Article details

Valk R.A. van der, Qin L., Moolenaar G.F. & Dame R.T. (2018), Quantitative determination of DNA bridging efficiency of chromatin proteins. In: Dame R.T. (Ed.) Bacterial Chromatin: Methods and Protocols. Methods in Molecular Biology no. 1837 New York, NY, U.S.A.: Humana Press. 199-209.
Doi: 10.1007/978-1-4939-8675-0_12

Chapter 12

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 novel biochemical assay capable of not only detecting DNA bridge formation, but also allowing for quantification of DNA bridging efficiency and the effects of physico-chemical conditions 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]. Although studies involving DNA looping have a rich history [3, 5–12], in recent years, numerous new insights have become available through the application of new biochemical and biophysical techniques.

Classically, DNA loops (DNA bridges) were studied through the use of electron microscopy and atomic force microscopy [13–16]. These techniques permit visualization of DNA bridges. However, these static images are incapable of resolving the

Remus T. Dame (ed.), *Bacterial Chromatin: Methods and Protocols*, Methods in Molecular Biology, vol. 1837,
https://doi.org/10.1007/978-1-4939-8675-0_12,

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 [17, 18] and determine biophysical properties of the DNA bridges, but it is difficult to quantitate the protein(s)-DNA bridging efficiency.

Here, we describe a novel 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 and a 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 ^{32}P radiolabeled DNA (prey DNA) is detected through liquid scintillation. The recovered ^{32}P radiolabeled DNA is a direct measurement of the amount of DNA bridges formed under these conditions.

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 [19].

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% Tween20.
5. Incubation buffer 10× (IB 10×): 100 mM Tris–HCl pH 8.0, 0.2% Tween20, 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.

2.2 Generation of DNA Substrates Using PCR

To generate a DNA substrate for TPM, it is advised to use Polymerase Chain Reaction. This reaction requires:

1. A DNA template containing the sequence of interest (*see* **Note 1**).
2. A Forward primer.
3. A Reverse primer.
4. Dream-Taq DNA polymerase 5 U/μL.
5. 2 mM Deoxyribose Nucleotide Triphosphate (dNTP).
6. 10× Dream Taq-polymerase reaction buffer.
7. Gene Elute PCR cleanup kit (Sigma-Aldrich).
8. Eppendorf® PCR tubes.
9. Biorad T100 Thermocycler or any other available PCR machine.
10. 1% agarose gel in 1× TBE.
11. Nanodrop® (Thermo Fisher).
12. DNA ladder.

2.3 Bridging Assay Equipment

1. Magnetic Eppendorf rack.
2. Eppendorf shaker.
3. Eppendorf rack.
4. Eppendorf pipettes.
5. Streptavidin coated Dynabeads.

2.4 Quantifying DNA Bridging Through Radioactivity

1. Liquid scintillator (HIDEX 300SL).
2. Counting vials.
3. 37 °C heat block.
4. 80 °C water bath.
5. Eppendorf® PCR tubes.
6. ATP, gamma ^{32}P.
7. Tabletop Eppendorf centrifuge.
8. T4 Polynucleotide Kinase 10 U/μL.
9. Mini G50 columns (GE Healthcare).

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× Taq polymerase buffer	5 μL
5 U/μL dream Taq polymerase	0.2 μL
H_2O	Add to total volume of 50 μL

1. Keep this reaction mix on ice as much as possible and initiate the PCR using the following protocol (*see* **Note 2**).

	Cycles	Temperature	Duration
Initial denaturation	1	95 °C	5 min
Denaturation	35	95 °C	30 s
Annealing		62 °C	30 s
Elongation		72 °C	4 min
Final elongation	1	72 °C	10 min
	1	15 °C	∞

2. Purify the PCR product using the GeneElute PCR cleanup kit.
3. Load 2 μL of the purified PCR product on a 1% agarose gel in TBE 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.
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 (*see* **Note 3**). 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 the DNA solution at −20 °C.

