Supplementary information for

Nonspecific interactions between SpCas9 and dsDNA sites located downstream of the PAM mediate facilitated diffusion to accelerate target search

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Contents:

Methods and Materials
Reference
Supplementary Figures 1-6
Supplementary Tables 1-8
Methods and Materials

Cas9 purification and labeling

*Streptococcus pyogenes* Cas9 (SpCas9) were expressed in *Escherichia coli* strain BL21 (DE3) using the expression plasmid pMJ806 (Addgene plasmid # 39312) (Jinek et al., 2012) and purified as previously described. Briefly, the cells were sonicated on ice and further clarified by centrifugation. The supernatant was first bound to Ni-NTA agarose (Qiagen) in lysis buffer (20 mM HEPES pH 7.5, 500 mM NaCl, 1 mM TCEP, 1 mM PMSF, 10% glycerol), and then eluted with 20 mM HEPES pH 7.5, 250 mM KCl, 1 mM TCEP, and 150 mM imidazole. After removing the His$_6$-MBP affinity tag by overnight digestion at 4°C with TEV protease, the protein was further purified by cation exchange with Source S column (GE Healthcare), eluted with a linear KCl gradient of 0.1 – 1 M KCl, followed by gel filtration chromatography (Superdex 200 Increase 5/150 GL, GE Healthcare) with 50 mM Tris-HCl pH 7.5, 150 mM KCl, 1 mM TCEP, and finally concentrated to 5~10 mg/mL. The SpCas9 variants designed for site-specific labeling were constructed by QuikChange site-directed mutagenesis with Q5® High-Fidelity DNA Polymerase (NEB) and confirmed by DNA sequencing. The variants were purified as described for the wild type proteins.

The SpCas9 variants were labeled as previously described. The labeling reactions were conducted in 50 mM Tris-HCl pH 7.5, 150 mM KCl, 1 mM TCEP containing 30 mM Cas9 and 500 mM maleimide-Cy5 (LumiProbe). After 2 hours incubation at room temperature (~25 °C), reactions were quenched by adding 10 mM DTT, and excess free dyes were removed by Sephadex G-25 column (Nap-5, GE Healthcare). Labeling efficiency was estimated by measuring A$_{280}$ and A$_{649}$. Proteins were all flash-frozen in liquid nitrogen and stored at -80°C.

In-vitro transcription and purification of RNA

The sgRNA was transcribed and purified as previously described. DNA templates carrying T7 promoter and sgRNA sequence were cloned into a pMV Vector. The plasmid was linearized with BamHI digestion and further recovered as a template for in vitro transcription via HiScribe™ T7 High Yield RNA Synthesis Kit (NEB). RNA was isolated by 15% denaturing PAGE and dissolved in stocking buffer (300 mM NaAc pH 5.2, 1 mM EDTA) at 55°C. The soluble fraction containing RNA is recovered by ethanol precipitation. The integrity and purity of RNA were confirmed by 1.2% agarose gel electrophoresis. The purified RNA sample was dissolved in nuclease-free H$_2$O (Invitrogen).

Nucleic acid labeling

Oligonucleotides were purchased from Sangon Biotech (Shanghai, China) with a C6 amine modification on the specific position as indicated in Table S3-4. Nucleic acids were labeled using N-hydroxysuccinimido (NHS)-ester derived fluorophores following procedures from manufacturers (LumiProbe). In brief, NHS-fluorophores and amino modified DNA or RNA strands were mixed at
20:1 molar ratio and kept at room temperature (~23°C) for two hours. Labeled nucleic acid were separated from excess free fluorophores through ethanol precipitation.

DNA Duplex (< 60 bp) substrates were generated by annealing equimolar amounts of labeled DNA strands and unlabeled complementary strands at 65°C for 1-2 min and then slowly cooling down to room temperature. Long dsDNAs (> 60 bp) were generated by PCR amplification with fluorophore labeled primers and recovered from gel electrophoresis. Fluorophores labeled crRNA and unlabeled tracrRNA was annealed at 1:1 molar ratio to form the crRNA/tracrRNA complex.

**In-vitro cleavage assay.**

The target-containing plasmid (Table S1) used in Fig. 1a was purchased from Qinglan Biotech (Jiangsu, China) and linearized into two fragments by PCR amplification, purified and recovered from 0.8~2% agarose gel. Primers (Table S1) used for linearization were purchased from Sangon Biotech (Shanghai, China). DNA substrates were prepared by mixing equimolar amounts of two linearized fragments in reaction buffer (50 mM Tris-HCl pH 7.5, 150 mM KCl, 5 mM MgCl2, 1 mM DTT). sgRNA was pre-heated in reaction buffer at 95°C for 5 min and slowly cooling down to room temperature. SpCas9/RNA complexes were reconstituted by mixing SpCas9 with a 4× molar excess of the sgRNA in reaction buffer and incubating at 25°C for 20 minutes. Cleavage assay were initiated at the time point when adding Cas9/RNA complex (final concentration 28 nM) into two linearized DNA fragments (final concentration 7 nM), and terminated by adding 6× DNA loading buffer (NEB). For cleavage assay presented in Figs. 1d-f and S1d, the reaction containing DNA substrates with a final concentration of 10 nM (molar ratio of DNA:Cas9/RNA=1:2). Percentage of cleaved DNA was resolved by agarose electrophoresis and apparent cleavage rates were extracted by single exponential curves. S.E.M. were estimated through three or more replicates.

