ChIP-exo Method for Identifying Genomic Location of DNA-Binding Proteins with Near-Single-Nucleotide Accuracy

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ABSTRACT

This unit describes the ChIP-exo methodology, which combines chromatin immunoprecipitation (ChIP) with lambda exonuclease digestion followed by high-throughput sequencing. ChIP-exo allows identification of a nearly complete set of the binding locations of DNA-binding proteins at near-single-nucleotide resolution with almost no background. The process is initiated by cross-linking DNA and associated proteins. Chromatin is then isolated from nuclei and subjected to sonication. Subsequently, an antibody against the desired protein is used to immunoprecipitate specific DNA-protein complexes. ChIP DNA is purified, sequencing adaptors are ligated, and the adaptor-ligated DNA is then digested by lambda exonuclease, generating 25- to 50-nucleotide fragments for high-throughput sequencing. The sequences of the fragments are mapped back to the reference genome to determine the binding locations. The 5′ ends of DNA fragments on the forward and reverse strands indicate the left and right boundaries of the DNA-protein binding regions, respectively. *Curr. Protoc. Mol. Biol.* 100:21.24.1-21.24.14. © 2012 by John Wiley & Sons, Inc.

Keywords: ChIP • ChIP-exo • binding location • genome-wide • lambda exonuclease

INTRODUCTION

This unit describes a method for determining the genomic locations of a known DNA-associated protein at near-nucleotide resolution using a combination of chromatin immunoprecipitation (ChIP) and lambda exonuclease digestion (exo) followed by high-throughput sequencing (Fig. 21.24.1). First, intact cells are treated with formaldehyde to cross-link *in vivo* protein-DNA and protein-protein interactions. After cell lysis, chromatin is subjected to sonication to shear the associated DNA to an appropriate size. The soluble chromatin is selectively immunoprecipitated with an antibody against the desired protein. Since size heterogeneity of sonicated DNA fragments is too variable to precisely demarcate genomic locations of DNA-bound proteins, a 5′→3′ exonuclease is employed to trim the DNA sequences on one strand to within a few base pairs of the cross-linking point. DNA sequences 3′ to the cross-linking point remain intact and are sufficiently long to be uniquely identified in the genome after sequencing.

Several enzymatic steps are performed while the immunoprecipitated protein-DNA adducts are still on the beads. The first step involves blunt-ended polishing of sonicated DNA fragment ends, the second step ligates the first DNA adaptor to both ends of the polished DNA, and the third step uses lambda and $RecJ_f$ exonucleases to trim the DNA. At this point, the DNA is eluted from the antibody beads, the protein-DNA cross-links are reversed, and the DNA is extracted. To complete library construction for high-throughput sequencing, the DNA is primer-extended and ligated with a second

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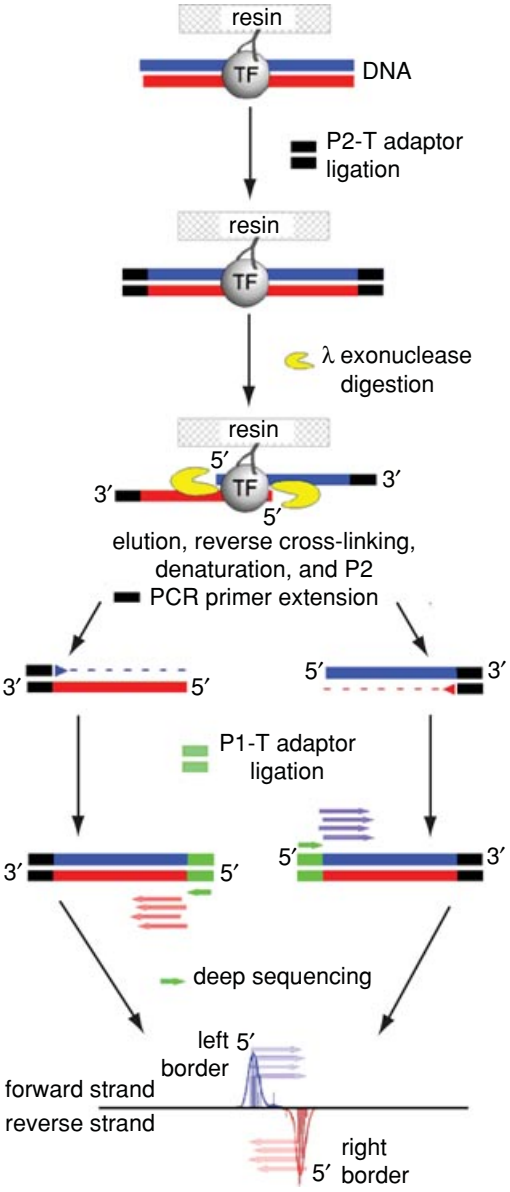

