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Mapping protein-DNA interactions using ChIP-exo and high-throughput sequencing

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Abstract

Chromatin immunoprecipitation (ChIP) provides a means of enriching DNA associated with transcription factors, histone modifications and indeed any other proteins for which suitably characterized antibodies are available. Over the years, sequence detection has progressed from quantitative real-time PCR and Southern blotting to microarrays (ChIP-chip) and now high-throughput sequencing (ChIP-seq). This progression has vastly increased the sequence coverage and data volumes generated. This in turn has enabled informaticians to predict the identity of multi-protein complexes on DNA based on the over-representation of sequence motifs in DNA enriched by ChIP with a single antibody against a single protein. In the course of the development of high-throughput sequencing, little has changed in the ChIP methodology until recently. In the last three years, a number of modifications have been made to the ChIP protocol with the goal of enhancing the sensitivity of the method and further reducing the levels of non-specific background sequences in ChIPped samples. In this chapter, we provide a brief commentary on these methodological changes and describe a detailed ChIP-exo method able to generate narrower peaks and greater peak coverage from ChIPped material.

1. Introduction

In 1988, Solomon *et al.* first described the mapping of protein-DNA interactions *in vivo* using formaldehyde as a crosslinking agent to stabilize these otherwise fragile interactions [1]. Ever since then, the chromatin immunoprecipitation (ChIP) protocol has only moderately changed, with the introduction of ultrasonic shearing devices and magnetic beads for separation being two notable exceptions. However, the downstream analysis of precipitated DNA fragments has been subject to immense changes and developments. Initially, the introduction of the ChIP-chip microarray technique allowed a first global and unbiased overview of the precipitated material [2]. Although this approach was a groundbreaking development, it was still far from optimal as it suffered from high noise, delivered relatively low resolution and was prone to artefacts. With the emergence of affordable high-throughput sequencing, first global ChIP-seq studies for both histone marks and transcription factors were published in 2007 [3,4]. The main advantage of ChIP-seq over the ChIP-chip technology is undoubtedly the resolution but other factors, such as the possibility to multiplex (i.e. simultaneous sequencing of multiple samples in a single lane using unique indices/barcodes) and reduced bias, should not be discounted.

However, the ability to quickly generate tens, and up to hundreds, of million reads per sample also imposes certain challenges on researchers, as these data require both storage space and novel computational approaches to interpret. As of today, there is no obligatory analysis pipeline or quality control when publishing ChIP-seq studies. Thus, studies vary in

their sequencing depth and quality as well as algorithms used for alignment and peak calling. In an effort to define uniform guidelines and make ChIP-seq experiments more reproducible between different labs, the ENCODE consortium recently published an updated version of their recommendations, which should be regarded as the gold standard [5].

In recent years, several minor and major modifications of the original ChIP-seq approach have emerged. These approaches generally try to tackle the biggest issue of conventional ChIP-seq, the requirement for large number of cells. We have compiled a list of the most popular modifications and listed their advantages over regular ChIP-seq (**Table 1**) [6-12].

Among these, the incorporation of 5'-3' strand-specific exonuclease digestion into the standard ChIP-seq procedure, ChIP-exonuclease (ChIP-exo), stands out as it does not necessarily aim at reducing input material but rather at improving signal to noise ratio, increasing plexity and cutting down hands-on time. ChIP-exo refines the conventional ChIP-seq by performing several on-bead enzymatic reactions prior to elution of the ChIPped DNA, thereby eliminating most of the time consuming AMPure XP beads cleanups (**Figure 1**). In addition, two on-bead exonuclease reactions digest DNA fragments until they reach protected areas (**Figure 2**). This greatly reduces fragment length and noise and allows for more multiplexing as it lowers the requirements for sequencing reads necessary for robust peak calling. In addition, ChIP-exo facilitates the high-resolution of TF binding locations and binding motifs by significantly reducing peak widths (**Figure 2**) [11]. Motif enrichment analysis of ChIP peaks remains a key tool in inferring the composition of multi-protein transcription complexes.

The method was originally developed by Rhee *et al.* in 2011 for the analysis of yeast transcription factor binding sites on the SOLID sequencing platform (Applied Biosystems) [11]. Recently, however, Serandour *et al.* developed an Illumina-based counterpart [12],

thereby enabling it to be compatible with the currently most widely used sequencing platform. Here, we focus on this application of this method to study androgen receptor (AR) binding in prostate cancer cells since ChIP-seq has become a vital instrument to define the AR transcriptome and several landmark studies have utilized this technique. These include ChIP-seq studies of the AR itself in cell lines and tumor tissue [13,14], ETS transcription factors, such as ERG [15], and pioneering factors, such as FoxA1 [16].

We provide a focused description of positive- and negative-control experiments to measure androgen-stimulated AR binding using ChIP-exo in combination with direct Illumina-based high throughput sequencing. However, this method is also more generally applicable to the study of AR and other transcription factors in other contexts.

2. Materials

2.1 Oligonucleotides

See **Table 2** for sequence information.

