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Circulating DNA from Plasma

User Manual

NucleoSpin® Plasma XS

February 2010 / Rev. 02

MACHEREY-NAGEL

Circulating DNA from Plasma

Protocol-at-a-glance (Rev. 02)

NucleoSpin® Plasma 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 3x Vortex 3 s Spin down briefly	Invert tube 3x Vortex 3 s Spin down briefly
4 Bind DNA		Load lysate 30 s 2,000 x *g* 5 s 11,000 x *g*	Load lysate 30 s 11,000 x *g*
5 Wash and dry silica membrane		1st wash 500 µl WB 30 s 11,000 x *g* 2nd wash 250 µl WB 3 min 11,000 x *g*	1st wash 500 µl WB 30 s 11,000 x *g* 2nd wash 250 µl WB 3 min 11,000 x *g*
6 Elute DNA		20 µl Elution Buffer 30 s 11,000 x *g*	20 µl Elution Buffer 30 s 11,000 x *g*
7 Removal of residual ethanol		8 min 90°C	*/*

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1 Components

1.1 Kit contents

	NucleoSpin® Plasma XS		
	10 preps	**50 preps**	**250 preps**
Cat. No.	**740900.10**	**740900.50**	**740900.250**
Binding Buffer BB	4.5 ml	22 ml	110 ml
Wash Buffer WB	10 ml	2 x 25 ml	250 ml
Elution Buffer*	5 ml	5 ml	13 ml
Proteinase K (lyophilized)**	6 mg	30 mg	2 x 75 mg
Proteinase Buffer PB	0.8 ml	1.8 ml	8 ml
NucleoSpin® Plasma XS Columns (red rings - plus 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 condistions 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® Plasma 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*.

2 Product description

2.1 The basic principle

The **NucleoSpin® Plasma XS** kit is designed for the efficient isolation of circulating DNA from human blood plasma. Fragmented DNA as small as 50 – 1000 bp can be purified with high efficiency. Due to a special funnel design the **NucleoSpin® Plasma XS Columns** allow very small elution volumes (5 – 30 µl) which results in highly concentrated DNA.

The protocol follows state-of-the-art bind-wash-elute procedures: After mixing of a plasma sample with the binding buffer, the mixture is applied to the **NucleoSpin® Plasma 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 of a slightly alkaline elution buffer of low ionic strength (5 mM Tris-HCl, pH 8.5).

2.2 Kit specifications

- The **NucleoSpin® Plasma XS** kit is recommended for the isolation of fragmented cell-free DNA from human EDTA plasma, serum, and bronchial lavage.
- The **NucleoSpin® Plasma XS** kit is designed for high recovery, especially of fragmented DNA in a range of 50 – 1000 bp.
- Up to 240 µl plasma can be used as sample material with a single column loading step. DNA yield strongly depends on the individual sample, but is typically in the range of 0.1 to 100 ng DNA per ml plasma. Up to 600 µl plasma can be used with three column loadings.
- 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 plasma samples.

Table 1: Kit specifications at a glance	
Parameter	**NucleoSpin® Plasma XS**
Sample material	Up to 200 µl EDTA plasma
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

DNA yield from human plasma

DNA amounts from less than 0.1 ng DNA per ml of plasma up to several 100 ng DNA per 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 is resulting from apoptotic cells. As a result, 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® Plasma 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.

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

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.

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.

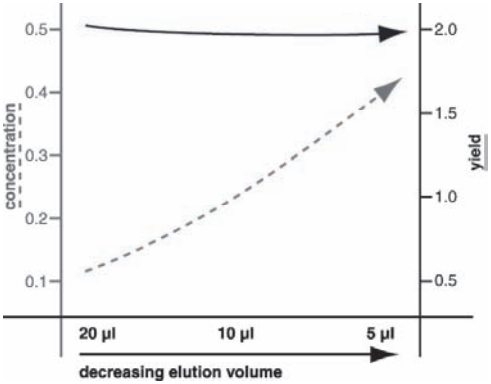


Figure 1: Correlation between elution volume and DNA concentration (NucleoSpin® Plasma XS Columns)

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 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.

* 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 DyNAmo™ Capillary SYBR® Green qPCR Kit (Finnzymes).

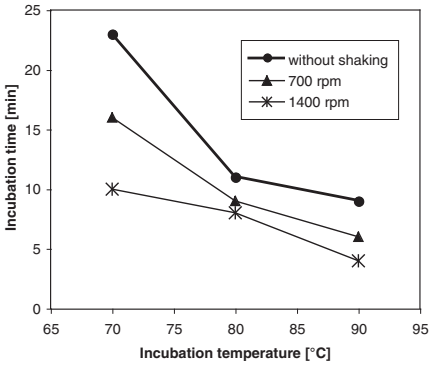


Figure 2: Removal of residual ethanol from the elution fraction by heat treatment.

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.

2.6 Stability of isolated DNA

Due to the typically low DNA content in plasma 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 frozen at -20°C for long term storage.

3 Storage conditions and preparation of working solutions

Attention:

The Buffer BB contains guanidine thiocyanate and ethanol! Wear gloves and goggles!

