TECHNICAL MANUAL

horizon™
INSPIRED CELL SOLUTIONS

CRISPRmod CRISPRi transcriptional repression system with synthetic guide RNA

Contents

1 Introduction to the CRISPR-Cas9 system for transcriptional repression

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 (Bhaya, et al., 2011).

CRISPR-Cas9 platform for transcriptional gene repression in mammalian cells

In addition to genome engineering applications in mammalian cells (Jinek et al., 2012), the *Streptococcus pyogenes* CRISPR-Cas9 system has been adapted to technologies for transcriptional regulation (Qi et al., 2013, Gilbert et al., 2013, Cheng et al., 2013). 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 (Gilbert et al., 2013, Gilbert et al., 2014), but recent publications have demonstrated improved transcriptional repression with a variety of different effectors (Yeo et al., 2018, Moghadam et al., 2020, Alerasool et al., 2020). Horizon's CRISPRi system utilizes a novel fusion protein comprised of repressor 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. Horizon's CRISPRi system was developed to provide robust and consistent gene repression when used in conjunction with synthetic guide RNA.

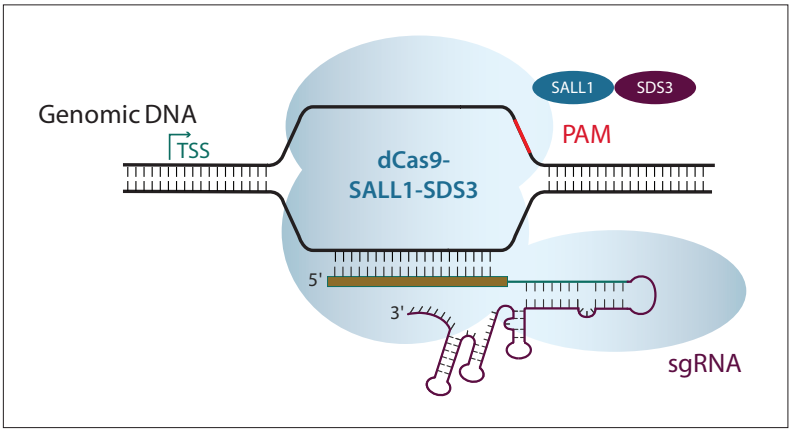

Figure 1. Diagram dCas9-SALL1-SDS3 with sgRNA targeting the region immediately downstream of a gene's transcription start site (TSS).

A CRISPRi guide RNA can be either synthetic CRISPR RNA (crRNA) complexed with a trans-activating CRISPR RNA (tracrRNA), or a single guide RNA (sgRNA) where the crRNA has been fused to the tracrRNA creating a chimeric structure (Figure 1). This protocol provides guidance for the single guide synthetic approach. For CRISPRi using expressed single guide RNA (sgRNA), please see this manual.

2 CRISPRmod CRISPRi workflow

To facilitate rapid generation of cell lines that constitutively express dCas9-SALL1-SDS3, the lentiviral dCas9-SALL1-SDS3 expression vector is packaged into particles, purified and concentrated for direct viral transduction. Subsequent transfection of synthetic or plasmid CRISPRi guide RNA or transduction of lentiviral CRISPRi sgRNA into dCas9-SALL1-SDS3 expressing cells results in target gene repression. Figure 2A summarizes the general experimental workflow.

Alternatively, CRISPRmod dCas9-SALL1-SDS3 mRNA can be used with synthetic CRISPRi guide RNA to directly transfect or electroporate cells, thereby eliminating the need to generate cell lines. Figure 2B summarizes the general experimental workflow with dCas9-SALL1-SDS3 mRNA. For more information regarding this method, please see these protocols.

