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Supporting Information

High-affinity and undissociated capillary electrophoresis for DNA strand exchange analysis

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Experimental Section

Chemicals and materials

Escherichia coli RecA was purchased from New England Biolabs (Ipswich, MA, USA). SSB and ATP were supplied by Promega (Madison, WI, USA) and Sigma-Aldrich (St. Louis, MO, USA), respectively. Ultrapure water (18.2 MΩ•cm) was generated using a Purelab Ultra system (ELGA LabWater, High Wycombe, UK). An uncoated, fused-silica capillary (25 μm i.d., 360 μm o.d.) was purchased from Yongnian Optical Fiber (Hebei, China). Single-use syringe filters (0.2-μm pore size) were purchased from Sartorius Stedim Biotech (Goettingen, Germany). All other reagents were of analytical or high-performance liquid chromatography grade and supplied by Beijing Chemical Reagents (Beijing, China).

DNA oligonucleotides

DNA oligonucleotides used for strand exchange were synthesized and purified by Sangon Biotech (Shanghai, China). The synthesized oligonucleotides were dissolved in annealing buffer containing 10 mM Tris–HCl (pH 8.0), 50 mM NaCl, and 0.1 mM EDTA. To prepare dsDNA probes, complementary strands were annealed by heating to 90 °C for 5 min and then slowly cooling to room temperature over 3 h, followed by purification with 12% native polyacrylamide gel electrophoresis. Detailed sequences and information concerning fluorescent labelling of these probes are provided in Table S1.

CE-LIF detection

CE-LIF analysis was performed using an in-house setup previously successfully applied to measure DNA–protein interactions.[1,2] Briefly, an uncoated, fused-silica capillary (25 μm i.d., 365 μm o.d.) was pre-activated with 0.2 M NaOH for 4 h and equilibrated with 1× Tris-glycine (TG) buffer [25 mM Tris and 192 mM glycine (pH 8.3)] for 2 h. Unless otherwise indicated, the capillary length was 26 cm, with an effective length of 20 cm from the inlet to the detection window. Prepared samples were electro-kinetically injected into the capillary using a voltage of 15 kV for 5 s, followed by separation in TG buffer at a voltage of 20 kV. After effective CE separation, the fluorophores were excited by a 543.5-nm laser at the detection window. Following image collection using a 10× objective lens and filtration using a 575 ± 15-nm filter, the emitted fluorescence was detected by a photomultiplier tube and processed using a specialized chromatography workstation (HW-2000;

Qianpu Software Co., Shanghai, China). To prevent possible clogging of the capillary, all solutions were filtered through a single-use syringe filter (0.2-μm pore size). After each run, the capillary was successively washed with 20 mM NaOH for 2 min, ultrapure water for 1 min, and TG buffer for 2 min. All operations were conducted at room temperature.

Kinetic CE analysis

TMR–ssDNA (10 nM) and SSB tetramers (100 nM) were pre-incubated in 1× Tris–HCl (TH) buffer [20 mM Tris-HCl, 10 mM Mg^{2+} (pH 7.4)] at 37 °C prior to CE-LIF analysis. The apparent binding affinity constant (K_a) and the apparent dissociation rate constant (k_{off}) were calculated using the following equations, as described previously:[3,4]

$$K_a = \frac{1 + (C + D)/F}{[SSB]_0\left(1 + \frac{F}{C + D}\right) - [DNA]_0} \quad (1)$$

$$k_{off} = ln(\frac{C + D}{C})/t_C \quad (2)$$

where *C*, *D*, and *F* represent the peak area of the undissociated ssDNA–SSB complex, ssDNA dissociated from the complex, and unbound ssDNA, respectively (Figure 1a), $[SSB]_0$ and $[DNA]_0$ are the total concentrations of SSB tetramer and ssDNA in the sample, respectively, and t_C is the migration time of the stable ssDNA–SSB complex.

Recombinase RecA-catalysed DNA strand exchange

To perform DNA strand-exchange reactions, an invading ssDNA (ss51 at the indicated concentration), a donor dsDNA (TMR–ds51, 50 nM), ATP (1.0 mM), and a recombinase (wild type RecA or RecA mutants, as indicated; 3.0 μM) were mixed in 1× TH buffer and incubated at 37 °C for 20 min. Tetrameric *E. coli* SSB (50 nM) was then added to interact with ssDNA and stop the strand-exchange reactions. Following an additional 10-min incubation at 37 °C, the samples were immediately subjected to CE-LIF analysis.

