Short Technical Reports

Fifty-One Kilobase HSV-1 Plasmid Vector Can Be Packaged Using a Helper Virus-Free System and Supports Expression in the Rat Brain

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ABSTRACT

Herpes simplex virus type 1 (HSV-1) plasmid vectors have a number of attractive features for gene transfer into neurons. In particular, the large size of the HSV-1 genome suggests that HSV-1 vectors might be designed to accommodate large inserts. We now report the construction and characterization of a 51 kb HSV-1 plasmid vector. This vector was efficiently packaged into HSV-1 particles using a helper virus-free packaging system. The structure of the packaged vector DNA was verified by both Southern blot and PCR analyses. A vector stock was microinjected into the rat striatum, the rats were sacrificed at 4 days after gene transfer, and numerous X-gal positive striatal cells were observed. This 51 kb vector was constructed using general principles that may support the routine construction of large vectors. Potential applications of such HSV-1 vectors include characterizing large promoter fragments or genomic clones and co-expressing multiple genes.

INTRODUCTION

Herpes simplex virus type 1 (HSV-1) plasmid vectors (5) or PCR products have a number of attractive features for gene transfer into neurons for both gene therapy of neurological disorders and the genetic analysis of neuronal physiology. Consequently, a growing number of investigators have used this vector system to modify neuronal physiology, and changes in behavior have been observed in some studies (4). Gene transfer with vector stocks prepared using a helper virus results in significant cytopathic effects and immune responses (7); therefore, we developed a helper virus-free packaging system for HSV-1 plasmid vectors (3). A set of 5 cosmids that represents the HSV-1 genome (2) was modified by deletion of the DNA cleavage/packaging sites (contained in the **a** sequences). Cotransfection of this modified cosmid set and an HSV-1 vector [contains HSV-1 origin of replication $(ori)_S$ and a DNA cleavage/packaging site] into fibroblast cells results in the packaging of vectors into HSV-1 particles, but no helper virus is produced (3). Upon injection into the rat brain, the helper virus-free vector stocks produce substantially less cytopathic effects and cell infiltration than previously observed with helper virus systems. The recent development of bacterial artificial chromosomes that harbor the HSV-1 genome may enhance the use of this system (17).

HSV-1 plasmid vectors have a number of potential advantages. In particular, the large size of the HSV-1 genome suggests that large vectors might be developed. HSV-1 plasmid vectors are packaged as head-to-tail concatamers that form a genome sized (~ 152 kb) DNA molecule. Thus, large vectors, such as 30 or 50 kb, that can form concatamers of approximately 150 kb might be correctly packaged. However, with helper virus packaging systems, competition between the vector and the helper virus is thought to result in a selective pressure for deletions in large vectors. Vectors (15 or 16 kb) have been correctly packaged using helper virus systems (10,16), but a 19 kb vector is subject to spontaneous deletions (10). However, a 30 kb vector that was stably maintained in a cell line using the Epstein-Barr virus (EBV) oriP-EBNA1 system has been successfully packaged using a helper virus (19). Because the helper virus-free packaging system may reduce or eliminate the competition between the vector and the helper virus during the packaging procedure, it is conceivable that large vectors might be successfully packaged in this system. Nonetheless, a 16 kb vector is the largest vector that has been reported to be packaged using this system (20).

In this study, we investigated the properties of a large HSV-1 vector in the helper virus-free system. We constructed a 51 kb HSV-1 plasmid vector that expresses the *Escherichia coli Lac*Z gene from a neurofilament heavy subunit (NFH) promoter and contains a 40 kb fragment of *Saccharomyces cerevisiae* DNA. This vector was packaged into HSV-1 particles using our helper virus-free packaging system (3,18), and the structure of the packaged vector DNA was verified by both PCR and Southern blot analyses. A vector stock was microinjected into the striatum, the rats were sacrificed at 4 days after gene transfer and X-gal positive cells (>500 per rat) were observed. The principles used to construct this 51 kb vector may support the routine construction of large vectors.

