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RNA Isolation from FFPE Samples

User Manual

NucleoSpin® FFPE RNA

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MACHEREY-NAGEL

RNA Isolation from FFPE Samples

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

NucleoSpin® FFPE RNA

		Protocol 5.1: **RNA** isolation with **Paraffin Dissolver**	Protocol 5.2: **RNA** isolation with **xylene**
	Supply sample	For appropriate sample quantity see section 2.4.	For appropriate sample quantity see section 2.4
1	**Deparaffinize sample**	400 µL Paraffin Dissolver 60°C, 3 min Mix hot sample	1 mL xylene RT, 2 min Mix
		Let sample cool down	Full speed, 2 min Discard supernatant 1 mL ~98% ethanol Mix Full speed, 2 min Discard supernatant Dry at 60°C, 3 - 10 min
2	**Lyse sample**	 100 µL FL 11,000 x *g*, 1 min 10 µL Proteinase K Mix 60°C, 15 min – 3 hours	100 µL FL – 10 µL Proteinase K Mix 60°C, 15 min – 3 hours
3	**Decrosslink**	 100 µL D-Link Mix gently 11,000 x *g*, 30 s 90°C, 15 min	100 µL D-Link Mix gently – 90°C, 15 min
4	**Adjust binding conditions**	 200 µL ~98% ethanol Mix 11,000 x *g*, 30 s	200 µL ~98% ethanol Mix –
5	**Bind RNA**	 Load aqueous (lower) phase 2,000 x *g*, 30 s	Load lysate 2,000 x *g*, 30 s
6	**Desalt silica membrane**	 100 µL MDB 11,000 x *g*, 30 s	100 µL MDB 11,000 x *g*, 30 s
7	**Digest DNA**	25 µL rDNase mixture RT, 15 min	25 µL rDNase mixture RT, 15 min
8	**Wash and dry silica membrane**	 1st 100 µL FW1 RT, 2 min 11,000 x *g*, 30 s 2nd 400 µL FW2 11,000 x *g*, 30 s 3rd 200 µL FW2 11,000 x *g*, 2 min	100 µL FW1 RT, 2 min 11,000 x *g*, 30 s 400 µL FW2 11,000 x *g*, 30 s 200 µL FW2 11,000 x *g*, 2 min
9	**Elute highly pure RNA**	 10 µL RNase-free H_2O 11,000 x *g*, 30 s	10 µL RNase-free H_2O 11,000 x *g*, 30 s

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1 Components

1.1 Kit contents

	NucleoSpin® FFPE RNA		
	10 preps	**50 preps**	**250 preps**
REF	**740969.10**	**740969.50**	**740969.250**
Paraffin Dissolver	5 mL	25 mL	125 mL
Lysis Buffer FL	1.8 mL	8 mL	30 mL
Decrosslink Buffer D-Link	1.8 mL	8 mL	30 mL
Wash Buffer FW1	2 x 1 mL	15 mL	2 x 15 mL
Wash Buffer FW2 (Concentrate)*	2 mL	7 mL	2 x 20 mL
Membrane Desalting Buffer MDB	1.8 mL	10 mL	50 mL
Reaction Buffer for rDNase	0.5 mL	3 mL	20 mL
rDNase, RNase-free (lyophilized)*	1 vial (size A)	1 vial (size C)	2 vials (size D)
Proteinase K (lyophilized)*	6 mg	30 mg	75 mg
Proteinase Buffer PB	0.8 mL	1.8 mL	8 mL
RNase-free H_2O	5 mL	15 mL	25 mL
NucleoSpin® FFPE Columns (light blue rings plus Collection Tubes)	10	50	250
Collection Tubes (2 mL)	20	2 x 50	2 x 250
Collection Tubes (1.5 mL)	10	50	250
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* For preparation of working solutions and storage conditions see section 3.

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

Reagents

- 96 – 100% ethanol (undenaturated ethanol is preferable)
- Optional for deparaffinization without Paraffin Dissolver: xylene or d-limonene (e.g., Roti®-Histol, Hemo-De), or mixtures of isoparafinic hydrocarbons (e.g., Roticlear®, Micro-Clear™, Neo-Clear®).

Consumables

- 1.5 mL microcentrifuge tubes (for sample lysis)
- Disposable pipette tips

Equipment

- Manual pipettors
- Centrifuge for microcentrifuge tubes
- Vortex mixer
- Thermal heating-block (adjustable to 60°C and 90°C)
- Personal protection equipment (e.g., lab coat, gloves, goggles)

1.3 About this User Manual

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

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

2 Product description

Formalin-fixed, paraffin-embedded (FFPE) tissue samples are routinely prepared from human surgical tissue samples by fixation with formalin and embedding in paraffin. Thin sections of FFPE samples are commonly subjected to histophathological analysis and remaining paraffin-tissue blocks are usually archived. Existing extensive archives of FFPE tissue samples represent a valuable source for retrospective studies of gene expression patterns and mutation analysis. However, the use of such samples for RNA analysis is limited due to chemical modification by formaldehyde and fragmentation of the RNA during tissue processing (sampling, fixing, embedding) and storage (state, time, temperature) of the samples. Standard RNA isolation procedures result in poor RNA yield or poor performance in downstream applications (e.g., RT-PCR). A special purification system taking the unique requirements into account is inevitably necessary for successful analysis of nucleic acids from FFPE samples.

