GENE-SWitCH

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

Protocol WP1
Teloprime Full-length cDNA amplification

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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 to be analysed within the GENE-SWitCH project are:

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

The three developmental stages to be examined 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) were sampled and total RNAs extracted.

To perform full-length cDNA preparation from the total RNA from the seven tissues of interest, we followed the protocol according to the Teloprime full-length cDNA amplification kit (v2) from Lexogen. Equal amounts of RNA from samples from each sex were combined for each tissue and species across all developmental stages, and all 42 samples (21 chicken and 21 pig) quantified using a Qubit fluorometer. 2 ug of each pooled RNA was used as starting material to produce the libraries. There is no difference in the protocols according to the developmental stage or sex or species of the animals, however three of the pig samples (lung and skin at 30dpf and muscle at 70 dpf) did not meet the required concentration of 2ug in 12 ul despite concentrating the samples using a speed vacuum. The final amounts of these three pooled RNA samples used for cDNA preparation were 1.55 ug, 1.36 ug and 1.34 ug respectively.

2. Protocol description

The TeloPrime Full-Length cDNA Amplification Kit V2 is a protocol for generating full-length cDNA from total RNA. It is based on Lexogen´s unique Cap-Dependent Linker Ligation (CDLL) and long reverse transcription (long RT) technology, and is highly selective for full-length RNA molecules that are both capped and polyadenylated. The cDNA is fully compatible with downstream Iso-Seq™ library preparation and long-read sequencing on Pacific Biosciences Instruments.

2.1 Required Reagents and Instruments

- TeloPrime Full-Length cDNA Amplification Kit V2 (013, Lexogen)
- AMPure PB Beads (100-265-900, PacBio)
- Dynamag rack (12321D, Invitrogen)
- SYBR Green I 10,000x in DMSO (S9430, Sigma-Aldrich,)
- DMSO (Sigma-Aldrich)
- Thermomixer (Starlab)

- Thermocycler MJ3
- qPCR machine (Light Cycler 480, Roche)
- 96-100% Ethanol
- 80% Ethanol
- 2 ml safe-lock Eppendorf tubes (cat. No 0030120.094)
- PCR tubes and lids
- 96-well qPCR plates (Thermo scientific)
- Sterile, RNase-free tips and P2-P1000 pipette
- Benchtop centrifuge (3,000 x g, rotor compatible with 96-well plates)
- Benchtop centrifuge (12,000 x g, rotor compatible with 1.5 ml and 2.0 ml micro-tubes)
- Mini spinner for PCR tubes (Starlab)
- Nanodrop spectrophotometer (Thermofisher)
- Qubit 4 Fluorometer (Thermofisher)
- DNA BR Qubit kit (Q32853, Thermofisher)
- RNA BR Qubit kit (Q10210, Thermofisher)
- Qubit tubes (Agilent)
- Tapestation 4200 Agilent
- Screen tapes and reagents for genomic DNA (5067-5365, 5067-5366, Agilent)
- TapeStation 4200 Loading Tips 112pk (5067-5599, Agilent)

2.2 TeloPrime cDNA preparation

2.2.1 First Strand cDNA Synthesis – Reverse Transcription

1. 1.34 – 2 µg pooled total RNAs were transferred to PCR tubes and topped up with RNA Buffer to the required 12 ul volume and mixed well by pipetting.
2. 2 µl of Reverse Transcription Primer (RTP) was added to each tube and mixed well by pipetting and then kept at room temperature.
3. A mastermix containing 4ul of Reverse Transcription mix (RT) and 1ul of enzyme E1/samples was prepared and mixed well by pipetting and then kept at room temperature.
4. Samples from step 2 were denatured for 30 seconds at 70°C in a thermocycler, then cooled down to 37°C, and incubated for 1 minute. 6 µl of mastermix from step 3 was added to each reaction, mixed by pipetting and then incubated for a further 2 minutes at 37°C, followed by 50 minutes at 46°C and then holding at 10°C. Samples were centrifuged before proceeding to the next step.
5. 160 ul of Column biding Buffer (CB1) was added to samples from step 4, mixed by pipetting and transferred to columns placed in 2 ml collection tubes. These were then centrifuged for 1 minute at 12000 x g at room temperature. The flow-through was discarded.
6. 200ul of Column Wash Buffer (CW) was added to each column and centrifuged for 1 minute at 12000 x g at room temperature. The flow-through was discarded and step 6 was repeated.
7. Columns from step 6 were centrifuged for 2 minutes at 12000 x g at room temperature and transferred to 1.5ml Eppendorf tubes.

