

MACHEREY-NAGEL

# User manual

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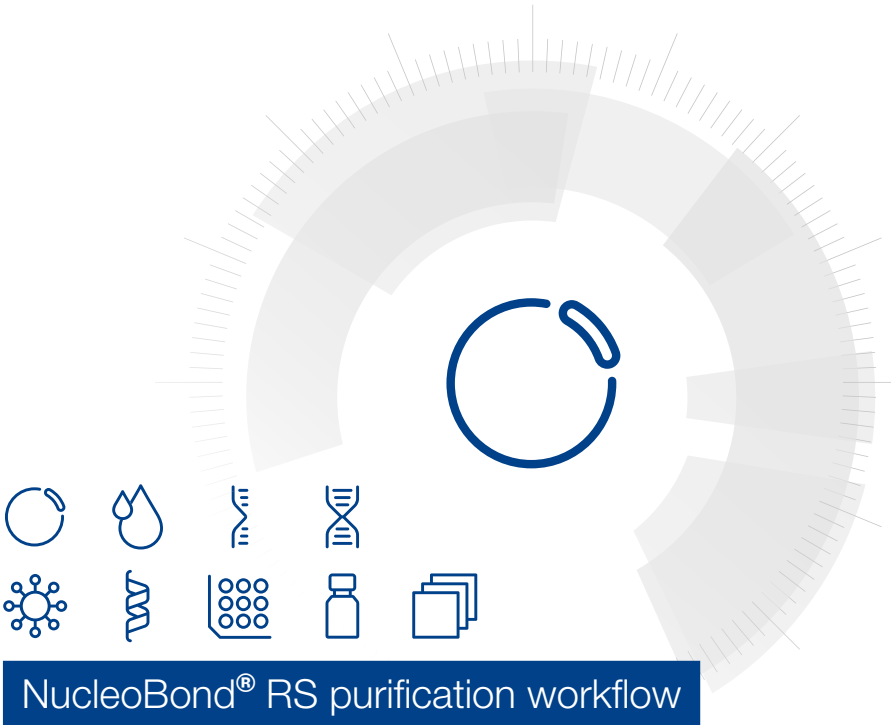
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## NucleoBond® RS purification workflow

- NucleoBond® RS columns

December 2024 / Rev. 06

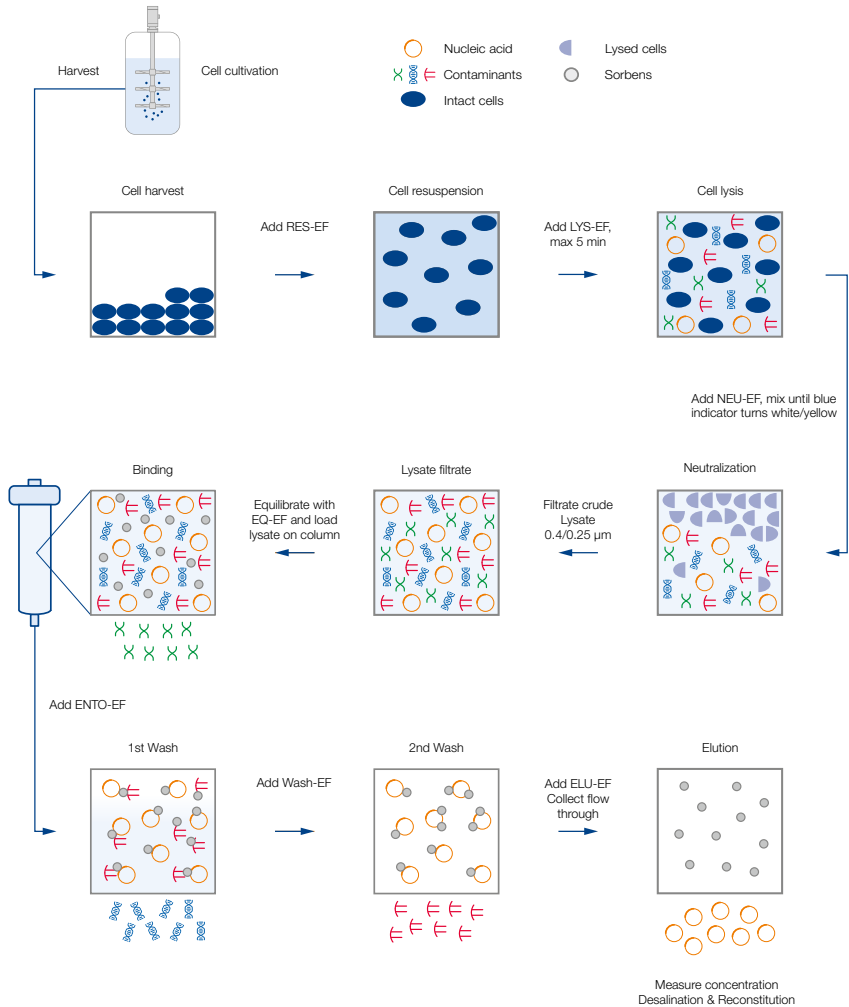
# NucleoBond® RS purification workflow

## Protocol at a glance (Rev. 06)

	RS 10	RS 50	RS 100	RS 200
<b>1–3</b> <b>Cell cultivation and harvest</b>	4 °C, 15 min 4,500–6,000 × g			
	max. recom: ODV: 3,000 CDW: 8 g typ. Yield: ~10 mg	max. recom: ODV: 15,000 CDW: 40 g typ. Yield: ~50 mg	max. recom: ODV: 30,000 CDW: 80 g typ. Yield: ~100 mg	max. recom: ODV: 54,000 CDW: 145 g typ. Yield: ~190 mg
<b>4–5</b> <b>Cell lysis</b>  <i>(Important: Check Buffer LYS-EF for precipitated SDS)</i>	60 mL Buffer RES-EF	300 mL Buffer RES-EF	600 mL Buffer RES-EF	1080 mL Buffer RES-EF
	60 mL Buffer LYS-EF	300 mL Buffer LYS-EF	600 mL Buffer LYS-EF	1080 mL Buffer LYS-EF
RT, max 5 min				
<b>6 Neutralization</b>	60 mL Buffer NEU-EF	300 mL Buffer NEU-EF	600 mL Buffer NEU-EF	1080 mL Buffer NEU-EF
	Mix thoroughly until colorless Incubate 15 min on ice!			
<i>Maximum recommended pump speed</i>	5 mL/min	10 mL/min	10 mL/min	10 mL/min
<b>7 Equilibration of the column</b>	30 mL EQ-EF	120 mL EQ-EF	250 mL EQ-EF	400 mL EQ-EF
<b>8 Clarification of the lysate</b>	Clarify and filtrate the crude lysate to 0.45/0.22 µm			
<b>9 Binding</b>	Load cleared lysate on NucleoBond® RS column			
<b>10 1<sup>st</sup> Wash</b>	30 mL Buffer ENTO-EF	150 mL Buffer ENTO-EF	300 mL Buffer ENTO-EF	550 mL Buffer ENTO-EF
<b>11 2<sup>nd</sup> Wash</b>	30 mL Buffer WASH-EF	150 mL Buffer WASH-EF	300 mL Buffer WASH-EF	550 mL Buffer WASH-EF
<b>13 Elution</b>	30 mL Buffer ELU-EF	120 mL Buffer ELU-EF	250 mL Buffer ELU-EF	400 mL Buffer ELU-EF
<b>14 Precipitation</b>	21 mL Isopropanol	84 mL Isopropanol	175 mL Isopropanol	280 mL Isopropanol
	Mix thoroughly 4,500–15,000 × g 4 °C, 15 min			
<b>15 Washing and drying</b>	5 mL 70 % EtOH 4,500–15,000 × g RT, 5 min	15 mL 70 % EtOH 4,500–15,000 × g RT, 5 min	25 mL 70 % EtOH 4,500–15,000 × g RT, 5 min	50 mL 70 % EtOH 4,500–15,000 × g RT, 5 min
	Approx. RT, 10–15 min	Approx. RT, 30–60 min	Approx. RT, 45–90 min	Approx. RT, 75–120 min
<b>16 Reconstitution</b>	Appropriate volume of TE-EF or H <sub>2</sub> O-EF.			
	It is recommended to choose the resuspension volume according to the requirements of the downstream application. (for var., @ 1 mg/mL)			
	8–10 mL	40–50 mL	80–100 mL	170–190 mL

# NucleoBond® RS purification workflow

## Protocol at a glance (Rev. 06)



# NucleoBond® RS RNase free purification workflow

## Protocol at a glance (Rev. 06)

	RS 10	RS 50	RS 100	RS 200
<b>1 – 3</b> <b>Cell cultivation and harvest</b>	4 °C, 15 min 4,500–6,000 × g			
	max. recom: ODV: 3,000 CDW: 8 g typ. Yield: ~10 mg	max. recom: ODV: 15,000 CDW: 40 g typ. Yield: ~50 mg	max. recom: ODV: 30,000 CDW: 80 g typ. Yield: ~100 mg	max. recom: ODV: 54,000 CDW: 145 g typ. Yield: ~190 mg
<b>4 – 5</b> <b>Cell lysis</b>  <i>(Important: Check Buffer LYS-EF for precipitated SDS)</i>	60 mL Buffer RES-EF	300 mL Buffer RES-EF	600 mL Buffer RES-EF	1080 mL Buffer RES-EF
	60 mL Buffer LYS-EF	300 mL Buffer LYS-EF	600 mL Buffer LYS-EF	1080 mL Buffer LYS-EF
<b>6 Neutralization</b>	RT, max 5 min			
	60 mL Buffer NEU-EF	300 mL Buffer NEU-EF	600 mL Buffer NEU-EF	1080 mL Buffer NEU-EF
	Mix thoroughly until colorless Incubate 15 min on ice!			
<i>Maximum recommended pump speed</i>	5 mL/min	10 mL/min	10 mL/min	10 mL/min
<b>7 Equilibration of the column</b>	30 mL EQ-EF	120 mL EQ-EF	250 mL EQ-EF	400 mL EQ-EF
<b>8 Clarification of the lysate</b>	Clarify the crude lysate (BottleTop Filter) or 1 – 5 µm (Capsule filter)			
<b>9 RNA precipitation</b>	Add 0.3 (v/v) isopropanol and mix thoroughly Incubate 15 min on ice!			
<b>10 RNA removal</b>	Clarify and filtrate the cloudy lysate to 0.45/0.22 µm			
<b>11 DNA precipitation</b>	Add 0.16 (v/v) isopropanol to the present lysate-isopropanol mixture volume.			
<b>12 Pellet wash</b>	Wash with 70 % Ethanol 4,500 – 15,000 × g RT, 5 min			
<b>13 Reconstitution binding parameters</b>	Reconstitution of pDNA with BMC-Buffer until pellet dissolved.			
<b>14 Binding</b>	Load RNA free lysate on NucleoBond® RS column			
<b>15 1<sup>st</sup> Wash</b>	75 mL Buffer ENTO-EF	375 mL Buffer ENTO-EF	750 mL Buffer ENTO-EF	1380 mL Buffer ENTO-EF
<b>16 2<sup>nd</sup> Wash</b>	75 mL Buffer WASH-E	375 mL Buffer WASH-EF	750 mL Buffer WASH-EF	1380 mL Buffer WASH-EF
<b>17 Elution</b>	30 mL Buffer ELU-EF	120 mL Buffer ELU-EF	250 mL Buffer ELU-EF	400 mL Buffer ELU-EF

# NucleoBond® RS RNase free purification workflow

## Protocol at a glance (Rev. 06)

	RS 10	RS 50	RS 100	RS 200
<b>18 Precipitation</b>	21 mL Isopropanol	84 mL Isopropanol	175 mL Isopropanol	280 mL Isopropanol
	Mix thoroughly 4,500 – 15,000 × g 4 °C, 15 min			
<b>19 Washing and drying</b>	5 mL 70 % EtOH 4,500 – 15,000 × g RT, 5 min Approx. RT, 10 – 15 min	15 mL 70 % EtOH 4,500 – 15,000 × g RT, 5 min Approx. RT, 30 – 60 min	25 mL 70 % EtOH 4,500 – 15,000 × g RT, 5 min Approx. RT, 45 – 90 min	50 mL 70 % EtOH 4,500 – 15,000 × g RT, 5 min Approx. RT, 75 – 120 min
<b>20 Reconstitution</b>	Appropriate volume of TE-EF or H <sub>2</sub> O-EF. It is recommended to choose the resuspension volume according to the requirements of the downstream application. (for var., @ 1 mg/mL)			
	8 – 10 mL	40 – 50 mL	80 – 100 mL	170 – 190 mL



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# 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 <sub>2</sub> O-EF	Endotoxin-free water	1000 mL
740382.1000 / .3000	BMC-EF	Binding mediating re-constitution buffer	1000 mL / 3000 mL
740553.5 (recommended)	Bottle Top Filter Type 2	Lysate filtration	5 pieces
740641 (recommended)	NucleoVac 96 Vacuum Regulator	Vacuum regulator 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 (Standard protocol)**

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 <sub>2</sub> O-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 (Standard protocol)**

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 <sub>2</sub> O-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 (Standard protocol)**

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 <sub>2</sub> O-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 (Standard protocol)**

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 <sub>2</sub> O-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 (Standard protocol)**

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)	30 mg	2	6

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

#### 1.3.1 Reagents

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

**Minimum amount of reagents and consumables required per preparation (standard protocol)**

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 7.0 kg/cm<sup>2</sup> (100 psi), typical during preparation: ~ 20 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 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, preventing 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 time-consuming centrifugation steps 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. The crude lysate passes through within 15 minutes. Discard the Bottle Top Filter and load the clear lysate to the NucleoBond® RS column.
- 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 will be successively filtered at decreasing pore sizes. At least one filtration step using 0.45 µm pore size is mandatory for reliable plasmid purification with the NucleoBond® RS

columns. It is recommended to use filter capsules with a pore size of 20, 5 and 0.45 µm, meeting quality criteria such as:

- USP< 85 >                      Biocompatibility: Bacterial endotoxin test
- USP< 87 >                      Biocompatibility: In-vitro cytotoxicity
- USP< 643 >                     Physiochemical: Conductivity
- USP< 645 >                     Physiochemical: Non-volatile residue & TOC
- USP< 788 >                     Cleanliness: Particulate matter
- 21CFR 2010.3 (b)            Cleanliness: Fiber shedding
- 21 CFR 177                      FDA indirect food additive

Manufacturers and distributors of capsule filters suitable for biological and bioprocess applications include 3M, Cobetter, International Filter Products, Saint-Gobain, and Whatman.

Below suitable 20 µm capsule filters of Cobetter are listed with increasing capacity:

- RS10                      Cobetter Filtration Cat. No.:    C01TTPFSA22000A1M
- RS50 – 100            Cobetter Filtration Cat. No.:    L05TTCHT2XA1
- RS200                    Cobetter Filtration Cat. No.:    L10TTCHT2XA1

Below suitable 5 µm capsule filters of Cobetter are listed with increasing capacity:

- RS10                      Cobetter Filtration Cat. No.:    KZ50LGFP0500M
- RS50 – 100            Cobetter Filtration Cat. No.:    C03KKCHV50A1
- RS200                    Cobetter Filtration Cat. No.:    L02KKCHV50A1

Below suitable 0.45 µm capsule filters of Cobetter are listed with increasing capacity:

- RS10                      Cobetter Filtration Cat. No.:    C01TTDPSHSL4522A1M
- RS50 – 100            Cobetter Filtration Cat. No.:    L05TTDPSHSL4522A1
- RS200                    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 clamps)
- Sanitary gaskets (silicone)

Connectors, supplementary material for preparation on a peristaltic pump:

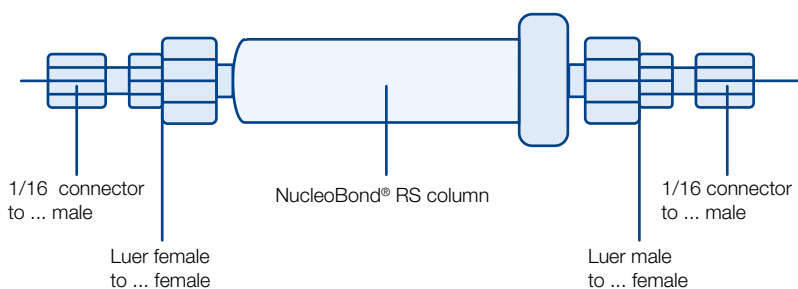
- Barbed Male Luer (Lock) to tubing connectors 1/16" and 1/8"
- Laboratory stand

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 example, the following connections can be used to operate NucleoBond® RS columns on an ÄKTA™ system. These can be purchased from Index-HS or other suppliers:

**Table 8: Three exemplary connection possibilities for NucleoBond® RS columns on FPLC systems**

Pieces needed	Connector name	Catalogue No. of supplier Index-HS <a href="https://www.index-hs.com/">https://www.index-hs.com/</a>
2	1/16 Connector to 10–32 male	F-120
1	Luer Male to 10–32 female	P-656
1	Luer female to 10–32 female	P-659
OR		
2	1/16 Connector to ¼-28 male	XP-206
1	Luer Male to ¼-28 female	P-675
1	Luer female to ¼-28 female	P-658

**Figure 1 Example of NucleoBond® RS column setup on FPLC 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 used, heat it overnight at 180 °C to destroy potential endotoxins on surface. Autoclaving does not inactivate endotoxins and is not recommended.
- 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<sub>2</sub>O-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.
- For research use only (RUO).

**Table 9: 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 10: NucleoBond® RS column binding capacity**

REF	Name	Theoretical binding capacity	ODV <sub>max</sub>	Cell wet weight <sub>max</sub>
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 specifically 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](http://www.mn-net.com)**.

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

If additional information are required, such as regulatory support files, please contact our Technical Support.

## 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  $\mu\text{g}$  DNA. FDA has approved several commercially available endotoxin measurement 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 various 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/ $\mu\text{g}$  are reported to be suitable. For standard transfection methods endotoxin levels of  $0.1 - 1.0$  EU/ $\mu\text{g}$  are typically suitable, and for highly sensitive applications, endotoxin levels of  $\leq 0.1$  EU/ $\mu\text{g}$ , 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  $\leq 0.01$  EU/ $\mu\text{g}$  DNA, facilitating compliance with such applications.

### 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 laboratory 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 with high temperature at  $> 250$  °C for 30 minutes or at 180 °C for

3 hours. Autoclaving does not inactivate endotoxins and is not recommended. 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 any plastic items used are pyrogen-free and check their suitability for used buffers. Components from these 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 the work area and equipment are clean and free from unnecessary materials, preventing 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.

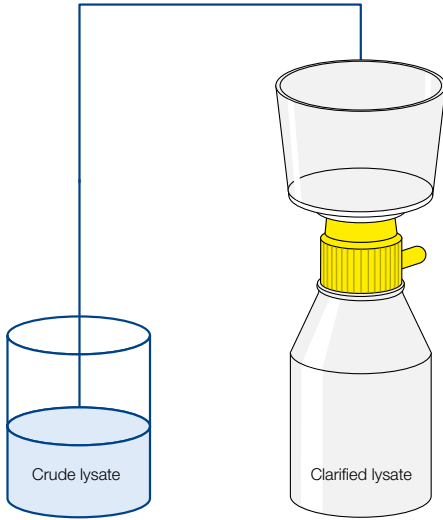
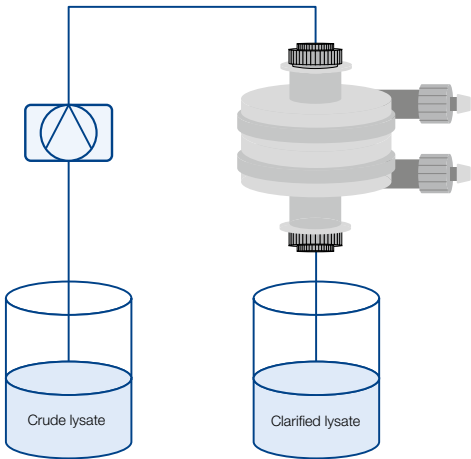


Figure 2 NucleoBond® RS Bottle Top Filter Type 2 setup



Unfiltered Crude lysate → 20 µm → 5 µm → 0.45 µm Clarified lysate

Figure 4 NucleoBond® RS capsule filter setup

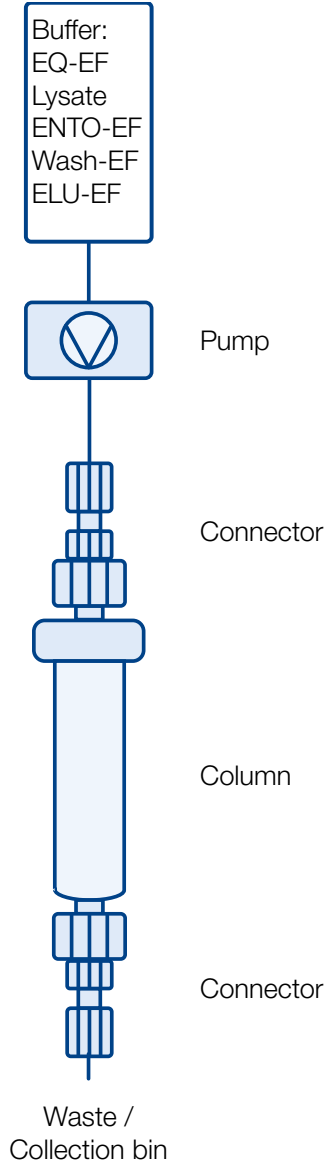


Figure 3 NucleoBond® RS preparative scale setup

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.

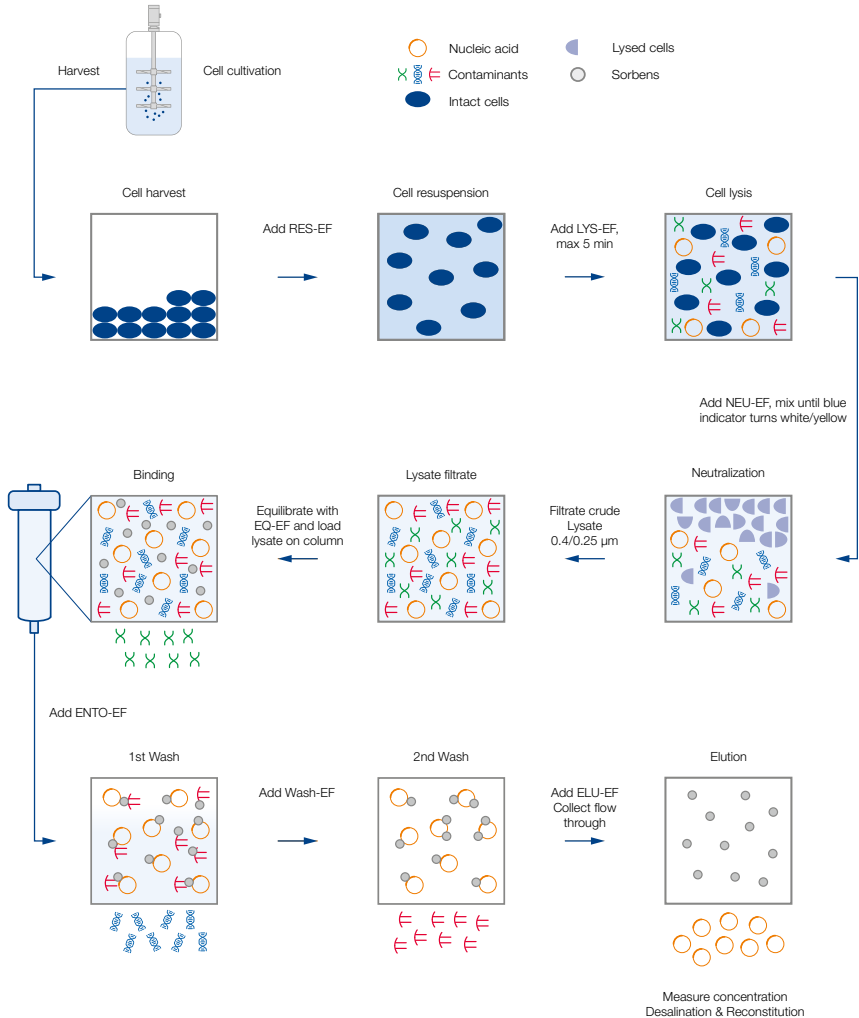


Figure 5 Scheme of the purification workflow of the NucleoBond® RS columns.

## 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<sub>600</sub> of 1 consists of  $1 \times 10^{12}$  cells and yields approximately 1.5–1.8 g cell wet weight. Overnight cultures grown in LB medium typically reach an OD<sub>600</sub> of 3–6 under vigorous shaking in flasks. Fermentation cultures typically reach an OD<sub>600</sub> 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<sub>600</sub>) and culture volume (V<sub>c</sub>) – two variables that are much easier to measure.

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

Note that for a correct OD determination the culture samples must be diluted when OD<sub>600</sub> 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<sub>600</sub> is then multiplied by the dilution factor 10/20 to obtain a theoretical OD<sub>600</sub> value. This OD<sub>600</sub> is used in Table 11 to determine the appropriate culture volume. Table 11 shows the recommended ODVs and the corresponding pairs of OD<sub>600</sub> and culture volume that can be easily handled using the given **NucleoBond® RS purification workflow**. For example, if the OD<sub>600</sub> 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 11: Recommended culture volumes for high-copy plasmids**

NucleoBond® RS column	Pellet wet weight	Rec. ODV	OD <sub>600</sub> = 2	OD <sub>600</sub> = 4	OD <sub>600</sub> = 6	OD <sub>600</sub> = 8	OD <sub>600</sub> = 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

**Table 11: Recommended culture volumes for high-copy plasmids**

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 11. 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:**

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

For example, if 500 mL of a high copy bacterial culture ( $\text{OD}_{600} = 6$ ) 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 **room-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<sub>2</sub>O-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<sub>2</sub>O-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.

## 5.11 RNase free support protocol

We have developed an additional RNase free protocol for the NucleoBond® RS columns. This protocol will assist in meeting increasingly stringent regulatory compliance requirements by eliminating the use of animal-derived materials. The RNase-free protocol generally follows the same procedure as the standard protocol, with the exception of significant alterations made to the lysate clarification process. It is advised to read the entire manual before applying the support protocol.

## 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.
- Using NucleoBond® RS RNase-free purification, store the BMC-EF buffer in a refrigerator. Buffer BMC-EF does not need to regain RT before usage.

## 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](http://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](https://qr.mn-net.com/qr/(241)740588)

QR-Code user manual









[qr.mn-net.com/qr/\(IFU\)740588](https://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<sup>2</sup> (100 psi) during use. Use only oven-baked glassware or pyrogen-free plasticware to avoid contamination with endotoxins. This is especially important during the elution, precipitation, and reconstitution steps.

	RS 10	RS 50	RS 100	RS 200
<b>1</b>	<b>Preparation of starter culture</b>			
	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.			
<b>2</b>	<b>Preparation of the fermentation culture</b>			
	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).			
	<i>Note:</i> To utilize the entire binding capacity of the <b>NucleoBond® RS columns</b> , 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. <b>The recommended culture volumes below are calculated to a final OD<sub>600</sub> of approximately 4</b> (see section 5.3).			
	V <sub>c</sub> = 500 mL	V <sub>c</sub> = 2,500 mL	V <sub>c</sub> = 5,000 mL	V <sub>c</sub> = 9,000 mL
<b>3</b>	<b>Harvest of bacterial cells</b>			
	Measure the OD <sub>600</sub> of the cell culture and determine the recommended culture volume.			
	ODV = OD <sub>600</sub> × V <sub>c</sub> Resuspension Vol. = ODV / 50			
	OD <sub>600</sub> = 6 V <sub>c</sub> = 500 mL ODV <sub>max</sub> = 3.000 Res. Vol. = 60 mL	OD <sub>600</sub> = 6 V <sub>c</sub> = 2,500 mL ODV <sub>max</sub> = 15.000 Res. Vol. = 300 mL	OD <sub>600</sub> = 6 V <sub>c</sub> = 5,000 mL ODV <sub>max</sub> = 30.000 Res. Vol. = 600 mL	OD <sub>600</sub> = 6 V <sub>c</sub> = 9,000 mL ODV <sub>max</sub> = 54.000 Res. Vol. = 1,080 mL
	Pellet the cells by centrifugation at <b>4,500–6,000 × g</b> for <b>≥ 10 min</b> at <b>4 °C</b> and carefully discard the supernatant completely.			
	At this stage it is recommended to perform an analytical check (see section 8.1) to optimize utilization of the NucleoBond® RS column.			
	<i>Note:</i> 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.			

