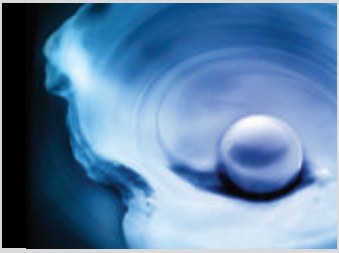


ISOLATE II
Biofluids RNA Kit
(Phenol free)

Product Manual

BIOLINE

A Meridian Life Science® Company

ISOLATE II Biofluids RNA Kit (Phenol free)

1. KIT CONTENTS

COMPONENT	50 Preps
ISOLATE II RNA Columns (black)	50
ISOLATE II Genomic DNA Removal Columns (white)	50
Collection Tubes (2ml)	100
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 Biofluids RNA Kit allows convenient processing of multiple samples in 20 minutes without the use of laborious methods such as CsCl ultracentrifugation or handling of toxic chemicals such as phenol/chloroform.

The ISOLATE II Biofluids RNA Kit is specially developed for the rapid phenol-free isolation of high quality total RNA from biofluids and viruses. Total RNA can be purified from blood, plasma, serum and other types of biofluids such as saliva, urine, semen and cerebrospinal fluid (CSF). Viral RNA can be isolated from the same sample sources, as well as cultured cells and tissue. The kit isolates all sizes of RNA from large mRNA, viral RNA and ribosomal RNA (rRNA) down to small RNAs such as microRNA (miRNA) and short interfering RNA (siRNA).

Isolation is based on a fast spin column format 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.

Samples are first lysed in the presence of guanidinium thiocyanate, a chaotropic salt which immediately deactivates endogenous RNases to ensure purification of intact RNA. DNA is then captured on a Genomic DNA Removal Column to remove contaminating genomic DNA. Ethanol is added to the flow-through and then applied to an RNA Column. The bound total RNA is washed to remove any impurities and eluted. The RNA is of the highest quality and integrity and is 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

Please read this manual carefully to familiarize yourself with the ISOLATE II Biofluids RNA 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 Biofluids RNA Kit is specially designed for the rapid isolation of total RNA including small RNAs (<200nt). The kit is compatible with biofluids such as blood, plasma, serum, saliva, urine, semen and CSF. Viral RNA can also be extracted from the same sample sources. The preparation time is approximately 20 minutes for 10 preps. The isolated RNA is of high-purity (A_{260}/A_{280} ratio: >1.9) and high integrity (RIN>9) for high quality samples (see below) and is ready for use in various downstream applications.

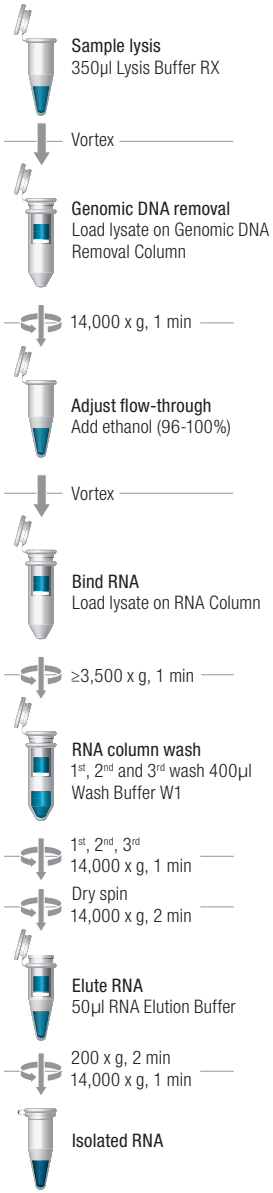
ISOLATE II BIOFLUIDS RNA COLUMN SPECIFICATIONS	
Max. binding capacity	50µg RNA
Max. column loading volume	650µl
RNA size distribution	All sizes, including small RNA <200 nucleotides
A_{260}/A_{280} ratio*	1.9-2.1
Typical RIN (RNA integrity number) †	>9
Max. amount of starting material:	
Biofluid (Blood/Saliva/Semen/Urine/CSF)	100µl
Plasma/Serum	200µl
Viruses	3 x 10^6 cells
	~20mg mammalian tissue
	100µl viral suspension or blood
Biofluid in Cotton Swab	One swab

* *Typically, the A_{260}/A_{280} ratio exceeds 1.9, indicating excellent RNA purity.*
† *Agilent 2100 Bioanalyzer (RNA 6000 assay). RNA integrity is highly dependent on sample quality.*

The following components are also included in the kit:

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

Biofluids RNA Isolation



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
- Sterile cotton swabs (for Nasal/Throat Swab Protocol)
- MS2 RNA (for Plasma/Serum Protocol)
- 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.

