Technical Manual

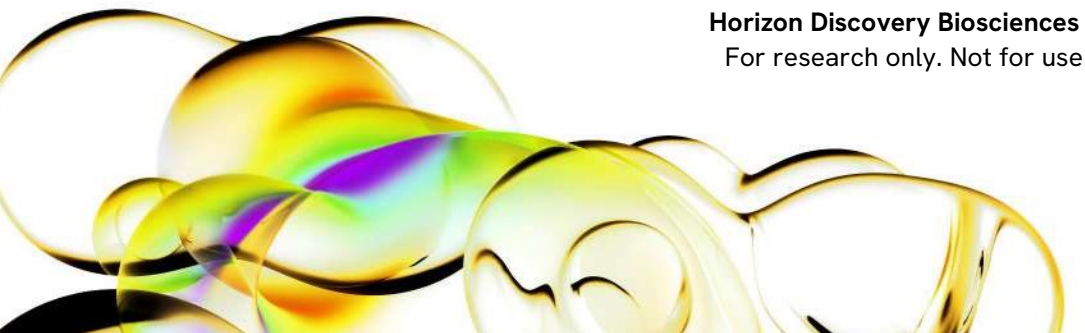
Transcriptional repression with the Dharmacon™ Strict-R™ Inducible CRISPRi Lentiviral System

Storage : -80°C

Volume : 25 µL per vial

Version: 0001

Revision date: 112025



Horizon Discovery Biosciences Ltd. (A Revvity Company)
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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. CRISPR-Cas9 platform for transcriptional repression in mammalian cells

In addition to genome engineering applications in mammalian cells[2], the Streptococcus pyogenes CRISPR-Cas9 system has been adapted to technologies for transcriptional regulation[3,4,5]. The nuclease activity of the S. pyogenes Cas9 was abolished by point mutations introduced into two catalytic residues (D10A and H840A) yielding a deactivated Cas9 (dCas9) that maintains the ability to bind to target DNA when guided by sequence-specific guide RNAs. When the dCas9 is fused to transcriptional regulators and guided to gene promoter regions, it induces RNA-directed transcriptional regulation. CRISPR-Cas9 based technologies for transcriptional regulation include CRISPR interference (CRISPRi) and CRISPR activation (CRISPRa).

CRISPRi systems initially utilized the Krüppel associated box (KRAB) domain from zinc finger protein 10 (KOX1) as a transcriptional repressor[4,6] but recent publications have demonstrated improved transcriptional repression with a variety of different effectors[7,8]. Revvity's CRISPRi system utilizes a novel fusion protein comprised of domains from two human transcriptional repressors, Sal-like protein 1 (SALL1) and Sin3 histone deacetylase corepressor complex component SDS3 (SDS3), fused to the C-terminal end of dCas9[9]. Horizon's CRISPRi system was developed to provide robust and consistent gene repression across the genome.

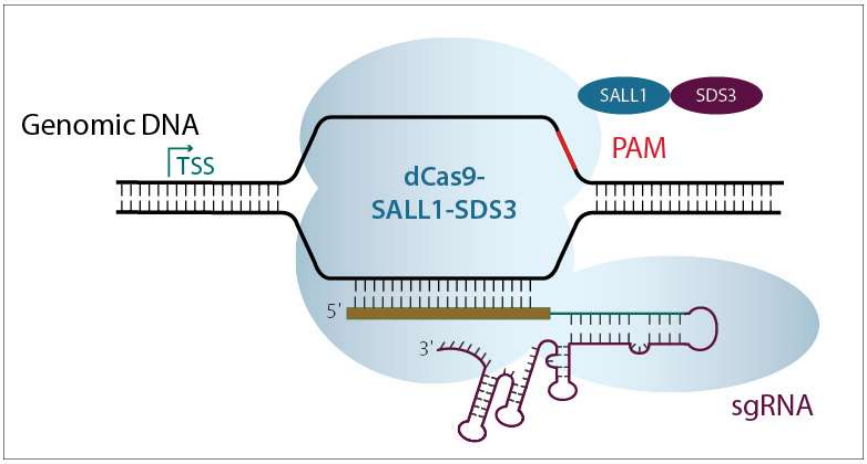

Figure 1. Illustration of dCas9-SALL1-SDS3 with sgRNA targeting the region immediately downstream of a gene's transcriptional start site (TSS).

