TECHNICAL MANUAL

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INSPIRED CELL SOLUTIONS

CRISPR-Cas9 Genome Engineering with Dharmacon™ Edit-R™ Inducible Lentiviral Cas9 Nuclease

Contents

1 Introduction to the CRISPR-Cas9 system for genome engineering

A. 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]

B. 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 named 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.

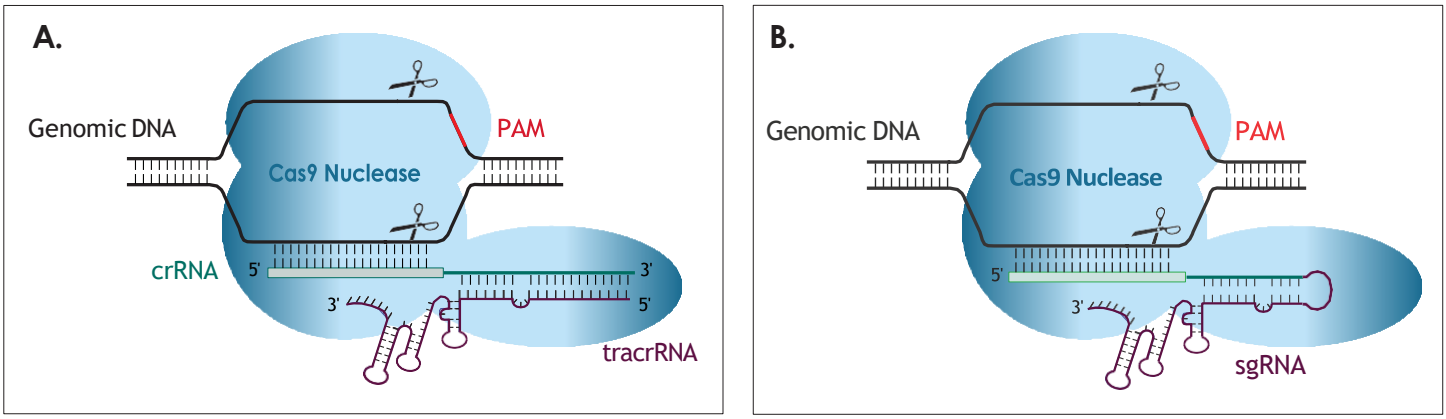

Figure 1. Illustration of CRISPR-Cas9 system. Cas9 nuclease (light blue), programmed by the crRNA (green):tracrRNA (blue) complex (A) or the sgRNA (B), cutting both strands of genomic DNA 5' of the PAM (red).

2 Genome engineering with Edit-R Inducible Lentiviral Cas9 Nuclease

Genome editing with the Edit-R Inducible Lentiviral Cas9 requires two critical components based on the *S. pyogenes* system: a vector for inducible Cas9 expression and a locus-specific vector for sgRNA expression designed to the target site of interest. Edit-R Inducible Lentiviral Cas9 is available as purified and concentrated lentiviral particles, ready for transduction in the cells of interest and easy generation of an inducible Cas9-expressing cell line. This vector is also available as endotoxin-free plasmid DNA for lentiviral packaging. Gene-specific Edit-R Lentiviral sgRNA or Edit-R Lentiviral sgRNA Pooled Libraries are provided as purified and concentrated lentiviral particles for single gene knockout or pooled screening experimental projects, respectively.

A. Edit-R Inducible Lentiviral Cas9 Nuclease for temporal control of Cas9 expression and gene editing

Edit-R Inducible Lentiviral Cas9 Nuclease reagent is designed to provide the researcher with an effective way to control the time of expression of the Cas9 nuclease in the cell of interest with an optimized doxycycline inducible system. The Edit-R Inducible Lentiviral Cas9 expression vector contains a human codon-optimized version of the *S. pyogenes cas9 (csn1)* gene under the control of a doxycycline inducible promoter **(TRE3G, Figure 2A)**. This platform is based on the Tet-On® 3G bipartite induction system: a tightly regulated system consisting of an optimized inducible RNA polymerase II promoter (TRE3G), which provides both minimal basal expression and potent activation upon induction with doxycycline.[6] The Tet-On 3G transactivator is constitutively expressed with the blasticidin resistance gene (Blast^R) as a bicistronic transcript with a 2A peptide sequence **(Figure 2A)** for selection of stable cells with the inducible Cas9 construct integration. In the presence of doxycycline, the constitutively expressed Tet-On 3G transactivator protein binds to the TRE3G promoter and activates the expression of Cas9 nuclease that is also encoded within the Edit-R Inducible Lentiviral Cas9 vector. Together, the Tet-On 3G protein and TRE3G promoter permit tight regulation of the Cas9 expression, including potent induction, even at the low doxycycline doses that are required for *in vivo* experiments.

