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Supplementary Information

The positional and numerical effect of *N*^{6}-methyladenosine in tracrRNA on the DNA cleavage activity of Cas9

Binh Thanh Tran[a,†], Seulgi Go[a,†], Kyoung-Ran Kim [a,†], Hak Suk Chung[a,b], Dae-Ro Ahn[a,b,*]

[a]Chemical and Biological Integrative Research Center, Korea Institute of Science and Technology (KIST), Hwarangno 14-gil 5, Seongbuk-gu, Seoul 02792, Republic of Korea

[b]Division of Bio-Medical Science and Technology, KIST School, University of Science and Technology (UST), Seoul 02792, Republic of Korea

[†]These authors contribute equally

[*]Corresponding author (E-mail: drahn@kist.re.kr)

Materials and methods

Materials

RNA phosphoramidites for natural sequences, controlled pore glass (CPG), and the chemicals used for solid phase oligonucleotide synthesis were purchased from Sigma-Aldrich (USA). N^6-methyladenosine phosphoramidite and the 5'-phosphate amidite were purchased from Glen Research (USA). The crRNA strand was purchased from Integrated DNA Technologies (USA). K562 cells were kindly provided by Dr. Gayeong Lim in Korea Institute of Science and Technology (KIST). HEK293 cells were obtained from the Korean Cell Line Bank (Seoul, Republic of Korea).

Synthesis of tracrRNAs

Head (42-mer) strands and 5'-phosphorylated tail (41-mer) strands were synthesized at 1 μmol scale by standard protocols for solid phase oligonucleotide synthesis using MerMade 4 DNA/RNA synthesizer (BioAutomation, USA). The RNA oligonucleotides were cleaved from CPG and deprotected by incubation in a mixture of 30% ammonia and ethanol (3:1) at 55°C for 16 h. After the solvents were removed by a rotary evaporator, the residue was suspended in 1 M tetrabutylammonium fluoride in tetrahydrofuran (Sigma-Aldrich, USA) and incubated at 25°C for 24 h. After removing the solvent, the residue was finally purified by 10% denaturing polyacrylamide gel electrophoresis (PAGE) following the reported protocol [1]. For enzymatic ligation, the reaction mixture (20 μL) containing head (1 μL, 20 μM) strands, tail strands (2 μL, 20 μM), T4 RNA Ligase 1 (1 μL, New England Biolabs, USA), ATP (1 μL, 1 mM, Thermo Fisher Scientific, USA), 15% PEG8000 (6 μL), and RNase Inhibitor (0.5 μL, New England Biolabs, USA) in the reaction buffer provided by the manufacturer were incubated at 37°C for 16 h.

TracrRNAs were purified as ligation products by 10% denaturing PAGE of the reaction mixture by following the reported protocol [1] (Fig. S1). The head and tail sequences used for synthesis of tracrRNAs containing m6As are presented in Table S1.

Expression and purification of Cas9.

pET-NLS-Cas9-6xHis was purchased from Addgene (plasmid #62934, USA). Recombinant Cas9 was expressed and purified as previously described [2].

***In vitro* DNA cleavage reactions**

The DNA plasmid pSMART-EGFP [3] was used as the substrate after linearization with Pvu I (New England Biolabs, USA) for the *in vitro* DNA cleavage experiments (Table S2). Cas9 (33 nM), linearized pSMART-EGFP (1 nM), and crRNA (33 nM, Table S2), tracrRNA (33 nM) were incubated in the reaction buffer (20 mM HEPES, pH6.5, 100 mM NaCl, 5 mM $MgCl_2$, 0.1 mM EDTA, 30 μL) at 37 °C for 60 min. The reaction was quenched by adding 6x gel loading buffer (19.8 mM Tris-HCl, pH 8.0, 66 mM EDTA, 0.017% SDS, 2.5% Ficoll®-400, 0.015% bromophenol blue, New England Biolabs, USA) and subsequently analyzed using 0.8% agarose gel electrophoresis. The gel was stained with SYBR™ Gold (Thermo Fisher Scientific, USA) and imaged using the iBright™ FL1000 (Thermo Fisher Scientific, USA). Band intensities of cleavage products and the substrate were quantified using ImageJ software (National Institutes of Health, USA). The cleavage efficiency (%) was calculated by 100 x (total band intensity of cleavage products)/[(total band intensity of cleavage products) + (band intensity of the substrate)]. All data were obtained by three independent experiments.