For cleavage assay towards short target-containing fragments (≤240 bp), the DNA substrates were labeled with NHS-Cy5 and quenched by 2 × formamide loading dye and resolved on 8~12.5% denaturing polyacrylamide gels containing 8 M urea and visualized by GE typhoon FLA9500 imaging system.

**Acquisition of single-molecule fluorescence data**

All smFRET experiments were performed at 25 ºC in the reaction imaging buffer (50 mM Tris-HCl pH 7.5, 150 mM KCl, 5 mM MgCl2, 1 mM DTT, 3 mg/mL glucose, 100 µg/mL glucose oxidase (Sigma-Aldrich), 40 µg/mL catalase (Roche), 1 mM cyclooctatetraene (COT, Sigma-Aldrich), 1 mM 4-nitrobenzylalcohol (NBA, Sigma-Aldrich), 1.5 mM 6-hydroxy-2,5,7,8-tetramethyl-chromane-2-carboxylic acid (Trolox, Sigma-Aldrich – added from a concentrated DMSO stock solution)). Single-molecule spectroscopic microscopy was performed on a home-built objective-type TIRF microscope, based on a Nikon Eclipse Ti-E with an EMCCD camera (Andor iXon Ultra 897), and solid state 532 nm excitation lasers (Coherent Inc. OBIS Smart Lasers) ². Fluorescence emission from the probes was collected by the microscope and spectrally separated by interference dichroic (T635lpxr, Chroma) and
bandpass filters, ET585/65m (Chroma, Cy3) and ET700/75m (Chroma, Cy5), in a Dual-View spectral splitter (Photometrics, Inc., Tucson, AZ). Hardwares were controlled and single molecule movies were collected using Cell Vision software (Beijing Coolight Technology).

Single-molecule fluorescence experiments

Biotinylated DNA strands and non-modified DNA strands used for single-molecule fluorescence assay (Table S3) were purchased from Sangon Biotech (Shanghai, China). DNA substrates were prepared by mixing equimolar amounts of biotinylated and non-modified DNA strands (≤ 60 bp) heating at 65°C for 1-2 min and then slowly cooling down to room temperature, or by PCR amplification (> 60 bp).

For single-molecule fluorescence assay, 200 pM biotinylated DNA was firstly immobilized to streptavidin-decorated slides. The excessive unbound DNAs were removed by washing with reaction imaging buffer. Single fluorophore labeled Cas9/RNA (100 nM : 400 nM) were pre-incubated at 25 °C in reaction buffer (50 mM Tris-HCl pH 7.5, 150 mM KCl, 5 mM MgCl₂, 1 mM DTT). To record the appearance time of Cas9/RNA, the collection of movies began 5 s prior to injection of 50 µL pre-incubated 5 nM labeled Cas9/RNA complex in reaction imaging buffer and carried out without further washing. All movies were acquired at 500 ms per frame. The association rates of Cas9/RNA towards different DNA substrates were determined by recording each appearance time upon individual stable binding event occurred and fitted with single exponential decay.

For single-molecule FRET assay, 200 pM Cy5-labeled dsDNA was firstly immobilized on surface. Cy3-labeled RNA was pre-incubated with Cas9 at a molar ratio of Cy3-RNA: Cas9 = 1:2 at 25 °C in reaction buffer. The collection of movies began 5 s prior to injection of 50 µL Cy3 labeled RNA/Cas9 complex with the final concentration of 5 nM. The movies were recorded at a time resolution of 200 ms per frame.

For single-molecule fluorescence experiments under low salt conditions, the buffer composition was 50 mM HEPES-NaOH pH 7.5, 10 mM NaCl, 2 mM MgCl₂ and 1 mM DTT.

Cryo-EM sample preparation and structural analysis for the spCas9 complex

DNA oligo (5'- GATATTCGTAGGCCGATAAAAGATGAGACGCTGGCGATTAGCTAATCGCC-AGCGTCTCATCTTTATGCGCTACGAATATC -3’) was annealed at 72°C to generate symmetric dsDNA target for Cas9. The wild type spCas9 protein was firstly incubated with gRNA by 1 to 1 ratio at 4°C for 30mins in the buffer of 20 mM HEPES-NaOH pH 7.5, 150 mM NaCl, 1 mM MgCl₂ and 1 mM DTT. Then the RNP was further incubated with dsDNA substrate by 1 to 1 ratio at 4°C for 2hr in the buffer of 20 mM HEPES-NaOH pH 7.5, 150 mM NaCl, 5mM EDTA and 1 mM DTT. No further purification was performed, and the final concentration of the complex was about 2.5 μM. We followed the same protocol for cryoEM grid preparation as described before 3. Around 3.7 µL droplets of the sample were placed onto C-flat grids (2/2μm). The grid freezing was processed in the FEI Vitrobot. Data were acquired using an FEI Titan Krios transmission electron microscope operated at 300 keV.
with a GIF energy filter with defocus ranging from −0.5 to −1.8 μm. 53 frame movies were recorded using SerialEM 4 on a Gatan K3 Summit direct electron detector operated in super-resolution mode(pixel size 0.57 Å) with a total dose of 53 e− Å² s⁻¹.

We followed similar protocol for data analysis as described before 5 but using the updated version of CryoSpaC V3.1.0 instead 6. Cas9 dimer was refined with either C1 symmetry or C2 symmetry. The EM map with C2 symmetry was used for model building and interpretation. CryoEM models were visualized and presented in UCSF-chimera 7. PDB model 5F9R 8 was used as the template to build the dimeric Cas9 complex in coot, and then the re-built model was refined with the EM map and validated in Phenix1.14 9.

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Figure S1. Representative gel images and intensity plots of time-dependent in-vitro cleavage assay of Cas9. Related to Figure 1.

a-c) Representative gel scanning images (upper) and intensity plot (lower) of time dependent cleavage assay towards D2188 DNA (a), D480 DNA (b), and D30 DNA (c), respectively. D2188 in (a) was cleaved into two fragments with equal length and was resolved by 1–2% agarose gel. Linearized supplemental 1708 bp non-target containing dsDNAs were added into D480 in (b) to maintain identical total length and base composition to (a), and the cleavage assay was resolved by 2% agarose gel. The D30 in (c) was labeled using NHS-Cy5 and the cleavage assay was resolved by 12.5% denaturing polyacrylamide gels, visualized by GE typhoon FLA9500 imaging. Linearized supplemental 2158 bp non-target containing dsDNAs were added.

D240 in (e), D120 DNA in (f), and D59 DNA in (g) were labeled with NHS-Cy5 and the cleavage assays were resolved by 8–12.5% denaturing polyacrylamide gels, visualized by GE typhoon
FLA9500 imaging. Linearized unlabeled supplemental fragments were added but were not visible in (e-g). h) Gel scanning images of time-dependent cleavage assay towards 617-bp DNA containing a centered target site with no PAM sites (substrate 1), with 10 PAMs flanked on non-target strand (substrate 2), and with 10 PAMs flanked on target strand (substrate 3). 3 or more replicates were performed and quantified for the cleavage assay on each dsDNA.
Figure S2. Single-molecule fluorescence assays on three different DNA series containing distinct target sites. Related to Figure 2.

a) Scheme of single-molecule fluorescence assay to capture apparent target search rates of Cas9 towards DN-PAM-36 DNAs. b-d) Distributions of appearance time of Cas9 on three series of DN-PAM-36 DNAs. DN-PAM-36<sup>81</sup> (b), DN-PAM-36<sup>82</sup> (c), and DN-PAM-36<sup>83</sup> (d) containing different target sites, which were listed and colored in red. Single exponential decay was used to extract average appearance time from injection until stable binding events of Cas9 on dsDNAs occurred.
Figure S3. Single-molecule FRET assay on dsDNA with biotinylation at the same end of dsDNA extension. Related to Figure 3.

a) Scheme of single-molecule FRET assay to capture apparent target search rates of Cas9 on D40-PAM-NT# DNAs with biotinylation on the 3’ end of the non-target strand. FRET pair uses the same design as indicated in Figure 2a. The distance between the PAM site and the 3’ end of the non-target strand ranges from 0 bp to 128 bp. b) Distributions of appearance time of Cas9 on D40-PAM-NT# DNAs, which were fitted by single exponential decay. c) Model scheme of single-molecule FRET assay of recording apparent target search rates of Cas9 on DN-PAM-28# DNAs with biotinylation on the 5’ end of the non-target strand. FRET pair is designed the same as shown in Figure 2d. The distance between the PAM site and the 5’ end of the non-target strand ranges from 20 bp (minimal space for Cas9/RNA/DNA complex formation according to structural information) to 148 bp. d) Distributions of appearance time of Cas9 on DN-PAM-28# DNAs, which were fitted by single exponential decay.
Figure S4. Single particle cryoEM analysis. Related to Figure 5.

