Technical Manual **CRISPR editing**

Genome engineering with the Dharmacon™ Strict-R™ Inducible Cas9 Lentiviral System

Storage : -80°C

Volume : 25 µL per vial

Version: 0001 **Revision date:** 112025

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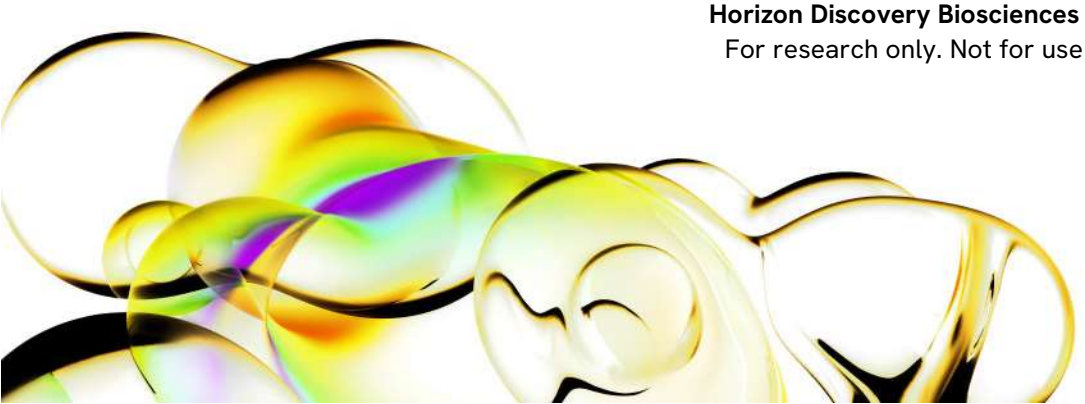

Contents

1 Introduction

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 breakthrough 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.

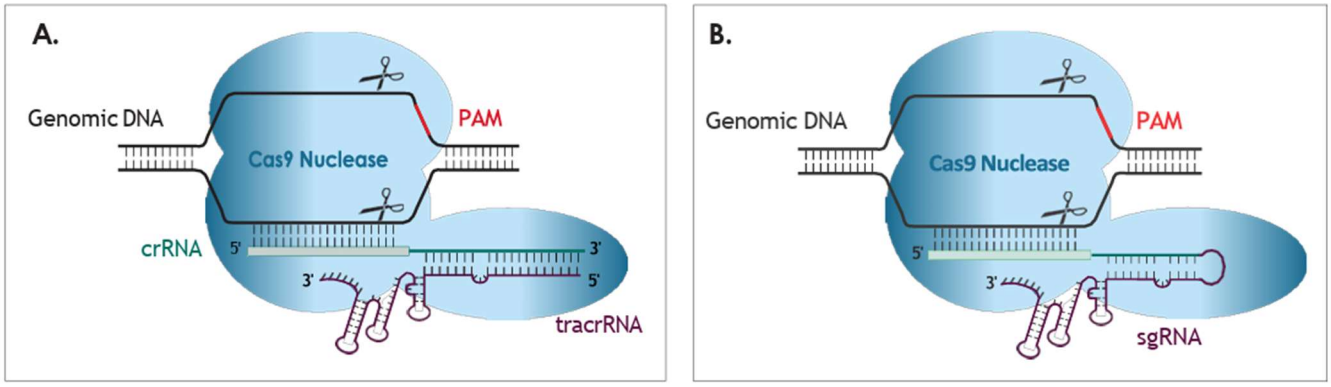

Figure 1. Illustration of CRISPR-Cas9 system. Cas9 nuclease (light blue), programmed by the crRNA (green): tracrRNA (blue) complex (A) or the sgRNA (B), cutting both strands of genomic DNA 5′ of the PAM (red).

2 The Strict-R inducible Cas9 system for temporal control of genome engineering

The Strict-R inducible Cas9 system is available as purified and concentrated lentiviral particles, ready for transduction and easy generation of an inducible Cas9-expressing cell line. To perform inducible gene editing experiments, gene-specific Edit-R sgRNA(s) are available as purified and titered lentiviral particles, lentiviral pooled libraries, or glycerol stocks to pair with the Strict-R inducible Cas9 system.