Figure 21.24.1 Scheme for ChIP-exo. After ChIP, 3′ sonicated ends are demarcated from the eventual exonuclease-treated 5′ end by ligating the P2-T adaptor to the ChIP DNA on the resin prior to exonuclease digestion. Then, 5′-to-3′ exonuclease trimming up to the site of cross-linking selectively eliminates the P2-T adaptor sequence attached at the 5′ end of each strand. After cross-link reversal, eluted single-stranded DNA is made double-stranded by P2 PCR primer extension. Finally, a P1-T adaptor is ligated to the exonuclease-treated end, and the resulting library is subjected to high-throughput sequencing. Mapping the 5′ ends of the resulting sequencing tags to the reference genome demarcates the exonuclease barrier and thus the precise site of protein-DNA cross-linking (adapted from Rhee and Pugh, 2011).

sequencing adaptor. After gel-purification and PCR, the amplified library is ready for high-throughput sequencing.

The method is similar for yeast and mammalian cells. The complete method is described for yeast (*Saccharomyces cerevisiae*) in the Basic Protocol, and minor modifications for mammalian cells are described in the Alternate Protocol.

IDENTIFICATION OF PROTEIN-DNA BINDING SITES IN *SACCHAROMYCES CEREVISIAE* BY ChIP-exo

BASIC PROTOCOL

This protocol was designed to detect genomic locations of a DNA-binding protein in *S. cerevisiae* at near-single-base resolution. It was developed for use with Applied Biosystems SOLiD sequencing instruments. Some minor differences for using the SOLiD System 5500 Series and the SOLiD System 2.0 are noted in the steps. Since the molecular biology of library construction in the SOLiD System 5500 Series is essentially the same as in the Illumina methodology, we infer, but have not tested, that Illumina adaptors should provide equivalent results.

Materials

Yeast culture
37% (w/v) formaldehyde
2.5 M glycine
ST buffer (see recipe)
Protease inhibitor cocktail tablets (Roche)
FA lysis buffer (see recipe)
20% (v/v) SDS
Antibody against protein of interest
Protein A− or G−Sepharose beads or equivalent (e.g., magnetic beads)
FA wash buffers 1, 2, and 3 (see recipes)
TE buffer (*APPENDIX 2*)
10 mM Tris-Cl, pH 7.5, 8.0, and 9.2 (*APPENDIX 2*)
NEBuffer 2 (New England BioLabs)
10× BSA (1 mg/ml)
3 and 25 mM dNTPs
3 mM dATP
Oligonucleotides for library construction (see Table 21.24.1)
3 U/μl T4 DNA polymerase (New England BioLabs)
10 U/μl T4 polynucleotide kinase with 10× buffer (New England BioLabs)
5 U/μl Klenow fragment (3′→5′ exo$^-$, New England BioLabs)
500 U/μl T4 DNA ligase with 10× buffer (New England BioLabs)
10 U/μl phi29 DNA polymerase with 10× buffer (New England BioLabs)
5 U/μl lambda exonuclease with 10× buffer (New England BioLabs)
30 U/μl $RecJ_f$ exonuclease (New England BioLabs)
ChIP elution buffer (see recipe)
20 μg/μl protease K (Roche)
25:24:1 (v/v/v) phenol/chloroform/isoamyl alcohol (Sigma)
70%, 75%, and 100% (v/v) ethanol
20 mg/ml glycogen (Roche)
AMPure magnetic beads (Agencourt)
5 U/μl *Taq* DNA polymerase with 10× buffer (New England BioLabs)

Shaking incubator (e.g., I-26R Incubator Shaker, New Brunswick)
200-ml centrifuge bottle with sealing cap (Nalgene)
RC6+ centrifuge with F10S-6X500Y rotor (Sorvall) or equivalent
2.0-ml conical tube, natural (USA Scientific)
Filtered pipet tips
0.5-mm zirconium silicate beads (Next Advance)
Mini-Beadbeater-96 (BioSpec)
22-G, 1.5-inch needle (Becton Dickinson)
13 × 100−mm borosilicate glass culture tube
Centrifuge 5810R with A-4-81 swinging-bucket rotor (Eppendorf) or equivalent

