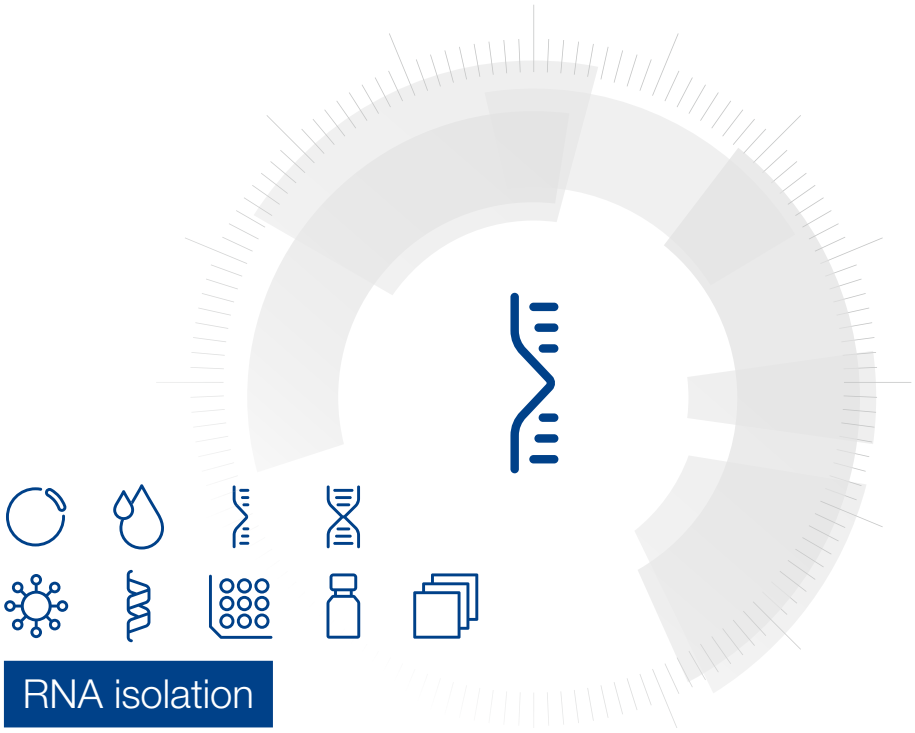
Bioanalysis

MACHEREY-NAGEL

User manual



RNA isolation

- NucleoSpin® RNA
- NucleoSpin® RNA Midi

November 2023 / Rev. 22

RNA isolation

Protocol at a glance (Rev. 22)

		Mini NucleoSpin® RNA	Midi NucleoSpin® RNA Midi
1	**Homogenize sample**	 30 mg	 100 mg
2	**Lyse cells**	 350 µL RA1 3.5 µL ß-mercaptoethanol Mix	 1.8 mL RA1 18 µL ß-mercaptoethanol Mix
3	**Filtrate lysate**	  11,000 x *g*, 1 min	  4,500 x *g*, 10 min
4	**Adjust RNA binding conditions**	350 µL 70 % ethanol Mix	1.8 mL 70 % ethanol Mix
5	**Bind RNA**	  Load sample 11,000 x *g*, 30 s	  Load sample 4,500 x *g*, 3 min
6	**Desalt silica membrane**	  350 µL MDB 11,000 x *g*, 1 min	  2.2 mL MDB 4,500 x *g*, 3 min
7	**Digest DNA**	 95 µL DNase reaction mixture RT, 15 min	 250 µL DNase reaction mixture RT, 15 min
8	**Wash and dry silica membrane**	 1st wash 200 µL RAW2 2nd wash 600 µL RA3 3rd wash 250 µL RA3 1st and 2nd  11,000 x *g*, 30 s 3rd  11,000 x *g*, 2 min	 1st wash 2.6 mL RAW2 2nd wash 2.6 mL RA3 3rd wash 2.6 mL RA3 1st and 2nd  4,500 x *g*, 3 min 3rd  4,500 x *g*, 5 min
9	**Elute highly pure RNA**	  60 µL RNase-free H_2O 11,000 x *g*, 1 min	  500 µL RNase-free H_2O RT, 2 min 4,500 x *g*, 3 min

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Table of contents

1 Components

1.1 Kit contents

	NucleoSpin® RNA		
REF	**10 preps 740955.10**	**50 preps 740955.50**	**250 preps 740955.250**
Lysis Buffer RA1	10 mL	25 mL	125 mL
Wash Buffer RAW2	13 mL	13 mL	80 mL
Wash Buffer RA3 (Concentrate)*	6 mL	12 mL	3 x 25 mL
Membrane Desalting Buffer MDB	10 mL	25 mL	125 mL
Reaction Buffer for rDNase	7 mL	7 mL	30 mL
rDNase, RNase-free (lyophilized)*	1 vial (size D)	1 vial (size F)	5 vials (size F)
RNase-free H_2O	13 mL	13 mL	60 mL
NucleoSpin® Filters (violet rings)	10	50	250
NucleoSpin® RNA Columns (light blue rings – plus Collection Tubes)	10	50	250
Collection Tubes (2 mL)	30	150	750
Collection Tubes (1.5 mL)	10	50	250
User manual	1	1	1

* For preparing of workings solutions and storage conditions see section 3.

Kit contents *continued*

	NucleoSpin® RNA Midi
REF	**20 preps 740962.20**
Lysis Buffer RA1	125 mL
Wash Buffer RAW2	80 mL
Wash Buffer RA3 (Concentrate)*	25 mL
Membrane Desalting Buffer MDB	50 mL
Reaction Buffer for rDNase	7 mL
rDNase, RNase-free (lyophilized)*	1 vial (size D)
RNase-free H_2O	13 mL
NucleoSpin® Filters Midi (plus Collection Tubes)	20
NucleoSpin® RNA Midi Columns (plus Collection Tubes)	20
Collection Tubes (15 mL)	20
User manual	1

* For preparing of workings 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 RA3)
- 70 % ethanol (to adjust RNA binding conditions)
- Reducing agent (ß-mercaptoethanol, **or** DTT (dithiothreithol), **or** TCEP (BisTris (Bis-(2-hydroxyethyl)-imino-tris(hydroxymethyl)-methane)) as supplement for Lysis Buffer RA1

Consumables

- 1.5 mL microcentrifuge tubes (NucleoSpin® RNA) or 15 mL tubes (NucleoSpin® RNA Midi)
- Sterile RNase-free tips

Equipment

- Manual pipettors
- NucleoSpin® RNA: centrifuge for microcentrifuge tubes
- NucleoSpin® RNA Midi: centrifuge for 15 mL tubes with a swing-out rotor and appropriate buckets capable of reaching 4,000–4,500 x *g*
- Equipment for sample disruption and homogenization (see section 2.3)
- Personal protection equipment (e.g., lab coat, gloves, goggles)

1.3 About this user manual

It is strongly recommended reading the detailed protocol sections of this user manual if the **NucleoSpin® RNA** or **NucleoSpin® RNA Midi** kit is used for the first time. 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.

