APPLICATION NOTE

A CRISPR-Cas9 gene engineering workflow: Generating functional knockouts using Dharmacon Edit-R Cas9 and synthetic crRNA and tracrRNA.

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Abstract

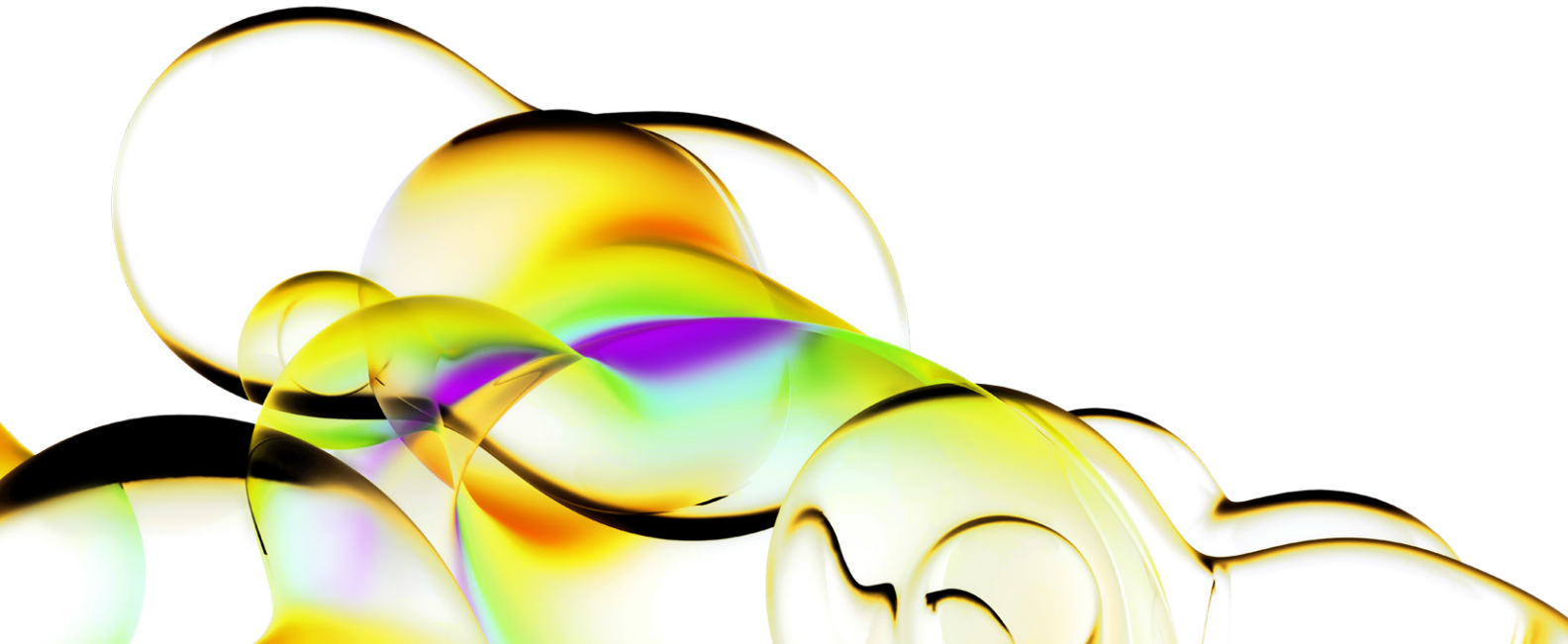
The CRISPR-Cas9 system is being widely used for genome engineering in many different biological applications. It was originally adapted from the bacterial Type II CRISPR system and utilizes a Cas9 endonuclease guided by RNA to introduce double-strand DNA breaks at specific locations in the genome. The **Dharmacon™ Edit-R™ CRISPR-Cas9 Gene Engineering** platform is comprised of Cas9 expressed from a plasmid, a long synthetic **tracrRNA**, and custom-designed **synthetic crRNA** to efficiently introduce gene editing events in mammalian cells. Here we demonstrate a complete workflow using the Dharmacon Edit-R platform, starting from optimization of the gene editing parameters and enrichment of edited cells, to clonal selection and verification of the specific genetic change by sequence analysis, and finally to confirmation of protein knockout.

