DANAGENE microRNA KIT

Ref. 0805 50 preps

1. INTRODUCTION

DANAGENE microRNA Kit provides a rapid and efficient method for the isolation and purification of small RNA molecules (<200 nt) from cultured animal cells, small tissue samples, and bacterial cells. These small RNAs include regulatory RNA molecules such as **microRNA (miRNA**) and **short interfering RNA (siRNA).**

Most commercial RNA purification kits do not recover RNA molecules smaller than < 200 nucleotides, using an approach consisting of two sequential filtrations with different ethanol concentrations, **an RNA fraction highly enriched in RNA species < 200 nucleotides can be obtained with of DANAGENE microRNA Kit.**

Features:

- **microRNA isolation from cultured cells and tissues.**
- **Fast protocol: 25 minutes.**
- **microRNA isolated without the use of harmful chemicals as phenol or chloroform.**
- **Efficient isolation of small RNA species using 2 column process, resulting in minimal contamination of larger RNA and gDNA.**
- **Elution volume: 30 µl.**
- **Purified RNA can be used in a number of downstream applications including: qPCR, reverse transcription PCR, Northern blotting, RNase protection and expression array assays.**

2. KIT COMPONENTS

	50 preps	Stockage
Lysis Buffer RNA	**20 ml**	Room temperature
Wash Buffer 1	**6 ml**	Room temperature
Wash Buffer 2*	**10 ml**	Room temperature
Nuclease-free water	**4 ml**	Room temperature
Large RNA removal column	**50 units**	Room temperature
microARN column	**50 units**	Room temperature
Collection tubes	**150 units**	Room temperature

(*) These solutions must be prepared as indicated in the Preliminary Preparations section of the protocol.

The RNA Lysis Buffer and Wash Buffer 1 contain guanidinium thiocyanate which is a potent irritant, wear protective gloves and goggles. Both buffers can form dangerous reactive components when combined with bleach.

2.1Equipment and additional reagents required

- Ethanol 100 %
- Microcentrifuge.
- Micropipettes
- RNase-free 1.5 ml or 2.0 ml microfuge tubes.
- Mechanical homogenizer.
- Liquid Nitrogen , mostar and pestle..
- β-mercaptoethanol.

3. PROTOCOL

3.1 Preliminary Preparations

- **Add 40 ml of Ethanol 100 % to the Wash Buffer 2**. Keep the container closed to avoid the ethanol evaporation.

- **OPTIONAL:** The use of β-mercaptoethanol in lysis is highly recommended for most animal tissues, particularly those know to have a high RNAse content (ex.pancreas), as well as for most plant tissues. It is also recommended for uses who whish to isolate RNA for sensitive downstream applications**. Add 10 µl of β-mercaptoethanol (provided by the user) to each 1 ml of Lysis Buffer RNA required**. Alternatively, the Lysis Buffer RNA can be used as provided.

3.2 General Remarks

Stabilization of RNA in biological samples

The RNA is not protected until the sample material freezes instantaneously or breaks / lyses in the presence of inhibitors or denaturing agents of the RNases.
Immediate stabilization of the DNA expression pattern is a prerequisite for accurate gene-expression analysis.

Methods for sample collection

• Use a freshly collected sample for immediate lysis and RNA purification.

• Samples can be stored in the lysis buffer after lysis at -80 ° C for one year, at 4º C for up to 24 hours or up to several hours at room temperature. Frozen samples in lysis buffer should be thawed slowly before starting RNA isolation.

• Freeze the sample in liquid N2 immediately after collection and store at 80ºC. This can be done with a mortar and pestle. Make sure that the sample does not thaw before contact with the lysis buffer.

• Samples can be submerged and stored in **DANAPROTECT SOLUTION or RNAlater**. Before using such samples, remove excess DANAPROTECT SOLUTION from the tissue before use.

Disruption and homogenization of starting materials for RNA isolation

Efficient disruption and homogenization of the starting material is an absolute requirement for all total RNA isolation procedures. Disruption and homogenization are two distinct steps.

Disruption: Complete disruption of cells walls and plasma membranes of cells and organelles is absolutely required to release all the RNA contained in the sample. Different samples requires different methods to achieve complete disruption. Incomplete disruption results in significantly reduced yields.

Homogenization: Is necessary to reduce the viscosity of the cells lysates produced by disruption. Homogenization shears the high-molecular-weight genomic DNA and other high-molecular-weight cellular components to create a homogeneous lysate. Incomplete homogenization results in efficient binding of RNA and therefore reduced yields.

