ChIP-Seq: A Method for Global Identification of Regulatory Elements in the Genome

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ABSTRACT

This unit describes ChIP-Seq methodology, which involves chromatin immunoprecipitation (ChIP) followed by high-throughput sequencing (Seq), and enables the genome-wide identification of binding sites of transcription factors (TFs) and other DNA-binding proteins. The process is initiated by cross-linking DNA and DNA-bound proteins. Subsequently, chromatin is isolated from nuclei and subjected to sonication. An antibody against a specific TF or DNA-binding protein is then used to immunoprecipitate specific DNA-TF complexes. ChIP DNA is purified, sequencing adapters are ligated, and 30- to 35-nucleotide (nt) sequence reads are generated. The sequence of the DNA fragments is mapped back to the reference genome for determination of the binding sites.

INTRODUCTION

ChIP-Seq has proven to be a valuable method for mapping transcription factor (TF) binding sites and chromatin modifications throughout the genome for any organism of interest whose genome has been sequenced. ChIP-Seq involves chromatin immunoprecipitation (ChIP) followed by high-throughput sequencing (Seq). The first successful application of ChIP identified the binding sites of RNA polymerase in a bacterial genome (Gilmour and Lis, 1984). With the emergence of high-throughput sequencing platforms like Illumina Genome Analyzer and SOLiD (see Chapter 7), and the availability of ChIP-grade antibodies, ChIP-Seq has become one of the most widely used methods for determining functional elements in the genome (Johnson et al., 2007; Robertson et al. 2007; Wacker and Kim, 2009). In general, when compared with ChIP-chip (ChIP followed by hybridization to a microarray; UNIT 21.13), ChIP-Seq has higher signal/noise ratios, is less expensive, and requires lower amounts of ChIP DNA for genome-wide analysis (Euskirchen et al., 2007). The Human ENCODE project (The ENCODE Project Consortium, 2007), recently launched by NHGRI, is working toward generating a comprehensive list of all the functional elements of the human genome using ChIP-Seq.

The protocol described in this unit was developed to perform ChIP-Seq with mammalian cells grown in culture (Fig. 21.19.1). The cells are grown in tissue culture flasks in appropriate medium and cross-linked using formaldehyde (see Basic Protocol 1). Although other cross-linking agents have been tested, formaldehyde remains the most widely used for ChIP. The cross-linked cells are then homogenized in hypotonic buffer to isolate nuclei. This step eliminates cytoplasmic proteins and significantly reduces the number of possible cross-reactive proteins. Nuclei are then exposed to lysis buffer, and the chromatin is sheared by sonication. Antibodies raised against a particular TF or DNA-binding protein of interest are used to immunoprecipitate specific DNA-protein complexes. ChIP DNA is then separated from proteins by reverse cross-linking, followed by RNase and proteinase K digestion.
Purified ChIP DNA is then prepared for sequencing on the Illumina Genome Analyzer (GA). Adapters are ligated to ChIP DNA and control Input DNA (not immunoprecipitated), and amplified by PCR for a limited number of cycles. Denatured ChIP DNA and Input DNA are loaded onto individual lanes of an Illumina flowcell, and a cluster of DNA molecules is generated by amplification of the DNA fragments. The clusters are then sequenced using fluorescently labeled modified nucleotides. The data from the Illumina GA are analyzed using a series of software programs called “eland” (provided by Illumina). The quality of the ChIP-Seq data is determined through analysis with a Genome Browser (Affymetrix or UCSC). A number of algorithms are available for scoring ChIP-Seq data (Jothi et al., 2008; Zhang et al., 2008; Rozowsk et al., 2009; Tuteja et al., 2009) and for performing further downstream analyses.

