix by pipetting to obtain a cell suspension.
15. Transfer the cells from each cell culture dish into a 15-mL tube containing 5 mL of DMEM.
16. Quantify cell concentrations using a cell counter and adjust to 25 cells/μL using DMEM.
17. Transfer the cells from the sample tubes into a sterile disposable reagent reservoir.
18. Transfer cell sample, 200 μL each (5000 cells), to the collagen-coated 96-well cell culture plate wells using a 12-channel pipette. For each of the genome-editing or control enzymes, three four-row units (i.e., three units of rows A–D or E–H of 96-well cell culture plates) are required to allow genome-editing assays with 48 gRNAs in triplicates.
19. Incubate the assay plates for 12–15 h in a cell culture incubator at 37 °C and 0.05% CO_2 and immediately proceed with the next step (*see* **Note 7**).

3.4 Massively Parallel Transfection of Genome-Editing Reagents

Here we describe massively parallel transfections of genome-editing reagents to HEK293T cells in the 96-well plates prepared as described in Subheading 3.3 with an efficient strategy for assembling different gRNAs and different genome-editing enzymes using a 12-channel pipette (Fig. 6). Each reaction transfects 120 ng of a genome-editing (or control) enzyme expression plasmid and 40 ng of a gRNA expression plasmid to the cells using PEI Max transfection reagent. After transfection, the cells are incubated for 3 days to induce genome editing. The assay conditions may need to be modified for other cell lines by changing input plasmid amounts, transfection reagent, cell density, and/or incubation time.

3.4.1 gRNA Reagent Preparation

1. Measure the DNA concentration of each gRNA expression plasmid using a spectrophotometer immediately before the transfection assay.
2. Adjust concentrations of gRNA plasmids to 25 ng/μL using ultrapure distilled water and assemble, 100 μL each, in a fresh 96-well PCR plate. Fill rows A–D of the PCR plate for

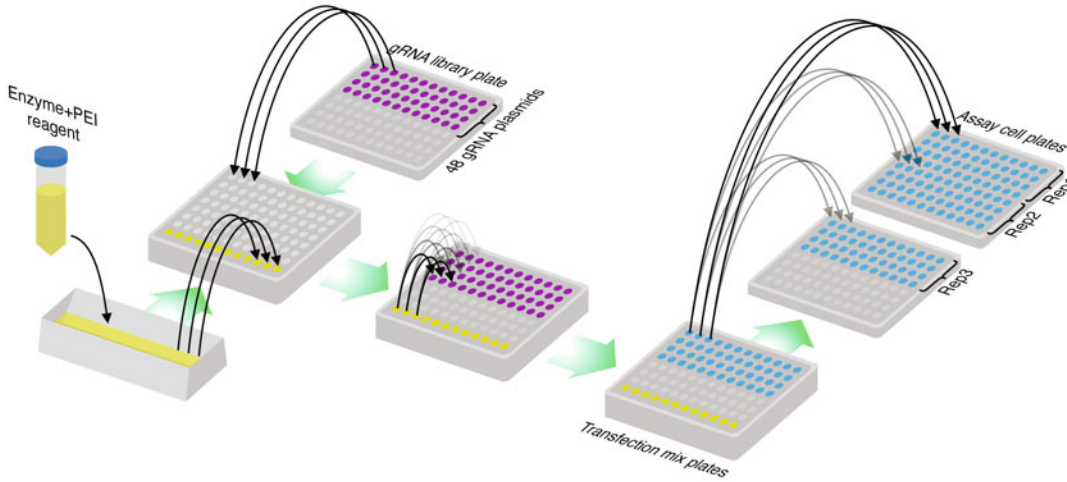

Fig. 6 Assembly and transfection of genome editing reagents for each enzyme

48 gRNAs, so that the well positions are in concordance with the well positions of the target amplification primers in the curated primer mix plate.

3. Seal the gRNA library plate with an optically clear adhesive PCR film.
4. Centrifuge the gRNA library plate at 1000 × *g* for 2 min. The plate can be stored long term at −20 °C.

3.4.2 Transfection

1. Prepare genome-editing enzyme plasmids and a control enzyme plasmid by midiprep.
2. Measure and adjust the DNA concentrations of each enzyme plasmid to 500 ng/μL (*see* **Note 8**).
3. To perform genome-editing assays for 48 gRNAs in triplicates, prepare 200× enzyme mix in a 15-mL sample tube for each of the genome-editing and control enzymes. Note that a sufficient excess of the reagent mix is necessary for the following two layers of aliquoting procedures.