A. The Dharmacon™ Strict-R™ Inducible Lentiviral Cas9 system

The Strict-R inducible Cas9 system is designed to provide the researcher with an effective way to control the time of expression of the Cas9 effector in the cell of interest with two highly cell-permeable small molecules. The Strict-R inducible Cas9 lentiviral expression vector contains a human codon-optimized version of the *S. pyogenes* dCas9 gene under the control of a doxycycline inducible promoter (TRE3G, Figure 2). This platform is based on the Tet-On® 3G bipartite induction system: a tightly governed system consisting of an optimized inducible RNA polymerase II promoter (TRE3G), which provides both low basal expression and potent activation upon introduction of doxycycline to cell media[6]. The Tet-On 3G transactivator is constitutively expressed with the blasticidin resistance gene (Blast^R), hygromycin resistance gene (Hyg^R) or green fluorescent protein (EGFP) as a bicistronic transcript with a 2A peptide sequence (Figure 2) for selection of stable cells with the inducible Cas9 construct integration.

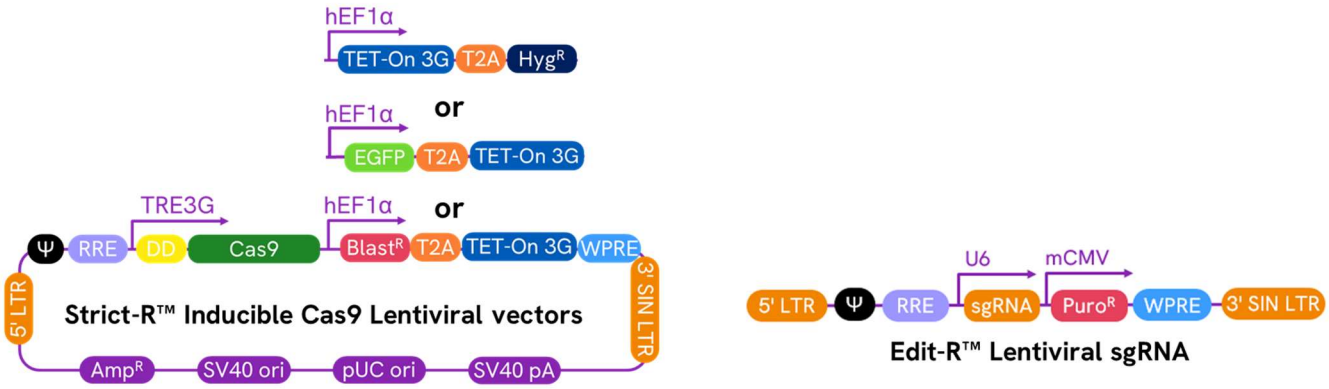

Figure 2. Schematic maps of the Strict-R inducible Cas9 lentiviral systems (right) and Edit-R Lentiviral sgRNA vectors (left).

Fusion of an FKBP12-derived destabilizing domain[7] (Figure 3) to the N-terminus of Cas9 adds a level of post-translation regulation to the Strict-R inducible Cas9 system. The destabilizing domain (degron) is intrinsically unstable and tags Cas9 nuclease for rapid proteasomal degradation[8]. However, addition of the cell-permeable ligand Shield1 stabilizes the degron-Cas9 fusion, permitting accumulation of the nuclease protein and subsequent complexing with expressed sgRNA to perform gene-specific editing. Together, the Tet-On 3G system and the FKBP12-derived destabilizing domain provide enhanced temporal regulation of Cas9 expression, minimizing basal target gene editing (leakiness) while maintaining potent induction.

B. Edit-R Lentiviral single guide RNA

Edit-R predesigned lentiviral sgRNA is expressed from a lentiviral vector under the control of a human U6 promoter. Puromycin resistance marker (Puro^R) is driven by the mouse CMV promoter and allows for rapid selection of cells with integrated sgRNA (Figure 2).

