Published in final edited form as:

Curr Protoc Mol Biol. ; 110: 21.31.1–21.31.25. doi:10.1002/0471142727.mb2131s110.

Mapping regulatory factors by immunoprecipitation from native chromatin

Guillermo A. Orsi^{1,2,6}, Sivakanthan Kasinathan^{3,4,6}, Gabriel E. Zentner³, Steven Henikoff^{3,5}, and Kami Ahmad^{1,*}

¹Department of Biological Chemistry and Molecular Pharmacology, Harvard Medical School, Boston, MA 02115, USA

²CNRS–UMR3664/Institut Curie - Centre de Recherche, Paris F-75248, France

³Basic Sciences Division, Fred Hutchinson Cancer Research Center, Seattle, WA 98109, USA

⁴Medical Scientist Training Program, University of Washington School of Medicine, Seattle, WA 98195, USA

⁵Howard Hughes Medical Institute, Seattle, WA 98109, USA

Abstract

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.

Keywords

chromatin; transcription factor; MNase-seq; transcription; ORGANIC

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), where 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

*correspondence to: kami_ahmad@hms.harvard.edu.

⁶These authors contributed equally to this work

Contact information: Department of BCMP, Harvard Medical School, Bldg. C1, Room 203, Boston, MA 02115, Tel: (617) 432-1906

KEY REFERENCES

Kasinathan et al., 2014; Orsi et al., 2014.

non-specific protein-DNA interactions, leading to false positive sites (Neph et al., 2012; Teytelman et al., 2013; Park et al., 2013; Poorey 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 (Krassovsky et al., 2011; Henikoff & Henikoff, 2012; Weber et al., 2014), RNA Polymerase II (Teves & Henikoff, 2011), chromatin remodelers (Zentner & 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, Support Protocol 1 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 this method can be used to obtain stringent maps of transcription factor binding with high resolution.

Materials

Solutions

- YPD culture medium
- Resuspension Buffer (see Recipes) 1.2 M sorbitol, 100 mM potassium phosphate buffer, pH 7.5, 0.5 M CaCl₂, 0.5 mM 2-mercaptoethanol
- Zymolyase (100T equivalent, ZymoResearch, Cat. No E1004)
- SPC Buffer (see Recipes) 1 M sorbitol, 200 mM PIPES, pH 6.3, 0.1 mM CaCl₂
- SPC Buffer with protease inhibitors (see Recipes) Same as above, with 1 mM phenylmethanesulfonyl fluoride (PMSF) and 10 ug/mL each of leupeptin, pepstatin A, and chymostatin (LPC)
- Ficoll Buffer 9% Ficoll-400, 20 mM PIPES, pH 6.3, 0.5 mM CaCl₂

- Liquid Nitrogen
- 1 M CaCl₂
- MNase (Sigma-Aldrich, Cat. No. N3755; 1 U MNase/5 uL nuclease-free water)
- 0.5 M EDTA, pH 8.0
- 1 M Tris, pH 8.0
- Potassium phosphate buffer, pH 7.5
- 1 M PIPES, pH 6.3
- 80 mM, 150 mM or 600 mM NaCl Extraction Buffer 70, 140, or 590 mM NaCl, 0.75 mM EDTA, 10 mM potassium phosphate buffer, pH 7.5, 0.1% Triton X-100.
- 5 M NaCl
- Anti-FLAG M2 magnetic beads (Sigma-Aldrich, Cat. No. A2220)
- IP Wash Buffer 10 mM phosphate buffer, pH 7.5, 0.75 mM EDTA, 70 mM NaCl
- RNase A (10 mg/mL, Thermo Scientific, Cat No EN0531)
- Proteinase K (20 mg/mL, Life Technologies, Cat. No. AM2542)
- Phenol/Chloroform/Isoamyl alcohol
- Glycogen (20 mg/mL, Life Technologies, Cat. No. 10814-010)
- 100% ethanol
- 70% ethanol
- TE_{0.1} buffer (see Recipes)
- Quant-iT PicoGreen dsDNA assay kit. (Life Technologies, Cat. No. P11496)

Materials

- DuPont Sorvall RC-5B Refrigerated centrifuge or equivalent.
- Refrigerated centrifuge with rotor adapters for 50mL and 15mL conical tubes
- Refrigerated microcentrifuge
- 500 mL Nalgene PPCO centrifuge bottle with sealing enclosure (Thermo, Cat No. 3141-0250)
- Spectrophotometer
- Liquid nitrogen container
- 37°C water bath
- Low-retention 50mL and 15mL conical tubes, 1.5mL microcentrifuge tubes, pipette tips.

- 20 gauge and 26 gauge needles with 5 mL syringe
- Nutator or end-over-end rotator in refrigerated 4°C room.
- Magnetic microcentrifuge tube rack

Preparation of beads for immunoprecipitation

- 1 Prior to beginning nuclei isolation, prepare beads for immunoprecipitation by washing 100 uL 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 in 1 mL PBS with 0.5% BSA in a microcentrifuge tube.
- 2 Resuspend beads in 250 uL BSA. Add desired amount of antibody (if not using anti-FLAG magnetic beads).
- 3 Incubate beads for at least 4 h at 4°C with agitation on Nutator.