a) The workflow for single particle cryoEM analysis. The dimer map refined with C2 symmetry was used for model building and interpretation. b) The EM map for Cas9 dimer was shown in different angles and sections. The Cas9 protein density was colored in grey, gRNA in tan, dsDNA in light blue. The two PAMs in dsDNA were colored in yellow, and the density for the stretching loop from Cas9 PI domain was colored in orange. c) The EM map for Cas9 monomer. The DNA density downstream of the PAM were not well resolved due to the structural flexibility. d) The gold-standard FSC curves reported by CryoSparc non-uniform refinement for Cas9 dimer with C2 symmetry.
Figure S5. Nuclease activity of Cas9 variants. Related to Figure 5.

a) Cleavage assays of Cas9 variants toward 10 nM Cy5 labeled target DNA. The cleaved products were resolved by 12.5% denaturing polyacrylamide gels and visualized by GE typhoon FLA9500 imaging system. b) Cleavage percentage of 100 nM pre-formed Cas9/sgRNA complex quantified from gel images in (a). The percentage was measured by imageJ and were quantified by Gaussian fitting.
Figure S6. Single molecule fluorescence assay of Cas9-EQR. Related to Figure 2.

a) Scheme of single-molecule fluorescence assay to capture apparent target search rates of Cas9-EQR on D48-PAM-N DNAs. The PAM sequence is 5’-TGAG-3’ that is recognized by Cas9-EQR. b) Distributions of appearance time of Cas9-EQR on D48-PAM-N DNAs, which were fitted by single exponential decay. c) Scheme of capturing apparent target search rates of Cas9-EQR on D N-PAM-36 DNAs. d) Distributions of appearance time of Cas9-EQR on D N-PAM-36 DNAs.
Figure S7. Dwell time distributions and binding rates of Cas9/RNA complex on dsDNAs containing no target sites. Related to Figure 5 and Figure S4.

a) 25 nM Cas9/RNA complex was introduced to surface immobilized 59 bp DNAs containing no target sites with no PAM (upper), 1 PAM (middle) and 3 PAMs (bottom). crRNA was labeled with Cy3 and pre-incubated with Cas9. 2.5 ms per frame was used to capture transient interactions. The weighted average dwell time (left) and appearance time (right) were extracted using double exponential decay.

b) Representative single-molecule fluorescence trajectories of Cas9/RNA transient interact with dsDNAs containing no target sites with no PAM (upper), 1 PAM (middle) and 3 PAMs (bottom). c) 25 nM Cas9/RNA complex was introduced to microscope flow channel without immobilized dsDNA. Almost no non-specific transient binding events was observed.

d) The average emission time of surface immobilized Cy3-labeled crRNA/Cas9 complex before photobleaching when using 2 μW/μm² excitation with 2.5 ms per frame. e) Scatter plot showing the binding rates ($k_{on}$) for each dsDNA containing multiple PAMs without target site. DNA sequences were listed in Table S3. Error bars represent standard error of the mean.
Table S1. Plasmid and primer sequences for in-vitro cleavage assay. Related to Figure 1.

| Name a                  | Sequences b                  |
|-------------------------|------------------------------|
| Target plasmid           |                              |
| plasmid (2188bp-F)       | TAGTGGCAATCTGACCGGCATTCTCGTGT |
| plasmid (2188bp-R)       | TGGAGTGAACGAAGATAGACAGCTGGT  |
| plasmid (30bp-F)         | GAGCGGAAATAGGAGCAGCGATGGCAT  |
| plasmid (30bp-R)         | TTGTTACTTCCGAGCTTCTCCATTTAT  |
| Vector-30-F              | CGTCACGCTCATATAGACCGTGC      |
| Vector-30-R              | AGCAGAGATTTCTCTCGTCTCTAG     |
| Substrate #1 | Substrate #2 | Substrate #3 |
|--------------|--------------|--------------|
| hep | hep | hep |
| | | |
TTTGCATACGCTGCTGCGCTGACGACATCACAAAATTGACGCTC
AAGTCAGAGCTGCGAAAGCTGACAGCACTATAAAGATAGCAGCGTTTG
CGCTGCAAGCTGCTCCTGCGGCTTCTGCTGTTGGCGAGCTGCGCTTTAGCGCA
TAGCTGTGCTGCTTTCTGCTTCGCGAAGCGTGCGCTTTCTCAATGCTCA
CGCTGTAGCCTATCTCAGTTCCGTGTAACCTGTCTGACAGTACGAT

*Sequences are listed from 5'-3'. Only non-target (NT) strands of dsDNA are shown. Protospacer sequences, PAM sequences and positions containing PAM at the complementary strands are underlined.*
Table S2. Cleavage rates of *in-vitro* cleavage assay. Related to Figure 1.

