illustra
RNAspin 96 RNA Isolation Kit

Product booklet

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1 Introduction

Product code

25050075 (4 × 96 purifications)

About

For the rapid extraction and purification of RNA from various samples.

Important

Read these instructions carefully before using the products.

Intended use

The products are intended for research use only, and shall not be used in any clinical or *in vitro* procedures for diagnostic purposes.

Safety

All chemicals should be considered as potentially hazardous. For use and handling of the products in a safe way, refer to the Safety Data Sheets.

CAUTION
The chaotrope in the Lysis Solution is harmful if ingested, inhaled or absorbed through the skin and can cause nervous system disturbances, severe irritation and burning. High concentrations are extremely destructive to the eyes, skin and mucous membranes of the upper respiratory tract. Gloves should always be worn when handling this solution.

Waste effluents from this kit should be decontaminated with bleach or detergent-based method. Decontamination with bleach may be reactive resulting in foam and emission of ammonia gas and should be carried out in an exhaust hood. Consult local safety regulations for the safe disposal of all waste.

Storage

Lyophilized RNase-free DNase I should be stored at +4°C upon arrival.

All other kit components should be stored at room temperature (20–25°C).

Expiry

For expiry date please refer to outer packaging label.

2 Components

Kit contents

Identification	Pack Size Product code	4 × 96 preps 25050075
Red	Lysis Solution (Red sticker)	125 mL
Yellow	Wash Buffer I (Yellow sticker)	360 mL
Grey	Wash Buffer II (Grey sticker)	2 × 90 mL. Add 360 mL ethanol to each bottle
Black	Wash Buffer III (Black sticker)	2 × 65 mL. Add 150 mL ethanol to each bottle
Green	DNase Reaction Buffer (Green sticker)	20 mL
Orange	DNase I, (lyophilized, RNase-free) (Orange sticker)	4 vials. Add 400 µL RNase-free H_2O
White	RNase-free H_2O (White sticker)	2 × 65 mL
	RNAspin RNA Binding Plate (blue)	4
	1.5mL Tubes[1] (RNase-free)	16
	Wash Plate[2] (including six paper sheets)	4

Identification	Pack Size	4 × 96 preps
	Product code	25050075
	Square-well Block	4
	Vacuum Elution Plate (U-Bottom) (including one self-adhering PE-foil)	4

[1] For DNase I working solution during automated use.

[2] Is not used when following the centrifuge protocol in *Standard protocol for manual purification of total RNA using a centrifuge, on page 24* for the isolation of total RNA.

Materials to be supplied by user

70% and 95–100% ethanol

β-Mercaptoethanol (β-ME)

Equipment to be supplied by user

Standard 1 mL polypropylene 96-well round-well block, such as Corning™ (Product code 3959) or 2 mL polypropylene 96-well round-well block such as Cytiva (Product code 77015200).

NucleoSpin RNA Filter Plate (Macherey-Nagel Product code 740711; recommended if processing tissue or large number of cells).

3 Description

Background

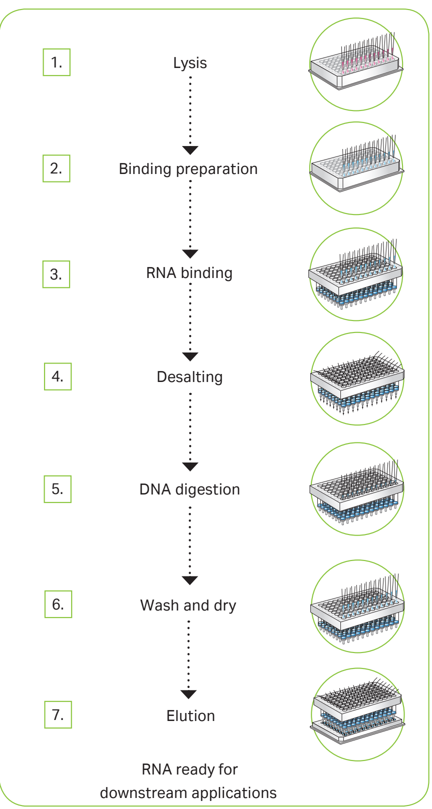
The illustra™ RNAspin 96 RNA Isolation Kit is designed for fast 96-well, small-scale isolation of total RNA from tissues or cells in the microtiter plate format using suitable vacuum manifolds (See Chapter Appendices) or suitable centrifuges. This kit can also be used in a fully automated flow with vacuum on common laboratory workstations (contact Technical Service for more information). The illustra RNAspin 96 RNA Isolation 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.

One of the most important aspects in the isolation process is to prevent the degradation of the RNA during the isolation procedure. With the illustra RNAspin 96 RNA Isolation Kit, cells are lysed by incubation in a solution containing large amounts of chaotropes. This Lysis solution immediately inactivates RNases, which are present in virtually all biological materials. The binding conditions are adjusted to 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 DNase I solution to the silica. Simple washing steps with two different buffers remove salts, metabolites and macromolecular cellular components. Finally, pure RNA is eluted under low ionic strength conditions with RNase-free H_2O.

RNA isolation using the illustra RNAspin 96 RNA Isolation 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. The kit contains sufficient reagents and columns for 4 × 96 (25050075) purifications.

The basic principle

Use of the illustra RNAspin Mini RNA Isolation Kit involves the following steps:



Step procedure

Step	Comments	Component
1. Lysis	Procedure depends on sample type	Lysis Solution (Red)
2. Binding preparation	Create appropriate binding conditions that favor adsorption of RNA to the silica membrane	Wash Buffer III (Black)
3. RNA Binding	Bind RNA to the silica membrane	RNAspin RNA Binding Plate
4. Desalting	Reduce the salt concentration and prepare for DNA digestion	Desalting Buffer (Blue)
5. DNA digestion	Digest the DNA on the column	DNase I (Orange)
6. Wash and dry	A washing step to remove contaminants from the membrane-bound RNA. These centrifugation steps use ethanol -based buffers	Wash Buffer I & II & III (Yellow, Grey, Black)
7. Elution	Elute high quality RNA in RNase-free water	RNase-free H_2O (White)

Product specifications

	Tissue		Cell culture	
	Vacuum	**Centrifuge**	**Vacuum**	**Centrifuge**
Sample size	10–30 mg tissue	30 mg tissue	2×10^6 cells	1×10^7 cells
Typical yield[1]	Up to 40 µg	Up to 100 µg	Up to 20 µg	Up to 100 µg
Elution volume	50 - 130 µL			
Effective binding capacity	100 µL			
RNA integrity	sharp rRNA bands with no substantial degradative bands visible 28S:18S = ~2:1 RNA Integrity Number (RIN) values ≥ 7			
RNA purity	A_{260}/A_{280} = 1.8–2.2			
Time/Prep[2]	70 min/96 preps			

[1] Actual yields will vary depending on sample and the growth phase.

[2] Actual time/prep will vary depending on user's experience with the protocol.

Typical output

The illustra RNAspin 96 RNA Isolation Kit provides reagents and consumables for the purification of up to 100 µg of highly pure total RNA suitable for direct use in downstream applications like Quantitative Reverse Transcriptase-PCR (RT-qPCR), Primer Extension, RNase Protection Assays, cDNA Synthesis and Microarray Analysis. The illustra RNAspin 96 RNA Isolation Kit 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 (*Fig. 1, on page 13*).

The final concentration of eluted RNA is 50–500 ng/µL, depending on the elution buffer volume and starting material (*Fig. 1, on page 13* and *Table 1, on page 14*). 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 (*Fig. 2, on page 14*). 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 5×10^5 cells, as determined by quantitative PCR (*Fig. 3, on page 15*).

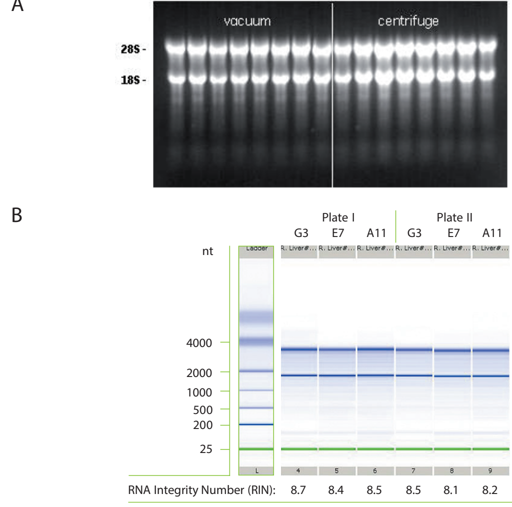
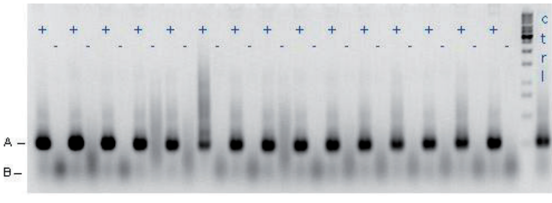


Fig 1. The illustra RNAspin 96 RNA Isolation Kit produces high quality RNA. rRNA bands are sharp, with the 28S band being approximately double the intensity of the 18S band, as well as having high RNA Integrity Number (RIN) values. (A) Total RNA was purified from 10 mg of liver tissue using illustra RNAspin 96 RNA Isolation Kit. 100 µL of RNase-free H_2O was dispensed onto the silica membrane for elution. 100 µL of RNA eluate was recovered by centrifugation, or 80 µL of RNA eluate was recovered using vacuum processing. 20/100 µL or 20/80 µL of each eluate was analyzed on a 1% formaldehyde agarose gel; (B) Total RNA from Rat liver was isolated with illustra RNAspin 96 RNA Isolation Kit centrifugation protocol, and 1 µL of 100 µL eluate from six independent samples was evaluated using the Agilent 2100 Bioanalyzer.

Table 1. Correlation between dispensed elution buffer volume and typical recoveries following a standard protocol.

	Dispensed elution buffer	50 μL	70 μL	90 μL	110 μL	130 μL
Recovered elution buffer	Vacuum	30 ± 5 μL	50 ± 5 μL	70 ± 5 μL	90 ± 5 μL	110 ± 5 μL
	Centrifuge	45 ± 5 μL	65 ± 5 μL	85 ± 5 μL	105 ± 5 μL	125 ± 5 μL



\+ : samples, total RNA prepared used the illustra RNAspin 96 RNA Isolation Kit from 5×10^5 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 2. The RT-PCR detection of total RNA shown by downstream agarose gel electrophoresis. HeLa cells (5×10^5) were pelleted in a 96-well cell culture plate. Total RNA was isolated using the illustra RNAspin 96 RNA Isolation Kit. 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 (20 μl each) were loaded on a 2% agarose gel shown here.

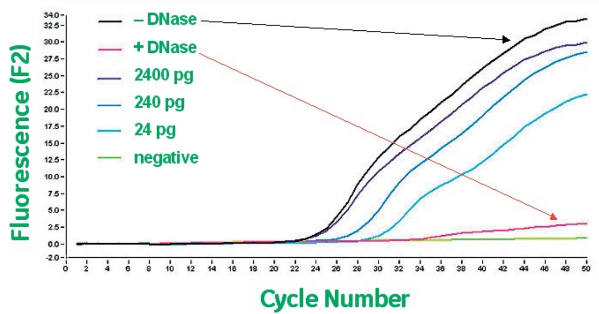


Fig 3. PCR detection of genomic DNA (362-bp fragment of GAPDH). 2 µL of each eluate (80 µL elution volume, total RNA preparation from 5 × 10^5 HeLa cells) was amplified with "LightCycler - DNA Amplification Kit Hybridization Probes" (Roche) (0.5 µmol GAPDH primer, 0.15 µM LCRed 640 hybridization probes, 50 cycles).

4 Protocols

Preparation of working solutions

See *Materials to be supplied by user, on page 6*.

DNase I (Orange)

CAUTION

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

Step	Action
1	Add 400 µl of RNase-free H_2O to the DNase I vial and incubate for 1 min at room temperature.
2	Gently swirl the vial to completely dissolve the DNase I.
3	Prepare DNase working solution by dilute dissolved DNase I with 2.8 mL DNase Reaction Buffer.
4	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 6 mo. 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.

Wash buffer II (Grey)

Wash buffer III (Black)

Add the indicated volume of 96–100% ethanol to Wash buffer II and Wash buffer III concentrates. Store both buffers at room temperature (20–25°C) for up to 1 yr.

Standard protocol for total manual purification of total RNA under vacuum

Lysis

Step	Action
1	Homogenization and lysis of cell suspension. **a.** For up to 2×10^6 cell, transfer cell suspension to a microtiter plate (< 280 µL) or square-well block (> 280 µL). **b.** Centrifuge for 5 min at 500 × g. Remove the supernatant completely. **c.** Add 130 µL Lysis Solution (Red) and 1.3 µL β-mercaptoethanol to the cells in each well. **d.** Pipet up-and-down repeatedly or vortex for 5 min to lyse the cells. ***Note:*** *To process greater than 1×10^6 cells, homogenization and filtration through NucleoSpin RNA Filter Plate (Macherey-Nagel Product code 740711) is recommended. See Preparation and storage of starting materials, on page 33. Use of 1% of β-mercaptoethanol is recommended, but not essential for most cell types.*

Step	Action
2	Homogenization and lysis of adherent cell cultures in 96-well format. **a.** Centrifuge for 5 min at 500 × g. Remove the culture medium completely. **b.** Add 130 µL Lysis Solution (Red) and 1.3 µL β-mercaptoethanol to the tissue in each well. **c.** Pipet up-and-down repeatedly or vortex for 5 min to lyse the cells
3	Homogenization and lysis of tissue. **a.** Add 300 µL Lysis Solution (Red) and 3 µL β-mercaptoethanol to each well. **b.** Add up to 30 mg frozen tissue to each well followed by homogenization. For commercial homogenizers, see *Preparation and storage of starting materials, on page 33*. **c.** Recommend to filtrate the lysate through NucleoSpin RNA Filter Plate (Macherey-Nagel Product code 740711, not provided). Or centrifuge homogenized tissue samples for 5 min at maximum speed, transfer supernatant to suitable plate.
4	Proceed with the next part of the protocol.

Binding preparation

Step	Action
1	Add 130 µL or 300 µL Wash Buffer III, depending on the volume of Lysis Solution, to each well.
2	Pipet up-and-down at least 10–15 times.
3	Prepare the vacuum manifold.
4	Proceed with the next part of the protocol.

RNA binding

Step	Action
1	Place an RNAspin RNA Binding Plate into the vacuum manifold's lid and apply samples to the wells.
2	Applying vacuum (~200 mbar, 1 min) until all lysates have passed through the columns. Release the vacuum.
3	Proceed with the next part of the protocol.

Desalting

Step	Action
1	Add 500 µL Wash Buffer II (Black) to each well.
2	Applying vacuum (-200 mbar, 3 min) until all the buffer has passed through the columns. Release the vacuum.
3	Proceed with the next part of the protocol.

DNA Digestion

Step	Action
1	Pipet 30 µL DNase I reaction mixture (10% DNase I (Orange) and 90% DNase Reaction Buffer (Green)) directly to the bottom of each well of the RNAspin RNA Binding Plate.

CAUTION
Do not touch the silica membrane with the pipette tips.

Step	Action
2	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.
3	Proceed with the next part of the protocol.

Wash and dry

Step	Action
1	Add 500 µL Wash Buffer I (Yellow) to each well of the RNAspin RNA Binding Plate.
2	Applying vacuum (~200 mbar, 1 min) until all the buffer has passed through the columns. Release the vacuum.
3	Add 800 µL Wash Buffer II to each well of the RNAspin RNA Binding Plate.

Step	Action
4	Applying vacuum (-200 mbar; 1 min) until all the buffer has passed through the columns Release the vacuum.
5	Add 500 µL Wash Buffer III to each well of the RNAspin RNA Binding Plate.
6	Applying vacuum (-200 mbar; 1 min) until all the buffer has passed through the columns. Release the vacuum.
7	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 ethanol-containing Wash buffer. Remove manifold lid, Wash Plate, and waste container from the vacuum manifold.
8	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.

Step	Action
9	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.

CAUTION

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

Step	Action
10	Finally, close the valve and release the vacuum.
11	Proceed with the next part of the protocol.

Elution

Step	Action
1	Place the Vacuum Elution Plate (U-Bottom) onto the vacuum manifold.
2	Pipet 50-130 µL RNase-free H_2O directly to the bottom of each well.
3	Incubate for 2 min at room temperature
4	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.

Standard protocol for manual purification of total RNA using a centrifuge

Lysis

Step	Action
1	Homogenization and lysis of cell suspension. **a.** For up to 2 × 10^6 cell, transfer cell suspension to a microtiter plate (< 280 µL) or square-well block (> 280 µL). **b.** Centrifuge for 5 min at 500 × g. **c.** Remove the supernatant completely. **d.** Add 130 µL Lysis Solution (Red) and 1.3 µL β-mercaptoethanol to the cells in each well. **e.** Pipet up-and-down repeatedly or vortex for 5 min to lyse the cells. ***Note:*** *To process greater than 1 × 10^6 cells, homogenization and filtration through NucleoSpin RNA Filter Plate (Macherey-Nagel Product code 740711) is recommended. See Preparation and storage of starting materials, on page 33. Use of 1% of β-mercaptoethanol is recommended, but not essential for most cell types.*

Step	Action
2	Homogenization and lysis of adherent cell cultures in 96-well format. **a.** Centrifuge for 5 min at 500 × g. Remove the culture medium completely. **b.** Add 130 µL Lysis Solution (Red) and 1.3 µL β-mercaptoethanol to the tissue in each well. **c.** Pipet up-and-down repeatedly or vortex for 5 min to lyse the cells
3	Homogenization and lysis of tissue **a.** Add 300 µL Lysis Solution (Red) and 3 µL β-mercaptoethanol to each well. **b.** Add up to 30 mg frozen tissue to each well followed by homogenization. For commercial homogenizers, see *Preparation and storage of starting materials, on page 33*. **c.** Recommend to filtrate the lysate through the RNAspin RNA Filter Plate (not provided) Or centrifuge homogenized tissue samples for 5 min at maximum speed, transfer supernatant to suitable plate.
4	Proceed with the next part of the protocol.

Binding preparation

Step	Action
1	Add 130 µL or 300 µL Wash Buffer III, depending on the volume of Lysis Solution, to each well.

Step	Action
2	Pipet up-and-down at least 10–15 times.
3	Proceed with the next part of the protocol.

RNA binding

Step	Action
1	Place an RNAspin RNA Binding Plate onto an Square-well Block.
2	Pipet up-and-down once, then transfer the lysates to the wells of the RNAspin RNA Binding Plate.
3	Centrifuge for 2 min at 5600–6000 × g.
4	Proceed with the next part of the protocol.

Desalting

Step	Action
1	Add 500 µL Wash buffer II (Grey) to each well.
2	Centrifuge for 2 min at 5600–6000 × g. Discard the flowthrough.
3	Proceed with the next part of the protocol.

DNA Digestion

Step	Action
1	Place the RNAspin RNA Binding Plate back onto the Square-well Block.
2	Pipet 30 µL DNase reaction mixture (10% DNase I (Orange) and 90% DNase Reaction Buffer (Green)) directly to the bottom of each well of the RNAspin RNA Binding Plate. Do not touch the silica membrane with the pipette tips.
3	Incubate at room temperature for 15 min.

CAUTION

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

Step	Action
4	Proceed with the next part of the protocol.

Wash and dry

Step	Action
1	Add 500 µL Wash Buffer I (Yellow) 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 × g

Step	Action
2	Add 800 µL Wash Buffer II (Grey) to each well of the RNAspin RNA Binding Plate and centrifuge for 2 min at 5600–6000 × g. Discard the flowthrough.
3	Add 500 µL Wash Buffer III (Grey) to each well of the RNAspin RNA Binding Plate
4	Centrifuge for 10 min at 5600–6000 × g .
5	Proceed with the next part of the protocol.

The ethanol in Wash Buffer III inhibits downstream enzymatic reactions and has to be completely removed before DNA elution.

Elution

Elution directly into a square-well block or roundwell 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).

Step	Action
1	Pipet 50–130 µL RNase-free H_2O (White) directly to the bottom of each well of the RNAspin RNA Binding Plate.

CAUTION
Make sure that all of the water gets in contact with the silica membrane and that the membrane is completely wetted.

Step	Action
2	Incubate for 2 min at room temperature and centrifuge at 5600–6000 × g for 3 min.

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

Prepare robotic workstation

Step	Action
1	Place the plastic equipment like plates and the assembled vacuum manifold at the locations as specified in the individual robotic programs.
2	Proceed with the next part of the protocol.

Prepare buffers

Step	Action
1	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.
2	Proceed with the next part of the protocol.

Harvest cells

Culture cells

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

Step	Action
1	Aliquots of up to 2×10^6 cells can be transferred into the wells of a 96-well microtiter plate.
2	Pellet cells by centrifugation at 500 × g for 5 min.
3	Remove the supernatant by pipetting and start the preparation.

Tissue samples:

For harvesting and homogenization of tissue samples please refer to *Preparation and storage of starting materials, on page 33*.

Incorporate samples with automated workflow

Step	Action
1	Place microtiter plate with samples at the appropriate position of the robotic workstation.
2	Proceed with the next part of the protocol.

Run automation program

Step	Action
1	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 working solution is pipetted into the middle of the well.
2	Proceed with the next part of the protocol.

Elution of purified total RNA

For increased RNA concentration, dispense at least 50 µL of RNase-free H_2O 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:

Protocol for total RNA clean-up from reaction mixtures

Prepare robotic workstation

Step	Action
1	Per 50 µL (1 volume) sample volume add 160 µL (3.2× the sample volume) Lysis Solution (Red) and 110 µL (2.2× the sample volume) ethanol (96–100%). It is possible to scale up the volumes. The total volume of Lysis Solution (Red) supplied in the kit is sufficient for a maximum of 300 µL Lysis Solution (Red) per well.
2	Proceed with the next part of the protocol.

Adjust RNA binding condition

Step	Action
1	Mix by pipetting up and down at least 15 times and transfer samples to the wells of the RNAspin RNA Binding Plate.

Step	Action
2	Proceed with Step 3 of the standard protocols (Bind RNA to silica membrane). ***Note:*** *DNase treatment might not be necessary, depending on starting material and downstream application.*

5 Appendices

Calculation of RPM from RCF

The appropriate centrifugation speed for a specific rotor can be calculated from the following formula:

$RPM = 1000 \times \sqrt{(RCF/1.12\,r)}$

Where RCF = relative centrifugal force; r = radius in mm measured from the center of the spindle to the bottom of the rotor bucket; and RPM = revolutions per min.

For example, if an RCF of 735 × g is required using a rotor with a radius of 73 mm, the corresponding RPM would be 3000.

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_2 immediately, and stored at -70°C, stored in a stabilizing agent, or processed as

soon as possible. Samples can be stored in Lysis Solution after disruption at -70°C for up to 1 yr, at +4°C for up to 24 h or up to several hours at room temperature. Frozen samples are stable for up to 6 mo. Frozen samples in Lysis Solution should be thawed slowly before starting with the isolation of total RNA.

Note: *Wear gloves at all times during the preparation. Change gloves frequently.*

For optimal homogenization and removal of particles when using larger cell numbers (> 2 × 10^6 cells) or amounts of tissue, filtration of the lysate is recommended. The NucleoSpin RNA Filter Plate (Macherey-Nagel Product code 740711) should be used for this purpose.

Cell culture

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

Cell cultures up to 2 × 10^6 cells can be processed using the vacuum protocol. Using a centrifuge, up to 1 × 10^7 cells can be processed. Transfer the cell suspension to a microtiter plate (< 280 µL) or square-well block (> 280 µL), and centrifuge for 5 min at 500 × 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 adding Lysis Solution in order to reduce the viscosity.

For larger cell numbers (> 2 × 10^6), increasing the volume of Lysis Solution 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 NucleoSpin RNA Filter Plate (Macherey-Nagel Product code 740711) to prevent the RNAspin RNA Binding Plate from becoming clogged. Filter the lysates through the NucleoSpin RNA Filter Plate before applying them to the RNAspin RNA Binding Plate for optimal homogenization and to hold back cell debris.

Tissue

When processing tissue or nuclease-rich cells, ensure the addition of 1% β-mercaptoethanol to Lysis Solution.

β-mercaptoethanol supports the inhibition of RNases. Depending on the type of sample, up to 30 mg tissue can be processed. In case the lysate is too viscous, add 300 µL Lysis Solution and a corresponding amount of Wash Buffer III.

For higher throughput in the 96-well format, add Lysis Solution 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 (*www.saaten-union.de*) or Geno/Grinder 2000 (*www.spexcsp.com*). Alternatively, samples may be homogenized individually by mortar and pestle grinding with liquid N_2 or rotor-stator homogenization.

Once lysed, the tissue lysate can be passed through the NucleoSpin RNA Filter Plate (Macherey-Nagel Product code 740711) for optimal homogenization and to hold back cell debris to prevent subsequent blockage of the RNAspin RNA Binding Plate. Rest the NucleoSpin RNA Filter Plate on a square-well block and apply the samples to the filter. Centrifuge at 5600–6000 × 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 Wash

Buffer III. 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 Wash Buffer III. Filtration through the NucleoSpin RNA 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 NucleoSpin RNA 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 Wash Buffer III to the flowthrough.

Vacuum setup

The RNAspin 96 RNA Isolation 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 (Product code 740680) is required. To process less than 96 samples with the RNAspin 96 RNA Isolation Kit, use a rubber pad or selfadhering PE Foil to cover up any non-used wells of the RNAspin RNA Binding Plate to guarantee a proper vacuum.

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 200–400 mbar (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.

Troubleshooting guide

Problem: RNA is degraded/no RNA obtained

Possible cause	Suggestions
RNase contamination	• 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 2 h at 250°C before use. • Do not return unused buffer from the trough reservoir into the stock bottle. • Use sterile tips with filter.
Sample material	• Sample material not fresh. Whenever possible, use fresh material.

Problem: Poor RNA quality or yield

Possible cause	Suggestions
Reagents not applied or prepared properly	• Add the indicated volume of RNase-free H_2O to the DNase I vial or 96–100% ethanol to buffer concentrates Wash Buffer II and Wash Buffer III, and mix.
Kit storage	• Store aliquots of the reconstituted DNase I at -20°C. • 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 bewetted completely. Ionic strength and pH influence A_{260} absorption as well as ratio A_{260}/A_{280}; thus, for absorption measurement , use 5 mM Tris-HCl pH 8.5 as diluent.

Possible cause	Suggestions
Sample material	• Sample material not stored properly. Whenever possible, use fresh material. If this is not possible, flash freeze the samples in liquid N_2 or treat with a stabilizing agent. Samples should always be kept at -80°C. Never allow tissues to thaw before addition of Lysis Solution. Perform disruption of samples in liquid N_2, if possible. • Insufficient disruption and/or homogenization of starting material. Ensure thorough sample disruption and use NucleoSpin RNA Filter Plate (Macherey-Nagel Product code 740711) for easy clean-up of disrupted starting material. • To process more than 10^6 cells, use a shaker or a commercial homogenizer for optimal homogenization of the starting material. • Too much starting material may lead to RNAspin 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

Possible cause	Suggestions
	by decreasing the amount of starting material and/or increasing the volumes of Wash Buffers, Wash Buffer I, Wash Buffer II, and Wash Buffer III, up to a maximum of 800 µL.

Problem: Clogged RNAspin Binding Plate

Possible cause	Suggestions
Sample material	• Use the NucleoSpin RNA Filter Plate (Machery-Nagel Product code 740711) to reduce the risk of clogging the RNAspin RNA Binding Plate. • To prevent clogging, reduce the sample amount, increase the time for vacuum processing or centrifugation steps, and/or increase the volume of Lysis Solution and Wash Buffer III. 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 Wash Buffer II).

Problem: Contamination of RNA with genomic DNA

Possible cause	Suggestions
DNase I not active	• Reconstitute and store lyophilized DNase I according to instructions given in Chapter Protocols.
DNase solution not properly applied	• Pipet DNase I solution directly onto the center of the silica membrane.
Too much cell material used	• Reduce quantity of cells or tissue used.
	• Increase mixing cycles after adding Wash Buffer III.
Carry-over of ethanol or salt	• Be sure to remove all of Wash Buffer III after the final washing step. Dry the RNAspin RNA Binding Plate for at least 10 min with maximum vacuum.
	• Check if Wash Buffer II has been equilibrated to room temperature before use. Washing at lower temperatures lowers the efficiency of salt removal by Wash Buffer II.
Store isolated RNA properly	• 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.

Problem: Vacuum manifold

Possible cause	Suggestions
Vacuum pressure is not sufficient	• Check if the vacuum manifold lid fits tightly on the manifold base when vacuum is turned on.

Problem: Buffers

Possible cause	Suggestions
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.

Problem: Cross contamination

Possible cause	Suggestions
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.

6 Quick Reference Protocol Cards

RNAspin 96 RNA Isolation Kit

- 1st time users of RNAspin kit should follow the detailed protocol in Chapter Protocols.
- The quick reference protocol is for experienced users only.
- Ensure no precipitate present in Lysis Solution.
- Ensure ethanol added to Wash Buffer II and III.

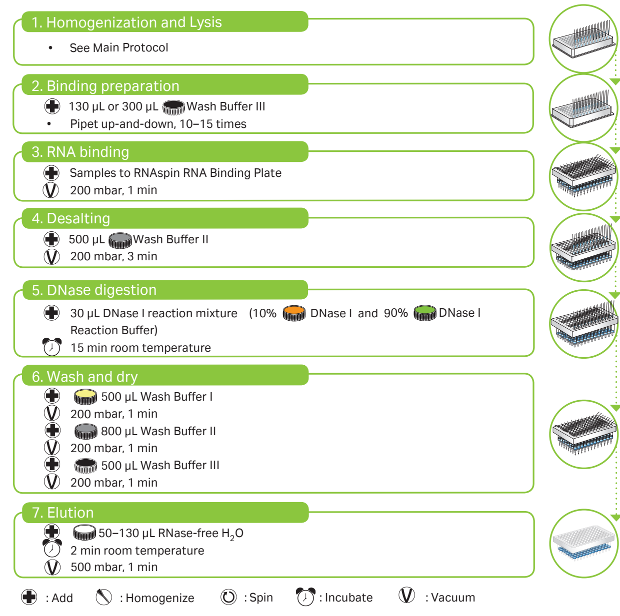


Fig 4. Standard protocol for the manual purification under vacuum

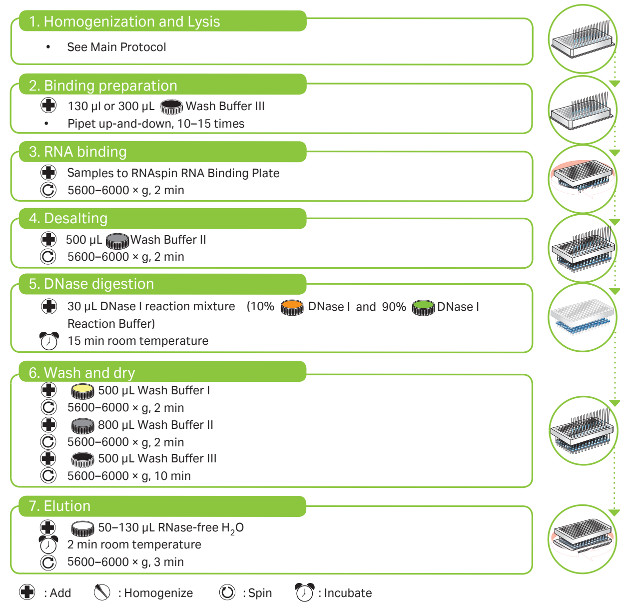


Fig 5. Standard protocol for the manual purification using a centrifuge

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