CHAPTER EIGHT

Genome Editing Using Cas9 Nickases

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Contents

Abstract

The RNA-guided, sequence-specific endonuclease Cas9 has been widely adopted as genome engineering tool due to its efficiency and ease of use. Derived from the microbial CRISPR (clustered regularly interspaced short palindromic repeat) type II adaptive immune system, Cas9 has now been successfully engineered for genome editing applications in a variety of animal and plant species. To reduce potential off-target mutagenesis by wild-type Cas9, homology- and structure-guided mutagenesis of *Streptococcus pyogenes* Cas9 catalytic domains has produced "nicking" enzymes (Cas9n) capable of inducing single-strand nicks rather than double-strand breaks. Since nicks are generally repaired with high fidelity in eukaryotic cells, Cas9n can be leveraged to mediate highly specific genome editing, either via nonhomologous end-joining or homology-directed repair. Here we describe the preparation, testing, and application of Cas9n reagents for precision mammalian genome engineering.

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1. INTRODUCTION

Targeted, rapid, and efficient genome editing using the RNA-guided Cas9 system is enabling the systematic interrogation of genetic elements in a variety of cells and organisms and holds enormous potential as a next-generation gene therapy (Hsu, Lander, & Zhang, 2014). In contrast to other DNA-targeting systems based on zinc-finger proteins (Klug, 2010) and transcription activator-like effectors (Boch & Bonas, 2010), which rely on protein domains to confer DNA-binding specificity, Cas9 forms a complex with a small-guide RNA that directs the enzyme to its DNA target via Watson–Crick base pairing. Consequently, the system is simple and fast to design and requires only the production of a short oligonucleotide to direct DNA binding to any locus.

The type II microbial CRISPR (clustered regularly interspaced short palindromic repeat) system (Chylinski, Makarova, Charpentier, & Koonin, 2014), which is the simplest among the three known CRISPR types (Barrangou & Marraffini, 2014; Gasiunas, Sinkunas, & Siksnys, 2014; Wiedenheft, Sternberg, & Doudna, 2012), consists of the CRISPR-associated (*Cas*) genes and a series of noncoding repetitive elements (direct repeats) interspaced by short variable sequences (spacers). These short ~30-bp spacers are often derived from foreign genetic elements such as phages and conjugating plasmids, and they constitute the basis for an adaptive immune memory of those invading elements (Barrangou et al., 2007). The corresponding sequences on the phage genomes and plasmids are called protospacers, and each protospacer is flanked by a short protospacer-adjacent motif (PAM), which plays a critical role in the target search and recognition mechanism of Cas9. The CRISPR array is transcribed and processed into short RNA molecules known as CRISPR RNAs (crRNA) that, together with a second short trans-activating RNA (tracrRNA) (Deltcheva et al., 2011), complex with Cas9 to facilitate target recognition and cleavage (Deltcheva et al., 2011; Garneau et al., 2010). Additionally, the crRNA and tracrRNA can be fused into a single guide RNA (sgRNA) to facilitate Cas9 targeting (Jinek et al., 2012).

The Cas9 enzyme from *Streptococcus pyogenes* (SpCas9), which requires a 5′-NGG PAM (Mojica, Diez-Villasenor, Garcia-Martinez, & Almendros, 2009), has been widely used for genome editing applications (Hsu et al., 2014). In order to target any desired genomic locus of interest that fulfills the PAM requirement, the enzyme can be "programmed" merely by

altering the 20-bp guide sequence of the sgRNA. Additionally, the simplicity of targeting lends itself to easy multiplexing, such as simultaneous editing of several loci by including multiple sgRNAs (Cong et al., 2013; Wang et al., 2013).

Like other designer nucleases, Cas9 facilitates genome editing by inducing double-strand breaks (DSBs) at its target site, which in turn stimulates endogenous DNA damage repair pathways that lead to edited DNA: homology-directed repair (HDR), which requires a homologous template for recombination but repairs DSBs with high fidelity, and nonhomologous end-joining (NHEJ), which functions without a template and frequently produces insertions or deletions (indels) as a consequence of repair. Exogenous HDR templates can be designed and introduced along with Cas9 and sgRNA to promote exact sequence alteration at a target locus; however, this process typically occurs only in dividing cells and at low efficiency.

