Video Article

Selection-dependent and Independent Generation of CRISPR/Cas9-mediated Gene Knockouts in Mammalian Cells

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Abstract

The CRISPR/Cas9 genome engineering system has revolutionized biology by allowing for precise genome editing with little effort. Guided by a single guide RNA (sgRNA) that confers specificity, the Cas9 protein cleaves both DNA strands at the targeted locus. The DNA break can trigger either non-homologous end joining (NHEJ) or homology directed repair (HDR). NHEJ can introduce small deletions or insertions which lead to frame-shift mutations, while HDR allows for larger and more precise perturbations. Here, we present protocols for generating knockout cell lines by coupling established CRISPR/Cas9 methods with two options for downstream selection/screening. The NHEJ approach uses a single sgRNA cut site and selection-independent screening, where protein production is assessed by dot immunoblot in a high-throughput manner. The HDR approach uses two sgRNA cut sites that span the gene of interest. Together with a provided HDR template, this method can achieve deletion of tens of kb, aided by the inserted selectable resistance marker. The appropriate applications and advantages of each method are discussed.

Video Link

The video component of this article can be found at https://www.jove.com/video/55903/

Introduction

Stable genetic alterations provide an advantage over transient methods of cellular perturbation, which can be variable in their efficiency and duration. Genomic editing has become increasingly common in recent years due to the development of target specific nucleases, such as zinc-finger nucleases[1,2,3,4,5], transcription activator-like effector nucleases (TALENs)[6,7,8,9] and RNA-guided nucleases derived from the clustered, regularly interspaced short palindromic repeats (CRISPR) system[10].

The CRISPR/Cas9 editing machinery is adapted from an immune system that bacteria and archaea use to defend against viral infections[11,12,13]. In this process, short, 20-30 nt fragments of invading viral sequence are incorporated into a genomic locus as "spacers" flanked by repeating units[14,15]. Subsequent transcription and RNA processing generates small CRISPR-associated RNAs[16] (crRNAs) that, together with a trans-activating crRNA[17] (tracrRNA), assemble with the effector Cas9 endonuclease. The crRNAs thus provide specificity to Cas9 targeting, guiding the complex to cleave complementary viral DNA sequences and preventing further infections[18,19]. Any "protospacer" sequence in the targeted DNA can serve as the source of the crRNA, as long as it is directly 5' to a short protospacer adjacent motif (PAM), NGG in the case of *S. pyogenes* Cas9[20]. The absence of the PAM sequence near the spacer in the host's CRISPR locus distinguishes between self and non-self, preventing targeting of the host. Because of its universality and flexibility, this biological system has been powerfully adapted for genomic editing, such that nearly any PAM-adjacent DNA site can be targeted. In this version, a further modification fused the crRNA and tracrRNA into a single guide RNA (sgRNA) component that is loaded into the Cas9 protein[21].

Upon expression of Cas9 and an sgRNA in eukaryotic cells, the Cas9 protein cleaves both DNA strands at the targeted locus. In the absence of a suitable region of homologous sequence, the cell fixes this break via non-homologous end joining (NHEJ)[22,23,24], which typically introduces small deletions or, rarely, insertions. When targeting an open reading frame, the repair likely leads to a translational frameshift that produces a non-functional protein product. In contrast, when provided with an exogenous template with large regions of homology, the cell may fix the double-strand break by homology directed repair[25,26]. This route allows for larger precise deletions, replacements or insertions in the genome, coupled with the introduction of excisable selection markers[27].

Here, we present protocols for generating knockout cell lines by either of these two CRISPR/Cas9 methods (**Figure 1A**). The NHEJ approach uses a single sgRNA cut site and selection-independent screening, and thus requires little upfront preparation. When using this method, guide RNAs complementary to exons near the 5' end of the transcript, which are most likely to produce a knockout, must be designed. Since the modifications to the genome in this case are small, screening for knockout clones is based on dot blots, where the protein product is assessed in a high-throughput manner. We use the generation of ELAV-like 1 protein (ELAVL1) knockout lines as an example. The second approach relies on homology directed repair (HDR) and uses two sgRNA cut sites that span the gene or region of interest, allowing for deletions of tens of kb. A plasmid with two regions of homology that flank the cleavage sites provides a replacement template (**Figure 1B**), introducing a selectable resistance marker that increases efficiency of knockout generation. This method can also be adapted to introduce gene modifications with

properly designed homology arms. In this case, the integration of a new DNA fragment allows for PCR based screening (**Figure 1C**). Here, we use the generation of Pumilio RNA binding family member 2 (PUM2) knockout lines as an example.

