CRISPRi^2: Simultaneous Gene Repression by Attacking Transcription and Translation

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1: Introduction

Gene repression plays a vital role in understanding gene function and regulating gene activity. However, existing methods for gene repression face significant challenges. Complete gene knockout, while effective in silencing genes, often disrupts the normal function of the specific protein that gene encoded for. This leads to imbalances in cellular processes and results in cellular death, rendering them unsuitable for further study (1). This limitation particularly pertains to essential genes because knocking out these genes results in cell death, thereby eliminating the ability to investigate specific functions or roles of those genes in cellular mechanisms. On the other hand, partial gene repression systems aim to prevent cell damage and death while suppressing gene expression. By inhibiting gene activity to a certain degree, partial repression allows for the study of gene function without disrupting important cellular processes (2). However this method also presents limitations to studying essential genes. Residual gene activity can complicate the interpretation of experimental results, because undetectable traces of gene expression can make it difficult to distinguish between partial repression and complete knockout of a gene (3). The residual gene activity can also affect studies of gene interactions because traces of gene expression can still influence cellular pathways or regulatory networks in ways that are difficult to control or predict.

These limitations highlight the need to develop a different approach to gene repression using CRISPR systems. CRISPR Interference (CRISPRi) utilizes a mutated version of a CRISPR associated protein (Cas) that is made catalytically inactive, known as *dCas* (Nuclease-deactivated CRISPR associated protein) which may overcome the challenges of gene knockout by enabling targeted, reversible gene repression while keeping cells viable for further study (1).

In typical CRISPR-Cas system uses, the Cas endonuclease will bind and cut targeted DNA sites to alter the sequence. Traditionally, guide RNA sequences are designed to identify specific DNA sequences, directing CRISPR-associated nucleases to introduce precise cleavages at these sites (4). CRISPRi uses a catalytically inactivated Cas endonuclease to repress gene expression without introducing breaks in DNA or RNA by binding to the target DNA preventing the transfer of genetic information. In the CRISPRi system, guide RNA sequences are

repurposed to guide the dCas protein to the target sequence in DNA. The dCas protein binds to this sequence, making the target sequence inaccessible for molecular machinery to bind to DNA at that sequence, such as RNA polymerase, transcription factors, and ribosomal units, by blocking access to the target (4). Additionally, CRISPRi is reversible, which creates an "on/off switch" to allow more successful studies of gene function. One CRISPRi system utilizes a dCas12a, which binds to DNA without cutting it and suppresses mRNA transcription by RNA polymerase with an efficiency of 92-99% (4). Another system utilizes dCas13a, which functions by binding to RNA rather than DNA to disrupt translation by interfering with ribosomal unit binding, effectively repressing protein synthesis with an efficiency up to 93% (6).

The hereinafter described project is working towards the development of CRISPRi^2: a system that combines the dCas12a and dCas13a CRISPRi systems to maximize gene repression possibilities. By employing the use of Cas12a and Cas13a, cells can be subject to multi-stage repression at both transcriptional and translational levels, allowing genetic repression to occur without killing cells.

2: Methods

To achieve a dual plasmid insertion in a single *E. coli,* each plasmid system must contain a different origin of replication (ORI). Two plasmid systems with the same ORI would compete for resources within the cell until a complete takeover occurred.

2.1 Design and preparation of the dCas13a plasmid

Beginning with the *Leptotrichia wadei* (*Lwa*) Cas13a plasmid, one catalytic site of the Cas13a protein was inactivated via site-directed mutagenesis through PCR (7). Two other sites were targeted in tandem using HIFI DNA assembly (New England Biolabs, Catalog #E5520S). Primers were designed to amplify the entire Cas13a plasmid to surround the gBlock (Integrated DNA Technologies), an orderable synthetic DNA strand containing the desired nucleotide mutations. The gBlock and Cas13a plasmid were ligated with HIFI Master Mix according to the NEBuilder HiFi DNA Assembly Reaction Protocol. Following sequence verification of the correct ligation with no off-target effects, the now dCas13a plasmid was amplified and subsequently ligated into the pUC19 plasmid backbone containing the pUC19 ORI and ampicillin resistance via Gibson assembly (8). After sequence verification of proper ligation, the dCas13a plasmid was banked at -80℃ and renamed pDNZ003.

