Chapter 10

Force-Activated DNA Substrates for In Situ Generation of ssDNA and Designed ssDNA/dsDNA Structures in an Optical-Trapping Assay

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Abstract

Single-molecule force spectroscopy can precisely probe the biomechanical interactions of proteins that unwind duplex DNA and bind to and wrap around single-stranded (ss)DNA. Yet assembly of the required substrates, which often contain a ssDNA segment embedded within a larger double-stranded (ds)DNA construct, can be time-consuming and inefficient, particularly when using a standard three-way hybridization protocol. In this chapter, we detail how to construct a variety of force-activated DNA substrates more efficiently. To do so, we engineered a dsDNA molecule with a designed sequence of specified GC content positioned between two enzymatically induced, site-specific nicks. Partially pulling this substrate into the overstretching transition of DNA (~65 pN) using an optical trap led to controlled dissociation of the ssDNA segment delineated by the two nicks. Here, we describe protocols for generating ssDNA of up to 1000 nucleotides as well as more complex structures, such as a 120-base-pair DNA hairpin positioned next to a 33-nucleotide ssDNA segment. The utility of the hairpin substrate was demonstrated by measuring the motion of *E. coli*. RecQ, a 3′-to-5′ DNA helicase.

Key words Optical tweezers, Optical trap, Helicase, DNA overstretching, Single-molecule force spectroscopy

1 Introduction

Single-molecule force spectroscopy (SMFS) has revealed important mechanistic details about the dynamics and energetics of individual protein–nucleic-acid complexes [1–4]. For proteins that bind to and move along DNA, SMFS assays leverage a variety of substrates, including purely double-stranded DNA (dsDNA), purely single-stranded DNA (ssDNA), or a combination of both. One particularly useful substrate consists of a DNA hairpin adjacent to a short ssDNA site embedded within a micron-scale dsDNA molecule that meets the geometric requirements of optical-

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trapping based assays (Fig. 1a). This hairpin-unwinding construct enables high-resolution studies of helicase motion due to its three-fold amplification of the measured unwinding signal [5]. Specifically, each base pair (bp) of the hairpin unwound results in 2 nucleotides (nt) of ssDNA being added to the overall substrate's contour length. The added contour length, which leads to the measured signal, is 1.12 nm (2 × 0.56 nm/nt) per base pair unwound, compared to the 0.34 nm decrease in signal from 1-bp steps of a surface-anchored helicase [6]. The utility of the hairpin-unwinding assay is not limited to optical-trapping studies of DNA helicases; it was originally developed for RNA helicases [5], has been applied to ribosomes [7], and adopted in magnetic-tweezers based assays [8–10]. Notwithstanding this utility and widespread application, assembling the required DNA substrate is inefficient and laborious, particularly when using the standard three-way hybridization scheme (Fig. 1b, left) [11, 12]. Moreover, in traditional surface-based assays, hairpin-unwinding assays are often low throughput since the unwinding of all the DNA molecules in the sample chamber starts once a limiting agent, typically ATP, is added [6, 13].

Here, we detail an efficient in situ method of generating hairpin-unwinding substrates starting from pure dsDNA by force-induced strand denaturation. This dsDNA sequence can, in most cases, be cloned into a bacterial plasmid, providing practically unlimited quantities of template for our final nicked dsDNA product. This scheme of using mechanical denaturation of dsDNA via a transiently elevated force (Fig. 1b, right and Fig. 1c) can be extended to generate kbp-scale segments of ssDNA. Besides essentially 100% efficiency in generating the desired substrate, in situ generation of the exposed ssDNA enables quantifying the initial binding of a protein and thereby its on-rate (k_{on}). Measurements of k_{on} are difficult in a standard optical-trapping assay initiated by adding a limiting component due to mechanical perturbations from fluid flows and/or a lack of a precisely defined start time. When using our force-activated substrates, the effective dead time can be <1 s since only a transient high force is needed. Importantly, the assay buffer can contain all necessary components (enzyme, Mg^{2+}, ATP, etc.) because many helicases do not initiate unwinding on dsDNA. Mechanical activation exposes a suitably sized segment of ssDNA that acts as a helicase loading site, after which unwinding of the adjacent duplex DNA can occur.

As background, force-induced strand displacement from dsDNA traditionally occurred at very high force (120–250 pN), a regime inaccessible in a typical optical trap but one that was easily achieved using an atomic force microscope (AFM). In the original assay, the dsDNA was rapidly stretched at 0.15–3 μm/s in a buffer with no added chemical denaturants [14]. The resulting force–extension curve first showed DNA's canonical overstretching transition at 65 pN [15, 16] and then a more subtle transition at very

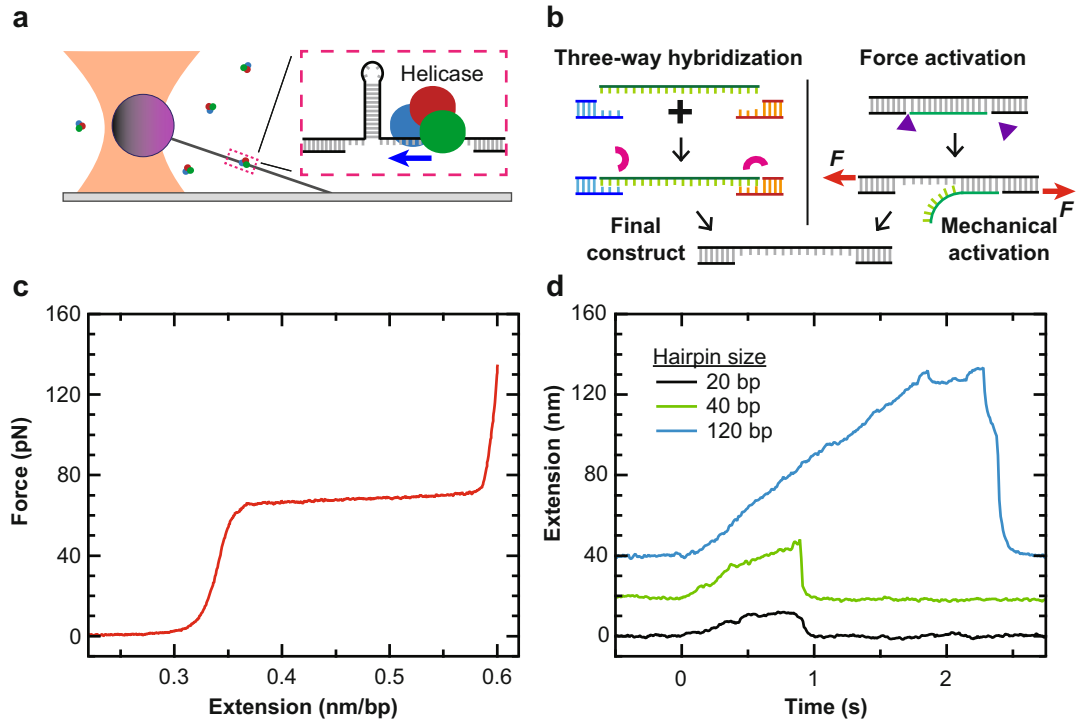

Fig. 1 (**a**) Cartoon depiction of a surface-based optical trapping assay. A single DNA molecule is stretched between an optically trapped, micron-sized bead and a functionalized surface. The inset depicts a helicase that has bound to a ssDNA section adjacent to the hairpin. The helicase proceeds to unwind the DNA hairpin. (**b**) Scheme comparing standard three-way hybridization and force activation methods of synthesizing a section of ssDNA (often a DNA hairpin). (*Left*) The three separate DNA pieces are joined with DNA ligase (*small red arcs*) to form the final construct. (*Right*) The bottom strand in the duplex is site-specifically nicked by DNA nicking enzymes (*purple triangles*). The ssDNA section between the nicks dissociates when the construct is force-activated at a user-defined time. (**c**) A force–extension curve of a 2-μm long dsDNA molecule. The overstretching transition occurs at ≈65 pN. (**d**) Three example helicase unwinding traces on hairpins of 20 bp (*black*), 40 bp (*green*), and 120 bp (*blue*) in length. Traces are offset vertically for clarity. ((**a**), (**b**), and (**c**) adapted from [25], which is in the public domain)

high force, which was the dissociation of one strand away from the other. Hence, strand displacement was quite distinct from overstretching, where a small increase in force around 65 pN leads to a 70% increase in DNA's extension (Fig. 1c). As was common at the time of the original AFM assay, nonspecific attachment was used to anchor the DNA both to a glass coverslip and the tip of the AFM cantilever, limiting both the duration of the assay and control over the exact length of the molecule stretched. Numerous subsequent studies using both optical tweezers and AFM showed that pulling dsDNA into or just past the overstretching transition did not result in strand dissociation [15–22], except when the dsDNA was ≤30 bp long [23]. A more recent work showed that pulling DNA through the overstretching transition and maintaining a high force

of 75 pN for ≥5 s could lead to strand displacement [22]. However, this high sustained force can lead to the rupture of the protein-based conjugation often used in anchoring DNA to either beads and/or surfaces (e.g., streptavidin–biotin and digoxigenin–anti-digoxigenin).

We hypothesized that kbp-sized segments of ssDNA could be induced to dissociate at ~65 pN in standard assay conditions by controlling the GC content of the DNA at a base-pair scale. This hypothesis was based on an improved understanding of DNA overstretching. Over the last decade, overstretching has been shown to arise from three distinct mechanisms that all occur at essentially the same force [19–21]: peeling from free ends or nicks, internal melting of base pairs, and a transition to S-DNA. The particular mechanism depends on the exact ionic conditions, temperature, and topology of the construct. For situations where peeling and/or melting occur but the strands do not separate at the end of overstretching, it was speculated that the short stretches of repeated GC base pairs, so-called GC islands, found in typical DNA sequences could suppress strand separation [24]. We therefore designed a 1000-bp sequence with exactly 50%-GC content but containing no adjacent GC base pairs. In a recent publication, we demonstrated force-induced strand displacement of this large ssDNA segment and of a more complicated but smaller hairpin-unwinding construct consisting of a 20-bp hairpin positioned adjacent to a 33-nt ssDNA segment [25]. For both substrates, strand displacement occurred after stretching them partially into the overstretching transition (and thus not applying a force above ~65 pN). Moreover, this force was only transiently applied (~0.1 s for the 20-bp hairpin and ~0.3 s for the 1000-bp construct). This force activation scheme resolved three important issues: (1) high sustained forces can lead to failure of protein-ligand bonds used in anchoring the DNA, (2) force-activation efficiency of natural DNA sequences decreases under ionic conditions that stabilize base-pair formation (e.g., 150 mM NaCl), and (3) more sophisticated DNA structures, such as the one needed for the hairpin-unwinding assay, had not been realized.

This chapter provides a protocol on engineering and synthesizing a variety of force-activated DNA constructs. In addition to the two aforementioned substrates, we extend these design principles to make longer DNA hairpins (40 and 120 bp) (Fig. 1d) and hairpins with segments of GC content varying from 0% to 75%. We also modified the helicase binding sites to allow study of both 3′-to-5′ and 5′-to-3′ polarity helicases. Finally, we detail the design and synthesis of a 120-bp hairpin with distinct runs of GC content of 0% and 50%. The first four sections of this chapter explain the design and synthesis process for the force-activated substrates. Subheading 3.1 explains the design of the 1-kbp 50%-GC construct, the 20-bp hairpins, and variations on the hairpin design.

Subheading 3.2 details the molecular biology techniques used to synthesize DNA templates for the final PCR amplification. Subheading 3.3 describes the protocol for the final PCR amplification of the substrate with labeled PCR primers, and Subheading 3.4 explains the site-specific nicking of the force-activated substrates. The last two sections describe application of the force-activated substrates in an optical-trapping assay. Subheading 3.5 describes the process of coupling the substrates to a PEG-coated glass coverslip by a mechanically robust covalent bond using copper-free click chemistry [26]. Finally, Subheading 3.6 details the process of activating the substrates in an optical-trapping assay.

2 Materials

2.1 Designing Force-Activated DNA Substrates

1. Secondary structure of ssDNA lengths was checked using OligoAnalyzer, an online analysis tool from Integrated DNA Technologies (IDT) (https://www.idtdna.com/pages/tools/oligoanalyzer).
2. Melting temperatures of dsDNA lengths were obtained using the nearest neighbor method from OligoCalc: Oligonucleotide Properties Calculator from Northwestern University (http://biotools.nubic.northwestern.edu/OligoCalc.html).

2.1.1 A 1-kbp Segment of Exactly 50% GC

1. Software for designing ~1000 nt long ssDNA sequences containing no adjacent GC base pairs such that no secondary structure forms (detailed in Subheading 3.1.1). The full designed sequence, cloned into plasmid pUC57, was ordered from GenScript Biotech Corporation.
2. All primers in this chapter were synthesized by IDT. Primers for the 1-kbp 50%-GC construct are listed in Table 1. The 1-kbp construct consisted of a 50%-GC sequence flanked by additional dsDNA which we refer to as the left and right handles. The 50%-GC sequence was synthesized with flanking recognition sites for restriction endonucleases PflMI and BbsI. This allows us to attach dsDNA handles by PCR using M13mp18 plasmid-based primers with either PflMI or BbsI. pUC57 primers were used to amplify the 50%-GC section. PflMI is a restriction endonuclease with its recognition sequence highlighted in cyan. BbsI recognition sequence is highlighted in green. M13mp18 plasmid sequence is shown in red. The DBCO (dibenzocyclooctyne) and biotin primers are used in the final preparative PCR reaction. Primers follow the same format, for example, L_1000GC50_F refers to the left handle of the 1000-bp 50%-GC construct forward primer.

