PROTOCOL

RNA extraction from tissue - using Bioruptor® (Standard/Plus) and RNA extraction kit

Introduction

Isolation of intact RNA is essential for many techniques used in gene expression analysis. Efficient disruption and homogenization of animal tissues are required to ensure high yield of RNA. Disruption releases RNA, while homogenization reduces sample viscosity to facilitate RNA purification. Diagenode's Bioruptor® Sonicator uses state-of-the-art ultrasound technology to efficiently disrupt and homogenize tissues in one step. Diagenode's RNA extraction reagent (included in the RNA extraction kit) is used as sonication medium and maintains the integrity of RNA while disrupting cells and dissolving cell components.

Bioruptor® together with RNA extraction reagent offer unique benefits for tissue disruption and homogenization:

- Fast protocol
- Non-contact minimizes contamination
- Efficient and reproducible
- Isothermal process
- Multiplexing capability of up to 6 samples in parallel (depends on Bioruptor® model)

General remarks before starting

- Minimize the time of tissue collection to prevent RNA degradation by RNases and from changes in RNA expression triggered by sample manipulation. RNA quality correlates to tissue-specific response to physiological stress both prior to and following tissue death.
- Dissected tissues can be snap-frozen in liquid nitrogen and stored at -80°C until RNA extraction. Alternatively, RNAlater solution can be used to protect RNA in unfrozen sample.
- This protocol has been validated for 20-50 mg of tissue. Do not use more tissue per sample as it might result in low quality RNA. For larger quantity, cut the tissue and proceed to disruption in separate tubes.
- When working with RNA extraction reagent, work in fume hood and use gloves and eye protection.
- When working with RNA, care must be taken to maintain an RNase-free environment starting with RNA purification and continuing through analysis. Wear gloves at all times to prevent RNase contamination from the surface of the skin or from dusty laboratory equipment. Change gloves frequently and keep tubes closed whenever possible. Keep isolated RNA on ice when aliquots are pipetted for downstream applications.

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PROTOCOL

Required materials and reagents

- Bioruptor® Standard or Plus (Diagenode, Cat. No. UCD-200, UCD-300) for tissue disruption and homogenization
- RNA extraction kit (Diagenode, Cat. No. AL-001-0050)
- Bioruptor® Water Cooler (Diagenode, Cat. No. BioAcc-Cool)
- Single Cycle Valve for Bioruptor® Plus (Diagenode, Cat. No. VB-100-0001)
- Tube holder pack for extraction kits (Diagenode, Cat No. O-ring-15)
- Liquid nitrogen or RNAlater solution (Ambion, Cat #7020) for tissue collection
- Isopropanol (Molecular Biology grade)
- Chloroform (Molecular Biology grade)
- 70 % ethanol (Molecular Biology grade)
- RNase-free water
- 2 ml RNase-free tubes
- Nanodrop and Agilent Bioanalyzer for quality assessment (optional)

Protocol

Tissue disruption and homogenization

1. Pre-cool Bioruptor to 4°C using the Bioruptor® Water Cooler (Cat. No. BioAcc-Cool) or crushed ice.
2. Prepare sonication tubes: add 1 ml of cold RNA extraction reagent to the pre-filled RNA extraction tubes.
   We highly recommend to use these tubes without reflecting bars.
3. Keep the tubes on ice.
4. Add 20-50 mg of snap-frozen tissue per tube containing RNA extraction reagent. Alternatively, RNAlater-treated samples can be used.
   If needed, cut the frozen tissue in a Petri dish placed on dry ice. Minimize the time required to do this, and do not allow sample to thaw before immersing into RNA extraction reagent.
5. Adjust the sample volume with RNA extraction reagent to final volume 2 ml and vortex vigorously.
6. Insert the aluminium rings to ensure an optimal position of the tube in tube holder during sonication (see picture). To guarantee homogeneity of sonication, the tube holders should always be completely filled with tubes.

