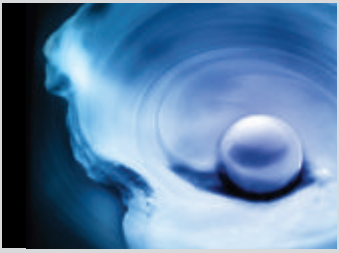

ISOLATE II
miRNA Kit
(Phenol free)

Product Manual

BIOLINE

A Meridian Life Science® Company

ISOLATE II miRNA Kit (Phenol free)

ISOLATE II miRNA Kit (Phenol free)

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1. KIT CONTENTS

COMPONENT	25 Preps
ISOLATE II Large RNA Removal Columns (white)	25
ISOLATE II miRNA Columns (black)	25
Collection Tubes (2ml)	50
Elution Tubes (1.7ml)	50
Lysis Buffer RX*	40ml
Wash Buffer W1† (concentrate)	38ml
DNase I Solution (RNase-free)	0.8ml
DNase I Reaction Buffer DRB	6ml
RNA Elution Buffer	6ml
Product Manual	1
Bench-Top Protocol	1

** Contains a guanidine salt. Not compatible with disinfectants containing bleach or acidic solutions. See safety information in section 4.*
† Before use, add 90ml of 96-100% ethanol and mark wash buffer bottle label.

2. DESCRIPTION

The ISOLATE II miRNA kit allows convenient processing of multiple samples in 25 minutes without the use of laborious methods such as CsCl ultracentrifugation or handling of toxic chemicals such as phenol/chloroform.

The ISOLATE II miRNA Kit is specially developed for the rapid phenol free isolation of highly enriched small RNA (<200nt) from cultured cells, mammalian tissue, bacteria, blood and other biofluids. Small RNA include regulatory RNA molecules such as microRNA (miRNA), short interfering RNA (siRNA), as well as transfer RNA (tRNA) and 5S ribosomal RNA (rRNA). Small RNAs, such as miRNAs and siRNAs are ~20-25 nucleotide RNAs involved in regulating gene expression by binding to messenger RNAs (mRNAs).

Isolation is based on a fast spin column format using a novel RNA affinity resin as the separation matrix so that small RNA are preferentially purified from other cellular components. This kit does not require the use of phenol or chloroform.

Samples are first lysed in the presence of guanidinium thiocyanate, a chaotropic salt which immediately deactivates endogenous RNases to ensure purification of intact RNA. Ethanol is added to the sample and then applied to a Large RNA Removal Column. The larger RNA molecules bind the column membrane, whilst smaller RNA species pass into the flow-through. Ethanol is then added to the flow-through and is applied to the miRNA Column where small RNAs bind the membrane and contaminants are efficiently washed away. High-quality small RNA are eluted and are ready for use in various applications, including:

- Real-Time PCR (qPCR)
- Reverse transcriptase PCR (RT-PCR)
- cDNA synthesis
- Next generation sequencing
- Northern blotting
- Microarray analysis

A simple series of wash and spin steps allows for larger RNA molecules from the same sample to be sequentially purified and used for a wide variety of downstream applications.

Please read this manual carefully to familiarise yourself with the ISOLATE II miRNA protocol before starting (also available on www.bioline.com). More experienced users can refer to the Bench-Top Protocol for quick referencing during the procedure.

3. STORAGE

Store DNase I at -20°C upon arrival. All other components should be stored at room temperature (18-25°C). Storage at lower temperatures may cause precipitation of salts.

4. SAFETY INFORMATION

When working with chemicals, always wear a suitable lab coat, gloves and safety glasses.

Lysis Buffer RX contains guanidinium thiocyanate. This chemical is harmful in liquid form when in contact with skin or ingested. If the solution is allowed to dry, the powder is harmful if inhaled.

CAUTION: Do not add bleach directly to solutions or sample preparation waste containing guanidinium salts. Reactive compounds and toxic gases can form. In the case of spillage, clean the affected area with a suitable laboratory detergent and water.

For detailed information, please consult the material data safety sheet (MSDS) available on our website at www.bioline.com.

5. PRODUCT SPECIFICATIONS

The ISOLATE II miRNA Kit is specially designed for the rapid isolation of highly enriched small RNA species (<200nt). Larger RNA species can be sequentially purified from the same sample. The kit is compatible with a wide range of tissues and cells and the preparation time is approximately 25 minutes for 10 preps. The isolated RNA is of high purity (A_{260}/A_{280} ratio: >1.9) for high quality samples (see below) and is ready to use in various downstream applications.

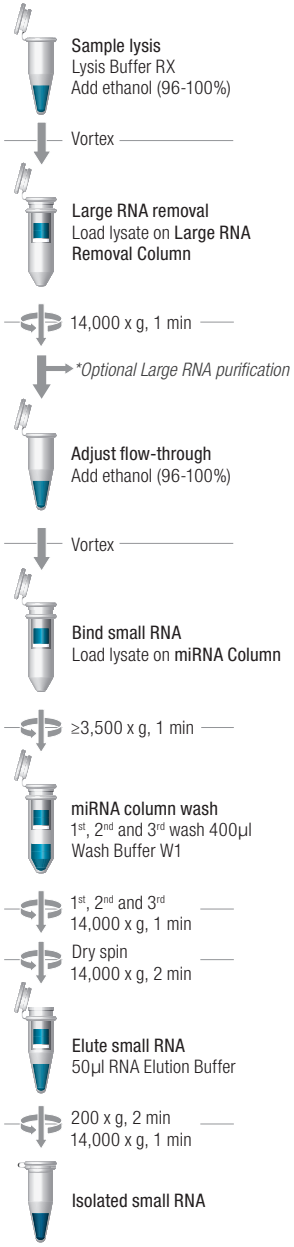
ISOLATE II miRNA COLUMN SPECIFICATIONS	
Max. binding capacity	50µg RNA
RNA size distribution	<200 nucleotides
A_{260}/A_{280} ratio*	1.9-2.1
Max. column loading volume	650µl
Min. elution volume	20µl
Max. amount of starting material	
Cultured cells (human/other mammalian)	3 x 10^6 cells
Tissue (human/other mammalian)	5-25 mg
Bacteria	1 x 10^9 cells
Blood and other biofluids	100µl

