Supplementary Information for

NgAgo possesses guided DNA nicking activity

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SUPPLEMENTAL NOTES

NgAgo has some nonspecific DNA cleavage activity

We hypothesized that NgAgo generates random guides in the host via DNA chopping[1], which co-purifies with NgAgo leading to apparent guide-independent activity *in vitro*. While we were able to confirm the presence of these random copurified guides (Supplementary Fig. 3c), we were unable to displace them with incubation at high temperature (55 °C) and reload with our target guides (reloading protocol). Subsequent testing had similar guide-independent cleavage activity with no evidence of increased linearized plasmid (Supplementary Fig. 3d). As refolded NgAgo had no cleavage activity, we used soluble NgAgo to study its function *in vitro* unless otherwise stated.

Previous studies have demonstrated that TtAgo can obtain random guides from the expression plasmid DNA via DNA chopping[1]. Thus, the observed guide-independent cleavage may indeed be guide-dependent as a result of chopping and subsequent guide loading with homologous DNA, which cannot be easily displaced as demonstrated in Supplementary Fig 3d. To examine this hypothesis, we completed the *in vitro* cleavage assay with a 'related' plasmid, pNCS-mNeonGreen (Supplementary Fig. 8a), and an 'unrelated' plasmid, p15-KanR (Supplementary Fig.8c). The unrelated plasmid, p15-KanR, shares no DNA homology with the NgAgo expression plasmid while the related plasmid, pNCS-mNeonGreen, has the same ampicillin resistance gene. NgAgo cleaved both related and unrelated plasmids independent of guide (Supplementary Fig. 8b and 8d), suggesting that the guide-independent cleavage activity of our purified NgAgo does not rely on pre-loaded DNA. These results confirmed that NgAgo has guide-independent cleavage activity *in vitro*, sharing similar properties with bacterial TtAgo[1] and archaeal MjAgo[2].

Persistence of guide for in vivo *assays*

As our guide sequences are 5'P ssDNA, they are exogenously supplied via transformation. Guides are supplied before NgAgo induction for all *in vivo* assays in order to minimize DNA chopping due to guide-free NgAgo. However, guide molecules cannot replicate and are depleted through cell division and degradation. To assay their persistence and determine whether or not they persist long enough to encounter induced NgAgo, we transformed D4PA-labelled red ssDNA (Supplementary Table 6) into cells expressing BFP. After 4 h when sufficient BFP has accumulated to be visible, labelled ssDNA still persists and is theoretically available for NgAgo binding (Supplementary Fig. 12).

NgAgo may represent a new class of mesophilic pAgos

To our knowledge, NgAgo is the first studied pAgo with a fused repA domain. This domain is essential for cleavage as evidenced in our cell-free *in vitro* studies and recombination assays. Interestingly, all repA domain-containing pAgos are from halophilic Archaea mesophiles, suggesting that the repA domain may have fused to pAgos in the last common ancestor of all halophiles. Moreover, single-stranded binding (SSB)

proteins have been demonstrated to participate in and enhance pAgo activity[3] suggesting that the repA domain at the N-terminus of NgAgo may be involved in the cleaving process without recruitment of endogenous SSB proteins. Further research, however, is needed to clarify the function of this repA domain.

SUPPLEMENTAL METHODS

In vitro activity assay

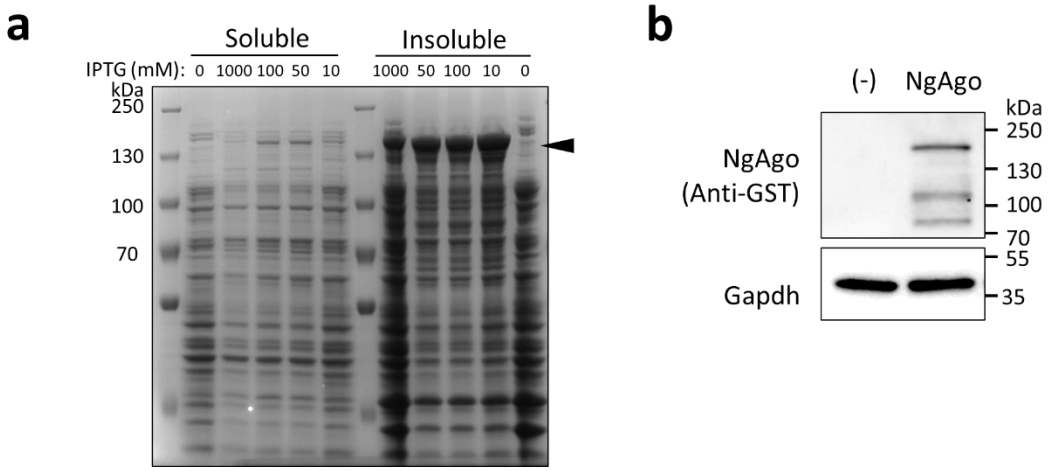
For the reloading protocol, 5 µg purified NgAgo was mixed with 1 µg total of phosphorylated single-stranded DNA (P-ssDNA) targeting mNeonGreen (Supplementary Table 6) and incubated at 55 °C for an hour. 200-300 ng of substrate plasmid DNA (pNCS-mNeonGreen) was then added to the sample. The final volume of the reaction was 50 µl (working concentration: 20 mM Tris-Cl, 300 mM KCl, 500 µM $MgCl_2$, and 2 mM DTT). The sample was then incubated at 37 °C for three hours. 0.8 units of Proteinase K (NEB, Ipswich, MA. Cat. No: P8107S) were added to the sample to digest the protein for 5 minutes at 37 °C. The nucleic acids were then isolated with the DNA Clean & Concentrator™-5 kit (Zymo Research, Irvine, CA. Cat. No: D4003T) according to manufacturer instructions and mixed with 6X loading dye containing SDS (Thermo Fisher S, Waltham, MA. Cat. No: R1151) before gel electrophoresis. The gel containing Sybrsafe (Thermo Fisher S, Waltham, MA. Cat. No: S33102) was visualized under a blue light (Azure Biosystems, Dublin, CA. Azure c400).

For our standard protocol, we incubated the same amount of guides and proteins at 37 °C for 30 minutes, and added the same amount of plasmid DNA (p15-KanR or pBSI-SceI(E/H)[4]) with 50 ul final volume (working concentration: 20mM Tris-Cl, 300mM NaCl, 250 uM $MgCl_2$, and 2mM DTT). The samples were incubated at 37 °C for an hour before Proteinase K treatment. The rest of the procedure is the same as the reloading protocol.