Some disruption methods simultaneously homogenize the sample (rotor-stator-homogenizer) while others require an additional homogenization step

3.3 Protocol for microRNA purification from human or animal tissues

Process samples of up to **25 mg** of fresh or frozen tissue. Grind the sample to a fine powder in the presence of liquid Nitrogen with a pestle and mortar.

1. Transfer the tissue into a mortar that contains an appropriate amount of liquid nitrogen to cover the sample. Grind the tissue thoroughly using a pestle. Allow the liquid nitrogen to evaporate, without allowing the tissue to thaw.

2. Transfer the sample to an RNase-free tube (not provided)) and add **400 µl de Lysis Buffer RNA** . Homogenize by passing the lysate 5-10 times through a 20-G (0.90 mm) gauge needle attached to a syringe or an electrical homogenizer.

3. Incubate at room temperature for 5 minutes.

4. Centrifuge for **3 minutes a maximum speed.**

5. Transfer de supernatant to another RNase-free microcentrifuge tube. **IMPORTANT:Note the volume of the supernatant/lysate.**

6. Add a volume of **100% ethanol** that is equivalent to (55 µl of 100 % ethanol is added to every 100 µl of supernatant/lysate). Mix by vortex for 10 seconds.

7. Assemble a **Large RNA removal column** with one the provided collection tubes.

8. Apply the clarified lysate with ethanol into the columns. **Centrifuge at 10.000 rpm for 1 minute.**

9. **Retain the liquid that has passed through the column and that is in the collection tube, which contains the small RNA species.**

10. Add **700 µl de ethanol 100% to the lysate .**Mix well.

11. Pass half of the lysate to a microRNA column with its collection tube. **Centrifuge at 10.000 rpm for 30 seconds.**

12. Place the column in a new collection tube. **Pass the other half and centrifuge at 10,000 rpm for 30 seconds.**

13. Add **100 µl of RNA Wash Buffer 1.** Centrifuge at maximum speed for 1 minute.

14. Add **700 µl of RNA Wash Buffer 2.** Centrifuge at maximum speed for 1 minute.

15. Centrifuge for **3 minutes** at **maximum speed (11.000 x g)** to completely dry the membrane. Place the column in a 1.5 ml (not supplied) microtube.

16. Remove the collection tube and insert the Spin Column in a 1.5 ml microtube **. Add 25-30 µl Nuclease-free water** into reservoir of Spin column. Dispense buffer directly onto the silica membrane. **Incubate at room temperature for 2 min.**
It is very important to add the elution buffer in the centre of the membrane to be completely wet.

17. **Centrifuge at 10.000 rpm for 60 seconds.** Collect 25-30 µl and redeposit in the centre of the membrane. This increases yield.

18. **Incubate 2 minutes and centrifuge at maximum speed for 1 minute.**

3.4 Protocol for microARN purification from cultured cells (< 1 x 10^7)

Adherent Cultured Animal Cells
Aspirate cell-culture medium and add an equal amount of PBS in order to wash the cells. Aspirate PBS. Add 0.1-0.3% trypsinin PBS and incubate for an appropriate time to detach the cells the dish surface. After cell detachment , add cell culture medium, transfer cells to an appropriate tube and pellet by centrifugation. Remove supernatant and continue with the point 1.

Suspension Cultured Animal Cells
Transfer cells (up to 1 x 10^7) to a 1.5 ml microcentrifuge tube. Harvest cells by centrifugation, remove the supernatant and continue with the point 1.

1. Add **400 µl of Lysis Buffer RNA**. Lyse the cells using up-down the micropipette to dissolve the cell pellet. Make sure that all pellets are dissolved before proceeding with the next step.

2. Add **210 µl ethanol 100%** to the lysate. Mix by vortex for 10 seconds.

3. Assemble a **Large RNA removal column** with one the provided collection tubes.

4. Apply the clarified lysate with ethanol into the columns. **Centrifuge at 10.000 rpm for 1 minute.**

5. **Retain the liquid that has passed through the column and that is in the collection tube, which contains the small RNA species.**

6. Add **700 µl de ethanol 100% to the lysate .**Mix well.

7. Pass half of the lysate to a microRNA column with its collection tube. **Centrifuge at 10.000 rpm for 30 seconds.**

8. Place the column in a new collection tube. **Pass the other half and centrifuge at 10,000 rpm for 30 seconds.**

9. Add **100 µl of RNA Wash Buffer 1.** Centrifuge at maximum speed for 1 minute.

10. Add **700 µl of RNA Wash Buffer 2.** Centrifuge at maximum speed for 1 minute.

16. Centrifuge for **3 minutes** at **maximum speed (11.000 x g)** to completely dry the membrane. Place the column in a 1.5 ml (not supplied) microtube.

12. Remove the collection tube and insert the Spin Column in a 1.5 ml microtube **. Add 25-30 µl Nuclease-free water** into reservoir of Spin column. Dispense buffer directly onto the silica membrane. **Incubate at room temperature for 2 min.**
It is very important to add the elution buffer in the centre of the membrane to be completely wet.

13. **Centrifuge at 10.000 rpm for 60 seconds.** Collect 25-30 µl and redeposit in the centre of the membrane. This increases yield.

14. **Incubate 2 minutes and centrifuge at maximum speed for 1 minute.**

3.5 Protocol for microARN purification from bacteria cultures.

Note: It is recommended that no more than 1 x 10^9 bacteria cells be used. Bacterial growth can be measured using a spectrophotometer, as a general rule, an E.coli culture containing 1 x 10^9 bacteria cells has an OD_{600} of 1.0.
Prepare the appropriate lysozyme-containing TE buffer , this solution should be prepared with sterile, Rnase free TE buffer, and kept on ice until needed. For Gram-negative the concentration of lysozyme will be of 1 mg/ml and for Gram-positive the concentration of lysozyme will be of 3 mg/ml.

1. Pellet bacteria by centrifugation at **14.000 x g (aprox. 14.000 rpm) for 1 minute.**
2. Decant supernantant, and carefully remove any remaining media by aspiration. Resuspended the bacteria in **100 µl of appropriate lysozyme-containing TE by** vortexing. Incubate at room temperature for 10 minutes.
3. Add **300 µl of Lysis Buffer RNA** . Lyse cells by vortex for 15 seconds.
4. Add **210 µl of ethanol 95-100%** to lysate. Mix by vortex for 10 seconds. Ensure that the entire pellet is completely dissolved before proceeding to the next step.
5. Assemble a **Large RNA removal column** with one the provided collection tubes.
6. Apply the clarified lysate with ethanol into the columns. **Centrifuge at 10.000 rpm for 1 minute.**
7. **Retain the liquid that has passed through the column and that is in the collection tube, which contains the small RNA species.**
8. Add **700 µl de ethanol 100% to the lysate .**Mix well.
9. Pass half of the lysate to a microRNA column with its collection tube. **Centrifuge at 10.000 rpm for 30 seconds.**
10. Place the column in a new collection tube. **Pass the other half and centrifuge at 10,000 rpm for 30 seconds.**
11. Add **100 µl of RNA Wash Buffer 1.** Centrifuge at maximum speed for 1 minute.
12. Add **700 µl of RNA Wash Buffer 2.** Centrifuge at maximum speed for 1 minute.
13. Centrifuge for **3 minutes** at **maximum speed (11.000 x g)** to completely dry the membrane. Place the column in a 1.5 ml (not supplied) microtube.
14. Remove the collection tube and insert the Spin Column in a 1.5 ml microtube **. Add 25-30 µl Nuclease-free water** into reservoir of Spin column. Dispense buffer directly onto the silica membrane. **Incubate at room temperature for 2 min.**
15. It is very important to add the elution buffer in the centre of the membrane to be completely wet.
16. **Centrifuge at 10.000 rpm for 60 seconds.** Collect 25-30 µl and redeposit in the centre of the membrane. This increases yield.
17. **Incubate 2 minutes and centrifuge at maximum speed for 1 minute.**

3.6 Protocol for microARN purification from yeast cultures

Prepare the appropriate amount of lyticase resuspension buffer have the following composition: 50mM Tris, pH 7.5, 10 mM EDTA, 1M Sorbital, 0.10 % β-mercaptoethanol and 1 unit / µl of lyticase. This solution should be prepared with sterile, RNase free reagents, and kept on ice until needed.

1. Pellet yeast for centrifugation **(aprox. 14.000 rpm) for 1 minute.**

2. Decant supernantant, and carefully remove any remaining media by aspiration. Resuspended the yeast in **100 µl of Lyticase resuspension buffer by vortexing**. Incubate at 37ºC for 10 minutes.

3. Add **300 µl of Lysis Buffer RNA** . Lyse cells by vortex for 15 seconds.

4. Add **210 µl of ethanol 95-100%** to lysate. Mix by vortex for 10 seconds. Ensure that the entire pellet is completely dissolved before proceeding to the next step.

