GE Healthcare

illustra
RNAspin 96 RNA Isolation Kit

Product booklet

Codes: 25-0500-74 (2 x 96 preps)
      25-0500-75 (4 x 96 preps)
      25-0500-76 (24 x 96 preps)
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1. Legal

Product use restriction.

The RNAspin 96 Kit components have been designed, developed, and sold for research purposes only. They are suitable for in vitro use only. No claim or representation is intended for its use to identify any specific organism or for clinical use (diagnostic, prognostic, therapeutic, or blood banking).

It is the responsibility of the user to verify the use of the RNAspin 96 Kits for a specific application range as the performance characteristic of this kit has not been verified to a specific organism.

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GE Healthcare UK Limited.
Amersham Place, Little Chalfont,
Buckinghamshire, HP7 9NA UK
2. Handling

2.1. Safety warnings and precautions

Warning: For research use only.
Not recommended or intended for diagnosis of disease in humans or animals. Do not use internally or externally in humans or animals.

Buffers RA1 and RA2 contain guanidine thiocyanate. Wear gloves and safety glasses.
All chemicals should be considered as potentially hazardous. We therefore recommend that this product is handled only by those persons who have been trained in laboratory techniques and that it is used in accordance with the principles of good laboratory practice. Wear suitable protective clothing such as laboratory overalls, safety glasses and gloves. Care should be taken to avoid contact with skin or eyes. In the case of contact with skin or eyes wash immediately with water. See material safety data sheet(s) and/or safety statement(s) for specific advice.

2.2. Storage
Store lyophilized **RNase-free DNase I** at +4°C on arrival (stable up to 1 year).
All other kit components should be stored at room temperature (20–25°C) and they are stable for up to one year. Storage at lower temperatures may cause precipitation of salts. If a salt precipitate is observed, incubate the bottle at 30-40°C for some minutes and mix well until all of the precipitate is redissolved.

2.3. Expiry
For expiry date please refer to outer packaging label.
3. Components

3.1. Kit contents

*Reagents highlighted with asterisk require prior preparation.

Table 3.1. RNAspin 96 kit contents

<table>
<thead>
<tr>
<th>Pack Size</th>
<th>2 x 96 preps</th>
<th>4 x 96 preps</th>
<th>24 x 96 preps</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cat. No.</td>
<td>25-0500-74</td>
<td>25-0500-75</td>
<td>25-0500-76</td>
</tr>
<tr>
<td>Buffer RA1</td>
<td>60ml</td>
<td>125ml</td>
<td>6 x 125ml</td>
</tr>
<tr>
<td>Buffer RA2</td>
<td>160ml</td>
<td>360ml</td>
<td>6 x 360ml</td>
</tr>
<tr>
<td>Buffer RA3 concentrate*</td>
<td>65ml</td>
<td>90ml</td>
<td>2 x 90ml</td>
</tr>
<tr>
<td>Buffer RA4 concentrate*</td>
<td>2 x 65ml</td>
<td>2 x 65ml</td>
<td>6 x 1265ml</td>
</tr>
<tr>
<td>DNase reaction buffer</td>
<td>7ml</td>
<td>20ml</td>
<td>6 x 20ml</td>
</tr>
<tr>
<td>DNase I*</td>
<td>2 vials</td>
<td>4 vials</td>
<td>24 vials</td>
</tr>
<tr>
<td>RNase-free water</td>
<td>65ml</td>
<td>2 x 65ml</td>
<td>6 x 1265ml</td>
</tr>
</tbody>
</table>

RNAspin RNA Binding Plate (blue) 2 4 24
1.5ml Tubes 2 8 16 96
Wash Plate (including six paper sheets) 2 4 24
Square-well Block 2 4 24
Vacuum Elution Plate, U-Bottom (including one self-adhering PE-foil) 2 4 24
Protocol 1 1 6 x 1

1 The kit for 24 x 96 preparations (Cat. No. 25-0500-76) consists of 6 x Cat. No. 25-0500-75
2 For DNase I working solution during automated use.
3 Is not used when following the centrifuge protocol in section 8.2 for the isolation of total RNA.
4 Use standard 1ml polypropylene 96-well round-well blocks, such as those from Macherey-Nagel (Cat. No. 740671, pack of 20) or Corning (Cat. No. 3959, pack of 5)
3.2. Materials to be supplied by user

- 70% and 95–100% ethanol
- β-mercaptoethanol

Standard 1ml polypropylene 96-well round-well block, such as those from Macherey-Nagel (Cat No. 740671, pack of 20) or Corning (Cat. No. 3959, pack of 5)

RNAspin 96 Filter Plate (Cat. No. 25-0500-88, recommended if processing tissue or large cell numbers)
4. Description
4.1 The basic principle