The k_{obs} values were estimated by curve-fitting of graph showing time dependent-cleavage efficiency of UM, HM, TM or FM (in Fig. 2c). For curve fitting, the Origin software ExpDecay1 model was used, and the values were derived using the formula

$$y = y0 + A1 * exp\left(\frac{-(x - x0)}{t1}\right)$$

where y0 is the offset, x0 is the center, A1 is the amplitude, and t1 is the time constant. The values obtained from three independent experiments for each sample are shown as mean±S.D. in the graph in Figure 2d.

Cellular gene disruption experiments

K562 and HEK293 cells were maintained in RPMI 1640 medium (Welgene, Gyeongsan, Korea) and Dulbecco's Modified Eagle Medium (DMEM) (Welgene), respectively. Both media were supplemented with 10% fetal bovine serum (FBS) (Welgene) and 1% penicillin-streptomycin (Welgene). The cells were incubated at 37°C in a humidified atmosphere containing 95% air and 5% CO_2.

To achieve cell transfection, 1 x 10^5 cells were harvested, washed with phosphate-buffered saline (PBS), resuspended in Neon Electroporation Buffer R (50 μL, Thermo Fisher Scientific), and mixed with RNP complexes (1 μg Cas9, 125 ng crRNA, 125 ng tracrRNA for HEK293 cells; 2 μg Cas9, 250 ng crRNA, 250 ng tracrRNA for K562 cells) in the buffer (50 μL). Cells in the mixture were subjected to electroporation at 1150 V with two pulses of 20 ms (HEK293 cells) or 1700 V with one pulse of 20 ms (K562 cells) using 100 μL Neon tip. After electroporation, cells were seeded into 12-well plates. HEK293 cells were incubated for 48 h, while K562 cells were incubated for 72 h.

Genomic DNA was extracted using the MagListo™ 5M Genomic DNA Extraction Kit (Bioneer, Daejeon, Korea), according to the manufacturer's instructions. The amplicons of the target loci were prepared by polymerase chain reaction (PCR) amplification of genomic DNA using Speed-Pfu Polymerase (NanoHelix, Daejeon, Korea) and following primers for the indicated genes: *RUNX1*: Forward 5′-TTAATAGGGCTTGGGGAGTC-3′, Reverse 5′-CTGCCATTTCATTACA GGC-3′, *EMX1*: Forward 5′-AGCTCAGCCTGAGTGTTG-3′, Reverse 5′-TCGTGGGTTTGTG GTTG-3′, *HEK3*: Forward 5′-AGACAGGGATCCCAGGGAAA-3′, Reverse 5′- GAGCTGCAC ATACTAGCCCC-3′. Amplicons of the target loci were sequenced using the Sanger sequencing method by Macrogen (Seoul, Korea). The sequencing data were analyzed by the Tracking of Indels by DEcomposition (TIDE) method [4], to estimate indel efficiency. The indel frequencies shown in Fig. 5 were obtained by subtraction of the indel frequency of the control cells (electroporation only) from the indel frequency of each RNP-treated cells.

Table S1. The sequences of crRNA, head, and tail strands. Red color indicates m6A.

CrRNA (5′ to 3′)		
GGAGCGCACCATCTTCTTCAGUUUUAGAGCUAUGCUGUUUUG		
TracrRNA	Sequences (5′ to 3′)	
UM	Head	GGAACCAUUCAAAACAGCAUAGCAAGUUAAAAUAAGGCUAGU
	Tail	Phos-CCGUUAUCAACUUGAAAAAGUGGCACCGAGUCGGUGCUUUU
FM	Head	GGAACCAUUCAAAACAGCAUAGCAAGUUAAAAUAAGGCUAGU
	Tail	Phos-CCGUUAUCAACUUGAAAAAGUGGCACCGAGUCGGUGCUUUU
HM	Head	GGAACCAUUCAAAACAGCAUAGCAAGUUAAAAUAAGGCUAGU
	Tail	Phos-CCGUUAUCAACUUGAAAAAGUGGCACCGAGUCGGUGCUUUU
TM	Head	GGAACCAUUCAAAACAGCAUAGCAAGUUAAAAUAAGGCUAGU
	Tail	Phos-CCGUUAUCAACUUGAAAAAGUGGCACCGAGUCGGUGCUUUU
SM1	Head	GGAACCAUUCAAAACAGCAUAGCAAGUUAAAAUAAGGCUAGU

