

# An enhanced assay to characterize anti-CRISPR proteins using a cell-free transcription-translation system

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## **ABSTRACT**

The characterization of CRISPR-Cas immune systems in bacteria was quickly followed by the discovery of anti-CRISPR proteins (Acrs) in bacteriophages. These proteins block different steps of CRISPR-based immunity and, as some inhibit Cas nucleases, can offer tight control over CRISPR technologies. While Acrs have been identified against a few CRISPR-Cas systems, likely many more await discovery and application. Here, we report a rapid and scalable method for characterizing putative Acrs against Cas nucleases using an *E. coli*-derived cell-free transcription-translation system. Using known Acrs against type II Cas9 nucleases as models, we demonstrate how the method can be used to measure the inhibitory activity of individual Acrs in under two days. We also show how the method can overcome non-specific inhibition of gene expression observed for some Acrs. In total, the method should accelerate the interrogation and application of Acrs as CRISPR-Cas inhibitors.

## 1. INTRODUCTION

Bacteria and bacterial viruses called bacteriophages have been engaged in an ongoing arms race for billions of years, resulting in each side developing offensive and defense capabilities to gain an upper hand over the other [1–3]. These capabilities have gifted biotechnology with an important battery of biological tools. Notably, bacteria evolved phylogenetically and functionally diverse adaptive immune systems, called CRISPR-Cas (Clustered Regularly Interspaced Short Palindromic Repeats and CRISPR-associated) systems, to recognize and eradicate the genetic material of bacteriophages and other foreign invaders [4–7]. This sequence-specific adaptive immunity is based on the acquisition of small fragments derived from the DNA of invading plasmids or bacteriophages into the system. These so-called spacers are separated by identical repeats in the CRISPR array. The array is transcribed and processed into individual CRISPR RNAs (also called guide RNAs, gRNAs) that complex with a Cas nuclease. The guide portion of the CRISPR RNA then directs the nuclease to bind complementary sequences flanked by a short but distinct genetic signature such as a protospacer-adjacent motif (PAM) [8]. Because the guide sequence derives from the spacer and therefore the invader's genetic material, the nuclease is programmed to seek out and destroy the same genetic material if found in the bacterium's cytoplasm. These same nucleases have been co-opted for programmable DNA and RNA binding and cleavage in many organisms, opening new opportunities for genome editing and many other applications [9–13].

As part of the arms race between bacteria and bacteriophages, bacteriophages have mounted their own countermeasures against CRISPR-Cas systems in the form of anti-CRISPR proteins (Acrs) [14–17]. These proteins inhibit different steps of adaptive immunity and vary in mechanism, such as inhibiting the proteins responsible for acquiring new spacers, competitively blocking DNA binding, or preventing the nuclease domains from cleaving the bound DNA [18–20]. The inhibitory effect appears to be altruistic, even for the nuclease-targeting Acrs: the bacteriophage encoding the Acr still succumbs to rapid attack by the CRISPR-associated (Cas) nuclease, but the Acr blocks the nuclease from attacking a

second wave of bacteriophage infections [21,22]. Aside from the natural roles of Acrs in bacteria-bacteriophage interactions, the nuclease-targeting Acrs also hold technological potential to tightly control the temporal and spatial activity of Cas nucleases in applications ranging from gene editing and gene drives to synthetic gene circuits and optogenetics [23,24].

Since the discovery of Acrs in 2013 [25], a growing number of Acrs have been reported that inhibit CRISPR-Cas systems from subtypes I-C, I-E, I-F, II-A, and II-C [16,25–32]. Each subtype further boasts a collection of genetically and structurally distinct Acrs (e.g. currently five distinct Acrs that inhibit Type II-C CRISPR-Cas systems) [33–35]. These Acrs have lacked any universal defining features aside from a tendency to sit adjacent to genes harboring helix-turn-helix motifs [26,33], confounding their identification. Furthermore, as over 30 subtypes of CRISPR-Cas systems have been identified and Acrs can inhibit a given system through different mechanisms of action, we have likely only observed the proverbial tip of the iceberg for Acrs in nature. There is thus a pressing need for rapid and scalable methods to characterize putative Acrs.

We recently reported the use of an *E. coli*-derived, cell-free transcription-translation (TXTL) system to characterize Cas nucleases and synthetic guide RNAs [36]. This TXTL system comprises a lysate energized by an ATP regeneration system and supplemented with the necessary building blocks (ribonucleosides and amino acids) to recapitulate translation and transcription through promoters specific to the *E. coli* core RNA polymerase and sigma factor 70 [37,38]. Adding linear or plasmid DNA encoding expression constructs leads to active transcription and translation of functional biomolecules within minutes to hours--all without protein purification or live cells [39,40]. As a lysate, the reaction conditions can be exquisitely controlled, whether by varying the amount of each DNA construct, adding different chemicals, or varying the incubation temperature. Finally, TXTL reactions can be conducted in a few microliters in microtiter plates, allowing scalable measurements such as when measuring expression of a fluorescent reporter on a fluorescence plate reader. In our recent work, we showed that expressing different Cas nucleases and guide RNAs targeting

an added GFP reporter plasmid allowed us to quantify nuclease activity over time [36]. Furthermore, co-expressing established or putative Acr's allowed us to quantify the ability of each Acr to inhibit nuclease activity, where a similar approach was separately used to identify novel Acrs that inhibit Type V-A Cas12a nucleases [36]. Through these experiments, we observed that co-expressing some Acrs partially or fully inhibited GFP expression, confounding our ability to measure inhibitory activity. Therefore, further improvements to the method were needed to overcome this non-specific inhibitory effect and expand our ability to assess a broader set of putative Acrs.

Here, we report an enhanced method to characterize Acrs and their inhibitory activity against different Cas nucleases with TXTL that circumvents the non-specific inhibitory effect of expressing some Acrs. The modification involved pre-expressing each Acr separately from the Cas nuclease and the guide RNA and then adding the pre-expressed Acr and nuclease:gRNA complex along with the targeted GFP reporter construct to a fresh TXTL reaction. By varying the added amount of the pre-expressed Acr, we could eliminate the non-specific inhibition of GFP expression while preserving inhibition of nuclease activity. Furthermore, to facilitate testing, we showed that the pre-expressed nuclease:gRNA complex or the Acr could be frozen for at least a week without substantially impacting the measured inhibitory activity of the Acr. The findings came from testing six different Acrs against the Cas9 nucleases from *Streptococcus pyogenes* and *Campylobacter jejuni*. In total, this method is expected to accelerate the discovery and characterization of Acrs to expand our knowledge of CRISPR-Cas immune systems and advance the capabilities of CRISPR technologies.

## 2. METHODS

Below we detail the TXTL-based method to measure the inhibitory activity of Acrs while eliminating non-specific loss of GFP expression. Note that the method specifically detects activities that block DNA cleavage by Cas nucleases (**Figure 1A**), although inhibition of DNA binding would also be detected. As part of the method, the nuclease and guide RNA are pre-

expressed separately from the Acr to ensure that the ribonucleoprotein is fully formed. For this work, we used single-guide RNAs (sgRNAs) that fuse a processed CRISPR RNA and tracrRNA from Type II CRISPR-Cas systems [41]. These two reactions are then diluted into a subsequent fresh TXTL reaction, and the targeted GFP reporter is added as a readout of cleavage activity (**Figure 1B**). Measuring reporter levels over time in a fluorescence plate reader then allows the quantification of the inhibitory activity of the tested Acr. A key aspect of the method is using different dilutions of the pre-expressed Acr, as small dilutions can prevent GFP expression non-specifically while large dilutions may not lead to any measurable inhibition of nuclease activity.

## 2.1. Design of the DNA expression constructs

The DNA used to express the Cas nuclease, guide RNAs, putative Acrs, and the GFP reporter can be in the form of a plasmid or linear DNA (e.g. gBlock from IDT). In either case, the constructs should allow strong expression in *E. coli* under exponential-growth -- the conditions in which the lysate for TXTL is prepared -- using strong constitutive promoters and, when expressing proteins, highly efficient ribosome-binding sites (RBS). For instance, the reporter plasmid (P70a-deGFP) used here relies on the consensus Sigma 70 promoter P70a to drive expression of a variant of eGFP that was optimized for TXTL [37]. Alternatively, genes can be expressed from a T7 promoter, although the TXTL reaction must be supplemented with a plasmid expressing the T7 RNA polymerase (e.g. P70a-T7RNAP). A transcriptional terminator is included at the end of the construct; we typically use the T500 terminator (see **SI Table 1**), although any rho-independent terminator from *E. coli* should be sufficient. If linear DNA is used (as we do to express Acrs), the TXTL reaction requires GamS protein or linear DNA encoding chi sites that block rapid degradation of linear DNA by RecBCD [39,42].

We recommend including some unstructured sequence between the end of the construct and the terminator, as a proximal rho-independent terminator was shown to inhibit processing of a transcribed array by Cas12a [ref Chunyu's paper]. The guide RNAs can be

encoded in different forms depending on the associated nuclease. The most important consideration is whether any additional processing factors need to be added. For instance, sgRNAs are sufficient to guide DNA targeting by Cas9, because they represent the processed version of CRISPR RNAs. By contrast, CRISPR arrays would require co-expression of the tracrRNA, while the RNase III native to *E. coli* and already present in the TXTL lysate would be responsible for cleaving the hybridized repeat:tracrRNA [43]. In general, other CRISPR nucleases (e.g. Cas12a, Cas13a) and effector complexes (e.g. Cascade) can process a transcribed array without accessory factors. In these cases, guide RNAs can be encoded as arrays or processed CRISPR RNAs. A number of techniques are available for constructing CRISPR arrays [43–50].