Certain applications—e.g., therapeutic genome editing in human stem cells—demand editing that is not only efficient but also highly specific. Nucleases with off-target DSB activity could induce undesirable mutations with potentially deleterious effects, an unacceptable outcome in most clinical settings. The remarkable ease of targeting Cas9 has enabled extensive off-target binding and mutagenesis studies employing deep sequencing (Fu et al., 2013; Hsu et al., 2013; Pattanayak et al., 2013) and chromatin immunoprecipitation in human cells (Kuscu, Arslan, Singh, Thorpe, & Adli, 2014; Wu et al., 2014). As a result, an increasingly complete picture of the off-target activity of the enzyme is emerging. Cas9 will tolerate some mismatches between its guide and a DNA substrate, a characteristic that depends strongly on the number, position (PAM proximal or distal), and identity of the mismatches. Off-target binding and cleavage may further depend on the organism being edited, the cell type, and epigenetic contexts.

These specificity studies, together with direct investigations of the catalytic mechanism of Cas9, have stimulated homology- and structure-guided engineering to improve its targeting specificity. The wild-type enzyme makes use of two conserved nuclease domains, HNH and RuvC, to cleave DNA by nicking the sgRNA-complementary and noncomplementary strands, respectively. A "nickase" mutant (Cas9n) can be generated by alanine substitution at key catalytic residues within these domains—SpCas9 D10A inactivates RuvC (Jinek et al., 2012), while N863A has been found to inactivate HNH (Nishimasu et al., 2014). Though an H840A mutation was also reported to convert Cas9 into a nicking enzyme, this mutant has

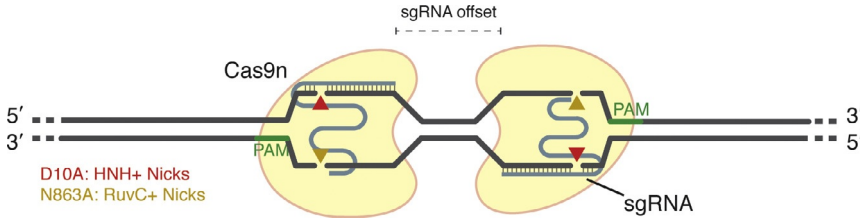

Figure 8.1 Diagram of Cas9n enzymes in a double-nicking configuration. Offset nicking with the D10A mutant, which retains only the catalytic activity of the HNH nuclease domain, generates 5′ overhang products in the target genome by nicking the sgRNA-complementary DNA strand (nicks represented with red (dark gray in the print version) triangles). Alternatively, Cas9n N863A selectively nicks the noncomplementary strand (nicks represented with yellow (gray in the print version) triangles). sgRNA offset is defined as the distance between the 5′ (or PAM-distal) end of each sgRNA. The PAM sequences, represented in green (light gray in the print version), are present in the target genome but not the sgRNA.

reduced levels of activity in mammalian cells compared with N863A (Nishimasu et al., 2014) (Fig. 8.1).

Because single-stranded nicks are generally repaired via the nonmutagenic base-excision repair pathway (Dianov & Hubscher, 2013), Cas9n mutants can be leveraged to mediate highly specific genome engineering. A single Cas9n-induced nick can stimulate HDR at low efficiency in some cell types, while two nicking enzymes, appropriately spaced and oriented at the same locus, effectively generate DSBs, creating 3′ or 5′ overhangs along the target as opposed to a blunt DSB as in the wild-type case (Mali et al., 2013; Ran et al., 2013). The on-target modification efficiency of the double-nicking strategy is comparable to wild type, but indels at predicted off-target sites are reduced below the threshold of detection by Illumina deep sequencing (Ran et al., 2013).

The following protocol describes protocols and considerations for the design and testing of nickase reagents for high-precision mammalian genome editing, including target selection, sgRNA construction, transfection, detection of Cas9-induced indel mutations using the SURVEYOR nuclease assay, and design and quantification of homology-directed insertions.

