Supporting Information

The impact of DNA topology and guide length on target selection by a cytosine-specific Cas9

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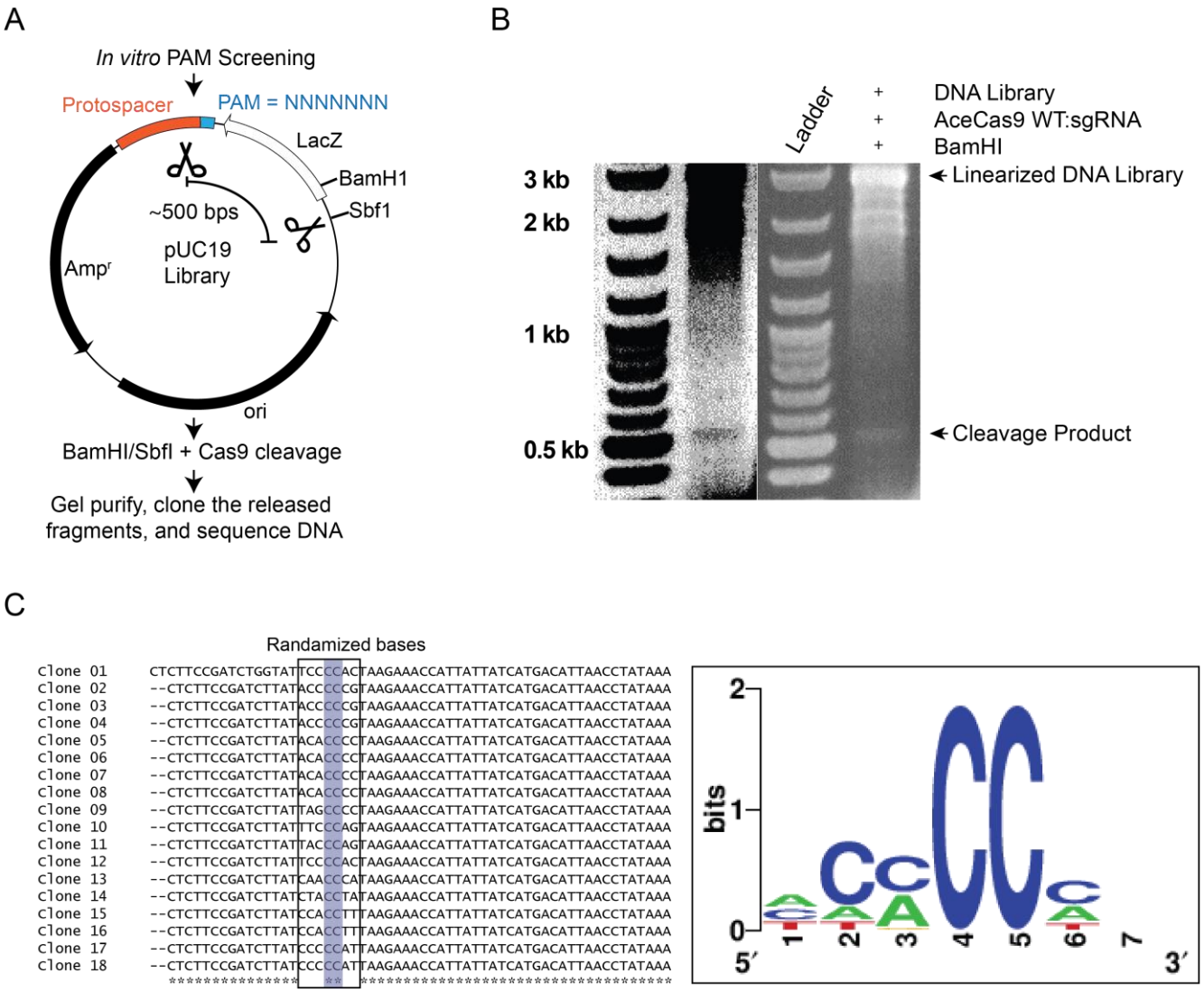


Figure S1. *In vitro* PAM Determination by DNA Library Cleavage Assay. Related to Figure 2 and 3.

(A) Experimental design of DNA library cleavage assay to determine PAM sequence for AceCas9.

(B) A smaller cleavage product (~500-bp) that contains PAM sequences was released after the double digestion cleavage with AceCas9 and BamHI. Color-inverted, contrast-adjusted image (left) shows the ~500-bp cleavage product from the original gel image (right).