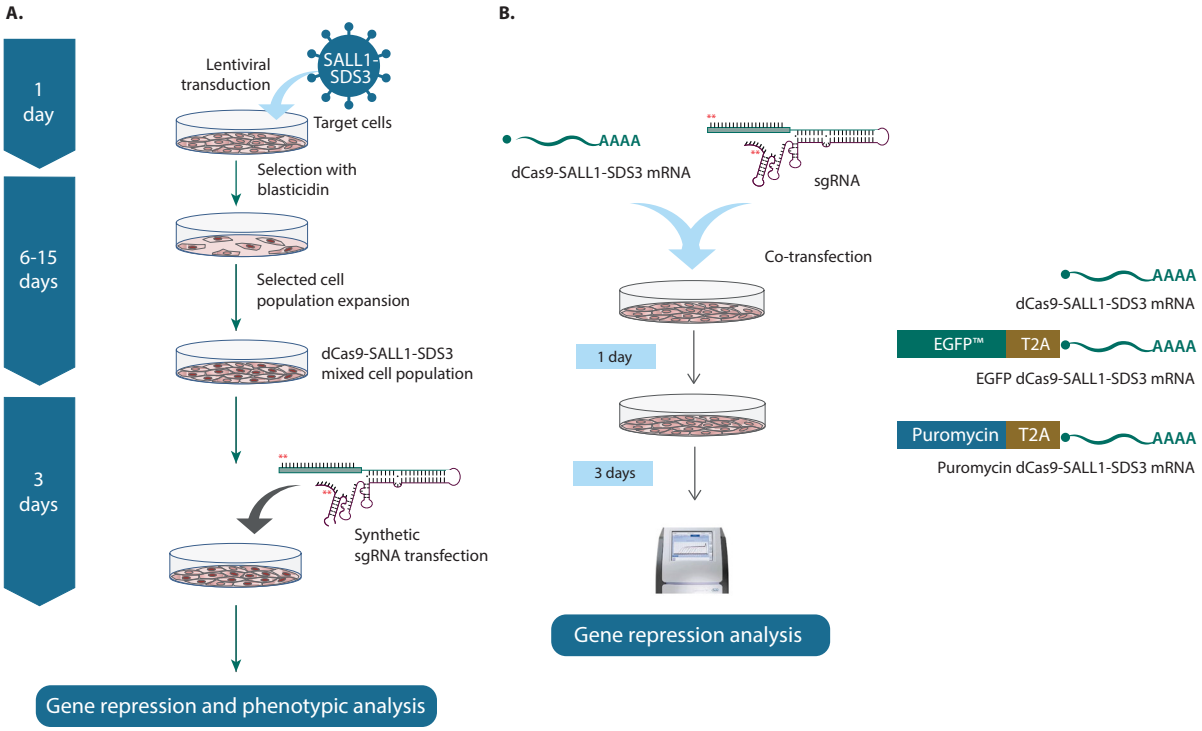

Figure 2. CRISPR interference workflow with synthetic sgRNA and lentiviral dCas9-SALL1-SDS3 (2A) or dCas9-SALL1-SDS3 mRNA (2B).

CRISPRmod CRISPRi Lentiviral dCas9-SALL1-SDS3 expression vectors

The lentiviral dCas9-SALL1-SDS3 expression vectors contain a human codon-optimized version of the catalytically inactive *S. pyogenes cas9 (csn1)* gene due to point mutations on the RuvC1 and HNH nuclease domains (D10A and H840A) in addition to a blasticidin resistance marker (BlastR). Expression is bicistronic with a 2A peptide sequence and under the control of a single promoter (Figure 3). A brief description of the lentiviral vector elements is provided in Table 1.

Several promoter options are available (Figure 3) enabling the researcher to choose a lentiviral vector with the most active promoter for their specific cells of interest. All lentiviral dCas9-SALL1-SDS3 expression vectors are supplied as lentiviral particles (≥ 1×10^7 TU/mL, ± 20%).

Table 1. Elements of the dCas9-SALL1-SDS3 expression vectors.

Vector element	Utility
dCas9-SALL1-SDS3	*S. pyogenes* dCas9-SALL1-SDS3 for transcriptional repression of targeted gene when programmed with a guide RNA
T2A	Self-cleaving peptide allows simultaneous expression of two proteins from a single transcript
Blast^R	Blasticidin resistance marker permits antibiotic selection of transduced mammalian cells
hCMV	Human cytomegalovirus immediate early promoter
mCMV	Mouse cytomegalovirus immediate early promoter
hEF1α	Human elongation factor 1 alpha promoter
5' LTR	5' Long Terminal Repeat necessary for lentiviral particle production and integration of the construct into the host cell genome
ψ	Psi packaging sequence allows lentiviral genome packaging using lentiviral packaging systems
RRE	Rev Response Element enhances titer by increasing packaging efficiency of full-length lentiviral genomes
WPRE	Woodchuck Hepatitis Post-transcriptional Regulatory Element enhances transgene expression in target cells
3' SIN LTR	3' Self-inactivating Long Terminal Repeat for generation of replication-incompetent lentiviral particles
SV40 pA	Simian virus 40 polyadenylation signal
pUC ori	pUC origin of replication
SV40 ori	Simian virus 40 origin of replication
Amp^R	Ampicillin resistance gene for vector propagation in *E. coli* cultures