	Tail	Phos-CCGUUAUCAACUUGAAAAAGUGGCACCGAGUCGGUGCUUUU
SM2	Head	GGAACCAUUCAAAACAGCAUAGCAAGUUAAAAUAAGGCUAGU
	Tail	Phos-CCGUUAUCAACUUGAAAAAGUGGCACCGAGUCGGUGCUUUU
SM3	Head	GGAACCAUUCAAAACAGCAUAGCAAGUUAAAAUAAGGCUAGU
	Tail	Phos-CCGUUAUCAACUUGAAAAAGUGGCACCGAGUCGGUGCUUUU
SM4	Head	GGAACCAUUCAAAACAGCAUAGCAAGUUAAAAUAAGGCUAGU
	Tail	Phos-CCGUUAUCAACUUGAAAAAGUGGCACCGAGUCGGUGCUUUU
SM5	Head	GGAACCAUUCAAAACAGCAUAGCAAGUUAAAAUAAGGCUAGU
	Tail	Phos-CCGUUAUCAACUUGAAAAAGUGGCACCGAGUCGGUGCUUUU
SM6	Head	GGAACCAUUCAAAACAGCAUAGCAAGUUAAAAUAAGGCUAGU
	Tail	Phos-CCGUUAUCAACUUGAAAAAGUGGCACCGAGUCGGUGCUUUU
SM7	Head	GGAACCAUUCAAAACAGCAUAGCAAGUUAAAAUAAGGCUAGU
	Tail	Phos-CCGUUAUCAACUUGAAAAAGUGGCACCGAGUCGGUGCUUUU
SM8	Head	GGAACCAUUCAAAACAGCAUAGCAAGUUAAAAUAAGGCUAGU
	Tail	Phos-CCGUUAUCAACUUGAAAAAGUGGCACCGAGUCGGUGCUUUU
SM9	Head	GGAACCAUUCAAAACAGCAUAGCAAGUUAAAAUAAGGCUAGU
	Tail	Phos-CCGUUAUCAACUUGAAAAAGUGGCACCGAGUCGGUGCUUUU
SM10	Head	GGAACCAUUCAAAACAGCAUAGCAAGUUAAAAUAAGGCUAGU
	Tail	Phos-CCGUUAUCAACUUGAAAAAGUGGCACCGAGUCGGUGCUUUU
SM11	Head	GGAACCAUUCAAAACAGCAUAGCAAGUUAAAAUAAGGCUAGU
	Tail	Phos-CCGUUAUCAACUUGAAAAAGUGGCACCGAGUCGGUGCUUUU
SM12	Head	GGAACCAUUCAAAACAGCAUAGCAAGUUAAAAUAAGGCUAGU
	Tail	Phos-CCGUUAUCAACUUGAAAAAGUGGCACCGAGUCGGUGCUUUU
SM13	Head	GGAACCAUUCAAAACAGCAUAGCAAGUUAAAAUAAGGCUAGU
	Tail	Phos-CCGUUAUCAACUUGAAAAAGUGGCACCGAGUCGGUGCUUUU
SM14	Head	GGAACCAUUCAAAACAGCAUAGCAAGUUAAAAUAAGGCUAGU
	Tail	Phos-CCGUUAUCAACUUGAAAAAGUGGCACCGAGUCGGUGCUUUU
SM15	Head	GGAACCAUUCAAAACAGCAUAGCAAGUUAAAAUAAGGCUAGU
	Tail	Phos-CCGUUAUCAACUUGAAAAAGUGGCACCGAGUCGGUGCUUUU
SM16	Head	GGAACCAUUCAAAACAGCAUAGCAAGUUAAAAUAAGGCUAGU
	Tail	Phos-CCGUUAUCAACUUGAAAAAGUGGCACCGAGUCGGUGCUUUU
SM17	Head	GGAACCAUUCAAAACAGCAUAGCAAGUUAAAAUAAGGCUAGU

