ROJETECHNOLOGIES

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RNSol H Reagent

Total RNA isolation based on silica technology

- MiniPrep

For RNA Isolation from

Animal Tissue (Fibrous and non-fibrous tissue)

Animal Cells

Bacteria Cells

PBMC (Peripheral Blood Mononuclear Cell)

Whole Blood

WBC (White Blood Cell)

By ROJE

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ROJETechnologies has been founded since 2014, and manufactures a wide range of molecular biology kits. We research, develop and create our products in order to make easier and more comfortable approaches to do research in molecular biology. Our target is offering high-quality affordable molecular and diagnostic Kits and reagents, comparable of the world leaders, to research centers, laboratories, clinics, hospitals and diagnostic centers all over the world.

Contents

Storage

Shipment condition is checked by ROJETechnologies. After arrival, RNSol H Reagent should be kept dry, at 2-8 °C. When storage condition is as directed, all reagents are stable until expiration date, as indicated on the kit box.

Intended Use

RNSol H Reagent is used to purify RNA and miRNA from animal tissue and cell, PBMC, whole blood, WBC and bacteria. Notice that, RNSol H Reagent is intended for molecular biology applications not for diagnostic use. We recommend all users to study RNA experiments guideline, before starting their work.

Guarantee & Warranty

ROJETechnologies guarantees the efficiency of all manufactured kits and reagents. For more information on choosing proper kits based on your needs, please contact our technical support team. If any product does not satisfy you, due to reasons other than misuse, please contact our technical support team. If problem is due to manufacturing process, ROJE team will replace the Kit for you.

Notice to Purchaser

This product is only for experiments and not for commercial use in any kind. No right to resell this kit or any components. For information about out licensing or distributors contact ROJE business team.

Warning and Precautions

Due to chemical material usage that may be hazardous, always make sure to wear suitable lab coat, disposable gloves, and protective eyewear. Material Safety Data Sheet (MSDS) for all products and reagents are provided. They are accessible online at www.rojetechnologies.com

Quality Control

MiRJia Kit is tested against predetermined experiments on a lot-to-lot basis according to ROJETechnologies ISO-certified quality management system, to ensure consistent product quality. For your information, the results of all experiments are accessible by addressing REF and Lot number on web at www.rojetechnoloes.com

Procedure

The RNSol H reagent is designed for isolating all form of RNA molecules from 0.5- 100 mg tissue samples or up to 1×107 animal cells and up to 2 x 109 bacteria. Fresh or frozen tissue samples should be lysed and homogenized. Aqueous phase is separated using chloroform. Then, RNA selectively will be isolated by the addition of chloroform. Contaminants removed by 75% ethanol. Pure RNA is finally eluted in nuclease free water. Isolated RNA is ready to use in downstream applications. It has A260/A280 ratios of 1.9–2.3 by spectrophotometer, confirming high purity.

Equipment & Reagents to Be Supplied by User

- Isopropanol
- Nuclease free Water
- Chloroform
- 1.5 ml RNase free microcentrifuge tubes
- Pipets and RNase free pipet tips
- RNase free microcentrifuge tube
- Benchtop refrigerated microcentrifuge
- Vortex
- Heat block / water bath
- TissueLyser/ Homogenizer/ Mortar and pestle

Applications

The isolated RNA and miRNA can be used in many downstream applications:

- RNA Sequencing
- cDNA synthesis
- Poly A+ RNA selection
- Microarrays
- RT-PCR and real-time RT-PCR
- Northern, dot blot analysis

Features

Specific features of RNSol H Reagent are listed here in Table 1.

Table 1: RNSol H Reagent features and specifications

Features	Specifications
Elution volume (µl)	30-100µl
Technology	Solution based
Main sample type	Animal cells and tissue /Bacteria cells/PBMC/Whole blood
Processing	Manual
Purification of total RNA, miRNA, Poly A+ mi-RNA, DNA or protein	Yes
Sample Amount	• 50-100 mg of tissue • or up to 10^7 cells and up to 10^9 bacteria cells • 0.5 to 10 ml of whole blood
Operation time per reaction (min)	Less than 90 Min
Typical yield For 50 Mg Tissue	200µg
Average purity	A260/A280= 1.9-2.3
Size of purified RNA	Total RNA
Enzyme	No

Recommended Starting Material

To reach optimized results it is better to follow as listed below. The size of the recommended starting material to use with determined RNSol H Reagent and chloroform volumes, are written in Table 2.

Table 2. Appropriate sample size and amount of RNSol H Reagent, chloroform and ethanol amount

Sample Size	RNSol H Reagent Amount	Isopropanol	Chloroform Amount
10-80 mg animal tissue, $(1\times10^6) – (8\times10^6)$ animal cells 1-2 ml whole blood	800µl	400µl	200µl
90-100 mg animal tissue $(9\times10^6) – (10^7)$ animal cells 2.5-10 ml whole blood 1 x 107 bacteria cell	1ml	500µl	200µl

Sample Storage and Preparation

Animal Tissue

Storage: Fresh or frozen tissues may be used for the procedure. For long storage of tissue samples, Tissues should be flash-frozen in liquid nitrogen and transferred immediately to a -70 °C freezer. Tissues may be stored at -70 °C for several months. When isolating RNA and miRNA from frozen tissues ensure that the tissue does not thaw during weighing or prior to RNA preparation. It is recommended to store tissue sample in RNaseLag for storage (refer to RNaseLag).

