

Chromatin preparation for ChIP-seq in *Oikopleura dioica*

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Abstract

Chromatin immunoprecipitation (ChIP) is a widely used molecular technique for binding site identification of DNA-associated proteins in many organisms. Although well established, its use in non-model organisms usually requires extensive protocol optimisation.

Here we describe an optimized protocol for collection, fixation, lysis and chromatin sonication of three developmental stages of the chordate model *Oikopleura dioica*, an emergent model organism for chordate developmental and evolutionary biology studies.

1 Introduction

To fully understand many biological processes and disease states it is essential to determine how proteins that interact with DNA regulate gene expression. This epigenetic information is complementary to genotype and expression data. This is possible through chromatin immunoprecipitation followed by high-throughput sequencing (ChIP-seq), the currently most widely used technology in a vast array of organisms [1].

The appendicularian *Oikopleura dioica* is an emergent model organism for research in chordate developmental biology due to its remarkably simple adult body with a small number of cells and small genome. It's peculiar use of different cell cycle modes throughout development is also a appealing feature to study in cell biology. In general, it also possesses features that make it a good experimental model: transparency, defined cell lineages and a short life cycle which can be fully completed in captivity [2].

An increasing number of molecular techniques has been developed for *Oikopleura* such as RNA interference and RNA *in situ* hybridization, but an optimized procedure for consistent chromatin extraction and preparation for ChIP-seq in an array of developmental stages were still lacking. Here we present an optimized protocol for collection, fixation, lysis and chromatin sonication of three developmental stages of *Oikopleura*.

2 Protocol

The following protocol has been developed using a Covaris S200 sonicator and chromatin fragment length optimized for Illumina high-throughput sequencing. Lists of the required and most relevant consumables and equipment can be seen in the appendices.

2.1 Collection of *O. dioica*

2.1.1 Tailbud stage

To collect *Oikopleura* in the tailbud developmental stage, a controlled *in vitro* fertilization was performed. Day six mature male animals were collected to a Petri dish with seawater and allowed to spawn. As all male animals spawned, sperm quality was visually inspected on a light microscope for motility. Day six mature females were individually collected to glass salliers with seawater and allowed to spawn

at 18°C. When spawned, 60 μ L of sperm solution was added to the salier which was washed five times with sea water after five minutes post fertilization. After 3-4 hours, all tailbud animals exhibiting normal development were collected to a 1.5 mL microtube.

2.1.2 Day two stage

Late day two *Oikopleura* were individually collected with the aid of a 2 mL plastic pipette to a 1 L beaker with clean seawater (no algae) to be allowed to empty the gut of any remaining algae as well as build a new clean house for 3 to 4 hours.

To achieve release of the houses, animals were individually collected again, this time using a mouth-pipette built from a 1 mL plastic pipette connected to a 50 mL falcon tube and poured into a glass recipient on ice with 0.125 mg/mL MS222 and allowed to sink to the bottom, where a third collection using a micropipette with 200 μ L tip transferred them to a 1.5 mL microtube.

2.1.3 Day six, immature stage

Immature day six animals with a visible gonad but naked-eye indistinguishable sexual features were collected from culture into a 500 mL plastic beaker with clean seawater with the aid of a 25 mL plastic pipette and from there to a 1.5 mL microtube with a 2 mL plastic pipette.

2.2 Chromatin extraction

2.2.1 Animal fixation

Careful consideration of the amount of animals to use for fixation is required. See section 3 for minimum recommended numbers.

Tailbud and day two animals were briefly spin at 5000 g for 10 seconds to be collected at the bottom of the microtube, while day six immature sank in seconds time. Seawater was exchanged for 0,5 mL PBS and 0,5 ml of either 1 or 2% freshly opened methanol-free formaldehyde in PBS to have a final fixative concentration of 0.5 or 1% respectively, and let rotating at 18°C for a time depending of the developmental stage, as shown in Table 1.