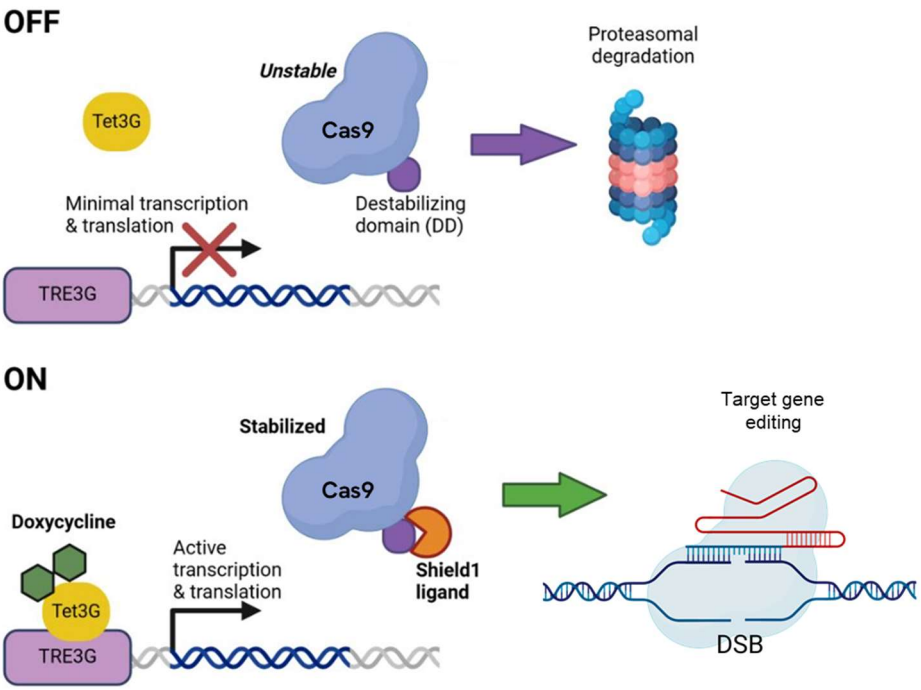

Figure 3. Diagram of the Strict-R inducible Cas9 lentiviral system. In the absence of doxycycline and Shield1, the system is "OFF." Leaky bursts of transcription from the TRE3G promoter result in the translation of degron-fused protein which is rapidly degraded by the proteasome. In the presence of doxycycline, the constitutively expressed Tet-On 3G transactivator protein binds to the TRE3G promoter and activates the expression of Cas9. The addition of Shield1 stabilizes Cas9 protein translated from the doxycycline-induced transcripts allowing Cas9 nuclease to accumulate and target gene editing to proceed. Diagram created with BioRender.com.

Each Edit-R 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. The functional elements for both Cas9 and sgRNA lentiviral vectors are listed and described in **Table 1**.

Table 1. Elements of the Strict-R inducible Cas9 lentiviral and Edit-R lentiviral sgRNA vectors.

Vector element	Utility
Cas9	*S. pyogenes* Cas9 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
DD	FKBP12-derived destabilizing domain (degron) provides small molecule post-translational regulation of Cas9
hEF1α	Human elongation factor 1 alpha short promoter
Blast^R	Blasticidin resistance marker permits antibiotic selection of transduced mammalian cells
Hyg^R	Hygromycin resistance marker permits antibiotic selection of transduced mammalian cells
EGFP	Green fluorescent protein reporter enables selection of transduced mammalian cells by FACS
T2A	Self-cleaving peptide allows for simultaneous expression of selectable marker 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
WPRE	Rev Response Element enhances titer by increasing packaging efficiency of full-length lentiviral genomes
U6	Human RNA polymerase III promoter U6
sgRNA	Optimized single guide RNA, a fusion of gene-specific crRNA with the tracrRNA scaffold
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
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

C. Overview of gene editing workflow with Strict-R inducible Cas9 system

Once inducible Cas9 cell lines are generated, these cells are transduced with gene-specific sgRNA lentiviral particles and subsequent gene editing/knockout can be obtained by treatment with doxycycline and Shield1 at predefined concentrations (see section 4.B.iii. Doxycycline and Shield1 induction). Figure 4 summarizes general experimental workflows to generate stable cell lines carrying the Strict-R inducible Cas9 system followed by transduction with sgRNA lentiviral particles and induction with doxycycline and Shield1 for phenotypic analysis.

