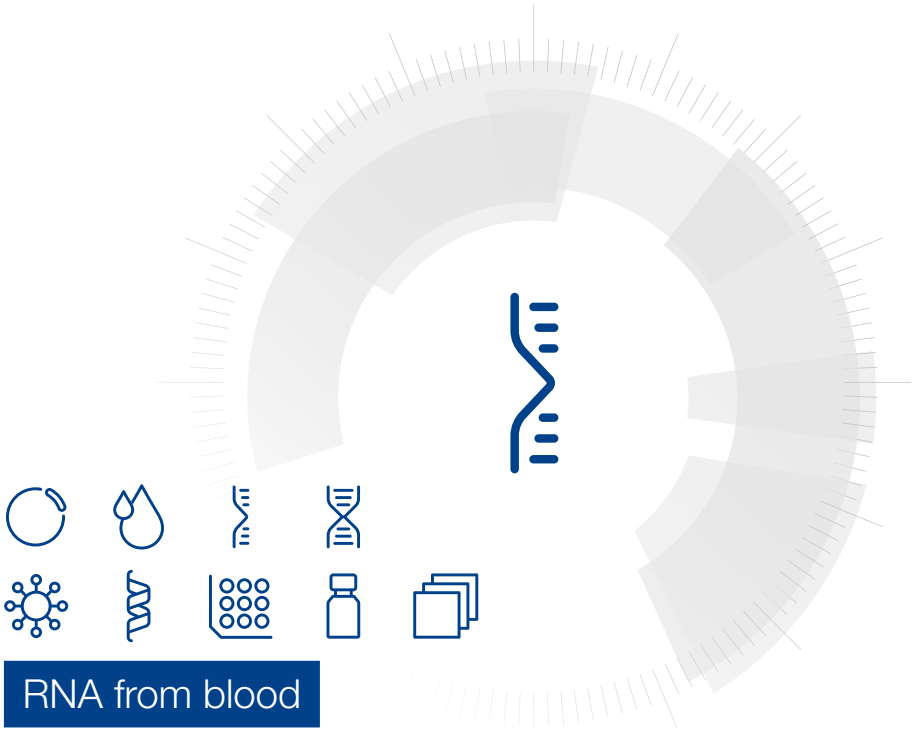
Bioanalysis

MACHEREY-NAGEL

User manual



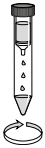
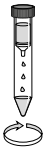
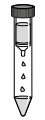
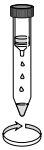
RNA from blood

- NucleoSpin® RNA Blood
- NucleoSpin® RNA Blood Midi

September 2023 / Rev. 08

RNA from blood

Protocol at a glance (Rev. 08)

	Mini NucleoSpin® RNA Blood **200 µL blood**	**Mini** NucleoSpin® RNA Blood **400 µL blood**	**Midi** **NucleoSpin® RNA Blood Midi** **1.3 mL blood**
1 Lyse blood	 200 µL blood 200 µL DL Mix 5 µL Pro. K RT, 3–15 min (shaking)	 400 µL blood 400 µL DL Mix 10 µL Pro. K RT, 3–15 min (shaking)	 1.3 mL blood 1.3 mL DL Mix 33 µL Pro. K RT, 3–15 min (shaking)
2 Adjust RNA binding conditions	200 µL 70 % ethanol Mix	400 µL 70 % ethanol Mix	1.3 mL 70 % ethanol Mix
3 Bind RNA	 Load sample 11,000 x *g*, 30 s	 Load sample stepwise 11,000 x *g*, 30 s	 Load sample 4,500 x *g*, 3 min
4 Desalt silica membrane	 350 µL MDB 11,000 x *g*, 30 s	 350 µL MDB 11,000 x *g*, 30 s	 1.2 mL MDB 4,500 x *g*, 3 min
5 Digest DNA	 95 µL rDNase RT, 15 min	 95 µL rDNase RT, 15 min	 240 µL rDNase RT, 15 min
6 Wash silica membrane	 200 µL RB2 600 µL RB3 250 µL RB3	 200 µL RB2 600 µL RB3 250 µL RB3	 1 mL RB2 3 mL RB3
1st and 2nd wash	 11,000 x *g*, 30 s	 11,000 x *g*, 30 s	 4,500 x *g*, 3 min
3rd wash	11,000 x *g*, 2 min	11,000 x *g*, 2 min	
7 Elute RNA	 60 µL RNase-free H_2O 11,000 x *g*, 30 s	 60 µL RNase-free H_2O 11,000 x *g*, 30 s	 200 µL RNase-free H_2O 4,500 x *g*, 3 min

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1 Components

1.1 Kit contents

	NucleoSpin® RNA Blood*	
REF	**10 preps 740200.10**	**50 preps 740200.50**
Lysis Buffer DL	25 mL	25 mL
Wash Buffer RB2	13 mL	13 mL
Wash Buffer RB3 (Concentrate)**	6 mL	12 mL
Membrane Desalting Buffer MDB	10 mL	25 mL
Reaction Buffer for rDNase	7 mL	7 mL
rDNase, RNase-free (lyophilized)**	1 vial (size C)	2 vials (size D)
Liquid Proteinase K	120 µL	600 µL
RNase-free H_2O	13 mL	13 mL
NucleoSpin® RNA Blood Columns (light blue rings -plus Collection Tubes)	10	50
Collection Tubes (2 mL, with lid) for lysis	10	50
Collection Tubes (1.5 mL) for elution	10	50
Collection Tubes (2 mL)	30	150
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* Patent pending

** For preparation of working solutions and storage conditions see section 3.

Kit contents continued

	NucleoSpin® RNA Blood Midi*
REF	**20 preps 740210.20**
Lysis Buffer DL	50 mL
Wash Buffer RB2	2 × 13 mL
Wash Buffer RB3 (Concentrate)**	25 mL
Membrane Desalting Buffer MDB	50 mL
Reaction Buffer for rDNase	7 mL
rDNase, RNase-free (lyophilized)**	2 vials (size D)
Liquid Proteinase K	800 µL
RNase-free H_2O	13 mL
NucleoSpin® RNA Blood Midi Columns (plus Collection Tubes)	20
Collection Tubes (15 mL) for lysis, elution, and washing steps	60
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* Patent pending

** 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 (to prepare Wash Buffer RB3)
- 70 % ethanol (to adjust RNA binding conditions)

Consumables

- Sterile RNase-free tips

Equipment

- Manual pipettors
- Vortex mixer
- Centrifuge for microcentrifuge tubes (NucleoSpin® RNA Blood)
- Centrifuge for 15 mL tubes with a swing-out rotor capable of reaching 4,500 x *g* (NucleoSpin® RNA Blood Midi)
- Personal protection equipment (e.g., lab coat, gloves, goggles)

1.3 About this user manual

It is strongly recommended to read the detailed protocol sections of this user manual if using the **NucleoSpin® RNA Blood** or **NucleoSpin® RNA Blood Midi** kits for the first time. However, experienced users may refer to the Protocol-at-a-glance. 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***.

2 Product description

2.1 The basic principle

The **NucleoSpin® RNA Blood** kits offer a direct total blood lysis from 200 – 400 µL (NucleoSpin® RNA Blood) or 400 – 1300 µL (NucleoSpin® RNA Blood Midi) whole blood collected in standard (e.g., EDTA) blood collection tubes. One of the most important aspects in RNA purification is to prevent RNA degradation during the isolation. With the **NucleoSpin® RNA Blood** method, leukocytes (the main source of RNA in whole blood) and other blood cells, are lysed by incubating the whole blood in a solution containing large amounts of chaotropic ions. This lysis buffer immediately inactivates RNases (which are present in virtually all biological materials) and creates appropriate binding conditions that favor adsorption of RNA to the silica membrane. A complex selective erythrocyte lysis and preparation of a leukocyte pellet is not necessary. Contaminating DNA, which is also bound to the silica membrane, is removed by a recombinant DNase solution (supplied) which is directly applied onto the silica membrane during the preparation. Simple washing steps with two different buffers remove salts, metabolites, and macromolecular cellular components. Pure RNA is finally eluted under low ionic strength conditions with RNase-free H_2O (supplied).

The RNA preparation using **NucleoSpin® RNA Blood** kits is performed at room temperature. A refrigerated centrifuge is not necessary. The eluate, however, should be handled with care because RNA is very sensitive to trace contaminations of RNases, often found on general lab ware, fingerprints, and dust. To ensure RNA stability, keep RNA frozen at -20 °C for short-term or -70 °C for long-term storage.

Simultaneous isolation of RNA and DNA (NucleoSpin® RNA / DNA Buffer Set)

The NucleoSpin® RNA / DNA Buffer Set (see ordering information) is a support set for RNA and DNA isolation in conjunction with NucleoSpin® RNA, NucleoSpin® RNA XS, NucleoSpin® miRNA, NucleoSpin® RNA Plant, NucleoSpin® RNA / Protein, and NucleoSpin® RNA Blood.

This patented technology enables successive elution of DNA and RNA from one NucleoSpin® Column with low salt buffer and water respectively. DNA and RNA are immediately ready for downstream applications.

2.2 Kit specifications

- **NucleoSpin® RNA Blood** kits are recommended for the isolation of RNA from whole blood (e.g., stabilized with EDTA, citrate, or heparin).
- The **NucleoSpin® RNA Blood** kits allow the purification of RNA with an A_{260}/A_{280} ratio typically exceeding 1.9 (measured in TE buffer, pH 7.5).
- The isolated RNA is ready to use for typical downstream applications (e.g., reverse transcriptase-PCR (RT-PCR)).
- RNA isolated with the NucleoSpin® RNA Blood kits is typically of high integrity. However, RNA integrity strongly depends on the sample quality.
- The amount of DNA contamination is significantly reduced during on-column digestion with rDNase. However, in very sensitive applications, it may be possible to detect traces of DNA. The probability of DNA detection with PCR increases with:
  1. the number of DNA copies per preparation: single copy target < plastidial / mitochondrial target < plasmid transfected into cells.
  2. decreasing PCR amplicon size.

Table 1: Kit specifications at a glance

Parameter	NucleoSpin® RNA Blood	NucleoSpin® RNA Blood Midi
Sample material	200 – 400 µL fresh or frozen whole blood (e.g., stabilized with EDTA, citrate, or heparin)	400 – 1300 µL fresh or frozen whole blood (e.g., stabilized with EDTA, citrate, or heparin)
Format	Mini spin column	Midi spin column
Fragment size	> 200 nt	> 200 nt
Typical yield	~ 7 µg (3 – 20 µg) per 1 mL blood from healthy subjects	~ 7 µg (3 – 20 µg) per 1 mL blood from healthy subjects
A_{260}/A_{280}	1.9 – 2.1	1.9 – 2.1
Elution volume	40 – 120 µL	200 – 400 µL
Binding capacity	200 µg	700 µg
Preparation time	55 min/6 preps (excl. lysis)	75 min/6 preps (excl. lysis)
Use	For research use only	For research use only

The **NucleoSpin® RNA Blood** kit contains one protocol that allows the use of 200 µL of whole blood by a total direct blood lysis and a second protocol for processing 400 µL of whole blood with a second loading step.

The NucleoSpin® RNA Blood Midi kit contains a protocol that allows 1.3 mL of whole blood by a total direct blood lysis.

If other volumes than 200 µL, 400 µL, or 1300 µL blood are used, adjust the volumes of Buffer DL and 70 % ethanol in step 1 and 2 of the corresponding protocol by maintaining the following ratio:

1:1:1 (sample / Buffer DL / 70 % ethanol)
Example: 300 µL blood +300 µL Buffer DL +300 µL 70 % ethanol

The volume of Proteinase K can be calculated as follows:
Blood volume µL / 40 = volume Proteinase K µL
Example: 300 µL blood / 40 = 7.5 µL Liquid Proteinase K

The isolated RNA can be used as a template in RT-PCR-reactions. Generally, 1 – 40 % of the eluate from RNA prepared with 200 – 400 µL blood is suitable as a template for RT-PCR. If possible, intron-spanning primers should be used for RT-PCR.

2.3 Handling, preparation, and storage of starting materials

NucleoSpin® RNA Blood kits are designed for isolation of total RNA from fresh, human whole blood. Whole blood should be collected in the presence of an anticoagulant, preferably EDTA, citrate, or heparin.

It is highly recommended to process blood samples within a few hours after collecting them (when EDTA, citrate, or heparin collection tubes are used). Samples should be stored at 4 °C for no longer than 24 hours. The mRNAs contained in blood cells have different stabilities. As a result, in order to ensure that the isolated RNA contains a representative distribution of mRNAs, blood samples should not be stored for long periods before isolating RNA.

If frozen blood samples have to be processed, aliquots of 200 µL, 400 µL, or 1300 µL, preferably, of frozen blood aliquots should be quickly thawed in the presence of 1 volume Lysis Buffer DL while shaking.

If intermediate storage of stabilized whole blood is necessary, it is recommended storing the lysates at -20 °C. For this, add the indicated volume of Lysis Buffer DL to the blood sample without adding Liquid Proteinase K. Store the lysates at -20 °C. After thawing, add Liquid Proteinase K and follow the protocol at step 1.

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

2.4 Elution procedures

It is possible to adjust the elution method and volume of RNase-free water used for the subsequent application of interest (refer to Table 1 regarding suitable ranges of elution volumes). In addition to the standard method described in the individual protocols (recovery rate about 70 – 90 %), there are several modifications possible:

- **High yield:** Perform two elution steps with the volume indicated in the individual protocol. About 90 – 100 % of bound nucleic acids will be eluted.
- **High yield and high concentration:** Elute with the standard elution volume and apply the eluate once more onto the column for re-elution.

Eluted RNA should be immediately placed and kept on ice for optimal stability and to prohibit omnipresent RNases (general lab ware, fingerprints, dust) from degrading the RNA. For short term storage, freeze at -20 °C, for long term storage, freeze at -70 °C.

3 Storage conditions and preparation of working solutions

Attention: *Buffers DL, RB2, and MDB contain chaotropic salts. Wear gloves and goggles!*

CAUTION: Buffers DL , RB2, and MDB contain guanidinium salts 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.

- For optimal stability (up to 1 year) it is recommended to store the lyophilized **rDNase (RNase-free)** at 4 °C upon arrival.
- Lysis Buffer DL is light sensitive during long time storage. Therefore, Lysis Buffer DL is provided in a black bottle. Short light exposure (several hours) does not affect the buffer.
- After first use, it is recommended to store Liquid Proteinase K at 4 °C or -20 °C.
- All other kit components should be stored at 15 – 25 °C and are stable until: see package lable. Storage at lower temperatures may cause precipitation of salts.
- Check that 70 % ethanol is available as additional solution to adjust RNA binding conditions.
- Check that 96 – 100 % ethanol is available as additional solution to prepare Wash Buffer RB3.

Before starting any **NucleoSpin® RNA Blood** protocol, prepare the following:

- **rDNase (RNase-free)**: Add indicated volume of Reaction Buffer for rDNase (see table below) to the rDNase vial and incubate for 1 min at room temperature. Gently swirl the vials 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 6 months. Do not freeze / thaw the aliquots more than three times. (Be careful when opening the vial as some particles of the lyophilisate may be attached to the lid.)
- **Wash Buffer RB3**: Add the indicated volume of 96 – 100 % ethanol (see table below) to Buffer RB3 Concentrate. Mark the label of the bottle to indicate that ethanol was added. Store Wash Buffer RB3 at 15 – 25 °C for up to one year.

	NucleoSpin® RNA Blood		NucleoSpin® RNA Blood Midi
REF	**10 preps 740200.10**	**50 preps 740200.50**	**20 preps 740210.20**
Wash Buffer RB3 Concentrate	6 mL Add 24 mL ethanol	12 mL Add 48 mL ethanol	25 mL Add 100 mL ethanol
rDNase, RNase-free (lyophilized)	1 vial (size C) Add 1 mL Reaction Buffer for rDNase	2 vials (size D) Add 2.5 mL Reaction Buffer for rDNase to each vial	2 vials (size D) Add 2.5 mL Reaction Buffer for rDNase to each vial

4 Safety instructions

When working with the **NucleoSpin® RNA Blood** kits 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 ***http://www.mn-net.com/msds***).

Caution: Guanidine hydrochloride in Buffer RB2, and guanidinium thiocyanate in Buffer DL 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® RNA Blood** kits 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 and Proteinase K 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 NucleoSpin® RNA Blood protocols

5.1 RNA isolation from 200 µL blood

Before starting the preparation:

- Check if Wash Buffer RB3 and rDNase were prepared according to section 3.
- The complete procedure should be performed at room temperature (18 – 25 °C).
- The use of blood collected in common blood collection tubes with anticoagulant (typically EDTA) is recommended. For frozen blood samples, see section 2.3.
- Check if 70 % ethanol is available to adjust binding conditions.
- Refer to section 2.2 if less than 200 µL whole blood is used.

1 Lyse blood

Provide **200 µL whole blood** in a Collection Tube (2 mL, with lid; provided).

200 µL blood

Add **200 µL Lysis Buffer DL** to the tube and close the lid. Mix. If necessary, shortly spin to clean the lid.

+200 µL DL

Add **5 µL Liquid Proteinase K** and close the lid.

+5 µL Liquid Proteinase K

Incubate **3 – 15 min** at **room temperature** (18 – 25 °C) vigorously shaking the tube on a shaker (e.g., Eppendorf Thermoshaker, 1,400 rpm) .

RT, 3 – 15 min

Centrifuge briefly to clean the lid (**~ 1** s at **~ 2,000 x *g***). Short spin only!

~ 2,000 x *g*, ~ 1 s

<u>Note:</u> Mixtures of blood and Lysis Buffer DL can be stored for up to five days at +4 °C or up to 2 weeks at -20 °C or below largely maintaining RNA quality and yield in the subsequent purification. After such an intermittend storage continue with addition of Liquid Proteinase K and incubation for 3 – 15 minutes at room temperature. Do not add proteinase K before such an intermittend storage!

2 Adjust RNA binding conditions

Add **200 µL 70 % ethanol** to the tube and mix vigorously.

+200 µL 70 % ethanol

Mix

<u>Note:</u> It is important to thoroughly mix the ethanol with the lysate. <u>Recommended:</u> Place tubes in a rack with lid. Close the rack lid and strongly shake the assembly. Alternatively, pipette the solution up and down ~ 5 times.

Centrifuge briefly to clean the lid (**~ 1 s** at **~ 2,000 x *g***). Short spin only!

~ 2,000 x *g*, ~ 1 s

3 Bind RNA

Adjust pipette to **610 µL** and transfer lysate into a **NucleoSpin® RNA Blood Column** placed in a Collection Tube.

Load lysate

Note: Do not pipette more than 650 µL into the spin column, this will cause the column to overflow! Avoid formation of foam and aerosols! Avoid wetting the rim (edge) of the column.

Centrifuge **30 s** at **11,000 x *g***. Discard flow-through and Collection Tube. Place the column in a new Collection Tube (2 mL; provided).

11,000 x *g*, 30 s

Note: Mixtures of blood and Lysis Buffer DL can be stored for up to five days at +4 °C or below largely maintaining RNA quality and yield in the subsequent purification. After such an intermittend storage continue with addition of Liquid Proteinase K and incubation for 3 – 15 minutes at room temperature. Do not add proteinase K before such an intermittend storage!

4 Desalt silica membrane

Add **350 µL MDB** (Membrane Desalting Buffer) onto the column and centrifuge **30 s** at **11,000 x *g***.

+350 µL MDB

11,000 x *g*, 30 s

Note: After centrifugation, the column can remain in the Collection Tube including the flow-through! The flow-through may be slightly brown. The flow-through can remain in the tube without disturbing DNA digestion.

5 Digest DNA

Add **95 µL rDNase** onto the column. Incubate at **room temperature** for **15 min**.

+95 µL rDNase

RT, 15 min

Note: Centrifugation after incubation is not necessary.

6 Wash and dry silica membrane

1 st wash

+200 µL RB2

11,000 x *g*, 30 s

Add **200 µL Buffer RB2** to the NucleoSpin® RNA Blood Column. Centrifuge for **30 s** at **11,000 x *g***. Discard flow-through and Collection Tube and place the column into a new Collection Tube (2 mL; provided).

Buffer RB2 will inactivate the rDNase.

2nd wash

+600 µL RB3

11,000 x *g*, 30 s

Add **600 µL Buffer RB3** to the NucleoSpin® RNA Blood Column. Centrifuge for **30 s** at **11,000 x *g***. Discard flow-through and place the column into a new Collection Tube (2 mL; provided).

Note: Make sure that residual buffer from the previous steps is washed away with Buffer RB3, especially if the lysate has been in contact with the inner rim of the column during loading of the lysate onto the column. For efficient washing of the inner rim flush it with Buffer RB3.

3rd wash

+250 µL RB3

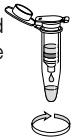
11,000 x *g*, 2 min

Add **250 µL Buffer RB3** to the NucleoSpin® RNA Blood Column. Centrifuge for **2 min** at **11,000 x *g***. In this step, ethanol is removed from the column.

Place the column into a nuclease-free Collection Tube (1.5 mL, supplied) and discard the Collection tube with flow-through from the previous step.

If for any reason the liquid level in the Collection Tube has reached the NucleoSpin® RNA Blood Column after centrifugation, discard flow-through, and centrifuge again.

7 Elute RNA



60 µL RNase-free H_2O

11,000 x *g*, 30 s

Add **60 µL RNase-free H_2O** (supplied) onto the column and centrifuge **30 s** at **11,000 x *g***. The RNA is eluted into the Collection Tube.

For alternative elution procedures, see section 2.4.

5.2 RNA isolation from 400 µL blood

Before starting the preparation:

- Check if Wash Buffer RB3 and rDNase were prepared according to section 3.
- The complete procedure should be performed at room temperature (18 – 25 °C).
- The use of blood collected in common blood collection tubes with anticoagulant (typically EDTA) is recommended. For frozen blood samples, see section 2.3.
- Check if 70 % ethanol is available to adjust binding conditions.
- Refer to section 2.2 if less than 400 µL whole blood is used.

1 Lyse blood

400 µL blood

Provide 4**00 µL whole blood** in a Collection Tube (2 mL, with lid; provided).

+400 µL DL

Add **400 µL Lysis Buffer DL** to the tube and close the lid. Mix. If necessary, shortly spin to clean the lid.

+10 µL Liquid Proteinase K

Add **10 µL Liquid Proteinase K** and close the lid.

RT, 3 – 15 min

Incubate **3 – 15 min** at **room temperature** (18 – 25 °C) vigorously shaking the tube on a shaker (e.g., Eppendorf Thermoshaker, 1,400 rpm) .

~ 2,000 x *g*, ~ 1 s

Centrifuge briefly to clean the lid (**~ 1** s at **~ 2,000 x *g***). Short spin only!

<u>Note:</u> Mixtures of blood and Lysis Buffer DL can be stored for up to five days at +4 °C or up to 2 weeks at -20 °C or below largely maintaining RNA quality and yield in the subsequent purification. After such an intermittend storage continue with addition of Liquid Proteinase K and incubation for 3 – 15 minutes at room temperature. Do not add proteinase K before such an intermittend storage!

2 Adjust RNA binding conditions

+400 µL 70 % ethanol

Mix

Add **400 µL 70 % ethanol** to the tube and mix vigorously.

<u>Note:</u> It is important to thoroughly mix the ethanol with the lysate. <u>Recommended:</u> Place tubes in a rack with lid. Close the rack lid and strongly shake the assembly. Alternatively, pipette the solution up and down ~ 5 times.

~ 2,000 x *g*, ~ 1 s

Centrifuge briefly to clean the lid (**~ 1 s** at **~ 2,000 x *g***). Short spin only!

3 Bind RNA

Transfer **610 µL** and transfer lysate into a **NucleoSpin® RNA Blood Column** placed in a Collection Tube.

Load 610 µL lysate

Note: Do not pipette more than 650 µL into the spin column, this will cause the column to overflow! Avoid formation of foam and aerosols! Avoid wetting the rim (edge) of the column.

Centrifuge **30 s** at **11,000 x *g***. Discard flow-through and Collection Tube. Place the column in a new Collection Tube (2 mL; provided).

11,000 x *g*, 30 s

Apply the remaining lysate into the NucleoSpin® RNA Blood Column.

Load residual lysate

Note: Do not pipette more than 650 µL into the spin column, this will cause the column to overflow! Avoid foam and aerosol formation! Avoid wetting the rim (edge) of the column.

Centrifuge **30 s** at **11,000 x *g***. Discard flow-through and Collection Tube. Place the column in a new Collection Tube (2 mL; provided).

11,000 x *g*, 30 s

4 Desalt silica membrane

Add **350 µL MDB** (Membrane Desalting Buffer) onto the column and centrifuge **30 s** at **11,000 x *g***.

+350 µL MDB

11,000 x *g*, 30 s

Note: After centrifugation, the column can remain in the Collection Tube including the flow-through! The flow-through may be slightly brown. The flow-through can remain in the tube without disturbing DNA digestion.

5 Digest DNA

Add **95 µL rDNase** onto the column. Incubate at **room temperature** for **15 min**.

+95 µL rDNase

RT, 15 min

Note: Centrifugation after incubation is not necessary.

6 Wash and dry silica membrane

1 st wash

+200 µL RB2

Add **200 µL Buffer RB2** to the NucleoSpin® RNA Blood Column. Centrifuge for **30 s** at **11,000 x *g***. Discard flow-through and Collection Tube and place the column into a new Collection Tube (2 mL; provided).

11,000 x *g*, 30 s

Buffer RB2 will inactivate the rDNase.

2nd wash

+600 µL RB3

Add **600 µL Buffer RB3** to the NucleoSpin® RNA Blood Column. Centrifuge for **30 s** at **11,000 x *g***. Discard flow-through and place the column into a new Collection Tube (2 mL; provided).

11,000 x *g*, 30 s

Note: Make sure that residual buffer from the previous steps is washed away with Buffer RB3, especially if the lysate has been in contact with the inner rim of the column during loading of the lysate onto the column. For efficient washing of the inner rim flush it with Buffer RB3.

3rd wash

+250 µL RB3

Add **250 µL Buffer RB3** to the NucleoSpin® RNA Blood Column. Centrifuge for **2 min** at **11,000 x *g***. In this step, ethanol is removed from the column.

11,000 x *g*, 2 min

Place the column into a nuclease-free Collection Tube (1.5 mL, supplied) and discard the Collection tube with flow-through from the previous step.

If for any reason the liquid level in the Collection Tube has reached the NucleoSpin® RNA Blood Column after centrifugation, discard flow-through, and centrifuge again.

7 Elute RNA

60 µL RNase-free H_2O

Add **60 µL RNase-free H_2O** (supplied) onto the column and centrifuge **30 s** at **11,000 x *g***. The RNA is eluted into the Collection Tube.

11,000 x *g*, 30 s

For alternative elution procedures, see section 2.4.

5.3 RNA isolation from SARSTEDT S-Monovette® RNA Exact

1 Prepare sample

Provide SARSTEDT S-Monovette® RNA Exact (containing 2.4 mL blood in 7.3 mL stabilizing solution).

Transfer 1.2 mL solution (whole blood stored in S-Monovette® RNA Exact) from the S-Monovette® RNA Exact and transfer into a 2 mL tube with lid (not provided).

1.2 mL content from S-Monovette RNA Exact

2 Lyse sample

Add **5 µL Liquid Proteinase K**.

+5 µL Liquid Proteinase K

Incubate **15 min** at **room temperature** vigorously shaking the tube.

RT, 15 min

Short spin to clean lid.

~ 2,000 x *g*, ~ 1 s

3 Bind RNA

Apply **600 µL** lysate onto the **NucleoSpin® RNA Blood Mini column** placed in a Collection Tube (provided). The lysate might start to flow through the column – this is o.k.

Load 600 µL lysate

Note: Do not pipette more than 650 µL into the spin column, this will cause the column to overflow! Avoid formation of foam and aerosols! Avoid wetting the rim (edge) of the column.

Centrifuge **30 s** at **11,000 x *g***. Discard flow-through and Collection Tube. Place the column in a new Collection Tube (2 mL; provided).

11,000 x *g*, 30 s

Apply the remaining lysate (approx. 600 µL) onto the NucleoSpin® RNA Blood Mini column.

Centrifuge **30 s** at **11,000 x *g***. Discard flow-through and Collection Tube. Place the column in a new Collection Tube (2 mL; provided).

11,000 x *g*, 30 s

After sample loading, continue with the standard protocol procedure in section 5.1 step 4 (page 13): Desalt Silica membrane (which is the addition of 350 µL MDB).

6 NucleoSpin® RNA Blood Midi protocol – RNA isolation from 1.3 mL blood

Before starting the preparation:

- Check if Wash Buffer RB3 and rDNase were prepared according to section 3.
- The complete procedure should be performed at room temperature (18 – 25 °C).
- The use of blood collected in common blood collection tubes with anticoagulant (typically EDTA) is recommended. For frozen blood samples, see section 2.3.
- Check if 70 % ethanol is available to adjust binding conditions.
- For centrifugation, a centrifuge with a swing-out rotor and appropriate buckets capable of reaching 4,500 x *g* is required.
- Refer to section 2.2 if less than 1.3 mL whole blood is used.

1 Lyse blood

1.3 mL blood

+1.3 mL DL

+33 µL Liquid Proteinase K

RT, 3 – 15 min

~ 2,000 x *g*, ~ 1 s

Provide **1.3 mL whole blood** in a 15 mL tube (provided).

Add **1.3 mL Lysis Buffer DL** to the tube and close the lid. Mix. If necessary, shortly spin to clean the lid.

Add **33 µL Liquid Proteinase K** and close the lid.

Incubate **3 – 15 min** at **room temperature** (18 – 25 °C) vigorously shaking the tube.

Centrifuge briefly to clean the lid (**~ 1 s** at **~ 2,000 x *g***). Short spin only!

Note: Mixtures of blood and Lysis Buffer DL can be stored for up to five days at +4 °C or up to 2 weeks at -20 °C or below largely maintaining RNA quality and yield in the subsequent purification. After such an intermittend storage continue with addition of Liquid Proteinase K and incubation for 3 – 15 minutes at room temperature. Do not add proteinase K before such an intermittend storage!

2 Adjust RNA binding conditions

+1.3 mL 70 % ethanol

Mix

~ 2,000 x *g*, ~ 1 s

Add **1.3 mL 70 %** ethanol to the tube and mix vigorously.

Note: It is important to thoroughly mix the ethanol into the lysate. Recommended: Vigorously shake for 5 s (e.g., on a vortexer at medium speed). Alternatively, pipette the solution up and down ~ 5 times.

If necessary, centrifuge briefly to clean lid (**~ 1 s** at **~ 2,000 x *g***). Short spin only!

This centrifugation step can be omitted if the lid is not wetted by the lysate. For example, mix by vortexing at medium speed or by pipetting up and down.

3 Bind RNA

Transfer the complete lysate **(~ 4000 µL)** into a NucleoSpin® RNA Blood Midi Column placed in a 15 mL Collection Tube.

Do not pipette more than 4000 µL into the Midi column, this will cause the column to overflow! Avoid foam and aerosol formation!

Centrifuge **3 min** at **4,500 x *g*** and leave the column in the tube with the flow-through.

Load max. 4000 µL lysate

4,500 x *g*, 3 min

4 Desalt silica membrane

Add **1.2 mL MDB (Membrane Desalting Buffer)** onto the column and centrifuge **3 min** at **4,500 x *g***.

Discard flow-through and Collection Tube and place the column in a new Collection Tube (15 mL, provided).

+1.2 mL MDB

4,500 x *g*, 3 min

5 Digest DNA

Add **240 µL rDNase** onto the column.

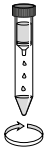
Incubate at **room temperature** for **15 min** (centrifugation after this incubation is not necessary).

+240 µL rDNase

RT, 15 min

6 Wash and dry silica membrane

1 st wash



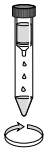
+1 mL RB2

Add **1 mL Buffer RB2** to the NucleoSpin® RNA Blood Midi Column. Centrifuge for **3 min** at **4,500 x *g***.

Leave the NucleoSpin® RNA Blood Midi Column in the tube with the flow-through.

4,500 x *g*, 3 min

2nd wash



+3 mL RB3

Add **3 mL Buffer RB3** to the NucleoSpin® RNA Blood Midi Column. Centrifuge for **3 min** at **4,500 x *g***.

Place the column into a nuclease-free Collection Tube (15 mL; provided) and discard the Collection Tube with flow-through from the previous step.