There are different ways to generate the expression constructs for anti-CRISPR proteins. One way is to amplify the target region from the originating DNA. A Sigma 70 or T7 promoter can be added, although residual transcription from the native promoter may also suffice [36,40]. Another approach is to synthesize the putative Acr as a linear gene fragment that can be used as-is in TXTL or cloned into a plasmid construct. We recommend using the gene fragment as-is, based on challenges we encounter when cloning Acrs into *E. coli*. When the Acrs are expressed under a strong constitutive promoter, one issue in particular is toxicity. These problems could also be resolved by using a promoter that is inducible or not normally active in *E. coli* (e.g. T7) or by exchanging the promoter after introduction into a plasmid. We use a standard expression construct encoding the strong, constitutive J23119 promoter, a strong ribosome-binding site, and the T500 terminator. The specific sequence for this construct is shown in **Figure 2**, where the encoded Acr (AcrIIA4) can be replaced with the coding region of a putative Acr codon-optimized for expression in *E. coli*. The sequence flanking the RBS may also need to be optimized using online tools such as the RBS calculator [51,52], to ensure efficient translation. The construct also encodes flanking primer sites so the synthesized DNA can be amplified.

We applied similar approaches to encode Cas nucleases and guide RNAs. In this work, each was encoded on an individual plasmid based on prior published work [36,39].

However, the nuclease and guide RNAs could be encoded in linear DNA. Note that the same promoter or plasmid backbone can be used for these constructs, as plasmid maintenance and stability are irrelevant in TXTL. When designing the guide sequence, we generally recommend targeting the P70a promoter driving expression of deGFP, as any Acrs that inhibit DNA cleavage but not binding will still yield loss of deGFP expression through transcriptional inhibition. To differentiate between binding and cleavage, a site upstream of the promoter can be targeted such that cleavage leads to RecBCD-dependent degradation of the plasmid and loss of deGFP expression [36]. However, this approach is incompatible with the use of RecBCD inhibitors and therefore requires all constructs to be cloned into plasmids.

In this article, GFP refers to deGFP, a truncated version of eGFP with the same fluorescent properties [40]. All oligonucleotides, plasmids, and gene-fragment sequences used to demonstrate the method can be found in the Appendix.

## **2.2. Preparation of DNA**

In our experience, DNA used in TXTL must be highly pure, as residual salts and other components can interfere with gene expression in TXTL. We normally prepare plasmid DNA first using a midiprep kit followed by a second purification using a PCR clean-up kit. PCR products can be purified in one step using a PCR clean-up kit (DNA clean and concentrator, #D4014, Zymogen). This step is also useful to re-concentrate DNA. DNA concentrations were measured with standard techniques, such as the spectrophotometer/fluorometer by DeNovix (DeNovix, DS-11 FX+). As a specific amount of DNA is used for every reaction, the stock concentration has to be set accordingly (**Tables 1-3**). If the concentration of the DNA was too low to meet these requirements, the corresponding plasmid was concentrated using a DNA purification kit.

## **2.3. RNA and protein production using TXTL**

The TXTL mix we use is based on an *E. coli* lysate, which can be prepared through well-established protocols [53] or purchased commercially (e.g. myTXTL mix available through Arbor Biosciences). The reaction lasts ~16 h for the constructs and template concentrations we used, so all experiments reported here were conducted for 16 h. However, shorter times can be used, particularly when assaying cleavage activity of the Cas nuclease. We also conduct all reactions at 29°C, the optimal temperature for deGFP production, although the temperature can be varied between 25°C to 42°C [36,37]. Measurements are performed in a fluorescence plate reader (e.g. BioTek NeoG2 plate reader) by recording GFP fluorescence every three minutes. If the TXTL reaction is being used only for end-point measurements or for protein/RNA pre-expression, then the reactions can be incubated in a thermocycler or an incubator. We pre-express the individual components in 12- $\mu$ l reactions in PCR tubes, and we conduct the cleavage assays in 3- $\mu$ l reactions in 96-well plates seeded using a Labcyte Echo 525 acoustic liquid dispensing system. The cleavage assays can also be pipetted manually, as described in **section 2.6**.

While the *E. coli*-based TXTL mix will be used throughout this protocol, many other TXTL mixes can be made or are commercially available. The protocol would need to be modified (e.g. solely using T7 promoters), although the general procedures outlined here should apply to any TXTL mix. We also use the Cas9 nucleases from *Streptococcus pyogenes* and *Campylobacter jejuni*, corresponding sgRNAs targeting the GFP reporter, and previously reported Acrs as examples within the protocol and experiments conducted to illustrate the method. However, the protocol can be readily adapted to test other Cas nucleases.

#### **2.4. Pre-expression of Cas9 and sgRNA**

Cas9 and a sgRNA are initially pre-expressed in the same TXTL reaction to enable the formation of an active Cas9-sgRNA complex without potential inhibition of gene expression by an Acr. As part of the reaction, plasmids encoding Cas9 and the sgRNA are added to the

TXTL premix, and the reaction is incubated at 29°C for 16 h to ensure maximal generation of the complex.