The pDNZ003 plasmid was grown overnight in LB media and isolated using the Qiagen QIAprep Spin Miniprep Kit. 1.5 ug of pDNZ003 was digested with 3ul of BsmBI-v2 (New England BioLabs) in a 50 ul reaction at 55℃ for 2 hours. The enzyme was then heat-inactivated at 80℃ for 20 minutes. The reaction was then held at 55℃ to prevent self-annealing.

2.2 Preparation of the dCas12a plasmid

From a previous study, pCRJ001 (dCas12a plasmid system) containing chloramphenicol resistance and the p15a ORI was grown overnight in LB media (4). After plasmid purification, 1.5ug of pCRJ001 was digested with 3ul of BsaI-HF®v2 (New England BioLabs) in a 50 ul reaction at 37℃ for 8 hours. The enzyme was then heat-inactivated at 80℃ for 20 minutes. The reaction was then held at 55℃ to prevent self-annealing.

2.3 CRISPR RNA Sequence Design and Preparation

We designed a total of ten CRISPR RNA (crRNA) sequences, five for the *dCas12a* system and five for the *dCas13a* system. These crRNAs were synthesized to complement target regions within genes of interest and cloned into plasmid backbones with necessary promoters and regulatory elements. Each single-stranded RNA (sgRNA) was diluted to 100uM.1 ul of each sgRNA was then combined with 1ul of its respective match, and EB buffer was added to 10uL. The reaction was heated to 94℃ and cooled to 25℃ in a -.5 ℃/s to ensure proper ligation. The specific targeting strategy for each crRNA is summarized in Tables 1 and 2 below.

Name	Target	Sequence	PAM
JSL004 JSL00_4_	RPO's	AGAT cacacaacatacgagccgga ATAC tccggctcgtatgttgtgtg	attc
JSL003 JSL00_3	Promoter/RPOD18	AGAT cactttatgcttccggctcg ATAC cgagccggaagcataaagtg	ttta
JSL001 JSL00_1	CRP/Promoter	AGAT atgtgagttagctcactcat ATAC atgagtgagctaactcacat	atta
JSL002 JSL00_2_	Promoter	AGAT ggcaccccaggctttacact ATAC agtgtaaagcctggggtgcc	atta
JSL005 JSL00_5_	Promoter	AGAT gctcactcattaggcacccc ATAC ggggtgcctaatgagtgagc	gtta

Table 1. dCas12a- crRNA design sequences and targeting regions.

Name	Target	Sequence
JSL00_6_ JSL006	RBS	TATC aacaatttcacacaggaggcagctatgag AAAC ctcatagctgcctcctgtgtgaaattgtt
JSL00_7_ JSL007	Start of start codon	TATC caggaggcagctatgagcaaaggagaaga AAAC tcttctcctttgctcatagctgcctcctg
JSL00_8_ JSL008	Middle of start codon	TATC atgagcaaaggagaagaacttttcactgg AAAC ccagtgaaaagttcttctcctttgctcat
JSL00_9_ JSL009	Middle of gene	TATC gacacaaactcgagtacaactttaactca AAAC tgagttaaagttgtactcgagtttgtgtc
JSL0_10_	Off gene (negative control)	TATC ggaccatggcggtcggtgcactttaggtg

JSL010		AAAC cacctaaagtgcaccgaccgccatggtcc

Table 2. dCas13a crRNA designs and targeting regions.

2.4 CRISPR RNA Insertion of dCas13a and dCas12a Plasmid Systems

Using the Golden Gate assembly method, we inserted each of the five crRNA sequences designed for the dCas12a plasmid. In a reaction, 75 ng of pCRJ001 was combined with each respective crRNA in a 1:5 molar ratio (9). In addition, 2ul of 2x Stick End Master Mix was added and the reaction was heated at 25℃ for 2 hours. This process was repeated for pDNZ003. Additionally, a negative control, consisting of plasmids devoid of any crRNA insert, was included for each system. In total, 12 constructs were created.

