NucleoBond® Xtra Maxi

Support Protocol for Large Constructs

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MACHEREY-NAGEL

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1 NucleoBond® Xtra Maxi - Support protocol for purification of BACs and other large constructs

1.1 Field of application

This support protocol can be used in combination with **NucleoBond® Xtra Maxi Kits** which are intended for the purification of plasmid DNA. Due to the size and low-copy nature of large constructs like BACs, PACs, P1 constructs, or cosmids the purification procedure has to be modified for optimal results.

This support protocol cannot replace a specialized kit for purification of large constructs like **NucleoBond® Xtra BAC** (currently under development). However, it might help if large constructs are to be purified occasionally.

Note: The buffer volumes included in the NucleoBond® Xtra kit will not be sufficient if a larger number of BAC preps is performed. Additional buffer volumes can be ordered, see ordering information.

1.2 Growth of bacterial cultures

Yield and quality of large construct DNA highly depend on the **type of culture media** and antibiotics, the bacterial host strain, the type of DNA, size, and copy number but also on the **growth conditions**.

For large constructs like BAC, PAC, P1, or cosmid DNA LB (Luria-Bertani) medium is strongly recommended. The cell culture should be grown to an OD 600 of 1.5-2.0 to prevent starvation of the cells and degradation of large constructs. Therefore, incubate at 32-37°C preferably 12-16 h over night. Use flasks of at least three or four times the volume of the culture volume and shake at 200-250 rpm to provide a growth medium fully saturated with oxygen.

Alternatively to LB, rich media like 2xYT (Yeast/Tryptone), TB (Terrific Broth) or CircleGrow can be used. In this case bacteria grow faster, reach the stationary phase much earlier than in LB medium (≤ 12 h), and reach higher cell masses. However, this does not necessarily yield more DNA. Overgrowing a culture might lead to a higher percentage of dead or starving cells and the resulting BAC, PAC, P1, or cosmid DNA might be partially degraded or contaminated with chromosomal DNA. To find the optimal culture conditions, the culture medium and incubation times have to be optimized for each host strain/large construct combination individually.

Cell cultures should be grown under **antibiotic selection** at all times to ensure large construct DNA propagation. Without this selective pressure, cells tend to lose a large construct during cell division. Since bacteria grow much faster without the burden of an additional chromosome, they take over the culture rapidly and the yield goes down regardless of the cell mass. Table 1 gives information on concentrations of commonly used antibiotics.

Table 1: Information about antibiotics according to Maniatis*			
Antibiotic	**Stock solution (concentration)**	**Storage**	**Working concentration**
Ampicillin	50 mg/ml in H_2O	-20°C	50-100 µg/ml
Chloramphenicol	34 mg/ml in EtOH	-20°C	25-170 µg/ml
Kanamycin	10 mg/ml in H_2O	-20°C	10-50 µg/ml
Streptomycin	10 mg/ml in H_2O	-20°C	10-50 µg/ml
Tetracycline	5 mg/ml in EtOH	-20°C	10-50 µg/ml

The ***E. coli* host strain** mostly influences the quality of the large construct DNA. Whereas strains like DH5α or XL1-Blue usually produce high quality super-coiled DNA, other strains like e.g. HB101 with high levels of endonuclease activity might yield lower quality large construct giving poor results in downstream applications like enzymatic restriction or sequencing. Upon problems with host strains like Top10, HB101, or its derivatives like TG1, JM100 a change to DH5α or XL1-Blue should be considered.

The **type of large construct**, especially the **size and the origin of replication (ori)** has a crucial influence on DNA yield. In general, the larger the construct or the cloned insert is, the lower is the expected DNA yield due to a lower copy number. Cosmids or BACs e.g. are maintained at copy numbers <20 down to even only 1, whereas vectors based on e.g. pUC, pBluescript or pGEM can be present in several hundred copies per cell.

Thus, all the above mentioned factors should be taken into consideration if a particular DNA yield has to be achieved. Culture volume and lysis procedures have to be adjusted accordingly. The prep to prep variation of yield varies much more compared to high-copy plasmid purification.