2.1 The basic principle

The **NucleoSpin® FFPE RNA** kit provides a convenient, reliable, and fast method to isolate RNA from formalin-fixed, paraffin-embedded (FFPE) tissue specimen. The procedure omits the use of flammable and malodorous xylene or d-limonene commonly used for deparaffinization. Further, the procedure omits the difficult removal of organic solvent from often barely visible tissue pellets, thus saving time. **NucleoSpin® FFPE RNA** employs the odorless Paraffin Dissolver (patent pending) and allows efficient lysis in a convenient two-phase system.

First, the paraffin of FFPE sections is dissolved in the Paraffin Dissolver. Tissue is then digested by proteinase to solubilize the fixed tissue and release nucleic acids into solution. Subsequently, heat incubation effectively eliminates crosslinking of RNA, maintaining its integrity. After addition of ethanol, the lysate is applied to the **NucleoSpin® FFPE Column**. RNA is bound to the silica membrane. Residual DNA remaining on the membrane is removed by convenient on-column rDNase digestion (RNase-free rDNase is supplied with the kit). Washing steps with two different buffers remove salts, metabolites, and macromolecular cellular components. Pure RNA is finally eluted under low ionic strength conditions in a small volume (10 µL) of RNase-free water, yielding highly concentrated RNA.

Nucleic acid preparation using **NucleoSpin® FFPE RNA** can be performed at room temperature. The eluate, however, should be treated with care. 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.

2.2 Kit specifications

- **NucleoSpin® FFPE RNA** is recommended for the isolation of total RNA from formalin-fixed, paraffin-embedded (FFPE) tissue samples, typically as thin sections (approx. 3 – 20 µm thickness). Thin sections mounted on glass slides can serve as sample material after transfer in a microcentrifuge tube. Formalin-fixed samples which are not embedded in paraffin can also be used as sample material by omitting the deparaffinization steps.

- **Sample amount:** The maximum sample size is determined by a) the amount of tissue and b) by the amount of paraffin.
  NucleoSpin® FFPE RNA is suitable for up to 5 mg tissue.
  The amount of paraffin is limited to 15 mg, when using the standard protocol with Paraffin Dissolver (ca. 7 sections of 10 µm x 250 mm²). However, larger amounts of paraffin samples may be processed by using either additional Paraffin Dissolver or by deparaffinization using xylene.

- **RNA yield** strongly depends on sample type, quality, amount, and time of storage. Further, measured yield may vary considerably among different quantification methods. Yield determined by absorption measurement at 260 nm or via a fluorescent dye (e.g., RiboGreen®) may deviate from values obtained by quantification via RT-PCR. Even quantification values obtained via RT-PCR with a short (e.g., 80 bp) and a long (e.g., 300 bp) amplicon may differ considerably. The deviation of quantification also depends on RNA size distribution as well as on efficiency of decrosslinking (or extent of remaining crosslinks). Please also see section 6.1 for considerations on determining RNA quality and quantity.

- **The innovative column design** with a funnel shaped thrust ring and a small silica membrane area allows elution of RNA in as little as 5 – 30 µL. Thus, eluted **RNA is highly concentrated** and RNA is ready-to-use for common downstream applications (e.g., RT-PCR).

- **RNA size distribution:** RNA isolated from formalin-fixed, paraffin-embedded tissue shows size distribution from 50 to 5,000 bases. Often short sized RNA from ca. 100 – 300 bases predominate, especially when the sample material is old. However, samples which were subjected to good tissue fixation, embedding, and storage conditions can yield RNA even larger than 5,000 bases.

- **RNA integrity:** RNA Integrity Numbers (RIN) according to Agilent 2100 Bioanalyzer assays depend on sample type and quality. In general the quality of RNA extracted from FFPE samples is poor. Typical RIN of RNA isolated with NucleoSpin® FFPE kits are in range of 2 – 6.

- **RNA preparation time** is approximately 70 min for 6 samples (including deparaffinization, 15 min lysis, and 15 min decrosslinking time).**rDNase** is supplied with the kit. DNA contaminations are removed by on-column digestion with rDNase. For most demanding downstream applications a subsequent digestion with rDNase in the eluate is also possible (see section 5.3).

Table 1: Kit specifications at a glance	
Parameter	**NucleoSpin® FFPE RNA**
Sample material*	Up to 7 sections, 10 µm surface of 250 mm^2
Typical yield	Strongly depends on sample quality and amount
Elution volume	5 – 30 µL
Binding capacity	110 µg
Maximum loading volume	600 µL
Preparation time	70 min/6 preps
Format	Mini spin column - XS design

2.3 Handling, preparation, and storage of starting materials

Many factors influence the yield and usability of RNA obtained from FFPE samples. The procedures of tissue sampling, post sampling delay before fixation, fixation time, embedding, and storage conditions have a high impact on RNA quality and yield. For more details about these aspects see, for example.: Chung JY *et al.* 2008; van Maldegem F *et al.* 2008; von Ahlfen S *et al.* 2007; Castiglione F *et al.* 2007; Leyland-Jones BR *et al.* 2008.

