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Introduction

E.Z.N.A.® Micro-Spin Total RNA Kit provides a rapid and easy method for the isolation of up to 50 µg of total RNA from small amount of cultured eukaryotic cells, tissues such as laser dissected samples (LDS) or fine needle aspirates (FNA). Normally, up to 5 x 10^5 eukaryotic cells or 5 mg tissue (amounts depend on the tissue used) can be used in a single experiment. The kit allows single or multiple, simultaneous processing of samples in less than 30 min. 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.® Micro-Spin Total 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.® Micro-Spin Total RNA Kits combine the reversible binding properties of HiBind® matrix, a new silica-based material with the speed of mini-column spin technology. A specifically formulated high salt buffer system allows RNA molecules greater than 200 bases to bind to the matrix. Cells or tissues are first lysed under denaturing conditions that practically inactivate RNases. After add the ethanol, samples are then applied to the HiBind® Micro-spin columns to which total RNA binds, while cellular debris and other contaminants are effectively washed away. High quality RNA is finally eluted in DEPC-treated sterile water.

Storage

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

*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

Product No.	R6831-00	R6831-01	R6831-02
Purifications	5	50	200
HiBind™ Micro-Spin Columns	5	50	200
2 ml Collection Tubes	10	100	400
QVL Buffer	5 ml	25 ml	100 ml
RNA Wash Buffer I	5 ml	50 ml	200 ml
RNA Wash Buffer II Concentrate	5 ml	12 ml	50 ml
DEPC-ddH_2O	1 ml	20 ml	60 ml
Carrier RNA	50ul	100 µg	100 µg
Instruction Manual	1	1	1

Before Starting

IMPORTANT	
	Wash Buffer II Concentrate must be diluted with absolute ethanol before use. Trial Sample R6831-00 Add 20 ml 100 % ethanol R6831-01 Add 48 ml 100% ethanol R6831-02 Add 200 ml 100% ethanol
	Carrier RNA must be dissolved before use. When processing < 5000 cells, Carrier RNA should be added to the lysate before homogenization. First dissolve the carrier RNA with 100µl DEPC-Teated Water to make 1µg/µl stock solution. To make working solution for 10 preps, add 5µl of stocking solution to 45µl of QVL Buffer and mix by pipetting. Take 5µl of this diluted solution, mix with 95µl QVL Buffer. This final concentration will be 5ng/ul. Each RNA isolation will need 4µl this solution.

Note: 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 QVL 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 QVL Lysis Buffer before use. Add 20 µl of 2-mercaptoethanol per 1 ml of QVL 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

For all RNA isolation process, it is absolutely critical to disrupt and homogenize the sample. Laser dissected samples (LDS) and fine needle aspirates (FNA) can be simultaneously disrupted and homogenized by vortexing. For other samples, select one of the following methods for disruption and homogenization.

A. Disruption with Liquid Nitrogen

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 QVL Lysis Buffer and continue with the procedure as outlined below.

B. Homogenization Using Homogenizer column or fine needle:

After interrupt tissue, lysate can be homogenized with Omega Homogenizer Spin Column (Product # HCR-03). **The lysate is loaded onto Omega Homogenizer Spin Column in a 2 ml collection tube**. Spin two minutes at a maximum speed in a microcentrifuge and the homogenized lysate collected. Use the Omega Homogenizer Spin Column is 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 lysate through a narrow needle (19-21 gauge) for 5-10 times.

C. Disruption and Homogenization with Rotor-Stator

Rotor-Stator is the most preferred method for disruption and homogenizing tissue samples if required equipments are available. Rotor-stator homogenizers effectively homogenize and homogenize most tissues in the present of QVL Lysis Buffe . 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 of small volumes in microfuge tubes. Such homogenizers are available from:

- Tekmar Inc., Cincinnati, OH (Tissuemizers®)
- BIOSPEC Products, Bartlesville, OK (Tissue-Tearor™)
- Craven Laboratories, Austin, TX.

D. Disruption and Homogenization with Beads Mills

Tissue sample can also be effectively disrupted and homogenized by rapid agitation in the

presence of beads and lysis buffer. Tissue samples are disrupted and simultaneously homogenized with the sheared and crushed action of the beads as they collide with cells.

A. Total RNA Isolation From Laser dissected samples:

Materials supplied by user:

- 2-mercaptoethanol
- 70% ethanol in DEPC-treated sterile distilled water
- Sterile RNase-free pipette tips and microcentrifuge tubes
- Disposable latex gloves

Procedure:

1. Collect samples into a 1.5 or 2ml centrifuge tube contains 65 µl or 300 µl QVL Buffer. **Remember to add 20 µl of 2-mercaptoethanol per 1 ml of QVL Buffer before use.**

2. **Adjust the sample volume to 75µl or 350µl with QVL Buffer**. When process small amount of cells (≤5000 cells). Add 20ng of carrier RNA (5µl working solution, see page 3 for instruction) to the lysate before homogenization.