* Maniatis T, Fritsch EF, Sambrook J: *Molecular cloning. A laboratory manual,* Cold Spring Harbor, Cold Spring, New York 1982.

1.3 Culture volume for large constructs

Due to the influence of growth media (TB, CircleGrow, 2xYT), growth conditions (shaking, temperature, incubation time), host strain or type of insert etc. the final amount of cells in a bacterial culture can vary over a wide range. By rule of thumb, 1 liter of *E. coli* culture with an OD_{600} of 1 consists of 1 x 10^{12} cells and yields about 1.5-1.8 g cell wet weight. Overnight cultures containing a large construct and grown in LB medium usually reach an OD_{600} of 3 under vigorous shaking in flasks. The expected DNA yield for a large construct is approximately 30-40 µg per gram cell wet weight.

It is therefore important to **adjust the cell mass rather than the culture volume** for the best large construct purification results. But since the cell mass or cell wet weight is tedious to determine it was replaced in this manual by the mathematical product of optical density at 600 nm (OD_{600}) and culture volume (Vol) - two variables that are much easier to measure.

ODV = OD_{600} x Vol [ml]

Note that for a correct OD determination the **culture samples have to be diluted if OD_{600} exceeds 0.5 in order to increase proportionally with cell mass**. For a well grown *E. coli* culture a 1:10 dilution with fresh culture medium is recommended. The measured OD_{600} is then multiplied with the dilution factor 10 to result in a theoretical OD_{600} value. This OD_{600} is used in Table 2 to determine the appropriate culture volume. Table 2 shows recommended ODVs and the corresponding pairs of OD_{600} and culture volume that can be easily handled using the standard kit protocol lysis buffer volumes. For example, if the OD_{600} of your *E. coli* culture is 2, use 900 ml culture for your BAC preparation.

Table 2: Recommended culture volumes for large constructs

NucleoBond® Xtra	Pellet wet weight	Rec. ODV = OD_{600} x Vol	Recommended culture volume for				
			OD_{600} = 2	OD_{600} = 3	OD_{600} = 4	OD_{600} = 5	OD_{600} = 6
Maxi	3.4 g	1800	900 ml	600 ml	450 ml	360 ml	300 ml

1.4 Cell lysis

The bacterial cell pellet is resuspended in Buffer RES and lysed by a sodium hydroxide/SDS treatment with Buffer LYS. Proteins, as well as chromosomal and large construct DNA are denatured under these conditions. RNA is degraded by DNase-free RNase A. **Large construct lysates have to be treated even more carefully than plasmid DNA lysates to avoid nicking and irreversible denaturation of the large constructs - therefore shaking or vortexing of the lysate must be avoided.** Neutralization Buffer NEU, containing potassium acetate, is then added to the lysate, causing SDS to precipitate as KDS (potassium dodecyl sulfate) and pulling down proteins, chromosomal DNA, and other cellular debris. The potassium acetate buffer also neutralizes the lysate. Circular DNA can revert to its native super-coiled structure and remains in solution.

The **NucleoBond® Xtra** buffer volumes according to the standard protocol are adjusted to ensure optimal lysis for culture volumes given in section 1.3, Table 2. Using too much cell material leads to inefficient cell lysis and precipitation and might reduce large construct yield and purity. Therefore, lysis buffer volumes should be increased when applying larger culture volumes.

By rule of thumb, calculate the necessary lysis buffer volumes for RES, LYS, and NEU as follows:

Vol. [ml] = Culture Volume [ml] x OD_{600} / 20

Note that this formula differs from the one for plasmid DNA to ensure maximum yield for large constructs.

For example, if 900 ml of a bacterial culture (OD_{600} = 2, ODV = 1800) is to be lysed, the appropriate volumes of lysis buffers RES, LYS, and NEU are 90 ml each. If more lysis buffer is needed than is provided with the kit, an additional buffer set including buffers RES, LYS, NEU, and RNase A can be ordered separately (see ordering information).