2.2 Cell culture and crosslinking

1. RPMI1640 media (Life technologies) supplemented with 10% fetal bovine serum (FBS) (Gibco)
2. Phenol red-free RPMI (Life technologies), supplemented with 10% charcoal dextran-stripped FBS (CSS) (Life technologies)
3. AR ligands, for example 5α-Dihydrotestosterone/5α-Androstan-17β-ol-3-one (DHT) (Sigma) or R1881 (Sigma)
4. Formaldehyde (e.g. Sigma)
5. 2.5M glycine solution

2.3 Harvesting, lysis and sonication of cells

1. Cell scrapers
2. PBS (Life technologies)
3. Rotating tube mixer at 4°C
4. Diagenode Bioruptor (Diagenode)
5. Proteinase inhibitor cocktail (Roche)

6. Lysis buffer 1 (LB1): 50mM HEPES-KOH (pH 7.5), 140mM NaCl, 1mM EDTA, 10% glycerol, 0.5% NP-40, 0.25% Triton-X – add 1x Proteinase inhibitor cocktail fresh before use
7. Lysis buffer 2 (LB2): 10mM Tris-HCl (pH 7.5), 200mM NaCl, 1mM EDTA, 0.5mM EGTA – add 1x Proteinase inhibitor cocktail fresh before use
8. Lysis buffer 3 (LB3): 10mM Tris-HCl (pH 7.5), 100mM NaCl, 1mM EDTA, 0.5mM EGTA, 0.1% Sodium-Deoxycholate, 0.5% SDS – add 1x Proteinase inhibitor cocktail fresh before use
9. 10% Triton-X in LB3

2.4 Immunoprecipitation

1. Dynabeads Protein A/G beads (Life technologies) (See **Note 1**)
2. Magnetic tube rack (e.g. Life technologies)
3. Antibody targeting your protein of interest (e.g. anti-AR N-20 Santa Cruz Biotechnology)
4. Non-specific control IgG (e.g. rabbit IgG)
5. PBS supplemented with 0.5% bovine serum albumin (BSA)

2.5 ChIP-exo

1. RIPA wash buffer: 50mM Hepes (pH 7.6), 1mM EDTA, 0.5M LiCl, 1% Igepal (NP-40), 0.7% Sodium Deoxycholate
2. 10mM Tris-HCl pH 8.0
3. NEB2 (NEB, supplied with RecJf exonuclease, see below)

4. ATP (NEB)
5. Deoxynucleotide (dNTP) Solution Mix (NEB)
6. NEBNext® End Repair Module (NEB)
7. Klenow fragment (NEB)
8. Thermomixer
9. Annealing buffer: 10mM Tris-HCl pH 8.0, 50mM NaCl, 1mM EDTA
10. Annealed P7 exo-adapter forward and reverse oligos (See **Note 2**)
11. T4 DNA ligase (NEB) 2000U/µl
12. Phi29 DNA polymerase (NEB)
13. In-house Phi29 DNA polymerase buffer: 50mM Tris-HCl pH 7.5, 10mM $MgCl_2$, 10mM $(NH_4)SO_4$, 1mM DTT. Filter, aliquot and store at -20°C.
14. Lambda exonuclease (NEB)
15. RecJf (NEB)

2.6 DNA elution and clean-up

1. ChIP elution buffer: 50mM Tris–HCl, pH 8, 10mM EDTA, 1% SDS
2. Proteinase K (20µg/µl)
3. TE buffer: 50mM Tris-HCl pH 8, 10mM EDTA, 1% SDS
4. Phenol – chloroform – isoamyl alcohol mixture (Sigma)
5. Phase Lock Gel Heavy tubes (5prime)
6. 5M NaCl
7. Glycogen (Life technologies) or suitable carrier for precipitation
8. Absolute ethanol
9. 75% ethanol
10. 10mM Tris-HCl pH 8.0

2.7 ChIP-exo amplification

1. P7 primer
2. AMPure XP beads (Beckman Coulter)
3. Annealed P5 exo-adapter forward and reverse oligos (See **Note 2**)
4. NEBNext High-Fidelity 2x PCR Master Mix (NEB)
5. PCR Primer mix 50μM each (1:1 mix of forward and reverse)
6. Certified Low Range Ultra Agarose (Biorad)
7. Dedicated electrophoresis equipment
8. TAE buffer (Sigma)
9. Ethidium bromide solution (Sigma)
10. NEB Low Molecular Weight DNA Ladder (NEB)
11. Transilluminator
12. Scalpels
13. MinElute Gel Extraction Kit (Qiagen)
14. Absolute isopropanol

2.8 Quality control

1. Oligonucleotide primers for genomic regions of interest
2. SYBR green master mix (e.g. Applied Biosystems)
3. PCR plates
4. Qubit system and dsDNA HS Assay (Life technologies)
5. Bioanalyzer system and DNA1000 or High Sensitivity DNA Kit (Agilent)

3. Methods

3.1 Cell culture

1. Maintain LNCaP cells in RPMI medium supplemented with 10% FBS in cell culture incubators (5% CO_2 at 37°C) and passage at a dilution of 1:3 when approaching confluence with trypsin/EDTA.

2. For ChIP-exo assays involving hormone starvation, split cells and spin down at 300 x g for 5min at RT. Subsequently, gently wash pellet with PBS and resuspend in phenol red-free RPMI supplemented with 10% CSS. Seed $6x10^6$ per 15cm dishes and use two 15cm dishes per ChIP-exo reaction/antibody, e.g. AR and IgG equals four plates in total.

3.2 ChIP-exo

3.2.1 Day 1 – Overnight preparation of bead-antibody complexes

1. 48h after seeding in CSS medium, wash 50-100µl Dynabeads (protein A/G depending on antibody species, see **Note 1**) per reaction in 2ml Eppendorf tubes three times with 1-2ml 0.5% BSA in PBS. Use a magnet to separate the magnetic beads in between washes. Incubate overnight with 5-10ug of specific antibody or IgG control in 300µl 0.5% BSA in PBS. We typically use 10µg antibody and 50µl Dynabeads per reaction but different antibodies might require different amounts.

3.2.2 Day 2 – Crosslinking, harvesting, sonication and immunoprecipitation

1. 72h after seeding (cells should be 70%+ confluent), replace cell culture media with CSS medium supplemented with androgens (e.g. 1nM DHT or R1881) or an equal volume of

ethanol (vehicle) and return cells to the incubator for 4h. Adjust the preparation of the antibody-bead complexes (Day 1) accordingly if longer treatment times are desired.

2. Aspirate medium and replace with 1% Formaldehyde in PBS (always prepare fresh). NB: Do not use serum in the crosslinking reaction as this influences the crosslinking efficacy of formaldehyde. Incubate swirling at RT for 10min. (See **Note 3**)

3. Quench the crosslinking reaction by adding glycine to a final concentration of 125mM (1/20 of 2.5M stock solution). Incubate swirling at RT for 5min.

4. On ice: Carefully wash plates twice with approximately 15ml ice-cold PBS. Scrape cells in PBS and transfer to 15ml Falcon. Spin at 2,000 x g and 4°C for 5min and aspirate supernatant.

5. Resuspend pellet in 15ml LB1 freshly supplemented with proteinase inhibitors and rotate at 4°C for 10min to lyse cells. Spin at 2,000 x g and 4°C for 5min and aspirate supernatant.

6. Resuspend pellet in 15ml LB2 freshly supplemented with proteinase inhibitors and rotate at 4°C for 5min to wash nuclei. Spin at 2,000 x g and 4°C for 5min and aspirate supernatant.

7. Resuspend pellet in 300µl LB3 freshly supplemented with proteinase inhibitors per 15cm plate used. Distribute 300µl each into Eppendorf tubes and rotate at 4°C for 10min.

8. Sonicate cells in a Diagenode Bioruptor for approximately 30 cycles of 30s ON, 30s OFF to shear chromatin to an average size of 200-300bp. (See **Note 4**)

9. Repool lysates and add 1/10 volume of 10% Triton-X in LB3 to quench SDS. Spin at 18,000 x g for 15min at 4°C and transfer supernatant to a new tube.

10. Optional step: Preclearing of chromatin (See **Note 5**)

11. If desired, take 25µl input control and store at -20°C until tomorrow.

12. Wash the Dynabead-antibody solution from day 1 three times with 0.5% BSA in PBS. After the third wash add identical amounts of sonicated chromatin to every tube with antibody-bead complexes and incubate on a 4°C rotator overnight.

3.2.3 Day 3 – Washing and on-bead enzymatic reactions

1. Using the magnet, wash the bead-antibody-protein-DNA complexes six times with 1-2 ml RIPA wash buffer. Properly resuspend beads between washes. For all subsequent steps, always prepare enzyme master mixes before washing beads with Tris-HCl pH 8.0. Do not let the beads dry out and do not keep them in Tris-HCl pH 8.0 for an extended period.

2. Set up the end repair master mix and wash the beads twice with 10mM Tris-HCl pH 8.0. Do not remove tubes from magnet or you will lose material. Make sure you have thoroughly removed all traces of RIPA or this will inhibit subsequent enzyme reactions. Remove all traces of Tris-HCl pH 8.0 prior to resuspending the beads in the enzyme mix.

3. Blunt DNA fragments using the following end-repair reaction. Add 100µl directly to the beads. Incubate at 30°C for 30min on a Thermomixer at 900rpm. NEB2 is used to maintain a 1mM DTT concentration (See **Note 6**)

10µl 10x NEB2 buffer

10µl ATP 10mM, final 1mM

1µl dNTPs 10mM, final 100µM

5µl End repair enzyme mix (NEB)

1µl Klenow fragment (NEB) 5U/µl

73µl H_2O

Total 100µl

4. An Illumina compatible P7 adapter is ligated to the blunted DNA ends.

Set up a master mix for the ligation of the P7 adapter and wash the beads twice with RIPA wash buffer and 10mM Tris-HCl pH 8.0 as explained above. Resuspend beads in 100µl reaction mix and incubate at 25°C for 60min on a Thermomixer at 900rpm.

10µl 10x NEB2 buffer

10µl ATP 10mM, final 1mM

15µl P7 adapter mix (See **Note 6**)

1µl T4 DNA ligase (NEB) 2000 U/µl

64µl H_2O

Total 100µl

5. Ligation of the P7 adapter leaves behind a nick, which needs to be closed.

Set up a master mix for the nick repair and wash the beads twice with RIPA and 10mM Tris-HCl pH 8.0 as explained above. Resuspend beads in 100µl reaction mix and incubate at 30°C for 20min on a Thermomixer at 900rpm.

1.5µl Phi29 DNA polymerase (NEB) 10 U/µl, 15 U final

1.5µl dNTPs 10mM, final 150uM

10µl 10x home-made Phi29 DNA polymerase buffer

87µl H_2O

Total 100µl

The in-house buffer is used to maintain a 1mM DTT concentration (See **Note 6**).

6. Lambda exonuclease is used to digest dsDNA in the 5'-3' direction until it reaches protected DNA (**Figure 2**).

Set up a master mix for the Lambda exonuclease digestion and wash the beads twice with RIPA and 10mM Tris-HCl pH 8.0 as explained above. Resuspend beads in 100µl reaction mix and incubate at 37°C for 30min on a Thermomixer at 900rpm.