3. **Mix the sample throughly by vortexing for 30 seconds to homogenize the sample.**

4. Add equal volume (75µl or 350µl)of 70% ethanol to the homogenized lysate. Mix well by pipetting.

5. Apply sample onto HiBind® Micro-Spin RNA column. The maximum capacity of the spin cartridge is 750 µl. A precipitate may form on addition of ethanol in step 2. Vortex and add the entire mixture to the column. With the spin column inside a 2ml collecting tube (supplied with kit), centrifuge at 10,000 x g for 15 seconds **at room temperature**. Discard flow-through and proceed to step 6.

6. **Place column in a clean 2ml collection tube** (supplied), and add 300 µl RNA Wash Buffer I. Centrifuge and discard flow-through. Reuse the collection tube in step 7 or step 8. If on-membrane DNase I digestion is desired, proceed step 7, otherwise go to step 8.

7. DNase digestion (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)

   a. 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:

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

   b. 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.

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

8. **Place column in a same 2ml collection tube** from step 6, add 500 µl RNA Wash Buffer I. **(If on-membrane DNase digestion was performed in the previous step, wait at least 5 minutes before proceeding).** Centrifuge at 10,000 x g for 30 seconds. Discard flow-through and re-use the collection tube.

9. **Place column in the same 2ml collection tube**, add 500 µl RNA Wash Buffer II diluted with ethanol. Centrifuge at 10,000 x g for 30 seconds. Dicard the flow-through and reuse the collection tube.

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

8. Wash column with a second 500 µl of Wash Buffer II as in step 9. Centrifuge and discard flow-through. Then with the collection tube empty, centrifuge the spin cartridge for **2 min at full speed** to completely dry the HiBind® matrix.

9. **Elution of RNA.** Transfer the column to a clean 1.5 ml microfuge tube (not supplied with kit) and elute the RNA with 15-20 µl of DEPC-treated water (supplied with kit). Make sure to add water directly onto center of membrane. Centrifuge 1 min at maximum speed.

   RNA may be eluted with a smaller (<15µl) volume of water to get higher RNA concentration. While reduced elution volume decrease total RNA yield. The total yield will be 20-30% less when the elution volume is less than 10µl.

B. Extraction of RNA from Micro-Dissected Formalin-Fixed tissues

Materials supplied by user:

- 96-100% ethanol
- β-Mercaptoethanol
- RNase-free filter pipette tips
- Microcentrifuge capable of 12,000 xG
- Disposable latex gloves
- RNase-Free 1.5 ml tubes
- Water bath or heat block preset at 55°C
- Proteinase K (20mg/ml)

Note: Equilibrate samples and QVL Lysis buffer to room temperature before start. All steps must be carried out at room temperature. Work quickly, but carefully.

Note: Depend on the process of the fixation protocol, storage condition, staining protocol, and the age of the sample, RNA can be highly degraded into small fragments

less than 300nt. Since Total RNA isolation protocol will remove most fragments less than 200nt. This could lead to overall lose of RNA if the sample is highly degraded.

1. Collect samples into a 1.5 or 2ml centrifuge tube contains 100µl QVL Buffer. **Remember to add 20 µl of 2-mercaptoethanol per 1 ml of QVL Buffer before use.**

2. **Adjust the sample volume to 150µl with QVL Buffer**. When process small amount of cells (≤5000 cells). Add 20ng of carrier RNA (5µl working solution, see page 3 for instruction) to the lysate before homogenization.

3. Add 195 µl DEPC-Treated water into the sample. Then add adding 5µl of Proteinase k (20mg/ml)

4. Incubate at 55 °C for 10 minutes.

5. Centrifuge at 10,000 x g for 5 minutes at room temperature. A small pellet of tissue debris will form and a thin layer or film can be seen on top of the supernatant.

6. **Transfer the supernatant (about 450µl) into a RNase-Free 1.5 or 2ml tube (not supplied).**
   Avoid to transfer any of pellet. Hold the pipett tip under the thin layer or film on top of the supernatant, if present. This layer will usually adhere to outside the tip and should not be transferred.

7. Add 0.5 volume of absolute ethanol (96-100%) to the cleared lysate, mix throughly by pipetting.

8. **Place HiBind® Micro-Spin RNA column into a 2ml collection tube** (supplied) and **carefully** apply sample from step 7 (including any precipitate) to the HiBind® RNA column.

