horizon

TECHNICAL MANUAL

Dharmacon™ CRISPRmod CRISPRa transcriptional activation system with expressed sgRNA

Contents

1 Introduction to the CRISPR-Cas9 system for transcriptional activation

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 activation 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). CRISPRa utilizes dCas9 fused to different transcriptional activation domains (Cheng et al., 2013, Gilbert et al., 2014, Tanenbaum et al., 2014, Konermann et al., 2015; Chavez et al., 2015), which can be directed to promoter regions by specifically designed guide RNA (depicted in Figure 1). The VPR activation system utilizes a fusion of three transcriptional activators (VP64, p65 and Rta) to the C-terminal end of dCas9 and demonstrates a robust gene activation in mammalian systems (Chavez et al., 2015). Unlike other CRISPRa systems that require expression of the transcriptional activators from separate vectors, the VPR system requires delivery of just two components to the cells of interest: dCas9-VPR and a guide RNA, making it easier to utilize across different biological applications.

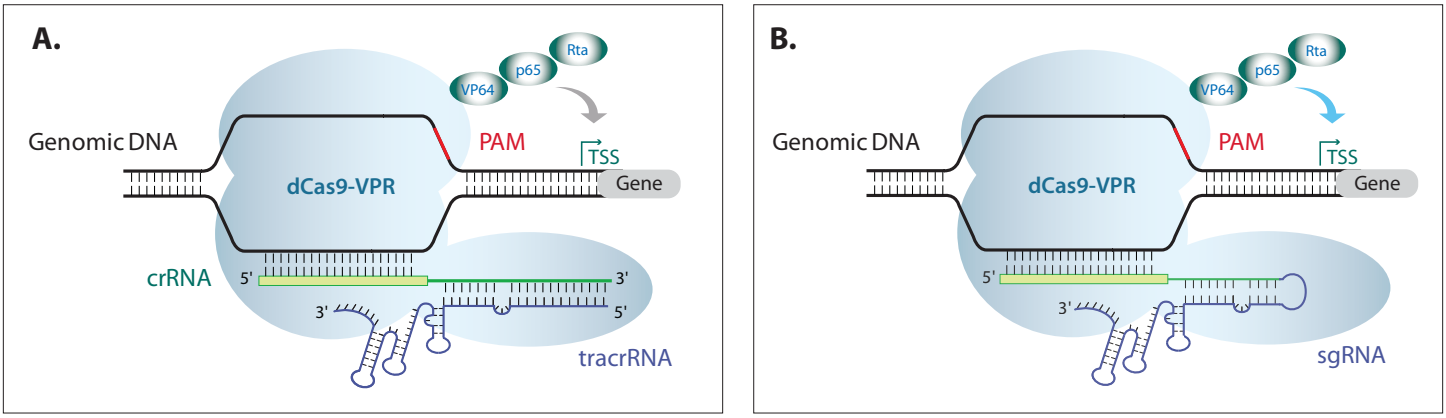

Figure 1. Diagram of dCas9-VPR with crRNA:tracrRNA or sgRNA targeting a gene's promoter region

A CRISPRa guide RNA can be either synthetic CRISPR RNA (crRNA) complexed with trans-activating CRISPR RNA (tracrRNA) (Figure 1A) or a single guide RNA (sgRNA) where the crRNA has been fused to the tracrRNA creating a chimeric structure (Figure 1B).

This protocol provides guidance for expressed single guide RNA (sgRNA). For CRISPRa using the two-part synthetic guide RNA approach (crRNA complexed with tracrRNA) please see this manual.