8. 19ul of RNA buffer was added to each column and incubated for 1 minute at room temperature and then centrifuged for two minutes at 12000 x g.

9. 18ul of eluted samples were transferred to new PCR tubes.

2.2.2 Double-Strand Specific Ligation

During this step, using a double-strand specific ligase, the adapter is attach to the cDNA in the hybrid by base-pairing of the 5' C to the cap structure of the RNA.

10. Mastermix containing 20 ul of Ligation mix (LM) and 2 ul of Enzyme E2 mix was added to each sample from step 9, mixed well and incubated in a thermocycler for 3 hours at 25 °C.

11. Samples were transferred to 1.5 ml tubes with 320 ul of CB1 buffer, mixed well by pipetting and transferred to a column placed in 2 ml collection tubes. This was followed by centrifugation for 1 minute at 12,000 x g at room temperature. The flow-through was discarded.

12. 400ul of CW buffer was added to the column, centrifuged for 1 minute at 12,000 x g and flow-through discarded. Step 12 was repeated and then columns were centrifuged again for 2 minutes at 12,000 x g.

13. Columns were transferred to new 1.5 ml tubes and 13 ul of RNA buffer was added and incubated for one minute at room temperature then centrifuged for two minutes at 12,000 x g

14. 11 ul of eluted samples were transferred to new PCR tubes.

2.2.3 Second strand synthesis

The full-length cDNA is converted to ds cDNA using the PCR Forward Primer (FP), which is included in the Second Strand Mix (SS).

15. A combination of 8 ul of second strand mix (SS) and 1 ul of Enzyme E3 was added to each sample from step 14 and 1 cycle of thermocycling was conducted with the following program: 90 seconds at 95.8 °C, 60 seconds at 62 °C, 5 minutes at 72 °C, then hold at 4 °C.

16. 160 ul of Column biding buffer (CB2) was added to samples from step 15 and applied onto columns placed in 2 ml collection tubes and then centrifuged from 1 minute at 12.000 x g at room temperature. Flow-through was discarded.

17. 200 ul of CW buffer was applied onto the columns and these were centrifuged for 1 minute at 12.000 x g at room temperature. Flow-through was discarded and Step 17 was repeated. The flow-through was removed and columns centrifuged again for 2 minutes at 12.000 x g.

18. Columns were transferred to new 1.5 ml tubes and 21 ul of DNA buffer was applied, incubated for 1 minute at room temperature and centrifuged for 2 minutes at 12.000 x g

19. 20 ul of eluted cDNA was transferred to new PCR tubes.

2.2.4 qPCR

An aliquot of the full-length ds cDNA from each sample from step 19 was used for a qPCR assay to determine the Optimal Endpoint PCR (OEP) cycle number for full-length cDNA

