AQUA-FAANG - Standard Operating Protocol -
ChIP-seq sample and library preparation protocol in carp embryos

Overview
This protocol describes the ChIP-seq sample and library preparation protocol used in crosslinked carp embryos samples.
This protocol is based on the µChIPmentation protocols optimised in Roslin Institute and Universidad de Santiago de Compostela (1, 2) and the Diagenode µChIPmentation Kit for Histones and Easy Shear Kit handbooks (3, 4) which have been adapted for carp embryos.

The protocol describes a method to prepare sheared chromatin fragments, perform immunoprecipitation and prepare libraries ready for sequencing, starting from crosslinked carp embryo material. Starting material can be achieved following the corresponding University of Birmingham Standard Operating Protocol in the FAANG portal (5).

Equipment/kits

- Sonication device (Bioruptor Pico)
- Rotatory system
- 1,5/2ml centrifuge set-up at 4ºC
- A 1.5ml plastic pestle
- Bioruptor Sonication tubes
- 1,5/2ml Eppendorf tubes
- Qiagen MinElute PCR Purification Cat. No. Kit 28004
- Diagenode Easy Shear Kit - Cat. No. C01020012
- Diagenode µChIPmentation Kit - Cat. No. C01011011
- Diagenode UDI for tagmented libraries – Set I, Cat. No. C0101134
- Diagenode UDI for tagmented libraries – Set II, Cat. No. C0101136
- H3K4me3 antibody - Cat. No. C15410003
- H3K27me3 antibody - Cat. No. C15410195
- H3K27ac antibody - Cat. No. C15410196
- Qubit device and reagents (High Sensitivity)
- 1.5/2ml LoBind tubes

Solutions/reagents

- PBS
- Proteinase inhibitor cocktail (PIC) solution: 1 PIC tablet in 10ml PBS, or 10µl PIC solution (Easy shear kit) and 2ml PBS
- Hanks Balanced Salt Solution (HBBS)
- RNase (100mg/ml)
- Proteinase K (20 mg/ml)
- Trypan blue

1. Cell lysis and chromatin shearing

1.1. Centrifuge samples at 850 RCF for 5 minutes at 4°C, discard the supernatant and wash embryos in PBS once by gently pipetting up and down.

1.2. Centrifuge samples at 850 RCF for 5 minutes at 4°C, discard the supernatant and dissociate embryos to single cells in PIC solution using a P200 pipette.

1.2.1. Early-stage embryos will dissociate easily by pipetting with P200 pipette

1.2.2. Late-stage embryos can be dissociated using a 1.5ml pestle first, followed by pipetting with a P200 pipette.

1.3. Quantify cells using a haemocytometer. Take a 10 µl aliquot of the cell suspension and mix with 10 µl of trypan blue. Load 10 µl of the mixture in the haemocytometer.

Aim for collection of the same number of cells across samples to make sonication optimisation easier.

1.4. Pellet the desired amount of cells by centrifuging an aliquot at 850 RCF for 5 minutes at 4°C.

1.4.1. Discard the supernatant and lyse the pellet in 315 µl of tL1 lysis buffer + 2 µl of 200X PIC. Mix by pipetting and vortex for 5-10 seconds.

1.4.2. Keep the samples on ice for 20-30 minutes to ensure complete lysis. A pale-yellow solution indicates full lysis. If the samples look cloudy, leave the tubes on the bench at room temperature for 2 minutes and vortex. If there are big chunks of tissue visible, mix by pipetting with a P200.

1.4.3. Add 185 µl of HBSS and mix.

1.4.4. Take out 100 µl of the lysate into a sonication tube. At this step, the remaining samples can be stored at -80°C.

1.5. Sonicate 100 µl. Sonication conditions should be optimised for each type of equipment and sample. For example, for Bioruptor Pico, 3 cycles with 30 seconds ON and 30 seconds OFF worked well for carp embryos at various stages, leading to chromatin fragments between 200-800 bp.

1.6. After sonication, centrifuge the lysate at 17000 RCF for 5 minutes at 15°C.

1.7. Carefully transfer the supernatant to a 1.5 ml DNA loBind tube. From this, a 15 µl aliquot can be used to test the shearing efficiency. Store the remaining chromatin at -80°C until needed for ChIP.
    1.7.1. To analyse chromatin shearing size range add 0.5 µl of RNase to the lysate.
    1.7.2. Incubate at 50°C for 1 hour on a PCR block
    1.7.3. Add 25 µl of tE1 and 2 µl of tE2, mix by pipetting.
    1.7.4. Reverse the crosslinking at 65°C for 4 our on a PCR block.
    1.7.5. Add 2 µl of proteinase K and incubate at 50°C for 1 hour on the PCR block.
    1.7.6. Purify the DNA using the Qiagen MinElute PCR purification kit, following the manufacturer's instructions.
    Before DNA purification, add 1 µl of glacial acetic acid per 250 µl of PB buffer. This will make the pH acid for efficient column binding.
    1.7.7. Measure the amount of chromatin in Qubit and the size distribution of chromatin fragments in Tapestation.

