Contents

Phenol-Chloroform Free Based Protocols

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 volume, is written in Table 1.

Table 1. Appropriate sample size and amount of RLB

Sample Type	Sample Size	Amount of RLB
Animal Cells	≤5 $X10^6$	350 µl
Animal Cells	5 $X\ 10^6$ To 1 $X\ 10^7$	600 µl
Animal Not Fibrous Tissues	0.5-10 Mg	350 µl
Animal Not Fibrous Tissues	10-30 Mg	600 µl
Animal Fibrous Tissues	0.5-30 Mg	300 µl
Bacteria Cells	≤5 $X10^8$	500 µl
Bacteria Cells	5 $X\ 10^8$ - 1 $X\ 10^9$	800 µl
Bacteria Cells	1 $X\ 10^9$ – 2 $X\ 10^9$	1000 µl
Whole blood	Up to 2 x 10^6 cells	350 µl
Whole blood	2 x 10^6 to 1 x 10^7 cells	600 µl

Protocol 1

Isolation of Total RNA (based on silica technology)

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 spin column, are carried out on ice.
- Before using the protocol for the first time, add 2-Mercaptoethanol to RLB Buffer, 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 buffer 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 (96–100%) to TW1 and TW2 buffers (TWB1 and TWB2), as indicated on the bottle, before using for the first time.
- 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, in main handbook, carefully.

Process

1. Remove the tissue from RNaseLag or use fresh tissue or snap-freeze tissue. Weight the intended tissue. Add appropriate RLB buffer to the tissue sample (Refer to the table 2).

Table 2. 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	350 µl
Soft Tissue (brain, liver and etc.)	Treated with RNaseLag	0.5-10 mg	600 µl
Soft Tissue (brain, liver and etc.)	Fresh or Snap Freeze and Treated with RNaseLag	10-30 mg	600 µl
Other Tissue Types	Fresh, Snap Freeze or Treated with RNaseLag	0.5-30 mg	600 µl

2. Disrupt and homogenize the tissue sample by selecting one of these ways:
   - After adding appropriate amount of RLB buffer, use Micropestle followed by homogenizer or syringe needle to homogenize the tissue.
   - After adding appropriate amount of RLB buffer, 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 buffer (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.

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.

3. 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.

4. Add one volume of 70% Ethanol (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.

5. Transfer up to 700 µl of the lysate, including any precipitate that may have formed, to a spin 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.

6. 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.

7. Add 700 µl TWB1 (TW1 buffer) to the spin column. Centrifuge for 1 min at 13000 rpm. Discard the flow-through.
8. Add 500 µl TWB2 (TW2 buffer) to the spin column. Centrifuge for 1 min at 13000 rpm. Discard the flow-through.
9. Add 500 µl TWB2 (TW2 buffer) to the spin column. Centrifuge for 3 min at 14000 rpm. Discard the collection tube with the flow-through.
10. Place the spin column in a new 1.5 ml microtube. Add 30-50 µl RNase-free water directly to the spin 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 spin 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 10 once more to obtain RNA with higher concentration.

Protocol 2

Isolation of Total RNA (based on silica technology)

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 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 spin column, are carried out on ice.
- Before using the protocol for the first time, add 2-Mercaptoethanol to RLB buffer, 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 buffer 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 (96–100%) to TW1 and TW2 buffers (TWB1 and TWB2), as indicated on the bottle, before using for the first time.
- 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, in main handbook, 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

1. 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 buffer to the tissue sample.
2. 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 buffer (see Table 1) 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.

3. Add 600 µl nuclease-free water and 15 µl RJ-Protease. pulse vortex for 15 s and incubate the microtube at 55˚C for 15 min.
4. 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.

5. 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.

6. Transfer up to 700 µl of the sample, including any precipitate that may have formed, to a spin 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.

7. 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.

8. Add 700 µl TWB1 (TW1 buffer) to the spin column. Centrifuge for 1 min at 13000 rpm. Discard the flow-through.
9. Add 500 µl TWB2 (TW2 buffer) to the spin column. Centrifuge for 1 min at 13000 rpm. Discard the flow-through.
10. Add 500 µl TWB2 (TW2 buffer) to the spin column. Centrifuge for 3 min at 14000 rpm. Discard the collection tube with the flow-through.
11. Place the spin column in a new 1.5 ml microtube. Add 30-50 µl RNase-free water directly to the spin 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 spin 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 (based on silica technology)

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 spin column, are carried out on ice.
- Before using the protocol for the first time, add 2-Mercaptoethanol to RLB buffer, 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 buffer 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 (96–100%) to TW1 and TW2 buffers (TWB1 and TWB2), as indicated on the bottle, before using for the first time.
- 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, in main handbook, carefully.

