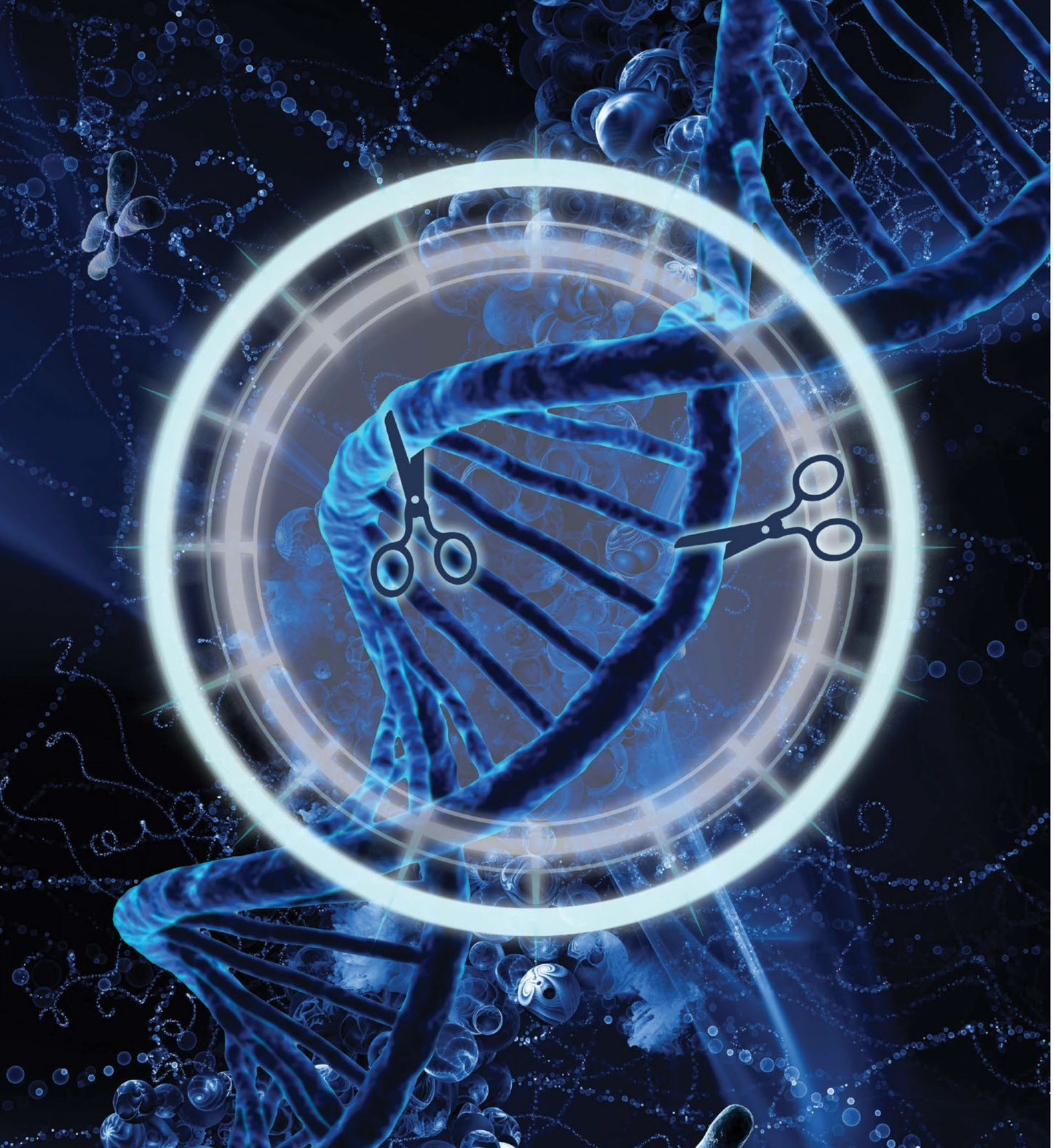
GE Healthcare

Specific, functional and scalable

Dharmacon™ Edit-R™ CRISPR-Cas9 Gene Editing Products



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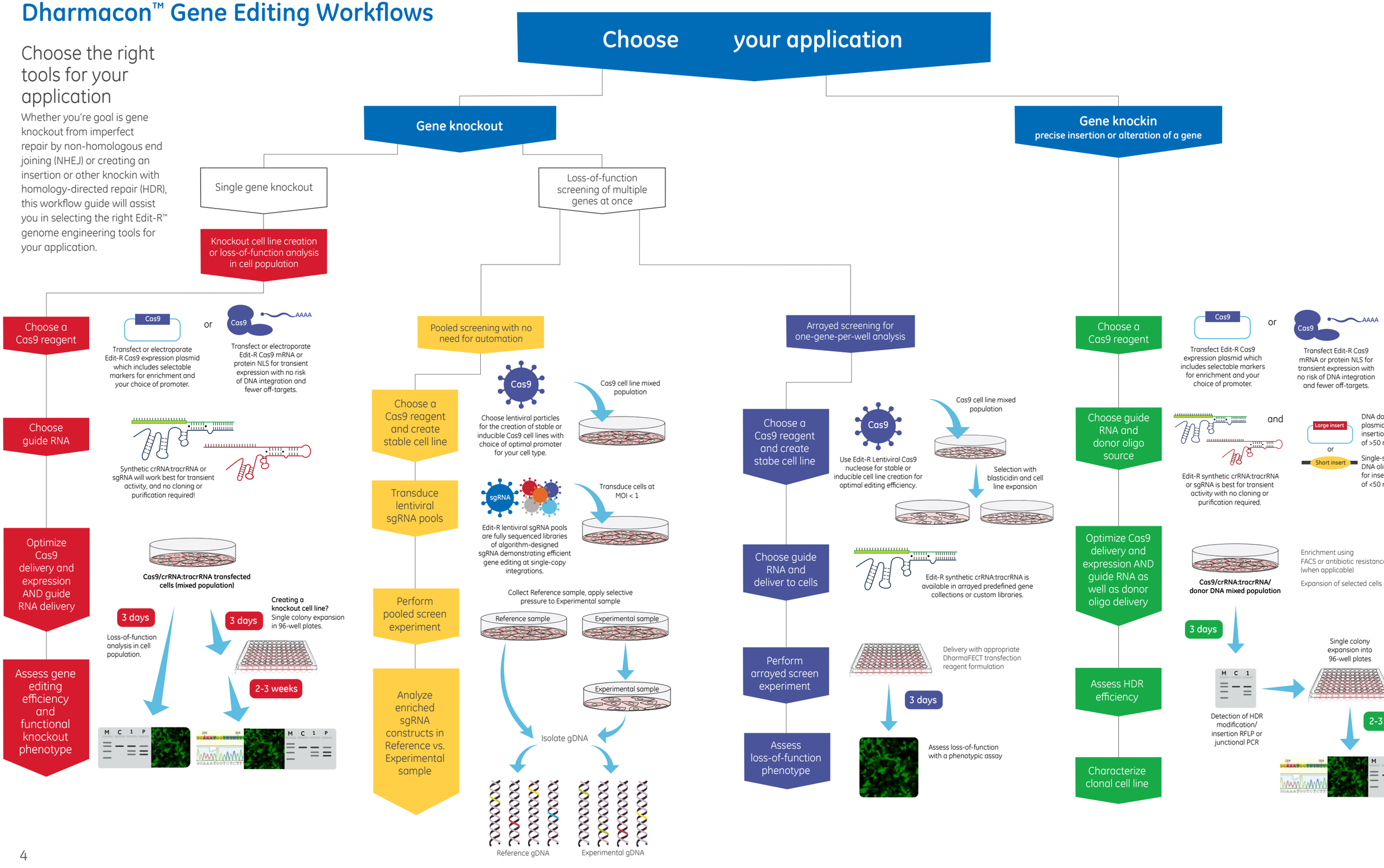
Application Notes

Dharmacon™ Gene Editing Workflows

Choose the right tools for your application

Whether you're goal is gene knockout from imperfect repair by non-homologous end joining (NHEJ) or creating an insertion or other knockin with homology-directed repair (HDR), this workflow guide will assist you in selecting the right Edit-R™ genome engineering tools for your application.



Introduction to CRISPR-Cas9 Genome Engineering

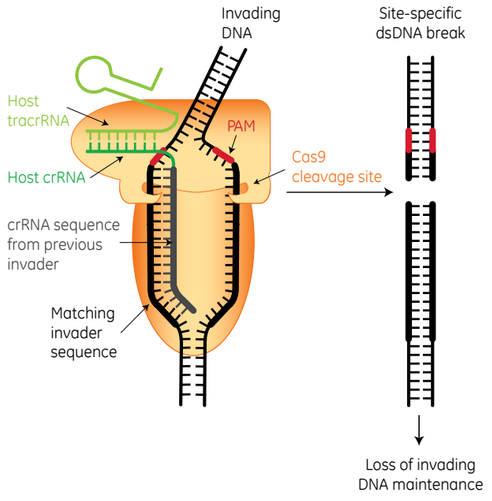
Interest in genome engineering of mammalian cells has been increasing in the past few years with the development of new tools to create DNA breaks at specific locations in the genome. Among these tools, the CRISPR-Cas9 system has gained significant interest due to its relative simplicity and ease of use compared to other genome engineering technologies.

CRISPR-Cas: An adaptive immunity defense mechanism in bacteria and archaea

The CRISPR (clustered regularly interspaced palindromic repeats)-Cas (CRISPR-associated proteins) system is an adaptive bacterial and archaeal defense mechanism that serves to recognize and cleave incoming foreign nucleic acids. Upon infection by bacteriophage or other foreign DNA elements, a host organism can incorporate short sequences from the invading genetic material, called protospacers, into a specific region of its genome (the CRISPR locus) between short palindromic DNA repeats of variable lengths. Multiple spacer-repeat units are clustered at the CRISPR locus to form the CRISPR array. The entire locus including the CRISPR array is transcribed by RNA polymerase into a primary transcript, the pre-CRISPR RNA (pre-crRNA), which is then processed into small, mature CRISPR RNAs (crRNAs) such that they include sequences complementary to the foreign, invading DNA. crRNAs guide a multifunctional protein or protein complex (the CRISPR-associated or Cas proteins) to cleave complementary target DNA that is adjacent to short sequences known as protospacer-adjacent motifs (PAMs). Thus, the organism acquires a way to protect itself from subsequent infection[1].

Engineering a CRISPR-Cas9 platform for mammalian genome editing

Many bacterial and archaeal CRISPR-Cas systems have been identified with diverse sets of mechanisms, Cas proteins, and multi-subunit complexes. In particular, the processes and key components of the *Streptococcus pyogenes* CRISPR-Cas9 system have been well-studied and adapted for genome engineering in mammalian cells. In S. pyogenes, only three components are required for targeted DNA cleavage at specific target sites adjacent to a PAM[2]: (1) the endonuclease Cas9, programmed by (2) a mature crRNA processed from transcription of the CRISPR locus/array which complexes with (3) another CRISPR locus-encoded RNA, the trans-activating CRISPR RNA (tracrRNA[3]). Upon site-specific double-strand DNA cleavage, a mammalian cell predominantly repairs the break through either non-homologous end joining (NHEJ) or homologous recombination (HR). NHEJ is often imperfect, resulting in small insertions and deletions (indels) that can cause nonsense mutations resulting in gene disruptions to produce gene knockouts[4]. This endogenous DNA break repair process, coupled with the highly tractable *S. pyogenes* CRISPR-Cas9 system, allows for a readily engineered platform to permanently disrupt gene function.



A Type II CRISPR-Cas9 system generally consists of the Cas9 nuclease complex programmed by tracrRNA and crRNA. The CRISPR-Cas9 system acts as an adaptive immune response system in bacteria resulting in degradation of invading DNA; it can be repurposed as a tool to introduce site-specific breaks in DNA for gene editing.

CRISPR-Cas9 Gene Editing Applications

The components of CRISPR-Cas9 genome engineering systems can be combined in multiple ways for various gene editing applications. The exact genomic changes that result can be determined by additional experiments using clonal cell lines. The Cas9 nuclease is programmed by a guide RNA (gRNA), which can take the form of a two-RNA system of a crRNA and tracrRNA, or a single guide RNA (sgRNA) where the crRNA and tracrRNA are connected into one long molecule. These guide RNAs can either be transcribed intracellularly, in vitro transcribed, or chemically synthesized and introduced to the cell through transfection or electroporation. Intracellular expression of Cas9 endonuclease can be accomplished by plasmid or integrated lentiviral expression vectors driven by constitutive or inducible promoters. Effective levels of intracellular Cas9 can also be delivered as mRNA or protein. When combined with synthetic crRNA and tracrRNA they provide a fully DNA-free genome engineering system to protect against potential integration events or ongoing off-targets.

Gene knockout

With the use of a target-specific synthetic crRNA and tracrRNA, or an sgRNA, locations within complex mammalian genomes can be targeted by the Cas9 endonuclease for a double-strand break[5]. These breaks can be repaired by endogenous DNA repair mechanisms through a process known collectively as NHEJ. Because NHEJ is error-prone, genomic deletions or insertions (indels) may result in frame shifts or premature termination to permanently silence, or knockout, target genes. Loss-of-function analysis studies may then be carried out, either in a population or single cells may be isolated to create a knockout cell line. It is important to be aware that the insertions and deletions resulting from NHEJ are random and can differ from allele to allele and cell to cell.

Embryonic stem cell and transgenic animals

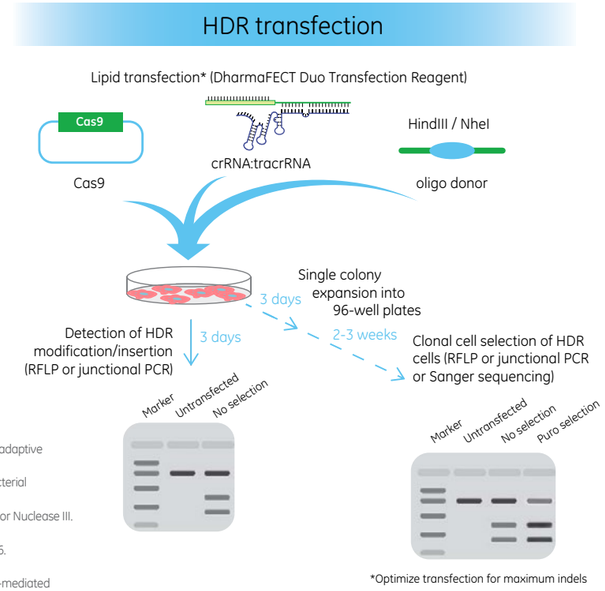
CRISPR-Cas systems can be used to rapidly and efficiently engineer one or multiple genetic changes to murine embryonic stem cells for the generation of genetically modified mice[6]. A similar approach has been used to genetically modify primate single cell embryos[7] and zebrafish[8]. An Application Note demonstrating successful gene knockout results following microinjection of Edit-R Cas9 mRNA with Edit-R synthetic crRNA and tracrRNA demonstrates the effectiveness of this approach for genetic manipulations in model organisms.

Homology-directed repair (HDR)

The CRISPR-Cas9 induced double-strand break can also be used to create a knockin, rather than a target gene knockout. The precise insertion of a donor template can alter the coding region of a gene to "fix" a mutation, introduce a protein tag, or create a new restriction site. Depending on the desired modification to a locus, there are many parameters which require optimization:

- Ideal cut site within the gene
- Length of homology arms
- Design and size of the insert

Edit-R synthetic crRNA reagents have been used alongside a single-stranded DNA to demonstrate a precise insertion by co-transfection of all components using DharmaFECT Duo Transfection Reagent.



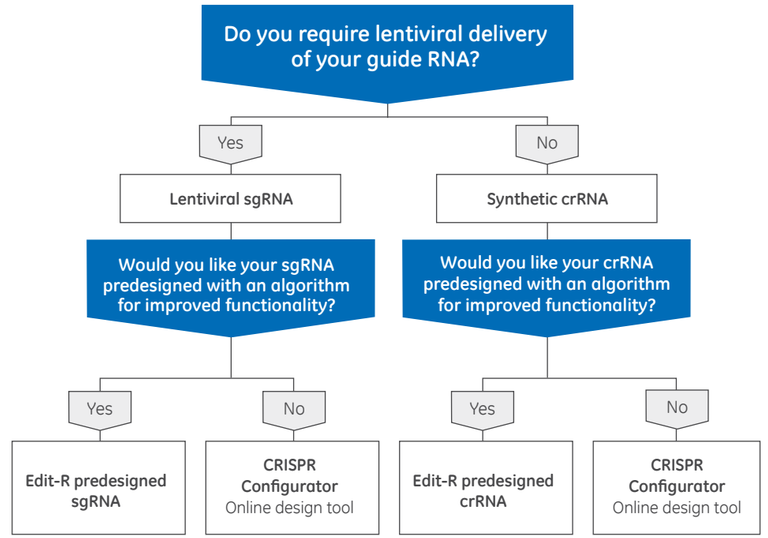
References and Recommended Reading

1. D. Bhaya, M. Davison, *et al.* CRISPR-Cas systems in bacteria and archaea: versatile small RNAs for adaptive defense and regulation. *Annu. Rev. Genet.* **45**, 273-297 (2011).
2. M. Jinek, K. Chylinski, *et al.* A Programmable Dual-RNA-Guided DNA Endonuclease in Adaptive Bacterial Immunity. *Science.* **337**(6096), 816-821 (2012).
3. E. Deltcheva, K. Chylinski, *et al.* CRISPR RNA maturation by trans-encoded small RNA and host factor Nuclease III. *Nature.* **471**(7340), 602-607 (2011).
4. Mali, P., *et al.*, RNA-guided human genome engineering via Cas9. *Science*, 2013. **339**(6121): p. 823-6.
5. Jinek, M., *et al.*, RNA-programmed genome editing in human cells. *Elife*, 2013. **2**: p. e00471.
6. Wang, H., *et al.*, One-step generation of mice carrying mutations in multiple genes by CRISPR/Cas-mediated genome engineering. *Cell*, 2013. **153**(4): p. 910-8
7. Niu, Y., *et al.*, Generation of Gene-Modified Cynomolgus Monkey via Cas9/RNA-Mediated Gene Targeting in One-Cell Embryos. *Cell*, 2014. **156**(4): p. 836-43.
8. http://dharmacon.gelifesciences.com/uploadedfiles/resources/edit-r-cas9-mrna-zebrafish-appnote.pdf

Guide RNA

Lentiviral and synthetic reagents
for targeted gene knockout

Which guide RNA is best for you?



Selection guide. While the best guide RNA for your experiment may heavily depend on your particular application or cell type, a few basic questions may help to point you in the right direction for product selection.

Follow the icons to your product solution

Pooled screening libraries:
Genome-scale or custom libraries of pooled high-titer lentiviral particles.

Arrayed screening libraries:
Available as predefined gene collections or cherry-pick libraries in 96-well microtiter plates.

Individual synthetic crRNA:
Select up to 5 CRISPR RNA (crRNA) per gene at a variety of quantities.

Gene families and pathways:
Predefined libraries arranged by gene family and/or function.

High-titer lentiviral particles:
Transduction-ready high-titer lentiviral particle format.

Species:
Human
Mouse
Rat

Overview

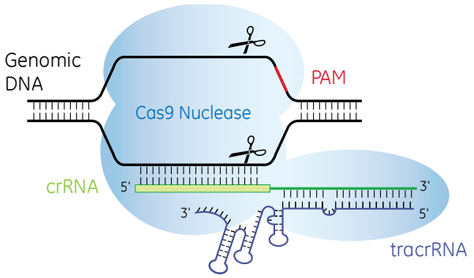
Guide RNAs program Cas9 nucleases to cut at a specific genomic location. For CRISPR-mediated gene knockout for functional analysis studies, the design of an effective, specific guide RNA is critical to achieving successful gene knockout.

Guide RNAs in the CRISPR-Cas9 system

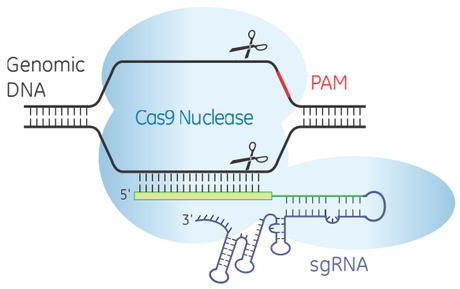
In addition to expression of the Cas9 nuclease, the CRISPR-Cas9 system requires a specific RNA moiety to recruit and direct the nuclease activity. These guide RNAs take one of two forms: a long, chemically synthesized trans-activating CRISPR RNA (tracrRNA) plus a synthetic CRISPR RNA (crRNA) designed to cleave the gene target site of interest (Figure A)

-or-

an expressed single guide RNA (sgRNA) that consists of both the crRNA and tracrRNA as a single construct (Figure B)



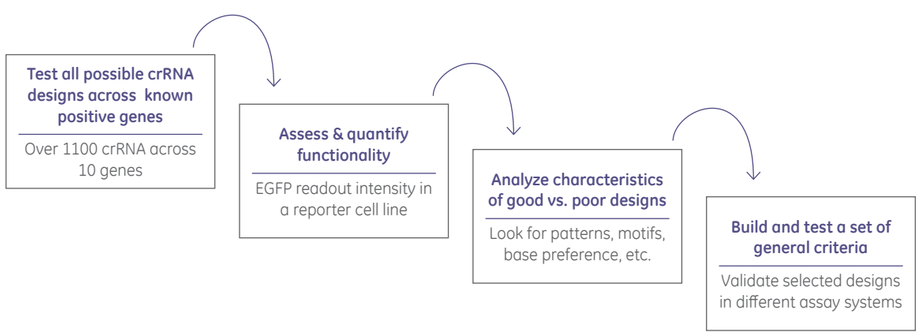
A. Illustration of Cas9 nuclease programmed by the crRNA:tracrRNA complex cutting both strands of genomic DNA 5' of the PAM



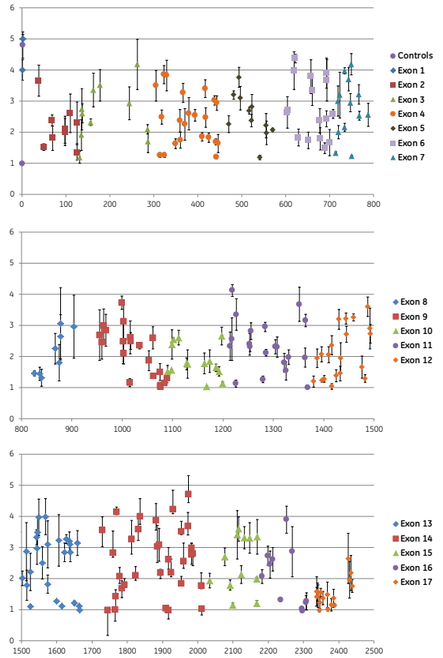
B. Illustration of Cas9 nuclease programmed by the sgRNA complex cutting both strands of genomic DNA 5' of the PAM

Edit-R™ CRISPR RNA functional algorithm

The goal of an algorithm for selection of crRNA is to identify target regions that are more likely to generate a functional knockout, not just double-strand breaks (DSBs). By assessing functional phenotypes for over 1100 designs, then validating our design rules in other assay systems, we have established rules for determining target sites that are more likely to give efficient cleavage and functional knockout with high specificity.