7. Sonicate samples in Bioruptor using the following settings:
   - Power: H position (High)
   - Sonication cycle: 30 seconds ON, 30 seconds OFF
   - Temperature: 4°C
   - Total sonication time: 1-3 cycles
8. Stop Bioruptor® after each cycle, vortex samples and visually check the sample for disruption.

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Please note that optimization might be required depending on sample format (fresh or frozen tissue), tissue type and tissue amount. The shortest sonication time should be chosen to prevent RNA degradation. Incomplete disruption may occur with fibrous tissues like muscles. Do not sonicate longer than 3 cycles to prevent low quality RNA.
Samples with high amount of blood, iron or hemoglobin may change the blue color of RNA extraction reagent.

9. Vortex tubes vigorously after sonication and incubate for 5 min at room temperature.

RNA isolation

10. Centrifuge samples at 3.000 rpm for 5 min at room temperature and transfer the supernatant to a new 2 ml RNase-free tube.
This step permits the complete dissociation of nucleoproteins complex
11. Add 0.4 ml of chloroform, vortex and centrifuge at 12.000 g for 10 min at 4°C.
Chloroform mixed with isoamyl alcohol should not be used.
Thorough mixing is important for subsequent phase separation
12. Transfer the colorless upper phase to a new 2 ml tube. Take care not to aspirate the DNA-containing white interface and organic blue phase.
If contamination of genomic DNA is expected, extract again by adding an equal volume of chloroform to the aqueous phase transferred to the new tube.
13. Add 0.8 ml of isopropanol, mix and centrifuge at 12.000 g for 10 min at 4°C. A gel-like pellet forms on the side and bottom of the tube.
14. Remove the supernatant and keep the pellet.
15. Add 1 ml of 70% ethanol, vortex samples and centrifuge for 10 min at 12.000 g
16. Remove the supernatant and air-dry the pellet for 5-10 min at room temperature. Do not over-dry the pellet.
17. Add RNase-free water (100-300 µl depending on expected RNA yield), resuspend carefully by pipetting. The solution can be incubated at 55-60 C° for 10 min if the pellet is hard to dissolve.
18. Take an aliquot for quantitation and quality analysis. Store RNA -80°C.

RNA quantitation and quality assessment

19. Quantify RNA using a Nanodrop and analyze ratio OD 260/280 and OD 260/230 to ensure the purity of RNA.
Ratio OD260/280 1.8-2.0 is considered good. A low ratio might indicate protein contamination. A ratio greater than 2.1 might indicate RNA degradation.
Ratio OD260/230 greater than 1.8 is considered good. A low value might indicate organic contamination.
20. Asess the integrity of RNA using the Agilent 2100 Bioananlyzer (or BioRad Experion system). .
RIN values threshold depends on the desired downstream experiments and should be correlated with the specific assay to be run (RT-PCR or microarray, for example).
The table below shows suggested applications for RNA within different RIN ranges:

RIN Value	RNA quality	Suggested application
1-4	degraded to low	PCR assays with short regions of amplification
4.1-6.9	moderate to regular	qRT-PCR applications
7.0-10.0	excellent to outstanding	Highly demanding gene array assays

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Example of tissue disruption

Figure 1.
An example of mouse brain disruption using RNA extraction tubes and RNA extraction reagent.

Left picture shows samples before sonication. Right picture shows disrupted sample.

