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# Mechanisms of the type V CRISPR-Cas12c system

By

Carolyn J Huang

A dissertation submitted in partial satisfaction of the

requirements for the degree of

Doctor of Philosophy

in

Molecular and Cell Biology

in the

Graduate Division

of the

University of California, Berkeley

Committee in charge:

Professor Jennifer Doudna, Chair
Professor Kathleen Collins
Professor Britt Glaunsinger
Professor Donald Rio

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## Abstract

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Professor Jennifer A. Doudna, Chair

CRISPR (clustered regularly interspaced short palindromic repeats)-Cas (CRISPR-associated) systems are a form of prokaryotic adaptive immunity against invading viruses or plasmids. In these systems, Cas nuclease effectors recognize and cleave foreign nucleic acids containing sequences that are complementary to their associated CRISPR RNA (crRNA) to trigger degradation of targeted sequences. For successful CRISPR interference to occur, the sequences encoding crRNAs must be first acquired from foreign genetic material. These acquired DNA sequences are then transcribed into pre-crRNAs and processed into mature crRNAs that can guide interference against the specific invaders. The type V CRISPR-Cas12c (V-C) system is a compact system that has unique 3' pre-crRNA processing activity and lacks a *cas2* gene that was previously thought to be essential during the acquisition stage for all CRISPR systems. Although the initial activities of this system have been analyzed, the mechanisms by which this system acquires functional spacer, produces its guide RNA, and restricts viral infection are not fully defined.

For my graduate work, I addressed the fundamental mechanisms of the type V-C system by determining the mechanisms of Cas12c-mediated pre-crRNA processing and interference. My work has revealed that Cas12c is an RNA-guided DNA binder that does not cleave target DNA. The repurposed RuvC domain of Cas12c exclusively processes pre-crRNA, and maturation of crRNA is essential for efficient Cas12c DNA targeting. Importantly, targeted Cas12c DNA binding can repress transcription and restrict phage infection, accomplishing antiviral interference without chemical attack on the invader. Additionally, I attempted to reconstitute type V-C adaptation *in vivo* in a heterologous host *Escherichia coli* with the goal of eventually identifying determinants for adaptational protospacer adjacent motif (PAM) selectivity and looking for evidence of primed type V-C adaptation. Together the investigation described here in this dissertation sheds light on the mechanisms of the compact V-C CRISPR system, allowing for comparison with more complex systems and provides insight into the evolutionary history of CRISPR systems and functions.

## Acknowledgements

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Last but not least, I would like to express my deepest gratitude to my family and friends who have been supportive throughout my entire graduate career and beyond. Their belief in me has kept my spirits and motivation high during this unforgettable journey, the memories of which I will treasure for the rest of my life.

## Table of Contents

**Abstract** .......... **1**
**Acknowledgements** .......... **i**
**Table of Contents** .......... **ii**
**List of Figures** .......... **iv**
**List of tables** .......... **v**
**Chapter 1 Introduction to CRISPR-Cas systems** .......... **1**
1.1 Introduction .......... 2
**Chapter 2 A naturally DNase-free CRISPR-Cas12c enzyme silences gene expression** .......... **10**
2.1 Abstract .......... 11
2.2 Introduction .......... 11
2.3 Materials and Methods .......... 12
2.3.1 Protein sequence alignment .......... 12
2.3.2 Generation of plasmids .......... 12
2.3.3 Nucleic acid preparation .......... 12
2.3.4 Protein expression and purification .......... 13
2.3.5 Pre-crRNA processing assays .......... 13
2.3.6 Radiolabeled DNA cleavage assays .......... 14
2.3.7 Fluorophore quencher (FQ)-labeled reporter assays .......... 14
2.3.8 Filter-binding assays .......... 15
2.3.9 Dual-color fluorescence interference assay .......... 15
2.3.10 Bacteriophage plaque assays .......... 16
2.4 Results .......... 16
2.4.1 Cas12c processes its pre-crRNA but cannot cleave DNA *in vitro* .......... 16
2.4.2 The Cas12c RuvC domain is responsible for pre-crRNA processing .......... 17
2.4.3 Cas12c catalyzes maturation of an engineered single-guide RNA .......... 18
2.4.4 RNA-guided DNA binding by Cas12c depends on pre-crRNA processing .......... 18
2.4.5 Cas12c blocks gene expression in cells without invoking target-activated DNase activity .......... 19
2.4.6 Naturally DNase-free Cas12c protects cells from bacteriophage infection .......... 21
2.5 Discussion .......... 57
2.6 Acknowledgments .......... 58
2.7 Author contributions .......... 59
**Chapter 3 Reconstituting type V-C CRISPR-Cas adaptation in *E. coli*** .......... **60**
3.1 Abstract .......... 61
3.2 Introduction .......... 61
3.3 Materials and Methods .......... 62
3.3.1 Construction of expression plasmids .......... 62
3.3.2 *In vivo* spacer acquisition assays .......... 63

3.3.3 Oligo protospacer electroporation....64
3.4 Results....64
3.4.1 Native V-C CRISPR locus in a plasmid did not lead to spacer acquisition....64
3.4.2 Codon-optimized V-C Cas1 and Cas12c induced at physiological expression levels did not lead to spacer acquisition....65
3.4.3 V-C Cas1 without Cas12c did not lead to spacer acquisition....65
3.4.4 Overexpression of V-C proteins did not lead to spacer acquisition....65
3.5 Discussion....103
3.6 Acknowledgments....103
**Bibliography....104**

**List of Figures**

Figure 1.1 Stages of CRISPR-Cas immunity in PAM-dependent systems ......................6
Figure 1.2 Adaptational PAM selectivity and primed adaptation ......................................7
Figure 1.3 Pre-crRNA processing mechanisms in dsDNA-targeting Class 2 CRISPR-Cas systems ..............................................................................................................9
Figure 2.1 Cas12c has no detectable target-activated nuclease activity *in vitro* ............23
Figure 2.2 The Cas12c RuvC domain processes pre-crRNA........................................24
Figure 2.3 Design and function of pre-sgRNAs for Cas12c ..........................................26
Figure 2.4 Tight RNA-guided DNA binding by Cas12c depends on pre-crRNA processing ..................................................................................................................27
Figure 2.5 Cas12c blocks gene expression *in vivo* without target-activated DNase activity ........................................................................................................................30
Figure 2.6 Cas12c protects cells from bacteriophage infection.....................................31
Figure 2.7 Proposed mechanism of the type V-C CRISPR-Cas12c system ..................33
Figure 2.8 Cas12c pre-crRNA processing is precise and occurs 3' to the spacer, Related to Figure 2.1 ..................................................................................................35
Figure 2.9 Cas12c has no detectable target-activated *trans*-cleavage activity on RNA, Related to Figure 2.1...................................................................................................36
Figure 2.10 The RuvC of Cas12c has no detectable target-activated *trans*-cleavage activity on DNA, Related to Figure 2.1 .........................................................................38
Figure 2.11 Sequence alignment of two Cas12c proteins, Related to Figure 2.1 ..........39
Figure 2.12 Dependence of pre-crRNA processing and binding on divalent metal ion, Related to Figure 2.2....................................................................................................40
Figure 2.13 Cas12c blocks gene expression *in vivo* without target-activated DNase activity in a strand-independent manner, Related to Figure 2.5....................................42
Figure 2.14 Effect of Cas12c-induced repression of essential gene expression in E. coli and the requirement of both tracrRNA and crRNA for ribonucleoprotein assembly, Related to Figure 2.5....................................................................................................43
Figure 3.1 Acquisition assay with V-C loci in native DNA sequences ...........................67
Figure 3.2 Acquisition assay with *E. coli*-codon-optimized V-C Cas1 and Cas12c induced at physiological expression level .....................................................................70
Figure 3.3 Acquisition assay with V-C Cas1 only..........................................................71
Figure 3.4 Acquisition assay with overexpressed V-C Cas1 and Cas12c......................73
Figure 3.5 Acquisition assay with overexpressed V-C Cas1 only ..................................75
Figure 3.6 Acquisition assay with overexpressed V-C Cas1 and Cas12c without tracrRNA.....................................................................................................................77

## List of tables

Table 2.1 List of plasmids, Related to Methods ............................................................ 48
Table 2.2 Oligonucleotide sequences, Related to Methods .......................................... 53
Table 2.3 Plasmids and oligonucleotides used in experiments, Related to Methods..... 56
Table 3.1 Summary of amino acid and guide RNA sequences of type V-C CRISPR-Cas systems used in this study, Related to Methods ........................................................... 81
Table 3.2 Expression plasmids used in this study, Related to Methods ........................ 99
Table 3.3 Oligonucleotide sequences, Related to Methods ........................................ 100
Table 3.4 Plasmids and oligonucleotides used in experiments, related to Methods .... 102

# Chapter 1 Introduction to CRISPR-Cas systems

## 1.1 Introduction

CRISPR (clustered regularly interspaced short palindromic repeats)-Cas (CRISPR-associated) systems are a form of adaptive immunity against invading mobile genetic elements, such as viruses or plasmids, found in an estimated 50% of bacteria and 87% of archaea (Makarova et al., 2015; Wright et al., 2016). In these systems, Cas nuclease effectors typically recognize and cleave foreign nucleic acids containing sequences complementary to their associated CRISPR RNAs (crRNAs) to trigger degradation of targeted nucleic acids (Figure 1.1). Currently, these CRISPR-Cas systems are categorized into two classes and six types, with class 1 (types I, III, IV) CRISPR systems using multi-subunit effector ribonucleoprotein complexes and class 2 (types II, V, VI) CRISPR systems using single Cas endonucleases as effectors (Koonin et al., 2017; Makarova et al., 2017a, 2017b, 2020; Shmakov et al., 2017). Every mature crRNA contains a repeat region that allows for the formation of an effector ribonucleoprotein complex and a spacer region that is complementary to the invading target nucleic acid. The programmability of the well-characterized type II CRISPR-Cas9 and type V CRISPR-Cas12a (V-A) systems to cleave at desired sites guided by their crRNAs has led to the widespread use of these single-effector Class 2 CRISPR systems for genome engineering applications (Knott and Doudna, 2018).

For effective RNA-guided CRISPR interference to occur, the DNA sequence encoding the crRNA must be first acquired from foreign genetic material during the adaptation (or acquisition) stage (Figure 1.1). During adaptation, fragments of foreign DNA are integrated as spacers into a CRISPR array, which is composed of identical repeat sequences interspaced by these spacer sequences. At the very early stage of a phage infection, a phage inserts its linear dsDNA, typically with unprotected ends, into a bacterium (Jackson et al., 2017). The host DNA repair complexes, such as RecBCD in Gram-negative bacteria and AddAB in Gram-positive bacteria, recognize free DNA ends and degrade the DNA until reaching a *chi* site, which is homologous recombinational hotspot octamer sequence that halts RecBCD degradation (Dillingham and Kowalczykowski, 2008; Lee and Sashital, 2022; Modell et al., 2017) (Figure 1.2). The free dsDNA ends in the phage genome that get recognized by DNA repair complexes can be additionally generated from double stranded breaks caused by restriction modification systems and stalled replication forks (Jackson et al., 2017; Lee and Sashital, 2022; Levy et al., 2015; Maguin et al., 2022) (Figure 1.2). Since bacterial genomes have higher density of *chi* sites than the genomes of mobile genetic elements (Lee and Sashital, 2022; Levy et al., 2015), mobile genetic elements are preferentially degraded, generating small DNA fragments that can be potential prespacer substrates for the adaptation machinery.

In DNA-targeting types I, II, and V CRISPR-Cas systems, target cleavage requires a short 2- to 5-nucleotide (nt)-long protospacer adjacent motif (PAM) next to the target sequence (reviewed in Gleditzsch et al., 2019). For these systems where target cleavage relies on PAM recognition, there must be a PAM-interacting component that selects for PAM-adjacent protospacers in their adaptation modules to ensure a functional immune response. The mechanism for adaptational PAM selectivity can be distinct for different systems (Figure 1.2). For example, in the type I-E system, the Cas1 component of the CRISPR integrase has inherent PAM-interacting residues and can preferentially select protospacers next to a PAM (Wang et al., 2015) (Figure 1.2). In systems with a Cas4

nuclease or Cas4 fusion protein, such as types I-D and I-G, Cas4 is required for PAM recognition (Hu et al., 2021; Kieper et al., 2018) (Figure 1.2). In type II-A systems, the PAM-interacting domain of the interference effector nuclease Cas9 serves as the adaptational PAM-recognizing component that allows for integration of functional pre-spacers adjacent to a PAM (Heler et al., 2015; Nussenzweig et al., 2019) and interacts with the Cas1-Cas2 integrase in complex with an adaptational accessory protein Csn2 (Heler et al., 2015; Ka et al., 2018; Wei et al., 2015) (Figure 1.2). Note that the nuclease activity of Cas9 is not required for spacer acquisition (Heler et al., 2015). Recently, the adaptational PAM selectivity for the V-A and V-B systems has also been reconstituted (Wu et al., 2022). The adaptational PAM recognition in these type V systems, which have a Cas4 or a Cas4/1 fusion, is similar in mechanism to those of type I systems with Cas4 such that, Cas4 is required for PAM selection in both type V-A and V-B systems and spacer trimming in type V-A (Wu et al., 2022). The diverse mechanisms the CRISPR acquisition machinery has developed to select and integrate functional protospacers adjacent to a PAM underscore its importance in functional immunity as well as prevention of autoimmunity.

Subsequent to obtaining an initial, functional spacer against a foreign DNA, the pre-existing spacer within a CRISPR array can direct interference against the specific foreign DNA, enabling more rapid acquisition of additional spacers from the same foreign DNA, and this process is known as primed adaptation (Datsenko et al., 2012; Jackson et al., 2017; Lee and Sashital, 2022; Nussenzweig et al., 2019; Swarts et al., 2012) (Figure 1.2). Primed spacer acquisition has been described for several type I and type II CRISPR-Cas systems (Lee and Sashital, 2022; Nussenzweig and Marraffini, 2020). For instance, during the type I-E and I-F interference stage, the CRISPR-associated complex for antiviral defense (Cascade) recruits the ATP-dependent helicase/nuclease Cas3 to degrade target DNA into ssDNA fragments, producing potential prespacer substrates that can be integrated into the CRISPR array (Künne et al., 2016; Lee and Sashital, 2022). Additionally, there is also evidence suggesting that Cascade recruits Cas3 with Cas1-Cas2, forming a primed acquisition complex that translocates along the foreign DNA unidirectionally and allowing Cas3 to directly transfer its degradation products to the Cas1-Cas2 integrase for spacer acquisition (Dillard et al., 2018; Lee and Sashital, 2022; Nussenzweig and Marraffini, 2020; Redding et al., 2015). In this case, new prespacer substrates are generated as the target DNA is being destroyed and are subsequently integrated into the CRISPR array. In contrast, type II primed adaptation is less understood but also similarly requires the nuclease activity of Cas9 (Nussenzweig et al., 2019). After Cas9 introduces double stranded breaks, Cas9 remains tightly bound to the free DNA ends at its cleavage sites and is believed to recruit the Cas1-Cas2 integrase with the help of Csn2 (Lee and Sashital, 2022; Wilkinson et al., 2019), allowing for more rapid spacer acquisition from the same foreign invader and thereby linking CRISPR interference to adaptation.

After acquiring spacer sequence from foreign nucleic acids to the CRISPR arrays by the CRISPR integrase complex, the CRISPR array is typically transcribed as a long precursor crRNA (pre-crRNA) that undergoes further processing into individual mature crRNAs (Figure 1.1), the mechanism of which can be distinct for different systems (Figure 1.3). For instance, type II CRISPR-Cas9 systems require a *trans*-activating crRNA (tracrRNA) for both the processing of pre-crRNA from its 3’ end by host factor RNase III

and Cas9-mediated CRISPR interference, making Cas9 systems dual-RNA guided systems (Deltcheva et al., 2011) (Figure 1.3A). In contrast, type V-A systems can process their own pre-crRNA without a tracrRNA using an active site in the WED (wedge) III domain of Cas12a, which is distinct from the RuvC domain that is responsible for RNA-guided dsDNA cleavage (Fonfara et al., 2016; Swarts et al., 2017) (Figure 1.3B). Similar to the V-A system, the type V Cas12j system does not require a tracrRNA, and the pre-crRNA is also processed from the 5' end within the repeat sequence by the effector protein itself (Pausch et al., 2020) (Figure 1.3C). Surprisingly, the RuvC domain of Cas12j, which cleaves the target dsDNA, also catalyzes pre-crRNA processing (Pausch et al., 2020). However, the full diversity of pre-crRNA processing and interference mechanisms in CRISPR-Cas systems remained to be described.

A previous study has shown that in the type V CRISPR-Cas12c (V-C) system (Figure 1.3D), the effector nuclease, Cas12c (also known as C2c3), can process its own pre-crRNA using a cleavage mechanism that is distinct from those observed for other known CRISPR-Cas systems (Harrington et al., 2020) (Figure 1.3). Cas12c processes its own pre-crRNA at the boundary of the spacer and the downstream (3') repeat sequence, and this activity requires a tracrRNA (Figure 1.3E). These Cas12c proteins were first identified in 2015 on small DNA fragments from marine and gut metagenomes with unknown hosts (Shmakov et al., 2015). The effector proteins of these V-C systems have a domain architecture resembling Cas12a and contain a RuvC-like nuclease domain with intact canonical DED active site residues. Currently, three V-C systems have been characterized *in vivo* in a heterologous host *Escherichia coli* (Yan et al., 2019). When using crRNA libraries targeting essential genes in *E. coli* or a plasmid with antibiotic resistance genes essential for bacterial survival in a *E. coli* negative screen, the interference activity of reconstituted CRISPR-Cas12c systems killed cells. Subsequently, the surviving bacteria were sequenced, and depleted spacers were mapped back to the sequence of the targeted genomic regions or plasmid (Yan et al., 2019). Through this screen, Yan et al. showed that WT Cas12c, but not RuvC-deactivated dCas12c, exhibited broad and symmetrical targeting of both DNA strands, the pattern of which is consistent with dsDNA interference activity (2019). Although the DNA interference activity *in vitro* was not reconstituted in this study, it was presumed that the RNA-guided dsDNA interference of Cas12c revealed from this *E. coli* screen was a result of RuvC-mediated target dsDNA cleavage (Yan et al., 2019). However, Harrington et al. additionally showed that although Cas12c can process its pre-crRNA *in vitro*, it was not able to cleave dsDNA or ssDNA *in vitro* under the conditions tested (2020).

The objective of my graduate work was to understand the fundamental mechanisms of adaptation, crRNA biogenesis, and interference stages of the type V CRISPR-Cas12c systems. By using biochemical techniques, including enzymatic assays, binding assays, *in vivo* CRISPR interference assays, I aimed to address the following questions in this work: How is the RuvC domain involved in Cas12c-mediated interference *in vivo* if not cleaving target DNA *in vitro*? How does Cas12c silence gene expression in a strand-independent manner *in vivo* with no evidence of DNA cleavage *in vitro*? Which domain is responsible for pre-crRNA processing? What determines PAM selectivity during acquisition of spacers that encode for these crRNAs? To answer these questions, I first explored the mechanism of Cas12c pre-crRNA processing by identifying the domain responsible for guide RNA processing and determining the active-site chemistry. I also

deciphered the mechanistic details of interference of this system using *in vitro* and *in vivo* assays, described in detail in Chapter 2. Lastly, I attempted to reconstitute type V-C adaptation *in vivo* with the goal of eventually identifying determinants of adaptational PAM selectivity, described in Chapter 3.

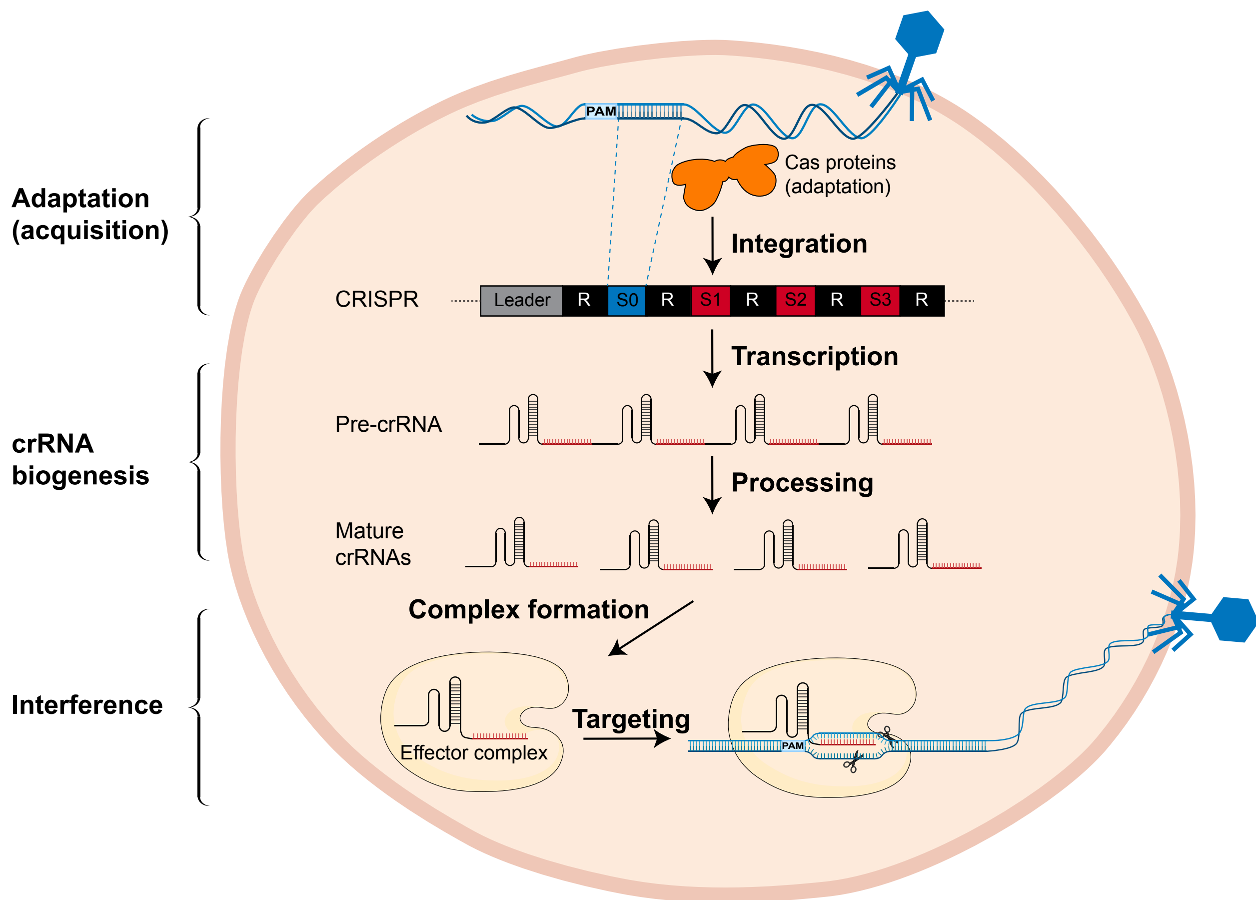



**Figure 1.1 Stages of CRISPR-Cas immunity in PAM-dependent systems**

CRISPRs are genomic regions in many bacteria and archaea that contain CRISPR arrays comprised of alternating repeat (R) and spacer (S) sequences. CRISPR-Cas immunity occurs in three stages, and this illustration focuses on those of DNA-targeting systems that require a protospacer adjacent motif (PAM). During adaptation, protospacers are selected and extracted from fragments of foreign nucleic acids that have a PAM. Cas proteins involved in adaptation then integrate protospacers as new spacers into a CRISPR array adjacent to the $1^{st}$ repeat sequence near the leader end. During the crRNA biogenesis stage, the CRISPR array is typically transcribed into a long pre-crRNA and processed by various mechanisms into mature crRNAs. During the interference stage, CRISPR-Cas ribonucleoprotein effector complexes are formed and target only foreign nucleic acids that are complementary to their crRNAs in a PAM-dependent manner.

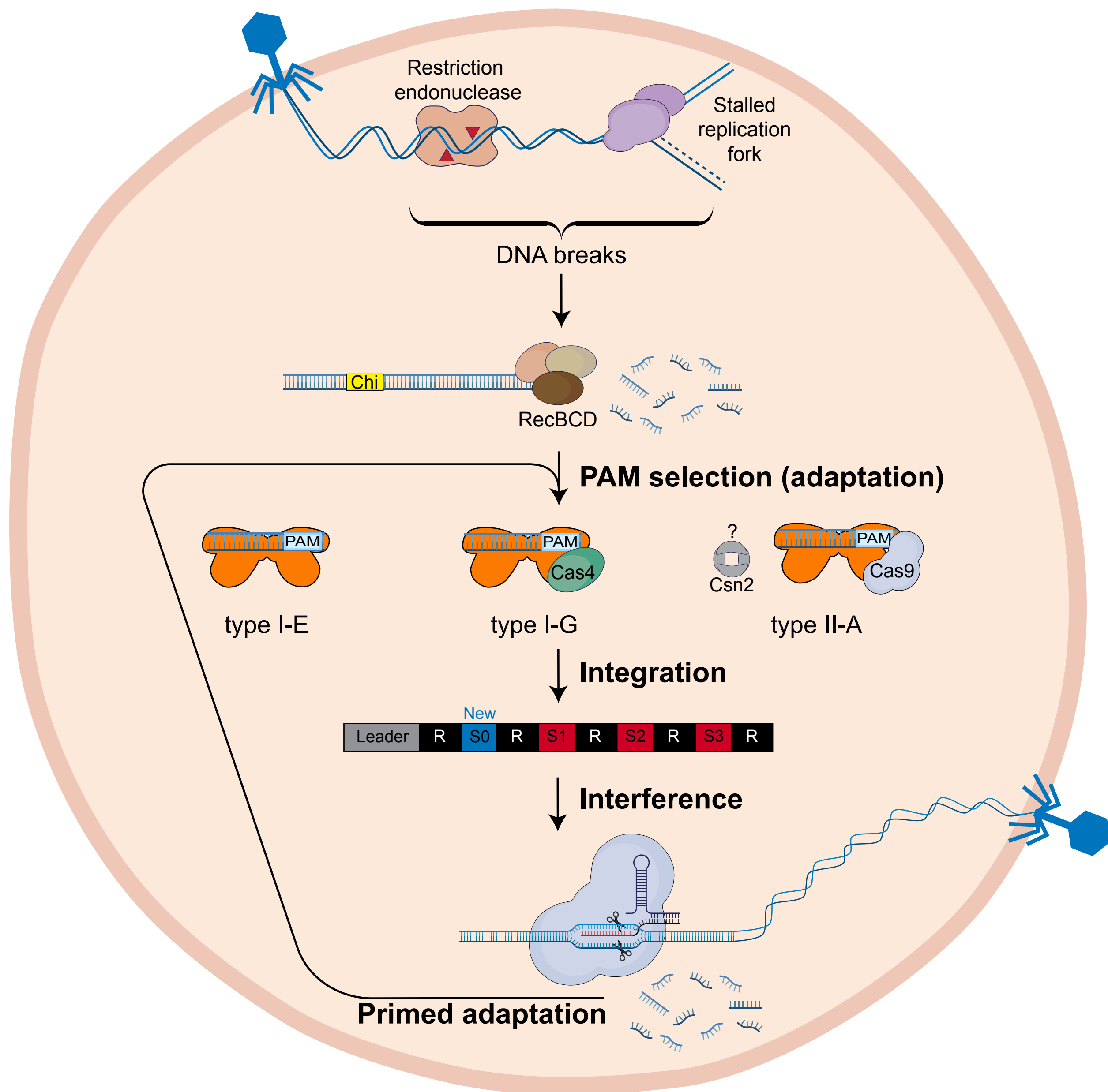



**Figure 1.2 Adaptational PAM selectivity and primed adaptation**

Upon injection of a phage genome into a bacterium, the phage genome is typically linear with unprotected dsDNA ends. Double stranded breaks in the phage genome can be additionally produced by restriction modification systems and stalled replication forks. The host DNA repair complexes, such as RecBCD, recognize free DNA ends and degrade the DNA (until reaching a *chi* site), generating small DNA fragments that can be prespacer substrates for the acquisition machinery. In CRISPR-Cas systems that require a protospacer adjacent motif (PAM) for interference, the mechanism for adaptational PAM selectivity can be distinct for different systems. For example, in the type I-E system, the Cas1 component of the CRISPR integrase has inherent PAM-interacting residues and

can preferentially select protospacers next to a PAM. In systems with a Cas4 nuclease or Cas4 fusion protein, such as type I-G, Cas4 is required for PAM recognition. In type II systems, Cas9 serves as the adaptational PAM-recognizing component that allows for integration of functional pre-spacers adjacent to a PAM. Csn2 is required for type II-A adaptation, but the exact function is unknown. After obtaining an initial spacer against a foreign DNA, the pre-existing spacer within a CRISPR array can direct interference against the specific foreign DNA, enabling more rapid acquisition of additional spacers from the same foreign DNA, and this process is known as primed adaptation.

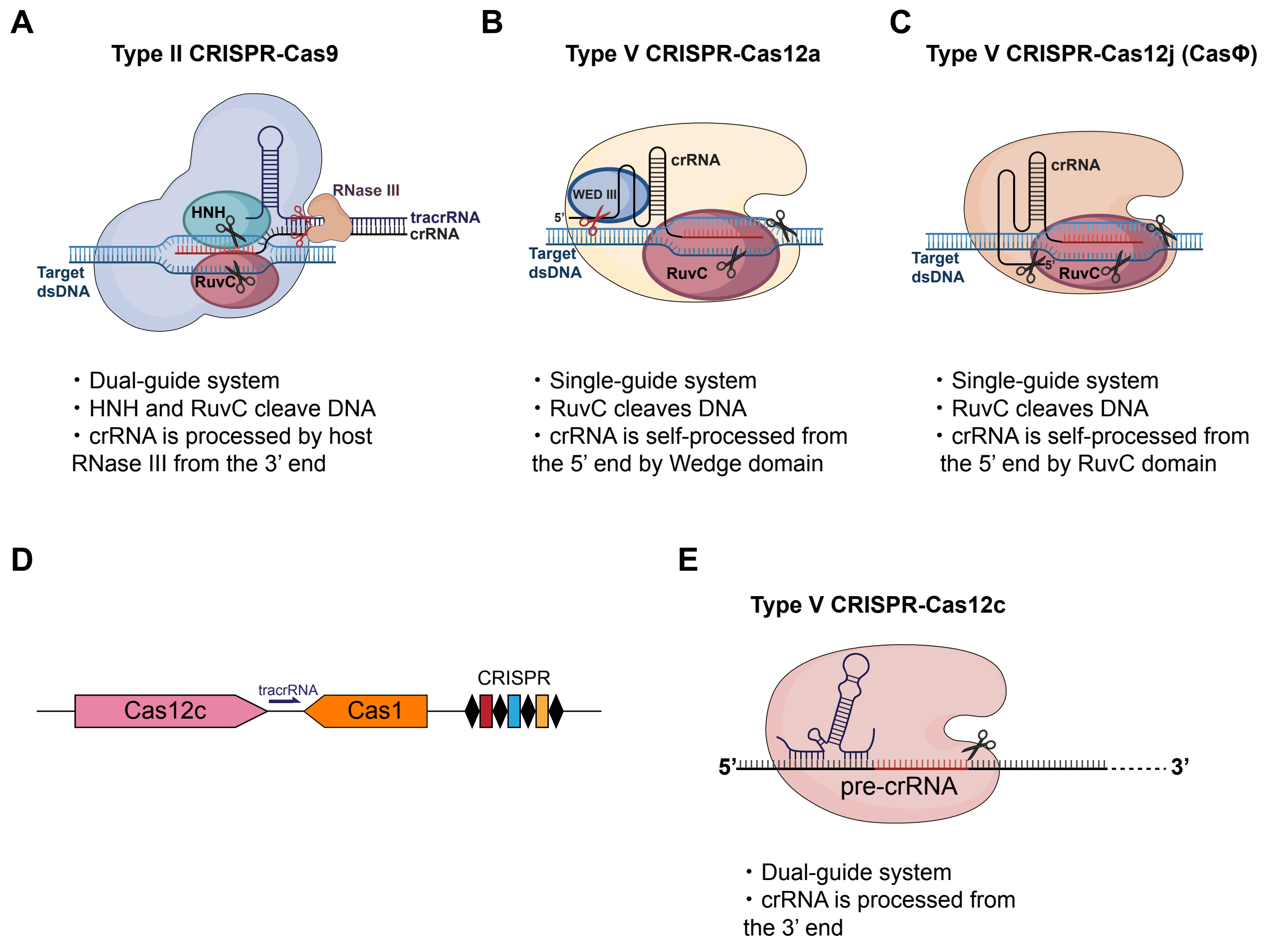



**Figure 1.3 Pre-crRNA processing mechanisms in dsDNA-targeting Class 2 CRISPR-Cas systems**

(A) In type II CRISPR-Cas9 systems where the HNH and RuvC domains of Cas9 together generate a blunt-end cleavage on the target dsDNA, the host factor RNase III processes the pre-crRNA from its 3' end when a *trans*-activating crRNA (tracrRNA) base-pairs with pre-crRNA.
(B) In the type V CRISPR-Cas12a (V-A) system, in which a single RuvC domain generates a staggered cut on the target dsDNA, the WED (wedge) III domain processes pre-crRNA on its 5' end.
(C) In the type V CRISPR-Cas12j (V-J) system, in which a RuvC domain generates a staggered cut on the target dsDNA, the RuvC domain also processes pre-crRNA on its 5' end.
(D) Diagram of a type V CRISPR-Cas12c (V-C) genomic locus.
(E) In type V-C system, the pre-crRNA is processed from its 3' end by Cas12c.
The repeat region of the crRNA is shown in black, while the spacer region is shown in red. Scissors indicate cleavage sites created by domains/enzymes indicated in the schematic.

# Chapter 2 A naturally DNase-free CRISPR-Cas12c enzyme silences gene expression

*Part of the work presented in this chapter has previously been published in the following manuscript: Huang, C. J., Adler, B. A., & Doudna, J. A. (2022). A naturally DNase-free CRISPR-Cas12c enzyme silences gene expression. *Molecular Cell*, *82*(11), 2148-2160.

## 2.1 Abstract

Used widely for genome editing, CRISPR-Cas enzymes provide RNA-guided immunity to microbes by targeting foreign nucleic acids for cleavage. We show here that the native activity of CRISPR-Cas12c protects bacteria from phage infection by binding to DNA targets without cleaving them, revealing that antiviral interference can be accomplished without chemical attack on the invader or general metabolic disruption in the host. Biochemical experiments demonstrate that Cas12c is a site-specific ribonuclease capable of generating mature CRISPR RNAs (crRNAs) from precursor transcripts. Furthermore, we find that crRNA maturation is essential for Cas12c-mediated DNA targeting. These crRNAs direct double-stranded DNA binding by Cas12c using a mechanism that precludes DNA cutting. Nevertheless, Cas12c represses transcription and can defend bacteria against lytic bacteriophage infection when targeting an essential phage gene. Together, these results show that Cas12c employs targeted DNA binding to provide antiviral immunity in bacteria, providing a native DNase-free pathway for transient antiviral immunity.

## 2.2 Introduction

CRISPR-Cas (clustered regularly interspaced short palindromic repeats, CRISPR associated) systems provide adaptive immunity in bacteria (Barrangou et al., 2007) and also function as powerful tools for genome editing in plants, animals, and microbes (Knott and Doudna, 2018). Fundamental to these systems are RNA-guided nucleases, including the Cas9 (type II) and Cas12 (type V) enzyme families that use CRISPR RNA (crRNA) to recognize foreign double-stranded DNA (dsDNA) by forming an R-loop structure in which ~20 nucleotides (nt) of the crRNA (the crRNA ‘‘spacer’’) base pair with the target strand (TS) of the target DNA, displacing the non-target strand (NTS) (Makarova et al., 2020; Swarts and Jinek, 2018). These Cas proteins also recognize a protospacer-adjacent motif (PAM), a short DNA sequence next to the crRNA-complementary sequence, to prevent autoimmunity and initiate R-loop formation for DNA interference (Gleditzsch et al., 2019).

Multiple studies identified a family of RuvC-containing Cas proteins, termed Cas12, whose variants exhibit diverse biochemical and cellular activities (Burstein et al., 2017; Shmakov et al., 2015; Yan et al., 2019). Among them, Cas12c (also known as C2c3), originally found in small DNA fragments from marine and gut metagenomes (Shmakov et al., 2015), shares certain features with other DNA-cutting Cas12 enzymes, but several aspects of this protein are enigmatic. First, although mature guide RNA production is known to be catalyzed by Cas12c with the requirement of a trans-activating crRNA (tracrRNA), the mechanism of RNA recognition and processing is not known (Harrington et al., 2020). Second, despite the demonstration of its gene targeting activity in *E. coli* and the presence of a RuvC nuclease domain with the canonical catalytic Asp-Glu-Asp motif (Yan et al., 2019), no enzymatic DNA cleavage activity was observed *in vitro* (Harrington et al., 2020). In addition, the lack of detectable DNase activity raised the question of whether or how Cas12c provides bacteria with antiviral protection.

We hypothesized that Cas12c might operate in bacteria using a DNase-free mechanism that is distinct from other Cas12 family members. To test this idea, we first

confirmed the inability of the RuvC active site to catalyze DNA cleavage and then tested its activity as a ribonuclease (RNase), revealing its function in site-specific precursor crRNA (pre-crRNA) processing. *In vitro* binding assays indicated that despite the lack of target DNA cleavage, Cas12c binds to target DNA through a canonical crRNA-mediated interaction. Cell-based assays in bacteria demonstrated robust repression of transcription by Cas12c without target-activated cleavage. In addition, Cas12c and crRNAs that recognize an essential phage gene protected bacteria from lytic bacteriophage infection. These findings show that a DNA-binding-only mechanism is capable of conferring immunity during viral attack. We conclude that Cas12c is a naturally occurring example of an RNA-guided DNA-binding enzyme that protects bacteria from infection by target binding rather than target degradation. Furthermore, its immune function is likely to be dependent on RuvC-mediated pre-crRNA processing in its natural host. These results suggest that other functional DNA-targeting CRISPR immune systems may lack enzymatic machinery and that transcriptional repression may play a larger role in adaptive immunity than previously thought.

## 2.3 Materials and Methods

### 2.3.1 Protein sequence alignment

Amino acid sequences of Cas12c1 and Cas12c_4 (the focus of this study) were compared by generating a global alignment using SnapGene Version 5.0.8 with the Needleman-Wunsch algorithm/BLOSUM-62. The alignment diagram was plotted using ESPript (Robert and Gouet, 2014). Percent identity and similarity from SnapGene are reported in the figure legend of Figure 2.11.

### 2.3.2 Generation of plasmids

For protein purification, Cas12c bacterial expression plasmid (10xHis-MBP-TEVcs-Cas12c) was originally described in the study conducted by Harrington et al., 2020 and modified (R965H) to match with the original protein sequence of Cas12c_4 in the JGI IMG database. For *in vivo* experiments, LbCas12a and Cas12c genes were cloned into a low-copy plasmid backbone that contains a SC101 origin of replication and a kanamycin resistance gene. The backbone of guide RNA plasmids was amplified from pBFC0423 (Knott et al., 2019) so that sgRNA or crRNA expression was under control of a strong constitutive promoter (proD). Dual guide RNA plasmids were constructed from Integrated DNA Technologies (IDT) gBlocks so that the expressions of most of the concatenated non-coding RNA sequence (Yan et al., 2019), which includes tracrRNA, and crRNA were under control of separate proD promoters in opposite directions. A list of plasmids is described in Table 2.1. Plasmid sequences and maps are available on addgene.org. To reprogram the guide RNA plasmids for targeting different loci, guide sequences were exchanged via Gibson Assembly or Golden Gate assembly to encode the guide for the selected target site (guide spacer sequences are listed in Table 2.1B).

### 2.3.3 Nucleic acid preparation

All DNA oligonucleotides were synthesized commercially (IDT). DNA oligonucleotides used in cleavage assays were purified by denaturing PAGE, ethanol-precipitated, and resuspended in water.

RNA substrates were either synthesized commercially (IDT) or generated through *in vitro* transcription, which was described previously (Cofsky et al., 2020). For *in vitro* T7 RNA polymerase transcription, double-stranded DNA templates were assembled from several overlapping DNA oligonucleotides (IDT) by PCR or by annealing a short oligonucleotide containing the T7 promoter sequence to a long ssDNA oligonucleotide template (IDT). Synthesized or transcribed RNA fragments were PAGE-purified and resuspended in RNA storage buffer (0.1 mM EDTA, 2 mM sodium citrate, pH 6.4). All oligonucleotide identities and sequences are listed in Table 2.2.

**2.3.4 Protein expression and purification**

Cas12c expression and purification were performed as previously described (Chen et al., 2018) with modifications. Briefly, *E. coli* Rosetta (DE3) (Novagen) containing Cas12c expression plasmids were grown in Terrific Broth supplemented with 100 µg/mL ampicillin and 20 µg/mL chloramphenicol to an OD600 of 0.4, cooled down on ice for 15 min before induction with 0.5 mM isopropyl β-D-thiogalactoside (IPTG) and overnight growth at 16°C. Cells were harvested by centrifugation, re-suspended in lysis buffer (50 mM HEPES-Na, pH 7.5, 500 mM NaCl, 20 mM imidazole, 1 mM TCEP, 5% (v/v) glycerol, 1 tablet of cOmplete Protease Inhibitor Cocktail/50 mL, 0.25 mg/mL lysozyme, and 0.5% TritonX-100) and lysed by sonication. After centrifugation, the soluble fraction of the lysate was loaded onto a Ni-NTA Superflow Cartridge (QIAGEN) pre-equilibrated in wash buffer (50 mM HEPES-Na, pH 7.5, 500 mM NaCl, 20 mM imidazole, 1 mM TCEP, 5% (v/v) glycerol). Bound proteins were washed with wash buffer until UV baseline and eluted in elution buffer (50 mM HEPES-Na, pH 7.5, 500 mM NaCl, 300 mM imidazole, 1 mM TCEP, 5% glycerol). The buffer of the eluate was exchanged to ion exchange buffer A (50 mM HEPES-K, pH 7.5, 200 mM KCl, 1 mM TCEP, 5% (v/v) glycerol) using a HiPrep 26/10 Desalting Column (Cytiva) before the addition of TEV protease. After overnight cleavage at 4°C, proteins were loaded into a MBPTrap HP column (Cytiva) connected to a HiTrap Heparin HP Column (Cytiva) and washed until UV baseline. The TEV-cleaved proteins were eluted from the Heparin column with a KCl gradient and concentrated to 2 mL before injection into a HiLoad 16/600 Superdex 200 pg column (Cytiva). The gel filtration buffer contained 20 mM HEPES-K, pH 7.5, 200 mM KCl, 1 mM TCEP, 5% (v/v) glycerol. Peak fractions were verified by SDS-PAGE, and the concentrations of purified proteins were determined using a NanoDrop 8000 Spectrophotometer (Thermo Scientific).

**2.3.5 Pre-crRNA processing assays**

Pre-crRNA processing assays were performed as previously described (Harrington et al., 2020). Briefly, the reactions were carried out in cleavage buffer containing 20 mM Tris-Cl (pH 7.5 at 37°C), 150 mM KCl, 5 mM $MgCl_2$, 1 mM TCEP, and 5% (v/v) glycerol. For radiolabeling experiments, the pre-crRNA substrates were 50-radiolabeled with T4 Polynucleotide Kinase (PNK) (NEB) in the presence of gamma $^{32}$P-ATP. In a typical pre-crRNA processing reaction, the concentrations of Cas12c, tracrRNA and $^{32}$P-labeled pre-crRNA substrates were 100 nM, 125 nM and 3 nM, respectively. Prior to the addition of Cas12c to the reaction, tracrRNA and pre-crRNA were pre-annealed in 1X Cleavage Buffer. Reactions were incubated at 37°C, and an aliquot of each reaction was quenched with 2x Quench Buffer (94% (v/v) formamide, 30 mM EDTA, 400 µg/mL heparin, 0.2% SDS, and 0.025% (w/v) bromophenol blue) at 0, 2, 5, 15, 30, and 60 min.

RNA hydrolysis ladders were prepared by incubating RNA probes in 1X RNA Alkaline Hydrolysis Buffer containing 50 mM Sodium Carbonate [Na2CO3], pH 9.2, and 1 mM EDTA at 95°C for 1, 5, and 15 mins before the addition of 2x Quench Buffer. Quenched reactions were incubated at 95°C for 3 min, and products were then resolved by denaturing PAGE (10% acrylamide:bis-acrylamide 19:1, 7 M urea, 1X TBE). Gels were dried (3 hr, 80°C) on a Model 583 Gel Dryer (Bio-Rad) and exposed to a phosphor screen. Phosphor screens were imaged on an Amersham Typhoon phosphorimager (GE Healthcare). Phosphorimages were quantified using ImageQuant software (GE Healthcare). In pre-sgRNA processing reactions, the concentrations of Cas12c and $^{32}$P-labeled pre-sgRNA substrates were 100 nM and 3 nM, respectively.

For experiments with 3' 6-FAM-labeled pre-crRNA, the concentrations of Cas12c, tracrRNA, and 3' 6-FAM-labeled pre-crRNA substrates were 2000 nM, 2200 nM and 200 nM, respectively. For termini chemistry analysis, 7 µL of the processing reaction products were treated with 10 units Quick CIP (NEB) in 1X CutSmart buffer (NEB) for 1 hr at 37°C. The 2X Quenching Solution for fluorophore experiments contained 97% formamide and 30 mM EDTA. Products were resolved by 20% denaturing PAGE gel and visualized with Amersham Typhoon Biomolecular Imager (GE Healthcare). Oligonucleotide identities are shown in Table 2.3.

### 2.3.6 Radiolabeled DNA cleavage assays

Radiolabeled DNA cleavage assays were performed as previously described (Harrington et al., 2020). Briefly, the reactions were carried out in the same cleavage buffer described above for pre-crRNA processing. The tested interference substrates of either target strand (TS) or non-target strand (NTS) were 5'-radiolabeled with T4 PNK (NEB) in the presence of gamma $^{32}$P-ATP. To form dsDNA substrates, the labeled substrate was annealed with excess cold TS or NTS depending on the labeled strand. The final concentrations of Cas12c, tracrRNA, crRNA, and $^{32}$P-labeled interference substrates were 100 nM, 125 nM, 125 nM and 3 nM, respectively. Before the addition of the labeled interference substrates at 37°C, Cas12c ribonucleoprotein was pre-complexed by incubating with its pre-annealed dual guide RNAs at 37°C for 5 min, then 25°C for 25 min. Reactions were incubated for 60 min and quenched with 2x Quench Buffer (described above for pre-crRNA processing). The quenched reactions were heated to 95°C for 5 min. The reaction products were resolved by 10% denaturing PAGE and phosphorimaging. Oligonucleotide identities are shown in Table 2.3.