	RS 10	RS 50	RS 100	RS 200
<b>4</b>	<b>Resuspension (Buffer RES-EF)</b>			
	Carefully resuspend the cell pellet completely in <b>Resuspension Buffer RES-EF (supplemented with RNase A)</b> in a suitable wide-mouth bottle with screw cap by shaking the suspension for 5–10 minutes. Ensure that the bacterial pellet is fully resuspended and free of any aggregates. Efficient cell lysis requires a homogeneous suspension!			
	Alternatively, the suspension can be stirred at low speed (approx. 30 rpm) for approx. one hour at RT.			
	<i>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 <b>additional buffer (see ordering information in section 9.2)</b>.</i>			
	<i>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).</i>			
	60 mL RES-EF	300 mL RES-EF	600 mL RES-EF	1,080 mL RES-EF
<b>5</b>	<b>Cell lysis (Buffer LYS-EF)</b>			
	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 <b>inverting</b> the flask <b>5–6 times</b> , to obtain a clear, highly viscous solution.			
	<b>Do not vortex the resulting lysate</b> , since this may release contaminating chromosomal DNA from cellular debris into the suspension. Incubate the mixture at room temperature for approx. 4–5 minutes.			
<b>!</b>	<i>Note: Prolonged exposure to alkaline conditions may irreversibly denature and degrade plasmid DNA and release contaminating chromosomal DNA into the lysate.</i>			
	<i>Note: 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)</i>			
	60 mL LYS-EF	300 mL LYS-EF	600 mL LYS-EF	1,080 mL LYS-EF

	RS 10	RS 50	RS 100	RS 200
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**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.



*Note:* In order to achieve optimal mixing, it is recommended that the container used for this step contain no more than two-thirds of its capacity. 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.

*Note:* 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 neutralization).

60 mL NEU-EF	300 mL NEU-EF	600 mL NEU-EF	1,080 mL NEU-EF
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**Incubate the crude lysate on ice for at least 15 minutes.**

> 15 min

**Equilibration of the column**

**7** 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.

*Note:* 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

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	RS 10	RS 50	RS 100	RS 200
<b>8</b>	<b>Clarification of lysate</b>			
	<p>Perform a lysate clarification that results in a 0.45 µm filtered lysate. Use the specified filtration procedures or an equivalent procedure that provides the same result. However, keep shear forces as low as possible to minimize DNA damage and contamination.</p> <p>Lysate clarification can be performed as a one-step procedure using <b>NucleoBond® Bottle Top Filters Type 2</b>. Connect a Bottle Top Filter Type 2 to a clean vacuum flask and a vacuum source.</p> <p>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. Discard the NucleoBond® Bottle Top Filter. Repeat the process, preferably using fresh Bottle Top Filters, until the crude lysate is completely filtered</p> <p>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 information in section 9.2).</p> <p><i>Note: 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).</i></p> <p><i>Tip:</i> 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 NucleoBond® Bottle Top Filter Type 2. The filters can also be rinsed with BMC-EF buffer after filtration.</p>			

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RS 10	RS 50	RS 100	RS 200
<b>Alternative clarification procedure:</b>			
<p>Three-stage capsule filter filtration: <b>Stage I (Pre-filtration):</b> Connect a ~ <b>20 µm filter capsule</b> 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.</p> <p>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 phase is pumped first onto the filter capsule, leaving the floating precipitate as the last part to be filtered. To do so, simply push the end of the tube all the way down to the bottom of the lysate flask.</p> <p>Using a flow rate of 20 mL/min, pump the raw lysate through the 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. The filtrate will still appear cloudy. Stop filtration if a break-through of particles occurs. Replace filter capsule in this case.</p> <p><i>Tip: Once the lysate is completely loaded, rinse the capsule filter with BMC-EF buffer for complete collection of remaining lysate in capsule filter. Centrifugation (≥ 4,500 x g, 30 minutes, 2–8 °C) can replace pre-filtration, but it is advisable to perform a pre-filtration to prolong the life of the fine filter.</i></p> <p><b>Stage II (Fine-filtration):</b> Replace the 20 µm pre-filter capsule with a ~ <b>1–5 µm filter capsule</b>. 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 fine-filter at a flow rate of <b>20 mL/min</b> and collect the flow-through.</p> <p><i>Tip: Once the lysate is completely loaded, rinse the capsule filter with BMC-EF buffer for complete collection of remaining lysate in capsule filter. Centrifugation (≥ 4,500 x g, 30 minutes, and 2–8 °C) can replace fine-filtration, but it is advisable to perform fine-filtration to prolong the life of the sterile filter.</i></p> <p><b>Stage III (Sterile-filtration):</b> Replace the glassfiber filter capsule with a <b>0.45 µm PES sterile filter capsule</b>. 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.</p> <p><i>Tip: Once the lysate is completely loaded, rinse the capsule filter with BMC-EF buffer for complete collection of remaining lysate in capsule filter.</i></p> <p><i>Note: 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.</i></p>			
5 mL / min	10 mL / min	10 mL / min	10 mL / min

	RS 10	RS 50	RS 100	RS 200
<b>9 Loading of lysate</b>				
	<p>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.</p> <p><i>Note: You may wish to save some or all of the flow through for analysis.</i></p> <p><b>!</b> <i>Note: If the column runs dry, rehydrate the column bed by re-equilibrating the column with EQ-EF buffer and continue with the protocol as normal.</i></p>			
	5 mL/min	10 mL/min	10 mL/min	10 mL/min
<b>10 1<sup>st</sup> Wash with Buffer ENTO-EF</b>				
	<p>Wash the NucleoBond® RS column with <b>endotoxin removal buffer ENTO-EF</b>.</p> <p><i>Note: If the column runs dry, rehydrate the column bed by re-equilibrating the column with EQ-EF buffer and continue with the protocol as normal.</i></p> <p><b>!</b></p>			
	30 mL ENTO-EF 5 mL/min	150 mL ENTO-EF 10 mL/min	300 mL ENTO-EF 10 mL/min	550 mL ENTO-EF 10 mL/min
<b>11 2<sup>nd</sup> Wash with Buffer WASH-EF</b>				
	<p>Wash the NucleoBond® RS column with <b>wash buffer WASH -EF</b>.</p> <p><i>Note: If the column runs dry, rehydrate the column bed by re-equilibrating the column with EQ-EF buffer and continue with the protocol as normal.</i></p> <p><b>!</b></p>			
	30 mL WASH-EF 5 mL/min	150 mL WASH-EF 10 mL/min	300 mL WASH-EF 10 mL/min	550 mL WASH-EF 10 mL/min
<b>12 Elution (Buffer ELU-EF)</b>				
	<p>Elute the plasmid DNA with <b>Elution buffer ELU-EF</b>. Collect the eluate at room temperature in an oven-baked glass or endotoxin-free plastic vessel.</p> <p>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.</p> <p><i>Note: If possible, continuously check the plasmid concentration at <math>A_{260}</math> during elution to obtain maximum yield and concentration by adjusting the elution volume. Store the eluate on ice during elution.</i></p>			

RS 10	RS 50	RS 100	RS 200
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

### 13 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.**

Incubate for 2 minutes at room temperature and pellet the DNA by centrifugation afterwards ( $\geq 4,500 \times g$  for  $\geq 15$  min at  $\leq$  room temperature, preferably at  $15,000 \times g$  for 30 min at  $4^\circ\text{C}$ ).