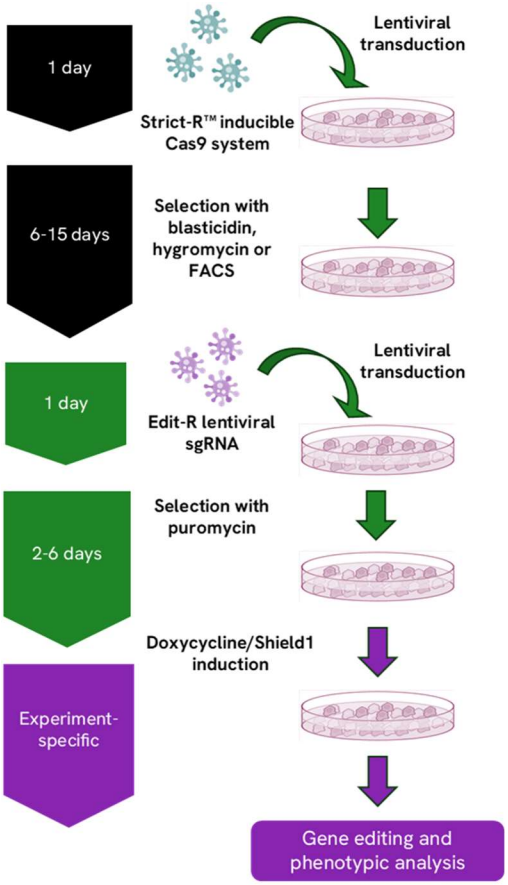

Figure 4. Gene overexpression workflow using the Strict-R inducible lentiviral Cas9 system.

3 Required materials for gene activation with the Strict-R inducible Cas9 system

A. Materials required

- Dharmacon™ Strict-R™ Inducible Cas9 Lentiviral System
  - Strict-R Inducible Hygromycin Cas9 Lentiviral Particles
  - Strict-R Inducible Blasticidin Cas9 Lentiviral Particles
  - Strict-R Inducible EGFP Cas9 Lentiviral Particles
- Edit-R™ Lentiviral sgRNA particles or virus produced from Edit-R Lentiviral sgRNA glycerol stock

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, or similar)
- Puromycin (Fisher Scientific, Cat #BP2956-100; InvivoGen, Cat #ant-pr-1, or similar)
- Hygromycin B (Fisher Scientific, Cat #BP29521MU; Gibco™, Cat #10687010, or similar)
- Doxycycline hyclate (Fisher Scientific, Cat #ICN19895510, or similar)
- Shield1 Ligand (Takara, Cat #632189, or similar)
- 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
- Optional: transduction enhancer such as LentiBOOST® (https://horizondiscovery.com/en/transfection-and-ancillary-reagents/products/lentiboost-lentivirus-transduction-enhancer) or Polybrene
- **Tetracycline-free (characterized or -system approved) fetal bovine serum** (Hyclone, Cat# SH30070.03T; Takara, Cat #631106)
- **Tet-free growth medium**: antibiotic-free cell culture medium (with tetracycline-free serum and/or supplements) recommended for maintenance of the cells
- **Tet-free 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.
- Optional: Live/Dead indicator such as Zombie Dyes (BioLegend®, https://www.biolegend.com/en-us/live-dead), Resazurin or similar metabolic assay
- Assay(s) for detecting gene editing

4 Assay optimization

A. Optimization of lentiviral transduction

While lentiviral particles exhibit broad cell tropism, the conditions for successful and efficient delivery can vary significantly. Parameters that may influence the efficiency of lentiviral transduction include, but are not limited to:

Transduction medium: When possible, the transduction of cells with lentiviral particles should be performed in a small volume of low-serum (0.5-2%) or serum-free medium. For cells sensitive to low serum conditions, transduction optimization can be performed in complete medium.

Transduction duration: Incubation time can vary between 4 and 24 hours and will depend on the cells of interest.

Transduction medium additives: Cationic polymers such as hexadimethrine bromide (Polybrene) or transduction enhancers such as LentiBOOST® may be added to enhance lentiviral particle binding to the cell surface. We recommend testing a range of concentrations for identification of optimal transduction efficiency with minimal or no cell toxicity.

Cell density at transduction: The density at which cells are seeded may also influence transduction efficiency. We recommend seeding cells at a range of densities for optimization of transduction efficiency.

Strict-R inducible Cas9 lentiviral particles co-expressing Hygromycin or Blasticidin resistance gene do not contain a fluorescent reporter. Therefore, Strict-R inducible Cas9 lentiviral particles co-expressing EGFP can be substituted for rapid optimization of transduction conditions.

