

Life science unlimited

Manual



innuPREP RNA Mini Kit

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1 Safety precautions

All due care and attention should be exercised in handling the materials and reagents contained in the kit. Always wear gloves while handling these reagents and avoid any skin contact! In case of contact, flush eyes or skin with a large amount of water immediately.

2 Storage conditions

The innuPREP RNA Mini Kit should be stored dry, at room temperature (14 – 25 °C) and is stable for at least 12 months under these conditions. Before every use make sure that all components have room temperature. If there are any precipitates within the provided solutions solve these precipitates by careful warming.

3 Function testing and technical assistance

The Analytik Jena AG guarantees the correct function of the kit for applications as described in the manual. The components of each innuPREP RNA Mini Kit were tested by isolation of total RNA from tissue samples and subsequent analysis on a Agilent Bioanalyzer.

We reserve the right to change or modify our products to enhance their performance and design. If you have any questions or problems regarding any aspects of the innuPREP RNA Mini Kit or other Analytik Jena AG products, please do not hesitate to contact us. For technical support or further information in Germany please dial +49 36 41 / 77 94 00. For other countries please contact your local distributor.

4 Product use and warranty

The user is responsible to validate the performance of the Analytik Jena AG kits for any particular use, since the performance characteristics of our kits have not been validated for any specific application. Analytik Jena AG kits may be used in clinical diagnostic laboratory systems after the laboratory has validated the complete diagnostic system as required by CLIA' 88 regulations in the U.S. or equivalents in other countries.

All products sold by Analytik Jena AG are subjected to extensive quality control procedures and are warranted to perform as described when used correctly. Any problems should be reported immediately.

**Note**

For research use only!

5 Kit components



Important

Kit components are stored at room temperature.

	10 extractions	50 extractions	250 extractions
Lysis Solution RL	6 ml	30 ml	125 ml
Washing Solution HS	3 ml (final volume 6 ml)	15 ml (final volume 30 ml)	70 ml (final volume 140 ml)
Washing Solution LS	2 ml (final volume 10 ml)	8 ml (final volume 40 ml)	40 ml (final volume 200 ml)
RNase-free Water	1,5 ml	6 ml	2 x 15 ml
Spin Filter D	10	50	5 x 50
Spin Filter R	10	50	5 x 50
Receiver Tubes (2.0 ml)	50	5 x 50	25 x 50
Elution Tubes (1.5 ml)	10	50	5 x 50
Manual	1	1	1
Initial steps	<ul style="list-style-type: none"> • Add 3 ml of 96-99.8 % ethanol to the bottle Washing Solution HS, mix thoroughly and keep the bottle always firmly closed! • Add 8 ml of 96-99.8 % ethanol to the bottle Washing Solution LS, mix thoroughly and keep the bottle always firmly closed! 	<ul style="list-style-type: none"> • Add 15 ml of 96-99.8 % ethanol to the bottle Washing Solution HS, mix thoroughly and keep the bottle always firmly closed! • Add 32 ml of 96-99.8 % ethanol to the bottle Washing Solution LS, mix thoroughly and keep the bottle always firmly closed! 	<ul style="list-style-type: none"> • Add 70 ml of 96-99.8 % ethanol to the bottle Washing Solution HS, mix thoroughly and keep the bottle always firmly closed! • Add 160 ml of 96-99.8 % ethanol to the bottle Washing Solution LS, mix thoroughly and keep the bottle always firmly closed!