### 2.3.7 Fluorophore quencher (FQ)-labeled reporter assays

Target-activated *trans*-cleavage activity was measured by performing the fluorophore quencher (FQ)-labeled reporter assays as previously described (Chen et al., 2018) with modifications. Briefly, the reactions were carried out either in the cleavage buffer described above for pre-crRNA processing [20 mM Tris-Cl (pH 7.5 at 37°C), 150 mM KCl, 5 mM $MgCl_2$, 1 mM TCEP, and 5% (v/v) glycerol] or in rCutSmart buffer (NEB). The final concentrations of Cas12c, guide RNA, dsDNA activator, FQ reporter substrates were 100 nM, 120 nM, 50 nM and 200 nM, respectively. Activator dsDNA duplex was prepared by annealing 1.5-fold molar excess of the NTS to TS in 1X reaction buffer. Cas12c ribonucleoprotein was pre-complexed by incubating Cas12c with its dual or single guide RNAs at 37°C for 5 min, then 25°C for 5 min before the incubation with dsDNA

activator at 37°C for 5 min, then 25°C for 25 min. RNaseAlert or DNaseAlert reporters (IDT) were added to initiate the *trans*-cleavage reactions, which were then incubated in a Cytation5 fluorescence plate-reader (BioTek) for 180 min at 37°C, with fluorescence measurements taken every 2 min (RNaseAlert reporter = excitation filter: 485 nm/20 bandpass, emission filter: 528 nm/20 bandpass. DNaseAlert reporter = excitation filter: 530 nm/25 bandpass, emission filter: 590 nm/35 bandpass).

### 2.3.8 Filter-binding assays

The filter-binding assays were performed as previously described (Cofsky et al., 2020). Briefly, complexes were formed in 1X binding buffer (20 mM Tris-Cl, pH 7.9 at 25°C, 150 mM KCl, 5 mM $MgCl_2$, 1 mM TCEP, 50 µg/mL heparin, 50 µg/mL bovine serum albumin, 5% glycerol). In a typical binary complex binding assay, Cas protein was first diluted down from 600 nM in series in binding buffer, added to a fixed concentration of tracrRNA (750 nM for all protein dilutions), and was incubated with radiolabeled RNA (100 pM) at 37°C for 5 min, then 25°C for at least 1 hr. For ternary complex binding assays, Cas protein was first diluted down from 600 nM in series in binding buffer, added to a fixed concentration of guide RNAs (750 nM), and incubated at 37°C for 5 min, then 25°C for 25 min. This complex was then added to the radiolabeled DNA probe (100 pM) and incubated at 37°C for 5 min, then 25°C for at least 1 hr. HT Tuffryn (Pall), Amersham Protran, and Amersham Hybond-N+ (GE Healthcare) membranes were equilibrated in 1X membrane wash buffer (20 mM Tris-Cl, pH 7.9 at 25°C, 150 mM KCl, 5 mM $MgCl_2$, 1 mM TCEP, 5% glycerol) and assembled on a vacuum dot-blot apparatus. The membranes were washed once with 30 µL 1X wash buffer before radioactive samples were applied to the membranes by low vacuum. Membranes were then washed once with 40 µL 1X wash buffer, air-dried, and analyzed by phosphorimaging. Data were quantified with ImageQuant TL Software (GE Healthcare) and fit to a binding isotherm using Prism (GraphPad Software). ‘‘Fraction bound’’ is defined as (background-subtracted volume of Protran spot)/(total background-subtracted volume of Protran spot + Hybond N+ spot). For DNA binding, data were best fit by a model that included an exponent on the concentration terms. The physical basis for this dependency is unknown. Equilibrium dissociation constants ($K_D$) and number of independent replicates (n) are reported in the figure legends, when appropriate. For assays testing complex assembly in an EDTA-containing buffer, 25 mM EDTA was substituted for 5 mM $MgCl_2$. Oligonucleotide identities are shown in Table 2.3.

### 2.3.9 Dual-color fluorescence interference assay

The strain used for all *in vivo* assays in this study was *E. coli* MG1655 with sfGFP and mRFP chromosomally integrated at the *nfsA* locus, originally described by Qi et al. (2013) and modified through the removal of the $kan^R$ cassette. Guide RNA plasmids were transformed into electrocompetent cells containing the Cas protein using a MicroPulser Electroporator (Bio-Rad). Cells were recovered for 100 min at 37°C in LB broth, and ten-fold serial dilutions were plated on LB agar supplemented with 50 µg/mL kanamycin and 34 µg/mL chloramphenicol in Nunc Rectangular Dishes (Thermo Scientific). Plates were incubated at 37°C for 13-17 hours. GFP images were taken on an Amersham Typhoon Biomolecular Imager (GE Healthcare) with a Cy2 525BP20 filter or on a ChemiDoc MP Imaging System (Bio-Rad) with Blue Epi illumination as excitation source and a 530/28

emission filter. RFP images were taken on the Typhoon Imager with a Cy3 570BP20 filter or on the ChemiDoc imager with Green Epi illumination as excitation source and a 605/50 emission filter. Colonies were visualized using the ChemiDoc imager with White Trans illumination as excitation source and a standard filter, and colony forming units (CFUs) were counted. For experiments comparing WT vs. dCas12c transcriptional repression levels, the GFP intensity of undiluted spots was quantified using Image Lab software (Bio-Rad) and divided by the CFU intensity of the same spots. This ratio is normalized by the ratio of the corresponding non-targeting guide sample so that % GFP intensity is 100% for non-targeting guide samples. Two-way ANOVA with Šídák's multiple comparison test was performed using Prism (GraphPad Software) for statistical analysis of GFP intensities. All assays were performed in triplicate, and independent transformation was performed for each replicate. Plasmid identities are shown in Table 2.3.

### 2.3.10 Bacteriophage plaque assays

Bacteriophage assays were conducted following a modified double agar overlay protocol (Knott et al., 2019) using phage λ cI857 (St-Pierre and Endy, 2008). Briefly, *E. coli* (NEB 10-beta) containing both Cas effector and gRNA plasmids (Table 2.3) were grown overnight 37°C, 200 rpm. To perform plaque assays, 100 µL of saturated overnight culture was mixed with molten LB Lennox top agar supplemented with appropriate antibiotics and decanted onto a corresponding LB Lennox Agar plate (to final overlay concentrations of 1% (w/v) tryptone, 0.5% (w/v) yeast extract, 0.5% (w/v) NaCl, 0.7% (w/v) agar, 50 µg/mL kanamycin sulfate, and 34 µg/mL chloramphenicol hydrochloride). For Cas12c-containing strains targeting *cro*-1 and Cas12c/dCas12c containing strains targeting highly expressed target *mcpE*, the overlay was also induced with 10 µM IPTG. This overlay was left to dry for 15 minutes under microbiological flame. Ten-fold serial dilutions of λ cI857 were performed in SM buffer (Teknova), and 2 µL of each dilution were spotted onto the top agar and allowed to dry for 10 minutes. Plaque assays were incubated at 37°C for 12-16 hours. After overnight incubation, plaques were scanned using a standard photo scanner and plaque forming units (PFUs) enumerated. In cases where individual PFUs were not enumerable, but clearings were observed at high phage concentrations, the most concentrated dilution at which no plaques/clearings were observed was counted as 1 PFU. Efficiency of plaquing (EOP) calculations for a given condition were performed by normalizing the mean of PFU for a condition to the mean PFU of a non-targeting control: $mean(PFU_{condition})/mean(PFU_{negativecontrol})$. All plaque assays were performed in biological triplicate (three experiments carried out on different days using independent bacterial cultures, independently prepared bottom and top agar, and freshly prepared bacteriophage dilutions).

## 2.4 Results

### 2.4.1 Cas12c processes its pre-crRNA but cannot cleave DNA *in vitro*

To confirm the repertoire of enzymatic activities that Cas12c has at its disposal for immune function, we began by reconstituting the previously reported pre-crRNA processing activity of Cas12c (Harrington et al., 2020) and probed it in greater detail. Unlike all other auto-processing Cas enzymes (Behler and Hess, 2020; East-Seletsky et al., 2016; Fonfara et al., 2016; Pausch et al., 2020; Yan et al., 2019; Zhang et al., 2018),

which cleave the pre-crRNA proximal to or within the repeat sequence that is directly bound by the protein, Cas12c cleaves its pre-crRNA 17 nt downstream (3') of the protein-bound repeat (Harrington et al., 2020) (Figure 2.1A). We confirmed the previously reported cleavage site, defined by a 17-nt distance downstream of the protein-bound repeat and showed that it is precise and robust to adjustments in sequence or length on either the 5' or 3' end of the pre-crRNA substrate (Figure 2.8).

Next, we tested the ability of Cas12c to perform target-activated DNA/RNA cleavage. We tested a variety of different target substrates, including dsDNA, single-stranded DNA (ssDNA), partially base-paired (''bubbled'') DNA, and single-stranded RNA (ssRNA). Cas12c failed to cleave these guide-RNA-complementary substrates under the conditions tested (Figure 2.1B). Additionally, although target-activated, non-specific DNA or RNA *trans*-cleavage activity is a property of other Cas12-family enzymes (Chen et al., 2018; Harrington et al., 2018, 2020; Pausch et al., 2020; Yan et al., 2019), including an ortholog of Cas12c (Cas12c1; Wang and Zhong, 2021), we did not detect such target-activated *trans*-cleavage activity for the Cas12c ortholog of focus (database identifier: Cas12c_4; Harrington et al., 2020) under all conditions tested (Figure 2.1C, Figure 2.9, & Figure 2.10). Sequence alignment indicates that these two Cas12c orthologs share limited sequence identity that could result in distinct enzymatic activities (Figure 2.11). These results led us to conclude that Cas12c_4 is an RNase but not a DNase.

### 2.4.2 The Cas12c RuvC domain is responsible for pre-crRNA processing

We hypothesized that the RuvC nuclease domain of this Cas12c, instead of cleaving DNA, catalyzes pre-crRNA processing. Consistent with this hypothesis, mutation of a RuvC metal-coordinating carboxylate (D928A; described in the study conducted by Yan et al., 2019) abolished Cas12c's pre-crRNA-processing activity (Figure 2.2A). To further test the role of the RuvC domain in pre-crRNA processing, we asked whether the chemical requirements and products of Cas12c's cleavage activity are consistent with known enzymatic features of RuvC function.

Because RuvC belongs to a group of enzymes that require divalent cations for activity (Yang and Steitz, 1995), we first investigated the metal-ion dependency of the Cas12c-catalyzed pre-crRNA processing reaction by performing the pre-crRNA processing reaction in the presence of the divalent metal-ion chelating reagent, EDTA. Results showed that Cas12c cannot process pre-crRNA without access to divalent cations (Figure 2.12A). However, further experimentation revealed that Cas12c also cannot bind to pre-crRNA in the presence of EDTA (Figure 2.12B), suggesting that $Mg^{2+}$ is likely required for ribonucleoprotein (RNP) complex formation. Therefore, metal-ion dependency is not a reliable indicator of the mechanism of catalysis in this case.

We next evaluated the nature of the RNA products generated by Cas12c-catalyzed pre-crRNA cleavage. General acid-base-catalyzed RNA cleavage employs the 2'-hydroxyl group upstream of the scissile phosphate as the attacking nucleophile, producing 5' and 3' cleavage products with 2',3'-cyclic phosphate and 5'-hydroxyl termini, respectively (Knott et al., 2017; Swarts et al., 2017) (Figure 2.2B). In metal-ion-dependent RNA cleavage reactions, water serves as the attacking nucleophile and the 3' cleavage product has a 5'-phosphate terminus (Pausch et al., 2020) (Figure 2.2B). To identify the 5'-terminal chemistry of the Cas12c-generated 3' cleavage product, we treated the mature crRNA with phosphatase and resolved the product by denaturing polyacrylamide gel

electrophoresis (PAGE). If a 5'-phosphate is present in the Cas12c-generated 3' cleavage product, phosphatase-catalyzed phosphate removal would be expected to reduce the electrophoretic mobility of the treated RNA. Conversely, the lack of a phosphatase-dependent mobility shift would indicate that no removable phosphate was present on the Cas12c-generated 3' cleavage product. We observed the reduced electrophoretic mobility of the 3' RNA cleavage fragment after phosphatase treatment (Figure 2.2C), consistent with a metal-ion-dependent catalytic mechanism and suggesting that the metal-ion-dependent RuvC domain of Cas12c is responsible for pre-crRNA processing.

Given the lack of detectable *cis*- or *trans*-DNA cleavage under all tested conditions, we further investigated the ability of the RuvC domain to cleave DNA by using a pre-crRNA substrate containing four 2'-deoxynucleotides spanning the pre-crRNA processing site (Figure 2.2D). This substrate was not cleavable by Cas12c, indicating that despite its ribonucleolytic activity, Cas12c's RuvC domain cannot cleave DNA, even when presented in the context of the established pre-crRNA substrate. We further demonstrated that only the 2'-hydroxyl group downstream of the scissile phosphate is required for pre-crRNA cleavage (Figure 2.2E). Notably, the upstream 2'-hydroxyl group, which is necessary for acid-base catalysis, is not required for pre-crRNA processing by Cas12c (Figure 2.2E), consistent with a metal-ion-dependent mechanism. Together, our data suggest that the role of the RuvC domain in Cas12c is only for pre-crRNA processing.

### 2.4.3 Cas12c catalyzes maturation of an engineered single-guide RNA

Because Cas12c is the first tracrRNA-requiring Cas protein found to catalyze autonomous pre-crRNA processing (Chylinski et al., 2013; Deltcheva et al., 2011; Fonfara et al., 2016; Pausch et al., 2020; Yan et al., 2019), we wondered whether Cas12c's dual guide RNAs (crRNA and tracrRNA) can be engineered as a single-guide RNA (sgRNA) without disrupting Cas12c-catalyzed pre-crRNA maturation. Based on the predicted secondary structure of the mature dual RNAs (Yan et al., 2019) (Figure 2.3A), we tested two different pre-sgRNA designs. In the first design, the 3' end of the tracrRNA was connected by a 5'-GAAA-3' linker sequence to the unprocessed 5' end of the crRNA (pre-sgRNA 1) (Figure 2.3A). In the second design, the 3' end of the tracrRNA was instead connected by the same linker to the processed 5' end of the crRNA (pre-sgRNA 2). We found that Cas12c processes pre-sgRNA 1 as efficiently as when the dual guide RNAs are not fused together (Figure 2.3B and Figure 2.3C), while pre-sgRNA 2 is processed less efficiently (Figure 2.3B and Figure 2.3C). The pre-sgRNA design 1 may be useful for future applications of Cas12c.

### 2.4.4 RNA-guided DNA binding by Cas12c depends on pre-crRNA processing

Having demonstrated that Cas12c does not perform target DNA cleavage (Figure 2.1B), we next asked whether Cas12c binds guide-RNA-complementary substrates. Filter-binding assays indicated that Cas12c binds target dsDNA with a $K_D$ in the low nanomolar range and has even stronger affinity for partially base-paired (''bubbled'') DNA (Figure 2.4A). These data suggest that like other Cas12 enzymes and Cas9, Cas12c forms an R-loop with complementary dsDNA (Cofsky et al., 2020; Jiang et al., 2016; Lim et al., 2016; Liu et al., 2019; Pausch et al., 2021; Stella et al., 2017; Swarts and Jinek, 2019; Takeda et al., 2021; Yang et al., 2016; Zhang et al., 2021). Cas12c also has moderate binding affinity for target ssDNA (~33 nM) but does not bind detectably to

ssRNA (Figure 2.4A). Filter-binding assays additionally demonstrated that target DNA binding is sequence-specific and PAM-dependent (Figure 2.4A).

The pre-crRNA processing activity of Cas12c suggested that crRNA maturation may be essential for target dsDNA binding. To explore this idea, we tested Cas12c's DNA-binding affinity when guided by either pre-crRNA or mature crRNA. WT Cas12c was able to bind to target dsDNA with comparable affinities in each case (Figure 2.4B). However, when the pre-crRNA contained phosphorothioates that prevent Cas12c-catalyzed processing (Figure 2.8C), Cas12c bound target dsDNA much more weakly (Figure 2.4B). Consistent with this observation, we found that Cas12c containing a catalytically deactivating mutation (dCas12c-D928A) was impaired for RNA-guided dsDNA binding when a pre-crRNA was used and behaved similarly to the WT Cas12c when a mature crRNA was used (Figure 2.4C). These results suggest that pre-crRNA processing is required for the function of the CRISPR-Cas12c system and that without pre-crRNA processing, Cas12c cannot bind target dsDNA efficiently (Figure 2.4D). Together, these findings imply that the functional role of the RuvC domain in this CRISPR system is distinct from its role in most other systems.

### 2.4.5 Cas12c blocks gene expression in cells without invoking target-activated DNase activity

Cas12c can target genes essential for survival in bacteria as detected by depletion of gene-targeting members of a crRNA library transformed into Cas12c-expressing *E. coli* (Yan et al., 2019). This observation could result from Cas12c-mediated cleavage of genomic targets *in vivo*, potentially with the help of *E. coli* host factors, or it could reflect Cas12c-mediated transcriptional silencing through targeted polymerase blockage. To distinguish between these possibilities, we developed a dual-color fluorescence interference assay using a strain of *E. coli* (Qi et al., 2013) in which reporter genes encoding green fluorescent protein (GFP) and red fluorescent protein (RFP) are integrated into the *E. coli* genome. Cas12c-directed cleavage of the chromosomally integrated GFP or RFP would be expected to be toxic to *E. coli* (Figure 2.5A) due to chromosome breaks (Cui and Bikard, 2016). In contrast, if Cas12c binds but does not cut target DNA, we expect to observe a loss of the genetically targeted fluorophore without cell death (Figure 2.5A), similar to the effect of CRISPR interference (CRISPRi) with a catalytically inactivated Cas9 or Cas12a (Kim et al., 2017; Qi et al., 2013; Zhang et al., 2017).

We used WT LbCas12a (WT Cas12a) and catalytically deactivated LbCas12a (dCas12a) as controls, which exhibited the expected behavior: expression of WT Cas12a killed the bacteria, while dCas12a silenced gene expression without cell death (Figure 2.5B and Figure 2.13A). In contrast, both WT and dCas12c, when guided by a GFP- or RFP-targeting sgRNA, silenced gene expression without killing cells (Figure 2.5C and Figure 2.13B). These results are consistent with biochemical data showing that Cas12c binds but does not cleave target DNA (Figure 2.4). In contrast to dCas12a, which blocks transcription elongation in a strand-specific manner (Figure 2.13A; also similarly reported in the studies conducted by Kim et al., 2017 and Zhang et al., 2017), Cas12c exhibited no strand bias when it was targeted to the coding DNA sequences of the fluorescent reporter genes (Figure 2.13B and Figure 2.13C). The lack of strand bias is consistent with the *in vivo* screen data from Yan et al. (2019) that revealed Cas12c-mediated interference

activity on both DNA strands of the targeted regions, which are bacterial essential genes.

We next tested whether Cas12c-induced repression of essential gene expression in *E. coli* causes cell death. In control experiments using either WT or catalytically deactivated Cas12a, we found that crRNAs targeting selected essential genes (that were described in the study conducted by Yan et al., 2019) cause cell death or growth defects (smaller colonies), although the magnitude of the effects depended on the crRNA and were enhanced by the target-activated nuclease activity of WT Cas12a (Figure 2.14A). To varying degrees, both WT and dCas12c produced effects similar to those observed using dCas12a when targeted to essential genes (Figure 2.14B). This finding suggests that DNA binding by Cas12c blocks transcription of essential genes, inducing cell death or reduced cell proliferation.

Previously, it was reported with a slightly different Cas12c ortholog that WT Cas12c, but not dCas12c, prevented growth of *E. coli* cells that expressed genome-targeting guide RNAs (Yan et al., 2019), leading to the deduction that Cas12c functions by a genome-cutting mechanism. Since our Cas12c construct (Cas12c_4) varies from the previously tested Cas12c2 (Yan et al., 2019) by seven amino acids, none of which are predicted RuvC active site residues, we also performed the CRISPRi assay using the Cas12c2 variant. We found that both WT Cas12c2 and dCas12c2, when expressed in cells containing targeting sgRNAs, repress expression of a chromosomally integrated GFP gene without killing the bacteria (Figure 2.5D). This finding supports our conclusion that both Cas12c_4 (focus of this study) and Cas12c2 (Yan et al., 2019) are RNA-guided DNA-binding proteins that do not cut DNA.

Notably, we found that expression of tracrRNA and pre-crRNA under separate promoters, rather than as a single pre-sgRNA transcript, produced a more apparent difference in GFP repression between the WT Cas12c2 and dCas12c2 (Figure 2.5D and Figure 2.5E). Since both tracrRNA and pre-crRNA are required for binding and processing by Cas12c (Figure 2.14C), these observations imply that guide RNA maturation limits Cas12c function in cells expressing the dual transcripts, perhaps due to differences in the assembly kinetics of a tripartite versus bipartite complex. This difference in repression between WT Cas12c2 and dCas12c2 was eliminated when the crRNA was produced using a self-cleaving HDV ribozyme at the end of the pre-crRNA transcript (Figure 2.5D and Figure 2.5E), suggesting that the WT Cas12c2/ dCas12c2 discrepancy reported previously (Yan et al., 2019) was due to a difference in pre-crRNA processing capability rather than target dsDNA cleavage activity. Consistent with this conclusion, Cas12c-independent dual RNA processing enabled more robust dCas12c_4-mediated gene repression that is comparable to the repression observed in the presence of unprocessed sgRNA (Figure 2.5F and Figure 2.5G). We noticed that Cas12c_4-mediated GFP repression is less sensitive to the dual versus sgRNA switch than Cas12c2 is, which may be explained if the seven-amino-acid difference yields a difference in the baseline pre-crRNA-processing capacity of each variant. Regardless of these differences between Cas12c2 and Cas12c_4, our data, together with those presented by Yan et al. (2019), are best explained by a model in which Cas12c does not cleave DNA. In addition, pre-crRNA processing is a prerequisite for efficient target dsDNA binding (Figure 2.4).

Exploiting its autonomous guide RNA maturation activity, we also tested the ability of Cas12c to induce multiplexed CRISPRi by co-expressing in a single transcript two pre-sgRNAs that target GFP and RFP, respectively (Figure 2.5H). Cell-based results showed

that in this experiment, Cas12c robustly repressed both RFP and GFP expressions (Figure 2.5H). This multiplexing property can be useful as a biotechnological tool, as demonstrated for other CRISPR-Cas enzymes (McCarty et al., 2020). Together, these *in vivo* data suggest that RNA-guided Cas12c binding is sufficient for robust transcriptional repression of targeted gene expression without target-activated DNase activity, and it can be used for biotechnological purposes.

**2.4.6 Naturally DNase-free Cas12c protects cells from bacteriophage infection**

In nature, CRISPR-Cas systems are thought to protect prokaryotes from viral infections based on RNA-guided cleavage of foreign nucleic acid, and dsDNase activity is the key enzymatic function of all known DNA-targeting CRISPR systems (Nussenzweig and Marraffini, 2020; Shmakov et al., 2017; Smargon et al., 2017). Having no detectable target-activated DNase activity (Figure 2.1, Figure 2.5, Figure 2.9, Figure 2.10), can Cas12c provide anti-phage immunity based solely on targeted DNA binding? We tested this possibility using a phage plaque assay in which 10-fold serial dilutions of lambda (λ) phage were plated on lawns of *E. coli* expressing Cas12 and guide RNA(s) that either target a gene in the phage λ genome or contain a non-targeting sequence. We theorized that a DNase-free Cas12 protein could successfully restrict phage infection when targeting a phage-essential gene, but not a non-essential gene. However, nuclease activity would be required to restrict phage infection when targeting a non-essential gene. We chose two phage-essential genes: *cro*, which encodes an early regulator essential for preventing lysogeny (Johnson et al., 1978), and the highly expressed gene *E* that encodes the major capsid protein, which is essential for virion formation (*mcpE*) (Murialdo and Becker, 1977). We also investigated the impact of targeting λ gene of unknown function, *ea47*, which is non-essential for λ's lytic cycle (Casjens and Hendrix, 2015). A schematic of all the guides tested and locations of their protospacers within *cro*, *mcpE*, and *ea47* are shown in Figure 2.6A.

Consistent with our expectations, we observed that WT Cas12a exhibits anti-phage activity independent of both gene essentiality and strand targeting (Figure 2.6B). In contrast, the RuvC-deactivated dCas12a could only restrict λ when targeting the template strand of essential gene *cro* (Figure 2.6C). For naturally DNase-active Cas12a, this DNase activity confers greater guide-flexibility for phage restriction than a transcriptional-interference mechanism conferred by dCas12a.

Conversely, we found that WT Cas12c paired with sgRNAs targeting phage-essential genes, *cro* and *mcpE*, can result in several thousand-fold reduction of plaques as compared with a non-targeting control (Figure 2.6D). In the context of phage defense, guide efficacy appears to be strand independent, but strongly correlated with relative guide position, where guides more distal to the start codon appear more effective (Figure 2.6D). In line with our model that Cas12c is a naturally DNase-free repressor, all four guides targeting λ non-essential gene, *ea47*, failed to yield any detectable phage restriction. Furthermore, the RuvC-deactivated dCas12c gave comparable results to WT Cas12c, suggesting that phage defense is occurring independent of RuvC activity (Figure 2.6E). When successful interference occurs, the extent of plaque reduction induced by Cas12c/dCas12c is often comparable with that observed with dCas12a (Figure 2.6C) but slightly less robust than WT Cas12a (Figure 2.6B). Together, these results establish Cas12c as the first demonstrated example of a natural DNA-targeting CRISPR RNase

that provides antiviral immunity without target-activated DNase activity.

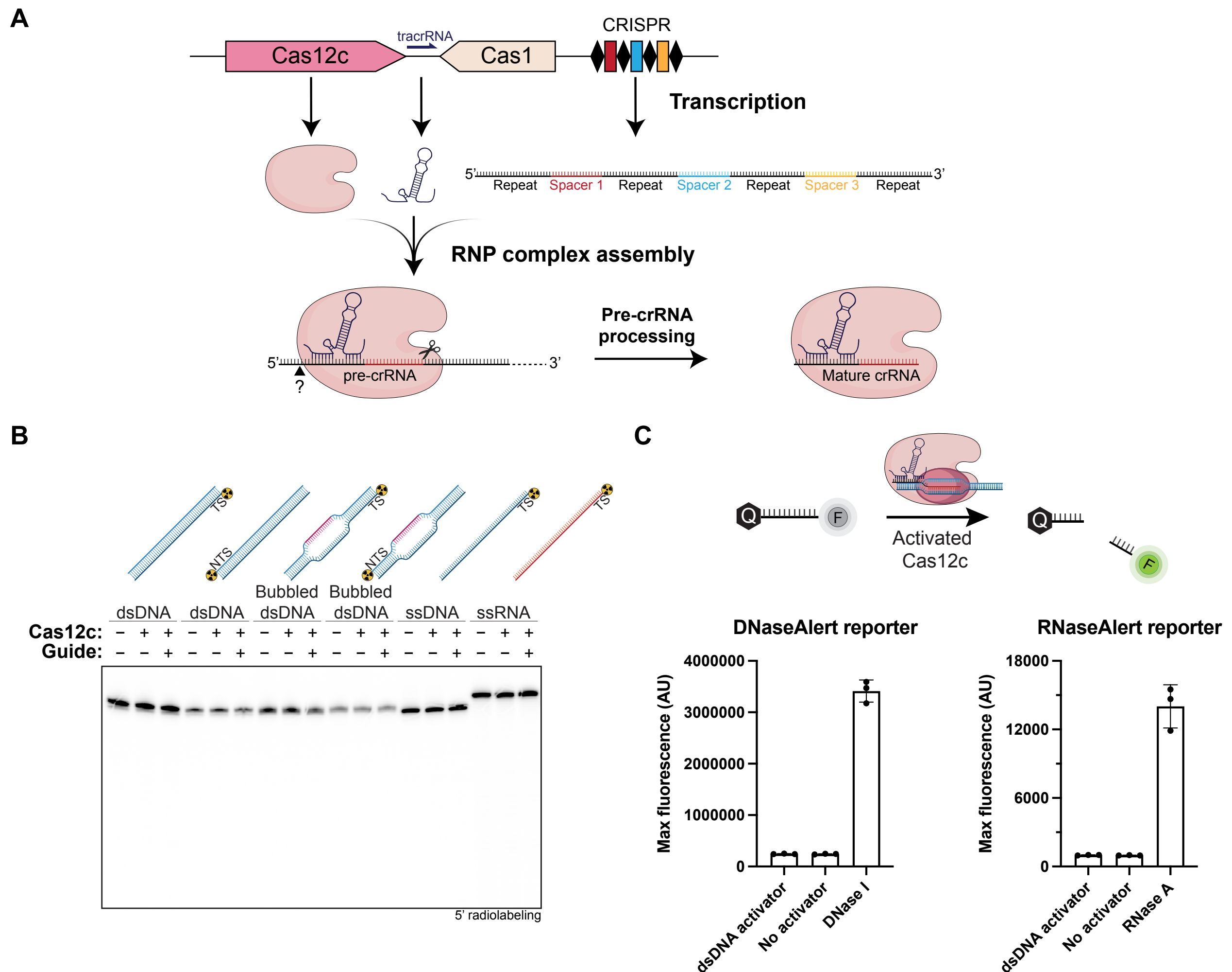



**Figure 2.1 Cas12c has no detectable target-activated nuclease activity *in vitro***

(A) Diagram of type V-C CRISPR-Cas12c genomic locus and 3’ pre-crRNA processing by Cas12c. The upstream repeat is processed at the 5’ end by unknown mechanisms, as indicated by the question mark.
(B) DNA cleavage assay targeting dsDNA, partially base-paired (‘‘bubbled’’) dsDNA (dsDNA containing 17 DNA-DNA base pair mismatches throughout the putative region of RNA complementarity), ssDNA, and ssRNA. TS, target strand; NTS, non-target strand.
(C) Quantification of maximum fluorescence signal generated from fluorophore quencher (FQ)-labeled reporter assays after incubating Cas12c:DualRNA:activator with DNaseAlert or RNaseAlert reporter substrates for 3 h at 37°C with DNase I or RNase A as positive controls, respectively. See also Figure 2.9 and Figure 2.10 for time course data. Data are represented as mean ± SD (n = 3).

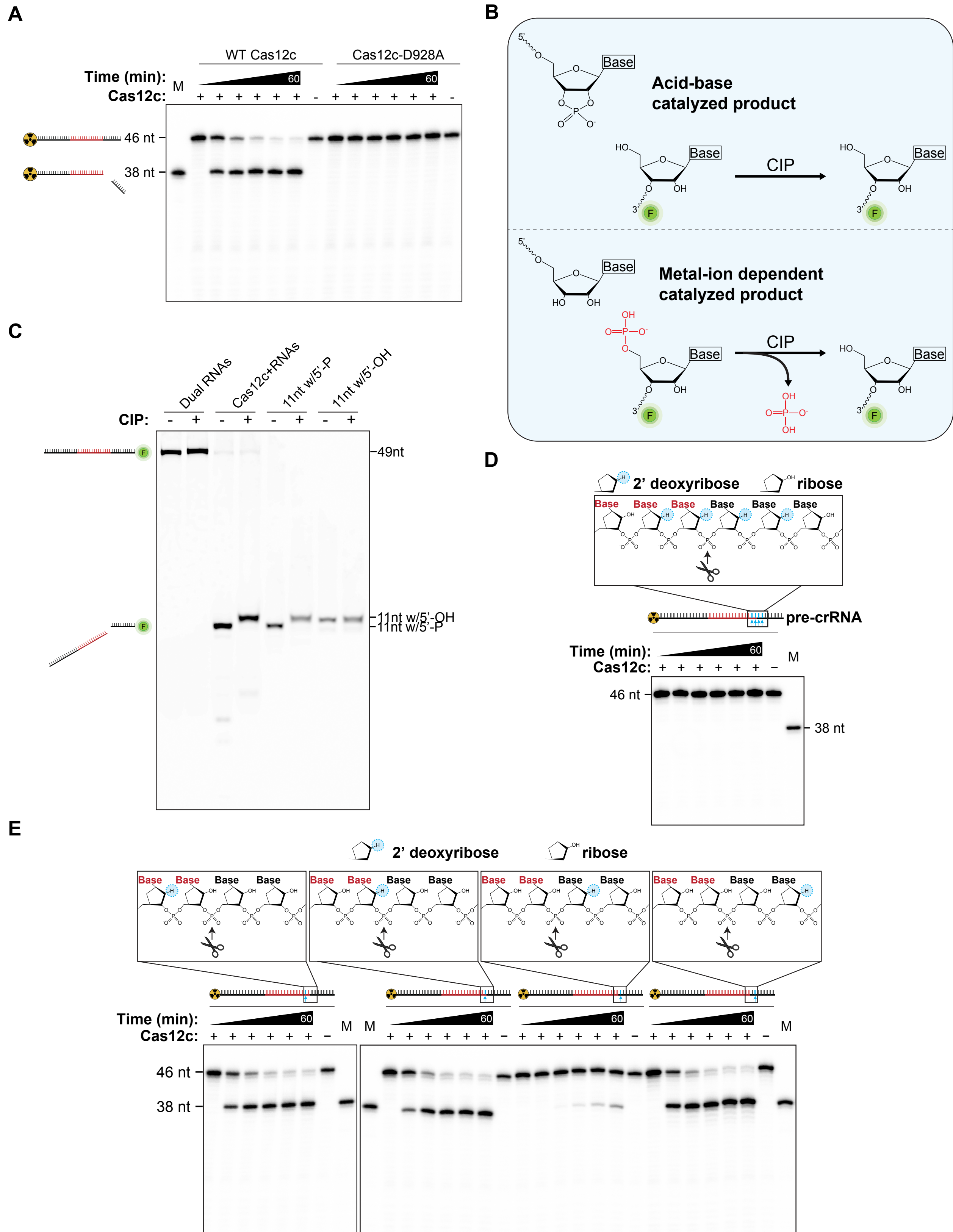



Figure 2.2 The Cas12c RuvC domain processes pre-crRNA

(A) Mutation of a RuvC catalytic residue (D928A) completely inactivated Cas12c's pre-crRNA processing activity.
(B) Schematics of the mature crRNA termini chemistry produced through acid-base catalysis or a metal-ion-dependent mechanism and respective outcomes of calf intestinal alkaline phosphatase (CIP) treatment on the 3' cleavage product.
(C) CIP phosphatase treatment identifies the 3' crRNA cleavage product to possess a 5'-phosphate terminus consistent with metal-ion-dependent cleavage. Customized oligonucleotides of the same 11-nt sequence with a 5'-phosphate or a 5'-hydroxyl terminus served as positive and negative controls, respectively. The pre-crRNA and controls were labeled with a 6-FAM fluorophore at the 3' end.
(D) Cas12c was not able to process a DNA/RNA hybrid pre-crRNA substrate containing four 2'-deoxynucleotides spanning the pre-crRNA processing site. The position corresponding to the scissile phosphate is indicated with a scissors icon.
(E) Pre-crRNA processing assays with modified pre-crRNAs containing a 2'-deoxynucleotide at indicated positions revealed a requirement of a ribose 2'-hydroxyl group in the nucleotide downstream of the scissile phosphate for pre-crRNA cleavage.

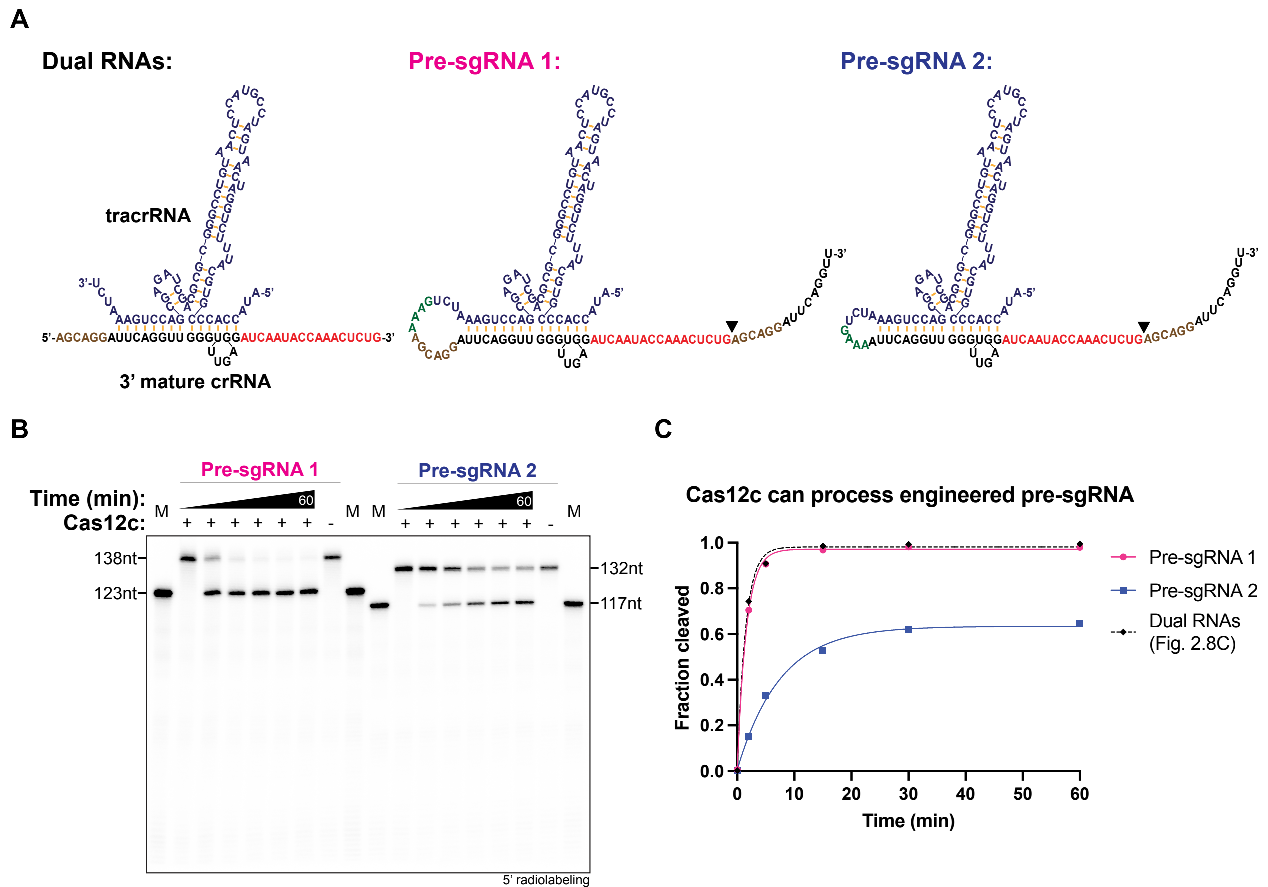
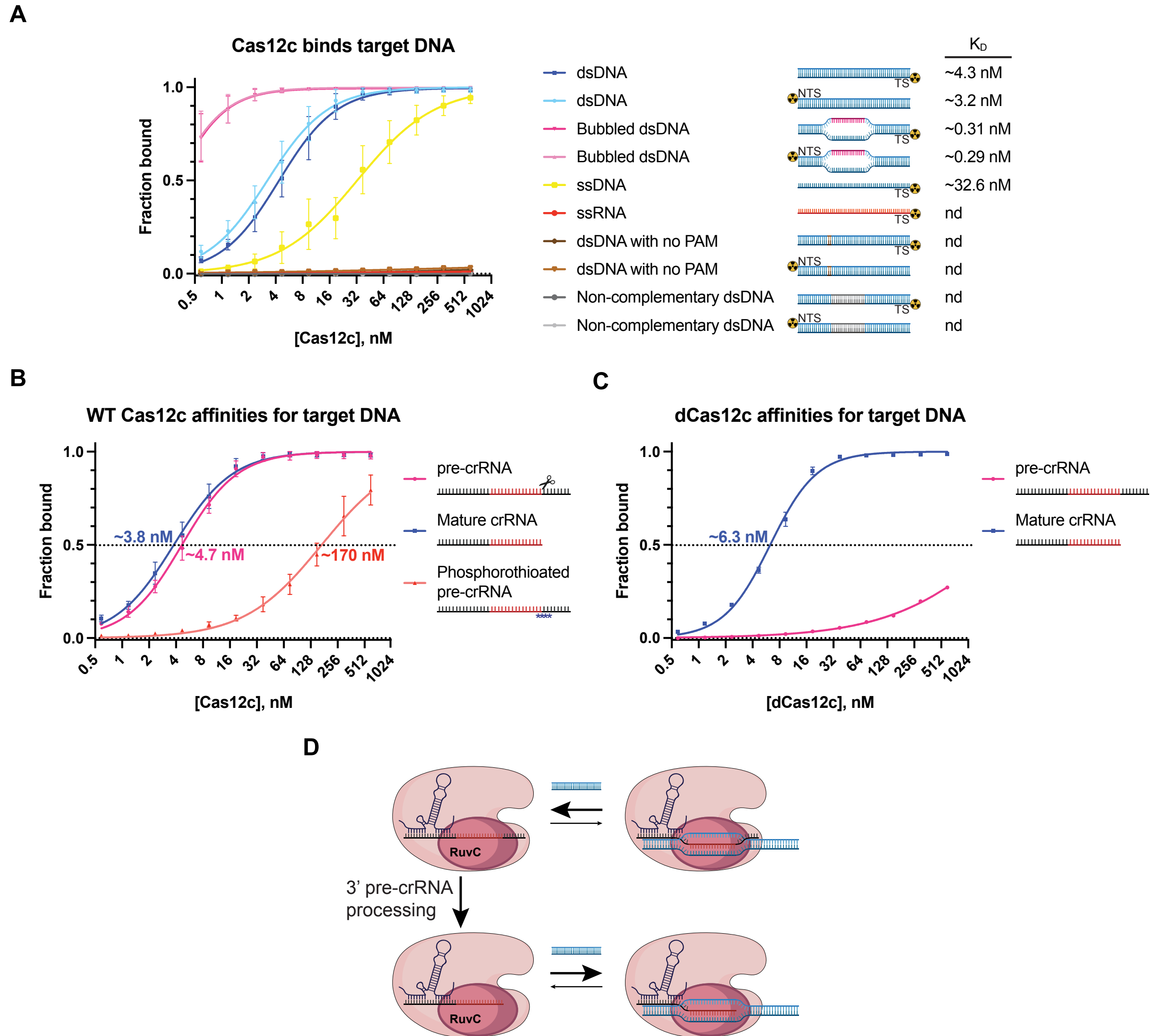



**Figure 2.3 Design and function of pre-sgRNAs for Cas12c**

(A) Schematic representation of dual guide RNA (Yan et al., 2019), pre-sgRNA 1, and pre-sgRNA 2. Spacer sequences are shown in red. The two pre-sgRNA designs differ by the inclusion or exclusion of the six nucleotides (shown in brown) present at the 5' end of the upstream repeat sequence (shown in black). These six nucleotides are processed *in vivo* based on RNA-seq data (Yan et al., 2019). The cleavage sites by Cas12c in pre-sgRNAs are represented by black triangles.
(B) RNA processing assay testing whether Cas12c can process the two different pre-sgRNAs.
(C) Pre-sgRNA cleavage efficiency compared with pre-crRNA cleavage from Figure 2.8C.




## Figure 2.4 Tight RNA-guided DNA binding by Cas12c depends on pre-crRNA processing

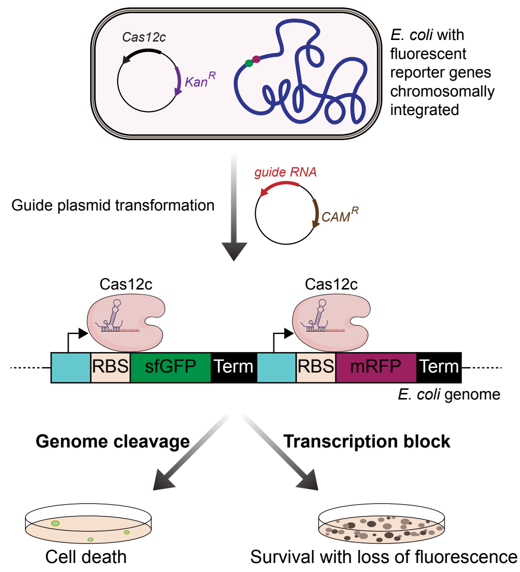
(A) Target DNA binding is sequence-specific and PAM-dependent. Data are from filter-binding assays with radiolabeled dsDNA, partially base-paired (''bubbled'') dsDNA, ssDNA, and ssRNA as a function of Cas12c protein concentration when supplied with a fixed concentration of dual RNAs. All filter-binding assay data in Figure 2.4 are represented as mean ± SD (n = 3) with estimated $K_D$ values reported as shown. nd, not determined.

(B) The dependence of dsDNA binding by Cas12c on pre-crRNA processing. Data are from filter-binding assays with radiolabeled dsDNA as a function of WT Cas12c protein concentration in the presence of tracrRNA and pre-crRNA, mature crRNA, or phosphorothioated pre-crRNA.

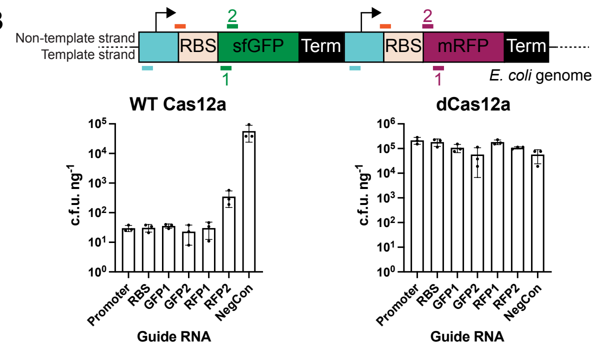
(C) Mature crRNA restores the ability of RuvC-deactivated dCas12c (D928A) to bind dsDNA. Data are from filter-binding assays with radiolabeled dsDNA as a function of dCas12c protein concentration in the presence of tracrRNA and either pre-crRNA or mature crRNA.

(D) Proposed model of DNA targeting by Cas12c. Maturation of crRNA is essential for high-affinity RNA-guided target dsDNA binding.