Protocol

1. Identification of Homology Regions Around the Desired Deletion

NOTE: Only necessary if using selection-based editing.

1. Select two regions, initially 1.5-2 kb, on either side of the desired deletion locus, which will serve as homology arms in the HDR template (**Figure 1A**). Identify regions that lack BsaI recognition sites on either strand (GGTCTC) to facilitate cloning. If BsaI sites are unavoidable, use an alternate type IIs restriction enzyme (BsmBI, SapI, BbsI) and modify the corresponding sites in the recipient plasmid (pUC19-BsaI), resistance marker donor plasmid (pGolden-Neo or pGolden-Hygro), and homology arm PCR product overhangs.

2. Generation of Cas9-sgRNA Expression Plasmids

1. Define the targeting site(s) for mutagenesis. For the single-cut, selection-free method, target 5' proximal coding exons to increase the probability of a non-functional mutation. For the two-cut method, select two sites that span as much of the gene as necessary.
   NOTE: In our experience, deletions up to 53 kb are efficiently created.
2. Input 200 to 300 bp of the targeted region sequence into the crispr.mit.edu design tool. For selection-based cloning, use 200-300 bp of the identified homology regions that are proximal to the deletion locus (**Figure 1A**). Select 2-3 of the highest-ranked sgRNAs per targeted region to account for differences in their activity, and to isolate independent clones that do not share the same potential off-target effects.
3. To design duplexed oligonucleotides with appropriate overhangs for insertion into the expression plasmid, omit the ending PAM sequence (NGG) and append a 5'-CACC overhang followed by a G to the top strand oligo. For the bottom strand oligo, append a 5'-AAAC overhang to the reverse-complemented target sequence, followed by a 3'-C, as illustrated below:

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sgRNA output:    5'-NNNNNNNNNNNNNNNNNNNNNGG-3'

TS:          5'-CACCGNNNNNNNNNNNNNNNNNNNN-3'
BS:               3'-CNNNNNNNNNNNNNNNNNNNNCAAA-5'
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4. Insert synthetic oligonucleotides corresponding to the sgRNAs into the pSpCas9(BB) plasmid using Golden Gate cloning[28] as described in the Zhang lab protocol[29].

3. Generation of Homology-directed Repair Template Plasmids

NOTE: Only necessary if using selection-based editing. The homology-directed repair template plasmid consists of a drug resistance cassette flanked by two regions that are complementary to the genome just outside the two sgRNA target sites (**Figure 1B**).

1. From the broader homology regions identified in step 1.1, select 800-1,000 bp[26] no more than 5-10 bp away from the designed Cas9 cut sites, to serve as the homology arms (**Figure 1A**). Be sure not to include the sgRNA target site and its PAM in the homology arms, as this will cause CRISPR/Cas9 cleavage of the template plasmid itself. If the homology arms do contain internal BsaI sites, use alternate sgRNA sites.
2. PCR amplify homology arms from genomic DNA using a high-fidelity DNA polymerase, per manufacturer's instructions, using forward and reverse primers with additional 5' sequence that introduces BsaI sites (GGTCTC) and unique overhangs on either end of the homology region. The overhangs must contain proper sequences to generate correct assembly order and orientation, as described below (**Figure 1B**):
   Left homology arm FP overhang: 5' GGGTCTCA**GGCC**
   Left homology arm RP overhang: 5' GGGTCTCT**CACG**
   Right homology arm FP overhang: 5' GGGTCTCA**GTCC**
   Right homology arm RP overhang: 5' GGGTCTCT**CCAC**
3. Check homology arms for appropriate amplification via gel electrophoresis before proceeding forward.
4. **Assemble the right and left homology arm regions with the resistance cassette into an HDR template plasmid by Golden Gate cloning[28]. Use pGolden-Neo or pGolden-Hygro plasmids[30] as the source of loxP-flanked resistance cassettes (loxP-PGK-Neo-pA-loxP or loxP-PGK-Hyg-pA-loxP). Use pUC19-BsaI, a modified pUC19 with BsaI sites in the multiple cloning region and eliminated BsaI sites elsewhere, as the recipient vector (available upon request). Use a ratio of 1:1:1:1 for the three inserts and vector.**
   1. Prepare the following reaction mixture[30]:

Right homology arm PCR product	0.06 pmol (30-40ng)
Left homology arm PCR product	0.06 pmol (30-40ng)
pGolden-Neo plasmid (100 ng/µL)	1 µL
pUC19-BsaI plasmid (100 ng/µL)	1 µL
2x T7 DNA ligase buffer	5 µL
BsaI (10 U/µL)	0.75 µL
T7 DNA ligase (3000 U/µL)	0.25 µL
Water	up to 10 µL

2. Use the following thermocycler parameters: 37 °C for 5 min, 20 °C for 5 min. Repeat for 30 cycles.
3. Treat the cloning product with an exonuclease per manufacturer's instructions to digest away any remaining linearized DNA.
4. Transform the reaction mixture into a competent *E. coli* strain according to the protocol supplied with the cells, and plate on ampicillin-containing dishes.

5. **To identify clones with the correct template assembly, perform bacterial colony PCR to amplify the homology arm subregions of the assembled insert (as the whole insert may be too large to amplify reliably). Use one primer annealing to the flanking plasmid sequence and a second primer complementary to the edge of the resistance cassette (Figure 1B, the sequence is common to both Neo and Hygro inserts), generating an assembly-dependent product that is approximately 200 bp longer than the contained homology arm:**
pUC19-BsaI-Left: 5' GGCTCGTATGTTGTGTGGAATTGTGAG
Resistance-Left: 5' AAAAGCGCCTCCCCTACCC
pUC19-BsaI-Right: 5' GCTATTACGCCAGCTGGCGAAA
Resistance-Right: 5' AAGACAATAGCAGGCATGCTGGG
   1. Pick bacterial colonies with sterile toothpicks and suspend the bacteria in 20 µL water. Heat at 95 °C for 15 min, and spin down in a tabletop centrifuge at maximal speed for 5 min. Place immediately on ice.
   2. Prepare the following PCR mixture[31]:

Dilute colony template	1.25 µL
10x Taq reaction buffer	1.25 µL
20mM dNTPs	0.25 µL
10 µM Forward primer	0.25 µL
10 µM Reverse primer	0.25 µL
Taq Polymerase	0.25 µL
Water	9 µL

   3. Use the following thermocycler parameters: 95 °C for 30 s, (95 °C 30 s, 61 °C for 30 s, 72 °C for 1 min/kb), repeat for 25 cycles, 72 °C for 2 min.
   4. Check PCR product for correct amplicon size by agarose gel electrophoresis.
   5. Optionally, miniprep plasmid DNA from individual colonies with correct insert size and sequence the homology arm regions by Sanger sequencing with the amplification primers mentioned above.

4. Transfection of CRISPR Components into Cultured Cells

1. Prior to transfection: culture T-REx293 cells in DMEM media supplemented with 10% FBS at 37 °C and 5% CO_2.
2. Plate cells onto 6-well plates and grow to approximately 70% confluency. Include a well for an untransfected control.
3. **Transfect cells with 2.5 µg total plasmid using a commercial transfection protocol. In parallel, maintain the untransfected control. NOTE: Transfection method and efficiency vary depending on cell type. Determine the appropriate transfection method for the system prior to the experiment.**
   1. For transfection of cells with selection, use 0.75 µg Cas9-sgRNA 1 (left cut), 0.75 µg Cas9-sgRNA 2 (right cut), and 1.0 µg homologous recombination template.
   2. For transfection of cells without selection, use 2.5 µg Cas9-sgRNA plasmid.

5. Drug Selection

1. 48 h after transfection, treat the cells with the appropriate drug (Neomycin/Hygromycin). Carry out selection until all cells in the untransfected control die (typically 3-5 days for Neo, 7-14 days for Hygro).
NOTE: Use concentrations of 500 µg/mL and 10 µg/mL for Neomycin and Hygromycin, respectively for T-REx293 cells. For other cell types, it may be useful to carry out a prior titration of drug on untransfected cells to determine the effective concentration.

6. Isolation of Clonal Populations

1. Grow cells to 100% confluency in the original well after selection. Seed cells into 96 well plates at a density of 0.33 cells per well.
NOTE: Seeding three 96 well plates is a good starting point to ensure isolation of more than one correct clone, but more or less may be needed depending on sgRNA and HDR efficiencies.
2. Observe colonies over a 2-4 week period, until colonies are visible to the eye. Pick visible colonies with a sterile pipette tip and reseed in new wells to encourage monolayer growth. When using suspension cells, even lower seeding densities can be used to ensure a greater proportion of single-colony wells.

