ChIP-qPCR of FLAG-tagged proteins in bacteria

Ge, P.; Rashid, F.Z.M.; Crémazy, F.G.E.; Dame, R.T.

Chapter 4

ChIP-qPCR of FLAG-Tagged Proteins in Bacteria

Pingzhuang Ge, Fatema-Zahra M. Rashid, Frédéric G. E. Crémazy, and Remus T. Dame

Abstract

DNA-protein interactions occur in biological processes such as genome replication, gene transcription, DNA repair, and chromatin compaction and organization. Mapping the distribution of the DNA-bound proteins on the chromosome is essential for understanding their associated biological process. Chromatin immunoprecipitation (ChIP) involves the antibody-mediated enrichment of DNA fragments bound by a target protein and has become one of the most powerful techniques for exploring the distribution of proteins on the chromosome. By incorporating quantitative polymerase chain reaction (qPCR) downstream of the ChIP assay, ChIP-qPCR was developed to describe binding profiles of DNA-associated proteins at a candidate locus. In this chapter, we describe ChIP-qPCR. We provide a step-by-step protocol for the preparation of a ChIP library of a 3× FLAG-tagged protein in bacteria, describe how downstream qPCR experiments can be performed with the appropriate controls, and explain how the data is analyzed. This chapter provides reliable technical guidance for ChIP-qPCR studies in bacteria.

Key words ChIP-qPCR, *Escherichia coli*, Nucleoid-associated proteins, 3× FLAG

1 Introduction

DNA-protein interactions play an important role in chromosome organization, regulating DNA transactions such as replication and transcription, and coordinating the segregation of sister chromosomes into daughter cells. For instance, tetramerization of MatP dimers bound to *matS* sites in the Ter macrodomain of *Escherichia coli* condenses Ter structurally facilitating chromosome segregation and Ter resolution prior to cell division [1, 2]. The nucleation of the ParB protein on *parS* sites and its subsequent multimerization recruits the ParA ATPase to initiate the process of sister chromosome segregation in *Caulobacter crescentus* and *Bacillus subtilis* [3]. The binding of the LacI protein to the *lac* operator sequence of the *lacZYA* operon represses its transcription by occluding RNA polymerase from the promoter [4]. In *E. coli*, the multimerization of histone-like nucleoid structuring protein (H-NS) on AT-rich

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_4,

regions of the chromosome silences genes and horizontally acquired genetic elements by RNA polymerase occlusion and road-blocking elongating RNA polymerase. The repressive effects can be overcome in response to an increase in osmolarity and temperature or a change in pH [5–7]. Hence, mapping the distribution of DNA-associated proteins along the chromosome is essential to understanding genome processes.

Chromatin immunoprecipitation (ChIP)—first reported in 1984 [8]—relies on the specificity of antibody-antigen reactivity to "fish" a target protein and the DNA to which it is bound from sheared chromatin in cell and tissue lysates. The DNA is then purified to prepare a library of genomic regions bound by the target protein (Fig. 1). By incorporating next-generation sequencing (NGS) downstream of ChIP (ChIP-seq), the DNA interaction profile of the target protein can be mapped on a genome-wide level [9]. To investigate local protein binding, quantitative PCR (ChIP-qPCR) is cheaper, customizable, and experimentally less challenging in terms of readout (Fig. 1).

An ideal ChIP assay involves the use of an antibody that reacts with the native target protein with no cross-reactivity. Developing ChIP-grade antibodies with such properties is time-consuming and often unrealistic owing to the presence of homologous proteins. Therefore, to streamline ChIP assays, target proteins are often translationally labeled with standardized artificial epitopes such as the FLAG (DYKDDDDK) tag [10] or Strep-tag II (WSHPQFEK) [11], among others, for which highly specific antibodies are commercially available. However, it must be noted that epitope tagging may interfere with the biochemical properties and, hence, physiological function of the target protein. Thus, additional assays investigating the impact of the epitope tag on the functionality of the target protein are necessary.

In this chapter we describe a protocol for the preparation of a ChIP library and its analysis with qPCR. We use *E. coli* as a representative organism and H-NS tagged with 3× FLAG (DYKDHDG-DYKDHDI-DYKDDDDK) at the C-terminal as the model protein.

2 Materials

2.1 Cell Fixation

1. Bacterial cell culture facility.
2. 37% formaldehyde, e.g., formaldehyde solution for molecular biology, 36.5–38% in H_2O (Sigma-Aldrich).
3. 2.5 M glycine.
4. 1× phosphate-buffered saline (1× PBS): 137 mM NaCl, 2.7 mM KCl, 10 mM Na_2HPO_4, 1.8 mM KH_2PO_4, pH 8.0. Chill on ice before use.