2 The Strict-R inducible CRISPRi system for temporal control of gene repression

The Strict-R inducible CRISPRi lentiviral system is available as purified and concentrated lentiviral particles, ready for transduction in the cells of interest and easy generation of an inducible dCas9-SALL1-SDS3-expressing cell line. Gene-specific CRISPRi Lentiviral sgRNA are provided as purified and concentrated lentiviral particles.

A. The Dharmacon™ Strict-R™ Inducible CRISPRi Lentiviral System

The Strict-R Inducible CRISPRi Lentiviral System is designed to provide the researcher with an effective way to control the time of expression of the dCas9-SALL1-SDS3 effector in the cell of interest with two highly cell-permeable small molecules. The Strict-R inducible CRISPRi lentiviral expression vectors contain a human codon-optimized version of the *S. pyogenes* dCas9 gene fused to the SALL1-SDS3 repressor system under the control of a doxycycline inducible promoter (TRE3G, Figure 3). 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 transcription and potent expression upon induction with doxycycline[10]. The Tet-On 3G transactivator is constitutively expressed with the hygromycin resistance gene (Hyg^R) or green fluorescent protein (EGFP) as a bicistronic transcript with a 2A peptide sequence (Figure 3) for selection of stable cells with the inducible dCas9-SALL1-SDS3 construct integration.

Fusion of an FKBP12-derived destabilizing domain[11] (Figure 2) to the N-terminus of dCas9-SALL1-SDS3 adds a level of post-translation regulation to the Strict-R inducible CRISPRi system. The destabilizing domain (degron) is intrinsically unstable and tags dCas9-SALL1-SDS3 for rapid proteasomal degradation[12]. However, addition of the cell-permeable ligand Shield1 stabilizes the degron-dCas9-SALL1-SDS3 fusion allowing expression to accumulate and target gene repression to occur in the presence of a gene-specific sgRNA. Together, the Tet-On® 3G system and the FKBP12-derived destabilizing domain provide enhanced temporal regulation of dCas9-SALL1-SDS3 expression, minimizing basal target gene repression (leakiness) while maintaining potent induction.

