Journal of Visualized Experiments

Genome editing in mammalian cell lines using CRISPR-Cas

--Manuscript Draft--

Article Type:	Invited Methods Article - JoVE Produced Video
Manuscript Number:	JoVE59086R2
Full Title:	Genome editing in mammalian cell lines using CRISPR-Cas
Keywords:	CRISPR; CAS9; Cas12a; Genome editing; non-homologous end joining; homology-directed repair
Corresponding Author:	Meng How Tan Nanyang Technological University Singapore, - SINGAPORE
Corresponding Author's Institution:	Nanyang Technological University
Corresponding Author E-Mail:	mh.tan@ntu.edu.sg
Order of Authors:	Kaiwen Ivy Liu
	Norfala-Aliah Binte Sutrisnoh
	Yuanming Wang
	Meng How Tan
Additional Information:	
Question	**Response**
Please indicate whether this article will be Standard Access or Open Access.	Standard Access (US$2,400)
Please indicate the **city, state/province, and country** where this article will be **filmed**. Please do not use abbreviations.	Singapore

NANYANG TECHNOLOGICAL UNIVERSITY SINGAPORE

School of Chemical and Biomedical Engineering
62 Nanyang Drive
N1.2-B2-33
Singapore 637459
Singapore

Agency for Science, Technology and Research SINGAPORE

Genome Institute of Singapore
60 Biopolis Street
Genome Building Level 7
Singapore 138672
Singapore

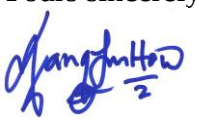
2 January 2019

Dear Dr. DSouza

We are pleased to submit to JoVE our revised manuscript, titled "**Genome editing in mammalian cell lines using CRISPR-Cas**". As requested, we have addressed all the editorial comments, with changes to our manuscript text highlighted using the "Track Changes" tool. The Table of Materials and one of the figures have been updated as well.

Please feel free to let us know if anything else is required. Thank you for your time.

Yours sincerely,



Meng How TAN, Ph.D.

TITLE:

Genome Editing in Mammalian Cell Lines using CRISPR-Cas

AUTHORS AND AFFILIATIONS:

Kaiwen Ivy Liu[1], Norfala-Aliah Binte Sutrisnoh[1], Yuanming Wang[1,2], Meng How Tan[1,2]

[1]Genome Institute of Singapore, Agency for Science Technology and Research, Singapore 138672, Singapore

[2]School of Chemical and Biomedical Engineering, Nanyang Technological University, Singapore 637459, Singapore

Corresponding Author:

Meng How Tan (mh.tan@ntu.edu.sg)

Email Addresses of Co-authors:

Kaiwen Ivy Liu (liukwi@gis.a-star.edu.sg)

Norfala-Aliah Binte Sutrisnoh (sutrisnohnb@gis.a-star.edu.sg)

Yuanming Wang (wangym@ntu.edu.sg)

KEYWORDS:

CRISPR, Cas9, Cas12a, genome editing, non-homologous end joining, homology-directed repair

SUMMARY:

CRISPR-Cas is a powerful technology to engineer the complex genomes of plants and animals. Here, we detail a protocol to efficiently edit the human genome using different Cas endonucleases. We highlight important considerations and design parameters to optimize editing efficiency.

ABSTRACT:

The clustered regularly interspaced short palindromic repeats (CRISPR) system functions naturally in bacterial adaptive immunity, but has been successfully repurposed for genome engineering in many different living organisms. Most commonly, the wildtype CRISPR associated 9 (Cas9) or Cas12a endonuclease is used to cleave specific sites in the genome, after which the DNA double-stranded break is repaired via the non-homologous end joining (NHEJ) pathway or the homology-directed repair (HDR) pathway depending on whether a donor template is absent or present respectively. To date, CRISPR systems from different bacterial species have been shown to be capable of performing genome editing in mammalian cells. However, despite the apparent simplicity of the technology, multiple design parameters need to be considered, which often leave users perplexed about how best to carry out their genome editing experiments. Here, we describe a complete workflow from experimental design to identification of cell clones that carry desired DNA modifications, with the goal of facilitating successful execution of genome editing experiments in mammalian cell lines. We highlight key considerations for users to take note of, including the choice of CRISPR system, the spacer length, and the design of a single-stranded oligodeoxynucleotide (ssODN) donor template. We envision that this workflow will be

useful for gene knockout studies, disease modeling efforts, or the generation of reporter cell lines.

INTRODUCTION:

The ability to engineer the genome of any living organism has many biomedical and biotechnological applications, such as the correction of disease-causing mutations, construction of accurate cellular models for disease studies, or generation of agricultural crops with desirable traits. Since the turn of the century, various technologies have been developed for genome engineering in mammalian cells, including meganucleases[1-3], zinc finger nucleases[4,5], or transcription activator-like effector nucleases (TALENs)[6-9]. However, these earlier technologies are either difficult to program or tedious to assemble, thereby hampering their widespread adoption in research and the industry.

In recent years, the clustered regularly interspaced short palindromic repeats (CRISPR)- CRISPR-associated (Cas) system has emerged as a powerful new genome engineering technology[10,11]. Originally an adaptive immune system in bacteria, it has been successfully deployed for genome modification in plants and animals, including humans. A primary reason why CRISPR-Cas has gained so much popularity in such a short time is that the element that brings the key Cas endonuclease, such as Cas9 or Cas12a (also known as Cpf1), to the correct location in the genome is simply a short piece of chimeric single guide RNA (sgRNA), which is straightforward to design and cheap to synthesize. After being recruited to the target site, the Cas enzyme functions as a pair of molecular scissors and cleaves the bound DNA with its RuvC, HNH, or Nuc domains[12-14]. The resulting double stranded break (DSB) is subsequently repaired by the cells via either the non-homologous end joining (NHEJ) or homology-directed repair (HDR) pathway. In the absence of a repair template, the DSB is repaired by the error-prone NHEJ pathway, which can give rise to pseudo-random insertion or deletion of nucleotides (indels) at the cut site, potentially causing frameshift mutations in protein-coding genes. However, in the presence of a donor template that contains the desired DNA changes, the DSB is repaired by the high fidelity HDR pathway. Common types of donor templates include single-stranded oligonucleotides (ssODNs) and plasmids. The former is typically used if the intended DNA changes are small (for example, alteration of a single base pair), while the latter is typically used if one wishes to insert a relatively long sequence (for example, the coding sequence of a green fluorescent protein or GFP) into the target locus.

The endonuclease activity of the Cas protein requires the presence of a protospacer adjacent motif (PAM) at the target site[15]. The PAM of Cas9 is at the 3' end of the protospacer, while the PAM of Cas12a (also called Cpf1) is at the 5' end instead[16]. The Cas-guide RNA complex is unable to introduce a DSB if the PAM is absent[17]. Hence, the PAM places a constraint on the genomic locations where a particular Cas nuclease is able to cleave. Fortunately, Cas nucleases from different bacterial species typically exhibit different PAM requirements. Hence, by integrating various CRISPR-Cas systems into our engineering toolbox, we can expand the range of sites that may be targeted in a genome. Moreover, a natural Cas enzyme can be engineered or evolved to recognize alternative PAM sequences, further widening the scope of genomic targets accessible to manipulation[18-20].

Although multiple CRISPR-Cas systems are available for genome engineering purposes, most users of the technology have relied mainly on the Cas9 nuclease from *Streptococcus pyogenes* (SpCas9) for multiple reasons. First, it requires a relatively simply NGG PAM, unlike many other Cas proteins that can only cleave in the presence of more complex PAMs. Second, it is the first Cas endonuclease to be successfully deployed in human cells[21-24]. Third, SpCas9 is by far the best characterized enzyme to date. If a researcher wishes to use another Cas nuclease, he or she would often be unclear about how best to design the experiment and how well other enzymes will perform in different biological contexts compared to SpCas9.

To provide clarity to the relative performance of different CRISPR-Cas systems, we have recently performed a systematic comparison of five Cas endonucleases – SpCas9, the Cas9 enzyme from *Staphylococcus aureus* (SaCas9), the Cas9 enzyme from *Neisseria meningitidis* (NmCas9), the Cas12a enzyme from *Acidaminococcus* sp. BV3L6 (AsCas12a), and the Cas12a enzyme from *Lachnospiraceae* bacterium ND2006 (LbCas12a)[25]. For a fair comparison, we evaluated the various Cas nucleases using the same set of target sites and other experimental conditions. The study also delineated design parameters for each CRISPR-Cas system, which would serve as a useful reference for users of the technology. Here, to better enable researchers to make use of the CRISPR-Cas system, we provide a step-by-step protocol for optimal genome engineering with different Cas9 and Cas12a enzymes (see **Figure 1**). The protocol not only includes experimental details but also important design considerations to maximize the likelihood of a successful genome engineering outcome in mammalian cells.

[Place **Figure 1** here.]

PROTOCOL:

1. Design of sgRNAs

1.1 Select an appropriate CRISPR-Cas system.

1.1.1 First, inspect the target region for the PAM sequences of all Cas9 and Cas12a nucleases that have been shown to be functional in mammalian cells[16,21-32]. Five frequently used enzymes are given in **Table 1** together with their respective PAMs.

NOTE: Besides the endonucleases listed in **Table 1**, there are other less commonly used Cas enzymes that have been successfully deployed in mammalian cells as well, such as a Cas9 nuclease from *Streptococcus thermophilus* (St1Cas9) that recognizes the PAM NNAGAAW. If the desired target site does not contain a known PAM, then one would not be able to use the CRISPR-Cas system for genome engineering.

[Place **Table 1** here.]

1.1.2 Second, consider any known properties of the target genomic locus or gene. Some properties to take into consideration include gene expression levels or chromatin accessibility and whether there are other closely related sequences as well.

NOTE: Certain enzymes are better suited for particular biological contexts. For example, to edit a repetitive genomic locus or a gene with several other close paralogs, it is recommended to use either AsCas12a (due to its lower tolerance for mismatches between the sgRNA and the target DNA than SpCas9 and LbCas12a) or SaCas9 (due to its requirement for longer spacers, which provides higher targeting specificity)[25].

1.2 Select a suitable spacer sequence. Identify as unique a sequence as possible to minimize the risk of off-target cleavage events, either by examining the target genome with BLAST[33] or by using several freely available online tools, such as: (a) Program from Feng Zhang's laboratory[34] (http://crispr.mit.edu/); (b) CHOPCHOP[35] (http://chopchop.cbu.uib.no/); (c) E-CRISP[36] (http://www.e-crisp.org/E-CRISP/); (d) CRISPOR[37] (http://crispor.tefor.net/); (e) Cas-OFFinder[38] (http://www.rgenome.net/cas-offinder/).

NOTE: The optimal length of the spacer can vary from 17 to 25 nucleotides (nt) inclusive, depending on which Cas enzyme is used (see **Table 1**). For Cas9, the spacer is upstream of the PAM, while for Cas12a, the spacer is downstream of the PAM. Additionally, HDR efficiency drops rapidly with increasing distance from the cut site. Hence, for precise DNA editing, position the sgRNA as close as possible to the intended modification site.

1.3 Synthesize DNA oligonucleotides with the appropriate overhangs for the CRISPR plasmid that is being used.

1.3.1 Add a G nucleotide in front of the spacer if the first position of the guide is not a G. Determine the reverse complementary sequence of the spacer. Add in the necessary overhangs for cloning purposes.

NOTE: By way of illustration, for the plasmids used in our evaluation study[25], the oligonucleotides to be synthesized are shown in **Table 2**. Use the example given in **Figure 2** as a guide, if necessary. Many CRISPR plasmids are available from commercial sources (e.g., Addgene). Some of the more popular plasmids are given in the **Table of Materials**.

[Place **Table 2** and **Figure 2** here.]

2. Cloning of oligonucleotides into a backbone vector

2.1 Phosphorylate and anneal sense and antisense oligonucleotides.

2.1.1 If the oligonucleotides are lyophilized, resuspend them to a 100 µM concentration in tris-ethylenediaminetetraacetic acid (EDTA) (TE buffer, see **Table of Materials**) or ddH_2O.

2.1.2 Prepare a 10 µL reaction mix containing 1 µL of sense oligonucleotide, 1 µL of antisense oligonucleotide, 1 µL of T4 DNA ligase buffer (10x), 1 µL of T4 polynucleotide kinase (PNK), and 6 µL of ddH_2O. Mix well by pipetting and place the reaction mix in a thermal cycler using the following parameters: 37 °C for 30 min, 95 °C for 5 min, and ramp down to 25 °C at 6 °C/min.

2.1.3 Dilute the reaction mix 1:100 in ddH_2O (e.g., 2 µL reaction mix + 198 µL ddH_2O).

2.2 Digest the CRISPR plasmid with an appropriate restriction enzyme.

NOTE: Cloning of sgRNAs typically rely on Golden Gate assembly with type IIs restriction enzymes. Different enzymes may be used for different CRISPR plasmids. For pSpCas9, use BbsI or BplI (see **Figure 3**). For pSaCas9, pNmCas9, pAsCas12a, and pLbCas12a, use BsmBI.

[Place **Figure 3** here.]

2.2.1 Prepare a 20 µL reaction mix containing 1 µg of circular plasmid vector, 2 µL of buffer (10x), 1 µL of restriction enzyme (e.g., BbsI, BplI, or BsmBI), and ddH_2O to a final volume of 20 µL. Incubate the reaction at 37 °C for 2.5 h.

2.2.2 Add 1 µL of shrimp alkaline phosphatase (SAP) to the reaction, and incubate at 37 °C for another 30 min.

2.2.3 Quench the reaction by adding 5 µL of 6x DNA loading dye (see **Table of Materials**), mix well, and resolve the reaction on a 0.8% agarose gel with 1x tris-acetate-EDTA (TAE) buffer. Then, excise the correct band and proceed to gel purify the linearized vector.

2.3 Ligate the annealed oligonucleotides into the digested CRISPR plasmid.

2.3.1 Prepare a 10 µL reaction mix: 50 ng of linearized vector, 1 µL of diluted annealed oligonucleotides, 1 µL of T4 DNA ligase buffer (10x), 1 µL of T4 DNA ligase, and ddH_2O to a final volume of 10 µL (see **Table of Materials**). Incubate the reaction at 16 °C overnight or at room temperature for 2 h.

NOTE: To speed up the ligation process, use concentrated T4 DNA ligase and incubate the reaction mix at room temperature for 15 min (see **Table of Materials**).

2.4 Transform ligated products into chemically competent *E. coli* cells (see **Supplementary File 1**). Spread the transformed bacterial cells on an LB agar plate with 100 µg/mL ampicillin.

2.5 Perform colony polymerase chain reaction (PCR) to identify the bacteria with insert.

2.5.1 Prepare two sets of sterile PCR strip tubes. In Set 1, add 4.7 µL of ddH_2O, while in Set 2, add 50 µL of LB broth with appropriate antibiotic (e.g., 100 µg/mL ampicillin).

2.5.2 With a sterile pipette tip, pick a colony off the plate, swipe it briefly in a Set 1 tube, and leave the tip in a Set 2 tube. Repeat for a few colonies, making sure to use different PCR tubes each time.

NOTE: Typically, screening four colonies is sufficient. However, this may vary depending on the cloning efficiency.

2.5.3 Add the following reagents to each Set 1 tube (for a 10 µL PCR): 5 µL of 2x PCR master mix (with loading dye, see **Table of Materials**), 0.15 µL of sense or antisense oligonucleotide (100 µM), 0.15 µL of primer (100 µM) targeting the CRISPR plasmid downstream or upstream of the sgRNA cassette respectively (see **Figure 3**).