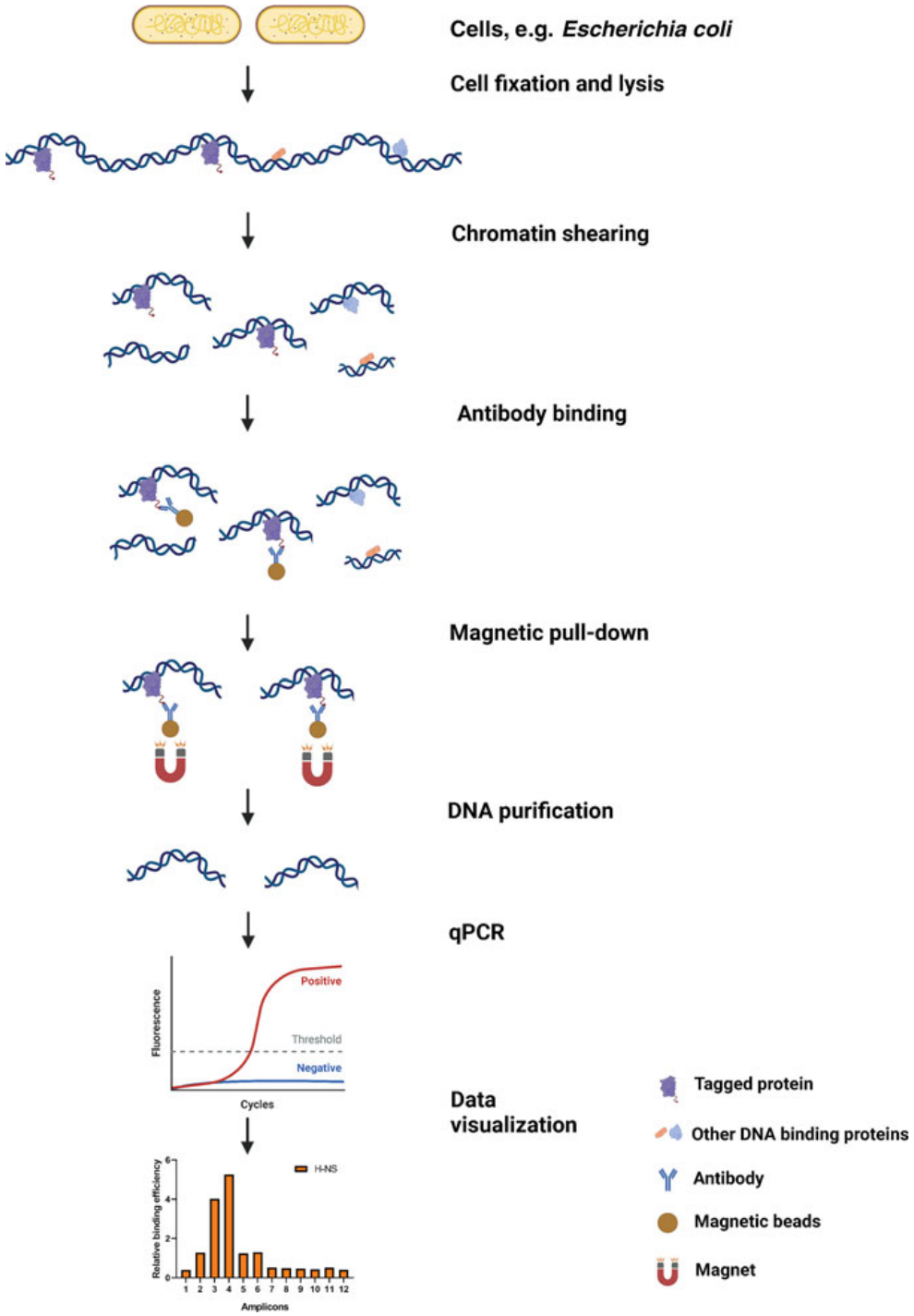

Fig. 1 ChIP-qPCR. DNA-protein interactions of the chromosome are "fixed" in the cell by treatment with formaldehyde. The fixed chromosome is extracted from the cells and fragmented by sonication. Next the target protein is immunoprecipitated. Often, the target protein is translationally tagged with a standardized epitope (e.g., 1× FLAG, 3× FLAG, and Strep-II, among others) that can be recognized by commercially available antibodies. The immunoprecipitated protein and the DNA fragments to which the proteins are bound are extracted from the reaction, and the DNA is purified to prepare the ChIP library. The binding profile of the target protein at a region of interest is investigated by quantifying selected loci within the region using qPCR

5. 50 mL conical tubes.
6. Roller bench.
7. Refrigerated centrifuge with a rotor for 50 mL tubes.
8. Micropipette set.
9. Serological pipettes.
10. Pipette pump.
11. Optional: 1.5 mL centrifuge tubes.
12. Optional: liquid nitrogen.
13. Optional: refrigerated centrifuge with a rotor for 1.5 mL tubes.
14. Optional: −80 °C or −70 °C freezer.

2.2 Chromatin Shearing and Shearing Analysis

1. iDeal ChIP-qPCR kit (Diagenode) (*see* **Note 1**). Lysis buffer iL1b, lysis buffer iL2, 200× protease inhibitor cocktail, and shearing buffer iS1b provided with the kit are required for chromatin shearing. Chromatin shearing analysis requires elution buffers iE1 and iE2.
2. Nuclease-free water (or miliQ water).
3. 25:24:1 phenol/chloroform/isoamy alcohol (*see* **Note 2**).
4. 1 mg/mL RNase A (*see* **Note 3**).
5. 100% and 70% ethanol. Keep cold.
6. 1.0 M NaOAc pH 8.0.
7. 5 mg/mL glycogen.
8. Isopropanol.
9. 1.5 mL centrifuge tubes.
10. Elongated Pasteur pipette. Heat up a ~1 cm region of the narrow section of a glass Pasteur pipette 1–2 cm away from the bore using a Bunsen burner. When the glass softens, pull apart the bore from the rest of the Pasteur pipette to produce a narrower channel in the pipette. Using tweezers, carefully break the pipette in the narrowed region to produce an elongated Pasteur pipette with a smaller bore (*see* **Notes 4** and **5**).
11. Micropipette set.
12. Roller bench.
13. Refrigerated centrifuge with a rotor for 1.5 mL tubes.
14. Bioruptor Plus® sonication device with a 1.5 mL tube holder (Diagenode).
15. Thermal shaker for 1.5 mL tubes, e.g., Eppendorf ThermoMixer® C with Eppendorf SmartBlock 1.5 mL.
16. Vortex mixer.
17. Molecular weight ladder for DNA with a size range between 100 and 1000 bp.

18. Agarose gel electrophoresis setup with gel trays that are at least 8 cm in length.
19. Optional: Bioruptor® Plus TPX microtubes (Diagenode) (*see* **Note 6**).

2.3 Immunoprecipitation

1. iDeal ChIP-qPCR kit (Diagenode) (*see* **Note 1**). ChIP-seq grade water, 5× ChIP Buffer iC1b, 5% BSA, DiaMag protein A-coated magnetic beads, 200× protease inhibitor cocktail, and wash buffers iW1, iW2, iW3, and iW4 provided with the kit are required for immunoprecipitation.
2. ChIP-grade anti-FLAG antibody (Diagenode) (*see* **Notes 7** and **8**).
3. 1.5 mL centrifuge tubes.
4. Micropipette set.
5. Thermal shaker for 1.5 mL tubes, e.g., Eppendorf ThermoMixer® C with Eppendorf SmartBlock 1.5 mL.
6. Magnetic rack for 1.5 mL centrifuge tubes.
7. Roller bench.

2.4 De-crosslinking and DNA Purification

1. iDeal ChIP-qPCR kit (Diagenode) (*see* **Note 1**). Proteinase K and DNA isolation buffer (DIB) provided with the kit are required for de-crosslinking and DNA purification.
2. Low-retention 1.5 mL centrifuge tubes.
3. Micropipette set.
4. Thermal shaker for 1.5 mL tubes, e.g., Eppendorf ThermoMixer® C with Eppendorf SmartBlock 1.5 mL.
5. Magnetic rack for 1.5 mL centrifuge tubes.
6. Fluorophore-based double-stranded DNA (dsDNA) quantitation system, e.g., Qubit® dsDNA HS Assay Kit and a Qubit® fluorometer (ThermoFisher Scientific) or QuantiFluor® dsDNA System and a Quantus™ fluorometer (Promega) (*see* **Note 9**).
7. −20 °C freezer.

2.5 Genomic DNA Preparation

1. Bacterial cell culture facility.
2. TES buffer: 50 mM Tris-HCl pH 7.5, 10 mM NaCl, 10 mM EDTA.
3. 30% Sarkosyl.
4. 1 mg/mL RNase A (*see* **Note 3**).
5. 20 mg/mL Proteinase K.
6. 4.0 M NH_4Oac.
7. 10 mM Tris pH 8.0.

8. 25:24:1 phenol/chloroform/isoamyl alcohol (*see* **Note 2**).
9. Chloroform (*see* **Note 2**).
10. Isopropanol.
11. 70% ethanol.
12. 1.5 mL centrifuge tubes.
13. Micropipette set.
14. Vortex mixer.
15. Benchtop centrifuge with a rotor for 1.5 mL tubes.
16. Elongated Pasteur pipette (*see* **Notes 4** and **5**).
17. Thermal shaker for 1.5 mL tubes, e.g., Eppendorf ThermoMixer® C with Eppendorf SmartBlock 1.5 mL.
18. Fluorophore-based dsDNA quantitation system, e.g., Qubit® dsDNA HS Assay Kit and a Qubit® fluorometer (ThermoFisher Scientific), or QuantiFluor® dsDNA System and a Quantus™ fluorometer (Promega) (*see* **Note 9**).
19. Agarose gel electrophoresis setup with gel trays that are at least 8 cm in length.

2.6 qPCR

1. DNA sequence analysis and primer designing software.
2. Intercalating dye-based quantitative PCR master mix, e.g., Luna® Universal qPCR Master Mix (New England Biolabs) (*see* **Note 10**).
3. 100 μM PCR primers dissolved in 10 mM Tris pH 8.0. Dilute the primers with nuclease-free water to a concentration of 10 μM before use. Primer design is described in Subheading 3.5.
4. Nuclease-free water.
5. Micropipette set.
6. Preferred: multi-channel pipette for 0.5–10 μL.
7. Low-retention micropipette tips.
8. Low-retention 1.5 mL centrifuge tubes.
9. 96-well PCR plates with white wells.
10. Optically transparent PCR plate sealing films.
11. Centrifuge with a rotor for 96-well PCR plates.
12. Real-time PCR detection system and analysis software, e.g., CFX96 Touch Real-Time PCR Detection System and the CFX Maestro software for CFX Real-Time PCR Instruments from Bio-Rad.
13. Spreadsheet software.

3 Method

3.1 Cell Fixation

1. Culture *E. coli* expressing a 3× FLAG-tagged target protein (*see* **Notes 11** and **12**).
2. Transfer a culture volume equivalent to 1×10^9 cells to a 50 mL conical tube at room temperature (*see* **Note 13**).
3. Add 37% formaldehyde at a ratio of 27.8 µL per 1 mL of culture to a final concentration of 1% formaldehyde.
4. Incubate the cells for 10 min at room temperature on a roller bench (*see* **Note 14**).
5. Add 2.5 M glycine to the fixation reaction at a ratio of 257.0 µL per 1 mL of culture to a final concentration of 0.5 M.
6. Incubate the tube for 5 min at room temperature on a roller bench.
7. Collect the cells by centrifugation at 3000 × g for 5 min at 4 °C. Pour off the supernatant.
8. Resuspend the cells in 20 mL of ice-cold 1× PBS.
9. Centrifuge the suspension for 5 min at 3000 × g at 4 °C to collect the cells. Discard the supernatant.
10. Repeat **steps 8** and **9** (Subheading 3.1) one more time.

    This is a pause point. Subheading 3.2 may be started immediately, but to continue the experiment at a later time:
11. Resuspend the cell pellet in 1.0 mL of ice-cold 1× PBS.
12. Transfer the cell suspension into a clean 1.5 mL centrifuge tube.
13. Centrifuge the tube at 10,000 × g for 2 min at 4 °C. Pipette off the supernatant.
14. Flash-freeze the cell pellet in liquid nitrogen and store the tube at −80 °C or −70 °C for up to 6 months.

3.2 Chromatin Shearing

1. Resuspend the cell pellet in 1 mL of ice-cold lysis buffer iL1b (iDeal ChIP-qPCR kit, Diagenode) (*see* **Note 15**).
2. Transfer the cell suspension into a clean 1.5 mL centrifuge tube. This is only necessary if Subheading 3.2 is continued from Subheading 3.1, **step 10**.
3. Incubate the suspension for 20 min on a roller bench at 4 °C.
4. Collect the cells by centrifugation at 3000 × g for 5 min at 4 °C. Remove the supernatant carefully with an elongated Pasteur pipette (*see* **Notes 4** and **5**).
5. Resuspend the cell pellet in 600 µL of ice-cold lysis buffer iL2 (iDeal ChIP-qPCR kit, Diagenode).
6. Incubate the cells for 10 min on a roller bench at 4 °C.

7. Centrifuge the cell suspension at 3000 × g for 5 min at 4 °C and remove the supernatant carefully with an elongated Pasteur pipette. Place the cell pellet on ice.
8. Prepare complete shearing buffer by mixing 1.5 µL of 200× protease inhibitor cocktail (iDeal ChIP-qPCR kit, Diagenode) with 298.5 µL of shearing buffer iS1b (iDeal ChIP-qPCR kit, Diagenode).
9. Resuspend the cell pellet from **step 7** in 300.0 µL of complete shearing buffer and incubate the preparation on ice for 10 min.
10. Optional: Transfer the preparation into a Bioruptor® Plus TPX microtube (Diagenode) (*see* **Note 6**).
11. Briefly spin the tube for 15 s to collect the content of the tube at the bottom.
12. Ensure that the water level in the Bioruptor® Plus sonication device is at the level recommended by the manufacturer and cool the device to 4 °C.
13. Place the tube in the Bioruptor® Plus 1.5 mL tube holder. Multiple ChIP library preparation samples can be sheared simultaneously. Fill all remaining spaces in the holder with tubes containing water. Ensure that the sample volume in all tubes in the holder is identical and that it is between 100 µL and 300 µL (*see* **Note 16**).
14. Shear the chromatin at a high power setting for nine cycles at 30 s "ON," 30 s "OFF." This program shears the chromatin to fragments ranging in length from 100 to 1000 bp, with a majority distributed between 150 and 500 bp (*see* **Note 17**) (Fig. 2).
15. Briefly spin the tube for 15 s.
16. Transfer the sonicated sample to a new 1.5 mL tube and centrifuge the tube at 10,000 × g for 10 min at 4 °C to pellet the cell debris (*see* **Note 18**).
17. Transfer the supernatant containing the sheared chromatin to a new 1.5 mL centrifuge tube.
    - Transfer 5.0 µL of the sonicated sample into a new 1.5 mL centrifuge tube for chromatin shearing analysis described in Subheading 3.3.
    - Keep 1.0–10.0 µL of the sonicated sample aside at 4 °C as the input sample. Continue processing the input sample at Subheading 3.5, **step 2** (*see* **Note 19**).
    - Use the remainder of the sonicated sample for immunoprecipitation (Subheading 3.4). Immunoprecipitation can be performed immediately. However, we recommend storing this sample at 4 °C until the chromatin shearing analysis (Subheading 3.3) has been performed to verify the size distribution of the sheared DNA fragments.