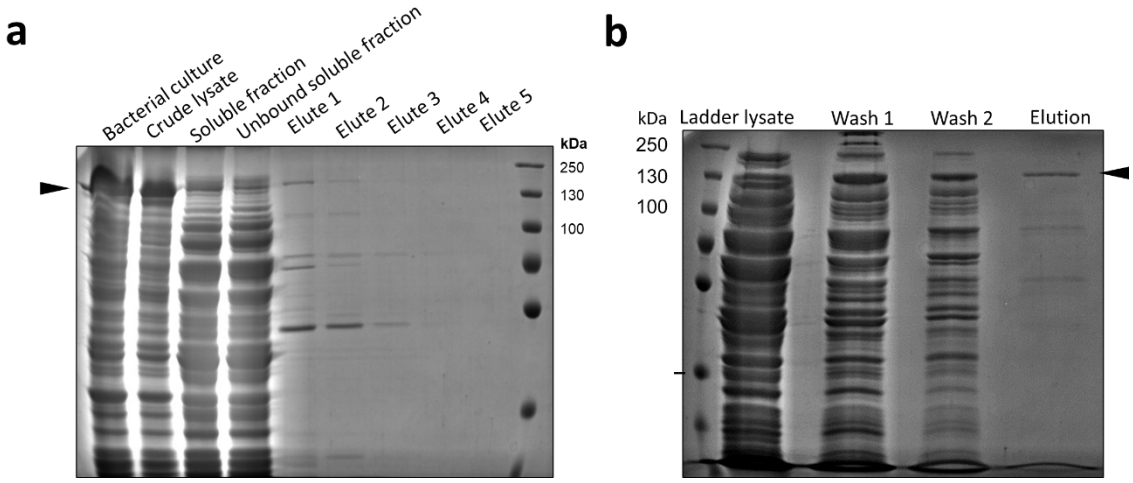
Electrophoretic mobility shift assay (EMSA)

5 µg of purified N-del and repA were incubated with 1 µg of mNeonGreen ssDNA guide in 50ul in buffer (working concentration: 20 mM Tris-Cl, 300 mM KCl, 500 µM $MgCl_2$, and 2 mM DTT) at 37 °C for an hour and treated with 0.8 units proteinase K for 5 minutes if needed before running with 20% TBE gel with 0.5X TBE buffer. Gels were stained with Sybr Gold (Thermo Fisher Scientific, Waltham, MA. Cat. No: S11494) before visualizing under a green fluorescent channel (Azure Biosystems, Dublin, CA. Azure c400). Positional marker 10/60 ladder (Coralville, IA. Cat. No: 51-05-15-01) was used in the EMSA assay.