2. TARGET SELECTION

SpCas9 targets can be any 20-bp DNA sequence followed at the 3′ end by 5′-NGG-3′. Our lab has developed an online tool that will accept a

region of interest as input and output a list of all potential sgRNA target sites within that region. Each sgRNA target site is then associated with a list of predicted genomic off-targets (http://tools.genome-engineering.org).

The tool also generates double-nicking sgRNA pairs automatically. The most important consideration for double-nicking sgRNA design is the spacing between the two targets (Ran et al., 2013). If the "offset" between two guides is defined as the distance between the PAM-distal (5′) ends of an sgRNA pair, an offset of −4 to 20 bp is ideal, though offsets as large as 100 bp can induce DSB-mediated indels. sgRNA pairs for double nicking should target opposite DNA strands.

3. PLASMID sgRNA CONSTRUCTION

sgRNA expression vectors can be constructed by cloning 20-bp target sequences into a plasmid backbone encoding a human U6 promoter-driven sgRNA expression cassette and a CBh-driven Cas9-D10A (pSpCas9n(BB), Addgene #48873). The N863A nickase can be exchanged with D10A in all cases. It is recommended to prepare this plasmid as an endotoxin-free maxiprep. The generalized oligos needed to clone a new target into pSpCas9n(BB) are described in Table 8.1 and can be purchased from Integrated DNA Technologies (IDT). Note that the PAM sequence required for target recognition by Cas9 is never present as part of the sgRNA itself.

1. To clone a target sequence into an sgRNA backbone vector, first resuspend sgRNA-fwd and sgRNA-rev oligos to 100 μ*M*. Note that these oligos include an appended guanine (lowercase) not present in the target site in order to increase transcription from the U6 promoter.

Table 8.1 General sgRNA cloning oligonucleotides

Primer	Sequence (5′ to 3′)	Description
sgRNA-fwd	CACCGNNNNNNNNNNNNNNNNNNN	Sticky overhang plus specific 20-bp genomic target to be cloned into sgRNA backbones
sgRNA-rev	AAACNNNNNNNNNNNNNNNNNNNC	Complementary annealing oligo for cloning new target into sgRNA backbones

2. Combine 1 μL of each oligo with 1 μL T4 ligation buffer, 10× (New England Biolabs (NEB) B0202S), 0.5 μL T4 PNK (NEB M0201S), and 6.5 μL ddH_2O for a 10 μL reaction total. Treat with polynucleotide kinase to add 5′ phosphate and anneal the oligos in a thermocycler with the following protocol: 37 °C for 30 min, 95 °C for 5 min, ramp down to 25 °C at 5 °C/min.
3. Dilute the annealed oligos (10 μL reaction) by adding 90 μL ddH_2O.
4. Set up a Golden Gate digestion/ligation with pSpCas9n(BB) and the annealed oligos as a cloning insert. The plasmid contains twin *Bsm*BI restriction sites in place of the sgRNA target sequence such that digestion leaves overhangs complementary to the annealed oligo overhangs. In a 25 μL reaction, combine 25 ng pSpCas9n(BB), 1 μL diluted annealed oligos from step 3, 12.5 μL Rapid Ligation Buffer, 2× (Enzymatics L6020L), 1 μL FastDigest *Bsm*BI (ThermoScientific FD1014), 2.5 μL 10× BSA (NEB B9001), 0.125 μL T7 Ligase (Enzymatics L6020L), and 7 μL ddH_2O.
5. A negative control should be performed using the same conditions as above and substituting the insert oligos with ddH_2O.
6. Incubate the ligation in a thermocycler for six cycles of 37 °C for 5 min, 20 °C for 5 min. The ligation is stable for storage at −20 °C.
7. Transform 2 μL of the ligation reaction into a competent *E. coli* strain using the appropriate protocol—the Stbl3 strain is recommended—plate onto ampicillin selection plates (100 μg/mL ampicillin), and incubate overnight at 37 °C. Typically, transformation occurs at high efficiency; no colonies form on the negative control plate, and hundreds form when the sgRNA oligos have been successfully cloned into the backbone.
8. 14 h later, pick two or more colonies from the transformation with a sterile pipette tip and use the bacteria to inoculate 3 mL LB or TB broth with 100 μg/mL ampicillin. Shake the culture at 37 °C for 14 h.
9. Isolate plasmid DNA from the cultures using the Qiagen Spin Miniprep Kit (27104) and determine the DNA concentration by spectrophotometry. These constructs can be Sanger sequence-verified through the sgRNA scaffold to confirm correct insertion of the target sequence. For optimal transfection conditions downstream, endotoxin-free plasmid should be prepared.

