Chapter 20

Quantitative Determination of DNA Bridging Efficiency of Chromatin Proteins

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Abstract

DNA looping is important for genome organization in all domains of life. The basis of DNA loop formation is the bridging of two separate DNA double helices. Detecting DNA bridge formation generally involves the use of complex single-molecule techniques (atomic force microscopy, magnetic or optical tweezers). Although DNA bridging can be qualitatively described, quantification of DNA bridging and bridging dynamics using these techniques is challenging. Here we describe a biochemical assay capable of not only detecting DNA bridge formation but also allowing for quantification of DNA bridging efficiency and the quantification of the effects of physicochemical conditions or protein interaction partners on DNA bridge formation.

Key words DNA bridging, DNA looping, DNA-DNA interactions, DNA-DNA cross-linking, DNA bridging proteins, Pull-down assay

1 Introduction

Three-dimensional organization of genomes affects and is affected by DNA transactions such as transcription regulation, replication, and recombination. In cells, a family of DNA-binding proteins, called chromatin proteins, is involved in the organization of the genome. These proteins wrap DNA around themselves, bend it, or bridge DNA, forming loops. DNA loops play a variety of roles in genome organization. These loops may operate locally with regulatory functions at specific single genes [1, 2] or over longer distances, enabling the organism to co-regulate genes that are in terms of genomic position far apart [3, 4]. Studies involving DNA looping have a rich history [4–13], with numerous new insights being available through the application of biochemical and biophysical techniques.

Remus T. Dame (ed.), *Bacterial Chromatin: Methods and Protocols*, Methods in Molecular Biology, vol. 2819,
https://doi.org/10.1007/978-1-0716-3930-6_20,

Classically, DNA loops (DNA bridges) were studied through the use of electron microscopy and atomic force microscopy [14–17]. These techniques permit visualization of DNA bridges, but these static images are incapable of resolving the formation of DNA bridges or its modulation. The advent of biophysical techniques such as magnetic and optical tweezers has made it possible to stretch bridged DNA molecules by applying force [18, 19] and determine biophysical properties of the DNA bridges, but it is difficult to quantitate the protein(s)-DNA bridging efficiency.

Here we describe an ensemble method for the quantification of protein-DNA bridging efficiency and its modulation by environmental conditions and other proteins. In this "bridging assay," we use streptavidin-coated paramagnetic beads coupled to 5′ biotin-labeled DNA (bait DNA). The DNA-coated beads are then incubated in the presence of ^{32}P radioactively labeled DNA (prey DNA) and a (potential) DNA bridging protein (or any di- or multivalent DNA-binding ligand). The beads are pulled down by using their magnetic properties, and the amount of recovered prey DNA is detected through liquid scintillation. The recovered prey DNA is a direct measurement of the efficiency of DNA bridge formation under these conditions. This method is compatible with other methods of DNA detection, for instance, based on detection of fluorescent intensity of DNA-bound intercalators or fluorescent end labels [20].

2 Materials

Prepare all solutions using ultrapure water (prepared by purifying deionized water, to attain a sensitivity of 18 MΩ-cm at 25 °C) and analytical-grade reagents. Prepare and store all reagents at −20 °C (unless indicated otherwise). You also need access to some routine biochemical techniques [21].

2.1 Stock Solutions

The following stock solutions are required to perform this experiment:

1. Phosphate-buffered saline (PBS): 12 mM $NaPO_4$ pH 7.4, 137 mM NaCl.
2. Renaturation buffer 10× (RB 10×): 200 mM Tris–HCl pH 9.5, 10 mM spermidine, 1 mM EDTA.
3. Labeling buffer (LB): 500 mM Tris–HCl pH 9.5, 100 mM $MgCl_2$, 40% glycerol.
4. Coupling buffer (CB): 20 mM Tris–HCl pH 8.0, 2 mM EDTA, 2 M NaCl, 2 mg/mL acetylated BSA, 0.04% Tween-20.

5. Incubation buffer 10× (IB 10×): 100 mM Tris–HCl pH 8.0, 0.2% Tween-20, 10 mg/mL acetylated BSA.
6. DNA storage buffer: 10 mM Tris–HCl pH 8.0, 50 mM KCl, 10 mM $MgCl_2$.
7. Stop buffer: 10 mM Tris–HCl pH 8.0, 1 mM EDTA, 200 mM NaCl, 0.2% SDS.
8. Wash buffer (WB): buffer corresponding with experimental conditions (i.e., 16 μL 1× experimental buffer, 2 μL DNA storage buffer, 2 μL buffer of protein sample).

