Electronic Supplementary Material (ESI) for Biomaterials Science.
This journal is © The Royal Society of Chemistry 2023

Supporting Information

Split-tracrRNA as an efficient tracrRNA system with an improved potential of scalability

Jihyun Park[1], Seong Jae Kang[1], Seulgi Go[1,2], Jeongmin Lee[1,3], Jinsu An[1,4], Hak Suk Chung[1,4], Cherlhyun Jeong[1,5], and Dae-Ro Ahn[1,4,*]

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

[2] *Department of Pharmacology, College of Medicine, Korea University, Seoul 02841, Korea*

[3] *Department of Life Sciences, Korea University, Seoul 02841, South Korea*

[4] *Division of Biomedical Science and Technology, KIST School, University of Science and Technology (UST), Hwarangno 14-gil 5, Seongbuk-gu, Seoul 02792, Korea*

[5] *KHU-KIST Department of Converging Science and Technology, Kyung Hee University, Seoul 02447, South Korea*

MATERIAL AND METHODS

Material

The DNA and RNA oligonucleotides used in this study were purchased from Integrated DNA Technologies (Coralville, IA, USA) and Bioneer (Daejeon, Republic of Korea) (Table S1). HeLa/EGFP cells were kindly provided by Dr. Sun Hwa Kim in Korea Institute of Science and Technology (KIST) (1). HEK293 cells were obtained from the Korean Cell Line Bank (Seoul, Republic of Korea).

Expression and purification of Cas9 and dCas9.

The pET-NLS-Cas9-6xHis plasmid was purchased from Addgene (Watertown, MA, USA, plasmid #62934). Cas9 was expressed and purified as previously described, with minor modifications (2). Pellets were harvested, resuspended in buffer A (50 mM Tris-HCl, pH 8.0, 0.5 M NaCl, 20 mM imidazole, and 5 mM 2-Mercaptoethanol (Bio-Rad), and lysed by sonication. After centrifugation at 10,000 × *g* for 40 min at 4°C, NLS-Cas9 was separated using Ni-NTA affinity chromatography. The eluted Cas9 was loaded onto a Hiprep SP HP 16/10 column (GE Healthcare Life Sciences, Chicago, IL, USA) and purified by a linear gradient of KCl from 0.15 M to 1 M in buffer B (20 mM HEPES, pH 7.5, 10 % glycerol, and 2 mM DTT). The eluted protein was concentrated, flash-frozen in liquid nitrogen, and stored at -80°C. The final purity and concentration of Cas9 were determined by SDS-PAGE and Bradford protein assays (Bio-Rad, Hercules, CA, USA), using bovine serum albumin as a protein standard. The catalytically inactive mutant dCas9 (D10A/H840A) was generated by quick-change site-directed mutagenesis following the manufacturer's protocol (Agilent, Santa Clara, CA, USA), as previously described (2), and purified following the same procedure as for Cas9.

In vitro DNA cleavage assays

The pSMART-EGFP plasmid encoding the enhanced green fluorescent protein (EGFP) was kindly provided by Prof. Jong Bum Lee (University of Seoul) (3) and was used as the DNA substrate for in vitro DNA cleavage assays (Table S3). Reaction mixtures (25 μL) containing Cas9 (40 nM), linearized pSMART-EGFP substrate (1 nM), crRNA (40 nM), and tracrRNA (40 nM) in the reaction buffer (20 mM HEPES pH6.5, 100 mM NaCl, 5 mM $MgCl_2$, 0.1 mM EDTA) were incubated at 37°C for 10 or 60 min. The reactions were quenched by adding 5 μL of 6× gel loading buffer (19.8 mM Tris-HCl, pH 8.0, 66 mM EDTA, 0.1 % SDS, 15 % Ficoll®-400, 0.09 % bromophenol blue, New England Biolabs, Ipswich, MA, USA) and were analysed by 1 % agarose gel electrophoresis. The gels were stained with SYBR Gold (Life Technologies, Grand Island, NY, USA) and imaged using an iBright™ FL1500 imaging system (Thermo Fisher Scientific, Carlsbad, CA, USA). Band intensities were quantified using ImageJ to analyze the cleavage level (%), which was determined as 100 × [sum of band intensities of cleavage products] / [sum of all band intensities]. Data represent the average values with the standard deviation of three independent experiments.

Fluorescence polarization-based binding assays

Fluorescein-labeled tracrRNA(67) (F-tracrRNA, 25 nM) was mixed with the Cas9/crRNA complex (200 nM) in a reaction buffer (20 mM HEPES, pH 6.5, 100 mM NaCl, 5 mM $MgCl_2$, 0.1 mM EDTA) to prepare an RNP complex. Then, to this RNP complex was added tracrRNA(67) or split-tracrRNAs at varying concentrations. The fluorescence polarization (FP) values of the mixtures were measured on an Appliskan™ (Thermo Fisher Scientific) using an excitation

wavelength of 485 nm and an emission wavelength of 525 nm. Statistical analyses and curve fitting was used to obtain IC_{50} values of split-tracrRNAs, using Sigmaplot™ (Systat Software, Chicago, IL, USA). To determine the K_d values for target DNA binding to the RNP complex (dCas9/crRNA/tracRNA), RNP complexes with split-tracrRNAs or tracrRNA(67) at varying concentrations were added to a solution of fluorescein-labeled target DNA duplex (F-DNA, 5′-GGG CGA GGA GCT GTT CAC CGG GGT GGT GCC-FAM-3′; 5′-GGC ACC ACC CCG GTG AAC AGC TCC TCG CCC-3′, 50 nM) in the binding mixture. To determine the K_d values for crRNA binding to the Cas9/tracrRNA complex, Cas9/tracrRNA or Cas9/split-tracrRNA complexes at varying concentrations were added to a solution of fluorescein-labeled crRNA (F-crRNA; 50 nM) in the binding buffer. The FP values of the mixtures were measured using this method. K_d values were determined by fitting the one-site binding hyperbola equation based on nonlinear regression using Sigmaplot™ (Systat Software).