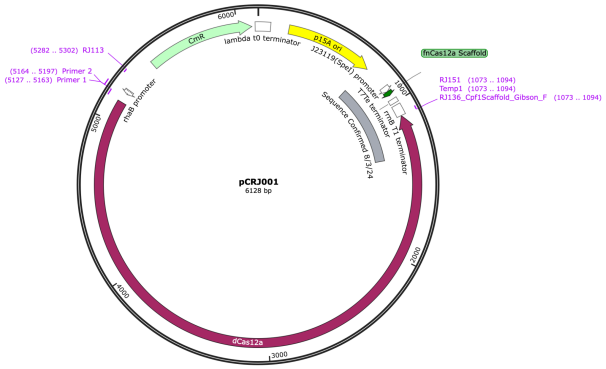

Figure 1. Plasmid Design of pCR001 (dCas12a)

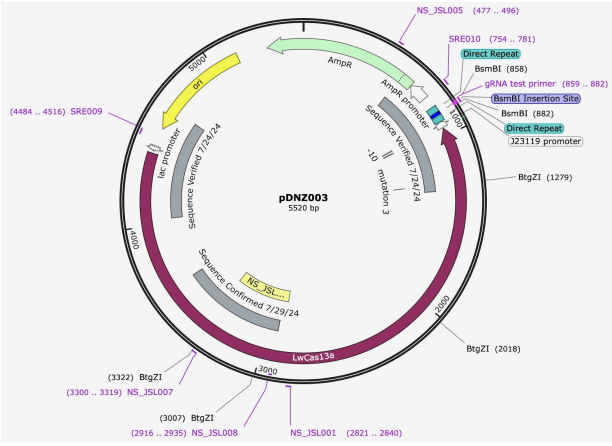

Figure 2. Plasmid Design of pDNZ003 (dCas13a)

2.5 Creation of Chemically Competent Nissle GFP

Nissle GFP was received as a gift in the form of a streaked plate from Dr. Nathan Crook (6). We prepared SOB medium (20 mM MgSO4) and SOC medium as per standard protocols. We then followed the Barrick Lab "Preparing Chemically Competent Cells using the Inoue Method" to completion. The aliquots were snap-frozen in liquid nitrogen and stored at -80°C. The following day, the cells were tested with 125ug of pUC19 control plasmid. The calculated transformation efficiency was found to be 2.42e2 transformants per ng of DNA.

2.6 Transformations into NEB 5α and Nissle GFP

Each of the 12 constructs was transformed into NEB® 5-alpha Competent E. coli (High Efficiency). Following sequencing verification of crRNA insertion with no off-target effects, sequences were banked at -80℃. The cells containing crRNA were then regrown overnight in an LB medium. After purification, plasmids were then dually transformed using 100ng of each plasmid system (200 ng total) per 50ul of Nissle GFP (6). The 275ul of the liquid culture was then grown on dual antibiotic plates.

2.7 Analysis Using Flow Cytometry

DCas12a plasmids in GFP strains were cultured overnight in LB medium (250 rpm, 37℃) and supplemented with 100 ug/mL of Chloramphenicol. Overnight cultures were inoculated to an optical density at 600 nm (OD_{600}) of 0.1 in two tubes of 3mL of fresh LB with Chloramphenicol medium, in one of the tubes, 2% w/v rhamnose was added to induce the promoter of the dCas12a (5). When measurements of dCas13a begin, cultures will be supplemented with lactose for single plasmid measurements and lactose and rhamnose for dual plasmid measurements as lactose is the inducer for the dCas13a promoter. Cultures were incubated for a total of 24 hours, at hours 0, 3, 9, and 24, 100 uL of culture was diluted in 1 mL of Phosphate Buffer Saline (PBS) pH 7.4 (ThermoFisher) and the geometric mean of 10,000 events per sample were measured. A blue solid-state laser (488 nM excitation), an optical filter at 530/30 nm for GFP fluorescence, and a 488/10 nm optical filter for side scatter (SSC) were used. Flow Cytometry Standard (FSC) files were analyzed using the Attune NxT Software (ThermoFisher). The geometric means of the fluorescence (in arbitrary units AU) were taken for the 10,000 events per sample.