	Tail	Phos-CCGUUAUCAACUUGAAAAAGUGGCACCGAGUCGGUGCUUUU
SM18	Head	GGAACCAUUCAAAACAGCAUAGCAAGUUAAAAUAAGGCUAGU
	Tail	Phos-CCGUUAUCAACUUGAAAAAGUGGCACCGAGUCGGUGCUUUU
SM19	Head	GGAACCAUUCAAAACAGCAUAGCAAGUUAAAAUAAGGCUAGU
	Tail	Phos-CCGUUAUCAACUUGAAAAAGUGGCACCGAGUCGGUGCUUUU
M1	Head	GGAACCAUUCAAAACAGCAUAGCAAGUUAAAAUAAGGCUAGU
	Tail	Phos-CCGUUAUCAACUUGAAAAAGUGGCACCGAGUCGGUGCUUUU
M2	Head	GGAACCAUUCAAAACAGCAUAGCAAGUUAAAAUAAGGCUAGU
	Tail	Phos-CCGUUAUCAACUUGAAAAAGUGGCACCGAGUCGGUGCUUUU
M3	Head	GGAACCAUUCAAAACAGCAUAGCAAGUUAAAAUAAGGCUAGU
	Tail	Phos-CCGUUAUCAACUUGAAAAAGUGGCACCGAGUCGGUGCUUUU
M4	Head	GGAACCAUUCAAAACAGCAUAGCAAGUUAAAAUAAGGCUAGU
	Tail	Phos-CCGUUAUCAACUUGAAAAAGUGGCACCGAGUCGGUGCUUUU
M5	Head	GGAACCAUUCAAAACAGCAUAGCAAGUUAAAAUAAGGCUAGU
	Tail	Phos-CCGUUAUCAACUUGAAAAAGUGGCACCGAGUCGGUGCUUUU
M6	Head	GGAACCAUUCAAAACAGCAUAGCAAGUUAAAAUAAGGCUAGU
	Tail	Phos-CCGUUAUCAACUUGAAAAAGUGGCACCGAGUCGGUGCUUUU

Table S2. The sequence of the substrate plasmid (pSMART-EGFP). The target sequence is colored in blue. The Pvu I restriction enzyme site is colored in red.

CCCGTGTAAA ACGACGGCCA GTTTATCTAG TCAGCTTGAT TCTAGCTGAT
CGTGGACCGG AAGGTGAGCC AGTGAGTTGA TTGCAGTCCA GTTACGCTGG
AGTCTGAGGC TCGTCCTGAA TGATATGCGA CCGCCGGAGG GTTGCGTTTG
AGACGGGCGA CAGATCGACA CTGCTCGATC CGCTCGCACC TAATACGACT
CACTATAGGG ATGCCACCAT GGATGGTGAG CAAGGGCGAG GAGCTGTTCA
CCGGGGTGGT GCCCATCCTG GTCGAGCTGG ACGGCGACGT AAACGGCCAC
AAGTTCAGCG TGTCCGGCGA GGGCGAGGGC GATGCCACCT ACGGCAAGCT
GACCCTGAAG TTCATCTGCA CCACCGGCAA GCTGCCCGTG CCCTGGCCCA
CCCTCGTGAC CACCCTGACC TACGGCGTGC AGTGCTTCAG CCGCTACCCC
GACCACATGA AGCAGCACGA CTTCTTCAAG TCCGCCATGC CCGAAGGCTA
CGTCCAGGAG CGCACCATCT TCTTCAAGGA CGACGGCAAC TACAAGACCC

