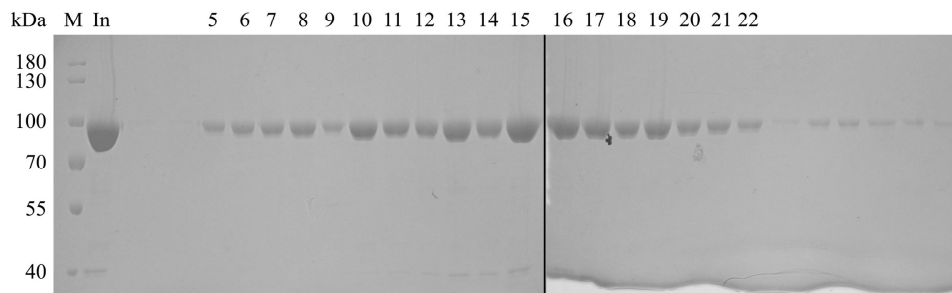
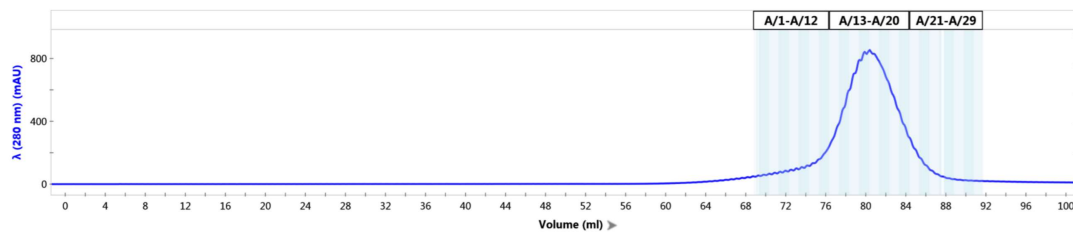


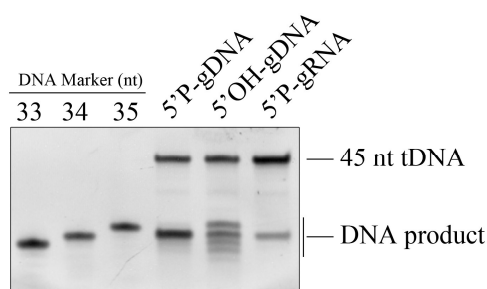
**A**

Protein	Source	D	E	D	X	Identity(%)
TtAgo	- <i>Thermus thermophilus</i>	AVGF <b>D</b> AGGR	AQAG <b>E</b> RIPO	LLLR <b>D</b> GRVP	LHL <b>A</b> DRLVK	17.95
CbAgo	- <i>Clostridium butyricum</i>	FIGL <b>D</b> VGTR	PQSG <b>E</b> KIAE	VIHR <b>D</b> GFSR	TGY <b>A</b> DKICK	22.26
NgAgo	- <i>Natronobacterium gregoryi</i>	FIGL <b>D</b> VSR	PQLG <b>E</b> KLQS	VIHR <b>D</b> GFMN	TAY <b>A</b> DQAST	21.34
RsAgo	- <i>Rhodobacter Sphaeroides</i>	VVGM <b>G</b> LAEL	ECEY <b>E</b> GYSD	RVVF <b>H</b> AHRP	IFYS <b>E</b> RIAE	14.06
MpAgo	- <i>Marinitoga piezophila</i>	YIGL <b>D</b> LSHD	LELN <b>E</b> KMNL	FILR <b>D</b> GRFI	LHI <b>A</b> NKVAL	18.34
LrAgo	- <i>Limnothrix rosea</i>	IVGL <b>D</b> VSRR	VIDG <b>E</b> ILPE	LIHR <b>D</b> GLFP	TTY <b>A</b> DKIST	21.09
IbAgo	- <i>Intestinibacter bartlettii</i>	YIGL <b>D</b> VCRE	HQSG <b>E</b> KIQI	VFHR <b>D</b> GINR	TTY <b>A</b> DLSSI	29.67
CbcAgo	- <i>Clostridium. butyricum</i> CWBI1009	FIGL <b>D</b> VGTR	PQSG <b>E</b> KIAE	VIHR <b>D</b> GFSR	TGY <b>A</b> DKICK	22.26
CpAgo	- <i>Clostridium perfringens</i>	FVGL <b>D</b> VGTR	PQNG <b>E</b> KINT	VIHR <b>D</b> GFSR	TGY <b>A</b> DKICK	22.21
SeAgo	- <i>Synechococcus elongates</i>	IIGF <b>D</b> TGTN	VQRG <b>E</b> TFSG	LLMR <b>D</b> GLVQ	LHL <b>A</b> DRSSK	18.70
PfAgo	- <i>Pyrococcus furiosus</i>	IIGF <b>D</b> VAPM	EQRG <b>E</b> SVDM	LLLR <b>D</b> GRIT	VHY <b>A</b> HKFAN	18.30
MjAgo	- <i>Methanocaldococcus jannaschii</i>	IMGL <b>D</b> TLGL	PAPG <b>E</b> RLHL	LFLR <b>D</b> GFIQ	IHY <b>A</b> DKFVK	15.77
hAgo2	- <i>Homo sapiens</i>	FLG <b>A</b> DVTHP	QHRQ <b>E</b> IIQD	IFYR <b>D</b> GVSE	AYY <b>A</b> HLVAF	14.97
KpAgo	- <i>Kluyveromyces Polysporus</i>	VLGS <b>D</b> VTHY	DGPG <b>E</b> EIIT	MYFR <b>D</b> GVSV	VYY <b>A</b> DLLCT	13.55
KmAgo	- <i>Kurthia massiliensis</i>	FIGL <b>D</b> VSHE	ILAG <b>E</b> KIDD	TIHR <b>D</b> GFWR	IHY <b>A</b> DLSAT	100.00
KmAgo_DM	- <i>Kurthia massiliensis</i> double mutant	FIGI <b>A</b> VSHE	ILAG <b>E</b> KIDD	TIHR <b>A</b> GFWR	IHY <b>A</b> DLSAT	

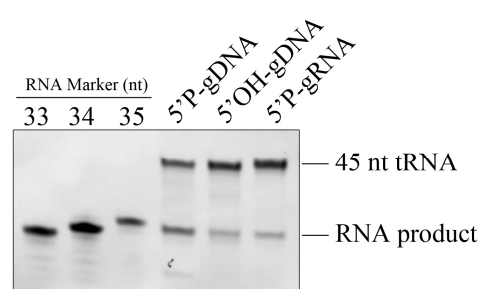
**B**



**C**

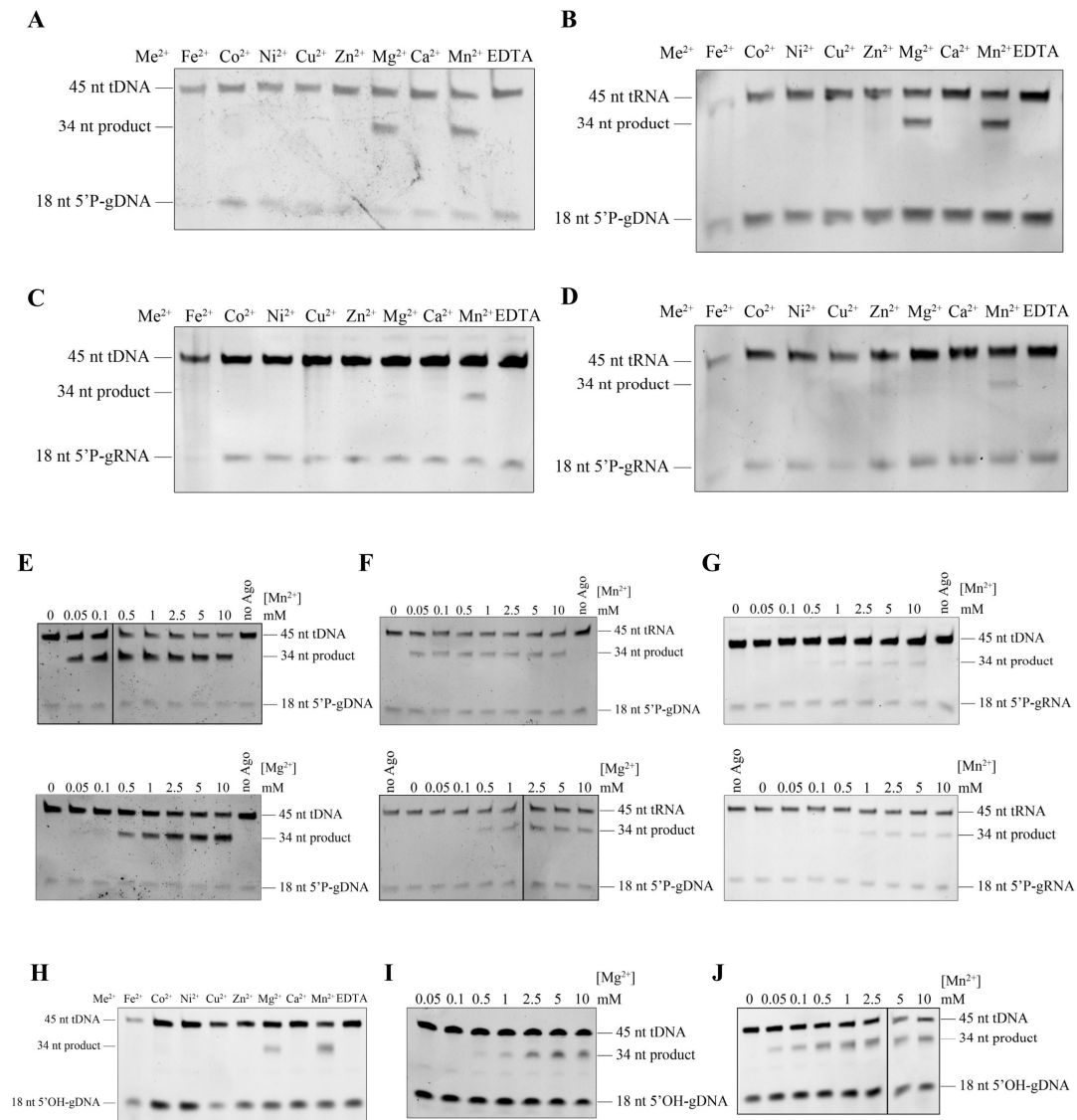


**D**

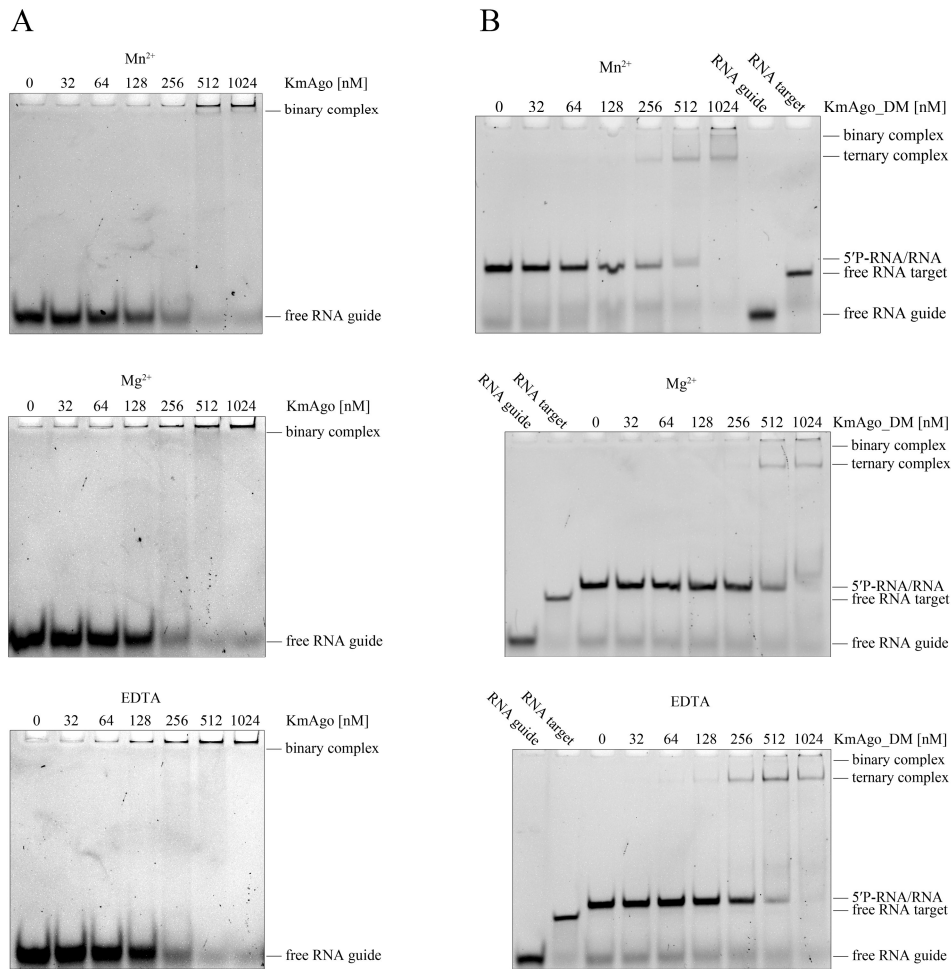


**Supplementary Figure S1.** KmAgo harbors a catalytic DEDD tetrad. (A) Multiple sequence alignment of KmAgo with several other biochemically or structurally characterized Ago proteins. The catalytically dead variant of the KmAgo protein (KmAgo\_DM) with amino acid substitutions within the catalytic tetrad is shown. The protein sequence identity of those Ago proteins between KmAgo are also shown. (B) Size exclusion diagram showing the elution peak of KmAgo (Upper panel). The purity of the purified KmAgo was determined using SDS-PAGE (Lower panel). M, marker; In, Heparin purified KmAgo; 5-22, chromatography fractions containing KmAgo. (C) Repeating experiment in Figure 1C with non-labeled tDNA. DNA marker

