Invitrogen™
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Instruction Manual

PureLink™ miRNA Isolation Kit

For rapid, efficient purification of small RNA molecules

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Table of Contents

Experienced Users Procedure

Introduction

This quick reference sheet is included for experienced users of the PureLink™ miRNA Isolation Kit. If you are a first time user, follow the detailed protocol in this manual.

Step	Action
Sample Preparation	**Mammalian Cells (up to 1x 10^6 cells)** 1. Harvest cells and add 300 µl Binding Buffer (L3) to cells. 2. Mix well by vortexing and add 300 µl 70% ethanol. Mix well. **Mammalian Tissue (up to 5 mg)** 1. Add 300 µl Binding Buffer (L3) and homogenize tissue using tissue homogenizer. 2. Centrifuge at 12,000 x g for 1 minute to remove any particulate materials. Transfer supernatant to a clean tube and add 300 µl 70% ethanol. Mix well by vortexing. **Plant Tissue (up to 100 mg)** 1. Grind tissue to a powder in liquid nitrogen and add 300 µl Binding Buffer (L3). Mix well by vortexing. 2. Centrifuge the lysate at 12,000 x g for 2 minutes to remove any particulate materials. Transfer supernatant to a clean tube and add 300 µl 70% ethanol. Mix well by vortexing. **Yeast Cells (up to 2x 10^7 cells)** 1. Harvest 1 ml fresh, log phase yeast cells (OD_{660} ~ 1.0) by centrifugation and resuspend cell pellet in 70 µl TE Buffer. 2. Add 30 units zymolase (lyticase) enzyme to the lysate and incubate for 30 minutes at 30 °C. 3. Add 300 µl Binding Buffer (L3) and 210 µl of 96-100% ethanol. Mix well by vortexing. **Bacterial Cells (up to 5x 10^6 cells)** 1. Harvest up to 5 x 10^6 *E. coli* cells by centrifugation and resuspend cell pellet in 90 µl TE with 1 mg lysozyme. Add 1 µl 5% SDS and mix well. 2. Incubate at room temperature for 5 minutes. 3. Add 300 µl Binding Buffer (L3) and add 210 µl 96-100% ethanol. Mix well by vortexing. **Note:** To process larger sample amount, see page 10 for a protocol using the TRIzol® Reagent.

Continued on next page

Experienced Users Procedure, Continued

Step	Action
Purification Procedure	The purification procedure is designed for use with a microcentrifuge capable of centrifuging >10,000 x g and can be completed in 10-15 minutes 1. **Add** prepared sample from previous page to a Spin Cartridge in a collection tube. 2. Centrifuge the Spin Cartridge at 12,000 × g for 1 minute. Total RNA is bound to the cartridge. **Keep the flow through.** 3. **Add** 96-100% ethanol to the flow through to a final concentration of 70%. Mix well by vortexing. 4. **Transfer** 700 µl sample to a **second** Spin Cartridge in collection tube. Centrifuge the Spin Cartridge at 12,000 x g for 1 minute. Small RNA molecules bind to the Spin Cartridge. Discard the flow through. 5. Repeat Step 4 for the remaining sample. Place the Spin Cartridge in the collection tube. 6. **Wash** the Spin Cartridge with 500 µl Wash Buffer (W5) with ethanol (page 13). Centrifuge at 12,000 × g for 1 minute. Discard the flow through. 7. **Repeat** wash step with 500 µl Wash Buffer (W5) once. 8. Discard the collection tube and place the Spin Cartridge in a Wash Tube supplied with the kit. 9. Centrifuge the Spin Cartridge at maximum speed for 1 minute to remove any residual Wash Buffer (W5). 10. Place the Spin Cartridge in a clean 1.7-ml Recovery Tube supplied with the kit. 11. **Elute** the RNA with 50-100 µl sterile RNase-free water supplied with the kit (add water to the center of the cartridge). 12. Incubate at room temperature for 1 minute. 13. Centrifuge the Spin Cartridge at maximum speed for 1 minute to elute RNA. *The Recovery Tube contains purified small RNA molecules*. Discard the Spin Cartridge. 14. Store purified RNA at -80°C or use RNA for the desired downstream application.

Kit Contents and Storage

Shipping and Storage

All components of the PureLink™ miRNA Isolation Kit are shipped at room temperature. Upon receipt, store all components at room temperature.

Contents

The components included in the PureLink™ miRNA Isolation Kit are listed below.

Sufficient reagents are provided in the kit to perform 25 reactions.

Component	Amount
Binding Buffer (L3)	45 ml
Wash Buffer (W5)	10 ml
Buffer (W4) for single column purification	3 ml
Sterile, RNase-free Water	5 ml
Spin Cartridges with Collection Tubes	50
Wash Tubes (2.0 ml)	50
Recovery Tubes (1.7 ml)	50

Product Qualification

The PureLink™ miRNA Isolation Kit is functionally qualified as described below.

