BI
Biological Industries

EZ-RNA II

Total RNA Isolation Kit (Without Chloroform)

Cat. No.: 20-410-100
Store at: 2-8°C

Instructions for Use

Product Description

EZ-RNA is a complete kit with ready-to-use reagents for the isolation of total RNA from samples of human, animal, plant, yeast, bacterial and viral origin. EZ-RN II is an improved version of the Chomczynski and Sacchi method [1] , which is based on disruption of cells in guanidine thiocyanate/detergent solution, followed by organic extraction and alcohol precipitation of the RNA, and which allows simultaneous processing of a large number of samples.1-Bromo-3-chloropropane (BCP) replaces chloroform, a highly volatile and toxic reagent used in molecular biology. Substituting BCP for chloroform in the EZ-RNA II kit, reduces toxic material handling without any adverse effects on the quality of isolated RNA, DNA or proteins. The resulting RNA is suitable for the isolation of Poly A+ RNA or for Northern Blotting, Dot Blotting, in vitro Translation, Molecular Cloning, RT-PCR and RNase Protection Assays, or other analytical procedures. DNA and proteins can also be recovered from the interphase and the organic phase of the same sample.

Kit Reagent

Cat. No.: **20-410-100A** Denaturing Solotion, 50ml
Contains:guanidine thiocyanate

Cat. No.: **20-410-100B** Water-saturated phenol, 40ml
Do not swirl. Make sure there are two phases. Take only from the organic (lower) phase, leaving the upper (aqueous) phase. Do not use if turbid.

Cat. No.: **20-410-100C** 1-Bromo-3-chloropropane (BCP), 9ml

Reagents Required But Not Supplied

RNA
- Isopropanol
- 75% Ethanol
- DEPC-Treated Water or 0.1mM EDTA

DNA
- Absolute Ethanol
- 0.1M Sodium Citrate in 10% Ethanol
- 75% Ethanol
- 8mM NaOH (fresh preparation)
- 1M Hepes, free acid

Proteins
- Isopropanol
- 0.3M Guanidine HCl in 95% Ethanol
- Absolute Ethanol
- 1% SDS

Precautions

EZ-RNA II contains phenol, which is poisonous, and guanidine thiocyanate, which is an irritant. Therefore, when working with EZ-RNA II, use gloves and eye protection, avoid contact with skin or clothing, and avoid inhaling vapor. In case of contact, wash immediately with plenty of water and seek medical advice.

1. Homogenization

1.1 Tissue

HHomogenize samples in the Denaturing Solution (0.5ml/50-100mg tissue) using homogenizer. Sample volume should not exceed 10% of the volume of the Denaturing Solution.

1.2 Cells

Cells grown in monolayer should be lysed directly in a culture dish using 0.5ml Denaturing Solution/10cm^2 f culture dish area. Pass the cell lysate several times through a pipette.

Cells grown in suspension should be first sedimented, then lysed in the Denaturing Solution (0.5ml Denaturing Solution/5-10x1^6 for animal, plant or yeast cells; or 10^7 for bacterial cells) by repeated pipetting.

2. Phase Separation

Store the homogenate for 5 minutes at room temperature. Then add 0.4ml Water-saturated phenol per 0.5ml Denaturing Solution. Shake vigorously. Add 0.09ml BCP per 0.5ml Denaturing solution. Shake vigorously for 15 seconds. Store the resulting mixture at room temperature for 10 minutes and then centrifuge at 12,000g for 15 minutes at 4°C.
* To increase yield, perform second extraction: Transfer the upper phase and interphase to a fresh tube, add Phenol and BCP of the above volume, and repeat centrifugation.

3. RNA Precipitation

Transfer the aqueous colorless (upper) phase to a fresh tube and store the interphase and the organic phase at 4°C for DNA isolation (if desired). Precipitate RNA from the aqueous phase by mixing with 0.5ml isopropanol per 0.5ml Denaturing Solution. Store at room temperature for 10 minutes and then centrifuge at 12,000g for 8 minutes at 4°C.
* To increase yield, store sample for 30 minutes - overnight at -20°C.