B. Edit-R Lentiviral sgRNA

The Edit-R Lentiviral sgRNA vector **(Figure 2B)** provides the gene-specific sgRNA required to direct Cas9 nuclease activity and is expressed under the control of the human U6 promoter. The Edit-R Lentiviral sgRNA vector expresses the puromycin resistance gene (Puro^R) driven by the mouse CMV promoter allowing for rapid selection of cells with integrated sgRNA. The functional elements for both Cas9 and sgRNA lentiviral vectors are listed and described in **Table 1**.

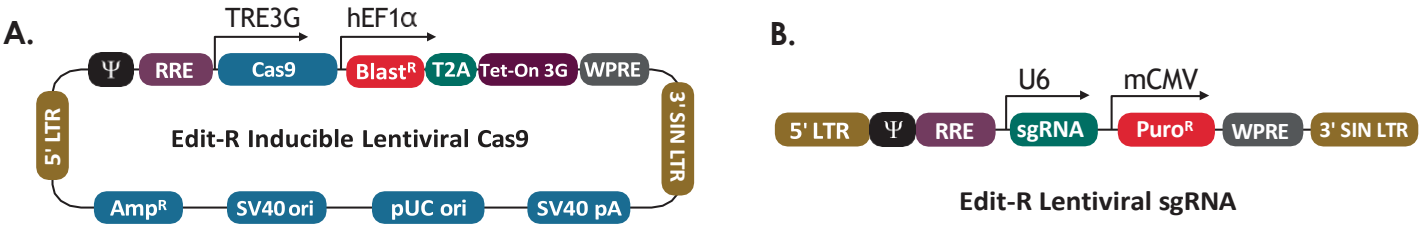

Figure 2. Schematic maps of the Edit-R Inducible Lentiviral Cas9 Nuclease (A) and sgRNA (B) vectors.

Table 1. Elements of the Edit-R Inducible Lentiviral Cas9 Nuclease and sgRNA expression vectors.

Vector Element	Utility
Cas9	Human codon-optimized S. pyogenes Cas9 nuclease for cleavage of targeted DNA when programmed with a sgRNA
TRE3G	Inducible promoter with Tetracycline Response Elements which is activated by the Tet-On 3G protein in the presence of doxycycline
hEF1α	Human elongation factor 1 alpha short promoter
Blast^R	Blasticidin resistance marker permits antibiotic selection of transduced mammalian cells
T2A	Self-cleaving peptide allows for simultaneous expression of blasticidin resistance and Cas9 proteins from a single transcript
Tet-On 3G	Tetracycline-regulated transactivator protein that binds to TRE3G promoter only in the presence of doxycycline.
U6	Human RNA polymerase III promoter U6
sgRNA	Chimeric form of the crRNA and tracrRNA that provides Cas9 with the DNA target site recognition
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

Each Edit-R predesigned lentiviral sgRNA is specific to the gene or genomic site of interest. The target-specific region of the sgRNA is comprised of 19-20 nucleotides identical to the genomic DNA (gDNA) target site, or protospacer, 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 (protospacer-adjacent motif). The predominant *S. pyogenes* PAM nucleotide sequence is NGG. Predesigned gene-specific sgRNAs can be ordered by searching for genes of interest on horizondiscovery.com or custom designed using the Dharmacon CRISPR Design Tool.

C. Overview of gene knockout workflow with Edit-R Inducible Lentiviral Cas9 Nuclease

Once inducible Cas9 cell lines are generated, these cells are transduced with gene-specific sgRNA lentiviral particles and subsequent gene knockouts can be obtained by treatment with doxycycline at a predefined concentration (see **Doxycycline induction** on page 10). Figure 3 summarizes general experimental workflows to generate stable cell lines carrying the inducible Cas9 followed by transduction with sgRNA lentiviral particles and induction with doxycycline for phenotypic analysis of gene knockout.