If less than the recommended amount of cells is to be used e.g. due to bad cell growth or limited culture volumes, less lysis buffer volumes can be used than given in the standard protocol (90 ml each). Calculate the necessary amount according to the above given formula. Note that the yield might then be significantly lower than 100 µg.

By using sufficient amounts of lysis buffer, lysis time can be limited to 3-4 minutes and should not exceed 5 minutes. Prolonged exposure to alkaline conditions can irreversibly denature and degrade especially large construct DNA and liberate contaminating chromosomal DNA into the lysate.

1.5 Difficult-to-lyse strains

For large construct purification of e.g. Gram-positive bacteria or strains with a more resistant cell wall it might be advantageous to start the preparation with a lysozyme treatment. Therefore, resuspend the cell pellet in Buffer RES containing **2 mg/ml lysozyme** and incubate at **37°C** for **30 minutes**. Proceed then with the lysis procedure according to the **NucleoBond® Xtra** standard protocol.

1.6 Washing of the column

The high salt concentration of the lysate prevents proteins and RNA from binding to the **NucleoBond® Xtra Column**. However, to remove all traces of contaminants and to purge the dead volume of the **NucleoBond® Xtra Column Filters** it is important to wash the column and the filter in two subsequent washing steps.

First apply Buffer EQU to the funnel rim of the filter to wash all residual lysate out of the filter onto the column. Do not just pour the buffer inside the filter. Then pull out and discard the column filter or remove the filter by turning the column upside down.

It is essential to wash the **NucleoBond® Xtra Column** without filter for a second time with Wash Buffer WASH. This ensures highest yields with best achievable purity. However, due to the expected small amounts of large construct DNA compared to the large binding capacity there is a lot of free space for RNA to be held back. If RNA contamination of large construct preparations is observed increase the WASH buffer volumes (double, triple). For additional Buffer WASH see ordering information.

1.7 Elution of large construct DNA

Elution is carried out under high-salt conditions and by a shift of pH from 7.0 to 9.0. Under these alkaline conditions the positive charge of the anion-exchange resin is neutralized and large construct DNA is released. To facilitate the dissociation of large construct DNA from the resin **ELU should be heated to 65-70°C**. The elution efficiency can be increased further by preventing the elution buffer from cooling down to fast. Therefore either **incubate the column at 50-60°C** during elution or apply the elution buffer in **smaller 1-2 ml portions (heated to 65-70°C)**.

1.8 Concentration of large construct DNA

For any downstream application it is necessary to precipitate the DNA and to remove salt and all traces of alcohol since they disturb or inhibit enzymatic activity needed for restriction or sequencing reactions.

All **NucleoBond® Xtra** eluates already contain enough salt for an isopropanol precipitation of DNA. Thus, the precipitation is started by directly adding 0.7 volumes of isopropanol. To prevent co-precipitation of salt, use **room-temperature (20-25°C) isopropanol** only and do not let the large construct DNA solution drop into a vial with isopropanol but **add isopropanol to the final eluate and mix immediately**.

DNA is then collected by centrifugation and washed with 70% ethanol according to the standard protocol. Attention should be paid at the drying step. All liquid should be allowed to evaporate completely at room temperature to reduce contamination of large construct DNA with ethanol. On the other hand any **over-drying** will render the DNA harder to dissolve and **should be avoided**.

To dissolve large constructs completely incubate in an appropriate volume of buffer **at 4°C over night**. Use only pipet tips with a large opening or **cut the tip** to increase the opening to prevent large constructs from shearing.

Concentration of BAC, PAC, or P1 constructs with **NucleoBond® Finalizer or Finalizer Large** is not recommended. The recovery drops with increasing size of the construct due to tighter binding of large DNA to the **NucleoBond® Finalizer** membrane (Use of NucleoBond® Finalizer is only recommended for vector sizes smaller than 50 kbp)!

2 NucleoBond® Xtra Maxi - Purification of BAC DNA

1 Prepare a starter culture

Inoculate a 3-5 ml starter culture of LB medium with a single colony picked from a freshly streaked agar plate. Make sure that plate and liquid culture contain the appropriate selective antibiotic to guarantee large construct propagation (see 1.2 for more information). Shake at 32-37°C and ~300 rpm for ~8 h.

2 Prepare large overnight cultures

Inoculate three 300 ml overnight cultures in 1000 ml Erlenmeyer flasks by diluting the starter culture 1/1000 into the given volumes of LB medium also containing the appropriate selective antibiotic. Refer to section 1.3 for larger culture volumes if the cultures are known to grow poorly.

Grow the cultures overnight at 32-37°C and 200-250 rpm for 12-16 h.

Note: To utilize the entire large binding capacity of the NucleoBond® Xtra Maxi Columns it is important to provide enough large construct DNA. If you are not sure about the large construct copy number and growth behavior of your host strain, increase the culture volume and decide in step 3 how much cells to use for the preparation. The culture volume recommended below is calculated for a final OD_{600} of around 2 and should yield around 100 µg of large construct DNA (see to section 1.3 for more information).

3 x 300 ml

3 Harvest bacterial cells

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

$V\ [ml] = 1800 / OD_{600}$

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

Note: It is of course possible to use more than the recommended amount of cells. In this case increase RES, LYS and NEU buffer volumes proportionally in steps 4, 5, and 7 (see section 1.4 for more information). Additional lysis buffer might have to be ordered separately (see ordering information for NucleoBond® Xtra Buffer Set I, section 3.2). It might be necessary to use a centrifuge for the lysate clarification in step 8 rather than the NucleoBond® Xtra Column Filters.

4 Resuspension (Buffer RES)

Resuspend the cell pellet completely in **Resuspension Buffer RES + RNase A** by pipetting up and down or vortexing the cells.

For an efficient cell lysis it is important that no clumps remain in the suspension.

Note: Increase RES buffer volume proportionally if more than the recommended cell mass is used (see section 1.4 for information on optimal cell lysis and section 1.5 regarding difficult-to-lyse strains).

90 ml

5 Cell lysis (Buffer LYS)

Check Lysis Buffer LYS for precipitated SDS prior to use. If a white precipitate is visible, warm the buffer for several minutes at 30-40°C until precipitate is dissolved completely. Cool buffer down to room temperature (20-25°C).

Add **Lysis Buffer LYS** to the suspension.

Mix gently by **inverting** the tube **5 times**. **Do not vortex** as this will shear and release contaminating chromosomal DNA from cellular debris into the suspension.

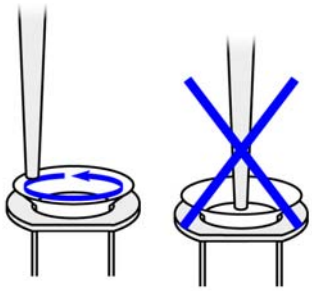
Incubate the mixture at room temperature (20-25°C) for **5 min**.

Note: Increase LYS buffer volume proportionally if more than the recommended cell mass is used (see section 1.4 for information on optimal cell lysis).

90 ml

6 Equilibration (Buffer EQU)

Equilibrate a NucleoBond® Xtra Maxi Column together with the inserted column filter with **Equilibration Buffer EQU**.



Apply the buffer onto the rim of the column filter as shown in the picture and make sure to wet the entire filter.

Allow the column to empty by gravity flow. The column does not run dry.

30 ml

7 Neutralization (Buffer NEU)

Add **Neutralization Buffer NEU** to the suspension and immediately mix the lysate gently by **inverting** the tube **10-15 times**. **Do not vortex.**

The flask or tube used for this step should not be filled more than two thirds to allow homogeneous mixing. Make sure to neutralize completely to precipitate all the protein and chromosomal DNA. The lysate should turn from a slimy, viscous consistency to a low viscosity, homogeneous suspension of an off-white flocculate.

Incubate lysate **on ice for 5 min**.

Note: Increase NEU buffer volume proportionally if more than the recommended cell mass is used (see section 1.4 for information on optimal cell lysis).

