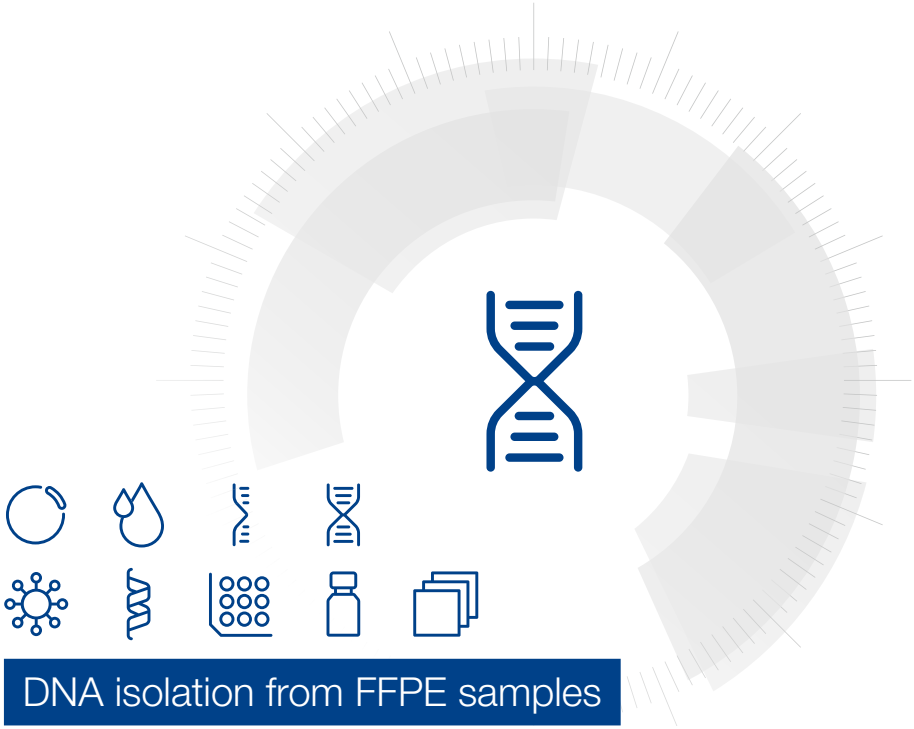
Bioanalysis

MACHEREY-NAGEL

User manual



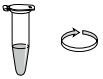
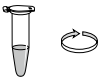
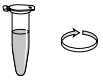
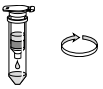
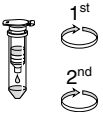
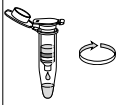
DNA isolation from FFPE samples

■ NucleoSpin® DNA FFPE XS

November 2023 / Rev. 05

DNA Isolation from FFPE Samples

Protocol at a glance (Rev. 05)

		NucleoSpin® DNA FFPE XS	
		Protocol 5.1: **DNA** isolation with **Paraffin Dissolver**	***Protocol 5.2:*** **DNA** isolation with **xylene**
Sample preparation		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	11,000 x *g*, 2 min Discard supernatant 1 mL ~ 98 % ethanol Mix 11,000 x *g*, 2 min Discard supernatant Dry at 60 °C, 3–10 min
2 Lyse sample		100 µL FL Mix vigorously 11,000 x *g*, 1 min 10 µL Proteinase K Mix lower phase RT, 3 hours or overnight	100 µL FL – 10 µL Proteinase K Mix RT, 3 hours or overnight
3 Decrosslink		100 µL D-Link Mix gently 11,000 x *g*, 30 s 90 °C, 30 min	100 µL D-Link Mix gently – 90 °C, 30 min
4 Adjust binding conditions		200 µL ~ 98 % ethanol Mix 1,000 x *g*, 1 s	200 µL ~ 98 % ethanol Mix –
5 Bind DNA		Load aqueous (lower) phase 2,000 x *g*, 30 s	Load lysate 2,000 x *g*, 30 s
6 Wash and dry silica membrane		1st 400 µL B5 11,000 x *g*, 30 s 2nd 400 µL B5 11,000 x *g*, 2 min	400 µL B5 11,000 x *g*, 30 s 400 µL B5 11,000 x *g*, 2 min
7 Elute DNA		20 µL BE 11,000 x *g*, 30 s	20 µL BE 11,000 x *g*, 30 s
8 *Optional: Remove residual ethanol*		90 °C, 8 min	90 °C, 8 min