2 Product description

2.1 The basic principle

One of the most important aspects in the isolation of RNA is to prevent degradation during the isolation procedure. With the **NucleoSpin® RNA** methods, cells are lysed by incubation in a solution containing large amounts of chaotropic ions. This lysis buffer immediately inactivates RNases – which are present in virtually all biological materials – and creates appropriate binding conditions which favor adsorption of RNA to the silica membrane. Contaminating DNA, which is also bound to the silica membrane, is removed by an rDNase solution which is directly applied onto the silica membrane during the preparation (RNase-free rDNase is supplied with the kit). 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** kits can be performed at room temperature. The eluate, however, should be treated 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, Protein, and DNA (NucleoSpin® RNA/DNA Buffer Set*, NucleoSpin® TriPrep*)

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® RNA Plant, or NucleoSpin® RNA / Protein.

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.

The combination of the NucleoSpin® RNA/DNA Buffer Set with NucleoSpin® RNA / Protein allows parallel isolation of RNA, DNA, and protein from one undivided sample.

The NucleoSpin® TriPrep kit features the purification of RNA, DNA, and protein from single undivided samples.

2.2 Kit specifications

- **NucleoSpin® RNA** kits are recommended for the isolation of RNA from cultured cells and tissue. Support protocols for the isolation of RNA from reaction mixtures, bacteria, and yeasts using the **NucleoSpin® RNA** kit are included. The **NucleoSpin® RNA** kits allow purification of pure RNA with an A_{260}/A_{280} ratio generally exceeding 1.9 (measured in TE buffer, pH 7.5).
- Even biological samples which are sometimes difficult to process will yield high quality RNA. Such samples are, for example, mouse tissue (liver, brain), different tumor cell lines, *Streptococci*, and *Actinobacillus pleuropneumoniae*.
- The isolated RNA is ready to use for applications like reverse transcriptase-PCR (RT-PCR), primer extension, or RNase protection assays.
- RNA isolated with NucleoSpin® RNA kits is of high integrity. RIN (RNA Integrity Number) of RNA isolated from fresh high quality sample material (e.g., eukaryotic cells or fresh mouse liver) generally exceeds 9.0. However, RNA integrity strongly depends on the sample quality. RNA integrity was examined using the Agilent 2100 Bioanalyzer in conjunction with the RNA 6000 Nano or Pico assay.
- The amount of DNA contamination is significantly reduced during on-column digestion with rDNase. Anyhow, in very sensitive applications it might be possible to detect traces of DNA. The **NucleoSpin® RNA** on-column DNA removal is tested with the following procedure: One million HeLa cells are subjected to RNA isolation according to the protocol. RNA eluate is used as template for PCR detection of a 1 kb fragment in a 30 cycle reaction. Generally no PCR fragment is obtained if the DNase is applied while a strong PCR fragment may be obtained if the DNase digestion is omitted. 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	NucleoSpin® RNA Midi
Technology	Silica membrane technology	Silica membrane technology
Use	For research use only	For research use only
Format	Mini spin column	Midi spin column
Processing	Manual handling and centrifugation	Manual handling and centrifugation
Sample material	< 5 x 10^6 cultured cells, < 10^9 bacterial cells, < 10^8 yeast cells, < 30 mg tissue	< 5 x 10^7 cultured cells, < 10^{10} bacterial cells, < 3 x 10^8 yeast cells, < 200 mg tissue
Fragment size	> 200 nt	> 200 nt
Typical yield	14 µg from 10^6 HeLa cells, 70 µg from 10^9 bacterial cells	180 µg from 10^7 HeLa cells, 620 µg from 4 x 10^7 HeLa cells
A_{260}/A_{280}	1.9–2.1	1.9–2.1
Typical RIN (RNA integrity number)	> 9	> 9
Elution volume	40–120 µL	500 µL
Preparation time	30 min/6 preps	80 min/4 preps
Binding capacity	200 µg	700 µg
Use	For research use only	

NucleoSpin® RNA

- The standard protocol (section 5.1) allows the purification of up to 70 µg RNA per **NucleoSpin® RNA Column** from up to 5 x 10^6 cultured cells or 30 mg of tissue (also see Table 1). The isolated RNA can be used as template in a RT-PCR-reaction. Generally, 1–10 % of the eluate of RNA prepared from 1 x 10^6 cells or 10 mg of tissue is sufficient as template for RT-PCR. If possible, intronspanning primers should be used for RT-PCR.
- The RNA prepared from such high amounts is generally free of residual DNA, although minute traces of DNA may remain in the preparation, if large amounts of material rich in nucleic acids are used. However, if the isolated RNA will be used as template in

a RT-PCR-reaction, we recommend to use lower quantities of sample (e.g., 1 x 10^6 cultured cells or 10 mg of tissue resulting in about 20 µg of RNA).

- The kit can be used for preparing RNA from different amounts of sample material. For optimal results the volume of Lysis Buffer RA1 (protocol step 1) and of ethanol (protocol step 3) should be adapted according to Table 2.

Table 2: Lysis adaptation

Sample	Amount	Volume of Lysis Buffer RA1 (protocol step 2)	Volume of Ethanol (protocol step 4)
Cultured animal or human cells (e.g., HeLa cells)	< 5 x 10^6	350 µL	350 µL
Human or animal tissue	< 20 mg 20 mg–30 mg*	350 µL 600 µL	350 µL 600 µL
Tissue stored in NucleoProtect® RNA or RNA*later*®	< 20 mg 20 mg–30 mg*	350 µL 600 µL	350 µL 600 µL
Samples known to be hard to lyse	< 5 x 10^7*	600 µL	600 µL

An additional loading step is required if 600 µL Buffer RA1 and ethanol is used (load the sample onto the column in two successive centrifugation steps).

Depending on sample type, the average yield is around 5–70 µg RNA (see Table 3). The A_{260} / A_{280} ratio generally exceeds 1.9, indicating purity of the RNA.

Table 3: Overview on average yields of RNA isolation using NucleoSpin® RNA

Sample	Average yield
8 x 10^4 HeLa cells	1.5 µg
4 x 10^5 HeLa cells	4 µg
1 x 10^6 HeLa cells	14 µg
2 x 10^6 HeLa cells	21 µg
2.5 x 10^6 HeLa cells	25 µg
5 x 10^6 HeLa cells	50 µg

* The volume of Lysis Buffer RA1 included in the kit is not sufficient to perform all preparations with 600 µL. If required, additional Lysis Buffer RA1 can be ordered separately (see ordering information, section 8.2).

NucleoSpin® RNA Midi

- The kit can be used for preparing RNA from different amounts of sample material. For optimal results the volume of Lysis Buffer RA1 (protocol step 1) and of ethanol (protocol step 3) should be adapted according to Table 4:

Table 4: Lysis adaptation

Sample	Amount	Volume of Lysis Buffer RA1 (protocol step 1)	Volume of Ethanol (protocol step 4)
Cultured animal cells (e.g., HeLa cells)	5×10^6–2×10^7 2×10^7–5×10^7	1.8 mL 3.6 mL	1.8 mL 3.6 mL
Animal tissue	30–100 mg 100–200 mg	1.8 mL 3.6 mL	1.8 mL 3.6 mL
Bacteria	1×10^9–5×10^9 2×10^9–1×10^{10}	1.8 mL 3.6 mL	1.8 mL 3.6 mL
Yeast	$< 3 \times 10^8$	3.6 mL	3.6 mL

An additional loading step is required if 3.6 mL Buffer RA1 and ethanol is used. If you isolate RNA from a certain kind of tissue the first time with the NucleoSpin® RNA Midi kit, we recommend starting with no more than 100 mg of tissue. Depending on the nature of the tissue, up to 200 mg can be processed. Do not use more than 200 mg of tissue to avoid clogging of the column.