Yeast tRNA and 10 bp DNA Ladder are purified using the kit as described in this manual. The purified small RNA is analyzed by gel electrophoresis.

Agarose gel electrophoresis must show the co-purification of yeast tRNA with 10 bp DNA Ladder fragments less than 40 bp and DNA fragments greater than 40 bp must not be detectable.

In addition, each kit component is free of ribonuclease contamination and is lot qualified for optimal performance.

Accessory Products

Additional Products

The following products are also available from Invitrogen. For more details on these products, visit our Web site at www.invitrogen.com or contact Technical Service (page 19).

Product	Quantity	Catalog no.
RNase *AWAY*®	250 ml	10328-011
UltraPure™ DEPC-treated Water	1 L	750023
UltraPure™ DNase/RNase-Free Distilled Water	500 ml	10977-015
Quant-iT™ RNA Assay Kit	1000 assays	Q-33140
Novex® TBE-Urea Gels, 15% (1.0 mm, 10 well)	1 box	EC6885BOX
Novex® TBE-Urea Gels, 10% (1.0 mm, 10 well)	1 box	EC6875BOX
Novex® TBE-Urea Sample Buffer (2X)	10 ml	LC6876
Novex® TBE Running Buffer (5X)	1 L	LC6675
Invitrogen Homogenizer (clarifier; differs from tissue homogenizer. See page 8)	50	12183-026
10 bp DNA Ladder	50 µg	10821-015
TrackIt™ 10 bp DNA Ladder	20 applications	10488-019
PureLink™ Micro-to-Midi Total RNA Purification System	50 reactions	12183-018
UltraPure™ Glycogen	100 µl	10814-010
TRIzol®	100 ml	15596-026

Introduction

Overview

Introduction

The PureLink™ miRNA Isolation Kit provides a rapid and efficient method to purify small RNA molecules from biological samples for functional analysis.

The kit is specifically designed to isolate high-quality small RNA molecules including tRNA, 5S rRNA, 5.8S rRNA and regulatory RNA molecules such as microRNA (miRNA) and short interfering RNA (siRNA). Using the kit to isolate small RNA molecules results in efficient removal of large RNA molecules such as 28S rRNA, 18S rRNA, and mRNA that can inhibit expression analysis of small RNA molecules. See below for an overview of the purification protocol.

The purified RNA is suitable for northern blot analysis and microarray analysis.

For information on small RNA molecules, see next page.

System Overview

The PureLink™ miRNA Isolation Kit is based on the selective binding of small RNA molecules to silica-based membrane in the presence of 70% ethanol.

The lysate is prepared from mammalian cells and tissue, plant tissues, *E. coli* cells and yeast cells using Binding Buffer (L3) containing guanidine isothiocyanate, a chaotrope capable of protecting the RNA from endogenous RNases. Ethanol is added to the lysate to a final concentration of 35% and the lysate is processed through the first Spin Cartridge. Large RNA molecules bind to the silica-based membrane in the cartridge while small RNA molecules are recovered in the flow through fraction.

Ethanol is added to the flow through fraction to a final concentration of 70% and the sample is loaded onto a second Spin Cartridge. The small RNA molecules bind to the silica-based membrane in the cartridge and impurities are removed by thorough washing with Wash Buffer. The RNA is then eluted in sterile, RNase free water.

Continued on next page

Overview, Continued

Small RNA Molecules

Small regulatory RNA molecules play an important role in regulation of gene expression in various organisms by binding to the target mRNAs through sequence complementation (Liu, 2004). Small RNA molecules include tRNA, 5S rRNA, and 5.8S rRNA and regulatory RNA molecules such as siRNA and miRNA.

siRNAs are ~21-23 nucleotide double-stranded RNA molecules involved in post-transcriptional gene silencing using the RNAi (RNA interference) pathway (Elbashir *et al.*, 2002). miRNAs are ~21-22 nucleotide double stranded RNA molecules that play important roles in the regulation of translation and degradation of target mRNAs through base pairing to partially complementary sites in the untranslated regions of the message (Lim, 2003).

Isolation and purification of small regulatory RNA molecules allows elucidation of biologically significant pathways for regulation of gene expression and requires enrichment of small RNA molecules from biological samples for functional analysis.

The standard protocols for isolating total RNA and mRNA are not optimized for isolation of small RNA molecules and result in the loss of substantial amounts of small RNA. Co-purification of larger RNA molecules such as 28S rRNA, 18S rRNA, and mRNA with small RNA inhibits expression analysis of small RNA.

The PureLink™ miRNA Isolation Kit is specifically designed for purification of small RNA with minimal contamination from large RNA molecules.

