Mapping Regulatory Factors by Immunoprecipitation from Native Chromatin

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Occupied Regions of Genomes from Affinity-purified Naturally Isolated Chromatin (ORGANIC) is a high-resolution method that can be used to quantitatively map protein-DNA interactions with high specificity and sensitivity. This method uses micrococcal nuclease (MNase) digestion of chromatin and low-salt solubilization to preserve protein-DNA complexes, followed by immunoprecipitation and paired-end sequencing for genome-wide mapping of binding sites. In this unit, we describe methods for isolation of nuclei and MNase digestion of unfixed chromatin, immunoprecipitation of protein-DNA complexes, and high-throughput sequencing to map sites of bound factors.
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INTRODUCTION

Mapping transcription factor (TF) binding sites is crucial to understanding gene regulatory networks. The majority of current genome-wide TF maps were obtained by cross-linked chromatin immunoprecipitation followed by high-throughput sequencing (X-ChIP-seq), using cross-linking reagents such as formaldehyde to preserve TF-DNA interactions. However, recent work has demonstrated that X-ChIP-seq can capture artifactual or transient nonspecific protein-DNA interactions, leading to false positive sites (Neph et al., 2012; Park et al., 2013; Poorey et al., 2013; Teytelman et al., 2013; Worsley Hunt and Wasserman, 2014). Here we describe a protocol for mapping TFs in *S. cerevisiae* and *D. melanogaster* cell lines using native chromatin immunoprecipitation (N-ChIP) called Occupied Regions of Genomes from Affinity-purified Naturally Isolated Chromatin (ORGANIC). By circumventing the need for cross-linking reagents and sonication, this procedure is selective for stable, direct protein-DNA interactions. ORGANIC profiling has been used to map nucleosomes (Henikoff and

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Henikoff, 2012; Krassovsky et al., 2012), RNA polymerase II (Teves and Henikoff, 2011), chromatin remodelers (Zentner and Henikoff, 2013; Zentner et al., 2013), TFs (Kasinathan et al., 2014), and TF-bound complexes (Orsi et al., 2014). Previous work has demonstrated that ORGANIC resolves the location of TFs at high resolution and provides details of multifactor complexes at binding sites (Kasinathan et al., 2014; Orsi et al., 2014). ORGANIC is also simple and relatively inexpensive and can thus be easily adopted.

The different sections in this unit describe the steps to perform ORGANIC, including DNA sequencing library construction, from *S. cerevisiae* and *Drosophila* cells. Basic Protocol 1 describes the procedure for N-ChIP of TFs from *S. cerevisiae*. Basic Protocol 2 analogously details the steps to perform N-ChIP from *Drosophila* cells. Basic Protocol 3 focuses on building barcoded libraries for paired-end sequencing. Finally, the Support Protocol provides a method to enrich immunoprecipitated samples for small DNA fragments corresponding to TF-bound sites.

BASIC PROTOCOL 1

NATIVE CHROMATIN IMMUNOPRECIPITATION OF TRANSCRIPTION FACTORS IN *S. CEREVISIAE*

This protocol describes the procedure for native chromatin immunoprecipitation of TF-DNA complexes in *S. cerevisiae*. Our previous work (Kasinathan et al., 2014) demonstrated that this method can be used to obtain stringent maps of transcription factor binding with high resolution.

Materials

Phosphate-buffered saline (PBS; see recipe) containing 0.5% bovine serum albumin (BSA)
Anti-FLAG M2 magnetic beads (Sigma-Aldrich, cat. no. A2220)
Saccharomyces cerevisiae growing in culture
YPD culture medium
Resuspension buffer (see recipe)
Zymolyase (100 T equivalent, ZymoResearch, cat. no E1004)
SPC buffer (see recipe) with and without protease inhibitors, cold
Ficoll buffer (see recipe), cold
Liquid N_2
1 M $CaCl_2$ (see recipe)
Sigma MNase (prepare 1 U MNase/5 µl nuclease free water; see recipe for 1 U/µl stock)
0.5 M EDTA, pH 8.0 (see recipe)
80 mM, 150 mM, or 600 mM NaCl extraction buffer (see recipe)
5 M NaCl (see recipe)
10% (v/v) Triton X-100
IP wash buffer (see recipe)
250 U/µl Benzonase (Sigma, cat. no. E1014)
10 mg/ml RNase A (Thermo Scientific, cat. no. EN0531)
10% (w/v) sodium dodecyl sulfate (SDS)
20 mg/ml proteinase K (Life Technologies, cat. no. AM2542)
25:24:1 phenol/chloroform/isoamyl alcohol (*UNIT 2.1A*)
Chloroform
20 mg/ml glycogen (Life Technologies, cat. no. 10814-010)
100% ethanol and 70% ethanol
$TE_{0.1}$ buffer (see recipe)
Quant-iT PicoGreen dsDNA assay kit (Life Technologies, cat. no. P11496)

DynaMag-2 magnetic microcentrifuge tube rack (Life Technologies)
Low-retention 50- and 15-ml conical tubes, 1.5-ml microcentrifuge tubes, and pipet tips
Nutator or end-over-end rotator in refrigerated 4°C room
500 ml Nalgene PPCO centrifuge bottle with sealing enclosure (Thermo Scientific, cat. no. 3141-0250)
DuPont Sorvall RC-5B refrigerated centrifuge, or equivalent
Refrigerated centrifuge with rotor adapters for 50-ml and 15-ml conical tubes
Refrigerated microcentrifuge
Spectrophotometer
Liquid N_2 container
20- and 26-G needles with 5-ml syringe
70°C and 100°C (boiling) water baths

Additional reagents and equipment for counting yeast cells [*UNIT 13.2* (Treco and Winston, 2008) and *UNIT 1.2* (Elbing and Brent, 2002)] and phenol extraction/ethanol precipitation of DNA (*UNIT 2.1A*; Moore and Dowhan, 2002)

Prepare beads for immunoprecipitation

1. Prior to beginning nuclei isolation, prepare beads for immunoprecipitation by washing 100 µl of either anti-FLAG or other magnetic beads (e.g., protein A and/or protein G beads, if TF of interest is not FLAG tagged) three times, each time using 1 ml PBS containing 0.5% BSA in a microcentrifuge tube, by removing the supernatant with the beads immobilized on a magnetic microcentrifuge tube rack.
2. Resuspend beads in 250 µl PBS containing 0.5% BSA. Add desired amount of antibody (if not using anti-FLAG magnetic beads).
3. Incubate beads for at least 4 hr at 4°C with agitation on a Nutator.

Isolate nuclei

4. Grow 500 ml *S. cerevisiae* culture to $OD_{600} = 0.6$ to 0.8 in YPD medium (see *UNITS 13.2 & 1.2*).
5. Transfer culture to centrifuge bottles.
6. Centrifuge 10 min at 2,700 × *g*, 4°C.
7. Discard the supernatant, wash cell pellet with 25 ml deionized water, and transfer to a 50-ml conical tube.
8. Centrifuge 10 min at 2,700 × *g*, 4°C.
9. Resuspend cells in 30 ml resuspension buffer.
10. Incubate for 15 min in 37°C water bath.
11. Add 500 µl 1 mg/ml Zymolase and incubate for 10 min in 37°C water bath.

    The extent of spheroplasting can be monitored by removing 20-µl aliquots of the spheroplasting reaction into 1 ml of 1% SDS and measuring OD_{600} (versus a 1% SDS blank). Spheroplasting is typically complete when OD_{600} is 10% to 20% of the starting value.

12. Centrifuge 5 min at 1000 × *g*, 4°C.
13. Wash pellet with 25 ml cold SPC buffer with protease inhibitors.
14. Centrifuge 5 min at 1000 × *g*, 4°C. Discard supernatant.
15. Repeat steps 13 and 14 once. Resuspend pellet in 0.5 ml SPC buffer without protease inhibitors.

16. Mix with 25 ml cold Ficoll buffer.
17. Centrifuge 10 min at 7500 × *g*, 4°C.
18. Wash pellet with 10 ml cold SPC buffer with protease inhibitors.
19. Centrifuge for 10 min at 4,200 × *g*, 4°C.
20. Repeat steps 16 and 17 once. Resuspend nuclei in 5 ml SPC with protease inhibitors.
21. Snap-freeze in liquid N_2.

    At this point, nuclei can be stored at −80°C.

Perform MNase digestion and chromatin extraction

22. Thaw nuclei samples on ice.
23. Prewarm samples for 5 min in a 37°C water bath.

    When processing multiple samples, move each sample to the water bath at 30-sec intervals.

24. Add 1 M $CaCl_2$ to 2 mM.
25. Add 2 U Sigma MNase. Mix by careful inversion.
26. Repeat steps 24 and 25 for each additional sample.
27. Incubate for 2.5 to 10 min at 37°C. Mix by careful inversion every 1 to 2 min.

    The MNase digestion time needs to be empirically defined depending on the factor of interest and the desired mapping resolution. See Figure 21.31.1 and the Commentary section below.

28. Stop the digestion reaction by adding 150 µl 0.5 M EDTA. Let chill on ice for 3 min.

    Stop the digestion reaction in each tube sequentially until all tubes are resting on ice.

29. While on ice, pass slurry four times through a 20-G needle, then four times through a 26-G needle.
30. Centrifuge 10 min at 9,400 × *g*, 4°C.
31. Collect the supernatant (fraction "S1") and keep on ice.
32. Resuspend the cell pellet in 5 ml 80, 150, or 600 mM NaCl extraction buffer and incubate for 4 hr at 4°C.

    The optimal salt concentration must be determined empirically for each TF of interest and greatly influences the final result. See the Commentary.

33. Centrifuge for 15 min at 15,800 × *g*, 4°C.
34. Collect the supernatant (fraction "S2") and keep on ice. Discard the pellet.
35. Adjust the NaCl and Triton X-100 concentrations of fraction S1 to match fraction S2 by adding the appropriate amounts of 5 M NaCl and 10% Triton X-100.
36. Combine fractions S1 and S2.

    A 50-µl aliquot can be used as an input protein sample and 100 µl can reserved as an input DNA sample.

