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SurePrep™ Small RNA Purification Kit

Product Cat. # BP2801-25

Instruction Manual

I. Introduction

A. Product Description

Traditional protocols for isolating total RNA and mRNA are not optimized for isolation of small RNA molecules resulting in the loss of substantial amounts of small RNA. Moreover, the co-purification of larger RNA molecules with small RNA will inhibit expression analysis of small RNA. However, the SurePrep™ Small RNA Purification Kit provides a rapid and efficient method to isolate and purify small RNAs (< 200 nucleotides) from larger RNAs in various biological samples. These small RNAs include regulatory RNA molecules such as microRNA (miRNA) and short interfering RNA (siRNA), as well as tRNA and 5S rRNA. Small RNA molecules are often studied due to their ability to regulate gene expression. miRNAs and siRNAs are typically 20-25 nucleotides long, and regulate gene expression by binding to target mRNAs that can affect their translation. Purified small RNA from the SurePrep kit is suitable for use in RT-PCR, northern blotting and microarray analysis.

B. Overview of Procedure

Purification is based on spin column chromatography using a proprietary resin as the separation matrix. The resin binds RNA in a manner that depends on ionic concentrations. The small RNA is preferentially purified from other cellular macromolecules such as ribosomal RNA and proteins without the use of phenol or chloroform. The process involves the use of two different spin columns: the *Large RNA Removal Column* and the *Small RNA Enrichment Column* (see flow chart on page 5).

Briefly, the cells or tissue of interest are first disrupted in a highly denaturing guanidine thiocyanate solution that simultaneously lyses cells and inactivates endogenous ribonucleases to ensure purification of intact RNA. Ethanol is then added to the sample lysate and if any precipitates are present the sample is centrifuged. The cleared lysate is then applied to the Large RNA Removal Column, and the larger RNA molecules will bind to the resin in the spin column while the smaller RNA species pass through into the flow-through. Ethanol is then added to the flow-through, and the sample is applied to the Small RNA Enrichment Column. The small RNA molecules will then bind to the resin, and any impurities are removed through a series of washes with the provided Wash Solution. The small RNA molecules are then eluted using the Elution Buffer, and are ready for use in various downstream applications.

C. Kit Specifications

Kit Specifications	
Column Binding Capacity	50 μg
Maximum Column Loading Volume	600 μL
Size of RNA Purified	< 200 nt
Maximum Amount of Starting Material: Animal Cells Animal Tissues Bacteria Plant Tissues	 3×10^6 cells 25 mg 5×10^8 cells 50 mg
Time to Complete 10 purifications	45 minutes

D. Advantages

- Fast and easy processing using rapid spin-column format
- No phenol or chloroform extractions
- Isolate all small RNA molecules (<200 nt)
- Minimal contamination from large RNA molecules and genomic DNA
- High quality small RNA can be used in various downstream applications

E. Kit Components

Component	Cat. # BP2801- 25 (25 preps)
Lysis Solution	2 x 20 mL
Wash Solution*	22 mL
RNA Elution Buffer	6 mL
Large RNA Removal Column	25
Small RNA Enrichment Column	25
Collection Tubes	50
Elution Tubes (1.7 mL)	50
Product Insert	1

*Prior to addition of 50 mL 95% ethanol

F. Storage Conditions and Product Stability

All solutions should be kept tightly sealed and stored at room temperature. These reagents should remain stable for at least 2 years in their unopened containers.

G. Precautions and Disclaimers

This kit is designed for research purposes only. It is not intended for human or diagnostic use.

Ensure that a suitable lab coat, disposable gloves and protective goggles are worn when working with chemicals. For more information, please consult the appropriate Material Safety Data Sheets (MSDS). The MSDS can be requested through our Customer Service.

H. Customer-Supplied Reagents and Equipment

You must have the following in order to use the SurePrep Small RNA Purification Kit.

For All Protocols

- Benchtop microcentrifuge
- β-mercaptoethanol
- 95 - 100% ethanol

For Animal Tissue Protocol

- Liquid nitrogen
- Mortar and pestle
- 25 gauge needle and syringe

For Bacterial Protocol

- Lysozyme-containing TE Buffer:

- For Gram-negative bacteria, 1 mg/mL lysozyme in TE Buffer
- For Gram-positive bacteria, 3 mg/mL lysozyme in TE Buffer

