Bioanalysis

MACHEREY-NAGEL

User manual

NucleoBond® RS purification workflow

- NucleoBond® RS columns

August 2023 / Rev. 01

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Table of contents

1 Components

1.1 Kit contents

Table 1: NucleoBond® RS columns

REF	Name	Theoretical binding capacity
743502	NucleoBond® RS 10 (5 columns)	up to 10 mg
743503	NucleoBond® RS 50 (1 column)	up to 50 mg
743504	NucleoBond® RS 100 (1 column)	up to 100 mg
743505	NucleoBond® RS 200 (1 column)	up to 200 mg

Every column package includes an insert that guides you to the latest user manual.

1.2 Recommended reagents, consumables and equipment available for purchase from MN

Note: The following reagents are recommended for optimal purification and are available as separate components.

Table 2: Reagents and consumables

REF	Name	Function	Volume
740397	Liquid RNase A	Degradation and removal of RNA (enzyme)	2.5 mL
740505.30	RNase A (lyophilized)		30 mg
740386.1000 / .3000	RES-EF	Resuspension buffer	1000 mL / 3000 mL
740387.1000 / .3000	LYS-EF	Lysis buffer	1000 mL / 3000 mL
740388.1000 / .3000	NEU-EF	Neutralization buffer	1000 mL / 3000 mL
740380.1000 / .3000	EQ-EF	Equilibration buffer	1000 mL / 3000 mL
740381.1000 / .3000	ENTO-EF	Endotoxin removal buffer	1000 mL / 3000 mL
740392.1000 / .3000	WASH-EF	General washing buffer	1000 mL / 3000 mL
740393.900 / .3000	ELU-EF	Elution buffer	900 mL / 3000 mL
740797.1 (optional)	TE-EF	Endotoxin-free TE buffer	1000 mL
740798.1 (optional)	H_2O-EF	Endotoxin-free water	1000 mL
740553.5 (recommended)	Bottle Top Filter Type 2	Lysate filtration	5 pieces
740641 (recommended)	NucleoVac 96 Vacuum Regulator	Vacuum manifold for controlling of vacuum	1 piece

1.2.1 Quantities of consumables required per preparation

The amount of buffer required for a preparation and the number of preparations per bottle of buffer are listed in the following tables. These tables are intended only as a rough guideline for the minimum amount of reagents that is required. Depending on culture characteristics and experimental setup (e.g. dead volume of tubing), a larger amount may be required.

Table 3: Required buffers in terms of possible preparations per bottle, as well as other necessary consumables for **NucleoBond® RS 10 column**.

Table 3: Required quantities of consumables for NucleoBond® RS 10 column

Buffer name	REF	Container volume	Required volume per preparation	Theoretical number of preps	Typical number of preps
RES-EF	740386.1000	1 L	60 mL	16.7	15
	740386.3000	3 L		50	46
LYS-EF	740387.1000	1 L	60 mL	16.7	15
	740387.3000	3 L		50	46
NEU-EF	740388.1000	1 L	60 mL	16.7	15
	740388.3000	3 L		50	46
EQ-EF	740380.1000	1 L	30 mL	33.3	28
	740380.3000	3 L		100	85
ENTO-EF	740381.1000	1 L	30 mL	33.3	28
	740381.3000	3 L		100	85
WASH-EF	740392.1000	1 L	30 mL	33.3	28
	740392.3000	3 L		100	85
ELU-EF	740393.900	0.9 L	30 mL	30	25
	740393.3000	3 L		100	85
TE-EF (optional)	740797.1	1 L	10 mL	100	95
H_2O-EF (optional)	740798.1	1 L		100	95
Bottle Top Filter Type 2 (recommended)	740553.5	5 pieces	1	5	5

Table 4: Required buffers in terms of possible preparations per bottle, as well as other necessary consumables for **NucleoBond® RS 50 column**.

Table 4: Required quantities of consumables for NucleoBond® RS 50 column

Buffer name	REF	Container volume	Required volume per preparation	Theoretical number of preps	Typical number of preps
RES-EF	740386.1000	1 L	300 mL	3.3	3
	740386.3000	3 L		10.0	9
LYS-EF	740387.1000	1 L	300 mL	3.3	3
	740387.3000	3 L		10.0	9
NEU-EF	740388.1000	1 L	300 mL	3.3	3
	740388.3000	3 L		10.0	9
EQ-EF	740380.1000	1 L	120 mL	8.3	8
	740380.3000	3 L		25.0	24
ENTO-EF	740381.1000	1 L	150 mL	6.7	6
	740381.3000	3 L		20.0	19
WASH-EF	740392.1000	1 L	150 mL	6.7	6
	740392.3000	3 L		20.0	19
ELU-EF	740393.900	0.9 L	120 mL	7.5	7
	740393.3000	3 L		25.0	24
TE-EF (optional)	740797.1	1 L	50 mL	20	19
H_2O-EF (optional)	740798.1	1 L		20	19
Bottle Top Filter Type 2 (recommended)	740553.5	5 pieces	2	2,5	2

Table 5: Required buffers in terms of possible preparations per bottle for **NucleoBond® RS 100 column**.

Table 5: Required quantities of consumables for NucleoBond® RS 100 column

Buffer name	REF	Container volume	Required volume per preparation	Theoretical number of preps	Typical number of preps
RES-EF	740386.1000	1 L	600 mL	1.7	1
	740386.3000	3 L		5	4
LYS-EF	740387.1000	1 L	600 mL	1.7	1
	740387.3000	3 L		5	4
NEU-EF	740388.1000	1 L	600 mL	1.7	1
	740388.3000	3 L		5	4
EQ-EF	740380.1000	1 L	250 mL	4	3
	740380.3000	3 L		12	11
ENTO-EF	740381.1000	1 L	300 mL	3.3	3
	740381.3000	3 L		10	9
WASH-EF	740392.1000	1 L	300 mL	3.3	3
	740392.3000	3 L		10	9
ELU-EF	740393.900	0.9 L	250 mL	3.6	3
	740393.3000	3 L		12	11
TE-EF (optional)	740797.1	1 L	100 mL	10	9
H_2O-EF (optional)	740798.1	1 L		10	9

Table 6: Required buffers in terms of possible preparations per bottle for **NucleoBond® RS 200 column**.

Table 6: Required quantities of consumables for NucleoBond® RS 200 column

Buffer name	REF	Container volume	Required volume per preparation	Theoretical number of preps	Typical number of preps
RES-EF	740386.1000	1 L	1200 mL	0.8	-
	740386.3000	3 L		2.5	2
LYS-EF	740387.1000	1 L	1200 mL	0.8	-
	740387.3000	3 L		2.5	2
NEU-EF	740388.1000	1 L	1200 mL	0.8	-
	740388.3000	3 L		2.5	2
EQ-EF	740380.1000	1 L	400 mL	2.5	2
	740380.3000	3 L		7.5	7
ENTO-EF	740381.1000	1 L	550 mL	1.8	1
	740381.3000	3 L		5.5	5
WASH-EF	740392.1000	1 L	550 mL	1.8	1
	740392.3000	3 L		5.5	5
ELU-EF	740393.900	0.9 L	400 mL	2.25	2
	740393.3000	3 L		7.5	7
TE-EF (optional)	740797.1	1 L	200 mL	5	4
H_2O-EF (optional)	740798.1	1 L		5	4

Table 7: Required amounts of RNase for Addition to Resuspension Buffer RES-EF.Either Liquid RNase A or RNase A (lyophilized) required.

Table 7: Required quantities of RNase A for correct supplementation of RES-EF

REF	Buffer name	Container Volume	Required amounts per L RES-EF	Required amounts per 3 L RES-EF
740397	Liquid RNase A	100 mg/mL in 2.5 mL	0.24 vials or 0.6 mL	0.72 vials or 1.8 mL
740505.30	RNase A (lyophilized)	60 mg	2	6

1.3 Recommended reagents, consumables and equipment to be supplied by user

1.3.1 Reagents

- Isopropanol (room-temperatured) , ACS or molecular biology grade
- 96 – 100 % ethanol (room-temperatured), non-denatured, ACS or molecular biology grade

Minimum amount of reagents and consumables required per preparation

Name	RS 10	RS 50	RS 100	RS 200
Isopropanol	21 mL	84 mL	175 mL	280 mL
Ethanol	5 mL	15 mL	25 mL	50 mL

1.3.2 Equipment and consumables

Peristaltic pump with a pump head, suitable for tubing with a tubing wall thickness of 1.6 mm.

- Flow rate: Minimum up to 10 mL/min (1.6 mm inner diameter tubing) and minimum up to 20 mL/min (3.1 mm inner diameter tubing).
- Pressure: up to 3.5 kg / cm^2 (50 psi) , typical 18 psi.
- Examples of tested pump and pump head combinations:
  - Lead Fluid BT100 L + YT15
  - Integra Biosciences DOSE IT Peristaltic Pump
  - Heidolph HeiFLOW Precision 01 + SP quick (SWS 1,6)

Alternatively, a FPLC or HPLC system can be used, e.g. ÄKTApure™ system, provided the lysate is already clarified. The specifications remain unchanged as for the peristaltic pump. A flow rate of at least 10 mL/min and a maximal backpressure of 100 psi are desirable.