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1 Components

1.1 Kit contents

	NucleoSpin® DNA FFPE XS		
REF	**10 preps 740980.10**	**50 preps 740980.50**	**250 preps 740980.250**
Paraffin Dissolver	5 mL	25 mL	125 mL
Lysis Buffer FL	8 mL	8 mL	4 × 8 mL
Decrosslink Buffer D-Link	8 mL	8 mL	30 mL
Wash Buffer B5 (Concentrate)*	6 mL	12 mL	50 mL
Proteinase K (lyophilized)*	6 mg	30 mg	75 mg
Proteinase Buffer PB	1.8 mL	1.8 mL	8 mL
Elution Buffer BE**	13 mL	13 mL	13 mL
NucleoSpin® DNA FFPE XS Columns (green rings plus Collection Tubes)	10	50	250
Collection Tubes (2 mL)	20	100	500
User manual	1	1	1

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

** Composition of Elution Buffer BE: 5 mM Tris/HCl, pH 8.5

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

Reagents

- 96 – 100 % ethanol (undenaturated ethanol is preferable) to prepare Wash Buffer B5 and to adjust binding conditions.
- Optional for deparaffinisation without Paraffin Dissolver:
  Xylene, d-Limonene, mixtures of isoparaffinic hydrocarbons, or similar reagents for deparaffinization.

Consumables

- 1.5 mL microcentrifuge tubes (for sample lysis and DNA elution)
- 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® DNA 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, 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 DNA analysis is limited due to chemical modification by formaldehyde and fragmentation of the DNA during tissue processing (sampling, fixing, embedding) and storage (humidity, time, temperature) of the samples. Standard DNA isolation procedures often result in low DNA yield or poor performance in downstream applications (e.g., PCR). A special purification system taking the unique requirements of FFPE tissue into account is inevitably necessary for successful analysis of nucleic acids from FFPE samples.

2.1 The basic principle

The **NucleoSpin® DNA FFPE XS** kit provides a convenient, reliable, and fast method to isolate DNA 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. **NucleoSpin® DNA FFPE XS** 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 DNA into solution. Subsequently, heat incubation with specially designed buffer effectively eliminates crosslinks from the previously released DNA. After addition of ethanol, the lysate is applied to the **NucleoSpin® DNA FFPE XS Column**. DNA is bound to the silica membrane. Two washing steps help remove salts, metabolites, and macromolecular cellular components. Pure DNA is finally eluted under low ionic strength conditions in a small volume (20 µL) of Elution Buffer BE, yielding highly concentrated DNA.

DNA preparation using **NucleoSpin® DNA FFPE XS** kits can be performed at room temperature. The eluate, however, should be treated with care, because the Elution Buffer BE does not contain DNase inhibitors like EDTA. To ensure DNA stability store frozen DNA at -20 °C.

2.2 Kit specifications

- The **NucleoSpin® DNA FFPE XS** kit is recommended for the isolation of DNA from formalin-fixed, paraffin-embedded (FFPE) tissue samples. Samples are typically thin sections (approx. 3 – 20 µm thickness) of human or animal origin usually obtained by tissue resection or biopsy.
- **Sample amount**: The maximum sample size is determined by a) the amount of tissue and b) by the amount of paraffin.
  NucleoSpin® DNA FFPE XS 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 (see also section 2.4).
- **DNA yield** strongly depends on the sample type, quality, quantity, and time of storage. Further, measured DNA yield may vary considerably between different quantification methods. Yield determined by absorption measurement at 260 nm or by a fluorescent dye (e.g., PicoGreen®) may deviate from values obtained by quantification with PCR. Even quantification values obtained via PCR with a short (e.g., 80 bp) and a long (e.g., 300 bp) amplicon may also differ considerably. The deviation of quantification also depends on DNA size distribution as well as on efficiency of decrosslinking (or extent of remaining crosslinks). Please also see section 6.1 for considerations on determining DNA quality and quantity.
- **The innovative column design** with a funnel shaped thrust ring and a small silica membrane area allows elution of DNA in as little as 5 – 30 µL. Thus, eluted **DNA is highly concentrated** and ready to use in all common downstream applications (e.g., PCR).
- **DNA size distribution**: DNA isolated from formalin-fixed, paraffin-embedded tissue shows size distribution from 50 to 5,000 bases. Often short sized DNA 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 DNA even larger than 5,000 bases.
- **DNA preparation time** strongly depends on the sample and the required lysis time. For best results lysis is performed at room temperature for at least three hours. For some kinds of sample a longer lysis (e.g., overnight) will even result in remarkably higher DNA yield.