Table 21.24.1 Oligonucleotides for Library Construction

Oligonucleotide	Supplier	Sequence
For Applied Biosystems SOLiD System 5500 Series		
P1-T Adaptor (15 μM)	Life Technologies	5′-CCACTACGCCTCCGCTTTCCTCTCTA TGGGCAGTCGGTGAT-3′ (41 bp)
		5′-TCACCGACTGCCCATAGAGAGGAAAGCGGA GGCGTAGTGGCC-3′ (42 bp)
P2-T Adaptor (15 μM)	Life Technologies	5′-CGCCTTGGCCGTACAGCAGCCTCTTACACAG AGAATGAGGAACCCGGGGCAGTT-3′ (55 bp)
		5′-CTGCCCCGGGTTCCTCATTCTCTGTGTAAGAG GCTGCTGTACGGCCAAGGCGT-3′ (53 bp)
Library PCR Primer 1 (20 μM)	Life Technologies	5′-CCACTACGCCTCCGCTTT CCTCTCTATG-3′ (28 bp)
Library PCR Primer 2 (20 μM)	Life Technologies	5′-CTGCCCCGGGTTCCTCATTCT-3′ (21 bp)
For Applied Biosystems SOLiD System 2.0		
P1 Adaptor (15 μM)	Life Technologies	5′-CCACTACGCCTCCGCTTTCCTCTC TATGGGCAGTCGGTGAT-3′ (41 bp)
		5′-ATCACCGACTGCCCATAGAGAGGAAAGCGGA GGCGTAGTGGCC-3′ (43 bp)
P2 Adaptor (15 μM)	Life Technologies	5′-AGAGAATGAGGAACCCGGGGCAGTT-3′ (25 bp)
		5′-CTGCCCCGGGTTCCTCATTCTCT-3′ (23 bp)
Library PCR Primer 1 (20 μM)	Life Technologies	5′-CCACTACGCCTCCGCTTTCCTCTCTATG-3′ (28 bp)
Library PCR Primer 2 (20 μM)	Life Technologies	5′-CTGCCCCGGGTTCCTCATTCT-3′ (21 bp)
For Illumina instruments[a]		
TruSeq Adaptor, Index 1, first ligation[b]	Illumina	5′-GATCGGAAGAGCACACGTCTGAACTCCAGTCAC ATCACGATCTCGTATGCCGTCTTCTGCTTG-3′ (63 bp)
TruSeq Universal Adaptor, second ligation[c]	Illumina	5′-AATGATACGGCGACCACCGAGATCTACACTCT TTCCCTACACGACGCTCTTCCGATCT-3′ (58 bp)

[a]Not tested with this protocol.
[b]Also requires reverse complement oligo with 3′ T overhang. Index 1 underlined.
[c]Also requires reverse complement oligo lacking 5′-A.

1.5-ml microcentrifuge tubes
Microcentrifuge(s) (e.g., models 5415R, 5424, and 5424R, Eppendorf)
15-ml conical polystyrene and polypropylene tubes
Bioruptor Standard (Diagenode)
Rotating wheel
Thermomixer Comfort 5355R, for 1.5-ml tubes (Eppendorf)
65°C heating block
Vacufuge Plus vacuum concentrator (Eppendorf)
0.5-ml PCR tubes (Eppendorf)
Thermal cycler (e.g., DNA Engine Thermal Cycler PTC-200, Bio-Rad)
DynaMag magnet (Invitrogen)

NOTE: Substitutes may be used for the indicated suppliers. Specific centrifuges and rotors are listed for convenience, but any centrifuge that uses similar-sized bottles and reaches the same *g* force can be used. The authors use a DNA Engine Thermal Cycler PTC-200, but any similar cycler should also work.

Harvest cells and perform cross-linking

1. For each sample, grow 250 ml yeast cells to an OD_{600} of ~0.8 to 1.0.
2. Add 6.75 ml of 37% formaldehyde (1% final) and grow cells for 15 min in a shaking incubator at 25°C.
3. To quench cross-linking, add 15.4 ml of 2.5 M glycine (0.15 M final) and shake for another 5 min at 25°C.
4. Transfer culture to a 200-ml centrifuge bottle with a sealing cap. Centrifuge 3 min at 2,820 × *g*, 4°C (e.g., in an RC6+ centrifuge with F10S-6X500Y rotor). Discard supernatant and place cells on ice.
5. Resuspend cells in 1 ml ice-cold ST buffer containing protease inhibitors and transfer to a 2.0-ml natural conical tube on ice. Centrifuge 2 min at 2,300 × *g*, 4°C, and discard supernatant. Repeat once more, then freeze cells in liquid nitrogen.

   Freezing is an integral part of the lysis procedure and should not be skipped. If needed, frozen cells may be stored at −80°C for up to 3 years.

Lyse cells

6. Thaw cells on ice and add 1 ml ice-cold FA lysis buffer containing protease inhibitors, followed by 1 ml of 0.5-mm zirconium silicate beads.
7. Lyse cells twice for 3 min in a Mini-Beadbeater-96 (total 6 min). Be sure to keep samples chilled and return to ice after lysing.
8. Quickly wipe off ice or water from the outside of the tube, then invert and puncture the bottom with a red-hot 22-G, 1.5-inch needle. Immediately place the punctured tube inside a 13 × 100−mm borosilicate glass culture tube on ice so that the 2.0-ml tube is hanging on the opening of the glass tube with the puncture hole pointing down.
9. Centrifuge 1 min at 200 × *g*, 4°C, in a swinging-bucket rotor, then place samples on ice. Confirm that the entire sample has been transferred to the glass tube before discarding the 2.0-ml tube containing the beads.
10. Resuspend the disrupted cells into the liquid layer by pipetting up and down with a 1-ml micropipettor, then transfer the suspension to a 1.5-ml microcentrifuge tube on ice.

    Lysed cells may be stored at −80°C for up to 3 years.

