horizon

TECHNICAL MANUAL

Dharmacon™ Edit-R™ CRISPR-Cas9 Gene Engineering with All-in-One Lentiviral Cas9 and sgRNA

Contents

1 Introduction to the CRISPR-Cas9 system for gene engineering

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

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

Engineering a CRISPR-Cas9 platform for mammalian genome editing

Many bacterial and archaeal CRISPR-Cas systems have been identified with diverse sets of mechanisms, Cas proteins and multi-subunit complexes. In particular, the processes and key components of the *Streptococcus pyogenes* CRISPR-Cas9 system have been well studied and adapted for genome engineering in mammalian cells. In *S. pyogenes*, only three components are required for targeted DNA cleavage at specific target sites adjacent to a PAM[2]: (1) The endonuclease Cas9, programmed by (2) a mature crRNA processed from transcription of the CRISPR locus/array which complexes with (3) another CRISPR locus-encoded RNA, the *trans*-activating CRISPR RNA (tracrRNA, Figure 1A)[3]. Alternatively, the crRNA can be fused to the tracrRNA creating a chimeric structure termed a single guide RNA (sgRNA, Figure 1B)[2].

Upon site-specific double-stranded DNA cleavage, a mammalian cell can repair such a break through either non-homologous end joining (NHEJ) or homology directed repair (HDR). NHEJ is often imperfect, resulting in small insertions and deletions (indels) that can cause nonsense mutations resulting in gene disruptions to produce gene knockouts[4,5]. 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.

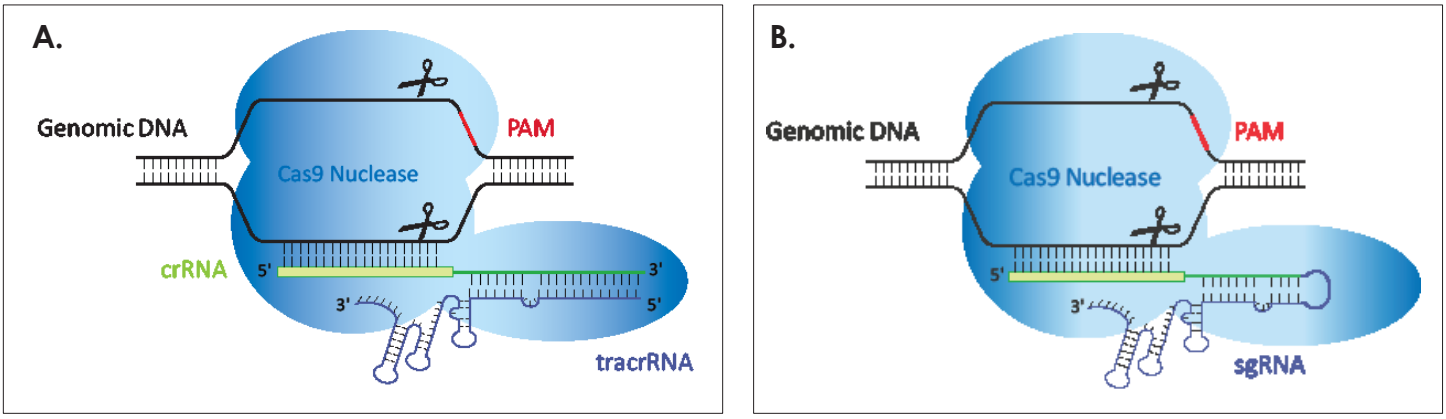

Figure 1. Illustration of CRISPR-Cas9 system. Cas9 nuclease (light blue), programmed by the crRNA (green):tracrRNA (blue) complex (A) or the sgRNA (B), cutting both strands of genomic DNA 5' of the PAM (red).

2 Gene editing using Edit-R All-in-one Lentiviral sgRNA

The Edit-R All-in-One Lentiviral Gene Engineering vector includes the two critical components based on the natural *S. pyogenes* system, the Cas9 nuclease and the gene-specific sgRNA. To facilitate rapid generation of cells lines that constitutively express Cas9 nuclease and the sgRNA, these vectors are packaged into lentiviral particles, purified and concentrated. Gene knockout cell lines can be obtained with the Edit-R All-in-one Lentiviral sgRNA by transduction with lentiviral particles followed by selection with puromycin. Figure 2 summarizes a general experimental workflow to generate stable cell lines expressing Cas9 and sgRNA followed by transduction with Edit-R All-in-one Lentiviral sgRNA lentiviral particles for phenotypic analysis of gene knockout.