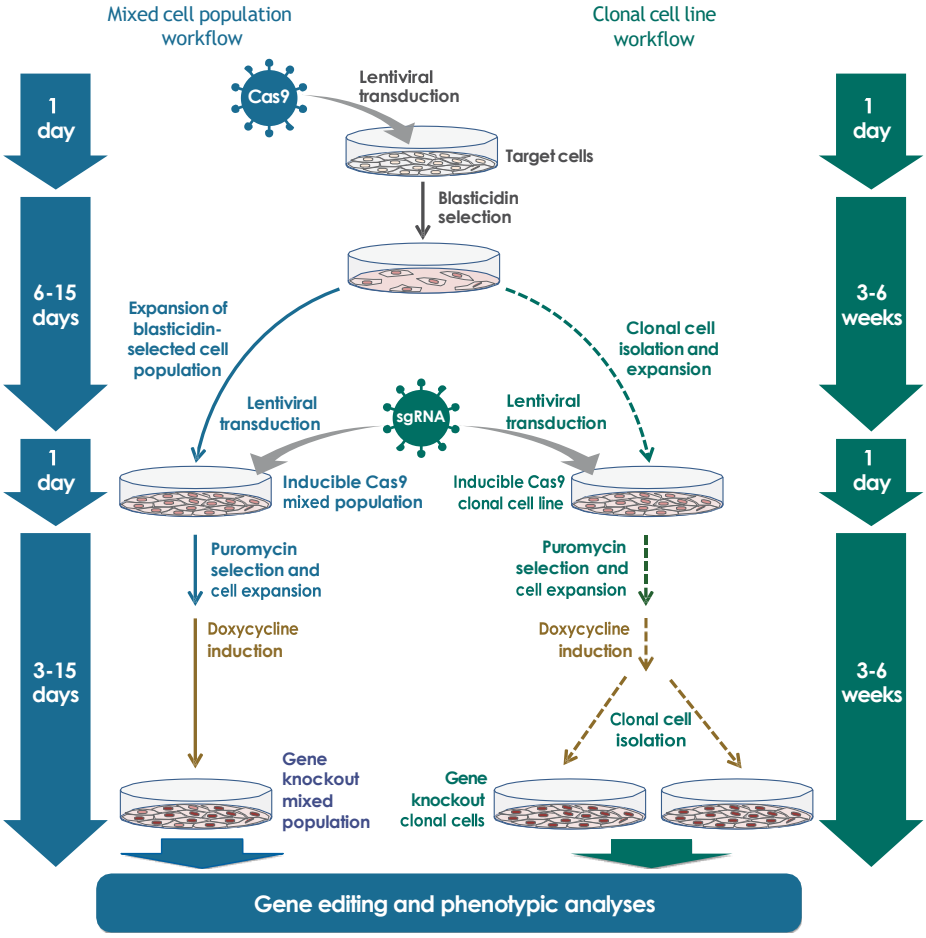

Figure 3. Gene knockout workflows using the Edit-R Inducible Lentiviral Cas9 with sgRNA system. Gene editing with Edit-R Inducible Lentiviral Cas9 and sgRNA can be done by induction of Cas9 expression with doxycycline following a mixed cell population approach (left side) or using isolated clonal cells lines (right side) when a defined genotype is required on each step for the phenotypic analysis.

D. Overview of gene knockout screening with Edit-R Inducible Lentiviral Cas9 Nuclease and Edit-R Lentiviral sgRNA Pooled Libraries

Gene knockout screens with the Edit-R Inducible Lentiviral Cas9 follows similar workflow and protocols of constitutive Edit-R Lentiviral Cas9 Nuclease with Edit-R Lentiviral sgRNA Pooled Libraries except for the additional step of doxycycline induction. Basic transduction and assay conditions need to be optimized prior to transduction with the Edit-R Lentiviral sgRNA Pooled Library, including the optimal concentration of doxycycline for the cell line of interest. Starting with the generation of stable cell lines by transduction with the Edit-R Inducible Lentiviral Cas9 particles, the expanded stable cell line population is subsequently transduced at low multiplicity of infection (MOI) with Edit-R Lentiviral sgRNA Pooled Library particles **(Figure 4)**. Individual cells in the resulting transduced population will contain single constructs integrated into their genomes. The experimental cell population **(Experimental sample, Figure 4)** is then induced with doxycycline for at least 72 hours and subjected to a selective pressure such that those constructs creating a gene disruption and a specific biological response can be identified. As a result of the selective pressure, cells expressing Cas9 and the sgRNA construct are either enriched or depleted in the cell population. The enriched/depleted sgRNAs “hits” are identified from isolated gDNA by PCR amplification with the Edit-R Pooled sgRNA Forward and Reverse Index PCR primers and analyzed by high-throughput sequencing on Illumina instrumentation. Direct identification of sgRNA sequences facilitates data analysis and ensures accurate target gene identification. See Edit-R Lentiviral sgRNA Pooled Screening Libraries for detailed information.

