OPTIMAL PARAMETERS FOR PLASMID DNA PREPARATION USING THE ALKALINE LYSIS METHOD

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Abstract: The great variety of different parameters found in the literature when preparing plasmid DNA from *Escherichia coli* makes difficult to established the best method in terms of yield, reproducibility and speed. In this study several parameters were addressed, including temperature and incubation time, and an clear, simple and affordable protocol which achieves relatively high yield of plasmid DNA was established.

INTRODUCTION

Molecular biology techniques have encountered a fast development in the last years, mainly because are used in various areas of research. One of the most important application is the preparation of plasmid DNA, which is to be further used in applications like cloning, transformation of bacteria, isolation of specifica DNA fragments or sequencing.

The first successful preparation of plasmid DNA was done by [1] in 1967 using ethydium-bromide in a dye-buoyant-density method. Since then, several methods have been described, based on ion exchange, gel-filtration chromatography or differential precipitation [2]. The best of them, in terms of simplicity and yield are those based differential precipitation. Two of them have become classic methods in molecular biology: the boiling method first described by [3] and the alkaline lysis method first described by [4].The last one is preferred over the boiling method because it yields a higher amount of DNA . The procedure takes advantage of the fact that plasmids are relatively small supercoiled DNA molecules and bacterial chromosomal DNA is much larger and less supercoiled. This difference in topology allows for selective precipitation of the chromosomal DNA and cellular proteins from plasmids and RNA molecules. The cells are lysed under alkaline conditions, which denatures both nucleic acids and proteins, and when the solution is neutralized by the addition of potassium acetate, chromosomal DNA and proteins precipitate because it is impossible for them to renature correctly (they are so large). Plasmids renature correctly and stay in solution, effectively separating them from chromosomal DNA and proteins. Although it is widely used, different sources describe different methods, using various reagents in different quantities. For example, the precipitation of plasmid DNA is done by [3] using isopropanol in a 1:1 ratio at -18 degrees C for 10 minutes, while [2] is using ethanol in a 2:1 ratio at room temperature for 2 minutes, [5] is using ice cold isopropanol in an 0,6:1 ratio at 4 ^{0}C and dr. Roderich Brandsch, in an unpublished protocol is using isopropanol 2:1 for 30 minutes in liquid nitrogen. One might be confused by this great variability and have difficulties in establishing the best protocol. In the current work we address the factors that influence the efficiency of plasmid DNA precipitation like: alcohol type, volume, incubation time, incubation temperature. Our final goal is to formulate a clear and simple protocol usable in our laboratory.

MATERIAL AND METHODS

Strains, plasmids and growing conditions

For plasmid propagation *Escherichia coli* XL1 Blue from Stratagene was used. The plasmids pH6EX3[6], pMAL[7], pBSKS[8] were a kind gift from prof. dr. R. Brandsh ……..

The microorganism was grown overnight on LB medium[2] supplemented with the appropriate antibiotics.

Mini-preparations of plasmid DNA

The alcaline lysis technique described by Sambrook et all [2] was used, with some modifications of the precipitation step. 5 ml of overnight-grown culture of *E. coli* harboring the desired plasmid were centrifuged for 10 minutes at 4000 rpm and the cells resuspended in 200 µl Resuspension buffer (25 mM Tris, 10 mM EDTA, pH 8). The pellet was completely resuspended by pipetting up and down several times and 400 µl Lysis solution (0,2 N NaOH and 1% SDS) was added. The complete lysis of the cells is proved by a clear solution. 300 µl of precipitation buffer was than added and the tube was mixed gently for 1-2 minutes and then incubated on room-temperature for 10 minutes. The tubes were centrifuged for 10 min at 13 000 rpm in an Haereus Biofuge table centrifuge and 700 µl of supernatant were transferred in a clean tube. The precipitation step was done by using various alcohols in different amounts at different temperatures as described in results. After the precipitation, the mixture was centrifuged for 10 minutes at maximum speed and the pellet was washed with 70% methanol. The precipitated DNA was resuspended in 40 µl resuspension buffer (10 mM TrisCl, 1Mm EDTA, pH 8)

Electrophoresis of DNA.

The agarose-gel electrophoresis in TBE (0.09 M Tris-borat; 0.002 M EDTA) buffer as described by [8] was used. 5 µl of

DNA solution was mixed with 5 µl DNA loading buffer (0.25 % albastru de bromphenol and 30% glycerol) and loaded on gel. All gels were 0,75% agarose with 0,5 microg/ml micrograms ethidium bromide. The gels were run in an horizontal apparatus produced by Renner Gmbh, Germany applying an current of 120 V for about 2 hours.

