Topic Introduction

# An Introduction to CRISPR Technology for Genome Activation and Repression in Mammalian Cells

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CRISPR interference/activation (CRISPRi/a) technology provides a simple and efficient approach for targeted repression or activation of gene expression in the mammalian genome. It is highly flexible and programmable, using an RNA-guided nuclease-deficient Cas9 (dCas9) protein fused with transcriptional regulators for targeting specific genes to effect their regulation. Multiple studies have shown how this method is an effective way to achieve efficient and specific transcriptional repression or activation of single or multiple genes. Sustained transcriptional modulation can be obtained by stable expression of CRISPR components, which enables directed reprogramming of cell fate. Here, we introduce the basics of CRISPRi/a technology for genome repression or activation.



## BACKGROUND

Targeted genome activation or repression is an important approach for engineering complex cellular functions, reprogramming cell fate and for disease modeling. In the past, RNA interference (RNAi) has been used as a major method for silencing the expression of genes in mammalian cells. RNAi uses base-pairing between small RNAs and mRNAs for triggering degradation of target transcripts (Chang et al. 2006). Protein-based tools such as zinc-fingers and transcription-activator-like effectors (TALEs) also provide customizable tools for site-specific perturbation of gene expression when fused to transcriptional activators or repressors (Kabadi and Gersbach 2014). However, these techniques have limited usefulness when compared with the emerging CRISPR technology owing to either high off-target effects (in the case of RNAi) or the difficulty experienced in their construction and delivery into cells (in the case of zinc fingers and TALEs). In contrast, the CRISPR technology offers a more efficient, robust, multiplexable, and designable approach for genome-wide activation or repression (Gilbert et al. 2013, 2014; Qi et al. 2013; Tanenbaum et al. 2014; Zalatan et al. 2015).

The CRISPR system for gene activation or repression has been repurposed from natural type II CRISPR systems in bacteria. We have named this CRISPR technology for gene regulation as "CRISPR interference" (CRISPRi for repression) or "CRISPR activation" (CRISPRa for activation). Both CRISPRi and CRISPRa use a catalytically inactive form of the Cas9 protein, termed dCas9, fused with transcriptional repressors and activators, respectively. Targeting of dCas9 to the genome is dictated by a single guide RNA (sgRNA) containing a designed 20-nucleotide sequence complementary to the DNA target, which is adjacent to a short DNA motif, termed the protospacer-adjacent motif (PAM; Fig. 1). Different homologs of Cas9 recognize different PAM sequences. For example, *Strepto-*

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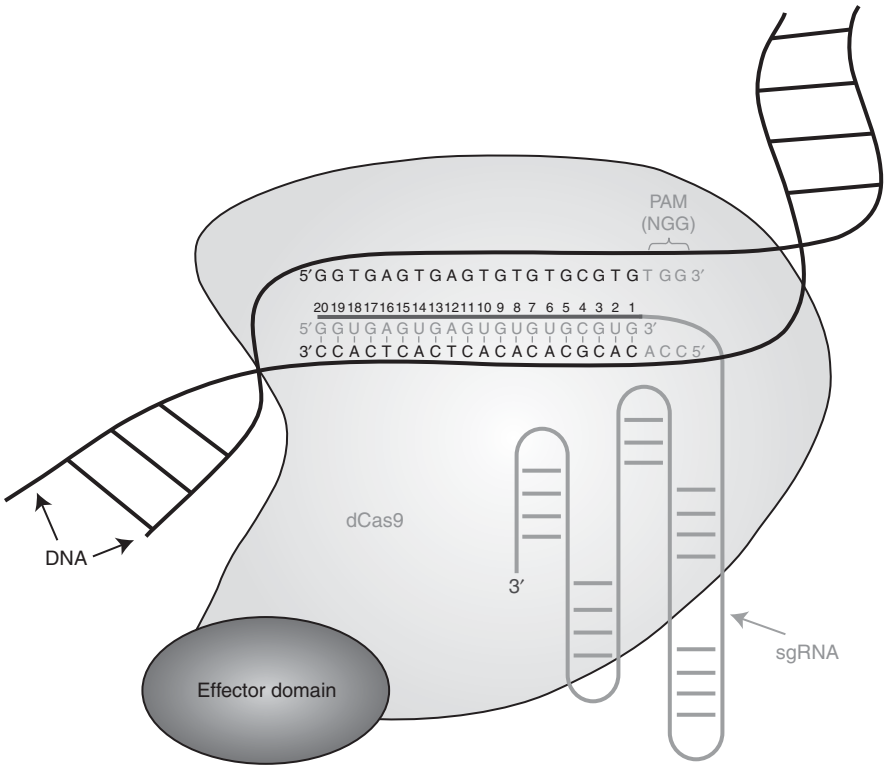


**FIGURE 1.** Illustration of sgRNA-guided DNA binding of dCas9. The diagram shows that a single guide RNA (sgRNA) can direct *S. pyogenes* nucleolytically deactivated Cas9 (dCas9, with D10A and H840A mutations) to a specific locus in the genome. The 20-bp guide sequence at the 5′ end of the sgRNA specifies the target sequence according to the rules of Watson–Crick base-pairing. On the target genomic DNA, the adjacent 5′-NGG PAM motif after the 20-bp target sequence is required to efficiently direct the dCas9–sgRNA complex to the genomic DNA. The numbers on top of the base-pairing targeting sequence indicate the nucleotide position relative to that of the adjacent PAM sequence. PAM, protospacer-adjacent motif; sgRNA, single guide RNA.

