

Developing Next-Generation dCas9-VP64 Systems for Temporal Activation Functionalities

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ABSTRACT: Recent developments have aimed to improve the efficiency and specificity of CRISPRa (activation) and CRISPRi (interference), different complexes involving the dCas9 enzyme, aiming to upregulate or downregulate genes through promoter binding. Allosteric regulation in the arCas9 system enables inducible and temporal control of CRISPR, using small molecules and corresponding ligand receptors. These engineered systems integrate ligand-binding domains (e.g., the estrogen receptor ESR1) directly onto the Cas9 or onto scaffold/effector domains. This permits activation of CRISPR based on the presence of specific signals (4-Hydroxytamoxifen in the case of ESR1), allowing precise ON/OFF switching. This allostery represents an improvement from the original CRISPR-Cas9 systems, which are constitutive in the presence of guide RNA (sgRNA) and often inadvertently create off-target effects. Building on the framework of ligand-based control and dCas9 gene regulation, we theorized a construct containing a dead allosterically regulated Cas9 (darC9) with effector domains. A darC9-effector domain platform could activate genes or inhibit regulators in the presence of ligands, allowing reversible and controlled triggers for various genetic engineering applications. Utilizing a Gibson assembly, we tested the combination of a recipient dCas9-VP64 plasmid with a sourced ESR1 to generate a darC9-VP64 molecule with precise ON/OFF capabilities. We completed an in silico design of a darC9-VP64 tool with theoretical imaging capabilities through GFP. We found that amplification of the 5' Gibson assembly fragment was successful, but we had challenges amplifying the ESR1 and 3' Gibson assembly fragment.

KEYWORDS: Biology, Molecular Biology, arCas9, CRISPR, dCas9.

■ Introduction

Since its inception, the Clustered Regularly Interspaced Short Palindromic Repeats (CRISPR) gene editing system has revolutionized the field of gene therapy and holds immense potential for many other fields in therapeutics and medicine. The system, popularized in 2012, is capable of locating and making cuts within the nucleus, using the Cas (CRISPR-associated protein) molecule (sourced from *Streptococcus pyogenes* or related bacteria) and short sequences called single-guide RNAs (sgRNAs). The Cas molecule employs its two nucleases, RuvC and HNH, to cleave the complementary and target strands of DNA, respectively.¹

Mutations of the Cas molecule at designated sites (D10A and H840A)² produce a dead Cas9 (dCas9), a molecule that is able to locate desired loci but is unable to make cuts, minimizing the off-target effects (unintended edits in other loci) of CRISPR. A derivative of this novel process is the CRISPRi and CRISPRa structures, which utilize CRISPR's locating abilities with a dCas9 in conjunction with an activation or deactivation domain to reversibly upregulate or downregulate a gene.

CRISPR interference (CRISPRi) works to downregulate a gene, utilizing a dCas9-sgRNA complex that binds to a complementary strand of a desired region, blocking transcription elongation using effector domains (such as KRAB). Conversely, CRISPR activation (CRISPRa) enables the targeting of any gene using specific and programmable sgRNA sequences to upregulate the selected gene. It localizes itself near a locus of interest and recruits transcription factors using activation ef-

factor domains (such as VP64 or p65) that are fused to one or both N- and C- termini of the dCas9. The effector domain recruits endogenous transcriptional activators, making downstream transcription of a protein-coding sequence more efficient, which successfully upregulates a gene.³

Advances to the first-generation dCas9-VP64 system form the second generation of CRISPR activation/Cas. The CRISPR SAM system (Synergistic Activation Mediator) combines dCas9 with specifically engineered sgRNAs to substantially boost transcription and activation. The dCas9 is fused with aptamers that bind to MS2 proteins, recruiting additional activation domains. SunTag, another second-generation CRISPRa approach, uses more VP64 per dCas9 with a repeated peptide scaffold, outperforming first-generation activation levels.^{4,5}

A further advancement to CRISPR is incorporating inducibility and allosteric regulation. Conditionally activated Cas9s are capable of 25-fold higher specificity in target genomic sites of the human cell than wild-type Cas9 (wtCas9).⁶ The first iteration of arCas9 (Oakes *et al.*)⁷ fuses a ligand-binding domain (LBD) to a Cas9 to create arCas9. The traditional ligand-binding domain used in arCas9 is an estrogen receptor ligand-binding domain (ER-LBD), usually an ESR1, that binds with 4-Hydroxytamoxifen (4-OHT).⁷ Other approaches to conditionally activated Cas involve chemically induced plasmids, or intein-mediated splits in the Cas9 molecule (Table 1).

