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A method for assaying DNA flexibility

Eliz Cirakli [a,b], Aakash Basu [b,*]

[a] Department of Chemistry, Durham University, Durham, UK
[b] Department of Biosciences, Durham University, Durham, UK

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ABSTRACT

The transcription, replication, packaging, and repair of genetic information ubiquitously involves DNA:protein interactions and other biological processes that require local mechanical distortions of DNA. The energetics of such DNA-deforming processes are thus dependent on the local mechanical properties of DNA such as bendability or torsional rigidity. Such properties, in turn, depend on sequence, making it possible for sequence to regulate diverse biological processes by controlling the local mechanical properties of DNA. A deeper understanding of how such a "mechanical code" can encode broad regulatory information has historically been hampered by the absence of technology to measure in high throughput how local DNA mechanics varies with sequence along large regions of the genome. This was overcome in a recently developed technique called loop-seq. Here we describe a variant of the loop-seq protocol, that permits making rapid flexibility measurements in low-throughput, without the need for next-generation sequencing. We use our method to validate a previous prediction about how the binding site for the bacterial transcription factor Integration Host Factor (IHF) might serve as a rigid roadblock, preventing efficient enhancer-promoter contacts in IHF site containing promoters in *E. coli*, which can be relieved by IHF binding.

1. Introduction

Almost all examples of DNA:protein interactions require some form of mechanical distortion of DNA, such as bending, stretching, twisting, or melting [1,2]. For examples, DNA bending occurs extensively during nucleosome formation [3] and during the packaging of dsDNA in viral capsids [4], while unwinding occurs during helicase action [5] or during transcription initiation by RNA polymerase [6]. Single-molecule methods such as optical tweezers and manetic tweezers have long been used to study DNA mechanics and DNA:protein interactions [7–9]. They have accurately provided measures of the average mechanical properties of DNA such as persistence length [10] and torsional rigidity [11]. Surprisingly, mechanical deformations of DNA below the persistence length have been evidenced in many DNA:protein interactions and in a variety of cellular processes such as regulation of gene expression, DNA replication, and DNA repair [1,12–14], suggesting that the energetics of such processes could be significantly modulated by the mechanical properties of DNA. Further, several earlier experiments have all suggested that the local mechanical and structural properties of DNA are sequence-dependent: compilation of structural data [12,15] as well as molecular dynamics simulations [16,17]suggest that local DNA shape is sequence-dependent, while dynamic cyclization of short ~ 100 bp DNA duplexes flanked with complementary overhangs show that looping rates significantly vary with sequence [18,19]. This observations have long suggested a hypothesis that sequence via its effects on local DNA mechanics, could encode regulatory information modulating the energetics of critical DNA:protein interactions genome-wide.

Historically, lack of a high-throughput method to directly measure how the local mechanical properties of DNA vary with local sequence across large regions of the genome has hindered understanding whether and to what extent DNA sequence might encode regulatory information via a "mechanical code". This barrier was recently alleviated by a novel genomic technique called loop-seq, capable of measuring in high-throughput, the bendabilities of ~ 100,000 different 100 bp DNA sequences that can be designed to span any large region of the genome [13]. In loop-seq, an initial library of DNA molecules with complementary overhangs is briefly permitted to undergo intramolecular cyclization. Unlooped molecules are enzymatically digested, resulting in an enrichment of the more flexible sequences in the digested library. The enrichment factor for each sequence is calculated via deep-sequencing of the original pool and the digested pool, and is used as a measure of cyclizability or bendability.

* Corresponding author.
E-mail address: aakash.basu@durham.ac.uk (A. Basu).

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Loop-seq was used to demonstrate that regulatory information with broad downstream impact in diverse organisms is encoded in the sequence-dependent mechanical properties of DNA [2,13,14]. Patterns of DNA bendability along genes in multiple organisms were shown to correlate with known nucleosome positions, confirming in high-throughput, earlier expectations that local DNA bendability impacts nucleosome organization. Loop-seq also suggested a role of DNA mechanics is aiding chromatin remodeling enzymes locate Transcription Start Sites (TSSs) and accordingly position critical promoter-proximal nucleosomes [13]. Loop-seq also suggested a role of sequence-dependent DNA flexibility in organizing nucleosomes around the binding sites of transcription factors, and in several non-nucleosomal contexts such as the binding of TATA Binding Protein (TBP) during transcription initiation, in DNA wrapping around topoisomerases, and is establishing bent DNA structures along regions of *C. elegans* genome [14].