2.2 Generation of DNA Substrates Using PCR

To generate a DNA substrate for the bridging assay, it is advised to use polymerase chain reaction (PCR). This reaction requires the following:

1. A DNA template containing the sequence of interest (*see* **Note 1**).
2. A forward primer 5′-labeled with biotin for bait DNA, unlabeled for prey DNA.
3. A reverse primer.
4. DNA polymerase 5 U/μL (*see* **Note 2**).
5. 2 mM deoxyribose nucleotide triphosphate (dNTP).
6. Polymerase reaction buffer.
7. GenElute PCR cleanup kit (Sigma-Aldrich).
8. Eppendorf® PCR tubes.
9. Bio-Rad T100 thermocycler or any other available PCR machine.
10. 1% agarose gel in 1× TAE.
11. NanoDrop® (Thermo Fisher).
12. DNA ladder.

2.3 Bridging Assay Equipment

1. Magnetic Eppendorf rack.
2. Eppendorf ThermoMixer C.
3. Eppendorf rack.
4. Eppendorf pipettes.
5. Streptavidin-coated Dynabeads (Invitrogen).

2.4 Quantifying DNA Bridging Through Radioactivity

1. Liquid scintillator (HIDEX 300SL).
2. Counting vials.
3. 37 °C heat block.
4. 92 °C heat block.
5. Eppendorf® PCR tubes.

6. ATP, gamma ^{32}P.
7. Tabletop Eppendorf centrifuge.
8. T4 Polynucleotide Kinase 10 U/μL.
9. Mini G50 columns (Cytiva).

3 Methods

3.1 Generation of DNA Substrates Using PCR

These reagents are combined in an Eppendorf® PCR tube according to the scheme below. These reactions must be done for both the standard and biotinylated primers.

Reagent	Final quantity
DNA template	1 ng
Forward primer	10 pmol
Reverse primer	10 pmol
2 mM dNTP	5 μL
10× polymerase buffer	5 μL
5 U/μL polymerase	0.2 μL
H_2O	Add to total volume of 50 μL

1. Initiate the PCR using the following protocol (*see* **Note 2**).

Temperature (°C)	Duration (s)	Cycles
98	30	
98 72 (−1 °C per cycle) 72	10 20 60	15×
98 57 72 72 12	10 20 60 600 ∞	25×

2. Purify the PCR product using the GenElute PCR cleanup kit.
3. Load 2 μL of the purified PCR product on a 1% agarose gel in TAE buffer alongside a DNA molecular weight marker for verification that a product of the expected length is formed. An example of a successful PCR and purification of the obtained PCR product is shown in Fig. 1.

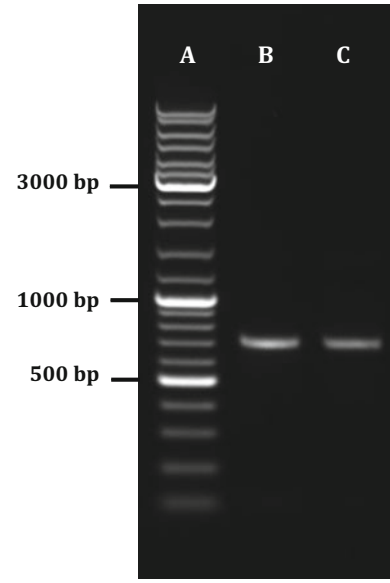

Fig. 1 Visualization of PCR product size by agarose gel electrophoresis. (A) 2 μL of the GeneRuler DNA molecular weight marker. (B) 2 μL of the purified PCR-generated unlabeled DNA (685 bp, ready for ^{32}P labeling). (C) 2 μL of the purified PCR-generated biotin-labeled DNA (685 bp)

4. Finally, the concentration of purified PCR-generated DNA needs to be determined accurately. Determine the concentration of the purified DNA by measuring UV absorbance at 260 and 280 nm. If no other method is available, the concentration of DNA can also be approximated using a DNA dilution series run on an agarose gel compared to a reference marker. Store DNA solution at −20 °C.