Table 1: Kit specifications at a glance

Parameter	NucleoSpin® DNA FFPE XS
Sample material*	Up to 7 sections, 10 µm, surface of 250 mm²
Typical yield	Strongly depends on sample quality and amount
Elution volume	5 – 30 µL
Maximum loading volume	600 µL
Format	Mini spin column – XS design
Use	For research use only

2.3 Handling, preparation, and storage of starting materials

Many factors influence the yield and usability of DNA obtained from FFPE samples. The procedure of tissue sampling, post sampling delay before fixation, fixation time, embedding, and storage conditions have a high impact on DNA quality and yield.

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

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

2.4 Quantities of FFPE sections

The standard protocol (section 5.1.) allows the preparation of FFPE samples with approximately 15 mg (ca. 17 µL) paraffin. This corresponds to:

~ 17 sections of 10 µm thickness and 100 mm² area

~ 7 sections of 10 µm thickness and 250 mm² area

~ 5 sections of 10 µm thickness and 325 mm² area

~ 4 sections of 10 µm thickness and 400 mm² area

~ 3 sections of 10 µm thickness and 575 mm² area

~ 2 sections of 10 µm thickness and 840 mm² area

~ 1 section of 10 µm thickness and 1680 mm² area

Larger amounts of paraffin can be dissolved by adding a higher volume of Paraffin Dissolver (REF 740968.25) to the sample initially (30 µL Paraffin Dissolver per mg paraffin), or by using xylene for deparaffinization as described in section 5.2. When using more than 400 µL Paraffin Dissolver per preparation, 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.

*Note: The NucleoSpin® DNA FFPE XS standard procedure is recommended for samples containing **up to 15 mg** paraffin (to ensure efficient dissolving of the paraffin with the*

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

indicated volume of Paraffin Dissolver) and ***up to 5 mg tissue*** *(to avoid an overloading of the membrane).*

- Three sections of 20 mm x 25 mm area and 10 µm thickness can contain up to 15 mg paraffin (especially, if only minor parts of the section contain tissue).
- One section of 20 mm x 25 mm area and 10 µm thickness does contain approximately 5 mg tissue, if the section contains area-wide tissue.

2.5 Elution procedures

High DNA 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 DNA, when small samples are processed. Such DNA samples may even require a subsequent concentration to be suitable for the desired application.

NucleoSpin® DNA FFPE XS kits allow efficient elution in very small volumes resulting in highly concentrated DNA. Elution volumes in the range of 5 – 30 µL are recommended, the default volume is 20 µL.

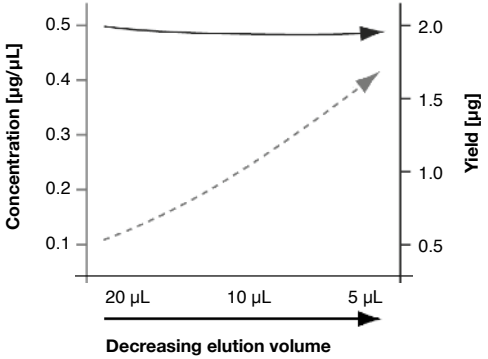

Figure 1 Correlation between elution volume and DNA concentration
(NucleoSpin® DNA FFPE XS Columns)

2.6 Stability of isolated DNA

Due to its composition, the Elution Buffer does not inhibit DNases, i.e., it does not contain substances (e.g., EDTA) to complex divalent cations. Therefore, be aware not to contaminate Elution Buffer with DNase!

For short term DNA solution may be stored at 0 – 4 °C and for long term storage at -20 °C is recommended.

* The maximum percentage of template volume in a PCR reaction may vary depending on the robustness of the PCR system; 40 % template volume were tested using LightCycler® PCR (Roche) with the DyNAmo™ Capillary SYBR® Green qPCR Kit (Finnzymes).

2.7 Removal of residual traces of ethanol for highest sensitivity in downstream applications

The default elution volume of **NucleoSpin® DNA FFPE XS** is 20 µL. The kit allows even lower elution volumes down to 5 µL to increase the DNA concentration (see section 2.5). Be aware that a reduction of the 20 µL default elution volume will also increase the concentration of residual ethanol in the eluate.

