PRODUCT: **RNAzol® BD for Blood** June 2017
Catalog No: RB 192
Storage: Store at room temperature

PRODUCT DESCRIPTION.

RNAzol® BD is a reagent for the isolation of total RNA, large RNA or small RNA from whole blood, plasma or serum of human or animal origin. The RNA can be used for RNA sequencing and gene expression analysis, as well as RT-PCR, qRT-PCR, microarrays, poly A+ selection or other molecular biology applications. In addition, the reagent allows for simultaneous isolation of RNA and DNA from the same biological sample. The outstanding effectiveness and versatility of RNAzol® BD makes it the most advanced and reliable reagent in this category.

RNAzol® BD has been used to determine RNA content in human blood, which averages 14 µg/ml of whole blood and ranges from 6 - 22 µg/ml (5). These levels of RNA are significantly higher than previously reported with other methods that average only 2 - 5 µg of total RNA per ml of human blood. RNAzol® BD provides an unsurpassed yield of either total RNA, large RNA (> 150 - 200 bases) or small RNA (< 150 - 200 bases). The small RNA constitutes about 15 - 30 % of the total RNA. RNAzol® BD provides an opportunity to isolate and investigate all RNA present in whole blood, and not a small fraction as isolated by other methods. RNAzol® BD isolates 20 - 150 µg of RNA/ml of animal blood depending on the species.

RNAzol® BD (US patents, 1, 2) is based on an improved single-step method to provide the highest yield and purity of isolated RNA, and is more effective than previous reagents (3, 4). RNAzol® BD is a monophase solution containing acidic phenol and guanidine thiocyanate. A blood, plasma or serum sample is lysed in RNAzol® BD and RNA is separated from other molecules in a single-step by precipitating DNA, proteins, polysaccharides and other cellular components. Pure RNA is precipitated from the aqueous supernatant by the addition of isopropanol. The RNA pellet is washed and solubilized. Use 1 ml of RNAzol® BD to process 0.5 ml of blood in less than one hour.

STABILITY/STORAGE: RNAzol® BD is stable at room temperature for at least two years from date of purchase.

SPECIAL HANDLING PRECAUTIONS.
RNAzol® BD contains phenol (poison, corrosive) and guanidine thiocyanate (irritant). CAUSES BURNS. Can be fatal. When working with RNAzol® BD, use gloves and eye protection (face shield, safety goggles). Do not get on skin or clothing. Avoid breathing fumes. Read the warning on the container and SDS. **In case of contact:** Immediately flush eyes or skin with a large amount of water for at least 15 minutes and if necessary seek medical attention.

I. PROTOCOLS FOR RNA ISOLATION.
Reagents required but not supplied: 1-bromo-3-chloropropane (BCP, MRC cat. BP151), acetic acid, isopropanol, ethanol and RNase-free water. We recommend the use of disposable polypropylene tubes (see MRC catalog). Other tubes should be tested to ensure integrity during centrifugation at 12,000 g with RNAzol® BD.

Isolation of total RNA - Abbreviated Protocol.

1. Lysis	- 1 ml blood + 2 ml RNAzol® BD + 27 µl acetic acid, shake 30 sec, 15 min at 37 - 40 C.
2. DNA/protein removal	- 3 ml lysate + 150 µl BCP, mix, store 15 min at RT, 12,000 g x 15 min at RT.
3. Total RNA precipitation	- 1.5 ml supernatant + 1.5 ml isopropanol, mix, store 15 min at RT, 12,000 g x 15 min.
4. RNA washes	- 0.4 - 0.8 ml 75% ethanol, mix, 12,000 g x 1 - 3 min at RT; wash twice.
5. RNA solubilization	- water or FORMAzol®.

Except for lysate incubation in Step 1, centrifugation and all other steps are performed at room temperature.

Isolation of large RNA and small RNA in separate fractions - Abbreviated Protocol.