2 CRISPRmod CRISPRa workflow

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

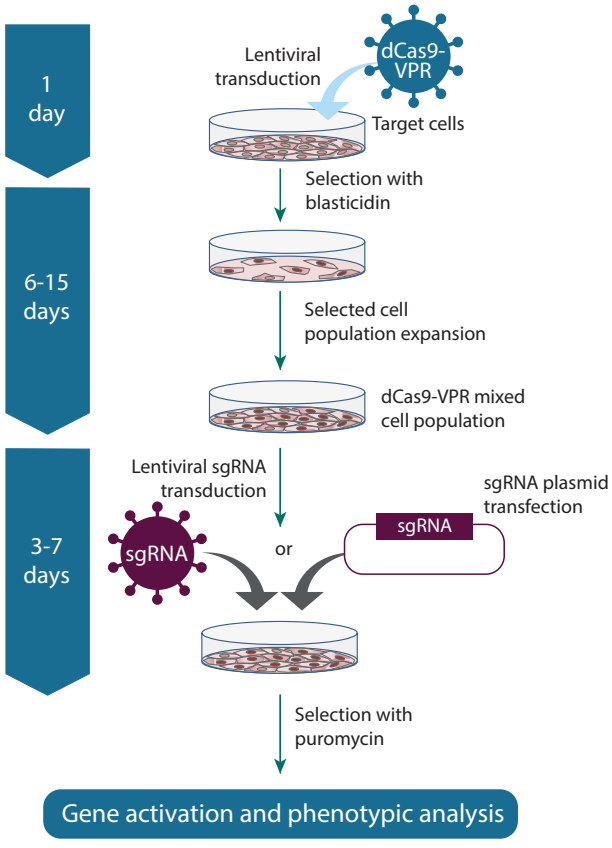

Figure 2. CRISPR activation workflow with lentiviral dCas9-VPR and expressed sgRNA.

CRISPRmod CRISPRa Lentiviral dCas9-VPR expression vectors

The CRISPRmod CRISPRa Lentiviral dCas9 -VPR 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) and the 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 specific cells of interest. All CRISPRmod CRISPRa Lentiviral dCas9-VPR expression vectors are supplied as lentiviral particles (≥ 1×10^7 TU/mL, ± 20%) or dried down, endotoxin-free plasmid DNA, ready for lentiviral packaging.

Table 1. Elements of the CRISPRmod CRISPRa Lentiviral dCas9-VPR expression vectors and sgRNAs.

Vector element	Utility
dCas9-VPR	*S. pyogenes* dCas9-VPR for gene activation of targeted DNA 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
U6	Human RNA polymerase III promoter U6
Puro^R	Puromycin resistance marker permits antibiotic selection of transduced or transfected mammalian cells
5' LTR	5' Long Terminal Repeat necessary for lentiviral particle production and integration of the construct into the host cell genome
ψ	Psi packaging sequence allows lentiviral genome packaging using lentiviral packaging systems
RRE	Rev Response Element enhances titer by increasing packaging efficiency of full-length lentiviral genomes
WPRE	Woodchuck Hepatitis Post-transcriptional Regulatory Element enhances transgene expression in target cells
3' SIN LTR	3' Self-inactivating Long Terminal Repeat for generation of replication-incompetent lentiviral particles
SV40 pA	Simian virus 40 polyadenylation signal
pUC ori	pUC origin of replication
SV40 ori	Simian virus 40 origin of replication
Amp^R	Ampicillin resistance gene
sgRNA	single guide RNA

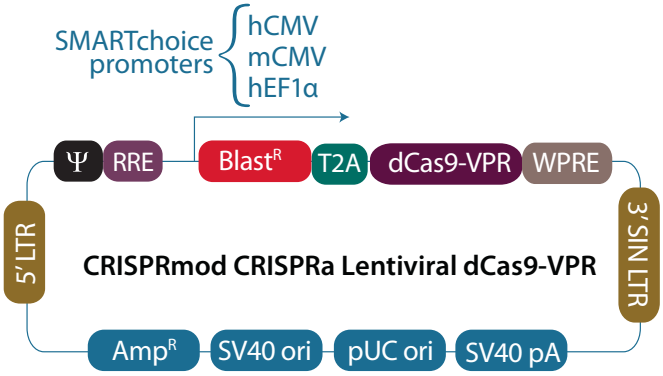

Figure 3. Schematic diagram of the CRISPRmod CRISPRa Lentiviral dCas9-VPR expression vectors.