Depending on sample type, the average yield is around 70–400 µg RNA (see Table 5). The A_{260}/A_{280} ratio indicating purity of the RNA generally exceeds 1.9.

Table 5:
Overview on average yields of RNA isolation using NucleoSpin® RNA Midi

Sample	Average yield
1×10^6 HeLa cells	20 µg
1×10^7 HeLa cells	160 µg
2×10^7 HeLa cells	330 µg
4×10^7 HeLa cells	620 µg
200 mg pig liver	450 µg
200 mg mouse liver	320 µg

2.3 Handling, preparation, and storage of starting materials

Work environment

Maintain an RNase-free work environment. Wear gloves at all times during the preparation. Change gloves frequently

Sample storage and RNase inhibition

RNases can rapidly degrade RNA within the samples if samples are not protected from RNase activity after harvest. The following methods are recommended to avoid RNA degradation:

- Use freshly harvested sample for immediate lysis and RNA purification.
- Submerge and store samples in NucleoProtect® RNA or similar stabilization solutions. Make sure to allow for complete permeation of the sample with the stabilization solution before freezing it. Remove excess stabilization solution from the sample prior to RNA isolation according to the stabilization solution user manual.
- Flash freeze sample in liquid N_2 immediately upon harvest and store at -70 °C. Frozen samples are stable up to 6 months. Mortar and pestle can be used to pulverize the sample in a frozen state. Make sure that the sample does not thaw prior to contact with lysis buffer.
- Store samples in Lysis Buffer RA1 after disruption at -70 °C for up to one year, at 4 °C for up to 24 hours or at room temperature for up to several hours. Samples frozen in Lysis Buffer RA1 should be thawed slowly before starting with the isolation of RNA.

Disruption and homogenisation of sample material

- **Cultured cells in suspension:**
  Collect cells by centrifugation, remove supernatant and immediately add Lysis Buffer RA1 according to step 2 of the standard protocol (see sections 5.1, 6.1).
- **Adherent cell cultures (lysis in culture dish):**
  Completely aspirate cell culture medium. Immediately add Lysis Buffer RA1 to the cell culture dish. Avoid incomplete removal of the cell culture medium in order to allow full lysis activity of the lysis buffer. Continue with lysate filtration (step 3 of the standard protocol).
- **Adherent cell cultures (lysis after trypsinization):**
  Aspirate cell culture medium and wash cells once with PBS. Aspirate PBS. Add 0.1–0.3 % trypsin in PBS and incubate for an appropriate time to detach the cells from the dish surface. After cell detachment, add medium, transfer cells to an appropriate tube (not supplied), and pellet by centrifugation for 5 min at 300 x *g*. Remove supernatant and continue with the addition of Lysis Buffer RA1 to the cell pellet.
- **Animal tissues:**
  It is essential for 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 tissues is grinding with a pestle and mortar. Grind the sample to a fine powder in the presence of liquid N_2. Take care that the sample does not thaw during or after

grinding or weighing and add the frozen powder to an appropriate aliquot of Buffer RA1 containing reducing agent, (e.g., ß-mercaptoethanol, DTT, or TCEP) and mix immediately. The broken-up tissue must then be homogenized with a NucleoSpin® Filter / Filter Midi (included in the kit) or by passing ≥ 5 times through a 0.9 mm syringe needle. Thawing of undisrupted animal tissue should be exclusively done in the presence of Buffer RA1 during simultaneous mechanical disruption, for example, with a rotorstator homogenizer. This ensures that the RNA is not degraded by RNases before the preparation has started. The spinning rotor disrupts and simultaneously homogenizes the sample by mechanical shearing of DNA within seconds up to minutes (homogenization time depends on sample). Take care to keep the rotor tip submerged in order to avoid excess foaming. Select a suitably sized homogenizer (5–7 mm diameter rotors can be used for homogenization in microcentrifuge tubes). Mechanical disruption and homogenization in Buffer RA1 (e.g., with a rotor stator homogenizer) is also recommended for tissue samples stored in stabilization solutions like NucleoProtect® RNA or RNA*later*®.

- **Bacteria and yeasts:**
  An enzymatic or mechanical lysis is required in most cases. For enzymatic lysis samples have to be incubated with lysozyme (bacteria) or lyticase/zymolase (yeast) solutions (see support protocols in section 5.2, 5.3). 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 RA1. 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. Alternatively, bacteria and yeast cells can be lysed mechanically by bead beating. Therefore, resuspend cell pellet in Lysis Buffer RA1, transfer the solution into a MN Bead Tube Type B and disrupt samples by bead beating (e.g., by using MN Bead Tube Holder on a Vortex Genie® 2). After lysis, homogenization is achieved by the use of a NucleoSpin® Filter or passing through a syringe-needle.

2.4 Elution procedures

It is possible to adapt elution method and volume of water used for the subsequent application of interest. 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 acid will be eluted.
- **High yield and high concentration:** Elute with the standard elution volume and apply the eluate once more onto the column for reelution.

Eluted RNA should immediately be put and always 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.

3 Storage conditions and preparation of working solutions

Attention:

Buffers RA1, RAW2, and MDB contain chaotropic salt. Wear gloves and goggles!

CAUTION: Buffers RA1, RAW2 and MDB contain chaotropic salt 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.

- Store lyophilized **rDNase (RNase-free)** at 4 °C on arrival (stable until: see package label).
- All other kit components should be stored at room temperature (15–25 °C) and are stable until: see package label. Storage at lower temperatures may cause precipitation of salts.
- Check that 70 % ethanol is available as additional solution to adjust RNA binding conditions in the lysate.
- Check that reducing agent (ß-ME, DTT, or TCEP) is available.

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

- **rDNase (RNase-free):** Add indicated volume of RNase-free H_2O (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 at least 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.) For temporary storage of dissolved rDNase during a working day always keep on ice.

In some cases the vial of rDNase may appear empty. This is due to lyophilized enzyme sticking to the septum. To avoid loss of rDNase, make sure to collect rDNase on the bottom of the vial before removing the plug.

- **Wash Buffer RA3:** Add the indicated volume of 96–100 % ethanol (see table on next page) to Buffer RA3 Concentrate. Mark the label of the bottle to indicate that ethanol was added. Wash Buffer RA3 can be stored at room temperature (18–25 °C) for at least one year.

	NucleoSpin® RNA		
REF	**10 preps** **740955.10**	**50 preps** **740955.50**	**250 preps** **740955.250**
Wash Buffer RA3 (Concentrate)	6 mL Add 24 mL ethanol	12 mL Add 48 mL ethanol	3 x 25 mL Add 100 mL ethanol to each vial
rDNase, RNase-free (lyophilized)	1 vial (size D) Add 120 µL RNase-free H_2O	1 vial (size F) Add 550 µL RNase-free H_2O	5 vials (size F) Add 550 µL RNase-free H_2O to each vial

	NucleoSpin® RNA Midi
REF	**20 preps** **740962.20**
Wash Buffer RA3 (Concentrate)	25 mL Add 100 mL ethanol
rDNase, RNase-free (lyophilized)	1 vial (size D) Add 540 µL RNase-free H_2O

4 Safety instructions

The following components of the NucleoSpin® RNA and NucleoSpin® RNA Midi kits contain hazardous contents.

When working with the **NucleoSpin® RNA** kit wear suitable protective clothing (e.g., lab coat, disposable gloves, and protective goggles).

For more information consult the appropriate Material Safety Data Sheets (MSDS available online at ***www.mn-net.com/msds***).

Caution: Guanidinium thiocyanate in buffer RA1 and guanidin hydrochlorid in buffer RAW2, 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** kit has not been tested for residual infectious material. A contamination of the liquid waste with residual infectious material is highly unlikely due to strong denaturing lysis buffer treatment but it cannot be excluded completely. Therefore, liquid waste must be considered infectious and should be handled and discarded according local safety regulations.

4.1 Disposal

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

5 NucleoSpin® RNA protocols

5.1 RNA purification from cultured cells and tissue

Before starting the preparation:

- Check if Wash Buffer RA3 and rDNase were prepared according to section 3.

1 Homogenize sample

Disrupt up to **30 mg** of **tissue** (for sample amounts see section 2.2; for homogenization methods see section 2.3).

Disrupt sample

Up to **5 x 10^6** eukaryotic cultured cells can be collected by centrifugation and lysed by addition of Buffer RA1 directly.

2 Lyse cells

Add **350 µL Buffer RA1** and **3.5 µL ß-mercaptoethanol** (ß-ME) to the cell pellet or to ground tissue and vortex vigorously.

+ 350 µL RA1

+ 3.5 µL ß-ME

For appropriate sample and lysis buffer amounts see section 2.2***.***

Note: As alternative to ß-ME the reducing agent DTT or TCEP may be used. Use a final concentration of 10–20 mM DTT or TCEP within the Lysis Buffer RA1.

3 Filtrate lysate

Reduce viscosity and clear the lysate by filtration through **NucleoSpin® Filter (violet ring)**: Place NucleoSpin® Filter in a Collection Tube (2 mL), apply the mixture, and centrifuge for **1 min** at **11,000 x *g*.**

11,000 x g, 1 min

The lysate may be passed alternatively ≥ 5 times through a 0.9 mm needle (20 gauge) fitted to a syringe.

In case of visible pellet formation (depending on sample amount and nature) transfer supernatant without any formed pellet to a new 1.5 mL microcentrifuge tube (not supplied).

Important: To process higher amounts of cells (> 1 x 10^6) or tissue (> 10 mg), the lysate should first be homogenized using the 0.9 mm needle (20 gauge), followed by filtration through NucleoSpin® Filters.

4 Adjust RNA binding conditions

Discard the NucleoSpin® Filter and add **350 µL ethanol (70 %)** to the homogenized lysate and mix by pipetting up and down (5 times).

+ 350 µL 70 % ethanol

Mix

Alternatively, transfer flowthrough into a new 1.5 mL microcentrifuge tube (not provided), add **350 µL ethanol (70 %)**, and mix by vortexing (2 x 5 s).

After addition of ethanol a stringy precipitate may become visible which will not affect the RNA isolation. Be sure to disaggregate any precipitate by mixing and load all of the precipitate on the column as described in step 5. Do not centrifuge the ethanolic lysate before loading it onto the column in order to avoid pelleting the precipitate.

5 Bind RNA

For each preparation take one **NucleoSpin® RNA Column (light blue ring)** placed in a Collection Tube. Pipette lysate up and down 2–3 times and **load the lysate** to the column. Centrifuge for **30 s** at **11,000 x *g***. Place the column in a new Collection Tube (2 mL).

Load lysate

11,000 x *g*, 30 s

Maximal loading capacity of NucleoSpin® RNA Columns is 750 µL. Repeat the procedure if larger volumes are to be processed.

6 Desalt silica membrane

Add **350 µL MDB** (Membrane Desalting Buffer) and centrifuge at **11,000 x *g*** for **1 min** to dry the membrane.

+ 350 µL MDB

11,000 x *g*, 1 min

Salt removal will make the following rDNase digest much more effective. If the column outlet has come into contact with the flowthrough for any reason, discard the flowthrough and centrifuge again for 30 s at 11,000 x *g*.

7 Digest DNA

Prepare DNase reaction mixture in a sterile 1.5 mL microcentrifuge tube (not provided): For each isolation, add **10 µL reconstituted rDNase** (also see section 3) to **90 µL Reaction Buffer for rDNase**. Mix by flicking the tube.

+ 95 µL rDNase reaction mixture

RT, 15 min

Apply **95 µL DNase reaction mixture** directly onto the center of the silica membrane of the column. Incubate at **room temperature** for 1**5 min**.

8 Wash and dry silica membrane

1st wash

Add **200 µL Buffer RAW2** to the NucleoSpin® RNA Column. Centrifuge for **30 s** at **11,000 x *g***. Place the column into a new Collection Tube (2 mL).

Buffer RAW2 will inactivate the rDNase.

+ 200 µL RAW2

11,000 x *g*, 30 s

2nd wash

Add **600 µL Buffer RA3** to the NucleoSpin® RNA Column. Centrifuge for **30 s** at **11,000 x *g***. Discard flowthrough and place the column back into the Collection Tube.

Note: Make sure that residual buffer from the previous steps is washed away with Buffer RA3, 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 RA3.

+ 600 µL RA3

11,000 x *g*, 30 s

3rd wash

Add **250 µL Buffer RA3** to the NucleoSpin® RNA Column. Centrifuge for **2 min** at **11,000 x *g*** to dry the membrane completely. Place the column into a nuclease-free Collection Tube (1.5 mL, supplied).

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

+ 250 µL RA3

11,000 x *g*, 2 min

9 Elute RNA

Elute the RNA in **60 µL RNase-free H_2O**, (supplied) and centrifuge at **11,000 x *g*** for 1 min.

If higher RNA concentrations are desired, elution can be done with 40 µL. Overall yield, however, will decrease when using smaller volumes.

+ 60 µL RNase-free H_2O

11,000 x *g*, 1 min

For further alternative elution procedures see section 2.4.

5.2 RNA preparation from up to 10^9 or 30 mg bacterial cells

Additional reagent to be supplied by user:

- Lysozyme or
- MN Bead Tubes Type B (see ordering information section 8.2)

Before starting the preparation:

- Check if Wash Buffer RA3 and rDNase were prepared according to section 3.

1 Homogenize

Two alternative protocols are given for homogenization of bacterial cells. Users may choose between an enzymatic digestion (A) or mechanical homogenization (B), depending on laboratory equipment and personal preference.

A) Homogenization by enzymatic digest

Resuspend the bacterial cell pellet (Gram-negative strains, up to 10^9 cells) in **100 µL TE** buffer (10 mM Tris-HCl, 1 mM EDTA; pH 8) containing 1 mg/mL lysozyme by vigorous vortexing. Incubate at **37 °C** for **10 min**.

For preparation of RNA from Gram-positive bacteria, resuspend cells in 100 µL TE containing 2 mg/mL lysozyme. It may be necessary to optimize incubation time and lysozyme concentration, depending on the bacterial strain.

Note: Due to the much higher concentration of genome equivalents in a nucleic acid preparation of bacteria compared with eukaryotic material, it may be necessary to use a lower quantity of cells for the preparation

Add **350 µL Buffer RA1** and **3.5 µL ß-mercaptoethanol** to the suspension and vortex vigorously.

For appropriate sample and lysis buffer amounts see section 2.2.

Note: As alternative to ß-ME the reducing agent DTT or TCEP may be used. Use a final concentration of 10–20 mM DTT or TCEP within the Lysis Buffer RA1.

B) Homogenization by MN Bead Tubes

Pellet cells (up to appx. 30 mg wet weight) by centrifugation and discard supernatant.

Add **350 µL Buffer RA1** to the cell pellet and vortex vigorously.

Note: Reducing agents such as ß-mercaptoethanol, DTT or TCEP are not necessary.

Transfer the resuspended cells into a MN Bead Tubes Type B and close the tube.

Disruption on the MN Bead Tube Holder:

Attach the MN Bead Tubes **horizontally** to a vortexer, for example, by taping or using a special adapter (e.g., MN Bead Tube Holder, see ordering information).

Vortex the samples at **full speed** and **room temperature** for **3 min**.

Disruption on a swing mill:

Alternatively place the MN Bead Tubes in a swing-mill and perform bead beating at 30 Hz for 1 min.

Note: In both cases we highly recommend to optimize the bead beating procedure (increase or decrease of disruption time) for your application and starting material in order to obtain a good balance of RNA yield and integrity.

Centrifuge the MN Bead Tube for 1 min at 11,000 x *g* to sediment the beads.

Recover the supernatant (lysate).

Proceed with step 3 of the NucleoSpin RNA standard protocol (section 5.1).

5.3 RNA preparation from up to 5 x 10^7 or 30 mg yeast cells

Additional reagents and components to be supplied by user:

- Reducing agent (ß-mercaptoethanol, or DTT (dithiothreithol), or TCEP (BisTris (Bis-(2-hydroxyethyl)-imino-tris(hydroxymethyl)-methane))
- Sorbitol and lyticase (or zymolase) for homogenization by enzymatic digestion
- MN Bead Tubes Type B and the MN Bead Tube Holder or a swing-mill for homogenization by mechanical disruption

Before starting the preparation:

- Check if Wash Buffer RA3 and rDNase were prepared according to section 3.

1 Homogenize sample

Two alternative protocols are given for homogenization of yeast cells. Users may choose between an enzymatic digestion (A) or mechanical homogenization (B), depending on laboratory equipment and personal preference. Homogenization by enzymatic digest is only recommended for fresh harvested cells, homogenization by mechanical disruption may also be performed with yeast cell pellets, stored at -70 °C for several months or yeast cells stabilized with NucleoProtect® RNA (see ordering information, section 8.2). Since this is highly dependent on the organism used, we recommend a comparison of frozen and fresh material in advance.

Note: Due to the much higher concentration of genome equivalents in a nucleic acid preparation of yeasts compared with cultured cells or tissue material, it may be necessary to use a lower quantity of cells for the preparation.

A) Homogenization by enzymatic digest

Harvest **2–5 mL** of **YPD culture** (5,000 x *g*; 10 min). Resuspend pellet in an appropriate amount of fresh prepared **sorbitol / lyticase buffer** (50–100 U lyticase or zymolase in 1 M sorbitol / 100 mM EDTA) and incubate at **30 °C** for **30 min**. Pellet the resulting spheroplasts by centrifugation (1,000 x *g*; 10 min).

Carefully discard supernatant.

Note: It may be necessary to optimize incubation time and lyticase / zymolase concentration, depending on the yeast strain.

Add **350 µL Buffer RA1** and **3.5 µL ß-mercaptoethanol** and vortex vigorously to lyse spheroplasts.

For appropriate sample and lysis buffer amounts see section 2.2.

Note: As alternative to ß-ME the reducing agent DTT or TCEP may be used. Use a final concentration of 10–20 mM DTT or TCEP within the Lysis Buffer RA1.

B) Homogenization by mechanical disruption

Pellet yeast cells (appx. 30 mg wet weight) by centrifugation and discard supernatant. Resuspend the cell pellet in **350 µL Lysis Buffer RA1**.

Note: Reducing agent such as ß-mercaptoethanol, DTT or TCEP is not required.

Transfer the resuspended cells into a MN Bead Tube Type B and close the tube.

Disruption on a swing mill:

Shake samples in a swing-mill at **30 Hz** for **10 sec**.

Disruption on the MN Bead Tube Holder:

Shake samples in the MN Bead Tube Holder for **3 min with maximum speed** at **room temperature**.

In both cases we highly recommend to optimize the bead beating procedure (increase or decrease of disruption time) for your application and starting material in order to obtain a good balance of RNA yield and integrity.

Centrifuge the MN Bead Tube for 1 min at 11,000 x *g* to sediment the beads.

Recover the supernatant (lysate).

Proceed with step 4 of the NucleoSpin® RNA standard protocol (section 5.1).

5.4 RNA preparation from paraffin embedded tissue*

Additional reagent to be supplied by user:

- Xylene

Before starting the preparation:

- Check if Wash Buffer RA3 and rDNase were prepared according to section 3.

A	Put **10 mg** of finely minced tissue into a 1.5 mL microcentrifuge tube (not provided). Add **300 µL xylene** and incubate 5 min with constant mixing at room temperature.
B	Centrifuge at **maximum speed** (13,000 rpm) for **3 min** to pellet the tissue. Discard the xylene.
C	Repeat the steps A and B twice, for a total of three xylene washes.
D	Add **300 µL of 96 % ethanol** to the tube and incubate **5 min** with constant mixing at **room temperature**.
E	Centrifuge at **maximum speed** (13,000 rpm) for **3 min** to pellet the tissue. Discard the ethanol.
F	Repeat steps D and E, for a total of two ethanol washes.
	Continue with step 1 of the NucleoSpin® RNA standard protocol (section 5.1). *Note: For high performance isolation of RNA from formalin-fixed, paraffin-embedded tissue the NucleoSpin® totalRNA FFPE (REF 740982, see ordering information, section 8.2) or NucleoSpin® totalRNA FFPE XS (REF 740969, see ordering information, section 8.2) is recommended.*

* Please also refer to: Annunziata Gloghini, Barbara Canal, Ulf Klein, Luigino Dal Maso, Tiziana Perin, Riccardo Dalla-Favera, and Antonino Carbone **RT-PCR Analysis of RNA Extracted from Bouin-Fixed and Paraffin-Embedded Lymphoid Tissues** J Mol Diagn 2004 6: 290–296 as one example for customer modification of the support protocol mentioned above.

5.5 Clean up of RNA from reaction mixtures

Before starting the preparation:

- Check that Wash Buffer RA3 was prepared according to section 3.

1 Prepare sample

Fill up RNA samples smaller than 100 µL with RNase-free H_2O to 100 µL.

If different samples with varying volumes between 100 and 200 µL are purified, RNA samples should be filled up with RNase-free H_2O to a uniform volume (e.g., 200 µL).

2 Prepare lysis-binding buffer premix

Prepare a Buffer RA1 - ethanol premix with ratio 1 : 1.

For **each 100 µL RNA sample,** mix **300 µL Buffer RA1** and **300 µL ethanol (96–100 %)**.

If multiple samples are processed, the preparation of a master-premix is recommended (e.g., 2 mL RA1 + 2 mL 98 % ethanol for approximately 6 NucleoSpin® RNA preparations).

3 Filtrate lysate

Not necessary!

4 Adjust RNA binding conditions

To **100 µL of RNA sample, add 600 µL (6 volumes) of Buffer RA1 - ethanol premix**. Mix sample with premix by vortexing.

If 200 µL of RNA samples are processed, add 1200 µL of RA1-ethanol premix.

Maximal loading capacity of NucleoSpin® RNA Columns is 750 µL. Repeat the procedure if larger volumes are to be processed.

After addition of ethanol, a stringy precipitate may become visible which will not affect the RNA isolation. Be sure to mix thoroughly and apply sample as homogenieous solution onto the column. For binding capacity of the columns, see Table 1*.*

Proceed with step 5, 8, and 9 of the NucleoSpin® RNA standard protocol (section 5.1). Steps 6 and 7 of the respective protocols may be omitted in this case.

As alternative products for RNA clean up, NucleoSpin® RNA Clean up and NucleoSpin® RNA Clean up XS are recommended (see ordering information, section 8.2).

5.6 RNA purification from Insects

1 Collect samples

Use fresh, shock-frozen (-70 °C.) or NucleoProtect* stabilized samples (approx. 1–10 mg of insect).

* Make sure to remove excess NucleoProtect® RNA solution from the sample before starting the RNA isolation procedure.

2 Resuspension

Add 350 µL Lysis Buffer RA1 without ß-mercaptoethanol or TCEP to a MN Bead Tube Type G and add the insect sample.

3 Homogenization and lysis

Perform bead beating using a retsch mill (30 Hz, 1–3 min) or MN Bead Tube Holder (full speed, 10 min).

4 Sedimentation of beads

Remove the steel balls from the bead tube (e.g. with a magnet), recover the lysate and apply it onto the NucleoSpin Filter (violet ring) according to step 3 of user manual section 5.1.

6 NucleoSpin® RNA Midi protocols

6.1 RNA purification from cultured cells and tissue

Before starting the preparation:

- Check that Wash Buffer RA3 and rDNase were prepared according to section 3.
- For centrifugation, a centrifuge with a **swing-out rotor** and appropriate buckets capable of reaching 4,000–4,500 x *g* is required.

1 Homogenize sample

Disrupt up to 100 mg of tissue (for sample amounts see section 2.2; for homogenization methods see section 2.3).

Disrupt sample

Up to **5 x 10^7** eukaryotic **cultured cells** are collected by centrifugation and lysed by addition of Buffer RA1 directly.

To choose an appropriate amount of starting material see section 2.2**.**

2 Lyse cells

Add **1.8 mL Buffer RA1** and **18 µL ß-mercaptoethanol** (ß-ME) to the disrupted material in a 15 mL centrifuge tube (not supplied) and vortex vigorously (use 3.6 mL Buffer RA1 and 36 µL ß-mercaptoethanol for large sample amounts; see section 2.2.)

+ 1.8 mLRA1

+ 18 µL ß-ME

Note: As alternative to ß-ME the reducing agent DTT or TCEP may be used. Use a final concentration of 10–20 mM DTT or TCEP within the Lysis Buffer RA1.

3 Filtrate lysate

Apply the lysate to a **NucleoSpin® Filter Midi** placed in a Collection Tube and centrifuge sample for **10 min** at **4,500 x *g***. This step will homogenize the sample by removal of residual insoluble material and simultaneous reduction of lysate viscosity.

4,500 x *g*, 10 min

In case of visible pellet formation (depending on sample amount and nature) transfer supernatant without any formed pellet to a new 15 mL centrifuge tube (not supplied).

If working with small amounts of cultured cells (e.g., < 1 x 10^7 HeLa cells) step 3 may be substituted by vigorous mixing of the sample.

4 Adjust RNA binding conditions

Discard the NueoSpin® Filter Midi and add **1.8 mL ethanol (70 %)** to the lysate in the Collection Tube and mix by vortexing 2 x 5 s (use 3.6 mL of 70 % ethanol if working with large sample amounts, see step 2 and section 2.2).

After addition of ethanol a stringy precipitate may become visible which will not affect the further procedure. Resuspend precipitates thoroughly before loading onto the NucleoSpin® RNA Midi Column.

+ 1.8 mL 70 % ethanol

Mix

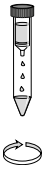
5 Bind RNA

Load the lysate-ethanol mixture (maximal 3.8 mL) onto a **NucleoSpin® RNA Midi Column**. Centrifuge for **3 min** at **4,500 x *g***.

If working with large sample amounts, apply the rest of the lysate-ethanol mixture (max. 3.8 mL) onto the column and centrifuge again.

If the lysate has not passed through the column, centrifuge again at 4,500 x g for 10 min.

In case of column-overloading incomplete flow through of the sample might be observed, for example, the membrane is still wet or some lysate has not passed through. Remove the lysate, which has not passed through the column, and continue with the next protocol step. Use less starting material and carefully remove insoluble material in step 3 next time.



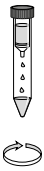
Load max. 3.8 mL lysate

4,500 x g, 3 min

6 Desalt silica membrane

Add **2.2 mL MDB** (Membrane Desalting Buffer) to the NucleoSpin® RNA Midi Column. Centrifuge for 3 min at **4,500 x *g***. Discard flow through.

If the silica membrane is not completely dry after centrifugation, centrifuge again at 4,500 x *g* for 10 min. This step will create optimal reaction conditions for the rDNase.



+ 2.2 mL MDB

4,500 x g, 3 min

7 Digest DNA

Prepare DNase reaction mixture: in a sterile microcentrifuge tube mix **235 µL Reaction Buffer for rDNase** and **25 µL reconstituted rDNase** (see section 3) per NucleoSpin® RNA Midi Column. Mix thoroughly but gently.

+ 250 µL rDNase reaction mixture

Digest with rDNase

Apply **250 µL DNase** reaction mixture directly onto the center of the silica membrane. Incubate at **room temperature** for **15 min**.

RT, 15 min

8 Wash and dry silica membrane

1st wash

Add **2.6 mL Buffer RAW2** to the NucleoSpin® RNA Midi Column. Incubate at room temperature for 2 min. Centrifuge for **3 min** at **4,500 x *g***. Discard flow through and place the column back into the Collection Tube.

+ 2.6 mL RAW2

4,500 x *g*, 3 min

Buffer RAW2 will inactivate the rDNase.

2nd wash

Add **2.6 mL Buffer RA3** to the NucleoSpin® RNA Midi Column. Centrifuge for **3 min** at **4,500 x *g***.

+ 2.6 mL RA3

4,500 x *g*, 3 min

The flow through has not to be discarded in this step. Leave the NucleoSpin® RNA Midi Column in the Collection Tube.

+ 2.6 mL RA3

4,500 x *g*, 5 min

3rd wash

Add **2.6 mL Buffer RA3** to the NucleoSpin® RNA Midi Column. Centrifuge for 5 min at **4,500 x *g*** to dry the membrane completely. Place the column into a fresh Collection Tube (15 mL, supplied).

9 Elute RNA

Pipette **500 µL RNase-free H_2O** (supplied) directly onto the center of the silica membrane. Incubate at **room temperature** for **2 min** and centrifuge for **3 min** at **4,500 x *g***.

+ 500 µL RNase-free H_2O

RT, 2 min

Reduction of elution volume will generally not result in an increased concentration of eluted nucleic acid with the NucleoSpin® RNA Midi kit (see section 2.4 for alternative elution procedures).

4,500 x *g*, 3 min

6.2 RNA preparation from up to 5 x 10^9 bacterial cells

Additional reagent to be supplied by user:

- Lysozyme or
- MN Bead Tubes Type B (see ordering information section 8.2)

Before starting the preparation:

- Check if Wash Buffer RA3 and rDNase were prepared according to section 3.

1 Homogenize

A) Homogenization by enzymatic digest

Resuspend the bacterial cell pellet (Gram-negative strains) in **200 µL TE** buffer (10 mM Tris-HCl, 1 mM EDTA; pH 8) containing **1 mg/mL lysozyme** by vigorous vortexing. Incubate at **37 °C** for **10 min**.

For preparation of RNA from Gram-positive bacteria, resuspend cells in 200 µL TE containing 2 mg/mL lysozyme. It may be necessary to optimize incubation time and lysozyme concentration, depending on the bacterial strain.

Note: Due to the much higher concentration of genome equivalents in a nucleic acid preparation of bacteria compared with eukaryotic material, it may be necessary to use a lower quantity of cells for the preparation

Add **1.8 mL Buffer RA1** and **1.8 µL ß-mercaptoethanol** to the suspension and vortex vigorously.

For appropriate sample and lysis buffer amounts see section 2.2.

Note: As alternative to ß-ME the reducing agent DTT or TCEP may be used. Use a final concentration of 10–20 mM DTT or TCEP within the Lysis Buffer RA1.

B) Homogenization by mechanical disruption

Pellet cells by centrifugation and discard supernatant.

Add glass beads (e.g., MN Beads Type B1 (Bulk), 40-70 mm, or MN Beads Type B2 (Bulk),0.3-0.4 mm, see ordering information).

Shake samples in a swing-mill at 30 Hz for **15 min**.

Note: Reducing agent such as ß-mercaptoethanol, DTT and TCEP might be dispensable (sample dependent).

Note: We highly recommend to optimize the bead beating procedure (increase or decrease of disruption time) for your application and starting material in order to obtain a good balance of RNA yield and integrity.

1 Filtrate lysate

Reduce viscosity and turbidity of the solution by filtration through NucleoSpin® Filter Midi. Place NucleoSpin® Filter Midi in Collection Tubes (2 mL), apply mixture, and centrifuge for **10 min** at **4,500 x *g***.

In case of visible pellet formation (depending on sample amount and nature) transfer supernatant without any formed pellet to a new 15 mL centrifuge tube (not supplied).

2 Adjust RNA binding conditions

Add **1.8 mL of ethanol (70 %)** to the lysate and mix by vortexing.

Proceed with step 5 of the NucleoSpin® RNA Midi standard protocol (section 6.1).

6.3 RNA preparation from up to 3 x 10^8 yeast cells

Additional reagents and components to be supplied by user:

- Reducing agent (ß-mercaptoethanol, or DTT (dithiothreithol), or TCEP (BisTris (Bis-(2-hydroxyethyl)-imino-tris(hydroxymethyl)-methane))
- Sorbitol and lyticase (or zymolase) for homogenization by enzymatic digestion or a swing-mill and glass beads for homogenization by mechanical disruption

Before starting the preparation:

- Check that Wash Buffer RA3 and rDNase were prepared according to section 3.

1 Homogenize sample

Two alternative protocols are given for homogenization of yeast cells. Users may choose between an enzymatic digestion (A) **or** mechanical homogenization (B), depending on laboratory equipment and personal preference. Homogenization by enzymatic digest is only recommended for fresh harvested cells, homogenization by mechanical disruption may also be performed with yeast cell pellets, stored at -70 °C for several months.

Note: Due to the much higher concentration of genome equivalents in a nucleic acid preparation of yeasts compared with cultured cells or tissue material, it may be necessary to use a lower quantity of cells for the preparation.

A) Homogenization by enzymatic digest

Harvest an appropriate amount of cells from **YPD culture (5,000 x *g*; 10 min)**. Resuspend pellet in an appropriate amount of fresh prepared sorbitol / lyticase buffer (50–100 U lyticase or zymolase in 1 M sorbitol / 100 mM EDTA) and incubate at **30 °C** for 30 min. Pellet the resulting spheroplasts by centrifugation (**1,000 x *g***; **10 min**).

Carefully discard supernatant.

It may be necessary to optimize incubation time and lyticase / zymolase concentration, depending on the yeast strain.

Continue with step 2.

OR

B) Homogenization by mechanical disruption

Harvest an appropriate amount of cells from **YPD culture** (**5,000 x *g***; **10 min**) and wash with ice-cold water. Resuspend the cell pellet in a mixture of 3.6 mL Buffer RA1 and 36 µL ß-mercaptoethanol.

Add corundum beads (e.g., MN Beads Type B (Bulk) or C (Bulk), see ordering information).

Shake samples in a swing-mill at 30 Hz for **15 min**.

Continue with step 3 Filtrate Lysate.

Note: Reducing agent such as ß-mercaptoethanol, DTT and TCEP might be dispensable (sample dependent).

Note: We highly recommend to optimize the bead beating procedure (increase or decrease of disruption time) for your application and starting material in order to obtain a good balance of RNA yield and integrity.

2 Lyse cells

Add **3.6 mL Buffer RA1** and **36 µL ß-mercaptoethanol** and vortex vigorously to lyse spheroplasts.

For appropriate sample and lysis buffer amounts see section 2.2**.**

Note: As alternative to ß-ME the reducing agent DTT or TCEP may be used. Use a final concentration of 10–20 mM DTT or TCEP within the Lysis Buffer RA1.

3 Filtrate lysate

Reduce viscosity and turbidity of the solution by filtration through **NucleoSpin® Filter Midi**. Place NucleoSpin® Filter Midi placed in Collection Tubes and centrifuge for **10 min** at **4,500 x** *g*.

In case of visible pellet formation (depending on sample amount and nature) transfer supernatant without any formed pellet to a new 15 mL centrifuge tube (not supplied).

4 Adjust RNA binding conditions

Discard the NucleoSpin® Filter Midi and add **3.6 mL 70 % ethanol** to the lysate in the Collection Tube and mix by vortexing.

Proceed with step 5 of the NucleoSpin® RNA Midi standard protocol (section 6.1).

6.4 Clean up of RNA from reaction mixtures

Before starting the preparation:

- Check that Wash Buffer RA3 was prepared according to section 3.

1 Prepare sample

Fill up RNA samples smaller than 500 µL with RNase-free H_2O to 500 µL.

2 Prepare lysis-binding buffer premix

Prepare a Buffer RA1 - ethanol premix with ratio 1 : 1.

For **each 500 µL RNA sample**, mix **1500 µL Buffer RA1** and **1500 µL ethanol (96–100 %)**.

If multiple samples are processed, the preparation of a master-premix is recommended (e.g., 2 mL RA1 + 2 mL 98 % ethanol for approximately 6 NucleoSpin® RNA preparations).

3 Filtrate lysate

Not necessary!

4 Adjust RNA binding conditions

To **500 µL of RNA sample, add 3000 µL (6 volumes) of Buffer RA1-ethanol premix**. Mix sample with premix by vortexing.