STRATEGIC PLANNING

A good sonication protocol and high-quality antibodies are key to the success of a ChIP-Seq experiment. The sonication protocol can vary from one cell line to another and requires optimization for each cell line under investigation. Optimization can easily be done by varying the duration of each burst and the number of cycles of sonication. Ideally, sonication should result in enrichment of DNA fragments between 100- and 1000-nt long. The length of each burst should be adjusted to keep the sample temperature no higher than 4°C during the entire sonication. Once the optimal length of each burst is determined,
the number of cycles of sonication can be varied to obtain optimal sonication conditions. Aliquots of sonicated chromatin collected at different cycle intervals, after reverse cross-linking and subsequent purification, can be run on an agarose gel to determine if optimum sonication conditions have been achieved. Over-sonication of chromatin can increase the yield of DNA fragments 100- to 1000-nt long, but might cause denaturation of proteins and may not be suitable for ChIP. A qPCR assay measuring the enrichment of known targets in ChIP DNA prepared using RNA polymerase II antibody can be used as a final test, and sonication conditions that will give highest level of enrichment (a minimum of five-fold) will define the optimal procedure.

The biggest challenge to identifying regulatory elements using ChIP-Seq is the availability of ChIP-grade antibodies (meaning the antibody can immunoprecipitate the protein of interest). Although many vendors provide information about the applications for which an antibody is tested, it is always a good practice to test the antibody by immunoprecipitation (IP; UNIT 10.16) and/or immunoblotting (UNIT 10.8) before performing ChIP. IP from nuclear extract produces cleaner blots compared to those from whole-cell extracts. Mass spectrometric analysis of IP products, or RNA interference followed by immunoblotting, can provide further validation of antibody specificity. As a control, one can use an RNA Pol II antibody (Covance, cat. no. MMS-126R) for IP/blot and mass spectrometry. Mass spectrometric analysis of Pol II IP products should detect the subunit of interest as well as other Pol II-specific subunits.

**PREPARATION OF ChIP DNA**

This protocol describes a method to prepare ChIP DNA from mammalian cells grown in culture. Cells grown in appropriate medium are first cross-linked with formaldehyde. The amino and imino groups of protein and DNA are cross-linked when cells are exposed to formaldehyde. This causes DNA to be pulled down along with proteins when immunoprecipitation is performed. Chromatin is isolated from cross-linked cells, sonicated, and subjected to immunoprecipitation. DNA is then separated from protein and purified for sequencing.

**Materials**

- Cells growing in appropriate growth medium (see APPENDIX 3F)
- 37% formaldehyde
- 2 M glycine (see recipe)
- 1× PBS with protease inhibitors: dissolve one Complete Protease Inhibitor tablet (Roche) in 22.5 ml ice-cold water, add 2.5 ml 10× PBS (APPENDIX 2) and vortex to dissolve; keep on ice
- Hypotonic buffer (see recipe), ice-cold
- 1× RIPA buffer (see recipe)
- Antibody for immunoprecipitation
- Protein A–Agarose, Fast Flow (Millipore)
- Elution Buffer 1 (see recipe)
- Elution Buffer 2 (see recipe)
- TE buffer (APPENDIX 2)
- 20 mg/ml proteinase K
- RNase (Qiagen, cat. no. 19101)
- QIAquick PCR purification kit (Qiagen) including QIAquick purification columns and elution buffer (Buffer EB)
- Nuclease-free water (for preparing reagents listed above)
- 15- and 50-ml conical tubes (Falcon)
- Clay Adams Nutator mixer
7-ml Dounce homogenizer (Kontes Glass)
2-ml microcentrifuge tubes, prechilled
Probe sonicator: e.g., Branson 250 Sonifier
65°C and 45°C water baths

Additional reagents and equipment for basic cell culture techniques (including 
trypsinization; APPENDIX 3F) and immunoprecipitation (UNIT 10.16)

**NOTE:** Use nuclease-free water in all recipes and protocol steps.

**NOTE:** Vary the volume of each reagent depending upon the number of ChIP DNAs in 
the experiment.

**NOTE:** The tissue culture cells need to be maintained in appropriate medium and in 
growth conditions optimal for healthy cells. Grow cells in freshly prepared medium, 
change medium every 2 to 3 days to avoid overgrowth of cells, and plate cells at appro-
priate density when passing cells. Also see APPENDIX 3F.

1. For ChIP-Seq experiments, plate sufficient cells to attain a density of $0.5 \times 10^6$ cells/ml at 24-hr post-plating.

   It is convenient to use $5 \times 10^7$ cells for each ChIP-Seq experiment, and a typical ex-
   periment should involve two to three biological replicates. The biological replicates are 
   prepared by splitting cells from a culture flask into two to three separate flasks, depending 
   on the number of replicates, and expanding cells from each flask separately.

