Chapter 26

Next-Generation Sequencing Applied to Flower Development (ChIP-Seq)

Emmanuelle Graciet[1*], Diarmuid Seosamh Ó'Maoiléidigh[1], and Frank Wellmer[1*]

[1] Smurfit Institute of Genetics, Trinity College Dublin, Dublin, Ireland

Correspondence: graciete@tcd.ie or wellmerf@tcd.ie; +353-1-896-3729 (phone); +353-1-679-8558 (FAX)

Summary

Over the past 20 years, classic genetic approaches have shown that the developmental program underlying flower development involves a large number of transcriptional regulators. However, the target genes of these transcription factors, as well as the gene regulatory networks they control, remain largely unknown. Chromatin immunoprecipitation coupled to next-generation sequencing (ChIP-Seq), which allows the identification of genome-wide transcription factor binding sites, has been successfully applied in many model organisms. The ChIP-Seq procedure involves chemical crosslinking of proteins to DNA, followed by chromatin fragmentation and immunoprecipitation of specific protein/DNA complexes. The regions of the genome bound by a specific transcription factor can then be identified after next-generation sequencing.

Keywords: Arabidopsis, flower development, ChIP-Seq, transcription factors

1. Introduction

The different developmental programs required to form specific organs are largely controlled at the transcriptional level, through the specific regulation of gene expression by transcription factors (1). In plants, genetic analyses have identified a large number of transcriptional regulators that are involved in the specification of the different floral organs and in the regulation of flower development ((2), reviewed in (3)). Although these studies have led to an understanding of how these transcription factors act, the molecular basis for their function, as well as the topology of the underlying gene regulatory networks, have remained largely unknown.

The development of chromatin immunoprecipitation (ChIP) in the 1980s by Varshavsky and colleagues (4) was an important step forward in the identification of regions of the genome that were bound by specific proteins. However, this method alone was not sufficient to identify, at a genome-wide scale, the binding sites of transcription factors. Recent advances in sequencing technologies, in particular the development of next-generation or ultra high-throughput sequencing (reviewed in (5)), and their coupling to ChIP (termed ChIP-Seq) has opened new possibilities to investigate, at a genome-wide scale, how transcription factors act to regulate complex developmental programs (6-10).

The use of whole-genome tiling arrays to identify the regions of the genome bound by a protein (termed ChIP-chip) has also been used for the genome-wide identification of transcription factors binding sites (11-13). However, this approach leads to lower resolution around the binding sites (12) and has been superseded by ChIP-Seq. This chapter will therefore focus on the use of next-generation sequencing, with emphasis on the use of an Illumina GAIIx sequencer.

2. Materials

2.1 Tissue Collection and Fixation

1. Liquid nitrogen

2. MC buffer: 10 mM sodium phosphate buffer pH 7.0, 50 mM NaCl, 0.1 M sucrose

3. Fixation buffer: 1% formaldehyde (w/v) in MC buffer (see Note 1)

4. Solution to stop fixation: 2.5 M glycine

5. Vacuum pump and vacuum dessicator

2.2 Chromatin Preparation

1. M1 buffer: 10 mM sodium phosphate buffer pH 7.0, 100 mM NaCl, 1 M hexylene glycol, 10 mM 2-mercaptoethanol, 1x Complete Protease Inhibitor cocktail (Roche) (See Note 2)

2. M2 buffer: 10 mM sodium phosphate buffer pH7.0, 100 mM NaCl, 10 mM $MgCl_2$, 0.5% Triton X100 (v/v), 1 M hexylene glycol, 10 mM 2-mercaptoethanol, 1x Complete Protease Inhibitor cocktail (Roche) (See Note 2)

3. M3 buffer: 10 mM sodium phosphate buffer pH7.0, 100 mM NaCl, 10 mM 2-mercaptoethanol, 1x Complete Protease Inhibitor cocktail (Roche) (See Note 2)

4. Lysis buffer: 50 mM Tris-HCl pH 8.1, 10 mM EDTA, 1% SDS (v/v), 1x Complete Protease Inhibitor cocktail (Roche) (See Notes 2 and 3)

5. ChIP dilution buffer: 16.7 mM Tris-HCl pH8.1, 167 mM NaCl, 1.2 mM EDTA, 1.1% Triton X-100 (v/v), 0.01% SDS (v/v), 1x Complete Protease Inhibitor cocktail (Roche) (See Note 2)