For Plant Protocol

- Liquid nitrogen
- Mortar and pestle
- 70% ethanol

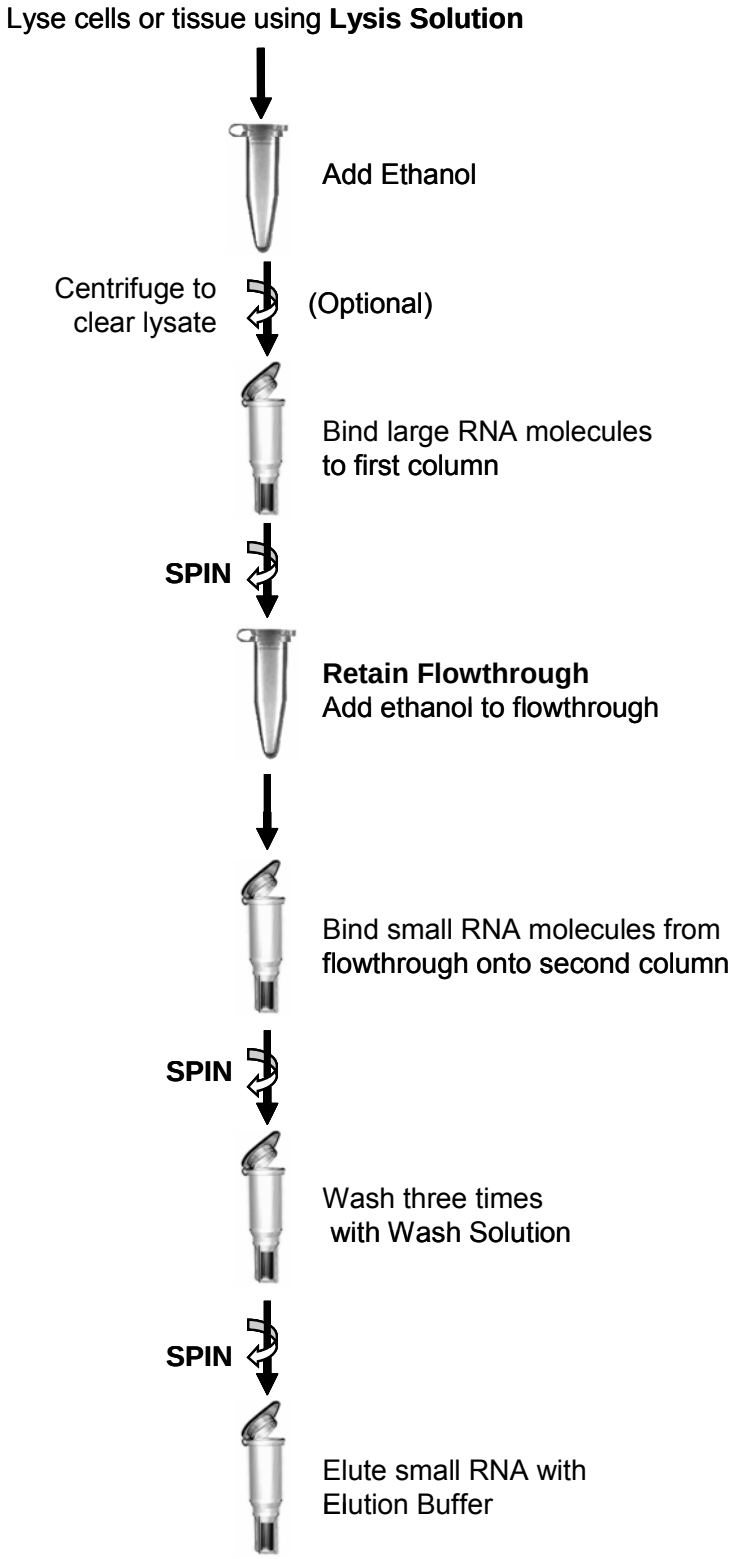
I. Working with RNA

RNases are very stable and robust enzymes that degrade RNA. Autoclaving solutions and glassware is not always sufficient to actively remove these enzymes. The first step when preparing to work with RNA is to create an RNase-free environment. The following precautions are recommended as your best defense against these enzymes.

- The RNA area should be located away from microbiological work stations.
- Clean, disposable gloves should be worn at all times when handling reagents, samples, pipettes, disposable tubes, etc. It is recommended that gloves are changed frequently to avoid contamination.
- There should be designated solutions, tips, tubes, lab coats, pipettes, etc. for RNA only.
- All RNA solutions should be prepared using at least 0.05% DEPC-treated autoclaved water or molecular biology grade nuclease-free water.
- Clean all surfaces with commercially available RNase decontamination solutions.
- When working with purified RNA samples, ensure that they remain on ice during downstream applications.

Flow Chart

Procedure for the SurePrep™ Small RNA Purification Kit



Purified Small RNA (miRNA, siRNA, tRNA, 8S rRNA, etc)

II. Purification of Small RNA from Biological Samples

A. Equipment Preparation

All centrifugation steps are carried out in a benchtop microcentrifuge. Various speeds are required for different steps, so please check your microcentrifuge specifications to ensure that it is capable of the proper speeds. All centrifugation steps are performed at room temperature. The correct rpm can be calculated using the formula:

$$RPM = \sqrt{\frac{RCF}{(1.118 \times 10^{-5})\,(r)}}$$

where *RCF* = required gravitational acceleration (relative centrifugal force in units of g); *r* = radius of the rotor in cm; and *RPM* = the number of revolutions per minute required to achieve the necessary *g*-force.

If you do not own a variable speed microcentrifuge consider purchasing Thermo Scientific's Sorvall Legend benchtop model that offers power, safety and convenience.

- Choice of 17,000 or 21,000 x g (for RNA purification using SurePrep kits, the microcentrifuge with 17,000 x g is sufficient)
- Holds 36 x 0.5 mL microtubes, 24 x 2 mL tubes or 8 x 8 PCR
- Unique ClickSeal™ bio-containment rotor lid for safe processing of infectious specimens plus adequate clearance of SurePrep spin columns
- Fast acceleration and deceleration speeds up your protocols
- Broad range of rotors supports virtually any application
- Intuitive controls and vivid display
- Highly resistant materials allow vigorous cleaning and autoclaving

Sorvall Legend MicroCentrifuges

Technical Specifications

	Sorvall Legend Micro 17 & 17R	**Sorvall Legend Micro 21 & 21R**
Max g-force:	17,000	21,100
Max RPM:	13,300	14,800
Noise level:	<55 dBA	<56 dBA
Time set range:	1 min - 99 min; 1 min increments	1 min - 99 min; 1 min increments
Temp set range:	Set from -9 °C to +40 °C; per 1 °C increment	Set from -9 °C to +40 °C per 1 °C increment

Ordering Information

	Cat. No.	Cat. No.
Sorvall Legend Micro 17/17R	230V 50/60Hz	120V 60 Hz
Sorvall Legend Micro 17, includes 24 x 1.5/2.0 mL rotor with ClickSeal bio-containment lid	75002430	75002431
Sorvall Legend Micro 17R, includes 24 x 1.5/2.0 mL rotor with ClickSeal bio-containment lid	75002440	75002441
Sorvall Legend Micro 21/21R	230V 50/60Hz	120V 60 Hz
Sorvall Legend Micro 21, incl. 24 x 1.5/2.0 mL rotor with ClickSeal bio-containment lid	75002435	75002436
Sorvall Legend Micro 21R, incl. 24 x 1.5/2.0 mL rotor with ClickSeal bio-containment lid	75002445	75002446

For detailed product specifications, information on additional rotors, lids, and adapters please visit www.thermo.com.

B. Protocols for Purification of Small RNA from Cultured Animal Cells

All centrifugation steps are carried out in a benchtop microcentrifuge at 14,000 x g (~12,000 RPM) except where noted. All centrifugation steps are performed at room temperature. Please be advised that all RPM values referenced in the protocols next to the actual g force are calculated for Sorvall Legend Microcentrifuge with rotor for 24 x 2 mL tubes.