3: Results

Using flow cytometry, initial results of dCas12a repression of the target gene were recorded showing plasmids with target sites JSL002 and JSL003 having the highest repression with percent expression of 30% and 29% respectively (Figure 3c). Percent repression was calculated by dividing the fluorescence measured when the dCas12a was induced and not

induced with 2% rhamnose, the inducer for the dCas12a promoter. These two gRNAs sit at the promoter at the -10 motif and RNA polymerase binding site (RPOD18) (Figure 4). JSL005 also showed comparable repression of 31% expression when induced which sits at the -35 promoter motif. JSL001, while it covers the -35 promoter motif and sits on the Cyclic AMP receptor protein (CRP) region, showed 77% expression, suggesting that a successful guide RNA must cover more of the promoter region to successfully repress expression. JSL004 also showed low repression with 59% expression; this guide RNA sits before the CRP region and therefore does not successfully block the RNA polymerase. To act as a reference, we used a dCas12a without a miscellaneous gRNA, that is it does not sit on the plasmid, expression levels of this plasmid show the typical expression level of the GFP gene without targeted CRISPRi and without expression of dCas12a at all. To measure the lowest level of repression, we used NEB5α, an E. coli strain without the GFP in the genome, to measure the lower levels of fluorescence possible to achieve. Due to differences in how cultures grew, slight variation in expression levels occurred, however expression of the target gene over time of our uninduced and uninduced plasmids showed relatively similar growth levels throughout the course of measurements (Figure 3a, 3b). In these results, we are also able to see that expression of dCas12a may have affected fluorescence on JSL001, JSL004 and our control dCas12a until around hour 6, where the expression of the GFP seems to increase dramatically after. To validate these results, measurements must be measured again under the same conditions to ensure similar results are observed. When comparing the expression of successful regions to the negative controls, JSL002, JSL003, and JSL005 showed highly successful repression of the target gene, with measurements comparable to the E. coli.

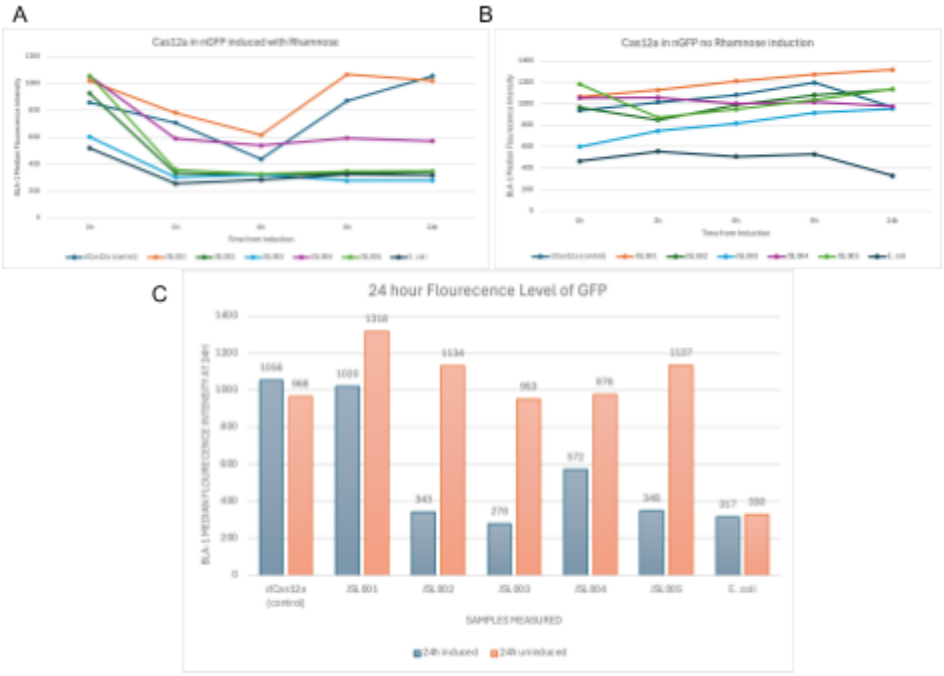

Figure 3: Fluorescence level of target gene when repressed by dCas12a

(a) Fluorescence levels when dCas12a is induced with 2% rhamnose over a 24 hour time period.