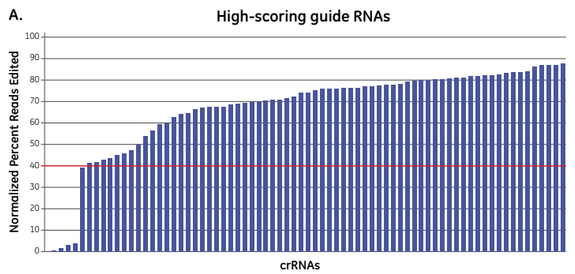


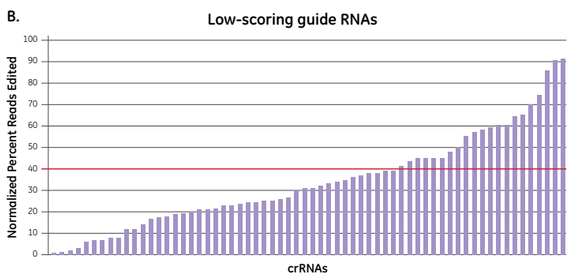
Phenotypic analysis of 10 genes with all possible crRNA designs was carried out to generate training data for the Edit-R algorithm. A GFP reporter system was used to quantify the degree of functional knockout. Assessment of functionality (y-axis) of synthetic crRNA along the length of the coding sequence (x-axis) of the *VCP* gene indicates no particular pattern as it relates to exonic position, reinforcing the importance of a design algorithm to identify characteristics of a functional guide RNA.



crRNA functionality is position and sequence dependent. Recombinant U2OS Ubi[G76V]-EGFP -Cas9 cells were transfected with 266 different synthetic crRNA:tracrRNA complexes targeting VCP gene along the length of the coding regions (x-axis) of the gene (using DharmaFECT 4 Transfection Reagent at 0.07 µL/well and 50 nM synthetic crRNA:tracrRNA final concentration). After 72 hours the EGFP fluorescence was measured (y-axis) using an Envision plate reader (Perkin Elmer).

crRNA with high scores from the Edit-R algorithm have higher cleavage efficiency than low-scoring designs. Ten crRNAs with high functional scores (A) and 10 crRNAs with low functional scores (B) for 10 genes were analyzed for editing by next generation sequencing. Ninety-three percent of the high-scoring crRNAs and only 32% of the low-scoring crRNAs showed > 40% of editing (indel formation). A HEK293T-Cas9 cell line was transfected with 50 nM crRNA:tracrRNA, using 0.25 µL/well of DharmaFECT 1 Transfection Reagent. Seventy-two hours post-transfection, cells were lysed and Nextera transposon-adapted amplicons spanning each crRNA site were generated for every treated sample as well as for a matched control amplicon from untransfected samples. Samples were indexed using the Nextera 96-well index kit and pooled for sequencing on a MiSeq instrument (paired end reads, 2 x 300 length). Reads that passed NGS quality filtering criteria were aligned to the reference file (Bowtie2 v2.1.0). Percent perfect reads were calculated and normalized to the control untransfected samples (Samtools v0.1.12a); the data is presented as normalized percent edited.





Edit-R™ Predesigned crRNA

Description

Edit-R crRNA is a chemically synthesized RNA, comprised of 20 nucleotides identical to the genomic DNA target site, or protospacer, followed by the required *S. pyogenes* repeat sequence that interacts with the tracrRNA, which is required for use with synthetic crRNA. Edit-R crRNAs are designed with an algorithm to improve functional knockout and specificity, and provide genome-wide coverage of human, mouse, or rat genes.

Benefits

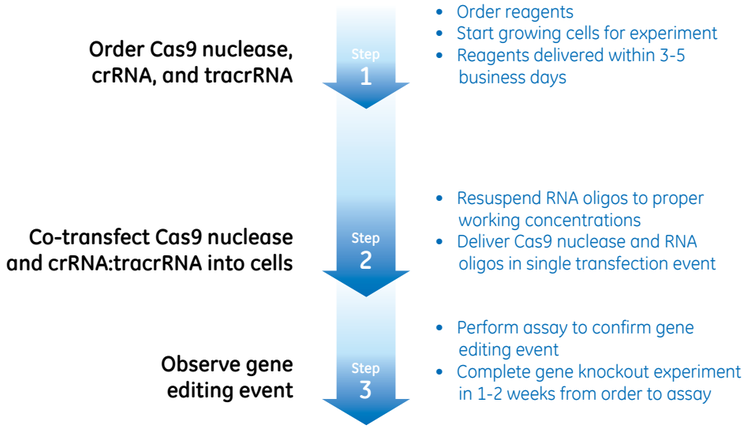
- Choose up to five algorithm-designed crRNA to target your gene of interest – no design steps required
- Predesigned Edit-R crRNA are designed with the Edit-R algorithm, which selects target sites more likely to give functional gene knockout
- Synthetic crRNA:tracrRNA are easily co-transfected with Cas9 protein, plasmid, or mRNA for great experimental flexibility
- Transfection of crRNA:tracrRNA into a Cas9-expressing cell line enables rapid, high-throughput analysis of multiple target sites per gene for any number of genes

Functional data

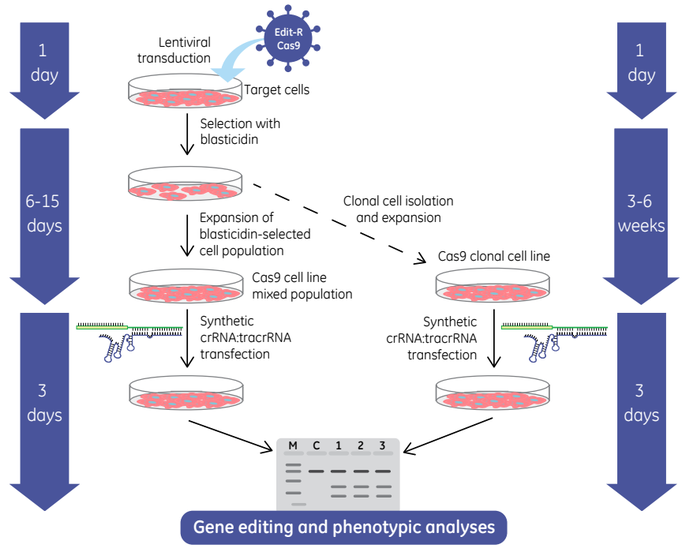
Required components for an Edit-R CRISPR-Cas9 gene editing experiment using synthetic crRNA:

- crRNA targeting a gene of interest; at least three unique constructs recommended
- Synthetic tracrRNA; which complexes with the crRNA to recruit Cas9 nuclease
- Expression of Cas9 nuclease to achieve the DSB that leads to insertions and deletions (indels)

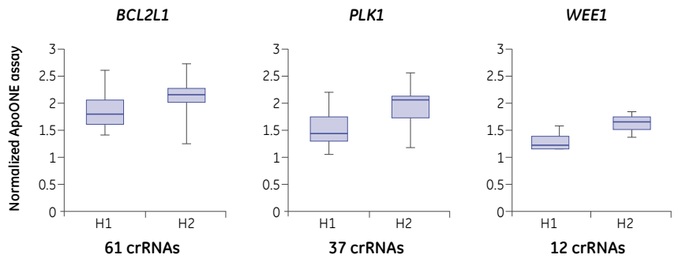
Rapid gene knockout results with Edit-R synthetic crRNA



1-2 weeks



Gene knockout workflow using the Edit-R Lentiviral Cas9 Nuclease Expression particles with synthetic crRNA and tracrRNA. To facilitate rapid generation of cell lines that constitutively express Cas9 nuclease, the Edit-R Lentiviral Cas9 Nuclease Expression vector is packaged into particles, purified and concentrated for direct transduction. Subsequent transfection of synthetic crRNA and tracrRNA into Cas9-expressing cell lines results in a higher percentage of edited cells in comparison to co-transfection of Cas9 plasmid DNA with synthetic crRNA and tracrRNA without enrichment.



crRNAs with high functional scores from the Edit-R algorithm show stronger phenotypes in ApoONE assay than low-scoring designs. For the box plot, the crRNAs were divided into bottom half (H1) and top half (H2) based on their Edit-R algorithm functional score (110 total data points represented). The medians, distribution of data between the lower and upper halves and the minimum and maximum values demonstrate that high-scoring crRNAs have increased functionality. U2OS-Proteasome cells with integrated Cas9 (under CAG promoter) were plated in 96-well plates at 10,000 cells per well. Twenty-four hours after plating, cells were transfected with 25 nM crRNA:tracrRNA using 0.2 µL/well of DharmaFECT 4 Transfection Reagent. Cells were analyzed for apoptosis 48 h after transfection using the ApoONE homogeneous assay (Promega).

Availability

Size nmol	Catalog No.
Edit-R Synthetic Human crRNA	
2 nmol	CR-HUMAN-XX-0002
5 nmol	CR-HUMAN-XX-0005
10 nmol	CR-HUMAN-XX-0010
20 nmol	CR-HUMAN-XX-0020
Edit-R Synthetic Mouse crRNA	
2 nmol	CR-MOUSE-XX-0002
5 nmol	CR-MOUSE-XX-0005
10 nmol	CR-MOUSE-XX-0010
20 nmol	CR-MOUSE-XX-0020
Edit-R Synthetic Rat crRNA	
2 nmol	CR-RAT-XX-0002
5 nmol	CR-RAT-XX-0005
10 nmol	CR-RAT-XX-0010
20 nmol	CR-RAT-XX-0020

*These are agnostic product identifiers. Actual catalog numbers are gene and species-specific (*e.g.*, CR-011580-04)

Edit-R™ Synthetic crRNA Controls

Description

Edit-R synthetic crRNA positive controls are available with mismatch detection assay primers to verify gene editing experimental conditions and estimate efficiency. They are recommended as positive controls for CRISPR-Cas9 experiments utilizing synthetic crRNA to optimize transfection conditions and verify Cas9 nuclease expression.

Edit-R synthetic crRNA non-targeting controls are recommended as negative controls for experiments using crRNAs in human, mouse, or rat cells. All Edit-R Non-targeting Controls are designed to have a minimum of three mismatches or gaps to all potential PAM-adjacent targets in human, mouse and rat genomes. Changes in viability or gene expression levels in cells treated with these controls likely reflect a baseline cellular response that can be compared to the levels in cells treated with target-specific crRNAs.

Benefits

Postitive controls and positive control kits:

- Species-specific crRNAs targeting well-characterized genes; kits additionally include mismatch detection assay primers, to determine the effectiveness of your gene editing conditions for maximal efficiency

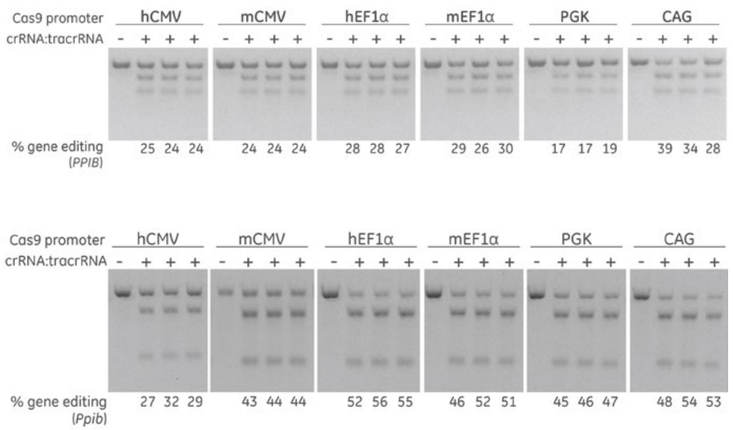
Non-targeting controls:

- Proprietary alignment tools used to verify at least 3 mismatches or gaps to any potential target in human, mouse or rat genomes
- Five different designs to choose from, to protect your system from potential off-target effects

Experimental data

Required components for an Edit-R CRISPR-Cas9 control gene engineering experiment using synthetic crRNA:

- Control CRISPR RNA (crRNA) construct
- Synthetic tracrRNA, which complexes with the crRNA to recruit Cas9 nuclease
- Expression of Cas9 nuclease to achieve the DSB that leads to insertions and deletions (indels)



Gene editing of *PPIB* in human and mouse cells quantified using a DNA mismatch detection assay. A human recombinant U2OS ubiquitin-EGFP proteasome cell line (Ubi[G76V]-EGFP) (A) and a mouse fibroblast (NIH/3T3) (B), were stably transduced with lentiviral particles containing Cas9 and a blasticidin resistance gene driven by the indicated promoters. A population of cells with stably integrated Cas9-Blast® was selected with blasticidin for a minimum of 10 days before transfections. Cells were transfected with 50 nM synthetic crRNA:tracrRNA targeting human *PPIB* or mouse *Ppib* using DharmaFECT 1 or DharmaFECT 3 Transfection Reagent, respectively. After 72 hours, the relative frequency of gene editing was calculated based on a DNA mismatch detection assay using T7EI and mismatch detection primers on genomic DNA extracted from the transfected cells.

Availability

Edit-R Synthetic Positive crRNA Controls

Edit-R *PPIB* Synthetic crRNA Control

Human	5 nmol	U-007000-05
	20 nmol	U-007000-20
Mouse	5 nmol	U-007100-05
	20 nmol	U-007100-20
Rat	5 nmol	U-007200-05
	20 nmol	U-007200-20

Edit-R Dnmt3b Synthetic crRNA Control

Human	5 nmol	U-007010-05
	20 nmol	U-007010-20
Mouse	5 nmol	U-007110-05
	20 nmol	U-007110-20
Rat	5 nmol	U-007210-05
	20 nmol	U-007210-20

Edit-R Synthetic Positive cRNA Control Kits
5 nmol each of a forward and reverse primer are included

Edit-R *PPIB* Synthetic crRNA Control Kit

Human	5 nmol	U-007050-05
	20 nmol	U-007050-20
Mouse	5 nmol	U-007150-05
	20 nmol	U-007150-20
Rat	5 nmol	U-007250-05
	20 nmol	U-007250-20

Edit-R DNMT3B Synthetic crRNA Control Kit

Human	5 nmol	U-007060-05
	20 nmol	U-007060-20
Mouse	5 nmol	U-007160-05
	20 nmol	U-007160-20
Rat	5 nmol	U-007260-05
	20 nmol	U-007260-20

Edit-R Synthetic crRNA Non-targeting Controls

Edit-R crRNA Non-targeting Control

Control #1	5 nmol	U-007501-05
	20 nmol	U-007501-20
Control #2	5 nmol	U-007502-05
	20 nmol	U-007502-20
Control #3	5 nmol	U-007503-05
	20 nmol	U-007503-20
Control #4	5 nmol	U-007504-05
	20 nmol	U-007504-20
Control #5	5 nmol	U-007505-05
	20 nmol	U-007505-20

Edit-R™ Predesigned Lentiviral sgRNA

Description

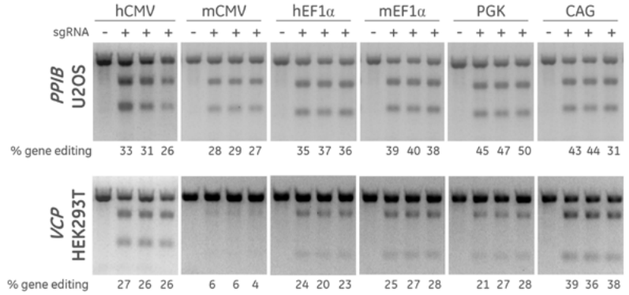
Edit-R Lentiviral sgRNAs express a chimeric structure comprised of a crRNA and tracrRNA fused through a short linker for the programming of Cas9 nuclease and creation of DNA double-strand breaks (DSBs). In the Edit-R Lentiviral sgRNA vector backbone, the gene-specific crRNA and tracrRNA are expressed under the control of a human U6 promoter, while expression of the puromycin resistance marker (Puro^R) is driven from the mouse CMV promoter and allows for rapid selection of cells with integrated sgRNA. Each Edit-R Lentiviral sgRNA is specific to the gene or genomic site of choice. The crRNA region of the sgRNA is comprised of 19-20 nucleotides identical to the genomic DNA target site followed by the non-variable sgRNA scaffold containing the tracrRNA sequence from *S. pyogenes*.

Designed with an algorithm to improve functional knockout and provide best-in-class specificity checking, predesigned sgRNAs enable high-confidence gene editing experiments without the need for laborious cloning or lentiviral packaging.

Benefits

- The Edit-R algorithm's alignment tool identifies mismatches AND gaps to optimize selection of highly specific target sequences to avoid off-targeting
- The Edit-R algorithm scores were developed on functional gene knockout rather than just quantifying indels in the genomic target DNA
- Predesigned Edit-R Lentiviral sgRNAs are supplied as concentrated high-titer particles for straightforward transduction directly into Cas9-expressing cells for efficient knockout at single-copy integration without need for cloning or packaging
- Purified, concentrated lentiviral particles can be directly transduced into difficult-to-transfect cells without the toxic cellular debris and contaminants found in supernatant preparations
- Save money by eliminating time-consuming cloning, packaging and titering steps

Functional data



High levels of gene editing are achieved with Edit-R Lentiviral sgRNA in multiple cell lines. A recombinant U2OS ubiquitin-EGFP proteasome cell line (Ubi[G76V]-EGFP) and HEK293T cells were transduced with lentiviral particles containing Cas9 and a blasticidin resistance gene. A population of stably integrated cells were selected with blasticidin for a minimum of 10 days before transduction with sgRNAs. Cells were transduced with sgRNA lentiviral particles at low MOI (0.3) to obtain cells with one integrant and selected with puromycin for seven days prior to analysis. The relative frequency of gene editing in the puromycin-selected cells was estimated from a DNA mismatch detection assay using T7 Endonuclease I.

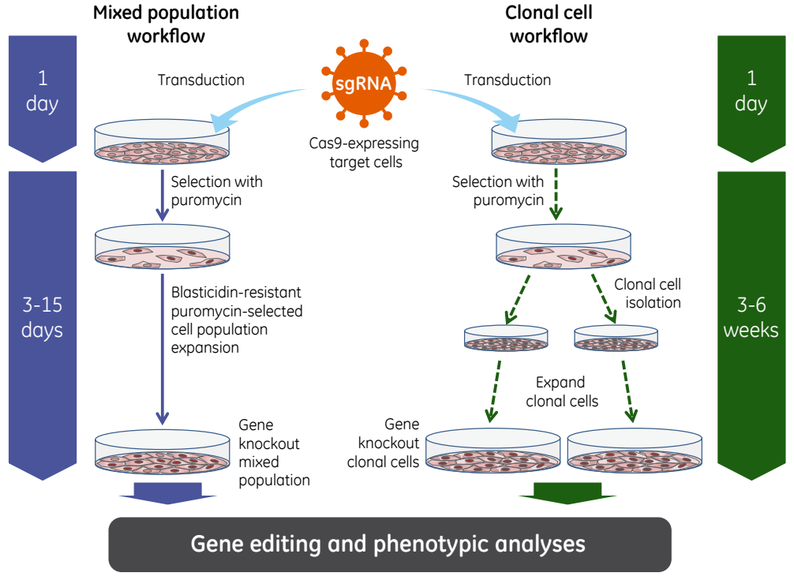
Availability

Size	Catalog No.
Edit-R Human Lentiviral sgRNA	
100 µL, 10^7 TU/mL	VSGH10142
200 µL, 10^7 TU/mL	VSGH10143
Set of 3 Edit-R Human Lentiviral sgRNA	
100 µL, 10^7 TU/mL	VSGH10148
200 µL, 10^7 TU/mL	VSGH10149
Edit-R Mouse Lentiviral sgRNA	
100 µL, 10^7 TU/mL	VSGM10144
200 µL, 10^7 TU/mL	VSGM10145
Set of 3 Edit-R Mouse Lentiviral sgRNA	
100 µL, 10^7 TU/mL	VSGM10150
200 µL, 10^7 TU/mL	VSGM10151
Edit-R Rat Lentiviral sgRNA	
100 µL, 10^7 TU/mL	VSGM10146
200 µL, 10^7 TU/mL	VSGM10147
Set of 3 Edit-R Rat Lentiviral sgRNA	
100 µL, 10^7 TU/mL	VSGM10152
200 µL, 10^7 TU/mL	VSGM10153

Edit-R Lentiviral sgRNA

Vector Element	Utility
mCMV	Mouse cytomegalovirus immediate early promoter
Puro^R	Puromycin resistance marker permits anitbiotic selection of transduced mammalian cells
5' LTR	5' Long Terminal Repeat necessary for lentiviral particle production and integration of the construct into the host cell genome
Ψ	Psi packaging sequence allows lentiviral genome packaging using lentiviral packaging systems
RRE	Rev Response Element enhances titer by increasing packaging efficiency of full-length lentiviral genomes
WPRE	Woodchuck Hepatitis Post-transcriptional Regulatory Element enhances transgene expression in target cells

Schematic map and table of vector elements of the Edit-R Lentiviral sgRNA vector.



