PLASMID ISOLATION FROM BACTERIA

Some fast step-by-step procedures tried out at the DSMZ

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Introduction

In virtually all bacterial species plasmids exist. These accessory genetic elements typically account for only a small fraction of a bacterial genome corresponding roughly to a range between 1 and 200 kb. Extremely large plasmids with sizes far beyond 200 kb are also known. Plasmids of more than 50 kb might be characterized as “large”, plasmids of less than 10 kb as “small”. The aim of this compilation is to describe some fast methods for small scale plasmid isolation leading to “crude lysates”, the quality of which being sufficient for analytical purposes, mainly agarose gel electrophoresis. Further downstream steps might require a higher quality of plasmid DNA and therefore, additional purification. By using few microliters of crude lysates for agarose gel electrophoresis, the electrophoretic separation allows conclusions on

* the presence of plasmid DNA,

* the number of different plasmid species,

* the determination of the molecular weight(s),

* the approximate copy number or

* the amount of plasmid DNA due to the band intensity and on

* the purity of the crude lysate.

The fast methods described here are often suitable for plasmid screenings from bacteria other than *E. coli* (but, including *E. coli*) and were tried out at the DSMZ. They don’t require the use of commercially available columns or reagent “kits” while kits are routinely and successfully used for usual molecular applications. Of course, modern kits are the result of in-depth DNA research and deliver highly purified DNA for downstream applications It must be pointed out that in some cases the ideal method of plasmid isolation can only be found out by a trial and error approach, especially for “difficult” strains or large plasmids.

Principles of Plasmid Isolation and General Instructions

The procedures are based on the fact that plasmids usually occur in the covalently closed circular (supercoiled) ccc configuration within the host cells. After gentle cell lysis all intracellular macromolecules have to be eliminated whereas plasmid DNA is enriched and purified. The smaller a plasmid the easier is the isolation of intact ccc molecules. DNA is very sensitive to mechanical stress, therefore shearing forces caused by mixing/vortexing or fast pipetting must be avoided as soon as cell lysis occurs. All mixing steps during and after cell lysis should be performed carefully by inverting the tubes several times (8-10 fold). Especially in case of larger plasmids it is recommended to cut off the ends of plastic pipette tips to minimize shearing forces. Gloves should be worn in order to prevent contamination with DNases. Autoclaved (DNase-free) buffer solutions, tubes and tips should be used. If phenotypic markers of a plasmid (e.g. antibiotic resistances) are known, it is recommended to grow the cells under selective pressure to avoid plasmid loss. If necessary,small plasmids of Escherichia coli can easily be amplified using chloramphenicol. This results in several thousand plasmid copies per cell leading to high DNA quantities (Clewell, 1972). Large plasmids are maintained with only one copy per host chromosome: visible DNA bands are more difficult to get.

For plasmid isolation, bacterial cultures should be grown to late logarithmic/early stationary phase. It is important to remove the supernatant completely after centrifugation from the cell pellets. Tris buffer is the typical buffering substance for DNA with buffering capacity in the slightly alkaline range in which DNA can also be stored best (pH 7.5-8.2). EDTA is an

important substance in plasmid preparations because it inhibits nuclease activity. For long-term storage, plasmid DNA should be frozen in aliquots of storage TE buffer. Repeated thawing and freezing of DNA should be avoided.

The plasmid isolation methods described here are brief step-by-step instructions with literature citations. In case of "difficult" plasmids in E.coli, the use of a rich medium like Terrific Broth (Tartof & Hobbs, 1987) can result in significant increase in plasmid yield. For many Gram-positive bacteria it is recommended to add 0.01 M L-threonine to the growth medium to weaken the cell wall and hence achieve easier enzymatic lysis.

Unless otherwise stated, centrifugation is done at appr. 3.000 x g for harvesting cells, centrifugation in microcentrifuge tubes is done at appr. 16.000 x g. Solution percentages are in w/v.

Ethanol precipitation of plasmid DNA

Measure the volume of the aqueous DNA solution and mix gently with (10% v/v) 3 M Na-acetate, pH 5.2, then add double of the total volume of pure ethanol (cooled to -20C), mix and leave for 10 min in crushed ice. Spin for at least 30 min at room temperature. DNA precipitation is not enhanced by long or low temperature incubation, whereas an extended centrifugation time results in good DNA recovery.

RNase treatment

Prepare 100 ml of the following sterile TE buffer: 0.01 M Tris, pH 7.5, 0.001 M EDTA. Mix 1 mg of RNase A with 1 ml of this TE buffer in an Eppendorf tube and incubate for 20 min in a boiling water bath to eliminate DNases. Cool to room temperature, add the RNase solution to the remaining 99 ml of the same TE buffer. This RNase buffer can be stored at 4°C for a long time and is a good storage buffer for plasmid DNA. RNase is a very stable enzyme and cleaves RNA within few minutes at room temperature.