(b) Fluorescence level when dCas12a is not induced over a 24 time period.
(c) Fluorescence level of all plasmids after 24 hours when induced and uninduced.

Results are still incomplete at this time as we work to develop the dCas13a plasmids to measure the best place on the genome repression can be observed. Based on the results of the dCas12a, the best regions observed cover the promoter region further upstream, sitting completely on the -35 or closer to the -10 and RPOD18. Therefore when targeting translation, we expect the best gRNA to be those that sit more directly on the ribosomal binding site (RBS) or further upstream, to prevent the ribosome from assembling or translating the protein. Future directions include creating and testing these dCas13a plasmids and creating dual plasmid systems with both the dCas12a and dCas13a to measure repression levels of CRISPRi[2]. While results are incomplete, based on our initial data we predict seeing significant levels of repression when combining the two CRISPRi systems to achieve complete repression.

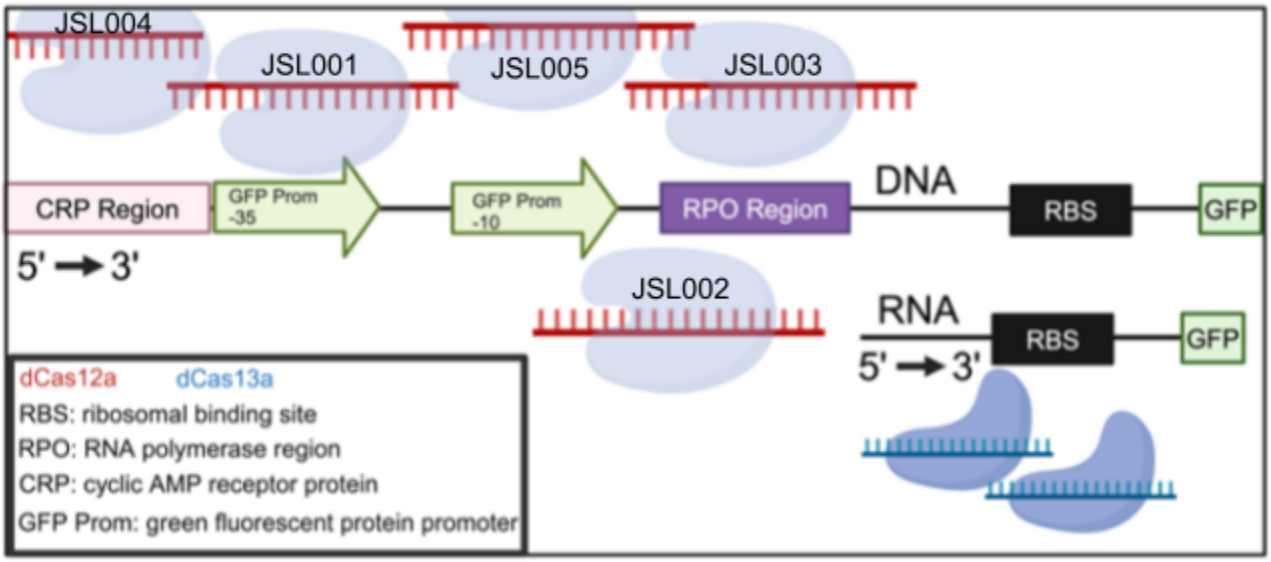

Figure 4: Placements of dCas12a and dCas13a on the gene of interest

4: Conclusion

The results on dCas12a-mediated repression give us a baseline understanding of the effectiveness of the target-specific plasmids tested, signifying that the success of repression strongly depends on the precise location of gRNA binding within the promoter region. The gRNAs that target the -10 motif and the RNA polymerase binding site, JSL002 and JSL003, achieved the highest repression levels, reducing expression to 30% and 29%, respectively. These findings highlight the critical importance of targeting key regulatory sequences within promoter regions to achieve substantial transcriptional repression. Similarly, JSL005, which targets the -35