- Material

- myTXTL Sigma 70 Master Mix Kit (Arbor Biosciences myTXTL Sigma 70 Master Mix Kit, Cat # 507096)
- plasmid encoding Cas9 (e.g. SpCas9 no tracr Cm p15a)
- plasmid encoding a targeting sgRNA (e.g. sgRNA-pos9 SpCas9) or a non-targeting sgRNA (e.g. sgRNA-non-targeting SpCas9)
- PCR tubes (e.g. Multiply® -µStrip Pro, Sarstedt, Cat # 72.991.002)
- Thermocycler or general purpose incubator

- Protocol

1. Calculate the volume of myTXTL that is needed according to the number of reactions. If a master mix is prepared, it has to be taken into account that about 10% more reaction volume than actually needed should be prepared due to inaccuracies during pipetting. The master mix was usually prepared containing either the targeting or non-targeting sgRNA plasmid.
2. Thaw the appropriate volume of myTXTL on ice. Briefly centrifuge if necessary to ensure that all liquid is at the bottom of the tube. If more than one tube is used, combine them before other reaction agents are added.
3. Prepare the reaction by adding the components listed in **Table 1** into a fresh PCR tube. Mix gently by lightly vortexing and then spin down the contents using a table-top centrifuge.
4. Place the 12-µl reactions in a thermocycler or incubator and incubate them for 16 h at 29°C (see **Note 1**).
5. The final pre-expression reactions can be stored frozen at -20°C for later use.

## 2.5. Pre-expression of Acrs

The Acrs are encoded on linear DNA and pre-expressed without any additional components. Therefore, GamS is added to the TXTL premix to block degradation of the linear DNA by RecBCD.

- Material

- myTXTL Sigma 70 Master Mix Kit (Arbor Biosciences myTXTL Sigma 70 Master Mix Kit, Cat # 507096)
- Gene fragment encoding the anti-CRISPR protein (e.g. construct encoding AcrIIA4 shown in **Figure 2**)
- GamS Purified Nuclease Inhibitor Protein (Arbor Biosciences, Cat # 501096)
- Thermocycler or general purpose incubator

- Protocol

1. Calculate the volume of myTXTL that is needed according to the number of reactions. If a master mix is prepared, it has to be taken into account that about 10% more reaction volume than actually needed should be prepared due to inaccuracies during pipetting. In this case it has shown to be useful to prepare a master mix without the DNA encoding the respective Acr.
2. Thaw the appropriate volume of myTXTL on ice. Briefly centrifuge if necessary to ensure that all the liquid is at the bottom of the tube. If more than one tube is used, combine them before other components are added.
3. Prepare the reaction by adding the components listed in **Table 2** to a fresh PCR tube. Mix gently by lightly vortexing and spin down using a table-top microcentrifuge.
4. Place the 12- $\mu$ l reactions in a thermocycler or incubator and incubate them for 16 h at 29°C (see **Note 1**).
5. The final pre-expression reactions can be stored frozen at -20°C for later use.

## 2.6. DNA cleavage assay

Once the pre-expression reactions are completed for Cas9, the sgRNA, and the Acr, the next step is measuring the inhibitory activity of the Acr using a GFP reporter construct targeted by the sgRNA. As part of the assay, the pre-expressed components are mixed with the reporter plasmid in a fresh aliquot of TXTL, and GFP production is measured over time using a fluorescence plate reader. A targeting sgRNA and a non-targeting sgRNA are used in the presence and absence of the Acr to quantify inhibitory activity and determine the extent to which the Acr inhibits GFP expression.

- Material

- myTXTL Sigma 70 Master Mix Kit (Arbor Biosciences myTXTL Sigma 70 Master Mix Kit, Cat # 507096)
- P70a-deGFP plasmid
- Reactions with pre-expressed Cas9, sgRNA and Acr
- 96-well plate (e.g. Corning® 96-well Clear V-Bottom, Cat # 3363)
- Fluorescence plate reader (e.g. Biotek Synergy Neo2 plate reader)
- Cover mat (e.g. Fisher Scientific, Corning™ Storage Mat III, Cat # 10428571)

- Protocol

1. Calculate the volume of myTXTL that is needed according to the number of reactions. If a master mix is prepared, it has to be taken into account that about 10% more reaction volume than actually needed should be prepared due to inaccuracies during pipetting.
2. Thaw the appropriate volume of myTXTL on ice. Briefly centrifuge if necessary, to ensure that all the liquid is at the bottom of the tube. If more than one tube is used, combine them before other reaction agents are added.
3. Prepare the reaction by adding the components listed in **Table 3** to a fresh PCR tube. Mix gently by vortexing and spin it down afterwards.

4. Load 5- $\mu$ l duplicates of each reaction in the bottom of a 96-well V bottom plate (see **Note 2**). Be careful not to introduce any bubbles.
5. Seal the plate with a cover mat to prevent evaporation over the course of the reaction.
6. Place the sealed plate in a plate reader to measure GFP fluorescence (Ex 485 nm, Em 528 nm). The plate reader should be pre-warmed to 29°C (see **Note 1**).
7. Incubate the reactions for 16 h at 29°C (see **Note 1**) and measure GFP fluorescence every three minutes.

## 2.7. Data Processing

The fluorescence data collected by the plate reader is next processed to extract the extent to which the Acr inhibited DNA binding and cleavage by Cas9.

- Protocol
1. Export the data in an Excel spreadsheet after the plate reader run is finished. It should include time points, temperature and fluorescence intensity values for each well and each time point.
  2. Subtract the background fluorescence. Background fluorescence was measured for each plate reader separately using a TXTL reaction containing a plasmid that doesn't encode deGFP. Fluorescence values were measured for 16 h at 29°C, identical to the measurement settings of the cleavage assay.
  3. If needed, calculate the deGFP concentration for each timepoint by using a deGFP standard curve (see **Note 3**).
  4. Calculate the average of the replicates and visualize the data on a graph by plotting the fluorescence over time.
  5. Calculate standard deviations for the replicates and use them to show error bars for each time point in the graph.

6. Calculate the fold-reduction for the reporter construct using the ratio of deGFP concentrations after 16 h of the reaction containing non-targeting sgRNA over the reaction containing targeting sgRNA.
7. Percent inhibition of DNA cleavage by the nuclease can be calculated using the following formula:

$$\% \text{ Inhibition of nuclease activity} = 100\% * \left( \frac{\frac{\text{GFP}_{t, \text{Acr}}}{\text{GFP}_{nt, \text{Acr}}} - \frac{\text{GFP}_{t,-}}{\text{GFP}_{nt,-}}}{1 - \frac{\text{GFP}_{t,-}}{\text{GFP}_{nt,-}}} \right)$$

where the values are for the measured GFP fluorescence with a targeting or non-targeting sgRNA (t/nt) and in the presence or absence of an Acr (Acr/-). By taking the ratio of fluorescence values for the targeting and non-targeting sgRNAs, any non-specific inhibition of GFP expression by the Acr would be assumed to equally impact the two samples and would therefore cancel out.

## 2.8. Troubleshooting

If cleavage of the reporter plasmid cannot be observed even in the reaction without any Acr, we recommend first confirming that the expression constructs are unmutated. In our experience, the nuclease construct can sometimes collect mutations in the *E. coli*, eliminating cleavage activity in TXTL. The expression of the nuclease can also be determined by Western blotting analysis if an affinity tag is included, although we have encountered instances in which no expression was observable by Western blotting analysis despite robust GFP silencing. Similarly, expression of the guide RNA can be detected by Northern blotting analyses [54]. Otherwise, there are multiple ways to potentially enhance cleavage activity. The concentration of the DNA components can be varied, such as increasing the concentration of the nuclease plasmid or reducing the concentration of the deGFP reporter plasmid. Separately, the temperature of the reaction can be varied between 25°C and 42°C (see **Note 1**) or the reaction can be supplemented with Mg<sup>2+</sup> previously

shown to improve the expression of some proteins [37]. It is also possible to vary the pre-expression time.

As mentioned earlier, the DNA has to be highly pure if it is to be used in a TXTL reaction. When no expression or cleavage of the deGFP plasmid can be observed, impurities in the DNA of one of the constituents may be inhibiting transcription and/or translation. Therefore, it is advisable to perform an additional DNA cleaning step in case of an error during the reaction. Also, too much anti-CRISPR protein can in some cases lead to inhibition of GFP expression, which can be adjusted by using a higher dilution of those Acrs.

## **2.9. Notes**

Note 1: The TXTL reactions can be conducted at temperatures between 25°C to 42°C [37]. While 29°C is optimal for deGFP production, some CRISPR nucleases exhibit higher expression and activity at other temperatures (e.g. MbCas12a) [36]. Therefore, it may be helpful to vary the reaction temperature to optimize nuclease activity prior to testing any Acrs. The duration of the reaction and the number of fluorescence measurements can also be adjusted.

Note 2: The protocol assumes that the DNA cleavage reaction will be prepared by manual pipetting. If a liquid handling system that can accurately transfer small volumes is used (e.g. the Labcyte Echo 525 used as part of the presented experiments), the reaction volumes can be scaled down to 2 - 3- $\mu$ l.

Note 3: Generating a calibration curve to calculate the concentration of deGFP protein from the fluorescence measurements [40] can ease comparisons between plate readers and research groups. The curve can be readily generated by obtaining purified recombinant eGFP (e.g. Hölzel Diagnostika, STA-201) and adding different concentrations to a final TXTL reaction with either no DNA or DNA that does not produce deGFP.

## **3. RESULTS AND DISCUSSION**

We had previously used TXTL to characterize twenty Acrs against a panel of five Cas9 nucleases. As part of these assays, the Acrs were co-expressed with Cas9, a targeting or non-targeting sgRNA, and the targeted deGFP reporter. While these assays allowed us to characterize the inhibitory range of most of the Acrs, we encountered Acrs that seemed to directly impact deGFP expression, independent of CRISPR-Cas activity. Two of the Acrs completely inhibited and three partially inhibited an increase in the fluorescence even in the presence of a non-targeting sgRNA [36]. Follow-up work demonstrated that the Acrs were not inhibiting the fluorescence output of pre-expressed deGFP, suggesting that the Acr expression was inhibiting gene expression. Because this effect could extend to expression of Cas9 and the targeting sgRNA, it confounded our ability to measure inhibition of nuclease activity. We therefore reasoned that pre-expressing the Cas9 and sgRNA separate from the Acr could prevent this confounding effect (**Figure 1B**). Furthermore, diluting the amount of pre-expressed Acr could help us reach a regime in which the Acr can selectively inhibit nuclease activity without impacting deGFP expression.

