Specific, functional and scalable

Dharmacon™ Edit-R™ CRISPR-Cas9 Gene Editing Products
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Choose the right tools for your application

Whether your 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.

Choose the right Edit-R® workflow guide will assist you in selecting the right Edit-R® genome engineering tools for your application.

**Gene knockout**

- Single gene knockout
- Loss-of-function screening of multiple genes at once

**Gene knockin**

- Precise insertion or alteration of a gene
- Experimental gDNA

**Optimize Cas9**

- Assess gene expression
- Directed repair (HDR)

**Assess gene delivery and expression**

- Choose a Cas9 reagent
- Choose guide RNA
- Optimize Cas9 delivery and expression

**And guide RNA delivery**

**Assess gene editing efficiency and functional knockout phenotype**

**Transduce lentiviral sgRNA pools**

- Transfect or electroporate
- Design and express
- After 3 days
- Isolate gDNA

**Choose guide RNA**

- Perform pooled screen experiment
- Analyze enriched gRNA constructs in Reference vs. Experimental sample

**Reference gDNA**

**Experimental gDNA**

**Reference sample**

**Experimental sample**

**Perform arrayed screen experiment**

**Isolate gDNA**

**Assess loss-of-function phenotype**

**Characterize clonal cell line**

**Gene knockin**

- Precise insertion or alteration of a gene
- Experimental gDNA

**Optimize Cas9**

- Assess HDR
- Delivery of appropriate Cas9 reagent

**Assess HDR efficiency**

- Analyze arrayed samples
- Select for HDR

**Characterize clonal cell line**

**Gene knockin**

- Precise insertion or alteration of a gene
- Experimental gDNA

**Optimize Cas9**

- Assess HDR
- Delivery of appropriate Cas9 reagent

**Assess HDR efficiency**

- Analyze arrayed samples
- Select for HDR

**Characterize clonal cell line**
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) system in bacteria and archaea is an adaptive and archaean 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) that include sequences complementary to the foreign, invading DNA. crRNAs guide a multifunctional 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.

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: (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). 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. 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.

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: direct cloning of 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 (DSB). 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 knock out, 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. A similar approach has been used to genetically modify primates single cell embryos and zebrafish. 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 knockout, rather than a targeted gene knockout. The precise insertion of a donor template using HDR may be isolated to create a knockin, rather than a target gene knockout. Depending on the desired modification to a locus, there are many parameters which require optimization:

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

Guide RNA
Lentiviral and synthetic reagents for targeted gene knockout

Which guide RNA is best for you?

Do you require lentiviral delivery of your guide RNA?
- Yes
  - Lentiviral sgRNA
- No
  - Synthetic crRNA

Would you like your sgRNA predesigned with an algorithm for improved functionality?
- Yes
  - CRISPR Configurator
  - Edit-R predesigned sgRNA
- No
  - CRISPR Configurator
  - Edit-R predesigned crRNA

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: Predesigned 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.

Test all possible crRNA designs across known positive genes

Over 1100 crRNA across 10 genes

Assess & quantify functionality

EGFP readout intensity in a reporter cell line

Analyze characteristics of good vs. poor designs

Look for patterns, motifs, base preference, etc.

Build and test a set of general criteria

Where selected designs in different assay systems

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 (Lm0764)-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 0.375 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).

Assessment of functionality (y-axis) of synthetic crRNA along the length of the coding sequence (x-axis) of the 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.
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 5'-pygones 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. Stabilizing modifications improve DNA-free editing.

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
• Chemically modified for improved stability

Functional data
Required components for an Edit-R CRISPR-Cas9 gene editing experiment using synthetic crRNA:
• Chemically modified for improved stability
• Transfection of crRNA:tracrRNA into a Cas9-expressing cell line enables rapid, high-throughput analysis of multiple target sites per gene
• Functional data

Rapid gene knockout results with Edit-R synthetic crRNA

Order Cas9 nuclease, crRNA, and tracrRNA
Co-transfect Cas9 nuclease and crRNA:tracrRNA into cells
Observe gene editing event

1-2 weeks

Gene editing and phenotypic analyses

Edit-R™ Predesigned crRNA Table

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</table>

*These are agnostic product identifiers. Actual catalog numbers are gene and species-specific (e.g., CM-011800-04).