Nuclei isolation

- 4 Grow 500 mL *S. cerevisiae* culture to OD₆₀₀= 0.6 – 0.8 in YPD.
- 5 Transfer culture to centrifuge bottles.
- 6 Centrifuge for 10 min at 2,700 x g.

All centrifugation steps should be carried at 4°C.

- 7 Discard the supernatant and wash cell pellet with 25 mL deionized H₂O and transfer to 50 mL conical tube.
- 8 Centrifuge for 10 min at 2,700 x g.
- 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 uL aliquots of the spheroplasting reaction into 1 mL of 1% SDS and measuring OD₆₀₀ (vs. 1% SDS blank). Spheroplasting is typically complete when OD₆₀₀ is 10–20% of the starting value.

- 12 Centrifuge for 5 min at 1,000 x g.
- 13 Wash pellet with 25 mL cold SPC buffer with protease inhibitors.
- 14 Centrifuge for 5 min at 1,000 x g.
- 15 Repeat steps 10 and 11 once. Resuspend pellet in 0.5 mL SPC Buffer without protease inhibitors.
- 16 Mix with cold 25 mL Ficoll buffer.
- 17 Centrifuge for 10 min at 7,500 x g.
- 18 Wash pellet with 10 mL cold SPC buffer with protease inhibitors.
- 19 Centrifuge for 10 min at 4,200 x g.

- 20 Repeat steps 16 and 17 once. Resuspend nuclei in 5 mL SPC with protease inhibitors.
- 21 Snap-freeze in liquid nitrogen.

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

MNase digestion and chromatin extraction

- 22 Thaw nuclei on ice.
- 23 Pre-warm for 5 min on 37°C water bath.

When processing multiple samples, move each sample to the water bath in 30 second intervals.

- 24 Add 1 M CaCl_2 to 2 mM.
- 25 Add 2 U Sigma-Aldrich MNase. Mix by careful inversion.
- 26 Repeat steps 21 and 22 for each additional sample.
- 27 Incubate for 2.5 min or 10 min at 37°C . Mix by careful inversion every 1–2 min.

The MNase digestion time needs to be empirically defined depending on the factor of interest and the desired mapping resolution. See Figure 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 gauge needle, then four times through a 26 gauge needle.
- 30 Centrifuge for 10 min at $9,400 \times g$.
- 31 Collect the supernatant (fraction “S1”) and keep on ice.
- 32 Resuspend the cell pellet in 5 mL 80, 150 or 600mM NaCl Extraction Buffer and incubate for 4 h 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 section below.

- 33 Centrifuge for 15 min at $15,800 \times g$.
- 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 amount of 5 M NaCl and 10% Triton X-100.
- 36 Combine fractions S1 and S2.

A 50 μL fraction can be used as an input protein sample and 100 μL can reserved as an input DNA sample.

Chromatin Immunoprecipitation

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

Alternatively, the supernatant can be reserved as an unbound sample.

- 40 Wash beads by resuspending in 1 mL IP Wash Buffer. Transfer to a low-retention 1.5 mL microcentrifuge tube.
- 41 Repeat wash step 37 twice.
- 42 Resuspend beads in 400 µL IP Wash Buffer.

At this point IP samples are ready to purify DNA or protein. 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 Benzonase to the protein sample and incubate 20 min at 37°C. Then add 1/4 volume of 4X SDS sample buffer and boil at 100°C 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 EDTA 0.5 M, 8 µL 5 M NaCl and 1 µL RNase A. Incubate 20 min at 37°C.
- 45 Add 20 µL 10% SDS and 4 µL Proteinase K. Incubate 20 min at 70°C.
- 46 Add 1 volume of Phenol/Chloroform/Isoamyl Alcohol. Vortex 30 sec, and centrifuge at 15,000g for 5 minutes.
- 47 Carefully pipette 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 Carefully pipette the supernatant and transfer to a new tube.
- 49 Add 2 µL 20 mg/mL glycogen. Precipitate by adding two volumes pure ethanol at –80°C for at least 30 min.
- 50 Centrifuge at 18,000 x g for 10 min at 4°C.
- 51 Wash with 1 mL 100% Ethanol, being careful not to disrupt pellet and centrifuge again at 18,000 x g for 10 min at 4°C.
- 52 Remove supernatant and let air dry for 10 min.
- 53 Resuspend sample in 25 µL TE_{0.1} buffer.
- 52 Measure concentration with a high-sensitivity assay (e.g., QuantIt PicoGreen dsDNA assay).

The total amount of DNA expected is in the range of 3–100 ng, with concentrations as low as 0.5 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 the previous section for yeast cells, the procedure itself is considerably different with regards to nuclei isolation and chromatin sample preparation.