7. Screening Candidates

1. **Screening candidates without the use of selection: dot blot**
   1. Grow individual clones to 50-100% confluency. Dislodge the monolayer of cells by pipetting within the well.

2. Aliquot 90 µL of the 100 µL total volume to a clean microcentrifuge tube. Spin down at 6000 rpm for 5 min, remove media, and lyse cell pellet in 10 µL of 1x Lysis Buffer or 1x SDS loading buffer (5x: 250 mM Tris-Cl pH 6.8, 8% SDS, 0.1% bromophenol blue, 40% glycerol, 100 mM DTT). Add 90 µL of new media to the remainder of the cells in the well to continue propagating.
3. Pipette 1 µL of cell lysate onto a dry nitrocellulose membrane to form a dot. Blot each sample twice on two separate membranes, creating two identical patterns of samples.
4. Block the membranes in 5% milk in TBST (Tris Buffered Saline + 0.01% Tween 20: 8 g NaCl, 0.2g KCl, 3 g Tris base, up to 1 L distilled water, pH 7.4)[31] for 1 h at room temperature (with rocking, here and throughout the procedure).
5. Blot using the primary antibody against the target protein on one membrane, and the primary antibody for a control protein (tubulin, GAPDH, or any other protein that is not expected to change) on the other. Use the recommended primary antibody dilution (0.2 µg/mL for ELAVL1 and 0.1 µg/mL for Pum2 in this case) in TBST + 5% milk. Incubate 1 h at room temperature.
6. Wash 3 times with TBST for 5 min.
7. Incubate each membrane with appropriate HRP-conjugated secondary antibody in TBST + 5% milk for 1 h at room temperature.
8. Wash 3 times with TBST for 5 min.
9. Apply chemiluminescence substrate solution (see Materials Table) following the manufacturer's instructions and image the blotted membrane on a digital chemiluminescence imager.
10. Quantify dot intensities for the target and control proteins using the appropriate software.
11. Eliminate candidates with low control protein signal, and calculate background-subtracted ratios of target to control protein intensities for the remaining candidates. Select the candidates with the lowest ratios for passage and further validation by western blot.

2. **Screening candidates with the use of selection: colony PCR**
    1. Collect cell lysate using a DNA prep kit (See **Materials Table**).
        1. Duplicate individual colonies in a new 96 well and grow until 100% confluency.
        2. Remove media from one set of the clones, and resuspend cells in 30 µL of extraction buffer from the kit. Transfer to a clean 1.5 mL microcentrifuge tube.
        3. Heat the solution to 96 °C for 15 min and let cool to room temperature.
        4. Add 30 µL of Stabilization buffer from the kit. Mix well.

    2. Identify wildtype and monoallelic/biallelic mutant lines by colony PCR.
        1. Design two separate sets of PCR primers (for the left and right side of the targeted locus) to amplify regions around the homology arms based on either successful or unsuccessful integration of the resistance cassette (**Figure 2C**). On each side, use a common primer (red) that anneals outside of the homology arm. Use it with a corresponding paired primer that is complementary to the endogenous sequence, spanning the homology region (blue), to test for the wild-type allele. Use another paired primer, complementary to the inserted resistance cassette (see primers in section 2.3), to test for the desired mutation.
        2. (Recommended) Validate the primer sets using cell lysate from the original selected bulk cell population, since it will contain a mixture of both WT and mutant alleles (**Figure 2D**).
        3. Prepare the following reaction mixture:

Cell lysate	0.5 µL
10x KOD buffer	1.25 µL
25mM MgSO4	0.75 µL
2 mM dNTPs	1.25 µL
10 µM Forward primer	0.375 µL
10 µM Reverse primer	0.375 µL
KOD polymerase	0.25 µL
Water	7.75 µL

        4. Use the following thermocycler conditions: 95 °C for 2 min, (95 °C for 20 s, Primer Tm for 10 s, 70 °C for 20 s/kb) repeat for 25 cycles.
        5. Visualize the PCR product by agarose gel electrophoresis.

    3. Repeat the colony PCR testing for each side of the predicted integration. Select and expand candidates that show presence of integration and no endogenous sequence product for the deleted region.
    4. Validate positive candidates by western blot and/or RT-qPCR.

8. Verify the Genomic Mutation by Sequencing

1. Isolate genomic DNA from mutant cell lines by phenol chloroform extraction[31].
2. PCR amplify the sgRNA target region, using primers with 5' overhangs that add BsaI restriction sites to each end.
   NOTE: For clones edited with HDR, where both alleles may be identical, the PCR product can be sequenced directly.
3. Clone the amplified region into pUC19-BsaI by Golden Gate cloning.
4. Transform the reaction mixture into a competent *E. coli* strain.
5. Miniprep plasmid DNA from 6-10 individual colonies and sequence the cloned region by Sanger sequencing.

Representative Results

For the generation of ELAVL1 knockout lines, a robust antibody was available, so editing using single sgRNAs (**Figure 1A**, left) was performed, followed by dot immunoblot. Three sgRNAs were transfected independently to compare efficiencies and to rule out off target effects in the resulting clones. After collecting and blotting cell lysates from clonal populations onto two nitrocellulose membranes, the blots were probed for both ELAVL1 and PUM2 (as a normalization control) (**Figure 2A**). The quantified ratio of ELAVL1 to PUM2 signal demonstrated a very broad, $>10^4$-fold range among the isolated lines (**Figure 2B**). Using this ratio as a selection criterion, we validated a number of lines using ELAVL1 western blots (**Figure 3A**). In most cases, clonal populations with the lowest relative ELAVL1 signal were correctly identified as knockouts (**Figure 2B**), confirming the predictive power of this quantification (despite considerable variability in the control signal, which likely arose from differing numbers of collected cells). Conversely, most of the clones with intermediate ratios exhibited ELAVL1 signal upon validation, potentially representing monoallelic deletion events. However, no general tiering of the ratios into WT, monoallelic and full (biallelic) mutant pools was observed, and even the ELAVL1-positive clones displayed a wide and continuous range of levels, likely due to clonal variability of expression. The overall effectiveness of ELAVL1 knockout generation using this method ranged from 6 to 36% among the sgRNAs.

For other gene targets (such as PUM2), the quality of the available antibodies prevents accurate screening of candidate clones by dot blot due to high background from cross-reactivity. Additionally, the efficiency of targeting some genomic loci may be substantially lower due to chromatin accessibility. In such cases, a selection-dependent method is appropriate to increase efficiency. Two sgRNAs targeting early and late coding regions of PUM2 were cotransfected with a homology-directed repair template in order to excise 45 kb of genomic DNA and replace it with a neomycin resistance cassette (**Figure 1A**, right). Assembly of the homology template is illustrated in **Figure 1B**. PCR primers were designed to amplify regions around the homology arms based on either successful or unsuccessful integration of the resistance cassette (**Figure 2C**), and were tested on untransfected and the bulk selected cells (left panel). As expected, in untransfected cells, only an amplicon corresponding to the endogenous state (top) was observed, while the bulk selected cells showed amplicons from both wild-type and mutant alleles. Cell lysates from generated clonal candidates were collected and screened for the presence of both the endogenous sequence and the resistance cassette. Candidate knockouts were identified by the presence of the resistance cassette amplicon alone, indicating biallelic deletion/integration (**Figure 2D**, stars). Clones with monoallelic insertions were also observed, and were fairly common, since such populations can also survive the selection process. These clones were indicated by the presence of both the endogenous sequence and the resistance cassette. In a few cases, clonal wildtypes at the tested locus were also observed, suggesting that random, non-sgRNA-assisted genomic integration of the homology-directed repair template occurs at a low frequency. Biallelic mutant lines were further validated by western blot (**Figure 3B**). The overall efficiency of knockout generation using the HDR procedure was 33%.

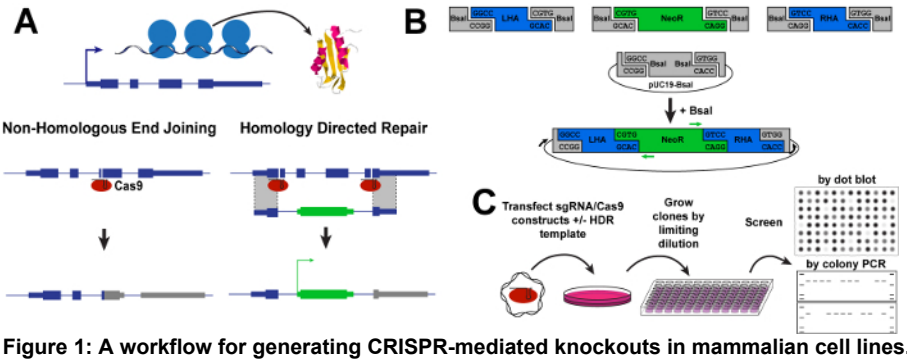

Figure 1: A workflow for generating CRISPR-mediated knockouts in mammalian cell lines. (**A**) Genome editing events produced by CRISPR/Cas9 cleavage and NHEJ (left) or HDR (right). The site of Cas9 cleavage is denoted by a white vertical line. (**B**) Components and assembly of the HDR template plasmid. The type IIs restriction enzymes used in Golden Gate cloning, such as BsaI, recognize an asymmetric DNA sequence and leave a staggered cut outside of the recognition sequence, allowing for the design of arbitrary overlapping segments for assembly (pictured). Arrows indicate the primer positions to check for correct plasmid assembly. (**C**) Workflow: after transfection (and drug selection if applicable), cells are plated at limiting dilutions in 96-well plates. Clonal populations are screened either by dot immunoblot (selection-independent, NHEJ) or colony PCR (selection-dependent, HDR). Please click here to view a larger version of this figure.

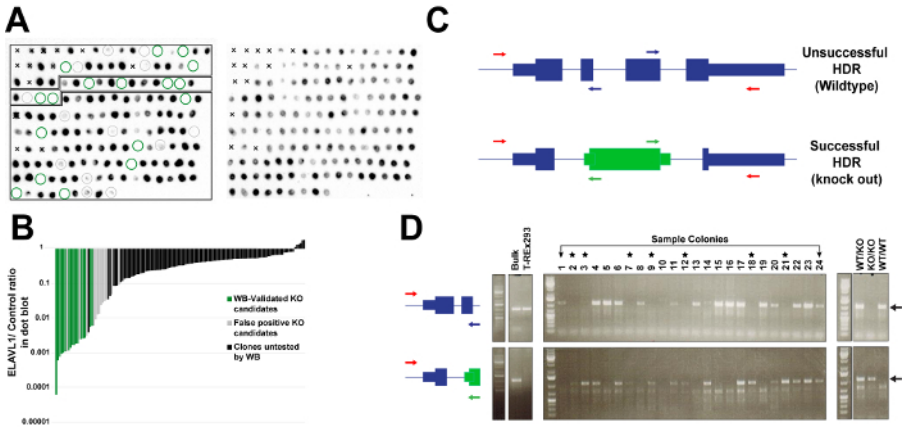

Figure 2: Screening candidate knockout lines by dot blots or colony PCR. (**A**) Dot immunoblot of isolated ELAVL1-targeted clones with ELAVL1 antibody (left), and separately with an antibody for a control protein, Pum2 (right). Clones that displayed little control signal, likely due to low input protein amounts, are marked with an X and excluded from further analysis. Clones with low ELAVL1 signal (circles) were further tested by western blots that confirmed (green) or invalidated (grey) the candidates. (**B**) Quantification of KO candidates from dot blot. Ratios of ELAVL1 to control protein signal in the dot blot shown in A, in increasing order. (**C**) Primer design for knockout/insertion testing via colony PCR. A primer complementary to the genomic locus outside of the homology arm region (red) is paired with a primer complementary to the endogenous sequence (blue) or to the resistance cassette (green), probing for the unedited state, or a deletion/insertion event, respectively. Separate primer pairs are designed to test both sides of the integration. (**D**) Example agarose gel of colony PCR results using primers spanning the upstream integration junction using endogenous inside primers (top) and knockout inside primers (bottom). Primers are first validated in both parental T-REx293 cells and bulk selected cells (left). Individual candidate clones (middle) and the expected band patterns for WT, KO and monoallelic deletion (WT/KO) clones (right). Biallelic knockout clones are indicated by a star. The correct amplification products are indicated by black arrows. Please click here to view a larger version of this figure.