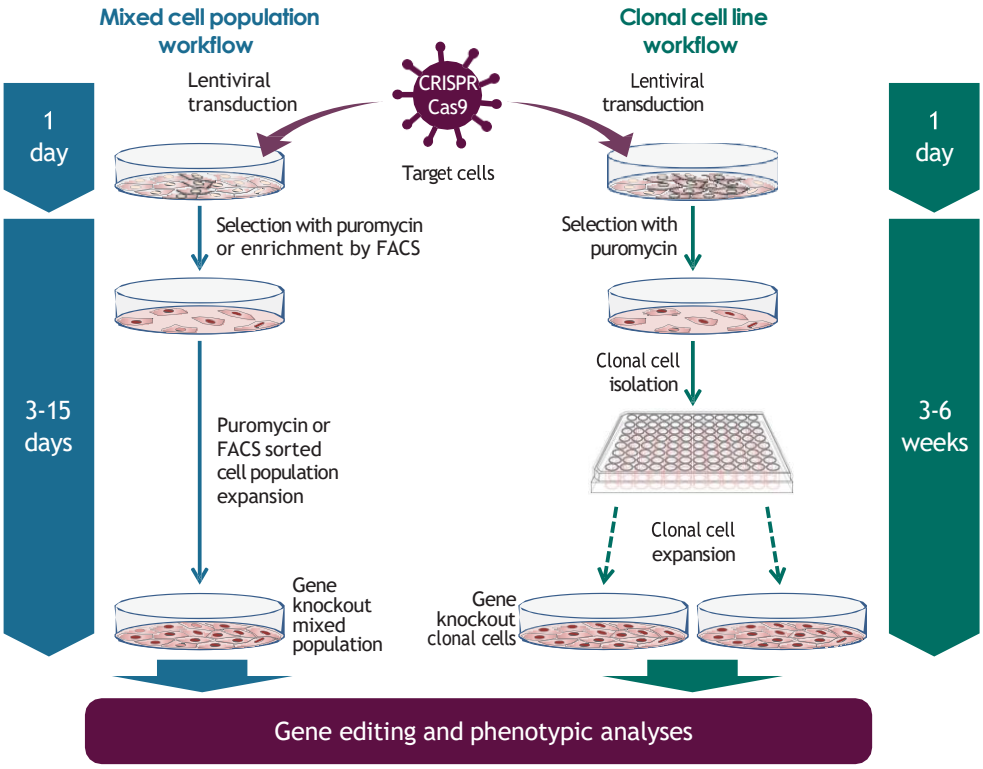

Figure 2. Gene knockout workflow using the Edit-R All-in-one Lentiviral sgRNA system. Gene editing with Edit-R All-in-one Lentiviral sgRNA can be done following the mixed cell populations approach (left side) typically for gene knockout screenings or the isolated clonal cell lines approach (right side) when a defined genotype is desired or required on each step for phenotypic analyses.

Edit-R All-in-one Lentiviral sgRNA vector

The Edit-R All-in-one Lentiviral sgRNA vector contains a human codon-optimized version of the *S. pyogenes cas9* (*csn1*) gene and the puromycin resistance marker (Puro^R). Both are expressed as a bicistronic transcript with a 2A peptide sequence linker under the control of a single promoter (Figure 3). Puro^R is placed upstream of the Cas9 coding region rather than on the 3' end so that no extra amino acids are added to the C-terminus, thus preserving the full Cas9 endonuclease activity. The gene-specific sgRNA is expressed under the control of a human U6 promoter. The optimized sgRNA scaffold used in the Edit-R All-in-one Lentiviral sgRNA can further improve gene knockout efficiency. A brief description of the lentiviral vector elements is listed in Table 1. Two promoter options are available (Figure 3) for selection of a Cas9 nuclease vector with the most active promoter for specific cells of interest.

Each Edit-R All-in-one Lentiviral sgRNA is specific to the gene or genomic site of choice. The crRNA region of the sgRNA is comprised of 19-20 nucleotides identical to the genomic DNA target site, followed by the non-variable sgRNA scaffold containing the tracrRNA sequence from *S. pyogenes*. The chosen genomic DNA target sequence must be immediately upstream of a PAM. The predominant *S. pyogenes* PAM nucleotide sequence is NGG. Predesigned, gene-specific all-in-one vectors can be ordered by searching for genes of interest on horizondiscovery.com, or custom-designed using the Dharmacon CRISPR Design Tool.