5. Assemble a **Large RNA removal column** with one the provided collection tubes.

6. Apply the clarified lysate with ethanol into the columns. **Centrifuge at 10.000 rpm for 1 minute.**

7. **Retain the liquid that has passed through the column and that is in the collection tube, which contains the small RNA species.**

8. Add **700 µl de ethanol 100% to the lysate .**Mix well.

9. Pass half of the lysate to a microRNA column with its collection tube. **Centrifuge at 10.000 rpm for 30 seconds.**

10. Place the column in a new collection tube. **Pass the other half and centrifuge at 10,000 rpm for 30 seconds.**

11. Add **100 µl of RNA Wash Buffer 1.** Centrifuge at maximum speed for 1 minute.

12. Add **700 µl of RNA Wash Buffer 2.** Centrifuge at maximum speed for 1 minute.

13. Centrifuge for **3 minutes** at **maximum speed (11.000 x g)** to completely dry the membrane. Place the column in a 1.5 ml (not supplied) microtube.

14. Remove the collection tube and insert the Spin Column in a 1.5 ml microtube **. Add 25-30 µl Nuclease-free water** into reservoir of Spin column. Dispense buffer directly onto the silica membrane. **Incubate at room temperature for 2 min.**

15. It is very important to add the elution buffer in the centre of the membrane to be completely wet.

16. **Centrifuge at 10.000 rpm for 60 seconds.** Collect 25-30 µl and redeposit in the centre of the membrane. This increases yield.

17. **Incubate 2 minutes and centrifuge at maximum speed for 1 minute.**

3.7 Protocol for microARN from plant tissues.

Note: The maximum recommended input of plant tissue is 30 mg o 5 x 10^6 plant cells. Both fresh and frozen plant samples can be used for this protocol. Samples should be flash frozen in liquid nitrogen and transfer immediately to a -70ºC freezer for long-term storage. Do not allow frozen tissues to thaw prior to grinding wit the mortar and pestle in order to ensure that the integrity of the RNA is not compromised.

1. Transfer the tissue into a mortar that contains an appropriate amount of liquid nitrogen to cover the sample. Grind the tissue thoroughly using a pestle. Allow the liquid nitrogen to evaporate, without allowing the tissue to thaw.

2. Transfer the sample to an RNase-free tube (not provided)) and add **400 µl de Lysis Buffer RNA** . Homogenize by passing the lysate 5-10 times through a 20-G (0.90 mm) gauge needle attached to a syringe or an electrical homogenizer.

3. Incubate at room temperature for 5 minutes.

4. Centrifuge for **3 minutes a maximum speed.**

5. Transfer de supernatant to another RNase-free microcentrifuge tube. **IMPORTANT:Note the volume of the supernatant/lysate.**

6. Add a volume of **100% ethanol** that is equivalent to (55 µl of 100 % ethanol is added to every 100 µl of supernatant/lysate). Mix by vortex for 10 seconds.

7. Assemble a **Large RNA removal column** with one the provided collection tubes.

8. Apply the clarified lysate with ethanol into the columns. **Centrifuge at 10.000 rpm for 1 minute.**

9. **Retain the liquid that has passed through the column and that is in the collection tube, which contains the small RNA species.**

10. Add **700 µl de ethanol 100% to the lysate .**Mix well.

11. 11.Pass half of the lysate to a microRNA column with its collection tube. **Centrifuge at 10.000 rpm for 30 seconds.**

12. Place the column in a new collection tube. **Pass the other half and centrifuge at 10,000 rpm for 30 seconds.**

13. Add **100 µl of RNA Wash Buffer 1.** Centrifuge at maximum speed for 1 minute.

14. Add **700 µl of RNA Wash Buffer 2.** Centrifuge at maximum speed for 1 minute.

15. Centrifuge for **3 minutes** at **maximum speed (11.000 x g)** to completely dry the membrane. Place the column in a 1.5 ml (not supplied) microtube.

16. Remove the collection tube and insert the Spin Column in a 1.5 ml microtube **. Add 25-30 µl Nuclease-free water** into reservoir of Spin column. Dispense buffer directly onto the silica membrane. **Incubate at room temperature for 2 min.**

It is very important to add the elution buffer in the centre of the membrane to be completely wet.

17. **Centrifuge at 10.000 rpm for 60 seconds.** Collect 25-30 μl and redeposit in the centre of the membrane. This increases yield.

18. **Incubate 2 minutes and centrifuge at maximum speed for 1 minute.**

4. PROBLEM GUIDE AND POSSIBLE ANSWER

For any doubts or additional questions about the protocol, please contact the technical service of DANAGEN-BIOTED S.L info@danagen.es