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RNA Blue

REAGENT FOR RAPID ISOLATION OF PURE AND INTACT RNA
(Cat. No. R011, R012, R013) rev. 04/2025

WARNING: RNA Blue contains phenol and some other toxic components. After contact with skin, wash immediately with plenty of detergent and water.

Description

RNA Blue is a ready to use reagent for progressive method of isolation of total RNA or for the simultaneous isolation of RNA, DNA and proteins from samples of human, animal, plant, yeast, bacterial and viral origin. Basis of the method has been described by Chomczynski and Sacchi (Anal. Biochem. 162, 156-159, 1987). This highly reliable technique performs well with small and large quantities of tissues or cultured cells, and allows simultaneous processing of a large number of samples.

RNA Blue reagent contains phenol and guanidine thiocyanate in a mono-phase solution. A biological sample is homogenized or lysed in RNA Blue reagent and the homogenate is separated into the aqueous and organic phases by 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 addition of isopropanol, washed with ethanol and solubilized. DNA and proteins are sequentially precipitated from the interphase and organic phase with ethanol and isopropanol, washed with ethanol and solubilized.

Technical data

Components an packing

- Bottles containing 25, 50 or 100 ml of RNA Blue.
- Detailed instructions for isolation of RNA, DNA and proteins from the same sample.

Storage

- Store at temperature 4 ± 3°C.

Quality control:

- Each batch of RNA Blue is controlled for performance in RNA isolation. When RNA is isolated according the recommended protocol, RNA is intact as can be documented by electrophoretic separation on formaldehyde gel and staining with ethidium bromide.

Cat. No.	Product name and specification	Amount
R011	RNA Blue	25 ml
R012	RNA Blue	50 ml
R013	RNA Blue	100 ml

Protocol

1. ISOLATION OF RNA

- Isolation of RNA requires to follow certain rules to avoid RNA degradation:
- Gloves should be used whenever working with RNA to protect samples from RNases present on hands.
- It is necessary to use only RNase-free solutions and sterile plasticware.
- RNA blue reagent possesses strong inhibitors of RNAses, which protect RNA from degradation. However, after step 1.2 (see below) RNAses could degrade isolated RNA and therefore it is important to protect samples against RNases. If glass tubes, pipettes and beakers are used , they must be exposed to high temperature (180^{o}C for 4 h). RNAase-free plastic ware should be used whenever possible. Otherwise plastic must be treated with 0.5 M NaOH for 10 min, extensively washed with RNase-free water and then autoclaved.

Other reagents required but not supplied

- Chloroform (without isoamyl alcohol or any other additives)
- Isopropanol,
- RNase-free water (e.g. PCR Ultra H_2O, Cat. No. P040),
- RNase-free 75% ethanol (e.g. PCR Et-OH, Cat. No. P044).

1.1. Cell homogenization

This step is done at room temperature (15 - 30^{o}C)

1.1.1. Cells in suspension. The cells grown in suspension should be sedimented first, and then lysed in RNA Blue reagent by repetitive pipetting. Use 1 ml of the reagent per 5-10 x 10^6 of animal, plant or yeast cells or per 10^7 bacterial cells. Washing cells before addition of RNA Blue reagent should be avoided as this increases the possibility of mRNA degradation. Disruption of some yeast and bacterial cells may require the use of a homogenizer.

1.1.2. Cells in monolayers. From the cells grown in monolayer the medium is sucked off and the attached cells are lysed by adding RNA Blue directly into a culture dish; 1 ml of the reagent is used per 10 cm^2 area. The cells are homogenized by pipetting RNA Blue reagent and transferred to 1.5 ml Eppendorf tube.

1.1.3 Solid tissues. Homogenize tissue samples in RNA Blue (50-100 mg tissue/ 1 ml RNA Blue) using Polytron homogenizer or other homogenizer. Volume of the tissues should be less than 10% of the volume of RNA Blue reagent used for homogenization.

1.2. Phase separation

The cell homogenate is stored for 5 min at room temperature to permit the complete dissociation of nucleoprotein complexes. Next, to 1 ml of RNA Blue homogenate add 0.2 ml chloroform. Cover the samples tightly and shake vigorously for 15 seconds. Store the resulting mixture at room temperature for 2-15 minutes and centrifuge at 12 000 x g for 15 min at 4^{o}C. After centrifugation, the sample is separated into a lower blue, phenol-chloroform phase, interphase, and the 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 RNA Blue reagent used for homogenization .

1.3. RNA precipitation

The aqueous phase is transferred into a fresh tube and RNA is precipitated by mixing with isopropanol. Use 0.5 ml of isopropanol per 1 ml of RNA Blue reagent used for the initial homogenization. Store samples at room temperature for 5-10 min and centrifuge at 12 000 x g for 8 min at 4^{o}C. RNA precipitate (often invisible before centrifugation) forms a gel-like pellet on the side and bottom of the tube.

1.4. RNA wash

After centrifugation, supernatant is removed and RNA pellet is washed once with 75% ethanol by vortexing and subsequent centrifugation at 7 500 x g for 5 minutes at 4 - 25^{o}C. Add at least 1 ml of 75% ethanol per 1 ml RNA Blue reagent used for the initial homogenization. If the RNA pellet accumulates on a side of the tube and has tendency to float, sediment the pellet at 12 000 x g.

1.5. RNA solubilization

After removal of supernatant, RNA pellet is air-dried for 5 min at air. It is important not to let the RNA pellet dry completely as this will greatly decrease its solubility. Do not dry RNA by centrifugation under vacuum. Dissolve RNA in PCR H_2O (Cat No. P042) by passing the solution a few times through a pipette tip, and incubating for 10-15 minutes at 55-60^{o}C.

RNA Blue reagent isolates a whole spectrum of RNA molecules, rarely observed in RNA preparations isolated by other methods. The ethidium bromide staining of RNA separated in agarose gel visualizes two predominant bands of small (~2 kb) and large (~5 kb) ribosomal RNA, low molecular weight (0.1-0.3 kb) RNA, and discrete bands of high molecular weight (7-15 kb) RNA.

The final preparation of total RNA is free of DNA and proteins and has an A260/280 ratio 1.6 - 1.9.
Expected yield:
A) tissues (µg RNA/mg tissue): liver, spleen, 6-10 µg; kidney, 3-4 µg; skeletal muscles, brain, 1-1.5 µg; placenta, 1-4 µg;
B) cultured cells (µg RNA/10^6 cells): epithelial cells, 8-15 µg; fibroblasts, 5-7 µg.

1.6. Notes and comments for RNA isolation

1.6.1. RNA molecules isolated

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1.6.2 Increasing Yield of RNA

To facilitate isolation of RNA from small samples (<10^6 cells or <10 mg tissue) perform lysis in 0.8 ml of RNA Blue reagent supplemented with 10 µl of RNA carrier (Cat. No. R 057). Next, add chloroform and proceed with the phase separation and other steps of isolation as described above.
The final preparation of total RNA is free of DNA and proteins and has an A260/280 ratio 1.6 - 1.9.

1.6.2. Storage

After homogenization (before addition of chloroform) samples can be stored at -70^{o}C for at least one month. The RNA precipitate (step 4, RNA WASH) can be stored in 75% ethanol at 4^{o}C for at least one week, or at least one year at -20 C.

1.6.3. Enhancing purity of isolated RNA

An additional isolation step may be required for samples with a high content of proteins, fat, polysaccharides or extracellular material such as muscles, fat tissue and tuberous parts of plants. Following homogenization, remove insoluble material from the homogenate by centrifugation at 12 000 x g for 10 min at 4^{o}C. The resulting pellet contains extracellular membranes, polysaccharides and high molecular weight DNA, while the supernatant contains RNA. In samples from fat tissue, an excess of fat collects as a top layer which should be removed. Transfer the clear supernatant to a fresh tube and proceed with the phase separation and other steps of RNA isolation as described above. High molecular weight DNA can be recovered from the pellet by following steps 2 and 3 of the DNA isolation protocol.

2. Isolation of DNA

The DNA is isolated from the interphase and the phenol phase separated from the initial homogenate as described in the RNA isolation protocol. Following precipitation and a series of washes, the DNA is solubilized in 8 mM NaOH, neutralized and used for analysis. The DNA isolated by RNA Blue reagent can be used for PCR, restriction and Southern blotting. In addition, full recovery of DNA from tissues and cultured cells permits the use of RNA Blue reagent for determination of the DNA content in analyzed samples.

Reagents required:

Absolute ethanol,
0.1 M Sodium citrate,
8 mM sodium hydroxide.

2.1. DNA precipitation

The remaining aqueous phase overlying the interphase (from step 1.2) is removed and DNA is precipitated from the interphase and organic phase by adding 0.3 ml of 100% ethanol per 1 ml of RNA Blue reagent used for the initial homogenization. Sample is mixed by inversion and stored at room temperature for 2-3 min. DNA is sedimented by centrifugation at 2 000 x g for 5 min at 4 C. Careful removal of the aqueous phase is critical for the quality of the isolated DNA.

