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RNJia Kit

- RNA isolation based on silica technology
- MiniPrep

For RNA Isolation from

Animal Tissue (Fibrous and non-fibrous tissue)

Animal Cells

Bacteria Cells

PBMC

WBC

Whole blood

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.

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Kit Content

Component	100 preps
RLB	2 x 50ml
TWB1 (concentrate)	2 x 16ml
TWB2 (concentrate)	2 x 15ml
Nuclease-free Water	10ml
HiPure DR Column	100
Collection Tube	100

Storage

Shipment condition is checked by ROJETechnologies. After arrival, all reagent should be kept dry at room temperature (15-25 °C). When storage condition is as directed, all reagents are stable until expiration date, as indicated on the kit box.

Intended Use

RNJia Kit provides the components and procedures necessary for purifying total RNA from animal tissue, cultured cells, PBMC, Whole blood, WBC and bacteria. Notice that, RNJia Kit 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

RNJia 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.

Description

RNJia Provides a time-saving, reliable and meticulous method for RNA isolation from various sample types including animal tissues, cultured cells, PBMC, WBC, whole blood and bacteria. This kit can be used for RNA isolation from bacteria, lipid and fibrous tissue, however we suggested to use RNJia Bacteria Kit, RNJia Lipid Kit and RNJia Fibrous kits, which are specialized for RNA isolation from bacteria, lipid and fibrous tissues to reach optimum results, however RNJia is capable of isolating RNA from these sample types if suitable enzymes are purchased. RNJia kit is based on spin column technology for isolation of concentrated, highly purified and intact RNA, without use of phenol and chloroform, which is suitable to use for variety of downstream processes such as Northern blot experiments, Real-time PCR, RNA sequencing, Microarray and etc.

Procedure

The RNJia is designed for isolating all RNA molecules larger than 200 nucleotides from 0.5-30 mg tissue samples or up to 1×10^7 animal cells. Fresh or frozen tissue samples should be lysed and homogenized. Then, RNA binding to the silica membrane is achieved selectively, by the addition of ethanol to the lysate. Contaminants removed by two specific kinds of washing buffers. Pure RNA is finally eluted in nuclease-free water. Isolated RNA is ready to use in downstream amplification. It has A260/A280 ratios of 1.9–2.3 by spectrophotometer, confirming high purity.

Equipment & Reagents to Be Supplied by User

- Absolute ethanol
- 1.5ml RNase free microcentrifuge tubes
- RNase free pipets and pipet tips
- Benchtop microcentrifuge

- Vortex
- Thermoblock or water bath
- TissueLyser/ Mortar and pestle (for animal tissue sample)
- Syringe and needles
- Homogenizer

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 MiRJia Kit are listed here in Table 1.

Table 1: MiRJia Kit features and specifications

Features	Specifications
Elution volume (µl)	30-100µl
Technology	Silica technology
Main sample type	Animal tissue/ cultured cell/ WBCs/ PBMC/ whole blood /bacteria
Processing	Manual
Sample Amount	Varies
Operation time per reaction (min)	Less than 30min
Typical yield (µg)	60µg
Average purity(A260/A280)	A260/A280= 1.9-2.3
Size of purified RNA	≥200 nucleotides

Enzyme	Only for fibrous tissue and bacteria should be prepared

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 RLB are written in Table 2.

Table 2. Appropriate sample size and amount of RLB

Sample Type	Sample Size	Amount of RLB
Animal cells	≤5 X106	350µl
Animal cells	5 X 10^6 To 1 X 10^7	600µl
Animal not fibrous tissues	0.5-10 Mg	350µl
Animal not fibrous tissues	10-30Mg	600µl
Animal fibrous tissues	0.5-30Mg	600µl
Bacteria cells	≤5 X10^8	500µl
Bacteria cells	5 X 10^8 - 1 X 10^9	800µl
Bacteria cells	1 X 10^9 – 2 X 10^9	1000µl
Whole blood	Up to 2 x 106 cells	350µl
Whole blood	2 x 10^6 to 1 x 10^7 cells	600µl

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 RLB, 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 RLB (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	• Homogenizer • Syringe and needles • Vortexing
Animal Cells, WBC, PBMC	• RLB • Vortexing	• Homogenizer • Syringe and needle
Bacteria Cells	• RLB • Vortexing	• Homogenizer • Syringe and needles

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 RNJia. 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 11 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 (5 ml). 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 (5 ml) 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 12 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 15 ml centrifuge tube containing 10 ml 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	40-45mm	3×10^6
	250-300mm	1×10^7
	650-750mm	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

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 RNJia. 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 5sec. Incubate at room temperature (15–25°C) for 5min.
- 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

RNJia 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.

Washing Buffer Preparation

Before the first use, add appropriate amount of ethanol (%96-100) to each washing buffer tube, then mix thoroughly to prepare washing buffer, refer to Table 6. Do not forget to tick the check box on the bottle label to indicate that ethanol has been added. Before each use mix reconstituted buffer by shaking. Storing at room temperature.

Table 6: Washing buffer preparation

Buffer Name	Concentrated Volume	Amount of Ethanol	Final Volume
TWB1	16m	24m	40m
TWB2	15m	45m	60m

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

- All centrifugation steps are carried out at room temperature (15–25°C) in a microcentrifuge.
- All steps, before applying sample into HiPure DR Column, are carried out on ice.
- Before using the protocol for the first time, add 2-Mercaptoethanol to RLB, %2 of RLB volume. 2-Mercaptoethanol is commercially available and due to safety issue, is not provided in the kit. It can be order separately, by ROJETechnologies (Cat No BU983034) or Sigma-Aldrich (Cat No M3148).
  Attention! It is recommended to prepare it freshly. It is not stable more than 2 weeks.
- If RLB forms precipitate, please warm it to 56°C until the precipitate has fully dissolved. This is due to storage condition and won't influence the efficiency of the buffer.
- Do 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.
- For frozen samples in RNaseLag, thaw them to room temperature. Avoid repeated freezing and thawing of samples, since it may cause RNA degradation.
- If working with RNA for the first time, read Appendix 1 carefully.

Process

- Remove the tissue from RNaseLag or use fresh tissue or snap-freeze tissue. Weight the intended tissue. Add appropriate RLB to the tissue sample (refer to the table 7).

Table 7: The appropriate amount of RLB

Sample type	Storage Condition	Weight	RLB amount
Soft tissue (brain, liver and etc.)	Fresh or snap freeze	0.5-10 mg	350µl

Soft tissue (brain, liver and etc.)	Treated with RNaseLag	0.5-10 mg	600µl
Soft tissue (brain, liver and etc.)	Fresh or snap freeze and treated with RNaseLag	10-30 mg	600µl
Other tissue types	Fresh, snap freeze or treated with RNaseLag	0.5-30 mg	600µl

- Disrupt and homogenize the tissue sample by selecting one of these ways:
  - After adding appropriate amount of RLB, use Micropestle followed by homogenizer or syringe needle to homogenize the tissue.
  - After adding appropriate amount of RLB, use 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 RLB (refer to the Table 7) and homogenize by passing the lysate 5-10 times through a blunt 20-gauge needle fitted to an RNase-free syringe.

    Note: Make sure that the disruption and homogenization is complete. For soft tissues like brain, the tissue will be disappeared completely, however for hard tissue like spleen or kidney; a little non-homogenized tissue might remain at the end of lysis step, which will not influence the other steps.

- Centrifuge the lysate for 3 min at 15000 rpm. Carefully remove the supernatant by pipetting, and transfer it to a new RNase-free microcentrifuge tube.

  Note: For some tissues the pellet is recognizable, however for others like brain, the pellet is very little or very clear, and is hard to be recognized. So be careful not to disrupt the pellet by pipette tip.

- Add one volume of %70 ethanol (molecular biology grade, nuclease-free) to the cleared lysate, and mix immediately by pulse vortexing for 15 s. Do not centrifuge.

  Note: If total RNA is required, add absolute ethanol instead of %70 ethanol to the cleared lysate.

  Note: Sometimes, precipitates may appear after ethanol addition. This does not disturb the procedure.

- Transfer up to 700 µl of the lysate, including any precipitate that may have formed, to a HiPure DR Column placed in a 2ml collection tube (supplied in the kit box). Centrifuge for 1 min at 13000 rpm. Discard the flow-through.

  Note: If the lysate does not pass the column, repeat the centrifugation once more at full speed for 1 min.

- Repeat the previous step by the remaining sample from step 4

  Note: If the lysis does not pass the column, repeat the centrifugation once more at full speed for 1 min.

- Add 700 µl TWB1 to the HiPure DR Column. Centrifuge for 1 min at 13000 rpm. Discard the flow-through.
- Add 500 µl TWB2 to the HiPure DR Column. Centrifuge for 1 min at 13000 rpm. Discard the flow-through.
- Add 500 µl TWB2 to the HiPure DR Column. Centrifuge for 3 min at 14000 rpm. Discard the collection tube with the flow-through.
- Place the HiPure DR Column in a new 1.5 ml microtube. Add 30-50 µl 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 11 once more to obtain RNA with higher concentration.

Protocol 2: Isolation of Total RNA (Animal tissues, fibrous tissue)

Sample Type: Animal tissues (fibrous tissue)

Some Tips to Know:

- It is possible to Isolate RNA from Fibrous tissue like lung, heart, and skin, however RJ-Protease (Cat No EB983121) and nuclease-free water (Cat No WA983014, WA983008, WA983009 and WA983010) should be ordered separately. However, RNJia Fibrous kit (Cat No RN983024, RN983025 and RN983026) is designed specifically to isolate RNA from fibrous tissue.

  Note: For isolation of RNA from skeletal muscle use RNSol H Reagent instead of RLB.

- Set Thermoblock or water bath at 55℃ before starting the process.
- All centrifugation steps are carried out at room temperature (15–25 °C) in a microcentrifuge.
- All steps, before applying sample into HiPure DR Column, are carried out on ice.
- Before using the protocol for the first time, add 2-Mercaptoethanol to RLB, %2 of RLB volume. 2-Mercaptoethanol is commercially available and due to safety issue, is not provided in the kit. It can be order separately, by ROJETechnologies (Cat No BU983034) or Sigma-Aldrich (Cat No M3148).
- Attention! It is recommended to prepare it freshly. It is not stable more than 2 weeks.
- If RLB forms precipitate, please warm it to 56°C until the precipitate has fully dissolved. This is due to storage condition and won't influence the efficiency of the buffer.
- Do 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.
- For frozen samples in RNaseLag, thaw them to room temperature. Avoid repeated freezing and thawing of samples, since it may cause RNA degradation.
- If working with RNA for the first time, read Appendix 1 carefully.
- Consider that the provided nuclease-free water in kit is intended to use as rehydration solution. It is recommended to buy nuclease-free water for consuming in lyses step.

Process

- Remove the tissue from RNaseLag or use fresh tissue or snap-freeze tissue. Weight the intended tissue, up to 30 mg. Add 300 µl RLB to the tissue sample.
- Disrupt and homogenize the tissue sample by selecting one of these ways:
    - After adding appropriate RLB amount, use Micropestle followed by homogenizer or syringe needle to homogenize the tissue.
    - After adding appropriate RLB 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 RLB (see Table 2) and homogenize by passing the lysate 5-10 times through a blunt 20-gauge needle fitted to an RNase-free syringe.

Note: Make sure that the disruption and homogenization is complete.

- Add 600 µl nuclease-free water and 15 µl RJ-Protease. Pulse vortex for 15 s and incubate the microtube at 55℃ for 15 min.
- Centrifuge the lysate for 3 min at 15000 rpm. Carefully remove the supernatant by pipetting, and transfer it to a new RNase-free microcentrifuge tube.

Note: Be careful not to disrupt the pellet by pipette tip.

- Add half volume of absolute ethanol to the cleared lysate, and mix immediately by pulse vortexing for 15 s. Do not centrifuge.

Note: Sometimes, precipitates may appear after ethanol addition. This does not disturb the procedure.

- Transfer up to 700 µl of the sample, including any precipitate that may have formed, to a HiPure DR Column placed in a 2 ml collection tube (supplied in the kit box). Centrifuge for 1 min at 13000 rpm. Discard the flow-through.

Note: If the lysate does not pass the column, repeat the centrifugation once more at full speed for 1 min.

- Repeat the previous step by the remaining sample from step 5.

Note: If the lysis does not pass the column, repeat the centrifugation once more at full speed for 1 min.

- Add 700 µl TWB1 to the HiPure DR Column. Centrifuge for 1 min at 13000 rpm. Discard the flow-through.
- Add 500 µl TWB2 to the HiPure DR Column. Centrifuge for 1 min at 13000 rpm. Discard the flow-through.
- Add 500 µl TWB2 to the HiPure DR Column. Centrifuge for 3 min at 14000 rpm. Discard the collection tube with the flow-through.
- Place the HiPure DR Column in a new 1.5 ml microtube. Add 30-50 µl 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 30-50 µl 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 11 once more to obtain RNA with higher concentration.

Protocol 3: Isolation of Total RNA (Animal cultured cell)

Sample Type: Animal cultured cell

Some tips to know:

- All centrifugation steps are carried out at room temperature (15–25 °C) in a microcentrifuge.
- All steps, before applying sample into HiPure DR Column, are carried out on ice.
- Before using the protocol for the first time, add 2-Mercaptoethanol to RLB, %2 of RLB volume. 2-Mercaptoethanol is commercially available and due to safety issue, is not provided in the kit. It can be order separately, by ROJETechnologies (Cat No BU983034) or Sigma-Aldrich (Cat No M3148).

  Attention! It is recommended to prepare it freshly. It is not stable more than 2 weeks.

- If RLB forms precipitate, please warm it to 56°C until the precipitate has fully dissolved. This is due to storage condition and won't influence the efficiency of the buffer.
- Do 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
- For frozen samples in RNaseLag, thaw them to room temperature. Avoid repeated freezing and thawing of samples, since it may cause RNA degradation.
- 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 8).

Table 8: 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 RLB (refer to Table 2), Vortex to mix.

    Note: Before adding RLB, 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 RLB to the cell-culture dish (refer to Table2). Collect the lysate with a rubber policeman. 25 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**.**
- Centrifuge the lysate for 3 min at 15000 rpm. Carefully remove the supernatant by pipetting, and transfer it to a new RNase-free microcentrifuge tube.

Note: The pellet is very little or very clear, and is hard to be recognized. So be careful not to disrupt the pellet by pipette tip.

- Add one volume of %70 ethanol (molecular biology grade, nuclease-free) to the cleared lysate, and mix immediately by pulse vortexing for 15 s. Do not centrifuge.
- Note: If total RNA is required, add absolute ethanol instead of %70 ethanol (molecular biology grade, nuclease-free) to the cleared lysate.

Note: Sometimes, precipitates may appear after ethanol addition. This does not disturb the procedure.

- Transfer up to 700 µl of the lysate, including any precipitate that may have formed, to a HiPure DR Column placed in a 2 ml collection tube (supplied in the kit box). Centrifuge for 1 min at 13000 rpm. Discard the flow-through.
- Note: If the lysate does not pass the column, repeat the centrifugation once more at full speed for 1 min.
- Repeat the previous step by the remaining sample from step 5.

Note: If the lysis does not pass the column, repeat the centrifugation once more at full speed for 1 min.

- Add 700 µl TWB1 to the HiPure DR Column. Centrifuge for 1 min at 13000 rpm. Discard the flow-through.
- Add 500 µl TWB2 to the HiPure DR Column. Centrifuge for 1 min at 13000 rpm. Discard the flow-through.
- Add 500 µl TWB2 to the HiPure DR Column. Centrifuge for 3 min at 14000 rpm. Discard the collection tube with the flow-through.
- Place the HiPure DR Column in a new 1.5 ml microtube. Add 30-50 µl 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 30-50 µl 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 11 once more to obtain RNA with higher concentration.

Protocol 4: Isolation of RNA (PBMC, WBC and Whole blood)

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

Some tips to know:

- All steps, before applying sample into HiPure DR Column, are carried out on ice.
- Before using the protocol for the first time, add 2-Mercaptoethanol to RLB, %2 of RLB volume. 2-Mercaptoethanol is commercially available and due to safety issue, is not provided in the kit. It can be order separately, by ROJETechnologies (Cat No BU983034) or Sigma-Aldrich (Cat No M3148).

  Attention! It is recommended to prepare it freshly. It is not stable more than 2 weeks.

- If RLB forms precipitate, please warm it to 56°C until the precipitate has fully dissolved. This is due to storage condition and won't influence the efficiency of the buffer.
- 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.
- If working with RNA for the first time, read Appendix 1 carefully.

Process

- Collect 0.5 to 1.5 ml blood into EDTA tubes. Add three volumes of RBC Lysis Buffer (RNase-free). Invert the tube 5 times and incubate at 4ºc for 10 min.
- Pulse vortex every 2 min during incubation to intersperse the sample.
- Collect the WBCs by centrifugation at 2700 x g for 10 min at 4ºC.
- Discard the supernatant; add two volumes of RBC Lysis Buffer (RNase-free) to the pellet, vortex until the pellet is dissolved completely.
- Collect the WBCs by centrifugation at 2700 g for 10 min at 4ºC.
- Discard the supernatant. Disrupt the cells by adding RLB (Supplemented with 2-βME). Loosen the cell pellet thoroughly by flicking the tube. Add the appropriate volume of RLB (see Table 2) and vortex or pipet to mix. After adding appropriate amount of RLB, use Micro pestle followed by homogenizer or syringe needle to homogenize the sample.
- **Note:** Incomplete loosening of the cell pellet may lead to inefficient lysis and reduced RNA yields.
- **Note:** For previously isolated PBMCs and WBCs, start from step 6.
- Centrifuge the lysate for 3 min at 15000 rpm. Carefully remove the supernatant by pipetting, and transfer it to a new RNase-free microcentrifuge tube.

- Add one volume of %100 ethanol to the homogenized lysate, and mix well by pulse vortexing for 15 s.

  Note: When purifying RNA from isolated cells, precipitates may be visible after addition of ethanol. This does not affect the procedure.

- Transfer up to 700 µl of the sample, including any precipitate that may have formed, to HiPure DR Column placed in a 2ml collection tube (supplied in the kit box). Close the lid gently, and centrifuge for 1min at 13000 rpm. Discard the flow-through. Reuse the collection tube in step 10.
- Add 700 µl TWB1 to the HiPure DR Column. Centrifuge for 1 min at 13000 rpm at room temperature. Discard the flow-through.
- Add 500 µl TWB2 to the HiPure DR Column. Centrifuge for 1 min at 13000 rpm at room temperature. Discard the flow-through.
- Add 500 µl 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 1.5 ml microtube. Add 30-50 µl 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 30-50 µl RNase-free water. Centrifuge for 1 min at 12000 rpm. However, it is possible to pass the flow-through from step 13 once more to obtain RNA with higher concentration.

Protocol 5: Isolation of RNA (Bacteria, gram negative and positive)

Sample Type: Bacteria (gram negative and positive)

Some Tips to Know:

- It is possible to Isolate RNA from bacteria by RNJia Kit, however RJ-Protease (Cat No EB983121) and Lysozyme (Cat No EB983017) should be ordered separately. RNJia Bacteria Kit (Cat No RN983020, RN983021, RN983022 and RN983023) is designed specifically to isolate RNA from Bacteria cells.
- This protocol needs to be improved by the user for intended bacterial species.
- Set Thermoblock or water bath at both temperature, 40℃ and 60℃ before starting the process.

- Before using the protocol for the first time, add 2-Mercaptoethanol to RLB, %2 of RLB volume. 2-Mercaptoethanol is commercially available and due to safety issue, is not provided in the kit. It can be order separately, by ROJETechnologies (Cat No BU983034) or Sigma-Aldrich (Cat No M3148).

  Attention! It is recommended to prepare it freshly. It is not stable more than 2 weeks.

- If RLB forms precipitate, please warm it to 56 °C until the precipitate has fully dissolved. This is due to storage condition and won't influence the efficiency of the buffer.
- Do 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.
- For frozen samples in RNaseLag, thaw them to room temperature. Avoid repeated freezing and thawing of samples, since it may cause RNA degradation.
- If working with RNA for the first time, read Appendix 1 carefully.
- In this protocol, the bacteria cell lysis is accomplished by lysozyme and RJ-Protease. Lysozyme should be prepared as a 20 mg/ml solution in nuclease-free TE buffer.

  Attention! It is recommended to prepare lysozyme freshly. It is not stable more than 3 weeks at 4 °C.

Process

- Calculate the bacteria cell number (refer to appendix 7). Collect the cell by centrifugation at 10000 rpm for 10 min. Discard the supernatant.
- Add 20 µl RJ-Protease and 200 µl of prepared lysozyme solution to the pellet. Resuspend by pulse vortexing for 15 s to completely solve the pellet. Incubate the lysate at 40℃ for 15 min. During the incubation, pulse vortex every 2 min for 10 s.
- Add appropriate amount of RLB (refer to Table 2), to the pellet. Vortex for 1 min and incubate at 40℃ for 5min.
- Add an appropriate absolute ethanol, invert several times (refer to Table 9).
- Transfer up to 700µl of the sample to a HiPure DR Column placed in a 2 ml collection tube (supplied in the kit box). Centrifuge for 1min at 13000 rpm. Discard the flow-through.

  Note: If the lysate does not pass the column, repeat the centrifugation once more at full speed for 1 min.

Table 9: Appropriate absolute ethanol amount for bacteria cell number

Bacteria Cell Number	Absolute Ethanol Amount
$<5 \times 10^8$	300µl
$5 \times 10^8 - 1 \times 10^9$	300µl
$>1 \times 10^9$	300µl

- Repeat the previous step by the remaining sample from step 4.

  Note: If the lysis does not pass the column, repeat the centrifugation once more at full speed for 1 min.

- Add 700 µl TWB1 to the HiPure DR Column. Centrifuge for 1 min at 13000 rpm. Discard the flow-through.
- Add 500 µl TWB2 to the HiPure DR Column. Centrifuge for 1 min at 13000 rpm. Discard the flow-through.
- Add 500 µl TWB2 to the HiPure DR Column. Centrifuge for 3 min at 14000 rpm. Discard the collection tube with the flow-through.
- Place the HiPure DR Column in a new 1.5 ml microtube. Add 30-50 µl RNase-free water directly to the HiPure DR Column membrane. Incubate at 60℃ for 5 min. 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 30-50 µl RNase-free water. Incubate at 60°C for 5 min. 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 10 once more to obtain RNA with higher concentration.

Protocol 6: Isolation of RNA (Bacteria, gram negative and positive)

Sample Type: Bacteria (gram negative and positive)

Protocol Type: Simultaneously mechanical and enzymatic lysis

Some tips to know:

- It is possible to Isolate RNA from bacteria by RNJia Kit, however RJ-Protease (Cat No EB983121) and Lysozyme (Cat No EB983017) should be ordered separately. RNJia Bacteria Kit (Cat No RN983020, RN983021, RN983022 and RN983023) is designed specifically to isolate RNA from Bacteria cells.

- This protocol needs to be improved by the user for intended bacterial species.
- Set Thermoblock or water bath at both temperature, 40℃ and 60℃ before starting the process.
- Before using the protocol for the first time, add 2-Mercaptoethanol to RLB, %2 of RLB volume. 2-Mercaptoethanol is commercially available and due to safety issue, is not provided in the kit. It can be order separately, by ROJETechnologies (Cat No BU983041) or Sigma-Aldrich (Cat No M3148).

  Attention! It is recommended to prepare it freshly. It is not stable more than 2 weeks.

- If RLB forms precipitate, please warm it to 56 °C until the precipitate has fully dissolved. This is due to storage condition and won't influence the efficiency of the buffer.
- Do 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.
- For frozen samples in RNaseLag, thaw them to room temperature. Avoid repeated freezing and thawing of samples, since it may cause RNA degradation.
- If working with RNA for the first time, read Appendix 1 carefully.
- In this protocol, the bacteria cell lysis is accomplished by lysozyme and RJ-Protease. Lysozyme should be prepared as a 20mg/ml solution in nuclease-free TE buffer.

  Attention! It is recommended to prepare lysozyme freshly. It is not stable more than 3 weeks at 4°C.

Process

- For each sample, weigh 25–50 mg acid-washed glass beads (150–600 µm diameter) in a 2 ml Safe-Lock tube.
- Calculate the bacteria cell number (refer to appendix 7). Collect the cell by centrifugation at 10000 rpm for 10 min. Discard the supernatant.
- Add 20 µl RJ-Protease and 200 µl of prepared lysozyme solution to the pellet. Resuspend by pulse vortexing for 15 s to completely dissolve the pellet. Incubate the lysate at 40℃ for 15 min. During the incubation, pulse vortex every 2 min for 10 s.
- Add appropriate amount of RLB (refer to Table 2), to the pellet. Vortex for 1 min then incubate at 40℃ for 5 min.
- Transfer the suspension into the 2 ml Safe-Lock tube containing the acid washed glass beads prepared in step 1. Disrupt the cells in the Tissuelyser for 5 min at maximum speed.

- Centrifuge at 14000 rpm for 1 min. Transfer the supernatant to a new microcentrifuge tube. Add appropriate amount of absolute ethanol (refer to Table 9), invert several times.
- Transfer up to 700 µl of the sample to a HiPure DR Column placed in a 2 ml collection tube (supplied in the kit box). Centrifuge for 1 min at 13000 rpm. Discard the flow-through.

  Note: If the lysate does not pass the column, repeat the centrifugation once more at full speed for 1 min.

- Repeat the previous step by the remaining sample from step 6.

  Note: If the lysis does not pass the column, repeat the centrifugation, once more at full speed for 1 min.

- Add 700 µl TWB1 to the HiPure DR Column. Centrifuge for 1 min at 13000 rpm. Discard the flow-through.
- Add 500 µl TWB2 to the HiPure DR Column. Centrifuge for 1 min at 13000 rpm. Discard the flow-through.
- Add 500 µl TWB2 to the HiPure DR Column. Centrifuge for 3 min at 14000 rpm. Discard the collection tube with the flow-through.
- Place the HiPure DR Column in a new 1.5 ml microtube. Add 30-50 µl RNase-free water directly to the HiPure DR Column membrane. Incubate at 60°C for 5 min. 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 30-50 µl RNase-free water. Incubate at 60°C for 5 min. 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.
	Insufficient disruption and homogenization	• If working with fibrous tissue, it is recommended to use RNJia Fibrous kit (Order by Cat No RN983024, RN983025 and RN983026) • If working with bacteria, it is recommended to use RNJia Bacteria Kit (Order by Cat No RN983020, RN983021, RN983022 and RN983023) • As a guide for better disruption and homogenization, based on sample type refer to suitable sample preparation guidelines.
	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 rehydration is incomplete	• Perform rehydration step once more, by adding another 30-50 µl nuclease-free 36 water to columns and before centrifugation, incubate 5 min at room temperature. • Check that all previous steps are done appropriately.
Degradation	Improper sample storage	• It is suggested to store samples in RNaseLag, refer to sample preparation section.
	Too thick sample for stabilization	• Cut large samples into slices less than 5 mm thick for stabilization in RNaseLag.

	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	• If working with fibrous tissue, it is recommended to use RNJia Fibrous Kit (Order by Cat No RN983024, RN983025 and RN983026). • If working with bacteria, it is recommended to use RNJia Bacteria Kit (Order by Cat No RN983020, RN983021, RN983022 and RN983023). • As a guide for better disruption and homogenization, based on sample type refer to suitable 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.
	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.
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
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.
	Centrifuge at low temperature	• The centrifugation temperature should be 20–25°C. Make sure that the centrifuge temperature is set at 25°C.

Ordering Information

Category	Product Name	Cat No.	Size
	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
	MiRJia Kit	RN003079	100 preps
	RNaseLag	RN983016	50ml
	RNZO	RN983048	25ml
	RNSol H Reagent	RN983061	100ml

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.

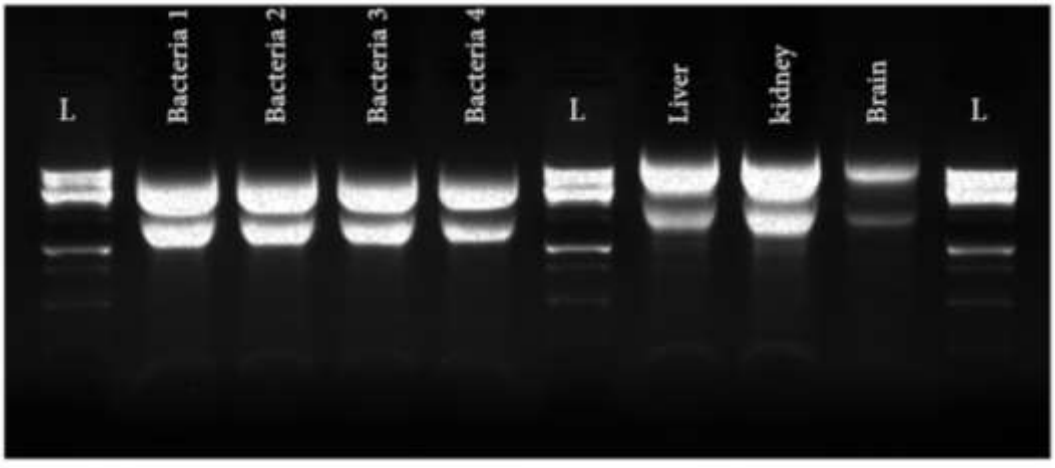

Figure 1. Isolated RNA using RNJia Kit, RNA was isolated from the indicated animal tissues and bacteria cells using the RNJia Kit. For each sample, 10 µg RNA was pretreated by ROJE Sample Buffer and analyzed on Denaturing Gel Electrophoresis (DGE).

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 by RNJia Kit

RNJia 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℃ for 30 min.
- Centrifuge for 10 min at 14000 rpm in a benchtop centrifuge. Discard the supernatant.

- Wash the pellet with 100 µl 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

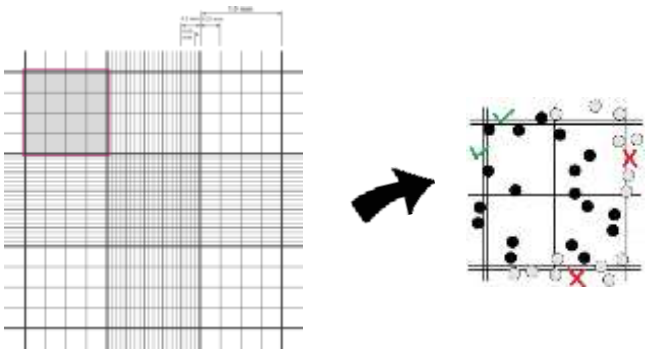

Figure2. Cell counting with hemocytometer

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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