promoter motif, showed a similar level of repression (31%), further demonstrating that targeting promoting motifs play a key role in successful repression.

In contrast, gRNAs that didn't target critical promoter motifs or key regions outside of these sites exhibited higher expression levels. JSL001, which overlaps the -35 promoter motif and the Cyclic AMP Receptor Protein (CRP) binding site, had a 77% expression level, suggesting that incomplete coverage of the promoter region allows residual transcriptional activity. Likewise, JSL004, which targets a region upstream of the CRP binding site, demonstrated limited repression with 59% expression, indicating that its position fails to block RNA polymerase effectively. These observations reinforce the conclusion that gRNA placement is a major determinant of dCas12a system efficiency.

To establish baseline fluorescence levels, controls were included: a dCas12a plasmid without a gRNA insert (to represent typical GFP expression without CRISPRi) and NEB 5α cells lacking GFP entirely (to represent the lowest achievable fluorescence levels). Although minor variations in fluorescence were observed across replicates due to differences in culture growth conditions, the overall trends remained consistent. Notably, fluorescence levels for JSL001, JSL004, and the control plasmid initially decreased from dCas12a expression but later increased after a significant time period, suggesting potential limitations in repression stability over time and regulatory cellular mechanisms that serve to restore gene expression levels.

Future directions include validating these results under identical conditions to ensure reproducibility and extending the study to the dCas13a plasmids designed. Based on the success of targeting key promoter motifs for transcriptional repression, it is anticipated that the most effective dCas13a gRNAs will target the ribosomal binding sites (RBS) or regions immediately upstream, where they can directly block ribosome assembly and the initiation of translation. While the results for dCas13a are pending, the initial data strongly suggest that integrating the dCas12a and dCas13a systems in dual-plasmid constructs will provide highly successful repression at both the transcriptional and translational levels and comprehensive gene silencing, paving the way for highly effective CRISPR interference strategies.

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Human Centered Design

The stakeholders of this project, agricultural researchers, biotech companies, and academic researchers, can utilize its findings to improve crop productivity. By leveraging CRISPR interference (CRISPRi) technology to precisely silence specific genes in plants, this work offers an innovative approach to enhancing desirable traits such as yield, drought resistance, and reduced susceptibility to pests. For example, CRISPRi technology can be used to silence the Knox gene, which plays a role in regulating plant development (10). This could lead to more uniform and efficient growth patterns, directly benefiting agricultural productivity. Further applications of this research could involve integrating CRISPRi into breeding programs for crops like wheat, rice, and corn, enabling precise genetic modulation without introducing permanent genome edits (11). This temporary and reversible gene silencing can help address concerns about unintended consequences or ecological risks associated with traditional genetic modification. It is important to consider how future work that involves this project must consider the ethical and biosafety rules that are intertwined with this work.

Biosafety

This project has met the biosafety rules that were defined by GOGEC. Our team and our research verified that the various aspects of the procedure did not interfere with the GOGEC Biosafety and Biosecurity Blacklist. Firstly, there was no experimentation that was conducted on human subjects or samples. Next, we used NEB5α *E. coli*, which is considered a Risk Group 1 microorganism. Additionally, although we used CRISPR technology, it was never used on any organism other than the NEB5α *E. coli* as previously stated. Lastly, our work did not help pathogens interfere with the immune system or interfere with the normal host cell's processes of replication, transcription, and translation. Although we are mutating coding sequences which interfere with the processes of replication, transcription, and translation, they are not being altered in a host organism and are instead solely altered in the pathogen itself.

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