3050 Spruce Street, St. Louis, MO 63103 USA
Tel: (800) 521-8956 (314) 771-5765 Fax: (800) 325-5052 (314) 771-5757
email: techservice@sial.com sigma-aldrich.com

Product Information

mirPremier® microRNA Isolation Kit

Catalog Numbers **SNC10, SNC50**
Store at Room Temperature

TECHNICAL BULLETIN

Procedures

Product Description

microRNAs (miRNAs) are a class of small RNA molecules, about 21 nucleotides (nt) in length, that regulate gene expression in a variety of manners, including translational repression, mRNA cleavage and deadenylation. Sigma's mirPremier microRNA Isolation Kit provides a rapid and efficient method for purifying and enriching miRNAs and other small RNAs from diverse biological sources, including mammalian cell cultures, animal tissues, plant tissues, and microbial cultures, without using hazardous organic extractions. In addition, the kit also can be used for isolating total RNA if messenger RNA or other large RNAs are of interest.

Small RNA Preparation

The mirPremier microRNA Isolation Kit employs a novel purification chemistry to isolate miRNAs and other small RNAs in a simplified and streamlined manner, without using phenol and chloroform. A biological sample is lysed in a lysis mix that releases small RNA and, at the same time, inactivates ribonucleases, as well as interfering secondary metabolites, that may exist in plant tissues. Large RNA and genomic DNA remain insoluble and are removed from the lysate along with other cellular debris in a short centrifugation.

Small RNA is then captured onto a silica binding column in the presence of alcohol. Residual impurities are removed by wash solutions, and purified small RNA is eluted in RNase-free water, ready for immediate use in RT-PCR, Northern blots, and other applications. Up to 20 μg of small RNA can be isolated in 30 minutes. Purified RNA is typically less than about 200 nt in length.

Total RNA Preparation

For total RNA isolation, a biological sample is lysed in a lysis solution that releases total RNA and, at the same time, inactivates ribonucleases, as well as interfering secondary metabolites, that may exist in plant tissues. After the removal of cellular debris, RNA is then captured onto a silica binding column with a unique binding solution, which effectively inhibits the binding of genomic DNA as well as polysaccharides. Residual impurities and most residual genomic DNA are removed by wash solutions, and purified RNA is eluted in RNase-free water, ready for immediate use in RT-PCR, Northern blots, and other applications. Up to 150 μg of total RNA can be isolated in 30 minutes.

It should be noted, however, that small RNA is not efficiently recovered in total RNA preparation, and therefore the total RNA preparation procedure is not recommended for recovering small RNAs.

If all traces of DNA must be eliminated from an RNA preparation, further treatment with DNase I is recommended. For total RNA preparation, DNase I digestion can be performed with the On-Column DNase I Digestion Set, Catalog Numbers DNASE10 and DNASE70, while the RNA is bound to the Binding Column during RNA purification (see Appendix), or with Amplification Grade DNase I, Catalog Number AMPD1, on the final RNA preparation. However, on-column DNase I digestion is not recommended for small RNA preparation. The final small RNA preparation can be treated with the Amplification Grade DNase I.

Reagents Provided	Catalog Number	10 Preps	50 Preps
microRNA Lysis Buffer	M1070	10 ml	50 ml
Lysis Solution	L8167	10 ml	50 ml
Binding Solution	L8042	2 x 10 ml	2 x 50 ml
Wash Solution 2 Concentrate	W3261	2.5 ml	15 ml
Elution Solution	E8024	1.5 ml	10 ml
Filtration Columns	C6866	10 each	50 each
Binding Columns	C6991	10 each	50 each
Collection Tubes, 2 ml	T5449	4 x 10 each	4 x 50 each

Reagents and equipment required, but not provided	Mammalian cell cultures	Mammalian tissues	Plant tissues	Yeast	Bacteria
2-Mercaptoethanol, Cat. No. M3148	Required	Required	Required	Required	Required
100% Ethanol, Cat. No. E7023	Required	Required	Required	Required	Required
Microcentrifuge and 2-ml tubes	Required	Required	Required	Required	Required
Rotor-stator homogenizer		Required			
Mortar and pestle			Required		
Liquid nitrogen and dry ice			Required		
55 °C heat block or water bath			Required		
Yeast Enzyme Stock Solution, Cat. No. Y0378 Yeast Lysis Solution, Cat. No. Y0253				Required	
Bacterial Enzyme Stock Solution, Catalog No. B7809 Bacterial Lysis Solution, Cat. No.B7934					Required
On-Column DNase I Digestion Set, Cat. Nos. DNASE10, DNASE70	Optional	Optional	Optional	Optional	Optional
DNase I, Amplification Grade, Cat. No. AMPD1	Optional	Optional	Optional	Optional	Optional

Precautions and Disclaimer
This product is for R&D use only, not for drug, household, or other uses. Please consult the Material Safety Data Sheet for information regarding hazards and safe handling practices.

Storage/Stability
Store the kit at room temperature. If any reagent forms a precipitate, warm at 65 °C to dissolve the precipitate and allow to cool to room temperature before use.

Avoiding RNase Contamination
RNases are ubiquitous and very stable enzymes and generally do not require cofactors for enzymatic activity. While endogenous RNases are denatured during the lysis step and removed subsequently during RNA purification, care must be taken to avoid introducing exogenous RNases during RNA preparation, especially during the final wash and elution steps. The work area and the pipette set must be free of RNases. Use RNase-free pipette tips, preferably those with an aerosol barrier. Always wear gloves and change them frequently. Keep bottles and tubes closed when not in use. Additional information on avoiding RNase contamination and working with RNA can be found in the references listed at the end of this technical bulletin.

Preparation Instructions
1. **Ethanol-diluted Wash Solution 2.** Add an appropriate amount of 100% ethanol (200 proof) to the bottle of Wash Solution 2 Concentrate according to the table below. Mix briefly and store the Ethanol-diluted Wash Solution 2 tightly capped to prevent the evaporation of ethanol.

Kit Size	Amount of Ethanol to Add
10-prep	10 ml
50-prep	60 ml

2. **Assemble Column and Collection Tube.** Insert a Filtration Column (blue retainer ring) into a 2 ml Collection Tube (provided) and close the lid. Likewise, insert a Binding Column (red retainer ring) into a 2 ml Collection Tube (provided) and close the lid. It should be noted that a Filtration Column is not required for mammalian cell cultures.

Procedures

I. Small RNA Isolation - Mammalian Cell Cultures

1. **Prepare Lysis Mix.** According to Table 1 below, add microRNA Lysis Buffer and Binding Solution to a clean tube and mix briefly. Supplement the solution with 10 µL of 2-mercaptoethanol per 1 ml of the solution. Use Table 2 to determine the total amount of the Lysis Mix sufficient for that day's use. It is best to prepare the Lysis Mix just prior to the start of the purification procedure. Keep the Lysis Mix at room temperature before use.

Table 1. Lysis Mix for Mammalian Cell Cultures

Component	Amount/ml Lysis Mix
microRNA Lysis Buffer (M1070)	700 µL
Binding Solution (L8042)	300 µL

Note: A Lysis Mix of 0.5 vol. of microRNA Lysis Buffer and 0.5 vol. of Binding Solution is also suitable for most cultures, especially attached cultures, and might increase the yield by 10-15%.

2. **Prepare Cell Samples**

a. **Suspension cell cultures.** Pellet suspension cultures or detached cells for 5 minutes at ≥300 x *g*. Remove the culture medium. Re-centrifuge the cell sample briefly and use a pipette tip to remove the last drop of liquid. Incomplete removal of the culture medium may adversely affect the result.

b. **Attached cell cultures.** Remove the culture medium and wash the culture with Hank's Balanced Salt Solution. Remove the wash fluid. Tilt the culture vessel to one side and use a pipette tip to remove the last drop of liquid. Incomplete removal of the culture medium may adversely affect the results.

3. **Lyse Cells**

a. **Pelleted cells.** Use Table 2 to determine the amount of the Lysis Mix needed for the cell sample. Vortex the cell pellet 1-2 seconds to loosen cells. Add the Lysis Mix to the cell pellet and vortex immediately but gently and briefly (2-3 seconds) to disrupt the cell pellet. Let the sample sit for ≥ 5 minutes and mix it 2-3 times in between by gentle shaking or flipping. Do not vortex the crude lysate after the cells have been lysed. Do not be concerned with the cellular debris that may be present in the crude lysate from some cell lines. Transfer the crude lysate to a 2-ml microcentrifuge tube (not provided).

Note: Excessively vortexing the crude lysate will shear genomic DNA and adversely affect the result. If the cell pellet has been stored at –70 °C, lyse the cells at 55 °C for 5 minutes instead of room temperature.

b. **Attached cells.** Use Table 2 to determine the amount of the Lysis Mix needed for the cell sample. Add the Lysis Mix to the culture. Swirl or rock the culture vessel to cover the surface with the Lysis Mix. Let the sample sit for ≥ 5 minutes and mix it 2-3 times in between by gently swirling or rocking the vessel. Tilt the culture vessel to one side to collect the crude lysate to a 2-ml microcentrifuge tube (not provided). Do not be concerned with the cellular debris in the crude lysate or on the vessel surface.

Table 2. Lysis Mix Volume Recommendation

Sample	Number of cells or culture vessel	Amount of Lysis Mix Required
Pelleted cells	0.1 –0.5 million	50-100 µL
	0.6-1 million	200 µL
	2 million	250 µL
	3 million	300 µL
	4 million	400 µL
	5 million	500 µL
	6 million	600 µL
	7 million	700 µL
Attached cells	24-well plate	150 µL/well
	12-well plate	200 µL/well
	6-well plate	300 µL/well
	25 cm^2 flask < 50% confluent	500 µL/flask
	25 cm^2 flask 50-70% confluent	600 µL/flask
	25 cm^2 flask 80-100% confluent	700 µL/flask

4. **Pellet Cellular Debris.** Centrifuge the sample at maximum speed (14,000 –16,000 x *g*) in a standard microcentrifuge for 5 minutes to remove cellular debris, genomic DNA, and large RNA. Transfer the supernatant to a clean 2-ml Collection Tube (provided). Do not disturb the pellet.
Note: If the pellet is too loose, centrifuge the sample again for 3-5 minutes, or use a Filtration Column (blue retainer ring) to filter the supernatant. When the starting culture is small, the pellet may not be readily visible. Mark the tube orientation before centrifugation to help avoid the pellet.

5. **Add Ethanol for RNA Binding.** Measure the volume of the clarified lysate with a pipette. Add 1.1 volumes of 100% ethanol to the clarified lysate (For example, add 550 µL of 100% ethanol to 500 µL of the clarified lysate) and mix immediately and thoroughly by vortex or inversion. Increase the amount of ethanol to 1.5 volumes for HeLa cultures. **Do not centrifuge the tube**.
   Note: For most cell lines, such as HEK293, 293T, CHO, K562, Jurkat, A549, and D17, the optimal amount of 100% ethanol to be combined with the clarified lysate is 1.1 volume. However, for some particular cell lines such as HeLa, the optimal amount of the ethanol is 1.5 volumes. You might want to compare these two ethanol volumes to determine which is optimal for your own cell lines.
6. **Bind RNA to Column.** Pipet 700 µL of the mixture into a Binding Column (red retainer ring) and centrifuge at maximum speed (14,000 –16,000 x g) for 30 seconds. Decant the flow-through liquid. Repeat the binding step with the remaining mixture.
7. **First Column Wash.** Add 700 µL of 100% ethanol into the column. Centrifuge at maximum speed (14,000 –16,000 x g) for 30 seconds.
8. **Second Column Wash.** Transfer the Binding Column into a fresh Collection Tube (provided). Add 500 µL of the Ethanol-diluted Wash Solution 2 into the column. Centrifuge at maximum speed (14,000 –16,000 x g) for 30 seconds. Discard the flow-through liquid and return the column to the Collection Tube.
9. **Third Column Wash.** Add another 500 µL of the Ethanol-diluted Wash Solution 2 into the column and centrifuge at maximum speed (14,000 –16,000 x g) for 30 seconds. Discard the flow-through liquid and return the column to the Collection Tube.
10. **Dry Column.** Centrifuge the column at maximum speed (14,000 –16,000 x *g*) for 1 minute to dry. Carefully remove the column-tube assembly from the centrifuge to avoid splashing the residual flow-through liquid to the dried column.
11. **Elute RNA.** Transfer the column to a new 2 ml Collection Tube (provided). Add 50 µL of Elution Solution directly onto the center of the filter inside the column. Reduce the volume of Elution Solution to 20-30 µL if the starting culture is small or if a more concentrated RNA sample is desired. Close the cap and let the tube sit for 1 minute. Centrifuge at maximum speed (14,000 –16,000 x g) for 1 minute to elute. Repeat the elution step by collecting the eluate in a pipette tip and reload the solution directly onto the center of the filter inside the column. Close the cap and let the tube sit for 1 minute. Centrifuge at maximum speed for 1 minute to elute. Purified RNA is now in the flow-through eluate and ready for immediate use or storage at –20 °C (short term) or –70 °C (long term).

II. Small RNA Isolation - Mammalian Tissues

1. **Prepare Lysis Mix.** According to Table 3 below, add microRNA Lysis Buffer and Binding Solution to a clean tube and mix briefly, and then add 100% ethanol and mix briefly. Supplement the solution with 10 µL of 2-mercaptoethanol per 1 ml of the solution. Prepare a volume of the Lysis Mix sufficient for that day's use. Each standard preparation requires 700 µL of the Lysis Mix. It is best to prepare the Lysis Mix just prior to the start of the purification procedure. Keep the Lysis Mix at room temperature before use.

Table 3. Lysis Mix for Mammalian Tissues

Component	Amount/ml Lysis Mix
microRNA Lysis Buffer (M1070)	630 µL
Binding Solution (L8042)	320 µL
100% ethanol	50 µL

2. **Lyse Tissue Samples.** Add 700 µL (for 20-40 mg tissue) or 350 µL (for < 20 mg tissue) of the Lysis Mix to the tissue sample and homogenize the tissue with a rotor-stator homogenizer for 45-50 seconds in moderate speed. Let the sample sit at room temperature for 3-5 minutes. Do not vortex the crude lysate.
   Note: Do not exceed 60 seconds of homogenization. Excessive shearing of DNA might cause DNA contamination in final prep.
3. **Pellet Cellular Debris.** Centrifuge the sample at maximum speed (14,000 –16,000 x *g*) in a standard microcentrifuge for 5 minutes to remove cellular debris, genomic DNA, and large RNA.
4. **Filter Lysate.** Pipet the lysate supernatant into a Filtration Column (blue retainer ring). Avoid the pellet. Centrifuge at maximum speed (14,000 – 16,000 x g) for 30 seconds. Save the clarified flow-through lysate.
5. **Add Ethanol for RNA Binding.** Measure the volume of the clarified lysate with a pipette. Add 1.1 volumes of 100% ethanol to the clarified lysate (For example, add 748 µL of 100% ethanol to 680 µL of the clarified lysate). Mix immediately and thoroughly by vortex or inversion. **Do not centrifuge the tube**.
   Note: Increase the amount of 100% ethanol to 1.5 volumes if process liver tissue or other tissues that may contain a high level of glycogen.
6. **Bind RNA to Column.** Pipet 700 µL of the mixture into a Binding Column (red retainer ring). Centrifuge at maximum speed (14,000 –16,000 x *g*) for 30 seconds. Decant the flow-through liquid. Repeat the binding step with the remaining mixture.
   Note: Liver tissues are rich in glycogen, and a small amount of glycogen may be retained on the top of the red retainer at the centrifugal side after each binding step. If this occurs, switch the tube centrifugation orientation in the following wash steps to facilitate the removal of the glycogen.
7. **First Column Wash.** Add 700 µL of 100% ethanol into the column. Centrifuge at maximum speed (14,000 –16,000 x *g*) for 30 seconds. Discard the flow-through liquid and return the column to the Collection Tube.
8. **Second Column Wash.** Add 500 µL of Binding Solution into the column. Centrifuge at maximum speed (14,000 –16,000 x *g*) for 30 seconds.
   Note: Repeat the Binding Solution wash step if processing pancreatic tissue or any tissue rich in ribonucleases.
9. **Third Column Wash.** Transfer the Binding Column into a fresh Collection Tube (provided). Add 500 µL of the Ethanol-diluted Wash Solution 2 into the column. Centrifuge at maximum speed (14,000 –16,000 x *g*) for 30 seconds. Discard the flow-through liquid and return the column to the Collection Tube.
10. **Fourth Column Wash.** Add another 500 µL of the Ethanol-diluted Wash Solution 2 into the column, and centrifuge at maximum speed (14,000 –16,000 x *g*) for 30 seconds. Discard the flow-through liquid and return the column to the Collection Tube.
11. **Dry Column.** Centrifuge the column at maximum speed (14,000 –16,000 x *g*) for 1 minute to dry. Carefully remove the column-tube assembly from the centrifuge to avoid splashing the residual flow-through liquid to the dried column.
12. **Elute RNA.** Transfer the column to a new 2 ml Collection Tube (provided). Add 50 µL of Elution Solution directly onto the center of the filter inside the column. Reduce the volume of Elution Solution to 20-30 µL if the starting material is small or if a more concentrated RNA sample is desired. Close the cap and let the tube sit for 1 minute. Centrifuge at maximum speed (14,000 –16,000 x *g*) for 1 minute to elute. Repeat the elution step by collecting the eluate in a pipette tip and reload the solution directly onto the center of the filter inside the column. Close the cap and let the tube sit for 1 minute. Centrifuge at maximum speed for 1 minute to elute. Purified RNA is now in the flow-through eluate and ready for immediate use or storage at –20 °C (short term) or –70 °C (long term).

III. Small RNA Isolation - Plant Tissues

1. **Prepare Plant Tissue Samples.** Harvest plant tissue and submerge it in liquid nitrogen as soon as possible to prevent RNA degradation. Grind the tissue to a fine powder in liquid nitrogen using a mortar and pestle. It should be noted that insufficient grinding of tissue would result in reduction in RNA yield. For best practice, place the mortar on dry ice and keep the plant material frozen at all times.
   After liquid nitrogen has evaporated from the frozen tissue powder, quickly weigh approximately 100 mg (90-110 mg) of the tissue powder in a 2-ml microcentrifuge tube (not supplied), pre-chilled on dry ice or in liquid nitrogen. Keep the weighed sample on dry ice or store at –70 °C before RNA purification. Do not exceed 110 mg of tissue powder per tube, especially with difficult-to-process plant tissues, such as pine needles.
   Note: For extremely difficult-to-process plant tissues, such as citrus leaves and old Red Maple leaves that may contain oily and/or gummy material, start with 50-70 mg per tube.
2. **Prepare Lysis Mix.** According to Table 4 below, add microRNA Lysis Buffer and Binding Solution to a clean tube and mix briefly, and then add 100% ethanol and mix briefly. Supplement the solution with 10 µL of 2-mercaptoethanol per 1 ml of the solution. Prepare a volume of the Lysis Mix sufficient for that day's use. Each standard preparation requires 750 µL of the Lysis Mix. It is best to prepare the Lysis Mix just prior to the start of the purification procedure. Keep the Lysis Mix at room temperature before use.

Table 4. Lysis Mix for Plant Tissues

Component	Amount/ml Lysis Mix
microRNA NA Lysis Buffer (M1070)	650 µL
Binding Solution (L8042)	300 µL
100% ethanol	50 µL

Note: The ethanol might be omitted from the Lysis Mix by increasing the microRNA Lysis Buffer to 0.7 volume. This might increase the total yield of small RNA. However, in some plant species, the residual DNA in the final prep may also increase when ethanol is omitted from the Lysis Mix.

3. **Lyse Tissue Samples.** Add 750 µL (for 50-100 mg tissue) or 380 µL (for < 50 mg tissue) of the Lysis Mix to the tissue powder and vortex immediately and thoroughly for 30 seconds. Incubate the sample at 55 °C for 5 minutes. Do not vortex the sample during or after the heat incubation.
   Note: If processing starch storage tissues that will absorb a large amount of liquid, increase the volume of the Lysis Mix to 1 ml per 100 mg of sample and incubate the sample at room temperature instead of 55 °C.
4. **Pellet Cellular Debris.** Centrifuge the sample at maximum speed (14,000 –16,000 x *g*) in a standard microcentrifuge for 5 minutes to remove cellular debris, genomic DNA, and large RNA.
5. **Filter Lysate.** Pipet the lysate supernatant into a Filtration Column (blue retainer ring) and centrifuge at maximum speed (14,000 –16,000 x *g*) for 1 minute to remove residual debris. Save the clarified flow-through lysate.
   Note: If there is a layer of floating material, position the pipette tip below the floating layer and away from the pellet before pipetting the supernatant. Do not be concerned with carry-over of particulates to the Filtration Column, but avoid taking the pellet. If all of the liquid has not passed through the Filtration Column after 1 minute of centrifugation, recentrifuge the column for 2-3 minutes.
6. **Add Ethanol for RNA Binding**. Measure the volume of the clarified lysate with a pipette. Add 1.1 volumes of 100% ethanol to the clarified lysate (For example, add 715 µL of 100% ethanol to 650 µL of the clarified lysate). Mix immediately and thoroughly by vortex or inversion. **Do not centrifuge the tube**.
   Note: The volume of the clarified lysate in a standard preparation may vary greatly from 600 and 700 µL depending on tissue type. Adding excess of ethanol (>1.2 volumes) to the clarified lysate may reduce the small RNA yield.
7. **Bind RNA to Column.** Pipet 700 µL of the mixture into a Binding Column (red retainer ring). Centrifuge at maximum speed (14,000 –16,000 x *g*) for 30 seconds. Decant the flow-through liquid. Repeat the binding step with the remaining mixture.
   Note: Do not be concerned with pigments that may accumulate on the filter surface after each binding step. These pigments will be washed off in the following washing steps.
8. **First Column Wash.** Add 700 µL of 100% ethanol into the column. Centrifuge at maximum speed (14,000 –16,000 x *g*) for 30 seconds. Discard the flow-through liquid and return the column to the Collection Tube.

9. **Second Column Wash.** Add 500 µL of Binding Solution into the column. Centrifuge at maximum speed (14,000 –16,000 x *g*) for 1 minute.
10. **Third Column Wash.** Transfer the Binding Column into a fresh Collection Tube (provided). Add 500 µL of the Ethanol-diluted Wash Solution 2 into the column. Centrifuge at maximum speed (14,000 –16,000 x g) for 30 seconds. Discard the flow-through liquid and return the column to the Collection Tube.
11. **Fourth Column Wash.** Add another 500 µL of the Ethanol-diluted Wash Solution 2 into the column and centrifuge at maximum speed (14,000 –16,000 x g) for 30 seconds. Discard the flow-through liquid and return the column to the Collection Tube.
12. **Dry Column.** Centrifuge the column at maximum speed (14,000 –16,000 x *g*) for 1 minute to dry. Carefully remove the column-tube assembly from the centrifuge to avoid splashing the residual flow-through liquid to the dried column.
13. **Elute RNA.** Transfer the column to a new 2 ml Collection Tube (provided). Add 50 µL of Elution Solution directly onto the center of the filter inside the column. Reduce the volume of Elution Solution to 20-30 µL if the starting material is small or if a more concentrated RNA sample is desired. Close the cap and let the tube sit for 1 minute. Centrifuge at maximum speed (14,000 –16,000 x *g*) for 1 minute to elute. Repeat the elution step by collecting the eluate in a pipette tip and reload the solution directly onto the center of the filter inside the column. Close the cap and let the tube sit for 1 minute. Centrifuge at maximum speed for 1 minute to elute. Purified RNA is now in the flow-through eluate and ready for immediate use or storage at –20 °C (short term) or –70 °C (long term).

IV. Small RNA Isolation - Yeast and Bacterial Cultures

1. Prepare Enzyme Digestion Solution

a. Yeast Enzyme Digestion Solution. For every 100 µL of yeast enzyme digestion solution, combine 90 µL of the Yeast Lysis Solution, Catalog No. Y0253, 10 µL of the Yeast Enzyme Stock Solution, Catalog No. Y0378, and 0.1 µL of 2-mercaptoethanol. Each sample will require 25 µL (for small RNA isolation) or 50 µL (for total RNA isolation) of the enzyme digestion solution.

b. Bacterial Enzyme Digestion Solution. For every 100 µL of bacterial enzyme digestion solution, combine 90 µL of the Bacterial Lysis Solution, Catalog No. B7934, and 10 µL of the Bacterial Enzyme Stock Solution, Catalog No. B7809. Each sample will require 25 µL (for small RNA I isolation) or 50 µL (for total RNA isolation) of the enzyme digestion solution.
Note: For *Staphylococcus* species, supplement the bacterial enzyme digestion solution with 20 units of Lysostaphin, Catalog No. L7386, per 100 µL of the enzyme digestion solution. For *Streptococcus* species, supplement the bacterial enzyme digestion solution with 25 units of Mutanolysin, Catalog No. M9901, per 100 µL of the enzyme digestion solution.

2. Prepare Lysis Mix. According to Table 5 below, add microRNA Lysis Buffer and Binding Solution to a clean tube and mix briefly. Supplement the solution with 10 µL of 2-mercaptoethanol per 1 ml of the solution. Prepare a volume of the Lysis Mix sufficient for that day's use. Each standard preparation requires 700 µL of the Lysis Mix. It is best to prepare the Lysis Mix just prior to the start of the purification procedure. Keep the Lysis Mix at room temperature before use.

Table 5. Lysis Mix for Yeast and Bacterial Cultures

Component	Amount/ml Lysis Mix
microRNA Lysis Buffer (M1070)	800 µL
Binding Solution (L8042)	200 µL

3. Harvest Cells

a. Yeast Cultures. Pellet up to 5 x 10^7 cells in a 2 ml microcentrifuge tube by centrifugation at ≥5,000 x *g* for 5 minutes. Remove the culture medium and recentrifuge the sample for 30 seconds. Remove the last drop of the medium with a pipette tip.

b. Bacterial Cultures. Pellet up to 1 x 10^9 cells in a 2 ml microcentrifuge tube by centrifugation at ≥ 5,000 x *g* for 5 minutes. Remove the culture medium and recentrifuge the sample for 30 seconds. Remove the last drop of the medium with a pipette tip.

4. Digest Cell Walls

a. Yeast Cultures. Add 25 µL of the yeast enzyme digestion solution to the cell pellet and resuspend the pellet by vortex. Let the sample sit for 10 minutes.

b. Bacterial Cultures. Add 25 µL of the bacterial enzyme digestion solution to the cell pellet and resuspend the pellet by vortex. Let the sample sit for 10 minutes.
Note: For gram-negative bacteria, such as *E. coli*, the enzyme digestion step can be omitted and the cell pellet can be lysed directly with the Lysis Mix.

5. Lyse Cells. Add 700 µL of the Lysis Mix to the digested sample and mix immediately but gently by inversion. Let the sample sit for ≥ 5 minutes and mix it gently 2-3 times in between. Do not vortex the crude lysate.

6. Remove Cellular Debris. Centrifuge the sample at maximum speed (14,000 –16,000 x *g*) for 5 minutes to remove cellular debris, genomic DNA, and large RNA.

7. Filter Lysate. Pipet the lysate supernatant into a Filtration Column (blue retainer ring). Avoid the pellet. Centrifuge at maximum speed for 30 seconds. Save the clarified flow-through lysate.

8. Add Ethanol for RNA Binding. Measure the volume of the clarified lysate with a pipette. Add 1.1 volumes of 100% ethanol to the lysate (For example, add 770 µL of 100% ethanol to 700 µL of the lysate). Mix thoroughly by vortex or inversion. **Do not centrifuge the tube**.

9. Bind RNA to Column. Pipet 750 µL of the mixture into a Binding Column (red retainer ring). Centrifuge at maximum speed (14,000 –16,000 x *g*) for 30 seconds. Decant the flow-through liquid. Repeat the binding step with the remaining mixture.

10. First Column Wash. Add 700 µL of 100% ethanol into the column. Centrifuge at maximum speed (14,000 –16,000 x *g*) for 30 seconds. Discard the flow-through liquid and return the column to the Collection Tube.

11. Second Column Wash. Add 500 µL of Binding Solution into the column. Centrifuge at maximum speed (14,000 –16,000 x *g*) for 30 seconds.

12. **Third Column Wash.** Transfer the Binding Column into a fresh Collection Tube (provided). Add 500 µL of the Ethanol-diluted Wash Solution 2 into the column. Centrifuge at maximum speed (14,000 –16,000 x *g*) for 30 seconds. Discard the flow-through liquid and return the column to the Collection Tube.
13. **Fourth Column Wash.** Add another 500 µL of the Ethanol-diluted Wash Solution 2 into the column and centrifuge at maximum speed (14,000-16,000 x *g*) for 30 seconds. Discard the flow-through liquid and return the column to the Collection Tube.
14. **Dry Column.** Centrifuge the column at maximum speed (14,000 –16,000 x *g*) for 1 minute to dry. Carefully remove the column-tube assembly from the centrifuge to avoid splashing the residual flow-through liquid to the dried column.
15. **Elute RNA.** Transfer the column to a new 2 ml Collection Tube (provided). Add 50 µL of Elution Solution directly onto the center of the filter inside the column. Reduce the volume of Elution Solution to 20-30 µL if a more concentrated RNA sample is desired. Close the cap and let the tube sit for 1 minute. Centrifuge at maximum speed (14,000 –16,000 x *g*) for 1 minute to elute. Repeat the elution step by collecting the eluate in a pipette tip and reload the solution directly onto the center of the filter inside the column. Close the cap and let the tube sit for 1 minute. Centrifuge at maximum speed for 1 minute to elute. Purified RNA is now in the flow-through eluate and ready for immediate use or storage at –20 °C (short term) or –70 °C (long term).

V. Total RNA Isolation - Mammalian Cell Cultures

1. **Prepare Lysis Solution/2-ME Mixture.** Transfer the Lysis Solution (L8167) to a clean tube and add 10 µL of 2-mercaptoethanol (2-ME) for every 1 ml of the Lysis Solution and mix briefly. Prepare a volume of the Lysis Solution/2-ME Mixture sufficient for that day's use.

Table 6. Lysis Solution/2-ME Mixture Volume Recommendation

Sample	Number of cells or culture vessel	Lysis Solution/2-ME Mixture volume
Pelleted cells	$< 1 \times 10^6$	100 µL
	$1\text{-}3 \times 10^6$	250 µL
	$4\text{-}7 \times 10^6$	500 µL
Attached cells	12-well plate	150 µL/well
	6-well plate	250 µL/well
	25 cm^2 flask	500 µL/flask

2. **Prepare Cell Samples.** See Step 2 in Small RNA Isolation from Mammalian Cell Cultures.
3. **Lyse Cells**

a. **Pelleted cells.** Vortex the cell pellet 1-2 seconds to loosen cells. Add the Lysis Solution/2-ME Mixture to the cell pellet according to Table 6 and vortex or pipette thoroughly until all clumps disappear. Let the sample sit for ≥ 5 minutes and mix it 2-3 times in between by vortex. Transfer the lysate to a 2-ml microcentrifuge tube (not provided). **Note**: If the cell pellet has been stored at –70 °C, lyse the cells at 55 °C for 5 minutes instead of room temperature.

b. **Attached cells**. Add the Lysis Solution/2-ME Mixture to the culture according to Table 6. Rock the culture vessel while tapping the side for a few seconds to cover the surface with the Lysis Solution/2-ME Mixture. Let the sample sit for ≥ 5 minutes and mix it 2-3 times in between by rocking or swirling. Tilt the culture vessel to one side to collect the lysate to a 2 ml microcentrifuge tube.

4. **Pellet Cellular Debris.** Centrifuge the sample at maximum speed (14,000 –16,000 x *g*) for 3 minutes to remove cellular debris.
5. **Filter Lysate.** Pipet the lysate supernatant into a Filtration Column (blue retainer ring). Avoid the pellet. Centrifuge at maximum speed (14,000 –16,000 x *g*) for 1 minute. Save the clarified flow-through lysate.
6. **Add Binding Solution.** Add 1.5 volumes of Binding Solution to the clarified lysate, based on the volume of the Lysis Solution/2-ME Mixture used to lyse the sample (For example, add 750 µL of Binding Solution to the clarified lysate if 500 µL of the Lysis Solution/2-ME Mixture has been used to prepare the lysate). Mix thoroughly by vortex or inversion. **Do not centrifuge**.
7. **Bind RNA to Column.** Pipet 700 µL of the mixture into a Binding Column (red retainer ring). Centrifuge at maximum speed (14,000 –16,000 x *g*) for 30 seconds. Decant the flow-through liquid. Repeat the binding step with the remaining mixture.
8. **First Column Wash.** Add 700 µL of 100% ethanol into the column. Centrifuge at maximum speed (14,000 –16,000 x *g*) for 30 seconds. Discard the flow-through liquid and return the column to the Collection Tube.
9. **Second Column Wash.** Add 500 µL of Binding Solution into the column. Centrifuge at maximum speed (14,000 –16,000 x *g*) for 1 minute. **Optional**: For on-column DNase I digestion, continue with the procedure described in Appendix.
10. **Third Column Wash.** Transfer the Binding Column into a fresh Collection Tube (provided). Add 500 µL of the Ethanol-diluted Wash Solution 2 into the column. Centrifuge at maximum speed (14,000 –16,000 x *g*) for 30 seconds. Discard the flow-through liquid and return the column to the Collection Tube.
11. **Fourth Column Wash.** Add another 500 µL of the Ethanol-diluted Wash Solution 2 into the column and centrifuge at maximum speed (14,000 –16,000 x *g*) for 30 seconds. Discard the flow-through liquid and return the column to the Collection Tube.
12. **Dry Column.** Centrifuge the column at maximum speed (14,000 –16,000 x *g*) for 1 minute to dry. Carefully remove the column-tube assembly from the centrifuge to avoid splashing the residual flow-through liquid to the dried column.
13. **Elute RNA.** Transfer the column to a new 2 ml Collection Tube (provided). Add 50-100 µL of Elution Solution directly onto the center of the filter inside the column. Close the cap and let the tube sit for 1 minute. Centrifuge at maximum speed (14,000 –16,000 x g) for 1 minute to elute. Repeat the elution step by collecting the eluate in a pipette tip and reload the solution directly onto the center of the filter inside the column. Close the cap and let the tube sit for 1 minute. Centrifuge at maximum speed for 1 minute to elute. Purified RNA is now in the flow-through eluate and ready for immediate use or storage at –20 °C (short term) or –70 °C (long term).

VI. Total RNA Isolation - Mammalian Tissues

1. **Prepare Lysis Solution/2-ME Mixture.** Transfer the Lysis Solution (L8167) to a clean tube and add 10 µL of 2-mercaptoethanol (2-ME) for every 1 ml of the Lysis Solution and mix briefly. Prepare a volume of the Lysis Solution/2-ME Mixture sufficient for that day's use.
2. **Lyse Tissue Samples.** Add 500 µL (for 10-40 mg tissue) or 250 µL (for < 10 mg tissue) of the Lysis Solution/2-ME Mixture to the tissue sample and homogenize the tissue with a rotor-stator homogenizer for 50 - 60 seconds or until no visible pieces remain. Let the sample sit for ≥ 5 minutes.
   Note: If processing spleen, thymus, liver, or any tissue that contain a high content of DNA, do not exceed 20 mg of tissue per 500 µl of the Lysis Solution/2-ME Mixture.
3. **Pellet Cellular Debris.** Centrifuge the sample at maximum speed (14,000 –16,000 x *g*) for 3 minutes to remove cellular debris.
4. **Filter Lysate.** Pipet the lysate supernatant into a Filtration Column (blue retainer ring). Avoid the pellet. Centrifuge at maximum speed (14,000 –16,000 x g) for 1 minute. Save the clarified flow-through lysate.
5. **Add Binding Solution.** Measure the volume of the clarified lysate with a pipette. Add 1.5 volumes of Binding Solution to the clarified lysate (For example, add 675 µL of Binding Solution into 450 µL of the clarified lysate). Mix thoroughly by vortex or inversion. **Do not centrifuge**.
   Note: If processing liver tissue or any tissue that is rich in glycogen, reduce the amount of Binding Solution to 1 volume to avoid clogging the column.
6. **Bind RNA to Column.** Pipet 700 µL of the mixture into a Binding Column (red retainer ring). Centrifuge at maximum speed (14,000 –16,000 x *g*) for 30 seconds. Decant the flow-through liquid. Repeat the binding step with the remaining mixture.
   Note: If all of the liquid has not passed through the column after 1 minute of centrifugation, recentrifuge the column 1-2 minutes.
7. **First Column Wash.** Add 700 µL of 100% ethanol into the column. Centrifuge at maximum speed (14,000 –16,000 x *g*) for 30 seconds. Discard the flow-through liquid and return the column to the Collection Tube.
8. **Second Column Wash.** Add 500 µL of Binding Solution into the column. Centrifuge at maximum speed (14,000 –16,000 x *g*) for 1 minute.
   Note: If the starting material is pancreatic tissue or any tissue highly rich in ribonucleases, repeat the column wash with another 500 µL of Binding Solution.
   For on-column DNase I digestion, continue with the procedure described in Appendix.
9. **Third Column Wash.** Transfer the Binding Column into a fresh Collection Tube (provided). Add 500 µL of the Ethanol-diluted Wash Solution 2 into the column. Centrifuge at maximum speed (14,000 –16,000 x *g*) for 30 seconds. Discard the flow-through liquid and return the column to the Collection Tube.
10. **Fourth Column Wash.** Add another 500 µL of the Ethanol-diluted Wash Solution 2 into the column and centrifuge at maximum speed (14,000 –16,000 x *g*) for 30 seconds. Discard the flow-through liquid and return the column to the Collection Tube.
11. **Dry Column.** Centrifuge the column at maximum speed (14,000 –16,000 x *g*) for 1 minute to dry. Carefully remove the column-tube assembly from the centrifuge to avoid splashing the residual flow-through liquid to the dried column.
12. **Elute RNA.** Transfer the column to a new 2 ml Collection Tube (provided). Add 50-100 µL of Elution Solution directly onto the center of the filter inside the column. Close the cap and let the tube sit for 1 minute. Centrifuge at maximum speed (14,000 –16,000 x g) for 1 minute to elute. Repeat the elution step by collecting the eluate in a pipette tip and reload the solution directly onto the center of the filter inside the column. Close the cap and let the tube sit for 1 minute. Centrifuge at maximum speed for 1 minute to elute. Purified RNA is now in the flow-through eluate and ready for immediate use or storage at –20 °C (short term) or –70 °C (long term).

VII. Total RNA Isolation - Plant Tissues

1. **Prepare Plant Tissue Samples.** See Step 1 in Small RNA Isolation from Plant Tissues.
2. **Prepare Lysis Solution/2-ME Mixture.** Transfer the Lysis Solution (L8167) to a clean tube and add 10 µL of 2-mercaptoethanol (2-ME) for every 1 ml of the Lysis Solution and mix briefly. Prepare a volume of the Lysis Solution/2-ME Mixture sufficient for that day's use.
3. **Lyse Tissue Sample.** Add 500 µL (for 50 –100 mg tissue) or 250 µL (for < 50 mg tissue) of the Lysis Solution/2-ME Mixture to the tissue powder and vortex immediately and thoroughly for 30 seconds. Incubate the sample at 55 °C for 5 minutes. Do not vortex the sample during or after the heat incubation.
   Note: If processing starch storage tissues that will absorb a large amount of liquid, increase the volume of the Lysis Solution/2-ME Mixture to 750 µL per 100 mg of sample and incubate the sample at room temperature instead of 55 °C.
4. **Pellet Cellular Debris.** Centrifuge the sample at maximum speed (14,000 –16,000 x *g*) for 3 minutes to pellet cellular debris.
   Note: Lipid storage tissues may produce a viscous lysate and may require longer centrifugation for pelleting the cellular debris.
5. **Filter Lysate.** Pipet the lysate supernatant into a Filtration Column (blue retainer ring) and centrifuge at maximum speed (14,000 –16,000 x *g*) for 1 minute to remove residual debris. Save the clarified flow-through lysate.
   Note: If there is a layer of floating particulates, position the pipette tip below the floating layer and away from the pellet before pipetting the supernatant. Do not be concerned with carry-over of the floating particulates to the Filtration Column, but avoid taking the pellet. If all of the liquid has not passed through the Filtration Column after 1 minute of centrifugation, recentrifuge the column for 2-3 minutes.
6. **Add Binding Solution.** Measure the volume of the clarified lysate with a pipette and add 1.5 volumes of Binding Solution to the clarified lysate (For example, add 675 µL of Binding Solution into 450 µL of the clarified lysate). Mix thoroughly by vortex or inversion. **Do not centrifuge**.
   Note: The volume of the clarified lysate may vary greatly from tissue to tissues. If the starting material is rich in starch or if the clarified lysate is viscous, reduce the amount of Binding Solution to 1 volume.
7. **Bind RNA to Column.** Pipet 700 µL of the mixture into a Binding Column (red retainer ring). Centrifuge at maximum speed (14,000 –16,000 x *g*) for 30 seconds. Decant the flow-through liquid. Repeat the binding step with the remaining mixture.
   Note: If all of the liquid has not passed through the column after 30 seconds of centrifugation, recentrifuge the column 1-2 minutes.
8. **First Column Wash.** Add 700 µL of 100% ethanol into the column. Centrifuge at maximum speed (14,000 –16,000 x *g*) for 30 seconds. Discard the flow-through liquid and return the column to the Collection Tube.
9. **Second Column Wash.** Add 500 µL of Binding Solution into the column. Centrifuge at maximum speed (14,000 –16,000 x *g*) for 1 minute.
   Optional: For on-column DNase I digestion, continue with the procedure described in Appendix.
10. **Third Column Wash.** Transfer the Binding Column into a fresh Collection Tube (provided). Add 500 µL of the Ethanol-diluted Wash Solution 2 into the column. Centrifuge at maximum speed (14,000 –16,000 x *g*) for 30 seconds. Discard the flow-through liquid and return the column to the Collection Tube.
11. **Fourth Column Wash.** Add another 500 µL of the Ethanol-diluted Wash Solution 2 into the column and centrifuge at maximum speed (14,000 –16,000 x *g*) for 30 seconds. Discard the flow-through liquid and return the column to the Collection Tube.
12. **Dry Column.** Centrifuge the column at maximum speed (14,000 –16,000 x *g*) for 1 minute to dry. Carefully remove the column-tube assembly from the centrifuge to avoid splashing the residual flow-through liquid to the dried column.
13. **Elute RNA.** Transfer the column to a new 2 ml Collection Tube (provided). Add 50-100 µL of Elution Solution directly onto the center of the filter inside the column. Close the cap and let the tube sit for 1 minute. Centrifuge at maximum speed (14,000 –16,000 x g) for 1 minute to elute. Repeat the elution step by collecting the eluate in a pipette tip and reload the solution directly onto the center of the filter inside the column. Close the cap and let the tube sit for 1 minute. Centrifuge at maximum speed for 1 minute to elute. Purified RNA is now in the flow-through eluate and ready for immediate use or storage at –20 °C (short term) or –70 °C (long term).