Gel visualization and densitometry

The gels were visualized and photographed under UV light using an Vilber-Lourmant UV-VIS transiluminator and an Sony DSC-H1 camera. The intensity of the spots were quantified using the ImageJ software from Național Institutes of Health, USA; http://rsb.info.nib.gov/ij. [9]. For each gel run, the same sample was considered as standard and the intensity of the spots were expressed as % from that sample. Each sample was run in triplicate and the mean and standard error was calculated using the Excel application from Microsoft. To remove any variability of the gel concentration and ethidium bromide concentration all the sample that were compared with each other were run on the same gel.

RESULTS AND DISCUSSIONS

Using isopropanol for precipitation results in the best yield.

Alcohol precipitation is an old and classical method for recovery and purification of biological molecules. These molecules are maintained in solution by the interaction of surface hydrophilic groups with the water solvent. Consequently, if the polarity of the solvent is reduced by the addition of an organic solvent less polar than water, the molecule will tend to become less soluble [10]. Usually for plasmid DNA precipitation ethanol or isopropanol are used [11]. They differ by one carbon extra carbon in the side chain and this is reflected also in their polarity (as an effect of the dielectric constant and dipole moment). In order to have a complete picture, we assayed 3 different alcohols with different numbers of carbons: methanol, ethanol and isopropanol. Their polarity differ mainly because of the dielectric constant: methanol has 32,6, ethanol 22,4 and isopropanol 18,3 [12]. In order to establish which one is a better choice, 2100 µl (3 volumes) of alcohol was used for precipitation of the plasmid DNA from 5 ml of culture as described in Material and Methods. The result of an minipreparation with pH6EX3 can be seen in figure 1, together with the densitogram.

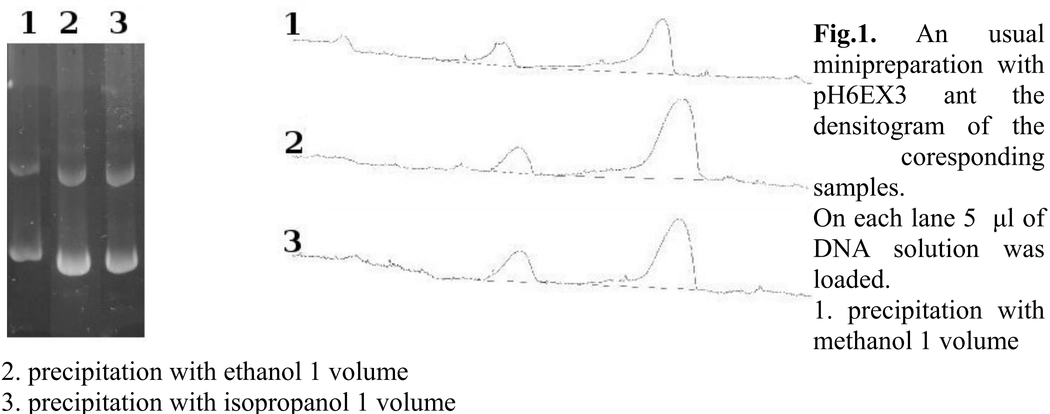

Fig.1. An usual minipreparation with pH6EX3 ant the densitogram of the coresponding samples.
On each lane 5 µl of DNA solution was loaded.
1. precipitation with methanol 1 volume
2. precipitation with ethanol 1 volume
3. precipitation with isopropanol 1 volume

The results obtained, expressed as procents of isopropanol sample are presented in tabel 1.

Table 1. Plasmid DNA yield when precipitating with 1 volume of methanol, ethanol or isopropanol.

Plasmid	pH6EX3		pMAL		pBSKS	
	Mean (%)	SE	Mean (%)	SE	Mean (%)	SE
Methanol	77.34	7..51	65.25	24.1	65.52	20.7
Ethanol	69.07	8.08	56.09	21.36	109.2	20.73
Isopropanol	168.43	8.79	59.1	21.28	129.13	17.21

As it can be easily seen, for the pH6EX3 and pBSKS plasmids, the best yield was obtained when using isopropanol, its low dielectric constant contributing to a more rapid decrease in terms of polarity of the alcohol: water mixture.

The volume of alcohol used for precipitation does not impose any restrictions in this microscale method. When it is scaled up though, using 3 volumes of alcohol for precipitation is time consuming and expensive. This is the reason why, we studied also the influence of the amount of alcohol used for precipitation on the precipitation yield. For the pH6EX3 and pBSKS plasmids, decreasing the volume from 2,1 ml (3 volumes) to 700 µl (1 volume) of alcohol resulted as expected in lower recovery. Still, the yield is quite good (data not shown) and using 1 volume of alcohol for precipitation is feasible for large scale purifications. Of economical reasons we decided to use 1 volume of alcohol in the further experiments.

For the pMAL plasmid, no matter of the alcohol type or volume used the yield was never bigger than 65%. This shows that this plasmid is in a smaller copy number than the other two and for higher yields chloramphenicol amplification would be required [13].

