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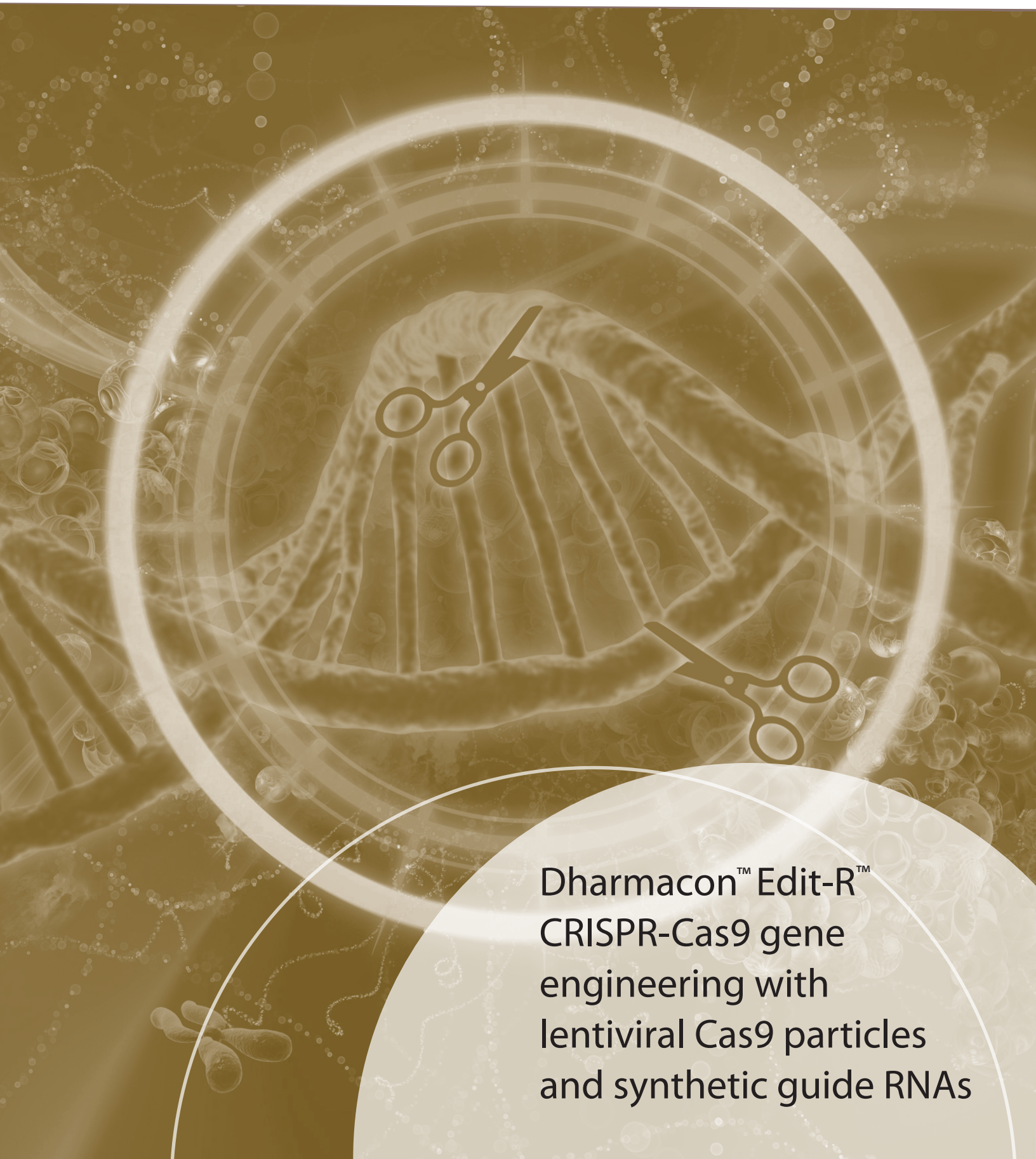

Dharmacon™ Edit-R™ CRISPR-Cas9 gene engineering with lentiviral Cas9 particles and synthetic guide RNAs

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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 homologous recombination (HR). NHEJ is often imperfect, resulting in small insertions and deletions (indels) that can cause nonsense mutations resulting in gene disruptions to produce gene knockouts.[4,5] 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.

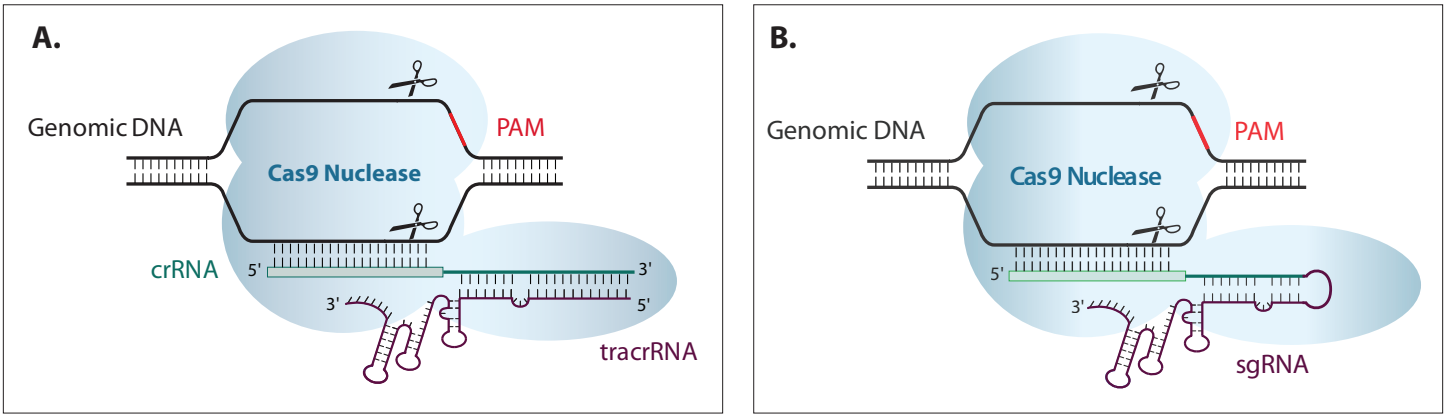

Figure 1. Illustration of CRISPR-Cas9 system. Cas9 nuclease (light blue), programmed by the crRNA (green):tracrRNA (purple) complex (A) or the sgRNA (B), cutting both strands of genomic DNA 5' of the PAM (red).

2 Gene editing using Edit-R Lentiviral Cas9 Nuclease particles with synthetic guide RNAs

The Edit-R platform includes these critical components based on the natural *S. pyogenes* system: a vector for Cas9 expression and chemically-synthesized guide RNA designed to the target site of interest. Synthetic guide RNA can be either a synthetic single guide RNA or a tracrRNA complexed with a gene-specific synthetic crRNA. To facilitate rapid generation of cell lines that constitutively express Cas9 nuclease, the Edit-R lentiviral Cas9 expression vector is packaged into particles, purified and concentrated for direct transduction. Subsequent transfection of synthetic guide RNA into Cas9-expressing cell lines results in a higher percentage of edited cells in comparison to co-transfection of a Cas9 expression plasmid DNA with guide RNA. Figure 2 summarizes a general experimental workflow to generate stable cell lines expressing the Cas9 nuclease that can then be transfected with synthetic guide RNAs for phenotypic analysis of gene knockout.

