ChipSeq protocol using Frozen Tissue

Overview:
Both for ATAC and ChIP libraries, the protocols for the tissues were slightly altered from the original AquaFAANG protocols to better serve their implementation in gilthead seabream's tissues. The changes in the ChIPseq protocol concern mostly the process of the tissue disruption and sonication For different tissues, different changes have been made. **All steps were performed on ice**.
All the buffers and solutions were prepared according to the original AquaFAANG instructions.

Tissue disruption: Liver, Brain, Head kidney, Gills, Female Mature Gonad
Steps 1-7 should not last more than 15 min, due to the degradation of the protein epitopes.

1. A suitable amount of each tissue (Table 1**Σφάλμα! Το αρχείο προέλευσης της αναφοράς δεν βρέθηκε.**) was weighed (while still frozen),and placed into an ice-cold dounce-homogenizer containing 4 ml cold PBS with PIC buffer where it was left to thaw completely in the buffer.
    - Gills were grinded in a mortar, which was kept on dry ice, until powder was obtained. The powder was then transferred in the dounce-homogenizer.
    - For mature female gonads the eggs were removed before homogenization. The removal was done manually by washing/mixing the tissue in dH2O and with the help of watchmaker forceps. The tissue was cut in small pieces in a petri dish with 1.2ml HB buffer 1x (on ice)
2. The tissue was homogenized using pestles A and B (loose and tight), until there was no resistance. The number of strokes varied on the tissue type and the persons' strength.
3. The solution was filtered through a 40um cell strainer in a 50 ml eppendorf tube.
    - Mature female gonads were not filtered.
4. The filtrated suspension was transferred in a 15ml eppendorf tube and centrifuged for 5 minutes at 3000rpm, 4^{o}C
5. The supernatant was discarded by quickly flipping the falcon tube (The pellet should be stuck on the tube walls).
    - In case the pellet didn't stick on the wall, the time and/or the speed of the centrifugation were increased. If the pellet was still detached from the tube, the supernatant was removed with a serological pipette.
6. The pellet was quickly flicked until it looked "melted". This process helps to break small tissue and cell lumps.

Tissue disruption: Male Mature Gonad

7. A suitable amount (Table 1**Σφάλμα! Το αρχείο προέλευσης της αναφοράς δεν βρέθηκε.**) was weighed (while still frozen),and placed into an 5ml tube containing 1 ml cold PBS*plus* where it was left to thaw completely in the buffer.
8. The tissue was squeezed with the help of a plastic pestle to release the sperm. Then it was vortexed full speed for 30 seconds.
9. The solution was filtrated through 40um cell strainer placed on the top of 50ml tube.
10. The tissue (on the top of the strainer) was rinsed with 500ul PBS*plus.*
11. The filtrate was discarded and the tissue pieces from the filter were transferred to the initial 5ml tube, where 1 ml of fresh PBS*plus* was added.

12. The steps 8-11 were repeated 4-6 times (to release almost all sperm).
13. The tissue pieces were transferred into a Douncer containing 4 ml cold PBS *plus.*
14. The tissue was homogenized using pestles A and B (loose and tight), until there was no resistance. The number of strokes varied on the tissue type and the persons' strength
15. The homogenized solution was transferred in a 15ml eppendorf tube and centrifuged for 5 min at 3000rpm, 4^{o}C.
16. The supernatant was discarded by quickly flipping the falcon tube (The pellet should be stuck on the tube walls).
    - In case the pellet didn't stick on the wall, the time and/or the speed of the centrifugation were increased. If the pellet was still detached from the tube, the supernatant was removed with a serological pipette.
17. The pellet was quickly flicked until it looked "melted". This process helps to break small tissue and cell lumps.

Crosslinking

18. 2.5ml of Formaldehyde solution 1% was added in the pellet at RT. The solution was incubated at RT under constant rotation for 8min.
19. Formaldehyde was inactivated with the addition of 360 ul Glycine 1M (0.125M final). The solution was then incubated at RT under constant rotation for 7min.
20. The solution was centrifuged for 5 min at 3000rpm, 4^{o}C.
21. The supernatant was discarded by quickly flipping the falcon tube (The pellet should be stuck on the tube walls). The supernatant containing formaldehyde was properly eliminated.
    - In case the pellet didn't stick on the walls, the time and/or the speed of the centrifugation were increased. If the pellet was still detached from the tube, the supernatant was removed with a serological pipette.
22. The pellet was quickly flicked until it looked "melted". This process helps to break small tissue and cell lumps.
23. 3-4ml of PBS with PIC were added in order to wash the pellet.
24. The solution was again centrifuged for 5 min at 3000rpm, 4^{o}C and an additional step of washing with PBS with PIC was done.