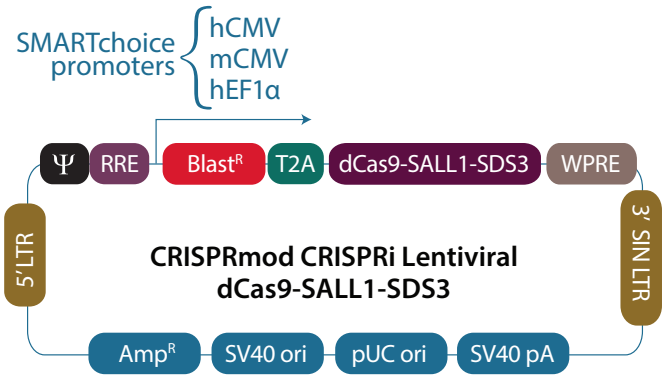

Figure 3. Schematic diagram of the Lentiviral dCas9-SALL1-SDS3 expression vectors.

CRISPRi synthetic single guide RNA (sgRNA) for transcriptional repression

CRISPRi synthetic sgRNA is a 100 nucleotide chimera fusing the crRNA and tracrRNA sequences with a 4 nt tetraloop sequence (Jinek et al., 2012). It is modified for nuclease resistance on both 5′ and 3′ ends of the molecule. CRISPRi sgRNAs are pre-designed, based on a published CRISPRi v2.1 algorithm (Horlbeck et al., 2016) and target genomic regions in the proximity of a transcriptional start site (TSS). The sgRNAs are available as three individual sgRNAs or a pool of three sgRNA for human protein coding genes. When more than one TSS exists for a gene, a second set of sgRNA reagents is available (labeled P2) to target the alternative start site.

3 CRISPRi protocol for transcriptional gene repression

In this workflow, lentiviral dCas9-SALL1-SDS3 particles are used to generate cells stably expressing dCas9-SALL1-SDS3 in a heterogenous or clonal population. These cells are then transfected with synthetic guide RNAs to obtain target gene repression.

CRISPRmod CRISPRi materials required

CRISPRmod Lentiviral dCas9-SALL1-SDS3

CRISPRmod Lentiviral dCas9-SALL1-SDS3 expression vectors are provided as concentrated, purified lentiviral particles for immediate transduction. Select the lentiviral dCas9-SALL1-SDS3 expression vector with the most active promoter in your cell line based on empirical testing or known promoter activity.

- CRISPRmod CRISPRi Lentiviral dCas9-SALL1-SDS3 particles with your choice of promoter:
  - CRISPRmod CRISPRi Lentiviral hCMV-Blast-dCas9-SALL1-SDS3 particles (Cat# VCAS12245)
  - CRISPRmod CRISPRi Lentiviral mCMV-Blast-dCas9-SALL1-SDS3 particles (Cat# VCAS12246)
  - CRISPRmod CRISPRi Lentiviral hEF1α-Blast-dCas9-SALL1-SDS3 particles (Cat# VCAS12247)

CRISPRmod CRISPRi guide RNA

- CRISPRmod CRISPRi synthetic sgRNA: Predesigned sgRNA for repression of your gene of interest
  - Human CRISPRi sgRNA pool (Cat #CF-HUMAN-XX) 2, 5, 10 nmol tubes
  - Human CRISPRi Set of 3 sgRNA (Cat #CQ-HUMAN-XX) 2, 5, 10 nmol tubes
  - Human CRISPRi sgRNA (Cat #CI-HUMAN-XX) 2, 5, 10 nmol tubes
  - The above catalog numbers are agnostic; actual products have gene-specific catalog numbers.

DharmaFECT Transfection Reagent for transfection of sgRNA

Each DharmaFECT formulation is chemically distinct to optimize delivery and viability across a wide variety of cell types. DharmaFECT 1 is the most universal reagent that works well across a wide variety of cell lines. Optimal conditions for siRNA may be used for sgRNA in a given cell line.