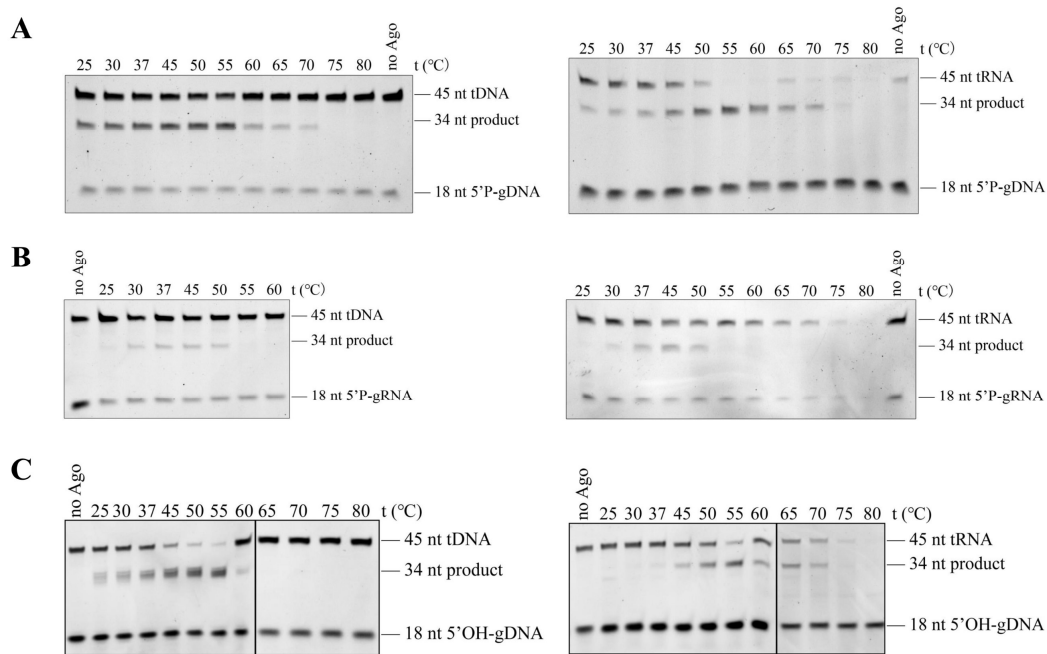
(33, 34, 35 nt) were partially hydrolyzed tDNA. (D) Repeating experiment in Figure 1D with non-labeled tRNA. RNA marker (33, 34, 35 nt) were partially hydrolyzed tRNA. The experiments in (C) and (D) were performed at the 4:2:1 KmAgo:guide:target molar ratio in reaction buffer containing  $Mn^{2+}$  ions for 30 min at 37 °C.



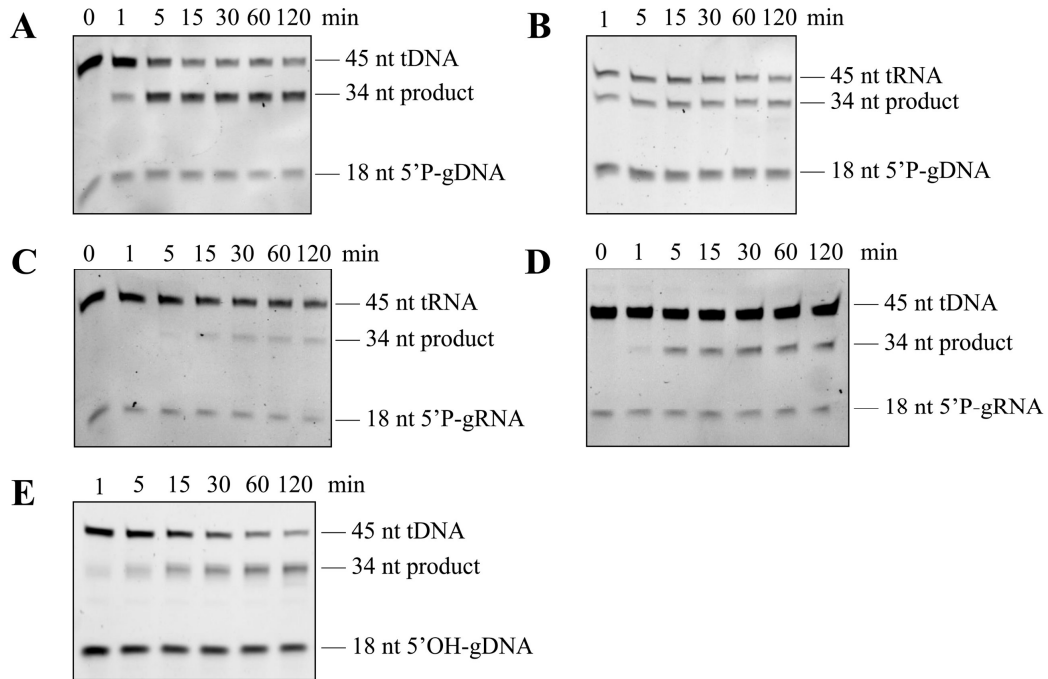
**Supplementary Figure S2.** Effects of different cations on KmAgo. (A) Effects of different cations on DNA cleavage activity mediated by 5'-P-gDNA. (B) Effects of different cations on RNA cleavage activity mediated by 5'-P-gDNA. (C) Effects of different cations on DNA cleavage activity mediated by 5'-P-gRNA. (D) Effects of different cations on RNA cleavage activity mediated by 5'-P-gRNA. (E) Effects of Mn<sup>2+</sup> concentration (Upper panel) and Mg<sup>2+</sup> concentration (Lower panel) on DNA cleavage activity mediated by 5'-P-gDNA. (F) Effects of Mn<sup>2+</sup> concentration (Upper panel) and Mg<sup>2+</sup> concentration (Lower panel) on RNA cleavage activity mediated by 5'-P-gDNA. (G) Effects of Mn<sup>2+</sup> concentration on DNA cleavage activity (Upper panel) and RNA cleavage activity (Lower panel) mediated by 5'-P-gRNA. (H) Effects of different cations on DNA cleavage activity mediated by 5'-OH-gDNA. (I) Effects of Mg<sup>2+</sup> concentration on DNA cleavage activity mediated by 5'-OH-gDNA. (J) Effects of Mn<sup>2+</sup> concentration on DNA cleavage activity mediated by 5'-OH-gDNA. All reactions were carried out for 15 min at 37 °C, at the 4:2:1 KmAgo:guide:target molar ratio (800 nM KmAgo preloaded with 400 nM guide, plus 200 nM target).



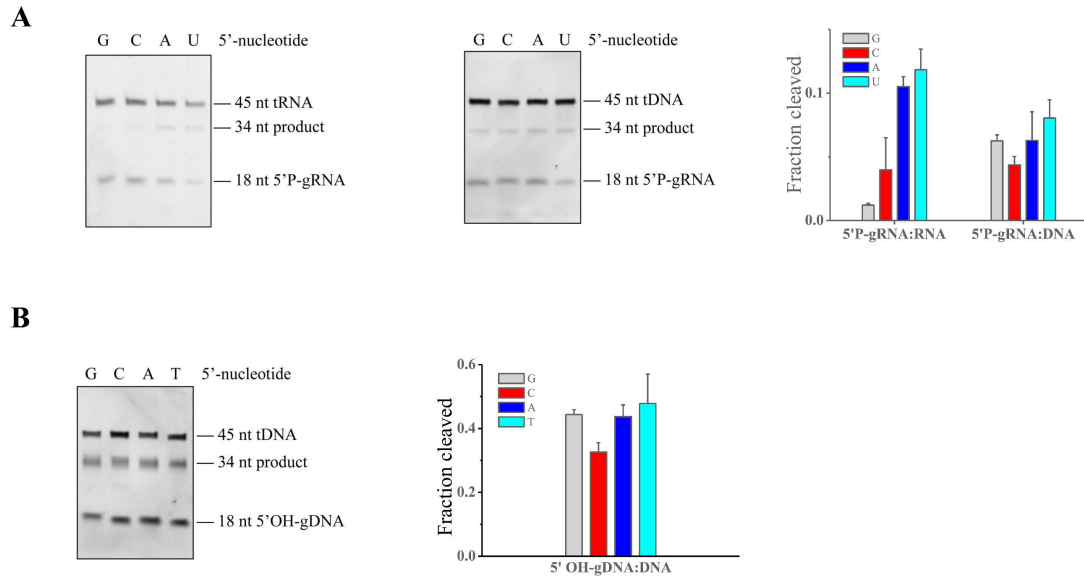
**Supplementary Figure S3.** Electrophoresis mobility shift assay (EMSA) of the binding of the KmAgo to 5'P-gRNA and 5'P-gRNA/tRNA duplex. (A) Effects of different cations on the loading of RNA guides to KmAgo. (Upper panel) 5 mM Mn<sup>2+</sup>, (Middle panel) 5 mM Mg<sup>2+</sup>, (Lower panel) 5 mM EDTA. (B) Effects of different cations on the loading of 5'P-gRNA/tRNA duplex to KmAgo\_DM. (Upper panel) 5 mM Mn<sup>2+</sup>, (Middle panel) 5 mM Mg<sup>2+</sup>, (Lower panel) 5 mM EDTA. The 5'P-gRNA and tRNA are 3' end FAM-labeled and 5' end FAM-labeled, respectively.



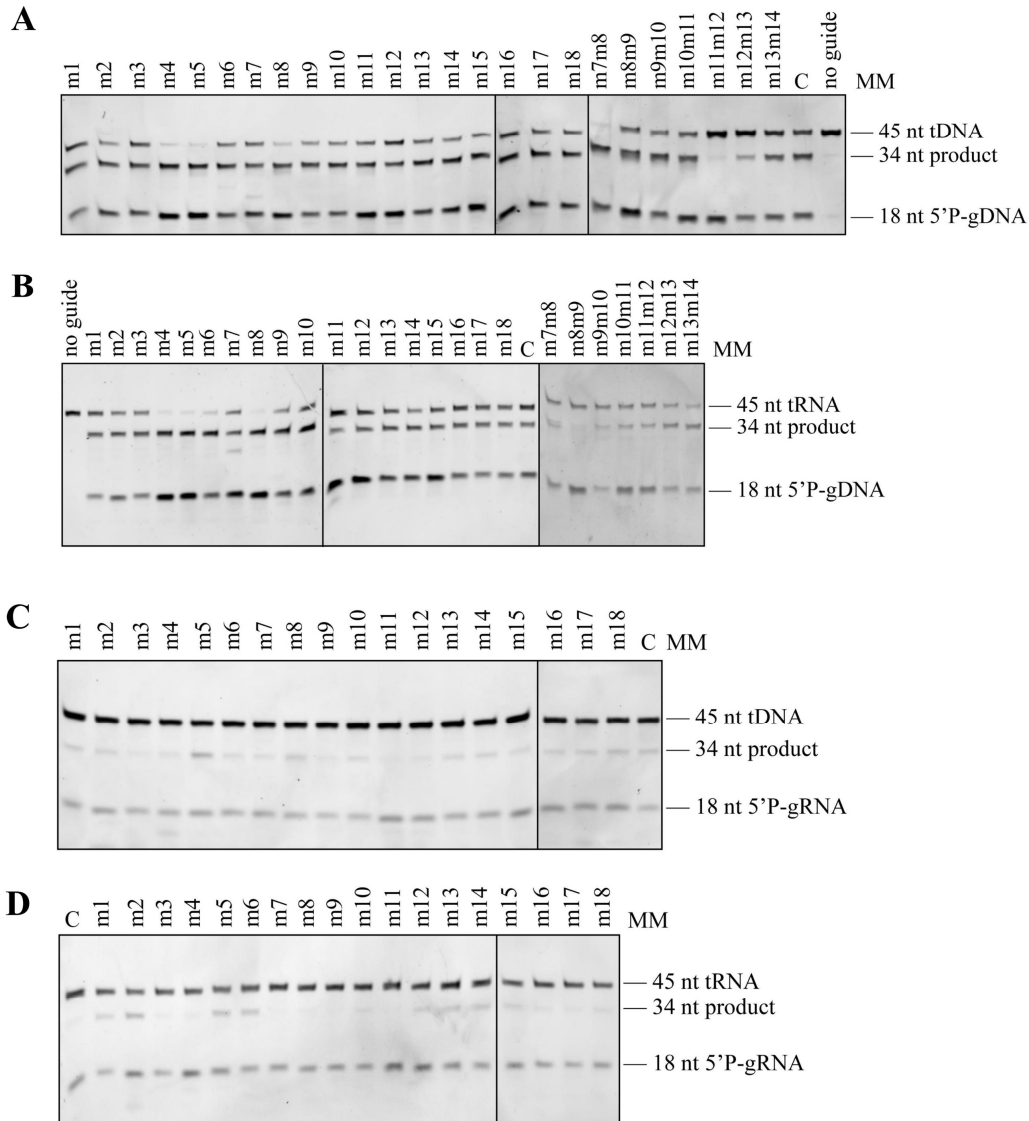
**Supplementary Figure S4.** Representative denaturing PAGE (one of three independent experiments) showing KmAgo activity with different temperatures. Cleavage efficiencies from three independent experiments were quantified and plotted against temperature (Figure 3). (A) Representative denaturing PAGEs (one of three independent experiments) showing effects of temperature on DNA cleavage activity (left panel) and RNA cleavage activity (right panel) using 5'P-gDNA. The no Ago reaction was carried out for 15 min at 80 °C. (B) Representative denaturing PAGEs (one of three independent experiments) showing effects of temperature on DNA cleavage activity (left panel) and RNA cleavage activity (right panel) using 5'P-gRNA. The no Ago reaction was carried out for 15 min at 37 °C. (C) Representative denaturing PAGEs (one of three independent experiments) showing effects of temperature on DNA cleavage activity (left panel) and RNA cleavage activity (right panel) using 5'OH-gDNA. The no Ago reaction was carried out for 15 min at 37 °C.