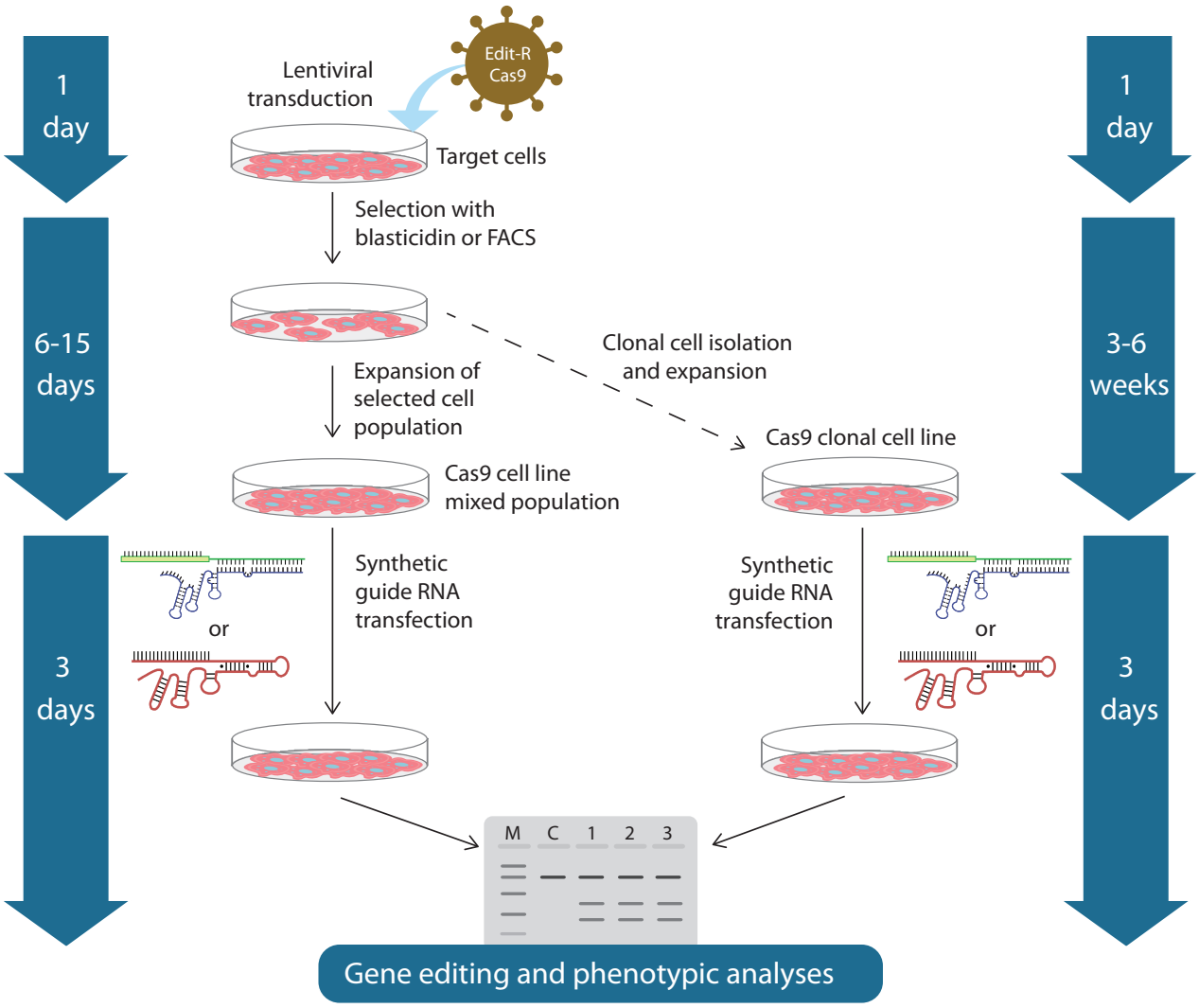

Figure 2. Gene knockout workflow using the Edit-R Lentiviral Cas9 Nuclease Expression particles with synthetic guide RNA. The workflow describes generating either a mixed cell population or clonally isolated Cas9-expressing cell line for transfection with guide RNA.

Edit-R Lentiviral Cas9 Nuclease Expression vectors

The Edit-R Lentiviral Cas9 Nuclease Expression vectors contain a human codon-optimized version of the *S. pyogenes cas9 (csn1)* gene and the blasticidin resistance marker (Blast^R). The Edit-R Lentiviral Fluorescent Cas9 Nuclease Expression vectors contain a human codon-optimized version of the *S. pyogenes cas9 (csn1)* and a fluorescent reporter (mKate2 or TurboGFP™). In both reporter vectors, expression is bicistronic with a 2A peptide sequence linker and under the control of a single promoter (Figure 3). Reporter genes are placed upstream of the Cas9 coding region rather than the 3' end so that no extra amino acids are added to the C-terminus, thus preserving the full Cas9 endonuclease activity. A brief description of the lentiviral vector elements are listed in Table 1.

Multiple promoter options are available (Figure 3) for selection of a lentiviral vector with the most active promoter for specific cells of interest. All Edit-R Lentiviral Cas9 Nuclease Expression vectors are supplied as lentiviral particles (≥ 1 × 10^7 TU/mL) or dried down, endotoxin-free plasmid DNA, ready for lentiviral packaging.

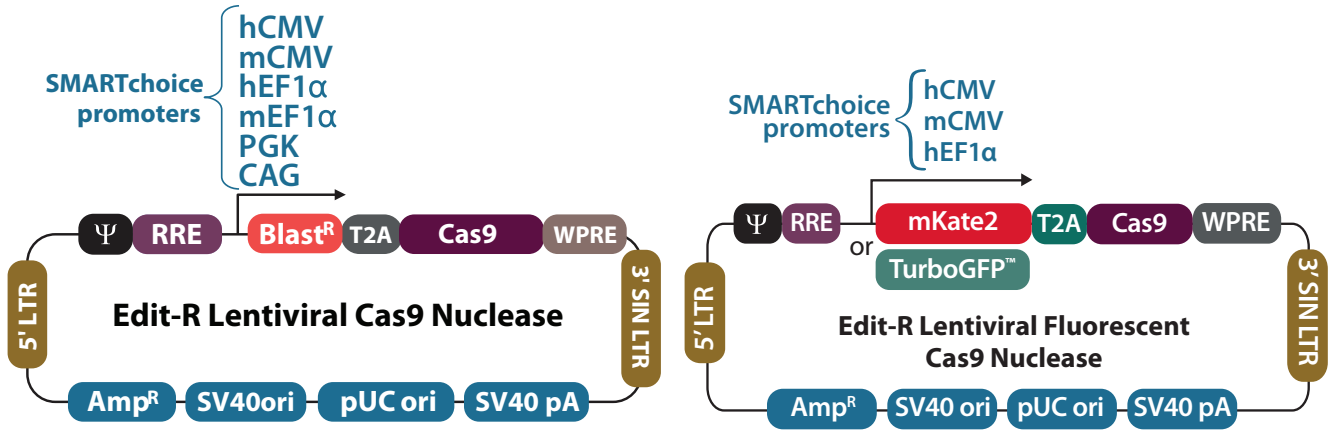

Figure 3. Schematic diagram of the Edit-R Lentiviral Cas9 Nuclease Expression vectors.

