PRODUCT: **TRI REAGENT®BD - RNA / DNA / PROTEIN ISOLATION REAGENT for BLOOD DERIVATIVES**
Cat. No: TB 126 March 2017
Storage: Store at 4 - 25 C.

PRODUCT DESCRIPTION

TRI Reagent® BD is a complete and ready to use reagent for isolation of total RNA or for the simultaneous isolation of RNA, DNA and proteins from serum, plasma or whole blood. TRI Reagent® BD is an adaptation of the popular single-step method of total RNA isolation (1,2) permitting fast and efficient processing of blood derivatives. This highly reliable technique performs well with small and large sample volumes, and permits simultaneous processing of a large number of samples. TRI Reagent® BD and the single-step method are subjects of the international patents.

TRI Reagent® BD combines phenol and guanidine thiocyanate in a mono-phase solution to facilitate the immediate and most effective inhibition of RNase. A sample of blood is lysed in TRI Reagent® BD and the lysate is separated into the aqueous and organic phases by the bromochloropropane or chloroform addition and centrifugation. RNA remains exclusively in the aqueous phase, DNA in the interphase, and proteins remain in the organic phase. RNA is precipitated from the aqueous phase by isopropanol, washed with ethanol and solubilized. DNA and proteins are sequentially precipitated from the interphase and organic phase by ethanol and isopropanol, washed of remaining impurities and solubilized.

STABILITY: TRI Reagent® BD is stable at 25 C for at least two years from the date of purchase (3).

SPECIAL HANDLING PRECAUTIONS

TRI Reagent® BD contains poison (phenol) and an irritant (guanidine thiocyanate). Causes burns. CAN BE FATAL. When working with TRI Reagent® BD **use gloves and eye protection** (shield, safety goggles). Do not get on skin or clothing. Avoid breathing vapor. Read the warning note on the bottle and SDS. **In case of contact**: Immediately flush eyes or skin with a large amount of water for at least 15 minutes and seek immediate medical attention.

I. ISOLATION OF RNA

The isolation of total RNA by TRI Reagent® BD from whole blood, plasma and serum **can be completed in 1 h, and the recovery of undegraded RNA is 30 - 150% higher than in other methods of RNA isolation.** TRI Reagent® BD isolates a whole spectrum of RNA molecules rarely observed in RNA isolated by other methods. Typically, column-based methods may artificially change the mRNA composition. TRI Reagent® BD is especially useful for isolating viral RNA. The isolated RNA can be used for northern analysis, dot blot hybridization, poly A^+ selection, in vitro translation, RNase protection assay, molecular cloning, and for polymerase chain reaction (PCR).

PROTOCOL

Reagents required, but not supplied: chloroform or 1-bromo-3-chloropropane (BCP, cat. no. BP 151), isopropanol, ethanol and 5 N acetic acid. We recommend the use of disposable polypropylene tubes provided by Molecular Research Center, Inc. Tubes from other suppliers should be tested to ensure integrity during centrifugation at 12,000 g with TRI Reagent® BD.
The protocol includes the following steps:

1. LYSIS	- 0.75 ml TRI Reagent BD + 0.2 - 0.25 ml whole blood, plasma or serum.
2. PHASE SEPARATION	- homogenate + 0.1 ml BCP or 0.2 ml chloroform.
3. RNA PRECIPITATION	- aqueous phase + 0.5 ml isopropanol.
4. RNA WASH	- 1 ml 75% ethanol.
5. RNA SOLUBILIZATION	- FORMAzol®, 0.5% SDS, or water.

The procedure is carried out at room temperature unless stated otherwise.

1. LYSIS

A. SERUM. Add 0.25 ml of serum and 1 - 4 µl of Polyacryl Carrier (cat. no. PC 152) to 0.75 ml of TRI Reagent BD, close the tube and shake the resulting mixture by hand or vortex.

B. WHOLE BLOOD or PLASMA. Add 0.2 ml of whole blood or plasma to 0.75 ml of TRI Reagent BD supplemented with 20 µl of 5 N acetic acid per 0.2 ml of whole blood or plasma. Close the tube and shake the resulting mixture by hand or vortex.

