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Supplementary Materials for

Systematic discovery of natural CRISPR-Cas12a inhibitors

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This PDF file includes:

Other Supplementary Materials for this manuscript include the following:

Materials and Methods

Bioinformatics with Self-Targeting Spacer Searcher (STSS)

The Self-Target Spacer Searcher is a cross-platform python script (available at https://github.com/kew222/Self-Targeting-Spacer-Search-tool/releases for public use) that accepts a search query for the NCBI Genomes database and returns a list of self-targeting spacers found within the genomes found from the query. The stepwise process is described below as used to produce the list of self-targeting spacers in Data S1. Many of the parameters specifically described below can be adjusted at runtime.

The search term 'Prokaryote' was provided to search NCBI's Genome database, which was linked to the nucleotide database via the assembly database to download all of the resulting genomes in fasta format. CRISPR arrays were then predicted for each genome using the CRISPR Recognition Tool (CRT) (*19*) using 18 and 45 as minimum and maximum repeat and spacer lengths, respectively, and a minimum repeat length of four. For each array that was predicted, the spacers were collected and used to BLAST (blastn with default settings and an e-value limit of 10^{-6}) all of the contigs within the array's assembly. Any hit to a contig in the assembly was considered a self-target, except for the DNA bases within all of the predicted arrays, plus an additional 500 bp from each end of the predicted array, which were ignored. Long stretches of degenerate bases were also artificially shortened to under 500 bp, as CRT is unable to process these sequences.

For each self-targeting spacer that was found, a set of data was collected about the source locus and the genomic self-target position. To collect these data, the Genbank file for each self-targeting genome was downloaded and all of the genes within 20 kb of the spacer within the array were compared to Hidden Markov Models (HMMs) for many of the known Cas proteins using HMMER v3 (*31*) with an e-value cutoff of 10^{-6} to call Cas proteins near the array. The list of Cas proteins was then used to try to predict the CRISPR subtype of the array based on the composition of the nearby Cas proteins, using previously coined definitions (*32,33*). The CRISPR subtype was predicted by enumerating the number of possible types each identified Cas protein could belong to and choosing the subtype with the great number of hits. The exact definitions chosen are provided can be found in CRISPR_definitions.py within STSS. Similarly, the Cas protein HMMs can also be also found within STSS.

After searching for Cas proteins, the repeats and spacers from a CRISPR array were also examined. First, all spacers in the self-targeting array were aligned with Clustal Omega (*34*) to check for conserved bases at each end of the spacer to determine if the array predicted by CRT miscalled the repeat sequence. If the array contained at least six repeats and a string of bases at either end contained 75% or more of the same base, those bases were assumed to be part of the repeat sequence and both the repeat and spacer sequences were adjusted appropriately. Arrays with four or five repeats used 100% as the cutoff to correct the repeat sequence (arrays with three or fewer repeats were not considered from the beginning). Additionally, if the length of the longest and shortest spacers within an array differed by more than 25%, the array was rejected as non-CRISPR, as they possibly represent a direct repeat sequence or other DNA feature (*35*). If passing the length variance filter, the consensus repeat sequence was determined using Biopython's dumb_consensus() method and any mutations/indels in the repeat sequences flanking the self-targeting spacer were reported.

To predict the subtype of CRISPR system the array of a self-targeting spacer belonged to (in addition to the protein method described above), the self-targeting spacer was compared to set a HMMs that were built from the REPEATS dataset from CRISPRmap (*36,37*) and additional multiple-sequence alignments from more recently discovered CRISPR systems, such as the type V and type VI systems (*22*,*33,38,39*). These HMMs are also available in STSS.

The orientation of the array was determined first using the direction provided in the repeat sequence HMMs if the consensus sequence produced a hit. Otherwise, the CRISPR array was assumed to be oriented such that it was downstream of the predicted Cas proteins, but only if a single subtype was predicted. If neither of these conditions were met, the array direction was left in the default orientation given by CRT (i.e. forward, on the top strand) and noted as such.

To analyze the genomic target of the self-targeting spacer, we took the spacer sequence (possibly corrected from the array analysis) and performed a gapless BLAST at the target site to force the comparison of mutations only and exclude indels in the alignment, as we would not expect bulging to occur within the Cas proteins. The gapless BLAST positions were used as the final alignments and nine bases up- and downstream of each target were reported as potential PAM sequences. Because of the possibility that the predicted CRISPR subtypes in earlier stages are incorrect (or there are multiple), and that there are myriad systems for which no PAM has been experimentally validated (especially in type II), no assumptions about what the expected

PAM was were made, nor which side of the protospacer it should occur on. At this stage, we performed a second heuristic filtering step to remove potential falsely predicted CRISPR arrays by checking the sequences up- and downstream of the protospacer and comparing them to the consensus repeat. If eight of the nine bases matched on either side of the protospacer, the potential self-target was rejected as being in a missed array or part of a direct repeat sequence or similar undesired feature (*35*) that escaped the length variance filter.

The last part of the STSS analysis was to check the contig the targeted DNA occurred in for the presence of MGEs. As part of the STSS pipeline, we searched for prophages in the contig using the online webserver provided by PHASTER (*20*) and noted if there were prophages present and which prophage the self-target occurred in if so. PHASTER analysis completed the STSS pipeline as written, but we also used the Islander Database (*21*) to locate predicted MGEs near the self-target sequence. Regardless whether an MGE was predicted or not, the feature (or features if the protospacer fell between genes) targeted by the self-targeting spacer was reported. If that gene was labeled as 'hypothetical protein', it was also analyzed for potential conserved sequences on NCBI's Conserved Domain Search webserver (*40*). All of the data collected in the steps described above was output in a tab-separated text format.

After the STSS data was collected, we performed a manual scan of the results to correct any potentially miscalled repeat/spacer sequences. Specifically, we looked for spacers that stood out as too long, too short, near the edges of contigs, etc. that would allow bad spacer calls from CRT to make it through our heuristic filters. To correct these potential miscalls, the predicted arrays were visually inspected and the most likely spacer was checked for self-targeting manually and the database updated if the original call was a mistake. The other area we examined was the unknown type II self-targeting spacers.

With the automated methods described above, we were unable to call type II-C separately from II-A or II-B, as it has no distinguishing proteins and its spacers are too similar to subtypes II-A and II-B to create an effective HMM. To overcome this drawback, we aligned the Cas9 protein of the CRISPR locus in question to a set of well-known Cas9s to determine its proper classification. We also manually inspected the spacer sequences, which tend to end in …AAT for type II-C, as opposed to …AAC for II-A. Last, we checked for the presence of Csn2, which is occasionally missed by the HMMs. Last, if the unknown type II array was determined to be subtype II-C, we also needed to manually adjust that orientation as II-C arrays are in the inverse

orientation relative to most CRISPR arrays (*41*). In most cases, identifying the proper subtype was obvious, those that were not obvious were left marked as unknown to prevent a potential mistake in the data. Finally, any data that was manually edited is noted in Data S1 with green highlighting.

To determine which genomes contained an Acr gene, a compiled list of the known Acr genes (*9*,*13*,*17*) was used to perform a BLAST against all NCBI genomes using a blastp search with an E-value limit of 10^{-3}. All genes passing this cutoff were annotated as anti-CRISPRs in this way for simplicity, as creating a precise definition of anti-CRISPR families was not our goal.

Analysis of self-targeting and anti-CRISPR co-occurrence

Self-targeting spacers derived from the type I-E and type I-F CRISPR system of *Pseudomonas aeruginosa*, type II-A system of *Listeria monocytogenes*, and type II-C system of *Neisseria meningitidis* were selected from the full STSS dataset to determine the level of co-occurrence. Self-targeting spacers were included as long as there was reasonable evidence that it belonged to one of the above four systems, using the identified Cas proteins and repeat sequences (via HMM or by inspection) as identifiers. Spacers whose target occurred on the edge of contig such that no PAM sequence was available were excluded. Genomes without protein annotations were also ignored.

In order for a self-targeting spacer to expected to be lethal it was required to meet three conditions: 1) all Cas surveillance proteins needs to be present (and not marked as a pseudogene), 2) no more than two mismatches in the target sequence, and 3) the target must have the correct PAM sequence. The PAM requirements differed for each system. The *L. monocytogenes* system was required to have a perfect NRG PAM and the *P. aeruginosa* systems required perfect PAMs of AAG or CC for the type I-E and I-F systems, respectively. Due to the longer requirement, of the NNNNGATT PAM for the type II-C system we allowed the *N. meningitidis* PAM sequence to contain either one mismatch or one indel.

Using the list of spacers, lists of genomes for each CRISPR system were compiled where each genome contained: at least one self-targeting spacer, at least one lethal self-targeting spacer, or at least one lethal self-targeting spacer and at least one anti-CRISPR.

Selecting genomes to search for Cas12 anti-CRISPRs

Within the results from STSS, there are many cases of predicted lethal self-targeting across the variety of CRISPR subtypes, especially in a number of type I and type II systems (fig. S2). However, we chose to focus mainly on Cas12 due to its increasing interest for medical applications and the lack of inhibitors described for type V systems.

In our results, we observed roughly 250 genomes containing self-targeting type V systems. Of this set, 17 and 19 genomes could be readily identified as type V-A or V-B, respectively. From this point, we chose to focus only on type V-A (Cas12a) as it is more commonly used for gene editing and diagnostic applications (*3,4,28,29*) and more mechanistic information is available for Cas12a than Cas12b.

From the 17 genomes with self-targeting type V-A CRISPR arrays, four were missing Cas12a, which would allow self-targeting to be stable without an anti-CRISPR present. From the remaining 13 genomes, two species had multiple genomes with self-targeting spacers that targeted a predicted internal prophage: *Francisella philomiragia* (3 strains) and *Moraxella bovoculi* (4 strains). Association with an MGE was a major selection point for choosing the organisms to screen as nearly all Acrs that have been discovered are associated with MGEs (*17*). Of the two species, *M. bovoculi* was more attractive because its PAM sequence had been previously determined to be TTV (*22*), which was present in its self-targeting genomes, meaning that its self-targeting spacers should cleave the genome and be lethal to the cell. Additionally, in the *F. philomiragia* genomes Cas12 was marked as a pseudogene. Also, there were multiple strains of *M. bovoculi* that could be screened with a greater spacer and MGE diversity than the *F. philomiragia* strains. Last, *M. bovoculi* is not a human pathogen and would be easier to handle.

Genomic DNA extraction

To extract gDNA, 4 mL of *M. bovoculi* cells (strains 22581, 33362, and 58069) were grown overnight in BHI media supplemented with 30 mM NaCl and pelleted. The pellets were resuspended in 300 µL of TE buffer, transferred to a 2 mL bead beating tube where 100 mg of 0.1 mm glass beads were added before beating for 90 seconds three times with 30 seconds on ice between each beating. The lysate was then used to purify the genomic DNA using the EZNA kit (Omega), following the manufacturer's instructions.

DNA preparation for transcription-translation (TXTL) reactions

The TXTL reactions contained up to four DNA components: the reporter plasmids (for GFP and RFP), a Cas12 genomic amplicon or plasmid, a gRNA plasmid, and an optional anti-CRISPR candidate amplicon or plasmid. The two reporter plasmids were minimal plasmids containing an Amp resistance gene, ColE1 origin, and a consensus *E. coli* σ^{70} promoter preceding either mRFP1 or superfolder GFP (SFGFP). The gRNA plasmids were built from the same vector as the reporter plasmids, except that the fluorescent reporters were replaced with LacI and a synthetic array following a P_{Lac} promoter containing either: three repeats interspersed with spacers targeting GFP and RFP or two repeats with a non-targeting (NT) spacer. For Cas12a expression, we prepared a genomic amplicon from *M. bovoculi* strain 22581 that contained Cas12a, Cas1, Cas2, and Cas4, stopping short of the genomic CRISPR array. Genomic amplicons or subfragments were generated using PCR (described below). Individual Acr candidate genes were cloned into the same vector as the reporter plasmids, replacing the reporter with TetR and a P_{Tet} promoter followed by the candidate protein with its genomic ribosome binding site and a strong terminator. See Table S3 for plasmid sequences.

To prepare the plasmids for TXTL, a 20 mL culture of *E. coli* containing one of the plasmids was grown to high density, then isolated across five preparations using the Monarch Plasmid Miniprep Kit (New England Biolabs), eluting in a total of 200 µL nuclease-free H_2O. 200 µL of AMPure XP beads (Beckman Coulter) were then added to each combined miniprep and purified according to the manufacturer's instructions, eluting in a final volume of 20 µL in nuclease-free H_2O.

All anti-CRISPR candidate amplicons and subfragments were prepared using 100 µL PCRs with either Q5, Phusion, or Taq LongAmp polymerase (all New England Biolabs), under various conditions to yield a strong band on an agarose gel such that the correct fragment length was greater than 95% of the fluorescence intensity of the lane on the gel. 100 µL of AMPure XP beads (Beckman Coulter) were then added to each reaction and purified according to the manufacturer's instructions, eluting in a final volume of 10 µL in nuclease-free H_2O. The Cas12a-containing amplicon was prepared the same way, except that the PCR was scaled to 500 µL and the resulting products were ethanol precipitated then dissolved in 100 µL of nuclease-free H_2O before the bead purification.

TXTL reactions

TXTL master mix was purchased from Arbor Biosciences and reactions were carried out in a total of 12 μL each. Each reaction contained 9 μL of TXTL master mix, 0.125 nM of each reporter plasmid, 1 nM of Cas12 amplicon, 2 nM of gRNA plasmid, 1 nM of genomic amplicon or Acr candidate plasmid, 1 μM of IPTG, 0.5 μM of anhydrotetracycline, and 0.1% arabinose. Additionally, we added 2 μM of annealed oligos containing six χ sites as described in Marshall, et al. (*40*) to protect against linear DNA degradation. Both reporters were always included to prevent the possibility of identifying false positives resulting from Acr candidates that generate fluorescence or differentially affect expression of one of the reporters.

The reactions were run at 29 °C in a TECAN Infinite Pro F200, measuring RFP (λ_{ex}: 580 nm, λ_{em}: 620 nM) and GFP (λ_{ex}: 485 nm, λ_{em}: 535 nm) fluorescence levels every three minutes for up to 10 hours. To plot kinetic data, the minimum measured value of the fluorescence (RFP or GFP) intensity was subtracted from each point on the curve (to compensate for early variations due to condensation on the sealing film), then the overall curve was normalized by the fluorescence level measured for the non-targeting negative control after 10 hours of reporter expression. Experiments in which the non-targeting or positive cleavage controls failed to produce the expected reporter expression levels were discarded and repeated.

%Inhibition for the TXTL assays was calculated from endpoint data (10 hours) using the following equation:

$$\%\text{Inhibition} = \frac{(\text{Fluor}_{10\ hr} - \text{Fluor}_{min}) - (\text{Fluor}_{no\ Acr,10\ hr} - \text{Fluor}_{no\ Acr,min})}{(\text{Fluor}_{NT,10\ hr} - \text{Fluor}_{NT,min}) - (\text{Fluor}_{no\ Acr,10\ hr} - \text{Fluor}_{no\ Acr,min})}$$

where: $\text{Fluor}_{10\ hr}$ is the fluorescence (RFP or GFP) measured at the 10-hour time point, Fluor_{min} is the minimum fluorescence measured, $\text{Fluor}_{no\ Acr,10\ hr}$ is the fluorescence of the no Acr positive control at the 10-hour time point, $\text{Fluor}_{no\ Acr,min}$ is the minimum fluorescence measured for the no Acr positive control, $\text{Fluor}_{NT,10\ hr}$ is the fluorescence of the non-targeting (NT) negative control at the 10-hour time point, and $\text{Fluor}_{NT,min}$ is the minimum fluorescence measured for the NT negative control.

Protein Purification

DNA encoding the sequences of the SpyCas9, MbCas12, AsCas12, and LbCas12 sequences were cloned into a custom vector containing, in order from the N-terminus: a 10x His tag, maltose binding protein (MBP), TEV protease cleavage site, the Cas12a sequence, and an optional C-terminal NLS sequence for proteins containing an NLS used in the gene editing assays. Protein purification proceeded largely as described in previous work (*43*). Briefly, each plasmid containing Cas12a or Cas9 was grown in *E. coli* Rosetta2 cells overnight in Lysogeny Broth and subcultured in Terrific Broth until the OD_{600} was between 0.6-0.8, after which protein production was induced with 375 μM IPTG and the cultures were grown at 16 °C for 16 hr. Cells were harvested and resuspended in Lysis Buffer (20 mM Tris-HCl pH 8.0, 500 mM NaCl, 10 mM imidazole, 0.5% Triton X-100, 1 mM TCEP, 1 mM PMSF, and Roche cOmplete protease inhibitor cocktail), lysed by sonication, and purified using Ni-NTA Superflow resin (Qiagen). The eluted proteins were cleaved with TEV protease overnight at 4 °C, then purified on a Heparin HiTrap column (GE) using cation exchange chromatography with a linear KCl gradient. The protein-containing fractions were pooled and concentrated before application over a Superdex 200 size exclusion column (GE), exchanging the proteins into the final storage buffer containing 20 mM HEPES-HCl, pH 7.5, 200 mM KCl, 1 mM TCEP, and 10% glycerol. Protein purity is shown in fig. S10.

Purification of the anti-CRISPR proteins was performed in the same way, except that a HiTrap Q column (GE) was used instead of a HiTrap Heparin column (GE) and AcrVA5 was purified using Superdex 75, not Superdex 200, during the size exclusion step.

Nucleic Acid purification for in vitro cleavage experiments

Cas12a gRNA templates for in vitro transcription were prepared by amplifying three overlapping DNA oligos purchased from IDT to create a template containing a T7 RNA polymerase promoter, the gRNA sequence, and the Hepatitis δ anti-genomic ribozyme. The templates were then transcribed and purified using standard methods after ribozyme cleavage and end-healing with PNK.

To produce the DNA target for the dsDNA cleavage experiments, cells containing a minimal vector with the ColE1 origin and AmpR gene were grown and miniprepped using the Monarch Plasmid Miniprep Kit (NEB), eluting with water. The plasmid was then linearized

using EcoRI, after which the enzyme was deactivated and the plasmid diluted to 50 nM in the 1X Cleavage Buffer for use in the in vitro cleavage experiments.

All DNA sequences can be found in table S3.

in vitro cleavage experiments

All dsDNA cleavage experiments were carried out in a 1X Cleavage Buffer that consisted of: 20 mM HEPES-HCl, pH 7.5, 150 mM KCl, 10 mM $MgCl_2$, 0.5 mM TCEP. gRNA sequences were first refolded by diluting the purified gRNA to 500 nM in 1X Cleavage Buffer, heating at 70 °C for 5 min then allowing to cool to room temperature. This was mixed with Cas12a protein diluted to 500 nM in 1X Cleavage Buffer at a 1:1 ratio and incubated at 37 °C for 10 min to form the RNP complex at 250 nM. To perform the cleavage reaction, a 9 uL mixture containing 5 nM of linearized plasmid and 0-1.25 μM (0 nM, 10 nM, 25 nM, 62.5 nM, 125 nM, 250 nM, 500 nM, 1.25 μM final) anti-CRISPR candidate protein was prepared then incubated at 37 °C for 10 min before adding preformed RNP to 25 nM to start the reaction. The reaction was incubated 30 min at 37 °C before quenching with 2 μL of 6X Quench Buffer (30% glycerol, 1.2% SDS, 250 mM EDTA). The cleaved/uncleaved DNA was resolved on a 1% agarose gel prestained with SYBR Gold (Invitrogen).

Mammalian cell culture

All mammalian cell cultures were maintained in a 37 °C incubator, at 5% CO_2. HEK293T (293FT; Thermo Fisher Scientific) human kidney cells and derivatives thereof were grown in Dulbecco's Modified Eagle Medium (DMEM; Corning Cellgro, #10-013-CV) supplemented with 10% fetal bovine serum (FBS; Seradigm #1500-500), and 100 Units/mL penicillin and 100 μg/mL streptomycin (100-Pen-Strep; Gibco #15140-122).

