RNA extraction from DRD Blood collection tubes using the miRNeasy Micro Kit

(Qiagen)

Materials

- DRD Blood collection tube
  - #DRDB_1: vacuum tube to collect 1 mL of venous blood in 3 mL of DRDB preservation buffer, or
  - #DRDB_2: microtube to collect 50 µL of capillary blood in 150 µL of DRDB preservation buffer
- miRNeasy Micro Kit *(#217084, Qiagen)*
- isopropanol *(#1096342511, Merck)*
- chloroform *(#032614.K2, Thermo Fisher Scientific)*
- ethanol 100 % *(#1009831011, Merck)*
- RNase-Free DNase set *(#79254, Qiagen)*
- basic vortex mixer *(#88882012, Thermo Fisher Scientific)* or equivalent
- mini centrifuge mySPIN12 *(#75004081, Thermo Fisher Scientific)* or equivalent centrifuge
- Sigma 4-16K, laboratory benchtop centrifuge (at 4 °C) *(#SC-00664370-01, Analis)* or equivalent centrifuge
- ERLAB filtration operator *(#64194-01, Erlab)* or equivalent fume hood
- safe lock tubes 2.0 mL *(#0030123620, Eppendorf)* or equivalent
- optional:
  - bacteriophage MS2 RNA (0.8 µg/µL) *(#10165948001, Merck)*
  - MS2 forward primer (GCTCTGAGAGCGGCTCTATTG) and reverse primer (CGTTATAGCGGACCGCGT) and optional probe (CCGAGACCAATGTGCGCCGTG) (O'Connell et al., Applied and Environmental Microbiology, 2006)

Note: This protocol is modified from the 'miRNeasy Serum/ Plasma Handbook' (April 2022, Qiagen) and 'miRNeasy Micro handbook' (March 2021, Qiagen), including DNase treatment according to page 42 'Procedure: DNase digestion for samples containing <1 µg total RNA approximately'.

Note: Buffer RWT should be prepared with isopropanol instead of ethanol. If the entire kit will not be used for RNA extraction from DRD Blood tubes, or if you use an open kit in which RWT was prepared with ethanol, it will be necessary to purchase additional Buffer RWT (1067933, Qiagen).

Protocol

1. Draw fresh blood into DRD Blood collection tube, or use DRD Blood tube stored at 4 °C (for up to 30 days) or at 25 °C (for up to 3 days), or thaw a tube stored frozen between -20 °C and -80 °C. Mix tube content by inverting 5 times.

   Note: **To maintain RNA integrity optimally, a frozen DRD Blood tube should be thawed in the fridge (4-8 °C). Consider approximately 2 hours for a full 5 mL tube (shorter times for smaller volumes). Make sure the entire content is liquid after thawing.**

2. Add 1000 µL QIAzol Lysis Reagent (optionally spiked-in with 16 µL MS2 RNA (3.2 fg/µL) as control) into a clean 2 mL microtube (not provided) and add 200 µL of stabilized blood. Mix by vortexing or pipetting up and down.

   Note: **The remainder of unused DRD Blood can be frozen. Limit freeze-thaw to 5 cycles. Aliquot DRD Blood into smaller volumes if more than 5 freeze-thaw cycles are expected.**

3. Incubate the microtube containing the lysate at room temperature (15-25 °C) for 5 min.
4. Add 200 µL chloroform to the tube containing the lysate and cap it securely. Vortex or shake vigorously for 15 s.
5. Incubate the tube containing the lysate at room temperature for 2-3 min.
6. Centrifuge for 15 min at 4025 RCF at 4 °C.

   Note: **After centrifugation, heat the centrifuge to room temperature if the same centrifuge is used for the next centrifugation step.**

   Note: **Upon centrifugation, the sample separates into 3 phases: an upper, colorless, aqueous phase containing RNA; a white interphase; and a lower, red, organic phase.**

7. Transfer 600 µL of the upper aqueous phase to a new 2 mL collection tube (not supplied). Avoid transfer of any interphase material. Add 900 µL of 100% ethanol and mix thoroughly by pipetting up and down several times. Do not centrifuge. Continue without delay with step 8.