Table 1
1-kbp 50%-GC construct primers

Name	Sequence (5′–3′)
L_1000GC50_F	ATT ACG GTC AAT CCG CCG
L_1000GC50_R	CGA GAT CCA TCC CAT GGT TGA GTC ACT GCT AAG TCA CTG CAT ATT CAC TGC CTT GAC AAG AAC CGG ATA TTC
R_1000GC50_F	CGC ATA GAA GAC TTG CAA TGA GAG AGT TGC AGC AAG C
R_1000GC50_R	AGT TGT TCC TTT CTA TTC TCA CT
pUC57_F	ACC GCA CAG ATG CGT AAG G
pUC57_R	GAA ACA GCT ATG ACC ATG ATT A
L_1000GC50_DBCO_F	5′DBCO-TEG-ATT ACG GTC AAT CCG CCG
R_1000GC50_Biotin_R	5′Biotin-TEG-GCC TCA ACC TCC TGT CAA TGC T

2.1.2 A 20-bp Hairpin with 33-nt Helicase Binding Site

Primers are listed in Table 2. The BstXI restriction sequence is highlighted gray; the Nb.BbvCI nick sequence is colored magenta, and the GC clamps are highlighted in yellow. M13mp18 plasmid sequence is again shown in red, and pBR322 sequence in gold. Helicase binding area is shown in blue. The 4X biotin primer is designed such that biotins are spaced every 9–10 bp within the primer sequence.

2.1.3 Extending to Longer Hairpins and Higher GC Content

1. 40-bp 50%-GC hairpin primers (*see* Table 3).
2. 20-bp 66%-, 75%-, and 100%-GC hairpin primers (*see* Table 4).
3. 40-bp 66%- and 75%-GC hairpin primers (*see* Table 5).
4. Reversed polarity (5′-to-3′) 40-bp hairpin primers (*see* Table 6).
   4XBiotin_adapt_F primer is an adapter sequence that contains two sections of sequence from M13mp18 (one in bold italics) that was used to make shorter handles (left handle decreased from 2.5 kbp to 1.3 kbp, right handle decreased from 3.0 kbp to 1.5 kbp) to decrease the mechanical compliance and therefore the thermal noise in our assay. Using an adapter allowed us to use the same L_4XBiotin_F primer, an expensive and unique oligo, in the final PCR reaction.

2.1.4 120-bp Hairpins with Distinct Runs of Different GC Content

1. Primers are listed in Table 7.

2.2 Assembling DNA Templates for Final PCR Amplification

1. Pairs of substrate primers from one of the previous sections.

Table 2
20-bp hairpin primers

Name	Sequence (5′–3′)
L_20GC50_F	TCA ATA ATC GGC TGT CTT TCC TTA TCA TTC GGG CGC TCG GCG TGC AGC CGT GCG GGC TTG TTC AGG ACT TAT CTA TTG TTG ATA AAC AG
L_20GC50_R	GAT GCC ATA AAA ATG GAT CCA GAC GTT GAC TCA AAT GCT GAG GGC GGG AGC CTG GCC GTG CCC GCT GAG AGA GTT GCA GCA AGC G
R_20GC50_F	TAA GCC ATT TTT ATG GAT CCA GAC GTT GAC TCC CCG CTG TCT AGA GGA TCC GAC TAT CGA CCT CAG CGC TTT AAT GCG GTA GTT TAT CAC AGT TAA ATT GC
R_20GC50_R	CGA AGG TAA CTG GCT TCA GCA GAG CGC AGA TAC CAA ATA CTG TCC
R_20GC50_DBCO_R	5′DBCO-TEG-CGA AGG TAA CTG GCT TCA GCA GAG CGC AGA TAC CAA
L_4XBiotin_F	5′Biotin-TEG-TCA ATA A-BiodT-C GGC TGT CT-BiodT TCC TTA TCA BiodT-TC

Table 3
40-bp 50%-GC hairpin primers

Name	Sequence (5′–3′)
L_40GC50_R	GAT GCC ATA AAA ATG GAT CCA GAC GTT GAC TCG TAC GTC AGT CGA TCT AGA CAA ATG CTG AGG GCG GGA GCC TGG CCG TGC CCG CTG AGA GAG TTG CAG CAA GCG
R_40GC50_F	TAA GCC ATT TTT ATG GAT CCA GAC GTT GAC TCG TAC GTC AGT CGA TCT AGA CCC CGC TGT CTA GAG GAT CCG ACT ATC GAC CTC AGC GCT TTA ATG CGG TAG TTT ATC ACA GTT AAA TTG
4XBiotin_adapt_F	CAT G ***TCA ATA ATC GGC TGT CTT TCC TTA TCA TTC*** CTT CTA AAT CCT CAA ATG TAT TAT CTA TTG ACG GCT C
pBR322_R	GC AGT CGC TTC ACG TTC GCT CG
R_DBCO_R	5′DBCO-TEG-GC AGT CGC TTC ACG TTC GCT CG

2.2.1 Initial PCR Amplification of DNA Handles

1. TE buffer: 10 mM Tris–HCl (pH 8.0), 1 mM EDTA.
2. KOD Hot Start DNA polymerase (Millipore-Sigma Novagen 71086-4).
3. Taq polymerase (Life Technologies 10342-053).

Table 4
20-bp 66%-, 75%-, and 100%-GC hairpin primers

Name	Sequence (5′–3′)
L_20GC100_R	CAT CCC ATA AAA ATG GCG CGC GCC GGC CCG CGG CAA ATG CTG AGG GCG GGA GCC TGG CCG TGC CCG CTG AGA GAG TTG CAG CAA GCG GTC
R_20GC100_F	TAA GCC ATT TTT ATG GCG CGC GCC GGC CCG CGG CTT TGC TGT CTA GAG GAT CCG ACT ATC GAC CTC AGC GCT TTA ATG CGG TAG TTT ATC ACA GTT AAA TTG C
L_20GC66_R	CAT CCC ATA AAA ATG GAG CAC GTC GTC CAG CAG CAA ATG CTG AGG GCG GGA GC
R_20GC66_F	TAA GCC ATT TTT ATG GAG CAC GTC GTC CAG CAG CTT TGC TGT CTA GAG GAT CCG AC
L_20GC75_R	CAT CCC ATA AAA ATG GCA CGC TCC GTC CCA CGG AGG CAA ATG CTG AGG GCG GGA G
R_20GC75_F	TAA GCC ATT TTT ATG GCA CGC TCC GTC CCA CGG AGG CTT TGC TGT CTA GAG GAT CCG AC

Table 5
40-bp 66%- and 75%-GC hairpin primers

Name	Sequence (5′–3′)
L_40GC66_R	CAT CCC ATA AAA ATG GAG CAC GTC GTC CAG CAG CAC CTC GTC GTG GAC CTG CAA ATG CTG AGG GCG GGA GC
R_40GC66_F	TAA GCC ATT TTT ATG GAG CAC GTC GTC CAG CAG CAC CTC GTC GTG GAC CTG CTT TGC TGT CTA GAG GAT CCG AC
L_40GC75_R	CAT CCC ATA AAA ATG GCA CGC TCC GTC CCA CGG AGG CTC CGT CGG TCG CAA ATG CTG AGG GCG GGA G
R_40GC75_F	TAA GCC ATT TTT ATG GCA CGC TCC GTC CCA CGG AGG CTC CGT CGG TCG CTT TGC TGT CTA GAG GAT CCG AC

4. 0.2-mL thin-walled PCR tubes.
5. 1.5-mL microcentrifuge tubes.
6. Ultrapure water (18.2 MΩ/UV irradiated/sterile filtered).
7. Metal PCR tube holder (optional, can also use a different tube holder, or just place tubes directly on crushed ice).
8. Hand vortex mixer (e.g., Scientific Industries Vortex-Genie 2 SI-0236).
9. M13mp18 plasmid DNA template (New England Biolabs N4018S).

Table 6
Reversed polarity (5′-to-3′) 40-bp hairpin primers

Name	Sequence (5′–3′)
L_5′-to-3′_R	GGA TCC ATA AAA ATG GAT CCA GAC GTT GAC TCG TAC GTC AGT CGA TCT AGA CGG GTT CGA TAG TCG GAT CCT CTA GAC AGC GCT GAG GTG AGA GAG TTG CAG CAA GCG
R_5′-to-3′_F	GGA TCC ATT TTT ATG GAT CCA GAC GTT GAC TCG TAC GTC AGT CGA TCT AGA CTT TCC TCA GCG CGG GCA CGG CCA GGC TCC CGC GCT TTA ATG CGG TAG TTT ATC ACA GTT AAA TTG C

Table 7
120-bp hairpin primers

Name	Sequence (5′–3′)
L_120GC50_R	CAT CGA TCC ATA AAA ATG GAT CCA GAC GTT GAC TCG TAC GTC AGT CGA TCT AGA CGA GCA TGA GCT GGT CGA CTC TCA TCA AGA GTG ACA TCT TGC TTC ACT GCT TGA CTG TAG CTG CAT ACC TGT GAC CAG AGT AAA TGC TGA GGG CGG GAG CCT G
R_120GC50_F	TAA GGA TCC ATT TTT ATG GAT CCA GAC GTT GAC TCG TAC GTC AGT CGA TCT AGA CGA GCA TGA GCT GGT CGA CTC TCA TCA AGA GTG ACA TCT TGC TTC ACT GCT TGA CTG TAG CTG CAT ACC TGT GAC CAG AGT CCC GCT GTC TAG AGG ATC CGA CTA TCG
L_120GC50/0/50_R	CAT CGA TCC ATA AAA ATG GAT CCA GAC GTT GAC TCG TAC GTC AGT CGA TCT AGA CAT AAT ATA ATT TAT AAT ATA ATT TAT AAT ATA ATT TAT TAC TTC ACT GCT TGA CTG TAG CTG CAT ACC TGT GAC CAG AGT AAA TGC TGA GGG CGG GAG CCT G
R_120GC50/0/50_F	TAA GGA TCC ATT TTT ATG GAT CCA GAC GTT GAC TCG TAC GTC AGT CGA TCT AGA CAT AAT ATA ATT TAT AAT ATA ATT TAT AAT ATA ATT TAT TAC TTC ACT GCT TGA CTG TAG CTG CAT ACC TGT GAC CAG AGT CCC GCT GTC TAG AGG ATC CGA CTA TCG
L_120_F	TCA ATA ATC GGC TGT CTT TCC TTA TCA TTC
R_120_F	GAT CCA TTT TTA TGG ATC CAG ACG TTG
L_120_R	GAT CCA TAA AAA TGG ATC CAG ACG TTG

10. pBR322 plasmid DNA template (New England Biolabs N3033S).
11. Thermocycler (e.g., Bio-Rad T100 Thermal Cycler).
12. Qiagen QIAquick PCR purification kit (Qiagen 28106).
13. Spectrophotometer (e.g., Thermo Scientific NanoDrop 2000).
14. Agarose (Sigma-Aldrich A9539).

15. Microwave.
16. TAE buffer (Tris–acetate–EDTA) buffer: 40 mM tris acetate (pH ≈ 8.3), 20 mM acetic acid, 1 mM EDTA (pH 8).
17. 6× DNA loading buffer: 0.25% bromophenol blue (w/v), 0.25% xylene cyanol (w/v), and 30% glycerol (v/v).
18. Gel electrophoresis setup (e.g., Thermo Scientific Owl Easy-Cast B1A Mini Gel Electrophoresis Systems, Thermo EC EC2060 power supply (max output: 2000 V, 600 mA, 300 W), Boekel Scientific Rocker II 260350).
19. Blue light transilluminator (e.g., the Invitrogen Safeimage2 G6600) for gel excisions.
20. UV transilluminator.
21. Clean razor blade.
22. Freeze 'N Squeeze DNA Gel Extraction Spin Columns (Bio-Rad 732-6165).
23. Microcentrifuge (e.g., Eppendorf Centrifuge 5424).
24. Millipore Amicon 0.5-mL Centrifugal Filter Ultracel 10 K (UFC501096).

2.2.2 Preparing the Substrate for Ligation Using Restriction Enzymes

(All restriction endonucleases in this chapter are purchased from New England Biolabs.)

1. PflMI.
2. BbsI.
3. BstXI.
4. Nb.BbvCI.
5. T4 DNA Ligase (New England Biolabs).
6. Manufacturer provided 10× Buffer for each enzyme.
7. Millipore Amicon 0.5-mL Centrifugal Filter Ultracel 30 K (UFC503096).

2.2.3 Cloning Substrate into E. coli for Efficiency and Accuracy

1. TOPO XL-2 Complete PCR Cloning Kit (Invitrogen K8050-10).
2. One Shot TOP10 Chemically Competent *E. coli* (Invitrogen C404010).
3. SOC recovery medium: 2% tryptone, 0.5% yeast extract, 10 mM NaCl, 2.5 mM KCl, 10 mM $MgCl_2$, 10 mM $MgSO_4$, 20 mM sterile glucose (pH 7.0).
4. Kanamycin stock (50 mg/mL = 1000× in water).
5. Bacto dehydrated agar (BD Biosciences 214010).
6. Luria Broth (LB): 15 g NaCl, 10 g Tryptone, 10 g Yeast Extract.

7. Petri dishes polystyrene 100 × 15 mm^2 (Falcon 351029).
8. Sterile glass culture tubes (20 mm × 150 mm).
9. Shaker incubator to grow colonies at 37 °C (200 rpm).
10. Qiagen QIAprep Spin Miniprep Kit (Qiagen 27106).
11. Qiagen Plasmid Plus Midi Kit (Qiagen 12943).

2.3 Final PCR Amplification with Labeled Primers

1. 4X biotin primer (4 biotin molecules spaced 9–10 bp apart starting at the 5′ end of the primer. Listed in Subheading 2.1.2 as "L_4XBiotin_F").
2. Dibenzocyclooctyne (DBCO) primer.

