GE Healthcare

Protocol

Dharmacon™ Edit-R™ Lentiviral sgRNA glycerol stocks

Product description:

The Dharmacon™ Edit-R™ Lentiviral sgRNA vector is part of the Edit-R CRISPR-Cas9 system for genome engineering. The purpose is to provide the researcher with the most effective tools to deliver a gene-specific sgRNA and, together with Cas9 expression, allow gene editing in cells. In addition to the Edit-R Lentiviral sgRNA, the Edit-R Lentiviral Gene Engineering platform also requires the Cas9 nuclease, which can be expressed in a Cas9-integrated cell line or from a Cas9 expression vector, such as the Edit-R Lentiviral Cas9 Nuclease or the Edit-R Cas9 Nuclease Expression plasmids (dharmacon.gelifesciences.com/gene-editing/crispr-cas9/cas9-nuclease).

In the Edit-R Lentiviral sgRNA vector, the gene-specific CRISPR RNA (crRNA) and the *trans*-activating CRISPR RNA (tracrRNA) are expressed as a chimeric single guide RNA (sgRNA) under the control of a human U6 promoter. Additionally, expression of the puromycin resistance marker (Puro^R) is driven from the mouse CMV promoter and allows for rapid selection of cells with integrated sgRNA or transiently transfected plasmid DNA.

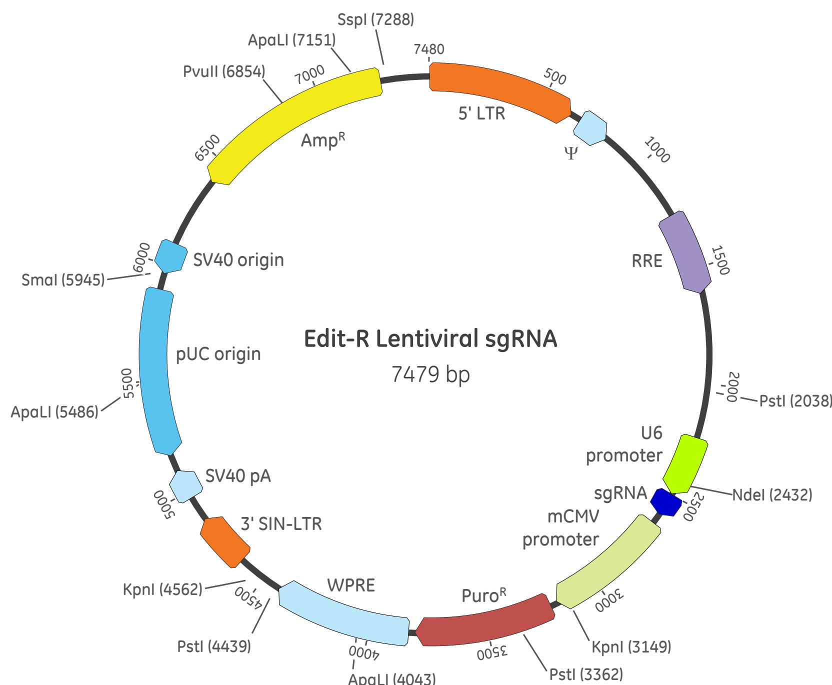
Edit-R Lentiviral sgRNA is available in glycerol stock or lentiviral particles format. If using lentiviral particles, please refer to the Edit-R CRISPR-Cas9 Gene Engineering with Lentiviral Cas9 and sgRNA Technical Manual for constitutive Cas9 expression or the Edit-R Inducible Lentiviral Cas9 Nuclease Technical Manual for inducible Cas9 expression.

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.

Additional information on the safety features incorporated in the Edit-R Lentiviral sgRNA vector and the Dharmacon™ Trans-Lentiviral™ packaging system can be found on page 3.