Continued on next page

Overview, Continued

Advantages

Using PureLink™ miRNA Isolation Kit to isolate small RNA molecules provides the following advantages:

- Rapid and efficient purification of high-quality small RNA molecules using spin column-based centrifugation
- Specifically designed to purify small RNA molecules including miRNA and siRNA from a variety of samples in less than 15 minutes
- Minimal contamination from large RNA molecules and genomic DNA
- Reliable performance of the purified small RNA in downstream applications

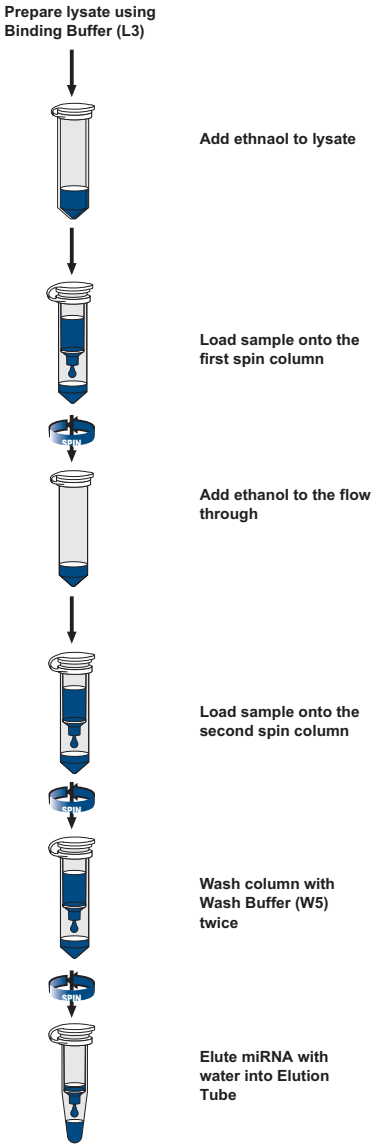
Specifications

Binding Capacity:	~1 mg nucleic acid
Starting Material:	Varies (see page 6)
Column Reservoir Capacity:	700 µl
Wash Tube Capacity:	2.0 ml
Recovery Tube Capacity:	1.7 ml
Centrifuge Compatibility:	Capable of centrifuging at >10,000 x g
RNA Yield:	Varies (see page 16)
RNA Recovery:	>80%

Experimental Overview

Introduction

The flow chart for purifying small RNA molecules using the PureLink™ miRNA Isolation Kit is shown below.



Methods

Preparing Lysates

Introduction

Instructions for preparing lysates from mammalian cells and tissues, plant tissues, yeast, and bacteria are described below.

To obtain high-quality RNA, follow the guidelines recommended below.

The PureLink™ miRNA Isolation Kit buffers contain guanidine isothiocyanate. Always wear a laboratory coat, disposable gloves, and eye protection when handling buffers.

Do not add bleach or acidic solutions directly to solutions containing guanidine isothiocyanate or sample preparation waste as it forms reactive compounds and toxic gases when mixed with bleach or acids.

General Handling of RNA

Observe the following guidelines to prevent RNase contamination:

- Use disposable, individually wrapped, sterile plastic ware
- Use only sterile, new pipette tips and microcentrifuge tubes
- Wear latex gloves while handling reagents and RNA samples to prevent RNase contamination from the surface of the skin
- Always use proper microbiological aseptic techniques when working with RNA
- Use RNase *AWAY*® Reagent (page viii) to remove RNase contamination from surfaces

Continued on next page

Preparing Lysates, Continued

Sample Amount and Quality

There are different protocols for preparing lysates depending on the starting material (sample). Based on your sample, choose an appropriate lysate preparation protocol from the table below.

To obtain high yield of small RNA molecules and minimize any degradation, collect the sample and proceed immediately to sample preparation or freeze the sample in liquid nitrogen immediately after collection.

To obtain the best results, use the appropriate protocol based on your sample and the recommended sample amount for purification as described in the table below.

If you wish to start with less amount of sample, use the appropriate protocol based on your sample without changing the volume of reagent used. Note that if you start with less amount of sample, the RNA yield may be lower.

Sample	Amount	Page no.
Mammalian cells	up to 1 x 10^6 cells	7
Mammalian tissues	up to 5 mg	8
Plant tissues	up to 100 mg	8
Yeast cells	2 x 10^7 cells	9
E. coli cells	5 x 10^6 cells	9
Dicing reaction	50 µl	11
Larger sample amount	--	10

Note

To minimize RNA degradation and to obtain optimal RNA yield, it is important to perform complete disruption of tissue in lysis buffer **quickly** and not to exceed the recommended starting amounts for various samples listed in the above table.

Using increased amount of starting material results in co-purification of large molecules such as 18S/28S rRNA, mRNA, and possibly gDNA that did not bind to the column during the first column screening, is retained as flow through, and co-purifies with small RNA molecules.

If you wish to process large amount of starting material, see page 10 for recommended conditions.

Continued on next page

Preparing Lysates, Continued

Materials Needed

- 96-100% ethanol
- 70% ethanol
- Sample (see previous page)
- Binding Buffer (L3) supplied with the kit
- TE buffer (10 mM Tris-HCl, 1 mM EDTA, pH 8.0)
- Zymolase (lyticase) enzyme for yeast lysate
- Lysozyme and 5% SDS for bacterial cell lysate
- Invitrogen Homogenizer (clarifier; for viscous or debris-containing samples)
- General tissue homogenizer for homogenizing mammalian and plant tissues
- TRIzol® Reagent and chloroform for processing large sample amount
- Sterile, DNase-free microcentrifuge tubes
- Water bath or heat block set at 30°C for yeast lysate

Mammalian Cells Lysate

Procedure to prepare lysate from mammalian cells is described below.

1. For adherent cells (up to 1 x 10^6 cells), remove the growth medium from the culture plate.

   For suspension cells (up to 1 x 10^6 cells), harvest the cells and centrifuge the cells at 250 x g for 5 minutes to pellet cells. Remove the growth medium.
2. Resuspend cells from Step 1 in 300 µl Binding Buffer (L3) supplied with the kit. Mix well by vortexing or pipetting up and down until the cells appear lysed.
3. Add 300 µl 70% ethanol to the cell lysate. Mix well by vortexing.
4. Proceed to the **Binding Step** (page 13).

Continued on next page

Preparing Lysates, Continued

Mammalian Tissue Lysate

Procedure to prepare lysate from mammalian tissues is described below.

1. Place ~5 mg of minced mammalian tissue into a sterile microcentrifuge tube.
2. Add 300 µl Binding Buffer (L3) supplied with the kit. Ensure the tissue is completely immersed in the buffer.
3. Homogenize the tissue using a tissue homogenizer.
4. Centrifuge the lysate at 12,000 x g for 5 minutes at room temperature to remove any particulate material.
5. Transfer the supernatant to a new, sterile microcentrifuge tube.

   Note: If the lysate is viscous or contains cell debris, clarify the lysate using the Homogenizer available from Invitrogen (page viii).
6. Add 300 µl 70% ethanol to the lysate. Mix well by vortexing.
7. Proceed to the **Binding Step** (page 13).

Plant Tissue Lysate

Procedure to prepare lysate from plant is described below.

1. Freeze the plant tissue in liquid nitrogen and grind the tissue to a powder.

   For soft tissue, cut the tissue into small pieces.
2. Add 300 µl Binding Buffer (L3) supplied with the kit to the tissue. For ground powder, vortex the powder until the powder is completely resuspended. For the soft tissue, homogenize using a tissue homogenizer.
3. Centrifuge the lysate at 12,000 x g for 2 minutes at room temperature to remove any particulate material.
4. Transfer the supernatant to another sterile microcentrifuge tube.

   Note: If the lysate is viscous or contains cell debris, clarify the lysate using a Homogenizer available from Invitrogen (page viii).
5. Add 300 µl 70% ethanol to the lysate. Mix well by vortexing.
6. Proceed to the **Binding Step** (page 13).

Continued on next page

Preparing Lysates, Continued

Yeast Lysate

Procedure to prepare lysate from yeast cells is described below.

1. Harvest 1 ml fresh, log-phase yeast cells (OD_{660} = 1.04) by centrifugation. If you are using a frozen cell pellet, proceed to Step 2.
2. Resuspend the cell pellet in 70 µl TE buffer (10 mM Tris-HCl, 1 mM EDTA, pH 8.0).
3. Add 30 units zymolase (lyticase) enzyme and incubate at 30°C for 30 minutes to lyse the cells.
4. Centrifuge at 12,000 x g for 5 minutes at room temperature to remove any particulate material.
5. Resuspend the pellet in 300 µl Binding Buffer (L3) supplied with the kit.
6. Add 210 µl 96-100% ethanol to the lysate. Mix well by vortexing.
7. Proceed to **Binding Step** (page 13).

E. coli Lysate

Procedure to prepare *E. coli* cell lysate is described below.

1. Prepare 90 µl TE buffer (10 mM Tris-HCl, 1 mM EDTA, pH 8.0) containing 1 mg lysozyme enzyme. Store on ice until use.
2. Harvest up to 5 x 10^6 *E. coli* cells by centrifugation. If you are using a frozen cell pellet, proceed to Step 3.
3. Resuspend the cell pellet in 90 µl TE buffer containing 1 mg lysozyme from Step 1.
4. Add 1 µl 5% SDS to the lysate and mix well by vortexing. Incubate at room temperature for 5 minutes.
5. Add 300 µl Binding Buffer (L3) supplied with the kit to the lysate. Mix well by vortexing.
6. Add 210 µl 96-100% ethanol to the lysate. Mix well by vortexing.
7. Proceed to the **Binding Step** (page 13).

Continued on next page

Preparing Lysates, Continued

Large Sample Amount

If you wish to process large sample amount as compared to the amount listed on page 6, use TRIzol® Reagent to prepare lysate as described below.

Lysate Preparation

Tissues

Homogenize tissue samples in 1 ml TRIzol® Reagent per 50-100 mg tissue using a tissue homogenizer.

Adherent Cells

Lyse cells directly in a culture dish by adding 1 ml TRIzol® Reagent to a culture dish and passing the cell lysate several times through a pipette. The amount of TRIzol® Reagent required is based on the culture dish area (1 ml per 10 cm^2) and not on the number of cells present.

Suspension Cells

Harvest cells and pellet cells by centrifugation. Use 1 ml of the TRIzol® Reagent per 5-10 × 10^6 animal, plant or yeast cells, or per 1 × 10^7 bacterial cells and lyse cells by repetitive pipetting.

Phase Separation

1. Incubate the lysate with TRIzol® Reagent at room temperature for 5 minutes to allow complete dissociation of nucleoprotein complexes.
2. Add 0.2 ml chloroform per 1 ml TRIzol® Reagent and shake the tube vigorously by hand for 15 seconds. Avoid vortexing the sample.
3. Incubate at room temperature for 2-3 minutes.
4. Centrifuge the sample at 12,000 x g for 15 minutes at 4°C.

   After centrifugation, the mixture separates into a lower red phenol-chloroform phase, an interphase, and a colorless upper aqueous phase. The volume of aqueous phase is ~600 µl.
5. Transfer ~ 400 µl of the colorless, upper phase containing RNA to a fresh tube.
6. Add 96-100% ethanol into 400 µl of the aqueous phase to obtain a final concentration to 35% (*e.g.* add 215 µl of 96-100% ethanol) and mix well by vortexing.
7. Proceed to the **Binding Step** (page 13).

Continued on next page

Preparing Lysates, Continued

Dicing Reaction

If you are purifying d-siRNA produced in the dicing reaction using the BLOCK-iT™ Dicer RNAi Kit (available from Invitrogen) or an equivalent kit, use the protocol below to prepare samples prior to purification.

To 300 µl dicing reaction, add 300 µl Binding Buffer (L3) and 300 µl 96-100% ethanol to obtain a final volume of 900 µl. Mix well by vortexing. Proceed to the **Binding Step**, page 13.

Note: You can also use 300 µl isopropanol instead of ethanol as suggested in the BLOCK-iT™ Dicer RNAi Kit manual for preparing the dicing reaction for purifying d-siRNA. For more details on the BLOCK-iT™ Dicer RNAi Kit, visit www.invitrogen.com or contact Technical Service (page 23).

Purification Procedure

Introduction

The purification procedure is designed for purifying small RNA molecules using spin column based centrifugation in a total time of **10-15 minutes**.

Materials Needed

- 96-100% ethanol
- Microcentrifuge capable of centrifuging >10,000 x g

Components supplied with the kit

- Wash Buffer (W5)
- Sterile, RNase-free Water (pH>7.0)
- Spin Cartridge in Collection Tubes
- Wash Tubes and Recovery Tubes

Follow the recommendations below to obtain the best results:

- Perform all centrifugation steps at room temperature
- Be sure to add ethanol to a final concentration of 70% and transfer sample to a second column to isolate small RNA molecules
- Perform the recommended wash steps to obtain high-quality RNA
- Pipet water in the center of the Spin Cartridge and perform a 1 minute incubation

Note

The purification procedure described in this section is designed for purifying small RNA molecules from tissues, cells, and dicing reaction using the two-column protocol.

If you are purifying small RNA molecules from a dicing reaction, an *optional* one-column protocol followed by ethanol precipitation of RNA is described on page 19.

If you wish to purify total RNA from the same sample, the total RNA is bound to the first spin column and can be washed and eluted using the reagents and protocol described in the PureLink™ Micro-to-Midi Total RNA Purification Kit (see page viii for ordering information). The manual can be downloaded from www.invitrogen.com or by contacting Technical Service (page 23).

Continued on next page

Purification Procedure, Continued

Elution Buffer

For two-column purification protocol, use sterile, RNase-free **water** supplied with the kit to elute small RNA molecules (page 14).

For one-column purification protocol, use **Buffer (W4)** for single column purification supplied with the kit to elute small RNA molecules (page 21).