Sonicate cells

11. Centrifuge cells 3 min at 2,300 × *g*, 4°C (e.g., in a model 5424R microcentrifuge), and discard the supernatant.
12. Add 1 ml ice-cold FA lysis buffer containing protease inhibitors and resuspend the pellet by pipetting up and down with a 1-ml micropipettor. Centrifuge 2 min at 2,300 × *g*, 4°C, and discard the supernatant. Repeat.
13. Add 1 ml ice-cold FA lysis buffer containing 0.2% SDS and protease inhibitors to the washed chromatin pellet and resuspend by pipetting up and down with a 1-ml micropipettor. Transfer to a 15-ml conical polystyrene tube on ice.

    Be sure that samples are transferred to a polystyrene tube (not to a polypropylene tube).

14. Shear chromatin to a median size of 250-300 bp sonicating in a Diagenode Bioruptor Standard (high power setting, 30 cycles of 30 sec on and 30 sec off). Keep in an ice-cold water bath during sonication. Transfer to a fresh 1.5-ml microcentrifuge tube on ice.

15. Centrifuge 10 min at 2,300 × *g*, 4°C, to pellet unbroken cells and debris. Transfer the supernatant to a fresh 1.5-ml microcentrifuge tube on ice and repeat centrifugation.

Sonicated chromatin may be stored at −80°C for up to 3 years.

Perform ChIP

16. Transfer sample to a 15-ml conical polypropylene tube and add 3 vol ice-cold FA lysis buffer with protease inhibitors to dilute the SDS concentration to 0.05%.

17. Add antibody against the protein of interest and incubate overnight at 4°C with shaking.

18. Using a wide-bore pipet tip (e.g., a 200-µl tip with the narrow end cut off), add 40 µl of a 50% Sepharose bead slurry and mix. Incubate 1.5 hr at 4°C on a rotating wheel.

19. Centrifuge 1 min at 94 × *g*, room temperature (e.g., in a model 5424 microcentrifuge), and remove the supernatant by aspiration.

20. Add 0.5 ml ice-cold FA lysis buffer containing protease inhibitors and resuspend beads by pipetting up and down with a wide-bore pipet tip. Transfer to a fresh 1.5-ml microcentrifuge tube and centrifuge 1 min at 94 × *g*, room temperature. Remove supernatant by aspiration.

21. Add 1 ml ice-cold FA lysis buffer containing protease inhibitors and incubate 5 min at room temperature on a rotating wheel. Pellet beads by centrifuging 1 min at 94 × *g*, room temperature and remove supernatant by aspiration. Repeat (total two washes).

22. Repeat step 21 using 1 ml each of the following buffers containing protease inhibitors in this order:

    ice-cold FA wash buffer 1 (high-salt)
    FA wash buffer 2
    FA wash buffer 3
    TE buffer.

23. Add 1 ml ice-cold 10 mM Tris-Cl, pH 8.0, containing protease inhibitors. Centrifuge 1 min at 94 × *g*, room temperature, and remove as much wash solution as possible without disturbing the beads.

ChIP material may be stored at 4°C for up to 3 days.

Perform on-bead enzymatic reactions

24. Assemble the polishing reaction as follows (total 60 µl) and incubate in a Thermomixer Comfort 5355R for 20 min at 12°C.

    20 µl chromatin-beads
    27 µl 10 mM Tris-Cl, pH 8.0 (final 10 mM)
    6 µl 10× NEBuffer 2 (final 1×)
    3 µl 10× BSA (final 1×, 100 µg/ml)
    3 µl 3 mM dNTPs (final 150 µM each)
    1 µl 3 U/µl T4 DNA polymerase (final 3 U).

    In all of the following enzymatic reactions, a Thermomixer is used for incubation with mixing. If a heating block is used instead of a Thermomixer, the tubes should be vortexed periodically to resuspend the beads.

25. Wash beads as in steps 21-22. Perform a final wash as in step 23 but use Tris-Cl at pH 7.5.

The following kinase reaction is optional, but may improve the yield of ligation. The beads are washed at pH 7.5 for optimal kinase activity. If the kinase reaction is skipped, perform the final wash at pH 8.0 and proceed to step 28.

For convenience, the protocol may be interrupted following any of the enzymatic reactions, as long as the beads have been fully washed (i.e., after any odd step from 25 to 39). Store washed chromatin-beads up to 1 day at 4°C.

26. Assemble the kinase reaction as follows (total 60 µl) and incubate for 30 min at 37°C.

    20 µl chromatin-beads
    33 µl 10 mM Tris-Cl, pH 7.5 (final 10 mM)
    6 µl 10× T4 polynucleotide kinase reaction buffer (final 1×)
    1 µl 10 U/µl T4 polynucleotide kinase (final 10 U).

27. Wash as in steps 21-23 with the final wash at pH 8.0.

    A-tailing reactions are needed only for SOLiD System 5500 series. For SOLiD System 2.0, perform the final wash at pH 7.5 and proceed to step 30.

28. Assemble the A-tailing reaction as follows (total 60 µl) and incubate for 30 min at 37°C.

    20 µl chromatin-beads
    31 µl 10 mM Tris-Cl, pH 8.0 (final 10 mM)
    6 µl 10× NEBuffer 2 (final 1×)
    2 µl 3 mM dATP (final 100 µM)
    1 µl 5 U/µl Klenow fragment (final 5 U).

29. Wash as in steps 21-23 with the final wash at pH 7.5.

30. Assemble the first adaptor ligation reaction (total 60 µl) and incubate for 90 min at 25°C.

    20 µl chromatin-beads
    28 µl 10 mM Tris-Cl, pH 7.5 (final 10 mM)
    6 µl 10× T4 DNA ligase buffer (final 1×)
    5 µl 15 µM P2-T or P2 adaptor (final 1.25 µM)
    1 µl 500 U/µl T4 DNA ligase (final 500 U).

    Use the P2-T adaptor for SOLiD System 5500 Series, and the P2 adaptor for SOLiD System 2.0.

31. Wash as in steps 21-23 with the final wash at pH 7.5.

32. Assemble the filling-in reaction (total 60 µl) and incubate for 20 min at 30°C.

    20 µl chromatin-beads
    18 µl 10 mM Tris-Cl, pH 7.5 (final 10 mM)
    12 µl 10× BSA (final 2×, 200 µg/ml)
    6 µl 10× phi29 DNA polymerase buffer (final 1×)
    3 µl 3 mM dNTPs (final 150 µM each)
    1 µl 10 U/µl phi29 DNA polymerase (final 10 U).

33. Wash as in steps 21-23 with the final wash at pH 7.5.

    The following kinase reaction can be skipped if 5′-phosphorylated adaptors were used in the first adaptor ligation (step 30). In this case, perform the final wash at pH 9.2 and proceed to step 36.

34. Assemble the kinase reaction (total 60 µl) and incubate for 30 min at 37°C.

    20 µl chromatin-beads
    33 µl 10 mM Tris-Cl, pH 7.5 (final 10 mM)
    6 µl 10× T4 polynucleotide kinase reaction buffer (final 1×)
    1 µl 10 U/µl T4 polynucleotide kinase (final 10 U).

35. Wash as in steps 21-23 with the final wash at pH 9.2.

36. Assemble the lambda exonuclease reaction (total 60 µl) and incubate for 30 min at 37°C.

    20 µl chromatin-beads
    32 µl 10 mM Tris-Cl, pH 9.2 (final 10 mM)
    6 µl 10× lambda exonuclease buffer (final 1×)
    2 µl 5 U/µl lambda exonuclease (final 10 U).

37. Wash as in steps 21-23 with the final wash at pH 8.0.

38. Assemble the $RecJ_f$ exonuclease reaction (total 60 µl) and incubate for 30 min at 37°C.

    20 µl chromatin-beads
    33 µl 10 mM Tris-Cl, pH 8.0 (final 10 mM)
    6 µl 10× NEBuffer 2 (final 1×)
    1 µl 30 U/µl $RecJ_f$ exonuclease (final 30 U).

39. Wash as in steps 21 and 22.

Elute chromatin from antibody beads

40. Add 450 µl ChIP elution buffer, mix, and incubate 15 min in a 65°C heating block to elute the chromatin precipitate from antibody beads.

    Other ChIP elution buffers can be used.

41. Centrifuge 1 min at 94 × *g*, room temperature, and transfer the eluate (450 µl) to a fresh 1.5-ml microcentrifuge tube.

    The eluate may be stored at 4°C for 1 day.

Reverse cross-linking and extract DNA

42. Add 1 µl of 20 µg/µl protease K and incubate at 65°C overnight to reverse the cross-links.

43. Add 450 µl ice-cold 25:24:1 (v/v/v) phenol/chloroform/isoamyl alcohol. Vortex vigorously for 1 min and separate phases by centrifuging 6 min at 18,400 × *g*, room temperature (e.g., in a model 5424 microcentrifuge).

44. Transfer aqueous (upper) phase to a fresh 1.5-ml microcentrifuge tube and add 1 ml ice-cold 100% ethanol followed by 1 µl of 20 mg/ml glycogen to aid precipitation. Mix thoroughly and incubate 1 hr at −80°C.

45. Centrifuge 15 min at 16.2 × *g*, 4°C (e.g., in a model 5415R microcentrifuge). Discard supernatant.

    The precipitates will form a very small and nearly invisible pellet.

46. Wash pellet with 500 µl ice-cold 75% ethanol, centrifuge for 5 min at 16.2 × *g*, 4°C, and discard supernatant.

47. Allow sample to dry for 20 min in a Vacufuge Plus vacuum concentrator.

48. Resuspend the precipitate in 11 µl TE buffer and transfer to a 0.5-ml PCR tube.

    Samples may be stored at −20°C for up to 3 years. Thaw before use.