2.2. DNA wash

Phenol-ethanol supernatant is removed and saved (at 4°C) for the protein isolation. DNA pellet is washed twice in a solution containing 0.1 M sodium citrate in 10% ethanol. Use 1 ml of the solution per 1 ml of RNA Blue reagent used for the initial homogenization. At each wash, store the DNA pellet in the washing solution for 30 minutes at room temperature (with periodic mixing) and centrifuge at 2 000 g for 5 mi at 4°C. Next, suspend the DNA pellet in 75% ethanol (1.5-2 ml of 75% ethanol per 1 ml RNA Blue reagent), store for 10 - 20 minutes at room temperature (with periodic mixing) and centrifuge at 2 000 g for 5 minutes at 4 - 25°C.

An additional wash in 0.1 M sodium citrate-10% ethanol solution is required for large pellets containing >200 µg DNA or large amounts of a non-DNA material.

2.3. DNA solubilization

DNA pellet is air dried by keeping tubes open for 5 min at room temperature. DNA pellet is dissolved in 8 mM NaOH by slowly passing through a pipette. Add an adequate amount of 8 mM NaOH to approach a DNA concentration of 0.2 - 0.3 µg/µl. Typically, add 0.3 - 0.6 ml 8 mM NaOH to the DNA isolated from 50 - 70 mg of tissue or 10^7 cells. The use of a mild alkaline solution assures full solubilization of the DNA pellet. At this stage, however, the DNA preparations (especially from tissues) still contain insoluble gel-like material (fragments of membranes etc.). Remove this material by centrifugation at 12 000 g for 10 minutes and transfer the resulting supernatant containing DNA to new tubes. A high viscosity of the supernatant indicates the presence of high molecular weight DNA. To adjust pH values of the samples , add the following amount of HEPES buffer to 1 ml of 8 mM NaOH with DNA>

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	HEPES (M)
8.4	66 µl (0.1M)
8.2	90 µl (0.1M)
8.0	115 µl (0.1M)
7.8	135 µl (0.1M)
7.5	180 µl (0.1M)
7.2	30 µl (1M)
7.0	42 µl (1M)

2.4. Notes and comments

2.4.1. Quantification of isolated DNA

An aliquot of the DNA solubilized in 8 mM NaOH is mixed with water and absorbance at A_{260} is determined. Calculate the DNA content assuming that one A_{260} unit equals 50 µg of 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 = 7.1 µg, rat = 6.5 µg and mouse = 5.8 µg.

Expected yield:

A) tissues (µg DNA/mg tissue); liver, kidney, 3-4 µg, skeletal muscles, brain, placenta 2-3 µg;

B) cultured human, rat and mouse cells 5-7 µg DNA/10^6 cells.

Atypical preparation of DNA isolated from tissues is composed of 60-100 kb DNA (70%) and ~ 20 kb DNA (30%). The preparation isolated from cultured cells contains >80% of 60-100 kb DNA and <20% ~ 20 kb DNA. The isolated DNA is free of RNA and proteins and has an A260/280 ratio >1.7.

2.4.2. Use of the isolated DNA for amplification by PCR

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

2.4.3. Use of isolated DNA for digestion by restriction enzymes.

Adjust the pH of the DNA solution to a required value using HEPES (see Table above). Alternatively, dialyze samples against 1 mM EDTA, pH 7 - 8. Carry out the DNA restriction for 3 - 24 h under optimal conditions for a specific restriction enzymes using 3-5 units of the enzyme per µg DNA. In a typical assay, 80-100% of the DNA preparation is digested by restriction enzymes.

2.4.4. Storage of isolated DNA

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.

3. Isolation of proteins

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

Reagents required but not supplied:

isopropanol
0.3 M guanidine hydrochloride in 95% ethanol,
95% ethanol
1% SDS
absolute ethanol

3.1. Protein precipitation

Proteins are precipitated from the phenol-ethanol supernatant (step 2.2; approximate volume 0.8 ml) with isopropanol. 1.5 ml of isopropanol is added per 1 ml of RNA Blue reagent used for the initial homogenization. Store samples for at least 10 minutes at room temperature and sediment the protein precipitate at 12 000 g for 10 min at 4°C.

3.2. Protein wash

Supernatant is removed and protein pellet is washed 3 times in a solution containing 0.3 M guanidine hydrochloride in 95% ethanol. 2 ml of the solution is added per 1 ml of RNA Blue reagent used for the initial homogenization. At each wash, samples in the washing solution are stored for 20 min at room temperature and centrifuge at 7 500 x g for 5 min at 4°C. Next, the protein pellet is wortexed with 2 ml of ethanol (100%), stored for 20 min at room temperature and centrifuge at 7 500 x g for 5 min at 4°C.