The detailed protocol for loop-seq has been published [20]. Here we describe the protocol from a modified point of view, which permits the quantification of the bendabilities of a few different short 100 bp DNA sequences, without requiring next-generation sequencing. The results also serve as an important control for the original loop-seq protocol. We also use the method to confirm an earlier hypothesis that the IHF binding site in IHF containing σ^{54} promoters in *E. coli* makes local DNA very rigid, preventing communication between promoter and enhancers [21]. IHF binding forces DNA in a tightly bent configuration, likely overcoming this roadblock to spontaneous bending, thus serving a regulatory role in gene expression.

2. Protocol for the gel-based low-throughput variant of loop-seq

2.1. Overview of the experiment

The premise of loop-seq and other DNA cyclization assays is that the rate of intramolecular cyclization of a short duplex DNA molecule flanked with complementary single-stranded overhangs serves as a measure of the bendability of the DNA molecule.

Loop-seq was developed as a high-throughput method to quantify the cyclizabilities of ~ 100,000 different short ~ 100 bp DNA sequences simultaneously [13,14,20]. Here we describe a variant of loop-seq that permits a gel-based readout of the cyclizabilities of a few different DNA sequences at a time. It does not require next-generation sequencing, which is costly, time consuming, and wasteful if the flexibilities of only a few sequences are required. While single-molecule Fluorescence Resonance Energy Transfer (smFRET) based methods have been applied to obtain looping kinetic curves of individual sequences [18], they require significant technology investments such as a single-molecule fluorescence microscope. Also, while ligation-based DNA cyclization protocols, where the complementary single-stranded overhangs are short (and hence ligation is required to obtain stably looped molecules) have long been used [19,22], ligase-based methods do not sample the equilibrium population of states where the ends of DNA are close to each other [18]. Rather, the substrate for ligase is a state where the ends are annealed. This state is extremely sensitive to unlooping rates because of the short overhang, and thus DNA cyclization measured in the ligase-based assay does not report on purely the looping rate [18]. Biologically, it is the looping rate that measures how quickly distal ends of DNA can be spontaneously brought in proximity, which can then be stabilized via protein interaction. The unlooping rate on the other hand, depends on the annealing between the strands, and is not of general biological relevance [18].

Loop-seq was previously used to quantify the Intrinsic Cyclizabilities (C0) of 12,472 different randomly specified DNA sequences [13]. From the calculated values, we identified two sequences, called minC0 and maxC0, which has the lowest and highest values of C0 respectively. Here we perform a modified gel-based variant of the loop-seq protocol that eliminates the need for high-throughput sequencing when measuring the cyclizabilities of a few sequences (i.e., this is a low-throughput method). We show that this method indeed reproduces the result that minC0 has a lower cyclizability than maxC0.

We also describe performing the gel-based loop-seq assay on two other DNA fragments called with_IHF_site and without_IHF_site. The with_IHF_site sequence contains a known 22 bp binding site of the bacterial transcription factor Integrative Host Factor (IHF) in the middle [21], whereas the without_IHF_site sequence is identical to it, except that the IHF binding site sequence has been replaced with random DNA of the same length. Previously published DNA cyclization studies indicated that the IHF binding site is rigid [21]. This was hypothesized to serve as a roadblock preventing spontaneous bending from bringing promoters in proximity of enhancers in IHF containing σ^{54} promoters in *E. coli*. IHF binding, which induces significant bending, was suggested to relieve this roadblock and allow transcription. We use the gel-based loop-seq protocol to suggest that indeed the with_IHF_site sequence is more rigid than the without_IHF_site sequence.

