Supplementary Information

High-yield purification of exceptional-quality, single-molecule DNA substrates

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Abbreviations used: CV, column volume; MT, magnetic tweezer; PCR, polymerase chain reaction

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Table SI.
Oligonucleotides and their uses

Oligonucleotide	Sequence	Use
PB 712	5’-biotin-dT-AGATGCTTTTCTGTGACTGG-3’	PCR-2; PCR-4
PB 713	5’-dIG-GTTCCTGGCCTTTTGCTGGC-3’	PCR-3; PCR-4
PB 714	5’-GATCGTCAGATGCCTTTTGGCATCTGAC-3’	MT substrate hairpin
PB 717	5’-biotin-TEG-TGGTAGGATCCGTCATAGCTTTAGCGATTTGGGACACTTCATCAAGACTTCCAGAGCAGCCGGAGACATATAGCTACAGGGGCC-3’	MT substrate fork
PB 718	5’-CCTGTAGCTATATGTCTCCGGCTGCTCTGGCCCCCCCCCCTGTGTGTGTGTGGTGTTGGTGGTTGCATACTTCCGGGAACGCAG-3’	MT substrate fork
PB 719	5’-AAAAAAGTGTTGTTTGGGTGTTGTTGTTTTGTTGTTGTTGTGTTGTGTGTTGTTTGCTGCGTTCCCGGAAGTATGCAACCAACAC-3’	MT substrate fork
PB 721	5’-CACTCATTAGGCACCCCAGG-3’	1.5 kb fragment
PB 722	5’-CTTCGCTATTACGCCAGCTGG-3’	1.5 kb fragment
PB 733	5’-AGATGCTTTTCTGTGACTGG-3’	PCR-1; PCR-3
PB 734	5’-GTTCCTGGCCTTTTGCTGGC-3’	PCR-1; PCR-2

Protocols to construct DNA substrates, substrate components, and purification.

Materials:

Reagents (presented in order of being used)

High-copy number plasmid template (scDNA plasmid)
PCR Primer PB 733 (IDT)
PCR Primer PB 734 (IDT)
PCR Primer PB 712 (IDT, PAGE purified)
PCR Primer PB 713 (IDT, HPLC purified)
2X PrimeSTAR MAX Premix (Takara)
TOSOH binding buffer (20 mM Tris-HCl (pH 9.0))
TOSOH elution buffer (20 mM Tris-HCl (pH 9.0), 2 M NaCl)
PCR Primer PB 721 (IDT)
PCR Primer PB 722 (IDT)
*Apa*I (50,000 U/mL, NEB)
*BamH*I-HF (20,000 U/mL, NEB)
10X Buffer 4 (NEB)
Substrate oligonucleotide PB 714 (IDT, PAGE purified)
Substrate oligonucleotide PB 718 (IDT, PAGE purified)
T4 Polynucleotide Kinase (T4 PNK, 10000 U/mL, NEB)
10X T4 PNK buffer (NEB)
ATP (GE Healthcare Life Sciences)
Substrate oligonucleotide PB 717 (IDT, HPLC purification)
Substrate oligonucleotide PB 719 (IDT, PAGE purification)
dATP (GE Healthcare Life Sciences)
dCTP (GE Healthcare Life Sciences)
digoxigenin-11-dUTP (Roche)
Klenow Fragment (3' to 5' Exo$^-$) (5000 U/mL, NEB)
10X Buffer 2 (NEB)
T4 DNA ligase (400000 U/mL, NEB)
10X T4 DNA ligase buffer (NEB)

Equipment

Thermocycler
Gel electrophoresis chambers and power supply
Biorad ChemiDoc XRS imaging system
Biorad duoflow system
TOSOH TSKgel DNA-stat column
Microcentrifuge
Speedvac concentrator
Spectrophotometer
Heat block
Typhoon phosphorimager system and Imagequant software

Procedures

A. 2.9 kb DNA substrate

Four different 2.9 kb DNA substrates were made. They are, 2.9 kb control DNA made with unmodified primers (Fig. 3 PCR-1); 2.9 kb DNA made with the left primer modified with 5'-biotin-dT (Fig. 3 PCR-2); 2.9 kb DNA with the opposite primer modified with 5'-digoxigenin (Fig. 3 PCR-3); 2.9 kb DNA with both 5'-biotin-dT and 5'-digoxigenin modified primers (Fig. 3 PCR-4).

1. Set up 5 optimized PCR reactions to make PCR-1, column purify, and use this as the template for subsequent PCR reactions.

(a). PCR reaction set up

Reagents for a 250 µL master mix (5 PCR reaction), add in the following order:

(i). Water: 109 µL
(ii). 2X PrimeSTAR MAX Premix: 125 µL
(iii). scDNA plasmid: 6 µL. The total amount is 5 ng or 1 ng of DNA per reaction
(iv). PCR Primer PB 733: 5 µL. Final concentration is 200 nM
(v). PCR Primer PB 734: 5 µL. Final concentration is 200 nM

Mix well and separate the master mix into 5, thin wall, 0.2 mL PCR tubes, 50 µL per tube.

(b). PCR cycling parameters

i. Denature at 98°C for 4 min
ii. Denature at 98°C for 10sec; anneal at 56°C for 5 sec and polymerize at 72°C for 15 sec.
iii. Repeat step (ii) 19 times.
iv. Cool to 4°C

After PCR, Evaluate the product quality by agarose gel electrophoresis. If product quality is acceptable, pool all PCR reactions.

(c). Column chromatography purification

Purify the pooled PCR products using column chromatography. Use the 2.8 mL TSKgel DNA-stat column at 0.5 mL/min flow rate throughout the whole process.