Gene knockoworkflow 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.

Gene editing and phenotypic analyses

BCL2L1
PLK1
WEE1

Gene knockoworkflow 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.

Gene editing and phenotypic analyses

BCL2L1
PLK1
WEE1

crRNAs with high functional scores from the Edit-R algorithm show stronger phenotypes in Apg0NE assay than low-scoring designs. For the box plot, the crRNAs were divided into bottom half (H2) and top half (H1) 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 O.I µl/well of DharmaFECT 4 Transfection Reagent. Cells were analyzed for apoptosis 48 h after transfection using the Apg0NE homogeneous assay (Promega).
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 minimal 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

Positive 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)

Availability

Edit-R Synthetic Positive crRNA Controls

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<tr>
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<th>Mouse</th>
<th>Rat</th>
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Edit-R Synthetic crRNA Control Kits

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Edit-R Non-targeting Controls

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<tr>
<td>Kit #2</td>
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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 (UB3276V5) EGFPl (A) and a mouse fibroblast NIH/3T3-Bliss (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-Bliss® 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.

Edit-R Synthetic Positive crRNA Control Kit

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Edit-R Synthetic crRNA Control Kit

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Edit-R Synthetic Negative crRNA Control Kit

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Edit-R Synthetic crRNA Non-targeting Controls

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Edit-R™ Synthetic crRNA Controls
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 (PuroR) 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 the mouse CMV promoter and allows for rapid selection of cells with integrated sgRNA.

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 supematant 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 reporter cell line (6×3G7/EFGp) and H1299/6 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 blasticidin-selected cells was estimated from a DNA in-silico detection assay using T7 Endonuclease I.

**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.

**Availability**

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**Experimental workflow:**

1. **Transduction:** Cas9-expressing target cells
2. **Selection with puromycin:** Blasticidin-resistant puromycin-selected cell population expansion
3. **Clonal cell isolation:** Gene knockout expanded clonal cells
4. **Gene editing and phenotypic analyses:** Gene knockout expanded clonal cells

**Mixed population workflow:**

1. **Transduction:** sgRNA
2. **Selection with puromycin:** Blasticidin-resistant puromycin-selected expanded population
3. **Gene knockout:** Mixed population
4. **Gene knockout clonal cells:** Gene knockout expanded clonal cells

**Clonal cell workflow:**

1. **Transduction:** sgRNA
2. **Selection with puromycin:** Blasticidin-resistant puromycin-selected expanded population
3. **Clonal cell isolation:** Gene knockout expanded clonal cells
**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**

Positive 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 lentiviral 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**

<table>
<thead>
<tr>
<th>sgRNA</th>
<th>hCMV</th>
<th>mCMV</th>
<th>hEF1α</th>
<th>mEF1α</th>
<th>PGK</th>
<th>CAS</th>
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<tbody>
<tr>
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<td>15</td>
<td>34</td>
<td>9</td>
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<td>HEK293T</td>
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<td>21</td>
<td>12</td>
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<tr>
<td>HEK293T</td>
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<td>25</td>
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<td>32</td>
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</table>

**Availability**

**Edit-R Lentiviral sgRNA Positive Controls**

<table>
<thead>
<tr>
<th>Species</th>
<th>sgRNA</th>
<th>MOI</th>
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<tbody>
<tr>
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**Edit-R Lentiviral sgRNA Non-targeting Controls**

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**Edit-R Lentiviral sgRNA Positive Control Kits**

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<th>Product Code</th>
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<tr>
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<tr>
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<td>2 x 25 µL lentiviral particles</td>
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<td>PPIB</td>
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**Edit-R Lentiviral sgRNA Non-targeting Control Kits**

<table>
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**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 shows that the hCMV and CAS 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.
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. 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, and is modified for stability.

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 transfecatable with standard reagents and can be co-transfected with Cas9 mRNA, protein, or plasmid
• Chemically modified for improved stability

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 tracrRNA:crRNA to recruit Cas9 nuclease rely on excellent purity and sequence fidelity for optimal activity. Instrument used: Acquity UPLC by Waters.

