

## Transcriptional Regulation with CRISPR/Cas9 Effectors in Mammalian Cells

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### Abstract

CRISPR/Cas9-based regulation of gene expression provides the scientific community with a new high-throughput tool to dissect the role of genes in molecular processes and cellular functions. Single-guide RNAs allow for recruitment of a nuclease-dead Cas9 protein and transcriptional Cas9-effector fusion proteins to specific genomic loci, thereby modulating gene expression. We describe the application of a CRISPR-Cas9 effector system from *Streptococcus pyogenes* for transcriptional regulation in mammalian cells resulting in activation or repression of transcription. We present methods for appropriate target site selection, sgRNA design, and delivery of dCas9 and dCas9-effector system components into cells through lentiviral transgenesis to modulate transcription.

**Key words** CRISPR/Cas9, Cas9-effectors, Transcriptional regulation, Gene activation, Gene repression

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### 1 Introduction

Dissection of individual gene function through targeted downregulation or overexpression greatly benefits from versatile systems that allow for easy manipulation of target genes. Loss-of-function and gain-of-function high-throughput screens for factors involved in cellular function are also highly desirable. CRISPR (clustered regularly interspaced short palindromic repeats)-associated (Cas) systems offer the opportunity to manipulate endogenous genes at the level of transcriptional regulation, allowing for insight into the role of individual genes and gene regulatory networks in their endogenous context.

The CRISPR/Cas system present in bacteria and archaea serves as an adaptive immune system detecting and silencing foreign DNA [1, 2]. CRISPR systems incorporate invading DNA sequences into the genome at CRISPR loci that are later transcribed and used to guide nucleases to cleave invading DNA. Type II CRISPR systems require a single CRISPR-associated (Cas) gene, Cas9, a trans-activating CRISPR RNA (tracrRNA) and a

CRISPR RNA (crRNA) to carry out this directed, sequence-specific DNA degradation [3, 4].

Type II CRISPR systems have recently been adapted for genome editing in mammalian cells, where a human codon-optimized version of the Cas9 protein introduced along with a crRNA and tracrRNA is capable of inducing site-specific double-strand breaks [5]. It is common to use a chimeric version of the crRNA and tracrRNA to form a single guide RNA (sgRNA), further simplifying the system to just two components consisting of the Cas9 protein and sgRNA to achieve site-specific recruitment and target cleavage [5, 6].

Target sites for CRISPR-mediated DNA cleavage are dependent on the presence of a specific protospacer adjacent motif (PAM) sequence 3' to the targeted genomic DNA sequence [3, 7]. PAM sequences differ between CRISPR systems, for example, for the species *Streptococcus pyogenes* (Sp) the PAM sequence is "NGG" [7] whereas for *Neisseria meningitidis* (Nm), the PAM sequence is "NNNNGATT" [8]. Since the Cas9 endonuclease cleaves target DNA only if the PAM sequence is present, the possible target sites are therefore defined by the CRISPR system used. However certain variations in the PAM sequences can be tolerated, including "NAG" for the Sp system and "NNNNGCTT" for the Nm system [9, 10], increasing the frequency of possible genomic target sites.

Several methods have been published describing the use of CRISPR-Cas9 systems for genome editing (e.g., [11]). However, since Cas9 can be guided to target specific genomic sequences, and effector proteins can be fused to Cas9, this programmable system is ideal for many applications beyond gene targeting through DNA cleavage. The protocol presented here focuses on CRISPR-based methods to manipulate gene regulation in mammalian cells using a Sp CRISPR-Cas9 system coupled to the Krueppel repressor associated box (KRAB) domain and the quadruple tandem repeat of the herpes simplex virus VP16 (VP64) transactivation domain [12–15]. In extension, the methods described can be easily transferred to CRISPR-Cas9 systems from other species as well as to Sp Cas9 coupled to different effectors.