MATERIALS AND METHODS

Vector Construction

pNFHlac, which contains a 0.6 kb fragment of the mouse NFH promoter from plasmid pH-615 (14), has been described (20).

A 51 kb HSV-1 vector (Figure 1) was constructed in several stages. First, two oligonucleotides (5′-AATTGTTAATT-AAGGCGCGCCGCGGCCGCGGGC-CCGGCGCCGAATTCCTCGAGTCT-AGAGGTACCGGATCCAAGCTTGC-AGAGATCTTTAATTAAGGCGCGC-CGCGGCCGCA-3′ and 5′-TCGATG-CGGCCGCGGCGCGCCTTAATTAA-AGATCTCTGCAAGCTTGGATCCG-GTACCTCTAGACTCGAGGAATTC-GGCGCCGGGCCCGCGGCCGCGG CGCGCCTTAATAAC-3′) were inserted between the *Eco*RI and *Sal*I sites of pBR322 (pBR-linker). Next, a 1.5 kb *Hin*dIII and *Bgl*II fragment containing the neo^r gene was inserted between the *Hin*dIII and *Bam*HI sites of pBR-linker (pBR-linker-neo). A cosmid (C8179) harboring 43 839 bp of *S. cerevisiae* DNA was obtained from ATCC (Rockville, MD, USA). Approximately 1 kb fragments that are about 40 kb apart in C8179 were isolated using PCR [5′ fragment, PCR primers 5′-CGGTGGG-CCCGATTAAGATAAGCGTCGGAG-GAGG-3′ and 5′-GCGGAATTCAAG-GCGATTGACGTATG-GTCAGC-3′ (nucleotides [nt] 176–199 and complementary to nt 1149–1162); 3′ fragment, PCR primers 5′-CCCGAATTCCG-GTCTCGAGGTGTCAACGATGCGT-ATGCCGACG-3′ and 5′-CGCGTCGA-CCCGTATAGTGTAGCGCCAGACA-

Table 1. The Titers Obtained from Helper Virus-Free Packaging of pNFHlac or pNFHlac-Sac

	Titers, Infectious Vector Particles/mL		
	pNFHlac	pNFHlac-Sac	
Experiment	Crude[a]	Crude[a]	Purified[b]
1	1.3×10^5	1.0×10^5	ND[c]
2	2.0×10^5	2.0×10^4	1.1×10^6
3	2.6×10^5	2.4×10^4	2.4×10^6

Experiment 1 was a small-scale packaging for an initial test of the vectors, and experiments 2 and 3 were large preparations to produce vector stocks for both DNA characterization and microinjection into the rat brain.

[a]Crude, the vector stock obtained from the packaging.

[b]Purified, the vector stock after purification (11).

[c]ND, not done.

CG-3′ (nt 39 166–39 189 and complementary to nt 40 090–40 114); *Apa*I, *Eco*RI, *Xho*I and *Sal*I sites are underlined]. The 5′ PCR product was digested with *Apa*I and *Eco*RI, the 3′ PCR product was digested with *Eco*RI and *Sal*I, and these 2 fragments were inserted between the *Apa*I and *Xho*I sites in pBR-linker-neo (pBR-neo-Sac).

Next, a 4.7 kb *Bam*HI fragment that contains the transcription unit from pNFHlac was inserted into the *Bam*HI site of pBR-linker-neo (pBR-NFHlacZ; two orientations are possible and pBR-NFHlacZ contains the 3′ end of the NFHlac transcription unit adjacent to