3.2 Radiolabeling DNA

1. Add 1.5 μL of RB 10× to 2 pmol of the purified DNA, and fill to a final volume of 15 μL using H_2O.
2. Prepare the kinase mix according to the following scheme:

Kinase mix component	Added volume per DNA labeling (μL)
LB 10×	2.5
50 mM DTT	2.5
ATP, gamma ^{32}P	2
10 U/μL Polynucleotide Kinase	1
H_2O	2

3. Incubate the DNA mix at 92 °C for 2 min and immediately put the sample on ice (*see* **Note 3**).

4. Add 10 μL of the kinase mix to the DNA sample and incubate at 37 °C for 30 min.
5. Stop the reaction by adding 1 μL of 0.5 M EDTA. Incubate the sample at 92 °C for 10 min to deactivate the kinase.
6. Quickly spin the sample down using a tabletop centrifuge.
7. Prepare the Mini G50 column by pre-incubating it in DNA storage buffer as described by the column manual.
8. Add 15 μL of DNA storage buffer to the DNA sample; obtain the minimal required volume of 40 μL for column purification.
9. Purify the labeled DNA using the G50 column.
10. Assess the volume of the purified DNA, and adjust to 100 μL using DNA storage buffer. The DNA should now have a final concentration of approximately 20 pmol/μL.
11. Fill a counting vial with 7 mL of H_2O.
12. Prepare 2 μL of the labeled DNA for liquid scintillation, by transferring it to a PCR tube and submerging it in the counting vial.
13. Determine the amount of counts per minute per vial.

3.3 Bridging Assay

The DNA bridging assay relies on the immobilization of bait DNA on magnetic beads and the capture and detection of ^{32}P-labeled prey DNA if DNA-DNA bridge formation occurs (*see* Fig. 2 for a schematic depiction of the assay).

1. Wash 6 μL of streptavidin-coated paramagnetic beads (henceforth referred to as "beads") per condition you wish to test with 100 μL of PBS on the magnetic rack (*see* **Note 4**).
2. Wash the beads with 100 μL of CB twice.
3. Resuspend the beads in 6 μL of CB and split the suspension in two samples.
4. Dilute 100 fmol of biotinylated DNA in a total volume of 3 μL using DNA storage buffer (one per sample).
5. Add the biotinylated DNA solution to half of the washed and resuspended beads. Add 3 μL DNA storage buffer only to the other half of suspended beads (Fig. 2, part c) (*see* **Note 5**).
6. Gently vortex the sample to ensure that the beads are resuspended.
7. Incubate the samples at 25 °C for 20 min in the Eppendorf ThermoMixer C at 1000 RPM.
8. Wash each bead suspension with 16 μL of 1× IB twice.
9. Resuspend each bead suspension in 16 μL of 1× IB.
10. Add 2 μL of radiolabeled DNA (with a minimum of 5000 counts per minute) to each sample.

