Protocol

Chromatin Characterization in *Xenopus laevis* Cell-Free Egg Extracts and Embryos

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Xenopus laevis development is marked by accelerated cell division solely supported by the proteins maternally deposited in the egg. Oocytes mature to eggs with concomitant transcriptional silencing. The unique maternal chromatin state contributing to this silencing and subsequent zygotic activation is likely established by histone posttranslational modifications and histone variants. Therefore, tools for understanding the nature and function of maternal and embryonic histones are essential to deciphering mechanisms of regulation of development, chromatin assembly, and transcription. Here we describe protocols for isolating pronuclear sperm chromatin from *Xenopus* egg extracts and hydroxyapatite-based histone purification from this chromatin. The histones purified through this method can be directly assembled into chromatin through in vitro assembly reactions, providing a unique opportunity to biochemically dissect the effect of histone variants, histone modifications, and other factors in chromatin replication and assembly. We also describe how to isolate chromatin from staged embryos and analyze the proteins to reveal dynamic developmental histone modifications. Finally, we present protocols to measure chromatin assembly in extracts, including supercoiling and micrococcal nuclease assays. Using these approaches, analysis of maternal and zygotic histone posttranslational modifications concomitant with cell-cycle and developmental transitions can be tested.

MATERIALS

It is essential that you consult the appropriate Material Safety Data Sheets and your institution's Environmental Health and Safety Office for proper handling of equipment and hazardous materials used in this protocol.

RECIPES: Please see the end of this protocol for recipes indicated by <R>. Additional recipes can be found online at <http://cshprotocols.cshlp.org/site/recipes>.

Reagents

Agarose/TAE gels (standard; Lonza SeaKem Agarose LE is preferable for MNase assays as the gels have a clearer appearance)

Chromatin assembly stop buffer <R>

DNA loading buffer containing RNase A <R>

Egg lysis buffer—chromatin isolation buffer (ELB-CIB) <R>

ELB-CIB with 0.5 M sucrose final concentration

ELB-CIB with 250 mM KCl final concentration

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Embryo lysis buffer (EmLB) <R>
Ethanol (100% and 70%)
Ethidium bromide
GlycoBlue (15 mg/mL)
HDB 2000 Buffer <R>
High salt buffer <R>
High salt phosphate buffer <R>
Hydroxyapatite resin (DNA Grade Bio-Gel HTP, Bio-Rad)
Laemmli SDS-PAGE sample buffer (1.5×)
Low salt phosphate buffer <R>
Medium salt buffer <R>
Micrococcal nuclease (MNase; Sigma-Aldrich)
MNase reaction buffer <R>

Use 90 μ L of the buffer containing 1 unit of MNase (Sigma-Aldrich) for each reaction. Prepare the mixture fresh.

MNase stop buffer <R>
NaOAc (3 M; pH 5.2)
Phenol/chloroform/isoamyl alcohol
Proteinase K (20 mg/mL)
RNase A (10 mg/mL)
SDS-PAGE gel (15% for checking histone fractions) and appropriate buffer
Spermine and spermidine (optional; see Step 3)
Topoisomerase-I relaxed pG5ML or similar plasmid (625 ng/ μ L)
Xenopus laevis egg (low-speed interphase supernatant/LSS or high-speed interphase supernatant/HSS)
or oocyte extracts (Banaszynski et al. 2010), or fertilized embryos (see Protocol: **Preparation of Cellular Extracts from *Xenopus* Eggs and Embryos** [Good and Heald 2018])
Xenopus laevis demembranated sperm chromatin (see Protocol: **Isolation and Demembranation of *Xenopus* Sperm Nuclei** [Hazel and Gatlin 2018])

Agarose gel electrophoresis apparatus
Beaker, 1L
Centrifuge with Fiberlite F15-8×50c, F14-14, or SS34 rotors (Thermo Scientific)
Cold room
Digital imaging system
Dounce homogenizer (5 mL or larger) with type B pestle
Econo-Pac chromatography column (Bio-Rad)
Falcon tubes (15 mL)
HB-6 swinging bucket rotor or equivalent
Liquid nitrogen and dewar
Microcentrifuge, fixed-angle and swinging-bucket microcentrifuge rotors with temperature control
and cooling
Microcentrifuge tube heating/cooling block
Microcentrifuge tubes (1.5 mL, flip-top)
6–8 kDa MWCO dialysis tubing (10 mm)
Pellet Pestle Cordless Motor
Pellet Pestles (Fisher)
Peristaltic pump and tubing
Protein concentrator with 3000 Da molecular weight cut off
SDS-PAGE gel electrophoresis apparatus
Sonicator with microtip
Water bath

