Total RNA Isolation from Skeletal Muscle

Sample preparation

1. Harvest skeletal muscle and place the sample in ice-cold TriPure (1mL for SO or WV, 1.5 mL for PL, 3 mL for TA muscle from one leg) in a 13-mL polypropylene tube.
2. Homogenize the sample on ice-water bath using a polytron with 3 bursts for 10 seconds each. Ensure the polytron is clean between samples by two 10-second bursts in clean water and/or disassembly followed by wiping with Kim wipe if needed.
3. Place the samples in a -80 °C freezer and store at -80 °C until ready for isolation of RNA. Otherwise, proceed immediately to step 4.

Isolation of RNA (with 1.5mL of Trizol, scale-down/up if needed). For large sample sets, perform isolation procedure on a maximum of 10 at one time to avoid RNA degradation.

4. Remove samples from -80 °C freezer and thaw at room temperature.
5. Transfer samples to 2 mL micro centrifuge tube and add 0.3 mL of chloroform (20% of TriPure volume) immediately after samples thawed.
6. Cap the tubes tightly and shake for 30 sec.
7. Incubate the mixtures at room temperature for 5 min.
8. Centrifuge the samples at 4°C for 15 min at 12,000×g.
9. Transfer the colorless upper aqueous phase (about 0.9mL) of each sample to a new 2 mL micro centrifuge tube. Take care to not disturb the partition between the two phases.
10. Repeat the extraction by adding 1 mL of chloroform to the transferred, separated aqueous phase of each sample.
11. Cap the sample tubes tightly and shake for 30 sec.
12. Centrifuge the samples at 4°C for 15 min at 12,000×g.
13. Transfer only the colorless, upper aqueous phase to a 2-mL micro centrifuge tube.
14. Add 1 mL of isopropanol to each of the final, isolated aqueous phase, cap tightly and mix by inverting.
15. Incubate at room temperature for 5 min.
16. Centrifuge the samples at 4°C for 10 min at 12,000×g.
17. Remove the supernatant carefully without disturbing the pellet of RNA.
18. Add 1 mL of room temperature RNase-free 70% ethanol.
19. Vortex briefly and centrifuge at 12,000×g for 5 min at 4°C.
20. Remove the supernatant carefully by pipetting and air-dry the sample for 10-30 min. Do not over dry the sample, which will make it very difficult to dissolve.
21. Add 10 µL of nuclease-free water to each sample.
22. Incubate the sample on ice for 20-60 min with vortex at 5 min intervals.
23. Determine the concentration of total RNA by diluting 1 µL of the sample in 79 µL of Tris-EDTA buffer and measure the absorbance at 260 nm in a spectrophotometer (it may vary depending on the cuvette used).

24. Determine the integrity of total RNA by electrophoresis of 1 µg of total RNA on a 1.0% agarose gel and staining with ethidium bromide for 18S:28S RNA.

Note: place samples in a storage box and store at -80 °C prior to further analysis.

RT-PCR

1. Prepare primer set as following:

Oligo-dT (cat#N420-01)	40 µL	40 µM
Random Primer (cat#48190-011)	2.64 µL	40 µM
DEPC-H2O	57.4 µL	

This primer set could be aliquoted in 50 µL and stored at -20°C.

2. Thaw RNA sample on ice.

3. Prepare RT reaction mixture as following for each sample in an RNase-free Eppendorf tube:

Total RNA (x µg/µL)	x µL (5µg)	0.1 µg/µL
dNTP (2.5mM each, cat#R725-01)	10 µL	0.5 µM
Primer set (40 µM)	2.5 µL	2 µM
DEPC-H2O	final vol to 33.75 µL	

4. Heat the samples at 85 °C for 3 min and chill them on ice.

5. Add the following reagents to each tube:

RT buffer (5×)	10 µL	1×
DTT (0.1 M)	2.5 µL	5 mM
RNase inhibitor (40 U/µL)	2.5 µL	2U/µL
SuperScript II (200 U/µL, cat#18064-022)	1.25 µL	5U/µL

This can be added as a cocktail of 16.25 µL each.

6. Mix the sample gently and incubate at 42 °C for 1 hour.

Heat the sample at 92 °C for 10 min to stop the enzymatic reaction. The sample can be stored at -20 °C prior to next PCR reaction.

8. Determine the integrity of cDNA by electrophoresis of 1 µL of total cDNA on a 1.2% agarose gel and staining with ethidium bromide for 18s cDNA. For each sample use the following volume for PCR reaction.

ddH_2O: 5.9µL
2x Taq: 7.5µL
18s F : 0.3µL
18s R : 0.3µL
cDNA : 1.0µL

Use the following parameters for the 18s PCR reaction:

94°C- 5 minutes
60°C- 30 seconds
72°C- 40 seconds
Repeat 12 cycles
72° - 5 minutes
hold: 12°C

Load the samples on the 1.2% agarose gel and run at 130V for 20 minutes.