For the default elution volumes a heat incubation of the eluate is recommended if the eluate comprises more than 20 % of the final PCR volume (incubate eluate with open lid for 8 min at 90 °C). Inhibition of sensitive downstream reactions can be avoided by this precautional measure.

In this context, please mind the remarks below:

a) An incubation of the elution fraction at higher temperatures will increase signal output in PCR. This is especially of importance if the template represents more than 20 % of the total PCR reaction volume (e.g., more than 4 µL eluate used as template in a PCR reaction with a total volume of 20 µL).

   The template may represent up to 40 %* of the total PCR reaction volume, if the eluate is incubated at 90 °C for 8 min as described above.

b) Typically 20 µL eluate will evaporate to 12 – 14 µL during heat incubation for 8 min at 90 °C. If higher final volumes are required, please increase the volume of elution buffer (e.g., from 20 µL to 30 µL).

c) An incubation of the elution fraction for 8 min at 90 °C will denature DNA. If non denatured DNA is required (for downstream applications other than PCR; e.g., ligation or cloning), we recommend incubating at a temperature below 80 °C for a longer time as most DNA has a melting point above 80 °C. Suggestion: incubate for 17 min at 75 °C.

d) The incubation of the eluate at higher temperatures may be adjusted according to Figure 2. The incubation time- and temperature conditions shown will reduce an elution volume of 20 µL to about 12 – 14 µL and will effectively remove traces of ethanol as described above.

e) If the initial volume of elution buffer applied to the column is less than 20 µL, heat incubation time should be reduced in order to avoid complete dryness. If the elution volume is for example 5 µL, a heat incubation of the eluate for 2 min at 80 °C will adequately remove residual ethanol.

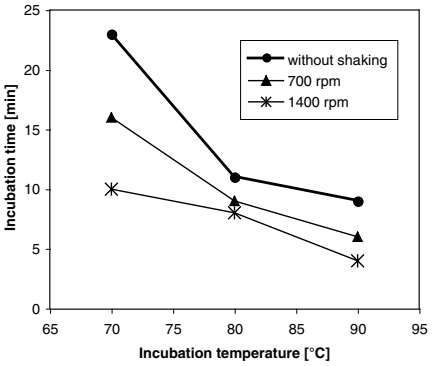

Figure 2 Removal of residual ethanol from the elution fraction by heat treatment. In order to obtain highest PCR sensitivity, heat incubation of the eluate is recommended. Heat incubation may be performed at temperatures of 70 – 90 °C in a heat block, with or without shaking. Effective conditions (temperature, time, and shaking rate) for ethanol removal can be read from the diagram; an initial volume of 20 µL will evaporate to 12 – 14 µL during the described incubation.

3 Storage conditions and preparation of working solutions

Attention:
Buffers FL contains chaotropic salts. Wear gloves and goggles!

- All 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.
- Check that 96 – 100 % ethanol is available (undenaturated ethanol is preferable) to adjust the binding conditions in the lysate and to prepare Wash Buffer B5 (see below).

Before starting protocol prepare the following:

- **Proteinase K**: Add the indicated volume of Proteinase Buffer PB (see following table or on the vial) to dissolve lyophilized Proteinase K. Proteinase K solution is stable at -20 °C for 6 months.
- **Wash Buffer B5**: Add the indicated volume of 96 – 100 % ethanol (see following table or on the bottle) to Buffer B5 Concentrate. Store Wash Buffer B5 at room temperature (18 – 25 °C) for up to one year.

	NucleoSpin® DNA FFPE XS		
REF	**10 preps 740980.10**	**50 preps 740980.50**	**250 preps 740980.250**
Wash Buffer B5 (Concentrate)	6 mL Add 24 mL 96 – 100 % ethanol	12 mL Add 48 mL 96 – 100 % ethanol	50 mL Add 200 mL 96 – 100 % ethanol
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

When working with the **NucleoSpin® DNA FFPE XS** 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***).

The waste generated with the **NucleoSpin® DNA FFPE XS** 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 and Proteinase K treatment but it cannot be excluded completely. Therefore, liquid waste must be considered infectious and should be handled and discarded according local safety regulations.

4.1 Disposal

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

5 Protocols

NucleoSpin® DNA FFPE XS 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). Both methods show same results and effiency.

Deparaffinization with Paraffin Dissolver: Section 5.1

Deparaffinization with xylene: Section 5.2

5.1 DNA purification from FFPE samples using Paraffin Dissolver

Before starting the preparation:

- Check if Proteinase K and Buffer B5 were prepared according to section 3.
- Check if 96 – 100 % ethanol is available.
- Set incubator(s) at 60 °C (for paraffin melting) and 90 °C (for decrosslink step).

Sample preparation

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

For appropriate sample amounts see section 2.4.

1 Deparaffinize sample

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

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

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

Note: Ideally, use a thermoshaker for incubation!

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).

+ 400 µL Paraffin Dissolver

60 °C, 3 min

Vortex hot sample

2 Lyse sample

Add **100 µL Buffer FL**.

Vortex vigorously.

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

Optional: The upper organic phase can be removed and discarded after centrifugation.

+ 100 µL FL

Mix

11,000 x *g*, 1 min

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

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.)

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.

+ 10 µL Proteinase K

Mix by pipetting up and down (lower phase)

Incubate at room temperature for 3 hours to lyse sample tissue.

If residual unlysed tissue particles are visible after 3 hours, add additional 10 µL Proteinase K solution and continue digestion for further 3 hours or overnight

Note: Yield of amplifiable DNA typically increases with elongated lysis time. During this incubation step protein is digested and DNA is released into solution.

RT, 3 hours

Vortex 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.

Vortex 5 s

3 Decrosslink

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

+ 100 µL D-Link

Vortex

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

11,000 x *g*, 30 s

Incubate at **90 °C** for exactly **30 min**.

90 °C, 30 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).

Note: This decrosslink step is necessary to remove the crosslinks (chemical modification caused by formalin) from the DNA, which was released into solution by the previous lysis step. Decrosslinked DNA generally shows better performance in downstream applications.

4 Adjust binding conditions

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

+ 200 µL ethanol

Vortex

Spin down briefly (approx. 1 s at 1,000 x *g*) to achieve complete phase separation.

1,000 x *g*, 1 s

Note: Avoid to centrifuge at much higher g-force, because nucleic acid might precipitate.

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

5 Bind DNA

Load aqueous (lower) phase

For each preparation, take one **NucleoSpin® DNA FFPE XS Column (green ring)** placed in a CollectionTube (2 mL).

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

It is recommended to pipette a volume of 450 µL on the 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.

Centrifuge for **30 s** at **2,000 x *g***. If the solution does not flow through completely, centrifuge for **30 s** at **11,000 x *g*** until the complete solution passed the column.

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 flowthrough and place the column in a new Collection Tube (2 mL).

6 Wash and dry silica membrane

+ 400 µL B5

1st wash

Add 400 µL Buffer B5 to the NucleoSpin® DNA FFPE XS Column.

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

11,000 x *g*, 30 s

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

2nd wash

+ 400 µL B5

Add 400 µL Buffer B5 to the NucleoSpin® DNA FFPE XS Column.

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

11,000 x *g*, 2 min

Discard the Collection Tube with flowthrough and place the column into a new nuclease-free microcentrifuge tube (1.5 mL, not provided).

7 **Elute DNA**

Pipette **20 µL Buffer BE** directly to the center of the silica membrane of the column.

Elution volume may be varied from 5 – 30 µL. For the correlation of elution volume, DNA concentration, and DNA yield see section 2.5.

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

+ 20 µL BE

11,000 x *g*, 30 s

8 ***Optional:* Remove residual ethanol**

Incubate the eluate (20 µL) with open lid for 8 min at 90 °C.

See section 2.7 for detailed information and recommendations for removal of residual ethanol.

90 °C, 8 min

5.2 DNA purification from FFPE samples with xylene deparaffinization

Before starting the preparation:

- Check if Proteinase K and Buffer B5 were prepared according to section 3.
- Check if 96 – 100 % ethanol is available.
- Check if xylene (or a similar reagent*) is available for deparaffinisation.
- Set incubator(s) at 60 °C (for ethanol evaporation) 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 Add **1 mL xylene** (or alternative reagent*) to the sample. Incubate at **room temperature** until the paraffin is completely dissolved (usually approx. 2 min) and vortex vigorously (10 s). Make sure that the paraffin is completely dissolved. Centrifuge for **2 min** at **11,000 x *g***. **Discard the supernatant** by pipetting. Do not remove any of the pellet.	 **1 mL xylene** **RT, 2 min** **Vortex**  **11,000 x *g*, 2 min** **Discard supernatant**
	Add **1 mL ethanol (96 – 100 %)** to the pellet and vortex (5 s). Centrifuge for **2 min** at **11,000 x *g***. **Discard the supernatant** by pipetting. Do not remove any of the pellet.	 **1 mL ethanol** **Vortex**  **11,000 x *g*, 2 min** **Discard supernatant**
	Incubate the open tube at **60 °C** for **3 – 10 min** to dry the pellet. It is important to evaporate all residual ethanol. Residual ethanol may reduce DNA yield.	**60 °C, 3 – 10 min**

* Examples of alternatives to xylene are: d-Limonene (e.g., Roti®-Histol, Hemo-De) or mixtures of isoparafinnic hydrocarbons (e.g., Roticlear®, Micro-ClearTM, Neo-Clear®).

2 Lyse sample

+ 100 µL FL

+ 10 µL Proteinase K

Vortex

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

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*).

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 **room temperature** for **3 hours** to lyse sample tissue.

RT, 3 hours

If residual unlysed tissue particles are visible after 3 hours incubation, add additional 10 µL Proteinase K solution and continue digestion for further 3 hours or overnight.

Note: Yield of amplifiable DNA typically increases with elongated lysis time. During this incubation step protein is digested and DNA is released into solution.

Vortex tube 5 s.

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

Set heating block to 90 °C (for subsequent decrosslink step).

3 Decrosslink

+ 100 µL D-Link

Vortex

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

Incubate at **90 °C** for exactly **30 min**.

90 °C, 30 min

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

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

Note: This decrosslink step is necessary to remove crosslinks (chemical modification caused by formalin) from the DNA which is released in solution by the previous lysis step. Decrosslinked DNA generally shows better performance in downstream applications.

4 Adjust binding conditions

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

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

+ 200 µL ethanol

Vortex

5 Bind DNA

For each preparation, take one **NucleoSpin® DNA FFPE XS Column** (green ring) placed in a Collection Tube (2 mL).

Load lysate

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

Load the lysate into the column.

Centrifuge for **30 s** at **2,000 x *g*.** If the solution does not flow through completely, centrifuge for **30 s** at **11,000 x *g*** until the complete solution passed the column.

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 flowthrough and place the column in a new Collection Tube (2 mL).

6 Wash and dry silica membrane

1st wash

Add **400 µL Buffer B5** to the NucleoSpin® DNA FFPE XS Column.

+ 400 µL B5

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

11,000 x *g*, 30 s

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

2nd wash

+ 400 µl B5

Add **400 µl Buffer B5** to the NucleoSpin® DNA FFPE XS Column.

11,000 x *g*, 2 min

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

Discard the Collection Tube with flowthrough and place the column into a nuclease-free Collection Tube (1.5 mL; not provided).

7 Elute DNA

Pipette **20 µL Buffer BE** directly to the center of the silica membrane of the column.

Elution volume may be varied from 5 – 30 µL. For the correlation of elution volume, DNA concentration, and DNA yield see section 2.5.

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

+ 20 µL BE

11,000 x *g*, 30 s

8 ***Optional:* Remove residual ethanol**

*Incubate the eluate (20 µL) with open lid for **8 min** at **90 °C**.*

See section 2.7 for detailed information and recommendations for removal of residual ethanol.

90 °C, 8 min

6 Appendix

6.1 Comments on DNA quality and quantity

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

Affected DNA 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)
- PCR
- Array analysis (e.g., DNA microarrays)

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

- **A high DNA yield**, as determined by A_{260} readings or by fluorescent dye (e.g., PicoGreen®) analysis does not necessarily result in good performance of the DNA in a PCR. DNA may be highly degraded (i.e., smaller fragments than the PCR target) or insufficiently decrosslinked.
- **Low or no DNA yield** as determined by A_{260} readings will most likely result in poor PCR results, but it is still possible to achieve a good performance. There may be a small amount DNA which is decrosslinked sufficiently and shows good reactivity.
- **DNA of high molecular weight** does not guarantee a good amplifiability in PCR or reactivity in other enzymatic reactions. DNA may be insufficiently decrosslinked although it has high molecular weight.
- **DNA of low molecular weight**, i.e. highly degraded DNA 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 (e.g., 80 – 150 bp) can be amplified successfully, especially if the DNA is well decrosslinked.