Cellular knock-out efficiencies of RNP complexes

HeLa/EGFP cells were cultured in Dulbecco′s Modified Eagle′s Medium (DMEM) (Welgene, Gyeongsan, Korea) supplemented with 10 % fetal bovine serum (FBS) (Welgene), 1 % penicillin streptomycin (Welgene) in a 37 °C, 5 % CO_2 incubator. The cells (approximately 10,000 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) in the buffer (50 µL). Cells in the mixture were subjected to electroporation at 1350 V for one pulse (30 ms). After electroporation, cells were seeded into 6-well plates, incubated for 48 h, and the proportion of EGFP-positive cells estimated using flow cytometry (GUAVA™, Millipore, Burlington, MA, USA). HEK293 cells were cultured

under the same conditions as those used for Hela-GFP cells. The target sequences and the primers used for target amplification are listed in Table S5. For transfection of HEK293 cells, the RNP complex was electroporated using a 100 μL Neon tip, at 1150 V, with two pulses of 20 ms each.

Gene disruption efficiencies at targets and off-targets

HEK293 and HeLa/EGFP cells were treated with RNP complexes by electroporation in the same manner as described above. 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 and off-target loci (Table S2) were prepared by polymerase chain reaction (PCR) amplification of genomic DNA using Q5 High-Fidelity DNA polymerase (New England Biolabs) and the DNA primers shown in Table S5. 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, to estimate gene disruption efficiency (4). The amplicons of off-target loci were sequenced by GeneWiz (South Plainfield, NJ, USA) using an Illumina next-generation sequencing (NGS) system. Briefly, for each sample, > 50 ng of purified PCR fragment was used for library preparation. These PCR products were treated with End Prep Enzyme Mix (New England Biolabs) for end repair, 5′ phosphorylation, and dA-tailing in one reaction, followed by T-A ligation to add adaptors to both ends. The adaptor-ligated DNA was then purified using DNA Clean Beads. A second PCR was carried out with the primers carrying sequences that can anneal to the flowcell to perform bridge PCR and index, allowing for multiplexing. The final library products for sequencing were then purified using beads and was subsequently qualified. The qualified libraries were sequenced with 300-bp paired-end reads (ca. 50,000 reads) on the Illumina Miseq System. The FASTQ files of NGS data were analyzed with

Cas-Analyzer (5) to estimate off-target gene disruption efficiencies. CRISPR RGEN Tools used for the analysis of NGS data is an open-source collaborative initiative that is available in the repository (http://www.rgenome.net/).

Single-molecule protein-induced fluorescence enhancement

A sample chamber for single-molecule protein-induced fluorescence enhancement (PIFE) imaging was assembled with a quartz slide glass, which had been coated with PEG-biotin as previously described (6), to prevent nonspecific binding of the Cas9-complex to the surface. NTS oligonucleotide containing an amine-functionalized thymidine at the 5′-end (Integrated DNA Technologies) was conjugated with Cy3 fluorescent dye, modified with an NHS ester (GE Healthcare) and purified using ethanol precipitation (6). The target strand (TS) was annealed to a non-target strand (NTS) to form a DNA duplex at 1 μM in T100 buffer (Tris 10 mM, NaCl 100 mM, pH 8.0). The TS/NTS DNA duplex (100 nt, 100 pM) (Table S4) was immobilized on the PEG-coated surface via streptavidin–biotin interactions. For the Cas9-crRNA-tracrRNA(s) complex (1:1:1(:1)) molar ratio), Cas9 was incubated with tracrRNA(s) and crRNA that were annealed in advance in NEBuffer™ 3 buffer (New England Biolabs Inc.) for 10 min at room temperature. The final concentration of the Cas9-crRNA-tracrRNA(s) complex used for the reaction was 40 nM. The reaction buffer consisted of 50 mM Tris-HCl, 100 mM KCl, and 10 mM $MgCl_2$. To minimize photo-bleaching and suppress photo-blinking, an oxygen scavenging system consisting of 3 mM Trolox (Merck, Madison, NJ, USA), 5 mM 3,4-dihydroxybenzoic acid (Sigma-Aldrich, St Louis, MO, USA), 1.64 U/mL recombinant protocatechuate 3,4-dioxygenase from bacteria (Oriental Yeast, Tokyo, Japan) was included in the reaction buffer. Fluorescence signals from single molecules were collected in a prism-type total internal reflection microscope with a

0.1-s time resolution. To measure the PIFE effects, the donor molecules were excited at 532 nm, and fluorescence signals from Cy3 were collected using a water immersion objective lens (UPlanSApo 60x, Olympus, Tokyo, Japan), filtered with emission filters (LP03-532RU and NF03-633E-25, Semrock, Rochester, NY, USA) to prevent scattered laser beams, separated by a dichroic mirror (635dcxr, Chroma Technology Corp, Bellows Falls, VT, USA), and imaged using an EMCCD (Andor, Belfast, UK). Real-time single-molecule PIFE data were analyzed using MATLAB 2016b (Mathworks, Natick, MA, USA) and OriginPro 8 (OriginLab, Northampton, MA, USA).

Table 1. Sequences of the target DNA, crRNA, and tracrRNAs used in this study (5′ to 3′).

EGFP target in cell	GGAGCGCACCATCTTCTTCA
CrRNA for gene disruption in cells	GGAGCGCACCAUCUUCUUCAGUUUUAGAGCUAUGCUGUUUUG
Target sequence for in vitro DNA cleavage	CGAGGAGCTGTTCACCGGGG
CrRNA for in vitro DNA cleavage	CGAGGAGCUGUUCACCGGGGGUUUUAGAGCUAUGCUGUUUUG
F-crRNA	CGAGGAGCUGUUCACCGGGGGUUUUAGAGCUAUGCUGUUUUG-FAM

TracrRNA	Strand	Sequence
tracrRNA(89)	-	GUUGGAACCAUUCAAAACAGCAUAGCAAGUUAAAAUAAGGCUAGUCCGUUAUCAACUUGAAAAAGUGCACCGAGUCGGUGCUUUUUUUU
tracrRNA(67)	-	AGCAUAGCAAGUUAAAAUAAGGCUAGUCCGUUAUCAACUUGAAAAAGUGGCACCGAGUCGGUGCUUU
F-tracrRNA(67)		AGCAUAGCAAGUUAAAAUAAGGCUAGUCCGUUAUCAACUUGAAAAAGUGGCACCGAGUCGGUGCUUU-FAM
tracrRNA(49+18)	Head	AGCAUAGCAAGUUAAAAUAAGGCUAGUCCGUUAUCAACUUGAAAAAGUG
	Tail	GCACCGAGUCGGUGCUUU
tracrRNA(41+26)	Head	AGCAUAGCAAGUUAAAAUAAGGCUAGUCCGUUAUCAACUUG
	Tail	AAAAAGUGGCACCGAGUCGGUGCUUU
tracrRNA(33+34)	Head	AGCAUAGCAAGUUAAAAUAAGGCUAGUCCGUUA
	Tail	UCAACUUGAAAAAGUGGCACCGAGUCGGUGCUUU
tracrRNA(18+49)	Head	AGCAUAGCAAGUUAAAAU
	Tail	AAGGCUAGUCCGUUAUCAACUUGAAAAAGUGGCACCGAGUCGGUGCUUU
tracrRNA(40+27)	Head	AGCAUAGCAAGUUAAAAUAAGGCUAGUCCGUUAUCAACUU
	Tail	GAAAAAGUGGCACCGAGUCGGUGCUUU
tracrRNA(42+25)	Head	AGCAUAGCAAGUUAAAAUAAGGCUAGUCCGUUAUCAACUUGA
	Tail	AAAAGUGGCACCGAGUCGGUGCUUU

tracrRNA(43+24)	Head	AGCAUAGCAAGUUAAAAUAAGGCUAGUCCGUUAUCAACUUGAA
	Tail	AAAGUGGCACCGAGUCGGUGCUUU
tracrRNA(44+23)	Head	AGCAUAGCAAGUUAAAAUAAGGCUAGUCCGUUAUCAACUUGAAA
	Tail	AAGUGGCACCGAGUCGGUGCUUU
tracrRNA(40+23)	Head	AGCAUAGCAAGUUAAAAUAAGGCUAGUCCGUUAUCAACUU
	Tail	AAGUGGCACCGAGUCGGUGCUUU
tracrRNA(40+23) -GC	Head	AGCAUAGCAAGUUAAAAUAAGGCUAGUCCGUUAUCACCCC
	Tail	GGGGGGCACCGAGUCGGUGCUUU
F-DNA		GGGCGAGGAGCTGTTCACCGGGGTGGTGCC-FAM
		GGCACCACCCCGGTGAACAGCTCCTCGCCC

Table S2. The target and off-target sequences in HeLa/EGFP and HEK293 cells used for evaluation of the gene disruption efficiency of split-tracrRNAs. The mismatched sequences are colored in red.

Gene	Sequence (5′ to 3′)	PAM
EGFP	GGAGCGCACCATCTTCTTCA	AGG
OT1	GGAGCACCCCATCTTCTTCG	AGG
OT2	GGAGCTCTCCATCTCCTTCA	AGG
DNMT1	GATTCCTGGTGCCAGAAACA	GGG
OT1	GCTTTCTGGTGCCAGGACCA	AGG
OT2	TATTCCTGGAGCCAGAAGCA	GGG
EMX1	GAGTCCGAGCAGAAGAAGAA	GGG
OT1	GAGTTAGAGCAGAAGAAGAA	AGG
OT2	GAGGCCGAGCAGAAGAAAGA	CGG
FANCF1	GGAATCCCTTCTGCAGCACC	TGG
OT1	GGAACCCCGTCTGCAGCACC	AGG
OT2	GGAGTCCCTCCTACAGCACC	AGG
HEK3	GGCCCAGACTGAGCACGTGA	TGG
OT1	AGCTCAGACTGAGCAAGTGA	GGG
OT2	CACCCAGACTGAGCACGTGC	TGG
RNF2	GTCATCTTAGTCATTACCTG	AGG
OT1	AATATGTTAGTCATTACCTG	AGG
OT2	GGTATCTAAGTCATTACCTG	TGG
RUNX1	GCATTTTCAGGAGGAAGCGA	TGG
OT1	GCATTTTCAGAAGGAAGCAA	GGG
OT2	ACAATCTCAGGAGGAAGGGA	GGG
VEGFA	GGTGAGTGAGTGTGTGCGTG	TGG
OT1	TGTGAGTAAGTGTGTGTGTG	TGG
OT2	AGCGAGTGGGTGTGTGCGTG	GGG

Table S3. The sequence of the substrate plasmid (pSMART-EGFP) used as the substrate for in vitro DNA cleavage assays. EGFP coding sequences are colored in blue. The Pvu I restriction enzyme site is colored in red. The target sequences are indicated in bold with a grey box.

CCCGTGTAAA	ACGACGGCCA	GTTTATCTAG	TCAGCTTGAT	TCTAGCTGAT	CGTGGACCGG
AAGGTGAGCC	AGTGAGTTGA	TTGCAGTCCA	GTTACGCTGG	AGTCTGAGGC	TCGTCCTGAA
TGATATGCGA	CCGCCGGAGG	GTTGCGTTTG	AGACGGGCGA	CAGATCGACA	CTGCTCGATC
CGCTCGCACC	TAATACGACT	CACTATAGGG	ATGCCACCAT	GGATGGTGAG	CAAGGG**CGAG**
GAGCTGTTCA	**CCGGGG**TGGT	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		

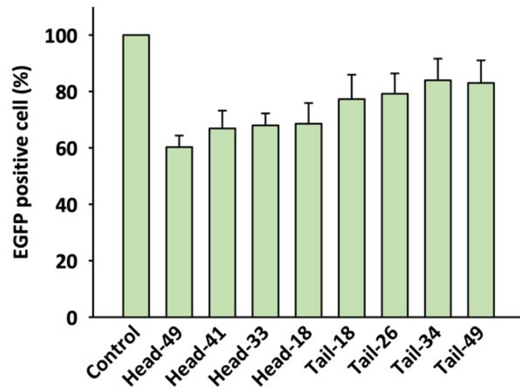
Table S4. TS and NTS sequences used in PIFE. The 20-nt target regions are colored in red.

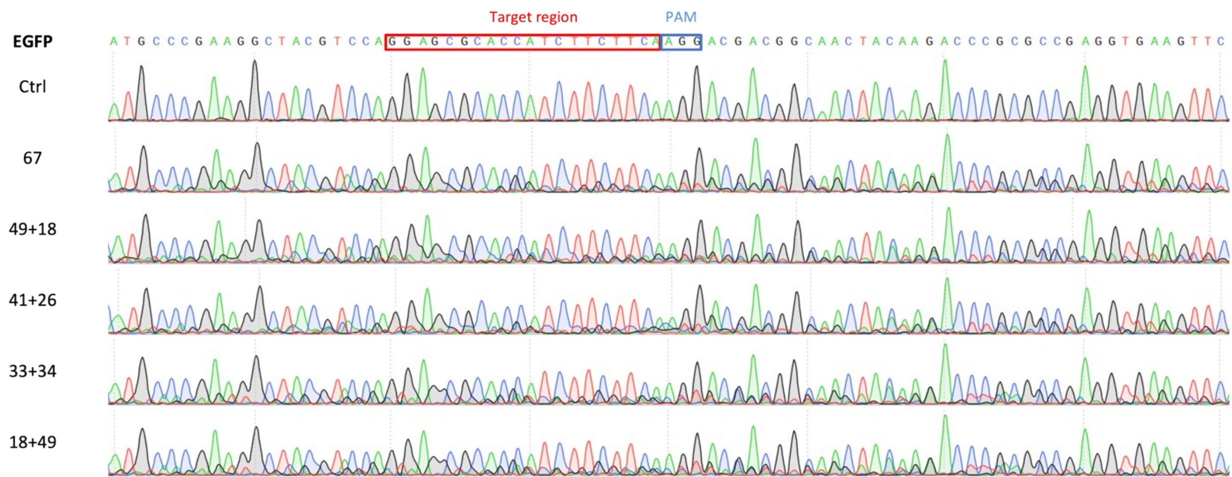
	Sequence (5′ to 3′)
TS	Biotin-TTTTTCTAAGAGCATGCGAATAGTTCCATGAAGAAGATGGTGCGCTCCTCTA
NTS	/iAmMC6T/AGAGGAGCGCACCATCTTCTTCATGGAACTATTCGCATGCTCTTAG

Table S5. Primer sequences used to prepare NGS samples.

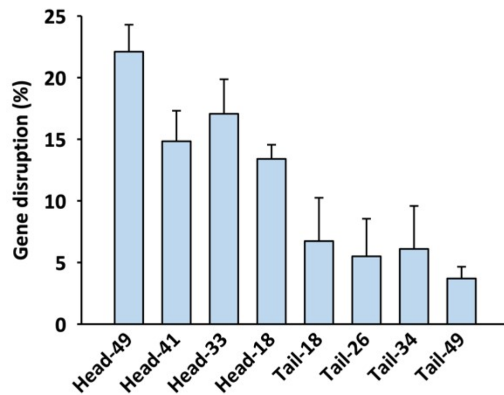
Gene	Primers	Sequence (5′ to 3′)
EGFP	Forward	GTAAACGGCCACAAGTTCAG
	Reverse	TTGAAGTCGATGCCCTTCAG
OT1	Forward	GTAAGCTGGAGAGCTCCGTA
	Reverse	TGAGACCAACCCTCAGTTGT
OT2	Forward	CCACCCCATGAAAATTGGAC
	Reverse	AGTCTCCTACCATCTTCCAC
DNMT1	Forward	CCAGAATGCACAAAGTACTGC
	Reverse	TCCTGATGGTCCATGTCTG
OT1	Forward	AGTGATGTTTCCCTTTGGGC
	Reverse	CTGTGGTCAAGGCTCAGATT
OT2	Forward	AGGCATTGCTGAATCTAAGGAC
	Reverse	TTGGCTCATGACTAACATTTGCTT
EMX1	Forward	AGCTCAGCCTGAGTGTTG
	Reverse	CTCGTGGGTTTGTGGTTG
OT1	Forward	CCCTGTCTTCCAGGAATGTG
	Reverse	CCGCTTGTCCATGTCTAGGAA
OT2	Forward	CACTTGCGGTTTGCACTG
	Reverse	ATCCCGACCTTCATCCCT
FANCF1	Forward	GGGCTTTTAAGTTGCCCAG
	Reverse	TCTTGCCTCCACTGGTTG
OT1	Forward	CATAACCCACTGAAGAAGCAGG
	Reverse	CCTGCTGCACTCTCTGAGTAT
OT2	Forward	TTGCACCTTCCCTCTACACA
	Reverse	CCCAGTGAGACCAGTTTGAG
HEK3	Forward	CTGCCTAGAAAGGCATGGAT
	Reverse	CCAGCCAAACTTGTCAACC

OT1	Forward	TTCAGTTGGCAGAAGCTGAG
	Reverse	CTGGGATGTTTGTGTTGCAC
OT2	Forward	TGTGCCAAAGGATCAACAGC
	Reverse	TGTTGTAGCAGGACGACTGT
RNF2	Forward	CACCCAGTACCTACCACAAA
	Reverse	TTCAGACCATAGCACTTCCC
OT1	Forward	TGAAAACTCAAATGTCCATCAAGTGA
	Reverse	CAAACAATAAACCCTCCTGCC
OT2	Forward	GCAAATGTATAAAACTTTCTGTAGTTACAGTTC
	Reverse	TGCCCAAATAGCTACGTATGAAATG
RUNX1	Forward	TTAATAGGGCTTGGGGAGTC
	Reverse	GCTGCCATTTCATTACAGGC
OT1	Forward	GGGAGGATGTTGGTTTTAGGG
	Reverse	TCTGTGGTAGCATCAACAGAGAA
OT2	Forward	AATCATCTCTCAGACCCCAC
	Reverse	GGGGTTGGTAAGCAGTCTTA
VEGFA	Forward	CCAGACTCCACAGTGCATA
	Reverse	TCCTGGACCCCCTATTTCT
OT1	Forward	ACTGGGGGAAAGTCAGCAGT
	Reverse	CCAGGACCATTTGAGGCACT
OT2	Forward	GAAGGCCGAGAGTGAGTGAG
	Reverse	CTAAAAACCCGCGAGGGGAA


a
EGFP positive cell (%)
100
80
60
40
20
0
Control
Head-49
Head-41
Head-33
Head-18
Tail-18
Tail-26
Tail-34
Tail-49

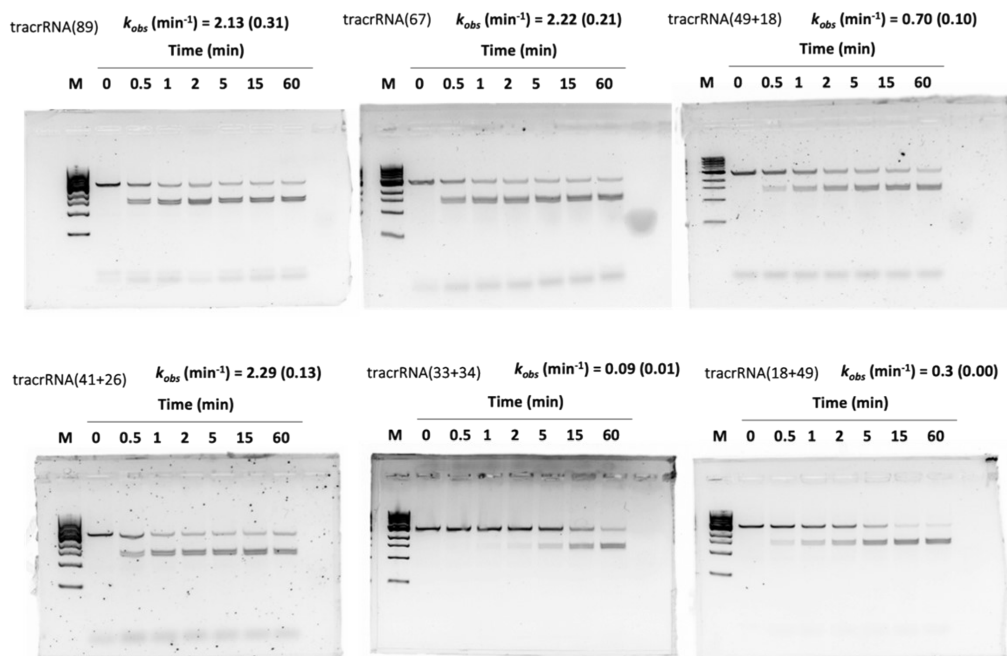

b
Target region
PAM
EGFP
ATGCCCGAAGGCTACGTCCAGGAGCGCACCATCTTCTTCAAGGACGACGGCAACTACAAGACCCGCGCCGAGGTGAAGTTC
Ctrl
67
49+18
41+26
33+34
18+49

c



d

Figure S1. (a) Cytometric analysis to quantify EGFP-positive HeLa cells after treatment with RNP complexes with various tracrRNAs. (b) Representative Sanger sequencing profiles used to estimate gene disruption levels shown in Fig. 1c. (c) TIDE analysis to evaluate the gene disruption efficiency of RNP complexes with various tracrRNAs. (d) Representative Sanger sequencing profiles used to estimate gene disruption levels shown in Fig. S1c.



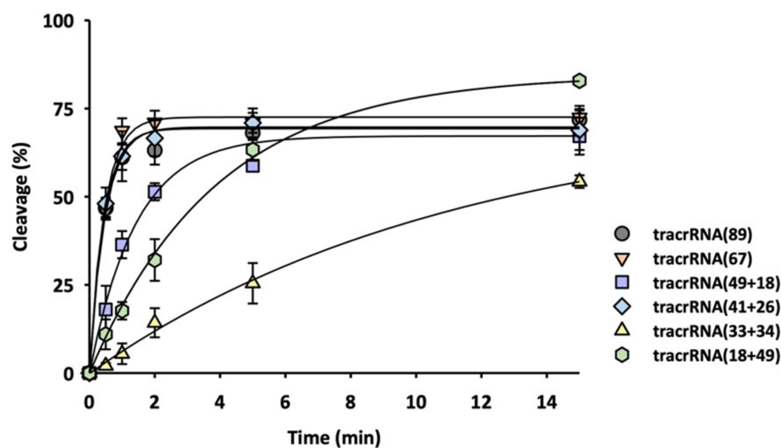


Figure S2. (a) PAGE analysis of in vitro DNA cleavage reactions performed with RNP complexes with split-tracrRNAs. M denotes size makers which are 1, 1.5, 2, and 3 kb from bottom to top. The cleavage rate (k_{obs}) in the right panel determined by curve fitting of (b) which was plotted with the data of three independent experiments. The numbers in the parenthesis denote the standard deviation.

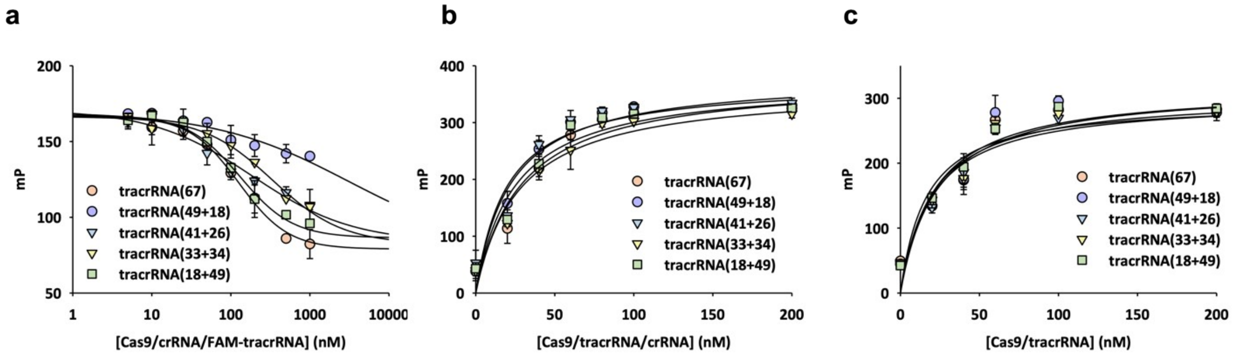
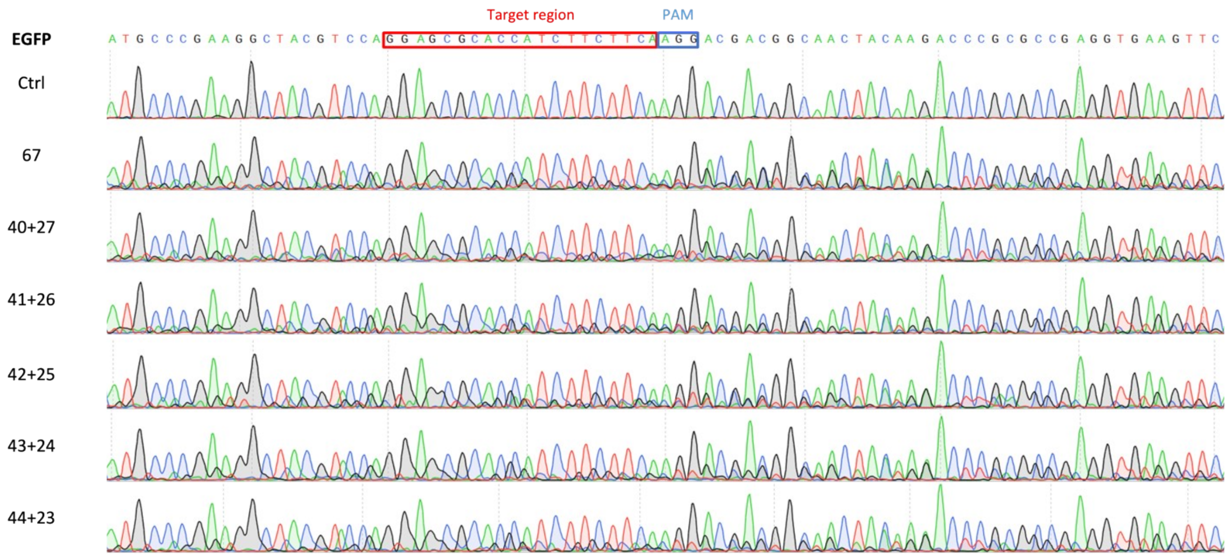
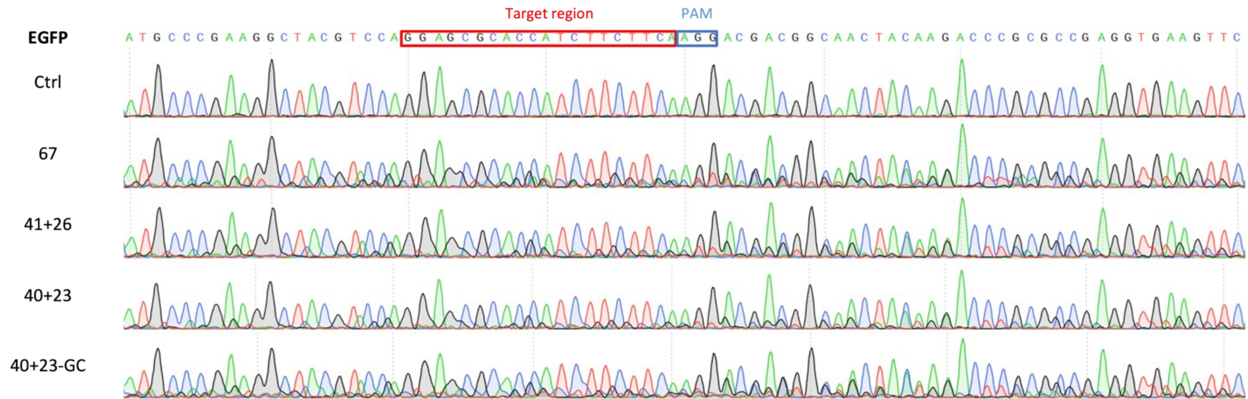


Figure S3. (a) Fluorescence polarization assays to estimate IC_{50} values of split-tracrRNAs against F-tracrRNA for binding to Cas9, (b) K_d values of F-DNA binding to RNP complexes with split-tracrRNAs, and (c) K_d values of F-crRNA binding to Cas9/split-tracrRNA complexes.

a



b



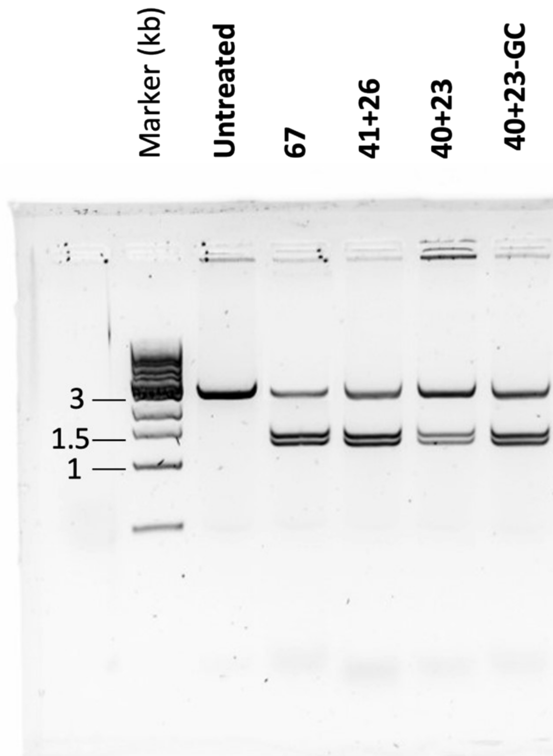
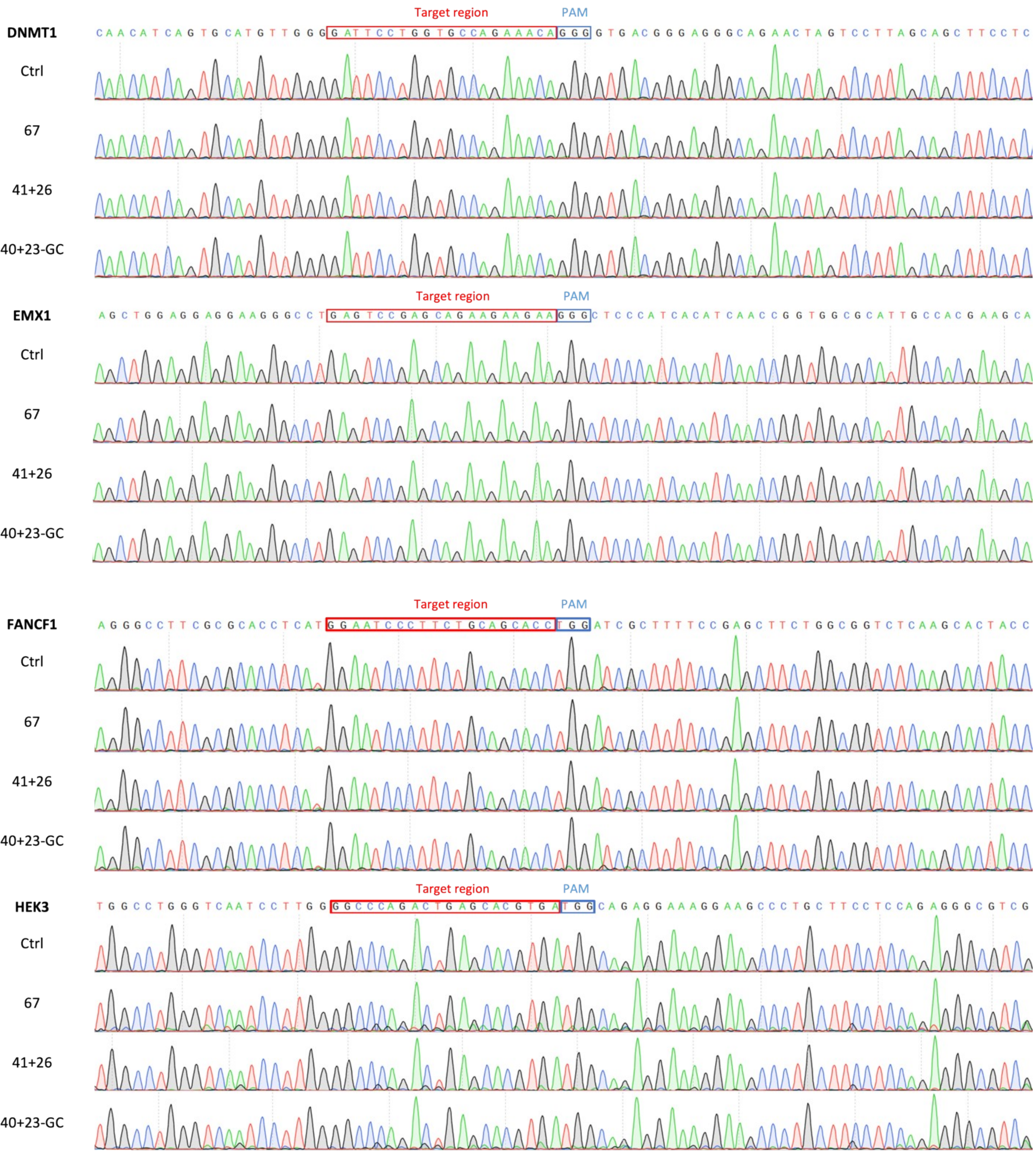


Figure S4. Representative Sanger sequencing profiles used to estimate gene disruption levels shown in (a) Fig. 3c and (b) Fig. 3f. (c) PAGE analysis of in vitro DNA cleavage reactions by RNP complexes with tracrRNA(67), tracrRNA(41+26), tracrRNA(40+23), and tracrRNA(40+23)-GC.



Target region
PAM
DNMT1
CAACATCAGTGCATGTTGGGGATTCCTGGTGCCAGAAACAGGGGTGACGGGAGGGCAGAACTAGTCCTTAGCAGCTTCCTC
Ctrl
67
41+26
40+23-GC
Target region
PAM
EMX1
AGCTGGAGGAGGAAGGGCCTGAGTCCGAGCAGAAGAAGAAGGGCTCCCATCACATCAACCGGTGGCGCATTGCCACGAAGCA
Ctrl
67
41+26
40+23-GC
Target region
PAM
FANCF1
AGGGCCTTCGCGCACCTCATGGAATCCCTTCTGCAGCACCTGGATCGCTTTTCCGAGCTTCTGGCGGTCTCAAGCACTACC
Ctrl
67
41+26
40+23-GC
Target region
PAM
HEK3
TGGCCTGGGTCAATCCTTGGGGCCCAGACTGAGCACGTGATGGCAGAGGAAAGGAAGCCCTGCTTCCTCCAGAGGGCGTCG
Ctrl
67
41+26
40+23-GC

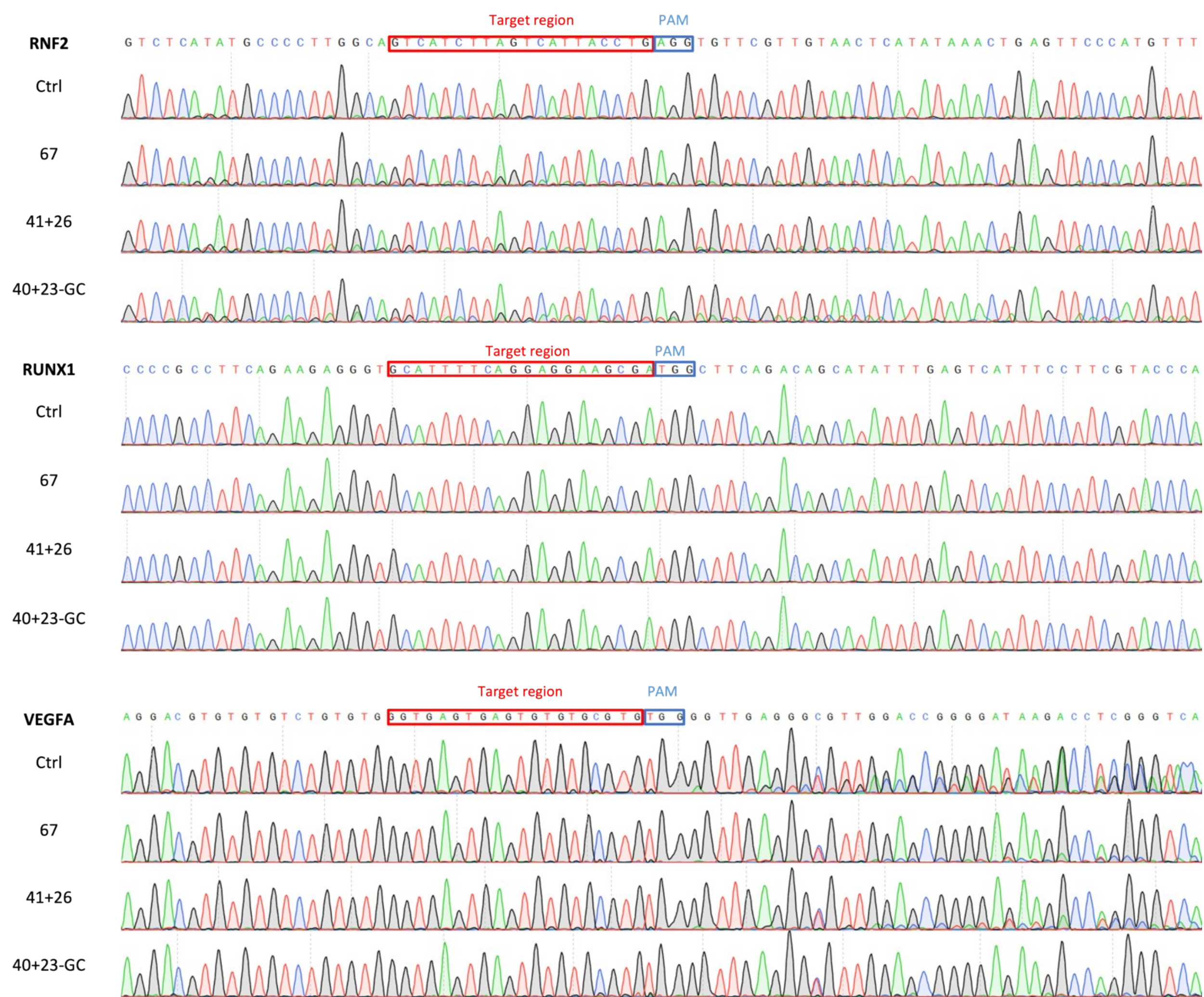


Figure S5. Representative Sanger sequencing profiles used to estimate gene disruption levels shown in Fig. 5a.

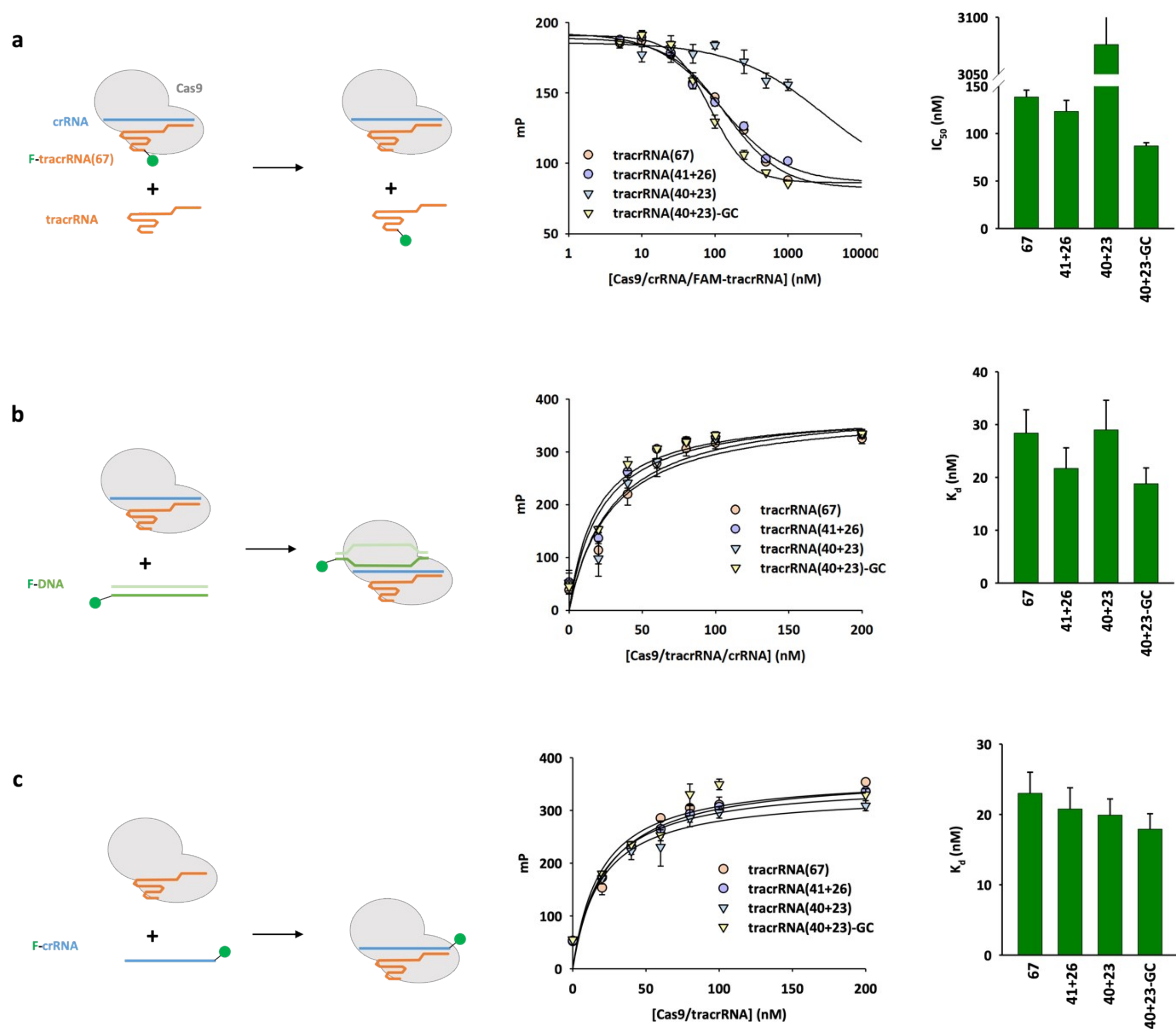


Figure S6. (a) Fluorescence polarization assays to estimate IC_{50} values of split-tracrRNAs against F-tracrRNA for binding to Cas9, (b) K_d values of F-DNA binding to RNP complexes with split-tracrRNAs, and (c) K_d values of F-crRNA binding to Cas9/split-tracrRNA complexes. The scheme for each binding event is presented in the left panel.

References for supporting information

1. Kim,H., Jeong,J., Kim,D., Kwak,G., Kim,S.H., Lee,J.B. (2017) Bubbled RNA-based cargo for boosting RNA interference. Adv. Sci., 4, 1600523.

2. Kim,H.Y., Kang,S.J., Jeon,Y., An,J., Park,J., Lee,H.J., Jang,J.-E., Ahn,J.S., Bang,D., Chung,H.S., Jeonga,C, and Ahn.D.-R. (2019) Chimeric crRNAs with 19 DNA residues in the guide region show the retained DNA cleavage activity of Cas9 with potential to improve the specificity. Chem. Commun., 55(24), 3552-3555.

3. Kim,H., Park,Y., Lee,J.B. (2015) Self-assembled messenger RNA nanoparticles (mRNA-NPs) for efficient gene expression. Sci. Rep., 5, 12737.

4. Brinkman,E.K., Chen,T., Amendola,M., Steensel,B.V. (2014) Easy quantitative assessment of genome editing by sequence trace decomposition. Nucleic Acids Res., 42, e168.

5. Park,J., Lim,K., Kim,J.-S., Bae,S. (2017) Cas-analyzer: an online tool for assessing genome editing results using NGS data. Bioinformatics, 33, 286-288.

6. Jeon,Y., Kim,D., Martín-López,J.V., Lee,R., Oh,J., Hanne,J., FishelR., Lee,J.-B. (2016) Dynamic control of strand excision during human DNA mismatch repair. Proc. Natl. Acad. Sci. U.S.A., 113, 3281-3286.