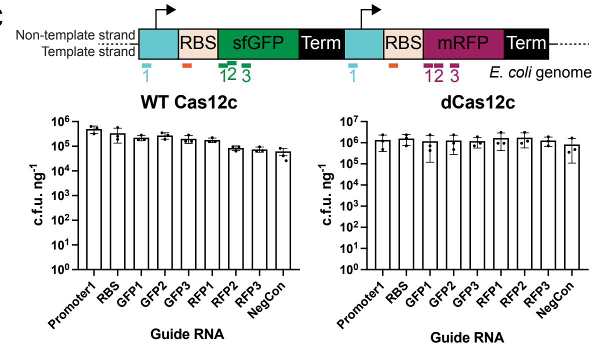
A



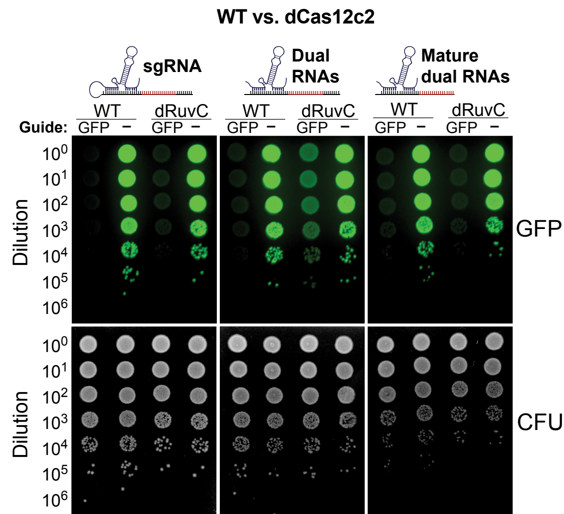

B



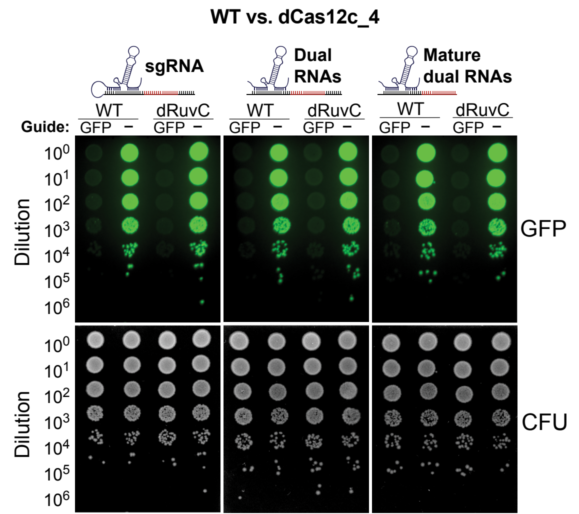

C



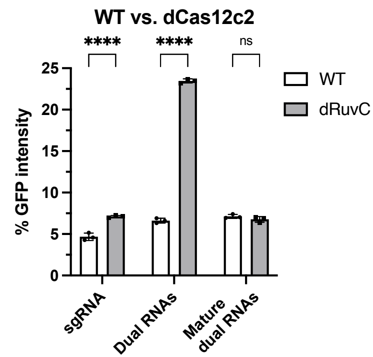

D



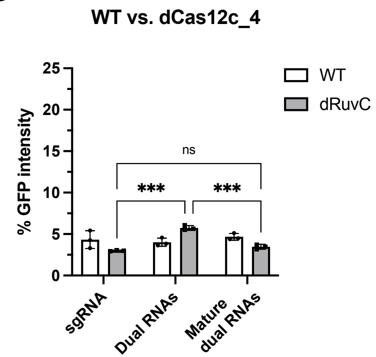

F




E




G




H

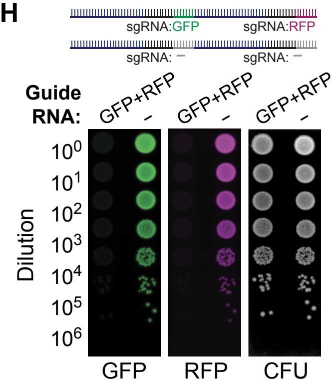


**Figure 2.5 Cas12c blocks gene expression *in vivo* without target-activated DNase activity**

(A) Graphical representation of the dual-color fluorescence interference assay used to distinguish target DNA binding from target DNA cleavage *in vivo*.
(B) Control fluorescence interference experiments with Cas12a. WT Cas12a with targeting crRNAs killed cells, while dCas12a with targeting crRNAs silenced gene expression (see Figure 2.13A) with no effect on survival, as compared with non-targeting negative controls (NegCon). The DNA regions targeted by different spacers (colored lines) used to repress the fluorescent reporters are indicated. c.f.u. ng-1, colony forming unit per nanogram of guide plasmids used in transformation. Data are represented as mean ± SD (n = 3).
(C) Fluorescence interference experiment with Cas12c. Both WT and dCas12c with targeting sgRNAs silenced gene expression (Figure 2.13B) without killing the cells, as compared with non-targeting controls (NegCon). Data are represented as mean ± SD (n = 3).
(D) Images of the fluorescence interference experiment with WT Cas12c2 (a variant described in the study conducted by Yan et al., 2019) and dCas12c2 when GFP-targeting or non-targeting (–) sgRNA, dual RNAs, or mature dual RNAs were used. dRuvC, RuvC-deactivated.
(E) Quantification of GFP intensities in targeting guide expressing cells relative to non-targeting guide expressing cells (from Figure 2.5D). Data are represented as mean ± SD (n = 3). Two-way ANOVA with Šídák's multiple comparison test was used for statistical analysis. ****p < 0.0001.
(F) Images of the fluorescence interference experiment with WT Cas12c_4 (the focus of this study) and dCas12c_4, when GFP-targeting or non-targeting (–) sgRNA, dual RNAs, or mature dual RNAs were used.
(G) Quantification of GFP intensities in targeting guide expressing cells relative to non-targeting guide expressing cells (from Figure 2.5F). Data are represented as mean ± SD (n = 3). Two-way ANOVA with Šídák's multiple comparison test was used for statistical analysis. ***p < 0.001.
(H) Cas12c can use sgRNAs targeting GFP and RFP co-expressed in a single transcript for multiplexed targeting. Two non-targeting (–) spacers were in place of the targeting spacers as a negative control. Images shown are representative of the effect seen in replicates (n = 3).

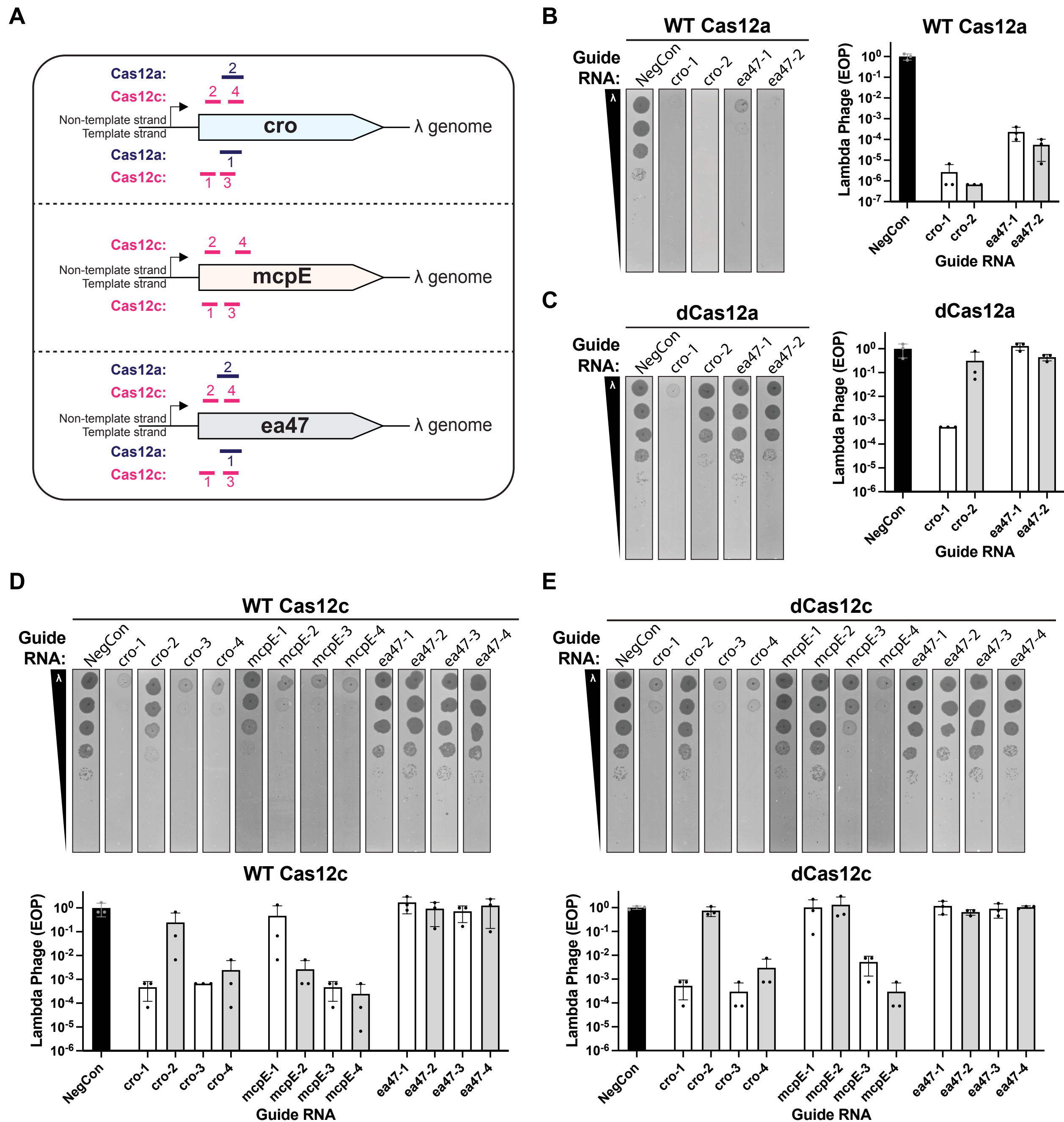



**Figure 2.6 Cas12c protects cells from bacteriophage infection**

(A) Graphic representation of the DNA regions targeted by different spacers (colored lines) used in phage plaque assays to repress the essential-for-virulence *cro*, *mcpE* genes, and the non-essential *ea47* gene in the lambda (λ) phage genome.
(B) 10-fold serial dilutions of phage lambda were spotted on lawns of *E. coli* strains expressing WT Cas12a and a lambda-targeting or a non-targeting guide RNA. Data show that WT Cas12a is capable of restricting phage infection when targeting phage lambda, regardless of target gene essentiality. The efficiency of plaquing (EOP) of lambda phage was calculated as the ratio of plaque forming units (PFUs) on targeting guide expressing

cells divided by the number of PFUs on non-targeting guide (NegCon; shown in black) expressing cells. Guides targeting the template strand are shown in white, and guides targeting the non-template strand are shown in gray.
(C) dCas12a is capable of restricting phage infection when targeting the phage-essential gene *cro*, but not non-essential gene *ea47*. Phage restriction mediated by dCas12a occurs in a strand-specific manner.
(D) WT Cas12c is capable of restricting phage infection when targeting phage-essential genes *cro* and *mcpE*, but not phage-non-essential gene *ea47*. Phage restriction mediated by WT Cas12c occurs with no significant strand bias. Guide RNAs more distal to the start codon appear to yield a stronger impact on phage restriction.
(E) dCas12c is capable of restricting phage infection when targeting phage-essential genes *cro* and *mcpE*, but not phage-non-essential gene *ea47*, suggesting that the RuvC catalytic activity is not essential for phage restriction. Akin to phage restriction mediated by WT Cas12c, phage restriction does not occur with significant strand bias, but guides more distal to the start codon yield stronger impacts. All EOP data in Figure 6 are represented as mean ± SD (n = 3). All phage assays were performed in the absence of an inducer, except for assays targeting the *cro*-1 (D) and *mcpE* (D and E), which were performed with 10 μM IPTG.

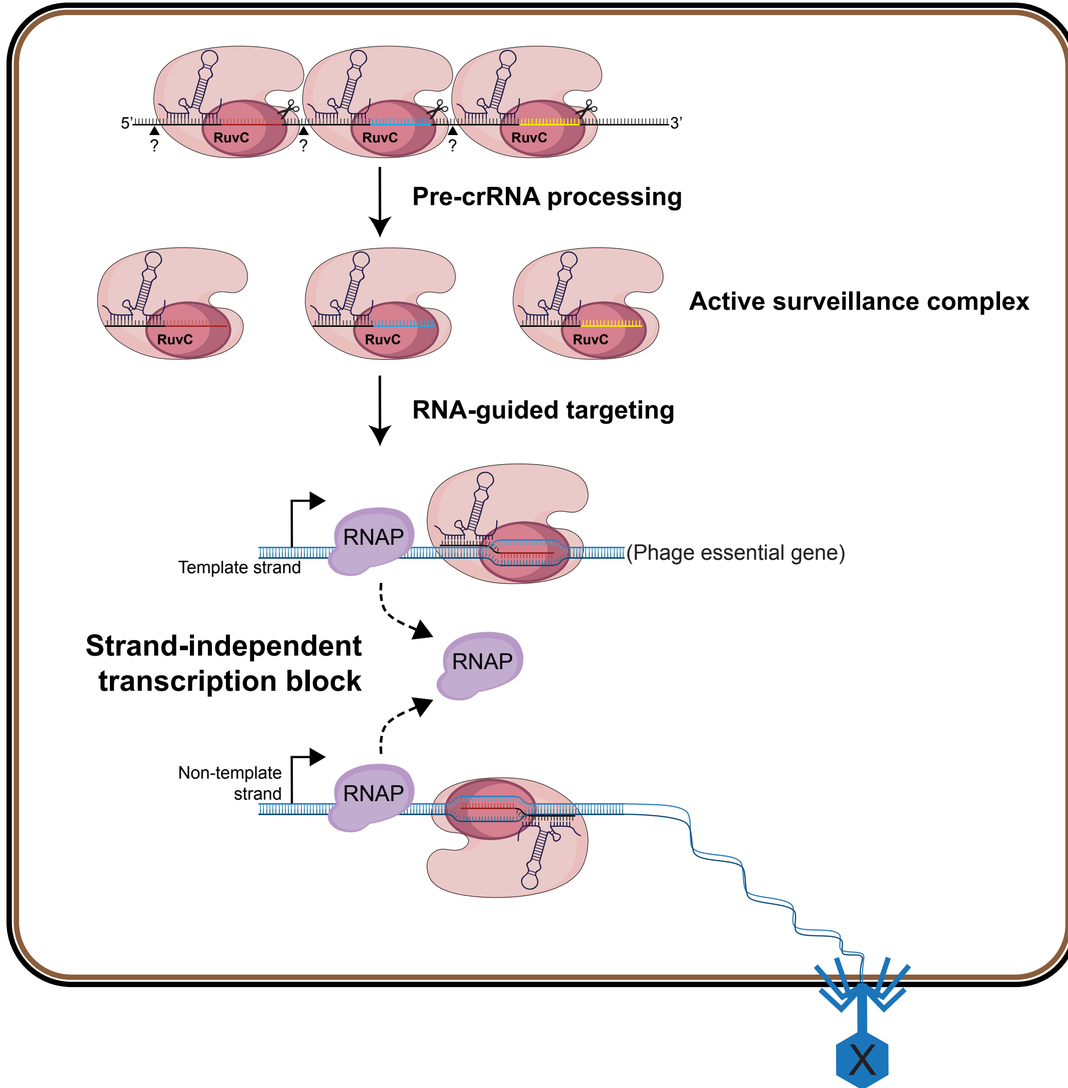
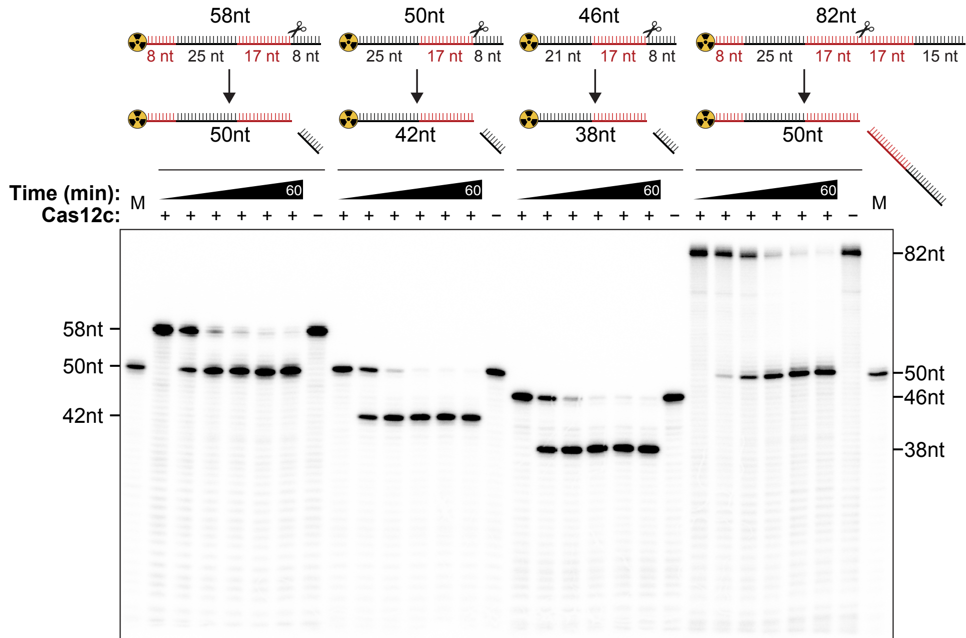
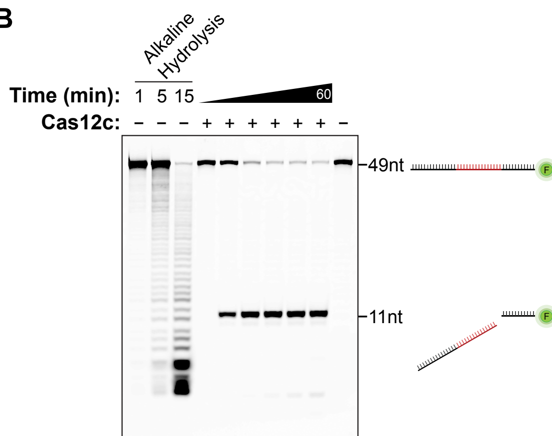
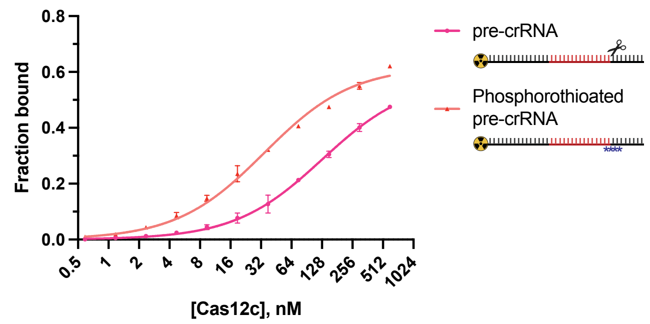



**Figure 2.7 Proposed mechanism of the type V-C CRISPR-Cas12c system**

After the CRISPR array is transcribed into a long precursor transcript, Cas12c, with the help of a tracrRNA, recognizes and binds to pre-crRNA. The long pre-crRNA is processed into smaller fragments by Cas12c and unknown enzymes (question mark), allowing Cas12c to bind target DNA molecules by base-pairing between the crRNA and the DNA target. Targeting either strand of the coding dsDNA (and promoter region) represses target gene expression by transcription block without target-activated DNase activity. Binding alone is sufficient to confer immunity against certain DNA populations, such as essential genes in phages, and this may be Cas12c's native immune mechanism.


A
58nt
8 nt 25 nt 17 nt 8 nt
50nt
50nt
25 nt 17 nt 8 nt
42nt
46nt
21 nt 17 nt 8 nt
38nt
82nt
8 nt 25 nt 17 nt 17 nt 15 nt
50nt
Time (min):
M
60
60
60
60
M
Cas12c:
58nt
50nt
42nt
82nt
50nt
46nt
38nt


B
Alkaline Hydrolysis
Time (min): 1 5 15
60
Cas12c:
49nt
11nt


D
Cas12c can bind phosphorothioated pre-crRNA
Fraction bound
0.8
0.6
0.4
0.2
0.0
0.5 1 2 4 8 16 32 64 128 256 512 1024
[Cas12c], nM
pre-crRNA
Phosphorothioated pre-crRNA

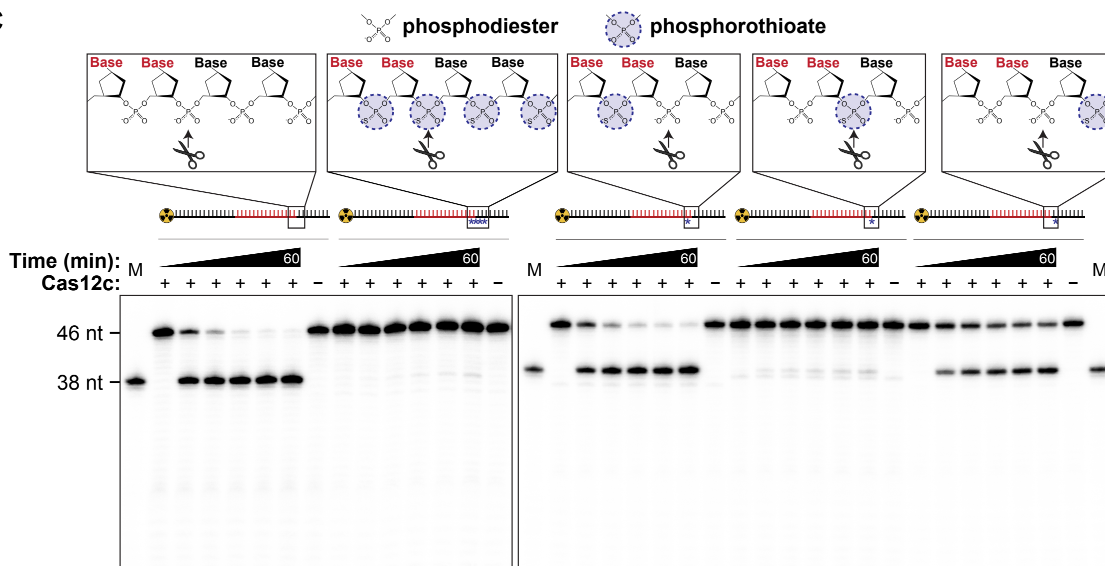

C
phosphodiester
phosphorothioate
Base
Time (min):
M
60
Cas12c:
46 nt
38 nt

**Figure 2.8 Cas12c pre-crRNA processing is precise and occurs 3' to the spacer, Related to Figure 2.1**

(A) Time courses of pre-crRNA processing by Cas12c when pre-crRNA has a 5' extension (which was tested in Harrington et al., 2020), the original length of 5' (upstream) protein-bound repeat (25 nt), a more processed version of the 5' repeat sequence, or two spacers in between the upstream and downstream repeat sequences. Repeat sequences are shown in black, and spacer sequences are shown in red. M = Marker. The results confirmed that Cas12c processes pre-crRNA at the expected position, that is 17 nt away from the upstream protein-bound repeat, regardless of the 3' (downstream) sequence.
(B) Time course of pre-crRNA processing by Cas12c when pre-crRNA was labeled with a 6-FAM fluorophore at the 3' end to further determine whether Cas12c severs the RNA at one site or more than one site. The result showed that Cas12c-mediated cleavage occurred at the position expected from the 5'-labeled experiments, indicating that Cas12c makes a single precise endonucleolytic cut between the spacer and the repeat. Figure 2.2C confirms that the product here is exactly 11 nt.
(C) Phosphorothioate inhibition of pre-crRNA processing to test the precision and rigidity of Cas12c-catalyzed RNA cleavage. Different variants of pre-crRNAs are shown, with purple circles indicating phosphorothioate linkages. The position corresponding to the scissile phosphate is indicated with a pair of scissors. M = Marker. A single phosphorothioate modification at the scissile phosphate group almost completely abolished Cas12c processing activity, and the effect was slightly stronger when the flanking linkages were simultaneously phosphorothioated. Note that none of the phosphorothioate modifications caused a shift in the position of cleavage, suggesting that Cas12c processes pre-crRNA using a strict and precise mechanism. Modification of the linkage 3' to the scissile phosphate reduced the extent of processing by ~40% at 60 mins, suggesting that one of the two non-bridging oxygens may be important for catalysis.
(D) Phosphorothioate inhibition of pre-crRNA processing was not due to defects in binding. Data are from filter-binding assays with radiolabeled unmodified or phosphorothioated pre-crRNA as a function of Cas12c protein concentration when supplied with a constant concentration of tracrRNA. Values represent mean ± SD (n = 2).

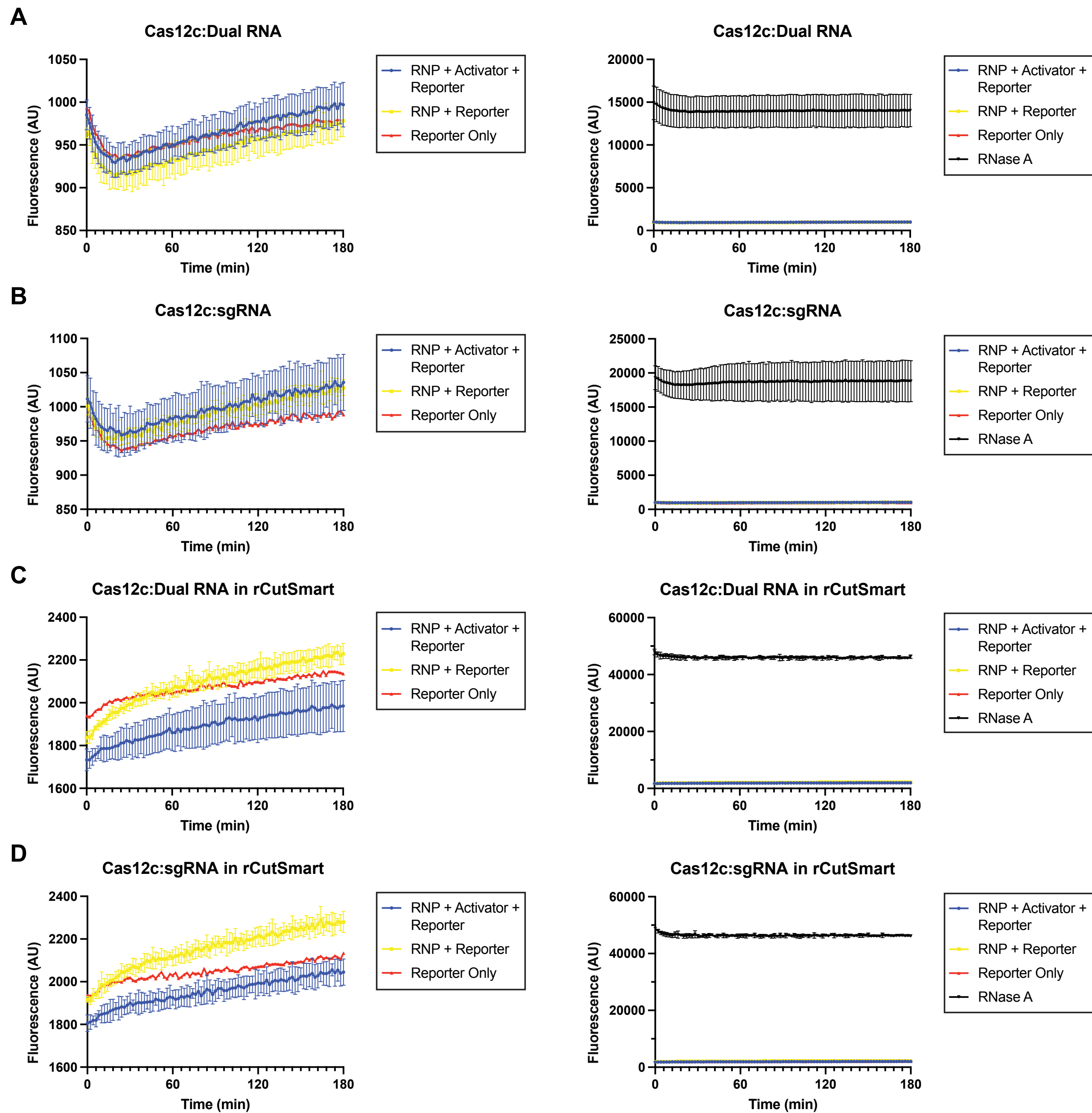



**Figure 2.9 Cas12c has no detectable target-activated *trans*-cleavage activity on RNA, Related to Figure 2.1**

Time courses of RNaseAlert substrate cleavage by Cas12c preassembled with dual RNA or sgRNA (described in Figure 2.3) and activated by target dsDNA, with fluorescence measurements taken every 2 min for 180 min at 37°C. The same data sets are presented without (left panels) or with (right panels) corresponding positive controls (RNase A). Reactions in (A) and (B) were carried out in the same cleavage buffer for pre-crRNA processing. Reactions in (C) and (D) were carried out in rCutSmart buffer, a buffer that a Cas12c ortholog (Cas12c1) was reported to be active in when measuring *trans*-cleavage activities (Wang and Zhong, 2021). Data are represented as mean ± SD (n = 3).

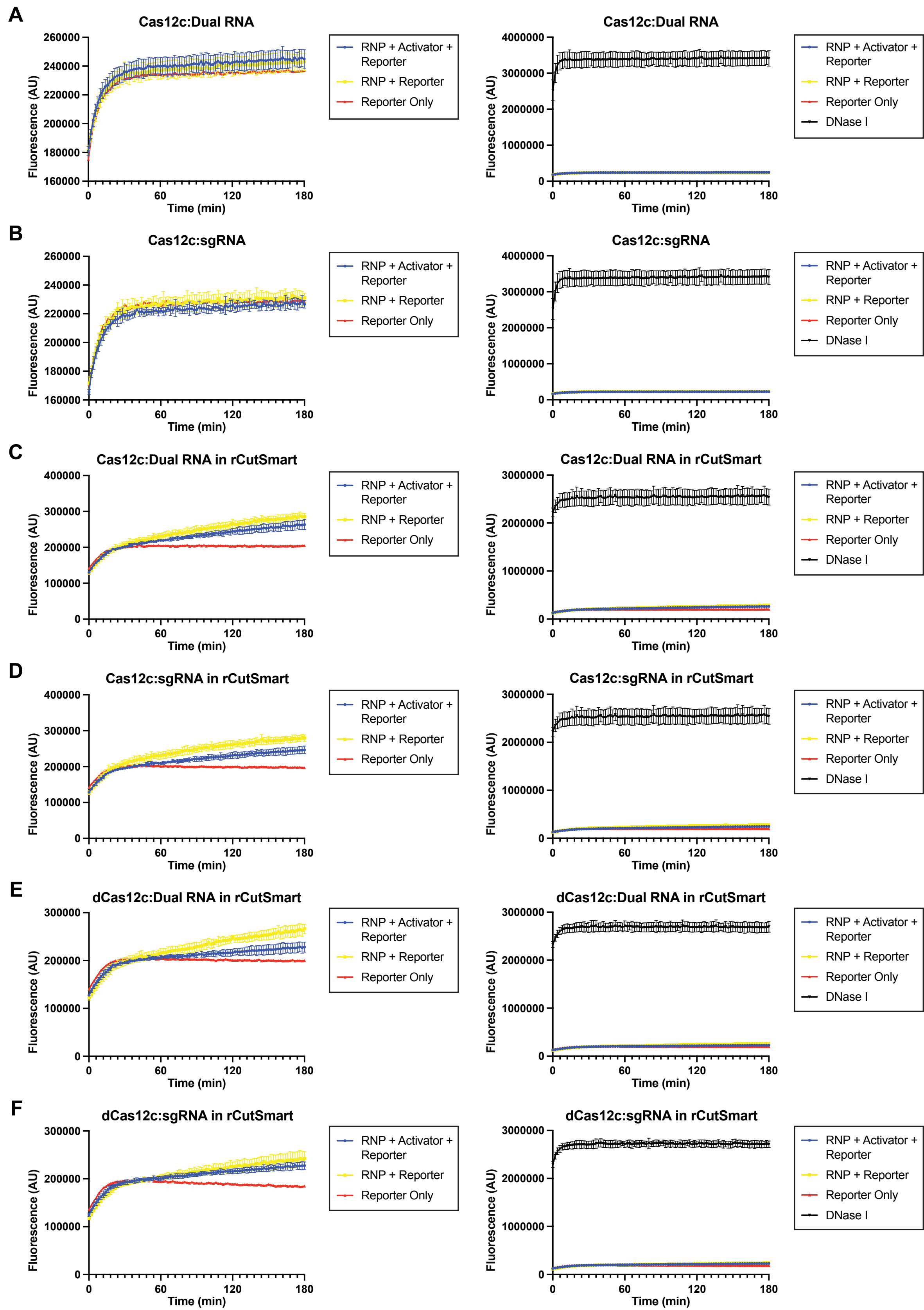

A
Cas12c:Dual RNA
B
Cas12c:sgRNA
C
Cas12c:Dual RNA in rCutSmart
D
Cas12c:sgRNA in rCutSmart
E
dCas12c:Dual RNA in rCutSmart
F
dCas12c:sgRNA in rCutSmart
Fluorescence (AU)
Time (min)
RNP + Activator + Reporter
RNP + Reporter
Reporter Only
DNase I

**Figure 2.10 The RuvC of Cas12c has no detectable target-activated *trans*-cleavage activity on DNA, Related to Figure 2.1**

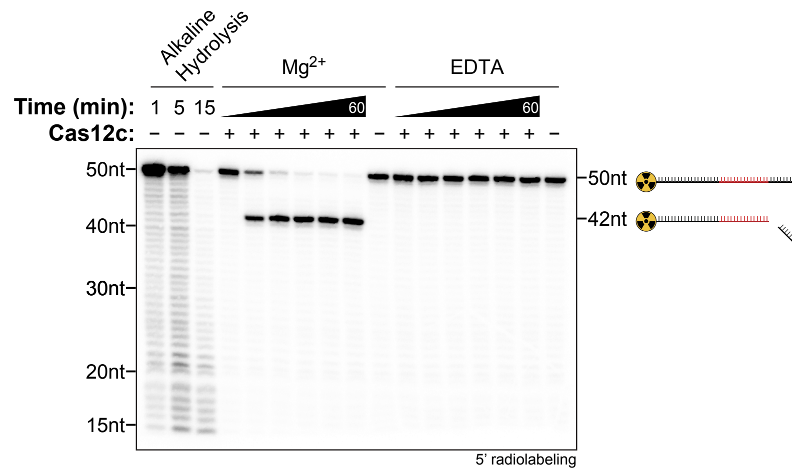
Time courses of DNaseAlert substrate cleavage by Cas12c (A-D) or RuvC-deactivated dCas12c-D928A (E&F) preassembled with dual RNA or sgRNA and activated by target dsDNA, with fluorescence measurements taken every 2 min for 180 min at 37°C. The same data sets are presented without (left panels) or with (right panels) corresponding positive controls (DNase I). Reactions in (A) and (B) were carried out in the same cleavage buffer for pre-crRNA processing and used the same positive control. Reactions in (C) and (D) were carried out in rCutSmart buffer and used the same positive control. In conditions with rCutSmart buffer, there was a small increase in fluorescence that was non-target-activated (C&D) and independent of the catalytic activity of the RuvC domain (E&F). Data are represented as mean ± SD (n = 3).

```
Cas12c_4    1 MTKHSIPLHAFRNSGADARKWKGRIALLAKRGKE..................TMRTLQFPLEMSEPEAAAI.NTTPFAVAYNAIEGTGKG
Cas12c1     1 MQTKKTHLHLI..SAKASRKYRRTIACLSDTAKKDLERRKQSGAADPAQELSCLKTIKFKLEV..PEGSKLPSFDRISQIYNALETIEKG

Cas12c_4   72 TLFDYWAKLHLAGFRFFPSGGAATIFRQQAVFEDASWNAAFCQQSGKDWPWLVPSKLYERFTKAPREVAKKDGSKKSIEFTQENVANESH
Cas12c1    87 SLSYLLFALILSGFRIFPNSSAAKTFASSSCYKNDQFASQIKEIFGEMVKNFIPSEL........ESILKKGRRKNNKDWTEENIKRVLN

Cas12c_4  162 VSLVGASITDKTPEDQKEFFLKMAGALAEKFDSWKSANEDRIVAMKVIDEFLKSEGLHLPSLENIAVKCSVETKPDNATVAW.HDAPMS.
Cas12c1   169 .SEFGRKNSEGSSALFDSFLSKFSQELFRKFDSWNEVNKKYLEAAELLDSMLASYGPFDSVCKMIGDSDSRNSLPDKSTIAFTNNAEITV

Cas12c_4  250 GVQNLAIGVFATCASRIDNIYDLNGGKLSKL..IQESATTPNVTALSWLFGKGLEYFR.........TTDIDTIMQD.FNIPASAKESIK
Cas12c1   258 DIESSVMPYMAIAA..LLREYRQSKSKAAPVAYVQSHLTTTNGNGLSWFFKFGLDLIRKAPVSSKQSTSDGSKSLQELFSVPDDKLDGLK

Cas12c_4  328 PLVESAQAIPTMTVL.GKK....NYAPFRPNFGGKIDSWIANYASRLMLLNDILEQIEPGFELPQALLD.NETLMSGIDMTGDELKELIE
Cas12c1   346 FIKEACEALPEASLLCGEKGELLGYQDFRTSFAGHIDSWVANYVNRLFELIELVNQLPESIKLPSILTQKNHNLVASLGLQEAEVSHSLE

Cas12c_4  412 AVYAWVDAAKQGLATLLGRGGNVDDAVQTFEQFSAMMDTLNGTLNTISARYVRAVEMAGKDEARLEKLIECKFDIPKW..CKSVPKLVGI
Cas12c1   436 LFEGLVKNVRQTLKKLAGIDISSSPNEQDIKEFYAFSDVLN.RLGSIRNQIENAVQTAKKDKIDLESAIEWK....EWKKLKKLPKLNGL

Cas12c_4  500 SGGLPKVEEEIKVMNAAFMDVRARMFVRFEEIAAYVASKGAGMDVYDALEKRELEQLKKLKSAVPERAHIQAYRAVLHRIGRAVQNCSEK
Cas12c1   521 GGGVPKQQELLDKALESVKQIRHYQRIDFERVIQWAVNEHCLETVPKFLVDAEKKKINK.ESSTDFAAKENAVRFLLEGIGAAARGKTDS

Cas12c_4  590 .TKQLFSSKVIEMGVFKNPSHLNNFIFNQKGAIYRSPFDRSRHAPYQLHADKLLKNDWMELLAEISTTLMASESTE.........QMEDA
Cas12c1   610 VSKAAYNWFVVNN..FLAKKDLNRYFINCQGCIYKPPYSKRRSLAFALRSD...NKDTIEVVWEKFETFYKEISKEIEKFNIFSQEFQTF

Cas12c_4  670 LRLERTRLQLQLSGLPDWEYPASLAKPDIEVEIQTALKMQLA....KDTVTSDVLQRTFNLYSSVLSGLTFKLLRRSFS.LKMRFSVADT
Cas12c1   695 LHLENLRMKLLLRRIQK.PIPAEIAFFSLPQEYYDSLPPNVAFLALNQEITPSEYITQFNLYSSFLNG.NLILLRRSRSYLRAKFSWVGN

Cas12c_4  755 TQLIYVPKDCD.WAIPKQYLQA.EGEIGIAARVVTESS.....PAKMVTEVEMKEP..KALGHFMQQAPHDWY....FDASLGGTQVAGR
Cas12c1   783 SKLIYAAKEARLWKIPNAYWKSDEWKMILDSNVLVFDKAGNVLPAPTLKKVCEREGDLRLFYPLLRQLPHDWCYRNPFVKSVGREK...N

Cas12c_4  832 IVEKGKEVGKER...KLVG..YRMRGNSAYKTVLDKSLVG..NTELSQCSMIIEIPYTQTVDADFRAQVQAGLPK..VSINLPVKETITA
Cas12c1   870 VIEVNKE.GEPKVASALPGSLFRLIGPAPFKSLLDDCFFNPLDKDLRECMLIVDQEISQKVEAQ...KVEASLESCTYSIAVPIRYHLEE

Cas12c_4  913 SNKDEQMLFDRFVAIDLGERGLGYAVFDAKTLELQES.....GHRPIKAITNLLNRTHHYEQRPNQRQKFQAKFNVNLSELRENTVGDVC
Cas12c1   956 PKVSNQ..FENVLAIDQGEAGLAYAVFSLKSIGEAETKPIAVGTIRIPSIRRLIHSVSTYRKKQRLQNFKQNYDSTAFIMRENVTGDVC

Cas12c_4  998 HQINRICAYYNAFPVLEYMVP..DRLDKQLKSVYESVTNRYIWSSTDAHKSARVQFWLGGETW.....EHPYLKSAKDKK.......PLV
Cas12c1  1044 AKIVGLMKEFNAFPVLEYDVKNLESGSRQLSAVYKAVNSHFLYFKEPGRDALRKQLWYGGDSWTIDGIEIVTRERKEDGKEGVEKIVPLK

Cas12c_4 1074 LSPGRGASGKGTSQTCSCCGRNPFD.LIKDMKPRA.KIAVVDGKAKLENSE..LKLFERNRESKDDMLARRHRNERAGMEQPLTPGNYTV
Cas12c1  1134 VFPGRSVSARFTSKTCSCCGRNVFDWLFTEKKAKTNKKFNVNSKGELTTADGVIQLFEADRSKGPKFYAR..RKERTPLTKPIAKGSYSL

Cas12c_4 1160 DEIKALLRANLRRAPKNRRTKDTTVSEYHCVFSDCG...KTMHADENAAVNIGGKFIADIEK...................
Cas12c1  1222 EEIERRVRTNLRRAPKSKQSRDTSQSQYFCVYKDCALHFSGMQADENAAINIGRRFLTALRKNRRSDFPSNVKISDRLLDN
```

**Figure 2.11 Sequence alignment of two Cas12c proteins, Related to Figure 2.1**

Global amino acid sequence alignment between Cas12c_4 (the focus of this study) and Cas12c1, which exhibits *trans*-cleavage activity on DNA (Wang and Zhong, 2021). These two orthologs share 28.2% sequence identity (highlighted in black) with 47.2% similarity (boxes around residues).

A




B

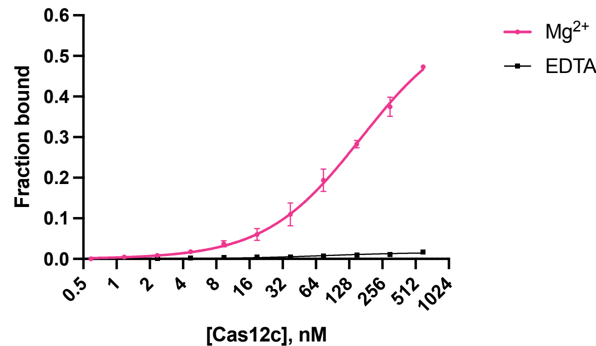
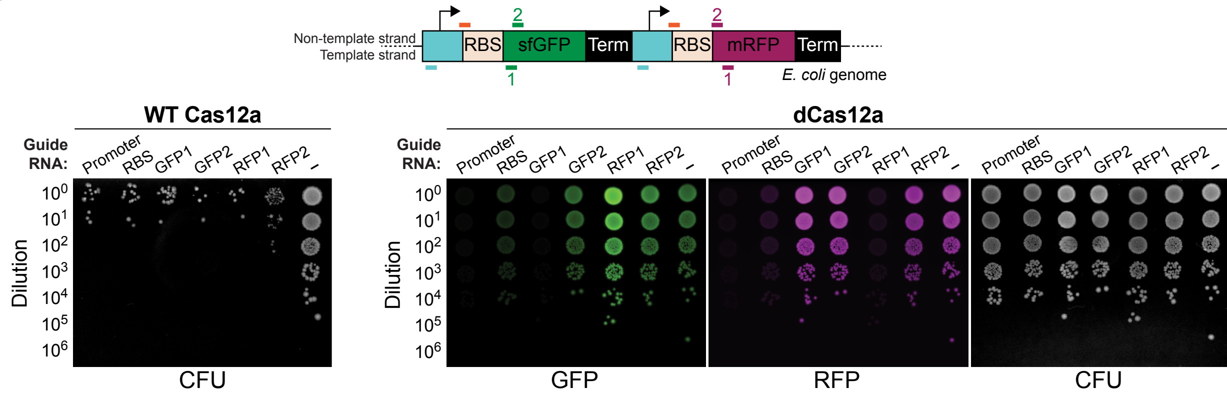


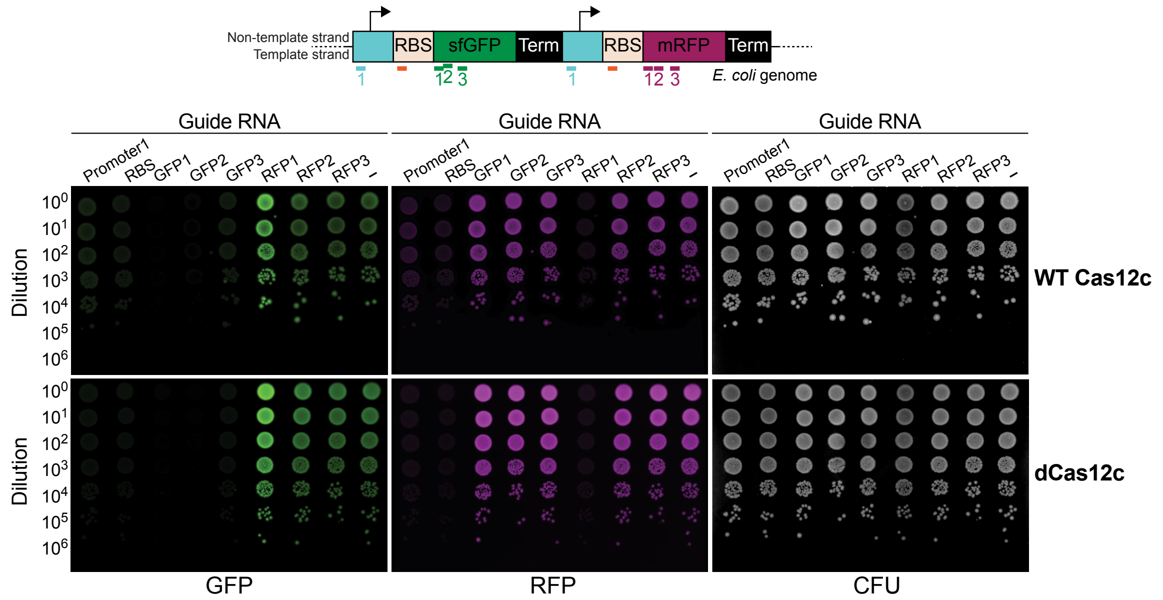

**Figure 2.12 Dependence of pre-crRNA processing and binding on divalent metal ion, Related to Figure 2.2**

(A) EDTA inhibits pre-crRNA processing. Data are from a time-course experiment testing the RNA processing activity of Cas12c in the presence of $Mg^{2+}$ or EDTA.
(B) EDTA inhibits pre-crRNA binding (binding is metal-ion dependent). Data are from filter-binding assays with radiolabeled pre-crRNA as a function of Cas12c protein concentration in the presence of $Mg^{2+}$ or EDTA, when supplied with a constant concentration of tracrRNA. Values represent mean ± SD (n = 2).