NOTE: The PCR product should ideally yield a product size of approximately 150 bp or larger, so that any positive bands are not mistaken as primer dimers.

2.5.4 Run the PCRs in a thermal cycler using the following parameters: 95 °C for 3 min, 95 °C for 30 s (step 2), 60 °C for 30 s (step 3), 72 °C for 30 s (step 4), repeat steps 2–4 for another 34 cycles, 72 °C for 5 min, and hold at 4 °C.

NOTE: The annealing temperature of 60 °C may need to be optimized for the primers designed. The elongation time of 30 s can also vary depending on the expected PCR amplicon size and the DNA polymerase used.

2.5.5 Resolve the reactions on a 1% agarose gel using 1x TAE buffer.

2.5.6 Inoculate a colony that yields a positive band in the PCR by transferring 50 µL of its culture from the corresponding Set 2 tube into a larger conical tube containing 5 mL LB with an appropriate antibiotic. Let the culture grow overnight in a 37 °C incubator-shaker.

2.5.7 Isolate plasmids from the overnight culture using a miniprep kit (see **Table of Materials**) and sequence the sample using the colony PCR primer that is not the sense or antisense oligonucleotide (hU6_forward or M13R(-20) in **Figure 3**).

NOTE: If necessary, perform a maxiprep of the sequence-verified CRISPR plasmid to obtain a larger amount for downstream experiments.

3. Design and synthesis of repair templates

NOTE: For precision genome engineering, a template specifying the desired DNA modifications needs to be provided together with the CRISPR reagents. For small DNA edits such as alteration of a single nucleotide, ssODN donor templates are most suitable (see section 3.1). For larger DNA edits such as insertion of a GFP tag 5’ or 3’ of a particular protein-coding gene, plasmid donor templates are most suitable (see section 3.2).

3.1 Design and synthesize a ssODN donor template (see **Figure 4**).

3.1.1 Determine the correct strand whose sequence the template should follow.

NOTE: Cas12a exhibits a preference for ssODNs of the non-target strand sequence, while Cas9 exhibits a preference for ssODNs of the target strand sequence instead[25] (see **Figure 4a**).

3.1.2 Ensure that the repaired sequence is not targetable by the selected Cas nuclease again. For example, mutate the PAM in such a way that there is no amino acid change or eliminate the PAM from the donor template if it has no functional consequence. Use the example given in **Figure 4b** as a guide, if necessary.

3.1.3 Decide if a symmetric or asymmetric donor template is desired. For symmetric donors, ensure that each homology arm flanking the DNA modification site is at least 17 nt long[25]. For asymmetric donor templates, use longer arms 5' of the desired DNA changes (see **Figure 4a**). Importantly, ensure that the shorter arm is around 37 nt in length, while the other homology arm is around 77 nt in length[25,39].

[Place **Figure 4** here.]

3.1.4 Synthesize the designed template as a piece of single-stranded DNA.

NOTE: Asymmetric ssODNs can, but do not always, exhibit higher HDR efficiency than symmetric ssODNs. In general, an asymmetric donor typically performs at least as well as a symmetric donor, when designed correctly. However, the asymmetric template costs much more because it is longer and hence requires polyacrylamide gel electrophoresis (PAGE) purification or a special synthesis procedure. Routine gene knockouts usually rely on the NHEJ repair pathway and do not require a repair template. However, if the knockout efficiency is low, design a ssODN donor template that contains a frameshift mutation and is at least 120 nt in length[25,40].

3.2 Design and clone an appropriate plasmid donor template. For example, it may contain a GFP sequence flanked by long arms that are homologous to the target genomic locus (see **Figure 5**).

3.2.1 Ensure that the modified sequence is not targetable by the selected Cas nuclease again. For example, the protospacer may be split by the inserted (GFP) tag. Alternatively, the PAM may be mutated or removed from the donor template in such a way that does not affect gene function.

3.2.2 Amplify the homology arms from genomic DNA using PCR. The length of each homology arm is typically 1000 to 1500 bp.

NOTE: To facilitate cloning, ensure that the forward primer for the left homology arm and the reverse primer for the right homology arm each has at least 20 nt overlapping sequence with a selected vector backbone. Additionally, ensure that the reverse primer for the left homology arm

and the forward primer for the right homology arm has some overlapping sequences with the epitope tag as well.

3.2.3 Clone the two homology arms and the (GFP) tag into the vector backbone using Gibson assembly[41] (see the **Table of Materials**). Verify the plasmid by Sanger sequencing using forward and reverse primers that are upstream and downstream of the donor template respectively.

NOTE: Sanger sequencing is widely and cheaply available as a commercial service. Send an aliquot of the plasmid together with the sequencing primers to a service provider.

3.2.4 Linearize the donor template with a restriction enzyme that cuts the plasmid only once either upstream of the left homology arm or downstream of the right homology arm.

NOTE: Recently, a double-cut donor, which is flanked by the sgRNA-PAM sequences and is released from the plasmid after cleavage by the corresponding Cas nuclease, has been shown to increase HDR efficiency[42]. When the sgRNA-PAM sequences are inserted upstream and downstream of the left and right homology arms respectively (for example by Gibson assembly), the homology arm length may be reduced to 300 bp and there is no need to linearize the plasmid.

[Place **Figure 5** here.]

4. Cell transfection

NOTE: The remaining parts of the protocol are written with HEK293T cells in mind. The culture medium used consists of Dulbecco Modified Eagle Medium (DMEM) supplemented with 4.5 g/L glucose, 10% fetal bovine serum (FBS), 2 mM L-glutamine, and 0.1% penicillin/streptomycin. Different steps of the protocol may have to be modified according to the actual cell line used. All cell culture work is done in a Class II Biosafety Cabinet to ensure a sterile work environment.

4.1 Seed 1.8 x 10^5 cells in a 24-well tissue culture plate one day prior to transfection.

4.1.1 Dissociate the cells by aspirating the media and then adding 150 µL 0.25% Trypsin-EDTA per well. Incubate the cells at 37 °C for 2 min.

4.1.2 Neutralize the trypsin by adding 150 µL (or 1x volume) of cell culture media. Transfer the cell suspension to a conical tube. Spin down the cells in a bench top centrifuge at 1000 x *g* for 5 min.

4.1.3 Aspirate the supernatant, and resuspend with 5 mL of cell culture media. In a separate centrifuge tube, aliquot 10 µL of 0.4% trypan blue solution. Then, add in 10 µL resuspended cells from step 4.1.2 and mix thoroughly.

4.1.4 Pipette 10 µL of the mixture (cells + trypan blue) into a hemocytometer. Proceed to count the cells manually or by using an automated cell counter.

4.1.5 Seed 1.8 x 10^5 cells into one well of a 24-well tissue culture plate.

4.2 Prepare a transfection mix containing either 500 ng of CRISPR plasmid (for NHEJ-mediated editing) or 300 ng of CRISPR plasmid and 300 ng of donor template (for HDR-mediated editing), according to the instructions provided with the transfection reagent (see **Table of Materials**). Incubate at room temperature for the recommended time duration (typically around 10–20 min).

4.3 Add the transfection mix to the cells in a dropwise fashion, and gently swirl the plate after.

4.4 Incubate at 37 °C in a humidified 5% CO_2 air incubator for 24 h (for NHEJ-based experiments) or 72 h (for HDR-based experiments).

5. Fluorescence activated cell sorting (FACS) of transfected cells

5.1 Dissociate the cells by aspirating the media and then adding 150 µL of 0.25% trypsin-EDTA per well. Incubate the cells at 37 °C for 2 min.

5.2 Neutralize the trypsin by adding 150 µL (or 1x volume) of cell culture media. Transfer the cell suspension to a centrifuge tube. Spin down the cells in a microcentrifuge at 235 x *g* for 5 min.

5.3 Aspirate the supernatant and resuspend the cells with 2% fetal bovine serum (FBS) in phosphate-buffered saline (PBS). Filter the cells through a 30 µm mesh or cell strainer in a 5 mL FACS tube.

5.4 Prepare another centrifuge tube with approximately 100 µL culture media or 2% FBS in PBS for collection of cells.

5.6 On the flow cytometer, gate the cells with non-transfected cells as a negative control. Sort and collect the transfected cells, according to which fluorescence marker is present on the CRISPR plasmid used. For example, if the plasmid carries a mCherry gene, sort for RFP-positive cells.

NOTE: Different CRISPR plasmids will have different selectable markers. The set of plasmids (pSpCas9, pSaCas9, pNmCas9, pAsCpf1, and pLbCpf1) used in this evaluation study carry either the orange fluorescent protein (OFP) or the mCherry gene.

6. Expansion of individual clones

6.1 Centrifuge the sorted cells in a bench top centrifuge at maximum speed (18,000 x *g*) for 5 min. Aspirate the supernatant and resuspend the pellet with 300 µL culture media. Seed 200 µL cells in a 24-well tissue culture plate and let them recover for a few days in a 37 °C incubator. Keep the remaining 100 µL cells for section 7.

6.2 Once the cells start becoming confluent, passage them according to steps 4.1.1–4.1.3. Seed the cells sparsely in a 100 mm tissue culture dish to allow sufficient space for individual colonies to grow. Incubate at 37 °C in a humidified 5% CO_2 air incubator.

NOTE: Try various dilutions. Single cells need sufficient space to grow as individual colonies. However, they also cannot be overly sparse, as some cell lines do not grow well when the number of cells are too few.

6.3 Once the colonies are starting to form, pick them under the microscope (with a 4x magnification) and place each clone into an individual well of a 24-well plate that contains cell culture media. Incubate at 37 °C in a humidified 5% CO_2 air incubator until the cells are becoming confluent.

NOTE: An alternative to serial dilutions and colony picking is to use flow cytometry to sort for single cells into a 96-well plate. However, this may not work for some cell lines that do not grow well when only one cell is present.

7. Evaluation of editing efficiency

7.1 Extract genomic DNA by centrifuging the remaining 100 µL sorted cells (from step 6.1) in a bench top centrifuge at maximum speed (18,000 x *g*) for 5 min. Aspirate the supernatant and proceed to isolate genomic DNA using an extraction kit (see **Table of Materials**).

7.2 Perform the T7 endonuclease I (T7EI) cleavage assay (see **Figure 6**).

7.2.1 Set up a 50 µL PCR containing 10 µL of PCR reaction buffer (5x), 1 µL of dNTP mix (10 mM), 2.5 µL of user-defined forward primer (10 µM), 2.5 µL of user-defined reverse primer (10 µM), 0.5 µL of DNA polymerase, 2–5 µL of genomic DNA template (depending on how many cells have been sorted), then top up to 50 µL with ddH_2O (see **Table of Materials**).

NOTE: The primers are designed to flank the target genomic locus and yield a PCR product of around 400–700 bp. Usually one primer is positioned closer to the cut site of the Cas enzyme than the other primer, so that the result of the T7EI assay is two distinct bands on an agarose gel (see **Figure 6**).

7.2.2 Run the PCR in a thermal cycler with the following parameters: 98 °C for 3 min, 98 °C for 30 s (step 2), 63 °C for 30 s (step 3), 72 °C for 30 s (step 4), repeat steps 2–4 for another 34 cycles, 72 °C for 2 min, and hold at 4 °C.

7.2.3 Resolve the reaction on a 2% agarose gel using 1x TAE buffer.

7.2.4 Excise the PCR product from the gel with a clean, sharp scalpel and purify the DNA using a gel extraction kit, according to manufacturer's instructions. Measure the concentration of the

PCR product using a spectrophotometer at a wavelength of 260 nm absorbance (see **Table of Materials**).

7.2.5 Prepare an assay mix containing 200 ng of DNA, 2 µL of T7EI reaction buffer (10x), and topped up to 19 µL with ddH_2O (see **Table of Materials**).

7.2.6 Re-anneal the PCR product in a thermal cycler using the following parameters: 95 °C for 5 min, ramp down to 25 °C at 6 °C/min, then hold at 4 °C.

7.2.7 Add 5 U T7EI to the re-annealed PCR product, mix well by pipetting, and incubate at 37 °C for 50 min.

7.2.8 Resolve the T7EI-digested DNA on a 2.5% agarose gel using 1x TAE buffer.

7.2.9 Image the gel, quantify the band intensities using ImageJ, and calculate rate of indel formation using the following formula:

$$Indel(\%) = \left(1 - \sqrt{1 - \frac{\mathrm{b} + \mathrm{c}}{\mathrm{a} + \mathrm{b} + \mathrm{c}}}\right) \times 100$$

where a represents the intensity of the intact PCR product and b and c correspond to the intensities of the cleavage products[43].

7.2.9.1 To quantify the intensity of a band in ImageJ, first draw a rectangular box around the band as close to its boundary as possible. Second, click on **Analyze** and then **Set Measurements**. Ensure that the **area**, **mean gray value**, and **integrated density** options are checked. Exit the settings window by clicking **ok**. Third, click on **Analyze** and then **Measure**. The mean or RawIntDen value is used as the band intensity.

[Place **Figure 6** here.]

7.3 Perform targeted amplicon sequencing (see **Figure 6**).

7.3.1 Design PCR primers to amplify the target genomic locus. Position one of the primers to be less than 100 bp but more than 20 bp from the protospacer.

NOTE: Typically, the total PCR product size is designed to be around 150–300 bp (see **Figure 6**).

7.3.2 Append additional sequences to the primers as follows: (a) 5'–GCGTTATCGAGGTC-NNNN-[Forward Primer] – 3'; (b) 5' - GTGCTCTTCCGATCT-[Reverse Primer]–3'.

7.3.3 Set up a 50 µL PCR reaction mix containing 10 µL of PCR reaction buffer (5x), 1 µL of dNTP (10 mM), 5 µL of primer a (10 µM), 5 µL of primer b (10 µM), 0.5 µL DNA polymerase, 2–5 µL genomic DNA template (depending on how many cells have been sorted), then top up to 50 µL with ddH_20.

7.3.4 Run the PCR in a thermal cycler with the following parameters: 98 °C for 3 min, 98 °C for 30 s (step 2), 63 °C for 30 s (step 3), 72 °C for 15 s (step 4), repeat steps 2–4 for another 34 cycles, 72 °C for 2 min, and hold at 4 °C.

7.3.5 Resolve the reaction on 2% agarose gel and purify the PCR product using a gel extraction kit according to manufacturer's instructions. Quantify the DNA using a spectrophotometer at a wavelength of 260 nm absorbance (see the **Table of Materials**).

7.3.6 Synthesize the following round 2 PCR primers: (c) 5'–AATGATACGGCGACCACCGAGATCTACACCCTACACGAGCGTTATCGAGGTC–3'; (d) 5'–CAAGCAGAAGACGGCATACGAGAT-[barcode]-GTGACTGGAGTTCAGACGTGTGCTCTTCCGATCT–3'

7.3.7 Set up a 20 µL PCR reaction mix containing 4 µL of PCR reaction buffer (5x), 0.4 µL of dNTP (10 mM), 2 µL of primer c (10 µM), 2 µL of primer d (10 µM), 0.2 µL of DNA polymerase, 2 µL of DNA template (from step 7.3.5, diluted 1:100), and 9.4 µL of ddH_2O.

NOTE: The dilution factor for the DNA template may vary depending on its original concentration. If the concentration is around 20–40 ng/µL, use a 1:100 dilution factor. In addition, pick a different barcode for each experimental sample, if the same primer a and primer b are used in step 7.3.3.

7.3.8 Run the PCR in a thermal cycler with the following parameters: 98 °C for 3 min, 98 °C for 30 s (step 2), 65 °C for 30 s (step 3), 72 °C for 30 s (step 4), repeat steps 2–4 for another 14 cycles, 72 °C for 2 min, and 4 °C hold.

7.3.9 Resolve 5 µL of each reaction on a 2% agarose gel to determine the success of the PCRs. Combine all the samples together (assuming that a different barcode has been used for each sample) and clean up the pooled DNA using a PCR purification kit according to manufacturer's instructions. If some of the PCRs exhibit more than one band (indicating the presence of non-specific products), perform an additional gel extraction step.

7.3.10 Sequence the library on a high throughput sequencing instrument (see **Table of Materials**) according to manufacturer's instructions to produce paired 151 bp reads. The read 1 sequencing primer is custom designed and has to be provided separately. Its sequence is as follows: Read1_seq: 5'–CCACCGAGATCTACACCCTACACGAGCGTTATCGAGGTC–3'. The read 2 sequencing primer and index sequencing primer are standard and are provided in the reagent cartridge.

NOTE: The T7EI assay and targeted amplicon sequencing are commonly used to check the efficiency of genome editing. However, other experiments may be performed to evaluate editing efficiency, depending on the type of DNA modifications introduced. For example, if a restriction site is created at the target site, a restriction fragment length polymorphism (RFLP) assay can be performed. It is similar to the T7EI assay except that a restriction endonuclease is used to digest the PCR product instead.

8. Screening of individual clones

8.1 From step 6.3, split the cells once they start getting confluent. For each individual clone, collect the remaining cells and extract genomic DNA according to step 7.1.

8.2 Perform the T7EI assay for all the individual clones according to section 7.2, except for one modification. Amplify the target genomic locus from wildtype cells and in step 7.2.5, instead of using 200 ng of test DNA only, mix 100 ng of test DNA with 100 ng of wildtype DNA.

NOTE: The reason for the modified step is that some clones may have undergone successful biallelic conversion and are homozygous mutants. In such cases, there will be no cleavage bands in the T7EI assay if the wildtype DNA is not mixed in.

8.3 Sequence the target site in the clones that exhibit cleavage bands in the T7EI assay.

8.3.1 Amplify the modified genomic locus according to steps 7.2.1–7.2.4.

8.3.2 Set up the following cloning reaction: 4 µL of PCR product, 1 µL of salt solution, 1 µL of TOPO vector (see the **Table of Materials**).

8.3.3 Mix gently by pipetting and incubate at room temperature for at least 5 min.

8.3.4 Transform 3 µL of the reaction mix into chemically competent *E. coli* cells (such as TOP10 or Stbl3) (see **Supplementary File 1**). Spread the transformed bacterial cells on an LB agar plate with 50 µg/mL kanamycin.

8.3.5 The next day, inoculate at least 10 colonies in LB liquid media containing 50 µg/mL kanamycin.

8.3.6 When the bacterial cultures are turbid, isolate the plasmids using a miniprep kit (see **Table of Materials**) and sequence them using the standard M13 forward or M13 reverse primer.

8.4 Perform a western blot (also known as immunoblot) to determine the absence or presence of the targeted protein (if the genome editing experiment involves knocking out a protein-coding gene via frameshift mutations). See **Supplementary File 1**.

NOTE: Other experiments can be performed to identify the clone carrying the desired genomic modifications. For example, a phenotypic assay can be performed if knocking out a particular gene is known to cause certain changes in cellular behavior.

REPRESENTATIVE RESULTS:

To perform a genome editing experiment, a CRISPR plasmid expressing a sgRNA targeting the locus-of-interest needs to be cloned. First, the plasmid is digested with a restriction enzyme

(typically a type IIs enzyme) to linearize it. It is recommended to resolve the digested product on a 1% agarose gel alongside an undigested plasmid to distinguish between a complete and partial digestion. As undigested plasmids are supercoiled, they tend to run faster than their linearized counterparts (see **Figure 7a**). Second, two oligonucleotides for the sgRNA have to be annealed and ligated into the cut plasmid. To determine if the oligonucleotides have been successfully cloned into the CRISPR plasmid backbone, colony PCR is performed (see **Figure 7b**). Positive clones are then inoculated before the plasmids are extracted and sent for Sanger sequencing.

[Place **Figure 7** here.]

Once the constructs have been sequence-verified, they can be transduced into a desired cell line (such as HEK293T). CRISPR plasmids often carry a selectable marker (e.g., an mCherry gene), thereby allowing successfully transduced cells to be selected. Fluorescence can be readily visualized under a microscope upon successful delivery of the plasmids (see **Figure 8a**). The cells are sorted by flow cytometry around 24–72 h post-transfection. The gating is set based on a non-transfected control (see **Figure 8b**). Part of the sorted cells are seeded on a tissue culture plate to allow for recovery.

[Place **Figure 8** here.]

While the sorted cells are recovering, the editing efficiency is evaluated to determine whether the experiment should be continued. Genomic DNA (gDNA) is isolated from the remaining un-plated cells. A T7EI assay is performed to check the cleavage efficiency, which is calculated from the intensities of the bands observed on an agarose gel (see **Figure 9a**). Additionally, if an HDR-based experiment is designed to incorporate a restriction site at the target locus, a RFLP assay can be performed with the corresponding restriction enzyme (see **Figure 9b**).

[Place **Figure 9** here.]

If the editing efficiency is acceptable (e.g., at least 5% by the T7EI assay), we proceed to identify individual clones that carry the desired DNA modifications. Plated cells, which have been left to recover, are seeded sparsely to allow for individual colonies to grow. Subsequently, individual colonies are picked and expanded, before gDNA is isolated from these individual clones. Another round of T7EI assay is performed to identify clones with modified DNA at the target site. TOPO cloning and Sanger sequencing are then performed to determine the exact sequences of all alleles. Ideally, for gene knockouts, the indels should not be in multiples of three, which do not cause frameshift mutations (see **Figure 10a**). To further validate that a protein-coding gene has been successfully inactivated, a western blot can be carried out to ensure that no targeted protein is present (see **Figure 10b**).

[Place **Figure 10** here.]

FIGURE AND TABLE LEGENDS:

Table 1: Some commonly used Cas enzymes with their cognate PAMs and optimal sgRNA lengths. N = Any nucleotide (A, T, G, or C); R = A or G; V = A, C, or G.

Table 2: Oligonucleotides required for cloning sgRNA sequences into CRISPR plasmids used in a recent evaluation study[25]. The overhangs are italicized.

Figure 1: An overview of the workflow to generate genome edited human cell lines.

Figure 2: An example illustrating how to select target sites and design oligonucleotides for cloning into CRISPR plasmids. The target genomic locus here is exon 45 of the human CACNA1D gene. The PAMs for SpCas9 and SaCas9 are NGG and NNGRRT respectively and are highlighted in red, while the PAM for AsCas12a and LbCas12a is TTTN and is highlighted in green. The red horizontal bar indicates the protospacer for SpCas9 and SaCas9, while the green horizontal bar indicates the protospacer for the two Cas12a enzymes.

Figure 3: An example of a CRISPR plasmid. (**a**) A map indicating different important features of the plasmid. Here, the EF-1a promoter drives the expression of Cas9, while the U6 promoter drives the expression of the sgRNA. Amp(R) indicates an ampicillin-resistance gene in the plasmid. (**b**) The sequence of the "BbsI-BplI cloning site" in the plasmid. The recognition sequence of BbsI is GAAGAC and is indicated in red, while the recognition sequence of BplI is GAG-N_5-CTC and is indicated in green. (**c**) Primers that can be used for colony PCR to check whether the sgRNA sequence has been successfully cloned into the plasmid. The hU6_forward primer is indicated by a purple arrow on the plasmid map, while the universal M13R(-20) primer is indicated by a pink arrow on the plasmid map.

Figure 4: Design of ssODN donor templates. (**a**) Schematic illustrating various possible designs. The red horizontal rectangles indicate the non-target (NT) strand, while the blue rectangles indicate the target (T) strand. In addition, the small green rectangles indicate the desired DNA modifications (such as single nucleotide changes). When a symmetric ssODN is used, the minimum length of each homology arm should be at least 17 nt (but can be longer). For asymmetric ssODNs, the 37/77 T ssODN appears to be optimal for SpCas9-induced HDR, while the 77/37 NT ssODN appears to be optimal for Cas12a-induced HDR. L = left homology arm; R = right homology arm. (**b**) A specific example to demonstrate how to design ssODN templates. Here, the target genomic locus is exon 45 of the human CACNA1D gene. The PAM for Cas9 is pink and underlined, while the PAM for Cas12a is brown and underlined. The goal is to create a missense mutation (highlighted in green) by converting AGU (encoding serine) to AGG (encoding arginine). To prevent re-targeting by Cas12a, the TTTC PAM is mutated to CTTC. Note that there is no change in amino acid (UAU and UAC both code for tyrosine). To further prevent re-targeting by Cas9, an AGU codon is replaced with a UCC codon (bold), both of which code for serine.

Figure 5: Design and cloning of a plasmid donor template. (**a**) The goal in this specific example is to fuse P2A-GFP to the C-terminus of the CLTA protein. The blue horizontal rectangle indicates the left homology arm, while the red horizontal rectangle indicates the right homology arm. Capital letters indicate protein-coding sequences, while lowercase letters indicate non-coding

sequences. The PAMs for SpCas9 and Cas12a are italicized and underlined. (**b**) A plasmid donor template that can be used to endogenously tag P2A-GFP at the C-terminus of CLTA. The provided primer sequences can be used to clone the plasmid by Gibson assembly. The PCR conditions are as follows: 98 °C for 3 min, 98 °C for 30 s (step 2), 63 °C for 30 s (step 3), 72 °C for 1 min (step 4), repeat steps 2–4 for another 34 cycles, 72 °C for 3 min, and hold at 4 °C. Black letters correspond to vector sequences, blue letters correspond to the left homology arm, green letters correspond to P2A-GFP, and red letters correspond to the right homology arm. Note that once the sequence encoding P2A-GFP is successfully integrated into the target locus, re-targeting by SpCas9 will not be possible, since only 9 nt of its protospacer (GTGCACCAG) will be left intact. Moreover, in order to prevent re-targeting by Cas12a, three basepairs immediately downstream of the STOP codon (in bold) are deleted from the plasmid sequence.

Figure 6: Checking cells for successful genome editing outcomes. (**a**) A schematic illustrating two commonly used assays, namely the mismatch cleavage assay with the T7 endonuclease I (T7EI) enzyme and next generation sequencing (NGS) or targeted amplicon sequencing. The blue horizontal rectangles indicate DNA and the yellow circles indicate modifications induced by the CRISPR-Cas system. Primers for the T7E1 assay are denoted in green, while primers for generating amplicons for NGS are denoted in red. (**b**) Design of primer sequences for the T7EI cleavage assay and for NGS. Here, the target genomic locus is exon 45 of the human CACNA1D gene. The intended modification site is indicated by an asterisk.

Figure 7: Cloning of sgRNA-expressing CRISPR plasmids. (**a**) Image showing digested and undigested plasmids after gel electrophoresis. Lanes 1–3 show plasmids that have been linearized completely by BbsI. Lane 4 shows the original undigested plasmid, which is supercoiled and hence migrates faster on an agarose gel. (**b**) Representative gel image of colony PCR products. Positive clones are marked by a green tick (indicating that oligonucleotides have been successfully ligated into the vector backbone), while negative clones are marked by a red cross.

Figure 8: Introduction of CRISPR reagents into human cells. (**a**) Representative microscopy image of HEK293T cells successfully transfected with a CRISPR plasmid bearing a mCherry marker (see the **Table of Materials**). Transfection efficiency can be estimated by the percentage of cells displaying red fluorescence. 10x magnification, scale bar represents 200 µm. (**b**) Representative FACS plots. Transfected cells (right panel) are gated against a non-transfected control (left panel).

Figure 9: Evaluating the extent of genome editing. (**a**) Representative gel image showing the results of a T7E1 assay. Lanes 1–8 represent different experimental samples. For each sample, the top band indicates uncut DNA, while the bottom two bands indicate the cleavage products. Varying NHEJ efficiencies can be observed among the samples. Digestion of PCR amplicons from non-transfected cells is used as a negative control and is marked by a "-". (**b**) Representative gel image showing the results of a RFLP assay. Lanes 1–6 represent different experimental samples. For each sample, the top band indicates uncut DNA, while the bottom two bands indicate the cleavage products after restriction digest. Varying HDR efficiencies can be observed among the samples.

Figure 10: Screening for gene knockouts. (**a**) Representative Sanger sequencing data. The original unmodified DNA sequence (top row) can be aligned with the sequencing result received (bottom row) to check whether any edits have taken place. Here, there is a 1 bp insertion and a 7 bp deletion at the target site (as depicted by the red boxes, which represent gaps in the alignment). (**b**) Representative western blot image. Here, several clones (expanded from single cells) are screened for the presence or absence of the GLUL protein. The red arrows indicate the clones where the GLUL gene has been successfully knocked out. "WT" stands for wildtype cells, where GLUL is highly expressed. ACTB (β-actin) serves as a loading control (see the **Table of Materials**).

DISCUSSION:

The CRISPR-Cas system is a powerful, revolutionary technology to engineer the genomes and transcriptomes of plants and animals. Numerous bacterial species have been found to contain CRISPR-Cas systems, which may potentially be adapted for genome and transcriptome engineering purposes[44]. Although the Cas9 endonuclease from *Streptococcus pyogenes* (SpCas9) was the first enzyme to be deployed successfully in human cells[21-24], enzymes from other bacterial species, including those shown in **Table 1**, have also been shown to function well as engineering tools in human[16,26-28]. Consequently, researchers keen to use the technology are often unsure which system to choose for their work and how to design their experiments for optimal results.

Here, we seek to assist CRISPR users in maximizing their usage of the technology. Fundamental to any genome editing experiment is the selection and cloning of an appropriate sgRNA for targeting. Work done by us and others reveal that different CRISPR-Cas systems are optimally active at different spacer lengths (see **Table 1**)[25]. Additionally, the researcher should choose which system to use based on knowledge of the target site. Generally, among the enzymes tested in our evaluation study, SpCas9 and LbCas12a exhibit the highest cleavage efficiency, especially in more highly expressed genes presumably as a result of more open and accessible chromatin. Hence, we recommend these two enzymes for routine genome editing experiments. However, we have found that both nucleases exhibit higher tolerance for mismatches between the sgRNA and targeted DNA than AsCas12a. Hence, if the researcher is targeting a gene with several closely related homologs or a repetitive region of the genome, we recommend using AsCas12a to minimize off-target effects. Alternatively, SaCas9 may also be utilized as it requires longer spacer lengths for robust activity, thereby offering higher intrinsic specificity than SpCas9 and LbCas12a.