The steps are the following:

i. Equilibrate the column with 12 mL of TOSOH binding buffer
ii. Load the pooled PCR reactions onto the column
iii. Wash the column with 6 mL of TOSOH binding buffer
iv. Elute the DNA using a linear gradient from 0 M to 1 M NaCl. The elution volume of the gradient is 28 mL (10 column volumes). Fraction

size is 500 μL

v. After elution, wash the column with 6 mL of TOSOH elution buffer.

vi. Equilibrate the column with 12 mL of TOSOH binding buffer

After elution, Evaluate the product quality by agarose gel electrophoresis and take the fractions which contain high purity product and precipitate with following steps：

(i). Add 5 μL 20 mg/mL glycogen and 1 mL 100 % ethanol, -80°C overnight (or at least 30 min)

(ii). Using microcentrifuge spin at 21,000×G for 30 min at 4°C, remove the supernatant and keep the pellet

(iii). Add 70 % ethanol, gently invert the tube and spin at room temperature, 13,500×G for 10 min

(iv). Remove supernatant and dry in a speedvac concentrator for 20 min

(v). Dissolve pellet in TE to a total volume of 100 μL to 200 μL and determine the product concentration using a spectrophotometer

2. PCR reamplification of PCR-1

Using PCR-1 purified by step 1 as the template, reamplify to create a large amount of each of the four 2.9 kb DNA substrates. The reagents for a 1000 μL master mix (20 PCR reaction), added in following order to make PCR-1 are:

(i). Water: 440 μL
(ii). 2X PrimeSTAR MAX Premix: 500 μL
(iii). PCR-1 template: 20 μL. The total amount is 20 ng or 1 ng per reaction
(iv). PCR Primer PB 733: 20 μL. Final concentration is 200 nM
(v). PCR Primer PB 734: 20 μL. Final concentration is 200 nM

Mix well and separate the master mix into 20 thin walled, 0.2 mL PCR tubes, 50 μL per tube. PCR amplify using the cycling parameters from step 1(b). Column purify the amplified DNA according to step 1(c).

3. PCR amplification of PCR-2

Reagents for a 1000 μL master mix (20 PCR reaction), add in the following order:

(i). Water: 440 μL
(ii). 2X PrimeSTAR MAX Premix: 500 μL
(iii). PCR-1 template: 20 μL. The total amount is 20 ng or 1 ng per reaction
(iv). PCR Primer PB 712: 20 μL. Final concentration is 200 nM
(v). PCR Primer PB 734: 20 μL. Final concentration is 200 nM

Mix well and separate the master mix into 20 thin wall, 0.2 mL PCR tubes, 50 μL per tube. PCR amplify using the cycling parameters from step 1(b). Column purify the amplified DNA according to step 1(c).

4. PCR amplification of PCR-3
Reagents for a 1000 μL master mix (20 PCR reaction), add in the following order:

(i). Water: 440 μL
(ii). 2X PrimeSTAR MAX Premix: 500 μL
(iii). PCR-1 template: 20 μL. The total amount is 20 ng or 1 ng per reaction
(iv). PCR Primer PB 733: 20 μL. Final concentration is 200 nM
(v). PCR Primer PB 713: 20 μL. Final concentration is 200 nM

Mix well and separate the master mix into 20 thin wall, 0.2 mL PCR tubes, 50 μL per tube. PCR amplify using the cycling parameters from step 1(b). Column purify the amplified DNA according to step 1(c).

5. PCR amplification of PCR-4
Reagents for a 1000 μL master mix (20 PCR reaction), add in the following order:

(i). Water: 440 μL
(ii). 2X PrimeSTAR MAX Premix: 500 μL
(iii). PCR-1 template: 20 μL. The total amount is 20 ng or 1 ng per reaction
(iv). PCR Primer PB 712: 20 μL. Final concentration is 200 nM
(v). PCR Primer PB 713: 20 μL. Final concentration is 200 nM

Mix well and separate the master mix into 20 thin-walled, 0.2 mL PCR tubes, 50 μL per tube. PCR amplify using the cycling parameters from step 1(b). Column purify the amplified DNA according to step 1(c).

6. Aliquot the purified DNA and store at -80°C.

B. 1.2 kb magnetic tweezer DNA substrate (MT substrate)
The 1.2 kb MT substrate consists of three parts: a central 1.2 kb fragment sandwiched between a hairpin on one side and a fork tagged with digoxigenin and biotin on the opposite side. The scheme to construct the MT substrate is presented in Figure 5.

8. Set up 5, optimized PCR reaction for the 1.5 kb fragment amplification. Use this as the template for subsequent PCR reaction.

Reagents for a 250 μL master mix, add in the following order:

(i). Water: 109 μL
(ii). 2X PrimeSTAR MAX Premix: 125 μL
(iii). scDNA plasmid: 6 μL. The total amount is 5 ng or 1 ng per reaction
(iv). PCR Primer PB 721: 5 μL. Final concentration is 200 nM
(v). PCR Primer PB 722: 5 μL. Final concentration is 200 nM

Mix well and separate the master mix into 5, thin walled, 0.2 mL PCR tubes, 50 μL per tube.

9. PCR cycling parameters

  i. Denaturing at 98°C for 4 min
  ii. Denaturing at 98°C for 10 sec; annealing at 60°C for 5 sec; polymerization at 72°C for 8 sec.
  iii. Repeat step (ii) 19 times.
  iv. Polymerization at 72°C for 10 min
  v. Cool to 4°C

After PCR, Evaluate the product quality by agarose gel electrophoresis. If product quality is acceptable, pool all PCR reactions.

10. Purify the 1.5 kb fragment using the same steps used in 1(c). The purified 1.5 kb product is used to create a pool of the 1.5 kb starting material.

11. Set up 60 PCR reactions for 1.5 kb DNA reamplification.

Reagents for a 3000 μL master mix, add in the following order:

(i). Water: 1320 μL
(ii). 2X PrimeSTAR MAX Premix: 1500 μL
(iii). Purified 1.5 kb DNA: 60 μL. The total amount is 60 ng or 1 ng per reaction
(iv). PCR Primer PB 721: 60 μL. Final concentration is 200 nM
(v). PCR Primer PB 722: 60 μL. Final concentration is 200 nM

Mix well and separate the master mix into 60, thin walled, 0.2 mL PCR tubes, 50 μL per tube.

12. Using the same PCR cycle parameters as in step 9 and purify the DNA by following the same steps in step 1 (c). The purified 1.5 kb starting material is used to make the 1.2 kb insert.

13. Restriction enzyme cleavage of the 1.5 kb fragment to make the 1.2 kb insert.

(a). Digest with ApaI

Reagents for a 500 μL digest mix, add in the following order:

  i. Water: 70 μL
  ii. 10X Buffer 4: 50 μL
  iii. 1.5 kb fragment: 370 μL. The total amount of DNA is 76 μg
  iv. ApaI: 10 μL. The final concentration of enzyme is 1 U/μL

Incubate the mixture at 25°C for 1 hr, followed by 30 min incubation at 37°C for 30 min to inactivate ApaI. Evaluate the product quality by agarose gel electrophoresis.

(b). Digest with BamHI-HF

Add 10 μL BamHI-HF to the mixture (the final concentration of enzyme is 0.4 U/μL), incubate at 37°C overnight. Evaluate the product quality by agarose gel electrophoresis.

14. Column chromatography purification

Purify the cleavage product using column chromatography. Use the 2.8 mL TSKgel DNA-stat column at 0.5 mL/min flow rate throughout the whole process.

The steps are the following:

i. Equilibrate the column with 12 mL of TOSOH binding buffer
ii. Load the product onto the column
iii. Wash the column with 6 mL of TOSOH binding buffer
iv. Wash the column with 6 mL of 0.6 M NaCl (30 % of TOSOH elution buffer)
v. Wash the column with 6 mL of 0.7 M NaCl (35 % of TOSOH elution buffer)
vi. Set a linear gradient from 0.78 M to 1 M NaCl. The volume of the gradient is 28 mL (10 column volume). Fraction size is 500 μL
vii. After elution, wash the column with 6 mL of TOSOH elution buffer.
viii. Equilibrate the column with 12 mL of TOSOH binding buffer

After elution, Evaluate the product quality by agarose gel electrophoresis and take the fractions which contain high purity product, precipitate with following steps：

(i). Add 5 μL 20 mg/mL glycogen and 1 mL 100 % ethanol, -80°C overnight (or at least 30 min)
(ii). Using microcentrifuge spin at 21,000×G for 30 min at 4°C, remove the supernatant and keep the pellet
(iii). Add 70 % ethanol, gently invert the tube and spin at room temperature, 13,500×G for 10 min
(iv). Remove supernatant and dry in a speedvac concentrator for 20 min
(v). Dissolve pellet in TE to a total volume of 100 μL to 200 μL and determine the product concentration using a spectrophotometer

Aliquot the product and store it at -20°C. 76 μg of 1.5 kb DNA was cleaved by *Apa*I and *BamH*I-HF. The predicted yield of 1.2 kb insert is 59 μg. As 50.49 μg of DNA was recovered, the yield is 86 %.

15. Phosphorylation of substrate oligonucleotides PB 714 and PB 718

PB 714 is used to make the hairpin while PB 718 is used to make the fork (Fig. 5).

(a). Phosphorylation of PB 714

Reagents for a 150 μL mixture, add in the following order:

(i). Water: 85.5 μL
(ii). 10X T4 PNK buffer: 15 μL
(iii). PB 714: 45 μL. The final concentration is 30 μM

(iv). ATP: 1.5 µL. The final concentration is 1.6 mM
(v). T4 PNK: 3 µL. The final concentration of enzyme is 0.2 U/µL

(b). Phosphorylation of PB 718
Reagents for a 150 µL mixture, add in the following order:
(i). Water: 85.5 µL
(ii). 10X T4 PNK buffer: 15 µL
(iii). PB 718: 45 µL. The final concentration is 30 µM
(iv). ATP: 1.5 µL. The final concentration is 1.6 mM
(v). T4 PNK: 3 µL. The final concentration of enzyme is 0.2 U/µL

For each oligonucleotide, incubate at 37°C for 30 min, followed by 20 min at 70°C to inactivate the T4 PNK.

16. Annealing reactions

Two separate annealing reactions are done. PB 714 is self-annealed to make the hairpin with a *Bgl*II tail. PB 718 is annealed to PB 717 and PB 719 to form the unmodified fork.

(a). Hairpin
Reagents for a 900 µL master mix, add in the following order:
(i). Water: 495 µL
(ii). $Mg(OAc)_2$: 75 µL. The final concentration is 10 mM
(iii). NaCl: 180 µL. The final concentration is 100 mM
(iv). PB 714: 150 µL. The final concentration is 5 µM

(b). Unmodified fork
Reagents for a 900 µL master mix, add in the following order:
(i). Water: 401 µL
(ii). $Mg(OAc)_2$: 75 µL. The final concentration is 10 mM
(iii). NaCl: 180 µL. The final concentration is 100 mM
(iv). PB 718: 150 µL. The final concentration is 5 µM
(v). PB 717: 47 µL. The ratio of PB 717 to PB 718 is 1.05:1
(vi). PB 719: 47 µL. The ratio of PB 719 to PB 718 is 1.05:1

Mix well and separate each master mix into 9, thin walled, 0.6 mL PCR tubes, 100 µL per tube. Use a 100°C-heat block to denature oligos. Add water to each well of the heat block and once the temperature reach to 95°C, place tubes in the water and incubate for 5 min. Remove the heat block and allow it to cool overnight to room temperature.