Quantification of strand-exchange efficiency

To accurately quantify strand-exchange efficiency by CE-LIF, we considered two factors: quantum yield and migration time.[5] In this study, both the quantum yield and migration time of TMR labelling of DNA changed due to the strand-exchange and SSB-binding reactions (Figures 4a and S5); however, neither parameters for the TMR-labelled donor dsDNA changed. Therefore, we

employed the change in fluorescence signal of TMR–ds51 to quantify the strand-exchange efficiency. Simultaneously, free TMR dye at a constant concentration was added to each sample as an internal standard (IS) to calibrate the detected peak area:

$$Strand\ exchange\ efficiency = 1 - \frac{[dsDNA]_{remained}}{[dsDNA]_{initial}} \times \frac{[IS]_{initial}}{[IS]_{remained}} \quad (3)$$

where $[dsDNA]_{initial}$ and $[dsDNA]_{remained}$ represent the detected peak areas of dsDNA before and after the DNA strand-exchange reaction, respectively, and $[IS]_{initial}$ and $[IS]_{remained}$ are the detected peak areas of IS before and after the DNA strand-exchange reaction, respectively.

DNA-mismatch detection

For DNA-mismatch detection, ss51 (5.0 nM), RecA (1.0 μM), ATP (1.0 mM), and TMR-labelled donor dsDNA (containing a various number of mismatches) (2.5 nM; see Table S1) were mixed in 1× TH buffer and incubated at 37 °C for 20 min. Tetrameric *E. coli* SSB (50 nM) was then added to interact with ssDNA and stop the strand-exchange reactions. Following an additional 10-min incubation at 37 °C, the samples were immediately subjected to CE-LIF analysis.

Fluorescence spectra measurements

Fluorescence spectra measurements were performed using a JASCO FP-8300 spectrofluorometer (Tokyo, Japan).[6,7] The excitation wavelength was set at 543.5 nm, and emission spectra were recorded from 555 nm to 630 nm. Both the excitation and emission slit bandwidths were set at 5.0 nm. The samples were pre-incubated in 1× TH buffer for 10 min. The final concentrations of free TMR or TMR-labelled DNA probes were 10 nM, and the final concentrations of SSB, RecA, and ATP were 100 nM, 3 μM, and 1 mM, respectively.

Statistical analysis

Statistical data are presented as the mean ± s.d. from three independent experiments (n = 3). Significance differences were determined according to a $P < 0.01$ or $P < 0.001$. Figures were produced using Origin 8.0 software (OriginLab Corp., Northampton, MA, USA).

Supporting Tables

Table S1. Sequences and fluorescent labels of DNA oligos.

ID	Sequence (5'→3')	Label
TMR–ss15	TTCTGGAGGAGACTG	5' TMR
ss15(−)	CAGTCTCCTCCAGAA	
TMR–ss21	TTCTGGAGGAGACTGGACGGA	5' TMR
ss21(−)	TCCGTCCAGTCTCCTCCAGAA	
TMR–ss30	TTCTGGAGGAGACTGGACGGATCTTAAGGT	5' TMR
ss30(−)	ACCTTAAGATCCGTCCAGTCTCCTCCAGAA	
TMR−ss51 (also FM−ss51)	TTCTGGAGGAGACTGGACGGATCTTAAGGTAGGAAACGCATGACATAGTAC	5' TMR
TMR-ss33	CCAGTCTCCGCCGGCCAGTGTTACCCTTAGAGC	22nd TMR-dT
ss45(-)	GCTCTAAGGGTAACACTGGCCGGCGGAGACTGGCAGACTCAGCAT	
Cy3–ss51	TTCTGGAGGAGACTGGACGGATCTTAAGGTAGGAAACGCATGACATAGTAC	5' Cy3
Cy5–ss51	TTCTGGAGGAGACTGGACGGATCTTAAGGTAGGAAACGCATGACATAGTAC	5' Cy5
ss51	TTCTGGAGGAGACTGGACGGATCTTAAGGTAGGAAACGCATGACATAGTAC	
ss51(−)	GTACTATGTCATGCGTTTCCTACCTTAAGATCCGTCCAGTCTCCTCCAGAA	
1MM ss51	TTCTGGAGGAGACTGGACGGATCTTTAGGTAGGAAACGCATGACATAGTAC	5' TMR
2MM ss51	TTCTGGAGGAGAGTGGACGGATCTTTAGGTAGGAAACGGATGACATAGTAC	5' TMR
3MM ss51	TTCTGGAGGAGAGTGGACGGATCTTTAGGTAGGAAACGGATGACATAGTAC	5' TMR
FM	FM-ss51/ss51(−)	
1MM	1MM-ss51/ss51(−)	
2MM	2MM-ss51/ss51(−)	
3MM	3MM-ss51/ss51(−)	

Notes: (1) dsDNA probes were prepared by annealing ssDNA probes with their complementary strands [indicated by "(−)"].

