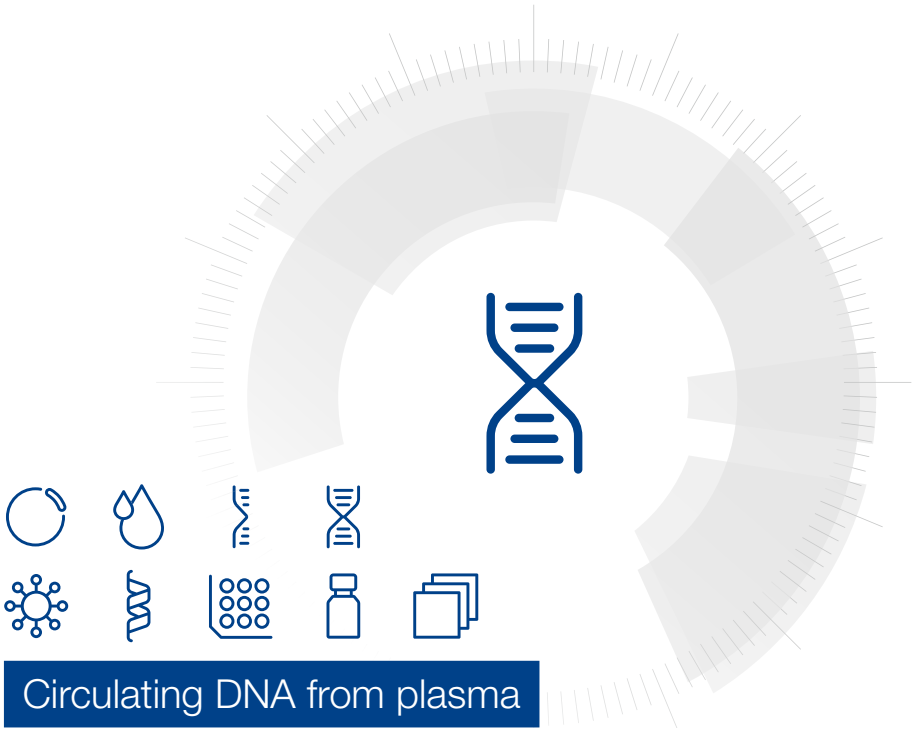
Bioanalysis

MACHEREY-NAGEL

User manual



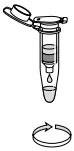
Circulating DNA from plasma

■ NucleoSpin® cfDNA XS

November 2023 / Rev. 10

Circulating DNA from plasma

Protocol at a glance (Rev. 10)

NucleoSpin® cfDNA XS			High Sensitivity protocol	Rapid protocol
1	**Prepare sample**		Use up to 240 µL plasma	Use up to 200 µL plasma
1a	***Optional: Proteinase K treatment***		*Add 20 µL Proteinase K* *Mix* *Incubate at 37 °C for 10 min*	/
2	**Adjust binding conditions**		Add 360 µL BB	Add 300 µL BB
3	**Mix sample**		Invert tube 3 x Vortex 3 s Spin down briefly	Invert tube 3 x Vortex 3 s Spin down briefly
4	**Bind DNA**		Load lysate 2,000 x *g*, 30 s 11,000 x *g*, 5 s	Load lysate 11,000 x *g*, 30 s
5	**Wash and dry silica membrane**		1st wash 500 µL WB 11,000 x *g*, 30 s 2nd wash 250 µL WB 11,000 x *g*, 3 min	1st wash 500 µL WB 11,000 x *g*, 30 s 2nd wash 250 µL WB 11,000 x *g*, 3 min
6	**Elute DNA**		20 µL Elution Buffer 11,000 x *g*, 30 s	20 µL Elution Buffer 11,000 x *g*, 30 s
7	**Removal of residual ethanol**		90 °C, 8 min	/