A Polymerase Chain Reaction (PCR) is a nucleic acid amplification technique that aims to create millions to billions of exact copies of a DNA target based on the number of cycles. Each cycle has a denaturation, annealing, and extension step.⁸

A Gibson assembly is a cloning procedure used to combine multiple features of different plasmids into one. A desired plasmid is produced by amplifying overlapping fragments (usually with PCR) and combining the amplified fragments with a 5' exonuclease, a DNA polymerase, and a DNA ligase. This allows for the desired sequence to bind with a linearized recipient plasmid at a specified target site.⁹ Both of these techniques are essential tools for biomolecular research.

Based on these prior efforts, we theorized the creation of a darC9-VP64 system that would be capable of inducible allosteric activation, permitting reversible upregulation of a target gene. A functional darC9-VP64 tool would be able to temporally activate desired genes after ligand administration, which would be particularly effective for *in vivo* therapeutic applications requiring the minimization of off-target effects.

We developed a system to produce a darC9-VP64 plasmid through an *in silico* Gibson cloning assembly using a host and recipient plasmid to produce ESR1 and dCas9-VP64, respectively. The Gibson cloning assembly involved three fragments: the region from AgeI (the upstream restriction site) to the ERBD insertion site, the ERBD, and the region from the ERBD insertion site to BamHI (the downstream restriction site). Experimentally, we saw successful PCR amplification of the AgeI fragment, but were unsuccessful for the remaining fragments, demonstrating that darC9-VP64 is a feasible tool.

■ Methods

Theoretical design and engineering review:

A literature review was conducted to assemble various approaches to engineering Cas9 systems. Queries were done in PubMed, using the keywords “inducible”, “Cas9”, “CRISPRi”, and “CRISPRa”. Three tables were assembled to summarize findings for each system: Table 3 for inducible approaches, Table 4 for CRISPRi, and Table 5 for CRISPRa, found in Appendix A.

Among the reviewed CRISPRa systems, first-generation dCas9-VP64 provides the weakest activation levels, requiring multiple gRNAs and producing off-target effects. Second-generation approaches such as VPR and SunTag improve activation potency through multiple effector domains, but suffer from variable efficiency. All reviewed systems rely on constitutive promoters, which provide no temporal control over gene activation. This gap motivates the development of a darC9-VP64 tool that can combine the activation functionality of CRISPRa with the allosteric control from a ligand-binding domain.

Plasmid templates:

Two plasmids were used as templates and recipients. dCAS9-VP64_GFP was a gift from Feng Zhang (Addgene plasmid #61422; <http://n2t.net/addgene:61422>; RRID:Addgene_61422). It was used as a recipient for the ESR1 insertion. pBLO1811_arC9_noNLS_human was a gift from David Savage (Addgene plasmid #74494; <http://n2t.net/addgene:74494>; RRID:Addgene_74494), which was used to source the ESR1.

Plasmid streaking and colony validation:

To conduct the plasmid streaking protocol, LB-agar plates containing 100 µg/mL ampicillin were labeled with the plasmid identification and date. A sterile pipette tip was used to collect cells from the plasmid agar stab, which were then inoculated onto the plate by streaking to establish a dense initial region. Serial dilution streaking was performed by transferring cells with fresh tips in a spiral pattern to promote the isolation of single colonies. The inoculated plates were incubated overnight at 37°C. Colony growth was assessed the following day, and plates were sealed with Parafilm and stored appropriately.

Thermal cycling:

The T100 BioRad Thermal Cycler was used for all thermal cycling applications during the procedure. The thermocycling conditions were as follows: denaturation at 95°C for 2 minutes; 33 cycles of 95°C for 30 seconds, primer annealing temperature for 30 seconds (Table 2), 72°C for 2 minutes and 15 seconds; followed by a final extension at 72°C for 5 minutes.

In silico:

Addgene was used for ordering and validating sequences. BLAST was used to validate and characterize plasmids sourced from Addgene, utilizing a tiling approach where sequences were broken up into 500bp chunks for more accurate queries. Benchling was used for sequence alignments to validate the dCas9-VP64 plasmid and the ESR1 plasmid (Figure 1). Benchling was also used for the Gibson assembly (link to final sequence can be found in Appendix B), annotation, and primer design.