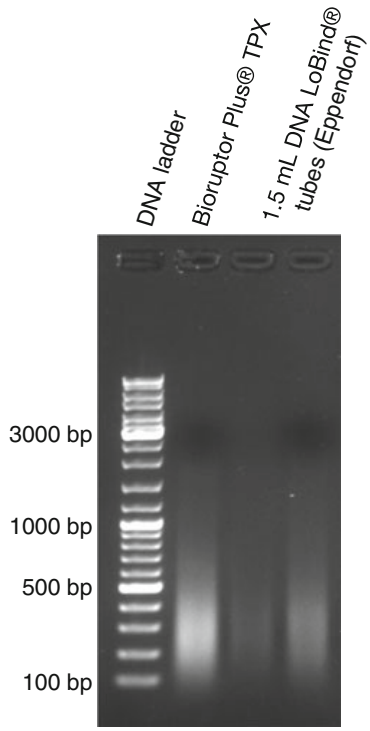

Fig. 2 Chromatin shearing efficiency. Chromatin shearing efficiency is comparable between the Bioruptor Plus® TPX microtubes and the Eppendorf 1.5 mL DNA LoBind® tubes. Nine shearing cycles of 30 s ON and 30 s OFF at a high power setting in a Bioruptor® Plus are sufficient to shear chromatin to a size distribution of 150–500 bp

3.3 Chromatin Shearing Analysis

There are four pause points in this section—before **step 1**, at **step 4**, in option 1 of **step 9**, and after **step 15**. Pause the experiment overnight at only one of the four points. Do not pause the experiment for longer so as to preserve the quality of the sheared chromatin (*see* Subheading 3.2, **step 17**) for immunoprecipitation.

1. Add 1.0 μL of 1 mg/mL RNase A to 5.0 μL of sheared chromatin.
2. Incubate the reaction for 1 h in a Thermomixer set at 37 °C.
3. Add 5.0 μL of elution buffer iE1 and 0.4 μl of elution buffer iE2 to the reaction. Mix thoroughly by pipetting.
4. Incubate the sample in a Thermomixer at 65 °C for 4 h to reverse formaldehyde-mediated crosslinks. To pause the experiment at this step, extend the incubation by up to 12 h.
5. Add 89.0 μL of nuclease-free water to the reaction and mix thoroughly (*see* **Note 20**).
6. Add 100.0 μL of 25:24:1 phenol/chloroform/isoamyl alcohol to the preparation and vortex for 10 s (*see* **Note 2**).

7. Centrifuge the tube at 12,000 × g for 10 min at room temperature (*see* **Note 2**).
8. Transfer the aqueous upper phase to a clean 1.5 mL centrifuge tube. Discard the organic (lower) phase (*see* **Note 2**).
9. ***Option 1***: Add 10.0 µL of 1.0 M NaOAc pH 8.0 and 2.5 µL of 5 mg/mL glycogen to the aqueous phase. Vortex to mix. Next, add 250 µL of cold 100% ethanol and mix by vortexing. Incubate the sample for 10 min at room temperature to precipitate DNA. To pause the experiment at this step, DNA precipitation can be performed overnight at −20 °C.

   Option 2 (***This option does not have a pause point***): Add 70.0 µL of 100% isopropanol to the aqueous phase. Mix by vortexing and incubate the sample for 10 min at room temperature.
10. Centrifuge the tube at 12,000 × g for 15 min at 4 °C. Remove the supernatant with an elongated Pasteur pipette (*see* **Notes 4** and **5**).
11. Add 500 µL of cold 70% ethanol to the pellet to wash. Centrifuge the tube at 12,000 × g for 10 min at 4 °C and remove the supernatant with an elongated Pasteur pipette (*see* **Notes 4** and **5**).
12. Air-dry pellet by placing the tube, opened, on a benchtop for 1–2 h. Alternatively, keep the opened tube in a Thermomixer at 40 °C for 30 min or at 60 °C for 10 min.
13. Dissolve the dried pellet in 10.0 µL of nuclease-free water.
14. Run the sample on a 1% agarose gel (*see* **Note 21**). Sheared DNA should appear as a smear between 100 and 1000 bp with the bulk of the fragments distributed between 150 bp and 500 bp (Fig. 2).

3.4 Immunoprecipitation (IP)

1. Prepare 1× ChIP Buffer iC1b as follows:
   - 499.2 µL of ChIP-seq grade water (iDeal ChIP-qPCR kit, Diagenode)
   - 128.0 µL of 5× ChIP Buffer iC1b (iDeal ChIP-qPCR kit, Diagenode)
   - 12.8 µL of 5% BSA (iDeal ChIP-qPCR kit, Diagenode).
2. Transfer 30.0 µL of DiaMag protein A-coated magnetic beads (iDeal ChIP-qPCR kit, Diagenode) to a clean 1.5 mL centrifuge tube.
3. Add 200.0 µL of 1× ChIP Buffer iC1b to the bead suspension and mix thoroughly by pipetting up and down several times.
4. Incubate the bead suspension in a Thermomixer set at 4 °C for 5 min at 300 rpm.