E. Overview of gene knockout with Edit-R Inducible Lentiviral Cas9 Nuclease and Edit-R synthetic crRNA:tracrRNA

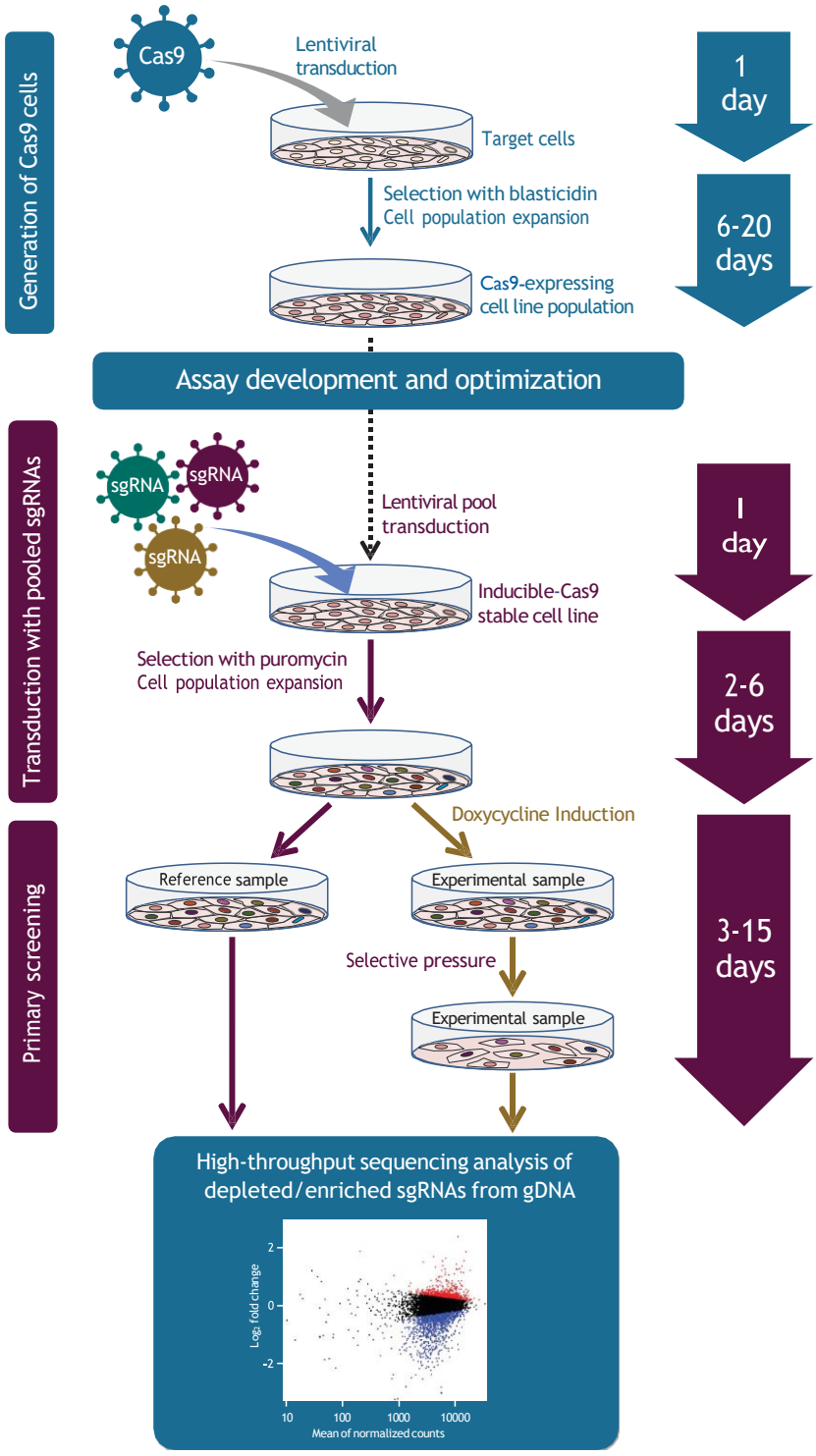

Figure 4. Gene knockout screening workflow using the Edit-R Lentiviral sgRNA Pooled Screening Library platform. An inducible Cas9 stable cell line (mixed or clonal cell population) is first generated with Edit-R Inducible Lentiviral Cas9 particles by selection with blasticidin. These cells are then transduced with lentiviral sgRNA pooled library and selected with puromycin. Transduced cells are split into reference and experimental populations for induction with doxycycline and application of a selective pressure and/or phenotypic selection. Genomic DNA is then isolated from the reference and experimental populations of transduced cells. Edit-R Pooled sgRNA Indexing PCR primers are used to amplify integrated constructs and add Illumina flow cell binding sequences. The resulting amplicons are sequenced on Illumina platform sequencers, using the Edit-R Pooled sgRNA Read 1 and Index Read Sequencing primers. The integrated sgRNA sequences in both reference and experimental samples are identified and relative abundance compared. sgRNA constructs that are enriched or depleted are identified as hits to be confirmed and studied further using individual Edit-R Lentiviral sgRNAs or synthetic crRNA:tracrRNA in additional phenotypic and/or biochemical assays.

Once inducible Cas9 cell lines are generated, these cells are induced with doxycycline at a predefined concentration at the experimentally optimal time and subsequently transfected with gene-specific Edit-R crRNA and tracrRNA and subsequent gene knockouts can be obtained. See the CRISPR-Cas9 Gene Engineering with Lentiviral Cas9 Particles and Edit-R Synthetic guide RNAs technical manual for more detailed protocols for synthetic crRNA:tracrRNA delivery. Figure 5 summarizes general experimental workflows to generate stable cell lines carrying the inducible Cas9 followed by induction with doxycycline and transfection with synthetic crRNA:tracrRNA for phenotypic analysis of gene knockout.