Formaldehyde was quenched with 250 mM glycine and tubes were again let rotating for 5 min at 18°C. Again by either spinning at 5000 g for 30 seconds or allowing animals to freely sink, animals were washed three times with cold PBS with protease inhibitors on ice. All solution was removed and animals were either used for dissection at this point (see section 2.2.2) or snap frozen in liquid nitrogen and stored at -80°C for future use.

Table 1: **Fixation conditions for each tested developmental stage of *Oikopleura dioica***

Stage	Fixative	Time (min)	Temperature (°C)
Tailbud	1%	5	18
Day two	0.5%	5	18
Day six immature	1%	10	18

2.2.2 Gonad dissection

Gonads of D6 immature animals, were extracted by dissection on a pre-cooled Petri dish using two fine needles in a binocular, simply by severing the gonad from the remaining body. This allows identification of sex, and therefore use of sex-specific gonads downstream. Gonads were separated according to sex in microtubes, snap frozen with liquid nitrogen and stored at -80°C for future use.

2.2.3 Cell lysis and chromatin sonication

Frozen animals pellets were thawed on ice and pooled with the aid of cold Lysis buffer to a total volume multiple of 130 μ L but never less than 390 μ L depending on the total amount of animal material.

Mechanical lysis was conducted with a fine needle passing the whole volume no less than three times through, and incubation for 15 minutes on ice.

Samples were sonicated using the instrument settings seen on Table 2, which sonicate the chromatin to a approximate gaussian distribution within 100-800 bp.

Whole sonicated lysates were centrifuged for 10 min, at 21000 g at 4°C and the chromatin-enriched supernatant was collect to a siliconized microtube.

Table 2: Settings used on the S200 Covaris sonicator for *Oikopleura dioica* chromatin

Setting	Value
Duty cycle	5%
Intensity	7.5
Processing time	5 minutes
Bath temperature	5-6 °C
Power mode	Frequency sweeping
Degassing mode	Continuous
Volume	130 μ L
AFA intensifer	Integrated
Water level	8

2.2.4 Chromatin quality assessment

To check the distribution of the sonicated chromatin fragments, 1% SDS, 100mM NaCl and 50 μ g/mL RNase A were added to a fraction of the chromatin and incubated for 30 min at 55°C. Proteinase K was added to 200 μ g/mL and samples incubated 90 min to O/N at 65°C to reverse crosslinks. DNA was purified with the phenol-chloroform method and precipitation with ethanol. Briefly, an equal volume of phenol:chloroform:isoamyl alcohol (25:24:1) was mixed to the samples, and after centrifugation 30 μ g glycogen, 300 mM sodium acetate (final concentrations) and 2.5 times the volume of cold absolute ethanol were added to the aqueous phase in a new microtube and incubated at -80°C for 1 hour. After centrifugation at 4°C, DNA pellets were washed with 70°C ethanol and dissolved in 3 to 5 μ L TE buffer.

DNA quality and amount were quantified using a Nanodrop instrument and loaded on an digital electrophoresis chip, to assess the distribution of DNA fragments with a Bioanalyzer instrument.

3 Results and Final Remarks

Chromatin prepared with the presented protocol can be used for ChIP-seq but possibly other downstream applications such as ChIP-chip. It is within the required range for Illumina high-throughput sequencing (100-800 bp), with the peak of distribution in the range around 100-400bp (see figure 1).

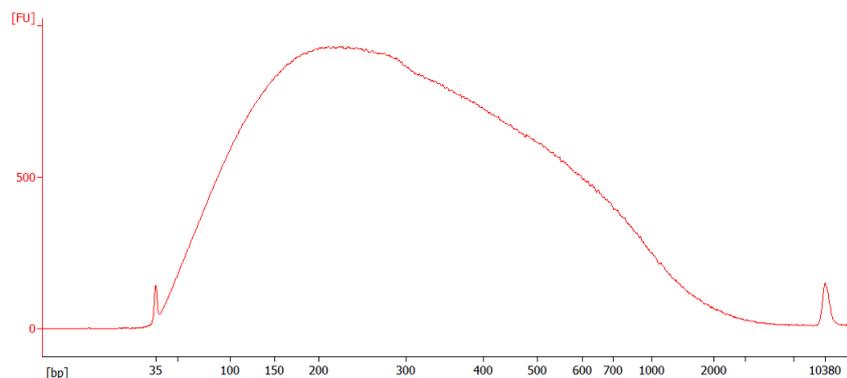


Figure 1: Chromatin prepared according to this protocol is appropriate for ChIP-seq. Electropherogram of DNA extracted from sonicated chromatin of D6 male gonads prepared as indicated in this protocol. Chromatin prepared from animals of other stages look similiar.

