Chapter 18

Spike-In Normalization of ChIP Data Using DNA–DIG–Antibody Complex

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Abstract

Chromatin immunoprecipitation (ChIP) is a widely used method to determine the occupancy of specific proteins within the genome, helping to unravel the function and activity of specific genomic regions. In ChIP experiments, normalization of the obtained data by a suitable internal reference is crucial. However, particularly when comparing differently treated samples, such a reference is difficult to identify. Here, a simple method to improve the accuracy and reliability of ChIP experiments by the help of an external reference is described. An artificial molecule, composed of a well-defined digoxigenin (DIG) labeled DNA fragment in complex with an anti-DIG antibody, is synthesized and added to each chromatin sample before immunoprecipitation. During the ChIP procedure, the DNA–DIG–antibody complex undergoes the same treatments as the chromatin and is therefore purified and quantified together with the chromatin of interest. This external reference compensates for variability during the ChIP routine and improves the similarity between replicates, thereby emphasizing the biological differences between samples.

Key words Chromatin immunoprecipitation (ChIP), Spike-in, Digoxigenin, DIG-11-dUTP, DNA–-DIG–antibody complex, Normalization, External reference

1 Introduction

Chromatin immunoprecipitation (ChIP) is a widely used method in molecular cell biology to identify the occupancy of DNA-bound proteins (such as histones, polymerases, and transcription factors) within the genome thereby pointing to the state and the function of DNA regions [1]. Next to identifying protein–DNA interaction directly, this method is also valuable to determine indirect interactions of proteins with the genome, whereby this indirect interaction can be mediated by other proteins or RNA (e.g., transcriptional co-activators, histone deacetylases or splicing factors, heterogeneous nuclear ribonucleoproteins (hnRNPs)) [2, 3]. The analysis of DNA sequences bound by the protein of interest is achieved by high-throughput sequencing for the whole genome (ChIP-seq) or by

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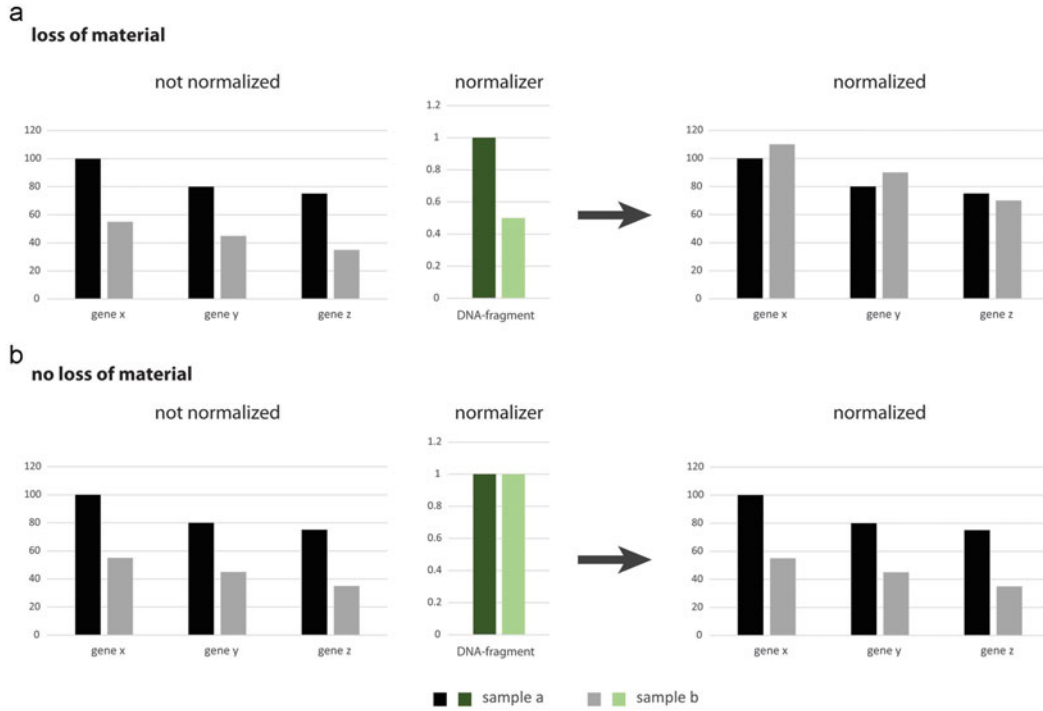