B. Selection and induction optimization

In general, gene editing with the Strict-R inducible Cas9 lentiviral system follows a three-step protocol (as shown in Figures 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 and Shield1 ligand to promote Cas9 expression and editing of the gene(s) target(s). For successful results, it is recommended that optimal concentrations of doxycycline, Shield1, blasticidin, hygromycin, and puromycin be determined prior to initiating the experimental project.

i. Blasticidin or Hygromycin selection

Strict-R inducible Blasticidin or Hygromycin Cas9 lentiviral particles confer resistance to blasticidin or hygromycin in transduced cells, respectively. Before transducing cells, determine the minimum concentration of chemical selection required to kill non-transduced cells between seven and fourteen days by generating a blasticidin or hygromycin kill curve. The blasticidin concentration range for many mammalian cells is 2-15 µg/mL and 50 – 1000 µg/mL for hygromycin.

ii. Fluorescence-activated cell sorting (FACS) selection

Strict-R Inducible EGFP Cas9 Lentiviral Particles vectors allow creation of stable cell lines by sorting cells positive for EGFP fluorescence after transduction using a cell sorting flow cytometer. For optimal Cas9 gene editing, cells expressing the highest level of EGFP often correlate with higher expression of gene-editing machinery and subsequently higher edit rates. However, for some sensitive cell types or to minimize background editing, cell populations with low or medium EGFP expression should be collected and assessed. For each EGFP population sorted, cells should be assessed for viability, growth and targeted gene editing. Additionally, clonal cell line generation can be achieved by plating single cells from bulk sorted EGFP cell populations or directly sorting single EGFP-expressing cells into 96-well plates.

iii. Puromycin selection

Edit-R Lentiviral sgRNA particles confer 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.

iv. Doxycycline and Shield1 induction

The Strict-R inducible Cas9 platform employs the Tet-On 3G induction system and FKBP12-derived destabilizing domain. These allow for robust Cas9 induction at doxycycline doses between 100 ng/mL and 1000 ng/mL and Shield1 doses between 250 nM and 500 nM in most cell lines. If desired, reduced expression of Cas9 may be obtained at doxycycline doses between 10 ng/mL and 100 ng/mL and/or Shield1 doses between 25 nM and 125 nM, 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 and Shield1 to produce maximal Cas9 expression with minimal effect on cell viability.

Edit-R Lentiviral sgRNA Positive Control particles and gene editing quantification by mismatch detection assay such as T7 Endonuclease (T7EI) can be used to create a dose-response curve for doxycycline and Shield1 in the Strict-R inducible Cas9 stable cell line. Alternatively, a dose-response curve for doxycycline and Shield1 can also be generated by estimating the amount of induced Cas9 protein by immunoblotting using an anti-Cas9 specific antibody. Due to the high affinity and sensitivity of the Tet-On 3G transactivator to doxycycline, maintain the cultured cells in Tet-free/-system approved serum medium until ready to induce Cas9 expression.

5 Protocol for inducible gene editing

A. Generation of stable cell lines with Strict-R Inducible Lentiviral Cas9 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 B for guidelines on other plate formats). Adjust proportionally to the number of cells, volumes, and reagent quantities when using a different sized culture dish. Permissivity to lentiviral delivery and optimal transduction conditions vary widely amongst cell types and must be determined empirically for each cell line of interest. A protocol describing how to determine functional titer by flow cytometry can be found in the appendix of the CRISPRmod CRISPRa All-in-One Lentiviral sgRNA Pooled Libraries - Technical Manual.

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.

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.

3. Thaw the Strict-R inducible Cas9 lentiviral particles on ice.

4. To prepare the Transduction Medium, gently mix the lentiviral particles and pipette the calculated volume into the base cell culture medium such that the total volume is 0.25 mL per well (see Appendix B for guidelines on other plate formats).

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:

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

Method 1 using Strict-R Inducible Lentiviral Cas9 with hygromycin or blasticidin selection

1. At 24-48 hours post-transduction, replace the culture medium with Tet-free selection medium containing the appropriate amount of selection antibiotic (blasticidin or hygromycin).

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 15 days and hygromycin 5 and 10 days, slow growing cells may take longer. If the cells become confluent, split the cells into a larger dish to allow proper selection (for example, split cells from 24-well to 6-well culture dishes).

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 editing with Edit-R Lentiviral sgRNA particles.

Method 2 using Strict-R Inducible EGFP Cas9 Lentiviral System

1. At 72 hours post-transduction, expand cells into a larger dish and continue to expand culture until enough cells are collected for cell sorting (500,000-10 million cells depending on generation of clonal cell line or population from cell line of interest).

2. Prepare cells for FACS according to commonly used protocols.

3. Sort cells with desired EGFP fluorescence intensity.

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 sorted or subclones generated from the bulk sort EGFP-expressing cell population, these cell lines will be a mixed population that on average have a single integration of Cas9 in their genomes.