Examples of total RNA profiles obtained from animal tissues

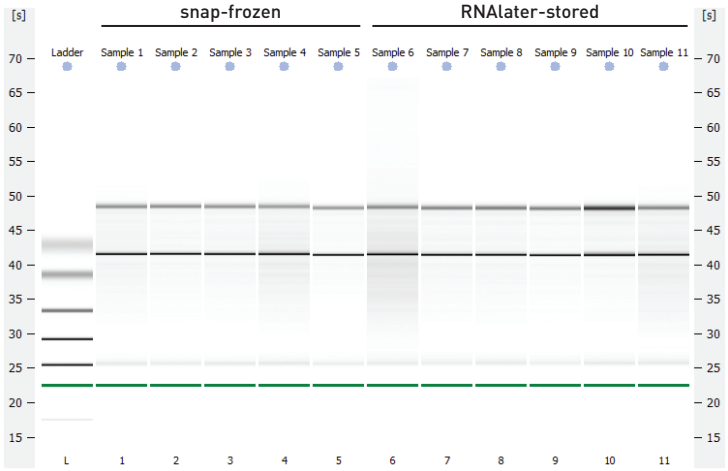


Figure 2.

Total RNA efficiently extracted from snap-frozen (lanes 1-5) and RNAlater-treated (lanes 6-11) mouse brain samples.

Tissue was disrupted with the Bioruptor® Standard (UCD-200). Total RNA was extracted as directed in the protocol and analyzed on BioAnalyzer (Agilent).

