AxyPrep Multisource Total RNA Midiprep Kit

For the purification of total RNA from animal tissues, plant tissues, cultured cells, bacteria, yeast and filamentous fungi

Kit contents, storage and stability

Cat. No.	AP-MD-MS-RNA-10	AP-MD-MS-RNA-25
Kit size	10 preps	25 preps
Lysate Filtration column	10	25
Midiprep RNA column	10	25
1.5 ml microfuge tube	20	50
Plastic wrench	1	1
Buffer R-I	50 ml	125 ml
Buffer R-II	20 ml	50 ml
Buffer W1A concentrate	48 ml	96 ml
Buffer W2 concentrate	48 ml	2 x 72 ml
Buffer TE (nuclease-free)	6 ml	10 ml
Protocol manual	1	1

All buffers are stable for a period of at least 12 months from the date of receipt when stored under ambient conditions. Please avoid exposure to direct sunlight or extremes in temperature.
Axygen Biosciences warrants the performance of this kit for a period of 12 months from the date of receipt when stored under the conditions specified.

Lysate Filtration column: Store at room temperature.
Midiprep RNA column: Store at room temperature.
Buffer R-I: Cell lysis buffer. Store at room temperature.
Buffer R-II: Neutralization buffer. Store at room temperature.
Buffer W1A concentrate: Wash buffer. Before use of the kit, add the amount of ethanol specified on the bottle label. Either 100% or 95% denatured ethanol can be used. Mix well and store at room temperature.
Buffer W2 concentrate: Desalting buffer. Before use of the kit, add the amount of ethanol specified on the bottle label. Either 100% or 95% denatured ethanol can be used. Mix well and store at room temperature.
Buffer TE (nuclease-free): Eluent. Contains 10 mM Tris-Cl and 0.1 mM EDTA, pH 7.5. Store at room temperature.

Introduction

AxyPrep Multisource Total RNA Midiprep Kit represents a new approach for total cellular RNA purification, which is designed to eliminate the problems associated with other column-type RNA kits, such as clogged columns and incomplete purification. Tissues and cells are first lysed by Cell Lysis Buffer R-I, which also inactivates any indigenous RNase activity. Proteins and genomic DNA are then precipitated by the addition of Neutralization Buffer R-II to the cell lysate. The Lysate Filtration column removes any remaining particulates and contains special media to remove residual traces of contaminating proteins. After addition of isopropanol to the supernatant, the total RNA is then bound to membranes within a detachable module at the end of the Midiprep RNA column for further washing and desalting. Highly purified, full-length total cellular RNA is then eluted in a small volume of TE (or DEPC-treated water) and is ready for use in any downstream application.* Each Midiprep RNA column has sufficient capacity to bind up to 0.8 mg of total cellular RNA. Yields will vary, depending upon amount and type of starting materials, lysis methods and efficiency, etc.

**Some applications may require the use of the AxyPrep RNase-free DNase Digestion kit to remove residual amounts of genomic DNA.*

Caution

Buffer R-I and Buffer W1A contain chemical irritants. When working with these buffers, always wear protective clothing such as safety glasses, gloves and laboratory coat. Be careful to avoid contact with eyes and skin. In the case of such contact, wash immediately with water. If necessary, seek medical assistance.

Equipment and consumables required

- Centrifuge capable of 8,000xg
- Fixed angle rotor to accommodate 15 ml centrifuge tubes
- Nuclease-free 15 ml centrifuge tubes (capable of centrifugation at 8,000xg)
- Microcentrifuge capable of 12,000xg
- Mortar and pestle
- Homogenizer (Dounce-type or motorized) optional
- Plastic syringe and 21-25-gauge syringe needle (see specific prep requirements)
- AxyVac Vacuum Manifold (#AP-VM) or other vacuum manifold
- Vacuum regulator
- Vacuum source (capable of –25-30 inches Hg)
- Liquid nitrogen
- 95-100% ethanol
- Isopropanol

Preparation before experiment

1). Before using the kit, add the amounts of 95-100% ethanol to the Buffer W1A concentrate and the Buffer W2 concentrate, specified on the bottle labels and mix well.

2). Use DEPC-treated materials whenever practical.

Homogenization Methods

Depending upon the starting material and individual preference, different methods can be employed to achieve physical disruption of the source material, cell lysis and shearing of the genomic DNA. These methods include: mortar and pestle, manual Dounce-type homogenizer, motorized rotor-stator homogenizer, etc. The specific method selected to achieve physical disruption is left to the individual preference of the end user. Physical disruption and homogenization will either occur simultaneously (homogenizers) or successively (mortar and pestle), depending upon the method selected. Homogenization occurs in the presence of Buffer R-I. During homogenization, the individual cells are lysed, releasing their contents. Homogenization also inactivates nucleases and shears the genomic DNA, reducing the viscosity of the lysate (homogenate). Shearing the genomic DNA and reducing the lysate viscosity is important to achieving optimal RNA yield and purity.

The protocols provided utilize a mortar and pestle, followed by the use of a syringe needle to achieve complete homogenization, obviating the requirement for special equipment. In the event that a homogenizer (either manual or motorized) is used, please follow the manufacturer's recommendations for achieving complete homogenization of the starting material. When using a homogenizer, freshly harvested tissue will generally have to be minced into small pieces in order to rapidly achieve complete homogenization and thoroughly nullify nuclease activity and preserve the integrity of the RNA.

IMPORTANT: The purification and handling of RNA requires particular attention to cleanliness to avoid contamination of work surfaces and laboratory equipments with nucleases. Please follow generally recommended practices for maintaining a nuclease-free work environment.

IMPORTANT: Grossly overloading the Midiprep RNA columns with excessive RNA will often result in significantly diminished yields and purity. Please follow all guidelines for amounts of starting material.

I. Purification of Total RNA from Animal Tissues

Animal tissues can be efficiently disrupted by any one of the following methods:

- Mortar and pestle (tissue is freshly harvested and flash-frozen with liquid nitrogen)
- Dounce-type homogenizer (freshly harvested tissue, minced)
- Motorized homogenizer (freshly harvested tissue, minced)

In addition to physically disrupting the tissue, subsequent homogenization is important to shear the genomic DNA and achieve complete release of the cellular RNA. Throughout these protocols, homogenization is achieved by passing the lysate several times through a syringe needle. Shearing the genomic DNA reduces the viscosity of the lysate and results in higher yields and purity of the total cellular RNA. When using a manual or motorized homogenizer, the freshly harvested tissue should be quickly minced on ice to increase the efficiency of the homogenization process. Freshly harvested tissue can also be flash-frozen with liquid nitrogen and pulverized using a mortar and pestle before using a homogenizer.

Please use the following guidelines:

RNA-rich tissues (e.g., liver)	use up to 300 mg
RNA-poor tissues (e.g., muscle)	use up to 500 mg
When processing <100 mg tissue	reduce R-I, R-II and isopropanol volumes by half
When processing >300 mg tissue	increase R-I, R-II and isopropanol volumes proportionally

1. Select 30-300 mg of freshly harvested animal tissue and immediately flash-freeze by immersion in liquid nitrogen. Place a pestle into a mortar and freeze by adding liquid nitrogen to the mortar. Transfer the frozen tissue to the mortar and rapidly and vigorously grind to a finely pulverized powder. Depending upon the rapidity with which the tissue is pulverized, it may be necessary to add small amounts of liquid nitrogen intermittently so that the tissue remains frozen.
   IMPORTANT: The tissue must remain frozen before and during grinding to prevent enzymatic degradation of the RNA.

2. Add 4 ml of Buffer R-I and continue to grind the tissue until the pulverized tissue and buffer are completely mixed. Quickly homogenize the sample by passing it 8-10x through a 10 ml syringe fitted with a 21-25-gauge needle. Be careful to minimize foaming. Transfer the homogenate to a nuclease-free 15 ml centrifuge tube (not provided).
   Note: Thorough homogenization of the lysate is essential for high yield and purity of the RNA. The fully homogenized lysate should flow easily dropwise through the syringe needle. A viscous, stringy lysate indicates incomplete shearing of the genomic DNA and further homogenization is required.

3. Add 1.5 ml of Buffer R-II and vortex for 15-30 seconds. Centrifuge at 8,000xg for 10 minutes at 4°C to pellet DNA and protein.
4. Transfer the supernatant from Step 3 into the Lysate Filtration column. Carefully insert the plunger into the Lysate Filtration column and push with a slow, steady motion. Discharge the filtrate into a nuclease-free15 ml centrifuge tube. Add 2.5 ml of isopropanol to the 15 ml centrifuge tube and mix by vortexing.
   Note: The supernatant should be carefully removed without disturbing the pellet. The transfer of pellet material may result in column clogging and contamination of the RNA with DNA and protein.

5. Attach the vacuum manifold base to a vacuum source. Use the plastic wrench to tighten the binding module(s) on the bottom of the Midiprep RNA column(s). Firmly position the Midiprep RNA column(s) into the complementary fittings on the manifold top. Transfer the binding solution from Step 4 to the Midiprep RNA column. Turn on the vacuum source and adjust to -25 inches Hg. Continue to apply the vacuum until no solution remains in the Midiprep RNA column. The RNA will bind to membranes within the binding module on the end of the Midiprep RNA column.
   IMPORTANT: The binding module on the bottom of the Midiprep RNA column must be fully seated before loading the lysate onto the column. Use the plastic wrench to tighten the module before proceeding.
   Note: -25 inches Hg is equivalent to -850-1,000 mbar or -12-15 psi.

6. Add 5 ml of Buffer W1A and draw all of the solution through the Midiprep RNA column.
   Note: Make sure that ethanol has been added into Buffer W1A concentrate. Make a notation on the bottle label for future reference.