Experimental workflow using Edit-R Lentiviral sgRNA. Edit-R Lentiviral sgRNA are transduced into a stable cell line expressing Cas9 nuclease for efficient gene knockout, even at low MOIs. The left side of the figure illustrates analysis of gene knockout in an enriched, but mixed population. The right side of the figure illustrates the ability to isolate single cells to expand and create clonal cell lines with a desired gene knockout.

Edit-R™ Lentiviral sgRNA Controls

Description

Edit-R Lentiviral sgRNA Positive controls are designed to target human, mouse and rat genes and can be included in all gene editing experiments to confirm successful transduction by observing the generation of insertions and deletions (indels) in your targeted genomic DNA using the available primers to run DNA mismatch detection assays.

Edit-R Lentiviral Non-targeting control sgRNAs are designed to establish experimental baselines and to distinguish sequence-specific biological effects from non-specific effects. CRISPR-Cas9 gene editing experiments require the use of negative control samples in order to accurately distinguish biological effects of sgRNA-induced editing of targeted genomic DNA from non-specific effects.

Benefits

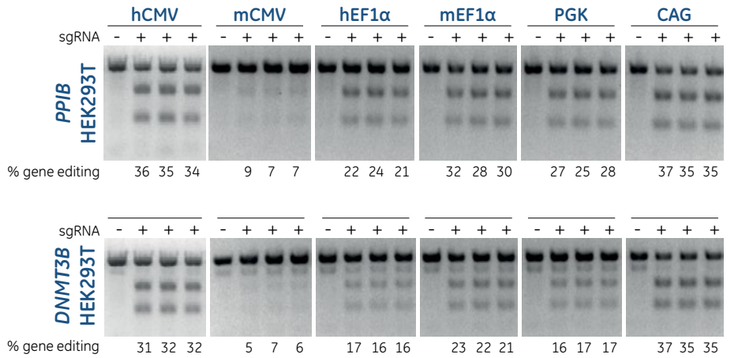
Postitive controls and positive control kits:

- Species-specific lentiviral sgRNAs targeting well-characterized genes, as well as mismatch detection assay primers, to determine the effectiveness of your gene editing conditions for maximal efficiency

Non-targeting controls:

- CRISPR guide RNA sequences cloned into the same optimized lentviral expression backbone as Edit-R gene targeting sgRNAs
- Provided as purified, concentrated lentiviral particles which avoids toxicity due to cellular debris and nucleases resulting from the packaging reaction and present in supernatants
- Designed and checked using an optimized alignment program for thorough specificity analysis of CRISPR guide RNA sequences, including detection of gapped alignments, which other alignment tools miss
- Verified bioinformatically to not be homologous to any genes in the human, mouse and rat genomes

Functional data



Edit-R Lentiviral sgRNA positive controls detect varying levels of gene editing in HEK293T cells due to differential expression of Cas9 under the control of different promoters. Using Edit-R Lentiviral sgRNA Positive controls and a mismatch detection assay to test efficiency of gene editing in HEK293T cells show that the hCMV and CAG promoters result in the highest levels of editing. HEK293T cells were stably transduced with lentiviral particles containing Cas9 and a blasticidin resistance gene. A population of stably integrated cells were selected with blasticidin for a minimum of 10 days before transduction with sgRNAs. Cells were transduced with positive control sgRNA lentiviral particles at low MOI to obtain cells with one integrant and selected with puromycin for 7 days prior to analysis. The relative frequency of gene editing in the puromycin selected cells was calculated from DNA mismatch detection assay using T7 Endonuclease I.

Availability

Edit-R Lentiviral sgRNA Positive Controls			
Human	DNMT3B	2 x 25 µL lentiviral particles	VSGH10230
	PPIB	2 x 25 µL lentiviral particles	VSGH10231
Mouse	DNMT3B	2 x 25 µL lentiviral particles	VSGH10232
	PPIB	2 x 25 µL lentiviral particles	VSGH10233
Rat	DNMT3B	2 x 25 µL lentiviral particles	VSGH10234
	PPIB	2 x 25 µL lentiviral particles	VSGH10235

Edit-R Lentiviral sgRNA Positive Control Kits			
Human	DNMT3B	2 x 25 µL lentiviral particles	VSGH11203
	PPIB	2 x 25 µL lentiviral particles	VSGH11204
Mouse	DNMT3B	2 x 25 µL lentiviral particles	VSGM11205
	PPIB	2 x 25 µL lentiviral particles	VSGM11206
Rat	DNMT3B	2 x 25 µL lentiviral particles	VSGR11207
	PPIB	2 x 25 µL lentiviral particles	VSGR11208

Edit-R Lentiviral sgRNA Non-targeting Controls		
Control #1	2 x 25 µL lentiviral particles	VSGC10215
Control #2	2 x 25 µL lentiviral particles	VSGC10216
Control #3	2 x 25 µL lentiviral particles	VSGC10217
Control #4	2 x 25 µL lentiviral particles	VSGC10218
Control #5	2 x 25 µL lentiviral particles	VSGC10219
Control #6	2 x 25 µL lentiviral particles	VSGC10220
Control #7	2 x 25 µL lentiviral particles	VSGC10221
Control #8	2 x 25 µL lentiviral particles	VSGC10222
Control #9	2 x 25 µL lentiviral particles	VSGC10223
Control #10	2 x 25 µL lentiviral particles	VSGC10224

Edit-R™ tracrRNA

Description

The Edit-R tracrRNA is a chemically synthesized and HPLC-purified long RNA molecule based on the published *S. pyogenes* tracrRNA sequence[1]. The Edit-R tracrRNA has been tested for efficient editing in multiple mammalian cell types. It is required for use with synthetic Edit-R crRNA to enable activation of the Cas9 nuclease.

The Dharmacon Edit-R CRISPR-Cas9 synthetic crRNA platform requires three components for gene editing in mammalian cells, based on the natural *S. pyogenes* system:

- Cas9 nuclease delivered as DNA plasmid, lentiviral vector, mRNA, or protein
- A chemically synthesized trans-activating CRISPR RNA (tracrRNA)
- A chemically synthesized CRISPR RNA (crRNA) designed to the gene target site of interest

Once delivered to the cell, the crRNA:tracrRNA complexes with Cas9 nuclease to generate site-specific, DNA double-strand breaks (DSBs). When DSBs are repaired through non-homologous end-joining (NHEJ), the resulting small insertions and deletions (indels) can cause nonsense mutations resulting in gene disruption to produce a functional knockout.

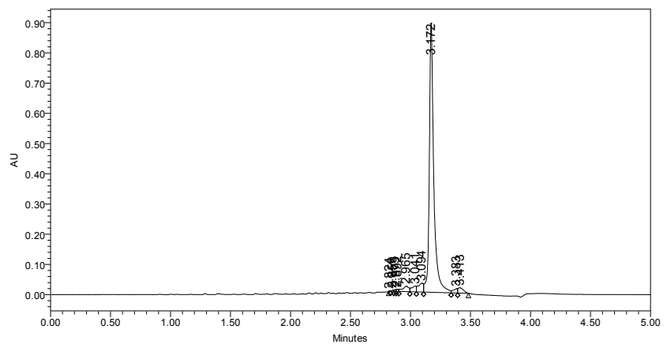
Benefits

- Proprietary 2'ACE chemistry enables highly reliable synthesis of long RNAs
- Edit-R tracrRNA is a universal component, compatible with Edit-R crRNA targeting any gene
- Synthetic crRNA:tracrRNA is easily transfectable with standard reagents and can be co-tranfected with Cas9 mRNA, protein, or plasmid

Availability

Size	Catalog No.
Edit-R CRISPR-Cas9 Synthetic tracrRNA	
5 nmol	U-002000-05
20 nmol	U-002000-20
50 nmol	U-002000-50

Functional data



UPLC trace demonstrating excellent purity of Dharmacon Edit-R synthetic tracrRNA. UPLC (ultra-performance liquid chromatography) analysis of Edit-R tracrRNA, a >70 nt purified RNA, demonstrates the high quality achieved routinely by Dharmacon 2'ACE chemical synthesis. CRISPR-Cas9 experiments using a crRNA:tracrRNA to recruit Cas9 nuclease rely on excellent purity and sequence fidelity for optimal activity. Instrument used: Acquity UPLC by Waters.

Reference:
1. M. Jinek, K. Chylinski, *et al.* A Programmable Dual-RNA-Guided DNA Endonuclease in Adaptive Bacterial Immunity. *Science*. **337**(6096), 816-821 (2012).

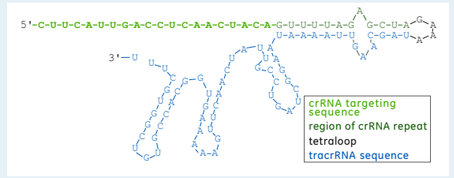
Benefits of synthetic sgRNA in gene editing

Although a natural synthetic dual RNA (crRNA:tracrRNA) system is very efficient and cost-effective for most applications, researchers working with *in vivo* and *ex vivo* models have indicated a preference for a sgRNA system.

The advantages to using a synthetic sgRNA compared to plasmid-expressed or *in vitro* transcribed (IVT) sgRNA include:

- A single oligonucleotide, arrives ready to use
- No cloning and sequencing steps or IVT reactions to perform
- Options for completely DNA-free gene editing when combined with Cas9 mRNA or Cas9 protein
- Potential for incorporation of chemical modifications

Sequence structure of a synthetic 99-mer sgRNA



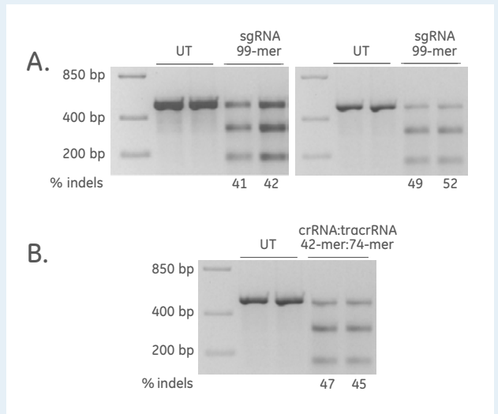
Sequence structure of sgRNA
Below is one example of how to design a sgRNA[1] for chemical synthesis:

- 20 nt targeting sequence
- 12 nt of the crRNA repeat sequence
- 4 nt of tetraloop sequence (underlined)
- 63 nt of tracrRNA sequence

5'- NNNNNNNNNNNNNNNNNNNNGUUUUAGAGCUAGAAA
UAGCAAGUUAAAAUAAGGCUAGUCCGUUAUCAACUUGAAAAAGUGGCACCGAGUCGGUGCUUU -3'

Ordering a synthetic sgRNA
Custom single-strand RNA synthesis ordering supports lengths up to 105 nt at the 0.4 µmol synthesis scale and is suitable for ordering single guide RNAs. It is recommended to include HPLC purification and 2'-deprotect/desalt to reduce the presence of non-full length RNAs in the final product. Final amounts typically achieved with this processing are 3-5 nmol but will vary depending on the RNA length.

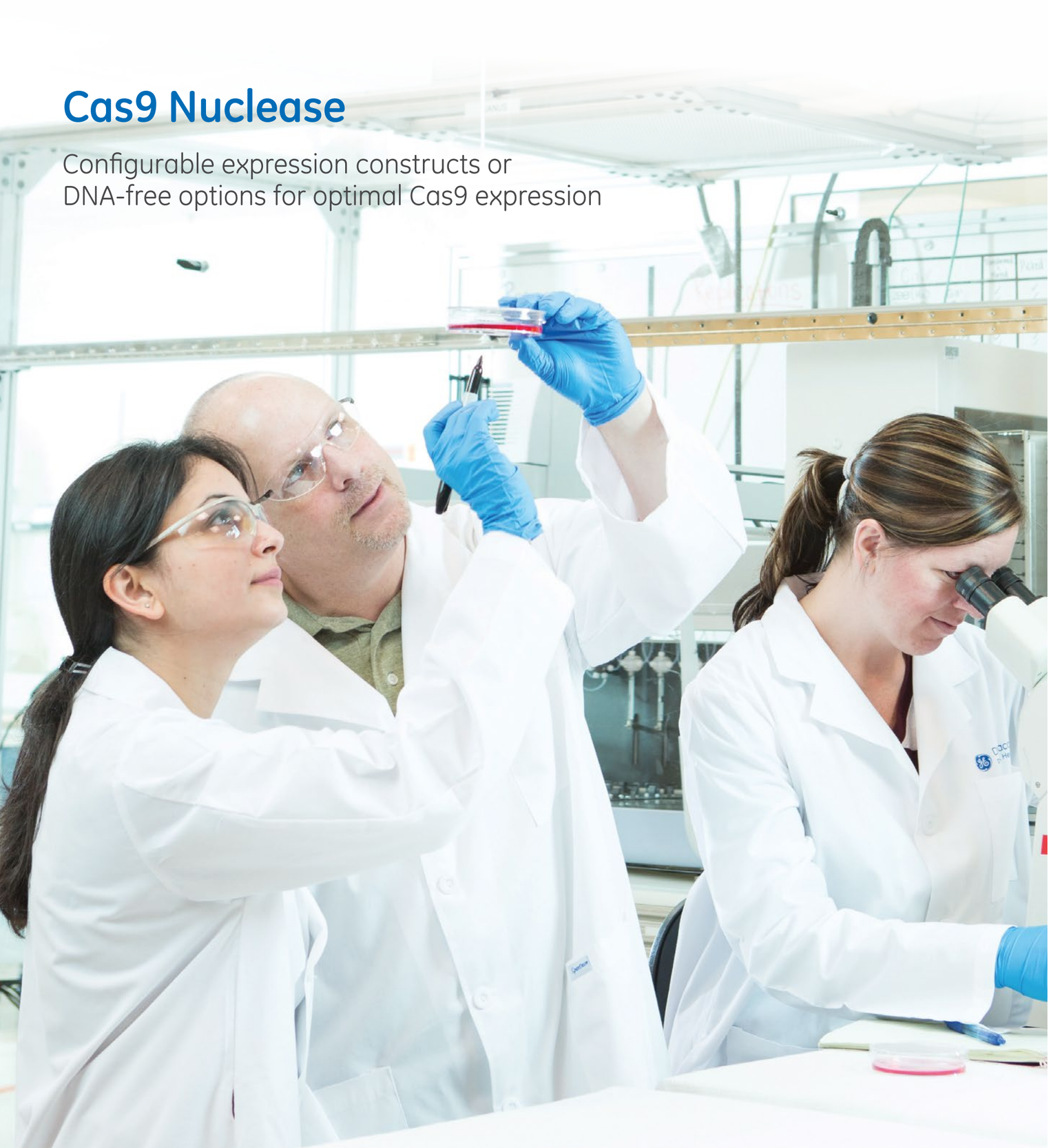
Efficient CRISPR-Cas9 gene editing achieved with synthetic sgRNA and synthetic crRNA:tracrRNA. 2'ACE chemistry was used to synthesize a 99-mer sgRNA[1] targeting *PPIB*, which was then purified by HPLC. A U2OS cell line stably expressing Cas9 nuclease from the CAG promoter was plated at 10,000 cells per well in 96-well format one day prior to transfection. sgRNA (25 nM) or synthetic crRNA:tracrRNA (25 nM) was transfected into duplicate wells using DharmaFECT™ 3 Transfection Reagent (0.25 µL/well). After 72 hours, direct cell lysis was amplified using primers surrounding the target site on the *PPIB* gene and gene editing efficiency estimated using a mismatch detection assay (Edit-R™ Synthetic crRNA Positive Controls - Protocol). The 99-mer synthetic sgRNA for target gene editing resulted in high efficiency indel formation (data shown are from two duplicate experiments; A), and high editing efficiency was also achieved for synthetic crRNA:tracrRNA (B).



Reference
1. Hsu *et al.* 2013, Briner *et al.* 2014

Cas9 Nuclease

Configurable expression constructs or
DNA-free options for optimal Cas9 expression



Overview

Cas9 nuclease in the CRISPR-Cas9 system

In Type II CRISPR-Cas systems, the CRISPR-associated enzyme Cas9 is an RNA-guided endonuclease that requires a CRISPR RNA (crRNA) and a trans-activating crRNA (tracrRNA) for genomic DNA target recognition and cleavage to generate DNA double-strand breaks (DSBs).

Choose from a variety of Cas9-expressing vectors

The Edit-R Cas9 Nuclease Expression vectors incorporate a human codon-optimized version of the *Streptococcus pyogenes* Type II cas9 (formerly csn1) gene. The activity of any given promoter controlling the transcription of Cas9 nuclease can differ greatly from one biological context to another, resulting in variable Cas9 expression levels and thus varying levels of DNA cleavage. Choosing an optimal promoter for your cell line or type will therefore affect the degree of gene editing in your experimentation. All vector-based Edit-R Cas9 Nuclease products are offered with six different, well-characterized cellular promoters from which you can choose (Table 1).

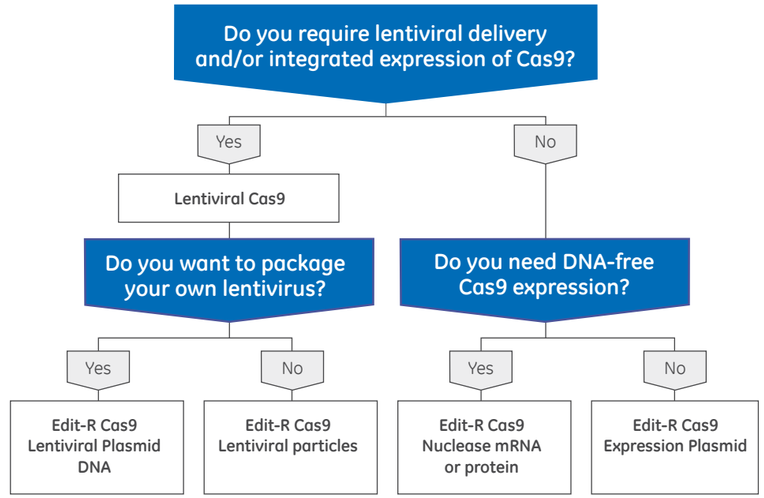
DNA-free Cas9 nuclease reagents

What does “DNA-free” CRISPR-Cas9 gene editing really mean? It means that your system uses no CRISPR-Cas9 components in the form of DNA vectors; each component is either RNA or protein. Starting with mRNA or protein as the source for Cas9 nuclease in genome engineering experiments has advantages for some applications. The use of DNA-based Cas9 (or guide RNA) expression systems carries with it the possibility of undesirable genetic alterations due to plasmid DNA integration at the cut site and constitutive expression of CRISPR-Cas9 reagents may generate increased off-target events. For this reason, a DNA-free gene editing system can be a good choice for creating engineered cell lines or working in animal models.

Promoter	Description
hCMV	human cytomegalovirus immediate early promoter
mCMV	mouse cytomegalovirus immediate early promoter
hEF1α	human elongation factor 1 alpha promoter
mEF1α	mouse elongation factor 1 alpha promoter
PGK	mouse phosphoglycerate kinase promoter
CAG	chicken beta actin hybrid promoter

Table 1. Six SMARTchoice promoter options for expressing Cas9 nuclease

Which Cas9 Nuclease is best for you?



Edit-R™ Cas9 Nuclease Expression Reagents

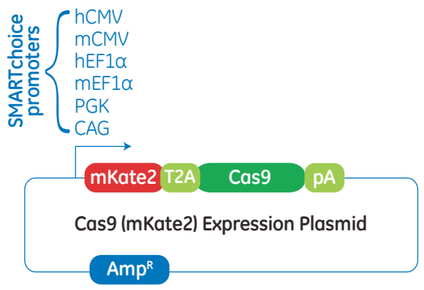
Description

Endotoxin-free, purified DNA for direct co-transfection with Edit-R synthetic crRNA and tracrRNA. Choose from three options to facilitate enrichment of Cas9-expressing cells.

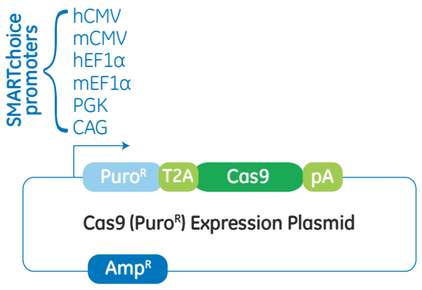
Benefits

- **Edit-R Cas9 Expression plasmids with mKate2:** Cas9 nuclease and the mKate2 fluorescent reporter are both expressed under the control of a single RNA pol II promoter, making this plasmid useful for downstream cell enrichment by FACS. Offered with your choice of six different promoters
- **Edit-R Cas9 Expression plasmids with Puro^R:** Cas9 nuclease and the puromycin-resistance marker are both expressed under the control of a single RNA pol II promoter, making this plasmid useful for downstream cell enrichment by antibiotic (puromycin) selection. Offered with your choice of six different promoters
- **Edit-R Cas9 Nuclease Expression plasmid with hCMV-Blast^R:** Cas9 nuclease expression is driven from a human cytomegalovirus (hCMV) promoter, and blasticidin resistance (Blast^R) is under the control of the Simian virus 40 (SV40) promoter. This simple vector is useful for those who do not need a fluorescent protein constitutively expressed in the cells of interest and prefer to enrich for Cas9-expressing cells through blasticidin treatment, especially if a longer antibiotic selection time is required

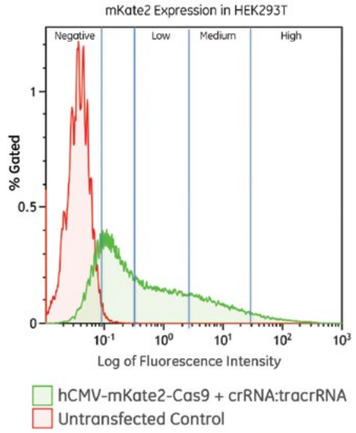
Functional data



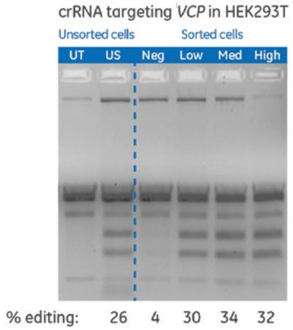
Vector elements and promoter options of Edit-R Cas9-mKate2 Expression Plasmid. The Edit-R Cas9-mKate2 plasmid expresses the monomeric transcript for red fluorescent protein mKate2 and the human codon-optimized Cas9 nuclease from *S. pyogenes*, driven by one of six choices of RNA pol II promoters. By linking expression of mKate2 to Cas9 nuclease using the self-cleaving peptide T2A, sorting mKate2-positive cells by FACS will enrich for Cas9-expressing cells and increase the percentage of cells which have undergone the gene editing event.