- DharmaFECT 1 Transfection Reagent (Cat #T-2001-01, 02, 03, 04)
- DharmaFECT 2 Transfection Reagent (Cat #T-2002-01, 02, 03, 04)
- DharmaFECT 3 Transfection Reagent (Cat #T-2003-01, 02, 03, 04)
- DharmaFECT 4 Transfection Reagent (Cat #T-2004-01, 02, 03, 04)

Additional materials required

The following additional materials are required but not supplied:

- 10 mM Tris pH 7.4, nuclease-free buffer (Tris buffer) solution (Dharmacon, Cat #B-006000-100)
- Multi-well tissue culture plates or tissue culture dishes
- Blasticidin S (Fisher Scientific, Cat #BP2647-25; InvivoGen, Cat #ant-bl-1)

Positive control CRISPRi sgRNA reagent:

- CRISPRmod PPIB CRISPRi sgRNA control (Cat #U-009250-XX-02 or -05)
- CRISPRmod PPIB CRISPRi sgRNA control pool (Cat #U-009250-XX-02 or -05)
- CRISPRmod SEL1L CRISPRi sgRNA control (Cat #U-009600-XX-02 or -05)
- CRISPRmod SEL1L CRISPRi sgRNA control pool (Cat #U-009600-XX-02 or -05)

Negative control sgRNA reagent:

- CRISPRmod CRISPRi sgRNA Non-targeting (Cat #U-009550-XX-02 or 05)
- CRISPRmod CRISPRi sgRNA Non-targeting control pool (Cat #U-009550-XX-02 or 05)

Base Medium: Appropriate antibiotic-free cell culture medium without serum

Growth Medium: Appropriate antibiotic-free cell culture medium (with serum and/or supplements) recommended for maintenance of the cells of interest

Selection Medium: Growth Medium supplemented with the appropriate concentration of antibiotics

Additional recommended materials:

- Materials for RNA isolation and quantitative RT-qPCR for gene expression analysis
- Assay for assessing cell viability

Generation of stable cell line expressing dCas9-SALL1-SDS3

The protocol described here is designed for rapid generation of a cell population where most of the cells have single integration of a lentiviral dCas9-SALL1-SDS3 proviral sequence in the genome.

Determining blasticidin concentration for selection of transduced cells

The CRISPRmod CRISPRi Lentiviral dCas9-SALL1-SDS3 expression vectors confer resistance to blasticidin in transduced cells. Before transducing cells, determine the minimum concentration of blasticidin required to kill non-transduced cells (in 3 to 10 days) by generating a blasticidin kill curve. The blasticidin concentration range for many mammalian cells is 2-15 µg/mL.

Transduction of cells with CRISPRmod CRISPRi Lentiviral dCas9-SALL1-SDS3 expression particles

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

If a different size culture dish is used, adjust the number of cells, volumes, and reagent quantities in proportion to the change in surface area (see Appendix Table 3 for suggested volumes of Transduction Medium per surface area of culture dishes).

Day 1:

1. Plate 5 × 104 cells per well in a 24-well plate using Growth Medium.
2. Incubate cells at 37 °C in a humidified CO_2 incubator overnight.

Optimal cell number for plating will vary with growth characteristics of specific cells and should be determined experimentally. Typically, cells should be at 60-80% confluency on the day of transduction.

Day 2:

1. Equilibrate the Base Medium to 37 °C.
2. Calculate the volume of lentiviral particles needed for transduction at MOI = 0.3 (see appendix).

The functional titer of Lentiviral CRISPRmod Lentiviral dCas9-SALL1-SDS3 expression particles (in HEK293T cells, as determined by qPCR) is reported on the Certificate of Analysis (C of A). We recommend an MOI ≤ 0.3 (adjusted for relative transduction efficiency in your cell type) to ensure single integration of the lentiviral dCas9-SALL1-SDS3. The relative transduction efficiency of your cell type will likely be lower than that of HEK293T cells.

3. Thaw the CRISPRmod CRISPRi Lentiviral dCas9-SALL1-SDS3 particles on ice.

Lentiviral particles are shipped on dry ice as 25 µL aliquots and must be stored at -80 °C. Under these conditions, lentiviral particles are stable for at least 12 months. Repeated freeze-thaw cycles should be avoided, as this is expected to negatively affect titer. Once thawed, unused lentiviral particles should be kept on ice, divided into smaller aliquots (if necessary) and immediately returned to -80 °C.

4. Once thawed, gently mix and pipette the calculated volume of lentiviral particles into 0.25 mL of the Base Medium (no serum) to create the Transduction Medium.
5. Remove the Growth Medium from the well and add the Transduction Medium containing the lentiviral particles (see Appendix for guidelines on other plate formats).
6. Incubate cells at 37 °C in a humidified CO_2 incubator for 4-6 hours.
7. At 4-6 hours post-transduction, add an additional 0.75 mL of Growth Medium (with serum) and resume incubation at 37 °C in a humidified CO_2 incubator.

If toxicity occurs with your cells, in step 7, replace the medium after 4-6 hours with fresh Growth Medium (with serum).