- Recommended: PharMed BPT tubing (Sain-Gobain or similar)
  - Recommended tubing material is made of TPE, is pyrogen-free and sterile. Depending on the application, verify that the tubing meets required regulatory compliances e.g. FDA and USP criteria.
- Tubing inner diameter: 1.6 mm (1/16") for NucleoBond® RS columns.
- Tubing inner diameter: 3.1 mm (1/8") for filtration cartridges.
- Tubing wall thickness must match the requirements of the pump head. A 1.6 mm tubing wall thickness is recommended.
- If an FPLC or HPLC system is used, please use suitable supported tubing.

Lysate clarification :

After alkaline lysis, the lysate must be clarified to remove cell debris and other solid particles to prevent clogging of the column. This can be done in several ways, two of which are described in this manual. Ultimately, it is important to achieve a clarification efficiency of sterile filtration with a pore size of at least 0.45 µm, while keeping shear forces as low as possible to avoid damaging the plasmid DNA and thereby compromising DNA integrity.

- Option 1 - Vacuum filtration setup:
  Use the vacuum operated NucleoBond® Bottle Top Filter Type 2 (REF 740553.5) to filter the lysate. The **NucleoBond® Bottle Top Filter Type 2** are specifically designed to make the separation of the bacterial lysate and SDS precipitate easy, fast, and convenient. In addition, the use of a Bottle Top Filter eliminates the time-consuming centrifugation step for lysate clarification. Attach the Bottle Top Filter to a suitable flask (e.g. Schott), add the bacterial lysate (up to 1,000 mL flow-through recommended) and apply vacuum. After 15 minutes, the solution will have passed through. Load the clear lysate to the NucleoBond® RS column and discard the Bottle Top Filter.
- Option 2 - Filter cartridges for in-line filtration with peristaltic pump:
  Filter cartridges can be used for a stepwise filtration or clarification of the lysate. The lysate is successively filtered at decreasing pore sizes in order to avoid the risk of clogging. At least one filtration step using 0.45 µm pore size is needed for reliable plasmid purification step. It is recommended to use filter capsules with a pore size of 20, 5 and 0.45 µm, which

are labeled as pyrogen-free (rec. meeting regulatory compliance USP < 85 > Bacterial Endotoxin Test) in order to minimize the entry of contamination and therefore high endotoxin levels. Examples of manufacturers of capsule filters suitable for biological and bioprocess applications including 3M, Cobetter, International Filter Products, Saint-Gobain and Whatman, among others.

Below are examples of 20 µm polypropylene filter capsules with increasing capacity:

- Cobetter Filtration Cat. No.: C01TTPFSA22000A1M
- Cobetter Filtration Cat. No.: L05TTCHT2XA1
- Cobetter Filtration Cat. No.: L10TTCHT2XA1

The following are examples of 5 µm fiberglass filters with increasing capacity:

- Cobetter Filtration Cat. No.: KZ50LGFP0500M
- Cobetter Filtration Cat. No.: C02KKCHV50A1
- Cobetter Filtration Cat. No.: L02KKCHV50A1

The following are examples of 0.45 – 0.22 µm PES filter capsules with increasing capacity:

- Cobetter Filtration Cat. No.: C01TTDPSHSL4522A1M
- Cobetter Filtration Cat. No.: L05TTDPSHSL4522A1
- Cobetter Filtration Cat. No.: L10TTDPSHSL4522A

Additional equipment needed for inline-filtration with listed filtration cartridges:

- Tri-Clamp, Mini Sanitary Flange (for use with filtration capsules described above, other capsules may require a different connection)
- 3/4” Tri-Clamp x 1/8” Hose Barb (for use with the above described filtration capsules, other capsules might need a different clamp)
- Sanitary gaskets (silicone)

Connectors, supplementary material for preparation on a peristaltic pump:

- Barbed Male Luer (Lock) to tubing connectors 1/16” and 1/8”
- Laboratory stand

Connectors, supplementary material for use on a liquid chromatography system (e.g. ÄKTA™):

Please note that the NucleoBond® RS column connectors consist of a 4.26 mm female luer connector and a Luerlock male connector. For instance, the following connections can be used to operate an ÄKTA™ system:

- 1/16 male connector
- Luerlock male to 1/16 female
- Union luerlock female to 1/16 female

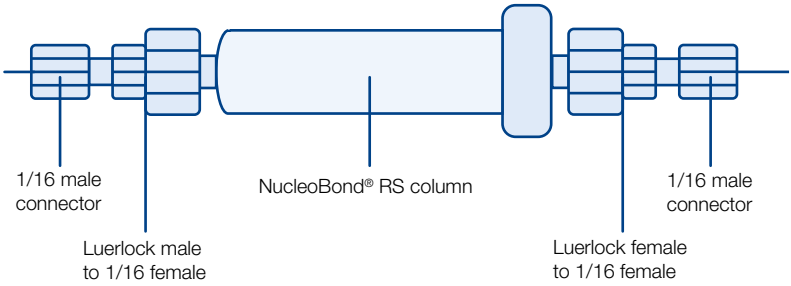

Figure 1 Exemplary setup of a NucleoBond® RS column attached to an ÄKTA™ system

- Waste containers for flow-through
- Endotoxin-free containers or vessels for the collection of filtrates and eluates
- Pyrogen-free or endotoxin-free plastic or glassware and pipette tips. If glassware is to be used, heat overnight at 180 °C to destroy endotoxins. Autoclaving does not inactivate endotoxins and is not recommended if the autoclave is also used to inactivate bacterial cultures.
- Wet ice or refrigerator

2 Kit specifications

NucleoBond® RS columns are designed for large-scale isolation of plasmid DNA from bacterial cultures using peristaltic pumps or FPLC systems. In combination with the optimized NucleoBond® buffer chemistry, resembling the NucleoBond® RS purification workflow the NucleoBond® RS columns are able to achieve very low endotoxin levels. After two efficient washing steps removing endotoxins and other contaminants, the plasmid DNA is eluted, precipitated, and easily reconstituted in H_2O-EF, TE-EF, or any other suitable endotoxin-free buffer for further use.

- All **NucleoBond® RS columns** are resistant to organic solvents such as alcohol, chloroform & phenol and are suitable for buffers containing denaturing agents such as formamide, urea, or detergents such as Triton X-100 or NP-40.
- **The resin of all NucleoBond® RS columns** can be used over a wide pH range (pH 2.5 – 9.0), and can remain in contact with buffers for several hours without changing its chromatographic properties.

Table 8: NucleoBond® RS column specification in combination with the NucleoBond® buffers

Parameter	Method	Criterion
Integrity	AGE	≥ 90 % ccc
Purity	UV spectrum	$A_{260/280}$ 1.8 – 2.0 $A_{260/230}$ 1.9 – 2.3
Endotoxin	LAL, rFC	≤ 0.01 EU / µg
Host protein	BCA	≤ 1 %
Host DNA	qPCR	≤ 1 %
RNA	AGE	Not visible at 200 ng DNA
	Fluorometric Quantification	≤ 2 %

Table 9: NucleoBond® RS column binding capacity

REF	Name	Theoretical binding capacity	ODV_{max}	Cell wet $weight_{max}$
743502	NucleoBond® RS 10 (5 columns)	up to 10 mg	3,000	8 g
743503	NucleoBond® RS 50 (1 column)	up to 50 mg	15,000	40 g
743504	NucleoBond® RS 100 (1 column)	up to 100 mg	30,000	80 g
743505	NucleoBond® RS 200 (1 column)	up to 200 mg	54,000	145 g

Note: Compatibility with other buffers, than the optimized NucleoBond® RS buffers has not been tested.

3 About this user manual

General information about endotoxins can be found in section 4. A detailed description of the NucleoBond® RS purification workflow and important information about cell growth, cell lysis and subsequent purification steps are provided in section 5.

Section 6 provides information on storage and buffer preparation, and section 7 provides information on safety instructions and disposal of the kit.

First-time users are strongly advised to read these sections thoroughly before using this kit. Experienced users may proceed directly to the purification protocols (section 8) or use the Protocol at a Glance for a quick reference.

It is recommended to read the instructions of this user manual carefully before use. All technical literature is also available on the Internet at ***www.mn-net.com***.

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

4 Endotoxins

4.1 Quantification of endotoxins

Endotoxins can be measured in several highly sensitive assays and are expressed in endotoxin units (EU). Endotoxin levels in plasmid DNA are usually expressed as EU per µg DNA. FDA has approved several commercially available endotoxin measurment assays. These include the gel clot, turbidimetric, colorimetric, chromogenic, and recombinant factor C (rFC) assays.