Materials

Solutions

- Complete Schneider's medium (see Recipes)
- PBS (see Recipes)
- TM2+ Buffer (see Recipes)
- TM2+I Buffer (see Recipes)
- 0.2 M CaCl₂
- MNase (see Recipes)
- 0.2 M EGTA
- TM2+IS (see Recipes)
- 80TM+IS (see Recipes)
- Antibody
- Protein G-coupled Magnetic Beads (Dynabeads, Life Technologies, Cat No 10004D)
- Benzonase (Sigma, Cat No E1014)
- 4X SDS sample buffer (Life Technologies, Cat No NP0007)
- 0.5 M EDTA
- 5 M NaCl
- RNase A (10 mg/mL, Thermo Scientific, Cat No EN0531)
- 10% SDS
- Proteinase K (20 mg/mL, Life Technologies, Cat. No. AM2542)
- Phenol/Chlorophorm/Isoamyl alcohol
- Glycogen (20 mg/mL, Life Technologies, Cat. No. 10814-010)
- 100% ethanol

- 70% ethanol
- Quant-iT PicoGreen dsDNA assay kit. (Life Technologies, Cat. No. P11496)

Materials

- T75 culture flasks, cell scrapers, serological pipettes
- Refrigerating 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 pipette tips
- 37°C heating block or water bath
- 26 ½ gauge needle with 1mL syringe
- Magnetic rack for microcentrifuge tubes

Nuclei isolation

- 1 Grow *Drosophila* S2 cells in T75 flasks with 15mL 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 pipette.
- 3 Count cells using a hemocytometer 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 samples 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 1,200 x g and wash the pellet with 10 mL cold PBS.

Carry all centrifugation steps at 4°C, or using a refrigerating centrifuge.

- 6 Centrifuge the cells again for 4 min at 1,200 x g, 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 3–5 min, vortexing at half maximum speed once a 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, 3:30 minutes has proved optimal. For *Drosophila* BG3 cells, 4 minutes is optimal.*

- 10** Centrifuge 10 min at 100 x g. 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+ by pipetting with a P1000 pipette.

The nuclei pellet should be readily resuspended after 5–10 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 x g and discard supernatant carefully. Resuspend in 800 µL TM2+I. Transfer to an microcentrifuge tube.

MNase digestion and chromatin preparation

- 13** Pre-warm nuclei sample for 3 min on 37°C heat block or water bath.

When processing multiple samples, move each sample to the heating block in 30 second intervals.

- 14** Add 4 µL 0.2 M CaCl₂. Quickly add 5 µL 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 poor-quality digest.

- 15** Repeat step 14 for each additional sample.

- 16** Incubate each sample for precisely 6 min. Mix by careful inversion every two minutes.

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

- 18** Centrifuge all samples for 10 min at 100 x g.

- 19** Carefully aspirate supernatant. Resuspend pellet with 800 µL TM2+IS.

- 20** Centrifuge 10 min at 100 x g.

- 21** Resuspend pellet in 400 µL 80TM+IS Buffer. Keep on ice.

- 22** Cavitate nuclei by drawing up and expelling the suspension with a 26 ½ gauge needle.

Draw up and expel firmly but slowly to avoid foaming.

- 23** Centrifuge 10 min at 100 x g.

- 24** Transfer 300 µL supernatant to a clean tube: this is the chromatin input.

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

Chromatin Immunoprecipitation

- 25** Add appropriate amount of antibody to the 300 μ L chromatin sample.

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

- 26** Incubate samples with agitation on Nutator overnight at 4°C.
- 27** Combine 40 μ L of magnetic bead slurry in 500 μ L 80TM+IS buffer. Let the solution equilibrate at room temperature for 3 min, capture beads in magnet, remove buffer, and repeat this step.
- 28** Add chromatin sample to beads. Carefully resuspend beads and chromatin with P1000 and incubate with agitation on Nutator for 2 h at 4°C.
- 29** Collect beads in magnetic 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 μ L 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 μ L Benzonase and incubate 20 min at 37°C. Then add 1/4 volume of 4X SDS sample buffer and boil at 100°C for 10 min. Place in magnet to remove beads and store supernatant at –20°C for later protein analysis.
- 32** Bring the volume of the DNA sample to 400 μ L with 80TM+IS. Add 8 μ L 0.5 M EDTA, 8 μ L 5 M NaCl and 1 μ L RNase A. Incubate 20 min at 37°C.
- 33** Add 20 μ L SDS 10% and 8 μ L Proteinase K. Incubate 10 min at 65°C.
- 34** Add 1 volume of Phenol/Chloroform/Isoamyl Alcohol. Vortex 30 sec, centrifuge at 15,000 x g for 5 minutes.
- 35** Carefully pipette 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, centrifuge at 15,000 x g for 5 minutes.
- 37** Carefully pipette the supernatant and transfer to a new tube.

- 38 Add 1/10 volume 3 M sodium acetate, and 1 μ L glycogen. Precipitate with 1mL ethanol at -20°C overnight.
- 39 Centrifuge at $18,000 \times g$ for 10 min at 4°C .
- 40 Wash with 70% ethanol, being careful not to disrupt pellet, centrifuge again at $18,000 \times g$ for 10 min at 4°C .
- 41 Remove supernatant and let air dry for 30 min.
- 42 Resuspend sample in 25 μ L TE_{0.1} buffer.
- 43 Measure concentration with a high-sensitivity assay.

The total amount of DNA expected is in the range of 3–100 ng, with concentrations as low as 0.5 ng/ μ L. 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, reproducible and can be used to build libraries from as little as 3 ng of total input DNA.