Fig. 1 Analysis of ChIP experiments to illustrate the importance of normalization. Here, a devised ChIP experiment with two independent samples (sample a and b) is shown. (**a**) Three different DNA regions (gene x, y, and z) are quantified in the immunoprecipitated samples by qPCR for both samples and shown relative to the input sample (*left panel*). The DNA–DIG–antibody complex was added as external reference to the chromatin extracts before immunoprecipitation and co-purified with the DNA of interest. The DNA fragment is analyzed by qPCR in sample a and b (in *green*, *middle panel*). In this scenario the DNA fragment is reduced half in sample b compared to sample a, indicating sample loss during chromatin isolation or DNA purification in sample b. When normalizing the data with the help of the external reference, no difference between sample a and b for gene x, y, and z can be determined (*right panel*). In (**b**) same experiment as in (**a**) is shown, but in this scenario no material was lost during the ChIP experiment. Therefore, the levels of the DNA fragment measured by qPCR are the same in sample a and b (*middle panel*). Using this reference for normalization, a clear difference between sample a and b is detected for gene x, y, and z

real-time PCR for specific regions of the genome (quantitative PCR, qPCR).

The ChIP method includes several critical aspects such as cross-linking of cells [4], specificity/suitability of antibodies [5, 6], and normalization issues that have to be addressed. Normalization is crucial to reduce experimentally induced variation between samples and thereby increases the reliability of ChIP data (Fig. 1). The use of an internal reference, in other words adjusting the data to an endogenous DNA region that is expected to be identical in all samples, is the favored normalization strategy, as this "normalizer" is already present in the sample from the beginning and thus treated exactly the same way as the chromatin of interest. In many experiments, however, the occupancy of a specific protein within the

genome is compared in diverse cell types or different conditions (e.g., depletion/overexpression of proteins, treatments with drugs). To compensate for variations between these different samples, the use of an internal reference is often precluded since a DNA sequence that is not affected by the experimental routine cannot be determined and/or global effects are expected. Here, an external reference that does not interfere with the ChIP experiment helps to assess biological differences between samples by improving the statistical resolution of the data. The ideal external normalizer should resemble the biological samples in such a way that it can be purified and quantified by the same methods as the chromatin. An interesting approach is to spike the chromatin with a low amount of chromatin from a different species before immunoprecipitation and use the foreign chromatin as reference [7]. This so-called spike adjustment procedure is however restricted to proteins of interest that share conserved epitopes in both species and are therefore recognized by the same antibody.

Here, the protocol for the synthesis and application of an artificial molecule that can be used as external reference in ChIP experiments is described [8]. For this purpose, a DNA sequence of bacterial origin lacking homologies in eukaryotes is labeled with digoxigenin (DIG) and incubated with a stoichiometric amount of an anti-DIG antibody. The formed complex, composed of the antibody and the DIG-labeled DNA, is crosslinked with formaldehyde and purified. At the beginning of the ChIP experiment, a fixed amount of the purified DNA–DIG–antibody complex is added to the harvested and sheared chromatin before the incubation with the antibody against the protein of interest. The addition of this artificial reference does not disturb the subsequent ChIP procedure, i.e., isolation of chromatin bound to antibodies with the help of protein A and/or G coupled to magnetic or sepharose beads, digestion of proteins and RNA, reversal of the crosslinking, and purification of the DNA (Fig. 2). Finally, all purified DNA sequences, including the DNA fragment of the spiked DNA–DIG–antibody complex, are quantified in both the starting material (input) and in the immunoprecipitated sample by qPCR or sequencing. Importantly, the DNA–DIG–antibody complex serves as normalizer to compensate for variations in different samples introduced during chromatin isolation, reversal of the crosslinking or DNA purification (Fig. 1). The normalization of the ChIP signals thus minimizes variability of technical origin between the individual samples and increases robustness and accuracy of the data. It is noteworthy however that variation referring to the quality of the chromatin cannot be corrected by this method.