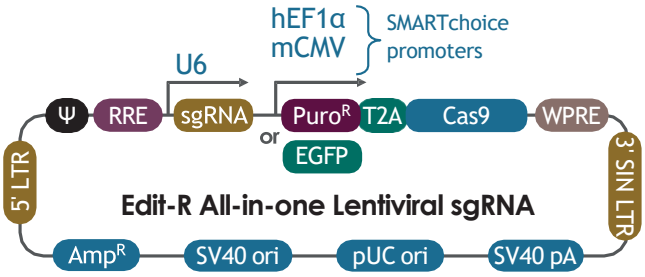

Figure 3. Schematic map of the Edit-R All-in-one Lentiviral sgRNA vector.

Table 1. Elements of the Edit-R All-in-one Lentiviral sgRNA vectors.

Vector element	Utility
Cas9	Human codon-optimized *S. pyogenes* Cas9 nuclease for cleavage of targeted DNA when programmed with a sgRNA
T2A	Self-cleaving peptide allows for simultaneous expression of puromycin resistance and Cas9 protein from a single transcript
Puro^R	Puromycin resistance marker permits antibiotic selection of transduced mammalian cells
mCMV	Mouse cytomegalovirus immediate early promoter
hEF1α	Human elongation factor 1 alpha short promoter
U6	Human RNA polymerase III promoter U6
sgRNA	Optimized single guide RNA, a fusion of gene-specific crRNA with the tracrRNA scaffold
5' LTR	5' Long Terminal Repeat necessary for lentiviral particle production and integration of the construct into the host cell genome
Ψ	Psi packaging sequence allows lentiviral genome packaging using lentiviral packaging systems
RRE	Rev Response Element enhances titer by increasing packaging efficiency of full-length lentiviral genomes
WPRE	Woodchuck Hepatitis Post-transcriptional Regulatory Element enhances transgene expression in target cells
3' SIN LTR	3' Self-inactivating Long Terminal Repeat for generation of replication-incompetent lentiviral particles
SV40 pA	Simian virus 40 polyadenylation signal
pUC ori	pUC origin of replication
SV40 ori	Simian virus 40 origin of replication
Amp^R	Ampicillin resistance gene for vector propagation in *E. coli* cultures

3 Protocol for gene engineering using Edit-R All-in-one Lentiviral sgRNA

The Edit-R All-in-one Lentiviral sgRNA Gene Engineering system utilizes Cas9 nuclease and the sgRNA in a single-step process to achieve efficient gene editing for phenotypic analyses in a population of cells or in isolated clonal cell lines.

Edit-R All-in-one Lentiviral sgRNA gene engineering materials required

Edit-R All-in-one Lentiviral sgRNA vectors are provided as concentrated, purified lentiviral particles for immediate transduction or as glycerol stocks for plasmid DNA preparation.

- Edit-R™ All-in-One Lentiviral sgRNA with SMARTchoice promoter options and predesigned sgRNA particles or custom glycerol stocks:
  - Edit-R™ Human All-in-one hEF1α Lentiviral sgRNA
  - Edit-R™ Human All-in-one mCMV Lentiviral sgRNA
  - Edit-R™ Mouse All-in-one hEF1α Lentiviral sgRNA
  - Edit-R™ Mouse All-in-one mCMV Lentiviral sgRNA

- Edit-R™ All-in-One Lentiviral CRISPR-Cas9 with SMARTchoice promoter options and custom sgRNA designed using the Dharmacon CRISPR Design Tool:
  - Edit-R™ Custom All-in-one hEF1α Lentiviral sgRNA
  - Edit-R™ Custom All-in-one mCMV Lentiviral sgRNA

Additional materials required

The following additional materials are required but not supplied:

- Multi-well tissue culture plates or tissue culture dishes
- Puromycin (Fisher Scientific, Cat #BP2956-100; InvivoGen, Cat #ant-pr-1)
- Resazurin cell viability reagent or similar
- Assay(s) for detecting gene engineering events in a cell population
- Edit-R All-in-one Lentiviral sgRNA Positive controls particles
- Edit-R All-in-one Lentiviral sgRNA Non-targeting Controls
- **Growth Medium:** antibiotic-free cell culture medium (with serum and/or supplements) recommended for maintenance of the cells of interest.
- **Transduction Medium:** the serum-free base cell culture medium containing lentiviral particles. Depending on the cell type, transduction additives, such as polybrene, and serum may be added.
- **Selection Medium:** Growth Medium supplemented with the appropriate concentration of puromycin.

Optional materials for transfection of plasmid DNA

- DharmaFECT™ kb transfection reagent (Dharmacon, Cat #T-2006-01)

Generation of a stable cell line expressing Edit-R All-in-one Lentiviral sgRNA

The protocol described here is designed for rapid generation of a cell population where most of the cells have a single copy of the Edit-R All-in-one Lentiviral sgRNA proviral sequence in the genome.