In order to utilize the CRISPR system to affect gene regulation, it is first necessary to inactivate the DNA cleavage activity of the Cas9 protein. Cas9 contains two nuclease domains, a RuvC-like domain and a HNH domain, the activities of which are responsible for performing the double strand break once the Cas complex is recruited to DNA [3]. Inactivation of Cas9 nuclease activity is therefore commonly achieved through mutation of key catalytic residues in both of the Cas9 nuclease domains (Sp system: amino acid changes D10A and H840A) creating a nuclease dead version of the Cas9 protein (dCas9) [3, 16]. dCas9 can then be used with an sgRNA to enable recruitment to specific genomic loci without inducing DNA cleavage.

sgRNA-directed dCas9 recruitment to DNA has been shown to interfere with gene transcription (CRISPRi) through steric hindrance by preventing transcriptional elongation, polymerase binding or transcription factor binding thereby influencing gene expression levels [16]. Recently, additional tools have been developed for the manipulation of gene regulation through fusion of dCas9 to effector domains as have previously been developed for TALE and zinc finger systems [17]. Coupling the dCas9 to effectors allows for site-specific delivery of any effector domain of interest, and many different effector domains have been successfully fused to dCas9 systems to modulate gene expression in mammalian cells. Positioned by the sgRNA-dCas9 complex, the effector domain can dictate the cellular response resulting in gene repression (CRISPRi) (e.g., KRAB, SID domains) or gene activation (CRISPRa) (e.g., VP16, VP64, p65AD domains) when targeted to regions upstream of transcriptional start sites [16, 18–25].

dCas9 effector-mediated gene regulation can be applied to a broad range of studies including influencing cellular states in stem cells through differentiation [24] to dissecting of the contribution of individual genomic elements or transcription factor binding sites to the regulation of a single gene. Critically, due to the speed and ease of sgRNA design, high-throughput approaches have been developed for gain- or loss-of-function screens in mammalian cells [26, 27].

Here, we describe a stepwise protocol for applying a CRISPR-Cas9 system from *Streptococcus pyogenes* for gene regulation in mammalian cells through a KRAB repressor domain or VP64 activation domain. The protocol includes preparation of a dCas9-effector system, design and cloning of sgRNAs (Subheadings 3.1–3.3) in addition to lentiviral production and transgenesis (Subheadings 3.4 and 3.5). These steps can be applied to other autologous CRISPR-Cas9 systems and can be utilized in a variety of cell types.

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## 2 Materials

### 2.1 Plasmids

1. dCas9 and dCas9-effector plasmids
  - pHAGE TRE-dCas9 (Addgene #50915)
  - pHAGE TRE-dCas9-VP64 (Addgene #50916)
  - pHAGE TRE-dCas9-KRAB (Addgene #50917)
  - pHAGE EF1alpha-dCas9-VP64 (Addgene #50918)
  - pHAGE EF1alpha-dCas9-KRAB (Addgene #50919)
2. sgRNA plasmids
  - pLenti Sp BsmBI sgRNA Puro (Addgene #62207)
  - pLenti Sp BsmBI sgRNA Hygro (Addgene #62205)
3. Lentiviral plasmids

pHDM-G (DNASU #235)  
 pHDM-Hgpm2 (DNASU #236)  
 pHDM-tat1b (DNASU #237)  
 pRC/CMV-rev1b (DNASU #246)

## **2.2 Molecular Biology Reagents**

1. DNA oligomers (25 nmol standard desalting conditions).
2. Molecular biology grade water.
3. ATP.
4. T4 polynucleotide kinase 10 U/ $\mu$ l.
5. BsmBI 10 U/ $\mu$ l.
6. Tris–acetate–EDTA (TAE) buffer.
7. Agarose.
8. QIAquick Gel Extraction Kit (Qiagen).
9. T4 DNA ligase 400 U/ $\mu$ l.
10. Stbl3 chemically competent *E. coli*.
11. Ampicillin sodium salt.
12. Luria broth (LB).
13. Luria agar.
14. QIAprep Spin Miniprep Kit (Qiagen).
15. EcoRI 20 U/ $\mu$ l.
16. QIAGEN Plasmid Maxi Kit (Qiagen).

