GENE-SWitCH

The regulatory GENomE of SWine and CHicken: functional annotation during development

Protocol WP1
ChIP-SEQ – ChIPmentation 96 samples

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Table of contents

1 Summary

GENE-SWitCH aims at identifying functional elements located in the genomes of pig and chicken working on seven different tissues at three different developmental stages. It requires a collection of samples corresponding to the selected tissues and developmental stages with associated metadata describing accurately the samples and the sampling process.

The seven tissues analysed in GENE-SWitCH are:

- Cerebellum
- Lung
- Kidney
- Dorsal skin
- Small intestine
- Liver
- Skeletal muscle

The three developmental stages are:

- Early organogenesis (E8 chick embryo and D30 pig foetuses)
- Late organogenesis (E15 chick embryo and D70 pig foetuses)
- Newborn piglets and hatched chicks

For each species and each developmental stage, 4 biological replicates (2 males and 2 females) are sampled.

There is no difference in the protocols according to the developmental stage of the animals, however, depending on the tissue, the amount of tissue used as a starting material for this protocol varies between 25 mg and 100 mg.

2 Chromatin extraction protocol description

Before starting

- Use the reagents from the Diagenode Chromatin shearing optimization kit – Low SDS (iDeal kit for Transcription Factors) (Cat N° C01020013) + more fixation buffer
- Define the volume of shearing buffer iS1b taking into account that you will need:
    a. 100 µl of sheared chromatin (containing the desired amount of tissue) per IP
    b. 20 µl of sheared chromatin for chromatin shearing assessment
    c. Add 5% excess of iS1b

- Approximately 120 mg of tissue
- Need 1 µg chromatin per IP

STEP 1 - Grind the frozen tissue using the tissue lyser II

- Keep the adapters of the tissue lyser at -80°c for at least 4hours (preferentially ON). Transport on dry ice
- Transfer the tissue pieces (+/- 120 mg) into a 2ml tube (done previously)
- Switch ON the Big centrifuge and set the temperature @4°C
- Put the Protease inhibitors @RT
- Prepare all the buffers (on ice), Fixation buffer under Fumehood (previously boocked)
- Add 2 steel balls (previously put on dry ice) and keep the samples on dry ice until grinding.
- Transfer the frozen tubes to the frozen adaptors and grind for 2minutes at 30strockes/sec.
- Freeze again in dry ice and grind for additional 2 min
- Store the samples at -80°C if not used immediately for chromatin preparation.

STEP 2 – Tissue Lysis and Chromatin Shearing

- Equilibrate the Fixation Buffer to room temperature before use, PBS on ice, PIC at RT
- In a fume hood, add 40.5 µl (or as calculated previously) of 37% formaldehyde to 1.5 ml of Fixation buffer (or as calculated previously) to get concentration of 1%. Use 1.5 ml of Fixation buffer for one chromatin preparation
- Thaw samples on ice for 2 minutes (keep into the tissue lyser support) and then add 1.5 ml of cross-linking solution with automatic pipet with 5 ml combitip
- Close tubes and put back the lid of the support, shake shortly and **set the timer to 13 min**
- Incubate for a total time in the Tissue lyser (15-'X'-2 min/4 strokes/s) ['X' is the remaining time in the timer]
  (prepare glycine at this step + robot pipet + switch ON big centri if not done previously)
- Add 150 µl of Glycine to the tissue suspension to stop the fixation.