The gel-based loop-seq protocol is described in Fig. 1 and is identical to the original loop-seq protocol previously published in detail [20], up to the stage where looping is performed and unlooped molecules are digested. Briefly, the chemically-synthesized 100 nt sequence whose cyclizability we want to calculate is PCR amplified with primers that add 10 bp on either end, resulting in 120 bp molecules. One of the primers also adds a biotin moiety for surface immobilization on to streptavidin coated magnetic beads. Following surface immobilization, the DNA is nicked *in situ* using a site-specific nicking enzyme whose recognition sequence has been engineered in the DNA sequence. The nicks occur 10 nt from the ends of each strand. Washing away with hot buffer results in immobilized molecules containing a central 100 bp duplex region, flanked by 10 nt single-stranded overhangs. Further, by design, these 10 nt overhangs are complementary. The bead solution is then split into two equal volumes, termed “sample” and “control”, which thus contain the same number of DNA molecules. One is subject to brief looping for 1 min in presence of 1 M sodium chloride, followed by digestion of unlooped molecules using the exonuclease RecBCD. The other is treated identically, expect no digestion enzyme is added to the digestion mixture. DNA in both the sample and control pools is PCR amplified, column purified under identical conditions, and eluted in the same volume. Equal volumes of the purified DNA from the PCR amplification of the “sample” and “control” fractions are run side-by-side on a 1% agarose gel. If the DNA sequence is highly cyclizable, more number of molecules in the “sample” fraction will have been able to loop under 1 min and thus survive the subsequent digestion step. In the theoretical limiting case of infinite cyclizability, the number of molecules surviving digestion in the “sample” fraction should equal the number of molecules in the “control” fraction. We thus use the ratio of the band intensity of DNA in the sample fraction lane to that in the control fraction lane as a proxy for cyclizability.

2.2. DNA oligos and reagents

1 The following are the sequences (5′ to 3′) that were initially obtained as PAGE purified single stranded oligos, chemical synthesis by Integrated DNA Technology (IDT).

maxC0: 5′ – TTT CTT CAC TTA TCT CCC ACC GTC CCC CGA TGG TCC ACA TGC TCC TTA GAA GAG CTA GCC GTC GAT AGA CCA TCC GGC AGA AGA CAA GGG AAC GAA ATA G – 3′.

minC0: 5′ – TTT CTT CAC TTA TCT CCC ACC GTC CGA ATC GCA AAA CGA TCA GGC CGA GTG ACC ATT CAA TTT TCT GTC AGA CTT GGC AGA AGA CAA GGG AAC GAA ATA G – 3′.

with_IHF_site: 5′ – TTT CTT CAC TTA TCT CCC ACC GTC CCT TTT GCA CGA TGG T**TT GCT TAT CAA TTT GTT GCA CC**G GCA ATT TAA AAG GGC AGA AGA CAA GGG AAC GAA ATA G – 3′ (the IHF binding site [21] is in boldface).

without_IHF_site: 5′ – TTT CTT CAC TTA TCT CCC ACC GTC CCT TTT GCA CGA TGG T**GC GCA TGA TAA CGC CTT TTA GG**G GCA ATT TAA

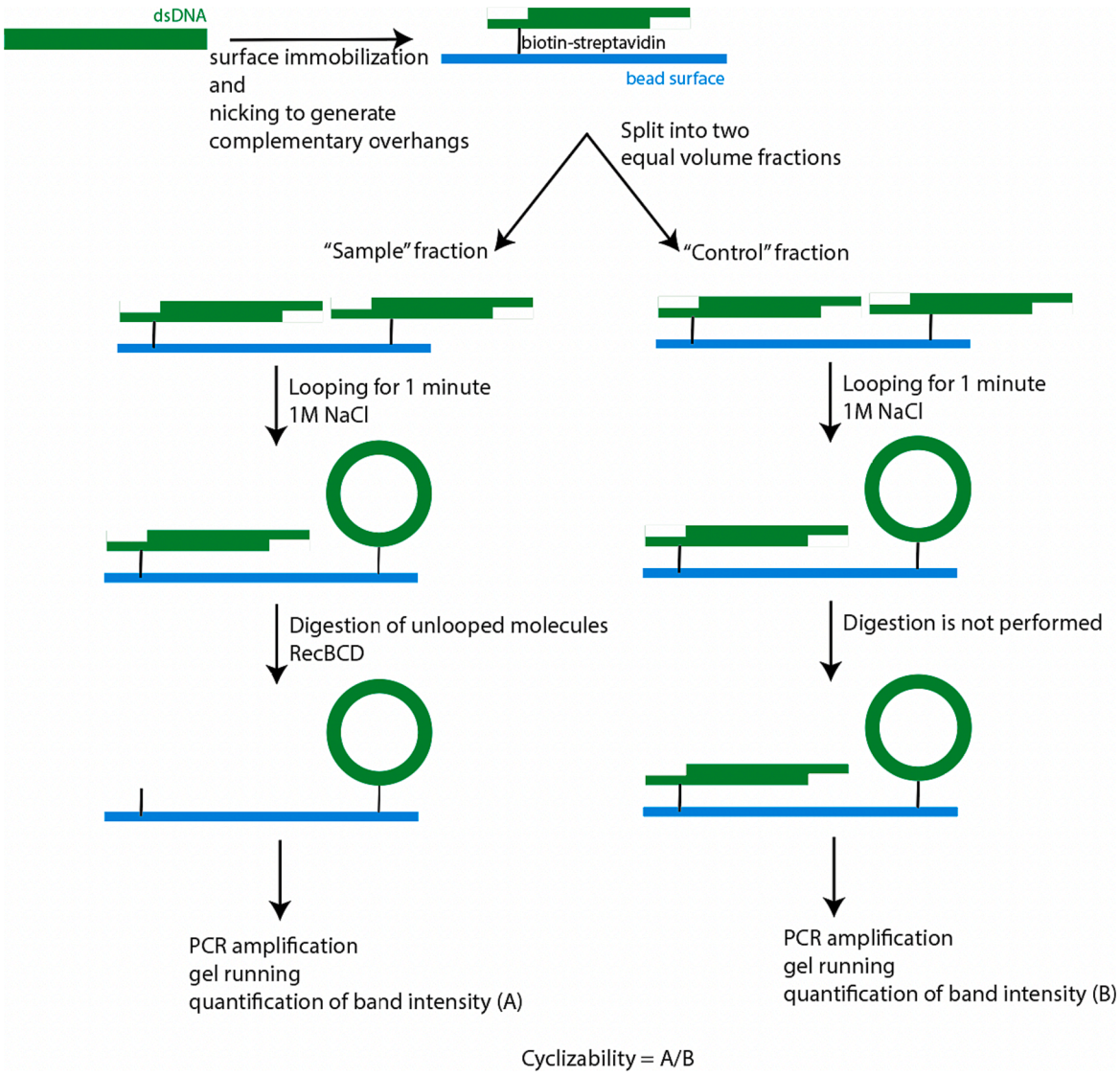

Fig. 1. Schematic of the gel-based variant of the loop-seq protocol. The DNA sequence of interest is chemically modified to have 10 nt single-stranded complementary overhangs on either end is surface immobilized on streptavidin-coated magnetic beads, exactly as done during loop-seq [20]. The bead solution is split into two equal-volume fractions termed "sample" and "control". Herein, for clarity, two DNA molecules are shown for each fraction, though there are millions of identical molecules. In each fraction, DNA is permitted to loop via annealing of the overhangs in presence of 1 M NaCl. Unlooped molecules are digested in the "sample", but not in the control. The surviving molecules in each fraction are PCR amplified and run on a gel to quantify band intensity. The ratio of DNA band intensity in the sample to that in the control is defined as the cyclization of the DNA sequence.

AAG GGC AGA AGA CAA GGG AAC GAA ATA G – 3′ (random DNA replacing the IHF binding site is in boldface).

These sequences are all 100 nucleotides (nt) in length. The left 25 nt and right 25 nt are identical, and serve as adapter sequences for subsequent PCR amplification steps.

2.3. PCR amplification primers

The following amplification primers were obtained from IDT:

NP1: 5′ – CAG AAT CCG TCG AAG AGC CTT ATC TCC CAC CGT CC – 3′.

NP2: 5′ – ACG GAT TCT GCG AAG AGC TTC CCT TG/iBiodT/ CTT CTG CC – 3′.

(/iBiodT/ refers to a biotin moiety attached to the thymine base).

2.4. Protocol

The protocol up to the looping and digestion stage closely follows the original loop-seq protocol [20]. Briefly, the four 100 bp DNA oligos were PCR amplified using primers NP1 and NP2, resulting in 120 bp products. These primers add a biotin moiety and overhangs that contain important DNA sequences for subsequent nicking and for forming the 10 nt long complementary single-stranded overhangs. The amplicons were attached to beads, nicked *in situ*, split into a sample and control fractions, looped, and digested (sample fractions) or not digested (control fraction) exactly as per the original loop-seq protocol [20]. The loop-seq protocol was followed as if the four individual DNA sequences were four individual libraries.