2.4 Site-Specific Nicking of the Force-Activatable Substrate

1. Nb.BsmI (5′...GAATG/CN...3′) (nicks complementary strand).
2. Nt.BspQI (5′...GCTCTTCN/...3′).
3. Nb.BbvCI (5′...CCTCA/GC...3′) (nicks complementary strand).
4. 10× alkaline agarose gel buffer: 0.5 M NaOH, 10 mM EDTA; make fresh every time (from Ch. 5, protocol 8 of [27]).
5. 6× alkaline gel loading dye: 300 mM NaOH, 6 mM EDTA, 0.25%w/v bromophenol blue, 30% w/v glycerol; make fresh every time.
6. Neutralization buffer: 1 M Tris–HCl (pH 7.6), 1.5 M NaCl; make fresh every time (from Ch. 5, protocol 8 of [27]).

2.5 Coupling Substrates to Coverslips Through Covalent Click Chemistry

1. Silane-PEG-azide, 600 Da [Nanocs Inc. PG2-AZSL-600, (200 mg vial)].
2. UV-ozone chamber (Novascan Technologies PSD-UV Benchtop UV-Ozone Cleaner) or hand-held UV lamp.
3. Chemical fume hood.
4. Large magnetic hot plate.
5. Fine balance scale.
6. KOH-cleaned glass coverslips (Corning 2980-224 borosilicate glass).
7. Diamond scribe.
8. ≈500 mL toluene (AR ACS Reagent Grade, Macron 8608-16).
9. ≈50 mL isopropanol (Fisher Chemical BPA4164).
10. Large glass petri dish with top (Pyrex 150 × 15 mm).
11. 10-mL and 100-mL graduated cylinders.
12. Four 250-mL beakers.
13. 10-mL beaker with cover (small petri dish).

14. Watch glass to cover 250-mL beaker (Pyrex 998575).
15. 1 small (≈12 mm long) and 1 medium magnetic stir bars (≈38 mm long).
16. Metal spatula.
17. Deposition buffer: 20 mM HEPES (pH 7.5), 20 mM KCl, 1 mM EDTA (pH 8).

2.6 Using the Constructs in an Optical-Trapping Assay

1. Optical-trapping instrument or, more generally, a single-molecule force spectroscopy instrument that can apply 65 pN of force.
2. Prepared sample with force-activated DNA substrates.
3. Assay buffer: 20 mM Tris–HCl (pH 7.5), 150 mM NaCl, 0.4% Tween 20.
4. Flow cell (*see* Subheading 3.8 of [28] for details on the construction).

3 Methods

3.1 Designing Force-Activated DNA Substrates

The first point to consider when designing a force-activated substrate is the sequence of the target region, which includes both the sequence of the force-activated ssDNA section as well as the placement of the two recognition sites for enzymatically nicking the same strand. The ssDNA section should be designed to suit your experiment (e.g., encode a hairpin-unwinding substrate for a helicase study or a specified length of unstructured ssDNA for ssDNA-binding protein studies). In the following subsections, we lay out a few of our group's designs, focusing on hairpin-unwinding substrates. Once the target sequence and the two nicking sites have been designed, the length of the final construct is extended to satisfy the length requirements of an optical-trapping assay by adding generic dsDNA sequence, here termed dsDNA "handles." For simplicity, we use sequences taken from a common plasmid DNA (e.g., M13mp18). To accommodate our optical-trapping assay, we use molecules that are 0.67–2 μm (2000–6000 bp) in length [29]. Additionally, we sometimes add in GC clamps (20–30 bp of 80–85% GC) to suppress peeling and stabilize the dsDNA handles near their ends or adjacent to nicking sites [25]. Construct plasmids described in the following sections were deposited with Addgene (https://www.addgene.org) and are available with the following accession numbers: hp-50%GC-20bp, 169196; hp-50%GC-40bp, 169197; hp-50%GC-40bp-Revbindsite, 169198; hp-66%GC-20bp, 169199; hp-66%GC-40bp, 169200; hp-75%GC-20bp, 169201; hp-75%GC-40bp, 169202; hp-100%GC-20bp, 169203; hp-50%GC-Left-half-120bp, 169204; hp-50%GC-Right-half-

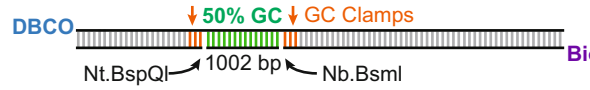

Fig. 2 A cartoon showing the design of the 1-kbp 50%-GC construct. The substrate is labeled with DBCO, a copper-free click chemistry reagent, on the left and biotin on the right. GC clamps (*orange*) help prevent peeling of the dsDNA. Nt.BspQI and Nb.BsmI nick the same strand in the positions indicated by the black arrows. (Adapted from [25], which is in the public domain)

120bp, 169205; hp-50-0-50%GC-Left-half-120bp, 169206; hp-50-0-50%GC-Right-half-120bp, 169207. The 50%GC-alternating-1kbp is available from the authors to avoid the introduction of an unwanted insertion.

3.1.1 A 1-kbp Segment of Exactly 50% GC

This section deals with the design of a substrate that releases a long segment of ssDNA upon force activation. We synthesized a 1000-bp sequence of 50% GC where the sequence strictly alternates between GC and AT base pairs 500 times (Fig. 2). This substrate was used as a proof-of-concept construct to demonstrate very long DNA segments could be force activated while only stretching partway through the overstretching transition. This construct removed the nearest-neighbor effects of two, three, or more consecutive GC bp that occur in natural DNA sequences. The exact sequence was designed using custom code that made the sequence in 10-bp segments by alternating between randomly selecting either an 'A' or 'T' and a 'G' or 'C'. The full 1000-bp sequence was run through a custom autocorrelation routine to ensure that no stable secondary structures would form, but any DNA secondary structure analysis program would work as well. After this sequence was defined (sequence shown in Table 8), we introduced recognition sites for the DNA nicking endonucleases Nt.BspQI and Nb.BsmI and GC-clamp sequences next to the nicking sites to stabilize subsequently appended DNA handles (Fig. 2). This de novo sequence was synthesized by GenScript. The DNA handles were appended to this sequence using standard DNA cloning based on two restriction endonucleases, PflmI and BbsI, and ligation.

To generate the substrate's DNA handles, we PCR amplified DNA segments with the primers listed in Subheading 2.1.1, which contain M13mp18 sequences with the addition of the PflMI recognition sequence in the left reverse primer (L_1000GC50_R), and the BbsI recognition sequence in the right forward primer (R_1000GC50_F). To construct this substrate (exact steps are detailed in later sections), we first PCR amplified the 50%-GC plasmid with pUC57 primers (we could also grow up more plasmid in *E. coli*). Next, the 50%-GC section and left handle were both cut with PflMI (37 °C for 3 h) and ligated together with T4 DNA ligase (16 °C for 16 h). The ligated product was gel-purified to

Table 8
Sequence of 1-kbp 50%-GC construct. PflmI and BbsI restriction sequences are colored cyan and green, respectively. Nt.BspQI and Nb.BsmI nick sequences are colored red, and both enzymes nick the sense strand. GC clamp sequences are colored yellow

Sequence (5′–3′):

ATACCATGGGATGGTATAGGGCGCTCGGCGTGCAGCCGTGCGGGCTCTTCGT
CACAGACACTGTGACACTGTCAGTGACTCTGAGAGACTGAGTGAGTGTGAGT
GAGTCAGTGACACAGTCACTCTGTCAGAGACACTCTGACAGTGAGAGTGTGA
CAGACTGTCTCAGAGTCAGACAGTCAGACTCTCTGAGTCACTGTCACTGACA
CTGACTCAGAGTCAGTCACTCAGTGAGTCTCTGTGTCTGTGTGTCTCTGACTG
TGACACTCTCTCTCTGTGTGAGACACTCAGACACACTCTGTGACAGAGTGTCA
CACTCACACTGTCTGACAGACACTGAGAGAGTCAGTGAGAGTCAGTGTGTCA
GACTGTGACTCAGTCAGTCACTGAGACTGTCTGACACTCTCTGAGACTGTGAG
TGAGTGTCTCAGAGACTGTGAGACTGTGTCAGTCTGAGTGACACTGACAGAG
ACTCAGTCAGAGACTCTCACACTCAGAGTGTCTGAGTGAGAGAGAGTCACTG
AGAGTCAGAGACTGACTGTCTCAGTGTGTCTGACAGTCACACTGACAGTCTCT
GACACTGTCTGTCAGAGTCTGTCACTGAGTGTCACAGACAGAGAGAGTGTGT
CACACTGTGACAGTCACAGTGTCAGTGTGTGACACTCACTCTGACTGACAGA
GTGTGAGAGACACACACACACTCACAGTGTGTGAGTCTGACTCAGTGAGAGT
GACAGTCTGTCTCTGTGTGTCTGACACTGACACACTGTGTGTGTGTGACTCAG
ACAGAGAGTGTGAGTCACTCAGACAGTGAGTGTGACTCACAGTCAGACACAG
AGTCACTGTGACTCTGACTGAGAGTCTCACTGTCTCTCTGACTCAGACTGTGT
GACTGACACACAGAGACTGTCTCACACTGAGACTCACACTGACTGAGAGACA
CTCTCACAGTGACAGTCAGTGACTGTGACTGAGAGAGACTCTGTGAGTCTGT
GAGTGAGAGACTGTCTGTGTCACACAGTGACACTGTGAGAGACTCAGAGAGA
GTCTGAGCATTCGCGATCCGTGCGGGCACGGCCAGGCTCCCGCATAGAAGAC
TTGCAATCAC

isolate it from unligated components. Then the PflmI ligated left handle-50%GC and the right handle were both cut in equimolar quantities with BbsI (37 °C for 4.5 h), ligated together, and gel-purified as before. The final product was cloned into pCR-XL-TOPO plasmid using the TOPO-XL cloning kit. Be cautious when growing up a plasmid with this sequence because we often got an unwanted duplication event (in the 50%-GC region) if we did not directly transform the plasmid and immediately grow up a culture from a single colony on an LB-agar plate. Thus, to avoid this duplication, we never started from a culture or a frozen glycerol stock for growing up the bacteria for a small- or large-scale plasmid preparation. After plasmid isolation, the plasmid sequence was verified using a commercial company (e.g., Quintara Biosciences or Genewiz); this plasmid was used as a template for the final PCR amplification with primers L_1000GC50_DBCO_F and R_1000GC50_Biotin_R to label the construct with biotin and

DBCO for surface/bead attachment. The final, labeled PCR products were purified by gel electrophoresis.

The advantage of cloning the ligated product into a plasmid is that it not only gives a very pure template for PCR but also gives enough product to verify the sequence and essentially an endless supply of template. As opposed to using a ligation product, PCR amplifying the final product from a plasmid template that has been sequenced guarantees that you always produce the same products. We have empirically found PCR amplification from a plasmid template yields a cleaner product—less extraneous bands when analyzed by gel electrophoresis—and more DNA. The final PCR reaction adds the chemical moieties used for anchoring the DNA via a 5′ label on the forward and reverse PCR primers—here biotin and DBCO, a copper-free click chemistry reagent.

3.1.2 A 20-bp Hairpin with 33-nt Helicase Binding Site

Our initial hairpin-unwinding substrate contained a 20-bp long hairpin capped with a 4-T loop (Fig. 3a). The hairpin sequence was exactly 50% GC and slightly modified from a previously published hairpin (20R50/4T) [30]. Adjacent (3′) to the hairpin was a 33-nt helicase landing site based on a previously published RecQ helicase study (oligo #3 in [31]). Assembling the full hairpin-unwinding construct is technically challenging. A 20-bp hairpin is thermodynamically stable and thus could interfere with PCR amplification if encoded into a single PCR primer as undesirable intra- and inter-primer structures can form (Fig. 3b). We solved this problem by encoding the two halves of the hairpin stem in two separate PCR primers that amplified two larger DNA molecules that separately formed the left and right handles. A restriction site was also engineered into the location of the tetraloop at the top of the hairpin in each primer, so that each half of the hairpin could be separately PCR amplified (Subheading 3.2.1), cut with the restriction enzyme (Subheading 3.2.2), and ligated together to form the final construct. Specifically, this process was implemented by using the nonpalindromic restriction endonuclease site of BstXI (Fig. 3c, d). Digesting the PCR products with BstXI (37 °C for 3 h) yielded complementary, nonpalindromic 4-nt ssDNA overhangs. The left and right halves were buffer exchanged with a Millipore Amicon 10 K filter, annealed, and ligated at the loop region of the hairpin with T4 DNA ligase (Fig. 3d). The ligated product (5529 bp) was purified by gel electrophoresis. The purified product was then cloned into pCR-XL-TOPO using the TOPO XL PCR cloning kit and transformed into One Shot TOP10 Chemically Competent *E. coli*. A colony from the LB-agar plate was grown up in 5 or 150 mL of LB + kanamycin. We then purified the plasmid containing the desired 20-bp hairpin product using a Qiagen QIAprep Spin Miniprep Kit or Qiagen Plasmid Plus Midi (for purifying larger amounts of plasmid up to 10 mg, usually in 150 mL).