**A**




**B**




**C**

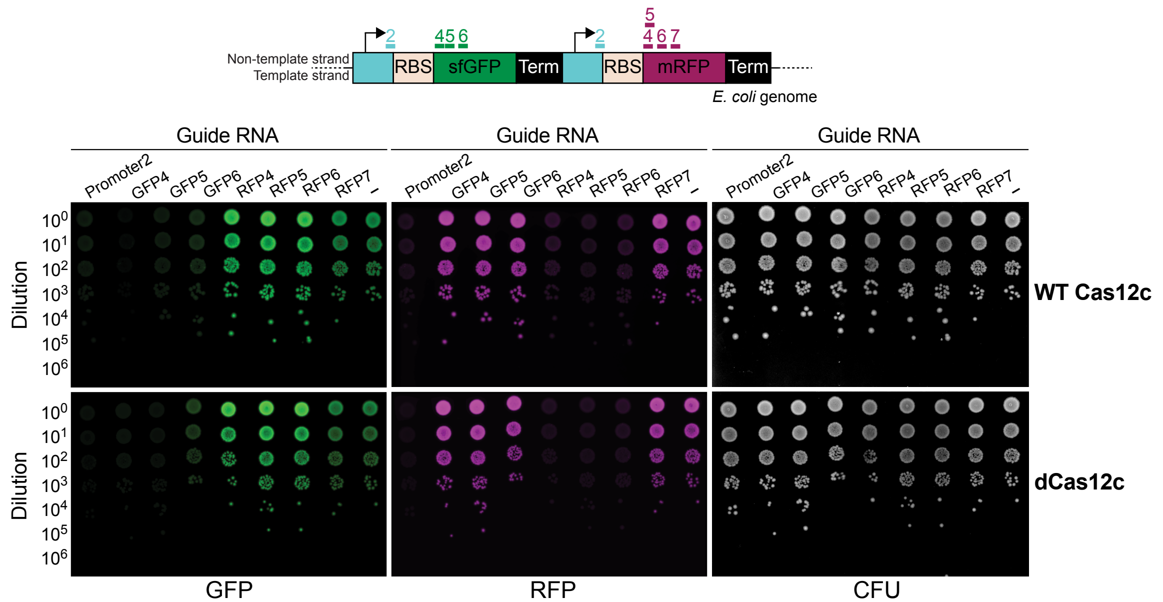


**Figure 2.13 Cas12c blocks gene expression *in vivo* without target-activated DNase activity in a strand-independent manner, Related to Figure 2.5**

(A) Images of fluorescence interference experiments of Cas12a. The DNA regions targeted by different spacers (colored lines) used to repress the fluorescent reporters are indicated. WT Cas12a with targeting crRNA killed cells, while dCas12a with targeting crRNA had no effect in survival but silenced gene expression, as compared with non-targeting (–) controls. The effect of CRISPRi with a dCas12a exhibits strand bias when blocking transcription elongation: dCas12a represses gene expression when it is targeted to the template strand (GFP1 and RFP1) but fails to do so when it is targeted to the non-template strand (GFP2 and RFP2) of the targeted coding DNA sequences.
(B) Images of fluorescence interference experiments with Cas12c targeting the template strand of the reporter genes. Both WT Cas12c and dCas12c with selected targeting sgRNAs silenced GFP and/or RFP expression without killing the cells, as compared with non-targeting (–) controls.
(C) Images of fluorescence interference experiments with Cas12c targeting the non-template strand of the reporter genes. Both WT and dCas12c with selected targeting sgRNAs silenced GFP and/or RFP expression without killing the cells, as compared with non-targeting (–) controls. All images shown in Figure S6 are representative of the effect seen in replicates (n = 3).

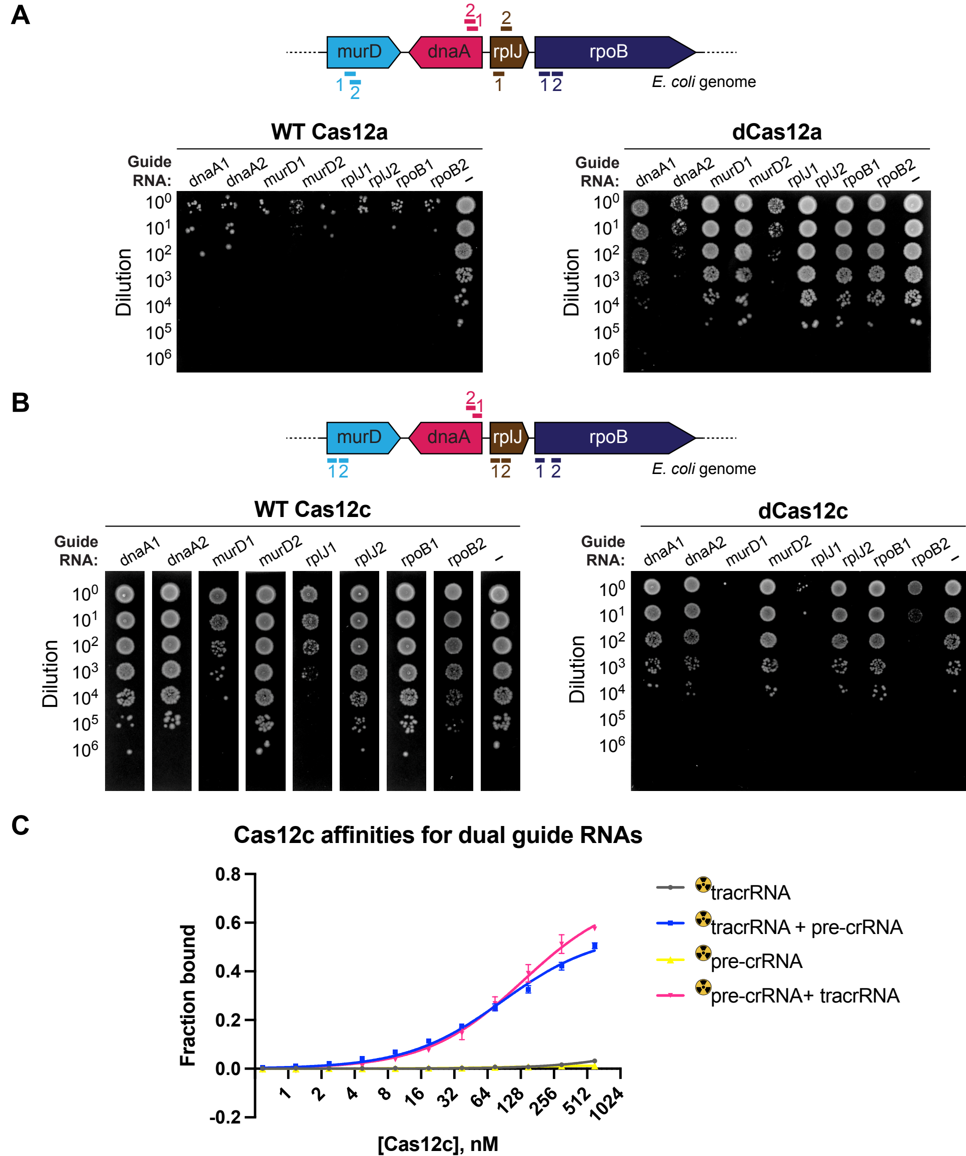



**Figure 2.14 Effect of Cas12c-induced repression of essential gene expression in E. coli and the requirement of both tracrRNA and crRNA for ribonucleoprotein assembly, Related to Figure 2.5**

(A) Interference assay to test whether Cas12a with crRNAs targeting essential host gene(s) can cause cell death. Data suggest that dCas12a with selected crRNAs (dnaA1, dnaA2, and rplJ1) could result in depletion of cells containing those guides, as compared with non-targeting (–) controls. WT Cas12a was included for comparison.
(B) Interference assay to test whether targeting WT or dCas12c to essential host genes can cause cell death. Data showed that both WT and dCas12c with selected crRNAs (murD1, rplJ1, and rpoB2) can cause cell death or slower growth, resulting in depletion

of cells containing those guides, as compared with non-targeting (–) controls. All images shown in Figure 2.14A&B are representative of the effect seen in replicates (n = 3).
(C) Cas12c binds to the complex from tracrRNA and crRNA. Data are from filter-binding assays with radiolabeled tracrRNA or crRNA as a function of Cas12c protein concentration when they were alone or when two RNAs were present in the binding reaction. Values represent mean ± SD (n = 2).

| Table 2.1A: Plasmids containing Cas protein | | | |
|---|---|---|---|
| **Plasmid ID** | **Description** | **Selection marker** | **Addgene ID** |
| pCJH002 | Bacterial protein expression plasmid of wild-type Cas12c_4. This is a R965H version of the Cas12c in Harrington et al., 2020. | Ampicillin | 183069 |
| pCJH003 | Bacterial protein expression plasmid of RuvC-deactivated Cas12c_4 (D928A version of pCJH002) | Ampicillin | - |
| pCJH019 | Wild-type Cas12c_4 in a plasmid containing a SC101 origin and *lac* promoter for *in vivo* interference assay | Kanamycin | 183071 |
| pCJH020 | RuvC-deactivated Cas12c_4 (D928A) in a plasmid containing a SC101 origin and *lac* promoter for *in vivo* interference assay | Kanamycin | - |
| pCJH021 | Wild-type Cas12c2 (Yan et al., 2019) in a plasmid containing a SC101 origin and *lac* promoter for *in vivo* interference assay | Kanamycin | 183072 |
| pCJH022 | RuvC-deactivated Cas12c2 (D928A) (Yan et al., 2019) in a plasmid containing a SC101 origin and *lac* promoter for *in vivo* interference assay | Kanamycin | - |
| pCJH027 | Wild-type LbCas12a (Chen et al., 2018) in a plasmid containing a SC101 origin and lac promoter for *in vivo* interference assay | Kanamycin | 183074 |
| pCJH028 | RuvC-deactivated LbCas12a (D832A) (Chen et al., 2018) in a plasmid containing a SC101 origin and lac promoter for *in vivo* interference assay | Kanamycin | - |

| Table 2.1B: Guide RNA plasmids for *in vivo* assays | | | | |
|---|---|---|---|---|
| **Plasmid ID** | **Description** | **Selection marker** | **Addgene ID** | **Spacer sequence** |
| pCJH007 | Plasmid expressing Cas12c sgRNA targeting the promoter of integrated GFP/RFP (1) | Chloramph enicol | - | ACAGCTAGCTCAGTCCT |
| pCJH008 | Plasmid expressing Cas12c sgRNA targeting the RBS sequence of integrated GFP/RFP | Chloramph enicol | - | AATTCATTAAAGAGGAG |
| pCJH009 | Plasmid expressing Cas12c sgRNA targeting GFP (1) | Chloramph enicol | 183070 | TCCCAATTCTTGTTGAA |
| pCJH010 | Plasmid expressing Cas12c sgRNA targeting GFP (2) | Chloramph enicol | - | AATTAGATGGTGATGTT |
| pCJH011 | Plasmid expressing Cas12c sgRNA targeting GFP (3) | Chloramph enicol | - | CACTACTGGAAAACTAC |
| pCJH012 | Plasmid expressing Cas12c sgRNA targeting RFP (1) | Chloramph enicol | - | GCGAGTAGCGAAGACGT |
| pCJH013 | Plasmid expressing Cas12c sgRNA targeting RFP (2) | Chloramph enicol | - | cgtttcaaagttcgtat |
| pCJH014 | Plasmid expressing Cas12c sgRNA targeting RFP (3) | Chloramph enicol | - | ggacatcctgtccccgc |
| pCJH015 | Plasmid expressing a Cas12c non-targeting sgRNA | Chloramph enicol | - | ttttttgtacttggtac |

| | | | | |
|---|---|---|---|---|
| pCJH026 | Plasmid expressing a Cas12c sgRNA targeting GFP and another sgRNA targeting RFP in one transcript for testing multiplexed repression | Chloramphenicol | 183073 | TCCCAATTCTTGTTGAA; GCGAGTAGCGAAGACGT |
| pCJH029 | Plasmid expressing Cas12a guide RNA targeting the promoter of integrated GFP/RFP | Chloramphenicol | - | ACAGCTAGCTCAGTCCTAGGTATAATAG |
| pCJH030 | Plasmid expressing Cas12a guide RNA targeting the RBS sequence of integrated GFP/RFP (non-template strand) | Chloramphenicol | - | ATGAATTCAGATCTATTATACCTAGGAC |
| pCJH031 | Plasmid expressing Cas12a guide RNA targeting GFP (1) | Chloramphenicol | 183075 | ACTGGAGTTGTCCCAATTCTTGTTGAAT |
| pCJH032 | Plasmid expressing Cas12a guide RNA targeting GFP (2) (non-template strand) | Chloramphenicol | - | TGCCCATTAACATCACCATCTAATTCAA |
| pCJH033 | Plasmid expressing Cas12a guide RNA targeting RFP (1) | Chloramphenicol | - | aaagttcgtatggaaggttccgttaacg |
| pCJH034 | Plasmid expressing Cas12a guide RNA targeting RFP (2) (non-template strand) | Chloramphenicol | - | ATAACGTCTTCGCTACTCGCCATGGTAC |
| pCJH035 | Plasmid expressing a Cas12a non-targeting guide RNA | Chloramphenicol | - | ttttttgtacttggtacagcaggattca |
| pCJH037 | Plasmid expressing Cas12c sgRNA targeting dnaA (1) | Chloramphenicol | - | TCACTTTCGCTTTGGCA |
| pCJH038 | Plasmid expressing Cas12c sgRNA targeting dnaA (2) | Chloramphenicol | - | GCAGCAGTGTCTTGCCC |
| pCJH039 | Plasmid expressing Cas12c sgRNA targeting murD (1) | Chloramphenicol | - | GCTGATTATCAGGGTAA |
| pCJH040 | Plasmid expressing Cas12c sgRNA targeting murD (2) | Chloramphenicol | - | TCGTCATTATCGGCCTG |
| pCJH041 | Plasmid expressing Cas12c sgRNA targeting rplJ (1) | Chloramphenicol | - | GCTTTAAATCTTCAAGA |
| pCJH042 | Plasmid expressing Cas12c sgRNA targeting rplJ (2) | Chloramphenicol | - | TTGCTGAAGTCAGCGAA |
| pCJH043 | Plasmid expressing Cas12c sgRNA targeting rpoB (1) | Chloramphenicol | - | GTTTACTCCTATACCGA |
| pCJH044 | Plasmid expressing Cas12c sgRNA targeting rpoB (2) | Chloramphenicol | - | GTAAACGTCCACAAGTT |
| pCJH045 | Plasmid expressing Cas12a guide RNA targeting dnaA (1) | Chloramphenicol | - | GCTTTGGCAGCAGTGTCTTGCCCGATTG |
| pCJH046 | Plasmid expressing Cas12a guide RNA targeting dnaA (2) | Chloramphenicol | - | GCAGCAGTGTCTTGCCCGATTGCAGGAT |
| pCJH047 | Plasmid expressing Cas12a guide RNA targeting murD (1) | Chloramphenicol | - | CTGCGTGGACTTTTTCCTCGCTCGCGGT |
| pCJH048 | Plasmid expressing Cas12a guide RNA targeting murD (2) | Chloramphenicol | - | CTCGCTCGCGGTGTGACGCCGCGCGTTA |
| pCJH049 | Plasmid expressing Cas12a guide RNA targeting rplJ (1) | Chloramphenicol | - | AATCTTCAAGACAAACAAGCGATTGTTG |
| pCJH050 | Plasmid expressing Cas12a guide RNA targeting rplJ (2) (non-template strand) | Chloramphenicol | - | GCTACTTCGCTGACTTCAGCAACAATCG |
| pCJH051 | Plasmid expressing Cas12a guide RNA targeting rpoB (1) | Chloramphenicol | - | CTCCTATACCGAGAAAAAACGTATTCGT |
| pCJH052 | Plasmid expressing Cas12a guide RNA targeting rpoB (2) | Chloramphenicol | - | GTAAACGTCCACAAGTTCTGGATGTACC |
| pCJH053 | Plasmid expressing Cas12c sgRNA targeting Lambda cro (1) | Chloramphenicol | - | gaacaacgcataaccct |
| pCJH054 | Plasmid expressing Cas12c sgRNA targeting Lambda cro (3) | Chloramphenicol | - | ggcaaaccaagacagct |

| pCJH055 | Plasmid expressing a Cas12c non-targeting sgRNA | Chloramphenicol | - | gctagcatgactggtgg |
|---|---|---|---|---|
| pCJH057 | Plasmid expressing Cas12a guide RNA targeting Lambda cro (1) | Chloramphenicol | - | ggcaaaccaagacagctaaagatctcgg |
| pCJH058 | Plasmid expressing a Cas12a non-targeting guide RNA | Chloramphenicol | - | gcggtgaagtcctgactgcgttcgctcg |
| pCJH059 | Plasmid expressing Cas12c tracrRNA and a GFP-targeting pre-crRNA separately | Chloramphenicol | 183076 | TCCCAATTCTTGTTGAA |
| pCJH061 | Plasmid expressing two Cas12c non-targeting sgRNAs connected in one transcript as a control for multiplexed repression | Chloramphenicol | - | ttttttgtacttggtac; tcgagtaaggatctcca |
| pCJH064 | Plasmid expressing Cas12c tracrRNA and a non-targeting pre-crRNA separately | Chloramphenicol | - | ttttttgtacttggtac |
| pCJH065 | Plasmid expressing Cas12c tracrRNA and GFP-targeting crRNA separately, with the crRNA ending with a HDV ribozyme sequence. | Chloramphenicol | 183077 | TCCCAATTCTTGTTGAA |
| pCJH066 | Plasmid expressing Cas12c tracrRNA and non-targeting crRNA separately, with the crRNA ending with a HDV ribozyme sequence. | Chloramphenicol | - | ttttttgtacttggtac |
| pCJH069 | Plasmid expressing Cas12c sgRNA targeting the promoter of integrated GFP/RFP (2) (non-template strand) | Chloramphenicol | - | AATTCAGATCTATTATA |
| pCJH070 | Plasmid expressing Cas12c sgRNA targeting GFP (4) (non-template strand) | Chloramphenicol | - | ATTCAACAAGAATTGGG |
| pCJH071 | Plasmid expressing Cas12c sgRNA targeting GFP (5) (non-template strand) | Chloramphenicol | - | TGCCCATTAACATCACC |
| pCJH072 | Plasmid expressing Cas12c sgRNA targeting GFP (6) (non-template strand) | Chloramphenicol | - | CAAATAAATTTAAGGGT |
| pCJH073 | Plasmid expressing Cas12c sgRNA targeting RFP (4) (non-template strand) | Chloramphenicol | - | ACGTCTTCGCTACTCGC |
| pCJH074 | Plasmid expressing Cas12c sgRNA targeting RFP (5) (non-template strand) | Chloramphenicol | - | ATAACGTCTTCGCTACT |
| pCJH075 | Plasmid expressing Cas12c sgRNA targeting RFP (6) (non-template strand) | Chloramphenicol | - | accgttaacggaacctt |
| pCJH076 | Plasmid expressing Cas12c sgRNA targeting RFP (7) (non-template strand) | Chloramphenicol | - | cggggacaggatgtccc |
| pCJH078 | Plasmid expressing Cas12c sgRNA targeting Lambda Cro (2) (non-template strand) | Chloramphenicol | - | cataatctttcagggtt |
| pCJH079 | Plasmid expressing Cas12c sgRNA targeting Lambda Cro (4) (non-template strand) | Chloramphenicol | - | atatacgccgagatctt |
| pCJH080 | Plasmid expressing Cas12c sgRNA targeting Lambda mcpE (1) | Chloramphenicol | - | ctggcggcaaatgagca |
| pCJH081 | Plasmid expressing Cas12c sgRNA targeting Lambda mcpE (3) | Chloramphenicol | - | atccgctgtttctgcgt |
| pCJH082 | Plasmid expressing Cas12c sgRNA targeting Lambda mcpE (2) (Non-template strand) | Chloramphenicol | - | ctcatttgccgccagca |

| | | | | |
|---|---|---|---|---|
| pCJH083 | Plasmid expressing Cas12c sgRNA targeting Lambda mcpE (4) (Non-template strand) | Chloramphenicol | - | aagggatagctctcacg |
| pCJH084 | Plasmid expressing Cas12c sgRNA targeting Lambda ea47 (1) | Chloramphenicol | - | gagagttttatgactaa |
| pCJH085 | Plasmid expressing Cas12c sgRNA targeting Lambda ea47 (3) | Chloramphenicol | - | ctcaaatgctgcattga |
| pCJH086 | Plasmid expressing Cas12c sgRNA targeting Lambda ea47 (2) (non-template strand) | Chloramphenicol | - | gtttttagtcataaaa |
| pCJH087 | Plasmid expressing Cas12c sgRNA targeting Lambda ea47 (4) (non-template strand) | Chloramphenicol | - | cagcatttgagcaagtg |
| pCJH088 | Plasmid expressing Cas12a guide RNA targeting Lambda Cro (2) (non-template strand) | Chloramphenicol | - | atatacgccgagatctttagctgtcttg |
| pCJH091 | Plasmid expressing Cas12a guide RNA targeting Lambda ea47 (1) | Chloramphenicol | - | tcgcacttgctcaaatgctgcattgata |
| pCJH092 | Plasmid expressing Cas12a guide RNA targeting Lambda ea47 (2) (non-template strand) | Chloramphenicol | - | agcaagtgcgataaatctttaagtcttc |

| **Table 2.1C: Guide RNA plasmids for Golden Gate Assembly** | | | | |
|---|---|---|---|---|
| **Plasmid Name** | **Description** | **Selection marker** | **Addgene ID** | **Spacer sequence** |
| Cas12c-sgRNA_BbsI_CAM | Cas12c sgRNA containing BbsI restriction recognition sites in spacer sequence for Golden Gate Assembly | Chloramphenicol | 183221 | tcgtcttccgaagactt |
| LbCas12a-gRNA_BbsI_CAM | LbCas12a gRNA containing BbsI restriction recognition sites in spacer sequence for Golden Gate Assembly | Chloramphenicol | 183222 | tcgtcttccttgtacttggtgaagacaa |

## Table 2.1 List of plasmids, Related to Methods

| Table 2.2A: ssDNA oligonucleotides PAGE-purified for assays | | | | |
|---|---|---|---|---|
| **ID** | **Description** | **Length** | **Sequence** | **Source** |
| dCJH0073 | Cas12c Non-target Strand DNA | 53 | GCCTGCCCGCAGATTGatcaataccaaactctgGCGGCGTAAACTTTCCAGTC | IDT |
| dCJH0074 | Cas12c Target Strand DNA | 53 | GACTGGAAAGTTTACGCCGCcagagtttggtattgatCAATCTGCGGGCAGGC | IDT |
| dCJH0086 | Cas12c Non-target Strand DNA with Non-cognate Protopacer | 53 | GCCTGCCCGCAGATTGttttttgtacttggtacGCGGCGTAAACTTTCCAGTC | IDT |
| dCJH0096 | Cas12c Non-target Strand DNA with Non-cognate PAM | 53 | GCCTGCCCGCAGATACatcaataccaaactctgGCGGCGTAAACTTTCCAGTC | IDT |
| dCJH0097 | Cas12c Target Strand DNA with Non-cognate PAM | 53 | GACTGGAAAGTTTACGCCGCcagagtttggtattgatGTATCTGCGGGCAGGC | IDT |
| dCJH0098 | Cas12c Target Strand DNA with Non-cognate Protopacer | 53 | GACTGGAAAGTTTACGCCGCgtaccaagtacaaaaaaCAATCTGCGGGCAGGC | IDT |

| Table 2.2B: ssDNA oligos used to generate the ssRNA through *in vitro* transcription (IVT) | | | | |
|---|---|---|---|---|
| **ID** | **Description** | **Length** | **Sequence** | **Source** |
| oCJH0017 | Used in a PCR reaction to generate the dsDNA template for IVT of rCJH007 | 58 | GGCTCTCCCTTAGCCATCCGAGTTTCCTCGGATGCCCAGGTCGGACCGCGAGGAGGTG | IDT |
| oCJH0031 | Used in a PCR reaction to generate the dsDNA template for IVT of rCJH006 | 47 | GTCGAAATTAATACGACTCACTATAGGTGGTATCTGATGAGGCCTTC | IDT |
| oCJH0032 | Used in a PCR reaction to generate the dsDNA template for IVT of rCJH006 | 43 | AGATTTCAGGTCGCTCTAGCTGCGCGCCCGGACATTGAGGTAC | IDT |
| oCJH0033 | Used in a PCR reaction to generate the dsDNA template for IVT of rCJH006 | 59 | GTATCTGATGAGGCCTTCGGGCCGAAACGGTGAAAGCCGTAATACCACCCGTGCATTTC | IDT |
| oCJH0034 | Used in a PCR reaction to generate the dsDNA template for IVT of rCJH006 | 53 | CCGGACATTGAGGTACGGATCATTGATCCAGAAATGCACGGGTGGTATTACGG | IDT |
| oCJH0035 | Used in PCR reactions to generate the dsDNA template for IVT of rCJH007, rCJH010 | 49 | GTCGAAATTAATACGACTCACTATAgggtaacgagcaggattcaggttg | IDT |
| oCJH0036 | Used in a PCR reaction to generate the dsDNA template for IVT of rCJH007 | 60 | aacgagcaggattcaggttgggtttgaggATCAATACCAAACTCTGagcaggatGGGTCG | IDT |
| oCJH0037 | Used in a PCR reaction to generate the dsDNA template for IVT of rCJH007 | 51 | GACCGCGAGGAGGTGGAGATGCCATGCCGACCCatcctgctCAGAGTTTGG | IDT |
| oCJH0042 | Used in a PCR reaction to generate the dsDNA template for IVT of rCJH010 | 42 | GGCTCTCCCTTAGCCATCCGAGTTTCCTCGGATGCCCAGGTC | IDT |
| oCJH0043 | Used in a PCR reaction to generate the dsDNA template for IVT of rCJH010 | 60 | taacgagcaggattcaggttgggtttgaggATCAATACCAAACTCTGGGGTCGGCATGGC | IDT |

| oCJH0051 | T7 promoter oligo in the forward direction for IVT of rCJH019, rCJH024, and rCJH040 | 17 | TAATACGACTCACTATA | IDT |
|---|---|---|---|---|
| oCJH0044 | Used in a PCR reaction to generate the dsDNA template for IVT of rCJH010 | 58 | TCGGATGCCCAGGTCGGACCGCGAGGAGGTGGAGATGCCATGCCGACCCCAGAGTTTG | IDT |
| oCJH0060 | Used in a PCR reaction to generate the dsDNA template for IVT of rCJH017 | 42 | GTCGAAATTAATACGACTCACTATAagcaggattcaggttgg | IDT |
| oCJH0061 | Used in PCR reactions to generate the dsDNA template for IVT of rCJH017, rCJH018 | 42 | GGCTCTCCCTTAGCCATCCGAGTTTCCTCGGATGCCCAGGTC | IDT |
| oCJH0062 | Used in a PCR reaction to generate the dsDNA template for IVT of rCJH017 | 60 | gcaggattcaggttgggtttgaggATCAATACCAAACTCTGagcaggatGGGTCGGCATG | IDT |
| oCJH0063 | Used in PCR reactions to generate the dsDNA template for IVT of rCJH017, rCJH018 | 53 | TCGGATGCCCAGGTCGGACCGCGAGGAGGTGGAGATGCCATGCCGACCCatcc | IDT |
| oCJH0064 | Used in a PCR reaction to generate the dsDNA template for IVT of rCJH018 | 60 | ATAggattcaggttgggtttgaggATCAATACCAAACTCTGagcaggatGGGTCGGCATG | IDT |
| oCJH0065 | Used in a PCR reaction to generate the dsDNA template for IVT of rCJH018 | 43 | GTCGAAATTAATACGACTCACTATAggattcaggttgggtttg | IDT |
| oCJH0066 | Used to anneal with the T7 promoter primer (oCJH0051) for IVT of rCJH019 | 99 | aacctgaatcctgctCAGAGTTTGGTATTGATCAGAGTTTGGTATTGATcctcaaacccaacctgaatcctgctcgttacccTATAGTGAGTCGTATTA | IDT |
| oCJH0071 | Used to anneal with the T7 promoter primer (oCJH0051) for IVT of rCJH024 | 155 | aacctgaatcctgctCAGAGTTTGGTATTGATcctcaaacccaacctgaatcctgctTTTCAGATTTCAGGTCGCTCTAGCTGCGCGCCCGGACATTGAGGTACGGATCATTGATCCAGAAATGCACGGGTGGTATCCTATAGTGAGTCGTATTA | IDT |
| oCJH0076 | Used in a PCR reaction to generate the dsDNA template for IVT of rCJH033 | 60 | ACTCACTATAGGCCAGTCCTGATGAGGCCTTCGGGCCGAAACGGTGAAAGCCGTAGACTG | IDT |
| oCJH0077 | Used in a PCR reaction to generate the dsDNA template for IVT of rCJH033 | 56 | TGAAAGCCGTAGACTGGAAAGTTTACGCCGCcagagtttggtattgatCAATCTGC | IDT |
| oCJH0078 | Used in a PCR reaction to generate the dsDNA template for IVT of rCJH033 | 52 | ggtattgatCAATCTGCGGGCAGGCGGGTCGGCATGGCATCTCCACCTCCTC | IDT |
| oCJH0079 | Used in a PCR reaction to generate the dsDNA template for IVT of rCJH033 | 50 | CATCTCCACCTCCTCGCGGTCCGACCTGGGCATCCGAGGAAACTCGGATG | IDT |
| oCJH0080 | Used in a PCR reaction to generate the dsDNA template for IVT of rCJH033 | 29 | GGCTCTCCCTTAGCCATCCGAGTTTCCTC | IDT |
| oCJH0081 | Used in a PCR reaction to generate the dsDNA template for IVT of rCJH033 | 31 | GTCGAAATTAATACGACTCACTATAGGCCAG | IDT |
| oCJH0087 | Used in PCR reactions to generate the dsDNA template for IVT of rCJH038, rCJH041 | 39 | GTCGAAATTAATACGACTCACTATAGGATACCACCCGTG | IDT |

| oCJH0088 | Used in PCR reactions to generate the dsDNA template for IVT of rCJH038, rCJH041 | 30 | GGCTCTCCCTTAGCCATCCGAGTTTCCTCG | IDT |
|---|---|---|---|---|
| oCJH0089 | Used in PCR reactions to generate the dsDNA template for IVT of rCJH038, rCJH041 | 42 | AGGATACCACCCGTGCATTTCTGGATCAATGATCCGTACCTC | IDT |
| oCJH0090 | Used in PCR reactions to generate the dsDNA template for IVT of rCJH038, rCJH041 | 56 | GATCAATGATCCGTACCTCAATGTCCGGGCGCGCAGCTAGAGCGACCTGAAATCTG | IDT |
| oCJH0091 | Used in a PCR reaction to generate the dsDNA template for IVT of rCJH038 | 44 | AGCGACCTGAAATCTGAAAagcaggattcaggttgggtttgagg | IDT |
| oCJH0092 | Used in PCR reactions to generate the dsDNA template for IVT of rCJH038, rCJH041 | 60 | caggttgggtttgaggATCAATACCAAACTCTGGGGTCGGCATGGCATCTCCACCTCCTC | IDT |
| oCJH0093 | Used in PCR reactions to generate the dsDNA template for IVT of rCJH038, rCJH041 | 51 | GCATCTCCACCTCCTCGCGGTCCGACCTGGGCATCCGAGGAAACTCGGATG | IDT |
| oCJH0094 | Used to anneal with the T7 promoter primer (oCJH0051) for IVT of rCJH040 | 149 | aacctgaatcctgctCAGAGTTTGGTATTGATcctcaaacccaacctgaatTTTCAGATTTCAGGTCGCTCTAGCTGCGCGCCCGGACATTGAGGTACGGATCATTGATCCAGAAATGCACGGGTGGTATCCTATAGTGAGTCGTATTA | IDT |
| oCJH0095 | Used in a PCR reaction to generate the dsDNA template for IVT of rCJH041 | 38 | AGCGACCTGAAATCTGAAAattcaggttgggtttgagg | IDT |

| **Table 2.2C: ssRNA oligos used in this study** | | | | |
|---|---|---|---|---|
| **ID** | **Description** | **Length** | **Sequence** | **Source** |
| rCJH006 | Cas12c tracrRNA | 75 | AUACCACCCGUGCAUUUCUGGAUCAAUGAUCCGUACCUCAAUGUCCGGGCGCGCAGCUAGAGCGACCUGAAAUCU | PCR IVT |
| rCJH007 | Cas12c 5' extended pre-crRNA (8nt random + **R***S* + 8nt of R) | 58 | GGGUAACG**AGCAGGAUUCAGGUUGGGUUUGAGG***AUCAAUACCAAACUCUG*AGCAGGAU | PCR IVT |
| rCJH010 | Mature Cas12c crRNA (for rCJH007) (8nt random + **R***S*) | 50 | GGGUAACG**AGCAGGAUUCAGGUUGGGUUUGAGG***AUCAAUACCAAACUCUG* | PCR IVT |
| rCJH017 | Cas12c pre-crRNA (**R***S* + 8nt of R) | 50 | **AGCAGGAUUCAGGUUGGGUUUGAGG***AUCAAUACCAAACUCUG*AGCAGGAU | PCR IVT |
| rCJH018 | Cas12c pre-crRNA (21-nt shortened **R** + *S* + 8nt of R) | 46 | **GGAUUCAGGUUGGGUUUGAGG***AUCAAUACCAAACUCUG*AGCAGGAU | PCR IVT |
| rCJH019 | Cas12c double spacer pre-crRNA (8nt random + **R***SS* + 15nt of R) | 82 | GGGUAACG**AGCAGGAUUCAGGUUGGGUUUGAGG***AUCAAUACCAAACUCUGAUCAAUACCAAACUCUG*AGCAGGAUUCAGGUU | IDT IVT |

| rCJH024 | Cas12c pre-sgRNA version 1 (tracrRNA + GAAA + **R**_S_ + 15nt R) | 138 | GGAUACCACCCGUGCAUUUCUGGAUCAAUGAUCCGUACCUCAAUGUCCGGGCGCGCAGCUAGAGCGACCUGAAAUCUGAAA**AGCAGGAUUCAGGUUGGGUUUGAGG**_AUCAAUACCAAACUCUG_AGCAGGAUUCAGGUU | IDT IVT |
|---|---|---|---|---|
| rCJH026 | 3' 6-FAM labeled Cas12c pre-crRNA (**R**_S_ + 11nt of R) | 49 | **GGAUUCAGGUUGGGUUUGAGG**_AUCAAUACCAAACUCUG_AGCAGGAUUCA/36-FAM/ | IDT |
| rCJH027 | 3' 6-FAM labeled 11nt R Cas12c with 5' P | 11 | /5Phos/AGCAGGAUUCA/36-FAM/ | IDT |
| rCJH028 | 3' 6-FAM labeled 11nt R Cas12c with 5' OH | 11 | AGCAGGAUUCA/36-FAM/ | IDT |
| rCJH029 | Multi-phosphorothioated rCJH018 Cas12c pre-crRNA (21-nt shortened **R** + _S_ + 8nt of R) | 46 | **GGAUUCAGGUUGGGUUUGAGG**_AUCAAUACCAAACUCU_*_G_*A*G*CAGGAU | IDT |
| rCJH030 | Phosphorothioated rCJH018 Cas12c pre-crRNA (21-nt shortened **R** + _S_ + 8nt of R) | 46 | **GGAUUCAGGUUGGGUUUGAGG**_AUCAAUACCAAACUCU_*_G_AGCAGGAU | IDT |
| rCJH031 | Phosphorothioated rCJH018 Cas12c pre-crRNA (21-nt shortened **R** + _S_ + 8nt of R) | 46 | **GGAUUCAGGUUGGGUUUGAGG**_AUCAAUACCAAACUCUG_*AGCAGGAU | IDT |
| rCJH032 | Phosphorothioated rCJH018 Cas12c pre-crRNA (21-nt shortened **R** + _S_ + 8nt of R) | 46 | **GGAUUCAGGUUGGGUUUGAGG**_AUCAAUACCAAACUCUG_A*GCAGGAU | IDT |
| rCJH033 | (ssRNA) Cas12c target strand (cleaved by HHrz and HDVrz) | 53 | GACUGGAAAGUUUACGCCGCCAGAGUUUGGUAUUGAUCAAUCUGCGGGCAGGC | PCR IVT |
| rCJH037 | Mature Cas12c crRNA (for rCJH018) (21-nt shortened **R** + _S_) | 38 | **GGAUUCAGGUUGGGUUUGAGG**_AUCAAUACCAAACUCUG_ | IDT |
| rCJH038 | Mature sgRNA version 1 (processed version of rCJH024) | 123 | GGAUACCACCCGUGCAUUUCUGGAUCAAUGAUCCGUACCUCAAUGUCCGGGCGCGCAGCUAGAGCGACCUGAAAUCUGAAA**AGCAGGAUUCAGGUUGGGUUUGAGG**_AUCAAUACCAAACUCUG_ | PCR IVT |
| rCJH039 | Partial DNA version of rCJH018 Cas12c pre-crRNA (21-nt shortened R + S + 8nt of R) | 46 | rGrGrArUrUrCrArGrGrUrUrGrGrGrUrUrUrGrArGrGrArUrCrArArUrArCrCrArArArCrUrC**(d)T(d)G(d)A(d)G**rCrArGrGrArU | IDT |
| rCJH040 | Cas12c sgRNA version 2 (tracrRNA + GAAA + 19nt **R**+ _S_ + 15nt R) | 132 | GGAUACCACCCGUGCAUUUCUGGAUCAAUGAUCCGUACCUCAAUGUCCGGGCGCGCAGCUAGAGCGACCUGAAAUCUGAAA**AUUCAGGUUGGGUUUGAGG**_AUCAAUACCAAACUCUG_AGCAGGAUUCAGGUU | IDT IVT |
| rCJH041 | Mature sgRNA version 2 (processed version of rCJH040) | 117 | GGAUACCACCCGUGCAUUUCUGGAUCAAUGAUCCGUACCUCAAUGUCCGGGCGCGCAGCUAGAGCGACCUGAAAUCUGAAA**AUUCAGGUUGGGUUUGAGG**_AUCAAUACCAAACUCUG_ | PCR IVT |

| | | | | |
|---|---|---|---|---|
| rCJH042 | Partial DNA version of rCJH018 Cas12c pre-crRNA (21-nt shortened R + S + 8nt of R) | 46 | rGrGrArUrUrCrArGrGrUrUrGrGrGrUrUrGrArGrGrArUrCrArArUrArCrCrArArArCrUrC**(d)T**rGrArGrCrArGrGrArU | IDT |
| rCJH043 | Partial DNA version of rCJH018 Cas12c pre-crRNA (21-nt shortened R + S + 8nt of R) | 46 | rGrGrArUrUrCrArGrGrUrUrGrGrGrUrUrGrArGrGrArUrCrArArUrArCrCrArArArCrUrCrU**(d)G**rArGrCrArGrGrArU | IDT |
| rCJH044 | Partial DNA version of rCJH018 Cas12c pre-crRNA (21-nt shortened R + S + 8nt of R) | 46 | rGrGrArUrUrCrArGrGrUrUrGrGrGrUrUrGrArGrGrArUrCrArArUrArCrCrArArArCrUrCrUrG**(d)A**rGrCrArGrGrArU | IDT |
| rCJH045 | Partial DNA version of rCJH018 Cas12c pre-crRNA (21-nt shortened R + S + 8nt of R) | 46 | rGrGrArUrUrCrArGrGrUrUrGrGrGrUrUrGrArGrGrArUrCrArArUrArCrCrArArArCrUrCrUrGrA**(d)G**rCrArGrGrArU | IDT |

Notes:
IDT IVT: A long ssDNA oligo from IDT was directly annealed with a T7 promoter oligo (oCJH0051) and transcribed
PCR IVT: Multiple IDT oligos were used in a PCR reaction to create the dsDNA template with a T7 promoter sequence for IVT, on which IVT was then performed
IDT: Oligos ordered from Integrated DNA Technologies
R = Repeat sequence; S = Spacer sequence

**Table 2.2D: DNA oligos used to assemble *in vitro* transcription templates**

| **oligo ID** | **IVT template assembled by PCR or direct annealing from DNA oligos:** | **ribozyme(s) in initial transcript?** |
|---|---|---|
| rCJH006 | oCJH0031, oCJH0032, oCJH0033, oCJH0034 | HHrz |
| rCJH007 | oCJH0035, oCJH0017, oCJH0036, oCJH0037 | HDVrz |
| rCJH010 | oCJH0035, oCJH0042, oCJH0043, oCJH0044 | HDVrz |
| rCJH017 | oCJH0060, oCJH0061, oCJH0062, oCJH0063 | HDVrz |
| rCJH018 | oCJH0065, oCJH0061, oCJH0064, oCJH0063 | HDVrz |
| rCJH019 | oCJH_0066, oCJH0051_T7oligo | None |
| rCJH024 | oCJH_0071, oCJH0051_T7oligo | None |
| rCJH033 | oCJH0080, oCJH0081, oCJH0076, oCJH0077, oCJH0078, oCJH0079 | HHrz, HDVrz |
| rCJH038 | oCJH0087, oCJH0088, oCJH0089, oCJH0090, oCJH0091, oCJH0092, oCJH0093 | HDVrz |
| rCJH040 | oCJH_0094, oCJH0051_T7oligo | None |
| rCJH041 | oCJH0087, oCJH0088, oCJH0089, oCJH0090, oCJH0095, oCJH0092, oCJH0093 | HDVrz |

Key:
HHrz: Hammerhead ribozyme on 5′ end of transcript
HDVrz: Hepatitis delta virus ribozyme on 3′ end of transcript

## Table 2.2 Oligonucleotide sequences, Related to Methods

| | Plasmid or oligo ID |
|---|---|
| **Figure 2.1B** | tracrRNA: rCJH006<br>crRNA: rCJH010<br>dsDNA (TS/NTS): dCJH0074+dCJH0073<br>Bubbled dsDNA (TS/bubbledNTS): dCJH0074+dCJH0086<br>ssDNA: dCJH0074<br>ssRNA : rCJH033 |
| **Figure 2.1C** | tracrRNA: rCJH006<br>crRNA: rCJH010<br>dsDNA (TS/NTS): dCJH0074+dCJH0073 |
| **Figure 2.2A** | pre-crRNA: rCJH018, tracrRNA: rCJH006<br>RNA marker: rCJH037 |
| **Figure 2.2C** | pre-crRNA: rCJH026<br>tracrRNA: rCJH006<br>RNA markers: rCJH027 and rCJH028 |
| **Figure 2.2D** | pre-crRNA: rCJH039<br>tracrRNA: rCJH006<br>RNA marker: rCJH037 |
| **Figure 2.2E** | Pre-crRNA: rCJH042, rCJH043, rCJH044, rCJH045<br>tracrRNA: rCJH006<br>RNA Marker: rCJH037 |
| **Figure 2.3B** | pre-sgRNA: rCJH024 and rCJH040<br>RNA markers: rCJH038 and rCJH041 |
| **Figure 2.4A** | tracrRNA: rCJH006<br>crRNA: rCJH010<br>dsDNA (TS/NTS): dCJH0074+dCJH0073<br>Bubbled dsDNA (TS/bubbledNTS): dCJH0074+dCJH0086<br>dsDNA with no PAM (NTS/TS): dCJH0096+dCJH0097<br>Non-complementary dsDNA (NTS/TS): dCJH0086+dCJH0098<br>ssDNA: dCJH0074<br>ssRNA : rCJH033 |
| **Figure 2.4B** | tracrRNA: rCJH006<br>pre-crRNA: rCJH018<br>Mature crRNA: rCJH037<br>Phosphorothioated pre-crRNA: rCJH029<br>dsDNA (5' radiolabeled NTS/TS): dCJH0073+dCJH0074 |
| **Figure 2.4C** | tracrRNA: rCJH006<br>pre-crRNA: rCJH018<br>Mature crRNA: rCJH037<br>dsDNA (5' radiolabeled NTS/TS): dCJH0073+dCJH0074 |
| **Figure 2.5B** | Cas plasmids: pCJH027 or pCJH028<br>Guide RNA plasmids: pCJH029, pCJH030, pCJH031, pCJH032, pCJH033, pCJH0034, and pCJH035 |
| **Figure 2.5C** | Cas plasmids: pCJH019 or pCJH020<br>sgRNA plasmids: pCJH007, pCJH008, pCJH009, pCJH010, pCJH011, pCJH012, pCJH013, pCJH014, and pCJH015 |
| **Figure 2.5D&E** | Cas plasmids: pCJH021 or pCJH022<br>sgRNA plasmids: pCJH009 and pCJH015<br>Dual RNA guide plasmids: pCJH059 and pCJH064 |

| | |
|---|---|
| | Dual RNA with HDVrz guide plasmids: pCJH065 and pCJH066 |
| **Figure 2.5F&G** | Cas plasmids: pCJH019 or pCJH020<br>sgRNA plasmids: pCJH009 and pCJH015<br>Dual RNA guide plasmids: pCJH059 and pCJH064<br>Dual RNA with HDVrz guide plasmids: pCJH065 and pCJH066 |
| **Figure 2.5H** | Cas plasmid: pCJH019<br>sgRNA plasmids: pCJH026 and pCJH061 |
| **Figure 2.6B&C** | Cas12a plasmids: pCJH027 (Figure 2.6B) or pCJH028 (Figure 2.6C)<br>Cas12a guide plasmids: pCJH057, pCJH088, pCJH091, pCJH092<br>Cas12a non-targeting guide plasmid: pCJH058 |
| **Figure 2.6D&E** | Cas12c plasmid: pCJH019 (Figure 2.6D) or pCJH020 (Figure 2.6E)<br>Cas12c sgRNA plasmids: pCJH053, pCJH078, pCJH054, pCJH079, pCJH080, pCJH082, pCJH081, pCJH083, pCJH084, pCJH086, pCJH085, pCJH087<br>Cas12c non-targeting sgRNA plasmid: pCJH055 |
| **Figure 2.8A** | Pre-crRNA: rCJH007, rCJH017, rCJH018, and rCJH019<br>tracrRNA: rCJH006<br>RNA marker: rCJH010 |
| **Figure 2.8B** | pre-crRNA: rCJH026<br>tracrRNA: rCJH006 |
| **Figure 2.8C** | Pre-crRNA: rCJH018, rCJH029, rCJH030, rCJH031, rCJH032<br>tracrRNA: rCJH006<br>RNA marker: rCJH037 |
| **Figure 2.8D** | Pre-crRNA: rCJH018 and rCJH029<br>tracrRNA: rCJH006 |
| **Figure 2.9A&C** | tracrRNA: rCJH006<br>crRNA: rCJH010<br>dsDNA (TS/NTS): dCJH0074+dCJH0073 |
| **Figure 2.9B&D** | sgRNA: rCJH038<br>dsDNA (TS/NTS): dCJH0074+dCJH0073 |
| **Figure 2.10A,C,E** | tracrRNA: rCJH006<br>crRNA: rCJH010<br>dsDNA (TS/NTS): dCJH0074+dCJH0073 |
| **Figure 2.10B,D,F** | sgRNA: rCJH038<br>dsDNA (TS/NTS): dCJH0074+dCJH0073 |
| **Figure 2.12** | pre-crRNA: rCJH017<br>tracrRNA: rCJH006 |
| **Figure 2.13A** | Cas plasmids: pCJH027 or pCJH028<br>Guide RNA plasmids: pCJH029, pCJH030, pCJH031, pCJH032, pCJH033, pCJH0034, and pCJH035 |
| **Figure 2.13B** | Cas plasmids: pCJH019 or pCJH020<br>sgRNA plasmids: pCJH007, pCJH008, pCJH009, pCJH010, pCJH011, pCJH012, pCJH013, pCJH014, and pCJH015 |
| **Figure 2.13C** | Cas plasmids: pCJH019 or pCJH020<br>sgRNA plasmids: pCJH069, pCJH070, pCJH071, pCJH072, pCJH073, pCJH074, pCJH075, pCJH076, and pCJH015 |

| | |
|---|---|
| **Figure 2.14A** | Cas plasmids: pCJH027 or pCJH028<br>Guide RNA plasmids: pCJH045, pCJH046, pCJH047, pCJH048, pCJH049, pCJH050, pCJH051, pCJH052, pCJH035 |
| **Figure 2.14B** | Cas plasmid: pCJH019 or pCJH020<br>sgRNA plasmids: pCJH037, pCJH038, pCJH039, pCJH040, pCJH041, pCJH042, pCJH043, pCJH044, pCJH015 |
| **Figure 2.14C** | pre-crRNA: rCJH017<br>tracrRNA: rCJH006 |

## Table 2.3 Plasmids and oligonucleotides used in experiments, Related to Methods

## 2.5 Discussion

CRISPR-Cas systems have evolved in diverse microbes to provide adaptive immunity against foreign nucleic acids. Until now, immunity provided by the class 2 single-effector type V CRISPR-Cas systems was thought to rely on RNA-guided nuclease activity that targets phage or other foreign molecules, typically at the level of dsDNA cutting. In this study, we show that the type V CRISPR effector Cas12c is an RNA-guided DNA-binding enzyme that does not cut target DNA or exhibit detectable target-activated *trans*-cleavage activity. We found that the RuvC domain of Cas12c, which was previously assumed to cut DNA based on bioinformatic and *in vivo* functional comparison with other type V CRISPR enzymes, instead exclusively catalyzes the maturation of pre-crRNA for targeted Cas12c DNA binding. Despite its lack of dsDNase activity, in cells this DNA-binding activity enables robust transcriptional repression of fluorescent reporter proteins in a strand-independent manner. Furthermore, this naturally DNase-free Cas12c enzyme can protect bacteria from lytic bacteriophage infection when targeting an essential phage gene. These results suggest that CRISPR systems can provide anti-phage immunity even in the absence of target-directed nuclease activity and that this is likely Cas12c's native mechanism (Figure 2.7).