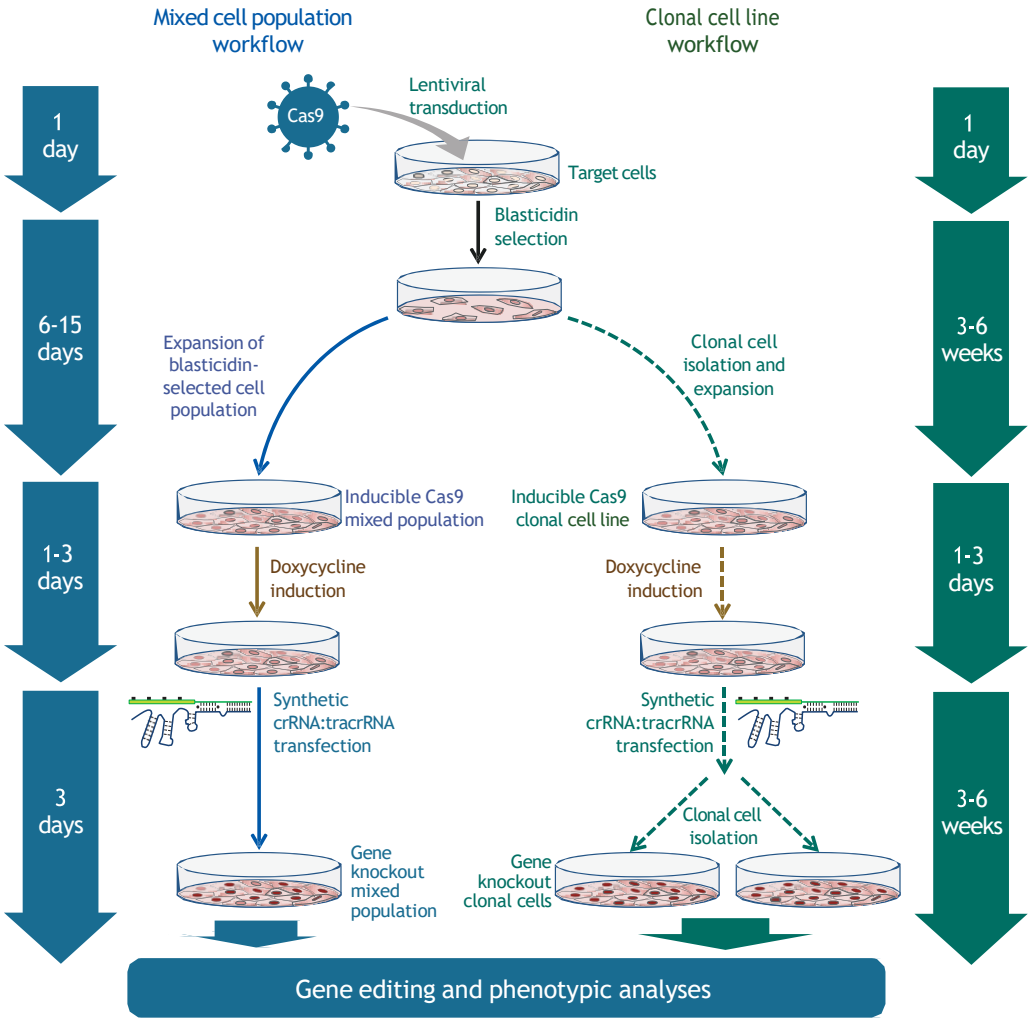

Figure 5: Gene knockout workflow using the Edit-R Inducible Lentiviral Cas9 particles with Edit-R synthetic crRNA and tracrRNA. The workflow describes generating either a mixed cell population or clonally isolated Cas9-expressing cell line for subsequent transfection with crRNA:tracrRNA.

3 Protocols for genome engineering using Edit-R Inducible Lentiviral Cas9 Nuclease

The Edit-R Inducible Lentiviral Cas9 expression particles are utilized to generate cell lines stably expressing Cas9 nuclease. These cells can subsequently be transduced with Edit-R Lentiviral sgRNA particles and later induced with doxycycline to achieve efficient gene editing for phenotypic analyses in a population of cells or in isolated clonal cell lines.

A. Materials required

Edit-R Inducible Lentiviral Cas9 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. Edit-R Lentiviral sgRNA particles are provided as concentrated, purified lentiviral particles for direct transduction.

- Edit-R Inducible Lentiviral hEF1α-Blast-Cas9 Nuclease Particles (Cat #VCAS11227) or
- Edit-R Inducible Lentiviral hEF1α-Blast-Cas9 Nuclease Plasmid DNA (Cat #CAS11229)
- Edit-R Lentiviral sgRNA particles (Cat #VSGXXXXXX-XXXXX) or
- Edit-R synthetic tracrRNA (Cat #U-002005-XX) and Edit-R synthetic crRNA, predesigned for your gene of interest in a variety of sizes, or designed and ordered using the Dharmacon CRISPR Design Tool.