7. IMPORTANT NOTES

The protocol steps are homogenization and lysis from different starting materials (section 8), genomic DNA removal (section 9) and purification of total RNA (section 10). Sections 9 and 10 apply to all starting materials. Optional DNase I treatment protocols are provided.

- Section 8 contains the lysate preparation protocols for various starting materials; ensure you follow the specific protocol for your sample.
- Section 9 contains the protocol to remove genomic DNA from all types of lysate.
- Section 10 describes the purification of total RNA from all types of lysate.
- For isolating RNA from plasma/serum samples, follow the dedicated protocol in section 11.
- The Appendix contains the optional protocols for additional DNase I treatment.

The ISOLATE II Biofluids RNA 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 **Genomic DNA Removal Column** (white ring) and the ISOLATE II **RNA 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 8.3.1.

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 8.3.2.

7.2.3 Lifting of adherent cells with trypsin

To trypsinize adherent growing cells, 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 8.3.2.

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) 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 to minutes. Homogenization time depends on sample size and toughness. The spinning rotor disrupts and homogenizes the sample simultaneously 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 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 tissue). 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 Solution and 100µl of DNase I Reaction Buffer DRB. Mix gently by inverting a few times.

Optional in-solution digestion: In a microcentrifuge tube, mix together 10µl of DNase I Reaction Buffer DRB, 2.5µl of DNase I Solution 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 Biofluids RNA Kit. Genomic DNA contamination is efficiently removed by the Genomic DNA Removal Column. However, if the sample contains high amounts of genomic DNA, additional treatment can be performed with the supplied DNase I. Two alternative protocols are provided. The on-column digestion protocol (see section 7.3.3 and Appendix A) efficiently removes genomic DNA sufficient for most applications. For highly 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 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 your starting material (see section 7.2). The subsequent steps detailed in section 9 (Genomic DNA Removal) and section 10 (Total RNA Purification) are the same in all cases.

Before you start:

- Ensure Lysis Buffer RX 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 **Genomic DNA Removal Column** (white ring) and the **RNA 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 BIOFLUIDS / VIRUSES

- This protocol is designed for the isolation of all sizes of RNA (including miRNA) from a wide variety of biofluids such as saliva, urine, semen, CSF and blood. We recommend the use of non-coagulated blood samples (including fresh and anticoagulant treated blood).
- Follow this protocol for isolating viral RNA from biofluids.
- Follow the protocol in section 11 for isolating total RNA or viral RNA from plasma or serum.
- It is recommended that no more than 100µl of biofluid is used in order to prevent clogging of the column.
- Biofluids derived from all human and animal sources are considered potentially infectious. All necessary precautions recommended by the appropriate authorities in the country of use should be taken when working with biofluids.

8.1.1 Protocol

1. Transfer up to 100µl of biofluid to a 1.5ml RNase-free microcentrifuge tube (user supplied).
2. Add 350µl of Lysis Buffer RX to the sample. Lyse cells by vortexing for 15s. Ensure that mixture becomes transparent before proceeding with the protocol. **Proceed to section 9.**

8.2 LYSATE PREPARATION FROM BIOFLUIDS / VIRUSES COLLECTED WITH NASAL OR THROAT SWABS

Before you start:

- This protocol is designed for the isolation of all sizes of RNA (including miRNA) from a wide variety of biofluids derived from all human and animal sources collected with nasal or throat swabs.
- Follow this protocol for isolating RNA from viruses collected with nasal or throat swabs.

8.2.1 Protocol

1. Add 600µl of Lysis Buffer RX to a 1.5ml RNase-free microcentrifuge tube (user supplied).
2. Gently brush a sterile, single-use cotton swab inside the nose or mouth.
3. Using sterile techniques, cut the shaft of the cotton tip and place the tip containing the collected cells into the microcentrifuge tube containing Lysis Buffer RX. Close tube and vortex gently. Incubate for 5 min at room temperature.
4. Using a pipette, transfer lysate into another 1.5ml RNase-free microcentrifuge tube (user supplied). Note the volume of the lysate. **Proceed to section 9.**

8.3 LYSATE PREPARATION FROM VIRUSES IN CULTURED CELLS

Before you start:

- This section contains the protocol to isolate integrated viral RNA from mammalian cells growing in culture.
- A maximum of 3 x 10^6 eukaryotic cells can be used as starting material. 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 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 to ensure that 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 8.3.2, step 3).

Additional reagents required:

- PBS (RNase-free).

8.3.1 Cells Growing in a Monolayer

1. Aspirate media and wash cell monolayer with an appropriate amount of PBS. Aspirate PBS.
2. Add 350µl of Lysis Buffer RX directly to culture plate.

   Note: This volume of lysis buffer can be used for a culture plate of ~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). **Proceed to section 9.**

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

8.3.2 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.
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 350µl of Lysis Buffer RX to the pellet. Lyse cells by vortexing for 15s. Ensure entire pellet is completely dissolved before proceeding to the next step. **Proceed to section 9.**

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

8.4 LYSATE PREPARATION FROM VIRUSES IN SUSPENSION

Before you start:

- Follow the protocol below for isolating RNA from free viral particles.
- It is recommended that no more than 100µl of viral suspension be used in order to prevent clogging of the column.

8.4.1 Protocol

1. Transfer up to 100µl of viral suspension to a 1.5ml RNase-free microcentrifuge tube (user supplied).
2. Add 350µl of Lysis Buffer RX. Lyse cells by vortexing for 15s. Ensure that mixture becomes transparent before proceeding with the protocol. **Proceed to section 9.**

8.5 LYSATE PREPARATION FROM VIRUSES IN MAMMALIAN TISSUE

Before you start

- The following protocol describes the isolation of viral RNA from small amounts of tissue (up to 20mg for most types of tissue). 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
Heart	5mg
Kidney, Liver, Lung, Spleen	20mg
Other tissues	10mg

- RNA in mammalian tissue 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.

8.5.1 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 600µl of Lysis 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) 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 to pellet any cell debris. Transfer the supernatant to another 1.5ml RNase-free microcentrifuge tube. Note the volume of the supernatant/lysate. **Proceed to section 9.**

9. GENOMIC DNA REMOVAL FROM ALL TYPES OF LYSATE

The following protocol for the removal of genomic DNA applies to all the different types of lysate. Ensure you use the correct column: the Genomic DNA Removal Column has a white ring.

9.1 Protocol

1. Assemble an ISOLATE II **Genomic DNA Removal Column** (white ring) with a Collection Tube (provided).
2. Apply up to 600µl of lysate prepared from section 8 onto the column and centrifuge for 1 min at 14,000 x g.

   Note: Ensure that 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.

3. Transfer the flow-through into a sterile RNase-free 1.5ml microcentrifuge tube (user supplied) for Total RNA Purification (see section 10). The flow-through contains the RNA and should be stored on ice or at -20°C until the Total RNA Purification protocol is carried out.
4. Discard the column with the bound genomic DNA. **Proceed to section 10.**

10. TOTAL RNA PURIFICATION FROM ALL TYPES OF LYSATE

The following protocol for the purification of total RNA apply to all the different types of lysate. Ensure you use the correct column: the ISOLATE II RNA Column has a black ring.

Before you start:

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

10.1 Binding RNA to Column

1. To every 100µl of flow-through from section 9, add 60µl of 96-100% ethanol. Mix by vortexing. For example, for 200µl of flow-through, add 120µl of ethanol.
2. Assemble an ISOLATE II **RNA Column** (black ring) with a provided Collection Tube.
3. Apply up to 600µl 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. Reassemble the spin column with its Collection Tube.
5. Depending on the lysate volume, repeat steps 3 and 4 as required.
6. **Optional:** The ISOLATE II Biofluids RNA Kit isolates Total RNA with minimal amounts of genomic DNA contamination. However, for sensitive applications, an optional on-column DNA removal protocol is provided in Appendix A. DNase I treatment should be performed at this point in the protocol with the supplied DNase I. For highly sensitive applications, in-solution DNase I treatment can be performed (see Appendix B).

10.2 RNA Column Wash

1. Apply 400µl of Wash Buffer W1 to the RNA 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 buffer 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 another 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.

10.3 RNA Elution

1. Place the RNA Column into a fresh 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 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 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 high concentration).

10.4 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.

11. SUPPORT PROTOCOL: TOTAL RNA PURIFICATION FROM PLASMA OR SERUM

Before you start:

- Plasma or serum of all human and animal subjects is considered potentially infectious. All necessary precautions recommended by the appropriate authorities in the country of use should be taken when working with plasma or serum.
- We recommend the use of this kit to isolate RNA from plasma or serum prepared by a standard protocol from non-coagulated, fresh whole blood using EDTA or sodium citrate as the anti-coagulant.
- Plasma prepared from fresh blood using heparin as an anti-coagulant is not suitable for use with this protocol. For heparin-prepared samples, follow the protocol in section 8.1 (Lysate Preparation from Biofluids/Viruses).
- Due to the relatively low DNA content in plasma, the Genomic DNA Removal Column is not necessary for this protocol.
- It is recommended that no more than 200µl of plasma or serum is used in order to prevent clogging of the column.
- Yields of RNA from plasma and serum is highly variable. In general, the expected yield could vary from 1 to 100ng per 100µl plasma or serum used. In addition, the expected A_{260}/A_{280} ratio as well as the A_{260}/A_{230} ratio will be lower (<1.8) than the normal acceptable range from other cells or tissues. Nonetheless, these isolated RNA can be effectively used in different downstream applications such as real-time PCR or microarrays.
- Avoid multiple freeze-thaw cycles of the plasma or serum sample. Aliquot out the appropriate volume for usage prior to freezing.
- It is important to work quickly during this procedure.

11.1 Lysate Preparation from Plasma/Serum

1. Transfer up to 200µl of plasma or serum to a 1.5ml RNase-free microcentrifuge tube (not supplied).
2. Add 300µl of Lysis Buffer RX to every 100µl of plasma or serum. Mix by vortexing for 10s.

3. Optional: Add 0.7µl of 0.8µg/µl MS2 RNA per sample.
   Note: The use of MS2 RNA can increase the consistency of downstream applications such as real-time-PCR and RT-PCR. However, the use of MS2 RNA is not recommended for applications involving global gene expression analysis such as microarrays or sequencing.
4. Add 400µl of 96-100% ethanol to every 400µl of lysate (equivalent to every 100µl plasma or serum used). Mix by vortexing for 10s.

11.2 Binding RNA to Column

1. Assemble an ISOLATE II **RNA Column** (black ring) with a provided Collection Tube.
2. Apply up to 600µl 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 through into the Collection Tube by inspecting the column. If the entire lysate volume has not passed through, spin for an additional minute at 14,000 x g.
3. Discard the flow-through. Reassemble the spin column with its Collection Tube.
4. Depending on the lysate volume, repeat steps 2 and 3 as required.

11.3 RNA Column Wash

1. Apply 400µl of 96-100% ethanol to the column and centrifuge for 1 min at 14,000 x g.
   Note: Ensure the entire wash buffer volume has passed through into the Collection Tube by inspecting the column. If the entire wash buffer volume has not passed through, spin for an additional minute at 14,000 x g.
2. Discard the flow-through and reassemble the spin column with its Collection Tube.
3. Repeat steps 1 and 2 to wash the column a second time.
4. Wash column a third time by adding 400µl of 96-100% ethanol 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. Spin the column for 2 min at 14,000 x g in order to dry the column thoroughly. Discard the Collection Tube.

11.4 RNA Elution

1. Place the column 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 50µl has not been eluted, spin the column at 14,000 x g for an additional minute to elute the RNA.
   Note: For maximum RNA recovery, it is recommended to apply a second volume of 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 high concentration).

11.5 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.

12. APPENDICES

The ISOLATE II Biofluids RNA Kit isolates total RNA with minimal amounts of genomic DNA contamination using the supplied Genomic DNA Removal Column. However, additional DNase I treatment may be required in certain cases e.g. the amount of genomic DNA in the sample exceeds the capacity of the Genomic DNA Removal Column, or performing a highly sensitive application. Two alternative protocols are given below for additional DNase I treatment.

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

The optional protocol provided below can be used for additional removal of residual DNA that may affect sensitive downstream applications.

12.1.1 Protocol

1. 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.
2. Perform the appropriate RNA isolation procedure for your starting material up to and including the "Binding RNA to Column" section (section 10.1, step 5).
3. 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.
4. 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 necessary, spin for an additional minute at 14,000 x g.
5. 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 I activity and to obtain maximum yields of RNA. This is particularly important for the isolation of small RNA species.
6. Incubate at room temperature (18-25°C) for 15 min. Without any further centrifugation, proceed directly to the second wash step in the RNA Column Wash section (section 10.2, step 3). Apply the wash buffer directly to the column containing the DNase I - buffer mix.

12.2 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

12.2.1 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.

13. TROUBLESHOOTING GUIDE

CLOGGED SPIN COLUMN (GENOMIC DNA REMOVAL COLUMN OR RNA COLUMN)	
POSSIBLE CAUSE	RECOMMENDED SOLUTION
Insufficient disruption or homogenization	Reduce amount of starting material used. Ensure sufficient lysis buffer is used for 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 kit specifications.
High amounts of genomic DNA present in sample	Lysate may be passed through a 20 gauge syringe needle 5-10 times in order to shear the genomic DNA before loading onto the column.
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 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.
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.
Ethanol not added to lysate	Ensure correct amount of ethanol was added to lysate before binding to column.
Sample material degraded	Store sample material properly. Use fresh material whenever possible; if not fresh, 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 appropriate amount of PBS in order to remove residual media from cells. Incomplete removal of medium will inhibit lysis of the cells and compromise efficiency of RNA isolation.
Low RNA content	RNA content can vary in different types of tissues and cells. Therefore, expected yields of RNA may vary greatly from different sources. Check the literature to determine expected RNA content of your starting material.
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 RNA Elution Buffer supplied in the kit for maximum RNA recovery.
LOW A_{260}/A_{230} RATIO	
POSSIBLE CAUSE	RECOMMENDED SOLUTION
Guanidinium salt carryover	Carefully load lysate onto columns, avoiding contamination between column and lid.
UNEXPECTED A_{260}/A_{230} RATIO	
POSSIBLE CAUSE	RECOMMENDED SOLUTION
Measurement falls below detection limit of spectrophotometer	To obtain a significant A_{260}/A_{280} ratio, initially measured A_{260} and A_{280} values must be significantly above the detection limit of the spectrophotometer used. An A_{280} value near the background noise of the spectrophotometer will result in unexpected A_{260}/A_{280} ratios.

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 that all steps are followed quickly in order to maintain the 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. Store at -20°C for short term storage or -80°C for long-term storage.
GENOMIC DNA CONTAMINATION	
POSSIBLE CAUSE	RECOMMENDED SOLUTION
Amount of genomic DNA in sample exceeds capacity of Genomic DNA Removal Column	Perform additional DNase I treatment using supplied DNase I. See Appendices A and B.
Incorrect lysis	Check protocol has been followed correctly.
Too much starting material	Reduce amount of starting material.
DNase I inactive	Store as recommended.
Oversensitive DNA detection	Use DNase I digestion in solution protocol.
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. Ethanol can interfere with many downstream applications.
RNA not washed three times with Wash Buffer	Ensure RNA Column is washed three times with Wash Buffer W1, in order to remove traces of salt from binding step. Salt in the 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. 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 Biofluids RNA Kit	50 Preps	BIO-52086

C. ASSOCIATED PRODUCTS

PRODUCT	PACK SIZE	CAT NO.
SensiFAST™ cDNA Synthesis Kit	50 Reactions	BIO-65053
MyTaq™ HS DNA Polymerase	250 Units	BIO-21111
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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