Several classes of substances and factors, such as chelating agents, divalent cations, proteins, lipids, denaturing agents, pH shifts or viscosity can affect endotoxin quantification. Some samples may interfere with the assay's ability to react with endotoxins due to a variety of chemical or physical factors. Chemical inhibition can be caused by chelation (e.g. EDTA), protein denaturation (e.g. fluorescein) or pH interference (e.g. pH outside the test range). Inhibition can be prevented by diluting the sample to the Maximum Valid Dilution (MVD) or Maximum Valid Concentration (MVC) of the endotoxin assay used.

4.2 Endotoxin limits for applications

Endotoxins are released from cells in small amounts during cell growth and in very large amounts during cell death and lysis, thus also during plasmid purification. Like intact cells the free lipopolysaccharide (LPS) molecules induce inflammatory reactions of the mammalian immune system. Therefore, removal from plasmid preparations to ensure high transfection rates and high viability of transfected cells is crucial. Due to their amphiphilic nature and their negative charge LPS behave like DNA and are co-purified with most common plasmid purification systems.

Depending on the downstream application, different amounts of endotoxins are still adequate. For molecular biology applications, standard plasmid isolation kits with > 10 EU/µg are reported to be suitable. For standard transfection methods endotoxin levels of 0.1 – 1.0 EU/µg are typically suitable, and for highly sensitive applications, endotoxin levels of ≤ 0.1 EU/µg, often referred as "endotoxin-free", are required. Even lower levels of endotoxin are required for pharmaceutical applications.

The NucleoBond® RS workflow is capable of achieving endotoxin levels of ≤ 0.01 EU/µg DNA, facilitating compliance with such requirements.

4.3 Endotoxin free working environment

Endotoxins can be found almost in all places, so endotoxin-free operation might represent a challenge. Beside to the microbiological culture itself, some typical sources of endotoxin contamination can be packaging components, raw materials or equipment used in product preparation. In addition, water used as a solvent or in processing and chemicals, can be contaminated with endotoxins. Control of microbiological and endotoxin contamination in the above potential sources, may be included in good practice.

Removing endotoxins from products is a labor-intensive process. Therefore, it is preferable to prevent contamination by keeping all components free of endotoxins rather than removing endotoxins once they are present. Be sure to use only endotoxin-free consumables, reagents, and glassware. Always wear gloves and handle equipment only when necessary. Endotoxin-free conditions can be easily lost due to poor handling, endotoxin-contaminated water and other materials.

If glassware is used, it can be reliably depyrogenated by dry heat sterilization. This is done by treating the glassware at 250 °C for 30 minutes or at 180 °C for 3 hours. Autoclaving does not inactivate endotoxins and is not recommended if the autoclave is also used to inactivate bacterial cultures. Another way to remove endotoxins from solid components is to rinse them with pyrogen-free water. It is possible to use oxidizing solutions and strong alkalis, again followed by rinsing with pyrogen-free water as well. Methods such as filtration, irradiation or ethylene oxide treatment are only moderately successful in reducing endotoxins.

Ensure that any plastic items used are pyrogen-free and check their suitability for the buffers. Components from the containers may leach into the buffers and interfere with the endotoxin tests, resulting in false positive or false negative results.

If compliance with Good Manufacturing Practice (GMP) is desired, there are further suggestions described by the EMA in the Guidelines on GMP specific to Advanced Therapy Medicinal Products (9.4 Prevention of cross-contamination in production).

Before starting the plasmid purification process, it is essential to ensure that the work area and equipment are clean and free from unnecessary materials to prevent mix-ups and to protect products from microbial and other contaminants, such as endotoxins. If necessary, to safeguard columns, solutions and buffers from contamination risks, take appropriate measures, such as segregated premises, dedicating specific production areas for different products, using closed systems, air-locks, and single use technologies. Employ appropriate cleaning procedures tailored to the purification/manufacturing process, with regular cleaning between batches.

5 NucleoBond® RS purification workflow

5.1 Schematic setup and procedure of NucleoBond® RS columns

Ideally, cultivation up to lysate clarification should be physically separated from plasmid isolation on the **NucleoBond® RS column** to minimize the risk of subsequent endotoxin contamination of the product. If this is not possible, pay particular attention to the recommendations for working in an endotoxin-free environment in section 4.3. The **NucleoBond® RS column** is mounted on a clamp that is attached to a laboratory stand. Adjust the height of the column as required.

An illustrative setup for clarifying crude lysate utilizing the NucleoBond® Bottle Top Filter Type 2 on a vacuum manifold is highlighted in Figure 2. The crude lysate can be conveniently emptied into the Bottle Top Filter. For easy transfer by pumping or simply pouring, the crude lysate can be cautiously agitated just before transfer.

A comparable method can be utilized by employing a peristaltic pump and capsule filters as illustrated in Figure 4. It is advised not to agitate the lysate during this process, and only load debris on the filter at the very end.

The NucleoBond® RS column setup for preparative scale is shown in Figure 3. The column is oriented vertically with a flow direction from top to bottom.

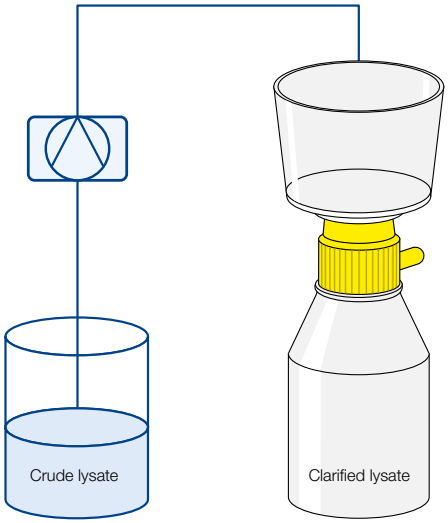

Figure 2 NucleoBond® RS Bottle Top Filter Type 2 setup

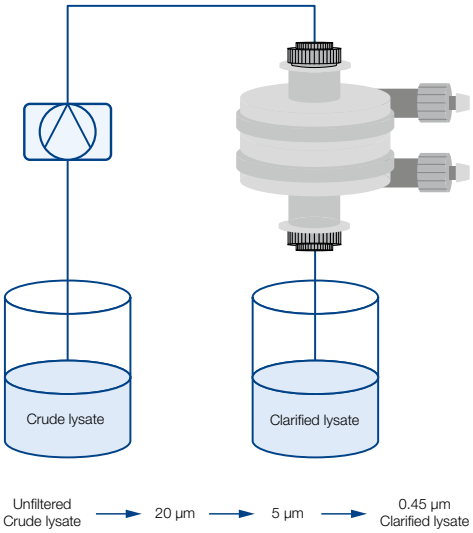

Figure 4 NucleoBond® RS capsule filter setup

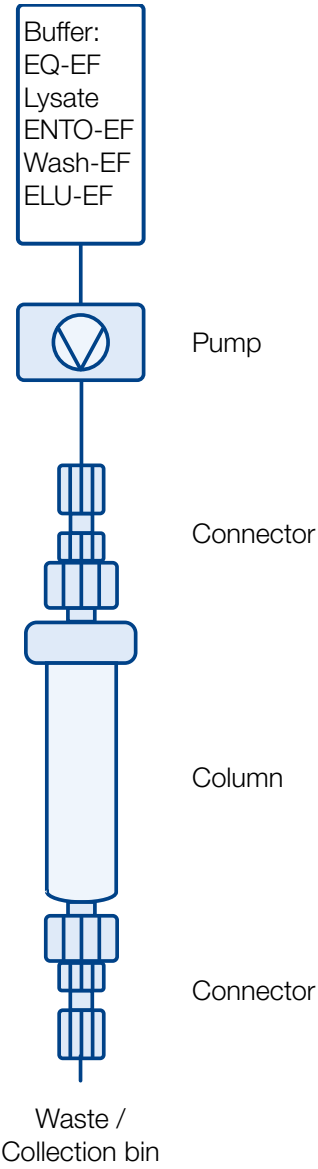

Figure 3 NucleoBond® RS preparative scale setup

The **NucleoBond® RS purification workflow** can be roughly divided into 11 steps. Cell Cultivation, Harvesting, Resuspension, Lysis, Neutralization, Lysate clarification, Column Equilibration, Lysate Binding, Wash 1, Wash 2 and Elution. The main steps affecting the product purification process are shown in the flow chart below.