Precise editing of a genomic locus requires the introduction of a repair template together with the CRISPR reagents. The template serves as an instruction manual to tell the cells how to repair the double-stranded break via the HDR pathway. Two common forms of template are a ssODN and a plasmid. The design of this repair template is a critical step for optimal HDR efficiency[25,39,42,45-47]. For ssODNs, the DNA strand from which the sequence of the ssODN is derived is important. AsCas12a and LbCas12a exhibit a preference for ssODNs of the non-target strand sequence, while SpCas9 exhibits a preference for ssODNs of the target strand sequence instead. Moreover, HDR efficiencies may potentially be improved further by utilizing asymmetric donors. However, the researcher has to be careful of which homology arm is extended (see **Figure 2**). Increasing the length of the wrong arm will end up degrading the HDR efficiency

instead. For plasmid donors, the template is often linearized by restriction digest before cell transduction. In addition, a "double-cut" plasmid donor, where spacer-PAM sequences are placed before and after the left and right homology arms respectively, has been shown to yield a higher HDR efficiency than a plasmid donor without the additional spacer-PAM sequences[42].

Besides preparation of the necessary CRISPR reagents with or without a repair template, generation of modified cell lines (e.g., containing specific gene knockouts) also involves cell transduction, single cell expansion, and screening of individual clones. The protocol outlines all the downstream steps to enable users to perform a complete genome editing experiment. Some details are cell line-specific. For example, some cell lines may be easy to transfect, while others may require extensive optimization with different transduction methods, including nucleofection and electroporation. Importantly, from our experiences, a critical step in the protocol is single-cell cloning. Certain cell types may not proliferate when they are seeded as a single cell in a well, perhaps due to a lack of secreted growth factors. For example, human embryonic stem cells and many patient-derived primary cell lines typically grow very poorly as isolated single cells. Hence, after transduction, we recommend plating cells sparsely and then picking individual colonies on a plate over using flow cytometry to sort single cells into different wells of a 96-well plate. There may also be a need to add pro-survival factors to the cell culture media, such as the ROCK inhibitor[48], to improve the recovery of dissociated cells. In addition, during the process of single-cell cloning, there may be occasions where two separate cells grow very close to each other and end up merging, thereby giving the false appearance of one colony only. In such situations, during the stages of determining the genomic mutations introduced, one may end up identifying three or more distinct alleles. Nevertheless, it is still possible to recover individual clones by sparsely plating these cells again and then picking additional individual colonies.

In conclusion, the CRISPR-Cas technology is revolutionizing biomedical and biotechnological research. However, it currently suffers from several limitations (such as restricted targeting range and large protein size) that hampers its widespread adoption in some applications, including gene therapy. Nevertheless, as time progresses, more and more naturally occurring Cas enzymes will be discovered in a wide range of microbial species and some of these new enzymes may have advantageous properties over existing nucleases. Detailed studies will then have to be performed to properly understand the design parameters of these novel CRISPR-Cas systems when they are deployed as genome and transcriptome engineering tools. Hence, while our protocol serves as a good reference for current genome editing efforts, it will need to be continuously updated with latest information about the new systems as they emerge.

ACKNOWLEDGMENTS:

M.H.T. is supported by an Agency for Science Technology and Research's Joint Council Office grant (1431AFG103), a National Medical Research Council grant (OFIRG/0017/2016), National Research Foundation grants (NRF2013-THE001-046 and NRF2013-THE001-093), a Ministry of Education Tier 1 grant (RG50/17 (S)), a startup grant from Nanyang Technological University, and funds for the International Genetically Engineering Machine (iGEM) competition from Nanyang Technological University.

DISCLOSURES:

The authors do not have competing financial interests.

REFERENCES:

1 Epinat, J. C. *et al.* A novel engineered meganuclease induces homologous recombination in yeast and mammalian cells. *Nucleic Acids Research.* **31** (11), 2952-2962, (2003).

2 Arnould, S. *et al.* Engineered I-CreI derivatives cleaving sequences from the human XPC gene can induce highly efficient gene correction in mammalian cells. *Journal of Molecular Biology.* **371** (1), 49-65, (2007).

3 Chapdelaine, P., Pichavant, C., Rousseau, J., Paques, F. & Tremblay, J. P. Meganucleases can restore the reading frame of a mutated dystrophin. *Gene Therapy.* **17** (7), 846-858, (2010).

4 Carroll, D. Genome engineering with zinc-finger nucleases. *Genetics.* **188** (4), 773-782, (2011).

5 Urnov, F. D., Rebar, E. J., Holmes, M. C., Zhang, H. S. & Gregory, P. D. Genome editing with engineered zinc finger nucleases. *Nature Reviews Genetics.* **11** (9), 636-646, (2010).

6 Miller, J. C. *et al.* A TALE nuclease architecture for efficient genome editing. *Nature Biotechnology.* **29** (2), 143-148, (2011).

7 Zhang, F. *et al.* Efficient construction of sequence-specific TAL effectors for modulating mammalian transcription. *Nature Biotechnology.* **29** (2), 149-153, (2011).

8 Boch, J. *et al.* Breaking the code of DNA binding specificity of TAL-type III effectors. *Science.* **326** (5959), 1509-1512, (2009).

9 Moscou, M. J. & Bogdanove, A. J. A simple cipher governs DNA recognition by TAL effectors. *Science.* **326** (5959), 1501, (2009).

10 Hsu, P. D., Lander, E. S. & Zhang, F. Development and applications of CRISPR-Cas9 for genome engineering. *Cell.* **157** (6), 1262-1278, (2014).

11 Sander, J. D. & Joung, J. K. CRISPR-Cas systems for editing, regulating and targeting genomes. *Nature Biotechnology.* **32** (4), 347-355, (2014).

12 Jinek, M. *et al.* A programmable dual-RNA-guided DNA endonuclease in adaptive bacterial immunity. *Science.* **337** (6096), 816-821, (2012).

13 Nishimasu, H. *et al.* Crystal structure of Cas9 in complex with guide RNA and target DNA. *Cell.* **156** (5), 935-949, (2014).

14 Yamano, T. *et al.* Crystal Structure of Cpf1 in Complex with Guide RNA and Target DNA. *Cell.* **165** (4), 949-962, (2016).

15 Swarts, D. C., Mosterd, C., van Passel, M. W. & Brouns, S. J. CRISPR interference directs strand specific spacer acquisition. *PLoS One.* **7** (4), e35888, (2012).

16 Zetsche, B. *et al.* Cpf1 is a single RNA-guided endonuclease of a class 2 CRISPR-Cas system. *Cell.* **163** (3), 759-771, (2015).

17 Sternberg, S. H., Redding, S., Jinek, M., Greene, E. C. & Doudna, J. A. DNA interrogation by the CRISPR RNA-guided endonuclease Cas9. *Nature.* **507** (7490), 62-67, (2014).

18 Hu, J. H. *et al.* Evolved Cas9 variants with broad PAM compatibility and high DNA specificity. *Nature.* **556** (7699), 57-63, (2018).

19 Kleinstiver, B. P. *et al.* Broadening the targeting range of Staphylococcus aureus CRISPR-

Cas9 by modifying PAM recognition. *Nature Biotechnology.* **33** (12), 1293-1298, (2015).
20 Kleinstiver, B. P. *et al.* Engineered CRISPR-Cas9 nucleases with altered PAM specificities. *Nature.* **523** (7561), 481-485, (2015).
21 Cong, L. *et al.* Multiplex genome engineering using CRISPR/Cas systems. *Science.* **339** (6121), 819-823, (2013).
22 Mali, P. *et al.* RNA-guided human genome engineering via Cas9. *Science.* **339** (6121), 823-826, (2013).
23 Jinek, M. *et al.* RNA-programmed genome editing in human cells. *Elife.* **2** e00471, (2013).
24 Cho, S. W., Kim, S., Kim, J. M. & Kim, J. S. Targeted genome engineering in human cells with the Cas9 RNA-guided endonuclease. *Nature Biotechnology.* **31** (3), 230-232, (2013).
25 Wang, Y. *et al.* Systematic evaluation of CRISPR-Cas systems reveals design principles for genome editing in human cells. *Genome Biology.* **19** (1), 62, (2018).
26 Ran, F. A. *et al.* In vivo genome editing using Staphylococcus aureus Cas9. *Nature.* **520** (7546), 186-191, (2015).
27 Hou, Z. *et al.* Efficient genome engineering in human pluripotent stem cells using Cas9 from Neisseria meningitidis. *Proceedings of the National Academy of Sciences U S A.* **110** (39), 15644-15649, (2013).
28 Kim, E. *et al.* In vivo genome editing with a small Cas9 orthologue derived from Campylobacter jejuni. *Nature Communications.* **8** 14500, (2017).
29 Edraki, A. *et al.* A Compact, High-Accuracy Cas9 with a Dinucleotide PAM for In Vivo Genome Editing. *Molecular Cell.* 10.1016/j.molcel.2018.12.003, (2018).
30 Chatterjee, P., Jakimo, N. & Jacobson, J. M. Minimal PAM specificity of a highly similar SpCas9 ortholog. *Science Advances.* **4** (10), eaau0766, (2018).
31 Muller, M. *et al.* Streptococcus thermophilus CRISPR-Cas9 Systems Enable Specific Editing of the Human Genome. *Mol Therapy.* **24** (3), 636-644, (2016).
32 Esvelt, K. M. *et al.* Orthogonal Cas9 proteins for RNA-guided gene regulation and editing. *Nature Methods.* **10** (11), 1116-1121, (2013).
33 Boratyn, G. M. *et al.* BLAST: a more efficient report with usability improvements. *Nucleic Acids Research.* **41** (Web Server issue), W29-33, (2013).
34 Hsu, P. D. *et al.* DNA targeting specificity of RNA-guided Cas9 nucleases. *Nature Biotechnology.* **31** (9), 827-832, (2013).
35 Montague, T. G., Cruz, J. M., Gagnon, J. A., Church, G. M. & Valen, E. CHOPCHOP: a CRISPR/Cas9 and TALEN web tool for genome editing. *Nucleic Acids Research.* **42** (Web Server issue), W401-407, (2014).
36 Heigwer, F., Kerr, G. & Boutros, M. E-CRISP: fast CRISPR target site identification. *Nature Methods.* **11** (2), 122-123, (2014).
37 Haeussler, M. *et al.* Evaluation of off-target and on-target scoring algorithms and integration into the guide RNA selection tool CRISPOR. *Genome Biology.* **17** (1), 148, (2016).
38 Bae, S., Park, J. & Kim, J. S. Cas-OFFinder: a fast and versatile algorithm that searches for potential off-target sites of Cas9 RNA-guided endonucleases. *Bioinformatics.* **30** (10), 1473-1475, (2014).
39 Richardson, C. D., Ray, G. J., DeWitt, M. A., Curie, G. L. & Corn, J. E. Enhancing homology-directed genome editing by catalytically active and inactive CRISPR-Cas9 using

asymmetric donor DNA. *Nature Biotechnology.* **34** (3), 339-344, (2016).
40 Richardson, C. D., Ray, G. J., Bray, N. L. & Corn, J. E. Non-homologous DNA increases gene disruption efficiency by altering DNA repair outcomes. *Nature Communications.* **7** 12463, (2016).
41 Gibson, D. G. *et al.* Enzymatic assembly of DNA molecules up to several hundred kilobases. *Nature Methods.* **6** (5), 343-345, (2009).
42 Zhang, J. P. *et al.* Efficient precise knockin with a double cut HDR donor after CRISPR/Cas9-mediated double-stranded DNA cleavage. *Genome Biology.* **18** (1), 35, (2017).
43 Ran, F. A. *et al.* Genome engineering using the CRISPR-Cas9 system. *Nature Protocols.* **8** (11), 2281-2308, (2013).
44 Shmakov, S. *et al.* Diversity and evolution of class 2 CRISPR-Cas systems. *Nature Reviews Microbiology.* **15** (3), 169-182, (2017).
45 Moreno-Mateos, M. A. *et al.* CRISPR-Cpf1 mediates efficient homology-directed repair and temperature-controlled genome editing. *Nature Communications.* **8** (1), 2024, (2017).
46 Lin, S., Staahl, B. T., Alla, R. K. & Doudna, J. A. Enhanced homology-directed human genome engineering by controlled timing of CRISPR/Cas9 delivery. *Elife.* **3** e04766, (2014).
47 Yang, L. *et al.* Optimization of scarless human stem cell genome editing. *Nucleic Acids Research.* **41** (19), 9049-9061, (2013).
48 Watanabe, K. *et al.* A ROCK inhibitor permits survival of dissociated human embryonic stem cells. *Nature Biotechnology.* **25** (6), 681-686, (2007).

Figure 1

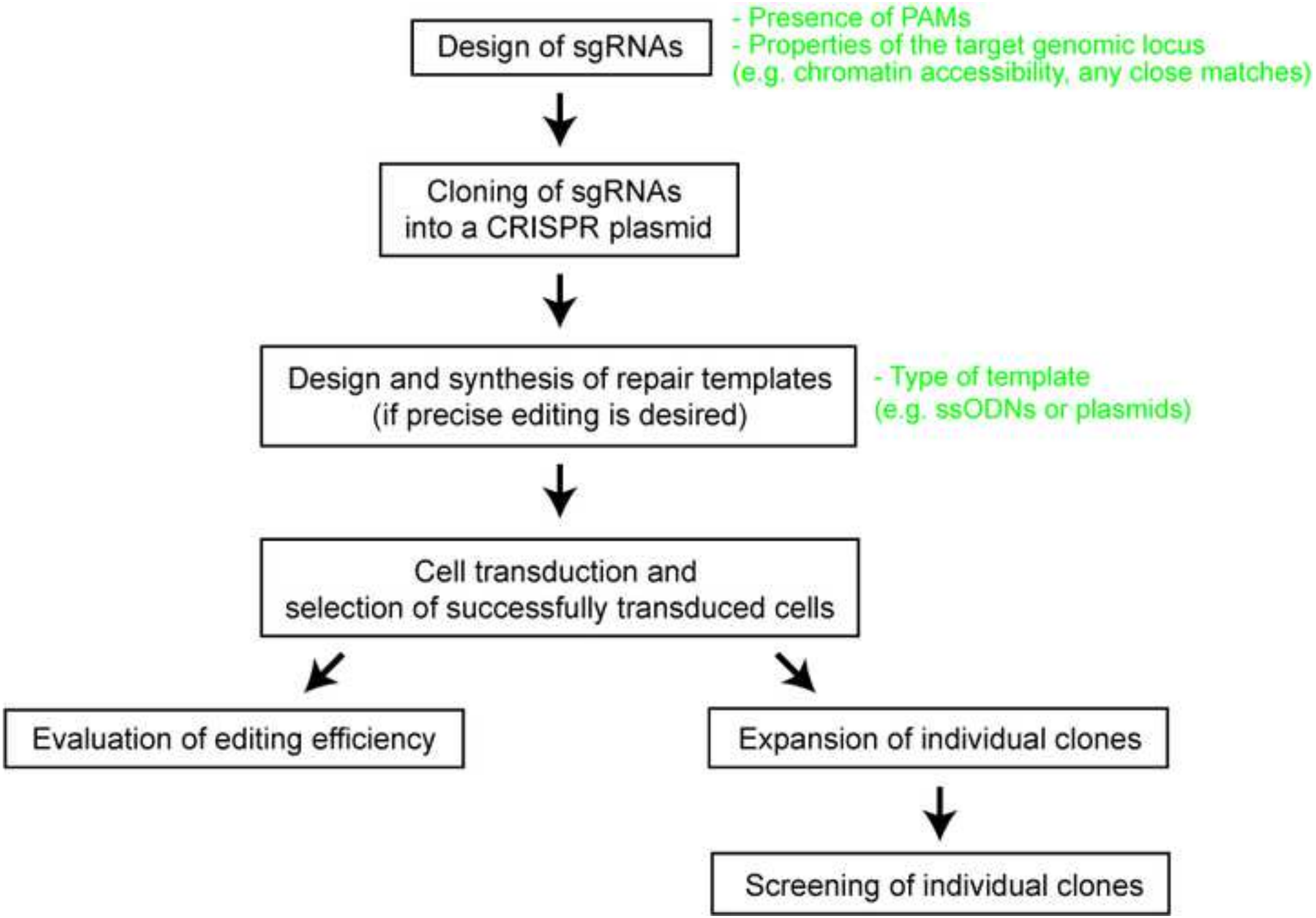
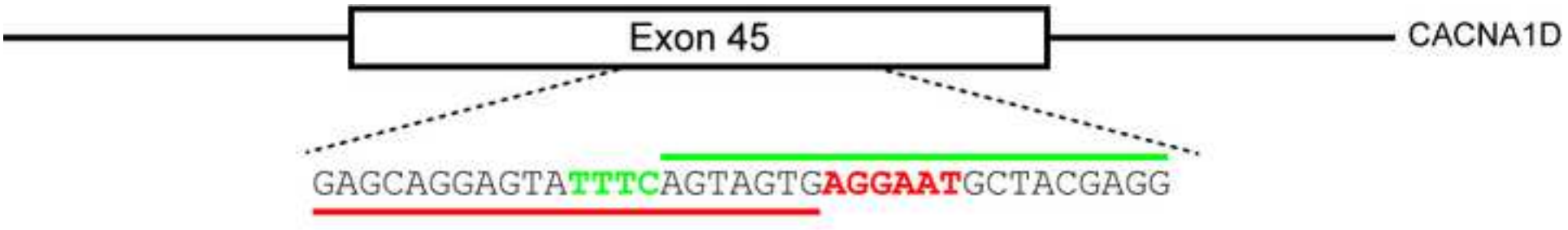