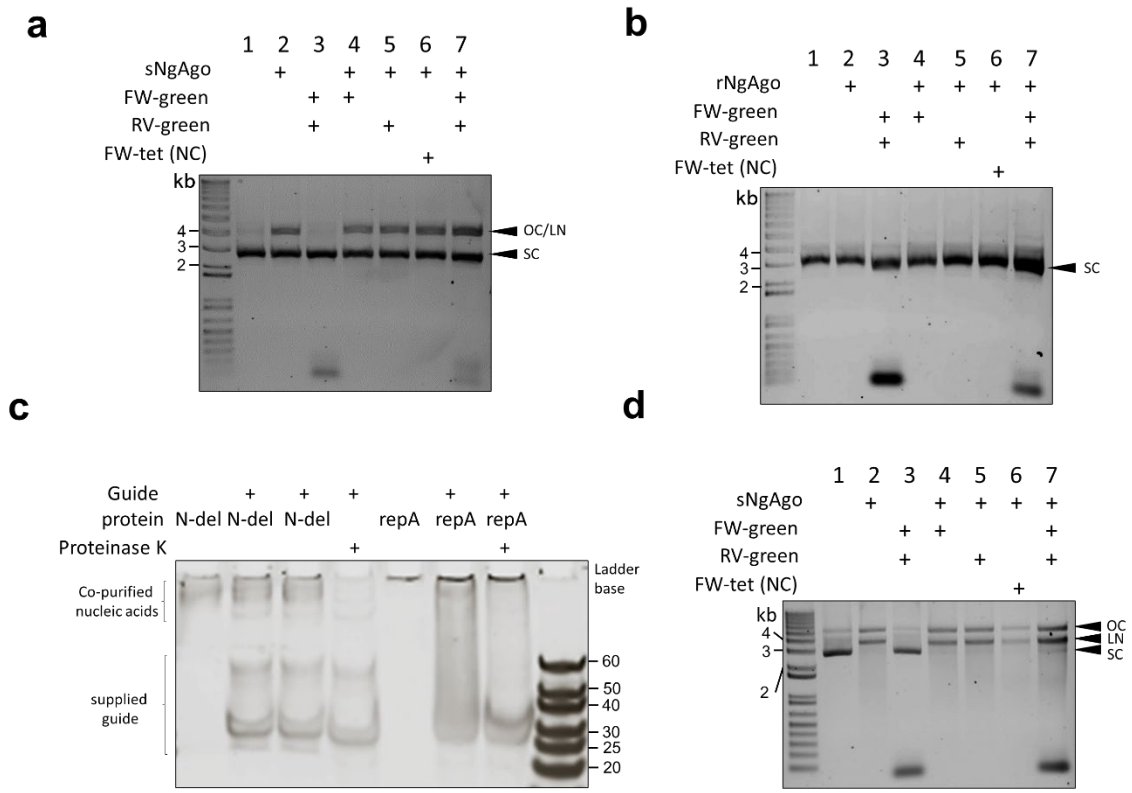
SUPPLEMENTARY DATA



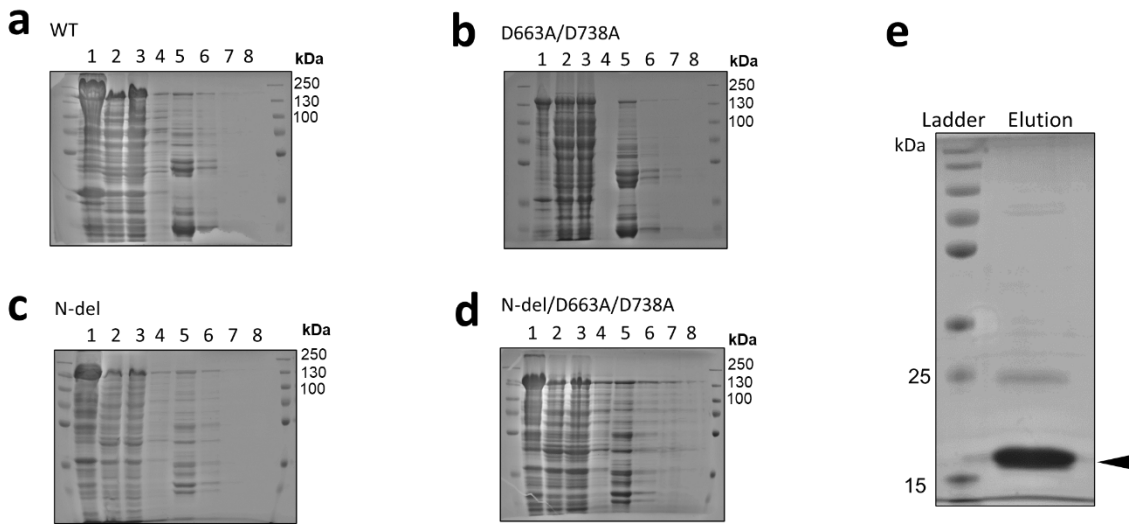
Supplementary Figure 1 | Optimization of soluble NgAgo protein expression. a, Different IPTG concentrations (1000 mM, 100 mM, 50 mM, and 10 mM) were used to induce GST-NgAgo expression in BL21 (DE3). Soluble and insoluble protein fractions were analyzed by SDS-PAGE to determine the optimal conditions for soluble NgAgo expression. **b**, Soluble GST-NgAgo expression with 100mM IPTG was probed with anti-GST antibody and a Gapdh internal control.



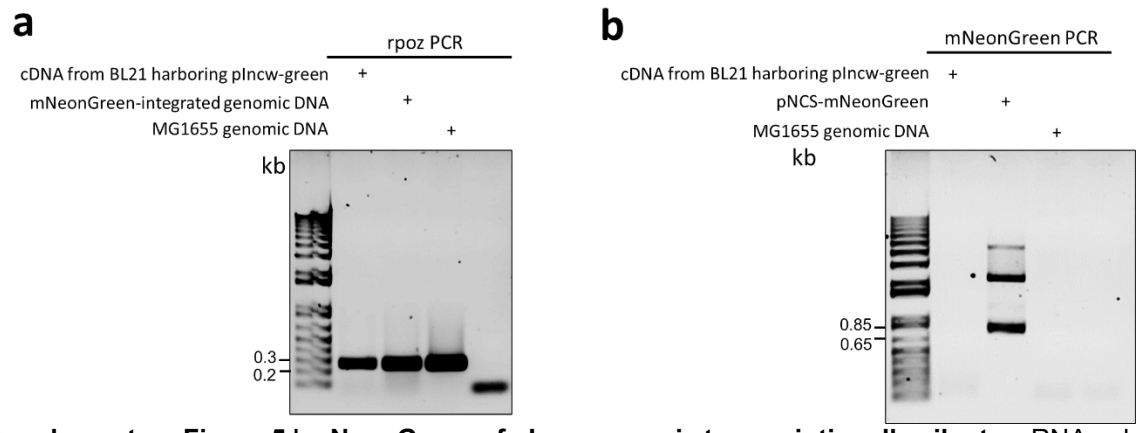
Supplementary Figure 2 | SDS-PAGE analysis of His-tag purified NgAgo variants. a, SDS-PAGE analysis of purified WT NgAgo from soluble fraction (sNgAgo). Elute 1 was used for *in vitro* assay. b, SDS-PAGE analysis of purified WT NgAgo from insoluble fraction after refolding (rNgAgo). Elution fraction was used for *in vitro* assay.



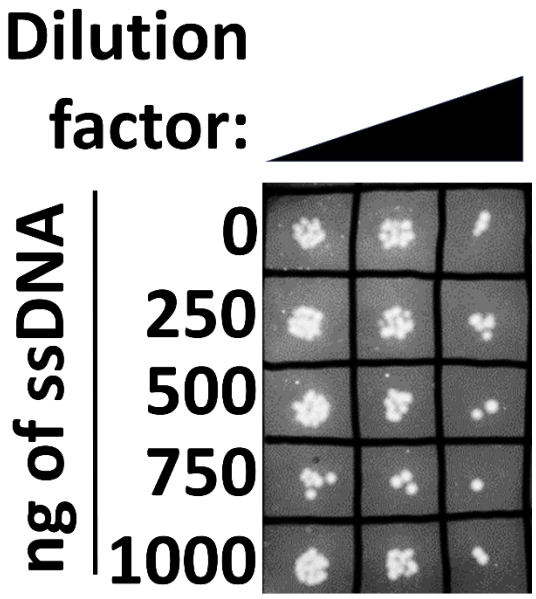
Supplementary Figure 3 | Soluble NgAgo variants nick and cut plasmids DNA in vitro. a, Soluble NgAgo (sNgAgo) nicks an cuts plasmids DNA regardless the presence of guide DNA. **b**, Refolded NgAgo, rNgAgo, has no effect on plasmids DNA regardless the presence of guide DNA. **c**, Electrophoretic mobility shift assay (EMSA) of N-del and repA domain with guides. N-del does not show band shifting while repA treatment shifts the bands. **d**, Soluble NgAgo (sNgAgo) nicks and cuts the plasmids DNA regardless the presence of guide DNA with Han's guide-reloading protocol. OC: open circular; LN: linear; SC: supercoiled.



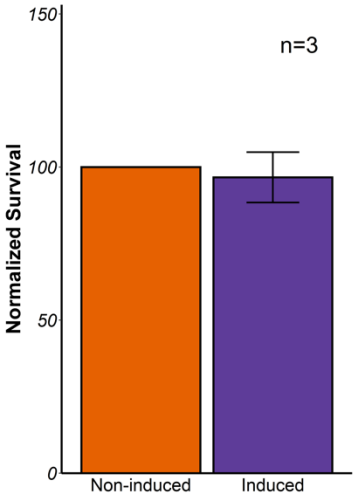
Supplementary Figure 4 | SDS-PAGE analysis of GST-tag purified soluble NgAgo variants. a, SDS-PAGE analysis of GST-tag purified WT NgAgo. **b**, SDS-PAGE analysis of GST-tag purified D663A/D738A. **c**, SDS-PAGE analysis of GST-tag purified N-del. **d**, SDS-PAGE analysis of GST-tag purified N-del/D663A/D738A. 1: whole cell lysate; 2: soluble fraction; 3: unbound soluble fraction; 4 washed fraction; 5-8: eluted fraction 1-4. **e**. His-tagged soluble repA