| Target dsDNA groups | Cleavage rate ($10^{-3} \text{s}^{-1}$) |
|---------------------|---------------------------------------|
| D30                 | $2.8 \pm 0.5$                         |
| D59                 | $6.2 \pm 0.7$                         |
| D120                | $8.3 \pm 0.5$                         |
| D240                | $8.3 \pm 0.5$                         |
| D480                | $9.8 \pm 0.5$                         |
| D960                | $9.3 \pm 0.5$                         |
| D2188               | $11.2 \pm 0.7$                        |
| Substrate #1        | $8.2 \pm 0.8$                         |
| Substrate #2        | $6.2 \pm 0.8$                         |
| Substrate #3        | $3.7 \pm 0.5$                         |

All results were averages of three repeated experiments.
Table S3. Sequence of sgRNA and DNA substrates used for single-molecule fluorescence assay. Related to Figures 2, 4, S1, S5 and S6.

| Description | Sequences * |
|-------------|-------------|
| sgRNA       | 5'-GAGCGACATGGTGTTCATGCTATTTTACTGATATGACTGACGCTGG-3' |
| sgRNA1      | 5'-Biotin-AGCGATGCTGGTCTCATCTGATTTTTACTGATATGACGCTGGTCGCATCAT3'-Biotin-3' |
| sgRNA2      | 5'-GCAGCAAGCACAAGCGCAATAAGATGAGACGCTGG-3' |
| sgRNA3      | 5'-Biotin-AGCGATGCTGGTCTCATCTGATTTTTACTGATATGACGCTGGTCGCATCAT3'-Biotin-3' |
|              | 5'-Biotin-AGCGATGCTGGTCTCATCTGATTTTTACTGATATGACGCTGGTCGCATCAT3'-Biotin-3' |
| D48-PAM-0   | 5'-Biotin-AGCGATGCTGGTCTCATCTGATTTTTACTGATATGACGCTGGTCGCATCAT3'-Biotin-3' |
| D48-PAM-7   | 5'-Biotin-AGCGATGCTGGTCTCATCTGATTTTTACTGATATGACGCTGGTCGCATCAT3'-Biotin-3' |
| D48-PAM-16  | 5'-Biotin-AGCGATGCTGGTCTCATCTGATTTTTACTGATATGACGCTGGTCGCATCAT3'-Biotin-3' |
| D48-PAM-32  | 5'-Biotin-AGCGATGCTGGTCTCATCTGATTTTTACTGATATGACGCTGGTCGCATCAT3'-Biotin-3' |
| D48-PAM-64  | 5'-Biotin-AGCGATGCTGGTCTCATCTGATTTTTACTGATATGACGCTGGTCGCATCAT3'-Biotin-3' |
| D48-PAM-128 | 5'-Biotin-AGCGATGCTGGTCTCATCTGATTTTTACTGATATGACGCTGGTCGCATCAT3'-Biotin-3' |
| D8-PAM-28   | 5'-GGAGCGATGCTGGTCTCATCTGATTTTTACTGATATGACGCTGGTCGCATCAT3'-Biotin-3' |
| D16-PAM-28  | 5'-GGAGCGATGCTGGTCTCATCTGATTTTTACTGATATGACGCTGGTCGCATCAT3'-Biotin-3' |
| D20-PAM-28  | 5'-GGAGCGATGCTGGTCTCATCTGATTTTTACTGATATGACGCTGGTCGCATCAT3'-Biotin-3' |
| D28-PAM-28  | 5'-GGAGCGATGCTGGTCTCATCTGATTTTTACTGATATGACGCTGGTCGCATCAT3'-Biotin-3' |
| D36-PAM-28  | 5'-GGAGCGATGCTGGTCTCATCTGATTTTTACTGATATGACGCTGGTCGCATCAT3'-Biotin-3' |
| D52-PAM-28  | 5'-GGAGCGATGCTGGTCTCATCTGATTTTTACTGATATGACGCTGGTCGCATCAT3'-Biotin-3' |
| D84-PAM-28  | 5'-GGAGCGATGCTGGTCTCATCTGATTTTTACTGATATGACGCTGGTCGCATCAT3'-Biotin-3' |
| D148-PAM-28 | 5'-GGAGCGATGCTGGTCTCATCTGATTTTTACTGATATGACGCTGGTCGCATCAT3'-Biotin-3' |
| D8-PAM-36   | 5'-CTAAAGCGAGACGCTGGTCTCATCTGATTTTTACTGATATGACGCTGGTCGCATCAT3'-Biotin-3' |
| D16-PAM-36  | 5'-CTAAAGCGAGACGCTGGTCTCATCTGATTTTTACTGATATGACGCTGGTCGCATCAT3'-Biotin-3' |
| D20-PAM-36  | 5'-CTAAAGCGAGACGCTGGTCTCATCTGATTTTTACTGATATGACGCTGGTCGCATCAT3'-Biotin-3' |
| D28-PAM-36  | 5'-CTAAAGCGAGACGCTGGTCTCATCTGATTTTTACTGATATGACGCTGGTCGCATCAT3'-Biotin-3' |
| D36-PAM-36  | 5'-CTAAAGCGAGACGCTGGTCTCATCTGATTTTTACTGATATGACGCTGGTCGCATCAT3'-Biotin-3' |
| D52-PAM-36  | 5'-CTAAAGCGAGACGCTGGTCTCATCTGATTTTTACTGATATGACGCTGGTCGCATCAT3'-Biotin-3' |
sgRNA guide sequence and target sites are underlined. #1, #2 and #3 refer to 3 different spacer sequences. PAM sequence is 5'-TGG-3' and underlined. Biotinylated DNA duplexes are named referring to the PAM location relative to the 5' and 3' end of the NT strand, and only non-target strand sequence is listed in this table. DNAs containing full-cognate target sequences paired with the spacers in sgRNA#1, sgRNA#2 or sgRNA#3 are designed based on the same principles and are not listed for clarity.
Table S4. Apparent target search rates of single fluorophore labeled Cas9 under different salt concentrations. Related to Figures 2, 4 and S1.