Keywords

CRISPR-Cas9, Gene Editing, Genome Engineering, Dharmacon Edit-R, Cas9, Clonal Selection, FACS, Immunoblot, Sanger Sequencing, Cloning, Transfection, Transfection Optimization

Introduction

The CRISPR (clustered regularly interspaced short palindromic repeats)-Cas (CRISPR-associated proteins) system is an adaptive bacterial and archaeal defense mechanism that serves to recognize and silence incoming



bacteriophage or other foreign nucleic acids[1].
While there are many bacterial and archaeal CRISPR-Cas systems that have been identified, the mechanism and key components of the *Streptococcus pyogenes* Type II CRISPR-Cas9 system have been well characterized and subsequently adapted for genome engineering in mammalian cells. In *S. pyogenes*, the Cas9 (CRISPR-associated 9) protein is the sole nuclease that cleaves the DNA when guided by two required small RNA sequences: the CRISPR RNA (crRNA), which binds the target DNA and guides cleavage, and the trans-activating crRNA (tracrRNA), which base-pairs with the crRNA and enables the Cas9-crRNA complex to form (Figure 1)[2,3].
Upon site-specific double-strand DNA cleavage, a mammalian cell can repair the break through either nonhomologous end joining (NHEJ) or homology directed repair (HDR). NHEJ is often imperfect, resulting in small insertions and deletions (indels) that can result in nonsense mutations or introduction of a stop codon to produce functional gene knockouts[4,5].
This endogenous DNA break repair process, coupled with the highly tractable *S. pyogenes* CRISPR-Cas9 system, allows for a readily engineered system to permanently disrupt gene function in mammalian cells.

The Dharmacon Edit-R CRISPR-Cas9 Gene Engineering platform includes the three components required for gene editing in mammalian cells: (1) a plasmid expressing a mammalian codon-optimized gene sequence encoding Cas9 nuclease, (2) a long, chemically synthesized tracrRNA, and (3) a synthetic crRNA designed to the target site of interest. The Dharmacon Edit-R Cas9 Nuclease Expression plasmids contain either the mKate2 fluorescent reporter (Evrogen, Moscow, Russia) or the puromycin resistance marker (Puro^R) under the same promoter as Cas9 to facilitate enrichment of Cas9-expressing cells, thus increasing the percentage of cells where editing has occurred.

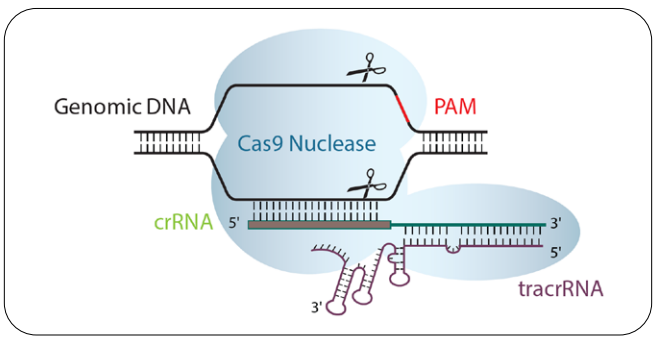


Figure 1: Illustration of Cas9 nuclease (light blue), programmed by the crRNA (green):tracrRNA (purple) complex, cutting both strands of genomic DNA 5′ of the PAM (red).