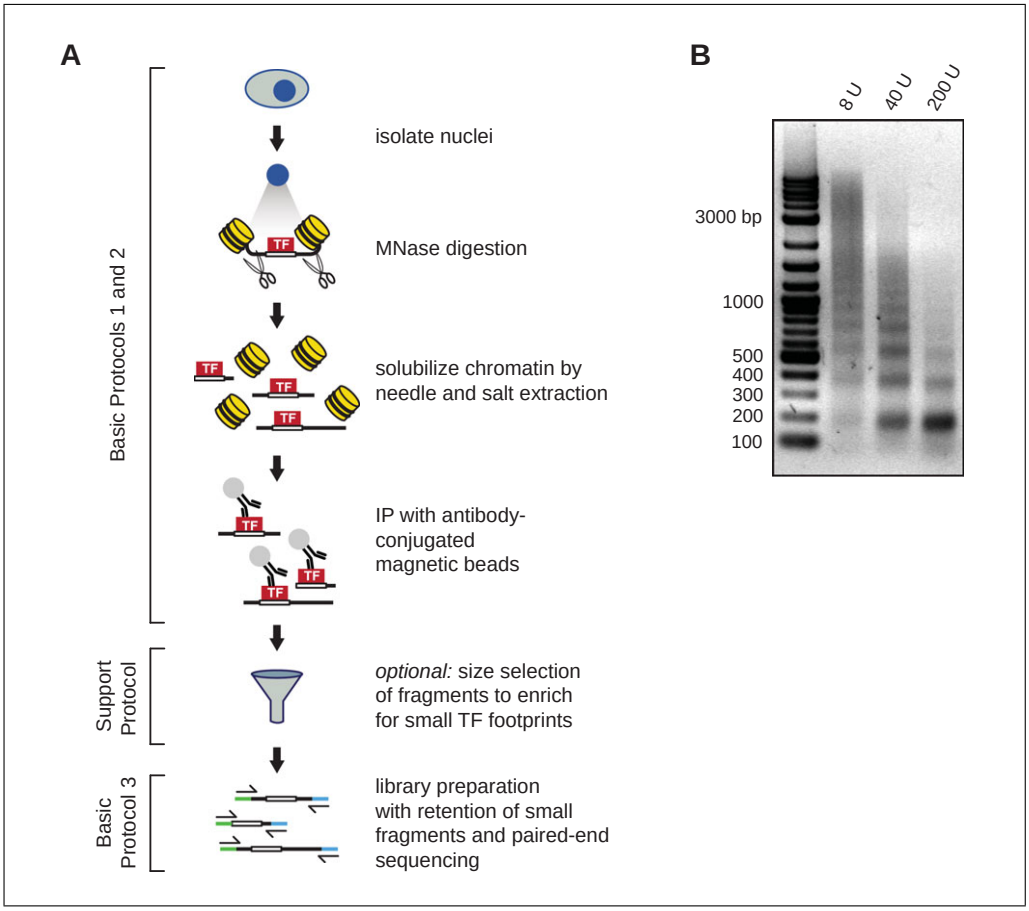

Figure 21.31.1 Overview of ORGANIC protocol and MNase digestion ladder. (**A**) ORGANIC profiling workflow. (**B**) Visualization of nucleosomal ladder and sub-nucleosomal fragments from *Drosophila* nuclei treated with increasing amounts of MNase. Digestion with 200 U produced an ideal ladder, with >75% mono-nucleosomal fragments, and a clear smear of sub-nucleosomal DNA.

Perform chromatin immunoprecipitation

37. Spin down magnetic beads prepared at the beginning of the protocol briefly at 500 × *g*, 4°C, remove supernatant, and combine magnetic beads and chromatin sample.
38. Incubate overnight at 4°C with agitation on a Nutator or end-over-end rotator.
39. Place samples on a magnetic microcentrifuge tube rack to collect beads. Discard the supernatant.

    Alternatively, the supernatant can be reserved as an unbound sample.
40. Resuspend beads in 1 ml IP wash buffer, transfer to a low-retention 1.5-ml microcentrifuge tube, place on a magnetic microcentrifuge tube rack to immobilize the beads, and remove the supernatant.
41. Wash beads twice by resuspending in 1 ml IP wash buffer, placing on a magnetic microcentrifuge tube rack to immobilize them, and removing the supernatant.
42. Resuspend beads in 400 µl IP wash buffer.

    At this point IP samples are ready for DNA or protein purification. As DNA yields are typically low, we recommend splitting the sample to keep the volume for protein preparation to a minimum.
43. Add 0.5 µl 250 U/µl Benzonase to the protein sample and incubate 20 min at 37°C. Then, add 1/4 volume of 4 × SDS sample buffer and heat in a 100°C

water bath for 10 min. Place in magnet to remove beads and store supernatant at −20°C.

44. Bring the volume of the DNA sample to 400 µl with IP wash buffer. Add 8 µl of 0.5 M EDTA, 8 µl of 5 M NaCl, and 1 µl of 10 mg/ml RNase A. Incubate 20 min at 37°C.

    See UNIT 2.1A *(Moore and Dowhan, 2002) for additional detail on DNA purification*

45. Add 20 µl of 10% SDS and 4 µl of 20 mg/ml proteinase K. Incubate 20 min at 70°C.

46. Add 1 volume of 25:24:1 phenol/chloroform/isoamyl alcohol. Vortex 30 sec, and centrifuge 5 min at 15,000 × *g*, 4°C.

47. Carefully pipet off the supernatant, being careful not to disrupt the organic layer, and transfer to a new tube.

    Phenol and chloroform can inhibit downstream reactions. Minimize contamination by leaving a small volume of aqueous layer.

48. Add 1 volume of chloroform. Vortex 30 sec, and centrifuge at 15,000 × *g*, 4°C. Carefully pipet off the supernatant and transfer to a new tube.

49. Add 2 µl of 20 mg/ml glycogen. Precipitate by adding two volumes of 100% ethanol at −80°C for at least 30 min.

50. Centrifuge 10 min at 18,000 × *g*, 4°C.

51. Wash with 1 ml 100% ethanol, being careful not to disrupt the pellet, and centrifuge again for 10 min at 18,000 × *g*, 4°C.

52. Remove supernatant and let air dry for 10 min.

53. Resuspend sample in 25 µl $TE_{0.1}$ buffer.

54. Measure concentration with a high-sensitivity assay (e.g., QuantIt PicoGreen dsDNA assay).

    The total amount of DNA expected is in the range of 3 to 100 ng, with concentrations as low as 0.2 ng/µl. We recommend using Quant-IT Picogreen as a high-sensitivity assay to measure DNA concentration in that range.

BASIC PROTOCOL 2

NATIVE CHROMATIN IMMUNOPRECIPITATION OF TRANSCRIPTION FACTORS IN *DROSOPHILA* CULTURED CELLS

The following protocol describes the native chromatin immunoprecipitation procedure starting with *Drosophila* cultured cells. Note that although the principles are essentially the same as those described in Basic Protocol 1 for yeast cells, the procedure itself is considerably different with regard to nuclei isolation and chromatin sample preparation.

Materials

Drosophila S2 cells (Drosophila Genomics Resource Center, DGRC)
Complete Schneider's medium (see recipe)
Phosphate-buffered saline (PBS; see recipe)
TM2+ buffer (see recipe)
10% (v/v) NP-40
TM2+I buffer (see recipe)
0.2 M $CaCl_2$ (see recipe)
40 U/µl USB Affymetrix MNase (see recipe)
0.2 M EGTA

TM2+IS buffer (see recipe)
80TM+IS buffer (see recipe)
Antibody directed against the regulatory factor of interest (the antibody efficiency may depend on antigen accessibility in native chromatin, and must be determined on a case-by-case basis; mouse, rat, and rabbit IgG antibodies are captured by protein G-coupled beads)
Protein G-coupled Magnetic Beads (Dynabeads, Life Technologies, cat no 10004D)
250 U/μl Benzonase (Sigma, cat. no. E1014)
4× SDS sample buffer (Life Technologies, cat no. NP0007)
0.5 M EDTA (see recipe)
5 M NaCl (see recipe)
10 mg/ml RNase A (Thermo Scientific, cat no. EN0531)
10% (w/v) sodium dodecyl sulfate (SDS)
20 mg/ml proteinase K (Life Technologies, cat. no. AM2542)
25:24:1 phenol/chloroform/isoamyl alcohol (*UNIT 2.1A*)
Chloroform
3 M sodium acetate (*APPENDIX 2*)
20 mg/ml glycogen (Life Technologies, cat. no. 10814-010)
100% and 70% ethanol
$TE_{0.1}$ buffer (see recipe)
Quant-iT PicoGreen dsDNA assay kit. (Life Technologies, cat. no. P11496)

75-cm^2 (T-75) culture flasks
Cell scrapers
Hemacytometer or automated cell counter
15- and 50-ml conical tubes
Refrigerated centrifuge with adaptors for 50-ml conical tubes, 15-ml conical tubes, and 1.5-ml microcentrifuge tubes
Low-retention 1.5-ml microcentrifuge tubes and pipet tips
37°C heating block or water bath
26-G ½-in. needle with 1-ml syringe
Nutator
DynaMag-2 magnetic microcentrifuge tube rack (Life Technologies)
65° and 100°C (boiling) water baths

Additional reagents and equipment for counting cells (*UNTI 1.2*; Elbing and Brent, 2002) and phenol extraction/ethanol preciptitation of DNA (*UNIT 2.1A*; Moore and Dowhan, 2002)

Isolate nuclei

1. Grow *Drosophila* S2 cells in 75-cm^2 (T-75) flasks with 15 ml Complete Schneider's medium.

   Grow cells to late log phase. Depending on cell density, one to two flasks will be needed for each chromatin sample.

2. Collect cells using a scraper and resuspend cells by gentle pipetting with a serological pipet.

3. Count cells using a hemacytometer (*UNIT 1.2*; Elbing and Brent, 2002) or an automated cell counter.

4. Transfer 2×10^8 cells to a 50-ml conical tube.

   Prepare as many 2×10^8–cell aliquots as needed for immunoprecipitation. Because of time-sensitive steps, we recommend processing no more than four samples at a time. Once chromatin is prepared (step 24), additional samples can be processed.