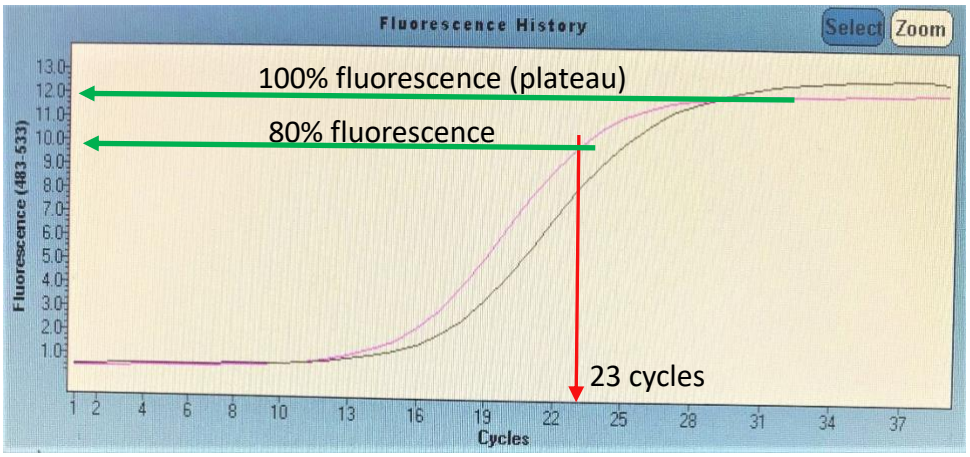
amplification. The assay was performed on a Light Cycler 480 SW 1.5 machine using white qPCR 96 well plates.

20. 16 ul of mastermix was prepared, containing 8 µl of TeloPrime PCR Mix (Telo PCR), 1 µl of PCR Forward Primer (FP), 1 µl of PCR Reverse Primer (RP), 1 µl of Enzyme Mix (E3), 1 µl of a 2xSYBR Green I dilution [(diluted 10,000x stock 1:5,000 in DMSO). The final concentration of SYBR Green I in the 20 µl PCR reaction must be 0.1x)] and 3 ul of DNA buffer was added to 4 ul of the samples from step 19 and mixed well and sealed with adhesive foil. The plate was centrifuged for 2 minutes at 3000 rpm.

21. Using a real-time PCR machine, 40 cycles were performed with the following program: one cycle of 95.8°C for 30 seconds, 50°C for 45 seconds, 72°C for 20 minutes; then 39 cycles of 95.8°C for 30 seconds, 62 °C for 30 seconds, 72°C for 20 minutes, and a final extension at 72 °C for 20 minutes and hold at 10°C.

22. The end-point PCR cycle number was determined at 80% of the maximum fluorescence value (plateau) on the qPCR amplification curve of each sample.

    Example of qPCR amplification curve and endpoint PCR calculation



    In theory, the endpoint PCR should be performed at 23 cycles for the 'pink' sample. However, the number seems to be high, so in practice every samplewith a value < 20 in the qPCR was amplified for 20 cycles.

2.2.5 Amplification of full-length cDNA

The endpoint PCR was performed to generate enough material for downstream long-read sequencing.

23. 16 ul of each sample from step 20 was divided into 4 equal volumes and end-point PCR was performed in parallel. A mastermix containing 8 µl of TeloPrime PCR Mix, 1 µl of the PCR Forward Primer, 1 µl of the PCR Reverse Primer, and 1 µl of Enzyme Mix E3 and 5 ul of DNA buffer was added to 4 ul of each sample. PCRs were conducted in a thermocycler with the following program: 1 cycle of 95.8 °C for 30 seconds, 50 °C for 45 seconds, 72 °C for 20 minutes, then XX number of cycles of 95.8 °C for 30 seconds, 62 °C for 30 seconds, 72 °C for 20 minutes, and a final extension at 72 °C for 20 minutes, hold at 10 °C.

2.2.6 Purification using PacBio AMPure PB Beads

The amplified transcripts were purified from PCR components that can interfere with quantification and downstream application. Before purification the beads were calibrated to room temperature and 80% EtOH freshly prepared.

24. Technical replicates from step 23 were combined, mixed and topped up with water to 200 ul. Then the samples were split in two volumes: 150 ul and 50 ul. 0.4x volume of AMPure beads was added to the 150 ul and 0.6x volume of beads were added to the 50 ul aliquots. The samples were mixed thoroughly by flicking the tube ~10x and briefly centrifuging for 1s to collect beads.