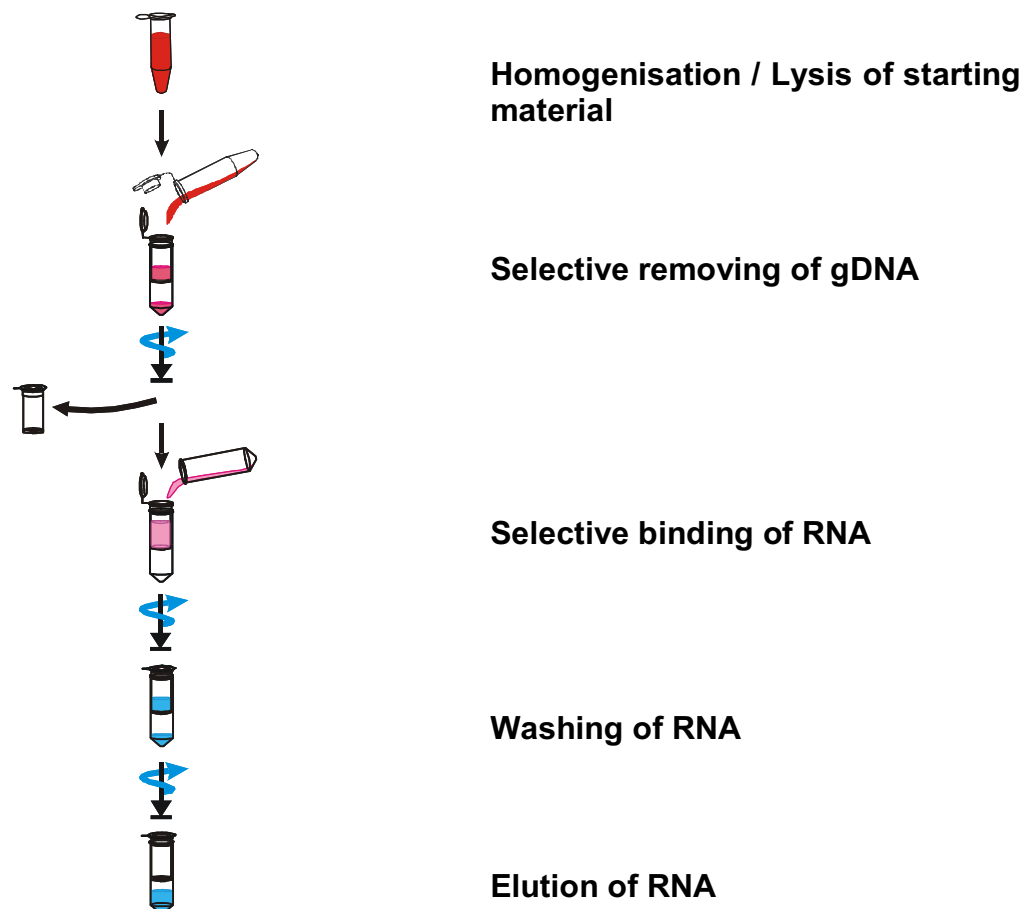
6 Recommended steps before starting

- Ensure that the Washing Solution HS and Washing Solution LS have been prepared according to the instruction (→ "Kit components" p. 4)
- Centrifugation steps should be performed at room temperature
- Avoid freezing and thawing of starting materials

7 Components not included in the kit

- DNase I; optional
- Lysozyme; optional
- ddH₂O
- TE-Buffer (10 mM Tris-HCl; 1 mM EDTA; pH 8.0); optional
- Reaction tubes
- Ethanol (70 %, 96-99.8 %)

8 General procedure for RNA extraction



9 Product specifications

1. Starting material:

- Eucaryotic cells (5×10^6)
- Tissue samples (up to 20 mg)
- gram+ and gram- bacteria (1×10^9)
- Biopsies
- Paraffine embedded tissue samples

2. Time for isolation:

Approximately 15 - 40 minutes

3. Typical yield:

Not determined. The yield depends on the type and the amount of the starting material.

4. Binding capacity:

Approximately: 100 μ g RNA

10 General notes and safety recommendations on handling RNA

RNA is far less stable than DNA. It is very sensitive to degradation by endogenous RNases in the biological material and exogenous RNases which are permanently present everywhere in the lab. To achieve satisfactory qualitative and quantitative results in RNA preparations, contaminations with exogenous RNases have to be reduced to a minimum in accordance with the following recommendations:

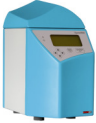
- Always wear latex or vinyl gloves while handling reagents and RNA samples to prevent RNase contaminations from surface of the skin or from dusty laboratory equipment.
- Change gloves frequently and keep tubes closed.
- Keep isolated RNA on ice.
- Reduce preparation time as much as possible.
- Use only sterile, disposable polypropylene tubes throughout the procedure (these tubes are generally RNase-free.)
- Non-disposable plastic ware should be treated before use to ensure that it is RNase-free. Plastic ware should be thoroughly rinsed with 0.1 M NaOH, 1 mM EDTA followed by RNase-free water. You can also take chloroform-resistant plastic ware rinsed with chloroform to inactivate RNases.
- All glassware should be treated before use to ensure that it is RNase-free. Glassware should be cleaned with detergent, thoroughly rinsed and oven baked at 240 °C for four or more hours before use. Autoclaving alone will not inactivate many RNases completely. Oven baking inactivates RNases and ensures that no other nucleic acids (such as plasmid DNA) are present on the surface of the glassware. You can also clean glassware with 0.1 % DEPC (diethyl pyrocarbonate). The glassware has to be immersed in 0.1 % DEPC solution for 12 hours at 37 °C and then it has to be autoclaved or heated to 100 °C for 15 min to remove residual DEPC.
- Electrophoresis tanks should be cleaned with detergent solution (e.g. 0.5 % SDS), thoroughly rinsed with RNase-free water, rinsed with ethanol and finally allowed to dry.
- All buffers have to be prepared with DEPC-treated RNase-free ddH₂O.
- Avoid handling bacterial cultures, cell cultures or other biological sources of RNases in the same lab where the RNA purification will be performed.
- Do not use equipment, glassware and plastic ware employed for other applications which might introduce RNase contaminations in the RNA isolation.

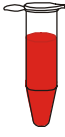
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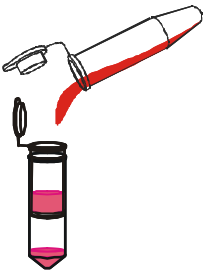

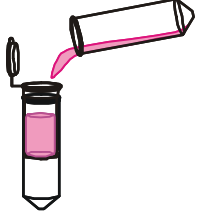



Protocol 1: RNA extraction from tissue samples

Recommended steps before starting

- Prepare Washing Solution HS and Washing Solution LS according to the instruction

- | | | | |
|-------------------------------|---|---|---|
| 1. Starting material | <ul style="list-style-type: none"> Tissue | <ul style="list-style-type: none"> Max. 20 mg | |
| 2. Homogenization and lysis | <ul style="list-style-type: none"> Homogenizer e.g. SpeedMill <p>or</p> <ul style="list-style-type: none"> Liquid nitrogen | 



 | <ul style="list-style-type: none"> <u>See also</u>: innuSPEED Tissue RNA Kit Add frozen starting material to homogenizer tube Add 450 µl RL and homogenize Add sample to a 1.5 ml tube Grind starting material to fine powder under liquid nitrogen Add sample to a 1.5 ml tube Add 450 µl RL <u>or</u> PL and lyse sample under continuous shaking Centrifuge: max. speed; 1 min |
| 3. Selective removing of gDNA |  |  | <ul style="list-style-type: none"> Spin Filter D to Receiver Tube Add supernatant to Spin Filter D 10.000 x g (12.000 rpm): 2 min Discard Spin Filter D Add equal volume 70 % ethanol (approx. 400 µl) to filtrate |
| 4. Selective binding of RNA | <p>New Receiver Tube</p>  |  | <ul style="list-style-type: none"> Spin Filter R to Receiver Tube Add sample to Spin Filter R 10.000 x g (12.000 rpm): 2 min |
| 5. Washing | <p>New Receiver Tube</p>  |  | <ul style="list-style-type: none"> Add 500 µl HS 10.000 x g (12.000 rpm): 1 min Add 750 µl LS 10.000 x g (12.000 rpm): 1 min |

Cut at the scattered line and laminate the card for a more convenient handling on the table top

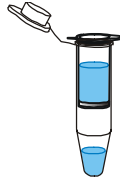
6. Remove Ethanol

New Receiver Tube



- Discard filtrate
- Spin Filter R to Receiver Tube
- Centrifuge: max speed, 2 min

7. Elution



- Spin Filter R to an Elution Tube
- Add 30-80 µl RNase-free water
- Incubation: 1 min @ RT
- 6.000 x g (8.000 rpm): 1 min

Order No.:

845-KS-2040010	10 reactions
845-KS-2040050	50 reactions
845-KS-2040250	250 reactions

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Publisher:

Analytik Jena AG
Konrad-Zuse-Straße 1
07745 Jena/ Germany

Phone +49 (0) 36 41 / 77-94 00
Fax +49 (0) 36 41 / 77-76 77 76



www.bio.analytik-jena.com
biosolutions@analytik-jena.com

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Protocol 2: RNA extraction from eucaryotic cells

Recommended steps before starting

- Prepare Washing Solution HS, Washing Solution LS according to the instruction

1. Starting material

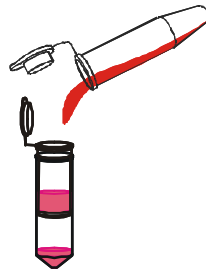
- Max. 5×10^6 cells

2. Lysis



- Add 400 μ l RL to cell pellet
- Incubation: 2 min @ RT
- Resuspend completely
- Incubation: 3 min @ RT

3. Selective removing of gDNA



- Spin Filter D to Receiver Tube
- Add supernatant to Spin Filter D
- 10.000 x g (12.000 rpm): 2 min
- Discard Spin Filter D
- Add equal volume 70 % ethanol (approx. 400 μ l) to filtrate

4. Selective binding of RNA

New Receiver Tube



- Spin Filter R to Receiver Tube
- Add sample to Spin Filter R
- 10.000 x g (12.000 rpm): 2 min

5. Washing

New Receiver Tube



- Add 500 μ l HS
- 10.000 x g (12.000 rpm): 1 min
- Add 750 μ l LS
- 10.000 x g (12.000 rpm): 1 min

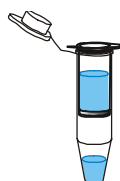
6. Remove Ethanol

New Receiver Tube



- Discard filtrate
- Spin Filter R to Receiver Tube
- 10.000 x g (12.000 rpm): 3 min

7. Elution



- Spin Filter R to an Elution Tube
- Add 30-80 μ l RNase-free water
- Incubation: 1 min @ RT
- 6.000 x g (8.000 rpm): 1 min

Order No.:

845-KS-2040010	10 reactions
845-KS-2040050	50 reactions
845-KS-2040250	250 reactions

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Analytik Jena AG
Konrad-Zuse-Straße 1
07745 Jena/ Germany

Phone +49 (0) 36 41 / 77-94 00
Fax +49 (0) 36 41 / 77-76 77 76



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biosolutions@analytik-jena.com

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Protocol 3: RNA extraction from bacterial cells

Recommended steps before starting

- Prepare Washing Solution HS, Washing Solution LS according to the instruction

1. Starting material

- Max. 1×10^9 cells

2. Pellet cells



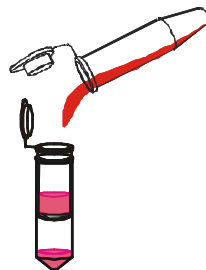
- 5.000 x g (7.500 rpm); 5 min
- Discard supernatant

3. Lysis



- Add 100 µl TE buffer
- Resuspend cell pellet
- Add Lysozyme (gram- 2 µl; gram+ 6 µl)
- Incubation (clear or viscous sol.)
- Add 450 µl RL
- Incubation: 3 min @ RT

4. Selective removing of gDNA



- Spin Filter D to Receiver Tube
- Add supernatant to Spin Filter D
- 10.000 x g (12.000 rpm): 2 min
- Discard Spin Filter D
- Add equal volume 70 % ethanol (approx. 600 µl) to filtrate

5. Selective binding of RNA

New Receiver Tube



- Spin Filter R to Receiver Tube
- 650 µl sample to Spin Filter R
- 10.000 x g (12.000 rpm): 1 min
- Add residual sample
- 10.000 x g (12.000 rpm): 1 min

6. Washing

New Receiver Tube



- Add 500 µl HS
- 10.000 x g (12.000 rpm): 1 min
- Add 750 µl LS
- 10.000 x g (12.000 rpm): 1 min

Cut at the scattered line and laminate the card for a more convenient handling on the table top

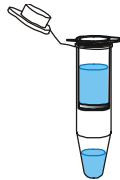
7. Remove Ethanol

New Receiver Tube



- Discard filtrate
- Spin Filter R to Receiver Tube
- 10.000 x g (12.000 rpm): 3 min

8. Elution



- Spin Filter R to an Elution Tube
- Add 30-80 µl RNase-free water
- Incubation: 1 min @ RT
- 6.000 x g (8.000 rpm): 1 min

Order No.:

845-KS-2040010	10 reactions
845-KS-2040050	50 reactions
845-KS-2040250	250 reactions

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Phone +49 (0) 36 41 / 77-94 00
Fax +49 (0) 36 41 / 77-76 77 76



www.bio.analytik-jena.com
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11 Protocol 1: RNA extraction from tissue samples



Important

Please note that up to 20 mg of tissue samples can be processed.
Avoid freezing and thawing of tissue samples!

1. Homogenization of starting material

Note: To maximize the final yield of total RNA a complete homogenization of tissue sample is important!

For the homogenization of tissue sample it is possible to use commercially available rotor-stator homogenizer or bead mills. It is also possible to disrupt the starting material using mortar and pestle in liquid nitrogen and grind the tissue sample to a fine powder.

A. Homogenization of the tissue sample using a rotor-stator homogenizer

1. Transfer the weighed amount of fresh or frozen starting material in a suitable reaction vessel for the homogenizer.
2. Add **450 µl Lysis Solution RL**.
3. Homogenize the sample.
4. Transfer the homogenized tissue sample into a 1.5 ml reaction tube and place the sample under Lysis Solution RL for longer storage at $-20\text{ }^{\circ}\text{C}$ or use the sample immediately for isolation of total RNA following the protocol step 2.

B. Disruption of the tissue sample using a mortar and pestle and liquid nitrogen

1. Transfer the weighed amount of fresh or frozen starting material under liquid nitrogen and grind the material to a fine tissue powder.
2. Transfer the powder into a 1.5 ml reaction tube. Don't allow the sample to thaw!
3. Add **450 µl Lysis Solution RL** and incubate the sample for appropriate time for a further lysis under continuous shaking.
4. Finally place the sample under Lysis Solution RL for longer storage at $-20\text{ }^{\circ}\text{C}$ or use the sample immediately for isolation of total RNA following protocol step 2.

-
2. After lysis spin down unlysed material by centrifugation at maximum speed for 1 minute. Place a Spin Filter D into a 2.0 ml Receiver Tube. Transfer the supernatant of the lysed sample onto the Spin Filter D. Centrifuge at 10.000 x g (12.000 rpm) for 2 minutes. Discard the Spin Filter D.

Do not discard the filtrate, because the filtrate contains the RNA!

Note: If the solution has not completely passed through the Spin Filter, centrifuge again at higher speed or prolong the centrifugation time.

3. Place a Spin Filter R into a new 2.0 ml Receiver Tube. Add an **equal volume** (appr. 400 µl) **of 70 % ethanol** to the filtrate from step 2. Mix the sample by pipetting up and down several times. Transfer the sample onto the Spin Filter R. Centrifuge at 10.000 x g (12.000 rpm) for 2 minutes.

Note: If the solution has not completely passed through the Spin Filter, centrifuge again at higher speed or prolong the centrifugation time.

Discard the 2.0 ml Receiver Tube with filtrate and place the Spin Filter R into a new 2.0 ml Receiver Tube.

4. Open the Spin Filter R and add **500 µl Washing Solution HS**, close the cap and centrifuge at 10.000 x g (12.000 rpm) for 1 minute. Discard the Receiver Tube with the filtrate. Place the Spin Filter R into a new 2.0 ml Receiver Tube.
5. Open the Spin Filter R and add **700 µl Washing Solution LS**, close the cap and centrifuge at 10.000 x g (12.000 rpm) for 1 minute. Discard the Receiver Tube with the filtrate. Place the Spin Filter R into a new 2.0 ml Receiver Tube.
6. Centrifuge at 10.000 x g (12.000 rpm) for 2 minutes to remove all traces of ethanol. Discard the 2.0 ml Receiver Tube.
7. Place the Spin Filter R into a 1.5 ml Elution Tube. Carefully open the cap of the Spin Filter R and add **30-80 µl RNase-free water**. Incubate at room temperature for 1 minute. Centrifuge at 6.000 x g (8.000 rpm) for 1 minute.



Note

Depending on the extracted yield or the needed concentration of total RNA you can also elute with different volumes of RNase-free water. A lower volume of RNase-free water increases the concentration of RNA and a higher volume of RNase-free water leads to an increased yield but a lower concentration of total RNA. Please note, that the minimum of RNase-free water should be 20 µl.

12 Protocol 2: RNA extraction from eucaryotic cells



Important

Please note that up to 5×10^6 cells can be processed.

1. Add **400 μ l Lysis Solution RL** to the cell pellet. Incubate for 2 minutes at room temperature. Re-suspend the cell pellet completely by pipetting up and down. Incubate the sample for further 3 minutes at room temperature.

Note: To maximize the final yield of total RNA a complete disruption and lysis of the cell pellet is important! No cell clumps should be visible after lysis step.

2. Place a Spin Filter D into a 2.0 ml Receiver Tube. Transfer the lysed sample onto the Spin Filter D. Centrifuge at $10.000 \times g$ (12.000 rpm) for 2 minutes. Discard the Spin Filter D.