9. Centrifuge at 10,000 x g for 30 seconds. Discard the flow-through and reuse the collection tube.

10. **Place column in a clean 2ml collection tube**, and add 300 µl RNA Wash Buffer I. Centrifuge at 10, 000 x g for 30 seconds. Discard flow-through and re-use the collection tube in step 12. If On-membrane DNase I digestion procedure is desired, proceed step 11, otherwise go to step 12.

11. DNase I Digestion (Optional): this is point to start On-membrane DNase I digestion. (See detail procedure on page 5)

12. **Place column in same 2ml collection tube** from setp 10, and add 500 µl RNA Wash Buffer I. **(If on-membrane DNase digestion was performed in the previous step, wait at least 5 minutes before proceeding).** Centrifuge at 10, 000 x g for 30 seconds. Discard the flow-through and re-use the collection tube.

13. **Place column in the same 2ml collection tube** from step 12, and add 500 µl RNA Wash Buffer II diluted with ethanol. Centrifuge at 10, 000 x g for 30 seconds. Discard the flow-through and re-use the collection tube. Reuse the collection tube in step 7.
   Note: Wash Buffer II Concentrate must be diluted with absolute ethanol before use. Refer to label on bottle for instructions

14. Wash column with a second 500 µl of Wash Buffer II as in step 5. Centrifuge at 10,000 x g and discard flow-through. Then with the collection tube empty, centrifuge the spin cartridge for **2 min at full speed** to completely dry the HiBind® matrix.

15. **Elution of RNA.** Transfer the column to a clean 1.5 ml microfuge tube (not supplied with kit) and elute the RNA with 15 µl of DEPC-treated water (supplied with kit). Make sure to add water directly onto column matrix. Centrifuge 1 min at maximum speed.

   RNA may be eluted with a smaller (<15µl) volume of water to get higher RNA concentration. While reduced elution volume decrease total RNA yield. The total yield will be 20-30% less when the elution volume is less than 10µl.

C. RNA Isolation From Animal tissue or cell culture

This method is designed for most animal tissues and culture cells. For RNA isolation from fibrous tissue, follow the specialized protocol on page 10. For laser dissected samples, please follow the protocol on page 5. All centrifugation step must be carried out at room temperature.

Materials supplied by user:

- 96-100% ethanol
- β-Mercaptoethanol
- RNase-free filter pipette tips
- Microcentrifuge capable of 12,000 xG
- Disposable latex gloves
- RNase-Free 1.5 ml tubes

1. Determine the starting amount of sample. Do not use more than $5x\ 10^5$ cells or 5mg tissue.

2. Lyse cells (< $5x\ 10^5$) or tissues (< 5mg) with 350 µl of QVL Lysis Buffer. **Remember to add 20 µl of 2-mercaptoethanol per 1 ml of QVL Buffer before use**

3. Disrupt the tissue or cells and Homogenize the lysate in QVL according to one of the methods on page 4. When process small amount of cells (≤5000 cells). Add 20ng of carrier RNA (5µl working solution, see page 3 for instruction) to the lysate before homogenization.

4. Add an equal volume 70% Ethanol to the lysate and mix thoroughly by vortexing.

5. Apply sample onto HiBind® RNA Micro-Spin column pre-inserted in a 2 ml collection tube. The maximum capacity of the spin cartridge is 750 µl. A precipitate may form on addition of ethanol in step 2. Vortex and add the entire mixture to the column. With the spin column inside a 2ml collecting tube (supplied with kit), centrifuge at 10,000 x g for 15 seconds **at room temperature**. Discard flow-through and the collection tube.

6. **Place column in a new 2ml collection tube (suppled)**, and add 300 µl RNA Wash Buffer I. Centrifuge and discard flow-through. Reuse the collection tube in step 7 or step 8. If on-membrane DNase I digestion is desired, proceed step 7, otherwise go to step 8.

7. DNase I Digestion (Optional): this is point to start On-membrane DNase I digestion. (See detail procedure on page 5)

8. **Place column in the same 2ml collection tube from step 6**, and add 500 µl RNA Wash Buffer I. **(If on-membrane DNase digestion was performed in the previous step, wait at least 5 minutes before proceeding).** Centrifuge and discard flow-through.

9. **Place column in the same 2ml collection tube** from step 8, and add 500 µl RNA Wash Buffer II diluted with ethanol. Centrifuge at 10, 000 x g for 30 seconds. Discard the flow-through and re-use the collection tube. Reuse the collection tube in next step.

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

10. Wash column with a second 500 µl of Wash Buffer II as in step 5. Centrifuge at 10,000 x g and discard flow-through. Then with the collection tube empty, centrifuge the spin cartridge for **2 min at full speed** to completely dry the HiBind® matrix.