4. LiCl Precipitation (optional)

Polysaccharides and other contaminants may be removed by LiCl precipitation of the RNA. Re-suspend the RNA pellet by mixing with 2.5M LiCl solution. Vortex if necessary. Store at -20°C for at least 30 minutes and then centrifuge at 10,000g for 15 minutes at 4°C.

5. RNA Wash

Remove supernatant and wash the RNA pellet (by vortexing) with 1ml 75% ethanol. Then centrifuge at 7,500g for 5 minutes at 4°C. The RNA precipitate can be stored in 75% ethanol at 4°C for one week or at -20°C for at least one year.

6. RNA Solubilization

Remove the ethanol wash and air-dry the RNA pellet for 5 minutes. Do not let the RNA pellet dry completely. Dissolve the RNA in 100µl of DEPC-treated water with 0.1mM EDTA, or in 0.5% SDS solution (prepared with DEPC-treated water) by incubating for 10-15 minutes at 55°C.

* **Important:** for best results in RT-PCR, dissolve the RNA in DEPC-treated water without EDTA (heat if necessary).

The final preparation of total RNA will be free of DNA and proteins, and will have a 260/280 O.D. ratio of 1.6 to 1.9.

Protocol for DNA Isolation

1. DNA Precipitation

Carefully remove the remaining upper aqueous phase and discard. Add 0.3ml of absolute ethanol per 0.5ml of Denaturing Solution, and mix by inversion. Store at room temperature for 3 minutes and then centrifuge at 2000g for 5 minutes at 4°C. Remove the phenol-ethanol supernatant and store at 4°C for protein isolation (if desired).

2. DNA Wash

Wash the DNA pellet twice in a solution containing 0.1M Sodium Citrate in 10% ethanol. Use 1ml of solution per 0.5ml Denaturing Solution. Store at room temperature for 30 minutes with occasional mixing, and then centrifuge at 2,000g for 5 minutes at 4°C. Dissolve the DNA pellet in 75% ethanol (1.5-2ml per 0.5ml Denaturing Solution). Store at room temperature for 10-20 minutes with occasional mixing, and then centrifuge at 2000g for 5 minutes at 4°C.

3. DNA Solubilization

Remove the ethanol wash and air-dry for 5 minutes. Dissolve the DNA in 8mM NaOH by careful pipetting. Add 0.3-0.6ml 8mM NaOH to DNA isolated from 50mg of tissue or 10^7 cells. To remove any insoluble material, centrifuge at 12,000g for 10 minutes and transfer the supernatant to a new tube. Samples can be stored at 4°C overnight. For prolonged storage, adjust sample to pH 7-8 (with 1M Hepes, free acid) and adjust the EDTA concentration to 1mM.

4. pH Adjustment of DNA Samples Dissolved in 8mM NaOH

For 1ml of 8mM NaOH, use the following amounts of 1M Hepes, free acid:

Final pH	7.0	7.2	7.5	7.8	8.0	8.4
1 M Hepes (µl)	42	30	18	13.5	11.5	9.5

5. Amplification of DNA by PCR

Following solubilization in 8mM NaOH, adjust the pH of the DNA sample to 8.4 with 1M Hepes, free acid. Add 0.1-1.0µg of the DNA sample to a PCR reaction mixture and perform the standard PCR protocol.

6. Digestion of DNA by Restriction Endonucleases

Adjust the pH of the DNA solution to a required value using 1M Hepes, free acid (see table). Use 3-5 units of enzymes per microgram of DNA. Use the conditions recommended by the enzyme manufacturer.

Protocol for Protein Isolation

1. Protein Precipitation

Precipitate proteins from the phenol-ethanol supernatant (step 2.1) with 1.5ml isopropanol per 0.5ml of Denaturing Solution used for the initial homogenization. Store samples for 10 minutes at room temperature and then centrifuge at 12,000g for 10 minutes at 4°C.

2. Protein Wash

Remove the supernatant and wash the pellet 3 times with 0.3M guanidine HCl in 95% ethanol. Use 2ml of wash solution per 0.5ml of Denaturing Solution for each wash. Store samples in wash solution for 20 minutes at room temperature. Centrifuge at 7,500g for 5 minutes at 4°C. After the final wash, add 2ml of absolute ethanol and vortex the protein pellet. Store for 20 minutes at room temperature and then centrifuge at 7500g for 5 minutes at 4°C.

3. Protein Solubilization

Air-dry the protein pellet for 10 minutes. Dissolve the pellet in 1% SDS solution by pipetting. Complete solubilization of the protein pellet may require incubation at 50°C. Remove any insoluble material by centrifugation at 10,000g for 10 minutes at 4°C and transfer the supernatant to a new tube. The proteins may be used immediately for Western Blotting or stored at -20°C.

Reference

(1) Chomczynski, P. and Sacchi, N., Anal. Biochem., 162:156-159 (1987)

Tissue/Sample Type	Recommendations
Bacterial Cells	When working with bacterial cells, it is better to isolate RNA from exponentially growing cells as opposed to stationary cultures. Disruption of some bacterial cells may require homogenization or vortexing. Isolation of RNA from Mycobacteria. Briefly: Grow cells to A600 of 0.5. Take 1ml, and centrifuge at 5000 rpm at 4°C for 5 minutes. Add 1.25ml of Denaturing Solution to cell pellet. To help break up the cell wall, a sonication for 15 seconds, followed by 3 cycles of freeze thawing should be performed. Remove cell debris by centrifugation for 2 minutes at 5000 rpm. Add 1.0ml of water-saturated phenol and shake vigorously. Add 0.225ml BCP and shake vigorously for 15 seconds. Precipitate with 0.6 volumes of isopropanol with 10µl of 100µg/ml glycogen as a carrier. The rest of the procedure is the same as the EZ-RNA II protocol.
Bone Tissue	Freeze the bone tissue in liquid nitrogen, then transfer the frozen tissue to a chilled mortar and pestle and grind it to a fine powder. Add Denaturing Solution to the powder and allow it to sit for 5 minutes at room temperature. Tissue should be further homogenized by vortexing. If interested in RNA isolation only, centrifuge prior to the addition of water saturated phenol and BCP, to get rid of the extracellular debris.
Cartilage	Pulverize the sample (frozen in liquid nitrogen) before homogenizing it. Centrifuge. The modified LiCl precipitation step should be used to remove proteoglycans and polysaccharides.
Fatty Tissue	Add 250mg tissue to 0.75ml Denaturing Solution. Incubate for 5 minutes at room temperature. Then add 0.6ml of water-saturated phenol and shake vigorously. Add 0.135ml BCP and shake vigorously for 15 seconds. Continue according to the original protocol.
Fungus	Isolation of RNA from fungus is difficult. RNases are abundant in fungi. Start with frozen tissue, powder it, add Denaturing Solution, homogenize again after addition of Denaturing Solution, and use an additional spin before adding water-saturated phenol and BCP.
Lymphocytes/ Monocytes	The use of FICOLL-HYPAQUE to isolate cells (lymphocytes/monocytes) does not interfere with the use of EZ-RNA II.
Mold (e.g., Aspergillus)	Freeze mold in liquid nitrogen, add Denaturing Solution and grind to powder with mortar and pestle. If DNA is not to be isolated from this sample, perform centrifugation after homogenization to remove extracellular material. If RNA solution is very viscous, try re-precipitation with high salt isopropanol precipitation.
Pancreas	Pulverize the tissue in liquid nitrogen. Once pulverized, perform the EZ-RNA II extraction with the tube on ice (in an ice water slurry). Use a tissumizer or dounce homogenizer (on ice). Once the RNA is purified, store the RNA in 100% formamide (not an aqueous solution) at -70°C. Resuspending RNA isolated from pancreas in water results in degradation even at -70°C.
Plants	Homogenize 15mg (total weight) leaf with ground glass homogenizer in 0.5ml Denaturing Solution. Proceed with rest of the EZ-RNA II protocol. RNA precipitation from tissue with high content of proteoglycans and/or polysaccharides: after precipitation of the RNA, re-suspend the RNA pellet by mixing with 2.5M LiCl solution, vortex if necessary and store at -20°C for at least 30 minutes. Centrifuge at 10,000g for 15 minutes at 4°C, and proceed with the isolation as described in the EZ-RNA II protocol. The modified precipitation effectively precipitates RNA and maintains proteoglycans and polysaccharides in a soluble form. This procedure should ONLY be used if the sample is known to have a high content of proteoglycans and polysaccharides. 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.