Notes Prior to Use

- Ensure that all solutions are at room temperature prior to use.
- There are 2 different spin columns provided with this kit; the Large RNA Removal Column and the Small RNA Enrichment Column. Ensure that the correct column is used for each step of the procedure.
- Prepare a working concentration of the **Wash Solution** by adding 50 mL of 95% ethanol (provided by the user) to the supplied bottle containing the concentrated **Wash Solution**. This will give a final volume of 72 mL. The label on the bottle has a box that may be checked to indicate that the ethanol has been added.
- Prepare an appropriate amount of Lysis Solution by adding 10 μL of β-mercaptoethanol (provided by the user) to each 1 mL of Lysis Solution required. β-mercaptoethanol is toxic and should be dispensed in a fume hood.
- The maximum recommended input of animal cells is 3 x 10^6. A hemocytometer can be used in conjunction with a microscope to count the number of cells. As a general guideline, a confluent 3.5 cm plate of HeLa cells will contain 10^6 cells.
- Cell pellets can be stored at -70°C for later use or used directly in the procedure. Determine the number of cells present before freezing.
- Frozen pellets should be stored for no longer than 2 weeks to ensure that the integrity of the RNA is not compromised.
- Frozen pellets should not be thawed prior to beginning the protocol. Add the Lysis Solution directly to the frozen cell pellet.
- It is important to work quickly during this procedure.

Cells Growing in a Monolayer

1. Cell Lysate Preparation

a. Aspirate media and wash cell monolayer with an appropriate amount of PBS. Aspirate PBS.
b. Add 300 μL of **Lysis Solution** directly to culture plate.
c. Lyse cells by gently tapping culture dish and swirling buffer around the plate surface for five minutes.
d. Transfer lysate to a microcentrifuge tube.
e. Add 40 μL of 95 – 100% ethanol (provided by the user) to the lysate**.** Mix by vortexing for 10 seconds.
f. If visible precipitates are present, spin lysate for 1 minute in a benchtop microcentrifuge to pellet any debris. Otherwise, proceed directly to step **2a** without centrifugation.

2. Large RNA Removal

a. Assemble a **Large RNA Removal Column** with one of the provided collection tubes.
b. Apply the clarified lysate with the ethanol (from step **1f**) onto the column and centrifuge for 1 minute. Retain the flow-through, which contains the small RNA species. If large RNA is to be isolated, retain the column and proceed to the optional **Large RNA Purification Protocol (Appendix A)**. Otherwise, discard the column.
c. Transfer the flow-through to an RNase-free microcentrifuge tube (not provided). The flow-through contains the small RNA species, thus take care not to discard this fraction.

3. Small RNA Capture

a. Add 450 µL of 95 – 100% ethanol (provided by the user) to the flow-through collected from step **2b.** Mix by vortexing for 10 seconds.
b. Assemble a **Small RNA Enrichment Column** with one of the provided collection tubes.
c. Apply half of the lysate mix with ethanol onto the spin column and centrifuge for 1 minute.
d. Discard the flowthrough and reassemble the spin column with the collection tube.
e. Repeat steps **3c** and **3d** to complete the capture of the small RNA.

4. Column Wash

a. Apply 400 µL of **Wash Solution** to the **Small RNA Enrichment Column** and centrifuge for 1 minute.

Note: Ensure the entire wash solution has passed through into the collection tube by inspecting the column. If the entire wash volume has not passed, spin for an additional minute.

b. Discard the flowthrough and reassemble the spin column with the collection tube.
c. Repeat steps **4a** and **4b** to wash column a second time.
d. Wash column a third time by adding another 400 µL of **Wash Solution** and centrifuging for 2 minutes.
e. Ensure that the column is dry. Spin for an additional minute, if necessary.
f. Discard the collection tube with the flowthrough.

5. Small RNA Elution

a. Place the **Small RNA Enrichment Column** into a fresh 1.7 mL Elution Tube provided with the kit.
b. Add 50 µL of **RNA Elution Buffer** to the column.

Note: For higher concentrations of RNA, a lower elution volume may be used. A minimum volume of 20 µL is recommended.

c. Centrifuge for 2 minutes at **200 x *g* (~1,500 RPM)**, followed by 1 minute at **14,000 x *g* (~12,000 RPM).** Note the volume eluted from the column. If the entire volume has not been eluted, spin the column at 14,000 x g (~12,000 RPM) for 1 additional minute.

Note: For maximum RNA recovery, it is recommended that a second elution be performed into a separate microcentrifuge tube (Repeat steps **5b** and **5c**).

6. Storage of RNA

The purified RNA sample may be stored at –20°C for a few days. It is recommended that samples be placed at –70°C for long term storage.

Cells Growing in Suspension and Lifted Cells

1. Cell Lysate Preparation

a. Transfer cell suspension to an RNase-free tube (not provided) and centrifuge at no more than 200 *x g* (~1,500 RPM) for 10 minutes to pellet cells.
b. Carefully decant the supernatant. A few microliters of media may be left behind with the pellet in order to ensure that the pellet is not dislodged.

c. Add 300 µL of **Lysis Solution** to the pellet. Lyse cells by vortexing for 15 seconds. Ensure that the entire pellet is completely dissolved before proceeding to the next step.
d. Add 40 µL of 95 – 100% ethanol (provided by the user) to the lysate. Mix by vortexing for 10 seconds.
e. If visible precipitates are present, spin lysate for 1 minute in a benchtop microcentrifuge to pellet any debris. Otherwise, proceed directly to step **2a** without centrifugation.

2. Large RNA Removal

a. Assemble a **Large RNA Removal Column** with one of the provided collection tubes.
b. Apply the clarified lysate with ethanol (from step **1e**) onto the column and centrifuge for one minute. Retain the flow-through which contains the small RNA species. If large RNA is to be isolated, retain the column and proceed to the optional **Large RNA Purification Protocol (Appendix A)**. Otherwise, discard the column.
c. Transfer the flow-through to an RNase-free microcentrifuge tube (not provided). The flow-through contains the small RNA, thus take care not to discard this fraction.

3. Small RNA Capture

a. Add 450 µL of 95 – 100% ethanol (provided by the user) to the flow-through collected from step **2b.** Mix by vortexing for 10 seconds.
b. Assemble a **Small RNA Enrichment Column** with one of the provided collection tubes.
c. Apply half of the lysate mix with ethanol onto the spin column and centrifuge for 1 minute.
d. Discard the flowthrough and reassemble the spin column with the collection tube.
e. Repeat steps **3c** and **3d** to complete the capture of the small RNA.