Do not discard the filtrate, because the filtrate contains the RNA!

Note: If the solution has not completely passed through the Spin Filter, centrifuge again at higher speed or prolong the centrifugation time.

3. Place a Spin Filter R into a new 2.0 ml Receiver Tube. Add an **equal volume** (appr. 400 μ l) of **70 % ethanol** to the filtrate from step 2. Mix the sample by pipetting sometimes up and down. Transfer sample onto the Spin Filter R. Centrifuge at $10.000 \times g$ (12.000 rpm) for 2 minutes.

Note: If the solution has not completely passed through the Spin Filter, centrifuge again at higher speed or prolong the centrifugation time.

Discard the 2.0 ml Receiver Tube with filtrate and place the Spin Filter R into a new 2.0 ml Receiver Tube.

4. Open the Spin Filter R and add **500 μ l Washing Solution HS**, close the cap and centrifuge at $10.000 \times g$ (12.000 rpm) for 1 minute. Discard the Receiver Tube with the filtrate. Place the Spin Filter R into a new 2.0 ml Receiver Tube.
5. Open the Spin Filter R and add **700 μ l Washing Solution LS**, close the cap and centrifuge at $10.000 \times g$ (12.000 rpm) for 1 minute. Discard the Receiver Tube with the filtrate. Place the Spin Filter R into a new 2.0 ml Receiver Tube.
6. Centrifuge at $10.000 \times g$ (12.000 rpm) for 3 minutes to remove all traces of ethanol. Discard the 2.0 ml Receiver Tube.
7. Place the Spin Filter R into a 1.5 ml Elution Tube. Carefully open the cap of the Spin Filter R and add **30-80 μ l RNase-free water**.

Incubate at room temperature for 1 minute. Centrifuge at 6.000 x g (8.000 rpm) for 1 minute.



Note

Depending on the extracted yield or the needed concentration of total RNA you can also elute with different volumes of RNase-free water. A lower volume of RNase-free water increases the concentration of RNA and a higher volume of RNase-free water leads to an increased yield but a lower concentration of total RNA. Please note, that the minimum of RNase-free water should be 20 µl.

13 Protocol 3: RNA extraction from bacterial cells



Important

Please note that up to 1×10^9 cells can be processed.

We recommend a preincubation of bacterial cells with Lysozyme or optionally other bacterial lysis proteins.

Stock solution of Lysozyme for gram(-) bacteria:

20 mg/ml in water; storage of Lysozyme stock solution in aliquots at $-20\text{ }^{\circ}\text{C}$.

Stock solution of Lysozyme for gram(+) bacteria:

50 mg/ml in water; storage of Lysozyme stock solution in aliquots at $-20\text{ }^{\circ}\text{C}$.

Prepare TE-Buffer: (10 mM Tris HCl / 1 mM EDTA; pH 8.0)

1. Spin down the bacterial cells by centrifugation at $5.000 \times g$ for 2–5 minutes. Discard the supernatant as complete as possible.
2. For gram(-) bacteria resuspend the cell pellet in **100 μl TE-Buffer** **and add 2 μl** of the corresponding **Lysozyme** stock solution. Pipette sometime up and down; the solution should become clear or viscous.
For gram(+) bacteria resuspend the cell pellet in **100 μl TE-Buffer** **and add 6 μl** of the corresponding **Lysozyme** stock solution. Pipette sometimes up and down; incubate until the solution becomes clear or viscous.

Note: The amount of Lysozyme and also the essential time for incubation may need to be diversified depending on bacterial strains. Read also the guideline of the Lysozyme supplier. A complete destruction of bacterial cell walls is important.

3. Add **450 μl Lysis Solution RL** to the sample and vortex vigorously or pipette sometimes up and down. Incubate the sample for further 3 minutes at room temperature.

Note: To maximize the final yield of total RNA a complete disruption and lysis of the cell pellet is important! No cell clumps should be visible after lysis step.

4. Place a Spin Filter D into a 2.0 ml Receiver Tube. Transfer the lysed sample onto the Spin Filter D. Centrifuge at $10.000 \times g$ (12.000 rpm) for 2 minutes. Discard the Spin Filter D.

Do not discard the filtrate, because the filtrate contains the RNA!

Note: If the solution has not completely passed through the Spin Filter, centrifuge again at higher speed or prolong the centrifugation time.