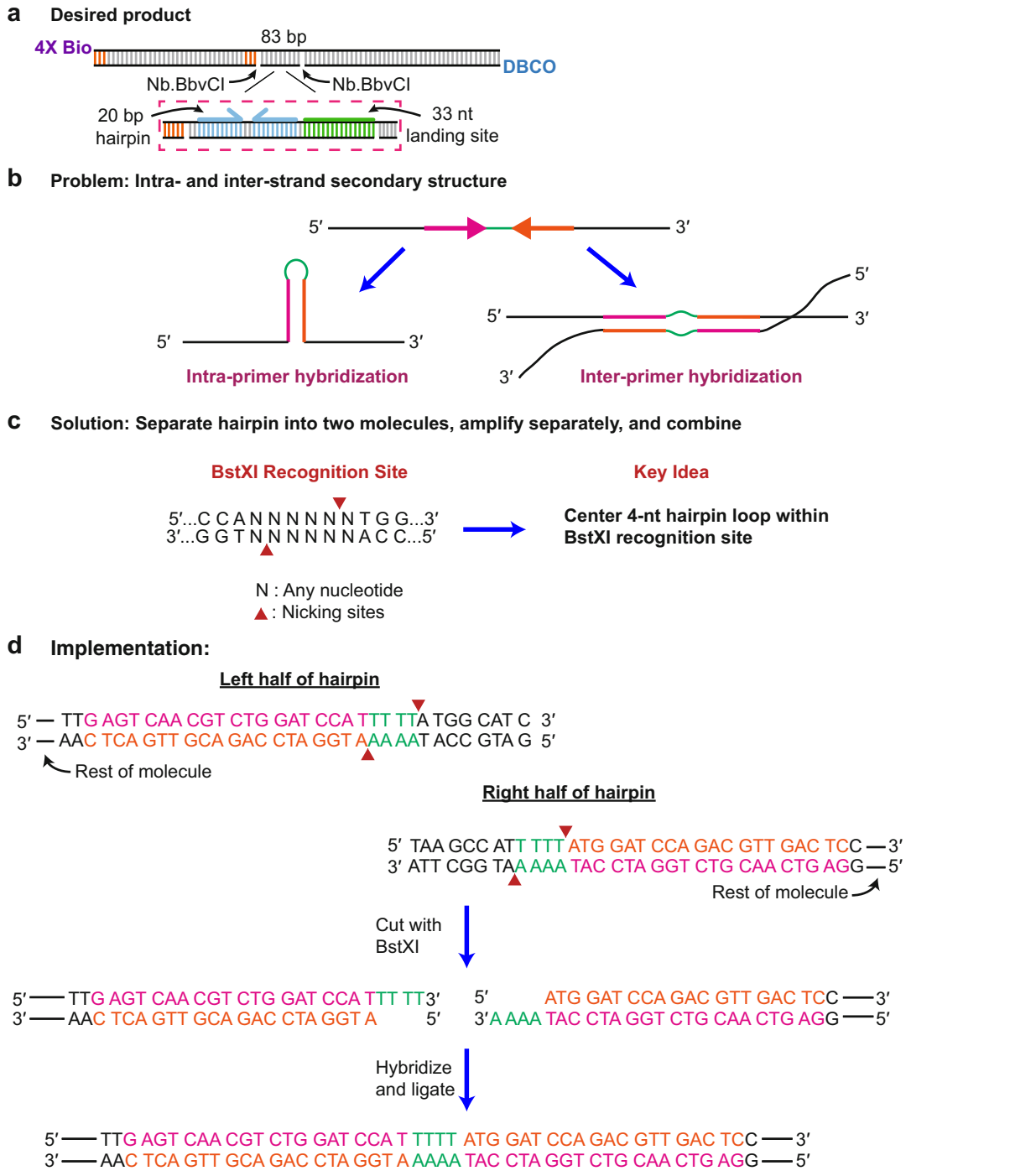

Fig. 3 Design scheme for the 20-bp hairpin. (**a**) The desired product encoding a 20-bp hairpin, helicase binding site, and nicks in the bottom DNA strand. (**b**) Cartoon illustrating the issues of using a PCR primer with the full hairpin sequence. The left (*pink*) and right (*orange*) halves of the hairpin have complementary sequence and allow both intra-primer and inter-primer hybridization. (**c**) A solution to this issue is using primers that each encode half the hairpin, where each primer contains the BstXI recognition sequence. BstXI's 12-bp recognition sequence contains 6 bp that can accommodate any sequence allowing us to design a nonpalindromic recognition sequence. BstXI cuts at the red triangles leaving a 4-nt overhang. (**d**) Sequence of steps to

Because the hairpin stem region interferes with the DNA sequencing reaction, it was necessary to cut the plasmid with BstXI and use primers a few hundred nucleotides upstream of the hairpin. This plasmid DNA was used as the template for the final PCR. As before, we purified the final product by gel electrophoresis and nicked the DNA with Nb.BbvCI. We used this force-activated construct to determine the on-rate for the RecQ helicase [25].

To assemble this 20-bp 50%-GC hairpin construct, we amplified the DNA by PCR using the primers and templates listed in Table 9. After purification, the resulting DNA left and right handles were each cut separately with BstXI, purified, and then ligated together. Finally, we cloned the entire sequence in pCR-XL-TOPO, grew it in *E. coli*, and isolated the plasmid. The final PCR amplification introduced the labels by using the L_4XBiotin_F and R_20GC50_DBCO_R primers.

3.1.3 Extending to Longer Hairpins and Higher GC Content

The above 20-bp hairpin is not much larger than the footprint of most helicases. As such, it is difficult to confidently determine unwinding rates due to boundary effects (i.e., nonstandard helicase–substrate interactions at the bottoms and tops of hairpins). To mitigate these effects, we extended the hairpin to 40 bp. The sequence of the first 20 bp of this larger hairpin was the same as our original 20-bp hairpin described above. The additional 20 bp were also 50% GC and checked for low secondary structure. It was amplified and assembled in the same fashion as the 20-bp hairpin. This 40-bp hairpin is our standard substrate for helicase-unwinding assays.

We also designed hairpin substrates with higher GC content to investigate the sequence dependence of helicase activity (Fig. 4a). Specifically, we designed 66%-GC and 75%-GC, 20-bp hairpins in a similar manner as described above (no more than 3 GC bp in a row). Despite our initial concerns on how increased GC content would affect activation reliability, we found that these 20-bp hairpins were successfully activated via the overstretching transition on all attempts at standard temperatures and salt conditions. In contrast, we made a 100%-GC 20-bp hairpin that never activated. We made 66%-GC and 75%-GC 40-bp hairpins, however only the 66%-GC hairpin successfully force-activated. Finally, we also redesigned our standard 40-bp hairpin so that the helicase landing site was on the opposite side of the hairpin (Fig. 4b), allowing us to study 5′-to-3′ polarity helicases (as opposed to the 3′-to-5′ polarity of the original 40-bp hairpin).

Fig. 3 (continued) produce the full dsDNA hairpin sequence. First, the left half and right halves are PCR amplified separately. Next, each product is cut with BstXI, purified, and then the two halves are hybridized and ligated together. The final construct is now ready to be amplified using labeled PCR primers and nicked. (Adapted from [25], which is in the public domain)

Table 9
Primers and templates for hairpin constructs

Hairpin Construct	Left handle Forward primer Reverse primer Template Product size	Right handle Forward primer Reverse primer Template Product size
20-bp 50%-GC hairpin	L_20GC50_F L_20GC50_R M13mp18 plasmid 2494 bp	R_20GC50_F R_20GC50_R pBR322 plasmid 3055 bp
40-bp 50%-GC hairpin	4XBiotin_adapt_F L_40GC50_R M13mp18 plasmid 1368 bp	R_40GC50_F pBR322_R pBR322 plasmid 1617 bp
20-bp 100%-GC hairpin	4XBiotin_adapt_F L_20GC100_R M13mp18 plasmid 1350 bp	R_20GC100_F pBR322_R pBR322 plasmid 1599 bp
20-bp 66%-GC hairpin	4XBiotin_adapt_F L_20GC66_R 20-bp 100%-GC hairpin plasmid 1350 bp	R_20GC66_F pBR322_R 20-bp 100%-GC hairpin plasmid 1599 bp
20-bp 75%-GC hairpin	4XBiotin_adapt_F L_20GC75_R 20-bp 100%-GC hairpin plasmid 1353 bp	R_20GC75_F pBR322_R 20-bp 100%-GC hairpin plasmid 1602 bp
40-bp 66%-GC hairpin	4XBiotin_adapt_F L_40GC66_R 20-bp 100%-GC hairpin plasmid 1368 bp	R_40GC66_F pBR322_R 20-bp 100%-GC hairpin plasmid 1617 bp
40-bp 75%-GC hairpin	4XBiotin_adapt_F L_40GC75_R 20-bp 100%-GC hairpin plasmid 1365 bp	R_40GC75_F pBR322_R 20-bp 100%-GC hairpin plasmid 1614 bp
Reversed polarity (5′-to-3′) 40-bp 50%-GC hairpin	4XBiotin_adapt_F L_5′-to-3′_R M13mp18 plasmid 1371 bp	R_5′-to-3′_F pBR322_R pBR322 plasmid 1614 bp
120-bp 50%-GC hairpin	L_120_F L_120GC50_R 40-bp 50%-GC hairpin plasmid 1447 bp	R_120GC50_F pBR322_R 40-bp 50%-GC hairpin plasmid 1700 bp
120-bp 50%/0%/50% GC hairpin	L_120_F L_120GC50/0/50_R 40-bp 50%-GC hairpin plasmid 1447 bp	R_120GC50/0/50_F pBR322_R 40-bp 50%-GC hairpin plasmid 1700 bp

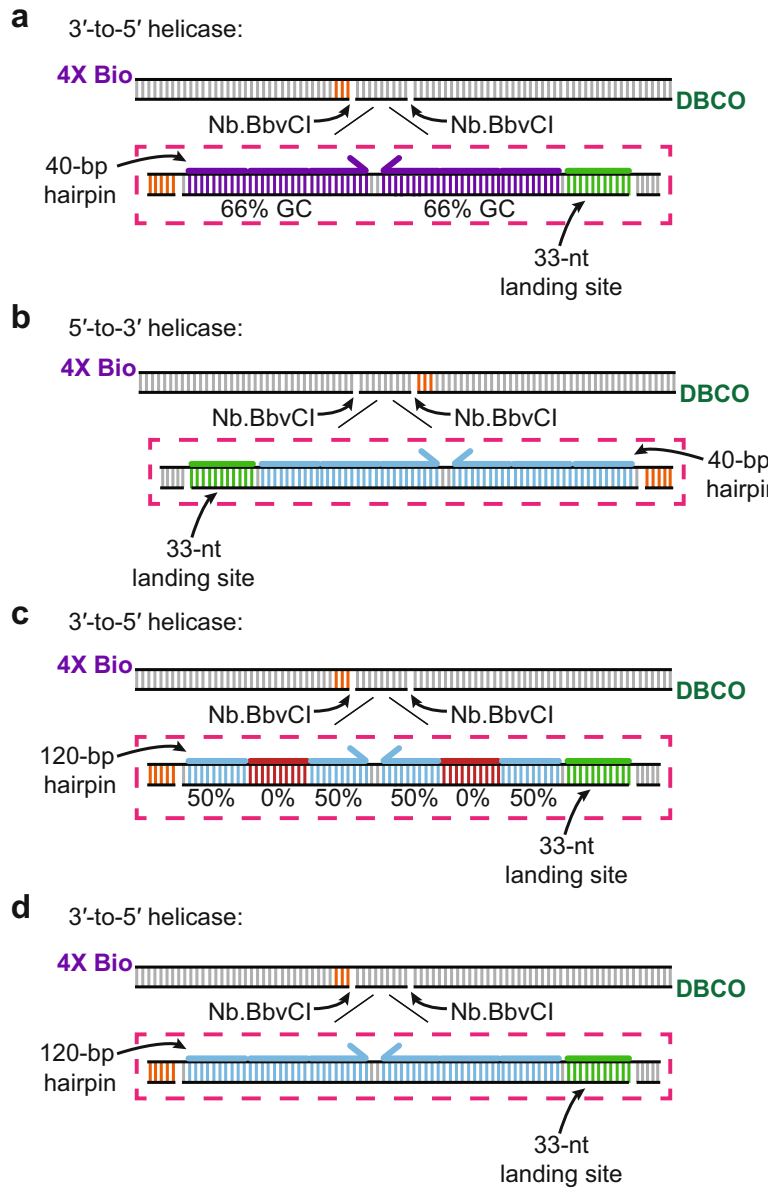

Fig. 4 Designs for several force-activated substrates. (**a**) Cartoon of the 40-bp 66%-GC construct. The inset shows the 40-bp hairpin (*purple*) with a 4 T loop at the top. A 33-nt landing site (*green*) is positioned directly adjacent to the hairpin stem where the helicase can bind. The molecule is labeled with a 4X biotin label on one end and a DBCO label on the other end. A GC clamp (*orange*) is included to prevent peeling. (**b**) A reversed polarity 40-bp hairpin. This hairpin is designed for helicases with a 5′-to-3′ translocation direction. (**c**) The 120-bp hairpin with distinct runs of 50%-GC and 0%-GC content. (**d**) Cartoon of the 120-bp 50%-GC construct

All hairpins are treated the same after assembling the initial sequence. To assemble the initial sequence, we amplified the left handle and right handle separately using the primers and templates listed in Table 9. Next, we cut each half with BstXI, purified them, and ligated them together. The resulting DNA was cloned into a plasmid using pCR-XL-TOPO (or pCR-XL-2-TOPO), grown in *E. coli*, and the plasmid was isolated. Finally, we introduced the labels via PCR with L_4XBiotin_F and R_DBCO_R (R_20GC50_DBCO_R for the 20-bp 50% GC hairpin) primers (*see* **Note 1**).

3.1.4 120-bp Hairpins with Distinct Runs of Different GC Content

We designed a 120-bp hairpin such that the first and last 40-bp segments were 50% GC while the intervening 40-bp segment was 0% GC (Fig. 4c). Using this construct, one can investigate the sequence dependence of helicase unwinding within a single construct, since the same individual enzyme sequentially unwinds different segments of GC content. The sequence of the first 40 bp of the hairpin is the same as the 50%-GC 40-bp hairpin described above. The subsequent 40-bp regions were designed at either 0% or 50% GC such that there are never three GC or AT bp together (except in the 0% GC region). We also minimized secondary structure such that no theoretical secondary hairpin stem of more than 8 bp formed using the hairpin tool on IDT OligoAnalyzer. This substrate successfully force-activated approximately 2/3 of the time. Fortunately, the force activation was not subtle: either a construct easily activated on the first try, or it never activated no matter how many pulls were made. We hypothesize that part of each batch was not nicked successfully but have not yet identified the root cause. We note the denaturing gel electrophoresis shows most of the DNA was properly nicked, although there was a dim band of improperly nicked molecules. Future work is expected to improve this efficiency. We also made a 120-bp 50%-GC hairpin unwinding substrate (Fig. 4d). We note that both complete substrates could not be cloned into a plasmid like the other constructs, most likely due to the extended secondary structure, which we hypothesize impeded DNA polymerase from amplifying a template containing the full sequence. Instead, the left and right halves of these substrates were amplified and then ligated together to form the template for the final PCR amplification.