Table 1. Elements of the Edit-R Lentiviral Cas9 Nuclease Expression vectors.

Vector element	Utility
Cas9	Human codon-optimized *S. pyogenes* Cas9 nuclease for cleavage of targeted DNA when programmed with guide RNA
T2A	Self cleaving peptide allows for simultaneous expression of two proteins from a single transcript
Blast^R	Blasticidin resistance marker permits antibiotic selection of transduced mammalian cells
mKate2	Red fluorescent protein reporter enables selection of transduced mammalian cells by FACS
TurboGFP	Green fluorescent protein reporter enables selection of transduced mammalian cells by FACS
hCMV	Human cytomegalovirus immediate early promoter
mCMV	Mouse cytomegalovirus immediate early promoter
hEF1α	Human elongation factor 1 alpha promoter
mEF1α	Mouse elongation factor 1 alpha promoter
PGK	Mouse phosphoglycerate kinase promoter
CAG	Human cytomegalovirus, chicken β-actin hybrid promoter
5' LTR	5' Long Terminal Repeat necessary for lentiviral particle production and integration of the construct into the host cell genome
Ψ	Psi packaging sequence allows lentiviral genome packaging using lentiviral packaging systems
RRE	Rev Response Element enhances titer by increasing packaging efficiency of full-length lentiviral genomes
WPRE	Woodchuck Hepatitis Post-transcriptional Regulatory Element enhances transgene expression in target cells
3' SIN LTR	3' Self-inactivating Long Terminal Repeat for generation of replication-incompetent lentiviral particles
SV40 pA	Simian virus 40 polyadenylation signal
pUC ori	pUC origin of replication
SV40 ori	Simian virus 40 origin of replication
Amp^R	Ampicillin resistance gene for vector propagation in *E. coli* cultures

Edit-R synthetic guide RNA

Edit-R trans-activating CRISPR RNA (tracrRNA)

The Edit-R tracrRNA is a chemically synthesized and HPLC-purified, long RNA based on a published *S. pyogenes* tracrRNA sequence.[2] It is modified for nuclease resistance and can be used with modified or unmodified Edit-R crRNA.

Edit-R CRISPR RNA (crRNA)

The Edit-R crRNA is a synthetic RNA, comprised of 20 nucleotides identical to the genomic DNA target site, or protospacer, followed by a fixed *S. pyogenes* repeat sequence that interacts with the tracrRNA. The chosen protospacer sequence in the target genomic DNA must be immediately upstream of a PAM in the genomic DNA. The predominant *S. pyogenes* PAM nucleotide sequence is NGG. Pre-designed crRNAs for gene knockout are available for human, mouse and rat coding genes on dharmacon.horizondiscovery.com. Custom crRNAs can be designed and ordered using the Dharmacon CRISPR Design Tool. It is modified for nuclease resistance and can be used with modified or unmodified Edit-R tracrRNA.

Edit-R synthetic single guide RNA (sgRNA)

Edit-R synthetic sgRNA is a 100 nucleotide chimera fusing the crRNA and tracrRNA sequences with a 4 nt tetraloop sequence[3]. It is modified for nuclease resistance on both 5' and 3' ends of the molecule. Edit-R synthetic sgRNAs can be ordered through the Dharmacon CRISPR Design Tool.

3 Protocol for gene engineering using Edit-R Lentiviral Cas9 Nuclease and synthetic guide RNAs

In this workflow, lentiviral Cas9 nuclease is utilized to generate cell lines stably expressing Cas9 nuclease. These cells can then be transfected with synthetic guide RNAs to achieve efficient gene editing for phenotypic analyses in a population of cells or in isolated clonal cell lines.

Edit-R CRISPR-Cas9 gene engineering materials required

- Edit-R™ Lentiviral Cas9 Nuclease (choose one):
  - » Dharmacon Edit-R Lentiviral Cas9 Nuclease Expression vectors are provided as concentrated, purified lentiviral particles for immediate transduction or as endotoxin-free plasmid DNA for direct transfection into a packaging cell line and production of your own lentiviral particles (Table 2).