**Typically, the A_{260}/A_{280} ratio exceeds 1.9, indicating excellent RNA purity.*

The following component is also included in the kit:

- DNase I (RNase-free) for eliminating genomic DNA contamination by on-column digestion or by digestion in solution (for the most sensitive applications).

Small RNA Isolation



**See section 10*

6. EQUIPMENT AND REAGENTS TO BE SUPPLIED BY USER

When working with chemicals, always wear a suitable lab coat, protective goggles and disposable gloves. Please consult the relevant MSDS from the product supplier for further information and see section 4.

The following may be supplied by the user:

- ß-mercaptoethanol (ß-ME)* (Optional for Lysis Buffer RX)
- 96-100% ethanol† (for Wash Buffer W1)
- Equipment for sample disruption and homogenization (see section 7.2). One or more of the following are required depending on chosen method:
  - o PBS (RNase-free) and trypsin
  - o Needle and syringe (both RNase free)
  - o Mortar and pestle
  - o Rotor-stator homogenizer
  - o Liquid nitrogen
- Molecular biology grade water
- RNase-free microcentrifuge tubes (1.5ml)
- Sterile RNase-free tips
- Benchtop microcentrifuge (capable of 14,000 x g)

** TCEP is also a suitable reducing agent instead of ß-ME.*
† Molecular biology grade ethanol is recommended. Do not use denatured alcohol which contains unwanted additives such as methanol and acetone.

For the bacterial protocol:

- Lysozyme-containing TE buffer;
  - o For Gram-negative bacteria, 1mg/ml lysozyme in TE buffer
  - o For Gram-positive bacteria, 3mg/ml lysozyme in TE buffer

For the yeast protocol:

- Resuspension buffer with lyticase:
  - o 50mM Tris pH 7.5, 10mM EDTA, 1M sorbitol, 0.1% ß-ME, 1unit/µl lyticase

7. IMPORTANT NOTES

The protocol steps are homogenization and lysis from different starting materials (section 8) and purification of small RNA (section 9). The steps in section 9 apply to all starting materials. The protocol in section 10 gives the option for users to sequentially purify the larger RNA species from the same sample. Optional DNase I treatment protocols are provided in the Appendix.

- Section 8 contains the lysate preparation protocols for different starting materials; ensure you follow the specific protocol for your sample.
- Section 9 contains the protocol to purify small RNA from all types of lysate.
- Section 10 contains the optional large RNA purification protocol for users also wishing to purify larger RNA species from the same sample.
- The Appendix contains the optional protocols for on-column or in-solution DNase I treatment.

The ISOLATE II miRNA Kit purification procedures can be performed at room temperature. Handle the eluted RNA carefully to avoid contamination by RNases, often found on labware, fingerprints and dust. For optimal RNA stability, keep RNA frozen at -20°C for short-term or -80°C for long-term storage. When working with RNA samples in downstream applications, keep the RNA solution on ice.

Two types of spin columns are provided with this kit: the ISOLATE II **Large RNA Removal Column** (white ring) and the ISOLATE II **miRNA Column** (black ring). Ensure the correct column is used for each step of the procedure.

All centrifugation steps are carried out in a benchtop microcentrifuge at 14,000 x g except where noted. Perform all centrifugation steps at room temperature.

Ensure that all solutions are at room temperature prior to use.

It is important to work quickly when purifying RNA (see hints and tips on working with RNA at www.bioline.com/isolate).

7.1 HANDLING AND STORING STARTING MATERIALS

RNA is not protected against digestion until the sample is flash frozen or disrupted in the presence of RNase inhibiting or denaturing reagents. Samples should be flash frozen in liquid nitrogen immediately and can be stored at -80°C for several months, or processed as soon as possible. Following disruption and homogenization in Lysis Buffer RX, samples can be kept at -80°C for up to one year, at 4°C for up to 24 hours or at room temperature for several hours. Frozen samples are stable for up to 6 months. Frozen samples in Lysis Buffer RX should be thawed slowly before starting the isolation of total RNA.

7.2 DISRUPTING AND HOMOGENIZING STARTING MATERIALS

For all RNA purification procedures, efficient disruption and homogenization of starting material is essential. To release all RNA contained in a sample, the total disruption of cell walls, plasma membranes and organelles must occur. Incomplete disruption results in reduced RNA yields. Homogenization reduces lysate viscosity following disruption and also facilitates efficient binding of RNA to the column membrane. Incomplete homogenization results in inefficient binding of RNA to the membrane and therefore reduced RNA yields.

7.2.1 Cells grown in a monolayer

Remove the cell culture medium completely. Incomplete removal of the medium will inhibit lysis of the cells and compromise the efficiency of RNA isolation. Wash with PBS and add Lysis Buffer RX immediately to the cell culture plate. Refer to step 2 of section 8.1.1.

7.2.2 Cells grown in suspension

Centrifuge an appropriate number of cells and remove all supernatant by aspiration. Wash with PBS and lyse by adding Lysis Buffer RX. Refer to step 2 of section 8.1.2.

7.2.3 Lifting of adherent cells with trypsin

To lift adherent growing cells using trypsin, first aspirate the cell culture medium. Add an equal volume of PBS to wash the cells and aspirate excess liquid. Add 0.1-0.3% trypsin in PBS to the washed cells. Incubate until the cells are detached. Add fresh sterile culture medium and transfer cells to an appropriate tube (not supplied). Pellet cells by centrifugation for 10 min at 200 x g. Remove supernatant and add Lysis Buffer RX to the cell pellet. Refer to section 8.1.2.

7.2.4 Disruption using a mortar and pestle

An RNase-free mortar and pestle can be used in combination with liquid nitrogen to disrupt and lyse frozen or fibrous tissue samples, which are often solid. Grind the frozen tissue into a fine powder and add liquid nitrogen as necessary. It is important to ensure the sample does not thaw during or after grinding. After grinding, transfer tissue powder into a liquid nitrogen-cooled tube and allow the liquid nitrogen to evaporate. Add Lysis Buffer RX with reducing agent to the powdered tissue and mix immediately. Homogenize the sample with a nuclease-free 20 gauge (0.9mm diameter) syringe needle.

7.2.5 Disruption and homogenization using a rotor-stator homogenizer

Rotor-stator type tissue homogenizers can homogenize, disrupt and emulsify mammalian tissue samples in the presence of lysis buffer in seconds or minutes. Homogenization time depends on sample size and toughness. The spinning rotor disrupts and homogenizes the sample by turbulence and mechanical shearing. Foaming can be minimized by keeping the rotor tip submerged. Select a suitably sized homogenizer: 5-7mm diameter rotors can be used for homogenization in microcentrifuge tubes.

7.3 BUFFER PREPARATION AND PARAMETERS

Ensure 96-100% ethanol is available. Prepare the following:

7.3.1 Preparing Wash Buffer W1 with ethanol

Add 90ml of 96-100% ethanol to the 38ml of Wash Buffer W1 Concentrate to give a final volume of 128ml.

Note: Mark the label of the bottle to indicate ethanol was added. Store Wash Buffer W1 at room temperature (18-25°C).

7.3.2 Preparing Lysis Buffer RX with ß-mercaptoethanol (optional)

Optional: The use of ß-mercaptoethanol (ß-ME) in lysis is highly recommended for most mammalian tissues, particularly those known to have a high RNase content (e.g. pancreatic tissues). It is also recommended for users who wish to isolate RNA for sensitive downstream applications. Add 10µl of ß-ME (provided by the user) to each 1ml of Buffer RX required. ß-ME is toxic and should be dispensed in a fume hood. Alternatively, Buffer RX can be used as provided.

Note: TCEP can also be used as an alternative reducing agent. Use TCEP at a final concentration of 10mM within Lysis Buffer RX.

7.3.3 Preparing DNase I (RNase-free) (optional)

Optional on-column digestion: For each on-column reaction to be performed, prepare a mix of 15µl of DNase I and 100µl of DNase Reaction Buffer DRB. Mix gently by inverting a few times.

Optional in-solution digestion: In a microcentrifuge tube, mix together 10µl of DNase Reaction Buffer DRB, 2.5µl of DNase I and up to 87.5µl of RNA solution. For lower starting volumes of RNA, bring the volume up to 100µl using RNase-free water. Gently swirl tube to mix solution.

Note: Do not vortex the DNase I as the enzyme is particularly sensitive to mechanical denaturation. Dispense into aliquots to avoid excessive freeze-thawing. Store aliquots at -20°C.

7.3.4 Elution parameters

Elute RNA using RNA Elution Buffer (included). The standard elution protocol can be modified for different applications.

- To achieve high yield, perform two successive elution steps with an elution volume described in the individual protocol (90-100% recovery rate). You may elute into the same or a different microcentrifuge tube depending on your application.
- For both high-yield and high-concentration, elute with the standard elution volume. Then re-apply eluate onto the column for re-elution into the same microcentrifuge tube.

Always place eluted RNA on ice immediately to prevent degradation by RNases. For short-term storage freeze at -20°C but for long-term storage freeze at -80°C.

7.4 ELIMINATING GENOMIC DNA CONTAMINATION

For most applications, genomic DNA is undetectable in preparations of RNA using the ISOLATE II miRNA Kit. Genomic DNA contamination is efficiently removed by on-column digestion with DNase I (see optional section 7.3.3 and Appendix A). The optional on-column DNase I digestion could be applied to both the small RNA purification as well as the optional large DNA purification. However, residual genomic DNA contamination may be detected in very sensitive applications e.g. probe-based real-time PCR. A DNase I digest in the eluate can be performed to remove even traces of contaminating DNA (see optional section 7.3.3 and Appendix B).

8. LYSATE PROTOCOLS

The steps for preparing the lysate in this section are different depending on the starting material. Please ensure you follow the correct procedure for preparing the lysate from your starting material (see section 7.2). The subsequent steps detailed in section 9 are the same in all cases.

