Contents

Recommended Starting Material

To reach optimized results it is better to follow as listed below. The size of the recommended material to use with determined lysis volumes are written in Table.

Table. Appropriate sample size and amount of RLB

Sample Type	Sample Size	Amount of RLB	Absolute Ethanol Amount
Bacteria Cells	≤5 X10^8	500 µl	300 µl
Bacteria Cells	5 X 10^8 - 1 X 10^9	800 µl	480 µl
Bacteria Cells	1 X 10^9 – 2 X 10^9	1000 µl	600 µl

Protocol 1

Isolation of RNA (based on silica technology)

Sample Type: Bacteria (gram negative and positive)

Protocol Type: Enzymatic lysis

Some Tips to Know:

- 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 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

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 buffer (refer to Table), 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).
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.

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 follow-through from step 10 once more to obtain RNA with higher concentration.

Protocol 2

Isolation of RNA (based on silica technology)

Sample Type: Bacteria (gram negative and positive)

Protocol Type: Simultaneously mechanical and enzymatic lysis

Some tips to know:

- 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. 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), 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), 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 follow-through from step 12 once more to obtain RNA with higher concentration.