Isolation of small and large RNA

User manual

NucleoSpin® miRNA

November 2011 / Rev. 02

MACHEREY-NAGEL

6.1: RNA purification from animal tissue and cultured cells: small and large RNA in one fraction (total RNA) S+L

Protocol-at-a-glance (Rev. 02)

NucleoSpin® miRNA

1 **Cell lysis**		< 30 mg animal tissue or < 10^7 cultured cells 300 µL ML 5 min RT
2 **Homogenization of the lysate**	 **Violet** ring	Load sample 1 min 11,000 x *g*
3 **Adjust binding conditions for large RNA and DNA**		150 µL ethanol (96–100 %) Vortex 5 s 5 min RT
4 **Bind large RNA and DNA**	 **Blue** or **green** ring	Load sample 1 min 14,000 x *g*

Large RNA bound to column

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

Small RNA in column flow-through

7 **Precipitate protein**		300 µL MP Vortex 5 s
8 **Collect/ remove protein precipitate**	 **White** ring	3 min 11,000 x *g* Load supernatant 1 min 11,000 x *g*
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** or **green** ring	Load sample stepwise 30 s 11,000 x *g*
11 **Wash and dry silica membrane**	 **Blue** or **green** ring	1st 600 µL MW1 30 s 11,000 x *g* 2nd 700 µL MW2 30 s 11,000 x *g* 3rd 250 µL MW2 2 min 11,000 x *g*
12 **Elute small and large RNA**	 **Blue** or **green** ring	30–100 µL RNase-free H_2O 1 min RT 30 s 11,000 x *g*

MACHEREY-NAGEL GmbH & Co. KG · Neumann-Neander-Str. 6–8 · 52355 Düren · Germany
Tel.: +49 24 21 969-270 · Fax: +49 24 21 969-199 · tech-bio@mn-net.com · ***www.mn-net.com***

6.1: RNA purification from animal tissue and cultured cells: small and large RNA in two fractions (S L) / small RNA (S)

Protocol-at-a-glance (Rev. 02)

NucleoSpin® miRNA

1	**Cell lysis**	< 30 mg animal tissue or < 10^7 cultured cells 300 µL ML 5 min RT
2	**Homogenization of the lysate**	**Violet** ring Load sample 1 min 11,000 x *g*
3	**Adjust binding conditions for large RNA and DNA**	150 µL ethanol (96–100 %) Vortex 5 s 5 min RT
4	**Bind large RNA and DNA**	**Blue** ring Load sample 1 min 14,000 x *g*

Large RNA bound to column

5	**Desalt silica membrane**	**Blue** ring 350 µL MDB 1 min 11,000 x *g*
6	**Digest DNA**	**Blue** ring 100 µL rDNase >15 min RT
11	**Wash and dry silica membrane**	**Blue** ring **1st** 600 µL MW1 30 s 11,000 x *g* **2nd** 700 µL MW2 30 s 11,000 x *g* **3rd** 250 µL MW2 2 min 11,000 x *g*
12	**Elute large RNA**	**Blue** ring 30–100 µL RNase-free H_2O 1 min RT 30 s 11,000 x *g*

Small RNA in column flow-through

7	**Precipitate protein**	300 µL MP Vortex 5 s
8	**Collect/ remove protein precipitate**	**White** ring 3 min 11,000 x *g* Load supernatant 1 min 11,000 x *g*
9	**Adjust binding conditions for small RNA**	800 µL MX Vortex 5 s
10	**Bind small RNA**	**Green** ring Load sample stepwise 30 s 11,000 x *g*
11	**Wash and dry silica membrane**	**Green** ring **1st** 600 µL MW1 30 s 11,000 x *g* **2nd** 700 µL MW2 30 s 11,000 x *g* **3rd** 250 µL MW2 2 min 11,000 x *g*
12	**Elute small RNA**	**Green** ring 30–100 µL RNase-free H_2O 1 min RT 30 s 11,000 x *g*

MACHEREY-NAGEL GmbH & Co. KG · Neumann-Neander-Str. 6–8 · 52355 Düren · Germany
Tel.: +49 24 21 969-270 · Fax: +49 24 21 969-199 · tech-bio@mn-net.com · ***www.mn-net.com***

6.2: RNA purification from plant tissue: small and large RNA in one fraction (total RNA) S+L

Protocol-at-a-glance (Rev. 02)

NucleoSpin® miRNA

1 Cell lysis		< 50 mg plant tissue 400 µL ML 5 min RT
2 Optional: ***Phenol :chloroform extraction***		*1 vol acidic phenol:chloroform* *Vortex 30 s* *10 min 11,000 x* g *Separate phases and proceed with step 4*
3 Clarification of the lysate	 **Violet** ring	Load sample 1 min 11,000 x *g*
4 Adjust binding conditions for large RNA and DNA		150 µL ethanol (96–100 %) Vortex 5 s 5 min RT
5 Bind large RNA and DNA	 **Blue** or **green** ring	Load sample 2 min 11,000 x *g*

Large RNA bound to column

Small RNA in column flow-through

6 Desalt silica membrane	 **Blue** or **green** ring	350 µL MDB 1 min 11,000 x *g*
7 Digest DNA	 **Blue** or **green** ring	100 µL rDNase >15 min RT

8 Adjust binding conditions for small RNA		800 µL MX Vortex 5 s

Binding of small RNA to column containing large RNA

9 Bind small RNA	 **Blue** or **green** ring	Load sample stepwise 30 s 11,000 x *g*
10 Wash and dry silica membrane	 **Blue** or **green** ring	**1st** 600 µL MW1 30 s 11,000 x *g* **2nd** 700 µL MW2 30 s 11,000 x *g* **3rd** 250 µL MW2 2 min 11,000 x *g*
11 Elute small and large RNA	 **Blue** or **green** ring	30–100 µL RNase-free H_2O 1 min RT 30 s 11,000 x *g*

MACHEREY-NAGEL GmbH & Co. KG · Neumann-Neander-Str. 6–8 · 52355 Düren · Germany
Tel.: +49 24 21 969-270 · Fax: +49 24 21 969-199 · tech-bio@mn-net.com · ***www.mn-net.com***

6.2: RNA purification from plant tissue: <u>small and large</u> RNA in two fractions S L / <u>small</u> RNA S

Protocol-at-a-glance (Rev. 02)

NucleoSpin® miRNA

1 Cell lysis		< 50 mg plant tissue 400 µL ML 5 min RT
2 Optional: ***Phenol :chloroform extraction***		*1 vol acidic phenol:chloroform* *Vortex 30 s* *10 min 11,000 x* g *Separate phases and proceed with step 4*
3 Clarification of the lysate	 **Violet** ring	Load sample 1 min 11,000 x *g*
4 Adjust binding conditions for large RNA and DNA		150 µL ethanol (96–100 %) Vortex 5 s 5 min RT
5 Bind large RNA and DNA	 **Blue** ring	Load sample 2 min 11,000 x *g*

<u>Large</u> RNA bound to column

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

<u>Small</u> RNA in column flow-through

8 Adjust binding conditions for small RNA		800 µL MX Vortex 5 s
9 Bind small RNA	 **Green** ring	Load sample stepwise 30 s 11,000 x *g*
10 Wash and dry silica membrane	 **Green** ring	**1st** 600 µL MW1 30 s 11,000 x *g* **2nd** 700 µL MW2 30 s 11,000 x *g* **3rd** 250 µL MW2 2 min 11,000 x *g*
11 Elute small RNA	 **Green** ring	30–100 µL RNase-free H_2O 1 min RT 30 s 11,000 x *g*

10 Wash and dry silica membrane	 **Blue** ring	**1st** 600 µL MW1 30 s 11,000 x *g* **2nd** 700 µL MW2 30 s 11,000 x *g* **3rd** 250 µL MW2 2 min 11,000 x *g*
11 Elute large RNA	 **Blue** ring	30–100 µL RNase-free H_2O 1 min RT 30 s 11,000 x *g*

MACHEREY-NAGEL GmbH & Co. KG · Neumann-Neander-Str. 6–8 · 52355 Düren · Germany
Tel.: +49 24 21 969-270 · Fax: +49 24 21 969-199 · tech-bio@mn-net.com · ***www.mn-net.com***

6.3: RNA purification in combination with TRIzol® lysis: small and large RNA in one fraction (total RNA) S+L

Protocol-at-a-glance (Rev. 02)

	NucleoSpin® miRNA	
1 Prepare sample		< 50 mg animal tissue, < 10^7 cultured cells, or < 50 µL liquid sample 800 µL TRIzol®
2 Cell lysis		Vortex 15 s 5 min RT
3 Phase separation		160 µL chloroform Vortex 15 s 3 min RT 15 min 12,000 x *g* 2–8 °C Transfer 400 µL aqueous upper phase
4 Adjust binding conditions		1000 µL MX Vortex 5 s
5 Bind small and large RNA	 **Blue** or **green** ring	Load sample stepwise 30 s 11,000 x *g*

(Steps 6 - 9 are not necessary with this protocol)

10 Wash and dry silica membrane	 **Blue** or **green** ring	**1st** 600 µL MW1 30 s 11,000 x *g* **2nd** 700 µL MW2 30 s 11,000 x *g* **3rd** 250 µL MW2 2 min 11,000 x *g*
11 Elute small and large RNA	 **Blue** or **green** ring	30–100 µL RNase-free H_2O 1 min RT 30 s 11,000 x *g*

MACHEREY-NAGEL GmbH & Co. KG · Neumann-Neander-Str. 6–8 · 52355 Düren · Germany
Tel.: +49 24 21 969-270 · Fax: +49 24 21 969-199 · tech-bio@mn-net.com · ***www.mn-net.com***

6.3: RNA purification in combination with TRIzol® lysis: small and large RNA in two fraction SL / small RNA S

Protocol-at-a-glance (Rev. 02)