- Incubate for 5 minutes at room temperature in the Tissue lyser (5 strokes/s).
- Centrifuge samples in the tissue lyser support at 850 x g for 5 minutes at 4°C. Gently discard the supernatant (using 1 ml pipet) and keep the pellet.
- Wash the pellet with 1.5 ml of ice-cold PBS and centrifuge samples at 850 x g for 5 minutes at 4°C. Gently discard the supernatant. Keep everything at 4°C or on ice from now on.
- Add 7.5 µl (or as previously calculated) of 200x protease inhibitor cocktail to 1.5 ml of ice-cold Lysis buffer iL1b.
- Add 1.5 ml of ice-cold complete Lysis buffer iL1b to the pellet and resuspend using the tissue lyser for 1 min at (10 strokes/s)
- Incubate @4°C for 20 minutes with gentle mixing on the thermomixer (600 rpm) using the 2 ml tubes support.
- Pellet the cells by centrifugation at 850 x g for 5 minutes at 4°C and discard the supernatant.
- Add 7.5 µl (or as calculated) of 200x protease inhibitor cocktail to 1.5 ml of ice-cold Lysis buffer iL2 (done)
- Add 1.5 ml of ice-cold complete Lysis buffer iL2 to the cell pellet and resuspend using the tissue lyser for 1 min at (10 strokes/s)
- Incubate in the thermomixer 2 ml tubes support for 10 min @4°C/1400 rpm
- Pellet the cells again by centrifugation at 850 x g for 5 minutes at 4°C and discard supernatant.
- Add 200x protease inhibitor cocktail (2.5 µl or as calculated) to Shearing buffer iS1b (500 µl/sample). Keep on ice.
- Add the complete Shearing buffer iS1b to the pellet. Resuspend the cells using the tissue lyser for 2 min at (5 strokes/s).
- Incubate in a thermomixer (5 min @ 4°C @ 1400 rpm)
- Can be stored @ -80°C at this stage-
- Make a Qc of the Bioruptor Pico2 to be used (gDNA @10ng/µl, 100 µl in 0.65 ml microtubes, 13 cycles 30sON/30sOFF) then run in FA NGS kit, Average size no more than 220 bp & CV less than 10 %
- Thaw tubes on ice if needed
- After complete thawing, short spin
- Split the cell suspension into aliquots of maximum 300 µl by transferring it to the appropriate 1.5 ml sonication microtubes.
- Incubate for 10 minutes on ice (no need if there is a lot of samples).
- Shear chromatin by sonication using the Bioruptor Pico using the following conditions unless otherwise stated:
    - 1 sample for 2 cycles
      Each cycle corresponds to 30'' ON 30'' OFF

!!!NB!!! If there is a lot of samples to shear, a 15 minutes break should be added between each 3x8 cycles run

- Briefly spin down the liquid in the samples for 15 seconds. Transfer samples to new 1.5 ml tubes and centrifuge at 13,000 rpm (16,000 x g) for 10 minutes. Collect the supernatant which contains the sheared chromatin.
- Take an aliquote of 20 µl chromatin and go to step 3

- Measure the concentration of the chromatin sample (1µl for Qubit BR) after launching the reverse crosslink

STEP 3 – Shearing efficiency analysis

- Take an aliquot of 20 µl of sheared chromatin (adjust the volume to 50µl by adding 30 µl iS1 buffer).
- Add 50 µl of elution buffer iE1.
- Add 4 µl of elution buffer iE2, mix thoroughly.
- Incubate samples at 65°C for 4h (or overnight) on thermomixer (900rmp).

  (during this time, measure the concentration of chromatin (1µl in Qubit ds DNA BR kit) and record them in the Excel sheet)

  The following day:
- In alternative, if the sample concentration could be low, use the Diapure kit for purification.

1. In a 1.5 ml microcentrifuge tube, add 5 volumes of ChIP DNA Binding buffer to each volume of sample (5:1). Mix briefly.

Example 1: Add 250 µl ChIP DNA Binding buffer to 50 µl cell lysate following DNA shearing, reverse cross-linking and Proteinase K digestion in TES (50 mM Tris-Cl, pH 8.0, 10 mM EDTA, 1% SDS) or 0.1M NaHCO3 containing 1% SDS.

Note: For clean-up of DNA from most enzymatic reactions, add five volumes of ChIP DNA Binding buffer to each volume of sample (i.e., 5:1).

Example 2: Add 600 µl ChIP DNA Binding buffer to 120 µl eluent in TES or 0.1M NaHCO3 containing 1% SDS buffers from chromatin-antibody-Protein A agarose-bead complexes followed by reverse cross-linking and Proteinase K digestion.

2. Transfer mixture to a provided Spin column in a Collection tube.

3. Centrifuge at ≥ 10,000 x g for 30 seconds. Discard the flow-through.

4. Add 200 µl DNA Wash buffer to the column. Centrifuge at ≥ 10,000 x g for 30 seconds. Repeat wash step.

5. Add 6-100 µl DNA Elution buffer directly to the column matrix. Transfer the column to a new 1.5 ml microcentrifuge tube and centrifuge at ≥ 10,000 x g for 30 seconds to elute the DNA.

Ultra-pure DNA is now ready for use for Library Preparation, PCR, arrays, DNA quantification, sequencing and other molecular applications.