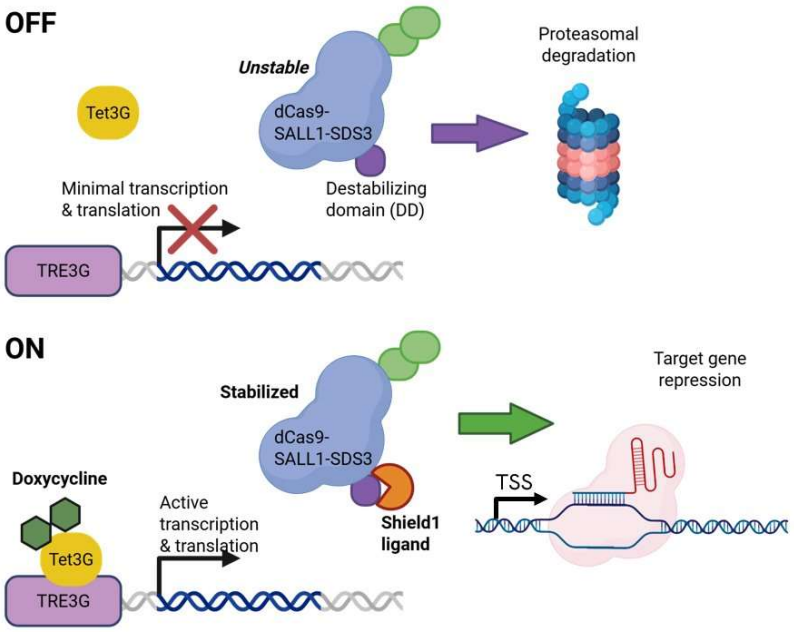

Figure 2. Diagram of the Dharmacon™ Strict-R™ Inducible CRISPRi Lentiviral System. In the absence of doxycycline and Shield1, the system is "OFF". Leaky bursts of transcription from the TRE3G promoter result in the translation of degron-fused dCas9-SALL1-SDS3 protein which is rapidly degraded by the proteasome. In the presence of doxycycline, the constitutively expressed Tet-On 3G transactivator protein binds to the TRE3G promoter and activates the expression of dCas9-SALL1-SDS3. The addition of Shield1 stabilizes dCas9-SALL1-SDS3 protein translated from the doxycycline-induced transcripts allowing dCas9-SALL1-SDS3 expression to accumulate and target gene repression to proceed. Diagram created with BioRender.com.

B. CRISPRmod CRISPRi Lentiviral guide RNA

CRISPRmod CRISPRi 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 3). CRISPRmod CRISPRi Lentiviral sgRNAs are designed based on a published CRISPRi algorithm[13] and target genomic DNA in the proximity of transcriptional start site (TSS). When more than one TSS exists for a gene, a second set of sgRNA reagents is available (labeled P2).

The protospacer region of the sgRNA is comprised of 19-20 nucleotides identical to the genomic DNA target site, followed by the non-variable sgRNA scaffold containing the tracrRNA sequence from *S. pyogenes*. The optimized sgRNA scaffold used in the CRISPRmod CRISPRi Lentiviral sgRNA can further improve gene repression efficiency. The functional elements for both dCas9-SALL1-SDS3 and sgRNA lentiviral vectors are listed and described in Table 1.

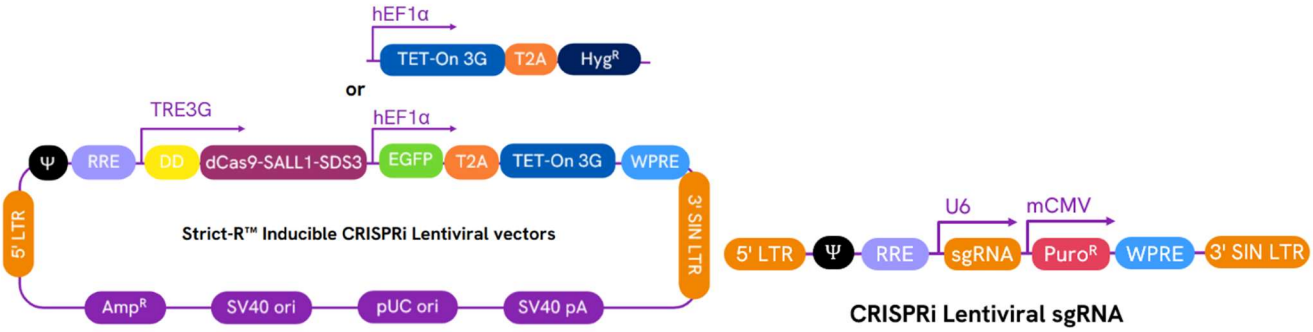

Figure 3. Schematic maps of the Strict-R Inducible CRISPRi Lentiviral and CRISPRmod CRISPRi Lentiviral sgRNA vectors.

Table 1. Elements of the Strict-R Inducible CRISPRi Lentiviral and CRISPRmod CRISPRi Lentiviral sgRNA vectors.

Vector element	Utility
dCas9-SALL1-SDS3	*S. pyogenes* dCas9-SALL1-SDS3 for gene repression of targeted DNA when programmed with a guide RNA
TRE3G	Inducible promoter with Tetracycline Response Elements which is activated by the Tet-On 3G transactivator in the presence of doxycycline
DD	FKBP12-derived destabilizing domain (degron) provides small molecule post-translational regulation of dCas9-SALL1-SDS3
hEF1α	Human elongation factor 1 alpha short promoter
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 transactivator 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 repression workflow with Strict-R inducible CRISPRi system

Once inducible dCas9-SALL1-SDS3 cell lines are generated, these cells are transduced with gene-specific sgRNA lentiviral particles and subsequent gene repression 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 CRISPRi system followed by transduction with sgRNA lentiviral particles and induction with doxycycline and Shield1 for phenotypic analysis.