5. Place a Spin Filter R into a new 2.0 ml Receiver Tube. Add an **equal volume** (appr. 600 μ l) **of 70 % ethanol** to the filtrate from step 4. Mix the sample by pipetting sometimes up and down.
6. **Transfer 650 μ l of the sample** onto the Spin Filter R. Centrifuge at 10.000 x g (12.000 rpm) for 1 minute. Discard the 2.0 ml Receiver Tube and place the Spin Filter R into a new 2.0 ml Receiver Tube. **Load the residual sample** on the Spin Filter R and centrifuge again at 10.000 x g (12.000 rpm) for 1 minute.

Note: If the solution has not completely passed through the Spin Filter, centrifuge again at higher speed or prolong the centrifugation time.

Discard the 2.0 ml Receiver Tube with filtrate and place the Spin Filter R into a new 2.0 ml Receiver Tube.

7. Open the Spin Filter R and add **500 μ l Washing Solution HS**, close the cap and centrifuge at 10.000 x g (12.000 rpm) for 1 minute. Discard the Receiver Tube with the filtrate. Place the Spin Filter R into a new 2.0 ml Receiver Tube.
8. Open the Spin Filter R and add **700 μ l Washing Solution LS**, close the cap and centrifuge at 10.000 x g (12.000 rpm) for 1 minute. Discard the Receiver Tube with the filtrate. Place the Spin Filter R into a new 2.0 ml Receiver Tube.
9. Centrifuge at 10.000 x g (12.000 rpm) for 3 minutes to remove all traces of ethanol. Discard the 2.0 ml Receiver Tube.
10. Place the Spin Filter R into a 1.5 ml Elution Tube. Carefully open the cap of the Spin Filter R and add **30-80 μ l RNase-free water**. Incubate at room temperature for 1 minute. Centrifuge at 6.000 x g (8.000 rpm) for 1 minute.



Note

Depending on the extracted yield or the needed concentration of total RNA you can also elute with different volumes of RNase-free water. A lower volume of RNase-free water increases the concentration of RNA and a higher volume of RNase-free water leads to an increased yield but a lower concentration of total RNA. Please note, that the minimum of RNase-free water should be 20 μ l.

14 Troubleshooting

Problem / probable cause	Comments and suggestions
<p>Clogged Spin Filter</p> <ul style="list-style-type: none"> • Insufficient disruption or homogenization 	<p>After lysis centrifuge lysate to pellet debris and continue with the protocol using the supernatant.</p> <p>Reduce amount of starting material.</p>
<p>Little or no total RNA eluted</p> <ul style="list-style-type: none"> • Insufficient disruption or homogenization • Incomplete elution 	<p>Reduce amount of starting material. Overloading reduces yield!</p> <p>Prolong the incubation time with RNase-free water to 5 minutes or repeat elution step once again.</p>
<p>DNA contamination</p> <ul style="list-style-type: none"> • Too much starting material • Incorrect lysis of starting material 	<p>Reduce amount of starting material.</p> <p>Use the recommended techniques for lysis of cell pellet.</p> <p>Perform DNase digest of the eluate containing the total RNA or perform a on column DNase digest step after binding of the RNA on Spin Filter R!</p>
<p>Total RNA degraded</p> <ul style="list-style-type: none"> • RNA source inappropriately handled or stored • RNase contamination of solutions; Receiver Tubes, etc. 	<p>Ensure that the starting material is fresh! Ensure that the protocol, especially the first steps, has been performed quickly.</p> <p>Use sterile, RNase-free filter tips. Before every preparation clean up the pipette, the devices and the working place. Always wear gloves!</p>
<p>Total RNA does not perform well in downstream applications (e.g. RT-PCR)</p> <ul style="list-style-type: none"> • Ethanol carryover during elution • Salt carryover during elution 	<p>Increase time for removing of ethanol.</p> <p>Ensure that Washing Solution HS and Washing Solution LS are at room temperature.</p> <p>Check up Washing Solution for salt precipitates. If there are any precipitate dissolves these precipitate by carefully warming.</p>

Analytik Jena AG
bio solutions
Konrad-Zuse-Strasse 1
07745 Jena / Germany

Phone +49 (0) 36 41 / 77 94 00
Fax +49 (0) 36 41 / 77 76 77 76

biosolutions@analytik-jena.com
www.bio.analytik-jena.com