Table 2. Edit-R Lentiviral Cas9 Nuclease particles and plasmids with SMARTchoice promoter options and selection options

Edit-R™ Lentiviral Cas9 Nuclease Expression Particles & Plasmids with SMARTchoice promoter options			
Promoter	Selection	Particles Cat. No.	Plasmids Cat. No.
hCMV	Blast^R	VCAS10124	CAS10136
	mKate2	VCAS11869	CAS11877
	TurboGFP™	VCAS11868	CAS11876
mCMV	Blast^R	VCAS10125	CAS10137
	mKate2	VCAS11863	CAS11871
	TurboGFP™	VCAS11862	CAS11870
hEF1α	Blast^R	VCAS10126	CAS10138
	mKate2	VCAS11865	CAS11873
	TurboGFP™	VCAS11864	CAS11872
mEF1α	Blast^R	VCAS10127	CAS10139
PGK	Blast^R	VCAS10128	CAS10140
CAG	Blast^R	VCAS10129	CAS10141

- Synthetic targeting guide RNA (choose one):
  - » Edit-R synthetic crRNA and tracrRNA oligos:
    a. crRNA, predesigned for your gene of interest in a variety of sizes, or designed and ordered using the Dharmacon CRISPR Design Tool
    b. tracrRNA, 5, 20 or 50 nmol (Cat #U-002005-XX)

    or
  - » Edit-R synthetic sgRNA, custom ordering using the Dharmacon CRISPR Design Tool
- DharmaFECT Transfection Reagent options. Each formulation is chemically distinct to optimize delivery and viability across a wide variety of cell types.
  - » DharmaFECT 1 (Cat #T-2001-XX)
  - » DharmaFECT 2 (Cat #T-2002-XX)
  - » DharmaFECT 3 (Cat #T-2003-XX)
  - » DharmaFECT 4 (Cat #T-2004-XX)

Additional materials required

The following additional materials are required but not supplied:

- 10 mM Tris pH 7.4, nuclease-free buffer (Tris buffer) solution (Dharmacon, Cat #B-006000-100)
- Multi-well tissue culture plates or tissue culture dishes
- Blasticidin S (Fisher Scientific, Cat #BP2647-25; InvivoGen, Cat #ant-bl-1) or flow cytometer
- Assay for assessing cell viability such as Resazurin Cell Viability Reagent
- Assay(s) for detecting gene engineering events in a cell population
- Positive control guide RNA (researcher defined)
- Growth Medium: antibiotic-free cell culture medium (with serum and/or supplements) recommended for maintenance of the cells of interest
- Transduction Medium: the base cell culture medium containing lentiviral particles (with transduction additives and serum, if necessary)
- Selection Medium: Growth Medium supplemented with the appropriate concentration of blasticidin

Generation of stable cell line expressing Cas9 nuclease

The protocol described here is designed for rapid generation of a cell population where most of the cells have a single copy of a lentiviral Cas9 nuclease 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.

Blasticidin selection

The Edit-R Lentiviral Cas9 Nuclease Expression vectors confer resistance to blasticidin in transduced cells. Before transducing cells, determine the minimum concentration of blasticidin required to kill non-transduced cells between three and 10 days by generating a blasticidin kill curve. The blasticidin concentration range for many mammalian cells is 2-15 µg/mL.

FACS selection

The Edit-R Lentiviral Fluorescent Cas9 Nuclease vectors allow creation of stable cell lines through selecting for positive fluorescent cells using a flow cytometer. For optimal Cas9 gene editing, cell populations with high fluorescence should be collected. Additionally, single cells with high fluorescence can be sorted into 96-well plates for clonal cell line generation.

Transduction of cells with Edit-R Lentiviral Cas9 Nuclease Expression particles

The protocol below provides the basic steps for transduction of the lentiviral particles into U2OS cells (as an example) using serum-free medium in a 24-well plate. Optimal transduction conditions vary widely amongst cell types and must be determined empirically for each cell line of interest.

If a different sized culture dish is used, adjust the number of cells, volumes, and reagent quantities in proportion to the change in surface area (see Appendix for suggested volumes of Transduction Medium per surface area of culture dishes).

Day 1:

1. Plate 5×10^4 cells per well in a 24-well plate using Growth Medium.

Optimal cell number for plating will vary with growth characteristics of specific cells and should be determined experimentally. Typically cells should be at 60-80% 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 Note below).

The functional titer of Edit-R Lentiviral Cas9 Nuclease Expression 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. The relative transduction efficiency of your cell type will likely be lower than that of HEK293T cells.

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 = lentiviral 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 = 1×10^7 TU/mL

Then,

$V = 0.3$ TU/cell $\times$ 100 000 cells/well $\div 1 \times 10^7$ TU/mL $\times$ 1000 = 3 µL of lentiviral stock per well.

3. Thaw the Edit-R Lentiviral Cas9 Nuclease Expression 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 base cell medium (no serum) to create 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).

Days 3-15:

Generation of stably expressing Cas9 cell lines can be achieved by two different methods, depending on the Edit-R Cas9 lentivirus used during the experiment:

Method 1 using Edit-R Lentiviral Fluorescent Cas9 Nuclease

1. At 72 hours post-transduction expand cells into a larger dish and continue to passage until enough cells are collected for cell sorting (500,000-10 million cells depending on generation of clonal cell line or population).
2. Prepare cells for FACS analysis according to commonly used protocols.
3. Sort cells as desired.

We recommend sorting the cells into fractions with low, medium, and high fluorescence expression levels. Additionally, ensure that each fraction has enough of the total cell population to be easily expanded for downstream applications and testing. Alternatively, single cells with high fluorescence can be sorted into 96-well plates for clonal cell line generation.

4. Once the sorted cells are growing normally, expand accordingly to freeze enough aliquots for your experimental project. Unless single cells were isolated, these cell lines will be a mixed population that on average have a single integration of Cas9 in their genomes.

Method 2 using Edit-R Lentiviral Cas9 Nuclease with blasticidin selection

1. At 24-48 hours post-transduction, replace the Transduction Medium with Selection Medium (Growth Medium containing the appropriate amount of blasticidin).

The appropriate antibiotic concentration is specific to each cell line and should be determined experimentally prior to selection using a kill curve. Blasticidin usually kills cells between 3 and 10 days, slow growing cells may take longer. If the cells become confluent, split the cells into a larger dish to allow proper blasticidin selection (for example, split cells from 24-well to 6-well culture dishes).

2. Once the selected cells are growing normally, expand accordingly to freeze enough aliquots for your experimental project. These cells will be a mixed population that on average have a single integration of Cas9 in their genomes.

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

Utilize the mixed population of Cas9-expressing cells obtained above for transfection with synthetic guide RNA and subsequent phenotypic knockout analysis for your gene of interest. If clonal cell lines are required for your application, we recommend that you isolate clonal cell lines for downstream experiments using protocols appropriate for your cells of interest.

Transfection of synthetic guide RNA

The following is an example protocol to deliver Edit-R synthetic sgRNA or Edit-R synthetic crRNA complexed with tracrRNA into adherent U2OS cells stably expressing Cas9 in 24-well plates.

Day 1:

1. Plate 1×10^5 U2OS-Cas9 cells per well in a 24-well plate using Growth Medium.

Optimal cell number for plating will vary with growth characteristics of specific cells and should be determined experimentally.

Day 2:

1. Prepare 2 µM synthetic guide RNA transfection complex from previously prepared 10 µM stocks.
   - » For crRNA:tracrRNA add 2 µL of 10 µM crRNA and 2 µL of 10 µM tracrRNA to 6 µL of Tris buffer (total volume is 10 µL).

     or

   - » For synthetic sgRNA add 2 µL of 10 µM synthetic sgRNA to 8 µL of Tris buffer (total volume is 10 µL).
2. In nuclease-free microcentrifuge tubes prepare the synthetic guide RNA complex by adding 6.25 µL of 2 µM guide RNA complex to 43.75 µL of serum-free medium (see Table 3 for volume recommendations for alternative plating formats).
3. Prepare a DharmaFECT 3 working solution in a separate tube by diluting 1 µL of DharmaFECT 3 reagent in 49 µL of serum-free medium and mixing gently. Incubate the tube for 5 minutes at room temperature.

The optimal DharmaFECT Transfection Reagent and concentration varies for different cell lines and is affected by the cell density. Easy-to-transfect cells and lower cell densities typically require a lower amount of DharmaFECT Transfection Reagent. For replicates, please prepare sufficient sample volumes for the number of replicates and to account for pipetting error.

4. Add 50 µL DharmaFECT working solution to each sample tube containing the crRNA:tracrRNA complex. This brings the total volume to 100 µL.
5. Immediately mix by pipetting gently up and down and incubate for 20 minutes at room temperature.
6. Prepare the Transfection Medium by adding 400 µL antibiotic-free complete Growth Medium to each sample to bring the total volume in each tube to 500 µL.
7. Remove medium from the wells of the 24-well plate containing cells and replace with 500 µL of the appropriate Transfection Medium to each well.
8. Incubate cells at 37 °C in a humidified CO_2 incubator for 48 to 72 hours before proceeding with the phenotypic assay or gene editing analysis (see Appendix). If homogeneous clonal cells are required, proceed with clonal cell isolation using the preferred protocol.

Table 3. Suggested volumes per well for transfection of Cas9-expressing cells with 25 nM synthetic guide RNA.