A fine equilibrium between sonication time and intensity must be achieved for efficient chromatin shearing. Reduced shearing will create DNA fragments of too large a length, while over-sonication causes the opposite but also reduced chromatin yield due to reduced fragment length (see figure 2).

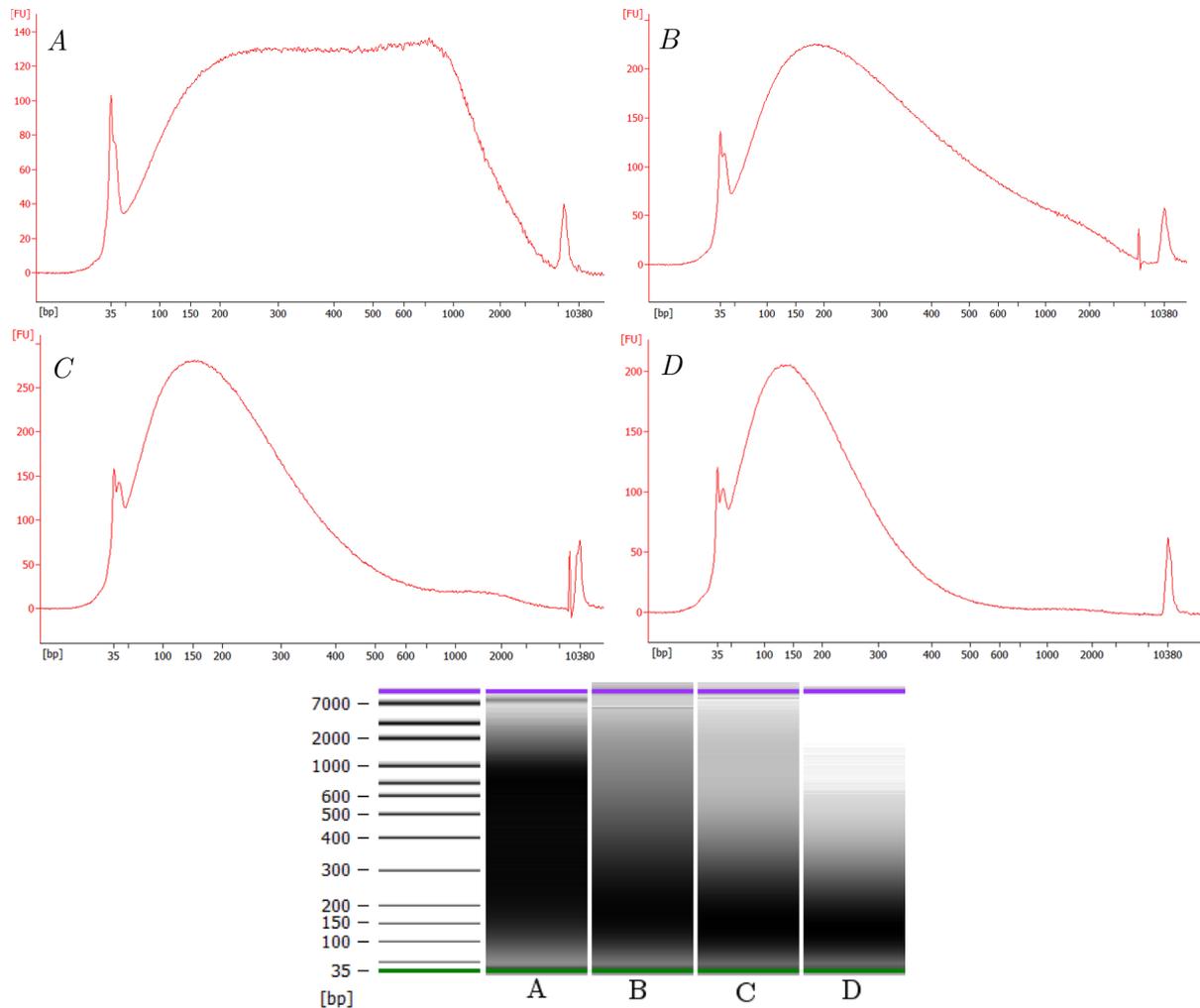


Figure 2: **Effect of duration of sonication on chromatin.** Male D6 male gonads chromatin sonicated for: A - 2; B - 5; C - 7.5; D - 10 minutes.

Fine tuning fixation conditions is imperative, for over-fixed chromatin is highly resistant to sonication (see figure 3) and is inappropriate for a ChIP assay. Evidence of over-fixation can be seen in a unusual peak in the distribution of DNA fragment length around 2-3 kb. Samples with residual amounts of over-fixed chromatin can still be used as long as the distribution of fragments in the correct range is several times superior to the over-fixed one.

One factor that can contribute to variable fixation results is the ratio between fixative molecules and material (animals) being fixed. Since fixative concentration is constant in the protocol, the number of animals used per fixation should as well remain constant. Nevertheless, this is a condition that is generally more critical when using a low number of animals (due to the increased probability of over-fixation of chromatin) than the opposite. A recommended minimal number of animals used for fixation is as follows: *a*) 10 batches of tailbud; *b*) 200 day 2; *c*) 10 day 6 immature animals.

It should be noted that there are sex-specific differences in the gonad tissues regarding fixation. A small amount of chromatin in the female sample is over-fixed when preparing chromatin with the described protocol (figure 4). Since fixation is performed on immature animals and sex determination is only done after fixation through dissection, fixation is optimal for male samples, but less for female.