**Supplementary Figure S5.** Representative denaturing PAGE showing the results of DNA and RNA cleavage kinetics in the presence of 5 mM  $\text{MnCl}_2$ . Assays were performed in three independent replicates, and time points were taken at 0, 1, 5, 15, 30, 60, and 120 min. Cleavage efficiencies from three independent experiments were quantified and plotted against time (Figure 3D). (A) Representative denaturing PAGE (one of three independent experiments) showing DNA cleavage kinetics using 5'P-gDNA. (B) Representative denaturing PAGE (one of three independent experiments) showing RNA cleavage kinetics using 5'P-gDNA. (C) Representative denaturing PAGE (one of three independent experiments) showing RNA cleavage kinetics using 5'P-gRNA. (D) Representative denaturing PAGEs (one of three independent experiments) showing DNA cleavage kinetics using 5'P-gRNA. (E) Representative denaturing PAGE (one of three independent experiments) showing DNA cleavage kinetics using 5'OH-gDNA.

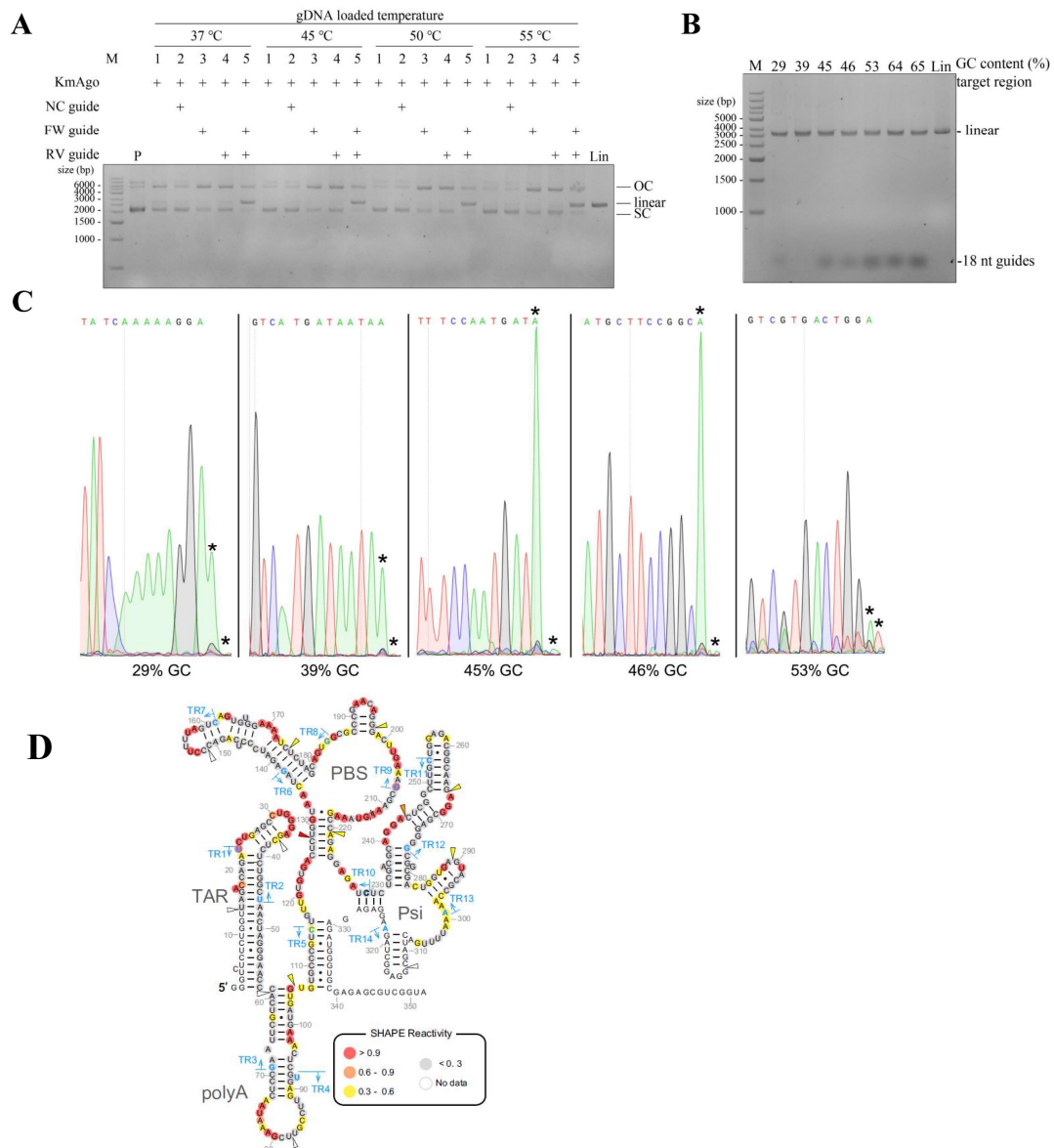


**Supplementary Figure S6.** Representative denaturing PAGEs (one of three independent experiments) showing KmAgo cleavage efficiencies with different nucleotides at the 5' end of the guide and their respective targets. Cleavage efficiencies from three independent experiments were quantified and plotted against 5' end nucleotide of the guide. (A) Representative denaturing PAGEs (one of three independent experiments) showing RNA cleavage efficiencies (left panel) and DNA cleavage efficiencies (middle panel) with different nucleotides at the 5' end of the 5'P-gRNA. Cleavage efficiencies from three independent experiments were quantified and plotted against 5' end nucleotide of the 5'P-gRNA (right panel). (B) Representative denaturing PAGE (one of three independent experiments) showing DNA cleavage efficiencies (left panel) with different nucleotides at the 5' end of the 5'OH-gDNA. Cleavage efficiencies from three independent experiments were quantified and plotted against 5' end nucleotide of the 5'OH-gRNA (right panel).



