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 pig Tissues

Authors: Hervé Acloque and Mayrone Mongellaz (INRAE)

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

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.

This protocol was used only for pig sample preparation.

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

Genomic DNAs with low 260/230 ratios have been purified a second time with the QIAamp DNA Mini Kit (250) (ref: 51306). We have partially modified the protocol "QIAamp® DNA Mini and Blood Mini Handbook" (cf : step 2.6 Second Purification DNA QIAamp).

2 Protocol description

2.1 Required Reagents and Instruments

- AllPrep® DNA/RNA/miRNA Universal kit (Qiagen ref 80224)
- QIAamp DNA Mini Kit (250) (Qiaen ref: 51306)
- RNA 6000 Nano Kit (Agilent ref: 5067-1511)
- Standard Sensitivity Genomic DNA Analysis Kit - 50Kb (Agilent ref: DNF-467-0500)
- 2 ml Tube and 2 ml centrifuge tubes
- Pipets and tips
- Fragment Analyzer (Agilent)
- BioAnalyzeur (Agilent)

- Nanodrop spectrophotometer (thermofischer)
- Thermoblock
- Tissuelyser II (Qiagen)
- Stainless steel beads with a diameter of 5 mm (Qiagen ref:69989)
- β-Mercaptoethanol
- Ethanol
- Reagent DX (reduce foam) (Qiagen ref:19088)
- Chlorophorm
- Cryotable (or box of dry ice)
- Cryoprotection gloves
- Sterile disposable Petri dishes (100 mm and 60mm)
- Disposable scalpels
- Sterile clamps with smooth ends, 10cm long
- Racks for 2 mL tubes
- A permanent marker to label the zip lock bags
- Paper towels
- Waste bag
- A cleaning spray against RNAse
- Weighting scales

2.2 Preparatory Step

Before starting the purification of genomic DNA and total RNA we we need to weight the adequate quantity of tissue and to perform tissue homogenization. Working on the cryotable at - 25°C (or above a box of dry ice), for each sample, we excise 20-30 mg of tissue, except for muscle where we cut pieces of 45-55 mg. Each piece of tissue is then placed into a centrifuge tube and stored at - 80°C before performing the homogenization step. Between each tissue, we took care to clean the forceps and scalpel with ethanol and to tare the pre-labelled centrifuge tube.

To perform tissue homogenization, we used the Tissuelyser II. , we added to each centrifuge tube containing the samples 600 µL of lysis buffer composed of 591µl of "RLT plus" buffer + 6 µL β - Mercaptoethanol + 3 µL reagent DX. For the muscle, we used 800 ul of lysis buffer composed of 788 RLT plus buffer + 8 µL β –Me + 4 µL of reagent DX. We then added a stainless steel beads (5 mm) into each tube and placed the centrifuge tubes in the adapters set (2 x 24) of the Tissuelyser and run 1 cycle of 2 min at 25 Hz. We repeated the run when we observed pieces of tissues (generally, for the back skin and muscle of **New Born piglet** we proceed to another cycle).

2.3 Purification of Total and miRNA (Allprep kit)

1. After the lysis, we centrifuged 1 min at full speed (13000rpm) the centrifuge tubes containing the homogenate.
2. We transferred only the homogenized supernatant to an AllPrep DNA Mini spin column placed in a 2 ml collection tube. We closed the lid gently and centrifuged for 30 s at full speed (13000rpm, maximum speed of 20,000 x g). *Note: Make sure that no liquid remains on the column membrane after centrifugation. If necessary, repeat the centrifugation until all liquid has passed through the membrane*.

3. We placed the AllPrep DNA Mini spin column in a new 2 ml collection tube and store at room temperature or at 4°C for DNA purification later. We transferred the flow-through into a new 2 ml microcentrifuge tube for RNA purification. *Note: Do not store the DNA-containing AllPrep DNA Mini spin column at room temperature or at 4ºC for long periods. Do not freeze the column. Storage, Quantification, and Determination of Quality of RNA and DNA.*

Only for Cerebellum, we added 150µl chloroform to the flow-through from step 3 and vortex thoroughly. We centrifuged at 4°C for 3 min at full speed (maximum speed of 20,000 x g) to separate the phases. We carefully transferred the aqueous phase into a new 2 ml microcentrifuge tube.

Only for Muscle, we transferred 400µL of the flow-through from step 3 into a second collection tube (2 collection tube for each sample) and followed the step write ● in blue. This step avoids overflowing the collection tube at the end of step number 7.