Equipment

METHOD

Five procedures are outlined here: three to isolate chromatin and histones from extract or embryos for immunoblotting (Wang et al. 2014), mass spectrometry of modifications and variants (Nicklay et al. 2009; Shechter et al. 2009; Wang et al. 2014), or for use in chromatin assembly (Onikubo et al. 2015); and two procedures for measuring histone deposition and chromatin assembly in cell-free extract assays (Wang and Shechter 2016). Low-protein binding tubes and tips substantially reduce variability in these assays. The hydroxyapatite purification of histones was based on (Schnitzler 2001). Refer to (Banaszynski et al. 2010) for *Xenopus* egg extract and sperm preparation.

Isolation of Sperm Pronuclear Chromatin

1. Split 10 mL of fresh interphase-arrested LSS with energy mix (Banaszynski et al. 2010) into five 15 mL falcon tubes and add demembrated sperm chromatin (see Protocol: **Isolation and Demembration of *Xenopus* Sperm Nuclei** [Hazel and Gatlin 2018]) to a final concentration of 4000/ μ L.
2. Incubate in a water bath for 60 to 90 min at 22°C to form sperm pronuclei.
3. Lyse pronuclei by mixing each 2 mL of assembly reaction with ELB-CIB for a 10 mL total reaction volume. Incubate for 10 min on ice. Used chilled buffers and ice from this step on.

Adding 1 mM spermine and spermidine to ELB-CIB buffer results in a tighter pellet that allows more facile collection of chromatin but is more difficult to shear in the subsequent steps for hydroxyapatite purification of histones. These polyamines should be included when an isolation of pronuclear chromatin is performed for immunoblotting, acid extraction, or mass spectrometry.

4. Carefully underlay the suspension with 1 mL ELB-CIB containing 0.5 M sucrose and centrifuge at 4000 RPM in an HB-6 swinging-bucket rotor for 5 min at 4°C.
5. Carefully remove the entire supernatant with a 1 mL pipet without disrupting the chromatin pellet at the bottom of the tube. Wash the pellet once with 1 mL ELB-CIB containing 250 mM KCl and spin at 13,000 RPM for 2 min at 4°C. Retain the pellet which contains the pronuclear chromatin and histones (Fig. 1A).

At this point, histones can be collected from the chromatin pellet using acid extraction for immunoblotting or mass spectrometry (Shechter et al. 2007; Wang et al. 2014). Alternatively, Steps (6–18) can be used to isolate intact, native histone octamers and subcomplexes as substrates for chaperone chromatin assembly activity assays (Fig. 1B), remodeling assays, or posttranslational modifying-enzyme assays (Wilczek et al. 2011).

Hydroxyapatite Purification of Pronuclear Histone Complexes

6. Resuspend and wash the chromatin pellet in 10 mL of medium salt buffer. Centrifuge at 10,000 RPM for 10 min at 4°C in a fixed-angle Fiberlite F15-8 $\times$ 50c or equivalent rotor. Retain the pellet.
7. Resuspend the pellet with 1 mL of high salt buffer and remove the solution to a Dounce homogenizer with type B pestle.
8. Rigorously Dounce 50 times on ice to shear the chromatin.
9. Transfer the solution into a 1.5 mL microcentrifuge tube and centrifuge at 10,000 RPM for 20 min at 4°C in a microcentrifuge. Retain the supernatant that contains the solubilized chromatin. Move immediately to Step 10 or immediately freeze at –80°C.

It is critical that the chromatin is not over-sheared as it leads to high DNA contamination in the final product. Running the sample on a 0.8% agarose gel after phenol-chloroform purification can be used to measure the efficiency of chromatin shearing, which should appear as a smear at the top of the gel; if lower molecular weight DNA is observed, reduce homogenization accordingly.