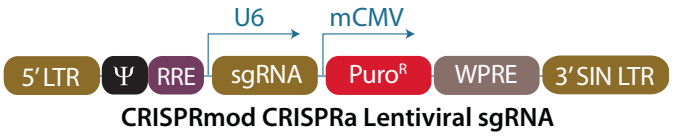

Figure 4. Schematic diagram of the CRISPRmod CRISPRa Lentiviral sgRNA.

CRISPRmod CRISPRa Lentiviral guide RNA

CRISPRmod CRISPRa 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 4). CRISPRmod CRISPRa sgRNAs are pre-designed, based on a published CRISPRa v2 algorithm (Horlbeck et al., 2016) and target genomic DNA in the proximity of transcriptional start site (TSS).

The protospacer region of the sgRNA comprises 19-20 nucleotides identical to the genomic DNA target site, followed by the non-variable sgRNA scaffold containing the tracrRNA sequence from *S. pyogenes* optimized for efficient gene activation. Four pre-designed CRISPRmod CRISPRa lentiviral sgRNAs per TSS are available for human and mouse protein coding genes. When more than one TSS exists for a gene, a second set of sgRNA reagents is available (labeled P2). CRISPRmod CRISPRa Lentiviral sgRNA vectors are supplied as concentrated, purified lentiviral particles (≥ 1×10^8 TU/mL , ± 20%) or glycerol stocks.

3 CRISPRmod CRISPRa protocol for transcriptional gene activation

In this workflow, CRISPRmod CRISPRa lentiviral dCas9-VPR particles are utilized to generate cell lines stably expressing dCas9-VPR in a population of cells or in isolated clonal cell lines. These cells can then be transduced with CRISPRmod CRISPRa lentiviral sgRNAs (for stable integration and expression of the sgRNA) or transfected with CRISPRmod CRISPRa lentiviral sgRNA expression plasmid (for transient expression of the sgRNA) to obtain transcriptional target gene activation.

CRISPRmod CRISPRa materials required

CRISPRmod CRISPRa Lentiviral dCas9-VPR

CRISPRmod CRISPRa Lentiviral dCas9-VPR expression vectors are provided as concentrated, purified lentiviral particles for immediate transduction or as endotoxin-free plasmid DNA for direct transfection into a packaging cell line and production of your own lentiviral particles. Select the CRISPRmod CRISPRa lentiviral dCas9-VPR nuclease vector with the most active promoter in your cell line based on empirical testing or known promoter activity.

- CRISPRmod CRISPRa Lentiviral dCas9-VPR particles with your choice of promoter:
  - CRISPRmod CRISPRa Lentiviral hCMV-Blast-dCas9-VPR particles (Cat # VCAS11918)
  - CRISPRmod CRISPRa Lentiviral mCMV-Blast-dCas9-VPR particles (Cat # VCAS11920)
  - CRISPRmodCRISPRa Lentiviral hEF1α-Blast-dCas9-VPR particles (Cat # VCAS11922)
- CRISPRmod CRISPRa Lentiviral dCas9 -VPR Expression plasmids with your choice of promoter:
  - CRISPRmod CRISPRa Lentiviral hCMV-Blast-dCas9-VPR plasmid (Cat # CAS11914)
  - CRISPRmod CRISPRa Lentiviral mCMV-Blast-dCas9-VPR plasmid (Cat # CAS11915)
  - CRISPRmod CRISPRa Lentiviral hEF1α -Blast-dCas9-VPR plasmid (Cat # CAS11916)

CRISPRmod CRISPRa guide RNA

- CRISPRmod CRISPRa lentiviral sgRNA are predesigned for activation for your gene of interest and are available as lentiviral particles or glycerol stocks.
  - CRISPRmod CRISPRa Lentiviral sgRNA particles (Cat # VSGH11888, VSGH11889, VSGH11891, VSGH11892, VSGM11894, VSGM11895, VSGM11897, VSGM11898)
  - CRISPRmod CRISPRa Lentiviral sgRNA glycerol stock (Cat# GSGH11887, GSGH11890, GSGM11893, GSGM11896)
- DharmaFECT Transfection Reagent (for transfection of the lentiviral sgRNA plasmids)
  - DharmaFECT kb Transfection Reagent (Cat # T-2006-01, -05)