The proposed DNA-binding-only mechanism of gene repression by Cas12c would require spacers that target transcribed regions of invading-essential genes for successful immunity in a native context (Figure 2.6 and Figure 2.7). We speculate that CRISPR-Cas12c immunity that limits protospacer targets to transcribed regions may be similarly limiting as the immunity offered by CRISPR systems that primarily target RNA transcripts such as the CRISPR-Cas13 systems (Abudayyeh et al., 2016; East-Seletsky et al., 2016). Although target-activated DNase activity may bolster immunity when directed to a transcribed region (see Cas12a in Figure 2.6B), these benefits must be weighed against the fitness costs associated with incidental host-directed nuclease activity (Weissman et al., 2020; Wimmer and Beisel, 2020). For example, if a native type V-C system accidentally acquires a self-targeting spacer, there is a lower chance of significant toxicity. Additionally, lack of native DNase activity exhibited by Cas12c is not a mere loss of function relative to Cas12a. As shown in Figure 2.6, Cas12c displays greater protospacer flexibility in strand-targeting and PAM constraints than the RuvC-deactivated dCas12a does, which is likely reflective of its evolutionary history. We speculate that the evolutionary trajectory of CRISPR-Cas12c systems has evolved to mitigate such tradeoff space while playing a role in immune defense.

All Cas12-family enzymes contain a RuvC catalytic center, an enzymatic domain resembling RNase H that catalyzes DNA or RNA phosphodiester cleavage by a ‘‘carboxylate-chelated two metal-ion’’ mechanism (Yang and Steitz, 1995). Enzymes thought to be ancestral to Cas12, including TnpB, possess RuvC domain-mediated DNA-cutting activity (Altae-Tran et al., 2021; Karvelis et al., 2021), implying that the observed RNA-cutting specificity of Cas12c's RuvC domain represents a lineage-specific departure from the ancestral state. Notably, the substrate specificities of RuvC domains found within different Cas12 enzymes are divergent. For example, Cas12a's RuvC domain cleaves DNA only, with a separate active site responsible for pre-crRNA processing (Fonfara et al., 2016). In contrast, the RuvC domain of CasPhi and Cas12g can cleave both DNA and RNA (Pausch et al., 2020; Yan et al., 2019). Based on reported *trans*-cleavage activity

on DNA by another Cas12c family member (Cas12c1; Wang and Zhong, 2021), the switch to exclusive use of RNA as a substrate by the Cas12c variants tested in this study may have occurred recently.

Repurposing the RuvC active site for exclusive RNA processing suggests that pre-crRNA processing is essential for function. Interestingly, we observed that RuvC-mediated pre-crRNA processing was required for high-affinity DNA binding *in vitro* but often not as important for transcriptional silencing *in vivo* in a heterologous host. A possible explanation for this observation is that guide RNA expression is under the control of a non-native strong promoter in our assays. If some transcripts undergo abortive transcription or cleavage by host nucleases, the resulting mature guide RNAs may be sufficient to direct unimpeded transcriptional silencing by Cas12c. The preservation of RuvC-catalyzed RNase activity in Cas12c suggests that crRNA maturation is an essential function of Cas12c in its natural host, perhaps due to lower pre-crRNA transcriptional levels or a lack of certain host nucleases.

Prior to this study, single-effector CRISPR-Cas systems that are DNA targeting yet DNase-free have only been described in the context of transcriptional regulation (Ratner et al., 2019; Workman et al., 2021) and transposase association (Strecker et al., 2019), with no indication of immune function. The results presented here suggest that contrary to previous assumptions, immunity against bacteriophage may be achieved solely through RNA-guided DNA binding. Other CRISPR systems for which no immune mechanism has been identified, such as some type IV multi-effector systems (Taylor et al., 2021), may work analogously to Cas12c. The targeting parameters of the DNase-free Cas12c orthologs, including mismatch tolerance and seed sequence, remain to be determined, and they could be distinct from those of DNA-cleaving Cas12 enzymes. In addition, the structural basis for the substrate specificity swap in Cas12c's RuvC active site will be fascinating to uncover. Nonetheless, the specific properties of this CRISPR-Cas12c system, including its minimal PAM requirement (TN), capacity for multiplexed targeting, and uniquely precise pre-crRNA processing activity, offer new tool development potential for transcriptional regulation and engineered base editing.

**Limitations of the study**

Since many type V-C CRISPR-Cas systems, including the focus of this study, were identified on short contigs from metagenomes with unknown hosts, we cannot fully rule out the possibility that a Cas12c that is DNase-free *in vitro* and *in vivo* in a heterologous host *E. coli* may be associated with other enzymatic activities in its natural hosts instead of simply being a DNA binder. Additionally, due to the unusually short spacer lengths (17–18 nt) in the native CRISPR arrays and limited availability of databases on mobile genetic elements, we were unable to identify the origins of the native spacers with confidence. It remains possible that although a DNA-targeting, but DNase-free, CRISPR-Cas12c protein is capable of anti-phage activity, it may have other functions instead of, or in addition to, being part of a prokaryotic adaptive immune system.

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### 2.7 Author contributions

Conceptualization, C.J.H. and J.A.D.; investigation, C.J.H. and B.A.A.; methodology, C.J.H., B.A.A., and J.A.D.; supervision, J.A.D.; writing, C.J.H., B.A.A., and J.A.D.

### Declaration of interests

J.A.D. is a cofounder of Caribou Biosciences, Editas Medicine, Scribe Therapeutics, Intellia Therapeutics, and Mammoth Biosciences. J.A.D. is a scientific advisory board member or consultant for Vertex, Caribou Biosciences, Intellia Therapeutics, Scribe Therapeutics, Mammoth Biosciences, Algen Biotechnologies, Felix Biosciences, The Column Group, Sixth Street, and Inari. J.A.D. is a Director at Altos Labs, Johnson & Johnson, and Tempus, and she has research projects sponsored by AppleTree Partners and Roche. J.A.D. is a member of Molecular Cell’s Advisory Board.

# Chapter 3 Reconstituting type V-C CRISPR-Cas adaptation in *E. coli*

## 3.1 Abstract

Acquiring functional spacer sequences from foreign nucleic acid to the CRISPR array during adaptation is an essential part of CRISPR-Cas immunity. Some type V CRISPR-Cas12c (V-C) systems are compact systems that interfere through a transcriptional-blocking mechanism without target-activated DNase activity, and regardless of the mechanism for interference, V-C systems lack a cas2 gene that was previously thought to be essential during the acquisition stage for all CRISPR systems. To eventually understand the basic principles underlying the type V-C PAM-specific spacer acquisition and look for evidence of primed type V-C adaptation in DNase-free V-C systems, I attempted to reconstitute the type V-C adaptation *in vivo* in a heterologous host *E. coli*. The results here showed that the multiple attempts using different plasmid constructs/expression strategies to reconstitute spacer acquisition *in vivo* have not been successful under the conditions tested, but varying induction conditions or detection methods may improve experimental outcomes in future studies. Understanding the mechanistic details of interference for type V-C systems can contribute to our understanding of the CRISPR-Cas adaptation process, which may advance the development of molecular recording tools.

## 3.2 Introduction

CRISPR (clustered regularly interspaced short palindromic repeats)-Cas (CRISPR-associated) systems are prokaryotic adaptive immunity against invading mobile genetic elements, such as viruses or plasmids (Wright et al., 2016). In these systems, Cas nuclease effectors typically recognize and cleave foreign nucleic acids containing sequences complementary to their associated crRNAs to trigger degradation of targeted nucleic acids. Before functional interference can occur, the spacer sequence from foreign nucleic acid fragments must first be acquired to CRISPR arrays by the CRISPR integrase complex, commonly Cas1 and Cas2, potentially with the help of other factors.

The type V CRISPR Cas12c (V-C) system is uniquely interesting for exploring different aspects of spacer acquisition, as it is a compact system that lacks Cas2, which was previously thought to be an essential structural component of the CRISPR integrases. However, Wright et al. showed that a V-C (6) Cas1 alone is sufficient for integration *in vitro*, which explains the unusually short spacers (~17-18 nt) in these V-C systems (2019). Interestingly, the three V-C systems characterized in the *E. coli* screen performed in Yan et al. 2019 appeared to carry out immunity function through various interference mechanisms (DNase-free or DNase-active). Kurihara et al. showed that, among these 3 orthologs, Cas12c1 (Cas12c_6) was DNase-active, while Cas12c2 (similar to Cas12c_4 in this study) and OspCas12c (from *Oleiphilus sp.* HI0009) were unable to cleave or nick circular or linear target DNA under the conditions tested (2022). One of these DNase-free Cas12c orthologs (Cas12c_4) has been proposed to be a natural CRISPRi enzyme, supported by the observations that it could bind to target DNA but exhibited no DNase activity *in vitro* or *in vivo* in a heterologous host *E. coli* (Huang et al., 2022). Targeted Cas12c DNA binding alone was sufficient to repress target gene transcription and restrict phage infection in *E. coli* in a strand-independent manner, and this was proposed to be the native function of these DNase-free CRISPR-Cas systems (Huang et al., 2022). Since

nuclease activity in several types I and II systems, including type II Cas9, is required for primed adaptation (Lee and Sashital, 2022; Nussenzweig et al., 2019), whether or not, and how, primed adaptation occur in DNase-free systems are questions yet to be answered.

Additionally, the mechanism by which the acquisition machinery selects functional protospacers flanked by a PAM during adaptation has not been explored in any type V-C systems. For effective interference to occur, DNA targets of the type V-C CRISPR-Cas system must contain a PAM sequence of TN/TG, depending on the homolog (Yan et al., 2019). The type V-C acquisition machinery must also select protospacers flanked by the same PAM during adaptation to ensure functional immunity. Since there are only two proteins, Cas1 and Cas12c, encoded in these minimal V-C CRISPR-Cas systems, it would be intriguing to test if the type V-C Cas1 recognizes the PAM, similar to type I Cas1 (Wang et al. 2015) or if Cas12c is essential for aiding the V-C Cas1 mini-integrase to select protospacers in a PAM-dependent manner, similar to the role of Cas9 in type II adaptation (Heler et al., 2015).

Without prior knowledge of the PAM sequence identities for interference (Yan et al., 2019), Wright et al. reconstituted the DNA integration activity of a type V-C Cas1 mini-integrase *in vitro* (2019). However, the integration events were promiscuous, suggesting that additional host factors or mechanisms were involved in specific spacer acquisition to the CRISPR array (Wright et al., 2019). These hypothetical host factors can potentially include an integration host factor (IHF), which is required for leader-proximal spacer integration *in vitro* and spacer acquisition *in vivo* in *Escherichia coli* type I-E CRISPR systems (Nuñez et al. 2016). I therefore reasoned that an *in vivo* adaptation assay might yield more specific integration events than *in vitro* experiments, in addition to the potential of testing primed adaptation capability. Since type V-C systems have been mostly found in uncultured microbes and from marine and gut metagenomic data with hosts that remained undetermined due to incomplete genome reconstruction (Shmakov et al. 2015; Burstein et al. 2017), I attempted to reconstitute V-C adaptation in a heterologous *E. coli* host.

To reconstitute type V-C adaptation *in vivo* in a heterologous host *E. coli*, I cloned the three type V-C CRISPR loci each containing *cas1* and *cas12c* genes as well as a minimal CRISPR array consisting of reduced number of repeat:spacer sequences flanked by ~200bp of the upstream and downstream native sequences into different vectors. Spacer acquisition assays were performed using multiple expression strategies including using native DNA sequences and codon-optimized sequences with inducible promoters. Assay results showed that my attempts to reconstitute type V-C adaptation *in vivo* in *E. coli* have not been successful, but varying expression conditions or other variables such as PCR strategies may yield positive outcomes in future studies.

## 3.3 Materials and Methods

### 3.3.1 Construction of expression plasmids

Three type V-C CRISPR-Cas systems with identified PAM for interference (Yan et al., 2019) were investigated in this study. For the V-C (4) system, Cas12c_4 in this study corresponds to what was described in Huang et al., 2022 and is similar to Cas12c2 described in Yan et al., 2019. For the V-C (6) system, Cas12c_6 in this study corresponds

to Cas12c1 described in Yan et al., 2019, and Cas1 (VC-6) corresponds to the Cas1 described in Wright et al., 2019.

For Figure 3.1, the native sequences of the three V-C CRISPR-Cas loci were cloned into a low-copy plasmid backbone that contains a SC101 origin of replication and a kanamycin resistance gene, with the only change being that the number of repeat:spacer pairs in the array was reduced to ~6.

For Figure 3.2, the coding sequences for all Cas1 and Cas12c were *E. coli*-codon optimized, and a two-plasmid expression system was used. On the first plasmid (p15A origin and $CAM^R$), the expression of Cas1 and Cas12c were under the control of the isopropyl β-D-thiogalactoside (IPTG)-inducible Lac promoter and the anhydrotetracycline (aTc)-inducible Tet promoter, respectively, while the expression of tracrRNA was under the control of a strong constitutive promoter, proD. On the second plasmid (SC101 origin and $Kan^R$), the miniature CRISPR array, including ~200 bp upstream (5') and downstream (3') flanking native sequences, was constitutively expressed under the control of a J23119 promoter.

For overexpression of the V-C proteins, another two-plasmid expression system was used. On the first plasmid (pCDFDuet-1 from Novagen with a CloDF13 origin and $Sm^R$), the expressions of Cas1 and Cas12c were under the control of separate T7 promoters. On the second plasmid (SC101 origin and $Kan^R$), the tracrRNA and the minimal CRISPR array including only one repeat and spacer (flanked by native neighboring sequences) were constitutively expressed in one single transcript under the control of a J23119 promoter.

These plasmids were originally constructed from Integrated DNA Technologies (IDT) gBlocks or ThermoFisher GeneArt Gene Synthesis. Different variants of the plasmids were produced by Gibson assembly. Sequences of the V-C systems and plasmids generated are described in Table 3.1 and Table 3.2.

### 3.3.2 *In vivo* spacer acquisition assays

Adaptation assays were performed as previously described (Kieper et al., 2018; Levy et al., 2015) with modifications. Briefly, a single colony of *E. coli* BL21 (DE3) Star (QB3-Macrolab, UC Berkeley) or BL21-AI (Invitrogen) harboring various combinations of plasmids coding for type V-C systems was inoculated in LB medium containing appropriate antibiotics (34 μg/mL chloramphenicol, 50 μg/mL Kanamycin, and/or 60 μg/mL Spectinomycin depending on the constructs). Cultures were aerated at 37 °C overnight. Each overnight culture was diluted 1:1000 in LB medium containing appropriate antibiotics and inducers (anhydrotetracycline hydrochloride, IPTG, and/or L-arabinose depending on the constructs and cell type) with inducer concentrations specified in each figure. These induced cultures were typically aerated at 37 °C for overnight unless otherwise specified in the figure. 400 μL of cells were harvested by centrifugation for 1 min at 13,000*g* and resuspended in 100 μL water. Samples were boiled at 98°C for 6 min and stored at -20°C until further analysis. Subsequently, 2-μL cell suspension (or 12.5 μL if cell culture was collected within 4 hours) was subjected to spacer detection PCR using primers described in Table 3.3 and Table 3.4., and PCR products were analyzed by agarose gel electrophoresis. Selected samples were SPRI bead-purified and sent to IGI Next-Generation Sequencing Core (UC Berkeley) for library prep and sequencing on an Illumina MiSeq. The plasmids used are described in Table

3.2 and Table 3.4.

### 3.3.3 Oligo protospacer electroporation

Oligo protospacer electroporation for adaptation assays was performed as previously described (Shipman et al., 2016) with modifications. Briefly, after inoculated cultures were grown with inducers with or without antibiotic selection (specified in each figure) at 37°C for 2 h, 1.5 mL of each culture was pelleted at 13,000*g* for 1.5 min at 4°C and re-suspended in 1 mL of ice-cold water. The pellet was washed twice more with ice-cold water, then pelleted a final time and re-suspended in 50 µL of a 3.2-µM solution of double-stranded oligonucleotides. Finally, the cell-oligo mixture was transferred to a 1mm gap cuvette and electroporated using a Bio-Rad MicroPulser Electroporator with Ec1 default settings. Only those conditions with an electroporation time constant > 4.0 ms were carried through to analysis. Immediately after electroporation, cells were transferred into a culture tube containing 3 mL of LB and grown for an additional 2 h or more (specified in each figure). A list of IDT-synthesized oligo protospacers used can be found in Table 3.3 and Table 3.4.

## 3.4 Results

### 3.4.1 Native V-C CRISPR locus in a plasmid did not lead to spacer acquisition

To attempt to reconstitute type V-C adaptation *in vivo*, I first cloned the three native V-C loci (Figure 3.1A) each containing Cas1, Cas12c, and CRISPR array and surrounding native sequences into a low-copy-number plasmid without changing any sequence except for reducing the number of repeat:spacer pairs in the array to ~6 (Figure 3.1B). Liquid culture of *E. coli* containing a native V-C locus in a plasmid was placed in a shaker at 37°C for overnight with antibiotic selection (Figure 3.1B). Afterwards, PCR was performed across the miniature array using a PCR primer set annealing to the 5' and 3' flanking sequences of the array, and the amplification products were analyzed by agarose gel electrophoresis to detect expanded arrays (Figure 3.1B). With this PCR strategy, the amplification products would contain both parental and expanded arrays. If spacer acquisition is reconstituted, a portion of the PCR product would be ~43 bp longer (for +1 new spacer), and I should observe a slower migration of expanded array on the agarose gel (Figure 3.1B).

In this experimental setup, the source of new spacers would derive from fragments of bacterial genome and plasmids. From experiments published on the *E. coli* type I-E CRISPR system under non-overexpressed conditions (Levy et al., 2015), I expected that with this PCR strategy and experimental setup, if there are expanded arrays, the events could be rare (~2% of the total lane volume). Additionally, for the nuclease-free orthologs, I hypothesized that acquiring self-spacers should not be detrimental to the host unless the spacer is from essential genes, which amount to only a small fraction of the genome (Gerdes et al., 2003; Martínez-Carranza et al., 2018). Therefore, I expected to observe expanded arrays in the DNase-free orthologs, VC-4 and V-C Osp, but not the DNase-active VC-6.

The result of the acquisition assay (Figure 3.1C) showed that there could be a faint band at expected +1 spacer position (+43 bp) on the agarose gel. Therefore, samples were sent for Next-Generation Sequencing. However, no expanded arrays were observed

after analyzing the sequencing data (Data not shown).

### 3.4.2 Codon-optimized V-C Cas1 and Cas12c induced at physiological expression levels did not lead to spacer acquisition

It was possible that the native sequences of the loci contained elements (such as promoter and ribosome binding site) that were too distant from *E. coli*, and *E. coli* was unable to express the protein or RNA required for adaptation. Therefore, I designed a two-plasmid expression system with the coding sequences for Cas1 and Cas12c being codon-optimized for *E. coli* (Figure 3.2). I aimed for physiological expression levels (not overexpression) for Cas 1 and Cas12c, which were under the control of synthetic promoters pLac and pTet, respectively (Figure 3.2). The tracrRNA was constitutively expressed from the same plasmid containing *Cas1* and *Cas12c*, while the CRIPSR array was under the control of another strong constitutive promoter on separate plasmid (Figure 3.2).

Agarose gel result showed that there were no expanded arrays (Figure 3.2) and that the shadows at expected +1 spacer (+43 bp) position existed even in uninduced conditions. Next-Generation Sequencing again confirmed the lack of new spacer acquisition under the conditions tested (Data not shown).

### 3.4.3 V-C Cas1 without Cas12c did not lead to spacer acquisition

To improve the assay setup, I used a minimal parental CRISPR array containing only one repeat:spacer sequence instead of the miniature array containing ~6 repeat:spacer pairs so that the expanded arrays, if existed, could be better observed when using agarose gel electrophoresis as the main detection method. In terms of experimental strategy, I tested if there was acquisition of new spacers occurring without the interference component, Cas12c. However, the results showed that after overnight incubation of the cultures with antibiotic selection, no expanded arrays were observed in agarose gel when there was no Cas12c encoded in the plasmids (Figure 3.3).

### 3.4.4 Overexpression of V-C proteins did not lead to spacer acquisition

Since overexpression of the *E. coli* type I-E CRISPR-Cas proteins Cas1 and Cas2 was shown to efficiently drive acquisition of new spacers (Levy et al., 2015; Shipman et al., 2016), I wondered if V-C Cas1 (and potentially Cas12c) should be overexpressed in order to observe adaptation *in vivo*. In this experiment, the expressions of Cas1 and Cas12c were under the control of separate T7 promoters on the same plasmid (Figure 3.4A). *E. coli* BL21-AI containing these inducible *Cas1* and *Cas12c* genes were induced for 2 hours before electroporating a 20-nt DNA duplex containing a native 18-nt spacer sequence and a TG PAM (Figure 3.4B). These 20-nt duplex oligos could serve as a source of new spacers, in addition to bacterial genome and plasmids. However, no expanded arrays were observed under the conditions tested (Figure 3.4C). Overexpression of Cas1 without Cas12c also did not result in expanded arrays under the conditions tested (Figure 3.5).

Although in type II-A systems, Cas9 with its tracrRNA is required for spacer acquisition (Heler et al., 2015), I explored the possibility that tracrRNA may be inhibitory for spacer acquisition in type V-C systems by performing the acquisition assay with Cas1 and Cas12c but without a tracrRNA (Figure 3.6A). Results from overnight expression

without antibiotic selection or when using the electroporation strategy without antibiotic selection suggest that these changes also did not allow for the reconstitution of type V-C spacer acquisition *in vivo* (Figure 3.6B and Figure 3.6C).

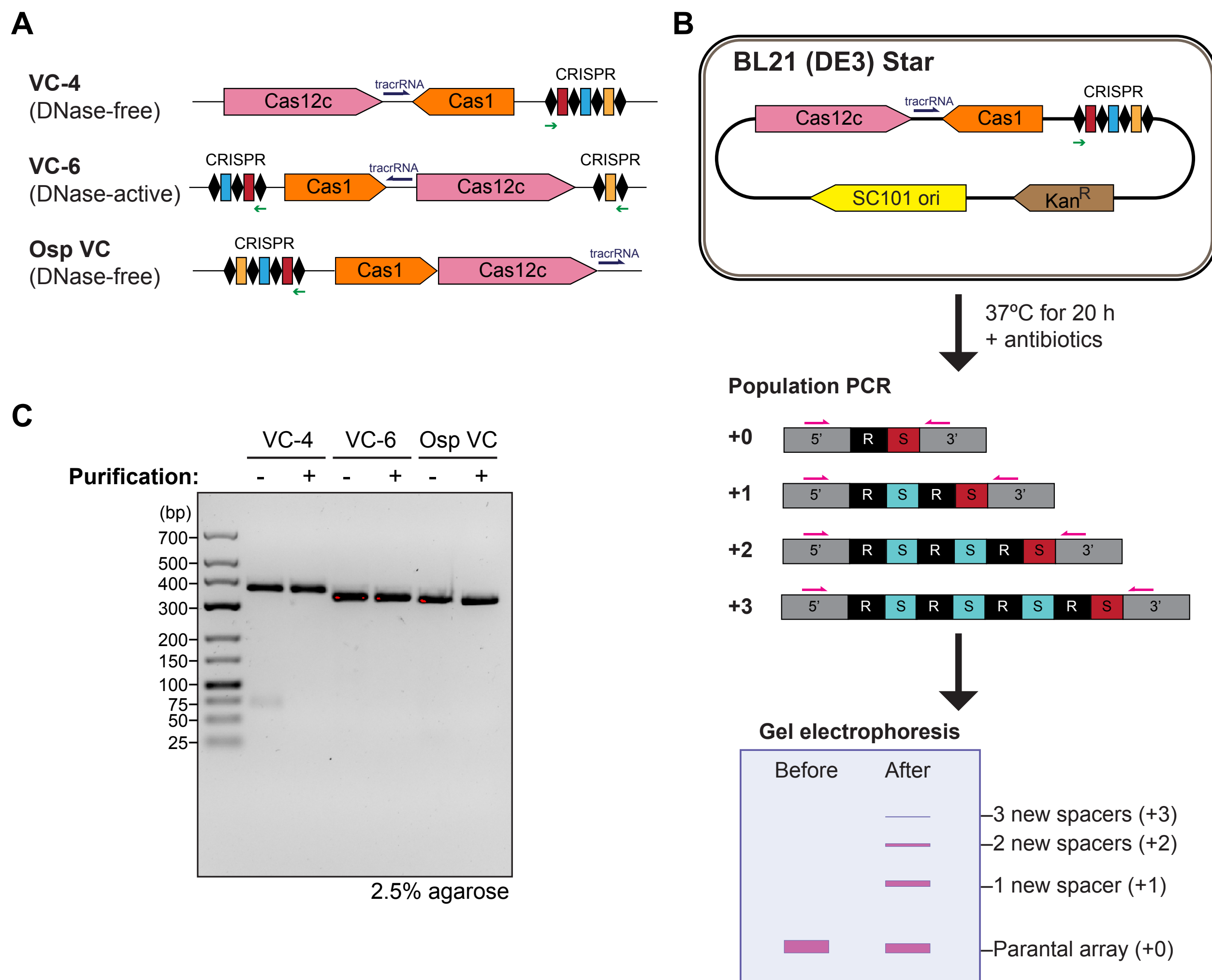
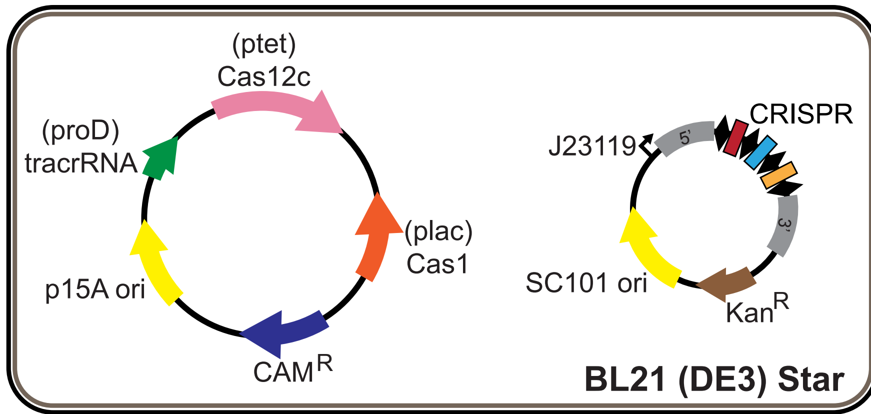


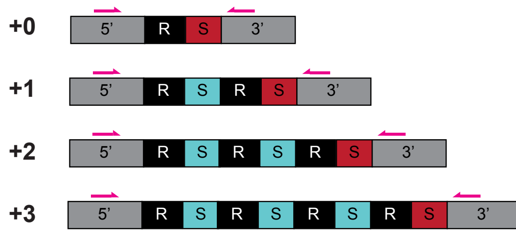

**Figure 3.1 Acquisition assay with V-C loci in native DNA sequences**

(A) Representation of the three V-C CRISPR-Cas systems with identified PAM for interference (Yan et al., 2019). Only two proteins, Cas1 and Cas12c, are encoded by these type V-C CRISPR loci. The status of DNase activity of each Cas12c effector was identified based on previous reports from Huang et al., 2022 and Kurihara et al., 2022. Osp = Oleiphilus sp. HI0009.
(B) Schematics of the experimental outline. The native sequence of each V-C locus was cloned into a single plasmid that was transformed into a heterologous *Escherichia coli* BL21(DE3) Star host. The miniature CRISPR array including ~6 repeat(R):spacer(S) sequences flanked by native neighboring sequences acted as the parental CRISPR array that could accept new spacer integration (blue rectangles in “Population PCR” section). After incubation at 37°C overnight with antibiotic selection, a PCR primer set annealing to the flanking sequences of the array were used to amplify the V-C arrays, and PCR products were analyzed by agarose gel electrophoresis to detect expanded arrays.
(C) Result of the acquisition assay described in Figure 3.1B. The amplicons of the parental arrays were 361 bp, 333 bp, and 328 bp for V-C (4), for V-C (6), and for V-C Osp,

respectively. Addition of 1 new spacer would add ~43 bp to the length of the original amplicon. Samples were SPRI bead-purified for Next-Generation Sequencing.




37ºC for 20 h
+ antibiotics + inducers



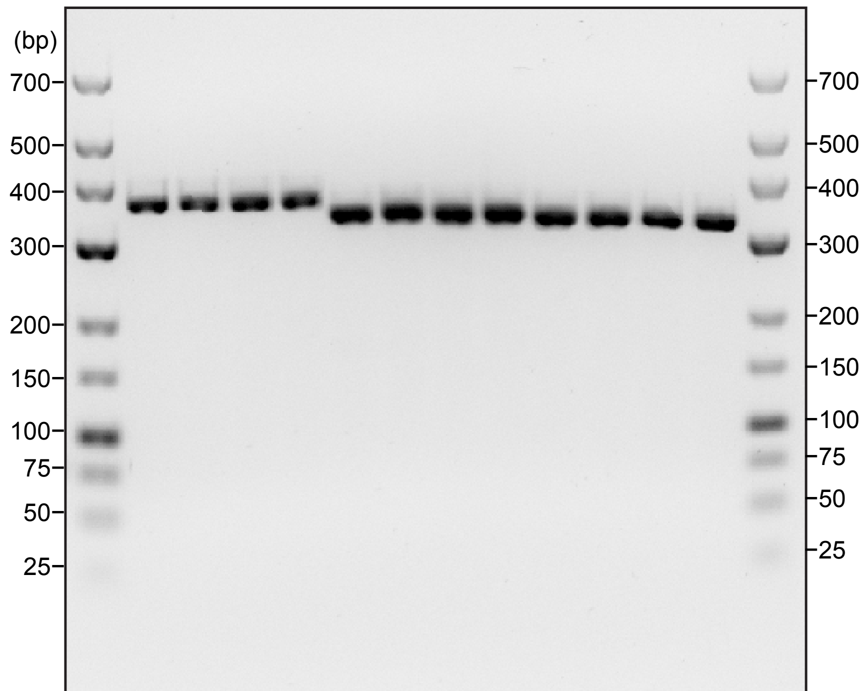
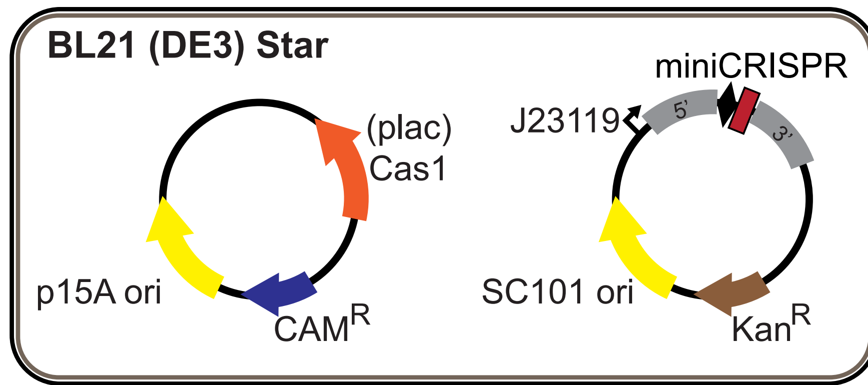
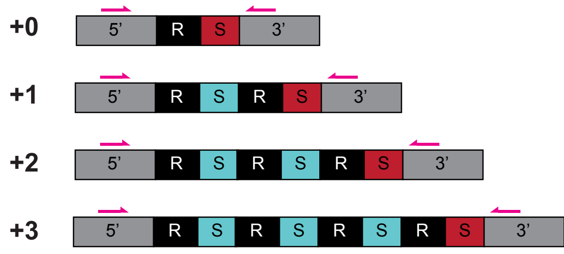



**Figure 3.2 Acquisition assay with *E. coli*-codon-optimized V-C Cas1 and Cas12c induced at physiological expression level**

Schematics of the experimental outline and outcomes. The coding sequences for Cas1 and Cas12c were *E. coli*-codon optimized and under the control of the isopropyl β-D-thiogalactoside (IPTG)-inducible Lac promoter and the anhydrotetracycline (aTc)-inducible Tet promoter, respectively. On the same plasmid, the expression of tracrRNA was under the control of a strong constitutive promoter, proD. The miniature CRISPR array, including 5' and 3' flanking native sequences, was constitutively expressed under the control of a J23119 promoter on a separate plasmid. DNA from overnight BL21(DE3) Star cultures containing these plasmids with appropriate antibiotics and various combinations of inducers was used as templates in PCR for analysis of spacer acquisition. Agarose gel electrophoresis result showed no difference between induced and uninduced samples. The amplicons of the parental arrays were 361 bp, 333 bp, and 328 bp for V-C (4), for V-C (6), and for V-C Osp, respectively. Addition of 1 new spacer would add ~43 bp to the length of the original amplicon.




37ºC for 17 h
+ antibiotics + 0.1 mM IPTG



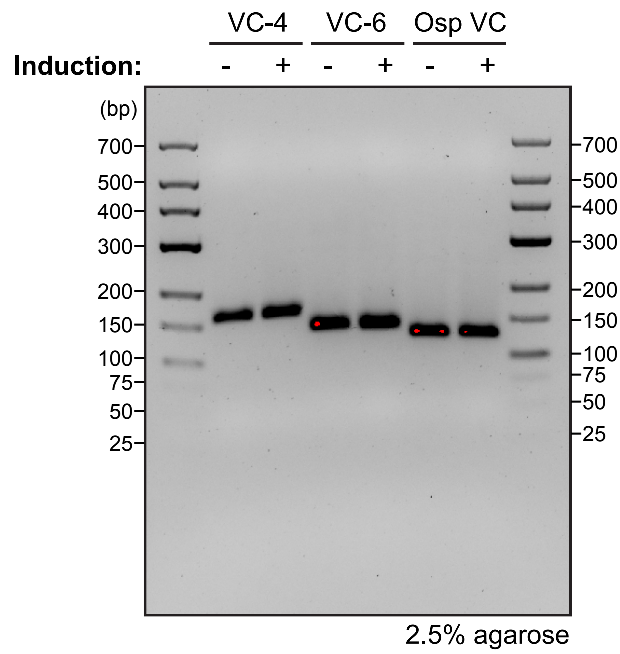




**Figure 3.3 Acquisition assay with V-C Cas1 only**

Schematics of the experimental outline and outcomes. The coding sequences for Cas1

orthologs were *E. coli*-codon optimized and were under the control of a Lac promoter. The minimal CRISPR array including only one repeat and spacer (flanked by native neighboring sequences) was constitutively expressed under the control of a J23119 promoter on a separate plasmid. DNA from overnight BL21(DE3) Star cultures containing these plasmids with appropriate antibiotics and 0.1 mM IPTG was used as templates in PCR for analysis of spacer acquisition. Agarose gel electrophoresis result showed no difference between induced and uninduced samples. The amplicons of the parental arrays were 159 bp, 137 bp, and 125 bp for V-C (4), for V-C (6), and for V-C Osp, respectively. Addition of 1 new spacer would add ~43 bp to the length of the original amplicon.

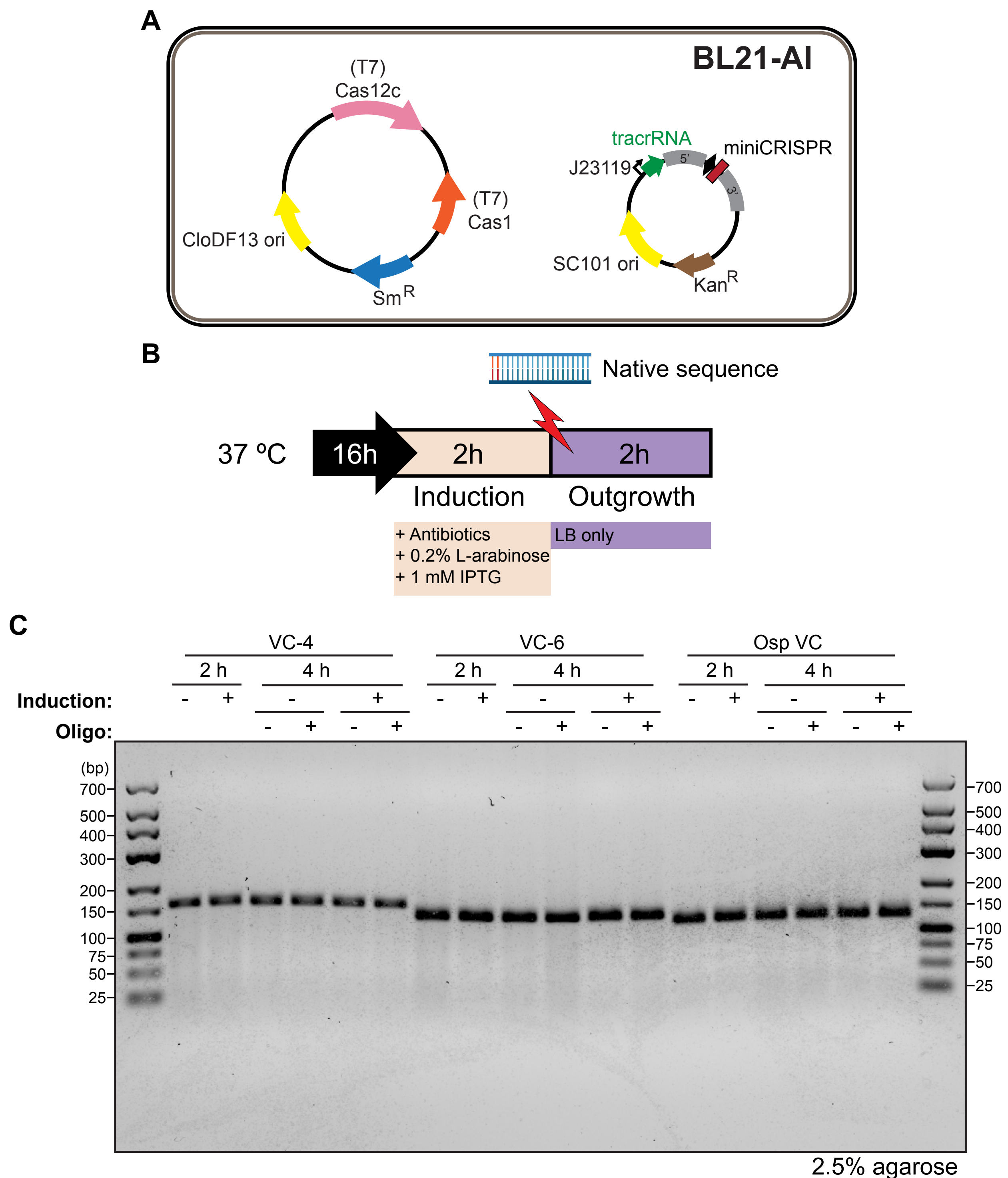



**Figure 3.4 Acquisition assay with overexpressed V-C Cas1 and Cas12c**

(A) The coding sequences for Cas1 and Cas12c were *E. coli*-codon optimized and cloned into pCDFDuet-1, under the control of separate T7 promoters. On the second plasmid, the tracrRNA and the minimal CRISPR array were constitutively expressed in one single

transcript under the control of a J23119 promoter.
(B) After BL21-AI cultures containing the plasmids described in Figure 3.4A were grown with 0.2% L-arabinose and 1 mM IPTG in the presence of appropriate antibiotics at 37°C for 2 h, cells were electroporated in solution containing double-stranded oligonucleotides. After electroporation, cells were grown for an additional 2 h in LB only. DNA from these samples collected at 2 h and 4 h was used as templates in PCR for analysis of spacer acquisition.
(C) Agarose gel electrophoresis result showed no difference between induced and uninduced samples at any time point, with or without oligo protospacer electroporated. The amplicons of the parental arrays were 159 bp, 137 bp, and 125 bp for V-C (4), for V-C (6), and for V-C Osp, respectively. Addition of 1 new spacer would add ~43 bp to the length of the original amplicon.

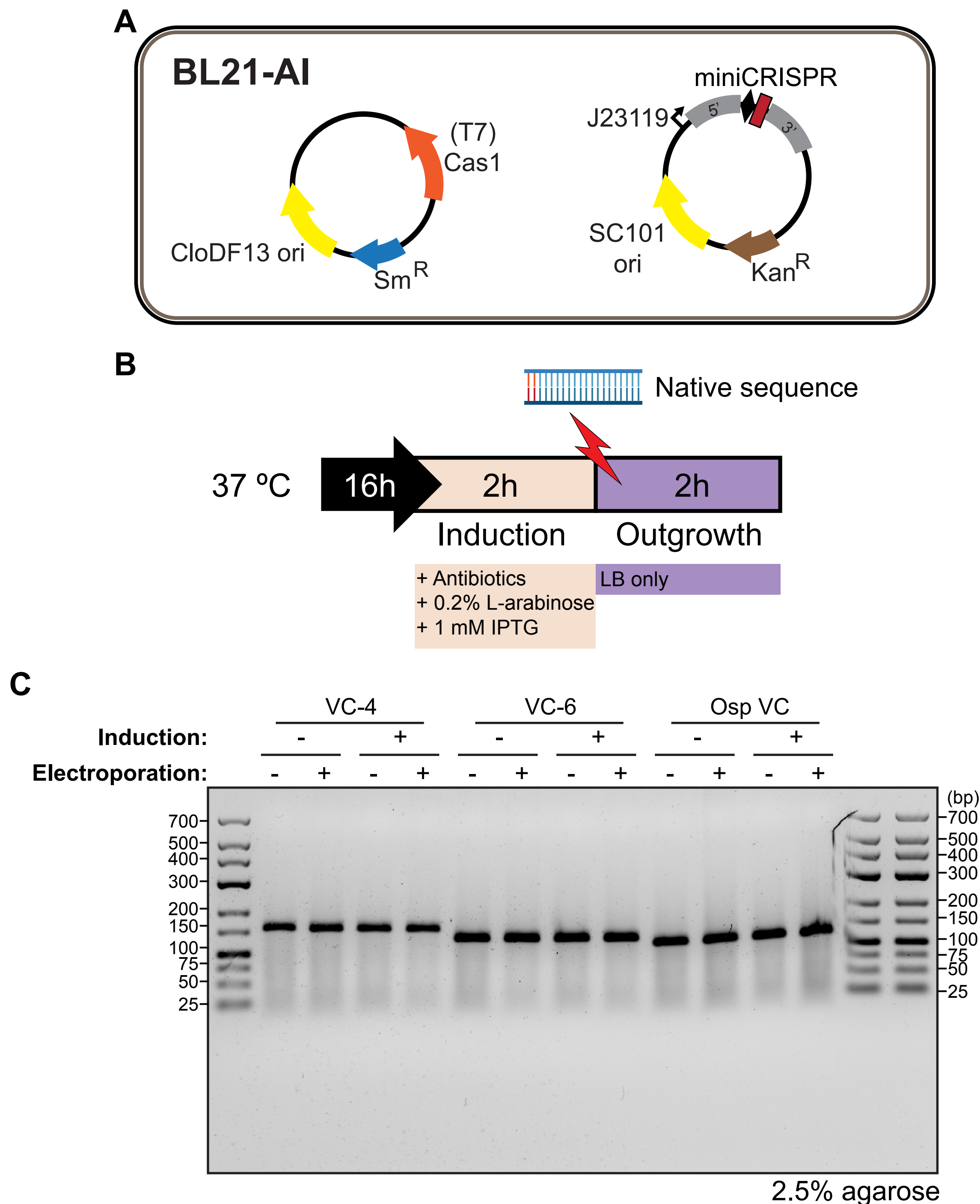



**Figure 3.5 Acquisition assay with overexpressed V-C Cas1 only**

(A) The coding sequences for Cas1 orthologs were *E. coli*-codon optimized and under the control of a T7 promoter. On the second plasmid, the minimal CRISPR array was constitutively expressed under the control of a J23119 promoter.
(B) After BL21-AI cultures containing the plasmids described in Figure 3.5A were grown with 0.2% L-arabinose and 1 mM IPTG in the presence of appropriate antibiotics at 37°C

for 2 h, cells were electroporated in solution containing double-stranded oligonucleotides. After electroporation, cells were grown for an additional 2 h in LB only. DNA from these samples collected at 4 h was used as templates in PCR for analysis of spacer acquisition. (C) Agarose gel electrophoresis result showed no difference between induced and uninduced samples at any time point, with or without oligo protospacer electroporated. The amplicons of the parental arrays were 159 bp, 137 bp, and 125 bp for V-C (4), for V-C (6), and for V-C Osp, respectively. Addition of 1 new spacer would add ~43 bp to the length of the original amplicon.