GCGCCGAGGT GAAGTTCGAG GGCGACACCC TGGTGAACCG CATCGAGCTG
AAGGGCATCG ACTTCAAGGA GGACGGCAAC ATCCTGGGGC ACAAGCTGGA
GTACAACTAC AACAGCCACA ACGTCTATAT CATGGCCGAC AAGCAGAAGA
ACGGCATCAA GGTGAACTTC AAGATCCGCC ACAACATCGA GGACGGCAGC
GTGCAGCTCG CCGACCACTA CCAGCAGAAC ACCCCCATCG GCGACGGCCC
CGTGCTGCTG CCCGACAACC ACTACCTGAG CACCCAGTCC GCCCTGAGCA
AAGACCCCAA CGAGAAGCGC GATCACATGG TCCTGCTGGA GTTCGTGACC
GCCGCCGGGA TCACTCTCGG CATGGACGAG CTGTACAAGT AAGGATCGAC
GAGAGCAGCG CGACTGGATC AGTTCTGGAC GAGCGAGCTG TCGTCCGACC
CGTGATCTTA CGGCATTATA CGTATGATCG GTCCACGATC AGCTAGATTA
TCTAGTCAGC TTGATGTCAT AGCTGTTTCC TGAGGCTCAA TACTGACCAT
TTAAATCATA CCTGACCTCC ATAGCAGAAA GTCAAAAGCC TCCGACCGGA
GGCTTTTGAC TTGATCGGCA CGTAAGAGGT TCCAACTTTC ACCATAATGA
AATAAGATCA CTACCGGGCG TATTTTTTGA GTTATCGAGA TTTTCAGGAG
CTAAGGAAGC TAAAATGAGT ATTCAACATT TCCGTGTCGC CCTTATTCCC
TTTTTTGCGG CATTTTGCCT TCCTGTTTTT GCTCACCCAG AAACGCTGGT
GAAAGTAAAA GATGCTGAAG ATCAGTTGGG TGCACGAGTG GGTTACATCG
AACTGGATCT CAACAGCGGT AAGATCCTTG AGAGTTTACG CCCCGAAGAA
CGTTTTCCAA TGATGAGCAC TTTTAAAGTT CTGCTATGTG GCGCGGTATT
ATCCCGTATT GACGCCGGGC AAGAGCAACT CGGTCGCCGC ATACACTATT
CTCAGAATGA CTTGGTTGAG TACTCACCAG TCACAGAAAA GCATCTCACG
GATGGCATGA CAGTAAGAGA ATTATGCAGT GCTGCCATAA CCATGAGTGA
TAACACTGCG GCCAACTTAC TTCTGGCAAC GATCGGAGGA CCGAAGGAGC
TAACCGCTTT TTTGCACAAC ATGGGGGATC ATGTAACTCG CCTTGATCGT
TGGGAACCGG AGCTGAATGA AGCCATACCA AACGACGAGC GTGACACCAC
GATGCCTGTA GCAATGGCAA CAACGTTGCG CAAACTATTA ACTGGCGAAC
TACTTACTCT AGCTTCCCGG CAACAATTAA TAGACTGGAT GGAGGCGGAT
AAAGTTGCAG GATCACTTCT GCGCTCGGCC CTCCCGGCTG GCTGGTTTAT
TGCTGATAAA TCTGGAGCCG GTGAGCGTGG GTCTCGCGGT ATCATTGCAG
CACTGGGGCC AGATGGTAAG CCCTCCCGCA TCGTAGTTAT CTACACGACG
GGGAGTCAGG CAACTATGGA TGAACGAAAT AGACAGATCG CTGAGATAGG
TGCCTCACTG ATTAAGCATT GGTAATGAGG GCCCAAATGT AATCACCTGG
CTCACCTTCG GGTGGGCCTT TCTTGAGGAC CTAAATGTAA TCACCTGGCT
CACCTTCGGG TGGGCCTTTC TGCGTTGCTG GCGTTTTTCC ATAGGCTCCG
CCCCCCTGAC GAGCATCACA AAAATCGATG CTCAAGTCAG AGGTGGCGAA
ACCCGACAGG ACTATAAAGA TACCAGGCGT TTCCCCCTGG AAGCTCCCTC
GTGCGCTCTC CTGTTCCGAC CCTGCCGCTT ACCGGATACC TGTCCGCCTT
TCTCCCTTCG GGAAGCGTGG CGCTTTCTCA TAGCTCACGC TGTAGGTATC
TCAGTTCGGT GTAGGTCGTT CGCTCCAAGC TGGGCTGTGT GCACGAACCC
CCCGTTCAGC CCGACCGCTG CGCCTTATCC GGTAACTATC GTCTTGAGTC
CAACCCGGTA AGACACGACT TATCGCCACT GGCAGCAGCC ACTGGTAACA
GGATTAGCAG AGCGAGGTAT GTAGGCGGTG CTACAGAGTT CTTGAAGTGG
TGGCCTAACT ACGGCTACAC TAGAAGAACA GTATTTGGTA TCTGCGCTCT
GCTGAAGCCA GTTACCTCGG AAAAAGAGTT GGTAGCTCTT GATCCGGCAA
ACAAACCACC GCTGGTAGCG GTGGTTTTTT TGTTTGCAAG CAGCAGATTA
CGCGCAGAAA AAAAGGATCT CAAGAAGATC CTTTGATTTT CTACCGAAGA
AAGGCCCA