5. Centrifuge the cells for 4 min at 1200 × *g*, 4°C, then wash the pellet with 10 ml cold PBS.
6. Centrifuge the cells again for 4 min at 1200 × *g*, 4°C, then resuspend in 2 ml ice-cold TM2+ buffer.
7. Transfer to a 15-ml conical and chill in ice for 1 min.
8. To release nuclei, add 120 µl 10% NP-40 and vortex gently at half maximum speed.
9. Let sit on ice for 3 to 5 min, vortexing at half maximum speed once per minute.

   The time needed for nuclei isolation varies depending on each cell type and should be determined empirically. Release of nuclei can be checked by light microscopy. If nuclei isolation is reliable and robust, this step can be timed. For Drosophila S2 and S2R+ cells, 3 min, 30 sec has proven optimal. For Drosophila BG3 cells, 4 min is optimal.

10. Centrifuge 10 min at 100 × *g*, 4°C. Discard supernatant.

    The nuclei pellet is loose and easily dislodged. Aspirate supernatant carefully without tilting the tube or decanting.

11. Resuspend in 2 ml TM2+ buffer by pipetting up and down a 1-ml (P1000) pipet tip.

    The nuclei pellet should be readily resuspended after 5 to 10 aspirations and form a translucent, homogeneous solution. If resuspension is difficult and clumps are seen in the solution, that may indicate excessive NP-40 treatment. Consider reducing the incubation time and using milder vortexing at step 9.

12. Centrifuge again 10 min at 100 × *g*, 4°C, and discard supernatant carefully. Resuspend in 800 µl TM2+I buffer. Transfer this nuclei sample to a 1.5-ml microcentrifuge tube.

Perform MNase digestion and chromatin preparation

13. Prewarm nuclei samples for 3 min on 37°C heat block or water bath.

    When processing multiple samples, move samples to the heating block at 30-sec intervals.

14. Add 4 µl 0.2 M $CaCl_2$. Quickly add 5 µl of 1 U/ 5 µl USB Affymetrix MNase. Mix by careful inversion.

    The mixture should be an opaque white homogeneous suspension. Precipitates or clumps can form when nuclei were improperly released (step 9), and this is predictive of a poor-quality digest.

15. Repeat step 14 for each additional sample.
16. Incubate each sample for precisely 6 min. Mix by careful inversion every 2 min.

    Extent of MNase digestion is a critical parameter and should be determined empirically as outlined in the Commentary section. An appropriate digest is shown in Figure 21.31.1.

17. Stop the digestion by adding 8 µl 0.2 M EGTA, mix well by inversion, and chill on ice for at least 2 min.

    Stop the digestion reactions in each tube sequentially until all tubes are resting on ice.

18. Centrifuge all samples for 10 min at 100 × *g*, 4°C.
19. Carefully aspirate supernatant. Resuspend pellet with 800 µl TM2+IS.
20. Centrifuge 10 min at 100 × *g*, 4°C.
21. Resuspend pellet in 400 µl of 80TM+IS buffer. Keep on ice.

22. Cavitate nuclei by drawing up and expelling the suspension with a 26-G, ½-in. needle.

    Draw up and expel firmly but slowly, to avoid foaming.

23. Centrifuge 10 min at 100 × *g*, 4°C.

24. Transfer 300 µl of the supernatant to a clean tube: this is the chromatin input.

    An additional 30 µl of supernatant can be collected as a DNA or protein input sample. To prepare DNA or proteins, refer to steps 31 to 43.

Chromatin immunoprecipitation

25. Add appropriate amount of antibody to the 300 µl chromatin sample.

    The amount depends on each antibody and must be determined empirically. A number of antibodies yield good results when using 1 to 5 µg for each immunoprecipitation reaction.

26. Incubate samples with agitation on a Nutator overnight at 4°C.

27. Combine 40 µl of protein G–coupled magnetic bead slurry in 500 µl 80TM+IS buffer. Let the solution equilibrate at room temperature for 3 min, capture beads on a magnetic rack for microcentrifuge tubes, remove buffer, and repeat this step.

28. Add chromatin sample to beads. Carefully resuspend beads and chromatin by pipetting up and down with a 1-ml (P1000) pipet tip, and incubate with agitation on Nutator for 2 hr at 4°C.

29. Collect beads in magnetic microcentrifuge tube rack and discard supernatant.

    Alternatively, the supernatant can be conserved as an unbound DNA or protein sample.

30. Briefly wash beads twice with cold 80TM+IS buffer. Resuspend beads in 50 µl 80TM+IS. Split the sample to prepare DNA and proteins.

    At this point IP samples are ready to purify DNA or protein. If sample is to be split to separately purify DNA and protein, we recommend reserving 10% of the sample for protein purification and 90% for DNA purification, as the DNA yields tend to be low.

31. To the protein sample, add 0.5 µl 250 U/µl Benzonase and incubate 20 min at 37°C. Add 1/4 volume of 4× SDS sample buffer and heat in a 100°C water bath for 10 min. Place in magnetic microcentrifuge tube rack to remove beads, and store supernatant at −20°C for later protein analysis.

32. Bring the volume of the DNA sample to 400 µl with 80TM+IS buffer. Add 8 µl 0.5 M EDTA, 8 µl 5 M NaCl, and 1 µl 10 mg/ml RNase A. Incubate 20 min at 37°C.

33. Add 20 µl of 10% SDS and 8 µl of 20 mg/ml proteinase K. Incubate 10 min at 65°C.

34. Add 1 volume of 25:24:1 phenol/chloroform/isoamyl alcohol. Vortex 30 sec, centrifuge 5 min at 15,000 × *g*, 4°C.

    See UNIT 2.1A (Moore and Dowhan, 2002) for additional detail on DNA purification.

35. Carefully pipet off the supernatant, being careful not to disrupt the organic layer, and transfer to a new tube.

    Phenol and chloroform can inhibit downstream reactions. Minimize contamination by leaving a small volume of aqueous layer.

36. Add 1 volume of chloroform. Vortex 30 sec, then centrifuge 5 min at 15,000 × *g*, 4°C.

37. Carefully pipet off the supernatant and transfer to a new tube.
38. Add 1/10 volume of 3 M sodium acetate and 1 μl of 20 mg/ml glycogen. Precipitate with 1 ml of 100% ethanol at −20°C overnight.
39. Centrifuge 10 min at 18,000 × *g*, 4°C.
40. Wash with 70% ethanol, being careful not to disrupt pellet, then centrifuge again for 10 min at 18,000 × *g*, 4°C.
41. Remove supernatant and let air dry for 30 min.
42. Resuspend sample in 25 μl $TE_{0.1}$ buffer.
43. Measure concentration with a high-sensitivity assay (e.g., QuantIt PicoGreen dsDNA assay).

The total amount of DNA expected is in the range of 3 to 100 ng, with concentrations as low as 0.5 ng/μl. We recommend using PicoGreen as a high-sensitivity assay to measure DNA concentration in that range.

BASIC PROTOCOL 3

LIBRARY PREPARATION FOR PAIRED-END ILLUMINA SEQUENCING

The following section describes the protocol used for preparation of libraries suited for Illumina paired-end sequencing. Particular steps are taken to retain short DNA fragments that are generally lost or selected against in other protocols. The procedure is efficient and reproducible, and can be used to build libraries from as little as 3 ng of total input DNA.

NOTE: Low-retention tubes and micropipettor tips should be used throughout library construction to minimize sample loss.

Materials

Commercial paired-end adapters and PCR primers from Illumina TruSeq (or custom-made adapters and primers can be used); adapters and primer sequences are as follows:

TruSeq Universal Adapter: 5′-AATGATACGGCGACCACCGAGATCTAC ACTCTTTCCCTACACGACGCTCTTCCGATC*T

TruSeq Indexed Adapter: 5′-[P]GATCGGAAGAGCACACGTCTGAACT CCAGTCACNNNNNNATCTCGTATGCCGTCTTCTGCTT*G

Library Enrichment Primers:

P5 PCR Primer: 5′ AATGATACGGCGACCACCGAG

P7 PCR Primer: 5′ CAAGCAGAAGACGGCATACGAG

In the above sequences [P] indicates a 5′-phosphate group on the indexed adapter; * indicates a phosphorothioate bond between the two last bases at the 3′ end of adapters; NNNNNN indicates a 6-base barcode for multiplexing sequencing reactions

TE buffer (*APPENDIX 2*)

UltraPure, nuclease free water

10× T4 ligase buffer (New England Biolabs, cat. no. B0202S) containing 10 mM dATP

dNTP mix: 10 mM each dNTP (Life Technologies, cat. no. 18427-013; also see *APPENDIX 2*)

5 U/μl T4 DNA polymerase (Invitrogen, cat. no.100004994)

5 U/μl Klenow fragment (DNA polymerase I large fragment; New England Biolabs, cat. no. M0210L)

10 U/μl T4 PNK (New England Biolabs, cat. no. M0201L)

25:24:1 phenol/chloroform/isoamyl alcohol (*UNIT 2.1A*; Moore and Dowhan, 2002)
Illustra microspin HR S-300 columns (GE Healthcare Life Sciences, cat. no. 27-5130-01)
10× NEBuffer 2 (New England Biolabs, cat. no. B7002S)
1 mM dATP (see recipe)
Klenow fragment (3′→5′ exo-; New England Biolabs, cat. no. M0212M)
Nuclease-free H_2O
2× Rapid Ligase Buffer (Enzymatics, cat. no. B1010L)
Rapid DNA Ligase (Enzymatics, cat. no. L6030-HC-L)
AMPure XP magnetic beads (Agencourt, cat. no. A63880)
70% ethanol
$TE_{0.1}$ buffer (see recipe)
KAPA HiFi HotStart DNA Polymerase with dNTPs (KAPA Biosystems, cat. no. KK2501)

Thermal cycler
Low-retention 1.5-ml and 0.5-ml microcentrifuge tubes and pipet tips
20°C water bath
SpeedVac evaporator
DynaMag-2 magnetic microcentrifuge tube rack (Life Technologies)

Prepare adapters

1. Mix equimolar amounts of TruSeq Universal Adapter and TruSeq Indexed Adapter in 1× TE buffer.

2. To anneal Universal and Indexed adapters, incubate the mix in a thermal cycler with the following program:

   Step 1: 95°C for 30 min
   Step 2: 70°C for 15 min.

3. Allow mixture to cool at room temperature for 15 min.

   Annealed adapters can be prepared in advance and stored at −20°C.

End repair

4. Use 3 to 100 ng of the DNA from step 3 for library preparation. Adjust volume of the sample to 38.5 µl with UltraPure, nuclease-free water.

   We recommend using the maximum amount of DNA possible. Low amounts of DNA will require additional cycles of library PCR amplification, which can lead to amplification biases.

5. Prepare the end repair master mix by combining the following for each sample:

   5 µl T4 ligase buffer with 10 mM dATP
   2 µl dNTP mix (10 mM each)
   1.5 µl T4 DNA polymerase (5 U/µl)
   0.5 µl Klenow fragment (DNA polymerase I, large fragment; 5 U/µl)
   2.5 µl T4 PNK (10 U/µl).

   Mix by careful pipetting, as Klenow fragment is sensitive to vortexing.

6. Add 11.5 µl of end repair master mix from step 5 to 38.5 µl of DNA. Mix by carefully pipetting up and down.

   Keep samples on ice while adding end repair mix to avoid uneven catalytic activity, which can lead to reduced quality of the end product.

7. Incubate in 20°C water bath for 30 min.

   Fill a thermally isolated bucket with fresh running water. This should be ~20°C, check temperature with a thermometer and adjust with ice water if necessary.

8. Add 50 µl 25:24:1 phenol/chloroform/isoamyl alcohol.

   For additional detail on DNA purification, see UNIT 2.1A (Moore and Dowhan, 2002).

9. Vortex ~30 sec.

10. Centrifuge 1 min at 18,000 × *g*, 4°C.

11. Prepare S-300 column according to manufacturer's instructions: Vortex to resuspend resin, snap off the bottom end of the column, loosen cap a quarter turn, insert column into waste tube, and centrifuge 1 min at 735 × *g*. Transfer column to a new 1.5-ml microcentrifuge tube, being careful not to disrupt the resin.

12. Pipet up the aqueous layer.

    It is important to avoid organic layer contamination. If organic solution is pipetted, it will settle at the end of the tip. Touch the tip to the side of the microcentrifuge tube to remove remaining organics from the aqueous.

13. Add the aqueous layer to the middle of the resin on the prepared S-300 spin column and centrifuge 2 min at 735 × *g*, 4°C.

14. Reduce volume to 35 µl in a SpeedVac evaporator with no heat.

    This takes 5 to 15 min. Compare the volume to a tube containing 35 µl. If short, bring to 35 µl volume with nuclease-free water.

    At this point, samples can be stored at −20°C.

Add 3′-A overhang

15. Prepare the 3′-A overhang addition (A-tailing) master mix by combining the following for each sample:

    5 µl 10× NEBuffer 2
    10 µl of 1 mM dATP
    0.3 µl Klenow 3′→5′ exo- fragment.

16. Add 15 µl of A-tailing master mix to 35 µl of DNA. Mix by carefully pipetting up and down.

    Hold samples on ice while adding 3′-A overhang addition mix to avoid uneven catalytic activity, which can lead to reduced quality of the end product.

17. Incubate at 37°C for 30 min

18. Extract with 25:24:1 phenol/chloroform/isoamyl alcohol and clean up with an S-300 column by repeating steps 8 to 13.

19. Place on ice and add appropriate amount of annealed adapters (from step 3) to give an estimated 10:1 adapter:insert molar ratio.

    Estimate the expected distribution of fragment lengths in the DNA sample to calculate the DNA molar amount (see Support Protocol, below). The median fragment size can be used as an approximation.

20. Reduce volume to 20 µl in SpeedVac (no heat; this takes 15 to 45 min. Compare volume to a tube containing 20 µl. Adjust volume to 20 µl using nuclease-free water if necessary.

Ligate adapter

21. Prepare adapter ligation master mix by combining, for each sample:

    25 µl 2× Rapid DNA ligase buffer
    5 µl Rapid DNA ligase.

22. Add 30 µl of ligation master mix to 20 µl of DNA from step 20. Mix by careful pipetting.

    Keep samples on ice to avoid uneven catalytic activity.

23. Incubate in 20°C water bath (as described in step 7) for 15 min.

24. Add 50 µl of Ampure XP magnetic bead slurry.

    Allow beads to come to room temperature and make sure they are well dispersed prior to use.

25. Mix well by carefully pipetting up and down. Incubate at room temperature for 5 min.

26. Place in magnetic rack for microcentrifuge tubes and wait until solution has cleared.

    This takes approximately 2 min.

27. Aspirate and discard the supernatant, being careful not to disrupt the beads.

28. Add 200 µl 70% ethanol and wait until cleared.

    This takes approximately 1 min.

29. Aspirate supernatant and discard. Repeat step 28.

30. Let air dry for 5 min on magnetic microcentrifuge tube rack.

    Ensure that all residual ethanol in the tube has evaporated. Do not over-dry.

31. Remove from magnet and disperse beads into 40 µl $TE_{0.1}$ buffer. Mix thoroughly by careful pipetting.

32. Place into magnetic microcentrifuge tube holder and wait until cleared.

    This takes approximately 1 min.

33. Aspirate supernatant into a new 1.5-ml microcentrifuge tube. Discard the beads.

    At this point, samples can be stored at −20°C.

Amplify library

Library enrichment

34. Prepare a PCR reaction master mix by combining the following (from the KAPA HiFi HotStart kit) for each sample:

    5.00 µl 5× KAPA HiFi Fidelity buffer
    0.75 µl KAPA dNTP mix (10 mM each)
    1.25 µl P5 primer (10 µM)
    1.25 µl P7 primer (10 µM)
    0.50 µl KAPA HiFi HotStart DNA polymerase (1 U/µl).

35. Add 8.75 µl of the PCR master mix to 16.25 µl of the Ampure-purified DNA from step 33.

36. Enrich for adapter-ligated DNA using the following PCR cycle program:

    a. 98°C, 45 sec.
    b. 98°C, 15 sec.
    c. Ramp to 60°C at 1°C/sec.
    d. 60° C, 20 sec.
    e. Ramp to 98°C at 1°C/sec.
    f. Got to step b, 9 cycles or 13 cycles.

       For input DNA samples <30 ng, run 14 cycles. For samples >30 ng, run 10 cycles.

    g. 72°C, 1 min.
    h. 8°C, indefinitely.

37. Add 25 μl of Ampure magnetic bead slurry to the tubes containing PCR products.

    Allow beads to come to room temperature and disperse well before use.

38. Clean up sample by repeating steps 25 to 32.

39. Transfer DNA library to a new 1.5-ml microcentrifuge tube. Discard beads.

    A volume of 5 μl of library can be used to check for correct size, proper PCR amplification and elimination of adapter dimers. Run in a 2% agarose gel for 1 hr at 75 V with a low-molecular-weight ladder. Take into consideration that adapter dimers add 126 bp to DNA inserts.

    Library concentration should be precisely determined using a high-sensitivity assay, such as Quant-IT PicoGreen. Follow Illumina recommendations for preparation of flow cell mixtures. Once a library has been successfully sequenced, it can be used as a reference for the amount of DNA to include in the flow cell.

SUPPORT PROTOCOL

SIZE SELECTION OF IMMUNOPRECIPITATED DNA FRAGMENTS

MNase digestion produces DNA fragments ranging in size from polynucleosomes (>150 bp) to short TF footprints (<100 bp), and immunoprecipitation from this material selectively enriches fragments bound by the factor of interest (Fig. 21.31.2A, B). In order to selectively sequence fragments bound by transcription factors, a simple solid-phase reversible immobilization (SPRI) bead-based approach can be used to enrich for short fragments. This protocol can be performed with commercially available beads (i.e., Ampure XP) or custom-made beads (see Rohland and Reich, 2012). Due to the possibility of lot-to-lot variability in SPRI beads, determining the optimal bead mixture-to-DNA ratio is recommended.

Materials

Agencourt AMPure XP beads (Beckman Coulter, cat. no. A63880) or equivalent custom made SPRI beads (see Commentary)
70% and 100% ethanol
$TE_{0.1}$ buffer, pH 8.0 (see recipe)20 mg/ml glycogen (Life Technologies, cat. no. 10814-010)
5 M NaCl (see recipe)
1× TBE (see recipe)
SYBR Gold nucleic acid gel stain (Life Technologies, cat. no. S-11494)
10% and 6% TBE acrylamide gels prepared according to standard methods
10-bp DNA ladder (Life Technologies, cat. no. 10821-015)

DynaMag-2 magnetic microcentrifuge tube rack (Life Technologies)
Siliconized low-retention microcentrifuge tubes.