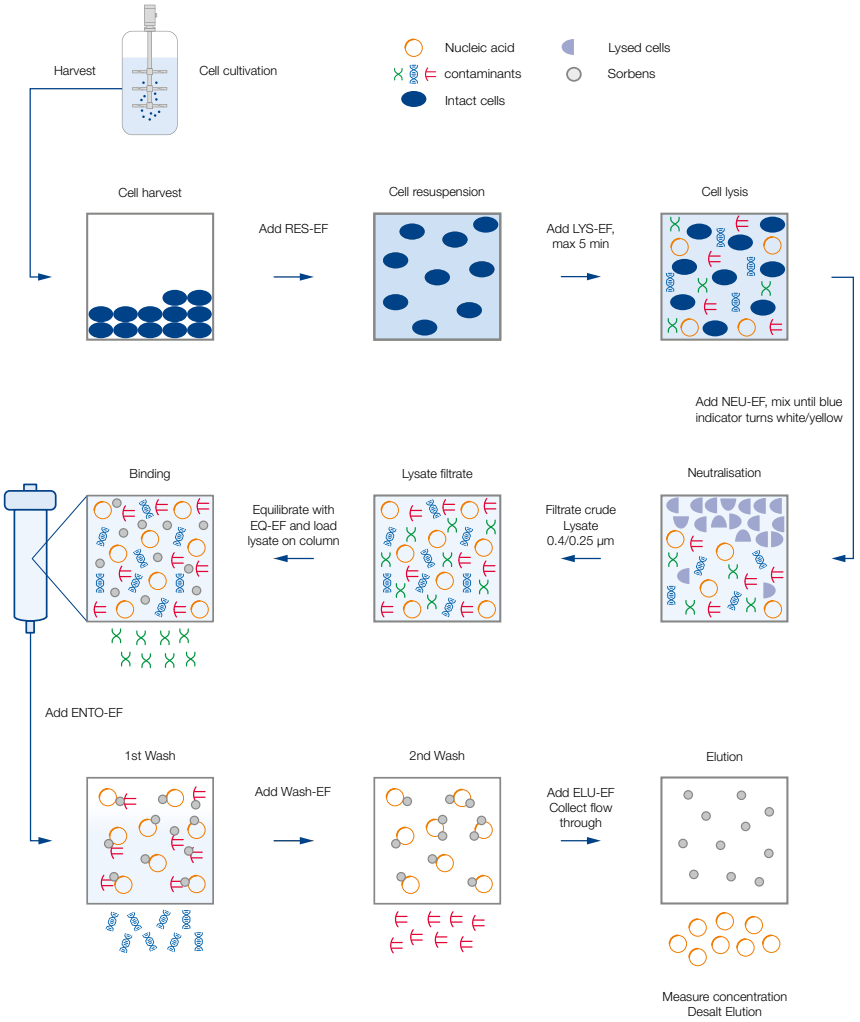

Figure 5 Scheme of the purification workflow of the NucleoBond® RS columns.

5.2 Growth of bacterial cultures

The yield and quality of plasmid DNA is highly dependent on the **type of culture media** and antibiotics, the bacterial host strain, the plasmid type, size, copy number and the **growth conditions**.

Overgrowth of a culture may lead to a higher percentage of dead or starving cells, resulting in plasmid DNA that is partially degraded or contaminated with chromosomal DNA. To determine the optimal culture conditions, culture media and incubation times must be optimized for each host strain/plasmid construct combination individually.

Bacterial cell cultures should always be grown under **antibiotic selection** at all times to ensure plasmid propagation. Without this selective pressure, cells tend to lose plasmid copies during cell division. Since bacteria grow much faster without the burden of a high-copy plasmid, they take over the culture quickly and the plasmid yield decreases regardless of the cell mass. In addition, the ***E. coli* host strain** and the **type of plasmid**, especially the **size and the origin of replication (ori)** have a critical effect on plasmid DNA yield.

Therefore, all of the above mentioned factors should be considered if a specific plasmid DNA yield needs to be achieved. Culture volumes and lysis procedures must be adjusted accordingly.

5.3 Culture volume for high-copy plasmids

Due to the influence of growth media, growth conditions (e.g. agitation speed, aeration, temperature), host strain, type of plasmid insert, etc., the final amount of cells in a bacterial culture can vary over a wide range. As a rule of thumb, 1 liter of *E. coli* culture with an OD_{600} of 1 consists of 1×10^{12} cells and yields approximately 1.5 – 1.8 g cell wet weight. Overnight cultures grown in LB medium typically reach an OD_{600} of 3 – 6 under vigorous shaking in flasks. Fermentation cultures typically reach an OD_{600} of 20 and higher. The expected DNA yield for a high copy plasmid can be approximately 1 mg per gram wet cell weight.

Therefore, it is important to **adjust the cell mass rather than the culture volume** in order to achieve the best plasmid purification results. **Using rich growth media without checking the cell amount can easily lead to overloading the column and thereby, loss of plasmid DNA quality and yield.** Since the cell mass or wet cell weight is tedious to determine, it has been replaced in this manual by the mathematical product of optical density at 600 nm (OD_{600}) and culture volume (Vc) – two variables that are much easier to measure.

$$\mathbf{ODV = OD_{600} \times V_c\ [mL]}$$

Note that for a correct OD determination the culture samples must be diluted when OD_{600} exceeds 0.5 in order to increase proportionally with cell mass. For a well grown *E. coli* culture a 1 :10 or 1:20 dilution with fresh culture medium is recommended. The measured OD_{600} is then multiplied by the dilution factor 10 / 20 to obtain a theoretical OD_{600} value. This OD_{600} is used in Table 10 to determine the appropriate culture volume. Table 10 shows the recommended ODVs and the corresponding pairs of OD_{600} and culture volume that can be easily handled using the given **NucleoBond® RS purification workflow**. For example, if the OD_{600} of your *E. coli* culture is 6, use 500 mL culture volume in combination with a **NucleoBond® RS 10** or 5 L culture volume in combination with a **NucleoBond® RS 100** preparation.

Table 10: Recommended culture volumes for high-copy plasmids

NucleoBond® RS column	Pellet wet weight	Rec. ODV	OD_{600} = 2	OD_{600} = 4	OD_{600} = 6	OD_{600} = 8	OD_{600} = 10
RS 10	8 g	3,000	1.5 L	0.75 L	0.5 L	0.375 L	0.3 L
RS 50	40 g	15,000	7.5 L	3.75 L	2.5 L	1.875 L	1.5 L
RS 100	80 g	30,000	15 L	7.5 L	5 L	3.75 L	3.0 L
RS 200	145 g	54,000	27 L	13.5 L	9 L	6.75 L	5.4 L

5.4 Cell lysis

The bacterial cell pellet is vigorously resuspended in Buffer RES-EF, supplemented with RNase A, and carefully lysed with Buffer LYS-EF. Addition of Buffer LYS-EF is performed by gently inverting the flask 6 – 10 times until the mixture appears uniformly blue discolored. Proteins, as well as chromosomal and plasmid DNA will be denatured under these conditions. RNA is degraded by DNase-free RNase A. Do not stir the resulting lysate since this may release contaminating chromosomal DNA from debris into the suspension. Neutralization Buffer NEU-EF is then added to the lysate, causing the neutralization of the lysate and precipitation of proteins, chromosomal DNA, and other cellular debris. This process is enhanced by cooling on ice. Plasmid DNA can revert to its native supercoiled structure and remain in solution.

The **NucleoBond® RS purification workflow** has been adapted to ensure optimal lysis for culture volumes, appropriate for high copy plasmids according to section 5.3, Table 10. Using too much cell material will result in inefficient cell lysis and precipitation, potentially causing reduced plasmid yields and purity. Therefore, lysis buffer volumes should be increased when applying larger culture volumes, e.g. low-copy plasmid purification.

As a rule of thumb, calculate the minimum required buffer volumes for RES-EF, LYS-EF, and NEU-EF as follows:

$$\text{Buffer volume [mL]} = V_c \text{ [mL]} \times OD_{600} / 50$$

For example, if 500 mL of a high copy bacterial culture (OD_{600} = 6) is to be lysed, the appropriate volumes of buffers RES-EF, LYS-EF, and NEU-EF are 60 mL each. By using sufficient amounts of lysis buffer, the lysis time can be limited to 4 minutes and should not exceed 5 minutes. Prolonged exposure to alkaline conditions may cause irreversible denaturation and degradation of plasmid DNA and releases contaminating chromosomal DNA into the lysate.

5.5 Lysate neutralization and LyseControl

Proper mixing of the lysate with Neutralization Buffer NEU-EF is of utmost importance for complete precipitation of SDS, protein, and genomic DNA. Incomplete neutralization will result in reduced yields. However, released plasmid DNA is very vulnerable at this point and shaking too long or too strongly will damage the DNA.

Therefore, **do not vortex or shake** vigorously but **gently shake or invert the flask 6 – 10 times,** until a fluffy off-white precipitate has formed and the **LyseControl** has turned **colorless** throughout the lysate without any trace of blue color.

5.6 Lysate clarification

After the alkaline lysis, the sample must be cleared from cell debris and precipitates to ensure high plasmid purity and a fast column flow rate. This can be achieved by using our **NucleoBond® Bottle Top Filter Type 2**, a stepwise sterile filtration with capsule filters, or any other filtration method that results in a filtration performance equivalent to a pore size of at least 0.45 µm.