(C) (Left) DNA sequences of 18 clones were aligned using Clustal Omega[1]. (Right) PAM sequence regions were extracted and aligned using WebLogo[2].

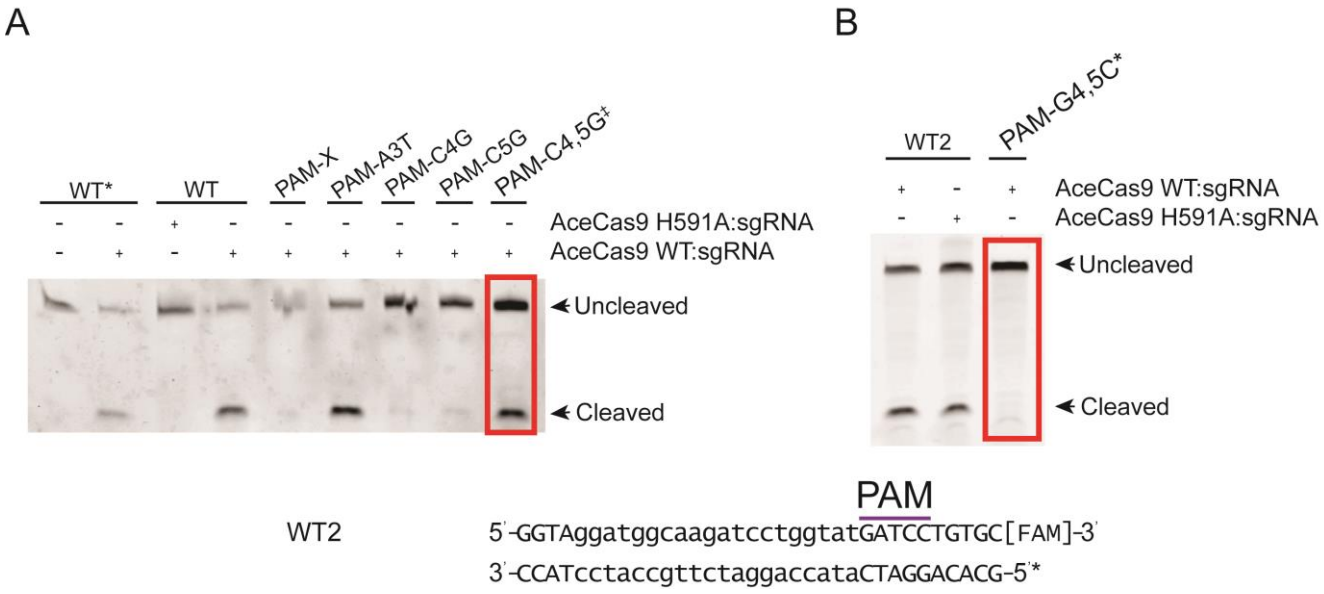


Figure S2. Oligo DNA Cleavage Activity of AceCas9 and AceCas9 mutants. Related to Figure 2.
(A) Various oligo DNA substrates containing HEX-labeled targeting strand were subjected to cleavage assay by AceCas9 and HNH-inactivated AceCas9 (H591A). The names of the substrates are identical to those defined in Figure 2. H591A AceCas9 was included as a control as it does not cleave the targeting strand of the WT dsDNA. Red box indicates the image used for PAM-C4,5G‡ substrate in Fig. 2B.

(B) A wild-type dsDNA with slightly different PAM sequence (WT2) and its mutant containing CC in place of GG pairs (PAM-G4,5C*), both with 6-FAM labeled on non-targeting DNA strand, were tested for cleavage by AceCas9 and the H591A mutant. H591A mutant cleaves the non-targeting DNA strand of WT2, suggesting that the RuvC domain of AceCas9 H591A remains active. Red box indicates the image used for PAM-G4,5C* substrate in Fig. 2B.