## **2.3 Cell Culture Reagents**

1. Cell line: human embryonic kidney (HEK) 293T/17 (ATCC # CRL-11268).
2. Dulbecco's Modified Eagle's Medium (DMEM) high glucose (4.5 g/L).
3. Fetal bovine serum (FBS).
4. GlutaMAX.
5. Nonessential amino acids (NEAA).
6. Sodium pyruvate.
7. 0.25 % trypsin.
8. 10 cm dish.
9. 6-well plate.
10. TransIT-293 transfection reagent (Mirus).
11. OptiMEM medium.
12. 10 ml syringes.
13. Millex-HV Syringe Filter Unit, 0.45  $\mu$ m, PVDF, 33 mm.
14. Lenti-X concentrator.
15. Puromycin 10 mg/ml.
16. G418 50 mg/ml.

17. Hygromycin B 50 mg/ml.
18. Doxycycline.
19. Phosphate buffered saline (PBS).
20. Formalin.
21. Trypan Blue.
22. PBST (1× PBS + 0.2 % Triton 100).
23. Donkey serum.
24. Rat anti-hemagglutinin (HA) antibody (3F10) (Roche).
25. Secondary antibody.
26. Hoechst 33342 10 mg/ml.
27. Target specific qPCR primers and/or antibody.

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## 3 Methods

### 3.1 Preparation of dCas9 and dCas9-Effector and sgRNA Plasmids

1. Obtain bacterial stocks of Sp dCas9 or dCas9-effector (KRAB and VP64) lentiviral plasmids available through Addgene. These plasmids are ready for use and are available as either constitutive or inducible versions (*see Note 1*).
2. Streak bacteria onto 100 µg/mL ampicillin LB-agar plates and grow overnight at 37 °C.
3. Pick a single colony and grow for 6–8 h at 37 °C with shaking at 250 rpm in 5 mL of LB media (containing 100 µg/mL ampicillin).
4. Transfer the 5 mL of culture to 250 mL of fresh LB media (containing 100 µg/mL ampicillin) and grow overnight with shaking at 250 rpm at 37 °C.
5. Prepare the plasmid DNA using the Qiagen Maxiprep kit according to the manufacturer's protocol for subsequent preparation in Subheading 3.3.
6. (Optional) Confirm plasmid integrity by diagnostic digest.
  - (a) Digest 1 µg of plasmid DNA with 1 µL of each of the restriction enzymes indicated in Table 1 for 1 h at 37 °C.
  - (b) Run the digest on a 1 % agarose gel to resolve the indicated band sizes

### 3.2 sgRNA Design

Guidelines for selecting genomic targets are discussed in **Note 2**. Since the efficiency of different sgRNAs in downstream applications may vary, we recommend designing multiple sgRNAs per target.

#### 3.2.1 Designing DNA Oligomers for Sp sgRNA Cloning Manually

1. Search for the PAM sequence (NGG) within the region of interest to identify possible target sites with the following genomic context GN<sub>19</sub>NGG. Since the sgRNA is expressed from a human U6 promoter, a G is required at the start of the sequence for expression.