5. Place the tube on a magnetic rack to capture the beads from suspension.
6. When the suspension is cleared, pipette off the supernatant.
7. Repeat **steps 3–6** of this section two more times.
8. Resuspend the beads in 30.0 µL of 1× ChIP Buffer iC1b and keep the beads at room temperature.
9. Prepare the ChIP reaction mix as follows:
   - 6.0 µL of 5% BSA (iDeal ChIP-qPCR kit, Diagenode)
   - 1.8 µL of 200× protease inhibitor cocktail (iDeal ChIP-qPCR kit, Diagenode)
   - 20.0 µL of 5× iC1b Buffer (iDeal ChIP-qPCR kit, Diagenode)
   - 4.0 µL of ChIP-grade FLAG antibody (Diagenode) (*see* **Note 7**)
   - 38.2 µL of ChIP-seq grade water.
10. Add 70.0 µL of the ChIP reaction mix to 30.0 µL of washed DiaMag protein A-coated magnetic beads (**step 8** of this section) and mix well by pipetting.
11. Incubate the tube for 2–4 h at 4 °C on a roller bench to coat the beads with the anti-FLAG antibody.
12. Add 250.0 µL of sheared chromatin (*see* Subheading 3.2, **step 17**) to the beads and mix thoroughly by pipetting. Incubate the tubes overnight at 4 °C on a roller bench.
13. Collect the magnetic beads—now bound to immunoprecipitated chromatin—by placing the tube on a magnetic rack until the suspension clears. This takes approximately 1 min.
14. Pipette off the supernatant carefully, making sure not to aspirate any beads.
15. Resuspend the beads in 350.0 µL of wash buffer iW1(iDeal ChIP-qPCR kit, Diagenode) and incubate the tube on a roller bench at 4 °C for 5 min.
16. Place the tube on a magnetic rack to collect the beads.
17. Remove the supernatant carefully.
18. Repeat **steps 15–17** of this section with wash Buffers iW2, iW3, and iW4 (iDeal ChIP-qPCR kit, Diagenode).
19. Close the 1.5 mL tube containing the beads (IP sample) to prevent them from drying out. Keep the sample at room temperature and proceed with Subheading 3.5.

3.5 De-crosslinking and DNA Purification

1. Add 2.0 μL of proteinase K (iDeal ChIP-qPCR kit, Diagenode) to 200.0 μL of DNA isolation buffer (DIB, iDeal ChIP-qPCR kit, Diagenode) to prepare complete DIB. Keep the preparation on ice.
2. Add 100.0 μL of complete DIB to the IP and input samples (Subheading 3.4, **step 19** and Subheading 3.2, **step 17**, respectively). Mix thoroughly.
3. Incubate the samples in a Thermomixer at 55 °C for 15 min followed by 100 °C for 15 min.
4. Transfer the input sample to a clean low-retention 1.5 mL centrifuge tube.
5. Place the tube containing the IP sample on a magnetic rack until the solution clears.
6. Transfer the supernatant containing the ChIP library to a clean low-retention 1.5 mL centrifuge tube.
7. Measure the concentration of ChIP library and input sample using a fluorophore-based double-stranded DNA (dsDNA) quantitation system (*see* **Note 9**).
8. Store the ChIP library and input sample at −20 °C until use.

3.6 Genomic DNA Preparation

1. Culture *E. coli* to stationary phase in 2.0 mL of medium. For this step, the culture can be considered to be in stationary phase when two consecutive OD_{600} measurements differ by less than 0.05. The OD_{600} measurements should be taken at least one exponential phase doubling time apart.
2. Collect the cells in 1.0 mL of the culture by centrifugation at 10,000 × g for 2 min at room temperature.
3. Remove the supernatant with an elongated Pasteur pipette.
4. Resuspend the cell pellet in 200.0 μL of TES buffer.
5. Add 7.5 μL of 30% sarkosyl, 2.0 μL of 1 mg/mL RNase A, and 1.0 μL of 20 mg/mL proteinase K to the cell suspension and mix thoroughly. Incubate the preparation at 37 °C for at least 1 h or, preferably, until the solution clears.
6. Add 200.0 μL of 4.0 M NH_4Oac to the lysate. Vortex to mix.
7. Add 400.0 μL of 25:24:1 phenol/chloroform/isoamyl alcohol to the lysate. Vortex for 10 s (*see* **Note 2**).
8. Centrifuge the tube at 12,000 × g for 10 min at room temperature (*see* **Note 2**).
9. Transfer the aqueous upper phase to a clean tube and discard the lower organic phase (*see* **Note 2**).
10. Repeat **steps 7–9** of this section one more time (*see* **Note 2**).
11. Add 400.0 μL of chloroform to the aqueous phase and vortex the preparation for 10 s (*see* **Note 2**).

12. Centrifuge the tube at 12,000 × g for 10 min at room temperature (*see* **Note 2**).
13. Transfer the aqueous upper phase to a clean tube and discard the lower organic phase (*see* **Note 2**).
14. Add 400.0 µL of isopropanol to the aqueous phase to precipitate DNA. Mix thoroughly by vortexing and incubate the sample at room temperature for 10 min.
15. Pellet the precipitated DNA by centrifugation at 14,000 × g for 20 min at room temperature.
16. Remove the supernatant with an elongated Pasteur pipette (*see* **Notes 4** and **5**).
17. Pipette 500.0 µL of 70% ethanol onto the pellet to wash.
18. Centrifuge the tube at 14,000 × g for 5 min at room temperature.
19. Remove the supernatant with an elongated Pasteur pipette (*see* **Notes 4** and **5**).
20. Air-dry the genomic DNA pellet by placing the opened tube on a benchtop for 1–2 h. Alternatively, place the opened tube in a Thermomixer at 40 °C for 30 min or at 60 °C for 10 min.
21. Dissolve the dried pellet in 100.0 µL of 10 mM Tris pH 8.0.
22. Measure the concentration of genomic DNA using a fluorophore-based double-stranded DNA (dsDNA) quantitation system (*see* **Note 9**).
23. Visualize the genomic DNA preparation on a 1% agarose gel. Genomic DNA runs as a heavy band at a position of >10 kb, with gDNA signal also detectable in the wells of the agarose gel. A smear between ~100 bp and ~3000 bp indicates RNA contamination. Should this be observed, add 100.0 µL Tris pH 8.0 and 2.0 µL of 1 mg/mL RNase to the preparation. Incubate the sample at 37 °C for 1 h and follow the protocol from **step 7** of this section.

