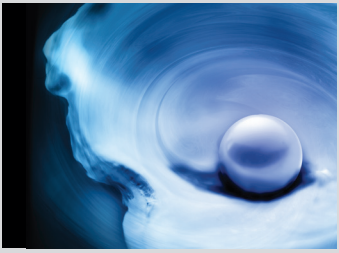


ISOLATE II
Plant miRNA Kit
(Phenol free)

Product Manual

BIOLINE

A Meridian Life Science® Company

ISOLATE II Plant miRNA Kit (Phenol free)

1. KIT CONTENTS

COMPONENT	25 Preps
ISOLATE II BioFilters	25
ISOLATE II Large RNA Removal Columns (white)	25
ISOLATE II miRNA Columns (black)	25
Collection Tubes (2ml)	50
Collection Tubes (2ml)	25
Elution Tubes (1.7ml)	50
Lysis Buffer RPX*	30ml
Wash Buffer W1† (concentrate)	18ml
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 Plant miRNA kit allows convenient processing of multiple samples in 25 minutes without the need for laborious methods such as CsCl ultracentrifugation or handling of toxic chemicals such as phenol/chloroform.

The ISOLATE II Plant miRNA Kit is specially developed for the rapid phenol-free isolation of small RNAs (<200nt) from plant cells and tissue. Small RNAs 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 which are involved in regulating plant gene expression by binding to messenger RNAs (mRNAs).

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

Plant samples are first lysed in the presence of guanidinium chloride, a chaotropic salt which immediately deactivates endogenous RNases to ensure purification of intact RNA. The lysate is cleared through the BioFilter, ethanol is added 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 RNAs 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 Plant 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 RPX contains guanidinium chloride. 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 Plant miRNA Kit is specially designed for the rapid and efficient isolation of high quality and highly enriched small RNA species (<200nt). Larger RNAs can be sequentially purified from the same sample. The kit is compatible with fresh or frozen plant cells and tissues. The preparation time is approximately 25 minutes for 10 preps. The isolated RNA is of high-purity (A_{260}/A_{280} ratio: >1.9) and is ready for use in various downstream applications.

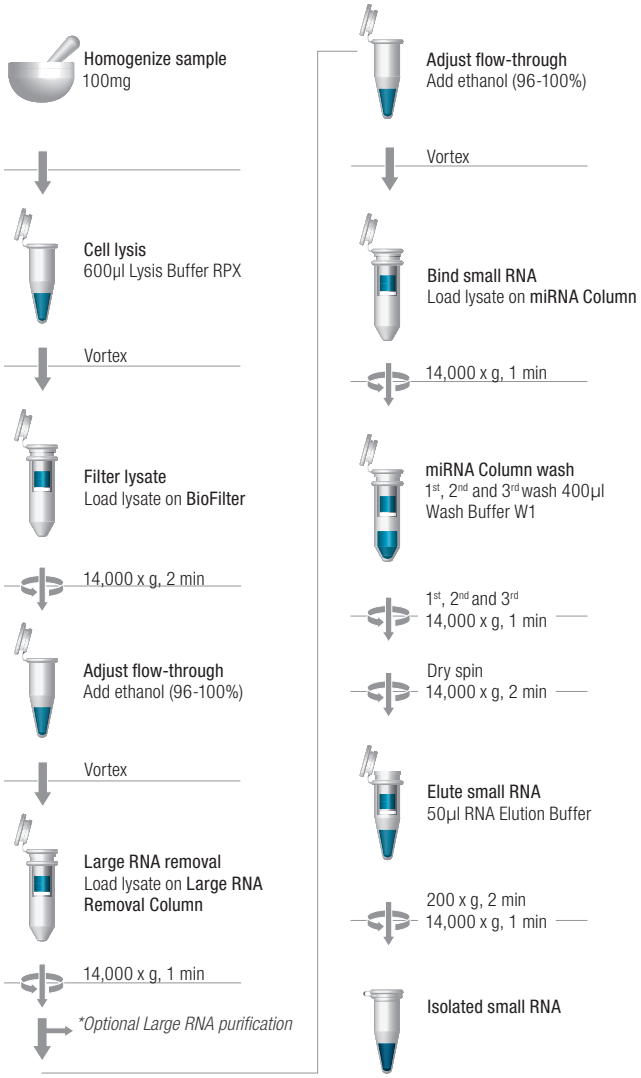
ISOLATE II PLANT miRNA COLUMN SPECIFICATIONS	
Max. binding capacity	50µg RNA
RNA size distribution	<200 nucleotides
A_{260}/A_{280} ratio*	1.9-2.1
Max. column loading volume	650µl
Min. elution volume	20µl
Max. amount of starting material	
Plant tissues	100 mg
Plant cells	5 x 10^6 cells

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

The following components are also included in the kit:

- ISOLATE II BioFilters for homogenization and reduction of lysate viscosity.
- DNase I (RNase-free) for eliminating genomic DNA contamination by on-column digestion or by digestion in solution (for the most sensitive applications).

Plant Small RNA Isolation



*See section 9

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:

- 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 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)

† Molecular biology grade ethanol is recommended. Do not use denatured alcohol which contains unwanted additives such as methanol and acetone.

7. IMPORTANT NOTES

The protocol steps are homogenization, lysis and purification of small RNA (section 8). The protocol in section 9 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 protocol to purify small RNA from plant cells and tissues.
- Section 9 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 Plant miRNA 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 RPX, 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 RPX should be thawed slowly before starting the isolation of total RNA.

Both fresh and frozen plant tissues can be used for this protocol. The recommended input is 100mg of plant tissue or 5 x 10^7 cells. However it can be reduced (to 30mg) or increased (up to 150mg) depending on customer's optimization based on a specific sample type.

7.2 DISRUPTING AND HOMOGENIZING STARTING MATERIALS

Plant material is generally a robust tissue type to work with, therefore the efficient mechanical disruption and homogenization of starting material is essential. To release all the RNA contained in a sample, the total disruption of cell walls, plasma membranes and organelles must occur. Incomplete disruption results in reduced RNA yields. For best results, homogenization is required to reduce lysate viscosity further but depends on chosen disruption method. Incomplete homogenization results in inefficient binding of RNA to the membrane and therefore reduced RNA yields. The lysis procedure is most effective with well-homogenized, powdered samples. Suitable methods include any type of commercial homogenizers (e.g. rotor-stator homogenizer) or bead mills using steel or glass beads. A mortar and pestle is also a commonly used technique to disrupt plant tissues.

7.2.1 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 plant 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 immediately after grinding. After grinding, transfer the tissue powder into a liquid nitrogen cooled tube and allow the liquid nitrogen to evaporate. Add Lysis Buffer RPX to the powdered tissue and vortex immediately. Homogenize the sample with an ISOLATE II BioFilter (supplied) or by passing through a user supplied nuclease-free 20 gauge (0.9mm) syringe needle 5-10 times.

7.2.2 Disruption and homogenization using a rotor-stator homogenizer

Rotor-stator type tissue homogenizers can rapidly homogenize, disrupt and emulsify plant tissue samples in the presence of lysis buffer in seconds or minutes. Homogenization time depends on sample size and toughness. Thawing of undisrupted plant tissue should only be performed in presence of lysis buffer to prevent degradation of RNA by RNases. 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 42ml of 96-100% ethanol to Wash Buffer W1 Concentrate to give a final volume of 60ml.

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 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.

7.3.3 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.

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 Plant miRNA Kit. Genomic DNA contamination is efficiently removed by on-column digestion with DNase I (see optional section 7.3.2 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 RNA 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 7.3.2 and Appendix B).

8. PURIFICATION OF SMALL RNA FROM PLANT CELLS AND TISSUE

Before you start:

- Both fresh and frozen plant tissues can be used for this protocol.
- For optimal results, the recommended input is 100mg of plant tissue or 5 x 10^7 cells. However, the input can be reduced (to 30mg) or increased (up to 150mg) depending on customer's optimization based on a specific sample type.
- Ensure Wash Buffer W1 is 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 Sample Homogenization and Lysis

1. Transfer ≤100 mg of plant tissue or a maximum of 5 x 10^7 plant cells into a mortar that contains enough liquid nitrogen to cover the sample. Grind sample into a fine powder using a pestle in liquid nitrogen.

   Note: If stored frozen samples are used, do not allow the samples to thaw before transferring to the liquid nitrogen. For handling and preparation methods see sections 7.1-7.2.

2. Allow the liquid nitrogen to evaporate without allowing the tissue to thaw.
3. Transfer the powder to a 1.5ml microcentrifuge tube (user supplied)
4. Add 600µl of Lysis Buffer RPX and vortex vigorously for 30s.
5. Assemble an ISOLATE II BioFilter with a Collection Tube (provided).
6. Pipette lysate onto the BioFilter and centrifuge for 2 min at 14,000 x g.
7. Pipette only the clear supernatant from the flow-through into an RNase-free 1.5ml microcentrifuge tube (user supplied). Note the volume of the supernatant/lysate.

   Note: Ensure only the clear supernatant is transferred, avoiding any of the debris at the bottom of the Collection Tube.

8. Add a volume of 96-100% ethanol that is a half of the lysate volume (e.g. 50µl of ethanol is added to every 100µl of lysate). Vortex to mix. **Proceed to section 8.2**.

8.2 Large RNA Removal

1. Assemble an ISOLATE II **Large RNA Removal Column** (white ring) with the provided Collection Tube.
2. Apply the ethanolic lysate onto the column (from section 8.1, step 8) and centrifuge for 1 min at 14,000 x g. Transfer the flow-through, which contains the small RNA into an RNase-free 1.5ml microcentrifuge tube (user supplied).

   Important note: *The flow-through contains the small RNA, therefore ensure 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 (see section 9)**. 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.*

8.3 Small RNA Capture

1. Add 1 volume of 96-100% ethanol to the small RNA containing flow-through collected in section 8.2, step 2. 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 a provided Collection Tube.
3. Apply half of the ethanolic lysate onto the column and centrifuge for 1 min 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 Plant 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.

8.4 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 column a second time.
4. Wash column a third time by adding 400µl of Wash Buffer W1 and centrifuge for 1 min at 14,000 x g.
5. Discard flow-through and reassemble 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.

8.5 Small RNA Elution

1. Place the ISOLATE II **miRNA Column** 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 minute 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 7.3.3 for further details on alternative elution procedures.

8.6 Storage of RNA

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

9. OPTIONAL LARGE RNA PURIFICATION PROTOCOL

Following purification of the small RNA in section 8, 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 8.2). This protocol should be carried out on the same day as freezing and thawing of the column is not recommended.

Before you start:

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

9.1 Large RNA Column Wash

1a. Reassemble the ISOLATE II **Large RNA Removal Column** (white ring) with the Collection Tube used in section 8.2 step 2.

   Optional: The ISOLATE II Plant 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 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 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 thoroughly dry the column. Discard the Collection Tube.

9.2 Large RNA Elution

1. Place the ISOLATE II **Large RNA Removal Column** into a sterile 1.7ml Elution Tube (supplied).
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 7.3.3 for further details on alternative elution procedures.

9.3 Storage of RNA

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

10. APPENDICES

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

The ISOLATE II Plant 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.

10.1.1 On-column DNase I treatment for small RNA

From captured small RNA, section 8.3, 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 through, spin for an additional minute at 14,000 x g.

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

   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 in order to ensure maximum DNase I - buffer 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 8.4). Apply the wash buffer directly to the column containing DNase I - buffer mix.

10.1.2 On-column DNase I treatment for large RNA

From captured large RNA, section 9.1, 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 through, spin for an additional minute at 14,000 x g.

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

   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 in order to ensure maximum DNase I - buffer 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 9.1). Apply the wash buffer directly to the column containing DNase I - buffer mix.

10.2 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/components required:

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

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 the bottom of the 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.

11. TROUBLESHOOTING GUIDE

CLOGGED SPIN COLUMN	
POSSIBLE CAUSE	RECOMMENDED SOLUTION
Insufficient disruption or homogenization	Reduce amount of starting material used. Ensure sufficient volume of Lysis Buffer is used for amount of cells or tissue. Ensure thorough disruption and use ISOLATE II BioFilters for homogenization of disrupted starting material.
Maximum number of cells or amount of tissue exceeded	Determine if amount of starting material falls within kit specifications.
Insufficient centrifugation	Increase centrifugation speed and time.
Centrifuge temperature too low	Ensure centrifuge remains at room temperature during protocol. Temperatures <15°C may cause precipitates to form that can cause the columns to clog.
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 amount of cells or tissue. Ensure thorough disruption and use ISOLATE II BioFilter for homogenization of disrupted starting material.
Large RNA Removal Column has become clogged	Do not exceed recommended amounts of starting materials. Amount of starting material may need to be decreased below recommended levels if column shows clogging. See also Clogged Spin Column above.
Flow-through from first binding step discarded	Flow-through from binding step with ISOLATE II 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 RNAs.
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.
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 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 silica membrane. Prepare and store reagents according to instructions given in section 7.3.
Different elution buffer used	Use RNA Elution Buffer supplied in kit for maximum RNA recovery.

RNA DEGRADED	
POSSIBLE CAUSE	RECOMMENDED SOLUTION
RNase contamination	Ensure an RNase-free working environment. (see online hints and tips a www.bioline.com/isolate). Discard any solutions contaminated with RNases 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 the 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 that RNA integrity is not compromised.
RNA not stored optimally	Always keep eluted RNA on ice to prevent degradation by RNases. Store at -20°C for short term storage or -80°C for long-term storage.
LOW A_{260}/A_{230} RATIO	
POSSIBLE CAUSE	RECOMMENDED SOLUTION
Guanidinium salt carryover	Carefully load lysate onto columns, avoiding contamination between column and lid.
GENOMIC DNA CONTAMINATION	
POSSIBLE CAUSE	RECOMMENDED SOLUTION
Incorrect lysis	Check 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 7.3.2)
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 7.3.2) to eliminate even traces of contaminating genomic DNA. In solution DNase I digestion is recommended for the most sensitive downstream applications.
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 it is applied to ISOLATE II 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 the input. Elution volume should first be adjusted to 300µl using Lysis Buffer RPX. Follow protocol, starting with addition of ethanol, centrifuging the lysate in order to pellet any debris, and applying clarified lysate to ISOLATE II 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. Ensure final dry spin during ethanol removal step is performed, in order to remove traces of ethanol prior to elution.
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 binding step. Salt in sample may interfere with downstream applications.
Salt carryover during elution	Ensure Wash Buffer WI 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. Store at -20°C for short term storage or -80°C for long-term storage.

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 Plant miRNA Kit	25 Preps	BIO-52084

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.

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