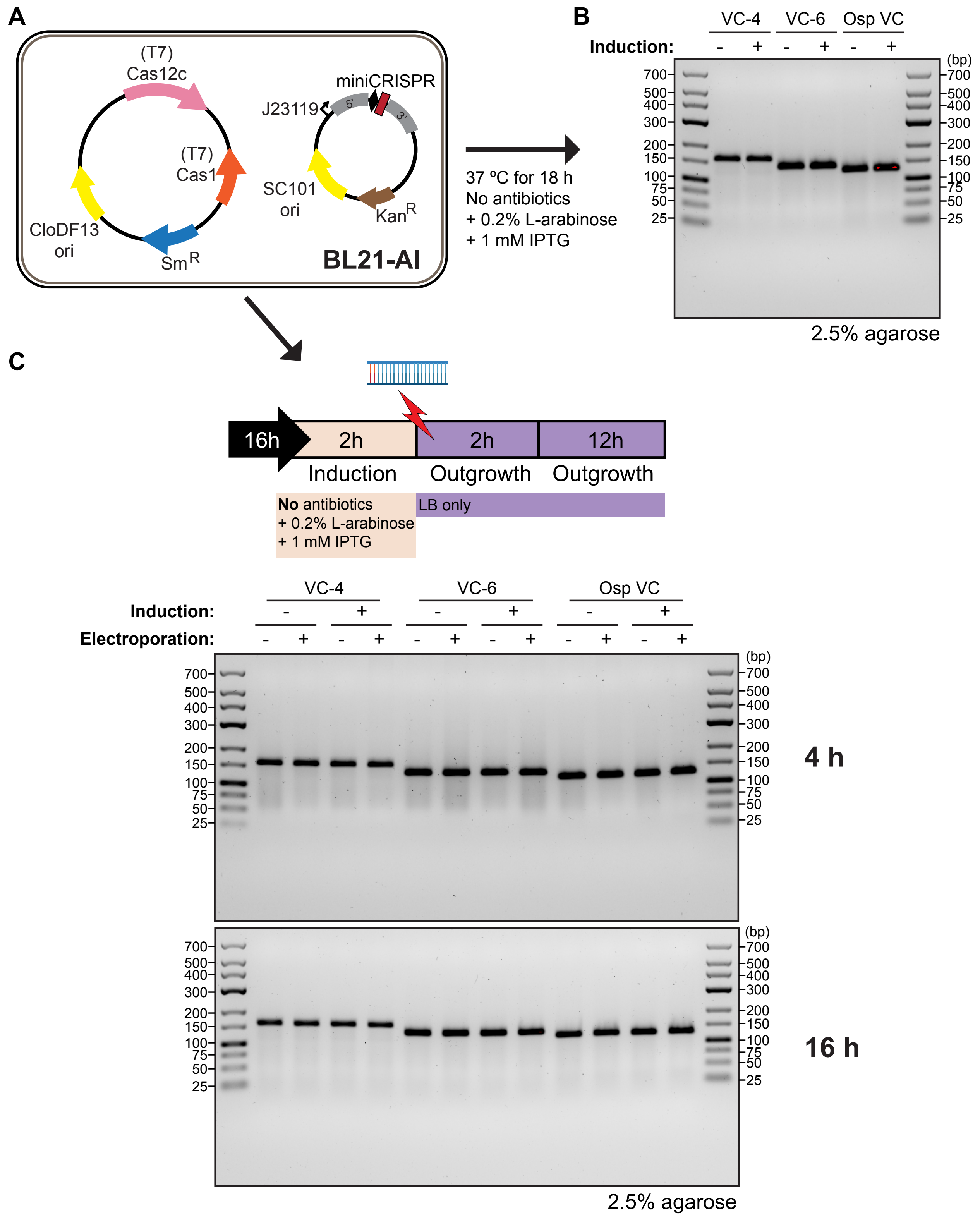



**Figure 3.6 Acquisition assay with overexpressed V-C Cas1 and Cas12c without tracrRNA**

(A) The coding sequences for Cas1 and Cas12c were *E. coli*-codon optimized and cloned into pCDFDuet-1, under the control of separate T7 promoters. On the second plasmid, the minimal CRISPR array was constitutively expressed under the control of a J23119 promoter.
(B) DNA from overnight BL21-AI cultures containing these plasmids described in Figure 3.6A with 0.2% L-arabinose and 1 mM IPTG (but no antibiotic selection) was used as templates in PCR for analysis of spacer acquisition. The result showed no difference between induced and uninduced samples.
(C) After BL21-AI cultures containing these plasmids described in Figure 3.6A were grown with 0.2% L-arabinose and 1 mM IPTG (in the absence of antibiotics) at 37°C for 2 h, cells were electroporated in solution containing double-stranded oligonucleotides. After electroporation, cells were grown for an additional 2 h or 14 h in LB only. DNA from these samples was used as templates in PCR for analysis of spacer acquisition. The results showed no difference between induced and uninduced samples at any time point, with or without oligo protospacer electroporated. The amplicons of the parental arrays were 159 bp, 137 bp, and 125 bp for V-C (4), for V-C (6), and for V-C Osp, respectively. Addition of 1 new spacer would add ~43 bp to the length of the original amplicon.

| **3.1A: Amino acid sequences of type V-C Cas1 and Cas12c** | | |
|---|---|---|
| **Protein** | **Amino Acid Sequence** | **Length (AA)** |
| Cas1 (VC-4) | MEISQFQHPDSIWTWKNIRGGRAALWIPYLNSIEPIQKSKAR<br>YKFVYKGGEVVCALKDIDFIMIYGGDAALSVGFLDDIGTHGV<br>VLAIHRRNMPRPLYLLPHTESDDTDILSAQILVRENLTRRCYV<br>ARTIIAQRIASFSWRIPIPGAVFKKLREARTLKSIRSIEAEWTK<br>RYWDAFYSNLSIDTTRRETHQVNSALDAGSKFLSGILLRWA<br>LFHKLSPTHGFLHEQTTYISLIYDLIEPYRIWIERAVDMAFQQ<br>TSNEDMLVATTISILKEMLLEDVYLPAFQKSTRRKNVLHAEV<br>LALRSYLAKETARLVIPVEGERIGGRPVKAGFAIPGAKK* | 336 |
| Cas12c_4 | MTKHSIPLHAFRNSGADARKWKGRIALLAKRGKETMRTLQF<br>PLEMSEPEAAAINTTPFAVAYNAIEGTGKGTLFDYWAKLHLA<br>GFRFFPSGGAATIFRQQAVFEDASWNAAFCQQSGKDWPW<br>LVPSKLYERFTKAPREVAKKDGSKKSIEFTQENVANESHVSL<br>VGASITDKTPEDQKEFFLKMAGALAEKFDSWKSANEDRIVA<br>MKVIDEFLKSEGLHLPSLENIAVKCSVETKPDNATVAWHDAP<br>MSGVQNLAIGVFATCASRIDNIYDLNGGKLSKLIQESATTPN<br>VTALSWLFGKGLEYFRTTDIDTIMQDFNIPASAKESIKPLVES<br>AQAIPTMTVLGKKNYAPFRPNFGGKIDSWIANYASRLMLLN<br>DILEQIEPGFELPQALLDNETLMSGIDMTGDELKELIEAVYAW<br>VDAAKQGLATLLGRGGNVDDAVQTFEQFSAMMDTLNGTLN<br>TISARYVRAVEMAGKDEARLEKLIECKFDIPKWCKSVPKLVG<br>ISGGLPKVEEEIKVMNAAFMDVRARMFVRFEEIAAYVASKG<br>AGMDVYDALEKRELEQLKKLKSAVPERAHIQAYRAVLHRIG<br>RAVQNCSEKTKQLFSSKVIEMGVFKNPSHLNNFIFNQKGAIY<br>RSPFDRSRHAPYQLHADKLLKNDWMELLAEISTTLMASEST<br>EQMEDALRLERTRLQLQLSGLPDWEYPASLAKPDIEVEIQT<br>ALKMQLAKDTVTSDVLQRTFNLYSSVLSGLTFKLLRRSFSLK<br>MRFSVADTTQLIYVPKDCDWAIPKQYLQAEGEIGIAARVVTE<br>SSPAKMVTEVEMKEPKALGHFMQQAPHDWYFDASLGGTQ<br>VAGRIVEKGKEVGKERKLVGYRMRGNSAYKTVLDKSLVGN<br>TELSQCSMIIEIPYTQTVDADFRAQVQAGLPKVSINLPVKETI<br>TASNKDEQMLFDRFVAIDLGERGLGYAVFDAKTLELQESGH<br>RPIKAITNLLNRTHHYEQRPNQRQKFQAKFNVNLSELRENT<br>VGDVCHQINRICAYYNAFPVLEYMVPDRLDKQLKSVYESVT<br>NRYIWSSTDAHKSARVQFWLGGETWEHPYLKSAKDKKPLV<br>LSPGRGASGKGTSQTCSCCGRNPFDLIKDMKPRAKIAVVD<br>GKAKLENSELKLFERNRESKDDMLARRHRNERAGMEQPLT<br>PGNYTVDEIKALLRANLRRAPKNRRTKDTTVSEYHCVFSDC<br>GKTMHADENAAVNIGGKFIADIEK* | 1218 |
| Cas1 (VC-6) | MDQGNQTIENQTINCDQHPDFLWTWKSNKRGSRVSVWLP<br>YFSQAKKIPRSKKWSVAYNGGSIEFDLKETDLIMFYGATGEL<br>PLEFLDDASKNGVMILIHRRNVLQPYVFYPSVIGDEEDILTKQ<br>IQFRTNERKRLYIAKTLIKKRLENMGSTIPISAPLLRQLSAAKS<br>IDEVRAIEANTTARYWNKWYENLNIETTRRKDHPINSALDAG<br>SKFIYGVILRWLVFHRFSPNHGFMHQPTSYPSLVYDLMEPF<br>RYMIENVCSAAWKRGERENSKIVALSLSFLKEELDKPCYVP<br>ATRQYVRKKNLLHGAVLALRSYLIGDMRKLVFPSEGVPNGG<br>RPIKASYKLPGSMYDVGRKPPEIKQKDEICFDEVSQEESEE* | 374 |

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| Cas12c_6 | MQTKKTHLHLISAKASRKYRRTIACLSDTAKKDLERRKQSGA ADPAQELSCLKTIKFKLEVPEGSKLPSFDRISQIYNALETIEK GSLSYLLFALILSGFRIFPNSSAAKTFASSSCYKNDQFASQIK EIFGEMVKNFIPSELESILKKGRRKNNKDWTEENIKRVLNSE FGRKNSEGSSALFDSFLSKFSQELFRKFDSWNEVNKKYLEA AELLDSMLASYGPFDSVCKMIGDSDSRNSLPDKSTIAFTNN AEITVDIESSVMPYMAIAALLREYRQSKSKAAPVAYVQSHLT TTNGNGLSWFFKFGLDLIRKAPVSSKQSTSDGSKSLQELFS VPDDKLDGLKFIKEACEALPEASLLCGEKGELLGYQDFRTSF AGHIDSWVANYVNRLFELIELVNQLPESIKLPSILTQKNHNLV ASLGLQEAEVSHSLELFEGLVKNVRQTLKKLAGIDISSSPNE QDIKEFYAFSDVLNRLGSIRNQIENAVQTAKKDKIDLESAIEW KEWKKLKKLPKLNGLGGGVPKQQELLDKALESVKQIRHYQR IDFERVIQWAVNEHCLETVPKFLVDAEKKKINKESSTDFAAK ENAVRFLLEGIGAAARGKTDSVSKAAYNWFVVNNFLAKKDL NRYFINCQGCIYKPPYSKRRSLAFALRSDNKDTIEVVWEKFE TFYKEISKEIEKFNIFSQEFQTFLHLENLRMKLLLRRIQKPIPA EIAFFSLPQEYYDSLPPNVAFLALNQEITPSEYITQFNLYSSF LNGNLILLRRSRSYLRAKFSWVGNSKLIYAAKEARLWKIPNA YWKSDEWKMILDSNVLVFDKAGNVLPAPTLKKVCEREGDL RLFYPLLRQLPHDWCYRNPFVKSVGREKNVIEVNKEGEPKV ASALPGSLFRLIGPAPFKSLLDDCFFNPLDKDLRECMLIVDQ EISQKVEAQKVEASLESCTYSIAVPIRYHLEEPKVSNQFENV LAIDQGEAGLAYAVFSLKSIGEAETKPIAVGTIRIPSIRRLIHSV STYRKKKQRLQNFKQNYDSTAFIMRENVTGDVCAKIVGLMK EFNAFPVLEYDVKNLESGSRQLSAVYKAVNSHFLYFKEPGR DALRKQLWYGGDSWTIDGIEIVTRERKEDGKEGVEKIVPLKV FPGRSVSARFTSKTCSCCGRNVFDWLFTEKKAKTNKKFNV NSKGELTTADGVIQLFEADRSKGPKFYARRKERTPLTKPIAK GSYSLEEIERRVRTNLRRAPKSKQSRDTSQSQYFCVYKDCA LHFSGMQADENAAINIGRRFLTALRKNRRSDFPSNVKISDRL LDN* | 1302 |
| Cas1 (VC-Osp) | MNDENKTKSHPNSIWYWCSGEKLWYLPYSGTPKRTSKSV WTVSGSFGQLDIDLYKTSHILFYGANETTGDLPLLFLSDCAK HSVSISIHRNHVSEPFVFYRNKTTDRDDLVTAQILHRENTKK SAYIARTIIRLQIKQRGYVIPSSFDDTKLLSSRNVDEVRTIEAT ASREYWRLYFKKLGLEGLGRRDDHVVVKALDATSHFLSGIM LRWVLSHELSPAHGFIHKSSSYPSLVYDLIEPLRWIPELAVF QSYLEFGEHRLLERSIERFKQMLDEPIHSEPTRQLVYRRTLI HGQVIALRHYLNGNMQRYLPPVEELAKERGRKRAVSYTLP GQIWKK* | 338 |
| OspCas12c | MTKLRHRQKKLTHDWAGSKKREVLGSNGKLQNPLLMPVKK GQVTEFRKAFSAYARATKGEMTDGRKNMFTHSFEPFKTKP SLHQCELADKAYQSLHSYLPGSLAHFLLSAHALGFRIFSKSG EATAFQASSKIEAYESKLASELACVDLSIQNLTISTLFNALTTS VRGKGEETSADPLIARFYTLLTGKPLSRDTQGPERDLAEVIS RKIASSFGTWKEMTANPLQSLQFFEEELHALDANVSLSPAF DVLIKMNDLQGDLKNRTIVFDPDAPVFEYNAEDPADIIIKLTA RYAKEAVIKNQNVGNYVKNAITTTNANGLGWLLNKGLSLLP VSTDDELLEFIGVERSHPSCHALIELIAQLEAPELFEKNVFSD | 1252 |

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|---|---|---|
| | TRSEVQGMIDSAVSNHIARLSSSRNSLSMDSEELERLIKSFQ IHTPHCSLFIGAQSLSQQLESLPEALQSGVNSADILLGSTQY MLTNSLVEESIATYQRTLNRINYLSGVAGQINGAIKRKAIDGE KIHLPAAWSELISLPFIGQPVIDVESDLAHLKNQYQTLSNEFD TLISALQKNFDLNFNKALLNRTQHFEAMCRSTKKNALSKPEI VSYRDLLARLTSCLYRGSLVLRRAGIEVLKKHKIFESNSELR EHVHERKHFVFVSPLDRKAKKLLRLTDSRPDLLHVIDEILQH DNLENKDRESLWLVRSGYLLAGLPDQLSSSFINLPIITQKGD RRLIDLIQYDQINRDAFVMLVTSAFKSNLSGLQYRANKQSFV VTRTLSPYLGSKLVYVPKDKDWLVPSQMFEGRFADILQSDY MVWKDAGRLCVIDTAKHLSNIKKSVFSSEEVLAFLRELPHRT FIQTEVRGLGVNVDGIAFNNGDIPSLKTFSNCVQVKVSRTNT SLVQTLNRWFEGGKVSPPSIQFERAYYKKDDQIHEDAAKRK IRFQMPATELVHASDDAGWTPSYLLGIDPGEYGMGLSLVSI NNGEVLDSGFIHINSLINFASKKSNHQTKVVPRQQYKSPYAN YLEQSKDSAAGDIAHILDRLIYKLNALPVFEALSGNSQSAAD QVWTKVLSFYTWGDNDAQNSIRKQHWFGASHWDIKGMLR QPPTEKKPKPYIAFPGSQVSSYGNSQRCSCCGRNPIEQLRE MAKDTSIKELKIRNSEIQLFDGTIKLFNPDPSTVIERRRHNLG PSRIPVADRTFKNISPSSLEFKELITIVSRSIRHSPEFIAKKRGI GSEYFCAYSDCNSSLNSEANAAANVAQKFQKQLFFEL* | |

| **3.1B: tracrRNA sequences of type V-C systems** | | |
|---|---|---|
| **System** | **tracrRNA sequence** | **Length (nt)** |
| VC-4 | AUACCACCCGUGCAUUUCUGGAUCAAUGAUCCGUACCU CAAUGUCCGGGCGCGCAGCUAGAGCGACCUGAAAUCU | 75 |
| VC-6 | AGAAACUCCCGACUUCCUCAGGAUGAAGUUGAUUCUUU AUCCAUACCUUGGUGCCGGGACGCCGAUUGAGGAAUG GGCGGCGCCUUCCAAAUUCUCAUUCCUCACU | 106 |
| VC-Osp | UCAUCGUUGGUAUGGCUGUGUUCUUCUGAACGCACAUC AUUCUCUUAGAGACGUCUACAUACCAACCCACGAUGGG GAGCGUGGACAACAAAAUCUCCCCACU | 103 |

| **3.1C: Repeat sequences of type V-C systems** | | |
|---|---|---|
| **System** | **Repeat sequence** | **Length (nt)** |
| VC-4 | AGCAGGAUUCAGGUUGGGUUUGAGG | 25 |
| VC-6 | CCCAUUAUUGGAAGGGUUUAUAAGG | 25 |
| VC-Osp | AGAAUUACUGAUGUUGUGAUGAAGGC | 26 |

**Table 3.1 Summary of amino acid and guide RNA sequences of type V-C CRISPR-Cas systems used in this study, Related to Methods**

| Plasmid ID/Name | Plasmid sequence |
|---|---|
| pCJH099_SC101_Native_VC-4_Kan | ATTACATCCGACAAACCAAGCTGCAAGGAGGACTTTGGCAGGTGctctcctgagtaggacaaatccgccgccctagacctagggtacgggttttgctgcccgcaa<br>acgggctgttctggtgttgctagtttgttatcagaatcgcagatccggcttcaggtttgccggctgaaagcgctatttcttccagaattgccatgatttttccccacgggaggcgtcactggctcccgtgt<br>tgtcggcagctttgattcgataagcagcatcgcctgtttcaggctgtctatgtgtgactgttgagctgtaacaagttgtctcaggtgttcaatttcatgttctagttgctttgttttactggtttcacctgttctatta<br>ggtgttacatgctgttcatctgttacattgtcgatctgttcatggtgaacagctttaaatgcaccaaaaactcgtaaaagctctgatgtatctatcttttttacaccgttttcatctgtgcatatggacagttttc<br>cctttgatatctaacggtgaacagttgttctacttttgtttgttagtcttgatgcttcactgatagatacaagagccataagaacctcagatccttccgtatttagccagtatgttctctagtgtggttcgttgtttt<br>tgcgtgagccatgagaacgaaccattgagatcatgcttactttgcatgtcactcaaaaattttgcctcaaaactggtgagctgaatttttgcagttaaagcatcgtgtagtgtttttcttagtccgttacgta<br>ggtaggaatctgatgtaatggttgttggtattttgtcaccattcatttttatctggttgttctcaagttcggttacgagatccatttgtctatctagttcaacttggaaaatcaacgtatcagtcgggcggcctc<br>gcttatcaaccaccaatttcatattgctgtaagtgtttaaatctttacttattggtttcaaaacccattggttaagccttttaaactcatggtagttattttcaagcattaacatgaacttaaattcatcaaggct<br>aatctctatatttgccttgtgagttttcttttgtgttagttcttttaataaccactcataaatcctcatagagtatttgttttcaaaagacttaacatgttccagattatattttatgaatttttttaactggaaaagata<br>aggcaatatctcttcactaaaaactaattctaatttttcgcttgagaacttggcatagtttgtccactggaaaatctcaaagcctttaaccaaaggattcctgatttccacagttctcgtcatcagctctctg<br>gttgctttagctaatacaccataagcattttccctactgatgttcatcatctgagcgtattggttataagtgaacgataccgtccgttctttccttgtagggttttcaatcgtggggttgagtagtgccacaca<br>gcataaaattagcttggtttcatgctccgttaagtcatagcgactaatcgctagttcatttgctttgaaaacaactaattcagacatacatctcaattggtctaggtgattttaatcactataccaattgaga<br>tgggctagtcaatgataattactagtccttttcctttgagttgtgggtatctgtaaattctgctagacctttgctggaaaacttgtaaattctgctagaccctctgtaaattccgctagacctttgtgtgttttttttg<br>tttatattcaagtggttataatttatagaataaagaaagaataaaaaaagataaaaagaatagatcccagccctgtgtataactcactactttagtcagttccgcagtattacaaaaggatgtcgca<br>aacgctgtttgctcctctacaaaacagaccttaaaaccctaaaggcttaagtagcaccctcgcaagctcgggcaaatcgctgaatattccttttgtctccgaccatcaggcacctgagtcgctgtctt<br>tttcgtgacattcagttcgctgcgctcacggctctggcagtgaatgggggtaaatggcactacaggcgccttttatggattcatgcaaggaaactacccataatacaagaaaagcccgtcacggg<br>cttctcagggcgttttatggcgggtctgctatgtggtgctatctgactttttgctgttcagcagttcctgccctctgattttccagtctgaccacttcggattatcccgtgacaggtcattcagactggctaatg<br>cacccagtaaggcagcggtatcatcaacaggcttacccgtcttactgtccctagtgTCAGATTTCTTACTTTATTCAGGCGATTACgtgttacaaccaattaaccaattctgatt<br>agaaaaactcatcgagcatcaaatgaaactgcaatttattcatatcaggattatcaataccatatttttgaaaaagccgtttctgtaatgaaggagaaaactcaccgaggcagttccataggatgg<br>caagatcctggtatcggtctgcgattccgactcgtccaacatcaatacaacctattaatttcccctcgtcaaaaataaggttatcaagtgagaaatcaccatgagtgacgactgaatccggtgaga<br>atggcaaaagcttatgcatttctttccagacttgttcaacaggccagccattacgctcgtcatcaaaatcactcgcatcaaccaaaccgttattcattcgtgattgcgcctgagcgaggcgaaatacg<br>cgatcgctgttaaaaggacaattacaaacaggaatcgaatgcaaccggcgcaggaacactgccagcgcatcaacaatattttcacctgaatcaggatattcttctaatacctggaatgctgttttc<br>ccggggatcgcagtggtgagtaaccatgcatcatcaggagtacggataaaatgcttgatggtcggaagaggcataaattccgtcagccagtttagtctgaccatctcatctgtaacatcattggca<br>acgctacctttgccatgtttcagaaacaactctggcgcatcgggcttcccatacaatcgatagattgtcgcacctgattgcccgacattatcgcgagcccatttatacccatataaatcagcatccat<br>gttggaatttaatcgcggcctggagcaagacgtttcccgttgaatatggctcataacaccccttgtattactgtttatgtaagcagacagttttattgttcatgatgatatatttttatcttgtgcaatgtaaca<br>tcagagattttgagacacaacgtggctttgttgaataaatcgaacttttgctgagttgaaggatcagCACCTGCCCGAACCAGAGACTTATCTCAGgttgcATCCGGAAT<br>TTGGCGGGGAATGTTTTGAATTTACGGCCTTCGTTGGAATAGAAAAAACCTGGTCACTCATAATAGTAAACCGGGTTGATTTATTTTA<br>TTCGAACAACTGAACTTGTTACCCTTATTATAATCTTTGCATAGAGTCACAAAACCATGAGCTGCTTTTCCATGCAGATACTGCGTGA<br>AGGGAGGGAAACCTTCCTCAAACCCACCTGAATACTACTTAGATTCAGCAACATGTTCCTCAAACCCAACCTTAATCCTGCTTTACC<br>ACATTCGAGTTTTCCTCAAACCCAACCTGAATCCTGCTCCAGCCGAGCACGCAGGCCCTCAAACCCAACCTGAATCCTGCTTAGGA<br>CGTTCCAAAATGCCTCAAACCCAACCTGAATCCTGCTCACCGTCCCTTATTTCGCCTCAAACCCAACCTGAATCCTGCTCGCAGGG<br>CGGAAAACCTTCCTCAAACCCAACCTGAATCCTGCTCGTTCATAATATAACATTATGTATTATCATGAAATTCATTTTTAACAGCCACC<br>CAGCTTGCATATGGAAATCTCACAATTTCAACATCCAGACTCTATTTGGACATGGAAAAACATCCGTGGTGGTCGAGCGGCACTTTG<br>GATACCCTACCTAAATTCGATTGAACCTATCCAAAAATCCAAAGCTCGCTATAAGTTCGTGTACAAAGGTGGTGAAGTTGTTTGTGC<br>TTTGAAGGATATCGACTTCATCATGATTTACGGCGGTGATGCGGCCCTCTCAGTGGGATTTCTGGATGATATTGGGACTCATGGGG<br>TGGTTCTTGCTATCCATCGTCGCAATATGCCTCGCCCTCTTTATTTGTTACCCCATACAGAATCGGATGACACCGATATTTTGTCAGC<br>TCAAATACTTGTCCGTGAGAACCTAACCCGCCGCTGCTATGTTGCTCGTACCATCATCGCACAACGCATCGCATCATTTTCTTGGCG<br>CATTCCTATTCCCGGGGCCGTTTTCAAGAAATTGCGAGAAGCGCGAACGCTCAAGTCTATTCGTTCCATTGAAGCCGAATGGACTA<br>AACGATACTGGGACGCTTTTTACTCCAATCTGTCAATCGATACTACTCGTCGAGAGACCCATCAGGTCAACAGCGCTCTGGATGCA<br>GGTTCAAAATTCTTGTCCGGCATTCTGTTGCGATGGGCTTTGTTCCACAAGCTATCGCCTACGCACGGTTTCCTGCATGAACAAACC<br>ACCTACATCTCGCTGATCTATGACTTGATTGAACCGTACAGAATATGGATTGAGCGTGCCGTGGATATGGCTTTCCAGCAAACATCC<br>AACGAGGACATGCTGGTTGCTACCACCATCTCTATTCTCAAAGAAATGCTTTTGGAGGATGTGTACCTTCCTGCATTCCAGAAAAGC<br>ACAAGGCGTAAAAATGTCTTGCATGCGGAAGTGTTGGCACTGCGCAGCTATCTCGCCAAGGAAACGGCAAGGCTTGTGATCCCAG<br>TCGAAGGTGAGCGTATCGGCGGTCGCCCGGTGAAAGCTGGCTTTGCCATCCCAGGCGCAAAGAAATAAACTCAAACTACAGGCAA<br>CAAAAAACCGGCTTTCGCCGGTTTTCGTGCAGATTTCAGGTCGCTCTAGCTGCGCGCCCGGACATTGAGGTACGGATCATTGATCC<br>AGAAATGCACGGGTGGTATGTGTGTATTATATCTTTTCTCGCACTTTGTGCAAACTATTTTTCGATATCTGCAATGAACTTTCCGCCG<br>ATATTCACTGCCGCATTCTCATCCGCGTGCATTGTCTTTCCGCAGTCTGAAAAGACACAGTGATACTCGCTTACGGTGGTGTCCTTG<br>GTGCGGCGATTCTTGGGCGCACGGCGCAGATTTGCACGCAGCAAAGCCTTGATCTCGTCGACCGTGTAATTGCCAGGCGTCAGC<br>GGCTGCTCCATCCCCGCTCGCTCATTGCGATGGCGACGAGCGAGCATATCGTCCTTGGACTCCCGGTTCCGTTCAAACAGTTTGA<br>GTTCGCTGTTCTCCAGTTTTGCTTTCCCGTCCACCACTGCGATCTTCGCTCTCGGTTTCATATCTTTGATCAGGTCGAACGGGTTCC<br>GGCCGCAGCAAGAACAGGTCTGGGATGTCCCCTTCCCGCTTGCTCCGCGCCCCGGCGACAGCACCAGTGGCTTCTTGTCCTTTG<br>CAGACTTCAGGTATGGGTGCTCCCATGTTTCTCCGCCCAGCCAAAACTGGACACGGGCAGATTTGTGGGCATCCGTCGATGACCA<br>GATGTAGCGGTTCGTAACCGATTCATAGACTGACTTGAGCTGTTTGTCCAAGCGGTCCGGTACCATGTATTCCAACACCGGAAAGG<br>CGTTGTAGTAAGCGCAGATGCGGTTGATCTGGTGACACACATCACCTACCGTGTTTTCGCGCAGTTCGGATAGGTTCACATTGAAC<br>TTTGCCTGGAACTTCTGGCGCTGGTTCGGACGCTGTTCATAGTGGTGGGTGCGATTGAGCAGATTGGTTATCGCCTTGATTGGGC<br>GATGACCGCTCTCCTGCAATTCGAGCGTTTTTGCATCAAACACTGCATACCCCAGACCGCGCTCACCCAGGTCGATCGCGACAAAG<br>CGGTCGAATAGCATCTGTTCGTCCTTATTGGACGCTGTGATTGTTTCCTTCACGGGCAGGTTGATGGACACCTTTGGCAGCCCGGC<br>CTGTACTTGTGCGCGGAAGTCCGCATCAACCGTCTGGGTGTACGGGATCTCGATGATCATCGAGCACTGACTGAGTTCGGTATTG<br>CCCACCAGCGACTTGTCCAACACAGTCTTGTAGGCAGAGTTCCCTCGCATTCTGTAGCCCACCAGCTTACGTTCTTTTCCGACTTC<br>CTTGCCTTTCTCGACAATGCGGCCGGCTACCTGTGTTCCGCCAAGCGAGGCATCGAAATACCAATCGTGTGGGGCTTGTTGCATG<br>AAATGGCCGAGCGCTTTTGGCTCTTTCATCTCAACTTCTGTCACCATTTTGGCCGGACTGCTTTCAGTGACCACACGCGCTGCAATA<br>CCGATCTCCCCTTCAGCCTGCAAGTATTGTTTGGGAATCGCCCAGTCGCAATCTTTTGGGACATAGATCAGTTGTGTCGTATCAGCA<br>ACGCTGAAGCGCATCTTCAGCGAGAACGAGCGGCGCAGCAGCTTGAAGGTCAGGCCAGACAGCACCGAGGAGTACAGATTGAAT<br>GTGCGTTGCAGCACATCCGAAGTCACTGTGTCTTTTGCCAGTTGCATCTTCAGCGCCGTCTGAATCTCGACTTCGATGTCCGGCTT<br>GGCCAAGCTTGCCGGGTATTCCCAATCCGGCAACCCGGACAGTTGCAGTTGCAGTCGTGTCCGTTCAAGACGCAGCGCATCTTCC<br>ATCTGTTCGGTCGATTCCGATGCCATCAAGGTAGTTGAGATCTCTGCCAGCAGTTCCATCCAATCATTCTTGAGCAGTTTGTCCGCA<br>TGGAGCTGATATGGCGCATGGCGAGACCGGTCGAAAGGAGAGCGATATATCGCGCCTTTCTGGTTGAAGATGAAGTTGTTCAGAT<br>GCGACGGATTTTTGAATACGCCCATCTCGATCACTTTGGACGAGAACAGTTGCTTGGTCTTTTCAGAACAGTTCTGGACGGCGCGG<br>CCAATACGGTGCAGAACAGCGCGGTAGGCTTGGATATGTGCCCGTTCCGGCACGGCAGACTTGAGCTTCTTAAGCTGTTCCAGCT<br>CTCGCTTCTCAAGCGCGTCATAGACATCCATCCCTGCGCCCTTGCTGGCCACATAAGCGGCGATCTCCTCGAATCTGACGAACATT<br>CTGGCACGCACATCCATGAATGCGGCATTCATCACCTTGATCTCTTCTTCGACCTTGGGCAGTCCACCTGAAATGCCGACAAGCTT<br>CGGTACCGACTTACACCATTTTGGGATGTCGAACTTGCACTCGATCAATTTTTCCAGTCTGGCCTCGTCCTTCCCCGCCATCTCGAC<br>AGCACGAACATAACGTGCGCTGATGGTATTCAAGGTCCCGTTCAGGGTATCCATCATTGCCGAGAACTGTTCGAATGTCTGTACCG<br>CATCGTCTACGTTGCCGCCCCTGCCCAGCAGGGTGGCCAATCCTTGTTTCGCTGCATCCACCCAGGCATAGACCGCCTCGATCAG<br>CTCTTTCAGCTCATCTCCGGTCATATCGATCCCGGACATCAGTGTCTCGTTGTCCAGTAGTGCTTGCGGCAATTCGAATCCCGGCT<br>CAATCTGCTCCAGAATGTCGTTCAGCAACATCAGGCGGGAAGCGTAGTTCGCGATCCATGAGTCGATTTTCCCGCCAAAGTTCGGA<br>CGGAACGGCGCGTAGTTCTTCTTCCCGAGCACAGTCATGGTCGGGATAGCCTGCGCGCTCTCCACCAACGGCTTGATCGATTCCT<br>TGGCTGATGCCGGGATATTGAAGTCCTGCATGATGGTATCGATGTCTGTGGTACGGAAATACTCCAGGCCTTTGCCGAACAACCAT<br>GAGAGTGCTGTGACGTTCGGGGTTGTGGCGCTTTCCTGGATGAGCTTGGACAGTTTTCCACCATTCAAATCGTAGATGTTGTCGAT<br>TCTGGATGCACAGGTTGCAAAAACCCCGATGGCGAGATTCTGCACGCCACTCATCGGAGCGTCGTGCCATGCCACGGTCGCATTA<br>TCAGGTTTCGTTTCCACAGAGCATTTGACTGCTATGTTTTCCAAGGAAGGCAGGTGCAGCCCTTCGCTTTTCAGAAATTCATCGATC 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|---|---|
| | ACCTTCATGGCGACGATGCGATCTTCATTGGCAGACTTCCATGAGTCGAACTTCTCTGCCAGTGCGCCGGCCATCTTCAGGAAGAA<br>TTCTTTCTGGTCTTCTGGTGTCTTGTCCGTGATGGATGCGCCTACCAGCGATACATGACTTTCGTTTGCCACGTTTTCTTGCGTGAA<br>TTCGATGCTCTTTTTGCTGCCGTCTTTCTTGGCCACCTCTCGCGGTGCCTTGGTGAAGCGCTCATAGAGTTTGCTTGGGACGAGCC<br>ACGGCCAATCTTTACCTGATTGCTGGCAGAACGCTGCATTCCAGCTCGCATCCTCGAATACCGCCTGCTGCCGGAAAATCGTCGCA<br>GCTCCACCGGACGGGAAGAAGCGGAATCCAGCCAGGTGCAGCTTCGCCCAGTAATCAAACAGGGTGCCTTTCCCTGTGCCTTCAA<br>TGGCGTTGTAGGCGACGGCGAATGGAGTGGTGTTGATGGCTGCTGCTTCAGGCTCGCTCATTTCGAGCGGAAACTGCAGTGTTCG<br>CATCGTTTCTTTGCCGCGCTTGGCCAGCAAAGCAATGCGGCCTTTCCATTTTCTGGCGTCTGCGCCGGAGTTGCGGAATGCGTGA<br>AGGGGTATTGAGTGTTTTGTCATTTCGTTTTGCTCTTTTTACTGGATACCAAAAATTCTACAGATACGTCTCATATTCCAAAGCGTTAT<br>TTACTGCCCGGCTACTTTCTTTCCTTGGGGATGTGGCACTTTCCCAGCGTCCACCTTCGCCCAGAATCCCGGCTTTGGTTTTACAAC<br>ACCCAGTTTTTTGCATCGCTTCCCAATC |
| pCJH100_SC101_Native_VC-6_Kan | 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|---|---|
| | CTTATCTGCGAGCTAAATTTAGTTGGGTGGGTAATAGTAAACTTATCTATGCGGCAAAAGAAGCTAGATTATGGAAAATTCCCAATG<br>CATATTGGAAATCGGACGAATGGAAGATGATCCTAGATTCTAATGTGCTGGTGTTTGATAAAGCCGGCAATGTTCTTCCGGCACCG<br>ACCCTGAAAAAGGTGTGTGAACGTGAAGGTGATCTGAGGCTTTTCTACCCACTTTTAAGACAACTCCCCCATGATTGGTGCTACAGA<br>AATCCCTTTGTGAAGAGCGTGGGCAGAGAAAAAAACGTCATTGAAGTTAACAAAGAAGGGGAACCTAAAGTTGCTTCGGCTTTACC<br>GGGATCTTTGTTCCGACTGATAGGTCCTGCACCTTTTAAATCTCTGCTGGATGATTGTTTCTTTAATCCTTTAGATAAGGACCTACGG<br>GAATGCATGCTTATTGTCGATCAGGAAATAAGCCAGAAGGTAGAAGCTCAGAAAGTGGAGGCCTCATTGGAATCATGTACTTATTCA<br>ATCGCAGTCCCGATTAGATACCACTTAGAGGAACCGAAGGTATCCAATCAATTTGAAAATGTTTTGGCTATTGATCAGGGAGAAGCC<br>GGACTTGCATATGCGGTGTTCTCTCTTAAGAGTATTGGTGAAGCGGAAACCAAGCCAATTGCGGTCGGTACAATCAGAATTCCGTC<br>CATTAGGCGATTGATTCATTCTGTCTCGACTTACAGAAAAAAGAAGCAGCGACTGCAAAACTTTAAGCAAAATTACGATTCAACAGC<br>CTTCATTATGCGTGAGAACGTGACAGGAGATGTATGCGCAAAGATTGTTGGTTTAATGAAGGAATTCAATGCGTTTCCAGTTCTGGA<br>ATATGACGTTAAAAATTTAGAGTCCGGAAGCAGACAGCTGTCAGCAGTCTATAAGGCTGTGAACTCCCACTTCTTGTACTTTAAGGA<br>GCCTGGCAGAGATGCATTAAGAAAGCAGCTTTGGTACGGCGGAGATTCTTGGACTATTGACGGAATTGAGATTGTGACAAGGGAG<br>AGGAAAGAGGACGGAAAAGAAGGGGTGGAAAAGATTGTTCCGCTGAAAGTTTTCCCCGGGCGTTCCGTTTCTGCTCGATTCACGA<br>GCAAGACCTGTTCCTGCTGCGGGAGGAATGTTTTTGATTGGCTCTTCACTGAAAAGAAAGCGAAGACAAATAAGAAGTTTAATGTAA<br>ACAGTAAAGGAGAACTTACGACTGCTGACGGCGTCATTCAGCTGTTTGAAGCAGATCGATCCAAAGGTCCTAAGTTTTATGCGAGA<br>AGAAAGGAGAGGACGCCCCTAACAAAGCCAATAGCAAAGGGCTCTTACTCTCTGGAAGAGATTGAAAGGCGTGTGCGCACAAATC<br>TAAGAAGAGCCCCTAAATCCAAGCAATCGAGAGATACGAGTCAGAGTCAATATTTTTGCGTTTATAAAGATTGTGCGCTTCATTTTTC<br>CGGCATGCAGGCAGACGAAAACGCTGCAATTAATATTGGGCGCCGGTTCCTTACTGCATTGAGAAAAAATCGCAGGAGTGATTTTC<br>CTTCGAACGTAAAGATATCTGACAGATTGTTA |
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| pCJH134_pCDFDuet_Cas12c-4_Cas1(VC-4)_Spec | 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TGGCGAAGGAGACTGCTCGCCTTGTCATTCCAGTAGAGGGAGAGCGTATCGGCGGACGCCCGGTGAAGGCAGGA<br>TTTGCAATTCCCGGCGCGAAAAAGTAATTAACCTAGGCTGCTGCCACCGCTGAGCAATAACTAGCATAACCCCTTGGGGCCTCTAA<br>ACGGGTCTTGAGGGGTTTTTTGCTGAAACCTCAGGCATTTGAGAAGCACACGGTCACACTGCTTCCGGTAGTCAATAAACCGGTAA<br>ACCAGCAATAGACATAAGCGGCTATTTAACGACCCTGCCCTGAACCGACGACCGGGTCATCGTGGCCGGATCTTGCGGCCCCTCG<br>GCTTGAACGAATTGTTAGACATTATTTGCCGACTACCTTGGTGATCTCGCCTTTCACGTAGTGGACAAATTCTTCCAACTGATCTGC<br>GCGCGAGGCCAAGCGATCTTCTTCTTGTCCAAGATAAGCCTGTCTAGCTTCAAGTATGACGGGCTGATACTGGGCCGGCAGGCGC<br>TCCATTGCCCAGTCGGCAGCGACATCCTTCGGCGCGATTTTGCCGGTTACTGCGCTGTACCAAATGCGGGACAACGTAAGCACTA<br>CATTTCGCTCATCGCCAGCCCAGTCGGGCGGCGAGTTCCATAGCGTTAAGGTTTCATTTAGCGCCTCAAATAGATCCTGTTCAGGA<br>ACCGGATCAAAGAGTTCCTCCGCCGCTGGACCTACCAAGGCAACGCTATGTTCTCTTGCTTTTGTCAGCAAGATAGCCAGATCAAT<br>GTCGATCGTGGCTGGCTCGAAGATACCTGCAAGAATGTCATTGCGCTGCCATTCTCCAAATTGCAGTTCGCGCTTAGCTGGATAAC<br>GCCACGGAATGATGTCGTCGTGCACAACAATGGTGACTTCTACAGCGCGGAGAATCTCGCTCTCTCCAGGGGAAGCCGAAGTTTC<br>CAAAAGGTCGTTGATCAAAGCTCGCCGCGTTGTTTCATCAAGCCTTACGGTCACCGTAACCAGCAAATCAATATCACTGTGTGGCTT<br>CAGGCCGCCATCCACTGCGGAGCCGTACAAATGTACGGCCAGCAACGTCGGTTCGAGATGGCGCTCGATGACGCCAACTACCTCT<br>GATAGTTGAGTCGATACTTCGGCGATCACCGCTTCCCTCATACTCTTCCTTTTTCAATATTATTGAAGCATTTATCAGGGTTATTGTC<br>TCATGAGCGGATACATATTTGAATGTATTTAGAAAAATAAACAAATAGCTAGCTCACTCGGTCGCTACGCTCCGGGCGTGAGACTGC<br>GGCGGGCGCTGCGGACACATACAAAGTTACCCACAGATTCCGTGGATAAGCAGGGGACTAACATGTGAGGCAAAACAGCAGGGC<br>CGCGCCGGTGGCGTTTTTCCATAGGCTCCGCCCTCCTGCCAGAGTTCACATAAACAGACGCTTTTCCGGTGCATCTGTGGGAGCC<br>GTGAGGCTCAACCATGAATCTGACAGTACGGGCGAAACCCGACAGGACTTAAAGATCCCCACCGTTTCCGGCGGGTCGCTCCCTC<br>TTGCGCTCTCCTGTTCCGACCCTGCCGTTTACCGGATACCTGTTCCGCCTTTCTCCCTTACGGGAAGTGTGGCGCTTTCTCATAGC<br>TCACACACTGGTATCTCGGCTCGGTGTAGGTCGTTCGCTCCAAGCTGGGCTGTAAGCAAGAACTCCCCGTTCAGCCCGACTGCTG<br>CGCCTTATCCGGTAACTGTTCACTTGAGTCCAACCCGGAAAAGCACGGTAAAACGCCACTGGCAGCAGCCATTGGTAACTGGGAG<br>TTCGCAGAGGATTTGTTTAGCTAAACACGCGGTTGCTCTTGAAGTGTGCGCCAAAGTCCGGCTACACTGGAAGGACAGATTTGGTT<br>GCTGTGCTCTGCGAAAGCCAGTTACCACGGTTAAGCAGTTCCCCAACTGACTTAACCTTCGATCAAACCACCTCCCCAGGTGGTTT<br>TTTCGTTTACAGGGCAAAAGATTACGCGCAGAAAAAAAGGATCTCAAGAAGATCCTTTGATCTTTTCTACTGAACCGCTCTAGATTTC<br>AGTGCAATTTATCTCTTCAAATGTAGCACCTGAAGTCAGCCCCATACGATATAAGTTGTAATTCTCATGTTAGTCATGCCCCGCGCC<br>CACCGGAAGGAGCTGACTGGGTTGAAGGCTCTCAAGGGCATCGGTCGAGATCCCGGTGCCTAATGAGTGAGCTAACTTACATTAA 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| | TTGCGTTGCGCTCACTGCCCGCTTTCCAGTCGGGAAACCTGTCGTGCCAGCTGCATTAATGAATCGGCCAACGCGCGGGGAGAGG<br>CGGTTTGCGTATTGGGCGCCAGGGTGGTTTTTCTTTTCACCAGTGAGACGGGCAACAGCTGATTGCCCTTCACCGCCTGGCCCTG<br>AGAGAGTTGCAGCAAGCGGTCCACGCTGGTTTGCCCCAGCAGGCGAAAATCCTGTTTGATGGTGGTTAACGGCGGGATATAACAT<br>GAGCTGTCTTCGGTATCGTCGTATCCCACTACCGAGATGTCCGCACCAACGCGCAGCCCGGACTCGGTAATGGCGCGCATTGCGC<br>CCAGCGCCATCTGATCGTTGGCAACCAGCATCGCAGTGGGAACGATGCCCTCATTCAGCATTTGCATGGTTTGTTGAAAACCGGAC<br>ATGGCACTCCAGTCGCCTTCCCGTTCCGCTATCGGCTGAATTTGATTGCGAGTGAGATATTTATGCCAGCCAGCCAGACGCAGACG<br>CGCCGAGACAGAACTTAATGGGCCCGCTAACAGCGCGATTTGCTGGTGACCCAATGCGACCAGATGCTCCACGCCCAGTCGCGTA<br>CCGTCTTCATGGGAGAAAATAATACTGTTGATGGGTGTCTGGTCAGAGACATCAAGAAATAACGCCGGAACATTAGTGCAGGCAGC<br>TTCCACAGCAATGGCATCCTGGTCATCCAGCGGATAGTTAATGATCAGCCCACTGACGCGTTGCGCGAGAAGATTGTGCACCGCC<br>GCTTTACAGGCTTCGACGCCGCTTCGTTCTACCATCGACACCACCACGCTGGCACCCAGTTGATCGGCGCGAGATTTAATCGCCG<br>CGACAATTTGCGACGGCGCGTGCAGGGCCAGACTGGAGGTGGCAACGCCAATCAGCAACGACTGTTTGCCCGCCAGTTGTTGTG<br>CCACGCGGTTGGGAATGTAATTCAGCTCCGCCATCGCCGCTTCCACTTTTTCCCGCGTTTTCGCAGAAACGTGGCTGGCCTGGTTC<br>ACCACGCGGGAAACGGTCTGATAAGAGACACCGGCATACTCTGCGACATCGTATAACGTTACTGGTTTCACATTCACCACCCTGAA<br>TTGACTCTCTTCCGGGCGCTATCATGCCATACCGCGAAAGGTTTTGCGCCATTCGATGGTGTCCGGGATCTCGACGCTCTCCCTTA<br>TGCGACTCCTGCATTAGGAAATTAATACGACTCACTATA |
| pCJH135_pCDFDuet_Cas12c-6_Cas1(VC-6)_Spec | GGGGAATTGTGAGCGGATAACAATTCCCCTGTAGAAATAATTTTGTTTAACTTTAATAAGGAGATATACCATGCAAACGAAAAAGAC<br>CCATCTGCATCTGATCTCGGCAAAAGCGAGTCGCAAGTACCGTCGTACGATTGCTTGCCTTAGTGATACTGCAAAAAAAGATCTGG<br>AACGTCGTAAGCAGTCCGGGGCGGCGGATCCTGCGCAAGAATTGAGTTGCTTGAAGACTATTAAATTCAAATTAGAAGTCCCCGAA<br>GGGTCTAAGCTTCCATCGTTTGACCGCATCAGTCAAATCTATAATGCACTGGAAACGATCGAAAAAGGGTCACTTTCTTACCTGTTG<br>TTTGCTTTAATCTTGTCGGGATTCCGCATTTTCCCGAACAGTAGCGCGGCTAAGACCTTCGCATCATCGTCGTGCTATAAGAATGAT<br>CAGTTCGCCTCACAAATTAAGGAAATCTTCGGGGAAATGGTAAAGAACTTCATCCCCTCTGAACTTGAGTCTATTCTGAAGAAGGGC<br>CGTCGCAAGAATAACAAAGATTGGACAGAAGAAAATATCAAGCGTGTGTTGAATTCAGAATTCGGACGTAAGAACAGCGAAGGCTC<br>TAGCGCTTTATTCGATTCGTTCCTGTCAAAGTTCAGTCAAGAGTTGTTTCGCAAATTTGACAGTTGGAACGAAGTTAACAAGAAGTAT<br>TTAGAAGCTGCCGAGCTTTTAGATTCTATGTTGGCCTCCTATGGTCCATTCGACAGCGTATGCAAAATGATTGGCGATTCGGACTCC<br>CGCAATTCACTTCCGGATAAATCCACCATTGCTTTTACCAACAACGCCGAGATCACTGTAGACATCGAATCCTCCGTCATGCCTTAC<br>ATGGCCATCGCTGCCTTACTGCGCGAATACCGCCAATCTAAGAGTAAAGCCGCGCCCGTTGCATACGTCCAATCTCATTTGACGAC<br>GACCAACGGAAACGGCCTGTCCTGGTTCTTCAAGTTTGGCTTAGACTTGATCCGCAAGGCACCAGTTTCAAGTAAGCAGTCAACAT<br>CCGATGGCTCTAAGTCTCTGCAGGAACTTTTCTCAGTACCCGACGATAAGCTGGATGGTCTTAAATTCATTAAGGAGGCTTGTGAAG<br>CGCTGCCAGAGGCGTCATTGTTGTGCGGAGAAAAAGGTGAACTGTTAGGATACCAAGACTTTCGTACTAGCTTTGCTGGGCATATT<br>GACTCTTGGGTCGCCAACTACGTGAATCGTTTATTTGAGTTGATCGAGTTAGTTAATCAATTGCCGGAGAGCATTAAGTTACCAAGT<br>ATCCTGACTCAGAAGAACCACAACTTGGTGGCTTCGTTAGGGCTGCAGGAAGCCGAGGTGAGCCACAGTCTTGAATTATTCGAAG<br>GGTTAGTTAAGAACGTCCGTCAAACACTGAAAAAGTTGGCAGGGATTGATATTTCATCGAGTCCAAATGAACAGGATATTAAAGAAT<br>TCTACGCGTTTTCCGATGTGTTGAATCGCCTTGGTTCAATCCGCAACCAGATTGAGAATGCGGTCCAGACCGCAAAAAAAGATAAAA<br>TCGACCTGGAGTCGGCTATTGAGTGGAAGGAATGGAAGAAATTAAAGAAGCTGCCGAAGCTTAATGGCCTTGGCGGTGGTGTACC<br>GAAGCAGCAGGAGTTGCTGGATAAGGCACTGGAGAGCGTCAAACAAATTCGCCATTATCAACGCATCGATTTCGAGCGCGTCATC<br>CAGTGGGCTGTTAACGAGCATTGCCTTGAAACCGTGCCCAAGTTTCTGGTTGACGCCGAGAAGAAAAAGATCAACAAAGAGTCATC<br>GACGGACTTCGCCGCTAAGGAAAACGCGGTCCGTTTTCTGTTGGAAGGCATTGGGGCAGCCGCGCGCGGGAAGACCGACAGTGT<br>CAGCAAAGCGGCCTACAACTGGTTTGTCGTCAATAACTTTCTTGCCAAAAAGGATTTAAATCGTTACTTTATTAATTGCCAGGGGTG<br>CATCTATAAGCCGCCATATTCCAAGCGCCGCAGCTTAGCGTTTGCCCTGCGCTCGGATAACAAGGACACAATCGAGGTAGTTTGGG<br>AGAAATTCGAAACTTTTTACAAAGAGATTTCAAAGGAAATTGAGAAATTTAACATCTTTTCTCAGGAGTTCCAAACTTTTCTGCACTTA<br>GAAAATCTTCGTATGAAGTTGCTGTTGCGCGTATCCAGAAACCCATCCCCGCCGAAATCGCGTTTTTTTCCTTACCACAAGAGTAT<br>TACGACTCACTTCCACCCAATGTAGCTTTCTTGGCCTTAAATCAGGAGATCACCCGAGCGAATACATCACACAATTTAATTTATACA<br>GCAGCTTTCTTAATGGAAACTTAATTTTGTTACGCCGTAGCCGTAGTTATCTTCGTGCTAAATTCAGTTGGGTCGGCAACAGCAAGC<br>TGATTTACGCGGCTAAGGAGGCACGTTTGTGGAAAATTCCAAACGCATACTGGAAGAGCGATGAATGGAAAATGATTTTAGACTCA<br>AACGTCTTAGTGTTCGACAAAGCAGGCAATGTTCTGCCCGCTCCTACTTTGAAAAAGGTGTGTGAGCGTGAGGGCGATCTTCGCTT<br>GTTTTATCCGCTGCTGCGTCAGTTGCCGCATGACTGGTGCTACCGTAACCCCTTTGTCAAGTCTGTGGGCCGTGAAAAAAACGTAA<br>TCGAAGTCAACAAGGAAGGAGAACCTAAAGTCGCGTCGGCTTTGCCCGGTTCTCTTTTCCGCCTGATTGGACCGGCCCCCTTTAAA<br>AGTTTGCTTGATGATTGTTTCTTCAATCCTCTGGATAAAGACTTGCGTGAATGTATGTTAATTGTGGACCAAGAAATCTCTCAAAAAG<br>TAGAGGCGCAGAAAGTCGAAGCTTCTCTGGAATCTTGCACATATAGTATCGCGGTGCCAATCCGTTACCACCTTGAGGAACCCAAA<br>GTGAGTAATCAATTCGAGAATGTACTGGCTATTGATCAAGGAGAGGCAGGACTGGCCTATGCGGTATTTAGTTTAAAAAGCATTGGT<br>GAAGCCGAAACTAAGCCCATTGCCGTCGGAACCATCCGTATTCCGTCCATTCGCCGCCTTATTCATAGTGTATCAACCTATCGCAAA<br>AAGAAACAGCGTTTGCAAAATTTCAAGCAGAATTATGATAGCACTGCCTTCATTATGCGCGAGAACGTGACAGGCGATGTATGTGC<br>CAAAATCGTCGGCCTGATGAAAGAGTTCAACGCATTCCCGGTCCTGGAATACGATGTGAAGAATTTGGAGTCCGGGTCTCGTCAGT<br>TAAGTGCAGTATACAAAGCAGTTAACAGCCACTTCTTGTATTTTAAAGAGCCAGGTCGCGATGCCTTGCGTAAGCAACTTTGGTACG<br>GAGGTGATTCATGGACGATTGACGGAATCGAGATTGTCACCCGTGAACGCAAAGAAGACGGAAAAGAAGGTGTTGAAAAAATCGTA<br>CCGCTGAAGGTGTTCCCAGGACGTTCAGTAAGCGCACGCTTTACATCTAAAACTTGCTCCTGCTGTGGTCGTAATGTGTTTGATTG<br>GCTGTTTACGGAAAAGAAGGCAAAAACCAATAAAAAGTTCAATGTTAACTCCAAAGGCGAATTGACCACAGCTGATGGAGTCATCCA<br>ACTGTTCGAAGCAGATCGCAGTAAGGGTCCAAAGTTTTACGCTCGTCGTAAAGAACGTACACCACTGACAAAACCCATTGCTAAGG<br>GTTCGTATTCCCTTGAAGAAATTGAACGCCGCGTCCGTACCAACTTGCGTCGTGCACCTAAAAGCAAACAGTCCCGTGATACCTCT<br>CAGTCTCAGTATTTCTGCGTGTATAAGGATTGTGCTCTGCACTTCTCCGGCATGCAGGCAGATGAAAATGCAGCAATTAACATTGGC<br>CGCCGCTTCCTGACTGCATTGCGCAAAAACCGTCGCAGCGATTTCCCGTCAAACGTTAAAATTTCTGACCGTTTATTAGATAATTAA<br>TGCTTAAGTCGAACAGAAAGTAATCGTATTGTACACGGCCGCATAATCGAAATTAATACGACTCACTATAGGGGAATTGTGAGCGGA<br>TAACAATTCCCCATCTTAGTATATTAGTTAAGTATAAGAAGGAGATATACATATGGACCAAGGCAACCAAACGATCGAAAACCAGAC<br>AATCAACTGCGACCAACACCCCGACTTCTTATGGACTTGGAAATCAAATAAACGTGGAAGCCGCGTTTCAGTGTGGTTACCCTACTT<br>TTCCCAAGCGAAAAAAATCCCGCGTTCTAAAAAGTGGAGTGTAGCTTACAACGGTGGTTCAATCGAGTTCGACCTGAAGGAAACAG<br>ACTTAATCATGTTTTACGGAGCTACAGGCGAGTTACCGCTTGAATTTCTGGACGACGCAAGTAAAAATGGCGTCATGATTCTGATTC<br>ACCGCCGTAACGTGCTGCAGCCCTATGTCTTTTATCCGAGCGTGATCGGGGATGAGGAAGATATTTTAACAAAGCAGATTCAATTTC<br>GTACCAACGAGCGTAAACGTCTTTATATTGCTAAAACGTTGATTAAGAAACGCTTAGAGAACATGGGCTCAACTATCCCGATCTCTG<br>CACCATTGTTGCGTCAATTATCTGCAGCCAAGTCGATCGACGAAGTGCGCGCTATCGAAGCCAACACAACCGCGCGCTACTGGAAT<br>AAGTGGTATGAAAACTTGAACATTGAAACAACTCGTCGTAAGGATCACCCTATCAATTCCGCCCTGGACGCCGGGTCAAAATTCATT<br>TACGGAGTGATTCTTCGTTGGTTGGTGTTCCATCGTTTCAGCCCCAACCATGGATTCATGCACCAACCTACATCGTATCCATCCTTG<br>GTGTACGACTTGATGGAGCCTTTTCGTTACATGATCGAGAACGTCTGTAGCGCCGCGTGGAAACGTGGGGAACGTGAGAACAGCA<br>AAATCGTCGCGTTGAGCCTTTCGTTCCTGAAAGAAGAGTTGGACAAGCCCTGCTACGTGCCCGCGACCCGCCAATACGTTCGTAA<br>GAAAAATCTTTTGCATGGCGCAGTTCTTGCTCTTCGCAGTTACCTTATTGGAGATATGCGTAAGTTAGTTTTTCCCTCGGAAGGAGT<br>TCCTAATGGGGGTCGCCCAATTAAAGCTTCATATAAATTGCCCGGTTCCATGTATGATGTTGGACGCAAGCCTCCCGAGATCAAAC<br>AAAAGGATGAGATCTGTTTCGATGAGGTGAGCCAGGAAGAATCCGAAGAATGATTAACCTAGGCTGCTGCCACCGCTGAGCAATAA<br>CTAGCATAACCCCTTGGGGCCTCTAAACGGGTCTTGAGGGGTTTTTTGCTGAAACCTCAGGCATTTGAGAAGCACACGGTCACACT<br>GCTTCCGGTAGTCAATAAACCGGTAAACCAGCAATAGACATAAGCGGCTATTTAACGACCCTGCCCTGAACCGACGACCGGGTCAT<br>CGTGGCCGGATCTTGCGGCCCCTCGGCTTGAACGAATTGTTAGACATTATTTGCCGACTACCTTGGTGATCTCGCCTTTCACGTAG<br>TGGACAAATTCTTCCAACTGATCTGCGCGCGAGGCCAAGCGATCTTCTTCTTGTCCAAGATAAGCCTGTCTAGCTTCAAGTATGACG<br>GGCTGATACTGGGCCGGCAGGCGCTCCATTGCCCAGTCGGCAGCGACATCCTTCGGCGCGATTTTGCCGGTTACTGCGCTGTAC<br>CAAATGCGGGACAACGTAAGCACTACATTTCGCTCATCGCCAGCCCAGTCGGGCGGCGAGTTCCATAGCGTTAAGGTTTCATTTAG<br>CGCCTCAAATAGATCCTGTTCAGGAACCGGATCAAAGAGTTCCTCCGCCGCTGGACCTACCAAGGCAACGCTATGTTCTCTTGCTT 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| | TTGTCAGCAAGATAGCCAGATCAATGTCGATCGTGGCTGGCTCGAAGATACCTGCAAGAATGTCATTGCGCTGCCATTCTCCAAAT<br>TGCAGTTCGCGCTTAGCTGGATAACGCCACGGAATGATGTCGTCGTGCACAACAATGGTGACTTCTACAGCGCGGAGAATCTCGC<br>TCTCTCCAGGGGAAGCCGAAGTTTCCAAAAGGTCGTTGATCAAAGCTCGCCGCGTTGTTTCATCAAGCCTTACGGTCACCGTAACC<br>AGCAAATCAATATCACTGTGTGGCTTCAGGCCGCCATCCACTGCGGAGCCGTACAAATGTACGGCCAGCAACGTCGGTTCGAGAT<br>GGCGCTCGATGACGCCAACTACCTCTGATAGTTGAGTCGATACTTCGGCGATCACCGCTTCCCTCATACTCTTCCTTTTTCAATATT<br>ATTGAAGCATTTATCAGGGTTATTGTCTCATGAGCGGATACATATTTGAATGTATTTAGAAAAATAAACAAATAGCTAGCTCACTCGG<br>TCGCTACGCTCCGGGCGTGAGACTGCGGCGGGCGCTGCGGACACATACAAAGTTACCCACAGATTCCGTGGATAAGCAGGGGAC<br>TAACATGTGAGGCAAAACAGCAGGGCCGCGCCGGTGGCGTTTTTCCATAGGCTCCGCCCTCCTGCCAGAGTTCACATAAACAGAC<br>GCTTTTCCGGTGCATCTGTGGGAGCCGTGAGGCTCAACCATGAATCTGACAGTACGGGCGAAACCCGACAGGACTTAAAGATCCC<br>CACCGTTTCCGGCGGGTCGCTCCCTCTTGCGCTCTCCTGTTCCGACCCTGCCGTTTACCGGATACCTGTTCCGCCTTTCTCCCTTA<br>CGGGAAGTGTGGCGCTTTCTCATAGCTCACACACTGGTATCTCGGCTCGGTGTAGGTCGTTCGCTCCAAGCTGGGCTGTAAGCAA<br>GAACTCCCCGTTCAGCCCGACTGCTGCGCCTTATCCGGTAACTGTTCACTTGAGTCCAACCCGGAAAAGCACGGTAAAACGCCACT<br>GGCAGCAGCCATTGGTAACTGGGAGTTCGCAGAGGATTTGTTTAGCTAAACACGCGGTTGCTCTTGAAGTGTGCGCCAAAGTCCG<br>GCTACACTGGAAGGACAGATTTGGTTGCTGTGCTCTGCGAAAGCCAGTTACCACGGTTAAGCAGTTCCCCAACTGACTTAACCTTC<br>GATCAAACCACCTCCCCAGGTGGTTTTTTCGTTTACAGGGCAAAAGATTACGCGCAGAAAAAAAGGATCTCAAGAAGATCCTTTGAT<br>CTTTTCTACTGAACCGCTCTAGATTTCAGTGCAATTTATCTCTTCAAATGTAGCACCTGAAGTCAGCCCCATACGATATAAGTTGTAA<br>TTCTCATGTTAGTCATGCCCCGCGCCCACCGGAAGGAGCTGACTGGGTTGAAGGCTCTCAAGGGCATCGGTCGAGATCCCGGTGC<br>CTAATGAGTGAGCTAACTTACATTAATTGCGTTGCGCTCACTGCCCGCTTTCCAGTCGGGAAACCTGTCGTGCCAGCTGCATTAAT<br>GAATCGGCCAACGCGCGGGGAGAGGCGGTTTGCGTATTGGGCGCCAGGGTGGTTTTTCTTTTCACCAGTGAGACGGGCAACAGC<br>TGATTGCCCTTCACCGCCTGGCCCTGAGAGAGTTGCAGCAAGCGGTCCACGCTGGTTTGCCCCAGCAGGCGAAAATCCTGTTTGA<br>TGGTGGTTAACGGCGGGATATAACATGAGCTGTCTTCGGTATCGTCGTATCCCACTACCGAGATGTCCGCACCAACGCGCAGCCC<br>GGACTCGGTAATGGCGCGCATTGCGCCCAGCGCCATCTGATCGTTGGCAACCAGCATCGCAGTGGGAACGATGCCCTCATTCAGC<br>ATTTGCATGGTTTGTTGAAAACCGGACATGGCACTCCAGTCGCCTTCCCGTTCCGCTATCGGCTGAATTTGATTGCGAGTGAGATAT<br>TTATGCCAGCCAGCCAGACGCAGACGCGCCGAGACAGAACTTAATGGGCCCGCTAACAGCGCGATTTGCTGGTGACCCAATGCGA<br>CCAGATGCTCCACGCCCAGTCGCGTACCGTCTTCATGGGAGAAAATAATACTGTTGATGGGTGTCTGGTCAGAGACATCAAGAAAT<br>AACGCCGGAACATTAGTGCAGGCAGCTTCCACAGCAATGGCATCCTGGTCATCCAGCGGATAGTTAATGATCAGCCCACTGACGC<br>GTTGCGCGAGAAGATTGTGCACCGCCGCTTTACAGGCTTCGACGCCGCTTCGTTCTACCATCGACACCACCACGCTGGCACCCAG<br>TTGATCGGCGCGAGATTTAATCGCCGCGACAATTTGCGACGGCGCGTGCAGGGCCAGACTGGAGGTGGCAACGCCAATCAGCAA<br>CGACTGTTTGCCCGCCAGTTGTTGTGCCACGCGGTTGGGAATGTAATTCAGCTCCGCCATCGCCGCTTCCACTTTTTCCCGCGTTT<br>TCGCAGAAACGTGGCTGGCCTGGTTCACCACGCGGGAAACGGTCTGATAAGAGACACCGGCATACTCTGCGACATCGTATAACGT<br>TACTGGTTTCACATTCACCACCCTGAATTGACTCTCTTCCGGGCGCTATCATGCCATACCGCGAAAGGTTTTGCGCCATTCGATGGT<br>GTCCGGGATCTCGACGCTCTCCCTTATGCGACTCCTGCATTAGGAAATTAATACGACTCACTATA |
| pCJH136_pCDFDuet_OspCas12c_Cas1(OspVC)_Spec | GGGGAATTGTGAGCGGATAACAATTCCCCTGTAGAAATAATTTTGTTTAACTTTAATAAGGAGATATACCATGACCAAGCTGCGTCA<br>TCGTCAGAAGAAGCTGACACATGATTGGGCAGGTTCGAAGAAACGCGAAGTTCTTGGATCCAATGGTAAGTTGCAAAATCCCCTTT<br>TAATGCCCGTGAAGAAGGGACAGGTAACTGAATTTCGCAAAGCATTCTCGGCTTACGCGCGTGCTACAAAGGGAGAGATGACGGA<br>TGGTCGCAAAAATATGTTTACGCACAGCTTCGAACCATTCAAAACAAAACCTTCTTTGCACCAGTGCGAGCTTGCAGATAAAGCATA<br>TCAATCTTTACATAGCTACCTGCCGGGTAGTCTGGCGCACTTTTTACTTTCAGCGCATGCGTTAGGCTTTCGTATCTTTTCTAAGAGT<br>GGCGAAGCAACTGCATTTCAAGCTTCATCAAAGATCGAAGCGTATGAGAGTAAATTAGCCAGCGAACTTGCGTGTGTAGACTTGTC<br>TATCCAAAATTTGACTATTTCGACGTTATTTAATGCACTTACAACTAGTGTCCGCGGCAAAGGCGAAGAAACGTCGGCAGACCCTTT<br>GATTGCACGTTTTTATACATTGCTTACAGGAAAGCCCCTGAGCCGTGACACACAGGGTCCTGAACGTGACTTAGCAGAGGTTATCT<br>CACGCAAAATTGCTAGTTCGTTCGGGACGTGGAAAGAAATGACTGCCAACCCGCTTCAGTCATTGCAGTTTTTCGAAGAAGAACTT<br>CACGCATTGGACGCTAACGTCAGTTTGAGCCCAGCTTTTGACGTTCTGATTAAAATGAACGACCTGCAGGGAGATTTAAAGAATCGT<br>ACTATCGTTTTCGACCCGGACGCCCCAGTGTTTGAGTACAATGCGGAAGATCCTGCTGACATTATTATCAAGCTGACGGCACGCTA<br>TGCCAAGGAGGCTGTAATCAAAAACCAAAACGTCGGAAATTACGTGAAAAACGCTATTACCACCACAAACGCTAATGGTTTAGGAT<br>GGCTTTTAAATAAGGGCTTATCTTTGCTGCCAGTATCTACAGATGACGAACTGTTAGAGTTCATTGGGGTCGAACGCTCTCATCCTT<br>CCTGTCACGCATTGATCGAACTGATCGCGCAATTAGAAGCTCCGGAATTGTTTGAAAAAAACGTCTTCAGCGACACACGCTCCGAG<br>GTCCAGGGTATGATTGACTCGGCGGTGTCGAACCATATCGCCCGCTTGTCAAGTAGTCGTAACAGTTTGAGTATGGATTCGGAAGA<br>GCTGGAGCGCCTGATTAAATCTTTTCAGATTCATACACCGCATTGCAGCCTGTTTATTGGAGCCCAGTCACTTTCGCAGCAACTTGA<br>GTCGCTGCCAGAAGCCCTGCAGTCTGGCGTTAATTCGGCCGATATTTTGCTTGGTAGCACTCAGTACATGCTTACGAACTCTTTAGT<br>CGAGGAGAGTATCGCGACCTATCAGCGTACTTTAAATCGCATCAACTACTTATCCGGCGTAGCGGGTCAGATCAACGGAGCCATTA<br>AGCGTAAAGCCATTGATGGTGAAAAAATTCATCTTCCTGCGGCCTGGTCTGAGCTGATCTCTTTACCGTTTATCGGTCAGCCTGTAA<br>TCGACGTCGAGAGTGACCTTGCACATTTGAAAAACCAATATCAGACTCTGAGTAACGAATTTGACACGTTAATCTCTGCATTGCAAA<br>AGAATTTCGACTTGAATTTCAATAAAGCTTTATTGAACCGTACCCAACATTTTGAAGCGATGTGTCGTTCAACGAAGAAGAACGCCCT<br>GTCCAAACCGGAGATTGTGTCTTACCGCGACTTACTGGCTCGTTTGACGTCGTGCTTATATCGCGGATCATTGGTACTGCGTCGCG<br>CAGGGATCGAAGTGCTGAAGAAACATAAAATTTTTGAATCTAACTCGGAGCTTCGTGAACACGTACATGAGCGTAAGCACTTTGTCT<br>TTGTGTCACCTTTGGATCGCAAAGCGAAGAAGCTTTTGCGCCTGACAGATTCACGCCCTGATCTGCTTCATGTCATCGACGAGATC<br>TTACAGCATGACAATCTGGAAAACAAGGACCGTGAATCGTTATGGCTTGTGCGCTCCGGCTACTTGTTGGCCGGGTTACCGGATCA<br>ATTGAGCTCATCATTTATCAATTTACCTATCATCACACAAAAGGGGGACCGCCGCTTGATCGACTTGATTCAATACGATCAAATCAAC<br>CGTGATGCTTTTGTGATGTTGGTTACTAGCGCTTTTAAGTCTAACCTTAGCGGCTTACAATACCGCGCCAACAAACAGTCCTTCGTC<br>GTCACCCGTACTCTGTCACCCTATCTTGGAAGTAAATTAGTCTACGTACCTAAAGACAAAGACTGGCTTGTGCCGTCACAGATGTTT<br>GAGGGGCGCTTCGCAGACATCCTGCAATCGGATTATATGGTGTGGAAAGATGCCGGACGCTTGTGTGTTATTGATACCGCCAAACA<br>CTTGTCCAACATTAAGAAATCCGTTTTTTCTTCTGAGGAGGTCTTGGCATTCCTTCGCGAACTTCCACATCGTACCTTCATTCAAACC<br>GAGGTCCGTGGATTGGGCGTTAACGTGGATGGCATCGCGTTTAATAATGGAGACATCCCAAGTTTAAAAACCTTCTCTAATTGTGTG<br>CAGGTAAAGGTTTCGCGTACCAACACCTCGTTAGTGCAGACATTGAACCGTTGGTTCGAAGGGGGAAAAGTGTCCCCACCGTCAAT<br>CCAATTCGAACGTGCGTATTATAAAAAAGACGATCAAATCCATGAGGACGCCGCCAAGCGCAAGATTCGTTTTCAGATGCCTGCTA<br>CTGAATTGGTCCACGCCAGTGATGATGCAGGCTGGACTCCCTCGTACTTACTTGGCATTGACCCAGGAGAATACGGCATGGGTCTT<br>TCCCTGGTATCTATTAATAATGGCGAGGTATTAGACAGCGGTTTCATTCACATCAATAGCCTGATTAATTTTGCGTCAAAGAAGTCAA<br>ATCACCAAACCAAGGTGGTTCCGCGTCAGCAGTATAAGAGTCCCTACGCAAACTATCTTGAGCAATCGAAGGATTCTGCTGCCGGA<br>GACATTGCTCATATCCTTGATCGTTTGATCTATAAACTGAATGCATTGCCCGTTTTTGAGGCCTTGTCCGGTAACAGTCAGTCGGCT<br>GCTGACCAGGTGTGGACGAAAGTTCTGTCCTTCTATACCTGGGGAGATAATGACGCTCAGAACTCAATTCGTAAACAGCATTGGTT<br>TGGCGCGAGTCATTGGGACATCAAGGGCATGTTGCGTCAGCCACCTACTGAGAAAAAGCCGAAACCTTACATTGCTTTCCCAGGTT<br>CTCAAGTGTCGAGCTATGGCAATTCGCAGCGCTGCTCTTGCTGCGGTCGCAATCCCATCGAACAACTTCGCGAAATGGCGAAGGA<br>TACCTCGATTAAAGAGCTGAAGATTCGCAATAGCGAGATCCAGTTATTTGATGGTACTATTAAACTGTTCAACCCCGATCCCTCTAC<br>CGTAATTGAGCGCCGCCGCCACAACCTGGGCCCTTCTCGTATCCCTGTCGCTGACCGTACTTTCAAAAACATTAGTCCGAGCTCAC<br>TGGAATTTAAAGAGCTGATCACGATTGTTTCGCGCTCAATTCGCCATTCACCTGAATTTATCGCCAAGAAGCGCGGCATTGGATCTG<br>AATACTTTTGTGCATATAGCGACTGCAATTCTTCACTGAACAGTGAAGCGAACGCTGCTGCGAACGTTGCACAGAAGTTCCAGAAG<br>CAACTTTTCTTCGAGTTGTAATGCTTAAGTCGAACAGAAAGTAATCGTATTGTACACGGCCGCATAATCGAAATTAATACGACTCACT<br>ATAGGGGAATTGTGAGCGGATAACAATTCCCCATCTTAGTATATTAGTTAAGTATAAGAAGGAGATATACATATGAACGACGAGAAC<br>AAGACAAAATCACATCCCAATAGCATCTGGTACTGGTGCAGCGGGGAAAAACTGTGGTATTTGCCCTACTCAGGAACACCTAAACG<br>TACAAGCAAGAGTGTATGGACCGTTTCCGGCTCATTCGGTCAGTTAGACATCGATTTATATAAGACGTCCCACATTTTATTCTACGG<br>GGCTAATGAAACCACCGGAGATCTTCCATTACTTTTCCTGAGCGATTGCGCCAAACACAGTGTATCTATCAGCATCCATCGTAACCA<br>CGTTTCGGAACCTTTCGTGTTCTATCGTAACAAGACGACTGATCGTGATGACTTGGTTACAGCACAAATTCTGCATCGTGAAAATAC<br>CAAAAAGAGCGCTTATATTGCGCGCACGATTATTCGTTTACAAATTAAACAGCGCGGTTATGTCATTCCCTCGTCATTTGATGATAC |