| Buffer | DNA substrates | Appearance rates of Cas9/RNA (μM⁻¹ s⁻¹) |
|--------|----------------|----------------------------------------|
|        | D48-PAM-0      | 0.20 ± 0.02                            |
|        | D48-PAM-8      | 0.36 ± 0.02                            |
|        | D48-PAM-16     | 0.88 ± 0.04                            |
|        | D48-PAM-32     | 1.36 ± 0.04                            |
|        | D48-PAM-64     | 1.84 ± 0.08                            |
|        | D48-PAM-128    | 1.72 ± 0.08                            |
|        | D8-PAM-28      | 0.44 ± 0.03                            |
|        | D16-PAM-28     | 0.59 ± 0.05                            |
|        | D20-PAM-28     | 0.69 ± 0.05                            |
|        | D28-PAM-28     | 0.74 ± 0.06                            |
|        | D36-PAM-28     | 0.77 ± 0.06                            |
|        | D52-PAM-28     | 0.83 ± 0.07                            |
|        | D84-PAM-28     | 0.71 ± 0.08                            |
|        | D148-PAM-28    | 0.75 ± 0.06                            |
| Buffer 1 | D20-PAM-8     | N.A                                    |
|        | D28-PAM-8      | N.A                                    |
|        | D148-PAM-8     | N.A                                    |
|        | D8-PAM-36₁     | 0.67 ± 0.04                            |
|        | D20-PAM-36₁    | 1.54 ± 0.06                            |
|        | D28-PAM-36₁    | 1.3 ± 0.1                              |
|        | D148-PAM-36₁   | 1.4 ± 0.1                              |
|        | D8-PAM-36₂     | 1.22 ± 0.06                            |
|        | D20-PAM-36₂    | 2.08 ± 0.04                            |
|        | D28-PAM-36₂    | 1.74 ± 0.12                            |
|        | D148-PAM-36₂   | 1.65 ± 0.08                            |
|        | D8-PAM-36₃     | 1.00 ± 0.05                            |
|        | D20-PAM-36₃    | 1.92 ± 0.28                            |
|        | D28-PAM-36₃    | 1.64 ± 0.12                            |
|        | D148-PAM-36₃   | 1.52 ± 0.08                            |
| Buffer 2 (low salt) | D20-PAM-8  | 1.14 ± 0.16                            |
|        | D148-PAM-8     | 2.4 ± 0.2                              |
|        | D48-PAM-0      | 0.9 ± 0.1                              |
|        | D48-PAM-128    | 3.3 ± 0.3                              |