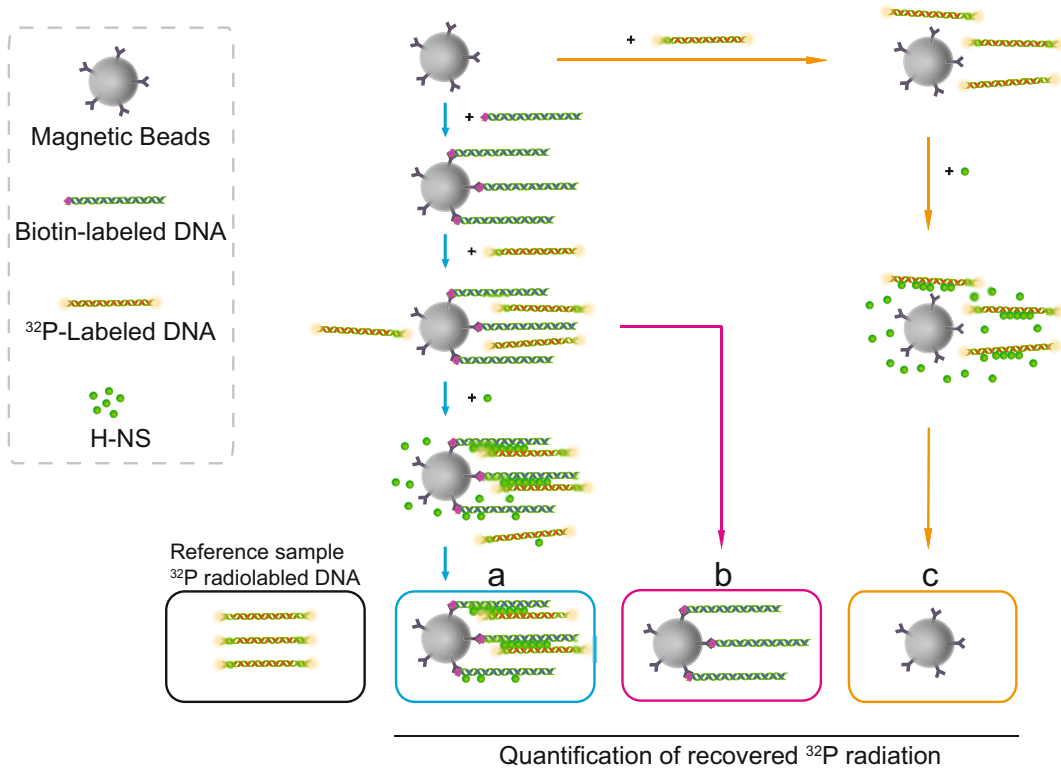

Fig. 2 Schematic depiction of the DNA bridging assay with the DNA bridging protein H-NS as an example. (a) A standard DNA bridging assay is shown by the blue arrows. Here streptavidin-coated paramagnetic beads are coupled to the bait DNA, 5′-labeled with biotin. The beads bound with DNA are then incubated in the presence of ^{32}P-radiolabeled DNA strand and H-NS. Next, using a magnetic rack, the beads are pulled down, and the amount of recovered ^{32}P-radiolabeled DNA (prey DNA) is quantified based on a reference ^{32}P-radiolabeled DNA sample. (b) The pink arrows indicate a standard negative control for the DNA bridging assay, in which no H-NS is added. This control checks the stability of both prey and bait DNA. No ^{32}P-radiolabeled DNA should be recovered for this sample (*see* **Note 4** if this is the case). (c) The orange arrows indicate a standard negative control for the assay in which the DNA bridging assay is performed in the absence of the bait DNA to test the stability of the protein. No ^{32}P-radiolabeled DNA should be recovered for this assay (*see* **Note 5** if this is the case)

11. Add 2 μL of the protein of interest to each sample except one. This sample is used as control for DNA integrity (Fig. 2, part b) (*see* **Notes 6** and 7).
12. Gently pipet the sample up and down to ensure that the beads are resuspended.
13. Incubate the samples at 25 °C for 20 min in the Eppendorf ThermoMixer C at 1000 RPM.
14. Gently wash the beads with 20 μL of WB.
15. Resuspend the beads in 20 μL stop buffer.
16. Transfer the sample to the liquid Cherenkov scintillation counter.

4 Results

4.1 DNA Bridging Efficiency as a Function of Protein Concentration

The protein concentration used in the assay determines the amount of DNA bridging observed. It is therefore essential to test a range of protein concentrations whenever a previously uncharacterized DNA bridging protein is investigated using the bridging assay. Here, we show an example (Fig. 3) from our recent study investigating the DNA bridging efficiency of the histone-like nucleoid structuring (H-NS) [22]. Using this assay, it was demonstrated that the DNA bridging efficiency of H-NS is highly dependent on protein concentration. Similar protein dependent behavior was shown for other proposed bacterial DNA bridging proteins like Rok, MvaT, and MucR [20, 23–25]. Recently, also the archaeal *M. jannaschii* histone MJ1647 was shown to be a DNA bridging protein [26].

4.2 DNA Bridging Efficiency of Proteins as a Function of Physicochemical Conditions and Protein-Protein Interactions

The DNA bridging assay allows for testing of the effect of altered physicochemical conditions. It has been shown previously that H-NS-mediated DNA bridging is strongly modulated by environmental factors such as osmotic stress [18, 22]. The DNA bridging assay revealed that increasing the amount of KCl in the buffer indeed effectively abolishes DNA bridging by H-NS (Fig. 4) [22]. This strong dependence of H-NS activity on environmental factors underlines the necessity to test different buffer conditions when testing new proteins. It is, however, important to verify that the DNA-binding activity of the protein is still intact under conditions that no DNA bridging is observed (*see* **Note 8**). Other physicochemical conditions that can be tested are (but not limited to) temperature, $MgCl_2$ concentration, and pH. It is useful to screen a wide range of conditions when a regulatory mechanism of DNA

