GENE-SWitCH

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

Protocol WP1
Purification of Genomic DNA and Total RNA, including small RNAs, from chicken Tissues

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Workpackage: WP1

Version: 1.0

Protocol associated with Deliverable(s):	D1.2 & D1.3
Submission date to FAANG:	11/11/2020, month M17

Research and Innovation Action, SFS-30-2018-2019-2020 Agri-Aqua Labs
Duration of the project: 01 July 2019 – 30 June 2023, 48 months

This project has received funding from the European Union's Horizon 2020 research and innovation programme under grant agreement No 817998

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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.

This protocol was used only for chicken sample preparation.

To perform the purification of total RNA from the 7 tissues of interest, we followed the protocol according to the AllPrep® DNA/RNA/miRNA Universal Handbook from Qiagen. There is no difference in the protocols according to the developmental stage of the animals, however, in the case of kidney tissue, some optimizations were necessary to extract the DNA and RNA. Each exception is written with a blue colour.

2 Protocol description

2.1 Required Reagents and Instruments

- AllPrep® DNA/RNA/miRNA Universal kit (80224, Qiagen)
- 96-100% Ethanol
- Isopropanol
- β-Mercaptoethanol (M-6250)
- Reagent DX (19088, Qiagen)
- 2 ml safe-lock Eppendorf (0030120.094, Eppendorf)
- Sterile, RNase-free tips and P2-P1000 pipette
- Bench centrifuge (Eppendorf)
- Mini spinner (Starlab)
- Fume Hood
- Sterile disposable Petri dishes
- Sterile disposable scalpels

- Weighing scales
- PPE
- Wet ice
- Dry ice
- Precellys lysing kit (P000918-LYSKO-A)
- Homogenizer Precellys 24 (Pequlab, Bertin technologies)
- Nanodrop spectrophotometer (Thermofischer)
- Qubit 4 Fluorometer (Thermofisher)
- DNA BR Qubit kit (Q32853, Thermofisher)
- RNA BR Qubit kit (Q10210, Thermofisher)
- Qubit tubes
- Tapestation 4200 Agilent
- Screen tapes and reagents for genomic DNA QC (5067-5365, 5067-5366, Agilent)
- Screen tapes and reagents for RNA QC (5067-5576, 5067-5577, Agilent)
- TapeStation 4200 Loading Tips 112pk (5067-5599, Agilent)

2.2 Preparatory Step

Before starting the extraction of DNA and RNA only sample stages E15 and hatched were weighed (12 -38 mg, with exception for kidneys: 2 – 9 mg). Four individuals of stage E8 for each tissue were pooled but not weighed. Each piece of tissue or pool was then transferred to chilled Precellys tubes and placed on dry ice.

To perform tissue homogenization, we used the Precellys 24 homogenizer. Before that, the RLT Plus lysis buffer was prepared according to the manufacturer instructions. For required volume of buffer the final concentration of β -Mercaptoethanol was 1% and reagent DX was 0.5%. To each tube containing the samples,600 µL of lysis buffer was added and for kidney samples 350 ul was added. The homogenization was performed at 1 cycle, 5000 rpm for 20 seconds. Then tubes were placed on wet ice for two minute and then centrifuged to reduce foam.

2.3 Purification of total and miRNA (Allprep kit)

1. The homogenized supernatants were transferred to AllPrep DNA Mini spin columns placed in 2 ml collection tubes. The samples were centrifuged for 30 s at 13000 rpm.
2. The AllPrep DNA Mini spin columns were put into new 2 ml collection tubes and stored at 4°C for DNA purification later.
3. The flow-through of each sample from step 1 was transferred into new 2 ml microcentrifuge tubes for further RNA purification.
4. 80 µl or ● 50 µL Proteinase K was added and mixed by pipetting to the samples from step 3 followed by 350 µl or ● 200 µL of 96–100% ethanol and mixed well.
5. The samples were incubated for 10 min at room temperature.
6. 750 µl or ● 500 µL of 96–100% ethanol was added and mixed well.

7. 700 µl of each sample, including any precipitate that may have formed, was added to an RNeasy Mini spin column placed in a 2 ml collection tube. The columns were centrifuged for 15 s at full speed. The flow-through was discarded.
8. Step 7 was repeated until the entire sample had passed through the RNeasy Mini spin column.
9. 500 µl of buffer RPE was added to the RNeasy Mini spin column and centrifuged for 15 s at full speed. The flow-through was discarded.
10. DNase treatment was performed. For each sample, a mix of 10ul of DNase I and 70 µl of buffer RDD was placed directly onto the RNeasy Mini spin column membrane and incubated for 15 minutes at room temperature.
11. 500 µl of buffer FRN was added to the spin column and centrifuged for 15 s at full speed.
12. The flow-through from step 11 was applied on the same RNeasy Mini spin columns in new 2 ml collection tubes and centrifuged for 15 s at full speed. The new flow-through was discarded.
13. 500 µl of buffer RPE was added to each RNeasy Mini spin column and centrifuged for 15 s at full speed. The flow-through was discarded.
14. 500 µl of 96–100% ethanol was added to each RNeasy Mini spin column and centrifuged for 2 min at full speed to wash the spin column membrane.
15. RNeasy Mini spin columns were placed into new 2 ml collection tubes and centrifuged at full speed for 2 min. This step was performed to eliminate any possible carryover of ethanol and to collect the residual flow-through that remains on the outside of the RNeasy Mini spin column after step 14.
16. RNeasy Mini spin columns were transferred into 1.5 ml collection tubes. 40 ul of RNase-free water was applied directly to the spin column membrane and centrifuged for 1 min at ≥8000 x g (≥10,000 rpm) to elute the RNA.
17. Eluted RNA from step 16 was applied again to the spin column membrane and centrifuged for 1 min at ≥8000 x g (≥10,000 rpm).
18. Total RNA including smallRNA was then quantify using Nanodrop, Qubit and tapestation. See section 2.5