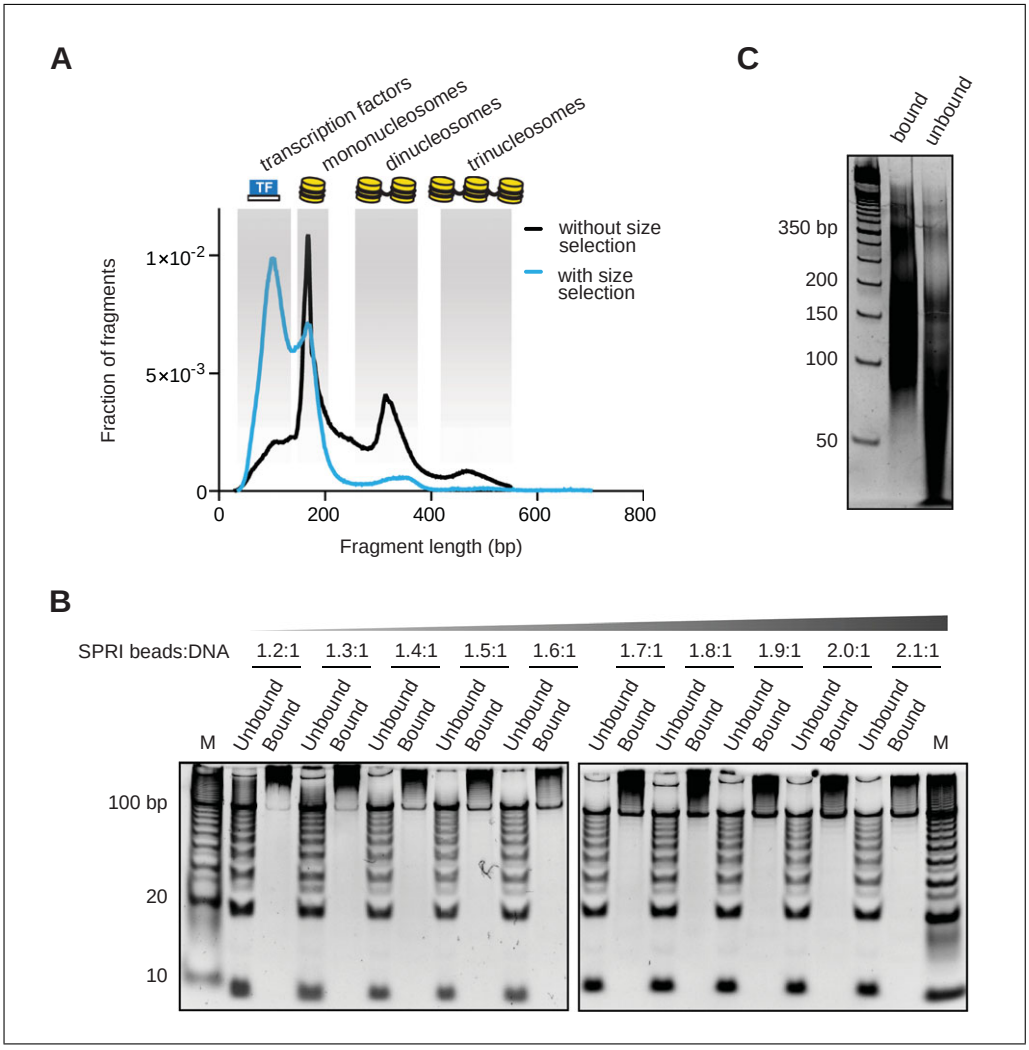

Figure 21.31.2 Size selection of ORGANIC IP DNA Fragments. (**A**) Example paired-end sequencing fragment length distributions from libraries made from non-size-selected and size-selected ORGANIC IP DNAs. Unbound refers to fraction not bound to SPRI beads. (**B**) Determination of optimal SPRI bead:DNA ratio by testing various bead:DNA ratios on 10-bp ladder and visualization of bead-bound (Bound) and supernatant (Unbound) fractions on 10% acrylamide gel. "M" indicates marker lanes. (**C**) Enrichment of different size classes in the SPRI bead-bound (Bound) and supernatant (Unbound) fractions visualized on a 6% acrylamide gel.

Low-retention 20-, 250-, and 1000-µl pipet tips with aerosol filter barriers
Refrigerated microcentrifuge.
Novex XCell SureLock Mini-Cell (Life Technologies, cat. no. EI0001) or equivalent electrophoresis equipment with power supply

Size selection of ORGANIC IP DNA fragments

1. Vortex SPRI beads until well mixed and add desired volume of SPRI beads to DNA sample. Pipet up and down ten times.

   In order to identify the optimal ratio of SPRI bead mixture to DNA, test various ratios of beads with respect to 10-bp ladder diluted to 0.5 µg. For example, ratios ranging from 1.2:1 to 2.1:1 (SPRI beads:DNA) were used in the experiment shown in Figure 21.31.2B.

   Follow size selection and ethanol precipitation procedures (see below) and visualize size-fractionated 10-bp ladder on 10% acrylamide gel stained with SYBR Gold. Choose a bead:DNA ratio that provides the best enrichment of fragments smaller than ~100 bp

in the Unbound fraction, with the most depletion of fragments >150 bp. For example, a bead:DNA ratio of 2.1:1 was chosen based on Figure 21.31.2B.

In this protocol, SPRI beads are used to capture undesired fragments larger than ~150 bp, while the desired fragments smaller than ~100 bp will remain in the Unbound fraction. Note that this is different from the use of Ampure SPRI beads in Basic Protocol 3.

2. Incubate at room temperature for 5 min.
3. Place samples on magnetic microcentrifuge tube rack.
4. Incubate at room temperature for 2 min.
5. Transfer supernatant to a new tube.

   This is the Unbound fraction, containing the desired short DNA fragments. This fraction can be used to prepare a sequencing library according to Basic Protocol 3. Proceed with steps 6 to 12 if recovery of large DNA fragments is desired.

6. Add 100 µl of 70% ethanol to microcentrifuge tubes containing beads.
7. Incubate for at room temperature for 30 sec.
8. Aspirate off ethanol and repeat step 7 once.
9. Allow beads to dry at room temperature for 2 min.
10. Remove tubes from magnetic microcentrifuge tube rack and disperse beads by adding 200 µl $TE_{0.1}$ buffer to beads and pipetting up and down ten times.
11. Place the tube back in the magnetic rack and wait until cleared.
12. Transfer supernatant to new tube.

    This is the bound, size-selected fraction.

Ethanol precipitation of size-selected fractions

13. Bring up volumes of Bound and Unbound fractions to 400 µl using $TE_{0.1}$.
14. Add 30 µg glycogen and 8 µl 5 M NaCl. Mix by inverting tubes.
15. Add 800 µl cold 100% ethanol and mix by inverting tubes.
16. Incubate overnight at –20°C.
17. Spin tubes in microcentrifuge at maximum speed for 30 min at 4°C.
18. Discard supernatant and wash pellet once with 1 ml 100% ethanol.
19. Microcentrifuge tubes 10 min at 25,000 × *g* (or maximum speed on standard refrigerated benchtop microcentrifuge), 4°C.
20. Discard supernatant and air dry pelleted DNA for 5 min at room temperature.
21. Resuspend DNA pellet in 30 µl $TE_{0.1}$.
22. To visualize Bound fractions, which should contain mainly fragments >150 bp, run DNA on a 6% polyacrylamide gel stained with SYBR Gold.

    Example results with both Bound and Unbound fractions after SPRI bead size selection are shown in Figure 21.31.2C.

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.

$CaCl_2$, 1 M

Make 1 M $CaCl_2$ stock solution by dissolving 110.98 g $CaCl_2$ in 80 ml distilled, deionized water. Bring to final volume of 100 ml and filter sterilize by passing solution through 0.2-μm filter. Solution can be stored at room temperature up to 1 year.

dATP, 1 mM

Dilute 1 μl 100 mM dATP (Promega, cat. no. U1201) in 99 μl UltraPure water. Store at −20°C up to 1 year or longer.

EDTA, pH 8.0, 0.5 M

Add 186.1 g disodium EDTA dihydrate to approximately 800 ml distilled, deionized water, and stir at room temperature. Add NaOH pellets to reach pH 8. Filter sterilize by passing solution through 0.2-μm filter and store at room temperature for up to 6 months.

Ficoll buffer

To make 25 ml Ficoll buffer, add 500 μl 1 M PIPES pH 6.3 stock solution (see recipe) and 12.5 μl 1 M $CaCl_2$ (see recipe) to a 50-ml conical tube. Add 15 ml sterile distilled, deionized water and 2.25 g Ficoll 400. Incubate at room temperature with agitation on a shaking platform until dissolved (this typically takes ~2 hr). Bring to 25 ml volume with sterile distilled, deionized water. Cool to 4°C in refrigerator before use. Ficoll buffer should be made fresh for each nuclei isolation.

Final concentrations: 9% (w/v) Ficoll 400, 20 mM PIPES, pH 6.3, and 0.5 mM $CaCl_2$.

IP wash buffer

To make 500 ml IP wash buffer, combine 5 ml potassium phosphate buffer, pH 7.5 (see recipe), 750 μl 0.5 M EDTA (see recipe), and 7 ml 5 M NaCl (see recipe). Bring volume to 500 ml with sterile, distilled deionized water. Store at 4°C for up to 1 year.

Final concentrations: 10 mM phosphate phosphate buffer, pH 7.5, 0.75 mM EDTA, and 70 mM NaCl.

LPC (leupeptin, pepstatin A, chymostatin), 10 mg/ml

Dissolve 10 mg each of leupeptin, pepstatin A, and chymostatin in 1 ml DMSO. Store at –20°C for up to 1 year.

NaCl, 5 M

Add 292.2 g NaCl to 1 liter water and stir at room temperature until dissolved. Filter sterilize by passing solution through 0.2 μm filter and store at room temperature for up to one year.

NaCl extraction buffer, 80 mM, 150 mM, or 600 mM

To make 100 ml extraction buffer, combine 100 μl Triton X-100, 1 ml potassium phosphate buffer pH 7.5 (see recipe), 150 μl 0.5 M EDTA (see recipe), and the appropriate volume of 5 M NaCl (see recipe) to achieve desired salt concentration. Bring to 100 ml with sterile distilled, deionized water. Store at 4°C for up to 6 months. Add 10 mg/ml LPC (see recipe) to 10 μg/ml final concentration (1:1000 dilution) and 100 mM PMSF (see recipe) to 1 mM final concentration (1:100 dilution) before use.

Final concentrations: 70, 140, or 590 mM NaCl, 0.75 mM EDTA, 10 mM potassium phosphate buffer, pH 7.5, 0.1% Triton X-100.

Phosphate-buffered saline (PBS)

Make 1 liter of 10× PBS by combining 800 ml double-distilled water with 80 g NaCl, 2 g KCl, 14.4 g Na_2HPO_4, and 2.4 g KH_2PO_4. Adjust pH to 7.4 with HCl, bring volume to 1 liter, and autoclave. Store up to 1 year at room temperature.

For 200 ml of 1× PBS, mix 20 ml of 10× PBS with 180 ml double-distilled water. Use within 1 month.

PIPES, pH 6.3, 1 M

Add 151.2 g PIPES (free acid) to 400 ml deionized water and stir at room temperature. While monitoring pH, add NaOH pellets during mixing until pH reaches ~6.0. Add 1 N NaOH until pH reaches 6.3, and bring volume up to 500 ml with distilled, deionized water. Filter sterilize by passing solution through 0.2-μm filter and store at 4°C for up to 6 months.

PMSF, 100 mM

Add 17.4 mg phenylmethylsulfonyl fluoride to 1 ml isopropanol and mix by inversion. Store at –20°C for up to 1 year (note that PMSF crystallizes upon freezing). Thaw at room temperature and vortex vigorously prior to use.

Potassium phosphate buffer, pH 7.5

Add 36 g K_2HPO_4 and 108 g KH_2PO_4 to a beaker with 800 ml distilled, deionized water and stir at room temperature until dissolved. Adjust pH to 7.5 using concentrated KOH or NaOH and bring volume up to 1 liter. Filter sterilize by passing solution through 0.2-μm filter and store at room temperature for up to 6 months.

Resuspension buffer

Combine the following volumes of stock solutions to make 30 ml resuspension buffer: 18 ml 2 M sorbitol (see recipe), 3 ml 1 M potassium phosphate buffer, pH 7.5 (see recipe), 15 μl 1 M $CaCl_2$ (see recipe), 15 μl 1 M 2-mercaptoethanol, and 8.97 ml sterile distilled, deionized water.

Resuspension buffer should be made fresh for each experiment because it contains 2-mercaptoethanol.

Final concentrations: 1.2 M sorbitol, 100 mM potassium phosphate buffer, pH 7.5, 0.5 M $CaCl_2$, and 0.5 mM 2-mercaptoethanol.

Schneider's medium, complete

Combine 450 ml Schneider's Drosophila Medium (Life Technologies, cat. no. 21720-001) with 50 ml fetal bovine serum (FBS; HyClone, cat. no. SH30070). Filter with a 0.2-μm pore size membrane under sterile conditions. Store at 4°C and use within 3 months.

Sorbitol, 2 M

Make 2 M sorbitol stock solution by dissolving 182.17 g sorbitol in 400 ml distilled, deionized water. Bring to final volume of 500 ml and filter sterilize by passing solution through 0.2-μm filter. Solution can be stored at room temperature up to 6 months.

Sigma MNase

Resuspend lyophilized Micrococcal Nuclease (Sigma-Aldrich, cat. no. N3755) in 50% glycerol (v/v) for a stock concentration of 1 U/μl. Aliquot and stock solution at −20°C for 1 year or longer.

SPC buffer

Combine the following volumes of stock solutions to make 100 ml SPC buffer: 50 ml 2 M sorbitol (see recipe), 2 ml 1 M PIPES pH 6.3 (see recipe), 10 µl 1 M $CaCl_2$ (see recipe), 48 ml sterile distilled, deionized water. SPC buffer can be stored for up to 6 months at 4°C.

Final concentrations: 1 M sorbitol, 200 mM PIPES, pH 6.3, 0.1 mM $CaCl_2$.

SPC Buffer with protease inhibitors: Same as above, with 1 mM phenylmethylsulfonyl fluoride (PMSF) and 10 µg/ml each of leupeptin, pepstatin A, and chymostatin diluted from 100 mM PMSF (see recipe) and 10 mg/ml LPC (see recipe), respectively.

$TE_{0.1}$ buffer

To make 500 ml, combine 0.5 ml Tris·Cl, pH 8 (see recipe), with 0.1 ml 0.5 M EDTA, pH 8 (see recipe) and bring volume to 500 ml with distilled, deionized water. Filter sterilize and store at room temperature for up to 6 months.

80TM + IS buffer

Make 100 ml 80T Buffer by combining 1.4 ml 5 M NaCl (see recipe), 1 ml 1 M Tris·Cl, pH 7.4 (see recipe), 200 µl 1 M $MgCl_2$, 1 ml 0.2 M EGTA, pH 8.0, 1 ml 10% Triton X-100, and 95.4 ml UltraPure water. This can be stored at room temperature up to 6 months.

On the day of the experiment, prepare 2 ml fresh 80TM + IS buffer by mixing 1.93 ml 80T buffer with 10 µl 100 mM PMSF (see recipe), 40 µl protease inhibitor cocktail (see recipe for TM2 + I buffer; add to 1× concentration), and 20 µl 0.2 M EGTA pH 8.0. Keep on ice and use immediately.

TM2+ buffer

For 500 ml of 10× TM2 buffer, mix 5 ml of 1 M Tris·Cl pH 7.4 (see recipe), 1 ml 1 M $MgCl_2$, and 494 ml UltraPure water. This stock solution can be stored at room temperature for up to 1 year.

On the day of the experiment, prepare 15 ml fresh TM2 + by mixing 1.5 ml 10× TM2 buffer, 75 µl 100 mM PMSF (see recipe), and 13.425 ml UltraPure water. Keep on ice and use within 3 hr.

TM2 + I buffer

Prepare 50× EDTA-free protease inhibitor cocktail by diluting one cOmplete, Mini, EDTA-free tablet (Roche, 11836170001) in 210 µl UltraPure H_2O. Vortex abundantly until tablet is fully dissolved. This can be stored at −20°C for 2 months. Vortex to resuspend when thawing.

On the day of the experiment, prepare 4 ml fresh TM2 + I buffer by mixing 3.92 ml TM2+ buffer (see recipe) with 80 µl of 50× inhibitor cocktail. Keep on ice and use immediately.

TM2 + IS

On the day of the experiment, prepare 2 ml fresh TM2 + IS buffer by mixing 1.96 ml TM2 + I buffer (see recipe) with 20 µl 0.2 M EGTA pH 8.0. Keep on ice and use immediately.

Tris·Cl, pH 8, 1 M

Dissolve 60.57 g Tris (hydroxymethyl) aminomethane in 400 ml distilled, de-ionized water by stirring at room temperature. Bring pH to 8.0 with concentrated HCl and adjust volume to 500 ml with distilled, deionized water. Filter sterilize and store at room temperature for up to 6 months.

USB Affymetrix MNase

Resuspend 15 kU of lyophilized USB Micrococcal Nuclease (Affymetrix, cat. no. 70196Y) in 375 μl of 50% (v/v) glycerol for a stock concentration of 40 U/μl. Aliquot and stock solution at −20°C for at least 1 year.

COMMENTARY

Background Information

Transcription factors (TFs) are DNA-binding proteins that control gene expression and help shape chromatin structure by coordinating the recruitment and activity of RNA polymerases and chromatin remodeling complexes. TFs specifically recognize short (~6- to 20-bp) DNA sequence motifs and often act cooperatively to bind regulatory elements. While consensus motifs for individual TFs can be defined by in vitro experiments, only a small fraction of all available motifs in the genome are bound in vivo (Biggin, 2011). Mapping of TF binding sites in vivo is most often achieved by cross-linked chromatin immunoprecipitation (X-ChIP). This method is based on the use of formaldehyde to covalently fix TF-DNA interactions, followed by fragmentation and solubilization of chromatin by sonication and immunoprecipitation of TF-DNA complexes with an antibody recognizing the TF of interest. Subsequent mapping of immunoprecipitated DNA sequences identifies TF binding sites genome-wide (X-ChIP-seq). The use of formaldehyde for cross-linking theoretically circumvents concerns that protein-DNA contacts may be disrupted after nuclei are disrupted and chromatin is solubilized. However, recent work has uncovered generalized biases in X-ChIP protocols stemming from cross-linking and sonication. These include frequent false positives and misleading quantitative measurements due to the preferential formation of protein-protein cross-links by formaldehyde (Jackson, 1978; Jackson, 1999) and the artifactual formation of protein-DNA cross-links in nucleosome-depleted, highly transcriptionally active regions (Park et al., 2013; Teytelman et al., 2013; Worsley Hunt and Wasserman, 2014).

An alternative to X-ChIP methods is chromatin immunoprecipitation with native chromatin (N-ChIP), which has been widely used to map the positions of histones and, more recently, RNA Polymerase II. N-ChIP maps of histones and Pol II take advantage of highly stable interactions between these proteins and DNA, allowing robust mapping without the use of cross-linking agents. Interactions between DNA and other types of proteins, such as chromatin remodelers and transcription factors, can be highly stable under appropriate experimental conditions (Lohman and Mascotti, 1992; Wilkins and Lis, 1998). ORGANIC profiling is based on the preservation and capture of native protein-DNA interactions, provided steps are taken to maintain protein-DNA interactions. In ORGANIC profiling, MNase digestion preferentially degrades DNA that is not associated with proteins, effectively footprinting protein-bound genomic regions and fragmenting chromatin. A single TF typically protects short fragments (20 to 40 bp), while regions simultaneously bound by multiple TFs yield larger fragments (60 to 80 bp; Orsi et al., 2014). Digested chromatin is solubilized and subjected to immunoprecipitation with an antibody against the factor of interest. The recovered DNA is subjected to paired-end sequencing to yield both genomic coordinates of factor-bound DNA and structural information on binding sites based on the size of recovered fragments (Orsi et al., 2014). We have used this method to successfully map structurally diverse TFs in yeast and *Drosophila* cells (Kasinathan et al., 2014; Orsi et al., 2014). In addition, we have demonstrated the utility of ORGANIC for mapping chromatin remodelers and protein complexes (Zentner and Henikoff, 2013; Zentner et al., 2013; Orsi et al., 2014), making ORGANIC a versatile method for characterizing the epigenome while avoiding cross-linking biases.

The quality and utility of ORGANIC maps depend on a few key parameters that should be carefully optimized, as detailed below.

Critical Parameters and Troubleshooting

Nuclei isolation

Chromatin fragmentation by MNase requires high-quality nuclei. Incomplete spheroplasting (in yeast) or permeabilization (in *Drosophila*) reduces the number of lysed cells and released nuclei. Excessive incubation in detergent-containing nuclei isolation buffers may result in clumping of nuclei and heterogeneous MNase digestion. Initially, and when working with a new strain or cell type, nuclei isolation can be monitored as described in Basic Protocols 1 and 2. Difficulty in resuspending nuclei prior to MNase treatment

is predictive of poor digests, and such samples should be discarded.

Quality control: MNase ladder

The extent of MNase digestion is critical for high-quality ORGANIC profiling. Light digestion increases biases due to local DNA accessibility and MNase sequence preferences, and also results in a large proportion of long fragments, possibly lowering mapping resolution. On the other hand, excessive digestion raises concerns that TF-DNA contacts may be disrupted, and structural information about individual sites may be lost. We recommend optimizing digestions by running a dilution series of MNase and visualizing the nucleosomal ladder from total nuclear DNA by agarose gel electrophoresis with ethidium bromide post-staining (Fig. 21.31.1). The ideal MNase ladder contains at least ~75% mononucleosome-sized DNA and a clear smear of shorter fragments, while di-nucleosomes and tri-nucleosomes are still detectable in low amounts. In our experience, once MNase conditions have been established for a given strain or cell type, they can be reliably used to obtain a reproducible ladder.

Quality control: Western blot

As with any immunoprecipitation experiment, a standard quality control consists of assessing efficient pulldown of the TF of interest by western blotting. The absence of cross-linking permits the separation of proteins from the immunopreciptate without additional steps. Efficient immunoprecipitation can be demonstrated by enrichment of the factor of interest in the immunoprecipitate (relative to IgG control) or depletion of the factor of interest in the unbound fraction after immunoprecipitation.

Immunoprecipitation buffer salt concentration

Stability of solubilized TF-DNA complexes is dependent on salt concentration. Low-salt buffers are more likely to prevent TF-DNA complex disruption, but may lead to decreased stringency. We have shown that the optimal salt concentration for achieving specific and complete recovery of binding sites can be determined experimentally for each target TF (Kasinathan et al., 2014). While we recommend using low-salt buffers in initial experiments, the optimal buffer conditions should ideally be determined for each TF by comparing mapping results from chromatin purified under a range of salt concentrations.

Fragment length distribution in the sequenced sample

Transcription factors typically protect DNA fragments ≤80 bp in MNase-digested chromatin. The abundance of paired-end reads ≤80 bp is critical for high-resolution mapping of individual binding sites. The Support Protocol can dramatically improve the ratio of these small fragments in the final sequencing library. Typical fragment length distributions from paired-end sequencing with and without size selection are shown in Figure 21.31.2A.

Sequencing depth

High-throughput DNA sequencing typically accounts for most of the cost of a genome-wide TF mapping experiment. The sequencing depth of ORGANIC samples is limiting for the interpretability of the data. We have obtained satisfactory TF maps in *Drosophila* with 40 million paired-end reads and in *S. cerevisiae* with 4 million paired-end reads, both from non-size-selected samples. If size selection is performed as described in the Support Protocol, 10 million reads can give satisfactory results in *Drosophila* samples. In both cases, samples can be multiplexed for sequencing.

MNase

While we find minimal lot-to-lot variability between different stocks of MNase, manufacturers may use different unit definitions. We recommend consistently using MNase from the same manufacturer and running pilot digests whenever trying new lots of enzyme or enzyme from a new supplier (see Fig. 21.31.1). We also recommend calibrating each new batch of MNase to ensure consistency of digestion.

Input requirements

The typical *Drosophila* ORGANIC input is prepared from 2×10^8 cells, which can be easily obtained from cultured cell lines. If ORGANIC yields are good, less input sample may be used. However, the cell concentration should not exceed the concentration recommended in Basic Protocol 2, because exceeding the limiting amount of magnesium in buffers, which stabilizes protein-DNA interactions, can compromise chromatin quality.

We typically perform ORGANIC profiling in budding yeast with 250 to 500 ml culture in logarithmic growth phase (OD_{600} = 0.6 to 0.8), which corresponds to approximately 7.5×10^9 cells, and immunoprecipitate using antibodies against FLAG or Myc epitope tags. However, depending on the abundance of the

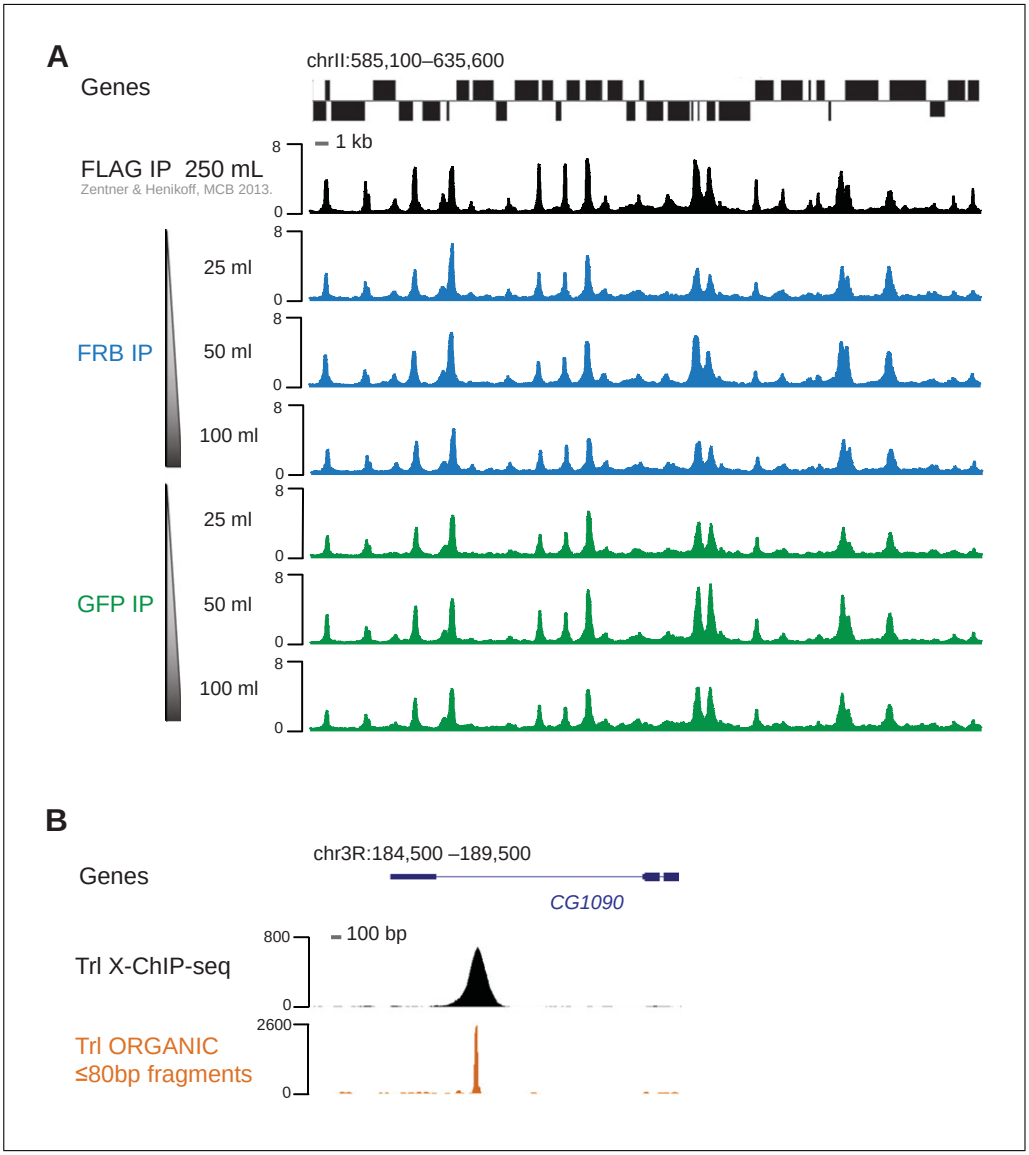

Figure 21.31.3 Anticipated results from ORGANIC profiling. (**A**) Robust ORGANIC profiling across a range of input cell numbers and immunoprecipitation epitopes in yeast. Example ~50 kb window showing comparison of ORGANIC profiles of the ATPase Mot1 generated using different input cell numbers and epitopes: 250 ml starting culture with FLAG-tagged Mot1 (black track); 25, 50, and 100 ml starting cultures with FRB-GFP-tagged Mot1 and immunoprecipitated with FRB antibody (blue tracks); and 25, 50, and 100 ml starting cultures with FRB-GFP-tagged Mot1 and immunoprecipitated with GFP antibody (green tracks). (**B**) High-resolution mapping of Trl with ORGANIC in Drosophila S2 cells. An example Trl-bound site revealed by X-ChIP-seq (black track) is resolved to a ~40-bp segment by ORGANIC (brown track). For the color version of this figure, go to *http://www.currentprotocols.com/protocol/mb2131*.

factor of interest, it may be possible to perform ORGANIC profiling using a smaller starting cell number and antibodies against different epitopes or the native, unmodified factor, provided that high-quality antibodies are available. In order to test the input requirement and dependence on antibody used, we carried out ORGANIC profiling at various starting cell numbers and with different antibodies using budding yeast strains harboring an FRB-GFP-tagged allele of the ATPase Mot1. ORGANIC profiles produced at starting culture volumes 10-fold lower (~7.5×10^8 cells) than the typical starting volume of 250 ml were robust and not sensitive to the antibody used for immunoprecipitation (Fig. 21.31.3A). Therefore, it is likely that ORGANIC profiling is compatible with low-input immunoprecipitation of transcription factors that are sufficiently expressed.

Reproducibility

We find robust reproducibility between ORGANIC experiments (Fig. 21.31.3A; Kasinathan et al., 2014). However, we recommend performing ORGANIC in biological replicates and processing and sequencing them in parallel.

Data analysis

Data analysis is critical for extracting interpretable information from ORGANIC, and is a complex task that is beyond the scope of this unit. However, publicly available tools such as the Galaxy Project suite (Goecks et al., 2010) can expedite the processing and mapping of sequencing data and constructing genome browser tracks for visualization. In addition, an abundant literature describes different approaches for discovery of statistically significant TF-bound sites from sequencing data (MacIsaac and Fraenkel, 2006; MacIsaac and Fraenkel, 2010). Some widely used tools may not be applicable to ORGANIC datasets because these tools do not utilize fragment size information obtained from paired-end sequencing and assume random fragmentation of the genome by sonication for statistical analysis. However, recent versions of utilities such as the MACS2 peak caller includes support for paired-end datasets (Zhang et al., 2008). We have previously described a simple approach to identifying binding sites from ORGANIC data based on setting a genome threshold for overlapping reads (Kasinathan et al., 2014). We anticipate improvements in these tools.

Anticipated Results

The protocols described in this unit enable the investigator to define, with high resolution, the genomic binding sites of TFs in a relatively simple, fast, and inexpensive manner. Basic Protocols 1 and 2 yield 3 to 100 ng of purified DNA after immunoprecipitation, used to prepare libraries for paired-end sequencing via a protocol that retains small fragments corresponding to TF footprints (Basic Protocol 3). Once sequencing reads have been mapped to a reference genome and these alignments converted to genome coverage files, the distribution of recovered fragments can be visualized using the publicly available UCSC Genome Browser (Kent et al., 2002) or other, similar utilities. Maps for TFs typically show discrete, narrow peaks with orders of magnitude enrichment over a negligible background (Fig. 21.31.3). Figure 21.31.3B shows a region in the *Drosophila* genome displaying ORGANIC and X-ChIP tracks for the factor Trl, underscoring the high specificity and resolution to be expected from ORGANIC relative to standard X-ChIP. TF-bound sites can be called across the genome by statistical methods as discussed above, and overrepresented DNA sequences can be discovered using motif-finding algorithms such as MEME (Bailey et al. 2009).

Time Considerations

The time required to complete ORGANIC and library construction is comparable to the time required to complete common X-ChIP protocols. The immunoprecipitation procedure itself (excluding the times required for obtaining the initial biological material, sequencing, and analyzing data), can be completed in ~4 days, with brief bench time on days 1 and 2. Basic Protocol 1 or 2 can be performed on days 1 to 3, and library construction described in Basic Protocol 3 on days 3 and 4. If fragment size selection based on the Support Protocol is desired, an additional day is required between immunoprecipitation and library construction, provided that preliminary experiments to determine optimal bead:DNA ratio have already been performed. The MNase ladder may be analyzed during days 1 and 2 to determine extent of digestion. Western blotting to monitor solubilization and immunoprecipitation may be performed on days 2 and 3, in parallel to other steps in the procedure. A detailed time frame for specific protocols is included below.

Basic Protocol 1

Buffer preparation: 1 hr; nuclei isolation, MNase digestion and chromatin extraction: ~8 total hr; ~4 hr of bench work.

Chromatin immunoprecipitation, DNA purification, and quantification: 48 total hr; ~3 hr of bench work.

Basic Protocol 2

Buffer preparation: 1 hr; nuclei isolation, MNase digestion, and chromatin extraction: 2 to 3 hr.

Chromatin immunoprecipitation, DNA purification and quantification: 48 total hr; ~3 hr of bench work.

Basic Protocol 3

End repair, 3′ A overhang addition and adapter ligation: 4-5 hr.

PCR library enrichment, clean-up and quantification: 3 hr.

Support Protocol

Once the optimal bead:DNA ratio is determined, the size selection can be completed in approximately 45 min.

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Literature Cited

Bailey, T.L., Boden, M., Buske, F.A., Frith, M., Grant, C.E., Clementi, L., Ren, J., Li, W.W., and Noble, W.S. 2009. MEME SUITE: Tools for motif discovery and searching. *Nucleic Acids Res.* (Web Server issue):W202-W208.

Biggin, M.D. 2011. Animal transcription networks as highly connected, quantitative continua. *Dev. Cell* 21:611-626.

Elbing, K. and Brent, R. 2002. Growth in liquid media. *Curr. Protoc. Mol. Biol.* 59:1.2.1-1.2.2.

Goecks, J., Nekrutenko, A., Taylor, J., and Galaxy Team. 2010. Galaxy: A comprehensive approach for supporting accessible, reproducible, and transparent computational research in the life sciences. *Genome Biol.* 11:R86.

Henikoff, S. and Henikoff, J.G. 2012. "Point" centromeres of Saccharomyces harbor single centromere-specific nucleosomes. *Genetics* 190:1575-1577.

Jackson, V. 1978. Studies on histone organization in the nucleosome using formaldehyde as a reversible cross-linking agent. *Cell* 15:945-954.

Jackson, V. 1999. Formaldehyde cross-linking for studying nucleosomal dynamics. *Methods* 17:125-139.

Kasinathan, S., Orsi, G.A., Zentner, G.E., and Ahmad, K., 2014. Henikoff, S. High-resolution mapping of transcription factor binding sites on native chromatin. *Nat. Methods* 11:203-209.

Kent, W.J., Sugnet, C.W., Furey, T.S., Roskin, K.M., Pringle, T.H., Zahler, A.M., and Haussler, D. 2002. The human genome browser at UCSC. *Genome Res.* 12:996-1006.

Krassovsky, K., Henikoff, J.G., and Henikoff, S. 2012. Tripartite organization of centromeric chromatin in budding yeast. *Proc. Natl. Acad. Sci. U.S.A.* 109:243-248.

Lohman, T.M. and Mascotti, D.P. 1992. Thermodynamics of ligand-nucleic acid interactions. *Methods Enzymol.* 212:400-424.

MacIsaac, K.D. and Fraenkel, E. 2006. Practical strategies for discovering regulatory DNA sequence motifs. *PLoS Comput Biol.* 2:e36.

Macisaac, K.D. and Fraenkel, E. 2010. Sequence analysis of chromatin immunoprecipitation data for transcription factors. *Methods Mol Biol.* 674:179-193.

Moore, D. and Dowhan, D. 2002. Purification and concentration of DNA from aqueous solutions. *Curr. Protoc. Mol. Biol.* 59:2.1.1-2.1.10.

Neph, S., Vierstra, J., Stergachis, A.B., Reynolds, A.P., Haugen, E., Vernot, B., Thurman, R.E., John, S., Sandstrom, R., Johnson, A.K., Maurano, M.T., Humbert, R., Rynes, E., Wang, H., Vong, S., Lee, K., Bates, D., Diegel, M., Roach, V., Dunn, D., Neri, J., Schafer, A., Hansen, R.S., Kutyavin, T., Giste, E., Weaver, M., Canfield, T., Sabo, P., Zhang, M., Balasundaram, G., Byron, R., MacCoss, M.J., Akey, J.M., Bender, M.A., Groudine, M., Kaul, R., and Stamatoyannopoulos, J.A. 2012. An expansive human regulatory lexicon encoded in transcription factor footprints. *Nature*. 489:83-90.

Orsi, G.A., Kasinathan, S., Hughes, K.T., Saminadin-Peter, S., Henikoff, S., and Ahmad, K. 2014. High-resolution mapping defines the cooperative architecture of Polycomb response elements. *Genome Res.* 24:809-820.

Park, D., Lee, Y., Bhupindersingh, G., and Iyer, V.R. 2013. Widespread misinterpretable ChIP-seq bias in yeast. *PLoS One.* 8: e83506.

Poorey, K., Viswanathan, R., Carver, M.N., Karpova, T.S., Cirimotich, S.M., McNally, J.G., Bekiranov, S., and Auble, D.T. 2013. Measuring chromatin interaction dynamics on the second time scale at single-copy genes. *Science* 342:369-372.

Rohland, N. and Reich, D. 2012. Cost-effective, high-throughput DNA sequencing libraries for multiplexed target capture. *Genome Res*. 22:939-946.

Teves, S.S. and Henikoff, S. 2011. Heat shock reduces stalled RNA polymerase II and nucleosome turnover genome-wide. *Genes Dev.* 25:2387-2397.

Teytelman, L., Thurtle, D.M., Rine, J., and van Oudenaarden, A. 2013. Highly expressed loci are vulnerable to misleading ChIP localization of multiple unrelated proteins. *Proc. Natl. Acad. Sci. U.S.A.* 110:18602-18607.

Treco, D. A. and Winston, F. 2008. Growth and manipulation of yeast. *Curr. Protoc. Mol. Biol.* 82:13.2.1-13.2.12.

Wilkins, R.C. and Lis, J.T. 1998. GAGA factor binding to DNA via a single trinucleotide sequence element. *Nucleic Acids Res.* 26:2672-2678.

Worsley Hunt, R. and Wasserman, W.W. 2014. Non-targeted transcription factors motifs are a systemic component of ChIP-seq datasets. *Genome Biol.* 15:412.

Zentner, G.E. and Henikoff, S. 2013. Mot1 redistributes TBP from TATA-containing to TATA-less promoters. *Mol. Cell Biol.* 33:4996-5004.

Zentner, G.E., Tsukiyama, T., and Henikoff, S. 2013. ISWI and CHD chromatin remodelers bind promoters but act in gene bodies. *PLoS Genet.* 9:e1003317.

Zhang, Y., Liu, T., Meyer, C.A., Eeckhoute, J., Johnson, D.S., Bernstein, B.E., Nusbaum, C., Myers, R.M., Brown, M., Li, W., and Liu, X.S. 2008. Model-based analysis of ChIP-Seq (MACS). *Genome Biol.* 9:R137.

Key References

Kasinathan et al., 2014. See above.

First description of the ORGANIC procedure and characterization of transcription factor binding in yeast and Drosophila. This paper developed the ORGANIC profiling protocol for native immunoprecipitation of Saccharomyces and Drosophila transcription factors, and compared it to cross-linking ChIP methods.

Orsi et al., 2014. See above.

This paper uses MNase-seq and ORGANIC profiling to describe the cooperative transcription factor complexes at Drosophila Polycomb Response Elements. Demonstrates the utility of ORGANIC to identify cooperative transcription factor binding and map transcription factor-bound complexes.