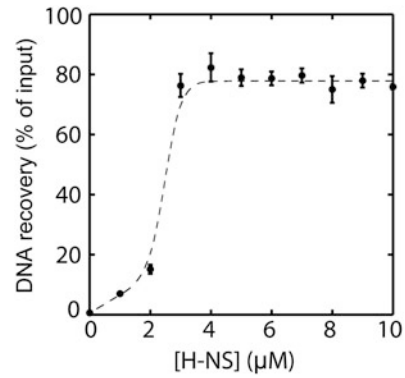

Fig. 3 DNA bridging as a function of H-NS concentration [22]. The experiments were performed in the presence of 10 mM Tris–HCl pH 8.0, 50 mM KCl, 10 mM $MgCl_2$, 5% (w/v) glycerol. Error bars indicate the standard deviation of a triplicate of assays

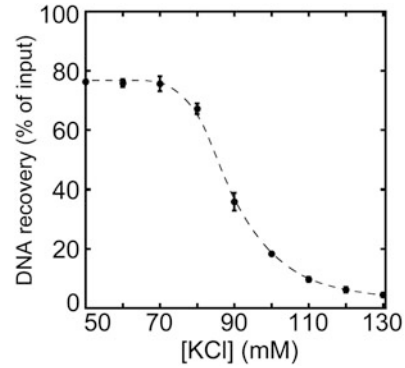

Fig. 4 Modulation of H-NS-DNA bridging by [K^+] [22]. The experiments were performed with 3.3 μM H-NS in 10 mM Tris–HCl pH 8.0, 10 mM $MgCl_2$, 5% w/v glycerol. Error bars indicate the standard deviation of a triplicate of experiments

bridging is unknown; absence of effects of altered physicochemical conditions might imply different mechanisms to modulate DNA bridging [23]. For instance, DNA bridging could be modulated by interaction with a protein partner or small molecules. Indeed, it has been shown that interactions with partner proteins can serve to enhance or reduce DNA bridging efficiency [22–24].

5 Notes

1. The length of the DNA substrate used can affect the efficiency of the assay. A 685 base pair DNA substrate was used in the experiments described here.
2. Conventional PCR protocols with fixed annealing temperatures may be used but need to be individually optimized for different primer-template combinations, synthesized primer lengths, and polymerases. In our experiments we routinely use a 685 bp DNA substrate for comparison with our other techniques (*see* Chapter 22). We use Phusion polymerase for high fidelity and fast synthesis of PCR product. In the past, we also had good experiences with the use of DreamTaq polymerase.
3. Snap-chilling your DNA before labeling ensures that the DNA remains single stranded and increases the efficiency of the kinase.
4. When washing the beads on the magnetic rack, pay attention to the following:
   (a) Keep the Eppendorf tubes in the magnetic rack, and incubate for at least 1 min to ensure that the beads are pelleted.

(b) When removing the supernatant, make sure to pipette slowly and not to disturb the pelleted beads with the pipette tip.

(c) Use a 0.5–10 Eppendorf micropipette to ensure that all liquid is removed from the sample.

(d) Gently pipette the new liquid onto the pelleted beads.

5. If ^{32}P-labeled DNA is recovered in the absence of biotin-labeled DNA, but in the presence of protein, it is likely caused by precipitation or aggregation of the protein. In these cases, it is best to:

   (a) Optimize the experimental buffer. Some proteins precipitate in suboptimal conditions. The conditions can vary greatly from protein to protein, so it is best to test a wide array of conditions (ion concentrations, pH, ion composition, etc.) and detergents until a suitable buffer has been found.

   (b) Use new beads as the streptavidin coating may decay over time, leading to inconclusive experiments.

6. Recovery of ^{32}P-labeled DNA in the absence of DNA bridging proteins may be an indication of nonspecific DNA-DNA interaction.

   In these cases, it is best to:

   (a) Check the integrity of DNA on a 1% agarose gel.

   (b) Reevaluate the experimental buffer as the absence of salt may cause larger DNA substrates to interact. Similar effects may occur at extreme pHs.

7. Depending on characteristics of the protein of interest (i.e., binding properties), the order of adding DNA and protein to the sample might yield a bias to the assay.

8. DNA binding of proteins is best confirmed with additional solution-based experiments such as microscale thermophoresis (*see* Chapter 17) or tethered particle motion (*see* Chapter 22).

Acknowledgments

This work was supported by the Netherlands Organization for Scientific Research [VICI 016.160.613/533 and OCENW.GROOT.2019.012 to RTD] and China Scholarship Council grant no. 201506880001 (LQ).

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