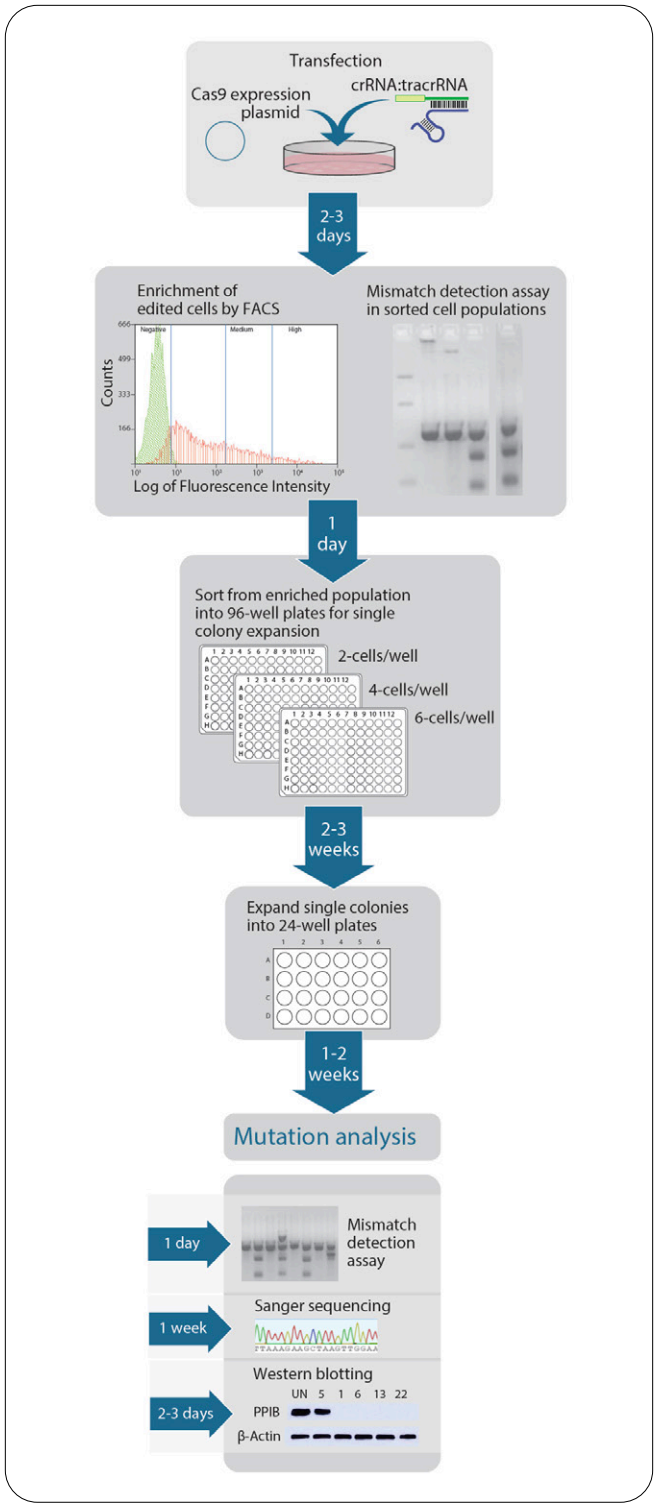


Figure 2: **A complete workflow using the Dharmacon Edit-R CRISPR-Cas9 gene engineering platform.** First, co-transfection was optimized for editing events and scaled up appropriately for FACS analysis. Next, transfected cells were enriched by FACS, and additionally the positive binned mKate2 cells were sorted into 96-well plates for single cell colony expansion. The sorted cell populations and the expanded colonies were assessed for mutations with a mismatch detection assay followed by Sanger sequencing to determine specific mutation events. Finally, protein knockout was confirmed with Western blot.

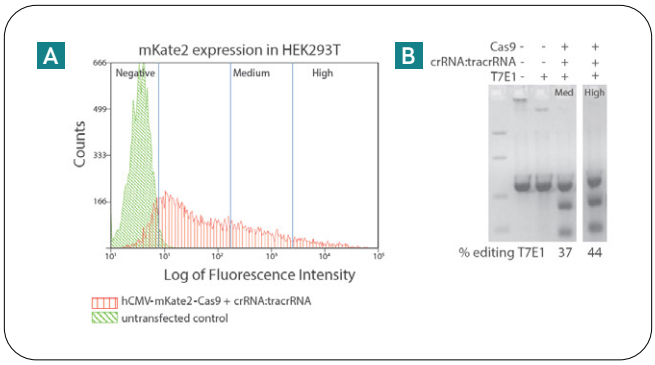


Figure 3: **FACS enriched cell populations show a high level of editing by mismatch detection assay (T7EI).** A. HEK293T cells were transfected with Dharmacon Edit-R hCMV-mKate2-Cas9 Expression plasmid and crRNA:tracrRNA targeting *PPIB*. Cells were sorted at 72 hours on a MoFlo XDP 100 instrument into three bins corresponding to negative, medium and high expression of the mKate2 fluorescent reporter. B. Mutation detection using T7EI was performed on sorted medium and high mKate2 cell populations compared to untransfected control (UT) cells with and without the endonuclease treatment. Samples were run on a 2% agarose gel and the level of editing was calculated using densitometry (% editing). An increase in gene editing is observed from the medium to the high fractions of sorted cells which correlates with the increased mKate2 expression.

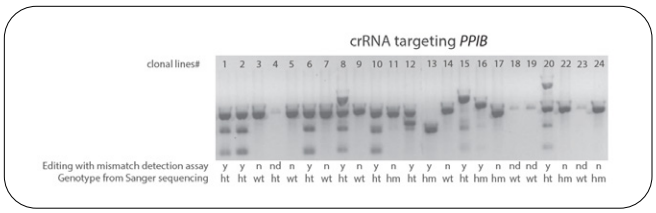


Figure 4: **Examples of mismatch detection analysis in FACS clonal lines of HEK293T cells.** Samples were run on a 2% agarose gel. The clones are numbered and the corresponding gene editing from mismatch detection assay is indicated as y (yes), n (no), or nd (not determined) along with genotype confirmed by Sanger sequencing below the gel image. The genotypes are abbreviated as follows: wt (wild type) indicates no detected mutations, ht (heterozygous) indicates there is a mutation in at least one allele and the other allele is either wt or a different mutation, and hm (homozygous) indicates that both alleles have the same mutation.

Additionally, multiple promoter options are available for Cas9 so that one can choose the plasmid containing the most active promoter in specific cells of interest for robust expression and maximal cleavage efficiency when co-transfected with Dharmacon Edit-R crRNAs and tracrRNA.