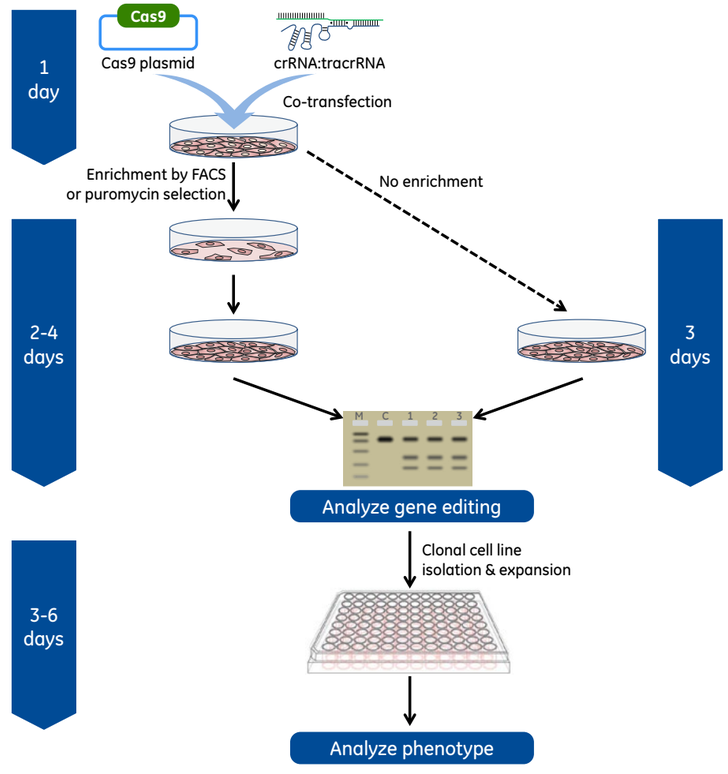
Vector elements and promoter options of Cas9-Puro^R Expression Plasmid.
The Edit-R Cas9-Puro^R plasmid expresses the puromycin-resistance selection marker and the human codon-optimized Cas9 nuclease from *S. pyogenes*, driven by one of six choices of RNA pol II promoters. By linking expression of the puromycin-resistance marker to Cas9 nuclease using the self-cleaving peptide T2A, selecting cells by treatment with puromycin will enrich for Cas9-expressing cells and thus increase the percentage of cells which have undergone the gene editing event.



Enrichment of Cas9-expressing U2OS cells using Cas9-mKate2 Expression plasmid by FACS results in increased target gene editing.
Enrichment of Cas9-expressing U2OS cells using Cas9-mKate2 expression plasmid by FACS results in increased percentage editing of human *PPIB*. U2OS cells were transfected with Cas9-mKate2 (with human CMV promoter) expression plasmid and synthetic tracrRNA:crRNA targeting the human *PPIB* gene. Cells were sorted at 72 hours post-transfection on a MoFlo XDP 100 instrument into three bins corresponding to negative, low, and high mKate2 fluorescence. SURVEYOR™ DNA mismatch assay was performed on sorted U2OS cells and percentage gene editing was compared with the unsorted (US) and control untransfected (UT) cells. The level of editing was calculated using densitometry (percentage editing). An increase in percentage gene editing is observed in the sorted cells, correlating with the increased mKate2 expression.



Gene knockout workflow using Cas9 Expression plasmid and Edit-R crRNA:tracrRNA . Gene editing with Edit-R Cas9 Nuclease Expression Plasmid and crRNA:tracrRNA is performed by co-transfecting all components with DharmaFECT Duo Transfection Reagent. One may then observe phenotypes directly (no enrichment), or enrich for transfected cells, either with cell sorting (with mKate2 plasmid) or puromycin selection (with PuroR plasmid). A DNA mismatch detection assay can be used to estimate gene editing efficiency prior to clonal cell line generation and characterization.



Availability

	Size µg	Catalog No.
Edit-R Cas9 Expression plasmids		
hCMV	120	U-004100-120
mCMV	120	U-004200-120
hEf1a	120	U-004300-120
mEf1a	120	U-004400-120
PGK	120	U-004500-120
CAG	120	U-004600-120
Edit-R mKate Transfection Optimization plasmid	120	U-003000-120
Edit-R CRISPR-Cas9 Nuclease Expression plasmid	120	U-001000-120

Edit-R™ Lentiviral Cas9 Nuclease Reagents

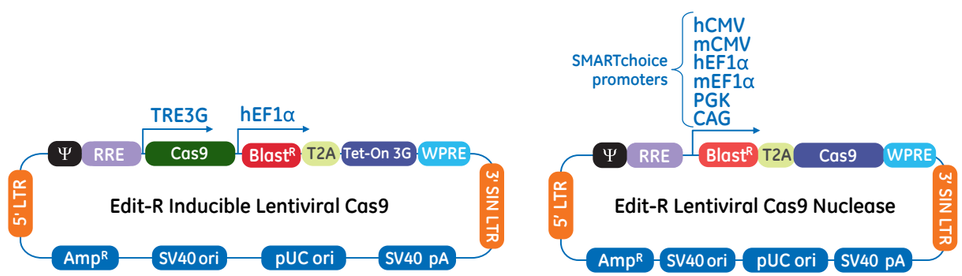
Description

Purified lentiviral particles or plasmid DNA for generation of stable Cas9 nuclease-expressing cell populations.

Benefits

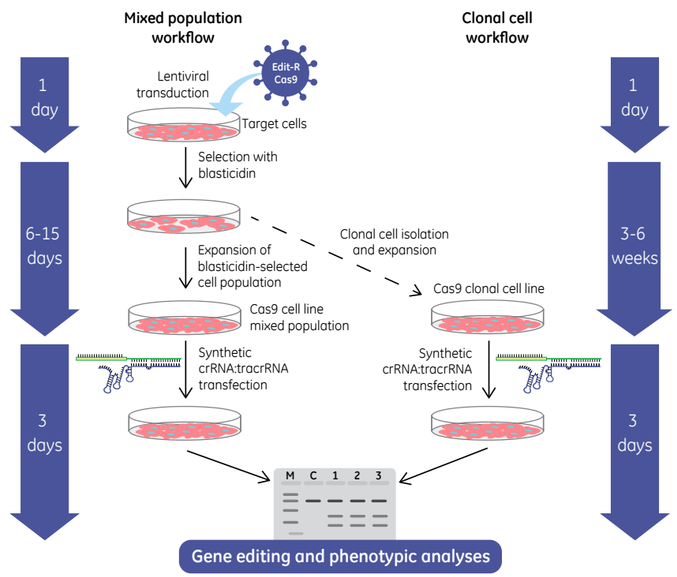
- Provided as concentrated, purified lentiviral particles for immediate transduction; 50 (2 x 25) µL, within 10% of minimum ≥ 1 × 10^7 TU/mL functional titer, by qPCR titering
- Also available as certified endotoxin-free plasmid DNA for direct transfection into a packaging cell line and production of your own lentiviral particles
- Customize your construct with one of six constitutive SMARTchoice promoters or an inducible promoter to ensure optimal Cas9 expression in your cell line of interest

Functional data

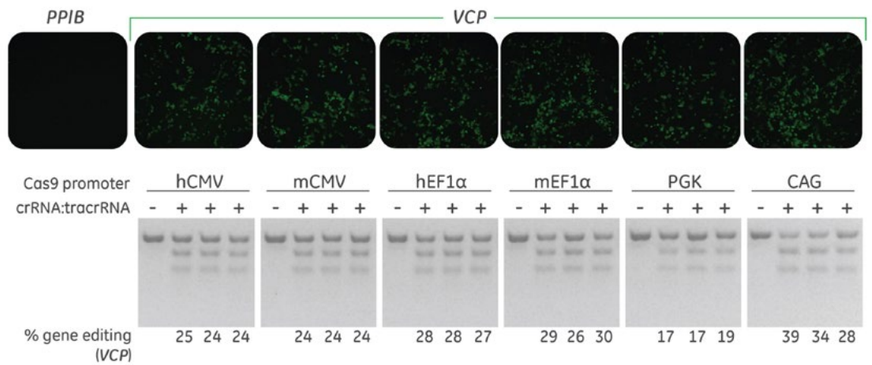


Vector Element	Utility
Cas9	Human codon-optimized *S. pyogenes* Cas9 nuclease for cleavage of targeted DNA when programmed with crRNA:tracrRNA complex
T2A	Self cleaving peptide allows for simultaneous expression of blasticidin resistance and Cas9 proteins from a single transcript
Blast^R	Blasticidin resistance marker permits antibiotic selection of transduced mammalian cells
hCMV	Human cytomegalovirus immediate early promoter
mCMV	Mouse cytomegalovirus immediate early promoter
hEF1α	Human elongation factor 1 alpha promoter
mEF1α	Mouse elongation factor 1 alpha promoter
PGK	Mouse phosphoglycerate kinase promoter
CAG	Human cytomegalovirus, chicken β-actin hybrid promoter
5' LTR	5' Long Terminal Repeat necessary for lentiviral particle production and integration of the construct into the host cell genome
ψ	Psi packaging sequence allows lentiviral genome packaging using lentiviral packaging systems
RRE	Rev Response Element enhances titer by increasing packaging efficiency of full-length lentiviral genomes
WPRE	Woodchuck Hepatitis Post-transcriptional Regulatory Element enhances transgene expression in target cells
3' SIN LTR	3' Self-inactivating Long Terminal Repeat for generation of replication-incompetent lentiviral particles
SV40 pA	Simian virus 40 polyadenylation signal
pUC ori	pUC origin of replication
SV40 ori	Simian virus 40 origin of replication
Amp^R	Ampicillin resistance gene for vector propagation in *E. coli* cultures

Schematic map and functional elements of the Edit-R Lentiviral Cas9 Nuclease Expression vector.



Gene knockout workflow using the Edit-R Lentiviral Cas9 Nuclease Expression particles with **synthetic crRNA and tracrRNA.** To facilitate rapid generation of cell lines that constitutively express Cas9 nuclease, the Edit-R Lentiviral Cas9 Nuclease Expression vector is available as packaged particles, purified and concentrated for direct transduction. Subsequent transfection of synthetic crRNA and tracrRNA into Cas9-expressing cell lines results in a higher percentage of edited cells in comparison to co-transfection of Cas9 plasmid DNA with synthetic crRNA and tracrRNA without enrichment.



Promoter strength varies across different cell types and will impact Cas9 expression and subsequent functional gene knockout. A recombinant U2OS ubiquitin-EGFP proteasome cell line (Ubi[G76V]-EGFP) was stably transduced with lentiviral particles containing Edit-R plasmid vectors expressing Cas9 nuclease and blasticidin resistance gene under the control of the indicated promoters. A population of cells with stably integrated Cas9-Blast^R was selected with blasticidin-treatment for a minimum of 10 days before transfections. Cells were transfected with 50 nM Edit-R synthetic crRNA:tracrRNA complex targeting *VCP*, a gene required for proteasome function, using DharmaFECT 3 Transfection Reagent. After 72 hours, transfected cells were examined for EGFP+ cells (upper panel) and the relative frequency of gene editing was estimated (lower panel) based on a DNA mismatch detection assay with T7 Endonuclease I.

Availability

	Size	Catalog No.
Edit-R Lentiviral Blast-Cas9 Nuclease Particles		
hCMV	50 µL, 10^7 TU/mL	VCAS10124
mCMV	50 µL, 10^7 TU/mL	VCAS10125
hEf1a	50 µL, 10^7 TU/mL	VCAS10126
mEf1a	50 µL, 10^7 TU/mL	VCAS10127
PGK	50 µL, 10^7 TU/mL	VCAS10128
CAG	50 µL, 10^7 TU/mL	VCAS10129
Inducible	50 µL, 10^7 TU/mL	VCAS11227
Edit-R Lentiviral Blast-Cas9 Nuclease Plasmid DNA		
hCMV	10 µg	CAS10136
mCMV	10 µg	CAS10137
hEf1a	10 µg	CAS10138
mEf1a	10 µg	CAS10139
PGK	10 µg	CAS10140
CAG	10 µg	CAS10141
Inducible	10 µg	Cas11229

Edit-R™ Cas9 Nuclease mRNA and protein NLS

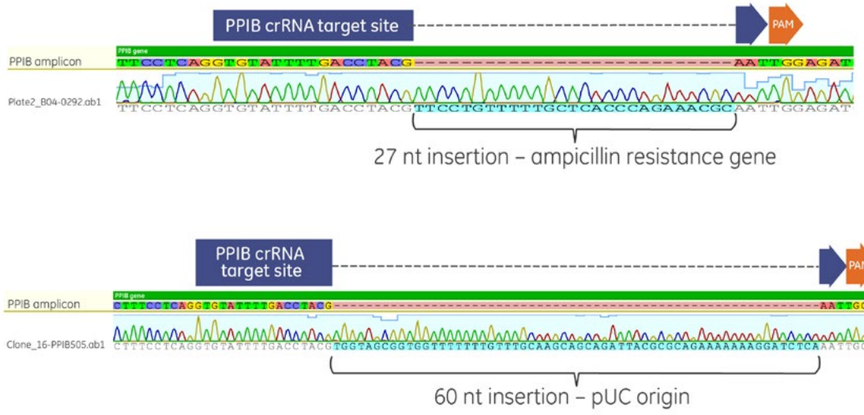
Description

Purified Cas9 Nuclease mRNA or protein can be co-transfected with synthetic crRNA and tracrRNA for a completely DNA-free genome engineering system. Edit-R Cas9 Nuclease mRNA is a highly pure, stable molecule with a 5' cap and a 3' poly(A) tail. Edit-R Cas9 Nuclease protein NLS is a highly pure, stable molecule and contains a nuclear localization signal (NLS) for targeted delivery in your cells.

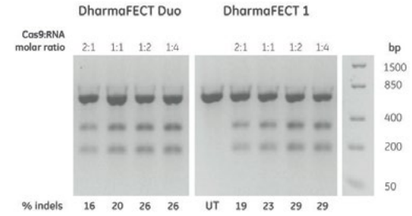
Benefits

- No exogenous DNA added to the system to ensure against the possibility of incorporating plasmid DNA into the host cell line's genome (see functional data)
- No issues with incompatabilities between promoter and cell line
- Transient expression of Cas9 nuclease, which may reduce off-targeting
- Can be co-transfected or electroporated with synthetic crRNA:tracrRNA

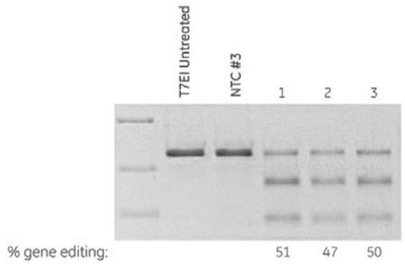
Functional data



Exogenous DNA may be introduced into the genome from CRISPR-Cas9 gene editing with plasmid components. HEK293T cells were plated in 6-well plates and transfected with hCMV-mKate2-Cas9 expression plasmid and crRNA:tracrRNA complex targeting the human *PPIB* gene in exon 2. Cells were harvested 72 hours post-transfection and sorted using the mKate2 fluorescent reporter. Fluorescent cells were plated at two, four or six individual cells per well in 96-well plates and further grown for clonal isolation. To precisely determine the genotype, Sanger sequencing was performed on PCR products amplified from gDNA spanning the crRNA target site and analyzed for indels. Various indels were observed, but notably, several of the clonal lines analyzed contained insertions homologous to components of the Cas9 expression plasmid. Similar observations were also reported in Hendel *et al.* (2014). See Application Note for more experimental details: http://dharmacon.gelifesciences.com/uploadedFiles/Resources/edit-r-experimental-workflow-appnote.pdf

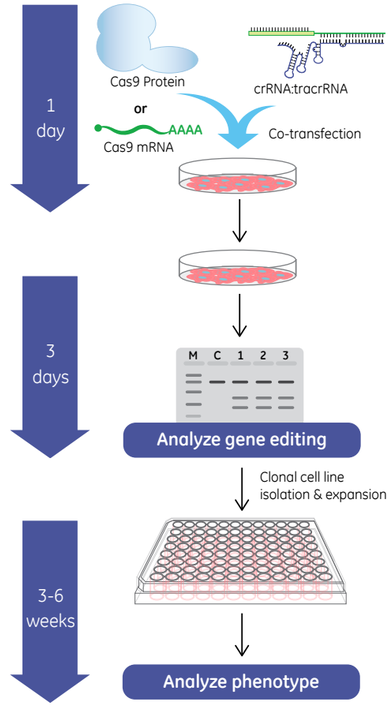


Efficient editing of *PSMD7* gene using Edit-R Cas9 protein NLS and synthetic crRNA:tracrRNA delivered with DharmaFECT Transfection Reagents. U2OS-(Ubi)EGFP cells were plated at 10,000 cells/well in 96-well plates and co-transfected using DharmaFECT Duo or DharmaFECT 1 Transfection Reagents with Edit-R Cas9 Nuclease protein NLS and synthetic crRNA:tracrRNA at the following ratios of Cas9:RNA nM (2:1 = 25:12.5, 1:1 = 25:25, 1:2 = 25:50, 1:4 = 25:100) targeting *PSMD7*. Cells were harvested 72 hours post-transfection and the relative frequency of gene editing was calculated based on a DNA mismatch detection assay with T7 Endonuclease I. UT = untreated sample, Ladder = FastRuler Low Range DNA Ladder (Thermo Scientific).



***PPIB* knockdown using electroporation of Edit-R Cas9 Nuclease mRNA and synthetic crRNA:tracrRNA in K562 cells.** K562 cells were plated at 10 M cells in a 150 mm cell culture plate. The next day, 2 million cells were collected and centrifuged at ~ 500 x g for 5 minutes. After centrifuging, cell pellets were resuspended in electroporation buffer and mixed with Cas9 mRNA (5 µg), then electroporated. Cells were transferred to one well of a 6-well plate for 6 hours, then collected and centrifuged at ~ 500 x g for 5 minutes at room temperature and electroporated with crRNA:tracrRNA (5.4 µM) synthetic positive control targeting *PPIB* (Cat #U-007503-xx) or Non Targeting Control #3 (Cat# U-007503-xx). Cells were harvested 72 hours post-electroporation and the relative frequency of gene editing was calculated based on a DNA mismatch detection assay with T7 Endonuclease I.

Gene editing with Edit-R Cas9 Nuclease DNA-free reagents and crRNA:tracrRNA is performed by co-transfecting all components with DharmaFECT Duo Transfection Reagent (or other DharmaFECT Transfection Reagent suitable to your specific cells of interest). One may then observe phenotypes directly. A DNA mismatch detection assay can be used to estimate gene editing efficiency prior to clonal cell line generation and characterization.



Availability

	Size	Catalog No.
Edit-R Cas9 Nuclease mRNA	20 µg	CAS11195
Edit-R Cas9 Nuclease protein NLS	500 pmol	CAS11200
Edit-R Cas9 Nuclease protein NLS	1000 pmol	CAS11201

CRISPR-Cas9 Screening Libraries

Pooled sgRNA or arrayed crRNA for high-throughput gene editing studies

Which CRISPR-Cas9 screening library is right for you?

When choosing a CRISPR-Cas9 screening platform, there are many considerations to determine the best tools for your experimental needs. The availability of instrumentation, analytical support, and assay type(s) must all be taken into account.

	Lentiviral pooled sgRNA libraries	Synthetic crRNA libraries
Format	Lentiviral particles in a small number of tubes	Synthetic crRNA arrayed in multi-well plates
Delivery to cells	Lentiviral transduction	Standard transfection reagents or electroporation
Assay types supported	Phenotypes can be investigated that result in changes in sgRNA abundance in the cell population and can be assessed by next generation sequencing. • Growth phenotypes (proliferation or survival) • Selectable by cell sorting (fluorescence or cell surface marker expression)	A wide range of cellular phenotypes (high content analysis, reporter or enzymatic assays, etc.) can be investigated due to one-gene-one-well layout. Editing efficiency in the population must be sufficient to allow for observation of the specific phenotype.
Screening hands-on time	Relatively low; can be carried out in a single culture dish	Scales up with the number of genes
Data analysis requirements	Next generation sequencing required for identification of sgRNA(s) and its abundance in the cell population	Phenotype is analyzed directly on a one-gene-per-well basis

Follow the icons to your product solution

Pooled screening libraries:
Genome-scale or custom libraries of pooled high-titer lentiviral particles

Libraries:
Available in 96-well or microtiter plates

Gene families and pathways:
Predefined libraries arranged by gene family and/or function and arrayed in 96-well microtiter plates

Species:

Human

Mouse

Rat

Edit-R™ crRNA Libraries

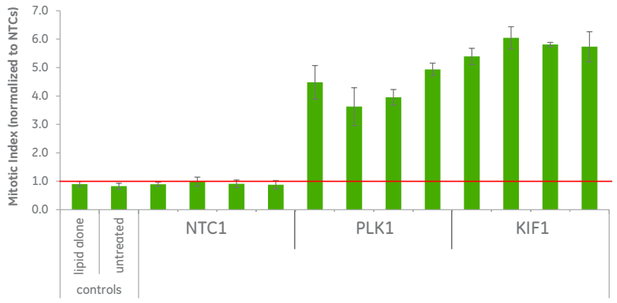
Description

Collections of algorithm-designed crRNAs targeting human and mouse gene families and pathways are assembled in arrayed libraries for rapid loss-of-function studies. Edit-R crRNA libraries enable rapid, high-throughput analysis of hundreds of genes with multiple target sites per gene. As opposed to pooled screening, this arrayed library format permits single-well analysis with nearly any phenotypic assay; including high-content assays and other morphological or reporter assays.

