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Introduction

E.Z.N.A.™ DNA/RNA Kit provides a rapid and easy method for the isolation of total RNA and genomic DNA from cultured cells and animal tissues. The kit allows single or simultaneous processing of multiple samples in less than 40 min. Normally, 1 x 10^6 - 1 x 10^7 eukaryotic cells or 25-30 mg tissue can be used in a single experiment. There is no need for phenol/chloroform extractions, and time-consuming steps such as CsCl gradient ultracentrifugation, and precipitation with isopropanol or LiCl, are eliminated.

RNA purified using the E.Z.N.A..™ DNA/RNA method is ready for applications such as RT-PCR*, Northern blotting, poly A^+ RNA (mRNA) purification, nuclease protection, and *in vitro* translation.

Principle

The E.Z.N.A..™ DNA/RNA Kit combines reversible binding properties of HiBind® RNA technology with a a specially designed buffer system which selective bind DNA to a DNA column before RNA isolation. Samples are first lysed and homogenized in a specially designed denature buffer (GTC), which immediately inhabit the activity of RNase and DNase. The lyste is then passed through a HiBind® DNA column which will selectively bind genomic DNA. After two quick wash steps, the purified DNA is eluted from HiBind® DNA column . The flow-through lysate fromthe HiBind® DNA column is then added with ethanol to create proper RNA binding conditions, the sample is then loaded into the HiBind® RNA column to bind RNA. After two wash steps, purified RNA is eluted with RNase-free water.

Storage

All components in the E.Z.N.A® DNA/RNA Kit should be stored at room temperature. During shipping and storage, crystals may form in the GTC Lysis Buffer, simply warm to 37°C to dissolve. All kit components are guaranteed for at least 12 months from the date of purchase.

*The PCR process is covered by U.S. Patents 4,683,195 and 4,683,202 (and international equivalents) owned by Hoffmann-LaRoche, Inc.

Kit Contents

	RNA Prep 5	RNA Prep 50	RNA Prep 200
Product Number	R6731-00	R6731-01	R6731-02
Purifications	5	50	200
Components			
HiBind™ RNA Columns	**5**	**50**	**200**
HiBind™ DNA Columns	**5**	**50**	**200**
2 ml Collection Tubes	**20**	**200**	**800**
GTC Lysis Buffer	**5 ml**	**40 ml**	**150 ml**
RNA Wash Buffer I	**5 ml**	**30 ml**	**125 ml**
RNA Wash Buffer II	**2 ml**	**12 ml**	**50 ml**
HB Buffer	**5 ml**	**30 ml**	**125 ml**
DNA Wash Buffer	**1.5 ml**	**15 ml**	**3 x 25 mL**
DEPC-ddH_2O	**1.0 ml**	**10 ml**	**40 ml**
Elution Buffer	**1.0 ml**	**5 ml**	**20 ml**
Instruction Manual	**1**	**1**	**1**

Before Starting

IMPORTANT

RNA Wash Buffer II must be diluted with absolute ethanol before use.

R6731-00	Add 8 ml 100% ethanol
R6731-01	Add 48 ml 100% ethanol
R6731-02	Add 200 ml 100% ethanol

DNA Wash Buffer must be diluted with absolute ethanol before use.

R6731-00	Add 6 ml 100% ethanol
R6731-01	Add 60 ml 100% ethanol
R6731-02	Add 100 ml 100% ethanol

Please take a few minutes to read this booklet thoroughly and become familiar with the protocol. Prepare all materials required before starting to minimize RNA degradation.

- Whenever working with RNA, always wear latex gloves to minimize RNase contamination. Use only clean RNase-free disposable plastic pipette tips when using the supplied reagents.
- During the procedure work carefully but quickly.

- Under cool ambient conditions, crystals may form in GTC Lysis Buffer. This is normal and the bottle should be warmed to redissolve the salt.
- 2-mercaptoethanol (ß-mercaptoethanol) is key in denaturing RNases and must be added to an aliquot of GTC Lysis Buffer before use. Add 20 µl of 2-mercaptoethanol per 1 ml of GTC Lysis Buffer. This mixture can be stored for 1 week at room temperature.
- All centrifugation steps must be carried out at 22°C-25°C.