A gentle filtration procedure is recommended in order to minimize shear forces acting on the vulnerable plasmid DNA.

Instead of centrifugation or stepwise filtration, the modified **NucleoBond® Bottle Top Filter Type 2** can be used as a single-step filtration to obtain ready-to-use sterile filtered lysate, without excessive effort. The filter is designed for fast passage of the crude lysate through the filter, allowing lysate volumes of up to 1,000 mL to be loaded without the risk of filter breakage or clogging.

Note: To utilize the full capacity of the filter, ensure that cell debris does not reach the bottom of the filter by continuously adding lysate to the top of the filter. If the floating cell debris does reach the bottom of the filter, the filter will clog considerably faster, resulting in a reduced flow rate and total throughput volume.

After clarification, the lysate can be loaded onto the **NucleoBond® RS column** without the risk of a gradual decrease in flow rate or column blockage. However, the maximum recommended flow rate of the column should be respected, especially during the binding step.

Many filters can be pre-wetted to reduce lysate loss and allow for bacterial lysate clarification. To further reduce lysate loss and extend filter life, the crude lysate can be centrifuged prior to filtration. The resulting supernatant is then applied to the filter. In case of purification of large DNA constructs such as PACs or BACs centrifugation should be avoided in order to reduce shear forces.

5.7 Washing of the column

The high salt concentration of the lysate prevents proteins and RNA from binding to the **NucleoBond® RS column**. However, to remove all traces of contaminants such as endotoxins and proteins, it is essential to wash the column in two subsequent wash steps (endotoxin wash with **Buffer ENTO-EF** and general wash with **Buffer WASH-EF**). This ensures the highest yield with the highest attainable purity.

5.8 Elution and concentration of plasmid DNA

Elution is performed under high salt conditions and by shifting the pH from 7.0 to 9.0. Under these alkaline conditions the positive charge of the anion exchange resin is neutralized and the plasmid DNA is released. For most subsequent application, it is necessary to precipitate the DNA and remove salt and any traces of alcohol. Failure to remove will result in interference or inhibition of these applications.

All **NucleoBond® RS column** eluates already contain sufficient salt for isopropanol precipitation of DNA. Therefore, the precipitation can be performed by direct addition of 0.7 volume of isopropanol. To prevent co-precipitation of salt, use only r**oom-temperature isopropanol** and do not add the plasmid DNA solution into a vial containing isopropanol. Instead, **add isopropanol to the final eluate and mix immediately**. Follow the steps 14 – 16 in the **NucleoBond® RS** purification workflow for desalting and concentration in section 8.2. Reconstitution of pure DNA is performed using H_2O-EF or slightly alkaline low salt buffer like TE-EF. Do not use pure water unless pH is definitely higher than 7.0.

5.9 Determination of DNA yield and quality

The **yield** of a plasmid preparation should be estimated before and after isopropanol precipitation in order to calculate the recovery after precipitation and to estimate the optimal reconstitution volume of the plasmid DNA. Simply use Elution Buffer ELU-EF or H_2O-EF / TE-EF as a blank in your photometric measurement.

The nucleic acid **concentration** of the sample can be calculated from its UV absorbance at 260 nm, where an absorbance of 1 (1 cm path length) corresponds to 50 µg DNA/mL. Note that the absolute measured absorbance should be between 0.1 and 0.7 to be in the linear part of the Lambert-Beer law. If necessary, dilute your sample in the appropriate buffer.

The plasmid **purity** can be checked by UV spectroscopy as well. An A_{260}/A_{280} ratio between 1.80 – 2.00 and an A_{260}/A_{230} ratio around 2.0 indicates pure plasmid DNA. An A_{260}/A_{280} ratio above 2.0 is a sign for too much RNA in your preparation, an A_{260}/A_{280} ratio below 1.8 indicates protein contamination.

Bacterial endotoxin testing can be performed with several commercially available tests such as chromogenic, turbidimetric and rFC assays kits.

5.10 Convenient stopping points

Cell pellets can easily be stored at -20 °C for several months.

Cleared lysates can be stored on ice or at 4 °C for several days.

For optimal performance the column purification should not be interrupted. However, the buffer flow through can be paused for several hours as the column will not run dry. This may result in a small loss of DNA yield.

The eluate can be stored at 4 °C for several days. Note that the eluate should be warmed to room temperature before precipitating the DNA. This will prevent co-precipitation of salt.

6 Storage conditions and preparation of working solutions

All components can be stored at 15 – 25 °C and are stable until: see component/package label.

Storage of Buffer LYS-EF below 20 °C may cause precipitation of SDS. If salt precipitate is observed, incubate the buffer at 30 – 40 °C for several minutes and mix well until all precipitate is completely redissolved. Allow to cool to room temperature before use.

Before using of the **NucleoBond® RS column,** prepare the following:

- Dissolve two vials of 30 mg lyophilized RNase A by adding of 1 mL of Buffer RES-EF. Wearing gloves is recommended. Pipette up and down until the RNase A is completely dissolved
- Transfer two vials of reconstituted RNase vials per 1 liter of RES-EF and mix well (final concentration 60 µg/mL). Record the date of RNase A addition. Store Buffer RES-EF with RNase A at 4 °C. The solution will be stable for at least 12 months at this temperature. *Alternatively, add 0.6 mL of Liquid RNase per 1 L of Buffer RES-EF. Save the remaining Liquid RNase for use in the next liter of the RES-EF buffer.*
- Prepare a 70 % ethanol solution with 96 – 100 % ethanol and endotoxin-free water for isopropanol precipitation.

7 Safety instructions

Wear appropriate protective clothing (e.g., lab coat, disposable gloves, and safety glasses) when working with the **NucleoBond® RS columns**. For more information, refer to the appropriate Material Safety Data Sheet (MSDS available online at ***www.mn-net.com/msds***).

NucleoBond® RS column waste has not been tested for residual infectious material. Contamination of liquid waste with residual infectious material is highly unlikely due to strong denaturing lysis buffer treatment, but it cannot be completely excluded. Therefore, liquid waste must be considered infectious and should be handled and disposed of according to local safety regulations.

7.1 Disposal

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

8 Protocol

8.1 Analytical check with NucleoSpin® Plasmid, Mini kit

Before starting the **NucleoBond® RS purification workflow**, it is recommended to check the cultivated cell material (yield & integrity) by purifying the plasmid DNA from 1 – 2 mL of culture with a **NucleoSpin® Plasmid, Mini** kit (not supplied). If 60 µg of plasmid DNA is obtained from 1 – 2 mL of fermentation broth, the **NucleoSpin® Plasmid Mini column** is overloaded. Dilute the fermentation broth or reduce the volume to within the spin column's specifications. If larger culture volumes (10 – 20 mL) are wished to be processed for the analytical check, the **NucleoBond® Xtra Midi** kit (REF 740410.50) is a suitable alternative.

The yield determined in the analytical check can be used to calculate the amount of fermentation broth required to optimize the utilization of the **NucleoBond® RS column**. The amount of broth used must be adjusted according to the maximum pellet weight and the maximum binding capacity of each column (see section 5.3).

Use the QR codes below to access additional information about the **NucleoSpin® Plasmid Mini** Kit (REF 740588.10/.50/.250). Follow the instructions of the attached protocol.

QR-Code product website

qr.mn-net.com/qr/(241)740588

QR-Code user manual

qr.mn-net.com/qr/(IFU)740588

8.2 NucleoBond® RS purification workflow

The following section contains detailed protocols for high copy plasmid purification.

The following **NucleoBond® RS purification workflow** is based on the use of 0,3 – 9 liters of Circlegrow culture (ODV 3.000 – 54.000), equivalent to up to 145 g of bacterial cells. **NucleoBond® RS columns** are intended for single use only. The pressure within the column should not exceed 7 kg/cm^2 (100 psi) during use. Use only oven-baked glassware or pyrogen-free plasticware for handling the column to avoid contamination with endotoxins. This is especially important during the elution, precipitation, and reconstitution steps.

	RS 10	RS 50	RS 100	RS 200
1	**Preparation of starter culture** Inoculate a 5 mL starter culture of LB medium with a single colony picked from a freshly streaked agar plate. Assure that the plate and liquid culture contain the appropriate selective antibiotic to ensure plasmid propagation. Shake at 37 °C and ~200 – 300 rpm for ~8 hours.			
2	**Preparation of the fermentation culture**  *Note: To utilize the entire binding capacity of the **NucleoBond® RS columns,** it is important to provide sufficient plasmid DNA. If the culture is known to grow poorly or the plasmid does not behave like a high copy plasmid, please refer to section 5.3 for larger culture volumes. If you are not sure of the plasmid copy number and growth behavior of your host strain, increase the culture volume and decide later in step 3 how many cells to use for the preparation. **The recommended culture volumes below are calculated to a final OD_{600} of approximately 4** (see section 5.3).* **!** Prepare an overnight bacterial culture by diluting an appropriate volume of starter culture into the appropriate volume of Circlegrow medium with selected antibiotics. Grow the culture overnight (12 – 16 h).			
	V_c = 500 mL	V_c = 2,500 mL	V_c = 5,000 mL	V_c = 9,000 mL

	RS 10	RS 50	RS 100	RS 200

3 Harvest of bacterial cells

Measure the OD_{600} of the cell culture and determine the recommended culture volume.

$$ODV = OD_{600} \times V_c$$
$$\text{Resuspension Vol.} = ODV / 50$$

!	OD_{600} = 6 V_c = 500 mL ODV_{max} = 3.000 Res. Vol. = 60 mL	OD_{600} = 6 V_c = 2,500 mL ODV_{max} = 15.000 Res. Vol. = 300 mL	OD_{600} = 6 V_c = 5,000 mL ODV_{max} = 30.000 Res. Vol. = 600 mL	OD_{600} = 6 Vc = 9,000 mL ODV_{max} = 54.000 Res. Vol. = 1,080 mL

Pellet the cells by centrifugation at **4,500 – 6,000 x *g*** for **≥ 10 min** at **4 °C** and carefully discard the supernatant completely.

Note: It is especially important to determine cell mass for rich growth media (e.g. PLASMID+®, Circlegrow™, Terrific Broth). Rich growth media can contain 5 – 10 times more cells per mL than regular growth media (e.g. LB). Using rich growth media without checking the cell amount can easily lead to overloading of the kit and loss of yield.

At this stage it is recommend to perform an analytical check (see section 8.1) to optimize utilization of the NucleoBond® RS column.

4 Resuspension (Buffer RES-EF)

Carefully resuspend the cell pellet completely in **Resuspension Buffer RES-EF (supplemented with RNase A)** in a suitable wide-mouth bottle with screw cap by shaking the suspension for 5 – 10 minutes.

Alternatively, the suspension can be stirred at low speed (approx. 30 rpm) for one hour at RT.

Check that the bacterial pellet is completely resuspended and that no aggregates remain. It is important for efficient cell lysis that no clumps remain in the suspension!

Note: It is of course possible to use larger culture volumes, for example if the plasmid does not behave like a typical high-copy vector (see section 5.3 for more information). In this case, increase the volumes of buffers RES-EF, LYS-EF and NEU-EF proportionally in steps 4, 5 and 6. It may be necessary to order ***additional buffer (see ordering information in section 9.2)****.*

Note: Increase the volume of buffer RES-EF proportionally if more than the recommended cell mass is used (see sections 5.3 and 5.4 for information on optimal cell resuspension).

60 mL RES-EF	300 mL RES-EF	600 mL RES-EF	1,080 mL RES-EF

	RS 10	RS 50	RS 100	RS 200
5	**Cell lysis (Buffer LYS-EF)**			
	Check Lysis Buffer LYS-EF for precipitated SDS prior to use. If a white precipitate is visible, heat the buffer at 30 – 40 °C for several minutes until the precipitate is completely dissolved. Allow the buffer to cool to room temperature. Add an equal volume of Lysis Buffer LYS-EF to the suspension. Immediately mix gently by **inverting** the flask **5 – 6 times**, to obtain a clear, highly viscous solution. **Do <u>not vortex</u> the resulting lysate**, since this may release contaminating chromosomal DNA from cellular debris into the suspension. Incubate the mixture at room temperature for approx. 4 – 5 minutes.			
!	Caution: Prolonged exposure to alkaline conditions may irreversibly denature and degrade plasmid DNA and release contaminating chromosomal DNA into the lysate. *<u>Note:</u> Increase the volume of buffer LYS-EF proportionally if more than the recommended cell mass is used (see section 5.4 for information on optimal cell lysis)*			
	60 mL LYS-EF	300 mL LYS-EF	600 mL LYS-EF	1,080 mL LYS-EF
6	**Neutralization (Buffer NEU-EF)**			
	Add an equal volume of precooled **Neutralization Buffer NEU-EF** to the suspension. Immediately mix the lysate gently by inverting the flask 6 – 8 times until a homogeneous suspension is obtained.			
!	The container used for this step should be no more than two-thirds filled to ensure homogeneous mixing. Ensure complete neutralization to precipitate cellular debris, proteins and chromosomal DNA. The lysate should change from a viscous consistency to a low viscosity, homogeneous suspension of an off-white flocculate. In addition, LyseControl should turn completely colorless with no trace of blue. *<u>Note:</u> Increase the volume of buffer NEU-EF proportionally if more than the recommended cell mass is used (see section 5.5 for information on optimal lysate neutralisation).*			
	60 mL NEU-EF	300 mL NEU-EF	600 mL NEU-EF	1,080 mL NEU-EF
7				
!	**Incubate the crude lysate on ice for at least 15 minutes.**			
	> 15 min			

	RS 10	RS 50	RS 100	RS 200
	Equilibration of the column			
8	Mount the column upright on a laboratory frame. Equilibrate the NucleoBond® RS column in an upward direction with buffer EQ-EF at a flow rate of 5 – 10 mL/min at room temperature.			
!	Check all fitting connections for tightness and use cable ties to prevent leakage.			
	If the column runs dry at any step during use, rehydrate the column bed by re-equilibrating the column with buffer EQ-EF.			
	30 mL EQ-EF	120 mL EQ-EF	250 mL EQ-EF	400 mL EQ-EF
	5 mL / min	10 mL / min	10 mL / min	10 mL / min
9	**Clarification of lysate**			
	Perform a lysate clarification that results in a 0.45 µm filtered lysate. Use the specified filtration procedures or an equivalent procedure that will give the same result. However, keep shear forces as low as possible to minimize DNA damage and contamination.			
	Lysate clarification can be performed as a one-step procedure using **NucleoBond® Bottle Top Filters Type 2**. Connect a Bottle Top Filter Type 2 to a clean vacuum flask and a vacuum source. Fill a Bottle Top Filter Type 2 with approximately 500 – 1,000 mL of crude lysate. Allow the precipitate to float up for 2 minutes and apply vacuum (reduction of atmospheric pressure: -400 to -600 mbar), until the solution passed through. A regulator can be used to apply the correct atmospheric pressure, e.g. our NucleoVac 96 Vacuum Regulator (see ordering information in section 9.2). Discard the NucleoBond® Bottle Top Filter. Repeat the process, preferably using new Bottle Top Filters, until the crude lysate is completely filtered.			
	Tip: Reduce lysate loss, filtration time and filter life by performing a centrifugation step prior to filtration. The crude lysate can be centrifuged at 3,000 – 5,000 x *g* at 4 °C for 5 – 10 minutes. After centrifugation, carefully remove the supernatant from the white precipitate and apply the lysate to a pre-moistened NucleoBond® Bottle Top Filter Type 2 with deionized water or 30 % NEU-EF buffer solution.			
	The clarified lysate can be stored on ice for several hours. **If precipitates continue to appear, re-filter the lysate before applying it to the NucleoBond® RS column.** We recommend the use of a 0.45 µm CA filter (e.g. NucleoBond® Bottle Top Filter Type 2, see ordering informationin section 9.2).			
	Alternative procedure:			
	Three-stage capsule filter filtration: Stage I (Pre-filtration): Connect a ~ 20 µm PP filter capsule on the inlet side (see supplier's literature) with a 3.1 mm (1/8", ID) tubing, using tri-clamp adapters and gaskets. Mount the capsule on a laboratory stand and position the outlet side over a clean flask, positioned below the outlet, to collect the filtration flow-through.			

RS 10	RS 50	RS 100	RS 200

Attach the tubing to the pump head according to the manufacturer's instructions and place the free end into the neutralized crude lysate. It is recommended that the clear bottom phase be the first part to be pumped onto the filter capsule, while the precipitate is the last part to be pumped. To do so, simply push the end of the tube all the way to the bottom of the lysate flask.

Using a flow rate of **20 mL/min**, pump the raw lysate through the PP pre-filter and collect the flow-through (take into account the settings of the pump with respect to the inner diameter of the tubing). Please keep in mind the specifications of the filter, particularly the maximum pressure limit. At this point the filtrate will still be cloudy. Stop filtration if a break-through of particles occurs. Replace filter capsule in this case.

Once the lysate is completely loaded, it is recommended to pump air through the filter capsule to completely collect remaining lysate in the capsule. Alternatively, rinse the capsule with 30 % NEU-EF buffer.

The pre-filtration can also be replaced by centrifugation (≥ 4,500 x *g*, 30 minutes, 2 – 8 °C)

Stage II (Fine-filtration): Replace the 20 µm PP filter capsule with a ~ **1 – 5 µm glassfiber filter capsule**. Replace the tubing and adapters accordingly. Please keep in mind the specifications of the filter, particularly the maximum pressure limit. Pump the pre-cleared lysate through the glassfiber filter at a flow rate of **20 mL/min** and collect the flow-through.

Once the lysate is completely loaded, it is recommended to pump air through the filter capsule to completely collect remaining lysate in the capsule. Alternatively, rinse the capsule with 30 % NEU-EF buffer. Centrifugation (≥ 4,500 x *g*, 30 minutes, 2 –8 °C) can replace fine-filtration, but it is advisable to perform fine-filtration to prolong the life of the sterile filter.

Stage III (Sterile-filtration): Replace the glassfiber filter capsule with a **0.45 µm PES sterile filter capsule**. Replace the tubing and adapters accordingly. Please keep in mind the specifications of the filter, particularly the maximum pressure limit. Pump the clarified lysate through the sterile filter at a flow rate of 20 mL/min and collect the flow-through.

!

Once the lysate is completely loaded, it is recommended to pump air through the filter capsule to completely collect remaining lysate in the capsule. Alternatively, rinse the capsule with 30 % NEU-EF buffer.

It is extremely important that the final filtration step is performed with a 0.45 µm pore size. Otherwise, residual precipitate will clog the NucleoBond® RS column.

5 mL / min	10 mL / min	10 mL / min	10 mL / min

	RS 10	RS 50	RS 100	RS 200

10 Loading of lysate

Load the cleared lysate from step 9 onto the NucleoBond® RS column (equilibrated with buffer EQ-EF) at the appropriate flow rate. Check the actual flow rate of the pump. Depending on the volume of the cleared lysate loaded on the NucleoBond® RS column, the flow rate may reduce which is a common observation during the binding phase. However, if the flow rate drops below half of the set rate, stop the pump and refer to the troubleshooting in section 9.1.

Depending on the volume of the cleared lysate, it is convenient to keep the cleared lysate stored in an icebox during the loading of the column overnight at 2 – 3 mL/min flow rate. Do not cool the column itself.

You may wish to save some or all of the flow through for analysis.

If the column runs dry during use, rehydrate the column bed by re-equilibrating the column with buffer EQ-EF.

5 mL / min	10 mL / min	10 mL / min	10 mL / min

11 1st Wash with Buffer ENTO-EF

Wash the NucleoBond® RS column with **endotoxin removal buffer ENTO-EF**.

If the column runs dry during use, rehydrate the column bed by re-equilibrating the column with Buffer EQ-EF.

30 mL ENTO-EF	150 mL ENTO-EF	300 mL ENTO-EF	550 mL ENTO-EF
5 mL / min	10 mL / min	10 mL / min	10 mL / min

12 2nd Wash with Buffer WASH-EF

Wash the NucleoBond® RS column with **wash buffer WASH -EF**.

If the column runs dry during use, rehydrate the column bed by re-equilibrating the column with Buffer EQ-EF.

30 mL WASH-EF	150 mL WASH-EF	300 mL WASH-EF	550 mL WASH-EF
5 mL / min	10 mL / min	10 mL / min	10 mL / min

	RS 10	RS 50	RS 100	RS 200

13 Elution (Buffer ELU-EF)

Elute the plasmid DNA with **Elution buffer ELU-EF**. Collect the eluate at room temperature in an oven-baked glass or endotoxin-free plastic vessel.

You may discard the first 1/8 of the eluate as this is the dead volume of the column. The following eluate contains the purified plasmid DNA.

It is recommended to precipitate the eluate as soon as possible (step 14). However, the eluate can be stored in a closed container on ice for several hours. In this case, the eluate should be pre-warmed to room temperature before precipitating the plasmid DNA.

If possible, continuously check the plasmid concentration at A_{260} during elution to obtain maximum yield with minimum elution volume by adjusting the elution volume. Store the eluate on ice during elution.

30 mL ELU-EF	120 mL ELU-EF	250 mL ELU-EF	400 mL ELU-EF
5 mL / min	10 mL / min	10 mL / min	10 mL / min

14 Precipitation (Isopropanol)

Note: It is highly recommended to determine the plasmid yield by measuring A_{260} before precipitating the DNA. This helps to select the best buffer volume in step 16 and allows calculating the recovery after precipitation.

Add 0.7 volume of **room temperature isopropanol** to precipitate the eluted plasmid DNA. **Mix thoroughly by shaking.**

Ensure that the temperature of the plasmid suspension does not exceed 25 °C to avoid reduced yield. Temperatures below 5 °C should be avoided to prevent salt precipitation.

0.7 volumes of isopropanol			
21 mL	84 mL	175 mL	280 mL

Incubate for 2 minutes at room temperature, then pellet the DNA by centrifugation afterwards (≥ 4,500 x *g* for ≥ 15 min at ≤ room temperature, preferably at 15,000 x *g* for 30 min at 4 °C).

Carefully discard the supernatant. Take care not to discard the DNA pellet.

	RS 10	RS 50	RS 100	RS 200
15	**Washing and drying (70 % EtOH)**			
	Add **70 % ethanol** at **room-temperature** to the pellet.			
	Mix briefly and centrifuge (≥ 4,500 x *g*, preferably ≥ 15,000 x *g* for 5 min at room temperature)**.**			
	Carefully remove ethanol completely from the container with a pipette tip. Allow the pellet to dry at **room temperature.**			
	Note: Plasmid DNA might be harder to dissolve when over-dried.			
	This step may be repeated once to reduce the risk of isopropanol carry-over.			
	5 mL	15 mL	25 mL	50 mL
	Until dry			
16	**Reconstitution (Buffer TE-EF or H_2O-EF)**			
	Dissolve the DNA pellet in an appropriate volume of endotoxin-free **Buffer TE-EF** or **H_2O-EF.** It is recommended to choose the resuspension volume according to the requirements of the downstream application. A standard value of a final concentration for sequence applications is 1 mg/mL. Depending on the type of container, dissolve by gentle pipetting up and down or by constant spinning in a sufficient volume of buffer for 10 – 180 min (3D shaker).			
	Determine the plasmid yield by UV spectrophotometry. Confirm plasmid integrity by agarose gel electrophoresi			
	var., @ 1 mg/mL			
	8 – 10 mL	40 – 50 mL	80 – 100 mL	170 – 190 mL

9 Appendix

9.1 Troubleshooting

If you are experiencing problems with reduced yield or purity, it is recommended to sequentially check which purification step of the procedure is causing the problem.

First, check the bacterial culture for sufficient growth (OD_{600}) in the presence of an appropriate selective antibiotic. Second, aliquots of the clarified lysate, flow-through, wash steps (ENTO-EF buffer, WASH-EF) and eluate can be retained for further analysis by agarose gel electrophoresis.

The following table describes some problems that may occur during preparation, explains the cause, and suggests how to resolve these issues:

Problem	Possible cause and suggestions
No or low plasmid DNA yield	*Plasmid did not propagate* • Check plasmid content in the cleared lysate. Use colonies from fresh plates for inoculation and add fresh selective antibiotic to plates and media. • Estimate plasmid content prior to large purifications by a quick NucleoSpin® Plasmid preparation. Alternatively, a preperation using NucleoBond® Xtra Midi is also applicable. *Alkaline lysis was inefficient* • Too much cell mass was used. Refer to section 5.3 – 5.7 regarding recommended culture volumes and lysis buffer volumes. Check plasmid content in the cleared lysate. • Check Buffer LYS-EF for SDS precipitation before use, especially after storage below 20 °C. If necessary incubate the bottle for several minutes at 30 – 40 °C and mix well until SDS is redissolved. Let Buffer LYS-EF cool down to RT again before use. *SDS- or other precipitates are present in the sample* • Incubation of cleared lysates for longer periods of time might lead to formation of new precipitate. If precipitate is visible, it is recommended to filter or centrifuge the lysate again directly before loading it onto the NucleoBond® RS column. *Sample/lysate is too viscous* • Too much cell mass was used. Refer to section 5.3 – 5.7 regarding recommended culture volumes and lysis buffer volumes. • Make sure to mix well after neutralization to completely precipitate SDS and chromosomal DNA. Otherwise, filtration efficiency go down, column backpressure increases, flow rate go down or column clogs and SDS prevents DNA from binding to the column. *pH or salt concentrations of buffers are too high* • Check plasmid content in the wash fractions. Keep all buffers tightly closed. Check and adjust pH of Buffer ENTO-EF (pH 6.5), WASH-EF (pH 7.0), and ELU-EF (pH 9.0) with HCl or NaOH if necessary. • Column overloaded with nucleic acids • Use a larger column or purify excess nucleic acids on a new column. Refer to the recommended culture volumes listed in section 5.3 at the beginning of the protocol. *Flow rates too high* • Do not exceed recommended flow rates for loading and eluting the plasmid DNA.