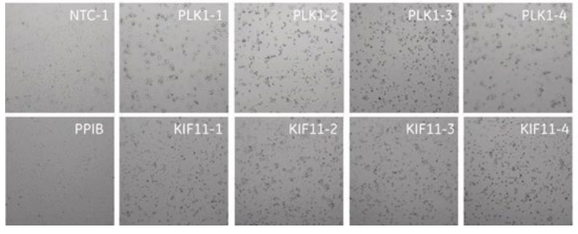
Benefits

- Edit-R predesigned crRNA are selected by the Edit-R algorithm as highly functional and specific to their target sequences for more robust, reliable gene knockout
- Catalog libraries provide four unique crRNA designs per gene, providing multiple data points for stratification of results
- Conveniently arrayed in 96-well plates and offered as gene family collections
- Also available as crRNA cherry-pick libraries; simply upload your own gene list and customize your plates

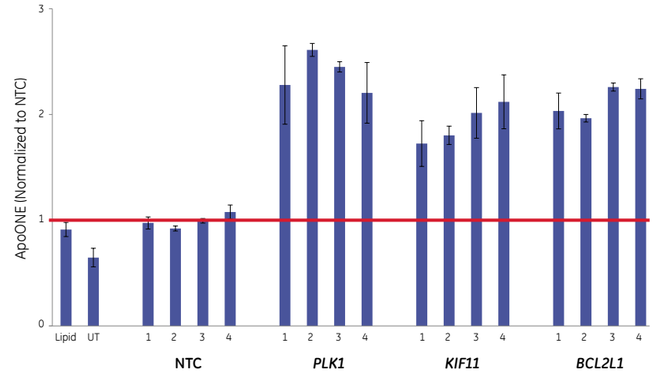
Functional data



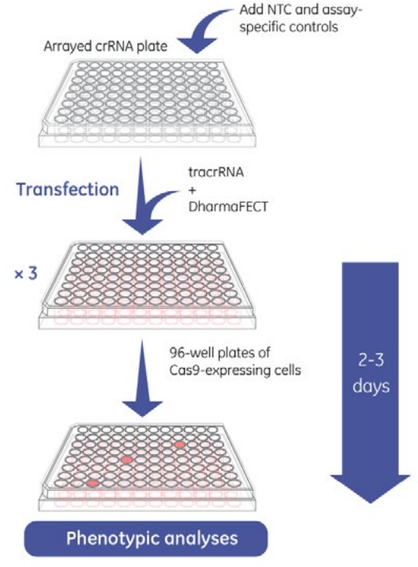
Increase of mitotic index upon knockout of PLK1 and KIF11 with synthetic crRNA:tracrRNA. U2OS-(Ubi)EGFP-Cas9 stable cells seeded at 5,000 cells/well in 96-well format were transfected the following day with four different crRNA:tracrRNA complexes at 25 nM concentration targeting PLK1 and KIF11. Four non-targeting crRNA controls (NTC), cells transfected with lipid alone (Lipid) or left untreated (UT) were used as negative controls. Cells were fixed at 48 hrs post-transfection and stained with anti Phospho-Histone H3 antibody (DY550 secondary antibody) and Hoechst 33342. Cell images were analyzed on the IN Cell Analyzer 2200 imaging system (GE Healthcare) and percent cells positive for Phospho-Histone H3 (Mitotic Index, MI) was normalized to the average MI of the negative NTC crRNAs.



Cell death phenotype observed following knockout of PLK1 and KIF11 with synthetic crRNA:tracrRNA. U2OS-(Ubi) EGFP-Cas9 stable U2OS-(Ubi)EGFP-Cas9 stable cell seeded at 10,000 cells/well in 96-well format were transfected the following day with four different crRNA:tracrRNA complexes targeting *PLK1* and *KIF11* at 25 nM concentration. Non-targeting crRNA #1 (NTC-1) or *PPIB* crRNA were used as negative controls. Bright filed images were taken at 48 hours post-transfection (20x, Leica DMIL microscope).



Knockout of essential genes by synthetic crRNA:tracrRNA results in apoptosis. U2OS-(Ubi) EGFP-Cas9 stable cell seeded at 10,000 cells/well in 96-well format were transfected the following day with four different crRNA:tracrRNA complexes at 25 nM concentration targeting *PLK1*, *KIF11* or *BCL2L1* or four non-targeting crRNA controls (NTC). Wells transfected with lipid alone (Lipid) or left untreated (UT) were also included as controls. The effects on apoptosis were assayed using Casp3/9 homogeneous assay (ApoONE, Promega) at 48 hours post-transfection. Data normalized to average of NTC (non-targeting) crRNA controls.



Knockout of essential genes by synthetic crRNA:tracrRNA results in apoptosis. Gene knockout workflow using Cas9-expressing cells with synthetic crRNA:tracrRNA. For optimal results in a crRNA library workflow, it is recommended to establish stable expression of the Cas9 nuclease to improve knockout efficiency. Subsequent transfection of crRNA:tracrRNA is very straightforward and can be carried out in a high-throughput manner.

Availability

	Size nmol	Catalog No.
Human Edit-R crRNA Library Set of 4 - 96 well		
Apotosis	0.5	GC-003900-05
Cell Cycle Regulation	0.5	GC-003200-05
Cytokine Receptors	0.5	GC-004000-05
Deubiquitinating Enzymes	0.5	GC-004700-05
DNA Damage Response	0.5	GC-006000-05
Epigenetics)	0.5	GC-006100-05
G Protein-Coupled Receptors	0.5	GC-003600-05
Ion Channels	0.5	GC-003800-05
Membrane Trafficking	0.5	GC-005500-05
Nuclear Receptors	0.5	GC-003400-05
Phosphatases	0.5	GC-003700-05
Proteases	0.5	GC-005100-05
Protein Kinases	0.5	GC-003500-05
Transcription Factors	0.5	GC-005800-05
Tyrosine Kinases	0.5	GC-003100-05
Ubiquitin Enzymes	0.5	GC-006200-05
Mouse Edit-R crRNA Library Set of 4 - 96 well		
Cell Cycle Regulation	0.5	GC-003200-05
Cytokine Receptors	0.5	GC-004000-05
Deubiquitinating Enzymes	0.5	GC-004700-05
DNA Damage Response	0.5	GC-006000-05
G Protein-Coupled Receptors	0.5	GC-003600-05
Ion Channels	0.5	GC-003800-05
Membrane Trafficking	0.5	GC-005500-05
Nuclear Receptors	0.5	GC-003400-05
Phosphatases	0.5	GC-003700-05
Proteases	0.5	GC-005100-05
Protein Kinases	0.5	GC-003500-05
Transcription Factors	0.5	GC-005800-05
Tyrosine Kinases	0.5	GC-003100-05
Ubiquitin Enzymes	0.5	GC-006200-05

Edit-R™ Lentiviral sgRNA Pooled Screening Libraries

Description

Edit-R Lentiviral sgRNA libraries are available for defined sub-libraries of gene families up to the entire genome for human and mouse; rat libraries and custom Edit-R Lentiviral sgRNA collections are available upon request.

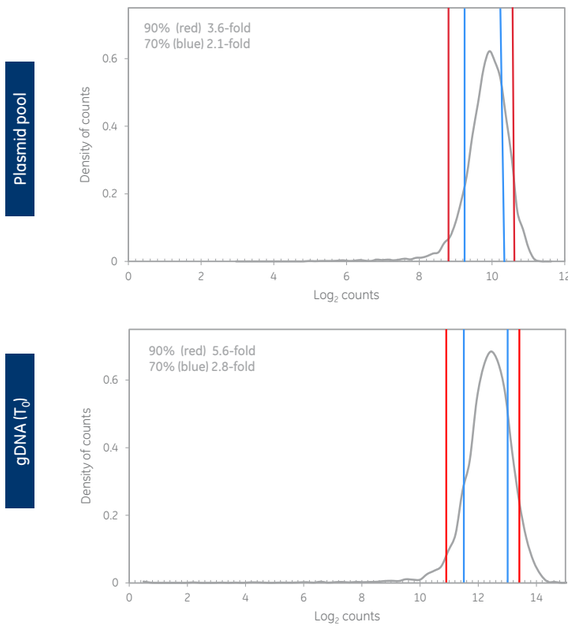
Each Edit-R Lentiviral sgRNA Pooled Screening Library includes:

- ≥ 5 x 10^8 TU/mL (± 20%) lentiviral particles in pre-aliquoted tubes
- 8 x 25 µL (200 µL total) for libraries ≤ 5000 constructs
- 16 x 25 µL (400 µL total) for libraries > 5000 constructs
- Five to ten sgRNAs per gene targeting coding genes in the NCBI Reference Sequence Database
- 100 non-targeting sgRNA negative controls bioinformatically confirmed to not align with any gene in the human, mouse and rat genomes
- Up to 340 gene-specific sgRNA positive controls targeting up to 34 protein-coding genes (10 sgRNA per gene); including common reference genes (*ACTB*, *GAPDH*, *LAMB1*, & *PPIB*)
- A data file containing complete library information, including: gene annotations, sgRNA target sequences, complete list of controls, and counts per millions of mapped reads

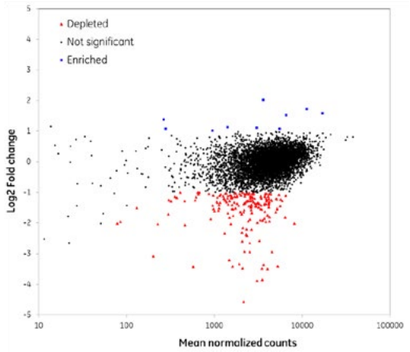
Benefits

- Efficient two-vector system that utilizes a lentiviral vector for Cas9 expression and a gene-specific vector for sgRNA expression
- Rationally designed Edit-R lentiviral sgRNAs efficient gene knockout with unparalleled specificity using a functionally validated proprietary algorithm
- Deep and broad coverage of 5 -10 sgRNAs per gene across the human, mouse and rat genomes for increased hit confidence and comprehensive genome screening
- Can be combined with the promoter flexibility of Edit-R Lentiviral Cas9 Nuclease Reagents for robust editing in biologically relevant cell types

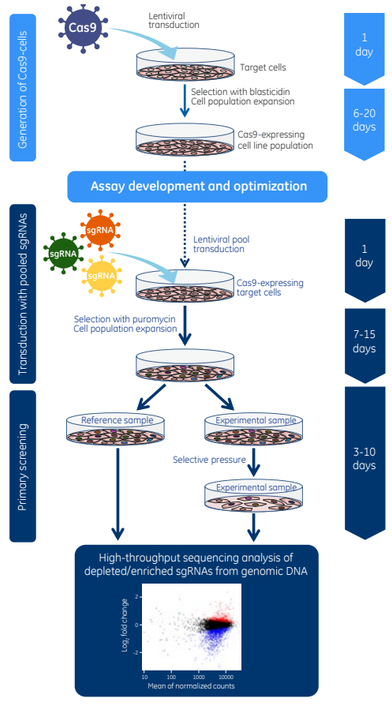
Functional data



High quality pooled screening begins with rigorous lentiviral sgRNA pooled library production. A pooled library comprised of 7519 lentiviral sgRNAs targeting human kinases was produced and the quality of the plasmid DNA library was verified by next generation sequencing (NGS). Counts per million mapped reads were obtained to determine percent recovery of input sgRNAs (99.5%) and the distribution (90% and 70% of the sgRNAs in the pool are within 3.6- and 2.1-fold of each other, respectively: Panel A). The pooled plasmid DNA library was then packaged into lentiviral particles and transduced at MOI 0.3 into U2OS cells from which genomic DNA was isolated at 24 hours post-transduction (T0), PCR-amplified and prepped for NGS. Distribution of lentiviral sgRNAs was not substantially affected, indicating rigorous production and maximized retention of constructs from production into pooled screening (lower panel).



Pooled lentiviral sgRNA human kinase library screen identifies high-confidence viability hits. A pooled lentiviral library comprised of 7079 Edit-R sgRNAs targeting 708 human kinases, 340 positive control sgRNAs targeting 34 essential genes and 100 non-targeting control sgRNAs was transduced into a Cas9-expressing U2OS cell line at MOI 0.3 and 1000-fold sgRNA representation with two biological replicates. At 24 hours post-transduction, the reference (T_0) cell populations were harvested and the experimental (T_1) cell populations were subjected to 2 µg/mL puromycin treatment (selective pressure). At eight days post-selection T_1 cell populations were harvested. Genomic DNA was harvested from both T_0 and T_1 samples and PCR-amplified with Edit-R Pooled sgRNA Forward and Reverse Index PCR primers for high-throughput sequencing on an Illumina platform. sgRNA abundance is shown as an MA plot of normalized count data. The sgRNAs that were significantly (adjusted p-value ≤ 0.05) higher and lower abundance and also showed > 2-fold abundance change are in red and blue, respectively.



CRISPR-Cas9 pooled lentiviral screening workflow. A Cas9-expressing stable cell line (mixed or clonal cell population) is first generated with Edit-R Lentiviral Cas9 Nuclease particles by selection with blasticidin. These cells are then transduced with lentiviral sgRNA pooled library and selected with puromycin. Transduced cells are split into reference and experimental populations for application of a selective pressure and/or phenotypic selection. Genomic DNA is isolated from the reference and experimental populations of transduced cells. Edit-R Pooled sgRNA Indexing PCR primers are used to PCR amplify integrated constructs and add Illumina flow cell binding sequences. The resulting amplicons are sequenced on Illumina platform sequencers, using the Edit-R Pooled sgRNA Read 1 and Index Read Sequencing primers. The integrated sgRNA sequences in both reference and experimental samples are identified and relative abundance compared. sgRNA constructs that are enriched or depleted are identified as hits to be confirmed and studied further using individual Edit-R Lentiviral sgRNAs in additional phenotypic and/or biochemical assays.

Availability	Catalog No.
Human Edit-R Lentiviral sgRNA Pooled Library*	
Apotosis	VSGH11126
Calcium Calmod Kinases	VSGH11122
Cell Cycle Reglulation	VSGH11123
Cytokine Receptors	VSGH11117
Deubiquitinating Enzymes	VSGH11121
DNA Damage Response	VSGH11114
Druggable Genome	VSGH11112
Epigenetics	VSGH11115
G Protein-Coupled Receptors	VSGH11106
Ion Channels	VSGH11108
MAP Kinases	VSGH11118
Membrane Trafficking	VSGH11119
Nuclear Receptors	VSGH11125
Phosphatases	VSGH11107
Proteases	VSGH11109
Protein Kinases	VSGH11105
Serine Proteases	VSGH11120
STE Kinases	VSGH11116
Tyrosine Kinases	VSGH11124
Ubiquitin Conjugation	VSGH11110
Whole Genome	VSGH11113
Mouse Edit-R Lentiviral sgRNA Pooled Library*	
Apotosis	VSGH11113
Calcium Calmod Kinases	VSGM11144
Cell Cycle Reglulation	VSGM11145
Cytokine Receptors	VSGM11139
Deubiquitinating Enzymes	VSGM11143
DNA Damage Response	VSGM11136
Druggable Genome	VSGM11134
Epigenetics	VSGM11137
G Protein-Coupled Receptors	VSGM11128
Ion Channels	VSGM11130
Membrane Trafficking	VSGM11141
Nuclear Receptors	VSGM11147
Phosphatases	VSGM11129
Proteases	VSGM11131
Protein Kinases	VSGM11127
Serine Proteases	VSGM11142
STE Kinases	VSGM11138
Tyrosine Kinases	VSGM11146
Ubiquitin Conjugation	VSGM11132
Whole Genome	VSGM11135
Rat Edit-R Lentiviral sgRNA Pooled Library*	
Apotosis	VSGR11170
Calcium Calmod Kinases	VSGR11166
Cell Cycle Reglulation	VSGR11167
Cytokine Receptors	VSGR11161
Deubiquitinating Enzymes	VSGR11165
DNA Damage Response	VSGR11158
G Protein-Coupled Receptors	VSGR11150
Ion Channels	VSGR11152
Membrane Trafficking	VSGR11163
Nuclear Receptors	VSGR11169
Phosphatases	VSGR11151
Proteases	VSGR11153
Protein Kinases	VSGR11149
Serine Proteases	VSGR11164
STE Kinases	VSGR11160
Tyrosine Kinases	VSGR11168
Ubiquitin Conjugation	VSGR11154
Whole Genome	VSGR11157
Edit-R Pooled sgRNA Indexing PCR and Sequencing Primer Kits	
Kit A	PRM10184
Kit B	PRM10185

*Size of all libraries: 200 µL, 10^8 TU/mL

Cherry-pick crRNA Libraries

Description

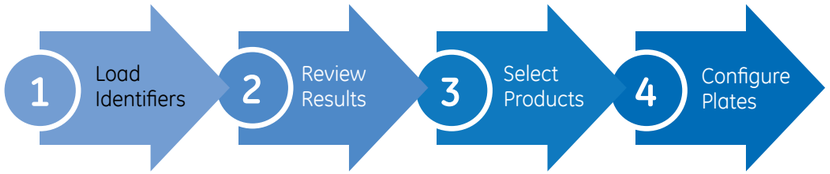
When Edit-R predefined crRNA Libraries do not match your experimental requirements, you can make your own custom crRNA library with our online Cherry-pick Library Plater. It offers the flexibility to configure a library of synthetic predesigned crRNA reagents for your unique individual experimental needs.

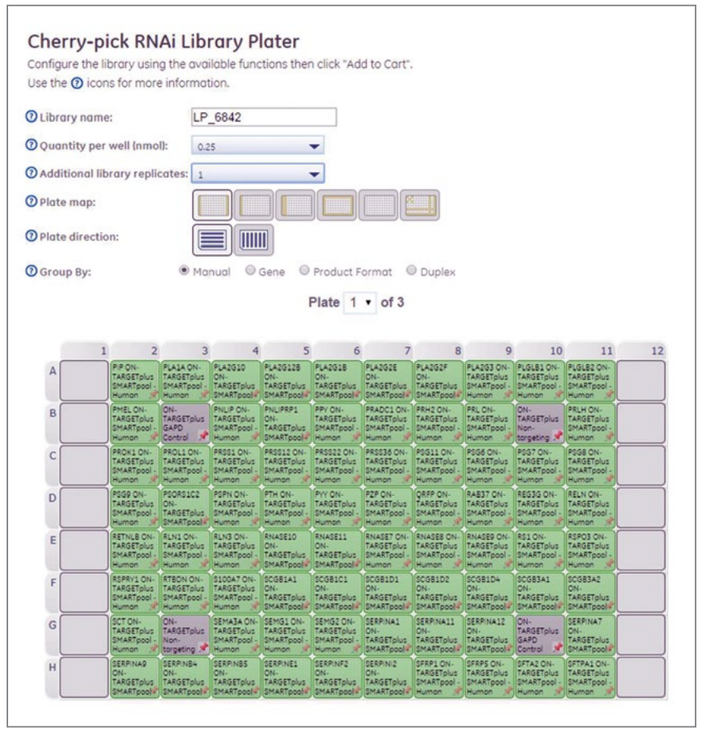
Benefits

- Choose up to five unique crRNA designs per gene for human or mouse
- Add positive and non-targeting controls anywhere on the plate
- Choose the quantity per well that best meets your experimental needs

Functional data

Once you have a gene list, assembling your customized cherry-pick library of synthetic crRNA reagents is simple:





1. **LOAD** gene identifiers for your desired crRNA products into our online Cherry-pick Library Plater. Go to http://dharmacon.gelifesciences.com/cherry-pick-libraries
   - NCBI Gene ID or Gene Symbol
   - Dharmacon product catalog numbers

2. **REVIEW** the results for accuracy and product availability

3. **SELECT** the number of crRNAs per gene to include on the plates

4. **CONFIGURE** the library plates for quantity, controls, replicates and layout

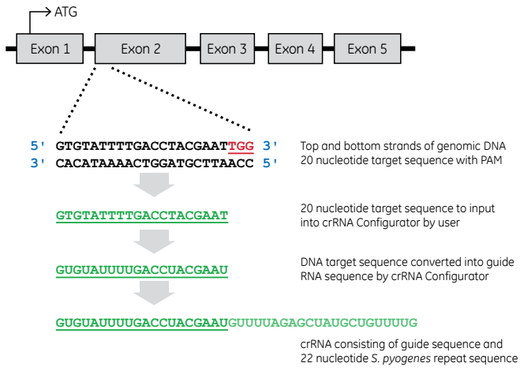
Then simply add to your cart!

CRISPR RNA Configurator

General principles for design of crRNAs

crRNAs can be designed to target a genomic sequence of interest using the following guidelines:

- Select a 20 nucleotide genomic DNA sequence within the target gene that is immediately 5' (upstream) of a PAM sequence (for example, NGG for *S. pyogenes*).
- The target sequence can be located on either strand of the genomic DNA.
- Perform a genomic alignment of the chosen target sequence to ensure that there are no identical or highly homologous sequences in the genome immediately 5' of the PAM sequence, especially within other coding regions of the genome[1]. Cas9 nuclease will cut both strands of DNA positioned three nucleotides upstream of the PAM.



An example of choosing a 20 nucleotide sequence targeting the human gene PPIB (chr15:64448014-64455354) for gene knockout. A target sequence directly 5' of the PAM (underlined and in red) is selected as the recognition site for the guide RNA. The user-selected 20 nucleotide sequence (underlined, bold and in green) is the necessary input for the CRISPR RNA Configurator, which will automatically convert DNA nucleotides to RNA and append the 22 nucleotide fixed *S. pyogenes* repeat sequence (in green) directly 3' of the user-defined input sequence to result in a 42 nucleotide gene-specific guide RNA. It can then be ordered as a lentiviral sgRNA or synthetic crRNA.

