

AxyPrep Multisource Total RNA Miniprep 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-MN-MS-RNA-4	AP-MN-MS-RNA-50	AP-MN-MS-RNA-250
Kit size	4 preps	50 preps	250 preps
Spin/vac mini column	4	50	250
2 ml Microfuge tube	4	50	250
1.5 ml Microfuge tube	8	100	500
Buffer R-I	3 ml	25 ml	125 ml
Buffer R-II	1 ml	10 ml	50 ml
Buffer W1A concentrate	2.4 ml	24 ml	120 ml
Buffer W2 concentrate	2.4 ml	24 ml	2 × 72 ml
Buffer TE	1 ml	6 ml	30 ml
Protocol manual	1	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.

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: Eluent. Contains 10 mM Tris-Cl and 0.1 mM EDTA, pH 7.5. Store at room temperature.

Introduction

AxyPrep Multisource Total RNA Miniprep Kit represents a new approach for total cellular RNA purification, which is designed to eliminate the problems associated with other spin 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 nullifies any indigenous RNase activity. Proteins and genomic DNA are

then precipitated by the addition of Neutralization Buffer R-II to the cell lysate. After addition of isopropanol to the supernatant, the Total RNA is then bound to a Spin/vac miniprep column for further washing and desalting. Highly purified and 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.

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

- Microcentrifuge capable of 12,000 × g
- 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

* Purifications can be carried out using either centrifugation in a conventional high-speed laboratory microcentrifuge or a vacuum manifold. Therefore, the use of a vacuum manifold is optional.

Preparation before experiment

- 1). Before the use of 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 equipment with nucleases. Please follow generally recommended practices for maintaining a nuclease-free work environment.

IMPORTANT: Grossly overloading the spin 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)

- 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 30 mg
RNA-poor tissues (e.g., muscle)	use up to 100 mg
When processing <20 mg tissue	reduce R-I, R-II and isopropanol volumes by half
When processing >40 mg tissue	increase R-I, R-II and isopropanol volumes proportionally

1. Select 20-40 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 400 µl 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 8-10× through a 1 ml syringe fitted with a 21-25-gauge needle. Be careful to minimize foaming. Transfer the homogenate to a

1.5 ml microfuge tube (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 150 μ l of Buffer R-II and vortex for 15-30 seconds. Centrifuge at 12,000 \times g for 5 minutes at 4°C to pellet DNA and protein.
4. Transfer the clarified supernatant into a 1.5 ml microfuge tube. Add 250 μ l of isopropanol 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.

Proceed with either the A (centrifugation) protocol or B (vacuum) protocol, below.

A. Using Centrifugation

1. Place a Spin/vac column into a 2 ml microfuge tube (provided) .Transfer the binding solution from Step 4 into the Spin/vac column. Centrifuge at 6,000 \times g for 1 minute at room temperature or 4°C.
2. Discard the filtrate from the 2 ml microfuge tube. Place the Spin/vac column back into the same 2 ml microfuge tube. Add 500 μ l of Buffer W1A to the Spin/vac column and centrifuge at 12,000 \times g for 1 minute.

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

3. Discard the filtrate and place the Spin/vac column back into the same 2 ml microfuge tube. Add 700 μ l of Buffer W2 and centrifuge at 12,000 \times g for 1 minute. Discard the filtrate from the 2 ml microfuge tube and repeat this wash with a second 700 μ l 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.

4. Discard the filtrate from the 2 ml microfuge tube. Place the Spin/vac column back into the 2 ml microfuge tube. Centrifuge at 12,000 \times g for 1 minute to remove residual wash solution.
5. Transfer the Spin/vac column into a clean 1.5 ml microfuge tube (provided). To elute the total RNA, add 70-100 μ l of Buffer TE to the center of the membrane. Let it stand for 1 minute at room temperature. Centrifuge at 12,000 \times g for 1 minute.