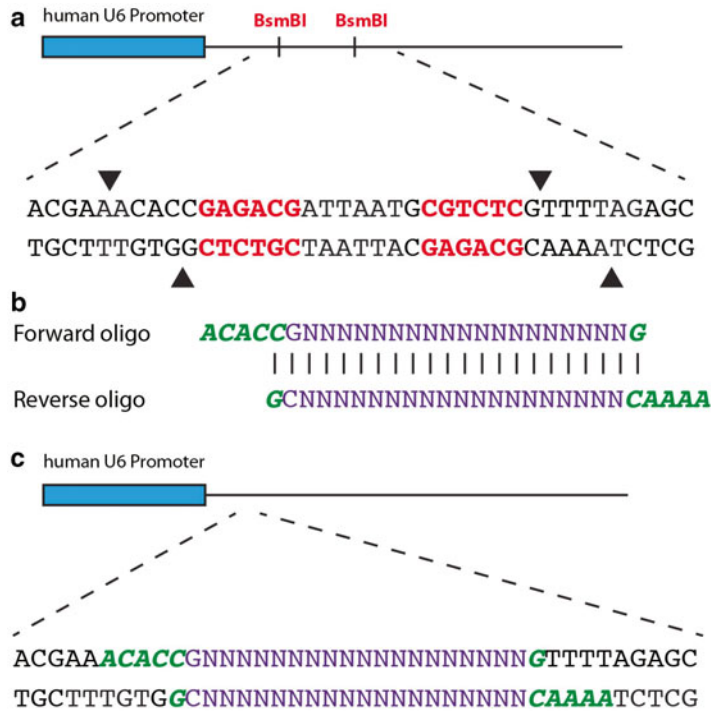
**Table 1**  
**List of plasmids and respective restriction enzymes**

Plasmid	Enzymes to digest with	Expected band sizes
pHAGE TRE-dCas9 (Addgene #50915)	AflIII	5919, 4385, 3953, 176
pHAGE TRE-dCas9-VP64 (Addgene #50916)	AflIII	5919, 4553, 3953, 176
pHAGE TRE-dCas9-KRAB (Addgene #50917)	AflIII	5919, 3953, 2760, 1824, 176, 17
pHAGE EF1alpha-dCas9-VP64 (Addgene #50918)	AflIII	6142, 3583, 3081
pHAGE EF1alpha-dCas9-KRAB (Addgene #50919)	AflIII	4355, 3583, 3081, 1824, 17
pLenti Sp BsmBI sgRNA Puro (Addgene #62207)	EcoRI, XhoI	7044, 1440
pLenti Sp BsmBI sgRNA Hygro (Addgene #62205)	XhoI	7743, 1550

2. BLAST the genomic context (GN<sub>19</sub>NGG) to confirm that there are no identical sequences in your genome.
3. Target site sequences with palindromes or poly-T -G or -A runs [26] should be avoided.
4. Order the following oligomers for sgRNA cloning into either sgRNA backbone vector. Be sure to omit the PAM sequence (NGG) and to include the overhang sequences to facilitate cloning into the BsmBI sites in the Addgene #62205 or #62207 sgRNA backbones (*see* Fig. 1).
  - (a) Forward oligo: ACACC (GN<sub>19</sub>) G
  - (b) Reverse oligo: AAAAC (N<sub>19</sub> complement C) G

### 3.2.2 Designing DNA Oligomers for Sp sgRNA Cloning Online

1. Many sgRNA design tools are now publically available to facilitate sgRNA design [9, 28–31]. DNA regions of interest can be fed into these tools to identify genomic targets predicted to have minimal off-target effects and to generate the target site sequences for a variety of mammalian species. It is important to ensure that the sgRNAs are designed for the Sp Cas9 system as sgRNAs designed for orthogonal CRISPR systems will have different PAM sequences and therefore would not be compatible.
2. In order to clone target site sequences into the Addgene #62205 or #62207 sgRNA backbones it is necessary to order oligomers containing the target site sequence (GN<sub>19</sub>) and to include the following overhang sequences:
  - (a) Forward oligo: ACACC (GN<sub>19</sub>) G
  - (b) Reverse oligo: AAAAC (N<sub>19</sub> complement C) G



**Fig. 1** Schematics for inserting annealed DNA oligomers into sgRNA expression backbones (Addgene #62207 and #62205). **(a)** Nucleotide sequence of backbone cloning region with BsmBI sites indicated in *bold red*. Digestion of the backbone with BsmBI results in DNA cuts at the positions indicated by *arrows*. **(b)** Annealed DNA oligomers containing the sgRNA target sequence. The overhangs for ligation are indicated in *bold green italics*. **(c)** Nucleotide sequence of ligation product containing oligomers inserted into sgRNA expression backbone

### 3.3 Cloning sgRNA into Lentiviral Expression Vector

1. Resuspend forward and reverse DNA oligomers for sgRNA cloning with molecular grade water at 100  $\mu\text{M}$ . Prepare the following reaction to phosphorylate the oligomers and anneal them into dsDNA fragments for ligation into an sgRNA backbone.