Perform primer extension and second adaptor ligation

49. Assemble the denaturing and primer extension reaction mixture as follows and add to the DNA sample (total 19 µl).

    2 µl 10× phi29 DNA polymerase buffer (final 1×)
    4 µl 10× BSA (final 1×, 100 µg/ml)
    1 µl 3 mM dNTPs (final 75 µM each)
    1 µl 20 µM library PCR primer 2 (final 1 µM).

    Incubate in a thermal cycler for 5 min at 95°C (denaturation) followed by 5 min at 62°C (primer annealing). Allow to cool to room temperature.

50. Add 1 µl of 10 U/µl phi29 DNA polymerase (total 20 µl) and incubate in the thermal cycler for 20 min at 30°C (primer extension) followed by 10 min at 65°C (heat inactivation).

51. Assemble the A-tailing reaction mixture as follows and add to the sample (total 30 µl).

    5 µl TE buffer
    3 µl 10× NEBuffer 2 (final 1×)
    1 µl 3 mM dATP (final 100 µM)
    1 µl 5 U/µl Klenow fragment (final 5 U).

    Incubate in a thermal cycler for 30 min at 37°C. Allow to cool to room temperature.

    A-tailing reactions are needed only for SOLiD System 5500 Series. For SOLiD System 2.0, skip this step and proceed to step 52.

52. Assemble the second adaptor ligation reaction mixture as follows and add to the sample.

    For SOLiD 5500 (total 40 µl):
    4 µl TE buffer
    4 µl 10× T4 DNA ligase buffer (final 1×)
    1 µl 15 µM P1-T adaptor (final 0.4 µM)
    1 µl 500 U/µl T4 DNA ligase (final 500 U).

    For SOLiD 2.0 (total 30 µl):
    5 µl TE buffer
    3 µl 10× T4 DNA ligase buffer (final 1×)
    1 µl 15 µM P1 adaptor (final 0.4 µM)
    1 µl 500 U/µl T4 DNA ligase (final 500 U).

    Incubate in a thermal cycler for 1 hr at 25°C. Allow the reaction to cool to room temperature.

    The buffer volumes are adjusted for SOLiD 2.0 because of the smaller volume without the A-tailing reaction.

    Samples may be stored at −20°C for up to 3 years. Thaw before use.

Perform AMPure purification

53. Transfer sample to a 1.5-ml microcentrifuge tube.

54. Gently shake AMPure bottle to resuspend magnetic particles. Add 72 µl (for 5500 Series) or 52 µl (for 2.0) AMPure beads to the sample and mix thoroughly by pipetting ten times.

55. Place the tube onto a DynaMag magnet for 2 min to separate beads from the solution, then aspirate and discard the cleared solution.

56. Add 200 µl of 70% ethanol to the beads, incubate for 30 sec at room temperature, and mix thoroughly by pipetting 10 times. Place the tube on the magnet for 2 min and remove the solution. Repeat twice.

57. Aspirate all cleared solution from the tube on the magnet and then dry beads for 20 min at room temperature. Be sure to remove all ethanol from the bottom of the tube.

58. Add 30 µl TE buffer, pH 8.0, and mix by pipetting ten times. Place the tube on the magnet for 2 min and transfer the 30 µl eluate to a fresh 0.5-ml PCR tube.

    Samples may be stored at −20°C for up to 3 years. Thaw before use.

Amplify by PCR

59. Assemble the PCR reaction mixture as follows:

    3 µl TE buffer
    4 µl 10× standard *Taq* reaction buffer (final 1×)
    0.5 µl 25 mM dNTPs (final 0.3 mM each)
    1 µl 20 µM library PCR primer 1 (final 0.5 µM)
    1 µl 20 µM library PCR primer 2 (final 0.5 µM)
    0.5 µl 5 U/µl *Taq* DNA polymerase (final 2.5 U).

    Add the PCR reaction mixture to the eluate (total 40 µl).

60. Carry out hot-start PCR using the following parameters:

Initial step:	5 min	95°C (denaturation)
12-25 cycles:	15 sec	95°C (denaturation)
	15 sec	62°C (annealing)
	1 min	72°C (extension)
Final step:	5 min	72°C (final extension).

Minimal cycling is desirable to avoid over-amplification. The number of cycles should be determined based on the amount of starting material used for ChIP.

The sample is now ready for next-generation sequencing following the manufacturer's instructions.

Samples may be stored at −20°C for up to 3 years until next-generation sequencing.

ALTERNATE PROTOCOL

IDENTIFICATION OF PROTEIN-DNA BINDING SITES IN MAMMALIAN CELLS BY ChIP-exo

The harvesting of mammalian cells and preparation of cross-linked, sonicated DNA that is suitable for ChIP-exo is the same as for any standard ChIP methodology, and thus any normal protocol that works for ChIP in mammalian cells may be used up through the sonication step. The immunoprecipitation step and subsequent enzymatic reactions for ChIP-exo are essentially the same as in the protocol for yeast cells, except that the buffers have been optimized for mammalian cells, as described below.