4. VALIDATION OF sgRNAs IN CELL LINES

This protocol describes the functional validation of sgRNAs in HEK293FT cells; culture and transfection conditions may vary for other cell types.

1. Maintain HEK293FT cells (Life Technologies R700-07) in sterile D10 media (DMEM, high glucose (Life Technologies 10313-039) supplemented with 10% vol/vol fetal bovine serum (Seradigm 1500-500) and 10 m*M* HEPES (Life Technologies 15630-080)). For optimal health, cells should be passaged every day at a ratio of 1:2–2.5 and always kept under 80% confluence.
2. Plate cells for transfection. Seed 120,000 cells per well of a 24-well tissue-culture treated plate in a total volume of 500 μL. Cultures and transfections can be proportionally scaled up or down for different formats based on growth surface area. For many adherent cell types, poly-D-lysine coated plastic may improve adherence and viability.
3. Check the plates after 18 h to determine the confluence of the cells—generally 90% is ideal. Lipofectamine 2000 (Life Technologies 11668109) reagent can be used to transfect DNA according to the manufacturer's protocol. For a 24-well plate, we do not transfect more than 500 ng/well DNA total.
4. To deliver one nicking pSpCas9n(sgRNA) plasmid, transfect 500 ng; for multiple nicking constructs, e.g., delivering 2 sgRNAs for double nicking, mix different constructs up to 500 ng at equimolar ratios before transfection.
5. It is important to include transfection controls, such as untransfected wells and GFP plasmid, as well as experimental controls, such as Cas9n without guides or guides alone, in these experiments. Transfecting in technical triplicates will facilitate analysis.
6. Within 6 h of transfection, change the media to 2 mL of fresh, prewarmed D10 media per well. At 24 h, estimate transfection efficiency by examining GFP-transfected wells. >80% of cells should be GFP positive.
7. Harvest the cells for genomic DNA extraction and/or downstream analysis at 48–72 h. If harvesting a 72-h time point, change the media again at 48 h to maintain optimal cell health.

When working with different cell types, alternative transfection reagents should be compared for efficiency and toxicity. It may also be informative to titrate pSpCas9n(sgRNA) in order to find the optimal transfection concentration with highest efficacy.

5. CELL HARVEST AND DNA EXTRACTION

1. Harvest cells in 24-well plate format by aspirating the medium completely and adding 100 μL of TrypLE Express reagent (Life Technologies 12604013) to facilitate dissociation.

2. Collect the cell suspension in a 1.5-mL Eppendorf tube and spin for 5 min at 1500 × *g*, aspirate the supernatant completely, and resuspend the cell pellet in 200 μL DPBS (Life Technologies 14190-250) to wash.
3. Spin the cell suspension again for 5 min at 1500 × *g* and resuspend in 50 μL QuickExtract (Epicentre QE09050).
4. Transfer the QuickExtract suspension to a 0.2-mL PCR tube and extract genomic DNA according to the following thermocycler protocol adapted from the manufacturer's instructions: 65 °C for 15 min, 98 °C for 10 min.
5. Centrifuge the reaction product to pellet cell debris and transfer cleared supernatant into a fresh tube for further analysis.
6. Determine the DNA concentration of the extraction by spectrophotometry and normalize to 100–200 ng/μL with ddH_2O.