4. Column Wash

a. Apply 400 µL of **Wash Solution** to the **Small RNA Enrichment Column** and centrifuge for 1 minute.

Note: Ensure the entire wash solution has passed through into the collection tube by inspecting the column. If the entire wash volume has not passed, spin for one additional minute.

b. Discard the flowthrough and reassemble the spin column with the collection tube.
c. Repeat steps **4a** and **4b** to wash column a second time.
d. Wash column a third time by adding another 400 µL of **Wash Solution** and centrifuging for 2 minutes.
e. Ensure that the column is dry. Spin for an additional minute, if necessary.
f. Discard the collection tube with the flow-through.

5. Small RNA Elution

a. Place the **Small RNA Enrichment Column** into a fresh 1.7 mL Elution Tube provided with the kit.
b. Add 50 µL of **RNA Elution Buffer** to the column.

Note: For higher concentrations of RNA, a lower elution volume may be used. A minimum volume of 20 µL is recommended.

c Centrifuge for 2 minutes at **200 x *g* (~1,500 RPM)**, followed by 1 minute at **14,000 x *g* (~12,000 RPM)**. Note the volume eluted from the column. If the entire volume has not been eluted, spin the column at 14,000 x g (~12,000 RPM) for one additional minute.

Note: For maximum RNA recovery, it is recommended that a second elution be performed into a separate microcentrifuge tube (Repeat steps **5b** and **5c**).

6. Storage of RNA

The purified RNA sample may be stored at –20°C for a few days. It is recommended that samples be placed at –70°C for long term storage.

C. Protocol for Purification of Small RNA from Animal Tissues

All centrifugation steps are carried out in a benchtop microcentrifuge at 14,000 x g (~12,000 RPM) except where noted. All centrifugation steps are performed at room temperature.

Notes Prior to Use

- Ensure that all solutions are at room temperature prior to use.
- There are 2 different spin columns provided with this kit; the Large RNA Removal Column and the Small RNA Enrichment Column. Ensure that the correct column is used for each step of the procedure.
- Prepare a working concentration of the **Wash Solution** by adding 50 mL of 95% ethanol (provided by the user) to the supplied bottle containing the concentrated **Wash Solution**. This will give a final volume of 72 mL. The label on the bottle has a box that may be checked to indicate that the ethanol has been added.
- Prepare an appropriate amount of Lysis Solution by adding 10 μL of β-mercaptoethanol (provided by the user) to each 1 mL of Lysis Solution required. β-mercaptoethanol is toxic and should be dispensed in a fume hood.
- RNA in animal tissues is not protected after harvesting until the tissues are disrupted and homogenized. Thus, it is important that the procedure is carried out as quickly as possible, particularly the Cell Lysate Preparation step.
- Fresh or frozen tissues may be used for the procedure. Tissues should be flash-frozen in liquid nitrogen and transferred immediately to a -70°C freezer for long-term storage. Tissues may be stored at -70°C for several months. Do not allow frozen tissues to thaw prior to grinding with the mortar and pestle in order to ensure that the integrity of the RNA is not compromised.
- It is recommended that no more than 25 mg of tissue be used, in order to prevent clogging of the column.

1. Cell Lysate Preparation

a. Excise the tissue sample from the animal.
b. Determine the amount of tissue by weighing. It is recommended that no more than 25 mg of tissue be used for the protocol.
c. Transfer the tissue into a mortar that contains an appropriate amount of liquid nitrogen to cover the sample. Grind the tissue thoroughly using a pestle.
d. Allow the liquid nitrogen to evaporate without allowing the tissue to thaw.
e. Add 400 μL of **Lysis Solution** to the tissue sample and continue to grind until the sample has been homogenized. Continue the lysis process by passing the lysate 5-10 times through a 25 gauge needle attached to a syringe.
f. Transfer the lysate into an RNase-free microcentrifuge tube (not provided).
g. Centrifuge at 14,000 x g for 2 minutes to pellet any cell debris. Transfer the supernatant to another RNase-free microcentrifuge tube. Note the volume of the supernatant/lysate.
h. Add a volume of 95-100% ethanol (provided by the user) that is equivalent to 15% of the lysate volume (15 μL of ethanol is added to every 100 μL of lysate). Vortex to mix.

2. Large RNA Removal

a. Assemble a **Large RNA Removal Column** with one of the provided collection tubes.
b. Apply the clarified lysate with the ethanol (from step **1h**) onto the column and centrifuge for 1 minute. Retain the flow-through which contains the small RNA species. If large RNA is to be isolated, retain the column and proceed to the optional **Large RNA Purification Protocol (Appendix A)**. Otherwise, discard the column.
c. Transfer the flow-through to an RNase-free microcentrifuge tube (not provided). The flow-through contains the small RNA, thus take care not to discard this fraction.

3. Small RNA Capture

a. Based on the lysate volume determined in step 1g, add 1.5 volumes of 95 – 100% ethanol (provided by the user) to the flowthrough collected in step **2c** (150 µL of ethanol is added to every 100 µL of flowthrough)**.** Mix by vortexing for 10 seconds.
b. Assemble a **Small RNA Enrichment Column** with one of the provided collection tubes.
c. Apply half of the lysate mix with ethanol onto the spin column and centrifuge for 1 minute.
d. Discard the flowthrough and reassemble the spin column with the collection tube.
e. Repeat steps **3c** and **3d** to complete the capture of the small RNA.

4. Column Wash

a. Apply 400 µL of **Wash Solution** to the **Small RNA Enrichment Column** and centrifuge for 1 minute.

Note: Ensure the entire wash solution has passed through into the collection tube by inspecting the column. If the entire wash volume has not passed, spin for an additional minute.

b. Discard the flow-through and reassemble the spin column with the collection tube.
c. Repeat steps **4a** and **4b** to wash column a second time.
d. Wash column a third time by adding another 400 µL of **Wash Solution** and centrifuging for 2 minutes.
e. Ensure that the column is dry. Spin for an additional minute, if necessary.
f. Discard the collection tube with the flow-through.

5. Small RNA Elution

a. Place the **Small RNA Enrichment Column** into a fresh 1.7 mL Elution tube provided with the kit.
b. Add 50 µL of **RNA Elution Buffer** to the column.

Note: For higher concentrations of RNA, a lower elution volume may be used. A minimum volume of 20 µL is recommended.

c. Centrifuge for 2 minutes at **200 x *g* (~1,200 RPM)**, followed by 1 minute at **14,000 x *g* (~12,000 RPM)**. Note the volume eluted from the column. If the entire 50 µL has not been eluted, spin the column at 14,000 x g (~14,000 RPM) for one additional minute.

Note: For maximum RNA recovery, it is recommended that a second elution be performed into a separate microcentrifuge tube (Repeat steps **5b and 5c**).

6. Storage of RNA

The purified RNA sample may be stored at –20°C for a few days. It is recommended that samples be placed at –70°C for long term storage.

D. Protocol for Purification of Small RNA from Bacteria

All centrifugation steps are carried out in a benchtop microcentrifuge at 14,000 x g (~12,000 RPM) except where noted. All centrifugation steps are performed at room temperature.

Notes Prior to Use

- Ensure that all solutions are at room temperature prior to use.
- There are 2 different spin columns provided with this kit; the Large RNA Removal Column and the Small RNA Enrichment Column. Ensure that the correct column is used for each step of the procedure.
- Prepare a working concentration of the **Wash Solution** by adding 50 mL of 95% ethanol (provided by the user) to the supplied bottle containing the concentrated **Wash Solution**. This will give a final volume of 72 mL. The label on the bottle has a box that may be checked to indicate that the ethanol has been added.
- Prepare an appropriate amount of Lysis Solution by adding 10 μL of β-mercaptoethanol (provided by the user) to each 1 mL of Lysis Solution required. β-mercaptoethanol is toxic and should be dispensed in a fume hood.
- Prepare the appropriate lysozyme-containing TE Buffer as indicated in Table 1. This solution should be prepared with sterile, RNAse-free TE Buffer, and kept on ice until needed. These reagents are to be provided by the user.
- It is recommended that no more than 5 x 10^8 bacterial cells be used in this procedure. Bacterial growth can be measured using a spectrophotometer. As a general rule, an *E. coli* culture containing 1 x 10^9 cells/mL has an OD_{600} of 1.0.
- For RNA isolation, bacteria should be harvested in log-phase growth.
- Bacterial pellets can be stored at -70°C for later use, or used directly in this procedure.
- Frozen bacterial pellets should not be thawed prior to beginning the protocol. Add the Lysozyme-containing TE Buffer directly to the frozen bacterial pellet (step **1c**).

1. Cell Lysate Preparation

a. Pellet bacteria by centrifuging at 14,000 *x g* (~12,000 RPM) for 1 minute.
b. Decant supernatant, and carefully remove any remaining media by aspiration.
c. Resuspend the bacteria thoroughly in 100 μL of the appropriate lysozyme-containing TE buffer (see Table 1) by vortexing. Incubate at room temperature for the time indicated in Table 1.
d. Add 200 μL of Lysis Solution and vortex vigorously for at least 10 seconds.
e. Add 50 μL of 95 – 100% ethanol (provided by the user) to the lysate. Mix by vortexing for 10 seconds.
f. If visible precipitates are present, spin lysate for 1 minute in a benchtop microcentrifuge to pellet any debris. Otherwise, proceed directly to step **2a** without centrifugation.

Table 1: Incubation Time for Different Bacterial Strains

Bacteria Type	Lysozyme Concentration in TE Bufffer	Incubation Time
Gram-negative	1 mg/mL	5 min
Gram-positive	3 mg/mL	10 min

2. Large RNA Removal

a. Assemble a **Large RNA Removal Column** with one of the provided collection tubes.
b. Apply the clarified lysate with the ethanol (from step **1f**) onto the column and centrifuge for 1 minute. Retain the flow-through which contains the small RNA species. If large

RNA is to be isolated, retain the column and proceed to the optional **Large RNA Purification Protocol (Appendix A)**. Otherwise, discard the column.

c. Transfer the flow-through to an RNase-free microcentrifuge tube (not provided). The flow-through contains the small RNA, thus take care not to discard this fraction.

3. Small RNA Capture

a. Add 450 µL of 95 – 100% ethanol (provided by the user) to the flowthrough collected from step **2b.** Mix by vortexing for 10 seconds.
b. Assemble a **Small RNA Enrichment Column** with one of the provided collection tubes.
c. Apply half of the lysate mix with ethanol onto the spin column and centrifuge for 1 minute.
d. Discard the flowthrough and reassemble the spin column with the collection tube.
e. Repeat steps **3c** and **3d** to complete the capture of the small RNA.

4. Column Wash

a. Apply 400 µL of **Wash Solution** to the **Small RNA Enrichment Column** and centrifuge for 1 minute.

Note: Ensure the entire wash solution has passed through into the collection tube by inspecting the column. If the entire wash volume has not passed, spin for an additional minute.

b. Discard the flow-through and reassemble the spin column with the collection tube.
c. Repeat steps **4a** and **4b** to wash column a second time.
d. Wash column a third time by adding another 400 µL of **Wash Solution** and centrifuging for 2 minutes.
e. Ensure that the column is dry. Spin for an additional minute, if necessary.
f. Discard the collection tube with the flow-through.

5. Small RNA Elution

a. Place the **Small RNA Enrichment Column** into a fresh 1.7 mL Elution tube provided with the kit.
b. Add 50 µL of **RNA Elution Buffer** to the column.

Note: For higher concentrations of RNA, a lower elution volume may be used. A minimum volume of 20 µL is recommended

c. Centrifuge for 2 minutes at **200 x *g* (~1,500 RPM)**, followed by 1 minute at **14,000 x *g* (~12,000 RPM).** Note the volume eluted from the column. If the entire volume has not been eluted, spin the column at 14,000 x g (~12,000 RPM) for one additional minute.

Note: For maximum RNA recovery, it is recommended that a second elution be performed into a separate microcentrifuge tube (Repeat steps **5b** and **5c**).

6. Storage of RNA

The purified RNA sample may be stored at –20°C for a few days. It is recommended that samples be placed at –70°C for long term storage.

E. Protocol for Purification of Small RNA from Plant

All centrifugation steps are carried out in a benchtop microcentrifuge at 14,000 x g (~12,000 RPM) except where noted. All centrifugation steps are performed at room temperature.

