Torsionally constrained DNA for single-molecule assays: an efficient, ligation-free method

D. Hern Paik,[1] Violet A. Roskens,[1] and Thomas T. Perkins*[1,2]

[1]JILA, National Institute of Standards and Technology and University of Colorado, Boulder, CO, 80309, U.S.A.

[2]Department of Molecular, Cellular, and Developmental Biology, University of Colorado, Boulder, CO, 80309, U.S.A.

*To whom correspondence should be addressed.

The authors wish it to be known that, in their opinion, the first two authors should be regarded as joint First Authors.

Supporting Information

Supplementary Protocol:

Here we provide step-by-step instructions for making megaprimer-labeled 6.6-kbp DNA. The procedure is broken down into four steps corresponding to those in Figure 2B.

Materials and Instruments

M13mp18 plasmid (NEB), BspH1(NEB), biotin-16-dUTP (Roche), digoxygenin-11-dUTP (Roche), dNTPs (Life Technologies), KOD Hot Start DNA Polymerase kit (Novagen), primer oligonucleotides (IDT; see Table S1 and Figure S1), 0.2 ml thin-walled tubes (Molecular BioProducts), QIAquick PCR Purification Kit (Qiagen), Thermocycler (Techne), NanoDrop (Thermo Scientific), Mini-Prep Cell Model 491 (Bio-Rad), Fraction Collector CF-2 (Spectra/Chrom), Mini-Pump Variable Flow (VWR), Owl Gel Electrophoresis System (Thermo Scientific), bioresearch grade agarose (RPI), 1kb DNA ladder (NEB), TAE buffer (pH 8.5, 40 mM Tris-acetate, 1 mM EDTA), TBE buffer (pH 8.3, 45 mM Tris-borate, 1 mM EDTA), TE buffer (pH 7.5, 10 mM Tris-Cl, 1 mM EDTA), 6× loading dye (30% glycerol, 0.09% Xylene Cyanol FF, 0.09% Bromophenol blue, 10 mM Tris-Cl, 1 mM EDTA), Freeze 'N Squeeze DNA Gel Extraction Spin Columns (Bio-Rad), Microfuge 18 Centrifuge (Beckman Coulter), Sybr Green (Invitrogen), UV transilluminator (VWR), Vortex Genie (Scientific Industries), doubly filtered water (NANOpure Diamond, Barnstead; 0.2 μm filter units, Thermo Scientific).

Table S1. Sequences of primer oligonucleotides used for making megaprimers.

Region	Label	Primer	Sequence (5′-3′)
509–533	None	Forward	ACGATTCCGCAGTATTGGACGCTA
890–912	Digoxigenin	Reverse	[5DigN]TTGCCCTGACGAGAAACACCAGAAC
1607–1633	Biotin	Forward	[BioTEG]AGTTGTTCCTTTCTATTCTCACTCCG
2000–2024	None	Reverse	AAGAGGCTGAGACTCCTCAAGAGA

Methods

STEP 1. Generating a linear template by linearizing M13mp18 plasmid

1.1. Prepare 100-μl reaction mixture in a PCR tube (0.2 ml thin-walled tube) in an ice bath using the volume listed below.

Table S2. 100-μl reaction mixture for linearizing M13mp18 plasmid

Reagent	Concentration	Volume (μl)	Final Amount
M13mp18	100 μg/ml	20	2 μg
NEB Buffer 4	10×	10	1×
BspH1 enzyme	10 U/μl	1	10 U
Water	—	69	—

1.2. Put the tube prepared in 1.1 in the thermocycler. Set the thermocycler as follow and run:

Endonuclease reaction: 37 °C for 1 h

Enzyme deactivation: 65 °C for 20 min
Hold: 4 °C

1.3. Conduct PCR purification using one column provided in the QIAquick kit for the 100-µl reaction from 1.2. The product is eluted with 60 µl of elution buffer [EB: 10mM Tris-Cl (pH 8.5)]. Measure the concentration by UV-Vis spectrometer (NanoDrop) and confirm linearization by agarose gel electrophoresis.

1.4. If residual circular M13mp18 is determined to be present by gel electrophoresis, then separate out the linearized template by gel purification and Freeze 'N Squeeze Columns (see STEP 4A for details). Quantify DNA.

1.5 We typically obtain 60 µl of 6 ng/µl.

STEP 2. Making biotin- and digoxigenin-labeled megaprimers

2.1. Prepare a ~8 mM dNTP mix with the desired amount of biotin-16-dUTP (B-dUTP) or digoxigenin-11-dUTP (D-dUTP). We used 100 mM dATP, dGTP, dCTP, and dTTP and 1 mM B-dUTP and D-dUTP solutions. See the table below for the volumes.