6. 10 mL syringe

7. Miracloth

8. Bioruptor (Diagenode)

9. Qubit and Quant-iT dsDNA HS assay kit (Invitrogen)

2.3 Checking Chromatin Size

1. RNase A (DNase-free)

2. Proteinase K

3. 100% ice-cold ethanol

4. 80% ice-cold ethanol

5. 3 M sodium acetate pH 5.2

6. Glycogen (from blue mussel)

2.4 Chromatin Pre-Clearing

1. Protein A or Protein G sepharose (See Note 4). We use protein A sepharose (GE#17-5280-01).

2. 0.5 mg/mL lipid-free bovine serum albumine (BSA) prepared in IP buffer (see below).

3. IP buffer: 50 mM Hepes pH7.5, 150 mM NaCl, 10 µM $ZnSO_4$, 5 mM $MgCl_2$, 1% Triton X-100 (v/v), 0.05% SDS (v/v)

4. Rotator at 4ºC

2.5 Chromatin Immunoprecipitation

1. Antibody against the protein of interest (See Note 5)

2. Protein A or Protein G Sepharose (See Note 4)

3. IP buffer (see above)

4. Elution buffer: 0.1 M glycine, 0.5 M NaCl, 0.05% Tween 20 (v/v); adjust to pH 2.8 using HCl

5. Neutralization solution: 1 M Tris base, pH9.0

6. Incubator shaker at 37ºC

2.6 DNA Purification

1. Proteinase K

2. RNase A (DNase free)

3. Phenol:chloroform:isoamyl alcohol solution (25:24:1)

4. Heavy Phase-lock tube

5. 100% ice-cold ethanol

6. 80% ice-cold ethanol

7. 3 M sodium acetate pH 5.2

8. Glycogen (from blue mussel)

9. TE buffer

10. Incubator or water bath at 65ºC

11. Qubit and Quant-iT dsDNA HS assay kit (Invitrogen)

2.7 ChIP validation using quantitative PCR (qPCR)

1. SYBR Green

2. Primers for negative loci (*i.e.* regions that are not bound by the protein of interest; see Table 1) and positive regions (*i.e.* loci that are known or suspected to be bound by the protein of interest)

2.8 Library Preparation for Illumina Sequencing

1. Illumina ChIP-Seq DNA Sample Prep Kit

2. Certified Low Range Ultra Agarose (Bio-Rad)

3. QIAquick Gel Extraction Kit (Qiagen)

4. QIAquick PCR Purification Kit (Qiagen)

5. MinElute PCR Purification Kit (Qiagen)

6. Dark Reader Transilluminator

7. DNA High Sensitivity Kit and Bioanalyzer (Agilent)

8. Qubit and Quant-iT dsDNA HS assay kit (Invitrogen)

8. SYBR Green

8. Primers for negative loci (*i.e.* regions that are not bound by the protein of interest; see Table 1) and positive regions (*i.e.* loci that are known or suspected to be bound by the protein of interest)

3. Methods

3.1 General considerations

Specific parameters need to be considered when designing a ChIP-Seq experiment (Fig. 1). One of the most important factors is the quality of the antibody used to immunoprecipitate the DNA-binding protein of interest. In order to avoid artifacts due to ectopic expression, the use of the endogenous promoter to express the gene encoding the protein of interest is recommended. The use of protein-specific antibodies avoids the need to generate plants with complex genetic backgrounds for the ChIP-Seq experiment. Protein-specific antibodies can be generated by inoculating animals (typically rabbits) using a recombinant full-length protein, a recombinant fragment of the protein or a peptide corresponding to a specific sequence in the protein of interest. Although the use of peptides as antigens has advantages, such as having the possibility to choose a unique region to avoid cross-reactivity with closely related proteins, we found that using larger fragments to immunize rabbits tends to yield better quality antibodies for ChIP. When generating your own polyclonal antibodies, it is worth testing the serum without any additional purification step in a ChIP-qPCR experiment. If the strength of the signal obtained by quantitative PCR is too low, the antibody of interest can be affinity-purified using either the recombinant protein or the peptide used to immunize the animal.