2µl Lambda exonuclease (NEB) 5 U/µl, 10 U final

10µl 10x Lambda exonuclease buffer

88µl H_2O

Total 100µl

7. RecJf exonuclease digests ssDNA in the 5’-3’ direction. This reaction is used to reduce noise.

Set up a master mix for the RecJf exonuclease digestion and wash the beads twice with RIPA and 10mM Tris-HCl pH 8.0 as explained above. Resuspend beads in 100µl reaction mix and incubate at 37°C for 30min on a Thermomixer at 900rpm.

1µl RecJf (NEB) 30 U/µl

10µl 10x NEB2 buffer

89µl H_2O

Total 100µl

8. Add 200µl ChIP elution buffer and 5µl Proteinase K (20µg/µl). Also include input you froze at -20°C. Add 175µl ChIP elution buffer and 5µl Proteinase K to 25µl input. Incubate overnight at 65°C on a Thermomixer at 900rpm.

3.2.4 Day 4/5 – DNA extraction, final enzymatic reactions and size selection

1. Phenol – chloroform – isoamyl alcohol mixture (PCI) and Phase Lock Gel Heavy tubes are used to clean up DNA to minimize losses.

Spin Phase Lock tubes at 14,000 x g for 1min to pellet gel. Add 200µl TE buffer to every sample and mix. Add an equal volume of PCI solution (400µl) and vortex thoroughly. Transfer to 2ml Phase Lock tube and spin at 14,000 x g for 5min at RT.

2. Transfer upper phase (approximately 400µl) to a new Eppendorf tube and add 16µl 5M NaCl (200mM final) and 1µl glycogen (20µg/µl), vortex briefly. Add 1ml EtOH abs. (ice-cold) and store at -80°C for at least 30min to facilitate precipitation.

3. Spin at 14,000 x g for 15 min at 4°C and discard supernatant.

4. Wash pellet with ice-cold 75% EtOH and spin again at 14,000 x g at 4°C for 15min.
Remove supernatant and let pellet air-dry for 10 min.

5. Elute pellet in 20µl 10mM Tris-HCl pH 8.0, incubate at 50°C for 10min and then transfer to clean PCR tubes.

6. Denature DNA at 95°C for 5min and put immediately on ice to avoid renaturing of DNA.
During the 5min, set up a master mix with the following components. Since DTT concentration does not matter anymore, commercial buffer can be used.

5µl P7 primer, 1µM stock (= 1:100 of 100µM)

5µl commercial Phi29 DNA polymerase buffer

20µl H_2O

Total 30µl

Add to the denatured DNA on ice and incubate at 65°C for 5min to anneal, and then cool down to 30°C for 2min.

7. Add to every well:

1µl Phi29 DNA polymerase (NEB) 10 U/µl

1µl dNTPs, 10mM

Incubate at 30°C for 20min followed by 65°C for 10min.

8. Briefly spin reaction and add 80µl of room-temperature AMPure XP beads (1.6 x volumes), incubate at RT for 15min. Place tubes on magnet and allow clearing for 15min. Remove and discard supernatant and wash beads twice with 80% EtOH. Remove all traces of EtOH and let beads air-dry for 5-10min.

Always prepare fresh EtOH every day, you do not want to lose any material due to lower concentrated EtOH and accidentally eluted DNA. Do not remove beads from magnet during washes or you will lose too much material.

9. Resuspend beads in 20µl RSB and wait for 2min. Place Eppendorf tubes in magnet, wait 5min and transfer 20µl supernatant to new PCR tubes.

10. An Illumina compatible P5 adapter is ligated to the DNA ends.

Set up the following master mix for the ligation of the P5 adapter and add 30ul to each sample

5µl T4 DNA ligase buffer 10x

1.5µl P5 adapter mix

1µl T4 DNA ligase (NEB) 2000 U/µl, final 2000 U

22.5µl H_2O

Total 30µl

Incubate at 25 °C for 60 min followed by 65 °C for 10 min.

10. PCR amplify DNA fragments using specific primers containing Illumina compatible index sequences ('barcodes') that allow multiplexing, i.e. simultaneous sequencing of several samples in one lane (See **Note 7**).

Set up a master mix with the following components:

25µl NEBNext High-Fidelity 2x PCR Master Mix (NEB)

0.5-1µl primer mix 50µM each (1:1 mix of index specific forward and universal reverse primer, **Table 2**)

4-4.5µl H_2O

Total 50µl

Run the following PCR program**.**

98°C for 30 s

10-18 cycles of

98°C 10 s

65°C 30 s

72°C 30 s

72°C 5 min

4°C forever

11. Briefly spin PCR reaction and add 50µl of room-temperature AMPure XP beads (1 volume), incubate at RT for 15min. Then place tubes on magnet and allow clearing for 15min. Remove and discard supernatant and wash beads twice with 80% EtOH. Remove all traces of EtOH and let beads air-dry for 5-10min.

12. Resuspend beads in 25µl RSB and wait for 2 min. Place Eppendorf tubes in magnet, wait 5 min and transfer 25µl supernatant to new PCR tubes.

13. Gel electrophoresis is used to isolate fragments of appropriate size.

Prepare 2% Agarose gel (2g Agarose per 100ml 1xTAE and add appropriate amounts of Ethidium bromide (EtBr) or another DNA dye). (See **Note 8**)

To prepare ladder: 8µl NEB Low Molecular Weight DNA Ladder + 3µl 50% glycerol in 1xTAE, load 11µl per lane

For samples: Add 10µl 50% glycerol in 1xTAE to 25µl ChIP-exo sample and load everything into one well.