Sonication

25. After the last washing step the pellet was re-suspended in complete sonication buffer. The amount of the complete sonication buffer varied, depending on the amount and type of tissue (Table 1).
26. The sonication solution was mixed by pipetting and then split into sonication aliquots in 1.5ml sonication tubes (1.5 ml TPX microtubes for Bioruptor® Plus, max volume: 300ul/tube), or in 15 ml sonication tubes (15 ml TPX tubes for Bioruptor® Plus, max volume: 2 ml/tube).
27. All the tissue samples were sonicated in Diagenode's Bioruptor® Plus device, with the following settings: intensity : HIGH, cycle rhythm (ON/OFF period):30sec/30 sec, total number of cycles: 18
    - The sonication was performed in 3 batches of 6 sonication cycles. Between the batches the ice was changed in the water bath and the samples were re-suspended, because the sonication material tends to settle down in the bottom of the tube.
28. After the sonication, all the aliquots were centrifuged at max speed for 10 min at 4^{o}C.
29. The supernatant (sonicated chromatin) from the aliquots was collected and merged in a single tube (single sample).
30. 20ul from each sample were removed for sonication control
31. The remaining sonicated chromatin was diluted by adding 3 volumes of complete IP buffer, and stored at -80^{o}C (for maximum 2 months).

Test of sonication

32. To the 20ul of sonicated chromatin from step 30, the following were added:
    67ul Elution Buffer
    5ul Proteinase K (20mg/ml)
    2ul RNAse A (100mg/ml)

6ul NaCl 5M

33. The reaction was incubated for 1h30min, at 68°C under agitation (500rpm in thermomixer).
34. After incubation the DNA was purified with a Qiagen DNA MinElute PCR purification kit, and eluted in 20ul Elution buffer.
35. 1ul was used to quantify the DNA concentration by Qubit BR.
36. From the rest of the sonication test, an amount was run in an agarose gel (GelRed staining), or in the bioanalyzer (High sensitivity DNA Chip assay), to assess the size profile.

Table 1: Amount of tissue and complete sonication buffer used for different tissue types.

Tissue type	mg of tissue	ml of complete sonication buffer
Liver, Brain	80-100	3.6
Gills	80-100	1.8
Head kidney	25-35	1.8
Mature ♂Gonad	400 (with sperm)	2.7
Mature ♀Gonad	150-200 (with eggs)	3

Washing of the beads for antibody coupling

37. In a 1.5ml eppendorf tube a mix of beads was prepared:
    45ul of protein A beads /IP reaction
    45ul of protein G beads /IP reaction

Example: If 5 IP reactions, then the mix contains 225ul protein A beads and 225ul protein G beads.

38. The tube containing the beads was placed on a magnetic rack until all beads were separated from the buffer (max 1', depending on the strength of the magnet).
39. The supernatant was removed without the beads being disturbed. The tube was removed from the magnet, in order not to allow the beads to dry.
40. 1ml complete IP buffer was added to the beads. The beads were mixed (washed) by inverting quickly the tube (the tube should remain cold).
41. When the beads solution became homogeneous the tube was placed back on the magnet. When the beads were completely separated, the whole magnet with tube was flipped to collect leftover beads solution that had remained on the lid.
42. As soon as the liquid became transparent (beads have been separated), the supernatant was removed and the tube was removed from the magnet.
43. Two additional washing steps with complete IP buffer were done.
44. After the last washing step complete IP buffer was added: 130 ul/IP reaction and the solution was mixed by pipetting.

Example: If 5 IP reactions, then add 650ul complete IP buffer.

45. The volume of beads was split in 1.5ml eppendorf tubes (1 tube for every different antibody used). Every tube contained 130ul beads solution for every reaction, and it was labeled according to the antibody it contained.

Example: If 5 IP reactions with 5 different antibodies, then the beads are split in 5 different tubes with every tube containing 130ul beads solution. Every tube is also labeled with the corresponding antibody which will be added.

Example: If 5 IP reactions with 2 different antibodies, then the beads are split in 2 different tubes. One tube will contain 390 ul beads solution (3 IP reactions), and the other will contain 160 ul beads solution (2 IP reactions).The tubes are labeled with the antibody they will contain.