1× reaction unit

Component	Volume (μL)
500 ng/μL genome-editing or control plasmid	0.24
25 ng/μL gRNA plasmid	1.6
1 mg/mL PEI Max	0.48
1× PBS	50.0
Total	52.32

200× reaction mix

Component	Volume (μL)
500 ng/μL genome-editing or control plasmid	48
1 mg/mL PEI max	320
1× PBS	10,000
Total	10,368

4. Mix the enzyme mixes by vortexing.
5. For each genome-editing (or control) enzyme, prepare a fresh 96-well round-bottom deep-well plate as the transfection mix plate and aliquot 800 μL of the enzyme mix to every well in row H (H1–H12).
6. Take gRNA expression plasmids, 5.5 μL each, from the gRNA library plate (48 samples) and apply to the corresponding positions of the transfection mix plates (rows A–D) for each genome-editing (or control) enzyme using a 12-channel pipette.
7. For each genome-editing (or control) enzyme, using a 12-channel pipette, take 180 μL of the enzyme mix from every well of row H in the transfection mix plate and apply it to row A of the same transfection mix plate and mix well by pipetting. Repeat the same procedure for destination rows B–D.
8. Seal the transfection mix plates with optically clear adhesive PCR films and incubate for 15 min at room temperature.
9. Retrieve the assay plates prepared in Subheading 3.3.2 from the cell culture incubator.
10. Apply transfection mixes, 52.32 μL each, to the designated wells of the assay plates using a 12-channel pipette. Note that each genome-editing (or control) enzyme assay requires three four-row units of the assay plates for genome-editing assays with 48 unique gRNAs in triplicates (i.e., a four-row unit of a transfection mix is stamped three times to one and a half 96-well assay plates).
11. Incubate the assay plates for 72 h in a cell culture incubator at 37 °C and 0.05% CO_2.

3.5 Template Genomic DNA Preparation

Here we describe genomic DNA template preparation for the amplicon sequencing procedure. The genome-editing treated cell samples in the 96-well assay plates are lysed with NaOH and boiling. and neutralized by Tris–HCl. This simple cell lysis is sufficient for the downstream amplicon sequencing library preparation procedure. Unlike column-based genomic DNA extraction protocols, this protocol processes one assay sample plate (96 samples) in 20 min with minimal hands-on time and also can be implemented for use with robotic liquid handling systems for further scalability.

1. Remove cell culture medium (about 250 μL each) from the assay plates using a 12-channel pipette (*see* **Note 9**).
2. Put 20 mL of freshly prepared 50 mM NaOH in a disposable reagent reservoir.
3. Using a 12-channel pipette, transfer 50 μL of the NaOH solution to every sample well of the assay plates.
4. Using a 12-channel pipette, carefully and gently transfer the entire lysed cell samples to fresh 96-well PCR plates. The cell lysate will be sticky. Slowly pipet the samples.
5. Seal the sample PCR plates with optically clear adhesive PCR films.
6. Centrifuge the sample PCR plates at 1050 × *g* for 2 min.
7. Incubate the sample PCR plates at 95 °C for 15 min, then cool to 4 °C using a thermal cycler.
8. Centrifuge the sample PCR plates at 1050 × *g* for 2 min.
9. Prepare 10 mL of 1 M Tris–HCl (pH 8.0) in a disposable reagent reservoir.
10. Remove the adhesive PCR films and carefully add 5 μL of the Tris–HCl solution into every sample well of the sample PCR plates for neutralization using a 12-channel pipette. The Tris–HCl volume should be 10% of the input NaOH volume.
11. Seal the sample PCR plates again with optically clear adhesive PCR films (*see* **Note 10**).
12. Centrifuge the sample PCR plates at 1050 × *g* for 2 min to spin down the remaining cell lysates on well walls and to remove air bubbles.
13. Proceed to the next step, or the cell lysate plates can be store at −20 °C for a few months or −80 °C for a long time.

3.6 Generation of a Multiplexed Amplicon Sequencing Library and Sequencing

In this final section, we describe parallel amplification of genome-editing target sites, pooling and indexing of the PCR products for each genome-editing (or control) enzyme, and generation of a multiplexed amplicon sequencing library with quality controls for one-shot Illumina sequencing to evaluate hundreds to thousands of genome-editing samples. Target genomic regions of genome-edited samples are PCR amplified using the corresponding primers screened in Subheading 3.1. Then, PCR products of similar sizes are all pooled for each genome-editing enzyme of each assay replicate and subjected to magnetic bead-based purification. The pooled PCR samples are reamplified by a second-round PCR to add Illumina sequencing adapters with unique indices. The indexed sequencing libraries are separately size-selected by agarose gel electrophoresis and quantified by qPCR (Fig. 7). Equal molar quantities of the indexed libraries are further pooled as a single multiplexed library and analyzed using an Illumina sequencer.