The magnetic beads in the eight fractions (sample and control fraction for each DNA sample) were pulled down and the buffer removed. It was replaced with PCR amplification buffer as follows:

2x KAPA Hifi Hot Start Ready Mix: 25 μl.
NP1 (100 μM): 1 μl.
NP2 (100 μM): 1 μl.
Water: 23 μl.

The beads in PCR mixture were transferred to PCR tubes. PCR

amplification was performed on each fraction as follows:

Step 1: 95 °C: 3 min.
Step 2: 95 °C: 20 s.
Step 3: 65 °C: 15 s.
Step 4: 72 °C: 15 s.
(loop over steps 2–4 16 times).
Step 5: 72 °C: 2 min.
Step 6: hold at 4 °C.

The beads were pulled down and the supernatant subject to PCR purification using the QIAquick PCR purification kit |(Qiagen). DNA was eluted in 30 μl Elution Buffer (QIAgen). Concentration was measured using a NanoDrop (Table 1).

All PCR products were diluted 20-fold in 10 mM tris pH 8.0. 1 μl of each diluted product was added to 19 μl 10 mM tris pH 8.0 and loaded on a SPBY gold stained 1% agarose E-gel (Invitrogen). The gel was run for 6 min and imaged (Fig. 2). Band intensities at the expected length of 120 bp were obtained using ImageJ [23] by plotting the intensity profile along the lane and summing the area under the peak that corresponds to the band after subtracting the background intensity along the lane. This procedure closely follows what is described in the imageJ manual (https://imagej.nih.gov/nih-image/manual/tech.html#analyze). As each product was exactly 120 bp long, band intensity was used as a measure of the concentration of the amplified DNA at 120 bp. For each sequence, cyclizability was defined as the ratio of the band intensity in the sample fraction to that in the control fraction (Table 1).

We indeed find that the maxC0 DNA has higher cyclizability than the minC0 DNA. This serves as an important control of the loop-seq protocol, and also demonstrates that this gel-based low-throughput readout that does not require next-generation sequencing.

We also find that DNA containing the IHF binding site is more rigid than DNA where the IHF site has been scrambled. This confirms earlier findings that the IHF site is rigid [21]. It is consistent with the hypothesis [21] that IHF binding, which significantly bends DNA [24], might serve to remove a roadblock in enhancer-promoter communication in IHF site containing σ^{54} dependent promoters in *E. coli*.

3. Future directions

The gel-based assay to visualize cyclizability offers a convenient method to perform cyclization measurements on a few different DNA sequence, without requiring next-generation sequencing or involved single-molecule fluorescence detection setups. It also retains the advantages of not using the traditional ligase-based cyclization assays with constructs containing short single-stranded overhangs [18].

In addition to querying the cyclizabilities of important sequences of interest, such as promoter sequences, and various transcription factor binding sites, this method may be of significant interest in determining how DNA damage, such as mismatches, insertions and deletions, and various chemical and epigenetic modifications to DNA such as methylation, impact its mechanical properties. The PCR steps prior to looping described in section 2.4, which ultimately serve to create constructs that have long single-stranded overhangs, would not retain the modifications like methylation, and would automatically "repair" lesions like mismatches. Loopable constructs can, however, be directly produced via annealing of two long DNA strands which have been chemically synthesized with all required modifications present. It can thus offer a convenient albeit low-throughput, option for assessing the flexibility contributions of various DNA modifications, and to develop hypotheses regarding how altered DNA flexibility allows DNA damage or other modifications to achieve a part of their downstream biological effects. Indeed mismatch repair proteins like MutS have been suggested to search for damaged DNA sites by scanning for the enhanced flexibility of DNA that mismatches likely incur [25].

Table 1
Calculated cyclizability (ratio of the band intensity of DNA in the sample to that in the control fraction band) of the four sequences, obtained by running equal volumes of the PCR product on a gel (Fig. 2).