- Elute the sample in 20 µl.
- Quantity the samples using the Qubit BR kit.
- Run samples (5-7 ng – dilute if necessary) on the Fragment Analyzer.

3 ChIP Protocol description

STEP 1 - IP

NB: All the buffers mentioned in this protocol are components of the Diagenode ChIPmentation kit for Histones (C01011009).

The Tagmentase used in this protocol is the Tagmentase from Diagenode (C01070012)

The Semi automated system mentioned in this protocol is the Viaflo from Integra

- Dilute 5x ChIP Buffer iC1 in ChIP-seq grade water to obtain 1x ChIP Buffer iC1.
  (ex: for 90 Ips, you need 9 ml 1x ChIP Buffer iC1)

  [1800 µl 5x ChIP Buffer iC1 + 7200 µl ChIP-seq grade water]

  Defreeze BSA and PIC

- Determine the number of IP reactions to be run. Take the required amount of Dia-Mag Protein A-coated magnetic beads and transfer it to clean 1.5 ml tubes. 30 µl of beads are required per IP and a maximum of 600 µl of beads should be transferred to a 1.5 ml tube.
  If you have bigger volume (ex: 30*90= 2700 µl, can be split into 3 tubes with 900 µl each)

- Wash the beads 4 times with 500 µl of ice-cold 1x ChIP Buffer iC1.
  To wash the beads, add 1x ChIP Buffer iC1, resuspend the beads by pipetting up and down several times and place the tubes in the DiaMag1.5 magnetic rack. Wait for 1 minute to allow the beads to be captured by the magnet and remove the supernatant. Repeat this 3 times.

- After the last wash, resuspend the beads in 1x ChIP Buffer iC1 adding the original volume of beads (this means 30 µl per IP, or 900 µl x 3 tubes for 90 IPs).
- Prepare the ChIP reaction mix as described below for 1 IP (**total volume = 200 µl per IP**). Use a 96 wells plate (Eppendorf or 4titude plates, don't use Greiner plates), each sample in a well.

Component	Volume 1 IP	Volume 90 IPs
BSA	4 µl	360 µl
200x PIC	1 µl	90 µl
5x ChIP buffer iC1	40 µl	3600 µl
DiaMag Protein A-coated beads	30 µl	2700 µl
ChIP-seq grade water	24 µl	2160 µl
Sheared diluted chromatin	100 µl	
ChIP-seq grade Antibody	X = Variable volume* (1 µg)	X*90

(*) volume antibody per IP (X)

H3K4me3 à 1 µl

H3K4me1 à 0.7 µl

H3K27me3 à 1 µl

H3K27ac à 0.35 µl

CTCF à 0.44 µl

IgG à 1 µl

- Seal the plate very well and incubate ON @ 4°C in a rotating wheel. You can use a tape in addition to elastics to ensure the plate will be fixed correctly during the incubation.

- Spin briefly the plate and Perform the washes in the Viaflo as following:

  - Add 200 µl of Wash buffer iW1
  - Select the Pipet/mix option in the Viaflo and chose the following parameters:
    Aspirate 10 µl, Aspirate speed 1

    Mix 180 µl

    Mix speed 4

    Cycles 30
  - Magnet for +/- 1 min until the supernatant is clear
  - Discard the supernatant using the Viaflo (Pipet mode: 220 µl; speed 4)
  - Add 200 µl of Wash buffer iW2
  - Select the Pipet/mix option in the Viaflo and chose the following parameters:
    Aspirate 10 µl, Aspirate speed 1

    Mix 180 µl

    Mix speed 4

    Cycles 30
  - Magnet for +/- 1 min until the supernatant is clear
  - Discard the supernatant using the Viaflo (Pipet mode: 220 µl; speed 4)
  - Add 200 µl of Wash buffer iW3
  - Select the Pipet/mix option in the Viaflo and chose the following parameters:
    Aspirate 10 µl, Aspirate speed 1

    Mix 180 µl

Mix speed 4

Cycles 30

- Magnet for +/- 1 min until the supernatant is clear
- Discard the supernatant using the Viaflo (Pipet mode: 220 µl; speed 4)
- Add 200 µl of **wash buffer tagW1**
- Select the Pipet/mix option in the Viaflo and chose the following parameters:

  Aspirate 10 µl, Aspirate speed 1

  Mix 180 µl

  Mix speed 5

  Cycles 30

- Magnet for +/- 1 min until the supernatant is clear
- Discard the supernatant using the Viaflo (Pipet mode: 220 µl; speed 4)

- Add 150 µl of **Wash buffer tagW1** to the beads. Resuspend using the Viaflo

  Aspirate 10 µl, Aspirate speed 1

  Mix 130 µl

  Mix speed 5

  Cycles 30

- Magnet until the supernatant is clear
- During this time, Prepare the ChIPmentation mix as described in the table below for the desired number of reactions. Mix thoroughly with a pipette.

