RNA extraction from DRD Blood collection tubes using VAMNE Magnetic Cell/Tissue Total RNA Kit

(Vazyme)

Introduction

We provide two protocols for extracting high-quality RNA from 200 µL stabilized blood. The protocols are compatible with blood from the DRD Blood venous collection tube (4 mL of stabilized blood) and the DRD Blood microtube (200 µL of stabilized blood). Protocol A is an automated procedure for bead-based purification; protocol B is the manual version. Both protocols are based on the kit's manual (RMA101-C2 version 24.1)

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 or transfer 50 µL of blood from EDTA or citrate tube into 150 µL of DRDB preservation buffer

    Note: **For maximal preservation of RNA stability, blood from a venous EDTA or citrate collection tube should be transferred immediately to the DRD Blood microtube. A disposable 50 µL pipette (#612-5849, Exact Volume Transfer Pipette, VWR) is a convenient way to transfer blood at the site of collection.**

- VAMNE magnetic Cell/Tissue total RNA kit (#RMA101-C2, Vazyme)
- glycogen (#10901393001, Roche)
- 2-mercaptoethanol(#M6250, Sigma)

  Note: **the 5 µL beta-mercaptoethanol can be effectively replaced with 20 µL TCEP 0.5 M (#77720 Thermo Fisher Scientific).**

- fume hood
- aluminum sealing film for microplates (e.g. #BR791381-100EA, BRAND) (for protocol A only)
- 96-well PCR plate (e.g. #BR781368-50EA, BRAND) (for protocol A only)
- plate shaker (for protocol A only)
- PurePrep 32 instrument (or similar device, e.g. Kingfisher) (for protocol A only)
  - tip combs (#MDPL00310200, Magtivio)
  - deep well plates (#MDPL00300050, Magtivio)

    Note: **Since 2-mercaptoethanol is used, the instrument should ideally be operated in a fume hood.**

- manual magnetic bead separator (for protocol B only)
- thermoshaker (e.g. Provocell, ESCO) (for protocol B only)
- 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)

Protocol A – 200 µL blood - automated

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.
2. Pipette 200 µL stabilized blood of the first 8 samples into the first column of the first deep well plate. For more samples, use column 7 of the same plate, and columns 1 and 7 of the second plate.

   Note: **The PurePrep 32 instrument can handle 32 extractions simultaneously using two deep-well plates. Adjust the setup (# of plates and position of samples and buffers according to the instructions of your magnetic bead purification system; see also kit manual RMA101-C2).**

3. For each sample, create the following preprocessing solution (in a fume hood).
   Make a master mix with 10% volume excess:
   - a. 30 µL proteinase K
   - b. 5 µL 2-mercaptoethanol
   - c. 5 µL glycogen (1 µg/µL)
   - d. 260 µL lysis buffer per sample
   - e. optional: add 3.2 fg MS2 RNA (~1500 copies)
4. Add 300 µL preprocessing solution to each sample in the plate.
5. Incubate for 10 minutes at room temperature whilst shaking at 400 rpm.
6. For each sample, combine 47 µL of RDD buffer with 3 µL of DNase I and mix gently.
   Make a master mix with 10% volume excess.
7. After incubation (step 5), fill the deep-well plate with DNase I mix, magnetic beads, wash and elution buffers as follows:

position	reagent	volume per well (µL)
column 1 and 7	*sample + preprocessing solution*	*500*
column 2 and 8	*beads + DNAse I mix*	*135 + 50*
column 3 and 9	*wash buffer 1*	*700*
column 4 and 10	*wash buffer 2*	*700*
column 5 and 11	*wash buffer 3*	*700*
column 6 and 12	*elution buffer*	*50-100*

Note: **Magnetic beads should be fully mixed before dispensing. Fifty µL is the minimal elution volume on most instruments. If you experience inconsistent results, increase to 75 µL.**

8. Place the deep well plate in the device in the correct orientation and insert the combs.
9. Run the total RNA extraction protocol. This can be obtained by contacting info@inactivblue.com or by manually entering the information as outlined in the table at the end of this protocol.
10. At the end of the automated procedure, transfer the eluate to a clean, RNase-free tube or plate for direct use in downstream experiments, or store it at -20 °C for short-term storage or at -80 °C for long-term storage.

step	position	name	mixing time (min)	adsorption time (s)	waiting time (min)	volume (µL)	mixing speed	temperature (°C)	mixing position	mixing amplitude	adsorption position	adsorption speed
1	*1*	*lysis*	*5*	*0*	*0*	*1000*	*8*	*58*	*10*	*80*	*0*	*10*
2	*2*	*beads*	*0.5*	*30*	*0*	*200*	*8*	*-*	*10*	*80*	*0*	*10*
3	*1*	*binding*	*2*	*60*	*0*	*1000*	*8*	*58*	*10*	*80*	*0*	*10*
4	*3*	*wash 1*	*1*	*30*	*0*	*700*	*8*	*-*	*10*	*80*	*0*	*10*
5	*2*	*DNase I*	*10*	*30*	*0*	*200*	*8*	*-*	*10*	*80*	*0*	*10*
6	*3*	*wash 1*	*1*	*30*	*0*	*700*	*8*	*-*	*10*	*80*	*0*	*10*
7	*4*	*wash 2*	*1*	*30*	*0*	*700*	*8*	*-*	*10*	*80*	*0*	*10*
8	*5*	*wash 3*	*1*	*30*	*5*	*700*	*8*	*-*	*10*	*80*	*0*	*10*
9	*6*	*elution*	*2.5*	*60*	*0*	*50-100*	*8*	*75*	*10*	*80*	*0*	*10*
10	*2*	*beads*	*0.1*	*0*	*0*	*200*	*8*	*-*	*10*	*80*	*0*	*10*