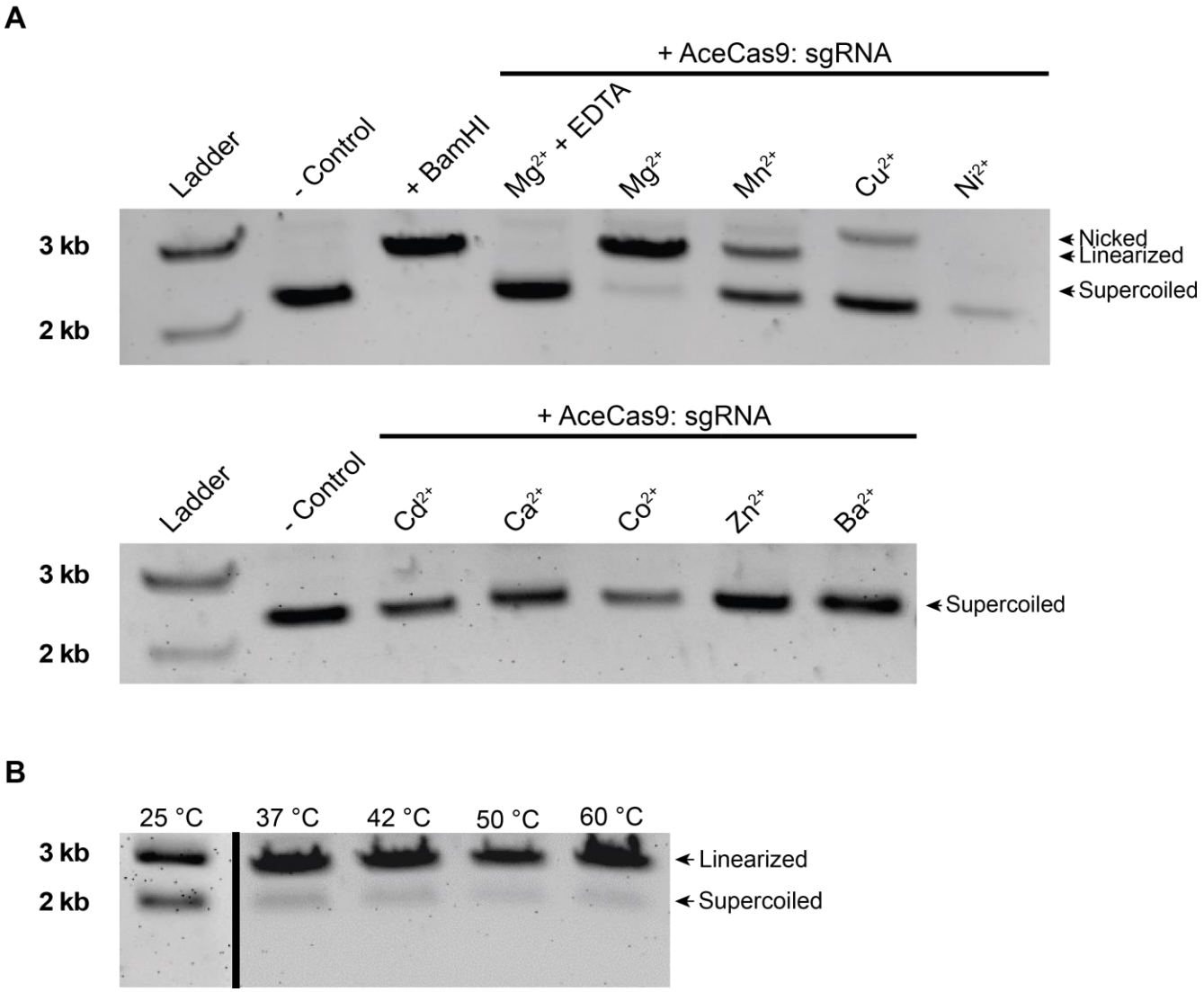


Figure S3. AceCas9 is Adaptable to Temperature Variation, but Selective to Metal Ions for Cleavage Activity. Related to Figure 3 and 5.

(A) A plasmid cleavage assay was performed with various divalent cations or divalent metal chelator. AceCas9 cleaves dsDNA plasmid in presence of Mg^{2+} and Mn^{2+}, while generating nicked DNA in presence of Cu^{2+}.

(B) AceCas9 linearized plasmid DNA in a broad range of temperature (25 – 60 °C). Reactions were performed identical to regular plasmid cleavage assay, with reaction tubes incubated at a temperature-equilibrated water bath for 60 minutes. Black line indicates the boarder of two separate gels.