Select the lentiviral Cas9 nuclease vector with the most active promoter in your cell line based on empirical testing or known promoter activity.

Puromycin selection

The Edit-R All-in-one Lentiviral sgRNA vectors confer resistance to puromycin in transduced cells. Before transducing cells, determine the minimum concentration of puromycin required to kill non-transduced cells by generating a puromycin kill curve. The puromycin concentration range for many mammalian cells is 0.5-10 µg/mL and it usually kills cells between two and seven days.

Transduction of cells with Edit-R All-in-one Lentiviral sgRNA particles

The protocol below provides the basic steps for transduction of the lentiviral particles into mammalian cells using serum-free medium in a 24-well plate. Permissivity to lentiviral delivery and optimal transduction conditions vary widely amongst cell types and must be determined empirically for each cell line of interest.

Day 1:

1. Based on experimentally-determined growth characteristics of the cell line being used, plate cells so they are at 40-80% confluency on the day of transduction.
2. Incubate cells at 37 °C in a humidified CO_2 incubator overnight.

Day 2:

1. Prepare the Transduction Medium and equilibrate the medium to 37 °C.
2. Calculate the volume of lentiviral particles needed for transduction at MOI = 0.3 (see Appendix).

The functional titer of Edit-R All-in-one Lentiviral sgRNA particles (in HEK293T cells, as determined by qPCR) is reported on the Certificate of Analysis (C of A). We recommend an MOI of 0.3 (adjusted for relative transduction efficiency in your cell type) to ensure single integration of the lentiviral Cas9 nuclease.

3. Thaw the Edit-R All-in-one Lentiviral sgRNA particles on ice.

Lentiviral particles are shipped on dry ice as 25 µL aliquots and must be stored at -80 °C. Under these conditions, lentiviral particles are stable for at least 12 months. Repeated freeze-thaw cycles should be avoided, as this is expected to negatively affect titer. Once thawed, unused lentiviral particles should be kept on ice, divided into smaller aliquots (if necessary) and immediately returned to -80 °C.

4. Once thawed, gently mix and pipette the calculated volume of lentiviral particles into the Transduction Medium.
5. Remove the Growth Medium from the well and add 0.25 mL of the Transduction Medium containing the lentiviral particles (see Appendix for guidelines on other plate formats).

6. Incubate cells at 37 °C in a humidified CO_2 incubator for 4-6 hours.
7. Approximately 4-6 hours post-transduction, add an additional 0.75 mL of Growth Medium (with serum) and resume incubation at 37 °C in a humidified CO_2 incubator.

If toxicity occurs with your cells, in step 7, replace the medium after 4-6 hours with fresh Growth Medium (with serum).

For puromycin resistance containing constructs
Days 3-15:

1. At 24-48 hours post-transduction, replace the medium with Selection Medium containing the appropriate amount of puromycin.
2. Replace the Selection Medium every 2-3 days and monitor the presence of dead cells daily
   a. If the cells become confluent, split the cells into a larger dish to allow proper puromycin selection (for example, split cells from 24-well to 6-well culture dishes).
3. Once the cells are growing normally in Selection Medium, expand the cells to freeze a sufficient number of aliquots for your experimental project or proceed to isolation of clonal cell lines.
   a. Expansion of the puromycin-resistant cells at this stage will generate a mixed population of cells having a single integration of the Cas9 nuclease and sgRNA in their genomes. This mixed population will carry a variety of insertions and deletions (indels) and can be used for phenotypic analysis of your gene knockout.
   b. If a defined mutation genotype is required, proceed to isolation of clonal cell lines using protocols appropriate for your cells of interest and determine the exact genotype by DNA sequencing.

Record the passage number and avoid working with stable cell populations at passage numbers exceeding 10 from frozen stock.

For fluorescent marker containing constructs
Days 3-15:

1. At 24-72 hours post-transduction, check the cells for expression of EGFP.

Visualizing the cells in media specifically designed for imaging may reduce background fluorescence.

   a. Cells can be subjected to enrichment by FACS or studied individually.
2. If cells have been subjected to enrichment of the fluorescent population, expand the cells to freeze a sufficient number of aliquots for your experimental project or proceed to isolation of clonal cell lines.
   a. Expansion of the fluorescent cells at this stage will generate a mixed population of cells having a single integration of the Cas9 nuclease and sgRNA in their genomes. This mixed population will carry a variety of insertions and deletions (indels) and can be used for phenotypic analysis of your gene knockout.
   b. If a defined mutation genotype is required, proceed to isolation of clonal cell lines using protocols appropriate for your cells of interest and determine the exact genotype by DNA sequencing.
3. Gene editing can be assessed in cells 48-72 hours post transduction.