B. Additional materials required

The following additional materials are required but not supplied:

- Multi-well tissue culture plates or tissue culture dishes
- Blasticidin S (Fisher Scientific, Cat #BP2647-25; InvivoGen, Cat #ant-bl-1).
- Puromycin (Fisher Scientific, Cat #BP2956-100; InvivoGen, Cat #ant-pr-1)
- Doxycycline hyclate (Fisher Scientific, Cat #ICN19895510, or similar)
- Resazurin cell viability reagent or similar
- Assay(s) for detecting gene engineering events in a cell population
- Choice of positive control sgRNA lentiviral particles:

  https://horizondiscovery.com/en/gene-editing/gene-editing-reagents/products/edit-r-lentiviral-sgrna-positive-controls-and-kits
- Choice of negative control sgRNA lentiviral particles:

  https://horizondiscovery.com/en/gene-editing/gene-editing-reagents/products/edit-r-lentiviral-sgrna-non-targeting-controls
- Tetracycline-free serum (Hyclone, Cat #SH30070.03T; Clontech, Cat #631106)
- **Tet-free growth medium:** antibiotic-free cell culture medium (with tetracycline-free 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 tetracycline-free serum, if necessary)
- **Tet-free selection medium:** Tet-free growth medium supplemented with the appropriate concentration of blasticidin and/or puromycin.

C. Selection and induction optimization

In general, gene editing with the Edit-R Inducible Lentiviral Cas9 follows a three-step protocol **(as shown in Figures 3 and 4)**: (1) transduction with lentiviral particles for generation of stable cell lines carrying a single copy of the inducible lentiviral Cas9 proviral sequence in the cell genome; (2) a second transduction with lentiviral sgRNA to integrate the second component of the CRISPR-Cas9 system; and, (3) induction with doxycycline to promote Cas9 expression and indel formation in the gene(s) target(s). For successful results, it is recommended that optimal concentrations of doxycycline, blasticidin and puromycin be determined prior to initiating the experimental project.

i. Doxycycline induction

The Edit-R Inducible Lentiviral Cas9 employs the Tet-On 3G induction system which allows robust Cas9 induction at doxycycline doses between 100 ng/mL and 1000 ng/mL in most cell lines. If desired, reduced expression of Cas9 may be obtained at doses between 10 ng/mL and 100 ng/mL, depending on the cell line of interest and experimental optimization to obtain consistent results. In some cell types, doses of doxycycline higher than 500 ng/mL may affect cell viability. Therefore, it is strongly recommended to generate a dose-response curve to determine the optimal concentration of doxycycline to produce maximal Cas9 expression with minimal effect on cell viability.

Edit-R Lentiviral sgRNA Positive Control particles and a DNA mismatch detection assay can be used to create a dose-response curve for doxycycline in the inducible Cas9 stable cell line previously generated. Alternatively, a dose-response curve for doxycycline can also be generated by estimating the amount of induced Cas9 protein by immunoblotting using an anti-Cas9 specific antibody. A plate layout to evaluate the induction response to multiple concentrations of doxycycline in an inducible Cas9 stable cell line is shown in **Figure 6**. Due to the high affinity and sensitivity of the Tet-On 3G transactivator to doxycycline, maintain the cultured cells in Tet-free growth medium until ready to induce Cas9 expression.

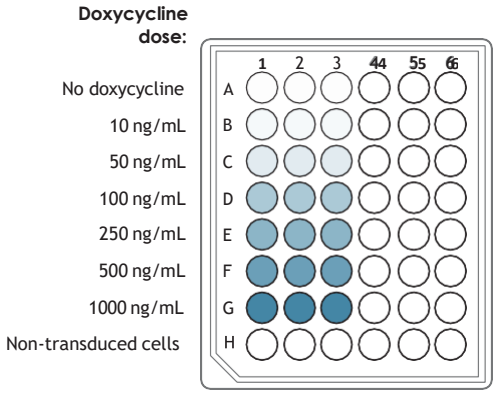

Figure 6. Plate layout for determination of the effect of increasing concentrations of doxycycline on Cas9 expression in stable cell lines.

ii. Blasticidin selection

The Edit-R Inducible Lentiviral Cas9 vector confers resistance to blasticidin in transduced cells. Before transducing cells, determine the minimum concentration of blasticidin required to kill non-transduced cells between three and ten days by generating a blasticidin kill curve (a detailed protocol is available in our webpage under Resources). The blasticidin concentration range for many mammalian cells is 2-15 µg/mL.

iii. Puromycin selection

The Edit-R Lentiviral sgRNA vector confers resistance to puromycin in transduced cells. Similar to blasticidin selection, before transducing cells, generate a puromycin kill curve to determine the minimum concentration of puromycin required to kill non-transduced cells between three and ten days. The puromycin concentration range for many mammalian cells is 1-10 µg/mL.

D. Generation of stable cell lines with Edit-R Inducible 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, tetracycline-free medium in a 24-well plate format (see Appendix for guidelines on other plate formats). Permissivity to lentiviral delivery and optimal transduction conditions vary widely amongst cell types and must be determined empirically for each cell line of interest. Adjust proportionally the number of cells, volumes, and reagent quantities when using a different sized culture dish.