Run gel at 120V for ~40 min.

14. In a dark room: Using a clean scalpel for every sample, excise bands between 200 and 300bp and transfer to a 2ml Eppendorf tube. Stick to a 400mg maximum and avoid excess transfer of gel that does not contain any DNA. The DNA should be visible and thus easy to

cut. Take a picture of the gel before and after excision to document excised range. (See **Note 9**)

15. DNA is extracted from the gel using the Qiagen MinElute gel extraction kit

a. Weigh gel slice and add 3 volumes of buffer QG (e.g. 300µl to 100mg of gel). Incubate at RT on a rotator/shaker for 10min to dissolve gel.
b. Add 1 gel volume of isopropanol, mix and transfer to 2ml column (max. loading capacity = 750µl, spin and reload if necessary).
c. Spin for 1min at 14,000 x g, discard flow through.
d. Add 500µl QG.
e. Spin for 1min at 14,000 x g, discard flow through.
f. Add 750µl PE and allow to stand for 3min at RT.
g. Spin for 1min at 14,000 x g, discard flow through.
h. Spin for 1min at 14,000 x g to dry column.
i. Transfer column to a new Eppendorf tube, add 20µl pre-warmed (50°C) EB and incubate at RT for 1min.
j. Spin for 1min at 14,000 x g.

This is now a ready-to-be-sequenced Illumina library and can be stored at -20°C for at least 6 months. The library is sequenced with single-end reads from the ligated P5 adapter and aligned to a reference genome prior to peak calling using a typical algorithm, such as Model-based analysis of ChIP-seq (MACS) [17].

16. If sequencing of input samples is desired, prepare input libraries using a conventional protocol, such as the Illumina TruSeq ChIP Sample Preparation Kit (Illumina, IP-202-1012).

17. Prior to sequencing, check DNA concentration and fragment distribution using the Qubit system and Agilent's Bioanalyzer and pool samples if desired. (See **Note 10**)

You can use a diluted library (1:10-1:100, depending on concentration) for analysis of enrichment using quantitative real-time PCR with oligonucleotides to your genomic region of interest. Calculate the fold enrichment over IgG using the 2ΔΔCt method.

4. Notes

1. Dynabeads Protein A and G beads exhibit species-dependent differences in affinities towards antibodies. Consult life technologies' homepage when planning your experiment.

2. To prepare annealed dsDNA P7 adapter mix, mix equal volumes of 100uM primer stocks P7 FWD and P7 REV (e.g. 100µl of both) (**Table 2**) with 4 volumes (e.g. 800µl) of annealing buffer (10mM Tris-HCl pH 8.0, 50mM NaCl, 1mM EDTA). Heat mixture to 95°C for 5min and then move it to room temperature for 2h. The mixture will gradually cool down and allow the oligos to anneal. This will yield dsDNA adapters used in this reaction. Aliquot and store at -20°C until ready to use. Annealed P5 exo-adapters are prepared in the same manner using the forward and reverse primers listed in **Table 2**.

3. Use this as a guideline only. Formaldehyde concentration and crosslinking duration might require optimization if ChIPped for other transcription factors. Using other crosslinking agents, such as imidoesters or NHD-esters, might be possible/necessary/applicable for your specific experiment. In addition, performing native ChIP, i.e. using no crosslinking agent can be possible but is generally only suitable for proteins that are very tightly attached to DNA.

4. The optimal sonication time and intensity depends on various factors, such as your sonication device, your cell line and your transcription factor of interest. Thus, this step requires optimization and optimal sonication time should be determined prior to starting the experiment to ensure optimal resolution. To assess shearing efficacy, take an aliquot (e.g. 20µl), reverse crosslink and clean-up DNA according to the steps mentioned under Day 3 –

Step 8. Subsequently, load varying amounts onto a 2% agarose gel and visualize under UV light.

5. If you experience high background in your reactions, it might be helpful to preclear the chromatin. To perform this, incubate it with 20µl Dynabeads for 1h at 4°C rotating and transfer the supernatant to a clean tube using the magnet prior to proceeding with the next step. This will clear your chromatin of fragments that bind unspecifically to the Dynabeads.

6. Do not use the NEB end repair buffer that comes with the mix. It contains 10mM DTT and will elute material. NEB2 contains only 1mM DTT and is also suitable for this reaction. The same applies to the Phi29 reactions where the commercial buffer contains 10mM DTT and a custom-made buffer is used instead.

7. When multiplexing (simultaneous sequencing of multiple samples in a single lane), follow Illumina's Adapter Tube Pooling Guidelines (Refer to **Table 3** for pooling strategies).

In general, ChIP-exo allows higher plexity than ChIP-seq and Serandour *et al.* successfully sequenced and demultiplexed up to twelve samples, each with at least 15 million reads on an Illumina HiSeq machine.

Both the optimal primer concentration and the amount of PCR cycles depend on the amount of ChIPped material. Thus, this step might require optimization in order to avoid too little material or over amplification, which might lead to large amounts of duplicate reads and underrepresented libraries during sequencing.

8. When loading the gel, always leave an empty well between every sample/marker to avoid cross-contamination. When using other DNA dyes than Ethidium bromide, such as SYBR gold, make sure to do a test run since many dyes are known to influence DNA migration.