It may be necessary to test MOIs higher than 0.3, and this needs to be empirically determined. If enrichment is not performed, or fluorescent cells not specifically analyzed, gene editing may not be detectable in cell populations transduced with relatively low MOIs. Record the passage number and avoid working with stable cell populations at passage numbers exceeding 10 from frozen stock.

4 Edit-R All-in-one Lentiviral custom sgRNA glycerol stocks

Important safety note
Please follow the safety guidelines for use and production of vector-based lentiviral particles as set by your institution's biosafety committee.
- **For glycerol stocks of E. coli containing lentiviral plasmids, BSL1 guidelines should be followed.**
- **For handling and use of lentiviral products to produce lentiviral particles, BSL2 or BSL2+ guidelines should be followed.**
- **For handling and use of lentiviral particle products, BSL2 or BSL2+ guidelines should be followed.**

Antibiotic resistance

Edit-R All-in-one Lentiviral sgRNA plasmids contain two antibiotic resistance markers (Table 2).

Table 2. Antibiotic resistances conveyed by Edit-R All-in-one Lentiviral sgRNA plasmid vectors.

Antibiotic	Concentration	Utility
Ampicillin (carbenicillin)	100 µg/mL	Bacterial selection marker (outside LTRs)
Puromycin	Variable	Mammalian selection marker

Plasmid preparation

Culture conditions for individual plasmid preparations

For plasmid preparation, grow all Edit-R All-in-one Lentiviral sgRNA clones at 37 °C in LB broth medium plus 100 µg/mL carbenicillin.

1. Upon receiving your glycerol stock(s) containing the Edit-R All-in-one Lentiviral sgRNA clone, store at -80 °C until ready to begin.
2. To prepare plasmid DNA, first thaw your glycerol stock culture and pulse vortex to resuspend any *E. coli* that may have settled to the bottom of the tube.
3. Take a 10 µL inoculum from the glycerol stock into 3-5 mL of LB broth medium with 100 µg/mL carbenicillin. Return the glycerol stock(s) to -80 °C.

If a large culture volume is desired, incubate the 3-5 mL culture for 8 hours at 37 °C with shaking and use as a starter inoculum. Dilute the starter culture 1:500-1:1000 into the larger volume.

4. Incubate at 37 °C for 18-19 hours with vigorous shaking.
5. Pellet the culture and begin preparation of plasmid DNA. Plasmid DNA can be isolated using a plasmid miniprep kit of your choice.
6. Edit-R All-in-one Lentiviral sgRNA plasmids are about 12 kb. See the vector maps (Appendix) for suggestions of restriction sites to confirm the vector integrity. Be aware that an additional restriction site may be present in the specific sgRNA target region.

Due to the tendency of lentiviral vectors to recombine, we recommend keeping the incubation times as short as possible and avoid subculturing. Return to your original glycerol stock for each plasmid preparation.

Packaging lentiviral particles

The Edit-R All-in-one Lentiviral sgRNA constructs are Tat-dependent; therefore, you **must** use a packaging system that expresses the *tat* gene. We recommend the Trans-Lentiviral shRNA packaging kit (Cat #TLP5912). The Trans- Lentiviral packaging system allows creation of replication-incompetent, HIV-1 based lentiviral particles that can be used to deliver and express your sgRNA of interest in either dividing or non-dividing mammalian cells, and it is based on the trans-lentiviral system developed by Kappes and Wu[6]. For protocols and information on packaging Edit-R Lentiviral sgRNA constructs with the Trans-Lentiviral packaging system, please see the product manual available on our website.

After packaging plasmid DNA, we recommend titering the lentiviral particles using a functional titration protocol such as limiting dilution with cell viability assay by crystal violet staining or genomic qPCR assay[7].

Transfection

Quantities and volumes should be scaled up according to the number of cells/well to be transfected (Table 3). This example is for transfection of Edit-R All-in-one Lentiviral sgRNA plasmid DNA in 24-well plate format.

1. In each well, seed ~ 5×10^4 adherent cells or ~ 5×10^5 suspension cells in 0.5 mL of growth medium 24 hours prior to transfection.

The recommended confluency for adherent cells on the day of transfection is 70-90%. Suspension cells should be in logarithmic growth phase at the time of transfection.

2. Dilute 1 µg of Edit-R All-in-one Lentiviral sgRNA plasmid DNA in 50 µL of DMEM or other serum-free growth medium
3. Gently mix DharmaFECT kb transfection reagent and add 3 µL to the diluted DNA. Mix immediately by pipetting.