Process

1. 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 3).

Table 3: 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 spin column, which finally reduce RNA yield.

2. Disrupt the cells by selecting one of these ways:
   a) adding appropriate volume of RLB buffer (refer to Table 1), Vortex to mix.

   Note: Before adding RLB buffer, 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 RLB to the cell-culture dish (refer to Table 1). 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 ablunt 20-gauge needle fitted to an RNase-free syringe.
    c. Pass the lysate through a shredder spin column by centrifuging at full speed for 2 min.
4. 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.

5. Add one volume of 70% Ethanol (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 (nuclease-free) to the cleared lysate.

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

6. Transfer up to 700 µl of the lysate, including any precipitate that may have formed, to a spin 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.
7. 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.

8. Add 700 µl TWB1 (TW1 buffer) to the spin column. Centrifuge for 1 min at 13000 rpm. Discard the flow-through.
9. Add 500 µl TWB2 (TW2 buffer) to the spin column. Centrifuge for 1 min at 13000 rpm. Discard the flow-through.
10. Add 500 µl TWB2 (TW2 buffer) to the spin column. Centrifuge for 3 min at 14000 rpm. Discard the collection tube with the flow-through.
11. Place the spin column in a new 1.5 ml microtube. Add 30-50 µl RNase-free water directly to the spin 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 spin 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 (based on silica technology)

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

Some tips to know:

- All steps, before applying sample into spin column, are carried out on ice.
- Before using the protocol for the first time, add 2-Mercaptoethanol to RLB buffer, 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 buffer 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 (96–100%) to TW1 and TW2 buffers (TWB1 and TWB2) as indicated on the bottle, before using for the first time.
- If working with RNA for the first time, read Appendix 1, in main handbook, carefully.

Process

1. Collect 0.5 to 1.5 ml blood into EDTA tubes. Add three volume of RBC Lysis Buffer (RNase-free). Invert the tube 5 times and incubate at 4ºc 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 4ºc.
4. Discard the supernatant, add two volume of RBC Lysis Buffer (RNase-free) to the pellet, vortex until the pellet is dissolved completely.
5. Collect the WBCs by centrifugation at 2700g for 10 min at 4ºc.
6. Discard the supernatant. Disrupt the cells by adding RLB buffer (Supplemented with 2-βME). Loosen the cell pellet thoroughly by flicking the tube. Add the appropriate volume of RLB (see Table 1) 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.
7. 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.
8. 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.

9. Transfer up to 700 µl of the sample, including any precipitate that may have formed, to spin 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.
10. Add 700 µl TWB1 (TW1 buffer) to the spin column. Centrifuge for 1 min at 13000 rpm at room temperature. Discard the flow-through.
11. Add 500 µl TWB2 (TW2 buffer) to the spin column. Centrifuge for 1 min at 13000 rpm at room temperature. Discard the flow-through.
12. Add 500 µl TWB2 (TW2 buffer) to the spin column. Centrifuge for 3 min at 14000 rpm at room temperature. Discard the collection tube with the flow-through.
13. Place the spin column in a new 1.5 ml microtube. Add 30-50 µl RNase-free water directly to the spin 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 spin 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 Total RNA (based on silica technology)

Sample Type: Bacteria (gram negative and positive)

Protocol Type: 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° C and 60° C before starting the process.
- Before using the protocol for the first time, add 2-Mercaptoethanol to RLB buffer, 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 buffer 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 (96–100%) to TW1 and TW2 buffers (TWB1 and TWB2), as indicated on the bottle, before using for the first time.

- 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, in main handbook, 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

1. Calculate the bacteria cell number (refer to appendix 7, in main handbook). Collect the cell by centrifugation at 10000 rpm for 10 min. Discard the supernatant.
2. 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˚C for 15 min. During the incubation, pulse vortex every 2 min for 10 s.
3. Add appropriate amount of RLB (refer to Table 1), to the pellet. Vortex for 1 min and incubate at 40˚C for 5 min.
4. Add an appropriate Absolute Ethanol, invert several times (refer to Table 4).
5. Transfer up to 700 µl of the sample to a spin 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.