*coccus pyogenes* Cas9 recognizes NGG or NAG, whereas *Neisseria meningitides* Cas9 recognizes NNNNGATT (Hou et al. 2013). The dCas9 fusion protein complexed with a sequence-specific sgRNA binds to the target DNA and is engineered such that it localizes a repressive or activating effector domain to turn down or turn on transcription of the target genes. Armed with CRISPRi- and CRISPRa-based approaches, investigators now have a platform for systematically interrogating gene expression.

## CRISPRi AND CRISPRa FOR TRANSCRIPTIONAL MODULATION

The CRISPR–dCas9 system provides a flexible and modular platform for recruiting different functional effector domains to essentially anywhere in the genome. This is mostly achieved by fusing dCas9 to different effector domains. Sometimes, recruitment of multiple effector domains is required for optimal genome-regulatory effectiveness.

### CRISPRi

It has been established that the dCas9 protein alone can efficiently silence gene expression in bacteria with a properly designed sgRNA (Qi et al. 2013). However, only moderate repression was observed using dCas9 alone in mammalian cells. Strong gene silencing was seen when fusing the dCas9 protein to the repressive KRAB (Kruppel-associated box) domain of Kox1 (Gilbert et al. 2013). Target genes can be stably silenced when cells stably express dCas9–KRAB and an sgRNA (Gilbert et al. 2013). When multiple sgRNAs are coexpressed, multiple genes can be silenced simultaneously (see below).




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Effective targeting sites of CRISPRi include enhancers, proximal promoters, and the coding region downstream from the transcription start site (TSS) of a gene (Gilbert et al. 2013; Kearns et al. 2014).

## CRISPRa

It has been shown that the dCas9 fusion with a transcription activator VP64 can activate a reporter gene relatively effectively (Cheng et al. 2013; Gilbert et al. 2013; Maeder et al. 2013; Mali et al. 2013; Perez-Pinera et al. 2013; Chakraborty et al. 2014; Kearns et al. 2014; Chavez et al. 2015). However, direct fusion of dCas9 to VP64 results in only very mild activation of endogenous target genes. For better activation of transcription, several systems have been developed. For example, fusing VP64 to both the amino and carboxyl terminus of dCas9 or fusing 10 copies of VP16 to dCas9 in each case enhanced activation (Cheng et al. 2013; Chakraborty et al. 2014). Chavez and colleagues generated a VP64–p65–Rta tripartite activator with dCas9 and showed that this construct enabled efficient endogenous gene activation (Chavez et al. 2015). All of these methods increased transcription activation of the target genes compared with that of the dCas9–VP64 system.

Additionally, a series of systems for indirect fusions of effector domains to CRISPR–Cas9 have been developed. For example, Konermann and colleagues appended two MS2 bacteriophage coat-protein-binding RNA motifs to two sgRNA stem loops. They coexpressed an MS2-activator (MS2–p65–HSF1) fusion protein together with dCas9–VP64 and modified sgRNA and observed more efficient transcription activation compared with dCas9–VP64 (Konermann et al. 2015). Tanenbaum and colleagues have developed a "SunTag" scaffold protein that can specifically recruit multiple copies of single-chain variable fragment (scFv), an artificial antibody fusion protein. When fusing scFv to a VP64 activator, multiple transcriptional activators can be recruited by dCas9–SunTag to the target DNA for very strong activation of endogenous genes (Gilbert et al. 2014; Tanenbaum et al. 2014).

Notably, CRISPRi and CRISPRa have low off-target effects. Using RNA-seq to assay the transcriptome, it has been shown that CRISPRi and CRISPRa can specifically modulate gene expression while inducing minimal off-target effects (Cheng et al. 2013; Gilbert et al. 2013, 2014; Perez-Pinera et al. 2013; Konermann et al. 2015).

We provide a working protocol for designing, cloning, and using sgRNAs for effective gene activation and repression in mammalian cells in Protocol: **CRISPR Technology for Genome Activation and Repression in Mammalian Cells** (Du and Qi 2016).