B. Using Vacuum

1. Attach the vacuum manifold base to a vacuum source. Firmly position the Spin/vac column(s) into the complementary fittings on the manifold top. Transfer the binding solution from Step 4 to the Spin/vac column. Turn on the vacuum source and adjust to -25 inches Hg. Continue to apply the vacuum until no solution remains in the Spin/vac column.

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

2. Add 500 μ l of Buffer W1A and draw all of the solution through the Spin/vac column.

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

3. Add 700 μ l of Buffer W2 along the wall of Spin/vac column to wash off residual Buffer W1A and draw all of the solution through the column. Repeat this wash with a second 700 μ l 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.

4. Transfer the Spin/vac column into a 2 ml microfuge tube (provided) and centrifuge at 12,000 \times g for 1 minute to remove residual wash solution.

5. Transfer the Spin/vac column into a clean 1.5 ml microfuge tube (provided). To elute the total RNA, add 70-100 μ l of Buffer TE to the center of the membrane. Let it stand for 1 minute at room temperature. Centrifuge at 12,000 \times g for 1 minute.

II. 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 or. 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 10-80 mg
For fibrous tissue (stems, etc.)	routinely process 100-150 mg
When processing <30 mg leaf tissue	reduce R-I, R-II and isopropanol volumes by half
When processing >80 mg leaf tissue	increase R-I, R-II and isopropanol volumes proportionally
When processing >150 mg fibrous tissue	increase R-I, R-II and isopropanol volumes proportionally

1. Select 30-150 mg of tissue from plant and immediately flash freeze by immersion in liquid nitrogen. Transfer to a mortar containing a small amount of liquid nitrogen. Grind rapidly and vigorously to form a finely pulverized powder.
2. Add 400 μ l of Buffer R-I and grind to form a homogenous mixture. Quickly homogenize the sample by passing it 8-10 \times through a 1 ml syringe fitted with a 18-23-gauge needle. Be careful to minimize foaming. Transfer the lysate to a 1.5 ml microfuge tube (provided).
3. Add 150 μ l of Buffer R-II and vortex for 15-30 seconds. Centrifuge at 12,000 \times g for 5 minutes at 4 $^{\circ}$ C to pellet DNA and protein.
4. Transfer the clarified supernatant to a 1.5 ml microfuge tube. Add 250 μ l of isopropanol and mix well 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.

Proceed with either the A (centrifugation) protocol or B (vacuum) protocol, below.

A. Using Centrifugation

1. Place a Spin/vac column into a 2 ml microfuge tube (provided). Transfer the binding solution from Step 4 into the Spin/vac column. Centrifuge at $6,000 \times g$ for 1 minute at room temperature or 4°C .
2. Discard the filtrate from the 2 ml microfuge tube. Place the Spin/vac column back into the same 2 ml microfuge tube. Add 500 μl of Buffer W1A to the Spin/vac column and centrifuge at $12,000 \times g$ for 1 minute.

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

3. Discard the filtrate and place the Spin/vac column back into the same 2 ml microfuge tube. Add 700 μl of Buffer W2 and centrifuge at $12,000 \times g$ for 1 minute. Discard the filtrate from the 2 ml microfuge tube and repeat this wash with a second 700 μl 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.

4. Discard the filtrate from the 2 ml microfuge tube. Place the Spin/vac column back into the 2 ml microfuge tube. Centrifuge at $12,000 \times g$ for 1 minute to remove residual wash solution.
5. Transfer the Spin/vac column into a clean 1.5 ml microfuge tube (provided). To elute the total RNA, add 70-100 μl of Buffer TE to the center of the membrane. Let it stand for 1 minute at room temperature. Centrifuge at $12,000 \times g$ for 1 minute.

B. Using Vacuum

1. Attach the vacuum manifold base to a vacuum source. Firmly position the Spin/vac column(s) into the complementary fittings on the manifold top. Transfer the binding solution from Step 4 to the Spin/vac column. Turn on the vacuum source and adjust to -25 inches Hg. Continue to apply the vacuum until no solution remains in the Spin/vac column.