Table 4. Appropriate absolute Ethanol amount for Bacteria Cell Number

Bacteria Cell Number	Absolute Ethanol Amount
<5 x 10^8	300 µl
5 x 10^8 – 1 x 10^9	480 µl
>1 x 10^9	600 µl

6. 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.

7. Add 700 µl TWB1 (TW1 buffer) to the spin column. Centrifuge for 1 min at 13000 rpm. Discard the flow-through.
8. Add 500 µl TWB2 (TW2 buffer) to the spin column. Centrifuge for 1 min at 13000 rpm. Discard the flow-through.
9. Add 500 µl TWB2 (TW2 buffer) to the spin column. Centrifuge for 3 min at 14000 rpm. Discard the collection tube with the flow-through.
10. Place the spin column in a new 1.5 ml microtube. Add 30-50 µl RNase-free water directly to the spin 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 spin 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 Total RNA (based on silica technology)

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° C and 60° C before starting the process.
- Before using the protocol for the first time, add 2-Mercaptoethanol to RLB buffer, 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 buffer 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 (96–100%) to TW1 and TW2 buffers (TWB1 and TWB2), as indicated on the bottle, before using for the first time.
- 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, in main handbook, 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

1. For each sample, weigh 25–50 mg acid-washed glass beads (150–600 µm diameter) in a 2 ml Safe-Lock tube.
2. Calculate the bacteria cell number (refer to appendix 7, in main handbook). Collect the cell by centrifugation at 10000 rpm for 10 min. Discard the supernatant.
3. 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˚C for 15 min. During the incubation, pulse vortex every 2 min for 10 s.

4. Add appropriate amount of RLB buffer (refer to Table 1), to the pellet. Vortex for 1 min then incubate at 40˚C for 5 min.
5. 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.
6. Centrifuge at 14000 rpm for 1 min. Transfer the supernatant to a new microcentrifuge tube. Add appropriate amount of Absolute Ethanol (refer to Table 4), invert several times.
7. Transfer up to 700 µl of the sample to a spin 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.
8. 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.
9. Add 700 µl TWB1 (TW1 buffer) to the spin column. Centrifuge for 1 min at 13000 rpm. Discard the flow-through.
10. Add 500 µl TWB2 (TW2 buffer) to the spin column. Centrifuge for 1 min at 13000 rpm. Discard the flow-through.
11. Add 500 µl TWB2 (TW2 buffer) to the spin column. Centrifuge for 3 min at 14000 rpm. Discard the collection tube with the flow-through.

12. Place the spin column in a new 1.5 ml microtube. Add 30-50 µl RNase-free water directly to the spin 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 spin 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.

Protocols Phenol-Chloroform Based Protocols

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, Chloroform and Ethanol volumes, are written in Table 5.

Table 5. Appropriate sample size and amount of RNSol, chloroform and Ethanol amount

Sample Size	RNSol Amount	Chloroform Amount
10 mg animal tissue, 1 × 10^6 animal cells	100 µl	100 µl
20 mg animal tissue 2 × 10^6 animal cells	200 µl	100 µl
30 mg animal tissue 3 × 10^6 animal cells 0.5-1 ml whole blood	300 µl	200 µl
40 mg animal tissue 4 × 10^6 animal cells	400 µl	200 µl
50 mg animal tissue 5 × 10^6 animal cells 1-3 ml whole Blood	500 µl	300 µl
60 mg animal tissue 6 × 10^6 animal cells	600 µl	300 µl
70 mg animal tissue 7 × 10^6 animal cells 3-5 ml whole Blood	700 µl	400 µl
80 mg animal tissue 8 × 10^6 animal cells	800 µl	400 µl
90 mg animal tissue 9 × 10^6 animal cells	900 µl	500 µl
100 mg animal tissue 10^7 animal cells 5-10 ml whole Blood	1ml	500 µl

Protocol 7

Isolation of Total RNA (based on silica technology)

Sample Type: Animal Tissues (fresh and frozen)

Some tips to know:

- All steps, before applying sample into spin column, are carried out on ice.
- Not forget to add the appropriate amount of Ethanol (96–100%) to TW1 and TW2 buffers (TWB1 and TWB2) as indicated on the bottle, before using for the first time.
- 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, in main handbook, carefully.

Process

1. Remove the tissue from RNaseLag or use fresh tissue. Determine the Weight of starting material and Add appropriate amount of RNSol Reagent to the tissue sample (refer to the Table 5).
2. Disrupt and homogenize the tissue sample by selecting one of these ways:
    - After adding appropriate amount of RNSol, use Micropestle followed by homogenizer or syringe needle to homogenize the tissue.

- After adding appropriate amount of RNSol, 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 (refer to Table 5) 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 5), 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 spin 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 (TW1 buffer) to the spin column. Centrifuge for 1 min at 13000 rpm at room temperature. Discard the flow-through.
9. Add 500 µl TWB2 (TW2 buffer) to the spin column. Centrifuge for 1 min at 13000 rpm at room temperature. Discard the flow-through.

10. Add 500 µl TWB2 (TW2 buffer) to the spin column. Centrifuge for 3 min at 14000 rpm at room temperature. Discard the collection tube with the flow-through.
11. Place the spin column in a new 1.5 ml microtube. Add 30-100 µl RNase-free water directly to the spin 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 spin 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 8

Isolation of Total RNA (based on silica technology)

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

Some tips to know:

- All steps, before applying sample into spin column, are carried out on ice.
- Not forget to add the appropriate amount of Ethanol (96–100%) to TW1 and TW2 buffers (TWB1 and TWB2) as indicated on the bottle, before using for the first time.
- 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, in main handbook, carefully.

Process

1. Collect 0.5 to 10 ml blood into EDTA tubes. Add three volume of RBC Lysis Buffer (RNase-free). Invert the tube 5 times and incubate at 4 ºc 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 4ºc.
4. Discard the supernatant, add two volume of RBC Lysis Buffer (RNase-free) to the pellet, vortex until the pellet is dissolved completely.
5. Collect the WBCs by centrifugation at 2700 x g for 10 min at 4ºc.
6. Discard the supernatant. Add appropriate amount of RNSol Reagent to the sample (refer to the table 5).

7. Disrupt and homogenize the sample by selecting one of these ways:

    - After adding appropriate amount of RNSol, use Micropestle followed by homogenizer or syringe needle to homogenize the cell pellet.
    - After adding appropriate amount of RNSol, 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 5), 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 spin 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.
14. Add 700 µl TWB1 (TW1 buffer) to the spin column. Centrifuge for 1 min at 13000 rpm at room temperature. Discard the flow-through.
15. Add 500 µl TWB2 (TW2 buffer) to the spin column. Centrifuge for 1 min at 13000 rpm at room temperature. Discard the flow-through.
16. Add 500 µl TWB2 (TW2 buffer) to the spin column. Centrifuge for 3 min at 14000 rpm at room temperature. Discard the collection tube with the flow-through.
17. Place the spin column in a new nuclease-free 1.5 ml microtube. Add 30-100 µl RNase-free water directly to the spin 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 spin 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 9

Isolation of Total RNA (based on silica technology)

Sample Type: Animal cultured cell

Some tips to know:

- All steps, before applying sample into spin column, are carried out on ice.
- Not forget to add the appropriate amount of Ethanol (96–100%) to TW1 and TW2 buffers (TWB1 and TWB2) as indicated on the bottle, before using for the first time.
- 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, in main handbook, carefully.

Process

1. 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 3).

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

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

a. Adding appropriate volume of RNSol (refer to Table 5), Vortex to mix.

Note: Before adding RNSol, 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 to the cell-culture dish (refer to Table 5). 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 spin 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 5), vigorously shake it for 30 s. Then pulse vortex for 15 s and incubate at -20°C for 2 min.
2. Centrifuge at 4°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 spin 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 µl TWB1 (TW1 buffer) to the spin column. Centrifuge for 1 min at 13000 rpm at room temperature. Discard the flow-through.
7. Add 500 µl TWB2 (TW2 buffer) to the spin column. Centrifuge for 1 min at 13000 rpm at room temperature. Discard the flow-through.
8. Add 500 µl TWB2 (TW2 buffer) to the spin column. Centrifuge for 3 min at 14000 rpm at room temperature. Discard the collection tube with the flow-through.
9. Place the spin column in a new 1.5 ml microtube. Add 30-100 µl RNase-free water directly to the spin 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 spin 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 12 once more to obtain RNA with higher concentration.