

### PLASMID ISOLATION FROM BACTERIA : SOME FAST PROCEDURES

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Over the past decades it became evident that virtually in all bacterial species plasmids exist. These accessory genetic elements are defined as autonomously replicating extrachromosomal DNA (Novick, 1980). Plasmids typically account for only a small fraction of a bacterial genome corresponding roughly to a range between 1 and 200 kilobase pairs. However, extremely large megaplasmids with sizes far beyond 200kb have also been detected such as in *Rhizobium* and others. Plasmids of more than 50 kb might be characterized as large plasmids whereas plasmids used as tools in molecular genetics are often smaller than 10 kb.

The aim of this TIS is to describe some fast methods for small scale plasmid isolation leading to crude cell lysates, the quality of which being sufficient for analytical purposes (agarose gel electrophoresis of the lysates or restriction analysis) and being the basis for further purification of the DNA. By using a few microliters of such a crude lysate of a strain 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.

These methods are especially suitable for plasmid screenings and have been tried at the DSM. They do not require the use of commercially available columns or reagent kits and are practicable for a normally equipped microbiological laboratory. It must be pointed out that in some cases the ideal method of plasmid isolation for a particular strain can only be found out by a trial and error approach.

### Principles of Plasmid Isolation and General Instructions

Plasmid isolation 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 is the easier is the isolation of intact ccc molecules. DNA is very sensitive to mechanical stress, therefore shearing forces caused by mixing or fast pipetting should be avoided as soon as cell lysis occurs. All mixing steps necessary during and after cell lysis should be performed manually by inverting the tubes several times (8-10 fold). Especially in case of larger plasmids it is recommended to cut off the very ends of the plastic pipette tips to minimise shearing forces. Gloves should be worn in order to prevent contamination with DNases. Autoclaved solutions, tubes and tips should be used. If phenotypic markers of a plasmid (e.g. antibiotic resistances) are known it is advantageous to grow the cells under selective pressure to avoid plasmid loss. If necessary, small plasmids of *Escherichia coli* can easily be amplified using chloramphenicol which results in several thousand plasmid copies per cell leading to high DNA quantities (Clewell, 1972). Large plasmids are maintained at the level of only one copy per host cell chromosome implying higher difficulties in getting visible DNA bands.

In general, for plasmid isolation the bacterial cultures should be grown to late logarithmic phase. It is important to remove the supernatant completely after centrifugation from the cell pellets. Tris buffer is the typical buffering substance for DNA having its 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 all plasmid preparations because it inhibits nuclease activity. For long-term storage, plasmid DNA should be frozen in aliquots of storage TE buffer and 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 Teriific Broth (Tartof & Hobbs, 1987) can result in a 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 3.000 x g for harvesting cells whereas centrifugations in microcentrifuge tubes are done at 16.000 x g. All 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) 3M 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 (A. Regensburger, pers. communication)*

Prepare 100ml of the following sterile TE buffer: 0.01M Tris, pH 7.5, 0.001 M EDTA. Mix 1mg of RNase A with 1ml 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 and 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 very long time and is a good storage buffer for plasmid DNA. The enzyme RNase is very stable and cleaves RNA within few minutes at room temperature.

### *Gel electrophoresis*

Immediately before loading the gel mix 8 $\mu$ l of the DNA sample with 2 $\mu$ l of loading buffer (0.05 M EDTA, 20% Ficoll, 0.25% bromophenol blue, in H<sub>2</sub>O).

When using a horizontal electrophoresis apparatus, mini-gels can be prepared as follows: about 25ml of 0.8-1.0% low electroendosmosis (EEO) agarose in TBE buffer (0.089M Tris, 0.089 M boric acid, 0.0025 M EDTA) are poured on a 10 x 7 cm glass slide. Depending on the 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 voltage for 6-2 hours. For visualization of DNA bands and photography the intercalating dye ethidium bromide is used (proper care should be taken as it is a carcinogenic compound). Add one drop of a 10 mg/ml stock solution to the staining tray containing water and the gel. Cover the tray with a lid. After staining for 30 min 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 and 5-10 mM EDTA at pH 7.5-8.0

## Isolation Techniques

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

-Centrifuge 1.5ml of culture in an Eppendorf tube and resuspend the pellet in 200µl of STET buffer (8.0% sucrose, 0.5% Triton X-100, 0.05 M EDTA, 0.05 M Tris-HCl, pH 8.0) containing 10µl of lysozyme (20mg/ml, freshly dissolved in H<sub>2</sub>O) and 20µl ZnCl<sub>2</sub> (1% in H<sub>2</sub>O).