Disruption and Homogenization of samples

Efficient disruption and homogenization of the sample is essential for successfully isolating total RNA. Completely disruption of the cell walls and plasma membrane is very important for releasing all the RNA containing in the samples. The purpose of homogenization is to reduce the viscosity of the cell lysates produced by cell disruption. Incompletely homogenization will reduce the binding of RNA to the RNA column and sometimes will clog the RNA column thus cause lower yield or no yield.

Disruption of Sample with Mortar and Pestle

Wear gloves and take great care when working with liquid nitrogen. Excise tissue and promptly freeze in a small volume of liquid nitrogen. Grind tissue with a ceramic mortar and pestle under approximately 10 ml of liquid nitrogen and pour the suspension into a pre-cooled 15 ml polypropylene tube. Unless the tube is pre-cooled (in liquid nitrogen), the suspension will boil vigorously possibly causing loss of tissue. When the liquid nitrogen has completely evaporated, add GTC Lysis Buffer and continue with the procedure as outlined below. After interrupt tissue, lysate can be homogenized with Homogenizer Spin Column (Product # HCR 002). **The lysate is loaded onto Homogenizer Spin Column in a 2 ml collection tube**. Spin two minutes at a maximum speed in a micro centrifuge and the homogenized lysate is collected. Use the Omega Homogenizer Spin Column is a fast and efficient way to homogenize the lysate without cross contamination of samples. The alternated way to homogenize the lysate is to use the syringe and needle. High molecular-weight DNA can be sheared by passing the lyrate through a narrow needle (19-21 gauge) for 10-20 times..

Disruption & homogenization of sample with Rotor-Stator Homogenizers

Rotor-stator homogenizers can effectively simultaneously disrupt and homogenize most samples. The process usually takes less than a minute depending on the tissue. Many Rotor-stator homogenizers operate with differently sized probes or generators that allow processing sample in 50ml tubes.

Disruption & homogenization of sample using Bead Milling

By using bead milling, cells and tissues can be disrupted and homogenized by rapid agitation in the presence of beads and lysis buffer. The optimal to use for RNA isolation are 0.5mm glass beads for yeast and unicellular cells, 4-8 mm beads for animal tissue samples.

Homogenization of lysate with Syringe Needle Method

High molecular weight DNA is responsible for the viscosity of cell lysates and can be shredded by passing the sample 10-20 times through a narrow needle (19-21 gauge).

E.Z.N.A DNA/RNA Isolation Protocol

A. Isolating Total RNA/DNA from Animal Cells

Materials supplied by user:

- 2-mercaptoethanol
- Microcentrifuge capable of at least 14,000 x g
- 70% ethanol in DEPC-treated sterile distilled water
- Sterile RNase-free pipette tips and 1.5ml centrifuge tubes
- Disposable latex gloves

Procedure:

1.**Determine the proper amount of starting material**: This is critical to use correct number of cells to obtain optimal RNA yield and purity . The maximum number of cells that can be processed with this DNA/RNA protocol depends on: 1). the specific RNA contents and type of cell lines; 2). The amount of GTC Buffer required for efficient lysis and maximum loading volume of the Hibind® RNA columns; 3). The DNA capacity of the Hibind® DNA columns. The maximum binding capacity of the HiBind® RNA column is 100µg and the maximum DNA capacity for HiBind® DNA column is 20µg . The maximum number of the cells that GTC lysis buffer used in the this protocol is 1 x 10^7. Use following table as a guideline to select correct starting material.

Average Yield of Total cellar RNA

Source	Number of Cells	RNA Yield (µg)
IC21	1 x 10^6	12
Hela	1 x 10^6	15
293HEK	1 x 10^6	10
HIN3T3	1 x 10^6	15

2. **Harvest Cells**: For cells grown in suspension: determine the number of cells. Pellet the appropriate number of cell by centrifuge at 500 x for 5 minutes. Aspirate the supernatant and continue the step 3 of this protocol.

   For cells grown in a monolayer: Cells grown in a monolayer in cell culture dish can be directly lysed in the dish or trypsinnized and collect the cell pellet before lysis. Cells grown in cell culture flask should be trypsinized and collect cell pellet prior to lysis.

3. **Disrupt cells (do not use more than 1 x 10^7 cells) with GTC Lysis Buffer**: For pelleted cells, loosen the cell pellet throughly by flicking the tube and add the appropriate amount of GTC Lysis Buffer based on table blow. To directly lyse the cell in the culture dish, add the appropriate amount if GTC Lysis Buffer directly to the dish. **Remember to add 20 µl of 2-mercaptoethanol per 1 ml of GTC Lysis Buffer before use.** .

GTC Lysis Buffer Volume for RNA Column

Number of Cells	Amount of GTC Lysis Buffer (µl)
< 5 x 10^6	350
5 x 10^6 - 1 x 10^7	700

4. **Homogenize cells by using one of the methods described on page 4-5.**

   Note: incomplete homogenization of the sample will cause lower yields and clogging of the column. It is recommended to homogenize the sample with rotor-stator homogenizers since it normally produce better yield.

5. **Transfer the homogenized lysate to a HiBind® DNA column placed in a 2ml collection tube**. Centrifuge at ≥13000 x g for 1 minute. Remove and save the column for later DNA isolation (steps 11-15). Use the flow-through for RNA isolation in next step.

   Note: make sure that all the liquid has passed through column after the centrifugation. If necessary, repeat the centrifugation until all liquid has passed through the membrane.

RNA Isolation

6. **Add an equal volume (350µl or 700µl) 70% ethanol to the flow-**

through and mix thoroughly by pipetting up and down 20 times. If the sample lost volume during homogenization, adjust the volume of ethanol accordingly.

7. **Apply sample onto HiBind® RNA spin column**. The maximum capacity of the spin column is 800 µl. (Larger volumes can be loaded successively.) A precipitate may form on addition of ethanol. Vortex and add the entire mixture to the column. With the spin column inside the 2ml collecting tube (supplied with kit), centrifuge at ≥10,000 x g for 1 min **at room temperature**. Discard flow-through and proceed to next step.

6. **Add 500µl RNA Wash Buffer I directly into the HiBind® RNA spin column.** Centrifuge at ≥ 10,000 x g for 1 minute to wash the column membrane. Discard flow-through and re-use the collecting tube.

7. **Place column into collection tube**, **and add 500µl RNA Wash Buffer II to the column**. Centrifuge at ≥10,000 x g for 1 minutes at room temperature. Discard flow-through and collection tube.

Note: Wash Buffer II Concentrate must be diluted with absolute ethanol before use. Refer to label on bottle for directions.

8. **Place the HiBind® RNA spin column into a new collection tube. Add 500µl RNA Wash Buffer II directly into the HiBind® RNA spin column.** Centrifuge at ≥ 10,000 x g for 1 minute to wash the spin column membrane. Discard flow-through and re-use the collecting tube.

9. Then with the collection tube empty, centrifuge the **HiBind® RNA spin column** for **2 minutes at maximum speed** to completely dry the HiBind® matrix.

10.. **Elution of RNA.** Transfer the column to a clean 1.5 ml centrifuge tube (Not supplied) and elute the RNA with 40-70µl of DEPC-treated water (supplied with kit). Make sure to add water directly onto column matrix. Centrifuge 1 min at 10,000 x g. A second elution may be necessary if the expected yield of RNA >30 µg.

Alternatively, RNA may be eluted with a greater volume of water. While additional elutions increase total RNA yield, the concentration will be lowered since more than 80% of RNA is recovered with the first elution. Pre-heating the water to 70°C before adding to column and incubating column 5 min at room temperature before centrifugation may increase yields.

DNA Isolation

11. **Place the DNA Binding column (from step 5) into a new 2ml collection tube.**

12. **Add 500µl HB Buffer to the column and centrifuge at ≥10,000 x g for 1 minute.** Discard the flow-through and re-use the collection tube.

13. **Add 700µl of DNA wash Buffer to the DNA Binding column**. Centrifuge at 10,000 x g for 30 seconds. Discard the flow-through and re-use the collection tube.

14. **Place the empty HiBind® DNA column into a same collection tube**. Open the lid of the column and centrifuge at full speed for 2 minutes to completely dry the membrane.

15. **Place the HiBind® DNA column into a new 1.5 ml centrifuge tube (not supplied)**. Add 50-100µl Elution Buffer directly to the center of the column membrane. Close the lids and centrifuge at maximum speed for 2 minutes to elute DNA.

B. Isolating Total RNA/DNA from Animal Tissues

Materials supplied by user:

- 2-mercaptoethanol
- Microcentrifuge capable of at least 14,000 x g
- 70% ethanol in DEPC-treated sterile distilled water
- Sterile RNase-free pipette tips and 1.5ml centrifuge tubes
- Disposable latex gloves

1. **Determine the proper amount of starting material**: This is critical to use the correct number of cells to obtain optimal RNA yield and purity . The maximum number of cells that can be processed with this DNA/RNA protocol varies depends on: 1). the specific RNA contents and type of cell lines; 2). The amount of GTC Buffer required for efficient lysis and maximum loading volume of the Hibind® RNA columns; 3). The DNA capacity of the Hibind® DNA columns. The maximum binding capacity of the HiBind® RNA column is 100µg and the maximum DNA removal capacity for HiBind® DNA column is 20µg . The maximum amount of tissue that GTC lysis buffer used in the this protocol is 30mg. Use following table as a guideline to select correct starting material. **If you have no information about the your starting material, use 10 mg as starting amount, base ob the yield and quality of RNA obtained from 10 mg, adjust the starting amount in the next purification.**

Average Yield of Total cellar RNA

Source	Amount of Tissue (mg)	RNA Yield (µg)
Mouse Tissue		
Brain	10	10
Kidney	10	30
Liver	10	45
Heart	10	5
Spleen	10	33
Lung	10	12
Pancreas	10	40
Thymus	10	20

2. Disrupt Tissue and homogenize the lysate in GTC Lysis Buffer using one of the described methods on page 4. **(Do not use more than 30mg tissue**). **Remember to add 20 µl of 2-mercaptoethanol per 1 ml of GTC Lysis Buffer before use.**

Note: incomplete homogenization of the sample will cause lower yields and clogging of the column. It is recommended to homogenize the sample with rotor-stator homogenizers since it normally produce better yield.

GTC Lysis Buffer Volume for RNA Column

Amount of Tissue (mg)	Amount of GTC Lysis Buffer (µl)
≤ 15	350
20- 30	700

3. **Centrifuge at 13,000 x g for 5 minutes.** Carefully transfer the cleared supernatant to a HiBind® DNA column pre-inserted in a 2ml collection tube**.**

Note: In some preparations, a fatty upper layer will form after the centrifugation, transferring any pellet or fatty layer may reduce the RNA yield or clog the column.

4. Centrifuge at ≥13000 x g for 1 minute. Remove and save the HiBind® DNA column for later DNA isolation

Note: make sure that all the liquid has passed through column after the centrifugation. If necessary, repeat the centrifugation until all liquid has passed through the membrane.

RNA Isolation

5. Add an equal volume (350µl or 700µl) 70% ethanol to the flow-through and mix thoroughly by pipetting. If the sample lost volume during homogenization, adjust the volume of ethanol accordingly.

6. Apply sample onto HiBind® RNA spin column. The maximum capacity of the spin column is 800µl. (Larger volumes can be loaded successively.) A precipitate may form on addition of ethanol. Vortex and add the entire mixture to the column. With the spin column inside the collection tube (supplied with kit), **centrifuge at ≥10,000 x g for 1** min **at room temperature**. Discard flow-through and re-use the collection tube.

7. **Add 500µl RNA Wash Buffer I directly into the HiBind® RNA spin column.** Centrifuge at ≥ 10,000 x g for 1 minute to wash the spin column membrane. Discard flow-through and re-use the collecting tube.

8. **Place column into collection tube**, **and add 500µl RNA Wash Buffer II to the column**. Centrifuge at ≥10,000 x g for 1 minutes at room temperature. Discard flow-through and collection tube.

Note: Wash Buffer II Concentrate must be diluted with absolute ethanol before use. Refer to label on bottle for directions

9. **Place the HiBind® RNA spin column into a new collection tube. Add 500µl RNA Wash Buffer II directly into the HiBind® RNA spin column.** Centrifuge at ≥ 10,000 x g for 1 minute to wash the spin column membrane. Discard flow-through and re-use the collection tube.