Component	Volume per reaction	Volume for 90 reactions
Tagmentation Buffer	29 µl	2610 µl
Tagmentase	1 µl	90 µl

- STEP 2 – Tagmentation and library prep

- When the supernatant is clear, discard it (using the pipet option in the Viaflo, 200 µl) and add 30 µl of ChIPmentation mix to each tube and gently resuspend the beads by pipetting.
- Incubate for 15 minutes at 37°C in the pre-heated thermocycler.

- Put the samples on ice and immediately add 150 µl of cold Wash Buffer tagW2 to each tube, gently shake the tubes to resuspend the beads and incubate for 5 minutes using the Viaflo:

Aspirate: 10 µL

Mix: 130 µL

Mix speed: 3

Cycles 30

- Briefly spin the tubes and place them in the 96x Magnet, wait until supernatant is clear and discard the supernatant.
- Add 150 µl of cold Wash Buffer tagW1 to each tube, gently shake the tubes to resuspend the beads and incubate for 5 minutes using the Viaflo:

Aspirate: 10 µL

Mix: 130 µL

Mix speed: 5

Cycles 30

- Briefly spin the tubes and place them in the 96x Magnet, wait until supernatant is clear and discard the supernatant.

- Remove the samples from the magnetic rack and add 10.5 µL of Stripping Reagent (= 20 mM EDTA) to the beads and resuspend by pipetting.

- Heat the samples 30 minutes at 50°C using a thermocycler.

- Make a mix with 945 µl MgCl2 and 2250 µl of 2x High-Fidelity Mastermix (it's for 90 IP reactions). Add 35.5 µL of this mix to each IP'ed and input samples and incubate as follows:

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

- Spin briefly and magnetize beads from the immunoprecipitated samples and transfer the supernatant to a new plate. Keep the samples at 4°C (on ice).

Library amplification

- Add 1 µl of the appropriate primer index pair (i5/i7) -already diluted to 10 µM- to each tube from the previous step and mix by pipetting.
- Briefly spin the tubes and run the PCR program (final volume 46 µL) described below.

Cycles	Temperature	Time
1	98°C	30 seconds
12	98°C	10 seconds
	63°C	30 seconds
	72°C	30 seconds
1	72°C	1 minute
1	10°C	∞

- Run 1µL into Fragment Analyzer to check if the library profile is sufficiently amplified. If not, add an appropriate number of cycles with the same PCR program as described above.
- Briefly spin the tubes before the purification.

STEP 3 – Purification (don't use the Viaflo, use 12 multichannel pipet)

- Carefully resuspend the room temperature AMPure XP beads by shaking and light vortexing until no pellet is visible at the bottom of the container.
- Estimate the library volume and add 1.8x volume of AMPure XP beads (for 46 µL, add 83 µL of AMPure XP beads).
- Mix by pipetting until resuspending the beads with the sample
- Incubate at room temperature for 10 minutes.
- Place the tube on the 96x Magnet and wait until the beads are completely bound to the magnet (~3 minutes).
- Carefully aspirate by pipette and discard the supernatant without disturbing the pellet.
- 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.
- Leaving the tube open, let the beads dry on the 96x Magnet for 5 minutes.
- Remove tubes from 96x Magnet and elute DNA by resuspending the beads in 20 µl of Resuspension Buffer.

- Mix by pipetting until resuspending all the beads in the Elution/resuspension buffer.
- Incubate for 10 minutes at room temperature.
- Place the tube on the 96x Magnet and wait until the beads are completely bound to the magnet (~3 minutes).
- Without disturbing the pellet, carefully aspirate and transfer the supernatant containing purified libraries to a new plate.
- Measure the cc° of the libraries and pass 5 ng into the Fragment Analyzer