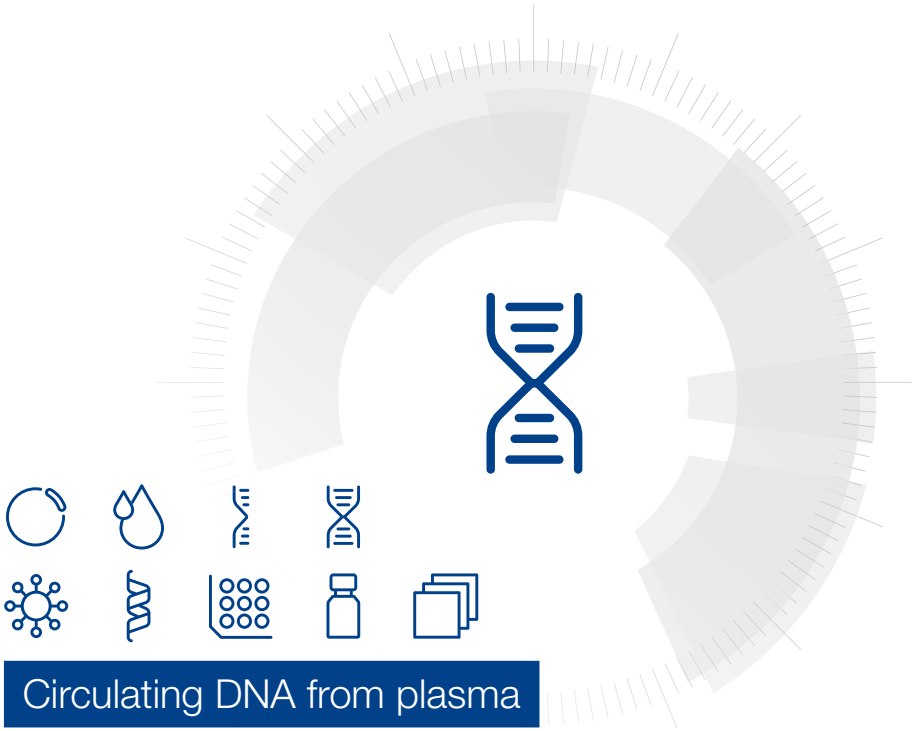
Bioanalysis

MACHEREY-NAGEL

User manual



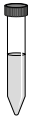
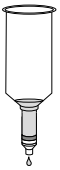
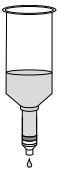
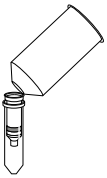
Circulating DNA from plasma

■ NucleoSnap® cfDNA

November 2023 / Rev. 04

Circulating DNA from plasma

Protocol at a glance (Rev. 04)

		NucleoSnap® cfDNA
1 Prepare sample	 	4,500 x *g*, 10 min
2 Lyse sample		45 µL Proteinase K 3 mL sample Mix RT, 5 min 3 mL VL Mix 56 °C, 5 min
3 Prepare Binding conditions		3 mL ethanol (96 – 100 %) Mix
4 Bind DNA	 Vacuum	500 µL CC 0.4 – 0.6 bar*, 30 s Load lysate 0.4 bar*, 5 – 15 min
5 Wash silica membrane	 Vacuum	1 mL VW1 0.2 – 0.4 bar*, 1 min 500 µL WB 0.2 – 0.4 bar*, 30 s
6 Dry silica membrane	 	Remove upper part and discard 11,000 – 20,000 x *g*, 3 min
7 Elute DNA	 	50 µL Elution Buffer RT, 3 min 11,000 x *g*, 1 min

* Reduction of atmospheric pressure

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Table of contents

1 Components

1.1 Kit contents

	NucleoSnap® cfDNA	
REF	**10 preps 740300.10**	**50 preps 740300.50**
Column Conditioner CC	13 mL	30 mL
Lysis Buffer VL	60 mL	275 mL
Wash Buffer VW1	13 mL	75 mL
Wash Buffer WB	10 mL	50 mL
Elution Buffer*	13 mL	13 mL
Liquid Proteinase K	800 µL	4 mL
NucleoSnap® cfDNA Columns	10	50
Collection Tubes (2 mL)	10	50
Collection Tubes (1.5 mL)	10	50
NucleoVac Mini Adapter	10	50
User manual	1	1

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

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

Reagents:

- 96 – 100 % ethanol

Consumables:

- 50 mL tubes for sample lysis
- Disposable pipette tips

Equipment:

- Vacuum manifold (e.g., NucleoVac 24 Vacuum Manifold; REF 740299; see ordering information section 7.2)
- Vacuum regulator (recommended, e.g., NucleoVac Vacuum Regulator; REF 740641, see ordering information section 7.2)
- Valves (recommended, e.g., NucleoVac Valves; REF 740298.24; see ordering information section 7.2)
- Centrifuge for microcentrifuge tubes
- Heating-block or water bath for 56 °C incubation of 50 mL tubes
- Manual pipettors
- Vortex mixer
- Personal protection equipment (lab coat, gloves, goggles)

1.3 About this user manual

It is strongly recommended for first time users to read the detailed protocol sections of the **NucleoSnap® cfDNA** kit before using this product. 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 online at ***www.mn-net.com***.

2 Kit specifications

- The **NucleoSnap® cfDNA** kit is recommended for the isolation of circulating cell-free DNA from human EDTA plasma, Cell-Free DNA BCT® plasma, or urine samples.
- The **NucleoSnap® cfDNA** kit is designed for high recovery of fragmented DNA ≥ 50 bp.
- Up to 5 mL plasma or urine can be used as sample material with a single column loading step. Sample volumes > 5 mL require an additional loading step.
- DNA yield from plasma and urine strongly depends on the individual sample. For human plasma it is typically in the range of 0.1 to 100 ng DNA per mL sample.
- Elution can be performed with as little as 20 – 50 µL elution buffer. DNA is ready to use for downstream applications like real-time PCR or others.
- The preparation time is approximately 50 min for 6 samples.

Table 1: Kit specifications at a glance

Parameter	NucleoSnap® cfDNA
Technology	Silica membrane technology
Format	NucleoSnap® Column
Sample material	Human EDTA/Cell-Free DNA BCT® plasma, urine
Sample amount	1 – 10 mL per preparation*
Typical yield	Sample dependent
Elution volume	20 – 100 µL
Preparation time	50 min/6 preps
Use	For research use only

* For processing larger volumes than 5 mL, Lysis Buffer VL and Proteinase K have to be purchased separately (see ordering information, section 7.2).

3 Product description

3.1 The basic principle

The **NucleoSnap® cfDNA** kit is designed for the efficient isolation of circulating free DNA from human blood plasma or urine. Fragmented DNA as small as 50 – 1000 bp is purified with high efficiency. The special snap-off design of the **NucleoSnap® cfDNA Columns** allows convenient processing of up to 5 mL sample at once (or 10 mL sample, which requires extra buffer; see ordering information) along with very small final elution volumes (20 – 50 µL), resulting in highly concentrated DNA.
The protocol follows state-of-the-art bind-wash-elute procedures: After mixing the sample with Liquid Proteinase K, lysis buffer and binding buffer, the mixture is applied to the **NucleoSnap® cfDNA Column**. Upon loading of the mixture onto the column and applying the vacuum, DNA binds to the silica membrane.
Two washing steps efficiently remove contaminating substances, such as PCR inhibitors. Subsequently, the upper part of the snap-off column is removed, and highly pure DNA is finally eluted with 20 – 50 µL of a slightly alkaline elution buffer of low ionic strength (5 mM Tris/HCl, pH 8.5) from the lower part of the snap-off column.

3.2 Size and yield of cfDNA

Usually, cfDNA concentrations in plasma are in a range of 0.1 ng DNA per mL of plasma to several 100 ng DNA per mL of plasma. The amount of circulating DNA in plasma/urine depends on the health condition of the donor, sampling and further handling of the sample, plasma preparation, DNA isolation method. cfDNA levels in samples obtained from cancer patients may be increased several fold.
A significant portion of the cfDNA in plasma originates from apoptotic cells. Therefore, a considerable percentage of this circulating 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 condition of the blood donor, blood sampling procedure, and handling of the sample.