## Modulation of Multiple Genes Using CRISPRi and CRISPRa

Multiple genes can be simultaneously activated or repressed by co-delivering multiple cognate sgRNAs, thus providing a powerful platform for analyzing the interaction of multiple genes (Cheng et al. 2013; Gilbert et al. 2013; Qi et al. 2013; Chavez et al. 2015; Konermann et al. 2015). To simultaneously activate and repress multiple genes in the same cell, scaffold RNAs (scRNAs) have been engineered by fusing sgRNAs to orthogonal protein-binding bacteriophage RNAs such as MS2, PP7, and Com (Zalatan et al. 2015). It has been shown that co-delivery of MCP–VP64 and COM–KRAB with a dCas9 protein allows simultaneous activation of *CXCR4* (a chemokine receptor) and repression of *B4GAL4NT1* (encoding β-1,4-*N*-acetyl-galactosaminyl transferase) in the same cell (Zalatan et al. 2015). Thus, engineered scRNAs provide a versatile platform for multigene modulation for recruiting diverse effectors to different genomic loci.

## Repression and Activation of Noncoding RNA Genes

In addition to protein-coding genes, CRISPRi can be harnessed to repress transcription of long noncoding RNAs (lncRNAs). For example, strong knockdown (>80%) of five tested lncRNAs (*H19*, *MALAT1*, *NEAT1*, *TERC*, and *XIST*) has been observed in human myelogenous leukemia K562 cells (Gilbert et al. 2014). These results showed that CRISPRi was able to repress lncRNA expression effectively, enabling further functional analysis of these noncoding genes. Konermann and colleagues have also shown that CRISPRa can activate long intergenic noncoding RNAs (lincRNAs) such as *TINCR*, *PCAT*, and *HOTTIP* (Konermann et al. 2015).

## Application of CRISPRi and CRISPRa for Reprogramming of Cell Fate

Expression of exogenous transcription factors has been used as a major approach for directed cell reprogramming (Ladewig et al. 2013). Now, CRISPR-based gene regulation provides a novel approach. For example, Kearns and colleagues showed that CRISPRi could modulate the differentiation of human pluripotent stem cells by using an sgRNA to repress the *OCT4* gene (Kearns et al. 2014). Furthermore, Chakraborty and colleagues showed that CRISPRa could induce the transdifferentiation of mouse embryonic fibroblasts into skeletal myocytes by activating transcription of the endogenous *Myod1* gene (Chakraborty et al. 2014). Finally, when paired with sgRNAs targeting the *OCT4* promoter, dCas9–VP192 has been used to replace the requirement for exogenous *OCT4* overexpression in a methodology for reprogramming human-induced pluripotent stem cells (Balboa et al. 2015).

In summary, CRISPRi and CRISPRa offer powerful approaches for repression and activation of endogenous genes, which is useful for studying gene functions, rewiring genetic networks, and reprogramming cell fates.

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Protocol

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Targeted modulation of transcription is necessary for understanding complex gene networks and has great potential for medical and industrial applications. CRISPR is emerging as a powerful system for targeted genome activation and repression, in addition to its use in genome editing. This protocol describes how to design, construct, and experimentally validate the function of sequence-specific single guide RNAs (sgRNAs) for sequence-specific repression (CRISPRi) or activation (CRISPRa) of transcription in mammalian cells. In this technology, the CRISPR-associated protein Cas9 is catalytically deactivated (dCas9) to provide a general platform for RNA-guided DNA targeting of any locus in the genome. Fusion of dCas9 to effector domains with distinct regulatory functions enables stable and efficient transcriptional repression or activation in mammalian cells. Delivery of multiple sgRNAs further enables activation or repression of multiple genes. By using scaffold RNAs (scRNAs), different effectors can be recruited to different genes for simultaneous activation of some and repression of others. The CRISPRi and CRISPRa methods provide powerful tools for sequence-specific control of gene expression on a genome-wide scale to aid understanding gene functions and for engineering genetic regulatory systems.

Cold Spring Harbor Protocols
www.cshprotocols.org

## MATERIALS

**It is essential that you consult the appropriate Material Safety Data Sheets and your institution's Environmental Health and Safety Office for proper handling of equipment and hazardous materials used in this protocol.**

**RECIPES: Please see the end of this protocol for recipes indicated by <R>. Additional recipes can be found online at http://cshprotocols.cshlp.org/site/recipes.**

### Reagents

Chemically competent *Escherichia coli* cells (e.g., One Shot TOP10 Cells from Life Technologies)
dCas9 expression vector(s) appropriate for experiment

- CRISPR activation (CRISPRa) dCas9–SunTag expression vectors

  *Two constructs are required: a lentiviral vector containing an SV40-promoter-driven dCas9 fusion between dCas9, 2X nuclear localization signal (NLS), 10X GCN4, and a P2A-tagBFP (Addgene 60903) and a lentiviral vector containing an SV40-promoter-driven fusion protein between the single chain variable fragment (scFv) for GCN4, a superfolder (sf) GFP, VP64, and 1X NLS (Addgene 60904).*

- CRISPR interference (CRISPRi) dCas9–KRAB expression vector

  *This comprises a lentiviral vector containing a spleen focus-forming virus SFFV-promoter-driven dCas9 fused to 2X NLS, a tagBFP and a KRAB domain (Addgene 46911).*