17. Fork modification
This step is required to add multiple digoxigenin-nucleotides to the fork on the 3' end of PB 718.

Reagents for a 1200 μL master mix, add in the following order:

(i). Water: 72 μL
(ii). 10X Buffer 2: 120 μL
(iii). Unmodified fork: 900 μL. The final concentration is 3.75 μM
(iv). dATP: 45 μL. The final concentration is 375 μM
(v). dCTP: 45 μL. The final concentration is 375 μM
(vi). digoxigenin-11-dUTP: 15 μL. The final concentration is 43.8 μM
(vii). Klenow Fragment (3' to 5' Exo-): 5 μL. The final concentration of enzyme is 0.02 U/μL

Mix well and separate the master mix into 12 tubes, 100 μL per tube, incubate at 37°C for 1 hr, followed by 20 min at 70°C to inactivate Klenow.

18. Ligation of the hairpin and modified fork to the purified 1.2 kb insert

Reagents for a 3000 μL master mix, add in the following order:

(i). Water: 230 μL
(ii). 10X T4 DNA ligase buffer: 300 μL
(iii). 1.2 kb insert: 300 μL. The total amount of DNA is 30 μg
(iv). Modified fork: 1200 μL. The ratio of the fork to 1.2 kb DNA is 100:1
(v). Hairpin: 900 μL. The ratio of the hairpin to 1.2 kb DNA is 100:1
(vi). *BamH*I-HF: 10 μL. The final concentration of the enzyme is 0.067 U/μL. *BamH*I-HF prevents 1.2 kb fragment self-ligation but does not cleave the hairpin: 1.2 kb fragment ligation site (*Bgl*II: *BamH*I-HF)
(vii). T4 DNA ligase: 60 μL. The final concentration of enzyme is 8 U/μL

Mix well and separate the master mix into 60 tubes, 50 μL per tube, incubate at 25°C overnight, the next day incubate at 70°C for 10 min to inactivate the T4 DNA ligase.

19. Using the same purification steps as in step 14 to remove spurious ligation fragment and excess fork/hairpin. The purified MT substrate quality can be Evaluated by ^{32}P-ATP labeling and agarose gel electrophoresis. Aliquot the MT substrate and store at -80°C.

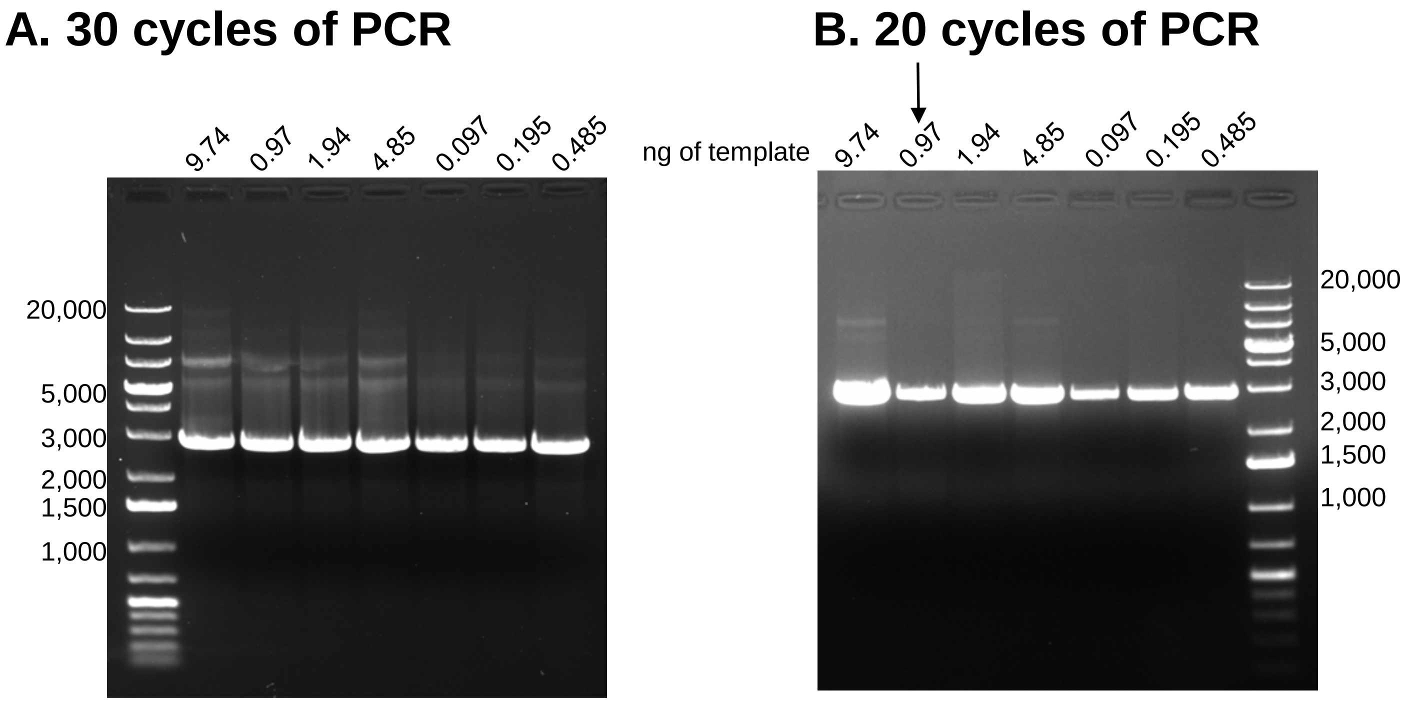
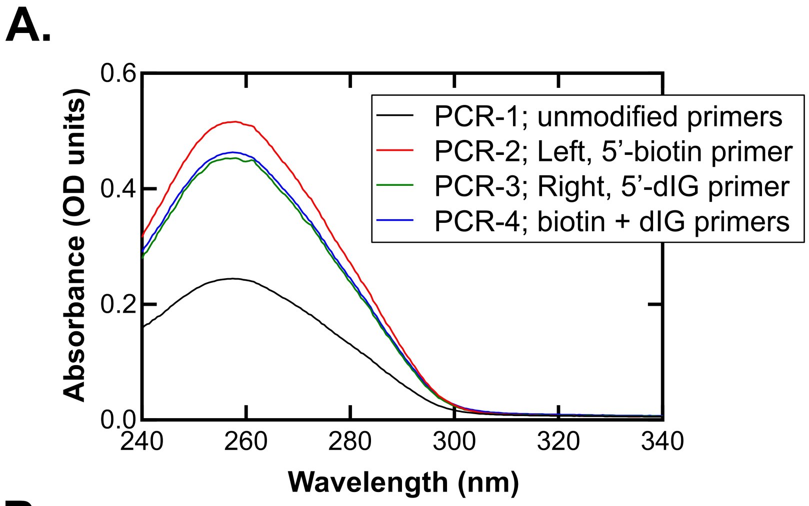