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1 Components

1.1 Kit contents

	NucleoSpin® cfDNA XS		
REF	**10 preps 740900.10**	**50 preps 740900.50**	**250 preps 740900.250**
Binding Buffer BB	5 mL	22 mL	110 mL
Wash Buffer WB	10 mL	50 mL	250 mL
Elution Buffer*	13 mL	13 mL	13 mL
Proteinase K (lyophilized)**	6 mg	30 mg	2 × 75 mg
Proteinase Buffer PB	1.8 mL	1.8 mL	8 mL
NucleoSpin® cfDNA XS Columns (red rings – incl. Collection Tubes)	10	50	250
Collection Tubes (2 mL)	20	100	500
User manual	1	1	1

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

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

1.2 Consumables and equipment to be supplied by user

Consumables

- 1.5 mL microcentrifuge tubes
- Disposable pipette tips

Equipment

- Manual pipettors
- Centrifuge for microcentrifuge tubes
- Vortex mixer
- Heating-block for incubation at 90 °C
- Personal protection equipment (lab coat, gloves, goggles)

1.3 About this user manual

The manual provides two procedures differing in the number of handling steps, speed and performance. The **high sensitivity procedure** is recommended if highest DNA yield and concentration is required. The **rapid procedure** is recommended if shortest preparation time is required.

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

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

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

2 Product description

2.1 The basic principle

The **NucleoSpin® cfDNA XS** kit is designed for the efficient isolation of circulating DNA from human blood plasma or other types of cell-free fluids. Fragmented DNA as small as 50 – 1000 bp can be purified with high efficiency. The special funnel design of the **NucleoSpin® cfDNA XS Columns** allows very small elution volumes (5 – 30 µL) resulting in highly concentrated DNA.

The protocol follows state-of-the-art bind-wash-elute procedures: After mixing of the sample with the binding buffer, the mixture is applied to the **NucleoSpin® cfDNA XS Column**. Upon loading of the mixture DNA binds to a silica membrane. Two subsequent washing steps efficiently remove contaminations and highly pure DNA is finally eluted with 5 – 30 µL elution buffer (5 mM Tris-HCl, pH 8.5).

2.2 Kit specifications

- The **NucleoSpin® cfDNA XS** kit is recommended for the isolation of fragmented cell-free DNA from human EDTA plasma, serum, and bronchial lavage. It has been successfully used for other cell-free fluids including urine (Outinen *et al.* 2012) and follicular fluid (Kansaku *et al.* 2018).
- The **NucleoSpin® cfDNA XS** kit is designed for high recovery, especially of fragmented DNA in a range of 50 – 1000 bp.
- Up to 240 µL sample can be used with a single column loading step. DNA yield strongly depends on the individual sample. For plasma it is typically in the range of 0.1 ng up to several 100 ng DNA per mL sample. Up to 720 µL sample can be used with three column loadings. If more than 240 µL sample is processed, additional Lysis Buffer BB is required (see ordering information).
- Elution can be performed with as little as 5 – 30 µL elution buffer. DNA is ready to use for downstream applications like real time PCR or others.
- The preparation time is approximately 15 – 30 min for 6 – 12 samples.

Forensic quality product:

NucleoSpin® cfDNA XS is certified as forensic quality product. Consumables used in forensics need to be treated carefully to prevent DNA contamination. MACHEREY-NAGEL therefore has a stringently controlled production process to avoid DNA contamination of consumables. Further, MACHEREY-NAGEL uses ethylene oxide (EO) treatment to remove amplifiable DNA, which might still be introduced during the manufacturing process. The NucleoSpin® cfDNA XS products (REF 740900.10/50/250) contain spin columns that are EO treated. This means, DNA of any kind, which might still be introduced into the spin columns during the production process, is inactivated by means of the treatment with ethylene oxide, in order to prevent the generation of accidental human profile by PCR amplification. Ethylene oxide treatment has been shown to be the method of choice to prevent DNA profiles due to DNA contamination (Shaw *et al.* 2008; Figure 1).