9. The excision range determines the libraries' resolutions. If lower resolutions are required, cut at higher range and vice versa. Do not cut below 200bp to avoid contamination with unconjugated primers.

10. When multiplexing (simultaneous sequencing of multiple samples in a single lane), it is essential that sample concentrations are roughly equal to ensure a similar sequencing depth for all samples. Thus, calculate mean fragment sizes using Agilent's Bioanalyzer and concentrations with the Qubit system (Life technologies). Subsequently, calculate the molarity of every sample and adjust them according to your sequencing facility's instructions.

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Figure Legends

Figure 1. Schematic workflow comparison of ChIP-seq and ChIP-exo from immunoprecipitation to DNA extraction.

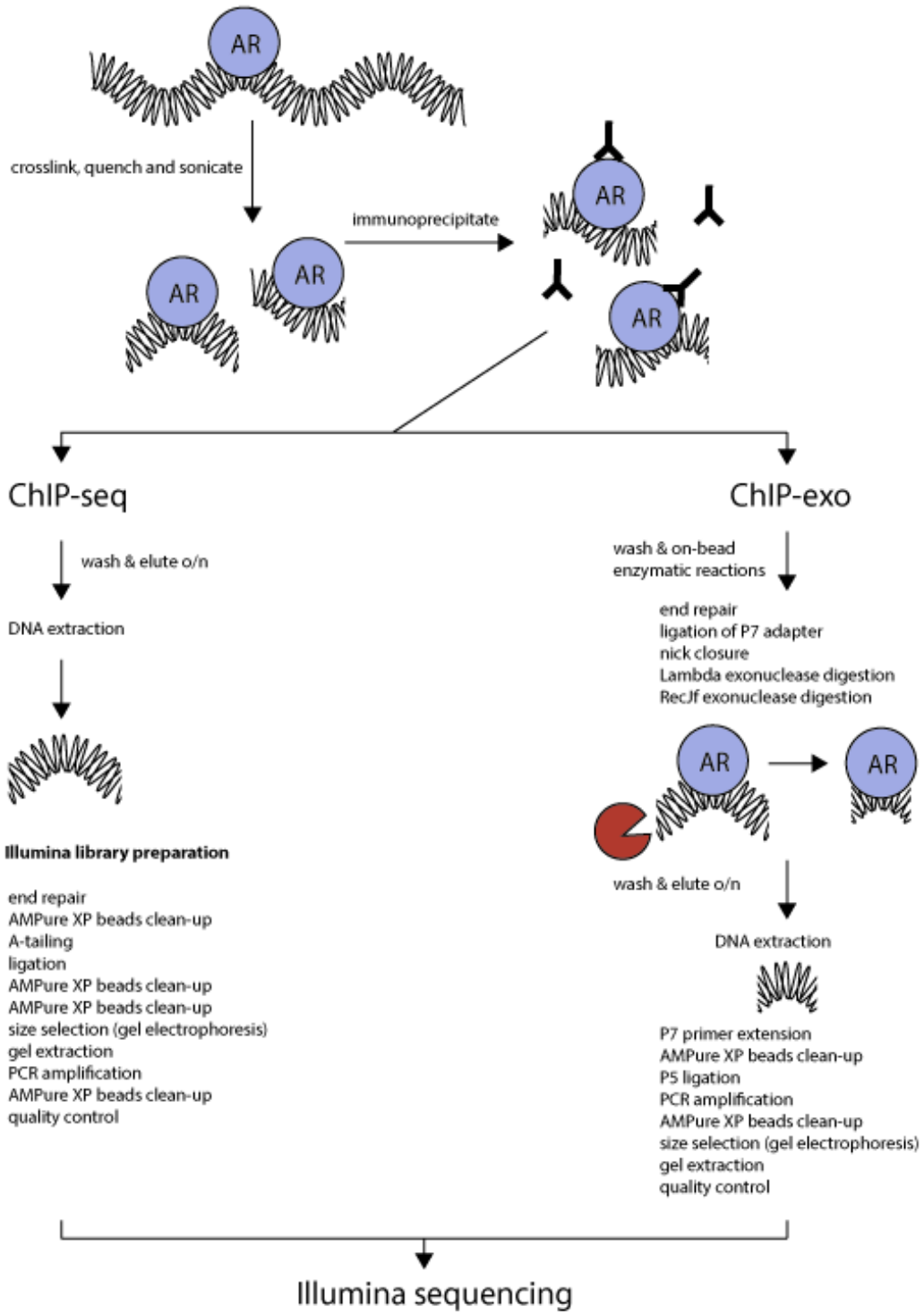
ChIP-exo and ChIP-seq share the initial steps including crosslinking, quenching, sonication and immunoprecipitation. Subsequently, in the conventional ChIP-seq protocol the precipitated DNA is eluted and extracted prior to Illumina TruSeq library preparation.
In the ChIP-exo protocol, however, the precipitated DNA remains linked to the magnetic beads. This allows rapid washing and on-bead enzymatic reactions, including two novel exonuclease digestions that reduce fragment length and background, which eliminates many of the time consuming AMPure XP bead cleanups.

Figure 2. Preparation of ChIPped DNA for sequencing using a conventional protocol or ChIP-exo.

