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Sequential $CaCl_2$, polyethylene glycol precipitation for RNase-free plasmid DNA isolation

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ABSTRACT

Functional genomics is facilitated by the ability to express genes in heterologous systems. In some cases function can be assayed by generation of in vitro transcripts of the unknown genes and expressing those transcripts in various expression systems. Plasmids bearing phage promoters are used to generate in vitro transcripts. Therefore, it is important to ensure that the template plasmid DNA is not contaminated with RNase from the isolation procedure. We have developed a plasmid purification protocol that does not utilize RNase yet yields pure plasmid DNA. The protocol combines the selective precipitation of RNA with 1.4 M $CaCl_2$, followed by a final selective precipitation of the plasmid DNA in a 10% polyethylene glycol (PEG), 250 mM NaCl solution. Purity of the resulting plasmid DNA was determined spectrophotometrically and by gel electrophoresis. No detectable contaminating RNA was observed in the plasmid DNA preparations. Inhibitory effects of the protocol were assayed by performing restriction analyses, sequencing, PCR, and in vitro transcription. These procedures were successful. The in vitro transcripts visualized by gel electrophoresis were found to be full length, thus indicating no significant endogenous RNase activity associated with the procedure.

Introduction

Although it is possible to utilize robotics to perform high-throughput plasmid purification [1], some laboratories are not positioned to afford such technologies. Most small laboratories rely on commercial kits for preparation of pure plasmid DNA which require RNase treatment to degrade contaminating RNA. Protocols using cesium chloride [2] or cesium trifluoroacetate [3] gradients can separate DNA from RNA but these are time-consuming and expensive procedures.

Size-selective precipitation of DNA fragments with polyethylene glycol (PEG)[1] is a long-established but underappreciated way of purifying DNA [4–7]. By decreasing the final PEG concentration in the presence of $MgCl_2$, Hartley and Bowen [6] demonstrated that they could selectively precipitate the larger fragments of a 50-bp DNA molecular weight marker. Schmitz and Riesner [7] showed that PEG_{6000} could efficiently precipitate plasmid DNA (90% efficiency) when used in combination with 500 mM NaCl. Efficient recovery did not require incubations at RT or on ice if the nucleic acid concentration was higher than 10 ng/ml.

Stepanov and Nyborg [8] in an attempt to avoid the use of RNase described a large-scale plasmid DNA isolation procedure that utilizes consecutive precipitation steps. They removed genomic DNA, proteins, and cell debris with lithium perchlorate in the presence of potassium acetate. Plasmid DNA and RNA were then precipitated from solution with 13% PEG, 10 mM $MgCl_2$ after incubation on ice for 1 h. The plasmid DNA obtained was contaminated with high molecular weight RNA which was removed by precipitation with 5 M LiCl. The small RNAs were removed with spermidine titration. The spermidine was eliminated from the plasmid DNA by precipitation with 0.3 M NH_4Cl. The protocol as described by Stepanov and Nyborg [8] achieved the preparation of pure plasmid DNA without contaminating RNA. However, it is a time-consuming protocol since it required as much as 6 h and the use of spermidine is a concern. Spermidine has been used successfully to precipitate nucleic acid [9,10]. However, the use of spermidine for plasmid purification as described by Stepanov and Nyborg [8] is viewed as both complicated and potentially devastating for downstream procedures such as in vitro transcription. The complication of the procedure is evident in the details provided by the authors in that care must be taken to follow the titration visually when increasing the spermidine concentration. The concern about spermidine use prior to certain applications and in particular in vitro transcription is based on the fact that spermidine, an organic polyamine, can bind both RNA and DNA. Failure to efficiently remove contaminating spermidine could result in precipi-

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[1] *Abbreviations used:* MOPS, 4-morpholinepropanesulfonic acid; PCR, polymerase chain reaction; PEG, polyethylene glycol; SDS, sodium dodecyl sulfate.

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tation of the template DNA required for in vitro transcription and, thus, failure to generate transcripts. We have developed a fast, efficient, and cost-effective protocol for obtaining pure plasmid DNA which does involve several precipitation steps. However, it does not require organics such as chloroform or phenol nor does it require the use of RNase or spermidine.

Our protocol involves the selective precipitation of high molecular weight RNA from the cleared lysate with 1.4 M $CaCl_2$, followed by isopropanol precipitation to concentrate the nucleic acids and remove the $CaCl_2$, followed by the selective precipitation of the plasmid DNA in 10% PEG, 250 mM NaCl. This protocol is fast, cost effective, and yields pure plasmid DNA that can be used in a variety of downstream applications.

Materials and methods

Recombinant plasmids

Various sized recombinant plasmids generated for structure function studies were used to test the efficacy of our protocol. Those recombinant plasmids consisted of the pET28a plasmid (5369 bp) with inserts of three different sizes: Int 261 (792 bp), Int 150 (459 bp), and Int 53 (168 bp). The T7 promoter and T7 terminator flank the inserts, thus facilitating the amplification and in vitro transcription of the cDNA inserts from purified plasmids.

Bacterial strain

Escherichia coli strain DH5α was used to maintain the recombinant plasmids. In preparation for plasmid DNA isolation, bacteria were grown overnight at 37 °C with vigorous shaking in LB medium containing 100 μg/ml kanamycin.

Alkaline lysis to generate cleared lysate

As with most of the plasmid DNA preparations, it has become routine to perform the alkaline lysis procedure as described by Sambrook et al. [3]. For our protocol, an aliquot (1.5 ml) of each of the overnight cultures was centrifuged at 12,000*g* for 5 min. The supernatant was removed and the pellet resuspended in 100 μl of 50 mM Tris/HCl, 10 mM EDTA, pH 8.0. To ensure high yields, care was taken to achieve complete resuspension. Lysis was achieved by addition of 200 μl of the lysis solution (0.2 N NaOH, 1% SDS) followed by gentle mixing. The resulting lysates were neutralized with 150 μl of 3 M KOAc, pH 5.5. Genomic DNA, proteins, and cell debris were removed from the plasmid DNA in solution by centrifugation at 12,000*g* for 10 min and the cleared lysate (supernatant) was transferred to a new microcentrifuge tube.

Removal of RNA from cleared lysate

Two salts (LiCl and $CaCl_2$) were tested for selective precipitation of RNA from the plasmid DNA in solution.

LiCl is widely used to precipitate high molecular weight RNA [1,3,11]. For removal of RNA by LiCl precipitation, 1 vol of 6 M LiCl was added to the cleared lysate to obtain a final concentration of 3 M LiCl The samples were mixed, left at room temperature for 10 min, and centrifuged at 12,000*g* for 10 min. The resulting supernatant containing the plasmid DNA was transferred to new tubes.

For removal of RNA by $CaCl_2$ precipitation [12], a stock solution of $CaCl_2$ at 4.2 M was added to the cleared lysate to obtain a final concentration of 1.4 M, and the samples were mixed followed by immediate centrifugation at 12,000*g* for 10 min. The supernatant containing predominantly plasmid DNA in solution was transferred to fresh tubes.

Plasmid DNA precipitation with polyethylene glycol

We chose to use PEG to selectively precipitate the plasmid DNA. A 20% PEG_{8000} stock solution was prepared and protected from light. The different PEG concentrations that were assayed for their ability to selectively precipitate the plasmid DNA were 5, 6, 7, 8.3, and 10%. Appropriate volumes of the stock solution were added to our samples to obtain those final concentrations. The different salts that were tested in combination with PEG were 15 mM $MgCl_2$, 15 mM NaCl, 250 mM NaCl.

Combination of $CaCl_2$ with PEG

In view of the results from the selective precipitation of RNA with $CaCl_2$ and of plasmid DNA with PEG, it was decided to use $CaCl_2$ and PEG in combination. After collecting the cleared lysates from the alkaline lysis step, a stock solution of 4.2 M of $CaCl_2$ was added to a final concentration of 1.4 M. The tubes were spun immediately at RT and the supernatants were transferred into a new tube. The nucleic acids were then precipitated by standard isopropanol precipitation (0.3 M sodium acetate, 0.6 vol isopropanol). After 10 min at RT, the nucleic acids were pelleted by centrifugation at 12,000*g* for 10 min. The pellets were washed with 70% ethanol, dried, and resuspended in 50 μl of TE. An equal volume of 20% PEG, 500 mM NaCl was added to the samples with vigorous mixing to achieve the final concentration of 10% PEG, 250 mM NaCl. Immediately after mixing, the samples were centrifuged at 12,000*g* at RT for 10 min. The supernatants were discarded and the pellets washed with 70% ethanol, dried, and resuspended in 30 μl of 10 mM Tris, pH 8.0, 1 mM EDTA, pH 8.0. Plasmid DNA concentrations were determined with a NanoDrop ND-1000.

PCR of inserts from plasmids precipitated with PEG

Each reaction volume was 100 μl composed of 10 μl of 10X buffer (NEB, standard buffer), 400 μM dNTP, 80 pmol each of T7 promoter primer (TAATACGACTCACTATAGGG) and T7 terminator primer (GCTAGTTATTGCTCAGCGG), 5 U *Taq* polymerase (NEB, *Taq* DNA polymerase with standard *Taq* buffer), and 25–50 ng of each plasmid. PCR were performed on the Applied Biosystems 2720 thermal cycler. Reactions were initiated with an incubation at 95 °C for 5 min, followed by 35 cycles of denaturation (95 °C for 30 s), annealing (52 °C for 30 s), and extension (72 °C for 40 s). A final extension was performed at 72 °C for 7 min. An aliquot (5 μl) of each reaction was resolved on a 1.5% agarose gel in TAE buffer (Tris acetate EDTA buffer).

Prior to in vitro transcription, the PCR products were precipitated by addition of 3 M sodium acetate to a final 0.3 M and an equal volume of isopropanol. Samples were incubated at room temperature for 10 min and pellets collected by centrifugation at 12,000*g* for 10 min. Copelleted salts were removed with addition of 70% ethanol followed by centrifugation at 12,000*g* for 10 min. The resulting pellets were dried under vacuum and each was resuspended in 10 μl of TE. Concentrations were determined spectrophotometrically with a NanoDrop ND-1000.

Sequencing

Two plasmids were sent to the Nevada Genomics Center (Reno, NV) for sequencing to make sure that the plasmids yielded by our procedure are suitable for sequencing. We followed the recommendations from the Center and sent the appropriate concentrations of plasmid along with the sequencing primer.