Availability

<table>
<thead>
<tr>
<th>Size</th>
<th>Catalog No.</th>
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<tbody>
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<tr>
<td>20 nmol</td>
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<tr>
<td>50 nmol</td>
<td>U-00205-50</td>
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</tbody>
</table>

Sequence structure of a synthetic 99-mer sgRNA

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 sgRNA

Below is one example of how to design a sgRNA 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

S5’- NNNNNNNNNNNNNNNNNNGUUUUAGAGCUAGAAA UAGCGAUGUUAAGAAUCGGUAGCCUGUUAACACUGUAGAAAAGUGGCACCGAGUCGGUGCUU-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/ deactivation 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 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 nM). For each sample, DNA 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
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 cas9) 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.

Which Cas9 Nuclease is best for you?

Do you require lentiviral delivery and/or integrated expression of Cas9?

- Yes
  - Lentiviral Cas9

- No
  - Edit-R Cas9 Nuclease mRNA or protein

Do you want to package your own lentivirus?

- Yes
  - Edit-R Cas9 Lentiviral/Plasmid DNA

- No
  - Edit-R Cas9 Lentiviral particles

Do you need DNA-free Cas9 expression?

- Yes
  - Edit-R Cas9 Expression Plasmid

- No
  - Edit-R Cas9 Lentiviral particles

Table 1. Six SMARTchoice promoter options for expressing Cas9 nuclease

<table>
<thead>
<tr>
<th>Promoter</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>hCMV</td>
<td>human cytomegalovirus immediate early promoter</td>
</tr>
<tr>
<td>mCMV</td>
<td>mouse cytomegalovirus immediate early promoter</td>
</tr>
<tr>
<td>hEF1α</td>
<td>human elongation factor 1 alpha promoter</td>
</tr>
<tr>
<td>mEF1α</td>
<td>mouse elongation factor 1 alpha promoter</td>
</tr>
<tr>
<td>PGK</td>
<td>mouse phosphoglycerate kinase promoter</td>
</tr>
<tr>
<td>CAG</td>
<td>chicken beta actin hybrid promoter</td>
</tr>
</tbody>
</table>

Cas9 Nuclease

Configurable expression constructs or DNA-free options for optimal Cas9 expression
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™**: 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™**: Cas9 nuclease expression is driven from a human cytomegalovirus (hCMV) promoter, and blasticidin resistance (Blast™) 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 Edit-R Cas9-Puro Expression Plasmid**
The Edit-R Cas9-Puro plasmid expresses the puromycin-resistance selection marker and the human codon-optimized Cas9 nucleic acid 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.

Availability

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<tr>
<td>CAG</td>
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<td>U-004500-120</td>
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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 Nelfx XQ® 100 instrument into three bins corresponding to negative, low, and high mKate2 fluorescence. SURVEYOR™ DNA mismatch assay was performed on selected 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 both (PuroR plasmid). A DNA mismatch detection assay can be used to estimate gene editing efficiency prior to clonal cell line generation and characterization.
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 × 10⁷ TU/mL functional titer, by qPCR titration
- 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**

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- Provided as concentrated, purified lentiviral particles for immediate transduction; 50 (2 x 25) µL, within 10% of minimum ≥ 1 × 10⁷ TU/mL functional titer, by qPCR titration
- 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

**Gene editing and phenotypic analyses**

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.

**Availability**

Edit-R Lentiviral Blast-Cas9 Nuclease Particles
- NCMV 10 µg CAS10124
- mCMV 10 µg CAS10125
- EF1α 10 µg CAS10126
- mEF1α 10 µg CAS10127
- FGK 10 µg CAS10128
- hEF1α 10 µg CAS10129
- CAS 10 µg CAS10126
- Inducible 50 µL, 10⁷ TU/mL VCAS11227

Edit-R Lentiviral Blast-Cas9 Nuclease Plasmid DNA
- NCMV 10 µg CAS10124
- mCMV 10 µg CAS10125
- EF1α 10 µg CAS10126
- mEF1α 10 µg CAS10127
- FGK 10 µg CAS10128
- hEF1α 10 µg CAS10129
- CAS 10 µg CAS10126
- Inducible 10 µg Cas11229

**Vector Element**

- Cas9
- mCMV
- EF1α
- mEF1α
- PKG
- CAG
- S'LTR
- RRE
- WPRE
- S'LTR
- SV40pA
- pC3 ori
- SV40 ori
- Amp*  

