AxyPrep microRNA (miRNA) Miniprep Kit

For the purification of miRNA from animal tissues, plant tissues and cultured cells

Kit contents, storage and stability

Cat. No.	AP-MN-MIRNA-50	AP-MN-MIRNA-250
Kit size	50 preps	250 preps
Spin/vac mini column	50	250
2 ml microfuge tube	50	250
1.5 ml microfuge tube	50	250
Buffer R-I	25 ml	125 ml
Buffer R-II	10 ml	50 ml
Buffer TE(DNase&RNase-free)	6 ml	30 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.

Buffer R-I: Cell lysis buffer. Store at room temperature.
Buffer R-II: Neutralization buffer. 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

The AxyPrep miRNA Miniprep Kit represents a new approach for cellular “micro” RNA (miRNA) purification. This kit 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 inactivates any indigenous RNase activity. Proteins and genomic DNA are then precipitated by the addition of Neutralization Buffer R-II to the cell lysate. After the addition of ethanol to the supernatant, larger RNAs, such as mRNA, 18s and 28s rRNA and DNA are removed by binding to a spin/vac column. miRNA and siRNA are then recovered by the addition of isopropanol to the filtrate, followed by centrifugation. Highly purified and enriched miRNA is then resuspended 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 R- II 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 12,000xg
- Mortar and pestle
- Homogenizer (Dounce-type or motorized) *optional*
- Plastic syringe and 21-25-gauge syringe needle (see specific prep requirements)
- Liquid nitrogen
- Isopropanol
- 95-100% ethanol
- Ice-cold 70% ethanol

Preparation before experiment

1). Use DEPC-treated materials whenever practical.
2). Prepare 70% ethanol; pre-chill to -20℃.

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 in conjunction with liquid nitrogen, 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 miRNA 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.

I. Purification of miRNA 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:

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 8-10x 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 μl of Buffer R-II and vortex for 15-30 seconds. Centrifuge at 12,000xg for 5 minutes at 4°C to pellet DNA and protein.
4. Transfer the clarified supernatant into a 1.5 ml microfuge tube (not provided). Add 180 μl of ethanol and mix by vortexing for 15 seconds.

   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. 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 6,000xg for 1 minute at room temperature or 4°C.

   Note: Keep the filtrate containing miRNA.
6. Discard the spin/vac column. Add 500 μl isopropanol to the filtrate, vortex for 15 seconds and centrifuge at 12,000xg for 10 minutes.
7. Discard the supernatant.

8. Add 700 μl ice-cold 70% ethanol and centrifuge at 12,000xg for 5 minutes.
9. Discard the supernatant and invert the microfuge tube on absorbent toweling to drain off residual solution. Dry the miRNA for 5-10 minutes at room temperature.

   Note: Make sure that no ethanol is left in the miRNA pellet after drying. Residual ethanol will inhibit many enzymatic reactions.

10. Add 70 μl of Buffer TE (DNase&RNase-free) to the 2 ml microfuge tube.

    Note: If the miRNA pellet appears difficult to resuspend, occasional gentle swirling or agitation may be required.

II. Purification of miRNA from Plant Tissues

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

- Mortal 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.

Protocol

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 μl of Buffer R-I and grind to form a homogenous mixture. Quickly homogenize the sample by passing it 8-10x 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 μl of Buffer R-II and vortex for 15-30 seconds. Centrifuge at 12,000xg for 5 minutes at 4°C to pellet DNA and protein.
4. Transfer the clarified supernatant into a 1.5 ml microfuge tube (not provided). Add 180 μl of ethanol and mix by vortexing for 15 seconds.

   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. 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 6,000xg for 1 minute at room temperature or 4°C.

   Note: Keep the filtrate containing miRNA.

6. Discard the spin/vac column. Add 500 μl isopropanol to the filtrate, vortex for 15 seconds and centrifuge at 12,000 x g for 10 minutes.
7. Discard the supernatant.
8. Add 700 μl ice-cold 70% ethanol and centrifuge at 12,000xg for 5 minutes.
9. Discard the supernatant and invert the microfuge tube on absorbent toweling to drain off residual solution. Dry the miRNA for 5-10 minutes at room temperature.