Days 3-15:

Generation of stably expressing dCas9-SALL1-SDS3 cell lines with blasticidin selection

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

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

2. Once the selected cells are growing normally, expand accordingly to freeze enough aliquots for your experimental project. These cells will be a mixed population that on average have a single integration of dCas9-SALL1-SDS3 in their genomes.

Record the passage number and avoid working with stable cell populations that exceed 10 passages from the frozen stock.

Utilize the mixed population of dCas9-SALL1-SDS3 expressing cells obtained above for transfection with synthetic CRISPRi guide RNAs or plasmid CRISPRi lentiviral sgRNAs or transduction of lentiviral CRISPRi sgRNAs for repression of your gene of interest. Alternatively, if clonal cell lines are required for your application, we recommend that you isolate clonal cell lines for downstream experiments using protocols appropriate for your cells of interest.

Transfection of synthetic CRISPRi guide RNAs

The following is an example protocol for delivery of CRISPRi synthetic sgRNA into adherent U2OS cells stably expressing dCas9-SALL1-SDS3. The protocol is provided for transfection in 96-well plates and the volumes are given for one well and a final 25 nM concentration of sgRNA. We suggest performing the transfection in triplicate wells and adjusting the volumes accordingly providing excess for the ease of pipetting. Transfection conditions vary between cell lines and should be determined empirically. For alternative plating formats see Table 2 for volume recommendations.

Day 1:

1. Plate 1×10^4 U2OS-dCas9-SALL1-SDS3 cells per well in a 96-well plate using Growth Medium.
2. Incubate cells at 37 °C in a humidified CO_2 incubator overnight.

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

Day 2:

1. Prepare 1 µM solution of CRISPRi synthetic sgRNA from previously prepared 10 µM stocks.
2. In nuclease-free microcentrifuge tubes (or deep-well 96-well plates for multiple targets) prepare the synthetic sgRNA by adding 2.5 µL of 1 µM sgRNA to 7.5 µL of serum-free medium.
3. Prepare a DharmaFECT transfection reagent working solution in a separate tube by diluting 0.2 µL of DharmaFECT 4 reagent in 9.8 µL of serum-free medium and mix gently. Incubate the tube for 5 minutes at room temperature.

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

4. Add 10 µL of DharmaFECT 4 working solution to each sample tube containing the sgRNA. This brings the total volume to 20 µL.
5. Immediately mix by pipetting gently up and down and incubate for 20 minutes at room temperature.
6. Prepare the Transfection Medium by adding 80 µL antibiotic-free complete Growth Medium to each sample to bring the total volume in each tube to 100 µL.
7. Remove medium from the wells of the 96-well tissue culture plate with cells and replace with 100 µL of the appropriate Transfection Medium in each well.
8. Incubate cells at 37 °C in a humidified CO_2 incubator for 48 to 72 hours before proceeding with the phenotypic assay or gene expression analysis (see Appendix).

Table 2. Suggested volumes per well for transfection of dCas9-SALL1-SDS3 expressing cells with 25 nM synthetic sgRNA

		Tube 1: sgRNA working solution (µL/well)		Tube 2: DharmaFECT transfection reagent working solution (µL/well)			
plating format (wells/plate	well surface area (cm^2/well)	1 µM synthetic sgRNA (µL)	Base Medium (serum free) (µL)	DharmaFECT (µL)	Base Medium (serum free) (µL)	Growth Medium (µL/well)	Final transfection volume (µL/well)
96	0.3	2.5	7.5	0.2	9.8	80	100
24	2	12.5	37.5	1	49	400	500
12	4	25	75	2	98	800	1000
6	10	50	150	5	195	1600	2000

4 Appendix

Optimization of transfection conditions for delivery of CRISPRi synthetic guide RNA

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

Our recommended ranges for transfection components in a 96 well plate are as follows:

- 0.05 to 0.8 µL/well of DharmaFECT 1, 2, 3 or 4
- 25 nM control sgRNA per well

At 48 to 72 hours post-transfection, perform a cell viability assay to determine the highest lipid concentration that has minimal cell toxicity (≥ 70% of cell viability is preferred). After assaying for cell viability, we recommend to carefully wash the cells once with Phosphate Buffered Saline (PBS) and proceed with gene expression analysis to determine the condition that produces highest gene repression. Use the determined optimal conditions for subsequent transfection of your selected dCas9-SALL1-SDS3 expressing cell lines with the CRISPRi synthetic guide RNA.