Before Starting

Add 40 ml 96-100% ethanol to 10 ml Wash Buffer (W5) included with the kit. Store the Wash Buffer (W5) with ethanol at room temperature.

Binding Step

1. Remove a Spin Cartridge in a Collection Tube from the package.
2. Add the lysate with Binding Buffer (L3) and ethanol (total volume is 600 µl) prepared as described on pages 5-11 to the Spin Cartridge.
3. Centrifuge the Spin Cartridge 12,000 × g for 1 minute at room temperature.
4. **Keep the flow through**. Add 96-100% ethanol to the flow through to obtain a final concentration of 70% ethanol and mix well by vortexing. **Note:** The lysate already contains 35% ethanol added during sample preparation.

   For example: To 600 µl lysate, add 700 µl 96-100% ethanol and mix well by vortexing.

   Note: To isolate total RNA from the same sample, place the Spin Cartridge into a new Wash Tube and process the Spin Cartridge for isolating total RNA as described in the PureLink™ Micro-to-Midi Total RNA Purification Kit (page viii).
5. Remove **another** Spin Cartridge in a Collection Tube from the package and transfer 700 µl sample from Step 4 to the Spin Cartridge.

Continued on next page

Purification Procedure, Continued

Binding Step, continued

6. Centrifuge the Spin Cartridge at 12,000 × g for 1 minute at room temperature.
7. Transfer remaining sample from Step 4 to the Spin Cartridge from Step 6 and centrifuge at 12,000 x g for 1 minute at room temperature.
8. Discard the flow through and place the Spin Cartridge into the collection tube.
9. Proceed to the **Washing Step**, below.

Washing Step

1. Add 500 µl Wash Buffer (W5) with ethanol (page 13) to the Spin Cartridge.
2. Centrifuge 12,000 × g for 1 minute at room temperature.
3. **Repeat** the wash step with 500 µl Wash Buffer (W5) with ethanol.
4. Discard the flow through and place the Spin Cartridge into the Wash Tube supplied with the kit.
5. Centrifuge the Spin Cartridge at maximum speed for 2-3 minutes at room temperature to remove any residual Wash Buffer. Discard the Wash Tube.
6. Proceed to the **Elution Step**, below.

Elution Step

1. Place the Spin Cartridge in a clean 1.7-ml Recovery Tube supplied with the kit.
2. Add 50-100 µl of Sterile, RNase-free water (pH >7.0) to the center of the Spin Cartridge.
3. Incubate at room temperature for 1 minute.
4. Centrifuge the Spin Cartridge at maximum speed for 1 minute at room temperature.
5. The Recovery Tube contains purified small RNA molecules. Remove and discard the cartridge.

   Based on the volume of elution buffer used for elution, the recovery of the elution volume will vary and is usually 90% of the elution buffer volume used.
6. Store small RNA at -80°C or use small RNA for the desired downstream application. You may determine the quality and quantity of the purified small RNA molecules as described on the next page.

Continued on next page

Analyzing RNA

RNA Yield

The quantity of the purified small RNA molecules is easily quantitated using UV absorbance at 260 nm or Quant-iT™ RNA Assay Kit.

UV Absorbance

1. Dilute an aliquot of the small sample in 10 mM Tris-HCl, pH 7.0. Mix well. Transfer to a cuvette (1-cm path length).

   Note: The RNA must be in a neutral pH buffer to accurately measure the UV absorbance.

2. Determine the OD_{260} of the solution using a spectrophotometer blanked against 10 mM Tris-HCl, pH 7.0.

Calculate the amount of total RNA using the following formula:

Total RNA (µg) = OD_{260} x 40 µg/(1 OD_{260} x 1 ml) x dilution factor x total sample volume (ml)

Quant-iT™ RNA Assay Kits

The Quant-iT™ RNA Assay Kit (page viii for ordering information) provides a rapid, sensitive, and specific method for RNA quantitation with minimal interference from DNA, protein, or other common contaminants that affect UV absorbance readings.

The kit contains a state-of-the-art quantitation reagent and pre-diluted standards for standard curve. The assay is performed in a microtiter plate format and is designed for reading in standard fluorescent microplate readers.

RNA Quality

Typically, small RNA molecules isolated using the PureLink™ miRNA Isolation Kit have an $OD_{260/280}$ of >1.8 when samples are diluted in Tris-HCl (pH 7.5). An $OD_{260/280}$ of >1.8 indicates that RNA is reasonably clean of proteins and other UV chromophores (heme, chlorophyl, etc.) that could either interfere with downstream applications or negatively affect the stability of the stored RNA.