We first asked if a pre-expressed Acr would still inhibit nuclease activity in TXTL. We selected as a test case AcrIIA4, a potent and well-characterized inhibitor of the *S. pyogenes* Cas9 (SpCas9) [15]. The Acr was encoded in linear DNA as shown in **Figure 2** and pre-expressed in TXTL for 16 h. In parallel, SpCas9 and a non-targeting sgRNA or a sgRNA targeting the non-template strand of the *degfp* gene were encoded on separate plasmids and were pre-expressed together for the same period of time. The pre-expressed AcrIIA4 was then diluted up to 1:10,000 in water and mixed with the pre-expressed SpCas9:sgRNA complex and the deGFP reporter plasmid in fresh TXTL mix using an acoustic liquid handling system (see **SI Method Echo 525 Cleavage Assay**). Production of deGFP was then monitored over time. The resulting fluorescence output is shown in **Figure 3**.

We found that, in the absence of added AcrIIA4, GFP production ceased after approximately one hour with the targeting sgRNA (**Figure 3**). In contrast, in the presence of AcrIIA4, GFP production was similarly high for both targeting and non-targeting sgRNAs. Therefore, AcrIIA4 actively inhibited Cas9 nuclease activity without non-specifically

impacting GFP production, as we found previously [36]. We also found that GFP production with a non-targeting sgRNA was lowest for the 1:10,000 dilution, although the explanation for this decrease remains unclear.

Interestingly, the pre-expressed AcrIIA4 completely inhibited cleavage by SpCas9 even when diluted by a factor of 100. Given that AcrIIA4 is known to act stoichiometrically by directly binding SpCas9 [15,55], these findings would suggest that the levels of pre-expressed AcrIIA4 are at least 100-fold higher than those of either SpCas9 or the sgRNA. More practically, these findings suggest that a pre-expressed Acr can be substantially diluted without compromising the observed inhibition of a Cas nuclease, offering a simple means to circumvent any non-specific inhibition of reporter expression.

As a further demonstration, we repeated the assay using the Cas9 from *Campylobacter jejuni* (CjCas9) based on our prior work [36,56]. Specifically, we tested the inhibitory activity of AcrIIC1 against this nuclease (**Figure 3C**). Our TXTL-based assay revealed strong inhibitory activity (58% for the 1:1 dilution). However, inhibition was lost for any dilutions larger than 1:10, potentially representing differences in expression and/or inhibitory activity compared to AcrIIA4.

One ramification of pre-expressing Cas9, the sgRNA, and the Acr is that the pre-expressed components could be stored prior to conducting the DNA cleavage assays. If so, then nuclease or potential Acrs could be mass-produced, aliquoted, frozen, and then used at later times (**Figure 4A**). To investigate this possibility directly, we stored the pre-expressed SpCas9:sgRNA complex and AcrIIA4 at -20°C. At this temperature, the TXTL mix is frozen. Each mix was then thawed after storage for one day or seven days and tested in the DNA cleavage assay using up to a 1:10,000-fold dilution of AcrIIA4 (**Figure 4B**). Freezing the pre-expressed mixes had at most a minor impact on the measured inhibition of cleavage by SpCas9, with the largest difference in inhibitory percentage of 14.7% (50.5% versus 65.2% for unfrozen and 1-day frozen AcrIIA4 diluted 1:1000) and p-values for statistical significance ranging between 0.016 and 0.94 (two-tailed T-test, n = 3). Therefore, pre-expressed nuclease:guide RNA complexes as well as Acrs can be stored at -20°C without substantially

impinging on their activity, although more testing will be required to determine whether this possibility extends to different Cas nucleases and Acrs.

With the method firmly established, we next focused on Acrs that partially or completely inhibited GFP expression in our prior assays even in the presence of a non-targeting sgRNA. Specifically, we selected AcrIIA3 and two homologs (AcrIIA3-2 and AcrIIA3-4). We also selected a homolog of AcrIIA4 (AcrIIA4-1) that appeared to partially inhibit the activity of Cas9's from *Campylobacter jejuni*, *Streptococcus thermophilus*, and *Neisseria meningitidis* but not SpCas9 [36]. Given that many of these appeared to inhibit the CjCas9, we included in the assays this Cas9 with an associated non-targeting sgRNA or sgRNA targeting upstream of the *gfp* target gene. We selected three dilutions of each pre-expressed Acr (1:1, 1:10, 1:100) because AcrIIA4 completely inhibited the activity of SpCas9 for all three dilutions (**Figure 3**). We also used the pre-expressed Acrs and Cas9:sgRNA complexes directly in the cleavage assays without freezing. The resulting output of the cleavage assays are shown in **Figure 5**.