10. Then with the collection tube empty, centrifuge the spin cartridge for **2 minutes at maximum speed** to completely dry the HiBind® matrix.

11. **Elution of RNA.** Transfer the column to a clean 1.5 ml centrifuge tube (Not supplied) and elute the RNA with 40-70µl of DEPC-treated water (supplied with kit). Make sure to add water directly onto column matrix. Centrifuge 1 min at 10,000 x g. A second elution may be necessary if the expected yield of RNA >30 µg.

12. **Place the DNA Binding column (from step 4) into a new 2ml collection tube.**

13. **Add 500µl HB Buffer to the column and centrifuge at ≥10,000 x g for 1 minute.** Discard the flow-through and re-use the collection tube.

14. **Add 700µl of DNA wash Buffer to the DNA Binding column**. Centrifuge at 10,000 x g for 30 seconds. Discard the flow-through and re-use the collection tube.

15. **Place the empty column into a same collection tube**. Open the lid of the column and centrifuge at full speed for 2 minutes to completely dry the membrane.

16. **Place the DNA Binding column into a new 1.5 ml centrifuge tube (not supplied)**. Add 50-100µl Elution Buffer directly to the center of the column membrane. Close the lids and centrifuge at maximum speed for 2 minutes to elute DNA.

Quantitation and Storage of RNA

To determine the concentration and purity of RNA, measure absorbance at 260 nm and 280 nm in a spectrophotometer. 1 O.D. unit measured at 260 nm corresponds to 40 µg of RNA per ml. DEPC-water is slightly acidic and can dramatically lower absorbance values. We suggest that you dilute the sample in a buffered solution (TE) for spectrophotometric analysis. The ratio of A_{260}/A_{280} of pure nucleic acids is 2.0, while for pure protein it is approximately 0.6. A ratio of 1.8-2.0 corresponds to 90%-100% pure nucleic acid. (Phenol has an absorbance maximum at 275 nm and can interfere with absorbance readings of DNA or RNA. Store RNA samples at -70°C in water. Under such conditions RNA prepared with the E.Z.N.A.® system is stable for more than a year.

RNA Quality

It is highly recommended that RNA quality be determined prior to all analyses. The quality of RNA can best be assessed by denaturing agarose gel electrophoresis and ethidium bromide staining. Two sharp bands should appear on the gel. These are the 28S and 18S (23S and 16S for bacteria) ribosomal RNA bands. If these band smear towards lower molecular weight RNAs, then the RNA has undergone major degradation during preparation, handling, or storage. Although RNA molecules less than 200 bases in length do not efficiently bind the HiBind matrix, a third RNA band, the tRNA band, may be visible when a large number of cells are used.

Troubleshooting Tips

Problem	Cause	Suggestion
Little or no RNA eluted	RNA remains on the column	• Repeat elution. • Pre-heat DEPC-water to 70° C prior to elution. • Incubate column for 10 min with water prior to centrifugation.
	Column is overloaded	• Reduce quantity of starting material.
Clogged column	Incomplete homogenization	• Completely homogenize sample. • Increase centrifugation time. • Reduce amount of starting material
Degraded RNA	Source	• Freeze starting material quickly in liquid nitrogen. • Do not store tissue culture cells prior to extraction unless they are lysed first. • Follow protocol closely, and work quickly.
	RNase contamination	• Ensure not to introduce RNase during the procedure. • Check buffers for RNase contamination.
Problem in downstream applications	Salt carry-over during elution	• Ensure Wash Buffer II Concentrate has been diluted with 4 volumes of 100% ethanol as indicated on bottle. • 1 X Wash Buffer II must be stored and used at room temperature. • Repeat wash with Wash Buffer II.
DNA contamination	HiBind DNA column is overloaded	• Reduce the amount of starting material. • Digest with RNase-free DNase and inactivate at 75°C for 5 min.
Low Abs ratios	RNA diluted in acidic buffer or water	• DEPC-treated water is acidic and can dramatically lower Abs260 values. Use TE buffer to dilute RNA prior to spectrophotometric analysis.