Here, a complete workflow (Figure 2) is demonstrated using the Dharmacon Edit-R CRISPR-Cas9 Gene Engineering platform to knock out *PPIB* in HEK293T cells. First, co-transfection of the mKate2-Cas9 expression vector with synthetic crRNA and tracrRNA was optimized for maximal editing efficiency. Next, Fluorescent Activated Cell Sorting (FACS) was used to enrich for mKate2-expressing cells. The mKate2-expressing cells were then sorted into 96-well plates for single cell colony expansion and analyzed for specific editing events. Cell expansions where functional *PPIB* knockout was expected based on Sanger sequencing were subsequently analyzed and confirmed by Western blot.

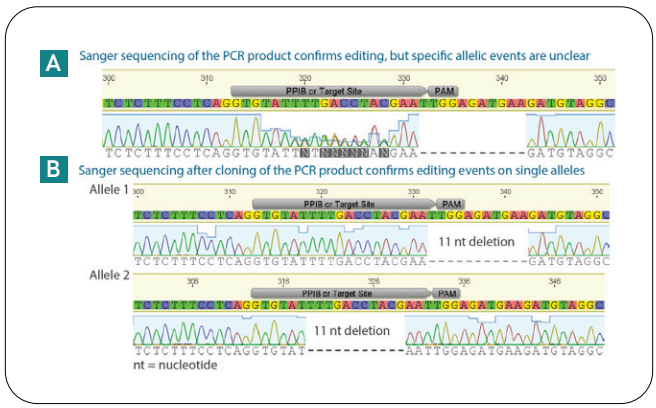


Figure 5: Sanger sequencing analysis on clonal line 1 confirms heterozygous mutations. A. Chromatogram from Sanger sequencing of purified PCR products. Mutation(s) are observed but allelic events are unclear. B. Chromatograms representing the 2 different mutations identified from cloning the PCR products into a blunt vector (Zero Blunt™ PCR Cloning Kit, Invitrogen), transforming into competent cells (One Shot™ TOP10 Chemically Competent E. coli, Invitrogen). Two different 11 base deletions that are inferred in A. and are confirmed in B. nt = nucleotide

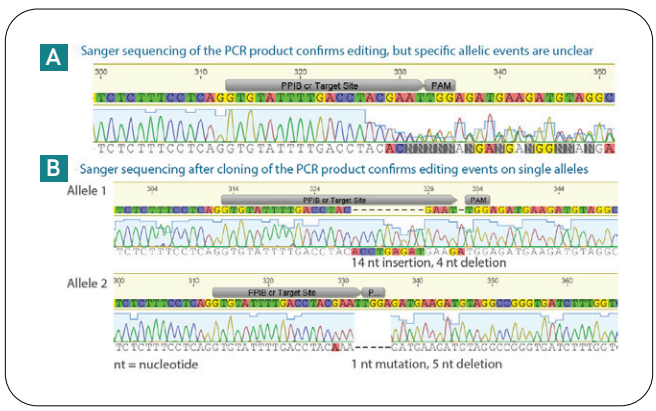


Figure 6: Sanger sequencing from clonal line 6 with heterozygous mutations. A. Chromatogram from Sanger Sequencing of purified PCR products after mutation detection analysis. Mutation(s) are observed but allelic events are unclear. B. Chromatograms representing the 2 different mutations identified from Sanger Sequencing after cloning PCR product into a blunt vector (Zero Blunt™ PCR Cloning Kit, Invitrogen, transforming into competant cells (One Shot™ TOP10 Chemically Competent *E. coli*, Invitrogen) and selecting 12 single colonies from each clone. Two different mutations that are inferred in A are confirmed in B. For allele 1 the mutations consist of a 14 nucleotide insertions and a 4 nucleotide deletion and for allele 2 the confirmed mutations are 1 nucleotide mutation from a G to A and a 5 nucleotide deletion. nt = nucleotide

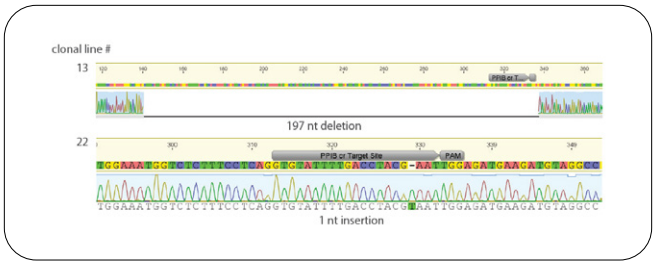


Figure 7: **Sanger sequencing analysis on clonal lines 13 and 22 confirm homozygous mutations.** Chromatograms from Sanger sequencing of purified PCR products after mutation detection analysis using T7EI. Mutations detected are observed to be the same on both alleles for clones 13 and 22. nt = nucleotide

Results

Transfection optimization for maximal editing efficiency: HEK293T cells were transfected with hCMV-mKate2-Cas9 expression plasmid and crRNA:tracrRNA complex targeting the human *PPIB* gene in exon 2. Transfection optimization was performed in 96-well plate format varying the cell density, transfection reagent amount, and the concentration of Dharmacon Edit-R components. The best conditions were determined to be > 80% cellviability and strongest visual detection of the mKate2 fluorescent reporter. Optimal experimental conditions were subsequently scaled up to a 6-well plate format to ensure sufficient cells for FACS enrichment.