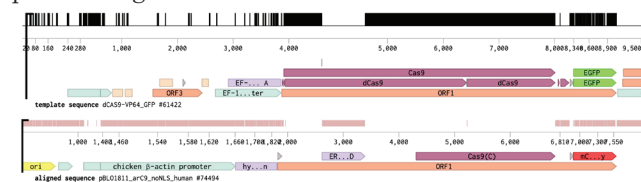


Figure 1: Sequence alignment of dCas9-VP64 #61422 and arC9 ESR1 template (see *Plasmid templates*). Black lines at the top represent “votes” (homologous pairs between the two sequences). Red bars above the plasmid annotations represent sections that are “skipped” by the alignment due to a lack of homology. Both Cas9 sections are entirely homologous except for the ERBD in the aligned sequence (bottom) and the D10A and H840A mutations in the template sequence (top).

Validation:

Validation was done by creating digestion mixtures that included designated amounts of CutSmart buffer, restriction enzymes BamHI and AgeI, and colony DNA. The mixture was then incubated at 37°C for 16 hours. The mixture was then loaded into a gel cast that ran at 180V for 4–5 hours. The gel was then imaged using the Invitrogen iBright 1500 and excised.

Throughout the process, gel electrophoresis was employed to validate digestion and PCR products, and primer design was iteratively refined based on experimental outcomes. The final steps involved isolating validated DNA fragments and preparing them for subsequent cloning procedures. This was done by visualizing the band under the iBright imager and

excising it using a scalpel. The gel was then digested with the Qiagen Gel Isolation Kit and its corresponding protocol.

PCR Setup & Conditions:

Primer working stock was set up as follows: 2.5 μ L of Taq buffer (1X); 0.5 μ L of dNTPs (200 μ M); 1 μ L of MgCl₂ (2 mM); 5 μ L of the FWD and REV primers at 1 μ M; 100 ng of gDNA; 0.25 μ L of Taq polymerase (1.25 U); and RNase-free water until the stock reached 25 μ L.

Results and Discussion

Theorized design:

We presumed that integration of a ligand-binding domain onto a catalytically inactive Cas9 fused to an effector domain enables inducible, precise gene expression or regulation with high temporal control. In the proposed design, binding of the small-molecule ligand 4-hydroxytamoxifen to an estrogen receptor ligand-binding domain (ER-LBD) triggers a conformational switch, shifting the complex to an ON state, and allows it to locate and subsequently recruit transcription factors.

In silico assembly of darC9-VP64 reveals a promising engineering strategy:

To engineer the darCas9-VP64 construct, a systematic *in silico* workflow was implemented, beginning with a review of plasmid design strategies. Three approaches were considered: 1. cloning VP64 onto a dCas9-ESR1 plasmid,⁷ 2. inserting ER-LBD into the dCas9-VP64 backbone, and 3. an approach involving site-directed mutagenesis of arCas9-VP64. Due to available resources and construct feasibility, the second approach was selected, utilizing the dCas9-VP64 plasmid as the recipient and using the arCas9 plasmid for the ER-LBD template. These vectors were chosen due to their compatibility for Gibson cloning, uniform delivery method, and corresponding restriction sites.

Key restriction sites, AgeI and BamHI, were identified as optimal insertion points for ER-LBD based on sequence alignment, and corresponding primers were designed to amplify these regions. The ER-LBD insertion site was identified at 231.⁷

A construct was designed to assemble darC9-VP64 in Benchling. dCas9-VP64 served as a backbone. The host plasmid was then digested at the AgeI and BamHI sites. One primer pair covered the region from AgeI to the ESR1 insertion site at 231, and the other primer pair covered the downstream region from 231 to BamHI. The individual fragments of the assembly are shown in Table 1. The primer pairs for PCR are shown in Table 2.

Based on the construct, a theoretical procedure was created: to assemble darC9-VP64, restriction enzymes would digest the recipient plasmid (dCas9-VP64) at the selected restriction sites (AgeI and BamHI) in order to create an opening for ERBD insertion. After restriction enzyme digestion, each fragment would be amplified with PCR, and then Gibson cloning would attach the fragments to assemble the completed plasmid.

Table 1: List of fragments from *in silico* Gibson assembly on Benchling. Color corresponds to Figure 3. Fragments 2-4 were amplified. "Start" and "End" refer to the base pair coordinates from each fragment's source plasmid.

#	Sequence	Source	Bin	Start	End
1	dCas9-VP64_GFP #61422	#61422	Backbone	8094	2760
2	AgeI to insertion site	#61422	3' overlap seq	2761	4595
3	ESR1 #74494	#74494	ER-LBD	1	774
4	Insertion site to BamHI	#61422	5' overlap seq	4596	8094

Table 2: Primer pairs for PCR reaction. Annealing temperatures were calculated using Benchling.

