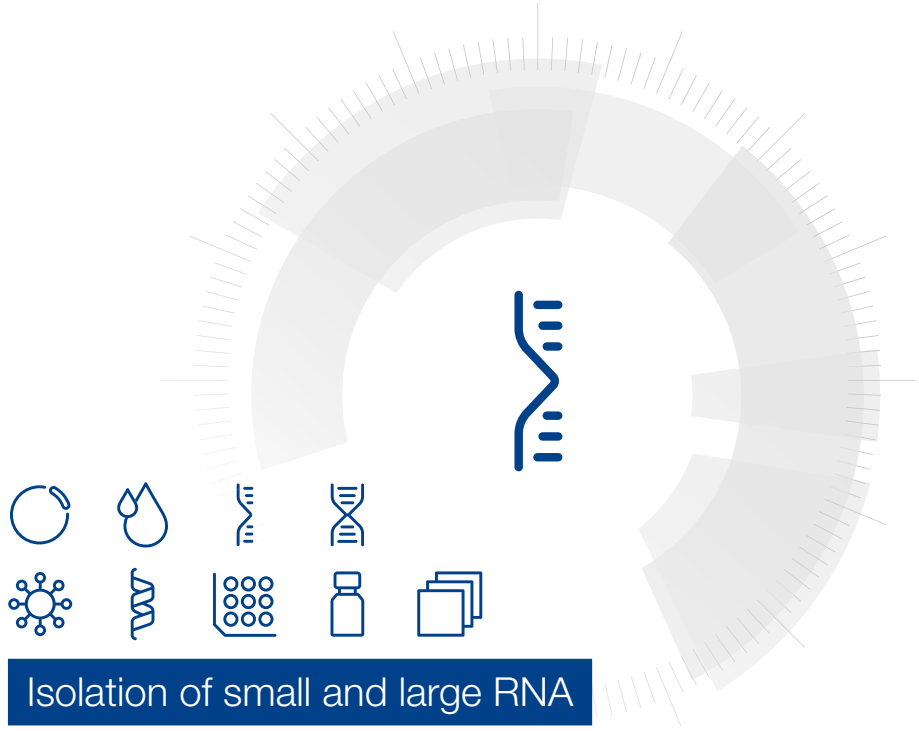
Bioanalysis

MACHEREY-NAGEL

User manual



Isolation of small and large RNA

- NucleoSpin® miRNA

September 2022 / Rev. 07

5.1 RNA purification from animal tissue, plant material and cultured cells

Protocol at a glance (Rev. 07)

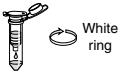
RNA purification from animal tissue, plant material and cultured cells

1 Cell lysis		Disrupt sample 300 µL ML RT, 5 min
2 Homogenization of the lysate	 Violet ring	Load sample 11,000 x *g*, 1 min Discard Filter Column
3 Adjust binding conditions for large RNA and DNA		150 µL 96–100 % ethanol Vortex 5 s RT, 5 min
4 Bind large RNA and DNA	 Blue ring	Load sample 11,000 x *g*, 1 min *Keep NucleoSpin® RNA Column and flow-through!*

Large RNA bound to column

5 Desalt silica membrane		350 µL MDB 11,000 x *g*, 1 min
6 Digest DNA		100 µL rDNase RT, > 15 min

Small RNA in column flow-through

7 Precipitate protein		*Flow-through* 300 µL MP Vortex 5 s 11,000 x *g*, 3 min
8 Remove residual debris	 White ring	Load supernatant 11,000 x *g*, 1 min Discard Filter Column
9 Adjust binding conditions for small RNA		800 µL MX Vortex 5 s

Binding of small RNA to column containing large RNA

10 Bind small RNA	 Blue ring	*Column from step 4* Load 600 µL sample 11,000 x *g*, 30 s Repeat two times
11 Wash and dry silica membrane	 1st	600 µL MW1 11,000 x *g*, 30 s
	 2nd	700 µL MW2 11,000 x *g*, 30 s
	 3rd	250 µL MW2 11,000 x *g*, 2 min
12 Elute RNA		30–100 µL RNase-free H_2O RT, 1 min 11,000 x *g*, 1 min

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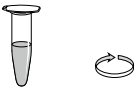
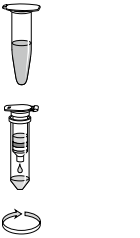
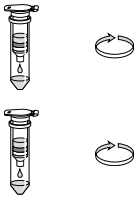
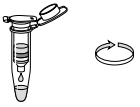
5.2 RNA purification using NucleoZOL

Protocol at a glance (Rev. 07)

RNA purification using NucleoZOL				
1 Cell lysis				500 µL NucleoZOL Disrupt sample RT, 5 min
2 Precipitate contaminants				200 µL RNase-free H_2O Vortex 5 s RT, 15 min 12,000 x *g*, 15 min Transfer 500 µL supernatant into a fresh Collection Tube (2 mL, lid)
3 Bind RNA		Blue ring		500 µL MX Vortex 5 s Load 500 µL sample 8,000 x *g*, 30 s Load remaining sample 8,000 x *g*, 30 s
4 Wash and dry silica membrane		1st 2nd	700 µL MW2 250 µL MW2	8,000 x *g*, 30 s 8,000 x *g*, 2 min
5 Elute RNA				30–100 µL RNase-free H_2O RT, 1 min 11,000 x g, 30 s

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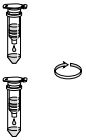
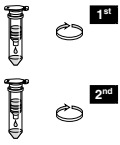
5.3 RNA clean-up

Protocol at a glance (Rev. 07)

RNA clean-up

1 Prepare sample		150 µL sample 150 µL ML Vortex 5 s
2 Adjust binding conditions for nucleic acids > 200 nt		200 µL 96–100 % ethanol Vortex 5 s RT, 5 min
3 Bind large nucleic acids	 Blue ring	Load sample 11,000 x g, 30 s *Keep NucleoSpin® RNA Column and flow-through!*

Optional: digest DNA		350 µL MDB 11,000 x g, 1 min 100 µL rDNase RT, > 15 min
4 Adjust binding conditions for nucleic acids > 200 nt		*Flow-through* 100 µL MP Vortex 5 s RT, 5 min 800 µL MX Vortex 5 s

5 Bind short nucleic acids		*Column from step 3* Load 700 µL sample 11,000 x *g*, 30 s Repeat
6 Wash and dry silica membrane	 1st 2nd	700 µL MW2 11,000 x *g*, 30 s 250 µL MW2 11,000 x *g*, 2 min
7 Elute RNA		30–100 µL RNase-free H_2O RT, 1 min 11,000 x *g*, 30 s

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1 Components

1.1 Kit contents

	NucleoSpin® miRNA		
REF	**10 preps 740971.10**	**50 preps 740971.50**	**250 preps 740971.250**
Lysis Buffer ML	5 mL	30 mL	125 mL
Protein Precipitation Buffer MP	5 mL	20 mL	100 mL
Binding Buffer MX	13 mL	60 mL	250 mL
Membrane Desalting Buffer MDB	10 mL	25 mL	125 mL
Reaction Buffer for rDNase	7 mL	7 mL	30 mL
rDNase, RNase-free (lyophilized)*	1 vial (size C)	2 vials (size C)	10 vials (size C)
Wash Buffer MW1	10 mL	35 mL	180 mL
Wash Buffer MW2 (Concentrate)*	6 mL	12 mL	50 mL
RNase-free H_2O	13 mL	13 mL	30 mL
NucleoSpin® Filters (violet rings)	10	50	250
NucleoSpin® RNA Columns (blue rings)	10	50	250
NucleoSpin® Protein Removal Columns (white rings)	10	50	250
Collection Tubes (1.5 mL)	10	50	250
Collection Tubes (2 mL)	10	50	250
Collection Tubes (2 mL, lid)	30	150	750
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* For preparation of working solutions and storage conditions see section 3.

1.2 Reagents, consumables and equipment to be supplied by user

Reagents

- 96 – 100 % ethanol
- Optional for RNA purification with phenol-based lysis: NucleoZOL (see ordering information)

Consumables

- 1.5 mL microcentrifuge tubes
- RNase-free disposable pipette tips

Equipment

- Manual pipettors
- Centrifuge for microcentrifuge tubes
- Optional: Equipment for sample disruption and homogenization (see section 2.4)
- Personal protection equipment (e.g., lab coat, gloves, goggles)

1.3 About this user manual

It is strongly recommended that first time users of the **NucleoSpin® miRNA** kit read the detailed protocol sections of this user manual. Experienced users, however, may refer to the protocol at a glance instead. The protocol at a glance is designed to be used only as a supplemental tool for quick referencing while performing the purification procedure.

All technical literature is available on the internet at ***www.mn-net.com***.

The protocols in this manual are designed for purification of total RNA in one fraction. **Please visit our website at *www.mn-net.com/Protocols* regarding support protocols for purification of small and large RNAs in separate fractions or for the purification of total nucleic acids including DNA.** Contact our Technical Support for information regarding changes of the current user manual compared to previous revisions.

2 Product description / intended use

2.1 Basic principle

The **NucleoSpin® miRNA** kit is designed for the purification of total RNA including miRNA from cells and a general RNA reaction clean up. Optionally, protein and DNA can be isolated as well. All steps including the centrifugation steps are performed at room temperature with no detectable RNase-activity once the cells are lysed in Buffer ML.

Sample material is lysed and stabilized in Lysis Buffer ML, containing denaturing salts and β-mercaptoethanol **(step 1)**. Depending on the sample material, additional mechanical disruption might be necessary to assist the disruption of hard-to-lyse cell walls and thus to reduce the time intrinsic RNases are active. For lipid-rich or hard-to-lyse tissue NucleoZOL, a phenol-based lysing substance, and NucleoSpin® Bead Tubes are available (see ordering information). See section 2.4 for additional information concerning mechanical cell disruption.

A filtration step with an inert filter removes unlysed remains of the sample and reduces the viscosity of the lysate **(step 2)**. High molecular weight DNA is sheared and hereby prepared for a more efficient DNase digest. The filtrate is further processed while the filter column is discarded.

Addition of ethanol **(step 3)** adjusts binding conditions for the binding of large RNA and DNA fragments above approximately 200 nt to the NucleoSpin® RNA Column **(step 4)** while small RNA of less than about 200 nt and proteins are in the flowthrough. This separation of small and large nucleic acids is necessary to guarantee a superior RNA purity as DNA and proteins can each be removed separately in a **patented** and most efficient way. Both the NucleoSpin® RNA Column with long nucleic acids and the flowthrough containing short nucleic acids and proteins are kept at this step.

If purification of **total nucleic acids including DNA** is desired, the following steps 5 and 6 are skipped, **see section 2.5** for further details. Otherwise a desalting step with Buffer MDB **(step 5)** prepares the NucleoSpin® RNA Column for the following on-column DNA digest **(step 6)**. During the ongoing DNase incubation, the flowthrough of step 4, containing small RNA and protein, is further processed. Addition of Buffer MP precipitates protein from the sample. Precipitated protein is removed by centrifugation **(step 7)**. If desired, the **protein** pellet can be redissolved and analyzed (**see section 2.6** for recommendations). Remaining protein is removed from the supernatant via filtration through a NucleoSpin® Protein Removal Column **(step 8)** leaving only small RNA in the flowthrough. The NucleoSpin® Protein Removal Column is discarded and the clear flowthrough combined with Buffer MX which adjusts binding conditions for small RNA to the NucleoSpin® RNA Column **(step 9)**.

After the DNase digest is completed the mixture of step 9 is loaded stepwise into the NucleoSpin® RNA Column **(step 10)**. If preparation of small and large RNA in separate fractions is desired, the mixture of step 9 can also be bound to a second NucleoSpin® RNA Column instead. In this case the following washing steps are performed for both columns in parallel.

Stringent washing steps with Wash Buffers MW1 and MW2 remove DNA fragments, contaminants and salts **(step 11)**. An optional third washing step with Buffer MW2

removes trace amounts of chaotropic salt carryover (see section 2.8 for details). Ethanol from Wash Buffer MW2 is removed by a prolonged centrifugation step.

Pure RNA is eluted in 30 – 100 µL of supplied RNase-free H_2O at **step 12**. See section 2.7 for details concerning the choice of elution volume. Eluted RNA is ready for both standard and demanding downstream applications.

2.2 Kit specifications

Kit specifications at a glance

Parameter	NucleoSpin® miRNA
Format	Mini spin columns
Processing	Manually via centrifuge
Target	RNA
CE certified	No, research use only
Sample material	< 10^7 cultured cells < 30 mg human / animal tissue < 30 mg plant material < 150 µL reaction mix
Binding capacity	200 µg (NucleoSpin® RNA Column)
Elution volume	30 – 100 µL
Preparation time	< 45 min (6 preps)
Typical yields	30 mg mouse liver: 100 µg 30 mg mouse kidney: 35 µg 30 mg mouse spleen: 48 µg 30 mg mouse lung: 27 µg 30 mg mouse heart: 24 µg 30 mg porcine liver: 80 µg 30 mg human brain: 11 µg 10^7 HeLa cells: 100 µg 30 mg wheat leaves: 25 µg

2.3 Amount of starting material

Ideally, the amount of starting material should be at the upper limit of the range in the table given above in order to achieve efficient purification of small and large RNA.

For quantitative RNA purification from starting material less than 3 mg tissue or 10^6 cells, it is advantageous to add 10 µg of Carrier RNA (see ordering information) before binding of small RNA to improve RNA binding. Prepare a Carrier RNA stock solution of 1 mg/mL in RNase-free H_2O. Add 10 µL of the stock solution to the cleared lysate after step 8 before adjusting binding conditions for small RNA with Buffer MX. Mix well before addition of Buffer MX and proceed with the protocol.

2.4 Preparation and storage of starting materials

RNA is not protected against digestion until the sample is flash frozen or disrupted in the presence of RNase inhibiting or denaturing agents. For long term storage it is recommended that samples are flash frozen and stored at -70 °C to -80 °C as soon as possible.

After disruption, samples can be stored in Lysis Buffer ML at -70 °C to -80 °C for up to one year, at -20 °C for up to 6 months, at +2 °C to +8 °C for up to 24 hours, or up to several hours at room temperature. Frozen samples in Lysis Buffer ML should be thawed slowly until no remaining salt crystals are visible before starting with the RNA isolation.

Wear gloves at all times during the preparation! Change gloves frequently! Use RNase-free equipment only!

Cultured tissue and cells can be collected by centrifugation (after trypsinization, if necessary). The cell pellet can be redissolved in Lysis Buffer ML where cells are lysed almost immediately. Nevertheless a change in expression profiles during long washing and centrifugation steps as well as during trysinization must be considered. Optionally, adherent cells can be lysed directly in the culture flask. Remove culture medium and wash the cells with Phosphate Buffered Saline (PBS) before addition Lysis Buffer ML.

- **Cells grown in monolayer**

  Remove culture medium completely and wash cells once with Phosphate Buffered Saline (PBS). Lyse cells by addition of **300 µL Buffer ML** for each **5×10^6 cells** directly to the culture disk and incubate for **5 min** at **room temperature**

  or

  Collect up to **10^7 cultured cells** after trypsinization by centrifugation, discard supernatant and add **300 µL Buffer ML**. Pipette up and down or vortex to lyse the cells.

- **Cells grown in suspension**

  Collect up to **10^7 cultured cells** by centrifugation, discard supernatant and add **300 µL Buffer ML**. Pipette up and down or vortex to lyse the cells.

  In all cases transfer exactly **300 µL lysate** to a **NucleoSpin® Filter Column** (violet ring) in a Collection Tube (2 mL, lid).

Animal and plant tissue is often solid and might be protected by a cell wall, which reduces the effectiveness of lysis buffers. Therefore, mechanical assistance is essential to quickly break up the cells and stabilize the RNA in Buffer ML. Different types of mechanical disruptors are available in the market, the most basic but also most effective method being grinding of the tissue with mortar and pestle under liquid nitrogen. Use prechilled material only when working with liquid nitrogen and do not let samples thaw.

Alternatively, dedicated tissue disruptors can be used to lyse and homogenize the sample. Depending on the system it may be necessary to increase the lysis volume or the sample amount to enable a proper functionality of the system. Increase both parameters proportionally. See ordering information for additional buffer ML.

- **Mortar and Pestle**

  Transfer sample material to a prechilled mortar and grind it using a prechilled pestle under constant addition of liquid nitrogen. Do not allow sample to thaw! Grind sample to a fine powder and transfer, using prechilled spatulas and tubes, up to **30 mg ground sample** to a 1.5 mL centrifuge tube (not supplied). Immediately add **300 µL Buffer ML** and vortex vigorously! Transfer lysate to a **NucleoSpin® Filter Column** (violet ring) in a Collection Tube (2 mL, lid).

- **Mechanical devices using shearing forces**

  Transfer sample material to a suitable or dedicated lysis tube and add **300 µL Buffer ML** for each **30 mg of sample**. Increase mass and volume proportionally, if needed. Disrupt sample according to the manufacturers' instructions. **Transfer 300 µL lysate** to a **NucleoSpin® Filter Column** (violet ring) in a Collection Tube (2 mL, lid).

- **Bead Tubes**

  Transfer up to **30 mg of sample** material to a **NucleoSpin® Bead Tube Type D or Type E** (not supplied, see ordering information) and add **450 µL Buffer ML** for **Type D** or **550 µL Buffer ML** for **Type E**.

  Vortex horizontally for **5 – 15 min** at room temperature e.g., on a MACHEREY-NAGEL Bead Tube Holder (not supplied, see ordering information) adapted to a suitable vortex basis (e.g., Vortex-Genie® II). **Transfer 300 µL lysate** to a **NucleoSpin® Filter Column** (violet ring) in a Collection Tube (2 mL, lid).

When using other sample disruption methods, be sure not to exceed a maximum of **30 mg sample material per 300 µL Buffer ML**. Do not dilute or mix Buffer ML with other fluids! Transfer exactly **300 µL lysate** to a **NucleoSpin® Filter Column** (violet ring) in a Collection Tube (2 mL, lid).

Bacteria and yeast have to be incubated in lysozyme or lyticase/zymolase solutions, respectively to break down the robust cell walls of these organisms. Sonication and mechanical disruption are alternatives for cell disruption. Avoid long incubation times to prevent changes in expression profiles.

NucleoZOL, a phenol-based lysis substance, is available separately (see ordering information). See protocol 5.2 for necessary adaptations.

2.5 Purification of total nucleic acids including DNA

The NucleoSpin® miRNA kit is suitable for purification of **total nucleic acids including DNA** and large RNA / small RNA as well as for the purification of denatured protein (see section 2.6). To enable a purification of DNA, the membrane desalting step with Buffer MDB and the DNase digest are omitted (steps 5 and 6 in section 5.1). It is important **not** to perform the membrane desalting step with **Buffer MDB** if purification of DNA is desired.

A separation of total nucleic acids into DNA and RNA can be achieved by an enzymatic digest of the split and eventually aliquoted eluate with DNase and RNase, respectively, or purification with the NucleoSpin® RNA/DNA Buffer Set (not supplied, see ordering information).

This kit is feasible for the simultanous extraction of small and large RNAs and the optional extraction of proteins.

The buffers and plastics provided by this kit do also suffice for 25 preps of separated isolation of small and large RNA in separate fractions.

Please see the ordering information regarding additional buffers and spin columns.

2.6 Analysis of the protein fraction

Buffer ML contains high amounts of chaotropic salt and β-mercaptoethanol. Furthermore, ethanol is added prior to the protein precipitation resulting in completely denatured protein. The precipitated protein pellet of the recommended amount of starting material might be difficult to resuspend, so if protein analysis is required, it could be advantageous not to use the protein pellet of the complete sample for further analysis but to remove an aliquot of 10 – 20 µL lysate before addition Buffer MP (between steps 4 and 6) and to precipitate this smaller portion with an adapted volume of Buffer MP separately (6.7 – 13.3 µL Buffer MP respectively).

Add 500 µL of 50 % ethanol to the protein pellet (no resuspension necessary) and centrifuge for 1 min at 11,000 x ***g***. Remove the ethanol completely and let the protein pellet dry at room temperature for 10 min.

Usually the denatured protein is dissolved in Laemmli buffer or a similar SDS-containing solution by incubating the sample at 90 °C for at least 5 minutes. Undissolved protein is removed by centrifugation and the solubilized protein can be used for downstream analysis.

Most protein quantification assays such as Bradford, Lowry, BCA, etc. do not work in the presence of SDS. For this purpose, MACHEREY-NAGEL offers the **Protein Quantification Assay** (see ordering information). It is designed for the determination of low protein concentrations in the presence of up to 10 % SDS, reducing agents, dyes like bromphenol blue or substances to increase the sample density like glycerol or sucrose. The kit also provides a Laemmli-like protein solubilization buffer PSB (Protein Solving Buffer) in which the precipitated protein can be dissolved, quantified, and used for SDS-PAGE.

2.7 Elution procedures

Higher elution volumes lead to higher RNA recovery, but lower RNA concentration. The optimal elution volume often depends on the kind of downstream application which dictates the necessity of a high yield with a high total volume or a high concentration for a limited amount of sensitive applications. Three elution volumes are suggested:

- **30 µL** for **high concentration** but reduced total yield
- **50 µL** for **medium** concentration and yield
- **100 µL** for **high yield** but lower concentration

30 µL are necessary as minimal elution volume to wet the silica entirely. A further decrease will result in significiantly less yield.

It is possible to **reload the eluate from the first elution step into the column and to use it as elution buffer for a second elution**. Multiple elution steps will increase the total yield.

To increase the yield and the concentration in a single elution step, heat the RNase-free water to 90 °C before elution. However, a high temperature leads to larger pipetting errors and consequently to higher variations in the final volume of the eluate.

2.8 Salt carry-over and low A_{260}/A_{230}

The silica membrane technology is based on the ability of chaotropic salts to dehydrate macromolecules and an affinity of dehydrated nucleic acids to silica. The most common chaotropic salt used for RNA purification is guanidinium thiocyanate which has got excellent characteristics for RNA binding and RNase-inactivation.

In contrast to guanidine hydrochloride, guanidine thiocyanate exhibits a strong absorption at wavelengths < 240 nm even in trace amounts below 1 mM.

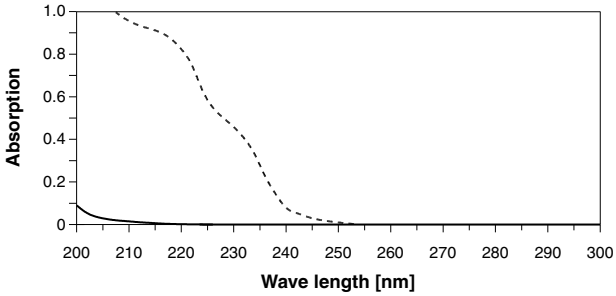


Figure 1 UV absorption spectra of 1 mM guanidine HCl (solid line) and 1 mM guanidine thiocyanate (dotted line).

Figure 1 demonstrates that guanidine thiocyanate concentrations even as low as 1 mM will increase the absorption at 230 nm by 0.5 resulting in a decreased A_{260}/A_{230}, especially in combination with low RNA concentrations (low A_{260}).

The concentration of contaminating chaotropic salt in eluates is usually substantially below 1 mM and has got no negative influence even on sensitive downstream applications.

Figure 2 shows a qPCR with effective chaotropic salt concentrations of 0 µM to 80 mM in the PCR reaction demonstrating that more than 20 mM of chaotropic salt must be present in the PCR reaction to negatively influence the amplification **which is 500-fold more than usually present**.

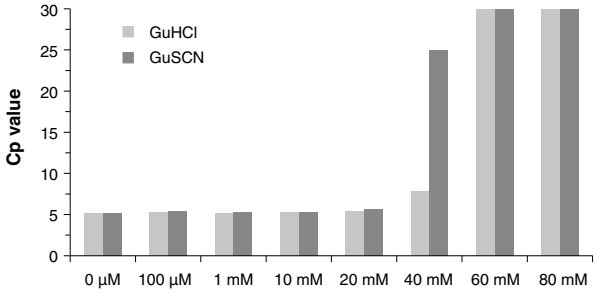


Figure 2 qPCR inhibition by GuHCl (light gray) and GuSCN (dark gray). A 164 bp DNA fragment was amplified from 5 ng pBS template with DyNAmo Capillary Master Mix (NEB) in a Lighcycler real-time PCR machine (Roche) in the presence of 0 – 80 mM GuHCl or GuSCN.

If it is still necessary to further reduce the chaotropic salt carry-over and hereby **to improve the A_{260}/A_{230}, a second washing step with 700 µL Buffer MW2 is recommended resulting in a total of three washes**. The supplied volume of Buffer MW2 will not be sufficient, additional Buffer MW2 must be ordered separately (see ordering information).

3 Storage conditions and preparation of working solutions

Attention:

Buffers ML, MDB, and MW1 contain chaotropic salt. Wear gloves and goggles!

CAUTION: Buffer ML, MDB, and MW1 contain guanidinium thiocyanate which can form highly reactive compounds when combined with bleach (sodium hypochlorite). DO NOT add bleach or acidic solutions directly to the sample-preparation waste.

Storage conditions

- Store lyophilized **RNase-free rDNase** at +4 °C on arrival (stable for at least one year).
- All other kit components should be stored at room temperature (15 – 25 °C) and are stable until: see package label. Storage at lower temperatures may cause precipitation of salts. If precipitation occurs, incubate the bottle for several minutes at about 30 – 40 °C and mix well until the precipitate is redissolved.

Before starting the first **NucleoSpin® miRNA** procedure prepare the following:

- **Wash Buffer MW2:** Add the indicated volumes of 96 – 100 % ethanol to the MW2 concentrate. Store buffer at room temperature for at least one year.
- **RNase-free rDNase:** Add the indicated volume of Reaction Buffer for rDNase to the rDNase vial and incubate for 1 min at room temperature. Gently swirl the vial to completely dissolve the rDNase. Be careful not to mix rDNase vigorously as rDNase is sensitive to mechanical agitation. Dispense into aliquots and store at -20 °C. The frozen working solution is stable for at least 6 months. Do not freeze/thaw the aliquots more than three times.

	NucleoSpin® miRNA		
REF	**10 preps 740971.10**	**50 preps 740971.50**	**250 preps 740971.250**
Wash Buffer MW2 (Concentrate)	6 mL Add 24 mL 96 – 100 % ethanol	12 mL Add 48 mL 96 – 100 % ethanol	50 mL Add 200 mL 96 – 100 % ethanol
RNase-free rDNase (lyophilized)	1 vial (size C) Add 3 mL **Reaction Buffer for rDNase**	2 vials (size C) Add 3 mL **Reaction Buffer for rDNase** to each vial	10 vials (size C) Add 3 mL **Reaction Buffer for rDNase** to each vial

4 Safety instructions

When working with the **NucleoSpin® miRNA** kit wear suitable protective clothing (e.g., lab coat, disposable gloves, and protective goggles). For more information consult the appropriate Material Safety Data Sheets (MSDS available online at *www.mn-net.com/msds)*

Caution: Guanidinium thiocyanate in Buffer ML can form highly reactive compounds when combined with bleach! Thus, do not add bleach or acidic solutions directly to the sample preparation waste.

The waste generated with the **NucleoSpin® miRNA** kit has not been tested for residual infectious material. A contamination of the liquid waste with residual infectious material is highly unlikely due to strong denaturing lysis buffer treatment but it cannot be excluded completely. Therefore, liquid waste must be considered infectious and should be handled and discarded according local safety regulations.

4.1 Disposal

Dispose hazardous, infectious or biologically contaminated materials in a safe and acceptable manner and in accordance with all local and regulatory requirements.

5 Protocols

5.1 RNA purification from animal tissue, plant material and cultured cells

Before starting the preparation:

- Check if 96 – 100 % ethanol is available
- Check if rDNase was prepared according to section 3
- Check if Wash Buffer MW2 was prepared according to section 3

1 Cell lysis

Disrupt sample

Tissue and plant material

Thoroughly disrupt up to **30 mg of sample** material in **300 µL Buffer ML** using mechanical devices. If necessary, increase sample amount and lysis buffer volume proportionally. Optimal lysis conditions need to be evaluated for each sample material individually.

+300 µL ML

RT
5 min

→ **See section 2.4 for details.**

Cultured cells

Lyse up to 10^7 cultured cells in 300 µL Buffer ML. Pipette up and down or vortex to lyse the cells.

→ **See Section 2.4 for details.**

Incubate **5 min** at **room temperature.**

2 Homogenization of the lysate

Load sample

Place a **NucleoSpin® Filter Column** (violet ring) into a Collection Tube (2 mL, lid). Load the lysate and centrifuge for **1 min** at **11,000 x *g*** to reduce viscosity and to clear the lysate from undissolved debris.

11,000 x *g*, 1 min

If a pellet is visible in the Collection Tube (2 mL, lid) after the centrifugation, transfer the supernatant to a fresh centrifuge tube (not supplied) without disturbing the pellet.

Alternative: samples without debris can be homogenized by passing them through a 0.9 mm needle (20 gauge), fittet to a syringe.

Discard the **NucleoSpin® Filter Column** (violet ring) and **proceed with the flowthrough**.

3 Adjust binding conditions for large RNA and DNA

Add exactly **150 µL 96 – 100 % ethanol** to **300 µL flowthrough** from step 2. Vortex immediately for **5 s**.

***Note:** After addition of ethanol a precipitate may become visible. Do not remove the precipitate and load it into the column at step 4!*

Incubate for **5 min** at **room temperature.**

300 µL lysate

+150 µL 96 – 100 % ethanol

Vortex 5 s

RT 5 min

4 Bind large RNA and DNA

Combine a **NucleoSpin® RNA Column** (blue ring) with a Collection Tube (2 mL, lid) and **load** the **sample** including any precipitate into the column.

Load sample

Centrifuge for **1 min** at **11,000 x *g***.

Keep both the **NucleoSpin® RNA Column** with bound large RNA and DNA **and** the **flowthrough** containing small RNA and proteins!

11,000 x *g*, 1 min

Place the NucleoSpin® RNA Column in a new Collection Tube (2 mL) without lid. Close the lid of the Collection Tube (2 mL, lid) with the saved flowthrough. Proceed with the NucleoSpin® RNA Column.

Note: If purification of total nucleic acids including DNA is desired, omit steps 5 and 6 and proceed directly to step 7. See section 2.5 for details.

5 Desalt silica membrane

Add **350 µL Buffer MDB** to the NucleoSpin® RNA Column (blue ring) and centrifuge for **1 min** at **11,000 x *g***.

+350 µL MDB

Discard flowthrough and place the column back into the collection tube.

11,000 x *g*, 1 min

6 Digest DNA

Add **100 µL rDNase** directly onto the silica membrane of the NucleoSpin® RNA Column (blue ring).

+100 µL rDNase

Incubate at **room temperature** until steps 7 – 10 are completed, but at least **15 min**.

RT > 15 min

7 Precipitate protein

Add **300 µL Buffer MP** to the saved flowthrough of step 4. Vortex for 5 s.

Centrifuge for **3 min** at **11,000 x *g*** to pellet protein.

***Note:** The protein pellet can be analyzed. Refer to section 2.6 for details.*

300 µL MP

Vortex 5 s

11,000 x *g*, 3 min

8 Remove residual debris

Place a **NucleoSpin® Protein Removal Column** (white ring) in a Collection Tube (2 mL, lid) and **load** the **supernatant** from step 7 into the column.

Centrifuge for **1 min** at **11,000 x *g***.

Discard the NucleoSpin® Protein Removal Column and **keep the flowthrough**.

Load supernatant

11,000 x *g*, 1 min

9 Adjust binding conditions for small RNA

Add **800 µL Buffer MX** to the flowthrough.

Vortex for **5 s**.

***Note:** After addition of Buffer MX a precipitate may be visible. Load all of the precipitate into the column at step 10.*

+800 µL MX

Vortex 5 s

10 Bind small RNA

Load 600 µL of the mixture from step 9 into the corresponding **NucleoSpin® RNA Column** (blue ring) already containing the large RNA from step 4.

Centrifuge for **30 s** at **11,000 x *g***.

Attention: Do not centrifuge the NucleoSpin® RNA Column with the rDNase reaction buffer before the mixture of step 9 is added.

Discard the flowthrough and place the column back into the collection tube.

Repeat this step two times to **load the remaining sample**.

Load 600 µL sample

11,000 x *g*, 30 s

Repeat once

Load remaining sample

11,000 x *g*, 30 s

11 Wash and dry silica membrane

1 st wash

Add **600 µL Buffer MW1** to the NucleoSpin® RNA Column.

+600 µL MW1

Centrifuge for **30 s** at **11,000 x *g***.

11,000 x *g*, 30 s

Discard flowthrough and place the column back into the collection tube.

2nd wash

Add **700 µL Buffer MW2** to the NucleoSpin® RNA Column.

+700 µL MW2

Centrifuge for **30 s** at **11,000 x *g***.

11,000 x *g*, 30 s

Discard flowthrough and place the column back into the collection tube.

Optionally: Repeat the 2nd wash step to remove trace amounts of chaotropic salt and thus to further increase $A_{260/230}$ ratios. See section 2.8 for details and ordering information for required additional Buffer MW2.

3rd wash

Add **250 µL Buffer MW2** to the NucleoSpin® RNA Column.

+250 µL MW2

Centrifuge for **2 min** at **11,000 x *g*** to dry the silica membrane.

11,000 x *g*, 2 min

Discard Collection Tube and place the NucleoSpin® RNA Column into a new Collection Tube (1.5 mL, lid).

12 Elute RNA

Add **30 µL** (high concentration), **50 µL** (medium concentration and yield) or **100 µL** (high yield) **RNase-free H_2O** to the NucleoSpin® RNA Column.

+30 – 100 µL RNase-free H_2O

Incubate for **1 min** at **room temperature.**

11,000 x *g*, 1 min

Centrifuge for **30 s** at **11,000 x *g***.

5.2 RNA purification using NucleoZOL

Before starting the preparation:

- NucleoZOL must be ordered separately, see ordering information
- Check if Wash Buffer MW2 was prepared according to section 3
- **Attention!** NucleoZOL contains phenol (corrosive liquid/poison) and guanidinium thiocyanate (irritant). Wear personal protection equipment at all times and take appropriate security measures for working with phenol! Carefully read and follow the hazard information and Safety Data Sheets supplied with NucleoZOL! Discard waste according to legal guidelines!

1 Cell lysis

+500 µL NucleoZOL

Disrupt sample

RT 5 min

Tissue and plant material

Thoroughly disrupt **50 mg sample** material in **500 µL NucleoZOL** using mechanical devices. Sample amount and NucleoZOL volume can be increased proportionally.

Transfer **500 µL lysate** to a Collection Tube (2 mL, lid).

Cultured cells

Cells grown in monolayer: Remove culture medium and lyse cells by addition of at least **1 mL NucleoZOL** to the culture disk (diameter 3.5 cm, 10 cm^2). Mix by pipetting up and down.

***Note:** An insufficient volume of NucleoZOL will lead to DNA contamination of the isolated RNA.*

Transfer **500 µL lysate** to a Collection Tube (2 mL, lid).

Alternatively, collect up to **10^7 cultured cells** after trypsinization by centrifugation, discard supernatant and add **500 µL NucleoZOL**. Pipette up and down or vortex to lyse the cells.

Cells grown in suspension: Collect up to **10^7 cultured cells** by centrifugation, discard supernatant and add **500 µL NucleoZOL**. Pipette up and down or vortex to lyse the cells.

Liquid samples

Use up to **200 µL liquid sample** with **500 µL NucleoZOL**.

Incubate **5 min** at **room temperature.**

2 Precipitate contaminants

Add **200 µL RNase-free H_2O** to the lysate in 500 µL NucleoZOL.

Vortex vigorously and incubate at **room temperature** for **15 min**.

Centrifuge samples for **15 min** at **12,000 x *g*.**

DNA, proteins and polysaccharides are pelleted, RNA remains in the supernatant.

Transfer **500 µL supernatant** into a fresh Collection Tube (2 mL, lid) without disturbing the pellet. Discard pellet and residual supernatant.

200 µL RNase-free H_2O

Vortex 5 s

RT 15 min

12,000 x *g*, 15 min

3 Bind RNA

Add **500 µL Buffer MX** to the transferred supernatant and **mix** by vortexing.

Combine a **NucleoSpin® RNA Column** (blue ring) with a Collection Tube (2 mL) and **load 500 µL** of the **sample** solution into the column.

Centrifuge for **30 s** at **8,000 x *g***.

Discard flowthrough and load the remaining sample.

Centrifuge for **30 s** at **8,000 x *g*** and discard flowthrough.

+500 µL MX

Vortex 5 s

Load 500 µL sample

8,000 x *g*, 30 s

Load remaining sample

8,000 x *g*, 30 s

4 **Wash and dry silica membrane**

1 st wash

Add **700 µL Buffer MW2** to the NucleoSpin® RNA Column.

+700 µL MW2

Centrifuge for **30 s** at **8,000 x *g***. Discard flowthrough.

8,000 x *g*, 30 s

Optionally: Repeat the 1 st wash step to remove trace amounts of chaotropic salt and thus to further increase $A_{260/230}$ ratios. See ordering information for required additional Buffer MW2.

2nd wash

Add **250 µL Buffer MW2** to the NucleoSpin® RNA Column.

+250 µL MW2

Centrifuge for **2 min** at **8,000 x *g***. Discard flowthrough.

8,000 x *g*, 2 min

5 **Elute RNA**

Place the NucleoSpin® RNA Column in a new Collection Tube (1.5 mL).

+30 – 100 µL RNase-free H_2O

Add **30 µL** (high concentration), **50 µ**L (medium concentration and yield) or **100 µL** (high yield) **RNase-free H_2O** to the NucleoSpin® RNA Column.

RT 1 min

Incubate for **1 min** at **room temperature.**

Centrifuge for **30 s** at **11,000 x *g***.

11,000 x *g*, 30 s

5.3 RNA clean up

Before starting the preparation:

- Check if 96 – 100 % ethanol is available
- Check if Wash Buffer MW2 was prepared according to section 3
- Optional: Check if rDNase was prepared according to section 3

1 Prepare sample

Add **150 µL Buffer ML** to **150 µL sample** reaction mixture and **vortex** for **5 s**.

***Note:** To purify less than 150 µL, adjust volume with RNase-free H_2O to 150 µL. To process more than 150 µL, increase volumes of buffers ML, MP and MX proportionally.*

150 µL sample

+150 µL ML

Vortex 5 s

2 Adjust binding conditions for nucleic acids > 200 nt

Add exactly **200 µL 96 – 100 % ethanol** and **vortex** for **5 s**.

Incubate at **room temperature** for **5 min**.

+200 µL 96 – 100 % ethanol

Vortex 5 s

RT 5 min

3 Bind large nucleic acids

Place a **NucleoSpin® RNA Column** (blue ring) in a Collection Tube (2 mL, lid) and **load the sample** into the column. Centrifuge for **30 s** at **11,000 x *g***.

Keep the flowthrough and the column!

Transfer the NucleoSpin® RNA Column containing the large nucleic acids to a new Collection Tube (2 mL) and save it for step 5.

Load sample

11,000 x *g*, 30 s

Optional: DNA-digest

Add **350 µL Buffer MDB** to the NucleoSpin® RNA Column (blue ring) and centrifuge for **1 min** at **11,000 x *g***.

+350 µL MDB

Discard flowthrough and place the column back into the collection tube.

11,000 x *g*, 1 min

Add **100 µL rDNase** directly onto the silica membrane of the NucleoSpin® RNA Column (blue ring).

+100 µL rDNase

Incubate at **room temperatur**e until steps 4 is completed but at least **15 min**.

RT > 15 min

4 **Adjust binding conditions for nucleic acids < 200 nt**

Flowthrough

Add **100 µL Buffer MP** to the flowthrough of step 3 and **vortex** for **5 s**.

+100 µL MP

Incubate for **5 min** at **room temperature.**

Vortex 5 s

Add **800 µL Buffer MX** and **vortex** for **5 s**.

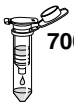
RT 5 min

+800 µL MX

Vortex 5 s

5 **Bind small nucleic acids**

Load 700 µL sample into the corresponding NucleoSpin® RNA Column containing the large nucleic acids.



Load 700 µL sample

Centrifuge for **30 s** at **11,000 x *g***.

11,000 x *g*, 30 s

Discard flowthrough and place the column back into the collection tube.

Repeat this step

Repeat this step to load the remaining sample.

6 Wash and dry silica membrane

1 st wash

+700 µL MW2

Add **700 µL Buffer MW2** to the NucleoSpin® RNA Column.

11,000 x *g*, 30 s

Centrifuge for **30 s** at **11,000 x *g***. Discard flowthrough.

Optionally: Repeat the 1 st wash step to remove trace amounts of chaotropic salt and thus to further increase $A_{260/230}$ ratios. See ordering information for required additional Buffer MW2.

2nd wash

+250 µL MW2

Add **250 µL Buffer MW2** to the NucleoSpin® RNA Column.

11,000 x *g*, 2 min

Centrifuge for **2 min** at **11,000 x *g***. Discard flowthrough.

7 Elute RNA

+30 – 100 µL RNase-free H_2O

RT 5 min

Place the NucleoSpin® RNA Column in a new Collection Tube (1.5 mL).

Add **30 µL** (high concentration), **50 µL** (medium concentration and yield) or **100 µL** (high yield) **RNase-free H_2O** to the NucleoSpin® RNA Column.

Incubate for **1 min** at **room temperature.**

11,000 x *g*, 30 s

Centrifuge for **30 s** at **11,000 x *g***.

6 Appendix

6.1 Troubleshooting

Problem	Possible cause and suggestions
Poor or no RNA yield	***Reagents not applied or restored properly*** • Always dispense exactly the buffer volumes given in the protocols! The correct ratios of buffers ML, MP, and ethanol are essential for optimal yield and purity. • Always follow the given instructions closely, with specific attention paid to order and mode of mixing (shaking, vortexing, etc). • Add the indicated volume of 96 – 100 % ethanol to Buffer MW2 Concentrate and mix thoroughly. • Store kit components at room temperature. Storage at lower temperatures may cause salt precipitation. If precipitation occurs, incubate the bottle for several minutes at about 30 – 40 °C and mix well until the precipitate is redissolved. • Keep bottles tightly closed in order to prevent evaporation or contamination. ***Sample material not stored properly*** • Whenever possible, use fresh material. Otherwise, flash freeze the samples in liquid nitrogen. Samples should always be kept at -70 °C. Never allow tissues to thaw before addition of lysis buffer. Perform disruption of samples in liquid nitrogen or lysis buffer. ***Insufficient disruption and/or homogenization of starting material*** • Ensure thorough sample disruption and use NucleoSpin® Filters for homogenization of disrupted starting material.
RNA is degraded	***RNase contamination*** • Create an RNase-free working environment. Wear gloves during all steps of the procedure. Change gloves frequently. Use sterile, disposable polypropylene tubes and filter tips. Keep tubes closed whenever possible during the preparation unless stated otherwise. Glassware should be oven-baked for at least 2 hours at 250 °C before use.

Problem	Possible cause and suggestions
Clogged NucleoSpin® RNA Columns	***Too much starting material***
	• Overloading may lead to decreased overall RNA yield due to binding of too much DNA. Reduce amount of sample material or use larger volume of lysis buffer.
	Insufficient homogenization of starting material
	• After cell lysis in Buffer ML and homogenization with NucleoSpin® Filters the lysate has to be clear and free of solid particles. If this is not the case, centrifuge the sample and transfer the clear supernatant to a new collection tube (not provided) without disturbing the pellet.
	Too much precipitated nucleic acids after addition of ethanol
	• Do not remove the precipitate (e.g., by centrifugation) since it contains large RNA.
	• Mix immediately after addition of ethanol to avoid too high local alcohol concentrations.
	• Rotate the NucleoSpin® RNA Column by 180 ° inside the centrifuge and repeat the loading step as often as necessary until all lysate has completely passed the column.
	• Increase centrifugation time and speed to load the sample.
	• Use NucleoSpin® Filters after ethanol addition to homogenize the lysate. Additional NucleoSpin® Filters can be ordered separately, see ordering information.
Clogged NucleoSpin® Protein Removal Column	***Too much protein precipitate or precipitate too fine***
	• Pellet the protein by centrifugation before loading the cleared lysate onto the NucleoSpin® Protein Removal Column.
	• Rotate the NucleoSpin® Protein Removal Column by 180 ° inside the centrifuge and repeat the protein removal step.
	• Increase centrifugation time and speed and repeat the protein removal step.
Contamination with genomic DNA	***Too much cell material used***
	• Reduce quantity of cells or tissue used.
	DNA detection system too sensitive
	• Use larger PCR targets (e.g., > 500 bp) or intron spanning primers for RNA analysis.

Problem	Possible cause and suggestions
Suboptimal performance of RNA in downstream experiments	***Carry over of ethanol or salt*** • Do not let the flowthrough touch the column outlet after the second MW2 wash. Make sure to centrifuge at the corresponding speed for the respective time in order to remove ethanolic Buffer MW2 completely. • Check if Buffer MW2 has been equilibrated to room temperature before use. Washing at lower temperatures lowers efficiency of salt removal. ***Store isolated RNA properly*** • Eluted RNA should always be kept on ice for optimal stability since trace contaminations of omnipresent RNases (general lab ware, fingerprints, dust) will degrade the isolated RNA. For short term storage freeze at -20 °C, for long term storage freeze at -70 °C.

6.2 Ordering information

Product	REF	Pack of
NucleoSpin® miRNA	740971.10 / .50 / .250	10 / 50 / 250 preps
NucleoZOL	740404.200	200 mL
NucleoSpin® Bead Tubes Type D (containing 3 mm steel balls)	740814.50	50
NucleoSpin® Bead Tubes Type E (contain a combination of 3 mm steel balls and 40 – 400 µm glass beads)	740815.50	50
Bead Tube Holder	740469	1 piece
Buffer ML	740973.30	30 mL
Buffer MW2 (concentrate)	740994.100	100 mL
RNase A	740505	100 mg
NucleoSpin® miRNA Column / Buffer Set (50 columns, 35 mL MW1, 20 mL MW2 Concentrate)	740304	1 set
Protein Quantification Assay	740967.50 / .250	50 / 250
Protein Solving Buffer Set (107 mg TCEP, 7.5 mL PSB)	740941	1 set
Carrier RNA (lyophilized)	740514	0.3 mg
rDNase Set (1 vial rDNase (size D), 7 mL Reaction Buffer for rDNase)	740963	1 set
NucleoSpin® Filters	740606	50
NucleoSpin® Collection Tubes (2 mL)	740600	1000
NucleoSpin® RNA / DNA Buffer Set	740944 / .10	100 / 10 preps

Visit ***www.mn-net.com*** for more detailed product information.

6.3 Product use restriction/warranty

WARRANTY DISCLAIMER

All MACHEREY-NAGEL products are designed for their intended use only. They are not intended to be used for any other purpose. The description of the intended use of the products can be found in the original MACHEREY-NAGEL product leaflets. Before using our products, please observe the instructions for use and the safety instructions from the respective Material Safety Data Sheet of the product.

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Last updated: 08/2022, Rev. 04

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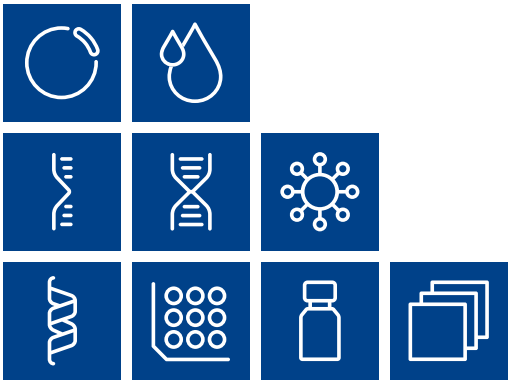
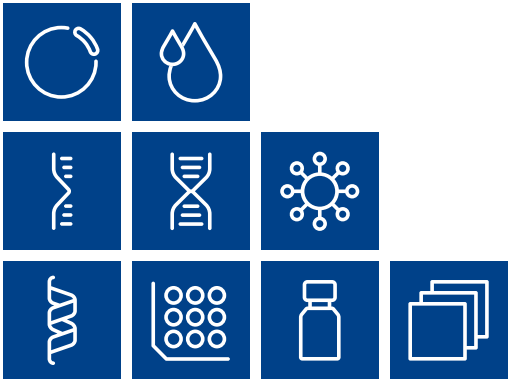


Plasmid DNA
Clean up
RNA
DNA
Viral RNA and DNA
Protein
High throughput
Accessories
Auxiliary tools



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