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# Plasmid Preparation

For plasmid preparations you always start off with a fresh bacterial colony. For mini-preps, all strains can be used. TG1 bacteria ($recA^+$, fast growing, ideal for cloning), as well as the usual XL-1 blue bacteria. If you aim for plasmid DNA, which will be used for sequencing or transfections, being prepaired with a column, recA-, like XL-1 or DH5 should be used.

Mini-preps should be inoculated into 5ml of TB-medium, or optional in LB-medium, midi-preps into 30ml and for maxi preps 80ml of medium are sufficient (plasmids with low copy number require larger volumes). Make sure the antibiotics in your medium and the resistance of your plasmid are consistent with each other! The right concentrations for antibiotics are either 200µg/ml ampicillin or 100µg/ml kanamycin, for other antibiotics refer to Maniatis.

**A) Mini-preps, without phenol** (This is standard for restriction analysis of plasmids. For cloning, DNA prepared this way must be further purified by ph./ chl. extraction)

- Fill 1-2ml of bacterial culture into an Eppendorf tube and spin for 1 min at 8.000 rpm (RT). Discard supernatant. (Extra bacterial culture might be used for additional preps, with phenol chlorophorm extraction, eventually)
- **completely** resuspend in 400 µl P1 (add fresh RNAse, final conc. 100 µg/ml) by vortexing
- add 400 µl P2, gently mix by invert the tube 2-4 time until the soltuion is clear, then incubate at RT for 2-3 min, but max. 5min before proceeding to the next step
- add 400 µl P3, gently mix by inverting 2-4 time, until white precipitate forms
- spin for 10 min. at 14.000 rpm (RT) (incubation for 1-2h before hand is possible)
- transfer clear supernatant into a fresh tube and add 0.7 Vol. isopropanol. Mix well and spin 10 min at 14.000 rpm (RT). Discard supernatant. Add 500 µl 70 % EtOH, mix and spin 2 min max speed. Discard supernatant, short spin to completely remove left over EtOH, dry pellet (but not overdry) and resuspend pellet in 50 µl TE.
- 1- 5 µl DNA are sufficient for gel-analysis or restriction.

**Buffer composition** (Qiagen- Protocol or Maniatis):

P1: 50 mM Tris-Cl pH 8, 10 mM EDTA

P2: 200 mM NaOH, 1 % SDS

P3: 3 M KAc pH 5.5

TE: 10 mM Tris-Cl pH 8, 0.1 mM EDTA

**This DNA is sufficient for restrictions, however cloning or labeling require high quality DNA. To this end, a ph/chl. extraction is the next step. Applications like in vitro transcription and DNA sequencing demand even further purification via columns (Machery Nagel).**

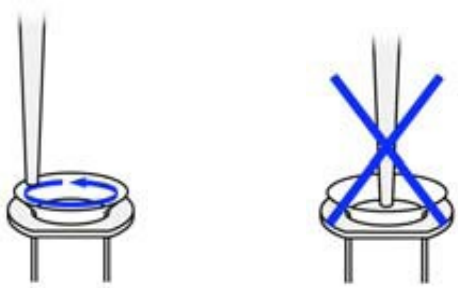
**Phenol/Chloroform purification of DNA:**

- Fill up the volume with TE up to 400 µl, add 1 µl Glykogen, 200 µl Phenol pH 7.5 and 200 µl chloroform. Vortex 20 sec and then spin for 2 min max speed.. Transfer supernatant into a new tube, be careful not to transfer the interphase or hints of phenol, otherwise repeat the previous step. Add 40 µl 3 M NaAc pH 5.5 an 800 µl 100% EtOH. Mix and spin 10 min at 14.000 rpm. Remove supernatant, wash DNA 1 x with 400 µl 70% EtOH, spin 5 min at 14.000 rpm. Completely remove EtOH and air dry pellet, then resuspend in 50 µl TE.
- 1- 5 µl DNA are sufficient for gel-analysis or restriction.
- To check DNA quality and amount, always run out uncut and linearized plasmid side by side.

