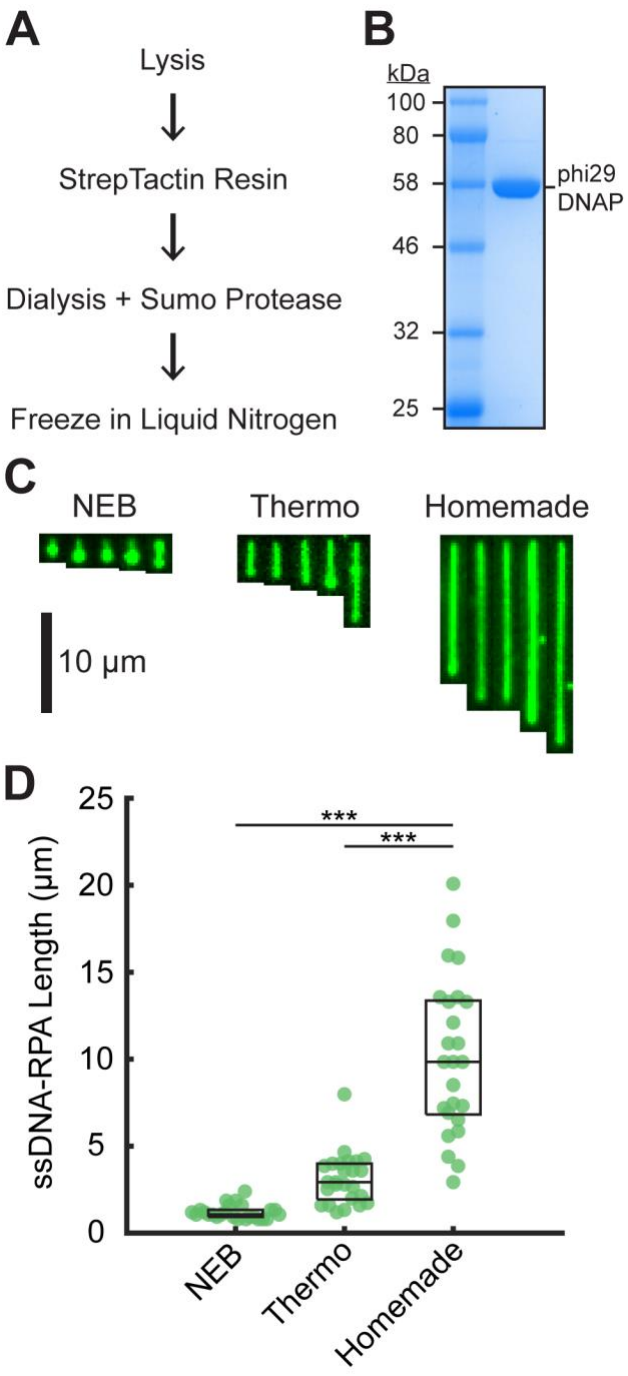

Figure S1: phi29 DNA Polymerase Purification and Analysis. (A) Purification and (B) Coomassie-stained SDS-PAGE gel of purified phi29 DNAP. (C) Comparison of representative RPA-GFP coated ssDNAs synthesized with homemade, and commercial NEB (catalog #M0269) and ThermoFisher (catalog #EP0091) phi29 DNAPs. (D) Quantification of 25 ssDNA molecules for each polymerase shown in (C). Boxplots denote the median, first and third quartiles of the data. Statistical significance was calculated using the Mann-Whitney U test. *** p-values < 1.0×10^{-07}.