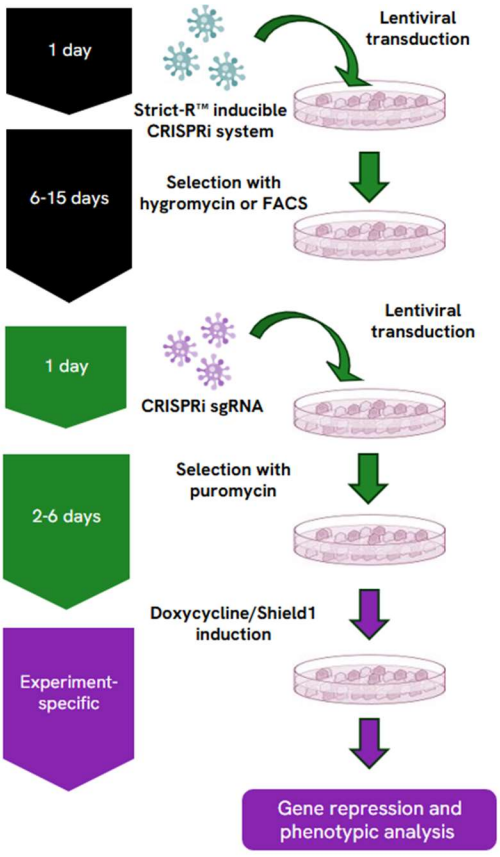

Figure 4. Gene repression workflow using the Dharmacon™ Strict-R™ Inducible CRISPRi Lentiviral System.

3 Required materials for gene repression with the Strict-R inducible CRISPRi system

A. Materials required

- Dharmacon™ Strict-R™ Inducible CRISPRi Lentiviral System
  - Strict-R Inducible Hygromycin dCas9-SALL1-SDS3 Lentiviral Particles
  - Strict-R Inducible EGFP dCas9-SALL1-SDS3 Lentiviral Particles
- CRISPRmod CRISPRi Lentiviral sgRNA particles

B. Additional materials required

The following additional materials are required but not supplied:

- Multi-well tissue culture plates or tissue culture dishes
- Hygromycin B (Fisher Scientific, Cat #BP29521MU; Gibco™, Cat #10687010, or similar)
- Puromycin (Fisher Scientific, Cat #BP2956-100; InvivoGen, Cat #ant-pr-1)
- 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/crisprmod/crispri/products/crispri-lentiviral-sgrna-positive-controls
- Choice of negative control sgRNA lentiviral particles: https://horizondiscovery.com/en/crisprmod/crispri/products/crispri-lentiviral-sgrna-non-targeting-controls
- 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 hygromycin 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 repression

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 Hyg dCas9-SALL1-SDS3 Lentiviral Particles do not contain a fluorescent reporter. Therefore, Strict-R Inducible EGFP dCas9-SALL1-SDS3 Lentiviral Particles can be substituted for rapid optimization of transduction conditions.

B. Selection and induction optimization

In general, gene repression with the Strict-R inducible CRISPRi system 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 dCas9-SALL1-SDS3 proviral sequence in the cell genome; (2) a second transduction with lentiviral sgRNA to integrate the second component of the CRISPRmod CRISPRi system; and, (3) induction with doxycycline and Shield1 ligand to promote dCas9-SALL1-SDS3 expression and downregulation of the gene(s) target(s). For successful results, it is recommended that optimal concentrations of doxycycline, Shield1, hygromycin, and puromycin be determined prior to initiating the experimental project.