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

*Note:* Ensure that the temperature of the plasmid suspension does not exceed  $25^\circ\text{C}$  to avoid reduced yield. Temperatures below  $5^\circ\text{C}$  should be avoided to prevent salt precipitation.

#### 0.7 volumes of isopropanol

21 mL	84 mL	175 mL	280 mL
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### 14 Washing and drying (70 % EtOH)

Add **70 % ethanol** at **room-temperature** to the pellet.

Mix briefly and centrifuge ( $\geq 4,500 \times g$ , preferably  $\geq 15,000 \times g$  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 or  $37^\circ\text{C}$** .

*Note:* Plasmid DNA might be harder to dissolve when over-dried.

*Note:* This step can optionally be repeated to reduce the risk of isopropanol carry-over.

5 mL	15 mL	25 mL	50 mL
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Until dry

### 15 Reconstitution (Buffer TE-EF or H<sub>2</sub>O-EF)

Dissolve the DNA pellet in an appropriate volume of endotoxin-free **Buffer TE-EF**.

Determine the plasmid yield by UV spectrophotometry.  
Confirm plasmid integrity by agarose gel electrophoresis.

*Note:* 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  $1 \text{ mg/mL}$ . 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).

var., @  $1 \text{ mg/mL}$

8–10 mL	40–50 mL	80–100 mL	170–190 mL
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### 8.3 NucleoBond® RS RNase free purification workflow

The NucleoBond® RS RNase free support protocol 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<sup>2</sup> (100 psi) during use. Use only oven-baked glassware or pyrogen-free plasticware to avoid contamination with endotoxins. This is especially important during the elution, precipitation, and reconstitution steps.

*Note: Additional NucleoBond® BottleTop Filter Type 2/Sterile-filter and additional BMC-EF buffer are needed for the preparation of the following RNase free support protocol.*

	RS 10	RS 50	RS 100	RS 200
1–7	Follow instructions steps 1–7 of the standard NucleoBond® RS purification workflow in chapter 8.2.			
	<b>Don't supplement RES-EF with RNase A!</b>			
8	<p><b>Clarification of lysate</b></p> <p>Perform a lysate clarification that results in at least &lt; 5 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.</p> <p>Lysate clarification can be performed as a one-step procedure using <b>NucleoBond® Bottle Top Filters Type 2</b>. Connect a Bottle Top Filter Type 2 to a clean vacuum flask and a vacuum source.</p> <p>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. Discard the NucleoBond® Bottle Top Filter. Repeat the process, preferably using fresh Bottle Top Filters, until the crude lysate is completely filtered.</p> <p><i>Note: 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).</i></p> <p><i>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 NucleoBond® Bottle Top Filter Type 2. The filters can also be rinsed with BMC-EF buffer after filtration.</i></p> <p>The clarified lysate can be stored on ice for several hours. <b>If precipitates continue to appear, re-filter the lysate before applying it to the NucleoBond® RS column.</b> We recommend the use of a 0.45 µm CA filter (e.g. NucleoBond® Bottle Top Filter Type 2, see ordering information in section 9.2).</p>			

RS 10	RS 50	RS 100	RS 200
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**Alternative procedure:**

**Three-stage capsule filter filtration: Stage I (Pre-filtration):** Connect a ~ **20 µm 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.

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 phase is pumped first onto the filter capsule, leaving the floating precipitate as the last part to be filtered. To do so, simply push the end of the tube all the way down to the bottom of the lysate flask.

Using a flow rate of **20 mL/min**, pump the raw lysate through the 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. The filtrate will still appear cloudy. Stop filtration if a break-through of particles occurs. Replace filter capsule in this case.

*Tip: Once the lysate is completely loaded, rinse the capsule filter with BMC-EF buffer for complete collection of remaining lysate in capsule filter. Centrifugation ( $\geq 4,500 \times g$ , 30 minutes, 2 –8 °C) can replace pre-filtration, but it is advisable to perform pre-filtration to prolong the life of the fine filter.*

5 mL/min	10 mL/min	10 mL/min	10 mL/min
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**9 Precipitation of RNA**

Add 0.3 (v/v) isopropanol to the clarified lysate and mix well. The lysate will turn cloudy.

**Incubate the cloudy lysate for at least 15 min on ice!**

**Note: Precipitates will form and accumulate on the bottom ground.**

	RS 10	RS 50	RS 100	RS 200
10	<b>Removal of RNA precipitate</b>			
	<p><b>Use another</b> a NucleoBond® Bottle Top Filter Type 2 in order to remove the precipitated RNA. <i>The flowrate may decrease faster compared to previous filtration step.</i></p>			
	<p><b>!</b> <i><u>Note:</u> It is extremely important to perform a final filtration step with 0.45 µm pore size. Doing not so may increase the risk of not removing precipitated RNA completely and clogging the NucleoBond® RS column.</i></p>			
	<p><b>Alternative procedure continued:</b></p>			
	<p><b>Stage II (Fine-filtration):</b> Replace the 20 µm filter capsule with a ~ <b>1 – 5 µm filter capsule</b>. 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 filter at a flow rate of <b>20 mL/min</b> and collect the flow-through.</p>			
	<p><i>Tip: Once the lysate is completely loaded, rinse the capsule filter with BMC-EF buffer for complete collection of remaining lysate in capsule filter. Centrifugation (<math>\geq 4,500 \times g</math>, 30 minutes, and 2–8 °C) can replace fine-filtration.</i></p>			
	<p><b>Stage III (RNA-removal &amp; Sterile-filtration):</b> Use a <b>0.45 µm sterile filter capsule to remove RNA and leftover lysate particles</b>. Replace the tubing and adapters accordingly. Please keep in mind the specifications of the filter, particularly the maximum pressure limit. Pump the lysate through the sterile filter at a flow rate of 20 mL/min and collect the flow-through.</p>			
	<p><b>!</b> <i>Tip: Once the lysate is completely loaded, rinse the capsule filter with BMC-EF buffer for complete collection of remaining lysate in capsule filter.</i></p>			
	<p><i><u>Note:</u> 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. Doing not so may increase the risk of not removing precipitated RNA completely and residual particles may clog the NucleoBond® RS column.</i></p>			
11	<b>Precipitation of plasmid DNA.</b>			
	<p>Precipitate the pDNA by adding isopropanol to a total ipa volume of 0.5–0.7 (v/v). This is achieved by adding 0.16 (v/v) isopropanol to the present lysate-isopropanol mixture volume.</p>			
	<p>Mix thoroughly by shaking.</p>			
	<p>Incubate for 2 minutes at room temperature, and pellet the DNA by centrifugation (<math>\geq 4,500 \times g</math> for <math>\geq 15</math> min at <math>\leq</math> room temperature, preferably at <math>15,000 \times g</math> for 30 min at 4 °C).</p>			
	<p><b>Carefully discard the supernatant. Take care not to discard the DNA pellet.</b></p>			

	RS 10	RS 50	RS 100	RS 200
<b>12</b>	<b>Washing and drying (70 % EtOH)</b>			
	Add 70 % ethanol at room-temperature to the pellet			
	Mix briefly and centrifuge ( $\geq 4,500 \times g$ , preferably $\geq 15,000 \times g$ for 5 min at room temperature). Carefully remove ethanol completely with a pipette tip. Allow the pellet to dry at room temperature or 37 °C for 15–30 minutes.			
	<b>Note: Plasmid DNA might be hard to dissolve if over-dried.</b>			
	5 mL	15 mL	25 mL	50 mL
	Until dry			
<b>13</b>	<b>Reconstitution of plasmid DNA until pellet dissolved</b>			
	Dissolve the crude DNA pellet in an appropriate volume of BMC-EF. It is recommended to choose the resuspension volume of initial lysate volume.			
	<i>Note: Concentrating plasmid DNA by using less than the original volume of lysate may reduce pDNA binding to the column, resulting in loss of plasmid.</i>			
	<i>Note: Store the BMC-EF Puffer in the refrigerator at 4–8 °C if not used. Buffer BMC-EF does not need to regain RT before usage. Do not exceed 24 °C/75°F temperature during the workflow!</i>			
	<i>Note: Depending on the type of container, dissolve the pellet by gentle pipetting, vortexing periodically or constant spinning for 10–180 min (3D shaker).</i>			
<b>14</b>	<b>Loading of lysate</b>			
	Load the cleared lysate onto the NucleoBond® RS column like instructed in standard protocol (8.2 step 9).			
	5 mL / min	10 mL / min	10 mL / min	10 mL / min
<b>15</b>	<b>1<sup>st</sup> Wash with Buffer ENTO-EF</b>			
<b>!</b>	Wash the NucleoBond® RS column with <b>endotoxin removal buffer ENTO-EF</b> .			
<b>■</b>	<i>Note: If the column runs dry, rehydrate the column bed by re-equilibrating the column with EQ-EF buffer and continue with the protocol as normal.</i>			
	75 mL ENTO-EF	375 mL ENTO-EF	750 mL ENTO-EF	1380 mL ENTO-EF
	5 mL / min	10 mL / min	10 mL / min	10 mL / min
<b>16</b>	<b>2<sup>nd</sup> Wash with Buffer WASH-EF</b>			
<b>!</b>	Wash the NucleoBond® RS column with <b>wash buffer WASH -EF</b> .			
<b>■</b>	<i>Note: If the column runs dry, rehydrate the column bed by re-equilibrating the column with EQ-EF buffer and continue with the protocol as normal.</i>			
	75 mL WASH-EF	375 mL WASH-EF	750 mL WASH-EF	1380 mL WASH-EF
	5 mL / min	10 mL / min	10 mL / min	10 mL / min
<b>17 – 20</b>	Follow the standard protocols instructions for further processing (8.2 steps 12 – 15).			

## 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 issues.

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
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No or low plasmid DNA yield	<p><i>Plasmid did not propagate</i></p> <ul style="list-style-type: none"> <li>• Check plasmid content in the cleared lysate. Use colonies from fresh plates for inoculation and add fresh selective antibiotic to plates and media.</li> <li>• Estimate plasmid content prior to large purifications by a quick NucleoSpin® Plasmid preparation. Alternatively, a preparation using NucleoBond® Xtra Midi is also applicable.</li> </ul>
	<p><i>Alkaline lysis was inefficient</i></p> <ul style="list-style-type: none"> <li>• 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.</li> <li>• 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.</li> </ul>
	<p><i>SDS- or other precipitates are present in the sample</i></p> <ul style="list-style-type: none"> <li>• 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.</li> </ul>
	<p><i>Sample/lysate is too viscous</i></p> <ul style="list-style-type: none"> <li>• Too much cell mass was used. Refer to section 5.3–5.7 regarding recommended culture volumes and lysis buffer volumes.</li> <li>• 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.</li> </ul>
	<p><i>pH or salt concentrations of buffers are too high</i></p> <ul style="list-style-type: none"> <li>• 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.</li> <li>• Column overloaded with nucleic acids</li> <li>• 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.</li> </ul>
	<p><i>Flow rates too high</i></p> <ul style="list-style-type: none"> <li>• Do not exceed recommended flow rates for loading and eluting the plasmid DNA.</li> </ul>

Problem	Possible cause and suggestions
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<p>NucleoBond® RS Column is blocked or very slow</p>	<p><i>Sample is too viscous</i></p> <ul style="list-style-type: none"> <li>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.</li> <li>Make sure to mix well after neutralization to completely precipitate SDS and chromosomal DNA.</li> </ul>
	<p><i>Lysate was not cleared completely</i></p> <ul style="list-style-type: none"> <li>Centrifuge at higher speed or for a longer period of time.</li> <li>The filtration capsule or NucleoBond® Bottle Top Filter Type 2 may have broken through. Change the capsule in time.</li> <li>Precipitates occur during storage. Clear lysate again before loading the column.</li> </ul>
	<p><i>Column overloaded with nucleic acids</i></p> <ul style="list-style-type: none"> <li>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</li> </ul> <p><i>High back pressure during purification</i></p> <ul style="list-style-type: none"> <li>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).</li> <li>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.</li> </ul> <p>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.</p>

Problem	Possible cause and suggestions
Genomic DNA contamination of plasmid DNA	<p data-bbox="300 212 575 231"><i>Lysis treatment was too harsh</i></p> <ul data-bbox="300 248 882 268" style="list-style-type: none"> <li data-bbox="300 248 882 268">• Make sure not to lyse in Buffer LYS-EF for more than 5 min.</li> </ul> <p data-bbox="300 285 984 331"><i>Lysate was mixed too vigorously or vortexed after lysis, allowing genomic DNA to shear off into lysis buffer</i></p> <ul data-bbox="300 349 984 459" style="list-style-type: none"> <li data-bbox="300 349 984 395">• Invert tube for only 5 times and mix gently. Do not vortex after addition of Buffer LYS-EF.</li> <li data-bbox="300 413 984 459">• Use larger tubes/flasks or reduce culture volume if the lysate is too viscous to mix properly.</li> </ul>
	<p data-bbox="300 483 586 502"><i>RNase digestion was inefficient</i></p> <ul data-bbox="300 520 917 592" style="list-style-type: none"> <li data-bbox="300 520 917 592">• 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.</li> </ul> <p data-bbox="300 609 754 628"><i>pH or salt concentration of wash buffer is too low.</i></p> <ul data-bbox="300 646 984 718" style="list-style-type: none"> <li data-bbox="300 646 984 718">• 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.</li> </ul> <p data-bbox="300 735 766 754"><i>Wash step with Buffer WASH-EF was not sufficient</i></p> <ul data-bbox="300 772 984 818" style="list-style-type: none"> <li data-bbox="300 772 984 818">• Double or triple washing volume with Buffer WASH-EF. Additional Buffer WASH-EF can be ordered separately (see ordering information).</li> </ul> <p data-bbox="300 836 760 855"><i>RNase free protocol: Sterile-filtration unsuccessful.</i></p> <ul data-bbox="300 873 934 919" style="list-style-type: none"> <li data-bbox="300 873 934 919">• Pay attention to filter breakthroughs. If necessary, filter again as a precaution.</li> </ul> <p data-bbox="300 936 782 956"><i>RNase free protocol: RNA precipitation unsuccessful</i></p> <ul data-bbox="300 973 855 1082" style="list-style-type: none"> <li data-bbox="300 973 855 1045">• Add Isopropanol in range of 0.3 – 0.35 (v/v) total volume: Add less isopropanol: RNA doesn't precipitate. Add more isopropanol: pDNA will be lost.</li> <li data-bbox="300 1062 822 1082">• Centrifuge for longer periods of time at higher speed.</li> </ul>
Low purity ( $A_{260}/A_{280} < 1.8$ )	<p data-bbox="300 1106 852 1125"><i>Only minimal amounts of DNA were loaded onto the column</i></p> <ul data-bbox="300 1142 949 1225" style="list-style-type: none"> <li data-bbox="300 1142 949 1189">• Too much free binding capacity requires more extensive washing – double washing step with Buffer ENTO-EF.</li> <li data-bbox="300 1206 580 1225">• Reduce lysis time &lt; 5 min.</li> </ul>