46. Antibodies were added to each tube containing beads solution:
    - H3K27ac: 0.9ul/ IP reaction

- H3K4me3: 0.9ul/ IP reaction
- H3K27me3: 2ul/ IP reaction
- H3K4me1: 0.8ul/ IP reaction
- CTCF: 0.6ul/ IP reaction

Example: If 5 IP reactions; two with the H3K27ac antibody and three with the CTCF antibody, then we should have one tube labeled "H3K27ac" and containing 260ul washed beads with 1.8 ul H3K27ac antibody (2*0.9ul), and a second tube labeled "CTCF" and containing 390ul washed beads with 1.8 ul CTCF antibody (3*0.6ul).

47. The tubes containing the beads with the antibodies were rotated at 4^{o}C, overnight, in order to get the antibodies coupled to the beads.

Washing of the beads for the pre-clearing of the chromatin

48. In a 1.5ml eppendorf tube a mix of beads was prepared:
30ul of protein A beads /IP reaction
30ul of protein G beads /IP reaction
Example: If 5 IP reactions , then the mix contains 150ul protein A beads and 150ul protein G beads.

46. Three washings with 1 ml complete IP buffer were done, as in the Washing of the beads for antibody coupling section.
47. After the last washing step 50ul complete IP buffer/IP reaction were added, and the beads were mixed by pipetting.

Example: If 5 IP reactions, 250ul complete IP is added to the beads.

48. The sonicated chromatin was taken from -80oC and left to thaw on ice (the chromatin had been diluted after sonication).
49. A volume that contains 6 ug of chromatin/ IP reaction is removed and transferred in a new tube. The volume is determined by the concentration that has been calculated during the test of sonication, and by the fact that the chromatin was diluted before storage in -80^{o}C.

Example: If 5 IP reactions , 30ug of chromatin were removed.

50. The chromatin and the washed beads were joined in one tube, which is rotated for 1h in RT, in order for the chromatin to be pre cleared.

Immunoprecipitation

51. While the chromatin was rotating, the tubes that contained the beads with the antibodies coupled (from step 47) were taken and washed 3 times with 1 ml complete IP buffer. The washing process was the same as the previous washing of the beads.
52. After the last wash complete IP buffer was added in every tube: 50ul IP buffer/ IP reaction and mixed by pipetting.

Example: If a tube contains an antibody for 3 reaction , ul complete IP buffer were added.

53. Tubes are labeled, so each tube will contain one IP reaction.

54. When the chromatin had been pre-cleared (from step 50), it was placed on the magnet until all the beads were attached to the tube wall. The supernatant was taken (containing pre-cleared chromatin) and split to the tubes that will contain the IP reaction (6ug chromatin in each tube, labeled in step 53).
55. Finally, to each of the tubes containing the chromatin, 50 ul of beads with coupled antibody were added, containing the corresponding antibody.

56. The tubes containing the beads with the coupled antibodies and the chromatin were rotated at 4°C, overnight for the chromatin to be immunoprecipitated.

Wash of the beads and chromatin recovery

The process of Wash of the beads and chromatin recovery was performed according to the original AquaFAANG protocol with 2 alterations.
One is that, during the step of chromatin elution the tubes were rotated in a 360° rotator, instead of vortexing in a thermomixer.
The second alteration was that the input control was not taken and decrosslinked from the stored chromatin in -80°C, but taken immediately from the test of sonication (step 34).

One-step decrosslinking

The process of One-step decrosslinking was performed according to the original AquaFAANG protocol with 2 alterations.
During MinElute PCR purification the DNA was eluted in 11 ul of low TE buffer (instead of 32ul), and only 1 ul was used for measuring the concentration in Qubit HS kit (instead of 2-6ul).

Library preparation

For this process Microplex v3 (Diagenode) kit was used.
In step 3 of the kit's protocol 18 cycles were used during library amplification step for all samples. Also as an input control sample, 100ng of DNA were used from the test of sonication (step 34) as template. For all the input Library amplification we used 12 cycles.
For Library Purification and size selection, NucleoMag kit for clean-up and size selection of NGS library prep reactions was used. We followed the "Protocol for DNA clean up and single size selection". For removing the small fragments the ratio (ratio = volume beads/volume sample) was 0.9. The final library was eluted in 22 ul low TE buffer.
The libraries' concentration was measured with Qubit Broad Range Kit. 4-5 ul of the libraries were run in an agarose gel (GelRed staining), or (in cases of lower concentrations) in the bioanalyzer (High sensitivity DNA Chip assay), to assess the size profile.
In case of an excess of long fragments in the samples (<250 bp), an additional round of beads size selection was done.