Acetic acid can be added before or after mixing TRI Reagent BD with blood samples. Prepare 5 N acetic acid by mixing 1 ml of glacial acetic acid (>99%) with 2.48 ml of water. The ratio of the sample volume to the reagent volume should be as indicated in the protocol above. Too large a sample volume will result in DNA contamination, and too a small sample volume will lower the yield of RNA.

2. PHASE SEPARATION

Store the lysed sample for 5 minutes at room temperature to permit the complete dissociation of nucleoprotein complexes. Supplement the lysate with 0.1 ml BCP or 0.2 ml of chloroform per 0.75 ml of TRI Reagent BD. Cover the sample tightly and shake vigorously for 15 seconds. Store the resulting mixture at room temperature for 2 - 5 minutes, and centrifuge at 12,000 g for 15 minutes at 4 C. Following centrifugation, the mixture separates into a lower brownish, phenol phase, an interphase, and a colorless upper aqueous phase. RNA remains exclusively in the aqueous phase whereas DNA and proteins are in the interphase and organic phase. The volume of the aqueous phase is about 60% of the volume of TRI Reagent BD used for lysis.

BCP is less toxic than chloroform and its use reduces the possibility of contaminating RNA with DNA (4). Chloroform used for phase separation should not contain isoamyl alcohol or any other additive.

It is important to perform centrifugation for phase separation in the cold (4 - 10 C). If performed at elevated temperatures, a residual amount of DNA may sequester in the aqueous phase. In this case, RNA can be used for northern analysis but it may not be suitable for PCR.

3. RNA PRECIPITATION

Transfer the aqueous phase to a fresh tube, and save the interphase and organic phase at 4 C for subsequent isolation of DNA and proteins. Precipitate RNA from the aqueous phase by mixing with isopropanol. Use 0.5 ml of isopropanol per 0.75 ml of TRI Reagent BD used for the initial lysis. Store samples at room temperature for 5 - 10 min and centrifuge at 12,000 g for 8 minutes at 4 - 25 C. RNA precipitate forms a gel-like or white pellet at the bottom of the tube.

4. RNA WASH

Remove the supernatant and wash the RNA pellet in 75% ethanol by vortexing. Add 1 ml of 75% ethanol per 0.75 ml of TRI Reagent BD. When the isolation is performed in large tubes (> 2 ml), add 1 ml of 75% ethanol to the RNA pellet and transfer the RNA-ethanol suspension to a microfuge tube. For the transfer, use a wide bore 1 ml pipette tip prepared by cutting 2 - 3 mm from the end of a plastic tip. Centrifuge the RNA suspension at 7,500 g for 5 minutes at 4 - 25 C. If the RNA pellet accumulates on the side of a tube or has a tendency to float, perform the centrifugation at 12,000 g.

5. RNA SOLUBILIZATION

Remove the ethanol wash and briefly air-dry the RNA pellet for 5 minutes. It is important to avoid completely drying the RNA pellet as this will greatly decrease its solubility. **Do not dry RNA by centrifugation under vacuum.** Dissolve RNA in FORMAzol® (stabilized formamide, cat. no. FO 121), water or 0.5% SDS by passing the solution a few times through a pipette tip and incubating for 10 - 15 minutes at 55 - 60 C. Water or the SDS solution used for RNA solubilization should be made RNase-free by diethyl pyrocarbonate (DEPC) treatment. RNA should be precipitated from FORMAzol with ethanol before using for RT-PCR.

6. RESULTS

Ethidium bromide staining of RNA separated in an agarose gel (or methylene blue staining of a hybridization membrane after RNA transfer) visualizes two predominant bands of small (~2 kb) and large (~5 kb) ribosomal RNA, low molecular weight (0.1 - 0.3 kb) RNA. The final preparation of total RNA is essentially free of DNA and proteins and has a 260/280 ratio 1.5 - 1.9. For RT-PCR analysis, DNAse treatment may be necessary for optimal results. Typically, 15 - 20 µg of total RNA can be isolated from 1 ml of human whole blood.

For optimal spectrophotometric measurements, RNA aliquots should be diluted with water or buffer with a pH > 7.5. Distilled water with a pH <7.0 falsely decreases the 260/280 ratio and impedes the detection of protein contamination in RNA samples (6).