Table S3. Reagent volumes for making dNTP mix with desired amount of labelled dUTPs

Mixing Ratio	dATP, dGTP, dCTP (µl)	dTTP (µl)	Labelled dUTP (µl)	Water (µl)
50% B-dUTP	1	0.5	50	0
25% D-dUTP	1	0.75	25	23
10% B-/D-dUTP	1	0.9	10	38

2.2. Prepare a 100-µl reaction mixture in a PCR tube on ice as described below.

Table S4. 100-µl reaction mixture for preparing biotin- or digoxigenin-labelled megaprimers

Reagent	Concentration	Volume (µl)	Final Concentration
Forward Primer	10 µM	3	0.3 µM
Reverse Primer	10 µM	3	0.3 µM
Linear M13mp18	6 ng/µl	6	0.36 ng/µl
KOD polymerase Buffer	10×	10	1×
$MgSO_4$	25 mM	6	1.5 mM
dNTP mix with labelled dUTP	8 mM	10	0.8 mM
KOD polymerase enzyme	1 U/µL	2	0.02 U/µl
Water	—	60	—

2.3. Put the tube prepared in 2.2 in the thermocycler. Set the thermocycler as follows and run:

Preheat lid: 105 °C
Initial denaturation: 95 °C for 2 min
Amplification: 35 × (95 °C for 20 s; 60 °C for 10 s; 70 °C for 20 s)
Final extension: 72 °C for 1 min
Hold: 10 °C

2.4. Conduct a PCR cleanup using one column provided in the QIAquick kit for the 100-µl reaction from 2.3. Megaprimers are eluted with 60 µl of EB. Quantify the megaprimers.

2.5. We typically obtain 60 µl of ~100 ng/µl (0.38 µM) and 60 µl of 120 ng/µl (0.45 µM) for the high-density and standard-density reactions, respectively.

STEP 3. Making 6.6 kbp DNA using megaprimers

Table S5. 100-µl (600-µl) reaction mixture for making 6.6 kbp DNA.

Reagent	**Concentration**	**Volume (µl)**	**Final Concentration**
Bio megaprimer	0.33 µM[†]	3 (~18)	0.01 µM
Dig megaprimer	0.33 µM[†]	3 (~18)	0.01 µM
Linear M13mp18	6 ng/µl	6 (36)	0.36 ng/µl
KOD polymerase Buffer	10×	10 (60)	1×
$MgSO_4$	25 mM	6 (36)	1.5 mM
dNTP mix	10 mM	8 (48)	0.8 mM
KOD polymerase enzyme	1 U/µL	2 (12)	0.02 U/µl
Water	—	62 (372)	—

[†]The concentrations of the megaprimers obtained from STEP 2 are between 0.33 and 0.45 µM. Dilute the megaprimer solutions to 0.33 µM.

3.1. Prepare 6 tubes of 100 µl, as described in Table S5.

3.2. Put the tubes prepared in 3.1 in the thermocycler. Set the thermocycler as follows and run.

Preheat Lid: 105 °C
Initial denaturation: 95 °C for 2 min
Amplification: 35 × (95 °C for 20 s; 60 °C for 10 s; 70 °C for 2 min 30 s)
Final extension: 72 °C for 1 min
Hold: 10 °C

3.3. Combine the 6 tubes and perform a PCR cleanup using one or two columns.

3.4. Elute the column with 60 µl of EB. Quantify the DNA. The eluted solution contains the remaining megaprimers as well as the desired target DNA (6.6 kbp).

3.5. The yields are from 8 to 14 µg.

STEP 4A: Purifying via agarose gel electrophoresis

4A.1. Prepare a 0.5 % agarose-TAE gel.

4A.2. Prepare a 120 µl loading sample: 3.5 µg of the PCR cleaned-up sample (from 3.6) and 20 µl of 6× loading dye. Add TE buffer to bring the final volume to 120 µl.

4A.3. Run at 100 V for ~1 h.

4A.4. Stain the gel with Sybr Green (in TAE) for 30 min, and then visualize the gel with a UV transilluminator (365 nm) and quickly (< 30 s) cut out the band at ~6.6 kbp. Mince the band into small cubes (~1 mm^3) and put them into the Freeze 'N Squeeze Column(s) and store the column(s) at -20 °C overnight.

4A.5. Collect the DNA in the frozen agarose gel by centrifuging the column (4A.4) at 15000 ×g for 3 min. To improve yield, we sometimes added an additional 200 µl of TAE to the column and it was stored at -20 °C overnight a second time and then re-centrifuged. At this point, the desired target DNA is in TAE with some Sybr Green and loading dye.