Notes Prior to Use

- Ensure that all solutions are at room temperature prior to use.
- There are 2 different spin columns provided with this kit; the Large RNA Removal Column and the Small RNA Enrichment Column. Ensure that the correct column is used for each step of the procedure.
- Prepare a working concentration of the **Wash Solution** by adding 50 mL of 95% ethanol (provided by the user) to the supplied bottle containing the concentrated **Wash Solution**. This will give a final volume of 72 mL. The label on the bottle has a box that may be checked to indicate that the ethanol has been added.
- Prepare an appropriate amount of Lysis Solution by adding 10 µL of β-mercaptoethanol (provided by the user) to each 1 mL of Lysis Solution required. β-mercaptoethanol is toxic and should be dispensed in a fume hood.
- The maximum recommended input of plant tissue is 50 mg or 5 x 10^6 cells.
- Both fresh and frozen plant tissues can be used for this protocol. Samples should be flash-frozen in liquid nitrogen and transferred immediately to a -70°C freezer for long-term storage. Do not allow frozen tissues to thaw prior to grinding with the mortar and pestle in order to ensure that the integrity of the RNA is not compromised.
- It is important to work quickly during this procedure.

1. Cell Lysate Preparation

a. Freeze ≤ 50 mg of plant tissue or 5 x 10^6 plant cells in liquid nitrogen.
b. Grind the samples into a fine powder using a mortar and pestle.

   Note: If stored frozen samples are used, do not allow the samples to thaw before transferring to the liquid nitrogen.

c. Transfer the powder and liquid nitrogen into an RNase-free microcentrifuge tube (not provided).
d. Allow the liquid nitrogen to evaporate, without allowing the tissue to thaw.
e. Immediately add 400 µL of **Lysis Solution** to the tissue sample and vortex to mix.
f. Centrifuge at 14,000 x g for 2 minutes to pellet any cell debris. Transfer the supernatant to another RNase-free microcentrifuge tube. Note the volume of the supernatant/lysate.
g. Add a volume of 95-100% ethanol (provided by the user) that is equivalent to 15% of the lysate volume (15 µL of ethanol is added to every 100 µL of lysate). Vortex to mix.

2. Large RNA Removal

a. Assemble a **Large RNA Removal Column** with one of the provided collection tubes
b. Apply the clarified lysate with the ethanol (from step **1g**) onto the column and centrifuge for 1 minute. Retain the flow-through which contains the small RNA species. If large RNA is to be isolated, retain the column and proceed to the optional **Large RNA Purification Protocol (Appendix A)**. Otherwise, discard the column.
c. Transfer the flow-through to an RNase-free microcentrifuge tube (not provided). The flow-through contains the small RNA, thus take care not to discard this fraction.

3. Small RNA Capture

a. Based on the lysate volume determined in step 1f, add 1.5 volumes of 95 – 100% ethanol (provided by the user) to the flowthrough collected in step **2c** (150 µL of ethanol is added to every 100 µL of flowthrough)**.** Mix by vortexing for 10 seconds.
b. Assemble a **Small RNA Enrichment Column** with one of the provided collection tubes.
c. Apply half of the lysate mix with ethanol onto the second spin column and centrifuge for 1 minute.
d. Discard the flowthrough and reassemble the spin column with the collection tube.

e. Repeat steps **3c** and **3d** to complete the capture of the small RNA.

4. Column Wash

a. Apply 400 µL of **Wash Solution** to the **Small RNA Enrichment Column** and centrifuge for 1 minute.

Note: Ensure the entire wash solution has passed through into the collection tube by inspecting the column. If the entire wash volume has not passed, spin for an additional minute.

b. Discard the flow-through and reassemble the spin column with the collection tube.
c. Repeat steps **4a** and **4b** to wash column a second time.
d. Wash column a third time by adding another 400 µL of **Wash Solution** and centrifuging for 2 minutes.
e. Ensure that the column is dry. Spin for an additional minute, if necessary.
f. Discard the collection tube with the flow-through.

5. Small RNA Elution

a. Place the **Small RNA Enrichment Column** into a fresh 1.7 mL Elution tube provided with the kit.
b. Add 50 µL of **RNA Elution Buffer** to the column.

Note: For higher concentrations of RNA, a lower elution volume may be used. A minimum volume of 20 µL is recommended

c. Centrifuge for 2 minutes at **200 x *g* (~1,500 RPM)**, followed by 1 minute at **14,000 x *g* (~12,000 RPM)** Note the volume eluted from the column. If the entire volume has not been eluted, spin the column at 14,000 x g (~12,000 RPM) for one additional minute.

Note: For maximum RNA recovery, it is recommended that a second elution be performed into a separate microcentrifuge tube (Repeat steps **5b** and **5c**).

6. Storage of RNA

The purified RNA sample may be stored at –20°C for a few days. It is recommended that samples be placed at –70°C for long term storage.

F. Assessing RNA Yield and Quality by UV Absorbance

The concentration and purity of an RNA solution can be determined by absorbance (A) measurements at 260 and 280 nm. A_{260} measurements are quantitative for relatively pure RNA preparations in microgram quantities. A_{260} readings cannot distinguish between DNA and RNA, however the ratio of A_{260}/A_{280} can be used as an indication of RNA purity. For example, contaminating proteins have a peak absorption at 280 nm that will reduce the A_{260}/A_{280} ratio.

a. Determine RNA concentration by diluting an aliquot of the purified RNA solution (e.g. 1:50 dilution) in TE (10 mM Tris and 1 mM EDTA, pH 7.4). Measure absorbance of the diluted sample in a 1 mL cuvette using a traditional UV-VIS spectrophotometer at 260 and 280 nm. The spectrophotometer should first be zeroed with the TE used to dilute the sample.
b. An A_{260} of 1.0 is equivalent to 33 µg RNA/mL. Calculate the RNA concentration in µg/mL as follows:

A_{260} x dilution factor x 33 = µg RNA/mL

c. The ratio of the readings at 260 and 280 nm (A_{260}/A_{280}) provides an estimate of the RNA purity with respect to contaminants that absorb in the UV range such as protein. Ratios of 1.8 to 2.1 indicate highly purified preparations of RNA. Contaminants such as protein that absorb at 280 nm will lower this ratio. However, RNA solutions with a ratio lower than 1.8 may function well in downstream applications such as RT-PCR and Northern blotting.

G. Assessing RNA Quality by Denaturing Gel Electrophoresis

The overall quality and size distribution of small RNA molecules purified with the SurePrep Kit can be evaluated by denaturing gel electrophoresis using 8 – 15% acrylamide TBE-urea gel or 2% agarose formaldehyde gel with ethidium bromide staining. The respective small RNAs should appear as distinct bands in the gel (Figure 1) generally < 200 nucleotides in size.

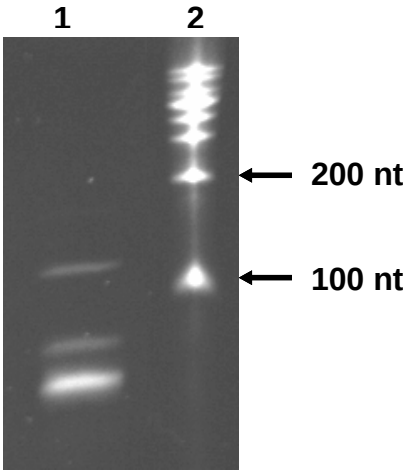


Figure 1. Small RNA isolated from 5 x 10^5 HeLa cells using the SurePrep™ Kit. Purified small RNA (lane 1) and a 100 nt RNA ladder (lane 2) were run on an 8% urea-PAGE gel.

III. Troubleshooting Guide

Problem	Possible Cause	Solution and Explanation
Poor RNA Recovery	Incomplete lysis of cells or tissue	Ensure that the appropriate amount of Lysis Solution was used for the amount of cells or tissue.
	Large RNA Removal Column has become clogged	Do not exceed the recommended amounts of starting materials. The amount of starting material may need to be decreased if the column shows clogging below the recommended levels. See also "Clogged Column" below.
	An alternative elution solution was used	It is recommended that the RNA Elution Buffer supplied with this kit be used for maximum RNA recovery.
	Low RNA content	Different tissues and cells have different RNA contents. Some tissues may not contain small RNA at detectable levels when processing the small sample sizes required for this procedure.
	Flow-through from the first binding step was discarded	The flow-through from the binding step with the Large RNA Removal Column contains the small RNA molecules, thus ensure that it is not inadvertently discarded.
	Ethanol was not added to the flow-through before binding to the Small RNA Enrichment Column	Ensure that the appropriate amount of ethanol was added to the flow-through from the first binding step before it is applied to the Small RNA Enrichment Column. This is imperative in order to capture the small RNA molecules.
	Ethanol was not added to the Wash Solution	Ensure that 50 mL of 95% ethanol is added to the supplied Wash Solution prior to use.
	Cell Culture: Cell monolayer was not washed with PBS	Ensure that the cell monolayer is washed with the appropriate amount of PBS in order to remove residual media from cells.
	Bacteria: All traces of media not removed	Ensure that all media is removed prior to the addition of the lysis solution through aspiration.

Problem	Possible Cause	Solution and Explanation
Clogged Column	Insufficient solubilization of cells or tissues	Ensure that the appropriate amount of Lysis Buffer was used for the amount of cells or tissue.
	Maximum number of cells or amount of tissue exceeds kit specifications	Refer to specifications to determine if amount of starting material falls within kit specifications
	Clarified lysate was not used for the binding step	Ensure that after the lysis step, the sample is centrifuged and only the clarified lysate is used in subsequent steps.
	High amounts of genomic DNA present in sample	The lysate may be passed through a 25 gauge needle attached to a syringe 5-10 times in order to shear the genomic DNA prior to loading onto the Large RNA Removal Column.
	Centrifuge temperature too low	Ensure that the centrifuge remains at room temperature throughout the procedure. Temperatures below 20°C may cause precipitates to form that can cause the columns to clog.
RNA is Degraded	RNase contamination	RNases may be introduced during the use of the kit. Ensure proper procedures are followed when working with RNA. Please refer to "*Working with RNA"* at the beginning of this user guide.
	Procedure not performed quickly enough	In order to maintain the integrity of the RNA, it is important that the procedure be performed quickly. This is especially important for the Cell Lysate Preparation Step in the Animal Tissue protocol, since the RNA in animal tissues is not protected after harvesting until it is disrupted and homogenized.
	Improper storage of the purified RNA	For short term storage purified RNA samples may be stored at –20°C for a few days. It is recommended that samples be stored at –70°C for longer term storage.
	Frozen tissues or pellets were allowed to thaw prior to disruption	Tissue samples should be flash-frozen in liquid nitrogen and transferred immediately to a -70°C freezer for long-term storage. Do not allow frozen tissues to thaw prior to grinding with the mortar and pestle in order to ensure that the integrity of the RNA is not compromised.

Problem	Possible Cause	Solution and Explanation
RNA does not perform well in downstream applications	RNA was not washed three times with the provided Wash Solution	Traces of salt from the binding step may remain in the sample if the Small RNA Enrichment Column is not washed three times with Wash Solution. Salt may interfere with downstream applications, and thus must be washed from the column.
	Ethanol carryover	Ensure that the dry spin under the Column Wash procedure is performed in order to remove traces of ethanol prior to elution. Ethanol is known to interfere with many downstream applications.
Genomic DNA contamination	Large amount of starting material used	Perform RNase-free DNaseI digestion on the RNA sample after elution to remove genomic DNA contamination.
Large RNA species present in elution (Figure 2)	Improper amount of ethanol added to the lysate before binding to the Large RNA Removal Column	Ensure that the appropriate amount of ethanol was added to the lysate before it is applied to the Large RNA Removal Column. This is imperative in order to capture the large RNA molecules onto the column.
	Too large amount of starting material used	Repeat purification using less starting material. Alternatively, the isolation procedure can be repeated using the elution as the input. The elution volume should first be adjusted to 300 µL using the provided Lysis Solution. The procedure can then be followed as written in the manual, starting with the addition of 40 µL of ethanol, centrifuging the lysate in order to pellet any debris, and applying the clarified lysate to the Large RNA Removal Column. Repeating the procedure should result in the removal of the large, contaminating RNA species.

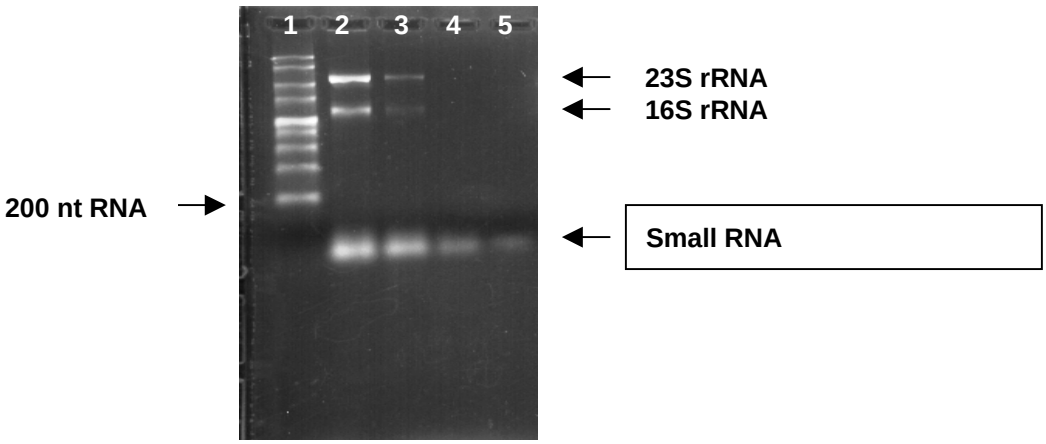


Figure 2. Small RNA (< 200 nt) isolated from different starting amounts of *E. coli* cells (Fisher strain FB5α, Cat. # BP4000-3). Large RNA species are efficiently removed from the small RNA elution (lanes 4, 5) when RNA is isolated from 2.5 x 10^8 cells. However, when the starting material is increased to 5 x 10^8 cells large RNA is found in the small RNA elution (lanes 2, 3) due to the binding capacity of the Large RNA Removal Column being exceeded. Lane 1 is a 1 kb RNA standard. Electrophoresis conditions: 1.5% formaldehyde-agarose gel run at 100 V for 70 min.

Note: The RNA content of a bacterial culture can vary due to the species or the growth conditions. We recommend starting with 5 x 10^8 cells. Depending on yield and purity, it may be possible to increase/decrease the number of bacteria in subsequent preparations.

IV. Related Products

A. Additional RNA Purification Kits

Catalog #	Product Description
BP2800-50	SurePrep™ TrueTotal™ RNA Purification Kit
BP2801-25	SurePrep™ Small RNA Purification Kit
BP2802-50	SurePrep™ RNA/DNA/Protein Purification Kit
BP2803-50	SurePrep™ Urine Exfoliated Cell RNA Purification Kit
BP2804-50	SurePrep™ Urine Bacterial RNA Purification Kit
BP2805-50	SurePrep™ Nuclear Or Cytoplasmic RNA Purification Kit
BP2806-50	SurePrep™ RNA/Protein Purification Kit
BP2807-50	SurePrep™ Leukocyte RNA Purification Kit
BP2809-50	SurePrep™ RNA Cleanup and Concentration Kit

B. Other Fisher BioReagents Functionally Tested for RNA Research

BP2484-50	Water, Sterile (DEPC-treated) 50mL
BP2484-100	Water, Sterile (DEPC-treated) 100mL
BP561-1	Water, Sterile (RNA Grade) 1L
BP2483-100	EDTA 0.5 M (DEPC-treated) 100mL
BP2483-1	EDTA 0.5 M (DEPC-treated) 1L
BP2483-500	EDTA 0.5 M (DEPC-treated) 500mL
BP2810-50	RiboLadder™ 100b RNA Standard with loading buffers
BP2811-50	RiboLadder™ 1Kb RNA Standard with loading buffers
BP3224-5	Optizyme™ Ribonuclease Inhibitor (Human Placental) 10,000U
BP3224-1	Optizyme™ Ribonuclease Inhibitor (Human Placental) 2,500U
BP3225-5	Optizyme™ Ribonuclease Inhibitor (Porcine) 10,000U
BP3225-1	Optizyme™ Ribonuclease Inhibitor (Porcine) 2,500U
BP3222-5	Optizyme™ Ribonuclease Inhibitor (Recombinant) 10,000U
BP3222-1	Optizyme™ Ribonuclease Inhibitor (Recombinant) 2,500U
BP3226-1	Optizyme™ Recombinant DNase I (RNase-free) 1,000U
BP3226-2	Optizyme™ Recombinant DNase I (RNase-free) 2,000U
BP176-100	2-Mercaptoethanol 100g
BP535-1	Lysozyme, Egg White 1g
BP535-5	Lysozyme, Egg White 5g
BP535-10	Lysozyme, Egg White 10g
BP2476-100	Tris-EDTA, 1X Solution, pH 7.4 100ml
BP2476-500	Tris-EDTA, 1X Solution, pH 7.4 500ml
BP160-100	Agarose, Low EEO, Multipurpose 100g
BP1360-100	Agarose, Low Melting, <1kb RNA 100g
BP1356-100	Agarose, Broad Separation Range for RNA 100g

V. Appendix A

Optional Large RNA Purification Protocol

1. Column Wash

a. Reassemble the **Large RNA Removal Column** with the collection tube used in Step **2b**
b. Apply 400 µL of **Wash Solution** to the **Large RNA Removal Column** and centrifuge for 1 minute.

Note: Ensure that the entire Wash Solution has passed through into the collection tube by inspecting the column. If the entire wash volume has not passed, spin for an additional minute.

c. Discard the flow-through and reassemble the spin column with the collection tube.
d. Repeat steps **1a** and **1b** to wash the column a second time.
e. Wash column a third time by adding another 400 µL of **Wash Solution** and centrifuging for 2 minutes.
f. Ensure that the column is dry. Spin for an additional minute, if necessary.
g. Discard the collection tube with the flowthrough.

2. Large RNA Elution

a. Place the **Large RNA Removal Column** into a fresh 1.7 mL Elution Tube provided with the kit.
b. Add 50 µL of **RNA Elution Buffer** to the column.
c. Centrifuge for 2 minutes at **200 x *g* (~1,500 RPM)**, followed by 1 minute at **14,000 x *g* (~12,000 RPM)**. Note the volume eluted from the column. If the entire volume has not been eluted, spin the column at 14,000 x g (~12,000 RPM) for one additional minute.

Note: For maximum RNA recovery, it is recommended that a second elution be performed into a separate microcentrifuge tube (Repeat steps **2b** and **2c**).

3. Storage of RNA

The purified RNA sample may be stored at –20°C for a few days. It is recommended that samples be placed at –70°C for long term storage.

Technical Support

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