Tissue/Sample Type	Recommendations
Serum	Add 3ml Denaturing Solution per ml serum. Close the tube and shake the solution by hand or vortex, ensuring that mixing is thorough. Store the homogenate for 5 minutes at room temperature then add 3ml Extraction Solution per 3ml Denaturing Solution. Shake vigorously for 15 seconds. Store the sample at room temperature for 10 minutes. Centrifuge at 12,000g for 15 minutes at 4°C. Continue from step 1.3 of the EZ-RNA II protocol.
Spleen, Liver	To avoid DNA contamination problems when isolating RNA from spleen or liver, it is suggested that a larger amount of EZ-RNA II be used (e.g., 0.5ml Denaturing Solution for 50mg tissue, instead of 0.5ml for 100mg tissue).
Whole Blood	Add 0.5ml Whole Blood to 1ml RBC Lysis Solution (Cat. No. 01-888-1). Allow to sit for 5-10 minutes at room temperature with gentle mixing. Centrifuge at 300g for 10 minutes. Repeat process if red blood cells are evident in the pellet. Add 0.5ml of EZ-RNA II to the leukocyte pellet, mix well and proceed with the rest of the protocol. Yield from 1ml blood is 10µg to 30µg. EZ-RNA II should be added to FRESH blood.
Yeast	Spin down cells, estimate the volume (in µl), and use X10 this volume of Denaturing Solution; Pipetting up and down may not be enough to crack the cells. Addition of 500mg of 0.4mm fine glass beads per 0.5ml Denaturing Solution, followed by vortexing will help. Typical yield is 1-5µg per 10^7 cells.

Assessing Yield of Total RNA

The yield of total RNA will vary depending on the tissue or cells from which it is obtained.

Tissue/Sample Type	Amount of Starting Material	Yield of Genomic RNA
Rat Liver	1mg	6µg
Rat Skeletal Muscle	1mg	0.9µg
Mouse Brain	1mg	1.25µg
Mouse Spleen	1mg	2.5µg
Mouse Testes	1mg	2.5µg
Mouse Thymus	1mg	0.85µg
Human Cerebellum	1mg	0.8µg
Human Prostate Yumor	1mg	1µg
MCF-7 Cell Line	$1x10^8$ cells	720µg
U251 Cell Line	$1x10^8$ cells	950µg
Kidney	1mg	3µg
Placenta	1mg	1-4µg
Epithelial Cells	10^6 cells	8-15µg
Fibroblast Cells	10^6 cells	5-7µg
Plant Poinsettia	1mg	0.7µg
Tobacco	1 mg	0.8µg
Yeast	10^7 cells	1-5µg
Bacteria	10^9 cells	3-5µg

Assessing Yield of Genomic DNA

The yield of genomic DNA will vary depending on the tissue or cells from which it is obtained.

Tissue/Sample Type	Amount of Starting Material	Yield of Genomic RNA
Liver	1mg	3-4µg
Kidney	1mg	3-4µg
Skeletal Muscle	1mg	2-3µg
Brain	1mg	2-3µg
Placenta	1mg	2-3µg
Human Cells	10^6 cells	5-7µg
Rat Cells	10^6 cells	5-7µg
Mouse Cells	10^6 cells	5-7µg
Lung	1mg	3-5µg

Problem	Possible Cause	Suggested Solution
Low Yield of RNA	RNA not completely solubilized	To increase solubilization, pipette RNA pellet repeatedly in SDS or DEPC-treated water. Heat sample at 55°C for 10 to 15 minutes. Do not allow RNA pellet to dry completely. Do not lyophilize or vacuum dry samples. Note: Clear pellet indicates over-drying. Do not centrifuge RNA above 12,000g.
	Sample not homogenized completely	Make sure no particulate matter remains. Be sure to incubate for 5 minutes at room temperature after homogenization.
	Some aqueous phase left	Perform second extraction on the remaining aqueous phase.
	Short precipitation	Extend the precipitation. Precipitate for 30 minute s-overnight at -20°C.
Degraded RNA	Sample manipulated too much before EZ-RNA II addition	Process tissue immediately after removal from animal. For cell culture samples, minimize washing steps. Add Denaturing Solution directly to plates. Do not trypsinize cells.
	Improper storage of RNA	Store isolated RNA at -70°C, not -20°C.
	Frozen tissue thawed in absence of Denaturing Solution	Add frozen tissue to Denaturing Solution.
	Aqueous solution or tubes used for procedure may not have been RNase free	Make sure to use only RNase free products.
	Formaldehyde used for the agarose gel electrophoresis may have a pH value below 3.5	Check the pH of the formaldehyde solution.
Low $A_{260/280}$ (<1.6)	Residual organic solvents in the RNA (phenol, chloroform)	Be sure not to carry any of the organic phase with the RNA sample. Precipitate the RNA again with ethanol.
	Sample not homogenized with sufficient Denaturing Solution	Use 0.5ml Denaturing Solution for up to 50mg tissue or 10^6 cells. Be sure to incubate sample for 5 minutes at room temperature after homogenization.

Problem	Possible Cause	Suggested Solution
	pH of solution is acidic	Dissolve sample in TE, or 1mM sodium phosphate buffer pH8, instead of water.
	A_{260} or A_{280} outside the linear range	Dilute sample to bring absorbency into linear range.
RNA contains some DNA	Part of the interphase was removed with the aqueous phase	Be sure not to take any of the interphase (contains the DNA) with the aqueous phase.
	Insufficient Denaturing Solution used	Use 0.5ml Denaturing Solution for 50mg tissue or 10^6 cells.
	Cells or tissue contained organic solvents	Be sure original sample does not contain organic solvents such as ethanol or DMSO.
	Insoluble materials were not removed before chloroform extraction	Remove any particulate material before chloroform addition. This material may trap DNA.
Aqueous phase containing RNA has color	For tissues with high fat content (i.e. skin), fat micelles do not completely separate to top of aqueous phase during centrifugation. Micelles pick up red color from Extraction and Phase Separation Solution.	Centrifuge sample before addition of water-saturated phenol and BCP, and remove fat layer on top.
	For samples with high amounts of blood, some iron or hemoglobin may remain in aqueous phase, giving a yellowish or maroon color.	Make sure not at the upper range of sample to Denaturing Solution ratio.
	Aqueous phase turns yellow upon addition of isopropanol	Try a fresh bottle of isopropanol. (Note: this color has not inhibited RT-PCR).
Cells do not detach from flask after addition of Denaturing Solution	This can be seen with some strongly adherent cells	After addition of Denaturing Solution, let cells sit 2 to 3 minutes. Scrape cells with a rubber policeman. Incubate several minutes. Collect cells and repeatedly pipette cells over flask surface. Then transfer homogenate to a tube.
Precipitate in bottom of the tube after addition of Extraction and Phase separation Solution and centrifugation	High amounts of polysaccharides or proteoglycans	LiCl precipitation. re-suspend the RNA pellet by mixing with 2.5M LiCl Solution. Vortex, store at -20°C for at least 30 minutes, and centrifuge.

Problem	Possible Cause	Suggested Solution
Low yield of DNA	Incomplete homogenization or lysis of sample	Make sure no particulate matter remains. Be sure to incubate for 5 minutes at room temperature after homogenization.
	DNA not completely solubilized	Make sure to use mild alkaline solution.
		Do not allow DNA to dry completely.
Low A_{260}/A_{280}	Phenol may not have been sufficiently removed from the DNA preparation.	Try one more wash of the DNA pellet with the 10% ethanol -0.1M sodium citrate solution.
Degradation of DNA	The tissue may have been immediately processed or frozen after removing from the animal	Process tissue immediately after removal from the animal.
	The sample used for isolation may heve been stored at -20°C instead of -70°C	Make sure to store the sample at -70°C as specified in the procedure.
	Sample may have been homogenized with a polytron or a similar high-speed homogenizer	Try to use a mild homogenizer.
DNA contains some RNA	Too much aqueous phase may have remained in the organic phase and interphase	Remove the remaining aqueous phase overlaying the interphase.
	The DNA pellet may not have been washed sufficiently with 10% ethanol - 0.1M sodium citrate solution	Try one more wash.

Problem	Possible Cause	Suggested Solution
Low yield of Protein	Sample may have been incompletely homogenized The final protein pellet may not have been dissolved	Make sure no particulate matter remains. Be sure to incubate for 5 minutes at room temperature after homogenization. To ensure solubilization, incubate the sample at 50°C.
Degradation of the Protein	The tissue may not have been immediately processed or frozen after removal from the animal	Process tissue immediately after removal from the animal.
Band deformation in PAGE	Protein pellet may not have been washed sufficiently	Perform an additional wash with ethanol.

EZ-RNA II Protocol at a glance

	RNA	DNA	Proteins
Homogenization	**0.5ml Denaturing Solution** 5 minutes after Homogenization		
Extraction and Phase separation	**Homogenate + 0.4ml Water-saturated phenol + 0.09ml BCP** 10 minutes /// 12,000g - 15 minutes - 4°C		
Precipitation	**Aqueous phase + 0.5ml isopropanot** 10 minutes /// 12,000g - 8 minutes - 4°C	**Organic phase (from RNA Exraction) + 0.3ml absolute ethanol** 3 minutes /// 2,000g - 5 minutes - 4°C	**phenol-Ethanol Supernatant (from DNA Precipitation step) + 1.5ml isopropanol** 10 minutes /// 12,000g - 10 minutes - 4°C
Wash	**1ml 75% Ethanol** 7,500g - 5 minutes - 4°C	**I. 1ml 0.1M sodium citrate in 10% ethanol - X2** 30 minutes /// 2,000g - 5 minutes - 4°C **II. 2ml 75% ethanol** 20 minutes /// 2,000g - 5 minutes - 4°C	**I. 2ml gnanidine-HC1, 0.3M in 95% ethanol - X3** **II. 2ml absolute ethanol** 20 minutes /// 7,500g - 5 minutes - 4°C
Solubilization	**Water with 0.1mM EDTA** 10-15 minutes - 55°C	**8mM NaOH**	**1% SDS solution** 10,000g - 10 minutes - 4°C

BI
Biological Industries
Culture of Excellence

Biological Industries Israel Beit Haemek Ltd.
Kibbutz Beit Haemek 25115, Israel
T.+972.4.9960595 F.+972.4.9968896
Email: info@bioind.com
www.bioind.com