Enrichment by FACS for cell populations with gene editing events: HEK293T cells were transfected and sorted 72 hours after transfection. Cells were sorted and binned into negative, medium, and high mKate2 fluorescent cell populations (Figure 3A). Gene editing of the medium and high mKate2 sorted cell populations were calculated to be 37% and 44%, respectively, using a mismatch detection assay, T7 Endonuclease I (T7EI). FACS was additionally used to plate medium and high mKate2 expression cell populations into 96-well plates such that two, four, and six individual cells were plated into each well and further grown for clonal isolation.

Clonal isolation: The clonal cell expansions were visually monitored for about two weeks, and 60 wells with single colonies were marked and grown until the cells were dense enough to transfer into a 24-well plate. More colonies were obtained from sorting four and six cells per well into the 96-well plates (18 and 15 colonies, respectively) than two cells per well (9 colonies). Of these 60 individual cell colonies, 42 were successfully expanded for mutational analysis.

Mutation analysis: DNA mismatch detection analysis was first performed to determine the presence of indels in the 42 clonal lines (examples in Figure 4). Genomic DNA (gDNA) spanning the crRNA target site was PCR amplified and analyzed with the mismatch detection assay using T7EI. Editing in at least one of two alleles is indicated by the cleaved bands under the primary PCR product. In these samples, the percent editing, typically between 40 and 50%, was calculated using densitometry (clonal lines 1, 2, 6, and 10). In some cases the PCR product was observed to be larger or smaller, indicative of longer insertions or deletions (for example clonal lines 8, 12, 13, 15, 17 and 20). To precisely determine the genotype and whether one or both alleles had been edited, Sanger sequencing was performed on PCR products amplified from gDNA spanning the crRNA target site. These data were reported as either wild type (wt), heterozygous (ht), or homozygous (hm) genotypes. A heterozygous genotype is indicated when there is a mutation in at least one allele while the other allele is either wt or a different mutation. A homozygous genotype is indicated when both alleles have the same mutation (for example clonal lines 11, 13, 16, 22, and 24; Figure 4) explaining the absence of cleaved bands in the mismatch detection assay.

Examples of Sanger sequencing results of clonal lines with heterozygous mutations are shown in Figures 5 and 6. The chromatograms of the PCR product encompassing the crRNA target site for clonal lines 1 and 6 are shown in Figures 5A and 6A. To better decipher the specific mutations, the same PCR products were cloned and 12 single bacterial colonies were sent for Sanger sequencing (Figures 5B and 6B). While HEK293T is an aneuploid cell line, only two mutations were identified with ratios of approximately 50:50 (data not shown) indicating that the cells have only two *PPIB* alleles (Chromosome 15). Both clonal lines contain frameshift mutations and were expected to result in protein disruption.

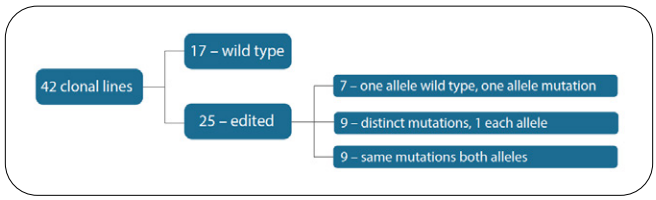


Figure 8: **A diagram characterizing mutations present in clonal lines.** Of the 42 clonal lines, 17 (40%) were wild type and 25 (60%) of them had clear mutations near the crRNA cut site. It was also observed that 18 of the 25 clonal lines with sequence confirmed mutations (72%) had mutations in both alleles.

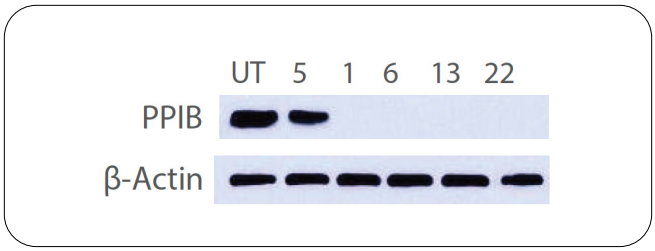


Figure 9: **Western blotting demonstrates complete knockout of PPIB.** Representative results of Western blot analysis showing seven clones compared to an untreated (UT) control. Clonal line 5 was characterized as wild type, while the other four clones had confirmed gene mutations. PPIB is only detected in the untreated control and wild-type samples while it is obliterated in the four mutants shown. β-Actin is the gel loading control.