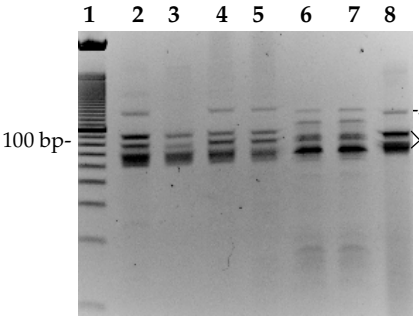
To visualize small RNA molecules, perform denaturing gel electrophoresis using 10% or 15% TBE acrylamide gels (page viii for ordering information). An example of denaturing TBE gel analysis is shown on the next page.

Expected Results

Results

An example of small RNA molecules isolated from various samples is shown in the figure below.

Small RNA molecules from various samples were isolated using the PureLink™ miRNA Isolation Kit as described in this manual. Samples (10 µl eluate) were analyzed on a Novex® TBE-Urea 15% Gel and RNA bands were visualized with ethidium bromide staining after electrophoresis.



Lane 1: 1 µl 10 bp DNA Ladder
Lane 2: HeLa cells
Lane 3: 293F cells
Lane 4: Rat spleen
Lane 5: Mouse liver
Lane 6: Spinach
Lane 7: MaV yeast cells
Lane 8: *E. coli* cells

Expected Yields

The yield of small RNA molecules obtained from various samples using the PureLink™ miRNA Isolation Kit is listed below. The RNA quantitation was performed with the Quant-iT™ RNA Assay Kit (page viii).

Material	Amount	Yield (µg)
HeLa cells	$1x10^6$	1.29
293F cells	$1x10^6$	1.95
Mouse liver	5 mg	0.71
Rat Spleen	5 mg	1.39
Spinach	60 mg	1.64
Yeast (*S. cerevisiae*)	$1x10^6$	6.21
Bacteria (*E. coli*)	$2x10^6$	0.55

Troubleshooting

Problem	Cause	Solution
Low RNA yield	Incomplete lysis or too much sample has clogged the filter	Decrease the amount of starting material used. For tissues, cut the tissue into smaller pieces and ensure the tissue is completely immersed in the Binding Buffer (L3) to obtain optimal lysis. Decrease the sample volume used, if cartridge is clogged or load the sample on 2 spin cartridges.
	Low RNA content	Various tissues have different small RNA content and some tissue may not contain any small RNA at detectable levels.
	Flow through from first cartridge discarded	**Do not** discard the flow through from the first cartridge. The flow through contains small RNA molecules.
	Incorrect binding conditions	For efficient binding of small RNA molecules, always **add** ethanol to the flow through to a final concentration of 70%.
	Ethanol not added to Wash Buffer (W5)	Be sure to add 96–100% ethanol to Wash Buffer (W5) as described on page 13.
	Incorrect elution conditions	Add water to the center of the cartridge and perform incubation for 1 minute with water before centrifugation. Be sure to use RNase-free water for elution using the two-column protocol and Buffer (W4) for single-column protocol.
	RNA quantitation performed with water	Be sure the RNA quantitation using UV absorbance is performed with 10 mM Tris-HCl, pH 7.0.(page 15) to accurately measure the UV absorbance.

Continued on next page

Troubleshooting, Continued

Problem	Cause	Solution
RNA degraded	RNA contaminated with RNase	Follow the guidelines on page 5 to prevent RNase contamination.
	Poor quality starting materials	Always use fresh samples or samples frozen at -80°C. For lysis, process the sample quickly to avoid degradation.
Genomic DNA contamination	Large sample amount loaded on the first column	Be sure to use the protocol with TRIzol® Reagent when processing large sample amount as described on page 10. Perform DNase I digestion on the RNA sample after elution to remove genomic DNA contamination.
Total RNA contamination	Processed large amount of starting material without using TRIzol® Reagent	Processing large amount of starting material using the standard protocols without TRIzol® Reagent will result in co-purification of large molecules such as 18S/28S rRNA and mRNA that did not bind to the first column. If you wish to process large amount of starting material, use TRIzol® Reagent to prepare samples as described on page 10.

Appendix

One-Column Protocol

Introduction

An optional one-column purification protocol is described below to purify siRNA from dicing reaction only.

Important

The one-column purification protocol is only applicable to samples obtained from dicing reaction.

The one-column protocol is not recommended with samples prepared from tissues or cells as the amount of sample used for purification may clog the spin cartridges, and the volume and amount of buffers used for wash and elution steps are not sufficient to produce high-quality RNA.

Experimental Outline

1. Add Binding Buffer (L3) and ethanol to the dicing reaction to adjust the conditions for binding.
2. Load the sample onto the Spin Cartridge to bind small RNA molecules.
3. Wash the Spin Cartridge with Wash Buffer (W5) containing ethanol to remove impurities.
4. Elute the siRNA with diluted Buffer (W4) containing ethanol and RNase-free water for single column purification.
5. Perform ethanol precipitation of siRNA from the eluate.