NucleoSpin® miRNA

1 Prepare sample		< 50 mg animal tissue, < 10^7 cultured cells, or < 50 µL liquid sample 800 µL TRIzol®
2 Cell lysis		Vortex 15 s 5 min RT
3 Phase separation		160 µL chloroform Vortex 15 s 3 min RT 15 min 12,000 x *g* 2–8 °C Transfer 400 µL supernatant
4 Adjust binding conditions for large RNA and DNA		200 µL ethanol (96–100 %) Vortex 5 s 5 min RT
5 Bind large RNA and DNA	 **Blue** ring	Load sample 30 s 11,000 x *g*

Large RNA bound to column

6 Desalt silica membrane	 **Blue** ring	350 µL MDB 1 min 11,000 x *g*
7 Digest DNA	 **Blue** ring	100 µL rDNase > 15 min RT
10 Wash and dry silica membrane	 **Blue** ring	1st 600 µL MW1 30 s 11,000 x *g* 2nd 700 µL MW2 30 s 11,000 x *g* 3rd 250 µL MW2 2 min 11,000 x *g*
11 Elute large RNA	 **Blue** ring	30–100 µL RNase-free H_2O 1 min RT 30 s 11,000 x *g*

Small RNA in column flow-through

8 Adjust binding conditions for small RNA		800 µL MX Vortex 5 s
9 Bind small RNA	 **Green** ring	Load sample stepwise 30 s 11,000 x *g*
10 Wash and dry silica membrane	 **Green** ring	1st 600 µL MW1 30 s 11,000 x *g* 2nd 700 µL MW2 30 s 11,000 x *g* 3rd 250 µL MW2 2 min 11,000 x *g*
11 Elute small RNA	 **Green** ring	30–100 µL RNase-free H_2O 1 min RT 30 s 11,000 x *g*

MACHEREY-NAGEL GmbH & Co. KG · Neumann-Neander-Str. 6–8 · 52355 Düren · Germany
Tel.: +49 24 21 969-270 · Fax: +49 24 21 969-199 · tech-bio@mn-net.com · ***www.mn-net.com***

6.4: Purification of siRNA and large dsRNA from DICER reactions: small and large RNA in two fractions

Protocol-at-a-glance (Rev. 02)

NucleoSpin® miRNA

1	**Prepare sample**	 150 µL DICER reaction mix 150 µL ML Vortex 5 s
2	**Adjust binding conditions for large dsRNA**	 200 µL ethanol (96–100 %) Vortex 5 s 5 min RT
3	**Bind large dsRNA**	 **Blue** ring Load sample 30 s 11,000 x *g*

Large dsRNA bound to column

6	**Wash and dry silica membrane**	 **Blue** ring **1st** 700 µL MW2 30 s 11,000 x *g* **2nd** 250 µL MW2 2 min 11,000 x *g*
7	**Elute large dsRNA**	 **Blue** ring 30–100 µL RNase-free H_2O 1 min RT 30 s 11,000 x *g*

siRNA in column flow-through

4	**Adjust binding conditions for siRNA**	 100 µL MP Vortex 5 s 5 min RT 800 µL MX Vortex 5 s
5	**Bind siRNA**	 **Green** ring Load sample stepwise 30 s 11,000 x *g*
6	**Wash and dry silica membrane**	 **Green** ring **1st** 700 µL MW2 30 s 11,000 x *g* **2nd** 250 µL MW2 2 min 11,000 x *g*
7	**Elute siRNA**	 **Green** ring 30–100 µL RNase-free H_2O 1 min RT 30 s 11,000 x *g*

MACHEREY-NAGEL GmbH & Co. KG · Neumann-Neander-Str. 6–8 · 52355 Düren · Germany
Tel.: +49 24 21 969-270 · Fax: +49 24 21 969-199 · tech-bio@mn-net.com · ***www.mn-net.com***

6.5: Fractionation of pre-purified RNA in small RNA and large RNA

Protocol-at-a-glance (Rev. 02)

NucleoSpin® miRNA

1	**Prepare sample**	 150 µL pre-purified RNA in water or low-salt elution buffer 150 µL ML Vortex 5 s
2	**Adjust binding conditions for large RNA**	 100 µL ethanol (96–100 %) Vortex 5 s 5 min RT
3	**Bind large RNA**	 **Blue** ring Load sample 30 s 11,000 x *g*

Large RNA bound to column

6	**Wash and dry silica membrane**	 **Blue** ring **1st** 700 µL MW2 30 s 11,000 x *g* **2nd** 250 µL MW2 2 min 11,000 x *g*
7	**Elute large RNA**	 **Blue** ring 30–100 µL RNase-free H_2O 1 min RT 30 s 11,000 x *g*

Small RNA in column flow-through

4	**Adjust binding conditions for small RNA**	 800 µL MX Vortex 5 s
5	**Bind small RNA**	 **Green** ring Load sample stepwise 30 s 11,000 x *g*
6	**Wash and dry silica membrane**	 **Green** ring **1st** 700 µL MW2 30 s 11,000 x *g* **2nd** 250 µL MW2 2 min 11,000 x *g*
7	**Elute small RNA**	 **Green** ring 30–100 µL RNase-free H_2O 1 min RT 30 s 11,000 x *g*

MACHEREY-NAGEL GmbH & Co. KG · Neumann-Neander-Str. 6–8 · 52355 Düren · Germany
Tel.: +49 24 21 969-270 · Fax: +49 24 21 969-199 · tech-bio@mn-net.com · ***www.mn-net.com***

Table of contents

1 Components

1.1 Kit contents

	NucleoSpin® miRNA		
	10 preps*	**50 preps***	**250 preps***
REF	**740971.10**	**740971.50**	**740971.250**
Lysis Buffer ML	5 mL	30 mL	125 mL
Protein Precipitation Buffer MP	5 mL	20 mL	100 mL
Binding Buffer MX	15 mL	2 x 30 mL	250 mL
Membrane Desalting Buffer MDB	10 mL	25 mL	125 mL
Reaction Buffer for rDNase	3 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	2 x 6 mL	50 mL
RNase-free H_2O	5 mL	15 mL	30 mL
NucleoSpin® Filters (violet rings)	10	50	250
NucleoSpin® RNA Columns (blue rings)	5	25	125
NucleoSpin® miRNA Columns (green rings)	5	25	125
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
User manual	1	1	1

* For detailed information on procedures and the corresponding number of preps see section 2.

** For preparation of working solutions and storage conditions see section 4.

1.2 Reagents, consumables, and equipment to be supplied by the user

Reagents

- 96–100 % ethanol
- Optional for RNA purifcation from plant tissue (section 6.2): Acidic (pH 4.5–4.7) phenol : choroform
- For RNA purification with TRIzol® lysis (section 6.3): TRIzol® or equivalent phenol solution (e.g., QIAzol®, TRI Reagent®), and chloroform

Consumables

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

Equipment

- Manual pipettors
- Centrifuge for microcentrifuge tubes
- Equipment for sample disruption and homogenization (see section 3.3)
- 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***.

Please contact Technical Service regarding information about changes of the current user manual compared to previous revisions.

Table 1: Protocol overview

Sample type	Sample size	Purification of	Section
Cultured cells	< 10^7	Small, large, total RNA, protein	6.1
Human/animal tissue	< 30 mg	Small, large, total RNA, protein	6.1
Plant tissue	< 50 mg	Small, large, total RNA	6.2
Any (in combination with TRIzol®, QIAzol®, or similar reagents)	< 50 µL (liquid samples) < 50 mg (animal/plant tissue) < 10^7 (cultured cells)	Small, large, total RNA	6.3
Reaction mix (e.g., DICER)	< 150 µL	siRNA, large dsRNA	6.4
Pre-purified RNA	< 150 µL	Small, large RNA	6.5

2 Kit specifications

- The **NucleoSpin® miRNA** kit is designed for the simultaneous isolation of small RNA (< 200 nt), large RNA (> 200 nt), and protein in three separate fractions (patent pending) from a large variety of sample materials (Table 1). For isolation of miRNA from body fluids like blood plasma, serum, saliva, urine, etc. please contact Technical Service for details.

- When small and large RNA is purified in two separate fractions, two binding columns are necessary, one to bind the large RNA (< 200 nt) and one to bind the small RNA. However, the **NucleoSpin® RNA Columns** (blue rings) and the **NucleoSpin® miRNA Columns** (green rings) only differ by their ring color for easier differentiation during the preparation. Therefore, the kits can be used either for 5, 25, 125 preps of fractionated RNA using two columns per prep as well as for 10, 50, 250 total RNA preparations with only one column per prep (Table 2).

- **NucleoSpin® Filters** (violet rings) are included for conveniently homogenizing and clearing of crude lysates.

- **NucleoSpin® Protein Removal Columns** (white rings) allow for fast and quantitative removal of the precipitated protein fraction to obtain highest purity of the small RNA fraction.

- The precipitated protein can be easily dissolved in Laemmli buffer and used for SDS-PAGE, Western Blot analysis, and protein quantification, for example with the MACHEREY-NAGEL Protein Quantification Assay (see ordering information).

- Residual genomic DNA is removed by on-column digest with the provided RNase-free recombinant **DNase**.

- No need for toxic and cumbersome phenol-chloroform extractions.

- The eluted RNA and miRNA are ready-to-use for all standard downstream applications, for example RT-PCR, Northern Blot, chip hybridization, etc.

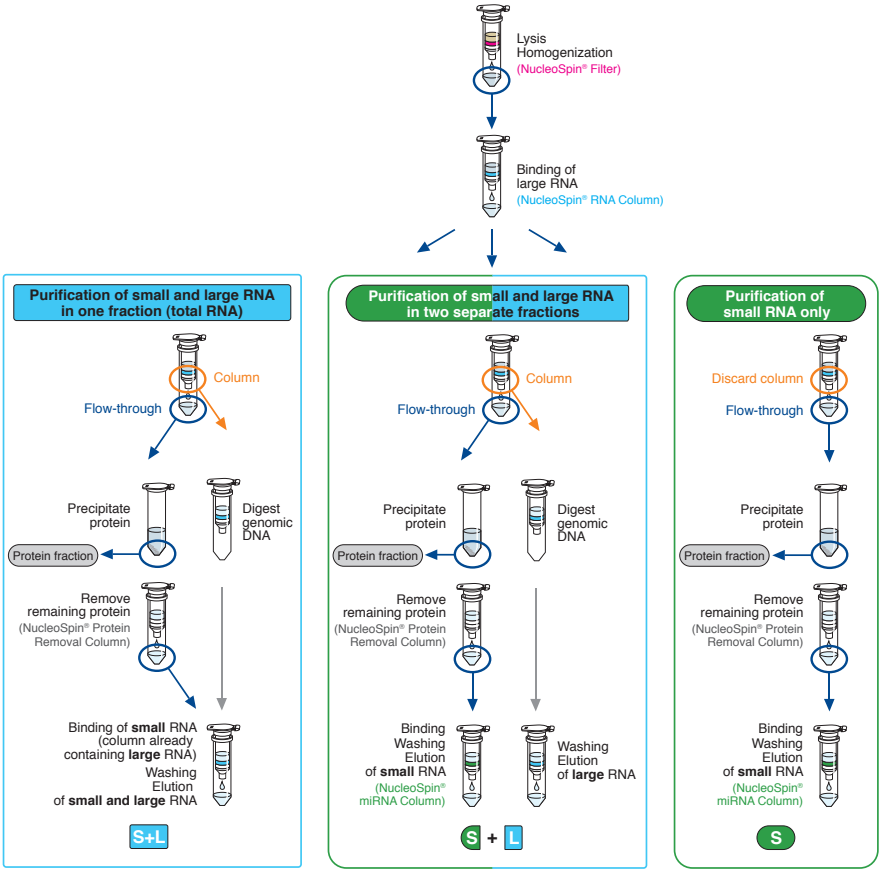

Figure 1: Overview of procedures

Table 2: Overview of procedures			
Purification of	**Small and large RNA in one fraction (total RNA)**	**Small and large RNA in two separate fractions**	**Small RNA only**
Follow protocol steps marked with	S+L	S L	S
NucleoSpin® RNA / miRNA Columns used	NucleoSpin® RNA **OR** NucleoSpin® miRNA	NucleoSpin® RNA **AND** NucleoSpin® miRNA	NucleoSpin® RNA **AND** NucleoSpin® miRNA
Kit contents sufficient for [number of preps]			
REF 740971.10	10 x total RNA*	5 x small RNA 5 x large RNA	5 x small RNA (large RNA is discarded)
REF 740971.50	50 x total RNA*	25 x small RNA 25 x large RNA	25 x small RNA (large RNA is discarded)
REF 740971.250	250 x total RNA*	125 x small RNA 125 x large RNA	125 x small RNA (large RNA is discarded)

Table 3: Kit specifications at a glance	
Parameter	**NucleoSpin® miRNA**
Sample size	< 10^7 cultured cells < 30 mg human/animal tissue < 50 mg plant material < 150 µL reaction mix
Fragment size small RNA	< 200 nt
Fragment size large RNA	> 200 nt
Binding capacity	200 µg (NucleoSpin® RNA/miRNA Column)
Elution volume	30–100 µL (NucleoSpin® RNA/miRNA Column)
Preparation time	< 45 min (6 preps animal tissue – small and large RNA) < 35 min (6 preps animal tissue – small RNA)

* With the protocol for isolation of small and large RNA in one fraction (total RNA) only one RNA binding column per preparation is used. The NucleoSpin® RNA Columns (blue rings) and the NucleoSpin® miRNA Columns (green rings) only differ by their ring color. Therefore, for isolation of total RNA please use either a NucleoSpin® RNA Column (blue ring) or a NucleoSpin® miRNA Column (green ring) for being able to perform the total number of 10, 50, and 250 preparations with the kits.

Table 4: Typical yields

Sample material	Amount	Protocol section	Yield total RNA	Yield fractionated RNA Large RNA	Small RNA
Mouse liver	30 mg	6.1	100 µg	105 µg	19 µg
Mouse kidney	30 mg	6.1	35 µg	31 µg	9 µg
Mouse spleen	30 mg	6.1	48 µg	36 µg	22 µg
Mouse lung	30 mg	6.1	27 µg	21 µg	9 µg
Mouse heart	30 mg	6.3	24 µg	19 µg	4 µg
Porcine liver	30 mg	6.1	80 µg	70 µg	13 µg
Human brain	30 mg	6.1	11 µg	10 µg	3 µg
Human brain	30 mg	6.3	17 µg	14 µg	3 µg
HeLa	10^7 cells	6.1	100 µg	100 µg	10 µg
Wheat leaves	50 mg	6.2	40 µg	25 µg	20 µg

3 Product description

3.1 The basic principle

The **NucleoSpin® miRNA** kit offers the unique feature (patent pending) to isolate separate fractions of large and small RNA as well as proteins without the need of cumbersome phenol/chloroform extraction. Gene expression can thus be analyzed on the level of transcription (mRNA or miRNA) and on the level of translated proteins.

The sample material is mechanically disrupted in Lysis Buffer ML. After addition of ethanol, DNA and large RNA (> 200 nt, e.g., 18S, 28S RNA, pri-miRNA) are bound to the NucleoSpin® RNA Column (blue ring). The DNA is digested on the column by an RNase-free rDNase. Afterwards, the large RNA can be washed and eluted with RNase-free water.

The flow-through of the first column still contains small RNA and protein. The protein is precipitated by Protein Precipitation Buffer MP, pelleted by centrifugation, and completely removed by filtering through a NucleoSpin® Protein Removal Column. The precipitated protein can be easily dissolved in Laemmli buffer and used for SDS-PAGE, Western Blot analysis, and protein quantification (see section 3.4 for more information).

After the protein precipitation step the cleared sample only contains small RNA (<200 nt/bp, for example, miRNA, pre-miRNA, tRNA, 5S RNA). After addition of Binding Buffer MX the small RNA is bound to the NucleoSpin® miRNA Column (green ring), washed, and eluted with RNase-free water.

Additionally, total RNA can be obtained as an alternative to the RNA fractionation by binding large and small RNA successively to just one NucleoSpin® RNA Column.

3.2 Amount of starting material and choice of procedure

Binding of RNA to the silica membrane follows a highly cooperative mechanism, i.e. RNA can bind the better to the membrane the more other RNA molecules are around thus working like carrier RNA.

Ideally, the amount of starting material should be at the upper limit of the range given in Table 3, for example 5 x 10^6–10^7 cultured cells or 15–30 mg of tissue. In this case the expected RNA yield will be high enough to allow an efficient binding.

If less sample material is available, the yield can be increased by following the total RNA purification procedure rather than isolating separate fractions of small and large RNA. Since the small RNA fraction is usually only 10–20 % of the total RNA, the small RNA is much more affected by the decreasing binding efficiency.

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 the binding step of small RNA to improve RNA binding. For this prepare a Carrier RNA stock solution of 1 µg/µL RNase-free H_2O (e.g., dissolve 0.3 mg Carrier RNA in

300 µL RNase-free H_2O). Add 10 µL of the stock solution to the cleared lysate before adjusting the binding conditions for small RNA with Buffer MX. Mix well before addition of Buffer MX and proceed with the corresponding protocol.

3.3 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 samples are flash frozen in liquid nitrogen immediately and stored at -70 °C or processed as soon as possible. After disruption samples can be stored in Lysis Buffer ML at -70 °C for up to one year, at +4 °C for up to 24 hours, or up to several hours at room temperature. Frozen samples are stable for at least up to 6 months. Frozen samples in Lysis Buffer ML should be thawed slowly before starting with the RNA isolation.

Wear gloves at all times during the preparation. Change gloves frequently. Use only RNase-free equipment and sterile filter tips.

Cultured tissue and cells can be collected by centrifugation (after trypsinization, if necessary) and directly lysed by adding Lysis Buffer ML according to the standard protocol. Adherent growing cells in a culture dish can also be lysed directly in the dish: Completely aspirate cell culture medium and continue immediately with the addition of Lysis Buffer ML to the cell culture. Remove the culture medium completely in order to guarantee full lysis activity of the lysis buffer.

Animal and plant tissue are often solid and must therefore be broken up mechanically prior to the chemical lysis. Depending on the disruption method, the viscosity of the lysed sample has to be reduced further for optimal results. It is essential for an efficient RNA preparation that all the RNA contained in the sample is released from the cells by disruption and that the viscosity of the sample is reduced by homogenization.

The most commonly used technique for disruption of animal and plant tissues is grinding with a **mortar and pestle**. Pre-cool mortar and pestle with liquid nitrogen. Grind the frozen sample thoroughly to a fine powder in the presence of liquid nitrogen. Take care that the sample does not thaw during or after grinding or weighing. Add the frozen powder to an appropriate aliquot of Lysis Buffer ML and mix immediately. The broken-up tissue must then be homogenized with a **NucleoSpin® Filter** (tissue and plant) or by passing ≥ 5 times through a 0.9 mm syringe needle (soft tissue). If the tissue contains a very large amount of DNA, for example spleen, which tends to precipitate upon addition of ethanol, NucleoSpin® Filters can be applied after ethanol addition to avoid clogging of the NucleoSpin® RNA Column (Additional NucleoSpin® Filters can be ordered separately, see ordering information).

Alternatively, **rotor-stator homogenizers, bead-mills** or other commercially available devices can be used to disrupt the sample material by spinning rotors or grinding with glass or steel beads. This has to be done exclusively in the presence of Lysis Buffer ML to ensure that the RNA is protected against RNases present in the sample. The mechanical force not only breaks up the sample but simultaneously homogenizes the sample by mechanical shearing of DNA within seconds or minutes (homogenization

time depends on sample). Take care to keep the rotor tip submerged in order to avoid excess foaming. Select suitably sized beads and homogenizers (5–7 mm diameter rotors can be used for homogenization in 1.5 mL centrifuge tubes).

Bacteria and yeast have to be incubated in lysozyme or lyticase/zymolase solutions, respectively. By this treatment, the robust cell walls of these organisms are digested or at least weakened, which is essential for effective cell lysis by Buffer ML. For microorganisms with extremely resistant cell walls – like some Gram-positive bacterial strains – it may be necessary to optimize the conditions of the treatment with lytic enzymes or the cultivation conditions. After lysis, homogenization is achieved by using a **NucleoSpin® Filter** or the syringe-needle method.

3.4 Analysis of the protein fraction

Protein isolation

The sample is lysed under chaotropic salt conditions and large RNA and DNA are removed by binding to a silica membrane. The remaining lysate only contains small RNA and protein which can be precipitated by Buffer MP.

Depending on the amount and nature of the sample material the protein can turn the lysate just a little turbid or it can appear as a white to brownish thick flocculate.

The precipitate can easily be pelleted by centrifugation and used for further analysis. The remaining protein in the sample is removed by filtration through a NucleoSpin® Protein Removal Column.

Choose optimal sample volume to precipitate protein

If the total protein of large sample amounts (e.g., 30 mg tissue, 10^6–10^7 cells) is precipitated according to the standard protocol, the protein pellet may be too large, which in turn may lead to problems in protein dissolution. Additionally, already very little amounts of dissolved protein are sufficient for SDS-PAGE analysis. Therefore, in this case it is recommended to take only a small amount of protein for further analysis to facilitate the dissolution:

- Precipitate the total protein from the entire sample with Buffer MP according to the protocol.
- **Before** the centrifugation step take a small portion (e.g., 10–20 µL) of the precipitated but still resuspended material to pellet the protein separately from this small portion.
- Pellet the protein from the **main sample** as well as from the **small portion** according to the protocol.
- Proceed with the supernatant of the main sample to remove the residual protein precipitate and isolate the small RNA according to the protocol. The supernatant from the small portion may be discarded or added to the supernatant of the main sample in order not to lose any small RNA.
- Use the protein pellet of the small portion for further analysis of the protein fraction.

Wash and dry protein pellet

Remove supernatant by pipetting as completely as possible. Add 500 µL of 50 % ethanol to the pellet (mixing or incubation is not necessary), and centrifuge 1 min at 11,000 x *g*. Again, remove supernatant completely. Open the lid and dry the protein pellet for 5–10 min at room temperature.

Protein dissolution

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

Dissolving a protein pellet becomes more difficult as the pellet size increases. Whereas a few minutes are enough to dissolve the total protein of 10^6 cultured cells in 50–100 µL buffer it might be almost impossible to dissolve total protein from 10^7 cells or 30 mg of tissue protein. In this case, precipitate smaller sample volumes as described above.

Protein analysis

Protein dissolved in SDS containing buffers like Laemmli buffer as described above can readily be used for SDS-PAGE. However, most protein quantification assays like 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.

3.5 Elution procedures

Standard elution procedures

Final RNA yield and concentration is highly dependent on the volume and temperature of the RNase-free water used for elution. Higher elution volumes lead to higher RNA recovery, however, they lower the final RNA concentration. The optimal choice often depends on the kind of downstream application. The three elution volumes suggested in the standard protocols are:

- **30 µL** for **high concentration**: 30 µL are necessary to wet the entire silica membrane and are sufficient to elute most of the RNA. High RNA concentrations can be obtained, however, the total RNA yield will not be optimal. Lowering the elution volume to < 30 µL leads to a further significant decrease in yield.
- **50 µL** for **medium concentration** and **good yield**.
- **100 µL** for **high yield**.

Modifications of the standard elution procedures

The following modifications can be used to further increase the yield and concentration:

- To increase the yield, perform a **second elution step** with fresh RNase-free water.
- To increase the yield and the concentration perform a **second elution step** with **reapplying the eluate** of the first elution step for a second time. Especially the yield of large RNA can be increased by this modification.
- To increase the yield and the concentration heat the water to **90 °C** before elution. This especially increases the yield of large RNA up to 20 %. However, a high temperature leads to larger pipetting errors and consequently to higher variations from prep to prep in the final volume of the eluate.

Elution of large and small RNA in one total RNA fraction

According to the standard protocols of this User Manual total RNA can be obtained by loading both, large RNA and small RNA onto the same column. Alternatively, there are two simple elution options to obtain a total RNA fraction:

- Follow the standard protocols to purify small and large RNA in two separate fractions and just **combine both eluates**. The resulting dilution of the RNA can be avoided by reducing the individual elution volumes for example from 100 µL to 50 µL (see above). Note that a slightly reduced yield might be the consequence.
- Elute the large RNA from the NucleoSpin® RNA Column according to the standard protocols. Instead of fresh RNase-free water **use this large RNA eluate to elute the small RNA** from the NucleoSpin® miRNA Column.

Eluted RNA should immediately be kept on ice for optimal stability because almost omnipresent RNases (general lab ware, fingerprints, dust) will degrade RNA. For short-term storage freeze at -20 °C, for long-term storage freeze at -70 °C.

4 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 (18–25 °C) and are stable for at least one year. 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 (18–25 °C) 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		
	10 preps	**50 preps**	**250 preps**
REF	**740971.10**	**740971.50**	**740971.250**
Wash Buffer MW2 (Concentrate)	6 mL Add 24 mL 96–100 % ethanol	2 x 6 mL Add 24 mL 96–100 % ethanol to each bottle	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

5 Safety instructions

The following components of the **NucleoSpin® miRNA** kits contain hazardous contents.
Wear gloves and goggles and follow the safety instructions given in this section.

CAUTION: Buffer ML, MW1, and MBD 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.

5.1 Risk and safety phrases

Component ***Inhalt***	**Hazard contents** ***Gefahrstoff***	**Hazard symbol** ***Gefahrstoff-symbol***	**Risk phrases** ***R-Sätze***	**Safety phrases** ***S-Sätze***
rDNase, RNase-free	rDNase, lyophilized *rDNase, lyophilisiert*	Xn	R 42/43	S 22-24
ML	Guanidinium thiocyanate 30–60 % *Guanidiniumthiocyanat 30–60 %*	Xn*	R 20/21/22-32-52/53	S 13-61
MX	Dioxan 10–90 % *Dioxan 10-90 %*	F Xi*	R 11-19-36/37-40-66	S 9-16-36/37-46
MW1	Guanidinium thiocyanate 1–15 % + ethanol 55–75 % *Guanidiniumthiocyanat 1–15 % + Ethanol 55-75 %*	F	R 11	S 7-16
MDB	Guanidinium thiocyanate 1–15 % + ethanol 5–20 % *Guanidiniumthiocyanat 1–15 % + Ethanol 5-20 %*		R 10	

* Hazard labeling not necessary if quantity per bottle below 125 g or mL (certificate of exemption according to 67/548/EEC Art. 25, 1999/45/EC Art. 12 and German GefStoffV § 20 (3) and TRGS 200 7.1). For further information see Material Safety Data Sheet.

Risk phrases

R 10	Flammable. *Entzündlich.*
R 11	Highly flammable. *Leichtentzündlich.*
R 19	May form explosive peroxides. *Kann explosionsfähige Peroxide bilden.*
R 20/21/22	Harmful by inhalation, in contact with the skin and if swallowed. *Gesundheitsschädlich beim Einatmen, Verschlucken und Berührung mit der Haut.*
R 32	Contact with acids liberates very toxic gas. *Entwickelt bei Berührung mit Säure sehr giftige Gase.*
R 36/37	Irritating to eyes and respiratory system. *Reizt die Augen und die Atmungsorgane.*
R 40	Possible risk of irreversible effects. *Verdacht auf krebserzeugende Wirkung.*
R 42/43	May cause sensitization by inhalation and skin contact *Sensibilisierung durch Einatmen und Hautkontakt möglich.*
R 52/53	Toxic to aquatic organisms, may cause long-term adverse effects in the aquatic environment. *Schädlich für Wasserorganismen, kann in Gewässern längerfristig schädliche Wirkungen haben.*
R 66	Repeated exposure may cause skin dryness or cracking. *Wiederholter Kontakt kann zu spröder oder rissiger Haut führen.*

Safety phrases

S 7	Keep container tightly closed. *Behälter dicht geschlossen halten.*
S 9	Keep container in a well-ventilated place. *Behälter an einem gut gelüfteten Ort aufbewahren.*
S 13	Keep away from food, drink and animal foodstuffs. *Von Nahrungsmitteln, Getränken und Futtermitteln fernhalten.*
S 16	Keep away from sources of ignition - No smoking! *Von Zündquellen fernhalten. – Nicht rauchen!*
S 22	Do not breathe dust. *Staub nicht einatmen.*
S 24	Avoid contact with the skin. *Berührung mit der Haut vermeiden.*
S 36/37	Wear suitable protective clothing and gloves. *Bei der Arbeit geeignete Schutzhandschuhe und Schutzkleidung tragen.*
S 46	If swallowed, seek medical advice immediately and show this container or label. *Bei Verschlucken sofort ärztlichen Rat einholen und Verpackung oder Etikett vorzeigen.*
S 61	Avoid release to the environment. Refer to special instructions/safety data sheet. *Freisetzung in die Umwelt vermeiden. Besondere Anweisungen einholen / Sicherheitsdatenblatt zu Rate ziehen.*

5.2 GHS classification

Only harmful features do not need to be labeled with H and P phrases until 125 mL or 125 g.

Mindergefährliche Eigenschaften müssen bis 125 mL oder 125 g nicht mit H- und P-Sätzen gekennzeichnet werden.