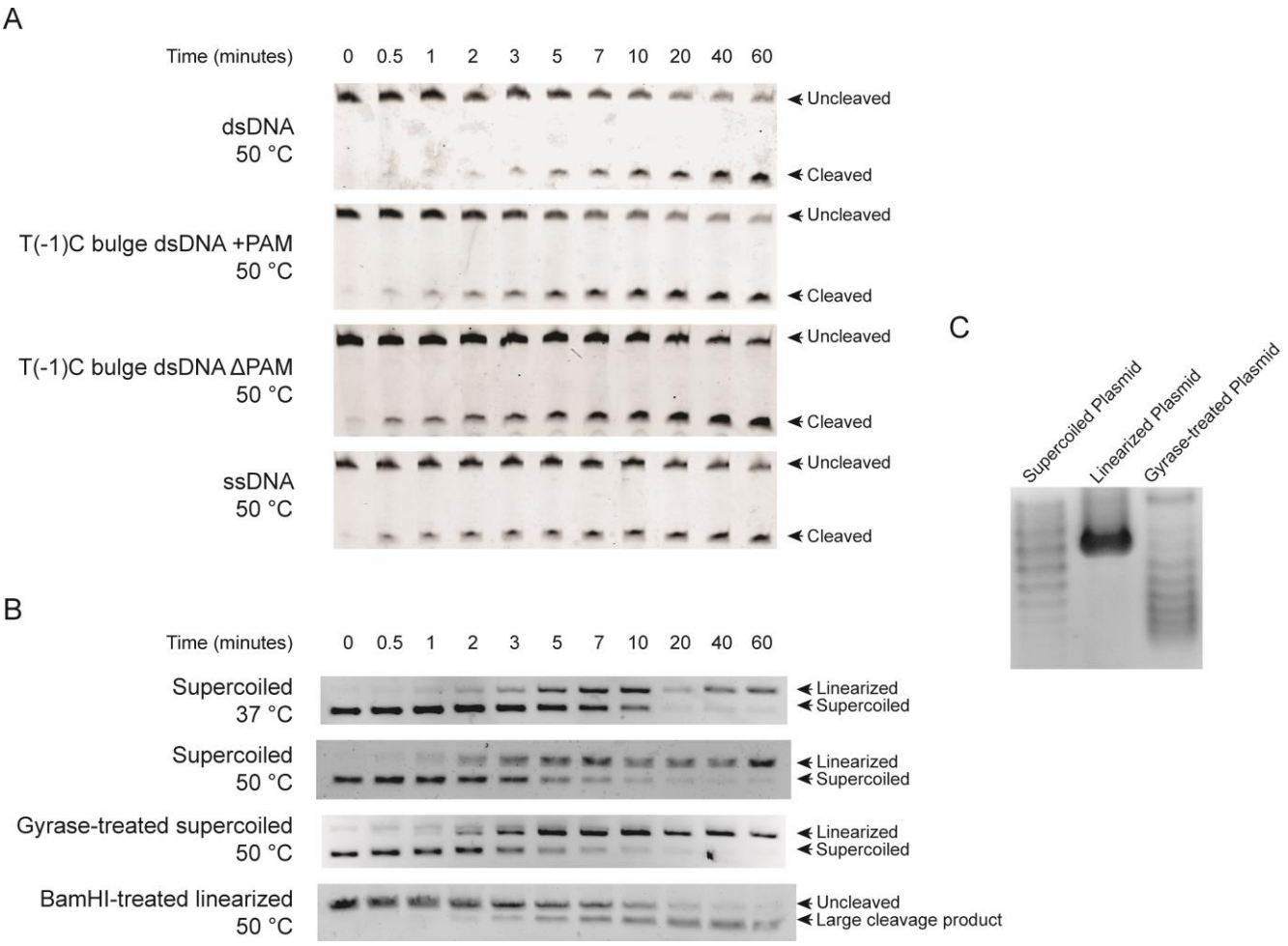


Figure S4. Single-turnover Kinetic Assays with Oligo and Plasmid DNA Substrates. Related to Figure 4.
(A and B) Representative gel images for each set of kinetics assays using (A) 30-nt oligonucleotide substrates or (B) 3-kb plasmid substrates. Pre-aliquoted samples were placed on ice and added gel loading buffer (with EDTA) at the indicated time points to stop the reaction. Each experiment was performed triplicate.
(C) pUC19 plasmids (supercoiled plasmid) treated with either BamHI (linearized plasmid) or *E.coli* gyrase (gyrase-treated plasmid) were resolved by 1% 1X TBE (with 10 μg/mL chloroquine, Sigma-Aldrich) agarose gel in 1X chloroquine-added TBE running buffer in 2.5 V/cm for 14 hours. Gel was rinsed in ddH_2O for 2 hours followed by incubation in SYBR Gold (Thermo Fisher Scientific) for 30 minutes. The gel was visualized by ChemiDoc XRS System (Bio-Rad). Chloroquine was added to help visualize supercoiling of the plasmid DNA substrates[3].