Materials Needed

- 96-100% ethanol (cold)
- 20 µg/µl Glycogen (page viii)
- 70% ethanol (cold)

Components supplied with the kit

- Binding Buffer (L3)
- Wash Buffer (W5)
- Buffer (W4) for single column purification
- Spin Cartridge with Collection Tube
- Wash Tubes and Recovery Tubes

Continued on next page

One-Column Protocol, Continued

Before Starting

Dilute Wash Buffer (W4) and (W5) as described below before staring the One-Column purification procedure.

- **Wash Buffer (W4)**: Add 1.5 ml of RNase-free water and 1.5 ml of 96-100% ethanol to the 3 ml Wash Buffer (W4) included with the kit. The diluted buffer (W4) will be used as Elution buffer in the One-Column protocol.
- **Wash Buffer (W5):** Add 40 ml 96-100% ethanol to 10 ml Wash Buffer (W5) included with the kit.
- Store the prepared Wash Buffer (W4) and (W5) with ethanol at room temperature.

Starting Material

The **One-Column Procedure** is designed for use with **up to 8 µg of RNA** in **50 µl** dicing reaction containing as starting material.

- If you wish to process **<8 µg RNA** in **<50 µl dicing reaction**, increase the volume of your starting material to 50 µl.
- If you wish to process **up to 8 µg** in **>50 µl dicing reaction**, adjust the buffer volumes of the procedure below accordingly. When loading the reaction mix with the increased volume to the column (Step 4 of the procedure, next page), perform multiple loading of the sample mix onto the Spin Cartridge (Step 4 and 5 of the procedure, next page). The reservoir capacity of the Spin Cartridge is ~700 µl.

  Note: Do not load more than 8 µg of RNA from a dicing reaction to the column.

Continued on next page

One-Column Protocol, Continued

One-Column Procedure

Follow the protocol below to purify small RNA from a dicing reaction.

1. Add 150 µl Binding Buffer (L3) to 50 µl dicing reaction volume and mix well to obtain a final volume of 200 µl.
2. Add 96-100% ethanol to the sample to obtain a final ethanol concentration of 75%.

   For example: To 200 µl of sample from Step 1, add 600 µl 96-100% ethanol to obtain a final volume of 800 µl.
3. Mix well.
4. Load sample mix onto the Spin Cartridge in a Collection Tube.

   Note: The reservoir capacity of the Spin Cartridge is ~700 µl. If the volume of your sample mix, after performing Step 3, exceeds 700 µl, perform multiple loading of the sample mix onto the Spin Cartridge (Step 4 and 5).
5. Centrifuge the Spin Cartridge at 12,000 x g for 1 minute at room temperature. Discard the flow through.
6. Wash the Spin Cartridge with 500 µl Wash Buffer (W5) with ethanol. Centrifuge the Spin Cartridge at 12,000 x g for 1 minute at room temperature.
7. Repeat wash step with Wash Buffer (W5) once.
8. Discard flow collection tube. Place the spin cartridge in the Wash Tube supplied with the kit.
9. Centrifuge the Spin Cartridge at 12,000 x g for 1 minute to remove any residual Wash Buffer (W5). Discard the Wash Tube.
10. Place the Spin Cartridge in the Recovery Tube supplied with the kit.
11. Add 100 µl Buffer (W4) for single-column purification supplied with the kit.
12. Incubate at room temperature for 1 minute.
13. Centrifuge the Spin Cartridge at 12,000 x g for 1 minute at room temperature. *The eluate contains siRNA.* Discard the cartridge.
14. Proceed to ethanol precipitation of siRNA, from the eluate, next page.

Continued on next page

One-Column Protocol, Continued

Ethanol Precipitation

Perform ethanol precipitation of siRNA from the eluate, as described below.

1. Add 200 µl cold 96-100% ethanol and 1 µl glycogen solution (20 µg/µl) to the eluate from Step 12, previous page.
2. Mix well and incubate at -20°C for 15 minutes.
3. Centrifuge the tube in a microcentrifuge at maximum speed for 15 minutes at 4°C.
4. Discard the supernatant carefully without disturbing the pellet and wash the pellet by resuspending the pellet in 0.5 ml cold 70% ethanol.
5. Centrifuge the tube in a microcentrifuge at maximum speed for 10 minutes at 4°C.
6. Discard the supernatant carefully without disturbing the pellet.
7. Air-dry the pellet for ~5 minutes.
8. Resuspend the dried pellet in 50 µl RNase-free water supplied with the kit. The pellet contains siRNA.
9. Store the siRNA at -80°C or use siRNA for the desired downstream application.

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Technical Service, Continued

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