Starting from a paraffin-embedded tissue block, samples should be sectioned under RNase-free conditions. Paraffin sections may be stored at +4°C or lower for several weeks without observable effects on RNA yield or usability. Long term storage of paraffin sections may have a negative effect on RNA due to air oxidation.

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

* When using the standard procedure with Paraffin Dissolver.
Processing large quantities is possible with protocol modifications, see section 2.4.

2.4 Quantities of FFPE sections

The procedure in section 5.1 describes the use of 400 µL Paraffin Dissolver per preparation, where up to approximately 15 mg (ca. 17 µL) of paraffin can be dissolved. This corresponds to:

~ 17 sections of 10 µm thickness and 100 mm^2 area
~ 7 sections of 10 µm thickness and 250 mm^2 area
~ 5 sections of 10 µm thickness and 325 mm^2 area
~ 4 sections of 10 µm thickness and 400 mm^2 area
~ 3 sections of 10 µm thickness and 575 mm^2 area
~ 2 sections of 10 µm thickness and 840 mm^2 area
~ 1 section of 10 µm thickness and 1680 mm^2 area

Larger amounts of paraffin can be dissolved by adding a higher volume of Paraffin Dissolver (REF 740968.25) to the sample (30 µL per mg paraffin), or by using xylene for deparaffinization as described in section 5.2. When using more of the Paraffin Dissolver, it is necessary to use a collection tube larger than 1.5 mL to enable removal of the lower, aqueous phase after the decrosslink step without spillage.

2.5 Elution procedures

High **RNA** concentration in the elution fraction is desirable for all typical downstream applications. With regard to limited volumes of reaction mixtures, high template concentration can be a crucial criterion. Due to a large default elution volume, standard kits often result in low concentrated RNA if only small samples are processed. Such RNA samples may even require a subsequent concentration to be suitable for the desired application.

NucleoSpin® FFPE kits allow efficient elution in very small volumes resulting in highly concentrated RNA. Elution volumes in the range of **5 – 30 µL** are recommended, the default volume is 10 µL.

2.6 Stability of isolated RNA

Eluted RNA should immediately be put on ice and always kept on ice during work for optimal stability. Contamination with almost omnipresent RNases (general lab ware, fingerprints, dust) may be a risk for isolated 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 FL, FW1, and MDB contain chaotropic salts. Wear gloves and goggles!

- 96 – 100% ethanol is required to adjust the binding conditions in the lysate.

Storage conditions:

- Store lyophilized rDNase and Proteinase K at +4°C upon arrival (stable up to 1 year).
- All other kit components should be stored at room temperature (18 – 25°C) and are stable up to one year. Storage at lower temperatures may cause precipitation of salts.

Before starting any **NucleoSpin® FFPE** protocol prepare the following:

- **rDNase:** Add indicated volume (see next page or on the vial) of RNase-free water to the rDNase vial and incubate for 1 min at room temperature. Gently swirl the vial to completely dissolve the rDNase. Be careful not to mix rDNase vigorously as rDNase is sensitive to mechanical agitation. Dispense into aliquots and store at -20°C. The frozen working solution is stable for 6 months. Do not freeze/thaw the aliquots more than three times.
- **Proteinase K:** Add the indicated volume (see next page or on the vial) of Proteinase Buffer PB to dissolve lyophilized Proteinase K. Proteinase K solution is stable at -20°C for 6 months.
- **Wash Buffer FW2:** Add the indicated volume (see next page or on the bottle) of 96 – 100% ethanol to Buffer FW2 Concentrate. Store Buffer FW2 at room temperature (18 – 25°C) for up to one year.

	NucleoSpin® FFPE RNA		
	10 preps	**50 preps**	**250 preps**
REF	**740969.10**	**740969.50**	**740969.250**
Wash Buffer FW2 (Concentrate)	2 mL Add 8 mL 96 – 100% ethanol	7 mL Add 28 mL 96 – 100% ethanol	2 x 20 mL Add 80 mL 96 – 100% ethanol to each bottle
rDNase, RNase-free (lyophilized)	1 vial (size A) Add 55 µL RNase-free H_2O	1 vial (size C) Add 230 µL RNase-free H_2O	2 vials (size D) Add 540 µL RNase-free H_2O to each vial
Proteinase K (lyophilized)	6 mg Add 260 µL Proteinase Buffer PB	30 mg Add 1.35 mL Proteinase Buffer PB	75 mg Add 3.35 mL Proteinase Buffer PB

4 Safety instructions – risk and safety phrases

The following components of the **NucleoSpin® FFPE RNA** kits contain hazardous contents.

Wear gloves and goggles and follow the safety instructions given in this section.