At the lowest dilution (1:1), AcrIIA3 partially and AcrIIA3-2 and AcrIIA3-4 strongly inhibited GFP expression (9%, 81%, and 55%, respectively, compared to no Acr in the presence of the non-targeting sgRNA), in line with the results from co-expressing all components at one time [36]. However, at the larger dilutions (1:10 and 1:100), deGFP levels returned to those in the absence of the Acr. The calculated inhibition of SpCas9 activity also diminished at these larger dilutions. The inhibition of SpCas9 activity appears to be real, though, as negligible inhibition was registered for these same Acrs against CjCas9. AcrIIA3, AcrIIA3-2, and AcrIIA3-4 therefore likely act weakly against SpCas9 or are poorly expressed in TXTL. We also speculate that the non-specific inhibition of GFP expression may represent a biological function of these putative Acrs, although more work is needed to explore this possibility.

We also assessed the inhibitory activity of the AcrIIA4 homolog AcrIIA4-1. Interestingly, this Acr at most marginally inhibited the activity of SpCas9 at the lowest dilution (8% compared to no Acr) and negligibly inhibited the activity of CjCas9 at all dilutions. We

had previously evaluated the inhibitory activity of this Acr against SpCas9 and CjCas9 using a prior version of the method in which the nuclease, sgRNA, Acr, and GFP reporter were expressed together [36]. Interestingly, we previously did not observe any inhibition of SpCas9, but we did observe notable inhibition of CjCas9. The differing output between our prior results and those reported here may be due to the impact of expressing the Acr separately from or together with the nuclease and sgRNA. Given the sequence similarity between AcrIIA4 and AcrIIA4-1, the poor inhibition of SpCas9 and negligible inhibition of CjCas9 better fits the ability of AcrIIA4 to only inhibit SpCas9, providing further evidence for the benefits of pre-expressing the Acrs separately from the nucleases and guide RNAs as part of the TXTL assay.

#### **4. Conclusions**

In total, we demonstrated a modified method using TXTL to characterize the inhibitory activity of putative Acrs against different Cas nucleases. The method, which relies on pre-expressing the Cas nuclease and guide RNA separately from the Acr, allowed us to freeze and store the pre-expressed components without an appreciable effect on their activity. Furthermore, by diluting the Acrs prior to conducting the DNA cleavage assay, we could reduce any non-specific effects on GFP expression while still quantifying inhibition of the Cas nuclease. Technically, the original Acr expression construct could have been diluted when expressing all components in the same reaction; however, it would be challenging to distinguish the specific inhibition of nuclease activity from the non-specific inhibition of nuclease and guide-RNA expression. We also note that the final concentration of Acr or the nuclease:guide-RNA complex cannot be readily quantified in the TXTL reaction, potentially leading to false negatives due to poor expression. However, the technique is much faster and scales more readily than any equivalent cell-based assay or any biochemical assay with purified components. Finally, we note that Acrs have been discovered that inhibit other mechanisms of CRISPR-Cas systems besides target binding and cleavage [19,20,57], where the method would need to be adapted to detect these other inhibitory activities. These

limitations aside, the method described here offers a powerful means to screen large sets of putative Acrs, providing a basis for further biochemical characterization and the application of Acrs for controlling CRISPR technologies.

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### **Competing interests statement**

The Noireaux laboratory receives research funds from Arbor Biosciences, a distributor of the myTXTL cell-free protein expression kit.

### **Appendix**

Supplementary Table S1 and a Supplementary method on using the Echo 525 liquid dispensing system can be found in the appendix file.

## Tables

**Table 1.** Components for pre-expression of Cas9 and a sgRNA in TXTL. The table states the reaction volume needed for one experiment. For the preparation of multiple pre-expression mixes for different nucleases or sgRNAs, prepare a master mix. Consider that about 10% more total volume should be calculated when preparing a master mix, due to inaccuracies during pipetting. The master mix for this reaction was usually prepared containing either the targeting or non-targeting sgRNA plasmid. If linear DNA is used instead of plasmids, add GamS in place of water to prevent degradation (see Table 2). We usually added the highest volume first. For identical volumes it is not important what component is added next, with the exception of GamS and linear DNA. If linear DNA is added to a reaction, make sure to add GamS first to prevent any degradation.

Component	Volume ( $\mu$ l)	Initial concentration (nM)	Final concentration (nM)
myTXTL	9	-	-
Cas9 plasmid	1	12	1
sgRNA plasmid	1	12	1
Water	1	-	-

**Table 2.** Components for pre-expression of Acrs. The table states the reaction volume needed for one experiment. If multiple Acrs are pre-expressed, prepare a master mix without DNA. Consider that about 10% more total volume should be calculated when preparing a master mix, due to inaccuracies during pipetting. In this case it has shown to be useful to prepare a master mix without the DNA encoding the respective Acr. Make sure to add GamS before adding any linear DNA to prevent degradation by RecBCD.