To assemble the 120-bp hairpins, we amplified the left and right halves using the primers and templates listed in Table 9. We then cloned each half separately into pCR-XL-TOPO plasmid. The left and right plasmids were isolated using a standard plasmid preparation. The left and right DNA molecules were amplified at preparative scale using the associated labeled primers (left half amplified with L_4XBiotin_F and L_120_R primers, right half amplified with R_DBCO_R and R_120_F primers (*see* **Note 2**). Finally, the left and right PCR products were purified and cut in

equimolar ratios with BstXI, and then ligated together. We then gel-purified the resulting product.

3.2 Assembling DNA Templates for Final PCR Amplification

The final PCR amplification for all constructs is done using primers labeled with DBCO and biotin to serve as attachment chemistries, as further described in Subheading 3.5. PCR is a common method of producing large amounts of DNA from a template. PCR combines template DNA, DNA primers, dNTPs (deoxyribonucleotide triphosphates), and a thermostable DNA polymerase in a buffered solution. DNA amplification occurs in three steps: (1) the temperature is raised to above the melting temperature of DNA to denature the dsDNA template into ssDNA, (2) the temperature is lowered to below the melting temperature of the primers, allowing the primers to anneal to the template ssDNA, and (3) the temperature is raised to the optimal temperature for polymerase activity so that the primed sequences are extended to their endpoints (ultimately determined by the DNA sequence of the other primer). Note the lowered temperature in step (2) is based on the melting temperature of the DNA primers: the temperature must be low enough for the primers to anneal to the template sequence, but not low enough to anneal to sequences that are not a perfect match. These three steps are typically repeated for ~30 cycles, resulting in exponential DNA amplification.

This section details the required steps to produce template DNA for the final PCR amplification where the chemical moieties for attachment are added. We first used PCR to create the left and right DNA handles and sections of the target region. These target regions are introduced by using primers that have 5′ sequence beyond the hybridization target on the template DNA. This added sequence is incorporated into the amplified product.

3.2.1 Initial PCR Amplification of DNA Handles

This section describes the PCR procedure used to amplify the DNA handles used in our force-activatable constructs. The sequences of the primers used are listed in Subheading 2.1. After PCR amplification, the products are purified with a Qiagen QIAquick PCR purification kit to remove polymerases and excess primers from the reaction mixture. Next, the QIAquick-purified DNA is run on a 1% agarose gel to ensure that a product with the correct length was produced and to separate it from any incorrectly sized DNA molecules. The desired band in the gel is excised (Fig. 5a) and run through a Freeze 'N Squeeze DNA gel extraction spin column. Finally, the DNA product is concentrated with a Millipore Amicon 0.5-mL Centrifugal Filter Ultracel 10K. DNA concentration is measured using a spectrophotometer (e.g., Nanodrop 2000).

We generally performed 100-μL PCR reactions using the high-fidelity polymerase KOD Hot Start DNA polymerase according to the manufacturer's protocol. We occasionally used Taq polymerase when KOD amplified poorly. There were some hairpin halves that

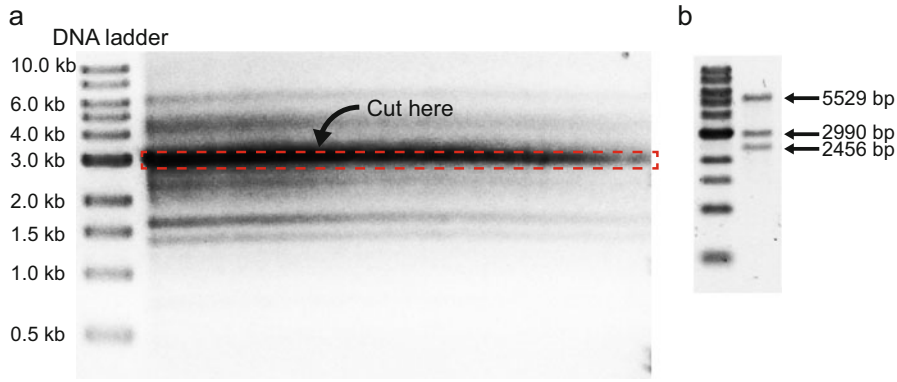

Fig. 5 (**a**) An example preparative gel for purification of the 40-bp 50%-GC hairpin with 4X biotin and DBCO labels (expected size 2.96 kbp). The dashed line indicates where the band of interest should be cut and subsequently extracted from the gel. (**b**) An alkaline denaturing gel for the 20-bp hairpin. The DNA molecule is separated into ssDNA strands. Longer sections of ssDNA run slower on the gel and are thus closer to the top end of the gel. This specific 20-bp hairpin is properly nicked because the top band corresponds to the unnicked DNA strand and the bottom two bands are the two halves of the nicked strand. The smaller pieces add up to the correct full length: 5529 nt (full unnicked construct) = 2290 nt + 2456 nt + 83 nt. The 83 nt section is complementary to the force-activatable hairpin section and is not visible on the gel

would not amplify with KOD but worked with Taq. All Taq polymerase PCR amplifications were done according to the manufacturer protocol, as well. DNA oligonucleotides for PCR primers and sequencing generally targeted a melting temperature $T_m \approx 60$ °C. To determine the melting temperature, we used OligoCalc with the nearest neighbor parameter, which affects the overall melting temperature (even though the total composition may be the same).

1. Follow the manufacturer conditions for the KOD Hot Start DNA polymerase protocol or the Taq polymerase with the following differences:
   (a) We use 100-μL PCR reactions instead of 50-μL.
   (b) All DNA oligonucleotides are diluted to 100 μM stock in TE and stored at 4 °C. A sub-stock is diluted to 10 μM in TE, which is the concentration we add to our PCR reactions.
   (c) We generally use 5 ng of plasmid DNA per 100-μL PCR reaction.
2. Set up enough parallel PCR amplifications to yield ~30 μg of crude PCR product (*see* **Note 3**, i.e., could be 4, 8, or 16 100-μL reactions, depending on the specific amplification being done). In a 0.2-mL thin-walled PCR tube, combine 10× PCR buffer, dNTP, $MgSO_4$ (or $MgCl_2$ for Taq polymerase), 30 pmol of each DNA primer, 5 ng of plasmid DNA, polymerase, and ultrapure water to 100 μL (with amounts according to manufacturer's protocol): *See* **Notes 4** and **5**.

3. Run the PCR thermocycler program, which will incubate the tubes at the specified temperatures for the specified times. Note that times needed by KOD are generally much shorter than those of Taq or other PCR polymerases.
   (a) 95 °C for 2 min (initial heat activation of the polymerase; also denatures all DNA present).
   (b) 95 °C for 20 s (denaturation of the dsDNA).
   (c) ≈60 °C for 10 s (annealing of primer to template ssDNA; we design our primers for $T_m \approx 60$ °C, *see* **Note 6**).
   (d) 70 °C extension step. Times for KOD: 10 s/kbp for <500 bp, 15 s/kbp for 500–1000 bp, 20 s/kbp for 1000–3000 bp, and 25 s/kbp > 3000 bp. Times for Taq: 1 min/kbp extended plus an additional 30–60 s (*see* **Note 7**).
   (e) Repeat **steps b**, **c**, and **d** 30 times.
   (f) 70 °C for 1 min (final extension to fill in any incomplete ends).
   (g) Hold at 10 °C indefinitely (instead of 4 °C to better preserve the life of the thermocycler's refrigeration system) until products can be removed. Store products at 4 °C until next steps can be performed.
4. Purify the PCR products using a Qiagen QIAquick PCR purification kit. This removes the primers and other small molecules from the sample. We followed the kit's protocol with the following exceptions: To ensure maximum DNA binding, we add 20 μL of 3 M sodium acetate (pH 5.2) to every 500 μL of PB buffer (from the Qiagen kit). It is easiest to add sodium acetate to a larger stock (50 mL) for use in many purification columns. For all preparative purifications, we washed the column with PE (from the Qiagen kit) three times instead of one time to remove any residual PB and free oligonucleotides.
5. Measure the DNA concentration using a spectrophotometer. This step is to ensure that you do not underload or overload a gel. There is also is no need to run a gel if the PCR reaction failed. We target loading ≈16 μg DNA per preparative gel (next step).
6. Given the likelihood of off-target products in the PCR reaction, use agarose gel electrophoresis to isolate the DNA of interest. In most cases, we use 1% agarose melted in TAE.
   (a) Add appropriate amount of 6× DNA loading buffer to the DNA. The sample buffer contains a dye to track the progress of the electrophoresis and a dense compound to keep the sample in the gel well.

(b) Load a maximum of 16 μg (200 μL max volume, including loading buffer) of PCR product in a preparative gel well on a 70 mm by 80 mm TAE agarose gel plate (Fig. 5a). For a preparative gel, the main well spans most of the gel alongside a small well for running a reference (1-kbp DNA ladder, load ~0.5 μg).

(c) Run the gel at 140 V for 45 min.

(d) Stain gel in ~10 μL (5000×) SYBR Green I per ~50 mL TAE. Allow gel to stain for ~1 h in a light proof container with slow rocking.

(e) Visualize stained gel (e.g., Invitrogen Safeimage2) using blue light transilluminator (instead of UV) to prevent DNA damage. Excise the band of interest with a razor blade (Fig. 5a).

7. Next, follow the Freeze 'N Squeeze protocol with the following modifications and notes:

(a) Dice up the band into ~1–2 mm cubes with the razor blade and load all the pieces into the Freeze 'N Squeeze spin column. Put in the −20 °C and "Freeze" for at least 1 h.

(b) Before proceeding to the "Squeeze" step, allow the spin column to thaw for 3 min.

(c) Spin for 3 min at 16,000 × *g* at room temperature. Save all liquid that has been "squeezed" out at the bottom of the tube.

(d) Add 100 μL of TE on top of the remaining gel slice bed. Spin again at 16,000 × *g* for 10 min at room temperature and add this flow through to the first spin flow through.

(e) If you have more diced up bands than will fit in a single tube, add them to the tube and repeat the above steps.

8. Pool all products together and concentrate the DNA by loading into a Millipore Amicon 0.5-mL Centrifugal Filter Ultracel 10 K. Add up to 400 μL of the DNA sample (do not overfill).

9. Spin at 14,000 × *g* for 5 min in a microcentrifuge. Discard flow-through at the bottom of the collection tube. Repeat until all the sample has been loaded.

10. To wash and buffer exchange the final DNA products:

(a) Wash the column twice with 400 μL TE or desired buffer at 14,000 × *g* for 5 min.

(b) Wash the column once with 400 μL TE or desired buffer at 14,000 × *g* for 20 min.

(c) Invert filter into a new collection tube, and spin at 1000 × *g* for 3 min to remove the concentrated product (usually ~40 μL) from the filter.

3.2.2 Preparing the Substrate for Ligation Using Restriction Enzymes

The PCR products are next digested with restriction enzymes. Restriction enzymes recognize a specific sequence of DNA and cleave the duplex (Fig. 3c, d). Cleaving for most restriction endonucleases generates "sticky ends," ssDNA overhangs that then can hybridize with complementary sequences. After hybridizing, the two pieces of DNA can be ligated together via DNA ligase forming covalent bonds in the phosphate backbone. In both halves for the hairpin constructs, we designed the overhang to be nonpalindromic (Fig. 3c, d). Specifically, the overhang is TTTT on the left handle and AAAA on the right handle. This assures that neither the left nor right handles can self-hybridize. For this protocol, we found that equimolar ratios of each component in the digestion worked well, and note that all steps prior to incubation should be done on ice:

1. For the cutting step, add to a PCR tube enough pure water so that the final volume will equal typically 30–50 μL, and always at least 20 μL (calculate water volume before starting based on all other ingredients) to reduce potential star effects—off target cutting—as discussed in manufacturer's instructions.
2. Add the 10× buffer appropriate to the restriction enzyme being used (e.g., Cutsmart for most enzymes, but NEBuffer 3.1 for PflMI) to the water and then briefly vortex for 5 s on a hand vortex mixer. Remember, always vortex thawed, concentrated enzyme buffers before use.
3. Calculate the molarity of the DNA components to be digested (and subsequently ligated) and add equimolar amounts of each component. For best results, we seek to ligate the maximum amount of material available. For example, we typically digest about 5–10 μg DNA (usually ~40 μL), so that we have plenty for one or two ligation reactions. We then buffer exchanged with a Millipore 30 K before ligation as described in the following steps.
4. Add to each reaction 5–10 units/μg DNA of restriction endonuclease. To prevent off-target cuts (i.e., "star activity"), do not exceed adding more than 5% enzyme of the total volume. Gently mix by repeatedly pipetting.
5. Incubate at 37 °C for 2–3 h for most enzymes. Can increase time or total units of enzyme if the enzyme is not cutting completely, as assayed by gel electrophoresis.
6. Remove buffer and small cleaved segments of DNA (<20 bp) using an Amicon-Millipore 30 K, washing 4 times with 10 mM Tris–HCl pH 8.0. Retain the supernatant. We avoid a

QIAquick cleanup kit because guanidinium has been reported to cause ligation reactions to fail.