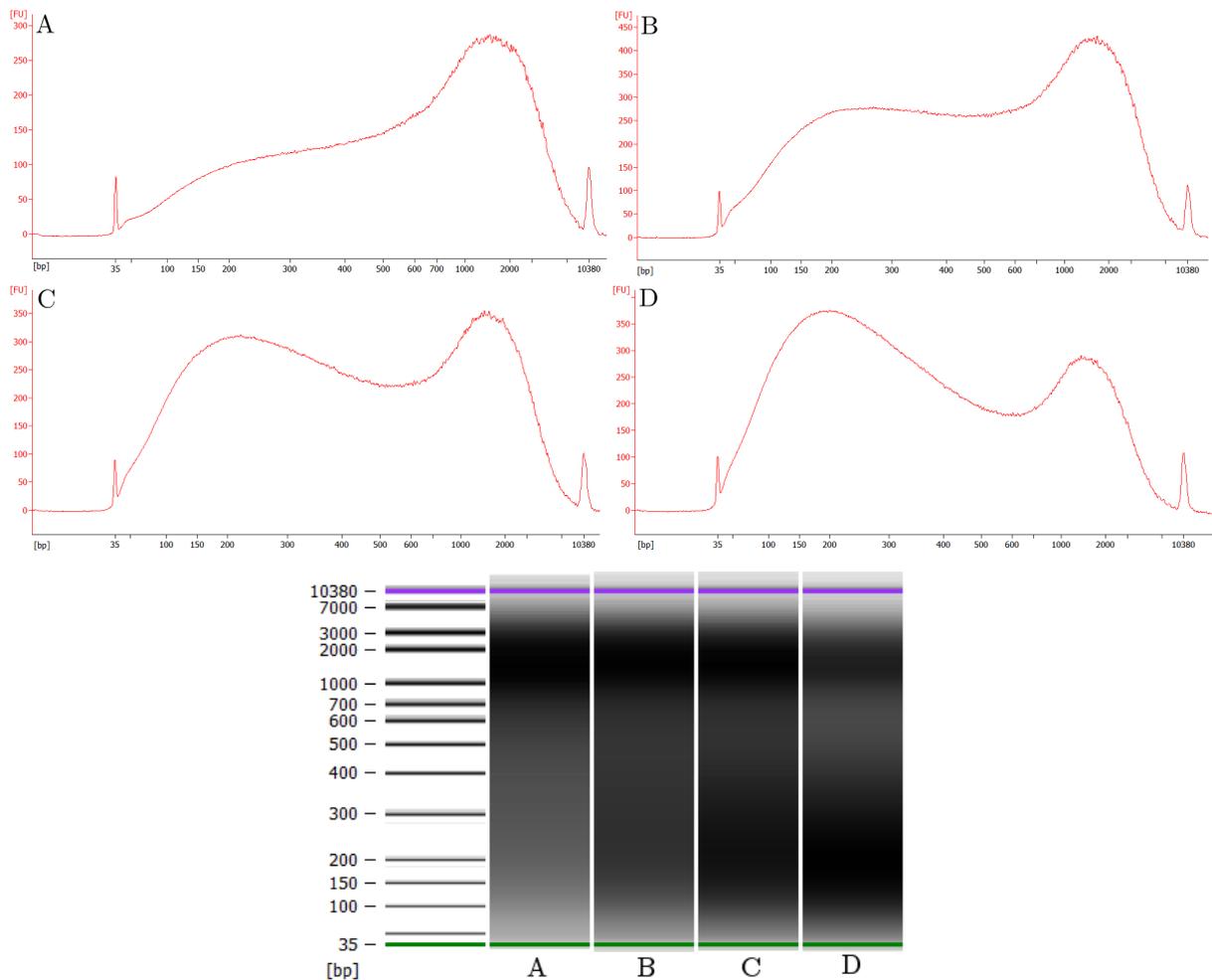


Figure 3: **Over-fixation impairs proper sonication of chromatin.** Chromatin from tailbud animals was fixed for 10 minutes with 1% formaldehyde and sonicated for: A - 2; B - 3; C - 4; D - 5 minutes.

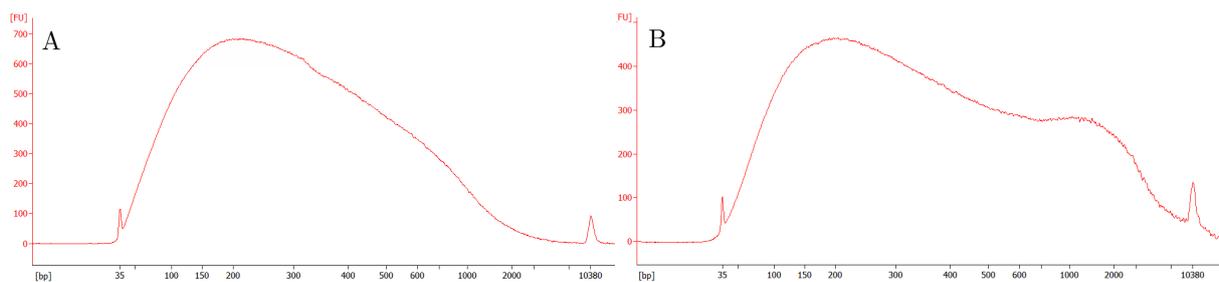


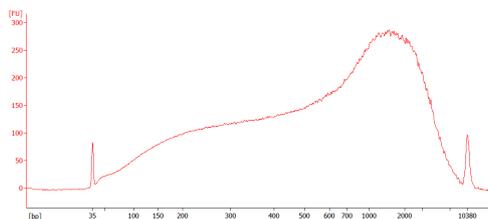
Figure 4: **Sex-specific differences in fixative permeability of D6 gonads.** Sonicated chromatin of D6 A) male or B) female gonads prepared as indicated in this protocol.

The use of a Bioanalyzer instrument is highly recommended due to its high sensitivity. Another great advantage is the possibility of using much lower amount of material during optimization steps.

This protocol may be adapted to other organisms, but considerable optimization of fixation and sonication conditions will likely need to be performed.

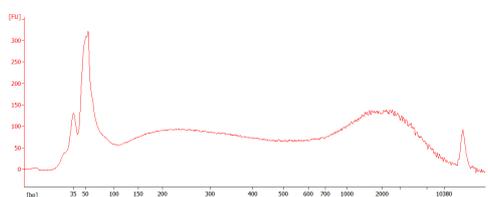
4 Troubleshooting

1. Fragment distribution has peak at high range (2-3 Kb)



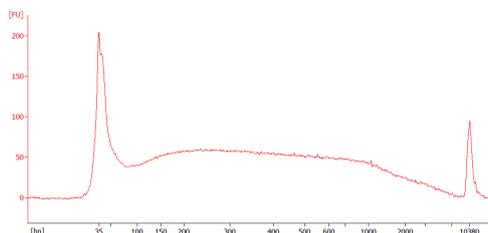
Chromatin is over-fixed - make sure formaldehyde is of good quality (methanol-free), opened freshly before use, concentration is correct and fixation time is accurate to the second.

2. Fragment distribution is bimodal regardless of sonication duration



Chromatin is partly over-fixed - make sure of all points mentioned in previous number, and that animal pellets are resuspended properly in the washes after fixation.

3. Fragment distribution too wide



Sonication is not fragmenting chromatin equally, creating fragments of variable sizes which don't stack as a narrow distribution. This is likely caused by insufficient intensity of sonication. Make sure the Covaris sonicator parameters used are according to the ones described here, and if yes try to increase intensity.

4. Low DNA yield

Chromatin might be over-sonicated, producing fragments of small length that are not usable for ChIP and are lost when purifying DNA. Try reducing sonication time.

5 Tips

Easy way to quantify protein

Total protein yield of extracted chromatin can be easily quantified with a Bradford assay and measured on a Nanodrop instrument. A standard curve made with protein standards (recommended range 0.1- 10 μg - triplicate measurements) is required, but can be reused. Add Coomassie reagent in a 30:1 ratio to standard/chromatin and use 2 μL for measurement.

6 Acknowledgments

We are much thankful to Rita Holdhus at the University of Bergen Microarray Core Facility in the Center for Medical Genetics and Molecular Medicine of the Haukeland University Hospital for access to the Covaris sonicator for this work.

7 References

- [1] T. S. Furey, “ChIP-seq and beyond: new and improved methodologies to detect and characterize protein-DNA interactions.,” *Nature reviews. Genetics*, vol. 13, pp. 840–52, Dec. 2012.
- [2] J.-M. Bouquet, E. Spriet, C. Troedsson, H. Otterå, D. Chourrout, and E. M. Thompson, “Culture optimization for the emergent zooplanktonic model organism *Oikopleura dioica*.,” *Journal of plankton research*, vol. 31, pp. 359–370, Apr. 2009.

Appendix A Consumables

Table 3: Consumables with particular relevance

Supplier	Consumable
Thermo Scientific	Formaldehyde ampoules, methanol-free
Sigma-Aldrich	Protease Inhibitor Cocktail - general use
Sigma-Aldrich	BD Precisionglide syringe needles - gauge 27, L 1/2 inches
Covaris	AFA glass 130 μ L microtubes
Sigma-Aldrich	Siliconized microtubes 1.7 mL capacity
Agilent	Bioanalyzer high sensitivity DNA Kit

Appendix B Instruments and equipment

Table 4: Instruments with particular relevance

Supplier	Instrument/equipment
Covaris	S200 focused sonicator
Agilent	2100 Bioanalyzer

Appendix C Buffers

PBS, pH 7.4 1.37 M NaCl; 27mM KCl;100 mM Na₂HPO₄; 1.8 mM KH₂PO₄

PBS-T 0.02% Tween-20 in PBS

Lysis buffer 150mM NaCl; 1% NP-40; 0.5% Na deoxycholate; 0.1% SDS; 50 mM Tris pH 8; 1mM EDTA

TE buffer 10 mM Tris pH 8; 1 mM EDTA