NOTES FOR RNA ISOLATION

1. To facilitate isolation of RNA from a small volume of blood derivatives (< 0.2 ml), perform lysis of samples in 0.75 ml of TRI Reagent® BD supplemented with 1 - 4 µl of Polyacryl Carrier (cat. no. PC-152). For sample volumes < 0.2 ml, adjust the volume to 0.2 ml with serum or use a smaller volume of the reagent. When isolating RNA from whole blood or plasma, add 10 µl of 5 N acetic acid per 0.1 ml of whole blood or plasma. Next, add BCP or chloroform and proceed with the phase separation and other steps of isolation as described above.
2. After lysis in TRI Reagent® BD but before BCP addition, samples can be stored at -70 C for at least two years. The RNA precipitate can be stored in 75% ethanol at 4 C for at least one week, or at least two years at -20 C.
3. Cellular RNA degrades rapidly when blood samples are stored at 4 C. For isolation of undegraded RNA, process blood samples immediately after collection, or aliquot and freeze at -70 with or without TRI Reagent® BD. Isolation of viral RNA can be performed with samples stored at 4 C for several days.
4. Hands and dust may be the major source of the RNase contamination. Use gloves and keep tubes closed throughout the procedure.
5. See also Troubleshooting Guide, poly A^+ RNA isolation and RT-PCR application notes on the last page of this booklet.

II. ISOLATION OF DNA BY TRI REAGENT® BD

The protocol for DNA isolation with TRI Reagent® BD is designed to overcome the heavy burden of proteins present in whole blood. DNA is precipitated from the interphase and the phenol phase obtained from the initial lysate as described in the RNA isolation protocol. Following precipitation, the DNA is solubilized in DNAzol®, re-precipitated and washed with ethanol. The final DNA preparation is solubilized in 8 mM NaOH, neutralized, and used for analysis. The isolated DNA can be used for PCR, restriction, Southern blotting, molecular cloning and other molecular biology applications.

PROTOCOL

Reagents required, but not supplied: isopropanol, ethanol, DNAzol® (cat. no. DN 127) and sodium hydroxide.
The protocol includes the following steps:

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1. DNA PRECIPITATION - phenol phase and interphase + 0.4 ml ethanol (per 0.75 ml TRI Reagent® BD)
2. DNA SOLUBILIZATION - 0.25 ml DNAzol®.
3. DNA PRECIPITATION - 0.125 ml ethanol, 2,000 g x 5 min.
4. DNA WASH - 1 ml 95% ethanol x 2 washes.
5. DNA SOLUBILIZATION - 8 mM NaOH.

The procedure is carried out at room temperature unless stated otherwise. The protocol describe isolation of DNA from the phenol phase and interphase of a sample lysed in 0.75 ml of TRI Reagent® BD.

1. DNA PRECIPITATION

Remove the remaining aqueous phase overlying the interphase. Precipitate DNA from the interphase and organic phase with ethanol. Add 0.4 ml of 100% ethanol per 0.75 ml of TRI Regent BD used for the initial lysis, and mix the sample by inversion. Store the sample for 2 - 3 minutes and sediment the DNA by centrifugation at 2,000 g for 5 minutes at 4 - 25 C. Careful removal of any residual aqueous phase is critical for the quality of the isolated DNA.
See Note 4 for an alternative DNA isolation procedure.

2. DNA SOLUBILIZATION

Remove the phenol-ethanol supernatant and save it at 4 C for the subsequent protein isolation. Add 0.25 ml of DNAzol® and dissolve the DNA precipitate by agitating the tube. When the isolation is performed in large (> 2 ml) tubes, transfer the DNA solution to a microcentrifuge tube.

3. DNA PRECIPITATION

Precipitate DNA by adding 0.125 ml of 100% ethanol to 0.25 ml of DNAzol®. Store tubes at room temperature for 2 - 5 minutes and sediment the DNA precipitate at 2,000 g for 3 minutes at 4 - 25 C.

4. DNA WASH

Wash the DNA precipitate twice with 0.8 - 1 ml of 95% ethanol. At each wash, suspend the precipitate by inverting the tubes, and centrifuge at 2,000 g for 1 - 3 minutes at 4 - 25 C. If the DNA forms a compact pellet, no centrifugation is necessary and the ethanol wash can be removed by decanting.