VIII. Total RNA Isolation - Yeast and Bacterial Cultures

1. **Prepare Lysis Solution/2-ME Mixture.** Transfer the Lysis Solution (L8167) to a clean tube and add 10 µL of 2-mercaptoethanol (2-ME) for every 1 ml of the Lysis Solution and mix briefly. Prepare a volume of the Lysis Solution/2-ME Mixture sufficient for that day's use.
2. **Prepare Enzyme Digestion Solution.** See Step 1 in Small RNA Isolation from Yeast and Bacterial Cultures.
3. **Harvest Cells.** See Step 3 In Small RNA Isolation from Yeast and Bacterial Cultures.
4. **Digest Cell Walls**

a. **Yeast Cultures.** Add 50 µL of the yeast enzyme digestion solution to the cell pellet and resuspend the pellet by vortex. Let the sample sit for 10 minutes.

b. **Bacterial Cultures.** Add 50 µL of the bacterial enzyme digestion solution to the cell pellet and resuspend the pellet by vortex. Let the sample sit for 10 minutes.

5. **Lyse Cells.** Add 500 µL of the Lysis Solution/2-ME Mixture to the digested sample and mix thoroughly by vortex or inversion. Let the sample sit for ≥ 5 minutes.
6. **Pellet Cellular Debris.** Centrifuge the sample at maximum speed (14,000 –16,000 x *g*) for 3 minutes to remove cellular debris.
7. **Filter Lysate.** Pipet the lysate supernatant into a Filtration Column (blue retainer ring). Centrifuge at maximum speed (14,000 –16,000 x *g*) for 30 seconds. Save the clarified flow-through lysate.
8. **Add Binding Solution.** Add 750 µL (about 1.5 volumes of the clarified lysate) of Binding Solution to the clarified lysate. Mix thoroughly by vortex or inversion. **Do not centrifuge**.
9. **Bind RNA to Column.** Pipet 700 µL of the mixture into a Binding Column (red retainer ring). Centrifuge at maximum speed (14,000 –16,000 x *g*) for 30 seconds. Decant the flow-through liquid. Repeat the binding step with the remaining mixture.
10. **First Column Wash.** Add 700 µL of 100% ethanol into the column. Centrifuge at maximum speed (14,000 –16,000 x *g*) for 30 seconds. Discard the flow-through liquid and return the column to the Collection Tube.
11. **Second Column Wash.** Add 500 µL of Binding Solution into the column. Centrifuge at maximum speed (14,000 –16,000 x *g*) for 1 minute. **Optional**: For on-column DNase I digestion, continue with the procedure described in Appendix.
12. **Third Column Wash.** Transfer the binding column into a fresh Collection Tube (provided). Add 500 µL of the Ethanol-diluted Wash Solution 2 into the column. Centrifuge at maximum speed (14,000 –16,000 x *g*) for 30 seconds. Discard the flow-through liquid and return the column to the Collection Tube.
13. **Fourth Column Wash.** Add another 500 µL of the Ethanol-diluted Wash Solution 2 into the column and centrifuge at maximum speed (14,000 –16,000 x *g*) for 30 seconds. Discard the flow-through liquid and return the column to the Collection Tube.
14. **Dry Column.** Centrifuge the column at maximum speed (14,000 –16,000 x *g*) for 1 minute to dry. Carefully remove the column-tube assembly from the centrifuge to avoid splashing the residual flow-through liquid to the dried column.
15. **Elute RNA.** Transfer the column to a new 2 ml Collection Tube (provided). Add 50-100 µL of Elution Solution directly onto the center of the filter inside the column. Close the cap and let the tube sit for 1 minute. Centrifuge at maximum speed (14,000 –16,000 x g) for 1 minute to elute. Repeat the elution step by collecting the eluate in a pipette tip and reload the solution directly onto the center of the filter inside the column. Close the cap and let the tube sit for 1 minute. Centrifuge at maximum speed for 1 minute to elute. Purified RNA is now in the flow-through eluate and ready for immediate use or storage at –20 °C (short term) or –70 °C (long term).

Results

Analysis of RNA

The concentration and quality of the RNA prepared can be determined by spectrophotometric analysis and agarose gel electrophoresis. The RNA sample can be measured directly on a NanoDrop instrument with a very small volume (1-2 μL) of the sample. For conventional spectrophotometers, dilute the RNA sample in TE buffer (10 mM Tris-HCl, 1 mM EDTA, pH 7-8) and measure the absorbance at 260, 280, and 320 nm. For best results, absorbance readings should be between 0.1 and 1.0 absorbance units (or within the linear range of the spectrophotometer). An absorbance of 1.0 at 260 nm corresponds to 40 μg/ml of RNA. The ratio of absorbance at 260 to 280 nm, calculated by $(A_{260} – A_{320})/(A_{280} – A_{320})$, is typically between 1.8 and 2.2.

RNA quality can be evaluated by gel electrophoresis, analysis on an Agilent Bioanalyzer, or by capillary electrophoresis. In small RNA preparation, tRNAs and 5S and 5.8S rRNAs should appear as discrete bands or peaks on 4% agarose gel or polyacrylamide gel. In total RNA preparation, the 23-28S (depending on species) large subunit ribosomal RNA and 16-18S (depending on species) small subunit ribosomal RNA should appear as discrete bands or peaks. In addition, there might be some minor bands or peaks of chloroplast and mitochondria ribosomal RNAs.

Expected Yield

The yield of RNA varies with tissue types, growth conditions, and developmental stages. In general, younger and more rapidly growing cells or tissues will contain more RNA. Plant root, stem, and starch storage organ generally contain less RNA. Approximately 1-2 μg of small RNA and 10-25 μg of total RNA per million mammalian cultured cells have been obtained with the mirPremier microRNA Isolation Kit.

Yields of 3-5 μg small RNA and 30-50 μg total RNA per 10 mg of tissue have been obtained from mouse liver. Yields of 5-15 μg small RNA and 40-100 μg total RNA per 100 mg of tissue have been obtained from pine needle, grape leaf, and tomato leaf. Yields of 6-8 μg small RNA and 50-70 μg total RNA have been obtained from $5x10^7$ yeast cells. Yields of 5-6 μg small RNA and 30-35 μg total RNA have been obtained from $1x10^9$ *E. coli* cells.

Appendix

On-Column DNase I Digestion

For total RNA preparation, significantly lower levels of residual DNA can be achieved by digestion with the On-Column DNase I Digestion Set, Catalog Numbers DNASE10 and DNASE70, as described below. For the most stringent removal of residual DNA, treatment of RNA preparations with DNase I, Amplification Grade, Catalog No. AMPD1, is recommended. On-column DNase I digestion is not recommended for small RNA preparation, because a significant amount of small RNAs may be released from the filter under the conditions used for digestion. However, small RNA preparation can be treated with Amplification Grade DNase I to remove residual DNA if desired.

1. After the Binding Column has been washed with the Binding Solution as described in the procedure. Transfer the Binding Column to a fresh Collection Tube.
2. Combine 10 μL of DNase I, Catalog No. D2816, with 70 μL of DNase I Digestion Buffer, Catalog No. D1566, for each preparation and mix gently by pipetting. Do not vortex the DNase I vial or the mixture of DNase I and DNase I digestion buffer. DNase I is sensitive to physical denaturation.
3. Add 80 μL of the DNase I/Digest Buffer mixture directly onto the center of the filter inside the Binding Column. Close the cap and incubate the sample at room temperature for 15 minutes.
4. Add 500 μL of Binding Solution into the column and centrifuge at maximum speed (14,000 –16,000 x *g*) for 30 seconds. Discard the flow-through liquid and return the column to the Collection Tube.
   Note: Reduce the volume of the Binding Solution for column washing in this step to 250 μL to conserve the reagent for other preps if the Binding Column has been washed twice with the Binding Solution, as is recommended for RNase-rich tissues, prior to the on-column DNase I digestion.
5. Continue with the washing step with the Ethanol-diluted Wash Solution 2 as described in the procedure.