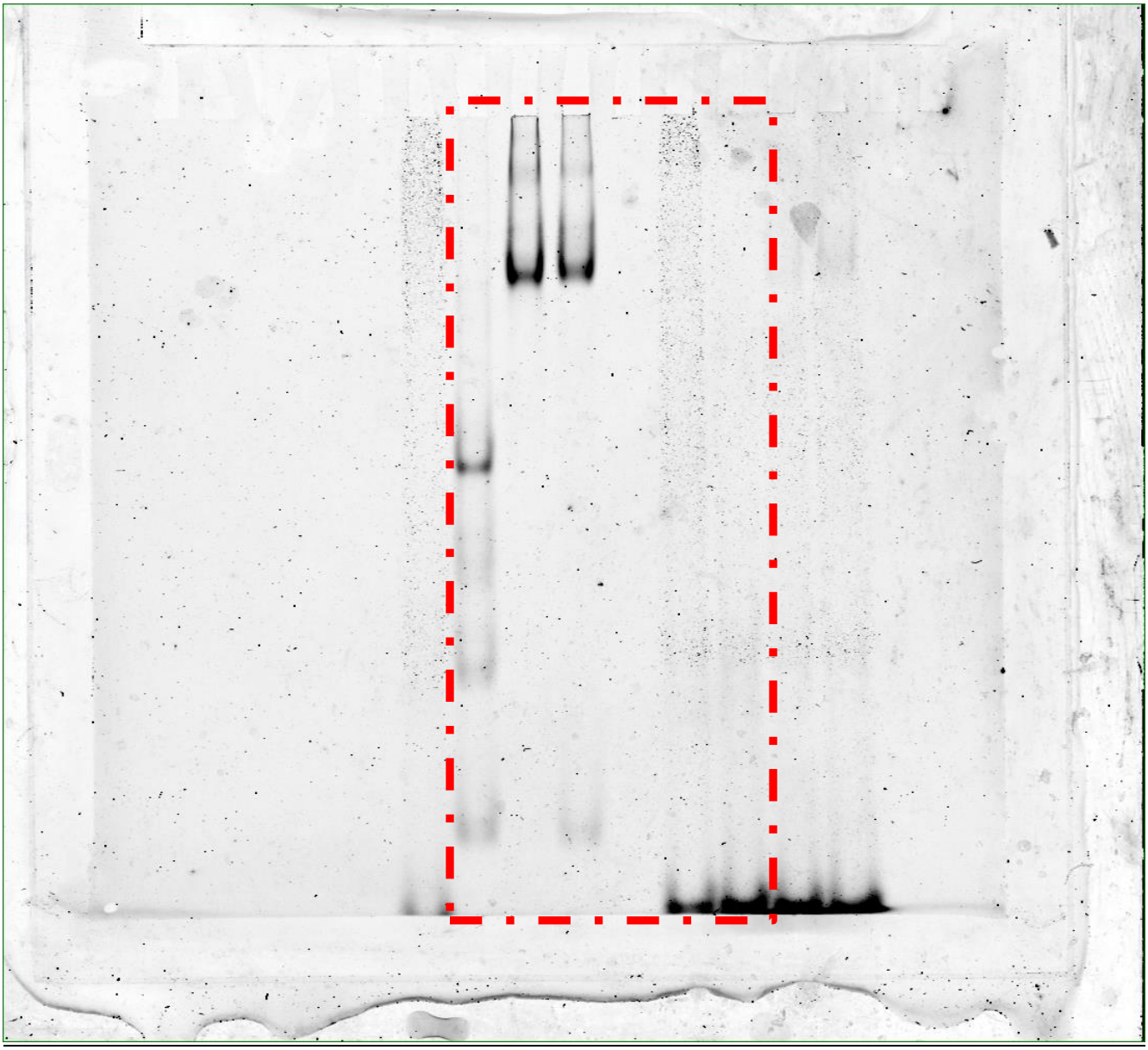
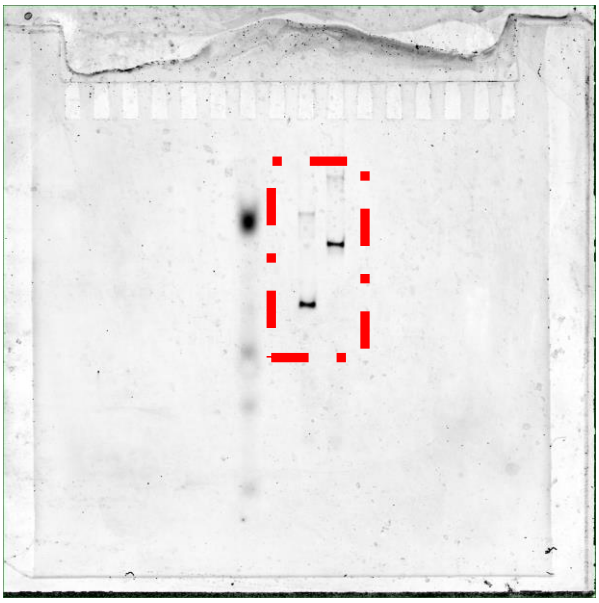


