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DSMZ

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 <u>small scale plasmid isolation</u> 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) <u>ccc configuration</u> 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 <u>shearing forces</u> 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