   A lower number of cells can be used depending on the abundance of the TF/DNA-binding 
   protein and the affinity of the antibody. The authors have successfully used $2 \times 10^7$ cells 
   for RNA Polymerase II ChIP-Seq analysis.

   It is important to grow and cross-link enough cells to complete a project—e.g., to com-
   plete a ChIP-Seq experiment involving five TFs, grow $2.5 \times 10^8$ cells for each replicate. 
   Depending on the source of the antibody, a control ChIP DNA using mouse, rabbit, or 
   goat normal IgG needs to be prepared for assessing the quality of the ChIP DNA.

**Prepare cross-linked cells**

**CAUTION:** Always perform the cross-linking procedure inside a hood to avoid exposure 
to formaldehyde vapor.

2. For cells grown in monolayer, use a typical trypsinization protocol (APPENDIX 3F) to 
detach cells from the flask. Resuspend the cells in fresh medium and proceed with 
the steps below to cross-link the cells.

3. Slowly add a volume of 37% formaldehyde equal to 1/36th of the volume of medium 
in which cells are suspended, to cross-link tissue culture cells with 1% formaldehyde 
for 10 min at room temperature. For example, add 2.8 ml 37% formaldehyde slowly 
to 100 ml medium while swirling/stirring cells at low speed. Cross-link for exactly 
10 min.

4. Add 2 M glycine to a final concentration of 0.125 M (swirl/stir cells at low speed) 
to neutralize the formaldehyde. For example, add 6.8 ml of 2 M glycine to 102.8 ml 
cross-linked cells and incubate at room temperature for 5 min.

5. Centrifuge 3 min at $1000 \times g$, room temperature, to pellet cells. Remove supernatant 
and add 10 ml of 1× PBS (with protease inhibitors) per $5 \times 10^7$ cells. Resuspend 
cells by pipetting up and down slowly. Transfer cells to a 15-ml Falcon tube.

6. Centrifuge 3 min at $1000 \times g$, room temperature, to pellet cells. Remove PBS, 
snap-freeze cells, and store at $-80^\circ C$.

   Cross-linked cells can be stored at $-80^\circ C$ at least for 1 year.
Prepare ChIP DNA

7. For each ChIP DNA preparation, remove one 15-ml tube containing $5 \times 10^7$ cross-linked cells from the $-80^\circ$C freezer. Add 5 ml of ice-cold 1× PBS containing protease inhibitors into each 15-ml Falcon tube of frozen cross-linked cells and thaw cells at 4°C for 1 hr using Nutator mixer.

8. Centrifuge the cells 3 min at 1000 × g, 4°C, remove PBS, and add 3 ml ice-cold hypotonic buffer. Resuspend cells by pipetting up and down and leave the tubes on ice to swell for 10 min.

9. After swelling cells for 10 min, use a 7-ml Dounce homogenizer, 30 strokes on ice, to break open the cells. Keep the total time to 15 min for the entire hypotonic lysis/homogenization steps.

10. Transfer equal volumes of lysates into two 2-ml microcentrifuge tubes that have been prechilled on ice. Microcentrifuge 5 min at 600 × g, 4°C, to pellet nuclei. Discard supernatant and wash nuclear pellet once with hypotonic buffer, centrifuging 5 min at 600 × g, 4°C, and removing the supernatant.

11. Resuspend nuclear pellets ($5 \times 10^7$) in 3 ml ice-cold 1× RIPA buffer. Transfer nuclear lysate to a 15-ml Falcon tube. Incubate for 30 min on ice, vortexing every 5 min.

   If the cell lysis is incomplete, debris will clog the sonicator tip in step 12 and cause foaming.

12. Sonicate each sample to shear the chromatin. For example, using a Branson 250 Sonifier, keep the power setting just below 7, the maximum output setting recommended for a microtip. Using 100% duty cycle, sonicate chromatin for 10 rounds, each round with 20-sec pulses. Incubate the tubes on ice for 2 min between rounds. While sonicating, keep the tip a few millimeters above the bottom of the Falcon tube to avoid foaming. Do not continue sonication if there is a lot of foam in the tube. Instead, transfer the nuclear lysate to a new 15-ml Falcon tube and continue sonication.

13. Transfer lysates to microcentrifuge tubes and clarify by centrifuging 15 min at 20,000 × g, 4°C. Pool supernatants in a 15-ml Falcon tube and bring the volume to 5 ml with 1× RIPA buffer.

14. Remove 200 μl lysate (Input DNA) and store overnight at 4°C. Process this Input DNA sample the next day along with ChIP DNA samples.

15. Immunoprecipitate (UNIT 10.16) DNA-protein complexes from sonicated chromatin using the optimum amount of antibody. As a control, perform immunoprecipitation using same amount of corresponding normal IgG and sonicated chromatin prepared from $5 \times 10^7$ cells. Incubate at 4°C on a Nutator for 12 to 16 hr (overnight).

   The optimum quantity of antibody required for ChIP can be determined by performing IP from a fixed quantity of lysate and varying quantities of antibody during the antibody validation step. IP products can be checked by immunoblot (UNIT 10.8). The quantity of antibody which yields the highest signal-to-noise ratio should be used for ChIP.

16. Gently shake the bottle of 50% Protein A–agarose to resuspend the slurry and remove 150 μl for each sample ($5 \times 10^7$ cells). Wash agarose twice with 1 ml of ice-cold 1× RIPA buffer containing protease inhibitor, PMSF, and DTT. For each wash, resuspend Protein A–agarose (cut the end off 1-ml pipet tips to enlarge the orifice and use them for resuspension), and microcentrifuge 1 min at 2600 × g, 4°C. Remove final supernatant. Resuspend beads in each tube to the original volume of the aliquot taken from the bottle (150 μl).
17. Add 150 μl slurry of Protein A agarose to each tube containing chromatin-Ab complex (from step 15) and incubate for 1 hr at 4°C in Nutator mixer. Centrifuging the tubes 3 min at 1000 × g, 4°C between washes, wash the protein A-agarose beads three times with 10 ml ice-cold RIPA buffer and once with ice-cold 1× PBS, incubating for 15 min at 4°C each time. Remove the final supernatant after centrifuging.

18. Resuspend Protein A–agarose beads in 1 ml ice-cold 1× PBS and transfer the beads to a 1.5-ml microcentrifuge tube. Add 300 μl ice-cold 1× PBS to the 15-ml tube to rinse, and add the residual beads to the bead suspension in the 1.5-ml microcentrifuge tube.

19. Centrifuge beads 1 min at 2600 × g, 4°C. Remove PBS completely, add 100 μl of Elution Buffer 1 to each tube, resuspend Protein A–agarose, and incubate at 65°C for 10 min with gentle mixing by tapping the bottom of the tube every 2 min.

   In this step, the DNA-protein complex bound to the beads is released into the elution buffer.

20. Centrifuge 1 min at 2600 × g, room temperature, and transfer the supernatant to a new microcentrifuge tube. Add 150 μl of Elution Buffer 2 to the protein A–agarose pellet in each tube. Incubate again at 65°C for 10 min with occasional gentle vortexing. Centrifuge 1 min at 2600 × g, room temperature, and combine the second eluate with the first one. Discard Protein A beads.

   In this step, any residual DNA-protein complex bound to the beads is released into the elution buffer.

21. Remove any residual Protein A–agarose by centrifuging the tube 2 min at 20,000 × g, room temperature. Carefully transfer the supernatant to a new 1.5-ml tube. Avoid transferring any beads.

22. Remove the Input DNA tube stored at 4°C (in step 14) and add 300 μl of Elution Buffer 1.

23. Reverse cross-link ChIP DNA and Input DNA overnight at 65°C.

24. Add 250 μl of 1× TE buffer containing 100 μg RNase to each DNA sample, and incubate for 30 min at 37°C.

25. Add 10 μl of 20 mg/ml proteinase K to each tube and incubate at 45°C for 30 min.

26. Purify ChIP DNA and Input DNA using Qiagen PCR purification columns. Elute ChIP DNA in 50 μl elution buffer (EB).

27. Assess the quality of the ChIP DNA (see Support Protocol).

**BASIC PROTOCOL 2**

**SEQUENCING ChIP DNA USING ILLUMINA GENOME ANALYZER**

To determine the binding sites of a TF or other DNA-binding protein, 30 to 35 nucleotides from one end of the ChIP DNA fragments are sequenced. This protocol describes preparation of ChIP samples for sequencing, in which adapters are added to blunt-ended ChIP DNA and amplified by PCR for limited number of cycles. A cluster of approximately 1000 DNA molecules is then generated by bridge amplification of individual ChIP DNA fragments and sequenced, e.g., using the procedure developed for the Illumina Genome Analyzer.
Materials

ChIP DNA sample from step 26 of Basic Protocol 1
End-It DNA End Repair Kit (Epicentre) containing:
2.5 mM dNTP Mix
10 mM ATP
End-Repair Enzyme Mix
End-Repair 10× Buffer
QIAquick PCR Purification Kit (Qiagen) including QIAquick columns and elution buffer (Buffer EB)
100 mM dATP (New England Biolabs)
Klenow fragment of DNA polymerase I (3’→5’ exo minus; New England Biolabs, cat. no. M0212) and NEBuffer 2 (New England Biolabs)
LigFast Ligase and 2× DNA ligase buffer (Promega)
Illumina Adapter Oligo Mix
QIAquick MinElute columns (Qiagen)
2% agarose E-gel (Invitrogen) with SYBR SAFE gel stain (also see UNIT 2.5A for general agarose gel electrophoresis protocols)
Qiagen Gel Extraction Kit
Illumina PCR Primers 1.1 and 2.1
2× Phusion HF Master Mix (New England Biolabs, cat. no. F531)
Illumina Genome Analyzer with flowcells, reagents, and cluster station
Eland data collection software (Illumina)

Additional reagents and equipment for agarose gel electrophoresis (UNIT 2.5A)

NOTE: Use nuclease-free water in all recipes and protocol steps.

End repair

Use two-thirds of the ChIP DNA and 100 ng Input DNA for library preparation. This protocol is optimized for 100 to 200 ng of DNA.

1. Combine and mix the following components in a microcentrifuge tube (total reaction volume, 50 μl):
   34 μl ChIP DNA to be end-repaired
   5 μl 10× End-Repair Buffer
   5 μl 2.5 mM dNTP Mix
   5 μl 10 mM ATP
   Sterile water to bring reaction vol to 49 μl
   1 μl End-Repair Enzyme Mix.

2. Incubate at room temperature for 45 min.


Add “A” base to 3’ ends

4. Combine and mix the following components (total reaction volume, 50 μl):
   34 μl end-repaired DNA (from step 3)
   5 μl 10× Klenow buffer (NEBuffer 2)
   10 μl 1 mM dATP
   1 μl Klenow enzyme (3’ to 5’ exo minus).

5. Incubate for 30 min at 37°C.

6. Purify DNA using QIAquick MinElute column, eluting in 12 μl of Buffer EB.
**Ligate adapters**

7. Dilute Illumina adapter oligo mix 1:10 with water.

   *Do not reuse diluted adapters.*

8. Combine and mix the following components in a microcentrifuge tube (total reaction volume, 30 μl):

   - 12 μl DNA from step 6
   - 15 μl 2× DNA ligase buffer
   - 1 μl adapter oligo mix (1:10)
   - 2 μl DNA ligase.

9. Incubate for 15 min at room temperature.

10. Purify DNA using QIAquick MinElute column, eluting in 20 μl of Buffer EB.

11. Run adapter-ligated DNA on a 2% agarose E-gel (with SYBR SAFE gel stain).

12. Excise gel piece containing DNA 150- to 300-bp in size with a disposable razor blade. Take photos of the gel before and after the gel slices are excised.

   *ChIP DNA quantity is usually low at this stage, and is not visible on the gel; however, a strong band of adapter close to ~100 bp might be visible. Run the E-gel for 30 min to obtain a good separation of the 100-bp and 200-bp bands of the DNA ladder. Avoid cutting gel slices close to the adapter band.*

13. Purify adapter-ligated ChIP DNA using Qiagen Gel Extraction Kit, eluting DNA in 25 μl Buffer EB.

**PCR-amplify ChIP DNA and perform size selection**

14. Dilute Illumina PCR primers (1.1 and 2.1) 1:1 with water.

15. Combine and mix the following components (50 μl total):

   - 23 μl adapter-ligated ChIP DNA (step 13)
   - 25 μl 2× Phusion HF Master Mix
   - 1 μl diluted PCR primer 1.1 (step 14)
   - 1 μl diluted PCR primer 2.1 (step 14).

16. Amplify using the following PCR protocol:

   - 1 cycle: 30 sec 98°C (initial denaturation)
   - 15 cycles: 10 sec 98°C (denaturation)
   - 30 sec 65°C (annealing)
   - 30 sec 72°C (extension)
   - 1 cycle: 5 min 72°C (final extension)
   - 1 cycle: indefinitely 4°C (hold).

17. Purify amplified DNA using QIAquick MinElute column, eluting in 20 μl of Buffer EB.

18. Run PCR-amplified DNA on a 2% agarose E-gel (with SYBR SAFE gel stain).

   *A bright smear of amplified DNA, 100- to 300-bp long, should be visible after this step.*

19. Excise DNA of 150 to 300 bp from the gel with disposable razor blades.

20. Purify the DNA from the agarose slices using Qiagen Gel Extraction Kit, eluting in 25 μl Buffer EB.
21. Measure DNA concentration (ng/μl) using a NanoDrop spectrophotometer.

A yield of 200 to 500 ng ChIP DNA is expected.

**Sequence adapter-ligated ChIP DNA**

22. Sequence the adapter-ligated ChIP DNAs using the Illumina Genome Analyzer.

This involves generation of millions of clusters of DNA molecules from each DNA sample and subsequent sequencing. The reagents required to generate clusters and perform the sequencing are purchased from Illumina.

**Generate clusters:** A cluster of ~1000 DNA molecules is first generated by isothermal amplification of a single ChIP DNA molecule hybridized to a primer on a flowcell. The flowcell has an appearance of a glass slide, which provides a surface for binding primers and allows access to fluids. Eight samples can be prepared at a time for sequencing using a single flowcell. The sample preparation is carried out in a semi-automated fluidic station called a “cluster station.” ChIP DNA is first denatured and allowed to hybridize to primers on the flowcell. This is followed by amplification of DNA and subsequent cleavage of one of the DNA strands. A sequencing primer is hybridized to the remaining DNA strands before the flowcell is transferred to the Illumina Genome Analyzer for sequencing.

Illumina technology is based on the “sequencing by synthesis” method. It involves the incorporation of four fluorescently labeled modified nucleotides, with a reversible termination property, by DNA polymerase followed by detection of the fluorescent signal in each sequencing cycle. The cleavage step removes the fluorescent tag and prepares the DNA molecules for incorporation of the next base. This completes one cycle of sequencing, and a total of 35 cycles of sequencing is typically performed. Approximately 20 million reads (35-nt sequences) are generated for each sample per run.

The sequence data is then processed using a set of data collection software called “eland,” which is provided by Illumina.

**ASSESS QUALITY OF ChIP DNA**

It is important to assess the quality of ChIP DNA before sequencing. The quality is determined by measuring the enrichment of known targets of a TF/DNA-binding protein by PCR or qPCR. An enrichment of five-fold or higher over ChIP DNA prepared using normal IgG is often detected for a good quality ChIP DNA. Forward and reverse PCR primers needs to be designed from around two to three previously characterized binding sites. If the binding site is not defined, multiple primer sets spanning the gene and the upstream/downstream sequences need to be designed.

**Materials**

- Input DNA from step 26 of Basic Protocol 1
- ChIP DNA prepared using TF-specific antibody (Basic Protocol 1)
- ChIP DNA prepared using normal IgG (Basic Protocol 1)
- Taq Master Mix (Qiagen)
- SYBR Mix for qPCR (Roche)
- Control Pol II primers:
  1. (FWD) 5’GGAAAGAGTGGTGCGGTAGC-3’
     (RVS) 5’-GGGATGTTCACCACCTGCTT-3’
  2. (FWD) 5’-CTGAGGGGCCACGGAAGAAA-3’
     (RVS) 5’-TGCCTGGGAACAACCGGTAGC-3’
- Additional reagents and equipment for agarose gel electrophoresis (*UNIT 2.5A*) or qPCR (*UNIT 15.8*)

**NOTE:** Use nuclease-free water in all recipes and protocol steps.
IgG 1
IgG2
IgG3
IgG4
ChIP 1
ChIP 2
ChIP 3
ChIP 4
input
ladder

**Figure 21.19.2** Evaluation of ChIP DNA quality by PCR. 1/50th of ChIP DNA prepared from $5 \times 10^7$ cells was assayed for enrichment of Pol II ChIP DNA at the promoter site of two target genes.