Gene expression analysis recommendations

RNA can be isolated using different methods per manufacturer's instructions. Quantitative RT-qPCR 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.

Multiplicity of Infection (MOI)

The equation to calculate a volume of lentiviral stock for a given MOI is:

$$V = MOI \times CN \div VT \times 1000$$

Where:

V = volume of lentiviral stock in µL

MOI = desired multiplicity of infection

CN = number of cells in the well at transduction

VT = lentiviral titer in TU/mL (indicated in the Certificate of Analysis) and multiplied by 1000 to convert the volume from mL to µL

For example, for a desired MOI of 0.3 and:

- Cell density of 100 000 cells per well at time of transduction
- Lentiviral titer = 1×10^7 TU/mL

Then,

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

Volume of Transduction medium per surface area in culture dishes

Table 3. Suggested volumes of Transduction Medium for different plate formats.

Tissue culture dish	Surface area per well (cm^2)	Suggested total serum-free medium volume per well (mL)
100 mm	56	5
6 well	9.4	1
12 well	3.8	0.5
24 well	1.9	0.25
96 well	0.3	0.05

Stability and storage

CRISPRmod CRISPRi Lentiviral dCas9-SALL1-SDS3 expression particles are shipped on dry ice as 25 µL aliquots and must be stored at -80 °C. Under these conditions, lentiviral particles are stable for at least 12 months. Repeated freeze-thaw cycles should be avoided, as this is expected to negatively affect titer. Once thawed, unused lentiviral particles should be kept on ice, divided into smaller aliquots (if necessary) and immediately returned to -80 °C.

Synthetic sgRNA

CRISPRmod CRISPRi synthetic guide RNA reagents are shipped as dried pellets at room temperature. Under these conditions, they are stable for at least four weeks. Upon receipt, synthetic guide RNA should be stored at -20 °C to -80 °C. Under these conditions, the reagents are stable for at least one year. Always resuspend synthetic guide RNA in nuclease-free Tris buffer. In solution and stored at -20 °C, the reagent aliquots are stable for at least one year. Avoid multiple freeze-thaw cycles. We recommend not exceeding four to five freeze-thaw cycles to ensure RNA integrity.

5 Frequently asked questions

How should I store my synthetic guide RNAs?

RNA oligonucleotides should be stored at -20 °C or -80 °C in a non-frost-free freezer, either as a dried pellet or resuspended in an RNase-free solution buffered to pH 7.4 to help with stability during freeze-thaw cycles. You may use our 10 mM Tris-HCl Buffer pH 7.4 for resuspension. We recommend that RNA oligonucleotides be resuspended to a convenient stock concentration (Synthetic guide RNA resuspension protocol) and stored in small aliquots to avoid multiple freeze-thaw cycles. RNA oligonucleotides should not go through more than five freeze-thaw cycles. If degradation is a concern, the integrity of the RNA oligonucleotides can be evaluated on an analytical PAGE gel

Can I use my siRNA transfection protocols to transfect CRISPRmod CRISPRi synthetic guide RNAs?

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

Can I use a transfection reagent other than DharmaFECT to deliver the sgRNA into my cells?

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

What is the best way to confirm that my gene is repressed?

We suggest using RT-qPCR to measure the relative change in target gene expression levels between samples treated with a non-targeting control and CRISPRi guide RNAs. RT-qPCR analysis can be completed with either the SYBR green method or probe based gene expression assays. Follow manufacturer's instructions for RNA isolation and RT-qPCR set up and use best practices to avoid cross-contamination during the RNA isolation, cDNA synthesis and qPCR set up. Use proper controls for RT-qPCR analysis (include no RNA samples, no reverse transcriptase samples, and no cDNA samples as negative controls). Additionally, when performing RT-qPCR for gene repression the expression level may drop to a level that is not detectable. In this case, when using the ΔΔCq method of analysis, an arbitrary value representing the detection limit of the qPCR instrument is used as a placeholder for "non-detectable" as a non-zero value is necessary to perform the calculation. In most cases this value will be between 35 and 40 depending on the number of programmed cycles and the instrument Cq determination method. We recommend adding additional cycles (up to 45 total) to standard qPCR cycling conditions.

Can the CRISPRmod CRISPRi system be used for gene repression in non-mammalian organisms, such as flies or worms?

The CRISPRmod CRISPRi system is designed for mammalian expression and has been tested in mammalian cells. The guide RNAs are predesigned to repress human genes. Custom guide RNAs could be ordered that target the genomes of other species, however we cannot predict the efficacy of using CRISPRmod lentiviral dCas9-SALL1-SDS3 particles and CRISPRmod synthetic guide RNA components, nor can we troubleshoot experiments performed in non-mammalian systems.