90 ml

8 Clarification and loading

Make sure to have a homogeneous suspension of the precipitate by **inverting the tube 3 times** directly before applying the lysate to the equilibrated NucleoBond® Xtra Column Filter to avoid clogging of the filter.

The lysate is simultaneously cleared and loaded onto the column. Refill the filter if more lysate has to be loaded than the filter is able to hold. Allow the column to empty by gravity flow.

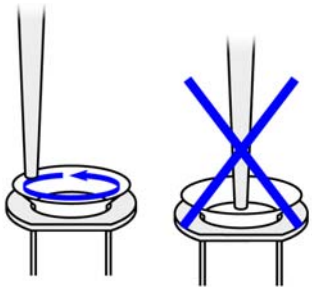
Alternative: The precipitate can be removed by centrifugation at ≥ 5,000 x *g* for at least 10 min, e.g. if more than the recommended cell mass was used. If the supernatant still contains suspended matter transfer it to a new tube and repeat the centrifugation, preferably at higher speed, or apply the lysate to the equilibrated NucleoBond® Xtra Column Filter.

This clarification step is extremely important since residual precipitate may clog the NucleoBond® Xtra Maxi Column. To load the column you can either apply the cleared lysate to the equilibrated filter or remove the unused filter beforehand. Allow the column to empty by gravity flow.

Note: You may want to save all or part of the flow-through for analysis (see section 3.1).

Optional: Final yield might be increased by reloading the lysate flow-through a second time especially if the amount of DNA is close to the binding capacity of the NucleoBond® Xtra Maxi Column.

9 Wash column filter and column (Buffer EQU)

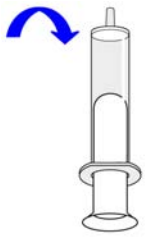


Wash the NucleoBond® Xtra Column Filter and NucleoBond® Xtra Maxi Column with **Equilibration Buffer EQU**.

Apply the buffer to the funnel shaped rim of the filter and make sure it is washing out the lysate which is remaining in the filter. Omitting this step or just pouring the buffer directly inside the funnel may reduce DNA yield.

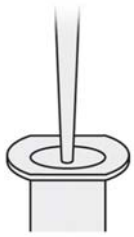
15 ml

10 Discard column filter



Either pull out the NucleoBond® Xtra Column Filter or discard it by turning the column upside down.

11 Wash column (Buffer WASH)



Wash the NucleoBond® Xtra Maxi Column with **Wash Buffer WASH**. It is important to remove the column filter before applying Buffer WASH to avoid a low purity.

45 ml

12 Elution (Buffer ELU)

Heat **Elution Buffer ELU** to 65-70°C.

Remove the waste container and place a 15 ml or 50 ml centrifuge tube (not provided) under the column. Elute the large construct DNA with hot **Elution Buffer ELU**.

Note: The elution efficiency can be increased by preventing the elution buffer from cooling down to fast. Therefore either incubate the column at 50-60°C during elution or apply the elution buffer in smaller 2-3 ml portions (heated to 65-70°C).

The overall yield can be increased even further by a second elution step with additional 10 ml of hot elution buffer.

Determine large construct yield by UV spectrophotometry before precipitating the DNA in order to adjust desired concentration of DNA in step 15 and calculate the recovery after precipitation.

15 ml

13 Precipitation

Add 0.7 volumes of **room-temperature isopropanol** to precipitate the eluted large construct DNA. Vortex well and let the mixture sit for **2 minutes**.

Centrifuge at ≥ **5,000 x *g*** for ≥**15 min** at ≤ **room temperature,** preferably at **15,000 x *g*** for **30 min** at **4°C**. Carefully discard the supernatant.

10.5 ml

14 Wash and dry DNA pellet

Add **room-temperature 70% ethanol** to the pellet and centrifuge at ≥ **5,000 x *g***, preferably ≥ **15,000 x *g*** for **5 min** at **room temperature** (20-25°C).