# Plasmid preparation (Midi and Maxi preparations) with Machery&Nagel columns

**Columns are expensive! Before running a maxi column, remove 1ml of the bacterial culture and prepare plasmid with method A. If the yield is ok, continue as outlined below.**

- For Midi and Maxi preparation one single colony should be picked out of the middle with a sterile tooth-pick and inoculated into 30ml/ 80ml of TB-medium (optional in LB-medium) for Midis and Maxi Preps in a 250/ 500 ml-flask, respectively. Allow to grow for max. 14h at 300-350 rpm (37°C)
- Spin bacteria at 5.000 rpm (4°C). (Pellets can be frozen at –20°C.)
- **Resuspension:** Resuspend the cell pellet completely in 8ml/ 12ml **resuspension buffer RES + RNase A (0.06mg/ml)** by pipetting the cells up and down. For an efficient cell lysis it is important that no clumps remain in the suspension.
- **Cell lysis:** 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 8ml/ 12ml **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**.
- **Equilibration:** Equilibrate a NucleoBond® Xtra column together with the inserted column filter with 12ml/ 25ml **equilibration buffer EQU**. Apply the buffer onto the rim of the column filter as shown in the picture. Allow the column to empty by gravity flow and make sure to wet the entire filter. The column does not run dry.



- **Neutralization:** Add 8ml/ 12ml **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. Immediately proceed with centrifugation step. **An incubation of the lysate is not necessary.**

- Remove precipitate by centrifugation at 18.000 rpm for 10 min (4°C) in Beckmann centrifuge. Precool rotor by running at 1.000 rpm at 4°C). Use sterile polyallomer centrifugation tubes. If the supernatant still contains suspended matter floating on top, carefully remove with blue Gilson tip or a piece of tissue. Then apply the lysate to the equilibrated NucleoBond® Xtra column filter. This clarification step is extremely important since residual precipitate may clog the NucleoBond® Xtra 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.
- **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.
- **Wash column filter and column (equilibration buffer EQU)** Wash the NucleoBond® Xtra column filter and NucleoBond ® Xtra column with 5ml/ 15ml **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 plasmid yield (note picture above).
- **Discard column filter:** Either pull out the NucleoBond® Xtra column filter or discard it by turning the column upside down.
- **Wash column (washing buffer WASH):** Wash the NucleoBond® Xtra column with 8ml/ 25ml **washing buffer WASH**. It is important to remove the column filter before applying the washing buffer to avoid low purity.
- **Elution:** Elute the plasmid DNA with 5ml/ 15ml **elution buffer ELU**.
- **Precipitation:** Add 3,5ml/ 10,5ml **room-temperature isopropanol** to precipitate the eluted plasmid DNA. Vortex well and let the mixture sit for **2 minutes**. Centrifuge at **18.000 rpm** for **10 min** at **RT (Beckmann)**. Carefully discard the supernatant. (high speed centrifuge, as above!)
- **Wash and dry DNA pellet:** Add 2ml/ 5ml **room-temperature 70% ethanol** to the pellet and centrifuge at **18.000 rpm** for 10 **min** at **room temperature** (20°C). Carefully remove ethanol

completely from the tube with a pipette tip. Allow the pellet to dry at **room temperature** (20-25°C). *Note: Plasmid DNA might be harder to dissolve when over-dried.*

- **Reconstitute DNA:** Dissolve the DNA pellet in an appropriate volume of buffer TE. Depending on the type of centrifugation tube, dissolve under gentle pipetting up and down or constant spinning in a sufficient (100-500 µl) amount of buffer for 10-60 min. Determine plasmid yield by UV spectrophotometry. Confirm plasmid integrity by agarose gel electrophoresis. Load 1 and 2 µl of uncut and linearized DNA on gel. Determine quality by spectrophotometry at $OD_{260/280}$. This should be ≥ 1.8. 1 OD = 50 µg/ml of double stranded DNA.

**Note:**

- The purity of the DNA after this method is sufficient for most methods. For transfections of ES-cells, as well as in vitro transcripton the DNA is restricted, followed by extraction with 0.8Vol. ph./chl. and once with chl.. Finally, precipitated with 0.1 Vol. 3 M NaAc pH 5.5 and 2 Vol. EtOH and washed 2 x with 70 % EtOH (for in vitro transcription DNA is resuspend in RNAse-free water).

Expectation: ~200 µg plasmid out of 30 ml LB culture.

- Low copy plasmids or a low yield need to be discussed with the boss or an experienced co-worker!
- For some purposes, LPS need to be removed from DNA. Refer to special protocol to do this.

## Plasmid Maxi Prep
## Quiagen Kit

before use, verify plasmid content by mini prep of ~ 1 ml culture.

**Same handling procedure as above. The difference comes from different column buffers, as required by Qiagen.**

resuspend pellet in 10 ml P1
add 10 ml P2, mix, 5’ @ RT
add 10 ml P3, mix, 20’ @ 0°C
equilibrate column 10 ml QBT
filter (paper) lysate directly onto column
wash 2x 30 ml QC

elute 15 ml QF

add 10,5 ml isopropanol

centrifuge 20.000g @ 4°C, 30’

wash pellet with 5 ml 70% EtOH

air dry ~10-15 min.

resuspend in appropriate volumn of TE.