The DNA–DIG external reference is universal and can be applied in any ChIP experiment containing chromatin from animal cells. The chosen DNA sequence is a part of the quinol *bo3* oxidase of *Escherichia coli* (*E. coli*) that is absent in eukaryotic genomes. The

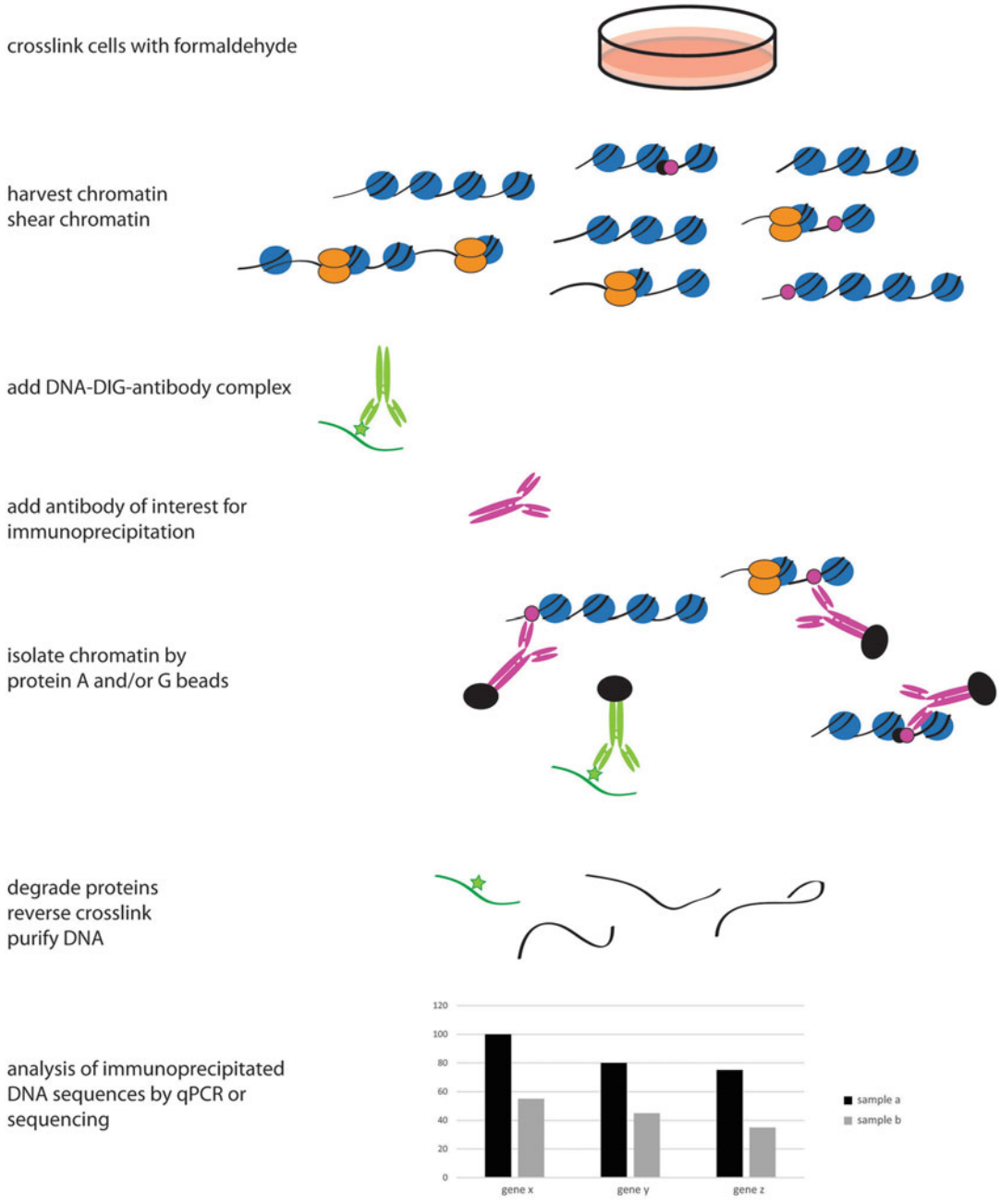

Fig. 2 Schematic overview of the use of an external reference in a ChIP experiment. A typical ChIP experiment starts by treating living cells with formaldehyde to crosslink proteins to DNA. In the next step, cells are harvested, the chromatin extracted and sheared by sonication or enzymatic digestion to obtain DNA fragments of about 500 bp. A constant amount of the DNA–DIG–antibody complex (in *green*) is then added as an external normalizer to each chromatin extract before immunoprecipitation with the antibody of interest (in *purple*). The DNA–DIG–antibody complex is co-isolated with the chromatin bound by the antibody of interest by means of magnetic or sepharose beads covered with protein A and/or G (*black circle*). In the end, proteins and RNA are degraded, the crosslinking reversed and the DNA is purified. The purified DNA sequences are finally quantified by qPCR or sequencing

designed primers for the qPCR do not amplify any sequences in chromatin extracts of HeLa and Drosophila S2 cells. DIG, which is used as an antigen conjugated to DNA, is exclusively found in the plant of the genus digitalis and the antibody does not cross react with any cellular protein in animal cells.

2 Materials

2.1 Production of the DNA Fragment

1. Distilled water.
2. dNTPs stock solution (10 mM each).
3. Taq DNA polymerase (e.g., from Thermo Fisher Scientific).
4. 10× buffer for Taq DNA polymerase.
5. 25 mM $MgCl_2$ (if not included in 10× buffer).
6. DNA template for PCR reaction: e.g., pETcyo plasmid [9].