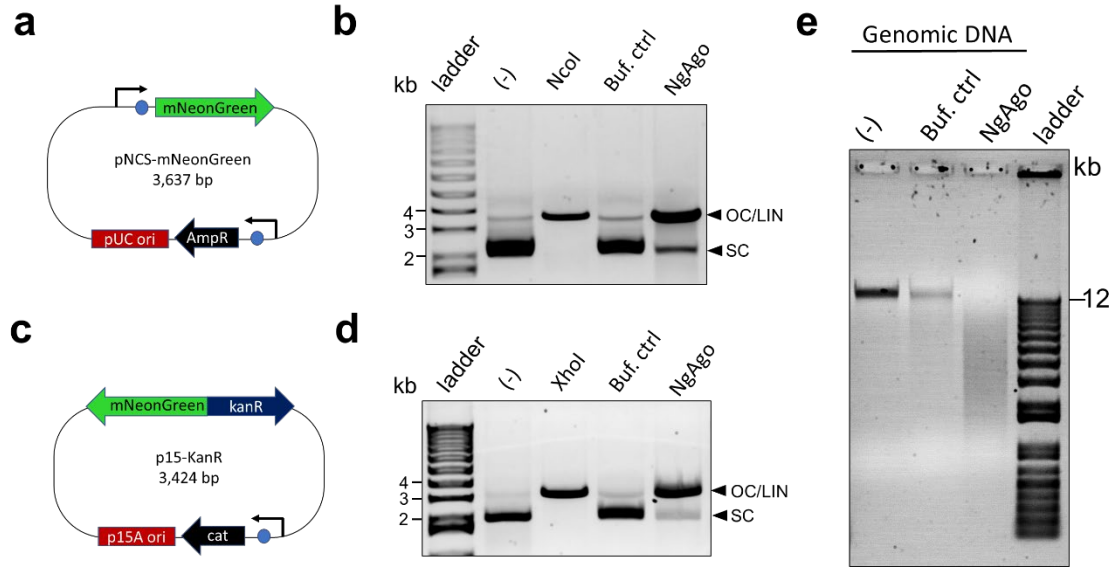
Supplementary Figure 5 | mNeonGreen of pIncw-green is transcriptionally silent. a, RNA polymerase subunit, *rpoz*, was amplified with cDNA from BL21 harboring pIncw-mNeonGreen, mNeonGreen-integrated genomic DNA, and WT genomic DNA. **b**, mNeonGreen was amplified with cDNA from BL21 harboring pIncw-mNeonGreen, pNCS-mNeonGreen plasmid DNA, and WT genomic DNA.



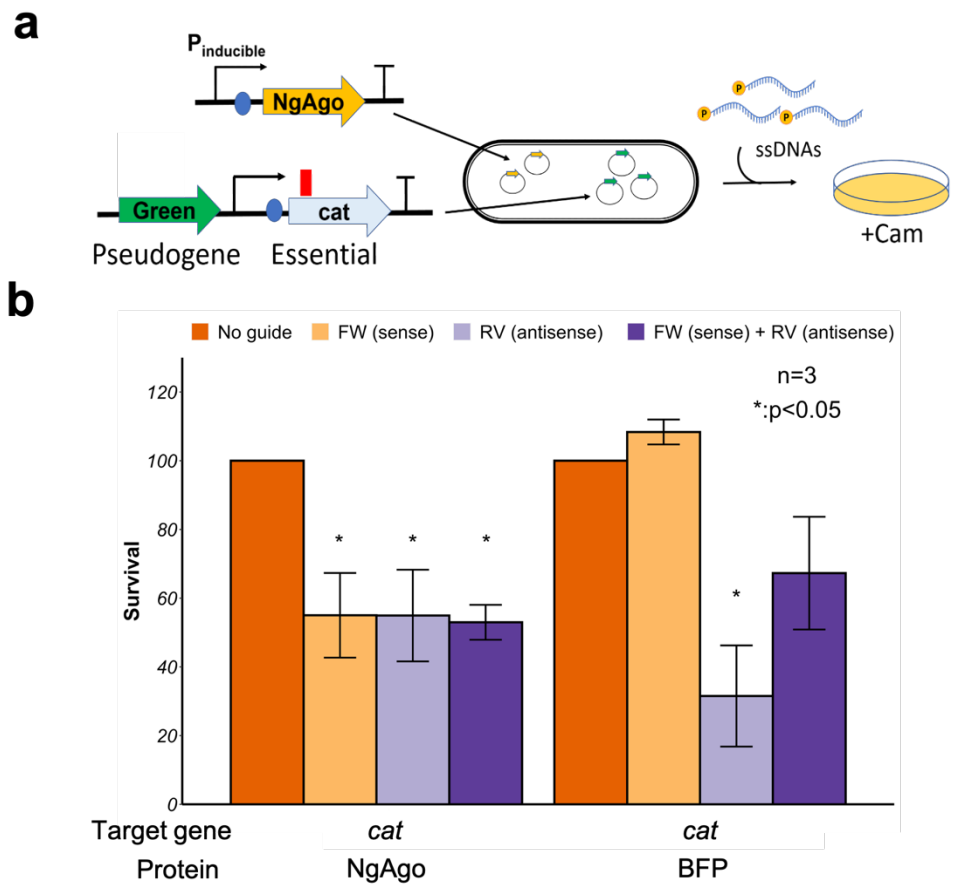
Supplementary Figure 6 | ssDNA guides are non-toxic. Different amounts of ssDNAs were transformed into BL21 and spot plated at varying dilutions (1000x, 2000x and 5000x). Ten microliter of each diluted sample was plated on LB.



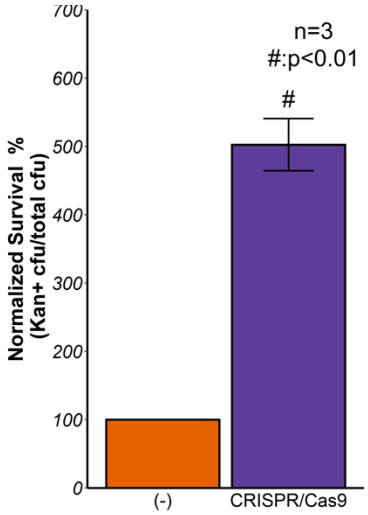
Supplementary Figure 7 | Off-target activity assessment with the two-plasmid system. The host strain harboring NgAgo expression plasmid and target plasmid are plated in the presence or absence of IPTG inducer and the number of colony forming units relative to the non-induced control was calculated as 'survival' . No significant change of survival is observed. Error bars are the standard errors generated from three replicates. Statistically significant results are indicated with * (p-value< 0.05, paired t-test).