If protein-specific antibodies of sufficient quality cannot be produced, the protein of interest may be expressed as a fusion with a tag (*e.g.* Green Fluorescent

Protein (GFP) or other epitope tags such as hemagglutinin (HA), myc, flag…). When using this strategy, it is important to check that the protein of interest remains functional after addition of the tag (*e.g.* by testing if the expression of the tagged protein is sufficient to rescue a mutant plant for the gene of interest). The choice of tag used, the sequence between the protein of interest and the tag (linker), as well as the position of the tag (at the N- or C-terminus of the protein) need to be considered carefully, as they can affect the function of the protein. Many companies sell ChIP-grade antibodies for a wide range of tags, but the quality of the antibodies can vary greatly from one batch to another, so it is important to test a batch before buying a large amount of antibody of the same batch for a ChIP-Seq experiment. Finally, polyclonal antibodies tend to perform better for ChIP-Seq than monoclonal antibodies, although the latter are usually more specific.

While the use of endogenous promoters is optimal, this can result in additional complications. For example, the gene of interest might only be expressed in a few cells, at very low levels, or at a specific stage of flower development, which makes ChIP-Seq more difficult, compared to genes that are expressed at higher levels throughout most of flower development. To overcome this problem, a constitutive promoter such as the Cauliflower Mosaic Virus 35S promoter has often been used. However, novel techniques such as the INTACT method allow the use of endogenous promoters for genes that are expressed at low levels or in a few cells only (14).

Another important factor to obtain good quality ChIP-Seq data is the choice of the negative control to filter background signal. The use of epitope-tagged proteins offers some advantages, as plants with a similar genotype as those used for ChIP-Seq, minus the tagged protein, can be used for background control. In this case, the background generated by binding of the antibody used to immunoprecipitate the

tagged protein to other DNA-binding proteins can be assessed directly. When using antibodies raised against the protein of interest, the choice of the negative control is more difficult, especially if the mutant plants for the gene of interest have phenotypes that are very different from the wild type. In this case, the pre-immune serum from the animal that produced the ChIP antibody can be used to generate the background control. When performing a ChIP for the background control, the DNA yield can be a problem, but this can be overcome by pooling together numerous ChIP experiments.

3.2 Tissue Collection and Fixation (Note 6)

1. Collect 0.5 mL of floral tissue in a 1.5 mL tube and freeze in liquid nitrogen (Note 7). The tissue can be stored at -80°C until use. The protocol below is for 0.5 mL tissue. All steps are performed at 4ºC unless otherwise stated.

2. Place the 1.5 mL tube containing 0.5 mL tissue on ice in a vacuum dessicator and add 1 mL of ice-cold Fixation buffer. Apply vacuum (800 mbar or until bubbles start to emerge from the tissue) for 15 min, then release vacuum and invert the tube 3 times. To ensure that the tissue is immersed in fixation buffer the tissue may be centrifuged at 9,500xg for 30 sec. Repeat vacuum infiltration for 15 min and mixing 3 more times (total fixation time: ~60 min) (Note 8).

3. Stop fixation by adding 50 μL of 2.5 M glycine (final glycine concentration: ~125 mM), mix well by inverting the tube 3 times and spin down at 9,500×g for 30 sec. Vacuum infiltrate on ice for 5 min.

4. Remove the fixation buffer and wash the tissue by adding 1 mL MC buffer, inverting the tube 3 times, followed by spinning down the tissue at 9,500×g for 30 sec. Repeat the wash step with MC buffer twice more. After the last wash, remove as much MC buffer as possible and snap freeze the tissue in liquid nitrogen (Note 9).

3.3 Chromatin Preparation

1. Add 100 µL M1 buffer and grind tissue on ice (Note 10) until the tissue suspension appears homogenous. Add 150 µL M1 buffer and grind the tissue further. Finally, add 1 mL of M1 buffer.

2. Put a circular piece of Miracloth (diameter ~3cm) into a 10-mL syringe. Pre-wet the miracloth with approx. 300 µL M1 buffer. Filter the solution through the miracloth. Squeeze the miracloth with the plunger. Repeat filtration once more with a fresh piece of miracloth.

3. Centrifuge the cell suspension at 7,700×g for 1 min at 5°C.

4. Remove the supernatant and resuspend the pellet in 0.9 mL M2 buffer. Centrifuge at 7,700×g for 1 min at 5°C. Repeat washes with M2 buffer twice more.