4A.6. Clean up the DNA solution (4A.5) with the QIAquick PCR Purification Kit. Elute the final product with 60 µl of EB. Qunatify 6.6 kbp DNA.

4A.7. Our best yield was 1.2 µg of target DNA (6.6 kbp) when 3.5 µg of the PCR cleaned-up sample from step 3.6 was gel-purified.

STEP 4B: Purification by continuous elution

4B.1. Assemble the column for gel pouring as directed by the Bio-Rad instructions.

4B.2. Prepare 0.8% and 0.25% agarose gels in TBE buffer.

4B.3. Pour a 9-cm long agarose separating gel (0.8%) in the column. Allow it to solidify at 4 °C for at least 20 min.

4B.4. Pour a 1-cm long agarose stacking gel (0.25%) on top of the separating gel (4B.3). Allow it to solidify at 4 °C for at least 20 min.

4B.5. Assemble the Mini-Prep Cell with the agarose gel column according to the Bio-Rad instructions. De-gas the TBE buffer before filling the appropriate chambers (voltage will be disrupted if air is present). Attach the Mini-Prep Cell to a peristaltic pump and a fraction collector.

4B.6. Load the sample (from 3.3, post-PCR cleanup) on top of the separating gel. Run at 65 V for 10 min to allow sample to enter the separating gel. Run at 50 V for 33 h.

4B.7. Collect fractions in 30-min intervals with the flow rate set at 18.7 µl/min. Perform an ethanol precipitation on each fraction. Re-suspend each fraction in 20 µl of EB.

4B.8. Analyze each fraction via gel electrophoresis. Combine all fractions containing the desired target DNA (6.6 kbp). Determine the concentration.

4B.9. Our best yield was 0.7 µg of the target DNA (6.6 kbp) when the sample from step 3.3 was gel purified via continuous elution.

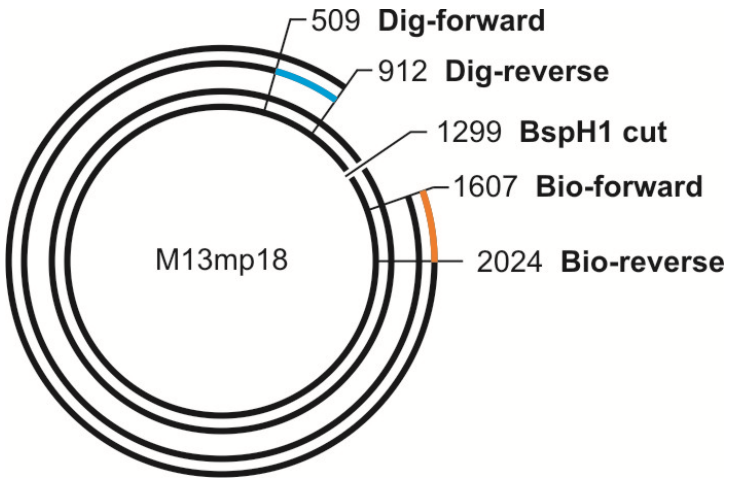

Figure S1. Locations of primers and restriction site for our DNA construct on the M13mp18 plasmid map. Our 6.6-kbp final dsDNA construct is depicted as the outer pair of arcs. The single-stranded digoxigenin and biotin megaprimers are represented as blue and orange arcs, respectively. The starting locations of the primers used to make the megaprimers are denoted by numbers giving their position within the M13mp18 plasmid.

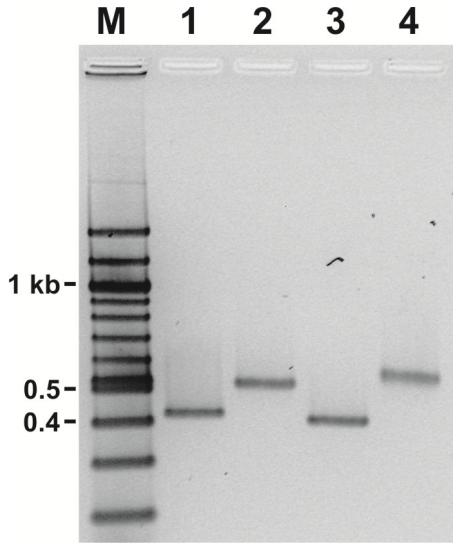

Figure S2. Gel shift of labeled megaprimers. Agarose gel electrophoresis of the high-density megaprimers shows biotin- and digoxigenin-labelled DNA segments shifted to higher molecular weights than the unlabelled megaprimers. Lane M: DNA ladder (100 bp, NEB); Lane 1: unlabelled 418-bp DNA segments Lane 2: biotin-labelled 418-bp DNA segments; Lane 3: unlabelled 404-bp segment; Lane 4: digoxigenin-labelled 404-bp DNA segment.