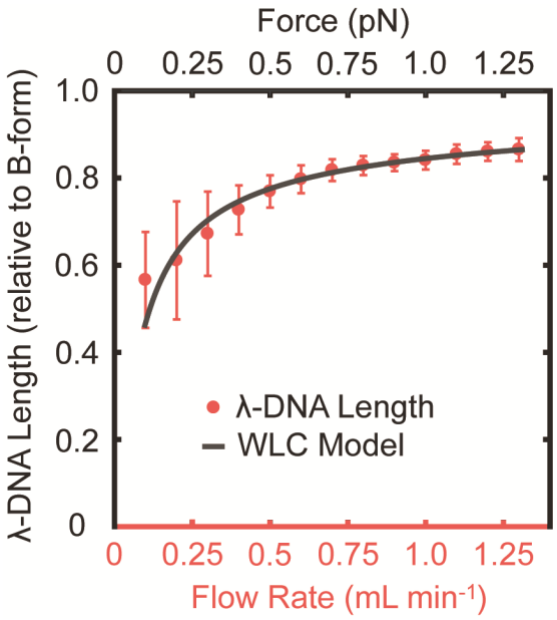

Figure S2: Calculation of Flow Extension. The relationship between applied flow rate and λ-DNA length (mean ± standard deviation) relative to λ-DNA B-form contour length, fitted to the Worm-Like Chain (WLC) model (solid line) with the fitted persistence length of 43 ± 3 nm and a contour length of 16.3 ± 0.1 μm.[1,2] The experiments described in this work were conducted at a 1 mL min^{-1} flow rate, providing about 1 pN of force on the DNA molecules. At this applied force, dsDNA molecules are extended to ~84% of the B-form contour length. Based on the crystallographic B-form inter-base distance of 0.34 nm, we calculate the inter-base distance at 84% extension to be 0.29 nm.

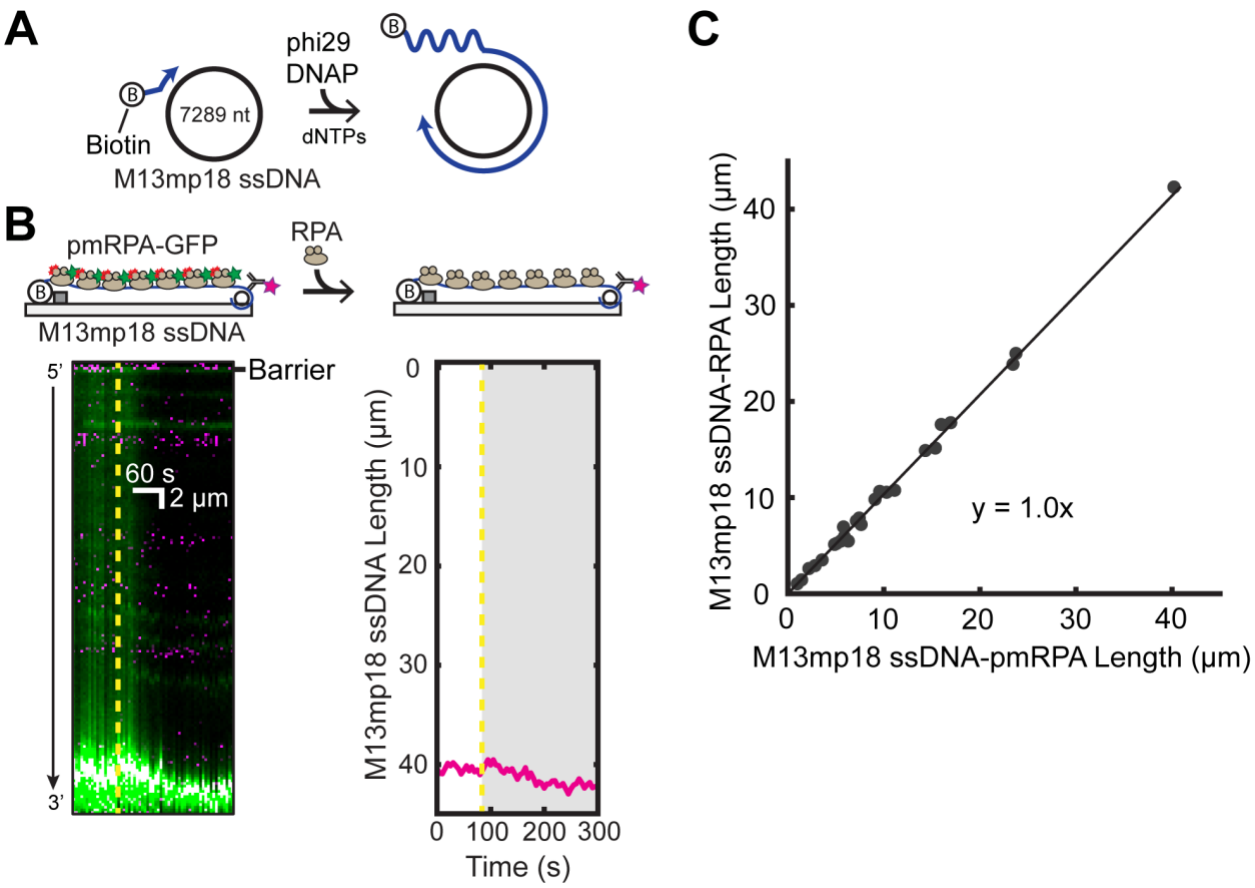

Figure S3: Analysis of RPA Binding Modes on M13mp18 ssDNA. (A) A biotinylated primer (blue) is annealed to M13mp18 ssDNA (black) to generate a substrate for rolling circle replication. ssDNA is synthesized by addition of phi29 DNAP and dNTPs. (B) Cartoon (top) and kymograph (left) of ssDNA-bound pmRPA-GFP (green) as it is replaced by wt RPA (unlabeled). The corresponding change in DNA length is shown in magenta. Dashed yellow line denotes when pmRPA-GFP was replaced with RPA in the buffer. Right: ssDNA end-label tracking. (C) Scatter plot of ssDNA length with pmRPA and RPA. Solid black line is a linear analysis of >3 flow cells (N=25 DNA molecules).

Table S1: Oligonucleotides used in this study.

Name	Sequence
Primer	5’ – /Bio/TC TCC TCC TTC T – 3’
Template	5’ – /Phos/AG GAG AAA AAG AAA AAA AGA AAA GAA GG – 3’
Complementary	5’ – AGG AGA AAA AGA AAA AAA GAA AAG AAG G – 3’
Atto647N-Complementary	5’ – /atto647N/AG GAG AAA AAG AAA AAA AGA AAA GAA GG – 3’
LAB07	5’ – /Phos/AGG TCG CCG CCC/Bio – 3’
LAB09	5’ – /Phos/GGG CGG CGA CCT/Dig – 3’
M13 primer	5’ – /Bio/TTT TTT TTT TTT TTT TTT TTT TTT TTT TTT GTA AAA CGA CGG CCA GT – 3’

Table S2: ssDNA length comparison of various end-labeling strategies.

	ssDNA (N)	Median (µm)	IQR	Median (knt)	IQR
ddUTP:dTTP = 1:5	31	2.3	0.8	12.0	4.0
ddUTP:dTTP = 1:50	25	3.7	3.8	18.5	18.0
ddUTP:dTTP = 1:500	27	3.0	3.5	15.2	16.9
anti-dsDNA	24	4.9	4.2	24.6	20.1

Table S3: Fluorescent labeling efficiency of various end-labeling strategies.

	Total ssDNA (N)	Labeled ssDNA (N)	Efficiency	S.D.
ddUTP:dTTP = 1:5	690	347	50%	8%
ddUTP:dTTP = 1:50	305	92	30%	2%
ddUTP:dTTP = 1:500	251	32	13%	2%
anti-dsDNA	162	107	66%	9%

SUPPLEMENTAL METHODS

Force Extension Curve: 48.5 kb λ-DNA (NEB N3011S) was annealed and ligated with biotinylated (LAB07: /5Phos/AGG TCG CCG CCC/3BioTEG, IDT) and digylated (LAB09: /5Phos/GGG CGG CGA CCT/3Dig_N, IDT) oligonucleotides. λ-DNA ends were visualized with a rabbit anti-digoxigenin primary antibody (Thermo 9H27L19) followed by incubation with a Qdot 705-labeled goat anti-rabbit secondary antibody (Thermo Q-11461MP). DNA extension was measured from flow rates of 0.1 ml min^{-1} to 1.3 mL min^{-1}. The resulting DNA extension was fit to the worm-like chain model:[1,3]

$$F = \frac{K_B T}{P}\left[\frac{1}{4}\left(1 - \frac{x}{L_0}\right)^{-2} - \frac{1}{4} + \frac{x}{L_0}\right]$$

Where F is the applied force, k_B is the Boltzmann constant, T is the absolute temperature, P is the persistence length, L_0 is the contour length of λ-DNA, and x is the measured extension of λ-DNA. The experimental data was fitted with persistence length of 43 ± 3 nm and contour length of 16.3 ± 0.1 μm, as previously described.[2]

M13mp18 ssDNA Curtains: Generation of M13mp18 ssDNA curtains was as previously described.[4,5] Briefly, a 100 μL reaction containing 10 μg of M13mp18 ssDNA (NEB N4040S) and 45 nM M13 primer (5' – /Bio/TTT TTT TTT TTT TTT TTT TTT TTT TTT TTT GTA AAA CGA CGG CCA GT, IDT) in 40 mM Tris-HCl [pH 8.0], 50 mM NaCl and 10 mM $MgCl_2$ was heated to 90ºC for 10 minutes. The reaction was cooled at a rate of -0.5°C min^{-1} to 20°C. The annealed product was further diluted with 200 μL buffer containing 10 mM Tris-HCl [pH 8.0], 50 mM NaCl and 5 mM $MgCl_2$. ssDNA was synthesized in 1x phi29 DNA Polymerase Reaction Buffer (NEB M0269S), 500 dNTPs (NEB N04467S), 0.2 mg mL^{-1} BSA (NEB B9000S), 10 nM annealed

product and 100 nM phi29 DNAP (purified in-house). The reaction was mixed and immediately injected on the flow cell and incubated at 30°C for 20 minutes. ssDNA synthesis was quenched by removing excess nucleotides and polymerase with BSA buffer (40 mM Tris-HCl pH 8.0, 1 mM $MgCl_2$, 1 mM DTT and 0.2 mg mL^{-1} BSA). ssDNA was end-labeled with mouse anti-dsDNA primary antibody (Thermo MA1-35346) followed by incubation with an ATTO647N-labeled goat anti-mouse secondary antibody (Sigma 50185). Ends were labeled after ssDNA was coated with pmRPA-GFP as the dsDNA antibody prevented ssDNA elongation.

SUPPLEMENTAL REFERENCES

(1) Smith, S. B.; Cui, Y.; Bustamante, C. Overstretching B-DNA: The Elastic Response of Individual Double-Stranded and Single-Stranded DNA Molecules. *Science* **1996**, *271* (5250), 795–799.

(2) Baumann, C. G.; Smith, S. B.; Bloomfield, V. A.; Bustamante, C. Ionic Effects on the Elasticity of Single DNA Molecules. *Proc. Natl. Acad. Sci.* **1997**, *94* (12), 6185–6190.

(3) Bustamante, C.; Marko, J. F.; Siggia, E. D.; Smith, S. Entropic Elasticity of Lambda-Phage DNA. *Science* **1994**, *265* (5178), 1599–1600.

(4) Gibb, B.; Silverstein, T. D.; Finkelstein, I. J.; Greene, E. C. Single-Stranded DNA Curtains for Real-Time Single-Molecule Visualization of Protein-Nucleic Acid Interactions. *Anal Chem* **2012**, *84* (18), 7607–7612.

(5) Qi, Z.; Greene, E. C. Visualizing Recombination Intermediates with Single-Stranded DNA Curtains. *Methods* **2016**, 1–13.