Troubleshooting Guide

Problem	Cause	Solution
Filtration Column is clogged.	Cellular debris pellet is transferred to the Filtration Column.	Avoid pipetting cellular debris pellet into the Filtration Column. If the pellet is too loose, repeat centrifugation for 3-5 minutes. If all of the liquid has not passed through the Filtration Column after first centrifugation, recentrifuge the column for 3-5 minutes.
	Plant sample is not heated at 55 °C during lysis.	Lyse the plant material at 55 °C for 5 minutes. Do not vortex the sample during or after the heat incubation.
	Starch rich plant sample is heated at 55 °C.	Lyse starch rich plant material at room temperature instead of 55 °C. Increase the volume of the Lysis Mix (small RNA isolation) or the Lysis Solution/2-ME Mixture (total RNA isolation) to lyse the sample.
	Plant material contains high level of lipids.	Increase the centrifugation time to 5-10 minutes to pellet the cellular debris and avoid transferring the floating layers of lipid materials into the Filtration Column. Increase the volume of the Lysis Mix (small RNA isolation) or the Lysis Solution/2-ME Mixture (total RNA isolation) to lyse the sample.
Binding Column is clogged.	Polysaccharides or plant fibers form aggregates after ethanol is added to the clarified lysate.	Do not transfer the aggregates into the Binding Column. Use a pipette tip to remove the aggregates before transferring the mixture to the Binding Column.
	The binding mixture is too viscous.	Reduce the Binding Solution to 1 volume of the clarified lysate when isolating total RNA from liver tissues or starch rich plant tissues. For future preparations, reduce the amount of the starting material. To salvage the current preparation, recentrifuge the column for 2-3 minutes. If clogging persists, use a pipette tip to remove the clogged liquid before proceeding to the wash step. Yield of RNA will be likely reduced.
Low yield or RNA degraded	The amount of 100% ethanol added to the clarified lysate is not optimal	For most starting materials, the optimal amount of 100% ethanol to be combined with the clarified lysate is 1.1 volume, and too much ethanol might result in reduction in RNA yield. However, for some starting materials, such as liver tissues and HeLa cultures, the optimal amount of the ethanol is 1.5 volumes. Compare these two ethanol volumes with the same starting material to determine which is optimal for your cell lines.
	Starting cells or tissues contain small amount of RNA	Yields will vary greatly between different types of cells or tissues. Older root, stem, and tuber generally contain a small amount of total RNA.
	Tissue or culture is too old.	Use cultures before they reach maximum density or become fully confluent, and harvest tissues as rapidly as possible.
	Too much starting material is used.	Do not exceed 110 mg of plant tissue powder per tube. For extremely difficult-to-process tissues, such as citrus leaf and old Red Maple leaves, start with 50-70 mg of plant tissue powder per tube. RNA yield will be greatly reduced or no RNA will be recovered if more than 110 mg of difficult-to-process plant material or more than 50-70 mg of extremely difficult-to-process plant material is used per standard isolation.
	Cells or tissues are insufficiently disrupted.	For small RNA isolation, mix the Lysis Mix with the cell pellet thoroughly but gently; lyse the sample at 55 °C if the cell pellet has been stored at –70 °C. For total RNA isolation, mix the Lysis Solution/2-ME Mixture with the cell pellet thoroughly by vortex until no cell clumps remain and lyse the sample at 55 °C if the cell pellet has been stored at –70 °C; homogenize tissues until no visible pieces remain. Grind plant tissue to a fine powder in liquid nitrogen. RNA yields are highly dependent on how well the tissue has been ground prior to RNA isolation.
	Starting material is too small.	Adjust the volume of the Lysis Mix (small RNA isolation) or the Lysis Solution/2-ME Mixture (total RNA isolation) according to the amount of the starting material.
	Elution Solution is inappropriately dispensed into the column.	Pipet Elution Solution directly onto the center of the filter surface inside the Binding Column. Let the solution absorb into the filter for 1-2 minutes before centrifugation. Repeat the elution step by reloading the eluate to the column

Troubleshooting Guide (continued)

Problem	Cause	Solution
Low yield or RNA degraded (continued)	Tissues are rich in RNases.	Tissues that are rich in RNases require immediate and thorough disruption in lysis solution in the presence of 2-mercaptoethanol. Wash the Binding Column twice each with 500 µL of Binding Solution.
	Improper storage.	Flash-freeze tissues in liquid nitrogen and store at –70 °C. Do not allow frozen tissues to thaw before they are disrupted in lysis solution in the presence of 2-mercaptoethanol.
Unacceptable level of residual DNA in purified RNA	Optional DNase I treatment is omitted.	Treat samples with the optional on-column DNase I digestion with the On-Column DNase I Digestion Set. See Appendix. Alternatively, treat the eluted RNA with Amplification Grade DNase I.
	Binding Column is overloaded.	For future preparations, use fewer cells or smaller tissue samples. Increase the volume of the Lysis Mix (small RNA isolation) or the Lysis Solution/2-ME Mixture proportionally as the amount of starting material increases.
	Excessive shearing of DNA in small RNA preparation	Avoid vortexing the cell pellet excessively in the Lysis Mix. Limit the tissue homogenization time to 40-50 seconds in moderate speed. Do not vortex the crude lysate after the cells have been lysed.
	Wrong lysis reagent is used	Use microRNA Lysis Buffer (M1070) for small RNA preparation. Use Lysis Solution (L8167) for total RNA preparation. Do not switch the solutions.
Downstream applications inhibited	Residual ethanol in eluate.	After the column-drying step, carefully remove the column-tube assembly from centrifuge to avoid splashing the residual flow-through liquid to the dried column. If the residual flow-through liquid does accidentally contact the dried column, recentrifuge the column again for 30 seconds before proceeding to the elution step.
	Residual salt in eluate.	Residual guanidine salt will inhibit enzymes. Transfer the Binding Column to a new Collection Tube (provided) before the wash with the Ethanol-diluted Wash Solution 2. Be sure to wash the column twice with 500 µL of the Ethanol-diluted Wash Solution 2.

References

1. Ausubel, F.M. et al., Current Protocols in Molecular Biology, John Wiley & Sons, NY, sections 4.1-4.10 (1995).
2. Farrell, Robert E., Jr. RNA Methodologies, 2nd Edition, Academic Press, NY, pp. 37-53 (1998).
3. Sambrook, J. et al. Molecular Cloning: A Laboratory Manual, 2nd ed., Cold Spring Harbor Laboratory Press, Plainview, NY, pp. 7.3-7.5 (1989).

Related Products

Deoxynucleotide (dNTP) Mix, Catalog No. D7295
Enhanced Avian Reverse Transcriptase, Catalog No. A4464
Ethidium bromide solution, 10 mg/ml, Catalog No. E1510
Luria Broth, Catalog No. L3522
MOPS-EDTA-Sodium Acetate Buffer, Catalog No. M5755
PerfectHyb Plus™ Hybridization Buffer, catalog No. H7033
Precast Agarose Gels, 1.25%, 8 well , Catalog No. P6222
RNA Sample Loading Buffers, Catalog Nos. R1386, R4268
RNaseZAP®, Catalog No. R2020
Taq DNA Polymerase, Catalog No. D1806
Transcript RNA Markers, 0.2-10 kb, Catalog No. R7020
Trypsin-EDTA solution (1x), Catalog No. T3924
YPD Broth, Catalog No. Y1375

JX,GS,PHC,NC, 03/10-1