Figure 4.1 shows an overview of an RNA isolation procedure using the RNAspin 96 Kit. One of the most important aspects in the isolation process is to prevent degradation of the RNA during the
isolation procedure. With the RNAspin 96 method, cells are lysed by incubation in a solution containing large amounts of chaotropic ions. This lysis buffer immediately inactivates RNases, which are present in virtually all biological materials, and creates appropriate binding conditions that favor adsorption of RNA to the silica membrane. Contaminating DNA, which is also bound to the silica membrane, is removed by the direct application of a DNase I solution to the silica membrane (RNase-free DNase I is supplied with the kit). Simple washing steps with three different buffers remove salts, metabolites and macromolecular cellular components. Finally, pure RNA is eluted under low ionic strength conditions with RNase-free water (supplied). RNA isolation using the RNAspin 96 Kit can be performed at room temperature. However, the eluate should be treated with care because RNA is very sensitive to trace contaminations of RNases, often found on general labware, fingers and dust. To preserve stability, keep the isolated total RNA frozen at −20°C for short-term or −80°C for long-term storage.

4.2 Kit specifications

The RNAspin 96 Kit is designed for fast 96-well, small-scale manual isolation of total RNA from tissues or cells in the microtiter plate format using suitable vacuum manifolds (see section 6) or suitable centrifuges. This kit can also be used in a fully automated flow with vacuum on common laboratory workstations (contact Technical Service for more information). The RNAspin 96 Kit can be used to process up to 96 samples simultaneously in less than 70 min. Actual automated processing time depends on the configuration of the workstation used.

Typical amounts of starting material and anticipated yields are shown in the Table 4.1. Please note that the yield of total RNA depends strongly on the starting material and on complete lysis/
homogenization. Results may vary. For more information on the lysis/homogenization process see section 7.

**Table 4.1. Typical Yields from Vacuum or Centrifuge Method**

<table>
<thead>
<tr>
<th></th>
<th>Vacuum</th>
<th>Centrifuge</th>
</tr>
</thead>
<tbody>
<tr>
<td>Maximum number of cells to be used</td>
<td>$2 \times 10^6$</td>
<td>$1 \times 10^7$</td>
</tr>
<tr>
<td>Yield up to</td>
<td>20µg</td>
<td>100µg</td>
</tr>
<tr>
<td>Maximum amount of tissue to be used</td>
<td>10-30mg</td>
<td>30mg</td>
</tr>
<tr>
<td>Yield up to</td>
<td>40µg</td>
<td>100µg</td>
</tr>
</tbody>
</table>

The kit provides reagents and consumables for the purification of up to 100µg of highly pure total RNA suitable for direct use in downstream applications like Quantitative Reverse Transcriptase-PCR (QRT-PCR), Primer Extension, RNase Protection Assays, cDNA Synthesis and Microarray Analysis. RNAspin 96 can be used under vacuum or in a centrifuge. The centrifugation method produces slightly higher yields because of the larger amount of starting material that can be used and the reduced dead volume of the membrane (Figure 4.2).

The final concentration of eluted RNA is 50–500 ng/µl, depending on the elution buffer volume and starting material (Figure 4.2 and Table 4.2). Elution is possible under vacuum and in a centrifuge without cross-contamination. To achieve this, vacuum settings during the elution have to be adjusted carefully (smooth elution) so no splattering of liquid occurs (Figure 4.3). Typically, the $A_{260}/A_{280}$ ratio is 1.9–2.1. The residual content of genomic DNA is less than 0.003% after isolation from more than 5 x $10^5$ cells, as determined by quantitative PCR (Figure 4.4).
Fig 4.2. The RNAspin 96 kits produce high quality RNA: rRNA bands are sharp, with the 28S band being approximately double the intensity of the 18S band, as well as having high RNA Integrity Number (RIN) values. (A) Total RNA was purified from 10mg of liver tissue using RNAspin 96. 100µl of RNase-free water was dispensed onto the silica membrane for elution. 100µl of RNA eluate was recovered by centrifugation, or 80µl of RNA eluate was recovered using vacuum processing. 20/100µl or 20/80µl of each eluate was analyzed on a 1% formaldehyde agarose gel; (B) Total RNA from rat liver was isolated with RNAspin 96 centrifugation protocol, and 1µl of 100µl eluate from six independent samples was evaluated using the Agilent 2100 bioanalyzer.
Table 4.2. Correlation between dispensed elution buffer volume and typical recoveries following a standard protocol

