FR-AgENCODE

Protocol: purification of total RNA from tissues

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1 Summary

FR-AgENCODE is a FAANG pilot project for the functional annotation of livestock genomes.

As part of the FAANG action (Functional Annotation of ANimal Genomes), the FR-AgENCODE project aims at improving the genomic annotation of four livestock species:

- cattle (Bos taurus)
- goat (Capra hircus)
- chicken (Gallus gallus)
- pig (Sus scrofa)

This is achieved by performing molecular assays on tissues and on sorted primary cells (CD4+ and CD8+ T lymphocytes) from 2 males and 2 females of each species. These assays include RNA-seq, ATAC-seq and Hi-C to characterize the transcriptome, the chromatin accessibility and the genome 3D topology in these cells, respectively.

On-going results from the project are available here:

http://www.fragencode.org/results.html

A first publication is also available here:

Multi-species annotation of transcriptome and chromatin structure in domesticated animals.

Foissac S, Djebali S, Munyard K, Vialaneix N, Rau A, Muret K, Esquerré D, Zytnicki M, Derrien T, Bardou P, Blanc F, Cabau C, Crisci E, Dhorne-Pollet S, Drouet F, Faraut T, Gonzalez I, Goubil A, Lacroix-Lamandé S, Laurent F, Marthey S, Marti-Marimon M, Momal-Leisenring R, Mompart F, Quéré P, Robelin D, Cristobal MS, Tosser-Klopp G, Vincent-Naulleau S, Fabre S, der Laan MP, Klopp C, Tixier-Boichard M, Acloque H, Lagarrigue S, Giuffra E.

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Here we describe the protocol used to purify total from the following tissues:

Cerebellum, lungs, kidney, ileum, duodenum, testis, skeletal muscle and gizzard.

To perform the purification of total RNA from these tissues, we followed the User manual of the "NucleoSpin RNA mini kit" from Macherey Nagel (https://www.mn-net.com/media/pdf/b0/51/ee/Instruction-NucleoSpin-RNA.pdf).

2 Protocol description

2.1 Required Reagents and Instruments

- NucleoSpin RNA, Mini kit for RNA purification (Macherey-Nagel ref 740955.250)
- 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 (Qiagen ref: 19088)
- Proteinase K (Qiagen 19131)
- 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 total RNA we weighted the adequate quantity of tissue and performed tissue homogenization. Working on a 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 50 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 650 µL of lysis buffer composed of 643 µl of RA1 buffer + 6.5 µL β-Mercaptoethanol + 0.5 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.
After the lysis, we centrifuged 1 min at full speed (13000rpm) the centrifuge tubes containing the homogenate.

For muscle samples, we transferred 600 µl of the homogenized supernatant to a new Eppendorf tube and we added 890µl of water and 10 µl of Proteinase K (>600 mAU/ml). The mixture is incubated 15min at 55°C. We transferred 1ml of the digested lysate to a new Eppendorf tube and we proceed to the next step.
Each lysate was then filtrated using the provided NucleoSpin Filter.

2.3 Purification of Total RNA

1. We transferred 600 µl of the homogenized supernatant to a new Eppendorf tube and added 600 µl of ethanol (70%) to the homogenized lysate and mix by pipetting up and down. For muscle samples, we added 500 µl of absolute ethanol to 1ml of the digested lysate and mixed by pipetting up and down.
2. We placed the NucleoSpin RNA Column in a 2 ml collection tube and transferred up to 600µl of the sample, including any precipitate that may have formed into the column. We closed the lid gently and centrifuged for 60s at 13,000 x g. We discarded the flow-through.
3. We repeated step 3 until the entire sample has passed through the RNeasy Mini spin column.
4. We added 350µl MDB Buffer to the column. We closed the lid gently and centrifuged for 60s at 13,000 x g. We discarded the flow-through and reused the collection tube.
5. We added 10µl DNase I stock solution to 90µl Reaction Buffer for rDNase and we mixed by flicking gently the tube.
6. We added the DNase I incubation mix (95µl) directly onto the centre of the silica membrane of the column and incubate at room temperature (20–30°C) for 15 min.
7. We then added 200µl Buffer RAW2 to the column. We closed the lid gently and centrifuged for 30s at 11,000 x g. We discarded the flow-through and placed the column into a new 2ml collection tube.
8. We added 600µl Buffer RA3 to the column. We closed the lid gently and centrifuge for 30s at 11,000 x g. We discarded the flow-through and reused the collection tube.
9. We added 250µl of Buffer RA3 to the column. We closed the lid gently and centrifuged for 2 min at 11,000 x g to dry the spin column membrane.
10. We placed the RNeasy Mini spin column into 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 11,000 x g to elute the RNA.
11. We applied the eluate (50µl) once more onto the column for reelution. We closed the lid gently and centrifuged for 1 min at 11,000 x g to elute the RNA.

2.4 Storage, Quantification, and Determination of Quality of total RNA

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.
RNA Integrity Numbers (RINs) were determined twice using a BioAnalyzer and a FragmentAnalyzer.