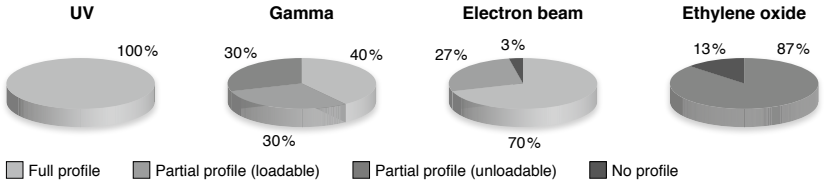

Figure 1 According to Shaw et al., 2008, Comparison of the effects of sterilization techniques on subsequent DNA profiling. Int J Legal Med 122: 29 – 33.

Full profile UV Gamma Electron beam Ethylene oxide Partial profile (loadable) Partial profile (unloadable) No profile 100 % 30 % 40 % 30 % 3 % 27 % 70 % 13 % 87 % Figure 1 According to Shaw *et al.*, 2008, Comparison of the effects of sterilization techniques on subsequent DNA profiling. Int J Legal Med 122: 29 – 33.

Table 1: Kit specifications at a glance

Parameter	NucleoSpin® cfDNA XS
Sample material	EDTA plasma, serum, bronchial lavage, urine, other cell-free fluids
Sample volume	standard volume: 240 µL (one column loading step) max. volume: 720 µL (three column loading steps)
Average yield	Typically in a range of 0.1 – 100 ng per mL plasma, depending on sample (depending on kind of patient samples, yield can be much higher).
Elution volume	5 – 30 µL
Preparation time	High sensitivity procedure: 22 – 27 min/6 preps Rapid procedure: 15 – 20 min/6 preps
Format	XS spin column
Use	For research use only

DNA yield from human plasma

DNA amounts from less than 0.1 ng DNA per mL of plasma up to several 100 ng DNA / mL of plasma have been reported (Chiu *et al.* 2006; Chun *et al.* 2006; Fatouros *et al.* 2006; Lazar *et al.* 2006; Rainer *et al.* 2006; Rhodes *et al.* 2006; Schmidt *et al.* 2005).

The content of DNA in plasma depends on: condition of the donor, sampling and handling of the blood, plasma preparation and DNA isolation method, DNA quantification method, and others.

Size of circulating DNA

A good portion of the cell-free DNA in plasma results from apoptotic cells. Therefore, a considerable percentage of this circulating nucleosomal DNA is known to be highly fragmented. However, the degree of fragmentation and the ratio of fragmented DNA to high molecular weight DNA depends on several parameters like origin of the DNA (e.g., fetal, tumor, microbial DNA), health of the blood donor, blood sampling procedure, and handling of the sample.

The performance of many downstream applications depends on the efficient isolation even of smallest DNA fragments (Chan *et al.* 2006, 2005, 2004, 2003; Deligezer *et al.* 2006; Giacona *et al.* 1998; Hanley *et al.* 2006; Hromadnikova *et al.* 2006; Jiang *et al.* 2006; Koide *et al.* 2005; Li *et al.* 2006, 2005, 2004; Wang *et al.* 2004). According to this the **NucleoSpin® cfDNA XS** purification system is designed for the efficient isolation of highly fragmented DNA in a range of 50 – 1000 bp. Within this range fragments are recovered with similar high efficiency.

2.3 Handling of sample material

Several publications indicate strong influence of blood sampling, handling, storage, and plasma preparation on DNA yield and DNA quality (Page *et al.* 2006; Sozzi *et al.* 2005; Chan *et al.* 2005; Lam *et al.* 2004; Jung *et al.* 2003). Therefore it is highly recommended keeping blood sampling procedure, handling, storage, and plasma preparation method constant in order to achieve highest reproducibility.

Samples can be isolated according to protocols described in literature (e.g., Chiu and Lo 2006; Birch *et al.* 2005) or according to the following recommendations:

Preparation of plasma from human EDTA blood

1. Centrifuge fresh blood sample for 10 min at 2,000 x *g*.
2. Remove the plasma without disturbing sedimented cells.
3. Freeze plasma at -20 °C for storage upon DNA isolation.
4. Thaw frozen plasma samples prior to DNA isolation and centrifuge for 3 min at ≥ 11,000 x *g* in order to remove residual cells, cell debris, and particulate matter. Use the supernatant for DNA isolation.

Preparation of other cell-free liquid samples (e.g., urine)

Clarify the sample by centrifugation (e.g., 5 min at 4,500 x *g*) in order to pellet cells or other solid particles suspended in the sample. Use only the supernatant for cfDNA isolation.

2.4 Elution procedures

The recommended standard elution volume is 20 µL. A reduction of the elution volume to 5 – 15 µL will increase DNA concentration, the total DNA yield is decreased by this reduction however. An increase of the elution volume to 30 µL or more will only slightly increase total DNA yield, but reduce DNA concentration. Figure 1 gives a graphic description of the correlation between elution volume and DNA concentration to help finding the optimized elution volume for your individual application.

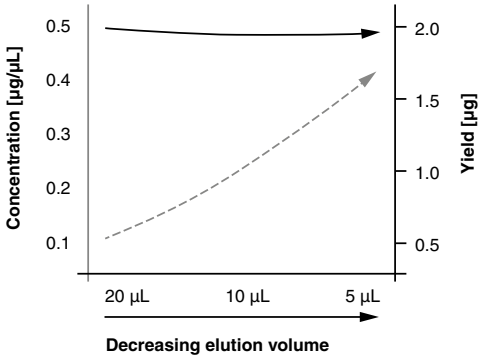

Figure 2 Correlation between elution volume and DNA concentration (NucleoSpin® cfDNA XS Columns)

2.5 Removal of residual traces of ethanol for highest PCR sensitivity

A reduction of the 20 µL standard elution volume will increase the concentration of residual ethanol in the eluate. For 20 µL elution volume a heat incubation of the elution fraction (incubate eluate with open lid for 8 min at 90 °C) is recommended if the eluate comprises more than 20 % of the final PCR volume, in order to avoid an inhibition of sensitive downstream reactions. In this context, please mind the remarks below:

- An incubation of the elution fraction at higher temperatures will increase signal output in PCR. This is of importance especially 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 increased temperature as described.

- A volume of 20 µL used for elution will evaporate to 12 – 14 µL during a heat incubation for 8 min at 90 °C. If a higher final volume is required, please increase the initial volume of elution buffer, for example from 20 µL to 30 µL.

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

* 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 LightCyclerTM PCR (Roche) with DyNAmoTM Capillary SYBR® Green qPCR Kit (Finnzymes).

- The incubation of the eluate at higher temperatures may be adjusted according to Figure 2. The incubation times and conditions shown will reduce an initial elution volume of 20 µL to about 12 – 14 µL and will effectively remove traces of ethanol as described above.
- If the initial volume of elution buffer applied to the column is less than 20 µL, time of heat incubation should be reduced to avoid complete dryness.

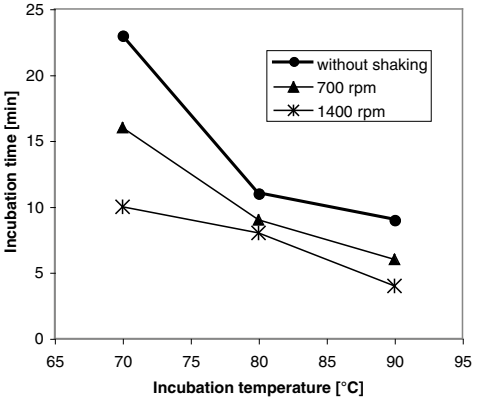

Figure 3 Removal of residual ethanol from the elution fraction by heat treatment.
In order to obtain highest PCR sensitivity, heat incubation of the eluate is recommended. Heat incubation may be performed at temperatures of 70 – 90 °C in a heat block with or without shaking. Effective conditions (temperature, 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.