Figure S1. PCR must be optimized to ensure high-quality substrate components are produced. The agarose gel results of PCR template titration experiments are shown. 50µl PCR reactions were done using PrimeStar-Max (Takara Biosciences) and varying amounts of template. Following PCR, 10µl of each reaction was mixed with gel loading dye and subjected to electrophoresis in a 1% agarose gel in 1xTAE buffer. (A), 30 PCR cycles, and (B), 20 PCR cycles. The arrow in (B) indicates the maximum amount of template that can be used to produce a high-quality product. The template used was a 4kB negatively supercoiled DNA template that was purified using an EZNA Plasmid Maxi Kit (Omega Bio-Tek).



B.

PCR type	260/280	Yield (µg)
1	1.85	36
2	1.88	51
3	1.87	45
4	1.87	46

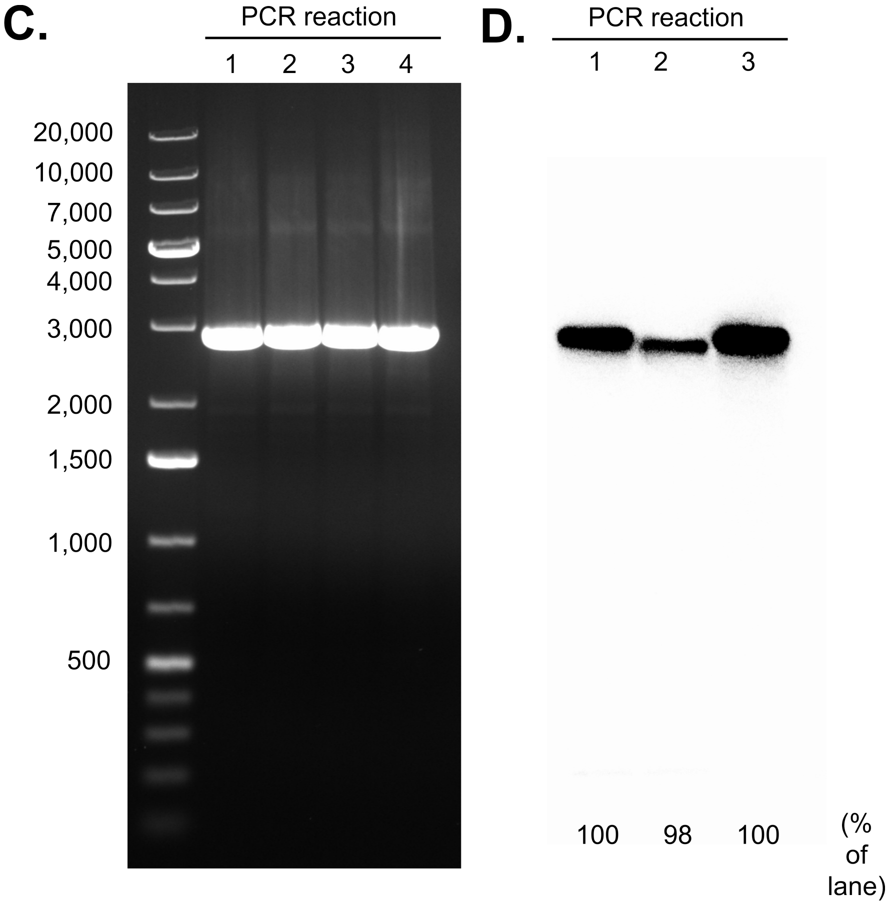
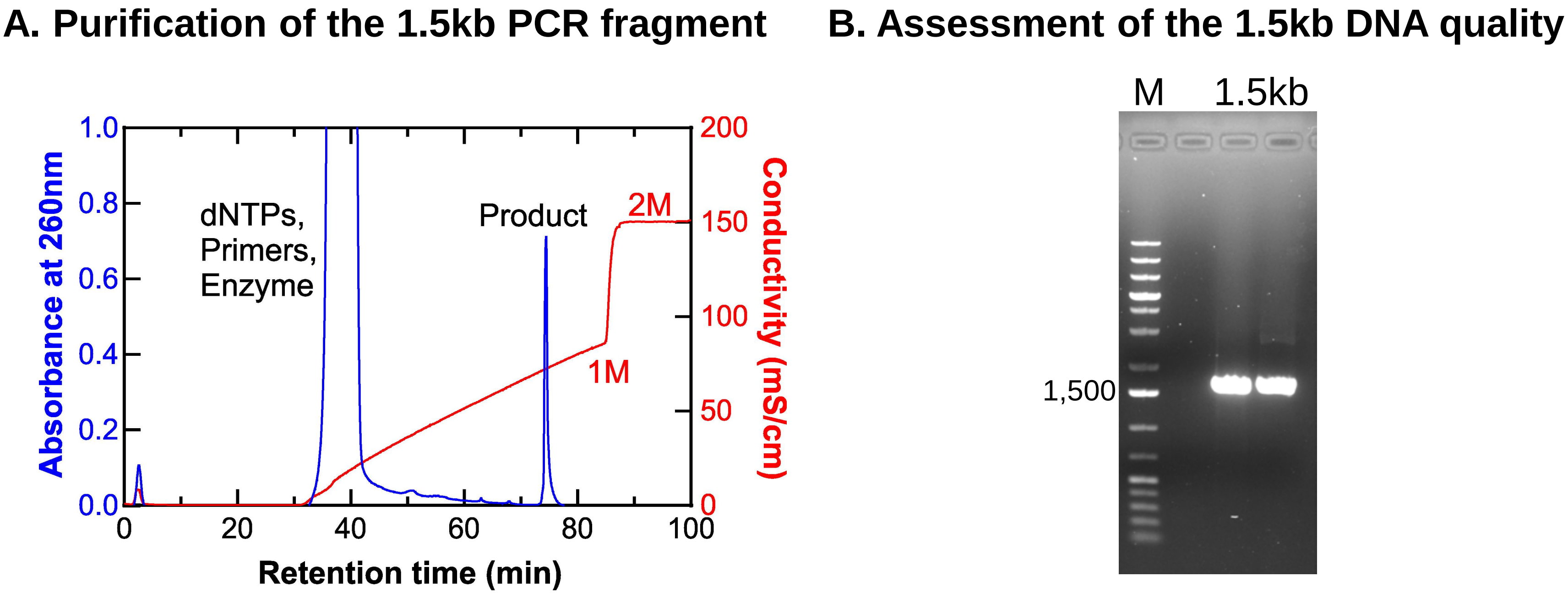