100 $\mu\text{M}$ Forward Oligo	2 $\mu\text{L}$
100 $\mu\text{M}$ Reverse Oligo	2 $\mu\text{L}$
10 $\times$ T4 polynuclease kinase buffer	2 $\mu\text{L}$
10 mM ATP	1 $\mu\text{L}$
T4 poly nuclease kinase	1 $\mu\text{L}$
Molecular grade water	12 $\mu\text{L}$
Total volume	20 $\mu\text{l}$

2. Incubate the reaction at 37  $^{\circ}\text{C}$  for 30 min.
3. Add 1  $\mu\text{L}$  of 5 M NaCl and 29  $\mu\text{L}$  Tris-EDTA (TE) Buffer

Heat the reaction in a heat block for 5 min at 95 °C, remove the block from the heat source and allow to cool slowly to room temperature. Alternatively, put in thermocycler, heat for 5 min at 95 °C and ramp to 25 °C at 0.1 °C/s

4. Dilute 1 µl of annealed sgRNA oligomers into 99 µl of TE buffer to prepare sample for ligation
5. Digest 2 µg of sgRNA backbone with 1 µL BsmBI overnight at 55 °C (*see Note 3*).
6. Run on a 1 % TAE agarose gel to resolve the 8500 bp fragment and detect potential uncut coiled plasmid
7. Gel purify the 8500 bp fragment using Qiagen Gel Extraction Kit
8. Elute in 30 µL elution buffer (EB) supplied with the Qiagen Gel Extraction Kit
9. Set up the following ligation overnight at 16 °C. Set up a ligation with no insert (annealed sgRNA oligomers) as a negative control.

Digested sgRNA backbone (from <b>step 8</b> )	100 ng
Annealed diluted sgRNA oligomers (from <b>step 4</b> )	4 µL
10× T4 DNA ligase buffer	2 µL
Molecular grade water	to 19 µL
T4 DNA ligase	1 µL

10. Transform 2 µL of ligation into Stb13 competent cells according to manufacturer's protocol. The use of Stb13 cells is critical, *see Note 4*.
11. Plate the transformed cells onto 100 µg/mL ampicillin LB-agar plates and grow overnight at 37 °C.
12. Check for colonies the following day. The negative control plate should have at least 10× fewer colonies than the plates where you have ligated in the annealed DNA oligomers. If the colony numbers are similar, this could indicate incomplete digestion of the sgRNA backbone.
13. Pick 4–6 colonies per ligation and grow overnight with shaking at 37 °C in 5 mL of LB culture (containing 100 µg/mL ampicillin).
14. Prepare the plasmid DNA using the Qiagen Miniprep kit according to the manufacturer's protocol
15. Perform a diagnostic restriction digestion to screen for the insertion of the annealed oligomers. Use the parental vector from **step 5**, Subheading **3.1** as a negative control.



- (a) Digest 1 µg plasmid DNA with 1 µl EcoRI-HF in NEB Buffer 2 at 37 °C for 1–2 h.
  - (b) Add 1 µl BsmBI to the reaction and incubate at 55 °C for 1–2 h.
  - (c) Run the digest on a 1 % agarose gel until 5500 and 3000 bp fragments are resolved in the negative control. If an insert is present, the BsmBI sites will have been removed and there will be a single 8500 bp fragment due to linearization with EcoRI.
16. (Optional) To verify cloning and to confirm the target site sequence, Sanger sequence the insert with a U6 forward sequencing primer (GACTATCATATGCTTACCGT)
  17. (Optional) Maxiprep verified clones for subsequent viral production using the Qiagen Maxiprep kit according to the manufacturer's protocol

### 3.4 Lentivirus Production

Both dCas9/dCas9-effector and sgRNA plasmids are third generation lentiviral vectors and can be packaged into lentiviral particles enabling delivery of both components of the dCas9 system into any cell type of interest.