Figure 2



SpCas9 and SaCas9

Sense oligo 5′-*CACC*GAGCAGGAGTATTTCAGTAGTG-3′
Antisense oligo 3′-CTCGTCCTCATAAAGTCATCAC*CAAA*-5′

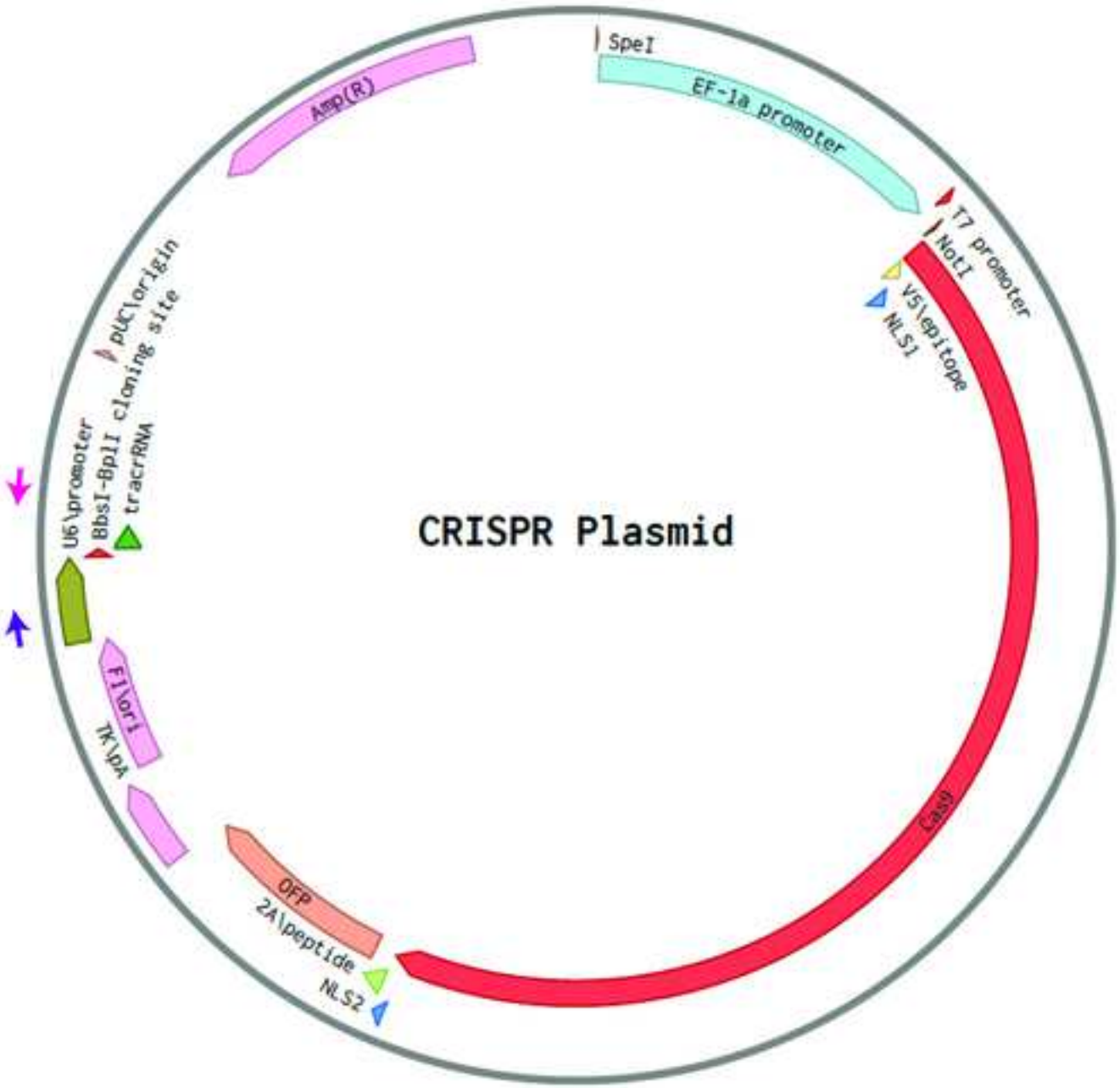
AsCas12a and LbCas12a

Sense oligo 5′-*AGAT*AGTAGTGAGGAATGCTACGAGG-3′
Antisense oligo 3′-TCATCACTCCTTACGATGCTCC*AAAA*-5′

Figure 3

Click here to access/download;Figure;Figure 3 v2 - SpCas9 plasmid map.tif

a



b

5′-AAACACCGG**GTCTTCGAG**CTAGC**CTCGAAGAC**CTGTTTTAG-3′
3′-TTTGTGGCC**CAGAAGCTC**GATCG**GAGCTTCTG**GACAAAATC-5′

c

Option 1

hU6_forward → : GAGGGCCTATTTCCCATGAT

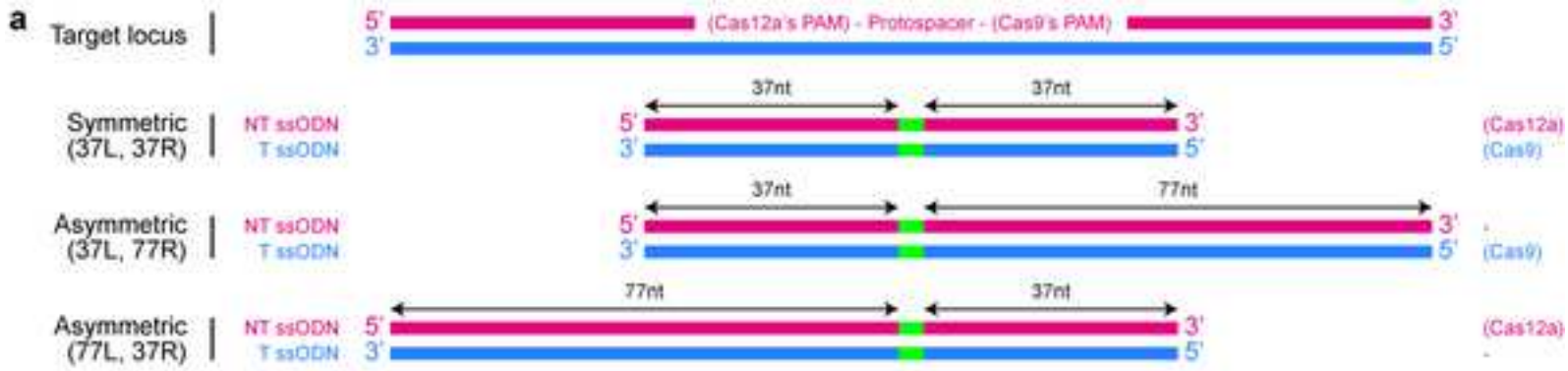
Reverse primer : Antisense oligo

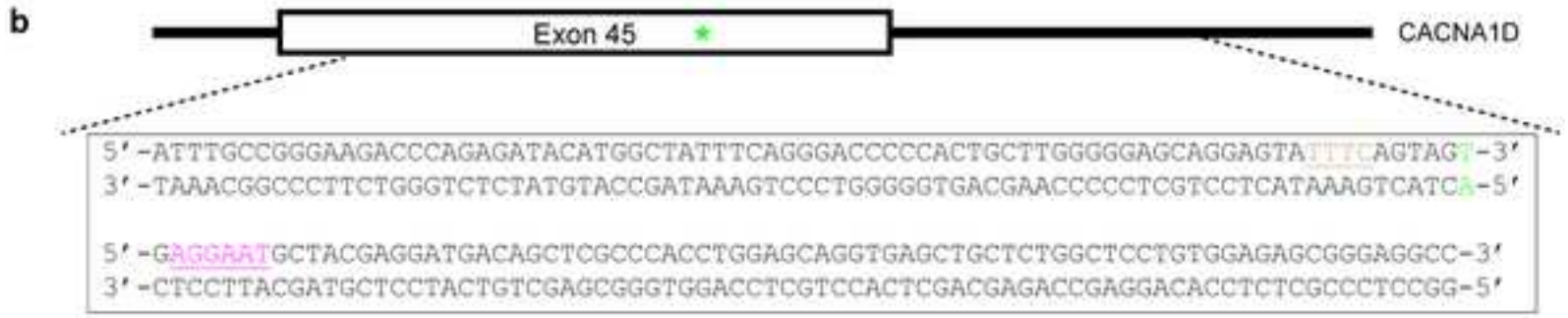
Option 2

Forward primer : Sense oligo

M13R(-20) ← : GCGGATAACAATTTCACACAGG

Figure 4





NT ssODN (37L, 37R)
5'-ACCCCCACTGCTTGGGGGAGCAGGAGTACTTCTCCAGG
GAGGAATGCTACGAGGATGACAGCTCGCCCACCTGGA-3'

T ssODN (37L, 37R)
5'-TCCAGGTGGGCGAGCTGTCATCCTCGTAGCATTCCTCC
CTGGAGAAGTACTCCTGCTCCCCCAAGCAGTGGGGGT-3'

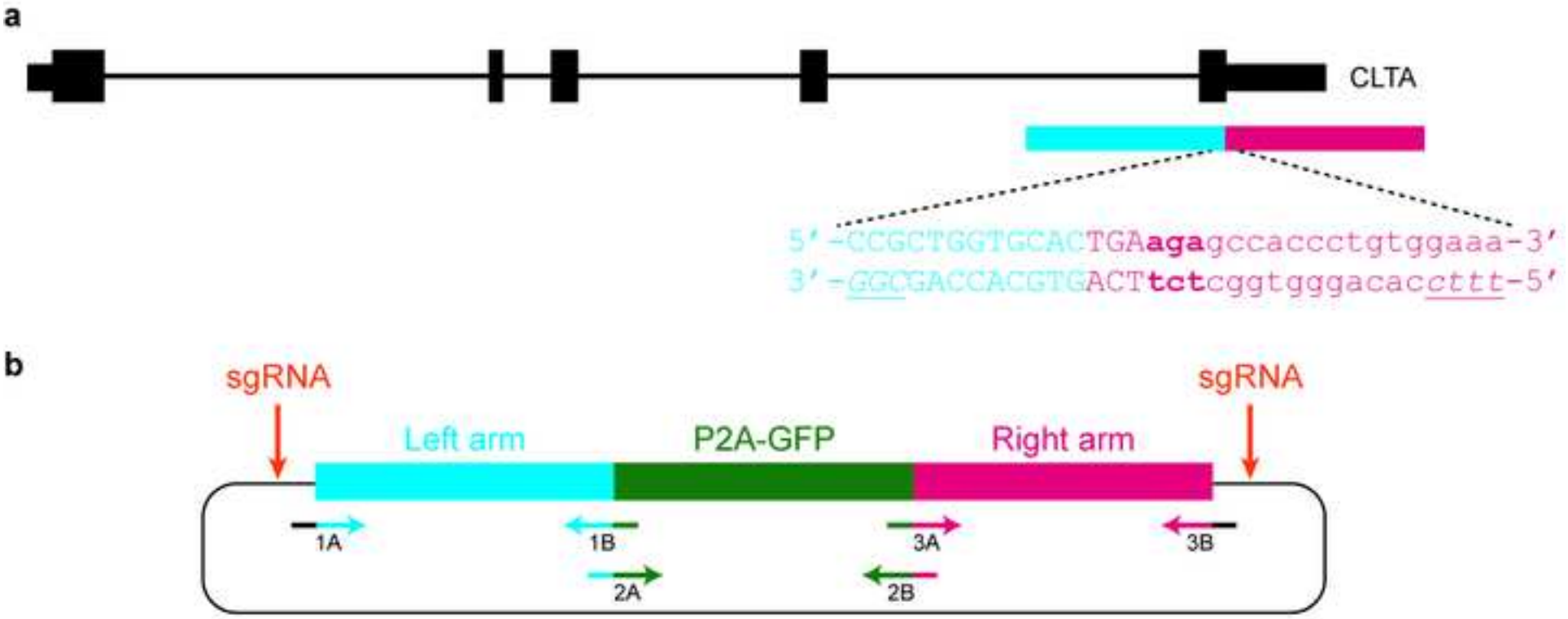
NT ssODN (37L, 77R)
(Do not use)

T ssODN (37L, 77R)
5'-GGCCTCCCGCTCTCCACAGGAGCCAGAGCAGCTCACCTGCTCCAGGTGGGCGAGCTGTCATCCTCGTAGCATTCCTCC
CTGGAGAAGTACTCCTGCTCCCCCAAGCAGTGGGGGT-3'

NT ssODN (77L, 37R)
5'-ATTTGCCGGGAAGACCCAGAGATACATGGCTATTTCAGGGACCCCCACTGCTTGGGGGAGCAGGAGTACTTCTCCAGG
GAGGAATGCTACGAGGATGACAGCTCGCCCACCTGGA-3'

T ssODN (77L, 37R)
(Do not use)