3.7 qPCR: Experimental Design

1. ***Recommended***: Identify the test, positive control, negative control, and internal control loci.
   - The test loci are the regions of the chromosome where the binding profile of the FLAG-tagged protein of interest is to be investigated.
   - The positive control locus is a region where the FLAG-tagged protein is known to be bound under the experimental conditions, for instance, at the *bglG* locus for FLAG-tagged H-NS [12].
   - The negative control locus is a site where the FLAG-tagged protein does not bind under the experimental conditions, for instance, *wzzE* and *appB* for FLAG-tagged H-NS [12].

- The internal control locus is a region at which the FLAG-tagged protein has the same binding profile under all experimental conditions. It is used as a normalization factor to compare different ChIP libraries. The internal control locus can be the positive control locus.

2. Design primer pairs to amplify ~100 bp amplicons at each region of interest. Design each primer with a melting temperature (Tm) of ~60 °C. Verify that the predicted Tm of any secondary structures that the primers can form is less than 45 °C and that the negative Gibbs free energy ($-\Delta G$) of any predicted primer homodimers and heterodimers is less than 15% of the $-\Delta G$ of complete hybridization (*see* **Note 22**).
3. Prepare the following templates for ChIP-qPCR:
   - ChIP library at a concentration of at least 0.1 ng/μL.
   - Input sample diluted to a concentration comparable to that of its corresponding ChIP library in a low-retention 1.5 mL centrifuge tube.
   - *E. coli* genomic DNA (*see* **Note 23**) diluted to 0.1×, 0.01×, 0.001×, and 0.0001× concentrations in low-retention 1.5 mL centrifuge tubes. A single dilution series is sufficient; however, we recommend in three replicates.
4. Set up the following samples in a 96-well plate (1.0 μL/sample):
   - At least three biological replicates of a ChIP library. Prepare three technical replicates for each biological replicate (count: nine samples) (*see* **Note 24**).
   - Input samples corresponding to every ChIP library tested above. Prepare three technical replicates for each input sample (count: three samples/ChIP library).
   - *E. coli* genomic DNA dilution series (count: 4–12 samples).
   - No template controls (count: three samples).
5. Thaw the reagents provided with the Luna® Universal qPCR master mix and place them on ice. Prepare the qPCR master mix for *n* samples (*see* **step 4** of this section) as below:
   - *n* * 5.5 μL of 2× Luna Universal qPCR Master Mix
   - *n* * 0.275 μL of 10 μM forward primer
   - *n* * 0.275 μL of 10 μM reverse primer
   - *n* * 3.85 μL of nuclease-free water
6. Transfer 9.0 μL of the qPCR master mix to 1.0 μL of sample in the 96-well plate (*see* **step 4**).
7. Seal the plate with an optically transparent PCR plate sealing film.

Table 1
qPCR thermal cycling parameters

Step	Temperature	Duration (mm:ss)	Cycles
Initial denaturation	95 °C	01:00	1
Denaturation	95 °C	00:15	45
Annealing/extension	60 °C	00:30	
Plate read (SYBR/FAM channel)	N/A	N/A	
Melt	60 °C	00:31	1
Ramp	60 °C	0:01	70
	+0.5 °C/cycle	+0:01/0.5 °C	
Plate read (SYBR/FAM channel)	N/A	N/A	

As per the manufacturer's instructions provided with the Luna® Universal qPCR Master Mix (New England Biolabs)

8. Briefly centrifuge the plate to remove bubbles and to collect the reaction at the bottom of the wells.
9. Run the qPCR on the CFX96 Touch Real-Time PCR Detection System using the program provided in Table 1.
10. Perform qPCRs for the test, positive, negative, and internal control loci.

3.8 qPCR: Data Analysis

A defined and validated internal control locus (*see* Subheading 3.7, **step 1**) is required to analyze qPCR data using the protocol described in this section. If such a control is unavailable, use the alternative data analysis method described in **Note 25** (*see* **Note 25**).

1. Export the qPCR data from the CFX96 Touch Real-Time PCR Detection System. The data file is in its compressed *.pcrd format. Decompress and open the file with the CFX Maestro software for CFX Real-Time PCR Instruments from Bio-Rad (available for both WindowsOS and macOS).
2. Set up a 96-well plate under the View/Edit menu.
   - Set the "Sample type" of wells that contain ChIP libraries and input samples as "Unknown" and fill in the "Target name" and "Sample name" for each well. The "Target name" is the locus being examined as defined by the primer pair used in the experiment, and the "Sample name" is the name of the ChIP library or input sample.
   - Set the "Sample type" of wells containing the *E. coli* genomic DNA dilutions as "Standard," and load the relative concentrations (0.1, 0.01, etc.) for each well in the input box marked "Load."

- Set the "Sample type" of the no template control reactions to "NTC" and include the "Target name" for these samples.

3. Verify the linear fit of the standard curve from the plot of the log of the input concentration against the quantification cycle (Cq) on the "Quantification" tab of the CFX Maestro software (Bio-Rad). The graph must have a negative slope, its correlation coefficient (R^2) should be ≥0.98, and the amplification efficiency (A.E.) of the reaction should lie between 1.8 and 2.2. Check that the range of the standard curve encompasses the Cq values of the ChIP libraries and input samples. If these criteria are not met, the standard curve cannot be used for quantification.
4. Verify the specificity of amplification in each well using the melting curve profiles on the "Melt Curve" tab of the CFX Maestro software (Bio-Rad). Each reaction should have a single melting peak and the melting temperature of identical amplicons should not differ by >0.5 °C. Verify the identity of the amplicons with Sanger sequencing of the product amplified in least two wells.
5. Verify that no amplification is detected in the no template control reactions.
6. Export the qPCR data files into a *.xlsx format using Export > Export All Data Sheets > Excel 2007 (*.xlsx) (*see* **Note 26**).
7. The "Quantification Cq Results" datasheet lists the starting quantity (SQ) of each "Unknown" sample (Column K) calculated from its Cq value and the standard curve. Calculate the average starting quantity (SQ_{avg}) of every ChIP library and input sample from the SQ values by taking a geometric average of its technical replicates. Calculate the error in the measurement of SQ_{avg} (σ_{SQavg}) by calculating the standard deviation of the technical replicates.
8. Assign the recovery of internal control locus by immunoprecipitation to be an arbitrary value of 1. Calculate the normalization factor, *F*, for each ChIP library using the SQ values of internal control locus using the formula below. If a validated internal control is unavailable, use the data analysis method described in **Note 25** (*see* **Notes 19** and **25**).

$$F = \left(\mathrm{SQ_{avg}\ input\ sample}\right) / \left(\mathrm{SQ_{avg}\ ChIP\ library}\right)$$