Figure S5. Electrophoresis mobility shift assay of ssDNA by AceCas9:sgRNA or HNH-inactivated AceCas9 (H591A):sgRNA. Related to Figure 1B.

The native polyacrylamide gel for the binding assay in Figure 1B. Red dotted line indicates the region of image that was used for the main text figure.

A



B

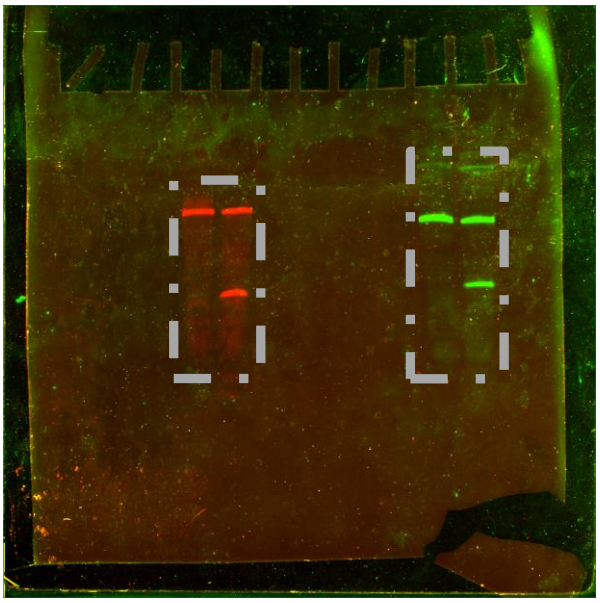


Figure S6. Formation and cleavage of oligo dsDNA by AceCas9:sgRNA. Related to Figure 2A.
(A) The non-labeled DNA shifted the HEX-labeled targeting DNA strand. Red dotted line indicates the region of image that was used in Figure 2A.
(B) Two oligo dsDNA were subjected to cleavage assay by AceCas9:sgRNA, related to Figure 2A. Red color bands are oligos with non-targeting DNA strand labeled by 6-FAM; green color bands are oligos with targeting DNA strand labeled by HEX. Grey dotted line indicates the region of images that were used in Figure 2A.

Table S1. List of Reported PAM Sequences for Cas9 orthologs. Related to Figure 2-3, and S1-S2

Organism (locus)	PAM Sequence	Type	Publication
Lactobacillus buchneri	NAAAAN	2-A	Anders et al., 2014[4]
Listeria innocua	NGG	2-A	Anders et al., 2014[4]
Streptococcus agalactiae	NGG	2-A	Lopez-Sanchez et al., 2012[5]
Streptococcus aureus	NNGRRNN	2-A	Ran et al., 2015[6]
Streptococcus mutans	NGG	2-A	Fonfara et al., 2014[7]
Streptococcus pasteurianus	NNGTGAN	2-A	Ran et al., 2015[6]
Streptococcus pyogenes	NGG	2-A	Jinek et al., 2012[8]
Streptococcus pyogenes	NAG	2-A	Mali et al., 2013[9]
Streptococcus thermophilus (CRISPR1)	NNAGAA	2-A	Deveau et al., 2008[10]
S.thermophilus (CRISPR1)	NNAGAAW	2-A	Horvath et al., 2008[11]
S.thermophilus (CRISPR1)	NNAAAAW	2-A	Fonfara et al., 2014[7]
Streptococcus thermophilus (CRISPR3)	NGGNG	2-A	Gasiunas et al., 2012[12]
S.thermophilus (CRISPR3)	NGG	2-A	Fonfara et al., 2014[7]
Treponema denticola	NAAAAN	2-A	Esvelt et al., 2013[13]
Francisella novicida	NG	2-B	Fonfara et al., 2014[7]
Acidothermus cellulolyticus	**NNNCC**	**2-C**	**This study**
Brevibacillus laterosporus	NNNNCND	2-C	Karvelis et al., 2015[14]
Campylobacter jejuni	NNNNACA	2-C	Fonfara et al., 2014[7]
Campylobacter lari	NNGGGNN	2-C	Ran et al., 2015[6]
Corynebacterium diphtheriae	NGGNNNN	2-C	Ran et al., 2015[6]
Neisseria cinerea	NNNNGAT	2-C	Ran et al., 2015[6]
Neisseria meningitidis	NNNNGATT	2-C	Zhang et al., 2013[15]
Parvibaculum lavamentivorans	NNNCAT	2-C	Ran et al., 2015[6]
Pasteurella multocida	GNNNCNNA	2-C	Fonfara et al., 2014[7]