5 ml

Carefully remove ethanol completely from the tube with a **pipette tip**. Allow the pellet to dry completely at **room temperature** (20-25°C).

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

5-10 min

15 Reconstitute DNA

Add an appropriate volume of **buffer TE or sterile H_2O to** dissolve the pellet. Less than 500 µl can be applied if the yield is expected to be very low.

500-1000 µl

Ideally incubate over night at 4°C to dissolve BAC DNA completely.

Avoid frequent pipetting up and down since large DNA constructs are prone to shearing. Rather shake the tube gently and use only pipette tips with a large opening or cut the tip to increase the opening.

4°C over night

If the dissolved DNA pellet is very viscous, add more buffer TE or H_2O to ensure complete dissolving and a correct quantification.

Determine large construct yield by UV spectrophotometry.

3 Appendix

3.1 Troubleshooting

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

First, the bacterial culture has to be checked for sufficient growth (OD_{600}) in the presence of an appropriate selective antibiotic (Table 1, section 1.2). **Second**, aliquots of the cleared lysate, the flow-through, the combined washing steps (Buffer EQU and Buffer WASH), and the eluate should be kept for further analysis by agarose gel electrophoresis.

Choose at least 1000 µl of the cleared lysate, flow-through, and combined washing steps as well as 200 µl of the eluate.

Precipitate the nucleic acids by adding 0.7 volumes of isopropanol. Centrifuge the samples, wash the pellets using 70% ethanol, centrifuge again, remove supernatant, air dry for 10 minutes, dissolve the DNA in 30 µl TE buffer, pH 8.0, and run 20-30 µl on a 1% agarose gel.

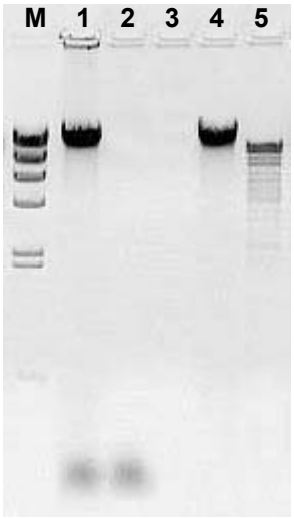
The exemplary gel picture (Figure 1) will help you to address the specific questions outlined in the following section more quickly and efficiently.

It shows e.g. the dominant large construct bands which should only be present in the eluate and in the lysate before loading to proof large construct production in your cell culture (lane 1). Large construct DNA found in the wash fractions, however, narrows down the problem to wrong or bad wash buffers (e.g. wrong pH, buffer components precipitated, evaporation of liquid due to wrong storage).

RNA might be visible as a broad band at the bottom of the gel for the lysate and the lysate flow-through samples (lanes 1 and 2). It might also occur in the wash fraction but must be absent in the eluate.

Genomic DNA should not be visible at all but would show up in the gel slot or right below indicating e.g. too harsh lysis conditions.

Figure 1 Exemplary analytical check of NucleoBond® Xtra Maxi purification of BAC samples
Large construct: 240 kbp BAC, bacterial strain: *E. coli* DH5α. 20 µl of each precipitated sample has been analyzed on a 1% agarose gel. Equal amounts of large construct DNA before (lane 1) and after (lane 4) purification using NucleoBond® Xtra Maxi are shown with a recovery of > 90%.



M: Marker λ *Hin*dIII
1: Cleared lysate, *large construct and degraded RNA*
2: Lysate flow-through, *no large construct DNA, but degraded RNA*
3: Wash flow-through, *no large construct DNA or residual RNA*
4: Eluate, *pure large construct DNA*
5: *Eco*RI restriction