<table>
<thead>
<tr>
<th>Dispersed elution buffer</th>
<th>50μl</th>
<th>70μl</th>
<th>90μl</th>
<th>110μl</th>
<th>130μl</th>
</tr>
</thead>
<tbody>
<tr>
<td>Recovered elution buffer</td>
<td>Vacuum</td>
<td>30±5μl</td>
<td>50±5μl</td>
<td>70±5μl</td>
<td>90±5μl</td>
</tr>
<tr>
<td></td>
<td>Centrifuge</td>
<td>45±5μl</td>
<td>65±5μl</td>
<td>85±5μl</td>
<td>105±5μl</td>
</tr>
</tbody>
</table>

+ : samples, total RNA prepared used RNAspin 96 from 5 x 10⁵ HeLa cells each.
– : control samples with water instead of cells (next to a well containing cells).
Ctrl: positive control
A: 200-bp PCR product of the GAPDH gene
B: primer

Fig. 4.3. The RT-PCR detection of total RNA shown by downstream agarose gel electrophoresis. HeLa cells (5 x 10⁵) were pelleted in a 96-well cell culture plate. Total RNA was isolated using RNAspin 96. A total of 30 samples were loaded in a checkerboard pattern onto the RNAspin RNA Binding Plate. Samples with and without RNA were used in LightCycler® analysis. The LightCycler assays (20µl each) were loaded on a 2% agarose gel shown here.
Fig. 4.4. PCR detection of genomic DNA (362-bp fragment of GAPDH). 2 µl of each eluate (80 µl elution volume, total RNA preparation from 5 x 10⁶ HeLa cells) was amplified with “LightCycler—DNA Amplification Kit Hybridization Probes” (Roche) (0.5 µmol GAPDH primer, 0.15 µM LC-Red 640 hybridization probes, 50 cycles).

Table 4.3. RNASpin 96 Technical specifications at a glance

<table>
<thead>
<tr>
<th>Animal Tissue</th>
<th>Cell Culture</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>Vacuum</strong></td>
<td><strong>Centrifuge</strong></td>
</tr>
<tr>
<td>Sample size</td>
<td>10-30 mg tissue</td>
</tr>
<tr>
<td>Typical yield</td>
<td>up to 40 µg</td>
</tr>
<tr>
<td>Elution volume</td>
<td>50-130 µl</td>
</tr>
<tr>
<td>Effective binding</td>
<td>100 µg</td>
</tr>
<tr>
<td>RNA integrity</td>
<td>sharp rRNA bands with no substantial degradative bands visible</td>
</tr>
<tr>
<td></td>
<td>28S:18S = 2.1</td>
</tr>
<tr>
<td>RNA Integrity Number (RIN) values ≥ 7</td>
<td></td>
</tr>
<tr>
<td>RNA purity</td>
<td>A₂₆₀/A₂₈₀ = 1.8 - 2.2</td>
</tr>
<tr>
<td>Time/Prep</td>
<td>70 min/96 preps</td>
</tr>
</tbody>
</table>
5. Preparation of working solutions

5.1 RNase-free DNase I

Avoid vigorous mixing of the DNase I enzyme because it is sensitive to mechanical agitation.

Add 400µl of RNase-free water to the DNase I vial and incubate for 1 min at room temperature. Gently swirl the vial to completely dissolve the DNase I. Dilute dissolved DNase with 2.8ml DNase Reaction Buffer.

To process less than a whole 96-well plate, thaw the frozen working solution and dispense into aliquots and store at –20°C. It is stable for six months. Do not freeze/thaw the aliquots more than three times. MnCl$_2$ in the DNase reaction buffer may cause a brownish precipitate upon storage. Unless it becomes dark brown, this will not affect the efficiency of the DNase reaction.

5.2 Buffer RA3 and Buffer RA4

Add the indicated volume of 96-100% ethanol to the RA3 and RA4 concentrates. Store both Buffer RA3 and RA4 at room temperature (20-25°C) for up to one year.

<table>
<thead>
<tr>
<th>Table 5.1. Reagent preparation</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cat. No.</td>
</tr>
<tr>
<td>----------</td>
</tr>
<tr>
<td>Buffer RA3 concentrate</td>
</tr>
<tr>
<td>ethanol</td>
</tr>
<tr>
<td>ethanol to each bottle</td>
</tr>
<tr>
<td>Buffer RA4 concentrate</td>
</tr>
<tr>
<td>ethanol</td>
</tr>
<tr>
<td>ethanol to each bottle</td>
</tr>
</tbody>
</table>

$^1$The kit for 24 x 96 preparations (25-0500-76) consists of 6 x 25-0500-75
6. Preparation of vacuum source

6.1 Vacuum hardware

The RNAspin 96 kit can be used with common vacuum manifolds. For manual processing under vacuum, the Macherey-Nagel NucleoVac® 96 or Qiagen QIAvac vacuum manifold is suggested. For positioning of the kit Wash Plate with certain manifolds, e.g. QIAvac, an adaptor such as Macherey-Nagel Frame (Cat. No. 740680) is required. To process less than 96 samples with the RNAspin 96 kit, use a rubber pad or self-adhering PE Foil to cover up any non-used wells of the RNAspin RNA Binding Plate to guarantee a proper vacuum.