2.6 Stability of isolated DNA

Due to the typically low DNA content and the resulting low total amount of isolated DNA, its fragmentation, and the absence of DNase inhibitors (the elution buffer does NOT contain EDTA) the eluates should be placed on ice for short term and at -20 °C for long term storage.

3 Storage conditions and preparation of working solutions

Attention: The Buffer BB contains chaotropic salt and ethanol! Wear gloves and goggles!

CAUTION: Buffer BB contains guanidinium thiocyanate which can form highly reactive compounds when combined with bleach (sodium hypochlorite). DO NOT add bleach or acidic solutions directly to the sample-preparation waste.

Storage conditions:

- All kit components can be stored at 15 – 25 °C and are stable until: see package label.
- If there is any precipitate present in the buffers, warm the buffer up to (25 – 37 °C) to dissolve the precipitate before use.

Before starting any **NucleoSpin® cfDNA XS** protocol prepare the following:

- Before first use of the kit, add the indicated volume of Proteinase Buffer PB to dissolve lyophilized **Proteinase K** (see bottle or table below). Proteinase K solution is stable at -20 °C for at least 6 months.

	NucleoSpin® cfDNA XS		
	10 preps	**50 preps**	**250 preps**
REF	**740900.10**	**740900.50**	**740900.250**
Proteinase K (lyophilized)	6 mg Add 260 µL Proteinase Buffer	30 mg Add 1.35 mL Proteinase Buffer	2 × 75 mg Add 3.35 mL Proteinase Buffer to each vial

4 Safety instructions

When working with the **NucleoSpin® cfDNA 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***).

Caution: Guanidine thiocyanate in Binding Buffer BB can form highly reactive compounds when combined with bleach! Thus, do not add bleach or acidic solutions directly to the sample preparation waste.

The waste generated with the **NucleoSpin® cfDNA 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

Before starting the preparation:

Equilibrate sample to room temperature (15 – 25 °C) and make sure that the sample is cleared from residual cells, cell debris, and particular matter (e.g., by centrifugation of the plasma sample for 3 min at ≥ 11,000 x *g*).

For the high sensitivity procedure: Set the thermal heating block to 75 – 90 °C for final ethanol removal (see section 2.6 for details).

5.1 High sensitivity protocol for the isolation of cfDNA

1 Prepare sample

Add **240 µL plasma or alternative cell-free fluid** to a microcentrifuge tube (not provided).

Less than 240 µL may be used. Adapt the binding buffer volume accordingly (see below).

240 µL sample

1a ***Optional:* Proteinase K treatment**

*Add **20 µL Proteinase K** to the sample, mix, and incubate at 37 °C for 10 min.*

Depending on the sample and the PCR conditions, the proteinase treatment provokes an increase of the PCR signal of 0.5 – 1.5 cycles, i.e. the cycle threshold (Ct-value)/crossing point (Cp-value) is reached 0.5 – 1.5 cycles earlier. The proteinase treatment may however alter the ratio of high to low molecular weight DNA.

Optional: + 20 µL Proteinase K

2 Adjust DNA binding conditions

Add **360 µL Buffer BB**.

If less than 240 µL sample is used, adjust the binding buffer volume accordingly. A ratio of 1:1.5 (v/v) for sample and binding buffer has to be ensured.

+ 360 µL BB

3 Mix sample

Invert the tube **3 x** and vortex for **3 s**. Centrifuge the tube briefly to clean the lid.

Mix sample

4 Bind DNA

For each sample, load the mixture **(620 µL)** to a **NucleoSpin® cfDNA XS Column** placed in a Collection Tube (2 mL).

Load lysate

Centrifuge at **2,000 x *g*** for **30 s**, increase centrifuge force to **11,000 x *g*** for further **5 s**. Discard Collection Tube with flowthrough and place column into new Collection Tube (provided).