Neither DNA yield, molecular weight, absorbance ratios, nor size distribution can reliably predict the performance in downstream PCR applications, especially if different purification and decrosslinking systems are compared.

The major quality indicator for DNA isolated from FFPE samples is its performance in the intended downstream application.

6.2 Troubleshooting

Problem	Possible cause and suggestions
DNA is degraded/no DNA obtained	*Poor sample quality* • Sample quality has a high impact on quality and amount of the DNA.
Poor DNA quality or yield	*Reagents not applied or restored properly* • Reagents not properly restored. Add the indicated volume of ethanol to Buffer B5 Concentrate and mix. Reconstitute and store 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 DNA to the silica membrane is only effective in the presence of ethanol. *Kit storage* • Reconstitute and store Proteinase K 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. *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 K digestion time* • Depending of the nature of the sample, an optimal digestion time from 3 to 16 hours has to be determined empirically. If residual unlysed tissue is still visible after 3 h continue the incubation for up to 16 hours. After the first 3 h incubation, additional Proteinase K may be added to the sample.

Problem	Possible cause and suggestions
Clogged NucleoSpin® DNA FFPE XS Column/ Poor DNA quality or yield	*Sample material* • Too much starting material was used. Overloading may lead to a decrease of DNA yield. Reduce the quantity of sample material or use larger volumes of Paraffin Dissolver and / or Lysis Buffer FL. • Insufficient disruption and / or homogenization of starting material. Only perform an overnight incubation, if the tissue was not completely digested after 3 hours.
Suboptimal performance of DNA in downstream experiments	*Carry-over of ethanol or salt* • Do not let the flowthrough touch the column outlet after the second wash with Buffer B5. Be sure to centrifuge at the corresponding speed for the respective time in order to remove ethanolic Buffer B5 completely. • Check if Buffer B5 has been equilibrated to room temperature before use. Washing at lower temperatures decreases efficiency of salt removal by Buffer B5. • Depending on the robustness of the used PCR system, PCR might be inhibited if too much eluate is applied. Use less eluate as template. *Store isolated DNA properly* • Eluted DNA should always be kept on ice for optimal stability since possible traces of DNases will degrade the isolated DNA.
Discrepancy between A_{260} quantification values and PCR quantification values	*Silica abrasion from the membrane* • Due to the typically low DNA content in small FFPE samples and the resulting low total amount of isolated DNA, a DNA 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 DNA 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., PicoGreen® fluorescent dye).
Unexpected A_{260}/A_{280} ratio	*Measurement not in the range of photometer detection limit* • In order to obtain a significant 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 unexpected A_{260}/A_{280} ratios.

6.3 Ordering information

Product	REF	Pack of
NucleoSpin® DNA FFPE XS	740980.10 / .50 / .250	10 / 50 / 250
NucleoSpin® totalRNA FFPE	740982.10 / .50 / .250	10 / 50 / 250
NucleoSpin® totalRNA FFPE XS	740969.10 / .50 / .250	10 / 50 / 250
NucleoSpin® Tissue XS	740901.10 / .50 / .250	10 / 50 / 250
Paraffin Dissolver	740968.25	25 mL
Paraffin Dissolver blue	740343.60	60 mL
Collection Tubes (2 mL)	740600	1000
Decrosslink Buffer D-Link	740979.30	30 mL

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

6.4 Product use restriction / warranty

All MACHEREY-NAGEL products are designed for their intended use only. They are not intended to be used for any other purpose. The description of the intended use of the products can be found in the original MACHEREY-NAGEL product leaflets. Before using our products, please observe the instructions for use and the safety instructions from the respective Material Safety Data Sheet of the product.

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

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

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

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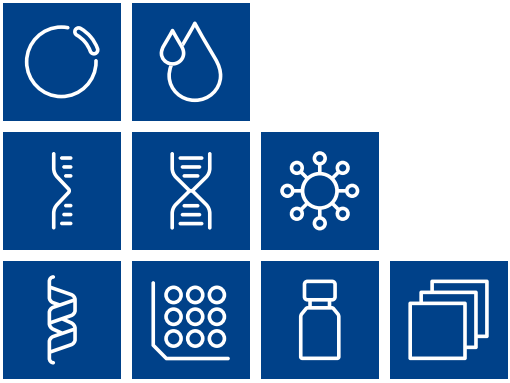

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