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| | GAAATTGCTTTCCTCTCGTAACGTTGATGAAGTACGTACCATTGAAGCCACCGCCTCGCGCGAATACTGGCGTTTGTATTTCAAGAA<br>GTTAGGGCTGGAAGGCCTGGGGCGCCGTGATGATCACGTCGTGGTAAAGGCATTGGACGCTACATCGCACTTTCTTTCGGGTATC<br>ATGTTACGCTGGGTTTTATCCCACGAGTTGTCCCCTGCGCATGGTTTCATTCACAAATCTTCTAGCTACCCCTCCCTGGTGTATGAT<br>CTTATTGAGCCACTTCGTTGGATTCCTGAACTGGCGGTTTTCCAGTCTTACTTGGAGTTCGGGGAGCATCGTTTGCTGGAACGCTC<br>AATCGAACGCTTCAAACAAATGCTGGACGAACCTATTCACTCCGAACCTACTCGCCAGTTGGTGTATCGTCGCACGTTAATTCACG<br>GCCAAGTCATTGCTTTGCGCCACTACTTAAATGGGAATATGCAACGCTATTTGCCCCCGGTAGAAGAATTGGCGAAGGAACGCGGA<br>CGTAAACGTGCAGTTTCCTACACACTGCCGGGCCAGATTTGGAAAAAGTAATTAACCTAGGCTGCTGCCACCGCTGAGCAATAACT<br>AGCATAACCCCTTGGGGCCTCTAAACGGGTCTTGAGGGGTTTTTTGCTGAAACCTCAGGCATTTGAGAAGCACACGGTCACACTGC<br>TTCCGGTAGTCAATAAACCGGTAAACCAGCAATAGACATAAGCGGCTATTTAACGACCCTGCCCTGAACCGACGACCGGGTCATCG<br>TGGCCGGATCTTGCGGCCCCTCGGCTTGAACGAATTGTTAGACATTATTTGCCGACTACCTTGGTGATCTCGCCTTTCACGTAGTG<br>GACAAATTCTTCCAACTGATCTGCGCGCGAGGCCAAGCGATCTTCTTCTTGTCCAAGATAAGCCTGTCTAGCTTCAAGTATGACGG<br>GCTGATACTGGGCCGGCAGGCGCTCCATTGCCCAGTCGGCAGCGACATCCTTCGGCGCGATTTTGCCGGTTACTGCGCTGTACCA<br>AATGCGGGACAACGTAAGCACTACATTTCGCTCATCGCCAGCCCAGTCGGGCGGCGAGTTCCATAGCGTTAAGGTTTCATTTAGCG<br>CCTCAAATAGATCCTGTTCAGGAACCGGATCAAAGAGTTCCTCCGCCGCTGGACCTACCAAGGCAACGCTATGTTCTCTTGCTTTT<br>GTCAGCAAGATAGCCAGATCAATGTCGATCGTGGCTGGCTCGAAGATACCTGCAAGAATGTCATTGCGCTGCCATTCTCCAAATTG<br>CAGTTCGCGCTTAGCTGGATAACGCCACGGAATGATGTCGTCGTGCACAACAATGGTGACTTCTACAGCGCGGAGAATCTCGCTCT<br>CTCCAGGGGAAGCCGAAGTTTCCAAAAGGTCGTTGATCAAAGCTCGCCGCGTTGTTTCATCAAGCCTTACGGTCACCGTAACCAGC<br>AAATCAATATCACTGTGTGGCTTCAGGCCGCCATCCACTGCGGAGCCGTACAAATGTACGGCCAGCAACGTCGGTTCGAGATGGC<br>GCTCGATGACGCCAACTACCTCTGATAGTTGAGTCGATACTTCGGCGATCACCGCTTCCCTCATACTCTTCCTTTTTCAATATTATTG<br>AAGCATTTATCAGGGTTATTGTCTCATGAGCGGATACATATTTGAATGTATTTAGAAAAATAAACAAATAGCTAGCTCACTCGGTCGC<br>TACGCTCCGGGCGTGAGACTGCGGCGGGCGCTGCGGACACATACAAAGTTACCCACAGATTCCGTGGATAAGCAGGGGACTAAC<br>ATGTGAGGCAAAACAGCAGGGCCGCGCCGGTGGCGTTTTTCCATAGGCTCCGCCCTCCTGCCAGAGTTCACATAAACAGACGCTT<br>TTCCGGTGCATCTGTGGGAGCCGTGAGGCTCAACCATGAATCTGACAGTACGGGCGAAACCCGACAGGACTTAAAGATCCCCACC<br>GTTTCCGGCGGGTCGCTCCCTCTTGCGCTCTCCTGTTCCGACCCTGCCGTTTACCGGATACCTGTTCCGCCTTTCTCCCTTACGGG<br>AAGTGTGGCGCTTTCTCATAGCTCACACACTGGTATCTCGGCTCGGTGTAGGTCGTTCGCTCCAAGCTGGGCTGTAAGCAAGAACT<br>CCCCGTTCAGCCCGACTGCTGCGCCTTATCCGGTAACTGTTCACTTGAGTCCAACCCGGAAAAGCACGGTAAAACGCCACTGGCA<br>GCAGCCATTGGTAACTGGGAGTTCGCAGAGGATTTGTTTAGCTAAACACGCGGTTGCTCTTGAAGTGTGCGCCAAAGTCCGGCTAC<br>ACTGGAAGGACAGATTTGGTTGCTGTGCTCTGCGAAAGCCAGTTACCACGGTTAAGCAGTTCCCCAACTGACTTAACCTTCGATCA<br>AACCACCTCCCCAGGTGGTTTTTTCGTTTACAGGGCAAAAGATTACGCGCAGAAAAAAAGGATCTCAAGAAGATCCTTTGATCTTTT<br>CTACTGAACCGCTCTAGATTTCAGTGCAATTTATCTCTTCAAATGTAGCACCTGAAGTCAGCCCCATACGATATAAGTTGTAATTCTC<br>ATGTTAGTCATGCCCCGCGCCCACCGGAAGGAGCTGACTGGGTTGAAGGCTCTCAAGGGCATCGGTCGAGATCCCGGTGCCTAAT<br>GAGTGAGCTAACTTACATTAATTGCGTTGCGCTCACTGCCCGCTTTCCAGTCGGGAAACCTGTCGTGCCAGCTGCATTAATGAATC<br>GGCCAACGCGCGGGGAGAGGCGGTTTGCGTATTGGGCGCCAGGGTGGTTTTTCTTTTCACCAGTGAGACGGGCAACAGCTGATT<br>GCCCTTCACCGCCTGGCCCTGAGAGAGTTGCAGCAAGCGGTCCACGCTGGTTTGCCCCAGCAGGCGAAAATCCTGTTTGATGGTG<br>GTTAACGGCGGGATATAACATGAGCTGTCTTCGGTATCGTCGTATCCCACTACCGAGATGTCCGCACCAACGCGCAGCCCGGACT<br>CGGTAATGGCGCGCATTGCGCCCAGCGCCATCTGATCGTTGGCAACCAGCATCGCAGTGGGAACGATGCCCTCATTCAGCATTTG<br>CATGGTTTGTTGAAAACCGGACATGGCACTCCAGTCGCCTTCCCGTTCCGCTATCGGCTGAATTTGATTGCGAGTGAGATATTTATG<br>CCAGCCAGCCAGACGCAGACGCGCCGAGACAGAACTTAATGGGCCCGCTAACAGCGCGATTTGCTGGTGACCCAATGCGACCAG<br>ATGCTCCACGCCCAGTCGCGTACCGTCTTCATGGGAGAAAATAATACTGTTGATGGGTGTCTGGTCAGAGACATCAAGAAATAACG<br>CCGGAACATTAGTGCAGGCAGCTTCCACAGCAATGGCATCCTGGTCATCCAGCGGATAGTTAATGATCAGCCCACTGACGCGTTG<br>CGCGAGAAGATTGTGCACCGCCGCTTTACAGGCTTCGACGCCGCTTCGTTCTACCATCGACACCACCACGCTGGCACCCAGTTGA<br>TCGGCGCGAGATTTAATCGCCGCGACAATTTGCGACGGCGCGTGCAGGGCCAGACTGGAGGTGGCAACGCCAATCAGCAACGAC<br>TGTTTGCCCGCCAGTTGTTGTGCCACGCGGTTGGGAATGTAATTCAGCTCCGCCATCGCCGCTTCCACTTTTTCCCGCGTTTTCGC<br>AGAAACGTGGCTGGCCTGGTTCACCACGCGGGAAACGGTCTGATAAGAGACACCGGCATACTCTGCGACATCGTATAACGTTACT<br>GGTTTCACATTCACCACCCTGAATTGACTCTCTTCCGGGCGCTATCATGCCATACCGCGAAAGGTTTTGCGCCATTCGATGGTGTC<br>CGGGATCTCGACGCTCTCCCTTATGCGACTCCTGCATTAGGAAATTAATACGACTCACTATA |
| pCJH140_SC101_J23119_tracrRNA(4)_VC-4MinArray_Kan | TTGACAGCTAGCTCAGTCCTAGGTATAATACTAGTGGATACCACCCGTGCATTTCTGGATCAATGATCCGTACCTCAATGTCCGGGC<br>GCGCAGCTAGAGCGACCTGAAATCTATATCCTTCAAAGCACAAACAACTTCACCACCTTTGTACACGAACTTATAGCGAGCTTTGGA<br>TTTTTGGATAGGTTCAATCGAATTTAGGTAGGGTATCCAAAGTGCCGCTCGACCACCACGGATGTTTTTCCATGTCCAAATAGAGTC<br>TGGATGTTGAAATTGTGAGATTTCCATATGCAAGCTGGGTGGCTGTTAAAAATGAATTTCATGATAATACATAATGTTATATTATGAA<br>CGAGCAGGATTCAGGTTGGGTTTGAGGAAGGTTTTCCGCCCTGCGAGGCCGTAAATTCAAAACATTCCCCGCCAAATTCCGGATAT<br>GCTTGGGCCCGAACAAAAACTCatctcagaagaggatctgaatagcgccgtcgaccatcatcatcatcatcattgagtttaaacgctctccagcttgggtgttttggcggatgagag<br>aagattttcagcctgatacagattaaatcagaacgcagaagcggtctgataaaacagaatttgcctggcggcagtagcgcggtggtcccacctgaccccatgccgaactcagaagtgaaacg<br>ccgtagcgccgatggtagtgtggggtctgccccatgcgagagtaggcaactgccaggcatcaaataaaacgaaaggctcagtcgaaagactgggcctttcgttttatctgttgtttgtcggtgaacg<br>ctctcctgagtaggacaaatccgccgccctagacctagggtacgggttttgctgcccgcaaacgggctgttctggtgttgctagtttgttatcagaatcgcagatccggcttcaggtttgccggctga<br>aagcgctatttcttccagaattgccatgatttttccccacgggaggcgtcactggctcccgtgttgtcggcagctttgattcgataagcagcatcgcctgtttcaggctgtctatgtgtgactgttgagct<br>gtaacaagttgtctcaggtgttcaatttcatgttctagttgctttgttttactggtttcacctgttctattaggtgttacatgctgttcatctgttacattgtcgatctgttcatggtgaacagctttaaatgcaccaa<br>aaactcgtaaaagctctgatgtatctatcttttttacaccgttttcatctgtgcatatggacagttttccctttgatatctaacggtgaacagttgttctacttttgtttgttagtcttgatgcttcactgatagatac<br>aagagccataagaacctcagatccttccgtatttagccagtatgttctctagtgtggttcgttgtttttgcgtgagccatgagaacgaaccattgagatcatgcttactttgcatgtcactcaaaaattttg<br>cctcaaaactggtgagctgaatttttgcagttaaagcatcgtgtagtgtttttcttagtccgttacgtaggtaggaatctgatgtaatggttgttggtattttgtcaccattcatttttatctggttgttctcaagttc<br>ggttacgagatccatttgtctatctagttcaacttggaaaatcaacgtatcagtcgggcggcctcgcttatcaaccaccaatttcatattgctgtaagtgtttaaatctttacttattggtttcaaaacccatt<br>ggttaagccttttaaactcatggtagttattttcaagcattaacatgaacttaaattcatcaaggctaatctctatatttgccttgtgagttttcttttgtgttagttcttttaataaccactcataaatcctcatag<br>agtatttgttttcaaaagacttaacatgttccagattatattttatgaattttttaactggaaaagataaggcaatatctcttcactaaaaactaattctaatttttcgcttgagaacttggcatagtttgtccac<br>tggaaaatctcaaagcctttaaccaaaggattcctgatttccacagttctcgtcatcagctctctggttgctttagctaatacaccataagcattttccctactgatgttcatcatctgagcgtattggttata<br>agtgaacgataccgtccgttctttccttgtagggttttcaatcgtggggttgagtagtgccacacagcataaaattagcttggtttcatgctccgttaagtcatagcgactaatcgctagttcatttgctttg<br>aaaacaactaattcagacatacatctcaattggtctaggtgattttaatcactataccaattgagatgggctagtcaatgataattactagtccttttcctttgagttgtgggtatctgtaaattctgctaga<br>cctttgctggaaaacttgtaaattctgctagaccctctgtaaattccgctagacctttgtgtgttttttttgtttatattcaagtggttataatttatagaataaagaaagaataaaaaaagataaaaagaat<br>agatcccagccctgtgtataactcactactttagtcagttccgcagtattacaaaaggatgtcgcaaacgctgtttgctcctctacaaaacagaccttaaaaccctaaaggcttaagtagcaccctc<br>gcaagctcgggcaaatcgctgaatattccttttgtctccgaccatcaggcacctgagtcgctgtctttttcgtgacattcagttcgctgcgctcacggctctggcagtgaatgggggtaaatggcacta<br>caggcgccttttatggattcatgcaaggaaactacccataatacaagaaaagcccgtcacgggcttctcagggcgttttatggcgggtctgctatgtggtgctatctgactttttgctgttcagcagttc<br>ctgccctctgattttccagtctgaccacttcggattatcccgtgacaggtcattcagactggctaatgcacccagtaaggcagcggtatcatcaacaggcttacccgtcttactgtccctagtgcttgg<br>attctcaccaataaaaaacgcccggcggcaaccgagcgttctgaacaaatccagatggagttctgaggtcattactggatctatcaacaggagtccaagcgagctctcgaaccccagagtccc<br>gctcagaagaactcgtcaagaaggcgatagaaggcgatgcgctgcgaatcgggagcggcgataccgtaaagcacgaggaagcggtcagcccattcgccgccaagctcttcagcaatatc<br>acgggtagccaacgctatgtcctgatagcggtccgccacacccagccggccacagtcgatgaatccagaaaagcggccattttccaccatgatattcggcaagcaggcatcgccatgggtca<br>cgacgagatcctcgccgtcgggcatgcgcgccttgagcctggcgaacagttcggctggcgcgagcccctgatgctcttcgtccagatcatcctgatcgacaagaccggcttccatccgagtacg<br>tgctcgctcgatgcgatgtttcgcttggtggtcgaatgggcaggtagccggatcaagcgtatgcagccgccgcattgcatcagccatgatggatactttctcggcaggagcaaggtgagatgaca<br>ggagatcctgccccggcacttcgcccaatagcagccagtcccttcccgcttcagtgacaacgtcgagcacagctgcgcaaggaacgcccgtcgtggccagccacgatagccgcgctgcctc<br>gtcctgcagttcattcagggcaccggacaggtcggtcttgacaaaaagaaccgggcgcccctgcgctgacagccggaacacggcggcatcagagcagccgattgtctgttgtgcccagtcat<br>agccgaatagcctctccacccaagcggccggagaacctgcgtgcaatccatcttgttcaatcatgcgaaacgatcctcatcctgtctcttgatcagatcttgatcccctgcgccatcagatccttgg<br>cggcaagaaagccatccagtttactttgcagggcttcccaaccttaccagagggcgccccagctggcaattccgacgtctaagaaaccattattatcatgacattaacctataaaaataggcgta<br>tcacgaggccctttggAATTC |