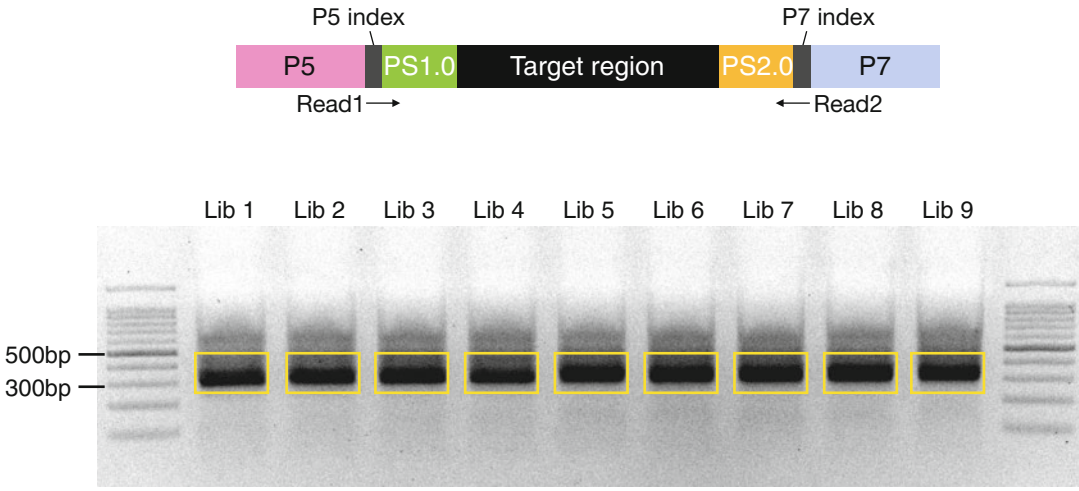

Fig. 7 Preparation of amplicon sequencing libraries. The first-round PCR product pools of different assay enzymes of different replicates are reamplified by a second-round PCR. The PCR products are then size-selected by agarose gel electrophoresis. The yellow boxes show the typical area for the size selection

After sequencing, sequencing reads can be demultiplexed into individual genome-editing assays according to their custom indices and endogenous target sequences. Sequencing reads of each assay sample can be used for mutation outcome pattern analyses.

3.6.1 PCR Amplification of the Target Regions

1. Thaw the curated primer mix plates (48 primer pairs) established in Subheading 3.1 at room temperature and centrifuge at 1000 × *g* for 2 min.
2. Retrieve the cell lysate plates established in Subheading 3.5 (thaw if frozen) and centrifuge at 1000 × *g* for 2 min.
3. To perform the following target amplification PCRs, prepare 180× PCR master mix for each genome-editing (or control) enzyme for a total of triplicate samples (scale the master mix volume with the number of genome-editing and control enzymes used).

   1× reaction unit

Component	Final concentration	Volume (μL)
5× Phusion HF buffer	1×	4
2 mM dNTPs	200 μM	2
10 μM primer mix	500 nM each	2
DNA template (cell lysate)		2
Phusion DNA polymerase		0.2
Ultrapure distilled water		9.8
Total		20

180× PCR master mix

Component	Volume (μL)
5× Phusion HF buffer	480
2 mM dNTPs	240
Phusion DNA polymerase	24
Ultrapure distilled water	1764
Total	2508

4. Transfer the PCR reaction mix into a disposable reagent reservoir placed on ice.
5. Prepare the same number of fresh 96-well PCR plates as the assay plates prepared in Subheading 3.3.2. Label the PCR plates to correspond to the assay plates. Using a 12-channel pipette, transfer the PCR master mix, 17 μL each, from the reservoir to all the wells of the PCR plates.
6. Using a 12-channel pipette, take 2 μL each from the four-row unit of the curated primer mix plate (rows A–D) and apply them to the top four-row unit (rows A–D) of the first PCR plate. Repeat the same procedure until both of the top and bottom four-row units of the all the PCR plates are filled up.
7. Using a 12-channel pipette, take 2 μL each from the cell lysate plates and apply them to the corresponding well positions of the PCR plates. Gently mix the samples by pipetting.
8. Seal the PCR plates with optically clear adhesive PCR films.
9. Centrifuge the PCR plates at 1000 × *g* for 2 min.
10. Perform the first-round target amplification PCR with the following thermal cycle conditions.

