FR-AgEncode: a French pilot project to enrich the annotation of livestock genomes

RNA Extraction Protocol

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July 2015

RNA ISOLATION from tissues

Protocol for RNA Isolation using TRIzol® Reagent with Phase Lock Gel-Heavy

Preliminary comment:
This protocol can be applied to both snapfrozen and RNAlater preserved tissues. However RNAlater should be removed by rinsing three times with DNase-RNase free water prior starting this protocol.

Required reagents
- TRIzol® Reagent (Life technologies; Cat # :15596-018)
- Phase Lock Gel Heavy (Fisher Scientific; Cat # :2302850)

1. Add 1 ml TRIzol Reagent for 50-100 mg of tissue and homogeneize with an ULTRA-TURRAX® (IKA-Werke) until obtaining an homogeneous suspension. To avoid cross-contamination among samples, the homogenizer should be decontaminated between samples with 0.2M NaOH washes followed by several washings with RNAse-free water.
2. Residual insoluble materials are remove by centrifugation at 12 000 x g for 10 minutes at 4°C.
3. In the meantime, pre-spin briefly the Phase Lock Gel-Heavy tubes to collect gel on tube bottoms (1500 x g for 1 minute).
4. Add the mixture "Trizol + homogenized tissue" to the tubes containing pre-spin Phase Lock Gel Heavy and incubate for 5 minutes at room temperature.
5. Add 0.2 ml chloroform per 1 ml TRIzol Reagent initially used. Cap tubes and shake vigorously for 15 seconds. Do not vortex!
6. Centrifuge samples at 12 000 x g for 10 minutes at 4°C.
7. Transfer aqueous phase containing RNA to a fresh tube.
Note: the organic phase can be stored at -20°C for further DNA extraction.
8. Precipitate RNA by adding 0.5 ml isopropyl alcohol per 1 ml TRIzol Reagent used initially. Mix samples by repeated inversion.
9. Store the tube at -20°C overnight.
10. Centrifuge samples for 30 minutes at 12 000 x g, 4°C.
11. Decant supernatant. Add 1 ml 75% ethanol per 1 ml TRIzol Reagent used initially to wash the RNA pellet. Mix samples to dislodge pellet, using a vortex if necessary.
12. Centrifuge samples at 12,000 x g for 15 minutes at 4°C.
13. Carefully decant supernatant. Briefly air-dry the RNA pellet to remove residual ethanol.
14. Resuspend RNA pellet in Molecular Biology Grade water.
15. Monitor RNA yield and purity by spectrophotometry (NanoDrop 2000).
16. Assess RNA integrity using an Agilent 2100 Bioanalyzer and RNA 6000 nano kits. RNA quality is evaluated by the RNA Integrity Number (RIN) value introduced by Agilent (Schroeder et al., 2006).

Note:
Expected RNA yield from liver (see Figure 1): Cattle: 2 µg/mg
Chicken: 7 µg/mg

DNase TREATMENT

Removal of contaminating genomic DNA from RNA samples using TURBOTM DNase

Important note:
Do not use more than 10 µg of RNA in 50 µl per reaction.

Required reagents

- TURBO™ DNase (AMBION; Cat #: AM2239)
- Phenol-Chloroform-Isoamyl Alcohol mixture (SIGMA-ALDRICH; Cat #: 77617)

For each 50 µl reaction add:
1. 50 U RNase inhibitor in the RNA sample (RNase stock 40 U/µl).
2. 10X TURBO™ DNase Buffer to 1X concentration in the RNA sample.
3. 1 µL TURBO ™ DNase (2 U) for up to 10 µg RNA in a 50 µL reaction, and incubate at 37°C for 30 minutes.
4. Extract the RNA sample with phenol/chloroform to inactivate the TURBO ™ DNase