**Supplementary Figure S7.** Representative denaturing PAGE (one of three independent experiments) showing effects of mismatches in the guide-target duplex on the slicing activity of KmAgo. Quantification of the data (means and standard deviations from three independent experiments) is shown on the Figure 6. Representative denaturing PAGE (one of three independent experiments) showing effects of mismatches in the gDNA-tDNA (Figure S6A), gDNA-tRNA (Figure S6B), gRNA-tDNA (Figure S6C), and gRNA-tRNA (Figure S6D) duplex on the slicing activity of KmAgo. C, control reactions with guide variants containing no substitutions; MM, mismatch position.





**Supplementary Figure S8.** (A) The effects of KmAgo-gDNA duplex preloaded temperature on plasmid cleavage activity. Plasmid cleavage assay performed by preloading KmAgo with the indicated 5'P-gDNAs at different temperatures for 30 min, followed by incubation with the target plasmid at 37 °C for 2 h, and analysis of the target plasmid by electrophoresis. gDNA preloaded temperature, the temperature of KmAgo-gDNA duplex formation stage; NC guide, C-gDNA; FW guide, 29GC-F; RV guide, 29GC-R; M, molecular weight marker; Lin, linearized plasmid; SC, supercoiled plasmid; OC, open circular plasmid. (B) KmAgo is unable to cleave linear plasmids. Plasmids were digested with *NdeI* or *Scal* before cleavage by two KmAgo-gDNA complexes. M, molecular weight marker; Lin, linearized plasmid. (C) Sanger sequencing analysis of pUC19 cleavage products by KmAgo-gDNA complexes. The position of cleavage site is indicated by the termination of primer extension in the sequencing reaction. Sequencing artifacts are shown with an asterisk above the corresponding peaks. (D) Secondary structure of HIV-1  $\Delta$ DIS 5'UTR predicted by SHAPE, which is copied from Figure 3C of Dayeh et al (1).

## Supplementary tables

**Table S1. List of let-7-derived sequences used in this study.**

Oligonucleotide name	Sequence (5'-3')	Description
FAM-tDNA	FAM- AAACGACGGCCAGTGCCAAGCTT ACTATACAACCTACTACCTCAT	5' FAM labeled T-tDNA
M1	FAM- AAACGACGGCCAGTGCCAAGCTT ACTATACAACC	5' FAM labeled 34 nt DNA
FAM-tRNA	FAM- AAACGACGGCCAGUGCCAAGCUU ACUAUACAACCUACUACCUCAU	5' FAM labeled U-tRNA
M2	FAM- AAACGACGGCCAGUGCCAAGCUU ACUAUACAACC	5' FAM labeled 34 nt RNA
FAM-gRNA	UGAGGUAGUAGGUUGUAU-FAM	3' FAM labeled U-gRNA
C-gDNA	CGAGGTAGTAGGTTGTAT	guide forms 5'-C pair with C-tDNA/C-tRNA
T-gDNA	TGAGGTAGTAGGTTGTAT	guide forms 5'-T pair with T-tDNA/T-tRNA
A-gDNA	AGAGGTAGTAGGTTGTAT	guide forms 5'-A pair with A-tDNA/A-tRNA
G-gDNA	GGAGGTAGTAGGTTGTAT	guide forms 5'-G pair with G-tDNA/G-tRNA
33nt DNA product	AAACGACGGCCAGTGCCAAGCTT ACTATACAAC	33 nt DNA marker
34nt DNA product	AAACGACGGCCAGTGCCAAGCTT ACTATACAACC	5' DNA product for 45 nt DNA target
35nt DNA product	AAACGACGGCCAGTGCCAAGCTT ACTATACAACCT	35 nt DNA marker
A-tDNA	AAACGACGGCCAGTGCCAAGCTT ACTATACAACCTACTACCTCTT	let-7 based 45 nt DNA target for A-gDNA/A-gRNA
G-tDNA	AAACGACGGCCAGTGCCAAGCTT ACTATACAACCTACTACCTCCT	let-7 based 45 nt DNA target for G-gDNA/G-gRNA
C-tDNA	AAACGACGGCCAGTGCCAAGCTT ACTATACAACCTACTACCTCGT	let-7 based 45 nt DNA target for C-gDNA/C-gRNA
T-tDNA	AAACGACGGCCAGTGCCAAGCTT ACTATACAACCTACTACCTCAT	let-7 based 45 nt DNA target for T-gDNA/U-gRNA
C-gRNA	CGAGGUAGUAGGUUGUAU	guide forms 5'-C pair with C-tDNA/C-tRNA
U-gRNA	UGAGGUAGUAGGUUGUAU	guide forms 5'-U pair with T-tDNA/T-tRNA