What is the formula for spectrophotometric quantification of synthetic sgRNA?

To quantify RNA, use Beer's Law: Absorbance (260 nm) =(ε)(concentration)(path length in cm), where ε, epsilon, is the molar extinction coefficient (provided on the Product Transfer Form supplied with the synthetic sgRNA order). When solved for the unknown, the equation becomes: Concentration = (Absorbance, 260 nm) / [(ε)(path length in cm)]. When a standard 10 mm cuvette is used, the path length variable in this equation is 1. If a different size of cuvette is used, e.g., a 2 mm microcuvette, then the path length variable is 0.2

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 repressor domains add an additional 533 amino acids to dCas9 which shift the molecular weight of dCas9-SALL1-SDS3 to approximately ~220 kDa. The protein could be detected using Cas9 antibodies (for example: Novus Biologicals cat#NBP2-36440).

Can I use Edit-R guide RNA for gene knockout or CRISPRmod predesigned guide RNA for gene activation in CRISPRi experiments?

The guide RNA designs for CRISPRi are different than the Edit-R guide RNAs for CRISPR-Cas9 knockout experiments and the CRISPRmod CRISPRa guide RNAs for gene activation. The guide RNA designs for CRISPRi are required to bind immediately downstream of the transcriptional start site and are based on apublished CRISPRi algorithm. Predesigned Edit-R guide RNAs are optimized for functional gene knockout and target the gene's coding region. Predesigned CRISPRmod CRISPRa guide RNAs are designed to bind upstream of the TSS and are based on a published algorithm specific to CRISPRa.

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

The published CRISPRi v2.1 algorithm (Horlbeck et al., 2016) used FANTOM and Ensembl databases to accurately predict the transcriptional start site (TSS) and some genes were identified as having alternative transcriptional start sites. The publication lists 10 guide RNA designs per TSS. For the CRISPRmod CRISPRi predesigned guide RNAs, we offer the top three guide RNAs for the primary TSS, and, when applicable, three guide RNAs for the secondary TSS. These are labeled as P1 and P2, respectively. If the CRISPRi 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, as which TSS is active and to what level, depends on your cell line. For a small number of genes Horlbeck et al., 2016 identified more than two TSS. We only offer P1 and P2 designs as catalog items, but we can generate the additional guide RNAs as a custom request using designs from the published algorithm.

How specific are the guide RNAs in targeting the gene of interest?

Several publications have shown CRISPRi to be highly specific by RNA-seq expression analysis, but CRISPRi is a new technology and off-targeting still needs to be explored in more detail. CRISPRi off-target effects can only occur when the guide RNA binds to the region proximal to the TSS of another gene , which dramatically decreases the potential off-target space. Furthermore, the published algorithm applies a filter for off-target binding, and takes into account chromatin structure, nucleosome position, and sequence features to accurately predict highly effective guide RNAs.

However, there might be examples of genes where the TSS for one gene is in close proximity to another gene's TSS. Investigation of the genomic location for your gene of interest and performing expression analysis to confirm repression of the target gene without having effects on other proximal genes might be important for proper interpretation of the phenotypic analysis.

Can I use CRISPRmod CRISPRi guide RNAs with dCas9-KRAB or other CRISPRi repressors?

Yes. The CRISPRmod CRISPRi system uses canonical guide RNAs that can be used with other similar systems that use canonical Cas9 guide RNAs, like KRAB-based repressors.

Can I use CRISPRmod CRISPRi guide RNAs with an aptamer-based CRISPRi system?

CRISPRmod CRISPRi guide RNAs cannot be used with CRISPRi systems that utilize guide RNAs modified with aptamer sequences to bring the repressors to the dCas9-gRNA binding site. The functionality of algorithm-designed guide RNAs is transferable between different CRISPRi systems, so the target sequence of a CRISPRi guide RNA could be utilized in a guide that is designed to recruit repressors and ordered as a custom RNA.

How do I choose between CRISPRmod Lentiviral dCas9-SALL1-SDS3 expression particles or CRISPRmod CRISPRi mRNA, which format works better?