10. Dialyze solubilized chromatin in 6–8 kDa MWCO dialysis tubing against 1 L of Low salt phosphate buffer for 4 h. Perform all procedures in a cold room with chilled buffers from this step on.
11. Hydrate 5 g of hydroxyapatite resin with 30 mL of low salt phosphate buffer and remove the fines by pouring off the top suspension after the bulk of the resin settles; repeat this step 3–4 times.
12. Connect the Econo-Pac chromatography column *outlet* to a peristaltic pump.

FIGURE 1. Isolation and use of *Xenopus* egg and embryo histones. (A) SDS-PAGE of hydroxyapatite purified histones from pronuclei assembled in *Xenopus* cell-free egg extract. A Coomassie-stained gel is shown on the *left*, while immunoblots for histones H2A, H4, and H2A.X.3 (also known as H2A.X-F) are shown on the *right*. (B) Recombinant *Xenopus* Nap1 chaperone was added to hydroxyapatite purified histones in the presence of a relaxed plasmid for the indicated times. The DNA was deproteinized and run on an agarose gel to measure supercoiling. (C) Fractionated stage 42 embryo cytoplasmic and nuclear protein was run on an SDS-PAGE, transferred to a membrane, and stained by Direct Blue 71 for subsequent immunoblotting. Enriched embryo histones are indicated on the *right*. Immunoblotting of chromatin-bound histones is possible from early (pre-MBT) embryos, depending upon the antibody (Wang et al. 2014).

It is essential that the column is connected to a pump as it will not flow by gravity. From this point on, it is crucial not to let the resin dry out.

13. Pour the hydrated hydroxyapatite resin into the chromatography column and wash with 10 mL of Low salt phosphate buffer.
14. Adjust the peristaltic pump to 0.5–1 mL/min flow rate. Stop the pump as all the wash buffer enters the resin.
15. Load the solubilized chromatin onto the resin by directly applying the sheared chromatin sample over the resin. Start the pump to load onto resin and stop the pump as all the solution enters the resin.
16. Wash the resin with 50 mL of low salt phosphate buffer using the pump.
This step is crucial for removing noncore histone-bound proteins, including linker histones. Adjust the volume of wash if linker histone contamination is observed in the final product. Alternatively, linker histones can be isolated from these washes. Separation of H2A-H2B and H3-H4 can also be achieved by eluting the histones using the phosphate buffer with a gradient of NaCl from 0.6 to 2.5 M. Since histones elute in a narrow range of NaCl (0.9 M for H2A-H2B and 1.1 M for H3-H4), the use of a prepacked hydroxyapatite column connected to a FPLC system is highly recommended for this purpose, as this system allows more facile monitoring of eluted proteins.
17. Elute core histones with four 8 mL volumes of high salt phosphate buffer.
Run each fraction on a SDS-PAGE gel to confirm the presence of histones, which primarily elute in the first two fractions.
18. Collect the fractions containing histones. Exchange the buffer to HDB2000 and concentrate using a protein concentrator per the manufacturer's instructions. Histones can be stored at -80°C for subsequent chromatin assembly activity assays (Fig. 1B).

Isolation of Chromatin from Staged Embryos

This procedure can be used to isolate chromatin and histones from staged embryos for immunoblotting of histone modifications and transcription factors (Wang et al. 2014).

19. Fertilize and culture embryos (see Protocol: **Preparation of Cellular Extracts from *Xenopus* Eggs and Embryos** [Good and Heald 2018]).
20. Collect embryos according to Nieuwkoop and Faber stages (Faber and Nieuwkoop 1994; Karpinka et al. 2015) into a microcentrifuge tube and remove excess buffer.
21. Flash-freeze aliquots of five embryos in a microcentrifuge tube in liquid nitrogen; at this point the samples can be saved at -80°C or used immediately in the next steps.
22. Add 100 μL of cold EmLB to each tube of five-staged and flash-frozen embryos and homogenize using a motorized Pellet Pestle within the tube.
23. Incubate the lysate on ice for 10 min.
24. Centrifuge the lysate in a microcentrifuge swinging-bucket rotor for 3 min at 1000g at 4°C to separate the nuclei.

Eggs, oocytes and embryos before stage 10 contain abundant egg yolk that should be moved out of the way with the pipet tip, and the tip wiped off with Kimwipes to avoid transferring the yolk, before collecting the supernatant after centrifugation.

