NucleoBond® RS RNase free support protocol

This protocol is only a supplement to the products general user manual. Please refer to the product manual for more detailed information regarding safety instructions, product-specific disclaimers, and especially preparations needed before starting the procedure. The latest version of the user manual is available at ***www.mn-net.com/usermanuals*** *or can be requested from our technical service (tech-bio@mn-net.com). Material safety data sheets (MSDS) can be downloaded from* ***www.mn-net.com/MSDS****.*

We have developed an additional RNase-free support protocol for the NucleoBond® RS columns. This is particularly important for emerging applications such as mRNA-based vaccines and gene therapy treatments, which rely heavily on high-quality plasmid DNA as starting material. The RNase free 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. However, significant adjustments occur in the lysate clarification process. It is advised to read the entire user manual before applying the support protocol.

NucleoBond® RS RNase free purification workflow

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

The 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 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	**Preparation of starter culture**			
	Inoculate a starter culture 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.			

	RS 10	RS 50	RS 100	RS 200

2 **Preparation of the fermentation culture**

Prepare an overnight bacterial culture by diluting an appropriate volume of starter culture into the appropriate volume of medium with selected antibiotics. Grow the culture overnight (12 – 16 h).

! *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).*

V_c = 500 mL	V_c = 2,500 mL	V_c = 5,000 mL	V_c = 9,000 mL

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.

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

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.

	RS 10	RS 50	RS 100	RS 200

4 Resuspension (Buffer RES-EF)

Carefully resuspend the cell pellet completely in **Resuspension Buffer RES-EF (no RNase A supplemented)** 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 one hour at RT.

Note: It is 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

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 not vortex 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.

Note: Prolonged exposure to alkaline conditions may irreversibly denature and degrade plasmid DNA and release contaminating chromosomal DNA into the lysate.

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)

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

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: 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.*

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

! **Incubate the crude lysate on ice for at least 15 minutes.**

> 15 min

7 **Equilibration of the column**

! 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

	RS 10	RS 50	RS 100	RS 200

8 **Clarification of lysate**

Perform a lysate clarification that results in at least < 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.

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. Discard the NucleoBond® Bottle Top Filter. Repeat the process, preferably using fresh Bottle Top Filters, until the crude lysate is completely filtered.

! 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).

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).

***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.*

RS 10	RS 50	RS 100	RS 200

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 (≥ 4,500 x g, 30 minutes, 2 – 8 °C) can replace pre-filtration.

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 **Removal of RNA precipitate**

Use a NucleoBond® Bottle Top Filter Type 2 in order to remove the precipitated RNA. *The flowrate may decrease faster compared to previous filtration step.*

! *Note: It is extremely important to perform a final filtration step with 0.45 µm pore size. Doing not so may increase the risk of leftover RNA and clogging the NucleoBond® RS column.*

Alternative procedure continued:

Stage II & III (RNA-removal & Sterile-filtration): Use a 5 µm and **0.45 µm filter capsule to remove RNA and leftover lysate particles**. Replace the tubing and adapters accordingly. Please keep in mind the specifications of the filter, particularly flowrate and maximum pressure limit. Pump the lysate through the sterile filter at a flow rate of 20 mL/min and collect the flow-through.

! *Tip: Once the lysate is completely loaded, rinse the capsule filter with BMC-EF buffer for complete collection of remaining lysate in capsule filter.*

Note: It is extremely important to perform a final filtration step with 0.45 µm pore size. Doing not so may increase the risk of leftover RNA and residual particles clogging the NucleoBond® RS column.

11 **Precipitation of plasmid DNA.**

Precipitate the pDNA by adding isopropanol to a total IPA volume of 0.5 – 0.7 (v/v).

This is accomplished by adding 0.16 (v/v) isopropanol to the current volume of the lysate-isopropanol mixture.

Incubate for 2 minutes at room temperature, and 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.

12 **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).

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

5 mL	15 mL	25 mL	50 mL
Until dry			

	RS 10	RS 50	RS 100	RS 200

13 **Reconstitution of plasmid DNA**

Dissolve the crude DNA pellet in an appropriate volume of BMC-EF.

Note: It is recommended to choose the resuspension volume of initial lysate volume. Depending on the type of container, dissolve by vortexing periodically or constant spinning for 10 – 180 min (3D shaker).

14 **Loading of lysate**

Load the re-suspended plasmid DNA onto the NucleoBond® RS column (equilibrated with buffer EQ-EF) at the indicated 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.

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

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.

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

15 **1st Wash with Buffer ENTO-EF**

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

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.

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

16 **2nd Wash with Buffer WASH-EF**

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

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.

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

	RS 10	RS 50	RS 100	RS 200

17 **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.

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.

Note: 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.

Note: 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

18 **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, 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.

Note: 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

	RS 10	RS 50	RS 100	RS 200
19	**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 or 37 °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
	Until dry			
20	**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.** 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 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 mg/mL			
	8 – 10 mL	40 – 50 mL	80 – 100 mL	170 – 190 mL

Troubleshooting

If you are experiencing difficulties please refer to the user manual chapter 9.