   Note: Make sure that no ethanol is left in the miRNA pellet after drying. Residual ethanol will inhibit many enzymatic reactions.

10. Add 70 μl of Buffer TE (DNase&RNase-free) to the 2 ml microfuge tube.

    Note: If the miRNA pellet appears difficult to resuspend, occasional gentle swirling or agitation may be required.

III. Purification of miRNA from Cultured Cells

This protocol is designed for the isolation of total RNA from up to $1x10^7$ mammalian cells grown in suspension, in monolayer or as a cell suspension isolated from animal tissues. If the number of cells is $\leqslant 2x10^6$, reduce the volumes of R-I, R-II, ethanol and isopropanol by half. All other buffer volumes should remain unchanged. If the number of cells is $>1x10^7$, the volumes of R-I, R-II, ethanol 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	$4\text{-}5\times10^4$
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	1×10^6
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 $2x10^6$-$1x10^7$cells in suspension and transfer into a 1.5 ml microfuge tube (provided). Centrifuge at 2,000xg for 5 minutes to pellet the cells. Discard the supernatant.

2. Add 400 μl of Buffer R-I. Lyse the cells by pipetting up and down 8-10x then homogenize the sample by passing it 8-10x through a 1 ml syringe fitted with a 21-25-gauge needle. Be careful to minimize foaming.
3. Add 150 μl of Buffer R-II and vortex for 15-30 seconds. Centrifuge at 12,000xg for 5 minutes at room temperature to pellet DNA and protein.

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 400 μl of Buffer R-I into each well. Lyse the cells by pipette up and down 8-10x, then homogenize the sample by passing it 8-10x through a 1 ml syringe fitted with a 21-25-gauge needle. Be careful to minimize foaming.
2. Transfer 400 μl of the cell homogenate to a 1.5 ml microfuge tube (provided).
3. Add 150 μl of Buffer R-II and vortex for 15-30 seconds. Centrifuge at 12,000xg for 5 minutes at room temperature to pellet DNA and protein.

Proceed with protocol, below

4. Transfer the clarified supernatant into a 1.5 ml microfuge tube (not provided). Add 180 μl of ethanol and mix by vortexing for 15 seconds.
   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. 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 6,000xg for 1 minute at room temperature or 4°C.
   Note: Keep the filtrate containing miRNA.

6. Discard the spin/vac column. Add 500 μl isopropanol to the filtrate, vortex for 15 seconds and centrifuge at 12,000xg for 10 minutes.
7. Discard the supernatant.
8. Add 700 μl ice-cold 70% ethanol and centrifuge at 12,000xg for 5 minutes.
9. Discard the supernatant and invert the microfuge tube on absorbent toweling to drain off residual solution. Dry the miRNA for 5-10 minutes at room temperature.
   Note: Make sure that no ethanol is left in the miRNA pellet after drying. Residual ethanol will inhibit many enzymatic reactions.

10. Add 70 μl of Buffer TE (DNase&RNase free) to the 2 ml microfuge tube.
    Note: If the miRNA pellet appears difficult to resuspend, occasional gentle swirling or agitation may be required.

Overview

Add sample and 400 μl of Buffer R-I — Lysis

Add 150 μl of Buffer R-II
Centrifuge at 12,000xg for 5 minutes — Neutralization

Add 180 μl of ethanol

Removal of large RNA

Add 500 μl of isopropanol — Precipitation

Add 700 μl of 70% ethanol — Washing

Add 70 μl of Buffer TE
(DNase&RNase-free) — Elution

Troubleshooting

1. Little or no miRNA eluted

- Inefficient cell lysis due to insufficient mixing of the samples with Buffer R-I
- 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 $A_{260/A280}$ value

- Inefficient cell lysis due to insufficient mixing of the samples with Buffer R-I
- Inadvertent transfer of pellet material to spin/vac column after Buffer R-II addition and centrifugation

3. Failure of miRNA precipitate/pellet

- Insufficient g-force or centrifugation time
- Be sure 100% isopropanol was used to precipitate miRNA

4. miRNA degraded

- Inappropriate handing 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 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.