Plasmid features	Position
5' LTR	1-635
Ψ	685-822
RRE	1252-1605
U6 promoter	2238-2501
sgRNA	2502-2597
mCMV promoter	2648-3185
Puro^R	3213-3812
WPRE	3833-4424
3' SIN-LTR	4628-4862
SV40 pA	4971-5101
pUC origin	5173-5896
SV40 origin	5967-6102
Amp^R	6410-7270

Restriction sites	Position
ApaLI	4043, 5486, 7151
KpnI	3149, 4562
NdeI	2432
PstI	2038, 3362, 4439
PvuI	6854
SmaI	5945
SspI	7288

Figure 1. Restriction map of the Edit-R Lentiviral sgRNA vector.

Table 1. Description of Edit-R Lentiviral sgRNA vector elements.

Vector Element	Utility
U6	Human RNA polymerase III promoter U6
sgRNA	Single guide RNA, a chimeric fusion of crRNA and tracrRNA
mCMV	Mouse cytomegalovirus immediate early promoter
$Puro^R$	Puromycin resistance marker permits antibiotic selection of transduced mammalian cells
5' LTR	5' Long Terminal Repeat necessary for lentiviral particle production and integration of the construct into the host cell genome
Ψ	Psi packaging sequence allows lentiviral genome packaging using lentiviral packaging systems
RRE	Rev Response Element enhances titer by increasing packaging efficiency of full-length lentiviral genomes
WPRE	Woodchuck Hepatitis Post-transcriptional Regulatory Element enhances transgene expression in target cells
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

Antibiotic resistance:

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

Table 2. Antibiotic resistances conveyed by Edit-R Lentiviral sgRNA plasmid.

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 Lentiviral sgRNA clones at 37 °C in LB broth medium plus 100 µg/mL carbenicillin.

1. Upon receiving your glycerol stock(s) containing the sgRNA(s), 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 Lentiviral sgRNA plasmid is 7.5 kb. See the vector map (Figure 1) 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.

Replication of plates:

Prepare target plates by dispensing ~ 160 µL of LB broth medium supplemented with 8% glycerol and 100 µg/mL carbenicillin.

Prepare Source Plates

1. Remove foil seals from the source plates while they are still frozen. This minimizes cross-contamination.
2. Thaw the source plates with the lid on. Wipe any condensation underneath the lid with a paper wipe soaked in ethanol.

Replicate

1. Gently place a disposable replicator in the thawed source plate and lightly move the replicator around inside the well to mix the culture. Make sure to scrape the bottom of the plate of the well.
2. Gently remove the replicator from the source plate and gently place in the target plate and mix in the same manner to transfer cells.
3. Dispose of the replicator.
4. Place the lids back on the source plates and target plates.

5. Repeat steps 1-4 until all plates have been replicated.
6. Return the source plates to the -80 °C freezer.
7. Place the inoculated target plates in a 37 °C incubator for 18-19 hours.
8. Freeze at -80 °C for long-term storage.

Transfection:

If the plasmid is transfected directly into cells for gene knockout, a Cas9 nuclease expression plasmid is also required for co-transfection unless a cell line stably expressing Cas9 nuclease is used. Consult our website for a list of available Edit-R Cas9 Nuclease expression plasmids.

Quantities and volumes should be scaled up according to the number of cells/well to be transfected (Table 3). This example is for co-transfection of equal amounts of Edit-R Lentiviral sgRNA and an Edit-R Cas9 Nuclease 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 0.5 µg each of Edit-R Lentiviral sgRNA and Cas9 Nuclease plasmid DNA (1 µg total 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 (GE Healthcare, Cat #T-2006-01) 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, cm²/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	$2.0\text{-}6.0 \times 10^4$ (1.0×10^5)	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.

Packaging lentiviral particles:

The Edit-R 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 or TLP5917). 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 (Kappes and Wu 2001). 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.

Edit-R Lentiviral sgRNA constructs do not express a fluorescent protein reporter. Therefore, 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 (Kutner *et al.* 2009).