#	Primer	Annealing Temp °C	nmol	μ L water for 100 μ M	Template DNA
1	Insertion-BamI FWD	73.2	16.74	167.4	dCAS9-VP64_GFP #61422
2	Insertion-BamI REV	75	33.47	334.7	dCAS9-VP64_GFP #61422
3	AgeI-Insertion FWD	73.1	30.87	308.7	dCAS9-VP64_GFP #61422
4	AgeI-Insertion REV	75	29.78	297.8	dCAS9-VP64_GFP #61422
5	ERBD FWD	75	30.27	302.7	pBLO1811_arC9_noN LS_human #74494
6	ERBD REV	72.9	27.23	272.3	pBLO1811_arC9_noN LS_human #74494

Gibson assembly of ESR1 into dCas9-VP64 finalizes the construct:

The dCas9-VP64 host was first validated and then digested at the AgeI and BamHI sites (Figure 3). The outputs of this process were the validation of the ordered dCas9-VP64 plasmid and the isolation of DNA between AgeI and BamHI for ESR1 insertion.

Initial PCR attempts to amplify the desired fragments encountered challenges, notably with the BamHI site, which can be attributed to insufficient elongation time for the larger 3kb product. Adjustments were made to the annealing temperature and PCR protocol, resulting in successful amplification at the AgeI site, but gel imaging (Figure 2) found that the ERBD insertion and the BamHI amplifications were unsuccessful. For BamHI, the incorrect DNA template was used. For the ERBD, we theorize that the failed reaction is due to improper annealing times and/or temperatures. Ongoing reflection on protocol modifications and next steps will be required to ensure reproducibility and technical rigor.

After successful amplification, Gibson cloning would ligate the three regions and the dCas9-VP64 backbone, creating darC9-VP64.

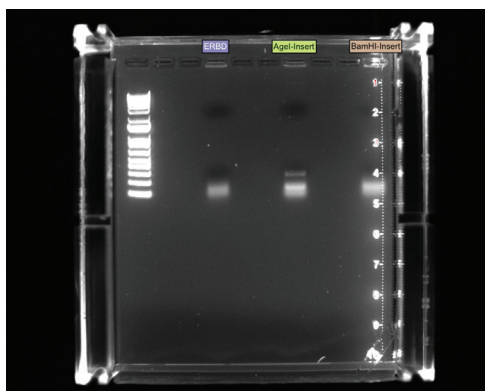


Figure 2: Image of amplification gel cast with ladder on left. Column 1 has ER-LBD primers (5/6 on Table 2), Column 2 has AgeI-insert primers (3/4), and Column 3 has BamHI-insert primers (1/2). Column 2 bands are accurate with amplified sequence length (1835bp).

The final darC9-VP64 construct mapped in Figure 4 is a product of the combination of the dCas9-VP64 and arCas9 plasmids. It features a dCas9 with an insertion of the ESR1 sequence at the 231 site.⁷ SV40 Nuclear Localization Signals (NLS) flank the darC9 sequence, allowing nuclear entry of the molecule. A GFP is attached, which allows for live cell imaging to track the activity of the complex. The plasmid contains multiple transcriptional boosters, including WPRE and a CMV enhancer. The EF-1 α promoter is directly upstream of the ORF containing the darC9-VP64 molecule, while the SV40 promoter comes after.

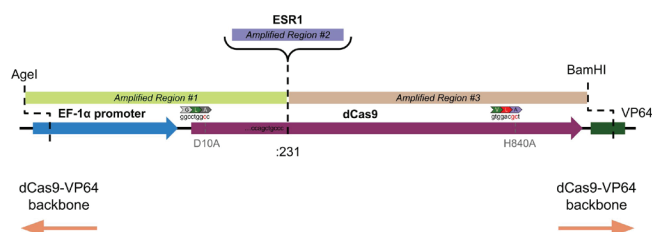


Figure 3: Linear map of darC9-VP64 from the AgeI to BamHI restriction sites, which were used for digestion, amplification, and ESR1 insertion. 3 regions were amplified: from AgeI to the 231 ESR1 insertion site; ESR1; and 231 to BamHI. Annotated are the D10A and H840A mutations that deactivate the RuvC and HNH domains on the Cas9.