Gel electrophoresis

Immediately before loading a gel, mix 8 µl of DNA sample with 2 µl of loading buffer (0.05 M EDTA, 20% Ficoll, 0.25% bromophenol blue, in H_20).

When using a horizontal electrophoresis apparatus (horizontal apparatus is the usual and better type of electrophoresis), for quick analytical gels, mini-gels on glass slides can be prepared as follows: about 25 ml of 0.8-1.0% low electroendosmosis (EEO) agarose in TBE buffer (0.089 M Tris, 0.089 M boric acid, 0.0025 M EDTA) are poured on a glass slide of approx. 10 x 7 cm. Depending on the electrophoresis comb used, up to 14 samples can be run. The same TBE buffer is used as electrophoresis buffer. Usually, the electrophoretic separation is done at 30-90 V for 2-6 hours (to be tried out). For visualization of DNA bands and photography, intercalating dyes like ethidium bromide are used: there are modern dyes with less hazardous potential like *SYBR Green (BIOZYM)* or others (proper care must be taken, carcinogenic/mutagenic). After staining for 30-60 minutes in the dark, DNA bands can be made visible under short wave length UV light. A fundamental description of the application of agarose gel electrophoresis is given by Meyers et al. (1976).

Abbreviations:

EDTA:	Ethylenedinitrilo tetra-acetic acid
SDS:	Sodium lauryl sulfate
Tris:	Tris (hydroxymethyl) aminomethane
storage TE buffer:	10-50 mM Tris, 5-10 mM EDTA, pH 7.5-8.0
RT:	room temperature

Isolation Techniques

1. Rapid boiling method for small plasmids in E. coli (Holmes & Quigley,1981; modified by Riggs & McLachlan, 1986)

- Centrifuge 1.5 ml of culture in Eppendorf tube and resuspend pellet in 200 µl of STET buffer (8.0% sucrose, 0.5% Triton X-100, 0.05 M EDTA, 0.05 M Tris-HCl, pH 8.0) containing 10 µl of lysozyme (20 mg/ml, freshly dissolved in H_2O) and 20 µl $ZnCl_2$ (1% in H_2O)
- Incubate at about 100°C for 45-55 sec, then cool on ice
- Centrifuge for 5 min and add supernatant to Eppendorf tube containing 480 µl of IS mix (400 µl isopropanol, 80 µl 5 M ammonium acetate). Incubate at RT for 20-30 min
- Centrifuge for 5 min, wash DNA pellet with 70% cold ethanol twice and dry in a vacuum dessiccator
- Resuspend pellet in 20 µl of storage TE buffer or in RNase buffer (see RNase treatment) before using for agrose gel electrophoresis

2. Hot alkaline method for all plasmid sizes and bacteria (Kado & Liu,1981), modified

- Centrifuge 2-3 ml of culture, resuspend pellet in 1 ml of solution containing 0.04 M Tris-acetate, pH 8.0 (adjust pH with glacial acetic acid) and 2 mM EDTA
- Add 2 ml of lysis buffer (0.05 M Tris, 3% SDS, pH 12.50, adjusted with 2 N NaOH) and mix
- Incubate at 60-68°C for 30-45 min (strain dependent)
- Add to hot samples 6 ml of phenol/chloroform (1:1) and mix gently to complete emulsification
- Separate phases by centrifugation at 10.000 x g for 15-20 min at RT and transfer the upper aqueous phase carefully (avoid interphase which contains debris) to new tube containing 1 volume of chloroform. Mix and centrifuge again for separation of phases
- Recover aqueous phase and use directly for agarose gel

3. Lysozyme method for various Gram-negative bacteria (Davis et al.,1980)

- Centrifuge 10 ml of culture, resuspend pellet in 1.4 ml of the following TE buffer: 0.01 M Tris, pH 8.5 and 1 mM EDTA. Transfer to Eppendorf tubes and spin for 3 min
- Resuspend pellet in 0.4 ml of solution (15% sucrose, 0.05 M Tris, pH 8.5, 0.05 M EDTA), mix vigorously, cool on ice
- Add 0.1 ml of freshly prepared lysozyme (5 mg/ml in TE buffer used above), mix carefully and incubate on ice for 20-40 min
- Add 0.3 ml of precooled Triton buffer (0.1% Triton X-100, 0.05 M Tris, pH
- 8.5, 0.05 M EDTA), incubate on ice for 20 min and centrifuge at 4°C for 4 min
- Transfer clear supernatant into new tube and add 4 µl of diethyloxydiformiate, mix gently
- Incubate for 15 min at 70°C, cool for 15 min to RT, then incubate on ice for 15 min
- Centrifuge for 4 min, transfer supernatant into new tube, fill up with ¬20°C ethanol for DNA precipitation, mix gently
- Centrifuge for at least 30 min at RT, dry pellet in vacuum dessiccator and resuspend in storage TE buffer or in RNase buffer (see RNase treatment) before use

4. Lysis of cells from single colonies on agarose gel (Eckhardt, 1978; Priefer, 1984), modified

- Transfer 1-2 freshly grown single colonies with a toothpick into 20 µl of cold buffer (0.025 M Tris, pH 8.0, 25% sucrose, 0.250 M EDTA, 7% Ficoll 400)
- Add 20 µl of freshly prepared lysis solution (0.1 mg/ml of lysozyme, 10 µl/ml of RNase A, in the above buffer), mix well and immediately fill 10-15 of the mixture into the well of an agarose gel which contains 0.5% SDS
- Add as “upper layer” onto the cell lysate 10 µl of the following solution: 0.025 M Tris, pH8.0, 10% SDS, 25% sucrose, 0.07% bromophenol blue
- After 15-30 min apply low voltage (half of usual voltage) for 30 min, then apply usual electrophoretical conditions

5. Plasmid isolation from Gram-positive bacteria, especially lactobacilli, with mutanolysin or lysozyme (Klaenhammer, 1984)

- Centrifuge 4 ml of culture and resuspend pellet in 10 ml of fresh medium. Incubate for 2 hrs at 37°C
- Centrifuge again and resuspend pellet in 1 ml of cold 25% sucrose, 0.05 M Tris, pH 7.5, 5 mM EDTA at 4°C
- Keep cell suspension in ice bath for 10 min, then add 75 µl of either mutanolysin or lysozyme (1 mg/ml in 0.05 M Tris, pH 7.5, 5 mM EDTA), mix and incubate in ice bath for 1hr (for some strains incubation at 37°C for 1hr is preferred)
- Centrifuge cells and add 500 µl of the following lysis solution to the pellet and mix well: 0.05 M Tris, 5 mM EDTA, 0.05 M glucose, 3% SDS; immediately before use mix 1.0 ml of this solution with 10 µl of 10 N NaOH
- Heat the sample at 62°C for 1 hr, then allow to cool slowly (approx.15 min) to RT, add 50 µl of 2 M Tris, pH 7.0, mix gently and add 70 µl of 5 M NaCl, mix gently
- Transfer into Eppendorf tube and extract with 500 µl of phenol which is saturated with 3% NaCl (mix gently until emulsification), leave at RT for 5 min. Add 300 µl of chloroform, mix gently
- Centrifuge for 5 min at RT for phase separation and take upper phase for extraction with 600 µl of chloroform:isoamylalcohol (24:1), leave at RT for 5 min, centrifuge and harvest aqueous phase for ethanol precipitation as usual

6. Lysis of Gram-positive bacteria with lysostaphin (Crosa et al., 1994), modified

- Centrifuge 10 ml of culture and resuspend the pellet in 0.5 ml of 7.5 mM NaCl, 0.05 M EDTA, pH 7.0
- Add lysostaphin to a final concentration of 15 µg/ml (double enzyme concentration might be necessary for some strains), incubate at 37°C for 30 min with gentle agitation, cool on ice
- For cell lysis add 0.75 ml of 0.4% deoxycholate, 1% Brij 58, 0.3 M EDTA, pH 8.0, mix gently and incubate on ice for 30 min
- Centrifuge at 23.000 x g for 20 min at 4°C, transfer supernatant into new tube, add 1.25 ml of H2O (addition of water might be superfluous)
- Add 4 µl of boiled RNase solution (1 mg/ml), incubate at 37°C for 1 hr; if necessary, for further purification perform a phenol/chloroform extraction (see Method 2)

7. Isolation procedure for all plasmid sizes from all bacteria (Crosa et al., 1994)

- Centrifuge 2 ml of a culture and wash pellet in 2 ml of the following TE buffer: 0.05 M Tris, pH 8.0, 0.01 M EDTA. Resuspend in 40 µl of the same TE buffer
- Fill 0.6 ml of freshly prepared lysis buffer (TE buffer used above with 4%SDS, pH adjusted to 12.45) into Eppendorf tube and add the cell suspension to the lysis buffer, mix gently
- Complete lysis by incubating at 37°C for 20-30 min
- Add 30 µl of 2 M Tris, pH 7.0 for neutralization, mix gently
- Add 024 ml of 5 M NaCl for precipitation of chromosomal DNA and protein and incubate on ice for 4 hrs
- Centrifuge for 10 min and transfer supernatant into new tube for ethanol precipitation (as usual) or for previous extraction with phenol/chloroform (see Method 2)

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