7. Add 7 ml of Buffer W2 along the wall of Midiprep RNA column to wash off residual Buffer W1A and draw all of the solution through the column. Repeat this wash step with a second 7 ml aliquot of Buffer W2.
   Note: Make sure that ethanol has been added into Buffer W2 concentrate. Make a notation on the bottle label for future reference.

8. Use the plastic wrench to detach the binding module from the Midiprep RNA column assembly and place it into a nuclease-free 1.5 ml microfuge tube (provided). Centrifuge at 12,000xg for 1 minute to remove residual wash solution.
9. Transfer the binding module to a fresh 1.5 ml microfuge tube (provided). To elute the total RNA, add 300 μl of Buffer TE (nuclease-free) to the center of the membrane. Let it stand for 1 minute at room temperature. Centrifuge at 12,000xg for 1 minute to collect the purified RNA sample.

Purification of Total RNA from Plant Tissues

Plant tissues can be efficiently disrupted by any one of the following methods:

- Mortar and pestle (tissue is freshly harvested and flash-frozen with liquid nitrogen)
- Dounce-type homogenizer (freshly harvested tissue, minced)
- Motorized homogenizer (freshly harvested tissue, minced)

In addition to physically disrupting the tissue, subsequent homogenization is important to shear the genomic DNA and achieve complete release of the cellular RNA. Throughout these protocols, homogenization is achieved by passing the lysate several times through a syringe needle or with a manual or motorized homogenizer. Shearing the genomic DNA reduces the viscosity of the lysate and results in higher yields and purity of the total cellular RNA. Due to the often fibrous nature of plant tissues, it is important to mince the starting material to increase the efficiency of manual or motorized homogenizers. Alternatively, freshly harvested plant tissues can be flash-frozen in liquid nitrogen and then pulverized with a mortar and pestle before homogenization.

Please use the following guidelines:

For leaf tissue	routinely process 80-500 mg
For fibrous tissue (stems, etc.)	routinely process 150-750 mg
When processing <200 mg leaf tissue	reduce R-I, R-II and isopropanol volumes by half
When processing >400 mg leaf tissue	increase R-I, R-II and isopropanol volumes proportionally
When processing >500 mg fibrous tissue	increase R-I, R-II and isopropanol volumes proportionally

1. Select 80-500 mg of freshly harvested animal tissue and immediately flash freeze by immersion in liquid nitrogen. Place a pestle into a mortar and freeze by adding liquid nitrogen to the mortar. Transfer the frozen tissue to the mortar and rapidly and vigorously grind to a finely pulverized powder. Depending upon the rapidity with which the tissue is pulverized, it may be necessary to add small amounts of liquid nitrogen intermittently so that the tissue remains frozen.

   IMPORTANT: The tissue must remain frozen before and during grinding to prevent enzymatic degradation of the RNA.

2. Add 4 ml of Buffer R-I and continue to grind the tissue until the pulverized tissue and buffer are completely mixed. Quickly homogenize the sample by passing it 8-10x through a 10 ml syringe fitted with a 18-23-gauge needle. Be careful to minimize foaming. Transfer the homogenate to a nuclease-free 15 ml centrifuge tube (not provided).

   Note: Thorough homogenization of the lysate is essential for high yield and purity of the RNA. The fully homogenized lysate should flow easily dropwise through the syringe needle. A viscous, stringy lysate indicates incomplete shearing of the genomic DNA and further homogenization is required.

3. Add 1.5 ml of Buffer R-II and vortex for 15-30 seconds. Centrifuge at 8,000xg for 10 minutes at 4°C to pellet the DNA and protein.
4. Transfer the supernatant from Step 3 into the Lysate Filtration column. Carefully insert the plunger into the Lysate Filtration column and push with a slow, steady motion. Discharge the filtrate into a nuclease-free 15 ml centrifuge tube. Add 2.5 ml of isopropanol to the 15 ml centrifuge tube and mix by vortexing.

   Note: The supernatant should be carefully removed without disturbing the pellet. The transfer of pellet material may result in column clogging and contamination of the RNA with DNA and protein.

5. Attach the vacuum manifold base to a vacuum source. Use the plastic wrench to tighten the binding module(s) on the bottom of the Midiprep RNA column(s). Firmly position the Midiprep RNA column(s) into the complementary fittings on the manifold top. Transfer the binding solution from Step 4 to the Midiprep RNA column. Turn on the vacuum source and adjust to -25 inches Hg. Continue to apply the vacuum until no solution remains in the Midiprep RNA column. The RNA will bind to membranes within the binding module on the end of the Midiprep RNA column.

   IMPORTANT: The binding module on the bottom of the Midiprep RNA column must be fully seated before loading the lysate onto the column. Use the plastic wrench to tighten the module before proceeding.

   Note: -25 inches Hg is equivalent to -850-1,000 mbar or -12-15 psi.

6. Add 5ml of Buffer W1A and draw all of the solution through the Midiprep RNA column.

   Note: Make sure that ethanol has been added into Buffer W1A concentrate. Make a notation on the bottle label for future reference.

7. Add 7 ml of Buffer W2 along the wall of Midiprep RNA column to wash off residual Buffer W1A and draw all of the solution through the column. Repeat this wash step with a second 7 ml aliquot of Buffer W2.

   Note: Make sure that ethanol has been added into Buffer W2 concentrate. Make a notation on the bottle label for future reference.

8. Use the plastic wrench to detach the binding module from the Midiprep RNA column assembly and place it into a 1.5 ml microfuge tube (provided). Centrifuge at 12,000xg for 1 minute to remove residual wash solution.
9. Transfer the binding module to a fresh 1.5 ml microfuge tube (provided). To elute the total RNA, add 300 μl of Buffer TE (nuclease-free) to the center of the membrane. Let it stand for 1 minute at room temperature. Centrifuge at 12,000xg for 1 minute.

Purification of Total RNA from Cultured Cells

This protocol is designed for the isolation of total RNA from up to $2x10^8$ mammalian cells grown in suspension, in monolayer or as a cell suspension isolated from animal tissues. If the number of cells is $\leq 1x10^7$, reduce the volumes of R-I, R-II and isopropanol by half. All other buffer volumes should remain unchanged. If the number of cells is $>1x10^8$, the volumes of R-I, R-II and isopropanol should be scaled up proportionally. Mammalian cells are lysed without the use of homogenizers or mortar and pestle. Generally, resuspension in lysis buffer, followed by pipetting up and down several times is sufficient for complete lysis. Complete homogenization and DNA shearing is then achieved by

passing the lysate several times through a syringe needle.

Table1. Describes the number of Hela cells growing in various culture vessels when cells are grown to confluence. It may be used as a guide for estimating the number of cells.

Table1. Estimated number of Hela cells grown in different vessels.

Vessels	Growth area (cm^2)	Cell number
Multi-well plate		
96-well	0.32-0.6	$4.0\text{-}5.0\times10^4$
48-well	1	1.3×10^5
24-well	2	2.5×10^5
12-well	4	5.0×10^5
6-well	9.5	1.2×10^6
Petri Dish		
35 mm	8	1.0×10^6
60 mm	21	2.5×10^6
100 mm	56	7.0×10^6
145-150 mm	145	2.0×10^7
Bottle		
40-50 ml	25	3.0×10^6
250-300 ml	75	1.0×10^7
650-750 ml	162-175	2.0×10^7
900 ml	225	3.0×10^7

1. Collect $1x10^7$-$1x10^8$cells in suspension and transfer into a 50 ml centrifuge tube. Centrifuge at 2,000xg for 5 minutes to pellet the cells. Discard the supernatant.
2. Add 4 ml of Buffer R-I and continue to grind the tissue until the pulverized tissue and buffer are completely mixed. Quickly homogenize the sample by passing it 8-10x through a 10 ml syringe fitted with a 21-25-gauge needle. Be careful to minimize foaming. Transfer the homogenate to a nuclease-free 15 ml centrifuge tube (not provided).

   Note: Thorough homogenization of the lysate is essential for high yield and purity of the RNA. The fully homogenized lysate should flow easily dropwise through the syringe needle. A viscous, stringy lysate indicates incomplete shearing of the genomic DNA and further homogenization is required.

3. Add 1.5 ml of Buffer R-II and vortex for 15-30 seconds. Centrifuge at 8,000xg for 10 minutes at 4°C to pellet DNA and protein.
4. Transfer the supernatant from Step 3 into the Lysate filtration column. Carefully insert the plunger into the Lysate filtration column and push with a slow, steady motion. Discharge the filtrate into a nuclease-free 15 ml centrifuge tube. Add 2.5 ml of isopropanol to the 15 ml centrifuge tube and mix by vortexing.

   Note: The supernatant should be carefully removed without disturbing the pellet. The transfer of pellet material may result in column clogging and contamination of the RNA with DNA and protein.

5. Attach the vacuum manifold base to a vacuum source. Firmly position the Midiprep RNA column(s) into the complementary fittings on the manifold top. Transfer the binding solution from Step 4 to the Midiprep RNA column. Turn on the vacuum source and adjust to -25 inches Hg. Continue to apply the vacuum until no solution remains in the Midiprep RNA column. The RNA will bind to membranes within the binding module on the end of the Midiprep RNA column.

   IMPORTANT: The binding module on the bottom of the Midiprep RNA column must be fully seated before loading the lysate onto the column. Use the plastic wrench to tighten the module before proceeding.

   Note: -25 inches Hg is equivalent to -850-1,000 mbar or -12-15 psi.

6. Add 5 ml of Buffer W1A and draw all of the solution through the Midiprep RNA column.
   Note: Make sure that ethanol has been added into Buffer W1A concentrate. Make a notation on the bottle label for future reference.

7. Add 7 ml of Buffer W2 along the wall of Midiprep RNA column to wash off residual Buffer W1A and draw all of the solution through the column. Repeat this wash step with a second 7 ml aliquot of Buffer W2.
   Note: Make sure that ethanol has been added into Buffer W2 concentrate. Make a notation on the bottle label for future reference.

8. Use the plastic wrench to detach the binding module from the Midiprep RNA column assembly and place it into a 1.5 ml microfuge tube (provided). Centrifuge at 12,000xg for 1 minute to remove residual wash solution.
9. Transfer the binding module to a fresh 1.5 ml microfuge tube (provided). To elute the total RNA, add 300 μl of Buffer TE (nuclease-free) to the center of the membrane. Let it stand for 1 minute at room temperature. Centrifuge at 12,000xg for 1 minute.

Purification of Total RNA from Bacteria

This protocol is designed for the isolation of RNA from $2x10^9$-$1x10^{10}$ bacterial cells. The following values may be used as a guide for estimating the number of bacterial cells. For an *E. coli* culture, an OD_{600} of $1=1x10^9$ cells/ml. If the number of bacterial cells is $\leq 2x10^9$, reduce the volumes of buffers R-I, R-II and isopropanol by half. All other buffer volumes remain unchanged. If the number of bacterial cells is $>1x10^{10}$, increase the volumes of R-I, R-II and isopropanol proportionally.

1. Collect $2x10^9$-1×10^{10}bacteria. Centrifuge at ≥6,000xg for 10 minutes to pellet the bacteria. Decant or pipette off as much of the supernatant as possible. Resuspend the bacterial pellet in 100 μl of PBS by vortexing. Transfer the sample to a mortar, completely frozen in liquid nitrogen. The bacterial suspension should freeze on contact. Grind rapidly and vigorously to form a finely pulverized powder.
   Note: If using a microfuge, simply centrifuge the bacteria for 2 minutes at top speed to pellet.

2. Add 4 ml of Buffer R-I and continue to grind the sample until the pulverized sample and buffer are completely mixed. Quickly homogenize the sample by passing it 8-10x through a 10 ml syringe fitted with a 21-25-gauge needle. Be careful to minimize foaming. Transfer the homogenate to a nuclease-free 15 ml centrifuge tube (not provided).
   Note: Thorough homogenization of the lysate is essential for high yield and purity of the RNA. The fully homogenized lysate should flow easily dropwise through the syringe needle. A viscous, stringy lysate indicates incomplete shearing of the genomic DNA and further homogenization is required.

3. Add 1.5 ml of Buffer R-II and vortex for 15-30 seconds. Centrifuge at 8,000xg for 10 minutes at 4°C to pellet DNA and protein.
4. Transfer the supernatant from Step 3 into the Lysate Filtration column. Carefully insert the plunger into the Lysate Filtration column and push with a slow, steady motion. Discharge the filtrate into a nuclease-free 15 ml centrifuge tube. Add 2.5 ml of isopropanol to the 15 ml centrifuge tube and mix

by vortexing.

Note: The supernatant should be carefully removed without disturbing the pellet. The transfer of pellet material may result in column clogging and contamination of the RNA with DNA and protein.

5. Attach the vacuum manifold base to a vacuum source. Firmly position the Midiprep RNA column(s) into the complementary fittings on the manifold top. Transfer the binding solution from Step 4 to the Midiprep RNA column. Turn on the vacuum source and adjust to -25 inches Hg. Continue to apply the vacuum until no solution remains in the Midiprep RNA column. The RNA will bind to membranes within the binding module on the end of the Midiprep RNA column.

   IMPORTANT: The binding module on the bottom of the Midiprep RNA column must be fully seated before loading the lysate onto the column. Use the plastic wrench to tighten the module before proceeding.

   Note: -25 inches Hg is equivalent to -850-1,000 mbar or -12-15 psi.

6. Add 5 ml of Buffer W1A and draw all of the solution through the Midiprep RNA column.

   Note: Make sure that ethanol has been added into Buffer W1A concentrate. Make a notation on the bottle label for future reference.

7. Add 7 ml of Buffer W2 along the wall of Midiprep RNA column to wash off residual Buffer W1A and draw all of the solution through the column. Repeat this wash step with a second 7ml aliquot of Buffer W2.

   Note: Make sure that ethanol has been added into Buffer W2 concentrate. Make a notation on the bottle label for future reference.

8. Use the plastic wrench to detach the binding module from the Midiprep RNA column assembly and place it into a 1.5 ml microfuge tube (provided). Centrifuge at 12,000xg for 1 minute to remove residual wash solution.
9. Transfer the binding module to a fresh 1.5 ml microfuge tube (provided). To elute the total RNA, add 300 μl of Buffer TE (nuclease-free) to the center of the membrane. Let it stand for 1 minute at room temperature. Centrifuge at 12,000xg for 1 minute.

Purification of Total RNA from Yeast

This protocol is designed for the isolation of total cellular RNA from $2x10^7$- $5x10^8$ yeast cells. The following values may be used as a guide to estimating the number of yeast cells. For yeast cultures, an OD_{600} of 1=$3x10^7$ cells/ml. If the number of yeast cells is $\leq 2x10^7$, reduce the volumes buffers R-I, R-II and isopropanol by half. All other buffer volumes remain unchanged. If the number of yeast cells is $>5x10^8$, the volumes of R-I, R-II and isopropanol should be increased proportionally.

There are two different methods which can be used to achieve the disruption and lysis of yeast. The **mechanical disruption method** (A. below) employs a mortar and pestle to grind yeast to form a fine powder, followed by homogenization with a syringe needle. The **enzymatic lysis method** (B. below) requires digestion of the cell walls with lyticase to convert the yeast to spheroplasts.

Follow either Protocol A (mechanical) or Protocol B (enzymatic), below.

A. Mechanical Disruption

1. Collect 2x10^7-5x10^8 yeast cells. Centrifuge at ≥6,000xg for 10 minutes to pellet the yeast. Decant or pipette off as much of the supernatant as possible. Resuspend the yeast pellet in 100 μl of PBS by vortexing. Transfer to a mortar, completely frozen with liquid nitrogen. The yeast suspension should freeze upon contact. Grind rapidly and vigorously to form a finely pulverized powder.
2. Add 4 ml of Buffer R-I and continue to grind the sample until the pulverized sample and buffer are completely mixed. Quickly homogenize the sample by passing it 8-10x through a 10 ml syringe fitted with a 21-25-gauge needle. Be careful to minimize foaming. Transfer the homogenate to a nuclease-free 15 ml centrifuge tube (not provided).

   Note: Thorough homogenization of the lysate is essential for high yield and purity of the RNA. The fully homogenized lysate should flow easily dropwise through the syringe needle. A viscous, stringy lysate indicates incomplete shearing of the genomic DNA and further homogenization is required.

3. Add 1.5 ml of Buffer R-II and vortex for 15-30 seconds. Centrifuge at 8,000xg for 10 minutes at 4°C to pellet DNA and protein.
4. Transfer the supernatant from Step 3 into the Lysate Filtration column. Carefully insert the plunger into the Lysate Filtration column and push with a slow, steady motion. Discharge the filtrate into a nuclease-free 15 ml centrifuge tube. Add 2.5ml of isopropanol to the 15 ml centrifuge tube and mix by vortexing.

   Note: The supernatant should be carefully removed without disturbing the pellet. The transfer of pellet material may result in column clogging and contamination of the RNA with DNA and protein.

5. Attach the vacuum manifold base to a vacuum source. Firmly position the Midiprep RNA column(s) into the complementary fittings on the manifold top. Transfer the binding solution from Step 4 to the Midiprep RNA column. Turn on the vacuum source and adjust to -25 inches Hg. Continue to apply the vacuum until no solution remains in the Midiprep RNA column. The RNA will bind to membranes within the binding module on the end of the Midiprep RNA column.

   IMPORTANT: The binding module on the bottom of the Midiprep RNA column must be fully seated before loading the lysate onto the column. Use the plastic wrench to tighten the module before proceeding.

   Note: -25 inches Hg is equivalent to -850-1,000 mbar or -12-15 psi.

6. Add 5 ml of Buffer W1A and draw all of the solution through the Midiprep RNA column.

   Note: Make sure that ethanol has been added into Buffer W1A concentrate. Make a notation on the bottle label for future reference.

7. Add 7 ml of Buffer W2 along the wall of Midiprep RNA column to wash off residual Buffer W1A and draw all of the solution through the column. Repeat this wash step with a second 7ml aliquot of Buffer W2.

   Note: Make sure that ethanol has been added into Buffer W2 concentrate. Make a notation on the bottle label for future reference.

8. Use the plastic wrench to detach the binding module from the Midiprep RNA column assembly and place it into a 1.5 ml microfuge tube (provided). Centrifuge at 12,000xg for 1 minute to

remove residual wash solution.

9. Transfer the binding module to a fresh 1.5 ml microfuge tube (provided). To elute the total RNA, add 300 μl of Buffer TE (nuclease-free) to the center of the membrane. Let it stand for 1 minute at room temperature. Centrifuge at 12,000xg for 1 minute.

B. Enzymatic Disruption

Prepare Buffer YE:

a) 1 M sorbitol
b) 0.1 M EDTA, pH 7.5

Just before use, add the following to Buffer YE:

- 0.1% β-mercaptoethanol
- 50 U lyticase per $1x10^7$ cells

1. Collect $2x10^7$-$5x10^8$ yeast cells. Centrifuge at ≥6,000xg for 10 minutes to pellet the yeast. Decant or pipette off as much of the supernatant as possible.
2. Resuspend the yeast pellet in 10 ml freshly prepared Buffer YE containing lyticase. Use 50 unit of lyticase for each $1x10^7$ yeast cells. Incubate for 20-30 minutes at 30°C with occasional gentle inversion to generate spheroplasts.
   IMPORTANT: Spheroplasts must be handled gently in the next step. Do not shake or agitate.
3. Pellet the spheroplasts by centrifuging for 5 minutes at 3,000xg. Carefully remove and discard the supernatant.
4. Add 4 ml of Buffer R-I and continue to grind the sample until the pellet and buffer are completely mixed. Quickly homogenize the sample by passing it 8-10x through a 10 ml syringe fitted with a 21-25-gauge needle. Be careful to minimize foaming. Transfer the homogenate to a nuclease-free 15 ml centrifuge tube (not provided).
   Note: Thorough homogenization of the lysate is essential for high yield and purity of the RNA. The fully homogenized lysate should flow easily dropwise through the syringe needle. A viscous, stringy lysate indicates incomplete shearing of the genomic DNA and further homogenization is required.
5. Add 1.5 ml of Buffer R-II and vortex for 15-30 seconds. Centrifuge at 8,000xg for 10 minutes at 4°C to pellet DNA and protein.
6. Transfer the supernatant from Step 3 into the Lysate Filtration column. Carefully insert the plunger into the Lysate Filtration column and push with a slow, steady motion. Discharge the filtrate into a nuclease-free 15 ml centrifuge tube (not provided). Add 2.5 ml of isopropanol to the 15 ml centrifuge tube and mix by vortexing.
   Note: The supernatant should be carefully removed without disturbing the pellet. The transfer of pellet material may result in column clogging and contamination of the RNA with DNA and protein.
7. Attach the vacuum manifold base to a vacuum source. Firmly position the Midiprep RNA column(s) into the complementary fittings on the manifold top. Transfer the binding solution from Step 4 to the Midiprep RNA column. Turn on the vacuum source and adjust to -25 inches Hg. Continue to apply the vacuum until no solution remains in the Midiprep RNA column. The RNA will bind to

membranes within the binding module on the end of the Midiprep RNA column.

IMPORTANT: The binding module on the bottom of the Midiprep RNA column must be fully seated before loading the lysate onto the column. Use the plastic wrench to tighten the module before proceeding.

Note: -25 inches Hg is equivalent to -850-1,000 mbar or -12-15 psi.

8. Add 5 ml of Buffer W1A and draw all of the solution through the Midiprep RNA column.
   Note: Make sure that ethanol has been added into Buffer W1A concentrate. Make a notation on the bottle label for future reference.

9. Add 7 ml of Buffer W2 along the wall of Midiprep RNA column to wash off residual Buffer W1A and draw all of the solution through the column. Repeat this wash step with a second 7ml aliquot of Buffer W2.
   Note: Make sure that ethanol has been added into Buffer W2 concentrate. Make a notation on the bottle label for future reference.

10. Use the plastic wrench to detach the binding module from the Midiprep RNA column assembly and place it into a 1.5 ml microfuge tube (provided). Centrifuge at 12,000xg for 1 minute to remove residual wash solution.
11. Transfer the binding module to a fresh 1.5 ml microfuge tube (provided). To elute the total RNA, add 300 μl of Buffer TE (nuclease-free) to the center of the membrane. Let it stand for 1 minute at room temperature. Centrifuge at 12,000xg for 1 minute.

Purification of Total RNA from Filamentous Fungi

When processing ≤100 mg of starting material, please reduce the volumes of buffers R-I, R-II and isopropanol by half. All other buffer volumes remain unchanged. When processing >500 mg, increase the volumes of R-I, R-II and isopropanol proportionally.

1. Select 100-500 mg of freshly harvested animal tissue and immediately flash freeze by immersion in liquid nitrogen. Place a pestle into a mortar and freeze by adding liquid nitrogen to the mortar. Transfer the frozen tissue to the mortar and rapidly and vigorously grind to a finely pulverized powder. Depending upon the rapidity with which the tissue is pulverized, it may be necessary to add small amounts of liquid nitrogen intermittently so that the tissue remains frozen.
   IMPORTANT: The tissue must remain frozen before and during grinding to prevent enzymatic degradation of the RNA.

2. Add 4 ml of Buffer R-I and continue to grind the tissue until the pulverized tissue and buffer are completely mixed. Quickly homogenize the sample by passing it 8-10x through a 10 ml syringe fitted with a 21-25-gauge needle. Be careful to minimize foaming. Transfer the homogenate to a nuclease-free 15 ml centrifuge tube (not provided).
   Note: Thorough homogenization of the lysate is essential for high yield and purity of the RNA. The fully homogenized lysate should flow easily dropwise through the syringe needle. A viscous, stringy lysate indicates incomplete shearing of the genomic DNA and further homogenization is required.