Step	Temperature	Time
1	98 °C	0'30
2	98 °C	0'10
3	60 °C	0'10
4	72 °C	1'00
Go to 2		(29 times)
5	72 °C	5'00
6	4 °C	∞

11. Centrifuge the first-round PCR sample plates at 1000 × *g* for 2 min.

12. Proceed to the bead-based DNA purification step, or store the PCR sample plates at −20 °C.

3.6.2 Purification of the First PCR Products

1. Retrieve AMPure XP beads from a fridge, settle to room temperature, and vortex thoroughly before use (*see* **Note 11**).
2. Centrifuge the first-round PCR sample plates at 1000 × *g* for 2 min.
3. Gently remove the plate seals.
4. For each genome-editing (or control) enzyme of each assay replicate, pool and combine the first-round PCR products, 3 μL each, in a 1.5-mL tube. This yields 144-μL PCR sample pools each containing amplicon products for 48 gRNA targeting regions.
5. Add 259.2 μL of AMPure XP beads (1.8× volume) to each first-round PCR sample pool and thoroughly mix by pipetting up and down several times without making bubbles.
6. Incubate the sample tubes at room temperature for 5 min to allow PCR products to bind to the beads.
7. Place the sample tubes on a magnetic stand and wait for 1 min to allow magnetic separation of beads.
8. Without removing the sample tubes from the magnetic stand, carefully remove all of the clear supernatants.
9. Add 500 μL of 70% EtOH to each sample tube on the magnetic stand and wait for 30 s.
10. Without removing the sample tubes from the magnetic stand, carefully remove all of the clear supernatants.
11. Repeat **steps 9** and **10** one more time. Make sure no EtOH remains in the sample tubes.
12. Remove the sample tubes from the magnetic stand and airdry for 1–3 min.
13. Apply 50 μL of ultrapure distilled water and gently mix with the magnetic beads by pipetting.
14. Incubate the sample tubes at room temperature for 2 min to allow PCR products to elute in the water solvent.
15. Place the sample tubes on the magnetic stand and wait for 2 min to allow magnetic separation of beads.
16. Without removing the samples tubes from the magnetic stand, transfer the clear supernatants each to a fresh 1.5-mL tube.
17. Measure DNA concentrations using a spectrophotometer.
18. Adjust sample concentrations to 10 ng/μL with ultrapure distilled water.

19. Proceed with the next pooled indexing PCRs, or store the first-round PCR pool samples at −20 °C.

3.6.3 Pooled Indexing PCR for Multiplexed Sequencing

1. Vortex the first-round PCR pool samples and spin down well using a benchtop centrifuge.
2. To perform the following second-round indexing PCR for each pooled sample, prepare 1.1× second-round PCR master mix. Scale up the volume of the master mix with the number of the first-round PCR pool samples (the number of genome-editing or control enzymes multiplied by three for assay replicates).

1× reaction unit

Component	Final concentration	Volume (μL)
5× Phusion HF buffer	1×	8
2 mM dNTPs	200 μM	4
10 μM P5 index primer	500 nM	2
10 μM P7 index primer	500 nM	2
First-round PCR pool		1
Phusion DNA polymerase		0.4
Ultrapure distilled water		22.6
Total		40

1.1× master mix

Component	Volume (μL)
5× Phusion HF buffer	8.8
2 mM dNTPs	4.4
Phusion DNA polymerase	0.44
Ultrapure distilled water	24.86
Total	38.5

3. Aliquot the PCR master mix, 35 μL each, to individually capped eight-strip PCR tubes.
4. Apply unique combinations of custom Illumina P5 and P7 index primers to the PCR reaction tubes. *See* Subheading 2.7 for the design of the custom Illumina index primers.
5. Apply the first-round PCR sample pools 1 μL each to the PCR reaction tubes and mix gently by pipetting. Make sure unique index pairs are assigned to the first-round PCR sample pools.
6. Spin down the PCR reaction tubes using a benchtop centrifuge.

7. Perform PCR with the following thermal cycle conditions.

Step	Temperature	Time
1	98 °C	0'30
2	98 °C	0'10
3	60 °C	0'10
4	72 °C	2'00
Go to 2		(14 times)
5	72 °C	5'00
6	4 °C	∞

8. Perform gel electrophoresis analysis with 2% agarose gel to check the rough yields and size distributions of the reamplified PCR products.
9. For each reamplified PCR product, cut the gel to obtain the PCR product in the expected size range (Fig. 7) and purify the amplicon sequencing libraries using a PCR purification kit (*see* **Note 12**).
10. Elute the size-selected samples into 20 μL of ultrapure distilled water and collect the eluate in a 1.5-mL LoBind DNA tube.
11. Check the concentration for each amplicon sequencing library using a spectrophotometer (*see* **Note 13**).
12. Proceed to the next step below, or store the amplicon sequencing libraries at −20 °C.

