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High-Throughput Method for Isolating Plasmid DNA with Reduced Lipopolysaccharide Content

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ABSTRACT

Isolating plasmid DNA from bacteria is a fundamental step in molecular biology. It is often accomplished by an alkaline lysis of bacteria and the subsequent adsorption of nucleic acids to silica oxide in the presence of chaotropic substances. Here we show that the addition of such chaotropic reagents is not required for the efficient DNA isolation with silica oxide. This surprising finding allowed us to purify plasmid

DNA with significantly less lipopolysaccharides (LPS), which is otherwise a common bacterial contaminant of silica oxide-isolated DNA and inhibits subsequent applications. In addition, we have implemented a precipitation step that altogether leads to a reduction of the LPS content by a factor of 900 relative to published methods. Our novel protocol facilitates an inexpensive high-throughput analysis of pure plasmids in a 96-well format without the addition of hazardous reagents.

INTRODUCTION

Purifying plasmid DNA from bacteria is a commonly used procedure that is the basis for cloning, sequencing, functional expression screens and newer applications like gene therapy. Each of these manipulations requires the isolation of plasmid DNA at high purity. Commercial anion exchange columns, even though they are expensive, are widely used for this purpose. Less expensive methods, such as the use of silica oxide, have the disadvantage that bacterial lipopolysaccharides (LPS or endotoxins) are copurified, which can interfere with downstream applications (2,6,7). Furthermore, silica oxide requires the use of chemicals, such as guanidine hydrochloride, that act as chaotropic substances by changing the structure of water and facilitate the binding of DNA to silica oxide (3). Because many of these applications require iterative plasmid isolations, we wanted to develop a protocol for a plasmid preparation in a 96-well format based on silica oxide as an established and inexpensive DNA binding matrix. At the same time, we sought to obviate the disadvantages of DNA isolation by silica oxide. Here, we describe the implementation of such an experimental protocol.

MATERIALS AND METHODS

Preparation of Silica Oxide

Silica oxide (Sigma, Deisenhofen, Germany) was dissolved in 250 mL water as a 50 mg/mL suspension. After incubation for 30 min, the fines were removed by suction, and the volume was reconstituted. The solution was

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treated by adding 150 μL HCl (37%) and was autoclaved.

Determination of LPS Content

We used a commercially available colorimetric test (BioWhittaker, Walkersville, MD, USA), to determine the LPS level. According to the manufacturer, the detection limit of this assay is 0.1 EU/mL endotoxin. We used internal controls to calibrate the tests.

Plasmid DNA Isolation in 96-Well Plates

A 96-well block containing 900 μL of LB medium with bacteria of an overnight culture in each well was centrifuged for 5 min at $3000\times g$ (Sigma Zentrifugen, Osterode am Harz, Germany). The supernatant was decanted, the block was inverted on Kimwipes® for 2–3 min and 170 μL F1 buffer (50 mM Tris-HCl/10 mM EDTA, pH 8.0) were added. The bacteria pellets were completely resuspended by vortex mixing for 10–20 min. Then, 170 μL F2 buffer (200 mmol/L NaOH, 1% SDS) were added, the block was sealed with tape (ScotchPad, 3M, Neuss, Germany), inverted and incubated at room temperature for 5 min. However, the 170 μL of 4°C cold buffer F3 (3 mol/L KOAc, pH 5.5) terminated the lysis. The 96-well block was then sealed with tape and inverted to mix the solutions. Ten microliters of an RNaseA solution (1.7 mg/mL) were added to digest excessive RNAs, incubated for 5 min at room temperature and then at -20°C for 10 min to efficiently precipitate proteins and genomic DNA.

The solution was again centrifuged for 10 min at $5800\times g$. The supernatant was decanted in new blocks, and the samples received 100 μL buffer F4 (2.5% SDS in isopropanol) to precipitate LPS. The block was vortex mixed for 5 min and incubated, first at 4°C for 15 min and then at -20°C for 15 min. The block was again centrifuged for 10 min at $5800\times g$, and the supernatant was added to robust, custom-made 96-well polyoxymethylene microtiter blocks. One-hundred and fifty microliters of silica oxide suspension were added and incubated for 20 min at room temperature. The plates were cen-

trifuged for 5 min at $5800\times g$, the supernatant was carefully decanted and 400 μL wash solution (acetone or 75% isopropanol, -20°C) were added. The plates were again vortex mixed for 30 s and centrifuged for 3 min at $5800\times g$. The wash step was repeated once. The pellets were first dried at room temperature for 5 min and then for 5 min in a vacuum chamber. They were subsequently resuspended in 75 μL LPS-free water at 60°C by vortex mixing for 30 s and centrifuged at maximum speed at $5800\times g$ for 10 min at 4°C to pellet the silica oxide. The supernatant contained the isolated plasmid DNA.

For DNA midipreps (100 mL bacteria culture) 240 mg silica oxide were used; for maxipreps (from 500 mL bacteria culture) 2.25 g silica oxide were applied to the lysate.

Transfection of Cells

The human embryonic cell line 293 was transfected as described (4) with calcium phosphate co-precipitation whose efficiency is sensitive to LPS. Transfection efficiencies were assayed with the plasmid pGreenLantern™ (Life Technologies, Gaithersburg, MD, USA), which encoded the green fluorescent protein (1).

RESULTS AND DISCUSSION

Silica oxide is known to interact with DNA in the presence of chaotropic substances (5). However, we unexpectedly discovered that, even without an added chaotropic reagent such as guanidine hydrochloride, there was no decline in the DNA binding capacity of silica oxide (Table 1). We stepwise reduced the concentration of guanidine hydrochloride, but even when we excluded this chemical altogether, we did not observe a significant drop in the yield of plasmid DNA (Table 1). We also found that chaotropic substances are not required for DNA binding to other silica-based DNA adsorption matrices such as commercially available spin columns (data not shown). The DNA binding is dependent on the presence of silica oxide and potassium acetate in the reaction (data not shown). Possibly, the high potassium acetate

Table 1. DNA Binding to Silica Oxide in the Presence of Different Concentrations of a Chaotropic Reagent

Guanidine Hydrochloride (M)	Plasmid DNA ($\mu\text{g/mL}$)
0	134.1 $\pm$ 46.9
0.5	135.3 $\pm$ 28.8
1	192.3 $\pm$ 48.3
1.5	161.3 $\pm$ 61.1
2	157.5 $\pm$ 23.5

Plasmid DNA (pGreen Lantern) was isolated from 1 mL of LB medium after an alkaline lysis of bacteria (*E. coli* SURE®; Stratagene, La Jolla, CA, USA). Different concentrations of the chaotropic substance guanidine hydrochloride were added as indicated, and the amount of DNA that could bind to 7.5 mg silica oxide was determined photometrically (A_{260}). Given are the means and standard deviations of three independent experiments for each condition.

concentration used to neutralize the alkaline lysis of bacteria is sufficient to facilitate the DNA binding to silica. The omission of a hazardous reagent such as guanidine hydrochloride is a major safety advantage of our protocol.

One of the most frequent contaminants of silica oxide-isolated DNA are lipopolysaccharides that are abundant in the membrane of bacteria and lead to significant reductions in transfection efficiency and toxic side effects in gene therapy (6,7). However, we observed that the exclusion of a chaotropic substance results in an LPS content of less than 50 U/ μg DNA (Table 2) in contrast to more than 1000 U/ μg with conventional silica oxide isolations (6). We also tested several washing conditions and found that a washing step with acetone removes LPS much more efficiently than the widely used isopropanol.

Furthermore, the absence of a chaotropic substance in our protocol allowed us to use additional reagents to purify the DNA, which would other-

Table 2. LPS Levels in 96-Well Plasmid Isolations after Acetone Wash and SDS Precipitation

Treatment	LPS (U/ μ g DNA)
Isoprop. Wash	26.5 $\pm$ 6.3
Isoprop. Wash +SDS	3.2 $\pm$ 0.6
Acetone Wash	7.9 $\pm$ 4.4
Acetone Wash + SDS	1.3 $\pm$ 0.4

Half of the 96-well plate was washed with acetone, the other half with 75% isopropanol. Half of each wash condition was additionally treated with SDS solution (2.5% in isopropanol). The LPS levels were determined and the means and standard deviations of three different wells from a 96-well plate for each condition are shown.

wise interfere with DNA binding. We introduced a simple precipitation step to further reduce the LPS concentration. To this end, we exploited the fact that LPS has an amphipathic character. Using SDS (2.5% in isopropanol), another amphipathic molecule that can be removed easily by coprecipitation in alcohol, we obtained a reduction in the LPS activity of the DNA isolations by a factor of eight relative to the acetone-washed control samples.

Combining the purification effects of the exclusion of guanidine hydrochloride, acetone wash and SDS precipitation, we achieved a reduction of the LPS content by a factor of 900 in comparison with published silica-based plasmid purifications (Reference 6 and Table 2). The level of LPS in our DNA isolations is comparable to CsCl- or anion exchange-purified plasmid DNA (6). In accordance, we have observed equal transfection efficiencies between our small scale DNA isolations and DNA purified by anion exchange columns (data not shown). With this protocol, we routinely obtain approximately 10 μ g of a high copy number plasmid from 900 μ L LB medium with an $A_{260/280}$ of 1.8 or better, 80% of which is in the supercoiled form (data

not shown). In addition to its use for a high-throughput format, this protocol could easily be upscaled for midi and maxi DNA preparations (data not shown). Therefore, it has the potential to considerably widen the application of silica oxide for the isolation of pure plasmid DNA.

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