6. SURVEYOR INDEL ANALYSIS

The SURVEYOR assay (Transgenomic 706025) is a method for detecting polymorphisms and small indels. DNA samples are PCR-amplified, and the products are heated to denature and cooled slowly to form heteroduplexes. Mismatched duplexes are then cleaved by the SURVEYOR nuclease, and cleavage products are analyzed by gel electrophoresis.

1. Perform PCR on genomic DNA. The primers for SURVEYOR PCR should ideally produce a clean ~500 bp amplicon in untransfected cell samples. Genomic PCR primers may be designed using software such as Primer3. Set up a 50 μL reaction containing 1 μL of each 10 μ*M* SURVEYOR primer, 10 μL Herculase II Reaction Buffer 5 × (Agilent 600675), 0.5 μL of 100 m*M* dNTP, 0.5 μL Herculase II Fusion Polymerase, 2 μL of 25 m*M* $MgCl_2$, and 36 μL ddH_2O. Denature for 20 s at 95 °C, anneal for 20 s at 60 °C, and extend for 20 s at 72 °C.
2. Note that, since SURVEYOR was designed to detect mutations, it is crucial to use a high-fidelity polymerase to avoid false positives.
3. Run 2 μL of the PCR product on a 1% agarose gel to ensure that a single product of expected size has formed.
4. Purify the PCR product using the QIAquick PCR Purification Kit (28104) according to the instructions provided. Measure the DNA concentration of the eluate by spectrophotometry and normalize to 20 ng/μL using ddH_2O.

5. Mix 18 µL of normalized PCR product with 2 µL Taq PCR buffer, 10 ×, for a 20 µL reaction total. Melt and rehybridize the products gradually in a thermocycler: melt at 95 °C for 10 min, then ramp the temperature down to 85 °C at a rate of −0.3 °C/s. Hold at 85 °C for 1 min, then ramp to 75 °C at 0.3 °C/s. Hold at 75 °C for 1 min, then ramp to 65 °C and so on, until the temperature reaches 25 °C. From 25 °C, ramp down to 4 °C at 0.3 °C/s and hold.
6. Mix 2.5 µL of 0.15 *M* $MgCl_2$, 0.5 µL ddH_2O, 1 µL SURVEYOR nuclease S, and 1 µL SURVEYOR enhancer S with all of the annealed product from step (5) for a 25 µL total reaction volume. Perform the