Prepare immediately prior to transfection. We recommend starting with 1 µg of DNA and 3 µL of DharmaFECT kb reagent per well in a 24-well plate (see scale-up Table 3). Subsequent optimization may further increase transfection efficiency depending on the cell line and transgene used.

4. Incubate 10 minutes at room temperature. Remove medium from wells and replace with 0.45 mL of fresh growth medium.

The transfection efficiency with DharmaFECT™ kb transfection reagent is equally high in the presence of serum. This is not the case with other transfection reagents.

5. Add 50 µL of the DharmaFECT kb reagent/DNA mixture gently to each well.
6. Gently rock the plate to achieve even distribution of the complexes.
7. Incubate at 37 °C in a CO_2 incubator.
8. Analyze the cells for gene editing or expected gene knockout phenotype 48-72 hours later, or longer if necessary depending on the experimental design and cell type.

Table 3. Scale-up ratios for transfection of adherent and suspension cells with DharmaFECT kb transfection reagent.

Tissue culture vessel	Growth area, cm^2/well	Volume of medium, mL	Adherent (suspension) cells to seed the day before transfection*	Amount of DNA		Volume of DharmaFECT kb, µL	
				µg**	µL***	Recommended	Range
96-well plate	0.3	0.1	$0.5\text{-}1.2 \times 10^4$ (2.0×10^4)	0.2	10	0.6	0.4-1.0
48-well plate	0.7	0.25	$1.0\text{-}3.0 \times 10^4$ (5.0×10^4)	0.5	25	1.5	0.8-2.2
24-well plate	2.0	0.5	$2.0\text{-}6.0 \times 10^4$ (1.0×10^4)	1.0	50	3.0	2.0-5.0
12-well plate	4.0	1.0	$0.4\text{-}1.2 \times 10^5$ (2.0×10^5)	2.0	100	6.0	3.9-9.0
6-well plate	9.5	2.0	$0.8\text{-}2.4 \times 10^5$ (4.0×10^5)	4.0	200	9.0	6.0-12.0
60 mm plate	20	3.0	$2.0\text{-}6.3 \times 10^5$ (1.0×10^6)	6.0	300	18.0	12.0-24.0

* These numbers were determined using HEK293T and U2OS cells. Actual values depend on the cell type.
** Amount of DNA and DharmaFECT kb transfection reagent used may require optimization.
*** The volume of DNA should be 1/10 of the volume of the culture medium used for dilution of the DNA.

5 Appendix

Gene editing assay recommendations

The most commonly used method for detection of insertions and deletions (indels) in a cell population is a mismatch detection assay such as T7 Endonuclease I (T7EI, NEB)[8,9,10]. When edited cells are expanded and clonal populations are obtained, the most commonly used method for confirming gene editing is Sanger sequencing[8].

Multiplicity of Infection (MOI)

The equation to calculate a volume of lentiviral stock for a given MOI is:

$V = MOI \times CN \div VT \times 1000$

Where:

V = volume of lentiviral stock in µL
MOI = desired multiplicity of infection
CN = number of cells in the well at transduction
VT = Viral titer in TU/mL (indicated in the Certificate of Analysis) and multiplied by 1000 to convert the volume from mL to µL

For example, for a desired MOI of 0.3 and:

- Cell density of 100 000 cells per well at time of transduction
- Lentiviral titer = 1×10^8 TU/mL

Then,

$V = 0.3$ TU/cell × 100 000 cells/well ÷ 1×10^8 TU/mL × 1000 = 0.3 µL of lentiviral stock/well.

Volume of Transduction Medium per surface area in culture dishes

Table 4. Suggested volumes of Transduction Medium per surface area per well of adherent cells..

Tissue culture dish	Surface area per well (cm2)	Suggested total serum-free-medium volume per well (mL)
100 mm	56	5
6 well	9.4	1
12 well	3.8	0.5
24 well	1.9	0.25
96 well	0.3	0.05

Stability and storage

Lentiviral particles

Edit-R All-in-one Lentiviral sgRNA particles are shipped on dry ice as 25 µL aliquots and must be stored at -80 °C. Under these conditions, lentiviral particles are stable for at least 12 months. Repeated freeze-thaw cycles should be avoided, as this is expected to negatively affect titer. Once thawed, unused lentiviral particles should be kept on ice, divided into smaller aliquots (if necessary) and immediately returned to -80 °C.

Glycerol stocks

Edit-R All-in-one Lentiviral custom sgRNA glycerol stocks are shipped as 150 µL *E. coli* culture in LB 8% glycerol medium at room temperature, with ice packs or dry ice depending on the estimated shipping delivery time. Upon receipt, the tubes should be stored at -80 °C. Under these conditions, the reagents are stable for at least one year.