		Tube 1: Synthetic guide RNA (µL/well)		Tube 2: Diluted DharmaFECT transfection reagent (µL/well)			
Plating format (wells/plate)	Well surface area (cm^2/well)	2 µM synthetic guide RNA	Serum-free medium (µL)	DharmaFECT (µL)	Serum-free medium (µL)	Growth medium (µL/well)	Final transfection volume (µL/well)
96	0.3	1.25	8.75	0.2	9.8	80	100
24	2	6.25	43.75	1	49	400	500
12	4	12.5	87.5	2	98	800	1000
6	10	25	175	5	195	1600	2000

4 Packaging Lentiviral Cas9 Nuclease plasmids into particles

Edit-R Lentiviral Cas9 Nuclease Expression plasmids are Tat dependent and requires a packaging system that expresses the *tat* gene. For packaging lentiviral constructs, we recommend the Dharmacon Trans-Lentiviral ORF Packaging System. For packaging protocols and additional information please consult the product manual.

Edit-R Lentiviral Blast-Cas9 Nuclease Plasmids do not express a fluorescent protein reporter, therefore, after packaging plasmid DNA, we recommend titering the lentiviral particles produced using a functional lentiviral titration protocol such as limiting dilution with cell viability assay by crystal violet staining or genomic qPCR assay.[14] Edit-R Lentiviral Fluorescent Cas9 Nuclease Expression plasmids have a fluorescent protein reporter and therefore, can be used to determine functional lentiviral titer in cells through observing fluorescence.

5 Appendix

Optimization of transfection conditions for delivery of Edit-R synthetic guide RNA

To obtain the highest transfection efficiency of the synthetic guide RNA with minimal effects on cell viability, we recommend carefully optimizing transfection conditions for each cell line using a synthetic control guide RNA. The transfection optimization can be easily performed in a 96-well format allowing for testing of multiple transfection conditions. Transfection conditions that have previously been optimized for small RNA delivery are a reasonable starting point for guide RNA complex transfection.

The optimization experiment should include two to three cell densities and a range of DharmaFECT Transfection Reagent volumes. Our recommended ranges for the different components are as follows:

- 0.05 to 0.8 µL/well of a 96-well plate DharmaFECT 1, 2, 3 or 4
- 25 nM control guide RNA complex per well

At 48 to 72 hours post-transfection, perform a cell viability assay to determine the highest lipid concentration that has minimal cell toxicity (≥ 70% of cell viability is preferred). After assaying for cell viability, we recommend to carefully wash the cells once with Phosphate Buffered Saline (PBS) and proceed with gene editing analysis (see below) to determine the condition that produces good editing efficiency. Use the optimal determined conditions for subsequent transfection of your selected Cas9-expressing cell lines with the Edit-R synthetic guide RNA.

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.[10,11,12] When edited cells are expanded and clonal populations isolated, the most commonly used method for confirming gene editing is Sanger sequencing.[11]

Volume of medium per surface area in culture dishes

Table 4. Suggested volumes of Transduction Medium for different plate formats.

Tissue culture dish	Surface area per well (cm^2)	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 Lentiviral Cas9 Nuclease Expression 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.

Plasmid DNA

Edit-R Lentiviral Cas9 Nuclease Expression plasmid DNA are shipped as dried pellets at room temperature. Under these conditions, they are stable for at least four weeks. Upon receipt, plasmid DNA should be stored at -20 °C to -80 °C. Under these conditions, the reagents are stable for at least one year. Always dissolve plasmid in nuclease-free solution, such as Tris buffer.

Synthetic guide RNA

Edit-R synthetic guide RNA reagents are shipped as dried pellets at room temperature. Under these conditions, they are stable for at least four weeks. Upon receipt, synthetic guide RNA should be stored at -20 °C to -80 °C. Under these conditions, the reagents are stable for at least one year.

Always resuspend synthetic guide RNA in nuclease-free Tris buffer. In solution and stored at -20 °C, the reagent aliquots are stable for at least one year. Avoid multiple freeze-thaw cycles. We recommend not exceeding four to five freeze-thaw cycles to ensure RNA integrity.

6 Frequently asked questions

How do I choose between the various Edit-R Lentiviral Cas9 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 using multiple lentiviral promoter-Cas9 constructs or selecting the best promoter empirically using the Dharmacon SMARTchoice Promoter Selection Plate Cat #SP-001000-01.

How should I store my synthetic guide RNAs?

RNA oligonucleotides should be stored at -20 °C or -80 °C in a non-frost free freezer, either as a dried pellet or resuspended in an RNase-free solution buffered to pH 7.4 to help with stability during freeze-thaw cycles. We recommend that RNA oligonucleotides be resuspended to a convenient stock concentration (Synthetic guide RNA resuspension protocol) and stored in small aliquots to avoid multiple freeze-thaw cycles. RNA oligonucleotides should not go through more than four to five freeze thaw cycles. If degradation is a concern, the integrity of the RNA oligonucleotides can be evaluated on an analytical PAGE gel.