Examples of sequencing results of clonal lines with homozygous editing events are shown in Figure 7. A 197 nucleotide deletion was identified in clonal line 13 while a single nucleotide insertion was identified in clonal line 22. Both clonal lines contain frameshift mutations and were expected to cause protein disruption.

The summary of the data analysis for the 42 clonal lines after mismatch detection assay and Sanger sequence analysis is shown in Table 1. Of the total clonal lines characterized, 17 (40%) were wild type and 25 (60%) had mutations near the crRNA cut site (Figure 8). Interestingly, 18 of the 25 edited clonal lines (72%) had mutations in both alleles indicating that the CRISPR-Cas9 technology is very efficient for gene editing of both alleles. A summary of specific insertions and deletions detected on each allele for all of the heterozygous and homozygous clonal lines is shown in Table 2.

Immunoblot analysis: Four clonal lines with mutations on both alleles and one wild type clonal line were further cultured and harvested for immunoblot analysis (Figure 9). PPIB is only detected in the parental HEK293T cells and a clonal line with a wild type phenotype, while it is not detected in the four edited clonal lines, validating functional gene knockout.

Table 1: **Mutational analysis on the 42 expanded clonal lines.** Editing was assessed using a mismatch detection assay and is reported as yes (y), no (n), or not determined (nd). The specific genotype and alleles edited was concluded from Sanger sequencing of PCR products surrounding the crRNA target site and reported as either wild type (wt) for no detected mutations or heterozygous (ht) and homozygous (hm) for mutations at the crRNA target site. A hm genotype indicates that both alleles have the same mutation, and a ht genotype is a mutation in at least one allele and the other allele is either wt or a different mutation.

Clonal line #	Editing	Genotype (Diploid)	Alleles
1	y	ht	both
2	y	ht	one
3	n	wt	wt
4	nd	ht	one
5	n	wt	wt
6	y	ht	both
7	n	wt	wt
8	y	ht	one
9	n	wt	wt
10	y	ht	one
11	n	hm	both
12	y	ht	both
13	y	hm	both
14	n	wt	wt
15	y	ht	both
16	y	hm	both
17	y	hm	both
18	nd	wt	wt
19	nd	wt	wt
20	y	ht	one
21	nd	wt	wt
22	n	hm	both
23	nd	wt	wt
24	n	hm	both
25	y	ht	one
26	y	hm	both
27	n	wt	wt
28	n	wt	wt
29	n	hm	both
30	y	ht	both
31	n	wt	wt
32	y	ht	both
33	n	wt	wt
34	n	wt	wt
35	n	wt	wt
36	n	wt	wt
37	y	ht	both
38	y	ht	one
39	y	ht	both
40	n	wt	wt
41	y	ht	both
42	y	hm	both

Discussion

Here we have demonstrated an experimental workflow for generating cell lines with a desired gene knockout using the Dharmacon Edit-R CRISPR-Cas9 Gene Engineering platform. Several experimental parameters influencing the efficiency of genome editing were optimized to minimize workflow timeline and cost of generation and isolation of a cell line with a specific gene knockout. Specifically, Cas9 levels and subsequent editing efficiency were increased with selection of a Cas9 nuclease expression plasmid with an active promoter for the cell line of interest and optimization of the co-transfection with the crRNA:tracrRNA. Further, gene editing was enriched with selection of fluorescent reporter-expressing cells. In this workflow, at least 40% of cells from FACS populations were shown to harbor mutations at the targeted site, using a mismatch detection assay, indicating almost 1 in 2 cells contained a gene editing event. Fewer individual clonal lines could be isolated to identify functional knockouts. With these careful considerations, we were able to easily obtain desired *PPIB* knockout clonal lines. Mutations were found in 25 of 42 clonal lines at the *PPIB* cleavage target site. Of the 25 edited clonal lines, 18 were mutated in both alleles. Surprisingly, 9 of these clonal lines were mutated identically on both alleles. One potential explanation for these occurrences could be that a mutagenic event from one of the alleles was transferred to the second (for example by inter-allelic gene conversion). Similar observations of a high percentage of homozygous indel mutations have been observed in other studies using the CRISPR-Cas9 system [6].

An additional explanation could be that one of the alleles was mutated to contain a very large deletion or insertion that was undetected because the PCR primers were designed to only amplify 505 nt spanning the crRNA target site. Clonal lines with the same mutation in both alleles may not be detected when using DNA mismatch detection assay to detect mutated clonal lines as these PCR products can reanneal and not be digested by the endonuclease.

From sequence alignment, it was determined that insertions in three clonal lines have significant homology to bacterial components of the Dharmacon Edit-R hCMV-mKate2-Cas9 Expression plasmid. The 27 nt insertion in clonal line 25 was derived from the ampicillin resistance gene. The 60 nt insertion in clonal line 16 was derived from the pUC origin. Finally, the 190 and 174 nt insertions in clonal line 15 were partially derived from the pUC origin. Similar observations were seen in Hendel et al [7].