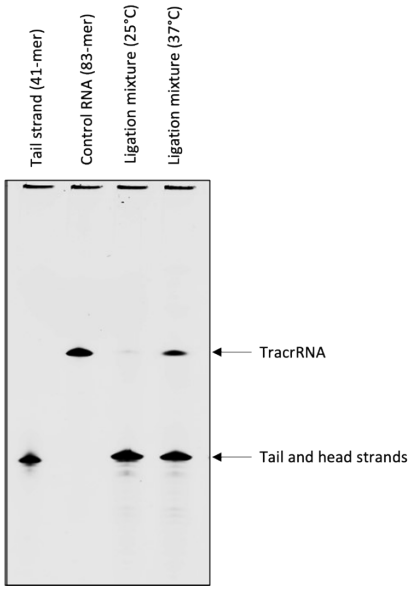

Figure S1. 10% denaturing PAGE analysis of the representative RNA ligation reaction

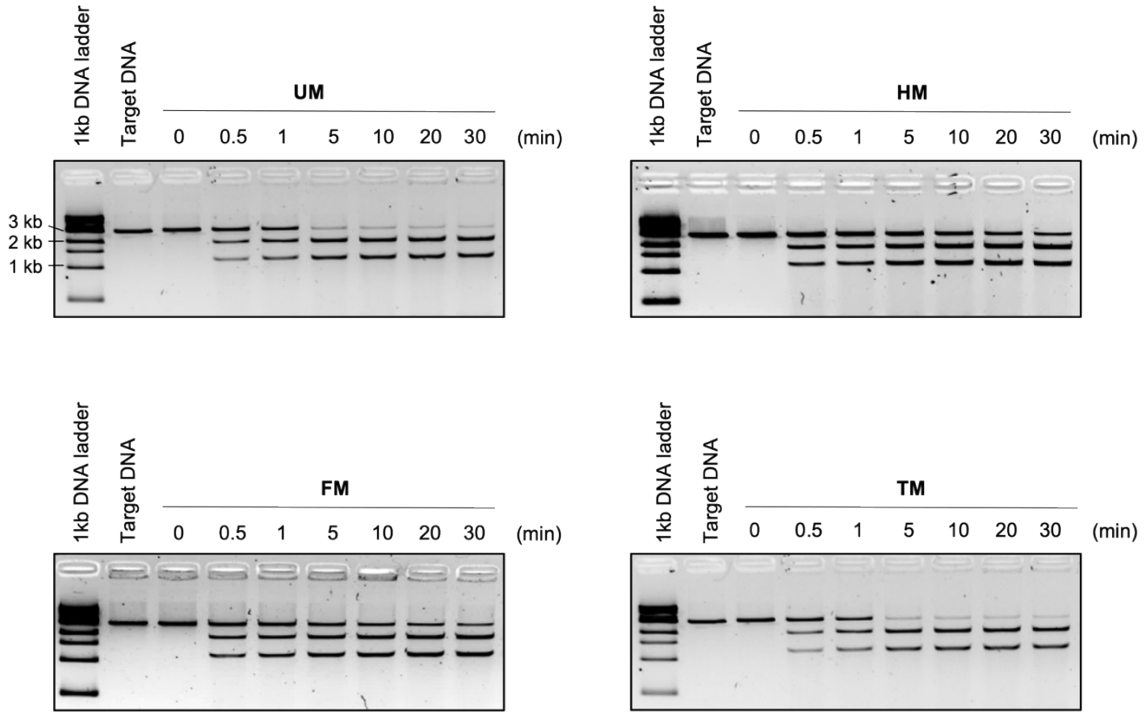

Figure S2. Agarose gel (0.8%) analysis of the DNA cleavage reactions by Cas9 with UM, FM, HM, or TM at various reaction time points.

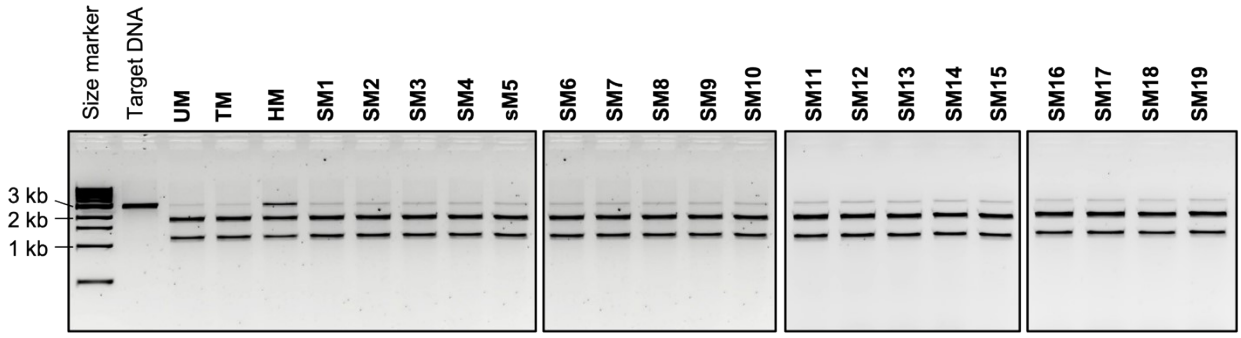