Before you start:

- Ensure Lysis Buffer RX and Wash Buffer W1 are prepared (see section 7.3).
- Ensure that all solutions are at room temperature before use.
- Two types of spin columns are provided with this kit: the **Large RNA Removal Column** (white ring) and the **miRNA Column** (black ring). Ensure the correct column is used for each step of the procedure.
- All centrifugation steps are carried out in a benchtop microcentrifuge at 14,000 x g except where noted. Perform all centrifugation steps at room temperature.
- It is important to work quickly during this procedure.

8.1 LYSATE PREPARATION FROM CULTURED CELLS

Before you start:

- For optimal results, it is recommended that up to 3 x 10^6 eukaryotic cells are used. A maximum of 5 x 10^6 cells may be used as starting material, but slight cross-contamination of genomic DNA in the RNA fraction may be observed.
- A hemocytometer can be used in conjunction with a microscope to count the number of cells. As a general guideline, a confluent 3.5cm plate of HeLa cells will contain approx. 10^6 cells.
- Cell pellets can be stored at -80°C for later use or used directly in the procedure. Determine the number of cells present before freezing.
- Frozen pellets should be stored for no longer than 2 weeks at -80°C to ensure the integrity of the RNA is not compromised.
- Frozen cell pellets should not be thawed prior to beginning the protocol. Add the Lysis Buffer RX directly to the frozen cell pellet (section 8.1.2, step 3).

Additional reagents/components required:

- PBS (RNase-free)

8.1.1 Cells growing in a monolayer

1. Aspirate both the media and the wash cell monolayer with an appropriate amount of PBS. Aspirate PBS.
2. Add 300µl of Lysis Buffer RX directly to culture plate.
   Note: This volume of lysis buffer can be used for a culture plate of up to 35mm in diameter or up to

one well of a 6-well culture dish.

3. Lyse cells by gently tapping culture dish and swirling buffer around plate surface for 5 min.
4. Transfer lysate to a 1.5ml RNase-free microcentrifuge tube (user supplied).

   Note: For input amounts greater than 10^6 cells, it is recommended that the lysate is passed 5-10 times through a nuclease-free 20 gauge (0.9mm) syringe needle at this point, in order to reduce the viscosity of the lysate prior to loading onto the column.

5. Add 150µl of 96-100% ethanol to the lysate. Mix by vortexing for 10s. **Proceed to section 9.**

8.1.2 Cells growing in suspension and lifted cells

1. Transfer cell suspension to a 1.5ml RNase-free microcentrifuge tube (user supplied) and centrifuge at no more than 200 x g for 10 min to pellet cells.

   Note: Refer to section 7.2.3 for protocol to lift cells using trypsin.

2. Carefully decant the supernatant to ensure that the pellet is not dislodged. Ensure that a few microliters of media are left behind with the pellet in order to ensure that the pellet is not dislodged.
3. Add 300µl of Lysis Buffer RX to the pellet. Lyse cells by vortexing for 15s. Ensure that the entire pellet is completely dissolved before proceeding to the next step.

   Note: For input amounts greater than 10^6 cells, it is recommended that the lysate is passed 5-10 times through a nuclease-free 20 gauge (0.9mm) syringe needle, in order to reduce the viscosity of the lysate prior to loading onto the column.

4. Add 150µl of 96-100% ethanol to the lysate. Mix by vortexing for 10s. **Proceed to section 9.**

8.2 LYSATE PREPARATION FROM MAMMALIAN TISSUE

Before you start:

- RNA in mammalian tissues is not protected from RNases after harvesting until the tissue is disrupted and homogenized. Therefore, it is important that the procedure is carried out as quickly as possible.
- Fresh or frozen tissues may be used for the procedure. Tissues should be flash-frozen in liquid nitrogen and transferred immediately to a -80°C freezer for long-term storage. Tissues may be stored at -80°C for several months. Do not allow frozen tissues to thaw prior to grinding with the mortar and pestle in order to avoid compromising the integrity of the RNA.
- Tissues stored in RNA*later*® are compatible with this protocol. Dry excessive liquid from tissue before starting.
- The maximum recommended input of tissue varies depending on the type of tissue being used. Please refer to Table 1 as a guideline for maximum tissue input amounts. If your tissue of interest is not included in the table, we recommend starting with an input of no more than 10mg.

TABLE 1: RECOMMENDED INPUT OF DIFFERENT TISSUES

Tissue	Max. Input Amount
Brain	25mg
Liver	20mg
Heart	5mg
Kidney, Spleen, Lung	10mg

8.2.1 Protocol

1. Determine amount of tissue by weighing.
2. Transfer the tissue into a mortar that contains enough liquid nitrogen to cover the sample. Grind thoroughly using a pestle.
3. Allow the liquid nitrogen to evaporate, without allowing the tissue to thaw.
4. Add 400µl of Buffer RX to the tissue sample and continue to grind until the sample has been homogenized. Homogenize by passing the lysate through a nuclease-free 20 gauge (0.9mm diameter) needle attached to a syringe 5-10 times.
5. Using a pipette, transfer the lysate into a 1.5ml RNase-free microcentrifuge tube (user supplied).
6. Spin the lysate for 2 min at 14,000 x g to pellet any cell debris. Transfer the supernatant to another 1.5ml RNase-free microcentrifuge tube (user supplied). Note the volume of the supernatant/lysate.
7. Add a volume of 96-100% ethanol equivalent to 50% of the lysate volume (e.g. Add 50µl of ethanol for every 100µl of lysate). Vortex briefly to mix. **Proceed to section 9.**

8.3 LYSATE PREPARATION FROM BLOOD AND OTHER BIOFLUIDS

Before you start:

- Blood derived from all human and animal sources is considered potentially infectious. All necessary precautions recommended by the appropriate authorities in the country of use should be taken when working with whole blood.
- We recommend the use of this kit to isolate RNA from non-coagulated blood samples (including fresh and anti-coagulant treated blood) and other biofluids such as semen, saliva and urine.
- It is recommended that no more than 100µl of blood or other biofluid sample is used in order to prevent clogging of the column.

8.3.1 Protocol

1. Transfer up to 100µl of non-coagulated blood/biofluid to a 1.5ml RNase-free microcentrifuge tube (user supplied).
2. Add 250µl of Lysis Buffer RX to the sample. Lyse cells by vortexing for 15s. Ensure that the mixture becomes transparent before proceeding with the protocol.
3. Add 150µl of 96-100% ethanol to the lysate. Mix by vortexing for 10s. **Proceed to section 9.**

8.4 LYSATE PREPARATION FROM BACTERIA

Before you start:

- Prepare the appropriate lysozyme-containing TE Buffer as indicated in Table 2. This solution should be prepared with sterile, RNase-free TE Buffer and kept on ice until needed. These reagents are to be provided by the user.
- It is recommended that no more than 10^9 bacterial cells are used as starting material in this procedure. As a general rule, an *E. coli* culture containing 1 x 10^9 cells/ml has an OD_{600} of 1.0.
- For RNA isolation, bacteria should be harvested in mid log-phase growth.
- Bacterial pellets can be used directly in this procedure or be stored at -80°C for later use.
- Frozen bacterial pellets should not be thawed prior to beginning the protocol. Add the lysozyme-containing TE Buffer directly to the frozen bacterial pellet (step 3).

TABLE 2: INCUBATION TIME FOR DIFFERENT BACTERIAL STRAINS

Bacteria Type	Lysozyme Concentration in TE Buffer	Incubation Time
Gram-negative	1mg/ml	5 min
Gram-positive	3mg/ml	10 min

8.4.1 Protocol

1. Pellet bacteria by centrifuging for 1 min at 14,000 x g.
2. Decant the supernatant and carefully remove any remaining media by aspiration.
3. Resuspend the bacteria by vortexing in 100µl of the appropriate lysozyme-containing TE Buffer (see Table 2) by vortexing. Incubate at room temperature for the time indicated in Table 2.
4. Add 200µl of Lysis Buffer RX and vortex vigorously for at least 10s.
5. Add 150µl of 96-100% ethanol to the lysate. Mix by vortexing for 10s. **Proceed to section 9.**

8.5 LYSATE PREPARATION FROM YEAST

Before you start:

- Prepare the appropriate amount of lyticase-containing Resuspension Buffer (see section 6), considering that 500µl of buffer is required for each preparation. This solution should be prepared with sterile, RNase-free reagents and kept on ice until needed. These reagents are to be provided by the user.
- It is recommended that no more than 10^7 yeast cells or 1ml of culture are used for this procedure.
- For RNA isolation, the yeast should be harvested in log-phase growth.
- Yeast can be used directly in this procedure or stored at -80°C for later use.
- Frozen yeast pellets should not be thawed prior to beginning the protocol. Add the lyticase-containing Resuspension Buffer directly to the frozen yeast pellet (step 3).

8.5.1 Protocol

1. Pellet yeast cells by centrifuging for 1 min at 14,000 x g
2. Decant the supernatant and carefully remove any remaining media by aspiration.
3. Resuspend yeast cells thoroughly in 500µl of lyticase-containing Resuspension Buffer by vortexing. Incubate at 37°C for 10 min.
4. Pellet spheroplasts for 3 min at 200 x g. Decant supernatant.
5. Add 300µl of Lysis Buffer TX and vortex vigorously for at least 10s. **Proceed to section 9.**

9. PURIFICATION OF SMALL RNA FROM ALL TYPES OF LYSATE

The remaining steps of the protocol for the purification of small RNA are the same from this point forward for all the different types of lysate.

Before you start:

- Ensure Wash Buffer W1 is prepared (see section 7.3)

9.1 Large RNA Removal

1. Assemble an ISOLATE II **Large RNA Removal Column** (white ring) with the Collection Tube (provided).
2. Apply the ethanolic lysate onto the column (from section 8) and centrifuge for 1 min at 14,000 x g. Transfer the flow-through, which contains the small RNA species into a RNase-free 1.5ml microcentrifuge tube (user supplied).
   Important note: *The flow-through contains the small RNA, therefore ensure that this fraction is not discarded.*
3. If the large RNA is to be isolated, retain the column and proceed to the **Optional Large RNA Purification Protocol (section 10)**. Otherwise, discard the column.
   Important note: *The Large RNA Removal Column can be kept at 4°C for several hours or ≤15 min at room temperature. Freezing and thawing is not recommended.*

9.2 Small RNA Capture

1. **For eukaryotic cells, blood, biofluid or bacteria:** Add 350µl of 96-100% ethanol to the small RNA containing flow-through collected in step 3. Vortex for 10s to mix.
   For mammalian tissue: Add 1 volume of 96-100% ethanol to the small RNA containing flow-through collected in step 3. For example, add 100µl of ethanol to every 100µl of collected flow-through. Vortex for 10s to mix.
2. Assemble an ISOLATE II **miRNA Column** (black ring) with the provided Collection Tube.
3. Apply half of the ethanolic lysate onto the column and centrifuge for 1 min at ≥3,500 x g.
   Note: Ensure the entire lysate volume has passed into the Collection Tube by inspecting the column. If the entire lysate volume has not passed through, centrifuge for an additional minute at 14,000 x g.
4. Discard the flow-through and reassemble the spin column with the Collection Tube.
5. Repeat steps 3 and 4 to complete the capture of the small RNA.
   Optional: The ISOLATE II miRNA Kit purifies small RNA with minimal amounts of genomic DNA contamination. However, for sensitive applications, an optional on-column DNA removal protocol is provided (see Appendix A). DNase I treatment should be performed at this point in the protocol with the supplied DNase I Solution and DNase I Reaction Buffer DRB.