4. We added 80µl or ● 54µL (in each tube collection) Proteinase K to the flow-through from step 3 or the aqueous phase (Cerebellum) and we mixed by pipetting.
5. We added 350µl or ● 234µL of 96–100% ethanol and we mixed well. Do not centrifuge.
6. We incubated the tubes for 10 min at room temperature.
7. We added 750µl ● 500µL of 96–100% ethanol and mixed well. Do not centrifuge.
8. We transferred up to 700µl of the sample, including any precipitate that may have formed, to an RNeasy Mini spin column placed in a 2 ml collection tube. We closed the lid gently and centrifuged for 15 s at full speed (maximum speed of 20,000 x g). We discarded the flow-through.
9. We repeated step 8 until the entire sample has passed through the RNeasy Mini spin column.
10. We added 500µl Buffer RPE to the RNeasy Mini spin column. We closed the lid gently and centrifuged for 15 s at full speed (maximum speed of 20,000 x g). We discarded the flow-through and reused the collection tube.
11. We added 10µl DNase I stock solution to 70µl Buffer RDD. We mixed by gently inverting the tube and centrifuged briefly to collect residual liquid from the sides of the tube.
12. We added the DNase I incubation mix (80µl) directly onto the RNeasy Mini spin column membrane and placed on the benchtop (20–30°C) for 15 min.
13. We added 500µl Buffer FRN to the RNeasy Mini spin column. We closed the lid gently and centrifuged for 15 s at full speed (maximum speed of 20,000 x g). We saved the flow-through for use in step 14. **IMPORTANT:** Do not discard the flow-through, as it contains small RNAs.
14. We placed the RNeasy Mini spin column in a new 2 ml collection tube. We applied the flow-through from step 13 to the spin column. We closed the lid gently and centrifuged for 15 s at full speed (maximum speed of 20,000 x g). We discarded the flow-through and reused the collection tube.
15. We added 500µl Buffer RPE to the RNeasy Mini spin column. We closed the lid gently and centrifuge for 15 s at full speed (maximum speed of 20,000 x g). We discarded the flow-through and reused the collection tube.

16. We added 500µl of 96–100% ethanol to the RNeasy Mini spin column. We closed the lid gently and centrifuged for 2 min at full speed (maximum speed of 20,000 x g) to wash the spin column membrane.
17. We placed the RNeasy Mini spin column into a new 2 ml collection tube and discarded the old collection tube with the flow-through. We centrifuged at full speed for 2 min. We performed this step to eliminate any possible carryover of ethanol or to collect the residual flow-through that remains on the outside of the RNeasy Mini spin column after step 16.
18. We placed the RNeasy Mini spin column in a new 1.5 ml collection tube. We added 50µl RNase-free water directly to the spin column membrane. We closed the lid gently and centrifuged for 1 min at ≥8000 x g (≥10,000 rpm) to elute the RNA.
19. We repeated step 18, using another 50µl RNase-free water directly to the spin column membrane. We then proceed to the purification of genomic DNA.

2.4 Purification of Genomic DNA (Allprep Kit)

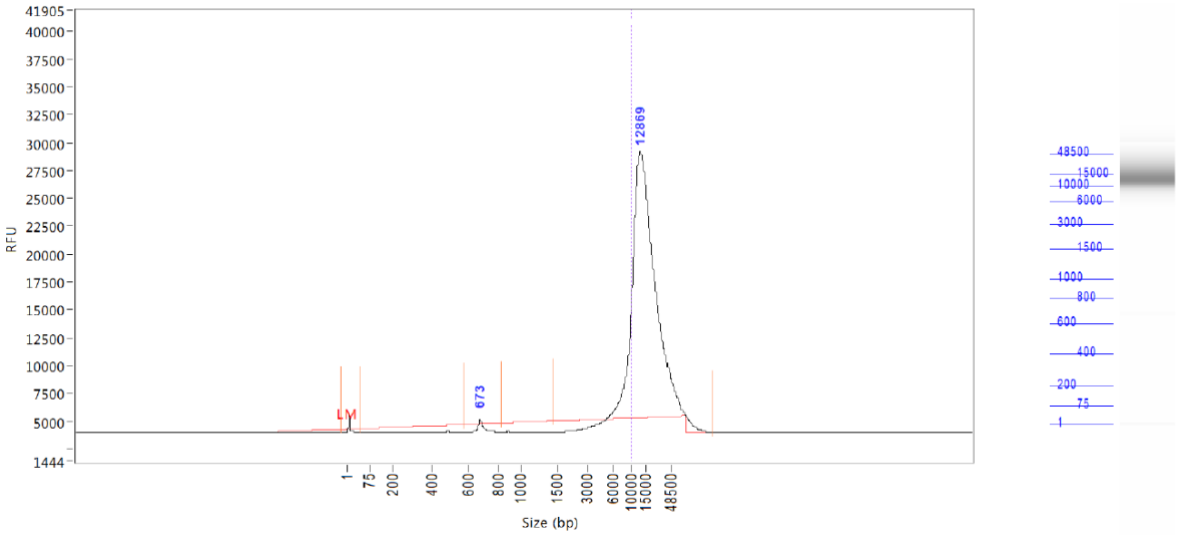
20. We added 350µl Buffer AW1 to the AllPrep DNA Mini spin column from step 3. We closed the lid gently and centrifuged for 30 s at full speed (maximum speed of 20,000 x g) to wash the spin column membrane. We discarded the flow-through. We reused the collection tube. *Note: Make sure that no liquid remains on the column membrane after centrifugation. If necessary, repeat the centrifugation until all liquid has passed through the membrane*.
21. We added 20µl Proteinase K to 60µl Buffer AW1, we mixed gently, and applied the mixture to the AllPrep DNA Mini spin column membrane.
22. We incubated for 5 min at room temperature.
23. We added 350µl Buffer AW1 to the AllPrep DNA Mini spin column. We closed the lid gently and centrifuged for 15 s at full speed (maximum speed of 20,000 x g) to wash the spin column membrane. We discarded the flow-through and reused the collection tube.
24. We added 500µl Buffer AW2 to the AllPrep DNA Mini spin column. We closed the lid gently and centrifuged for 2 min at full speed to wash the spin column membrane. We discarded the flow-through and reused the collection tube.
25. We centrifuged 1 min at full speed. We performed this step to eliminate any possible carryover of ethanol or to collect the residual flow-through that remains on the column.
26. We placed the AllPrep DNA Mini spin column into a new 1.5 ml collection tube. We added 50 µl Buffer EB directly to the spin column membrane and we closed the lid. We incubated at room temperature (15–25°C) for 1 min and then centrifuged for 1 min at ≥8000 x g (10,000 rpm) to elute the DNA.
27. We repeated step 26, using another 50µl Buffer EB directly to the spin column membrane.