Buffer 1: 50 mM Tris-HCl pH 7.5, 150 mM KCl, 5 mM MgCl₂, 1 mM DTT; Buffer 2: 50 mM HEPES-NaOH pH 7.5, 10 mM NaCl, 2 mM MgCl₂, 1 mM DTT. All results were averages of three repeated experiments. #1, #2 and #3 refer to 3 different spacer sequences listed in Table S3.
| Description | Sequences  
|-------------|------------------|
| 5’Cy3-crRNA | 5’-(Cy3)GAUGAGACCGGCUUUUAGAGCUAUGCUUUGG-3’ |
| 3’Cy3-crRNA | 5’-GAUGAGACCGGCUUUUAGAGCUAUGCUUUGG(Cy3)-3’ |
| tracrRNA     | 5’-GGAGACAAUCCAAACAGCAUGAGCAGAUUUAAAAAAGGCUAGUCGUUUAAUCAACUUGAAGUUUGCCAGUGGUUGGCAGUUAACGUG-3’ |
| 5’Cy3-sgRNA  | 5’-(Cy3)GAUGAGACCGGCUUUUAGAGCUAUGCUUUGGAAACAAAAACAGCAUGAGCAGAUUUAAAUAGGCUAGUCGUUUAAUCAACUUGAAGUUUGCCAGUGGUUGGCAGUUAACGUG-3’ |
| D40-PAM-0    | 5’-Biotin-TTCGATCTCATAATCTGACAT(Cy5)ACGCATAAAGATGAGACCGCTG-3’ |
| D40-PAM-8    | 5’-Biotin-TTCGATCTCATAATCTGACAT(Cy5)ACGCATAAAGATGAGACCGCTGTCGATCAT-3’ |
| D40-PAM-16   | 5’-Biotin-TTCGATCTCATAATCTGACAT(Cy5)ACGCATAAAGATGAGACCGCTGTCGATCATTTTACTG-3’ |
| D40-PAM-32   | 5’-Biotin-TTCGATCTCATAATCTGACAT(Cy5)ACGCATAAAGATGAGACCGCTGTCGATCATTTTACTGACGACAGCCTGTCGCAAGCGT-3’ |
| D40-PAM-64   | 5’-Biotin-TTCGATCTCATAATCTGACAT(Cy5)ACGCATAAAGATGAGACCGCTGTCGATCATTTTACTGACGACAGCCTGTCGCAAGCGT-3’ |
| D40-PAM-128  | 5’-Biotin-TTCGATCTCATAATCTGACAT(Cy5)ACGCATAAAGATGAGACCGCTGTCGATCATTTTACTGACGACAGCCTGTCGCAAGCGT-3’ |
| D40-PAM-0f   | 5’-TTGCATCTCATAATCTGACAT(Cy5)ACGCATAAAGATGAGACCGCTG-3’ |
| D40-PAM-8f   | 5’-TTGCATCTCATAATCTGACAT(Cy5)ACGCATAAAGATGAGACCGCTGTCGATCAT-3’ |
| D40-PAM-128f | 5’-TTGCATCTCATAATCTGACAT(Cy5)ACGCATAAAGATGAGACCGCTGTCGATCATTTTACTGACGACAGCCTGTCGCAAGCGT-3’ |
| D8-PAM-28    | 5’-TGAGAGCGCTGTCGATCATATTA(Cy5)ACTGACTATGAGTGCACAGCGA-3’ |
| D16-PAM-28   | 5’-CATAAAGATGAGACGCTGTCGATCATATTA(Cy5)ACTGACTATGAGTGCACAGCGA-3’ |
| D20-PAM-28   | 5’-GACGCATAAAGATGAGACGCTGTCGATCATATTA(Cy5)ACTGACTATGAGTGCACAGCGA-3’ |
| D28-PAM-28   | 5’-ATCTGACAGACGCTGTCGATCATATTA(Cy5)ACTGACTATGAGTGCACAGCGA-3’ |
| D36-PAM-28   | 5’-ATCTCATAAATCTGACAGACGCTGTCGATCATATTA(Cy5)ACTGACTATGAGTGCACAGCGA-3’ |
| D52-PAM-28   | 5’-CTTAAGGCAGCTGTCGATCATATTA(Cy5)ACTGACTATGAGTGCACAGCGA-3’ |
| D84-PAM-28   | 5’-ATCTGACAGACGCTGTCGATCATATTA(Cy5)ACTGACTATGAGTGCACAGCGA-3’ |
| D148-PAM-28  | 5’-ATCTGACAGACGCTGTCGATCATATTA(Cy5)ACTGACTATGAGTGCACAGCGA-3’ |

* Cognate sequences between RNA and dsDNA are underlined. PAM sequence is 5’-TGG-3’ and underlined.

Biotinylated DNA duplexes are named referring to the target and PAM location relative to the 5’ and 3’ end of the NT strand, Cy3 or Cy5 labeling positions are indicated. and only non-target strand, Cy3 or Cy5 labeling positions is listed in this table. For DN-PAM-28 series, the Cy5 was labeled at the corresponding position at the target strand.
* indicates the biotinylation of dsDNA on the opposite end.
Table S6. Apparent target search rates of Cas9 captured by smFRET assay under different salt concentrations. Related to Figures 3, 4 and S2.

| Buffer   | DNA substrates | Appearance rates of Cas9/RNA (μM⁻¹ s⁻¹) |
|----------|----------------|----------------------------------------|
| Buffer 1 | D40-PAM-0      | 0.22 ± 0.05                           |
|          | D40-PAM-8      | 0.43 ± 0.04                           |
|          | D40-PAM-16     | 1.4 ± 0.1                             |
|          | D40-PAM-32     | 2.0 ± 0.2                             |
|          | D40-PAM-64     | 2.5 ± 0.2                             |
|          | D40-PAM-128    | 2.8 ± 0.2                             |
|          | D8-PAM-28      | 0.56 ± 0.05                           |
|          | D16-PAM-28     | 0.5 ± 0.1                             |
|          | D20-PAM-28     | 0.9 ± 0.1                             |
|          | D28-PAM-28     | 1.2 ± 0.1                             |
|          | D36-PAM-28     | 1.2 ± 0.1                             |
|          | D52-PAM-28     | 1.2 ± 0.1                             |
|          | D84-PAM-28     | 1.37 ± 0.08                           |
|          | D148-PAM-28    | 1.22 ± 0.07                           |
|          | D40-PAM-0#     | N. A                                  |
|          | D40-PAM-8#     | 0.29 ± 0.03                           |
|          | D40-PAM-128#   | 2.2 ± 0.2                             |
|          | D20-PAM-28#    | 1.33 ± 0.05                           |
|          | D28-PAM-28#    | 2.0 ± 0.1                             |
|          | D148-PAM-28#   | 2.2 ± 0.2                             |
| Buffer 2 | D40-PAM-0      | 1.8 ± 0.2                             |
| (low salt)| D40-PAM-8      | 1.8 ± 0.2                             |
|          | D40-PAM-128    | 5.6 ± 0.6                             |
|          | D20-PAM-28     | 2.0 ± 0.2                             |
|          | D28-PAM-28     | 3.2 ± 0.5                             |
|          | D148-PAM-28    | 8.3 ± 0.7                             |

Buffer 1: 50 mM Tris-HCl pH 7.5, 150 mM KCl, 5 mM MgCl₂, 1 mM DTT; Buffer 2: 50 mM HEPES-NaOH pH 7.5, 10 mM NaCl, 2 mM MgCl₂, 1 mM DTT. All results were averages of three repeated experiments.

# indicates the biotinylation of dsDNA on the opposite end.
Table S7. Apparent target search rates of Cas9 variants with disruptive non-specific interactions with dsDNA. Related to Figures 5

| Cas9 variants | DNA substrates | Appearance rates of Cas9/RNA (μM⁻¹ s⁻¹) |
|---------------|----------------|-----------------------------------------|
| Cas9-WT       | D40-PAM-0      | 0.20 ± 0.02                             |
|               | D40-PAM-128    | 2.9 ± 0.2                               |
| Cas9-K1153A   | D40-PAM-0      | 0.22 ± 0.02                             |
|               | D40-PAM-128    | 1.4 ± 0.2                               |
| Cas9-6A       | D40-PAM-0      | 0.20 ± 0.03                             |
|               | D40-PAM-128    | 0.62 ± 0.08                             |

All results were averages of three repeated experiments.
Table S8. Apparent target search rates of Cas9 variants which recognized alternative PAM sequences. Related to Figures S5.

| Cas9 variants | DNA substrates | Appearance rates of Cas9/RNA (μM⁻¹ s⁻¹) |
|---------------|----------------|----------------------------------------|
|               | D48-PAM-0      | 0.0027 ± 0.0006                        |
|               | D48-PAM-8      | 0.004 ± 0.001                          |
|               | D48-PAM-16     | 0.014 ± 0.001                          |
|               | D48-PAM-32     | 0.016 ± 0.002                          |
|               | D48-PAM-64     | 0.017 ± 0.001                          |
|               | D48-PAM-128    | 0.017 ± 0.001                          |
| Cas9-EQR      | D8-PAM-36      | 0.003 ± 0.01                           |
|               | D16-PAM-36     | 0.004 ± 0.001                          |
|               | D8-PAM-36      | 0.005 ± 0.02                           |
|               | D28-PAM-36     | 0.008 ± 0.01                           |
|               | D36-PAM-36     | 0.011 ± 0.001                          |
|               | D52-PAM-36     | 0.011 ± 0.001                          |
|               | D84-PAM-36     | 0.011 ± 0.001                          |
|               | D148-PAM-36    | 0.011 ± 0.001                          |
|               | D48-PAM-0      | 0.05 ± 0.01                            |
|               | D48-PAM-8      | 0.09 ± 0.01                            |
|               | D48-PAM-128    | 0.61 ± 0.01                            |
| Cas9-VQR      | D8-PAM-36      | 0.067 ± 0.004                          |
|               | D16-PAM-36     | 0.23 ± 0.01                            |
|               | D20-PAM-36     | 0.22 ± 0.01                            |
|               | D148-PAM-36    | 0.22 ± 0.01                            |

PAM sequence was designed as 5’-TGAG-3’ for Cas9-EQR and Cas9-VQR variants. All results were averages of three repeated experiments.