Figure S3. Agarose gel (0.8%) analysis of the DNA cleavage reactions by Cas9 with SM1 – SM19 for 60 min.

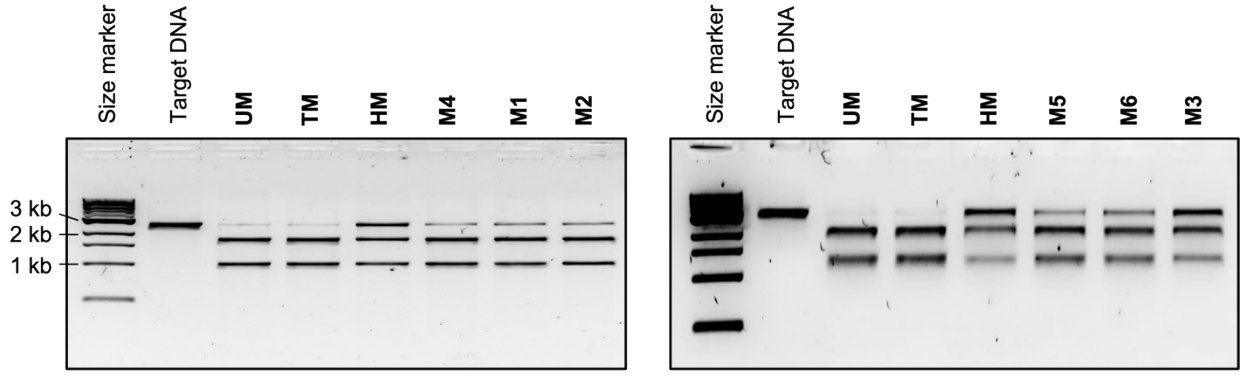

Figure S4. Agarose gel (0.8%) analysis of the DNA cleavage reactions by Cas9 with M1 – M6 for 60 min.

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