Using the Dharmacon Edit-R CRISPR-Cas9 Gene Engineering Platform we were able to attain complete gene knockout efficiently by enriching for cells with gene editing events and screening a small number of clonal lines.

Conclusion

The Dharmacon Edit-R CRISPR-Cas9 Gene Engineering platform simplifies the workflow of permanent gene knockout. The synthetic crRNA:tracrRNA complex eliminates the need for cloning of a single guide RNA expression vector. The Cas9 Nuclease Expression plasmids, with multiple promoter options provide flexibility to select a promoter for robust expression in the cells of choice and thus achieve high cleavage efficiency when co-transfected with crRNA and tracrRNA. The Dharmacon Edit-R Cas9 Nuclease Expression plasmids have either an mKate2 reporter or Puro^R marker to facilitate enrichment of Cas9-expressing cells, thus increasing the percentage of cells with insertions and/or deletions.

Table 2: **Specific allelic mutations observed from Sanger sequencing**. Genotypes are listed as homozygous indicating that both alleles have the same mutation and heterozygous indicating there is a mutation in at least one allele and the other allele is either wt or a different mutation. Specific nucleotide lengths of insertions and deletions around the *PPIB* editing site are indicated.

Genotype	Clonal line #	Allele 1		Allele 2	
		Insertion	Deletion	Insertion	Deletion
Heterozygous	1	-	11	-	11
	2	-	3	wt	wt
	4	-	5	wt	wt
	6*	14	4	-	5
	8	213	4	wt	wt
	10	-	31	wt	wt
	12	10	133	-	14
	15	190	-	174	-
	20	-	435	wt	wt
	25	27	1	wt	wt
	30	11	6	-	1
	32	-	7	-	2
	37	1	-	-	43
	38	3	11	wt	wt
	39	-	2	-	12
	41	143	-	-	8
Homozygous	11	-	35	-	35
	13	-	197	-	197
	16	60	-	60	-
	17	1	38	1	38
	22	1	-	1	-
	24	-	3	-	3
	26	-	37	-	37
	29	-	2	-	2
	42	-	126	-	126

*Additionally a one nucleotide mutation is observed.

Materials and methods

Tissue culture: HEK293T cells were maintained in normal growth medium per manufacturer's recommendations (ATCC, Cat #CRL-11268).

Cell co-transfection: Transfection optimization for HEK293T cells was performed in a 96-well tissue culture plate by varying the cell density, transfection reagent amount and plasmid amount. Optimal conditions were identified to be cells seeded at 20,000 cells per well one day prior to transfection, 200 ng Dharmacon Edit-R hCMV-mKate2-Cas9 Expression plasmid (Dharmacon, Cat #U-004100-120), 50 nM tracrRNA (Dharmacon, Cat #U-002000-120), and 50 nM custom designed crRNA targeting human *PPIB* (Cat #CTM-28425; target sequence = GTGTATTTTGACCTACGAAT) with 0.6 µL Dharmacon™ DharmaFECT™ Duo Transfection Reagent (Dharmacon, Cat #T-2010-02) in a total volume of 100 µL.

For FACS analysis, cells were transfected in a 6-well tissue culture plate. Here, cells were seeded at 500,000 cells/well one day prior to transfection. For one well of a 6-well plate, 5 µg Dharmacon Edit-R hCMV-mKate2-Cas9 Expression plasmid with 50 nM tracrRNA and 50 nM crRNA were co-transfected into HEK293T cells using 15 µL DharmaFECT Duo Transfection Reagent in a total volume of 2.5 mL. A total of three wells were transfected to insure a sufficient number of cells for FACS.

FACS analysis: HEK293T cells were trypsinized, resuspended in cell sorting medium at 10^7 cells/mL, and stored on ice until sorting. Cells were sorted on a Moflo XDP 100 cell sorting instrument by the Flow Cytometry Core, University of Colorado Cancer Center [Cancer Center Support Grant (P30CA046934)] into tubes and 96-well plates, using FBS enriched medium (FBS:HEK293T medium at 1:1 ratio).

Clonal isolation: FACS was used to plate medium-and high-mKate2 fluorescent positive cells into 96-well culture plates with two, four, and six cells per well. For each number of cells per well (two, four, and six), two plates of cells were seeded. Cells were grown and monitored for single colonies per well for two weeks and each colony was expended into one well of a 24-well tissue culture plate in medium containing 1% Pen/Strep to avoid contamination due to non-sterile conditions of the flow sorting instrument. One hundred thousand cells were harvested for immediate use and the remaining cells were frozen and banked in 5% DMSO (~1×10^6 cells/vial).