2.4 Purification of Genomic DNA (Allprep Kit)

19. 350 µl of buffer AW1 was added to each AllPrep DNA Mini spin column from step 2 and centrifuged for 30 s at full speed to wash the spin column membrane. The flow-through was discarded.
20. A mix of 20 µl Proteinase K and 60 µl buffer AW1 was applied to the AllPrep DNA Mini spin column membrane and incubated for 5 min at room temperature.
21. 350 µl of buffer AW1 was added to the AllPrep DNA Mini spin column and centrifuged for 15 s at full speed to wash the spin column membrane. The flow-through was discarded.
22. 500 µl of buffer AW2 was added to the AllPrep DNA Mini spin column and centrifuged for 2 min at full speed to wash the spin column membrane. The flow-through was discarded.

23. Columns were then transferred to new collection tubes and centrifuged again for 1 min at full speed. This step allowed elimination of any possible carryover of ethanol and collection of the residual flow-through that remained on the column.
24. AllPrep DNA Mini spin columns were placed into 1.5 ml collection tubes and 50 ul (E8 and hatched) or 40ul (E15) of EB buffer were added directly to the spin column membrane and incubated at room temperature for 1 min and then centrifuged for 1 min at ≥8000 x g (10,000 rpm) to elute the DNA.
25. Step 24 was repeated using another 50µl or 40ul of buffer EB respectively directly to the spin column membrane.
26. QC of the genomic DNA was performed using Qubit and tapestation. See section 2.5.

2.5 Storage, Quantification, and Determination of Quality of DNA and RNA

The DNAs are stored at -24°C and RNAs are stored at - 80°C.

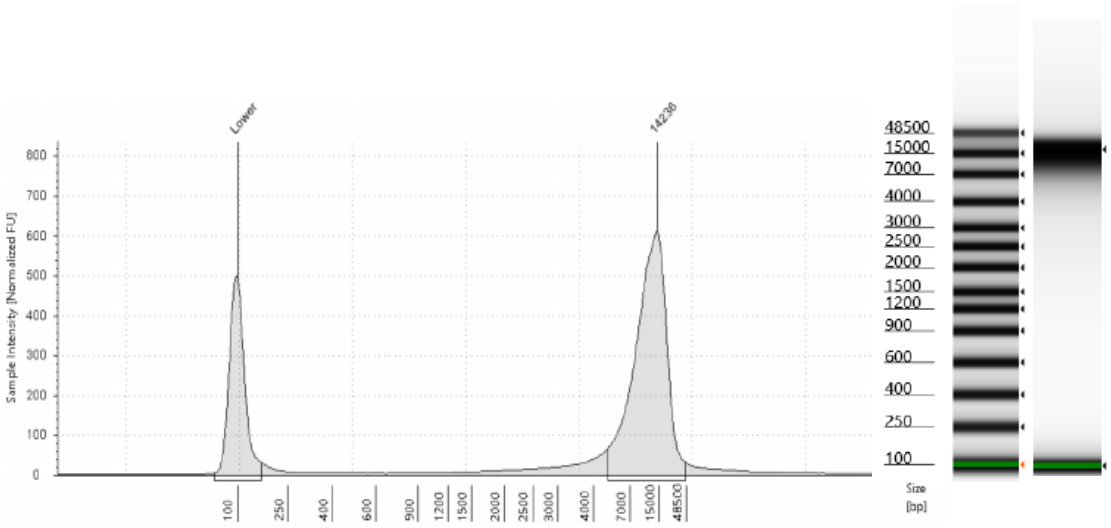
The DNA and RNA concentrations were performed using a Qubit 4 Fluorometer. The samples were prepared based on manufacturer protocol. For accuracy, all samples were measured in duplicate and the average of the two measurements was used for further application.

Additionally, for all RNA samples 260/280 and 260/230 ratios were measured on the Nanodrop.

To determine the quality of the DNAs and RNAs, a tapstation 4200 was used.

For DNA QC, genomic DNA screen tapes were applied. The DIN number was >7 across all the samples with few exceptions were the DIN number was >6.

Typical profile for genomic DNA on a tapestation:



For RNA QC, RNA screen tapes were used. The samples were prepared according to manufacturer protocol. The RIN scores across all samples were >9 with only one exception when the RIN was > 6.

Typical profile for RNA on a tapestation: