Total RNA isolation from FFPE Samples

User manual

NucleoSpin® totalRNA FFPE
NucleoSpin® totalRNA FFPE XS

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Total RNA from FFPE samples
Protocol-at-a-glance (Rev. 04) – page 1

	NucleoSpin® totalRNA FFPE	NucleoSpin® totalRNA FFPE XS
1 Sample preparation	Insert FFPE section(s) in a microcentrifuge tube	Insert FFPE section(s) in a microcentrifuge tube
2 Deparaffinize sample	 1 mL Paraffin Dissolver 56 °C, 5 min Vortex hot sample 16,000 x *g*, 2 min	 1 mL Paraffin Dissolver 56 °C, 5 min Vortex hot sample 16,000 x *g*, 2 min
	 170 µL MLF 16,000 x *g*, 2 min	 140 µL MLF 16,000 x *g*, 2 min
	Remove Paraffin Dissolver	Remove Paraffin Dissolver
3 Lyse sample A Quick protocol (perform method 3A or 3B)	 15 µL Proteinase K Mix gently 56 °C, 15 min	 12 µL Proteinase K Mix gently 56 °C, 15 min
	 15 µL MKA Vortex 0 °C, 5 min 16,000 x *g*, 5 min	 12 µL MKA Vortex 0 °C, 5 min 16,000 x *g*, 5 min
	 Transfer sample 80 °C, 15 min	 Transfer sample 80 °C, 15 min
Lyse sample B Protocol for difficult to lyse cells (perform method 3A or 3B)	 15 µL Proteinase K Mix gently 56 °C, 90 min	 12 µL Proteinase K Mix gently 56 °C, 90 min
	 15 µL MKA Vortex 0 °C, 5 min 16,000 x *g*, 5 min	 12 µL MKA Vortex 0 °C, 5 min 16,000 x *g*, 5 min
	 Transfer sample	 Transfer sample
4 Adjust binding conditions	 500 µL MX Vortex RT, 1 min	 400 µL MX Vortex RT, 1 min

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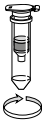
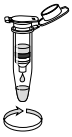
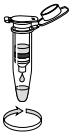
Total RNA from FFPE samples
Protocol-at-a-glance (Rev. 04) – page 2

	NucleoSpin® totalRNA FFPE	NucleoSpin® totalRNA FFPE XS
5 Bind RNA	 Load sample 16,000 x *g*, 15 s	 Load sample 16,000 x *g*, 15 s
6 Wash and dry silica membrane	1st  700 µL MW2 16,000 x *g*, 15 s	1st  400 µL MW2 16,000 x *g*, 15 s
	2nd  250 µL MW2 16,000 x *g*, 1 min	2nd  250 µL MW2 16,000 x *g*, 1 min
7 *Optional: Digest DNA*	 50 µL rDNase RT, 15 min	 25 µL rDNase RT, 15 min
	 100 µL MX RT, 1 min 16,000 x *g*, 15 s	 50 µL MX RT, 1 min 16,000 x *g*, 15 s
	1st  700 µL MW2 16,000 x *g*, 15 s	1st  400 µL MW2 16,000 x *g*, 15 s
	2nd  250 µL MW2 16,000 x *g*, 1 min	2nd  200 µL MW2 16,000 x *g*, 1 min
8 Elute highly pure RNA	 30–50 µL RNase-free H_2O RT, 1 min 16,000 x *g*, 1 min	 30–50 µL RNase-free H_2O RT, 1 min 16,000 x *g*, 1 min

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1 Components

1.1 Kit contents

	NucleoSpin® totalRNA FFPE		
REF	**10 preps 740982.10**	**50 preps 740982.50**	**250 preps 740982.250**
Paraffin Dissolver	15 mL	60 mL	300 mL
Lysis Buffer MLF	10 mL	10 mL	50 mL
Precipitation Buffer MKA	1 mL	1 mL	10 mL
Binding Buffer MX	13 mL	60 mL	250 mL
Reaction Buffer for rDNase	7 mL	7 mL	30 mL
Wash Buffer MW2 (Concentrate)*	6 mL	25 mL	100 mL
RNase-free H_2O	13 mL	13 mL	13 mL
rDNase, RNase-free (lyophilized)*	1 vial (size A)	1 vial (size C)	5 vials (size C)
Liquid Proteinase K	0.6 mL	0.8 mL	5 mL
NucleoSpin® RNA Columns (light blue rings)	10	50	250
Collection Tubes (2 mL)	10	50	250
Collection Tubes (1.5 mL)	10	50	250
User manual	1	1	1

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

1.1 Kit contents *continued*

	NucleoSpin® totalRNA FFPE XS		
REF	**10 preps 740969.10**	**50 preps 740969.50**	**250 preps 740969.250**
Paraffin Dissolver	15 mL	60 mL	300 mL
Lysis Buffer MLF	10 mL	10 mL	50 mL
Precipitation Buffer MKA	1 mL	1 mL	10 mL
Binding Buffer MX	13 mL	60 mL	250 mL
Reaction Buffer for rDNase	7 mL	7 mL	30 mL
Wash Buffer MW2 (Concentrate)*	6 mL	25 mL	100 mL
RNase-free H_2O	13 mL	13 mL	13 mL
rDNase, RNase-free (lyophilized)*	1 vial (size A)	1 vial (size C)	3 vials (size C)
Liquid Proteinase K	0.2 mL	0.8 mL	3 x 1.25 mL
NucleoSpin® RNA FFPE XS Columns (light blue rings)	10	50	250
Collection Tubes (2 mL)	10	50	250
Collection Tubes (1.5 mL)	10	50	250
User manual	1	1	1

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

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

Reagents

- 96–100 % ethanol (preferably undenaturated ethanol)

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 56 °C and 80 °C)
- Personal protection equipment (lab coat, gloves, goggles)

1.3 About this user manual

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

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

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

2 Product description

Formalin-fixed and paraffin-embedded (FFPE) tissue is commonly used in histopathological analysis. Recently, there is more and more interest in also investigating DNA modifications, RNA expression or miRNA profiles of old, archived FFPE samples. However, fixation, embedding and storage lead to crosslinking and fragmentation of RNA. Especially crosslinks cannot be detected by standard quality control assays, such as gel electrophoresis, spectrophotometry or microfluidic analysis, but the efficiency of enzymatic reactions is significantly reduced, for example in RT-PCR. Standard RNA purification procedures do not remove these chemical modifications and therefore result in low RNA yield or poor downstream application performance. The **NucleoSpin® totalRNA FFPE (XS)** procedure implements buffers and procedural steps to efficiently decrosslink nucleic acids and yield high quality RNA for the most demanding applications.

2.1 The basic principle

The **NucleoSpin® totalRNA FFPE (XS)** kits provide a convenient, reliable, and fast method to isolate RNA from formalin-fixed, paraffin-embedded (FFPE) tissue. Odorless and non-toxic Paraffin Dissolver (patent pending) replaces the flammable and odorous xylene or d-limonene commonly used for deparaffinization.

The tissue sample is then heat incubated with Proteinase K to digest the fixed tissue, release nucleic acids, and gently remove crosslinks. Optimal binding conditions for even small RNA (e.g., miRNA) are adjusted and the lysate is applied to the **NucleoSpin® RNA Column / NucleoSpin® RNA FFPE XS Column**. RNA is bound to the silica membrane. Residual DNA remaining on the membrane is removed by convenient on-column rDNase digestion. Washing steps remove salts, metabolites, and macromolecular cellular components. Pure RNA is finally eluted in a small volume of RNase-free water, yielding highly concentrated RNA.

Nucleic acid preparation using **NucleoSpin® totalRNA FFPE (XS)** 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® totalRNA FFPE (XS) 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). Formalin-fixed samples which are not embedded in paraffin can also be used as sample material by omitting the deparaffinization steps.

The sample size can be up to ~10 sections (1–20 µm) of FFPE. The amount of embedded tissue can be up to 50 mg for **NucleoSpin® totalRNA FFPE** or up to 5 mg for **NucleoSpin® totalRNA FFPE XS** (1x 10 µm section with 1 cm^2 tissue is approximately 1 mg).

RNA yield strongly depends on sample type, quality, and amount. Furthermore, 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 see, for example, Chung J.Y. et al. (2008); van Maldegem F. et al. (2008); von Ahlfen S. et al. (2007); Castiglione F. et al. (2007); Leyland-Jones B.R. et al. (2008).

RNA concentration: RNA can be eluted highly concentrated and ready-to-use in a small volume of 30–50 µL **(NucleoSpin® totalRNA FFPE)** or even 5–30 µL **(NucleoSpin® totalRNA FFPE XS)**.

RNA size distribution: RNA isolated from formalin-fixed, paraffin-embedded tissue shows size distribution from 15 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® totalRNA FFPE (XS) kits are in range of 2–6.

rDNase is supplied with the kit for a convenient removal of DNA by on-column digestion. For more demanding downstream applications, DNA can also be digested in solution as described in section 5.2.

Table 1: Kit specifications at a glance

Parameter	NucleoSpin® totalRNA FFPE	NucleoSpin® totalRNA FFPE XS
Technology	Silica-membrane technology	Silica-membrane technology
Format	Mini spin columns	Mini spin columns – XS design
Sample material	Up to ~10 sections with up to 50 mg of tissue	Up to ~10 sections with up to 5 mg of tissue
Typical yield	Strongly depends on sample quality and amount	Strongly depends on sample quality and amount
Elution volume	30–50 µL	5–30 µL
Preparation time	70 min/6 preps (90 min including optional rDNase digest)	70 min/6 preps (90 min including optional rDNase digest)

3 Storage conditions and preparation of working solutions

Storage conditions:

- Store lyophilized rDNase and Proteinase K at 4 °C upon arrival (stable for at least 1 year).
- All other kit components should be stored at room temperature (18–25 °C) and are stable for at least one year. Storage at lower temperatures may cause precipitation of salts. If precipitation occurs, incubate the bottle for several minutes at about 30–40 °C and mix well until the precipitate is redissolved.

Before starting any **NucleoSpin® totalRNA FFPE (XS)** protocol prepare the following:

- **RNase-free rDNase:** Add the indicated volume of Reaction Buffer for rDNase to the rDNase vial and incubate for 1 min at room temperature. Gently swirl the vial to completely dissolve the rDNase. Be careful not to mix the enzyme too 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.
- **Wash Buffer MW2:** Add the indicated volumes of 96–100 % ethanol to the MW2 concentrate. Stored at room temperature (18–25 °C), the buffer is stable for at least one year.

	NucleoSpin® totalRNA FFPE		
REF	**10 preps** **740982.10**	**50 preps** **740982.50**	**250 preps** **740982.250**
Wash Buffer MW2 (Concentrate)	6 mL Add 24 mL 96–100 % ethanol	25 mL Add 100 mL 96–100 % ethanol	100 mL Add 400 mL 96–100 % ethanol
RNase-free rDNase (lyophilized)	1 vial (size A) Add 0.75 mL **Reaction Buffer for rDNase**	1 vial (size C) Add 3 mL **Reaction Buffer for rDNase**	5 vials (size C) Add 3 mL **Reaction Buffer for rDNase** to each vial

	NucleoSpin® totalRNA FFPE XS		
REF	**10 preps** **740982.10**	**50 preps** **740982.50**	**250 preps** **740982.250**
Wash Buffer MW2 (Concentrate)	6 mL Add 24 mL 96–100 % ethanol	25 mL Add 100 mL 96–100 % ethanol	100 mL Add 400 mL 96–100 % ethanol
RNase-free rDNase (lyophilized)	1 vial (size A) Add 0.75 mL **Reaction Buffer for rDNase**	1 vial (size C) Add 3 mL **Reaction Buffer for rDNase**	3 vials (size C) Add 3 mL **Reaction Buffer for rDNase to each vial**

4 Safety instructions

The following components of the **NucleoSpin® totalRNA FFPE (XS)** kits contain hazardous contents.

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

GHS classification

Only harmful features do not need to be labeled with H and P phrases up to 125 mL or 125 g.
Mindergefährliche Eigenschaften müssen bis 125 mL oder 125 g nicht mit H- und P-Sätzen gekennzeichnet werden.

Component *Inhalt*	Hazard contents *Gefahrstoff*	GHS symbol *GHS-Symbol*	Hazard phrases *H-Sätze*	Precaution phrases *P-Sätze*
Liquid Proteinase K	Proteinase K 1–3 % *Proteinase K 1–3 %* CAS 39450–01–6l	 WARNING *ACHTUNG*	317	261, 272, 280, 302+352, 333+313, 363
rDNase	rDNase 90–100 % *rDNase 90–100 %* CAS 9003–98–9	 DANGER *GEFAHR*	317, 334	261, 272, 280, 302+352, 304+340, 333+313, 342+311, 363
MX	Dioxane 40–90 % *Dioxan 40–90 %* CAS 123–91–1	 DANGER *GEFAHR*	225, 319, 335, 351, EUH019, EUH066	201, 202, 210, 261, 264, 271, 280, 304+340, 305+351+338, 308+313, 312, 337+313, 370+378, 403+233, 403+235, 405

Hazard phrases

H225	Highly flammable liquid and vapour. *Flüssigkeit und Dampf leicht entzündbar.*
H317	May cause an allergic skin reaction. *Kann allergische Hautreaktionen verursachen.*
H319	Causes serious eye irritation. *Verursacht schwere Augenreizung.*
H334	May cause allergy or asthma symptoms or breathing difficulties if inhaled. *Kann bei Einatmen Allergie, asthmaartige Symptome oder Atembeschwerden verursachen.*
H335	May cause respiratory irritation. *Kann die Atemwege reizen.*

H351	Suspected of causing cancer <state route of exposure if it is conclusively proven that no other routs of exposure cause the hazard>. *Kann vermutlich Krebs erzeugen (Expositionsweg angeben, sofern schlüssig belegt ist, dass diese Gefahr bei keinem anderen Expositionsweg besteht).*
EUH019	May form explosive peroxides. *Kann explosionsfähige Peroxide bilden.*
EUH066	Repeated exposure may cause skin dryness or cracking. *Wiederholter Kontakt kann zu spröder oder rissiger Haut führen.*

Precaution phrases

P201	Obtain special instructions before use. *Vor Gebrauch besondere Anweisungen einholen.*
P202	Do not handle until all safety precautions have been read and understood. *Vor Gebrauch alle Sicherheitsratschläge lesen und verstehen.*
P210	Keep away from heat, hot surfaces, sparks, open flames and other ignition sources. No smoking. *Von Hitze, heissen Oberflächen, Funken, offenen Flammen sowie anderen Zündquellenarten fernhalten. Nicht rauchen.*
P261	Avoid breathing dust/fume/gas/mist/vapours/spray. *Einatmen von Staub/Rauch/Gas/Nebel/Dampf/Aerosol vermeiden.*
P264	Wash … thoroughly after handling. *Nach Handhabung … gründlich waschen.*
P271	Use only outdoors or in a well-ventilated area. *Nur im Freien oder in gut belüfteten Räumen verwenden.*
P272	Contaminated work clothing should not be allowed out of the workplace. *Kontaminierte Arbeitskleidung nicht außerhalb des Arbeitsplatzes tragen.*
P280	Wear protective gloves/protective clothing/eye protection/face protection. *Schutzhandschuhe/Schutzkleidung/Augenschutz/Gesichtsschutz tragen.*
P302+352	IF ON SKIN: Wash with plenty of water/… *BEI BERÜHRUNG MIT DER HAUT: Mit viel Wasser/… waschen.*
P304+340	IF INHALED: Remove person to fresh air and keep comfortable for breathing. *BEI EINATMEN: Die Person an die frische Luft bringen und für ungehinderte Atmung sorgen.*
P305+351+338	IF IN EYES: Rinse cautiously with water for several minuts. Remove contact lenses, if present and easy to do. Continue rinsing. *BEI KONTAKT MIT DEN AUGEN: Einige Minuten lang behutsam mit Wasser ausspülen. Eventuell vorhandene Kontaktlinsen nach Möglichkeit entfernen. Weiter ausspülen.*
P308+313	IF exposed or concerned: Get medical advice/attention. *BEI Exposition oder falls betroffen: Ärztlichen Rat einholen/ärztliche Hilfe hinzuziehen.*
P312	Call a POISON CENTER/doctor/…/ if you feel unwell. *Bei Unwohlsein GIFTINFORMATIONSZENTRUM/Arzt/… anrufen.*
P333+313	If skin irritation or rash occurs: Get medical advice/attention. *Bei Hautreizung oder -ausschlag: Ärztlichen Rat einholen/ärztliche Hilfe hinzuziehen.*
P337+313	If eye irritation persists: Get medical advice/attention. *Bei anhaltender Augenreizung: Ärztlichen Rat einholen/ärztliche Hilfe hinzuziehen.*
P342+311	If experiencing respiratory symptoms: Call a POISON CENTER/doctor/… *Bei Symptomen der Atemwege: GIFTINFORMATIONSZENTRUM/Arzt/… anrufen.*

P363	Wash contaminated clothing before reuse. *Kontaminierte Kleidung vor erneutem Tragen waschen.*
P370+378	In case of fire: Use … to extinguish. *Bei Brand: … zum Löschen verwenden.*
P403+233	Store in a well-ventilated place. Keep container tightly closed. *An einem gut belüfteten Ort aufbewahren. Behälter dicht verschlossen halten.*
P403+235	Store in a well-ventilated place. Keep cool. *An einem gut belüfteten Ort aufbewahren. Kühl halten.*
P405	Store locked up. *Unter Verschluss aufbewahren.*

For further information please see Material Safety Data Sheets (***www.mn-net.com***).
*Weiterführende Informationen finden Sie in den Sicherheitsdatenblättern (**www.mn-net.com**).*

⚠ The symbol shown on labels refers to the precaution phrases of this section.

Das auf Etiketten dargestellte Symbol weist auf die P-Sätzen dieses Kapitels hin.

5 Protocols

5.1 NucleoSpin® totalRNA FFPE

Before starting the preparation:

Check that rDNase and Buffer MW2 were prepared according to section 3.

Set incubator(s) to 56 °C (for paraffin melting and lysis step) and 80 °C (for decrosslink step).

Please note that lysis step 3A is the standard method for most common sample materials while lysis step 3B is utilized for difficult to lyse cells

1 Sample preparation

Insert FFPE section(s) in a microcentrifuge tube (not provided with the kit).

2 Deparaffinize sample

Add **1 mL Paraffin Dissolver** to the sample.

+ 1 mL Paraffin Dissolver

Incubate **5 min** at **56 °C** (to melt the paraffin).

56 °C, 5 min

Vortex the hot sample.

Vortex hot sample

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

Centrifuge sample for **2 min** at **16,000 x *g*.**

16,000 x *g*, 2 min

! *<u>Attention:</u> Check Buffer MLF prior to use! If a white precipitate is visible, heat the buffer for several minutes at 30–40 °C until the precipitate is completely dissolved and mix thoroughly.*

+ 170 µL MLF

Add **170 µL Buffer MLF**. Do not mix!

Centrifuge sample for **2 min** at **16,000 x *g***.

16,000 x *g*, 2 min

Remove and discard Paraffin Dissolver by pipetting it off.

Remove Paraffin Dissolver

<u>Note:</u> Slight residues of Paraffin Dissolver do not affect the following steps.

3 **A) Lyse sample – method A**
(perform method 3 A or 3 B) "Quick protocol"

Add **15 µL Proteinase K**.

Mix by gently shaking or pipetting up and down. Do not vortex!

Incubate for 15 **min** at **56 °C** to lyse sample tissue.

If tissue is still visible, continue incubation until sample is digested.

+ 15 µL Proteinase K
Mix gently
56 °C, 15 min

Add **15 µL Buffer MKA** and vortex briefly.

Incubate for **5 min** on **ice**.

Centrifuge for **5 min** at **16,000 x *g***.

+ 15 µL MKA
Vortex
0 °C, 5 min

16,000 x *g*, 5 min

Transfer clear supernatant to a new 1.5 mL microcentrifuge tube (not provided).

Incubate at **80 °C** for exactly **15 min**.

Shorter incubation or lower temperature leads to incomplete decrosslinking. Longer incubation might lead to degradation of RNA.

Transfer sample
80 °C, 15 min

3 **B) Lyse sample – method B**
(perform method 3 A or 3 B) "Protocol for diffcult to lyse samples"

Add **15 µL Proteinase K**.