Problem	Possible cause and suggestions
No or low large construct DNA yield	*Large construct did not propagate* • Check large construct content in the cleared lysate (see Figure 6). Use colonies from fresh plates for inoculation and add selective antibiotic to plates and media. *Alkaline lysis was inefficient* • Too much cell mass was used. Refer to section 1.3-1.4 regarding recommended culture volumes and lysis buffer volumes. Check large construct content in the cleared lysate (see Figure 1). • Check Buffer LYS 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 re-dissolved. *SDS- or other precipitates are present in the sample* • Load the crude lysate onto the NucleoBond® Xtra Column Filter inserted in the NucleoBond® Xtra Maxi Column. This ensures complete removal of SDS precipitates. 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® Xtra Maxi Column. *Sample/lysate is too viscous* • Too much cell mass was used. Refer to section 1.3-1.4 regarding recommended culture volumes and lysis buffer volumes. • Make sure to mix well after neutralization to completely precipitate SDS and chromosomal DNA. Otherwise, filtration efficiency and flow rate go down and SDS prevents DNA from binding to the column. *pH or salt concentrations of buffers are too high* • Check large construct content in the wash fractions (see Figure 1). Keep all buffers tightly closed. Check and adjust pH of Buffer EQU (pH 6.5) and ELU (pH 9.0) with HCl or NaOH if necessary.

Problem	Possible cause and suggestions
NucleoBond® Xtra Column Filter clogs during filtration	*Culture volumes are too large* • Refer to section 1.3-1.4 regarding recommended culture volumes and larger lysis buffer volumes. *Precipitate was not resuspended before loading* • Invert crude lysate at least 3 times directly before loading. *Incomplete precipitation step* • Make sure to mix well after neutralization to completely precipitate SDS and chromosomal DNA.
NucleoBond® Xtra Maxi Column is blocked or very slow	*Sample is too viscous* • Do NOT attempt to purify lysate prepared from a culture volume larger than recommended with standard lysis buffer volumes. Incomplete lysis does not only block the column but can also significantly reduce yields. Refer to section 1.3 for recommended culture volumes and section 1.4 for larger culture volumes and adjusted lysis buffer volumes. • Make sure to mix well after neutralization to completely precipitate SDS and chromosomal DNA. *Lysate was not cleared completely* • Use NucleoBond® Xtra Column Filter or centrifuge at higher speed or for a longer period of time. • Precipitates occur during storage. Clear lysate again before loading the column.
RNA contamination of large construct DNA	*RNase digestion was inefficient* • RNase was not added to Buffer RES or stored improperly. Add new RNase to Buffer RES (see section 3.2 for ordering information). *pH or salt concentration of wash buffer is too low* • Check RNA content in the wash fractions (see Figure 1). Keep all buffers tightly closed. Check pH of Buffer EQU (pH 6.5) and WASH (pH 7.0) and adjust with HCl or NaOH if necessary. • For BAC purifications the stringency of Buffer WASH can be increased by adjusting the pH to 7.5 in order to remove RNA more efficiently.

Problem	Possible cause and suggestions
Genomic DNA contamination of large construct DNA	*DNA visible in the pocket of an agarose gel was mistaken for genomic DNA* • Genomic DNA contamination is usually too low to be seen on an agarose gel. DNA in the gel loading pockets usually is denatured large construct DNA. Don't grow the culture to stationary phase. Allow for longer re-hybridization after neutralization (i.e. increase incubation after addition of Buffer NEU) and use more TE buffer or H_2O to dissolve large construct completely. *Lysis treatment was too harsh* • Make sure not to lyse in Buffer LYS for more than 5 min. *Lysate was mixed too vigorously or vortexed after lysis* • Invert tube for only 5 times. Do not vortex after addition of LYS. • Use larger tubes or reduce culture volumes for easier mixing. *Remaining small amounts of genomic DNA interfere with downstream application* • Digest linear DNA with Exonuclease (not provided). Note that not only genomic DNA will be digested but also linear or nicked large construct DNA. The overall yield will be significantly lower.
Low purity (A_{260}/A_{280} < 1.8)	*NucleoBond® Xtra Column Filter was not removed before second washing step* • Protein content too high due to inefficient washing. Remove the NucleoBond® Xtra Column Filter **before** performing the second washing step with Buffer WASH. *Buffer WASH was used instead of Buffer EQU for the first wash* • Buffer EQU has to be used to wash out the NucleoBond® Xtra Column Filter to avoid SDS carryover. *Only minimal amounts of DNA were loaded onto the column* • Excess free binding capacity requires more extensive washing – double washing step with Buffer WASH. • Reduce lysis time < 5 min.