Preparation: Sample preparation is composed of 2 steps, disruption and homogenization. First step is disruption of cell walls, plasma membranes of cells and organelles. Inefficient disruption results in RNA yield reduction. This can be done with one or cooperation of two methods depending on the tissue type. These methods include RNSol H Reagent, Mortar and Pestle, Tissuelyser etc. Viscosity reduction can be accomplished by Homogenization. The aim is to create a homogeneous lysate. Sometimes these 2 steps happen simultaneously. For more information refer to Table 3.

> **Note:** After disruption and homogenization in RNSol H Reagent (lysis buffer), samples can be stored at –70°C for months.

Table 3. Disruption and homogenization for different sample types

Sample type	Disruption	Homogenization
Animal Tissue	TissueRuptor/TissueLyser Mortar and pestle	TissueRuptor/TissueLyser Syringe and needles
Animal Cells, WBC, PBMC	RNSol H Reagent Vortexing	Homogenizer Syringe and needles
Bacteria Cells	RNSol H Reagent Vortexing	Homogenizer Syringe and needles

> **Optional:** Homogenizing step is optional. It means that using homogenizer or syringe needle to homogenize the lysate can be omitted from the process.

RNaseLag

For optimum result, it is recommended to store tissue samples in RNaseLag. RNaseLag is a Reagent which stabilizes RNA in tissues and cells.

Procedure

- Cut the animal tissue sample into slices less than 5mm thick, as quickly as possible.
- Completely immerse the tissue pieces in the collection vessel containing RNaseLag.

  Note: Make sure to use the appropriate volume of RNaseLag, so weight your sample before starting the procedure and use 10µl RNaseLag per 1mg of tissue.

- The sample is ready for archival storage at conditions shown in Table 4.
- After storage, for RNA isolation continue with appropriate protocol for the chosen sample type.

Table 4. Storage conditions and procedures after RNaseLag treatment.

Storage condition	Protocol
2–8°C	Incubate the prepared sample (in RNaseLag) for up to 4 weeks at 2–8°C.
15-25°C	Incubate the prepared sample (in RNaseLag) for up to 7 days at 15-25°C.
37°C	Incubate the prepared sample (in RNaseLag) for up to 1 days at 37°C.
–20°C	First, incubate the prepared sample (in the RNaseLag) overnight at 2–8°C. Then transfer it to –20°C for storage.
–80°C	First, incubate the prepared sample (in the RNaseLag) at 2–8°C. Then remove the tissue from the reagent, and transfer it to –80 °C for long storage.

Sample Storage and preparation

Animal cells

Storage: Fresh or frozen samples may be used by RNall. Frozen samples can be kept at -80°C for long time. As a guide, storage preparation stock and conditions are written here.

Cell selection: First, ensure that the cells are in their best possible condition. Select cultures near the end of log phase growth (approximately %90 confluent) and change their medium 24 hours prior to harvesting. Carefully examine the culture for signs of microbial contamination. Facilitate this by growing cultures in antibiotic-free medium for several passages prior to testing. This allows time for any hidden, resistant contaminants (present in very low numbers) to reach a higher, more easily detected level. Samples of these cultures are then examined microscopically and tested by direct culture for the presence of bacteria, yeasts, fungi, and mycoplasmas.

Cell harvesting: Remove all dissociating agents by washing or inactivation (especially important when using serum-free medium). Centrifugation, when absolutely necessary,

should only be hard enough to obtain a soft pellet; 100 x g for 5 to 6 minutes is usually sufficient. Count and then dilute or concentrate the harvested cell suspension to twice the desired final concentration, which is usually 4 to 10 million viable cells per milliliter. An equal volume of medium containing the cryoprotective agent at twice its final concentration will be added later to achieve the desired inoculum. Keep the cells chilled to slow their metabolism and prevent cell clumping. Avoid alkaline pH shifts by gassing with CO2 when necessary.

Cryoprotection: Cryoprotective agents are necessary to minimize or prevent the damage associated with slow freezing. DMSO is most often used at a final concentration of 5 to %15 (v/v). Always use reagent or other high purity grades that have been tested for suitability. Sterilize by filtration through a 0.2-micron nylon membrane in a polypropylene or stainless-steel housing and store in small quantities (5ml). Some cell lines are adversely affected by prolonged contact with DMSO. This can be reduced or eliminated by adding the DMSO to the cell suspension at 4°C and removing it immediately upon thawing. If this does not help, lower the concentration or try glycerol or another cryoprotectant. Glycerol is generally used at a final concentration of between 5 and %20 (v/v). Sterilize by autoclaving for 15 minutes in small volumes (5ml) and refrigerate in the dark. Although less toxic to cells than DMSO, glycerol frequently causes osmotic problems, especially after thawing. Always add it at room temperature or above and remove slowly by dilution. High serum concentrations may also help cells survive freezing. Replacing standard media-cryoprotectant mixtures with %95 serum and %5 DMSO may be superior for some overly sensitive cell lines, especially hybridomas. Add cryoprotective agents to culture medium (without cells) immediately prior to use to obtain twice the desired final concentration (2X). Mix this 2X solution with an equal volume of the harvested cell suspension to obtain the inoculum for freezing. This method is less stressful for cells, especially when using DMSO as the cryoprotectant.

Cooling rate: The cooling rate used to freeze cultures must be just slow enough to allow the cells time to dehydrate, but fast enough to prevent excessive dehydration damage. A cooling rate of -1°C to -3°C per minute is satisfactory for most animal cell cultures. Larger cells, or cells having less permeable membranes may require a slower freezing rate since their dehydration will take longer. Transfer from the cooling chamber or device to the final storage location must be done quickly to avoid warming of the vials. Use an insulated container filled with dry ice or liquid nitrogen as a transfer vessel to ensure that the cells remain below -70°C.

Thawing: Remove the vial from its storage location and carefully place the vessel in warm water, agitate gently until completely thawed. Rapid thawing (60 to 90 seconds at 37°C)

provides the best recovery for most cell cultures; it reduces or prevents the formation of damaging ice crystals within cells during rehydration.

Recovery: Since some cryoprotective agents may damage cells upon prolonged exposure, remove the agents as quickly and gently as possible. Several approaches are used depending on both the cryoprotective agents and characteristics of the cells. Most cells recover normally if they have the cryoprotective agent removed by a medium change within 6 to 8 hours of thawing. Transfer the contents of the vial to a T-75 flask or other suitable vessel containing 15 to 20 milliliters of culture medium and incubate normally. As soon as a majority of the cells have attached, remove the medium containing the now diluted cryoprotective agent and replace with fresh medium.

For cells that are sensitive to cryoprotective agents, removing the old medium is easily accomplished by gentle centrifugation. Transfer the contents of the vial to a 15ml centrifuge tube containing 10ml of fresh medium and spin for 5 minutes at 100 x g. Discard the supernatant containing the cryoprotectant and resuspend the cell pellet in fresh medium. Then transfer the cell suspension to a suitable culture vessel and incubate normally.

When glycerol is used as the cryoprotectant, the sudden addition of a large volume of fresh medium to the thawed cell suspension can cause osmotic shock, damaging or destroying the cells. Use several stepwise dilutions with an equal volume of warm medium every 10 minutes before further processing to give the cells time to readjust their osmotic equilibrium.

Preparation: It is crucial to use the correct amount of starting material. RNA content can vary greatly from cell to cell. Therefore, counting cells is the most important step before starting the procedure (for more information refer to appendix 7). However, as a guide, the number of HeLa cells after confluent growth obtained in various culture vessels, is given in Table 5. After counting and selecting the intended cell volume, refer to Table 3 for disruption and homogenization guide.

Table 5. Number of HeLa cells in various culture vessels

Vessel Type		Cell Number
Dishes	35mm	1×10^6
	60mm	2.5×10^6
	100mm	7×10^6
	145-150mm	2×10^7
Flask	40-45ml	3×10^6
	250-300ml	1×10^7
	650-750ml	2×10^7
Multiwell-plates	96-wells	$4\text{-}5 \times 10^4$

	48-wells	1×10^5
	24-wells	2.5×10^5
	12-wells	5×10^5
	6-wells	1×10^6

Maximize RNA Yield

To obtain higher yield of RNA, it is important to follow protocols carefully and pay attention to sample size table and its suitable lysis buffer amount recommended for your samples. Notice that all samples must be completely homogenized during cell lysis for maximum yields. It is good to know that:

- Yield and quality of the purified RNA depends on sample storage conditions. For best results, it is recommended to use fresh samples, however for long storage, it is better to treat sample in RNaseLag (for more information refer to RNaseLag).
- Avoid freezing and thawing samples, which may result in decreasing RNA yield, as compared to isolating RNA from fresh samples.

Sample preparation

Bacteria

Typical yields of RNA will vary depending on the cell density of the bacterial culture and the bacterial species, hence before starting, it's recommended to determine your bacterial species. As a guide, bacteria culture preparation and storage conditions are written here.

Storage

Fresh or frozen bacteria samples may be used by RNall. Frozen samples can be kept at -80°C for a long time. As a guide, storage, preparation of stock and conditions are written here.

Bacteria culture

The following protocol is for inoculating an overnight culture of liquid LB with bacteria**.**

- Prepare liquid Luria-Bertani (LB)

To make 400 mL of LB, weigh out the following into a 500 mL glass bottle:

- 4 g NaCl
- 4 g Tryptone
- 2 g Yeast Extract

- and dH2O to 400 mL

Loosely close the cap on the bottle and then loosely cover the entire top of the bottle with aluminum foil. Autoclave and allow to cool to room temperature. Now screw on the top of the bottle and store the LB at room temperature.

- Use a single, well-isolated colony from a fresh Luria-Bertani (LB) agar plate to inoculate 1–10 ml of LB medium.
- Loosely cover the culture with sterile aluminum foil or a cap that is not airtight.
- Incubate bacterial culture at 37°C for 12-18 hour in a shaking incubator

Storing condition

- Autoclave microcentrifuge tube or 1-3 ml screw cap.
- Grow a fresh overnight culture of the strain in broth. Do not grow the cultures too long. Bacteria strains should be grown to late log phase.
- Label the tube with the strain and date.
- Either 5% to 10% DMSO or glycerol can be used as cryopreservation in the culture medium. Glycerol is usually prepared in aqueous solution at double the desired final concentration for freezing. It is then mixed with an equal amount of cell suspension.
- Aliquot 1 to 1.8 ml of bacteria to each vial and seal tightly with screw cap.
- Allow the cells to equilibrate in the freeze medium at room temperature for a minimum of 15 min but no longer than 40 min. After 40 min, the viability may decline if DMSO is used as the cryoprotectant.
- Place the vials into a pre-cooled (4°C), controlled rate freeze chamber and place the chamber in a mechanical freezer at -70 °C for at least 24 hours.
- Quickly transfer the vials to liquid nitrogen or at -130 °C freezer. After 24 hours at -130 °C, remove one vial, restore the bacteria in the culture medium and check viability and sterility.

Recovery of cryopreserved cells

- Prepare a cultured vessel that contains at least 10 ml of the appropriate growth medium equilibrate for both temperature and pH.
- Remove the vial containing the strain of interest and thaw by gentle agitation in a 37 °C water bath (or a bath set at the normal growth temperature for that bacterial strain). Thaw the strain rapidly until all ice crystals have been melted (approximately 2 min).
- Remove the vial from the bath and decontaminate it by dipping in or spraying with 70% ethanol. Unscrew the top of the vial and transfer the entire content to the prepared growth

medium. Examine the cultures after an appropriate length of time. If the broth shows growth in 1-2 days, streak a plate from the broth and verify that is the correct strain.

Preparation: It is crucial to use the correct amount of starting material. RNA content can vary greatly between different bacteria types. So, counting cells is the most important step before starting the procedure. The input bacterial cell amount should not exceed 2 × 109 cells. For example, for E. coli, depending on culture growth, this is equivalent to 0.5 - 1.0 mL of an overnight culture. It is not recommended to exceed 1 mL of culture for this procedure. It is important to measure bacterial growth by spectrophotometer before starting the protocol. (For cell counting guideline refer to appendix 7). After counting and selecting the intended cell volume, refer to Table 3 for disruption and homogenization guide.

RNaseLag

For optimum results, it is recommended to store your samples in RNaseLag. RNaseLag is a Reagent, which provides in vivo stabilization of RNA in bacteria to ensure reliable gene expression analysis.

Procedure

- Calculate the required volume of bacterial culture (refer to Appendix 7).
- Add two volumes of RNaseLag into a tube (order by Cat No RN983016 or RN983017).
- Add one volume of bacterial culture to the tube. Mix by vortexing for 5 sec. Incubate at room temperature (15–25°C) for 5 min.
- Centrifuge for 10 min at 4000 rpm at universal centrifuge.

  Note: Sometimes the pellet is too clear to be recognized, it is due to RNaseLag treatment, and will not affect the ongoing process.

  - Decant the supernatant.

  Pellets can be stored at –20 to –30 °C for up to 2 weeks or at –70 °C for up to one month. For RNA isolation, thaw pellets at room temperature (15–25 °C) and proceed the appropriate RNA isolation protocol.

Genomic DNA Contamination

RNall is designed to selectively isolate the RNA. However, if further DNA removal is intended, it is recommended to use DNase Treatment kits, which are available from different suppliers.

Before start

Not forget to add the appropriate amount of ethanol (molecular biology grade, 96–100%) to TWB1 and TWB2 as indicated on the bottle, before using for the first time. Refer to washing buffer preparation section.

Maximize RNA Yield

To obtain higher yield of RNA, it is important to follow protocols carefully and pay attention to sample size table and its suitable lysis buffer amount recommended for your samples. Notice that all samples must be completely homogenized during cell lysis for maximum yields. It is good to know that:

- Yield and quality of the purified RNA depends on sample storage conditions. For best results, it is recommended to use fresh samples, however for long storage, it is better to treat sample in RNaseLag (for more information refer to RNaseLag).
- Avoid freezing and thawing samples, which may result in decreasing RNA yield, as compared to isolating RNA from fresh samples.

Protocols

Protocols Phenol-Chloroform Based Protocols

Protocol 1: Isolation of Total RNA (Animal Tissues, fresh and frozen)

Sample Type: Animal tissues (fresh and frozen)

Some tips to know

- Do not allow non-stabilized tissues to thaw while weighing or handling prior to disruption.
- Set Thermoblock or water bath at 60℃ before starting the process.
- If working with RNA for the first time, read Appendix 1 carefully.

Process

- Remove the tissue from RNaseLag or use fresh tissue. Determine the weight of starting material and add appropriate RNSol H Reagent to the tissue sample (refer to the Table 2).
- Disrupt and homogenize the tissue sample by selecting one of these ways:
    - After adding appropriate RNSol H Reagent amount, using TissueLyser or homogenizer to disrupt and homogenize the sample simultaneously.
    - Grind the weighed tissue in liquid nitrogen carefully with a cold mortar and pestle. Transfer tissue powder into an RNase-free, liquid-nitrogen–cooled, 1.5 ml

microcentrifuge tube. Let the liquid nitrogen to evaporate completely (but do not allow the tissue to thaw). Add the appropriate volume of RNSol H Reagent (refer to the Table 2).

Optional: If samples have a high fat content, centrifuge the lysate for 5 min at 12000× g at 4–10°C, then transfer the clear supernatant to a new tube.

- Incubate for 5 min at room temperature.
- Add appropriate amount of chloroform (refer to Table 2), then vigorously pulse vortex for 15 s and incubate at room temperature for 3 min.
- Centrifuge at 4℃ for 15 min at 13000 rpm.
- Transfer the aqueous phase (the upper phase) to a new tube. Be careful to avoid interfering the interphase.
- Add appropriate amount of isopropanol (refer to Table 2). Invert several times.
- Incubate at room temperature for 10 min.
- Centrifuge at 4℃ for 10 min at 13000 rpm.
- Discard the supernatant and add 750 µl 75% ethanol (nuclease-free) to the pellet. Vortex the sample briefly then centrifuge at 4℃ for 5 min at 12000 rpm.
- Remove the supernatant completely, and briefly air-dry the RNA pellet (for 10 min).
- Add 30-100 µl nuclease-free water. Pipetage several times to dislodge the RNA pellet then incubate 10 min at 60℃. Afterward, pipetage until the pellet dissolved completely.

Protocol 2: Isolation of Total RNA (PBMC, WBC and Whole blood)

Sample Type: PBMC (Peripheral Blood Mononuclear Cell), Whole blood, WBC (white Blood Cell)

Some tips to know

- Set Thermoblock or water bath at 60℃ before starting the process.
- If working with RNA for the first time, read Appendix 1 carefully.

Process

- Collect 0.5 to 10ml blood into EDTA tubes. Add three volumes of Nuclease-free RBC Lysis Buffer. Invert the tube 5 times and incubate at 4 ºc for 10 min.
- Pulse vortex every 2 min during incubation to intersperse the sample.
- Collect the WBCs by centrifugation at 2700 x g for 10min at 4ºc.

- Discard the supernatant, add two volumes of Nuclease-free RBC Lysis to the pellet, vortex until the pellet is dissolved completely.
- Collect the WBCs by centrifugation at 2700 x g for 10 min at 4ºc.
- Discard the supernatant. Add appropriate amount of RNSol H Reagent to the sample (refer to the Table 2).
- Disrupt the cell pellet by vortex for 1 min then incubate at room temperature for 5 min.
- Add appropriate amount of chloroform (refer to Table 2), then vigorously pulse vortex for 15 s and incubate at room temperature for 3 min.
- Centrifuge at 4℃ for 15 min at 13000 rpm.
- Transfer the aqueous phase (the upper phase) to a new tube. Be careful to avoid interfering the interphase.
- Add appropriate amount of isopropanol (refer to Table 2). Invert several times.
- Incubate at room temperature for 10 min.
- Centrifuge at 4℃ for 10 min at 13000 rpm.
- Discard the supernatant and add 750 µl 75% ethanol (nuclease-free) to the pellet. Vortex the sample briefly then Centrifuge at 4℃ for 5 min at 12000 rpm.
- Remove the supernatant completely, and briefly air-dry the RNA pellet (for 10 min).
- Add 30-100 µl nuclease-free water. Pipetage several times to dislodge the RNA pellet then incubate 10 min at 60℃. Afterward, pipetage until the pellet dissolved completely.

Protocol 3: Isolation of Total RNA (cultured cell)

Sample Type: Cells grown in monolayer; Cells grown in suspension

Some tips to know:

- Do not allow non-stabilized samples to thaw while weighing or handling prior to disruption.
- Set Thermoblock or water bath at 60℃ before starting the process.
- If working with RNA for the first time, read appendix 1 carefully.

Process

- Remove the cultured cells from RNaseLag or use fresh cultured cells. Determine the number of cells. Pellet the appropriate number of cells by centrifuging for 5 min at 300 x g. Carefully remove all supernatant by aspiration (for information about sample preparation refer to table 6).

Table 7: Lysis of attached monolayer and suspension cells

Cultured Cell Type	Lysis Type	Description
Cells grown in a monolayer	Lyse directly in the cell-culture vessel	Determine the number of cells. Completely aspirate the cell-culture medium.
	Trypsinize and collect cells at 300 x g	Determine the number of cells. Aspirate the medium and wash the cells with PBS. Aspirate the PBS and add %0.1–0.25 trypsin in PBS. After the cells detach from the surface, add medium (containing serum to inactivate the trypsin), transfer the cells to an RNase-free centrifuge tube, and centrifuge at 300 x g for 5 min. Completely aspirate the supernatant, and continue the process.
Cells grown in suspension	Collect cells at 300 x g	Determine the number of cells. Pellet the appropriate number of cells by centrifuging for 5 min at 300 x g. Carefully remove all supernatant by aspiration.

Note: Incomplete removal of cell-culture medium will inhibit complete lysis and binding to HiPure DR Column, which finally reduce RNA yield.

- Disrupt the cells by selecting one of these ways:
  - Adding appropriate volume of RNSol H Reagent (refer to Table 2), Vortex to mix.

    Note: Before adding RNSol H Reagent, flick the tube to loosen the cell pellet thoroughly. Make sure that loosening the cell occurs completely to avoid inefficient lysis and reduced RNA yields.

  - For direct lysis of cells grown in a monolayer, add the appropriate amount of RNSol H Reagent to the cell-culture dish (refer to Table2). Collect the lysate with a rubber policeman. Transfer the lysate into a microcentrifuge tube. Vortex to mix, and ensure that no cell clumps are visible.
- Homogenize the lysate by:
  - Homogenize the lysate for 30 s using a homogenizer.
  - Pass the lysate at least 5 times through a blunt 20-gauge needle fitted to an RNase-free syringe.
  - Pass the lysate through a shredder HiPure DR Column by centrifuging at full speed for 2 min.

    Optional: Homogenizing step is optional. It means that using homogenizer or syringe needle to homogenize the lysate can be omitted from the process.
- Add appropriate amount of chloroform (refer to Table 2), vigorously shake it for 30 s. Then pulse vortex for 15 s and incubate at -20˚C for 2 min.

- Centrifuge at 4°C for 12 min at 13000 rpm.
- Transfer the aqueous phase (the upper phase) to a new tube. Be careful to avoid interfering the interphase.
- Add one and half volume of absolute ethanol to the separated aqueous phase. Pulse vortex for 30 s.
- Transfer the solution to a HiPure DR Column placed in a 2ml collection tube (supplied in the kit box). Centrifuge for 1 min at 13000 rpm at room temperature. Discard the flow-through.
- Add 700µl TWB1 to the HiPure DR Column. Centrifuge for 1 min at 13000 rpm at room temperature. Discard the flow-through.
- Add 500µl TWB2 to the HiPure DR Column. Centrifuge for 1 min at 13000 rpm at room temperature. Discard the flow-through.
- Add 500µl TWB2 to the HiPure DR Column. Centrifuge for 3 min at 14000 rpm at room temperature. Discard the collection tube with the flow-through.
- Place the HiPure DR Column in a new 1.5ml microtube. Add 30-100µl RNase-free water directly to the HiPure DR Column membrane. Centrifuge for 1 min at 12000 rpm to elute the RNA.

> **Note:** If the expected RNA yield is more than the yield from pervious step, put the HiPure DR Column on a new microtube and add another 30-100µl RNase-free water. Centrifuge for 1 min at 12000 rpm. The yield will be nearly same as previous step. However, it is possible to pass the flow-through from step 12 once more to obtain RNA with higher concentration.

Troubleshooting

Here we try to cover as many problems as you may see in using this product, however scientists in ROJE Technical Support Team are eager to answer all your questions. Do not hesitate to contact us for more information.

Symptoms	Problem	Suggestion
Low yield	Inappropriate sample storage condition	• Avoid freezing and thawing of samples, which results in reduced RNA Yield. • For better results, it is recommended to store samples in RNaseLag.

	Incomplete cell lysis	• Too much starting material results in low RNA yield. To optimize the results, refer to Table 2.
	Ethanol from the washing buffer is present in elution	Preform another centrifugation before rehydration step to ensure no remaining trace of ethanol on column. Carefully remove the column from the collection tube so that the column does not contact the flow-through.
	RNA elution is incomplete	• Perform rehydration step once more, by adding another 30-100µl rehydration buffer to the column and before centrifugation, incubate 5 min at room temperature. • Check that all previous steps are done appropriately.
Degradation	Too thick sample for stabilization	• Cut large samples into slices less than 5mm thick for stabilization in RNaseLag.
	Improper sample storage	• It is suggested to store samples in RNaseLag, refer to sample preparation section.
	Frozen sample used for stabilization	• For stabilization in RNaseLag, use fresh samples.
	Storage duration in RNaseLag exceeded	• Refer to Table 4.
	RNase contamination	• All buffers have been tested and are guaranteed RNase-free. RNases can be introduced during use. Refer to appendix 1 for more information.
Low 260/280 ratio	Insufficient disruption and homogenization	• As a guide for better disruption and homogenization, based on sample type refer to sample preparation guidelines.
	RNA was diluted in low pH water	• Use 10 mM Tris-HCl with pH ≥ 7.5, or nuclease free water with pH ≥ 7.5.
	DNA contamination	• Follow precisely the respective protocol, If RNA purification is still problematic further do DNase treatment. • RNSol pH might alter during storage. Check the RNSol H Reagent pH it should be around 4.2.
	Protein contamination	• This is often due to exceeding the amount of starting material. Follow precisely the respective

		protocol; if RNA purification is still problematic further reduce the amount of starting material. • Remove the aqueous phase precisely.
DNA contamination in downstream application	No DNase treatment	• Perform DNase treatment.
	No incubation with TWB1	• Incubate the spin column for 5 min at room temperature after addition of TWB1 and before centrifuging
	Check RNSol pH	• RNSol H Reagent pH might alter during storage. Check the RNSol H Reagent pH it should be around 4.2.
Not performing well in downstream application	Ethanol carryover	• Preform another centrifugation before rehydration step to ensure no remaining of ethanol on column.
	Salt carryover	• Between washing steps, eliminate remaining flow-through from the rim of collection tube, by blotting on clean paper towels.
Clogged Column	Maximum amount of tissue exceeds kit specifications	• Refer to specifications to determine if the amount of starting material falls within kit specifications.
	The sample is too large	• Use fewer starting material. The problem can be solved by increasing the g-force and/or centrifuging for a longer period of time until the lysate passes through the column.

Ordering Information

Category	Product Name	Cat No.	Size
	MiRJia Kit	RN003079	100 preps
	RNJia Kit	RN983006	100 preps
	RNJia Fibrous Kit	RN983025	50 preps
	RNJia Bacteria Kit	RN983022	50 preps
	RNJia Phenol-Free PB Kit	RN983057	100 preps
	MiRJia Lipid Kit	RN003078	100 preps
	RNSol H Reagent	RN983061	100ml

	RNaseLag	RN983016	50ml
	RNZO	RN983018	25ml
	RNA Loading Set	LD983007	100 Preps

Technical Assistance

- ROJETechnologies guarantee your complete satisfaction. ROJE technical support team is composed of highly trained, experienced scientists; who can troubleshoot most problems you face. Our technical support team can offer expert advice which may help you select a suitable product.
- Contact our technical support at any time by selecting one of these ways:
- Through our telephone and fax number; +982191070705.
- You can submit your question directly to ROJE Technical Support Team from our website (www.ROJETechnologies. com).
- Or send your questions to this email address, technicalsupport@rojetechnologies.com.

Appendix 1: Handling RNA

RNA is highly sensitive to degradation due to the ubiquitous presence of RNases in the environment. unlike DNases, which require metal ions for activity, RNases do not require cofactors for their enzymatic activity and can maintain activity even after prolonged boiling or autoclaving. Since RNases are active enzymes, and are too hard to be deactivated, do not use any plasticware or glassware without first removing possible RNase pollution. Special precautions should be taken when working with RNA. All reagents and equipment must be specially treated to inactivate RNases prior to use.

The following are some tips and techniques to remember when working with RNA:

General Tips

- Always wear latex or vinyl gloves while handling reagents and RNA samples to prevent RNase contamination from the surface of the skin or from dusty laboratory mobilizations. These materials are generally RNase-free and do not require pretreatment to inactivate RNase.
- Treat surfaces of benches with commercially available RNase inactivating agents such as RNZO (Order by cat No RN983018 and RN983019).
- Treat non-disposable glassware and plasticware before use, to ensure that it is RNase-free. Thoroughly rinse plasticware with 0.1N NaOH/1mM EDTA, and RNase-free water.
- Use disposable RNase-free plasticware to decrease the possibility of sample contamination.

- Keep purified RNA on ice when aliquots are pipetted for downstream applications.
- Glassware should be immersed in freshly prepared %0.1 (v/v) DEPC in water or ethanol for several hours, followed by draining and autoclaving.

Appendix 2: RNA Storage Condition

RNA samples are commonly stored at −20 °C or −80 °C, eluted in RNase-free water. Under these conditions, RNA is not degraded after 1 year.

RNA Quality

The ratio of absorbance at 260nm and 280nm is used to assess the purity of RNA using UV absorption, by a spectrophotometer or Nanodrop. For pure RNA, A260/A280 ratios should be around 2.1. If the ratio is noticeably lower in either case, it may indicate the presence of protein or other contaminants that absorb strongly at or near 280nm. High A260/A280 purity ratios are not indicative of an issue.

Appendix 3: RNA Integrity

The integrity of RNA molecules is important for experiments that try to reflect the snapshot of gene expression of RNA isolation. The least expensive method for checking RNA integrity is to run the RNA on a %1 DGE agarose gel and examine the ribosomal RNA (rRNA) bands. The upper ribosomal band (28S in eukaryotic cells and 23S in bacterial cells) should be about twice the intensity of the lower band (18S in eukaryotic cells and 16S in bacterial cells) and should be sharp and tight.

Denaturing agarose gel electrophoresis

Formaldehyde is used to keep the RNA from denaturation, and when RNA is heated in the presence of formaldehyde all secondary structures are eliminated. Therefore, gel electrophoresis of RNA in agarose gels containing formaldehyde provides a good denaturing gel system. Formaldehyde is a potential carcinogen. Take appropriate safety measures and wear gloves when handling.

Gel preparation

- Prepare %1.2-2 gel with TAE 1x buffer (%5 formaldehyde).
- Pour the gel using a comb that will form wells large enough to accommodate at least 20µl.
- Assemble the gel in the tank.
- Use TAE 1x buffer (%5 formaldehyde) as the tank buffer.

RNA sample preparation

- Add 10µg of purified RNA to 6µl of ROJE sample buffer (in RNA Loading Set, order by cat No LD983007) and incubate at 70 °C for 3 min.
- Add 3µl Hinari to the mixture of RNA and sample buffer and mix well by pipetting.
- Then run the mixture in the prepared gel.

Electrophoresis

Load the gel and electrophorese at 5-6 V/cm until the loading dye at least has migrated 2/3 the length of gel.

Results

Visualize the gel on a UV transilluminator. The 28S and 18S ribosomal bands should be sharp and intense. The intensity of the 28S band should be twice more than the 18S band. The following Figure shows different RNA samples, isolated by RNJia kit and run by DGE.

Note: If DNA contamination is present, the high molecular weight smear will be appeared above the 28S band.

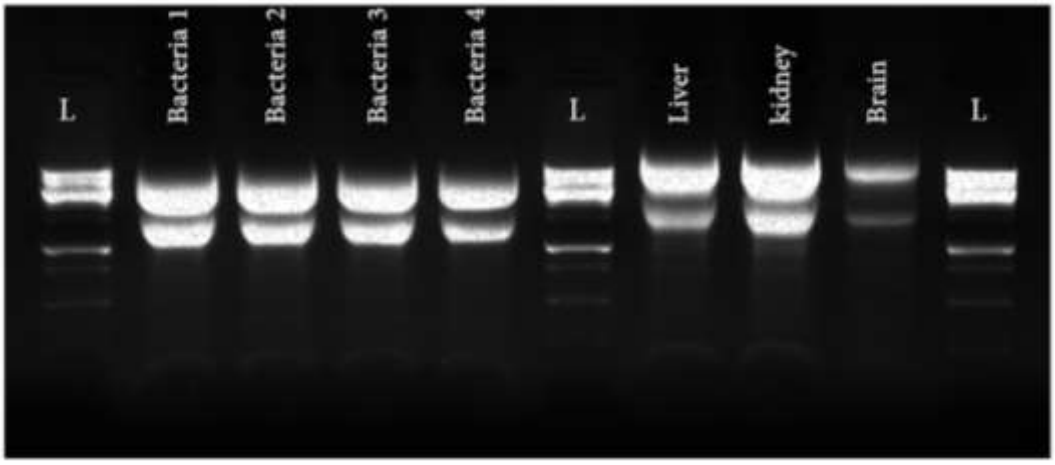

Figure 1. Isolated RNA using RNJia Kit, RNA was isolated from the indicated animal tissues and bacteria cells using the RNJia Kit. For each sample, 10 µg RNA was pretreated by ROJE Sample Buffer and analyzed on Denaturing Gel Electrophoresis (DGE).