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dNTPs (10 mM)
Double-distilled water ($ddH_2O$), sterile and nuclease-free
Dulbecco's modified Eagle's medium (DMEM), high-glucose (Life Technologies 11965-092)
Fetal bovine serum (FBS)
Gel electrophoresis reagents
- Agarose gels (1%, w/v)
- DNA ladder
- Ethidium bromide
- Tris-acetate-EDTA (TAE) buffer (50×) <R>

HEK293T cells (ATCC CRL-11268)

*HEK293T cells (or other cells derived from HEK293T cells) are required for lentivirus production in Steps 26–31. In addition, they are used here as an example of target cells in Steps 32–36. Other target cells may be used as appropriate for the experiment.*

In-Fusion HD Cloning Kit (Clontech 011614)
iQ SYBR Green Supermix (Bio-Rad 170-8880)
iScript cDNA Synthesis Kit (Bio-Rad 170-8890)
Lentiviral packaging plasmids pCMV-dR8.91 and pMD2.G (Addgene 12259)

*At the time of this writing, pCMV-dR8.91 is not avialable from Addgene. Alternatively, a lower version of the plasmid is available from Addgene (pCMV-dR8.2; Addgene 8455). Its use will not affect this protocol.*

Lysogeny broth (LB) with carbenicillin (liquid medium and agar plates) <R>
Mirus TransIT-LT1 Transfection Reagent (Mirus MIR 2300)
Opti-MEM Reduced-Serum Medium (Life Technologies 31985-062)
Penicillin-Streptomycin (100×), presterilized (Life Technologies 15070-063)
Phusion High-Fidelity Polymerase and 5× Phusion HF Buffer (New England BioLabs M0536L)
Polybrene (optional; see Step 33)
Primers
- PCR primers, one of which (sgRNA-F) contains the gene-specific sgRNA target sequence

  Forward primer (sgRNA-F): 5′-CCCTTGGAGAACCACCTTGTTGGN$_{(19)}$GTTTAAGAGCTATGCTGGAAACAGCA-3′

  Reverse primer (sgRNA-R): 5′-GATCCTAGTACTCGAGAAAAAAAGCACCGACTCGGTGCCAC-3′

*For sgRNA target sequence selection, see Steps 1–3.*

- Sequencing primer: 5′-GAGGCTTAATGTGCGATAAAAGA-3′

  *This primer binds to the mouse U6 promoter and is used to confirm the generation of sgRNA expression constructs in Step 21.*

- Target gene-specific primers for qRT-PCR (see Step 40)

QIAGEN Plasmid Midi Kit (QIAGEN 12143)

*It is important to use an endotoxin-free midiprep kit when purifying plasmid DNA for better transfection efficiency into mammalian cells.*

QIAprep Spin Miniprep Kit (QIAGEN 27106)
QIAquick Gel Extraction Kit (QIAGEN 28706)
QIAquick PCR Purification Kit (QIAGEN 28106)
Restriction enzymes BstXI, XhoI, and DpnI
RNeasy Plus Mini Kit (QIAGEN 74134)
Single guide RNA (sgRNA) expression vector

*This comprises a lentiviral vector containing the mouse U6 promoter driving sgRNA expression (Addgene 51024). It also contains an expression cassette consisting of a cytomegalovirus (CMV) promoter, a puromycin-resistance gene cassette, and an mCherry gene for selection purposes.*

Trypsin-EDTA (0.05%) (e.g., Life Technologies 25300-054)

## Equipment

Access to sequencing facility (see Step 21)
BD FACSAria II Cell Sorter (BD Biosciences) equipped with lasers and filters for detecting mCherry, EGFP, and tagBFP
CFX96 Real-Time PCR Detection System (Bio-Rad 185-5195)
$CO_2$ incubator at 37°C and 5% $CO_2$ for mammalian cell culture
Computer with Internet-connected web browser
Conical tubes
Digital gel-imaging system
Erlenmeyer flasks (250 mL)
Gel electrophoresis system
Glass tubes (25-mm)
Incubators at 37°C for growing bacteria (one standard and one capable of shaking at 200 rpm)
Microcentrifuge
Microcentrifuge tubes
Microplate for qRT-PCR
NanoDrop 8000 UV-Vis Spectrophotometer (Thermo Scientific)
PCR tubes (0.2-mL)
Six-well tissue-culture plates
Syringe filter (0.45-μm), sterile
Syringe, sterile
Thermocycler



## METHOD

*We have implemented a computational tool, termed CRISPR-ERA ("editing, repression, and activation") for automated design of sgRNAs for given mammalian organisms, such as mouse, rat, and human (Liu et al. 2015). The CRISPR-ERA algorithm aligns the designed sgRNA to the whole genome and reports potential off-target sites as defined by possession of fewer than three mismatches. The tool is freely available at http://CRISPR-ERA.stanford.edu. If using CRISPR-ERA, skip Steps 1–5.*

### Selection of sgRNA Targets in the Genome

1. Determine the DNA sequence of the target gene using an available genome database—for example, the UCSC genome browser (Kent et al. 2002).
2. Obtain annotation information of the target gene, including the location of the transcription start site (TSS).
3. Search for patterns of $GN_{(19)}$NGG around the TSS, wherein $GN_{(19)}$ is the binding site of the sgRNA and NGG is the protospacer adjacent motif (PAM), which is required for efficient DNA binding of *Streptococcus pyogenes* Cas9.

   *Our sgRNA expression construct uses a mouse U6 promoter, which requires a G at the very 5′ end for effective transcription. Therefore, we search for $GN_{(19)}$ as the binding site of the sgRNA. If another promoter is used, it is likely that the first nucleotide will be different.*

   *The recommended window of the target DNA is −50 to +300 bp relative to the TSS for CRISPR interference (CRISPRi) for gene repression, or −400 to −50 bp for CRISPR activation (CRISPRa). Usually, multiple sgRNA-binding sites within the target window of the gene need to be tested to define the most efficient targeting site for repression or activation.*

   *Many mammalian genes possess transcript isoforms with different TSSs. In this case, different sgRNAs need to be designed for each transcript. Currently, there is no direct evidence that the activities of CRISPRi and CRISPRa are sensitive to the DNA strand or GC content (Gilbert et al. 2014).*

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## Design of sgRNA Sequences

4. Ensure that the base-pairing sequence on the sgRNA is the reverse complement of the $GN_{(19)}$ sequence identified in Step 3.
5. Analyze the specificity of the target sequence in the genome use the basic local alignment search tool (BLAST; http://blast.ncbi.nlm.nih.gov) (Bhagwat et al. 2012).

   *The BLAST algorithm enables the specificity of sgRNA targeting in the genome to be analyzed when not using the CRISPR-ERA tool.*
6. Generate the full-length sgRNA by appending $GN_{(19)}$ 3′ to the rest of the optimized sgRNA sequence (5′-$GN_{(19)}$GUUUAAGAGCUAUGCUGGAAACAGCATAGCAAGUUUAAAUAAGGCUAGUCCGUUAUCAACUUGAAAAAGUGGCACCGAGUCGGUGCUUUUUUU-3′) (Chen et al. 2013).
7. Confirm that the $GN_{(19)}$ target sequence does not contain any transcription termination sequence for the U6 promoter (Paul et al. 2002).

## Preparation of sgRNA Expression Constructs

8. Generate the sgRNA backbone by digesting the empty sgRNA expression vector with restriction enzymes BstXI and XhoI for 4–16 h at 37°C according to the manufacturer's instructions.
9. Separate the digested sgRNA backbone products by electrophoresis through a 1% (w/v) agarose gel in 1× TAE buffer. Stain the gel with ethidium bromide, and visualize the bands using a digital gel imaging system. Compare the bands to those of a proper DNA ladder, and confirm that the band representing the sgRNA backbone DNA is ~8 kb.
10. Gel-purify the sgRNA backbone DNA using a QIAquick Gel Extraction Kit according to the manufacturer's instructions. Store the DNA at −20°C until use in Step 15.
11. Perform PCR as follows, using primers that contain the 20-nt target sequences identified in Steps 1–3.

    i. Assemble the following reaction (volumes shown are for one reaction) in a 0.2-mL PCR tube on ice.

| | |
|---|---|
| 0.5 µL | Empty sgRNA expression vector (undigested) as template (100 ng/µL) |
| 2.5 µL | Forward primer (sgRNA-F) (10 µM) |
| 2.5 µL | Reverse primer (sgRNA-R) (10 µM) |
| 2 µL | dNTPs (10 mM) |
| 0.5 µL | Phusion High-Fidelity Polymerase (2 U/µL) |
| 10 µL | Phusion HF Buffer (5×) |
| 32 µL | Nuclease-free water |
| 50 µL | Total volume |

    ii. Perform PCR with the following cycling conditions:

| | | |
|---|---|---|
| 1 cycle | 98°C | 30 sec |
| 25 cycles | 98°C | 10 sec |
| | 62°C | 30 sec |
| | 72°C | 10 sec |
| 1 cycle | 72°C | 5 min |
| 1 cycle | 4°C | Forever |

12. Confirm that the PCRs successfully amplified a ~150-bp DNA product by separating 5 µL of the PCR products on a 1% agarose gel as in Step 9.

13. Add 1 µL of DpnI (20 U/µL) into each PCR and then incubate for 1 h at 37°C.

    *Treatment of DpnI will digest the PCR templates.*

14. Purify the PCRs using a QIAquick PCR Purification Kit by following the manufacturer's instructions. Store the DNA at −20°C until use in Step 15.

15. Measure the concentrations of the purified sgRNA backbone DNA (from Step 10) and PCR fragments (from Step 14) using a NanoDrop UV-Vis 8000 Spectrophotometer.

16. Ligate the PCR fragments to the sgRNA backbone DNA using an In-Fusion HD Cloning Kit.

    i. Assemble the cloning reaction.

| | |
|---|---|
| 1 µL | In-Fusion HD Enzyme Premix (5×) |
| 50 ng | Linearized sgRNA backbone DNA (from Step 10) |
| 25 ng | Purified PCR fragments (from Step 14) |
| *x* µL | $ddH_2O$ |
| 5 µL | Total volume |

    ii. Incubate the reaction for 15 min at 50°C using a thermocycler.

    iii. Place on ice for 5 min. Store at −20°C until use in Step 17.

17. Transform chemically competent *E. coli* cells with the products of the ligation reactions. Follow the manufacturer's instructions for the *E. coli* cells. Spread transformed *E. coli* cells onto LB agar plates supplemented with 100 µg/mL carbenicillin. Incubate the plates overnight in a 37°C incubator.

18. Transfer single colonies into 25-mm glass tubes containing 5 mL of LB medium supplemented with 100 µg/mL carbenicillin. For each colony, use a sterile pipette tip to touch the colony, and then swirl the tip in the LB medium to dissolve the colony. Incubate overnight in a 37°C shaking incubator, swirling at 200 rpm.

19. Transfer 0.5 mL of bacterial culture into a 250-mL Erlenmeyer flask containing 50 mL of LB medium with 100 µg/mL carbenicillin. Incubate overnight in a 37°C shaking incubator, swirling at 200 rpm.

20. Extract the plasmid DNA from the remaining 4.5 mL of bacterial culture using a QIAprep Spin Miniprep Kit according to the manufacturer's instructions.

21. Send the extracted plasmid DNA for sequencing with the sequencing primer.

22. After the plasmid is verified by sequencing, extract DNA from the 50-mL bacterial culture using a QIAGEN Plasmid Midi Kit according to the manufacturer's instructions. Store the DNA at −20°C until use in Step 27.

## Preparation of dCas9 Expression Vectors

23. Transform chemically competent *E. coli* cells with the dCas9 expression vectors appropriate for the experiment (CRISPRi or CRISPRa). Spread transformed *E. coli* cells onto LB agar plates supplemented with 100 µg/mL carbenicillin. Incubate the plates overnight in a 37°C incubator.

24. Transfer a single colony into 50 mL of LB medium supplemented with 100 µg/mL carbenicillin. Incubate overnight in a 37°C shaking incubator, swirling at 200 rpm.

25. Extract DNA using a QIAGEN Plasmid Midi Kit according to the manufacturer's instructions. Store the DNA at −20°C until use in Step 27.

## Packaging of dCas9 and sgRNA Expression Constructs into Lentiviral Particles

*If more lentiviruses are required, scale up the cell numbers, DNA amounts, and transfection reagent volumes used here.*

26. On the day before transfection, seed a six-well tissue-culture plate with 2–3 × $10^5$ HEK293T cells in 2 mL of high-glucose DMEM containing 10% (v/v) FBS per well. Incubate overnight at 37°C and 5% $CO_2$.

    *HEK293T cells can be maintained in regular high-glucose DMEM medium supplemented with 10% (v/v) FBS, 100 U/mL streptomycin, and 100 µg/mL penicillin and regularly passaged using 0.05% (w/v) trypsin–EDTA. However, antibiotic-free DMEM is required during transfection and virus collection to achieve better efficiency.*

27. Twenty-four hours after plating the cells, prepare the transfection complexes as follows.

    i. Combine the following DNA samples.

    | | |
    |---|---|
    | 1.32 µg | pCMV-dR8.91 (lentiviral packaging plasmid) |
    | 165 ng | pMD2.G (lentiviral packaging plasmid) |
    | 1.51 µg | dCas9 or sgRNA expression construct |

    *Nontargeting sgRNA vector or dCas9 without fusion vector can be used in parallel as a negative control.*

    ii. Add this 3-µg DNA mixture into 250 µL of Opti-MEM Reduced-Serum Medium in a microcentrifuge tube. Mix well by pipetting up and down.

    iii. Add 7.5 µL of Mirus TransIT-LT1 Transfection Reagent into the same tube. Mix well by pipetting up and down.

    iv. Allow transfection complexes to form for 30 min at room temperature.

28. Remove 250 µL of medium from each well in the six-well plate.

29. Add the ~250-µL mixture from Step 27.iv into one well in the six-well plate. Mix well by rocking the plate gently back and forth. Incubate for 24 h at 37°C and 5% $CO_2$.

    *Cells will begin producing viruses 24 h after transfection.*

30. After the 24-h incubation, replace the transfection medium with 2.5 mL of fresh DMEM with 10% FBS.

    *If the target cells to be infected have any additional medium requirements, replace the transfection medium with 2.5 mL of special growth medium for the target cells.*

31. Use a sterile syringe to harvest the viral supernatant 24–48 h after medium replacement. Filter the medium through a 0.45-µm syringe filter into a conical tube to avoid transferring HEK293T cells.