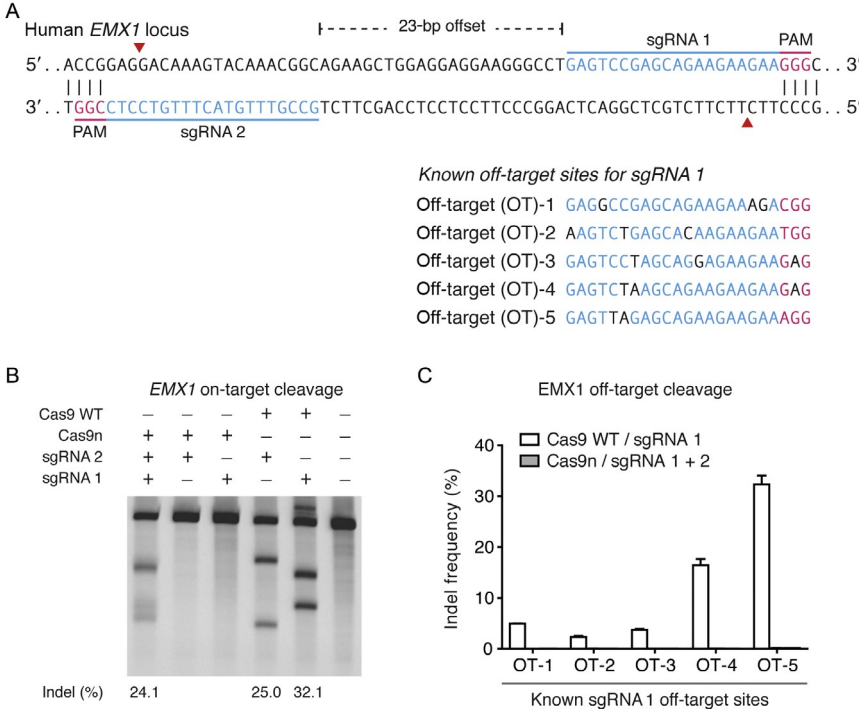

Figure 8.2 Double nicking reduces off-target modification. (A) Diagram of a Cas9n D10A double-nicking sgRNA pair designed for the human EMX1 locus. Guide sequences are shown in blue, demonstrating a 23-bp offset. The PAM is shown in pink, and nicking sites are represented by red triangles. Five known genomic off-target sites (Hsu et al., 2013) for sgRNA 1 are listed. (B) Example SURVEYOR results showing modification of the EMX1 locus by Cas9 WT and Cas9n along with sgRNA 1 and/or 2. (C) Deep sequencing quantification of off-target modifications at five known off-target sites by Cas9 WT and sgRNA 1 or Cas9n with sgRNAs 1 and 2. *Adapted with permission from Ran et al. (2013).*

digestion by incubating the reaction at 42 °C for 30 min. Samples that have mutations within the rehybridized PCR amplicons will be cleaved by SURVEYOR.

7. The digestion products can be mixed with an appropriate loading dye and visualized by electrophoresis on a 4–20% polyacrylamide TBE gel (see example, Fig. 8.2B).
8. Genome modification rates can be estimated first by calculating the relative intensities of digestion products *a* and *b* and the undigested band *c*. The frequency of cutting f_{cut} is then given by $(a+b)/(a+b+c)$. The following formula, based on the binomial probability distribution of duplex formation, estimates the percentage of indels in the sample.

$$\%\ \text{indel} = \left(1 - \sqrt{(1 - f_{\text{cut}})}\right) 100$$

7. HDR AND NON-HDR INSERTION USING Cas9n

A single-stranded oligodeoxynucleotide (ssODN) has a high efficiency as a template for homologous recombination, though linearized plasmid vectors can also be used. In some cell types, a single nickase may stimulate a targeted homologous repair event in the presence of a donor template. In others, such as human embryonic stem cells, a double-stranded break mediated by double nicking may be required to promote efficient HDR (Ran et al., 2013). The considerations for choosing double-nicking sgRNA pairs for HDR are similar to those for gene knockdown by NHEJ, with the additional requirement that one of the nicks must occur within ~20 bp of the HDR insertion site. In 293FT cells, double-nicking-mediated HDR can be comparably efficient to wild-type Cas9-mediated HDR.

Nicking Cas9 enzymes are well suited to generating highly precise modifications. Since HDR typically occurs at low efficiency in the best cases, we also provide pSpCas9n plasmids encoding the polycistronic 2A linker followed by GFP and puromycin markers (Addgene #48140 and 48141) in order to facilitate enrichment of modified cells.

HDR in mammalian cells proceeds via the generation of 3′ overhangs followed by strand invasion of a homologous locus by the 3′ end. It is therefore possible that the generation of 3′ overhang products by N863A-mediated double nicking could increase HDR efficiency.

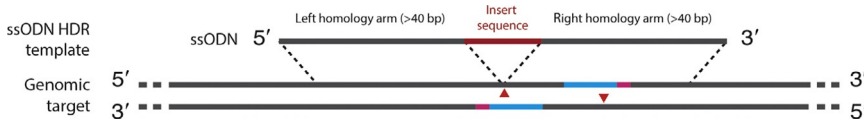

Figure 8.3 General design of ssODN HDR templates. The ssODN consists of an insertion sequence (red (dark gray in the print version)) flanked by homology arms on the left and right sides (at least 40 bp each). The homology between the ssODN and its targeting region is indicated by black dashes. Double-nicking Cas9 target sites are shown in blue (light gray in the print version), and their corresponding PAM sequences are shown in pink (light gray in the print version). Nicking sites are represented by red (dark gray in the print version) triangles.