Figure S2. The purified substrate components are of high quality. (A), Spectrophotometric analysis of column-purified DNA products following ethanol precipitation. (B), Assessment of the quality and yield of DNA. (C), Agarose gel analysis of purified products. 5 µg of each DNA was loaded per lane. (D), 5’-end labeling further demonstrates DNA quality. Purified PCR products from reactions 1-3 were 5’-end-labeled using γ-^{32}P-ATP, and subjected to electrophoresis in an agarose gel identical to that used in panel C. The gel was subsequently dried, exposed to a phosphorimager screen, scanned and quantitated. The values at the bottom of each lane correlate with the amount of signal in the purified DNA band.


A. Purification of the 1.5kb PCR fragment

B. Assessment of the 1.5kb DNA quality

Figure S3. Large amounts of high-quality substrate components are easily purified. (A). The elution profile of the purification of the 1.5 kb fragment is shown. 60 PCR reactions were pooled, loaded onto the column in TOSOH binding buffer and eluted with a linear gradient from 0-1M NaCl. (B). Agarose gel electrophoresis demonstrates the high quality of the purified 1.5 kb fragment. 5 µL of each peak fraction in the elution profile in (A) were subjected to electrophoresis in a 1% agarose gel and stained with ethidium Bromide.

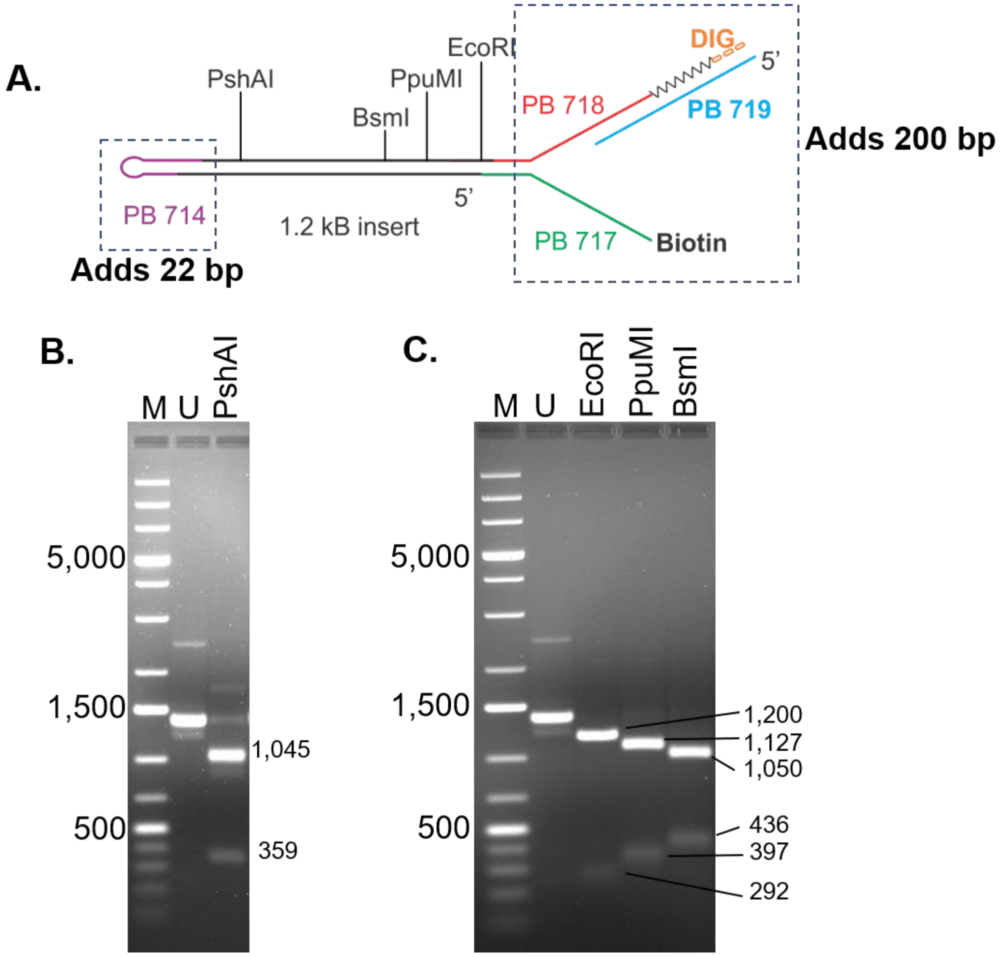


Figure S4. Restriction enzyme mapping confirms the MT substrate structure. (A). Schematic of the MT substrate with restriction enzyme cleavage sites is shown. (B) and (C). Restriction enzyme mapping confirms the substrate structure. (B). *PshA*I cleaves the substrate into a 1,045 and a 359 bp fragment with the hairpin ligated to the smaller fragment. (C). Multiple restriction enzyme cleavage reactions were required to show that the fork was ligated to the opposite end of the 1.2 kb fragment. The smaller fragments produced in each cleavage are longer by approximately 220 bp, consistent with the presence of the fork. M, marker and U, uncut DNA.

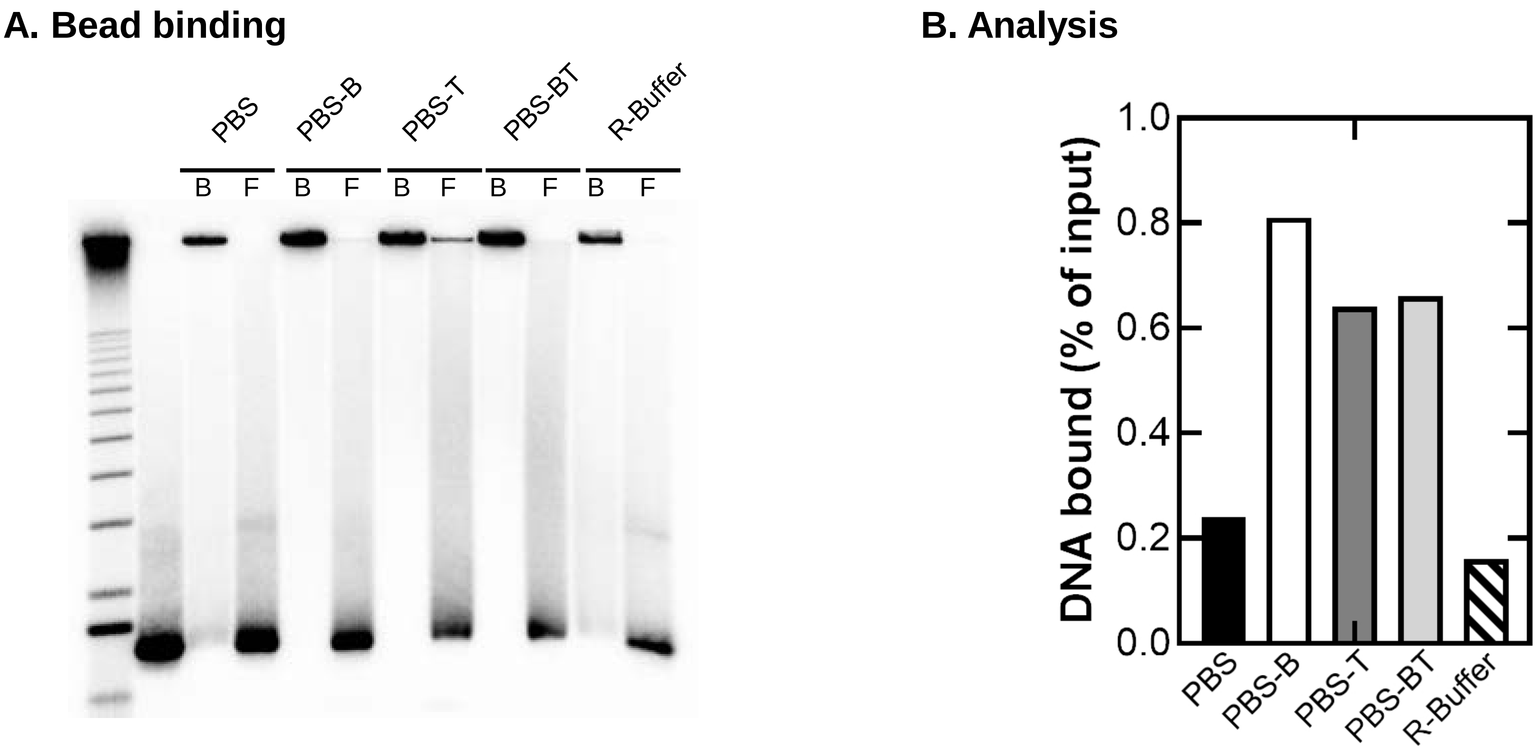
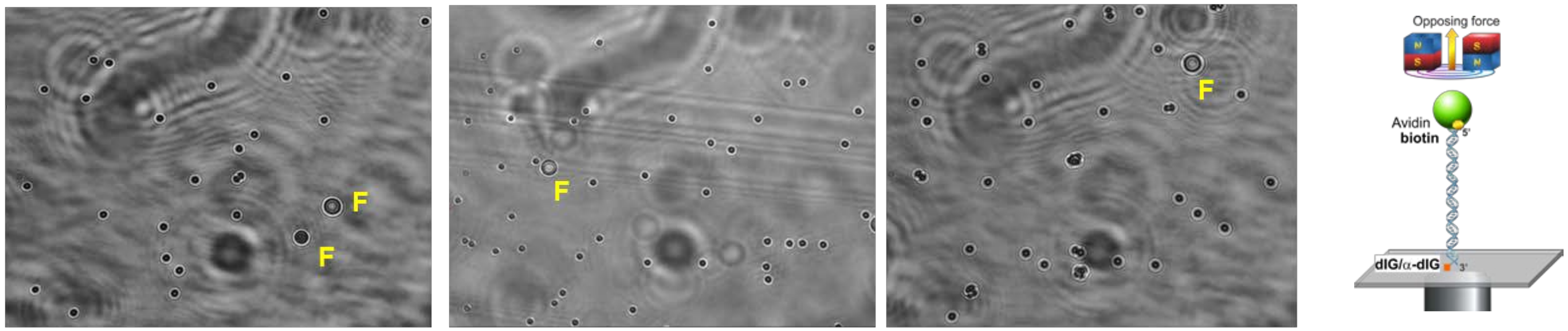