HEK293T and HEK-RT1 cells were tested for absence of mycoplasma contamination (UC Berkeley Cell Culture facility) by fluorescence microscopy of methanol fixed and Hoechst 33258 (Polysciences #09460) stained samples.

Lentiviral vectors

A lentiviral vector referred to as pCF525, expressing an EF1a-driven polycistronic construct containing a hygromycin B resistance marker, P2A ribosomal skipping element, and a

fluorescence marker (mTagBFP2, mCherry) or an AcrVA (AcrV1, AcrV4, AcrV5), was loosely based on pCF204 (Oakes*, Fellmann*, et al., submitted). In brief, to make the backbone more efficient, the f1 bacteriophage origin of replication and bleomycin resistance marker were removed. Within the provirus, the original expression cassette was replaced by the above described EF1a-driven HygroR-P2A-GOI (gene-of-interest) polycistronic constructs using custom oligonucleotides (IDT), gBlocks (IDT), standard cloning methods, and Gibson assembly techniques and reagents (NEB). Vector sequences are provided (table S4).

Lentiviral transduction

Lentiviral particles were produced in HEK293T cells using polyethylenimine (PEI; Polysciences #23966) based transfection of plasmids. HEK293T cells were split to reach a confluency of 70-90% at time of transfection. Lentiviral vectors were co-transfected with the lentiviral packaging plasmid psPAX2 (Addgene #12260) and the VSV-G envelope plasmid pMD2.G (Addgene #12259). Transfection reactions were assembled in reduced serum media (Opti-MEM; Gibco #31985-070). For lentiviral particle production on 6-well plates, 1 µg lentiviral vector, 0.5 µg psPAX2 and 0.25 µg pMD2.G were mixed in 0.4 mL Opti-MEM, followed by addition of 5.25 µg PEI. After 20-30 min incubation at room temperature, the transfection reactions were dispersed over the HEK293T cells. Media was changed 12 h post-transfection, and virus harvested at 36-48 h post-transfection. Viral supernatants were filtered using 0.45 µm cellulose acetate or polyethersulfone (PES) membrane filters, diluted in cell culture media if appropriate, and added to target cells. Polybrene (5 µg/mL; Sigma-Aldrich) was supplemented to enhance transduction efficiency, if necessary.

Mammalian gene editing inhibition assay

For rapid and reliable assessment of genome editing efficiency of various CRISPR-Cas variants in mammalian cells, we previously established a fluorescence-based genome editing reporter cell line referred to as HEK-RT1 (Oakes*, Fellmann*, et al., submitted). In brief, HEK293T human embryonic kidney cells were transduced at low-copy with the amphotropic pseudotyped RT3GEPIR-Ren.713 retroviral vector (*44*), comprising an all-in-one Tet-On system enabling doxycycline-controlled GFP expression. Single clones were isolated and individually assessed. HEK-RT3-4 cells were derived from the clone that performed best in these tests. Since

HEK-RT3-4 are puromycin resistant, monoclonal HEK-RT1 reporter cell lines were derived by transient transfection of HEK-RT3-4 cells with a pair of vectors encoding Cas9 and guide RNAs targeting the puromycin resistance gene, followed by identification and characterization of monoclonal derivatives that are puromycin sensitive and show doxycycline inducible and reversible GFP fluorescence. HEK-RT1 cells were derived from the clone that performed best in these tests.

To test the effect of genomic integration and expression of anti-CRISPR-Cas12a candidates (AcrVAs) in mammalian cells, HEK-RT1 were stably transduced with lentiviral vectors (pCF525) encoding AcrVA1, AcrVA4, AcrVA5, mTagBFP2 or mCherry. Transduced HEK-RT1 target cell populations were selected 48 h post-transduction using hygromycin B (400 µg/mL; Thermo Fisher Scientific #10687010). The derived polyclonal HEK-RT1-AcrVA1, HEK-RT1-AcrVA4, HEK-RT1-AcrVA5, HEK-RT1-mTagBFP2 and HEK-RT1-mCherry genome protection and editing reporter cell lines were then used to quantify gene editing inhibition by flow cytometry after transient transfection with CRISPR-Cas RNPs programmed with guide RNAs targeting the GFP reporter. RNP transfections were carried out using Lipofectamine 2000 (Thermo Fisher Scientific). Specifically, HEK-RT1 derived reporter cells were seeded in 24-well plates at 30% confluency 3-8 h prior to transfection. For each sample, the RNP complex was formed by mixing a 10 µL complexing solution containing 10 µM Cas9/Cas12 NLS-tagged protein, 12 µM eGFP-targeting gRNA, 20 mM HEPES pH 7.5, 0.6 mM TCEP, 160 mM KCl, and 8 mM $MgCl_2$ that was incubated at 37 °C for 10 min. The RNPs were mixed with 25 µL Opti-MEM (Gibco #31985-070) and 1.6 µL Lipofectamine 2000 was mixed with 25 µL Opti-MEM in a separate tube. Diluted RNPs were added to the diluted Lipofectamine 2000, incubated 15 min at room temperature, and co-incubated with the respective reporter cells. For SpyCas9 the eGFP-targeting guide RNAs were sg1 (ctgaagttcatctgcaccac) or sg2 (cagggtcagcttgccgtagg); for AsCas12a/LbCas12a/MbCas12a the eGFP-targeting guide RNA was cr1 (cgtcgccgtccagctcgacc).

GFP expression in HEK-RT1 derived reporter cells was induced by 24 h of doxycycline (1 µg/mL; Sigma-Aldrich) treatment starting at 24 h post-transfection. Percentages of GFP-positive cells were quantified by flow cytometry (Attune NxT, Thermo Fisher Scientific), routinely acquiring 30,000 events per sample. Non-transfected and non-induced reporter cells were used for normalization.

Analysis of genome editing efficiency in mammalian cells by T7E1 assay

The T7 endonuclease 1 (T7E1) assay is a semi-quantitative method to determine genome editing efficiencies at a specific locus. Among other nucleic acid structures, T7 endonuclease 1 recognizes and cleaves non-perfectly matched DNA. Such mismatched DNA can arise from hybridization of wild-type (wt) and mutant DNA strands derived from CRISPR-Cas mediated indels. To prepare for T7E1 assays, genomic DNA (gDNA) was extracted from edited and control cells by lysing cell pellets in QuickExtract DNA Extraction Solution (Epicentre, #QE09050), followed by incubation at 65°C for 20 min, and at 98°C for 20 min. Loci of interest were PCR amplified from gDNA using Q5 High Fidelity DNA Polymerase (New England Biolabs, #M0491). The GFP locus in HEK-RT1 cells was amplified using the primers MSCV5' (oCF114, cccttgaacctcctcgttcgacc) and GFP-rev (oCF136, tattttatcgattgtcgccctcgaacttcacc) with annealing at 71°C, yielding 869 bp products (without indels). PCR products (5 µl of a 50 µl reaction) mixed with Gel Loading Dye, Purple (New England Biolabs, #B7025S) were visualized on a 1.5% TAE agarose gel containing SYBR Safe DNA Gel Stain (Thermo Fisher Scientific, #S33102, 1:10000) and imaged using a ChemiDoc MP imaging system (Bio-Rad) to verify size and purity. A 100 bp DNA ladder (New England Biolabs, #N3231S) was used as reference. For T7E1 assays, approximately 200 ng unpurified PCR product (containing Q5 reaction buffer) were denatured and rehybridized to yield wt-mutant heteroduplexes in a 10 µl reaction supplemented with 50 mM KCl. Denaturation and annealing were carried out in a thermocycler with the following settings: 95°C for 10 minutes, 85°C-25°C gradient over 13 minutes, and hold at 4°C. The rehybridized PCR products were then digested in 20 µl reactions by adding 7.5 µl H_2O, 2.0 µl NEBuffer 2 (New England Biolabs, #B7002S), and 0.5 µl T7 endonuclease 1 (New England Biolabs, #M0302); and incubation in a thermocycler at 37°C for 30 min, and hold at 4°C. Digested amplicons (15 µl of a 20 µl reaction) were mixed with Gel Loading Dye, Purple (New England Biolabs, #B7025S), run on a 1.5% TAE agarose gel containing SYBR Safe DNA Gel Stain (Thermo Fisher Scientific, #S33102, 1:10000), and imaged using a ChemiDoc MP imaging system (Bio-Rad) to visualize the genome editing efficiency. A 100 bp DNA ladder (New England Biolabs, #N3231S) was used as reference.

Supplementary Figures S1-S14

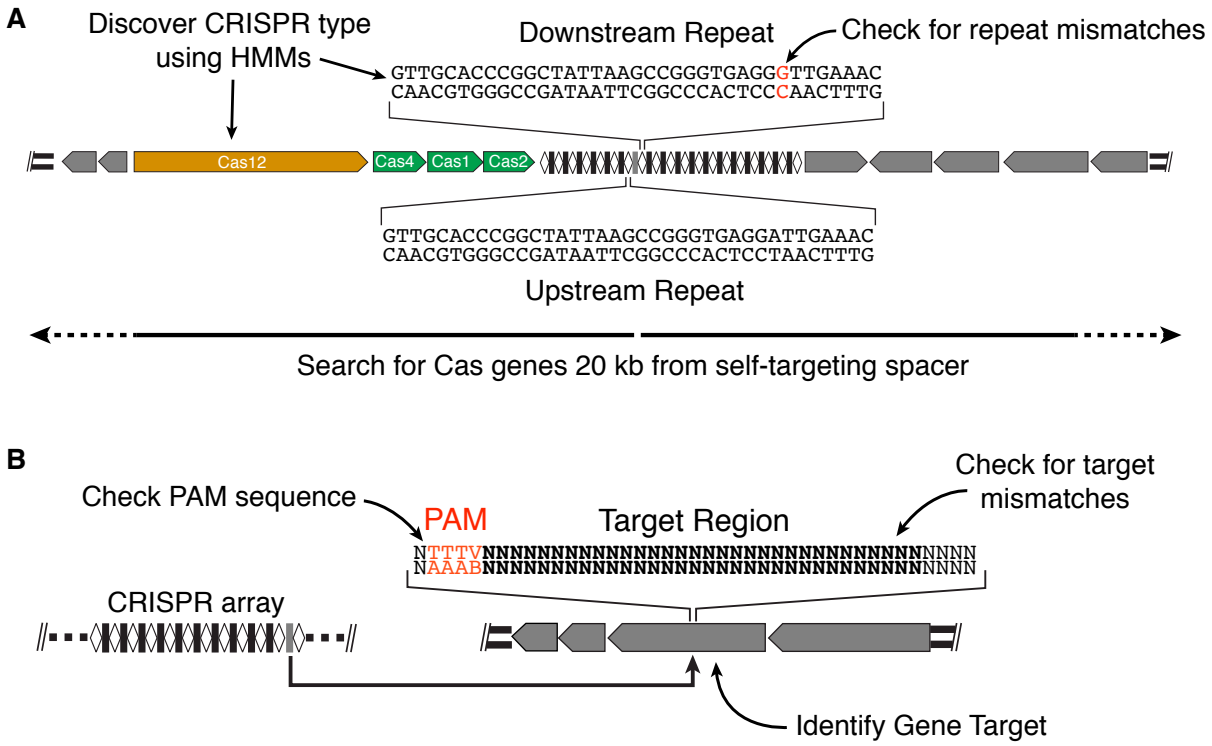


Fig. S1. Visualization of STSS locus and target searching. (**A**) After a self-targeting spacer is predicted with BLAST, information about the CRISPR locus is collected. First, Cas genes are identified within 20 kb of the self-targeting spacer using Hidden Markov Models (HMMs) to predict the CRISPR subtype of the locus. Second, the consensus repeat is determined and any indels or mutations in the up/downstream repeats from the self-targeting spacer are noted. The repeats are also used to predict the CRISPR subtype with a separate set of HMMs. (**B**) The region in the genome that the self-targeting spacer targets is also examined. The up/downstream sequences are reported to identify possible protospacer adjacent motif (PAM) sequences. Any mutations in the target sequence are reported as well as what gene products are targeted by the spacer.

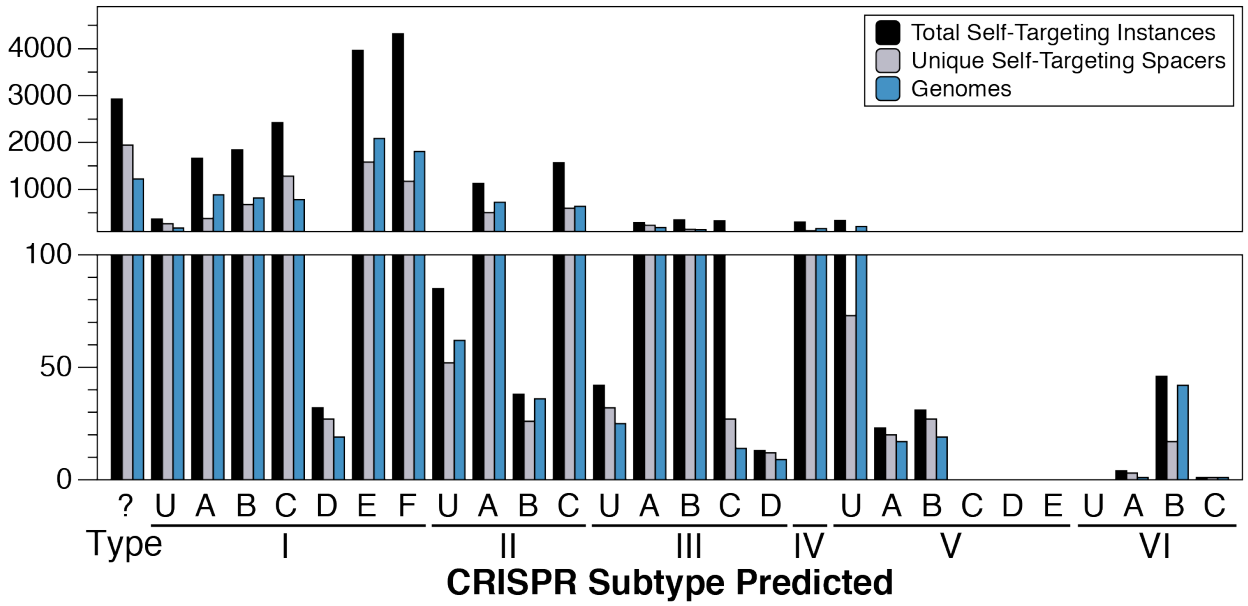


Fig. S2. Overview of STSS results. In total 22,125 instances of self-targeting were predicted by STSS, distributed across most CRISPR subtypes (black). After removing redundant spacers, we observed 8,917 unique spacers (gray). The number of genomes containing at least one self-targeting spacer of a given CRISPR subtype (blue) is lower than the number of self-targeting instances, as many genomes contain multiple self-targeting spacers. It is important to note that any genome may have multiple self-targeting CRISPR systems, causing some genomes to be counted more than once in the chart representation above. Additionally, the STSS prediction is a best guess and inaccuracies are likely, especially in the type III and IV systems, which are less well-defined than the other types and frequently have Cas proteins from other subtypes predicted nearby.

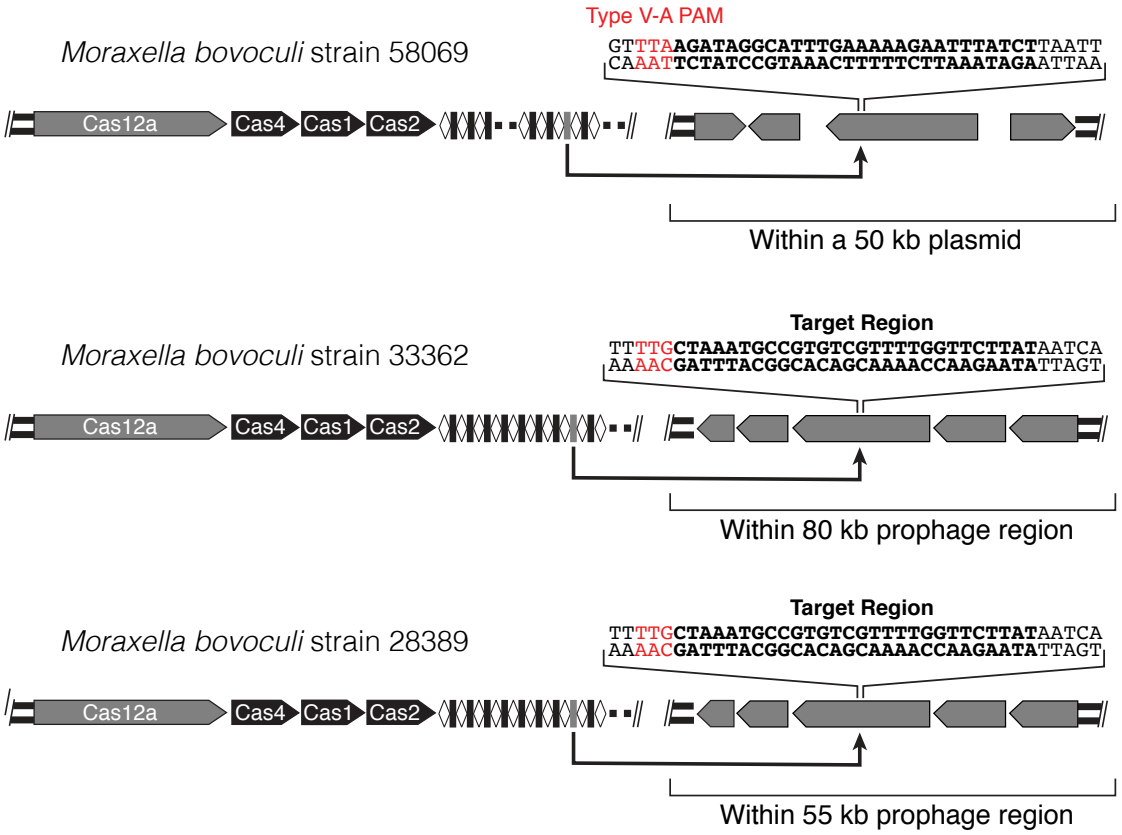


Fig. S3. Other *Moraxella bovoculi* type V-A self-targeting genomes. In total, four strains of *M. bovoculi* were found that contained a type V-A self-targeting CRISPR system: strains 22581 (Fig. 1D), 58069, 33362, and 28389 (above). The self-target in strain 58069 targets a plasmid associated with the genome and not the genome itself, while the self-target in *M. bovoculi* strains 33362 and 28389 is the same sequence that falls within an 80 kb or 55 kb prophage region, respectively. *M. bovoculi* strain 28389 was not screened for Acr genes in this study because we did not have access to the strain.