6 Frequently asked questions

How do I choose between the two Edit-R All-in-one Lentiviral sgRNA promoter options?

Choose the promoter option that has been demonstrated, either by your own experimental observations or through references in the published literature, to actively express a transgene in your cells of choice. For optimal experimental confidence (or if such information is not available), consider testing both options using the Edit-R All-in-one Lentiviral sgRNA Positive Controls.

What is the best way to confirm that my gene is knocked out?

Mismatch detection assays tell you that editing occurred in the cell population. Clonal cell isolation followed by DNA sequencing of the region of interest and determination of the protein functionality are necessary to confirm the gene knockout.

Can I order the Edit-R All-in-one Lentiviral sgRNA vector as plasmid DNA?

No. Our Edit-R All-in-one Lentiviral sgRNA vectors are made to each specific customer order and are sold exclusively as lentiviral particles or glycerol stocks.

Where can I find the sequence of an individual sgRNA construct?

The target sequence of an individual sgRNA construct is provided on the product insert and can be found online under your account Order History at horizondiscovery.com.

What primer can I use to confirm the sgRNA sequence?

For confirmation of the sgRNA sequence, we recommend the following primer (forward): 5'-GGCCTATTTCCCATGATTC-3'

What packaging cell line should I use for making lentiviral particles?

Any HEK293T cell line carrying the Large T antigen can be used for packaging lentiviral particles.

Can I use any 2nd generation packaging system with the Edit-R All-in-One Lentiviral sgRNA vector?

The Edit-R All-in-One Lentiviral sgRNA vector is Tat dependent, so a packaging system that expresses the *tat* gene is required.

Where can I purchase puromycin?

Puromycin is available from Fisher Scientific Cellgro (Cat #BP2956-100) or Invivogen (Cat #ant-pr-1).

What are benefits of transfecting plasmid directly vs transducing lentivirus?

Plasmid transfection is preferred for applications where one does not want to maintain an integrated copy of the vector in the genome. It may also be useful for quick qualitative assessment of design efficacy. Lentiviral transduction is preferred when single-copy, or controlled levels of editing machinery integration is required. In general, lentiviral integration does provide higher editing efficiency than transient transfection.

What are the maximum excitation and emission wavelengths for EGFP?

Fluorescent reporter	Excitation wavelength	Emission wavelength
EGFP	488 nm	507 nm

Can the Edit-R All-in-one Lentiviral sgRNA system be used for gene knockout in non-mammalian organisms, such as flies or worms?

Our predesigned Edit-R All-in-one Lentiviral sgRNA vectors are intended for mammalian expression and thus have only been tested in mammalian cells. We have a free tool to design Edit-R custom All-in-one lentiviral sgRNAs for non-mammalian species, however, as with all custom designed products, we cannot predict their efficacy: CRISPR-Design-Tool.

How do I know if Cas9 is expressed after introducing the Edit-R All-in-one Lentiviral sgRNA vectors?

We recommend performing a functional assay, such as a T7EI mismatch assay, to determine the presence of Cas9. For initial optimization, we recommend using one of our positive control kits (including detection primers) and performing a mismatch assay to assess the editing efficiency.

We strongly recommend performing mismatch detection assays when using any gene-specific Edit-R All-in-one Lentiviral sgRNA to determine editing efficiency for all experiments, even after the delivery conditions have been optimized.

Additionally, as the Cas9 nuclease is under the same promoter as EGFP/puromycin resistance marker, expression of EGFP or puromycin resistance would indicate the simultaneous expression of Cas9 nuclease. We

routinely select and enrich for Cas9 expressing cells using antibiotic resistance or EGFP expression, respectively.

How do I choose between Edit-R All-in-one Lentiviral sgRNA containing Puromycin and EGFP?

The Edit-R All-in-one Lentiviral sgRNA containing PuroR will confer resistance to puromycin in transduced cells and long term selection is used to generate stable cell lines. As the amount of puromycin and time needed for selection may vary between cell lines, performing a puromycin kill curve before the transducing cells is recommended. Edit-R All-in-one Lentiviral sgRNA vectors containing EGFP display green fluorescence in transduced cells and assays such as FACS can be used to enrich for cells containing successful lentiviral construct integration. The equipment being used (e.g lasers, filters, etc) should be suitable for detecting EGFP (see "excitation and emission wavelengths for EGFP").

Depending on the cell type, both EGFP expression and Puromycin resistance should appear 24-72 hours post-transduction. As FACS enrichment can be done in a matter of hours while puromycin selection can take several days, vectors containing EGFP may be preferable if the cells of interest have a short lifespan.