2,000 x *g*, 30 s

The maximal column volume is approximately 600 µL. Do not apply a higher volume in order to avoid spillage. If larger sample volumes have to be processed, the loading step may be repeated. Be aware of an increased risk of membrane clogging in case of multiple column loading steps. If the solution has not completely passed the column, centrifuge for an additional 60 s at 11,000 x g.

11,000 x *g*, 5 s

5 Wash and dry silica membrane

1st wash

+ 500 µL WB

Pipette **500 µL Buffer WB** onto the NucleoSpin® cfDNA XS Column. Centrifuge for **30 s** at **11,000 x *g***. Discard Collection Tube with flowthrough and place the column into new Collection Tube (provided).

11,000 x *g*, 30 s

2nd wash

+ 250 µL WB

Add **250 µL Buffer WB** to the NucleoSpin® cfDNA XS Column. Centrifuge for **3 min** at **11,000 x *g***. Discard Collection Tube with flowthrough and place the column into a 1.5 mL microcentrifuge tube for elution (not provided).

11,000 x *g*, 3 min

6 Elute DNA

+ 20 µL Elution Buffer

Add **20 µL Elution Buffer** to the NucleoSpin® cfDNA XS Column. Centrifuge for **30 s** at **11,000 x *g***.

Elution volume may be varied in range of 5 – 30 µL. For a correlation of elution volume, DNA concentration, and DNA amount eluted from the column see section 2.4.

11,000 x *g*, 30 s

7 Removal of residual ethanol

Incubate elution fraction with open lid for **8 min** at **90 °C**.

90 °C, 8 min

See section 2.5 for further comments and alternative incubation times and temperatures for a removal of residual ethanol.

5.2 Rapid protocol for the isolation of cfDNA

The rapid procedure represents a good compromise between DNA yield and concentration as well as ease and speed of nucleic acid extraction.

1 Prepare sample

Add **200 µL plasma or alternative cell-free fluid** to a microcentrifuge tube (not provided).

Less than 240 µL may be used. Adapt the binding buffer volume accordingly (see below).

200 µL sample

2 Adjust DNA binding conditions

Add **300 µL Buffer BB**.

If less than 200 µL sample is used, adjust the binding buffer volume accordingly. A ratio of 1:1.5 (v/v) for sample and binding buffer has to be ensured.

+ 300 µL BB

3 Mix sample

Invert the tube **3 x** and vortex for **3 s**. Centrifuge the tube briefly to clean the lid.

Mix sample

4 Bind DNA

For each sample, load the mixture **(500 µL)** to a **NucleoSpin® cfDNA XS Column** placed in a Collection Tube (2 mL).

Centrifuge at **11,000 x *g*** for **30 s**. Discard Collection Tube with flowthrough and place column into new Collection Tube (provided).

Load lysate

The maximal column volume is approximately 600 µL. Do not apply a higher volume in order to avoid spillage. If larger sample volumes have to be processed, the loading step may be repeated. Be aware of an increased risk of membrane clogging in case of multiple column loading steps. If the solution has not completely passed the column, centrifuge for an additional 60 s at 11,000 x g.

11,000 x *g*, 30 s

5 Wash and dry silica membrane

1st wash

Pipette **500 µL Buffer WB** onto the NucleoSpin® cfDNA XS Column. Centrifuge for **30 s** at **11,000 x *g***. Discard Collection Tube with flowthrough and place the column into new Collection Tube (provided).

+ 500 µL WB

11,000 x *g*, 30 s

2nd wash

Add **250 µL Buffer WB** to the NucleoSpin® cfDNA XS Column. Centrifuge for **3 min** at **11,000 x *g***. Discard Collection Tube with flowthrough and place the column into a 1.5 mL microcentrifuge tube for elution (not provided).