7. Oligonucleotides for amplification of DNA fragment: Forward oligo: 5′-gtgcgcgaacgtactgatta-3′ and reverse oligo: 5′-agatagcgatccagggtcaa-3′. There is no special requirement for purification of the oligonucleotides.
8. Tubes for PCR.
9. PCR machine.
10. Equipment and reagents for agarose gel electrophorese: agarose, TAE buffer (40 mM Tris, 20 mM acetic acid, 1 mM EDTA), ethidium bromide, loading buffer, molecular-weight size marker.
11. DNA purification kit, e.g., illustra GFX PCR DNA and Gel Band Purification Kit (GE Healthcare Life Sciences).
12. UV spectrophotometer/NanoDrop instrument.

2.2 Digoxigenin Labeling and Production of DNA–DIG–Antibody Complex

1. 100 mM dATP, 100 mM dCTP, 100 mM dGTP, 100 mM dTTP.
2. Digoxigenin-11-2′-deoxy-uridine-5′ triphosphate (DIG-11-dUTP), alkali-stable (Roche).
3. Anti-digoxigenin (DIG) antibody, e.g., mouse monoclonal anti-DIG antibody (Abcam).
4. PBS buffer: 137 mM NaCl, 2.7 mM KCl, 10 mM Na_2HPO_4, 2 mM KH_2PO_4.
5. Rotating wheel.
6. 37% formaldehyde.
7. 1 M glycine (pH not adjusted).
8. Protein concentrator cut off 100 kDa, e.g., Nanosep 100K omega centrifugal devices (Pall Gelman Laboratory).

2.3 DNA–DIG–Antibody Complex in a ChIP-qPCR Experiment

1. Reagents and equipment for ChIP experiment.
2. Reagents and equipment for qPCR: qPCR machine, qPCR tubes, 2× master mix for qPCR, e.g., KAPA SYBR Fast qPCR Master Mix (KAPA Biosystems).
3. Oligonucleotides for qPCR: NP-F: 5′-tattgcttccttcccaattctg-3′ and NP-R: 5′-gtcaacaacgcgacggtaa-3′. There is no special requirement for purification of the oligonucleotides.

3 Methods

3.1 Pre-experiments to Get a Suitable DNA Fragment

Choose a DNA sequence that is not present in the genome of the organism of interest and thus does not interfere in the ChIP experiment. Here, a part of the *bo3* oxidase operon from *E. coli* which is absent in the genome of eukaryotes is used [9]. For choosing alternative DNA sequence *see* **Notes 1** and **2**.