***Additional Materials** (also see Basic Protocol)*

Cross-linked, sonicated DNA from mammalian cell culture (*UNIT 21.19*)
IP buffer
Mixed micelle buffer (see recipe)
High-salt buffer (see recipe)
Detergent buffer (see recipe)

1. Transfer 2.5 × 10^6 cell equivalents of cross-linked, sonicated DNA to a 15-ml polypropylene tube.
2. Add antibody against the protein of interest, mix, and incubate on a rotating wheel overnight at 4°C.
3. Using a wide-bore pipet tip (e.g., a 200-µl tip with the narrow end cut off), add 40 µl of a 50% Sepharose bead slurry and mix. Incubate 1.5 hr at 4°C on a rotating wheel.
4. Centrifuge for 1 min at 94 × *g*, room temperature, and remove the supernatant by aspiration.
5. Add 0.5 ml ice-cold IP buffer containing protease inhibitors and resuspend beads by pipetting up and down with wide-bore pipet tip. Transfer to a fresh 1.5-ml microcentrifuge tube and centrifuge 1 min at 94 × *g*, room temperature. Remove supernatant by aspiration.
6. Add 1 ml ice-cold mixed micelle buffer containing protease inhibitors and incubate 5 min at room temperature on a rotating wheel. Pellet beads by centrifuging 1 min at 94 × *g*, room temperature, and remove the supernatant by aspiration. Repeat (total two washes).
7. Repeat step 6 using the following buffers containing protease inhibitors in this order:

   ice-cold mixed micelle buffer
   high-salt buffer
   detergent buffer
   TE buffer.

8. Add 1 ml ice-cold 10 mM Tris-Cl, pH 8.0, containing protease inhibitors. Centrifuge 1 min at 94 × *g*, room temperature, and remove as much wash solution as possible without disturbing the beads.
9. Proceed with ChIP-exo as described (see step 24 of the Basic Protocol), but after each on-bead reaction, wash the beads as in steps 6-7 above instead of steps 21-22 of the Basic Protocol.

   After each TE buffer wash, the final wash in Tris-Cl of the indicated pH is still performed to optimize the subsequent enzyme reaction.

REAGENTS AND SOLUTIONS

Use only molecular-biology-grade water (e.g., DNase-, RNase-, and protease-free, deionized, distilled) in all recipes and protocol steps. For common stock solutions, see ***APPENDIX 2****; for suppliers, see* ***SUPPLIERS APPENDIX****.*

ChIP elution buffer

25 mM Trizma
2 mM EDTA, pH 8.0
200 mM NaCl
0.5% (w/v) SDS
Store up to 5 years at room temperature

Detergent buffer

10 mM Tris-Cl, pH 8.0 (*APPENDIX 2*)
10 mM EDTA
250 mM LiCl
0.5% Nonidet P-40 (NP-40, Ipegal)
0.5% sodium deoxycholate
Store up to 1 year at −4°C

FA lysis buffer

50 mM HEPES, pH 8.0
2 mM EDTA, pH 8.0
150 mM NaCl
1% Triton X-100
0.1% sodium deoxycholate
Store up to 1 year at −4°C

FA wash buffer 1 (high-salt)

50 mM HEPES, pH 8.0
2 mM EDTA, pH 8.0
1 M NaCl
1% Triton X-100
0.1% sodium deoxycholate
Store up to 1 year at −4°C

FA wash buffer 2

50 mM HEPES, pH 8.0
2 mM EDTA, pH 8.0
0.5 M NaCl
1% Triton X-100
0.1% sodium deoxycholate
Store up to 1 year at −4°C

FA wash buffer 3

10 mM Tris-Cl, pH 8.0 (*APPENDIX 2*)
2 mM EDTA
25 mM LiCl
1% Nonidet P-40 (NP-40, Ipegal)
1% sodium deoxycholate
Store up to 1 year at −4°C

High-salt buffer

25 mM HEPES, pH 8.0
5 mM Tris-Cl, pH 8.0 (*APPENDIX 2*)
250 mM NaCl
0.5 mM EDTA
0.05% sodium deoxycholate
0.5% Triton X-100
Store up to 1 year at −4°C

Immunoprecipitation (IP) buffer

100 mM NaCl
50 mM Tris-Cl, pH 8.0 (*APPENDIX 2*)

continued

5 mM EDTA
0.33% (w/v) SDS
1.5% Triton X-100
Stored up to 1 year at 4°C

Mixed micelle buffer

20 mM Tris-Cl, pH 8.0 (*APPENDIX 2*)
5 mM EDTA
150 mM NaCl
5.2% (w/v) sucrose
0.2% (w/v) SDS
1% Triton X-100
Store up to 1 year at −4°C

ST buffer

10 mM Tris-Cl, pH 7.5 (*APPENDIX 2*)
100 mM NaCl
Store up to 1 year at −4°C

COMMENTARY

Background Information

Proteins bind to DNA to regulate genes, so it is important to determine where proteins are bound in the genome to understand how they regulate gene expression (Struhl, 1995; Ptashne and Gann, 1997; Lee et al., 2002). ChIP is the most widely used method to identify where DNA-binding proteins are located on the genome. However, some nonspecific binding occurs during ChIP, creating background signals (Peng et al., 2007; Rozowsky et al., 2009; Tuteja et al., 2009). Moreover, the size heterogeneity of randomly sheared ChIP DNA technically limits mapping resolution. The ChIP-exo methodology, described in this unit, can precisely map genomic binding locations of a protein at near-single-base resolution and eliminate most background signal (Rhee and Pugh, 2011).