Storage conditions:

- All kit components can be stored at room temperature (18 – 25°C) and are stable up to 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 any **NucleoSpin® Plasma 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 6 months.

	NucleoSpin® Plasma XS		
	10 preps	**50 preps**	**250 preps**
Cat. No.	**740900.10**	**740900.50**	**740900.250**
Proteinase K (lyophilized)	6 mg Add 260 µl Proteinase Buffer	30 mg Add 1.35 µl Proteinase Buffer	2 x 75 mg Add 3.35 ml Proteinase Buffer to each vial

4 Safety instructions – risk and safety phrases

The following components of the **NucleoSpin® Plasma XS** 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
BB	Guanidine hydrochloride + ethanol <45%	Xn*	Flammable - Harmful by inhalation, in contact with the skin, and if swallowed	R 10-20/21/22	S 7-13-16
WB	Ethanol <60%	F*	Highly flammable	R 11	S 7-16
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

Risk phrases

R 10	Flammable
R 11	Highly flammable
R 20/21/22	Harmful by inhalation, in contact with the skin, and if swallowed
R 36/37/38	Irritating to eyes, respiratory system, and skin
R 42	May cause sensitization by inhalation

Safety phrases

S 7	Keep container tightly closed
S 13	Keep away from food, drink, and animal feedstuffs
S 16	Keep away from sources of ignition - No Smoking!
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

* Hazard labeling not neccessary 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.

5 Protocols

Before starting the preparation:

- Equilibrate sample to room temperature (18 – 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°C – 90°C for final ethanol removal (see section 2.6 for details).

5.1 High Sensitivity protocol for the isolation of DNA from plasma

1 Prepare sample

Add **240 µl plasma** to a microcentrifuge tube (not provided).

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

240 µl plasma

1 a Optional: Proteinase K treatment

*Add **20 µl Proteinase K t**o the plasma sample, mix, and incubate at 37°C for 10 min.*

Depending on the plasma sample and the PCR conditions, the proteinase treatment of the plasma sample provokes a 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 plasma is used, adjust the binding buffer volume accordingly. A ratio of 1:1.5 (v/v) for plasma and binding buffer has to be ensured.

+ 360 µl BB

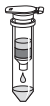
3 Mix sample

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 **(600 µl)** to a **NucleoSpin® Plasma 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 flow-through and place column into new Collection Tube (provided).

30 s
2,000 x *g*

5 s
11,000 x *g*

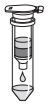
The maximal column volume is approximately 600 µl. Do not apply a higher volume in order to avoid spillage. If larger plasma sample volumes have to be processed, the loading step may be repeated. Be aware of an increased risk of membrane clogging in case of multiple column loading steps. If the solution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

5 Wash and dry silica membrane

1st wash

+ 500 µl WB

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



30 s
11,000 x *g*

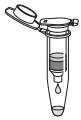
2nd wash

+ 250 µl WB

Add **250 µl Buffer WB** to the NucleoSpin® Plasma XS Column. Centrifuge for **3 min** at **11,000 x *g***. Discard Collection Tube with flow-through and place the column into a 1.5 ml microcentrifuge tube for elution (not provided).

3 min
11,000 x *g*

6 Elute DNA



+ 20 µl
Elution Buffer

Add **20 µl Elution Buffer** to the NucleoSpin® Plasma 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.

30 s
11,000 x *g*

7 Removal of residual ethanol

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

8 min
90°C

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 DNA from plasma

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** to a microcentrifuge tube (not provided).

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

200 µl plasma

2 Adjust DNA binding conditions

Add **300 µl Buffer BB**.

If less than 200 µl plasma is used, adjust the binding buffer volume accordingly. A ratio of 1:1.5 (v/v) for plasma 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.

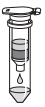
Mix sample

4 Bind DNA

For each sample, load the mixture **(500 µl)** to a **NucleoSpin® Plasma XS Column** placed in a Collection Tube (2 ml).

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

The maximal column volume is approximately 600 µl. Do not apply a higher volume in order to avoid spillage. If larger plasma 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.



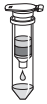
Load lysate

30 s
11,000 x *g*

5 Wash and dry silica membrane

1st wash — **+ 500 µl WB**

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



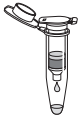
30 s
11,000 x *g*

2nd wash — **+ 250 µl WB**

Add **250 µl Buffer WB** to the NucleoSpin® Plasma XS Column. Centrifuge for **3 min** at **11,000 x *g***. Discard Collection Tube with flow-through and place the column into a 1.5 ml microcentrifuge tube for elution (not provided).

3 min
11,000 x *g*

6 Elute DNA



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

+ 20 µl
Elution Buffer

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.

30 s
11,000 x *g*

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 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 2.2).
Column clogging	*Sample contains residual cell debris or cells* • The plasma sample may have contained residual cells or cell debris. Make sure to use only clear plasma 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 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	Cat. No.	Pack of
NucleoSpin® Plasma XS	740900.10/.50/.250	10/50/250
Collection Tubes (2 ml)	740600	1000

6.3 References

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