The Dharmacon™ CRISPR RNA Configurator for custom guide RNA design

Although the Edit-R predesigned crRNA and sgRNA are highly functional and available genome-wide for human, mouse, and rat, there are CRISPR experiments and applications that require more specialized guide RNA designs:

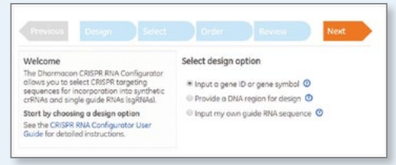
- Knockin (using homology-directed repair) of a DNA donor at a particular cut site
- Gene knockout in other model organisms (non-human, mouse, or rat)
- Targeting a particular protein domain, transcript, or exon
- Knockout of a microRNA or long noncoding RNA (lncRNA)

In cases like these, you can design a guide RNA yourself, using the principles outlined above, or use an online design tool for assistance. The advantages of the CRISPR Configurator are:

- An advanced option to limit target sequences to only selected transcript variants
- Design to protein-coding, microRNA, or lncRNA genes by simply entering an identifier
- Unmatched rigor in our alignment tool for improved specificity checking to reduced off-targets. Guide RNA sequences with perfect or high identity to other regions in the genome are excluded

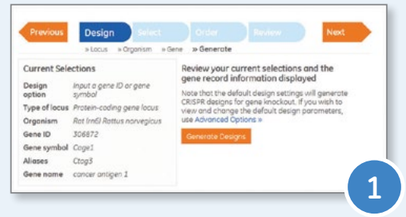
Simplified online design and ordering of CRISPR guide RNAs

The tools in the CRISPR RNA Configurator allow you to quickly and easily generate guide RNA sequences for ordering, either as synthetic crRNA or lentiviral sgRNA. There are three options:



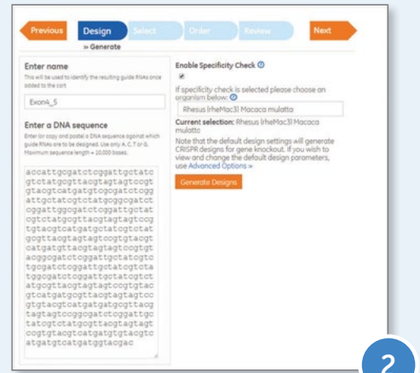
1

Option 1. Input a gene ID or gene symbol
To use the CRISPR RNA Configurator to generate designs for your desired target gene, indicate the locus type (microRNA, lncRNA, or protein-coding) and species, then enter a gene identifier. Advanced options are available for greater flexibility in specificity checking, allowed PAM and GC content. A list of candidate DNA target sites will be generated. Review the sequences listed in order from earliest to latest exon, and check the boxes next to your selected designs. You may then add them to your cart as synthetic crRNA or lentiviral sgRNA.



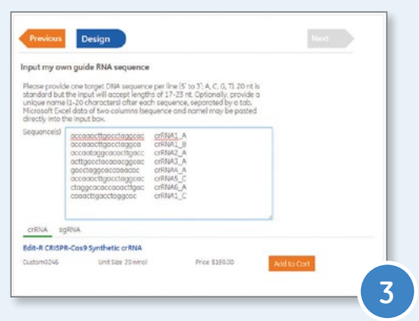
2

Option 2. Provide a DNA region for design
If you have a specific genomic region you wish to target, or are working in a species that is not included in the CRISPR RNA Configurator, you may provide a gene sequence or genomic DNA region as the basis for guide RNA design. Paste or enter the DNA sequence in the space provided (maximum 10,000 bases) and designs will be generated for all available PAM sites. Review the sequences listed in order from earliest to latest in the DNA sequence, and check the boxes next to your selected designs. You may then add them to your cart as synthetic crRNA or lentiviral sgRNA.



3

Option 3. Input my own guide RNA sequence
If you already know the sequence of the crRNA or lentiviral sgRNA you wish to order, select this option and simply enter the sequence in the space provided. While 20 nt is the typical target length, you have the option of entering any guide of 17-23 nt in length. The 22 nt spacer sequence will automatically be appended to your guide sequence. If you wish to order more than one guide RNA, you may enter or paste multiple target sequences (names optional) in the space provided. You may then add the entered guide RNA(s) to your cart as synthetic crRNA or lentiviral sgRNA.



Delivery Solutions for Gene Editing

Transfection reagents optimized for your specific application and cell type

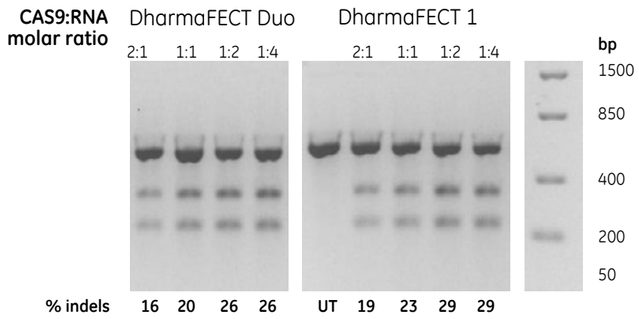
DharmaFECT™ Transfection Reagents

Description

DharmaFECT Transfection Reagents are specifically formulated for delivery of small RNAs. Typical results demonstrate higher efficiency with lower cytotoxicity than reagents designed for use with plasmids. While DharmaFECT 1 reagent is the most all-purpose reagent, DharmaFECT 2, 3 and 4 reagents offer distinct formulations to provide highly effective delivery of synthetic crRNA:tracrRNA, synthetic sgRNA, siRNA and microRNA reagents in a wide variety of cells

Benefits

- Ideal for single-gene studies or high throughput crRNA screens
- Low toxicity even at a broad range of experimental conditions for maximum experimental flexibility
- Co-transfect crRNA:tracrRNA (or synthetic sgRNA) with Cas9 nuclease mRNA, protein, or plasmid using DharmaFECT Duo reagent, specifically formulated for co-transfection



Efficient co-transfection of Cas9 protein and synthetic crRNA:tracrRNA by DharmaFECT Transfection Reagents results in effective editing of PSMD7 gene in U2OS-(Ubi) EGFP cells. U2OS-(Ubi)EGFP cells were plated at 10,000 cells/well in 96-well plates and co-transfected using DharmaFECT Duo or DharmaFECT 1 Transfection Reagents with Edit-R Cas9 Nuclease protein NLS and synthetic crRNA:tracrRNA at the following ratios of Cas9:RNA nM (2:1 = 25:12.5, 1:1 = 25:25, 1:2 = 25:50, 1:4 = 25:100) targeting PSMD7. Cells were harvested 72 hours post-transfection and the relative frequency of gene editing was calculated based on a DNA mismatch detection assay with T7 Endonuclease I. UT = untreated sample, Ladder = FastRuler Low Range DNA Ladder (Thermo Scientific).

Availability

	Catalog No.
DharmaFECT 1 Transfection Reagents	
0.2 mL	T-2001-01
0.75 mL	T-2001-02
1.5 mL	T-2001-03
5 × 1.5 mL	T-2001-04
2 × 10 mL	T-2001-07A
DharmaFECT 2 Transfection Reagents	
0.2 mL	T-2002-01
0.75 mL	T-2002-02
1.5 mL	T-2002-03
5 × 1.5 mL	T-2002-04
2 × 10 mL	T-2002-07A
DharmaFECT 3 Transfection Reagents	
0.2 mL	T-2003-01
0.75 mL	T-2003-02
1.5 mL	T-2003-03
5 × 1.5 mL	T-2003-04
2 × 10 mL	T-2003-07A
DharmaFECT 4 Transfection Reagents	
0.2 mL	T-2004-01
0.75 mL	T-2004-02
1.5 mL	T-2004-03
5 × 1.5 mL	T-2004-04
2 × 10 mL	T-2004-07A
DharmaFECT Set of 4 Transfection Reagents	
4 × 0.2 mL	T-2005-01
4 × 0.75 mL	T-2005-02
4 × 1.5 mL	T-2005-03

DharmaFECT™ Duo Transfection Reagent

Description

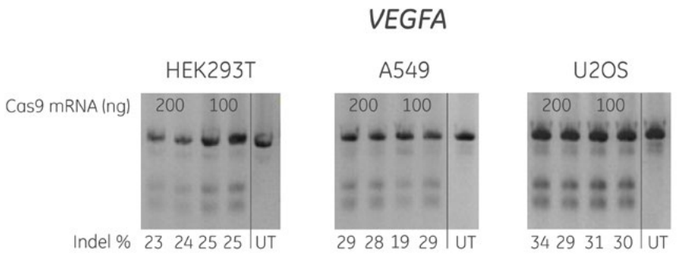
Optimized for co-transfection, DharmaFECT Duo Transfection Reagent provides highly effective delivery of plasmid protein, or mRNA with siRNA, microRNA, synthetic sgRNA, or crRNA:tracrRNA reagents. Useful for experiments utilizing reporter genes, RNAi rescue, or Edit-R Cas9 expression plasmid. Also effective for co-transfection of crRNA:tracrRNA with Cas9 mRNA or protein.

Benefits

- Effective delivery of both DNA plasmid and RNA from a single formulation
- Efficient transfection without compromising cell viability
- Potent RNAi results together with plasmid expression
- Recommended for use with Edit-R Cas9 Nuclease Expression plasmids, mRNA, or protein, with synthetic crRNA:tracrRNA

Availability

	Catalog No.
DharmaFECT Duo Transfection Reagent	
0.2 mL	T-2010-01
0.75 mL	T-2010-02
1.5 mL	T-2010-03
5 × 1.5 mL	T-2010-04



Co-transfection of Edit-R Cas9 Nuclease mRNA with crRNA:tracrRNA results in efficient gene editing in three cell lines. HEK293T, A549 and U2OS cells were plated at 20,000 cells/well in 96-well plates and co-transfected using DharmaFECT Duo Transfection Reagent with Edit-R Cas9 mRNA (200 or 100 ng) and synthetic crRNA:tracrRNA (50 nM) targeting *VEGFA*. Cells were harvested 72 hours post-transfection and the relative frequency of gene editing was calculated based on a DNA mismatch detection assay with T7 Endonuclease I.

A CRISPR-Cas9 gene engineering workflow: generating functional knockouts using Edit-R™ Cas9 and synthetic crRNA and tracrRNA

Maren M. Gross, Žaklina Strezoska, Melissa L. Kelley, Dharmacon, now part of GE Healthcare, Lafayette, CO, USA

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 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, Edit-R, Cas9, Clonal Selection, FACS, Immunoblot, Sanger Sequencing, Cloning, Transfection, Transfection Optimization

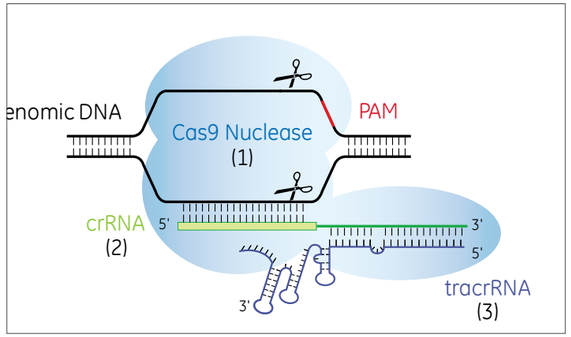


Figure 1. Illustration of Cas9 nuclease (light blue), programmed by the crRNA (green):tracrRNA (purple) complex, cutting both strands of genomic DNA 5' of the PAM (red).

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 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 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. 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 Edit-R crRNAs and tracrRNA.

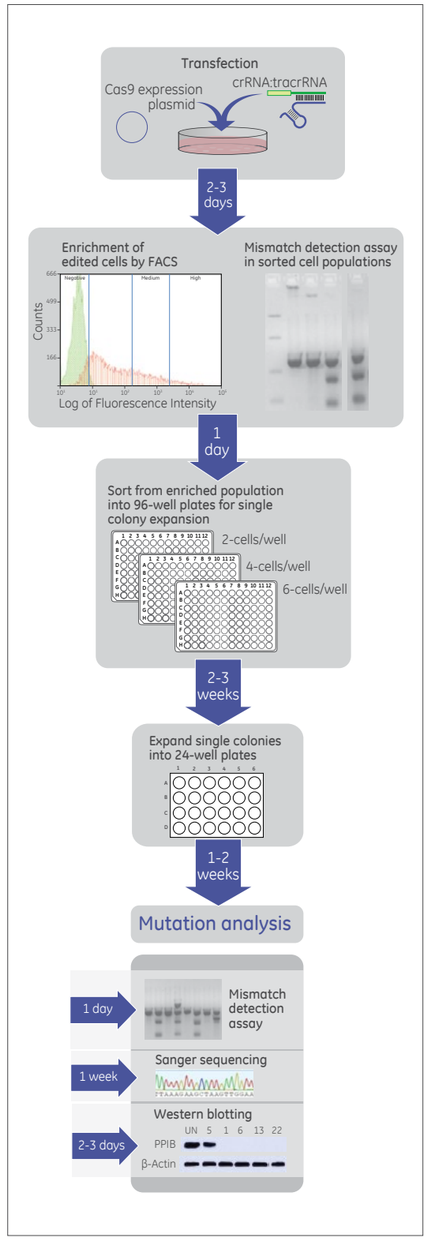


Figure 2. A complete workflow using the Edit-R CRISPR-Cas9 gene engineering platform. First, co-transfection was optimized for editing events and scaled up appropriately for FACS analysis. Next, transfected cells were enriched by FACS, and additionally the positive binned mKate2 cells were sorted into 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.

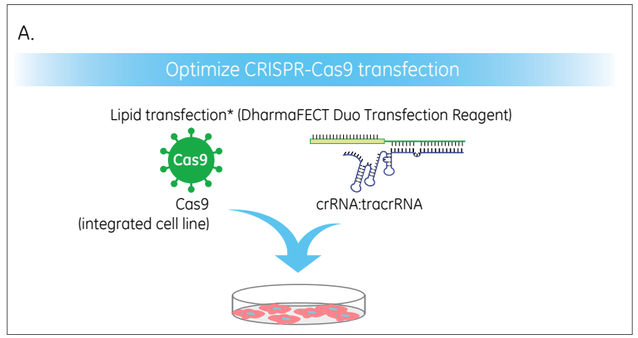


Figure 3. FACS enriched cell populations show a high level of editing by mismatch detection assay (T7EI). **A.** HEK293T cells were transfected with Edit-R hCMV_mKate2-Cas9 Expression plasmid and crRNA:tracrRNA targeting *PPIB*. Cells were sorted at 72 hours on a MoFlo XDP 100 instrument into three bins 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.

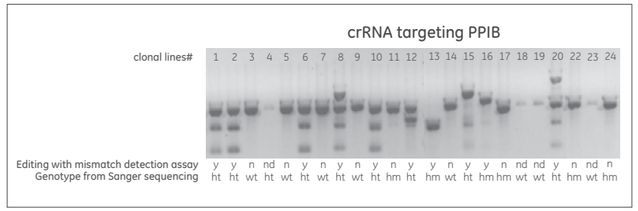


Figure 4. Examples of mismatch detection analysis in FACS clonal lines of HEK293T cells. Samples were run on a 2% agarose gel. The clones are numbered and the corresponding gene editing from mismatch detection assay is indicated as y (yes), n (no), or nd (not determined) along with genotype confirmed 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.

Here, a complete workflow (Figure 2) is demonstrated using the 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.

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 Edit-R components. The best conditions were determined to be > 80% cell viability 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.

Application Note

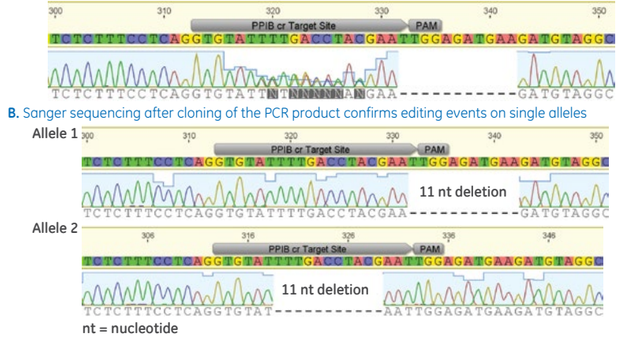


Figure 5. Sanger sequencing analysis on clonal line 1 confirms heterozygous mutations. **A.** Chromatogram from Sanger sequencing of purified PCR products. Mutation(s) are observed but allelic events are unclear. **B.** Chromatograms representing the 2 different mutations identified from cloning the 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

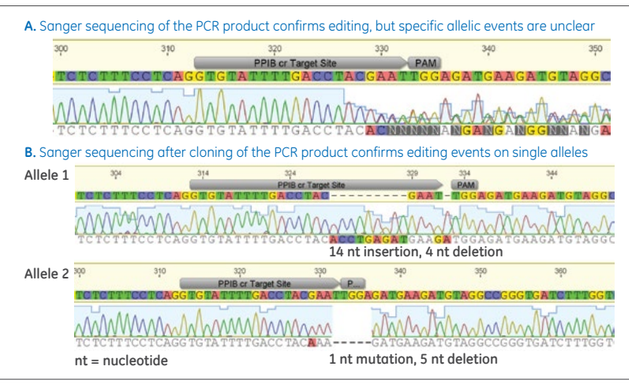


Figure 6. Sanger sequencing from clonal line 6 with heterozygous mutations. **A.** Chromatogram from Sanger Sequencing of purified PCR products after mutation detection analysis. Mutation(s) are observed but allelic events are unclear. **B.** Chromatograms representing the 2 different mutations identified from Sanger Sequencing after cloning PCR product into a blunt vector (Zero Blunt™ PCR Cloning Kit, Invitrogen, transforming into competent 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

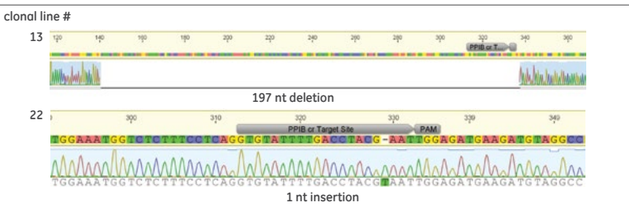


Figure 7. Sanger sequencing analysis on clonal lines 13 and 22 confirm homozygous mutations. Chromatograms from Sanger sequencing of purified PCR products after mutation detection analysis using T7EI. Mutations detected are observed to be the same on both alleles for Clones 13 and 22. nt = nucleotide

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.

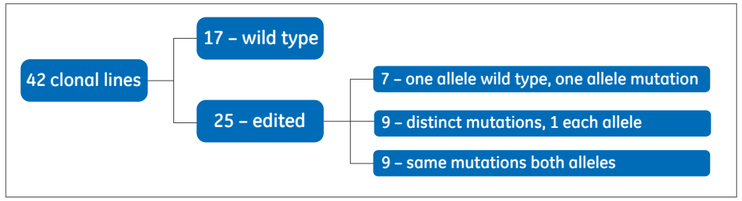


Figure 8. A diagram characterizing mutations present in clonal lines. Of the 42 clonal lines, 17 (40%) were wild type and 25 (60%) of them had clear mutations near the crRNA cut site. It was also observed that 18 of the 25 clonal lines with sequence confirmed mutations (72%) had mutations in both alleles.

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.

Discussion

Here we have demonstrated an experimental workflow for generating cell lines with a desired gene knockout using the 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 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 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.

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.

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

Application Note

Conclusion

The 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 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.

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 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 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 million 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).

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.

Geno-type	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.

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: One hundred thousand 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. Fifty 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). Twelve 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 TM 4X LDS sample buffer and NuPAGETM 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 anti-rabbit 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 anti-mouse 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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Application Note

Homology-directed repair with Dharmacon™ Edit-R™ CRISPR-Cas9 reagents and single-stranded DNA oligos

John A. Schiel, Eldon Chou, Maren Mayer, Emily M. Anderson, and Anja van Brabant Smith, Dharmacon, part of GE Healthcare, Lafayette, CO, USA

Abstract

CRISPR (clustered regularly interspaced short palindromic repeats)-Cas9 (CRISPR-associated protein 9) is a revolutionary tool that utilizes an RNA-guided nuclease for efficient site-directed genome engineering in various eukaryotic systems. The double-strand breaks (DSBs) created by CRISPR-Cas9 are repaired in the cell by two predominant mechanisms: imprecise non-homologous end joining (NHEJ) and precise homology-directed repair (HDR). The Dharmacon™ Edit-R™ CRISPR-Cas9 gene engineering platform consists of both synthetic and expressed guide RNAs. In conjunction with Cas9 expression plasmids and lentiviral particles, it supports multiple experimental workflows. Here we demonstrate an innovative workflow to guide the insertion of 10-12 nucleotides into a gene of interest by HDR with the Edit-R CRISPR-Cas9 system, Dharmacon DharmaFECT™ Duo Transfection Reagent, and a single-stranded donor DNA oligo (ssDNA).

Keywords

Homology-directed repair, homologous recombination, non-homologous end joining, gene editing, genome engineering, Edit-R, Cas9, CRISPR, donor oligo, donor DNA, lipid transfection, homology arms, insertion, repair, DSB, knockin

Introduction

The bacterial adaptive immune system, the CRISPR-Cas9 system, can be repurposed for targeted gene editing of mammalian genomes.[1,2,3] Derived from *Streptococcus pyogenes*, the system uses the Cas9 nuclease protein that complexes with a tracrRNA and a targeting crRNA (which contains a 20 nucleotide guide sequence complementary to the genomic target of interest).[3] The genomic target is amenable to Cas9 targeting if it is upstream of a PAM (protospacer adjacent motif), which for *S. pyogenes* is NGG. The Edit-R CRISPR-Cas9 system mimics the endogenous bacterial system by using a synthetic tracrRNA and custom synthetic crRNA to guide Cas9 to a specific target site in order to create a DSB three nucleotides upstream of the PAM sequence. Once the DSB occurs, the mammalian cell utilizes endogenous mechanisms to repair the broken genomic DNA, the most common methods being NHEJ or HDR. While NHEJ repair of the DSB is often imprecise and error-prone due to the generation of random insertions and deletions (indels), HDR faithfully copies the genetic information from a related DNA sequence, such as a sister chromatid or a foreign donor DNA sequence (plasmid or DNA oligo). By using single-stranded DNA (ssDNA) donors, one can rapidly design and synthesize a donor DNA sequence containing the desired changes to the genomic location of interest.

The Edit-R CRISPR-Cas9 synthetic guide RNA experimental workflow (Figure 1) consists of (1) a plasmid expressing a mammalian codon-optimized gene sequence encoding Cas9, (2) a synthetic crRNA designed to target a genomic site of interest, and (3) a synthetic tracrRNA. The use of chemically synthesized RNA components allows facile generation of indels through NHEJ which can result in gene disruption (knockout). However, this same workflow can also be combined with (4) an ssDNA donor oligo for rapid genome engineering using HDR. Here, we first demonstrate the application of Edit-R crRNA:tracrRNA in an experimental workflow (Figure 2) including ssDNA oligos to knock in short DNA sequences into two different genomic regions of interest. Second, we establish lipid-based transfection as an effective technique to accomplish HDR experiments with knockin efficiencies as high as 25%. Last, we optimize ssDNA oligo homology arm length to provide DNA donor oligo design recommendations for use with the Edit-R genome engineering platform. The methods presented within this application note can be applied to HDR-based insertion of epitope tags such as a FLAG™ tag, SNPs, precise stop codons, and amino acid changes in the active site of enzymes.