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

2. Add 500 μl of Buffer W1A and draw all of the solution through the Spin/vac column.

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

3. Add 700 μl of Buffer W2 along the wall of Spin/vac column to wash off residual Buffer W1A and draw all of the solution through the column. Repeat this wash with a second 700 μl 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.

4. Transfer the Spin/vac column into a 2 ml microfuge tube (provided) and centrifuge at $12,000 \times g$ for 1 minute to remove residual wash solution.

5. Transfer the Spin/vac column into a clean 1.5 ml microfuge tube (provided). To elute the total RNA, add 70-100 μ l of Buffer TE to the center of the membrane. Let it stand for 1 minute at room temperature. Centrifuge at $12,000 \times g$ for 1 minute.

III. Purification of Total RNA from Cultured Cells

This protocol is designed for the isolation of total RNA from up to 1×10^7 mammalian cells grown in suspension, in monolayer or as a cell suspension isolated from animal tissues. If the number of cells is $\leq 2 \times 10^6$, 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 $> 1 \times 10^7$, 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	$4-5 \times 10^4$
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	1×10^6
60 mm	21	2.5×10^6
100 mm	56	7×10^6
145-150 mm	145	2×10^7
Bottle		
40-50 ml	25	3×10^6
250-300 ml	75	1×10^7
650-750 ml	162-175	2×10^7
900 ml	225	3×10^7

a. Cells grown in suspension or cell suspension freshly-isolated from animal or human tissues:

1. Collect $2 \times 10^6 - 1 \times 10^7$ cells in suspension and transfer into a 1.5 ml microfuge tube (provided). Centrifuge at $2,000 \times g$ for 5 minutes to pellet the cells. Discard the supernatant.
2. Add 400 μ l of Buffer R-I. Lyse the cells by pipetting up and down 8-10 \times then homogenize the sample by passing it 8-10 \times through a 1 ml syringe fitted with a 21-25-gauge needle. Be careful to minimize foaming.
3. Add 150 μ l of Buffer R-II and vortex for 15-30 seconds. Centrifuge at $12,000 \times g$ for 5 minutes at room temperature to pellet DNA and protein.
4. Transfer the supernatant to a 1.5 ml microfuge tube. Add 250 μ l of isopropanol and mix well 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.

Proceed with either the A (centrifugation) protocol or B (vacuum) protocol, below.

b. Cells grown in a monolayer in a 96-well, 24-well, 12-well or 6-well plate:

1. Discard as much of the supernatant as possible, then add 300 μ l of Buffer R-I into each well.
2. Pipette up and down 8-10 \times , then homogenize the sample by passing it 8-10 \times through a 1 ml syringe fitted with a 21-25-gauge needle. Be careful to minimize foaming.
3. Transfer 300 μ l of the cell homogenate to a 1.5 ml microfuge tube (provided).
4. Add 110 μ l of Buffer R-II and vortex for 15-30 seconds. Centrifuge at 12,000 \times g for 5 minutes at room temperature to pellet DNA and protein.
5. Transfer the supernatant to a new 1.5 ml microfuge tube. Add 200 μ l of isopropanol and mix well 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.

Proceed with either the A (centrifugation) protocol or B (vacuum) protocol, below.

A. Using Centrifugation

1. Place a Spin/vac column into a 2 ml microfuge tube (provided). Transfer the binding solution from Step 4 (for suspension cells protocol) or Step 5 (for monolayer cells protocol) into the Spin/vac column. Centrifuge at 6,000 \times g for 1 minute at room temperature or 4 $^{\circ}$ C.
2. Discard the filtrate from the 2 ml microfuge tube. Place the Spin/vac column back into the same 2 ml microfuge tube. Add 500 μ l of Buffer W1A to the Spin/vac column and centrifuge at 12,000 \times g for 1 minute.

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

3. Discard the filtrate and place the Spin/vac column back into the same 2 ml microfuge tube. Add 700 μ l of Buffer W2 and centrifuge at 12,000 \times g for 1 minute. Discard the filtrate from the 2 ml microfuge tube and repeat this wash with a second 700 μ l 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.

4. Discard the filtrate from the 2 ml microfuge tube. Place the Spin/vac column back into the 2 ml microfuge tube. Centrifuge at 12,000 \times g for 1 minute to remove residual wash solution.
5. Transfer the Spin/vac column into a clean 1.5 ml microfuge tube (provided). To elute the total RNA, add 70-100 μ l of Buffer TE to the center of the membrane. Let it stand for 1 minute at room temperature. Centrifuge at 12,000 \times g for 1 minute.

B. Using Vacuum

1. Attach the vacuum manifold base to a vacuum source. Firmly position the Spin/vac column(s) into the complementary fittings on the manifold top. Transfer the binding solution from Step 4 (for suspension cells protocol) or Step 5 (for monolayer cells protocol) into the Spin/vac column. Turn on the vacuum source and adjust to -25 inches Hg. Continue to apply the vacuum until no solution remains in the Spin/vac column.

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

2. Add 500 μ l of Buffer W1A and draw all of the solution through the Spin/vac column.
Note: Make sure that ethanol has been added into Buffer W1A concentrate. Make a notation on the bottle label for future reference.
3. Add 700 μ l of Buffer W2 along the wall of Spin/vac column to wash off residual Buffer W1A and draw all of the solution through the column. Repeat this wash with a second 700 μ l 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.
4. Transfer the Spin/vac column into a 2 ml microfuge tube (provided) and centrifuge at 12,000 \times g for 1 minute to remove residual wash solution.
5. Transfer the Spin/vac column into a clean 1.5 ml microfuge tube (provided). To elute the total RNA, add 70-100 μ l of Buffer TE to the center of the membrane. Let it stand for 1 minute at room temperature. Centrifuge at 12,000 \times g for 1 minute.

IV. Purification of Total RNA from Bacteria

This protocol is designed for the isolation of RNA from $0.5-2 \times 10^9$ bacterial cells. The following values may be used as a guide for estimating the number of bacterial cells. For an E.coli culture, an OD₆₀₀ of 1= 1×10^9 cells/ml. If the number of bacterial cells is $<0.5 \times 10^9$, 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 $>2 \times 10^9$, increase the volumes of R-I, R-II and isopropanol proportionally.

1. Collect $0.5-2 \times 10^9$ bacteria. Centrifuge at $\geq 6,000 \times g$ for 10 minutes to pellet the bacteria. Decant or pipette off as much of the supernatant as possible. Resuspend the bacterial pellet in 50 μ l 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 400 μ l of Buffer R-I and grind to form a homogenous mixture. Quickly homogenize the sample by passing it 8-10 \times through a 1 ml syringe fitted with a 21-25-gauge needle. Be careful to minimize foaming. Transfer the lysate to a 1.5 ml microfuge tube(provided).
3. Add 150 μ l of Buffer R-II and vortex for 15-30 seconds. Centrifuge at 12,000 \times g for 5 minutes at room temperature to pellet DNA and protein.
4. Transfer the supernatant to a 1.5 ml microfuge tube. Add 250 μ l of isopropanol and mix well 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.

Proceed with either the A (centrifugation) protocol or B (vacuum) protocol, below.

A. Using Centrifugation

1. Place a Spin/vac column into a 2 ml microfuge tube (provided) .Transfer the binding solution

from Step 4 into the Spin/vac column. Centrifuge at $6,000\times g$ for 1 minute at room temperature or 4°C .

2. Discard the filtrate from the 2 ml microfuge tube. Place the Spin/vac column back into the same 2 ml microfuge tube. Add $500\ \mu\text{l}$ of Buffer W1A to the Spin/vac column and centrifuge at $12,000\times g$ for 1 minute.

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

3. Discard the filtrate and place the Spin/vac column back into the same 2 ml microfuge tube. Add $700\ \mu\text{l}$ of Buffer W2 and centrifuge at $12,000\times g$ for 1 minute. Discard the filtrate from the 2 ml microfuge tube and repeat this wash with a second $700\ \mu\text{l}$ 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.

4. Discard the filtrate from the 2 ml microfuge tube. Place the Spin/vac column back into the 2 ml microfuge tube. Centrifuge at $12,000\times g$ for 1 minute to remove residual wash solution.
5. Transfer the Spin/vac column into a clean 1.5 ml microfuge tube (provided). To elute the total RNA, add $70\text{-}100\ \mu\text{l}$ of Buffer TE to the center of the membrane. Let it stand for 1 minute at room temperature. Centrifuge at $12,000\times g$ for 1 minute.

B. Using Vacuum

1. Attach the vacuum manifold base to a vacuum source. Firmly position the Spin/vac column(s) into the complementary fittings on the manifold top. Transfer the binding solution from Step 4 to the Spin/vac column. Turn on the vacuum source and adjust to -25 inches Hg. Continue to apply the vacuum until no solution remains in the Spin/vac column.

Note: -25 inches Hg is equivalent to $-850\text{-}1,000$ mbar or $-12\text{-}15$ psi.

2. Add $500\ \mu\text{l}$ of Buffer W1A and draw all of the solution through the Spin/vac column.

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

3. Add $700\ \mu\text{l}$ of Buffer W2 along the wall of Spin/vac column to wash off residual Buffer W1A and draw all of the solution through the column. Repeat this wash with a second $700\ \mu\text{l}$ 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.

4. Transfer the Spin/vac column into a 2 ml Microfuge tube (provided) and centrifuge at $12,000\times g$ for 1 minute to remove residual wash solution.
5. Transfer the Spin/vac column into a clean 1.5 ml microfuge tube (provided). To elute the total RNA, add $70\text{-}100\ \mu\text{l}$ of Buffer TE to the center of the membrane. Let it stand for 1 minute at room temperature. Centrifuge at $12,000\times g$ for 1 minute.

V. Purification of Total RNA from Yeast

This protocol is designed for the isolation of total cellular RNA from 2×10^6 - 5×10^7 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 $1=3 \times 10^7$ cells/ml. If the number of yeast cells is $\leq 5 \times 10^6$, 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 $> 2 \times 10^7$, 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.

a. Mechanical Disruption

1. Collect $0.5-2 \times 10^7$ yeast cells. Centrifuge at $\geq 6,000 \times g$ for 10 minutes to pellet the yeast. Decant or pipette off as much of the supernatant as possible. Resuspend the yeast pellet in 50 μ l 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 400 μ l of Buffer R-I. Quickly homogenize the sample by passing it 8-10 \times through a 1 ml syringe fitted with a 21-25-gauge needle. Be careful to minimize foaming. Transfer the homogenate to 1.5 ml microfuge tube (provided).
3. Add 150 μ l of Buffer R-II. Vortex for 30 seconds. Centrifuge at $12,000 \times g$ for 5 minutes at room temperature.
4. Transfer the supernatant to a 1.5 ml microfuge tube. Add 250 μ l of isopropanol and mix well.
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.

b. Enzymatic Disruption

Prepare Buffer YE:

- 1 M sorbitol
- 0.1 M EDTA, pH 7.5

Just before use, add the following to Buffer YE:

- 0.1% β -mercaptoethanol
- 50 U lyticase per 1×10^7 cells

1. Collect $0.5-2 \times 10^7$ yeast cells. Centrifuge at $\geq 6,000 \times g$ for 10 minutes to pellet the yeast. Decant or pipette off as much of the supernatant as possible.
2. Resuspend yeast in 1 ml freshly prepared Buffer YE containing lyticase. Use 50 unit of lyticase for each 1×10^7 yeast cells. Incubate for 20-30 min 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 $3,000 \times g$. Carefully remove and discard the supernatant.

4. Add 400 μ l of Buffer R-I to the spheroplast pellet and vortex vigorously. Quickly homogenize the sample by passing it 8-10 \times through a 1 ml syringe fitted with a 21-25-gauge needle. Be careful to minimize foaming. Transfer it to 1.5 ml microfuge tube (provided).
5. Add 150 μ l of Buffer R-II and vortex for 30 seconds. Centrifuge at 12,000 \times g for 5 minutes at room temperature to pellet the DNA and protein.
6. Transfer the supernatant to a 1.5 ml microfuge tube. Add 250 μ l of isopropanol and mix well.
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.

Proceed with either the A (centrifugation) protocol or B (vacuum) protocol, below.

A. Using Centrifugation

1. Place a Spin/vac column into a 2 ml microfuge tube (provided). Transfer the binding solution from Step 4 (for mechanical disruption protocol) or Step 6 (for enzymatic disruption protocol) into the Spin/vac column. Centrifuge at 6,000 \times g for 1 minute at room temperature or 4°C.
2. Discard the filtrate from the 2 ml microfuge tube. Place the Spin/vac column back into the same 2 ml microfuge tube. Add 500 μ l of Buffer W1A to the Spin/vac column and centrifuge at 12,000 \times g for 1 minute.
Note: Make sure that ethanol has been added into Buffer W1A concentrate. Make a notation on the bottle label for future reference.
3. Discard the filtrate and place the Spin/vac column back into the same 2 ml microfuge tube. Add 700 μ l of Buffer W2 and centrifuge at 12,000 \times g for 1 minute. Discard the filtrate from the 2 ml microfuge tube and repeat this wash with a second 700 μ l 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.
4. Discard the filtrate from the 2 ml microfuge tube. Place the Spin/vac column back into the 2 ml microfuge tube. Centrifuge at 12,000 \times g for 1 minute to remove residual wash solution.
5. Transfer the Spin/vac column into a clean 1.5 ml microfuge tube (provided). To elute the total RNA, add 70-100 μ l of Buffer TE to the center of the membrane. Let it stand for 1 minute at room temperature. Centrifuge at 12,000 \times g for 1 minute.

B. Using Vacuum

1. Attach the vacuum manifold base to a vacuum source. Firmly position the Spin/vac column(s) into the complementary fittings on the manifold top. Transfer the binding solution from Step 4 (for mechanical disruption protocol) or Step 6 (for enzymatic disruption protocol) into the Spin/vac column. Turn on the vacuum source and adjust to -25 inches Hg. Continue to apply the vacuum until no solution remains in the Spin/vac column.
Note: -25 inches Hg is equivalent to -850-1,000 mbar or -12-15 psi.
2. Add 500 μ l of Buffer W1A and draw all of the solution through the Spin/vac column.
Note: Make sure that ethanol has been added into Buffer W1A concentrate. Make a notation on the bottle label for future reference.

3. Add 700 μ l of Buffer W2 along the wall of Spin/vac column to wash off residual Buffer W1A and draw all of the solution through the column. Repeat this wash with a second 700 μ l 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.
4. Transfer the Spin/vac column into a 2 ml microfuge tube (provided) and centrifuge at 12,000 \times g for 1 minute to remove residual wash solution.
5. Transfer the Spin/vac column into a clean 1.5 ml microfuge tube (provided). To elute the total RNA, add 70-100 μ l of Buffer TE to the center of the membrane. Let it stand for 1 minute at room temperature. Centrifuge at 12,000 \times g for 1 minute.

VI. Purification of Total RNA from Filamentous Fungi

When processing <30 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 >100 mg, increase the volumes of R-I, R-II and isopropanol proportionally.