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

Materials

Reagents and solutions

- 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'-

AATGATACGGCGACCACCGAGATCTACACTCTTTCCCTACACGACGCTCTTCCG
ATC*T

TruSeq Indexed Adapter:

5'-

[P]GATCGGAAGAGCACACGTCTGAACTCCAGTCACNNNNNNNATCTCGTATGCCG
TCTTCTGCTT*G

Library Enrichment Primers:

P5 PCR Primer:

5' AATGATACGGCGACCACCGAG

P7 PCR Primer:

5' CAAGCAGAAGACGGCATACGAG

*[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.*

- Ultra Pure, nuclease free water
- 10X T4 ligase buffer (New England Biolabs, Cat. No. B0202S)
- dNTP mix, 10mM each (Life Technologies, Cat. No. 18427-013)
- T4 DNA Polymerase (Invitrogen, Cat. No.100004994)
- Klenow fragment (New England Biolabs, Cat. No. M0210L)
- T4 PNK (New England Biolabs, Cat. No. M0201L)
- Phenol/chloroform/isoamyl alcohol
- Illustra microspin HR S-300 columns (GE Healthcare Life Sciences, Cat. No. 27-5130-01)
- 10X NEBuffer 2 (New England Biolabs, Cat. No. B7002S)
- 1 mM ATP (see Recipes)
- Klenow exo- (New England Biolabs, Cat. No. M0212M)
- 2X 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} (see Recipes)
- KAPA HiFi HotStart DNA Polymerase with dNTPs (KAPA Biosystems, KK2501)

Materials

- Thermocycler
- Low retention 1.5 mL and 0.5 mL microcentrifuge tubes and pipette tips.
- Speedvac
- 37°C heat block or water bath.
- Magnetic rack for microcentrifuge tubes.

Prepare adapters

- 1 Mix equimolar amounts of TruSeq Universal Adapter and TruSeq Indexed Adapter in 1X TE buffer.
- 2 To anneal Universal and Indexed adapters, incubate mix in a thermocycler 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 15min.

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

End Repair

- 4 Use 3–100 ng of DNA for library preparation. Adjust volume of the sample to 38.5µL with Ultra Pure, 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 ATP
 - 2 µL dNTP mix (10 mM each)
 - 1.5 µL T4 DNA polymerase (5 U/µL)
 - 0.5 µL Klenow 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 to 38.5 µL of DNA. Mix by careful pipetting.

Hold 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 phenol/chloroform/isoamyl alcohol.
- 9 Vortex ~30 sec.
- 10 Centrifuge for 1 min at 18,000 x g.
- 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, centrifuge 1 min at 735 x g. Transfer column to a new 1.5 mL microcentrifuge tube being careful not to disrupt the resin.

- 12** Pipette up the aqueous layer.

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

- 13** Add the aqueous layer to the middle of the resin on a S-300 spin column and centrifuge 2 min at 735 x g.

- 14** Reduce volume to 35 μ L in Speedvac with no heat.

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

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

Addition of 3'-A overhang

- 15** Prepare the 3'-A overhang addition master mix by combining the following for each sample:

- 5 μ L 10X Buffer 2
- 10 μ L 1 mM dATP
- 0.3 μ L Klenow exo-nuclease

- 16** Add 15 μ L of A-tailing master mix to 35 μ L of DNA. Mix by careful pipetting.

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 Phenol/Chloroform/Isoamyl alcohol and clean up with an S-300 column by repeating steps 8–13.

- 19** Place on ice and add appropriate amount of annealed adapters, 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 below, Supporting Protocol 1). The median fragment size can be used as an approximation.

- 20** Reduce volume to 20 μ L in Speedvac (no heat). This takes 15–45 min. Compare volume to a tube containing 20 μ L. Adjust volume to 20 μ L using nuclease-free water if necessary.

Adapter ligation

- 21** Prepare adapter ligation master mix by combining for each sample:

- 25 μ L 2X Rapid DNA ligase buffer
- 5 μ L DNA ligase
- 22** Add 30 μ L of ligation master mix to 20 μ L of DNA. Mix by careful pipetting.

Hold 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 μ L of Ampure Magnetic bead slurry.

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

- 25** Mix well by careful pipetting. Incubate at room temperature for 5 min.
- 26** Place in magnetic tube holder and wait until cleared.

This takes approximately 2 min.

- 27** Aspirate and discard the supernatant being careful not to disrupt the beads.
- 28** Add 200 μ L 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 magnet.

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

- 31** Remove from magnet and disperse beads into 40 μ L TE_{0.1}. Mix thoroughly by careful pipetting.
- 32** Place into magnetic 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.

Library amplification

Library enrichment

- 34** Prepare a PCR reaction master mix by combining the following for each sample:

5.00 μ L 5X KAPA HiFi Fidelity buffer

0.75 μ L KAPA dNTP mix (10 mM each)

1.25 μ L P5 primer (10 uM)

1.25 μ L P7 primer (10 uM)

0.50 μ L KAPA HIFI HotStart DNA polymerase (1U/uL)

35 Add 8.75 μ L of KAPA PCR master mix to 16.25 μ L of Ampure-purified DNA

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

Step 1) 98°C 45 sec

Step 2) 98°C 15 sec

Step 3) Ramp to 60°C @ 1°C/sec

Step 4) 60° C 20 sec

Step 5) Ramp to 98°C at 1°C/sec

Step 6) Got to step 2, 9x or 13x

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

Step 7) 72°C 1 min

Step 8) 8°C forever

37 Add 25 μ L of Ampure magnetic bead slurry to PCR tubes.

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

38 Clean up sample by repeating steps 25–32.

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

A volume of 5 μ L 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 h at 75V with a low-molecular weight ladder. Take into consideration that adapter dimers add 126bp 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 1 - 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 (Figure 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 Roland & Reich, 2012). Due to the possibility for lot-to-lot variability in SPRI beads, determining the optimal bead mixture-to-DNA ratio is recommended.