Table S2. List of oligos DNA used in this study. Related to Figure 1-5 and S1-SS

Name (Purpose)	Identification	Sequence	Figures
20-nt Spacer sgRNA DNA Template (for *in vitro* transcription)	Top Strand	5'-*TAATACGACTCACTATA*-3'	N/A
	Bottom Strand	5'-GAACCCCCTCGCTGCTGCGAGGGGGTGAAGAA TGCGACCCCACGAAGGGGTCTTGCTAGGTAGCC TTTTCAGGCTCCCCAGCataccaggatcttgccatcc*TATAGT GAGTCGTATTA*-3'	
24-nt Spacer sgRNA DNA Template (for *in vitro* transcription)	Bottom Strand	5'-GAACCCCCTCGCTGCTGCGAGGGGGTGAAGAA TGCGACCCCACGAAGGGGTCTTGCTAGGTAGCC TTTTCAGGCTCCCCAGCataccaggatcttgccatcctacc*TAT AGTGAGTCGTATTA*-3'	N/A
Q5 Site-directed Mutagenesis Primers (Generation of PAM Library)	Forward	5'-TAAGAAACCATTATTATCATGACATTAACCTA TAAA-3'	Fig. S1A
	Reverse	5'-NNNNNNNataccaggatcttgccatccGACGTCAGGTGG CACTTTTCG-3'	
Q5 SDM Primers (Generation of PAM^{WT})	Forward	5'-tcctggtatACACCaagcttGGCTGTTTTGGCGGATG-3'	Fig. 6
	Reverse	5'-tcttgccatcctacctctagaGCGTGATATTACCCTGTTAT C-3'	
Q5 SDM Primers (Generation of PAM$^{C(4,5)T}$)	Reverse	5'-tcctggtatACATTaagcttGGCTGTTTTGGCGGATG-3'	Fig. 6
Q5 SDM Primers (Generation of 22-nt Spacer sgRNA)	Forward	5'-GGTGGCTGAGATCAGCCACTTCGCTGGGGAGC CTGAAAAG-3'	Fig. 6
	Reverse	5'-TATAGTGAGTCGTATTAATTTCGATTATGCGGC -3'	
Q5 SDM Primers (Generation of 24-nt Spacer sgRNA)	Forward	5'-GGTAggatggcaagatcctggtatGC-3'	Fig. 6
Q5 SDM Primers (Generation of 26-nt Spacer sgRNA)	Forward	5'-ggcggcagtagcgcggtggtcccaccGCTGGGGAGCCTGA AAAG-3'	Fig. 6
Sequencing primers (PAM Library, Plasmid DNA cleavage)	Forward	5'-CTTTCACCAGCGTTTCTGGGTGA-3'	Fig. 3B
	Reverse	5'-GCCTGAATGGCGAATGGCGCCTG-3'	
Sequencing primers (PAM^{WT}, PAM$^{C(4,5)T}$)	Forward	5'-GGCCAGTGCACGTCTGCTGTC-3'	Fig. 6
	Reverse	5'-CGGATTTGTCCTACTCAGGAGAGCG-3'	
WT (oligo cleavage assay, kinetic analysis)	Non-targeting	5'-ggatggcaagatcctggtatCCACCTTAGC-3' 3'-cctaccgttctaggaccataGGTGGAATCG-HEX-5'	Fig. 2A, 2B, , 4A, 4B, S2A, S3A
	Targeting		
PAM-A3T (oligo cleavage assay)	Non-targeting‡	5'-ggatggcaagatcctggtatCC**T**CCTTAGC-3' 3'-cctaccgttctaggaccataGG**A**GGAATCG-HEX-5'	Fig. 2B, S2A
	Targeting*		
PAM-C4G (oligo cleavage assay)	Non-targeting‡	5'-ggatggcaagatcctggtatCCA**G**CTTAGC-3' 3'-cctaccgttctaggaccataGGT**C**GAATCG-HEX-5'	Fig. 2B, S2A
	Targeting*		
PAM-C5G (oligo cleavage assay)	Non-targeting‡	5'-ggatggcaagatcctggtatCCAC**G**TTAGC-3' 3'-cctaccgttctaggaccataGGTG**C**AATCG-HEX-5'	Fig. 2B, S2A
	Targeting*		
PAM-C4,5G‡ (oligo cleavage assay)	Non-targeting‡	5'-ggatggcaagatcctggtatCCA**GG**TTAGC-3'	Fig. 2B, S2A
PS-1.b (cleavage assay)	Non-targeting‡	5'-ggatggcaagatcctggta**g**CCACCTTAGC-3'	Fig. 2B, 4B, S3A
PAM-X (oligo cleavage assay, kinetic analysis)	Non-targeting‡	5'-ggatggcaagatcctggtat**GATTC**A**C**AGC-3' 3'-cctaccgttctaggaccata**CTAAG**T**G**TCG-HEX-5'	Fig. 2B, S2A
	Targeting*		