1. Lysis	- 1 ml blood + 2 ml RNAzol® BD + 27 µl acetic acid., shake 30 sec, 15 min at 37 - 40 C.
2. DNA/protein removal	- 3 ml lysate + 150 µml BCP, mix, store 15 min at RT, 12,000 g x 15 min at RT.
3. Large RNA precipitation	- 1.5 ml supernatant + 0.525 ml isopropanol, mix, store 15 min at RT, 12,000 g x 15 min.
4. Large RNA washes	- 0.4 - 0.8 ml 75% ethanol, mix, 12,000 g x 1 - 3 min at RT; wash twice.
5. Large RNA solubilization	- water or FORMAzol®.
6. Small RNA precipitation	- large RNA supernatant + 0.975 ml isopropanol, mix, store 30 min, 12,000 g x15 min.
7. Small RNA washes	- 0.8 ml 70% isopropanol, mix, 12,000 g x 3 min at RT; wash twice.
8. Small RNA solubilization	- water or FORMAzol®.

Except for lysate incubation (Step 1) and small RNA precipitation (Step 6), all steps are performed at room temperature.

1. LYSIS.
Collect whole blood into a vacuum tube (preferentially with EDTA as an anticoagulant) and immediately transfer blood aliquots to tubes containing RNAzol® BD and acetic acid. Add 1 ml of blood to 2 ml of the reagent and 27 µl of glacial acetic acid. Tightly cover the tube and vigorously shake the blood - RNAzol® BD mixture for 30 seconds. Please see Note 4 For RNA isolation from plasma or serum.
Vigorous shaking is important to ensure efficient removal of DNA from the aqueous phase.
The resulting lysate can be stored at -70 C for at least 2 years and at -20 C at least 6 months. After removing stored lysate from the freezer, thaw samples with shaking.

Alternatively, store aliquots of blood at -70 C. For example, transfer 1 ml aliquots of blood into 5 ml tubes and store at -70 C. For processing, add 2 ml of RNAzol® BD and 27 µl of glacial acetic acid to the frozen blood without thawing. Place the tubes in warm water (> 55 C) and thaw the contents with shaking.
Do not thaw blood samples without the reagent. This will lead to RNA degradation.
Blood adheres to glass and plastic surfaces during pipetting. This results in 5 - 10% loss in blood volume. A correction can be made by determining the weight of aliquoted blood samples. Human blood density is 1.06 g/ml.

2. DNA, PROTEIN AND POLYSACCHARIDE PRECIPITATION.
This protocol describes processing 3 ml of the lysate (1 ml blood + 2 ml RNAzol® BD) using 5 ml tubes. Incubate the lysate for 15 minutes at 37 - 40 C (or room temperature for a frozen lysate after thawing). Following incubation, supplement 3 ml of lysate with 150 µl of BCP (1-bromo-3-chloropropane). Tightly cover the tube and vigorously shake the mixture for 30 seconds. Store the sample for 15 minutes at room temperature and centrifuge the lysate at 12,000 g for 15 minutes at room temperature. Following centrifugation, DNA, proteins and polysaccharides are sequestered in the interphase and phenol phase at the bottom of the tube. RNA remains soluble in the aqueous supernatant at the top of the tube.
Centrifugation of the lysate at temperatures below 15 C results in a decrease of RNA yield.
Remove the remaining aqueous phase and store the interphase/phenol phase at 4 or -20 C for DNA isolation.

3. RNA PRECIPITATION.
Step 3A isolates total RNA in one precipitation step. Steps 3B and 3C isolate large RNA and small RNA in separate fractions in two sequential precipitation steps.
Samples containing < 0.5 ml of blood require addition of 1 - 2 µl of Polyacryl Carrier (PC 152) to improve RNA recovery and sample handling.
See Note 3 regarding the isolation of low quantities of RNA (< 5 µg).

3A. TOTAL RNA PRECIPITATION.
This step precipitates total RNA containing large nuclear RNA, rRNA, mRNA, tRNA, small RNA and microRNA.