Edit-R Lentiviral sgRNA vectors are not compatible with third generation packaging systems due to the requirement of the expression of *tat*, which third generation systems do not contain. We recommend the Trans-Lentiviral shRNA packaging system (Cat #TLP5912, #TLP5917) for use with Dharmacon lentiviral vectors.

Generation of a stable cell line:

For generation of a stable cell line expressing Cas9 nuclease and a sgRNA construct, we recommend using the Edit-R Lentiviral Cas9 Nuclease and sgRNA packaged into lentiviral particles format. See the Edit-R CRISPR-Cas9 Gene Engineering with Lentiviral Cas9 and sgRNA technical manual for protocols.

Additional safety information:

Historically, the greatest safety risk associated with a lentiviral delivery platform stems from the potential generation of recombinant viruses that are capable of autonomous replication. The Edit-R Lentiviral sgRNA minimizes these hazards to the greatest degree by combining a disabled lentiviral genome with the proprietary Trans-Lentiviral packaging process. Starting with the HXB2 clone of HIV-1 (GenBank, Accession #K03455), the lentiviral backbone has been modified to eliminate all but the most essential genetic elements necessary for packaging and integration (such as 5' LTR, Psi sequences, polypurine tracts, Rev responsive elements and 3' LTR). The resultant self-inactivating (SIN) vector greatly reduces the probability of producing recombinant particles and limits cellular toxicity often associated with expression of HIV genes.

Additional safety features can be incorporated by the packaging process itself. Generation of Edit-R Lentiviral sgRNA particles requires a packaging step during which the expression construct containing the silencing sequence is enclosed in a lentiviral capsid. Gene functions that facilitate this process (such as those encoded by the structural genes *gag, pol, env,* etc.) are distributed amongst multiple helper plasmids that do not contain significant regions of homology. This tactic further minimizes the probability of recombination events that might otherwise generate lentiviral particles capable of autonomous replication. Among commercially available lentiviral vector systems, the Trans-Lentiviral packaging system offers a superior safety profile as the packaging components are separated onto five plasmids. Additionally, expression of *gag-pro* and *tat-rev* are under the control of the conditional tetracycline-responsive promoter element (TRE), limiting expression of these lentiviral components strictly to the packaging cell line. A detailed description of the Trans-Lentiviral packaging system can be found in Wu *et al*. 2000.

With these safety measures in place, Edit-R Lentiviral sgRNA particles can be employed in standard Biosafety Level 2 tissue culture facilities.

Any investigator who purchases Dharmacon lentiviral vector products is responsible for consulting with their institution's health and biosafety group for specific guidelines on the handling of lentiviral vector particles. Further, each investigator is fully responsible for obtaining the required permissions for the acceptance of lentiviral particles into their local geography and institution.

- In the U.S., download 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), here.
- See also: NIH Guidelines for Research Involving Recombinant DNA Molecules (NIH Guidelines), downloadable here.
- For Biosafety Considerations for Research with Lentiviral Vectors, see the NIH OBA website.

Frequently asked questions (FAQs):

Question	Answer
Where can I find the sequence of an individual sgRNA construct?	The sequence of an individual sgRNA construct is provided on the product insert and can be found online under your account Order History at dharmacon.gelifesciences.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' The sgRNA sequence is: GN_{19}GTTTTAGAGCTAGAAATAGCAAGTTAAAATAAGGCTAGTCCG TTATCAACTTGAAAAAGTGGCACCGAGTCGGTGCTTTTTTT Where N_{19} is your target sequence.
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 (e.g., HEK293T Cat #HCL4517). We recommend the Trans-Lentiviral shRNA packaging kit (Cat #TLP5912 or TLP5917). This packaging kit allows creation of a replication-incompetent (Zufferey *et al.* 1998), HIV-1-based lentiviral particle that can be used to deliver and express your sgRNA of interest in either dividing or non-dividing mammalian cells. The Trans-Lentiviral packaging kit is based on the trans-lentiviral system developed by Kappes (Kappes and Wu 2001). For protocols and information on packaging lentiviral sgRNA with our Trans-Lentiviral shRNA packaging kit, please see the product manual available here.
Can I use any 2nd generation packaging system with the Edit-R Lentiviral sgRNA vector?	The Edit-R Lentiviral sgRNA vector is Tat dependent, so a packaging system that expresses the *tat* gene is required. We recommend the Trans-Lentiviral shRNA packaging kit (Cat #TLP5912 or TLP5917), which utilizes HEK293T cells.
Where can I purchase puromycin?	Puromycin is available from Fisher Scientific Cellgro (Cat #BP2956-100) or Invivogen (Cat #ant-pr-1).
How many transfections are available in each volume size of DharmaFECT kb transfection reagent?	The number of transfections that can be performed depends on the size of the culture dish used and the volume size of DharmaFECT kb transfection reagent purchased. For example, if you purchase 1 mL of DharmaFECT kb transfection reagent, then you can perform 333 transfections wells in 24-well plate format. See Table 3 for additional information.