The different formats of dCas9-SALL1-SDS3 are tailored towards different research needs. Generating stable cell lines using the CRISPRmod Lentiviral dCas9-SALL1-SDS3 expression particles and subsequently transfecting synthetic guide RNAs is optimal for use with short timepoint assays in cell lines that are amenable to transfection and lentiviral transduction. Alternatively, one may transduce the CRISPRmod Lentiviral dCas9-SALL1-SDS3 expression particles and lentiviral sgRNA sequentially if long-lasting repression is desired.

If lentiviral transductions are not an option in your cell type of interest, a co-transfection or co-electroporation of CRISPRmod dCas9-SALL1-SDS3 mRNA and CRISPRmod synthetic sgRNA can be performed. We offer two formats of CRISPRi mRNA that enable enrichment of transfected populations, PuroR dCas9-SALL1-SDS3 mRNA and EGFP dCas9-SALL1-SDS3 mRNA. For more information regarding these methods, please refer to the "CRISPRmod mRNA electroporation protocol" and "fluorescent CRISPRmod mRNA-enrichment protocol".

What level of repression should I expect? How long does target gene repression last?

The level of transcriptional repression will vary depending on your target gene, cell line, and timepoint of analysis. Target gene repression is maximal between 48 and 96 hours after transfection of CRISPRmod CRISPRi synthetic sgRNAs and can persist for five to seven days post-transfection. Alternatively, CRISPRmod CRISPRi Lentiviral sgRNAs can be used to enact sustained repression for assays requiring longer timepoints.

Is the level of transcriptional repression correlated with basal expression of the gene?

The level of target gene repression does not appear to correlate with basal expression levels. However, it can be difficult to detect changes in expression of genes transcribed at very low basal levels and CRISPRi reagents cannot be used to target genes that are not expressed. Therefore, it is still important to know the endogenous level of expression of the target gene(s) in the cell line you plan to work in.

Do pooled synthetic guide RNAs perform better than individual guide RNAs? Can I multiplex guide RNAs to simultaneously repress multiple genes?

Using our pools of predesigned synthetic guide RNAs to target your gene of interest ensures maximal repression as pools generally perform as well as, if not better than, the most active individual guide RNA. Individual guide RNAs targeting multiple genes can also be pooled together (multiplexed) without substantially compromising the levels CRISPRi-induced repression. If you intend on multiplexing more than four guides, we recommend testing each CRISPRi sgRNA individually to determine the lowest functional working concentration.

6 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. Qi, L.S., *et al.*, Repurposing CRISPR as an RNA-guided platform for sequence-specific control of gene expression. *Cell.* **152**(5): p. 1173-83 (2013)
4. L.A. Gilbert, *et al.*, CRISPR-mediated modular RNA-guided regulation of transcription in eukaryotes. *Cell.* **154**(2): p. 442-51 (2013).
5. A.W. Cheng, *et al.*, Multiplexed activation of endogenous genes by CRISPR-on, an RNA-guided transcriptional activator system. *Cell Res.* **23**(10): p. 1163-71 (2013).
6. NC. Yeo, *et al.*, An enhanced CRISPR repressor for targeted mammalian gene regulation. *Nat Methods.* **15**(8): p. 611-616 (2018).
7. F. Moghadam, *et al.*, Synthetic immunomodulation with a CRISPR super-repressor in vivo. *Nat Cell Biol.* **22**(9): p. 1143-1154 (2020).
8. N. Alerasool, *et al.*, An efficient KRAB domain for CRISPRi applications in human cells. *Nat Methods.* **17**(11): p. 1093-1096 (2020).
9. M. A. Horlbeck *et al.*, Compact and highly active next-generation libraries for CRISPR-mediated gene repression and activation. eLife. 5, e19760 (2016).
10. L. A. Gilbert *et al.*, Genome-Scale CRISPR-Mediated Control of Gene Repression and Activation. *Cell.* **159**, 647–661 (2014).

7 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 lentiviral particle products.

Any investigator who purchases 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 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.
Horizon Discovery
2650 Crescent Drive
Lafayette, CO 80026 USA
Tel. +1 800 235 9880; +1 303 604 9499
Fax: +1 800 292 6088; +1 303 604 9680
Email: technical@horizondiscovery.com

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.
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), April 2019

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 abovementioned 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).

8 Limited use licenses

The gene editing Products, use and applications, are covered by pending and issued patents. Certain Label licenses govern the use of the products; these can be found at 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.

For more information

To find the contact information in your country for your technology of interest, please visit us at **horizondiscovery.com/contact-us**

Horizon Discovery, 8100 Cambridge Research Park, Waterbeach, Cambridge, CB25 9TL, United Kingdom

horizon™ INSPIRED CELL SOLUTIONS

V10221