3.6.4 Multiplexed Amplicon Sequencing

1. Retrieve the amplicon sequencing libraries (and thaw if frozen).
2. Vortex the sample tubes well and spin down using a benchtop centrifuge.
3. Using KAPA Library Quantification Kits (Illumina), quantify the absolute concentrations of the amplicon sequencing libraries according to the manufacturer's instruction (*see* **Note 14**).
4. Pool amplicon sequencing libraries in a 1.5-mL LoBind DNA tube so that each library is represented equally.
5. Using KAPA Library Quantification Kits (Illumina), quantify the absolute concentration of the multiplexed library according to the manufacturer's instruction.
6. Analyze the multiplexed amplicon sequencing library with 20% PhiX spike-in using an Illumina sequencer (*see* **Note 15**). The library can be stored long term at −20 °C (*see* **Note 16**).

4 Notes

1. We used pSI-356 v2 in our previous studies [4, 35]. pSI-356 v2 encodes the human U6 promoter and 2-kb filler sequence sandwiched by two BsmBI cloning sites followed by the gRNA scaffold sequence. The filler DNA fragment is replaced with a target spacer-encoding fragment to obtain a gRNA expression plasmid.
2. Prepare a fresh NaOH solution immediately before the experiment every time.
3. For better transfection efficiency, use low passage HEK293T cells that have undergone no more than 10 passages since their establishment.
4. PCR efficiency might be affected by the amount of cell lysate. The template cell lysate concentration may need to be optimized.
5. To make the gRNA construction easy with a 12-channel pipette, order forward and reverse ssDNA oligos in the 96-well format. Obtain corresponding forward and reverse ssDNA oligos in the same positions of two separate plates. We also recommend obtaining the ssDNA oligos dissolved in 100 μM water or TE instead of a dry shipping option that requires the additional steps of dissolving and normalizing the ssDNA oligo samples.
6. Two to three colonies are usually sufficient to obtain a correct plasmid clone.
7. Before proceeding to the next step, use a microscope to confirm that the seeded cells are uniformly distributed in the well and adhered to the flat well bottom.
8. Midiprep from a 150–200 mL bacterial culture with 12–15 h incubation at 37 °C should be sufficient to obtain a genome-editing enzyme expression plasmid for transfection assays with 48 gRNAs in triplicates several times. Note that some genome-editing enzyme expression plasmids are toxic for bacterial growth and may need further optimization in this step.
9. The experiment can be stopped here. After completely removing the culture medium, the assay plates can be stored at −20 °C.
10. Cell lysate samples are sticky. PCR films need to be removed gently to avoid cross-well sample contaminations.
11. AMPure XP beads are sufficient to remove residual primers and primer dimers from the first-round PCR products. We recommend size-selection using agarose gel electrophoresis when strong nonspecific band signals are observed in a gel. The

purity of a first-round PCR product greatly affects the result of the downstream second-round PCR reamplification with custom Illumina index adapters.

12. The second-round PCR products usually show smear bands on a gel in a range of ±50 bp from the expected product size. According to our amplicon sequencing data, we assume this may be caused by indel mutations induced by genome editing. We recommend to size-select a wider area of the PCR bands than what is for usual for molecular cloning.
13. If the measured concentration is more than 10 ng/μL by spectrophotometry, adjust the sample concentration to 5–10 ng/μL with ultrapure distilled water so that the sample quantity is within the range of the of standards in the downstream qPCR.
14. Because the size distribution of the amplicon sequencing library can be expected by design, we prefer to use qPCR for library quantification and normalization rather than other quantification methods, such as TapeStation or BioAnalyzer.
15. The amplicon sequencing library should be sequenced (paired-end) with 20%–30% PhiX spike-in because the library complexity for each sequencing cycle is relatively low, and this is known to restrict sequencing cluster identification, at least slightly, in an Illumina sequencer. From 20,000 to 50,000 sequencing reads for each genome-editing assay is usually sufficient for further data analyses. We usually choose MiSeq Micro (total output five million reads) for less than 200 genome editing assays or MiSeq v3 (total output 25 million reads) for larger assays.
16. DNA binds to the tube wall even if LoBind tubes are used. The sequencing library should be quantified by qPCR no more than 2–3 days before the sequencing run.

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