#### 3.4.1 Lentivirus Production

1. HEK293T/17 cells are maintained in DMEM supplemented with 10 % FBS, 1 % GlutaMAX, 1 % NEAA, and 1 % sodium pyruvate. Cells are passaged every 3–4 days at around 70 % confluence. Do not allow the cells to become confluent.
2. The day before transfection, passage the cells with 0.25 % trypsin, and plate at  $1.3 \times 10^5$  cells/cm<sup>2</sup> or six million cells for each 10 cm dish. For alternatives *see* **Note 5**.
3. 2 h before transfection, feed the cells with fresh media
4. For the transfection, pipette DNA (dCas9-effector or sgRNA plasmids with packaging plasmids) in the following ratio 20:2:1:1:1 (Expression plasmid: pHDM-G: pHDM-Hgpm2: pHDM-tat1b: pRC/CMV-rev1b)

Plasmid	Desired (ng)
dCas9 or sgRNA	12,000
pHDM-G (DNASU #235)	1200
pHDM-Hgpm2 (DNASU #236)	600
pHDM-tat1b (DNASU #237)	600
pRC/CMV-rev1b (DNASU #246)	600
Total DNA	15,000

5. Transfect the cells with TransIT-293 transfection reagent in Opti-MEM according to manufacturer's protocol.

- (a) Dilute the DNA in 1 mL of OptiMEM in a 1.5 mL centrifuge tube
  - (b) Add 45  $\mu$ L of TransIT-293 transfection reagent to the center of the tube
  - (c) Mix gently using a 1 mL pipette
  - (d) Let incubate at room temperature, undisturbed, for 20 min
  - (e) Plate drop-wise onto cells and gently shake the plate to distribute the transfection mixture
6. The following day change the media on the transfected cells
  7. 48 h after transfection, harvest the virus by passing the media through a 0.45  $\mu$ M filter. Store the collected virus at  $-80^{\circ}\text{C}$ .
  8. (Optional) Feed the cells with an additional 10 mL of fresh media and harvest the virus 24 h later repeating **step 7**.
  9. (Optional) To obtain higher viral titers, virus can be concentrated immediately after harvest using Lenti-X concentrator according to the manufacturer's protocol. Alternatively, virus can be concentrated by centrifuging at  $100,000\times g$  for 90 min at  $4^{\circ}\text{C}$ .

**3.4.2 Viral Titer:  
Calculating Multiplicity  
of Infection (MOI)**

1. Maintain HEK293T/17 cells as in Subheading **3.4.1**
2. The day before infection plate  $5\times 10^4$  HEK293T/17 cells/well onto a 6-well plate.
3. The day of infection, thaw lentivirus stock on ice, and change the media on the cells. Add 10 or 1  $\mu$ L of virus to each well; be sure to leave one well uninfected as a negative control
4. 24 h after infection, change the media on infected cells
5. 48 h after infection, passage the cells at various densities (e.g., 1:10 and 1:100) onto a 10-cm dish and add selection reagent (depending on the lentiviral vector used this may vary, *see Note 6*)
6. Allow cells to grow until there are no cells left on the negative control plate and cells are large enough to visualize (usually 5–7 days), changing the media supplemented with selection reagent every 2–3 days
7. Carefully wash cells with PBS and fix with formalin solution for 30 min at room temperature. Wash with PBS, then stain with 2 mL of trypan blue for 10 min, and wash  $2\times$  with PBS. Count the number of colonies and calculate the viral titer:

$$\text{Titer (viral units / ml)} = \frac{(\# \text{colonies after selection})}{(\text{Dilution factor from } \mathbf{step\ 5} \text{ e.g. } 1/100)(\text{volume virus added from } \mathbf{step\ 3} / 1000)}$$