| pCJH141_SC101_J23119_tracrRNA(6)_VC-6MinArray_Kan | TTGACAGCTAGCTCAGTCCTAGGTATAATACTAGTGGAGAAACTCCCGACTTCCTCAGGATGAAGTTGATTCTTTATCCATACCTTG<br>GTGCCGGGACGCCGATTGAGGAATGGGCGGCGCCTTCCAAATTCTCATTCCTCACTTGATTGTTTGATTTTCGATTGTTTGATTTCC<br>CTGGTCCATATTTATTTAACTTTTTAATATATTTTCTATCAAGTTTAATCACATATTAAACAAATTTATTATACTTTTATAATTGACTTTCA<br>AATAGATATTTGCTAGTCTCCCATTATTGGAAGGGTTTATAAGGAACATATGCAAACAGAGAACCCAAAGTAAAGAAGACCTGCTTT<br>CTAGACGTCCATTGTTGATCCGGGAAGGATCGGTCTTCTGCGTCCCTAATTTATCGTATATGACCATCATTGCAGCGTCTCCGGACT<br>CCGGAGACGCTCTTCCTCAAGCTTTCTGTGATGAAGGCTCTTTCAGTTTCCCGGCGATAGCGCCTTTTCAATGACACCTAAACAAG<br>CCAGCAATGCTTGGGCCCGAACAAAAACTCatctcagaagaggatctgaatagcgccgtcgaccatcatcatcatcatcattgagtttaaacgctctccagcttgggtgttttg<br>gcggatgagagaagattttcagcctgatacagattaaatcagaacgcagaagcggtctgataaaacagaatttgcctggcggcagtagcgcggtggtcccacctgaccccatgccgaactca<br>gaagtgaaacgccgtagcgccgatggtagtgtggggtctgccccatgcgagagtaggaactgccaggcatcaaataaaacgaaaggctcagtcgaaagactgggcctttcgttttatctgttgtt<br>tgtcggtgaacgctctcctgagtaggacaaatccgccgccctagacctagggtacgggttttgctgcccgcaaacgggctgttctggtgttgctagtttgttatcagaatcgcagatccggcttcagg<br>tttgccggctgaaagcgctatttcttccagaattgccatgatttttccccacgggaggcgtcactggctcccgtgttgtcggcagctttgattcgataagcagcatcgcctgtttcaggctgtctatgtgt<br>gactgttgagctgtaacaagttgtctcaggtgttcaatttcatgttctagttgctttgttttactggtttcacctgttctattaggtgttacatgctgttcatctgttacattgtcgatctgttcatggtgaacagcttt<br>aaatgcaccaaaaactcgtaaaagctctgatgtatctatcttttttacaccgttttcatctgtgcatatggacagttttccctttgatatctaacggtgaacagttgttctacttttgtttgttagtcttgatgcttc<br>actgatagatacaagagccataagaacctcagatccttccgtatttagccagtatgttctctagtgtggttcgttgtttttgcgtgagccatgagaacgaaccattgagatcatgcttactttgcatgtca<br>ctcaaaaattttgcctcaaaactggtgagctgaatttttgcagttaaagcatcgtgtagtgtttttcttagtccgttacgtaggtaggaatctgatgtaatggttgttggtattttgtcaccattcatttttatctgg<br>ttgttctcaagttcggttacgagatccatttgtctatctagttcaacttggaaaatcaacgtatcagtcgggcggcctcgcttatcaaccaccaatttcatattgctgtaagtgtttaaatctttacttattggtt<br>tcaaaacccattggttaagccttttaaactcatggtagttattttcaagcattaacatgaacttaaattcatcaaggctaatctctatatttgccttgtgagttttcttttgtgttagttcttttaataaccactcata<br>aatcctcatagagtatttgttttcaaaagacttaacatgttccagattatattttatgaatttttttaactggaaaagataaggcaatatctcttcactaaaaactaattctaatttttcgcttgagaacttggca<br>tagtttgtccactggaaaatctcaaagcctttaaccaaaggattcctgatttccacagttctcgtcatcagctctctggttgctttagctaatacaccataagcattttccctactgatgttcatcatctgagc<br>gtattggttataagtgaacgataccgtccgttctttccttgtaggttttcaatcgtggggttgagtagtgccacacagcataaaattagcttggtttcatgctccgttaagtcatagcgactaatcgctagt<br>tcatttgctttgaaaacaactaattcagacatacatctcaattggtctaggtgattttaatcactataccaattgagatgggctagtcaatgataattactagtccttttcctttgagttgtgggtatctgtaaa<br>ttctgctagacctttgctggaaaacttgtaaattctgctagaccctctgtaaattccgctagacctttgtgtgttttttttgtttatattcaagtggttataatttatagaataaagaaagaataaaaaaagata<br>aaaagaatagatcccagccctgtgtataactcactactttagtcagttccgcagtattacaaaaggatgtcgcaaacgctgtttgctcctctacaaaacagaccttaaaaccctaaaggcttaagta<br>gcaccctcgcaagctcggcaaatcgctgaatattccttttgtctccgaccatcaggcacctgagtcgctgtctttttcgtgacattcagttcgctgcgctcacggctctggcagtgaatgggggtaaa<br>tggcactacaggcgccttttatggattcatgcaaggaaactacccataatacaagaaaagcccgtcacgggcttctcagggcgttttatggcgggtctgctatgtggtgctatctgactttttgctgttc<br>agcagttcctgccctctgattttccagtctgaccacttcggattatcccgtgacaggtcattcagactggctaatgcacccagtaaggcagcggtatcatcaacaggcttacccgtcttactgtcccta<br>gtgcttggattctcaccaataaaaaacgcccggcggcaaccgagcgttctgaacaaatccagatggagttctgaggtcattactggatctatcaacaggagtccaagcgagctctcgaacccc<br>agagtcccgctcagaagaactcgtcaagaaggcgatagaaggcgatgcgctgcgaatcgggagcggcgataccgtaaagcacgaggaagcggtcagcccattcgccgccaagctcttca<br>gcaatatcacgggtagccaacgctatgtcctgatagcggtccgccacacccagccggccacagtcgatgaatccagaaaagcggccattttccaccatgatattcggcaagcaggcatcgcc<br>atgggtcacgacgagatcctcgccgtcgggcatgcgcgccttgagcctggcgaacagttcggctggcgcgagcccctgatgctcttcgtccagatcatcctgatcgacaagaccggcttccatc<br>cgagtacgtgctcgctcgatgcgatgtttcgcttggtggtcgaatgggcaggtagccggatcaagcgtatgcagccgccgcattgcatcagccatgatggatactttctcggcaggagcaaggtg<br>agatgacaggagatcctgccccggcacttcgcccaatagcagccagtcccttcccgcttcagtgacaacgtcgagcacagctgcgcaaggaacgcccgtcgtggccagccacgatagccgc<br>gctgcctcgtcctgcagttcattcagggcaccggacaggtcggtcttgacaaaaagaaccgggcgcccctgcgctgacagccggaacacggcggcatcagagcagccgattgtctgttgtgc<br>ccagtcatagccgaatagcctctccacccaagcggccggagaacctgcgtgcaatccatcttgttcaatcatgcgaaacgatcctcatcctgtctcttgatcagatcttgatcccctgcgccatcag<br>atccttggcggcaagaaagccatccagtttactttgcagggcttcccaaccttaccagagggcgccccagctggcaattccgacgtctaagaaaccattattatcatgacattaacctataaaaat<br>aggcgtatcacgaggccctttggAATTC |
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| pCJH142_SC101_J23119_tracrRNA(OspVC)_VC-OspMinArray_Kan | TTGACAGCTAGCTCAGTCCTAGGTATAATACTAGTGGTCATCGTTGGTATGGCTGTGTTCTTCTGAACGCACATCATTCTCTTAGAG<br>ACGTCTACATACCAACCCACGATGGGGAGCGTGGACAACAAAATCTCCCCACTGTTGGCCAAAAGAACCAGAAACCGTCCAAACA<br>GATTTAGATGTTCTTTTAGGTGTCCCAGAGTAAGGCAAATACCATAGTTTCTCACCAGAACACCAATACCAAATTGAATTTGGGTGG<br>CTTTTTGTTTTATTTTCGTCATTCATATCCAATACTTGTATCACTATATAACTACCATTGCAATGGGTTTTGTATGCGCATAGAATTACT<br>GATGTTGTGATGAAGGCCTAACCGTGCAAGTGGCAGAATAGCGAAAGCTGTGATGAAGACCTATGCTTGGGCCCGAACAAAAACT<br>Catctcagaagaggatctgaatagcgccgtcgaccatcatcatcatcatcattgagtttaaacgctctccagcttgggtgttttggcggatgagagaagattttcagcctgatacagattaaatcag<br>aacgcagaagcggtctgataaaacagaatttgcctggcggcagtagcgcggtggtcccacctgaccccatgccgaactcagaagtgaaacgccgtagcgccgatggtagtgtggggtctgc<br>cccatgcgagagtaggaactgccaggcatcaaataaaacgaaaggctcagtcgaaagactgggcctttcgttttatctgttgtttgtcggtgaacgctctcctgagtaggacaaatccgccgccct<br>agacctagggtacgggttttgctgcccgcaaacgggctgttctggtgttgctagtttgttatcagaatcgcagatccggcttcaggtttgccggctgaaagcgctatttcttccagaattgccatgattttt<br>tccccacgggaggcgtcactggctcccgtgttgtcggcagctttgattcgataagcagcatcgcctgtttcaggctgtctatgtgtgactgttgagctgtaacaagttgtctcaggtgttcaatttcatgtt<br>ctagttgctttgttttactggtttcacctgttctattaggtgttacatgctgttcatctgttacattgtcgatctgttcatggtgaacagctttaaatgcaccaaaaactcgtaaaagctctgatgtatctatcttttt<br>acaccgttttcatctgtgcatatggacagttttccctttgatatctaacggtgaacagttgttctacttttgtttgttagtcttgatgcttcactgatagatacaagagccataagaacctcagatccttccgta<br>tttagccagtatgttctctagtgtggttcgttgtttttgcgtgagccatgagaacgaaccattgagatcatgcttactttgcatgtcactcaaaaattttgcctcaaaactggtgagctgaatttttgcagtta<br>aagcatcgtgtagtgtttttcttagtccgttacgtaggtaggaatctgatgtaatggttgttggtattttgtcaccattcatttttatctggttgttctcaagttcggttacgagatccatttgtctatctagttcaact<br>tggaaaatcaacgtatcagtcgggcggcctcgcttatcaaccaccaatttcatattgctgtaagtgtttaaatctttacttattggtttcaaaacccattggttaagccttttaaactcatggtagttattttca<br>agcattaacatgaacttaaattcatcaaggctaatctctatatttgccttgtgagttttcttttgtgttagttcttttaataaccactcataaatcctcatagagtatttgttttcaaaagacttaacatgttccag<br>attatattttatgaatttttttaactggaaaagataaggcaatatctcttcactaaaaactaattctaatttttcgcttgagaacttggcatagtttgtccactggaaaatctcaaagcctttaaccaaaggat<br>tcctgatttccacagttctcgtcatcagctctctggttgctttagctaatacaccataagcattttccctactgatgttcatcatctgagcgtattggttataagtgaacgataccgtccgttctttccttgtagg<br>ttttcaatcgtggggttgagtagtgccacacagcataaaattagcttggtttcatgctccgttaagtcatagcgactaatcgctagttcatttgctttgaaaacaactaattcagacatacatctcaattg<br>gtctaggtgattttaatcactataccaattgagatgggctagtcaatgataattactagtccttttcctttgagttgtgggtatctgtaaattctgctagacctttgctggaaaacttgtaaattctgctagacc<br>ctctgtaaattccgctagacctttgtgtgttttttttgtttatattcaagtggttataatttatagaataaagaaagaataaaaaaagataaaaagaatagatcccagccctgtgtataactcactactttag<br>tcagttccgcagtattacaaaaggatgtcgcaaacgctgtttgctcctctacaaaacagaccttaaaaccctaaaggcttaagtagcaccctcgcaagctcggcaaatcgctgaatattccttttg<br>tctccgaccatcaggcacctgagtcgctgtctttttcgtgacattcagttcgctgcgctcacggctctggcagtgaatgggggtaaatggcactacaggcgccttttatggattcatgcaaggaaact<br>acccataatacaagaaaagcccgtcacgggcttctcagggcgttttatggcgggtctgctatgtggtgctatctgactttttgctgttcagcagttcctgccctctgattttccagtctgaccacttcggatt<br>atcccgtgacaggtcattcagactggctaatgcacccagtaaggcagcggtatcatcaacaggcttacccgtcttactgtccctagtgcttggattctcaccaataaaaaacgcccggcggcaac<br>cgagcgttctgaacaaatccagatggagttctgaggtcattactggatctatcaacaggagtccaagcgagctctcgaaccccagagtcccgctcagaagaactcgtcaagaaggcgataga<br>aggcgatgcgctgcgaatcgggagcggcgataccgtaaagcacgaggaagcggtcagcccattcgccgccaagctcttcagcaatatcacgggtagccaacgctatgtcctgatagcggtc<br>cgccacacccagccggccacagtcgatgaatccagaaaagcggccattttccaccatgatattcggcaagcaggcatcgccatgggtcacgacgagatcctcgccgtcgggcatgcgcgcc<br>ttgagcctggcgaacagttcggctggcgcgagcccctgatgctcttcgtccagatcatcctgatcgacaagaccggcttccatccgagtacgtgctcgctcgatgcgatgtttcgcttggtggtcga<br>atgggcaggtagccggatcaagcgtatgcagccgccgcattgcatcagccatgatggatactttctcggcaggagcaaggtgagatgacaggagatcctgccccggcacttcgcccaatagc<br>agccagtcccttcccgcttcagtgacaacgtcgagcacagctgcgcaaggaacgcccgtcgtggccagccacgatagccgcgctgcctcgtcctgcagttcattcagggcaccggacaggtc<br>ggtcttgacaaaaagaaccgggcgcccctgcgctgacagccggaacacggcggcatcagagcagccgattgtctgttgtgcccagtcatagccgaatagcctctccacccaagcggccgga<br>gaacctgcgtgcaatccatcttgttcaatcatgcgaaacgatcctcatcctgtctcttgatcagatcttgatcccctgcgccatcagatccttggcggcaagaaagccatccagtttactttgcagggc<br>ttcccaaccttaccagagggcgccccagctggcaattccgacgtctaagaaaccattattatcatgacattaacctataaaaataggcgtatcacgaggccctttggAATTC |
| pCJH143_pCDF-1b_Cas1(VC-4)_Spec | GGGGAATTGTGAGCGGATAACAATTCCCCTGTAGAAATAATTTTGTTTAACTTTAATAAGGAGATATACCATGGAAATCAGTCAATTC<br>CAACATCCAGACAGTATCTGGACTTGGAAGAATATTCGTGGTGGTCGTGCAGCGCTTTGGATCCCTTATTTGAATTCCATTGAGCCA<br>ATCCAAAAGTCTAAGGCGCGTTACAAGTTCGTTTATAAAGGCGGTGAAGTGGTGTGCGCCCTGAAAGACATCGATTTCATTATGATT<br>TATGGAGGTGACGCTGCCCTTTCCGTTGGTTTTTGGACGACATCGGTACGCATGGCGTAGTTTTGGCTATCCATCGCCGTAACAT<br>GCCACGTCCCTTATACTTGCTTCCGCACACGGAGTCAGATGACACTGATATCTTATCAGCTCAGATCCTGGTACGCGAGAACCTTA<br>CTCGCCGCTGTTACGTGGCACGTACGATTATCGCCCAACGCATCGCGTCATTTAGCTGGCGCATCCCCATTCCGGGTGCAGTATTC<br>AAGAAACTGCGCGAGGCCCGCACACTGAAGTCGATTCGCTCTATTGAAGCGGAATGGACAAAACGTTACTGGGACGCTTTTTATTC<br>AAATTTATCGATCGACACGACACGTCGCGAGACCCATCAGGTTAATTCTGCTCTTGACGCCGGAAGCAAATTTCTGTCAGGTATTTT |

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|---|---|
| | GTTGCGCTGGGCTTTGTTTCATAAGTTATCGCCGACGCACGGGTTCTTACATGAACAGACCACATACATTTCGCTGATCTACGATTT<br>AATCGAGCCGTACCGCATCTGGATTGAACGTGCTGTTGACATGGCATTCCAACAAACCAGTAATGAGGATATGTTAGTAGCTACAA<br>CAATCAGTATCCTTAAGGAGATGCTGCTTGAGGATGTATATCTGCCAGCCTTCCAAAAATCAACTCGCCGCAAGAATGTCCTGCATG<br>CGGAAGTTTTGGCACTGCGTTCCTATTTGGCGAAGGAGACTGCTCGCCTTGTCATTCCAGTAGAGGGAGAGCGTATCGGCGGACG<br>CCCGGTGAAGGCAGGATTTGCAATTCCCGGCGCGAAAAAGTAATTAACCTAGGCTGCTGCCACCGCTGAGCAATAACTAGCATAAC<br>CCCTTGGGGCCTCTAAACGGGTCTTGAGGGGTTTTTTGCTGAAACCTCAGGCATTTGAGAAGCACACGGTCACACTGCTTCCGGTA<br>GTCAATAAACCGGTAAACCAGCAATAGACATAAGCGGCTATTTAACGACCCTGCCCTGAACCGACGACCGGGTCATCGTGGCCGG<br>ATCTTGCGGCCCCTCGGCTTGAACGAATTGTTAGACATTATTTGCCGACTACCTTGGTGATCTCGCCTTTCACGTAGTGGACAAATT<br>CTTCCAACTGATCTGCGCGCGAGGCCAAGCGATCTTCTTCTTGTCCAAGATAAGCCTGTCTAGCTTCAAGTATGACGGGCTGATAC<br>TGGGCCGGCAGGCGCTCCATTGCCCAGTCGGCAGCGACATCCTTCGGCGCGATTTTGCCGGTTACTGCGCTGTACCAAATGCGG<br>GACAACGTAAGCACTACATTTCGCTCATCGCCAGCCCAGTCGGGCGGCGAGTTCCATAGCGTTAAGGTTTCATTTAGCGCCTCAAA<br>TAGATCCTGTTCAGGAACCGGATCAAAGAGTTCCTCCGCCGCTGGACCTACCAAGGCAACGCTATGTTCTCTTGCTTTTGTCAGCA<br>AGATAGCCAGATCAATGTCGATCGTGGCTGGCTCGAAGATACCTGCAAGAATGTCATTGCGCTGCCATTCTCCAAATTGCAGTTCG<br>CGCTTAGCTGGATAACGCCACGGAATGATGTCGTCGTGCACAACAATGGTGACTTCTACAGCGCGGAGAATCTCGCTCTCTCCAG<br>GGGAAGCCGAAGTTTCCAAAAGGTCGTTGATCAAAGCTCGCCGCGTTGTTTCATCAAGCCTTACGGTCACCGTAACCAGCAAATCA<br>ATATCACTGTGTGGCTTCAGGCCGCCATCCACTGCGGAGCCGTACAAATGTACGGCCAGCAACGTCGGTTCGAGATGGCGCTCGA<br>TGACGCCAACTACCTCTGATAGTTGAGTCGATACTTCGGCGATCACCGCTTCCCTCATACTCTTCCTTTTTCAATATTATTGAAGCAT<br>TTATCAGGGTTATTGTCTCATGAGCGGATACATATTTGAATGTATTTAGAAAAATAAACAAATAGCTAGCTCACTCGGTCGCTACGCT<br>CCGGGCGTGAGACTGCGGCGGGCGCTGCGGACACATACAAAGTTACCCACAGATTCCGTGGATAAGCAGGGGACTAACATGTGA<br>GGCAAAACAGCAGGGCCGCGCCGGTGGCGTTTTTCCATAGGCTCCGCCCCTCCTGCCAGAGTTCACATAAACAGACGCTTTTCCGG<br>TGCATCTGTGGGAGCCGTGAGGCTCAACCATGAATCTGACAGTACGGGCGAAACCCGACAGGACTTAAAGATCCCCACCGTTTCC<br>GGCGGGTCGCTCCCTCTTGCGCTCTCCTGTTCCGACCCTGCCGTTTACCGGATACCTGTTCCGCCTTTCTCCCTTACGGGAAGTGT<br>GGCGCTTTCTCATAGCTCACACACTGGTATCTCGGCTCGGTGTAGGTCGTTCGCTCCAAGCTGGGCTGTAAGCAAGAACTCCCCG<br>TTCAGCCCGACTGCTGCGCCTTATCCGGTAACTGTTCACTTGAGTCCAACCCGGAAAAGCACGGTAAAACGCCACTGGCAGCAGC<br>CATTGGTAACTGGGAGTTCGCAGAGGATTTGTTTAGCTAAACACGCGGTTGCTCTTGAAGTGTGCGCCAAAGTCCGGCTACACTGG<br>AAGGACAGATTTGGTTGCTGTGCTCTGCGAAAGCCAGTTACCACGGTTAAGCAGTTCCCCAACTGACTTAACCTTCGATCAAACCA<br>CCTCCCCAGGTGGTTTTTTCGTTTACAGGGCAAAAGATTACGCGCAGAAAAAAAGGATCTCAAGAAGATCCTTTGATCTTTTCTACT<br>GAACCGCTCTAGATTTCAGTGCAATTTATCTCTTCAAATGTAGCACCTGAAGTCAGCCCCATACGATATAAGTTGTAATTCTCATGTT<br>AGTCATGCCCCGCGCCCACCGGAAGGAGCTGACTGGGTTGAAGGCTCTCAAGGGCATCGGTCGAGATCCCGGTGCCTAATGAGT<br>GAGCTAACTTACATTAATTGCGTTGCGCTCACTGCCCGCTTTCCAGTCGGGAAACCTGTCGTGCCAGCTGCATTAATGAATCGGCC<br>AACGCGCGGGGAGAGGCGGTTTGCGTATTGGGCGCCAGGGTGGTTTTTCTTTTCACCAGTGAGACGGGCAACAGCTGATTGCCCT<br>TCACCGCCTGGCCCTGAGAGAGTTGCAGCAAGCGGTCCACGCTGGTTTGCCCCAGCAGGCGAAAATCCTGTTTGATGGTGGTTAA<br>CGGCGGGATATAACATGAGCTGTCTTCGGTATCGTCGTATCCCACTACCGAGATGTCCGCACCAACGCGCAGCCCGGACTCGGTA<br>ATGGCGCGCATTGCGCCCAGCGCCATCTGATCGTTGGCAACCAGCATCGCAGTGGGAACGATGCCCTCATTCAGCATTTGCATGG<br>TTTGTTGAAAACCGGACATGGCACTCCAGTCGCCTTCCCGTTCCGCTATCGGCTGAATTTGATTGCGAGTGAGATATTTATGCCAG<br>CCAGCCAGACGCAGACGCGCCGAGACAGAACTTAATGGGCCCGCTAACAGCGCGATTTGCTGGTGACCCAATGCGACCAGATGC<br>TCCACGCCCAGTCGCGTACCGTCTTCATGGGAGAAAATAATACTGTTGATGGGTGTCTGGTCAGAGACATCAAGAAATAACGCCGG<br>AACATTAGTGCAGGCAGCTTCCACAGCAATGGCATCCTGGTCATCCAGCGGATAGTTAATGATCAGCCCACTGACGCGTTGCGCG<br>AGAAGATTGTGCACCGCCGCTTTACAGGCTTCGACGCCGCTTCGTTCTACCATCGACACCACCACGCTGGCACCCAGTTGATCGG<br>CGCGAGATTTAATCGCCGCGACAATTTGCGACGGCGCGTGCAGGGCCAGACTGGAGGTGGCAACGCCAATCAGCAACGACTGTT<br>TGCCCGCCAGTTGTTGTGCCACGCGGTTGGGAATGTAATTCAGCTCCGCCATCGCCGCTTCCACTTTTTCCCGCGTTTTCGCAGAA<br>ACGTGGCTGGCCTGGTTCACCACGCGGGAAACGGTCTGATAAGAGACACCGGCATACTCTGCGACATCGTATAACGTTACTGGTT<br>TCACATTCACCACCCTGAATTGACTCTCTTCCGGGCGCTATCATGCCATACCGCGAAAGGTTTTGCGCCATTCGATGGTGTCCGGG<br>ATCTCGACGCTCTCCCTTATGCGACTCCTGCATTAGGAAATTAATACGACTCACTATA |
| pCJH144_pCDF-1b_Cas1(VC-6)_Spec | GGGGAATTGTGAGCGGATAACAATTCCCCTGTAGAAATAATTTTGTTTAACTTTAATAAGGAGATATACCATGGACCAAGGCAACCA<br>AACGATCGAAAACCAGACAATCAACTGCGACCAACACCCCGACTTCTTATGGACTTGGAAATCAAATAAACGTGGAAGCCGCGTTT<br>CAGTGTGGTTACCCTACTTTTCCCAAGCGAAAAAAATCCCGCGTTCTAAAAAGTGGAGTGTAGCTTACAACGGTGGTTCAATCGAGT<br>TCGACCTGAAGGAAACAGACTTAATCATGTTTTACGGAGCTACAGGCGAGTTACCGCTTGAATTTCTGGACGACGCAAGTAAAAAT<br>GGCGTCATGATTCTGATTCACCGCCGTAACGTGCTGCAGCCCTATGTCTTTTATCCGAGCGTGATCGGGGATGAGGAAGATATTTT<br>AACAAAGCAGATTCAATTTCGTACCAACGAGCGTAAACGTCTTTATATTGCTAAAACGTTGATTAAGAAACGCTTAGAGAACATGGG<br>CTCAACTATCCCGATCTCTGCACCATTGTTGCGTCAATTATCTGCAGCCAAGTCGATCGACGAAGTGCGCGCTATCGAAGCCAACA<br>CAACCGCGCGCTACTGGAATAAGTGGTATGAAAACTTGAACATTGAAACAACTCGTCGTAAGGATCACCCTATCAATTCCGCCCTG<br>GACGCCGGGTCAAAATTCATTTACGGAGTGATTCTTCGTTGGTTGGTGTTCCATCGTTTCAGCCCCAACCATGGATTCATGCACCAA<br>CCTACATCGTATCCATCCTTGGTGTACGACTTGATGGAGCCTTTTCGTTACATGATCGAGAACGTCTGTAGCGCCGCGTGGAAACG<br>TGGGGAACGTGAGAACAGCAAAATCGTCGCGTTGAGCCTTTCGTTCCTGAAAGAAGAGTTGGACAAGCCCTGCTACGTGCCCGCG<br>ACCCGCCAATACGTTCGTAAGAAAAATCTTTTGCATGGCGCAGTTCTTGCTCTTCGCAGTTACCTTATTGGAGATATGCGTAAGTTA<br>GTTTTTCCCTCGGAAGGAGTTCCTAATGGGGGTCGCCCAATTAAAGCTTCATATAAATTGCCCGGTTCCATGTATGATGTTGGACGC<br>AAGCCTCCCGAGATCAAACAAAAGGATGAGATCTGTTTCGATGAGGTGAGCCAGGAAGAATCCGAAGAATGATTAACCTAGGCTGC<br>TGCCACCGCTGAGCAATAACTAGCATAACCCCTTGGGGCCTCTAAACGGGTCTTGAGGGGTTTTTTGCTGAAACCTCAGGCATTTG<br>AGAAGCACACGGTCACACTGCTTCCGGTAGTCAATAAACCGGTAAACCAGCAATAGACATAAGCGGCTATTTAACGACCCTGCCCT<br>GAACCGACGACCGGGTCATCGTGGCCGGATCTTGCGGCCCCTCGGCTTGAACGAATTGTTAGACATTATTTGCCGACTACCTTGG<br>TGATCTCGCCTTTCACGTAGTGGACAAATTCTTCCAACTGATCTGCGCGCGAGGCCAAGCGATCTTCTTCTTGTCCAAGATAAGCCT<br>GTCTAGCTTCAAGTATGACGGGCTGATACTGGGCCGGCAGGCGCTCCATTGCCCAGTCGGCAGCGACATCCTTCGGCGCGATTTT<br>GCCGGTTACTGCGCTGTACCAAATGCGGGACAACGTAAGCACTACATTTCGCTCATCGCCAGCCCAGTCGGGCGGCGAGTTCCAT<br>AGCGTTAAGGTTTCATTTAGCGCCTCAAATAGATCCTGTTCAGGAACCGGATCAAAGAGTTCCTCCGCCGCTGGACCTACCAAGGC<br>AACGCTATGTTCTCTTGCTTTTGTCAGCAAGATAGCCAGATCAATGTCGATCGTGGCTGGCTCGAAGATACCTGCAAGAATGTCATT<br>GCGCTGCCATTCTCCAAATTGCAGTTCGCGCTTAGCTGGATAACGCCACGGAATGATGTCGTCGTGCACAACAATGGTGACTTCTA<br>CAGCGCGGAGAATCTCGCTCTCTCCAGGGGAAGCCGAAGTTTCCAAAAGGTCGTTGATCAAAGCTCGCCGCGTTGTTTCATCAAG<br>CCTTACGGTCACCGTAACCAGCAAATCAATATCACTGTGTGGCTTCAGGCCGCCATCCACTGCGGAGCCGTACAAATGTACGGCCA<br>GCAACGTCGGTTCGAGATGGCGCTCGATGACGCCAACTACCTCTGATAGTTGAGTCGATACTTCGGCGATCACCGCTTCCCTCATA<br>CTCTTCCTTTTTCAATATTATTGAAGCATTTATCAGGGTTATTGTCTCATGAGCGGATACATATTTGAATGTATTTAGAAAAATAAACA<br>AATAGCTAGCTCACTCGGTCGCTACGCTCCGGGCGTGAGACTGCGGCGGGCGCTGCGGACACATACAAAGTTACCCACAGATTCC<br>GTGGATAAGCAGGGGACTAACATGTGAGGCAAAACAGCAGGGCCGCGCCGGTGGCGTTTTTCCATAGGCTCCGCCCTCCTGCCA<br>GAGTTCACATAAACAGACGCTTTTCCGGTGCATCTGTGGGAGCCGTGAGGCTCAACCATGAATCTGACAGTACGGGCGAAACCCG<br>ACAGGACTTAAAGATCCCCACCGTTTCCGGCGGGTCGCTCCCTCTTGCGCTCTCCTGTTCCGACCCTGCCGTTTACCGGATACCTG<br>TTCCGCCTTTCTCCCTTACGGGAAGTGTGGCGCTTTCTCATAGCTCACACACTGGTATCTCGGCTCGGTGTAGGTCGTTCGCTCCA<br>AGCTGGGCTGTAAGCAAGAACTCCCCGTTCAGCCCGACTGCTGCGCCTTATCCGGTAACTGTTCACTTGAGTCCAACCCGGAAAA<br>GCACGGTAAAACGCCACTGGCAGCAGCCATTGGTAACTGGGAGTTCGCAGAGGATTTGTTTAGCTAAACACGCGGTTGCTCTTGAA<br>GTGTGCGCCAAAGTCCGGCTACACTGGAAGGACAGATTTGGTTGCTGTGCTCTGCGAAAGCCAGTTACCACGGTTAAGCAGTTCC<br>CCAACTGACTTAACCTTCGATCAAACCACCTCCCCAGGTGGTTTTTTCGTTTACAGGGCAAAAGATTACGCGCAGAAAAAAAGGATC<br>TCAAGAAGATCCTTTGATCTTTTCTACTGAACCGCTCTAGATTTCAGTGCAATTTATCTCTTCAAATGTAGCACCTGAAGTCAGCCCC<br>ATACGATATAAGTTGTAATTCTCATGTTAGTCATGCCCCGCGCCCACCGGAAGGAGCTGACTGGGTTGAAGGCTCTCAAGGGCATC<br>GGTCGAGATCCCGGTGCCTAATGAGTGAGCTAACTTACATTAATTGCGTTGCGCTCACTGCCCGCTTTCCAGTCGGGAAACCTGTC |

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|---|---|
| | GTGCCAGCTGCATTAATGAATCGGCCAACGCGCGGGGAGAGGCGGTTTGCGTATTGGGCGCCAGGGTGGTTTTTCTTTTCACCAG<br>TGAGACGGGCAACAGCTGATTGCCCTTCACCGCCTGGCCCTGAGAGAGTTGCAGCAAGCGGTCCACGCTGGTTTGCCCCAGCAG<br>GCGAAAATCCTGTTTGATGGTGGTTAACGGCGGGATATAACATGAGCTGTCTTCGGTATCGTCGTATCCCACTACCGAGATGTCCG<br>CACCAACGCGCAGCCCGGACTCGGTAATGGCGCGCATTGCGCCCAGCGCCATCTGATCGTTGGCAACCAGCATCGCAGTGGGAA<br>CGATGCCCTCATTCAGCATTTGCATGGTTTGTTGAAAACCGGACATGGCACTCCAGTCGCCTTCCCGTTCCGCTATCGGCTGAATT<br>TGATTGCGAGTGAGATATTTATGCCAGCCAGCCAGACGCAGACGCGCCGAGACAGAACTTAATGGGCCCGCTAACAGCGCGATTT<br>GCTGGTGACCCAATGCGACCAGATGCTCCACGCCCAGTCGCGTACCGTCTTCATGGGAGAAAATAATACTGTTGATGGGTGTCTG<br>GTCAGAGACATCAAGAAATAACGCCGGAACATTAGTGCAGGCAGCTTCCACAGCAATGGCATCCTGGTCATCCAGCGGATAGTTAA<br>TGATCAGCCCACTGACGCGTTGCGCGAGAAGATTGTGCACCGCCGCTTTACAGGCTTCGACGCCGCTTCGTTCTACCATCGACAC<br>CACCACGCTGGCACCCAGTTGATCGGCGCGAGATTTAATCGCCGCGACAATTTGCGACGGCGCGTGCAGGGCCAGACTGGAGGT<br>GGCAACGCCAATCAGCAACGACTGTTTGCCCGCCAGTTGTTGTGCCACGCGGTTGGGAATGTAATTCAGCTCCGCCATCGCCGCT<br>TCCACTTTTTCCCGCGTTTTCGCAGAAACGTGGCTGGCCTGGTTCACCACGCGGGAAACGGTCTGATAAGAGACACCGGCATACT<br>CTGCGACATCGTATAACGTTACTGGTTTCACATTCACCACCCTGAATTGACTCTCTTCCGGGCGCTATCATGCCATACCGCGAAAG<br>GTTTTGCGCCATTCGATGGTGTCCGGGATCTCGACGCTCTCCCTTATGCGACTCCTGCATTAGGAAATTAATACGACTCACTATA |
| pCJH145_pCDF-1b_OspCas1(VC)_Spec | GGGGAATTGTGAGCGGATAACAATTCCCCTGTAGAAATAATTTTGTTTAACTTTAATAAGGAGATATACCATGAACGACGAGAACAA<br>GACAAAATCACATCCCAATAGCATCTGGTACTGGTGCAGCGGGGAAAAACTGTGGTATTTGCCCTACTCAGGAACACCTAAACGTA<br>CAAGCAAGAGTGTATGGACCGTTTCCGGCTCATTCGGTCAGTTAGACATCGATTTATATAAGACGTCCCACATTTTATTCTACGGGG<br>CTAATGAAACCACCGGAGATCTTCCATTACTTTTCCTGAGCGATTGCGCCAAACACAGTGTATCTATCAGCATCCATCGTAACCACG<br>TTTCGGAACCTTTCGTGTTCTATCGTAACAAGACGACTGATCGTGATGACTTGGTTACAGCACAAATTCTGCATCGTGAAAATACCA<br>AAAAGAGCGCTTATATTGCGCGCACGATTATTCGTTTACAAATTAAACAGCGCGGTTATGTCATTCCCTCGTCATTTGATGATACGAA<br>ATTGCTTTCCTCTCGTAACGTTGATGAAGTACGTACCATTGAAGCCACCGCCTCGCGCGAATACTGGCGTTTGTATTTCAAGAAGTT<br>AGGGCTGGAAGGCCTGGGGCGCCGTGATGATCACGTCGTGGTAAAGGCATTGGACGCTACATCGCACTTTCTTTCGGGTATCATG<br>TTACGCTGGGTTTTATCCCACGAGTTGTCCCCTGCGCATGGTTTCATTCACAAATCTTCTAGCTACCCCTCCCTGGTGTATGATCTT<br>ATTGAGCCACTTCGTTGGATTCCTGAACTGGCGGTTTTCCAGTCTTACTTGGAGTTCGGGGAGCATCGTTTGCTGGAACGCTCAAT<br>CGAACGCTTCAAACAAATGCTGGACGAACCTATTCACTCCGAACCTACTCGCCAGTTGGTGTATCGTCGCACGTTAATTCACGGCC<br>AAGTCATTGCTTTGCGCCACTACTTAAATGGGAATATGCAACGCTATTTGCCCCCGGTAGAAGAATTGGCGAAGGAACGCGGACGT<br>AAACGTGCAGTTTCCTACACACTGCCGGGCCAGATTTGGAAAAAGTAATTAACCTAGGCTGCTGCCACCGCTGAGCAATAACTAGC<br>ATAACCCCTTGGGGCCTCTAAACGGGTCTTGAGGGGTTTTTTGCTGAAACCTCAGGCATTTGAGAAGCACACGGTCACACTGCTTC<br>CGGTAGTCAATAAACCGGTAAACCAGCAATAGACATAAGCGGCTATTTAACGACCCTGCCCTGAACCGACGACCGGGTCATCGTG<br>GCCGGATCTTGCGGCCCCTCGGCTTGAACGAATTGTTAGACATTATTTGCCGACTACCTTGGTGATCTCGCCTTTCACGTAGTGGA<br>CAAATTCTTCCAACTGATCTGCGCGCGAGGCCAAGCGATCTTCTTCTTGTCCAAGATAAGCCTGTCTAGCTTCAAGTATGACGGGC<br>TGATACTGGGCCGGCAGGCGCTCCATTGCCCAGTCGGCAGCGACATCCTTCGGCGCGATTTTGCCGGTTACTGCGCTGTACCAAA<br>TGCGGGACAACGTAAGCACTACATTTCGCTCATCGCCAGCCCAGTCGGGCGGCGAGTTCCATAGCGTTAAGGTTTCATTTAGCGC<br>CTCAAATAGATCCTGTTCAGGAACCGGATCAAAGAGTTCCTCCGCCGCTGGACCTACCAAGGCAACGCTATGTTCTCTTGCTTTTGT<br>CAGCAAGATAGCCAGATCAATGTCGATCGTGGCTGGCTCGAAGATACCTGCAAGAATGTCATTGCGCTGCCATTCTCCAAATTGCA<br>GTTCGCGCTTAGCTGGATAACGCCACGGAATGATGTCGTCGTGCACAACAATGGTGACTTCTACAGCGCGGAGAATCTCGCTCTCT<br>CCAGGGGAAGCCGAAGTTTCCAAAAGGTCGTTGATCAAAGCTCGCCGCGTTGTTTCATCAAGCCTTACGGTCACCGTAACCAGCAA<br>ATCAATATCACTGTGTGGCTTCAGGCCGCCATCCACTGCGGAGCCGTACAAATGTACGGCCAGCAACGTCGGTTCGAGATGGCGC<br>TCGATGACGCCAACTACCTCTGATAGTTGAGTCGATACTTCGGCGATCACCGCTTCCCTCATACTCTTCCTTTTTCAATATTATTGAA<br>GCATTTATCAGGGTTATTGTCTCATGAGCGGATACATATTTGAATGTATTTAGAAAAATAAACAAATAGCTAGCTCACTCGGTCGCTA<br>CGCTCCGGGCGTGAGACTGCGGCGGGCGCTGCGGACACATACAAAGTTACCCACAGATTCCGTGGATAAGCAGGGGACTAACAT<br>GTGAGGCAAAACAGCAGGGCCGCGCCGGTGGCGTTTTTCCATAGGCTCCGCCCTCCTGCCAGAGTTCACATAAACAGACGCTTTT<br>CCGGTGCATCTGTGGGAGCCGTGAGGCTCAACCATGAATCTGACAGTACGGGCGAAACCCGACAGGACTTAAAGATCCCCACCGT<br>TTCCGGCGGGTCGCTCCCTCTTGCGCTCTCCTGTTCCGACCCTGCCGTTTACCGGATACCTGTTCCGCCTTTCTCCCTTACGGGAA<br>GTGTGGCGCTTTCTCATAGCTCACACTGGTATCTCGGCTCGGTGTAGGTCGTTCGCTCCAAGCTGGGCTGTAAGCAAGAACTCC<br>CCGTTCAGCCCGACTGCTGCGCCTTATCCGGTAACTGTTCACTTGAGTCCAACCCGGAAAAGCACGGTAAAACGCCACTGGCAGC<br>AGCCATTGGTAACTGGGAGTTCGCAGAGGATTTGTTTAGCTAAACACGCGGTTGCTCTTGAAGTGTGCGCCAAAGTCCGGCTACAC<br>TGGAAGGACAGATTTGGTTGCTGTGCTCTGCGAAAGCCAGTTACCACGGTTAAGCAGTTCCCCAACTGACTTAACCTTCGATCAAA<br>CCACCTCCCCAGGTGGTTTTTTCGTTTACAGGGCAAAAGATTACGCGCAGAAAAAAAGGATCTCAAGAAGATCCTTTGATCTTTTCT<br>ACTGAACCGCTCTAGATTTCAGTGCAATTTATCTCTTCAAATGTAGCACCTGAAGTCAGCCCCATACGATATAAGTTGTAATTCTCAT<br>GTTAGTCATGCCCCGCGCCCACCGGAAGGAGCTGACTGGGTTGAAGGCTCTCAAGGGCATCGGTCGAGATCCCGGTGCCTAATG<br>AGTGAGCTAACTTACATTAATTGCGTTGCGCTCACTGCCCGCTTTCCAGTCGGGAAACCTGTCGTGCCAGCTGCATTAATGAATCG<br>GCCAACGCGCGGGGAGAGGCGGTTTGCGTATTGGGCGCCAGGGTGGTTTTTCTTTTCACCAGTGAGACGGGCAACAGCTGATTG<br>CCCTTCACCGCCTGGCCCTGAGAGAGTTGCAGCAAGCGGTCCACGCTGGTTTGCCCCAGCAGGCGAAAATCCTGTTTGATGGTGG<br>TTAACGGCGGGATATAACATGAGCTGTCTTCGGTATCGTCGTATCCCACTACCGAGATGTCCGCACCAACGCGCAGCCCGGACTC<br>GGTAATGGCGCGCATTGCGCCCAGCGCCATCTGATCGTTGGCAACCAGCATCGCAGTGGGAACGATGCCCTCATTCAGCATTTGC<br>ATGGTTTGTTGAAAACCGGACATGGCACTCCAGTCGCCTTCCCGTTCCGCTATCGGCTGAATTTGATTGCGAGTGAGATATTTATGC<br>CAGCCAGCCAGACGCAGACGCGCCGAGACAGAACTTAATGGGCCCGCTAACAGCGCGATTTGCTGGTGACCCAATGCGACCAGA<br>TGCTCCACGCCCAGTCGCGTACCGTCTTCATGGGAGAAAATAATACTGTTGATGGGTGTCTGGTCAGAGACATCAAGAAATAACGC<br>CGGAACATTAGTGCAGGCAGCTTCCACAGCAATGGCATCCTGGTCATCCAGCGGATAGTTAATGATCAGCCCACTGACGCGTTGC<br>GCGAGAAGATTGTGCACCGCCGCTTTACAGGCTTCGACGCCGCTTCGTTCTACCATCGACACCACCACGCTGGCACCCAGTTGAT<br>CGGCGCGAGATTTAATCGCCGCGACAATTTGCGACGGCGCGTGCAGGGCCAGACTGGAGGTGGCAACGCCAATCAGCAACGACT<br>GTTTGCCCGCCAGTTGTTGTGCCACGCGGTTGGGAATGTAATTCAGCTCCGCCATCGCCGCTTCCACTTTTTCCCGCGTTTTCGCA<br>GAAACGTGGCTGGCCTGGTTCACCACGCGGGAAACGGTCTGATAAGAGACACCGGCATACTCTGCGACATCGTATAACGTTACTG<br>GTTTCACATTCACCACCCTGAATTGACTCTCTTCCGGGCGCTATCATGCCATACCGCGAAAGGTTTTGCGCCATTCGATGGTGTCC<br>GGGATCTCGACGCTCTCCCTTATGCGACTCCTGCATTAGGAAATTAATACGACTCACTATA |

**Table 3.2 Expression plasmids used in this study, Related to Methods**

| Oligo ID | Sequence | Length |
|---|---|---|
| oCJH0230 | GCTCTTCCGATCTTGCAAGCTGGGTGGCTGTT | 32-mer |
| oCJH0231 | GCTCTTCCGATCTCCCACCTGAATACTACTTAGATTCA GCAACATG | 46-mer |
| oCJH0232 | GCTCTTCCGATCTTTGACTTTCAAATAGATATTTGCTAG TCTCCC | 45-mer |
| oCJH0233 | GCTCTTCCGATCTCAATGGACGTCTAGAAAGCAGGTC | 37-mer |
| oCJH0234 | GCTCTTCCGATCTTTGCAATGGGTTTTGTATGCGCAT | 37-mer |
| oCJH0235 | GCTCTTCCGATCTGCCTTCACCACATTAATTCTATCAG ATCGG | 43-mer |
| oCJH0277 | GCTCTTCCGATCTGGCGGGGAATGTTTTGAATTTACG G | 38-mer |
| oCJH0278 | GCTCTTCCGATCTGCATAGGTCTTCATCACAGCTTTCG C | 39-mer |
| oCJH0324 | (duplex) TGAGCTGGATGCCGTTCCAC | 20-bp |
| oCJH0326 | (duplex) TGAGAAAAGCCAAATCGCAA | 20-bp |
| oCJH0328 | (duplex) TGGTTAGACCCATCTCGTTA | 20-bp |

**Table 3.3 Oligonucleotide sequences, Related to Methods**

| | **Plasmid or oligo ID** |
|---|---|
| **Figure 3.1** | VC-4:<br>Plasmid: pCJH099<br>Primers: oCJH0230 and oCJH0231<br><br>VC-6:<br>Plasmid: pCJH100<br>Primers: oCJH0232 and oCJH0233<br><br>VC-Osp:<br>Plasmid: pCJH101<br>Primers: oCJH0234 and oCJH0235 |
| **Figure 3.2** | VC-4:<br>Plasmids: pCJH105 and pCJH112<br>Primers: oCJH0230 and oCJH0231<br><br>VC-6:<br>Plasmids: pCJH106 and pCJH113<br>Primers: oCJH0232 and oCJH0233<br><br>VC-Osp:<br>Plasmids: pCJH107 and pCJH114<br>Primers: oCJH0234 and oCJH0235 |
| **Figure 3.3** | VC-4:<br>Plasmids: pCJH120 and pCJH123<br>Primers: oCJH0230 and oCJH0277<br><br>VC-6:<br>Plasmids: pCJH121 and pCJH124<br>Primers: oCJH0232 and oCJH0233<br><br>VC-Osp:<br>Plasmids: pCJH122 and pCJH125<br>Primers: oCJH0234 and oCJH0278 |
| **Figure 3.4** | VC-4:<br>Plasmids: pCJH134 and pCJH140<br>Duplex oligo used in electroporation: oCJH0324<br>Primers: oCJH0230 and oCJH0277<br><br>VC-6:<br>Plasmids: pCJH135 and pCJH141<br>Duplex oligo used in electroporation: oCJH0326<br>Primers: oCJH0232 and oCJH0233<br><br>VC-Osp:<br>Plasmids: pCJH136 and pCJH142 |

| | |
|---|---|
| | Duplex oligo used in electroporation: oCJH0328<br>Primers: oCJH0234 and oCJH0278 |
| **Figure 3.5** | VC-4:<br>Plasmids: pCJH143 and pCJH123<br>Duplex oligo used in electroporation: oCJH0324<br>Primers: oCJH0230 and oCJH0277<br><br>VC-6:<br>Plasmids: pCJH144 and pCJH124<br>Duplex oligo used in electroporation: oCJH0326<br>Primers: oCJH0232 and oCJH0233<br><br>VC-Osp:<br>Plasmids: pCJH145 and pCJH125<br>Duplex oligo used in electroporation: oCJH0328<br>Primers: oCJH0234 and oCJH0278 |
| **Figure 3.6** | VC-4:<br>Plasmids: pCJH134 and pCJH123<br>Duplex oligo used in electroporation: oCJH0324<br>Primers: oCJH0230 and oCJH0277<br><br>VC-6:<br>Plasmids: pCJH135 and pCJH124<br>Duplex oligo used in electroporation: oCJH0326<br>Primers: oCJH0232 and oCJH0233<br><br>VC-Osp:<br>Plasmids: pCJH136 and pCJH125<br>Duplex oligo used in electroporation: oCJH0328<br>Primers: oCJH0234 and oCJH0278 |

**Table 3.4 Plasmids and oligonucleotides used in experiments, related to Methods**

### 3.5 Discussion

In my attempts to reconstitute type V-C adaptation *in vivo* in a heterologous host *E. coli*, I cloned three type V-C CRISPR loci each containing *cas1* and *cas12c* genes as well as their respective minimal CRISPR arrays consisting of a reduced number of repeat:spacer sequences flanked by ~200bp of the upstream and downstream native sequences into different vectors. *In vivo* spacer acquisition assays were performed when using native sequences of these systems or codon-optimized sequences with synthetic inducible promoters. Assays were also performed when expressing Cas1 alone or co-expressing Cas1 with Cas12c in the presence or absence of its tracrRNA, under physiological expression or overexpression conditions, and with or without electroporation of synthetic protospacers with a PAM. Agarose gel results showed that the attempts to reconstitute type V-C CRISPR-Cas adaptation in a heterologous host *E. coli* have not been successful under the conditions tested. Some possible reasons include expression conditions and sensitivity of the detection method used. Varying the induction level, time, temperature for protein expression, or changing the synthetic strong promoter that control the expression of CRISPR minimal arrays, may improve the experimental outcomes in the future. Additionally, there may be some amplified arrays, the intensity of which may fall below the detection limit of agarose gels. Degenerated primers that preferentially amplify expanded compared with unexpanded arrays (Heler et al., 2015; Kieper et al., 2018, 2019) can be utilized in future studies to overcome such limitations.

However, it remains possible that spacer acquisition requires native host factors that may not exist in *E. coli*. Additionally, in the native host, co-occurring CRISPR-Cas systems may provide the necessary factors for adaptation *in vivo*. Therefore, computationally identifying putative native host factors from metagenomic data and experimentally screening some candidates may also be ways to improve the outcomes of this investigation. If type V-C adaptation is reconstituted *in vivo* in *E. coli* or other culturable microbes, further experiments can be performed to identify determinants of type V-C adaptational PAM selectivity and look for evidence of primed type V-C adaptation in DNase-free orthologs. Understanding the mechanistic details of type V-C adaptation can lay the foundation for many future studies to explore the interactions between the interference and acquisition complexes, including studies of the potential protein-protein interactions (if exists) between Cas1 and Cas12c, as well as structural determinations. These comprehensive insights from future work will contribute to our understanding of the CRISPR adaptation process, which may advance the development of molecular recording tools through the utilization of the natural property in CRISPR-Cas adaptation to store DNA-based information within living organisms on demand.

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