Materials

Reagents and solutions

- Agencourt AMPure XP beads (Beckman Coulter, Cat. No. A63880) or equivalent custom made SPRI beads (see **Commentary**)
- Glycogen (20 mg/mL, Life Technologies, Cat. No. 10814-010)
- 5 M NaCl
- 100% ethanol
- 70% ethanol
- TE_{0.1} pH 8.0 (*see Recipes*)
- 1X TBE (*see Recipes*)
- 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)

Materials

- DynaMag-2 magnetic microcentrifuge tube rack.
- Siliconized low-retention microcentrifuge tubes.
- Low-retention 20, 250, and 1000 μ L filter tips.
- Refrigerated microcentrifuge.
- Vortex mixer.
- 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 to 10 bp ladder diluted to 0.5 μ g. For example, ratios ranging from 1.2:1 to 2.1:1 (SPRI beads:DNA) were used in the experiment shown in Figure 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 bead:DNA ratio that provides best enrichment of fragments smaller than ~100 bp in the Unbound fraction with most depletion of fragments >150 bp. For example, a bead:DNA ratio of 2.1:1 was chosen based on Figure 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 6–11 if recovery of large DNA fragments is desired.

- 6 Add 100 μ L 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 rack and disperse beads by adding 200 μ L TE_{0.1} to beads and pipetting up and down ten times.
- 10 Place the tube back in the magnetic rack and wait until cleared.
- 11 Transfer supernatant to new tube.

This is the bound, size-selected fraction.

Ethanol precipitation of size-selected fractions

- 12 Bring up volumes of Bound and Unbound fractions to 400 μ L using TE_{0.1}.
- 13 Add 30 μ g glycogen and 8 μ L 5M NaCl. Mix by inverting tubes.
- 14 Add 800 μ L cold pure ethanol and mix by inverting tubes.
- 15 Incubate overnight at -20°C .
- 16 Spin tubes in microcentrifuge at maximum speed for 30 min at 4°C .
- 17 Discard supernatant and wash the pellet once with 1 mL 100% ethanol.
- 18 Centrifuge tubes at 25,000 $\times g$ (or max speed on standard refrigerated benchtop microcentrifuge) for 10 min at 4°C .
- 19 Discard supernatant and air dry pelleted DNA for 5 min at room temperature.
- 20 Resuspend DNA pellet in 30 μ L TE_{0.1}.

To visualize Bound fractions, which should contain mainly fragments >150 bp, run DNA on 6% acrylamide gel stained with SYBR Gold. Example results with both Bound and Unbound fractions after SPRI bead size selection are shown in Figure 2C.

RECIPES

2 M Sorbitol

Make 2 M sorbitol stock solution by dissolving 182.17 g sorbitol in 400 mL distilled, de-ionized water. Bring to final volume of 500 mL and filter sterilize by passing solution through 0.2 μ m filter. Solution can be stored at room temperature for six months.

1 M CaCl₂

Make 1 M stock CaCl₂ solution by dissolving 110.98 g CaCl₂ in 80 mL distilled, de-ionized water. Bring to final volume of 100 mL and filter sterilize by passing solution through 0.2 μ m filter. Solution can be stored at room temperature for one year.

Potassium phosphate buffer, pH 7.5

Add 36g K₂HPO₄ and 108g KH₂PO₄ to beaker with 800 mL distilled, de-ionized water and stir at room temperature until dissolved. Adjust pH to 7.5 using concentrated KOH or NaOH and bring up volume to 1 L. Filter sterilize by passing solution through 0.2 μ m filter and store at room temperature for up to six months.

1 M PIPES, pH 6.3

Add 151.2 g PIPES (free acid) to 400 mL de-ionized 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 up volume to 500 mL with distilled, de-ionized water. Filter sterilize by passing solution through 0.2 μ m filter and store at 4°C for up to six months.

0.5 M EDTA, pH 8.0

Add 186.1 g EDTA to approximately 800 mL distilled, de-ionized water and stir at room temperature. Add NaOH pellets to reach pH 8. Filter sterilize by passing solution through 0.2 μ m filter and store at room temperature for up to six months.

5 M NaCl

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

100 mM PMSF

Add 17.4 mg phenylmethylsulfonyl fluoride to 1 mL isopropanol and mix by inversion. Store at –20°C for up to one year. Note that PMSF crystallizes upon freezing. Thaw at RT and vortex vigorously prior to use.

10 mg/mL LPC

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

Resuspension Buffer

Combine the following volumes of stock solutions to make 30 mL Resuspension Buffer: 18 mL 2 M sorbitol, 3 mL 1 M potassium phosphate pH 7.5, 15 μ L 1 M CaCl_2 , 15 μ L 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.