9.3 miRNA Column Wash

1. Apply 400µl of Wash Buffer W1 to the ISOLATE II **miRNA Column** (black ring) and centrifuge for 1 min at 14,000 x g.
   Note: Ensure the entire wash buffer volume has passed into the Collection Tube by inspecting the column. If the entire wash volume has not passed through, centrifuge for an additional minute at 14,000 x g.
2. Discard the flow-through and reassemble the spin column with the Collection Tube.
3. Repeat steps 1 and 2 to wash the column a second time.
4. Wash the column a third time by adding 400µl of Wash Buffer W1 and centrifuge for 1 min at 14,000 x g.
5. Discard the flow-through and reassemble the spin column with its Collection Tube.
6. Centrifuge for 2 min at 14,000 x g in order to dry the column thoroughly. Discard the Collection Tube.

9.4 Small RNA Elution

1. Place the ISOLATE II **miRNA Column** (black ring) into a sterile 1.7ml Elution Tube (supplied).
2. Add 50µl of RNA Elution Buffer to the column.
   Note: For more concentrated RNA, use a lower volume of RNA Elution Buffer (a minimum of 20µl is recommended).
3. Centrifuge for 2 min at 200 x g, followed by 1 min at 14,000 x g. Note the volume eluted from the column. If the entire volume has not been eluted, spin column for an additional 1 min at 14,000 x g to elute the RNA.
   Note: For maximum RNA recovery, it is recommended to apply a second volume of 20-50µl RNA Elution Buffer and elute into the same microcentrifuge tube (repeat steps 2 and 3). Alternatively, re-apply the first eluate onto the column and re-elute into the same microcentrifuge tube (for higher concentration). See section 7.3.4 for further details on alternative elution procedures.

9.5 Storage of RNA

The isolated RNA can be stored at -20°C for a few days or at -80°C for long-term storage.

10. OPTIONAL LARGE RNA PURIFICATION PROTOCOL

Following purification of the small RNA in section 9, this section contains the optional large RNA purification protocol for users also wishing to purify larger RNA species from the same sample. The ISOLATE II Large RNA Removal Column can be kept at 4°C for several hours or ≤15 minutes at room temperature (see section 9.1). This optional procedure should be carried out on the same day as the rest of the protocol detailed in this manual. Freezing and thawing of the ISOLATE II Large RNA Removal Column is not recommended.

Before you start:

- Ensure Wash Buffer W1 is prepared (see section 7.3)

10.1 Large RNA Column Wash

1a. Reassemble the ISOLATE II **Large RNA Removal Column** (white ring) with the Collection Tube used in section 9.1 step 2.

Optional: The ISOLATE II miRNA Kit purifies large RNA with minimal amounts of genomic DNA contamination. However, for sensitive applications, an optional on-column DNA removal protocol is provided (see Appendix A). DNase I treatment should be performed at this point in the protocol with the supplied DNase I and reaction buffer.

1b. Apply 400µl of Wash Buffer W1 to the spin column and centrifuge for 1 min at 14,000 x g.

Note: Ensure that the entire wash buffer volume has passed into the Collection Tube by inspecting the column. If the entire wash volume has not passed through, centrifuge for an additional minute at 14,000 x g.

2. Discard the flow-through and reassemble the spin column with the Collection Tube.
3. Repeat steps 1b and 2 to wash the column a second time.
4. Wash the column for a third time by adding 400µl of Wash Buffer W1 and centrifuge for 1 min at 14,000 x g.
5. Discard the flow-through and reassemble the spin column with its Collection Tube.
6. Centrifuge the column for 2 min in order to dry the column thoroughly. Discard the Collection Tube.

10.2 Large RNA Elution

1. Place the ISOLATE II **Large RNA Removal Column** (white ring) into a fresh 1.7ml Elution Tube.
2. Add 50µl of RNA Elution Buffer to the column.
3. Centrifuge for 2 min at 200 x g, followed by 1 min at 14,000 x g. Note the volume eluted from the column. If the entire volume has not been eluted, spin the column for an additional minute at 14,000 x g to elute the RNA.

Note: For maximum RNA recovery, it is recommended to apply a second volume of 50µl RNA Elution Buffer and elute into the same microcentrifuge tube (repeat steps 2 and 3). Alternatively, re-apply the first eluate onto the column and re-elute into the same microcentrifuge tube (for higher concentration). See section 7.3.4 for further details on alternative elution procedures.

10.3 Storage of RNA

The isolated RNA can be stored at -20°C for up to three days or at -80°C for long-term storage.

11. APPENDICES

11.1 APPENDIX A: OPTIONAL ON-COLUMN DNASE I TREATMENT PROTOCOL

The ISOLATE II miRNA Kit isolates total RNA with minimal amounts of genomic DNA contamination. However, an optional protocol is provided below for maximum removal of residual DNA that may affect sensitive downstream applications. This procedure could be applied to both the small RNA purification as well as the optional large RNA purification.