For additional frequently asked questions (FAQs), please visit here.

Troubleshooting:

For help with transfection or transduction of your lentiviral constructs, please email technical support at ts.dharmacon@ge.com with the answers to the questions below, your sales order or purchase order number and the catalog number or clone ID of the construct with which you are having trouble.

1. Are you using direct transfection or transduction into your cell line?
2. What was the 260/280 ratio of DNA? Over 1.8?
3. What was the transfection efficiency if you used direct transfection? What transfection reagent was used?
4. Were positive and negative controls used (such as our PPIB or DNMT3B validated positive control and the validated non-targeting negative control)?
5. What were the results of the controlled experiments?
6. How was knockout measured (for example, DNA mismatch detection assay or western blot analysis)?
7. What is the abundance and the half-life of the protein? Does the protein have many isoforms?
8. What packaging cell line was used if you are using transduction rather than transfection?
9. What was your lentiviral titer in your cells?
10. What was your MOI?
11. Did you maintain the cells in puromycin selection medium after transfection or transduction?
12. How much time elapsed from transfection/transduction to puromycin selection?

If transfection into your cell line is unsuccessful, you may need to consider the following list of factors influencing successful transfection.

1. Concentration and purity of plasmid DNA and nucleic acids—determine the concentration of your DNA using 260 nm absorbance. Avoid cytotoxic effects by using pure preparations of nucleic acids.
2. Insufficient mixing of transfection reagent or transfection complexes.
3. Transfection in serum-containing or serum-free medium—our studies indicate that the transfection efficiency with DharmaFECT transfection reagent is equally high in the presence of serum. This is not the case with other transfection reagents.
4. We do not recommend antibiotics (e.g., pen-strep) in the transfection complexing medium.
5. Cell history, density, and passage number—it is very important to use healthy cells that are regularly passaged and in growth phase. The highest transfection efficiencies are achieved if cells are plated the day before; however, adequate time should be given to allow the cells to recover from the passaging (generally > 12 hours). Plate cells at a consistent density to minimize experimental variation. If transfection efficiencies are low or reduction occurs over time, thawing a new batch of cells or using cells with a lower passage number may improve the results.

References:

Cited references and additional suggested reading

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2. Jinek, M., Chylinski, K., *et al.* (2012). A programmable dual-RNA-guided DNA endonuclease in adaptive bacterial immunity. *Science* **337**, 816-821.
3. Kappes, J.C., and Wu, X. (2001). Safety considerations in vector development. *Somat. Cell Mol. Genet.* **26**, 147-58.
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7. Wu, X., Wakefield, J.K., *et al.* (2000). Development of a novel trans-lentivral vector that affords predictable safety. *Mol. Ther.* **2**, 47-55.
8. Zufferey, R., Dull, T., *et al.* (1998). Self-inactivating lentivirus vector for safe and efficient in vivo gene delivery. *J. Virol.* **72**, 9873-9880.

Label licenses:

The sgRNA and 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 gelifesciences.com/webapp/wcs/stores/servlet/catalog/en/GELifeSciences-us/about-us/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.

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