Transfer 1.5 ml of the aqueous supernatant from Step 2 to a new tube. Leave a 2 - 3 mm layer of supernatant above the interphase/phenol phase to avoid disturbing the lower phases. Mix the transferred supernatant with an equal volume of isopropanol (1.5 ml) to precipitate total RNA. Store the sample for 15 minutes at room temperature and centrifuge at 12,000 g for 15 minutes at room temperature. RNA precipitate forms a white gel-like pellet at the bottom of a tube. Immediately after centrifugation, the RNA pellet adheres to the side of the tube and most of the supernatant can be removed by decanting. Remove the remaining supernatant from the tube with a micropipette.
It is safe to collect up to 85% of the aqueous supernatant without disturbing the interphase/phenol phase.
The aqueous supernatant has a density of 1.06 g per ml. If necessary, verify the volume of supernatant used by its weight.
1 ml of the supernatant is equivalent to 0.55 ml of blood.
1 ml of the supernatant-isopropanol solution is equivalent to 0.275 ml of blood in the total RNA precipitation.

3B. LARGE RNA PRECIPITATION.
This step precipitates RNA > 150 - 200 bases, including large nuclear RNA, rRNA and mRNA. Due to multiple splicing sites, some small RNA can be detected in both the large and small RNA fractions.

Transfer 1.5 ml of the aqueous supernatant from Step 2 to a new tube. Leave a 2 - 3 mm layer of the supernatant above the interphase/phenol phase to avoid disturbing the lower phases. Mix the transferred supernatant with 0.525 ml (0.35 volumes) of isopropanol to precipitate the large RNA fraction. Store sample for 15 minutes at room temperature and centrifuge at 12,000 g for 15 minutes at room temperature. RNA precipitate forms a clear gel-like pellet at the bottom of a tube, which becomes white and visible during the ethanol wash step. Immediately after centrifugation, the RNA pellet adheres to side of the tube and most of the supernatant can be removed by decanting. Remove the remaining supernatant from the tube with a micropipette. Retain the removed supernatant in a new tube and store at -20 or -70 C for subsequent isolation of the small RNA fraction from this isopropanol-supernatant.
1 ml of the supernatant is equivalent to 0.55 ml of blood.
1 ml of the supernatant-isopropanol solution is equivalent to 0.407 ml of blood in the large RNA fraction precipitation.

3C. SMALL RNA PRECIPITATION.
This step precipitates RNA < 150 - 200 bases including small rRNA, tRNA, small RNA and microRNA down to 10 bases.

Supplement the 2.025 ml of supernatant obtained in Step 3B with 0.975 ml (0.481 volumes) of isopropanol. This makes a total volume of 3 ml with a final isopropanol concentration of 50% (v/v). Mix well and store samples at room temperature or 4 C for 30 minutes. Sediment precipitated RNA at 12,000g for 15 minutes at room temperature. RNA forms a white pellet at the bottom of a tube.

4. RNA WASH.
When using larger volume tubes, it is beneficial to transfer the RNA pellet into a microcentrifuge tube for the wash steps. Add 0.4 - 0.8 ml of wash solution. Gently resuspend the RNA pellet and transfer it to a microcentrifuge tube using a 1 ml pipette tip. Before transfer, cut the end of the tip to create a sufficiently large opening to collect the RNA pellet into the tip.

4A. WASHING TOTAL RNA OR LARGE RNA FRACTION PELLET.
Use 75% ethanol (v/v) for wash steps. Use 0.4 ml for washes in microcentrifuge tubes; for washes in larger tubes use 0.5 ml of ethanol wash per 1 ml of supernatant used for precipitation. Mix the pellet in the wash solution and centrifuge at 12,000 g for 1 - 3 minutes. After each wash, use a pipette tip to remove the remaining wash solution. Perform two washes.

4B. WASHING SMALL RNA FRACTION PELLET.
Use 70% isopropanol (v/v) for wash steps. Use 0.4 ml for washes in microcentrifuge tubes; for washes in larger tubes use 0.5 ml of isopropanol wash per 1 ml of supernatant used for precipitation. Mix the pellet and store for 2 - 3 minutes, followed by centrifugation at 12,000 g for 2 - 3 minutes. After each wash, use a pipette to remove the remaining wash solution. Perform two washes.