Problem	Possible cause and suggestions
No nucleic acid pellet formed after precipitation	<p data-bbox="300 212 432 233"><i>Pellet was lost</i></p> <ul data-bbox="300 248 981 323" style="list-style-type: none"> <li>• 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.</li> </ul> <p data-bbox="300 339 826 360"><i>Plasmid DNA might be smeared over the wall of the tube.</i></p> <ul data-bbox="300 376 911 424" style="list-style-type: none"> <li>• Dissolve DNA with an appropriate volume of endotoxin-free reconstitution buffer by rolling the container for at least 30 min.</li> </ul> <p data-bbox="300 440 583 461"><i>Nucleic acid did not precipitate</i></p> <ul data-bbox="300 477 981 563" style="list-style-type: none"> <li>• Check type and volumes of precipitating solvent. Make sure to use at least 0.7 volumes of isopropanol and mix thoroughly</li> <li>• Centrifuge for longer periods of time at higher speed</li> </ul> <p data-bbox="300 579 981 627"><i>RNase free protocol: Isopropanol addition for RNA precipitation was too high.</i></p> <ul data-bbox="300 643 981 683" style="list-style-type: none"> <li>• Add Isopropanol in range of 0.3–0.35 (v/v). Add more and pDNA will be lost.</li> </ul>
Nucleic acid pellet is opaque or white instead of clear and glassy	<p data-bbox="300 707 510 727"><i>Co-precipitation of salt</i></p> <ul data-bbox="300 743 981 935" style="list-style-type: none"> <li>• 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.</li> <li>• 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.</li> </ul>
Nucleic acid pellet does not resuspend in buffer	<p data-bbox="300 959 493 979"><i>Pellet was over-dried</i></p> <ul data-bbox="300 995 981 1070" style="list-style-type: none"> <li>• 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).</li> </ul> <p data-bbox="300 1086 684 1107"><i>Co-precipitation of salt or residual alcohol</i></p> <ul data-bbox="300 1123 981 1171" style="list-style-type: none"> <li>• Wash the pellet again with 70 % ethanol, or increase the reconstitution buffer volume.</li> </ul> <p data-bbox="300 1187 645 1208"><i>Insoluble particles in redissolved DNA</i></p> <ul data-bbox="300 1224 981 1297" style="list-style-type: none"> <li>• Centrifuge the redissolved DNA to pellet the insoluble particles and transfer supernatant to a new tube. Insoluble particles do not affect DNA quality.</li> </ul>

Problem	Possible cause and suggestions
Purified plasmid does not perform well in subsequent reactions	<i>Plasmid DNA is contaminated with chromosomal DNA or RNA</i>
	<ul style="list-style-type: none"> <li>• Refer to the detailed troubleshooting above.</li> </ul>
	<i>Plasmid DNA is contaminated with residual alcohol</i>
	<ul style="list-style-type: none"> <li>• 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 and dry DNA pellet completely.</li> </ul>
	<i>DNA is degraded</i>
	<ul style="list-style-type: none"> <li>• Make sure that your entire equipment (pipettes, centrifuge tubes, etc.) is clean and nuclease-free.</li> </ul>
	<ul style="list-style-type: none"> <li>• Do not lyse the sample with Buffer LYS-EF for more than 5 min.</li> </ul>
	<i>DNA is irreversibly denatured</i>
	<ul style="list-style-type: none"> <li>• 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.</li> </ul>
	<i>Endotoxin level is too high</i>
Endotoxin level is too high	<ul style="list-style-type: none"> <li>• Refer to the detailed troubleshooting below.</li> </ul>
	<i>See section 4.3 for recommendations for work in endotoxin-free environments</i>
	<i>Too much cell mass was used</i>
	<ul style="list-style-type: none"> <li>• Decrease cell mass or better increase lysis buffer volumes.</li> </ul>
	<i>Inefficient endotoxin removal</i>
	<ul style="list-style-type: none"> <li>• Apply all washing buffers in the correct order: ENTO-EF, WASH-EF.</li> <li>• Increase washing volume of Buffer ENTO-EF by 25 – 50 %.</li> </ul>
	<i>Contamination of DNA product after purification</i>
	<ul style="list-style-type: none"> <li>• 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.</li> </ul>
	<ul style="list-style-type: none"> <li>• Use only the tested endotoxin-free buffers.</li> </ul>

## 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 <sub>2</sub> O-EF (nuclease free)	740798.1	1000 mL
BMC-EF	740382.1000 / .3000	1000 mL / 3000 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 customer 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.

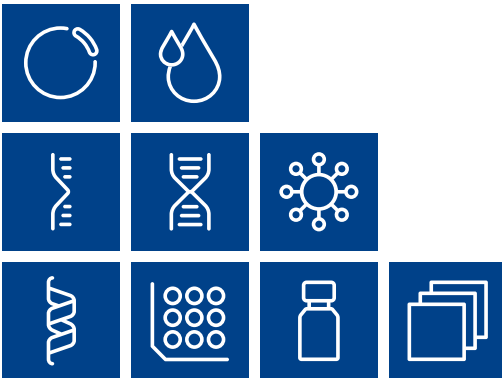
Products and their application are subject to change. Therefore, please contact our Technical Service Team for the latest information on MACHEREY-NAGEL products. You may also contact your local distributor for general scientific information. Descriptions in MACHEREY-NAGEL literature are provided for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

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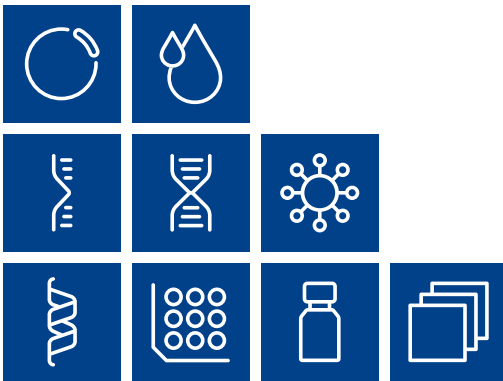
Please contact:  
MACHEREY-NAGEL GmbH & Co. KG  
Tel.: +49 24 21 969-333  
support@mn-net.com

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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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Clean up  
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