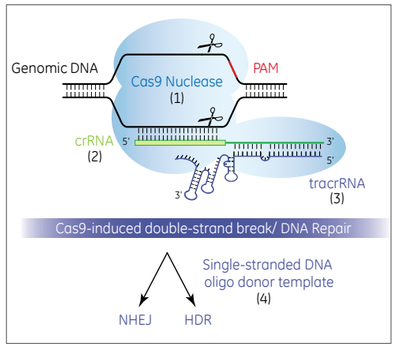

Figure 1. Edit-R CRISPR-Cas9 genome engineering. Illustration of Cas9 nuclease (1) bound to the synthetic crRNA (2):tracrRNA (3) complex and targeted to genomic DNA by the guide sequence in the crRNA, adjacent to the PAM (red). Repair of DSBs can be achieved by NHEJ in the absence of a donor template, or by HDR when a donor template such as a single-stranded DNA oligo (4) is present.

Results

Genome engineering using CRISPR-Cas9 requires expression of the Cas9 nuclease with the crRNA and tracrRNA. This can be achieved by co-transfection of a plasmid expressing Cas9 and crRNA:tracrRNA (Figure 2A), or by creation of a cell line in which the Cas9 cassette is delivered using lentiviral particles and stably integrated and expressed prior to transfection with crRNA:tracrRNA. For HDR and creation of knockins, it is important to optimize experimental conditions to obtain the maximal levels of DSBs. Therefore, experiments to optimize DSBs without a donor template should be performed (Figure 2A) to assess and maximize levels of gene editing. These experiments should be performed prior to experiments using the donor template (Figure 2B) and be used as a preliminary assessment for overall levels of DSB generation.

Genome engineering at the VCP locus: A crRNA was designed to target exon 2 of the human *VCP* gene and insert a DNA sequence containing two restriction sites for detection of the knockin (Figure 3A). First, a U2OS cell line was co-transfected with a hCMV-Puro^R-Cas9 nuclease expression plasmid and the synthetic crRNA:tracrRNA complex using DharmaFECT Duo Transfection Reagent according to transfection conditions optimized for maximal indel formation (Figure 2A). Transfected cell populations were either not selected or selected with puromycin to enrich for Cas9 expression. Additionally, U2OS cells stably expressing Cas9 were transfected with the *VCP* crRNA:tracrRNA. All cell populations were assessed 72 hours post-transfection with a DNA mismatch detection assay using T7 endonuclease I (T7EI) to estimate relative indel formation (Figure 3B).

The above transfections were also performed in the presence of a ssDNA donor oligo containing homology arms and a 12 nucleotide sequence for insertion that contains two adjacent restriction enzyme sequences (Figure 3A). Transfected cells were assayed for knockin of the inserted sequence 72 hours post-transfection by a Restriction Fragment Length Polymorphism (RFLP) assay (Figure 3C). The highest amounts of HDR knockin were observed in conditions corresponding to the highest amount of gene editing as measured by indels using the mismatch detection assay (Figure 3B and C). This result highlights the importance of optimizing experimental conditions to ensure maximal creation of CRISPR-Cas9-mediated DSBs.

Genome engineering at the *EMX1* locus: DharmaFECT Duo Transfection Reagent was used to co-transfect U2OS cells with a hCMV-Puro^R-Cas9 nuclease expression plasmid and synthetic crRNA:tracrRNA complex targeting exon 3 of the human *EMX1* gene, and to transfect synthetic crRNA:tracrRNA into a Cas9-integrated U2OS cell line (Figure 4A). Transfections were performed as described above, including a ssDNA donor oligo. Total gene editing (indel %) and HDR knockin in the non-selected, puromycin-selected, and Cas9-integrated U2OS cell populations were analyzed 72 hours post-transfection (Figure 4B and C). Consistent with the results from targeting *VCP*, the highest amounts of HDR knockin at the *EMX1* locus were observed in conditions corresponding to the highest amount of gene editing as measured by indel formation using the mismatch detection assay.

Concentration of synthetic single-stranded donor DNA oligo: In order to determine the optimal concentration of the donor template for HDR, we performed a dose curve of the ssDNA oligo targeting the *EMX1* locus. Non-selected and puromycin-selected U2OS cells were transfected with fixed amounts of the hCMV-Puro^R-Cas9 nuclease expression plasmid (200 ng/well), synthetic crRNA:tracrRNA complex (25 nM final/well), and increasing amounts of ssDNA donor oligo (0.5, 1.0, 2.5, 5.0, 10.0, 20.0, 40.0, 60.0, and 100.0 nM final/well) in a 96-well plate. Cas9-integrated U2OS cells were transfected with fixed amounts of the synthetic crRNA:tracrRNA complex (25 nM final/well), and increasing amounts of ssDNA donor oligo (0.5, 1.0, 2.5, 5.0, 10.0, 20.0, 40.0, 60.0, and 100.0 nM final/well) in a 96-well plate. Cells were harvested 72 hours post-transfection and HDR knockin was detected using the RFLP assay. Both non-selected and puromycin-selected U2OS cell populations display the highest levels of HDR knockin using a final concentration range of 2.5 nM to 10.0 nM ssDNA donor oligo (Figure 5). Similarly, the Cas9-integrated U2OS cell line exhibited the highest levels of HDR knockin using a final concentration range of 2.5 nM to 10.0 nM ssDNA donor oligo (Figure 5).

Effect of homology arm length on HDR efficiency: When targeting *VCP* or *EMX1* (Figures 3 and 4), the ssDNA oligo donor sequences contained 30 nucleotides of homology on each arm. In order to determine how homology arm length can affect HDR efficiency, Cas9-integrated U2OS cells were transfected with complexed crRNA:tracrRNA and ssDNA donor oligos with increasing homology arm lengths. Donor DNA oligo homology arms were designed to have 10 nucleotides of homology per arm, increasing in 10 nucleotide increments up to 70 nucleotides total per homology arm. Cell populations were assayed at 72 hours post-transfection and the percent HDR knockin was determined by RFLP (Figure 6). HDR was detected with homology arms as short as 20 nucleotides at a knockin percentage comparable to the longer homology arms that were tested (30-70 nucleotides).

Discussion

The Edit-R CRISPR-Cas9 genome engineering platform employing chemically synthesized RNAs is a quick and easy method to test multiple guide sequences for optimizing % indel formation through NHEJ, and achieving functional gene knockouts. Here, we have demonstrated the ability to utilize the same experimental workflow to rapidly create knockins through the HDR pathway simply by including a single-stranded DNA oligo donor template. We have further demonstrated the use of lipid-based transfection using DharmaFECT Duo Transfection Reagent to achieve precise sequence insertion through the HDR pathway, whereas, in the literature, this is commonly achieved through expensive electroporation methods and use of costly Cas9 nuclease reagents. Recommended

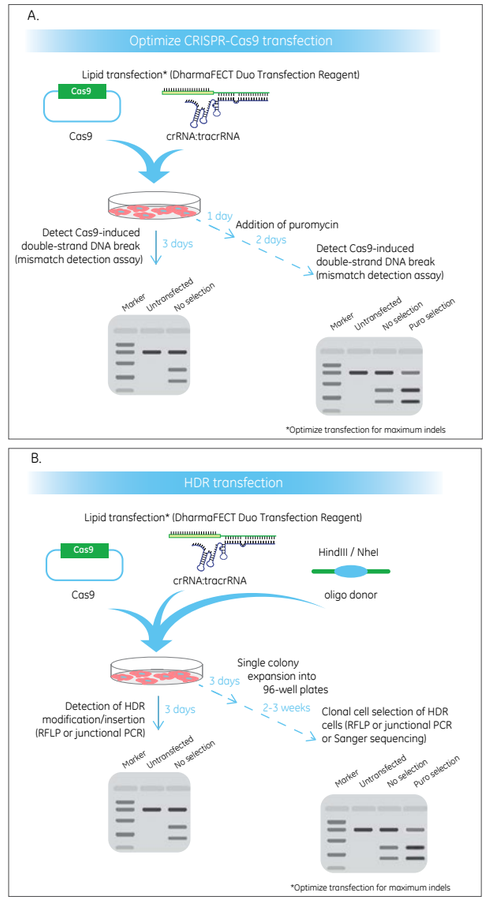

Figure 2. HDR-based experimental workflow for co-transfection of synthetic RNAs, Cas9 expression plasmid and synthetic DNA oligo donor. A Cas9 expression plasmid co-transfection workflow with DharmaFECT Duo Transfection Reagent. Detection of double-strand breaks **(A)** prior to performing the HDR transfection **(B)** ensures maximal double-strand breaks for HDR repair. Optimization of maximal DSBs (A and dashed arrows) is necessary to increase the amount of DSBs to be repaired by HDR.

Genome engineering at the VCP locus: A crRNA was designed to target exon 2 of the human *VCP* gene and insert a DNA sequence containing two restriction sites for detection of the knockin (Figure 3A). First, a U2OS cell line was co-transfected with a hCMV-Puro^R-Cas9 nuclease expression plasmid and the synthetic crRNA:tracrRNA complex using DharmaFECT Duo Transfection Reagent according to transfection conditions optimized for maximal indel formation (Figure 2A). Transfected cell populations were either not selected or selected with puromycin to enrich for Cas9 expression. Additionally, U2OS cells stably expressing Cas9 were transfected with the *VCP* crRNA:tracrRNA. All cell populations were assessed 72 hours post-transfection with a DNA mismatch detection assay using T7 endonuclease I (T7EI) to estimate relative indel formation (Figure 3B).

Application Note

experimental conditions for performing similar successful sequence insertion experiments using these Edit-R CRISPR-Cas9 reagents and workflows are shown in Table 1.

Optimizing gene editing experimental conditions using a mismatch detection assay is essential before performing HDR applications because of the generally lower baseline rate of HDR when compared to NHEJ for repair of double-strand DNA breaks. One method for increasing the percent editing in a population of transfected cells is to subject the cells to the selective pressure of antibiotic resistance, *e.g.*, puromycin selection. We have observed that transfected cell populations selected for Cas9 expression by application of puromycin correlated with higher levels of knockin through HDR. Furthermore, we have observed higher levels of knockins when using a cell line that stably expresses Cas9 nuclease. We observed that when comparing the genomic engineering of the two targets presented in this study, the mismatch detection assay and the RFLP assay results did not correlate, which is consistent with the overall lower efficiencies of HDR compared to the NHEJ. Yet, for each gene target tested, the highest amount of gene editing, as estimated by mismatch detection, is associated with the highest amount of HDR knockin. It is important to note that careful transfection optimization is a critical process to maximize HDR and decrease the overall effort of screening individual clonal lines to find the correct HDR-induced modification. For example, one would need to screen ten times more individual clonal lines when HDR knockin levels are observed at 1% compared to 10%. Additionally, expression of HDR machinery in cell lines and cell types can vary, which can affect HDR efficiency.

To this end, we were able to identify the optimal amounts of ssDNA donor

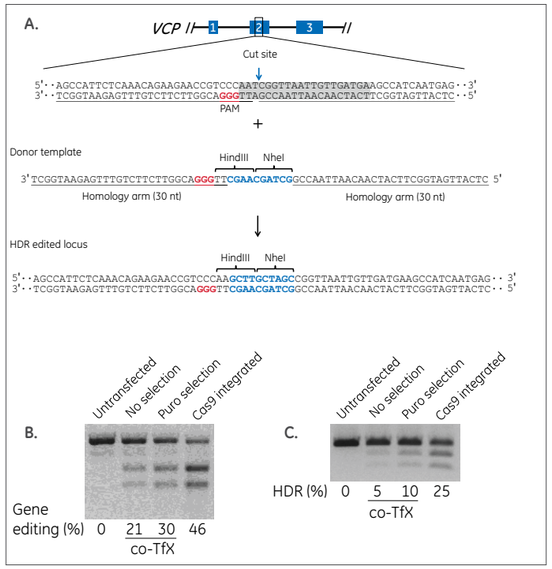

Figure 3. Gene editing at the *VCP* locus. Expressed Cas9 and synthetic crRNA:tracrRNA were used to target the *VCP* locus for HDR genome engineering in U2OS cells. **A.** Schematic of the *VCP* locus. Exons are indicated by the blue boxes. The guide sequence in the synthetic crRNA is indicated by the gray highlighted region, the PAM is indicated in red, and the cut site is indicated by the blue arrow. The inserted sequence is indicated in blue. **B.** Analysis of the efficiency of indel mutations using mismatch detection analysis of untransfected cells and transfected cells without (no selection) and with (puro selection) puromycin selection or using a Cas9-expressing cell line (Cas9 integrated). The percent editing is indicated below the gels. **C.** Analysis of the efficiency of HDR-based insertion of a HindIII and NheI restriction enzyme sites introduced using a 30 nucleotide homology arm DNA oligo. Percent HDR editing was calculated using an RFLP assay with NheI digestion of VCP PCR amplicons. co-Tfx = co-transfection.

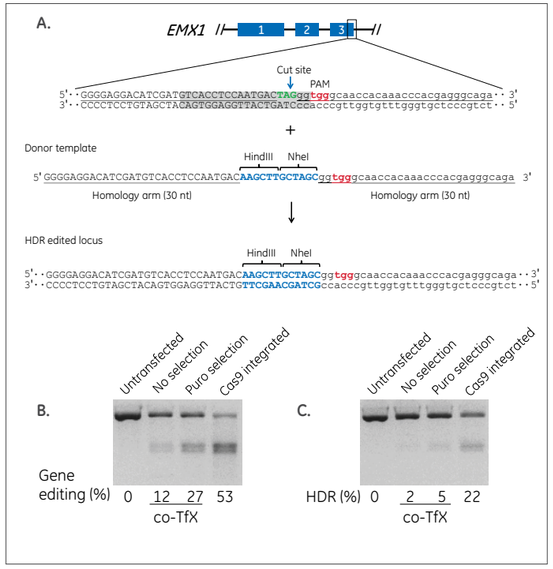

Figure 4. Gene editing at the *EMX1* locus. Expressed Cas9 and synthetic crRNA:tracrRNA were used to target the *EMX1* locus for HDR genome engineering in U2OS cells. **A.** Schematic of the *EMX1* locus. Exons are indicated by the blue boxes and uppercase lettering; introns are indicated by the lowercase lettering. The guide sequence in the crRNA is indicated by the gray highlighted region, the PAM is indicated in red, and the cut site is indicated by the blue arrow. The inserted sequence is indicated in blue, and the endogenous stop codon is green. **B.** Analysis of the efficiency of indel mutations using mismatch detection analysis of untransfected cells and transfected cells without (no selection) and with (puro selection) puromycin selection or using a Cas9-expressing cell line (Cas9 integrated). The percent editing is indicated below the gels. **C.** Analysis of the efficiency of HDR insertion of a HindIII and NheI restriction enzyme sites introduced using a 30 nucleotide homology arm DNA oligo. Percent HDR editing was calculated using an RFLP assay with NheI digestion of *EMX*1 PCR amplicons. co-Tfx = co-transfection.

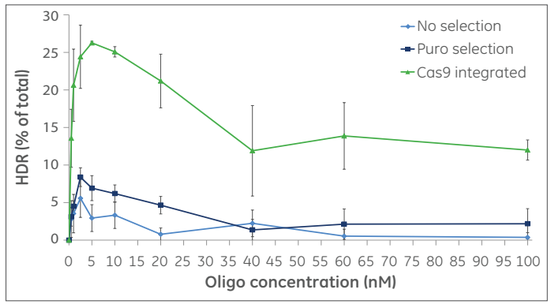

Figure 5. Donor DNA oligo concentration optimization. DharmaFECT Duo Transfection Reagent was used to co-transfect U2OS cells with a Cas9 expression plasmid, *EMX*1 crRNA:tracrRNA, and increasing concentrations of a donor DNA oligo with 30 nucleotide homology arms. Alternatively, a Cas9-integrated U2OS cell line was transfected with crRNA:tracrRNA to target *EMX*1, and increasing concentrations of a donor DNA oligo with 30 nucleotide homology arms. The RFLP assay was used to determine the amount of HDR knockin for each concentration of donor DNA oligo in each transfection. Data presented is from three independent transfections.

required (ranging from 2.5 nM to 10.0 nM) for maximal levels of HDR in U2OS, for both co-transfection of Cas9 plasmid plus synthetic crRNA:tracrRNA complex, and transfection of synthetic crRNA:tracrRNA complex into a Cas9-integrated cell line (Figure 5). Our data show that HDR is possible with donor homology arms as short as 20 nucleotides for each arm. However, the variability of the knockin is potentially greater at this arm length; therefore, we recommend each individual homology arm length to be at least 30 nucleotides flanking the desired insert.

When designing donor oligos for HDR, one must consider where the Cas9 DSB occurs with respect to the homology arms of the oligo donor. If the CRISPR protospacer and PAM sequence occurs in one of the homology arms of the oligo donor, silent mutations must be made at one of the two guanine bases of the NGG PAM and/or SNPs in the protospacer sequence proximal to the PAM to prevent Cas9 from cutting the donor oligo-repaired HDR site. Lastly, it has been found that SNP correction rates are as much as four-fold lower when the DSB occurs 100 bp away from the SNP;[4] thus, try to design your crRNA to create a Cas9-mediated DSB as close to the insertion site as possible for maximal HDR efficiency.[5]

Conclusion

The Edit-R CRISPR-Cas9 genome engineering platform simplifies the experimental workflow by removing tedious cloning steps prior to testing gene knockout, gene deletion and HDR. With careful optimization of editing efficiency in each desired cell line, precise insertion of short sequences using synthetic DNA oligo donors can be achieved utilizing the HDR cellular process.

Materials and Methods

Lipid transfection: U2OS and Ubi-GFP U2OS-Cas9 cells (Thermo Scientific™ BioImage™ Proteasome Redistribution Assay Cat #R0402102) were seeded in a 96-well plate at 10,000 cells per well. Edit-R synthetic crRNAs and tracrRNA were individually resuspended in 10 mM Tris-HCl (pH7.5), to 100 µM. crRNA and tracrRNA were combined and the RNA was further diluted to 2.5 µM using 10 mM Tris-HCl (pH7.5). A final concentration of 25 nM crRNA:tracrRNA complex (25 nM of each crRNA and tracrRNA) was used for transfection. Cells were transfected with 0.4 µL/well DharmaFECT Duo Transfection Reagent (Dharmacon Cat #T-2010-03) using 200 ng of Cas9 plasmid (for co-transfections) and 25 nM crRNA:tracrRNA complex. A final concentration range of 0.5 to 100 nM ssDNA donor oligo was used for transfections.

Puromycin selection: Twenty-four hours post-transfection, cells were exposed to 1.5 µg/mL of puromycin (Invivogen Cat #ant-pr-5). Cells surviving selection were harvested, analyzed, and are referred to as Puro selection.

ssDNA donor oligos: DNA oligos were synthesized in-house using standard solid-phase DNA synthesis and desalting procedures. Each DNA oligo used contained 2 phosphorothioate linkages on the first two and last two DNA bases. Oligos were ethanol precipitated and resuspended (100 µM) in 10 mM Tris-HCl (pH7.5). Oligos were further diluted to 1 µM prior to transfection.

Genomic DNA isolation and DNA mismatch detection assay: Genomic DNA was isolated 72 hours post-transfection by direct lysis of the cells in Phusion™ HF buffer (Thermo Scientific Cat #F-518L) proteinase K (Thermo Scientific Cat #EO0491) and RNase A (Thermo Scientific Cat #EN0531) for 1 hour at 56 °C followed by heat inactivation at 96 °C for 5 minutes. PCR was performed with primers flanking the cleavage sites. PCR products (500 ng) were treated with T7 endonuclease I (T7EI; NEB Cat #M0302L) for 25 minutes at 37 °C and the samples were separated on a 2% agarose gel. Percent editing in each sample was calculated using ImageJ software (NIH, imagej.nih.gov/ij, 1997-2014).

Restriction Fragment Length Polymorphism assay: Genomic isolation was performed as described above 72 hours post-transfection. PCR was performed with primers flanking the restriction enzyme knockin site and outside of the homology arms used for each donor DNA oligo. PCR products were further purified and eluted in water. PCR products (500 ng) were digested in FastDigest Buffer and 1 U of FastDigest NheI enzyme (Thermo Scientific Cat FD0974) for 30 minutes at 37 °C and the samples were separated on a 2% agarose gel. HDR knockin for each sample was calculated using ImageJ software (NIH, imagej.nih.gov/ij, 1997-2014).

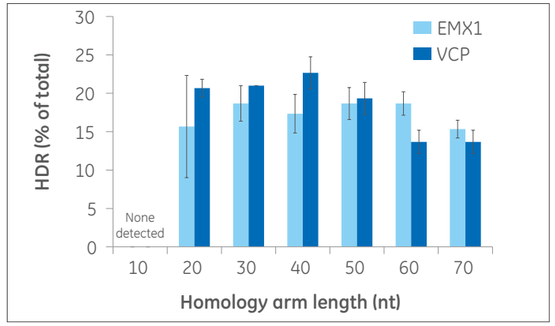

Figure 6. Optimizing homology arm length for maximal HDR knockin. DharmaFECT Duo Transfection Reagent was used to transfect a Cas9-integrated U2OS cell line with crRNA:tracrRNA targeting *EMX1* or *VCP* and a donor DNA oligo. Different donor DNA oligos were used, each with increasing homology arm length. A RFLP assay was performed on three independent transfections to determine HDR-based knockin for each donor DNA oligo with different homology arm lengths.