    *The total volume will be ~2 mL after filtering. Lentiviral particles can be stored for up to 1 wk at 4°C, or snap-frozen in liquid nitrogen and stored for several months at −80°C. However, we recommend using the lentiviruses immediately after collection.*

## Transduction of Target Cells with dCas9 and sgRNA Lentiviral Particles

*In the following, the use of HEK293T cells is given as an example. For other types of cells, modify the procedure (e.g., cell number and growth medium) as appropriate.*

32. Sixteen hours before transduction, seed a six-well tissue-culture plate with 1.5–2 × $10^5$ HEK293T cells in 2 mL of high-glucose DMEM supplemented with 10% FBS per well. Incubate at 37°C and 5% $CO_2$.

33. Replace the medium with 1 mL of DMEM containing 10% FBS and 1 mL of filtered viral supernatant. Incubate overnight at 37°C and 5% $CO_2$.

    *Depending on the virus titration, the viral supernatant can be diluted with growth medium for the target cells. Polybrene can be used to promote the infection efficiency with proper concentration; however, it is toxic for some types of cells, including HEK293T cells.*

34. Replace the viral supernatant with 2 mL of fresh DMEM with 10% FBS, and incubate for 48 h at 37°C and 5% $CO_2$.

*Cells usually will express dCas9 protein 48 h after addition of lentiviruses. However, for repression experiments, we suggest collecting cells at least 72 h after infection to minimize the interference by preexisting target gene mRNA. If necessary, split the cells when they reach 80%–90% confluence before sorting.*

35. Use a BD FACSAria II Cell Sorter to collect the cells.

    - For the CRISPRi system, collect cells positive for both blue fluorescent protein (BFP) and mCherry.

      *The BFP-positive cells should express dCas9 protein, and mCherry-positive cells should express sgRNA.*

    - For the CRISPRa (dCas9–Suntag) system, collect cells that are positive for BFP, mCherry, and GFP.

      *The GFP-positive cells should express scFv-sfGFP-VP64 fusion protein.*

36. Incubate the collected cells at 37°C and 5% $CO_2$.

    *After the cells are grown up, analyze the expression levels of target genes by qRT-PCR as described in Steps 37–40.*

## Quantification of the Effects of CRISPRi or CRISPRa on Gene Expression in Target Cells

37. Extract total RNA from the cells infected using an RNeasy Plus Mini Kit according to the manufacturer's instructions.

    *Typically, 0.5–1 × $10^6$ cells (50%–80% confluence of cells in one well in a six-well plate) are sufficient for total RNA extraction.*

38. Measure the concentrations of the total RNA samples using a NanoDrop spectrophotometer.

39. Synthesize cDNA using an iScript cDNA Synthesis Kit.

    i. Set up the cDNA synthesis reaction.

    | | |
    |---|---|
    | 4 µL | iScript reaction mix (5×) |
    | 1 µL | iScript reverse transcriptase |
    | 1 µg | Total RNA template |
    | *x* µL | Nuclease-free water |
    | 20 µL | Total volume |

    ii. Incubate the reaction as follows (e.g., using a thermocycler):

    | | | |
    |---|---|---|
    | 1 cycle | 25°C | 5 min |
    | 1 cycle | 42°C | 30 min |
    | 1 cycle | 85°C | 5 min |
    | 1 cycle | 4°C | Forever |

    iii. Store the DNA at −20°C until use in Step 40.i.

40. Analyze the cDNA levels of target genes using a standard qRT-PCR protocol.

    i. Set up the PCR in a microplate using the iQ SYBR Green Supermix according to the manufacturer's instructions.

    | | |
    |---|---|
    | 10 µL | iQ SYBR Green Supermix (2×) |
    | 1.2 µL | Forward primer (target gene-specific; 5 µM) |
    | 1.2 µL | Reverse primer (target gene-specific; 5 µM) |
    | 0.25 µL | Template (cDNA from Step 39) |
    | 7.35 µL | Nuclease-free water |
    | 20 µL | Total volume |

    *The amount of template cDNA can be scaled up or down according to the expression levels of the target genes in the cells. Housekeeping genes—for example, GAPDH, encoding glyceraldehyde-3-phosphate dehydrogenase—should be used as references.*

ii. Run the following real-time PCR profile in a CFX96 Real-Time PCR Detection System.

| | | |
|---|---|---|
| 1 cycle | 95°C | 2–3 min |
| 39 cycles | 95°C | 10–15 sec |
| | 55°C–60°C | 15–30 sec |
| | 72°C | 30 sec |
| Melt curve (optional) | 55°C–95°C (in 0.5°C increments) | 10–30 sec |

iii. Analyze the qRT-PCR data by standard methods to obtain the relative transcriptional expression levels of the target genes regulated by CRISPRi/a.