Component	Hazard contents	Hazard symbol		Risk phrases	Safety phrases
rDNase, RNase-free	rDNase, lyophilized	✖ Xi*	May cause sensitization by inhalation and skin contact	R 42/43	S 22-24
Proteinase K	Proteinase K, lyophilized	✖ Xn Xi**	Irritating to eyes, respiratory system and skin - May cause sensitization by inhalation	R 36/37/38-42	S 22-24-26-36/37
FW1	Guanidinium hydrochloride + ethanol < 25%	✖ Xn*	Flammable - Harmful if swallowed - Irritating to eyes and skin	R 10-22-36/38	S 7-16
MDB	Guanidinium thiocyanate <10% + ethanol <10%	**	Flammable	R 10	S 7-16
Paraffin Dissolver***	Mineral oil distillate	✖ Xn**	Harmful: May cause lung damage if swallowed - Repeated exposure may cause skin dryness or cracking	R 65-66	S 62

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

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

*** Disposal considerations for Paraffin Dissolver: Please observe local regulations for collection and disposal of waste and contact waste disposal company, where you will obtain information on disposal (waste code number 16 05 06).

Risk phrases

R 10	Flammable
R 20/21/22	Harmful by inhalation, in contact with the skin, and if swallowed
R 22	Harmful if swallowed
R 36/38	Irritating to eyes and skin
R 36/37/38	Irritating to eyes, respiratory system and skin
R 42	May cause sensitization by inhalation
R 42/43	May cause sensitization by inhalation and skin contact
R 65	Harmful: May cause lung damage if swallowed
R 66	Repeated exposure may cause skin dryness or cracking

Safety phrases

S 7	Keep container tightly closed
S 16	Keep away from sources of ignition - No smoking!
S 22	Do not breathe dust
S 24	Avoid contact with the skin
S 26	In case of contact with eyes, rinse immediately with plenty of water and seek medical advice
S 36/37	Wear suitable protective clothing and gloves
S 62	If swallowed, do not induce vomitting; seek medical advice immediately and show this container or label

5 Protocols

NucleoSpin® FFPE kits offer two different methods for sample deparaffinization. One utilizes the Paraffin Dissolver (included in the kit) and one utilizes xylene or comparable organic solvents (not supplied with the kit).

Deparaffinization with Paraffin Dissolver:	Section 5.1 (recommended)
Deparaffinization with xylene:	Section 5.2

5.1 RNA purification from FFPE samples using Paraffin Dissolver

Before starting the preparation:

- Check if rDNase, Proteinase K, and Buffer FW2 were prepared according to section 3.
- Check if 96 – 100% ethanol is available.
- Set incubator(s) at 60°C (for paraffin melting and lysis step) and 90°C (for decrosslink step).

Sample preparation

Insert FFPE section(s) in a microcentrifuge tube (not supplied).

For appropriate sample amounts see section 2.4.

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1 Deparaffinize sample

Add **400 µL Paraffin Dissolver** to the sample.

+ 400 µL Paraffin Dissolver

Incubate **3 min** at **60°C** (to melt the paraffin).

60°C 3 min

Vortex the sample immediately (at 60°C) at a vigorous speed to dissolve the paraffin.

Vortex hot sample

Cool down sample to room temperature.

Make sure that paraffin completely melts during the heat incubation step and mix well after melting to completely dissolve the paraffin.

Insufficient mixing of the heated sample may cause recurrence of solid paraffin particles. Make sure the sample does not comprise more than 15 mg paraffin or adjust the volume of Paraffin Dissolver (see section 2.4).

For samples comprising more than 15 mg paraffin, use 30 µL Paraffin Dissolver per 1 mg paraffin. If more than 400 µL Paraffin Dissolver is necessary, place sample in a 2 mL tube (not provided).

2 Lyse sample

Add **100 µL Buffer FL**.

+ 100 µL FL

Vortex vigorously.

Vortex

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

11,000 x *g*
1 min

Two phases will be formed: a lower (aqueous) phase and an upper (organic) phase. Tissue material will be transferred to the lower (aqueous) phase.

Pipette **10 µL Proteinase K** solution directly into the lower (aqueous) phase.

+ 10 µL Proteinase K

Mix the aqueous phase by pipetting up and down several times. (Pipette only the lower, aqueous phase up and down. Avoid mixing lower phase and upper phase excessively).

Mix by pipetting up and down

Make sure that the Proteinase K is mixed well with the lysis buffer.

If multiple samples are processed, preparation of a Buffer FL/ Proteinase K premix is recommended. Add 110 µL of the premix to the reaction tube, mix, and centrifuge to achieve phase formation and to transfer the tissue into the aqueous (lower) phase. Pipette aqueous phase up and down several times in order to disperse the tissue in the lysis buffer.

Incubate at **60°C** for **15 min – 3 hours** to lyse sample tissue.

60°C
15 min – 3 hours

If residual unlysed tissue particles are visible after 15 min incubation continue the incubation for up to 3 hours. If a large portion of sample is still undigested, add additional 10 µL Proteinase K solution and continue digestion for further 3 hours. An overnight incubation is only recommended if the tissue is not well digested within 2 x 3 hours.
RNA is generally sensitive to autolysis. Thus, a short incubation time is preferable.

Vortex 5 s. **Vortex**

Set heating block to 90°C.

Convenient stopping point: at this point, the procedure can temporarily be stopped. If pausing, we recommend to store the samples at -20°C.

3 Decrosslink

+ 100 µL D-Link

Add **100 µL Decrosslink Buffer D-Link** to the tube and **vortex** gently to mix Buffer D-Link into the aqueous (lower) phase.

Vortex

11,000 x *g* **30 s**

Centrifuge at **11,000 x *g*** for **30 s** to obtain phase formation.

Incubate at **90°C** for exactly **15 min**.

90°C **15 min**

Vortex 5 s and let cool down to room temperature (approx. 2 min).

Vortex

If necessary, spin down briefly to clear the lid (approx. 1 s at 1,000 x g).

4 Adjust binding conditions

+ 200 µL ethanol

Add **200 µL ethanol (96 – 100%)** to the tube and **mix** by vortexing (2 x 5 s).

Vortex

11,000 x *g* **30 s**

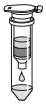
Centrifuge for **30 s** at **11,000 x *g*** to achieve complete phase separation.

The ethanol will merge with the aqueous (lower) phase only.

5 Bind RNA

For each preparation, take one **NucleoSpin® FFPE Column (light blue ring)** placed in a Collection Tube.

Pipette aqueous (lower) phase completely into the NucleoSpin® FFPE Column.



Load aqueous (lower) phase

It is recommended to pipette a total volume of 450 µL on the spin column, to ensure that the complete aqueous (lower) phase is transferred (the volume of the aqueous phase is approx. 410 µL). Small carry-over of the organic (upper) phase has no negative effect on the binding procedure.

2,000 x *g*
30 s

Centrifuge for **30 s** at **2,000 x *g*.**

The recommended centrifugation at 2,000 x g *is more efficient than centrifugation at 11,000 x* g.

Discard Collection Tube with flow-through and place the column in a new Collection Tube (2 mL).

6 Desalt silica membrane

Add **100 µL MDB (Membrane Desalting Buffer**) and centrifuge at **11,000 x *g*** for **30 s**.

+ 100 µL MDB

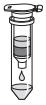
It is not necessary to use a fresh Collection Tube after this centrifugation step.

11,000 x *g*
30 s

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

7 Digest DNA

Prepare rDNase reaction mixture in a sterile microcentrifuge tube (not provided): For each isolation, add **3 µL reconstituted rDNase** (see section 3) to **27 µL Reaction Buffer for rDNase**. Mix by flicking the tube.



+ 25 µL rDNase reaction mixture

Apply **25 µL rDNase reaction mixture** directly onto the center of the silica membrane of the column. Close the lid. Incubate at **room temperature** for **15 min**.

RT
15 min

It is not necessary to use a new Collection Tube after the incubation step.

8 Wash and dry silica membrane

1st wash

+ 100 µL FW1

Add **100 µL Buffer FW1** to the NucleoSpin® FFPE Column. Incubate for **2 min** at **RT**.

RT
2 min

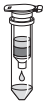
Centrifuge for **30 s** at **11,000 x *g***.

11,000 x *g*
30 s

Discard Collection Tube with flow-through and place the column into a new Collection Tube (2 mL).

Buffer FW1 will inactivate the rDNase.

2nd wash



+ 400 µL FW2

Add **400 µL Buffer FW2** to the NucleoSpin® FFPE Column.

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

11,000 x *g*
30 s

Discard flow-through and place the column back into the Collection Tube.

3rd wash

Add **200 µL Buffer FW2** to the NucleoSpin® FFPE Column.

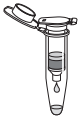
+ 200 µL FW2

Centrifuge for **2 min** at **11,000 x *g*** to dry the membrane. Discard the Collection Tube with flow-through and place the column into a nuclease-free 1.5 mL Collection Tube (provided).

11,000 x *g*
2 min

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

9 Elute highly pure RNA



+ 10 µL H_2O, RNase-free

Elute the RNA in **10 µL H_2O** (RNase-free; supplied) and centrifuge at **11,000 x *g*** for **30 s**.

11,000 x *g*
30 s

If higher RNA concentration or higher elution volume is desired, elution volume may be varied in the range of 5 – 30 µL.

5.2 RNA purification from FFPE samples with xylene deparaffinization

Before starting the preparation:

- Check if rDNase, Proteinase K, and Buffer FW2 were prepared according to section 3.
- Check if 96 – 100% ethanol is available.
- Set incubator(s) at 60°C (for ethanol evaporation and lysis step) and 90°C (for decrosslink step).

Sample preparation

Insert FFPE section(s) in a microcentrifuge tube (not supplied).

For appropriate sample amounts see section 2.4.

1 Deparaffinize sample

1 mL xylene

Add **1 mL xylene** (or alternatives, see section 1.2) to the sample.

RT
2 min

Incubate at **room temperature** until the paraffin is completely dissolved (usually approx. **2 min**) and vortex vigorously (10 s).

Vortex

Full speed
2 min

Centrifuge for **2 min** at **full speed**.

Discard supernatant

Discard the supernatant by pipetting. Do not remove any of the pellet.

1 mL ethanol

Add **1 mL ethanol (96 – 100%)** to the pellet and vortex (5 s).

Vortex

Centrifuge for **2 min** at **full speed**.

Full speed
2 min

Discard the supernatant by pipetting. Do not remove any of the pellet.