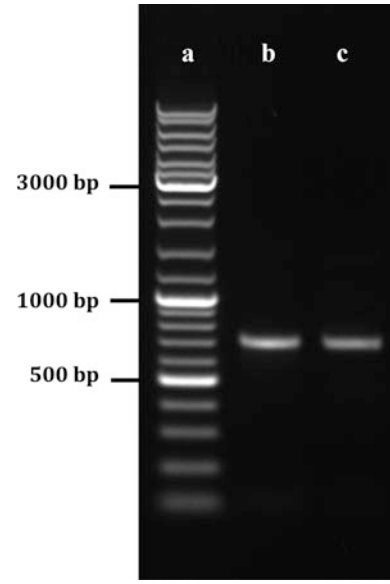

Fig. 1 Visualization of PCR product size by agarose gel electrophoresis. (**a**) 2 μL of the GeneRuler DNA molecular weight marker. (**b**) 2 μL of the purified PCR-generated unlabeled DNA (685 bp, ready for ^{32}P labeling). (**c**) 2 μL of the purified PCR-generated biotin-labeled DNA (685 bp)

3.2 Radio-labeling 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 80 °C for 2 min and immediately put the sample on ice (*see* **Note 4**).
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 75 °C for 15 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. Purify the labeled DNA using the G50 column.
9. Assess the volume of the purified DNA and fill it to 100 μL using DNA storage buffer. The DNA should now have a final concentration of approximately 20 pmol/μL.
10. Fill a counting vial with 7 mL of H_2O.
11. Prepare 2 μL of the labeled DNA for liquid scintillation, by transferring it to a PCR tube and submerging it in the counting vial.
12. Determine the amount of counts per minute per vial.
13. Quantify the counts per μL of labeled DNA

3.3 Bridging Assay

The DNA-bridging assay relies on the immobilization of bait DNA on magnetic microparticles 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).

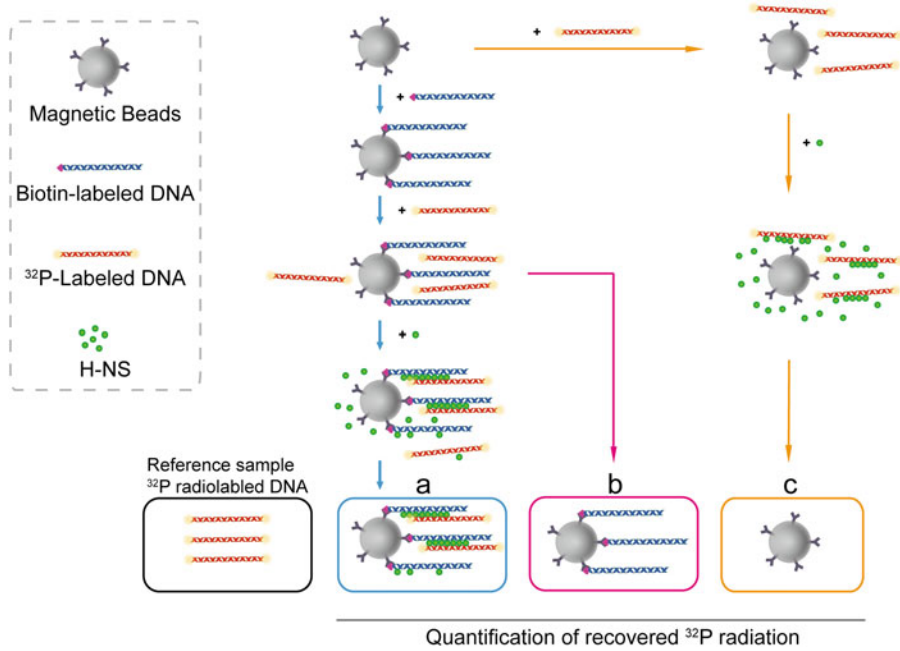

Fig. 2 Schematic depiction of the DNA bridging assay with the H-NS DNA bridging protein. A standard DNA bridging assay is shown by the blue arrows. Here streptavidin-coated paramagnetic beads are coupled to the bait DNA, 5′ labeled with biotin. The beads bound with DNA are then incubated in the presence 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 5** 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 6** if this is the case)

1. Wash 3 μL of streptavidin-coated paramagnetic beads (henceforth referred to as "beads") per condition you wish to test with 50 μL of PBS on the magnetic rack (*see* **Note 7**).
2. Wash the beads with 50 μL of CB twice.
3. Resuspend the beads in 3 μL of CB.
4. Dilute 100 pmol of biotinylated DNA in a total volume of 3 μL using DNA storage buffer (one per sample).
5. Add the biotinylated DNA solution to the washed and resuspended beads.
6. Gently vortex the sample to ensure that the beads are resuspended.
7. Incubate the samples at 25 °C for 20 mins in the Eppendorf shaker at 1000 rpm.
8. Wash the beads with 16 μL of 1× IB twice.
9. Resuspend the beads in 16 μL of 1× IB.
10. Add 2 μL of the protein of interest.
11. Add 2 μL of radiolabeled DNA (with a minimum of 5000 counts per minute).
12. Gently vortex the sample to ensure that the beads are resuspended.
13. Incubate the samples at 25 °C for 20 min in the Eppendorf shaker at 1000 rpm.
14. Gently wash the beads with bridged protein-DNA complexes with 20 μL of IB.
15. Resuspend the beads in 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 protein (H-NS) [20]. Using this assay, it was demonstrated that the DNA-bridging efficiency of H-NS is highly dependent on protein concentration.

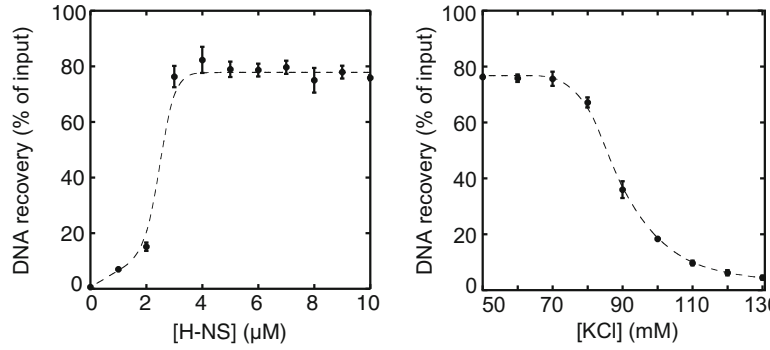

Fig. 3 DNA bridging as a function of H-NS concentration [20]. The experiments were performed in the presence of 10 mM Tris–HCl pH 8.0, 50 mM KCl, 10 mM $MgCl_2$, 5% (w/v) glycerol. Error bars indicate the standard deviation of a triplicate of experiments

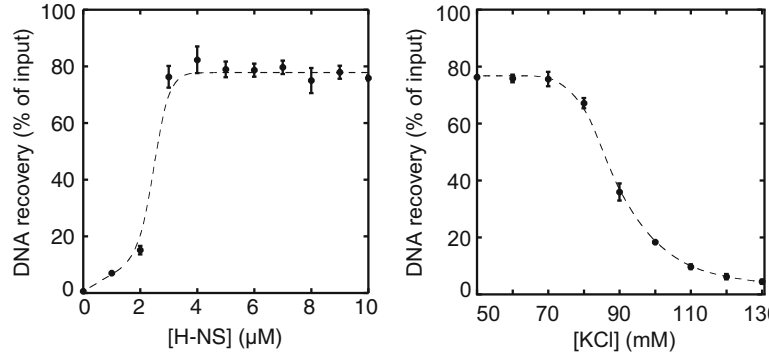

Fig. 4 Modulation of H-NS-DNA bridging by $[K^+]$ [20]. The experiments were performed with 3,3 μM H-NS in 10 mM Tris–HCl pH 8.0, 10 mM $MgCl_2$, 5% w/v glycerol. Error bars indicate the standard deviation of a triplicate of experiments

4.2 DNA Bridging Efficiency of H-NS as a Function of Physico-Chemical Conditions

The DNA-bridging assay allows for facile testing of the effect of altered physico-chemical conditions. It has been shown previously that H-NS-mediated DNA bridging is strongly modulated by environmental factors such as osmotic stress [17, 20]. The DNA-bridging assay revealed that increasing the amount of KCl in the buffer indeed effectively abolishes DNA bridging by H-NS (Fig. 4) [20].

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**).

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. This PCR protocol was optimized for a 685 bp DNA substrate using a Biorad T100 Thermocycler. Some optimization may be required for different thermocyclers or substrates of different lengths.
3. When determining the concentration of the DNA it may be advantageous to use a Nanodrop® as this technique requires a very small volume.
4. Snap-chilling your DNA before labeling the DNA remains single-stranded and increases the efficiency of the Kinase.
5. Recovery of ^{32}P labeled DNA in the absence of DNA-bridging proteins may be an indication of DNA aggregation.

   In these cases, it is best to:

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

   (b) Re-evaluate the experimental buffer as the absence of salt may cause larger DNA substrates to aggregate. Similar effects may occur at extreme pH's.

   (c) Use new beads as the streptavidin coating may decay over time, leading to inconclusive experiments.
6. If ^{32}P Labeled DNA is recovered in the absence of biotin labeled DNA, and not in the situation described in **Note 5**, 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, *see* **Note 5c**.
7. 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.

8. DNA binding of proteins is best confirmed with additional solution-based experiments such as Microscale thermophoresis (*see* Chapter 11) or Tethered particle motion (*see* Chapter 14).

Acknowledgments

This work was supported by grants from the Netherlands Organization for Scientific Research [VICI 016.160.613] and the Human Frontier Science Program (HFSP) [RGP0014/2014].

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