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)
- Puromycin (Fisher Scientific, Cat #BP2956-100; InvivoGen, Cat #ant-pr-1)

Positive control lentiviral sgRNA:

- CRISPRmod CRISPRa Human POU5F1 Lentiviral sgRNA (Cat # VSGH11902, GSGH11904)
- CRISPRmod CRISPRa Human TTN Lentiviral sgRNA (Cat # VSGH11899, GSGH11901)
- CRISPRmod CRISPRa Mouse Pou5f1 Lentiviral sgRNA (Cat # VSGM11908, GSGM11910)
- CRISPRmod CRISPRa Mouse Ttn Lentiviral sgRNA (Cat # VSGM11905, GSGM11907)

CRISPRmod CRISPRa with expressed sgRNAs Technical Manual

Negative control guide RNA:

- CRISPRmod CRISPRa sgRNA Non-targeting Control (Cat # VSGC11911, GSGC11913)
- 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-VPR

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

Determining blasticidin concentration for selection of transduced cells

The CRISPRmod CRISPRa Lentiviral dCas9-VPR 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 CRISPRa Lentiviral dCas9-VPR 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 sized culture dish is used, adjust the number of cells, volumes, and reagent quantities in proportion to the change in surface area (see Appendix for suggested volumes of Transduction Medium per surface area of culture dishes).

Day 1:

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

The functional titer of CRISPRmod CRISPRa Lentiviral dCas9-VPR 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-VPR. The relative transduction efficiency of your cell type will likely be lower than that of HEK293T cells.

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

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

Where:

V = volume of lentiviral stock in µL

MOI = desired multiplicity of infection

CN = number of cells in the well at transduction

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

For example, for a desired MOI of 0.3 and:

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

Then,

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

3. Thaw the CRISPRmod CRISPRa Lentiviral dCas9-VPR 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-VPR 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 CRISPRmod CRISPRa dCas9-VPR in their genomes.

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

Utilize the mixed population of dCas9-VPR expressing cell line obtained above for transfection with synthetic CRISPRmod CRISPRa guide RNAs or transduction of CRISPRmod CRISPRa lentiviral sgRNAs for activation of your gene of interest. If clonal cell lines are required for your application, we recommend that you isolate clonal cell lines for downstream experiments using protocols appropriate for your cells of interest.

Transduction of CRISPRmod CRISPRa lentiviral sgRNA

The following is an example protocol for transduction of CRISPRmodCRISPRa Lentiviral sgRNA expression particles for gene activation. The protocol is provided for transduction in 96-well plates at MOI of 0.3 into adherent U2OS cells stably expressing dCas9-VPR. Efficiency of lentiviral delivery and optimal transduction conditions vary widely between cell types and must be determined empirically for each cell line of interest. For alternative plating formats see Table 2 for volume recommendations.

Determining puromycin concentration for selection

The CRISPRmod CRISPRa Lentiviral sgRNA vector confers resistance to puromycin in transduced cells. Before transducing cells, generate a puromycin kill curve to determine the minimum concentration of puromycin required to kill non-transduced cells between two and five days. The puromycin concentration range for many mammalian cells is 1-10 µg/mL.

Day 1:

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

Day 2:

The functional titer of CRISPRmod CRISPRa Lentiviral sgRNA 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) for single integration of the lentiviral sgRNA. Higher MOI could be used as well but will generate a population of cells with different number of integrations.

3. Thaw the CRISPRmod CRISPRa Lentiviral sgRNA particles on ice.
4. 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 50 µL per well. We recommend performing the transduction in triplicate wells and adjusting the volumes accordingly (adding excess for the ease of pipetting).
5. Remove the Growth Medium from the well and add 50 µL of the Transduction Medium containing the lentiviral particles prepared in step 4 (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 50 µL of Growth Medium (with serum) per well and resume
8. Incubation at 37 °C in a humidified CO_2 incubator.

Day 3-7:

1. At 24-48 hours post-transduction, replace the medium with Selection Medium containing the appropriate amount of puromycin.