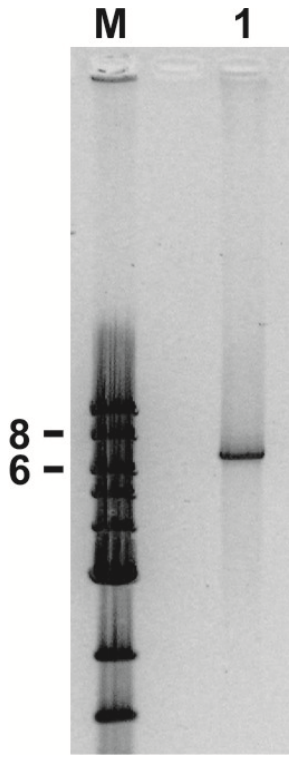

Figure S3. Agarose gel electrophoresis showing 6.6-kbp DNA product with 10% labeling. Lane M: DNA ladder (1kb, NEB); Lane 1: final PCR product (6,592 bp) after gel purification.

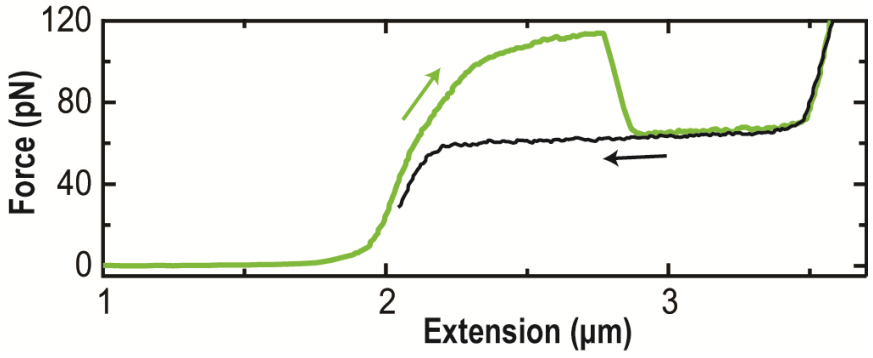

Figure S4. Force-extension curve of our PCR-based DNA (6.6 kbp) showing an abrupt force drop from 110 to 65 pN. This drop indicates that the torsional constraint has failed during stretching (*green*). The DNA stays torsionally unconstrained for the remaining stretching relaxation (*black*) processes.

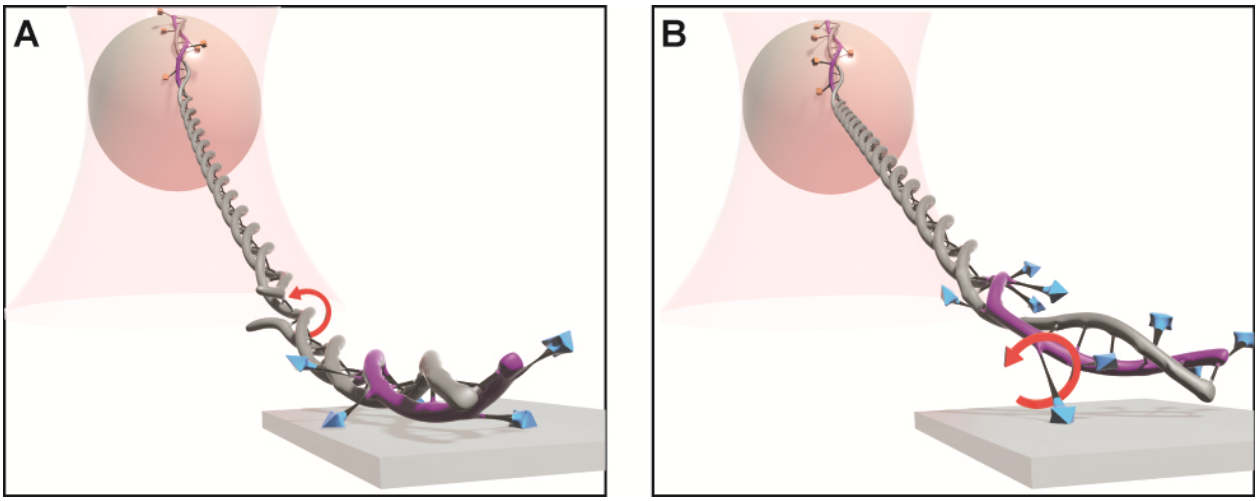

Figure S5. Two examples of torsionally unconstrained DNA tether in single-molecule optical-trapping assay. Although DNA is multiply labeled at both ends, the presence of a nick (A) or a single attachment (B) to either surface will result in a torsionally unconstrained DNA tether.