5. DNA SOLUBILIZATION

Air dry the DNA pellet by keeping tubes open for 5 - 10 minutes at room temperature. Dissolve DNA in 8 mM NaOH by slowly passing the pellet through a pipette. Add an adequate amount of 8 mM NaOH to approach a DNA concentration of 0.1 - 0.3 µg/µl. Typically, add 0.1 ml of 8 mM NaOH to the DNA isolated from 1 ml of whole blood. The use of a mild alkaline solution assures full solubilization of the DNA precipitate.

QUANTITATION OF DNA

For optimal spectrophotometric measurements, DNA aliquots should be diluted with water or buffer with a pH > 7.5. Distilled water with a pH < 7.0 falsely decreases the 260/280 ratio and impedes the detection of protein in RNA samples (6).

Calculate the DNA content assuming that one A_{260} unit equals 50 µg double-stranded DNA/ml. For calculation of the cell number in analyzed samples assume that the amount of DNA per 10^6 of diploid cells of human, rat and mouse origin equals: 7.1 µg, 6.5 µg and 5.8 µg, respectively (5). A preparation of DNA isolated from whole blood contains > 80% of 60 - 100 kb DNA and < 10% of 20 kb DNA. The isolated DNA is free of RNA and proteins and has a 260/280 ratio > 1.6. Typical yield is 10 - 20 µg DNA/ml of human blood.

AMPLIFICATION OF DNA BY PCR

Following solubilization in 8 mM NaOH, adjust the pH of the DNA sample to 8.4 using HEPES (see Table). Add an aliquot of the sample (typically 0.1 - 1 µg DNA) to a PCR reaction mix and perform PCR according to your standard protocol.

DIGESTION OF DNA BY RESTRICTASES

Adjust the pH of the DNA solution to a required value using HEPES (see Table). Alternatively, dialyze samples against 1 mM EDTA, pH 7 - 8. Carry out the DNA restriction for 3 - 24 hours under optimal conditions for a specific restriction enzyme using 3 - 5 units of the enzyme per µg DNA. In a typical assay, 90% - 100% of the DNA preparation is digested by restrictases.

Adjustment of pH in DNA samples solubilized in 8 mM NaOH.

For 1 ml of 8 mM NaOH use the following amounts of 0.1 M or 1 M HEPES (free acid):

Final pH - 0.1 M HEPES (µl)		Final pH - 1 M HEPES (µl)	
8.4	86	7.2	23
8.2	93	7.0	32
8.0	101		
7.8	117		
7.5	159		

NOTES FOR DNA ISOLATION

1. If necessary, the phenol phase and interphase can be stored at 4 C overnight. Samples suspended in 75% ethanol can be stored at 4 C for a long period of time (months). Samples solubilized in 8 mM NaOH can be stored overnight at 4 C. For prolonged storage, adjust samples to pH 7 - 8 and supplement with 1 mM EDTA.
2. The isolation protocol can be modified if the DNA is isolated only for quantitative purposes. The phenol phase and the interphase can be stored at 4 C for a few days or at -70 C for a few months.
3. If the expected yield of DNA is < 10 µg, the use of Polyacryl Carrier (cat. no. PC 152) is recommended to assure full recovery of DNA. Add 1 - 4 µl of Polyacryl Carrier to the interphase/organic phase and precipitate DNA with ethanol as described in the protocol.
4. This alternative procedure replaces steps 1 - 3 of the DNA Isolation procedure and can be performed without the use of DNAzol®. Prepare a back extraction buffer containing: 4 M guanidine thiocyanate, 50 mM sodium citrate and 1 M Tris (free base). Following phase separation (RNA Isolation Procedure Step 2), remove any remaining aqueous phase overlying the interphase and add back extraction buffer to the interphase - organic phase mixture. Use 0.5 ml of back extraction buffer per 0.75 ml of TRI Reagent® BD used for the initial lysis. Vigorously mix the sample by inversion for 15 seconds and store for 10 minutes at room temperature. Perform phase separation by centrifugation at 12,000 g for 15 minutes at 4 C. Transfer the upper aqueous phase containing DNA to a clean tube and save the interphase and organic phase at 4 C for subsequent protein isolation. Precipitate DNA from the aqueous phase by adding 0.4 ml of isopropanol per 0.75 ml of TRI Reagent® BD used for the initial lysis. Mix the sample by inversion and store for 5 minutes at room temperature. If the expected DNA yield is less than 20 µg, add 1 - 4 µl of Polyacryl Carrier to the aqueous phase prior to isopropanol addition and mix. Sediment DNA by centrifugation at 12,000 g for 5 minutes at 4 - 25 C and remove the supernatant. Wash the pellet with ethanol as described in the DNA Wash Step 4 and proceed with DNA solubilization as described in Step 5.
5. Also see the Troubleshooting Guide on the reverse side of the last page of this booklet.