6.2 Vacuum setup

Establish a reliable vacuum source for the vacuum manifold. The manifold may be used with a vacuum pump, house vacuum, or water aspirator. We recommend a vacuum of 200–400mbar (pressure difference). The use of a vacuum regulator is recommended. Alternatively, adjust vacuum so that during purification, the sample flows through the column with a rate of 1–2 drops per second. Depending on the amount of sample used, the vacuum times might have to be increased for complete filtration.
7. Handling, preparation, and storage of starting materials

RNA is not protected against digestion until the sample material is flash frozen or disrupted in the presence of RNase inhibiting or denaturing agents. Therefore, it is important that the samples are flash frozen in liquid N\textsubscript{2} immediately, and stored at -70°C, stored in a stabilizing agent, or processed as soon as possible. Samples can be stored in lysis buffer RA1 after disruption at -70°C for up to one year, at +4°C for up to 24 h or up to several hours at room temperature. Frozen samples are stable for up to 6 months. Frozen samples in buffer RA1 should be thawed slowly before starting with the isolation of total RNA.

Wear gloves at all times during the preparation. Change gloves frequently.

For optimal homogenization and removal of particles when using larger cell numbers (>2 x 10\textsuperscript{6} cells) or amounts of tissue, filtration of the RA1 lysate is recommended. The RNAspin RNA Filter Plate (not included in the kit; Cat. No. 25-0500-88) should be used for this purpose.

7.1 Cell culture

Recall that standard 96-well plates will not accommodate a total volume >280µl.

Cell cultures up to 2 x 10\textsuperscript{6} cells can be processed using the vacuum protocol. Using a centrifuge, up to 1 x 10\textsuperscript{7} cells can be processed. Transfer the cell suspension to a microtiter plate (<280µl) or square-well block (>280µl), and centrifuge for 5 min at 500 x g.

The supernatant has to be removed completely. For adherent cell cultures in a 96-well format, make sure that the culture medium is completely removed. Take the cell pellet into step 1 of the standard protocol.
To process greater than $10^6$ cells, it is recommended that you use a commercial homogenizer after lysis with buffer RA1 in order to reduce the viscosity (see section 7.2 for homogenizer information). For larger cell numbers (>2 x $10^6$), increasing the volume of RA1 may also aid in reducing sample viscosity. In such cases, it will be necessary to use a square-well block to allow for the volume increase.

It is also recommended that you use the \textit{RNAspin Filter Plate} to prevent the \textit{RNAspin RNA Binding Plate} from becoming clogged. Filter the lysates through the \textit{RNAspin Filter Plate} before applying them to the \textit{RNAspin RNA Binding Plate} for optimal homogenization and to hold back cell debris (see section 8).

\subsection*{7.2 Tissue}

When processing tissue or nuclease-rich cells, ensure the addition of 1% β-mercaptoethanol to buffer RA1. β-mercaptoethanol supports the inhibition of RNases.

Depending on the type of sample, up to 30mg (see also Table 4.1) can be processed. In case the lysate is too viscous, add 300µl buffer RA1 and a corresponding amount of buffer RA4. For higher throughput in the 96-well format, add lysis buffer RA1 to frozen or stabilized tissue collected in a round or square-well block or tube strips and immediately disrupt tissue with an appropriate homogenizer. Several commercial homogenizers are available for 96-well homogenization: CrushExpress™ (Saaten-Union Resistenzlabor GmbH, Germany, www.saaten-union.de) or Geno/Grinder™ 2000 (SPEX CertiPrep® USA, www.spexcsp.com). Alternatively, samples may be homogenized individually by mortar and pestle grinding with liquid N\textsubscript{2} or rotor-stator homogenization. Once lysed, the tissue lysate can be passed through the \textit{RNAspin Filter Plate} for optimal homogenization and to hold back cell debris.
to prevent subsequent blockage of the RNAspin RNA Binding Plate. Rest the RNAspin Filter Plate on a square-well block and apply the samples to the filter. Centrifuge at 5600–6000 x g until all the samples have passed through the filter. Start the RNA purification procedure with the filtrate collected in the square-well block and adding buffer RA4. Alternatively, centrifuge samples for 5 min at maximum g-forces, transfer the supernatant to a microtiter or square-well plate and proceed with the standard protocol adding buffer RA4. Filtration through the RNAspin Filter Plate can also be performed under vacuum. Transfer the samples to the filter plate and apply vacuum until all the samples have passed through the filter. Start the RNA purification procedure with the filtrate collected in the square-well block. Please note that the dead volume of the RNAspin Filter Plate will be greater than that for processing under centrifugation, thus processing under vacuum is only recommended when complete automation is desired. Proceed with adding buffer RA4 to the flow-through.
8. Protocols

8.1. RNAspin 96—Standard protocol for the manual purification of total RNA under vacuum

1. Lyse cells or tissue

Cell cultures:
Add 130µl RA1/1% β-mercaptoethanol [vol/vol] to the cells in each well of a multiwell cell-culture plate. Cells can be lysed by pipetting up and down repeatedly or by vortexing for 5 min.

If an increased volume of buffer RA1 is used during this step (see section 7.2), make sure that you use a square-well block to accommodate the larger volume. Also ensure that the RA4 buffer volume is increased proportionately in step 3 [Prepare binding].

Use of β-mercaptoethanol is recommended, but not essential for most cell types.

Tissue:
Add 300µl RA1/1% β-mercaptoethanol [vol/vol] to the sample. Refer to section 7.2 for tissue homogenization.
We recommended that you use the RNAspin RNA Filter Plate for the clarification of tissue lysates.

2. Prepare binding
If an increased volume of buffer RA1 was used during the lysis step make sure you increase the volume of RA4 accordingly. RA1 and RA4 have to be used in the same volume ratio.
Add 130µl or 300µl RA4, depending on the RA1 volume, to each well of the cell-culture plate. Mix by pipetting up and down at least 10-15 times.

3. Prepare the vacuum manifold

4. Transfer crude lysates to RNAspin RNA Binding Plate
Place an RNAspin RNA Binding Plate into the vacuum manifold’s lid and apply samples to the wells.

5. Bind RNA to silica membrane
Apply vacuum until all lysates have passed through the columns (~200 mbar; 1 min). Release the vacuum.
6. Desalt silica membrane
Desalt the membrane by adding 500µl RA3 to each well and applying vacuum (-200 mbar; 3 min) until all the buffer has passed through the columns. Release the vacuum.

7. DNase I incubation
Pipette 30 µl DNase I reaction mixture directly to the bottom of each well of the RNAspin RNA Binding Plate.

Do not touch the silica membrane with the pipette tips.
Incubate at room temperature for 15 min.

Be sure that all of the DNase reaction mixture gets into contact with the silica membrane, and that the membrane is completely wetted.

8. Wash silica membrane
Add 500µl RA2 to each well of the RNAspin RNA Binding Plate. Apply vacuum (-200 mbar; 1 min) until all the buffer has passed through the columns. Release the vacuum.
Add 800µl RA3 to each well of the RNAspin RNA Binding Plate. Apply vacuum (-200 mbar; 1 min) until all the buffer has passed through the columns. Release the vacuum.

Add 500µl RAA to each well of the RNAspin RNA Binding Plate. Apply vacuum (-200 mbar; 1 min) until all the buffer has passed through the columns. Release the vacuum.

9. Remove Wash Plate

After the final washing step, close the valve, release the vacuum and remove the RNAspin RNA Binding Plate. Rest it on a clean paper towel to remove residual EtOH-containing wash buffer. Remove manifold lid, Wash Plate, and waste container from the vacuum manifold.

10. Dry RNAspin RNA Binding Plate

Remove any residual washing buffer from the RNAspin RNA Binding Plate. If necessary, tap the outlets of the RNAspin
RNA Binding Plate onto a clean paper sheet (supplied with the Wash Plate) or soft tissue until no drops come out. Insert the RNAspin RNA Binding Plate into the lid and close the manifold. Build the vacuum up with the valve closed. Once the maximum vacuum (~600 mbar) is achieved, open the valve and apply vacuum for at least 10 min to dry the membrane completely. This step is necessary to eliminate traces of ethanol. The ethanol in buffer RA4 inhibits downstream enzymatic reactions and has to be completely removed before DNA elution.

Finally, close the valve and release the vacuum.

11. Elute highly pure total RNA

Place the Elution Plate (U-Bottom) onto the vacuum manifold. Pipette 50–130µl RNase-free water directly to the bottom of each well. Incubate for 2 min at room temperature. Build the vacuum up with the valve closed. Once the
maximum vacuum (-500 mbar) is achieved, open the valve and apply vacuum for 1 min.
Alternatively, elution into tube strips or standard PCR plates is possible. For elution into tube strips, place the tube strip inside the manifold. Elution into PCR plates can be performed by placing a PCR plate onto a square-well block (not provided) resting in the manifold.

8.2. RNAspin 96—Standard protocol for the manual purification of total RNA using a centrifuge

1. Lyse cells or tissue
Cell cultures:
Add 130µl RA1/1% β-mercaptoethanol (vol/vol) to the cells in each well of a multiwell cell-culture plate. Cells can be lysed by pipetting up and down repeatedly or by vortexing for 5 min.

If an increased volume of buffer RA1 is used during this step, make sure that you use a square-well block to accommodate the larger
volume. Also ensure that the RA4 buffer volume is increased proportionately in step 3 (Prepare binding).

Use of β-mercaptoethanol is recommended, but not essential for most cell types.

Tissue:
Add 300µl RA1/1% β-mercaptoethanol [vol/vol] to the sample. For homogenization of tissue samples please refer to section 7.2.

We recommend the use of the RNASpin RNA Filter Plate for the clarification of tissue lysates.

2. Prepare binding

If an increased volume of buffer RA1 was used during the lysis step make sure you increase the volume of RA4 accordingly. RA1 and RA4 have to be used in the same volume ratio.

Add 130µl or 300µl RA4, depending on the RA1 volume, to each well of the cell-culture plate. Mix by pipetting up and down at least 10-15 times.
3. Transfer crude lysates
Place the RNAspin RNA Binding Plate onto an RNAspin Square-well Block. Pipette up and down once then transfer the lysates to the wells of the RNAspin RNA Binding Plate.

4. Bind RNA to silica membrane
Centrifuge for 2 min at 5600-6000 x g.

5. Desalt silica membrane
Desalt the membrane by adding 500µl RA3 to each well and centrifuge for 2 min at 5600-6000 x g. Discard the flow-through.
6. DNase I incubation

Place the RNAspin RNA Binding Plate back onto the Square-well Block. Pipette 30µl DNase reaction mixture directly to the bottom of each well of the RNAspin RNA Binding Plate. Do not touch the silica membrane with the pipette tips. Incubate at room temperature for 15 min.

Be sure that all of the DNase reaction mixture gets into contact with the silica membrane, and that the membrane is completely wetted.

7. Wash silica membrane

Add 500µl RA2 to each well and place the RNAspin RNA Binding Plate with the Square-Well Block into the swinging-bucket rotor and centrifuge for 2 min at 5600–6000 x g.

Add 800µl RA3 to each well of the RNAspin RNA Binding plate and centrifuge for 2 min at 5600–6000 x g. Discard the flow-through.
Add 500µl RA4 to each well of the RNAspin RNA Binding plate and centrifuge for 10 min at 5600–6000 x g.

The ethanol in buffer RA4 inhibits downstream enzymatic reactions and has to be completely removed before DNA elution.

8. Elute highly pure total RNA

Elution directly into a square-well block or round-well block (not provided) is possible. Rest the RNAspin RNA Binding Plate on top of a square- or round-well block.

If you want to elute into a PCR plate, place the PCR plate between the RNAspin RNA Binding Plate and a square-well block (not provided).

Pipette 50-130µl RNase-free water directly to the bottom of each well of the Binding Plate. Make sure that all of the water gets in contact with the silica membrane and that the membrane is completely
wetted. Incubate for 2 min at room temperature and centrifuge at 5600–6000 x g for 3 min.

8.3. Standard protocol for automated purification of total RNA using common laboratory automation workstations

1. Prepare robotic workstation
Place the plastic equipment like plates and the assembled vacuum manifold at the locations as specified in the individual robotic programs.

2. Prepare buffers
Add sufficient buffer to the reservoirs or place the buffer bottles at the corresponding positions on the robot worktable. Calculate the needed buffer volumes plus 10% overage and fill the reservoirs appropriately. Buffers are delivered in sufficient, but limited amounts and should not be wasted. Do not return unused buffer into the bottle.

3. Harvest cells
Culture cells:
Aliquots of up to 2 x 10⁶ cells can be transferred into the wells of a 96-well microtiter plate. Pellet cells by centrifugation (5 min, 500 x g), remove the supernatant by pipetting and start the preparation.

Tissue samples:
For harvesting and homogenization of tissue samples please refer to section 7.2.
4. **Incorporate samples with automated workflow**
Place microtiter plate with samples at the appropriate position of the robotic workstation.

5. **Run automation program**
Select method for total RNA purification and start the run. Seal unused wells with self-adhering PE Foil. Use disposable filter tips for the transfer of sample to the Rnaspin RNA Binding Plate. All other steps can be processed with needles. Adjust vacuum times and strength, if necessary. Make sure that the solution of DNase I is pipetted into the middle of the well.

6. **Elution of purified total RNA**
For increased RNA concentration, dispense at least 50µl of RNase-free water to the membrane. Lower volumes of elution buffer may cause variable results. By using higher volumes of dispensed water, the concentration of eluted RNA will decrease, but the efficiency of elution will increase. Alternatively, the elution can be performed in a centrifuge to reduce the volume of water needed for elution thus increasing the concentration of the RNA.

Stop the protocol after the final washing step with RA4. Remove the Rnaspin RNA Binding Plate and tap it on a sheet of filter paper to remove residual wash buffer. Place the Rnaspin RNA Binding Plate on top of a rack with tube strips. Centrifuge at maximum speed for 10 min (5600–6000 x g).

8.4. **Support protocol for clean-up of total RNA**

1. **Clean-up**
Per 50µl (1 volume) sample volume add 160µl (3.2 times the sample volume) RA1 and 110µl (2.2 times the sample volume) ethanol (96-100%).
It is possible to scale up the volumes. The total volume of RA1 supplied in the kit is sufficient for a maximum of 300µl RA1 per well.

2. Adjust RNA binding conditions

Mix by pipetting up and down at least 15 times and transfer samples to the wells of the RNASpin RNA Binding plate.

Proceed with step 4 of the standard procedure (Bind RNA to silica membrane).

Note: DNase treatment might not be necessary, depending on starting material and downstream application.
9. Appendix
9.1. Troubleshooting guide

<table>
<thead>
<tr>
<th>Problem: RNA is degraded/no RNA obtained</th>
<th>Possible cause</th>
<th>Suggestions</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>RNase contamination</em></td>
<td></td>
<td>• Create an RNase-free working environment. Wear gloves during all steps of the procedure, and change gloves frequently. Use of sterile, disposable polypropylene tubes is recommended. Keep tubes closed whenever possible during preparation. Glassware should be oven-baked for at least 2h at 250°C before use. • Do not return unused buffer from the trough reservoir into the stock bottle. • Use sterile tips with filter</td>
</tr>
<tr>
<td><em>Sample material</em></td>
<td></td>
<td>• Sample material not fresh. Whenever possible, use fresh material</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Problem: Poor RNA quality or yield</th>
<th>Possible cause</th>
<th>Suggestions</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Reagents not applied or prepared properly</em></td>
<td></td>
<td>• Reagents not properly prepared. Add the indicated volume of RNase-free water to the DNase I vial and 96–100% ethanol to buffer concentrates RA3 and RA4 and mix.</td>
</tr>
<tr>
<td><em>Kit storage</em></td>
<td></td>
<td>• Store aliquots of the reconstituted DNase I at −20°C.</td>
</tr>
<tr>
<td>Possible cause</td>
<td>Suggestions</td>
<td></td>
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<td>-------------------------</td>
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</tbody>
</table>
| Kit storage cont.       | • Store other kit components at room temperature. Storage at low temperatures may cause salt precipitation.  
• Keep bottles tightly closed in order to prevent evaporation or contamination. |
| Suboptimal elution      | • Be sure that all of the water gets into contact with the silica membrane. No water drops should stick to the walls of the columns. The membrane has to be wetsed completely. |
| Sample material         | • Sample material not stored properly. Whenever possible, use fresh material. If this is not possible, flash freeze the samples in liquid N₂ or treat with a stabilizing agent. Samples should always be kept at −80°C. Never allow tissues to thaw before addition of buffer RA1. Perform disruption of samples in liquid N₂, if possible.  
• Insufficient disruption and/or homogenization of starting material. Ensure thorough sample disruption and use RNAspin 96 Filter Plate for easy clean-up of disrupted starting material.  
• To process more than 10⁶ cells, use a |
### Possible cause  |  Suggestions
---|---
Sample material cont. | shaker or a commercial homogenizer for optimal homogenization of the starting material.
- Too much starting material may lead to RNA Binding Plate clogging or reduced RNA quality or yield. For clogging issues, see below. RNA quality and yield problems relating to too much sample material may be addressed by decreasing the amount of starting material and/or increasing the volumes of wash buffers RA2, RA3, and RA4, up to a maximum of 800µl.