5. RNA SOLUBILIZATION.
Dissolve the RNA pellet, without drying, in water or FORMAzol® (MRC, FO 121). For solubilization in water, vortex the RNA pellet at room temperature for 3 - 5 minutes. Tubes and water used for the RNA solubilization should be RNase-free. For solubilization in FORMAzol®, mix the solution for 10 minutes on a vortex platform.
Drying the RNA pellet is not recommended as this greatly decreases its solubility.
Avoid injury to hand joints by using a vortex equipped with a platform attachment to hold the tubes.

Total RNA precipitation yields all classes of RNA. Expected yield is 8 - 22 µg of total RNA and 5 - 16 µg of the large RNA fraction per 1 ml of human blood with a normal white cell count. Human blood with an elevated white cell count may yield > 20 µg total RNA/ml blood. RNAzol® BD isolates 20 - 90 µg of RNA/ml from animal blood.

The results of RT-qPCR can be calculated per µg of RNA and/or per ml of blood. Due to the wide range of RNA content in whole blood, calculation of gene activity per ml of blood more accurately reflects accumulation of a gene product in the circulation. (5) This calculation is especially important when evaluating expression of signaling molecules/cytokines such as interleukins, TGFs and TNFs.

The total RNA or large RNA fraction has a 260/280 ratio of 1.7 - 2.0 and a 260/230 ratio of about 2. The RIN value of human blood mixed with the reagent immediately after collection is about 7.0 - 7.5 for total RNA and 7.0 - 8.5 for the large RNA fraction. The small RNA fraction has a 260/280 ratio of about 1.7 and 260/230 ratio of about 1.5. Typically for human blood, the small RNA fraction constitutes 15 - 30 % of the total RNA. This corresponds to 2 - 5 µg of small RNA per 1 ml blood.
For accurate OD measurement using a cuvette, use diluent with a slightly alkaline pH (6). Use 3 mM NaOH or a buffer with a pH >8. Typically, distilled water has an acidic pH.

II. NOTES FOR RNA ISOLATION.

1. The mixing of blood with RNAzol® BD immediately after collection is critical to the integrity and yield of RNA.
2. RNA isolation can be interrupted and samples can be stored as indicated below:

- A sample lysed in RNAzol® BD (Step 1) can be stored at room temperature for at least 3 hours, at 4 C for at least 24 hours, at -20 C for at least 6 months, and at -70 C for at least 2 years. To thaw samples, incubate them at 37 - 50 C for 5 - 10 minutes with intermittent mixing.
- The RNA precipitate can be stored in 75% ethanol overnight at room temperature, for at least one week at 4 C, or at least one year at -20 C.

3. When isolating low quantities of RNA (< 5 µg), residual supernatant adhering to the side of the tube can lower the A260/280 ratio. After centrifugation to obtain the RNA pellet, remove the supernatant from the tube. Briefly centrifuge the tube again to collect residual supernatant and remove it with a micropipette prior to washing steps. Similarly, centrifuge and remove any residual alcohol after the final wash step.
4. For RNA isolation from plasma or serum: Add 1 ml of plasma or serum to 2 ml of RNAzol BD and 10 µl of glacial acetic acid. Vigorously shake for 15 - 30 seconds. (Optionally add 1 µl of Polyacryl Carrier.) Add 150 µl of BCP to the lysate, shake vigorously for 15 - 30 seconds and store for 15 minutes at room temperature. Centrifuge at 12,000 g for 15 minutes. Proceed with total RNA precipitation (Step 3A) and wash (Step 4A). Solubilize the RNA in 10 - 20 µl of water. RNA yield is below the Nanodrop spectrophotometry sensitivity. Use approximately 50% of the RNA product in RT-PCR to obtain detectable reaction product in 25 - 50 cycles, depending on primers and target abundance.