Mismatch detection assay: 100,000 cells per sample were lysed in Phusion™ GC buffer (Thermo Scientific, Cat #F-549S) with 10 µL each of Proteinase K (Thermo Scientific, Cat #- EO0492) and RNase A (Thermo Scientific, Cat #EN0531) and incubated for 1 hour at 56 °C. 50 µL PCR reactions were carried out using 1 µL Phusion Hot Start II DNA Polymerase (Thermo Scientific, Cat #F-549S), 5X Phusion HF buffer (Thermo Scientific, Cat #F-549S), 200 µM each dNTP (Thermo Scientific, Cat #F-549S), 0.2 µM forward and reverse primers (Forward 5′-GAACTTAGGCTCCGCTCCTT-3′, Reverse 5′-CTCTGCAGGTCAGTTTGCTG-3′) and 5 µL direct cell lysis template. Touchdown PCR and an annealing program with the following thermal cycling steps were run for each sample: Denature/enzyme activation at 98 °C for 3 minutes followed by 10 cycles of 98 °C for 10 seconds, 72 °C for 15 seconds -1 °C/cycle, and 72 °C for 30 seconds then 25 cycles of 98 °C for 10 seconds, 62 °C for 15 seconds and 72 °C for 30 seconds and final extension at 72 °C for 10 minutes. Samples were heated to 95 °C for 10 minutes and slowly cooled to re-anneal. Then, 10 µL of annealed PCR products were combined with 5 units of T7EI enzyme and NEBuffer 2 (New England Biolabs, Cat #M0302L) and incubated at 37 °C for 25 minutes. Three µL of 6X Orange Loading Dye (Thermo Scientific, Cat #R0631) was added to the T7EI reactions and the entire volume was loaded and run on a 2% agarose gel. The level of editing was calculated using densitometry (% editing) in ImageJ following Luke Miller's method at lukemiller.org/index.php/2010/11/analyzing-gels-and-western-blots-with-image-j.

Sanger sequencing: Unincorporated primers and dNTPs were removed from the PCR products using spin column purification. Purified PCR products were sent for Sanger sequencing (Eurofins). For clonal lines with heterozygous mutations, the PCR product was cloned into the pCR-Blunt vector using the Zero Blunt™ PCR Cloning Kit (Invitrogen, Cat # K2700). 12 colonies were picked into a 96-well plate with LB medium containing 100 µg/mL carbenicillin and 8% glycerol, grown overnight, frozen, and sent for plasmid preparation and Sanger sequencing (ObliqueBio). Geneious version 6.1.8 (geneious.com)[8] was used for all Sanger sequencing analysis.

Immunoblotting: Cells were lysed on ice in Mammalian Protein Extraction Reagent (Thermo Scientific, Cat #78501) with 1 µL HALT Protease Inhibitor Cocktail (Thermo Scientific, Cat #87785). Protein concentration was determined using the Protein Assay BCA kit (Thermo Scientific, Cat #23227). Protein samples (7 µg) were denatured in NuPAGE™ 4X LDS sample buffer and NuPAGE™ Sample Reducing Agent (10X) (Life Technologies, Cat #NP0008, # NP0009) and heated to 70 °C for 5 minutes before running on a Novex™ 4-20% Tris Glycine Mini Protein Gel (Life Technologies, Cat #EC6025BOX) at 125 V for 85 minutes. The protein was wet transferred to a 0.45 µM nitrocellulous membrane in the Criterion™ Blotter (BioRad, Cat#170-4071). The membranes were blocked for 20 minutes in SuperBlock™ (PBS formulation) (Thermo Scientific, Cat #37515). Primary antibody [anti-rabbit PPIB polyclonal 1:800 dilution (Abcam, Cat #16045)] was diluted in SuperBlock overnight at 4 °C. Membranes were washed four times for 5 minutes in 0.05% Tween diluted in PBS. Secondary antibody [goat antirabbit IgG (H+L) Secondary Antibody, HRP conjugate (Thermo Scientific, Cat #32460)] was diluted 1: 20,000 in SuperBlock (PBS formulation) and incubated for 1 hour at room temperature. The membranes were then submerged in Super Signal West Dura Substrate

(Thermo Scientific, Cat #34016) solution shaking for 5 minutes and exposed to film. The membrane was stripped with Restore Western Stripping Buffer (Thermo Scientific, Cat #21059) for 15 minutes and rinsed in PBS before re-probing for β-Actin. Primary antibody [(anti-mouse-beta Actin polyclonal (Abcam, Cat #6276)] was diluted 1:2000 in SuperBlock (PBS formulation) applied to membranes for 2 hours at room temperature. Membranes were washed four times for 5 minutes in 0.05% Tween diluted in PBS. Secondary antibody [goat antimouse IgG (H+L) Secondary Antibody, HRP conjugate (Thermo Scientific, Cat #32430)] was diluted 1:20,000 in SuperBlock (PBS formulation) and shaking for 1 hour at room temperature. The membranes were then submerged in Super Signal West Dura Substrate shaking for 5 minutes and exposed to film.

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