Addition of blasticidin (to continue to selection for dCas9-VPR integrated cells) to the Selection Medium containing puromycin is optional. If the cells become confluent, split the cells into a larger dish to allow proper antibiotic selection (for instance, split cells from 96-well to 24-well culture dishes).

2. Replace the Selection Medium every 2-3 days and monitor the accumulation of dead cells daily.
3. Once the cells are growing normally in Selection Medium, expand the cells to freeze sufficient number of aliquots for your experimental project and/or proceed with the phenotypic assay and gene expression analysis (see Appendix).

Expansion of the puromycin/blasticidin-resistant cells at this stage will generate a mixed population of cells containing a single integration of the CRISPRmod CRISPRa lentiviral sgRNA in their genomes, in addition to the single integration of dCas9-VPR. For higher level of transcriptional gene activation, a higher MOI of the of CRISPRmod CRISPRa 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 activation.

Packaging CRISPRmod CRISPRa Lentiviral dCas9-VPR plasmids into particles

CRISPRmod CRISPRa Lentiviral dCas9-VPR expression plasmids are Tat dependent and require a packaging system that expresses the *tat* gene. For packaging of lentiviral constructs, we recommend the Dharmacon Trans-Lentiviral ORF Packaging System. For packaging protocols and additional information please consult the product manual.

CRISPRmod CRISPRa Lentiviral Blast-dCas9-VPR Plasmids do not express a fluorescent protein reporter, therefore, after packaging of plasmid DNA, we recommend titering the lentiviral particles using a functional lentiviral titration protocol such as limiting dilution with cell viability assay by crystal violet staining or genomic qPCR assay.

CRISPRmod CRISPRa Lentiviral sgRNA glycerol stocks

CRISPRmod CRISPRa Lentiviral sgRNA is provided as glycerol stock for plasmid isolation. Check the CRISPRmod CRISPRa sgRNA glycerol stock protocol for plasmid preparation and packaging into lentiviral particles. For plasmid preparation, grow all CRISPRmod CRISPRa Lentiviral sgRNA clones at 37 °C in LB broth medium supplemented with 100 µg/mL carbenicillin. The CRISPRmod CRISPRa Lentiviral sgRNA plasmids could be used for transfection of dCas9-VPR stable cells (see DharmaFECT kb protocol for recommendations). Alternatively, Lentiviral sgRNA plasmids could be used for co-transfection with dCas9-VPR expression plasmids for transient over-expression and target gene activation.

Co-transfection of dCas9-VPR plasmid and CRISPRmod CRISPRa lentiviral sgRNA plasmid

For transcriptional gene activation in short-term assays or if lentiviral transductions are not possible, a co-transfection of CRISPRmod CRISPRa lentiviral dCas9-VPR and sgRNA expression plasmids can be performed (Figure 5).

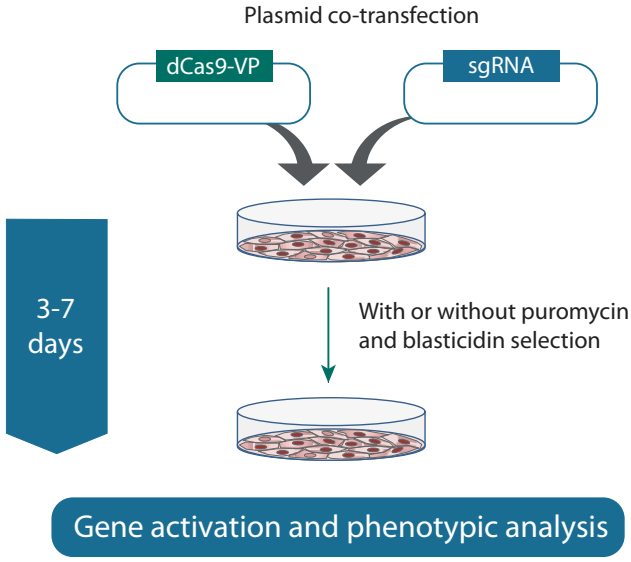

Figure 5. CRISPRa workflow for plasmid co-transfection of lentiviral dCas9-VPR and expressed sgRNA.