Problem	Possible cause and suggestions
No nucleic acid pellet formed after precipitation	*Pellet was lost* • Handle the precipitate with care. Decant solutions carefully. Determine DNA yield in Buffer ELU in order to calculate the amount of large construct DNA that should be recovered after precipitation. *Large construct DNA might be smeared over the wall of the tube* • Dissolve DNA with an appropriate volume of reconstitution buffer by rolling the tube for at least 30 min, better several hours. *Nucleic acid did not precipitate* • Check type and volumes of precipitating solvent. Make sure to use at least 0.7 volumes of isopropanol and mix thoroughly. • Centrifuge for longer periods of time at higher speed.
Nucleic acid pellet is opaque or white instead of clear and glassy	*Co-precipitation of salt* • Check isopropanol purity, and perform precipitation at room temperature (20-25°C) but centrifuge at 4°C. Do not let the eluate drip from the column into isopropanol but add isopropanol to the final eluate and mix immediately. • Try to dissolve the pellet in Buffer WASH, and reload onto the same NucleoBond® Xtra Maxi Column. Wash the column several times with Buffer WASH before loading.
Nucleic acid pellet does not resuspend in buffer	*Pellet was over-dried* • Try to dissolve at higher temperatures for a longer period of time (e.g. 2 h at 37°C or overnight at RT), preferably under constant spinning (3D-shaker). *Co-precipitation of salt or residual alcohol* • Wash the pellet again with 70% ethanol, or increase the reconstitution buffer volume.

Problem	Possible cause and suggestions
Purified large construct does not perform well in subsequent reactions	*Large construct is contaminated with chromosomal DNA or RNA* • Refer to the detailed troubleshooting above. *Large construct is contaminated with residual alcohol* • Large construct pellet was not dried completely before dissolving. Precipitate DNA again by adding 1/10 volume of 3 M NaAc pH 5.0 and 0.7 volumes of isopropanol. Proceed with the precipitation protocol in this manual und dry DNA pellet completely.
Purified large construct does not perform well in subsequent reactions *(continued)*	*DNA is degraded* • Use only pipette tips with a large opening for dissolving large constructs to avoid shearing of the DNA, e.g. cut off 1 cm of the pipette tip to increase the opening. • Make sure that your entire equipment (pipettes, centrifuge tubes etc.) is clean and nuclease-free. • Do not lyse the sample with Buffer LYS for more than 5 min. *DNA is not completely re-hybridized* • Increase incubation after neutralization (addition of Buffer NEU). *DNA is not completely dissolved* • Use more TE buffer or H_2O to dissolve large construct DNA pellet after precipitation.

3.2 Ordering information

Product	Cat. No.	Pack of
NucleoBond® Xtra Maxi	740414.10/.50/.100	10/50/100 preps
NucleoBond® Xtra Combi Rack	740415	1
NucleoBond® Xtra Buffer Set I (Buffer RES, LYS, NEU, RNase A)	740417	1
Buffer RES (without RNase A)	740363.1000	1000 ml
Buffer LYS	740329.1000	1000 ml
Buffer NEU	740348.1000	1000 ml
Buffer EQU	740317.1000	1000 ml
Buffer WASH	740375.1000	1000 ml
Buffer ELU	740316.600	600 ml
RNase A	740505	100 mg
RNase A	740505.50	50 mg

3.3 Product use restriction / warranty

NucleoBond® Xtra Maxi kit components were developed, designed and sold **for research purposes only**. They are suitable ***for in vitro uses only***. No claim or representation is intended for its use to identify any specific organism or for clinical use (diagnostic, prognostic, therapeutic, or blood banking).

It is rather the responsibility of the user to verify the use of the **NucleoBond® Xtra Maxi** kit for a specific application range as the performance characteristic of this kit has not been verified to a specific organism.

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