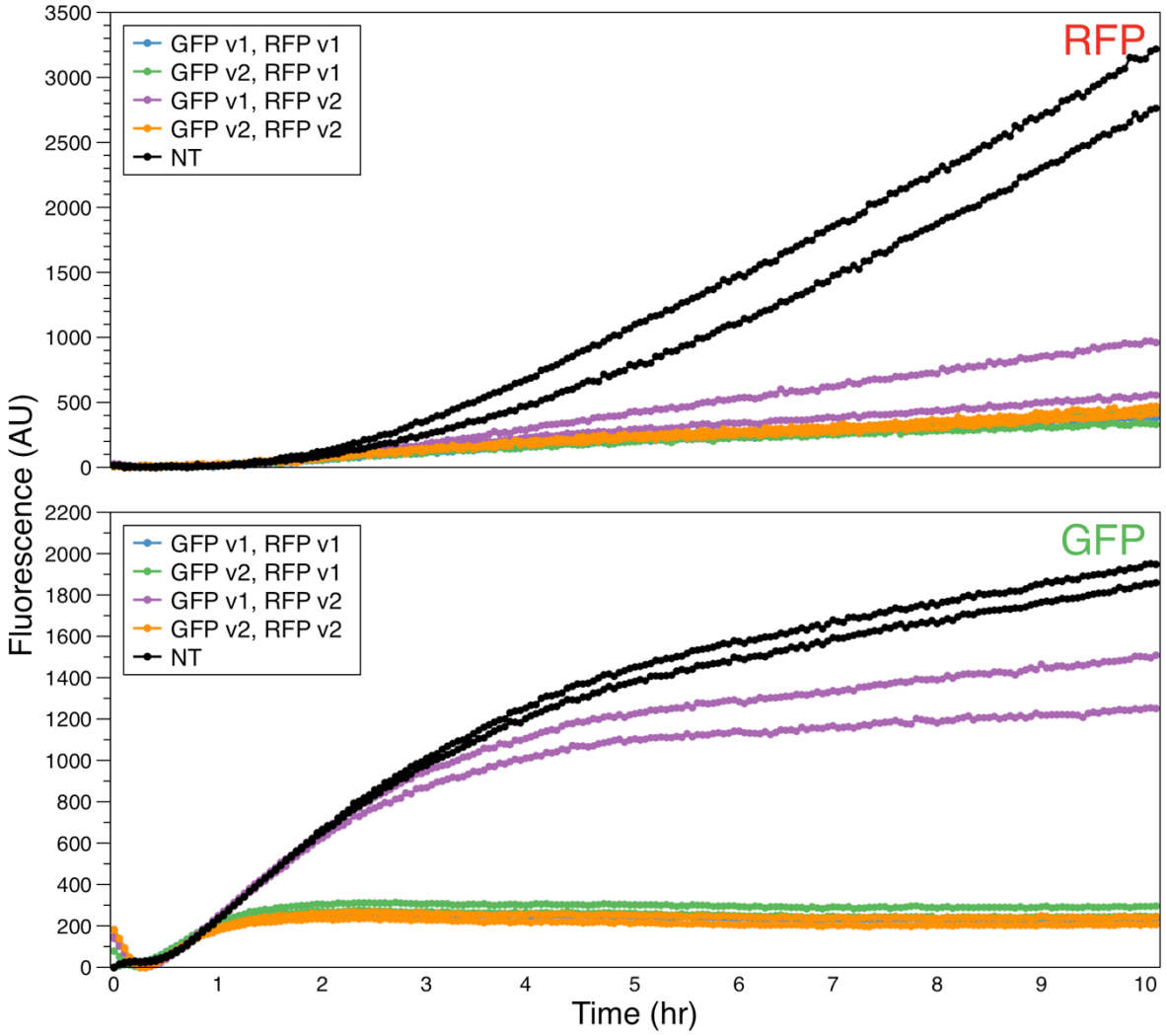
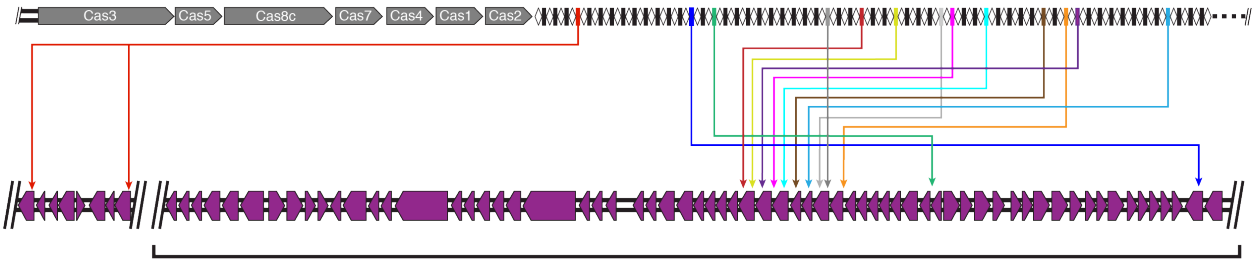


Fig. S4. MbCas12a gRNA optimization for TXTL. To find the optimal gRNA pair to produce the highest dynamic range for both reporters, we tested four combinations of two different gRNAs for each reporter plasmid. All four of combinations effectively reduced RFP expression, however, the combination of GFP gRNA version 1 and RFP gRNA version 2 did not strongly inhibit GFP expression. The GFP v2, RFP v1 gRNAs were selected for all other TXTL experiments in this work.

A *Moraxella bovoculi* strain 58069



B

Spacer Sequences	PAM	Target Mutations?
ACCCCGTTATCTGCCACGGTGGCGTTGGCTTTGT	TTC	Perfect match
TGGTGAATTTTCTTTTGAGATGCAGTCTGATGAA	TTC	Perfect match
AGCCAAGCTGGTTCGGTTGCCCTTGCCTTTGGAT	TTC	Perfect match
ATCGGTTTTGCATTCGGCTAAGGATTTGGGTGTA	TTC	Perfect match
ATAATCAGGGTGTTTTGAATTAACTGGCATAATG	TTC	...g.....
TATCTCTTCAGCTTGCTCACGCCAACCCGCCTGC	TTC	Perfect match
ACTTCGCAACATTGGCTATCCAAGTAACGCAAAC	TTC	Perfect match
CCAACACGCCTAAGACACGATGACTTGTTTTTAG	TTC	Perfect match
CATTCGCCGAGCTATTGTCGTTATATCACGGAAA	TCA	Perfect match
CAAAGACTGCTTTTTAAGCCAATCATAGTAGCTA	TTC	Perfect match
ATTTTTAAGCACCACGCCATAATCGCCAAACACC	TTC	Perfect match
TTTTTCTTGATCGATAGACGACTGATTAAACAAG	TTC	Perfect match
TTTTTCTTGATCGATAGACGACTGATTAAACAAG	TTC	Perfect match

Fig. S5. Type I-C self-targets in *M. bovoculi* strain 58069. (**A**) In addition to the type V-A self-target present in *Moraxella bovoculi* strain 58069, there are an additional 13 self-targeting spacers found in the type I-C system, targeting 14 locations in the genome. Twelve of the targeted positions are in a predicted prophage region. (**B**) Sequences of each self-targeting spacer, its neighboring PAM sequence, and any mismatches to the target.

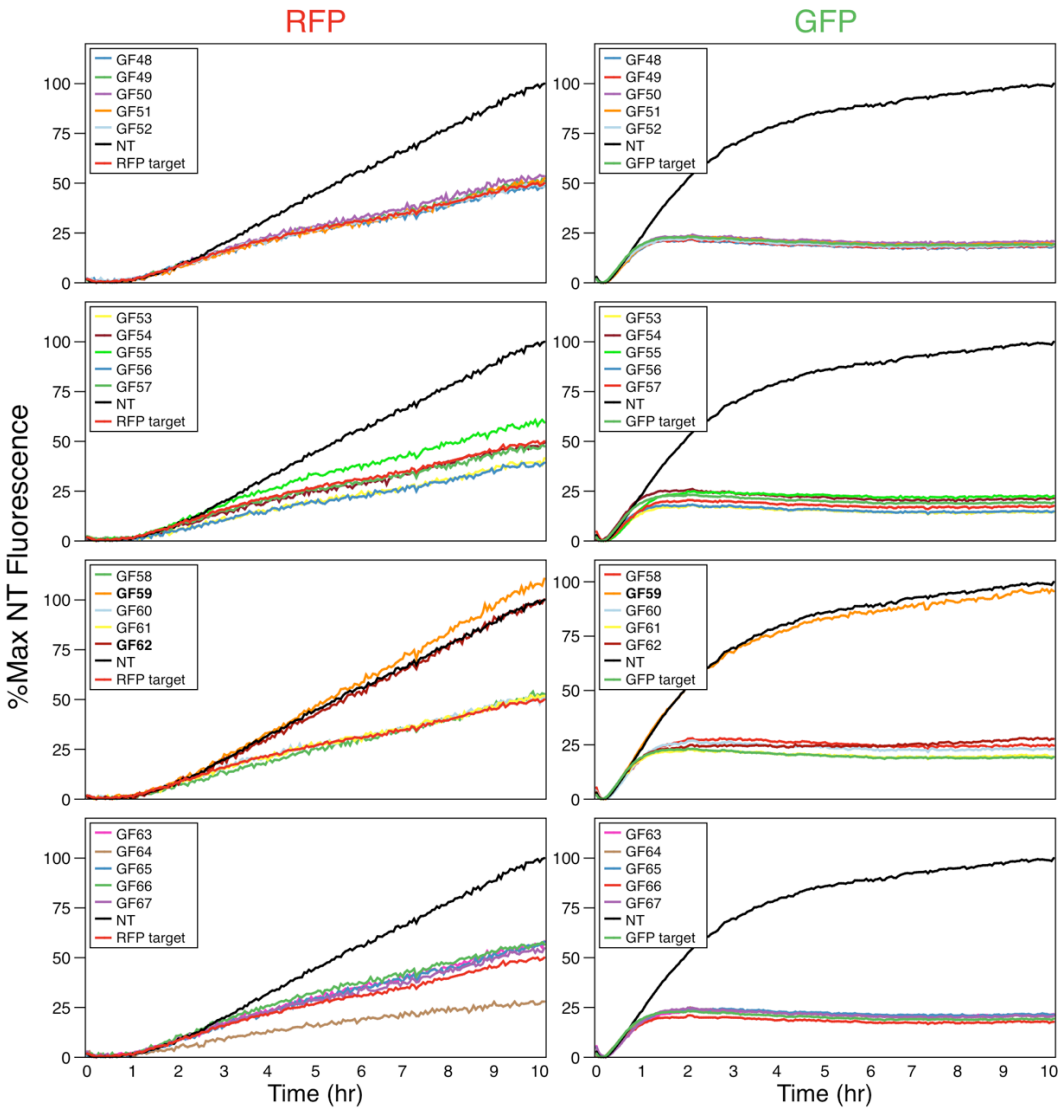


Fig. S6. Kinetic data for *M. bovoculi* 22581 genomic fragments. RFP and GFP fluorescence measured over the course of 10 hours, normalized to the average maximum level of expression in the reactions with the non-targeting gRNA plasmid (n=3). Each reaction contains MbCas12a genomic amplicon, reporter plasmids, gRNA plasmid, and either no Acr genomic fragment (NT – non-targeting, or GFP/RFP targeting) or the Acr genomic fragment indicated from *M. bovoculi* strain 22581. Bold text indicates high reporter expression, potentially associated with an anti-CRISPR. Both reporters are only highly expressed when genome fragment (GF) 59 is present.

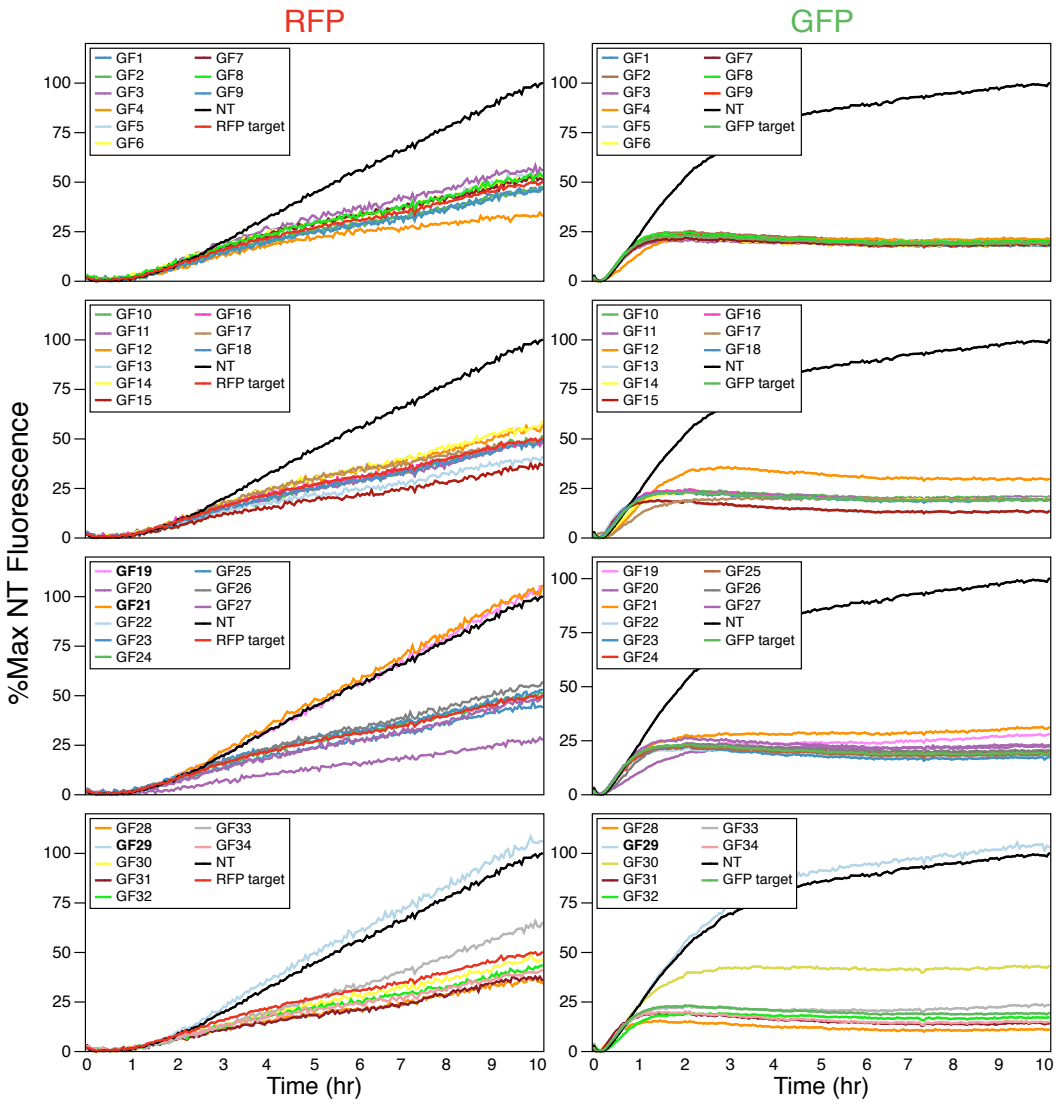


Fig. S7. Kinetic data for *M. bovoculi* 33362 genomic fragments. RFP and GFP fluorescence measured over the course of 10 hours, normalized to the average maximum level of expression in the reactions with the non-targeting gRNA plasmid (n=3). Each reaction contains MbCas12a genomic amplicon, reporter plasmids, gRNA plasmid, and either no Acr genomic fragment (NT – non-targeting, or GFP/RFP targeting) or the Acr genomic fragment indicated from *M. bovoculi* strain 33362. Bold text indicates high reporter expression, potentially associated with an anti-CRISPR. Both reporters are only highly expressed when genome fragment (GF) 29 is present.

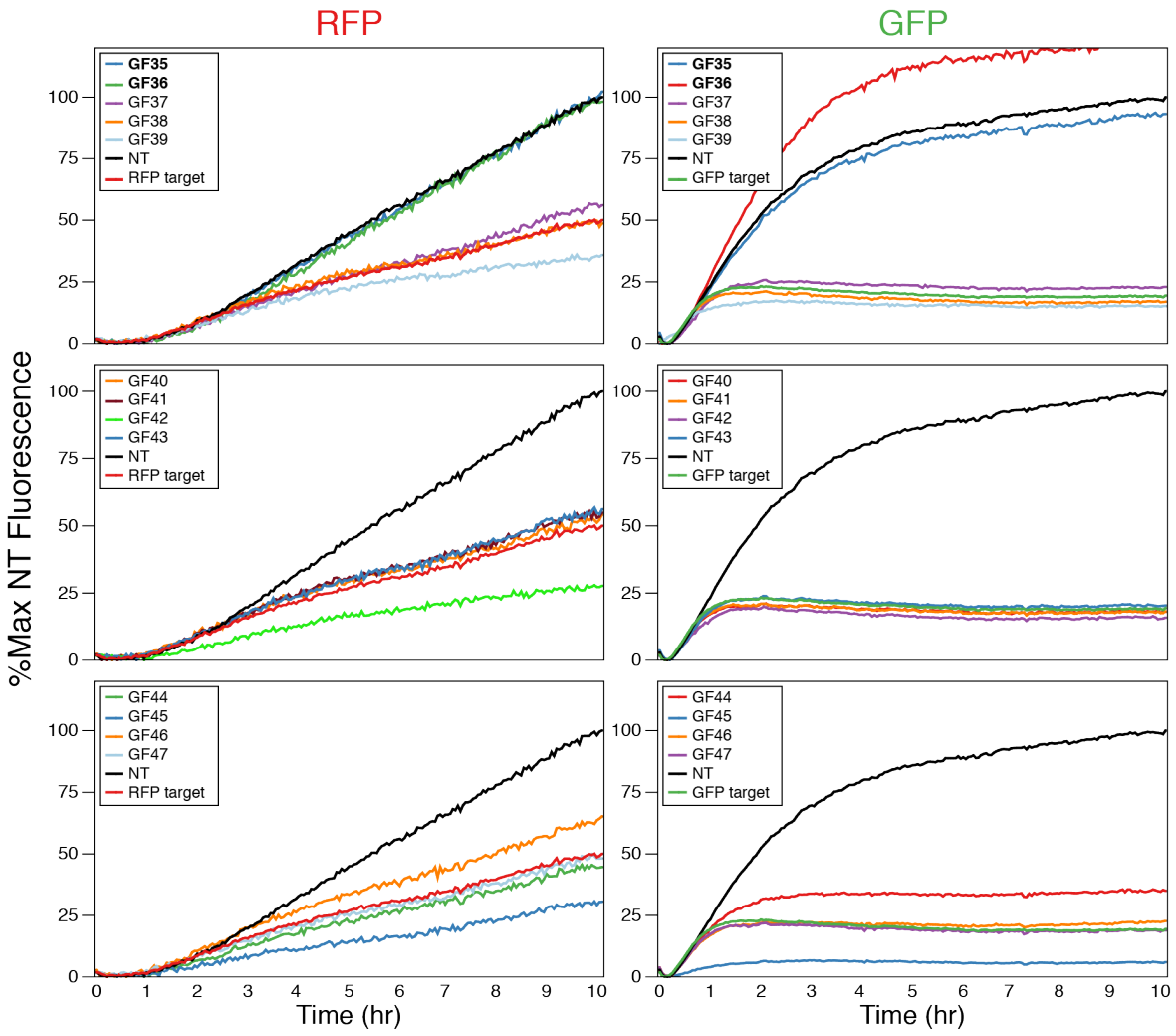


Fig. S8. Kinetic data for *M. bovoculi* 58069 genomic fragments. RFP and GFP fluorescence measured over the course of 10 hours, normalized to the average maximum level of expression in the reactions with the non-targeting gRNA plasmid (n=3). Each reaction contains MbCas12 genomic amplicon, reporter plasmids, gRNA plasmid, and either no Acr genomic fragment (NT – non-targeting, or GFP/RFP targeting) or the Acr genomic fragment indicated from *M. bovoculi* strain 58069. Bold text indicates high reporter expression, potentially associated with an anti-CRISPR. Both reporters are only highly expressed when genome fragments (GF) 35 or 36 are present.

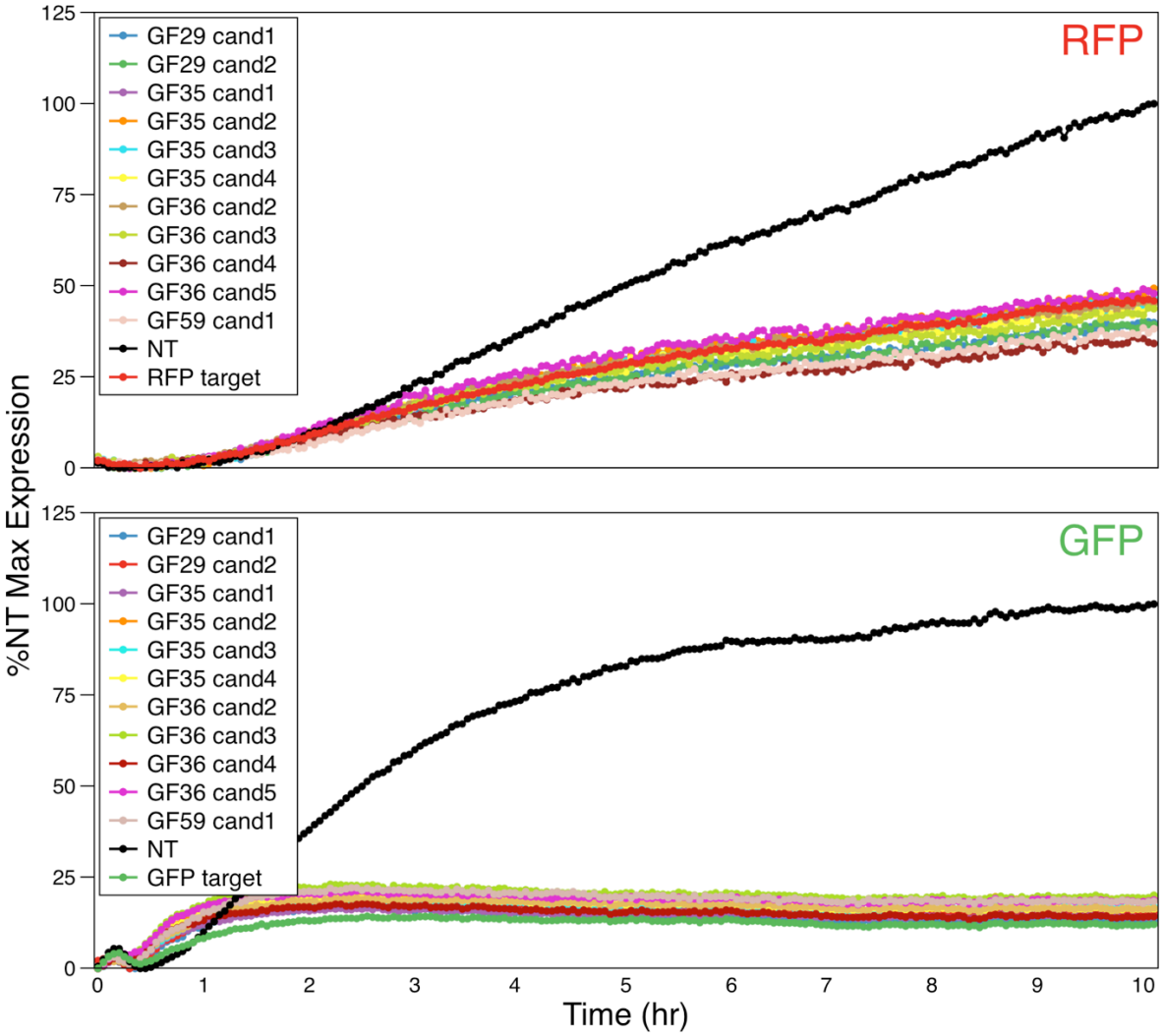


Fig. S9. Kinetic data for Acr candidates not exhibiting activity. Of the anti-CRISPR candidates tested, only three exhibited anti-CRISPR activity in TXTL and are shown in Figure 2E-F. The fluorescence measurements for the other tested candidates that did not exhibit inhibitory activity over the 10 hour TXTL run are shown above.

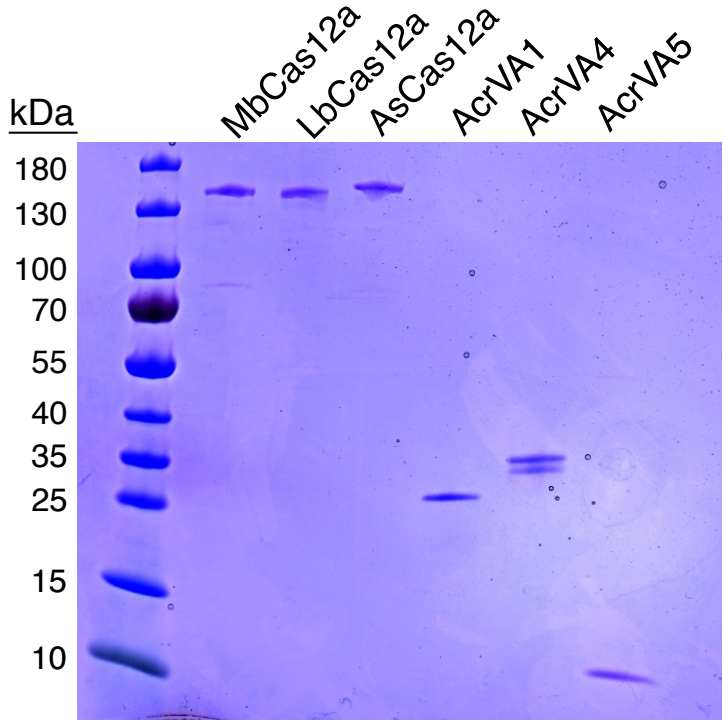
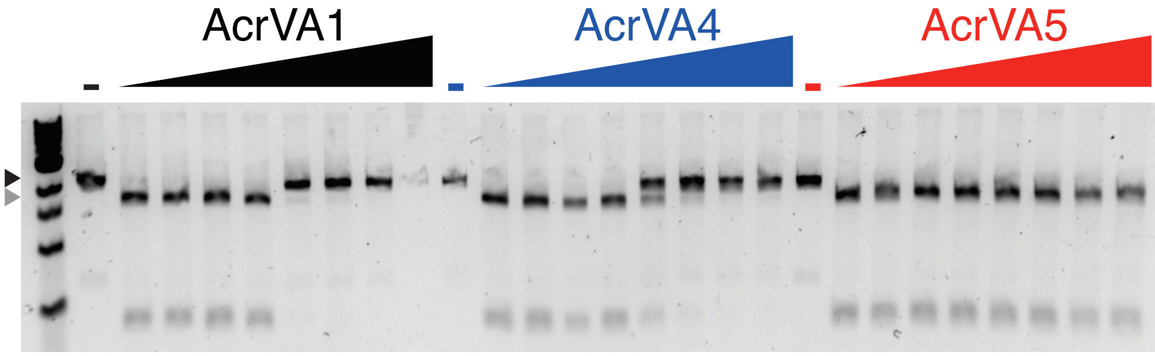


Fig. S10. Purification of Cas12a and AcrVA proteins. SDS-PAGE gel of Cas12a and AcrVA proteins used for the *in vitro* cleavage assays. MbCas12a is from *Moraxella bovoculi* strain 58069, LbCas12a is from *Lachnospiraceae* bacterium ND2006, and AsCas12a is from *Acidaminococcus* sp. BV3L6.

MbCas12a (strain 58069)




```
AsCas12a          1 MTQFEGFTNLYQVSKTLRFELIPQGKTLKHIQEQGFIEEDKARNDHYKELKPIIDRIYKTYADQCLQLVQLDWENLSAAIDSY----RKE
LbCas12a          1 MSKLEKFTNCYSLSKTLRFKAIPVGKTQENIDNKRLLVEDEKRAEDYKGVKKLLDRYYLSFINDVLHSIKL--KNLNNYISLF---RKKT
MbCas12a 58069    1 -MLFQDFTHLYPLSKTVRFELKPIGRTLEHIHAKNFLSQDETMADMYQKVKVILDDYHRDFIADMMGEVKL--TKLAEFYDVYLKFRKNP
MbCas12a 33362    1 -MLFQDFTHLYPLSKTVRFELKPIGKTLEHIHAKNFLNQDETMADMYQKVKAILDDYHRDFIADMMGEVKL--TKLAEFYDVYLKFRKNP

AsCas12a         87 KTEETRNALIEEQATYRNAIHDYFIGRTDNLTDAINKRHAEIYKGLFKAELF-NGKVLKQLGTVTTTEHEN-----ALLRSFDKFTTYFS
LbCas12a         86 RTEKENKELENLEINLRKEIAKAFKG-------------NEGYKSLFK----------KDIIETILPEFLDDKDEIALVNSFNGFTTAFT
MbCas12a 58069   88 KDDELQKQLKDLQAVLRKESVKPIGN---------GGKYKAGHDRLFGAKLFKDGKELGDLAKFVIAQEGKSSPKLAHLAHFEKFSTYFT
MbCas12a 33362   88 KDDGLQKQLKDLQAVLRKEIVKPIGN---------GGKYKAGYDRLFGAKLFKDGKELGDLAKFVIAQEGESSPKLAHLAHFEKFSTYFT

AsCas12a        171 GFYENRKNVFSAEDISTAIPHRIVQDNFPKFKENCHIFTRLITAVPSLRE-HFENVKKAIGIFVSTSIEEVFSFPFYNQLLTQTQIDLYN
LbCas12a        153 GFFDNRENMFSEEAKSTSIAFRCINENLTRYISNMDIF----EKVDAIFDKHEVQEIKEKILNSDYDVEDFFEGEFFNFVLTQEGIDVYN
MbCas12a 58069  169 GFHDNRKNMYSDEDKHTAIAYRLIHENLPRFIDNLQILTTIKQKHSALYD-QIINELTASGL--DVSLASHLDG--YHKLLTQEGITAYN
MbCas12a 33362  169 GFHDNRKNMYSDEDKHTAIAYRLIHENLPRFIDNLQILATIKQKHSALYD-QIINELTASGL--DVSLASHLDG--YHKLLTQEGITAYN

AsCas12a        260 QLLGGISREAGTEKIKGLNEVLNLAIQKNDETAHIIASLPHRFIPLFKQILSDRNTLSFILEEFKSDEEVIQSFCKYKTLLRNENVLETA
LbCas12a        239 AIIGGFVTESGE-KIKGLNE-YI---NLYNQ--KTKQKLP-KFKPLYKQVLSDRESLSFYGEGYTSDEEVLEVFRNT--LNKNSEIFSSI
MbCas12a 58069  254 RIIGEVNG-------------YT---NKHNQICHKSERIA-KLRPLHKQILSDGMGVSFLPSKFADDSEMCQAVNEF--YRHYADVFAKV
MbCas12a 33362  254 TLLGGISGEAGSRKIQGINELIN---SHHNQHCHKSERIA-KLRPLHKQILSDGMGVSFLPSKFADDSEVCQAVNEF--YRHYADVFAKV

AsCas12a        350 EAL---FNELNSIDLTHIFISH-KKLETISSALCDHWDTLR-----------NALYERRIS-----ELTGKITKSAKE---------KVQ
LbCas12a        319 KKLEKLFKNFDEYSSAGIFVKNGPAISTISKDIFGEWNVIR--------DKWNAEYDDIHLKKKA-VVTEKYEDDRRKSFKKIGSF-SLE
MbCas12a 58069  325 QSL---FDGFDDHQKDGIYVEH-KNLNELSKQAFGDFALLGRVLDGYYVDVVNPEFNERFAKAKTDNAKAKLTKEKDKFIKGVHSLASLE
MbCas12a 33362  338 QSL---FDGFDDYQKDGIYVEY-KNLNELSKQAFGDFALLGRVLDGYYVDVVNPEFNERFAKAKTDNAKAKLTKEKDKFIKGVHSLASLE

AsCas12a        411 RSLKHEDINLQEIISAAGKELSEAFK----------QKTSEILSHAHAALDQPLPT---TLKKQEE--------KEILKSQLDSLLGLYH
LbCas12a        399 QLQEY----ADADLSVVEK-LKEI-------IIQKVDEIYKVYGSSEKLFDADFVL-EKSLKKNDAV-------VAIMKDLLDSVKSFEN
MbCas12a 58069  411 QAIKHHTARHDDESVQAGK-LGQYFKHGLAGVDNPIQKIHNNHSTIKGFLERERPAGERALPKIKSGKNPEMTQLRQLKELLDNALNVAH
MbCas12a 33362  424 QAIEHYTARHDDESVQAGK-LGQYFKHGLAGVDNPIQKIHNNHSTIKGFLERERPAGERALPKIKSDKSPE---IRQLKELLDNALNVAH

AsCas12a        480 LLDWFAVDES-NEVDPEFSARLTGIKLEMEPSLSFYNKARNYATKKPYSVEKFKLNFQMPTLASGWDVNKEKNNGAILFVKNGLYYLGIM
LbCas12a        469 YIKAFFGEGKETNRDESFYGDFVLAYDILLKVDHIYDAIRNYVTQKPYSKDKFKLYFQNPQFMGGWDKDKETDYRATILRYGSKYYLAIM
MbCas12a 58069  500 FAKLLMTKTTLDNQDGNFYGEFGVLYDELAKIPTLYNKVRDYLSQKPFSTEKYKLNFGNPTLLNGWDLNKEKDNFGVILQKDGCYYLALL
MbCas12a 33362  510 FAKLLTTKTTLHNQDGNFYGEFGALYDELAKIATLYNKVRDYLSQKPFSTEKYKLNFGNPTLLNGWDLNKEKDNFGVILQKDGCYYLALL

AsCas12a        569 PKQKGRYKALSFEPTEKTSEGFDKMYYDYFPDAAKMIPKCSTQLKAVTAHFQTHTTPILLSNNFIEPLEITKEIYDLNNPEKEPKKFQTA
LbCas12a        559 --DKKYAKCLQKIDKDDVNGNYEKINYKLLPGPNKMLPK-------------------VFFSKKWM-----------AYYNPSEDIQKIYKN
MbCas12a 58069  590 --DKAHKKVFDNAPNTGKNV-YQKMIYKLLPGPNKMLPR-------------------VFFAKSNL-----------DYYNPSAELLDKYAQ
MbCas12a 33362  600 --DKAHKKVFDNAPNTGKSV-YQKMIYKLLPGPNKMLPK-------------------VFFAKSNL-----------DYYNPSAELLDKYAQ

AsCas12a        659 YAKKTGDQKGYREALCKWIDFTRDFLSKYTKTTS-IDLSSLRPSSQYKDLGEYYAELNPLLYHISFQRIAEKEIMDAVETGKLYLFQIYN
LbCas12a        619 GTFKKGDMFNLNDC-HKLIDFFKDSISRYPKWSNAYDF-NFSETEKYKDIAGFYREVEEQGYKVSFESASKKEVDKLVEEGKLYMFQIYN
MbCas12a 58069  649 GTHKKGDNFNLKDC-HALIDFFKAGINKHPEWQN-FGF-KFSPTSSYRDLSDFYREVEPQGYQVKFVDINADYIDELVEQGQLYLFQIYN
MbCas12a 33362  659 GTHKKGDNFNLKDC-HALIDFFKAGINKHPEWQH-FGF-KFSPTSSYQDLSDFYREVEPQGYQVKFVDINADYINELVEQGQLYLFQIYN

AsCas12a        748 KDFAKGHHGKPNLHTLYWTGLFSPENLAKTSIKLNGQAELFYRPKS--RMKRMAHRLGEKMLNKKLKDQKTPIPDTLYQELYDYVNHRLS
LbCas12a        707 KDFSDKSHGTPNLHTMYFKLLFDENNHGQ--IRLSGGAELFMRRASLKKEELVVHPANSPIANKNPDNPKK------------------
MbCas12a 58069  736 KDFSPKAHGKPNLHTLYFRALFSEDNLANPIYKLNGEAQIFYRKASLGMNETTIHRAGEILENKNPDNPKE------------------
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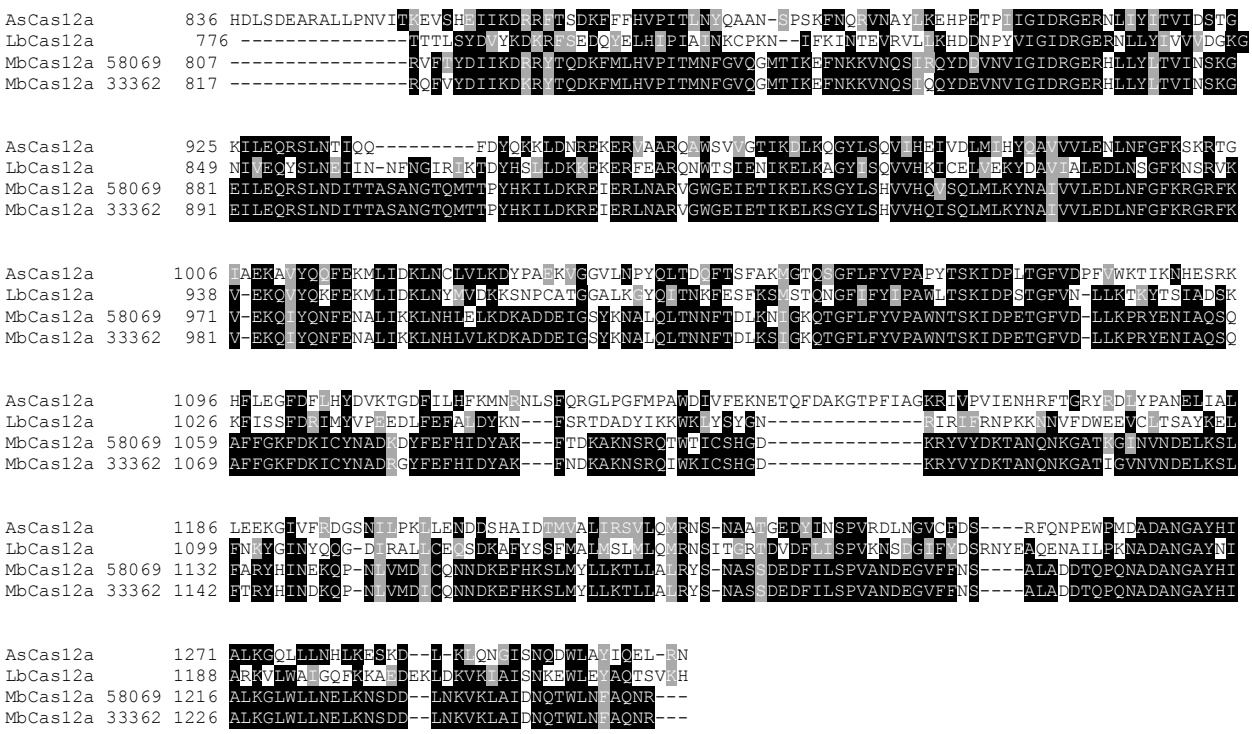

Fig. S11. MbCas12a from strain 58069 is not inhibited by AcrVA5. While the MbCas12a from strain 33362 is inhibited by AcrVA5 (Fig. 3), AcrVA5 is unable to inhibit the close homolog (99% identity) in strain 58069. The reason for this differential activity between such close homologs is unknown, but may be related to a short deletion in the helical I domain of the 58069 MbCas12a (positions 262-274 in the 33362 MbCas12a).

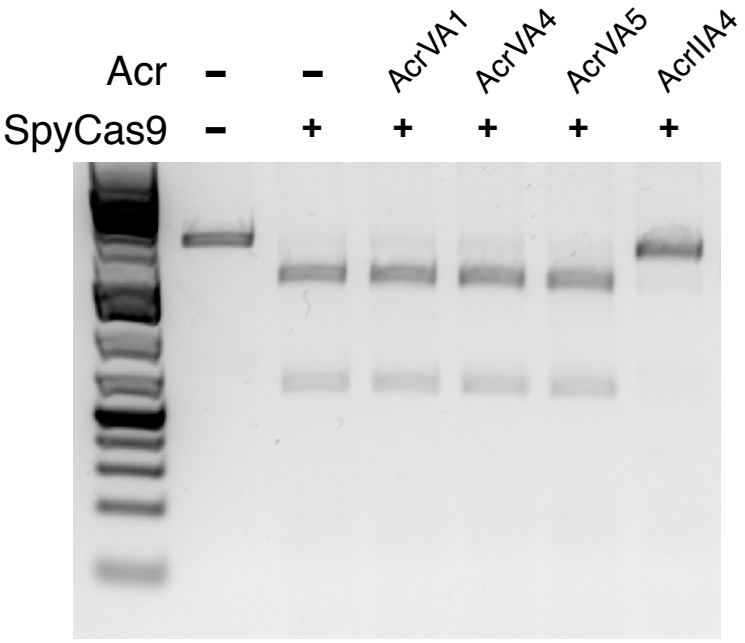

Fig. S12. AcrVA proteins do not inhibit SpyCas9 cleavage. Each of the three AcrVA proteins were incubated with SpyCas9 RNP at ratio of 50:1 (Acr:Cas9 RNP) and did not inhibit dsDNA cleavage. AcrIIA4, a SpyCas9 inhibitor (*16*), was able to prevent target cleavage under these conditions.

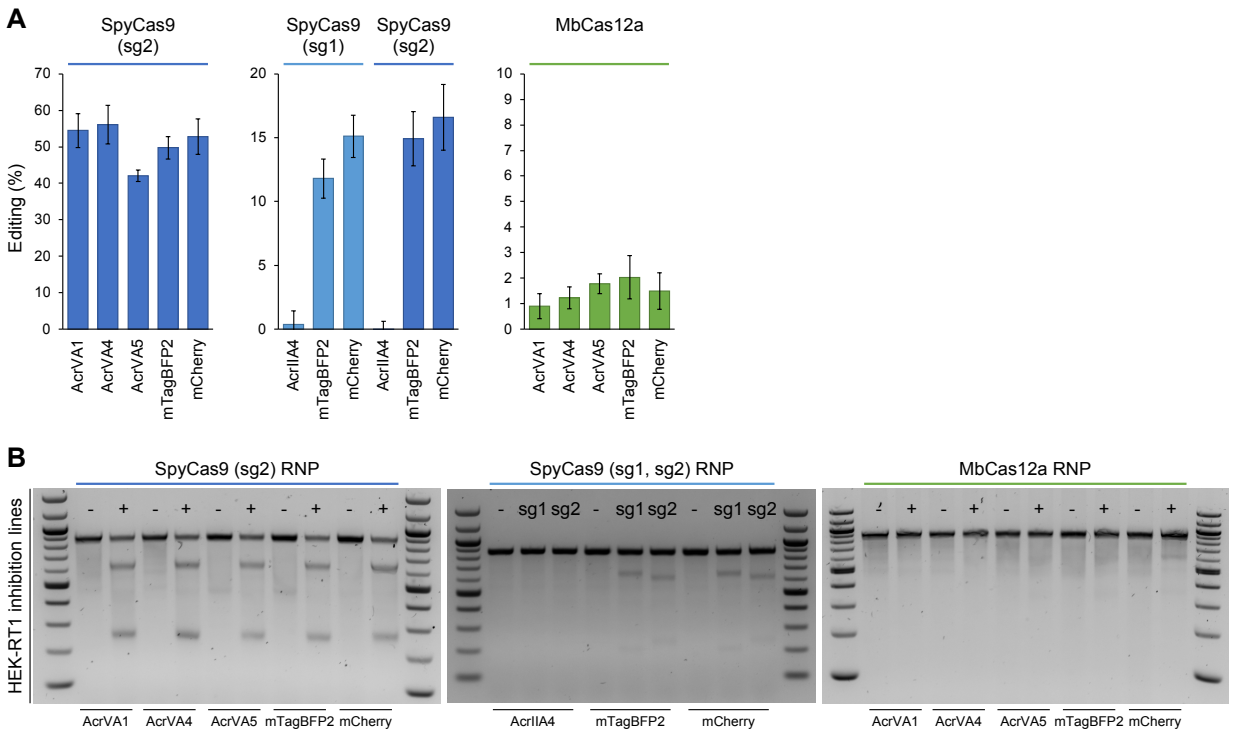

Fig. S13. AcrVA proteins do not inhibit SpyCas9 genome editing in human cells. (**A**) Quantification of genome editing in human reporter cell lines stably expressing the indicated CRISPR-Cas12a inhibitors (AcrVAs), a CRISPR-Cas9 inhibitor (AcrIIA4) or negative controls (mTagBFP2, mCherry). Reporter cell lines were transiently transfected with MbCas12a or SpyCas9 RNPs targeting the fluorescence reporter. For SpyCas9 two separate sgRNAs (sg1, ctgaagttcatctgcaccac; sg2, cagggtcagcttgccgtagg) were used, complementing data with sg1 shown in Figure 4. At 24 h post-transfection, the GFP reporter was induced by doxycycline (1 µg/mL) treatment for 24 h, followed by flow cytometry assessment of editing efficiency. Error bars indicate standard deviations of triplicates. (**B**) Biochemical analysis of AcrVA-mediated inhibition of genome editing by Cas12a, and AcrIIA4-mediated inhibition of genome editing by Cas9, in representative samples shown in (A). Editing at the GFP locus was assessed by the T7 endonuclease 1 (T7E1) assay.

A

anti-CRISPR	Species	Accession #	%Identity to *	%Query Coverage	Size (aa)	pI
AcrVA1	*Moraxella bovoculi*	WP_046701302.1*	100%	100%	170	4.4
	Moraxella bovoculi	WP_046697118.1	92%	38%	66	4.0
	Eubacterium eligens	WP_046701302.1	27%	97%	423	7.6
	Eubacterium eligens	OLA16786.1	26%	97%	425	8.5
	Eubacterium eligens	CDA41774.1	26%	97%	425	8.4
AcrVA4	*Moraxella bovoculi*	WP_046699156.1*	100%	100%	234	5.5
	Moraxella bovoculi	WP_046701923.1	99%	100%	234	5.7
	Moraxella atlantae	WP_067336174.1	34%	54%	204	9.2
AcrVA5	*Moraxella bovoculi*	WP_046699157.1*	100%	100%	92	3.9

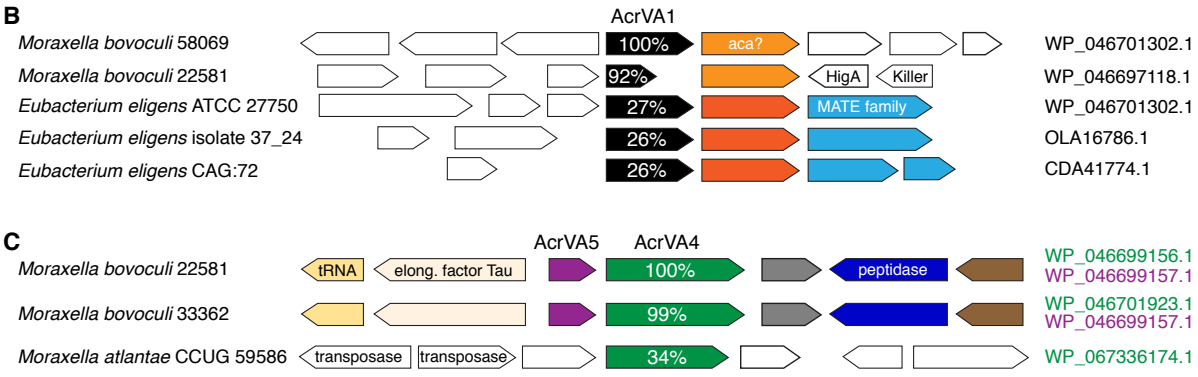

Fig. S14. AcrVA orthologs in the NCBI database. (**A**) Table of orthologs for AcrVA1, AcrVA4, and AcrVA5 found using PSI-BLAST and their basic properties. (**B**) Gene neighbors of AcrVA1 orthologs in different species in the NCBI database. While there are few examples of AcrVA1 found in the NCBI database, the downstream protein (GF36 candidate 2) can be found in many Gram-negative bacteria and may represent a new anti-CRISPR associated (aca) gene. It did not exhibit anti-CRISPR activity in our assays. (**C**) Gene neighbors of AcrVA4 and AcrVA5. No significant alignments were determined for AcrVA5, which is next to AcrVA4 in all genomes available, except for *M. atlantae*, which only contains a more distant ortholog of AcrVA4. Thus, without screening, AcrVA4 and AcrVA5 would have been unlikely to be discovered. Orthologs are indicated with the same color and percent identities to the anti-CRISPR indicated with an asterisk are labeled in both (A) and (B).

Genomic Fragment #	*M. bovoculi* strain	Forward primer sequence	Reverse primer sequence	Length (kb)
MbCas12 amplicon	22581	caaaaaccgatttcttaggaagtcgg	ggggtatttttaatgatttttgagagattagc	6.2
GF1	33362	ccatgataataaatccagtgcctttaacagc	gagcttgggtaaccacaggcg	9.5
GF2	33362	gtctttcaatacccaacgcttgc	gcaacaaatgccaaatggcgatatgat	8.2
GF3	33362	gcttccaaaacttcttcattcatttaaatctcc	tcattaaaaatacaaacaccgtcaatattggc	10.2
GF4	33362	gtaggcttttgtcattggtttttctcaaattttg	ccatatgccacccctacacaaac	9.5
GF5	33362	gatggagcggtggtaagatggg	ccaaaataactcatattatcctccaaaatattcaatc	9.1
GF6	33362	gggaatgtgagggcatgattaatgg	ggttttatattggctcatttaattttccttaatggg	8.3
GF7	33362	gccgtcaattaaacacaccaaatccaaatg	acaggcattgatagagatgccgatg	9.9
GF8	33362	ataggctttgggctaagcgtg	ggagtttcatttttttgcgtaatcctatgga	8.4
GF9	33362	gcatgctctcattgacatcatcagtc	ggtttttatttatctgtcgttgccgtaaaac	5.1
GF10	33362	gttgtcaagttatgattagtgtattgatattgtaac	gcgtaggctctctttcatggtatttataaac	10.3
GF11	33362	gcatgggcagaaatattcatctggtag	tcgggtttgaaataagccccagtatg	9.4
GF12	33362	tccataccttctcctataataggagtatgtcag	gttatctttgttaattccattcataattactctaccttc	6.0
GF13	33362	caataccttttggtgatggttcaagcc	ccacttattcgatgaggaaattgattatataaatctataacc	2.9
GF14	33362	gaagttcttgtaacaaagctatattagcatcg	gaagtttataaactcaatagcgaattgacttgg	10.6
GF15	33362	ggctaaacaaaaggagcttattgatggt	cctaattattcggtgaatttatactacaattatttgtgc	4.7
GF16	33362	gaatataaaagcatgatattttggcgaaatgtc	tgcatccataagacccacctatatataaaagttc	9.8
GF17	33362	cctgttatcatgtgaatatccccaattctacc	gggcggtatatattacggcagataaac	9.8
GF18	33362	gtgaatgtttttaaactcataagtatcccatcaacg	gatgagatgactagaaaaaatcttaacatttaagc	9.7
GF19	33362	gcttaaatgttaagatttttctagtcatctcatc	ccactttatcattaacttactaggattgattatgcc	9.2
GF20	33362	ggcataatcaatcctagtaagttaatgataaagtgg	gaaactaaaaaccccctagcgcc	10.8
GF21	33362	gtttgcagggtaaatcaacacgatg	catcggctcgctcctaaagttaatcc	3.6
GF22	33362	gtttaaattgtttgtcatttcttgccttttg	aaaatatatcaaataaccaaacataatccaccaacc	11.0
GF23	33362	ggggtttggtgagcttcatttatg	cttcatcaaatacacaggctaagtcctac	11.4
GF24	33362	caaaaaagcccattaggaaagttcc	ccaaacaaaaacgcatatcatcaaaacag	11.5
GF25	33362	cggtaaaatcaatgaatagcaagccgtatc	ccattctattagtatatagttaaagcgccatg	7.8
GF26	33362	ggtgggtctgaggtagtaaaaaacctac	cgcttaaaatagctcccataagcttc	5.0
GF27	33362	gctttttattgttttaaatcaatgggttgtaag	gggggttgatttaattagatgtcaatctac	2.7
GF28	33362	ggaaaaactgaaaggatattttactcatttgatttagg	caaagctccagaatggattaaagatgattg	4.1
GF29	33362	tgcttgacttaatataatatacattatataatacaaccatcaa	acctagcccacaccaccaac	2.6
GF30	33362	aaataaagccagtagctatctactggc	gtgctttacgtgaaattaaatacagaaaccc	7.1
GF31	33362	cacagcaattaccaactaaaatttaaaataaatttgg	gctattaaccctgctgcttgattag	8.2
GF32	33362	gctttatggttgttatttttagcagatttttagat	gcttatgtttcaagggtttttgcttg	7.6
GF33	33362	cgcgatggtttatatgtgattggc	gacaccctaaatttagggtaaaattataccaaccta	8.9
GF34	33362	gtagattgacatctaattaaatcaaccccc	catgccttgttctagtaagggaagagtt	9.8
GF35	58069	tgcaatatcgaattaaaaaacgttggtttttatcg	tctcatactccagtttgcgtgttaaatttagg	4.7
GF36	58069	agcatgatttgccttgtttgttggtataagg	ctgtactagcaagtgacgataaagtcagc	3.6
GF37	58069	tgagattttgtttcatggtttctccttgg	gacgacaaggaggcttattgtatggc	8.4
GF38	58069	ccccataaaaaaaaccgcccaaaaagg	cctaagatatttgaaaaagtttttgattagccc	7.8
GF39	58069	gctttcatcaagttttagtttcataacaaactcc	gggggagtaaaaatggctaaaaaacaaa	8.8
GF40	58069	cgtaaaaagtaactttaaactttctcattattcgc	taattcatatttgggttgccagtctcgtc	9.5

GF41	58069	gttactagatgatgaaatggctaagatgattggag	cattggtagaataacttgccatgatgattacc	6.2
GF42	58069	tgtaaatatgagagattgacgcagctgtg	gcaaaatcagattttgcgtcaagacc	7.7
GF43	58069	gaactcgaacagaaatctaacttccga	caaacttaaagacgcgctaactgc	7.5
GF44	58069	tatgtgccaagacaatctgtgggg	aaactgattcataaaaatattcctacttttgtttgttc	6.1
GF45	58069	ccagcactaagaaattttgacgaaaac	cggcgattcattatttaatggaaaattatg	2.1
GF46	58069	ggcactttataggcacaaaaaaaagcg	tgctacatgattaagggatacagactttaatc	2.6
GF47	58069	gataaaacaaaaaagcccattaaaattatggg	aaggcgtttaaaatcgctaaaaagggt	2.5
GF48	22581	cactaaaaatcatcaagtatttttaagtgtcataaacc	caaccataaaaaaccattaaatcttgtacagtc	9.6
GF49	22581	gttgtatgcgttcatgaatcatcatttc	tgcccattttgggcttatttgctgt	9.5
GF50	22581	ataatcatctcctagtgataagctggcacg	gttcaatttctaaagtgttcatatcatcccca	9.7
GF51	22581	gagctacgaccatatgagtaaacacg	gcgagttagggtggttaggtt	13.0
GF52	22581	caatgtttggacgattagttttgcg	gtacaaatatattttaagttaaacgggataaaatttggc	11.4
GF53	22581	gcttgttttggaaaaatagttacaaaattaaatcttatcg	cattgatgacacttttccagtctgga	10.3
GF54	22581	caatttttaagcggtctttaaattcggtcatag	caattcatcggtgaataatgttggtctgc	6.5
GF55	22581	attggcgaaaaacatttagagaaaatagggg	cccaacaatgttgcaaaaataagccc	10.7
GF56	22581	ccttgatgatatttggctggatggc	gcgagttagggtggttaggtt	9.0
GF57	22581	caatgtttggacgattagttttgcg	cgaatttttggctaggatgccgtg	2.6
GF58	22581	cgtcatgctaggcttgttttgcc	ggcgtgcattcgctgagattgc	9.9
GF59	22581	acccgagcggtagccaaagg	caattacttatgaataaatggcgtaaattggg	1.6
GF60	22581	tgcaaaatctctttaatttcagatatttgtattaaaaatttg	ggttaaactgcctcaaggcagg	10.6
GF61	22581	cctgtacaatgagccgtttttttgc	attctcaagacgacgtataagatcgtg	9.0
GF62	22581	agtcagtcatatgagctcctatgagac	cagatgattgatgaagcggtgctgg	6.4
GF63	22581	ggcaagtcttgaccgccgtcaa	cggatttcggttgcaccgtataagatac	9.8
GF64	22581	agaagcgagcgatctttgatga	ggtcaaaaaggcgatacaccgtttac	9.4
GF65	22581	catgaatttctccaataaaaaagcactcattacg	gctcatctcacgaaaattccaatcc	11.7