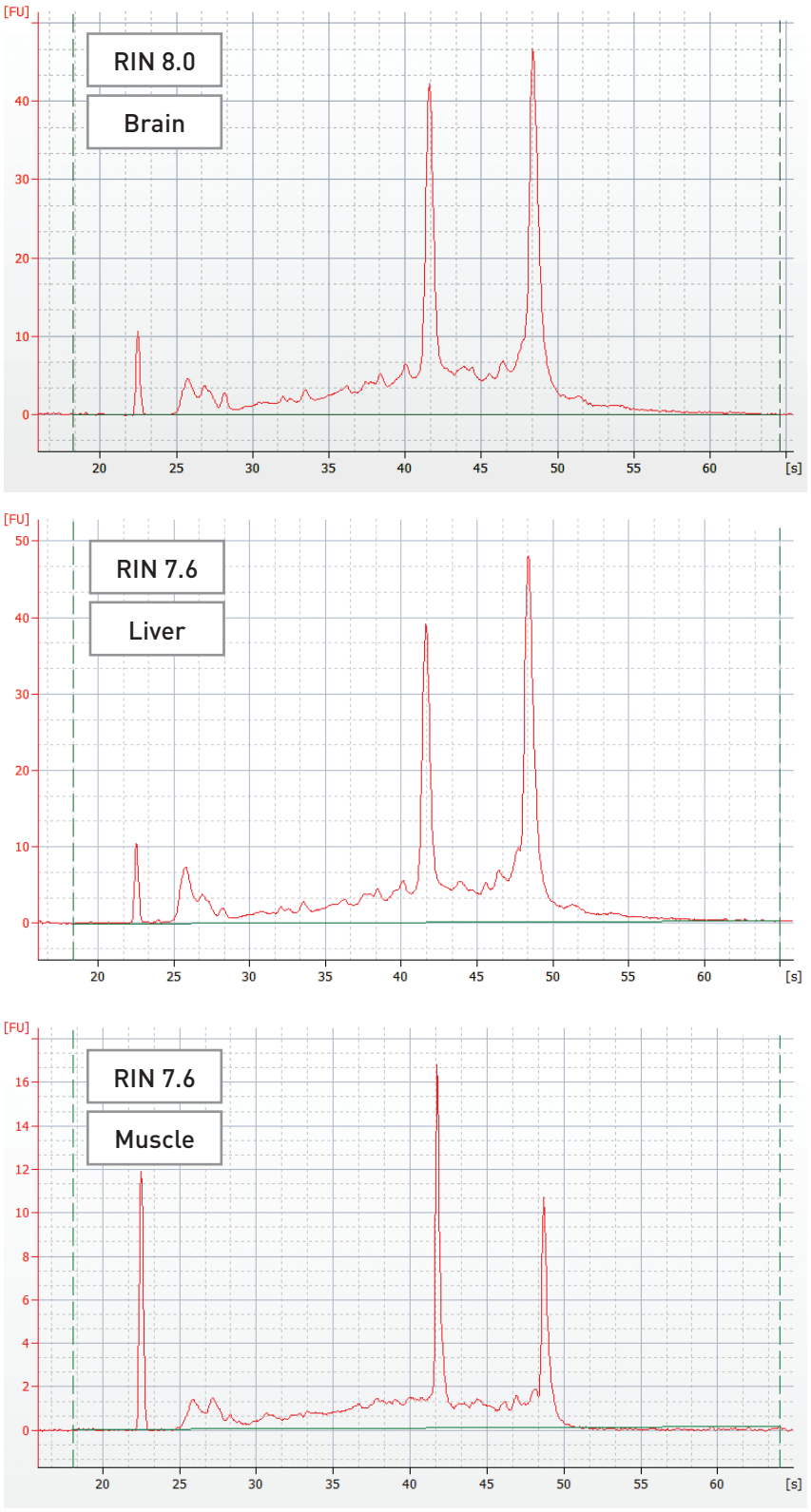


Figure 3.

Efficient extraction of pure RNA with high RIN.

Total RNA profiles from mouse brain (upper panel), liver (middle panel) and skeletal muscle (bottom panel). Tissues were disrupted with Bioruptor Plus (UCD-300) as described in the protocol and analyzed on BioAnalyzer (Agilent). Note that small RNAs are present in all profiles indicating that the RNA is largely intact.

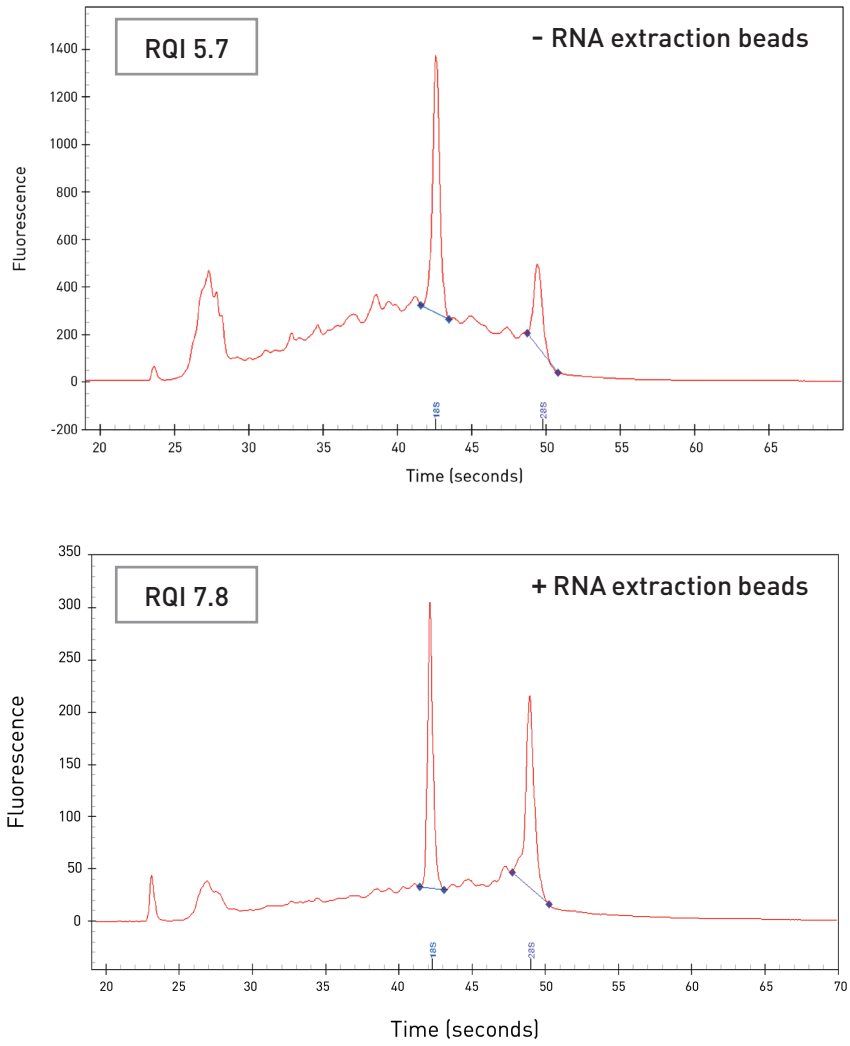
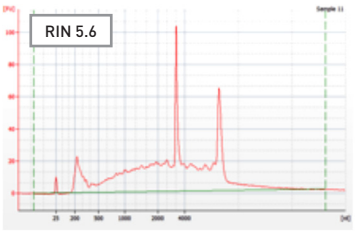