Maximal loading capacity of NucleoSpin® RNA Midi Columns is 4000 µL. Repeat the procedure if larger volumes are to be processed.

After addition of ethanol a stringy precipitate may become visible which will not affect the RNA isolation. Be sure to mix thoroughly and apply sample as homogenious solution onto the column. For binding capacity of the columns, see Table 1*.*

Proceed with step 5, 8, and 9 of NucleoSpin® RNA Midi standard protocol (section 6.1). Steps 6 and 7 of the respective protocols may be omitted in this case.

As alternative products for RNA clean up, NucleoSpin® RNA Clean up and NucleoSpin® RNA Clean up XS are recommended (see ordering information).

7 NucleoSpin® RNA / NucleoSpin® RNA Midi protocols

7.1 RNA preparation from NucleoProtect® RNA or RNA*later*® treated samples

Before starting the preparation:

- Check that Wash Buffer RA3 and rDNase were prepared according to section 3.

1	**Prepare sample** Remove NucleoProtect® RNA/RNA*later*® solution. Cut an appropriate amount of tissue.
2	**Lyse cells** Add **350 µL** (NucleoSpin® RNA) / **1.8 mL** (NucleoSpin® RNA Midi) **Buffer RA1** and **3.5 µL** (NucleoSpin® RNA) / **18 µL** (NucleoSpin® RNA Midi) **ß-mercaptoethanol** to the sample. Disrupt the sample material by using, for example, rotor-stator homogenizers (for homogenization methods see section 2.3).
	Proceed with step 3 (filtrate lysate) of the NucleoSpin® RNA standard protocol (section 5.1) or NucleoSpin® RNA Midi standard protocol (section 6.1).

7.2 rDNase digestion in solution

The on-column rDNase digestion in the standard protocol is already very efficient and thus resulting 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 repurification of the RNA (in order to remove buffer, salts, DNase and digested DNA) are usually required.

The high quality, recombinant, RNase-free DNase (rDNase) in the NucleoSpin® RNA kits facilitates such a digestion in solution in order to remove even traces of contaminating DNA.

A **Digest DNA (Reaction setup)**

Add **6 µL Reaction Buffer for rDNase** and **0.6 µL rDNase** to **60 µL eluted RNA**.

(Alternatively premix 100 µL Reaction Buffer for rDNase and 10 µL rDNase and add 1/10 volume to one volume of RNA eluate).

Gently swirl the tube in order to mix the solution. Spin down gently (approx. 1 s at 1,000 x *g*) to collect every droplet of the solution at the bottom of the tube.

B **Incubate sample**

Incubate for **10 min** at **37 °C**.

C **Repurify RNA**

Repurify RNA with a suitable RNA cleanup procedure, for example by use of the NucleoSpin® RNA Clean up, NucleoSpin® RNA Clean up XS kits (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 pellets and resuspend RNA in RNase-free H_2O.

8 Appendix

8.1 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 RNase-free H_2O to rDNase vial and 96 % ethanol to Buffer RA3 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 section 3. • 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 absorption 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
Poor RNA quality or yield *(continued)*	*Sample material* • Sample material not stored properly. Whenever possible, use fresh material. If this is not possible, flash freeze the samples in liquid N_2. Samples should always be kept at -70 °C. Never allow tissues to thaw before addition of Buffer RA1. Perform disruption of samples in liquid N_2. Alternatively, store tissue in NucleoProtect®RNA or similar protective reagents. • Insufficient disruption and / or homogenization of starting material. Ensure thorough sample disruption and use NucleoSpin® Filters / Filters Midi for easy homogenization of disrupted starting material.
Low A_{260} / A_{230} ratio	*Carry-over of guanidinium thiocyanate* • Carefully load the lysate to the NucleoSpin® RNA Column and try to avoid a contamination of the upper part of the column and the column lid. • Make sure that a sufficient amount / concentration of RNA is used for quantification so that the A_{230} value is significantly higher than the background level.
Clogged NucleoSpin® Column / Poor RNA quality or yield	Sample material • Too much starting material used. Overloading may lead to decreased overall yield. Reduce amount of sample material or use larger volume of Buffer RA1. • Insufficient disruption and / or homogenization of starting material. Ensure thorough sample disruption and use NucleoSpin® Filters / Filters L for easy homogenization of disrupted starting material.
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. *Too much cell material used* • Reduce quantity of cells or tissue used.

Problem	Possible cause and suggestions
Contamination of RNA with genomic DNA *(continued)*	*DNA detection system too sensitive* The amount of DNA contamination is effectively reduced during the on-column digestion with rDNase. However, it can not 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 / Plant system is checked by the following procedure: One million HeLa cells are subjected to RNA isolation according to the protocol. RNA eluate is used as 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 support protocol 7.2 for subsequent rDNase digestion in solution.
Suboptimal performance of RNA in downstream experiments	*Carry-over of ethanol or salt* • Do not let the flowthrough touch the column outlet after the second Buffer RA3 wash. Be sure to centrifuge at the corresponding speed for the respective time in order to remove ethanolic Buffer RA3 completely. • Check if Buffer RA3 has been equilibrated to room temperature before use. Washing at lower temperatures lowers efficiency of salt removal by Buffer RA3. *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.

8.2 Ordering information

Product	REF	Pack of
NucleoSpin® RNA	740955.10 / .50 / .250	10 / 50 / 250
NucleoSpin® RNA Midi	740962.20	20
NucleoSpin® miRNA	740971.10 / .50 / .250	10 / 50 / 250 preps
NucleoSpin® RNA / Protein	740933.10 / .50 / .250	10 / 50 / 250
NucleoSpin® TriPrep	740966.10 / .50 / .250	10 / 50 / 250
NucleoSpin® RNA Clean up	740948.10 / .50 / .250	10 / 50 / 250
NucleoSpin® RNA XS	740902.10 / .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
Buffer RA1	740961 / .500	60 / 500 mL
rDNase Set	740963	1 set
TCEP	740395.107	107 mg
NucleoProtect® RNA	740400.50 / .250	50 / 250 / 500
MN Bead Tubes Type B	740812.50	50
MN Bead Tubes Type B1 (Bulk) 40–70 mm glass beads	740809.B.5000	750 g
MN Bead Tubes Type B2 (Bulk) 0.3–0.4 mm glass beads	740812.B.1000	750 g
MN Bead Tubes Type C	740813.50	50
MN Bead Tubes Type C (Bulk) 1 mm corundum beads	740813.B.250	200 g
MN Bead Tube Holder	740469	1
NucleoSpin® Filters	740606	50
Collection Tubes (2 mL)	740600	1000

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

8.3 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.

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Please contact:

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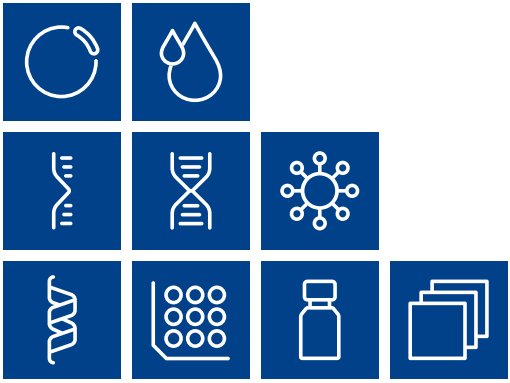

www.mn-net.com

MACHEREY-NAGEL

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