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**ROJETECHNOLOGIES**

# MiRJia Kit

Total RNA isolation based on silica technology

- MiniPrep

## For RNA Isolation from

Animal Tissue (Fibrous and non-fibrous tissue)

Animal Cells

PBMC (Peripheral Blood Mononuclear Cell)

Whole Blood

WBC (White Blood Cell)

**By ROJE**  
**Edition, 2020**

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	50 preps	100 preps
<b>RNSol H Reagent</b>	50 ml	100 ml
<b>TWB1 (concentrate)</b>	16 ml	2 x 16 ml
<b>TWB2 (concentrate)</b>	15 ml	2 x 15 ml
<b>Nuclease-free Water</b>	5 ml	10 ml
<b>HiPure DR Column</b>	50	100
<b>Collection Tube</b>	50	100

## Storage

Shipment condition is checked by ROJETechnologies. After arrival, RNSol H Reagent should be kept dry, at 2-8 °C and all remaining reagent should be kept 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

MiRJia Kit provides the components and procedures necessary for purifying RNA and miRNA from animal tissue and cell, PBMC, whole blood and WBC. Notice that, RNall 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](http://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](http://www.rojetechnoloes.com).

## Description

MiRJia Kit Provides a time-saving, reliable and meticulous method for total RNA isolation (including miRNA) from various sample types including animal tissues and cells. MiRJia Kit is based on spin column technology for isolation of concentrated, highly purified and intact total RNA, which is suitable to be used for a variety of downstream applications such as Northern blot experiments, Real-time PCR, RNA Sequencing, Microarray etc. As in process, phenol and chloroform are applied, it is recommended to use RNJia Kit for situations in which the purified RNA larger than 200 bp will be used in sensitive downstream applications.

## Procedure

The MiRJia Kit is designed for isolating all form of RNA molecules from 0.5-100 mg tissue samples or up to  $1 \times 10^7$  cells. Fresh or frozen tissue samples should be lysed and homogenized. Aqueous phase is separated using chloroform. Then, RNA binding to the silica membrane is achieved selectively, by the addition of ethanol to the lysate. Contaminants are removed by two specific kind of washing buffers. Pure miRNA and total RNA is finally eluted in nuclease free water. Isolated RNA (miRNA or total 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

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

- Benchtop refrigerated microcentrifuge (while RNSol H Reagent is used)
- 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

<b>Features</b>	<b>Specifications</b>
Elution volume (µl)	50-100 µl
Technology	Silica technology
Main sample type	Animal cells and tissue /PBMC
Processing	Manual
Purification of total RNA, miRNA, Poly A+ mi-RNA, DNA or protein	Yes
Sample Amount	0.5-100 Mg
Operation time per reaction (min)	Less than 45 Min
Typical yield (µg)	100 µg
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	Chloroform Amount
10-80 mg animal tissue, ( $1 \times 10^6$ ) – ( $8 \times 10^6$ ) animal cells 1-2 ml whole blood	800 $\mu$ l	200 $\mu$ l
90-100 mg animal tissue ( $9 \times 10^6$ ) – ( $10^7$ ) animal cells 2.5-10 ml whole blood	1ml	300 $\mu$ l

## 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	<ul style="list-style-type: none"> <li>• TissueRuptor/TissueLyser</li> <li>• Mortar and pestle</li> </ul>	<ul style="list-style-type: none"> <li>• TissueRuptor/TissueLyser</li> <li>• Syringe and needles</li> </ul>
Animal Cells, WBC, PBMC	<ul style="list-style-type: none"> <li>• RNSol H Reagent</li> <li>• Vortexing</li> </ul>	<ul style="list-style-type: none"> <li>• Homogenizer</li> <li>• Syringe and needles</li> </ul>

### ***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**

1. Cut the animal tissue sample into slices less than 5mm thick, as quickly as possible.
2. 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.
3. The sample is ready for archival storage at conditions shown in Table 4.
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 CO<sub>2</sub> 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  $-1^{\circ}\text{C}$  to  $-3^{\circ}\text{C}$  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  $-70^{\circ}\text{C}$ .

**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  $37^{\circ}\text{C}$ ) 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 15 ml centrifuge tube containing 10 ml of fresh medium and spin for 5 minutes at  $100 \times g$ . 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

<b>Vessel Type</b>		<b>Cell Number</b>
Dishes	35 mm	$1 \times 10^6$
	60 mm	$2.5 \times 10^6$
	100 mm	$7 \times 10^6$
	145-150 mm	$2 \times 10^7$
Flask	40-45 ml	$3 \times 10^6$
	250-300 ml	$1 \times 10^7$
	650-750 ml	$2 \times 10^7$
Multiwell-plates	96-wells	$4.5 \times 10^4$
	48-wells	$1 \times 10^5$
	24-wells	$2.5 \times 10^5$
	12-wells	$5 \times 10^5$
	6-wells	$1 \times 10^6$

## 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.

## Genomic DNA Contamination

MiRJia Kit is designed to selectively isolate total RNA. However, if further DNA removal is intended, it is recommended to use DNase treatment kits, which are available from different suppliers.

## 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	16 ml	24 ml	40 ml
TWB2	15 ml	45 ml	60 ml

## 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 steps, before applying sample into HiPure DR Column, are carried out on ice.
- 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, thaw them to room temperature. Avoid repeated thawing and freezing of samples due to decreasing in RNA yield.
- If working with RNA for the first time, read appendix 1 carefully.

#### **Process**

1. Remove the tissue from RNaseLag or use fresh tissue. Determine the Weight of starting material and Add appropriate amount of RNSol H Reagent to the tissue sample (refer to the Table 2).
2. Disrupt and homogenize the tissue sample by selecting one of these ways:
  - After adding appropriate amount of RNSol H Reagent, use Micropestle followed by homogenizer or syringe needle to homogenize the tissue.
  - After adding appropriate amount of RNSol H Reagent, 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) and homogenize by passing the lysate 5-10 times through a blunt 20-gauge needle fitted to an RNase-free syringe.

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

3. 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.
4. Centrifuge at 4°C for 12 min at 13000 rpm.
5. Transfer the aqueous phase (the upper phase) to a new tube. Be careful to avoid interfering the interphase.
6. Add one and half volume of absolute ethanol to separated aqueous phase. Pulse vortex for 30 s.
7. Transfer the solution to a HiPure DR Column placed in a 2 ml collection tube (supplied in the kit box). Centrifuge for 1 min at 13000 rpm at room temperature. Discard the flow-through.
8. Add 700 µl TWB1 to the HiPure DR Column. Centrifuge for 1 min at 13000 rpm at room temperature. Discard the flow-through.
9. Add 500 µl TWB2 to the HiPure DR Column. Centrifuge for 1 min at 13000 rpm at room temperature. Discard the flow-through.
10. 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.
11. Place the HiPure DR Column in a new 1.5 ml microtube. Add 30-100 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 30-100 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 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:**

- All steps, before applying sample into HiPure DR Column, are carried out on ice.
- 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, thaw them to room temperature. Avoid repeated thawing and freezing of samples due to decreasing in RNA yield.
- If working with RNA for the first time, read appendix 1 carefully.

### **Process**

1. Collect 0.5 to 10 ml blood into EDTA tubes. Add three volume of Nuclease-free RBC Lysis Buffer. Invert the tube 5 times and incubate at 4 °c for 10 min.
2. Pulse vortex every 2 min during incubation to intersperse the sample.
3. Collect the WBCs by centrifugation at 2700 x g for 10min at 4°C.
4. Discard the supernatant, add two volume of Nuclease-free RBC Lysis to the pellet, vortex until the pellet is dissolved completely.
5. Collect the WBCs by centrifugation at 2700 x g for 10 min at 4°C.
6. Discard the supernatant. Add appropriate amount of RNSol H Reagent to the sample (refer to the Table 2).
7. Disrupt and homogenize the sample by selecting one of these ways:
  - After adding appropriate amount of RNSol H Reagent, use Micropestle followed by homogenizer or syringe needle to homogenize the cell pellet.
  - After adding appropriate amount of RNSol H Reagent, use TissueLyser or homogenizer to disrupt and homogenize the sample simultaneously.

**Optional:** Homogenizing step is optional. It means that using homogenizer or syringe needle to homogenize the lysate can be omitted from the process.
8. After passing through the syringe for 5-10 times, pulse vortex for 1 min, incubate for 10 min at room temperature.

**Note:** During isolating RNA from PBMC, it is necessary to thoroughly homogenize the sample and it is recommended to homogenize by passing the lysate 5-10 times through a blunt 20-gauge needle fitted to an RNase-free syringe.

9. Add appropriate amount of chloroform (refer to Table 2), vigorously shake it for 30 s. Then pulse vortex for 15 s and incubate at room temperature for 5 min.
10. Centrifuge at 4°C for 12 min at 13000 rpm.
11. Transfer the aqueous phase (the upper phase) to a new tube. Be careful to avoid interfering the interphase.
12. Add 400 µl absolute ethanol to the separated aqueous phase. Pulse vortex for 30 s.
13. Transfer the solution to a HiPure DR Column placed in a 2 ml collection tube (supplied in the kit box). Centrifuge for 1 min at 13000 rpm at room temperature. Discard the flow-through.
1. Add 700 µl TWB1 to the HiPure DR Column. Centrifuge for 1 min at 13000 rpm at room temperature. Discard the flow-through.
2. Add 500 µl TWB2 to the HiPure DR Column. Centrifuge for 1 min at 13000 rpm at room temperature. Discard the flow-through.
3. 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.
4. Place the HiPure DR Column in a new nuclease-free 1.5 ml microtube. Add 30-100 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 30-100 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 17 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 steps, before applying sample into HiPure DR Column, are carried out on ice.
- 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, thaw them to room temperature. Avoid repeated thawing and freezing of samples due to decreasing in RNA yield.
- If working with RNA for the first time, read appendix 1 carefully.

**Process**

1. Remove the cultured cells from RNaseLag or use fresh cultured cells. Determine the number of cells (refer to Table 5). 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 7).

**Table 7:** Lysis of attached monolayer and suspension cells

<b>Cultured Cell Type</b>	<b>Lysis Type</b>	<b>Description</b>
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.

2. Disrupt the cells by selecting one of these ways:

- a. 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.

- b. 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.

3. Homogenize the lysate by:

- a. Homogenize the lysate for 30 s using a homogenizer.
- b. Pass the lysate at least 5 times through a blunt 20-gauge needle fitted to an RNase-free syringe.
- c. 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.

1. Add appropriate amount of chloroform (refer to Table 2), vigorously shake it for 30 s. Then pulse vortex for 15 s and incubate at  $-20^{\circ}\text{C}$  for 2 min.
2. Centrifuge at  $4^{\circ}\text{C}$  for 12 min at 13000 rpm.
3. Transfer the aqueous phase (the upper phase) to a new tube. Be careful to avoid interfering the interphase.
4. Add one and half volume of absolute ethanol to the separated aqueous phase. Pulse vortex for 30 s.
5. Transfer the solution to a HiPure DR Column placed in a 2 ml collection tube (supplied in the kit box). Centrifuge for 1 min at 13000 rpm at room temperature. Discard the flow-through.
6. Add 700  $\mu\text{l}$  TWB1 to the HiPure DR Column. Centrifuge for 1 min at 13000 rpm at room temperature. Discard the flow-through.

7. Add 500  $\mu$ l TWB2 to the HiPure DR Column. Centrifuge for 1 min at 13000 rpm at room temperature. Discard the flow-through.
8. Add 500  $\mu$ l TWB2 to the HiPure DR Column. Centrifuge for 3 min at 14000 rpm at room temperature. Discard the collection tube with the flow-through.
9. Place the HiPure DR Column in a new 1.5 ml microtube. Add 30-100  $\mu$ l 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 30-100  $\mu$ l 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
<b>Low yield</b>	Inappropriate sample storage condition	<ul style="list-style-type: none"> <li>• Avoid freezing and thawing of samples, which results in reduced RNA Yield.</li> <li>• For better results, it is recommended to store samples in RNaseLag.</li> </ul>
	Incomplete cell lysis	<ul style="list-style-type: none"> <li>• Too much starting material results in low RNA yield. To optimize the results, refer to Table 2.</li> </ul>
	Ethanol from the washing buffer is present in elution	<ul style="list-style-type: none"> <li>• Perform another centrifugation before rehydration step to ensure no remaining trace of ethanol on column.</li> <li>• Carefully remove the column from the collection tube so that the column does not contact the flow-through.</li> </ul>
	RNA elution is incomplete	<ul style="list-style-type: none"> <li>• Perform rehydration step once more, by adding another 30-100 µl rehydration buffer to the column and before centrifugation, incubate 5 min at room temperature.</li> <li>• Check that all previous steps are done appropriately.</li> </ul>
<b>Degradation</b>	Too thick sample for stabilization	<ul style="list-style-type: none"> <li>• Cut large samples into slices less than 5 mm thick for stabilization in RNaseLag.</li> </ul>
	Improper sample storage	<ul style="list-style-type: none"> <li>• It is suggested to store samples in RNaseLag, refer to sample preparation section.</li> </ul>
	Frozen sample used for stabilization	<ul style="list-style-type: none"> <li>• For stabilization in RNaseLag, use fresh samples.</li> </ul>

	Storage duration in RNaseLag exceeded	<ul style="list-style-type: none"> <li>Refer to Table 4.</li> </ul>
	RNase contamination	<ul style="list-style-type: none"> <li>All buffers have been tested and are guaranteed RNase-free. RNases can be introduced during use. Refer to appendix 1 for more information.</li> </ul>
<b>Low 260/280 ratio</b>	Insufficient disruption and homogenization	<ul style="list-style-type: none"> <li>As a guide for better disruption and homogenization, based on sample type refer to sample preparation guidelines.</li> </ul>
	RNA was diluted in low pH water	<ul style="list-style-type: none"> <li>Use 10 mM Tris-HCl with pH <math>\geq</math> 7.5, or nuclease free water with pH <math>\geq</math> 7.5.</li> </ul>
	DNA contamination	<ul style="list-style-type: none"> <li>Follow precisely the respective protocol, If RNA purification is still problematic further do DNase treatment.</li> <li>RNSol pH might alter during storage. Check the RNSol H Reagent pH it should be around 4.2.</li> </ul>
	Protein contamination	<ul style="list-style-type: none"> <li>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.</li> <li>Remove the aqueous phase precisely.</li> </ul>
<b>DNA contamination in downstream application</b>	No DNase treatment	<ul style="list-style-type: none"> <li>Perform DNase treatment.</li> </ul>
	No incubation with TWB1	<ul style="list-style-type: none"> <li>Incubate the spin column for 5 min at room temperature after addition of TWB1 and before centrifuging</li> </ul>
	Check RNSol pH	<ul style="list-style-type: none"> <li>RNSol H Reagent pH might alter during storage. Check the RNSol H Reagent pH it should be around 4.2.</li> </ul>

<b>Not performing well in downstream application</b>	Ethanol carryover	<ul style="list-style-type: none"> <li>• Perform another centrifugation before rehydration step to ensure no remaining of ethanol on column.</li> </ul>
	Salt carryover	<ul style="list-style-type: none"> <li>• Between washing steps, eliminate remaining flow-through from the rim of collection tube, by blotting on clean paper towels.</li> </ul>
<b>Clogged Column</b>	Maximum amount of tissue exceeds kit specifications	<ul style="list-style-type: none"> <li>• Refer to specifications to determine if the amount of starting material falls within kit specifications.</li> </ul>
	The sample is too large	<ul style="list-style-type: none"> <li>• 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.</li> </ul>

## **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**

1. 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.
2. Treat surfaces of benches with commercially available RNase inactivating agents such as RNZO (Order by cat No RN983018 and RN983019).
3. 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.
4. Use disposable RNase-free plasticware to decrease the possibility of sample contamination.
5. Keep purified RNA on ice when aliquots are pipetted for downstream applications.
6. 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  $-20^{\circ}\text{C}$  or  $-80^{\circ}\text{C}$ , eluted in RNase-free water. Under these conditions, RNA is not degraded after 1 year.

### **RNA Quality**

The ratio of absorbance at 260 nm and 280 nm 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 280 nm. 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**

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

#### **RNA sample preparation**

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

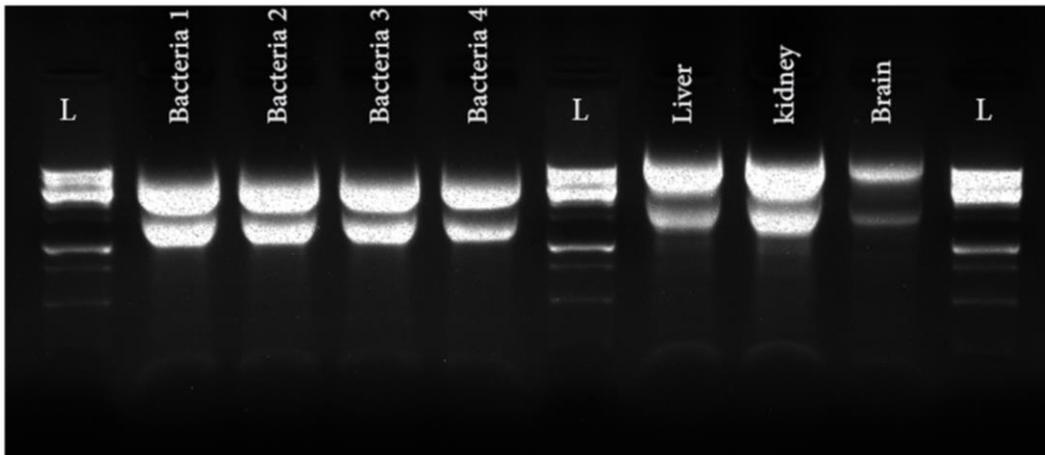
#### **Electrophoresis**

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:

1. 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.
2. 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

MiRJa Kitis 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

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

**Note:** For easier resuspension, do not over dry the pellet.

6. 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 0.5 ml suspension of cells would be removed from the Petri dish and mixed with 0.5 ml Trypan Blue solution in an Eppendorf or small test tube. Trypan Blue is a stain that selectively stains dead cells.

### **Loading Sample**

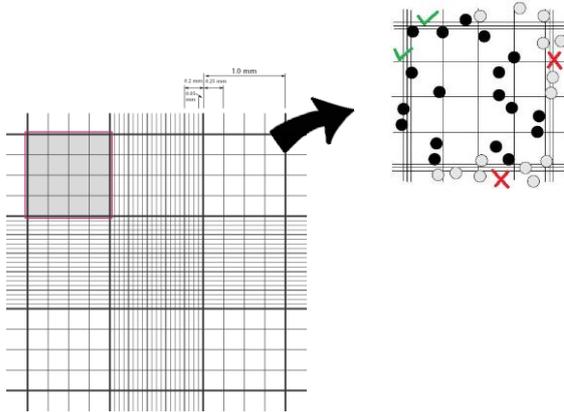
Load your sample quickly and smoothly. It is important to handle the hemocytometer and the cover slip carefully. Never place your fingers on the reflective surface of the slide. Always clean the slide before you load the sample by rinsing the slide and cover slip with 70-95% ethanol. Air-dry or gently 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 10  $\mu$ l 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:

$$\text{Average number of cells} \times \text{dilution factor} \times 10^4$$



**Figure2.** Cell counting with hemocytometer

### Appendix 8: Preparation of Phosphate Buffered Saline (PBS)

Dissolve the components in about 800 mL dH<sub>2</sub>O. 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
137 mM	NaCl	8 g
2.7 mM	KCl	0.2 g
10 mM	Na <sub>2</sub> HPO <sub>4</sub>	1.42 g
1.8 mM	KH <sub>2</sub> PO <sub>4</sub>	0.25 g

## Ordering Information

Category	Product Name	Cat No.	Size
<b>RNA Technologies</b>	MiRJia Kit	MI983001	50 preps
	MiRJia Kit	MI983002	100 preps
<b>Relevant products</b>	RNJia Kit	RN983005	50 preps
	RNJia Kit	RN983006	100 preps
	RNall Kit	RN983009	50 preps
	RNall Kit	RN983010	100 preps
	RNJia Fibrous Kit	RN983025	50 preps
	RNJia Fibrous Kit	RN983026	100 preps
	RNJia Bacteria Kit	RN983022	50 preps
	RNJia Bacteria Kit	RN983023	100 preps
	RNJia Phenol-Free PB Kit	RN983051	50 preps
	RNJia Phenol-Free PB Kit	RN983052	100 preps
	MiRJia Lipid Kit	MI983016	50 preps
	MiRJia Lipid Kit	MI983017	100 preps
	MiRJia PB Kit (No RBC Lysis Buffer)	MI983006	50 preps
	MiRJia PB Kit (No RBC Lysis Buffer)	MI983007	100 preps
	MiRJia PB Kit (With RBC Lysis Buffer)	MI983005	50 preps
	MiRJia PB Kit (With RBC Lysis Buffer)	MI983010	100 preps
	RNSol H Reagent	RN983060	50 ml
	RNSol H Reagent	RN983061	100 ml
	RNaseLag	RN983016	50 ml
	RNaseLag	RN983017	100 ml
	RNZO	RN983018	250 ml
	RNZO	RN983019	500 ml
	RNA Loading Set	LD983007	100 Preps
	2-Mercaptoethanol	BU983041	5 ml
	2-Mercaptoethanol	BU983034	10 ml

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