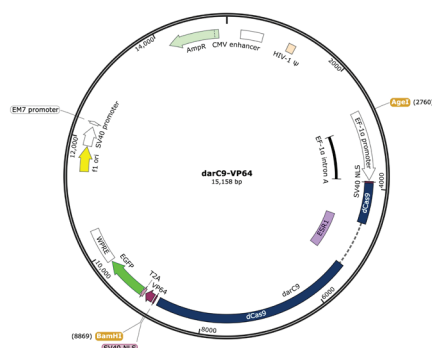


Figure 4: Circular map of darC9-VP64 with selected features and restriction sites.

Conclusion

The creation of a darC9-VP64 platform through Gibson cloning and the successful amplification of one of the three

fragments demonstrates the plausibility of constructing a dead allosterically regulated Cas9-VP64 tool that could reversibly upregulate a gene after 4-OHT activation.

We attempted to actualize this tool, but we realized that amplification of the ESR1 domain is especially difficult due to GC-rich sections. We successfully amplified the AgeI to ESR1 insertion site section, but could not validate the ESR1 and downstream sections. We theorize that this is due to low primer annealing rates, as a result of imprecise temperatures. Further optimization is required for amplification of the unsuccessful regions. We believe that this can be done through more precise PCR thermal cycles.

Inducible transcriptional activation would be engineered using a dCas9-VP64 fusion gated by an ER-LBD. After sgRNA base pairing, the dCas9-VP64 complex would recognize the target promoter and position itself at the locus. In the absence of the ligand (4-OHT), ESR1 would constrain the complex in an OFF conformation. Administration of 4-OHT would cause an allosteric transformation that permits recruitment of transcription factors. This would enable reversible, time-specific, and tunable control of gene expression.

A functioning darC9-VP64 platform has high potential in therapeutics and gene editing due to its precision. It combines the allostery of arCas9, which has high pertinence for *in vivo* applications requiring incredibly precise timing,⁷ with the reversibility and less destructive nature of regulatory CRISPR activation. Compared to other approaches to allostery, such as intein splicing, darC9-VP64 offers greater reversibility, as intein-mediated splicing is a permanent, one-way reaction once triggered. This makes darC9-VP64 better suited for applications requiring variable, temporally precise gene regulation, such as in developmental biology or therapeutics. This system is especially well-suited for applications requiring polygenetic manipulation, as it is especially effective for the coordinated and repeatable regulation of multiple genes.

Future work involving darC9-VP64 will focus on optimizing PCR conditions for the ESR1 and flanking fragments to complete the Gibson assembly and validate the full darC9-VP64 construct through cell assays.

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■ Appendix A

Below are the summary tables for the literature review mentioned in the Methods section. Some important acronyms included in the following tables include sgRNA, standing for

short guide RNA, used in CRISPR editing to direct nucleases to desired loci; AAV, standing for Adeno-associated viral vector; and KRAB, standing for Krüppel-associated box, a transcription repressor domain.

Table 3: Compilation of approaches for inducible (conditionally activated) Cas9 systems.

Inducible CRISPR system	Activation domain	Delivery	Control	Limitations/drawbacks	Reference(s)
Intein-mediated split	Cas9 halves fused to split inteins, becoming functional after dimerization in the presence of chemical ligands (4-OHT)	Dual recombinant AAV vectors	Full Cas9 expressed after intein-mediated trans-splicing	Binding efficiency of 4-OHT reduced slightly	10,11
Rapamycin-mediated split	dCas9-FRB (N) & dCas9-FKBP-VP64 (C) inteins that reassemble in the presence of rapamycin-induced FRB-FKBP dimerization.	Dual recombinant AAV vectors	Rapamycin induces dimerization and allows full Cas9 expression	Relies on rapamycin. Potential off-target effects.	11, 12, 13
Near-infrared (NIR) photoactivatable	Cyanine photobleaching activates rapamycin, inducing Cas9 split-dimerization	Dual recombinant AAV vectors	NIR illumination leads to dimerization	Low penetration depth of NIR	13
lgRNA*	Aptamer inserted into sgRNA. Ligand stabilizes complex, allowing for functional dCas9	Cas9 and sgRNA plasmid (produces endogenous lgRNA)	lgRNA aptamer binding turns Cas9-sgRNA on/off	High design complexity. Potential cross-reactivity	14
Aptazyme-embedded sgRNAs (sgRNAs)	Self-cleaving aptazyme fused to sgRNA. Ligand binding triggers cleavage, releasing the steric blockade strand	Plasmid with dCas9 and sgRNA cassettes, transfection	Small molecules (theophylline/guanine) cause aptazyme cleavage	High design complexity. Slower kinetics	12
Photoactivatable CRISPR	Light-inducible heterodimerization, photocleavable oligonucleotides, etc. (UV, visible, NIR)	Plasmid expression; electroporation <i>in vivo</i> (mouse muscle)	Far red light leads to dimerization, allowing antagonist gene activation + multiplex control	Phototoxicity, limited tissue penetration, slow activation, high design complexity, temperature sensitivity	15,16
Allosteric intein split	Same as intein split but with estrogen receptor. Conformational change	Dual recombinant AAV vectors	Conformational change allows for reversibility after Cas9 expression	Inconsistent intein splicing	6
ER-LBD arCas9	ER-LBD inserted into Cas9. 4-OHT induces conformational change, removing steric blockade	Plasmid transfection	After 4-OHT binding, ER-LBD switches into antagonist conformation, releasing the REC2 gate	Moderate background activity without inducer. Activation limited by ligand presence & domain arrangement	17,18