Day 1:

1. Plate 5 × 10^4 cells per well in a 24-well plate using Tet-free growth medium.
2. Incubate cells at 37 °C in a humidified CO_2 incubator overnight.

Days 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.
3. Thaw the Edit-R Inducible Lentiviral Cas9 expression particles **on ice**.
4. Once thawed, gently mix and pipette the calculated volume of lentiviral particles into the transduction medium.
5. Remove the Tet-free growth medium from the well and add 0.25 mL of the transduction medium containing the lentiviral particles.
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 Tet-free growth medium (with tetracycline-free serum) and resume incubation at 37 °C in a humidified CO_2 incubator. If toxicity occurs with your cells, replace the medium after 4-6 hours with fresh Tet-free growth medium.

Days 3-15:

1. At 24-48 hours post-transduction, replace the medium with Tet-free selection medium containing the appropriate amount of blasticidin.
2. Replace the Tet-free selection medium every 3-4 days and monitor the presence of dead cells daily. To allow proper blasticidin selection, split the cells into a larger dish if the cells become confluent.
3. Once the cells are growing normally in Tet-free selection medium, expand the cells to freeze a sufficient number of aliquots for your experimental project.

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

Utilize the mixed population of inducible-Cas9 cell line obtained above for gene knockout screening with Edit-R Lentiviral sgRNA Pooled Libraries or for single gene knockout with Edit-R Lentiviral sgRNA particles or Edit-R synthetic crRNAs. If clonal cell lines are desired for downstream experiments, proceed with isolation of clonal cell lines using protocols appropriate for your cells of interest after step 2.

E. Gene editing with Edit-R Lentiviral sgRNA particles

To create gene knockouts into the generated inducible Cas9 stable cells with sgRNA lentiviral particles follow the protocols described in the Edit-R CRISPR-Cas9 Gene Engineering with Lentiviral Cas9 and sgRNA technical manual keeping in mind the following recommendations below:

- Maintain the cells in tetracycline-free medium during transduction with sgRNA particles and selection with puromycin.
- To create the gene knockouts, induce the expression of Cas9 with the addition of an appropriate amount of a freshly prepared doxycycline solution. Induce the cells with doxycycline for at least 24 hours. Gene editing events should be detected within 3 days of induction. A higher percentage of gene editing will be achieved within 5 to 7 days or longer induction period, depending of the cell of interest.

F. Gene knockout screening with Edit-R Lentiviral sgRNA Pooled Libraries

Detailed protocols for gene knockout screening with the generated inducible Cas9 stable cells using pooled sgRNA libraries are described in the Edit-R Lentiviral sgRNA Pooled Libraries technical manual. Carefully follow the recommendation below to avoid Cas9 expression leakiness prior to induction with doxycycline:

- Maintain the cells in tetracycline-free medium during transduction with the lentiviral sgRNA pooled library particles, selection with puromycin and expansion of the cell population. When splitting the cell population, seed enough cells to maintain the desired fold-representation (recommended 200 times, or higher, the number of constructs in the pooled library) in the reference and experimental samples.
- To create the gene knockouts, induce the expression of Cas9 with the addition of an appropriate amount of a freshly prepared doxycycline solution. Induce the cells with doxycycline for at least 24 hours. Gene editing events should be detected within 3 days of induction. A higher percentage of gene editing will be achieved within 5 to 7 days or longer induction period, depending of the cell of interest.

G. Gene editing with Edit-R synthetic guide RNAs

To create gene knockouts into the generated inducible Cas9 stable cells with synthetic guide RNAs follow the protocols described in the CRISPR-Cas9 Gene Engineering with Lentiviral Cas9 Particles and Edit-R synthetic guide RNAs technical manual keeping in mind the following recommendations below:

- Maintain the cells in tetracycline-free medium prior to induction of Cas9 expression.
- To create the gene knockouts, induce the expression of Cas9 with the addition of an appropriate amount of a freshly prepared doxycycline solution at least 24 hours prior to transfection of the synthetic crRNA:tracrRNA complex. Gene editing events should be detected within 3 days of transfection.

4 Packaging Edit-R Inducible Lentiviral Cas9 Nuclease plasmids

Edit-R Inducible Lentiviral Cas9 expression plasmids are Tat-dependent and require 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 Trans-Lentiviral product manual.

Edit-R Inducible Lentiviral Cas9 expression plasmid does 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.[7]

5 Appendix

A. 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) or SURVEYOR® Mutation Detection Kit (Integrated DNA Technologies).[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.[9,10]

B. Volume of transduction medium per surface area in culture dishes

Table 2. Suggested volumes of Tet-free transduction medium per surface area per well of adherent cells.

Cell 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

C. Stability and storage

i. Lentiviral particles

Edit-R Inducible Lentiviral Cas9 and 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.

ii. Plasmid DNA

Edit-R Inducible Lentiviral Cas9 expression plasmid DNA reagents 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 nuclease-free 10 mM Tris pH 7.4 (Dharmacon Cat #B-006000-100).