Component *Inhalt*	Hazard contents *Gefahrstoff*	GHS symbol *GHS Symbol*	Hazard phrases *H-Sätze*	Precaution phrases *P-Sätze*
rDNase, RNase-free	rDNase, lyophilized *rDNase, lyophilisiert*	 Danger *Gefahr*	317, 334	261, 280, 302+352, 304+341, 333+313, 342+311, 363
ML	Guanidinium thiocyanate 30–60 % *Guanidiniumthiocyanat 30–60 %*	 Warning *Achtung*	302, 412, EUH031	260, 273, 301+312, 330
MX	Dioxan 10–90 % *Dioxan 10-90 %*	 Danger *Gefahr*	225, 319, 335, 351, EUH019, EUH066	202, 210, 261, 280, 304+340, 305+351+338, 308+313, 312, 337+313, 403+233, 403+235, 405
MW1	Guanidinium thiocyanate 1–15 % + ethanol 55–75 % *Guanidiniumthiocyanat 1–15 % + Ethanol 55-75 %*	 Danger *Gefahr*	225	210, 233, 403+235
MDB	Guanidinium thiocyanate 1–15 % + ethanol 5–20 % *Guanidiniumthiocyanat 1–15 % + Ethanol 5-20 %*	 Warning *Achtung*	226	210, 233, 403+235

Hazard phrases

H 225	Highly flammable liquid and vapour. *Flüssigkeit und Dampf leicht entzündbar.*
H 226	Flammable liquid and vapour. *Flüssigkeit und Dampf entzündbar.*
H 302	Harmful if swallowed. *Gesundheitsschädlich bei Verschlucken.*
H 317	May cause an allergic skin reaction. *Kann allergische Hautreaktionen verursachen.*
H 319	Causes serious eye irritation. *Verursacht schwere Augenreizung.*
H 334	May cause allergy or asthma symptoms or breathing difficulties if inhaled. *Kann bei Einatmen Allergie, asthmaartige Symptome oder Atembeschwerden verursachen.*
H 335	May cause respiratory irritation. *Kann die Atemwege reizen.*
H 351	Suspected of causing cancer. *Kann vermutlich Krebs erzeugen.*
H 412	Harmful to aquatic life with long lasting effects. *Schädlich für Wasserorganismen, mit langfristiger Wirkung.*
EUH019	May form explosive peroxides. *Kann explosionsfähige Peroxide bilden.*
EUH031	Contact with acids liberates toxic gas. *Entwickelt bei Berührung mit Säure giftige Gase.*
EUH066	Repeated exposure may cause skin dryness or cracking. *Wiederholter Kontakt kann zu spröder oder rissiger Haut führen.*

Precaution phrases

P 202	Do not handle until all safety precautions have been read and understood. *Vor Gebrauch alle Sicherheitsaratschläge lesen und verstehen.*
P 210	Keep away from heat / sparks / open flames / hot surfaces – No smoking. *Von Hitze / Funken / offener Flamme / heißen Oberflächen fernhalten. Nicht rauchen.*
P 233	Keep container tightly closed. *Behälter dicht verschlossen halten.*
P 260	Do not breathe vapours. *Dampf nicht einatmen.*
P 261	Avoid breathing dust. *Einatmen von Staub / Dampf vermeiden.*
P 273	Avoid release to the environment. *Freisetzung in die Umwelt vermeiden.*
P 280	Wear protective gloves / eye protection. *Schutzhandschuhe / Augenschutz tragen.*
P 301+312	IF SWALLOWED: Call a POISON CENTER or doctor / physician if you feel unwell. *BEI VERSCHLUCKEN: Bei Unwohlsein GIFTINFORMATIONSZENTRUM oder Arzt anrufen.*
P 302+352	IF ON SKIN: Wash with plenty of soap and water. *BEI BERÜHRUNG MIT DER HAUT: Mit viel Wasser und Seife waschen.*
P 304+340	IF INHALED: Remove victim to fresh air and keep at rest in a position comfortable for breathing.

	Bei Einatmen: Bei Atembeschwerden an die frische Luft bringen und in einer Position ruhigstellen, die das Atmen erleichtert.
P 304+341	IF INHALED: If breathing is difficult, remove to fresh air and keep at rest in a position comfortable for breathing. *BEI EINATMEN: An die frische Luft bringen und in einer Position ruhigstellen, die das Atmen erleichtert.*
P 305+351+338	IF IN EYES: Rinse continuously with water for several minutes. Remove contact lenses if present and easy to do – continue rinsing. *BEI KONTAKT MIT DEN AUGEN: Einige Minuten lang behutsam mit Wasser spülen. Vorhandene Kontaktlinsen nach Möglichkeit entfernen. Weiter ausspülen.*
P 308+313	IF exposed or concerned: Get medical advice / attention. *BEI Exposition oder falls betroffen: Ärztlichen Rat Einholen / ärztliche Hilfe hinzuziehen.*
P 312	Call a POISON CENTER or doctor / physician if you feel unwell. *Bei Unwohlsein GIFTINFORMATIONSZENTRUM oder Arzt anrufen.*
P 330	Rinse mouth. *Mund ausspülen.*
P 333+313	If skin irritation occurs: Get medical advice / attention. *Bei Hautreizung oder -ausschlag: Ärztlichen Rat einholen / ärztliche Hilfe hinzuziehen.*
P 337+313	Get medical advice / attention. *Bei anhaltender Augenreizung: Ärztlichen Rat einholen / ärztliche Hilfe hinzuziehen.*
P 342+311	If experiencing respiratory symptoms: Call a POISON CENTER or doctor / physician. *Bei Symptomen der Atemwege: GIFTINFORMATIONSZENTRUM oder Arzt anrufen.*
P 363	Wash contaminated clothing before reuse. *Kontaminierte Kleidung vor erneutem Tragen waschen.*
P 403+233	Store in a well ventilated place. Keep container tightly closed. *Behälter dicht verschlossen an einem gut belüfteten Ort aufbewahren.*
P 403+235	Store in a well ventilated place. Keep cool. *Kühl an einem gut belüfteten Ort aufbewahren.*
P 405	Store locked up. *Unter Verschluss aufbewahren.*

For further information please see Material Safety Data Sheets ***(www.mn-net.com).***

Weiterführende Informationen finden Sie in den Sicherheitsdatenblättern ***(www.mn-net.com)****.*

6 Protocols

6.1 RNA purification from animal tissue and cultured cells

Before starting the purification, check that 96–100 % ethanol is available.

For the miRNA purification three different procedures are possible:

1) S+L total RNA isolation – small RNA and large RNA in one single fraction
2) S L separated isolation – isolation of small and large RNA in two separate fractions
3) S miRNA isolation – isolation of small RNA fraction only

Please note that for the separation of small and large RNA (procedure 2) and 3)), two columns are used for one isolation. Both procedures result in 25 isolations for each fraction (50 prep kit). If procedure 2 or 3 are used exclusively, a separate column/buffer set is available to realize 50 isolations for each fraction (see ordering information).

1 Cell lysis

S+L S L See section 3.3 for more information on homogenization methods.

S Animal tissue

Thoroughly grind **animal tissue** under **liquid nitrogen** to a fine powder. Transfer up to **30 mg** to a 1.5 mL microcentrifuge tube (not provided) and add **300 µL Buffer ML**. Pipette up and down (> 5 times) or vortex to lyse the cells.

Disrupt sample

Alternatively, add **300 µL Buffer ML** to **30 mg animal tissue** and use a **rotor-stator, bead-mill,** or other devices to disrupt the cells.

+ 300 µL ML

Incubate for **5 min** at **room temperature (18–25 °C)**.

RT
5 min

Cultured animal cells

Collect up to **10^7 cultured cells** by centrifugation and add **300 µL Buffer ML**. Pipette cells up and down (> 5 times) or vortex to lyse the cells.

2 Homogenization of the lysate

S+L S L S

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

Load sample

11,000 x *g*
1 min

Upon pellet formation in the collection tube (depending on amount/nature of the sample) transfer the supernatant to a new 1.5 mL microcentrifuge tube (not provided) without disturbing the pellet.

Alternative: Soft samples can be homogenized by passing them > 5 times through a 0.9 mm needle (20 gauge) fitted to a syringe.

3 Adjust binding conditions for large RNA and DNA

S+L S L S

Discard the NucleoSpin® Filter. Add **exactly 150 µL 96–100 % ethanol** to **300 µL** homogenized lysate, close the lid, and vortex immediately for **5 s**.

300 µL lysate

+ 150 µL 96–100 % ethanol

Vortex 5 s

Incubate for **5 min** at **room temperature (18–25 °C)**.

RT
5 min

<u>Note</u>: After addition of ethanol a precipitate may become visible. Do not centrifuge the ethanolic lysate and be sure to load all of the precipitate onto the column in step 4.

<u>Note</u>: More than 300 µL of the lysate can be processed, however, the volumes of ethanol (step 3), Buffer MP (step 7) and Buffer MX (step 9) have to be increased proportionally and multiple loading steps might be necessary.

4 Bind large RNA and DNA

S+L S L S

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

Centrifuge for **1 min** at **14,000 x *g***.

Note: For total RNA purification S+L, both types of binding columns (blue or green ring) can be used. If the column clogs due to a large amount of precipitated nucleic acid, turn the NucleoSpin® RNA Column inside the centrifuge by 180° and repeat the spinning step at full speed. Repeat this as often as necessary until all liquid has completely passed the membrane. Additionally, NucleoSpin® Filters can be used to homogenize the sample after addition of ethanol (Additional NucleoSpin® Filters can be ordered separately, see ordering information).

Load sample

14,000 x *g*
1 min

S+L Purification of small and large RNA

S L **Save the flow-through** containing the small RNA for step 7. Transfer the NucleoSpin® RNA Column into a new Collection Tube (2 mL) and proceed with **→ step 5** to digest the DNA on the column.