Figure 5



(1A) CLTA_Left_Forward: agaggatctactagtcatatggattCTGTAGAGCCTCTCAGGGCCTATGAC

(1B) CLTA_Left_Reverse: TAGCTCCGGATCCGTGCACCAGCGGGGCCTG

(2A) P2A-GFP_Forward: CCCGCTGGTGCACGGATCCGGAGCTACTAATTTC

(2B) P2A-GFP_Reverse: acagggtggc---TCACTTGTACAGCTCGTCCATG

(3A) CLTA_Right_Forward: CGAGCTGTACAAGTGA---gccaccctgtggaaacac

(3B) CLTA_Right_Reverse: gagctcggtacccggggatccgatttgccacctcccacctcaag

Figure 6

Click here to access/download;Figure;Figure 6 v2 - T7E1 and deep seq.tif

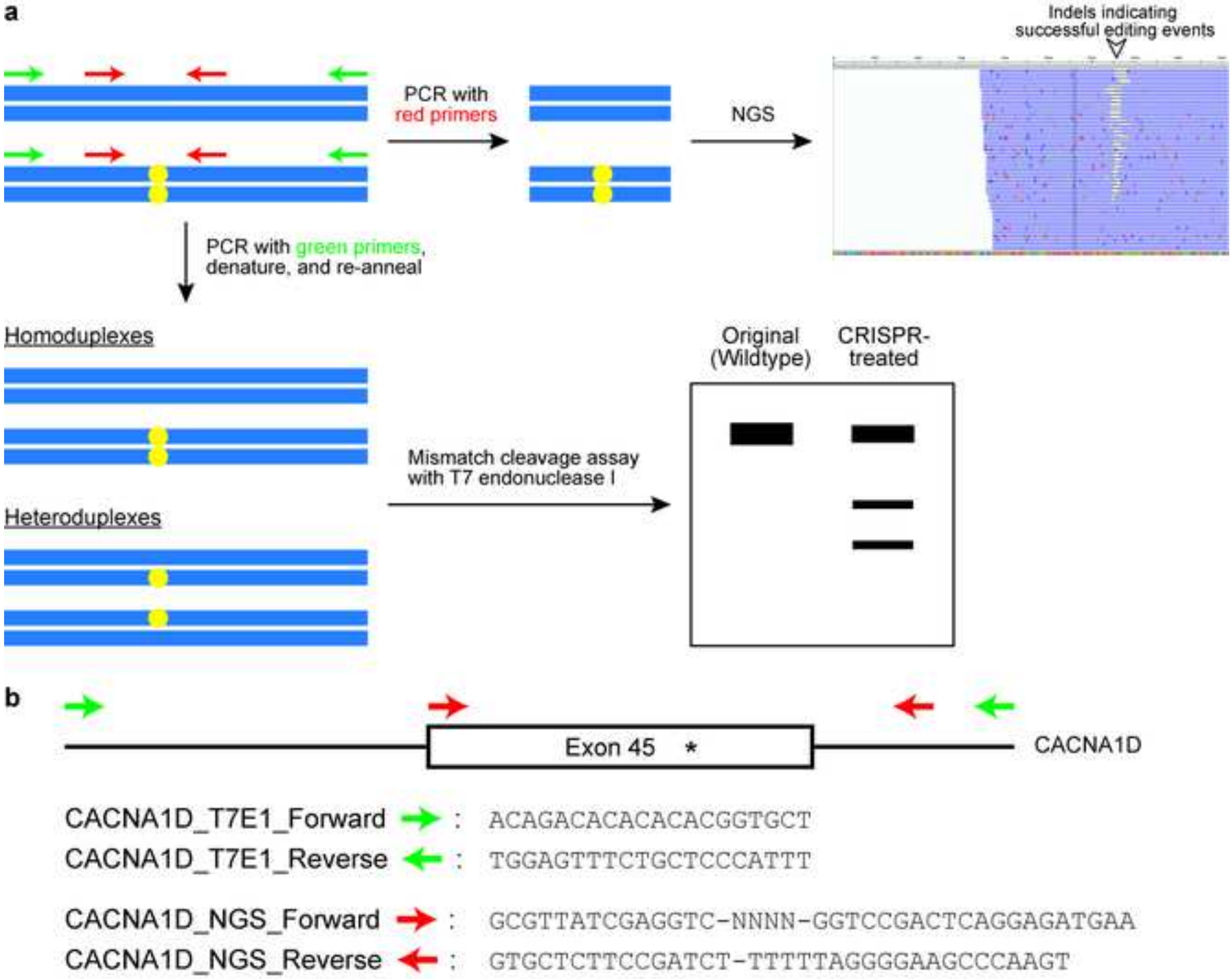


Figure 7

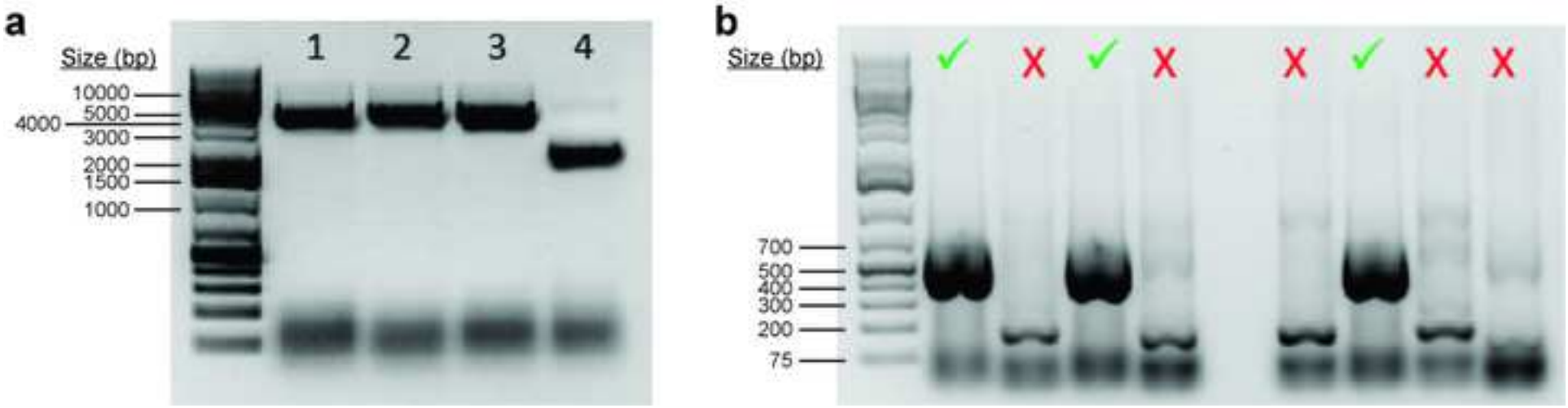


Figure 8

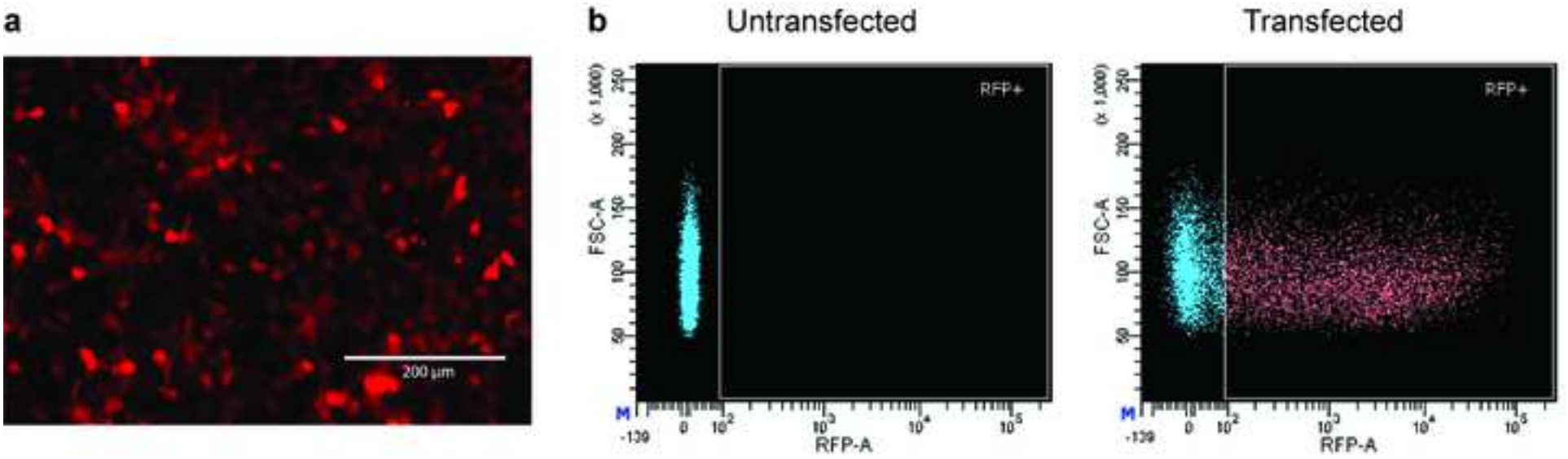


Figure 9

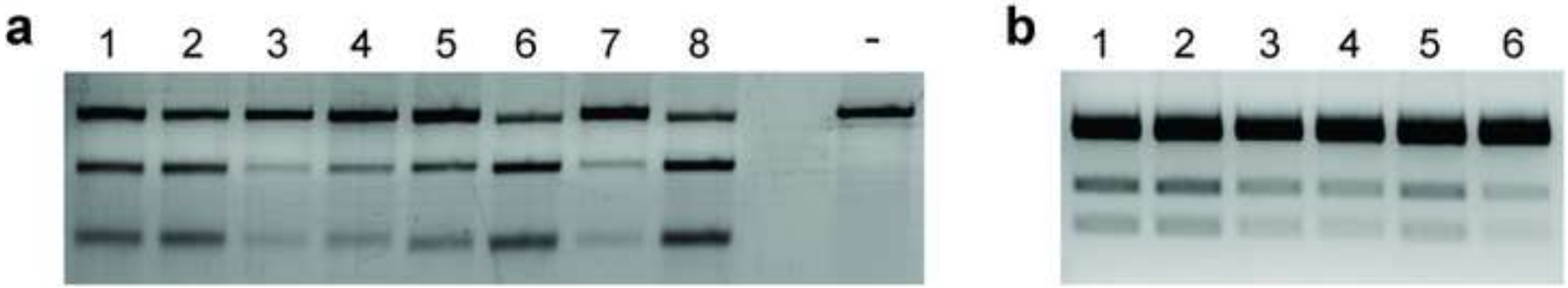


Figure 10

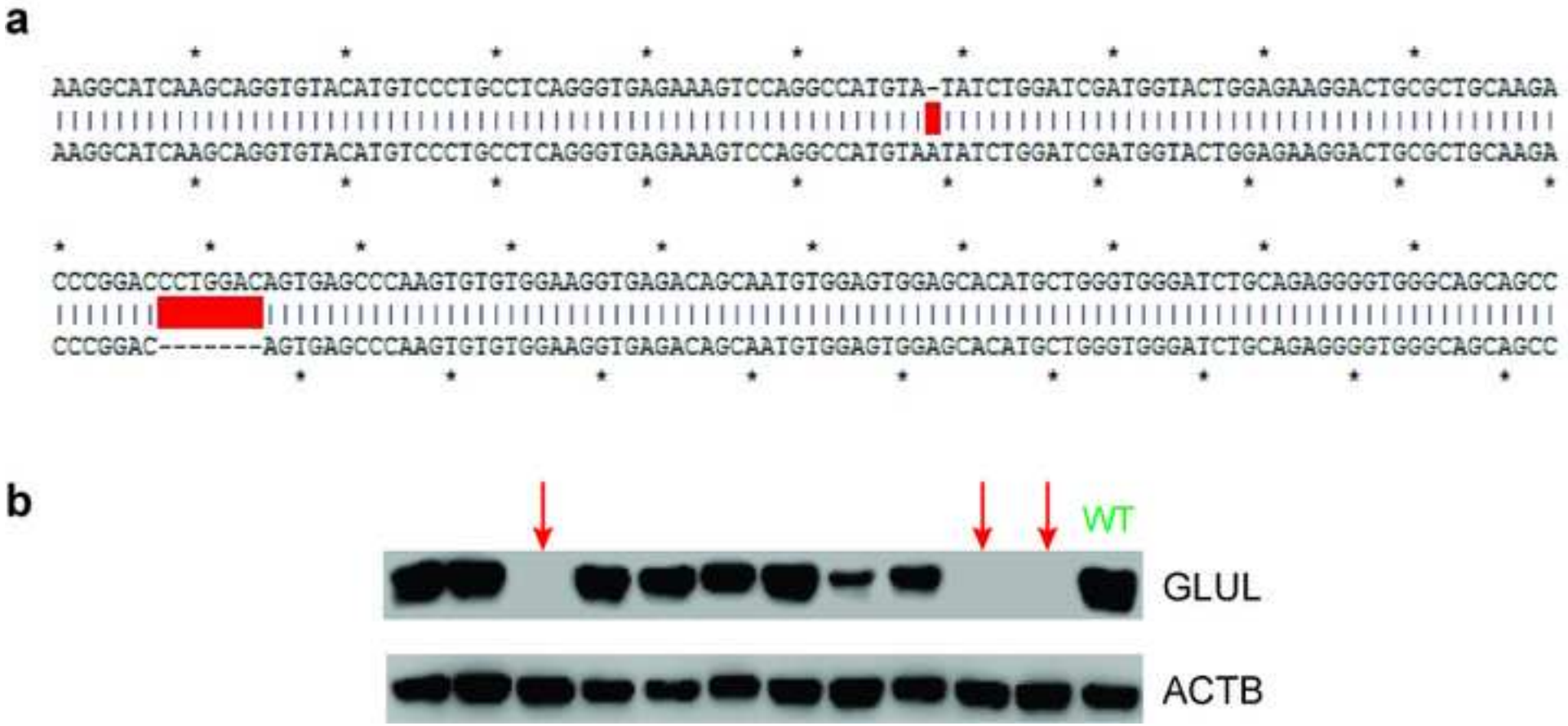


Table 1

Cas Endonuclease	PAM	Optimal spacer length
SpCas9	NGG	17-22 nt inclusive
SaCas9	NNGRRT	≥ 21 nt
NmCas9	NNNNGATT	≥ 19 nt
AsCas12a and LbCas12a	TTTV	≥ 19 nt

Table 2

CRISPR Plasmid	Sequence
pSpCas9 and pSaCas9	Sense: 5' - *CACC*(G)NNNNNNNNNNNNNNNNNNNN - 3' Antisense: 3' - (C)NNNNNNNNNNNNNNNNNNNN*CAAA* - 5'
pNmCas9	Sense: 5' - *CACC*(G)NNNNNNNNNNNNNNNNNNNN - 3' Antisense: 3' - (C)NNNNNNNNNNNNNNNNNNNN*CAAC* - 5'
pAsCas12a and pLbCas12a	Sense: 5' - *AGAT*NNNNNNNNNNNNNNNNNNNN - 3' Antisense: 3' - NNNNNNNNNNNNNNNNNNNN*AAAA* - 5'

Name of Material/ Equipment	Company	Catalog Number
T4 Polynucleotide Kinase (PNK)	NEB	M0201
Shrimp Alkaline Phosphatase (rSAP)	NEB	M0371
Tris-Acetate-EDTA (TAE) Buffer, 50X	1st Base	BUF-3000-50X4L
Tris-EDTA (TE) Buffer, 10X	1st Base	BUF-3020-10X4L
BbsI	NEB	R0539
BsmBI	NEB	R0580
T4 DNA Ligase	NEB	M0202
Quick Ligation Kit	NEB	M2200
Rapid DNA Ligation Kit	Thermo Scientific	K1423
Zero Blunt TOPO PCR Cloning Kit	Thermo Scientific	451245
NEBuilder HiFi DNA Assembly Master Mix	NEB	E2621L
One Shot Stbl3 Chemically Competent E.Coli	Thermo Scientific	C737303
LB Broth (Lennox), powder	Sigma Aldrich	L3022
LB Broth with Agar (Lennox), powder	Sigma Aldrich	L2897
SOC media	-	-
Ampicillin (Sodium), USP Grade	Gold Biotechnology	A-301
REDiant 2X PCR Mastermix	1st Base	BIO-5185
Agarose	1st Base	BIO-1000
T7 Endonuclease I	NEB	M0302
Plasmid DNA Extraction Miniprep Kit	Favorgen	FAPDE 300
Dulbecco's Modified Eagle Medium (DMEM), High Glucose	Hyclone	SH30081.01
L-Glutamine, 200mM	Gibco	25030
Penicillin-Streptomycin, 10, 000U/mL	Gibco	15140
0.25% Trypsin-EDTA, 1X	Gibco	25200
Fetal Bovine Serum	Hyclone	SV30160
Phosphate Buffered Saline, 1X	Gibco	20012
jetPRIME transfection reagent	Polyplus Transfection	114-75
QuickExtract DNA Extraction Solution, 1.0	Epicentre	LUCG-QE09050
ISOLATE II Genomic DNA Kit	Bioline	BIO-52067
Q5 High-Fidelity DNA Polymerase	NEB	M0491
Deoxynucleotide (dNTP) Solution Mix	NEB	N0447

6X DNA Loading Dye	Thermo Scientific	R0611
Protease Inhibitor Cocktail, Set3	Merck	539134
Nitrocellulose membrane, 0.2µm	Bio-Rad	1620112
Tris-glycine-SDS buffer, 10X	Bio-Rad	1610772
Tris-glycine buffer, 10X	1st base	BUF-2020
Ponceau S solution	Sigma Aldrich	P7170
TBS, 20X	1st base	BUF-3030
Tween 20	Sigma Aldrich	P9416
Skim Milk for immunoassay	Nacalai Tesque	31149-75
WesternBright Sirius-femtogram HRP	Advansta	K12043
Antibody for β-actin (C4)	Santa Cruz Biotechnology	sc-47778
MiSeq system	Illumina	SY-410-1003
NanoDrop spectrophotometer	Thermo Scientific	ND-2000
Qubit fluorometer	Thermo Scientific	Q33226
EVOS FL Cell Imaging System	Thermo Scientific	AMF4300
CRISPR plasmid: pSpCas9(BB)-2A-GFP (PX458)	Addgene	48138
CRISPR plasmid: pX601-AAV-CMV::NLS-SaCas9-NLS-3xHA-bGHpA	Addgene	61591
CRISPR plasmid: xCas9 3.7	Addgene	108379
CRISPR plasmid: pX330-U6-Chimeric_BB-CBh-hSpCas9	Addgene	42230
CRISPR plasmid: hCas9	Addgene	41815
CRISPR plasmid: eSpCas9(1.1)	Addgene	71814
CRISPR plasmid: VP12 (SpCas9-HF1)	Addgene	72247

Comments/Description
Dilute to 1X before use. The 1X solution contains 40 mM Tris, 20 mM acetic acid, and 1 mM EDTA.
Dilute to 1X before use. The 1X solution contains 10 mM Tris (pH 8.0) and 1 mM EDTA.
400,000 units/ml
An alternative to T4 DNA Ligase.
An alternative to T4 DNA Ligase.
The salt solution comes with the TOPO vector in the kit.
Kit for Gibson assembly.
Reconstitute in ddH_20, and autoclave before use
Reconstitute in ddH_20, and autoclave before use
2.5 mM KCl, 10 mM $MgCl_2$, 20 mM glucose in 1 L of LB Broth
4.5 g/L Glucose, no L-glutamine, HEPES and Sodium Pyruvate
FBS is heat inactivated before use at 56 ^{o}C for 30 min
An alternative to QuickExtract

10 mM Tris-HCl (pH 7.6) 0.03% bromophenol blue, 0.03% xylene cyanol FF, 60% glycerol, 60 mM EDTA
Dilute to 1X before use. The 1x solution contains 25 mM Tris, 192 mM glycine, and 0.1% SDS.
Dilute to 1X before use. The 1x solution contains 25 mM Tris and 192 mM glycine.
Dilute to 1X before use. The 1x solution contains 25 mM Tris-HCl (pH 7.5) and 150 mM NaCl.
Lot number: C0916
Single vector system: The gRNA is expressed from the same plasmid.
Single vector system: The gRNA is expressed from the same plasmid.
Dual vector system: The gRNA is expressed from a different plasmid.
Single vector system: The gRNA is expressed from the same plasmid.
Dual vector system: The gRNA is expressed from a different plasmid.
Single vector system: The gRNA is expressed from the same plasmid.
Dual vector system: The gRNA is expressed from a different plasmid.

jove JOURNAL OF VISUALIZED EXPERIMENTS
1 Alewife Center #200
Cambridge, MA 02140
tel. 617.945.9051
www.jove.com

ARTICLE AND VIDEO LICENSE AGREEMENT

Title of Article: Genome editing in mammalian cell lines using CRISPR-Cas

Author(s): Kaiwen Ivy Liu, Norfala-Aliah Binte Sutrisnoh, Yuanming Wang, Mxng How Tan

Item 1: The Author elects to have the Materials be made available (as described at http://www.jove.com/publish) via:

☒ Standard Access ☐ Open Access

Item 2: Please select one of the following items:

☒ The Author is **NOT** a United States government employee.

☐ The Author is a United States government employee and the Materials were prepared in the course of his or her duties as a United States government employee.

☐ The Author is a United States government employee but the Materials were NOT prepared in the course of his or her duties as a United States government employee.

ARTICLE AND VIDEO LICENSE AGREEMENT

1. **Defined Terms.** As used in this Article and Video License Agreement, the following terms shall have the following meanings: "**Agreement**" means this Article and Video License Agreement; "**Article**" means the article specified on the last page of this Agreement, including any associated materials such as texts, figures, tables, artwork, abstracts, or summaries contained therein; "**Author**" means the author who is a signatory to this Agreement; "**Collective Work**" means a work, such as a periodical issue, anthology or encyclopedia, in which the Materials in their entirety in unmodified form, along with a number of other contributions, constituting separate and independent works in themselves, are assembled into a collective whole; "**CRC License**" means the Creative Commons Attribution-Non Commercial-No Derivs 3.0 Unported Agreement, the terms and conditions of which can be found at: http://creativecommons.org/licenses/by-nc-nd/3.0/legalcode; "**Derivative Work**" means a work based upon the Materials or upon the Materials and other pre-existing works, such as a translation, musical arrangement, dramatization, fictionalization, motion picture version, sound recording, art reproduction, abridgment, condensation, or any other form in which the Materials may be recast, transformed, or adapted; "**Institution**" means the institution, listed on the last page of this Agreement, by which the Author was employed at the time of the creation of the Materials; "**JoVE**" means MyJove Corporation, a Massachusetts corporation and the publisher of The Journal of Visualized Experiments; "**Materials**" means the Article and / or the Video; "**Parties**" means the Author and JoVE; "**Video**" means any video(s) made by the Author, alone or in conjunction with any other parties, or by JoVE or its affiliates or agents, individually or in collaboration with the Author or any other parties, incorporating all or any portion of the Article, and in which the Author may or may not appear.

2. **Background.** The Author, who is the author of the Article, in order to ensure the dissemination and protection of the Article, desires to have the JoVE publish the Article and create and transmit videos based on the Article. In furtherance of such goals, the Parties desire to memorialize in this Agreement the respective rights of each Party in and to the Article and the Video.

3. **Grant of Rights in Article.** In consideration of JoVE agreeing to publish the Article, the Author hereby grants to JoVE, subject to **Sections 4** and **7** below, the exclusive, royalty-free, perpetual (for the full term of copyright in the Article, including any extensions thereto) license (a) to publish, reproduce, distribute, display and store the Article in all forms, formats and media whether now known or hereafter developed (including without limitation in print, digital and electronic form) throughout the world, (b) to translate the Article into other languages, create adaptations, summaries or extracts of the Article or other Derivative Works (including, without limitation, the Video) or Collective Works based on all or any portion of the Article and exercise all of the rights set forth in (a) above in such translations, adaptations, summaries, extracts, Derivative Works or Collective Works and(c) to license others to do any or all of the above. The foregoing rights may be exercised in all media and formats, whether now known or hereafter devised, and include the right to make such modifications as are technically necessary to exercise the rights in other media and formats. If the "Open Access" box has been checked in **Item 1** above, JoVE and the Author hereby grant to the public all such rights in the Article as provided in, but subject to all limitations and requirements set forth in, the CRC License.

jove VISUALIZED JOURNAL OF EXPERIMENTS
1 Alewife Center #200
Cambridge, MA 02140
tel. 617.945.9051
www.jove.com

ARTICLE AND VIDEO LICENSE AGREEMENT

4. **Retention of Rights in Article.** Notwithstanding the exclusive license granted to JoVE in **Section 3** above, the Author shall, with respect to the Article, retain the non-exclusive right to use all or part of the Article for the non-commercial purpose of giving lectures, presentations or teaching classes, and to post a copy of the Article on the Institution's website or the Author's personal website, in each case provided that a link to the Article on the JoVE website is provided and notice of JoVE's copyright in the Article is included. All non-copyright intellectual property rights in and to the Article, such as patent rights, shall remain with the Author.

5. **Grant of Rights in Video – Standard Access.** This **Section 5** applies if the "Standard Access" box has been checked in **Item 1** above or if no box has been checked in **Item 1** above. In consideration of JoVE agreeing to produce, display or otherwise assist with the Video, the Author hereby acknowledges and agrees that, Subject to **Section 7** below, JoVE is and shall be the sole and exclusive owner of all rights of any nature, including, without limitation, all copyrights, in and to the Video. To the extent that, by law, the Author is deemed, now or at any time in the future, to have any rights of any nature in or to the Video, the Author hereby disclaims all such rights and transfers all such rights to JoVE.

6. **Grant of Rights in Video – Open Access.** This **Section 6** applies only if the "Open Access" box has been checked in **Item 1** above. In consideration of JoVE agreeing to produce, display or otherwise assist with the Video, the Author hereby grants to JoVE, subject to **Section 7** below, the exclusive, royalty-free, perpetual (for the full term of copyright in the Article, including any extensions thereto) license (a) to publish, reproduce, distribute, display and store the Video in all forms, formats and media whether now known or hereafter developed (including without limitation in print, digital and electronic form) throughout the world, (b) to translate the Video into other languages, create adaptations, summaries or extracts of the Video or other Derivative Works or Collective Works based on all or any portion of the Video and exercise all of the rights set forth in (a) above in such translations, adaptations, summaries, extracts, Derivative Works or Collective Works and (c) to license others to do any or all of the above. The foregoing rights may be exercised in all media and formats, whether now known or hereafter devised, and include the right to make such modifications as are technically necessary to exercise the rights in other media and formats. For any Video to which this **Section 6** is applicable, JoVE and the Author hereby grant to the public all such rights in the Video as provided in, but subject to all limitations and requirements set forth in, the CRC License.

7. **Government Employees.** If the Author is a United States government employee and the Article was prepared in the course of his or her duties as a United States government employee, as indicated in **Item 2** above, and any of the licenses or grants granted by the Author hereunder exceed the scope of the 17 U.S.C. 403, then the rights granted hereunder shall be limited to the maximum rights permitted under such statute. In such case, all provisions contained herein that are not in conflict with such statute shall remain in full force and effect, and all provisions contained herein that do so conflict shall be deemed to be amended so as to provide to JoVE the maximum rights permissible within such statute.

8. **Protection of the Work.** The Author(s) authorize JoVE to take steps in the Author(s) name and on their behalf if JoVE believes some third party could be infringing or might infringe the copyright of either the Author's Article and/or Video.

9. **Likeness, Privacy, Personality.** The Author hereby grants JoVE the right to use the Author's name, voice, likeness, picture, photograph, image, biography and performance in any way, commercial or otherwise, in connection with the Materials and the sale, promotion and distribution thereof. The Author hereby waives any and all rights he or she may have, relating to his or her appearance in the Video or otherwise relating to the Materials, under all applicable privacy, likeness, personality or similar laws.

10. **Author Warranties.** The Author represents and warrants that the Article is original, that it has not been published, that the copyright interest is owned by the Author (or, if more than one author is listed at the beginning of this Agreement, by such authors collectively) and has not been assigned, licensed, or otherwise transferred to any other party. The Author represents and warrants that the author(s) listed at the top of this Agreement are the only authors of the Materials. If more than one author is listed at the top of this Agreement and if any such author has not entered into a separate Article and Video License Agreement with JoVE relating to the Materials, the Author represents and warrants that the Author has been authorized by each of the other such authors to execute this Agreement on his or her behalf and to bind him or her with respect to the terms of this Agreement as if each of them had been a party hereto as an Author. The Author warrants that the use, reproduction, distribution, public or private performance or display, and/or modification of all or any portion of the Materials does not and will not violate, infringe and/or misappropriate the patent, trademark, intellectual property or other rights of any third party. The Author represents and warrants that it has and will continue to comply with all government, institutional and other regulations, including, without limitation all institutional, laboratory, hospital, ethical, human and animal treatment, privacy, and all other rules, regulations, laws, procedures or guidelines, applicable to the Materials, and that all research involving human and animal subjects has been approved by the Author's relevant institutional review board.

11. **JoVE Discretion.** If the Author requests the assistance of JoVE in producing the Video in the Author's facility, the Author shall ensure that the presence of JoVE employees, agents or independent contractors is in accordance with the relevant regulations of the Author's institution. If more than one author is listed at the beginning of this Agreement, JoVE may, in its sole

jove VISUALIZED EXPERIMENTS
1 Alewife Center #200
Cambridge, MA 02140
tel. 617.945.9051
www.jove.com

ARTICLE AND VIDEO LICENSE AGREEMENT

discretion, elect not take any action with respect to the Article until such time as it has received complete, executed Article and Video License Agreements from each such author. JoVE reserves the right, in its absolute and sole discretion and without giving any reason therefore, to accept or decline any work submitted to JoVE. JoVE and its employees, agents and independent contractors shall have full, unfettered access to the facilities of the Author or of the Author's institution as necessary to make the Video, whether actually published or not. JoVE has sole discretion as to the method of making and publishing the Materials, including, without limitation, to all decisions regarding editing, lighting, filming, timing of publication, if any, length, quality, content and the like.

12. **Indemnification.** The Author agrees to indemnify JoVE and/or its successors and assigns from and against any and all claims, costs, and expenses, including attorney's fees, arising out of any breach of any warranty or other representations contained herein. The Author further agrees to indemnify and hold harmless JoVE from and against any and all claims, costs, and expenses, including attorney's fees, resulting from the breach by the Author of any representation or warranty contained herein or from allegations or instances of violation of intellectual property rights, damage to the Author's or the Author's institution's facilities, fraud, libel, defamation, research, equipment, experiments, property damage, personal injury, violations of institutional, laboratory, hospital, ethical, human and animal treatment, privacy or other rules, regulations, laws, procedures or guidelines, liabilities and other losses or damages related in any way to the submission of work to JoVE, making of videos by JoVE, or publication in JoVE or elsewhere by JoVE. The Author shall be responsible for, and shall hold JoVE harmless from, damages caused by lack of sterilization, lack of cleanliness or by contamination due to the making of a video by JoVE its employees, agents or independent contractors. All sterilization, cleanliness or decontamination procedures shall be solely the responsibility of the Author and shall be undertaken at the Author's expense. All indemnifications provided herein shall include JoVE's attorney's fees and costs related to said losses or damages. Such indemnification and holding harmless shall include such losses or damages incurred by, or in connection with, acts or omissions of JoVE, its employees, agents or independent contractors.