One interesting point is that, when using isopropanol in different amounts for precipitation, the way the plasmid DNA runs on gels varies. As it can be seen in figure 2 for the pH6EX3 plasmid, increasing the volume of isopropanol used from 700 µl to 2,1 ml results in a thicker upper band. This is probably due to the fact that the number of molecules in a more compact state increases because of a less polar environment.

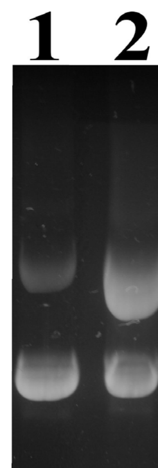

Fig. 2. Different running pattern of the pH6EX3 plasmid.
1. The precipitation was carried out using 1 volume of isopropanol
2. The precipitation was carried out using 3 volumes of isopropanol

Our main concern was the recovery of the plasmid DNA and thereby the purity of the DNA was not investigated. During the preparation of DNA the intense contamination by RNA described by [3] was also observed, indicating that an intermediate step of RNA-se digestion would be required. The amount of salt precipitated with the alcohols, as well as the ability of restriction enzymes to cleave this DNA would require further investigations.

The influence of temperature on plasmid DNA precipitation.
Usually, precipitation using organic solvents is carried at low temperatures to minimize denaturation. Plasmid DNA, because of its closed circle structure and small dimensions it is a more robust molecule, and thereby it is less exposed to damage. Still, as we have seen, in their protocols for plasmid DNA preparation, different authors use a variety of temperatures ranging from -196 ^{0}C (liquid nitrogen) to 24-25 ^{0}C (room temperature). Gerstein [11] indicates that low temperature are to be used when the DNA concentration is lower then 0,25 mg/ml , but room temperature is enough to efficiently precipitate DNA above this concentration. Given this variability, we tried to established which is the best temperature for precipitation with ethanol and isopropanol in order to achieve the highest yield. To precipitate the DNA, 700 µl of alcohol was used, and the mixture was incubated for 2 hours at different temperatures . The results obtained with pH6EX3 can be observed in figure 3.

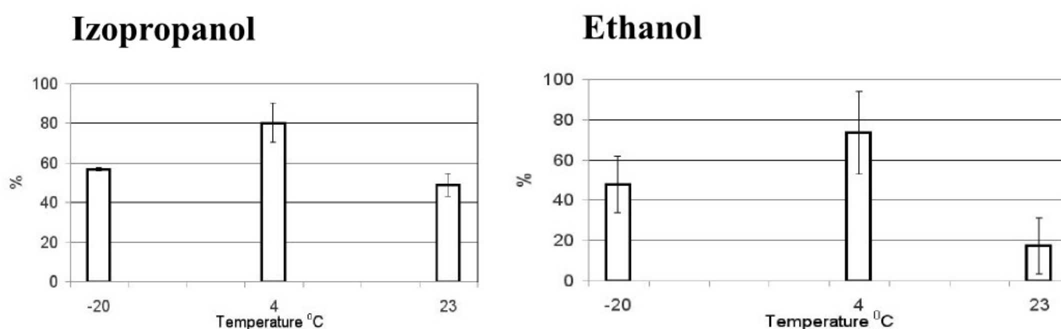

Fig.3. The influence of incubation temperatures on the plasmid DNA recovery.

Surprisingly, the biggest amount of DNA precipitate at 4 ^{0}C, and not at -20 ^{0}C as one would expect with both the alcohols used. This is in good accordance with the fact that the salting-out effect is directly proportional with the temperature [10]. Still, in our case, this direct link could not be observed as the recovery at 23 ^{0}C is low. This could actually indicate that the preparation is contaminated with DNA-ases.

The influence of incubation time plasmid DNA precipitation.
Our goal was to establish an reproducible, high-yield, cheap and fast method which would allow high throughput screening of recombinant plasmid. The time of incubation of the alcohol/extract mixture is essential for the amount of DNA precipitated. Different times were assayed, ranging from 30 minutes to 16 hours (over night). Disregarding the alcohol used, ethanol or methanol, the results were the same. The same amount of plasmid DNA was recovered, indicating that only 30 minutes are enough to completely precipitate the DNA using 1 volume ethanol or isopropanol. Further reducing of the incubation time was not required, as this way the complete protocol starting from bacterial culture and finishing with the DNA solution takes 2 to 3 hours.

CONCLUSIONS

Isopropanol was found to have an higher recovery over methanol and ethanol when used to precipitate plasmid DNA. Both using 1 volume or 3 volumes of alcohol give an high yield, the

best being obtained when using 3 volumes.
The best temperature for precipitation was found to be 4 ^{0}C and an incubation time of 30 minute is enough to achieve an relatively high recovery of plasmid DNA.
Using this parameters, the complete protocol takes about 2-3 hours to complete and assures an relatively high yield and reproducibility.

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