This method uses lambda exonuclease digestion, which processively degrades a DNA strand in the 5′→3′ direction until a protein-DNA cross-linking point is encountered. Thus, a protein covalently cross-linked to DNA would block strand-specific degradation by exonuclease, thereby creating a homogeneous 5′ border at a fixed distance from the bound protein. Un-cross-linked nonspecific DNA is largely eliminated by exonuclease treatment.

Critical Parameters

Although lambda exonuclease digests 5′ DNA up to the protein-DNA cross-linking points, smaller starting sizes of sonicated DNA fragments yield best results. In yeast cells, a median size of 250-300 bp DNA fragments is preferred after sonication.

Lambda exonuclease is a highly processive enzyme that acts in the 5′-to-3′ direction to remove 5′ mononucleotides from double-stranded DNA (Little, 1981). Therefore, it degrades only one strand of double-stranded DNA, leaving a single-stranded DNA product. Since the preferred substrate is 5′-phosphorylated double-stranded DNA, a prior kinase reaction (Basic Protocol, step 34) will yield optimal lambda exonuclease activity.

$RecJ_f$ is a single-strand-specific exonuclease that acts in the 5′-to-3′ direction to remove 5′ mononucleotides (Lovett and Kolodner, 1989). Therefore, it acts on the product generated by lambda exonuclease. Unbound DNA can adsorb nonspecifically to Sepharose beads during immunoprecipitation, producing background sequencing tags throughout the genome, which decrease the sensitivity of ChIP. The combination of lambda and $RecJ_f$ exonucleases removes a substantial portion of this background.

When PCR is performed, minimal cycling is desirable to avoid over-amplification (i.e., selective amplification of certain sequences). The optimal number of cycles should be determined based on the amount of starting material used for ChIP, and can be tested by using 12, 15, 18, 21, and 25 cycles and checking the PCR products on an agarose gel.

Troubleshooting

If most of the material is not converted to double-stranded DNA after primer extension (Basic Protocol, steps 49-50), this might be due to the low efficiency of the first adaptor ligation (step 30), which can be checked

by ligation-mediated PCR using PCR primer 2 (Dai et al., 2000). In addition, the presence of ChIP material can be checked by locus-specific PCR (Rychlik et al., 1990) for known binding locations. If different primer sequences are used for primer extension (steps 49-50) or PCR amplification (steps 59-60), try varying the annealing temperature, which is 62°C for both reactions.

If sequencing data show relatively low complexity and many repeat sequencing reads, try increasing the number of cells used for ChIP from the recommended 250 ml yeast cells (OD_{600} ~0.8 to 1.0) and 2.5×10^6 mammalian cells. The ratio of cells to antibody can also be titrated, depending on the protein of interest, the antibody efficiency, and the accuracy of the antibody concentration. Reducing the number of PCR cycles may also help reduce repeat sequencing reads.

If the signal from the adaptor dimers on the final PCR product (step 58) is too strong (i.e., by comparing with non-template control [PCR reaction buffer without sample DNA]), size selection is recommended to reduce the signal. Size selection on a polyacrylamide gel can be performed after the AMPure purification step (between steps 58 and 59), and allows for exclusion of unligated adaptor DNA. Reducing the number of PCR cycles may also help reduce selective amplification of adaptor dimer DNA sequences.

Anticipated Results

Starting with sonicated chromatin fragments with a median size of 250-300 bp, the median size of the final PCR product (step 60) should be approximately 225-250 bp, consisting of 125-150 bp of DNA fragment and 97 bp of ligation adaptors (Rhee and Pugh, 2012). It is possible that other minor products from adaptor dimers will also be visible.

Time Considerations

The Basic Protocol can be completed in 3.5 days. Harvesting cells, cross-linking, and quenching require 1 day. Cell lysis and sonication require half a day. ChIP, on-bead enzymatic reactions, and elution require 1 day. Reverse cross-linking, phenol/chloroform extraction, adaptor ligation, and PCR require 1 day.

Acknowledgments

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Key Reference

Rhee and Pugh, 2011. See above.

First description of ChIP-exo method for various transcription factors in species ranging from yeast to human. This paper shows the proof of principle of ChIP-exo, identifying a nearly complete set of genomic binding sites at near-single-nucleotide resolution.