8. Expected titers for sgRNAs are  $10^6$  to  $10^7$  viral particles/ml, and for dCas9-effectors are  $\sim 10^4$  viral particles/ml

### 3.5 Virus Delivery/ Functional Assay

To determine the downstream effects of sgRNA-guided Cas9-effectors we commonly monitor cellular RNA and protein levels of target gene. Experiments should be designed to include negative controls, these could include sgRNAs designed to target a gene desert region or a sequence not present in the genome of interest. In the case of effector-based repression, it may be beneficial to include a dCas9 without a fused effector to distinguish between effector-mediated and steric hindrance effects.

#### 3.5.1 Generation of Stable dCas9, dCas9- Effector Cell Lines

1. Passage desired cell type.
2. Incubate the cells with the concentrated dCas9 or dCas9-effector viruses.
3. 24 h after infection, change the media following lentiviral handling safety procedures.
4. 48 h after infection, change the media following lentiviral handling safety procedures and start selection to generate stable cell lines (*see* **Notes 6–8**). The required amount of time for selection will vary with cell line and selection cassette.
5. Following completion of selection, lines may be subcloned or a pool of stably transduced cells can be used for future experiments.
6. (Optional) Expression of dCas9 or dCas9-effector can be confirmed through immunofluorescence detection of the HA-epitope tag. If doxycycline-inducible dCas9-effectors are used, cells must be maintained in 2 µg/ml doxycycline.
  - (a) Wash cells with PBS, then fix in formalin solution for 30 min at room temperature.
  - (b) Wash 1× with PBS, then 1× with PBST.
  - (c) Incubate with blocking buffer (5 % donkey serum in PBST) for 30 min at room temperature
  - (d) Dilute HA antibody in blocking buffer and stain for 3 h at room temperature. The HA antibody dilution should be optimized by lot.
  - (e) Wash 3× PBST and incubate with appropriate secondary antibody for 2 h at room temperature.
  - (f) Wash 3× PBST and incubate with 4 µg/ml Hoechst for 5 min at room temperature.
  - (g) Wash 1× with PBS and visualize on microscope.

#### 3.5.2 Transduction of sgRNA Lentivirus

1. Passage stably transduced dCas9 and dCas9-effector cell lines.
2. Incubate the cells with sgRNA lentiviruses. We usually infect cells with an MOI of ~1.
3. 24 h after infection, change the media following lentiviral handling safety procedures.

4. 48 h after infection, change the media following lentiviral handling safety procedures and start puromycin (for plasmid #62207) or hygromycin (for plasmid #62205) selection if desired.

### 3.5.3 Assessment of sgRNA Function

1. Following sgRNA transduction, successful CRISPRi or CRISPRa will result in relative loss or gain of target gene transcripts. Effects on gene expression can be assessed by quantitative PCR methods comparing test sgRNAs to negative control sgRNAs. The time required for the dCas9 or dCas9-effector to take effect may vary and a timecourse of the functional readout should be established for each new cell type.