Supplementary Figure 8 | Soluble wildtype NgAgo nicks and cuts DNA in the absence of guide DNA. **a**, Plasmid map of the related plasmid, pNCS-mNeonGreen. This plasmid shares the same ampicillin antibiotic resistant gene with the NgAgo expression plasmid. **b**, Agarose gel analysis of wildtype NgAgo-treated pNCS-mNeonGreen, showing the degraded DNA product. **c**, Plasmid map of the unrelated plasmid, p15-KanR. This plasmid does not share genetic elements with the NgAgo expression plasmid. **d**, Agarose gel analysis of wildtype NgAgo-treated p15-KanR, showing the degraded DNA product. **e**, Agarose gel analysis of wildtype NgAgo-treated MG1655 genomic DNA, showing the degraded DNA product.



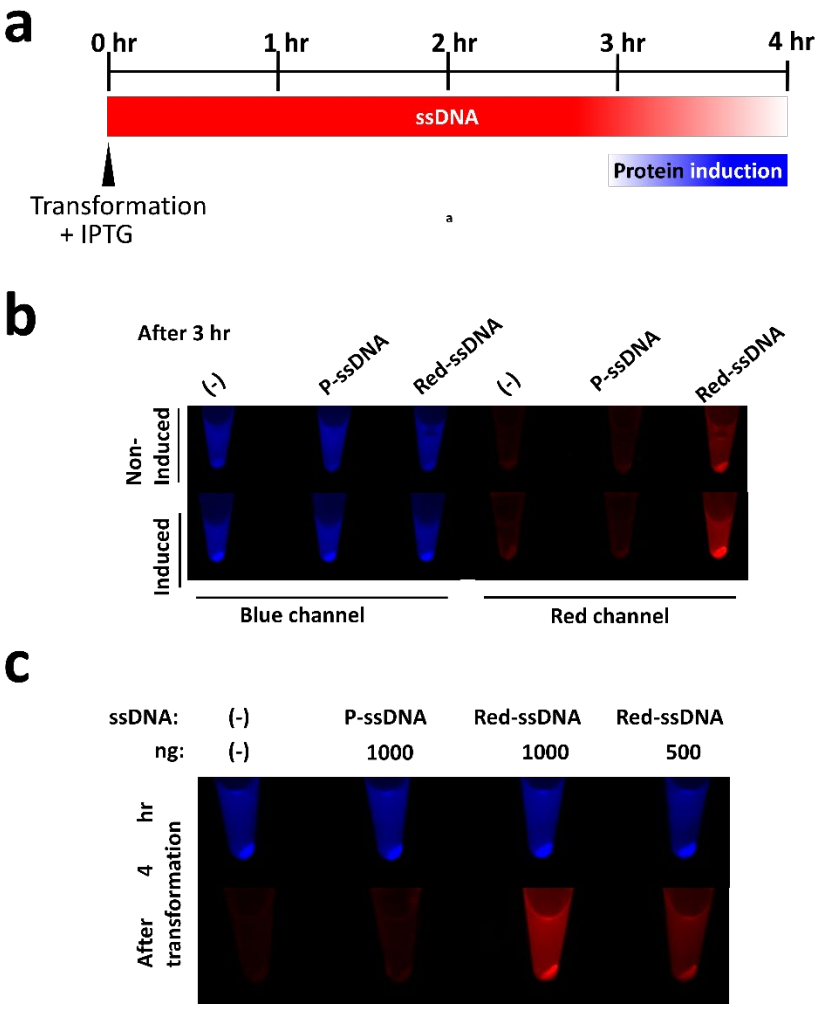
Supplementary Figure 9 | NgAgo can be programmed to target DNA in *E. coli*. a, Workflow of testing NgAgo function in *E. coli*. Two plasmids system used to test the function of NgAgo. One plasmid harbors NgAgo driven by T7 inducible promoter while the other low-copy plasmid serves as the target of NgAgo, including an untranscribed pseudogene, mNeonGreen. **b**. Targeting of the essential gene with any guide reduces survival suggesting a guide-induced effect. Using a BFP protein control reduces survival only in the presence of reverse guide, which is consistent with enhanced degradation of DNA-RNA hybrid duplexes.



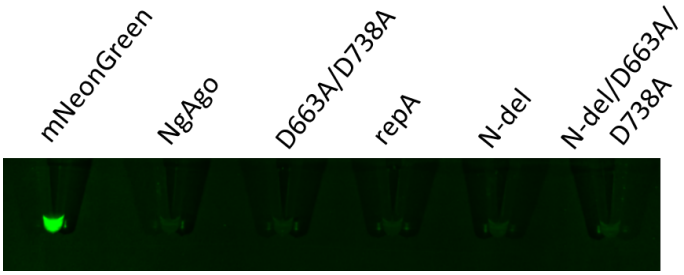
Supplementary Figure 10 | CRISPR/Cas9 enhances homologous recombination in *E.coli*. Induction of CRISPR/Cas9 enhance homologous recombination for 400%. Kanamycin positive colonies are normalized by total colony forming units. Error bars are the standard errors generated from three replicates. Statistically significant results are indicated with * (p-value< 0.05, paired t-test).

Conditions	N=1 gene-editing assay	N=2 gene-editing assay	N=3 gene-editing assay
BFP (-)	2 colonies	2 colonies	1 colony
NgAgo (-)	2 colonies	2 colonies	1 colony
NgAgo FW	2 colonies	2 colonies	1 colony
NgAgo RV	2 colonies	2 colonies	1 colony
NgAgo FW + RV	2 colonies	2 colonies	1 colony

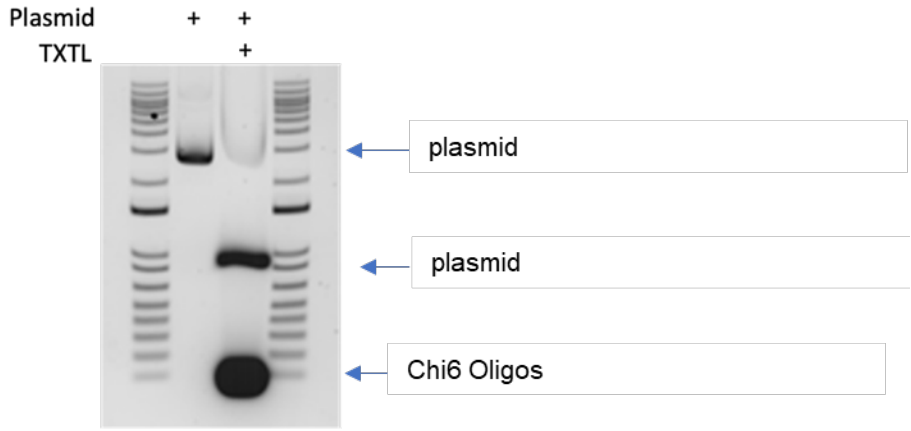
Supplementary Figure 11 | NgAgo-mediated gene-editing hosts have expected sequence. Representative sequencing of KanR$^+$ colonies confirm that observed KanR phenotypes are the result of recombination that inserts the J23100 promoter and RBS (B0034). Five colonies across three independent experiments were sequenced via Sanger sequencing.



Supplementary Figure 12 | Persistence testing of transformed guide DNA. a, Timeline of the experimental procedure. BL21 (DE3) harboring inducible BFP expression plasmid was made electrocompetent and transformed with D4PA-labelled Red-ssDNA. After transformation, cells were resuspended in SOC in the presence of 0.1mM IPTG. **b**, BFP expression is detected after 3hr transformation. **c**, Red-ssDNA is still present after 4 hours transformation.



Supplementary Figure 13 | Production of mNeonGreen and NgAgo variants. Cell-free system-produced mNeonGreen and NgAgo variants, including D663A/D738A, repA, N-del, and N-del/D663A/D738A, are visualized for green fluorescence to confirm successful expression in the mNeonGreen positive control.



Supplementary Figure 14 | TXTL reaction mix accelerates plasmid migration. TXTL Cell-free expression mix induces significant shift in plasmid mobility.

Supplementary Table 1. Top 10 hits of NgAgo in Phyre 2 search.

Structure ID	Structure source	Protein	Probability	Identity with NgAgo
5GUH	PDB	Silkworm PIWI-clade Argonaute Siwi	100%	15%
4EI3	PDB	Homo sapiens Argonaute2	100%	18%
3HO1	PDB	Thermus thermophilus Argonaute N546 mutant	100%	19%
4F1N	PDB	Kluyveromyces polysporus Argonaute	100%	14%
3DLB	PDB	Thermus thermophilus Argonaute	100%	19%
2F8S	PDB	Aquifex aeolicus Argonaute	100%	16%
5G5T	PDB	Methanocaldcoccus janaschii Argonaute	100%	15%
1U04	PDB	Pyrococcus furiosus Argonaute	100%	12%
5AWH	PDB	Rhodobacter sphaeroides Argonaute	100%	14%
5THE	PDB	Vanderwaltozyma polyspora Argonaute	100%	17%

Supplementary Table 2. Top 10 hits of NgAgo in HHpred search.

Structure ID	Protein	Probability	E-value	Identity to NgAgo
5GUH	silkworm PIWI-clade Argonaute Siwi	100%	1e-86	15%
4Z4D	Homo sapiens Argonaute2	100%	3.4e-77	16%
4F1N	Kluyveromyces polysporus Argonaute	100%	3e-77	17%
4NCB	Thermus thermophilus Argonaute	100%	2.5e-68	17%
5G5S	Methanocaldcoccus janaschii Argonaute	100%	2.6e-68	12%
1YVU	Aquifex aeolicus Argonaute	100%	3.9e-68	16%
1U04	Pyrococcus furiosus Argonaute	100%	1.2e-66	14%
5I4A	Marinitoga piezophila Argonaute	100%	1.6e-65	14%
6D92	Rhodobacter sphaeroides Argonaute	100%	1.9e-55	14%
5THE	Vanderwaltozyma polyspora Argonaute	100%	1.7e-56	17%

Supplementary Table 3. Top 10 hits of repA domain of NgAgo in Phyre 2 search. A non-OB fold domain match was eliminated in this table.

Structure ID	Source	Protein	Probability	Identity to NgAgo
2KEN	PDB	Methanosarcina mazei OB domain of MM0293	95.8%	12%
3DM3	PDB	Methanocaldococcus jannaschii repA	95.2%	23%
2K50	PDB	Methanobacterium thermoautotrophicum repA-related protein	94.6%	12%
1O7I	PDB	Sulfolobus solfataricus ssb	94.4%	16%
1FGU	PDB	Homo sapiens REPA	92.3%	15%
d1jmca2	SCOP	Homo sapiens RPA70	92%	15%
4OWX	PDB	Homo sapiens SOSS complex subunit B1	91.8%	15%
3E0E	PDB	Methanococcus maripaludis repA	78.2%	20%
2K75	PDB	Thermoplasma acidophilum OB domain of Ta0387	67.2%	14%
d1wjja_	SCOP	Arabidopsis thaliana hypothetical protein F20O9.120	66.0%	16%

Supplementary Table 4. Top 10 hits of repA domain of NgAgo in HHpred search. A non-OB fold domain match was eliminated in this table.

Ranking	Structure ID	Protein	Probability	E-value
27	4OWT	Homo sapiens SOSS1 subunit B1	94.68%	0.06
28	1WJJ	Arabidopsis thaliana hypothetical protein F20O9.120	94.65%	0.086
29	1O7I	Sulfolobus solfataricus single-stranded DNA binding protein chain B	94.0%	0.28
30	2K50	Methanobacterium thermoautotrophicum repA	92.46%	0.036
31	3DM3	Methanocaldococcus jannaschii repA	91.96%	0.65
33	3E0E	Methanococcus maripaludis repA	88.18%	2.5
34	1YNX	Saccharomyces cerevisiae repA	87.6%	1.3
35	5D8F	Homo sapiens SOSS complex subunit B1	84.78%	6.7
36	1JMC	Homo sapiens RPA70	82.12%	4.7
37	4HIK	Schizosaccharomyces pombe Pot1pC	81.44%	5.1

Supplementary Table 5. DNA primers used in this study. Restriction enzyme recognition sites are underlined and indicated in the primer name.

Name	Sequences (5'>3')	Template	Used to construct
5' NcoI 3xG Ago	ATCACCATGGGTGG CGGTATGGTGCCAAA AAAGAAGAG	Nls-NgAgo-GK	pET-GST-Ago-His
3' XhoI Ago	ATCACTCGAGCTTAC TTACATATGGATCCC GG		
NdeI HIS-Ago 5	TATACATATGGGTCA CCATCATCATCACCA TTCATCGCATCACCA TCACCATCACGTGCC AAAAAAGAAGAG	Nls-NgAgo-GK	pET-His-Ago
XhoI rmNdeI Ago 3'	ATATCTCGAGTTACT TACTTACGTATGGAT CCCGG	Nls-NgAgo-GK	pET-His-Ago
XhoI STOP repA 3'	CTAACTCGAGTTACT CGACGGTCGTCTGG	Nls-NgAgo-GK	pET-His-repA
D663A 3'	CGGGGTAGCTCCGA GAGACCGCAATCCC AATGAACATATC	pET-His-Ago	NgAgo mutant
D663A 5'	GATATGTTCATTGGG ATTGCGGTCTCTCGG AGCTACCCCG	pET-His-Ago	
D738A 5'	CGACCCATATCGTCA TCCACCGTGCGGGC TTCATGAACGAAGAC CTCGAC	pET-His-Ago	NgAgo mutant
D738A 3'	GTCGAGGTCTTCGTT CATGAAGCCCGCAC GGTGGATGACGATAT GGGTCG	pET-His-Ago	
XbaI KanR 5'	ATGGTCTAGAATGGG ATCGGCCATTG	pTKIP-neo	kanR-mNeonGreen cassette integration
BamHI KanR 3'	ATTTGGATCCTTAGA AGAACTCGTCAAGAA GGC	pTKIP-neo	
XbaI tGreen 5'	CCATTCTAGACCATG GTAGATGGCTCCG	pNCS-mNeonGreen	
XhoI Green uni 3'	TGATCTCGAGAGAGA ATATAAAAAGCCAGA TTATTAATCCGGCTTT TTTATTATTTTTACTT GTACAGCTCGTCCAT GC	pNCS-mNeonGreen	
XbaI tKanR 5'	TAGCTCTAGAGAAAG AGGAGAAATACTAGA TGGGATCGGCCATT G	pTKIP-neo	donor plasmid p15-KanR-PtetRed
EcoRI tKanR 3'	ATATGAATTCGATAC TTTCTCGGCAGGAGC	pTKIP-neo	
XbaI J23100 tGreen 5'	TTTCTCTAGAGCTAG CACTGTACCTAGGAC TGAGCTAGCCGTCAA	pNCS-mNeonGreen	

	CCATGGGAAGCCAC ATC		
XhoI Green uni 3'	TGATCTCGAGAGAGA ATATAAAAAGCCAGA TTATTAATCCGGCTTT TTTATTATTTTTACTT GTACAGCTCGTCCAT GC	pNCS-mNeonGreen	
XhoI pTet 5'	ATCACTCGAGTCCCT ATCAGTGATAGAGAT TGACATCCCTATCAG TGATAGAGATACTGA GCACTCTAG	pTK-Red	
XhoI DT exo 3'	TGATCTCGAGAAAAA AAAACCCCGCCGAA GCGGGGTTTTTTTTT TCATCGCCATTGCTC C	pTK-Red	
PEP1	TTACTTGTACAGCTC GTCCATG	pNCS-mNeonGreen	-
PEP12	GAATTCGAAGCTTGA TCCG	pNCS-mNeonGreen	-
PEP2	AGCGGATCCTTATCG TCA	pNCS-mNeonGreen	-
PEP22	GATGGTGAGCAAGG GC	pNCS-mNeonGreen	-
Adapter P-BacF	P-GGATTATTCATACCG TCCCA 3' dideoxylated	-	-
BacF RV	TGGGACGGTATGAAT AATCC	-	-

Supplementary Table 6. DNA guides used in this study.

Name of 5' phosphorylated guide DNA	Sequences (5' to 3')	Target
FW p-tetA	P-GGATTGGCCTTATCATGCCAGTCT	*tetA*
RV p-tetA	P-AGACTGGCATGATAAGGCCAATCC	*tetA*
FW p-mNeonGreen	P-TTAACTACCGCTACACCTACGAGG	*mNeonGreen*
RV p-mNeonGreen	P-CCTCGTAGGTGTAGCGGTAGTTAA	*mNeonGreen*
FW p-arpB	P-ATACAGCAGCATGTCCCCTTAGTC	*arpB*
RV p-arpB	P-GACTAAGGGGACATGCTGCTGTAT	*arpB*
D4PA-labelled Red-ssDNA	D4PA-TTAACTACCGCTACACCTACGAGG	*N/A*

Supplementary Table 7. Nicked/cut sites of NgAgo variants using PEP1 and PEP12.

Wildtype NgAgo			
Sequence ID	**Sequences (5' to 3')**	**Number of read**	**Target Similarity**
1	…CTCCCTTACT•GTTAACTACCGCTACACCTACGAGG…	20507	100.00%
2	…AAGCCAATGG•CGGCTAACTATCTGAAGAACCAGCC…	9	16.67%
3	…CGGCTAACTA•TCTGAAGAACCAGCCGATGTACGTG…	397	25.00%
4	…TATTACGCCA•GATCCGGATATAGTTCCTCCTTTCA…	99	33.34%
D663A/D738A			
1	…AAGACCGAGC•TCAACTTCAAGGAGTGGCAAAAGGC…	577	25.00%
2	…AAGGCCTTTA•CCGATGTGATGGGCATGGACGAGCT…	209	29.17%
3	…AACTGTTGGG•AAGGGCGATCGGTGCGGGCCTCTTC…	276	25.00%
4	…TCCTTTCGGG•CTTTGTTAGCAGCCGGATCAAGCTT…	71	29.17%
repA			
1	…GCCTTTACCG•ATGTGATGGGCATGGACGAGCTGTA…	24618	37.50%
2	…TTCCCTGCTG•ACGGTCCTGTGATGACCAACTCGCT…	24618	16.67%
3	…GGAGCTCAAG•CACTCCAAGACCGAGCTCAACTTCA…	133	50.00%
4	…AGCAGCCAAC•TCAGCTTCCTTTCGGGCTTTGTTAG…	133	41.67%
5	…TTTCGGGCTT•TGTTAGCAGCCGGATCAAGCTTCGA…	96	37.50%
N-del			
1	…AACGACAAAA•CCATCATCAGTACCTTTAAGTGGAG…	7453	29.14%
2	…AAAGGCCTTT•ACCGATGTGATGGGCATGGACGAGC…	7453	33.34%
3	…GTGGCAAAA•GGCCTTTACCGATGTGATGGGCATG…	397	33.34%
4	…CCTTTACCGA•TGTGATGGGCATGGACGAGCTGTAC…	794	20.83%
5	…CAACTTCAAG•GAGTGGCAAAAGGCCTTTACCGATG…	39	25.00%
6	…CAAGGAGTGG•CAAAAGGCCTTTACCGATGTGATGG…	6	25.00%
7	…TGGCGGCTAA•CTATCTGAAGAACCAGCCGATGTAC…	5	29.14%
8	…TTACCGATGT•GATGGGCATGGACGAGCTGTACAAG…	11	25.00%
9	…GTTATGCTAG•TTATTGCTCAGCGGTGGCAGCAGCC…	7453	25.00%
10	…AGCTTCCTTT•CGGGCTTTGTTAGCAGCCGGATCAA…	397	29.14%
11	…CTTTCGGGCT•TTGTTAGCAGCCGGATCAAGCTTCG…	39	20.83%
12	…TTTCGGGCTT•TGTTAGCAGCCGGATCAAGCTTCGA…	9	37.50%

Guide targeting region is underlined while cut/nicked sites are labelled with "•".

Supplementary Table 8. Nicked/cut sites of NgAgo variants using PEP2 and PEP22.

Wildtype NgAgo			
Sequence ID	**Sequences (5' to 3')**	**Number of read**	**Target Similarity**
1	…TCTGTACGAC•GATGACGATAAGGATCCGCTCGAGA…	316	41.67%
2	…GATCTGTACG•ACGATGACGATAAGGATCCGCTCGA…	316	29.17%
3	…GGTGCCCTGA•CCCACCATGTCAAAGTCCACACCGT …	316	25.00%
4	…GAGAGGCCAT•GTTATCCTCCTCGCCCTTGCTCACC…	316	37.50%

5	…CATCCCGTCA•GGGTAGGGCAGGTACTGATGGAAGC…	316	37.50%
D663A/D738A			
1	…GCGCCCAATA•CGCAAACCGCCTCTCCCCGCGCGTT…	20345	25.00%
2	…GGTCGGGATC•TGTACGACGATGACGATAAGGATCC…	20345	33.33%
3	…GGGAGAGAGG•CCATGTTATCCTCCTCGCCCTTGCT…	20345	29.17%
4	…ATGTTATCCT•CCTCGCCCTTGCTCACCATCTCGAG…	20345	33.33%
repA			
1	…GGGTCGGGAT•CTGTACGACGATGACGATAAGGATC…	11873	41.67%
2	…CCGTTGATGG•AGCCAAAGATGTGTAACTCATGTGT…	11873	29.17%
3	…ATGTTATCCT•CCTCGCCCTTGCTCACCATCTCGAG…	11873	33.33%
4	…GGGAGAGAGG•CCATGTTATCCTCCTCGCCCTTGCT…	11873	29.17%
N-del			
1	…GGATCTGTAC•GACGATGACGATAAGGATCCGCTCG…	4963	29.17%
2	…CTGTACGACG•ATGACGATAAGGATCCGCTCGAGAT…	1247	33.33%
3	…GGTCGGGATC•TGTACGACGATGACGATAAGGATCC…	1462	33.33%
4	…GGGTCGGGAT•CTGTACGACGATGACGATAAGGATC…	1247	41.67%
5	…GATCTGTACG•ACGATGACGATAAGGATCCGCTCGA…	1247	29.17%
6	…TAGCATGACT•GGTGGACAGCAAATGGGTCGGGATC…	230	20.83%
7	…GGGATCTGTA•CGACGATGACGATAAGGATCCGCTC…	230	33.33%
8	…ATGGGTCGGG•ATCTGTACGACGATGACGATAAGGA…	230	29.17%
9	…AATGGGTCGG•GATCTGTACGACGATGACGATAAGG…	230	45.83%
10	…GGGTCGGGAT•CTGTACGACGATGACGATAAGGATC…	230	41.67%
11	…CCGCGAAATT•AATACGACTCACTATAGGGAGACCA…	98	37.50%
12	…TCTGTACGAC•GATGACGATAAGGATCCGCTCGAGA…	247	41.67%
13	…CGGGATCTGT•ACGACGATGACGATAAGGATCCGCT…	247	29.17%
14	… AAATGGGTCG•GGATCTGTACGACGATGACGATAAG…	91	20.83%
15	…ATCATCATCA•TGGTATGGCTAGCATGACTGGTGGA…	169	29.17%
16	…TGTGTCGCTG•GGAGAGAGGCCATGTTATCCTCCTC …	1477	12.50%
17	…CCATGTTATC•CTCCTCGCCCTTGCTCACCATCTCG…	1984	29.17%
18	…AGGCCATGTT•ATCCTCCTCGCCCTTGCTCACCATC…	1247	33.33%
19	…CTGGGAGAGA•GGCCATGTTATCCTCCTCGCCCTTG…	1247	25.00%
20	…GGAGAGAGGC•CATGTTATCCTCCTCGCCCTTGCTC…	1477	33.33%
21	…GCCATGTTAT•CCTCCTCGCCCTTGCTCACCATCTC…	1247	25.00%
22	…TGTTATCCTC•CTCGCCCTTGCTCACCATCTCGAGC…	1247	33.33%
23	…CATGTTATCC•TCCTCGCCCTTGCTCACCATCTCGA…	2494	37.50%
24	…GTTATCCTCC•TCGCCCTTGCTCACCATCTCGAGCG…	328	37.50%
25	…ATGTGTAACT•CATGTGTCGCTGGGAGAGAGGCCAT…	98	33.33%
26	…ATGTTATCCT•CCTCGCCCTTGCTCACCATCTCGAG…	247	33.33%
27	…ATGTGTCGCT•GGGAGAGAGGCCATGTTATCCTCCT…	247	25.00%
28	…GGGAGAGAGG•CCATGTTATCCTCCTCGCCCTTGCT…	786	29.17%
29	…GAGAGGCCAT•GTTATCCTCCTCGCCCTTGCTCACC…	169	37.50%

Guide targeting region is underlined while cut/nicked sites are labelled with "•".

WORK CITED

1. Swarts, D. C. *et al.* Autonomous Generation and Loading of DNA Guides by Bacterial Argonaute. *Molecular Cell* **65**, 985-998.e6 (2017).
2. Zander, A. *et al.* Guide-independent DNA cleavage by archaeal Argonaute from Methanocaldococcus jannaschii. *Nature Microbiology* **2**, 17034 (2017).
3. Hunt, E. A., Evans Jr, T. C. & Tanner, N. A. Single-stranded binding proteins and helicase enhance the activity of prokaryotic argonautes in vitro. *PloS One* **13**, e0203073 (2018).
4. Niu, Y., Tenney, K., Li, H. & Gimble, F. S. Engineering variants of the I-SceI homing endonuclease with strand-specific and site-specific DNA-nicking activity. *Journal of Molecular Biology* **382**, 188–202 (2008).