Protocol B – 200 µL - manual

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.
2. Pipette 200 µL DRDB stabilized blood into a 1.5 mL microtube.
3. For each sample create the preprocessing solution (in a fume hood):
   - a. 30 µL proteinase K
   - b. 5 µL 2-mercaptoethanol
   - c. 5 µL glycogen (1 µg/µL)
   - d. 260 µL lysis buffer per sample
   - e. optional: add 3.2 fg MS2 RNA (~1500 copies)
4. Add 300 µL pre-processing solution to each sample.
5. Homogenize and incubate for 5 minutes at room temperature.
6. Add 500 µL of binding solution and incubate at 58 °C for 5 minutes whilst shaking at 1000 RPM.
7. For each sample, combine 47 µL RDD with 3 µL DNase I and mix gently.
8. Add 135 µL of binding beads and 50 µL of DNAse I solution to a fresh tube.
9. Place the tube on a magnetic separator for 2 minutes. Remove the DNAse I and bead buffer and transfer to a fresh tube.
10. Remove the tube from the magnetic separator.
11. Add the lyzed sample (1 mL, step 6) to the beads. Homogenize thoroughly. Incubate at 58 °C for 5 minutes whilst shaking at 1000 RPM.
12. Place the tube on a magnetic separator for 2 minutes. Discard the supernatant.
13. Remove the tube from the magnetic separator.
14. Add 350 µL wash 1 solution to the beads and homogenize.
15. Place the tube on a magnetic separator for 2 minutes. Discard the supernatant.
16. Remove the tube from the magnetic separator.
17. Add 150 µL of DNase I solution and bead buffer (from step 9). Incubate for 10 minutes at room temperature with occasional homogenization (e.g. vortex or thermoshaker).
18. Place the tube on a magnetic separator for 2 minutes. Discard the supernatant.
19. Remove the tube from the magnetic separator.
20. Add 350 µL wash 1 solution to the beads and homogenize.
21. Place the tube on a magnetic separator for 2 minutes. Discard the supernatant.
22. Remove the tube from the magnetic separator.
23. Add 700 µL wash 2 solution to the beads and homogenize.
24. Place the tube on a magnetic separator for 2 minutes. Discard the supernatant.
25. Remove the tube from the magnetic separator.
26. Add 700 µL wash 3 solution to the beads and homogenize.
27. Place the tube on a magnetic separator for 2 minutes. Discard the supernatant.
28. Let the beads dry for 5 minutes at room temperature.
29. Remove the tube from the magnetic separator.
30. Add 50-100 µL of elution buffer to the beads and incubate at 75 °C for 2.5 minutes whilst shaking at 600 RPM.

31. Place the tube on a magnetic separator for 2 minutes.

32. Transfer the eluate to a clean, RNase-free tube or plate for direct use in downstream experiments, or store at -20 °C for short-term storage or at -80 °C for long-term storage.

Note: **If traces of magnetic beads remain in the eluate, they can be removed with a magnetic stand.**

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 may 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 10 and 15 ng/µL (for 200 µL input and 50 µL elution buffer).

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, 2019).**

Note: **If a higher RNA concentration is required for the intended application, the RNA can be precipitated (Walker and Lorsch, 2013) or concentrated using an ultrafiltration column (e.g., Vivacon 500, 10K MWCO Hydrosart, #VN01H02, Sartorius; or Nanosep centrifugal device with Omega membrane 10K, #OD010C34, Cytiva) or an ultrafiltration plate (e.g., AcroPrep Advance 96 multi-well filter plate - 350 µL, Omega 10K MWCO, #8033, Cytiva).**

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.

References

- Becker et al., Methods, 2010 (https://doi.org/10.1016/j.ymeth.2010.01.010)
- Koetsier and Cantor, New England Biolabs Technote, 2019 (https://bit.ly/42UC6wP)
- Okamoto and Okabe., Int J Mol Med, 2000 (https://doi.org/10.3892/ijmm.5.6.657)
- O'Connell et al., Applied and Environmental Microbiology, 2006 (https://doi.org/10.1128/AEM.72.1.478-483.2006)
- Shim et al. Biopreserv Biobank, 2010 (https://doi.org/10.1089/bio.2010.0016)
- Unger et al., Anal Biochem, 2019 (https://doi.org/10.1016/j.ab.2019.02.002)
- Vermeulen et al., Nucleic Acids Research, 2011 (https://doi.org/10.1093/nar/gkr065)
- Walker and Lorsch, Methods Enzymol., 2013 (https://doi.org/10.1016/b978-0-12-420037-1.00019-1)
- Wilfinger et al., Biotechniques, 1997 (https://doi.org/10.2144/97223st01)

FertiPro NV
Industriepark Noord 32
B-8730 Beernem
Belgium

VAT BE0448 767 035
+32 (0)50 79 18 05
info@inactivblue.com
www.inactivblue.com