SPC Buffer

Combine the following volumes of stock solutions to make 100 mL SPC Buffer: 50 mL 2 M sorbitol, 2 mL 1 M PIPES pH 6.3, 10 μ L 1 M CaCl_2 , 48 mL sterile distilled, de-ionized water. SPC Buffer can be stored for up to six months at 4°C.

SPC Buffer with protease inhibitors

Same as above, with 1 mM phenylmethanesulfonyl fluoride (PMSF) and 10 μ g/mL each of leupeptin, pepstatin A, and chymostatin (LPC) diluted from 100 mM PMSF and 10 mg/mL LPC stock solutions, respectively.

Ficoll Buffer

To make 25 mL Ficoll Buffer, add 500 μ L 1 M PIPES pH 6.3 stock solution and 12.5 μ L 1 M CaCl_2 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 shaking platform until dissolved. This typically takes ~2 hrs. 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.

80 mM, 150 mM or 600 mM NaCl Extraction Buffer

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

IP Wash Buffer

To make 500 mL IP Wash Buffer, combine 5 mL potassium phosphate buffer, pH 7.5, 750 μ L 0.5 M EDTA, and 7 mL 5 M NaCl and bring volume to 500 mL with sterile, distilled de-ionized water. Store at 4°C for up to one year.

1 M Tris, pH 8

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, de-ionized water. Filter sterilize and store at room temperature for up to six months.

TE_{0.1}

To make 500 mL, combine 0.5 mL Tris pH 8 with 0.1 mL 0.5 M EDTA pH 8 and bring volume to 500 mL with distilled, de-ionized water. Filter sterilize and store at room temperature for up to six months.

Complete Schneider's medium

Combine 450 mL (Gibco, Cat No 21720-001) with 50 mL Fetal Bovine Serum (HyClone Cat No SH30070). Filter with a 0.2 µm pore size membrane in sterile conditions. Store at 4°C and use within 3 months.

PBS

Make 1 L 10X PBS by combining 800 mL double distilled H₂O with 80 g NaCl, 2 g KCl, 14.4 g Na₂HPO₄ and 2.4 g KH₂PO₄. Adjust pH to 7.4 with HCl, bring volume to 1 L, autoclave to preserve for up to 1 year at room temperature.

For 200 mL PBS 1X, mix 20 mL PBS 10X with 180 mL double distilled H₂O. Use within 1 month.

TM2+ Buffer

For 500 mL TM2 10X buffer, mix 5 mL 1 M Tris-HCl pH 7.4, 1 mL 1 M MgCl₂ and 494 mL ultra pure H₂O. 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 10X TM2 buffer, 75 µL 100 mM PMSF and 13.42 mL ultra pure H₂O. Keep on ice and use within 3 hours.

TM2+I Buffer

Prepare 50X EDTA-free Protease inhibitor cocktail by diluting one cOmplete, Mini, EDTA-free tablet (Roche, 11836170001) in 210 µL ultra pure H₂O. 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 above) with 80 µL 50X inhibitor cocktail. Keep on ice and use immediately.

MNase

Resuspend 15 kU of lyophilized USB MNase (Affymetrix, Cat No 70196Y) in 375 µL 50% Glycerol for a stock concentration of 40 U/µL. Aliquot and stock solution at -20°C for at least 1 year.

TM2+IS

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

80TM+IS

Make 100 mL 80T Buffer by combining 1.4 mL 5 M NaCl, 1 mL 1 M Tris-HCl pH 7.4, 200 μ L 1 M MgCl₂, 1 mL 0.2 M EGTA pH 8.0, 1 mL 10% Triton X-100, 95.4 mL ultra pure H₂O. This can be stored at room temperature for 6 months.

On the day of the experiment, prepare 2 mL fresh 80TM+IS buffer by mixing 1.93 mL 80T buffer with 10 μ L 100 mM PMSF, 40 μ L protease inhibitor cocktail (see above) and 20 μ L 0.2 M EGTA pH 8.0. Keep on ice and use immediately.

1 mM dATP

Dilute 1 μ L 100 mM dATP (Promega, U1201) in 99 μ L ultra pure water. Store at -20°C for at least a 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 (~6–20 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 (X-ChIP). Subsequent mapping of immunoprecipitated DNA sequences identifies TF binding sites genome-wide. 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 crosslinks by formaldehyde (Jackson, 1999; Jackson, 1978) and the artifactual formation of protein-DNA cross-links in nucleosome-depleted, highly transcriptionally active regions (Teytelman et al. 2013; Park 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 & Mascotti, 1992; Wilkins & 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 (20–40 bp), while regions simultaneously bound by multiple TFs yield larger fragments (60–80 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 demonstrated the utility of ORGANIC for mapping chromatin remodelers and protein complexes (Orsi, *et al.* 2014; Zentner, *et al.* 2013; Zentner & Henikoff, 2013), 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 (Figure 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 immunoprecipitate 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 ~80bp is critical for high-resolution mapping of individual binding sites. Support Protocol 1 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 Fig. 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 Support Protocol 1, 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 Figure 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, 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 250–500 mL culture in logarithmic growth phase ($OD_{600} = 0.6–0.8$), 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 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 ($\sim 7.5 \times 10^8$ cells) than the typical starting volume of 250 mL were robust and not sensitive to the antibody used for immunoprecipitation (Figure 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 (Figure 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 & Fraenkel, 2010; MacIsaac & Fraenkel, 2006). 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 peakcaller 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 3–100 ng of purified DNA after immunoprecipitation, which are used to prepare libraries for paired-end sequencing using 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 (Figure 3). Figure 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 over-represented DNA sequences 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 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 short bench time on days 1 and 2. Basic Protocols 1 or 2 can be performed on days 1–3 and library construction described in Basic Protocol 3 on days 3 and 4. If fragment size selection based on Support Protocol 1 is desired, an additional day is required between immunoprecipitation and library construction, provided 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 hour

Nuclei isolation, MNase digestion and chromatin extraction: ~8 total hours, ~4 hours of bench work.

Chromatin Immunoprecipitation, DNA purification and quantification: 48 total hours, ~3 hours of bench work.

Basic protocol 2: Buffer preparation: 1 hour

Nuclei isolation, MNase digestion and chromatin extraction: 2–3 hours

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

Basic protocol 3: End repair, 3' A overhang addition and adapter ligation: 4–5 hours

PCR library enrichment, clean-up and quantification: 3 hours

Support protocol 1: Once the optimal bead:DNA ratio is determined, the size selection can be completed in approximately 45 minutes.

ACKNOWLEDGEMENTS

GAO is supported by a Marie Curie Actions International Outgoing Fellowship (IOF-300710) from the European Union 7th Frame- work Program, Research Executive Agency. SK is supported by the Bob & Micki Flowers ARCS Endowment from the Seattle Chapter of the ARCS Foundation and grant 1F30 CA186458 from the US National Institutes of Health. GEZ is supported by the Fred Hutchinson Cancer Research Center Chromosome Metabolism and Cancer Training grant NIH 5T32 CA009657. Work in the laboratory of KA is supported by NIH grant R01GM098349. SH is supported by the Howard Hughes Medical Institute.

LITERATURE CITED

Bailey TL, Boden M, Buske FA, Frith M, Grant CE, Clementi L, Ren J, Li WW, Noble WS. MEME SUITE: tools for motif discovery and searching. *Nucleic Acids Res.* 2009 Jul.37:W202–W208.

(Web Server issue) Epub 2009 May 20. PubMed PMID: 19458158; PubMed Central PMCID: PMC2703892. [PubMed: 19458158]

Biggin MD. Animal transcription networks as highly connected, quantitative continua. *Dev Cell*. 2011 Oct 18; 21(4):611–626. Review. PubMed PMID: 22014521. [PubMed: 22014521]

Goecks J, Nekrutenko A, Taylor J, Galaxy Team. Galaxy: a comprehensive approach for supporting accessible, reproducible, and transparent computational research in the life sciences. *Genome Biol*. 2010; 11(8):R86. Epub 2010 Aug 25. PubMed PMID: 20738864; PubMed Central PMCID: PMC2945788. [PubMed: 20738864]

Henikoff S, Henikoff JG. “Point” centromeres of *Saccharomyces* harbor single centromere-specific nucleosomes. *Genetics*. 2012 Apr; 190(4):1575–1577. Epub 2012 Jan 10. PubMed PMID: 22234856; PubMed Central PMCID: PMC3316665. [PubMed: 22234856]

Jackson V. Studies on histone organization in the nucleosome using formaldehyde as a reversible cross-linking agent. *Cell*. 1978 Nov; 15(3):945–954. PubMed PMID: 569554. [PubMed: 569554]

Jackson V. Formaldehyde cross-linking for studying nucleosomal dynamics. *Methods*. 1999 Feb; 17(2):125–139. PubMed PMID: 10075891. [PubMed: 10075891]

Kasinathan S, Orsi GA, Zentner GE, Ahmad K, Henikoff S. High-resolution mapping of transcription factor binding sites on native chromatin. *Nat Methods*. 2014 Feb; 11(2):203–209. Epub 2013 Dec 15. PubMed PMID: 24336359; PubMed Central PMCID: PMC3929178. [PubMed: 24336359]

Kent WJ, Sugnet CW, Furey TS, Roskin KM, Pringle TH, Zahler AM, Haussler D. The human genome browser at UCSC. *Genome Res*. 2002 Jun; 12(6):996–1006. PubMed PMID: 12045153; PubMed Central PMCID: PMC186604. [PubMed: 12045153]

Krassovsky K, Henikoff JG, Henikoff S. Tripartite organization of centromeric chromatin in budding yeast. *Proc Natl Acad Sci U S A*. 2012 Jan 3; 109(1):243–248. Epub 2011 Dec 19. PubMed PMID: 22184235; PubMed Central PMCID: PMC3252899. [PubMed: 22184235]

Lohman TM, Mascotti DP. Thermodynamics of ligand-nucleic acid interactions. *Methods Enzymol*. 1992; 212:400–424. PubMed PMID: 1518457. [PubMed: 1518457]

MacIsaac KD, Fraenkel E. Practical strategies for discovering regulatory DNA sequence motifs. *PLoS Comput Biol*. 2006 Apr.2(4):e36. PubMed PMID: 16683017; PubMed Central PMCID: PMC1447654. [PubMed: 16683017]