Table 4: Compilation of approaches for dCas9 interference (CRISPRi) systems.

CRISPRi system	Interference domain	Delivery	Control	Limitations/drawbacks	Reference(s)
Foundational steric blockade (Qi et al.)	dCas9:sgRNA complex bound to non-template DNA strand, interfering with transcriptional regulation mechanisms	Two plasmids holding dCas9 and sgRNA chimera, complex assembly	aTc-inducible promoter on Cas9 plasmid; sgRNA constitutive	Moderate repression (~46%) in mammalian cells. Reversibility can take hours to days	3,15,19
dCas9-KRAB	dCas9:effector domain complex attaches to target DNA strand, repressor domain recruits transcription repressors	Lentiviral transduction of two vectors, dCas9-repressor domain and sgRNA	Constitutive promoters (SFFV, U6 RNA Pol III)	Variable repression (~75%)	19,20
KRAB-MeCP2	KRAB + MeCP2 tail fused to dCas9	Lentiviral dual-vector	Constitutive promoter	Much larger; MeCP2 can have off-target effects	21
ZIM3-KRAB (next gen)	Potent ZIM3 + KRAB	Single lentiviral or PiggyBac	Constitutive promoter	Less explored approach. ZIM3 primate restricted	22
dCas9-TET1	Fusion of catalytic demethylation domain to dCas9	Transfection, electroporation, or lentiviral transduction	sgRNA constitutive	Imprecise demethylation	23

Table 5: Compilation of approaches for dCas9 activation (CRISPRa) systems.

CRISPRa system	Activation domain	Delivery	Control	Limitations/drawbacks	Reference(s)
dCas9 with activation domains	dCas9:sgRNA complex bound to non-template DNA strand, improving transcription efficiency with activation domains (VP64 originally)	Plasmid transfection	Constitutive promoters CMV & U6	Weaker activation, multiple gRNAs required. Off-target effects. Specified architecture & domains.	24,25
dCas9-p300	p300 HAT (histone acetyltransferase) core domain	Plasmid transfection	Constitutive promoters CMV & U6	Larger fusion protein. Does not work equally at all loci. Specified optimization	26
dCas9 with serial activation domains VPR	VP64-p65-Rta (VPR) domain with dCas9. Serial activation domains more effective	Plasmid transfection, lentiviral vectors	Constitutive promoters	Large fusion protein. Variable efficiency. Possible use of multiple gRNAs.	5
dCas9 with SunTag	dCas9 fused with SunTag scaffold for multiple effector domains, improving activation 15-20-fold	Plasmid transfection	Weak promoters	Low expression. Potential interference with protein function due to size.	5
SAM (Synergistic Activation Mediator)	dCas9 + VP64 + MS2-p65-HSF1 complex, which recruits additional activation domains	Plasmid transfection, lentiviral vectors	Constitutive promoters	Requires co-expression of multiple components. Decrease in activation efficiency during high multiplexing. Variable gene activation	27
SPH (SunTag-p65-HSF1)	SunTag scaffold with p65-HSF1 (from SAM system)	PiggyBac transposon, AAV/lentiviral plasmid	Cre recombinase inducible	Difficult delivery due to large size. Variable gene activation.	28
Multiplexed CRISPRa	Multiple gRNAs targeting promoters or enhancers	Lentiviral vectors, piggyBac transposon systems, AAV vectors, plasmid transfection	Various	Off-target effects increase from additional gRNAs.	24,25