III. ISOLATION OF PROTEINS BY TRI REAGENT® BD

Proteins are isolated from the phenol-ethanol supernatant obtained after precipitation of DNA with ethanol (DNA Isolation Protocol Step 1). The resulting preparation can be analyzed for the presence of specific proteins by Western blotting (2, 8, 9).

PROTOCOL

Reagents required but not supplied: guanidine hydrochloride, ethanol, isopropanol, acetone, glycerol, SDS, urea and tributylphosphine (Sigma T 7567).
The protocol includes the following steps:

1. PROTEIN PRECIPITATION	- 0.2 - 0.5 ml phenol-ethanol supernatant (1 volume) + acetone (3 volumes)
2. PROTEIN WASH	- 1 ml of guanidine hydrochloride/ethanol/glycerol wash solution, 3 x 10 min.; - 1 ml ethanol/glycerol solution, 1 x 10 min.
3. PROTEIN SOLUBILIZATION	- 1% SDS, 10M Urea or other suitable solvent

The procedure is carried out at room temperature unless stated otherwise. This protocol describes the isolation of proteins from the phenol-ethanol supernatant obtained from a sample homogenized in 0.75 ml of TRI Reagent® BD.

1. PROTEIN PRECIPITATION

Aliquot a portion of the phenol-ethanol supernatant (0.2 - 0.5 ml, 1 volume) into a microfuge tube. Precipitate proteins by adding 3 volumes of acetone. Mix by inversion for 10 - 15 seconds to obtain a homogeneous solution. Store samples for 10 minutes at room temperature and sediment the protein precipitate at 12,000 g for 10 minutes at 4 C (See Notes 1and 2).

2. PROTEIN WASH

Decant the phenol-ethanol-acetone supernatant and disperse the protein pellet in 0.5 ml of 0.3 M guanidine hydrochloride in 95% ethanol + 2.5 % glycerol (V:V). Disperse the pellet using a pipette tip, syringe needle or a small conical Teflon pestle (Fisher K749515-0000) attached to a mechanical stirrer (~30 sec @ 800-1000 RPM). After dispersing the pellet, add another 0.5 ml aliquot of the guanidine hydrochloride/ethanol/glycerol wash solution and store for 10 minutes at room temperature. Sediment the protein at 8,000 g for 5 minutes. Decant the wash solution and perform two more washes in 1 ml each of the guanidine/ethanol/glycerol wash solution. Disperse the pellet by vortexing in each wash to efficiently remove residual phenol. Perform the final wash in 1 ml of ethanol containing 2.5 % glycerol (V:V). At the end of the 10 minute ethanol wash, sediment the protein at 8,000 g for 5 minutes at 4 C. Decant the alcohol, invert the tube and dry the pellet for 7 - 10 minutes at room temperature (See Note 3).

3. PROTEIN SOLUBILIZATION

Option 1. After briefly air-drying the protein pellet, add a suitable solvent such as 1% SDS, 10 M urea, or another suitable detergent-based solvent to the protein pellet (9). Use 0.2 ml of solvent per 10 - 20 mg of tissue sample (See Note 4). Gently disperse and solubilize the pellet for 15 - 20 minutes by "flicking" the tube or pipetting as required. The addition of a suitable reducing agent such as tributylphosphine (2.5% of solution volume) will improve protein yield in most preparations. For immediate use in Western analysis, heat the solution for 3 minutes at 100 C and sediment any insoluble material by centrifugation at 10,000 g for 5 minutes at room temperature. Transfer the supernatant to a clean tube and use immediately for Western blotting (See Note 5). Otherwise, store the solubilized proteins at -20 C and perform the heating, centrifugation or other preparatory steps at the time of use.