-Incubate at about 100C for 45-55 sec and then cool on ice

-Centrifuge for 5 min and add the supernatant to an Eppendorf tube containing 480µl of IS mix (400µl isopropanol, 80µl 5M ammonium acetate). Incubate at room temperature for 20-30min

-Centrifuge for 5 min, wash the DNA pellet with 70% cold ethanol twice and dry in a vacuum dessicator

-Resuspend the pellet in 20µl 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 2-3ml of culture, resuspend the pellet in 1ml of solution containing 0.04M Tris-acetate, pH 8.0 (adjust pH with glacial acetic acid) and 0.002 M EDTA

-Add 2ml of lysis buffer (0.05M Tris, 3% SDS, pH 12.60, adjusted with 2N NaOH) and mix

-Incubate at 60-68 C for 30-45 min (strain dependent)

-Add to hot samples 6ml of phenol/chloroform (1:1) and mix gently to complete emulsification

-Separate phases by centrifugation at 10.000 x for 15-20 min at room temperature 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 the aqueous phase and use directly for agarose gel

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

-Centrifuge 10ml of culture, resuspend the pellet in 1.4ml of the following TE buffer: 0.01M Tris, pH 8.5 and 0.001 M EDTA. Transfer to

Eppendorf tubes and spin for 3min

-Resuspend pellet in 0.4ml of solution (15% sucrose, 0.05M Tris, pH 8.5, 0.05M EDTA), mix vigorously, cool on ice

-Add 0.1ml of freshly prepared lysozyme (5mg/ml in TE buffer used above), mix carefully and incubate on ice for 20-40min

-Add 0.3ml of precooled Triton buffer (0.1% Triton X-100, 0.05 M Tris, pH 8.5 0.05M EDTA) incubate on ice for 20 min and centrifuge at 4C for 4min.

-Transfer clear supernatant into new tube and add 4 $\mu$ l of diethyloxidiformiate, mix gently

-Incubate for 15min at 70C, cool for 15min to room temperature and then put on ice for 15min

-Centrifuge for 4min, transfer supernatant into new tube and fill up with -20C ethanol for DNA precipitation, mix

-Centrifuge for at least 30 min at room temperature, dry pellet in a 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 by M. Jahnke, pers. communication*

-Transfer 1-2 freshly grown single colonies with a toothpick into 20 $\mu$ l of cold buffer (0.025 M Tris, pH 8.0, 25% sucrose, 0.250 M EDTA, 7% Ficoll 400)

-Add 20 $\mu$ l of freshly prepared lysis solution (0.1mg/ml of lysozyme, 10.0 $\mu$ l/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 $\mu$ l of (0.025 M Tris, pH8.0, 10% SDS, 25% sucrose, 0.07% bromophenol blue)

-After 15-30min apply low voltage (half of the normal voltage) for 30 min, then apply the usual electrophoretical conditions

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

-Centrifuge 4ml of culture and resuspend the pellet in 10ml of fresh medium. Incubate for 2hrs at 37C

-Centrifuge again and resuspend pellet in 1 ml of cold solution (25% sucrose, 0.05M Tris, pH 7.5, 0.005 M EDTA at 4C)

-Keep cell suspension for 10 min in ice bath, then add 75µl of either mutanolysin or lysozyme (1mg/ml in 0.05M Tris, pH 7.5, 0.005 M EDTA), mix and incubate in ice bath for 1hr (for some strains incubation at 37C for 1hr is better)

-Centrifuge cells and add 500µl of lysis solution (0.05M Tris, 0.005 M EDTA, 0.05 M glucose, 3% SDS-immediately before use mix 1.0ml of this solution with 10 µl of 10 N NaOH) to the pellet and mix well

-Heat the sample at 62C for 1hr, then allow to cool slowly (approx. 15min) to room temperature, add 50µl of 2M Tris, pH 7.0, mix gently and add 70µl of 5 M NaCl, mix gently

-Transfer into Eppendorf tube and extract with 500µl of phenol which is saturated with 3% NaCl (mix gently until emulsification), leave at room temperature for 5 min. Add 300µl of chloroform, mix gently

-Centrifuge for 5 min at room temperature for phase separation and take upper phase for extraction with 600µl of chloroform : isoamylalcohol (24:1), leave at room temperature for 5min , centrifuge and harvest aqueous phase for ethanol precipitation as usual.

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

-Centrifuge 10ml of culture and resuspend the pellet in 0.5ml of 0.0075 M NaCl, 0.05 M EDTA, pH 7.0

-Add lysostaphin to a final concentration of 15µg/ml (double enzyme concentration might be necessary for some strains), incubate at 37C for 30min with gentle agitation, cool on ice.

-For cell lysis add 0.75ml of 0.4% deoxycholate, 1% Brij 58, 0.3 M EDTA, pH 8.0, mix gently and incubate on ice for 30min.

-Centrifuge at 23.000 x g for 20min at 4C and transfer supernatant into new tube, add 1.25ml of H<sub>2</sub>O (addition of water might be superfluous)

-Add 4µl of boiled RNase solution (1mg/ml), incubate at 37C for 1hr ; 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 2ml of a culture and wash pellet in 2ml of the following TE buffer: 0.05 M Tris, pH 8.0, 0.01 M EDTA. Resuspend in 40µl of the same TE buffer

-Fill 0.6ml 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 37C for 20-30 min

-Add 30µl of 2M Tris, pH 7.0 for neutralization, mix gently

-Add 0.24ml of 5M NaCl for precipitation of chromosomal DNA and protein and incubate on ice for 4hrs

-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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