3.3. Protein solubilization

The protein pellet is air dried by keeping tubes open for 5 - 10 min at room temperature. Proteins are dissolved in 1% SDS by pipetting. Incubation of samples at 50°C may be required to efficiently extract soluble proteins from the pellet. The pellet is composed of soluble proteins and insoluble material such as tissue membranes, extracellular matrix etc. Insoluble material is sedimented by centrifugation at 10.000 x g for 10 min at 4°C and supernatant is transferred into a fresh tube. Protein solution is used immediately for Western blotting or stored at -20°C.

4. ALTERNATIVE PROTOCOL

The critical step in protein isolation is extraction of proteins from the pellet at the end of the protocol. The following is an alternative approach allowing efficient recovery of proteins: a) dialyze the phenol-ethanol supernatant against three changes of 0.1% SDS at 4°C, b) centrifuge the dialysate at 10 000 g for 10 minutes at 4 C, c) use the clear supernatant for Western blotting.

NOTES and COMMENTS

1. The protein pellet suspended in 0.3 M guanidine hydrochloride-95% ethanol or in ethanol can be stored for at least one month at 4°C or for at least one year -20°C.

2. The information on storage of samples at earlier steps of processing is provided in Notes and Comments in the RNA and DNA isolation protocols.

4. TROUBLESHOOTING GUIDE.

4.1 RNA isolation

Low yield: a) incomplete homogenization or lysis of samples, b) incomplete solubilization of the final RNA pellet.

$A_{260/280}$ ratio < 1.65: a) too small volume of the reagent used for sample homogenization, b) following homogenization, samples were not stored for 5 minutes at room temperature, c) contamination of the aqueous phase with phenol phase, d) incomplete solubilization of the final RNA pellet.

RNA degradation: a) tissues were not immediately processed or frozen after removing from animal, b) samples used for isolation, or the isolated RNA preparations were stored at -20°C instead of at -70°C, c) cells were dispersed by

trypsin digestion, d) aqueous solutions or tubes used for solubilization of RNA were not RNase-free, e) formaldehyde used for agarose-gel electrophoresis had a pH below 3.5.

DNA contamination: a) too small volume of the reagent was used for sample homogenization, b) samples used for the isolation contained organic solvents (ethanol, DMSO), strong buffers or alkaline solution.

Proteoglycan and polysaccharide contamination. The following modification of RNA precipitation (step 3) removes these contaminating compounds from the isolated RNA. Add to the aqueous phase 0.25 ml of isopropanol followed by 0.25 ml of a high salt precipitation solution (0.8 M sodium citrate and 1.2 M NaCl) per 1 ml of RNA Blue reagent used for the homogenization. Mix the resulting solution, centrifuge and proceed with the isolation as described in the protocol. The modified precipitation effectively precipitates RNA while maintaining polysaccharides and proteoglycans in a soluble form. To isolate pure RNA from plant material containing a very high level of polysaccharides the modified precipitation should be combined with an additional centrifugation of the initial homogenate described in notes in the RNA isolation protocol.

4.2. DNA isolation

Low yield: a) incomplete homogenization or lysis of samples, b) incomplete solubilization of the final DNA pellet.

$A_{260/280}$ ratio < 1.70: phenol was not sufficiently removed from the DNA preparation. Add one more wash of the DNA pellet with the 10% ethanol-0.1 M sodium citrate solution.

DNA degradation: a) tissues were not immediately processed or frozen after removing from animal, b) samples were homogenized with a Polytron or other high speed homogenizer.

RNA contamination: a) too large 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.

4.3. 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 removing from animal.
Band deformation in PAGE: insufficient wash of the protein pellet.

5. ISOLATION OF POLY A+ RNA

1. Following RNA precipitation with isopropanol (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 according to a standard protocol of Aviv and Leder (Proc Natl Acad Sci USA, 1972, 69, 1408-1412).

6. RT-PCR APPLICATION NOTES

1. The additional centrifugation described in Notes and Comments to the RNA isolation protocol (note # 5) further eliminates possibility of DNA contamination in RNA extracted by RNA Blue reagent.

2. 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.

3. For efficient RT-PCR with RNA Blue reagent-isolated RNA we recommend RNA Blue RT-PCR kit containing all key components for RNA isolation, reverse transcription of RNA to cDNA and PCR amplification of cDNA. Detailed protocol of RT-PCR is also included (Cat. No. R001).