25. Collect 80 μL supernatant as the cytoplasmic fraction (four embryo equivalent), dilute to 200 μL in $1.5\times$ Laemmli sample buffer, boil, and store at -80°C .
26. Wash the pellet in 200 μL of EmLB twice, spinning for 3 min at 1000g at 4°C after each wash and suspend the pellet in 200 μL of $1.5\times$ Laemmli buffer as the nuclear fraction.
27. Sonicate the nuclear fractions three times with 30 sec on, 30 sec off at 30% amplitude to solubilize chromatin on ice in the cold room. Boil the nuclear fractions for 5 min at 95°C and store at -80°C .

Dilute the cytoplasmic supernatant and chromatin fractions five- to 10-fold prior to loading on SDS-PAGE for immunoblots (Fig. 1C).

Plasmid Chromatin Assembly Reaction and Supercoiling Analysis in Extract

These next two procedures can be used to study how chromatin assembly pathways function in cell-free extract (Wang and Shechter 2016). There are batch-to-batch variations of egg extract chromatin assembly efficiency. Test each batch of egg extract after preparation using a time course from 30 min to 3 h of DNA incubation.

28. Add Topoisomerase-I relaxed pG5ML vector to a final concentration of 25 ng/ μL in egg or oocyte extract containing $1\times$ energy mix (Banaszynski et al. 2010). Tap the capped tube to mix and incubate at 23°C .

The typical reaction volume is 25–100 μL depending on the type and number of assay points desired, as in Step 29.

29. At desired time points (e.g., 0, 15, 30, 60, 90, and 120 min), aliquot 5 μL of reaction into 200 μL of Chromatin assembly stop buffer and incubate for 30 min at 37°C . Alternatively, or in parallel, continue to Step 34 for micrococcal nuclease analysis (skip Steps 30–33).
30. Add 5 μL of 20 mg/mL proteinase K (final 50 $\mu\text{g}/\text{mL}$) and incubate for 1 h at 56°C .
31. Purify plasmid DNA through standard procedures of Phenol/Chloroform extraction and Ethanol/NaOAc precipitation in the presence of 1 μL GlycoBlue.
32. Dissolve the DNA pellet in 12 μL of DNA loading buffer containing RNase A.
33. Analyze the DNA supercoiling on a 0.8% agarose-TAE gel at 25 V overnight in the cold room. Continue with Step 39 (Fig. 2A).

See Troubleshooting.

FIGURE 2. Chromatin assembly in egg extract. (A) A relaxed plasmid assembled in interphase extract for the indicated times was deproteinized and run on an agarose gel to measure supercoiling. (B) A plasmid assembled in interphase egg extract was digested with 1 Unit of MNase for the times indicated. Extracted DNA was run on an agarose gel and visualized with ethidium bromide. Size standards are shown on the *right*. Observed nucleosomes (indicated on *left*) had a repeat length <200 bps.

Micrococcal Nuclease Analysis

34. (Continued from Step 29). Transfer 11 μ L of the chromatin assembly reaction into a fresh microcentrifuge tube with 90 μ L MNase reaction buffer containing 1 unit MNase and incubate for 5 to 6 time points at room temperature for an empirically determined time.

See Troubleshooting regarding determination of MNase time course.

The MNase reaction is very sensitive. It is important to start the timer right after the addition of the reaction product into MNase reaction buffer and simply invert the tube twice to mix.

35. Add 110 μ L MNase stop buffer to stop the reaction.
36. Add 8 μ L RNase A (10 mg/mL) to each reaction and incubate overnight at 37°C.
37. Follow Steps 30–32 above.
38. Load the final product on a 2% agarose gel and run at 100 V for 60 min at room temperature.
39. Stain the gel with ethidium bromide and image on a digital imaging system for quantification (Fig. 2B).

See Troubleshooting.

TROUBLESHOOTING

Problem (Step 33 or 38): Speckles or shadows on 2% agarose gel.

Solution: Since the quantity of DNA for mono- or di-nucleosomes is <25 ng, background staining can lower the resolution of the nucleosome ladder. It is important to clean the electrophoresis apparatus thoroughly before casting the gel. Make sure to filter the buffer and dissolve the agarose completely, swirling the flask until it dissolved completely and avoid bubbles when pouring the agarose.

Problem (Steps 34 and 39): No nucleosome ladder observed after MNase digestion.

Solution: Perform a time course analysis of the supercoiling assay and use the optimal time point for assembly that produces multiple nucleosomal repeats in the MNase assay (e.g., 0 to 30 min in MNase). If the MNase digestion is too fast or too slow, one can fine tune the reaction by adjusting the concentration of CaCl₂ or MNase.

DISCUSSION

The methods presented here are appropriate for both in vitro and in vivo analysis of histones and chromatin from *Xenopus laevis* oocyte and egg extracts as well as from staged embryos. The extracts can be further manipulated by immunodepletion, drugs, or other means prior to probe specific roles of targeted proteins on chromatin assembly pathways. Embryos can be microinjected with morpholinos or drugs to similarly target developmentally regulated chromatin assembly pathways and these techniques used to probe their developmental function on chromatin. Finally, these methods can be combined with chromatin immunoprecipitation (ChIP) qPCR or ChIP-sequencing and qRT-PCR or RNA-sequencing to test chromatin-based mechanisms of the regulation of gene expression and genome integrity.

RECIPES

Chromatin Assembly Stop Buffer

Reagent	Concentration
Tris (pH 8.0)	10 mM
EDTA	20 mM
SDS	0.5%
RNase A	0.1 mg/mL

Store for up to 2 yr at 25°C (without RNase A).

DNA Loading Buffer Containing RNase A

Reagent	Concentration
Tris (pH 7.5)	10 mM
EDTA	1 mM
Glycerol	5%
RNase A	10 µg/mL

Add a very small amount of bromophenol blue if desired. (This can make the loading easier, but make sure that the color does not interfere with DNA signal.) Store for up to 2 yr at 25°C (without RNase A).

Egg Lysis Buffer–Chromatin Isolation Buffer (ELB–CIB)

Reagent	Concentration
HEPES (pH 7.8)	10 mM
KCl	50 mM
MgCl ₂	2.5 mM
Sucrose	0.25 M
DTT	1 mM
Triton X-100	0.1%
HALT Phosphatase Inhibitor Cocktail (Pierce)	1×
Protease Inhibitor Cocktail (Roche)	1×
Sodium butyrate	10 mM
Prepare fresh for each use.	

Embryo Lysis Buffer (EmlB)

Reagent	Concentration
Tris (pH 7.5)	10 mM
NaCl	200 mM
MgCl ₂	5 mM
NP-40	0.5%
Na butyrate	5 mM
HALT Phosphatase Inhibitor Cocktail (Pierce)	1×
Protease Inhibitor Cocktail (Roche)	1×
Cycloheximide	100 µg/mL
Prepare fresh for each use.	

HDB 2000 Buffer

Reagent	Concentration
Tris (pH 8.0)	20 mM
NaCl	2 M
EDTA	1 mM
Glycerol	5%
β-mercaptoethanol	10 mM
Prepare fresh for each use.	

High Salt Buffer

Reagent	Concentration
HEPES (pH 7.5)	20 mM
NaCl	400 mM
EDTA	1 mM
Glycerol	5%
PMSF	1 mM
Prepare fresh for each use.	

High Salt Phosphate Buffer

Reagent	Concentration
Sodium phosphate (pH 6.8)	20 mM
NaCl	2.5 M
β -mercaptoethanol	5 μ M
PMSF	1 mM
Prepare fresh for each use.	

Low Salt Phosphate Buffer

Reagent	Concentration
Sodium phosphate (pH 6.8)	20 mM
NaCl	600 mM
β -mercaptoethanol	5 μ M
PMSF	1 mM
Prepare fresh for each use.	

Medium Salt Buffer

Reagent	Concentration
HEPES (pH 7.5)	20 mM
NaCl	400 mM
EDTA	1 mM
Glycerol	5%
PMSF	1 mM
Prepare fresh for each use.	

MNase Reaction Buffer

Reagent	Concentration
HEPES (pH 8.0)	10 mM
KCl	50 mM
MgCl ₂	5 mM
CaCl ₂	3 mM
DTT	1 mM
NP-40	0.1%
Glycerol	8%
Prepare fresh for each use.	

MNase Stop Buffer

Reagent	Concentration
EDTA	40 mM
EGTA	2 mM
SDS	1%
Store for up to 2 yr at 25°C.	

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