Discard supernatant

Incubate the open tube at **60°C** for **3 – 10 min** to dry the pellet.

60°C
3 – 10 min

It is important to evaporate all residual ethanol. Residual ethanol may reduce RNA yield.

2 Lyse sample

Add **100 µL Buffer FL** and **10 µL Proteinase K** to the pellet. Vortex vigorously (5 s).

+ 100 µL FL

+ 10 µL Proteinase K

If multiple samples are processed, preparation of a Buffer FL/ Proteinase K premix is recommended. Add 110 µL of the premix to the pellet.

Centrifuge briefly (approx. 1 s at 1,000 x *g*).

Vortex

Solid section residuals at the tube wall should be flushed back into the solution by pipetting. Pipette solution up and down in order to homogenize sections.

Incubate at **60°C** for **15 min – 3 hours** to lyse sample tissue.

If residual unlysed tissue particles are visible after 15 min incubation continue the incubation for up to 3 hours. If a large portion of sample is still undigested, add additional 10 µL Proteinase K solution and continue digestion for further 3 hours. An overnight incubation is only recommended if the tissue is not well digested within 2 x 3 hours.

60°C 15 min - 3 hours

RNA is generally sensitive to autolysis. Thus, a short incubation time is preferable.

Vortex tube 5 s.

Set heating block to 90°C.

Convenient stopping point: At this point, the procedure can temporarily be stopped. If pausing, we recommend to store the samples at -20°C.

3 Decrosslink

+ 100 µL D-Link

Add **100 µL Decrosslink Buffer D-Link** to the lysate and vortex vigorously (5 s).

Vortex

Incubate at **90°C** for exactly **15 min**.

Subsequently, vortex 5 s and cool down to room temperature for approx. 2 min.

90°C 15 min

If necessary, spin down briefly to clear the lid (approx. 1 s at 1,000 x g).

4 Adjust binding conditions

Add **200 µL ethanol (96 – 100%)** to the tube and **mix** by vortexing (2 x 5 s).

+ 200 µL ethanol

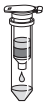
Vortex

Spin down briefly to clear the lid (approx. 1 s at 1,000 x *g*).

5 Bind RNA

For each preparation, take one **NucleoSpin® FFPE Column (light blue ring)** placed in a Collection Tube.

Pipette lysate up and down two times before loading the lysate.



Load lysate

Load the lysate into the column.

Centrifuge for **30 s** at **2,000 x *g***.

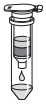
2,000 x *g* 30 s

The recommended centrifugation at 2,000 x g *is more efficient than centrifugation at 11,000 x* g.

Discard Collection Tube with flow-through and place the column in a new Collection Tube (2 mL).

6 Desalt silica membrane

Add **100 µL MDB (Membrane Desalting Buffer**) and centrifuge at **11,000 x *g*** for **30 s**.



+ 100 µL MDB

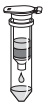
It is not necessary to use a fresh Collection Tube after this centrifugation step.

11,000 x *g* 30 s

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

7 Digest DNA

Prepare rDNase reaction mixture in a sterile microcentrifuge tube (not provided): For each isolation, add **3 µL reconstituted rDNase** (see section 3) to **27 µL Reaction Buffer for rDNase**. Mix by flicking the tube.



+ 25 µL rDNase reaction mixture

Apply **25 µL rDNase reaction mixture** directly onto the center of the silica membrane of the column. Close the lid. Incubate at **room temperature** for **15 min**.

RT 15 min

It is not necessary to use a new Collection Tube after the incubation step.

8 Wash and dry silica membrane

1st wash

+ 100 µL FW1

Add **100 µL Buffer FW1** to the NucleoSpin® FFPE Column. Incubate for **2 min** at **RT**.

RT 2 min

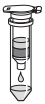
Centrifuge for **30 s** at **11,000 x *g***.

11,000 x *g* 30 s

Discard Collection Tube with flow-through and place the column into a new Collection Tube (2 mL).

Buffer FW1 will inactivate the rDNase.

2nd wash



+ 400 µL FW2

Add **400 µL Buffer FW2** to the NucleoSpin® FFPE Column.

Centrifuge for **30 s** at **11,000 x *g***. Discard flow-through and place the column back into the Collection Tube.

11,000 x *g* 30 s

3rd wash

Add **200 µL Buffer FW2** to the NucleoSpin® FFPE Column.

+ 200 µL FW2

Centrifuge for **2 min** at **11,000 x *g*** to dry the membrane. Discard the Collection Tube with flow-through and place the column into a nuclease-free 1.5 mL Collection Tube (provided).

11,000 x *g* 2 min

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

9 Elute highly pure RNA

Elute the RNA in **10 µL H_2O** (RNase-free; supplied) and centrifuge at **11,000 x *g*** for **30 s**.

If higher RNA concentration or higher elution volume is desired, elution volume may be varied in the range of 5 – 30 µL.