11. **Elution of RNA.** Transfer the column to a clean 1.5 ml microfuge tube (not supplied with kit) and elute the RNA with 15 µl of DEPC-treated water (supplied with kit). Make sure to add water directly onto column matrix. Centrifuge 1 min at maximum speed.
    RNA may be eluted with a smaller (<15µl) volume of water to get higher RNA concentration. While reduced elution volume decrease total RNA yield. The total yield will be 20-30% less when the elution volume is less than 10µl.

D. Extraction of RNA from Fibrous tissues (skeletal muscle, heart and aorta tissue, et al.)

Materials supplied by user:

- 96-100% ethanol
- β-Mercaptoethanol
- RNase-free filter pipette tips
- Microcentrifuge capable of 12,000 xG
- Disposable latex gloves
- RNase-Free 1.5 ml tubes
- Water bath or heat block preset at 55°C
- Proteinase K (20mg/ml)

Note: Equilibrate samples and QVL buffer to room temperature before start. All steps must be carried out at room temperature. Work quickly, but carefully.

1. Excise tissue from animal or from storage.

2. Weight up to 5mg tissue and immediately place it into a 1.5 ml tube for disruption and homogenization.

3. Add 150 µl QVL Buffer and disrupt tissue and homogenize lysate in QVL buffer by using methods described on page 4.

   Note: Incomplete homogenization will cause clogging of the spin column and lead to significantly lower yield. Generally, disruption and homogenization by using mortar and pestle or needle and syringe can generate lower yield. It is recommenced to use a rotor stator homogenizer or beads milling methods for animal tissues.

4. Pipet 290 µl RNase-Feee water to each homogenate. Add 5 µl OB Protease and mix through by pipetting.

5. Incubate at 55 °C for 10 minutes.

6. Centrifuge at 10,000 x g for 5 minutes at room temperature. A small pellet of tissue debris will form and a thin layer or film can be seen on top of the supernatant.

7. Transfer the supernatant into a RNase-Free 2 ml microtube.
   Note: Avoid to transfer any of pellet. Hold the pipett tip under the thin layer or film on top of the supernatant, if present. This layer will usually adhere to outside the tip and should not be transferred.

8. Add 0.5 volume (275 µl) of absolute ethanol (96-100%) to the cleared lysate, mix throughly by pipetting.

9. **Place HiBind® RNA column into a 2ml collection tube** (supplies) and **carefully** apply 700µl samples from step 8 (including any precipitate) to the HiBind® RNA column.

10. Centrifuge at 10,000 x g for 30 seconds. Discard the flow-through and collection tube.

11. Place the **HiBind® RNA column** into a new 2ml collection tube. Add 300µl RNA wash Buffer I into the column. Centrifuge at 10,000 x g for 30 seconds. Discard the flow-through and re-use the collection tube in step 13.

12. DNase I Digestion (Optional): this is point to start Dnase digestion. (See detail protocol in page 6)

13. **Place column into the 2ml collection tube from step 11**, and add 500 µl RNA Wash Buffer I. **(If on-membrane DNase digestion was performed in the previous step, wait at least 5 minutes before proceeding).** Centrifuge at 10,000 x g for 30 seconds. Discard flow-through and re-use the collection tube.

16. **Place column in the same 2ml collection tube**, then add 500 µl RNA Wash Buffer II diluted with ethanol. Centrifuge at 10,000 x g for 30 seconds. Discard flow-through and re-use the collection tube.

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

17. Wash column with a second 500 µl of Wash Buffer II as in step 5. Centrifuge at 10,000 x g and discard flow-through. Then with the collection tube empty, centrifuge the spin cartridge for **2 min at full speed** to completely dry the HiBind® matrix

18. **Elution of RNA.** Transfer the column to a clean 1.5 ml microfuge tube (not supplied with kit) and elute the RNA with 15 µl of DEPC-treated water (supplied with kit). Make sure to add water directly onto column matrix. Centrifuge 1 min at maximum speed.
RNA may be eluted with a smaller (<15µl) volume of water to get higher RNA concentration. While reduced elution volume decrease total RNA yield. The total yield will be 20-30% less when the elution volume is less than 10µl.

DNA Contamination

Generally HiBind® RNA spin column technology will efficiently removes most of the DNA without DNase treatment. However, no RNA extraction procedure can completely remove genomic DNA. For sensitive work (such as RT-PCR or differential display) we suggest that you perform on-column DNase I digestion (OBI cat# E1091) or treat the eluted RNA with RNase-free DNase. Also for RT-PCR, use intron-spanning primers that allow easy identification of DNA-contamination.

Quantization 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. However, the E.Z.N.A.® Total RNA Kit eliminates the use of phenol and avoids this problem.) 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.

Trouble Shooting

Problem	Cause	Solution
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		• **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.**