Figure 4.

Pre-filled RNA extraction tubes improve tissue disruption and RNA quality.

Experion (BioRad) traces of total RNA obtained from mouse liver using Bioruptor and the RNA extraction kit without (upper panel) or with RNA extraction beads (bottom panel). Note that only 2 cycles are required for complete tissue disruption using RNA extraction beads vs 15 cycles without RNA extraction beads. RNA extracted from a sample disrupted in the presence of RNA extraction beads shows significantly higher RQI.

Troubleshooting guide

Problem	Possible cause	Suggested solution
Low yields Expected yield of RNA per mg of tissue: *Liver 4-10 µg* *Skeletal muscle 0.3-1 µg* *Brain 2-5 µg* HeLa cells 6-15 µg per 1X10^6 cells	RNA is not solubilized completely	Do not allow pellet to dry completely. Do not lyophilize or vacuum dry sample.
Degraded RNA	Sample manipulated too much before freezing or RNAlater stabilization Improper storage of RNA Frozen tissue thawed in absence of RNA extraction reagent Sample sonicated too much	Process tissue immediately after dissection Store RNA at -80°C avoid thaw-freeze cycles Add frozen tissue immediately to RNA extraction reagent Avoid long sonication.1-2 cycles are enough. Do not sonicate longer even if some particulate matter remains.
Low RIN value due to high baseline 	DNA/protein contamination is possible	Be sure not to take any of the interphase (contains DNA) with the aqueous phase Do not use more than 50 mg for 2 ml of RNA extraction reagent. Remove any particulate material before chloroform addition Treat with RNase-free DNase I (see additional protocol)
Low ratio OD 260/280	Protein contamination	Too much tissue used. Do not use more than 50 mg for 2 ml of RNA extraction reagent. Be sure not to carry any organic phase with the RNA sample (step 9 in the protocol)

Low ratio OD 260/230	Organic contamination (chloroform, polysaccharides etc)	Too much tissue used. Do not use more than 50 mg for 2 ml of RNA extraction reagent. Be sure not to carry any organic phase with the RNA sample (step 9 in the protocol)
RNA contains some DNA	Part of the interphase was removed with the aqueous phase Too much tissue used for 2 ml of RNA extraction reagent Insoluble material was not removed before extraction	Be sure not to take any of the interphase (contains DNA) with the aqueous phase Do not use more than 50 mg for 2 ml of RNA extraction reagent. Remove any particulate material before chloroform addition For use in PCR and qRT-PCR, treatment with DNase I (RNase-free) is recommended

Additional protocol for DNase I treatment

- Combine up to 10 µg of RNA, 1 µl of RNase-free DNase (1 U/µl), 5 µl of 10X DNase buffer, 1 µl of RNasin (optional) and RNase-free water to final volume of 50 µl
- Incubate sample for 15-30 min at room temperature
- Add EDTA to final concentration 2 mM
- Extract RNA samples with 100 µl of RNA extraction reagent and 20 µl of chloroform. Use 80 µl of isopropanol to precipitate RNA
- Wash the pellet once with 70% ethanol, air-dry and resuspend in RNase-free water