2. Chromatin immunoprecipitation and tagmentation

In this step, follow the instructions in the Diagenode µChIPmentation Kit handbook (Version 5 06_2024) starting on page 28 (Magnetic immunoprecipitation and tagmentation) and up to page 31.
We used approximately 100 ng of chromatin per ChIP reaction.
Make sure to keep input (non-precipitated) samples as further control.

OPTION A, B and C

STEP 3

Magnetic immunoprecipitation and tagmentation

 Day 1-2  1h overnight incubation, 1.5 hours

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3.1 Determine the number of IP reactions to be run including the negative and positive control IPs. Take the required amount of DiaMag Protein A-coated magnetic beads and transfer it to a clean 1.5 ml tube. **10 µl of beads are required per IP**.

3.2 Wash the beads 4 times with **50 µl of ice-cold Beads Wash Buffer tBW1 per IP**. To wash the beads, add tBW1, resuspend the beads by pipetting up and down several times and place the tubes in the magnetic rack. Wait for 1 minute to allow the beads to be captured by the magnet and remove the supernatant. Repeat this 3 times.

3.3 After the last wash, resuspend the beads in Beads Wash Buffer tBW1 adding the original volume of beads (this means 10 µl per IP).

3.4 Prepare the Immunoprecipitation mix as described in the table below. Add **150 µl** of **Immunoprecipitation mix** to each chromatin sample.

Component	Volume per reaction
HBSS	50 µl
ChIP Buffer tC1	100 µl
200x Protease Inhibitor Cocktail (black cap)	0.75 µl

Set aside **4 µl** of each sample to be used as an **input sample** and keep at 4°C.

3.5 Add the specific antibody to each tube.

NOTE: *The required amount of antibody per IP varies. Check the supplier's recommendation or perform a titration curve using different amounts of antibody. If a positive control IP is included, use 0.5 µg of the H3K4me3 positive control antibody (white cap). If a negative control IP is included, use 0.5 µg of Rabbit IgG (white cap).*

3.6 Add **10 µl of the washed magnetic beads** to each tube.

3.7 Incubate overnight at 4°C on a rotator.

3.8 Perform the washes as follows: briefly spin the tubes and place them in the magnetic rack. Wait for 1 minute and remove the supernatant. Add **150 µl of Wash Buffer tW1**: gently shake the tubes to resuspend the beads and incubate for 5 minutes on the rotator at 4°C. Repeat the washing step as described above once with Wash Buffer tW2, tW3 and tagW1, respectively.

3.9 Prepare the ChIPmentation mix as described in the table below for the desired number of reactions, including the inputs. Mix thoroughly with a pipette. Keep on ice until used.

Component	Volume per reaction
Tagmentation Buffer (yellow cap)	19 µl
Tagmentase (loaded) (yellow cap)	1 µl

3.10 Put the tubes from step 3.8 on the DiaMag02. Wait until supernatant is clear and discard the supernatant.

3.11 Add **20 µl** of **ChIPmentation mix** to each IP tube and gently resuspend the beads by pipetting.

3.12 Add **20 µl** of **ChIPmentation mix** and **1 µl** of **$MgCl_2$** (blue cap) to each input sample and gently mix by pipetting.

3.13 Incubate IP and input samples for 10 minutes at 37°C in the preheated thermocycler. After 5 minutes of incubation, briefly mix the tubes to resuspend the beads.

NOTE: *The recommended tagmentation time is 10 minutes, but the optimal time can vary depending on the cell number and the antibody used. See "Remarks before starting" section for more details.*

3.14 Put the samples on ice and immediately add **150 µl of cold Wash Buffer tagW2** to each IP samples and **1 µl of 0.5 M EDTA** (not provided in the kit) to the input samples. Keep the input on ice until step 4.2. Gently shake the IP samples to resuspend the beads and incubate for 5 minutes on the rotator at 4°C.

3.15 Briefly spin the IP samples and place them in the DiaMag02, wait until supernatant is clear and discard the supernatant.

3.16 Add **150 µl of cold Wash Buffer tagW1** to each IP samples, gently shake the tubes to resuspend the beads and incubate for 5 minutes on the rotator at 4°C.

3.17 Briefly spin the tubes and place them in the DiaMag02, wait until supernatant is clear and discard the supernatant.

OPTION A, B and C

STEP 4

Stripping, end repair, reverse cross-linking

1 Day 2 30 minutes

4.1 Remove the strip from magnetic rack, add **10.5 µl of Stripping reagent** to the beads and resuspend by pipetting.

4.2 Heat the immunoprecipitated and input samples 30 minutes at 50°C using a thermocycler.

4.3 Add **10.5 µl $MgCl_2$** (blue cap), **4 µl of ChIP-seq grade water** and **25 µl of 2x High-Fidelity Mastermix** (violet cap) to each IP'ed. Add **1 µl of 0.5 M $MgCl_2$** (not provided in the kit) to the input samples. Take 25 µl of input and add **25 µl of 2x High-Fidelity Mastermix** (violet cap). Incubate IP'd samples and input as follows:

Step	Temperature	Time
End repair	72°C	5 minutes
Reverse cross-linking	95°C	10 minutes
	Cooling at 4°C (or ice)	

4.4 Magnetize beads from the immunoprecipitated samples and transfer the supernatant to a new 0.2 ml tube. Keep the samples at 4°C (or on ice).

NOTE: *The total volume of each IP or input sample is 50 µl.*

3. Library amplification and purification

As previously tested, we recommend skipping the pre-amplification qPCR step to determine the total number of cycles per sample, as this results in unnecessary loss of sample. Given the amount of chromatin used for this project per ChIP reaction, most samples will need 12-15 PCR cycles for amplification.

Our library preparation was performed using the Diagenode UDI for tagmented libraries kit sets I and II.
To follow the manufacturer's instructions on library preparation, please refer to Diagenode µChIPmentation Kit handbook (Version 5 06_2024) starting on page 32 (skipping steps 5.2 to 5.6) and up to page 34.

OPTION A, B and C

STEP 5

Library amplification

2 Day 2 2.5 hours

Determination of the optimal cycle number for the enrichment PCR

NOTE: *For this step only 2 µl of each library will be used.*

5.1 Dilute 5x the primers with the Primers Dilution Buffer before using them.

5.2 Prepare the **Quantification Mix** as described in the table below for the number of desired reactions. Mix by pipetting and keep on ice until use.

Component	Volume per reaction
Primer Pair S1 (diluted)	0.4 µl
2x High-Fidelity Mastermix (violet cap)	5 µl
100x SYBR	0.1 µl
Nuclease-free water	2.5 µl

5.3 Dispense 8 µl of the Quantification Mix into 0.2 ml tubes or strips according to the number of libraries.

5.4 Add 2 µl of IP'ed or input DNA to each tube and mix by pipetting.

5.5 Briefly spin the tubes and run the PCR program described below.

Cycles	Temperature	Time
1	98°C	30 seconds
25	98°C	10 seconds
	63°C	30 seconds
	72°C	30 seconds
1	72°C	1 minute
1	10°C	∞

Keep the IP'ed and input DNA on ice during the qPCR.

5.6 Analyse the Ct values. The optimal cycle number for the amplification of the rest of the ChIPmentation DNA is typically Ct +1.

NOTE: *The Ct value is highly dependent on the thermocycler you use, as well as the way you analyze the qPCR results. Thus when using the kit for the first time you may need to verify that the Ct+1 rule applies well in your conditions.*

5.7 Add **2 µl** of the **diluted Primer Pair** with the appropriate index in each tube from step 4.3 and mix by pipetting.

NOTE: *The tubes already contain the mastermix as it was added at step 4.2.*

5.8 Briefly spin the tubes and run the PCR program described below.

Cycles	Temperature	Time
1	98°C	30 seconds
X (Ct rounded up +1)	98°C	10 seconds
	63°C	30 seconds
	72°C	30 seconds
1	72°C	1 minute
1	10°C	∞

NOTE: *After amplification it is possible to use 1µl of library to run on a sizing device while keeping the samples on ice. It permits to check that enough material was generated. If needed additional amplification cycles can then be performed. However higher yields may come at the expense of reduced sequencing quality. Therefore we recommend not using more than 18 cycles in order to avoid an over-amplification.*

OPTION A, B and C

STEP 6

Clean-up

2 Day 2 45 minutes

6.1 Carefully resuspend the room temperature AMPure XP beads by shaking and light vortexing until no pellet is visible at the bottom of the container.

6.2 Estimate the library volume and add **1.8x volume of AMPure XP beads** (e.g. for a sample volume of 50 µl, add 90 µl of beads). Mix by pipette 8 – 10 times until the mixture is homogeneous.

6.3 Incubate at room temperature for 10 minutes.

6.4 Place the tube on the DiaMag02 and wait until the beads are completely bound to the magnet (~2 minutes).

6.5 Carefully aspirate by pipette and discard the supernatant without disturbing the pellet.

6.6 Wash the beads pellet 2 times as follows:

- With the tubes on the magnet, add **100 µl** of freshly prepared **80% ethanol** without disturbing the bead pellet and wait for 5 seconds.
- Carefully aspirate by pipette and discard the supernatant without disturbing the pellet.

6.7 Leaving the tube open, let the beads dry on the DiaMag02 for 5 minutes.

6.8 Remove tubes from DiaMag02 and elute DNA by resuspending the beads in **20 µl** of **Resuspension Buffer**.

6.9 Incubate for 10 minutes at room temperature.

6.10 Place the tube on the DiaMag02 and wait until the beads are completely bound to the magnet (~2 minutes).

6.11 Without disturbing the pellet, carefully aspirate and transfer the supernatant containing purified libraries to a new tube.

4. Library quality control

Measure the concentration of DNA libraries using the Qubit high sensitivity kit. Further library controls can be performed through qPCR tests, using positive and negative ChIP regions as targets.

Bibliography

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