Appendix 4: DNA Contamination

Different methods exist for monitoring genomic DNA contamination, for example, using OD measurement or via agarose gel electrophoresis. Nevertheless, genomic DNA traces which are not detectable by the above methods, can produce nonspecific results in sensitive downstream applications. The co-amplification of cDNA and contaminating genomic DNA produce one or more additional PCR fragments that can be traced via agarose gel electrophoresis.

strategies to solve the problem:

- Design PCR primers for span exon boundaries, which includes one or more intronic sequence motifs in the genomic DNA, results in amplification of cDNA template.
- Design a minus control reaction with reverse transcriptase. In that specific reaction, instead of the reverse transcriptase enzyme, water is added to the reaction mixture. PCR product in this control reaction indicate genomic DNA contamination.

Appendix 5: Protein Isolation

MiRJia Kit is specified for RNA isolation. However, protein can be extracted simultaneously. The denatured protein can be used for SDS-PAGE, western blotting, 2D gel electrophoresis and etc.

Equipment to be supplied by user

- Centrifuge
- Acetone
- Ethanol, molecular biology grade

Process

- Prepare cell lysate (appropriate to sample type) and centrifuge it through a HiPure DR Column, as described in all protocols.
- Do not discard the flow-through. Transfer it to a new clean microtube and Add 4 volumes of ice-cold acetone to it.
- Incubate at -20˚C for 30 min.
- Centrifuge for 10 min at 14000 rpm in a benchtop centrifuge. Discard the supernatant.
- Wash the pellet with 100µl ice-cold ethanol and air-dry.

  Note: For easier resuspension, do not over dry the pellet.
- Resuspend the pellet in the appropriate buffer according to downstream reaction.

Appendix 6: Convert RPM to RCF (centrifuge)

All centrifugation steps are performed at room temperature. The correct rpm can be calculated using the formula:

$$RPM = \sqrt{\frac{RCF}{(1.118 \times 10^{-5})(r)}}$$

Where **RCF** = required gravitational acceleration (relative centrifugal force in units of g); **r** = radius of the rotor in cm; and **RPM** = the number of revolutions per minute required to achieve the necessary g-force.

Appendix 7: Cell Count by a Hemocytometer

Sample preparation

First, resuspend the cell in flask, and then quickly remove your aliquot of cells. For a cell culture growing in a Petri dish, you need to resuspend the cells growing on the bottom of the dish by gently using a pipette to remove cells and media from a dish and then gently expelling them back into the dish. This aspiration of the contents of the Petri dish should be completed several times, each time expelling the cells and media while moving the pipette across the bottom of the dish to gently discharge cells growing on the entire surface. After resuspension, the aliquot is quickly removed from the vessel before the cells settle to the bottom again. For cell culture applications cells are often stained with Trypan Blue so that dead cells can be distinguished from live cells. For example, a 0.5ml suspension of cells would be removed from the Petri dish and mixed with 0.5ml Trypan Blue solution in an Eppendorf or small test tube. Trypan Blue is a stain that selectively stains dead cells.

Loading Sample

Load your sample quickly and smoothly. It is important to handle the hemocytometer and the cover slip carefully. Never place your fingers on the reflective surface of the slide. Always clean the slide before you load the sample by rinsing the slide and cover slip with %70-95 ethanol. Air-dry or gently wipe the slide and cover slip with lens paper. Place the clean and dry slide on your work surface and place the coverslip on top to cover the reflective surfaces. If you have diluted your cells in a test tube, invert the tube several times to resuspend the cells. Using a micropipette, quickly and smoothly without interruption, add 10µl of your cell suspension (or 1 drop from a transfer pipette) to the V-shaped groove on each side of the hemocytometer. If your sample moves into the gutters, you may not have loaded the sample in the correct location or you may have used too large of an aliquot.

Estimating cell density

count all of cells within each of the four large quadrants in the four corners of each counting chamber on the hemocytometer (see Figure 2). Count all of the cells within each quadrant except those on the far-right edge and lower bottom edge.

Calculate the cell density by this formula:

Average number of cells × dilution factor× 10^4

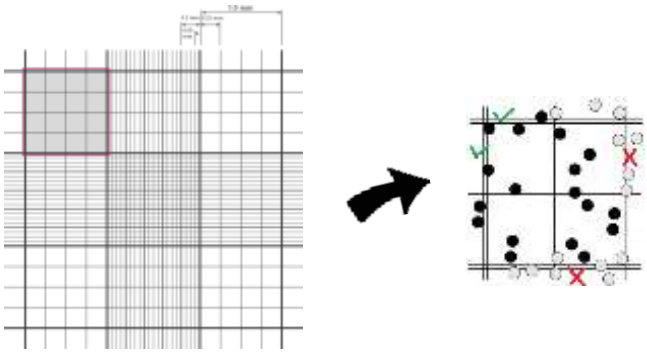

Figure2. Cell counting with hemocytometer

Appendix 8: Preparation of Phosphate Buffered Saline (PBS)

Dissolve the components in about 800mL dH2O. Adjust the pH to 7.4 with HCl. Adjust the volume to one liter. Sterilize by autoclaving. Store at room temperature.

Table 7. PBS preparation

Concentration	Component	G/litter
137mM	NaCl	8g
2.7mM	KCl	0.2g
10mM	Na2HPO4	1.42g
1.8mM	KH2PO4	0.25g

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