6 Frequently asked questions

Can I culture my cells in doxycycline continuously to treat this as a constitutive Cas9 expression vector?

Yes.

Do I need to generate a stable cell line expressing Tet-on first?

No, Edit-R Inducible Lentiviral Cas9 particles carry all necessary elements for selection and inducible expression. Cell lines capable of inducible Cas9 expression are generated with a single transduction.

Can I use tetracycline to induce expression of Cas9 in stable cell lines created with the Edit-R Inducible Lentiviral Cas9 construct?

No, Tet-On 3G has been optimized for binding doxycycline, and it responds poorly to tetracycline.

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

S. pyogenes CRISPR-Cas9 system has been successful in many non-mammalian species. However, we have designed the Edit-R Inducible Lentiviral Cas9 system for mammalian expression and thus have only tested in mammalian cells. Before moving into non-mammalian systems, you would need to establish the following:

1. Compatibility of lentiviral delivery with your cell type of interest.
2. Compatibility of Cas9 codon translation (Edit-R Cas9 is human codon-optimized).
3. Effective expression of the Tet-On 3G transactivator by the hEF1α promoter in the cell type of interest.
4. Functional level of Cas9 nuclease expression upon induction with doxycycline in the cell type of interest.

Can I co-transduce the Edit-R Inducible Lentiviral Cas9 and my sgRNA particles to generate my gene knockout faster?

Although this is not the recommended protocol, transduction of both the Edit-R Inducible Lentiviral Cas9 and the sgRNA particles can be performed at the same time. If co-transduction is necessary, it is recommended to extend the dual antibiotic selection to the longest selection time period previously determined for the individual antibiotics in your cells. Cells should be grown in tetracycline-free medium until ready to induce Cas9 expression and consequent gene knockout with doxycycline.

Can I use the Edit-R synthetic crRNA and synthetic tracrRNA with my inducible Cas9 stable cell line?

Yes. However, it is recommended to induce Cas9 expression for at least 24 hours prior to transfection with synthetic crRNA and tracrRNA to achieve a high percentage of gene editing in a cell population. The optimal conditions for induction and transfection should be empirically tested.

Can the ploidy of my cell line affect the results of my CRISPR-Cas9 gene editing experiment?

When using a CRISPR-Cas9 system to disrupt gene function, it is important to know as much as possible about the gene you are targeting and your cells of interest. In particular, the ploidy of your cells, the gene copy number and the presence of any SNPs are important considerations. In normal diploid cells, obtaining mutations on both alleles may be required for a complete knockout and an observable phenotype. Virtually all cancer lines and many immortalized cell lines exhibit aneuploidy and as such, mutation of multiple alleles may be necessary. The presence of SNPs and multiple genomic locations should also be taken into account when testing custom or predesigned crRNAs, as they can affect whether a complete knockout can be achieved. Expansion of clones from single cells followed by sequence validation to verify the desired mutation on all alleles is the most rigorous approach for confirming complete gene knockout.

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.

My DNA mismatch detection assay shows a low percentage of editing. Why is that?

Mutation analysis assays, which utilize mismatch-specific DNA endonucleases such as T7EI, rely on PCR amplification of a genomic DNA target site and subsequent observation of cleavage, commonly by gel electrophoresis. While these assays are a straightforward approach for detecting insertions and deletions (indels) introduced by CRISPR-Cas9 gene editing, sensitivity varies between endonucleases and generally underestimates editing efficiency for several reasons:

1. Cas9 cleavage followed by DNA repair through non-homologous end-joining (NHEJ) results in deletions, insertions, and mutations of various sizes. Mismatch DNA endonuclease cleavage can produce smeared bands on a gel which are not easily visualized or quantified.
2. Mismatch DNA endonuclease digestion can lead to non-specific cuts that degrade the PCR product and reduce the intensity of the desired bands, especially at longer incubation times.
3. If the CRISPR-Cas9 gene editing generates large inserts or deletions, primer binding sites can be impacted and the mutation thus will not be detected by the DNA mismatch assay.

Can I transfect the Edit-R Inducible Lentiviral Cas9 nuclease plasmid into my cells?

We do not recommend transfection due to the fact that transfection does not provide ability to control copy number and integration efficiency. The Edit-R Inducible Lentiviral Cas9 nuclease plasmid has been optimized for use and delivery as lentiviral particles.

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. R. Loew, *et al.* Improved Tet-responsive promoters with minimized background expression. *BMC Biotechnol.* **10**, 81 (2010).
7. 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. 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).
10. D. Li, *et al.* Heritable gene targeting in the mouse and rat using a CRISPR-Cas9 system. *Nat. Biotechnol.* **31**, 681-683 (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 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).

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 (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) (https://osp.od.nih.gov/wp-content/uploads/NIH_Guidelines.pdf).

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 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.

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