In a first step, the DNA sequence is amplified and purified. To check whether the chosen DNA sequence is suitable as external reference in ChIP experiments, the amplified product is detected with nested primers in a qPCR run.

1. Pipette the following reagents in a PCR tube (total volume is 100 µl; the sequences of the primers are listed in Subheading 2.1 above):

H_2O	76 µl
10× buffer (for Taq DNA polymerase)	10 µl
$MgCl_2$ (25 mM)	6 µl
dNTP (10 mM each)	2.5 µl
pETcyo plasmid (10 ng/µl)	1 µl (*see* **Note 3**)
forward oligo (20 µM)	2 µl
reverse oligo (20 µM)	2 µl
Taq DNA polymerase (5 U/µl)	0.5 µl

2. Run a PCR reaction according to the following scheme:

   1× (95 °C—5 min); 35× (95 °C—30 s, 55 °C—30 s, 72 °C—30 s); 1× (72 °C—7 min).
3. Analyze 5 µl of the PCR reaction on an 1.5% agarose gel (*see* **Note 4**).
4. Purify the PCR product by a PCR purification kit.
5. Run a real-time PCR (quantitative PCR, qPCR) with the purified PCR product as template and nested primers for the DNA fragment (NP-F and NP-R). A typical reaction in a qPCR experiment consists of 10 µl 2× master mix (containing a suitable buffer, $MgCl_2$, SYBR green, dNTPs, and Taq DNA

polymerase, e.g., KAPA SYBR Fast qPCR Master Mix), 3 µl H_2O, 2 µl DNA template, and 5 µl forward and reverse oligonucleotides (NP-F and NP-R, 4.5 µM each, oligonucleotide sequences are listed in Subheading 2.3 above). Use different dilutions of the PCR fragment to monitor the reaction efficiency of the qPCR and analyze the melting curves to confirm the suitability of the nested primers. Include in the same run H_2O instead of DNA as a non-template control. In addition, take genomic DNA of the organism of interest instead of the PCR product as template to ensure that the nested primers do not amplify any sequence of the chromatin in the ChIP experiments (no signal above background should be detected, *see* **Note 5**).

3.2 Labeling of DNA with Digoxigenin by PCR

The chosen and tested (*see* Subheading 3.1) DNA sequence is labeled by PCR using the commercially available DIG-labeled nucleotide digoxigenin-11-2′-deoxy-uridine-5′ triphosphate (DIG-11-dUTP) which is incorporated into newly synthesized DNA instead of dTTPs (*see* **Note 6**).

1. Mix 5 µl dATP (100 mM), 5 µl dCTP (100 mM), 5 µl dGTP (100 mM), 4.5 µl dTTP (100 mM), and 30.5 µl H_2O to prepare a stock of dNTP (final concentration: 10 mM dATP, 10 mM dCTP, 10 mM dGTP, and 9 mM dTTP).
2. Set up a PCR reaction with spiked DIG-11-dUTPs which is integrated into the PCR product (total volume 100 µl; the sequences of the primers are listed above in Subheading 2.1):

H_2O	75 µl
10× buffer (for Taq DNA polymerase)	10 µl
$MgCl_2$ (25 mM)	6 µl
dNTP mix from **step 1**	2 µl
DIG-11-dUTP (1 mM)	1 µl
pETcyo plasmid (10 ng/µl)	1 µl
forward oligo (20 µM)	2 µl
reverse oligo (20 µM)	2 µl
Taq DNA polymerase (5 U/µl)	1 µl

3. Run a PCR reaction according to the following scheme:

   1× (95 °C—5 min); 35× (95 °C—30 s, 55 °C—30 s, 72 °C—30 s); 1× (72 °C—7 min).
4. Purify the PCR reaction by a PCR purification kit to remove nucleotides including DIG-11-dUTPs (*see* **Note 7**).
5. Measure the concentration of the purified PCR product by UV spectrophotometry, e.g., NanoDrop instrument.

3.3 Production of DNA–DIG–Antibody Complex

1. The amount of anti-DIG antibody needed for preparing the DNA–DIG–antibody complex is calculated so that each DIG molecule of the DNA fragment is bound by an antibody, assuming that every IgG antibody recognizes two DIG molecules. Take the formula below to calculate the amount of antibody that is used for the DNA–DIG–antibody complex formation (*see* **Note 8** for clarification):

$$\text{ng (antibody)} = \frac{\text{ng(DNA fragment)} \times \text{kDa (antibody)}}{\text{kDa (DNA fragment)}} \times \frac{\text{DIG/DNA fragment}}{2}.$$

2. Incubate the purified, DIG-labeled DNA fragment (PCR product from Subheading 3.2) with the calculated amount of the anti-DIG antibody in PBS for 2 h on a rotating wheel at room temperature. A typical reaction contains 500 ng DNA fragment and 2000 ng antibody in 100 µl PBS.
3. Add 5.4 µl formaldehyde (37%) to the tube (*see* **Note 9**).
4. Incubate for 10 min at room temperature.
5. Add 12.5 µl 1 M glycine to quench the aldehyde groups and terminate the crosslinking reaction.
6. Incubate for 10 min at room temperature.
7. To remove residual formaldehyde, wash the DNA–DIG–antibody complex with PBS by a spin column with a cut off of 100 kDa (*see* **Note 10**), e.g., Nanosep 100K omega centrifugal devices.
8. Dilute the DNA–DIG–antibody complex in PBS to a final volume of 1 ml.
9. Aliquot the complex (20 µl) and store at −80 °C (*see* **Note 11**).

3.4 Test the Specificity and Working Concentration of DNA–DIG–Antibody Complex

1. Prepare chromatin for a typical ChIP experiment according to your protocol (*see* **Note 12**).
2. Divide the chromatin into five tubes (tube 1–5).
3. Add different amounts of the DNA–DIG–antibody complex to the tubes containing chromatin (tubes 1–3): In an initial test experiment, use 0, 5, and 50 µl of the complex (*see* **Note 13**). As negative controls, add the DNA fragment only and the anti-DIG antibody only into two separate tubes with chromatin (tubes 4 and 5).
4. Incubate for 15 min on a rotating wheel in the cold room.
5. Remove 1/100 of the sample from each tube and transfer to a new tube (inputs 1–5).
6. Add magnetic or sepharose beads that are covered with protein A and/or G to the tubes and incubate for 1 h on a rotating wheel in the cold room (*see* **Note 14**).

7. Wash beads according to your ChIP protocol.
8. Digest the proteins and RNA as well as reverse the crosslink according to your standard ChIP protocol. Include here the input samples from **step 5**.
9. Purify the DNA by phenol–chloroform extraction or by purification columns.
10. Run a qPCR with the purified samples (input and immunoprecipitated material) and use the nested primers NP-F and NP-R to amplify the DNA sequence of the DNA–DIG–antibody complex. A typical reaction in a qPCR experiment consists of 10 µl 2× master mix (containing a suitable buffer, $MgCl_2$, SYBR green, dNTPs, and the Taq DNA polymerase), 3 µl H_2O, 2 µl DNA template, and 5 µl forward and reverse oligonucleotides (NP-F and NP-R, 4.5 µM each, oligonucleotide sequences are listed Subheading 2.3). In the same run, include H_2O instead of DNA as a non-template control.
11. Analyze the threshold cycle values (Ct) of the DNA fragment by setting the input value to 1 and by calculating the immunoprecipitated material relative to the corresponding input sample. No signal should be obtained for the "antibody only control" and for the immunoprecipitated "DNA only control" sample.

3.5 The DNA–DIG–Antibody Complex in a ChIP Experiment

During a ChIP experiment, the DNA–DIG–antibody complex is added to the reaction after shearing of the chromatin and before the incubation with the antibody of interest. It is crucial that this external reference is already present in the input sample (starting material), therefore ensure that the complex is added before the input is removed from the sample. The DNA–DIG–antibody complex does not disturb the ChIP experiment and the entire ChIP procedure remains the same (Fig. 2).