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## 4 Notes

1. dCas9, dCas9-KRAB and dCas9-VP64 plasmids are available through Addgene as doxycycline-inducible versions with a neomycin selection cassette (#50915 and #50916) and constitutively active versions with a puromycin selection cassette (#50917, #50918, #50919). Additional dCas9-effector systems can also be acquired from Addgene or principle investigators. If using the inducible version dCas9, doxycycline must be added to the media at 2  $\mu\text{g}/\text{ml}$  after infection with dCas9 lentivirus to induce dCas9 expression. All vectors contain the dCas9 or dCas9-effector followed by a HA-epitope tag which can be used to detect expression of the dCas9. sgRNA plasmids for cloning are available with either a puromycin (#62207) or a hygromycin (#62205) selection cassette. Any of the dCas9 plasmids can be used with either of the sgRNA plasmids, however using a dCas9 lentiviral vector containing a selection cassette different from the sgRNA lentiviral vector will allow for selection of both components in a single cell.
2. Choosing an appropriate genomic target depends on the effector being used. When using dCas9 alone (without effector) to interfere with gene expression, genomic targets should either overlap or be downstream of the transcriptional start site. Successful CRISPRi effector mediated repression has been achieved by targeting dCas9KRAB within a  $-200$  to  $+300$  bp window of the transcriptional site [26, 32]. Targeting dCas9KRAB downstream of transcriptional start sites may result in a stronger repressive effect due to the combination of both KRAB-mediated repression and dCas9 mediated steric hindrance [26]. For CRISPRa, recent studies indicate that target sites within a  $-400$  to  $+1$  bp window of the transcriptional start site is optimal for dCas9-VP64 gene mediated activation [26,

- 32]. Designing multiple sgRNAs against a target and thereby recruiting multiple dCas9 or dCas9-effectors to the same region can increase the overall levels of activation [19–21, 24].
3. Esp3I (10U/ $\mu$ l), is an isoschizomer of BsmBI and can also be used to prepare the sgRNA backbone in place of BsmBI in Subheading 3.3, **step 5**. Digest (0.5  $\mu$ L/ $\mu$ g of plasmid) overnight at 37 °C.
  4. Due to the highly repetitive sequences found in lentiviral vectors (the repeat sequences in the LTR), Stbl3 cells are preferred for cloning. These cells reduce the rate of recombination caused by these repetitive sequences and conserve the integrity of the plasmid over time. The DNA yield is also found to be higher in these cells.
  5. The CRISPR-effector system can be used for screening purposes and the lentiviral production can be scaled down into a 96-well format using 100 ng of total DNA per well for transfection (scaled from 15  $\mu$ g of total DNA in **step 2**, subheading 3.4.1). In our experience, precoating plates with 5  $\mu$ g/ml fibronectin prior to plating the HEK293T cells for viral production will allow for comparable virus titers between large and small well sizes. For high throughput virus production we do not filter the virus but rather freeze directly after harvesting.
  6. The required dose and timing of selection reagents will vary with cell line. Typical timing and dose of common selection agents for mammalian cells are indicated but dose and concentration need to be optimized by cell type:

Concentration of selection reagent	Time required to complete selection
0.5–2 $\mu$ g/ml Puromycin	2 days
25–50 $\mu$ g/ml Hygromycin	4 days
50–300 $\mu$ g/ml G418 (Neomycin)	6 days

If each component cannot be selected for (e.g., when transducing multiple sgRNAs or using a puromycin resistant dCas9 plasmid with a puromycin resistant sgRNA plasmid) then the MOI in Subheading 3.5.2, **step 2** can be increased to attain cells expressing all components.

7. Some cell lines are difficult to transduce and may benefit from a pre-incubation step with lentiviral particles before plating (Subheading 3.5). For mouse and human ESCs, we perform a 3-h incubation with virus in low attachment plates containing a minimal amount of media, prior to plating for continued culture. Alternatively, for some cell lines transduction efficiency

may be improved by the addition of polybrene during transduction.

8. Due to the large size of some dCas9-effector constructs, it may be technically challenging to generate high titers of dCas9-effector lentivirus. If avoidance of lentiviral delivery of dCas9-effectors is desirable, cotransfection of dCas9-effectors together with sgRNAs is sufficient to modulate gene expression [19]. Alternatively, stable dCas9-effector lines can be generated through random or target integration of a linearized plasmid. Continuous selection can be used to ensure maintained expression of the dCas9-effectors. The sgRNAs can then be delivered to these cell lines via lentiviral infection or by transfection.

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