+ 10 µL H_2O, RNase-free

11,000 x *g* 30 s

5.3 Support protocol NucleoSpin® FFPE RNA: DNA digestion in the RNA eluate

Comments on DNA removal:

Although the on-column rDNase digestion in the standard protocol is very efficient, there are still certain applications which require even lower quantities of residual DNA. The 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 quantities 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 degrade contaminating DNA. This requires stringent RNase control and subsequent repurification of the RNA (in order to remove buffer, salts, DNase, and digested DNA).

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

A **Digest DNA (Reaction setup)**

Prepare enzyme-buffer premix: add **1 µL rDNase** to **10 µL Reaction Buffer for rDNase**.

Add 1/10 volume of enzyme-buffer premix to the eluted RNA (e.g., to 10 µL RNA add 1 µL of the premix comprising buffer and enzyme).

B **Incubate** for **10 min** at **37°C**.

C **Inactivate rDNase**

After rDNase digestion in the RNA eluate, incubate the sample for **5 min** at **75°C** to inactivate the rDNase. Subsequently, keep the sample on ice.

In most cases a further purification (in order to remove inactivated rDNase, buffer and salts) is not necessary, because the rDNase works in a highly dilute buffer and is inactivated during heat incubation. If nevertheless a repurification is required, NucleoSpin® RNA Clean-up XS is recommended (see ordering information).

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6 Appendix

6.1 Comments on RNA quality and quantity

Due to tissue fixation, nucleic acids in FFPE samples are commonly fragmented and chemically modified by formaldehyde. These modifications cannot be detected by standard quality control assays, such as gel electrophoresis, spectrophotometry, fluorometry, or microfluidics analysis. However, efficiency of enzymatic reactions with chemically modified RNA is significantly decreased.

Affected RNA analysis methods and applications are for example:

- Spectrophotometry, (e.g., absorption measurement A_{230}, A_{260}, A_{280})
- Fluorometry (e.g., RiboGreen®)
- Denaturing agarose gel electrophoresis
- Mirofluidics analysis (e.g., Agilent 2100 Bioanalyzer, BioRad´s Experion Automated Electrophoresis System)
- RT-PCR
- Array analysis (e.g., microarrays)

The following aspects should be considered when applying one of the listed methods, especially when comparing efficiency of different RNA isolation procedures and usability of the isolated RNA:

- **A high RNA yield**, as determined by A_{260} readings or by fluorescent dye (e.g., RiboGreen®) analysis does not necessarily result in good performance of the RNA in an RT-PCR. RNA may be highly degraded (i.e., smaller than the RT-PCR target or insufficiently decrosslinked).

- **Low or no RNA yield** as determined by A_{260} readings will most likely result in poor RT-PCR results, but it is still possible to achieve a good performance. There may be a small amount RNA which is decrosslinked sufficiently and shows good reactivity.

- **A high RNA integrity** does not guarantee a good amplifiability of RNA in RT-PCR or reactivity in other enzymatic reactions. RNA may be insufficiently decrosslinked but still show a high RIN (RNA Integrity Number; Agilent) or RQI (RNA Quality Indicator; BioRad) value.

- **A low RNA integrity**, i.e. highly degraded RNA with fragment sizes exclusively below 200 nucleotides will certainly not enable amplification of fragments exceeding this size. However, it is still likely that small sized target sequences can be amplified successfully, especially if they are well decrosslinked.

Neither RNA yield, nor RIN, RQI, absorbance ratios, or size distribution can reliably predict the performance in downstream RT-PCR applications, especially if different purification and decrosslinking systems are compared.

The major quality indicator for RNA isolated from FFPE samples is its performance in RNA profiling analysis (i.e., in RT-PCR or microarray experiments.)

6.2 Troubleshooting

Problem	Possible cause and suggestions
Incomplete paraffin dissolution in xylene	*Hard-to-solve paraffin* • Incubate sample 5 min in xylene at 60°C and mix. Centrifuge 2 min at maximum speed and remove supernatant. If paraffin is still not dissolved completely, add again 1 mL xylene, incubate 2 min at 60°C, centrifuge at maximum speed, and discard supernatant.
Poor RNA quality or yield	*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 sample quality* • Sample quality very much influences the obtainable RNA amount and quality. For aspects concering sample harvest, fixation, embedding, and storage refer to: Castiglione *et al.* 2007, Chung *et al.* 2008, Leyland-Jones *et al.* 2008, von Ahlfsen *et al.* 2007, von Maldegem *et al.* 2008. *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 FW2 Concentrate and mix. Reconstitute and store lyophilized rDNase and Proteinase K 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 kit components as described in section 3. • Keep bottles tightly closed in order to prevent evaporation or contamination.

Poor RNA quality or yield *(continued)*	*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 also see: - 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. *Proteinase digestion time* • Depending of the nature of the sample, an optimal digestion time from 15 min to 2 x 3 hours has to be determined empirically. If tissue residues are still visible after 15 min continue the incubation for up to 3 hours. If a large portion of the sample still remains undigested, continue digestion overnight. An overnight incubation is not recommended if the tissue digested well within 2 x 3 hours.
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 FL. • Insufficient disruption and/or homogenization of starting material. Make sure that the lysate is clear before loading the lysate on the column. If the column is clogged, try spinning at full speed for two minutes in order to save the sample.
Contamination of RNA with genomic DNA	*rDNase not active* • Reconstitute and store lyophilized rDNase according to instructions given in section 3. *rDNase solution not properly applied* • Pipette rDNase solution directly onto the center of the silica membrane and close the lid in order to press the solution into the membrane. *Too much cell material used* • Reduce quantity of cells or tissue used.