Macisaac KD, Fraenkel E. Sequence analysis of chromatin immunoprecipitation data for transcription factors. *Methods Mol Biol*. 2010; 674:179–193. PubMed PMID: 20827592; PubMed Central PMCID: PMC3922641. [PubMed: 20827592]

Neph S, Vierstra J, Stergachis AB, Reynolds AP, Haugen E, Vernot B, Thurman RE, John S, Sandstrom R, Johnson AK, Maurano MT, Humbert R, Rynes E, Wang H, Vong S, Lee K, Bates D, Diegel M, Roach V, Dunn D, Neri J, Schafer A, Hansen RS, Kutayavin T, Giste E, Weaver M, Canfield T, Sabo P, Zhang M, Balasundaram G, Byron R, MacCoss MJ, Akey JM, Bender MA, Groudine M, Kaul R, Stamatoyannopoulos JA. An expansive human regulatory lexicon encoded in transcription factor footprints. *Nature*. 2012 Sep 6; 489(7414):83–90. PubMed PMID: 22955618; PubMed Central PMCID: PMC3736582. [PubMed: 22955618]

Orsi GA, Kasinathan S, Hughes KT, Saminadin-Peter S, Henikoff S, Ahmad K. High-resolution mapping defines the cooperative architecture of Polycomb response elements. *Genome Res*. 2014 May; 24(5):809–820. Epub 2014 Mar 25. PubMed PMID: 24668908; PubMed Central PMCID: PMC4009610. [PubMed: 24668908]

Park D, Lee Y, Bhupindersingh G, Iyer VR. Widespread misinterpretable ChIP-seq bias in yeast. *PLoS One*. 2013 Dec 9.8(12):e83506. eCollection 2013. PubMed PMID: 24349523; PubMed Central PMCID: PMC3857294. [PubMed: 24349523]

Poorey K, Viswanathan R, Carver MN, Karpova TS, Cirimotich SM, McNally JG, Bekiranov S, Auble DT. Measuring chromatin interaction dynamics on the second time scale at single-copy genes. *Science*. 2013 Oct 18; 342(6156):369–372. Epub 2013 Oct 3. PubMed PMID: 24091704; PubMed Central PMCID: PMC3997053. [PubMed: 24091704]

Rohland N, Reich D. Cost-effective, high-throughput DNA sequencing libraries for multiplexed target capture. *Genome Res*. 2012 May; 22(5):939–946. Epub 2012 Jan 20. PubMed PMID: 22267522; PubMed Central PMCID: PMC3337438. [PubMed: 22267522]

- Teves SS, Henikoff S. Heat shock reduces stalled RNA polymerase II and nucleosome turnover genome-wide. *Genes Dev.* 2011 Nov 15; 25(22):2387–2397. PubMed PMID: 22085965; PubMed Central PMCID: PMC3222904. [PubMed: 22085965]
- Teytelman L, Thurtle DM, Rine J, van Oudenaarden A. Highly expressed loci are vulnerable to misleading ChIP localization of multiple unrelated proteins. *Proc Natl Acad Sci U S A.* 2013 Nov 12; 110(46):18602–18607. Epub 2013 Oct 30. PubMed PMID: 24173036; PubMed Central PMCID: PMC3831989. [PubMed: 24173036]
- Wilkins RC, Lis JT. GAGA factor binding to DNA via a single trinucleotide sequence element. *Nucleic Acids Res.* 1998 Jun 1; 26(11):2672–2678. PubMed PMID: 9592153; PubMed Central PMCID: PMC147603. [PubMed: 9592153]
- Worsley Hunt R, Wasserman WW. Non-targeted transcription factors motifs are a systemic component of ChIP-seq datasets. *Genome Biol.* 2014 Jul 29.15(7):412. [Epub ahead of print] PubMed PMID: 25070602. [PubMed: 25070602]
- Zentner GE, Henikoff S. Mot1 redistributes TBP from TATA-containing to TATA-less promoters. *Mol Cell Biol.* 2013 Dec; 33(24):4996–5004. Epub 2013 Oct 21. PubMed PMID: 24144978; PubMed Central PMCID: PMC3889552. [PubMed: 24144978]
- Zentner GE, Tsukiyama T, Henikoff S. ISWI and CHD chromatin remodelers bind promoters but act in gene bodies. *PLoS Genet.* 2013; 9(2):e1003317. Epub 2013 Feb 28. PubMed PMID: 23468649; PubMed Central PMCID: PMC3585014. [PubMed: 23468649]
- Zhang Y, Liu T, Meyer CA, Eeckhoutte J, Johnson DS, Bernstein BE, Nusbaum C, Myers RM, Brown M, Li W, Liu XS. Model-based analysis of ChIP-Seq (MACS). *Genome Biol.* 2008; 9(9):R137. Epub 2008 Sep 17. PubMed PMID: 18798982; PubMed Central PMCID: PMC2592715. [PubMed: 18798982]

Figure 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 units produced an ideal ladder, with >75% mono-nucleosomal fragments, and a clear smear of sub-nucleosomal DNA.

Figure 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.

Figure 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 ~40bp segment by ORGANIC (brown track).

Author Manuscript

Author Manuscript

Author Manuscript

Author Manuscript