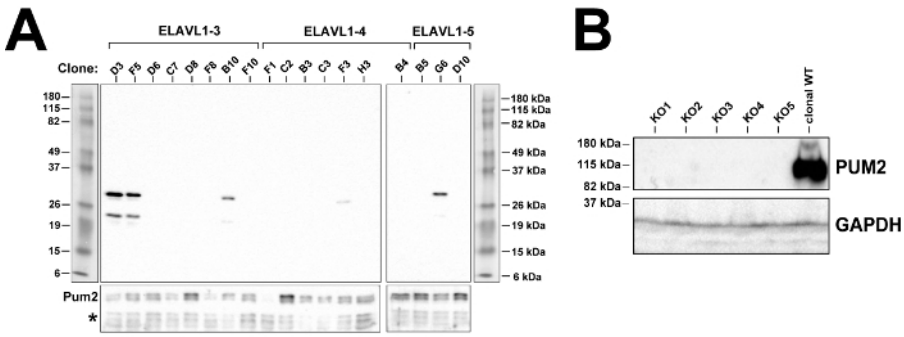

Figure 3: Validation of knockout candidates by Western blot. (**A**) Western blot for ELAVL1 (top) of a subset of clones identified by dot blot. PUM2 was blotted on the same membrane as a loading control (bottom). (**B**) Western blot for PUM2 (top) of a subset of clones identified by colony PCR. GAPDH was blotted on the same membrane as a loading control (bottom). Please click here to view a larger version of this figure.

Discussion

The CRISPR/Cas9 system has allowed for efficient generation of stable genomic modifications, which provide a more consistent alternative to other transient manipulation methods. Here, we have presented two methods for rapid identification of CRISPR/Cas9 gene knockouts in mammalian cell lines. Both methods require little cellular material, so testing can be done in early stages of clonal culture, saving time and reagents. To increase the efficiency of both methods, we recommend testing multiple sgRNAs, as efficiencies vary depending on sequence and genomic location. In addition, using multiple sgRNAs is useful in identifying off-target effects by detecting outlier phenotypes as a result of mutations in non-targeted genes. Effective transfection and Cas9 cleavage will greatly increase chances of generating knockouts. Alternatively, transfecting the cells directly with commercially sourced sgRNA/Cas9 ribonucleoprotein complexes[32,33] may expedite the protocol and lower off-target effects.

Dot blots are well-suited to assess protein knockouts caused by the small genomic alterations due to NHEJ. Since only a single sgRNA/Cas9 construct is necessary, this approach may be the quickest in obtaining null lines, and the blot procedure circumvents additional steps, expediting the screening. The normalized immunoblot protein signal serves as a reliable predictor of successful validation (**Figure 2B**). This protocol is ideal for generation of knockouts with minimal genomic perturbation. Alternatively, this method of screening can be adapted to probe for protein addition or modification, such as transgenic proteins or protein tags. However, this screening method requires an antibody with high specificity to the protein of interest, as cross-reactivity leads to high background in dot blots. This method is naturally limited to mutations targeting protein coding regions, as the final output depends on the presence/absence of a protein product. It is important to also note that mutations caused by NHEJ can lead to a variety of protein outcomes, including a protein with constitutively active, partial or dominant negative function, or a functional protein lacking the detection antibody epitope. To minimize these issues, it is helpful to target the Cas9 cut as early into the coding region as possible, in order to increase the chances of generating a fully non-functional protein. Additionally, targeting genomic regions that are

less accessible to the Cas9 machinery can produce low deletion yields in the absence of selection. Finally, knockouts that confer a selective disadvantage may be difficult to obtain by this method for similar reasons.

Using homology-directed repair mechanisms together with CRISPR/Cas9 cleavage allows for much larger and more specific genomic manipulations, which can include both large scale deletions (delineated by two sgRNA cleavages) and insertions. Since two cleavage events and a precise repair process need to take place, integration of a drug resistance marker substantially increases knockout yields using this method. Although dot blot detection can also be used, screening by colony PCR is more generally applicable to accurately detect correct integration/knockout candidates with such large genomic perturbations. Further, testing by PCR allows for identification of clones with imperfect and incomplete integrations, which can provide insight into the extent and nature of the HDR process itself. Additionally, the introduced marker can be subsequently removed (by transient Cre expression or addition in membrane-permeable form), leaving a minimal, 34 bp deletion scar (loxP site) and permitting future re-use of the selection marker. The flexibility of this approach increases with the use of multiple resistance markers, allowing for the generation of multiple cumulative protein knockouts. It is important when applying this method that the absence of product is further validated, for example by RT-qPCR of the expected transcript. Since this screen can be antibody independent, this method is easily applied to protein knockouts without a robust antibody, as well as to detect changes that do not fall in protein coding genes.

Disclosures

The authors declare that they have no competing financial interests.

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