GF66	22581	gggttttgggattttgtgcttcatg	cactccgctaccaaatccatttgg	10.0
GF67	22581	tctgaccttgtcgtggcaatc	gaaacaaccaccaaccgagcc	10.0

Table S1. List of genomic fragments screened for Acr genes in TXTL. The primer sequences and genomic DNA template that define each genomic fragment tested for anti-CRISPR activity in TXTL are shown above. Fragments that rescued expression of both reporter plasmids are shown in bold.

Acr candidate	*M. bovoculi* strain	Nucleic acid sequence
GF29 candidate 1	22581	acgattaaataggaagcaaataatgcccgaaaatctatccaacttgccgctgatcgtcaaaatcatcggcgtaatcatcggcgcagtctttgcactcacattgacgggtgacatcgacactgacggcaagctcaagctgagcttgggcgtgctcatcaaaatcgcgtttagtgcgtatttggcttcttggcaggcgcatggctcattgagtatatgggctggggtcattggtcgcacgcaagccacggctttgtgatgatgctgtgcagcgtgttcggcatgacattagtcggcgcaatctatcaagcgattaagctgtcaacgaccaataagacgccaagtgagattgtcactgaggttaaagacacatttaaagcgattttcaaataa
GF29 candidate 2	22581	acggggtttttttattggagtgaattatgattaaaaaaatccaaaccatcatcggtgccacacctgacggcatttggggtaatcaatctatccatgccctaagagtggcattatcaaagggtgtggttatccccatcaccaaaaacatcacgctaaacgagctgttggcaagtcagacggcggcaaggcgtggtattgataacatgccagatgccaaggtgcttaataatctgatcgaatcggcggtcaatctttggcagccagcgcgtgacatcttgggtcagcctgtattcattacgagtggctatcgcagtccacggctaaatagcgccatcggtggggcgaaaaacagcgcccacatacatgggctagcaattgattttcgtgcgcccacctttgggacaacacggcaaatcgtcgcacatctaaccaaagagcttaaaaagcgtggcatcaagtacgaccagattatccttgaatatccaaactctacgggtagctgggtgcatttgggctataagcacccgagcggtagccaaaggggcagggtttacgatagcttaa
GF35 candidate 1	58069	aaacaggagaataaaccatgttatcagactatctaaataaactatctgatgatttaaccaccagtcaaaccgtgcttaaacaaatcagccaagccagcaataacagccgtgtatcaaatgcgctggataaaatcgccagtaggctagaagttcacgccagccaaattcaaaagctggcagaccacgccagcacccaaaaaaagtag
GF35 candidate 2	58069	aacaggggaagcatgagccagcgcaaagccatttttgattatctgatgacggggcagaccctagacaatcaaaaagcctacaagctttttaatatgttttgcctatcccagcgcgtgagtgatttacggttaatctatggcatacccgttcaaagttgcttgattaatctaccaaatggtaagcggttttcatcatattggctggaacgtgattatattgccaaagttaaagcgggcgagattgaaagcgtttggggcgcaaatcttgccacgccttaa
GF35 candidate 3	58069	atttaggagcgtatcacatgaaccaaagcaattcacaaaccagcctacccaaagacggcatgagccgattagcccagctactaccattcttgccaatcggtaaaagcactgtttgggcatgggtaaaggctggcaaattcccccagccaatcaaactaagccccaccgtgaccgtatggcgtaacagcgatattcatacatggttaaacaaccaacaggggaagcatga
GF35 candidate 4	58069	accaacaaacaaggcaaatcatgctaacagataccgccataaaacggttaaagccgtccactaattgcaccccaaataagcctgataagcattcagacggcaacggcttacaattaatagtaagaccaacaggaacaaaagtttggttggttgcctatcgctatcatggcagacaaaccaatatcacattagggcgttatcctacaattagcctacaacaagcacgcctacaagccttggagataaaacagaagttagcacaaggtatagaccctaagaccgctaagcctaatacggtgctatttggcgatatagcgaacgaataccacacccaaagagaccgtaacaacccaatcaataagggcaaatacaccgtatcgaaagttacgcacaaaaaggatttgagccaatataataatgacattgccccacatatcgcccatttggacataaacgctgttacgcctgtgatgatactagatattgccaaacgcattgaaaagcgtggagcgtatgacatggcaaaacgagccattaggctaataggggcaatattccgccatgctagggataaggggttatatgaccgcctaccgccaacagacggctggaaaaacgcctaaccaaacgaaaacaagaacactttgcgcgcctagaatttcacgaattacccccaattctttcccatgttcatcattccacttgtgaaccgcttaccaaacttgcctttaaatttatttgcctgacctttgtgcgcaccatagaaatgcgctttatgcaatgggcggaaattgattgggataactacctttggcgaataccgcccgagcgcatgaaaatgggtaagccccatatcgtaccacttgccccacaagccatagagatttacacaaattaaggcaatggggctaagtgatgaatttgtgttttataaccccaaaaccaaaaagcccgttagtgagaattttctaacacaggcgttaaagcgtttgggctatcaagggcgaatgacgggacacgggtttaggggcattgccagcaccaaattacatgaattgcaatacaatcatgagtgtatagaattacaattagcccacgccaaagcggataaagtcagcatggcatacaatgggcggaacacttgccctaccgcgtgcagatgatgaaagaatgggcaaagctgatagaacacgcttgtcaatga
GF36 candidate 1 (AcrVA1)	58069	aaactggagtatgagattatgagcaaagcaatgtatgaagcgaaagagcgttacgccaagaaaaaaatgcaagaaaatacaaaaattgatacgctaacagatgaacagcatgatgcactagcgcagctttgtgcttttcgccataaattccactctaacaaagattctttatttttgagtgaaagtgcattttctggtgaattttcttttgagatgcagtctgatgaaaatagcaagcttagagaagttggcttaccaacaatcgaatggtcattttatgataattcccacatcccggatgattcatttagagaatggtttaattttgctaattattcagaattgtcggaaaccatccaggaacaaggcttagagctagacctagatgatgatgaaacctatgaacttgtgtatgatgaactatatacagaggctatgggcgaatatgaagagctaaatcaagatattgaaaaatacctaagacgaattgatgaagagcatggtacacaatactgtccaacagggtttgcaagattaagataa
GF36 candidate 2	58069	aagataagggataacacatgcaccacaccatcgcaagaatgaacgcctttaataaggcgtttgccaacgccaaagactgctacaaaaaaatgcaagcttggcatttgttgaataagccaaaacacgccttttccccatgcaaaacaccccagctcttgataatgggctggcggcgctgtatgaattgcgggggcgggaaagaagacgcacacatactatctatcttaagccgtctgtatctatacggcgcttggcgaaatacgcttggcatttaccagcttgatgaagagatcatcaaagattgcaaagagttaccagatgatacaccgacaagtatttttttgaatttgcctgactggtgcgtttatgttgatatatcatcagcccaaattgccacttttgatgatggcgttgccaagcacatcaaggggttttgggcgatatatgatattgttgagatgaacggcattaatcatgacgtacttgactttgttgtcgataccgacacagacgataacgtctatgtaccgcaaccgttcatcttgtcatcagggcagagcgtggcggaggttttggattatggcgcatcattgtttgacgatgacacctccaatacctaatcaaggggcttttgccctacttgttatggctgtgtgttgcagaacctgatattacatacaagggattacctgtcagccgcgaagagc

		taactagacccaagcatagcatcaacaaaaaaactggtgcgttcgtcacgcccagcgaaccttttatttatcagattggcgagcggttgggcagtgaagtcagacgttatcaaagcattattgacggtgaacagaagcggaacagaccgcacaccaaacgcccccatatccgccgtgggcattggcatggatattggcaaggtacaggacaagccaaagaatttagagtgcgctggcaaccagctgtctttgtgaacagcgggcgtgtaagcagttaa
GF36 candidate 3	58069	acacaaattggagtacgatgatgaacaatcttaaaaaaaccgccatcactcacgacggtgtatttgcttataaaaacaccgaaaccgtgattggttctgttggaagaaatgatattgtgatggctattgatgccacgcatggcgaatttaacgacaaaaactttattatttatgctgaccaccaacggcaaccccatctatcttggctatgcttatcttgatgataacaacgacgctcatattgatttagccgtgggggcttgcaatgaagatgatgattttgacgaaaaagaaatccatgaaatgattgcagaacaaatggagcttgctaagcgctatcaagagttgggcgataccgttcatggcacaacaagacttgcatttgatgatgacggttatatgacggttagactagaccaacaagcctaccccgattatcgcccagaaaatgatgacaaacacataatgtggagagctttggcattgacagccaccggtaaagaactagaagttttttggttggtagaagactacgaagacgaagaagttaatagttgggattttgacatcgcagatgactggcgcgagttgtga
GF36 candidate 4	58069	acgcggtaaaatataggtaaaatcaatggtaggcaaatcaaagatagattggcaaagcatagattggacaaagacaaacgcccaaatcgcccaagagtgcgggcgtgcttataataccgtttgtaaaatgcgtggtaaattgggtaaaagccatcaaggggcaaaatcgccccgaaaggacaagggcatatcacgcccacagccgcaccttaaccgcctagagtatcaagccttagcgactgccaaggctaaggcaagcccaaaagcaggacgatttgagactaataccaaggctaagacttggacactcaaatcacctgataacaagacttacacctttaccaatcttatgcactttgtgcgcaccaatccgcacttgtttgaccctgacgatgtggtctggcggacaaaatcaaatggcgtggagtggtgtcgtgcgtctagcggtcttgctttactcgctaaacgaaaaaaagccccttaagctggaaaggctggcgattaatctcactaaccaaggacaacaaatga
GF36 candidate 5	58069	actaaccaaggacaacaaatgaacaacccaaaaaccccctgaatacacccgaaaagcgataagagcgtacgaaaaaaaccttgttcgcaagtctgtaacctttgacgtacgaaaagatgatgacatggaattactcaagatgattgaacaagatgggcggacgtttgcacagattgcacgcaccgccctgcttgaacatttacaaaaataa
GF59 candidate 1	22581	ccagtcaaaaacaaaaagtcaagaataaattatgagcgacccaaaaaccccctgaatacacccgaaaagccatcagagcgtacgaaaaaaaccttgttcgtaagtctgtgactttcgatgtgcgtaagcctgatgacatggcattgcttgagatgattgaacaagatgggcgtgcattcgctgagattgcgcgcaccgccctgcttgaacacctgcagaaataa
GF59 candidate 2 (AcrVA4)	22581	atggtcgcttgatgagatggagctaaaaaaatgtatgaaatcaaactaaatgacacccttatacatcagacagatgatagagtgaacgcgtttgtggcttatcgctacctattaagacgtggcgatctaccaaaatgcgagaacatcgcaagaatgtattatgatggcaaggttattaagactgacgttattgaccacgacagtgttcacagtgacgaacaagccaaggttagtaacaacgacatcattaagatggcaatttccgaacttggcgtgaataacttcaagtcgctaatcaagaagcaaggctatccgttcagcaatgggcatattaatagttggttcacagatgacccagtcaaatccaagacaatgcacaatgacgaaatgtatttggttgtgcaggcactgattagagcatgcataattaaagagattgatttatatacagagcaattatataacatcattaagagcctgccgtacgacaaacgcccaaatgttgtatatagtgaccaaccattagacccgaataatcttgatttatccgaacctgaattatgggcggagcaggtgggcgaatgtatgagatatgcacacaacgaccaaccttgcttctacattggttcgactaagcgtgagttgcgtgttaattatattgtgcctgtaatcggtgtgagagatgagatagaacgtgtaatgacactggaagaagtgcgaaatttacacaaataa
GF59 candidate 3 (AcrVA5)	22581	gtactaaggtgcaggagttaagaaaatgaaaatcgaactatcaggcggatatatctgctacagcattgaagaagatgaagttaccattgacatggtagaggttaccaccaaacgtcaaggcattggcagtcagctaattgatatggttaaggatgtagcccgtgaagttggcttgccaattggtctatatgcctacccccaagatgacagcattagccaagaagacttgattgagttttattttttctaatgactttgagtatgaccccagatgatgtagatggtcgcttgatgagatggagctaa

Table S2. List of individual genes screened. Each gene that was individually cloned into the pTet expression vector and tested for anti-CRISPR activity is listed above. Underlined sequences represent the predicted open reading frames. Each plasmid sequence tested in TXTL can be derived by placing these sequences after the pTet Acr gene expression plasmid sequence (table S3).

DNA/RNA	Sequence
Chi6 DNA (forward)	TCACTTCACTGCTGGTGGCCACTGCTGGTGGCCACTGCTGGTGGCCACTGCTGGTGGCCACTGCTGGTGGCCACTGCTGGTG GCCA
Chi6 DNA (reverse)	TGGCCACCAGCAGTGGCCACCAGCAGTGGCCACCAGCAGTGGCCACCAGCAGTGGCCACCAGCAGTGAA GTGA
SFGFP sequence	atgagcaaaggagaagaacttttcactggagttgtcccaattcttgttgaattagatggtgatgttaatgggcacaaatttt ctgtccgtggagagggtgaaggtgatgctacaaacggaaaactcacccttaaatttatttgcactactggaaaactacctgt tccgtggccaacacttgtcactactctgacctatggtgttcaatgcttttcccgttatccggatcacatgaaacggcatgac tttttcaagagtgccatgcccgaaggttatgtacaggaacgcactatatctttcaaagatgacgggacctacaagacgcgtg ctgaagtcaagtttgaaggtgataccttgttaatcgtatcgagttaaagggtattgattttaaagaagatggaaacattct tggacacaaactcgagtacaactttaactcacacaatgtatacatcacggcagacaaacaaaagaatggaatcaaagctaac ttcaaaattcgccacaacgttgaagatggttccgttcaactagcagaccattatcaacaaaatactccaattggcgatggcc ctgtccttttaccagacaaccattacctgtcgacacaatctgtcctttcgaaagatcccaacgaaaagcgtgaccacatggt ccttcttgagtttgtaactgctgtgggattacacatggcatggatgagctctacaaa
mRFP1 sequence	atggcgagtagcgaagacgttatcaaagagttcatgcgtttcaaagttcgtatggaaggttccgttaacggtcacgagttcg aaatcgaaggtgaaggtgaaggtcgtccgtacgaaggtactcagaccgctaaactgaaagttaccaaaggtggtccgctgcc gttcgcttgggacatcctgtccccgcagttccagtacggttccaaagcttacgttaaacacccggctgacatcccggactac ctgaaactgtccttcccggaaggtttcaaatgggaacgtgttatgaacttcgaagacggtggtgttgttaccgttacccagg actcctccctgcaagacggtgagttcatctacaaagttaaactgcgtggtactaacttcccgtccgacggtccggttatgca gaaaaaaaccatgggttgggaagcttccaccgaacgtatgtacccggaagacggtgctctgaaaggtgaaatcaaaatgcgt ctgaaactgaaagacggtggtcactacgacgctgaagttaaaaccacctacatggctaaaaaaccggttcagctgccgggtg cttacaaaaccgacatcaaactggacatcacctcccacaacgaagactacaccatcgttgaacagtacgaacgtgctgaagg tcgtcactccaccggtgcttaa
MbCas12 repeat	gtctaacgacctttttaaatttctactgtttgtagat
GFP spacer sequence	CACTGGAGTTGTCCCAATTCTTGT
RFP spacer sequence	AAAGTTCGTATGGAAGGTTCCGTT
MbCas12 IVT template primer 1	taatacgactcactataggctaacgacctttttaaatttctactgtttg
MbCas12 IVT template primer 2	tttccaatgatgagcactttatctacaaacagtagaaatttaaaaggtcg
LbCas12 IVT template primer 1	taatacgactcactataggtttcaaagattaaataatttctactaagtg
LbCas12 IVT template primer 2	tttccaatgatgagcactttatctacacttagtagaaattatttaatctttgaaac
AsCas12 IVT template primer 1	taatacgactcactataggtcaaaagacctttttaatttctactc
AsbCas12 IVT template primer 2	tttccaatgatgagcactttatctacaagagtagaaattaaaaaggtcttttgac
Cas12 gRNA template reverse primer	ctcccttagccatccgagtggacgacgtcctccttcggatgcccaggtcggaccgcgaggaggtggagatgccatgccgacc ctttccaatgatgagcac
MbCas12 AmpR gRNA	ggctaacgacctttttaaatttctactgtttgtagataaagtgctcatcattggaaa
LbCas12 AmpR gRNA	ggtttcaaagattaaataatttctactaagtgtagataaagtgctcatcattggaaa
AsCas12 AmpR gRNA	ggtcaaaagacctttttaatttctactcttgtagataaagtgctcatcattggaaa

Linear DNA target	aattctaaagatctttgacagctagctcagtcctaggtataatactagtgcctctacctgcttcggccgataaagccgacga taatactcccaaagcccgccgaaaggcgggctttttttttggatccttactcgagtctagactgcaggcttcctcgctcactg actcgctgcgctcggtcgttcggctgcggcgagcggtatcagctcactcaaaggcggtaatacggttatccacagaatcagg ggataacgcaggaaagaacatgtgagcaaaaggccagcaaaaggccaggaaccgtaaaaaggccgcgttgctggcgtttttc cacaggctccgcccccctgacgagcatcacaaaaatcgacgctcaagtcagaggtggcgaaacccgacaggactataaagat accaggcgtttcccccctggaagctccctcgtgcgctctcctgttccgaccctgccgcttaccggatacctgtccgcctttct cccttcgggaagcgtggcgctttctcatagctcacgctgtaggtatctcagttcggtgtaggtcgttcgctccaagctgggc tgtgtgcacgaaccccccgttcagcccgaccgctgcgccttatccggtaactatcgtcttgagtccaacccggtaagacacg acttatcgccactggcagcagccactggtaacaggattagcagagcgaggtatgtaggcggtgctacagagttcttgaagtg gtggcctaactacggctacactagaagaacagtatttggtatctgcgctctgctgaagccagttaccttcggaaaaagagtt ggtagctcttgatccggcaaacaaaccaccgctggtagcggtggtttttttgtttgcaagcagcagattacgcgcagaaaaa aaggatctcaagaagatcctttgatcttttctacggggtctgacgctcagtggaacgaaaactcacgttaagggattttggt catgagattatcaaaaaggatcttcacctagatccttttaaattaaaaatgaagttttaaatcaatctaaagtatatatgag taaacttggtctgacagttaccaatgcttaatcagtgaggcacctatctcagcgatctgtctatttcgttcatccatagttg cctgactccccgtcgtgtagataactacgatacgggagggcttaccatctggccccagtgctgcaatgataccgcgagaccc acgctcaccggctccagatttatcagcaataaaccagccagccggaagggccgagcgcagaagtggtcctgcaactttatcc gcctccatccagtctattaattgttgccgggaagctagagtaagtagttcgccagttaatagtttgcgcaacgttgttgcca ttgctacaggcatcgtggtgtcacgctcgtcgtttggtatggcttcattcagctccggttcccaacgatcaaggcgagttac atgatcccccatgttgtgcaaaaaagcggttagctccttcggtcctccgatcgttgtcagaagtaagttggccgcagtgtta tcactcatggttatggcagcactgcataattctcttactgtcatgccatccgtaagatgcttttctgtgactggtgagtact caaccaagtcattctgagaatagtgtatgcggcgaccgagttgctcttgcccggcgtcaatacgggataataccgcgccaca tagcagaactttaaaagtgctcatcattggaaaacgttcttcggggcgaaaactctcaaggatcttaccgctgttgagatcc agttcgatgtaacccactcgtgcacccaactgatcttcagcatcttttactttcaccagcgtttctgggtgagcaaaaacag gaaggcaaaatgccgcaaaaaagggaataagggcgacacggaaatgttgaatactcatactcttcctttttcaatattattg aagcatttatcagggttattgtctcatgagcggatacatatttgaatgtatttagaaaaataaacaaataggggttccgcgc acatttccccgaaaagtgccacctgacgtctaagaaaccattattatcatgacattaacctataaaaataggcgtatcacga ggcagaatttcagataaaaaaaatccttagctttcgctaaggatgatttctgg
