GENE-SWitCH

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

Protocol WP5
Purification of Total RNA from liver and skeletal muscle, from piglets and foetuses 70dpf

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1 Protocol description

1.1 Required Reagents and Instruments

- RNeasy mini kit (Qiagen, 74106)
- RNase-free DNase set (Qiagen, 79256)
- 2 ml Tube, 2 ml centrifuge tubes and 5ml tubes (eppendorf)
- Pipets and tips
- Centrifuge
- Nanodrop spectrophotometer (thermofischer)
- M-tubes (Miltenyi Biotech, 130-093-236)
- GentleMACS dissociator (Miltenyi Biotech 130-093-235)
- Buffer RLT (Qiagen, 79216)
- β-Mercaptoethanol
- Ethanol
- Reagent DX (reduce foam) (Qiagen ref:19088)
- Proteinase K (Qiagen, 19133)
- RNase-free water
- 96-100% ethanol
- Dry ice
- Cryoprotection gloves
- Box with ice
- Sterile disposable Petri dishes (100 mm)
- Disposable scalpels
- Tweezers
- Disposable sterile skin punches (Robbins Instruments, RBP-40, 4mm)
- Racks for 2 and 5 mL tubes
- A permanent marker
- Paper towels
- Waste bag
- A cleaning spray against RNAse
- Weighting scale
- Timer

1.2 Notes before starting:

- Unless otherwise indicated, perform the procedure, as well as all centrifugation steps, at room temperature (15–25°C). Work quickly.
- Add 10 µl β-mercaptoethanol (β-ME) per 1 ml Buffer RLT before use. Buffer RLT containing β-ME can be stored at room temperature for up to 1 month.
- Add 4 volumes of ethanol (96–100%) to Buffer RPE for a working solution.
- Prepare DNase I stock solution. Dissolve the lyophilized DNase I in 550 µl RNase-free water by injecting the RNase-free water into the vial using an RNase-free needle and syringe. Mix gently by inverting the vial. Do not vortex. Store DNase I as single-use aliquots at –20°C for up to 9 months or at 2–8°C for up to 6 weeks. Do not refreeze after thawing.

- When disrupting and homogenizing tissues in Buffer RLT, excessive foaming may occur. This foaming is substantially reduced by adding Reagent DX to Buffer RLT Plus at a final concentration of 0.5% (v/v) before starting disruption and homogenization. Reagent DX has been carefully tested with RNeasy Plus Kits, and has no effect on RNA purity or on downstream applications such as real-time RT-PCR. Buffer RLT Plus containing Reagent DX can be stored at room temperature (15–25°C) for at least 9 months. Reagent DX is supplied separately (cat. no. 19088).
- Muscle specific in blue, as muscle tissue needed some additional steps to obtain RNA
- Before starting the purification of total RNA we need to weight the adequate quantity of tissue to perform tissue homogenization. Working above a box of dry ice, for each sample, we took a biopsy of the middle of the frozen tissue using a disposable biopsy punch. With a clean tweezer, the tissue was quickly removed from the biopsy punch, and a slice was cut out of the middle of the biopsy using a clean scalpel. For liver a piece weighing 20-30 mg of tissue was used, for muscle we cut pieces of 45-55 mg. Each piece of tissue was then placed into a M-tube containing RLT (+B-ME and Reagent DX) buffer on ice. 2 tubes were homogenized at the same time, and placed on ice after homogenization until 6 tubes were centrifuged at the same time (step 2 below).

1.3 Purification of Total RNA (RNeasy mini prep kit)

1. Disrupt and homogenize ≤30 (for muscle 45-55) mg tissue in 500/800 µl Buffer RLT (+ B-ME) (+ Reagent DX) using the M-tubes and the Miltenyi gentle MACS at program RNA_02 (Tightly close M Tube and turn the tube upside down in one quick move ensuring that the sample material reaches the area of the rotor/stator). If solution contains particles repeat this step.
2. Centrifuge at 10.000g for 3 minutes at 4°C, transfer supernatant to a new 2ml (5ml, avoid transferring any of a pellet if formed) eppie. (For **liver**: continue with step 8)
3. Add 108 µl Proteinase K, mix by pipetting
4. Add 468µL of 96–100% ethanol and mix well. Do not centrifuge.
5. Incubate the tubes for 10 min at room temperature.
6. Add 1000µL of 96–100% ethanol and mix well. Do not centrifuge.
7. Transfer up to 700µl of the sample, including any precipitate that may have formed, to a RNeasy Mini spin column placed in a 2 ml collection tube. Close the lid gently and centrifuge for 15 s at 15 s at ≥8000 x g. Discard the flow-through. Repeat step until complete lysate is used. Continue with step 10.
8. Add 0.5 volumes of 96–100% ethanol, and mix. Do not centrifuge.
9. Transfer 700 µl of sample to RNeasy Mini column (in a 2 ml collection tube). Close lid, centrifuge for 15 s at ≥8000 x *g* and discard flow-through. Repeat step until complete lysate is used.
10. Add 350 µl Buffer RW1 to RNeasy column. Close lid, centrifuge for 15 s at ≥8000 x *g* and discard flow-through.
11. Mix 10 µl DNase stock solution with 70 µl Buffer RDD, add to RNeasy membrane and incubate for 15 min at 20–30°C.

12. Add 350 µl Buffer RW1 to RNeasy column. Close lid, centrifuge for 15 s at ≥8000 x *g* and discard flow-through.
13. Add 500 µl Buffer RPE to RNeasy column. Close lid, centrifuge for 15 s at ≥8000 x *g* and discard flow-through.
14. Add 500 µl Buffer RPE to RNeasy column. Close lid, centrifuge for 2 min at ≥8000 x *g*.

Optional: Place RNeasy column in new 2 ml tube, close lid and centrifuge at full speed for 1 min.

15. Place RNeasy column in new 1.5 ml tube. Add 50 µl RNase-free water directly onto the membrane, close lid and centrifuge for 1 min at ≥8000 x *g*.

1.4 Storage and Quantification of RNA

The RNA is stored at -80°C in 2 aliquots. Quantification is performed with a Nanodrop to determine the concentration of each purification and the 260/230, 260/280 ratios.