i. Hygromycin selection

Strict-R Inducible Hyg dCas9-SALL1-SDS3 Lentiviral Particles confer resistance to hygromycin in transduced cells. Before transducing cells, determine the minimum concentration of hygromycin required to kill non-transduced cells between seven and fourteen days by generating a hygromycin kill curve. The hygromycin concentration range for many mammalian cells is 50 – 1000 µg/mL.

ii. Fluorescence-activated cell sorting (FACS) selection

The Strict-R Inducible EGFP dCas9-SALL1-SDS3 Lentiviral vector allow creation of stable cell lines through selecting positive fluorescent cells using a cell sorting flow cytometer. For optimal CRISPRi repression, cells expressing the highest level of EGFP often correlate with higher expression of dCas9-SALL1-SDS3 and subsequently higher transcriptional repression. However, for some sensitive cell types or to minimize background repression, cell populations with low or medium EGFP expression may be collected and assessed. Collecting cells with high, medium and low fluorescence cell populations can be helpful to test to find which population works best for downstream target repression.

iii. Puromycin selection

CRISPRmod CRISPRi Lentiviral sgRNA particles confer resistance to puromycin in transduced cells. Similar to hygromycin selection, before transducing cells, generate a puromyicin 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 CRISPRi platform employs the Tet-On 3G induction system and FKBP12-derived destabilizing domain. These allow for robust dCas9-SALL1-SDS3 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 dCas9-SALL1-SDS3 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 dCas9-SALL1-SDS3 expression with minimal effect on cell viability.

CRISPRmod CRISPRi Lentiviral sgRNA Positive Control particles and quantitative RT-PCR can be used to create a dose-response curve for doxycycline and Shield1 in the inducible dCas9-SALL1-SDS3 stable cell line. Alternatively, a dose-response curve for doxycycline and Shield1 can also be generated by estimating the amount of induced dCas9-SALL1-SDS3 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 growth medium until ready to induce dCas9-SALL1-SDS3 expression.

5 Protocol for inducible gene repression

A. Generation of stable cell lines with Strict-R Inducible dCas9-SALL1-SDS3 Lentiviral 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 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 CRISPRi 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 CRISPRi dCas9-SALL1-SDS3 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 dCas9-SALL1-SDS3 cell lines can be achieved by two different methods, depending on the Strict-R Inducible dCas9-SALL1-SDS3 lentivirus used during the experiment:

Method 1 using Strict-R Inducible Lentiviral dCas9-SALL1-SDS3 particles with hygromycin selection

1. At 24-48 hours post-transduction, replace the medium with Tet-free selection medium containing the appropriate amount of hygromycin.

The appropriate antibiotic concentration is specific to each cell line and should be determined experimentally prior to selection using a kill curve. Hygromycin usually kills cells between 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 hygromycin selection (for example, split cells from24-well to 6-well culture dishes)

2. Replace the Tet-free selection medium every 3-4 days and monitor the presence of dead cells daily. To allow proper hygromycin 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 dCas9-SALL1-SDS3 cell line obtained above for gene repression with CRISPRmod CRISPRi Lentiviral sgRNA particles.

Method 2 using Strict-R Inducible Lentiviral EGFP Strict-R Inducible dCas9-SALL1-SDS3 particles

1. At 72 hours post-transduction, expand until enough cells are collected for cell sorting (500,000-10 million cells).

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

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.

3. Sort cells with desired EGFP fluorescence intensity

4. Once the sorted cells are growing normally, expand accordingly to freeze enough aliquots for your experimental project.

B. Transduction of CRISPRmod CRISPRi lentiviral sgRNA

The following is an example protocol for transduction of CRISPRmod CRISPRi Lentiviral sgRNA expression particles for inducible gene repression. The protocol is provided for transduction in 24-well plates at MOI of 0.3 into adherent U2OS cells stably expressing the Strict-R inducible CRISPRi lentiviral system. Efficiency of lentiviral delivery and optimal transduction conditions vary widely between cell types and must be determined empirically for each cell line of interest.

Day 1:
1. Plate 5 × 10^4 inducible dCas9-SALL1-SDS3 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 CRISPRmod CRISPRi Lentiviral sgRNA particles on ice.

2. Once thawed, 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).

3. Remove the Growth 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 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 medium with Selection Medium containing the appropriate amount of puromycin.

Addition of hygromycin (to continue to selection for inducible dCas9-SALL1-SDS3 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 sufficient number of aliquots for your experimental project and/or proceed with dCas9-SALL1-SDS3 induction.

Expansion of the puromycin-resistant cells at this stage will generate a mixed population of cells containing a single integration of the CRISPRmod CRISPRi lentiviral sgRNA in their genomes, in addition to the single integration of inducible dCas9-SALL1-SDS3. For higher level of transcriptional gene repression, a higher MOI of the CRISPRmod CRISPRi Lentiviral sgRNA particles could be used to generate cells with multiple sgRNA integrations followed by isolation of clonal lines that have high level of gene repression.

C. Gene repression with the Strict-R inducible CRISPRi system

To initiate target gene repression, induce the expression of dCas9-SALL1-SDS3 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. Transcriptional repression should be detected within 48 hours of induction. Target gene repression can be sustained by replacing medium every 1-2 days with freshly prepared doxycycline and Shield1 containing growth medium.

6 Appendix

A. Gene expression analysis recommendations

RNA can be isolated using different methods per manufacturer's instructions. Quantitative RT-PCR analysis can be performed using gene expression assays according to manufacturer's instructions. Use the expression of a housekeeping gene for normalization of the expression of the gene of interest. Follow best practices for RT-qPCR analysis with appropriate number of technical replicates and proper controls.

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 CRISPRi Lentiviral and CRISPRmod CRISPRi 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 CRISPRi Lentiviral particles carry all necessary elements for selection and inducible expression. Cell lines capable of inducible dCas9-SALL1-SDS3 expression are generated with a single transduction.

Can I use tetracycline to induce expression of dCas9-SALL1-SDS3 in stable cell lines created with Strict-R Inducible CRISPRi Lentiviral Particles?

No, Tet-On 3G has been optimized for binding doxycycline, and it responds poorly to tetracycline resulting in suboptimal dCas9-SALL1-SDS3 induction.

Can I use the CRISPRmod CRISPRi synthetic sgRNA with my inducible dCas9-SALL1-SDS3 stable cell line?

Yes. However, it is recommended to induce dCas9-SALL1-SDS3 expression for at least 24 hours prior to transfection with synthetic sgRNAs to achieve robust gene repression. The optimal conditions for induction and transfection should be empirically tested.

How will Shield1 treatment impact my cells?

Shield1 is a well characterized small molecule ligand that does not elicit an appreciable transcriptional response[14]. 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 for EGFP?

EGFP fluorescence allows for the identification of transduced cells. If fluorescence-activated cell sorting (FACS) is to be performed, please ensure that the lasers and filters are suitable for the EGFP marker.

Fluorescent reporter	Excitation wavelength	Emission wavelength
EGFP	488 nm	507 nm

What is the size of the dCas9-SALL1-SDS3 protein and what antibody do you recommend for confirmation of expression of the dCas9-SALL1-SDS3?

The SALL1-SDS3 repressors add an additional 533 amino acids to dCas9 which shift the molecular weight of the dCas9-SALL1-SDS3 to approximately ~220 kDa. The protein could be detected using Cas9 antibodies (for example: BioLegend® cat # 844302).

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 of 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 Section9, Lentiviral particle product safety level information).

Can I order the Strict-R Inducible CRISPRi Lentiviral expression vectors as plasmid DNA or glycerol stocks?

No. Our Strict-R Inducible CRISPRi 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.

When performing CRISPRi, what level of transcriptional repression should I expect?

The level of transcriptional repression will vary depending on your target gene, cell line, and timepoint of analysis. Target gene repression is maximal ~72 hours after induction of dCas9-SALL1-SDS3 expression and can be sustained by replacing medium every 1-2 days with freshly prepared doxycycline and Shield1 containing growth medium.

How specific are the CRISPRmod CRISPRi sgRNAs in targeting the gene of interest?

Multiple publications have shown CRISPRi to be highly specific by RNA-seq expression analysis. Remember that for CRISPRi off-targeting, the guide RNA needs to bind to the promoter region of another gene to have an off-target effect, dramatically decreasing the potential off-target space. Furthermore, the guide RNAs are designed based on published algorithms that incorporate chromatin, nucleosome position, and sequence features to accurately predict highly effective guide RNAs and apply a filter for off-target binding. However, there might be examples of genes where the promoter region for one gene is in close proximity to another gene's promoter region. Investigation of the genomic locations of the hits of your screen might be important for proper interpretation of the results.

What if a gene has more than one transcriptional start site?

The published CRISPRi algorithm[13] used FANTOM and Ensembl databases to predict the transcriptional start site (TSS) more accurately. Some genes were identified as having alternative distal TSSs that could not be efficiently repressed from a single locus. The publication lists 10 gRNA designs per TSS. For the CRISPRmod CRISPRi predesigned guide RNAs, we offer the top guide RNAs for the primary TSS, and, when applicable, another set of guide RNAs for the secondary TSS. These are labeled as P1 and P2, respectively. If the CRISPRmod guide RNAs for your gene do not have a P2 designation, then only a single start site is predicted for that gene. If your gene has both P1 and P2 guide RNAs, it might be beneficial to test both for your experiment. Which TSS is active and to what level depends on your cell line.

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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).

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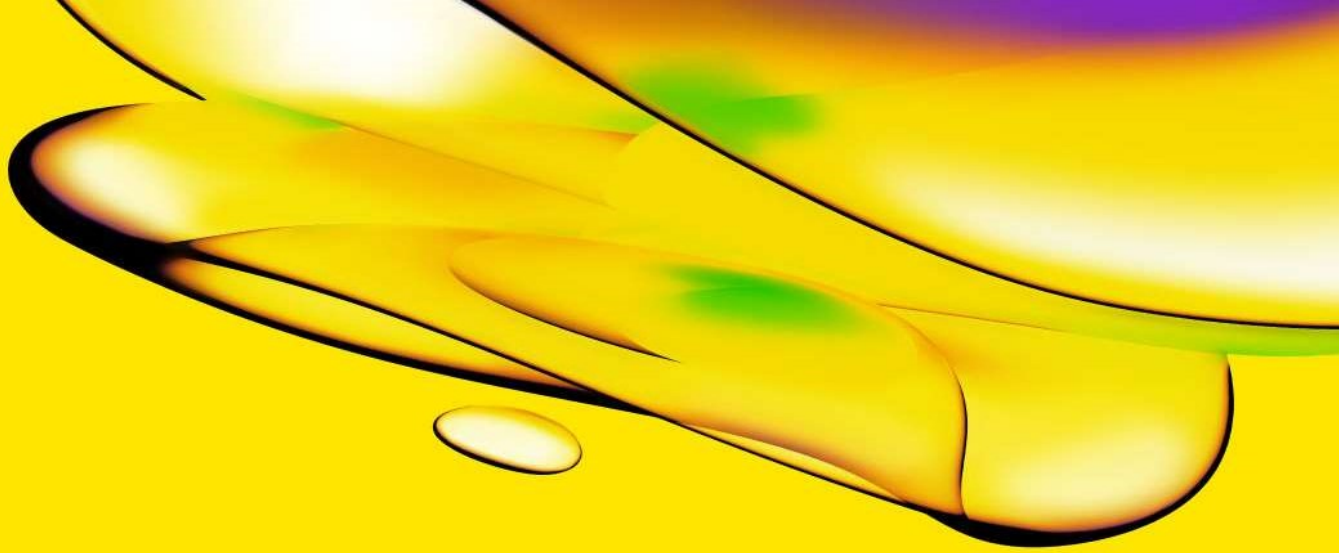

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