7 References

1. D. Bhaya, *et al*. CRISPR-Cas systems in bacteria and archaea: versatile small RNAs for adaptive defense and regulation. *Annu. Rev. Genet.* **45**, 273-297 (2011).
2. M. Jinek, *et al*. A programmable dual-RNA-guided DNA endonuclease in adaptive bacterial immunity. *Science* **337**, 816-821 (2012).
3. E. Deltcheva, *et al*. CRISPR RNA maturation by trans-encoded small RNA and host factor Nuclease III. *Nature* **471**, 602-607 (2011).
4. P. Mali, *et al*. RNA-guided human genome engineering via Cas9. *Science* **339**, 823-826 (2013).
5. T.R. Sampson, and D.S. Weiss. Exploiting CRISPR/Cas systems for biotechnology. *Bioessays* **36**, 34-38 (2014).
6. J.C. Kappes, and X. Wu. Safety considerations in vector development. *Somat. Cell Mol. Genet.* **26**, 147-58 (2001).
7. D. Reyon, *et al*. Engineering designer transcription activator-like effector nucleases (TALENs) by REAL or REAL-Fast assembly. *Curr. Protoc. Mol. Biol.* **100**, 12.15.1-12.15.14 (2012).
8. D.Y. Guschin, et al. A rapid and general assay for monitoring endogenous gene modification. *Methods Mol. Biol.* **649**, 247-256 (2010).
9. L. Cong, *et al*. Multiplex genome engineering using CRISPR/Cas systems. *Science* **339**, 819-823 (2013).

8 Lentiviral particle product safety level information

This lentiviral particle product safety level information constitutes Product Documentation according to clause 1 of the Product Terms and Conditions. It is applicable to all Dharmacon lentiviral particle products.

Any investigator who purchases Dharmacon lentiviral particle products is responsible for consulting with their institution's health and biosafety personnel for specific guidelines on the handling of lentiviral vector particles. Furthermore, each investigator is fully responsible for obtaining the required permissions for research use and the acceptance of replication-incompetent SIN lentiviral vectors and replication-defective lentiviral particles into their local jurisdiction and institution.

The Products are solely for internal research use (as set forth in the Product Terms and Conditions) in laboratories where the containment measures stated below and in applicable laws and regulations are met. Products may not be used for diagnostic, therapeutic or other commercial purposes and may not be administered to humans for any purpose or to animals for therapeutic purposes. The Products are replication-incompetent, self-inactivating (SIN) and non-pathogenic

(do not cause infectious human disease).

For questions concerning the design or production of the products, please contact our technical support team.
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In the US:

For US guidance on containment for lentiviral vectors, please refer to:

1. The Recombinant DNA Advisory Committee (RAC) guidelines for research with lentiviral vectors.
2. The U.S. Department of Health and Human Services Centers for Disease Control and Prevention and National Institutes of Health, Biosafety in Microbiological and Biomedical Laboratories (BMBL);
3. The NIH Guidelines For Research Involving Recombinant DNA Molecules (NIH Guidelines), April 2016

In the EU:

For the EU directives, please consult the following:

1. Council Directive 2009/41/EC of the European Parliament and of the Council of 6 May 2009 on the contained use of genetically modified micro-organisms. (revised version of Directive 90/219/EEC of the European Parliament and of the Council of 23 April 1990 on the contained use of genetically modified micro-organisms, amended by Council Directive 98/81/EC of 26 October 1998); and
2. Council Directive 2001/18/EC of the European Parliament and of the Council of 12 March on the deliberate release into the environment of genetically modified organisms and repealing Council Directive 90/220/EEC.

In Germany:

Required Containment Measures: The containment requirements as stated in the German Genetic Safety Ordinance (Gentechnik-Sicherheitsverordnung) of Safety Level 2* or higher have been assigned to the handling of the abovementioned lentiviral vector particles. Please note a higher Security Level might be required if the lentiviral vector particles are used for genetic engineering operations with other products which require a higher Security Level.
*Safety Level 2: activities of low risk for human health and the environment by the state of scientific knowledge (Stand der Wissenschaft).

For the German regulations, please consult the following:

1. German Genetic Engineering Act (Gentechnikgesetz - GenTG); and
2. Genetic Engineering Safety Ordinance (Gentechnik-Sicherheitsverordnung - GenTSV).

9 Limited use licenses

The gene editing Products, use and applications, are covered by pending and issued patents. Certain Label licenses govern the use of the products; these can be found at Dharmacon Licensing Statements. It is each Buyer's responsibility to determine which intellectual property rights held by third parties may restrict the use of Products for a particular application. Please review the Label Licenses governing all use of the Products.

If you have any questions, contact

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