- For each on-column digest to be performed, prepare a DNase I - buffer mix by adding 15µl of the supplied DNase I Solution to 100µl of DNase I Reaction Buffer DRB. Mix gently by inverting the tube a few times. Do not vortex.
- Perform the appropriate RNA isolation procedure for your starting material up to and including the Binding RNA to Column step.

11.1.1 On-column DNase I treatment for small RNA

From captured small RNA, section 9.2, step 5:

1. Apply 400µl of Wash Buffer W1 to the column and centrifuge for 2 min at 14,000 x g. Discard the flow-through. Reassemble the spin column with its Collection Tube.

   Note: Ensure the entire wash buffer volume has passed through into the Collection Tube by inspecting the column. If the entire wash volume has not passed, spin for an additional minute at 14,000 x g.

2. Apply 115µl of the DNase I - buffer mix to the column and centrifuge for 1 min at 14,000 x g.

   Note: Ensure the entire volume of DNase I - buffer mix passes through the column. If needed, spin for an additional minute at 14,000 x g.

3. After the previous centrifugation step, pipette the flow-through present in the Collection Tube back onto the top of the column.

   Note: This step must be performed to ensure maximum DNase activity and to obtain maximum yields of RNA. This is particularly important for the isolation of small RNA species.

4. Incubate at room temperature (18-25°C) for 15 min. Without any further centrifugation, proceed directly to the second wash step in the RNA Wash section (step 3 of section 9.3). Apply the wash buffer directly to the column containing the DNase I - buffer mix.

11.1.2 On-column DNase I treatment for large RNA

From captured large RNA, section 10.1, step 1a:

1. Apply 400µl of Wash Buffer W1 to the column and centrifuge for 2 min at 14,000 x g. Discard the flow-through. Reassemble the spin column with its Collection Tube.

   Note: Ensure the entire wash buffer volume has passed through into the Collection Tube by inspecting the column. If the entire wash volume has not passed, spin for an additional minute at 14,000 x g.

2. Apply 115µl of the DNase I - buffer mix to the column and centrifuge for 1 min at 14,000 x g.

   Note: Ensure the entire volume of DNase I - buffer mix passes through the column. If needed, spin for an additional minute at 14,000 x g.

3. After the previous centrifugation step, pipette the flow-through present in the Collection Tube back onto the top of the column.
   Note: This step must be performed to ensure maximum DNase activity and to obtain maximum yields of RNA. This is particularly important for the isolation of small RNA species.
4. Incubate at room temperature (18-25°C) for 15 min. Without any further centrifugation, proceed directly to the second wash step in the RNA Wash section (step 3 of section 10.1). Apply the wash buffer directly to the column containing the DNase I - buffer mix.

11.2 APPENDIX B: OPTIONAL DNASE I TREATMENT OF PURIFIED RNA IN SOLUTION PROTOCOL

The on-column DNase I digestion results in minimal residual DNA, undetectable in most downstream applications. For the most sensitive applications, DNA digestion in solution is recommended to eliminate even traces of contaminating DNA. Stringent RNase control is needed, as well as RNA repurification to remove buffer, salts, DNase I and digested DNA.

Additional reagents required:

- Sodium acetate (3M, pH 5.2)
- Ice-cold 70% ethanol

11.2.1 Protocol

1. In a microcentrifuge tube, mix together 2.5µl of the supplied DNase I Solution, 10µl of DNase I Reaction Buffer DRB and up to 87.5µl of eluted RNA. If using a lower starting volume of RNA solution, bring the volume up to 100µl using RNase-free water.
2. Gently swirl tube to mix solution. Gently spin down (approx. 1s at 1000 x g) to collect solution at bottom of tube.
3. Incubate at room temperature (18-25°C) for 10 min.
4. Repurify the RNA with a suitable RNA clean-up procedure, e.g. using ethanol precipitation.

Ethanol precipitation step

- Add 1/10th volume of sodium acetate (3M, pH 5.2).
- Add between 2.5 and 3 volumes of 96-100% ethanol to one volume of sample. Mix thoroughly.
- Precipitate for one hour at -20°C or overnight at -20°C.
  Note: Choose longer incubation times if the sample has a low RNA concentration. Shorter incubation times are sufficient for high RNA concentrations.
- Centrifuge at maximum speed for 10 min.
- Wash the RNA pellet with ice-cold 70% ethanol.
- Dry the RNA pellet and resuspend the RNA in RNase-free water.

12. TROUBLESHOOTING GUIDE

CLOGGED SPIN COLUMN	
POSSIBLE CAUSE	RECOMMENDED SOLUTION
Insufficient disruption or homogenization	Reduce amount of starting material used. Ensure sufficient lysis buffer is used for the amount of cells or tissue. Ensure thorough disruption; use appropriate equipment such as needle and syringe, mortar and pestle, or rotor stator homogenizer if required (see section 7.2).
Maximum number of cells or amount of tissue exceeded	Determine if amount of starting material falls within the kit specifications.
Insufficient centrifugation	Increase centrifugation speed and time.
Centrifuge temperature too low	Ensure centrifuge remains at room temperature during protocol. Temperatures below 15°C may cause precipitates to form that can cause the columns to clog.
RNA DEGRADED	
POSSIBLE CAUSE	RECOMMENDED SOLUTION
RNase contamination	Ensure an RNase-free working environment (see online hints and tips at www.bioline.com/isolate). Discard any solutions contaminated with RNase during use.
Inappropriate handling and storing of starting material	Ensure proper handling and storage of samples. Ensure all steps are followed quickly in order to maintain integrity of RNA in the sample.
Frozen tissues or pellets allowed to thaw prior to disruption	Tissue samples should be flash-frozen in liquid nitrogen and transferred immediately to a -80°C freezer for long-term storage. Do not allow frozen tissues to thaw prior to grinding with mortar and pestle in order to ensure RNA integrity is not compromised.
RNA not stored optimally	Always keep eluted RNA on ice to prevent degradation by RNases.
GENOMIC DNA CONTAMINATION	
POSSIBLE CAUSE	RECOMMENDED SOLUTION
Incorrect lysis	Check that protocol has been followed correctly.
Too much starting material	Reduce amount of starting material.
DNase I inactive	Store as recommended.
On-column DNase I digestion step not performed	Perform on-column DNase I treatment protocol provided (see Appendix A and section 7.3.3)