Mix by gently shaking or pipetting up and down. Do not vortex!

Incubate for **90 min** at **56 °C** to lyse sample tissue.

If tissue is still visible, continue/ increase incubation time up to overnight until sample is digested.

+ 15 µL Proteinase K
Mix gently
56 °C, 90 min

Add **15 µL Buffer MKA** and vortex briefly.

Incubate for **5 min** on **ice**.

Centrifuge for **5 min** at **16,000 x *g***.

+ 15 µL MKA
Vortex
0 °C, 5 min

16,000 x *g*, 5 min

Transfer clear supernatant to a new 1.5 mL microcentrifuge tube (not provided).

Transfer sample

4 Adjust binding conditions

Add **500 µL Buffer MX** and **mix** by vortexing (2 x 5 s).

Incubate for **1 min** at **room temperature** (18–25 °C).

+ 500 µL MX

Vortex

RT, 1 min

5 Bind RNA

Place a **NucleoSpin® RNA Column** in a new **Collection Tube (2 mL)**.

Load sample onto the column and centrifuge for **15 s** at **16,000 x *g***.

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

Load sample

16,000 x *g*, 15 s

6 Wash and dry silica membrane

1st wash

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

Centrifuge for **15 s** at **16,000 x *g***.

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

+ 700 µL MW2

16,000 x *g*, 15 s

2nd wash

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

Centrifuge for **1 min** at **16,000 x *g*** to dry the membrane completely.

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

+ 250 µL MW2

16,000 x *g*, 1 min

7 *Optional:* Digest DNA

Add **50 µL rDNase** directly onto the silica membrane of the NucleoSpin® RNA Column.

Incubate at **room temperature** (18–25 °C) for **15 min**.

+ 50 µL rDNase

RT, 15 min

Add **100 µL Buffer MX**.

+ 100 µL MX

Incubate for **1 min** at **room temperature** (18–25 °C).

RT, 1 min

Centrifuge for **15 s** at **16,000 x *g***.

16,000 x *g*, 15 s

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

1st wash

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

+ 700 µL MW2

Centrifuge for **15 s** at **16,000 x *g***.

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

16,000 x *g*, 15 s

2nd wash

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

+ 250 µL MW2

Centrifuge for **1 min** at **16,000 x *g*** to dry the membrane completely.

16,000 x *g*, 1 min

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

8 Elute highly pure RNA

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

+ 30–50 µL RNase-free H^2O

Add 30 µL (for high concentration) or 50 µL (for high yield) RNase-free H_2O to the column.

RT, 1 min

Incubate for **1 min** at room temperature (18–25 °C).

16,000 x *g*, 1 min

Centrifuge for **1 min** at **16,000 x *g***.

Keep the eluted RNA on ice or freeze at -20 °C (short-term storage) or -70 °C (long-term storage).

5.2 NucleoSpin® totalRNA FFPE XS

Before starting the preparation:

Check that rDNase and Buffer MW2 were prepared according to section 3.

Set incubator(s) to 56 °C (for paraffin melting and lysis step) and 80 °C (for decrosslink step).

Please note that lysis step 3A is the standard method for most common sample materials while lysis step 3B is utilized for difficult to lyse cells

1 Sample preparation

Insert FFPE section(s) in a microcentrifuge tube (not provided with the kit).

2 Deparaffinize sample

+ 1 mL Paraffin Dissolver

Add **1 mL Paraffin Dissolver** to the sample.

Incubate **5 min** at **56 °C** (to melt the paraffin).

56 °C, 5 min

Vortex the hot sample.

Vortex hot sample

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

16,000 x *g*, 2 min

Centrifuge sample for **2 min** at **16,000 x *g***.

! *Attention: Check Buffer MLF prior to use! If a white precipitate is visible, heat the buffer for several minutes at 30–40 °C until the precipitate is completely dissolved and mix thoroughly.*

+ 140 µL MLF

Add **140 µL Buffer MLF**. Do not mix!

Centrifuge sample for **2 min** at **16,000 x *g***.

16,000 x *g*, 2 min

Remove and discard Paraffin Dissolver by pipetting it off.

Remove Paraffin Dissolver

Note: Slight residues of Paraffin Dissolver do not affect the following steps.