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

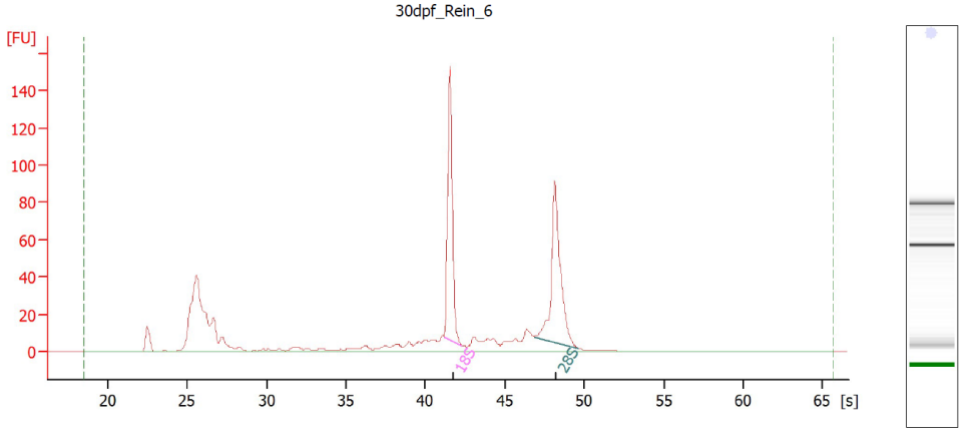
The DNAs and RNAs are stored at - 80°C. Quantification is performed with a Nanodrop to determine the concentration of each purification and the 260/230, 260/280 ratios. The DNAs with low 260/230

ratio (lower than 1.8) has been purified a second time with the QIAamp DNA Mini Kit (ref: 51306). We have partially modified the protocol "QIAamp® DNA Mini and Blood Mini Handbook" (cf : 2.6 Second purification DNA QIAamp).
To determine the quality of the DNAs we used a Fragment Analyser and "Standard Sensitivity Genomic DNA Analysis Kit - 50Kb" (ref: DNF-467-0500) while for the RNAs we used a BioAnalyser and performed RNA chips from the "RNA 6000 Nano Kit" (ref: 5067-1511).

Typical profile for genomic DNA on a Fragment Analyzer:



Typical profile for total RNA on a Bioanalyzer:



2.6 Second Purification DNA (QIAamp)

This protocol was carried out only for DNAs with low 260/230 ratios (lower than 1.8). This protocol follows the QIAamp® DNA Mini Kit handbook however, some steps have been modified to fit our experiments.

1. We pipetted 100µL of your DNA purification.
2. We added 80µl of buffer ATL.
3. We added 20µL of proteinase K and mixed by vortexing and incubated 20 min at 56°C.

4. We added 200µL of buffer AL and mixed thoroughly by vortexing for 15s.
5. We added 200µL ethanol (96-100%), vortex for 15s and briefly centrifuged the tube to remove drops from the lid.
6. We carefully applied the mixture from step 5 (including the precipitate) to the QIAamp Mini spin column. We centrifuged at 6000 x g (8000 rpm) for 1 min and discarded the tube containing the filtrate.
7. We added 500µl Buffer AW1 and centrifuged at 6000 x g (8000 rpm) for 1 min. We discarded the collection tube containing the filtrate.
8. We added 500µl Buffer AW2 and centrifuged at full speed (20,000 x g; 14,000 rpm) for 3 min.
9. We placed the QIAamp Mini spin column into a new 2 ml collection tube and discarded the old collection tube with the filtrate. We centrifuged at full speed for 1 min. This step helps to eliminate the chance of possible Buffer AW2 carryover.
10. We placed the QIAamp Mini spin column into a clean 1.5 ml microcentrifuge tube, and discarded the collection tube containing the filtrate. We added 50µl Buffer AE and incubated at room temperature for 1 min, and then centrifuged at 6000 x g (8000 rpm) for 1 min. We repeated this step.