3. Add 1.5 ml of Buffer R-II and vortex for 15-30 seconds. Centrifuge at 8,000xg for 10 minutes at 4°C to pellet DNA and protein.
4. Transfer the supernatant from Step 3 into the Lysate filtration column. Carefully insert the plunger into the Lysate filtration column and push with a slow, steady motion. Discharge the filtrate into a nuclease-free 15 ml centrifuge tube. Add 2.5 ml of isopropanol to the 15 ml centrifuge tube and mix by vortexing.

   Note: The supernatant should be carefully removed without disturbing the pellet. The transfer of pellet material may result in column clogging and contamination of the RNA with DNA and protein.

5. Attach the vacuum manifold base to a vacuum source. Firmly position the Midiprep RNA column(s) into the complementary fittings on the manifold top. Transfer the binding solution from Step 4 to the Midiprep RNA column. Turn on the vacuum source and adjust to -25 inches Hg. Continue to apply the vacuum until no solution remains in the Midiprep RNA column. The RNA will bind to membranes within the binding module on the end of the Midiprep RNA column.

   IMPORTANT: The binding module on the bottom of the Midiprep RNA column must be fully seated before loading the lysate onto the column. Use the plastic wrench to tighten the module before proceeding.

   Note: -25 inches Hg is equivalent to -850-1,000 mbar or -12-15 psi.

6. Add 5 ml of Buffer W1A and draw all of the solution through the Midiprep RNA column.

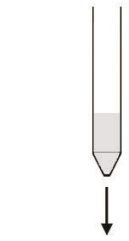
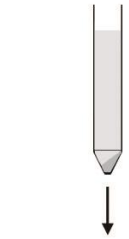
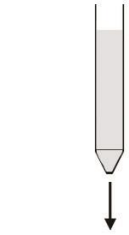
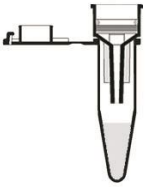
   Note: Make sure that ethanol has been added into Buffer W1A concentrate. Make a notation on the bottle label for future reference.

7. Add 7 ml of Buffer W2 along the wall of Midiprep RNA column to wash off residual Buffer W1A and draw all of the solution through the column. Repeat this wash step with a second 7 ml aliquot of Buffer W2.

   Note: Make sure that ethanol has been added into Buffer W2 concentrate. Make a notation on the bottle label for future reference.

8. Use the plastic wrench to detach the binding module from the Midiprep RNA column assembly and place it into a 1.5 ml microfuge tube (provided). Centrifuge at 12,000xg for 1 minute to remove residual wash solution.
9. Transfer the binding module to a fresh 1.5 ml microfuge tube (provided). To elute the total RNA, add 300 μl of Buffer TE (nuclease-free) to the center of the membrane. Let it stand for 1 minute at room temperature. Centrifuge at 12,000xg for 1 minute.

Overview

Add sample and 4 ml of Buffer R-I		Lysis
Add 1.5 ml of Buffer R-II Centrifuge at 8,000xg for 10 minutes		Neutralization
Filter the supernatant with Lysate Filtration column		Particulate & protein removal
Add 2.5 ml of isopropanol		Binding to Midiprep RNA column
Add 5 ml of Buffer W1A Add 7 ml of Buffer W2 Add 7 ml of Buffer W2		Washing
Add 0.3 ml of Buffer TE(nuclease-free)		Elution

Troubleshooting

1.Little or no RNA eluted

- Too much starting material. Overloading significantly reduces yield. Reduce the amount of starting material.
- Incomplete removal of supernatant after pelleting of cultured cells. Ensure complete removal of the supernatant after harvesting cells.
- Buffer temperatures are too low. All buffers must be equilibrated to room temperature before starting the procedure.

2.Low A_{260}/A_{280} value

- Inefficient cell lysis due to insufficient mixing of the samples with Buffer R-I.
- Buffer W1A or Buffer W2 prepared incorrectly with the correct amount of ethanol.
- Inadvertent transfer of pellet material to spin/vac column after Buffer R-II addition and centrifugation.

3.RNA degraded

- Inappropriate handling of starting material. Ensure that cells have been properly handled and that the RNA extraction has been performed without interruptions, especially the initial steps involving cell lysis.
- RNase contamination: check for RNase contamination of buffers. Although all buffers have been tested and are guaranteed RNase-free, RNase can be introduced during use. Be certain not to introduce any RNase during the procedure or later handling.

Warranty/Disclaimer

Axygen Biosciences warrants that this kit will perform as indicated for the specified application for a period of up to 12 months from the date of receipt when components are stored in the specified manner and used according to the instructions provided. In using this product, the customer agrees that Axygen Biosciences shall not be held liable for any direct or indirect damages, including, but not limited to, personal injury, property damage or lost profits (or other economic loss) resulting from the use or inability to use this product. In the event that this product fails to perform in the specified manner, remedial measures on the part of Axygen Biosciences shall be limited to the replacement of this product and will be implemented at the discretion of Axygen Biosciences.