*We use the $2^{-\Delta\Delta Ct}$ method to obtain the relative mRNA expression level of the CRISPRi or CRISPRa sample vs. a control sample, where $\Delta\Delta Ct = \Delta Ct$(CRISPRi/a sample)$-\Delta Ct$(control sample, e.g., nontargeting sgRNA sample), and where $\Delta Ct$(sample) = Ct(any sample)–Ct(endogenous housekeeping gene).*

## DISCUSSION

To date, several tools have been developed to functionally interrogate gene expression. RNAi has been shown to disrupt gene expression by triggering the degradation of target mRNAs (Chang et al. 2006). However, the technique is somewhat limited in its application owing to off-target effects and through being restricted to cytosolic target mRNAs (Jackson et al. 2003; Adamson et al. 2012; Sigoillot et al. 2012). Protein-based tools are difficult to be designed, cloned, and delivered into target cells. The complex programming and limited targeting sites also restrict the application of zinc fingers and tools based on transcription-activator-like effectors (TALEs) for perturbing the expression of multiple genes. Loss-of-function approaches based on genome editing, such as CRISPR–Cas9, cause irreversible frameshift disruptions, cytotoxic double-stranded DNA breaks, and in-frame insertion–deletions (indels) arising from error-prone DNA repair. These could limit the ability of the CRISPR technique to completely abolish the function of genes and noncoding RNAs (Huang et al. 1996; Jackson 2002; Koike-Yusa et al. 2014; Shalem et al. 2014; Wang et al. 2014).

In contrast, RNA-guided DNA targeting of the dCas9 protein to a specific locus provides a programmable platform to modulate genome status while generating minimal off-target effects. Fusion of different effector domains to dCas9 enables transcriptional repression (CRISPRi) or activation (CRISPRa) of specific target genes. CRISPRi and CRISPRa enable inducible and reversible modulation of specific endogenous gene expression within an intact biological system. The modulation of the transcription of single or multiple genes can be specifically achieved by delivery of multiple sgRNAs (Gilbert et al. 2013, 2014; Qi et al. 2013; Tanenbaum et al. 2014; Zalatan et al. 2015). By using scRNA, transcriptional activation or repression of different target genes can be achieved simultaneously in the same cell (Zalatan et al. 2015). Recently, CRISPRa has been used to effectively activate expression of target genes in plants and flies (Lin et al. 2015; Lowder et al. 2015). Furthermore, Kleinstiver and colleagues have modified *S. pyogenes* Cas9 (spCas9) to recognize alternative PAM sequences (other than NGG) by using a selection-based approach in bacterial cells (Kleinstiver et al. 2015). This provides researchers an expanded targetable sequence space in the genome for using CRISPR–dCas9. Thus, owing to its simplicity and flexibility, CRISPRi or CRISPRa can facilitate genome-scale perturbation of gene expression (Gilbert et al. 2014; Konermann et al. 2015).

However, the detailed mechanism underlying how CRISPRi and CRISPRa components interact with local transcriptional machinery and epigenetic factors is not well established. We usually design three to five sgRNAs for each target transcript and choose the best one for functional analysis. The reason why some of the designed sgRNAs have no function and why the efficiency of different designed sgRNAs varies is not clear. Knowledge of the mechanism would assist the efficiency of designing functional sgRNAs. Moreover, the spCas9 protein, which is widely used for transcriptional modulation, is a large molecule that is difficult to clone and package with the necessary regulatory elements into a size-restricted virus, such as the adeno-associated virus (AAV) that has been generally

used for gene therapy. In contrast, the smaller ortholog *Staphylococcus aureus* Cas9 (saCas9) has been shown to edit the targets efficiently and to be compatible with the AAV system, which has also been engineered as a transcriptional activating system (SAM) (Nishimasu et al. 2015; Ran et al. 2015). Currently, the gene-regulatory tools based on the *S. aureus* dCas9 are being developed for more-efficient transcriptional repression and activation. Thus, in summary, CRISPRi and CRISPRa based on different species of Cas9 or its homologs provide a versatile platform to manipulate and interrogate gene expression systematically.

## RECIPES

### *Lysogeny Broth (LB) with Carbenicillin*

| Reagent | Quantity |
|---|---|
| Agar (for plates only) | 20 g |
| NaCl | 10 g |
| Tryptone | 10 g |
| Yeast extract | 5 g |

Prepare the above-listed ingredients in 1 L of deionized water. Adjust the pH to 7.0 with 5 N NaOH. Autoclave for 20 min at 15 psi (1.05 kg/cm$^2$). Cool to ~60°C and add carbenicillin (final concentration 100 µg/mL). Pour the medium into Petri dishes (~25 mL per 100-mm plate). Store the LB plates at 4°C; they will keep for at least 4 mo.

### *Tris-Acetate-EDTA (TAE) Buffer (50×)*

| Reagent | Final concentration (1×) |
|---|---|
| Tris base | 40 mM |
| EDTA | 2 mM |
| Acetic acid | 20 mM |

Adjust to pH 8.5 and dilute to 1× with Milli-Q $H_2O$ before use.



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# CRISPR Technology for Genome Activation and Repression in Mammalian Cells

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