Contamination of RNA with genomic DNA *(continued)*	*DNA detection system too sensitive* • The amount of DNA contamination is significantly reduced during the on-column digestion with rDNase. Still, it can not be guaranteed that the purified RNA is 100% free of DNA, therefore in very sensitive applications it might be possible to detect DNA. DNA detection with PCR increases with: - the number of DNA copies per preparation: single copy target < plastidial/mitochondrial target < plasmid transfected into cells. - decrease in PCR amplicon size. • Use larger PCR targets (e.g., >500 bp) or intron spanning primers if possible. • Use support protocol for subsequent rDNase digestion in the eluate (section 5.5).
Suboptimal performance of RNA in downstream experiments	*Carry-over of ethanol or salt* • Do not let the column flow-through touch the column outlet after the second Buffer FW2 wash. Be sure to centrifuge at the recommended speed and time in order to remove ethanolic Buffer FW2 completely. • Check if Buffer FW2 has been equilibrated to room temperature before use. Washing at lower temperatures lowers efficiency of salt removal by Buffer FW2. • Depending on the robustness of the RT-PCR system used, RT-PCR might be inhibited if complete eluates are used as template for RT-PCR. Use less eluate as template. *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.

Discrepancy between A_{260} quantification values and PCR quantification values	*Silica abrasion from the membrane* • Due to the typically low RNA content in very small samples and the resulting low total amount of isolated RNA, a RNA quantification via A_{260} absorption measurement is often hampered due to the low sensitivity of the absorption measurement. When performing absorption measurements close to the detection limit of the photometer, the measurement may be influenced by minor amounts of silica abrasion. In order to prevent incorrect A_{260}-quantification of small RNA amounts centrifuge the eluate for 30 s at > 11,000 x *g* and take an aliquot for measurement without disturbing any sediment. Alternatively, use a silica abrasion insensitive RNA quantification method (e.g., RiboGreen® fluorescent dye).
Unexpected A_{260}/A_{280} ratio	*Measurement not in the range of photometer detection limit* • In order to obtain a reliable A_{260}/A_{280} ratio it is necessary that the initially measured A_{260} and A_{280} values are significantly above the detection limit of the photometer used. An A_{280} value close to the background noise of the photometer will cause non reliable A_{260}/A_{280} ratios.

6.3 Ordering information

Product	REF	Pack of
NucleoSpin® FFPE RNA	740969.10/.50/.250	10/50/250 preps
NucleoSpin® FFPE RNA/DNA*	740978.10/.50/.250	10/50/250 preps
NucleoSpin® RNA XS	740902.10/.50/.250	10/50/250 preps
NucleoSpin® RNA Clean-up XS	740903.10/.50/.250	10/50/250 preps
NucleoSpin® RNA II	740955.10/.50/.250	10/50/250 preps
NucleoSpin® RNA/Protein	740933.10/.50/.250	10/50/250 preps
NucleoSpin® TriPrep*	740966.10/.50/.250	10/50/250 preps
NucleoSpin® RNA/DNA Buffer Set*	740944	Suitable for 100 preps
rDNase Set	740963	1
Paraffin Dissolver	740968.25	25 mL
Decrosslink Buffer D-Link	740979.30	30 mL
NucleoSpin® Filters	740606	50
Collection Tubes (2 mL)	740600	1000

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

* DISTRIBUTION AND USE IN THE USA IS PROHIBITED FOR PATENT REASONS.

6.4 References

Castiglione F. *et al.* (2007): Real-time PCR analysis of RNA extracted from formalin-fixed and paraffin-embeded tissues: effects of the fixation on outcome reliability. Appl Immunohistocehm Mol Morphol 15(3): 338-342.

Chung J. Y. *et al.* (2008): Factors in tissue handling and processing that impact RNA obtained from formalin-fixed, paraffin-embedded tissue. Journal of Histochemistry & Cytochemistry. 56(11): 1033-1042.

Koch I. *et al.* (2006): Real-time quantitative RT-PCR shows variable, assay-dependent sensitivity to formalin fixation: implications for direct comparison of transcript levels in paraffin-embedded tissues. Diagn Mol Pathol. 15(3): 149-156.

Leyland-Jones B. R. *et al.* (2008): Recommendations for collection and handling of specimens from group breast cancer clinical trials. J. Clin. Oncol 26(34): 5638-5644.

Penland S. K. *et al.* (2007): RNA expression analysis of formalin-fixed paraffin-embedded tumors. Laboratory investigation 87: 383-391.

von Ahlfen S. *et al.* (2007): Determinants for RNA quality from FFPE samples. PLoS ONE. Issue 12, e1261.

von Maldegem F. *et al.* (2008): Effects of processing delay, formalin fixation, and immunohistochemistry on RNA recovery from formalin-fixed paraffin-embedded tissue sections. Diagn Mol Pathol. 17(1): 51-58.

6.5 Product use restriction / warranty

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