+ 250 µL WB

11,000 x *g*, 3 min

6 Elute DNA

Add **20 µL Elution Buffer** to the NucleoSpin® cfDNA XS Column. Centrifuge for **30 s** at **11,000 x *g***.

Elution volume may be varied in range of 5 – 30 µL. For a correlation of elution volume, DNA concentration, and DNA amount eluted from the column see section 2.4.

+ 20 µL Elution Buffer

11,000 x *g*, 30 s

6 Appendix

6.1 Troubleshooting

Problem	Possible cause and suggestions
Low DNA yield	*Low DNA content of the sample* • The content of cell-free DNA in human plasma and other cell-free bodily fluids may vary over several orders of magnitude. DNA contents from approximately 0.1 – 100 ng DNA per mL of plasma have been reported (see remarks in section 2.2). • If the DNA concentration is measured with double strand specific dyes, e.g., PicoGreen, and step 7 “Removal of residual ethanol” with incubation at 90 °C is performed, the measured yield is below the actual value. This is due to the denaturation of DNA during the heat incubation step and the double strand specificity of certain DNA dyes, e.g., PicoGreen.
Column clogging	*Sample contains residual cell debris or cells* • The sample may have contained residual cells or cell debris. Make sure to use only cleared samples (see remarks in section 2.3).
No increase of PCR signal despite of an increased volume of eluate used as template in PCR	*Residual ethanol in eluate* • Please see the detailed description of removal of residual traces of ethanol in section 2.5.
Discrepancy between A_{260} quantification values and PCR quantification values	*Silica abrasion from the membrane* • Due to the typically low DNA content in plasma (and other cell free fluids) 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 DNA quantification method (e.g., PicoGreen® fluorecent 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 unexpeced A_{260}/A_{280} ratios.

6.2 Ordering information

Product	REF	Pack of
NucleoSpin® cfDNA XS	740900.10 / .50 / .250	10 / 50 / 250
NucleoSpin® DNA cfDNA Midi	740303.48	48
NucleoSpin® DNA cfDNA Midi Core Kit	740302.48	48
NucleoSnap® cfDNA	740300.10 / .50	10 / 50
NucleoSpin® 96 cfDNA	740873.1 / .4	1 × 96 / 4 × 96
NucleoSpin® 96 cfDNA Core Kit	740874.1 / .4	1 × 96 / 4 × 96
Buffer BB	740394.22	22 mL
Buffer WB	740331	250 mL
Collection Tubes (2 mL)	740600	1000

Note: This product has been formerly distributed under the name NucleoSpin® Plasma XS. The product code (REF) and kit content have not been changed.

6.3 References

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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.

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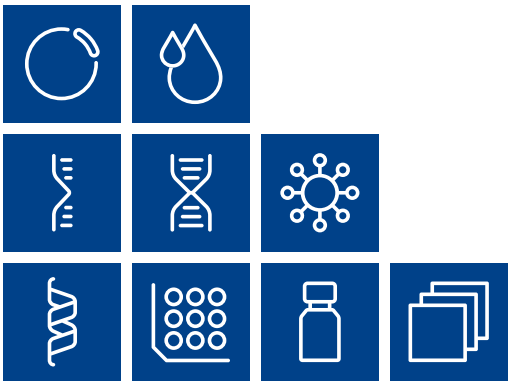

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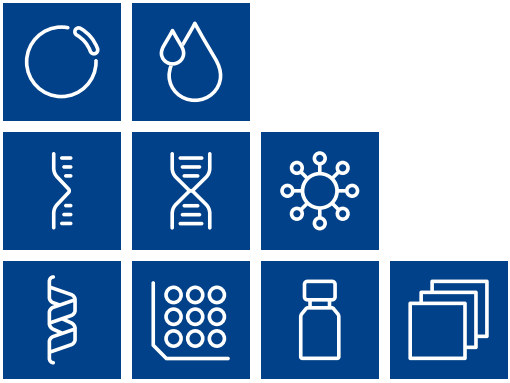

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