III. RNA ISOLATION TROUBLESHOOTING GUIDE.

Low yield. a) incomplete solubilization of the final RNA pellet, b) after collection, blood sample was not immediately mixed with the reagent, c) too much blood was used for the volume of the reagent, d) RNAzol® BD - blood - BCP lysate was centrifuged at a temperature < 15 C.
260/280 ratio < 1.6. a) ineffective wash of the RNA pellet. Mix samples more vigorously and store samples with 75% ethanol for 2 - 3 minutes at room temperature, b) acidic water was used for the OD measurement, c) incomplete solubilization of the RNA pellet, d) insufficient volume of reagent was used for lysis.
RNA degradation. a) blood was not immediately mixed with the reagent, b) blood was not immediately frozen after collection, c) aqueous solutions or tubes used for solubilization of RNA were not RNase-free.
DNA contamination. a) too much blood was used for the volume of the reagent, b) RNAzol® BD - blood - BCP lysate was centrifuged at a temperature < 15 C.

The RNAzol® BD method can be cited by referring to: RNAzol® BD Brochure, 2017, Molecular Research Center, Inc. Cincinnati, OH. RNAzol®, DNAzol® and FORMAzol® are registered trademarks of Molecular Research Center, Inc.

IV. ISOLATION OF DNA BY RNAzol® BD.

The protocol for DNA isolation with RNAzol® BD is based on re-extraction of DNA sequestered into the interphase/phenol phase during RNA isolation. This method allows for analysis of RNA and DNA derived from the same sample. The isolated DNA can be used for PCR, sequencing and other molecular biology applications. The isolated high-molecular weight DNA is not a good substrate for restriction enzymes.

V. PROTOCOL FOR DNA ISOLATION.

Reagents required, but not supplied: 1-bromo-3-chloropropane (BCP, MRC cat. no. BP 151), isopropanol, ethanol, 4 M guanidine thiocyanate solution (MRC cat. no. GT 192), Polyacryl Carrier (MRC cat. no. PC152) and 3M NaOH.

Isolation of DNA - Abbreviated Protocol.

DNA isolation from the phenol phase/interphase derived from 1 ml of whole blood lysed in 2 ml of RNAzol® BD.

1. DNA re-extraction	- phenol phase/interphase from1ml blood + 1 ml 4M guanidine thiocyanate + 100 µl 3M NaOH - add 5 µl carrier, shake, store 10 min at RT, shake.
2. DNA separation	- add 150 µl BCP, shake, 12,000 g x 10 min at RT.
3. DNA precipitation	- aqueous supernatant + 1 vol isopropanol, mix, store 15 min at RT, 12,000 g x 15 min at RT.
4. DNA washes	- 75% ethanol, mix, 12,000 g x 1 - 3 min at RT.
5. DNA solubilization	- water or TE buffer.

This procedure is carried out at room temperature. Calculation of solution volumes is based on the volume of blood used for RNA isolation.

1. DNA ISOLATION.

Remove the remaining aqueous phase overlying the interphase/phenol phase obtained from Step 2 of the RNA isolation procedure. Extract DNA by adding to the interphase/phenol phase derived from 1 ml of blood: 1 ml of DNA Extraction Solution (MRC cat. no. GT 192; 4M guanidine solution), 0.1 ml of 3M NaOH and 2 µl Polyacryl Carrier (MRC cat. no. PC 152). Tightly cover the tube and vigorously shake for 30 seconds to obtain a fine suspension. Store the mixture for 10 minutes at room temperature and shake for 30 seconds at the end of incubation.

This step solubilizes the DNA precipitated during RNA isolation.

Use a smaller scale processing to quantify DNA. Transfer a 1 ml aliquot of the extraction mixture into a 1.5 ml microcentrifuge tube and process the mixture as described in Note 3 for DNA Isolation below.

2. DNA SEPARATION.

Add to the extraction mixture 150 µl of BCP, tightly cover the tube and vigorously shake for 30 seconds. Centrifuge at 12,000 g for 10 minutes at room temperature. During this step, DNA is sequestered into the upper aqueous supernatant while proteins, carbohydrates and other molecules remain in the lower phenol phase.

3. DNA PRECIPITATION.

Transfer 1 ml the aqueous supernatant into a new 5ml tube and precipitate DNA by mixing with 1 volume of isopropanol. Store at room temperature for 15 minutes and sediment the DNA precipitate at 12,000 g for 15 minutes at room temperature.

1 ml of the supernatant is equivalent to 0.75 ml of blood.