3 A) Lyse sample – method A
(perform method 3 A or 3 B) "Quick protocol"

+ 12 µL Proteinase K

Add **12 µL Proteinase K**.

Mix by gently shaking or pipetting up and down. Do not vortex!

Mix gently

	Incubate for **15 min** at **56 °C** to lyse sample tissue. *If tissue is still visible, continue incubation until sample is digested.*		**56 °C, 15 min**
	Add **12 µL Buffer MKA** and vortex briefly. Incubate for **5 min** on **ice**. Centrifuge for **5 min** at **16,000 x *g***.	 	**+ 12 µL MKA** **Vortex** **0 °C, 5 min** **16,000 x *g*, 5 min**
	Transfer clear supernatant to a new 1.5 mL microcentrifuge tube (not provided). Incubate at **80 °C** for exactly **15 min**. *Shorter incubation or lower temperature leads to incomplete decrosslinking. Longer incubation might lead to degradation of RNA.*		**Transfer sample** **80 °C, 15 min**
3	**B) Lyse sample – method B** (perform method 3 A or 3 B) "Protocol for diffcult to lyse samples" Add **12 µL Proteinase K**. Mix by gently shaking or pipetting up and down. Do not vortex! Incubate for **90 min** at **56 °C** to lyse sample tissue. *If tissue is still visible, continue/ increase incubation time up to overnight until sample is digested.*		**+ 12 µL Proteinase K** **Mix gently** **56 °C, 90 min**
	Add **12 µL Buffer MKA** and vortex briefly. Incubate for **5 min** on **ice**. Centrifuge for **5 min** at **16,000 x *g***.	 	**+ 12 µL MKA** **Vortex** **0 °C, 5 min** **16,000 x *g*, 5 min**
	Transfer clear supernatant to a new 1.5 mL microcentrifuge tube (not provided).		**Transfer sample**
4	**Adjust binding conditions** Add **400 µL Buffer MX** and **mix** by vortexing (2 x 5 s). Incubate for **1 min** at **room temperature** (18–25 °C).		**+ 400 µL MX** **Vortex** **RT, 1 min**

5 Bind RNA

Place a **NucleoSpin® RNA FFPE XS Column** in a new **Collection Tube (2 mL)**.

Load sample

Load sample onto the column and centrifuge for **15 s** at **16,000 x *g***.

16,000 x *g*, 15 s

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

6 Wash and dry silica membrane

1st wash

Add **400 µL Buffer MW2** to the NucleoSpin® RNA FFPE XS Column.

+ 400 µL MW2

Centrifuge for **15 s** at **16,000 x *g***.

16,000 x *g*, 15 s

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

2nd wash

Add **200 µL Buffer MW2** to the NucleoSpin® RNA FFPE XS Column.

+ 200 µL MW2

Centrifuge for **1 min** at **16,000 x *g*** to dry the membrane completely.

16,000 x *g*, 1 min

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

7 *Optional:* Digest DNA

Add **25 µL rDNase** directly onto the silica membrane of the NucleoSpin® RNA FFPE XS Column.

+ 25 µL rDNase

Incubate at **room temperature** (18–25 °C) for **15 min**.

RT, 15 min

Add **50 µL Buffer MX**.

+ 50 µL MX

Incubate for **1 min** at **room temperature** (18–25 °C).

RT, 1 min

Centrifuge for **15 s** at **16,000 x *g***.

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

16,000 x *g*, 15 s

1st wash

Add 400 µL Buffer MW2 to the NucleoSpin® RNA FFPE XS Column.

+ 400 µL MW2

Centrifuge for 15 s at 16,000 x *g*.

16,000 x *g*, 15 s

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

2nd wash

Add 200 µL Buffer MW2 to the NucleoSpin® RNA FFPE XS Column.

+ 200 µL MW2

Centrifuge for 1 min at 16,000 x *g*.

16,000 x *g*, 1 min

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

16,000 x *g*, 5 min

Centrifuge for 5 min at 16,000 x *g* to dry the membrane.

8 Elute highly pure RNA

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

+ 5–30 µL RNase-free H_2O

Add **5 µL** (for high concentration) to **30 µL** (for high yield) **RNase-free H_2O** to the column.

RT, 1 min

Incubate for **1 min** at **room temperature** (18–25 °C).

16,000 x *g*, 1 min

Centrifuge for **1 min** at **16,000 x *g***.

Keep the eluted RNA on ice or freeze at -20 °C (short-term storage) or -70 °C (long-term storage).

5.3 DNA digestion in the RNA eluates

Comments on DNA removal:

Although the on-column rDNase digest in the standard protocol is very efficient, there are still certain applications which require even lower quantities of residual DNA.

For example, RT-PCR reactions with primers that do not differentiate between cDNA (derived from RNA) and contaminating genomic DNA.

DNA digestion in solution can efficiently degrade contaminating DNA. This requires stringent RNase control and optionally repurification of the RNA (in order to remove buffer, salts, DNase, and digested DNA).

The high quality, RNase-free, recombinant DNase (rDNase) provided with the kit facilitates such a digestion in solution.

A **Digest DNA (Reaction setup)**

Add **1/10 volume of rDNase** (dissolved in Reaction Buffer for rDNase) to the eluted RNA (e.g., add 3 µL enzyme to 30 µL RNA eluate).

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

C **Inactivate rDNase**

Incubate the sample for **5 min** at **75 °C** to inactivate the rDNase. Put the sample on ice.

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

6 Appendix

6.1 Troubleshooting

Problem	Possible cause and suggestions
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 sterile, disposable polypropylene tubes and filter strips. 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 F. et al. (2007), Chung J.Y. et al. (2008), Leyland-Jones B.R. et al. (2008), von Ahlfsen S. et al. (2007), von Maldegem F. et al. (2008). *Reagents not applied or restored properly* • Always dispense exactly the buffer volumes given in the protocols! • Always follow the given instructions closely, with specific attention paid to order and mode of mixing (shaking, vortexing, etc). • Add the indicated volume of 96–100 % ethanol to Buffer MW2 Concentrate and mix thoroughly. • Store kit components at room temperature (18–25 °C). Storage at lower temperatures may cause salt precipitation. If precipitation occurs, incubate the bottle for several minutes at about 30–40 °C and mix well until the precipitate is redissolved. • Keep bottles tightly closed in order to prevent evaporation or contamination.

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) and Wilfinger W.W. et al. (1997). *Proteinase digestion time* • Depending of the nature of the sample, an optimal digestion time from 15 min to 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 3 hours.
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. • Use larger PCR targets (e.g., > 500 bp) or intron spanning primers for RNA analysis. • Use support protocol for subsequent rDNase digestion in the eluate (section 5.2).

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 MW2 wash. Be sure to centrifuge at the recommended speed and time in order to remove ethanolic Buffer MW2 completely. • Check that Buffer MW2 has been equilibrated to room temperature before use. Washing at lower temperatures lowers efficiency of salt removal. • 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.

6.2 Ordering information

Product	REF	Pack of
NucleoSpin® totalRNA FFPE	740982.10/.50/.250	10/50/250 preps
NucleoSpin® totalRNA FFPE XS	740969.10/.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	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
rDNase Set	740963	1
Paraffin Dissolver	740968.25	25 mL
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.

6.3 References

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Manchester K.L. (1995): Value of A_{260}/A_{280} ratios for measurement of purity of nucleic acids. Biotechniques 19, 208–209.

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

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Wilfinger W.W. et al. (1997): Effect of pH and ionic strength on the spectorophotometric assessment of nucleic acid purity. Biotechniques 22, 474–481.

6.4 Product use restriction/warranty

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

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

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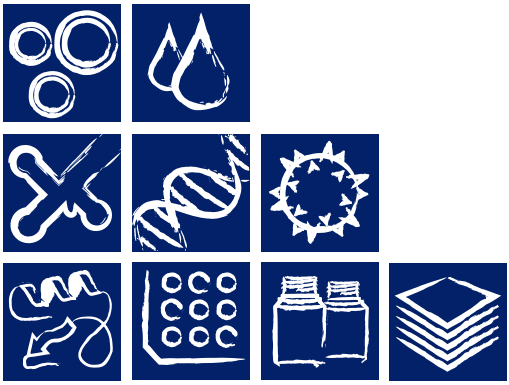

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