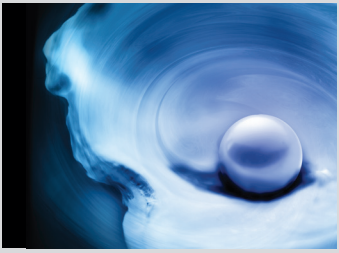


ISOLATE II
RNA/DNA/Protein Kit
(Phenol free)

Product Manual

BIOLINE

A Meridian Life Science® Company

ISOLATE II RNA/DNA/Protein Kit (Phenol free)

1. KIT CONTENTS

COMPONENT	50 Preps
ISOLATE II RNA/Protein Columns (black)	50
ISOLATE II DNA Columns (white)	50
Elution Tubes (1.7 mL)	150
Collection Tubes (2 mL)	150
Lysis Buffer TX*	40 mL
Wash Buffer W1† (concentrate)	2 x 38 mL
Wash Buffer W2†† (concentrate)	18 mL
Wash Buffer W3	30 mL
DNase I Reaction Buffer DRB	6 mL
DNase I Solution	0.8 mL
RNA Elution Buffer	6 mL
DNA Elution Buffer	15 mL
Protein Elution Buffer	8 mL
Protein Binding Buffer	8 mL
Protein Neutralization Buffer	4 mL
Protein Loading Dye†††	2 mL
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 90 mL of 96 - 100% ethanol to each of the bottles containing 38 mL of Wash Buffer W1.
††Before use, add 42 mL of 96 - 100% ethanol to the supplied bottle of 18 mL Wash Buffer W2.
††† Before use, add 93 mg of DTT.

2. DESCRIPTION

The ISOLATE II RNA/DNA/Protein Kit provides a reliable and rapid phenol-free method for the sequential isolation and purification of high quality total RNA (including small RNA), genomic DNA and proteins from a single sample of cultured cells, mammalian tissue, biofluids (including saliva, urine, semen and blood), bacteria, yeast, fungi or plants. The ISOLATE II RNA/DNA/Protein Kit is especially useful for researchers who are isolating macromolecules from precious, difficult to obtain or small samples such as biopsy materials or single foci from cell cultures, as it eliminates the need to fractionate the sample. Furthermore, analysis becomes more reliable since the RNA, DNA and proteins are derived from the same sample.

The protocol prevents degradation of the RNA by mixing the sample with guanidinium thiocyanate, a chaotropic salt which immediately deactivates RNases. Genomic DNA is captured and purified on a DNA Column. Ethanol is then added to the flow-through of the DNA purification step and the solution is loaded onto a RNA/Protein Column. Total RNA, including microRNAs (miRNAs), will bind to the column while proteins pass into the flow-through. The proteins present in the flow-through can be loaded directly onto an SDS-PAGE gel for visual analysis, or further purified. This kit does not require the use of phenol or chloroform.

The kit is ideal for researchers who are interested in investigating the genome, transcriptome and proteome of a single sample. The purified nucleic acids and proteins are of the highest purity and can be used in a wide variety of downstream applications including gene expression profiling, miRNA profiling, siRNA gene silencing studies, novel biomarker discovery, genotyping, transgenic analysis and cell line characterization.

Bioline provides a wide range of other isolation kits for purification of RNA and DNA from different sample sources, please see www.bioline.com/isolate.

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

3. STORAGE

Store DNase I at -20°C upon arrival. Store the Protein Loading Dye at room temperature (18-25°C); after the addition of DL-dithiothreitol (DTT), store at -20°C. All other components should be stored at room temperature. 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 TX 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 RNA/DNA/Protein Kit is specially designed for more reliable analysis since the RNA, DNA and proteins are derived from the same sample. The preparation time is approximately 30 minutes for 10 preps. The purified RNA is of the highest integrity and can be used in real-time PCR, reverse transcription PCR, Northern blotting, RNA-seq assays and expression microarrays. The genomic DNA can be used in PCR, real-time PCR, next generation sequencing and genotyping applications. The purified proteins can be used for various applications including SDS-PAGE analysis, Western blots, 2-D gels and mass spectrometry.

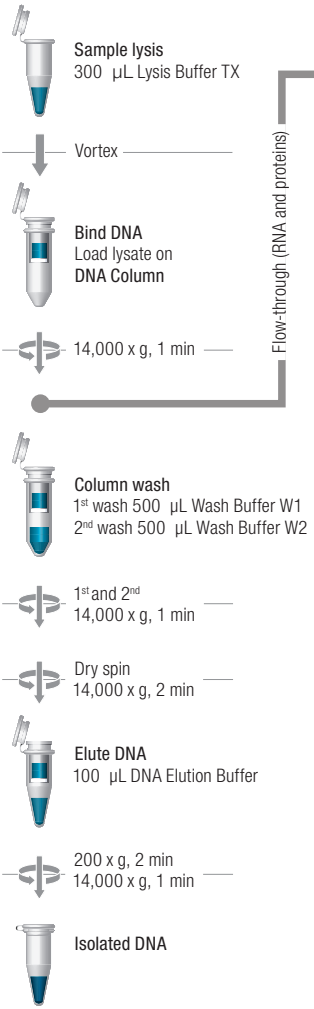
ISOLATE II RNA/DNA/PROTEIN COLUMN SPECIFICATIONS	
Max. binding capacity	50 µg for RNA 20 µg for DNA 200 µg for protein
Max. column loading volume	650 µL
RNA size distribution	All sizes, including small RNA
DNA size distribution	≥ 30 kb
Max. amount of starting material:	
Cultured cells (human/other mammalian)	5 x 10^6 cells
Tissue (human/other mammalian)	25 mg (depending on tissue)
Biofluid (saliva, urine, semen and blood)	100 µL
Bacteria	1 x 10^9 cells
Yeast	1 x 10^8 cells
Fungi	50 mg
Plant tissues	50 mg
Average yields*	
HeLa Cells (1 x 10^6 cells)	15 µg RNA
HeLa Cells (1 x 10^6 cells)	8 µg DNA
HeLa Cells (1 x 10^6 cells)	150 µg protein

** Average yields will vary depending upon a number of factors including species, growth conditions used and developmental stage*

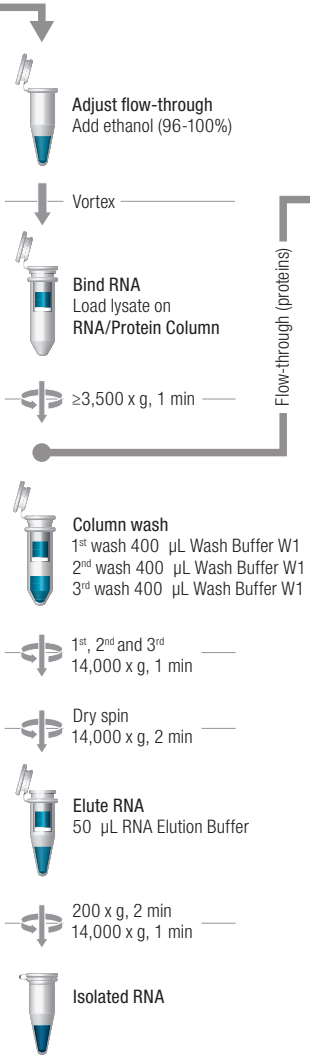
The following component is also included in the kit:

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

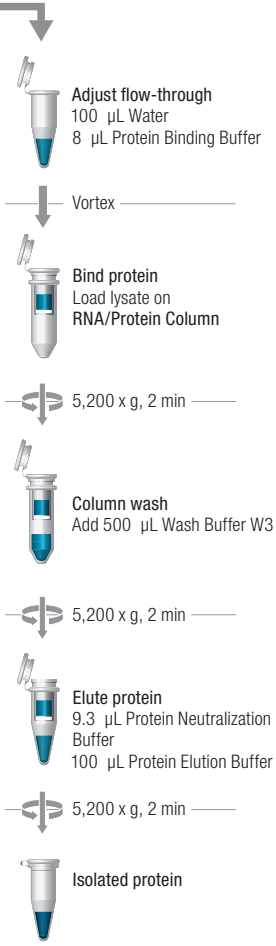
DNA Isolation



RNA Isolation



Protein Isolation



6. EQUIPMENT AND REAGENTS TO BE SUPPLIED BY USER

When working with chemicals, always wear a suitable lab coat, protective goggles and 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:

For all protocols

- ß-mercaptoethanol (ß-ME)* (Optional for Lysis Buffer TX)
- 96-100% ethanol† (for Wash Buffers W1 and W2)
- DL-dithiothreitol (DTT) or tris(2-carboxyethyl)phosphine (TCEP) (for Protein Loading Dye)
- Equipment for sample disruption and homogenization (see section 7.2). One or more of the following are required depending on chosen method:
  - PBS (RNase-free) and trypsin
  - Needle and syringe (both RNase free)
  - Mortar and pestle
  - Rotor-stator homogenizer
  - Liquid nitrogen
- Molecular biology grade water
- RNase-free microcentrifuge tubes (1.5 mL)
- 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:
  - For Gram-negative bacteria, 1 mg/ml lysozyme in TE buffer
  - For Gram-positive bacteria, 3 mg/ml lysozyme in TE buffer

For the yeast protocol:

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

7. IMPORTANT NOTES

The ISOLATE II RNA/DNA/Protein procedure is written in four steps. The protocol steps are: homogenization and preparation of lysate; purification of genomic DNA; purification of total RNA and isolation of protein.

- 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 purify genomic DNA.
- Section 10 contains the protocol to purify total RNA.
- Section 11 contains the protocol to isolate total proteins.
- The appendices contains the optional protocols for acetone precipitation of proteins, and on-column or in-solution DNase I treatment of total RNA.

Please note, the protocols in sections 9 to 11 apply to all the different starting materials.

The ISOLATE II RNA/DNA/Protein 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 **DNA Column** (white ring) and the ISOLATE II **RNA/Protein 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. All centrifugation steps are performed 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 long-term storage, or processed as soon as possible. Following disruption and homogenization in Lysis Buffer TX, 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 TX should be thawed slowly before starting the isolation of total RNA.

7.2 DISRUPTING AND HOMOGENIZING STARTING MATERIALS

For all RNA purification procedures, the efficient disruption and homogenization of the

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 TX 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 TX. 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 the cells to an appropriate tube (not supplied). Pellet the cells by centrifugation (10 minutes at 200 x g). Remove supernatant and add Lysis Buffer TX 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 TX with reducing agent to the powdered tissue and mix immediately. Homogenize the sample with a nuclease-free 20 gauge (0.9 mm) 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: 5-7 mm diameter rotors can be used for homogenization in microcentrifuge tubes.

7.2.6 Disruption and homogenization by enzymatic digest (bacteria/yeast)

For effective yeast RNA isolation, zymolase/lyticase treatment is required to degrade polysaccharides and proteins in the cell wall prior to cell lysis (section 8.5). For bacterial RNA isolation, lysozyme treatment is necessary to ensure efficient cell lysis and RNA release from the cells (section 8.4). For preparation of RNA from microorganisms with difficult to disrupt cell walls e.g. certain gram-positive bacteria, optimizing treatment conditions with lytic enzymes or culture conditions may be required. Following lysis, perform homogenization with a syringe and needle.

7.3 BUFFER PREPARATION AND PARAMETERS

Ensure 96-100% ethanol and reducing agents (ß-ME or TCEP for Lysis Buffer TX and DTT for Protein Loading Dye) are available. Prepare the following:

7.3.1 Preparing Wash Buffer W1 with ethanol

Add 90 mL of 96-100% ethanol to the 38 mL of Wash Buffer W1 Concentrate in each of the supplied bottles to give a final volume of 128 mL.

Note: Mark the label of the bottle to indicate ethanol was added. Store Wash Buffer W1 at room temperature (18-25°C). Wash Buffer W1 is used for both RNA and DNA Purification.

7.3.2 Preparing Wash Buffer W2 with ethanol

Add 42 mL of 96-100% ethanol to the 18 mL of Wash Buffer W2 Concentrate to give a final volume of 60 mL.

Note: Mark the label of the bottle to indicate ethanol was added. Store Wash Buffer W2 at room temperature (18-25°C).

7.3.3 Preparing Protein Loading Dye

Add 93 mg of DL-dithiothreitol (DTT, user supplied) to the Protein Loading Dye.

Note: Mark the label of the bottle to indicate DTT was added. Store at -20°C after the addition of DTT.

7.3.4 Preparing Lysis Buffer TX (optional)

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

Note: TCEP may be used in place of ß-ME. Use TCEP at a final concentration of 10 mM within Lysis Buffer TX.

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

Optional on-column digestion: For each on-column reaction to be performed, prepare a mix of

15 µL of DNase I and 100 µL of DNase Reaction Buffer DRB. Mix gently by inverting a few times.

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

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.

7.3.6 Elution parameters (DNA and RNA)

Elute DNA or RNA using the DNA or RNA Elution Buffers respectively. The standard elution protocol can be modified for different applications.

- To achieve higher 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.

Eluted DNA may be stored at 4°C for a few days however for long-term storage freeze at -20°C or -80°C.

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.

7.3.7 Elution parameters (Protein)

Elute proteins using the provided Protein Elution Buffer (10 mM sodium phosphate pH 12.5). Approximately 95% of bound protein is recovered in the first elution. If desired, a second elution step with Protein Elution Buffer may be carried out. This should be collected into a different tube (to which Protein Neutralization Buffer is pre-added) to prevent dilution of the first elution.

7.4 ELIMINATING GENOMIC DNA CONTAMINATION

For most applications, genomic DNA is undetectable in preparations of RNA using the ISOLATE II RNA/DNA/Protein Kit. Genomic DNA contamination is efficiently removed by on-column digestion with DNase I (see optional section 7.3.5 and Appendix B). 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 7.3.5 and Appendix C).

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 sections 9-11 are the same in all cases.

Before you start:

- Ensure Lysis Buffer TX is prepared (see section 7.3).
- Ensure that all solutions are at room temperature before use.
- 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 1 x 10^6 eukaryotic cells are used. A maximum of 5 x 10^6 cells may be used as starting material, however slight cross-contamination of genomic DNA in the RNA fraction may be observed in input ranges over 1 x 10^6 cells.
- A hemocytometer can be used in conjunction with a microscope to count the number of cells. As a general guideline, a confluent 3.5 cm 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 TX directly to the frozen cell pellet (section 8.1.2, step 4).

Additional reagents 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 300 µL of Lysis Buffer TX directly to the culture plate.
   Note: This volume of lysis buffer can be used for a culture plate of ~35 mm 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 1.5 mL RNase-free microcentrifuge tube (user supplied). **Proceed to section 9.1.**

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

Optional: Lysis can be enhanced at this point by passing the lysate through a 25 gauge needle attached to a syringe 5-10 times or heating the lysate at 55°C.

8.1.2 Cells growing in suspension and lifted cells

1. Transfer cell suspension to a 1.5 mL 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. Wash the cell pellet with an appropriate amount of PBS. Centrifuge at 200 x g for another 5 min.
3. Carefully decant the supernatant. Ensure that a few microliters of PBS are left behind with the pellet in order to ensure that the pellet is not dislodged.
4. Add 300 µL of Lysis Buffer TX to the pellet. Lyse cells by vortexing for 15s. Ensure that the 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 (0.9 mm) syringe needle 5-10 times, in order to reduce the viscosity of the lysate prior to loading onto the column.

   Optional: Lysis can be enhanced at this point by passing the lysate through a 25 gauge needle attached to a syringe 5-10 times or heating the lysate at 55°C.

8.2 LYSATE PREPARATION FROM MAMMALIAN TISSUE

Before you start:

- 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.
- 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 below, we recommend starting with an input not exceeding 10 mg.

TABLE 1: RECOMMENDED INPUT OF DIFFERENT TISSUES	
Tissue	Max. Input Amount
Brain	25 mg
Heart	5 mg
Kidney, Liver, Lung, Spleen	20 mg
Other tissues	10 mg

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 300 µL of Lysis Buffer TX 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 (0.9 mm) needle attached to a syringe 5-10 times.
5. Using a pipette, transfer the lysate into a 1.5 mL RNase-free microcentrifuge tube (user supplied).
   Optional: Lysis can be enhanced at this point heating the lysate at 55°C.
6. Spin the lysate for 2 min at 14,000 x g to pellet any cell debris. Transfer the supernatant to another 1.5 mL RNase-free microcentrifuge tube (user supplied). Note the volume of the supernatant/lysate. **Proceed to section 9.**

8.3 LYSATE PREPARATION FROM BIOFLUIDS

Before you start:

- This protocol is designed for processing a wide variety of biofluids such as saliva, urine, semen and blood. We recommend the use of this kit with non-coagulated blood samples (including fresh or anti-coagulant treated blood).
- It is recommended that no more than 100 µL of biofluid is used in order to prevent clogging of the column.
- Biofluids 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 biofluids.

8.3.1 Protocol

1. Transfer up to 100 µL of biofluid to a 1.5 mL RNase-free microcentrifuge tube (user supplied).
2. Add 300 µL of Lysis Buffer TX to the sample. Lyse cells by vortexing for 15s. Ensure that mixture becomes transparent before proceeding with the protocol. **Proceed to section 9.**
   Optional: The lysate could be heated at 55°C for 10 minutes to enhance lysis.

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 log-phase growth.
- Bacterial pellets can be used directly in this procedure or 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).

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. Optional: The lysate could be heated at 55°C for 10 minutes to enhance lysis.
3. Resuspend the bacteria thoroughly in 100 µL 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 300 µL of Lysis Buffer TX and vortex vigorously for at least 10s. **Proceed to section 9.** Optional: The lysate could be heated at 55°C for 10 minutes to enhance lysis.

TABLE 2: INCUBATION TIME FOR DIFFERENT BACTERIAL STRAINS

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

8.5 LYSATE PREPARATION FROM YEAST

Before you start:

- Prepare the appropriate amount of lyticase-containing Resuspension Buffer (see section 6), considering that 500 µL 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 1 mL 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 500 µL 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 300 µL of Lysis Buffer TX and vortex vigorously for at least 10s. **Proceed to section 9.** Optional: The lysate could be heated at 55°C for 10 minutes to enhance lysis.

8.6 LYSATE PREPARATION FROM FUNGI

Before you start:

- Fresh or frozen fungi may be used for this procedure. Fungal tissue should be flash-frozen in liquid nitrogen and transferred immediately to a -80°C freezer for long-term storage. Fungi 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 ensure that the integrity of the RNA is not compromised.
- It is recommended that no more than 50 mg of fungal tissue is used for this procedure in order to prevent clogging of the column.

8.6.1 Protocol

1. Determine the amount of fungal tissue by weighing. It is recommended that no more than 50 mg of fungal cells are used for the protocol.
2. Transfer the sample into a mortar that contains enough liquid nitrogen to cover the sample. Grind thoroughly using a pestle.
   Note: At this stage the ground sample may be stored at -80°C, such that the RNA purification can be performed at a later time.
3. Allow the liquid nitrogen to evaporate, without allowing the tissue to thaw.
4. Add 300 µL of Lysis Buffer TX to the tissue sample and continue to grind until the sample has been homogenized.
5. Using a pipette, transfer the lysate into an 1.5 mL RNase-free microcentrifuge tube (user supplied).
   Optional: The lysate could be heated at 55°C for 10 minutes to enhance lysis.
6. Spin the lysate for 2 min to pellet any cell debris. Transfer the supernatant to another 1.5 mL RNase-free microcentrifuge tube (user supplied). Note the volume of the supernatant/lysate. **Proceed to section 9.**

Optional: The lysate could be heated at 55°C for 10 minutes to enhance lysis.

8.7 LYSATE PREPARATION FROM PLANT

Before you start:

- The maximum recommended input of plant tissue is 50 mg or 5 x 10^6 plant cells.
- Both fresh and frozen plant samples can be used for this protocol. Samples should be flash-frozen in liquid nitrogen and transferred immediately to a -80°C freezer for long-term storage. Do not allow frozen tissues to thaw prior to grinding with the mortar and pestle in order to ensure that the RNA integrity is not compromised.

8.7.1 Protocol

1. Transfer ≤50 mg of plant tissue or 5 x 10^6 plant cells into a mortar that contains an appropriate amount of liquid nitrogen to cover the sample. Grind the 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.
2. Allow the liquid nitrogen to evaporate, without allowing the tissue to thaw.
3. Add 600 µL of Lysis Buffer TX to the tissue sample and continue to grind until the sample has been homogenized.
4. Using a pipette, transfer the lysate into an RNase-free microcentrifuge tube (user supplied). Optional: The lysate could be heated at 55°C for 10 minutes to enhance lysis.
5. Spin lysate for 2 min at 14,000 x g to pellet any cell debris. Transfer the supernatant to another 1.5 mL RNase-free microcentrifuge tube (user supplied). Note the volume of the supernatant/lysate. **Proceed to section 9.**

9. GENOMIC DNA PURIFICATION FROM ALL TYPES OF LYSATE

The following steps of the protocol for genomic DNA purification are the same for all the different types of lysate. Ensure you use the correct column: the ISOLATE II **DNA Column** has a white ring.

Before you start:

- Ensure Wash Buffer W1 and W2 are prepared (see section 7.3).

9.1 Binding to DNA Column

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

3. **Retain the flow-through for Total RNA Purification (see section 10).** The flow-through contains the RNA and proteins and should be stored on ice or at -20°C until the Total RNA Purification protocol is carried out.
4. Reassemble the spin column with the Collection Tube.

9.2 Genomic DNA Wash

1. Apply 500 µL of Wash Buffer W1 to the column and centrifuge for 1 min at 14,000 x g. Discard the flow-through.
2. Apply 500 µL of Wash Buffer W2 to the column and centrifuge for 1 min at 14,000 x g. Discard the flow-through.
3. Spin the column for 2 min at 14,000 x g in order to dry the column thoroughly. Discard the Collection Tube.

9.3 DNA Elution

1. Place the column into a fresh 1.7 mL Elution Tube.
2. Add 100 µL of DNA 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 1 min at 14,000 x g.

   Note: For maximum DNA recovery, it is recommended to apply a second volume of 100 µL DNA 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).

9.4 Storage of DNA

The isolated DNA can be stored at 4°C for a few days. It is recommended that samples are held at -20°C or -80°C for long-term storage.

10. TOTAL RNA PURIFICATION FROM ALL TYPES OF LYSATE

The following steps of the protocol for total RNA purification are the same for all the different types of lysate. Ensure you use the correct column: the ISOLATE II **RNA/Protein Column** has a black ring.

For sensitive applications that require the complete removal of genomic DNA, an optional on-column DNase I treatment can be performed after completion of section 10.1 step 6 (see Appendix B). Alternatively, an in solution DNase I treatment can be performed after RNA elution (see Appendix C).

10.1 Binding RNA to RNA/Protein Column

1. To every 100 µL of flow-through from section 9.1, step 3, add 60 µL of 96-100% ethanol. Mix by vortexing.
   Note: For example, for 300 µL of flow-through, add 180 µL of 96-100% ethanol.
2. Assemble an ISOLATE II **RNA/Protein Column (black ring)** with the Collection Tube provided.
3. Apply up to 600 µL 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 1 min at 14,000 x g.
4. **Retain the flow-through for Protein Isolation (see section 11)**. The flow-through contains the proteins and should be stored on ice or at -20°C until the Protein Isolation protocol is carried out.
5. Depending on your lysate volume, repeat steps 3 and 4 if necessary. The flow-throughs should be combined and retained in the same microcentrifuge tube.
6. Reassemble the spin column with the Collection Tube.
7. **Optional:** The ISOLATE II RNA/DNA/Protein 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 B. 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 C).

10.2 RNA Wash

1. Apply 400 µL of Wash Buffer W1 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 1 min at 14,000 x g.
2. Discard the flow-through and reassemble the column with the Collection Tube.
3. Wash the column a second time by adding another 400 µL of Wash Buffer W1 and centrifuge for 1 min at 14,000 x g.
   Note: If performing the optional on-column DNase I treatment (see Appendix B), resume the protocol at this point, by performing this second wash step.
4. Discard the flow-through and reassemble the spin column with its Collection Tube.
5. Wash the column a third time by adding 400 µL of Wash Buffer W1 and centrifuge for 1 min at 14,000 x g.
6. Discard the flow-through and reassemble the spin column with its Collection Tube.
7. Spin the column for 2 min at 14,000 x g in order to thoroughly dry the column. Discard the Collection Tube.

10.3 RNA Elution

1. Place the column into a fresh 1.7 mL Elution Tube (supplied).
2. Add 50 µL 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 1 min at 14,000 x g.

   Note: For maximum RNA recovery, particularly for samples that are known to contain large amounts of RNA, it is recommended that a second elution be performed 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.

4. **Retain the column for Protein Isolation (Proceed to section 11).**

10.4 Storage of RNA

The isolated RNA can be stored at -20°C for up to three days. It is recommended that samples are kept at -80°C for long-term storage.

11. TOTAL PROTEIN ISOLATION FROM ALL CELL TYPES

Before you start:

- At this stage, the proteins that are present in the flow-through from the RNA Binding Step (section 10.1, step 4) can be processed by one of the following three options:
  1. Run directly on an SDS-PAGE gel with the loading dye provided for visual analysis
  2. Column purification (recommended) (see section 11.1)
  3. Acetone precipitation (see Appendix A, section 12.1))
- Check that 93 mg of DTT (user supplied) has been added to the 2 mL of Protein Loading Dye. The Protein Loading Dye should be stored at -20°C after the addition of DTT.
- To run directly on a gel, the provided Protein Loading Dye should be used instead of a regular SDS-PAGE loading buffer in order to prevent any precipitates from forming. Add 1 volume of the Protein Loading Dye to the sample and boil for 2 min before loading.
- For acetone precipitation, please refer to the supplementary protocol provided in Appendix A.
- Column purification of the proteins is recommended. Please follow sections 11.1 to 11.4 below.

11.1 pH adjustment of lysate

1. Transfer the flow-through from the RNA Binding Step (section 10.1, step 4) to a separate 1.5 mL microcentrifuge tube (user supplied).
2. For every 100 µL of flow-through, add 100 µL of molecular biology grade water.

   Note: For example, to purify the entire flow-through of 480 µL, add 480 µL molecular biology grade water.

3. For every 100 µL of flow-through, add 8 µL of Protein Binding Buffer. Mix contents well.

   Note: For an entire flow-through of 480 µL, add 40 µL of Protein Binding Buffer. Depending on the type and amount of input, slight precipitation may occur, which will not affect the purification procedure.

11.2 Protein binding

1. Apply up to 600 µL of the pH-adjusted protein sample onto the ISOLATE II **RNA/Protein Column (black ring)** and centrifuge for 2 min at 5,200 x g. Inspect the column to ensure that the entire sample has passed through into the Collection Tube. If necessary, spin for an additional 3 min at 5,200 x g.
2. Discard the flow-through. Reassemble the spin column with its Collection Tube.
   Note: You can save the flow-through into a fresh tube for assessing the protein's binding efficiency.
3. Depending on your sample volume, repeat steps 1 and 2 until the entire protein sample has been loaded onto the column.

11.3 Column wash

1. Apply 500 µL of Wash Buffer W3 to the column and centrifuge for 2 min at 5,200 x g.
2. Discard the flow-through and reassemble the spin column with its Collection Tube.
3. Inspect the column to ensure that the liquid has passed through into the Collection Tube. There should be no liquid in the column. If necessary, spin for an additional minute at 5,200 x g to dry.

11.4 Protein elution and pH adjustment

1. Add 9.3 µL of Protein Neutralization Buffer to a fresh 1.7 mL Elution Tube (supplied).
2. Transfer the spin column from the Column wash procedure (section 11.3) into the Elution Tube.
3. Apply 100 µL of the Protein Elution Buffer to the column and centrifuge for 2 min at 5,200 x g to elute bound protein.

   Note: Approximately 95% of bound protein is recovered in the first elution. If desired, a second elution using 50 µL of Protein Elution Buffer may be carried out. This should be collected into a different tube (to which 4.6 µL of Protein Neutralization Buffer is pre-added) to prevent dilution of the first elution.

12 APPENDICES

12.1 APPENDIX A: ACETONE PRECIPITATION OF PROTEINS

Acetone precipitation allows for the concentration of a protein sample and removal of small interfering species, such as salts and detergents for downstream applications.

1. Add 4 volumes of ice-cold acetone to the flow-through from the RNA Binding Step (section 10.1, step 4).
2. Incubate for 15 min on ice or at -20°C.
3. Centrifuge for 10 min at 14,000 x g. Discard the supernatant and allow the pellet to air-dry.
   Note: At this point the pellet can be washed with 100 µL of ice cold ethanol and again air-dried.
4. Resuspend the pellet in the buffer suited to your downstream application.

12.2 APPENDIX B: OPTIONAL ON-COLUMN DNASE I TREATMENT

The ISOLATE II RNA/DNA/Protein 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.

1. For each on-column digest to be performed, prepare a DNase I-buffer mix by adding 15 µL of the provided DNase I Solution to 100 µL 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 6).
3. Apply 400 µL 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 has passed through into the Collection Tube by inspecting the column. If the entire wash volume has not passed through, spin for an additional 1 min at 14,000 x g.

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

   Note: Ensure that the entire volume of DNase I-buffer mix passes through the column. If needed, spin for an additional 1 min 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 in order to ensure maximum DNase 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 Wash section (section 10.2, step 3). Apply the wash buffer directly to the column containing the DNase I - buffer mix.

12.3 APPENDIX C: OPTIONAL DNASE I TREATMENT OF PURIFIED RNA IN SOLUTION

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

1. In a microcentrifuge tube, mix together 2.5 µL of the supplied DNase I Solution, 10 µL of DNase I Reaction Buffer DRB and up to 87.5 µL of eluted RNA. If using a lower starting volume of RNA solution, bring the volume up to 100 µL using RNase-free water.
2. Gently swirl the tube to mix the solution. Gently spin down (approx. 1s at 1000 x g) to

collect the solution at 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 RNA in the RNase-free water.

13. TROUBLESHOOTING GUIDE

LOW RNA YIELD OR QUALITY	
POSSIBLE CAUSE	RECOMMENDED SOLUTION
Incomplete lysis of cells or tissue	Ensure an appropriate amount of Lysis Buffer TX was used for amount of cells or tissue. If necessary, increase volume of Lysis Buffer TX used. Increase lysis incubation time.
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 the recommended levels. See also Clogged Spin Column below.
An alternative elution buffer was used	It is recommended that RNA Elution Buffer supplied with this kit is used for maximum RNA recovery.
Ethanol not added to the lysate	Ensure 90 mL of 96-100% ethanol is added to Wash Buffer W1 provided prior to use.
Low RNA content	Different tissues and cells have different RNA contents and thus expected yield of RNA will vary greatly between sources. Please check literature to determine expected RNA content of your starting material.
Cell Culture: Cell monolayer was not washed with PBS	Ensure cell monolayer is washed with an appropriate amount of PBS in order to remove residual media from cells.
Yeast: Lyticase not added to Resuspension Buffer	Ensure an appropriate amount of lyticase is added when making the Resuspension Buffer.
Bacteria and Yeast: All traces of media not removed	Ensure all media is removed prior to the addition of Lysis Buffer TX through aspiration.

CLOGGED SPIN COLUMN	
POSSIBLE CAUSE	RECOMMENDED SOLUTION
Insufficient solubilization of cells or tissues	Reduce amount of starting material used. Ensure sufficient lysis buffer is used for the amount of cells or tissue.
Maximum number of cells or amount of tissue exceeded	Determine if amount of starting material falls within kit specifications.
Centrifuge temperature too low	Ensure centrifuge remains at room temperature throughout procedure. Temperatures below 15°C may cause precipitates to form that can cause the columns to clog.
Insufficient centrifugation	Increase centrifugation speed and time.
RNA DEGRADED	
POSSIBLE CAUSE	RECOMMENDED SOLUTION
RNase contamination	RNases may be introduced during use of kit. Ensure proper procedures are followed when working with RNA. Tips with working with RNA can be found on www.bioline.com/isolate
Procedure not performed quickly enough	In order to maintain integrity of the RNA, it is important that procedure is performed quickly. This is especially important for Lysate Preparation steps in the mammalian tissue protocol, since RNA in mammalian tissues is not protected until it is disrupted and homogenized. After the DNA binding step, flow-through should be kept on ice or -20°C if an RNA purification step is not carried out immediately.
Improper storage of the purified RNA	For short term storage, RNA samples may be stored at -20°C for a few days. It is recommended that samples are kept at -80°C for longer term storage.
Frozen tissues or cell pellets were allowed to thaw prior to RNA isolation	Do not allow frozen tissues to thaw prior to grinding with the mortar and pestle in order to ensure RNA integrity is not compromised.
Lysozyme or lyticase used may not be RNase-free	Ensure that the lysozyme and lyticase being used with this kit are RNase-free, in order to prevent possible problems with RNA degradation.
Tissue samples were frozen improperly	Samples should be flash-frozen in liquid nitrogen and transferred immediately to a -80°C freezer for long-term storage.
RNA DOES NOT PERFORM WELL IN DOWNSTREAM APPLICATIONS	
POSSIBLE CAUSE	RECOMMENDED SOLUTION
RNA not washed twice with Wash Buffer	Traces of salt from binding step may remain in the sample if column is not washed twice with Wash Buffer W1. Salt may interfere with downstream applications and must be washed from column.
Ethanol carryover	Ensure that the dry spin in RNA Wash procedure is performed, in order to remove traces of ethanol prior to elution. Ethanol is known to interfere with many downstream applications.

LOW DNA YIELD	
POSSIBLE CAUSE	RECOMMENDED SOLUTION
Incomplete lysis of cells or tissue	Ensure an appropriate amount of Lysis Buffer TX was used for the amount of cells or tissue. Incubate Lysis Buffer TX for an extra 5 min to assist lysis.
DNA elution is incomplete	Ensure that centrifugation for 1 min at 14,000 x g is performed following centrifugation for 2 min at 200 x g. Also, ensure that entire volume of DNA Elution Buffer passed through and is eluted from column.
GENOMIC DNA SHEARED	
POSSIBLE CAUSE	RECOMMENDED SOLUTION
Old sample	Ensure sample is not too old, as old samples often yield only degraded DNA.
Sample repeatedly frozen and thawed	Samples should not be repeatedly frozen and thawed, as this tends to increase likelihood of isolating degraded DNA.
CONTAMINATION OF RNA WITH GENOMIC DNA	
POSSIBLE CAUSE	RECOMMENDED SOLUTION
Number of cells or amount of tissue used is close to the maximum recommended amount	When the maximum recommended amount of cells or tissues is used for this procedure, some cross-contamination of genomic DNA in the RNA fraction may be observed. Reduce the starting amount of cells or tissues below maximum recommendation in order to avoid this problem. Recommended amounts of starting material to use for optimal kit performance are given in each section of protocol. Alternatively, carry out DNase I treatment as described in Appendix B (on-column treatment) or in Appendix C (in solution protocol).
POOR PROTEIN RECOVERY	
POSSIBLE CAUSE	RECOMMENDED SOLUTION
Incorrect pH adjustment of sample	Ensure pH of starting protein sample is adjusted to pH 3.5 or lower after Protein Binding Buffer has been added and prior to binding to column. If necessary, add additional Protein Binding Buffer.
Low protein content in the starting materials	Run a 20 µL fraction from flow-through (after RNA binding) on an SDS-PAGE gel to estimate amount of protein present in sample. In addition, use entire flow-through in protein purification procedure.
PROTEIN DEGRADED	
POSSIBLE CAUSE	RECOMMENDED SOLUTION
Eluted protein solution was not neutralized	Add 9.3 µL of Protein Neutralization Buffer to each 100 µL of eluted protein in order to adjust pH to neutral. Some proteins are sensitive to high pH, such as elution buffer at pH 12.5.
Eluted protein was not neutralized quickly enough	If eluted proteins are not used immediately, degradation will occur. We recommend adding Protein Neutralization Buffer in order to lower pH.

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 RNA/DNA/Protein Kit	50 Preps	BIO-52085

C. ASSOCIATED PRODUCTS

PRODUCT	PACK SIZE	CAT NO.
TRIsure™	100 mL	BIO-38032
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
HyperPAGE Protein Marker	50-100 Lanes	BIO-33066

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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