Can I use my siRNA transfection protocols to transfect Edit-R synthetic guide RNAs?

Previously optimized protocols to transfect synthetic siRNA into your cells of interest can be a good starting point for transfection of synthetic guide RNA.

Can I use a different transfection reagent other than DharmaFECT to deliver the Edit-R components into my cells?

We cannot predict the transfection ability of other transfection reagents, nor can we troubleshoot experiments performed with any reagent other than DharmaFECT Transfection Reagents. However, other suitable transfection reagents designed for RNA transfection could be utilized provided transfection conditions are carefully optimized for each cell line of interest.

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 recommended to confirm the gene knockout.

Can the Edit-R lentiviral Cas9 system be used for gene knockout in non-mammalian organisms, such as flies or worms?

We have designed the Edit-R Cas9 plasmids and lentiviral particles for mammalian expression (and thus have only tested) in mammalian cells. We cannot predict the efficacy of using Edit-R lentiviral Cas9 particles and Edit-R synthetic guide RNA components, nor can we troubleshoot experiments performed in non-mammalian systems.

Can I co-transfect the Edit-R synthetic guide RNA components with the Edit-R Lentiviral Cas9 Nuclease Expression plasmid that I bought as DNA rather than making lentiviral particles?

The Edit-R Lentiviral Cas9 Nuclease Expression vectors are intended to be used as lentiviral particles to generate stable cell lines expressing Cas9. If you intend to perform genome engineering by transient transfection, we recommend the non-lentiviral Edit-R Cas9 Nuclease Expression plasmids, which are smaller in size and intended for co-transfection with the synthetic guide RNAs.

Where can I obtain the Edit-R lentiviral Cas9 expression vector maps?

Cas9 expression plasmid maps can be obtained upon request from Technical Support (ts.dharmacon@horizondiscovery.com) or +1 800 235 9880 toll-free; 303 604 9499).

What filters are recommended for sorting cells using mKate2 or TurboGFP™?

mKate2 has an excitation maximum of 588nm and emission maximum of 633nm. Recommended Omega Optical filter sets are QMAX-Red, XF102-2, Texas red, or similar. TurboGFP™ has an excitation maximum of 482nm and emission maximum of 502nm. Recommended filter sets are EGFP, FITC, and other green dyes. Recommended Omega Optical filter sets are QMAX-Green, XF100-2, XF100-3, XF115-2, and XF116-2.

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. Y. Fu, *et al.* High-frequency off-target mutagenesis induced by CRISPR-Cas nucleases in human cells. *Nat. Biotechnol.* **31**, 822-826 (2013).
7. P.D. Hsu, *et al.* DNA targeting specificity of RNA-guided Cas9 nucleases. *Nat. Biotechnol.* **31**, 827-832 (2013).
8. T. Wang *et al.* Genetic screens in human cells using the CRISPR-Cas9 system. *Science.* **343**, 80-84 (2014).
9. A.R. Bassett *et al.* Highly efficient targeted mutagenesis of Drosophila with the CRISPR/Cas9 system. *Cell Rep.* **4**, 220-228 (2013).
10. D.Y. Guschin, *et al.* A rapid and general assay for monitoring endogenous gene modification. *Methods Mol. Biol.* **649**, 247-256 (2010).
11. 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).
12. L. Cong, *et al.* Multiplex genome engineering using CRISPR/Cas systems. *Science.* **339**, 819-823 (2013).
13. J.C. Kappes, X. Wu and J.K. Wakefield. Production of trans-lentiviral vector with predictable safety. *Methods Mol. Med.* **76**, 449-465 (2003).
14. R.H. Kutner, X. Y. Zhang and J. Reiser. Production, concentration and titration of pseudotyped HIV-1-based lentiviral vectors. *Nat. Protoc.* **4**, 495-505 (2009).

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 the following lentiviral particle products:

- Edit-R™ Lentiviral CRISPR-Cas9 Nuclease Expression particles

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 to 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).

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 using and the acceptance of replication incompetent SIN lentiviral vectors and replication-defective lentiviral particles into their local jurisdiction and institution.

For questions concerning the design or production of the products, please contact our Molecular Biology 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 (https://osp.od.nih.gov/wp-content/uploads/Lenti_Containment_Guidance.pdf);
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) (http://osp.od.nih.gov/office-biotechnology-activities/biosafety/nih-guidelines).

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 (GentechnikSicherheitsverordnung) of Safety Level 2* or higher have been assigned to the handling of the above-mentioned 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 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

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