    The aim of this step is to get two fractions of transcripts: a shorter one (above 600 bp) and a longer one (above 1000 bp). These are combined together in equal molarities at the end. This allows us to keep a balance between shorter and longer transcripts and improve sequencing efficiency.

25. Samples were incubated by shaking for 20 minutes at 550 rpm at room temperature. These were then centrifuged and placed in a Dynamag rack for 5 minutes to collect the beads on the side of the tube.

26. Clear supernatant was removed by pipetting to avoid disturbing the bead pellet and 1.5 ml of 80% EtOH was added to each tube and incubated at room temperature for 30 s.

27. Ethanol was removed and a fresh volume added before incubation for 30 s after which ethanol was again removed.

28. Tubes were transferred from the magnetic rack and centrifuged to pellet the beads. These were again placed on the rack and any residual ethanol was gently removed and open tubes left for 30 - 60 s to allow beads to air-dry.

29. To the 0.6x fraction, 27 ul and to the 0.4x fraction, 22ul of Zymo Elution buffer was added and placed on the bench for 5 minutes to allow elution of the DNA from the beads.

30. Samples were centrifuged to pellet beads and then transferred onto the magnetic rack for a further 5 minutes to obtain a clear supernatant.

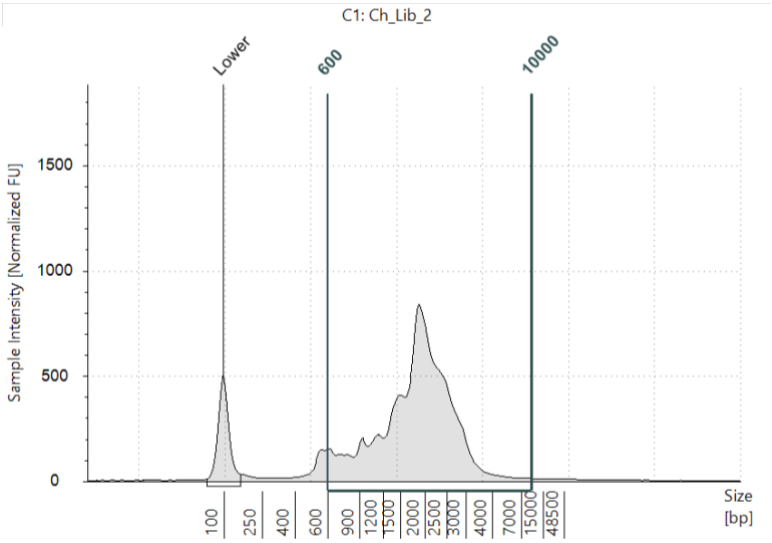
31. Both fractions of samples were checked on a tapestation and the software used to calculate molarity of each sample which were then combined together.

2.2.7 Quality and quantity measurements

Quality control of the amplified full-length cDNA was carried out with three available methods. 1. The concentrations were calculated using a Qubit 4 Fluorometer (Thermo scientific). The samples were prepared according to the manufacturer's protocol using the BR DNA Qubit kit (Thermo scientific). For accuracy, 2 ul of samples were used for measurements.

3. To determine the quality and size distribution of the cDNAs, genomic screen tapes were used with the TapeStation 4200.

Example of cDNA profile on the TapeStation:



4. A Nanodrop spectrophotometer was used to check the purity of the samples and to confirm that samples were not contaminated with guanidine salts. Guanidine isothiocyanate is a component of the binding buffers used in the protocol and, if present in samples, could affect the sequencing outcome. Any traces of the salt would be seen as an additional peak at 230 nm wavelength.
   None of the 42 samples showed salt contamination.

   Example of cDNA profile from the Nanodrop spectrophotometer: