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Introduction

E.Z.N.A.™ DNA/RNA/Protein Isolation Kit is designed to purify total RNA and genomic DNA simultaneously from a single biological sample source. Up to 100 µg of total RNA and DNA can be isolated from cultured eukaryotic cells and tissues. The kit allows single or multiple, simultaneous processing of samples in less than 30 min. Normally, 1 x 10^6 - 1 x 10^7 eukaryotic cells, or 20-30 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 this method is ready for applications such as RT-PCR*, Northern blotting, poly A^+ RNA (mRNA) purification, nuclease protection, and *in vitro* translation. DNA purified from this methods can be used for most of downstream applications such as PCR, Southernblot, and genotyping.

Principle

The E.Z.N.A.™ DNA/RNA/Protein Isolation Kit uses the reversible binding properties of HiBind® matrix, a new silica-based material, combined with the speed of mini-column spin DNA/RNA isolation technology to isolate high quality of DNA and RNA from single sample source. Samples are first lysed and homogenized with a specifically formulated high salt buffer which will immediately inactive the nucleases to ensure the intact of DNA and RNA. The lysate is then passed through the HiBind® DNA column which will bind genomic DNA . After two quick wash steps, the pure, ready-to-use DNA is eluted with low salt Elution Buffer or water. The flow-through lysate after the HiBind DNA column is collected and mixed with ethanol to create proper binding condition for RNA. The sample is then loaded to a HiBind RNA column, where the RNA is bound to the membrane and the contaminants are efficiently washed way. Purified RNA is then eluted with is finally eluted in DEPC-treated sterile water.

Storage and Stability

E.Z.N.A.™ DNA/ RNA/Protein Kits should be stored at room temperature. During shipment crystals may form in the Buffer GTC. Warm to 37°C to dissolve. All E.Z.N.A..™ DNA/RNA/Protein Isolation Kit components are guaranteed for at least 24 months from the date of purchase when stored at 22-25°C.

Kit Contents

Product Number	R6734-00	R6734-01	R6734-02
Purification times	5 Preps	50 Preps	200 Preps
HiBind™ RNA Columns	5	50	200
HiBind™ DNA Columns	5	50	200
2 ml Collection Tubes	15	150	600
Buffer GTC	5 ml	40 ml	150 ml
RNA Wash Buffer I	5 ml	50 ml	200 ml
RNA Wash Buffer II Concentrate	2 ml	12 ml	50 ml
Buffer HB	5 ml	30 ml	120 ml
DNA Wash Buffer Concentrate	5 ml	20 ml	40 ml
Elution Buffer	5 ml	20 ml	60 ml
DEPC-ddH_2O	1.0 ml	10 ml	40 ml
Instruction Manual	1	1	1

Before Starting

IMPORTANT

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

R6734-00	Add 8 ml 96-100% ethanol
R6734-01	Add 48 ml 96-100% ethanol
R6734-02	Add 200 ml 96-100% ethanol

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

R6734-00	Add 20 ml 96-100% ethanol
R6734-01	Add 80 ml 96-100% ethanol
R6734-02	Add 160 ml 96-100% ethanol

Please take a few minutes to read this booklet thoroughly and become familiar with the protocol.

- 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.
- Under cool ambient conditions, crystals may form in Buffer GTC. This is normal and the bottle should be warmed to redissolve the salt.
- 2-mercaptoethanol is key in denaturing RNases and must be added to an aliquot of Buffer GTC before use. Add 20 µl of 2-mercaptoethanol per 1 ml of Buffer GTC. 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. Homogenization shears the genomic DNA and other high molecular weight cell components to create a homogeneous lysate. 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 Buffer GTC 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 with by passing the lyrate through a narrow needle (19-21 gauge) for 5-10 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 present 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/Protein Isolation Protocol

A. Simultaneously Isolating DNA and RNA 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 yield and purity with HiBind® DNA/RNA column. The maximum number of cells that can be processed on a HiBind® RNA and DNA column varies depends on the specific DNA and RNA contents and type of cell lines. The maximum binding capacity of the HiBind® RNA and DNA column is 100µg. The maximum number of the cells that Buffer GTC used in the this protocol is 1 x 10^7. Use following table as a guideline to select correct starting material. If information for DNA/RNA contents is not available, we recommend to use less than 5 x 10^6 cells as starting amount and increase the starting cell amount after few successful preparations.

Average Yield of Total cellar RNA

Source	Number of Cells	DNA Yield	RNA Yield
IC21	1 x 10^6	7 µg	12 µg
Hela	1 x 10^6	6 µg	15 µg
293HEK	1 x 10^6	6 µg	10 µg
HIN3T3	1 x 10^6	8 µg	15 µg

2. **Harvest Cells**:

   For cells grown in suspension: determine the number of cells. Pellet the appropriate number of cell by centrifuge at 500 x g 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 collet 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 Buffer GTC: For pelleted cells, loose the cell pellet throughly by flicking the tube and add the appropriate amount of Buffer GTC based on table blow. To directly lyse the cell in the culture dish, add the appropriate amount if Buffer GTC directly to the dish. **Remember to add 20 µl of 2-mercaptoethanol per 1 ml of Buffer GTC before use.**

Buffer GTC Volume VS Number of Cells

Number of Cells	Amount of Buffer GTC (µl)
< 5 x 10^6	500
5 x 10^6 - 1 x 10^7	700

3. **Homogenize cells with a rotor-stator homogenizer or until the samples is uniformly homogenized**. Alternately, sample can be homogenized by vortexing combine with syringe needle method (described on page 4).
   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.

4. **Transfer the homogenized cell lysate to a HiBind® DNA Column pre-inserted in a 2 ml collection tube (supplied).** Close the cap of the column gently, centrifuge at ≥13,000 x g for 1 minute. Make sure that all cell lysate is passed through the column, centrifuge again if necessary.

5. **Place the HiBind® DNA column into a new 2 ml collection tube and store at room temperature for later DNA isolation in step 12-15**. Do not freeze the HiBind® DNA column.

Total RNA Isolation

6. **Add 0.5 volume (250µl or 350µl) absolute ethanol (96-100%) to the flow-through lysate from step 5 and mix thoroughly by vortexing** at maxi speed for 15 seconds. If the sample lost volume during homogenization, adjust the volume of ethanol accordingly.

7. **Apply the mixture from step 6 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 13,000 x g for 1 min at room temperature.

Transfer the flow through into a new tube for Protein Isolation in step 16.

8. **Wash column with by pipetting 500µl RNA Wash Buffer I directly into the HiBind® RNA spin column**. Centrifuge at 13,000 x g for 1 minute at room temperature. Discard the flow-through and re-use the 2 ml collecting tube.

   Note: This the starting point if on-membrane DNase I digestion (page 12).

9. **Add 500µl RNA Wash Buffer II diluted with absolute to the column and centrifuge at 13,000 x g for 1 minutes at room temperature**. Discard flow-through and re-use the collection tube in next step.

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

10. **Add 500µl RNA Wash Buffer II to the column and centrifuge at 13,000 x g for 1 minutes at room temperature**. Discard flow-through and re-use the collection tube. Then with the collection tube empty, centrifuge the column for 2 min at maximum speed(≥13,000 x g) 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 30-70µl of DEPC-treated water (supplied with kit). Make sure to add water directly onto column matrix. Centrifuge 1 min at 13,000 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.

Genomic DNA Isolation

12. **Add 500 µl Buffer HB to the HiBind® DNA column pre-inserted in a collection tube from step 5. Centrifuge at 13,000 x g for 1 min at room temperature.** Discard the flow-through and re-use the collection tube.

13. **Add 700 µl DNA Wash Buffer diluted with absolute ethanol to the HiBind® DNA column pre-inserted in a collection tube from step 5. Centrifuge at 13,000 x g for 1 min at room temperature.** Discard the flow-through and re-use the collection tube.

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

14. **Place the HiBind® DNA column into the collection tube from previous step, centrifuge at maximum speed(≥13,000 x g) for 2 minutes to dry the spin column membrane.**

15. **Elution of DNA.** Transfer the column to a clean 1.5 ml centrifuge tube (Not supplied) and elute the DNA with 50-100µl of Elution Buffer (supplied with kit). Make sure to add water directly onto column matrix. Incubate at room temperature for 2 minutes. Centrifuge for 1 min at 13,000 x g. A second elution may be necessary if the expected yield of DNA >30 µg.

Protein Isolation

16. **Add 4 volume of ice-cold acetone to the flow through from step 7.** Vortex to mix well.

17. **Incubate at -20°C for 30 minutes.**

18. Centrifuge at maximum speed for 10min at 4°C .

19. Wash the pellet with 1 ml ice-cold absolute ethanol.

20. Centrifuge at maximum speed for 3 min at 4°C .

21. Discard the liquid and air dry.

22. Dissolve the protein with Buffer for your downstream applictaion.

B. Simultaneously Isolating DNA and RNA 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 correct amount of tissue to obtain optimal yield and purity with HiBind® RNA/DNA column. The maximum amount of tissue that can be processed on a HiBind® RNA/DNA column varies depends on the specific RNA contents and type of tissue. The Maximum binding capacity of the HiBind® RNA and DNA column is 100µg. The maximum tissue that Buffer GTC used in the Total RNA protocol is 30 mg. 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	DNA Yield	RNA Yield
Mouse Tissue			
Brain	10 mg	8 µg	10 µg
Kidney	10 mg	20 µg	30 µg
Liver	10 mg	20 µg	45 µg
Heart	10 mg	8 µg	5 µg
Spleen	10 mg	60 µg	33 µg
Lung	10 mg	20 µg	12 µg
Pancreas	10 mg	50 µg	40 µg
Thymus	10 mg	60 µg	20 µg

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

Buffer GTC Volume for RNA Column

Amount of Tissue (mg)	Amount of Buffer GTC (µl)
≤ 15	500
20- 30	700

3. **Centrifuge at 13,000 x g for 5 minutes at room temperature.**
Note: In some preparations, a fatty upper layer will form after the centrifugation, will form, transferring any pellet or fatty layer may reduce the RNA yield or clog the column.

4. **Carefully transfer the cleared supernatant to a HiBind® DNA Column pre-inserted in a 2 ml collection tube (supplied).** Close the cap of the column gently, centrifuge at ≥13,000 x g for 1minute at room temperature. Make sure that all cell lysate is passed through the column, centrifuge again if necessary.

5. **Place the HiBind® DNA column into a new 2 ml collection tube and store at room temperature for later DNA isolation in step 12-15**. Do not freeze the HiBind® DNA column.

Total RNA Isolation

6. **Add 0.5 volume (250 µl or 350 µl) absolute ethanol (96-100%) to the flow-through lysate from step 5 and mix thoroughly by vortexing** at maxi speed for 15 seconds. If the sample lost volume during homogenization, adjust the volume of ethanol accordingly. For maximum maxi, add 1/3 volume of absolute ethanol to flow through when processing liver tissue.

7. **Apply the mixture from step 6 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 13,000 x g for 1 min at room temperature. Transfer the flow through into a new tube for Protein Isolation in step 16.

8. **Wash column with by pipetting 500µl RNA Wash Buffer I directly into the**

HiBind® RNA spin column. Centrifuge at 13,000 x g for 1 minute at room temperature. Discard the flow-through and re-use the 2 ml collecting tube.

Note: This the starting point if on-membrane DNase I digestion (page 12).

9. **Add 500µl RNA Wash Buffer II diluted with absolute to the column and centrifuge at 13,000 x g for 1 minutes at room temperature**. Discard flow-through and re-use the collection tube in next step.

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

10. **Add 500µl RNA Wash Buffer II to the column and centrifuge at 13,000 x g for 1 minutes at room temperature**. Discard flow-through and re-use the collection tube. Then with the collection tube empty, centrifuge the column for 2 min at maximum speed(≥13,000 x g) 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 30-70µl of DEPC-treated water (supplied with kit). Make sure to add water directly onto column matrix. Centrifuge 1 min at 13,000 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.

Genomic DNA Isolation

12. **Add 500 µl Buffer HB to the HiBind® DNA column pre-inserted in a collection tube from step 5. Centrifuge at 13,000 x g for 1 min at room temperature.** Discard the flow-through and re-use the collection tube.

13. **Add 700 µl DNA Wash Buffer diluted with absolute ethanol to the HiBind® DNA column pre-inserted in a collection tube from step 5. Centrifuge at 13,000 x g for 1 min at room temperature.** Discard the flow-through and re-use the collection tube.

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

14. **Place the HiBind® DNA column into the collection tube from previous step, centrifuge at maximum speed(≥13,000 x g) for 2 minutes to dry the spin column membrane.**

15. **Elution of DNA.** Transfer the column to a clean 1.5 ml centrifuge tube (Not supplied) and elute the DNA with 50-100µl of Elution Buffer (supplied with kit). Make sure to add water directly onto column matrix. Incubate at room temperature for 2 minutes. Centrifuge for 1 min at 13,000 x g. A second elution may be necessary if the expected yield of DNA >30 µg.

Protein Isolation

16. **Add 4 volume of ice-cold acetone to the flow through from step 7.** Vortex to mix well.

17. **Incubate at -20°C for 30 minutes.**

18. Centrifuge at maximum speed for 10min at 4°C .

19. Wash the pellet with 1 ml ice-cold absolute ethanol.

20. Centrifuge at maximum speed for 3 min at 4°C .

21. Discard the liquid and air dry.

22. Dissolve the protein with Buffer for your downstream applictaion.

C. DNase I digestion Protocol (Optional)

Since HiBind® RNA resin and spin-column technology actually removes most of DNA without the DNase treatment, it is not necessary to do DNase digestion for most downstream applications. However, certain sensitive RNA applications might require further DNA removal. Following steps provide on-membrane DNase I digestion:(see DNase I cat.# E1091for detail information)

1. Follow the standard protocol until the samples **completely** pass through the HiBind RNA column (step1-8). Prepare the following:

2. For each HiBind® RNA column, prepare the DNase I digestion reaction mix as follows:

OBI DNase I Digestion Buffer	73.5 µl
RNase-free DNase I (20 Kunitz unites/µl)	1.5 µl
Total volume	75 µl

Note:

a. **DNase I is very sensitive for physical denaturaion, so do not vortex this DNase I mixture. Mix gently by inverting the tube. Prepare the fresh DNase I digestion mixture before RNA isolation.**
b. **OBI DNase I digestion buffer is supplied with OBI RNase-free Dnase set**.
c. **Standard Dnase buffers are not compatible with on-membrane Dnase digestion.**

3. Pipet 75 µl of the DNase I digestion reaction mix directly onto the surface of HiBind® RNA resin in each column. Make sure to pipet the Dnase I digestion mixture directly onto the membrane. DNase I digestion will not be complete if some of the mix stick to the wall or the O-ring of the HiBind® RNA column.

4. Incubate at room temperature(25-30°C) for 15 minutes.

5. **Place column into a new 2 ml centrifuge tube**, and add 400µl RNA Wash Buffer I. **Place the column at room temperature for 2 minutes.** Centrifuge at 13,000 x g for 1 minutes and discard flow-through. Reuse the collection tube in step 6.

6. **Place column in the same 2 ml centrifuge tube**, and add 500 µl RNA Wash Buffer II diluted with ethanol. Centrifuge at 13,000 x g for 1 minutes and discard flow-through. Reuse the collection tube in step 7.

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

7. Wash column with a second 500µl of RNA Wash Buffer II. Centrifuge and discard flow-through. Then with the collection tube empty, centrifuge the spin cartridge for **2 min at maximum speed** to completely dry the HiBind® matrix.

8. **Elution of RNA.** Transfer the column to a clean 1.5 ml microfuge tube (not supplied with kit) and elute the RNA with 30- 70 µl of DEPC-treated water (supplied with kit). Make sure to add water directly onto column matrix. Let it stand for 1 minute. Centrifuge 2 min at 13,000 x g to elute RNA. A second elution may be necessary if the expected yield of RNA >30 µg.

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.

Problem	Cause	Suggestion
DNA contamination		• 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.
Little or no DNA eluted	Column is overloaded	• Reduce quantity of starting material.
	DNA is lost during washing step	• Make sure to add ethanol to the DNA wash Buffer.