PCR product	Cyclizability (ratio of band intensity)
maxC0 samplemaxC0 control	0.79 ± 0.06 (N = 3)
minC0 sampleminC0 control	0.20 ± 0.08(N = 3)
with_IHF_site samplewith_IHF_site control	0.50 ± 0.08 (N = 3)
without_IHF_site samplewithout_IHF_site control	0.89 ± 0.06 (N = 3)

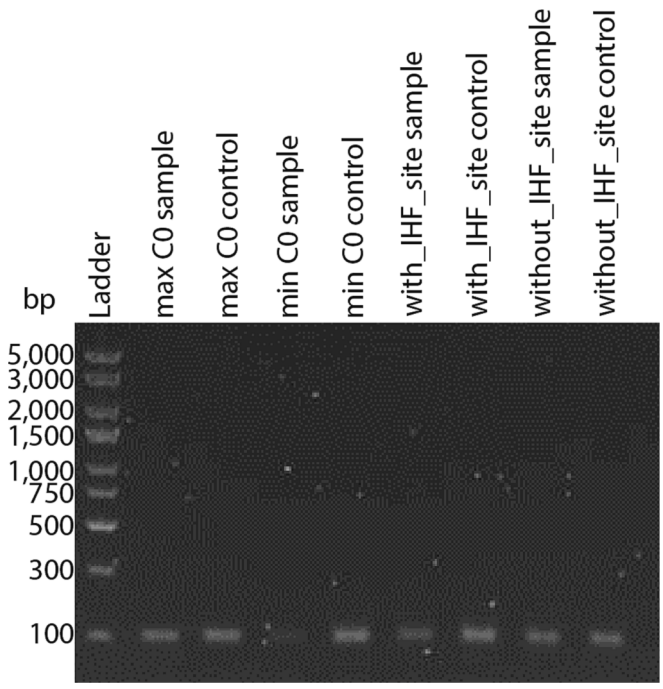

Fig. 2. The 8 fractions (sample and control fractions of the four DNA sequences) were PCR amplified after looping and digestion and diluted 20-fold. 1 μl of each of the diluted samples along with a ladder, were run on a 1% pre-cast agarose gel (Invitrogen). Band intensities were quantified and cyclizabilities were calculated (Table 1). The expected size of all 8 fractions is 120 bp.

4. Appendix

4.1. Equipment list

1. Nano drop 2000 (Thermo Scientific): for measuring DNA concentration.
2. Centrifuge (Eppendorf).
3. Rare earth magnetic stand (New England Biolabs S1506S): for pulling down magnetic beads and exchanging buffers.
4. Thermocycler (Eppendorf MasterCycler Gradient X2).
5. E-gel iBASE version 1.4.0 and E-gel EX Agarose gels 2% (Invitrogen).

4.2. Reagent list and brief uses

1 PCR purification kit: QIAquick PCR purification kit (Qiagen catalogue number 28106).

4. Streptavidin coated magnetic beads: Dynabeads MyOne Streptavidin T1 (Thermo Fisher Scientific catalogue number 65601). Biotinylated DNA were bound here and nicking, looping, and digestion were performed *in situ*.

5 Polymerase for PCR: KAPA Hifi Hot Start Ready Mix (Roche). This polymerase was used for all PCR reactions.

6. Nicking enzyme and buffer: Nt.BspQ1 and NEBuffer 3.1 (New England Biolabs catalogue number R0644S). Nicking of immobilized duplex DNA resulted in nicks 10 nt from ends of either strand.

Subsequent washing in 50 °C buffer resulted in the denaturation of the 10 nt bits, resulting immobilized molecules with 10 nt complementary overhangs.

7. Digestion enzyme and buffer: RecBCD and NEBuffer 4 (New England Biolabs catalogue number M0345S). RecBCD digests unlooped DNA molecules in the "sample" fraction.

8. Buffers: The following buffers are used in various stages including binding of DNA to beads, nicking, performing loop-seq. Their detailed use has been published earlier [20].

T50BSA: 95 % T50 (50 mM NaCl, 10 mM tris pH 8.0), 1 mg/ml BSA.

T10BSA: 95 % T10 (10 mM NaCl, 10 mM tris pH 8.0), 1 mg/ml BSA.

T2.5BSA: 95 % T2.5 (2.5 mM NaCl, 10 mM tris pH 8.0), 1 mg/ml BSA.

Looping buffer: 1 M NaCl, 20 mM tris pH 8.0 1 mg/ml BSA.

Digestion buffer: 1x NEBuffer 4 (New England Biolabs), 1 mM ATP, 0.333 units/ml RecBCD (New England Biolabs catalogue number M0345S).

Software: ImageJ was used to quantify band intensity (Fig. 2).

Author contributions

EC carried out all experiments, analysed the data, and prepared the figures. EC and AB wrote the paper. AB designed and supervised the research.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

Data will be made available on request.

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