

Procedure for RNA isolation from human muscle or fat

Reagents, all Rnase free:

20% SDS
DEPC-H₂O
Rnase ZAP
75% EtOH
Trizol
Chloroform
Isopropanol
0.8M NaCitrate/1.2M NaCl
TE buffer, pH 7.0

1. Homogenizer-probe is soaked O/N in 20 ml of 1% SDS (1 ml 20% SDS + 19 ml DEPC-H₂O).
2. Weigh samples (quickly).

Sample #						
Mass (mg)						

3. Rinse the probe extremely well with 5 ml of DEPC-H₂O x 2, then with Rnase ZAP, DEPC-H₂O, 75% EtOH and then Trizol. 10-15 sec for each cleaning step.

Homogenize (24,000 r/min) 50-100 mg samples in 2-4 ml Trizol on ice (beaker with ice+H₂O) for 45 sec, place back on ice for 45 sec, then repeat x 2. **KEEP SAMPLES ON ICE AT ALL TIMES!**

Company protocols suggest volume dependent upon sample size: 100-150 mg in 3 ml Trizol, 150-200 mg in 4 ml Trizol, but we have found that yield from 50-100 mg samples improves with greater volume, and thus use 4 ml Trizol.

***If you are isolating RNA from adipose tissue, add Rnase free glycogen (Ambion) at final concentration of 250 ug/ml as carrier to increase yield.

Between samples, rinse with 5 ml 75% EtOH, DEPC-H₂O and then Trizol (5-10 sec).

Sample #						
ml Trizol						

4. Centrifuge 12,000 x g for 10 min (5-6000 rpm, 4°C, SS34 rotor).
5. Transfer supernatant to fresh tubes and incubate 5 min at RT.
6. Add 0.2 ml of chloroform per 1 ml of Trizol used in step 4. Cap tubes securely and shake vigorously by hand for 15 sec. Incubate at RT for 2-3 min.

Sample #						
ml chloroform						

7. Centrifuge samples at 12,000 x g for 15 min at 4°C.
8. Carefully transfer the aqueous phase to a fresh tube – **it is important NOT to get any of the interface** (RNA is exclusively in the upper phase) – leave behind more! If you get even a bit of the interface, you will have protein contamination and poor yield from your RT reactions.
9. Add 0.25 ml of isopropanol and 0.25 ml of 0.8M Na citrate/1.2M NaCl per 1 ml Trizol added in step 4. Incubate for 10 min at RT.

Sample #						
ml isopropanol						
ml 0.8M citrate/1.2M NaCl						

10. Centrifuge at 12,000 x g for 10 min at 4°C.
11. Remove the supernatant. Add/wash with 75% EtOH ($V_{\text{EtOH}} = V_{\text{Trizol}}$ added in step 3). Spin 5 min at 12,000 x g.
12. Remove EtOH and add 1 ml 75% EtOH and transfer (including pellet) to an Eppendorf and spin at max rpm in at microcentrifuge at RT for 5 min.
13. Decant the EtOH carefully using a pipet. Spin down again for ~ 5 sec in microcentrifuge at max rpm at RT, and remove the remaining EtOH using a pipet and sterile cotton swabs.
14. Leave samples to dry on the bench for 15-20 min (DO NOT overdry the pellet!).
15. Add 25 μl DEPC-H₂O and mix gently using vortex. Spin down briefly on microcentrifuge at RT, and leave to dissolve on bench at RT for 10-20 min. If you are having difficulty with resuspending your pellet, consider incubation at 60°C for 5 min or add additional DEPC-H₂O to resuspend.
16. If you stop here, keep samples at –80°C.
17. Vortex samples and spin down for a few seconds and measure OD₂₆₀ (498 μl TE + 2 μl sample; Dilution factor = 500/2; blank 500 μl TE).

Concentration ($\mu\text{g}/\mu\text{l}$) = OD₂₆₀ x 40 x dilution factor x 1/1000

Yield = Concentration ($\mu\text{g}/\mu\text{l}$) x volume (μl)

RNAeasy Protocol – Total RNA clean-up with Dnase treatment

Reagents:

RNeasy Mini Kit (50)	Qiagen	Cat. no. 74104	\$180
Rnase-free Dnase set (50)	Qiagen	Cat. no. 79254	\$62
β-Mercaptoethanol			
Absolute Ethanol			
TE buffer			

Note that you should not apply more than 100 ug of RNA to each column (capacity of column). Try to do the Rneasy cleanup quickly (without interruptions).

Before starting:

1. Add β-Mercaptoethanol (β-ME) to the RLT Buffer (if starting with at new kit), 10 μl β-ME per ml of the RLT Buffer (IN THE HOOD!).
2. Add 4 volumes of 100% EtOH to the RPE Buffer (If starting with new kit, 44 ml 100% EtOH to 11 ml RPE Buffer.)
3. Pre-heat the Rnase-free water to 65°C.

Procedure:

1. Adjust samples to a volume of 100 μl with RNase –free water.

Sample #						
μl sample						
μl H2O						

Total vol. = 100 μl

The following should be done in the hood!!!

2. Add 350 μl RLT Buffer to the samples and mix by pipetting, then add 250 μl 100% EtOH and mix well by pipetting.
3. Apply entire sample (700 μl) to an Rneasy mini spin column sitting in a 2 ml collection tube. Centrifuge for 15 sec at max rpm in a microcentrifuge at RT.
4. Reapply filtrate again to the column and repeat the centrifugation. Discard flow-through and the collection tube. Transfer column to new tube.
5. Pipet 350 μl RW1 Buffer into the column and centrifuge for 15 sec at max rpm to wash. Discard the flow-through.