1. ssODN homology arms should be designed to be as long as possible, with at least 40 nucleotides of homology on either side of the sequence to be introduced. The Ultramer service provided by IDT allows the synthesis of oligos up to 200 bp in length. Homology templates should be diluted to 10 μ*M* and stored at −20 °C (see design example, Fig. 8.3).
2. Delivery by nucleofection is optimal for ssODNs. The 4D Nucleofector X Kit S (Lonza V4XC-2032) can be used for HEK293FT cells seeded in 6-well tissue-culture-treated plates. The manufacturer provides an optimal protocol for nucleofection of these and other cell types.
3. Mix 500 ng total pSpCas9n(sgRNA) plasmids with 1 μL of 10 μ*M* ssODN for nucleofection.

8. ANALYSIS OF HDR AND INSERTION EVENTS

HDR outcomes can be assessed and utilized in a variety of ways. Here, the FACS isolation of clonal pSpCas9n(sgRNA)-GFP 293FT cells is described. It is important to note that FACS procedures can vary between cell types.

1. Prepare FACS media (D10 without phenol red to facilitate fluorescence sorting): DMEM, high glucose, no phenol red (Life Technologies 31053-028) supplemented with 10% vol/vol fetal bovine serum and 10 m*M* HEPES supplemented with 1% penicillin–streptomycin (Life Technologies 15140122).
2. Prepare 96-well plates for clone sorting by adding 100 μL standard D10 media to each well.
3. 24 h after the transfection in Sections 7.2 and 7.3, aspirate the medium completely and dissociate the cells using sufficient TrypLE Express to cover the growth surface minimally.

4. Stop trypsinization by adding D10 medium, transfer the cells to a fresh 15-mL tube, and continue triturating gently 20 times. It is critical that the cells are in a single-cell suspension before proceeding.
5. Spin the cells for 5 min at 200 × *g*, aspirate the supernatant completely, and resuspend the pellet thoroughly and carefully in 200 μL FACS medium.
6. Filter the cells through a cell strainer (BD Falcon 352235) to filter out cell aggregates and place the cells on ice.
7. Sort single cells in the plates prepared in (2). The FACS machine can be gated on GFP + cells in order to enrich for transfected cells. Wells can be visually inspected to check for the presence of one cell.
8. Incubate and expand the cells for 2–3 weeks, changing media to fresh D10 as necessary.
9. When cells exceed 60% confluence, clonal populations can be passaged into replica plates containing fresh D10 media. Dissociate cells, passage 20% of the cells into replica plates, and conserve 80% for DNA extraction as described in Section 5.
10. Genotyping can be performed by PCR amplification of the locus of interest, PCR purification, and Sanger sequencing of the products.

9. TROUBLESHOOTING

1. Colonies form on the negative control plate while cloning targets into pSpCas9n.
   a. The presence of negative colonies generally indicates an incomplete restriction digestion of the backbone plasmid. The Golden Gate reaction can be extended for 20–25 cycles in order to increase the efficiency of digestion. The amount of restriction enzyme used can be increased, though the volume of enzyme should not exceed 20% of the total reaction volume.
   b. Retransform the Cas9 backbone plasmid, isolate a new preparation of plasmid DNA, and sequence-verify the restriction site.
2. The transfection efficiency of Cas9 reagents is low.
   a. Low transfection efficiency may be the norm for some cell lines, and especially primary cells or stem cell lines. Cell populations can be enriched for transfected cells by using pSpCas9n(BB)-GFP or pSpCas9n(BB)-Puro plasmids to FACS on GFP fluorescence or perform antibiotic selection.

3. Double nicking does not produce indels.
   a. The individual double-nicking sgRNAs should be tested with the wild-type context to ensure that each of them functions separately as a valid Cas9 guide.
   b. Check the spacing of the sgRNA pair. Double nicking performs optimally when the guides are spaced 20 bp apart or less, and the guides should be oriented such that their respective 5′ PAM sequences face away from each other.
4. Efficiency of HDR is low.
   a. Silent mutations may be introduced within the target site on the ssODN to prevent cleavage of the successfully recombined genomic site.

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