DNase inactivation (phenol chloroform extraction):
1. Add 1 volume of Phenol-Chloroform-Isoamyl Alcohol mixture.
2. Centrifuge 5 000 x g 10 minutes at 4°C.
3. Transfer the aqueous phase to a new tube.
4. Add 1 volume of chloroforme.
5. Centrifuge 5 000 x g 10 minutes at 4°C.
6. Transfer the aqueous phase to a new tube.
7. Add 3M sodium acetate (pH 5.2) equal to 1/10th the volume of your sample to your RNA sample and add 1 µl glycogen.
8. Add 2.5 volumes of 99% ethanol.
9. Incubate overnight at -20ºC.
10. Spin at 12 000 x g 30 minutes at 4ºC.
11. Decant the 99% ethanol, and wash the nucleic acid pellet with 75% ethanol twice.
12. Spin at 12 000 x g 10 minutes at 4ºC.
13. Dry the pellet and resuspend in Molecular Biology Grade water.
14. Monitor RNA yield and purity by spectrophotometry (NanoDrop 2000).
15. Assess RNA integrity using an Agilent 2100 Bioanalyzer and RNA 6000 nano kits. RNA quality is evaluated by the RNA Integrity Number (RIN) value introduced by Agilent (see Figure 2).

Note:
In our conditions, DNase treatment does not affect significantly the RNA yield. DNAse treatment can also be performed by other reagents/protocols (e.g. RNase-Free DNase Set, Qiagen, Cat # 79254).

SMALL RNA ISOLATION

Isolation of large RNA and small RNA from total RNA samples using mirVana miRNA Isolation kit

Required reagents:
- mirVana miRNA Isolation kit (Ambion ; cat#:1560)
- Phenol-Chloroform-Isoamyl Alcohol mixture (SIGMA-ALDRICH; Cat #: 77617)

Before start:
- Add 21 mL of 99% ethanol to miRNA Wash Solution 1. Mix well.
- Add 40 mL of 99% ethanol to Wash Solution 2/3. Mix well.

1. Mix 50–100 µg of total RNA with 5 volumes of Lysis/Binding Buffer. If the RNA sample volume is <30 µL, add water to bring the sample to 30 µL, and then add 150 µL of Lysis/Binding Solution.
2. Add 1/10 volume of miRNA Homogenate Additive to the RNA mixture from the previous step, and mix well by vortexing or inverting the tube several times. Leave the mixture on ice for 10 min.
3. Add 1/3 volume of 99% ethanol to the RNA mixture from the previous step. Mix thoroughly by vortexing or inverting the tube several times.
4. For each sample, place a filter cartridge into one of the collection tubes supplied.
5. Pipet the lysate/ethanol mixture onto the filter cartridge. Up to 700 µL can be applied to a filter cartridge at a time. For sample volumes greater than 700 µL, apply the mixture in successive applications to the same filter.
6. Centrifuge for 1 min at 5 000 g.
7. If the volume of the RNA mixture is >700 µL, transfer the flow-through to a fresh tube, and repeat until all of the RNA mixture is through the filter. Pool the collected filtrates if multiple passes were done, and measure the total volume of the filtrate. Important: keep this filtrate on ice until processing.

***Note**: At this point, the collected filtrate contains the **small RNA fraction** (<200 nt) while the filter cartridge contains the **large RNA fraction**. depleted of small RNAs.*

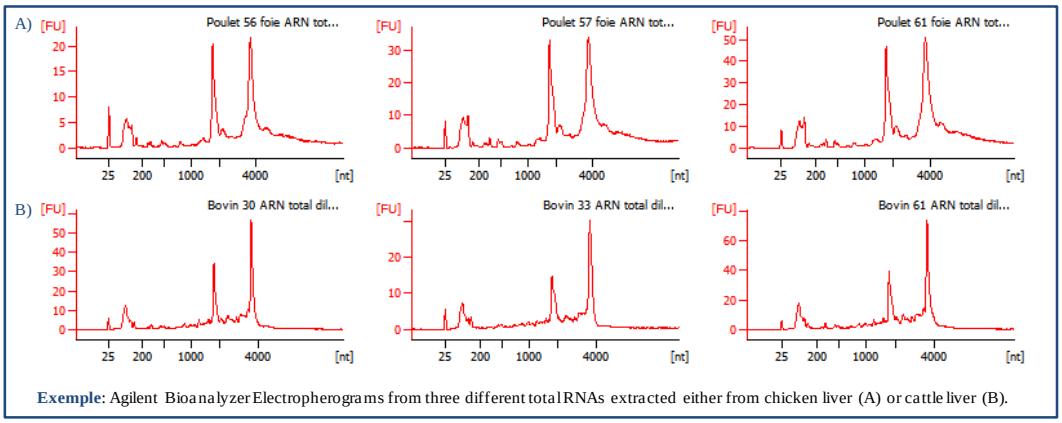
The large RNA fraction can be recovered by treating the filter cartridge (from step 7) as described below:

8. Centrifuge the filter cartridge at 10 000 g for 1 minute to remove residual fluid from the filter.
9. Apply 700 µL miRNA Wash Solution 1 to the filter cartridge and centrifuge at 10 000 g for 10 seconds. Discard the flow-through from the collection tube and replace the filter cartridge into the same collection tube.
10. Apply 500 µL Wash Solution 2/3 and draw it through the filter cartridge as in the previous step.
11. Repeat with a second 500 µL aliquot of Wash Solution 2/3.
12. After discarding the flow-through from the last wash, replace the filter cartridge in the same collection tube and centrifuge the filter cartridge at 10 000 g for 1 minute to remove residual fluid from the filter.
13. Transfer the filter cartridge into a fresh collection tube. Apply 100 µL of pre-heated (95°C) Elution Solution to the center of the filter and close the cap. Incubate at room temperature for 2 min and centrifuge for 1 minute at 10 000 g to recover the RNA. Collect the eluate (which contains the large RNA) and store it at –20°C or below.

The small RNA fraction can be recovered by treating the filtrates (from step 7) as described below:
14. Add 2/3 volume room temperature 99% ethanol to the filtrate (from step 7) and mix thoroughly.
15. For each sample, place a new filter cartridge into a new collection tube.

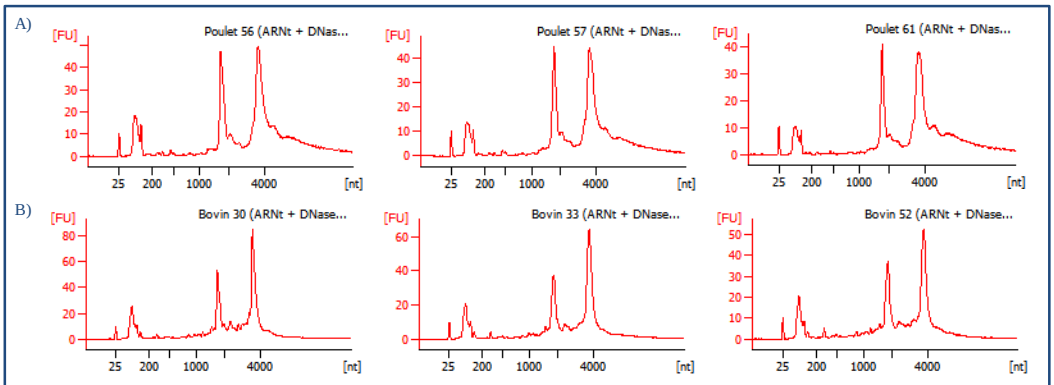
16. Pipet the filtrate/ethanol mixture onto the new filter cartridge (step 15). Up to 700µL can be applied to a filter cartridge at a time. For sample volumes greater than 700 µL, apply the mixture in successive applications to the same filter.
17. Centrifuge the filter cartridge at 5 000 g for 1 minute to pass the mixture through the filter.
18. Discard the flow-through and repeat the step 16 until all of the filtrate/ethanol mixture is passed through the filter. Reuse the collection tube for the washing steps.
19. Apply 700 µL miRNA Wash Solution 1 to the filter cartridge and centrifuge at 5 000 g for 1 minute. Discard the flow-through from the collection tube and replace the filter cartridge into the same collection tube.
20. Apply 500 µL Wash Solution 2/3 and draw it through the filter cartridge as in the previous step.
21. Repeat with a second 500 µL aliquot of Wash Solution 2/3.
22. After discarding the flow-through from the last wash, replace the filter cartridge in the same collection tube and centrifuge the filter cartridge at 10 000 g for 1 minute to remove residual fluid from the filter.
23. Transfer the filter cartridge into a fresh collection tube. Apply 50 µL of pre-heated (95°C) Elution Solution to the center of the filter and close the cap. Incubate at room temperature for 2 min and centrifuge for 1 minute at 10 000 g to recover the RNA.
24. Repeat the step 23 with a second 50 µL aliquot of pre-heated (95°C) Elution Solution.
25. Collect the eluate (which contains the small RNA) into the same collection tube and store it at –20°C or below.