5. Wash the pellet once with 0.9 mL M3 buffer and centrifuge at 2,000xg, 5 min, 5°C (Note 11).

6. Resuspend the pellet in 200 µL Lysis buffer by pipetting and incubate 10 min on ice (Note 12).

7. Add 800 µL ChIP dilution buffer and resuspend chromatin by pipetting until the extract is homogenous again.

8. Split sample in 3x300 µL aliquots and sonicate the sample to solubilize the chromatin and shear DNA into fragments around 300 bp in size. Use the Bioruptor at high intensity and carry out 12 cycles of sonication (*i.e.* in 30 sec pulses, with 30 sec in between) (Note 13).

9. Centrifuge the fragmented chromatin at 12,000×g for 10 min at 5°C to pellet insoluble material. Pool the supernatants and keep 100 µL to check chromatin size.

10. Measure the DNA concentration using the Qubit (Note 14).

3.4 Checking Chromatin Size

1. Take the 100 μL aliquot of sonicated chromatin from step 10 above and add 150 μL TE buffer.

2. Add 1.5 μL RNase and incubate 30 min at 37°C

3. Add 1.5 μL proteinase K and incubate 30 min at 55°C

4. Precipitate the DNA by adding 1 μL glycogen, 0.1 Vol. sodium acetate pH5.4 and 2.5 Vol. ice-cold 100% ethanol. Mix by pipetting and incubate at -20°C for at least 30 min.

5. Centrifuge 15 min at 18,000×g and 5°C

6. Remove supernatant and add 1 mL ice-cold 80% ethanol

7. Centrifuge 5 min at 18,000×g, 5°C

8. Remove supernatant and let the pellet dry

9. Resuspend the pellet in 15 μL water

10. Run the purified DNA on a 1.5% agarose gel. The DNA should be around 300 bp in average (Fig. 2) (see Note 13 if the DNA is not at the expected size).

3.5 Chromatin Pre-Clearing

1. To equilibrate the protein A sepharose, repeat five times the following procedure: add 1 mL IP buffer to 120 to 500 μL protein A sepharose slurry (120 μL of protein A sepharose slurry is sufficient for one IP). Invert the tube to resuspend the beads and centrifuge 1 min at 2,400×g, 4°C. Remove the supernatant and repeat.

2. Block the protein A sepharose by adding 1 mL of 0.5 mg/mL lipid-free BSA. Keep on a rotator at 4°C for 20 min. Centrifuge 2,400×g, 4°C for 1 min and remove the

supernatant. Estimate as accurately as possible the volume of beads and add an equal amount of IP buffer to obtain a 50% slurry.

3. Add 1.1 mL IP buffer to 900 µL sheared chromatin and spin twice at 18,000×g for 10 min at 5°C (transfer the supernatant to a new tube in between the 2 centrifugation steps). Keep the chromatin on ice.

4. Add 60 µL of BSA-blocked protein A sepharose per 2 mL of chromatin. Incubate on a rotator for 90 min at 4°C.

5. Centrifuge 1 min at 18,000×g, 4°C and keep the supernatant. If there were several tubes for pre-clearing, pool together the supernatants before taking the 'input' aliquot. Keep the remaining resin prepared in step 1 of this section at 4°C.

6. Remove 100 µL aliquot as 'input' fraction.

3.6 Immunoprecipitation

1. Aliquot the pre-cleared chromatin in 2 mL tubes (~1.8 mL per tube). Add the antibody specific to the protein of interest to the pre-cleared chromatin and incubate 1 hr to overnight at 4°C on a rotator (Note 15).

2. Centrifuge at 18,000×g, 10 min, 4°C to remove precipitated material.

3. Add 60 µL protein A beads (50% slurry) to each supernatant and incubate on a rotator for 2 hrs at 4°C.

4. Centrifuge 1 min at 2,400×g, 4°C and discard the supernatant.

5. Add 1 mL IP buffer and keep the tubes on their sides in ice while shaking for 8 min (a rotator in a cold room could also be used). Centrifuge 1 min at 2,400×g, 4°C and discard the supernatant. Repeat this step 4 times more.

6. Elute the protein–DNA complexes from the beads by adding 100 µL ice-cold elution buffer, incubate for 1 min at 37°C while shaking vigorously, and centrifuge

for 1 min at maximum speed. Transfer the eluate to a 2 mL tube and add 50 µL 1 M Tris pH 9 to neutralize. Elute from the beads twice more, but for the last elution step incubate for 4 min at 37°C (final combined volume of eluate will become ~450 µL).

7. Spin the eluate at maximum speed at 4°C for 2 min. Transfer eluate (protein–DNA complexes) to a new 2 mL tube without touching any pellet that may have been formed (*i.e.* residual beads of protein A sepharose).

8. Add 4 µL proteinase K to the eluate and incubate overnight at 37°C. Also add 330 µL TE to the 'input' DNA sample (step 6, section 3.5) and add 4 µL proteinase K. Trest the 'input' sample similarly as the eluate obtained after ChIP.

9. Add 2 µL DNAse-free RNAse to the DNA samples and incubate 30 min at 37°C.

10. Add another 4 µL proteinase K and incubate at 65°C for at least 6 hrs to reverse crosslinks.

3.7 DNA Purification

1. Add 500 µL phenol:chloroform:isoamyl alcohol, vortex and transfer to a pre-spun Heavy Phaselock tube

2. Centrifuge at 12,000xg for 10 min at 4°C

3. Precipitate DNA with 1 µL glycogen, 0.1 Vol. sodium acetate pH5.4 and 2.5 Vol. ice-cold ethanol

4. Incubate overnight at -20°C

5. Spin at maximum speed 15 min at 4°C

6. Wash pellet by adding 80% ethanol and spin 5 min at maximum speed and 4°C

7. Remove any ethanol and dry pellet

8. Resuspend pellet in 50 µL 1xTE pH 8.0. Incubate 20 min at 37°C. If multiple immunoprecipitation reactions need to be pooled, transfer the solution from the first

tube into the next DNA pellet. Incubate again 20 min at 37°C and repeat until all pellets have been resuspended.

9. Measure the DNA concentration using 3 µL DNA and the Qubit (Note 16). This DNA is ready for library preparation without any additional purification step.

3.8 ChIP validation using quantitative PCR (qPCR)

Before sequencing the ChIP DNA, it is better to check the quality of the ChIP by qPCR, using oligonucleotides for positive regions (*e.g.* known binding sites) and for negative regions (regions of the genome not expected to be bound). For the latter, we usually use 4 different sets of primers (Table 1) and use the average of the signal obtained for these negative regions ('average background') to calculate the enrichment at a specific locus relative to the average background.

When designing primers for qPCR, aim at amplicons that are between 60 and 200 bases. We usually aim at a primer annealing temperature of 60°C, although this is not always possible.

1. Prepare a master mix containing both primers at a final concentration of 5 µM each
2. Dilute the ChIP DNA to 50 pg/µL and the input DNA to 5 ng/µL (Note 17)
3. Dilute the SYBR green (for one reaction, use 5 µL SYBR green and add 3 µL H_2O)
4. Combine the following reagents (for 1 reaction)

DNA	1 µL
Primer mix	1 µL
Diluted SYBR green	8 µL

5. Run the following qPCR program:

Initial denaturation (1 cycle): 95°C, 10 min

Quantification (45 cycles): 95°C, 10 sec; 60°C, 20 sec; 72°C; X sec (where X is determined by the size of the largest amplicon)

6. Determine the Cp values

7. Data analysis

For a specific region of the genome, the amount of DNA is normalized using the input sample, so that:

enrichment at a specific locus = percentage of input DNA relative to total DNA used for ChIP (using this protocol: 5%) × $2^{\Delta Cp}$, with $\Delta Cp = Cp_{input} - Cp_{ChIP}$

To calculate the enrichment relative to the average background:

(a) calculate average background = (sum of enrichment values for all negative regions) / (number of negative regions tested)

(b) Calculate the relative enrichment = (enrichment at locus of interest) / (average background)

3.9 Library Preparation for Illumina Sequencing

Once a satisfactory ChIP sample has been obtained (**Note 18**), a sequencing library can be prepared. The ultimate goal of the library preparation protocol is to attach adapter sequences to the ChIP DNA, which allows PCR amplification using adapter sequence-specific primers. The ChIP sample requires several steps of processing before amplification. First, single-stranded DNA overhangs that are present in the ChIP sample must be converted into phosphorylated blunt ends. This allows the addition of single base adenine overhangs to the 3` end of the DNA fragments. The DNA adapter sequences include single base thymine overhangs, which allow their ligation to the modified ChIP DNA fragments. After PCR amplification of the adapter-ChIP sample, the sample can be size-selected. Finally, the quality of library is

estimated by quantification of the DNA concentration, size verification of the amplified DNA and by qPCR. A detailed account of these steps is provided below.