4,500 x *g*, 3 min

Caution! *Ethanol carry-over may impair downstream analysis (e.g., C_p retardation)! Therefore, removal of the column tube assembly from the centrifuge, transport to the lab bench, placement of it onto the bench and removal of the column from the tube should be done with great care in order to avoid any contamination of the column outlet with the flow-through/ethanol. To avoid any risk of wash buffer / ethanol contamination of the sample, discard wash buffer flow-through from the tube, re-insert the column into the tube and perform a further centrifugation (1 – 3 min 4,500 x g) in order to securely dry the column. Alternatively, use a fresh collection tube (not provided) for a secure column drying centrifugation.*

7 Elute RNA

+200 µL RNase-free H_2O

Add **200 µL RNase-free H_2O** (supplied) onto the column. Centrifuge for **3 min** at **4,500 x *g***. The RNA is eluted into the Collection Tube.

An additional elution step with **200 µL** fresh elution buffer will increase the total yield by approximately 25 %.

4,500 x *g*, 3 min

7 Appendix

7.1 rDNase digestion in solution

The on-column rDNase digestion in the standard protocol is already very efficient and results in minimal residual DNA. This DNA will not be detectable in most downstream applications. Despite this, there are still certain applications which require even lower contents of residual DNA. However, removal of DNA to a completely undetectable level is challenging and the efficiency of an on-column DNA digestion is sometimes not sufficient for downstream applications requiring lowest residual content of DNA.

A typical example for such a demanding application is an RT-PCR reaction in which the primer molecules do not differentiate between cDNA (derived from RNA) and contaminating genomic DNA. Especially, if

- high copy number targets are analyzed (e.g., multi gene family, mitochondrial, plastidal or plasmid targets (from transfections))
- the target gene is of a very low expression level
- the amplicon is relatively small (< 200 bp).

DNA digestion in solution can efficiently destroy contaminating DNA. However, stringent RNase control and subsequent re-purification of the RNA (in order to remove buffer, salts, rDNase, and digested DNA) are usually required.

The high quality, recombinant, RNase-free DNase (rDNase) in the NucleoSpin® RNA Blood kits can also be used for a digestion in solution in order to remove trace amounts of contaminating DNA.

1 **Digest DNA (Reaction setup)**

Add **0.5 µL rDNase** per **10 µL eluted RNA** and mix moderately.

Centrifuge briefly (**~ 1 s** at **~ 2,000 x *g***) to collect all liquid in the lower part of the tube.

Note: This step is important to ensure that every droplet of the RNA comes into contact with the rDNase to ensure efficient DNA digestion.

2 **Incubate sample**

Incubate for **10 min** at **37 °C**.

3 **Repurify RNA**

Repurify RNA with a suitable RNA cleanup procedure, for example, using the NucleoSpin® RNA Clean-up kit (see ordering information) or by ethanol precipitation.

Ethanol precipitation, exemplary:

Add **0.1 volume** of **3 M sodium acetate**, **pH 5.2** and **2.5 volumes of 96 – 100 % ethanol** to **one volume of sample**. Mix thoroughly.

Incubate **several minutes** to **several hours** at **-20 °C** or **4 °C**.

Note: Choose long incubation times if the sample contains low RNA concentration. Short incubation times are sufficient if the sample contains high RNA concentration.

Centrifuge for **10 min** at **maximum speed**.

Wash RNA pellet with **70 % ethanol**.

Dry RNA pellet and resuspend RNA in RNase-free H_2O.

7.2 Troubleshooting

Problem	Possible cause and suggestions
RNA is degraded / no RNA obtained	*RNase contamination* • Create an RNase-free working environment. Wear gloves during all steps of the procedure. Change gloves frequently. Use of sterile, disposable polypropylene tubes is recommended. Keep tubes closed whenever possible during the preparation. Glassware should be oven-baked for at least 2 hours at 250 °C before use.
Poor RNA quality or yield	*Reagents not applied or restored properly* • Reagents not properly restored. Add the indicated volume of Reaction Buffer for rDNase and 96 % ethanol to Buffer RB3 Concentrate and mix. Reconstitute and store lyophilized rDNase according to instructions given in section 3. • Sample and reagents have not been mixed completely. Always vortex vigorously after each reagent has been added. • No ethanol has been added after lysis. Binding of RNA to the silica membrane is only effective in the presence of ethanol. *Kit storage* • Reconstitute and store lyophilized rDNase according to instructions given in section3. • Store other kit components at room temperature. Storage at low temperatures may cause salt precipitation. • Keep bottles tightly closed in order to prevent evaporation or contamination. *Ionic strength and pH influence A_{260} absorption as well as ratio A_{260}/A_{280}* • For adsorption measurement, use 5 mM Tris pH 8.5 as diluent. Please see also: -Manchester, K L. 1995. Value of A_{260}/A_{280} ratios for measurement of purity of nucleic acids. Biotechniques 19, 208 – 209. -Wilfinger, W W, Mackey, K and Chomczyski, P. 1997. Effect of pH and ionic strength on the spectrophotometric assessment of nucleic acid purity. Biotechniques 22, 474 – 481.

Problem	Possible cause and suggestions
Clogged NucleoSpin® Column / Poor RNA quality or yield	*Sample material* • Bad sample quality. Make sure blood is collected into a standard blood collection tube (e.g., EDTA tube) according to the manufacturer's instructions; using fresh blood is always recommended. Sample should be stored at 4 °C for no longer than 24 hours. Freeze sample if it is not possible to process within one day. *Inappropriate lysis / binding conditions* • Do not premix Liquid Proteinase K with Lysis Buffer DL. • Make sure to vigorously shake during lysis incubation – shaking is essential for the procedure! • Make sure to use 70 % ethanol in this procedure to adjust binding conditions.
Contamination of RNA with genomic DNA	*rDNase not active* • Reconstitute and store lyophilized rDNase according to instructions given in section 3. *DNase solution not properly applied* • Pipette rDNase solution directly onto the center of the silica membrane. *High leukocyte number* • The higher the leukocyte number, the higher the risk to detect residual DNA in the eluted RNA. To avoid this, use less blood. *DNA detection system too sensitive* • The amount of DNA contamination is effectively reduced during the on-column digestion with rDNase. However, it cannot be guaranteed that the purified RNA is 100 % free of DNA. Therefore, in very sensitive applications it might still be possible to detect DNA. The NucleoSpin® RNA system was checked by the following procedure: One million HeLa cells are subjected to RNA isolation. RNA eluate is used as a template for PCR detection of a 1 kb fragment in a 30 cycle reaction. Generally, no PCR product is obtained while skipping the DNase digest usually leads to positive PCR results. • The probability of DNA detection with PCR increases with: -the number of DNA copies per preparation: single copy target < plastidial / mitochondrial target < plasmid transfected into cells -decreasing of PCR amplicon size. • Use larger PCR targets (e.g., > 500 bp) or intron spanning primers if possible. **Use protocol 7.1 for subsequent rDNase digestion in solution.**

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 Buffer RB3 wash. Be sure to centrifuge at the corresponding speed for the respective time in order to remove ethanolic Buffer RB3 completely. • Check if Buffer RB3 has been equilibrated to room temperature before use. Washing at lower temperatures lowers efficiency of salt removal by Buffer RB3. *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.

7.3 Ordering information

Product	REF	Pack of
NucleoSpin® RNA Blood	740200.10 / .50	10 / 50
NucleoSpin® RNA Blood Midi	740210.20	20
NucleoSpin® 8 RNA Blood	740220 / .5	12 × 8 / 60 × 8
NucleoSpin® 96 RNA Blood	740225.2 / .4	2 × 96 / 4 × 96
NucleoSpin® miRNA Plasma	740981.10 / .50 / .250	10 / 50 / 250
NucleoSpin® RNA Clean-up	740948.10 / .50 / .250	10 / 50 / 250
NucleoSpin® RNA Clean-up XS	740903.10 / .50 / .250	10 / 50 / 250
NucleoSpin® RNA / DNA Buffer Set	740944	Suitable for 100 preps
NucleoSpin® RNA	740955.10 / .50 / .250	10 / 50 / 250
NucleoSpin® RNA Midi	740962.20	20
NucleoSpin® RNA / Protein	740933.10 / .50 / .250	10 / 50 / 250
NucleoSpin® TriPrep*	740966.10 / .50 / .250	10 / 50 / 250
NucleoSpin® miRNA	740971.10 / .50 / .250	10 / 50 / 250
NucleoSpin® RNA XS	740902.10 / .50 / .250	10 / 50 / 250
rDNase Set	740963	1 set
Collection Tubes (2 mL)	740600	1000
Liquid Proteinase K	740396	5 mL

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

7.4 Product use restriction / warranty

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.

This MACHEREY-NAGEL product is carrying documentation stating specifications and other technical information. MACHEREY-NAGEL warrants to meet the stated specifications. The provided warranty is limited to the data specifications and descriptions as given in the original MACHEREY-NAGEL literature. No other statements or representations, written or oral, by MACHEREY-NAGEL's employees, agents or representatives, except written statements signed by a duly authorized officer of MACHEREY-NAGEL are authorized. They should not be relied upon by the costumer and are not a part of a contract of sale or of this warranty.

Liability for all possible damages that occur in any connection with our products is limited to the utmost minimum as stated in the general business terms and conditions of MACHEREY-NAGEL in their latest edition which can be taken from the company's website. MACHEREY-NAGEL does not assume any further warranty.

Products and their application are subject to change. Therefore, please contact our Technical Service Team for the latest information on MACHEREY-NAGEL products. You may also contact your local distributor for general scientific information. Descriptions in MACHEREY-NAGEL literature are provided for informational purposes only.

Last updated: 08/2022, Rev. 04

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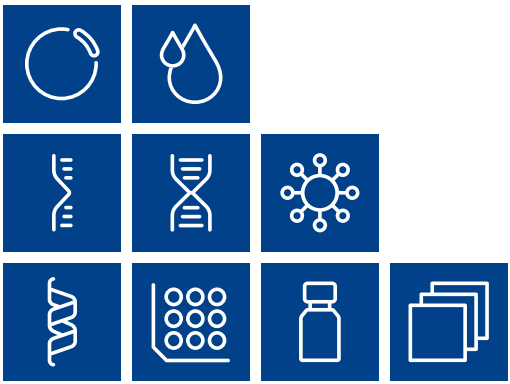
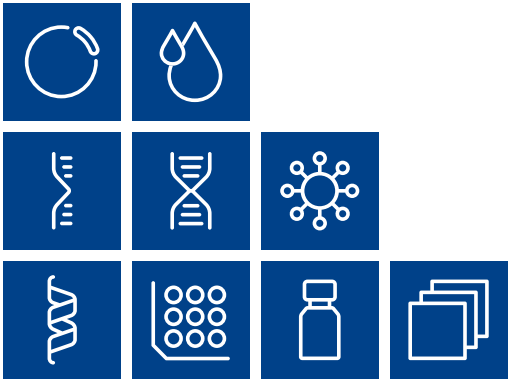


Plasmid DNA
Clean up
RNA
DNA
Viral RNA and DNA
Protein
High throughput
Accessories
Auxiliary tools



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