B. Transduction of Edit-R lentiviral sgRNA

The following is an example protocol for transduction of Edit-R lentiviral sgRNA particles for gene editing. This protocol provides transduction conditions for 24-well plates at an MOI 0.3 into adherent U2OS cells selected for the integration of Strict-R inducible Cas9 lentiviral system. A detailed protocol can be found here: Edit-R CRISPR-Cas9 Gene Engineering with Lentiviral Cas9 and sgRNA technical manual. Keep in mind the following recommendations:

▪ Maintain the cells in tetracycline-free medium during transduction with sgRNA particles and selection with puromycin.

▪ Efficiency of lentiviral delivery and optimal transduction conditions can vary widely between cell types and must be determined empirically for each cell line of interest.

▪ To create the gene knockouts, induce the expression of Cas9 with the addition of an appropriate amount of freshly prepared doxycycline and Shield1 solution. Induce the cells with induction medium 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 on the cell line of interest.

Day 1:
1. Plate 5 × 10^4 inducible Cas9 stable 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.

Day 2:
1. Thaw the Edit-R Lentiviral sgRNA particles on ice.

2. Once thawed, to prepare the transduction medium, gently mixing the appropriate volume of titered Edit-R lentiviral sgRNA particles (to generate an MOI 0.3) by pipette into the calculated volume of base cell culture medium (without serum) such that the total volume is 0.25 mL per well (see Appendix B for guidelines on other plate formats).

3. Remove the cell culture maintenance medium from the well and add 0.25 mL of the transduction medium containing the lentiviral particles prepared in step 2.

4. Incubate cells at 37 °C in a humidified CO_2 incubator for 4-6 hours.

5. At 4-6 hours post-transduction, add an additional 0.75 mL of Tet-free growth medium (with Tet-free or Tet-system approved serum) per well 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 3-7:

1. At 24-48 hours post-transduction, replace the growth medium with selection medium containing the appropriate concentration of puromycin.

Addition of blasticidin or hygromycin (to continue to selection for inducible Cas9 integrated cells) to the selection medium containing puromycin is optional.

2. Replace the Tet-free selection medium every 2-3 days and monitor the accumulation of dead cells daily.

3. Once the cells are growing normally in Tet-free selection medium, expand the cells to freeze enough aliquots for your experimental project and/or proceed with Cas9 induction. Transduction of the stable Strict-R inducible Cas9 system cell line at MOI 0.3 generated a mixed population of Edit-R sgRNA expressing cells with an average of one integration per cell.

C. Gene editing with the Strict-R inducible Cas9 system

To initiate targeted gene editing, induce the expression of Cas9 with the addition of an appropriate amount of freshly prepared doxycycline and Shield1 containing growth media (section 4.B.iii). Induce the cells with doxycycline and Shield1 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 on the cell line of interest.

6 Appendix

A. Gene editing assay recommendations

The most commonly used method for detection of insertions and deletions (indels) in a cell population requires PCR-amplification of the genomic region surrounding the target edit site. The PCR amplicon using predesigned

primers can be subjected to a mismatch detection assay such as T7 Endonuclease I (T7EI or TIDE) or next generation sequencing (NGS) to verify the efficiency of gene editing. When edited cells are expanded and clonal populations are obtained, the most commonly used method for confirming gene editing is Sanger sequencing.[9,10] After preliminary optimization and editing at the sequence level has been confirmed, expanded or clonal cell populations may also be assessed by phenotypic assays, such as western blot or flow cytometry, for target gene knockout.

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 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

Strict-R inducible Cas9 lentiviral and Edit-R sgRNA particles are shipped on dry ice and must be stored at -80 °C. Under these conditions, lentiviral particles are stable for at least 12 months from receipt. 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.

7 Frequently asked questions

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

No, Strict-R inducible Cas9 lentiviral 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 Strict-R inducible Cas9 lentiviral particles?

No, Tet-On 3G has been optimized for binding doxycycline, and it responds poorly to tetracycline resulting in suboptimal Cas9 induction.

Can I use the Edit-R synthetic sgRNA 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 sgRNAs to achieve robust gene activation. 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 there has been a gene edit (or indel)?

Mismatch detection assays, like T7EI or TIDE, inform 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.

How will Shield1 treatment impact my cells?

Shield1 is a well characterized small molecule ligand that does not elicit an appreciable transcriptional response[12]. However, as an additional control, cells expressing a non-targeting control can be treated with doxycycline/Shield1 to ensure exposure does not impact target gene expression or cause a cellular phenotype.

What are the maximum excitation and emission wavelengths and laser/filter requirements for detecting and sorting EGFP?

If fluorescence-activated cell sorting (FACS) is to be performed, please ensure that the lasers and filters are suitable for the EGFP marker, an example laser and filter that would work for this are provided.

Fluorescent reporter	Excitation wavelength	Emission wavelength	Cytometer laser	Bandpass filter
EGFP	488 nm	507 nm	488 nm blue	e.g. 510/20 nm or 530/30 nm

Are Dharmacon™ lentiviral particle products safe to use in the laboratory? What precautions should be taken when handling lentiviral particles?

Lentiviral delivery systems have been employed in many research laboratories worldwide without incident. Handling lentiviral products requires extensive experience with cell culture techniques. It is vital that the protocols provided by Revvity and the safety guidelines described for appropriate handling and storage are fully understood and followed precisely (see Section 9, Lentiviral particle product safety level information).

Can I order the Strict-R inducible Cas9 lentiviral expression vectors as plasmid DNA or glycerol stocks?

No. Our Strict-R inducible Cas9 lentiviral expression vectors are sold exclusively as lentiviral particles.

How should lentiviral particle products be stored?

All lentiviral particle products must be stored at -80 °C. If necessary, the particles can be aliquoted upon the first thaw to convenient volumes and the aliquots stored at -80 °C to minimize the number of future freeze-thaws. However, we recommend avoiding multiple freeze-thaw cycles as much as possible.

How are lentiviral particle products shipped?

Lentiviral particle products are shipped on dry ice for overnight domestic delivery or priority international for delivery outside of the U.S.

8 References

1. 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).
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9 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 Scientific 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).

10 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.

To find the contact information in your country for your technology of interest, please visit us at
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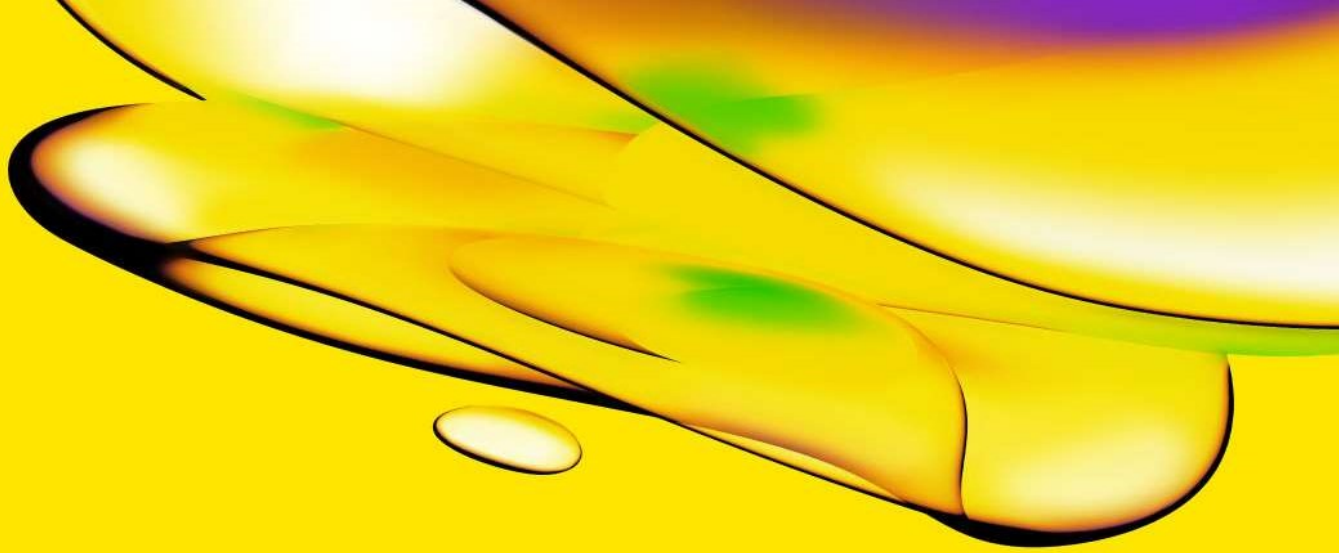

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