1. To convert the single stranded DNA overhangs to blunt phosphorylated ends, combine the ChIP DNA (**Note 19**) and the following reagents as outlined below and incubate for 30 minutes at 20°C.

ChIP DNA	30 μl
Sterile Water	10 μl
T4 DNA ligase buffer with 10mM ATP	5 μl
10mM dNTP mix	2 μl
T4 DNA polymerase	1 μl
Klenow DNA polymerase	1 μl
T4 PNK	1 μl
Total reaction volume	50 μl

2. Purify the blunting reaction using a QIAquick PCR purification kit. Elute the purified DNA in 34 μl of EB.

3. To incorporate an adenine base to the 3` end of the blunt phosphorylated DNA fragments, combine the DNA from step 2 with the following reagents. Incubate the reaction for 30 minutes at 37°C.

Blunt phosphorylated ChIP DNA	34 μl
Klenow Buffer	5 μl
dATP	10 μl
Klenow exo (3` to 5` minus)	1 μl
Total reaction volume	50 μl

4. Purify Step 3 using a MinElute PCR purification kit. Elute the purified DNA in 10 μl of EB.

5. To ligate the adapter DNA sequences to the ChIP DNA, combine the DNA from step 4 to the following reagents and incubate for 15 min at room temperature (**Note 20**).

'A' overhang ChIP DNA	10 μl
DNA ligase buffer	15 μl
Diluted adapter mix	1 μl
DNA ligase	4 μl
Total reaction volume	30 μl

6. Purify Step 5 using a QIAquick PCR purification kit. Elute the purified DNA in 36 μl of EB.

7. For sequencing, suitable concentrations of DNA are required. Therefore, the ChIP sample must be amplified using a high fidelity enzyme and adapter-specific DNA oligonucleotides, as described below.

Adapter-DNA	36 μl
5x Phusion buffer	10 μl
10 mM dNTP mix	1.5 μl
PCR primer 1.1	1 μl
PCR primer 2.1	1 μl
Phusion polymerase	0.5 μl
Total reaction volume	50 μl

PCR amplification protocol: (a) 30 seconds at 98°C, (b) (10 seconds at 98°C, 30 seconds at 65°C, 30 seconds at 72°C) for 18 cycles, (c) 5 minutes at 72°C. (d) Hold at 4°C.

8. Make a 2% agarose gel using Certified Low Range Agarose and ethidium bromide. After adding loading dye, load the PCR mix directly into a lane of the gel. Load a 100 bp and a 1 kb ladder to estimate the size of the DNA fragments that were amplified. Run a low current through the gel for at least 6 h.

9. Gel extract the entire smear of DNA that has been amplified (**Note 21**).

10. Purify Step 9 using a QIAquick Gel extraction kit. Elute the purified DNA in 30 μl of EB.

11. Repeat step 8 (**Note 22**).

12. Repeat step 9.

13. Purify Step 12 using a QIAquick Gel extraction kit (**Note 23**). Elute the purified DNA in 15 μl of EB.

14. Measure the DNA concentration using 1 μl of DNA from step 13 and the Qubit.

15. Estimate the size range that has been extracted by using the Agilent Bioanalyzer with the Agilent High Sensitivity DNA Kit (**Note 24**).

16. Estimate the quality of the library as described in section 3.8 (**Note 25**).

17. Dilute the prepared library to 10 nM (**Note 26**). Prepare the flow-cell using 4 pM final concentration following the Illumina protocol.

4. Notes

1. Use stabilized formaldehyde (*e.g.* Sigma #252549) and do not use the stock solution if a precipitate has formed. Add the formaldehyde to the fixation buffer immediately before use.

2. Add hexylene glycol, 2-mercaptoethanol and the Complete Protease Inhibitor cocktail just before use.

3. Keep the Lysis buffer at room temperature, as the SDS tends to precipitate.

4. Protein A and protein G have different affinities for antibodies from different organisms (*e.g.* protein G has higher affinity for specific mouse IgGs, compared to protein A), so the type of resin needs to be chosen carefully depending on the antibody used to immunoprecipitate the protein of interest.

5. The quality of the antibody is essential for a successful ChIP assay. Many companies sell 'ChIP-grade' antibodies, but they need to be tested carefully, keeping in mind that different batches can vary greatly. Antibodies against the protein of interest can also be custom-made using the usual protocols. Their efficiency for ChIP needs to be tested carefully.

6. All buffers should be ice-cold, except the Lysis buffer.

7. The amount of tissue indicated is for inflorescence tissue collected from the floral induction system described in Chapter 17 of this book. ChIP assays can also be carried out using whole inflorescences, but this usually results in a lower DNA yield after chromatin preparation. Hence, the volume of tissue collected should be adjusted to the type of tissue used for ChIP.

8. Be sure to resuspend any tissue that has been lodged at the bottom of the tube. If tissue remains lodged in the lid, the tubes can be spun for 30 sec at 9,500×g and 4ºC before applying vacuum again.

9. After this step, the tissue can be stored at -80ºC for long-term storage.

10. You might want to let everything defrost slightly after freezing in liquid nitrogen.

11. The pellet contains the purified nuclei.

12. If the tissue has not been fixed properly, when resuspending the nuclei pellet with the Lysis buffer, the solution will become very viscous.

13. We find the sonication step works better using tubes from Diagenode (#M-50001). Keep the Bioruptor in the cold room and check the temperature of the water bath before shearing the chromatin. The starting temperature should be at 4ºC or slightly below but will increase once sonication has begun. The time of sonication can be adjusted depending on the size of the DNA obtained (*e.g.* increase the number of cycles if the average size of the DNA fragments obtained is too high).

14. A chromatin concentration over 50 ng/µL of chromatin per 0.5 mL of inflorescence-like tissue is normal. At this stage, the chromatin can be frozen at -80°C until further use.

15. The amount of antibody used, as well as the incubation time with the antibody, need to be optimized for each antibody. If the DNA yield is low at the end of the procedure, a longer incubation with the antibody (up to overnight at 4°C), higher amounts of antibody per ChIP can be tested. In general, we find that the quality of the antibody is the most important parameter.

16. The yield after immunoprecipitation depends on several factors, such as protein abundance, as well as quality and timing of the tissue collection. Using GFP-tagged transcription factors such as APETALA3, PISTILLATA or AGAMOUS, a total DNA yield over 2 ng is normal.

17. If the yield after ChIP is very low, the qPCR can be carried out with less DNA (down to 25 pg for the ChIP DNA and 1 ng for the input).

18. The fold-enrichments expected after ChIP can vary depending on the protein being investigated, the genomic regions that are being tested and the antibody being used. We found that average fold-enrichments of 10 (when comparing 16 different

positively bound regions to the average of 4 negatively bound regions) were sufficient to produce high quality sequencing libraries. Additionally, we have successfully prepared and sequenced ChIP libraries from as little as 5 ng total of starting ChIP DNA. However, a higher concentration, if available, is desirable. We normally aim for a starting concentration of 10 ng.

19. The phenol-chloroform and ethanol precipitated ChIP DNA can be used directly to generate the sequencing library. An additional column purification prior to end repair can be performed if desired, however, this step is unnecessary.

20. Allowing the ligation reaction to run for longer may increase the number of adapter-DNA molecules formed, however, our results indicate that 15 minutes at room temperature is sufficient. If the ligation reaction time is increased, incubate the tubes at 16°C. We have successfully used as little as a 1:40 dilution of the adapter mix to prepare the sequencing libraries, however, a 1:10 dilution has also been used successfully. The choice of dilution should be based on the starting concentration of DNA used and practice rounds using INPUT DNA, if possible.

21. We found that size-selecting pre-defined ranges of DNA produced poorer quality libraries. Therefore, we advise that the entire DNA smear be extracted. The largest range of DNA we ultimately sequenced ranged from 150 bp to 500 bp. When visualizing the PCR-amplified DNA, oligonucleotide dimers and adapters are often visible at the bottom of the gel. If the oligonucleotide dimers or adapters are very close to the DNA you wish to extract, allow the DNA to migrate further.

22. A second gel extraction dramatically reduces the presence of oligonucleotide dimers and adapters.

23. Do not heat the gel in order to dissolve it. The column will need to be loaded several times with the dissolved gel. After the dissolved gel solution has been filtered

through the binding column, add 500 μL of Buffer QG to the column and discard the flow-through. Resume the Qiagen gel extraction protocol.

24. Oligonuleotide dimers and adapters can be observed and their concentration relative to the rest of the sample can be estimated by using the Agilent Bioanalyzer with the DNA High Sensitivity Kit. After the second gel extraction, contaminations should be at a minimum.

25. We have observed a compression of fold-enrichment values once the sequencing library has been prepared, relative to the starting material used. If possible, a sequencing library should also be prepared with a negative control ChIP or the INPUT DNA. This will normalize for any sequence-specific problems.

26. Use the average size of DNA indicated on the Bioanalyzer to estimate the concentration.

5. References

1. Kaufmann K*, et al.* (2010) Regulation of transcription in plants: mechanisms controlling developmental switches. *Nature reviews. Genetics* 11(12):830-842.
2. Bowman JL*, et al.* (1991) Genetic interactions among floral homeotic genes of Arabidopsis. *Development* 112(1):1-20.
3. Krizek BA & Fletcher JC (2005) Molecular mechanisms of flower development: an armchair guide. *Nature reviews. Genetics* 6(9):688-698.
4. Solomon MJ & Varshavsky A (1985) Formaldehyde-mediated DNA-protein crosslinking: a probe for in vivo chromatin structures. *Proc Natl Acad Sci U S A* 82(19):6470-6474.
5. Metzker ML (2010) Sequencing technologies - the next generation. *Nature reviews. Genetics* 11(1):31-46.
6. Johnson DS*, et al.* (2007) Genome-wide mapping of in vivo protein-DNA interactions. *Science* 316(5830):1497-1502.
7. Kaufmann K*, et al.* (2010) Orchestration of floral initiation by APETALA1. *Science* 328(5974):85-89.
8. Yant L*, et al.* (2010) Orchestration of the floral transition and floral development in Arabidopsis by the bifunctional transcription factor APETALA2. *Plant Cell* 22(7):2156-2170.
9. Wuest SE*, et al.* (2012) Molecular basis for the specification of floral organs by APETALA3 and PISTILLATA. *Proc Natl Acad Sci U S A* 109(33):13452-13457.

10. Gerstein MB*, et al.* (2012) Architecture of the human regulatory network derived from ENCODE data. *Nature* 489(7414):91-100.
11. Morohashi K & Grotewold E (2009) A systems approach reveals regulatory circuitry for Arabidopsis trichome initiation by the GL3 and GL1 selectors. *PLoS genetics* 5(2):e1000396.
12. Kaufmann K*, et al.* (2009) Target genes of the MADS transcription factor SEPALLATA3: integration of developmental and hormonal pathways in the Arabidopsis flower. *PLoS biology* 7(4):e1000090.
13. Mathieu J*, et al.* (2009) Repression of flowering by the miR172 target SMZ. *PLoS biology* 7(7):e1000148.
14. Deal RB & Henikoff S (2010) A simple method for gene expression and chromatin profiling of individual cell types within a tissue. *Developmental cell* 18(6):1030-1040.

Figure Captions

Figure 1. Overview of the ChIP procedure. Different steps of the ChIP-Seq protocol are outlined, including a timeline for the procedure. Steps shaded in grey usually require optimization, depending on the protein of interest and the antibody used.

Figure 2. Gel electrophoresis of fixed chromatin after fragmentation by sonication. A 1.5% agarose gel was used to separate the sheared chromatin. The size of the DNA ladders are indicated on either side of the gel. The average size for the chromatin is around 300 bp.

Tables

Table 1: genomic regions and primers used to calculate the average background (9).

Gene ID	Alias	Forward primer (5’ -> 3’)	Reverse primer (5’ -> 3’)
At4g26930	REF1	TCTCCGACCTTTCTTCACACCCATTCC	GTCTCCGCTTAGGAGCACGAAAGCTATC
At4g39400	BRI1	ACCCAGCACTAAACAGAAGATCAG	CCCAACCACCTATCTCTTGATTCTC
At5g09810	ACTIN	CGTTTCGCTTTCCTTAGTGTTAGCT	AGCGAACGGATCTAGAGACTCACCTTG
At4g03870	Mu	GATTTACAAGGAATCTGTTGGTGGT	CATAACATAGGTTTAGAGCATCTGC