Continued

Name (Purpose)	Identification	Sequence	Figures
PS-1.bX (cleavage assay, kinetic analysis)	Non-targeting‡	5'-ggatggcaagatcctggta**cGATTCAC**AGC-3'	Fig. 2B, 4B, S3A
WT2 (oligo cleavage assay)	Non-targeting	5'-GGTAggatggcaagatcctggtatGATCCTGTGC-FAM-3'	Fig. S2B
	Targeting	3'-CCATcctaccgttctaggaccataCTAGGACACG-5'	
PAM-G(4,5)C* (oligo cleavage assay)	Targeting*	3'-CCATcctaccgttctaggaccataCTA**CC**ACACG-5'	Fig. 2B, S2B
pPAM-C4T (Q5 Primers)	Forward	5'-TAAGAAACCATTATTATCATGACATTAAC-3'	Fig. 3A
	Reverse	5'-AT**GA**TGTataccaggatcttg-3'	
pPAM-C5T (Q5 Primer)	Reverse	5'-AT**A**GTGTataccaggatcttg-3'	Fig. 3A
pPAM-C4,5T (Q5 Primer)	Reverse	5'-AT**AA**TGTataccaggatcttg-3'	Fig. 3A
pPAM-C4G (Q5 Primer)	Reverse	5'-AT**G**CTGTataccaggatcttg-3'	Fig. 3A
pPAM-C5G (Q5 Primer)	Reverse	5'-AT**C**GTGTataccaggatcttg-3'	Fig. 3A
pPAM-C4,5G (Q5 Primer)	Reverse	5'-AT**CC**TGTataccaggatcttg-3'	Fig. 3A
pPST(-1)G (Q5 Primer)	Reverse	5'-ATGGTGT**c**taccaggatcttg-3'	Fig. 5
pPSWT (Q5 Primers)	Forward	5'-GGTAggatggcaagatcctggtatAC-3'	Fig. 4C, 5, S3B, S4
	Reverse	5'-GACGTCAGGTGGCACTTTT-3'	
pPSG(-4)A (Q5 Primer)	Forward	5'-GGTAggatggcaagatcctg**a**tatACACC-3'	Fig. 5
pPSC(-8)T (Q5 Primer)	Forward	5'-GGTAggatggcaagat**t**ctggtatACACC-3'	Fig. 5
pPSG(-20)A (Q5 Primer)	Forward	5'-GGTA**a**gatggcaagatcctggtatACACC-3'	Fig. 5
pPSG(-19,-20)A (Q5 Primer)	Forward	5'-GGTA**aa**atggcaagatcctggtatA-3'	Fig. 5
Spacer Oligos for BPK764 (Generation of SpyCas9 sgRNA targeting positive-selection plasmid)	Forward	5'-ATAGGAGGTAggatggcaagatcctgg-3'	Fig. 6
	Reverse	5'-AAACccaggatcttgccatccTACCTC-3'	

- *Italic and underline*, T7 promoter sequence; red, tetraloop; cyan, PAM region; lower case, protospacer sequence; **bold**: mutation relative to WT sequence; asterisk(*), mutation on targeting DNA strand; double dagger(‡), mutation on non-targeting DNA strand.

Supplemental reference:

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