13. **Fees.** To cover the cost incurred for publication, JoVE must receive payment before production and publication the Materials. Payment is due in 21 days of invoice. Should the Materials not be published due to an editorial or production decision, these funds will be returned to the Author. Withdrawal by the Author of any submitted Materials after final peer review approval will result in a US$1,200 fee to cover pre-production expenses incurred by JoVE. If payment is not received by the completion of filming, production and publication of the Materials will be suspended until payment is received.

14. **Transfer, Governing Law.** This Agreement may be assigned by JoVE and shall inure to the benefits of any of JoVE's successors and assignees. This Agreement shall be governed and construed by the internal laws of the Commonwealth of Massachusetts without giving effect to any conflict of law provision thereunder. This Agreement may be executed in counterparts, each of which shall be deemed an original, but all of which together shall be deemed to me one and the same agreement. A signed copy of this Agreement delivered by facsimile, e-mail or other means of electronic transmission shall be deemed to have the same legal effect as delivery of an original signed copy of this Agreement.

A signed copy of this document must be sent with all new submissions. Only one Agreement is required per submission.

CORRESPONDING AUTHOR

Name:	Tan Meng How
Department:	Stem Cells and Regenerative Biology
Institution:	Genome Institute of Singapore
Title:	Senior Research Scientist
Signature:	[signature]
Date:	11 Sept 2018

Please submit a **signed** and **dated** copy of this license by one of the following three methods:

1. Upload an electronic version on the JoVE submission site
2. Fax the document to +1.866.381.2236
3. Mail the document to JoVE / Attn: JoVE Editorial / 1 Alewife Center #200 / Cambridge, MA 02140

We thank the editor and reviewers for their comments, which have improved our protocol.

Editorial comments:

Changes to be made by the author(s) regarding the manuscript:

1. Please take this opportunity to thoroughly proofread the manuscript to ensure that there are no spelling or grammar issues.

We have proofread the manuscript to eliminate any noticeable language errors.

2. Please define all abbreviations (TE, PNK, SAP, SOC, etc.) before use.

We have defined all abbreviations on first use.

3. JoVE cannot publish manuscripts containing commercial language. This includes trademark symbols ((tm)), registered symbols (®), and company names before an instrument or reagent. Please remove all commercial language from your manuscript and use generic terms instead. All commercial products should be sufficiently referenced in the Table of Materials and Reagents. You may use the generic term followed by "(see table of materials)" to draw the readers' attention to specific commercial names. Examples of commercial sounding language in your manuscript are: One Shot Stbl3, Gibson assembly, QuickExtract, NanoDrop, Qubit, Fluidigm, Access Array System, Illumina MiSeq, etc.

As suggested, we have used generic terms followed by “(see List of Materials)” to draw the readers’ attention to specific commercial items.

4. Please revise the protocol text to avoid the use of any personal pronouns (e.g., "we", "you", "our" etc.).

We have revised the text to avoid the usage of any personal pronouns in the protocol text.

5. Please revise the protocol to contain only action items that direct the reader to do something (e.g., "Do this," "Ensure that," etc.). The actions should be described in the imperative tense in complete sentences wherever possible. Avoid usage of phrases such as "could be," "should be," and "would be" throughout the Protocol. Any text that cannot be written in the imperative tense may be added as a "Note." Please include all safety procedures and use of hoods, etc. However, notes should be used sparingly and actions should be described in the imperative tense wherever possible.

We have gone through the protocol to ensure that it contains primarily actions items described in imperative tense. We have also included safety procedures, when necessary.

6. Lines 162-170: Please include these in a Table.

We have created a Table for the oligonucleotide sequences.

7. Please add more details to your protocol steps. There should be enough detail in each step to supplement the actions seen in the video so that viewers can easily replicate the protocol. Please ensure you answer the "how" question, i.e., how is the step performed? Alternatively, add references to published material specifying how to perform the protocol action. See examples below.

As requested, we have added more details to some of our protocol steps.

8. 2.1.1, 2.4.3: Please provide composition of TE buffer, SOC media, etc. If they are purchased, please cite the Table of Materials.

As requested, we have provided the compositions in the Table of Materials.

9. 2.2.4: Please provide the composition of loading dye.

As requested, we have provided the composition of the loading dye in the Table of Materials.

10. 5.1: Please specify the reaction temperature.

As requested, we have specified the reaction temperature.

11. 5.2: What volume of cell culture is added?

As requested, we have specified the volume.

12. 6.3: Please specify growth conditions.

As requested, we have specified the growth conditions.

13. Lines 431, 470: What wavelengths are measured?

As requested, we have specified the wavelengths.

14. 7.2.9: Please describe how to quantify the band intensities using ImageJ.

As requested, we have described how to quantify band intensities in ImageJ.

15. After you have made all the recommended changes to your protocol (listed above), please highlight 2.75 pages or less of the Protocol (including headings and spacing) that identifies the essential steps of the protocol for the video, i.e., the steps that should be visualized to tell the most cohesive story of the Protocol.

Since George Church's laboratory has recently published a JoVE article on "CRISPR Guide RNA Cloning for Mammalian Systems", we propose to focus on more downstream parts of our protocol. In particular, we have highlighted the parts on expanding individual clones and evaluating editing efficiency using the T7EI assay.

16. Please highlight complete sentences (not parts of sentences). Please ensure that the highlighted part of the step includes at least one action that is written in imperative tense.

We have highlighted complete sentences that contain actionable items.

17. Please include all relevant details that are required to perform the step in the highlighting. For example: If step 2.5 is highlighted for filming and the details of how to perform the step are given in steps 2.5.1 and 2.5.2, then the sub-steps where the details are provided must be highlighted.

We have highlighted all relevant details for filming.

18. Figure 4: Please use the micro symbol µ instead of u for the scale bar unit.

As requested, we have used the micro symbol µ.

19. Figure 8: Please explain the red boxes in the figure legend.

As requested, we have explained the red boxes in the figure legend.

20. Table of Equipment and Materials: Please provide lot numbers and RRIDs of antibodies, if available.

As requested, we have provided information about the β-actin antibody.

21. Discussion: Please also discuss critical steps within the protocol and any limitations of the technique.

We apologize for the lack of clarity. We have now explicitly stated the critical steps (design of donor template and single cell cloning) as well as current limitations of the CRISPR-Cas technology (last paragraph of discussion section).

22. References: Please do not abbreviate journal titles.

As requested, we have listed the journal titles in full.

Reviewers' comments:

Reviewer #1:

Manuscript Summary:

In this manuscript by Liu et al., the authors provide a step-by-step protocol describing the detailed procedure of CRISPR genome editing in the mammalian cell lines. The text is mostly clearly written. However, in some places, the lack of illustration or specific examples makes it hard to grasp the methodology.

We apologise for the lack of clarity. We have included some specific examples to aid in the understanding of our protocol.

Major Concerns:

1. Table 1. The N, R and V symbols should be specifically explained in the footnote. In addition, the position of PAM domain (5' or 3') should be specified.

As requested, we have explained the N, R, and V symbols and specified the PAM positions.

2. For application purpose, the authors may consider providing a list of all Crispr plasmids available from Addgene, or commercial sources.

As requested, we have added a note to direct the reader to all the CRISPR plasmids available on Addgene and have also included a table listing the more popular ones.

3. Page 3, under 1.3.3, the Sense and antisense sequences should be drawn in a reverse complement session to improve visualization. As a further clarification, the authors can use an example of one particular gRNA target, highlighting the target of spaces in its genomic regions with PAM clearly indicated.

As requested, we have now drawn the sense and antisense sequences in a reverse complementary fashion (Table 2). We have also given an example of one target genomic locus (Figure 2).

4. Page 4, session 2.2, an example of Cas9 plasmid feature map and cloning site will be helpful.

As requested, we have provided a Cas9 plasmid map with its cloning sites (Figure 3).

5. Page 5, session 2.5.4, it will be helpful to give an example of primer sequences together with their relative locations on the Cas9 vector.

As requested, we have provided some primer sequences and their locations on the plasmid map (Figure 3).

6. Page 6, first line, an example of PCR primer sequences should be given.

The primers used for colony PCR can be used for Sanger sequencing as well. We have noted this in the protocol text.

7. Page 6, 3.1.1, an example of the sequence of ssODN donor template should be given.

As requested, we have provided examples of ssODN sequences (Figure 4).

8. In Figure 1, the ssODN sequences should be shown as single-stranded rather than double stranded. To facilitate interpretation, the authors may consider indicating the Cas12a or Cas9 preference for each type of donor templates in the same figure.

We apologize for the lack of clarity. The ssODNs are depicted as single-stranded. What we have meant to convey, is that the sequence of the ssODN template can be derived from either of the two DNA strands. We hope that the updated figure panel (now labelled as Figure 4a) and its associated legend are clearer now. We have also indicated the Cas12a and Cas9 preferences in the figure.

9. Page 7, 3.2.1, an example of the sequence of plasmid donor template should be given.

Instead of simply listing out a long plasmid sequence, we have created an illustrative example, which we hope will be more useful to readers (Figure 5).

10. Page 7, 3.2.2, an example of the sequences of the primers should be given.

As requested, we have provided an example of primer sequences for cloning a plasmid donor template (Figure 5).

11. Page 7, 4.2, "...according to manufacturer's instructions". Do the authors here refer to instructions on transfection? An example of transfection reagent should be given here.

Yes, we are referring to instructions on the transfection. We have modified the text to improve its clarity. An example of a transfection reagent is given in the List of Materials.

12. Page 9, 7.2.2, an example of the primer sequences should be given.

As requested, we have provided an example of the primer sequences in Figure 6.

13. Page 9, 7.2.5, what buffer?

We apologise for the lack of clarity. We are referring to the buffer that comes with the T7 endonuclease I and have updated the protocol text accordingly.

14. Page 10, 7.3.2, an example of the primer sequences should be given.

As requested, we have provided an example of the primer sequences in Figure 6.

Minor Concerns:

1. Total page numbers are off.

We will work with the editor to resolve the page numbers.

Reviewer #2:

Manuscript Summary:

In this manuscript, Tan et al. introduced a workflow of CRISPR gene editing toolkit which has great potential in the future genetic modification research work. Particularly, they described several endonucleases of CRISPR like Cas9, Cas12a, etc. They compared the pros and cons of different endonucleases and explained the rational of choosing favorable endonuclease sufficiently. In the method section, they provided the detailed steps of sequence design, transfection, efficiency evaluation, etc. They also discussed the possible issues and how to improve efficiency. The manuscript is well organized and illustrated the workflow explicitly.

We thank the reviewer for the kind remarks.

Minor Concerns:

Here are two suggestions:

1. Since the manuscript is describing the workflow of using CRISPR for gene editing, perhaps a flowchart such as a tree diagram is more straightforward than a paragraph in words to demonstrate how to choose endonuclease, how to select spacer sequence. The original protocol is perfect. Another tree diagram with branches can show how to make decision when they have different purposes or needs. It's easier to get the whole idea.

As requested, we have provided a flowchart in our revised manuscript (Figure 1).

2. I got the question about how the workflow works in primary cells until I read your brief discussion on stem cells in discussion section. I'm quite interested if you may share a little more experience in primary cells genetic modification because primary cells such as T cells, stem cells or other immune cells are very appealing to clinical therapy for now.

As requested, we have shared some of our experiences with primary cells in the discussion section.

Reviewer #3:

Manuscript Summary:

Authors do a very good job to lay out the basic protocol for performing CRIPSR-Cas9 mediated genetic manipulation. This will be very useful to the uninitiated people when they

attempt the method.

We thank the reviewer for the kind remarks.

Minor Concerns:

For 2.3.1: Authors can modify the protocol to mention that quick ligase enzyme can be used for ligation of insert into vector as it only takes 15 min at room temperature for ligation and saves time.

We thank the reviewer for the suggestion and have added it to the manuscript text.

Also while transforming bacteria if the selection cassette is ampicillin, then one need not incubate bacteria at 37 C after adding SOC. Cells can be directly plated on ampicillin containing agar plates to save time.

We thank the reviewer for the suggestion and have added it to the manuscript text.

Supplementary Methods

1. Bacterial Transformation

1.1 Add 3 µL ligation or TOPO reaction mix to 40 µL of chemically competent *E. coli* cells (see **Table of Materials**) and leave on ice for 30 min.

1.2 Heat shock the bacteria at 42 °C for 45 s, then leave on ice for 2 min.

1.3 Add 250 µL of Super Optimal broth with Catabolite repression (SOC) media and recover the bacteria in a 37 °C incubator-shaker for 1 h.

1.4 Spread 80 µL transformed bacterial cells on a pre-warmed Luria broth (LB) agar plate with the appropriate antibiotics. Incubate the plate at 37 °C overnight.

NOTE: Check which antibiotic-resistant cassette is present in the cloning plasmid. Ampicillin is commonly used, but some plasmids may rely on kanamycin, gentamycin, chloramphenicol, or spectinomycin.

NOTE: If ampicillin is used, the transformed bacteria may be directly plated onto antibiotic-containing agar plates immediately after heat shock without the 1 h recovery at 37 °C.

2. Western blot

2.1 Dissociate the cells as described in steps 5.1–5.3 of protocol and aspirate the supernatant.

2.2 Wash the pellet once with cold PBS, and spin them down in a bench top centrifuge at maximum speed (18,000 x g) for 5 min. Discard the supernatant and resuspend the cell pellet in cold RIPA buffer. Add protease inhibitor (see **Table of Materials**) to protect the proteins from degradation. Sonicate the cells for 10 cycles (30 s ON, 30 s OFF).

NOTE: Different protease inhibitors can be used, including aprotinin, leupeptin, pepstatin-A, and phenylmethylsulfonyl fluoride (PMSF). Commercially available cocktail tablets may also be used.

2.3 Determine the protein concentration using bovine serum albumin as a standard. Different methods can be used, including the Bradford assay and the bicinchoninic acid (BCA) assay.

2.4 Boil each cell lysate in 4x Laemmli sample buffer at 100 °C for 5 min.

2.5 For each sample, load 10 µg of protein into one empty well of an sodium dodecyl sulfate (SDS)-PAGE gel. Run the gel for 1–2 h at 100 V using 1x tris-glycine-SDS (TGS) buffer.

2.6 Transfer the electrophoresed proteins onto a nitrocellulose or polyvinylidene fluoride (PVDF) membrane using 1x tris-glycine (TG) buffer with 10% ethanol.

2.7 Stain the membrane with Ponceau S solution to check that the proteins have been properly transferred. Wash off the stain with water. Block the membrane for 1 h at room temperature using 5% skimmed milk (blocking buffer) and then remove the blocking buffer.

2.8 Dilute the primary antibody with blocking buffer and add it to the membrane. Incubate the membrane at 4 °C overnight with gentle shaking. The dilution factor depends on the antibody and may have to be optimized.

2.9 Remove the primary antibody solution. Wash three times with 0.1% polysorbate in tris-buffered Saline (TBST) at 10 min intervals.

2.10 Remove the 0.1% TBST. Dilute the conjugated secondary antibody with blocking buffer based on supplier's recommendations, then add it to the membrane. Incubate the membrane at room temperature for 2 h with gentle shaking.

2.11 Remove the secondary antibody solution. Wash three times with 0.1% TBST at 10 min intervals. Add a chemiluminescent reagent (see **Table of Materials**) to the membrane according to manufacturer's instructions and visualize the membrane using an appropriate imaging system.