Component	Volume ( $\mu$ l)	Initial concentration (nM)	Final concentration (nM)
myTXTL	9	-	-
Acr	1.2	40	4
GamS	0.8	30,000	2,000
Water	1	-	-

**Table 3.** DNA cleavage assay in TXTL. Final reaction to characterize Cas nuclease specificity by analyzing GFP expression over time. When performing experiments with different amounts of Acrs, dilute the samples beforehand with nuclease-free water. For the preparation of multiple reactions, prepare a master mix. Consider that about 10% more total volume should be calculated when preparing a master mix, due to inaccuracies during pipetting. Always add the deGFP plasmid last, to make sure that the nuclease doesn't cleave the target DNA before the Acr can inhibit the nuclease.

Component	Volume ( $\mu$ l)	Initial concentration (nM)	Final concentration (nM)
myTXTL	9	-	-
pre-expressed Cas9 & sgRNA	1	-	-
pre-expressed Acr	1	-	-
P70a-deGFP plasmid	1	12	1

## Figure legends

**Figure 1.** Overview of the enhanced TXTL-based method for assessing the inhibitory activity of putative Acrs against Cas nucleases. **(A)** The assay is designed to specifically measure inhibition of a Cas nuclease binding and cleaving its target. The target is placed within the promoter or transcribed region of a deGFP reporter gene, providing a direct, fluorescent readout of binding and cleavage activity. **(B)** Individual steps of the inhibition assay. The Cas nuclease and guide RNA (gRNA) are pre-expressed together separately from the putative Acr by adding linear or plasmid DNA encoding each component to a TXTL reaction. The reactions are then mixed in a fresh TXTL reaction with the reporter plasmid, and deGFP fluorescence is monitored over time in a fluorescence plate reader. Different dilutions of the pre-expressed Acr can be made to rule out potential non-specific inhibition of deGFP expression. The extent of inhibition is quantified based on the end-point fluorescence levels for reactions conducted with a targeting or non-targeting gRNA with or without an added Acr.

**Figure 2.** Schematic of the linear DNA template for expressing Acrs in TXTL. The construct includes a strong constitutive promoter for expression in *E. coli*, a strong ribosome-binding site (RBS), the coding region of the putative Acr, and a rho-independent terminator. Flanking primer sites (brown arrows) are included to amplify the construct. The coding region of the displayed *acr* gene (encoding AcrIIA4) can be replaced with any putative *acr*, although the RBS may need to be altered to maintain the translation strength. The sequence can be readily ordered as a gene fragment from most commercial suppliers. See the Appendix for an editable version of the sequence.

**Figure 3.** Applying the enhanced method to measure inhibition of DNA cleavage by SpCas9 and CjCas9. **(A)** Time courses of DNA cleavage assays using different dilutions of the pre-

expressed AcrIIA4. Each assay included the deGFP reporter plasmid, SpCas9 pre-expressed with a targeting (T, blue) or non-targeting (NT, green) sgRNA, and the pre-expressed AcrIIA4 diluted up to 10,000-fold. The dark line and light regions indicate the mean and S.D. of three independent cleavage assays. **(B)** and **(C)** Quantified inhibition (top) and GFP expression (bottom) based on the end-point measurements. Experiments were conducted with (B) SpCas9 and AcrIIA4 or with (C) CjCas9 and AcrIIC1. Values at the end of the 16-hour reaction were used to calculate the extent of inhibition of DNA cleavage by either nuclease. The bars in the top plot represent the mean and S.D. of three independently mixed TXTL reactions, while each set of dots in the bottom plot represents the final fluorescence values used to calculate the % inhibition of nuclease activity. The colored bar indicates the mean value for each set of fluorescence measurements.

**Figure 4.** Limited impact of freezing and storing pre-expressed AcrIIA4 and SpCas9:sgRNA complex. **(A)** Freezing and storing the TXTL pre-expression reactions. The reactions are frozen and stored at -20°C. At a later time, they can be thawed and used in the DNA cleavage assays. **(B)** Assessing the impact of freezing pre-expressed AcrIIA4 and SpCas9:sgRNA complex. The same pre-expression mix was used immediately (not frozen) or stored frozen for one day (1 d frozen) or seven days (7 d frozen) prior to conducting the DNA cleavage assay. Reactions were conducted with different dilutions of the unfrozen or frozen samples of pre-expressed AcrIIA4. The calculated inhibition is based on the end-point fluorescence measurements from a 16-hour cleavage assay. Values represent the mean and S.D. of three independent cleavage assays.

**Figure 5.** Measuring the inhibitory activity of five Acrs against two Cas9 nucleases. DNA cleavage assays were conducted using the indicated Cas9 and Acr pre-expressed for 16 h and immediately used in the DNA cleavage assay. See **Figure 3B** for more details. The bars

in the top plot represent the mean and S.D. of three independently mixed TXTL reactions, while each set of dots in the bottom plot represents the final fluorescence values used to calculate the % inhibition of nuclease activity.