Problem	Possible cause and suggestions
NucleoBond® Bottle Top Filter Type 2 clogs during filtration or is very slow	*Culture volumes are too large* • Please do not load more than 500 mL – 1,000 mL of crude lysate on a NucleoBond® Bottle Top Filter Type 2 in order to avoid clogging. If clogging still occurs, try loading only 500 mL on the Bottle Top Filter. *Precipitate was not resuspended before loading* • Invert crude lysate at least 3 times directly before loading and wait for 1 – 2 minutes before applying the vacuum. *Incomplete precipitation step* • Make sure to mix well after neutralization to completely precipitate SDS and chromosomal DNA

Problem	Possible cause and suggestions
NucleoBond® RS Column is blocked or very slow	*Sample is too viscous* • Do NOT attempt to purify lysate prepared from a culture volume larger than recommended for any given column size with standard lysis buffer volumes. Incomplete lysis not only blocks the column but can also significantly reduce yields. Refer to section 5.3 – 5.5 for recommended culture volumes, larger culture volumes and adjusted lysis buffer volumes. • Make sure to mix well after neutralization to completely precipitate SDS and chromosomal DNA. *Lysate was not cleared completely* • Centrifuge at higher speed or for a longer period of time. • The filtration capsule or NucleoBond® Bottle Top Filter Type 2 may have broken through. Change the capsule in time. • Precipitates occur during storage. Clear lysate again before loading the column. *Column overloaded with nucleic acids* • *Use a larger column or purify excess nucleic acids on a new column. Refer to the recommended culture volumes listed in the table at the beginning of each protocol* *High back pressure during purification* • Cleared lysate contains particulate matter. Make sure that there is no cell debris in the lysate. Particulate matter may clog the inlet frit or inline filters. If necessary, repeat filtration or centrifugation steps. Be sure to equilibrate and use the column in the correct direction (see protocol and scheme). • Do not skip incubation of the crude lysate on ice! Incubation significantly reduces the formation of new solid particles by precipitation of salts or SDS. These are largely responsible for the occurring clogging of the columns. If the flow rate decreases dramatically over time (to less than half of the set flow rate), the column is on the verge of blockage. In this case please stop the binding step immediately and continue with the next step of the protocol. The column is rinsed free in the subsequent wash step and the flow rate begins to normalize again.

Problem	Possible cause and suggestions
Genomic DNA contamination of plasmid DNA	*Lysis treatment was too harsh* • Make sure not to lyse in Buffer LYS-EF for more than 5 min. *Lysate was mixed too vigorously or vortexed after lysis, allowing genomic DNA to shear off into lysis buffer* • Invert tube for only 5 times and mix gently. Do not vortex after addition of Buffer LYS-EF. • Use larger tubes/flasks or reduce culture volume if the lysate is too viscous to mix properly.
RNA contamination of plasmid DNA	*RNase digestion was inefficient* • RNase was not added to Buffer RES-EF or stored improperly. Add new RNase to Buffer RES-EF. See section 9.2 for ordering information. *pH or salt concentration of wash buffer is too low.* • Check RNA content in the wash fractions. Keep all buffers tightly closed. Check pH of EQ-EF (6.5), ENTO-EF (6.5), and WASH-EF (7.0) and adjust with HCl or NaOH if necessary. *Wash step with Buffer WASH-EF was not sufficient* • Double or triple washing volume with Buffer WASH-EF. Additional Buffer WASH-EF can be ordered separately (see ordering information).
Low purity (A_{260}/A_{280} < 1.8)	*Only minimal amounts of DNA were loaded onto the column* • Too much free binding capacity requires more extensive washing – double washing step with Buffer ENTO-EF. • Reduce lysis time < 5 min.
No nucleic acid pellet formed after precipitation	*Pellet was lost* • Handle the precipitate with care. Decant solutions carefully. Determine DNA yield in Buffer ELU-EF in order to calculate the amount of plasmid DNA that should be recovered after precipitation. *Plasmid DNA might be smeared over the wall of the tube.* • Dissolve DNA with an appropriate volume of endotoxin-free reconstitution buffer by rolling the container for at least 30 min. *Nucleic acid did not precipitate* • Check type and volumes of precipitating solvent. Make sure to use at least 0.7 volumes of isopropanol and mix thoroughly • Centrifuge for longer periods of time at higher speed

Problem	Possible cause and suggestions
Nucleic acid pellet is opaque or white instead of clear and glassy	*Co-precipitation of salt* • Check isopropanol purity, and perform precipitation at room temperature but centrifuge at 4 °C. Do not let the eluate drip from the column into isopropanol but add isopropanol to the final eluate and mix immediately. • Try resuspending the pellet in Buffer ENTO-EF, and reload onto the same NucleoBond® RS Column. Wash the column several times with Buffer WASH-EF before loading.
Nucleic acid pellet does not resuspend in buffer	*Pellet was over-dried* • Try to dissolve at higher temperatures for a longer period of time (e.g., 2 h at 37 °C or overnight at RT), preferably under constant spinning (3D-shaker). *Co-precipitation of salt or residual alcohol* • Wash the pellet again with 70 % ethanol, or increase the reconstitution buffer volume. *Insoluble particles in redissolved DNA* • Centrifuge the redissolved DNA to pellet the insoluble particles and transfer supernatant to a new tube. Insoluble particles do not affect DNA quality.
Purified plasmid does not perform well in subsequent reactions	*Plasmid DNA is contaminated with chromosomal DNA or RNA* • Refer to the detailed troubleshooting above. *Plasmid DNA is contaminated with residual alcohol* • Plasmid DNA was not dried completely before redissolving. Precipitate DNA again by adding 1/10 volume of 3 M NaAc pH 5.0 and 0.7 volumes of isopropanol. Proceed with the precipitation protocol in this manual und dry DNA pellet completely. *DNA is degraded* • Make sure that your entire equipment (pipettes, centrifuge tubes, etc.) is clean and nuclease-free. • Do not lyse the sample with Buffer LYS-EF for more than 5 min. *DNA is irreversibly denatured* • A denatured plasmid band runs faster on the gel than the supercoiled conformation. Do not lyse the sample after addition of Buffer LYS-EF for more than 5 min. *Endotoxin level is too high* • Refer to the detailed troubleshooting below.

Problem	Possible cause and suggestions
Endotoxin level is too high	*See section 4.3 for recommendations for work in endotoxin-free environments* *Too much cell mass was used* • Decrease cell mass or better increase lysis buffer volumes. *Inefficient endotoxin removal* • Apply all washing buffers in the correct order: ENTO-EF, WASH-EF. • Increase washing volume of Buffer ENTO-EF by 25 – 50 %. *Contamination of DNA product after purification* • Use only new pyrogen- or endotoxin-free plastic ware and pipette tips. Endotoxins tend to stick to glass ware and are hard to remove. If glass ware is to be used, heat at 180 °C for at least 3 hours or at 250 °C for 30 minutes to destroy endotoxins. • Use only the tested endotoxin-free buffers.

9.2 Ordering information

Product	REF	Pack of
NucleoBond® RS 10	743502	5 columns
NucleoBond® RS 50	743503	1 column
NucleoBond® RS 100	743504	1 column
NucleoBond® RS 200	743505	1 column
RES-EF	740386.1000 / .3000	1000 mL / 3000 mL
LYS-EF	740387.1000 / .3000	1000 mL / 3000 mL
NEU-EF	740388.1000 / .3000	1000 mL / 3000 mL
EQ-EF	740380.1000 / .3000	1000 mL / 3000 mL
ENTO-EF	740381.1000 / .3000	1000 mL / 3000 mL
WASH-EF	740392.1000 / .3000	1000 mL / 3000 mL
ELU-EF	740393.900 / .3000	900 mL / 3000 mL
TE-EF	740797.1	1000 mL
H_2O-EF (nuclease free)	740798.1	1000 mL
Liquid RNase A	740397	2.5 mL
RNase A (lyophilized)	740505.30	30 mg
NucleoBond® Bottle Top Filter Type 2	740553.5	5 pieces
NucleoVac 96 Vacuum Regulator	740641	1 piece
NucleoBond® Xtra Midi kit	740410.10	10 preps
NucleoSpin® Plasmid, Mini kit	740588.50	50 preps

9.3 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.

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

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

Last updated: 08/2023, Rev. 01

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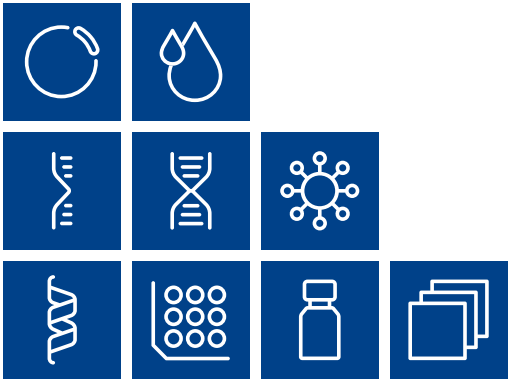
Li, Y. and Boraschi, D. (2016) 'Endotoxin contamination: a key element in the interpretation of nanosafety studies', Nanomedicine, 11:3, pp. 269 – 287, doi.org/10.2217/nnm.15.196



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