6. Add 10 μl Dnase stock solution to 70 μl RDD Buffer. Mix by gently inverting the tube – do not vortex!
7. Pipet the Dnase I incubation mix (80 μl) directly onto the Rneasy silica-gel membrane, and place on bench top for 15 min.
8. Pipet 350 μl RW1 Buffer into the column, and centrifuge for 15 sec at max rpm at RT. Discard the flow-through.
9. Add 500 μl RPE Buffer and centrifuge for 15 sec at max rpm at RT in the microcentrifuge. Discard the flow-through and re-use the collection tube in the next step.
10. Pipet 500 μl RPE Buffer onto the spin column. Centrifuge for 2 min at max rpm in the microcentrifuge at RT to dry the Rneasy membrane.
11. Transfer the spin column to a new 2 ml collection tube and discard the old collection tube with the filtrate. NO EtOH CARRY-OVER!!!
12. Centrifuge for 1 min at max rpm at RT.
13. Transfer the Rneasy column into a 1.5 ml Eppendorf tube. Pipet 50 μl of the pre-heated (65°C) Rnase-free H₂O directly to the center of the column membrane. Let it sit on the column for 1-2 min. Centrifuge for 1 min at max rpm at RT.
14. Apply another 50 μl of the pre-heated H₂O to the center of the membrane. Let it sit for 1-2 min. Centrifuge for 1 min at max rpm at RT. $V_{\text{total}} = 100 \mu\text{l}$.
15. Vortex samples and spin down for a few seconds and measure OD₂₆₀ (498 μl TE + 2 μl sample; Dilution factor = 500/2; blank 500 μl TE). Concentration is need for determining the amount of RNA to use for the formaldehyde gel.

$$\text{Concentration } (\mu\text{g}/\mu\text{l}) = \text{OD}_{260} \times 40 \times \text{dilution factor} \times 1/1000$$

$$\text{Yield} = \text{Concentration } (\mu\text{g}/\mu\text{l}) \times \text{volume } (\mu\text{l})$$

Ethanol precipitation of samples

Reagents (all Rnase free):

3M NaAc or 7.5 M NH₄Ac (ammonium acetate)
Abs. EtOH
75% EtOH
Glycogen 5 mg/ml
TE buffer

Procedure:

1. Take out 1 µg RNA for the formaldehyde gel and proceed with the rest of the sample.
2. Add 2.5 x vol. of 100% EtOH and ½ x vol of 7.5 M NH₄ Ac and 2 ul Glycogen to the samples and leave to precipitate for at least 2-4 hrs (but preferably overnight) at -80°C.
2. Spin for 30 min at max rpm in the microcentrifuge at 4°C.
3. Remove the supernatant. Add 500 µl 75% EtOH (-20°C) and spin for 5 min at max rpm at 4°C.
4. Remove the supernatant and repeat the washing step.
5. Remove the supernatant. Air-dry the pellet for 7-8 min.
6. Dissolve pellet in 12-20 µl DEPC-H₂O depending on the size of the pellet (start with 12 µl, if unable to dissolve, progressively add 1 µl aliquots) and your planned downstream uses. For cDNA reaction for arrays, maximum volume of RNA you can add to reaction is 8 ul, so you want to keep sample very concentrated.
7. Measure OD₂₆₀ (1µl sample + 499 µl TE)

Samples ready for RT/IVT-PCR.

Use 5-10 µg (not more) for RT.

Choose amount you will use for RT.

Try to save several µg for future PCR and other experiments.

Precipitate this remaining RNA and store @ -80°C.

4. Formaldehyde gel

<u>Samples:</u>	<u>RNA Ladder:</u>	<u>Running Buffer:</u>	<u>1% Agarose gel:</u>
1 ug RNA (add DEPC-H ₂ O~3ul)	4 ul Ladder (1 ug/ul)	440 ml ddH ₂ O	1 g Agarose
2 ul EtBr (1 mg/ml)	2 ul EtBr (1 mg/ml)	25 ml 10x MOPS	85 ml DEPC-H ₂ O
2 ul Formaldehyde dye	2 ul Formaldehyde dye	5 ml Formaldehyde (37%)	10 ml 10x MOPS
9 ul Running Buffer	6 ul Running Buffer		

Gel preparation:

1. In an Erlenmeyer flask dissolve 1 g agarose in 85 ml DEPC-H₂O and 10 ml 10x MOPS (this is for a small-size gel, scale up if using a larger tray).
2. Microwave “slow” for 2 min. Swirl the solution every 30 sec. Cool in a bath to 50° or let it sit for app. 10 min.
3. In the fume hood add 2.5 ml Formaldehyde (37%) (to denature) with sterile pipette and cover with Kimwipes immediately and swirl.
4. Pour solution into tray (pre-washed with with RNA ZAP and DEPC-H₂O) – remove bubbles with Kimwipes. Let it sit for 1 hr. Pour 1x MOPS from the sides to cover the gel.

Loading:

1. Add the above-mentioned 4 ingredients to an Eppendorf tube (Rnase-free) for each sample and ladder respectively.
2. Heat at 67° for 10 min and put on ice
3. Load samples. Run gel @ 75 V in 1x MOPS for 40 min (3/4 down the gel).
4. View the gel under UV light and take a picture (for Affy Core lab)