In the traditional Illumina-based library preparation, partially double-stranded adapters are ligated to the extracted DNA. PCR amplification and subsequent single-ended high-throughput sequencing result in two shifted populations of reads, one derived from the original top strand, the other one from the original bottom strand. In the ChIP-exo protocol, however, the novel exonuclease reaction digests DNA until it reaches protected DNA (i.e. shielded transcription factor binding sites). This results in two overlapping populations of sequencing reads as the sequencing adapters are ligated directly up- and downstream of the protected areas. Thus, ChIP-exo allows increased multiplexing through better coverage of

the actual transcription factor binding sites and generates narrower peak widths, which improves motif enrichment analysis.

Figure 1


AR
crosslink, quench and sonicate
immunoprecipitate
AR
AR
AR
AR
ChIP-seq
ChIP-exo
wash & elute o/n
DNA extraction
Illumina library preparation
end repair
AMPure XP beads clean-up
A-tailing
ligation
AMPure XP beads clean-up
AMPure XP beads clean-up
size selection (gel electrophoresis)
gel extraction
PCR amplification
AMPure XP beads clean-up
quality control
wash & on-bead
enzymatic reactions
end repair
ligation of P7 adapter
nick closure
Lambda exonuclease digestion
RecJf exonuclease digestion
AR
AR
wash & elute o/n
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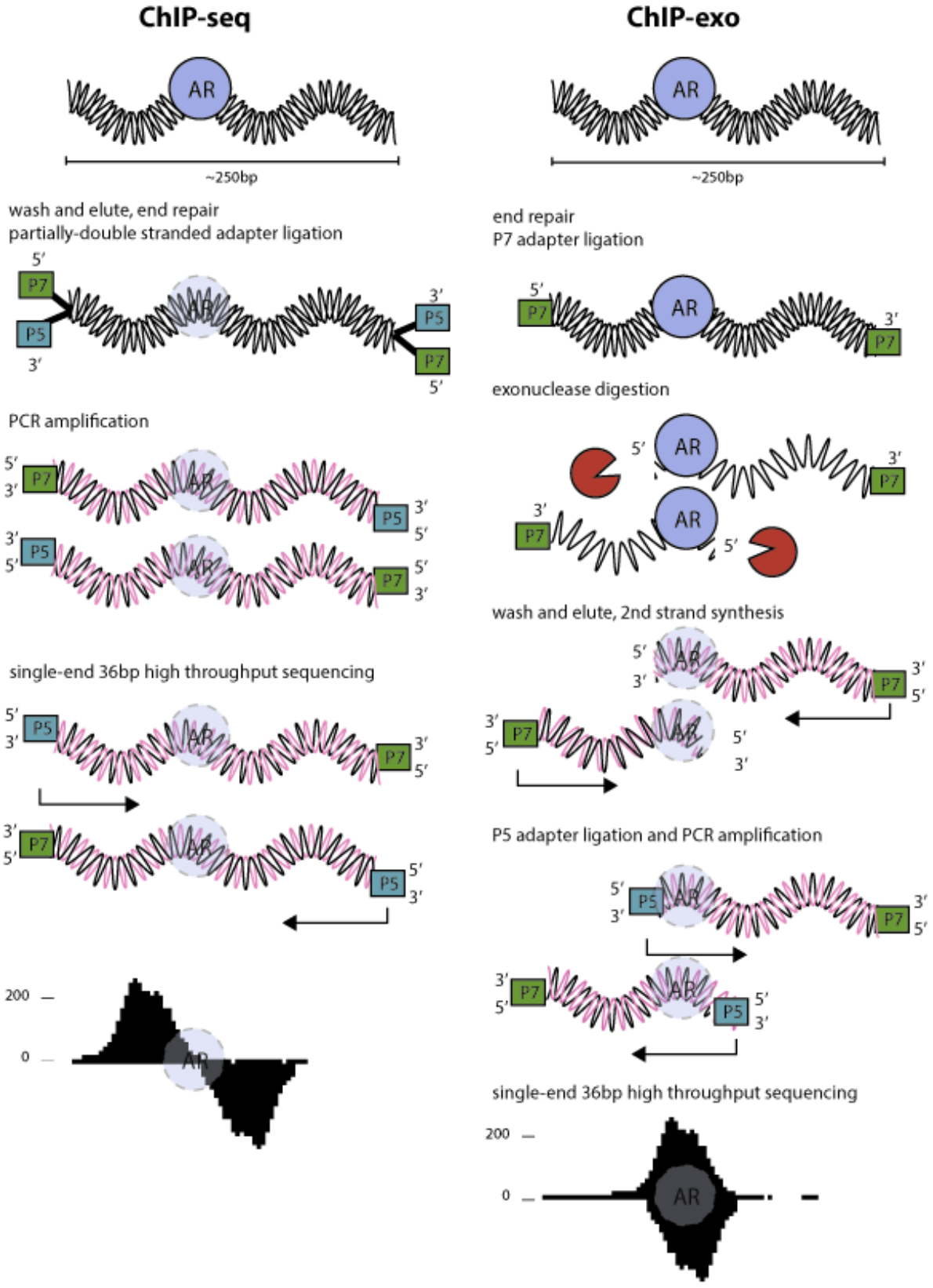
P5 ligation
PCR amplification
AMPure XP beads clean-up
size selection (gel electrophoresis)
gel extraction
quality control
Illumina sequencing

Figure 2


ChIP-seq
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~250bp
wash and elute, end repair
partially-double stranded adapter ligation
5′
P7
P5
3′
3′
P5
P7
5′
PCR amplification
single-end 36bp high throughput sequencing
200
0
ChIP-exo
AR
~250bp
end repair
P7 adapter ligation
exonuclease digestion
wash and elute, 2nd strand synthesis
P5 adapter ligation and PCR amplification
single-end 36bp high throughput sequencing
200
0