   Note: **A precipitate may form upon addition of ethanol, but this will not affect the procedure.**

8. Pipet up to 700 µL of the sample, including any precipitate that may have formed, into an RNeasy MinElute spin column in a 2 mL collection tube (supplied). Close the lid gently and centrifuge at 4025 RCF for 15 s at room temperature. Discard the flow-through. Reuse the collection tube in step 9.
9. Repeat step 8 using the remainder of the sample. Discard the flow through. Reuse the collection tube in step 10.
10. Pipet 350 µL Buffer RWT (prepared with 45 mL isopropanol) into the RNeasy Mini Spin Column and centrifuge for 15 s at 4025 RCF to wash. Discard the flow-through. Reuse the collection tube in step 12.
11. For each sample, add 10 µL DNase I stock solution to 70 µL Buffer RDD. Mix by gently inverting the tube. Do not vortex.

    Note: **Make sufficient DNase I incubation mix for all samples to be extracted.**

12. Pipet the DNase I incubation mix (80 µL) directly onto the RNeasy Mini Spin Column membrane and incubate at 20-30 °C for 15 min.

    Note: **Make sure to pipet the DNase I incubation mix directly onto the RNeasy membrane. DNase digestion will be incomplete if part of the mix sticks to the walls or the O-ring of the RNeasy Mini Spin Column.**

13. Pipet 500 µL Buffer RWT (prepared with isopropanol) into the RNeasy Mini Spin Column and centrifuge for 15 s at 4025 RCF. Keep the flow-through.
14. Reapply the flow-through to the RNeasy Mini Spin Column and centrifuge for 15 s at 4025 RCF. Discard the flow-through.
15. Pipet 500 µL of 80% ethanol onto the RNeasy MinElute spin column. Close the lid gently and centrifuge for 2 min at 4025 RCF to wash the spin column membrane. Discard the collection tube with the flow-through.
16. Place the RNeasy MinElute spin column into a new 2 mL collection tube (supplied). Open the lid of the tube with the spin column, and centrifuge at 4025 RCF for 5 min to dry the membrane. Discard the collection tube with the flow-through.
17. Place the RNeasy MinElute spin column in a new 1.5 mL collection tube (supplied). Add 16 µL RNase-free water directly to the center of the spin column membrane. Close the lid gently, and centrifuge for 2 min at 4025 RCF to elute the RNA.

Quality control

1. Intact RNA is expected, visualized by clear presence of 18S and 28S ribosomal RNA bands, respectively ~2 kb and 5 kb in length. RNA integrity values (RIN) depend on instrument and RNA concentration, and should be in the range of 6.5-8.

   Note: **Low RNA concentration and the presence of small RNAs (e.g. microRNAs) generally result in somewhat lower RIN values**

   Note: **While RIN ≥ 5 is recommended for RT-qPCR quantification (Becker et al., Methods, 2010), other studies indicate that the level of acceptable RNA integrity depends on factors such as the expression difference, the target abundance, the intra-group variability, the sensitivity to degradation of the target, and the gene expression measurement method (Vermeulen et al., Nucleic Acids Research, 2011).**

2. Using UV spectrophotometry, the RNA eluate concentration is expected between 20 and 35 ng/µL.

   Note: **While frequently reported, 260/280 and 260/230 absorbance ratios are not reliable metrics of nucleic acid purity, especially for concentrations ≤ 20 ng/µL and when eluted in water (Wilfinger et al., Biotechniques, 1997; Shim et al. Biopreserv Biobank, 2010; Okamoto et al., Int J Mol Med, 2000; Unger et al., Anal Biochem, 2019; Koetsier and Cantor, New England Biolabs Technote, 2019: https://bit.ly/42UC6wP).**

3. As the same amount of MS2 spike-in RNA was (optionally) added to the lysate, it can be used to evaluate RNA extraction efficiency and general purity of the RNA (absence of inhibitors). When doing RT-qPCR, the same MS2 Cq value is expected when using the same input volume of RNA. An endogenous reference RNA can also be used for functional RNA qualification, but because of known biological differences of endogenous reference genes, more variability is expected when comparing such Cq values across different blood samples.

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