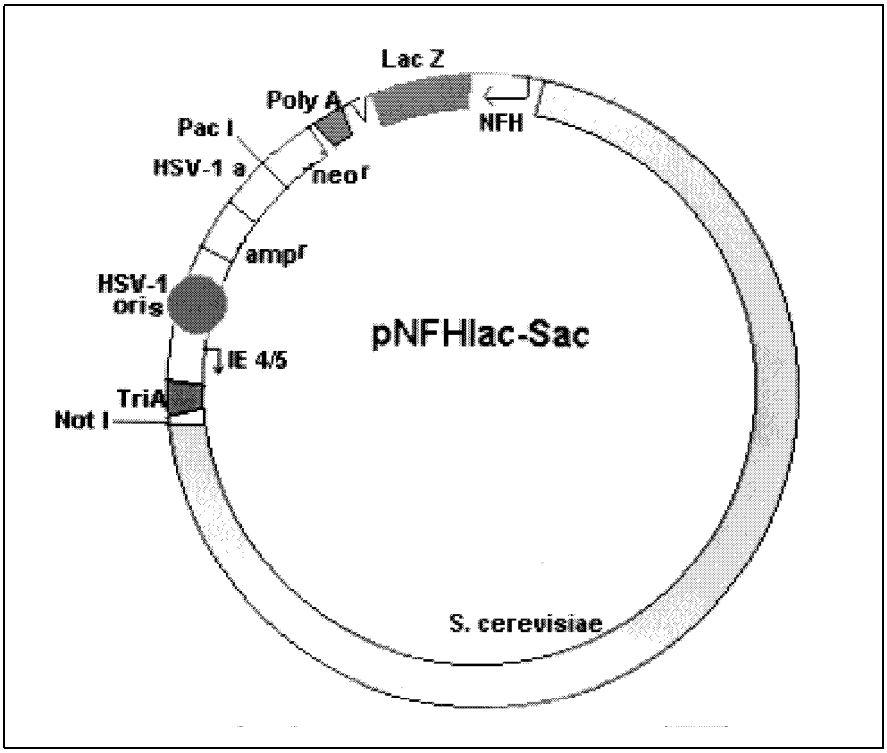

Figure 1. A diagram of pNFHlac-Sac. pNFHlac-Sac contains the NFHlac transcription unit from pNFHlac (20), a 40 kb fragment of *S. cerevisiae* DNA, an HSV-1 vector fragment and the neo^r gene. The NFHlac transcription unit is composed of the NFH promoter, the *Lac*Z gene, an intron and a polyadenylation site (poly A). The HSV-1 vector fragment contains the HSV-1 ori$_s$, an HSV-1 **a** sequence, the amp^r gene and the Col E1 origin of DNA replication. The ori$_s$ fragment contains an HSV-1 IE 4/5 promoter that is followed by three polyadenylation sites (TriA) (12). A selection for the neo^r gene was used in the final step in the construction (see vector construction). The *Pac*I and *Not*I sites are shown.

the 5′ end of the neo^r gene). We combined a 2.6 kb *Pvu*I and *Xba*I fragment from pBR-neo-Sac (the PCR products and a portion of the amp^r gene), a 3.1 kb *Pvu*I and *Not*I fragment from pBR-linker and a 6.2 kb *Not*I and *Xba*I fragment from pBR-NFHlacZ (the NFHlac transcription unit and the neo^r gene) to yield pBR-Sac-NFHlac-neo. A 40 kb fragment of *S. cerevisiae* DNA was inserted into pBR-Sac-NFHlac-neo (pBR-Sac40kb-NFHlac-neo) by in vivo recombination cloning in *E. coli* BJ5183 cells (1,9). The three fragments used in this cloning were pBR-Sac-NFHlac-neo digested with *Xho*I and treated with calf intestinal phosphatase, the 6.1 kb *Xmn*I fragment from C8179 (nt 140–6258) and the 36.9 kb *Spe*I fragment from C8179 (nt 3917– 40 771).

Miniprep DNA was isolated from the candidates, analyzed by restriction endonuclease digestion and DNA from a correct candidate was transferred into JM109 for large-scale preparation of DNA. Next, a *Hin*dIII and *Bam*HI fragment that contains 3 polyadenylation sites (TriA cassette) (12) was inserted between the *Hin*dIII site and a *Xho*II site (at the boundary between the HSV-1 **a** sequence and the polyadenylation site) in pHSVpUC (6) to obtain pHSVpUC/TriA. Two oligonucleotides (5′-CTAGTGAATTCGGATCCTTAATTAAGTCGACGC-3′ and 5′-GGCCGCGTCGACTTAATTAAGGATCCGAATTCA-3′) were inserted between the *Spe*I and *Not*I sites of pHSVpUC/TriA (pHSVpUC-linker-I), and then two oligonucleotides (5′-GGCCGCGATATCCGATCGGGATCCGGCGCGCCTTAATTAAC-3′ and 5′-GATCGTTAATTAAGGCGCGCCGGATCCCGATCGGATATCGC-3′) were inserted between the *Not*I and *Bam*HI sites of pHSVpUClinker-I (pHSVpU-Clinker-II).

The 46 kb *Pac*I and *Not*I fragment from pBR-Sac40kb-NFHlac-neo was inserted into pHSVpUC-linker-II that had been digested with the same enzymes to yield pNFHlac-Sac (ligation reactions contained 2:1, 1:1 or 1:2 molar ratios of vector to insert and DNA concentrations of 0.8, 1.2, 1.6 or 2.0 ng/μL). Candidates were isolated in DH5α Competent cells™ (Life Technologies, Gaithersburg, MD, USA) on plates that contained 25 μg/mL neo, grown in LB medium that contained 50 μg/mL amp and 25 μg/mL neo, and miniprep DNA was analyzed by restriction endonuclease digestion. Because large-scale DNA preparations from DH5α cells contained deletions, miniprep DNA was transformed into Epicurian coli® JM109, SURE® and XL-10 Gold™ cells (all from Stratagene, La Jolla, CA, USA) and MAX EFFICIENCY HB101™ cells (Life Technologies), and pNFHlac-Sac was stably maintained only in HB101. Finally, the pNFHlac-Sac DNA was isolated using the Qiagen maxiprep procedure.

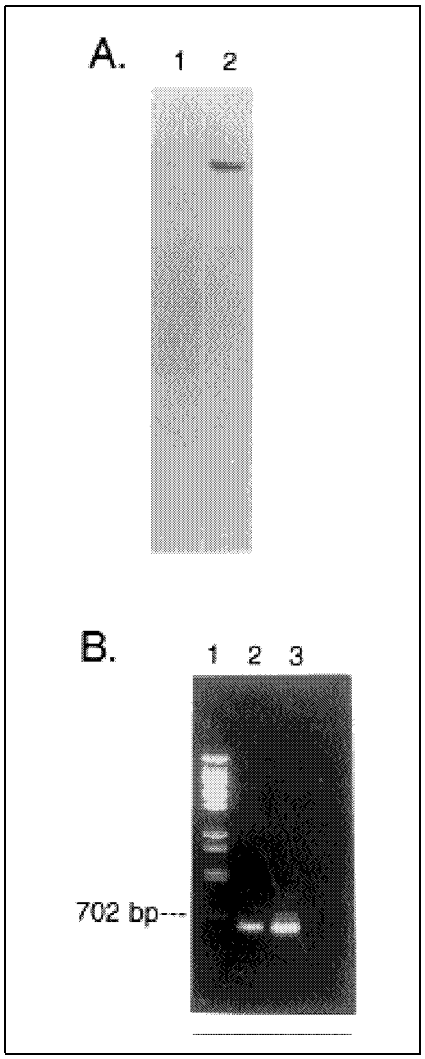

Figure 2. Analysis of the structure of pNFHlac-Sac DNA packaged into HSV-1 particles. DNA was isolated from a pNFHlac-Sac vector stock. (A) DNAs were digested with *Pac*I, electrophoresed on an agarose gel and subjected to Southern blot analysis using a hybridization probe from the *S. cerevisiae* DNA fragment. pNFHlac-Sac contains one *Pac*I site; the predicted size of the band is 51 kb. Lane 1, pNFHlac-Sac vector stock; lane 2, pNFHlac-Sac plasmid DNA. (B) DNAs were subjected to PCR analysis using primers from the *Lac*Z gene. The predicted size of PCR products is 582 bp. Lane 1, standards (λDNA digested with *Bst*EII); lane 2, pNFHlac-Sac vector stock; and lane 3, pHSVlac (5) plasmid DNA.

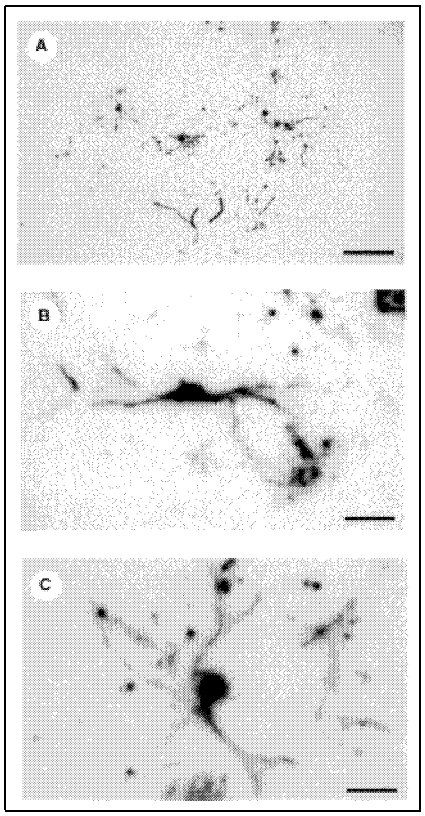

Figure 3. X-gal positive striatal cells from rats sacrificed at 4 days after microinjection of pNFHlac-Sac vector stock into the striatum. Expression of β-gal was detected with X-gal (16). (A) A low-power view of the striatum. Numerous X-gal positive cells and processes are visible. (B and C) High-power views that show X-gal positive cells with large cell bodies and extended processes that are characteristic of striatal neurons. Scale bars: A, 491 μm; B, 115 μm; and C, 77 μm.

Packaging Vectors into HSV-1 Particles

Vectors were packaged into HSV-1 particles using the helper virus-free packaging system in 2-2 cells (15) as modified to improve the titers (18). Vector stocks were purified and concentrated as described (11). They were titered by counting the number of X-gal positive cells obtained at one day after infection of baby hamster kidney fibroblast cells (BHK21). At one day after infection, we observed more X-gal positive cells using BHK cells than us-

ing PC12 cells (not shown). Consequently, BHK cells were used to titer the vectors, even though the expression from the NFH promoter in these fibroblast cells represents ectopic expression that may decline at longer times after gene transfer (not shown) and use of this ectopic expression may introduce some variability into the titers.

Analyses of Vector DNA in the Vector Stocks

DNA was isolated from vector particles, digested with *Pac*I, displayed on a 0.35% agarose gel and transferred to a nylon membrane. The hybridization probe was the *S. cerevisiae* DNA 5′ PCR product (see vector construction) labeled with dioxygenin-11-dUTP by random hexamer priming (DIG High Prime Labeling and Detection Starter Kit I; Roche Molecular Biochemicals, Indianapolis, IN, USA). Hybridization and immunological detection were performed according to the manufacturer's instructions.

*Lac*Z sequences were detected by PCR using the following primers: primer set 1, 5′-TCTGTATGAACGGTCTGGTCTTTGC-3′ [nt 1802–1826 (8)] and 5′-CCATCAGTTGCTGTTGACTGTACG-3′ (complementary to nt 2882 –2905); primer set 2, 5′-GTTGATTGAACTGCCTGAACTACC-3′ (nt 2034 –2057) and 5′-CACTTCAACATCAACGGTAATCG-3′ (complementary to nt 2594–2616). The PCR conditions have been described (16).

Stereotactic Injection of HSV-1 Vectors into the Rat Brain

Male Sprague Dawley rats (100–125 g) were used for these experiments. Vector stocks were delivered by stereotactic injection (2 sites, 3 µL/site) into striatum. Both hemispheres; anterior-posterior (AP) 0.8, medial-lateral (ML) 2.5, dorsal-ventral (DV) 5.5; AP 1.8, ML 2.5, DV 5.5; AP is relative to bregma, ML is relative to the sagittal suture and DV is relative to the bregma-lambda plane (13). The rats are smaller than those used for the atlas (13), and the injections sites were verified by cresyl violet staining. These studies were approved by the Children's Hospital IACUC.