(2) ss51(−) is the complementary strand for all of fluorescently labelled ss51 probes.

(3) ss51 was used as the invading ssDNA for DNA strand-exchange reactions.

(4) 1MM, one mismatch; 2MM, two mismatches; 3MM, three mismatches; FM, fully matched.

Supporting Figures

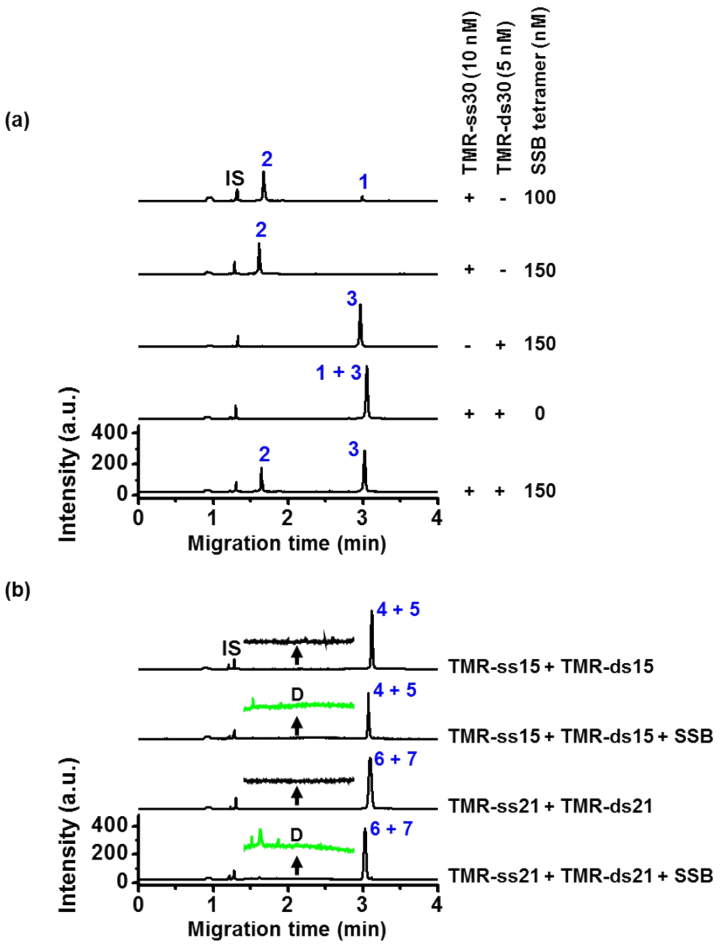

Figure S1. SSB-binding-mediated CE separation of ssDNA and dsDNA. (a) Complete resolution of ssDNA (30 nt) from dsDNA in the presence of higher concentrations of SSB. Peaks 1, 2, and 3 indicate unbound TMR–ss30, SSB-bound TMR–ss30, and TMR–ds30, respectively. The concentrations of DNA and SSB-binding protein are indicated. (b) Non-separation of ssDNA (15 nt and 21 nt) from dsDNA. Peaks 4, 5, 6, and 7 indicate unbound TMR–ss15, TMR–ds15, TMR–ss21, and TMR–ds21, respectively. The inserted green curve (D) indicates the dissociated fraction of ssDNA from the complex. TMR-labelled probes:10 nM; and SSB tetramer: 100 nM.

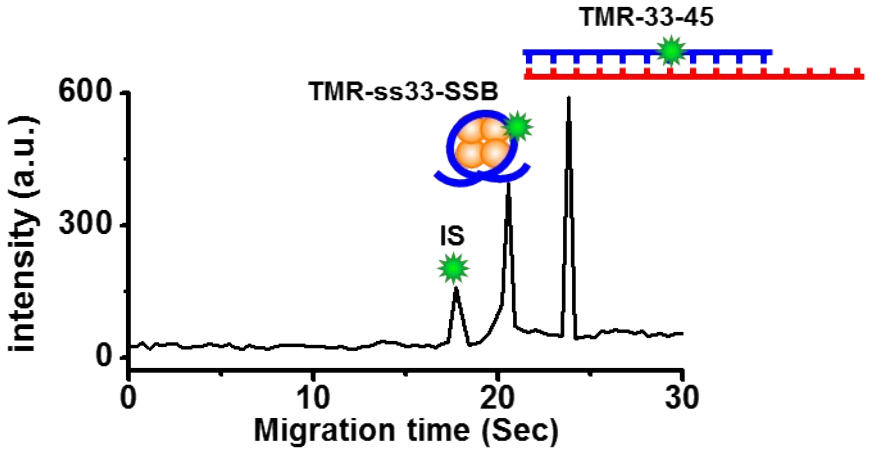

Figure S2. Fast separation of ssDNA and dsDNA for hybridization assays via stable SSB binding. The TMR-33-45 probe was prepared by annealing the unlabelled ss45(−) (45 nt) with a complementary TMR–ss33 (33 nt) (see Table S1). TMR-ss33: 10 nM; TMR-33-45: 5 nM, and SSB tetramer: 50 nM. The capillary length was 16 cm, with an effective length of 8 cm.

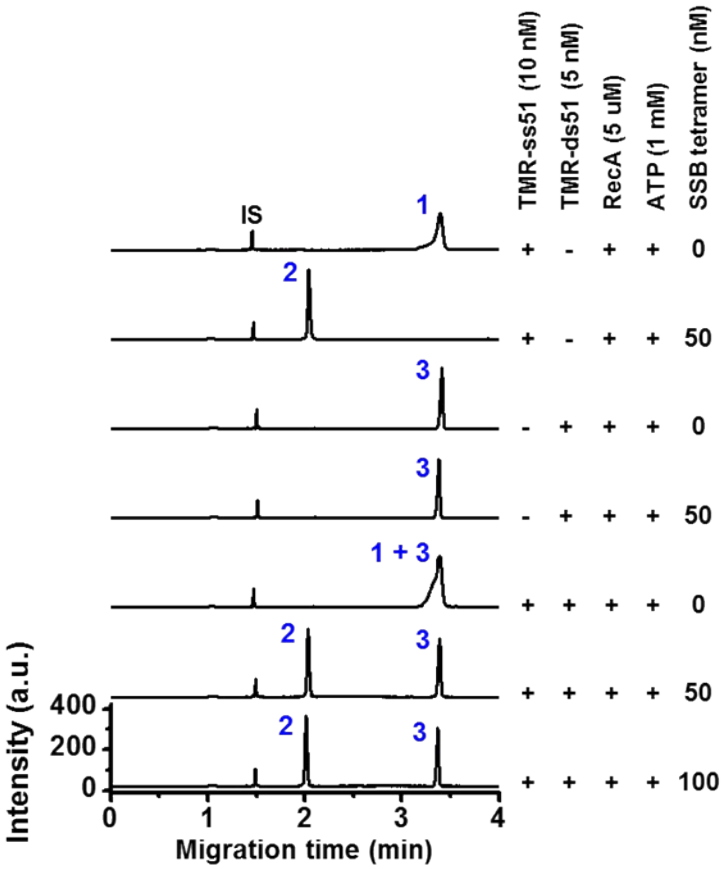

Figure S3. SSB selectively binds ssDNA and forms a stable and undissociated ssDNA–SSB complex in the presence of RecA and ATP, which are required for DNA strand-exchange reactions. TMR-labelled DNA probes (10 nM TMR–ss51, 5.0 nM TMR–ds51, or both) were first mixed with 3.0 μM RecA and 1.0 mM ATP in 1× TH buffer and incubated at 37 °C for 10 min prior to addition of SSB tetramer. Peaks 1, 2, and 3 indicate fractions of TMR–ss51–RecA, TMR–ss51-SSB complex, and free TMR–ds51, respectively.

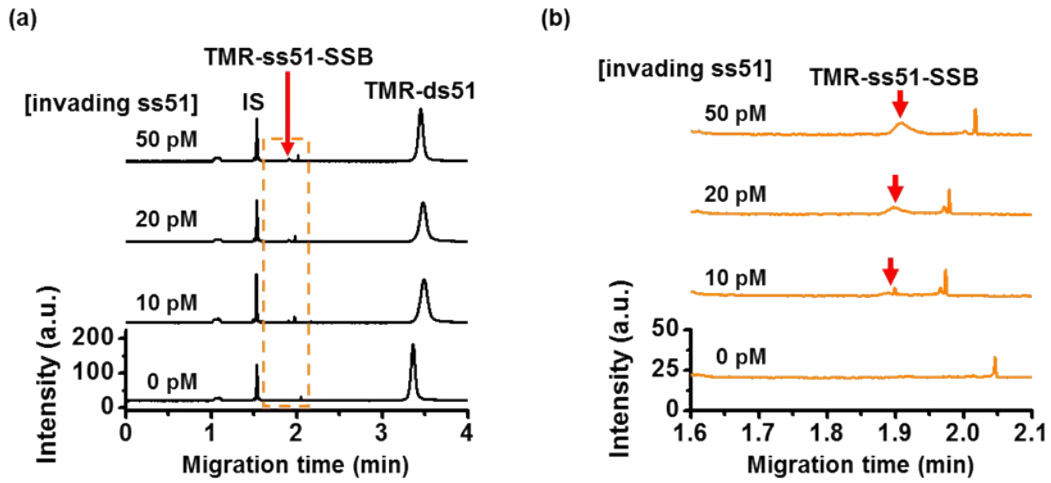

Figure S4. Ultra-high sensitivity of SSB-binding-mediated CE-LIF assays for detection of RecA-catalysed DNA strand-exchange reactions. (a) Electropherograms obtained from CE-LIF detection. (b) The dashed box shows 8× higher-resolution areas of the electropherograms. The products (i.e., TMR–ss51–SSB complex) of NA strand-exchange reactions are denoted by red arrows. The concentrations of TMR–ds51, RecA, ATP, and SSB were 5.0 nM, 3.0 μM, 1.0 mM, and 50 nM, espectively. The concentration of invading ss51 (0–50 pM) is indicated in each panel.

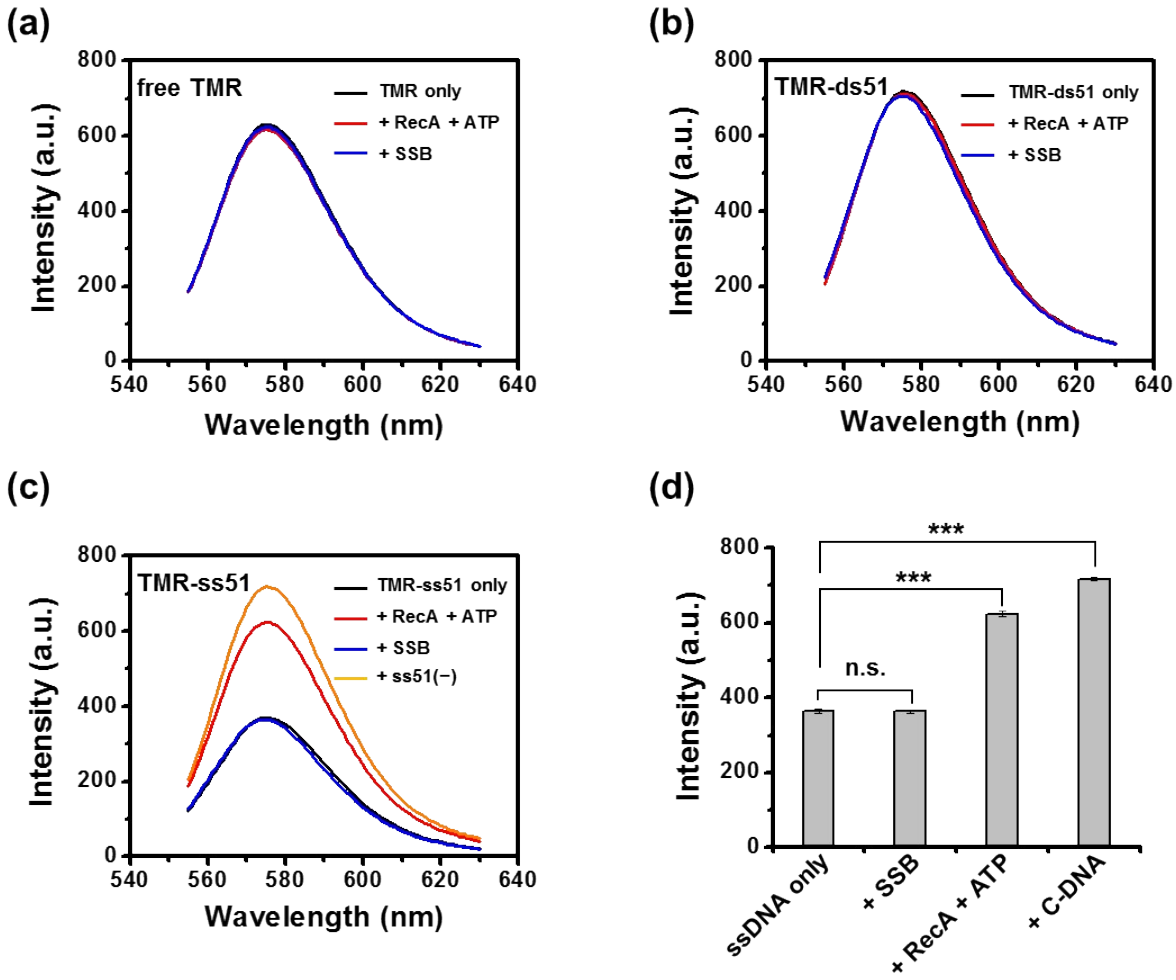

Figure S5. The fluorescence spectra of TMR-labelled DNA oligonucleotides. (a and b) Addition of RecA and SSB did not influence the fluorescence spectra of free TMR (a) or TMR-labelled dsDNA (i.e., TMR-ds51) (b). (c) Addition of RecA and ATP or complementary ssDNA [i.e., ss51(−)] enhanced the fluorescence of TMR-labelled ssDNA (i.e. TMR-ss51) probe, whereas the addition of SSB did not affect the fluorescence of TMR–ss51. (d) Fluorescence intensity at 575 nm quantified from the spectra shown in (c). n.s., not significant ($P > 0.05$); ***$P < 0.001$. The concentration of free TMR or TMR-labelled DNA probes was 10 nM. The concentrations of SSB, RecA, ATP, and ss51(−) were 100 nM, 3 μM, 1 mM, and 20 nM, respectively. Of note, ss51(−) is a complementary strand to ss51 (see Supporting Table S1).

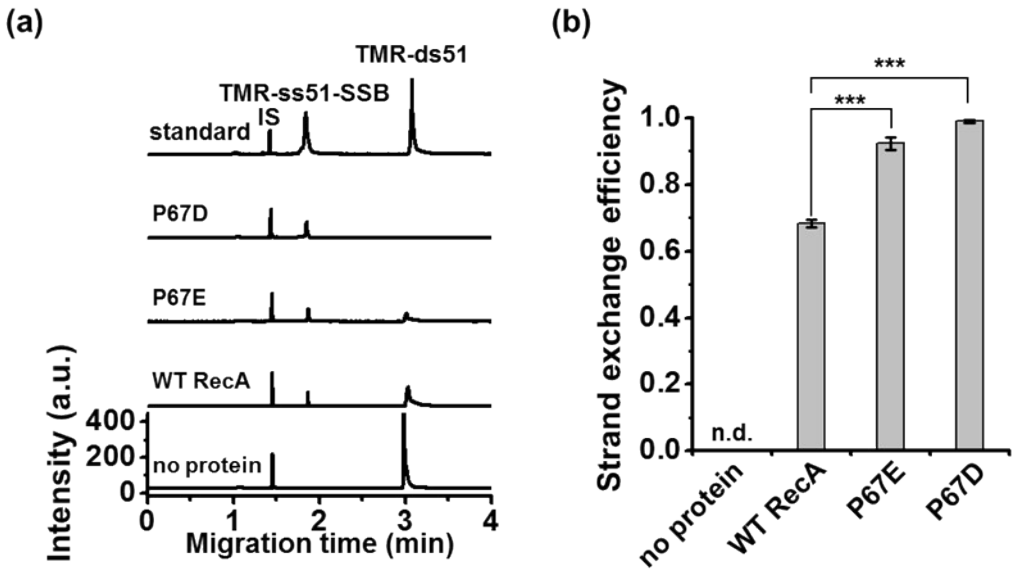

Figure S6. Stable SSB-binding-mediated CE-LIF assessment of the activities of RecA mutants. (a) Electropherograms obtained from stable SSB-binding-mediated CE-LIF analysis of DNA strand exchange catalysed by WT RecA or RecA mutants (P67E and P67D). (b) Bar plot of strand-exchange efficiency quantified from the electropherograms shown in (a). The concentrations of ss51, TMR–ds51, RecA proteins (WT, P67E, or P67D), ATP, and SSB were 5 nM, 5 nM, 3 μM, 1 mM, and 50 nM, respectively. ***P < 0.001. Data represent the mean ± s.d. from three independent experiments (n = 3). WT, wild type.

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