To perform a co-transfection of CRISPRmod CRISPRa dCas9-VPR expression plasmid and CRISPRmod CRISPRa dCas9-VPR plasmid, refer to DharmaFECT kb protocol.

The recommended plasmid ratio for co-transfection of two plasmids is 1:1 (100-200 ng total DNA per well in 96-well plates).

Blasticidin and puromycin selection can be used to eliminate non-transfected cells or create a stable cell population (see DharmaFECT kb protocol for more details).

4 Appendix

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.

Volume of medium per surface area in culture dishes

Table 2. Suggested volumes of Transduction Medium for different plate formats.

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

Stability and storage

Lentiviral particles

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

Plasmid DNA

CRISPRmod CRISPRa Lentiviral dCas9-VPR Expression plasmid DNA are shipped as dried pellets at room temperature. Under these conditions, they are stable for at least four weeks. Upon receipt, plasmid DNA should be stored at -20 °C to -80 °C. Under these conditions, the reagents are stable for at least one year. Always dissolve plasmid in nuclease-free solution, such as Tris buffer.

5 Frequently asked questions

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

We suggest using RT-qPCR to measure the relative change in target gene expression levels between samples treated with non-targeting control and CRISPRmod CRISPRa 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, no cDNA samples negative controls). Additionally, when performing RT-qPCR for gene activation the expression level may go from not detectable to expressed. 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 place holder 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.

How do I choose between the different methods for CRISPRa described in the protocol?

The different methods for CRISPRa described in the protocol are tailored towards different phenotypic readouts. Generating stable cell lines using the CRISPRmod CRISPRa Lentiviral dCas9-VPR Expression plasmids and subsequently transducing guide RNAs is optimal for long-term assays with stable integration of the guide RNA. Transfection of the guide RNA in CRISPRmod CRISPRa Lentiviral dCas9-VPR stable cells allows the development of short timepoint assays (3-7 days). This method also allows the transfection of multiple sgRNA plasmids per target for increased activation. Note that puromycin selection is required to remove non-transfected cells, if necessary for analysis and there likely will be variability in the transcriptional activation among the transfected cells. If lentiviral transductions are not an option, then a plasmid co-transfection with CRISPRmod CRISPRa Lentiviral dCas9-VPR Expression plasmids and CRISPRmod CRISPRa expressed sgRNA plasmid can be performed and used in short-term assays. Note that puromycin and blasticidin selection are required to remove non-transfected cells, if necessary for analysis and there likely will be variability in the transcriptional activation among the transfected cells.

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

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

Can the CRISPRmod CRISPRa guide RNAs be used for transfection in cells that use other CRISPRa systems?

The CRISPRmod CRISPRa guide RNAs can work with other systems that use standard guide RNAs, like SunTag, but it is not compatible with systems that use gRNAs modified with aptamers to bring the activators to the dCas9 binding site, like the SAM CRISPRa system. For these systems, our CRISPRmod MS2 tracrRNA can be paired with CRISPRmod CRISPRa synthetic crRNA reagents for gene activation.

Can I co-transduce the CRISPRmod CRISPRa Lentiviral dCas9-VPR Expression and my sgRNA particles in my cell lines for faster gene activation results?

Although this is not the recommended protocol, transduction of both the CRISPRmod CRISPRa Lentiviral dCas9 VPR expression and the sgRNA particles can be performed at the same time. If co-transduction is necessary, it is recommended to extend the dual antibiotic selection to the longest selection time period previously determined for the individual antibiotics in your cells. We cannot predict the outcome, nor troubleshoot any issues, resulting from co-transduction of dCas9-VPR and CRISPRmod CRISPRa lentiviral sgRNA particles.

How do I choose between the various CRISPRmod CRISPRa Lentiviral dCas9-VPR promoter options?

Choose the promoter option that has been demonstrated, either by your own experimental observations or through references in the published literature, to actively express a transgene in your cells of choice. For optimal experimental confidence or if such information is not available, consider testing CRISPRmod CRISPRa dCas9-VPR expression vectors with different promoters using positive control CRISPRmod CRISPRa guide RNAs.

How can I identify the best transduction conditions of CRISPRmod CRISPRa Lentiviral dCas9-VPR and single guide RNA particles in my cells?