A volume of supernatant used for precipitation also can be estimated by its weight, 1.10 g/ml.

4. DNA WASH.

It is beneficial to transfer the DNA pellet from a large tube into a microcentrifuge tube. After removing the supernatant, gently suspend the DNA pellet in 0.6 - 0.8 ml of 75% ethanol in the large tube. Transfer the DNA pellet to a microcentrifuge tube using a 1 ml pipette tip. Before transfer, cut the end of the tip to create a sufficiently large opening to collect the pellet into the tip. After transfer, sediment the DNA pellet by centrifugation at 12,000 g for 1 - 3 minutes at 4 - 25 C. Wash the DNA pellet a second time with 75% ethanol and centrifuge at 12,000 g for 1 - 3 minutes.

5. DNA SOLUBILIZATION.

Dissolve the DNA pellet in water or a buffer with pH > 7.5 by vortexing or passing the pellet through a micropipette. Add an adequate amount of solution to approach a DNA concentration of 0.1 - 0.3 µg DNA/µl. For small scale isolations use 150 µl water to dissolve DNA pellet. Depending on the intensity of vortexing, it takes 15 - 30 minutes to dissolve the DNA pellet. It takes 15 to 30 minutes to fully hydrate and dissolve the DNA pellet derived from up to 2 ml of blood. Larger DNA pellets require a longer dissolution time. For faster dissolution, add to the DNA aqueous NaOH to a final concentration of 0.5mM. In 0.5 mM NaOH, OD of DNA at 260 remains the same as in water while the 260/280 ratio increases by 10% and the 260/230 ratio decreases by 10%.

6. QUANTITATION OF DNA.

Calculate the DNA content assuming that one A260 unit equals 50 µg double-stranded DNA/ml. For calculation of the cell number in samples, assume that the amount of DNA per 10^6 diploid cells of human, rat and mouse origin equals 6.6 µg, 6.5 µg and 5.8 µg, respectively.

A preparation of DNA isolated from whole blood contains predominantly DNA >100 kb. The isolated DNA is free of RNA and proteins and has a 260/280 ratio >1.8 and 260/230 ratio 1.7 - 2. For human blood, typical recovery of DNA is 35 - 45 µg DNA/ml. 95 - 100 % recovery of DNA is achieved by precipitating DNA in a 1.5 ml microcentrifuge tube (Note 3).

VI. NOTES FOR DNA ISOLATION

1. If necessary, the interphase/phenol phase can be stored at 4 C overnight. Longer storage of the interphase/phenol phase significantly decreases DNA yield. Do not store samples at room temperature. Alternatively, store the interphase/phenol phase at -20 or -70 C. To thaw the samples, add 4M guanidine thiocyanate and 3M NaOH to the frozen sample. Hand shake the sample in 30 - 40 C water until thawed and immediately perform DNA isolation. Storage of interphase/phenol phase at room temperature decreases DNA yield.
2. DNA samples suspended in 75% ethanol can be stored at 4 C for a longer period of time (months). Samples solubilized in water can be stored overnight at 4 C. For prolonged storage, store samples in 1mM Na-EDTA.
3. For a small scale isolation, transfer a 1 ml aliquot of the phenol-guanidine extraction mixture (Step IV-1) into a 1.5 ml microcentrifuge tube. 1 ml of this mixture contains 0.41 ml blood. Supplement the mixture with 61.5 µl BCP, shake for 30 seconds and centrifuge at 12,000 g for 10 minutes at room temperature. Transfer 400 µl of the resulting aqueous supernatant to a new tube, add 400 µl of isopropanol and mix. Store samples for 15 minutes at room temperature and sediment the precipitated DNA at 12,000g for 15 minutes at room temperature. Wash the samples with 75% ethanol and centrifuge at 12,000 g for 1 - 3 minutes at room temperature. Dissolve samples in 150 µl water by vortexing for 15 minutes. Use a vortex platform to hold the tubes. 95 - 100 % recovery of DNA is achieved. Using this protocol, 95 - 100 % of DNA is recovered from blood samples used for RNA extraction.

VI. REFERENCES.

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