A-gRNA	AGAGGUAGUAGGUUGUAU	guide forms 5'-A pair with A-tDNA/A-tRNA
G-gRNA	GGAGGUAGUAGGUUGUAU	guide forms 5'-G pair with G-tDNA/G-tRNA
C-tRNA	AAACGACGGCCAGUGCCAAGCUU ACUAUACAACCUACUACCUCGU	let-7 based 45 nt RNA target for C-gRNA/C-gDNA
U-tRNA	AAACGACGGCCAGUGCCAAGCUU ACUAUACAACCUACUACCUCAU	let-7 based 45 nt RNA target for U-gDNA/U-gRNA
A-tRNA	AAACGACGGCCAGUGCCAAGCUU ACUAUACAACCUACUACCUCUU	let-7 based 45 nt RNA target for A-gDNA/A-gRNA
G-tRNA	AAACGACGGCCAGUGCCAAGCUU ACUAUACAACCUACUACCUCUU	let-7 based 45 nt RNA target for G-gDNA/G-gRNA
33nt RNA product	AAACGACGGCCAGUGCCAAGCUU ACUAUACAAC	33 nt RNA marker
34nt RNA product	AAACGACGGCCAGUGCCAAGCUU ACUAUACAACC	5' RNA product for 45 nt RNA target
35nt RNA product	AAACGACGGCCAGUGCCAAGCUU ACUAUACAACCU	34 nt RNA marker
gDNA_mm1	GGAGGTAGTAGGTTGTAT	guide forms mismatched pair in position 1 with C-tDNA/C-tRNA
gDNA_mm2	CCAGGTAGTAGGTTGTAT	guide forms mismatched pair in position 2 with C-tDNA/C-tRNA
gDNA_mm3	CGTGGTAGTAGGTTGTAT	guide forms mismatched pair in position 3 with C-tDNA/C-tRNA
gDNA_mm4	CGACGTAGTAGGTTGTAT	guide forms mismatched pair in position 4 with C-tDNA/C-tRNA
gDNA_mm5	CGAGCTAGTAGGTTGTAT	guide forms mismatched pair in position 5 with C-tDNA/C-tRNA
gDNA_mm6	CGAGGAAGTAGGTTGTAT	guide forms mismatched pair in position 6 with C-tDNA/C-tRNA
gDNA_mm7	CGAGGTTGTAGGTTGTAT	guide forms mismatched pair in position 7 with C-tDNA/C-tRNA
gDNA_mm8	CGAGGTACTAGGTTGTAT	guide forms mismatched pair in position 8 with C-tDNA/C-tRNA
gDNA_mm9	CGAGGTAGAAGGTTGTAT	guide forms mismatched pair in position 9 with C-tDNA/C-tRNA
gDNA_mm10	CGAGGTAGTTGGTTGTAT	guide forms mismatched pair in position 10 with C-tDNA/C-tRNA
gDNA_mm11	CGAGGTAGTACGTTGTAT	guide forms mismatched pair in position 11 with C-tDNA/C-tRNA
gDNA_mm12	CGAGGTAGTAGCTTGTAT	guide forms mismatched pair in position 12 with C-tDNA/C-

		tRNA
gDNA_mm13	CGAGGTAGTAGGATGTAT	guide forms mismatched pair in position 13 with C-tDNA/C-tRNA
gDNA_mm14	CGAGGTAGTAGGTAGTAT	guide forms mismatched pair in position 14 with C-tDNA/C-tRNA
gDNA_mm15	CGAGGTAGTAGGTTCTAT	guide forms mismatched pair in position 15 with C-tDNA/C-tRNA
gDNA_mm16	CGAGGTAGTAGGTTGAAT	guide forms mismatched pair in position 16 with C-tDNA/C-tRNA
gDNA_mm17	CGAGGTAGTAGGTTGTTT	guide forms mismatched pair in position 17 with C-tDNA/C-tRNA
gDNA_mm18	CGAGGTAGTAGGTTGTAA	guide forms mismatched pair in position 18 with C-tDNA/C-tRNA
gDNA_m7m8	CGAGGTTCTAGGTTGTAT	guide forms mismatched pair in position 7 and 8 with C-tDNA/C-tRNA
gDNA_m8m9	CGAGGTACAAGGTTGTAT	guide forms mismatched pair in position 8 and 9 with C-tDNA/C-tRNA
gDNA_m9m10	CGAGGTAGATGGTTGTAT	guide forms mismatched pair in position 9 and 10 with C-tDNA/C-tRNA
gDNA_m10m11	CGAGGTAGTACGTTGTAT	guide forms mismatched pair in position 10 and 11 with C-tDNA/C-tRNA
gDNA_m11m12	CGAGGTAGTTCCTTGTAT	guide forms mismatched pair in position 11 and 12 with C-tDNA/C-tRNA
gDNA_m12m13	CGAGGTAGTTGCATGTAT	guide forms mismatched pair in position 12 and 13 with C-tDNA/C-tRNA
gDNA_m13m14	CGAGGTAGTTGGAAGTAT	guide forms mismatched pair in position 13 and 14 with C-tDNA/C-tRNA
gRNA_mm1	GGAGGUAGUAGGUUGUAU	guide forms mismatched pair in position 1 with C-tDNA/C-tRNA
gRNA_mm2	CCAGGUAGUAGGUUGUAU	guide forms mismatched pair in position 2 with C-tDNA/C-tRNA

gRNA_mm3	CGUGGUAGUAGGUUGUAU	guide forms mismatched pair in position 3 with C-tDNA/C-tRNA
gRNA_mm4	CGACGUAGUAGGUUGUAU	guide forms mismatched pair in position 4 with C-tDNA/C-tRNA
gRNA_mm5	CGAGCUAGUAGGUUGUAU	guide forms mismatched pair in position 5 with C-tDNA/C-tRNA
gRNA_mm6	CGAGGAAGUAGGUUGUAU	guide forms mismatched pair in position 6 with C-tDNA/C-tRNA
gRNA_mm7	CGAGGUUGUAGGUUGUAU	guide forms mismatched pair in position 7 with C-tDNA/C-tRNA
gRNA_mm8	CGAGGUACUAGGUUGUAU	guide forms mismatched pair in position 8 with C-tDNA/C-tRNA
gRNA_mm9	CGAGGUAGAAGGUUGUAU	guide forms mismatched pair in position 9 with C-tDNA/C-tRNA
gRNA_mm10	CGAGGUAGUUGGUUGUAU	guide forms mismatched pair in position 10 with C-tDNA/C-tRNA
gRNA_mm11	CGAGGUAGUACGUUGUAU	guide forms mismatched pair in position 11 with C-tDNA/C-tRNA
gRNA_mm12	CGAGGUAGUAGCUUGUAU	guide forms mismatched pair in position 12 with C-tDNA/C-tRNA
gRNA_mm13	CGAGGUAGUAGGAUGUAU	guide forms mismatched pair in position 13 with C-tDNA/C-tRNA
gRNA_mm14	CGAGGUAGUAGGUAGUAU	guide forms mismatched pair in position 14 with C-tDNA/C-tRNA
gRNA_mm15	CGAGGUAGUAGGUUCUAU	guide forms mismatched pair in position 15 with C-tDNA/C-tRNA
gRNA_mm16	CGAGGUAGUAGGUUGAAU	guide forms mismatched pair in position 16 with C-tDNA/C-tRNA
gRNA_mm17	CGAGGUAGUAGGUUGUUU	guide forms mismatched pair in position 17 with C-tDNA/C-tRNA
gRNA_mm18	CGAGGUAGUAGGUUGUAA	guide forms mismatched pair in position 18 with C-tDNA/C-tRNA
8nt C-gDNA	CGAGGTAG	8 nt guide pair with C-tDNA/C-tRNA
9nt C-gDNA	CGAGGTAGT	9 nt guide pair with C-tDNA/C-