pTet Acr gene expression plasmid (genes inserted at **xxxx**) (Addgene 115669)	gaattcaactgcaggcactgcccatggacctcggtaccgaatagctagccggtaatgcattcgctagagctcctaaagcatg cgacctgcaaccggtctgtcacgtacgtcgccaccgtcgacgtcgttcgtaagtagcctagataaataaaataatcagttaa ccgcgagccccatgcgagagtagggaactgccaggcatttcagccaaaaaacttaagaccgccggtcttgtccactaccttg cagtaatgcggtggacaggatcggcggttttcttttctcttctcaaccgccgggagcggatttgaacgttgcgaagcaacgg cccggagggtggcgggcaggacgcccgccataaactgccaggcatcaaattaagcagaaggccatcctgacggatggccttt ttgcgtttctacaaactctgcggtaatacggttatccacagaatcaggggataacgcaggaaagaacatgtgagcaaaaggc cagcaaaaggccaggaaccgtaaaaaggccgcgttgctggcgtttttccacaggctccgccccctgacgagcatcacaaaa atcgacgctcaagtcagaggtggcgaaacccgacaggactataaagataccaggcgtttcccccctggaagctccctcgtgcg ctctcctgttccgaccctgccgcttaccggatacctgtccgcctttctcccttcgggaagcgtggcgctttctcatagctca cgctgtaggtatctcagttcggtgtaggtcgttcgctccaagctgggctgtgtgcacgaaccccccgttcagcccgaccgct gcgccttatccggtaactatcgtcttgagtccaacccggtaagacacgacttatcgccactggcagcagccactggtaacag gattagcagagcgaggtatgtaggcggtgctacagagttcttgaagtggtggcctaactacggctacactagaaggacagta tttggtatctgcgctctgctgaagccagttaccttcggaaaaagagttggtagctcttgatccggcaaacaaaccaccgctg gtagcggtggttttttgtttgcaagcagcagattacgcgcagaaaaaaggatctcaagaagatcctttgatcttttctac ggggtctgacgctcagtggaacgaaaactcacgttaagggattttggtcatgagattatcaaaaggatcttcacctagatc cttttaaattaaaaatgaagttttaaatcaatctaaagtatatatgagtaaacttggtctgacagttaccaatgcttaatca gtgaggcacctatctcagcgatctgtctatttcgttcatccatagttgcctgactccccgtcgtgtagataactacgatacg ggagggcttaccatctggccccagtgctgcaatgataccgcgagacccacgctcaccggctccagatttatcagcaataaac cagccagccggaagggccgagcgcagaagtggtcctgcaactttatccgcctccatccagtctattaattgttgccgggaag ctagagtaagtagttcgccagttaatagtttgcgcaacgttgttgccattgctacaggcatcgtggtgtcacgctcgtcgtt tggtatggcttcattcagctccggttcccaacgatcaaggcgagttacatgatcccccatgttgtgcaaaaaagcggttagc tccttcggtcctccgatcgttgtcagaagtaagttggccgcagtgttatcactcatggttatggcagcactgcataattctc ttactgtcatgccatccgtaagatgcttttctgtgactggtgagtactcaaccaagtcattctgagaatagtgtatgcggcg accgagttgctcttgcccggcgtcaatacgggataataccgcgccacatagcagaactttaaaagtgctcatcattggaaaa cgttcttcggggcgaaaactctcaaggatcttaccgctgttgagatccagttcgatgtaacccactcgtgcacccaactgat cttcagcatcttttactttcaccagcgtttctgggtgagcaaaaacaggaaggcaaaatgccgcaaaaagggaataagggc gacacggaaatgttgaatactcatactcttcctttttcaatattattgaagcatttatcagggttattgtctcatgagcgga tacatatttgaatgtatttagaaaaataaacaaatagggttccgcgcacatttccccgaaaagtgccacctgacgtctaag aaaccattattgtggatataccttactcgagttagccttgataggacgtcttaagacccactttcacatttaagttgttttt ctaatccgcatatgatcaattcaaggccgaataagaaggctggctctgcaccttggtgatcaaataattcgatagcttgtcg taataatggcggcatactatcagtagtaggtgtttccctttcttctttagcgacttgatgctcttgatcttccaatacgcaa cctaaagtaaaatgccccacagcgctgagtgcatataatgcattctctagtgaaaaaccttgttggcataaaaaggctaatt gattttcgagagtttcatactgtttttctgtaggccgtgtacctaaatgtacttttgctccatcgcgatgacttagtaaagc acatctaaaacttttagcgttattacgtaaaaaatcttgccagctttccccttctaaagggcaaaagtgagtatggtgccta tctaacatctcaatggctaaggcgtcgagcaaagcccgcttatttttttacatgccaatacaatgtaggctgctctacaccta gcttctgggcgagtttacgggttgttaaaccttcgattccgacctcattaagcagctctaatgcgctgttaatcactttact tttatctaatctagacatcattaattcctaatttttgttgacactctatcgttgatagagttatttttaccactccctatcag tgatagagaaaa**xxxx**

Cas12 non-targeting gRNA plasmid for TXTL	gaattctaaagatctggcacgtaagaggttccaactttcaccataatgaaacatactagagaaagaggagaaatactagatg gtgaatgtgaaaccagtaacgttatacgatgtcgcagagtatgccggtgtctcttatcagaccgtttcccgcgtggtgaacc aggccagccacgtttctgcgaaaacgcgggaaaaagtggaagcggcgatggcggagctgaattacattcccaaccgcgtggc acaacaactggcgggcaaacagtcgttgctgattggcgttgccacctccagtctggccctgcacgcgccgtcgcaaattgtc gcggcgattaaatctcgcgccgatcaactgggtgccagcgtggtggtgtcgatggtagaacgaagcggcgtcgaagcctgta aagcggcggtgcacaatcttctcgcgcaacgcgtcagtgggctgatcattaactatccgctggatgaccaggatgccattgc tgtggaagctgcctgcactaatgttccggcgttatttcttgatgtctctgaccagacacccatcaacagtattattttctcc catgaagacggtacgcgactgggcgtggagcatctggtcgcattgggtcaccagcaaatcgcgctgttagcgggcccattaa gttctgtctcggcgcgtctgcgtctggctggctggcataaatatctcactcgcaatcaaattcagccgatagcggaacggga aggcgactggagtgccatgtccggttttcaacaaaccatgcaaatgctgaatgagggcatcgttcccactgcgatgctggtt gccaacgatcagatggcgctgggcgcaatgcgcgccattaccgagtccgggctgcgcgttggtgcggatatctcggtagtgg gatacgacgataccgaagacagctcatgttatatcccgccgttaaccaccatcaaacaggattttcgcctgctggggcaaac cagcgtggaccgcttgctgcaactctctcagggccaggcggtgaagggcaatcagctgttgcccgtctcactggtgaaaaga aaaaccaccctggcgcccaatacgcaaaccgcctctccccgcgcgttggccgattcattaatgcagctggcacgacaggttt cccgactggaaagcgggcaggctgcaaacgacgaaaactacgctttagtagcttaataactctgatagtgctagtgtagatc cctactagagccaggcatcaaataaaacgaaaggctcagtcgaaagactgggcctttcgttttatctgttgtttgtcggtga acgctctctactagagtcacactggctcaccttcgggtgggcctttctgcgtttatatattgcttagaataatcgatctgcg gccgcagagagtgtagcttacctagtcatcgaaagctttgctacagcggatagaattgtgagcggataacaattgacattgt gagcggataacaagatactactagtgtctaacgacctttttaaatttctactgtttgtagatcgatgtgacatcaagtgctac ggggtctaacgacctttttaaatttctactgtttgtagatcaaagcccgccgaaaggcgggctttttttttgtggatatacctt actcgagttagccttgatagattgtctgattcgttaccaattatgacaacttgacggctacatcattcacttttttcttcaca accggcacggaactcgctcgggctggccccggtgcatttttttaaataccgcgagaaatagagttgatcgtcaaaaccaaca ttgcgaccgacggtggcgataggcatccgggtggtgctcaaaagcagcttcgcctggctgatacgttggtcctcgcgccagc ttaagacgctaatccctaactgctggcggaaaagatgtgacagacgcgacggcgacaagcaaacatgctgtgcgacgctggc gatatcaaaattgctgtctgccaggtgatcgctgatgtactgacaagcctcgcgtacccgattatccatcggtggatggagc gactcgttaatcgcttccatgcgccgcagtaacaattgctcaagcagatttatcgccagcagctccgaatagcgcccttccc cttgcccggcgttaatgatttgcccaaacaggtcgctgaaatgcggctggtgcgcttcatccgggcgaaagaaccccgtatt ggcaaatattgacggccagttaagccattcatgccagtaggcgcgcggacgaaagtaaacccactggtgataccattcgcga gcctccggatgacgaccgtagtgatgaatctctcctggcgggaacagcaaaatatcacccggtcggcaaacaaattctcgtc cctgatttttcaccaccccctgaccgcgaatggtgagattgagaatataacctttcattcccagcggtcggtcgataaaaaa atcgagataaccgttggcctcaatcggcgttaaacccgccaccagatgggcattaaacgagtatcccggcagcaggggatca ttttgcgcttcagccatacttttcatactcccgccattcagagaagaaaccaattgtccatattgcatcagacattgccgtc actgcgtcttttactggctcttctcgctaaccaaaccggtaaccccgcttattaaaagcattctgtaacaaagcgggaccaa agccatgacaaaaacgcgtaacaaaagtgtctataatcacggcagaaaagtccacattgattatttgcacggcgtcacactt tgctatgccatagcatttttatccataagattagcggatcctacctgacgctttttatcgcaactctctactgtttctccat atatcggatccttagtaaacctgcaggcactgcccatggacctcggtaccgaatagctagccggtaatgcattcgctagagc tcctaaagcatgcgacctgcaaccggtctgtcacgtacgtcgccaccgtcgacgtcgttcgtaagtagcctagataaataaa ataatcagttaaccgcgagccccatgcgagagtagggaactgccaggcatcaaataaaacgaaaggctcagtcgaaagactg ggcctttcgttttatctgttgtttgtcggtgaacgctctcctgagtaggacaaatccgccgggagcggatttgaacgttgcg aagcaacggcccggagggtggcgggcaggacgcccgccataaactgccaggcatcaaattaagcagaaggccatcctgacgg atggccttttgcgtttctacaaactctgcggtaatacggttatccacagaatcaggggataacgcaggaaagaacatgtga gcaaaaggccagcaaaaggccaggaaccgtaaaaaggccgcgttgctggcgtttttccataggctccgccccctgacgagc atcacaaaaatcgacgctcaagtcagaggtggcgaaacccgacaggactataaagataccaggcgtttcccccctggaagctc cctcgtgcgctctcctgttccgaccctgccgcttaccggatacctgtccgcctttctcccttcgggaagcgtggcgctttct catagctcacgctgtaggtatctcagttcggtgtaggtcgttcgctccaagctgggctgtgtgcacgaacccccgttcagc ccgaccgctgcgccttatccggtaactatcgtcttgagtccaacccggtaagacacgacttatcgccactggcagcagccac tggtaacaggattagcagagcgaggtatgtaggcggtgctacagagttcttgaagtggtggcctaactacggctacactaga aggacagtatttggtatctgcgctctgctgaagccagttaccttcggaaaaagagttggtagctcttgatccggcaaacaaa ccaccgctggtagcggtggtttttttgtttgcaagcagcagattacgcgcagaaaaaaaggatctcaagaagatcctttgat cttttctacggggtctgacgctcagtggaacgaaaactcacgttaagggattttggtcatgagattatcaaaaaggatcttc acctagatccttttaaattaaaaatgaagttttaaatcaatctaaagtatatatgagtaaacttggtctgacagttaccaat gcttaatcagtgaggcacctatctcagcgatctgtctatttcgttcatccatagttgcctgactccccgtcgtgtagataac tacgatacgggagggcttaccatctggccccagtgctgcaatgataccgcgagacccacgctcaccggctccagatttatca gcaataaaccagccagccggaagggccgagcgcagaagtggtcctgcaactttatccgcctccatccagtctattaattgtt gccgggaagctagagtaagtagttcgccagttaatagtttgcgcaacgttgttgccattgctacaggcatcgtggtgtcacg ctcgtcgtttggtatggcttcattcagctccggttcccaacgatcaaggcgagttacatgatcccccatgttgtgcaaaaaa gcggttagctccttcggtcctccgatcgttgtcagaagtaagttggccgcagtgttatcactcatggttatggcagcactgc ataattctcttactgtcatgccatccgtaagatgcttttctgtgactggtgagtactcaaccaagtcattctgagaatagtg tatgcggcgaccgagttgctcttgcccggcgtcaatacgggataataccgcgccacatagcagaactttaaaagtgctcatc attggaaaacgttcttcggggcgaaaactctcaaggatcttaccgctgttgagatccagttcgatgtaacccactcgtgcac ccaactgatcttcagcatcttttactttcaccagcgtttctgggtgagcaaaaacaggaaggcaaaatgccgcaaaaaaggg aataagggcgacacggaaatgttgaatactcatactcttcctttttcaatattattgaagcatttatcagggttattgtctc atgagcggatacatatttgaatgtatttagaaaaataaacaaatagggggttccgcgcacatttccccgaaaagtgccacctg acgtctaagaaaccattattatcatgacattaacctataaaaataggcgtatcacgaggcagaatttcagataaaaaaaatc cttagctttcgctaaggatgatttctg

Cas12 GFP, RFP gRNA plasmid for TXTL (Addgene 115661)	gAATTCTAAAGATCTGGCACGTAAGAGGTTCCAACTTTCACCataatgaaacatactagagaaagaggagaaatactagatg gtgaatgtgaaaccagtaacgttatacgatgtcgcagagtatgccggtgtctcttatcagaccgtttcccgcgtggtgaacc aggccagccacgtttctgcgaaaacgcgggaaaaagtggaagcggcgatggcggagctgaattacattcccaaccgcgtggc acaacaactggcgggcaaacagtcgttgctgattggcgttgccacctccagtctggccctgcacgcgccgtcgcaaattgtc gcggcgattaaatctcgcgccgatcaactgggtgccagcgtggtggtgtcgatggtagaacgaagcggcgtcgaagcctgta aagcggcggtgcacaatcttctcgcgcaacgcgtcagtgggctgatcattaactatccgctggatgaccaggatgccattgc tgtggaagctgcctgcactaatgttccggcgttatttcttgatgtctctgaccagacacccatcaacagtattatttttctcc catgaagacggtacgcgactgggcgtggagcatctggtcgcattgggtcaccagcaaatcgcgctgttagcgggcccattaa gttctgtctcggcgcgtctgcgtctggctggctggcataaatatctcactcgcaatcaaattcagccgatagcggaacggga aggcgactggagtgccatgtccggttttcaacaaaccatgcaaatgctgaatgagggcatcgttcccactgcgatgctggtt gccaacgatcagatggcgctgggcgcaatgcgcgccattaccgagtccgggctgcgcgttggtgcggatatctcggtagtgg gatacgacgataccgaagacagctcatgttatatcccgccgttaaccaccatcaaacaggattttcgcctgctggggcaaac cagcgtggaccgcttgctgcaactctctcagggccaggcggtgaagggcaatcagctgttgcccgtctcactggtgaaaaga aaaaccaccctggcgcccaatacgcaaaccgcctctccccgcgcgttggccgattcattaatgcagctggcacgacaggttt cccgactggaaagcgggcaggctgcaaacgacgaaaactacgctttagtagcttaataactctgatagtgctagtgtagatc cctactagagccaggcatcaaataaaacgaaaggctcagtcgaaagactgggcctttcgttttatctgttgtttgtcggtga acgctctctactagagtcacactggctcaccttcgggtgggcctttctgcgtttatatattgcttagaataatcgatctGCG GCCGCagagagtgtagcttacctagtcatcgaAAGCTTtgctacagcggatagaattgtgagcggataaCAATTGACATTGT GAGCGGATAACAAGATACTACTAGTGTCTAACGACCTTTTAAATTTCTACTGTTTGTAGATaaagttcgtatggaaggttcc gttGTCTAACGACCTTTTAAATTTCTACTGTTTGTAGATtgcccattaacatcaccatctaatGTCTAACGACCTTTTAAAT TTCTACTGTTTGTAGATcaaaGCCCGCCgaaaGGCGGGCtttttttttgtggatatacctTACTCGAGTtagccttgatagat tgtctgattcgttaccaattatgacaacttgacggctacatcattcacttttttcttcacaaccggcacggaactcgctcggg ctggccccggtgcatttttttaaataccgcgagaaatagagttgatcgtcaaaaccaacattgcgaccgacggtggcgatag gcatccgggtggtgctcaaaagcagcttcgcctggctgatacgttggtcctcgcgccagcttaagacgctaatccctaactg ctggcggaaaagatgtgacagacgcgacggcgacaagcaaacatgctgtgcgacgctggcgatatcaaaattgctgtctgcc aggtgatcgctgatgtactgacaagcctcgcgtacccgattatccatcggtggatggagcgactcgttaatcgcttccatgc gccgcagtaacaattgctcaagcagatttatcgccagcagctccgaatagcgcccttcccttgcccggcgttaatgatttg cccaaacaggtcgctgaaatgcggctggtgcgcttcatccgggcgaaagaaccccgtattggcaaatattgacggccagtta agccattcatgccagtaggcgcgcggacgaaagtaaacccactggtgataccattcgcgagcctccggatgacgaccgtagt gatgaatctctcctggcgggaacagcaaaatatcacccggtcggcaaacaaattctcgtccctgatttttcaccaccccctg accgcgaatggtgagattgagaatataacctttcattcccagcggtcggtcgataaaaaaatcgagataaccgttggcctca atcggcgttaaacccgccaccagatgggcattaaacgagtatcccggcagcaggggatcattttgcgcttcagccatacttt tcatactcccgccattcagagaagaaaccaattgtccatattgcatcagacattgccgtcactgcgtcttttactggctctt ctcgctaaccaaaccggtaaccccgcttattaaaagcattctgtaacaaagcgggaccaaagccatgacaaaaacgcgtaac aaaagtgtctataatcacggcagaaaagtccacattgattatttgcacggcgtcacactttgctatgccatagcatttttat ccataagattagcggatcctacctgacgctttttatcgcaactctctactgtttctccatatatcGGATCCTTAGTaaACCT GCAGGcACTGCCCATGGacctCGGTACCGaataGCTAGCCggtaATGCATTCgctAGAGCTCCtaaaGCATGCgacctGCAA CCGGTctgtcaCGTACGtcgccaccGTCGACgtcgttcgtaagtagcctagataaataaaataatcagttaaccgcgagccc catgcgagagtagggaactgccaggcatcaaataaaacgaaaggctcagtcgaaagactgggcctttcgttttatctgttgt ttgtcggtgaacgctctcctgagtaggacaaatccgccgggagcggatttgaacgttgcgaagcaacggcccggagggtggc gggcaggacgcccgccataaactgccaggcatcaaattaagcagaaggccatcctgacggatggcctttttgcgtttctaca aactctGCGGTAATAcggttatccacagaatcaggggataacgcaggaaagaacatgtgagcaaaaggccagcaaaaggcca ggaaccgtaaaaaggccgcgttgctggcgtttttccataggctccgccccctgacgagcatcacaaaaatcgacgctcaag tcagaggtggcgaaacccgacaggactataaagataccaggcgtttcccccctggaagctccctcgtgcgctctcctgttccg accctgccgcttaccggatacctgtccgcctttctcccttcgggaagcgtggcgctttctcatagctcacgctgtaggtatc tcagttcggtgtaggtcgttcgctccaagctgggctgtgtgcacgaaccccccgttcagcccgaccgctgcgccttatccgg taactatcgtcttgagtccaacccggtaagacacgacttatcgccactggcagcagccactggtaacaggattagcagagcg aggtatgtaggcggtgctacagagttcttgaagtggtggcctaactacggctacactagaaggacagtatttggtatctgcg ctctgctgaagccagttaccttcggaaaaagagttggtagctcttgatccggcaaacaaaccaccgctggtagcggtggttt ttttgtttgcaagcagcagattacgcgcagaaaaaaaggatctcaagaagatcctttgatcttttctacggggtctgacgct cagtggaacgaaaactcacgttaagggattttggtcatgagattatcaaaaggatcttcacctagatccttttaaattaaa aatgaagttttaaatcaatctaaagtatatatgagtaaacttggtctgacagttaccaatgcttaatcagtgaggcacctat ctcagcgatctgtctatttcgttcatccatagttgcctgactccccgtcgtgtagataactacgatacgggagggcttacca tctggccccagtgctgcaatgataccgcgagacccacgctcaccggctccagatttatcagcaataaaccagccagccggaa gggccgagcgcagaagtggtcctgcaactttatccgcctccatccagtctattaattgttgccgggaagctagagtaagtag ttcgccagttaatagtttgcgcaacgttgttgccattgctacaggcatcgtggtgtcacgctcgtcgtttggtatggcttca ttcagctccggttcccaacgatcaaggcgagttacatgatcccccatgttgtgcaaaaaagcggttagctccttcggtcctc cgatcgttgtcagaagtaagttggccgcagtgttatcactcatggttatggcagcactgcataattctcttactgtcatgcc atccgtaagatgcttttctgtgactggtgagtactcaaccaagtcattctgagaatagtgtatgcggcgaccgagttgctct tgcccggcgtcaatacgggataataccgcgccacatagcagaactttaaaagtgctcatcattggaaaacgttcttcggggc gaaaactctcaaggatcttaccgctgttgagatccagttcgatgtaacccactcgtgcacccaactgatcttcagcatcttt tactttcaccagcgtttctgggtgagcaaaaacaggaaggcaaaatgccgcaaaaaagggaataagggcgacacggaaatgt tgaatactcatactcttcctttttcaatattattgaagcatttatcagggttattgtctcatgagcggatacatatttgaat gtatttagaaaaataaacaaatagggggttccgcgcacatttccccgaaaagtgccacctgacgtctaagaaaccattattat catgacattaacctataaaaatagggcgtatcacgaggcagaatttcagataaaaaaaatccttagctttcgctaaggatgat ttctg

Table S3. DNA and RNA sequences used for TXTL and *in vitro* experiments. Primer sequences used for creating genomic amplicons are listed in table S1 and are not included here.

Vector name	Key features	Gene-of-interest (GOI)	GOI CDS sequence	Full vector sequence	Vector length
pCF525-AcrVA1 (Addgene 115662)	EF1a-HygroR-P2A-AcrVA1	AcrVA1	ATGTCAAAGGCTATGTATGAAGCCAAAGAACGATATGCGAAGAAGAAGATGCAGGAGAACACTAAGATAGACA CTCTCACCGATGAGCAGCATGACGCTCTCGCCCAGCTTTGCGCGTTTAGACATAAGTTTCATTCTAACAAAGATT CCCTGTTTCTTTCCGAATCAGCGTTCAGTGGGGAGTTTAGTTTCGAGATGCAGTCTGACGAGAACTCCAAGTTGA GGGAAGTAGGACTCCCCACAATCGAATGGTCCTTTTACGACAATTCTCACATACCCGACGACAGCTTTAGAGAGT GGTTCAATTTTGCTAACTATTCTGAGCTTTCAGAAACAATACAAGAGCAGGGTCTTGAACTCGACCTGGATGATG ACGAAACCTACGAGCTGGTCTATGATGAGCTTTATACAGAGGCGATGGGAGAATACGAGGAATTGAACCAGGAT ATAGAGAAATACCTTCGCAGAATAGATGAGGAACATGGCACGCAATACTGTCCAACTGGTTTTGCGCGGCTGAG ATAA	See A below	8637
pCF525-AcrVA4 (Addgene 115663)	EF1a-HygroR-P2A-AcrVA4	AcrVA4	ATGTATGAGATCAAGCTGAACGATACATTGATACACCAAACTGATGACAGAGTGAATGCCTTCGTGGCCTATCG CTATCTTCTGCGGCGCGGCGATTTGCCTAAATGTGAAAACATAGCGCGGATGTACTATGACGGTAAAGTCATCAA AACAGACGTGATTGACCATGATAGTGTTCACTCCGACGAGCAAGCTAAAGTATCTAATAACGACATAATCAAAA TGGCCATATCTGAGCTTGGTGTTAATAATTTCAAAAGTCTTATAAAGAAACAAGGTTACCCATTCAGCAATGGCC ACATCAACTCTTGGTTCACTGACGACCCGGTGAAGTCCAAAACTATGCATAACGACGAGATGTACCTTGTTGTTC AGGCACTTATAAGGGCTTGCATAATCAAGGAAATTGACCTGTATACTGAACAACTGTATAACATTATCAAGAGTC TGCCCTATGACAAACGACCCAATGTAGTATACTCAGACCAACCTCTGGACCCCAACAATCTGGACCTGTCCGAAC CCGAGCTTTGGGCTGAACAGGTAGGCGAGTGCATGCGGTATGCTCATAATGACCAGCCTTGTTTCTATATAGGTA GCACAAAGAGAGAACTTAGAGTCAACTACATTGTCCCCGTCATCGGCGTAAGGGATGAAATAGAGCGGGTTATG ACTCTGGAAGAGGTACGAAATCTTCATAAGTAA	See B below	8817
pCF525-AcrVA5 (Addgene 115664)	EF1a-HygroR-P2A-AcrVA5	AcrVA5	ATGAAGATCGAGCTTTCCGGGGGGTATATTTGTTACAGCATTGAGGAAGACGAAGTTACTATAGACATGGTAGA GGTCACGACTAAACGCCAGGGGATAGGCTCTCAGCTTATAGATATGGTAAAGGATGTGGCTAGAGAAGTAGGTC TCCCGATAGGTCTTTATGCGTATCCTCAAGATGACAGTATTAGTCAGGAGGATCTGATCGAGTTTTACTTCAGTA ATGATTTTGAGTATGACCCAGACGATGTCGATGGACGCCTTATGCGCTGGTCTTAA	See C below	8391
pCF525-AcrIIA4 (Addgene 115795)	EF1a-HygroR-P2A-AcrIIA4	AcrIIA4	ATGAATATTAATGACTTAATTAGAGAAATCAAAAACAAAGATTACACAGTGAAATTGAGTGGTACGGATAGCAA TAGTATCACACAGCTAATTATTCGCGTTAATAATGATGGCAACGAGTATGTAATTTCTGAAAGTGAAAATGAATC AATCGTTGAAAAATTCATCTCTGCATTCAAAAACGGTTGGAATCAAGAATACGAGGATGAAGAAGAATTTTATA ATGACATGCAAACAATCACCTTAAAAAGTGAGTTGAACTAA	See D below	8376
pCF525-mTagBFP2 (Addgene 115797)	EF1a-HygroR-P2A-mTagBFP2	mTagBFP2	TCTGAACTCATCAAGGAGAACATGCACATGAAACTCTACATGGAGGGGACCGTAGACAATCATCACTTCAAGTG TACaAGTGAAGGGGAGGGGAAGCCTTACGAAGGGACTCAAACTATGAGAATTAAGGTTGTCGAGGGCGGCCCCC TTCCCTTTGCGTTTGACATCCTCGCCACcAGTTTCTTGTATGGTTCCAAGACGTTTATAAATCATACACAGGGAAT ACCTGACTTCTTTAAACAAAGTTTTCCAGAGGGTTTCACATGGGAAAGGGTTACCACTTATGAGGATGGCGGAGT ACTCACCGCTACCCAAGATACATCTCTGCAAGATGGTTGCCTGATTTACAATGTAAAAATCCGAGGAGTTAACTT TACATCCAATGGTCCTGTTATGCAGAAAAAGACATTGGGGTGGGAAGCATTTACAGAAACGCTCTACCCCGCCG ACGGGGGCTTGGAGGGCCGCAATGACATGGCTCTCAAATTGGTAGGTGGTAGTCATCTTATCGCGAACGCGAAA ACTACCTACCGATCCAAGAAGCCCGCTAAAAATCTCAAAATGCCGGTCTATTACGTGGACTACAGACTGGAACG CAAGGAAGCTAACAACGAGACATACGTTGAGCAACATGAAGTGGCCGTGGCCAGATATTGCGATCTGCCGAGTA AGCTCGGGCACAAATTGAACTAA	See E below	8805
pCF525-mCherry (Addgene 115796)	EF1a-HygroR-P2A-mCherry	mCherry	ATGGTGAGCAAGGGCGAGGAGGATAACATGGCCATCATCAAGGAGTTCATGCGCTTCAAGGTGCACATGGAGG GCTCCGTGAACGGCCACGAGTTCGAGATCGAGGGCGAGGGCGAGGGCCGCCCCTACGAGGGCACCCAGACCGC CAAGCTGAAGGTGACCAAGGGTGGCCCCCTGCCCTTCGCCTGGGACATCCTGTCCCCTCAGTTCATGTACGGCTC CAAGGCCTACGTGAAGCACCCCGCCGACATCCCCGACTACTTGAAGCTGTCCTTCCCCGAGGGCTTCAAGTGGGA GCGCGTGATGAACTTCGAGGACGGCGGCGTGGTGACCGTGACCCAGGACTCCTCCCTGCAGGACGGCGAGTTCA TCTACAAGGTGAAGCTGCGCGGCACCAACTTCCCCTCCGACGGCCCCGTAATGCAGAAGAAaACCATGGGCTGG GAGGCCTCCTCCGAGCGGATGTACCCCGAGGACGGCGCCCTGAAGGGCGAGATCAAGCAGAGGCTGAAGCTGA AGGACGGCGGCCACTACGACGCTGAGGTCAAGACCACCTACAAGGCCAAGAAGCCCGTGCAGCTGCCCGGCGC CTACAACGTCAACATCAAGTTGGACATCACCTCCCACAACGAGGACTACACCATCGTGGAACAGTACGAACGCG CCGAGGGCCGCCACTCCACCGGCGGCATGGACGAGCTGTACAAGTAA	See F below	8823

	Full vector sequence
A	gtcgacggatcgggagatctcccgatcccctatggtgcactctcagtacaatctgctctgatgccgcatagttaagccagtatctgctccctgcttgtgtgttggaggtcgctgagtagtgcgcgagcaaaatttaagctacaacaaggcaaggcttgaccgacaattgcatgaagaatctgcttag ggttaggcgttttgcgctgcttcgcgatgtacgggccagatattgacattgattattgactagttattaatagtaatcaattacggggtcattagttcatagcccatatatggagttccgcgttacataacttacggtaaatggcccgcctggctgaccgcccaacgacccccgcccattgacgtcaataa tgacgtatgttcccatagtaacgccaatagggactttccattgacgtcaatgggtggagtatttacggtaaactgcccacttggcagtacatcaagtgtatcatatgccaagtacgccccctattgacgtcaatgacggtaaatggcccgcctggcattatgcccagtacatgaccttatgggactttcc tacttggcagtacatctacgtattagtcatcgctattaccatggtgatgcggttttggcagtacatcaatgggcgtggatagcggtttgactcacggggatttccaagtctccaccccattgacgtcaatgggagtttgttttggcaccaaaatcaacgggactttccaaaatgtcgtaacaactccgccc cattgacgcaaatgggcggtaggcgtgtacggtgggaggtctatataagcagcgcgttttgcctgtactgggtctctctggttagaccagatctgagcctgggagctctctggctaactagggaacccactgcttaagcctcaataaagcttgccttgagtgcttcaagtagtgtgtgcccgtctgtt gtgtgactctggtaactagagatccctcagacccttttagtcagtgtggaaaatctctagcagtggcgcccgaacagggacttgaaagcgaaagggaaaccagaggagctctctcgacgcaggactcggcttgctgaagcgcgcacggcaagaggcgaggggcggcgactggtgagtacg ccaaaaattttgactagcggaggctagaaggagagagatgggtgcgagagcgtcagtattaagcgggggagaattagatcgcgatgggaaaaaattcggttaaggccaggggaaagaaaaaatataaattaaaacatatagtatgggcaagcagggagctagaacgattcgcagttaatcc tggcctgttagaaacatcagaaggctgtagacaaatactgggacagctacaaccatcccttcagacaggatcagaagaacttagatcattatataatacagtagcaaccctctattgtgtgcatcaaaggatagagataaaagacaccaaggaagctttagacaagatagaggaagagcaaaaca aaagtaagaccaccgcacagcaagcggccgctgatcttcagacctggaggaggagatatgagggacaattggagaagtgaattatataaatataaagtagtaaaaattgaacgattaggagtagcacccaccaaggcaaagagaagagtggtgcagagagaaaaaagagcagtgggaata ggagctttgttccttgggttcttgggagcagcaggaagcactatgggcgcagcgtcaatgacgctgacggtacaggccagacaattattgtctggtatagtgcagcagcagaacaatttgctgagggctattgaggcgcaacagcatctgttgcaactcacagtctggggcatcaagcagctcca ggcaagaatcctggctgtggaaagatacctaaaggatcaacagctcctggggatttggggttgctctggaaaactcatttgcaccactgctgtgccttggaatgctagttggagtaataaatctctggaacagatttggaatcacacgacctggatggagtgggacagagaaattaacaattacaca agcttaatacactccttaattgaagaatcgcaaaaccagcaagaaaagaatgaacaagaattattggaattagataaatgggcaagtttgtggaattggttaacaataacaaattggctgtggtatataaaattattcataatgatagtaggaggcttggtaggtttaagaatagtttttgctgtactttctat agtgaatagagttaggcagggatattcaccattatcgtttcagacccacctcccaaccccgaggggacccgacaggcccgaaggaatagaagaagaaggtggagagagagacagagacagatccattcgattagtgaacggatcggcactgcgtgcgccaattctgcagacaaatggcagt attcatccacaattttaaaagaaaaggggggattgggggtacagtgcaggggaaagaatagtagacataatagcaacagacatacaaactaaagaattacaaaaacaaattacaaaaattcaaaattttcgggtttattacagggacagcagagatccagtttggttaattaagctagctaggtctt gaaaggagtgggaattggctccggtgcccgtcagtgggcagagcgcacatcgcccacagtccccgagaagttgggggaggggtcggcaattgatccggtgcctagagaaggtggcgcggggtaaactgggaaagtgatgtcgtgtactggctccgcctttttcccgagggtgggggag aaccgtatataagtgcagtagtcgccgtgaacgttcttttcgcaacgggtttgccgccagaacacaggtaagtgccgtgtgtggttcccgcgggcctggcctctttacgggttatggcccttcgctgccttgaattacttccactggctgcagtacgtgattcttgatcccgagcttcgggttggaagt gggtgggagagttcgaggccttgcgcttaaggagccccttcgcctcgtgcttgagttgaggcctggcctgggcgctggggccgccgcgtgcgaatctggtggcaccttcgcgcctgtctcgctgctttcgataagtctctagccatttaaaatttttgatgacctgctgcgacgctttttttctggcaa gatagtcttgtaaatgcgggccaagatctgcacactggtatttcggttttggggccgcgggcggcgacggggcccgtgcgtcccagcgcacatgttcggcgaggcggggcctgcgagcgcggccaccgagaatcggacgggggtagtctcaagctggccggcctgctctggtgcctggc ctcgcgccgccgtgtatcgccccgccctgggcggcaaggctggcccggtcggcaccagttgcgtgagcggaaagatggccgcttcccgccctgctgcagggagctcaaaatggaggacgcggcgctcgggagagcgggcgggtgagtcacccacacaaaggaaaaggggcctttcc gtcctcagccgtcgcttcatgtgactccacggagtaccgggcgccgtccaggcacctcgattagttctcgagcttttggagtacgtcgtctttaggttgggggagggggttttatgcgatggagtttccccacactgagtgggtggagactgaagttaggccagcttggcacttgatgtaattctccttt ggaatttgccctttttgagtttggatcttggttcattctcaagcctcagacagtggttcaaagttttttttcttccatttcaggtgtcgtgaaccggttctagagcgctgccaccatgGCCaaaaagcctgaactcaccgcgacgtctgtcgagaagtttctgatcgaaaagttcgacagcgtGtccgacc tgatgcagctctcggagggcgaagaatctcgtgctttcagcttcgatgtaggagggcgtggatatgtcctgcgggtaaatagctgcgccgatggtttctacaaagatcgttatgtttatcggcactttgcatcggccgcgctcccgattccggaagtgcttgacattggggagttcagcgagagcct gacctattgcatctcccgccgtgcacagggtgtcacgttgcaagacctgcctgaaaccgaactgcccgctgttctgcagccggtcgcggaggccaatggatgcgatcgctgcggccgatcttagccagacgagcgggttcggcccattcggaccgcaaggaatcggtcaatacactacatggc gtgatttcatatgcgcgattgctgatccccatgtgtatcactggcaaactgtgatggacgacaccgtcagtgcgtccgtcgcgcaggctctcgatgagctgatgctttgggccgaggactgcccgaagtccggcacctcgtgcacgcggatttcggctccaacaatgtcctgacggacaatggc cgcataacagcggtcattgactggagcgaggcgatgttcggggattcccaatacgaggtcgccaacatcttcttctggaggccgtggttggcttgtatggagcagcagacgcgctacttcgagcggaggcatccggagcttgcaggatcgccgcggctccgggcgtatatgctccgcattggt cttgaccaactctatcagagcttggttgacggcaatttcgatgatgcagcttgggcgcagggtcgatgcgacgcaatcgtccgatccggagccgggactgtcgggcgtacacaaatcgcccgcagaagcgcggccgtctggaccgatggctgtgtagaagtactcgccgatagtggaaaccg acgccccagcactcgtccgagggcaaaggaaggatccggcgcaacaaacttctctctgctgaaacaagccggagatgtcgaagagaatcctggaccgATGTCAAAGGCTATGTATGCAAGAACGATATGCGAAGAAGAAGATG CAGGAGAACACTAAGATAGACACTCTCACCGATGAGCAGCATGACGCTCTCGCCCAGCTTTGCGCGTTTAGACATAAGTTTCATTCTAACAAAGATTCCCTGTTTCTTTCCGAAT CAGCGTTCAGTGGGGAGTTTAGTTTCGAGATGCAGTCTGACGAGAACTCCAAGTTGAGGGAAGTAGGACTCCCCACAATCGAATGGTCCTTTTACGACAATTCTCACATACCCGA CGACAGCTTTAGAGAGTGGTTCAATTTTGCTAACTATTCTGAGCTTTCAGAAACAATACAAGAGCAGGGTCTTGAACTCGACCTGGATGATGACGAAACCTACGAGCTGGTCTAT GATGAGCTTTATACAGAGGCGATGGGAGAATACGAGGAATTGAACCAGGATATAGAGAAATACCTTCGCAGAATAGATGAGGAACATGGCACGCAATACTGTCCAACTGGTTT TGCGCGGCTGAGATAAacgcgttaagtcgacaatcaacctctggattacaaaatttgtgaaagattgactggtattcttaactatgttgctccttttacgctatgtggatacgctgctttaatgcctttgtatcatgctattgcttcccgtatggctttcattttctcctccttgtataaatcctggtt gctgtctctttatgaggagttgtggcccgttgtcaggcaacgtggcgtggtgtgcactgtgtttgctgacgcaacccccactggttggggcattgccaccacctgtcagctcctttccgggactttcgctttcccctccctattgccacggcggaactcatcgccgcctgccttgcccgctgctggac aggggctcggctgttgggcactgacaattccgtggtgttgtcggggaaatcatcgtcctttccttggctgctcgcctgtgttgccacctggattctgcgcgggacgtccttctgctacgtcccttcggccctcaatccagcggaccttccttcccgcggcctgctgccggctctgcggcctcttccgc gtcttcgccttcgccctcagacgagtcggatctccctttgggccgcctccccgcgtcgactttaagaccaatgacttacaaggcagctgtagatcttagccactttttaaaagaaaaggggggactggaagggctaattcactcccaacgaagacaagatctgcttttttgcttgtactggtctctctg gttagaccagatctgagcctgggagctctctggctaactagggaacccactgcttaagcctcaataaagcttgccttgagtgcttcaagtagtgtgtgcccgtctgttgtgtgactctggtaactagagatccctcagacccttttagtcagtgtggaaaatctctagcagggcccgtttaaacccgctg atcagcctcgactgtgccttctagttgccagccatctgttgtttgcccctcccccgtgccttccttgaccctggaaggtgccactcccactgtccttttcctataaaaatgaggaaattgcatcgcattgtctgagtaggtgtcattctattctggggggtggggcaggacagcaagggggagg attgggaagacaatagcaggcatgctggggatgcggtgggctctatggcttctgaggcggaaagaaccagccattaatgaaaagaaccagccattaatgaatcggccaacgcgcggggagaggcggtttgcgtattgggcgctcttccgcttcctcgctcactgactcgctgcgctcggtcgtt cggctgcggcgagcggtatcagctcactcaaaggcggtaatacggttatccacagaatcaggggataacgcaggaaagaacatgtgagcaaaaggccagcaaaaggccaggaaccgtaaaaggccgcgttgctggcgttttccataggctccgcccccctgacgagcatcacaaaaat cgacgctcaagtcagaggtggcgaaacccgacaggactataaagataccaggcgtttccccctggaagctccctcgtgcgctctcctgttccgaccctgccgcttaccggatacctgtccgcctttctcccttcgggaagcgtggcgctttctcatagctcacgctgtaggtatctcagttcggtgt aggtcgttcgctccaagctgggctgtgtgcacgaaccccccgttcagcccgaccgctgcgccttatccggtaactatcgtcttgagtccaacccggtaagacacgacttatcgccactggcagcagccactggtaacaggattagcagagcgaggtatgtaggcggtgctacagagttcttgaa gtggtggcctaactacggctacactagaagaacagtatttggtatctgcgctctgctgaagccagttaccttcggaaaaagagttggtagctcttgatccggcaaacaaaccaccgctggtagcggtggtttttttgtttgcaagcagcagattacgcgcagaaaaaaaggatctcaagaagatcctt 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Table S4. Lentiviral vectors for gene editing experiments. Each gene of interest (GOI) was cloned into a lentiviral vector for stable transduction of HEK283T cells and assessment of gene editing inhibition.

Data S1. (separate file) Compiled STSS results. Each line represents an instance of self-targeting predicted by STSS. Information about each instance is labeled within the file. Cells marked with green were manually edited.