Option 2. Dialyze the phenol-ethanol supernatant (DNA Isolation Protocol Step 1) in a suitable, regenerated cellulose dialysis tubing against three changes of 0.1% SDS at 4 C. Centrifuge the dialysate at 10,000 g for 10 minutes at 4 C and use the clear supernatant for Western blotting.

NOTES FOR PROTEIN ISOLATION

1. Isopropanol may replace acetone during protein precipitation but total recovered protein yield may be reduced by 5 - 10 % (8).
2. Limiting the volume of phenol – ethanol supernatant to 0.2 - 0.5 ml per tube will produce a smaller, more manageable protein pellet and improve protein yield.
3. In general, protein pellets suspended in 0.3 M guanidine hydrochloride/ethanol/glycerol wash solution or in ethanol/glycerol wash solution can be stored for at least one month at 4 C or one year -20 C. Individual proteins may display different sensitivity to long-term storage and optimal storage conditions should be established for sensitive and labile proteins.
4. The solubility and stability of specific proteins can be influenced by different detergent solutions (9). To obtain optimal results in various experimental applications, investigators may solubilize small amounts of protein in different solvents and determine which solution best addresses their unique experimental objectives.
5. Solubilized protein may form insoluble aggregates during storage at -20 C. Prior to western analysis, thaw the samples at 25 C for 10 - 15 minutes. Heat the solubilized protein sample for 3 minutes at 100 C, pipette the solution and remove insoluble protein by centrifugation as outlined in the protocol.

IV. TROUBLESHOOTING GUIDE.

RNA ISOLATION

Low yield. a) incomplete homogenization or lysis of samples, b) incomplete solubilization of the final RNA pellet.
260/280 ratio < 1.6. a) too small a volume of the reagent used for sample homogenization, b) acidic water was used for the spectrophotometric measurement, c) contamination of the aqueous phase with phenol phase, d) incomplete solubilization of the final RNA pellet.
RNA degradation. a) samples were not immediately processed or frozen after collection, b) samples used for isolation, or the isolated RNA preparations were stored at -20 C instead of at -70 C, c) aqueous solutions or tubes used for solubilization of RNA were not RNase-free, d) formaldehyde used for agarose-gel electrophoresis had a pH below 3.5.
DNA contamination. a) too small a volume of the reagent was used for sample lysis, b) phase separation was performed at temperatures above 10 C.

DNA ISOLATION

Low yield. a) incomplete homogenization or lysis of samples, b) incomplete solubilization of the final DNA pellet.
260/280 ratio < 1.70. a) phenol was not sufficiently removed from the DNA preparation, b) acidic water was used for the spectrophotometric measurement, c) incomplete solubilization of the DNA pellet, d) insoluble particles remain in the DNA preparation.
DNA degradation. a) blood samples were stored at room temperature, b) too much mechanical force applied during lysis.
RNA contamination. a) too large a volume of aqueous phase remained with the interphase and organic phase, b) DNA pellet was not sufficiently washed with 10% ethanol-0.1 M sodium citrate solution.

PROTEIN ISOLATION

Low yield. a) incomplete homogenization or lysis of samples, b) incomplete solubilization of the final protein pellet.
Protein degradation. Tissues were not immediately processed or frozen after collection.
Band deformation in PAGE. Insufficient wash of the protein pellet.

V. ISOLATION OF POLY A^+ RNA

Following RNA precipitation with isopropanol (RNA Isolation Protocol Step 3), the RNA pellet can be dissolved in a poly A^+ binding buffer and poly A^+ RNA selection can be performed on an oligo-dT column, or using any commercial product according to a standard protocol of Aviv and Leder (Proc Natl Acad Sci USA, 1972, 69, 1408-1412).

VI. RT-PCR APPLICATION NOTES

A more complete evaporation of ethanol is required for RNA samples used in RT-PCR. This is especially important for small volume samples (5 - 20 µl) which, if not dried sufficiently, may contain a relatively high level of ethanol.

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