9. Calculate the relative binding efficiency (*RBE*) of the FLAG-tagged protein for every test locus as follows:

$$\mathrm{RBE} = \left(F^{*}\ \mathrm{SQ_{avg}\ ChIP\ library}\right) / \left(\mathrm{SQ_{avg}\ input\ sample}\right)$$

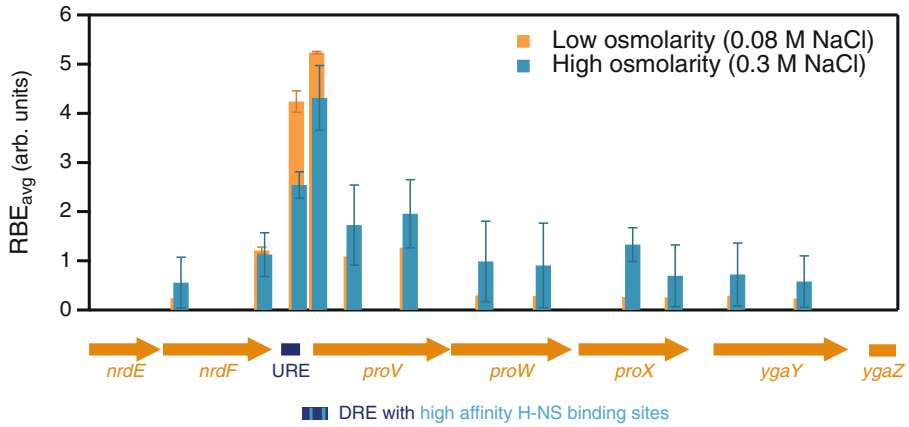

Fig. 3 Data representation. ChIP-qPCR data is represented as a graph of relative binding efficiency (*RBE*) or average relative binding efficiency (RBE_{avg}) against the position of the target locus on the chromosome. The graph presented here shows the distribution of H-NS-3XFLAG at the H-NS-regulated, osmoresponsive *proVWX* operon of *E. coli* and its flanking regions during exponential growth in a low osmolarity medium (0.08 M NaCl, orange bar graph) and in a high osmolarity medium (0.3 M NaCl, blue bar graph). The *bglG* gene was used as a positive control and as an internal control. The annotation below the graph represents the organization of the *proVWX* operon and its flanking genes as orange arrows, the upstream and downstream regulatory elements (URE and DRE) of *proVWX* through which H-NS exerts its regulatory effect on the operon as navy blue bars, and the two high-affinity H-NS binding sites of the DRE as light blue lines

10. Calculate the average RBE (RBE_{avg}) for every test locus by calculating the geometric average of the RBEs calculated for biological replicates. Calculate the standard deviation in RBE_{avg} (σ_{RBEavg}) as a measure of error.
11. Calculate the RBE and RBE_{avg} values for the positive and negative control loci as described in **steps 7–13** of this section. The positive control locus should have positive RBE and RBE_{avg} values. The negative control locus should not be detected in the ChIP library or its RBE and RBE_{avg} values should be low.
12. Visualize the relative binding profile of the FLAG-tagged protein at target loci by plotting a graph of RBE or RBE_{avg} on the *y*-axis versus the position of the locus on the chromosome on the *x*-axis (Fig. 3).

4 Notes

1. The manufacturer's protocol for the use of the iDeal ChIP-qPCR kit (Diagenode) is described for eukaryotic cells. Here, we describe an adapted protocol for bacteria. ChIP-qPCR can also be performed with reagents and kits provided by other suppliers with optimization.
2. 25:24:1 phenol/chloroform/isoamy alcohol and chloroform are toxic. These reagents can cause severe irritation or burns

upon contact with skin and eyes and if inhaled. Prolonged or repeated exposure to the reagents can cause organ damage. Use the reagents in a fume hood with appropriate protective equipment including a lab coat, gloves, and goggles.

3. RNase A is commercially available as 10–20 mg/mL stock solutions. Dilute the stock solution with nuclease-free water or as recommended by the supplier to a final concentration of 1 mg/mL.
4. An elongated Pasteur pipette is used to aspirate the supernatant fraction after centrifugation. It has a narrower bore than micropipette tips that reduces the likelihood of aspirating the pellet into the waste while removing the supernatant. It is especially useful when very small pellets are expected.
5. If glass Pasteur pipettes are unavailable or if elongated pipettes are difficult to prepare, a syringe may be used.
6. Bioruptor Plus® TPX microtubes are recommended by the manufacturer for use with the Bioruptor plus® sonication device. The harder polymethylpentene plastic improves the transmission of ultrasound to the sample. For our experimental setup, 1.5 mL DNA LoBind® tubes (Eppendorf) performed comparably (Fig. 2).
7. The antibody selected for chromatin immunoprecipitation should be compatible with the tag on the target protein. For instance, anti-FLAG antibodies are suitable for FLAG-tagged target proteins and anti-Strep antibodies for StrepII-tagged targets.
8. The immunoprecipitation protocol described in this chapter (Subheading 3.4) involves, first, coating DiaMag protein A-coated magnetic beads with an anti-FLAG antibody and, next, using the beads to immunoprecipitate and enrich the FLAG-tagged protein. Anti-FLAG-coated magnetic beads are also commercially available (e.g., anti-FLAG® M2 Magnetic Beads from Sigma-Aldrich) and can be directly used for immunoprecipitation.
9. Fluorophore-based dsDNA quantitation systems rely on the fluorescent signal produced when a dsDNA-specific intercalating dye inserts into the molecule. Such systems perform better than approaches that rely on UV absorption at 260 nm. UV absorption measurements do not exclude the signal from macromolecules such as proteins and glycogen that may be present in an impure dsDNA solution.
10. Using homemade qPCR master mixes with intercalating dyes is not recommended. Commonly used dyes such as SYBR impair polymerase activity, and their fluorescent signal is heavily

influenced by minor differences in the reaction buffer. As a result, the quality of homemade qPCR master mixes is inconsistent.