1. Add the DNA–DIG–antibody complex to the chromatin before the addition of the antibody.
2. Follow your standard ChIP protocol.
3. Finally, quantify the DNA sequence in the starting material (input) and in the immunoprecipitated material by the nested oligos NP-F and NP-R in a qPCR run (*see* also **step 10** in Subheading 3.4). These values can be used for normalization of the gene/DNA of interest as external reference and can therefore compensate for variations between the samples (e.g., loss during the purification of DNA) (Fig. 1, *see* **Note 15**).

4 Notes

1. Since the chosen DNA fragment is added to the chromatin extract during the ChIP experiment and quantified by qPCR or sequencing, it is essential that the DNA sequence does not cross-react with any sequence in the genome of the organism of interest. Therefore, homology searches between the chosen DNA sequence and the genome of the favorite organism should be performed (e.g., Blastn).
2. The DNA fragment should have a minimum length of 70 nt to enable robust quantification with nested primers by qPCR.
3. For the PCR, a plasmid containing the quinol *bo3* oxidase (pETcyo) was used. However, it is possible to use any other DNA sequence that does not cross-react with sequences in the genome of your organism of interest.
4. A single DNA band of 78 bp should be present on the agarose gel if using the indicated plasmid and oligonucleotides.
5. To investigate unspecific binding of the oligonucleotides to the genomic DNA of the favorite organism, the DNA should mimic the chromatin extract of a typical ChIP experiment. Therefore, the DNA should be sheared by sonication or by enzymatic digestion as in ChIP experiments. If available, a sample/input from a previous ChIP experiment can be used.
6. The ratio between DIG-11-dUTPs and dTTPs can be varied for different labeling extent of the resulting PCR product. Ensure that at least one DIG-labeled dUTP is incorporated per DNA fragment. Here, a ratio between DIG-11-dUTPs and dTTPs of 1:18 was chosen, i.e., on average, 1.4 dTTPs per ssDNA sequence are exchanged by DIG-11-dUTPs (26 thymidines are present in the 78 nt DNA sequence).
7. A small aliquot of the PCR reaction can be analyzed on an analytical 1.5% agarose gel, as described for **step 3** in Subheading 3.1.
8. The amount of the DNA fragment (ng) is measured by a UV spectrophotometer (*see* **step 4**; Subheading 3.2). The molecular weight of an IgG antibody is approximately 150 kDa and the molecular weight of the DNA fragment is estimated by multiplying the number of base pairs with 650 Da, the average molecular weight of a base pair (here: 78 bp $\times$ 650 Da = 50.7 kDa). In addition, the formula takes into consideration the amount of incorporated DIG-labeled dUTP per DNA fragment (here, since the DNA fragment is double-stranded: $2 \times 1.4 = 2.8$) and the two binding sites for DIG per antibody.

9. Crosslinking was performed with 2% formaldehyde. The crosslinking can be modulated by the amount of formaldehyde or by other crosslinking reagents. It is reasonable to use the same crosslinking conditions as for the chromatin in your standard ChIP experiment.
10. Using a concentrating unit with a cut off of 100K, DNA fragments that are not bound by an antibody will be washed out, decreasing background signals.
11. Analysis of the stability of the DNA–DIG–antibody complex showed that the complex must be stored at −80 °C or at −20 °C in the presence of glycerol [8]. It is therefore essential to prepare small aliquots of the complex and to avoid repeated freezing–thawing cycles.
12. Treat the chromatin extracts according to your standard ChIP protocol: pre-clear the samples if necessary and use the buffer including detergents that is required for the antibody incubation.
13. The signals of the normalizer DNA fragment measured by qPCR should be in the same range as the signals for the gene/DNA of interest. Therefore, depending on the experiment, the amount of the DNA–DIG–antibody complex has to be adjusted.
14. Take the same amount of beads and incubation time as in your standard ChIP protocol.
15. If the ChIP experiment is analyzed by high-throughput sequencing (ChIP-seq), the DNA fragment will also be sequenced and can therefore be used as reference to compensate for variability between samples.

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