Figure S5. Column purification yields high quality, functional substrate DNA for magnetic tweezer experiments (A), The MT substrate binds to streptavidin-coated magnetic beads as expected. To visualize the substrate, the DNA was 5'-end-labeled with γ-^{32}P-ATP. The quality of the final product is evident in lane 2, where the single band corresponds to 98% of the signal in the lane. To assess bead binding, the labeled substrate was bound to 1 µm, streptavidin-coated magnetic beads (Dynal), for 30 minutes in the following buffers: PBS; PBS-B (+ 1mg/ml BSA); PBS-T (+0.05% Tween 20); PBS-BT (BSA + Tween 20) and R-buffer, Dynal's recommended buffer. Following binding, tubes were placed on a magnetic separator, the supernatant removed (free DNA) and beads resuspended in the same buffer volume. Then aliquots of beads (B) or supernatant (F, free DNA) were subjected to electrophoresis in a 1% agarose gel in1xTAE buffer. (B), Analysis of the gel in A. DNA bound is expressed as a fraction of the input DNA present in lane 2 in panel B.

A. Tethering of the 2.9kB fragment control DNA



B. Tethering of the MT substrate DNA

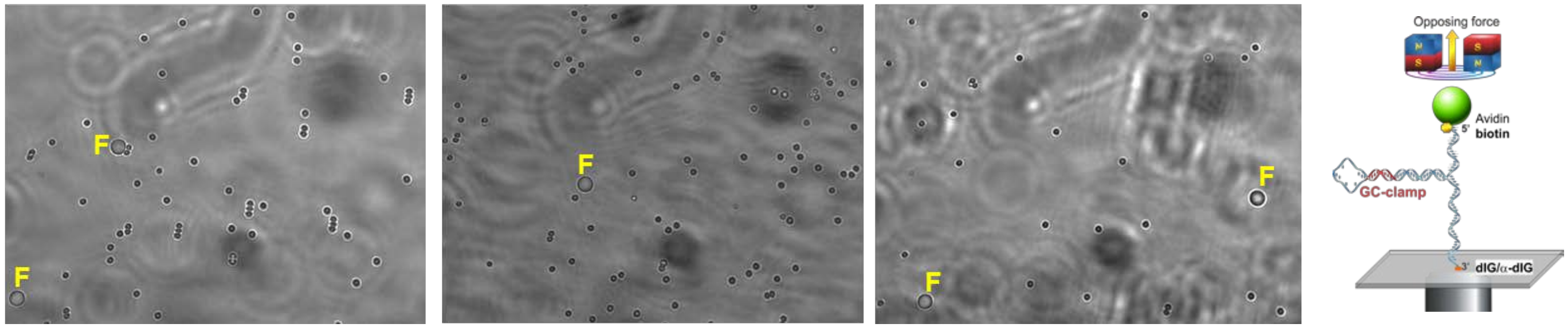


Figure S6. The MT substrate functions as expected in a magnetic tweezer experiment. (A). Tethering of the control, double-labeled DNA. The DNA came from PCR reaction 4 from Supplementary Figure 2. This DNA molecule is 2.9kb in length and has biotin at one end and dIG at the other. (B). Tethering of the MT substrate. F, fixed, 3µm polystyrene beads used as a reference. For both DNA molecules, DNA was bound to prewashed 1µm magnetics beads in PBS+BSA and then injected into a flow cell constructed of mylar and a coverslip. The coverslip was coated with anti-dIG antibody prior to the introduction of the DNA-bead complex. The schematics to the right of each panel indicate the DNA substrate tethered in the flow cell.