Tables

Table 1: Recent modifications to ChIP-seq methodology

Method	Adaptation	Sensitivity	Advantages/disadvantages	Target proteins	References
Linear Amplification of DNA (LinDA)	in vitro transcription instead of PCR, single tube reactions	5,000-10,000 cells	reduced PCR induced bias, higher sensitivity	TF (ERα) and histones (H3K4me3)	[6]
micro/nano ChIP-seq	various, e.g. single tube reactions, restriction enzymes	~10,000 cells	higher sensitivity	TF (CTCF) and histones (various)	[7,8]
PAT-ChIP (pathology tissue)	ChIP-seq of formalin-fixed, paraffin-embedded (FFPE) samples, includes deparaffinization and MNase digestion	10µM FFPE sections	higher sensitivity, allows ChIP-seq from FFPE samples	TF (CTCF), Pol2 and histones (various)	[9]
carrier-assisted ChIP	histones or mRNA as carrier to facilitate immunoprecipitation and degraded prior to library preparation	10,000	used on core needle biopsy samples, high sensitivity	TF (ERα)	[10]
ChIP-exo	additional exonuclease digestion of unprotected DNA straightens peaks and reduces noise	not tested, several millions used	higher plexity, narrower peaks, reduced noise	TF (ERα), not applicable for histones	[12,11]

Table 2. Oligonucleotide sequences

Primer	Sequence
P7 exo-adapter reverse (5' Phos = phosphorylated 5' end)	5' Phos-GTGACTGGAGTTCAGACGTGTGCTCTTCCGATC-OH 3'
P7 exo-adapter forward	5' OH-GATCGGAAGAGCACACGTCT-OH 3'
P5 exo-adapter reverse	5' OH-AGATCGGAAGAGCG-OH 3'
P5 exo-adapter forward	5' OH-TACACTCTTTCCCTACACGACGCTCTTCCGATCT-OH 3'
P7 primer	5' OH-GACTGGAGTTCAGACGTGTGCT-OH 3'
PCR Primer universal reverse (* = Phosphorothioates S-linkage)	5' OH-AATGATACGGCGACCACCGAGATCTACACTCTTTCCCTACACG*A-OH 3'
PCR Primer index 2 forward (* = Phosphorothioates S-linkage)	5' OH-CAAGCAGAAGACGGCATACGAGATACATCGGTGACTGGAGTTCAGACGTGTGC*T-OH 3'
PCR Primer index 4 forward (* = Phosphorothioates S-linkage)	5' OH-CAAGCAGAAGACGGCATACGAGATTGGTCAGTGACTGGAGTTCAGACGTGTGC*T-OH 3'
PCR Primer index 5 forward (* = Phosphorothioates S-linkage)	5' OH-CAAGCAGAAGACGGCATACGAGATCACTGTGTGACTGGAGTTCAGACGTGTGC*T-OH 3'
PCR Primer index 6 forward (* = Phosphorothioates S-linkage)	5' OH-CAAGCAGAAGACGGCATACGAGATATTGGCGTGACTGGAGTTCAGACGTGTGC*T-OH 3'
PCR Primer index 7 forward (* = Phosphorothioates S-linkage)	5' OH-CAAGCAGAAGACGGCATACGAGATGATCTGGTGACTGGAGTTCAGACGTGTGC*T-OH 3'
PCR Primer index 12 forward (* = Phosphorothioates S-linkage)	5' OH-CAAGCAGAAGACGGCATACGAGATTACAAGGTGACTGGAGTTCAGACGTGTGC*T-OH 3'
PCR Primer index 13 forward (* = Phosphorothioates S-linkage)	5' OH-CAAGCAGAAGACGGCATACGAGATTTGACTGTGACTGGAGTTCAGACGTGTGC*T-OH 3'
PCR Primer index 14 forward (* = Phosphorothioates S-linkage)	5' OH-CAAGCAGAAGACGGCATACGAGATGGAACTGTGACTGGAGTTCAGACGTGTGC*T-OH 3'
PCR Primer index 15 forward (* = Phosphorothioates S-linkage)	5' OH-CAAGCAGAAGACGGCATACGAGATTGACATGTGACTGGAGTTCAGACGTGTGC*T-OH 3'
PCR Primer index 16 forward (* = Phosphorothioates S-linkage)	5' OH-CAAGCAGAAGACGGCATACGAGATGGACGGGTGACTGGAGTTCAGACGTGTGC*T-OH 3'
PCR Primer index 18 forward (* = Phosphorothioates S-linkage)	5' OH-CAAGCAGAAGACGGCATACGAGATGCGGACGTGACTGGAGTTCAGACGTGTGC*T-OH 3'
PCR Primer index 19 forward (* = Phosphorothioates S-linkage)	5' OH-CAAGCAGAAGACGGCATACGAGATTTTCACGTGACTGGAGTTCAGACGTGTGC*T-OH 3'

Table 3: Illumina's Adapter Tube Pooling Guidelines

Plexity	Option	Adapters
2	1	index 6 and index 12
	2	index 5 and index 19
3	1	index 2, index 7 and index 19
	2	index 5, index 6 and index 15
	3	2-plex option with any other adapter
4	1	index 5, index 6, index 12 and index 19
	2	index 2, index 4, index 7 and index 16
	3	3-plex option with any other adapter
5 or higher	1	4-plex option with any other adapter