Successful transduction of cells depends on cell type, cell density, passage number, MOI during transduction, purity of the lentiviral preparation and the presence and/or absence of reagents that facilitate transduction (for example, polybrene). If you do not know what the best conditions for transduction of your cell type of interest are, optimization can be performed in a 96-well plate format prior to your experiments, testing cell density, presence or absence of serum, presence or absence of transduction additives (polybrene) and the duration of transduction (6 hours or overnight) prior to addition of growth medium to your cells.

Can I use the Edit-R predesigned guide RNA for gene knockout in CRISPRa experiments?

No, the CRISPRmod CRISPRa guide RNA designs for CRISPRa are different than the CRISPRmod CRISPRa guide RNAs for CRISPR-Cas9 knockout experiments. The guide RNA designs for CRISPRa are required to bind upstream of the transcriptional start site and are based on a published CRISPRa algorithm. Pre-designed CRISPRmod CRISPRa guide RNAs are optimized for functional gene knockout with the algorithm and target the gene's coding region.

Where can I obtain the CRISPRmod CRISPRa Lentiviral dCas9-VPR expression vector or CRISPRmod CRISPRa Lentiviral sgRNA vector maps?

CRISPRmod CRISPRa lentiviral dCas9-VPR and sgRNA plasmid maps can be obtained upon request from Technical Support.

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

The VPR activators add additional 536 amino acids to dCas9 which shift the molecular weight of the dCas9-VPR to approximately ~220 kDa. The protein could be detected using Cas9 antibodies (for example: Novus Biologicals cat #NBP2-36440).

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

The published CRISPRa v2 algorithm (Horlbeck et al., 2016) used FANTOM and Ensembl databases to predict the transcriptional start site (TSS) more accurately. Some genes (6.8%) were identified as having alternative transcriptional start sites. The publication lists 10 gRNA designs per TSS. For the CRISPRmod CRISPRa predesigned guide RNAs, we offer the top four guide RNAs for the primary TSS, and, when applicable, four guide RNAs for the secondary TSS. These are labeled as P1 and P2, respectively. If the CRISPRmod CRISPRa 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. For a small number of genes (0.1%) 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 based on designs from the published algorithm.

How specific are the CRISPRmod CRISPRa gRNAs in targeting the gene of interest?

Several publications have shown CRISPRa to be highly specific by RNA seq expression analysis, but CRISPRa is a new technology and off-targeting still needs to be explored in more detail. Keep in mind that for CRISPRa off-targeting, the guide RNA needs to bind to the promoter region of another gene in order to have an off-target effect, which dramatically decreases the potential off-target space. Furthermore, the guide RNAs are designed based on a published algorithm that incorporates chromatin, nucleosome position, and sequence features to accurately predict highly effective guide RNAs and also applies 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 location for your gene of interest and performing expression analysis to confirm activation of the target gene without having effects on other proximal genes might be important for proper interpretation of the phenotypic analysis.

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. A. Chavez *et al.*, Highly efficient Cas9-mediated transcriptional programming. *Nat. Methods.* **12**, 326–328 (2015).
7. M. A. Horlbeck *et al.*, Compact and highly active next-generation libraries for CRISPR-mediated gene repression and activation. eLife. 5, e19760 (2016).
8. L. A. Gilbert *et al.*, Genome-Scale CRISPR-Mediated Control of Gene Repression and Activation. *Cell.* **159**, 647–661 (2014).
9. M. E. Tanenbaum, L. A. Gilbert, L. S. Qi, J. S. Weissman, R. D. Vale, A protein-tagging system for signal amplification in gene expression and fluorescence imaging. *Cell.* **159**, 635–646 (2014).
10. S. Konermann *et al.*, Genome-scale transcriptional activation by an engineered CRISPR-Cas9 complex. *Nature.* **517**, 583–588 (2015).

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 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 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 2016

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

©2021 The Horizon logo and other trademarks are the property of Horizon Discovery Limited, unless otherwise stated. DHARMACON and EDIT-R are trademarks of Dharmacon Inc.

V41121