1. Dilute Input DNA from step 26 of Basic Protocol 1 to 10 ng/μl. Set up PCR reactions with (1) Input DNA, (2) ChIP DNA prepared using TF-specific antibody, and (3) ChIP DNA prepared using normal IgG. Set up PCRs in a total volume of 50 μl.

   - 25 μl 2× Taq Master Mix
   - 1 μl 10 μM Primer Mix
   - 1 μl template DNA
   - 23 μl H2O.

2. PCR amplify DNA using the following conditions

   1 cycle: 4 min 94°C (initial denaturation)
   29 cycles: 30 sec 94°C (denaturation)
   30 sec 58°C (annealing; see below)
   30 sec 72°C (extension)
   1 cycle: 10 min 72°C (final extension)
   1 cycle: indefinitely 4°C (hold).

   **Annealing temperature will vary depending on the $T_m$ of the primers (see UNIT 15.1 for more information on determining PCR parameters).**

3a. For gel analysis: Load 10 to 20 μl PCR product on 1.5% to 2.0% agarose gel (Fig. 21.19.2).

   *The percentage of agarose will depend on the size of the PCR product (see UNIT 2.5A).*

3b. For qPCR analysis: Perform a qPCR assay (SYBR green–based) using ChIP DNAs and primer sets designed from the sequence of the target gene.

   *qPCR protocols are provided in UNIT 15.8.*

**REAGENTS AND SOLUTIONS**

*Use nuclease-free water in all recipes and protocol steps. For common stock solutions, see APPENDIX 2; for suppliers, see APPENDIX 4.*

**DTT, 1 M**

Dissolve 1.54 g DTT in 10 ml 1× PBS. Store 500-μl aliquots at −20°C.

**Elution Buffer 1**

Add 0.2 ml 50× TE buffer (see APPENDIX 2 for 1× recipe) and 0.5 ml 20% (w/v) SDS to 9.3 ml RNase/DNase-free water. Prepare fresh; keep at room temperature.
**Elution Buffer 2**

Add 6.7 ml of Elution Buffer 1 (see recipe) to 3.3 ml of 1× TE buffer (*APPENDIX 2*). Prepare fresh; keep at room temperature.

**Glycine, 2 M**

Dissolve 15 g glycine in 100 ml PBS (*APPENDIX 2*). Prepare fresh before use.

**Hypotonic buffer**

To prepare 50 ml of hypotonic solution, place two Complete Protease Inhibitor tablets (Roche) in 37.5 ml ice-cold nuclease-free water, vortex to dissolve the tablets, then add:

1.0 ml 1 M HEPES, pH 7.9
0.5 ml 1 M KCl
0.1 ml 0.5 M EDTA, pH 8.0 (*APPENDIX 2*)
10.0 ml 50% (v/v) glycerol

Mix well and store up to 3 months at 4°C. Add 50 μl of 1 M DTT (see recipe) and 250 μl of 100 mM PMSF just before use.

**PMSF, 100 mM**

Dissolve 0.87 g PMSF in 50 ml isopropanol, store 200-μl aliquots up to 1 year at −20°C.

Some vendors sell PMSF in solution.

**RIPA buffer, 1×**

Place two Complete Protease Inhibitor tablets (Roche) in 45 ml ice-cold water, vortex to dissolve, and add 5.0 ml 10× RIPA buffer (Millipore, cat no. 20-188). Mix well and keep the tube on ice. Add 50 μl of 1 M DTT (see recipe) and 250 μl of 100 mM PMSF just before use.

**COMMENTARY**

**Background Information**

Since the completion of the sequencing of the human genome, a number of functional approaches have been taken to understand how the genome functions. Gene expression is a dynamic and complex process and is regulated by multi-protein transcriptional machinery involving protein-DNA and protein-protein interactions. The proteins include TFs, histones, enhancers, suppressors, and others. Each TF can regulate expression of many genes by binding near the transcription start site and can play important roles in defining the physiological state of a cell. ChIP-Seq is used to determine the in vivo binding sites of a TF and locations of chromatin modifications.

ChIP has become a widely used method for determining binding sites of proteins to DNA, since the study of RNA polymerase occupancy on bacterial genes in 1984 (Gilmour and Lis, 1984). Initially, Southern blot and PCR analysis was used to characterize a limited number of binding sites. Iyer et al. (2001) and Ren et al. (2000) used microarray-based detection technology to map genome-wide binding sites of TFs (ChIP-chip) for the first time. The advent of high-throughput DNA sequencing technologies has enabled a sequencing-based readout (ChIP-Seq), which has a number of advantages over ChIP-chip. ChIP-Seq methodology is simple, fast, and cost-effective. ChIP-Seq does not require physical existence of tiling arrays, but does require genome sequence information for mapping the sequences. ChIP-Seq is more sensitive and more reproducible and provides higher resolution.

**Critical Parameters and Troubleshooting**

The success of a ChIP-Seq experiment depends on many factors:

1. A key requirement for ChIP is the use of an antibody that can immunoprecipitate the target protein. Many commercially available
Figure 21.19.3  Signal map of Pol II ChIP DNA. (A) Signal map of two biological replicates of Pol II ChIP DNA and control IgG ChIP DNA. Enrichment of Pol II ChIP DNA, but not IgG ChIP DNA, is shown over an entire chromosome. (B) Illustrates a close-up view of Pol II binding near the transcription start site (TSS) of a RefSeq gene.

antibodies do not work in the immunoprecipitation assay. This is most probably due to the failure of many antibodies to recognize native proteins, as well as insufficient specificity. The protein of interest in the immunoprecipitate should represent at least 50% of the signal when assayed by immunoblot. Further validation of the antibody by either RNA interference assay or mass spectrometry is recommended. Cells treated with siRNA or shRNA designed from the sequence of the target gene are expected to have reduced expression of the protein of interest when assayed by immunoblotting.
2. It is important to establish a sonication protocol that results in enrichment of DNA fragments of 100 to 1000 nucleotides. The size distribution of sonicated DNA fragments needs to be visualized by agarose gel electrophoresis. Over-cross-linking might make the DNA resistant to sonication. It is important to make sure that the protein structure integrity is not compromised by the sonication protocol, to ensure recognition by the antibody. ChIP DNA that is over-sonicated often fails to show enrichment of target DNAs. When establishing a sonication protocol, ChIP DNA should be prepared using a known ChIP-grade antibody like the RNA Polymerase II antibody, and enrichment of known targets should be verified by PCR or qPCR.

3. ChIP DNA quality should always be measured by either PCR or qPCR. A minimum of 5-fold enrichment of target DNA should be observed in the ChIP DNA sample when compared to an IgG control. Sonicated Input DNA can be also be used as a control for the PCR assay.

**Anticipated Results**

Once the ChIP samples are sequenced and analyzed, the data can be visualized using a genome browser. An example of what can be expected from a good-quality ChIP DNA sample is shown in Figure 21.19.3. In the absence of any enrichment of target DNA in a ChIP DNA sample, mapped reads will look like background evenly distributed throughout each chromosome. Nonspecific peaks generated from amplification of a random DNA fragment, which will look perfectly rectangular, can be observed when an insufficient amount of template DNA is used for amplification.

**Time Considerations**

It takes approximately a week to prepare ChIP DNA and the corresponding library for sequencing. A total of 4 to 6 samples can be processed at once. The cross-linking step (Day 1) for attached cells can take several hours, depending on the number of cells needed for the experiment. Cross-linking cells in suspension takes less time, usually only a few hours. Preparation of sonicated chromatin and setup of the chromatin immunoprecipitation takes another day (Day 2). Typically, Day 3 involves the separation and purification of the immunoprecipitated chromatin and performance of the reverse cross-linking step. ChIP DNA is purified and assayed for enrichment by PCR or qPCR on Day 4. Preparation of ChIP-Seq library starts on Day 4 and continues on Day 5.

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**Literature Cited**