1. Select 30-100 mg of filamentous fungi and flash-freeze by immersing in liquid nitrogen. Transfer to a mortar, completely frozen in liquid nitrogen. Grind rapidly and vigorously to form a finely pulverized powder.
Note: The starting material can also be processed with either a manual or motorized homogenizer.
2. Add 400 μ l of Buffer R-I and grind to form a homogenous mixture. Quickly homogenize the sample by passing it 8-10 \times through a 1 ml syringe fitted with a 21-25-gauge needle. Be careful to minimize foaming. Transfer the lysate to a 1.5 ml microfuge tube (provided).
3. Add 150 μ l of Buffer R-II and vortex for 15-30 seconds. Centrifuge at 12,000 \times g for 5 minutes at room temperature to pellet DNA and protein.
4. Transfer the supernatant fluid to a 1.5 ml microfuge tube. Add 250 μ l of isopropanol and mix well 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.

Proceed with either the A (centrifugation) protocol or B (vacuum) protocol, below.

A. Using Centrifugation

1. Place a Spin/vac column into a 2 ml microfuge tube (provided). Transfer the binding solution from Step 4 into the Spin/vac column. Centrifuge at 6,000 \times g for 1 minute at room temperature or 4 $^{\circ}$ C.
2. Discard the filtrate from the 2 ml microfuge tube. Place the Spin/vac column back into the same 2 ml microfuge tube. Add 500 μ l of Buffer W1A to the Spin/vac column and centrifuge at 12,000 \times g for 1 minute.

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

3. Discard the filtrate and place the Spin/vac column back into the same 2 ml microfuge tube. Add 700 μ l of Buffer W2 and centrifuge at 12,000 \times g for 1 minute. Discard the filtrate from the 2 ml microfuge tube and repeat this wash with a second 700 μ l 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.
4. Discard the filtrate from the 2 ml microfuge tube. Place the Spin/vac column back into the 2 ml microfuge tube. Centrifuge at 12,000 \times g for 1 minute to remove residual wash solution.
5. Transfer the Spin/vac column into a clean 1.5 ml microfuge tube (provided). To elute the total RNA, add 70-100 μ l of Buffer TE to the center of the membrane. Let it stand for 1 minute at room temperature. Centrifuge at 12,000 \times g for 1 minute.

B. Using Vacuum

1. Attach the vacuum manifold base to a vacuum source. Firmly position the Spin/vac column(s) into the complementary fittings on the manifold top. Transfer the binding solution from Step 4 to the Spin/vac column. Turn on the vacuum source and adjust to -25 inches Hg. Continue to apply the vacuum until no solution remains in the Spin/vac column.
Note: -25 inches Hg is equivalent to -850-1,000 mbar or -12-15 psi.
2. Add 500 μ l of Buffer W1A and draw all of the solution through the Spin/vac column.
Note: Make sure that ethanol has been added into Buffer W1A concentrate. Make a notation on the bottle label for future reference.
3. Add 700 μ l of Buffer W2 along the wall of Spin/vac column to wash off residual Buffer W1A and draw all of the solution through the column. Repeat this wash with a second 700 μ l 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.
4. Transfer the Spin/vac column into a 2 ml microfuge tube (provided) and centrifuge at 12,000 \times g for 1 minute to remove residual wash solution.
5. Transfer the Spin/vac column into a clean 1.5 ml microfuge tube (provided). To elute the total RNA, add 70-100 μ l of Buffer TE to the center of the membrane. Let it stand for 1 minute at room temperature. Centrifuge at 12,000 \times g for 1 minute.

Overview

Add sample and 400 μ l of Buffer R-I



Lysis



Add 150 μ l of Buffer R-II
Centrifuge at 12,000 \times g for 5 minutes



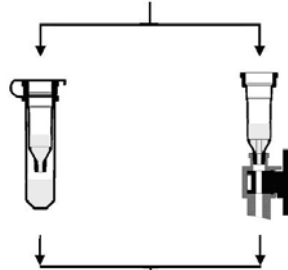
Neutralization



Add 250 μ l of isopropanol



Binding



Add 500 μ l of Buffer W1A
Add 700 μ l of Buffer W2
Add 700 μ l of Buffer W2

Binding

Washing

Add 70-100 μ l of Buffer TE (RNase-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 too low. All buffers must be equilibrated to room temperature before starting the procedure

2. Low A260/A280 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 stored in the specified manner indicated 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.