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DNA Isolation from FFPE Samples

User Manual

NucleoSpin® FFPE DNA

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MACHEREY-NAGEL

DNA Isolation from FFPE Samples

Protocol-at-a-glance (Rev. 01)

NucleoSpin® FFPE DNA

		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 11,000 x *g*, 30 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® FFPE DNA		
	10 preps	**50 preps**	**250 preps**
REF	**740980.10**	**740980.50**	**740980.250**
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 B5 (Concentrate)*	2 mL	12.5 mL	50 mL
Proteinase K (lyophilized)*	6 mg	30 mg	75 mg
Proteinase Buffer PB	0.8 mL	1.8 mL	8 mL
Elution Buffer BE**	5 mL	5 mL	15 mL
NucleoSpin® FFPE DNA Columns (green rings plus Collection Tubes)	10	50	250
Collection Tubes (2 mL)	20	100	500
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* 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® FFPE DNA** 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 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® FFPE DNA** 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 barley visible tissue pellets. **NucleoSpin® FFPE DNA** 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® FFPE DNA 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® FFPE DNA** 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® FFPE DNA** 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® FFPE DNA 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 mm^2). 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® FFPE DNA**
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
Maximum loading volume	600 µL
Format	Mini spin column – XS design

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 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

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

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.

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® FFPE DNA 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.

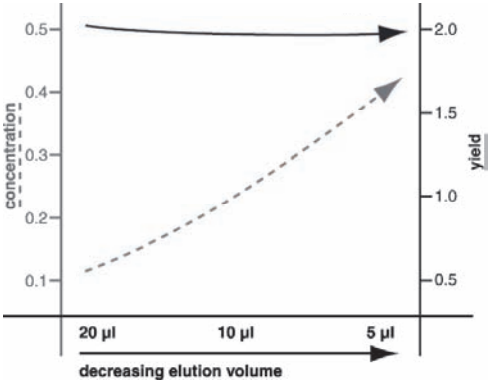


Figure 1: Correlation between elution volume and DNA concentration (NucleoSpin® FFPE DNA Columns)

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.

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

The default elution volume of **NucleoSpin® FFPE DNA** 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.

* 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).

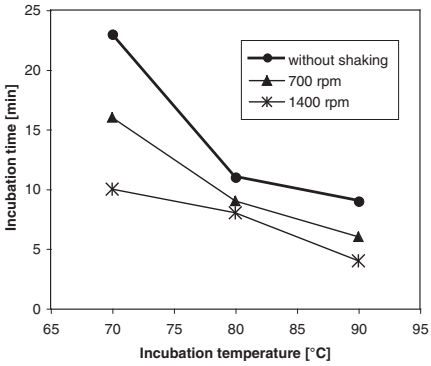


Figure 2: Removal of residual ethanol from the elution fraction by heat treatment.

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 FL1 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® FFPE DNA		
	10 preps	**50 preps**	**250 preps**
REF	**740980.10**	**740980.50**	**740980.250**
Wash Buffer B5 (Concentrate)	2 mL Add 8 mL 96 – 100% ethanol	12.5 mL Add 50 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 – risk and safety phrases

The following components of the **NucleoSpin® FFPE DNA** 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
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
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

Risk phrases

R 36/37/38	Irritating to eyes, respiratory system and skin
R 42	May cause sensitization by inhalation
R 65	Harmful: May cause lung damage if swallowed
R 66	Repeated exposure may cause skin dryness or cracking

Safety phrases

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

* 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).

5 Protocols

NucleoSpin® FFPE DNA 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.

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.

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).

+ 400 µL Paraffin Dissolver

60°C 3 min

Vortex hot sample

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**.

Vortex vigorously.

+ 100 µL FL

Mix

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.

11,000 x *g*
1 min

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

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.)

+ 10 µL Proteinase K

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

Mix by pipetting up and down (lower phase)

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 **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

RT
3 hours

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 5 s.

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.

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

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**.

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

90°C
30 min

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

Vortex

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

+ 200 µL ethanol

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

Vortex

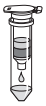
Centrifuge for **30 s** at **11,000 x *g*** to achieve complete phase separation.

11,000 x *g*
30 s

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

5 Bind DNA

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



Load aqueous (lower) phase

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

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 Wash and dry silica membrane

1st wash

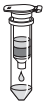
+ 400 µL B5

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

11,000 x *g*
30 s

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

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



2nd wash

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

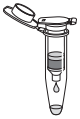
+ 400 µL B5

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

11,000 x *g*
2 min

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

7 Elute DNA



+ 20 µL BE

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.

11,000 x *g*
30 s

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

8 Optional: Remove residual ethanol

90°C
8 min

*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.

5.2 DNA purification from FFPE samples with xylene deparaffinization

Before starting the preparation:

- Check if Proteinase 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.

1 mL xylene

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

RT
2 min

Vortex

Make sure that the paraffin is completely dissolved.

Centrifuge for **2 min** at **11,000 x *g***.

11,000 x *g*
2 min

Discard supernatant

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

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

1 mL ethanol

Vortex

Centrifuge for **2 min** at **11,000 x *g***.

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

11,000 x *g*
2 min

Discard supernatant

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

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

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.

RT
3 hours

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

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

Vortex

Incubate at **90°C** for exactly **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*)*

90°C
30 min

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 x 5 s).

+ 200 µL ethanol

Vortex

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

5 Bind DNA

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

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 Wash and dry silica membrane

1st wash

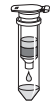
Add **400 µL Buffer B5** to the NucleoSpin® FFPE DNA Column.

+ 400 µL B5

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).



2nd wash

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

+ 400 µl B5

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

11,000 x *g*
2 min

Discard the Collection Tube with flow-through and place the column into a nuclease-free Collection Tube (1.5 mL; provided).

7 Elute DNA

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

+ 20 µL BE

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

11,000 x *g*
30 s

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

8 Optional: Remove residual ethanol

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

90 °C
8 min

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

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.

Clogged NucleoSpin® FFPE DNA 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. Perform only 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 flow-through 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® FFPE DNA	740980.10/.50/.250	10/50/250
NucleoSpin® FFPE RNA/DNA*	740978.10/.50/.250	10/50/250
NucleoSpin® FFPE RNA	740969.10/.50/.250	10/50/250
NucleoSpin® Tissue XS	740901.10/.50/.250	10/50/250
Paraffin Dissolver	740968.25	25 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.

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

6.4 Product use restriction / warranty

NucleoSpin® FFPE DNA kit components were developed, designed, distributed, and sold **FOR RESEARCH PURPOSES ONLY.** They are suitable **FOR *IN-VITRO* USES ONLY**. No claim or representation is intended for its use to identify any specific organism or for clinical use (diagnostic, prognostic, therapeutic, or blood banking).

It is rather the responsibility of the user to verify the use of the **NucleoSpin® FFPE DNA** kit for a specific application range as the performance characteristic of this kit has not been verified to a specific organism.

This MACHEREY-NAGEL product is shipped with documentation stating specifications and other technical information. MACHEREY-NAGEL warrants to meet the stated specifications. MACHEREY-NAGEL´s sole obligation and the customer´s sole remedy is limited to replacement of products free of charge in the event products fail to perform as warranted. Supplementary reference is made to the general business terms and conditions of MACHEREY-NAGEL, which are printed on the price list. Please contact us if you wish an extra copy.

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