Ethanol Precipitation (same for Small and Large RNA)
26. Add to the eluted RNA: 1/10 volumes of 3M sodium acetate (pH 5.2) and add 1 µl glycogen.
27. Add 2.5 volumes of 99% ethanol and incubate overnight at -20ºC.
28. Spin at 12 000 g in the 4ºC centrifuge for 30 minutes.
29. Decant the 99% ethanol, and wash the nucleic acid pellet with 75% ethanol.
30. Spin at 12,000 g in the 4ºC centrifuge for 10 minutes.
31. Dry the pellet and resuspend in Molecular Biology Grade water.
32. Monitor RNA yield and purity by spectrophotometry (NanoDrop 2000).
33. Assess RNA integrity using an Agilent 2100 Bioanalyzer and RNA 6000 nano kits. RNA quality is evaluated by the RNA Integrity Number (RIN) value introduced by Agilent (see Figure 3).



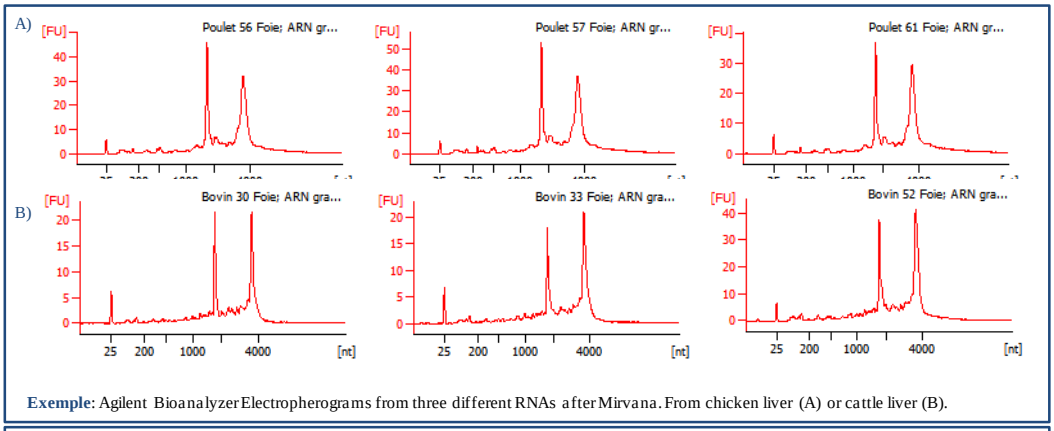
Exemple: Agilent Bioanalyzer Electropherograms from three different total RNAs extracted either from chicken liver (A) or cattle liver (B).

Figure 1

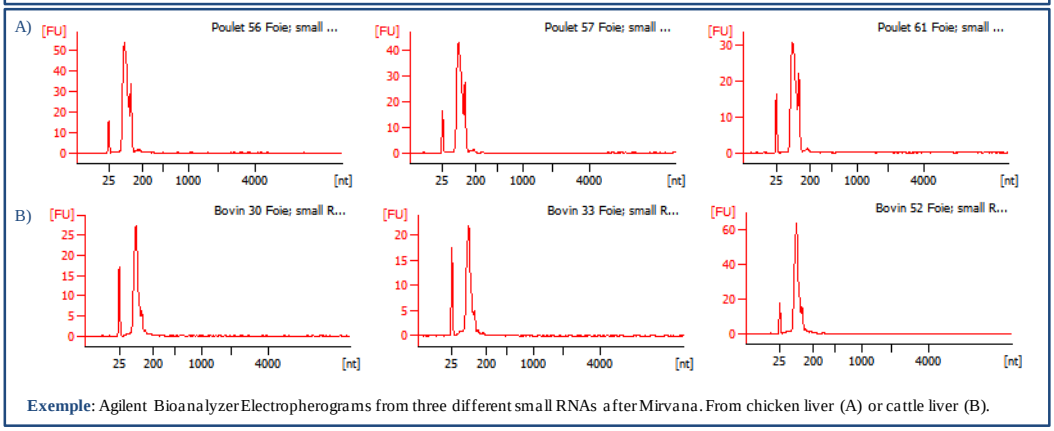


Exemple: Agilent Bioanalyzer Electropherograms from three different total RNAs after Dnase treatment. From chicken liver (A) or cattle liver (B).

Figure 2



Exemple: Agilent Bioanalyzer Electropherograms from three different RNAs after Mirvana. From chicken liver (A) or cattle liver (B).



Exemple: Agilent Bioanalyzer Electropherograms from three different small RNAs after Mirvana. From chicken liver (A) or cattle liver (B).

Figure 3