**Utility**

- 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
- BlastR
- Blasticidin resistance marker permits antibiotic selection of transduced mammalian cells
- HCMV
- Human cytomegalovirus immediate early promoter
- mCMV
- Mouse cytomegalovirus immediate early promoter
- NEF1α
- Mouse elongation factor 1 alpha promoter
- mEF1α
- Mouse elongation factor 1 alpha promoter
- PKG
- Mouse phosphoglycerate kinase promoter
- CAG
- Human cytomegalovirus, chicken β-actin hybrid promoter
- S'LTR
- 5' Long Terminal Repeat necessary for lentiviral particle production and integration of the construct into the host cell genome. Its 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
- S'LTR
- Self-inactivating Long Terminal Repeat for generation of replication-incompetent lentiviral particles
- SV40pA
- Simian virus 40 polyadenylation signal
- pC3 ori
- pC3 origin of replication
- SV40 ori
- Simian virus 40 origin of replication
- Amp*
- Ampicillin resistance gene for vector propagation in E. coli cultures

**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 (U2OS-TU(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-BlastR was selected with blasticidin treatment for a minimum of 10 days before transfections. Cells were transduced with 50 nM Edit-R synthetic crRNA:tracrRNA complex targeting U26 and the relative frequency of gene editing was estimated (lower panel) based on a DNA mismatch detection assay with T7 Endonuclease I.
**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 incompatibilities 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. K562 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 12 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](http://dharmacon.gelifesciences.com/uploadedFiles/Resources/edit-r-experimental-workflow-appnote.pdf)

**Availability**

<table>
<thead>
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<th>Product</th>
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<tr>
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<td>1000 pmol</td>
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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.

<table>
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<th>Synthetic crRNA libraries</th>
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<td>Synthetic crRNA arrayed in multi-well plates</td>
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<td>Lentiviral transduction</td>
<td>Standard transfection reagents or electroporation</td>
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<tr>
<td><strong>Assay types supported</strong></td>
<td>Phenotypes can be investigated that result in changes in sgRNA abundance in the cell population and can be assessed by next generation sequencing.</td>
<td>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.</td>
</tr>
<tr>
<td></td>
<td>Growth phenotypes (proliferation or survival)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Selectable by cell sorting (fluorescence or cell surface marker expression)</td>
<td></td>
</tr>
<tr>
<td><strong>Screening hands-on time</strong></td>
<td>Relatively low; can be carried out in a single culture dish</td>
<td>Scales up with the number of genes</td>
</tr>
<tr>
<td><strong>Data analysis requirements</strong></td>
<td>Next generation sequencing required for identification of sgRNA(s) and its abundance in the cell population</td>
<td>Phenotype is analyzed directly on a one-gene-per-well basis</td>
</tr>
</tbody>
</table>

---

**CRISPR-Cas9 Screening Libraries**

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

---

Which CRISPR-Cas9 screening library is right for you?

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 pre-designed 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
• Catalog libraries provide four unique crRNA designs per gene, providing multiple data points for stratification of results
• 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. EGFP-Cas9 stable cells seeded at 5,000 cells/well in 96-well format were transfection 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 hours 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.

Knockout of essential genes by synthetic crRNA:tracrRNA results in apoptosis. U2OS-(Ub)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.

Availability

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<tr>
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<tr>
<td>Cell Cycle Regulation</td>
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<td>GC-003200-05</td>
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<tr>
<td>Cytokine Receptors</td>
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<td>GC-004000-05</td>
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<tr>
<td>Deubiquitinating Enzymes</td>
<td>0.5</td>
<td>GC-003700-05</td>
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<td>DNA Damage Response</td>
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<td>GC-003300-05</td>
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<tr>
<td>Epigenetics</td>
<td>0.5</td>
<td>GC-005000-05</td>
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<tr>
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Mouse Edit-R crRNA Library Set of 4 - 96 well

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<td>Cytokine Receptors</td>
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<td>DNA Damage Response</td>
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<td>Proteases</td>
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<td>GC-003900-05</td>
</tr>
<tr>
<td>Tumor suppressors</td>
<td>0.5</td>
<td>GC-005100-05</td>
</tr>
</tbody>
</table>

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.
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.

- Can be combined with the promoter flexibility of Edit-R Lentiviral Cas9 Nuclease Reagents for robust editing in biologically relevant cell types
- Libraries and custom Edit-R Lentiviral sgRNA collections are available upon request.
- 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.

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 pooled 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 1.6- 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 for U2OS cells from which genomic DNA was isolated at 24 hours post-transduction (T0), PCR-amplified with two biological replicates. At 48 hours post-transduction, the reference (T0) cell populations were harvested and subjected to 2 µg/mL puromycin treatment (selective pressure). At eight days post-selection, T1 cell populations were harvested. 

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, sequences. The resulting amplicons are sequenced on Illumina platform sequencers and/or biochemical assays.

Availability

- Human Edit-R Lentiviral sgRNA Pooled Library
  - Apoptosis: VSGH11125
  - Calcium Cytoskeleton: VSGH11122
  - Cell Cycle Regulation: VSGH11123
  - Cytokine Receptors: VSGH11117
  - Deubiquitinating Enzymes: VSGH11133
  - DNA Damage Response: VSGH11114
  - Drug resistance: VSGH11113
  - Epigenetics: VSGH11112
  - G Protein-Coupled Receptors: VSGH11106
  - Ion Channels: VSGH11108
  - MAP Kinases: VSGH11118
  - MPRs: VSGH11119
  - Membrane Trafficking: VSGH11119
  - Nuclear Receptors: VSGH11147
  - Phosphatases: VSGH11129
  - Proteins: VSGH11131
  - Protein Kinases: VSGH11132
  - Proteases: VSGH11133
  - Serine Proteases: VSGH11134
  - STE Kinases: VSGH11135
  - Tyrosine Kinases: VSGH11136
  - Ubiquitin Conjugation: VSGH11137
  - Whole Genome: VSGH11138

- Mouse Edit-R Lentiviral sgRNA Pooled Library
  - Apoptosis: VSGM11151
  - Calcium Cytoskeleton: VSGM11144
  - Cell Cycle Regulation: VSGM11145
  - Cytokine Receptors: VSGM11139
  - Deubiquitinating Enzymes: VSGM11144
  - DNA Damage Response: VSGM11136
  - Drug resistance: VSGM11114
  - Epigenetics: VSGM11137
  - G Protein-Coupled Receptors: VSGM11132
  - Ion Channels: VSGM11130
  - Membrane Trafficking: VSGM11141
  - Nuclear Receptors: VSGM11147
  - Phosphatases: VSGM11129
  - Proteins: VSGM11131
  - Protein Kinases: VSGM11127
  - Serine Proteases: VSGM11142
  - STE Kinases: VSGM11138
  - Tyrosine Kinases: VSGM11146
  - Ubiquitin Conjugation: VSGM11132
  - Whole Genome: VSGM11133

- Rat Edit-R Lentiviral sgRNA Pooled Library
  - Apoptosis: VSRG11130
  - Calcium Cytoskeleton: VSRG11106
  - Cell Cycle Regulation: VSRG11107
  - Cytokine Receptors: VSRG11161
  - Deubiquitinating Enzymes: VSRG11133
  - DNA Damage Response: VSRG11136
  - Drug resistance: VSRG11113
  - G Protein-Coupled Receptors: VSRG11132
  - Ion Channels: VSRG11131
  - Membrane Trafficking: VSRG11133
  - Nuclear Receptors: VSRG11139
  - Phosphatases: VSRG11135
  - Proteins: VSRG11135
  - Protein Kinases: VSRG11140
  - Serine Proteases: VSRG11164
  - STE Kinases: VSRG11136
  - Tyrosine Kinases: VSRG11138
  - Ubiquitin Conjugation: VSRG11134
  - Whole Genome: VSRG11137

- Edit-R Pooled sgRNA Indexing PCR and Sequencing Primer Kits
  - Kit A: VRM11084
  - Kit B: VRM11085

*List of all genes (adjusted p-value ≤ 0.05) higher and lower abundance and also showed > 2-fold abundance change are in red and blue, respectively.
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

cRNAs 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.

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

1. **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).
2. **Cas9 nuclease will cut both strands of DNA positioned three nucleotides upstream of the PAM.**

   ![Diagram](image.png)

   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 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.

**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, IncRNA, 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 exon, 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 RNAs to your cart as synthetic crRNA or lentiviral sgRNA.

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:

• Knockout (using homology-directed repair) of a DNA donor at a particular cut site
• Targeting a particular protein domain, transcript, or exon
• Knockout of a microRNA or long noncoding RNA (IncRNA)

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 IncRNA 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
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

Availability

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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 (molar ratio 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).
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

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

Abstract

The CRISPR-Cas9 system is a widely used tool 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 Editing, Edit-R, Cas9, Clonal Selection, FACS, Immunoblot, Sanger Sequencing, Cloning, Transfection, Transfection Optimization

Introduction

The CRISPR (clustered regularly interspaced short palindromic repeat) system of Streptococcus pyogenes is a type II CRISPR-Cas system that has been genetically engineered to enable sequence-specific endonuclease activity (2). The CRISPR-Cas9 system encompasses the Cas9 protein and a class of short, non-coding RNA molecules (crRNAs) that guide Cas9 to specific genomic locations. When bound to a guide RNA, Cas9 forms a complex with the tracrRNA and efficiently cleaves DNA, leading to double-strand breaks (DSBs) in the target site of interest. The Edit-R CRISPR-Cas9 platform is comprised of Edit-R CRISPR-Cas9 nuclease expression plasmids, a long synthetic tracrRNA, and custom-designed crRNA.

Transfection Optimization

Transfection optimization is performed in 96-well plate conditions 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.

Results

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 CRISPR-Cas9 expression vector with synthetic crRNA and tracrRNA was optimized for maximal editing efficiency. Next, a 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 colonies where functional PPIB knockout was expected based on Sanger sequencing were subsequently analyzed and confirmed by Western blot.

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

Figure 2. A complete workflow using the Edit-R CRISPR-Cas9 gene engineering platform. First, co-transfection was optimized for editing events and scaled up appropriately for FACS analysis. Next, transfection 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. (A) HEK293T cells were transfected with Edit-R CRISPR-Cas9 expression plasmid and crRNA/tracrRNA targeting PPIB. Cells were sorted at 72 hours on a MoFlo Aria II. (B) Examples of T7EI analysis in FACS clonal lines of HEK293T cells. (C) Detection of HDR with mismatch detection assay. 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 CRISPR-Cas9 expression vector with synthetic crRNA and tracrRNA was optimized for maximal editing efficiency. Next, a 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 colonies where functional PPIB knockout was expected based on Sanger sequencing were subsequently analyzed and confirmed by Western blot.
Enrichment by FACS for cell populations with gene editing events: HEK293T cells were transplanted and sorted 12 hours after transfection. Cells were sorted and binned into naive, 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 (Figure 7E). FACS was additionally specific to wt and wt type, with mKate2 expression cell populations into 96-well plates such that four, and six individual cells were plated into each well and further grown for clonal isolation. Clonal isolation: The clonal cells 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 CRISPR 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 examples, the percent editing, typically between 40 and 50%, was calculated using densitometry (clonal lines 1, 2, and 6, and 10). In some cases, PCR products need to be larger or smaller, indicative of longer insertions or deletions for example clonal lines 12, 13, 15, 27 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 each individual cell population. These data were reported as either wild type (wt), heterozygous (ht), or homozygous (hm) genotypes. A heterozygous genotype is indicative when both alleles have been edited, at least one allele while the other allele is either wt, or a different mutation. A homozygous genotype is indicated when both alleles lack an editing event, for example clonal lines 11, 13, 15, and 24. Figure 4 (examples) shows that cloning is not always as expected and deletions detected on one 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 the selection of a NoCas9 expression plasmid, with an active promoter for the cell line of interest and optimization of the co-transfection with the sgRNA and crRNA. Further, gene editing was enriched with selection of fluorescent reporter-expressing cells. In this study, all but 5 colonies carrying the CRISPR target site were digested by the endonuclease. The cloning was successful in 25 of 42 clonal lines at the PIPB cleavage target site. Of the 25 edited clonal lines, 18 were mutated in both alleles. Surprisingly, 9 of these clonal lines were mutated identically in 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.

An additional explanation could be that one of the alleles was mutated to contain a very large deletion or insertion that was not detected because the new sequences were designed to amplify 500 bp spanning the CRISPR 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 the PCR products can rearrange and not digest at the cleavage site, indicating that the CRISPR target site is not preserved. From sequence alignment, it was determined that insertions in three clonal lines have significant homology to bacterial components of the Edit-R CRISPR-Cas9 Gene Engineering Platform. 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.
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 agent amount and planned 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-Caldwell Expression plasmid (Thermo, Cat #K-RT-2010-02) in a total volume of 100 µL. For transfection, 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 a 4-well plate, 5 µL Edit-R hCMV_mKate2-Caldwell Expression plasmid with 50 µg transfection reagent and 50 µg lipofectamine 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 ensure a sufficient number of cells for FACS.

FACS analysis: HEK293T cells were trypsinized, resuspended in cold PBS containing 10 mM EDTA, 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 of 2.5 mL. A total of three wells were transfected to ensure a sufficient number of cells for FACS.

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 #PK-5100) 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 GC buffer (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.

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 #PK-5100) 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 GC buffer (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: Denaturation/annealing reaction at 98 °C for 1 minute followed by 10 cycles of 98 °C for 10 seconds, 72 °C for 15 seconds then 62 °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 at 50 °C for 10 minutes and slowly cooled to room temperature. Then, 10 µL of amplified PCR products were combined with 5 units T7E1 enzyme and NEBuffer 2 (New England Biolabs, Cat #M0210S) and incubated at 37 °C for 25 minutes. Three µL of 6X Orange Loading Dye (Thermo Scientific, Cat #MO1011) was added to the T7E1 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.

References


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

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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 Cas9-CRISPR are repaired in the cell by two predominant mechanisms: non-homologous end joining (NHEJ) and precise homology-directed repair (HDR). HDR, the Edit-R CRISPR-Cas9 gene engineering platform consists of both synthesized 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 repurposed donor oligo for rapid genome engineering using HDR. Here, we first demonstrate an ssDNA donor oligo workflow (Figure 1). The Edit-R CRISPR-Cas9 synthetic guide RNA experimental workflow (Figure 1) allows facile generation of indels through NHEJ which can result in gene disruption (Figures 2A and 2B). Consistent with the results from targeting VCP, the highest amounts of HDR knockin in 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 oligos: 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 CRISPR-Cas9 nuclease expression plasmid (200 ng/well), synthetic crRNA:tracrRNA complex (25 nM final/well), and increasing amounts of ssDNA donor oligos (2.5, 5.0, 10.0, 20.0, and 60.0 ng/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 1-5 nM of a 100 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 to 10.0 nM ssDNA donor oligo (Figure 5).

Introduction
The bacterial adaptive immune system, the CRISPR-Cas-system, can be Reprogrammed for targeted gene editing of mammalian genomes.

Keywords
Homology-directed repair, homologous recombination, non-homologous end joining, CRISPR genome engineering, HDR, donor DNA, lipid transfection, homology arms, insertion, repair, DSB knockin

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

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 into the genome. HDR genome engineering platform employing chemically synthesized RNAs is a quick and easy way to test multiple guide sequences for optimizing HDR, as measured by 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 donor oligo and a Cas9 expression plasmid. We have further demonstrated the use of lipid-based transfection using DharmaFECT DUO Transfection Reagent for achieving precise sequence insertion through the HDR pathway, whereas, in the literature, this is commonly achieved through expensive electroporation methods, making HDR genome editing expensive and low-throughput. Recommended workflow is shown for performing similar successful sequence insertion experiments using these Edit-R CRISPR-Cas9 reagents and workflows 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 is 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 nucleases. We observed that when comparing the genomic engineering of the two transfected targets presented in this study, the mismatch detection assay and the RFLP assay results did not correlate, which is consistent with the overall lower efficiency 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 effect of screening individual clones to find the correct HDR induced modification. For example, one could need to screen ten times more individual clones 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 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 oligo resides within the homing arms of the oligo donor; silent mutations must be made at one of the two guanine bases of the NGG PAM and/or SHH 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 DSF occurs 100 bp away from the SNP; thus, to try to design your crRNA to create a Cas9-mediated DSF close to the insertion site as possible for maximal HDR efficiencies.

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 efficacy 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 U2OS-GFP-U2OS-cells (Thermo Scientific) BioImage. Promoterase RedCellulation Assay Cat #R0402102 were seeded in a 96-well plate at 10,000 cells per well. Edit-R synthetic crRNAs and tracrRNAs were individually resuspended in 20 µM Tris-HCl (pH 7.5), 50 µM crRNA and tracrRNA were combined and the final concentration was diluted to 2.5 µM using 20 µM Tris-HCl (pH 7.5). A final concentration of 25 nM of crRNA/tracrRNA complex (25 nM of each of crRNA and tracrRNA) was used in transfer. Cells were transfected with DharmaFECT (Dharmacon Cat #47-0100-25) using 200 ng of crRNA plasmid (for co-transfections) and 25 nM of crRNA/tracrRNA complex. A final concentration range of 0.1 to 100 µM puromycin donor oligo was used for transfection.

Puromycin selection: Twenty-four hours post-transfection, cells were collected and harvested, analyzed, and are referred to as Pure Selection.

ssDNA donor oligos: DNA oligos were synthesized in house using standard solid-phase DNA synthesis and desalting procedures. Each DNA oligos contained 2 phosphotriester linkages on the first two and last two DNA bases. Oligos were ethanol precipitated and resuspended (100 µg in 10 µl Tris-HCl (pH 7.5)). Oligos were further diluted to 1 µg/prior to tranfection.

Genomic DNA isolation and DNA mismatch detection assay: Genomic DNA was isolated 72 hours post-transfection by direct lysis of the cells in Phusionâ"∕â"∕HF buffer (Thermo Scientific Cat #SF100) proteinase K (Thermo Scientific Cat #K12511) and phenol:chloroform. A 5 µl sub-sample was used for reaction. PCR products were separated on a 2% agarose gel. Percent editing in each sample was calculated using ImageJ software (NIH, imagej.nih.gov/ij, 1997-2014).

Table 1. Recommendations for HDR-based gene editing using Edit-R express 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 transfection using Edit-R Cas9 Lentiviral Particles

DharmaFECT Duo Transfection Reagent (optimized concentration for each cell line)
» 2.5-10 nM Edit-R synthetic crRNA/tracrRNA

Oligo concentrations:
» U2OS cells (co-transfection): 2.5-10 nM (puro donor DNA oligo)
» U2OS-Cas9 cells (integrated): 2.5-10 nM (puro 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.

Restriction Fragment Length Polymorphism assay: Genomic isolation was performed by the high-salt method. Genomic DNA was digested with 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).

References


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

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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 Dharmaco 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 showed 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 embryos, 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 ability 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:PH-GFP; sox10:tagRFP) transgenic zebrafish line express both GFP and RFP. 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 line 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 zebrafish 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 (+/- lane). Genomic DNA was prepared 2 days post-injection and PCR was performed with primers flanking the cleavage sites. A DNA mismatch assay with T701 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 V5X spinning disk confocal system and Velocity 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 (15.4 % bar for reference).
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)
- 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 expresses both red and green fluorescent proteins under the Sox10 promoter.

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:

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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 (NEB; Cat #M0302S) at 37 °C for 15 minutes. 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

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

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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 crCRISPs. 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 nucleases under the CAG promoter (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 knockouts 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 4 transfection reagent concentration 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 PBG synthetic crRNA Control (Cat #IK-007910-05) was used as a positive control for gene editing and Edit-R or RNA targeting a protease gene PSMD7 was used as a positive control for the phenotypic assay. The crRNA was co-transfected with an equimolar amount of synthetic tracrRNA at a final concentration of 0.02 µL/well, using a range of DharmaFECT 4 transfection reagent from 0.02-0.3 µL/well. After incubation of the RNA:tracrRNA with the transfection reagent 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°C, 5% CO2 incubator for 72 hours. A resazurin viability assay and a DNA mismatch assay using T7E1 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 or RNA 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 PMI 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 and 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 1). As expected, a strong correlation was observed between the optimal conditions for PSMD7 and PMI. 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 (>5 fold increase of EGFP fluorescence compared to untreated cells) as well as excellent cell viability (>80%).

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.

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