### Clogged RNAspin Binding Plate

| Possible cause | Suggestions |
---|---
Sample material | • Use the RNAspin Filter Plate to reduce the risk of clogging the Binding Plate  
- To prevent clogging due to too much the sample amount, reduce the sample amount, increase the time for vacuum processing or centrifugation steps, and/or increase the volume of buffers RA1 and RA4.  
- If clogging still occurs during the run, take the remaining lysate off the RNAspin RNA Binding Plate, discard it, and proceed with the desalting step (with buffer RA3).
### Contamination of RNA with genomic DNA

<table>
<thead>
<tr>
<th>Possible cause</th>
<th>Suggestions</th>
</tr>
</thead>
<tbody>
<tr>
<td>DNase I not active</td>
<td>• Reconstitute and store lyophilized DNase I according to instructions given in section 5.</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Possible cause</th>
<th>Suggestions</th>
</tr>
</thead>
<tbody>
<tr>
<td>DNase solution not properly applied</td>
<td>• Pipette DNase I solution directly onto the center of the silica membrane.</td>
</tr>
<tr>
<td>Too much cell material used</td>
<td>• Reduce quantity of cells or tissue used.</td>
</tr>
<tr>
<td></td>
<td>• Increase mixing cycles after adding binding buffer RA4</td>
</tr>
</tbody>
</table>

### Suboptimal performance of RNA in downstream applications

<table>
<thead>
<tr>
<th>Possible cause</th>
<th>Suggestions</th>
</tr>
</thead>
<tbody>
<tr>
<td>Carry-over of ethanol or salt</td>
<td>• Be sure to remove all of ethanol buffer RA4 after the final washing step. Dry the RNAspin RNA Binding Plate for at least 10 min with maximum vacuum.</td>
</tr>
<tr>
<td></td>
<td>• Check if buffer RA3 has been equilibrated to room temperature before use. Washing at lower temperatures lowers the efficiency of salt removal by RA3.</td>
</tr>
<tr>
<td>Store isolated RNA properly</td>
<td>• Eluted RNA should always be kept on ice for optimal stability since trace contaminations of omnipresent RNases will degrade the isolated RNA. For short-term storage, freeze at –20°C, for long-term storage, freeze at –80°C.</td>
</tr>
<tr>
<td>Possible cause</td>
<td>Suggestions</td>
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<td>-------------------------------</td>
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</tr>
<tr>
<td>Vacuum pressure is not sufficient</td>
<td>• Check if the vacuum manifold lid fits tightly on the manifold base when vacuum is turned on.</td>
</tr>
</tbody>
</table>
| Buffer volumes are not enough | • Buffers are provided in sufficient, but limited amounts. Calculate the needed buffer volumes with a 10% overage amount and pour these into the reservoirs.  
• Do not return unused buffer from reservoir to the stock bottle. |
| Splashing                     | • Reduce the vacuum strength during the elution step. Alternatively, a round-well block can be used for collecting the eluate, if a higher vacuum strength is required during the elution. |
| Transfer of sample solution to the Binding Plate | • Make sure that no liquid drops from the tips while moving the tips above the binding plate. |
Total RNA purification from cultured cells and tissue with illustra
RNAspinn 96 Kit using centrifugation
Protocol Card

1. **Lyse cells or tissue**
   - Clarification of the lysate with an RNAspin Filter Plate is recommended in the case of tissue samples or a large number of cells.
   - 130µl RA1, mix
   - 300µl RA1, mix

2. **Prepare binding**
   - 130µl RA4, mix
   - 300µl RA4, mix

3. **Transfer crude lysates to the RNAspin RNA Binding Plate**

4. **Bind RNA to silica membrane**
   - 5600–6000 x g, 2 min
5. **Desalt silica membrane**

- 500µl RA3
- 5600–6000 x g, 2 min

6. **DNase I incubation**

- Be sure to pipette solution directly onto membrane without touching the tip to membrane
- 30µl DNase I solution
- RT 15 min

7. **Wash silica membrane**

- 500µl RA2
- 5600–6000 x g, 2 min
- 800µl RA3
- 5600–6000 x g, 2 min
- 500µl RA4
- 5600–6000 x g, 10 min

8. **Elute highly pure total RNA**

- 50–130µl RNase-free water
- RT 2 min
- 5600–6000 x g, 3 min
Total RNA purification from cultured cells and tissue with illustra RNAspin 96 Kit using a vacuum manifold Protocol Card.

Harvest cells
500 x g, 5 min

2. lyse cells or tissue
Clarification of the lysate with an RNAspin Filter Plate is recommended in the case of tissue samples or a large number of cells

3. Prepare binding

130μl RA1, mix
300μl RA1, mix

OR

130μl RA4, mix
300μl RA4, mix

3. Transfer crude lysates to the RNAspin RNA Binding Plate (blue)

4. Bind RNA to silica membrane of the RNAspin RNA Binding Plate
apply vacuum ca. -0.2 bar 1 min
5. Desalt silica membrane by washing

6. DNase incubation
   - Make sure to pipette solution directly onto membrane without touching the tip to membrane

7. Wash silica membrane

8. Remove wash plate

9. Dry RNAspin RNA Binding Plate

10. Elute highly pure total RNA

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