Table 1. Recommendations for HDR-based gene editing using Edit-R expressed Cas9 and synthetic crRNA:tracrRNA in 96-well cell culture dishes

Cells seeded at a density that gives 70-90% confluency on transfection day » Stable cell lines can be created by transduction using Edit-R Cas9 Lenti-viral Particles
DharmaFECT Duo Transfection Reagent (optimized concentration for each cell line)
25 nM/well Edit-R synthetic crRNA:tracrRNA
Oligo concentration: » U2OS cells (co-transfection): 2.5-10 nM/well donor DNA oligo » U2OS-Cas9 cells (integrated): 2.5-10 nM/well donor DNA oligo
30 nucleotide homology arms for small insertions (< 50 nucleotides)

Note: Conditions were optimized in U2OS cell lines. Conditions may need optimization in your cells of interest.

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Microinjection of zebrafish embryos using Dharmacon™ Edit-R™ Cas9 Nuclease mRNA, synthetic crRNA, and tracrRNA for genome engineering

Alex J. Blasky,* Amanda Haas, John A. Schiel, Rytis Prekeris,* Melissa L. Kelley, Dharmacon, now part of GE Healthcare, Lafayette, CO, USA *University of Colorado - Anschutz Medical Campus, Department of Cell & Developmental Biology, Denver, CO, USA

Abstract

The rapid advances being made in gene editing utilizing the CRISPR (clustered regularly interspaced short palindromic repeats)-Cas9 (CRISPR-associated protein 9) system have led to increasing interest in the application of this technology in zebrafish embryos for gene knockout. Using the Dharmacon™ Edit-R CRISPR-Cas9 DNA-free genome engineering platform, zebrafish embryos were injected with Cas9 Nuclease mRNA, synthetic tracrRNA and synthetic crRNA designed to target GFP in a zebrafish stable transgenic line. A mismatch detection assay was used to estimate gene editing efficiency, and successful functional protein knockout was confirmed by loss of GFP fluorescence.

Keywords

CRISPR-Cas9, gene editing, genome engineering, Cas9 mRNA, Edit-R, Cas9, direct injection, microinjection, zebrafish embryos, zebrafish

Introduction

The zebrafish (*Danio rerio*) is quickly becoming a preferred model system for biomedical research due to its high degree of sequence and functional homology with humans and relative ease of use in the laboratory. The zebrafish model is a genetically tractable system that undergoes a rapid external development, and therefore was initially utilized to study early developmental events. Currently, zebrafish research has expanded to a wide variety of basic science and clinical research settings, including modeling human genetic disease.[1] Zebrafish as a model for human disease is enhanced by the considerable genomic resources that exist, including a database of characterized mutant lines (zfin.org), and a complete and annotated genome.

Zebrafish are a relatively low cost research model, are highly amenable to genetic manipulation, and the molecular tools necessary to perform the targeted genomic editing are already available. As a result, zebrafish are an increasingly popular choice for target genomic modification using the CRISPR-Cas9 gene editing system.

Here we demonstrate DNA-free gene knockout of GFP in a stable transgenic zebrafish line by microinjection of the three components of the CRISPR-Cas9 system: Cas9 Nuclease mRNA, synthetic crRNA and synthetic tracrRNA.

Results

To determine the efficacy of the Edit-R CRISPR-Cas9 DNA-free genome engineering platform in zebrafish, approximately 100 single-cell stage embryos were microinjected with Cas9 mRNA and synthetic crRNA:tracrRNA, and approximately 50 control embryos were injected with Cas9 mRNA only. Survival was assessed at 1 and 2 days post-fertilization (dpf). Injected embryos had a high survival rate (WT = 97%, n=100; Control Injected = 96%, n=52; CRISPR Injected = 93%, n=94). At 2 dpf genomic DNA was extracted from eight of the embryos and analyzed for gene editing efficiency using a mismatch detection assay. Six of the eight embryos microinjected with all three components of the CRISPR-Cas9 system show estimated gene editing efficiency of 9-20% (Figure 1). Detecting functional knockout of GFP was performed by imaging the zebrafish microinjected with Cas9 mRNA and crRNA:tracrRNA and comparing them to the embryos that were not injected. Figure 2 shows GFP and RFP fluorescence of neural crest cells. The zebrafish embryo that was not injected shows expression of both GFP and RFP in transgenic cells (Figure 2A). Following microinjection of CRISPR-Cas9 components targeting GFP, a loss or decrease in GFP fluorescence is observed in the zebrafish embryo, confirming successful gene knockout.

Discussion

Zebrafish are an excellent model system for studying genetic manipulations and relating these experimental results to human diseases. Here we demonstrated the utility of using a completely DNA-free gene editing system for *in vivo* microinjection of zebrafish embryos to knock out GFP in a stable transgenic line. Neural crest cells in the Tg(sox10:tagRFP; sox10:PH-GFP) transgenic zebrafish line express both RFP and GFP. Using the Edit-R CRISPR-Cas9 genome engineering platform with Cas9 mRNA and synthetic crRNA:tracrRNA, GFP was targeted for gene editing and functional protein knockout. A mismatch detection assay showed that 75% of the microinjected zebrafish embryos resulted in efficient gene editing (Figure 1). Importantly, since 75% of analyzed embryos that were injected showed gene editing, it is likely that the germ cell [one of thousands of cells at 24 hours post-fertilization (hpf)] has been mutated and can be used for generation of a stable transgenic line for further scientific interrogation. Additionally, we confirmed GFP knockout *in vivo* using fluorescent microscopy. Analysis of neural crest cells 24 hpf revealed highly mosaic expression of GFP in embryos microinjected with gene editing components (Figure 2). The successful gene editing demonstrated here with DNA-free CRISPR-Cas9 components shows one of the many new possibilities using CRISPR-Cas9 gene editing in model systems to find correlations to human diseases.

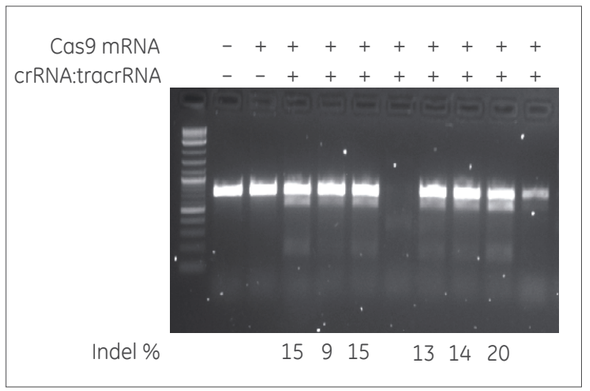

Figure 1. Zebrafish embryos microinjected with Edit-R Cas9 Nuclease mRNA and synthetic crRNA:tracrRNA have detectable editing events. Zebrafish embryos were microinjected with Edit-R Cas9 mRNA only (+/- lane) or with Edit-R Cas9 mRNA plus crRNA:tracrRNA targeting GFP (+ lanes). Genomic DNA was prepared 2 days post-injection and PCR was performed with primers flanking the cleavage sites. A DNA mismatch assay with T7EI was performed and the samples were separated on a 2% agarose gel. Percent of insertions and deletions due to gene editing (Indel %) was estimated using ImageJ software and is shown at the bottom of the lanes. Targeted DNA cleavage using Cas9 mRNA programmed with crRNA:tracrRNA targeting GFP was achieved in 75% of the zebrafish embryos analyzed.

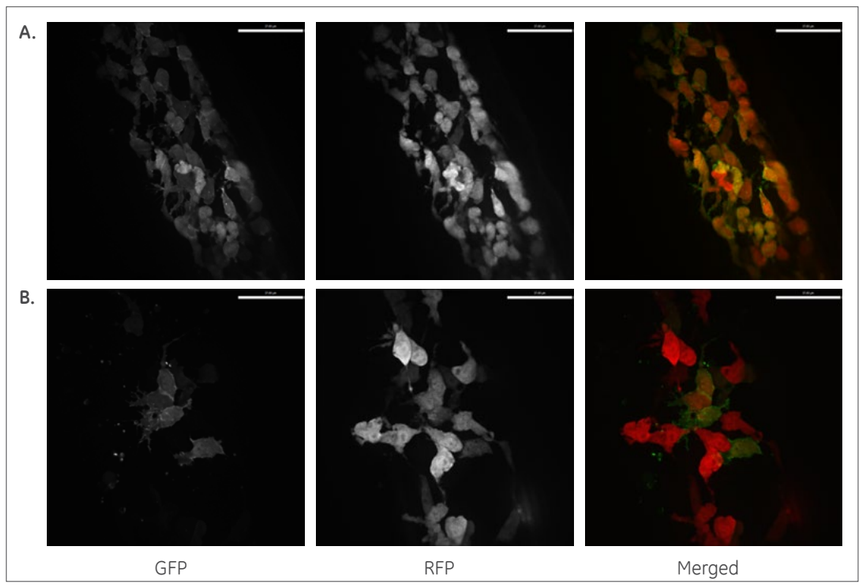

Figure 2. GFP knockout *in vivo* using Edit-R CRISPR-Cas9 DNA-free system. Dorsal view of Tg(Sox10:(PH)GFP; Sox10:tagRFP) zebrafish neural tube at 24 hpf. Images were collected on a Zeiss Axio Observer microscope equipped with a PerkinElmer UltraVIEW VoX spinning disk confocal system and Volocity imaging software (PerkinElmer) **A.** Neural crest cells expressing both GFP and RFP in zebrafish embryos injected with Cas9 mRNA only. **B.** Neural crest cells in embryos injected with Cas9 and crRNA:tracrRNA targeting transgenic gfp display mosaic GFP expression as a result of functional protein knockout. (37 µM bar for reference)

Application Note

Materials and methods

Materials required:

- Edit-R Cas9 Nuclease mRNA (Cat #CAS11195)
- Edit-R tracrRNA (Cat #U-002000-xx)
- Edit-R synthetic crRNA targeting GFP (Dharmacon custom synthesis; previously published sequence[2])
- Microinjection setup (Microinjector, injection needles, injection trays)
- Single-cell zebrafish embryos, stable transgenic line Tg(sox10:tagRFP; sox10:PH-GFP)

Zebrafish microinjection: Prior to injections, a mixture containing the GFP crRNA (25 pg), tracrRNA (100 pg) and Cas9 mRNA (100 pg) was prepared on ice (see below). The injection mixture remained on ice until loading into the microinjection needle to improve mRNA stability. At the single-cell stage, cells were injected with 1-2 nL of the injection mix into the stable transgenic line Tg(sox10:tagRFP; sox10:PH-GFP), which express both red and green fluorescent proteins under the Sox10 promoter.

Reagent	Volume	Final Concentration
KCl (2 M)	1.0 µL	0.2 M
Phenol Red (Sigma Cat #P0290)	1.0 µL	N/A
tracRNA (1.0 µg/µL)	1.0 µL	100 pg/nL
crRNA (GFP) (0.25 µg/µL)	1.0 µL	25 pg/nL
Cas9 mRNA (0.2 µg/µL)	5.0 µL	100 pg/nL
Water, nuclease free (Cat # B-003000-WB-100)	1.0 µL	N/A
Total Volume	**10.0 µL**	

Mismatch detection assay using T7 Endonuclease I: Genomic DNA was prepared from un-injected and injected embryos at 2 days post-fertilization (dpf) following a previously established method (https://zfin.org/zf_info/zfbook/chapt9/9.3.html). We PCR amplified a short genomic region (~ 700 bp) flanking the target site from the genomic DNA using Phusion® High-Fidelity PCR Master Mix with HF Buffer (NEB Cat# M0531S) and GFP-specific primers (FWD: ATGGTGAGCAAGGGCGAGGAG and REV: CATGCCGAGAGTGATCCCGGC). Thermal cycling conditions were as follows:

Cycle steps	Temperature	Time	Cycle(s)
Initial denaturation	98 °C	1:00 min	1
Denaturation	98 °C	0:10 sec	34
Touchdown annealing	65°C - 1 °C/cycle	0:10 sec	
Extension	72 °C	0:30 sec	
Final extension	72 °C	5:00 min	1

The PCR amplicons were purified using DNA Clean & Concentrator™-5 (Zymo Research Cat #D4003). To estimate the mutation rate for the GFP target site, a total of 200 ng of the purified PCR amplicon was denatured and slowly reannealed to facilitate heteroduplex formation. The reannealing procedure consisted of a 5 minute denaturing step at 95 °C, followed by cooling to 85 °C at -2 °C/second and further to 25 °C at -0.1 °C/second. The reannealed amplicon was then digested with 10 units of T7 Endonuclease I (T7EI; NEB Cat #M0302S) at 37 °C for 15 minute. The reaction was stopped by adding 1 µL of 0.5 M EDTA. The entire sample was resolved by electrophoresis through a 2% agarose gel and visualized by ethidium bromide staining. The band intensity was quantified using ImageJ software (National Institutes of Health, Bethesda, MD) for estimation of gene editing efficiency.

References

1. G.J. Lieschke and P.D. Currie. Animal models of human disease: zebrafish swim into view. *Nat. Rev. Genet.* **8**, 353–367 (2007).
2. L.-E. Jao, S. R. Wente and W. Chen. Efficient multiplex biallelic zebrafish genome editing using a CRISPR nuclease system. *Proc. Natl. Acad. Sci. U.S.A.* **110**, 13904-13909 (2013).

Optimization of reverse transfection of Dharmacon™ Edit-R™ synthetic crRNA and tracrRNA components with DharmaFECT™ transfection reagent in a Cas9-expressing cell line

Elena Maksimova, Žaklina Strezoska, Melissa L. Kelley and Anja van Brabant Smith, GE Healthcare, Lafayette, CO, USA

Introduction

Dharmacon™ Edit-R™ predesigned crRNA libraries enable rapid, high-throughput analysis of hundreds of genes with multiple target sites per gene. An arrayed library format permits single-well phenotypic analysis, including high-content assays and other morphological or reporter assays. Identification of relevant hits and successful screening outcomes from these types of assays depend on high transfection efficiency.

To obtain the highest transfection efficiency of the Edit-R synthetic crRNA:tracrRNA components with minimal negative effects on cell viability, it is recommended to carefully optimize transfection conditions for each cell line using positive control crRNAs. A reverse transfection method can be used to increase the throughput and reproducibility of a screen, and to more easily implement automated systems to carry out transfections.

Here we present an example of transfection optimization in reverse transfection format using a recombinant U2OS reporter cell line stably expressing Cas9 nuclease under the CAG promotor (Ubi[G76V]-EGFP-Cas9 cells). An un-cleavable ubiquitin moiety (Gly76Val) fused to EGFP allows constitutive degradation of the EGFP protein (and low basal fluorescence), while disruption of the proteasome components by functional protein knockout leads to accumulation of EGFP and detectable fluorescence.

Reverse transfection optimization

A full protocol for reverse transfection of synthetic crRNA is available here (http://dharmacon.gelifesciences.com/uploadedFiles/Resources/edit-r-reverse-transfect-array-crrna-plates-protocol.pdf). A transfection optimization experiment should include two to three cell densities and a range of DharmaFECT™ transfection reagent volumes. In this example, U2OS Ubi[G76V]-EGFP-Cas9 cells were trypsinized, then diluted to 5000, 10000 or 20000 cells per 80 µL in growth medium. Edit-R PPIB synthetic crRNA Control (Cat #UK-007050-05) was used as a positive control for gene editing and Edit-R crRNA targeting a proteasome gene *PSMD7* was used as a positive control for the phenotypic assay. The crRNAs were co-transfected with an equimolar amount of synthetic tracrRNA at a final concentration of 25 nM, using a range of DharmaFECT 4 transfection reagent from 0.02-0.3 µL/well. After incubation of the crRNA:tracrRNA with the transfection regent for 20 minutes at room temperature, the transfection mixture was transferred to triplicate wells of tissue culture plates and the suspended cells were added on top of the transfection complex. Transfected cells were incubated in a 37 ºC, 5% CO_2 incubator for 72 hours. A resazurin viability assay and a DNA mismatch assay using T7EI endonuclease were performed 72 hours post-transfection, and EGFP fluorescence was read on the Envision plate reader (Perkin Elmer) for the *PSMD7* crRNA phenotypic assay control.

Results

Transfection conditions previously determined for delivery of siRNAs in a cell line of interest can be used as a starting point for transfection optimization with Edit-R crRNA and tracrRNA. Here we used DharmaFECT 4 transfection reagent that was previously determined to provide the highest siRNA transfection efficiency for these U2OS cells. A range of DharmaFECT 4 transfection reagent concentrations was assessed to deliver synthetic crRNA:tracrRNA targeting *PPIB* for the highest gene editing efficiency and lowest impact on cell viability (Figure 1). The optimal transfection reagent concentration depended on cell density (0.02, 0.04 or 0.12 µL/well of DharmaFECT 4 for 5000, 10000 or 20000 cells per well, respectively), leading to high gene editing efficiency and little effect on cell viability.

A range of cell densities and DharmaFECT 4 transfection reagent concentrations were also assessed with synthetic crRNA:tracrRNA targeting *PSMD7* for gene knockout using the EGFP fluorescence intensity and cell viability (Figure 2). As expected, a strong correlation was observed between the optimal conditions for *PSMD7* and *PPIB* crRNAs. The use of 20000 cell per well in 96-well format with 0.12 µL/well of DharmaFECT 4 transfection reagent resulted in the most robust conditions with high editing efficiency (> 6 fold increase of EGFP fluorescence compared to untreated cells) as well as excellent cell viability (> 80%).

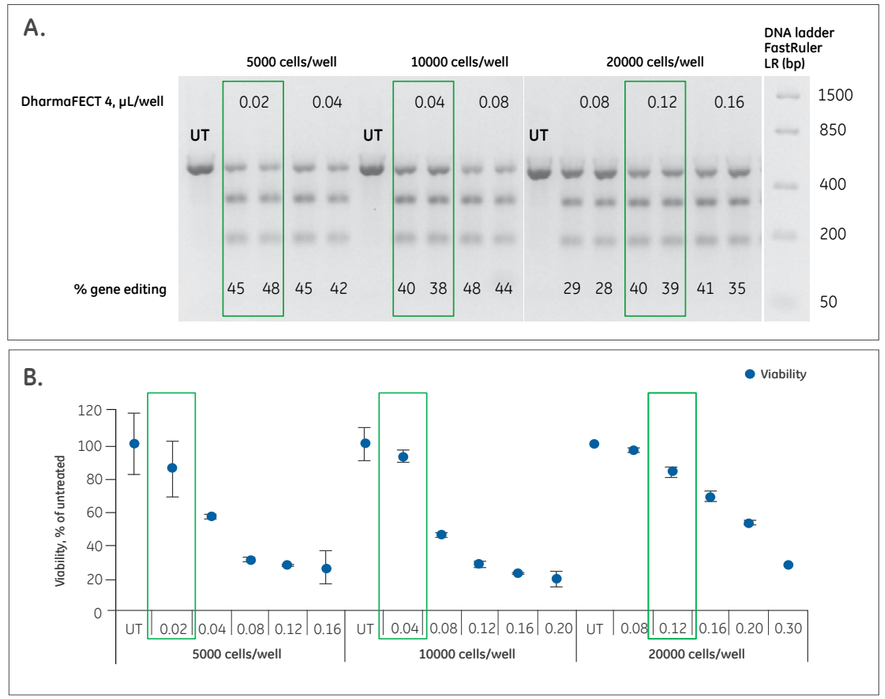

Figure 1. Identification of optimal reverse transfection conditions using PPIB crRNA positive control.
A. *PPIB* gene editing efficiency assessed with DNA mismatch assay using T7EI 72 hours post-transfection (duplicate wells are shown). The amount of transfection reagent required for successful gene editing varies with cell density. FastRuler™ Low Range DNA Ladder (Thermo Scientific, Cat #SM1103). **B.** Cell viability measured with resazurin assay 72 hours post-transfection. Optimal amount of transfection reagent per well will result in cell viability of 80% or higher. Best conditions based on gene editing efficiency and viability are boxed in green. UT = untreated control, 0.02, 0.04, 0.08, 0.12, 0.16, 0.20, 0.30 = µL/well of DharmaFECT 4 transfection reagent.

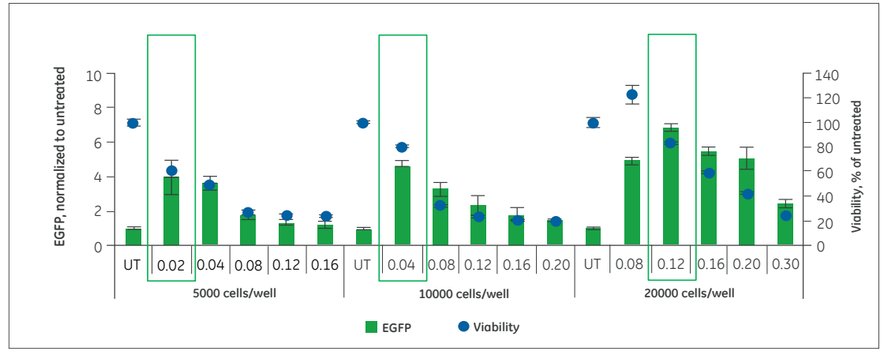

Figure 2. Phenotypic EGFP readout of PSMD7 functional gene knockout. For all cell densities, the optimal transfection conditions determined with *PPIB* crRNA (Figure 1) correlate with the best functional gene knockout, as indicated by EGFP readout. The best conditions for each cell density based on functional gene knockout efficiency and viability are boxed in green. UT = untreated control, 0.02, 0.04, 0.08, 0.12, 0.16, 0.20, 0.30 = µL/well of DharmaFECT 4 transfection reagent.

Conclusion

Arrayed crRNA libraries allow for functional genetic screens using a wide range of phenotypic readouts, including high-content imaging, therefore further facilitating the discovery of genes with roles in different biological processes.

A reverse transfection method for delivery of arrayed synthetic crRNA:tracrRNA libraries allows for functional gene knockout screening in an automated, high throughput manner. Determining the optimal transfection conditions to obtain the highest editing efficiency with minimal effects on cell viability is of utmost importance for the success of the screen.

Transfection conditions need to be optimized for every cell line keeping in mind the assay time-point and phenotypic assay requirements, as different cell densities and growth characteristics affect the transfection efficiency. Transfection conditions that induce changes in cell viability and/or provide insufficient delivery of the targeting agent can mislead researchers toward false interpretations of data, which in the long run, are time consuming and costly.

Application Note

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Technical Support: ts.dharmacon.eu@ge.com
0800 515 313