		tRNA
10nt C-gDNA	CGAGGTAGTA	10 nt guide pair with C-tDNA/C-tRNA
11nt C-gDNA	CGAGGTAGTAG	11 nt guide pair with C-tDNA/C-tRNA
12nt C-gDNA	CGAGGTAGTAGG	12 nt guide pair with C-tDNA/C-tRNA
13nt C-gDNA	CGAGGTAGTAGGT	13 nt guide pair with C-tDNA/C-tRNA
14nt C-gDNA	CGAGGTAGTAGGTT	14 nt guide pair with C-tDNA/C-tRNA
15nt C-gDNA	CGAGGTAGTAGGTTG	15 nt guide pair with C-tDNA/C-tRNA
16nt C-gDNA	CGAGGTAGTAGGTTGT	16 nt guide pair with C-tDNA/C-tRNA
17nt C-gDNA	CGAGGTAGTAGGTTGTA	17 nt guide pair with C-tDNA/C-tRNA
19nt C-gDNA	CGAGGTAGTAGGTTGTATA	19 nt guide pair with C-tDNA/C-tRNA
20nt C-gDNA	CGAGGTAGTAGGTTGTATAG	20 nt guide pair with C-tDNA/C-tRNA
21nt C-gDNA	CGAGGTAGTAGGTTGTATAGT	21 nt guide pair with C-tDNA/C-tRNA
25nt C-gDNA	CGAGGTAGTAGGTTGTATAGTAAG C	25 nt guide pair with C-tDNA/C-tRNA
30nt C-gDNA	CGAGGTAGTAGGTTGTATAGTAAG CTTGGC	30 nt guide pair with C-tDNA/C-tRNA
40nt C-gDNA	CGAGGTAGTAGGTTGTATAGTAAG CTTGGCACTGGCCGTC	40 nt guide pair with C-tDNA/C-tRNA
12nt C-gRNA	CGAGGUAGUAGG	12 nt guide pair with C-tDNA/C-tRNA
13nt C-gRNA	CGAGGUAGUAGGU	13 nt guide pair with C-tDNA/C-tRNA
14nt C-gRNA	CGAGGUAGUAGGUU	14 nt guide pair with C-tDNA/C-tRNA
15nt C-gRNA	CGAGGUAGUAGGUUG	15 nt guide pair with C-tDNA/C-tRNA
16nt C-gRNA	CGAGGUAGUAGGUUGU	16 nt guide pair with C-tDNA/C-tRNA
17nt C-gRNA	CGAGGUAGUAGGUGUA	17 nt guide pair with C-tDNA/C-tRNA
19nt C-gRNA	CGAGGUAGUAGGUUGUAUA	19 nt guide pair with C-tDNA/C-tRNA

20nt C-gRNA	CGAGGUAGUAGGUUGUAUAG	20 nt guide pair with C-tDNA/C-tRNA
21nt C-gRNA	CGAGGUAGUAGGUUGUAUAGU	21 nt guide pair with C-tDNA/C-tRNA
25nt C-gRNA	CGAGGUAGUAGGUUGUAUAGUAGC	25 nt guide pair with C-tDNA/C-tRNA
30nt C-gRNA	CGAGGUAGUAGGUUGUAUAGUAGCUUGGC	30 nt guide pair with C-tDNA/C-tRNA
40nt C-gRNA	CGAGGUAGUAGGUUGUAUAGUAGCUUGGCACUGGCCGUC	40 nt guide pair with C-tDNA/C-tRNA

**Table S2. Sequences of HIV-1  $\Delta$ DIS 5'UTR (1).**

5' pGGGUCUCUCUGGUUAGACCAGAUCUGAGCCUGGGAGCUCUCUGGCCUAACUAGG  
GAACCCACUGCUUAAGCCUCAAUAAAGCUUGCCUUGAGUGCUCUAAAGUAGUGUGGC  
CCGUCUGUUGUGUGACUCUGGUAAACUAGAGAUCUCCUCAGACCCUUUUAGUCAGUGUG  
GAAAUCUCUAGCAGUGGGCGCCCGAACAGGGACUUGAAAGCGAAAGUAAAGCCAGAG  
GAGAUUCUCUCGACGCAGGACUCGGCUUGCUGGAGACGGCAAGAGGGCGAGGGGGCGGC  
GACUGGUGAGUACGCCAAAAUUUUGACUAGCGGAGGCUAGAAGGAGAGAGAUGGGU  
GCGAGAGCGUCGGUA

**Table S3. List of gDNAs targeting HIV-1  $\Delta$ DIS 5'UTR.**

gDNA#	Sequence (5'-3')	Target region	5' product length (nt)
gDNA_1	ACTCAAGGCAAGCTTTAT	70-87	82
gDNA_2	GACGGGCACACACTACTT	93-110	105
gDNA_3	CTAGTTACCAGAGTCACA	116-133	128
gDNA_4	GACTAAAAGGGTCTGAGG	139-156	151
gDNA_5	CACTGCTAGAGATTTTCC	162-179	174
gDNA_6	CTTTCAAGTCCCTGTTTCG	185-202	197
gDNA_7	GATCTCCTCTGGCTTTAC	208-225	220
gDNA_8	GCAAGCCGAGTCCTGCGT	231-248	243
gDNA_9	CCCCTCGCCTCTTGCCGT	254-271	266
gDNA_10	TTTGGCGTACTCACCAGT	277-294	289
gDNA_11	TCTAGCCTCCGCTAGTCA	300-317	312

**Table S4. List of gDNAs targeting plasmid pUC19.**

gDNA	Sequence (5'-3')	GC content of target region (%)
29GC-F	TCAAAAAGGATCTTCACC	29
29GC-R	TAGGTGAAGATCCTTTTT	29
39GC-F	AAGAAACCATTATTATCA	39
39GC-R	CATGATAATAATGGTTTC	39
45GC-F	AAAAGTGCTCATCATTGG	45
45GC-R	TTCCAATGATGAGCACTT	45
46GC-F	CAACATACGAGCCGGAAG	46

46GC-R	TGCTTCCGGCTCGTATGT	46
53GC-F	CAGGGTTTTCCAGTCAC	53
53GC-R	TCGTGACTGGGAAAACCC	53
64GC-F	CGCTCGGTCGTTCCGGCTG	64
64GC-R	CGCAGCCGAACGACCGAG	64
65GC-F	TCGTGCGCTCTCCTGTTC	65
65GC-R	CGGAACAGGAGAGCGCAC	65



## REFERENCES

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