LOW RNA YIELD OR QUALITY	
POSSIBLE CAUSE	RECOMMENDED SOLUTION
Insufficient disruption or homogenization	Reduce amount of starting material used. Ensure sufficient volume of Lysis Buffer is used for the amount of cells or tissue.
Column has become clogged	Do not exceed recommended amounts of starting materials. Amount of starting material may need to be decreased if the column shows clogging below recommended levels. See also Clogged Spin Column above.
Flow-through from first binding step discarded	The flow-through from binding step with the Large RNA Removal Column contains small RNA and must be retained.
Ethanol not added to flow-through before binding to miRNA Column	Ensure correct amount of ethanol was added to flow-through from first binding step before it is applied to miRNA Column. This is necessary to capture small RNA.
Sample material degraded	Store sample material properly. Use fresh material whenever possible; if not, flash-freeze sample in liquid nitrogen. Always keep samples at -80°C. Always add Lysis Buffer before thawing sample. Disrupt samples in liquid nitrogen and ensure tubes are kept chilled.
Cell culture not washed with PBS	Ensure cultured cells are washed with an appropriate amount of PBS in order to remove residual media from cells. Incomplete removal of the medium will inhibit lysis of cells and compromise efficiency of RNA isolation.
Bacteria: traces of media remain	Ensure all media is removed by aspiration before adding Lysis Buffer RX.
Low RNA content	RNA content can vary in different types of tissues and cells. Some tissues may not contain small RNA at detectable levels when processing the small sample sizes required for this protocol.
Reagents not properly prepared	Add 96-100% ethanol to Wash Buffer W1 concentrate. Ethanol is required to create effective binding conditions for RNA to the silica membrane. Add reducing agent ß-ME or TCEP to Lysis Buffer RX (see section 7.3.2). Prepare and store reagents according to instructions given in section 7.3.
Different elution buffer used	Use supplied RNA Elution Buffer in the kit for maximum RNA recovery.
Residual genomic DNA contamination remaining after on-column DNase I digest performed	Perform in-solution DNase I treatment protocol provided (see Appendix B and section 7.3.3) to eliminate even traces of contaminating genomic DNA. In solution DNase I digestion is recommended for most sensitive downstream applications (e.g. probe-based real-time PCR).

LOW A_{260}/A_{230} RATIO	
POSSIBLE CAUSE	RECOMMENDED SOLUTION
Guanidinium salt carryover	Carefully load lysate onto columns, avoiding contamination between column and lid.
LARGE RNA SPECIES PRESENT IN ELUTION	
POSSIBLE CAUSE	RECOMMENDED SOLUTION
Ethanol incorrectly added to the lysate	Ensure appropriate amount of ethanol is added to lysate before the lysate is applied to Large RNA Removal Column in order to capture large RNAs onto column.
Large amount of starting material used	Repeat protocol using less starting material. Alternatively, repeat protocol using eluate as input. Elution volume should first be adjusted to 300µl using Lysis Buffer provided. Follow protocol, starting with addition of ethanol, centrifuging lysate in order to pellet any debris, and applying clarified lysate to the Large RNA Removal Column. Repeating procedure should result in removal of large contaminating RNA species.
RNA DOES NOT PERFORM WELL IN DOWNSTREAM APPLICATIONS	
POSSIBLE CAUSE	RECOMMENDED SOLUTION
Ethanol carryover during elution	Increase centrifugation time for ethanol removal step.
RNA not washed three times with Wash Buffer	Ensure miRNA Column is washed three times with Wash Buffer W1, in order to remove traces of salt from the binding step. Salt in sample may interfere with downstream applications.
Salt carryover during elution	Ensure Wash Buffer W1 is at room temperature. Washing at lower temperatures reduces efficiency of salt removal. Check both solutions for salt precipitates. Resuspend any visible precipitate by gentle warming.
RNA not stored optimally	Always keep eluted RNA on ice to prevent degradation by RNases.

A. TECHNICAL SUPPORT

For technical assistance or more information on these products, please email us at tech@bioline.com

B. ORDERING INFORMATION

PRODUCT	PACK SIZE	CAT NO.
ISOLATE II miRNA Kit	25 Preps	BIO-52083

C. ASSOCIATED PRODUCTS

PRODUCT	PACK SIZE	CAT NO.
SensiFAST™ SYBR® No-ROX Kit	200 Reactions	BIO-98002
SensiFAST™ Probe No-ROX Kit	200 Reactions	BIO-86002

D. PRODUCT WARRANTY AND DISCLAIMER

Bioline warrants that its products will conform to the standards stated in its product specification sheets in effect at the time of shipment. Bioline will replace any product that does not conform to the specifications. The warranty limits Bioline's liability to only the replacement of the product.

NOTES

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