S Purification of small RNA only

If you do not want to purify the large RNA fraction, discard the NucleoSpin® RNA Column, save the flow-through containing the small RNA and proceed with **→ step 7**.

5 Desalt silica membrane

S+L S L 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 the flow-through and place the column back into the collection tube.

11,000 x *g*
1 min

6 Digest DNA

S+L S L Add **100 µL rDNase** directly onto the silica membrane of the NucleoSpin® RNA Column (blue ring). Do not close the lid.

+ 100 µL rDNase

Incubate at **room temperature (18–25 °C)** until steps 7–10 are completed but at least **15 min**.

RT
> 15 min

7 Precipitate protein

S+L S L S

***Note*: The isolated protein can be easily dissolved in Laemmli buffer and used for SDS-PAGE, Western Blots, and protein quantification. See section 3.4 for detailed information.**

Add **300 µL Buffer MP** to the saved flow-through of step 4 containing only protein and small RNA, close the lid, and vortex for **5 s**.

+ 300 µL MP

Vortex 5 s

8 Collect/remove protein precipitate

S+L S L S

Centrifuge for **3 min** at **11,000 x *g*** to pellet protein. Use the protein pellet for further analysis of the protein fraction.

Place a **NucleoSpin® Protein Removal Column** (white ring) in a Collection Tube (2 mL, lid) and pipette or pour the supernatant containing small RNA and residual protein precipitate onto the column.

3 min
11,000 x *g*

Load supernatant

Centrifuge for **1 min** at **11,000 x *g*** to remove the residual protein precipitate. Discard the NucleoSpin® Protein Removal Column and **keep the flow-through.**

11,000 x *g*
1 min

Alternative: If the protein is not to be used, the entire sample including any precipitate can be pipetted or poured onto the NucleoSpin® Protein Removal Column and separated by centrifuging for 3 min at 11,000 x g.

9 Adjust binding conditions for small RNA

S+L S L S

Add **800 µL Buffer MX**, close the lid, and vortex for **5 s**.

Note: After addition of Buffer MX a precipitate may become visible. Do NOT centrifuge the mixture and be sure to load all of the precipitate onto the column in step 10.

+ 800 µL MX

Vortex 5 s

Note: The yield of small RNA from <10^6 cells or <3 mg tissue can be increased by addition of 10 µg Carrier RNA (see section 3.2 for detailed information).

10 Bind small RNA

S+L Purification of total RNA in one single fraction

Load **600 µL** sample onto the **NucleoSpin® RNA Column** (blue ring) already containing the large RNA from step 6.

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

Discard the flow-through and place the column back into the collection tube.

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

Proceed with → **step 11**.

Load 600 µL sample

11,000 x *g* 30 s

Load 600 µL sample

11,000 x *g* 30 s

Load remaining sample

11,000 x *g* 30 s

S L / S Purification of small RNA only or small and large RNA in two separate fractions

Place a **NucleoSpin® miRNA Column** (green ring) in a Collection Tube (2 mL) and load **725 µL** sample onto the column. Centrifuge for **30 s** at **11,000 x *g***.

Discard the flow-through and place the column back into the collection tube.

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

Load 725 µL sample

11,000 x *g* 30 s

Load remaining sample

11,000 x *g* 30 s

11 Wash and dry silica membrane

S+L S L S

From this step on the **NucleoSpin® RNA Column** containing **large RNA** or **total RNA** and the **NucleoSpin® miRNA Column** containing small RNA can be processed simultaneously in the same way.

+ 600 µL MW1

1st wash

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

11,000 x *g*
30 s

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

Discard flow-through and place the column back into the collection tube.

2nd wash

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

+ 700 µL MW2

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

11,000 x *g*
30 s

Discard flow-through and place the column back into the collection tube.

3rd wash

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

+ 250 µL MW2

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

If the liquid in the collection tube has touched the NucleoSpin® RNA/miRNA Column after the 3rd wash, discard flow-through and centrifuge again.

11,000 x *g*
2 min

Note: The same collection tube is used throughout the entire washing procedure to reduce plastic waste. If new collection tubes are to be used for each step, see ordering information.

12 Elute RNA

S+L
S L
S

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

Note: Yield and concentration of isolated RNA are highly dependent on the elution volume. See section 3.5 for detailed information and alternative elution procedures.

Add **30 µL** (for high concentration), **50 µL** (for medium concentration and yield) or **100 µL** (for high yield) **RNase-free H_2O** to the column.

Do not close the lid. Incubate for **1 min** at **room temperature (18–25 °C)**.

Close the lid and centrifuge for **30 s** at **11,000 x *g***.

+ 30–100 µL RNase-free H_2O

RT 1 min

11,000 x *g* 30 s

6.2 RNA purification from plant tissue

Before starting the purification, check that 96–100 % ethanol is available.

For the miRNA purification three different procedures are possible:

1) S+L total RNA isolation – small RNA and large RNA in one single fraction
2) S L separated isolation – isolation of small and large RNA in two separate fractions
3) S miRNA isolation – isolation of small RNA fraction only

Please note that for the separation of small and large RNA (procedure 2) and 3)), two columns are used for one isolation. Both procedures result in 25 isolations for each fraction (50 prep kit). If procedure 2 or 3 are used exclusively, a separate column/buffer set is available to realize 50 isolations for each fraction (see ordering information).

1 Cell lysis

Disrupt sample

S+L See section 3.3 for more information on homogenization methods.

S L

S Thoroughly grind **plant tissue** under **liquid nitrogen** to a fine powder. Transfer up to **50 mg** to a 1.5 mL microcentrifuge tube (not provided) and add **400 µL Buffer ML**. Pipette up and down (>5 times) or vortex to lyse the cells.

+ 400 µL ML

Alternatively, add **400 µL Buffer ML** to **50 mg plant tissue** and use a **rotor-stator, bead-mill**, or other devices to disrupt the cells.

Incubate for **5 min** at **room temperature (18–25 °C)**.

RT 5 min

2 *Optional: Phenol:chloroform extraction*

S+L S L S *Note: The organic extraction might help to improve the lysis efficiency and yield for difficult sample material.*

Add **1 volume** of **acidic (pH 4.5–4.7) phenol : chloroform** to the sample.

Vortex thoroughly for **30 s**.

Centrifuge for **10 min** at **11,000 x *g*** to separate the phases.

Transfer the upper aqueous phase without any traces of phenol:chloroform to a new 1.5 mL microcentrifuge tube (not provided).

Proceed directly with **→ step 4**.

Optional:

+ 1 vol acidic phenol: chloroform

Vortex 30 s

11,000 x *g* 10 min

Separate phases

3 Clarification of the lysate

S+L S L S Place a **NucleoSpin® Filter** (violet ring) in a Collection Tube (2 mL, lid). Load the mixture and centrifuge for **1 min** at **11,000 x *g*** to clear the lysate from undissolved debris.

Upon pellet formation in the collection tube (depending on amount/nature of the sample) transfer the supernatant to a new 1.5 mL microcentrifuge tube (not provided) without disturbing the pellet.

Load sample

11,000 x *g* 1 min

4 Adjust binding conditions for large RNA and DNA

S+L S L S Discard the NucleoSpin® Filter. Add **exactly 150 µL 96–100 % ethanol** to **400 µL flow-through**, close the lid, and vortex immediately for **5 s**.

Incubate for **5 min** at **room temperature (18–25 °C)**.

Note: After addition of ethanol a white or green precipitate may become visible. Do not centrifuge the ethanolic lysate and be sure to load all of the precipitate onto the column in step 5.

Note: More than 400 µL of the lysate can be processed, however, the volumes of ethanol (step 4) and Buffer MX (step 8) have to be increased proportionally and multiple loading steps might be necessary.

400 µL flow-through

+ 150 µL 96–100 % ethanol

Vortex 5 s

RT 5 min

5 Bind large RNA and DNA

S+L S L S

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

Load sample

Note: For total RNA purification S+L*, both types of binding columns (blue or green ring) can be used.*

Centrifuge for **2 min** at **11,000 x *g***. Repeat the centrifugation step at full speed if not all lysate has passed the membrane.

11,000 x *g*
2 min

S+L Purification of small and large RNA

S L **Save the flow-through** containing the small RNA for step 8. Transfer the NucleoSpin® RNA Column into a new Collection Tube (2 mL) and proceed with → **step 6** to digest the DNA on the column.

S Purification of small RNA only

If you do not want to purify the large RNA fraction, discard the NucleoSpin® RNA Column, save the flow-through containing the small RNA and proceed with → **step 8**.

6 Desalt silica membrane

S+L S L 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 the flow-through and place the column back into the collection tube.

11,000 x *g*
1 min

7 Digest DNA

S+L S L Add **100 µL rDNase** directly onto the silica membrane of the NucleoSpin® RNA Column (blue ring). Do not close the lid.

+ 100 µL rDNase

Incubate at **room temperature (18–25 °C)** until step 8 is completed but at least **15 min**.

RT
> 15 min

8 Adjust binding conditions for small RNA

S+L S L Add **800 µL Buffer MX** to the flow-through of step 5 including any pellet that may have formed.

+ 800 µL MX

S Close the lid, and vortex for **5 s** to mix and resuspend any pellet completely.

Vortex 5 s

<u>Note</u>: The yield of small RNA from < 3 mg tissue can be increased by addition of 10 µg Carrier RNA (see section 3.2 for detailed information).

9 Bind small RNA

Load 600 µL sample

S+L Purification of total RNA in one single fraction

Load **600 µL** sample onto the **NucleoSpin® RNA Column** (blue ring) already containing the large RNA from step 7.

11,000 x *g*
30 s

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

Load remaining sample

Discard the flow-through and place the column back into the collection tube.

30 s
11,000 x *g*

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

Proceed with → **step 10**.

S S L Purification of small RNA only or small and large RNA in two separate fractions

Load 700 µL sample

Place a **NucleoSpin® miRNA Column** (green ring) in a Collection Tube (2 mL) and load **700 µL** sample onto the column.