7. Next, ligate the two separately cut DNA molecules. For a 50-μL reaction, add enough pure water to a PCR tube to have the total volume equal 50 μL. Then add 5 μL of 10× New England Biolabs Ligation buffer.
8. Add the ~1 pmol of digested and Millipore-cleaned DNA to the reaction mixture. This is usually 20–30 μL, because we usually use a total volume of 30–50 μL.
9. Finally, add maximum amount of T4 DNA ligase such that the total does not exceed 5% of the final volume. Mix gently by pipetting.
10. Ligate at 16 °C for 16 h.
11. The ligated product is purified via a 1% agarose/TAE gel, extracted with Freeze 'N Squeeze, and cleaned with a Millipore 10 K filter, as described above.

3.2.3 Cloning Substrate into E. coli for Efficiency and Accuracy

The fully ligated construct can now be used as a template for the final PCR labeling with DBCO and biotin. However, for several reasons, it is a better option to first clone it into a plasmid (e.g., a pCR-XL-2-TOPO plasmid). First, the current DNA product is a mixture of our desired DNA template along with some incomplete or misprimed amplifications. The cloned plasmid template will only contain our desired sequence and subsequent PCR amplifications using this plasmid as a template will produce cleaner DNA. Second, we can essentially make an unlimited amount of template using the replication machinery inside the *E. coli* cell. This replication is also less likely to produce errors in the sequence than PCR. Lastly, because we have a large amount of plasmid template available, we can more easily verify the sequence of the template by sequencing (e.g., Quintara Biosciences or Genewiz) before making the final labeled construct.

Here we describe the steps required to clone the desired sequence into a bacterial plasmid, transform the plasmid into competent *E. coli*, grow up bacterial culture, and isolate the plasmid from the bacteria. Be sure to collect any bacterial waste (e.g., pipette tips, extra LB media with bacteria) and dispose of appropriately.

1. Use the TOPO XL (or TOPO XL-2) PCR Cloning Kit to clone the desired sequence into a bacterial plasmid. This plasmid uses kanamycin resistance for selection (*see* **Note 8**).
2. Take 10–50 ng of the DNA product and perform the Topo Cloning reaction for 30 min following the manufacturer's protocol.

3. Keep at 0–4 °C. The sample is now ready to be transformed into competent *E. coli*. There are many commercially available, chemically competent *E. coli* strains suitable for transformation. Since we are only interested in plasmid propagation and not protein production, we chose a nonexpression cell line. The TOPO kit includes One Shot TOP10 chemically competent *E. coli* cells, but others including XL10-Gold are similarly efficient for transformation.
4. Start the transformation (general standard method, check specifications for cell type you use):
   (a) Thaw a 50-μL vial of chemically competent *E. coli,* stored at −80 °C, on ice for 10–15 min.
   (b) Add 1.5–2 μL of the TOPO plasmid mixture into the 50-μL thawed vial of One Shot cells. Mix by flicking the tube gently with your fingertips for 10–15 s. Do not mix with a pipette.
   (c) Incubate undisturbed on ice for 30 min.
   (d) Heat shock the cells for exactly 30 s at 42 °C in a water bath without shaking.
   (e) Immediately transfer the tubes to ice and incubate for 2 min.
   (f) Add 250 μL SOC recovery medium at room temperature.
   (g) Shake horizontally at 225 rpm at 37 °C for 1 h.
5. Plate 100 μL of each transformation on a prewarmed (room temperature) kanamycin-LB agar plate. These plates are made by melting 15 g Bacto agar in 1 L of LB via autoclaving. Just before pouring the plates (LB-agar is still warm to the touch), add 1 mL of kanamycin stock (50 mg/mL) to the LB-agar. Gently mix by swirling and pour 25 mL into each standard plastic 100 mm × 15 mm petri dish and let cool. We use sterile plates, pipette tips, and tubes for these steps.
6. Incubate the plates at 37 °C overnight.
7. The next morning, add 5 μL of kanamycin stock (50 mg/mL) or the appropriate antibiotic for selection with your plasmid to 5 mL of sterile LB in sterile glass culture tubes (20 mm × 150 mm). You may want 6–8 of these.
8. Pick clonal colonies with a sterile pipet tip and drop in the tube with the LB + antibiotic. Usually we pick 6–8 colonies because some will have unwanted mutations, duplications, deletions, or will be missing the desired sequence.
9. Incubate your culture tubes on a shaker at 37 °C (200 rpm) overnight.
10. The next day, add 1.5 mL of the overnight bacteria culture into a 1.5-mL microcentrifuge tube.

11. Spin down each tube at 9400 × *g* for 3 min in a microcentrifuge. Remove and discard the supernatant.
12. The remaining pellet can either be stored at −20 °C or processed immediately using a Qiagen QIAprep Spin Miniprep Kit to isolate the plasmid (we follow the manufacturer's protocol exactly, using the microcentrifuge method). A single 1.5 mL Miniprep is almost always enough for sequencing. We typically have 2–12 μg plasmid from a Miniprep, and sequencing requires ~500 ng DNA.
13. After isolation, measure the concentration of the plasmid on a NanoDrop or other spectrophotometer.

We select multiple clones to assure that one of the plasmids we isolate contains the correct sequence. We confirm the sequence in two ways: first by PCR amplification and then by DNA sequencing. A PCR reaction of the correct sequence should yield the desired length product, but this does not show point mutations. Plasmids that produce a PCR product of the correct size should be submitted for DNA sequencing to verify the sequence is correct. Sequencing the entire hairpin region is usually difficult (only rarely sequences all the way through the hairpin without cutting), if not impossible. We have had success when we cut the final plasmid with the restriction endonuclease BstXI, which isolates each half of the hairpin from each other, because hairpin structure impedes sequencing, and then use a primer which is usually about 300 bp upstream of the hairpin loop.

3.3 Final PCR Amplification with Labeled Primers

Once the sequence of the plasmid template DNA has been verified, it can now be used in a final PCR amplification using primers containing DBCO and biotin. The DBCO is used to covalently attach the DNA to functionalized surfaces (Subheading 3.5) while the biotin attaches the DNA to streptavidin-coated microspheres for optical trapping. Briefly, we use these attachment chemistries due to their mechanical strength, specificity, and simplicity. Both coupling schemes are easy to implement and provide bonds that can resist the high forces (~65 pN) needed for force-activation by overstretching. For further mechanical strength, we often replace a single biotin labeled primer with a primer with four biotins spaced every 9–10 bp (4X biotin) so that the attachment does not rupture during overstretching. Using these labeled primers, this final PCR reaction is done in a similar manner to the PCR described previously in Subheading 3.2.1, albeit slightly less efficiently: perform 12, 100-μL PCR reactions with KOD polymerase, QIAquick with 3× PE wash, gel-purify, concentrate (Millipore 10 K), and do a final QIAquick (3× PE wash) to get rid of residual agarose.

3.4 Site-Specific Nicking of the Force-Activatable Substrate

The final step in synthesizing force-activatable substrates is to site-specifically nick the dsDNA at the two recognition sequences flanking the target region. We found that each nicking enzyme needed different conditions for best performance, as shown in **steps 1** and **2** below. One notable point is how to deactivate the nicking enzymes after incubation. The standard method is heat deactivation at 80 °C for 20 min. This is suitable for substrates with longer segments between the two nicks but may thermally melt smaller segments. For example, we found heat deactivation thermally melted ~1/3 of the 83-nt, nicked strands in the 20-bp hairpin construct. Therefore, we replaced heat deactivation with EDTA deactivation (10 mM final concentration).

To verify the DNA was nicked properly, we analyze the nicked construct on an alkaline denaturing agarose gel. The denaturing gel protocol separates the DNA duplex into ssDNA such that longer sections of ssDNA migrate slower through the gel. You should see bands that correspond to each expected ssDNA segment (*see* Fig. 5b). The following explains how we prepare and run an alkaline denaturing gel.

1. For the 1000-bp 50%-GC construct, nick DNA (≈1–2.5 μg in 50 μL) (as in **steps 1** and **2** of Subheading 3.2.2, first add pure water and appropriate 10× buffer). For Nt.BspQI, use 50 units per μg DNA at 50 °C for 4 h. Without additional purification, digest with Nb.BsmI, use 2 units per μg DNA at 65 °C for 1 h. Use heat deactivation after each nicking enzyme reaction for the 1000-bp construct.
2. For the hairpin constructs, add 5 units of Nb.BbvCI per μg of DNA [≈5–10 μg in 30–50 μL (for preparative-scale nicking) for the 40-bp hairpin] and nick at 37 °C for 1–2 h (first add pure water and appropriate 10× buffer). Deactivate enzymes using EDTA for the hairpin constructs (10 mM final concentration).
3. After nicking the full batch of DNA, clean only 200 ng of the total nicked DNA with a QIAquick kit to remove the BSA-filled buffer, since BSA can adversely affect the way the DNA runs on the agarose gel. Do not QIAquick the bulk of the DNA to be used for the optical trap since the guanidine (a strong denaturant) in the QIAquick protocol will most likely denature and remove the short ssDNA between the nick sites.
4. Elute the QIAquick product in a small volume (25 μL) of TE (use ~12–25 μL for gel).
5. Make a fresh solution of 50 mL 10× alkaline agarose gel buffer.
6. For a 70 × 80 mm^2 agarose gel (50 mL), melt 0.45 g of agarose in 50 mL of water (0.9% agarose gel) in a microwave. Just before pouring (cooled to ≈50 °C), add 5 mL of the 10×

alkaline agarose gel buffer and gently mix before casting the gel (avoid bubbles).

7. Add ~100–200 ng (~12–25 μL) of the nicked DNA to 2.4–5 μL of 6× alkaline gel loading dye.
8. Dilute the remaining 45 mL of 10× alkaline agarose gel buffer with 405 mL water, mix well, and use this as the running buffer for the gel. Load the nicked DNA with loading dye, along with 100 ng of 1-kbp DNA ladder into the alkaline gel lanes and run the gel slowly at 45 V for 3.5 h.
9. Neutralize the gel with two 30-min emersions in 50-mL neutralization buffer with slow rocking.
10. Stain with SYBR Green I or SYBR Gold for 1 h with slow rocking and visualize bands on a standard UV transilluminator. For the hairpin, one should see a very strong unnicked strand and two lighter fragments that add up to the size of the unnicked strand (*see* Fig. 5b). There is also a very short ssDNA segment that is not visualized on the gel because brightness on a gel is proportional to the amount of material not its molar concentration.

3.5 Coupling Substrates to Coverslips Through Covalent Click Chemistry

With the protocol for designing and manufacturing force-activated DNA substrates complete, these last two sections describe how we use them in surface-coupled optical-trapping assays (Fig. 6). This section explains how to functionalize glass coverslips for covalent click-chemistry reactions with labeled substrates. The final section then demonstrates the substrates in action in an optical-trapping assay. Note that these sections are not mandatory; different attachment protocols and specifics of the single-molecule assay will vary from group to group and depend on the force-spectroscopy instrument used.

Our surface-coupling protocol has been previously published and uses copper-free click chemistry to create a covalent bond between the DNA and the coverslip [32]. The following protocol describes the functionalization of a coverslip using silane-PEG-azide, which is then reacted with DBCO-labeled DNA to covalently anchor the DNA to the surface [26]. This protocol thus both passivates the surface with PEG and enables mechanically robust site-specific attachment via the azide moiety. Once functionalized, we use the glass to create a simple single-channel flow cell [28]. Of course, other surface-based sample geometries suited to specific application can be used instead. It may also be possible to perform this process on silica beads [33].

This protocol uses only a single reaction: a silanization reaction between activated SiO_2 groups on the coverslip surface and the silane (SiH_4) moiety of silane-PEG-azide molecules dissolved in a toluene–isopropanol solvent (Fig. 7a). To improve efficiency, we

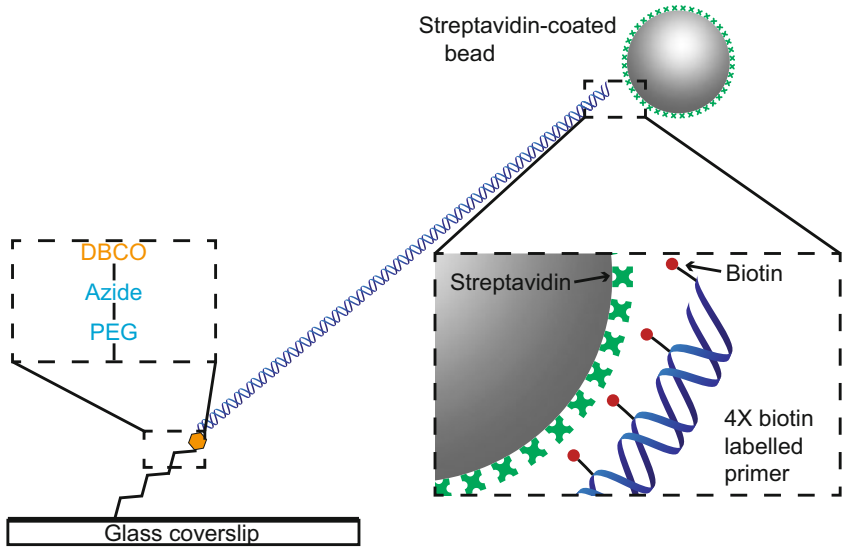

Fig. 6 Schematic showing the site-specific attachment of the DNA molecule to the optically trapped bead and the surface. The glass coverslip is functionalized with silane-PEG-azide that covalently bonds with the DBCO-labeled DNA. The optically trapped bead is coated with streptavidin which binds with the biotin labeled DNA. The inset shows the 4X biotin where biotins are spaced every 9–10 bp on the end of the DNA

first UV-ozone treat the glass coverslips to displace surface protons and make the coverslip more reactive. We also silanize in a solvent of primarily toluene at 60 °C to increase the speed of the reaction while preventing H_2O-based side reactions [34]. On that point, water directly competes with SiO_2 to react with silane, so reducing the amount of water present in this protocol is essential. To do so, we "condition" all glassware by rinsing with toluene, to remove any potential residual water.

1. Take a batch of 10 clean coverslips (such as those produced in a KOH etch described in ref. [28]) and use a diamond scribe to make some distinctive mark (such as an "X") in the bottom right corner of each coverslip. With this mark, the two sides of the coverslip can be distinguished, allowing us to tell which side is directly exposed to the UV-ozone.
2. Place the coverslips in a holder, such as a large glass petri dish, with their marked sides up. Leave space for a stir bar in the center of the petri dish used in the silanization step. Place this in a UV-ozone chamber and expose the coverslips for 30–45 min (*see* **Note 9**).
3. While the coverslips are irradiated, prepare the reaction solution containing silane-PEG-azide (SPA). First, gather the necessary glassware: one petri dish top, one 100-mL graduated cylinder, one 10-mL graduated cylinder, four mid-sized (~250-mL) beakers to serve as stock and waste beakers for toluene and

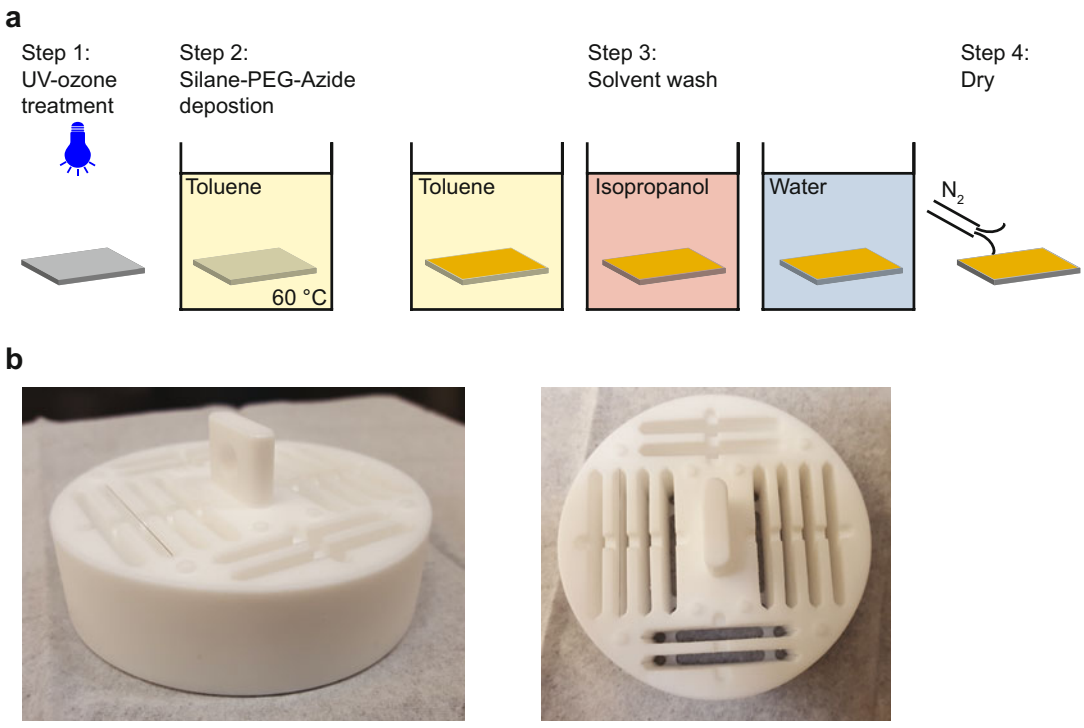

Fig. 7 (**a**) Schematic showing the process of functionalizing coverslips. Step 1 is UV-ozone treatment. Step 2 is the silane-PEG-azide deposition process that occurs in toluene at 60 °C. Step 3 is the solvent wash where the coverslips are sequentially rinsed in toluene, isopropanol, and then water. Finally, Step 4 is drying the coverslips with dry nitrogen gas. (**b**) (*Left*) Side view of a custom-made Teflon coverslip holder. The holder holds up to 12 coverslips and makes the process of functionalizing coverslips more efficient. (*Right*) Top view of the coverslip holder

isopropanol solvents, a 10-mL beaker and appropriate cover (small petri dish), and a watch glass. Also, gather two small magnetic stir bars and a thin metal spatula. Condition all components with toluene.

4. Preheat a large magnetic hot plate for a 60 °C solution temperature (*see* **Note 10**) and take the vial of SPA out of its −20 °C storage freezer. Let it thaw for ~5 min (moisture condenses in the vial if it is opened cold).
5. Place 10-mL beaker on fine balance and use spatula to weigh out ~10 mg of SPA into beaker (*see* **Note 11**). Place small stir bar into beaker.
6. Measure out 66 mL of toluene into 100-mL cylinder and 2 mL of isopropanol into 10-mL cylinder. We strive to make an ~0.15 mg/mL solution of SPA (empirically, 2-fold increase or decrease in concentration does not affect the end result).
7. Pour 2 mL isopropanol into SPA beaker (*see* **Note 12**). If necessary, agitate with spatula to aid dissolving process. Cover

beaker and place on magnetic hot plate. Stir at 800 rpm for 2 min.

8. Using a 10-mL cylinder, pour 2 mL toluene into the 10-mL SPA beaker. Stir at 800 rpm for 2 min.
9. Pour the contents of the 10-mL SPA beaker into a conditioned 250-mL beaker (could be the emptied stock toluene beaker), and subsequently pour in 66 mL toluene from 100-mL cylinder. Use some of this toluene to rinse out the 10-mL beaker two or three times and pour the toluene rinse into the 250-mL beaker. If necessary, remove small stir bar with tweezers and replace with medium-sized stir bar.
10. Cover beaker with watch glass, and stir on magnetic hot plate at 600 rpm for 5 min.
11. At this point, remove the petri dish with coverslips from the UV-ozone chamber (careful: it will be hot!). Place it on the hot plate and put the small stir bar in its center.
12. Slowly pour 70–100 mL of SPA solution into dish, trying not to move the coverslips. Cover with petri dish top and stir at 400 rpm. Let silanization proceed for 30 min.
13. After silanization has finished, gather four 250-mL beakers. Put toluene into the first, isopropanol into the second, and deionized water into the third and fourth. Fill enough to easily dunk coverslips.
14. Use tweezers remove each coverslip from silanization mixture and immerse each coverslip sequentially in the four beakers (toluene, isopropanol, water, water) for 20 s each (*see* **Note 13**). Transfer through air *very* quickly, as PEG clumps can form on the coverslip otherwise, particularly for the initial transfer into the toluene rinse. Put into secondary holder (such as a new petri dish or Teflon holder). Dry coverslips with N_2 gas and store at 4 °C. *See* Fig. 7a.
15. When ready, construct your flow chamber as normal with this functionalized coverslip. Consider prewashing the flow chamber to ensure no particulates linger on the coverslip. Make sure to use the functionalized side of coverslip (side with the distinctive mark facing inside the flow chamber). This side was face-up in the UV-ozone chamber and therefore likely to be more active, though either side may work.
16. Incubate DBCO-labeled DNA in the azide-functionalized coverslip flow chamber. For our assays, we incubate 100 pM DNA in 15 μL (volume of flow cell) at room temperature in deposition buffer for 1 h (*see* **Note 14**).
17. Wash sample with assay buffer to remove excess DNA and continue sample preparation as normal.

As a final note, we increased the efficiency and cost-effectiveness of this protocol by designing a large Teflon holder for the coverslips (Fig. 7b). This block holder has small slits in it to hold the coverslips, and a partially opened bottom to allow the stir bar underneath to mix the solution. Otherwise, its large size is meant to displace fluid so that less of the (admittedly expensive) SPA needs to be used per batch. A major benefit of this holder is that during the wash steps (toluene, isopropanol, water, water), all of the coverslips can be washed simultaneously by using a glass hook to submerge the holder into larger solvent wash beakers rather than one at a time with tweezers. We see SPA reduce in effectiveness over time (a vial typically lasts several months), so we store aliquots at −20 °C (*see* **Note 11**) to extend the lifetime of the SPA as much as possible.

3.6 Using the Constructs in an Optical-Trapping Assay

Once a flow cell is mounted within an optical trap, individual molecules can be force-activated. To do this, the molecules must be pulled some fraction of the way into the overstretching transition. The required fractional distance into the overstretching transition and speed needed to reliably activate a construct is easily determined empirically for a given construct design and buffer condition. This section provides some examples of force activation for a few of the substrates described in previous sections.

For the 1000-bp 50%-GC construct, we found that rapidly pulling the molecule 50% of the way through the full overstretching transition at $v = 2$ μm/s and immediately reversing direction was sufficient to activate all molecules tested at 22 °C and 150 mM NaCl (Fig. 8a). The initial stretching of the construct (*black*) produces a standard dsDNA force–extension curve with the expected contour length. The subsequent force–extension curve during relaxation shows hysteresis, including a set of abrupt upward force jumps consistent with reannealing of peeled ssDNA reforming dsDNA in the handle portion of the construct (*cyan*). Putting in GC clamps at the free ends and adjacent to the nicking sites can suppress this peeling. The next stretching curve does not superimpose upon the first. Using the known different mechanical properties of ssDNA and dsDNA, analysis confirms that the activated substrate now contains the expected 1000-nt segment [25]. Besides this standard procedure, we found that increasing the pulling speed from 2 μm/s to 20 μm/s decreased the percentage of activated molecules to ~70%. We also showed that pulling this construct less than 30% of the way through the overstretching transition did not lead to activation. From these data, it appears pulling slower and farther increases the chance of successful activation, as one would expect. We did not thoroughly test temperature variability, but we did perform some salt-dependent activation tests. We found that this construct could be successfully activated in conditions up to 150 mM NaCl + 5 mM $MgCl_2$ when pulling 50% of the way into

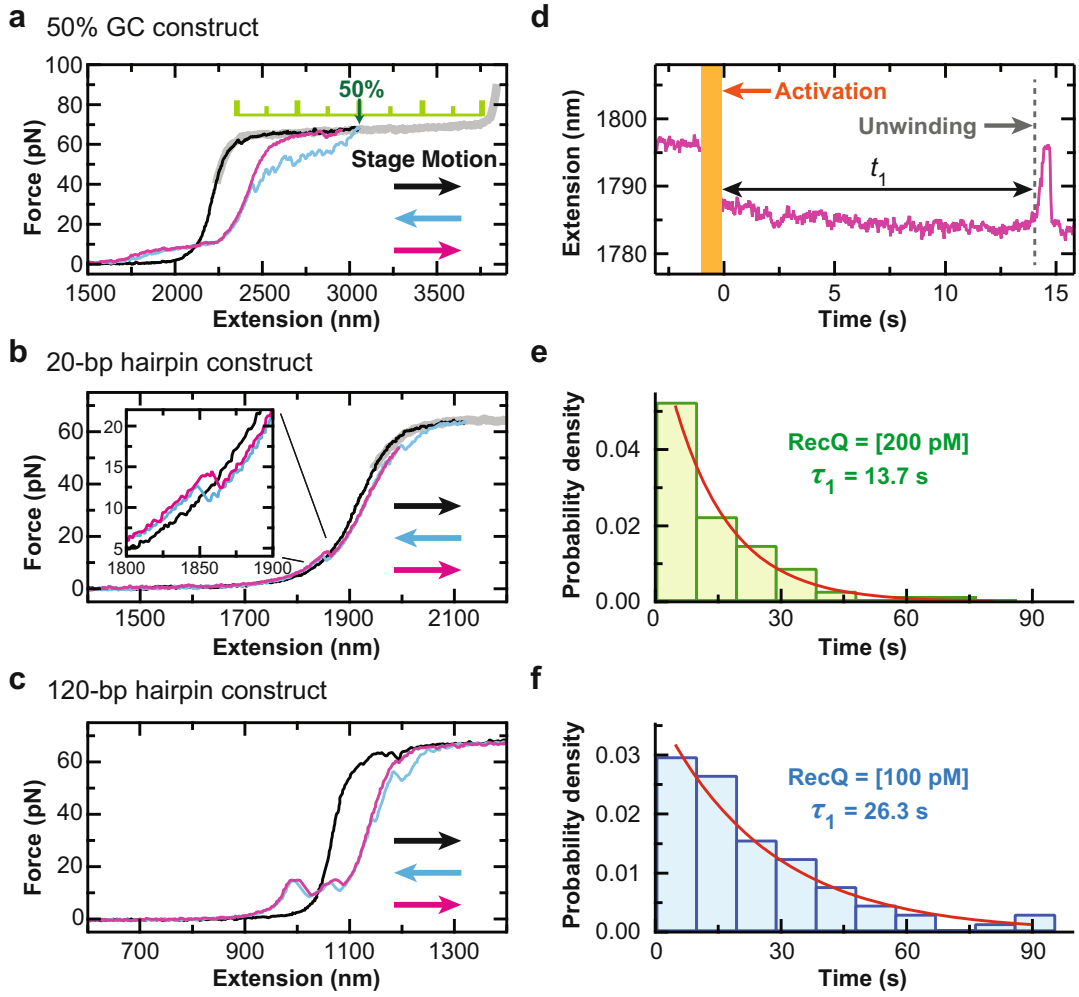

Fig. 8 Optical-trapping records showing force activation. (**a**) Force-activating the 1000-bp 50%-GC construct. A force–extension curve pulling the DNA 50% of the way through the overstretching transition. The dsDNA molecule was pulled (*black*) and then relaxed back (*cyan*) to zero force. The relaxation curve shows that the ssDNA dissociated and the molecule was successfully force-activated. This is confirmed by a subsequent pull (*magenta*) showing that the molecule is permanently active. (**b**) Force-activating the 20-bp hairpin. The molecule is stretched ~7% into the overstretching transition (*black*), relaxed back down to zero force (*cyan*), and pulled a second time (*magenta*). The inset shows a zoomed-in view of the hairpin transition. (**c**) 120-bp hairpin with distinct runs of 50% GC, 0% GC, and 50% GC going through pulling (*black*), relaxation (*cyan*), and a second pull (*magenta*). Two unfolding events are observed: the first corresponding to the 40 bp of 50% GC and the 40 bp of 0% GC unfolding, and the second corresponding to the last 40 bp of 50% GC unfolding. (**d**) An extension-vs.-time trace of a 20-bp hairpin showing force activation (*orange bar*) followed by a loading time (t_1) before the start of RecQ helicase unwinding activity (*black dashed line*). (**e**, **f**) Probability density histogram-vs.-loading time for RecQ at 200 pM and 100 pM concentration, respectively. The histogram is fit to a single exponential function (*red*). ((**a**), (**b**), (**d**)–(**f**) adapted from [25], which is in the public domain)

the overstretching transition at 2 μm/s, but higher ionic conditions of 150 mM NaCl + 10 mM $MgCl_2$ did not lead to activation. At lower ionic conditions, we showed that substrates in 25 mM NaCl (no divalent) showed no spontaneous activation prior to pulling.

The smaller 20-bp hairpin construct was easier to activate and required pulling only ~7% of the way into the overstretching transition (at $v = 2$ or 5 μm/s) (Fig. 8b). Performing a stretch-relax-stretch assay on this substrate shows the same general force–extension behavior as the 1000-nt substrate: the initial pull is fully dsDNA with the right contour length, the retraction curve has some hysteresis from partially peeled free ends zipping back together at high force (40–50 pN), and both the retraction and second stretch curves are displaced from the initial curve, showing permanent change. We also see the characteristic abrupt hairpin zipping (upon retraction) and unzipping (upon stretching), akin to the pioneering studies of RNA hairpins [35]. Importantly, this activation procedure was required to dissociate the 83-bp segment; pulling at forces just below the transition (≈57 pN) will not lead to activation. Note again that while the nicked sections are very stable within a single-molecule assay, they *can* be dissociated by molecular biology treatments that weaken base-pair stability. Specifically, we found that incubating the substrates at 80 °C for 20 min after nicking (to deactivate the restriction enzymes) caused some nicked sections to dissociate from their substrates. We therefore avoid this process in all force-activated hairpin substrate protocols, and instead deactivate the enzymes by addition of 10 mM ETDA into the buffer. The variant hairpins described above also activated similarly, with the exception that the higher GC-content hairpins required a slightly longer distance into the overstretching transition to ensure activation.

For the 120-bp hairpins with varying GC levels, activation proceeds similarly to the other hairpins (Fig. 8c). For the 50%–0%–50% construct, it is interesting to note that its hairpin unzipping signature has two peaks: the first 80 bp unzip together (the 40 bp of 50% GC and the 40 bp of 0% GC) followed by the second 40 bp of 50% GC. As noted in its design section, some molecules will not activate, no matter how many attempts are made or under what conditions. We assume this is due to being improperly nicked, but we have not yet fully resolved this issue. The two-thirds of molecules that activated were easily identifiable and allowed us to proceed with our assay. Figure 8d–f show an example of an extension versus time trace for an on-rate experiment [25]. This type of experiment makes excellent use of the force-activatable construct's ability to have a precisely defined start time in the experiment.

4 Notes

1. One can easily design different primers in the M13mp18 and pBR322 sequences to make the dsDNA handles longer or shorter to suit different geometric constraints.
2. We used slightly different primer nomenclature as we reused primers for two different 120-bp hairpin constructs.
3. Often, we need to PCR amplify more than one 100-μL reaction tube. The more material you start with, the better chance you have of getting the desired final assembled product. For these cases, we make a large batch by mixing all the components in a larger 1.5-mL tube and then aliquoting 100 μL into each PCR tube. We typically mix slightly more than the exact total amount needed. For example, prepare 430 μL for 4 reaction tubes (30 μL extra), 830 μL for 8 tubes, etc.
4. The 10× PCR buffer, $MgSO_4$, and the dNTPs can be warmed in your hand or a metal block at room temperature for a few minutes to speed up the thawing process. After it is completely thawed, keep in the ice bucket.
5. It is important to minimize the time enzymes are exposed to room temperature. If you have the enzyme stored in a bench-top enzyme cooler (with space for ~20 tubes), take the whole cooler out of the freezer and set on your lab bench next to your ice bucket. Only lift the polymerase tube out of the enzyme holder enough to pipette out the required 2 μL. You can leave the bottom of the polymerase tube resting on the cold holder. Once you are finished with the enzyme, put the holder back in the −20 °C freezer.
6. We typically design primers to have a melting temperature of ≈60 °C when using a nearest neighbor model. While 55 °C is a typical value to use, we prefer 60 °C as it produces fewer misannealed byproducts (at the cost of less product overall). You can adjust upward the annealing temperature of your PCR reaction if you get undesired products. For example, you can try 65 °C if you have a false primer band on a gel, or 50–55 °C if you have trouble amplifying product.
7. While it rarely hurts to add more extension time to ensure that extension fully completes, remember that the polymerase has a finite lifetime. For Taq polymerase, the manufacturer's protocol suggests an extension time of 90 s per kbp of template DNA, but the rule that has worked well in our lab is 60 s/kbp plus an additional constant time (30–60 s).
8. TOPO XL was used in our original work, but it has been discontinued. We have shifted to using TOPO XL-2.

9. In the past, our group performed this process with a UV hand lamp for 60 min. If you do not have access to a UV-ozone chamber, a hand lamp will work, though exposure time may need to be extended.
10. The set temperature of a hot plate is obviously not always the same as the real temperature of an equilibrated solution on its surface. Be sure to calibrate your hot plate so that the end solution temperature of the toluene is 60 °C.
11. We find that aliquoting the smaller molecular weight (600 Da), gel-phased silane-PEG-azide using a high-viscosity pipette dramatically improves the reproducibility of the protocol. Unfortunately, this is difficult to do with the larger molecular weight (3400 Da), solid/powdery SPA. We aliquot the SPA into disposable glass vials that we preclean with isopropanol and dry with N_2 gas (to ensure no water remains). These aliquots are stored in a −20 °C freezer. Another benefit of aliquots is that you do not have to weigh out the SPA each time you do the protocol.
12. We use 2 mL isopropanol to initially dissolve the low molecular weight SPA, even though it has some water content. We found this to be an acceptable trade for the dramatically increased dissolving power of isopropanol compared to toluene, especially for the small molecular weight, gel-phase SPA batches.
13. We find that using two tweezers is a very effective way to retrieve coverslips from a solution-filled petri dish. Use one pair of tweezers to prod a coverslip up against the wall of the dish, then use the other pair to grab it and start the washing process. This technique takes practice to do well, so consider trying it with just normal coverslips and water first so as not to waste components.
14. We empirically found that the monovalent salt concentration in the deposition buffer can affect how much DNA binds to sample surfaces. Strangely, the optimal salt concentration changes between batches of silane-PEG-azide for unknown reasons. For some batches of SPA, the micron-sized beads used for trapping seem to nonspecifically adsorb much more frequently to the surface. Increasing the monovalent salt concentration of the working buffer typically fixes this issue.

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Mention of commercial products is for information only; it does not imply NIST's recommendation or endorsement. T.T.P. is a staff member of NIST's quantum physics division.

References

1. Bustamante C, Bryant Z, Smith SB (2003) Ten years of tension: single-molecule DNA mechanics. Nature 421:423–427
2. Chemla YR (2010) Revealing the base pair stepping dynamics of nucleic acid motor proteins with optical traps. Phys Chem Chem Phys 12:3080–3095
3. Chaurasiya KR, Paramanathan T, McCauley MJ, Williams MC (2010) Biophysical characterization of DNA binding from single molecule force measurements. Phys Life Rev 7: 299–341
4. Zhou J, Schweikhard V, Block SM (2013) Single-molecule studies of RNAPII elongation. Biochim Biophys Acta 1829:29–38
5. Dumont S, Cheng W, Serebrov V, Beran RK, Tinoco IJ, Pyle AM, Bustamante C (2006) RNA translocation and unwinding mechanism of HCV NS3 helicase and its coordination by ATP. Nature 439:105–108
6. Perkins TT, Li HW, Dalal RV, Gelles J, Block SM (2004) Forward and reverse motion of single RecBCD molecules on DNA. Biophys J 86:1640–1648
7. Wen JD, Lancaster L, Hodges C, Zeri AC, Yoshimura SH, Noller HF, Bustamante C, Tinoco I (2008) Following translation by single ribosomes one codon at a time. Nature 452:598–603
8. Ribeck N, Kaplan DL, Bruck I, Saleh OA (2010) DnaB helicase activity is modulated by DNA geometry and force. Biophys J 99: 2170–2179
9. Manosas M, Xi XG, Bensimon D, Croquette V (2010) Active and passive mechanisms of helicases. Nucleic Acids Res 38:5518–5526
10. Seol Y, Strub MP, Neuman KC (2016) Single molecule measurements of DNA helicase activity with magnetic tweezers and t-test based step-finding analysis. Methods 105:119–127
11. Paik DH, Roskens VA, Perkins TT (2013) Torsionally constrained DNA for single-molecule assays: an efficient, ligation-free method. Nucleic Acids Res 41:e179
12. Lipfert J, Koster DA, Vilfan ID, Hage S, Dekker NH (2009) Single-molecule magnetic tweezers studies of type IB topoisomerases. Methods Mol Biol 582:71–89
13. Perkins TT (2014) Angstrom-precision optical traps and applications. Annu Rev Biophys 43: 279–302
14. Rief M, Clausen-Schaumann H, Gaub HE (1999) Sequence-dependent mechanics of single DNA molecules. Nat Struct Biol 6: 346–349
15. Smith SB, Cui Y, Bustamante C (1996) Overstretching of B-DNA: the elastic response of individual double-stranded and single stranded DNA molecules. Science 271:795–799
16. Cluzel P, Lebrun A, Heller C, Lavery R, Viovy JL, Chatenay D, Caron F (1996) DNA: an extensible molecule. Science 271:792–794
17. McCauley MJ, Williams MC (2007) Mechanisms of DNA binding determined in optical tweezers experiments. Biopolymers 85: 154–168
18. Paik DH, Seol Y, Halsey WA, Perkins TT (2009) Integrating a high-force optical trap with gold nanoposts and a robust gold-DNA bond. Nano Lett 9:2978–2983
19. van Mameren J, Gross P, Farge G, Hooijman P, Modesti M, Falkenberg M, Wuite GJ, Peterman EJ (2009) Unraveling the structure of DNA during overstretching by using multicolor, single-molecule fluorescence imaging. Proc Natl Acad Sci U S A 106:18231–18236
20. Zhang X, Chen H, Le S, Rouzina I, Doyle PS, Yan J (2013) Revealing the competition between peeled ssDNA, melting bubbles, and S-DNA during DNA overstretching by single-molecule calorimetry. Proc Natl Acad Sci U S A 110:3865–3870
21. King GA, Gross P, Bockelmann U, Modesti M, Wuite GJ, Peterman EJ (2013) Revealing the competition between peeled ssDNA, melting bubbles, and S-DNA during DNA overstretching using fluorescence microscopy. Proc Natl Acad Sci U S A 110:3859–3864
22. Candelli A, Hoekstra TP, Farge G, Gross P, Peterman EJ, Wuite GJ (2013) A toolbox for generating single-stranded DNA in optical tweezers experiments. Biopolymers 99: 611–620
23. Morfill J, Kuhner F, Blank K, Lugmaier RA, Sedlmair J, Gaub HE (2007) B-S transition in short oligonucleotides. Biophys J 93: 2400–2409
24. Clausen-Schaumann H, Rief M, Tolksdorf C, Gaub HE (2000) Mechanical stability of single DNA molecules. Biophys J 78:1997–2007
25. Okoniewski SR, Uyetake L, Perkins TT (2017) Force-activated DNA substrates for probing

individual proteins interacting with single-stranded DNA. Nucleic Acids Res 45: 10775–10782

26. Walder R, LeBlanc M-A, Van Patten WJ, Edwards DT, Greenbert JA, Adhikari A, Okoniewski SR, Sullan RMA, Rabuka D, Sousa MC, Perkins TT (2017) Rapid characterization of a mechanically labile α-helical protein enabled by efficient site-specific bioconjugation. J Am Chem Soc 139:9867–9875
27. Sambrook JF, Russell DW (eds) (2001) Molecular cloning: a laboratory manual, 3rd edn. Cold Spring Harbor, New York
28. Okoniewski SR, Carter AR, Perkins TT (2017) A surface-coupled optical trap with 1-bp precision via active stabilization. Methods Mol Biol 1486:77–107
29. Seol Y, Li J, Nelson PC, Perkins TT, Betterton MD (2007) Elasticity of short DNA molecules: theory and experiment for contour lengths of 0.6-7 μm. Biophys J 93:4360–4373
30. Woodside MT, Behnke-Parks WM, Larizadeh K, Travers K, Herschlag D, Block SM (2006) Nanomechanical measurements of the sequence-dependent folding landscapes of single nucleic acid hairpins. Proc Natl Acad Sci U S A 103:6190–6195
31. Harmon FG, Kowalczykowski SC (1998) RecQ helicase, in concert with RecA and SSB proteins, initiates and disrupts DNA recombination. Genes Dev 12:1134–1144
32. Eeftens JM, van der Torre J, Burnham DR, Dekker C (2015) Copper-free click chemistry for attachment of biomolecules in magnetic tweezers. BMC Biophys 8:9
33. Stigler J, Ziegler F, Gieseke A, Gebhardt JCM, Rief M (2011) The complex folding network of single calmodulin molecules. Science 334: 512–516
34. Kastantin M, Langdon BB, Chang EL, Schwartz DK (2011) Single-molecule resolution of interfacial fibrinogen behavior: effects of oligomer populations and surface chemistry. J Am Chem Soc 133:4975–4983
35. Liphardt J, Onoa B, Smith SB, Tinoco IJ, Bustamante C (2001) Reversible unfolding of single RNA molecules by mechanical force. Science 292:733–737