The **NucleoSnap® cfDNA** purification system is designed for the efficient isolation of highly fragmented DNA in a range of 50 – 1000 bp. Within this range, both small and large fragments are recovered with high efficiency.

3.3 Handling of sample material

Circulating DNA yield and quality is highly influenced by sampling, handling, storage, and plasma/urine preparation. It is highly recommended to perform these steps as uniformly as possible in order to achieve highest reproducibility.

Samples can be isolated according to the following recommendation:

Preparation of plasma samples from human EDTA blood

1 Centrifuge fresh blood sample for 10 min at 2,000 x *g*.

2 Transfer the plasma without disturbing sedimented cells and particles into a fresh tube.

3 Freeze plasma at -20 °C for storage until cfDNA isolation.

4 Thaw frozen plasma samples prior to DNA isolation and centrifuge for 3 min at ≥ 11,000 x *g* in a mini centrifuge for small plasma volumes or 10 min at 4,500 x *g* in a tabletop centrifuge for larger volumes of plasma in order to remove residual cells, cell debris, and particulate matter. Use the supernatant for DNA isolation.

Preparation of plasma from Cell-Free DNA BCT®
Please follow the procedures recommended in the Cell-Free DNA BCT® user manual.

Preparation of urine samples
Clarify the urine 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. The centrifugation step will reduce the amount of genomic DNA in the final eluate. Use only the supernatant for cfDNA isolation.

3.4 Elution procedures

The recommended standard elution volume is 50 µL. A reduction of the elution volume to 20 µL will increase DNA concentration, but the total DNA yield will decrease by this elution procedure. An increase of the elution volume to 100 µL or more will only slightly increase total DNA yield, but reduces DNA concentration.

3.5 Stability of isolated DNA

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

4 Storage conditions and preparation of working solutions

Attention: *Buffers VL and VW1 contain chaotropic salt! Wear gloves and goggles!*

CAUTION: Buffer VL and VW1 contain guanidinium hydrochloride 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 room temperature (18 – 25 °C) and are stable for at least one year.
- 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 the **NucleoSnap® cfDNA** protocol:

- No buffers or enzymes have to be prepared – all kit components are ready to use.
- Set up the vacuum manifold (please follow the instructions provided by the manufacturer).
- **Liquid Proteinase** is ready to use. After first use, store Liquid Proteinase K at 4 °C or -20 °C.

5 Safety instructions

When working with the NucleoSnap® cfDNA 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 http://***www.mn-net.com/msds***).

Caution: Guanidine hydrochloride in buffer VL and VW1 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 NucleoSnap® cfDNA 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.

5.1 Disposal

Dispose hazardous, infectious or biologically contaminated materials in a safe and acceptable

manner and in accordance with all local and regulatory requirements.

6 Protocols for the isolation of cell-free DNA

6.1 Protocol for the isolation of cfDNA from 1 – 5 mL EDTA plasma or urine

The procedure below describes the isolation of cell-free DNA from 3 mL human EDTA plasma or urine. 1 – 5 mL sample can be processed as needed by adjusting the volumes of Proteinase K, lysis buffer, and ethanol as follows:

Sample volume [mL]	Proteinase K [µL]	Buffer VL [mL]	Ethanol [mL]
1	15	1	1
2	30	2	2
3	45	3	3
4	60	4	4
5	75	5	5

Before starting the preparation:

- Prepare plasma or urine sample according to section 3.3.
- Set water bath or heating block for 50 mL tubes to 56 °C (for lysate incubation).
- Set up vacuum manifold (please follow the instructions provided by the manufacturer).

Protocol for 3 mL sample

1 **Prepare sample**

Plasma: Centrifuge plasma for at least **10 min** at **4,500 x *g*** in order to remove residual cells and cell debris. Use supernatant, discard sediment.

Urine: Centrifuge urine for at least **5 min** at **4,500 x *g*** in order to remove residual cells and cell debris. Use supernatant, discard sediment.

Note: The supernatant may still contain suspended matter (e.g., lipids). This floating material does not interfere with further processing.

4,500 x *g*, 10 min

2 **Lyse sample**

Add **45 µL Proteinase K** to a 50 mL tube. Add **3 mL sample** to the tube. Carefully **mix** the contents of the tube without moistening the tube lid.

Incubate **5 min** at **room temperature** (18 – 25 °C). Add **3 mL Buffer VL** to the tube. **Mix** the tube contents by vortexing for approximately 20 s in order to obtain a homogenous lysate.

Incubate tube at **56 °C** for **5 min** (e.g., in a water bath).

+ 45 µL Proteinase K

+ 3 mL sample

Mix

RT, 5 min

+ 3 mL VL

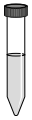
Mix

56 °C, 5 min

3 **Prepare binding conditions**

Carefully open the tube lid and add **3 mL ethanol** (96 – 100 %).

Mix the tube contents by vortexing for approximately 10 s in order to obtain a homogenous lysate.



+ 3 mL ethanol (96 – 100 %)

Mix

4 **Bind DNA**

+ 500 µL CC

0.4 – 0.6 bar*, 30 s

Load lysate

0.4 bar*, 5 – 15 min

Place the NucleoSnap® cfDNA Column onto a vacuum manifold (do not apply vacuum yet).

Note: The use of reusable NucleoVac Stop-cocks is recommended (not provided, see ordering information, section 7.2)

Add **500 µL Column Conditioner CC** to the column.

Apply vacuum (**approximately 0.4 – 0.6 bar*** differential pressure) until the solution has passed the column (usually several seconds). Release vacuum.

Note: Perform adding Column Conditioner CC and applying vacuum 1 – 5 min before adding the lysate to the NucleoSnap® Column.

Apply the **lysate from step 3** to the NucleoSnap® Column.

Apply vacuum (**approximately 0.4 bar*** differential pressure). Wait until the lysate has passed through the column. Approximate duration:

for 3 mL lysate (corresponding to 1 mL sample): up to 5 min;

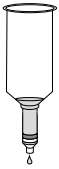
for 9 mL lysate (corresponding to 3 mL sample): up to 10 min;

for 15 mL lysate (corresponding to 5 mL sample): up to 15 min.

The vacuum can be adjusted to ≥ 0.6 bar* at the end of this step in case the lysate has not yet completely passed the column.

Close stopcocks for each column as soon as the lysate has passed the column!

5 **Wash silica membrane**



+ 1 mL VW1

0.2 – 0.4 bar*, 1 min

+ 500 µL WB

0.2 – 0.4 bar*, 30 s

Add **1 mL Buffer VW1** to the column.
Apply vacuum (**approximately 0.2 – 0.4 bar*** differential pressure) until the wash buffer has completely passed through the column. Approximate duration is up to one minute. Release vacuum.

Add **500 µL Buffer WB** to the column.
Apply vacuum (**approximately 0.2 – 0.4 bar*** differential pressure) until the wash buffer has passed through the column. Approximate duration is 30 s. Release vacuum.

* Reduction of atmospheric pressure

6 **Dry silica membrane**

Remove the NucleoSnap® Column from the vacuum manifold and insert it into a Collection Tube (2 mL, provided). **Remove the upper part of the column and discard** it.

Note: Tightly hold the upper part of the column in one hand and the labeled Collection Tube (2 mL) holding the NucleoSnap® Column in the other hand. Carefully snap off the upper part of the column. Do not label the lower (spin) part of the NucleoSnap® Column because the ink might be washed off.

Centrifuge the lower part of the column in the Collection Tube for **3 min** at **full speed** (> 11,000 x *g* up to 20,000 x *g*). Discard the Collection Tube with residual flow through and insert the column into a fresh Collection Tube (1.5 mL, provided).

Remove upper part and discard

11,000–20,000 x *g*, 3 min

7 **Elute DNA**

Add **50 µL Elution Buffer** to the column. Incubate **3 min** at **room temperature**. Centrifuge for **1 min** at **11,000 x *g***.

Store eluted DNA at 4 °C for short-term and at -20 °C for long-term storage.

Note: For alternative elution procedures see section 3.4.

50 µL Elution Buffer

RT, 3 min

11,000 x *g*, 1 min

* Reduction of atmospheric pressure

6.2 Protocol for the isolaton of cfDNA from 6 – 10 mL EDTA plasma or urine

6 – 10 mL sample can be processed as needed by adjusting the volumes of Proteinase K, lysis buffer, and ethanol as follows:

Sample volume [mL]	Proteinase K [µL]	Buffer VL [mL]	Ethanol [mL]
6	90	6	6
7	105	7	7
8	120	8	8
9	135	9	9
10	150	10	10

Note: For processing volumes larger than 5 mL, Lysis Buffer VL and Proteinase K have to be purchased separately (see ordering information, section 7.2).

Continue with step 4 of protocol 6.1.

6.3 Protocol for the isolaton of cfDNA from 1 – 5 mL plasma from Cell-Free DNA BCT® (Streck, Inc.)

Step 2 (lyse sample) of the standard protocol has to be modified as follows:

Add **45 µL Proteinase K** to a 50 mL tube. Add **3 mL plasma** to the tube. Carefully mix the content of the tube without moistening the tube lid. Incubate **15 min** at **room temperature** (18 – 25 °C). Add **3 mL Buffer VL** to the tube. **Mix** the tube content by vortexing for approximately 20 s in order to obtain a homogenous lysate.

Incubate tube at **56 °C** for **60 min** (e.g., in a water bath).

Continue with step 3 of protocol 6.1.

7 Appendix

7.1 Troubleshooting

Problem	Possible cause and suggestions
Low DNA yield	*Low DNA content of the sample* • The content of cell-free DNA in human plasma or urine may vary over several orders of magnitude. DNA contents from approximately 0.1 – 1000 ng DNA per mL of plasma have been reported (see remarks in section 3.2). *Inaccurate yield determination* • If the DNA concentration is measured with double strand specific dyes, e.g., PicoGreen®, make sure not to heat the eluted DNA before measurement. Due to denaturation of DNA during the heat incubation step and the double strand specificity of certain DNA dyes, e.g., PicoGreen®, results might be inaccurate.
Column clogging	*Sample contains residual cell debris or cells* • The sample may have contained residual cells or cell debris. Make sure to use only samples that have been centrifuged in order to remove cells and cell debris (see remarks in section 3.3).
Discrepancy between A_{260} quantification values and PCR quantification values	*Silica abrasion from the membrane* • Due to the typically low DNA content in plasma / urine 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 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 unexpected A_{260}/A_{280} ratios.

7.2 Ordering information

Product	REF	Pack of
NucleoSnap® cfDNA	740300.10 / 50	10 / 50 preps
NucleoVac 24 Vacuum Manifold	740299	1
NucleoVac Mini Adapter	740297.100	100
NucleoVac Valves	740298.24	24
NucleoSpin® cfDNA XS	740900.10 / 50 / 250	10 / 50 / 250 preps
NucleoMag® cfDNA	744550.1 / .4	1 x / 4 × 48 preps
NucleoSpin® 96 cfDNA	740873.1 / .4	1 x / 4 × 96 preps
NucleoSpin® cfDNA Midi	740303.48	1 × 48 preps
Collection Tubes (2 mL)	740600	1000
Lysis Buffer VL	740833.200	200 mL
Liquid Proteinase K	740396	5 mL

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

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

7.3 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.

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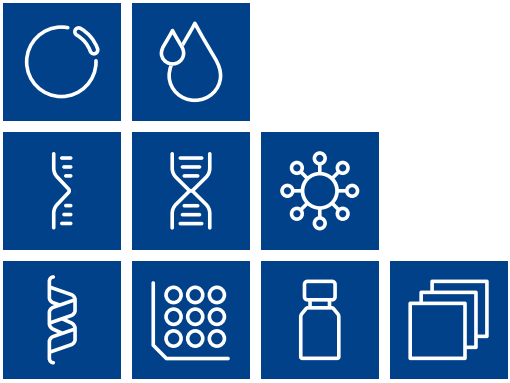

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