11. As a cross-reactivity control, a strain of an identical genetic background expressing the wild-type variant of the target protein should be processed in parallel. This control establishes whether the antibody used for the ChIP experiment specifically immunoprecipitates the FLAG-tagged target protein (Subheading 3.4). It verifies the validity of the qPCR signal (Subheadings 3.7 and 3.8).
12. The FLAG-tagged protein may either be expressed from a plasmid in a strain where the chromosomal gene has been knocked out, or a FLAG tag may be transcriptionally fused to the chromosomal gene and expressed from the endogenous locus. A λ-red recombination technique to prepare *E. coli* knock-outs and knock-ins is described in refs. 13, 14. Also *see* Chapter 11.
13. An OD_{600} of 1 corresponds to approximately 8 * 10^8 *E. coli* cells per mL.
14. Cell fixation with 1% formaldehyde at room temperature for 10 min is optimized for ChIP assays in *E. coli*. Tweak the concentration of formaldehyde or the temperature and duration of treatment to process other cell types with this protocol.
15. Some bacteria, for example, *Mycobacteroides abscessus*, are resistant to chemical lysis. In this case, cells may be broken using a bead beater.
16. Up to 25.0 µg of DNA can be sheared per sample.
17. In our experimental setup, nine cycles of 30 s ON and 30 s OFF are sufficient to shear chromatin to a size distribution of 150–500 bp. Sonication is a fickle technique. Hence, this step will require optimization for every new setup.
18. Sonication is damaging to sample tubes and they may break while pelleting the cell debris. To prevent sample loss, transfer the sonicated material to a new 1.5 mL centrifuge tube.
19. To use a "classical" alternative to the qPCR data analysis protocol provided in Subheading 3.8, calculate the input sample as a percentage of the total sheared chromatin. For instance, for a sheared sample volume of 300.0 µL, an input sample of 3.0 µL corresponds to 1% input. Using the alternative data analysis protocol (*see* **Note 25**) is suitable when the positive, negative, and internal control loci (Subheading 3.7) of the FLAG-tagged protein are unknown.
20. This step increases the sample volume for treatment with 25:24:1 phenol/chloroform/isoamyl alcohol and chloroform. This reduces sample loss during the extraction steps.

21. Some gel loading dyes mask the fluorescent signal from nucleic acid stains during UV exposure. Use loading dye solutions that do not produce a shadow, or load the DNA samples using 10% glycerol without a dye.
22. The melting temperature (Tm) of the primers and the negative Gibbs free energy ($-\Delta G$) of primer hybridization can be calculated using the OligoAnalyzer™ Tool supported by Integrated DNA Technologies (https://www.idtdna.com/pages/tools/oligoanalyzer) or Primer3 (https://primer3.org).
23. The concentration of the genomic DNA template should be between 100 and 1000 ng/μL.
24. ChIP libraries prepared for multiple experimental conditions can be tested in parallel.
25. To analyze the qPCR data using a "classical" protocol, adjust the cycle of quantitation (Cq) value corresponding to 1% input sample ($Cq_{1\%}$) to 100% ($Cq_{100\%}$) as follows:

$$Cq_{100\%} = Cq_{1\%} - \log_2(100)$$

Next, calculate the percentage enrichment (%Enr) of the target site as follows:

$$\%Enr = A.E.^{(Cq100\% - CqChIP)}$$

26. Data can also be exported as a *.csv file. This format is accessible to all spreadsheet software.

Acknowledgments

This work was supported by the Netherlands Organization for Scientific Research [OCENW.GROOT.2019.012 to RTD] and China Scholarship Council grant no. 202103250002 (PG).

References

1. Mercier R, Petit MA, Schbath S et al (2008) The MatP/matS site-specific system organizes the terminus region of the E. coli chromosome into a macrodomain. Cell 135(3):475–485. https://doi.org/10.1016/j.cell.2008.08.031
2. Dupaigne P, Tonthat NK, Espéli O et al (2012) Molecular basis for a protein-mediated DNA-bridging mechanism that functions in condensation of the E. coli chromosome. Mol Cell 48(4):560–571. https://doi.org/10.1016/j.molcel.2012.09.009
3. Jalal ASB, Le TBK (2020) Bacterial chromosome segregation by the ParABS system. Open Biol 10(6):200097. https://doi.org/10.1098/rsob.200097
4. Gilbert W, Müller-Hill B (1967) The lac operator is DNA. Proc Natl Acad Sci USA 58(6):2415–2421. https://doi.org/10.1073/pnas.58.6.2415
5. Wade JT, Struhl K, Busby SJ et al (2007) Genomic analysis of protein-DNA interactions in bacteria: insights into transcription and chromosome organization. Mol Microbiol 65(1):21–26. https://doi.org/10.1111/j.1365-2958.2007.05781.x

6. Dame RT, Rashid FM, Grainger DC (2020) Chromosome organization in bacteria: mechanistic insights into genome structure and function. Nat Rev Genet 21(4):227–242. https://doi.org/10.1038/s41576-019-0185-4
7. van der Valk RA, Vreede J, Qin L et al (2017) Mechanism of environmentally driven conformational changes that modulate H-NS DNA-bridging activity. elife 6. https://doi.org/10.7554/eLife.27369
8. Gilmour DS, Lis JT (1984) Detecting protein-DNA interactions in vivo: distribution of RNA polymerase on specific bacterial genes. Proc Natl Acad Sci USA 81(14):4275–4279. https://doi.org/10.1073/pnas.81.14.4275
9. Park PJ (2009) ChIP-seq: advantages and challenges of a maturing technology. Nat Rev Genet 10(10):669–680. https://doi.org/10.1038/nrg2641
10. Hopp TP, Prickett KS, Price VL et al (1988) A short polypeptide marker sequence useful for recombinant protein identification and purification. Nat Biotechnol 6(10):1204–1210. https://doi.org/10.1038/nbt1088-1204
11. Schmidt TGM, Skerra A (2007) The Strep-tag system for one-step purification and high-affinity detection or capturing of proteins. Nat Protoc 2(6):1528–1535. https://doi.org/10.1038/nprot.2007.209
12. Uyar E, Kurokawa K, Yoshimura M et al (2009) Differential binding profiles of StpA in wild-type and h-ns mutant cells: a comparative analysis of cooperative partners by chromatin immunoprecipitation-microarray analysis. J Bacteriol 191(7):2388–2391. https://doi.org/10.1128/jb.01594-08
13. Datsenko KA, Wanner BL (2000) One-step inactivation of chromosomal genes in *Escherichia coli* K-12 using PCR products. Proc Natl Acad Sci USA 97(12):6640–6645. https://doi.org/10.1073/pnas.120163297
14. Kolmsee T, Hengge R (2011) Rare codons play a positive role in the expression of the stationary phase sigma factor RpoS (σ(S)) in Escherichia coli. RNA Biol 8(5):913–921. https://doi.org/10.4161/rna.8.5.16265