11,000 x *g*
30 s

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

Load remaining sample

Discard the flow-through and place the column back into the collection tube.

11,000 x *g*
30 s

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

10 Wash and dry silica membrane

S+L S L S From this step on the **NucleoSpin® RNA Column** containing **large RNA** or **total RNA** and the **NucleoSpin® miRNA Column** containing small RNA can be processed simultaneously in the same way.

+ 600 µL MW1

1st wash

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

11,000 x *g* 30 s

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

Discard flow-through and place the column back into the collection tube.

2nd wash

+ 700 µL MW2

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

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

11,000 x *g* 30 s

Discard flow-through and place the column back into the collection tube.

3rd wash

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

+ 250 µL MW2

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

If the liquid in the collection tube has touched the NucleoSpin® RNA/miRNA Column after the 3rd wash, discard flow-through and centrifuge again.

11,000 x *g* 2 min

Note: The same collection tube is used throughout the entire washing procedure to reduce plastic waste. If new collection tubes are to be used for each step, see ordering information.

11 Elute RNA

S+L
S L
S

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

Note: Yield and concentration of isolated RNA are highly dependent on the elution volume. See section 3.5 for detailed information and alternative elution procedures.

+ 30–100 µL RNase-free H_2O

Add **30 µL** (for high concentration), **50 µL** (for medium concentration and yield) or **100 µL** (for high yield) **RNase-free H_2O** to the column.

RT
1 min

Do not close the lid. Incubate for **1 min** at **room temperature (18–25 °C)**.

11,000 x *g*
30 s

Close the lid and centrifuge for **30 s** at **11,000 x *g***.

6.3 RNA purification in combination with TRIzol® lysis

Before starting the purification, check that 96–100 % ethanol is available.

For the miRNA purification three different procedures are possible:

1) S+L total RNA isolation – small RNA and large RNA in one single fraction
2) S L separated isolation – isolation of small and large RNA in two separate fractions
3) S miRNA isolation – isolation of small RNA fraction only

Please note that for the separation of small and large RNA (procedure 2) and 3)), two columns are used for one isolation. Both procedures result in 25 isolations for each fraction (50 prep kit). If procedure 2 or 3 are used exclusively, a separate column/buffer set is available to realize 50 isolations for each fraction (see ordering information).

1 Prepare sample

S+L S L S See section 3.3 for more information on homogenization methods.

Disrupt sample

Animal or plant tissue

Thoroughly **grind sample** under **liquid nitrogen** to a fine powder. Transfer up to **50 mg** to a 1.5 mL microcentrifuge tube (not provided) and add **800 µL TRIzol®**.

+ 800 µL TRIzol®

Alternatively, add **800 µL TRIzol®** to up to **50 mg** sample and use a **rotor-stator**, a **bead-mill** or other devices to disrupt sample.

Liquid samples or cultured cells

Add **800 µL TRIzol®** to up to **50 µL liquid sample** or up to **10^7 cultured cells**.

2 Cell lysis

S+L S L S Vortex the sample for **15 s**. Incubate for **5 min** at **room temperature (18–25 °C)**.

Vortex 15 s

RT 5 min

3 Phase separation

+ 160 µL chloroform

S+L Add **160 µL chloroform**, close the lid, and vortex for **15 s**.

Vortex 15 s

S L Incubate for **3 min** at **room temperature (18–25 °C)**.

RT 3 min

S Centrifuge for **15 min** at **2–8 °C** and **12,000 x *g*.**

12,000 x *g* 2–8 °C 15 min

Transfer **400 µL** of the clear aqueous upper phase to a new Collection Tube (2 mL, lid).

Transfer 400 µL aqueous upper phase

4 Adjust binding conditions

S+L Purification of total RNA in one single fraction

+ 1000 µL MX

Add **1000 µL Buffer MX** to **400 µL clear supernatant**, close the lid, and vortex for **5 s**.

Vortex 5 s

S L S Purification of small RNA only or small and large RNA in two separate fractions

+ 200 µL 96–100 % ethanol

Add **exactly 200 µL 96–100 % ethanol** to **400 µL clear supernatant**, close the lid, and vortex for **5 s**.

Vortex 5 s

Incubate for **5 min** at **room temperature (18–25 °C)**.

RT 5 min

5 Bind large RNA and DNA

S+L S L S Place a **NucleoSpin® RNA Column** (blue ring) in a Collection Tube (2 mL, lid) and load up to **700 µL** of the sample onto the column.

Load sample

11,000 x *g* 30 s

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

<u>Note</u>: For total RNA purification S+L, both types of binding columns (blue or green ring) can be used.

S+L Purification of total RNA in one single fraction **without** DNA digest

Discard the flow-through and load the remaining sample onto the NucleoSpin® RNA Column.

Load remaining sample

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

Discard the collection tube and the flow-through.

11,000 x *g* 30 s

Place the NucleoSpin® RNA Column into a new Collection Tube (2 mL) and proceed with **→ step 10**.

S L Purification of small and large RNA **without** DNA digest

Note: DNA removal by phenol (e.g., TRIzol®) extraction is not quantitative but sufficient for most downstream applications.

Transfer the NucleoSpin® RNA Column into a new Collection Tube (2 mL) and save it for step 10.

Proceed with the flow-through containing the small RNA with **→ step 8**.

S L Purification of small and large RNA **with** DNA digest

Note: DNA removal by phenol (e.g., TRIzol®) extraction is not quantitative. Some downstream applications, for example RT-PCR require an additional DNase treatment.

Save the flow-through containing the small RNA for step 8. Transfer the NucleoSpin® RNA Column into a new Collection Tube (2 mL) and proceed with **→ step 6** to digest the DNA on the column.

S Purification of small RNA only

If you do not want to purify the large RNA fraction, discard the NucleoSpin® RNA Column, save the flow-through containing the small RNA and proceed with **→ step 8**.

---

6 Desalt silica membrane

+ 350 µL MDB

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

Discard the flow-through and place the column back into the collection tube.

11,000 x *g* 1 min

---

7 Digest DNA

S L

Add **100 µL rDNase** directly onto the silica membrane of the NucleoSpin® RNA Column (blue ring). Do not close the lid.

+ 100 µL rDNase

Incubate at **room temperature (18–25 °C)** until steps 8–9 are completed but at least **15 min**.

RT >15 min

The NucleoSpin® RNA Column is used again in step 10.

8 Adjust binding conditions for small RNA

S L
S

Add **800 µL Buffer MX** to the flow-through of step 5, close the lid, and vortex for **5 s**.

+ 800 µL MX

Vortex 5 s

9 Bind small RNA

Load 700 µL sample

S L
S

Place a **NucleoSpin® miRNA Column** (green ring) in a Collection Tube (2 mL) and load **700 µL** sample onto the column.

11,000 x *g* 30 s

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

Load remaining sample

Discard the flow-through and place the column back into the collection tube.

11,000 x *g* 30 s

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

10 Wash and dry silica membrane

S+L
S L
S

From this step on the **NucleoSpin® RNA Column** containing **large RNA** or **total RNA** and the **NucleoSpin® miRNA Column** containing **small RNA** can be processed simultaneously in the same way.

+ 600 µL MW1

1st wash

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

11,000 x *g* 30 s

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

Discard flow-through and place the column back into the collection tube.

2nd wash

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

+ 700 µL MW2

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

Discard flow-through and place the column back into the collection tube.

11,000 x *g* 30 s

3rd wash

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

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

+ 250 µL MW2

If the liquid in the collection tube has touched the NucleoSpin® RNA/miRNA Column after the 3rd wash, discard flow-through and centrifuge again.

11,000 x *g* 2 min

Note: The same collection tube is used throughout the entire washing procedure to reduce plastic waste. If new collection tubes are to be used for each step, see ordering information.

11 Elute RNA

S+L
S L
S

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

Note: Yield and concentration of isolated RNA are highly dependent on the elution volume. See section 3.5 for detailed information and alternative elution procedures.

+ 30–100 µL RNase-free H_2O

Add **30 µL** (for high concentration), **50 µL** (for medium concentration and yield) or **100 µL** (for high yield) **RNase-free H_2O** to the column.

RT 1 min

Do not close the lid. Incubate for **1 min** at r**oom temperature (18–25 °C)**.

11,000 x *g* 30 s

Close the lid and centrifuge for **30 s** at **11,000 x *g***.

6.4 Purification of siRNA and large dsRNA from DICER reactions

Before starting with the preparation check that 96 – 100% ethanol is available.

Follow S L to purify siRNA and large dsRNA in two separate fractions.
Refer to section 2, Table 2 for details regarding the corresponding number of preparations that can be performed.

1 Prepare sample

S L Add **150 µL Buffer ML** to **150 µL DICER reaction mix** and vortex for **5 s**.

Note: To purify less than 150 µL, adjust volume with RNase-free water to 150 µL. To process more than 150 µL, increase Buffer ML (step 1), MP (step 4), and MX (step 4) proportionally.

+ 150 µL ML

Vortex 5 s

2 Adjust binding conditions for large dsRNA

S L Add **exactly 200 µL 96–100 % ethanol**, close the lid, and vortex for **5 s**.

Incubate for **5 min** at **room temperature (18–25 °C)**.

+ 200 µL 96–100 % ethanol

Vortex 5 s

RT 5 min

3 Bind large dsRNA

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

Transfer the NucleoSpin® RNA Column containing the **larger dsRNA** to a new Collection Tube (2 mL) and save it for step 6.

Load sample

11,000 x *g*
30 s

4 Adjust binding conditions for siRNA

+ 100 µL MP

Vortex 5 s

Add **100 µL Buffer MP** to the flow-through of step 3, close the lid, and vortex for **5 s**.

RT
5 min

Incubate for **5 min** at **room temperature (18–25 °C)**.

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

+ 800 µL MX

Vortex 5 s

5 Bind siRNA

Load 700 µL sample

Place a **NucleoSpin® miRNA Column** (green ring) in a Collection Tube (2 mL) and load **700 µL** sample onto the column.

11,000 x *g*
30 s

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

Load remaining sample

Discard the flow-through and place the column back into the collection tube.

11,000 x *g*
30 s

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

6 Wash and dry silica membrane

From this step on the **NucleoSpin® RNA Column** containing **large dsRNA** and the **NucleoSpin® miRNA Column** containing **siRNA** can be processed simultaneously in the same way.

1st wash

+ 700 µL MW2

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

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

11,000 x *g*
30 s

Discard flow-through and place the column back into the collection tube.

2nd wash

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

+ 250 µL MW2

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

11,000 x *g* 2 min

If the liquid in the collection tube has touched the NucleoSpin® RNA/miRNA Column after the 2nd wash, discard flow-through and centrifuge again.

Note: The same collection tube is used throughout the entire washing procedure to reduce plastic waste. If new collection tubes are to be used for each step, see ordering information.

7 Elute RNA

+ 30–100 µL RNase-free H_2O

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

Note: Yield and concentration of isolated RNA are highly dependent on the elution volume. See section 3.5 for detailed information and alternative elution procedures.

RT 1 min

Add **30 µL** (for high concentration), **50 µL** (for medium concentration and yield) or **100 µL** (for high yield) **RNase-free H_2O** to the column.

Do not close the lid. Incubate for **1 min** at **room temperature (18–25 °C)**.

11,000 x *g* 30 s

Close the lid and centrifuge for **30 s** at **11,000 x *g***.

6.5 Fractionation of pre-purified RNA in small RNA and large RNA

Before starting with the preparation check that 96 – 100 % ethanol is available.

Follow S L to purify siRNA and large dsRNA in two separate fractions.
Refer to section 2, Table 2 for details regarding the corresponding number of preparations that can be performed.

1 Prepare sample

S L Add **150 µL Buffer ML** to **150 µL pre-purified RNA** and vortex for **5 s**.

Note: To purify less than 150 µL, adjust volume with RNase-free water to 150 µL. To process more than 150 µL, increase Buffer ML (step 1), MP (step 4), and MX (step 4) proportionally.

+ 150 µL RNA solution

Vortex 5 s

2 Adjust binding conditions for large RNA

S L Add **exactly 100 µL 96–100 % ethanol**, close the lid, and vortex for **5 s**.

Incubate for **5 min** at **room temperature (18–25 °C)**.

+ 100 µL 96–100 % ethanol

Vortex 5 s

RT 5 min

3 Bind large dsRNA

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

Load sample

Transfer the NucleoSpin® RNA Column containing the **large RNA** to a new Collection Tube (2 mL) and save it for step 6.

11,000 x *g*
30 s

4 Adjust binding conditions for small RNA

+ 100 µL MP

S L Add **100 µL Buffer MP** to the flow-through of step 3, close the lid, and vortex for **5 s**.

Vortex 5 s

Incubate for **5 min** at **room temperature (18–25 °C)**.

RT 5 min

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

+ 800 µL MX

Vortex 5 s

5 Bind small RNA

Load 700 µL sample

S L Place a **NucleoSpin® miRNA Column** (green ring) in a Collection Tube (2 mL) and load **700 µL** sample onto the column.

11,000 x *g* 30 s

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

Load remaining sample

Discard the flow-through and place the column back into the collection tube.

11,000 x *g* 30 s

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

6 Wash and dry silica membrane

S L From this step on the **NucleoSpin® RNA Column** containing **large dsRNA** and the **NucleoSpin® miRNA Column** containing **siRNA** can be processed simultaneously in the same way.

1st wash

+ 700 µL MW2

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

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

11,000 x *g* 30 s

Discard flow-through and place the column back into the collection tube.

2nd wash

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

+ 250 µL MW2

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

11,000 x *g* 2 min

If the liquid in the collection tube has touched the NucleoSpin® RNA/miRNA Column after the 2nd wash, discard flow-through and centrifuge again.

Note: The same collection tube is used throughout the entire washing procedure to reduce plastic waste. If new collection tubes are to be used for each step, see ordering information.

7 Elute small RNA

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

+ 30–100 µL RNase-free H_2O

Note: Yield and concentration of isolated RNA are highly dependent on the elution volume. See section 3.5 for detailed information and alternative elution procedures.

RT 1 min

Add **30 µL** (for high concentration), **50 µL** (for medium concentration and yield) or **100 µL** (for high yield) **RNase-free H_2O** to the column.

Do not close the lid. Incubate for **1 min** at **room temperature (18–25 °C)**.

11,000 x *g* 30 s

Close the lid and centrifuge for **30 s** at **11,000 x *g***.

7 Appendix

7.1 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 (18–25 °C). 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 if possible.

Problem	Possible cause and suggestions
Suboptimal performance of RNA in downstream experiments	*Carry-over of ethanol or salt* • Do not let the flow-through 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 (18–25 °C) 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.

7.2 Ordering information

Product	REF	Pack of
NucleoSpin® miRNA	740971.10/.50/.250	10/50/250 preps
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

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

7.3 Product use restriction/warranty

NucleoSpin® miRNA kit components are intended, developed, designed, and sold FOR RESEARCH PURPOSES ONLY, except, however, any other function of the product being expressly described in original MACHEREY-NAGEL product leaflets.

MACHEREY-NAGEL products are intended for GENERAL LABORATORY USE ONLY! MACHEREY-NAGEL products are suited for QUALIFIED PERSONNEL ONLY! MACHEREY-NAGEL products shall in any event only be used wearing adequate PROTECTIVE CLOTHING. For detailed information please refer to the respective Material Safety Data Sheet of the product! MACHEREY-NAGEL products shall exclusively be used in an ADEQUATE TEST ENVIRONMENT. MACHEREY-NAGEL does not assume any responsibility for damages due to improper application of our products in other fields of application. Application on the human body is STRICTLY FORBIDDEN. The respective user is liable for any and all damages resulting from such application.

DNA/RNA/PROTEIN purification products of MACHEREY-NAGEL are suitable for *IN VITRO*-USES ONLY!

ONLY MACHEREY-NAGEL products specially labeled as IVD are also suitable for *IN VITRO*-diagnostic use. Please pay attention to the package of the product. *IN VITRO*-diagnostic products are expressly marked as IVD on the packaging.

IF THERE IS NO IVD SIGN, THE PRODUCT SHALL NOT BE SUITABLE FOR *IN VITRO*-DIAGNOSTIC USE!

ALL OTHER PRODUCTS NOT LABELED AS IVD ARE NOT SUITED FOR ANY CLINICAL USE (INCLUDING, BUT NOT LIMITED TO DIAGNOSTIC, THERAPEUTIC AND/OR PROGNOSTIC USE).

No claim or representations is intended for its use to identify any specific organism or for clinical use (included, but not limited to diagnostic, prognostic, therapeutic, or blood banking). It is rather in the responsibility of the user or - in any case of resale of the products - in the responsibility of the reseller to inspect and assure the use of the DNA/RNA/protein purification products of MACHEREY-NAGEL for a well-defined and specific application.

MACHEREY-NAGEL shall only be responsible for the product specifications and the performance range of MN products according to the specifications of in-house quality control, product documentation and marketing material.

This MACHEREY-NAGEL product is shipped with documentation stating specifications and other technical information. MACHEREY-NAGEL warrants to meet the stated specifications. MACHEREY-NAGEL´s sole obligation and the customer´s sole remedy is limited to replacement of products free of charge in the event products fail to perform as warranted. Supplementary reference is made to the gene components not manufactured by MACHEREY-NAGEL, or damages resulting from such non-MACHEREY-NAGEL components or products.

MACHEREY-NAGEL makes no other warranty of any kind whatsoever, and SPECIFICALLY DISCLAIMS AND EXCLUDES ALL OTHER WARRANTIES OF ANY KIND OR NATURE WHATSOEVER, DIRECTLY OR INDIRECTLY, EXPRESS

OR IMPLIED, INCLUDING, WITHOUT LIMITATION, AS TO THE SUITABILITY, REPRODUCTIVITY, DURABILITY, FITNESS FOR A PARTICULAR PURPOSE OR USE, MERCHANTABILITY, CONDITION, OR ANY OTHER MATTER WITH RESPECT TO MACHEREY-NAGEL PRODUCTS.

In no event shall MACHEREY-NAGEL be liable for claims for any other damages, whether direct, indirect, incidental, compensatory, foreseeable, consequential, or special (including but not limited to loss of use, revenue or profit), whether based upon warranty, contract, tort (including negligence) or strict liability arising in connection with the sale or the failure of MACHEREY-NAGEL products to perform in accordance with the stated specifications. This warranty is exclusive and MACHEREY-NAGEL makes no other warranty expressed or implied.

The warranty provided herein and the data, specifications and descriptions of this MACHEREY-NAGEL product appearing in MACHEREY-NAGEL published catalogues and product literature are MACHEREY-NAGEL´s sole representations concerning the product and warranty. No other statements or representations, written or oral, by MACHEREY-NAGEL´s employees, agent or representatives, except written statements signed by a duly authorized officer of MACHEREY-NAGEL are authorized; they should not be relied upon by the customer and are not a part of the contract of sale or of this warranty.

Product claims are subject to change. Therefore please contact our Technical Service Team for the most up-to-date information on MACHEREY-NAGEL products. You may also contact your local distributor for general scientific information. Applications mentioned in MACHEREY-NAGEL literature are provided for informational purposes only. MACHEREY-NAGEL does not warrant that all applications have been tested in MACHEREY-NAGEL laboratories using MACHEREY-NAGEL products. MACHEREY-NAGEL does not warrant the correctness of any of those applications.

Last updated: 07/2010, Rev. 03

Please contact:
MACHEREY-NAGEL GmbH & Co. KG
Tel.: +49 24 21 969-270
tech-bio@mn-net.com