Histology

Four days after gene transfer, the rats were anesthetized with chloral hydrate (400 mg/kg, intraperitoneal) and then perfused with 50 mL PBS followed by 200 mL of 4% paraformaldehyde in PBS. The brains were postfixed in 4% paraformaldehyde in PBS (for 4 h at 4°C), cryoprotected in 25% sucrose in PBS (for 2 days at 4°C), and 25 µm coronal sections were cut on a freezing microtome. Enzymatic staining and immunohistochemistry were performed on free-floating sections. Expression of β-galactosidase (β-gal) was detected by incubation with X-gal (16); the X-gal reaction was carried out for 3 h at room temperature at pH 7.9.

Using these reaction conditions, rats that received PBS displayed no staining in the striatum, but faintly positive cells were occasionally observed in the red nucleus and in brain vasculature endothelium. Every fourth section was analyzed for expression of β-gal and approximately 10 of these sections contained the X-gal positive cells. Cell counts were performed under 40× magnification.

RESULTS AND DISCUSSION

Conventional plasmid construction strategies are difficult to use on large molecules; therefore, we used the following strategy (detailed in Materials and Methods) to construct a 51 kb HSV-1 plasmid vector. First, we constructed a small plasmid that contained the neo^r gene, the NFHlac transcription unit and two approximately 1 kb fragments of *S. cerevisiae* DNA that are approximately 40 kb distant from each other. In vivo recombination cloning in *E. coli* BJ5183 (1,9) was then used to insert an approximately 40 kb *S. cerevisiae* DNA fragment. These plasmids lacked HSV-1 vector sequences because the HSV-1 ori$_s$ and **a** sequences contain direct repeats that are not stable during the in vivo recombination cloning procedure.

A DNA fragment containing the NFHlac, neo^r and *S. cerevisiae* sequences was then inserted into a HSV-1 vector by DNA ligation, and candidates were isolated using a selection for the

neo^r gene. The resulting 51 kb HSV-1 vector was designated pNFHlac-Sac (Figure 1).

pNFHlac-Sac and pNFHlac (10.2 kb), as a control, were packaged into HSV-1 particles using a helper virus-free packaging system. In three experiments (Table 1), the titers of pNFHlac-Sac were similar to, or tenfold lower than, the titers of pNFHlac.

DNA was isolated from one stock of pNFHlac-Sac, digested with *Pac*I and subjected to Southern blot analysis using a hybridization probe from the *S. cerevisiae* DNA fragment. The pNFHlac-Sac (51 kb) contains one *Pac*I site. The pNFHlac-Sac (51 kb) is likely to be packaged as a concatamer of 3 copies to yield a DNA molecule close to the size of the 152 kb genome of HSV-1, and *Pac*I should cut each concatamer one time to yield 51 kb fragments. Because of the large size of the predicted DNA fragment, the transfer from the gel to the membrane was inefficient (measured by ethidium bromide staining; not shown). Nonetheless, the results (Figure 2A) demonstrated that the DNA packaged into HSV-1 particles contained a large band. This large band co-migrated with the standard; the standard was pNFHlac-Sac plasmid DNA (isolated from *E. coli*) that had been digested with *Pac*I. This result suggests that pNFHlac-Sac was correctly packaged into vector particles; however, we cannot exclude the possibility of small internal deletions that were too small to affect the apparent mobility of the band in the Southern blot analysis. The presence of *Lac*Z sequences in the DNA isolated from vector particles was demonstrated by performing PCR using primers from the *Lac*Z gene. The results (Figure 2B) showed a band of the predicted size that had a mobility similar to the PCR products obtained using pHSVlac (5) plasmid DNA as the template.

The pNFHlac-Sac vector stock was microinjected into the striatum of 3 rats, the rats were sacrificed at 4 days after gene transfer, and the brains were subjected to X-gal staining. A low-power photomicrograph (Figure 3A) shows numerous X-gal positive striatal cells, and high power views (Figure 3, B and C) demonstrate that some of these X-gal positive cells display neuronal morphology. The presence of some X-gal positive cells that lacked neuronal morphology may reflect either limitations of the vector system or that this 600 bp fragment of the NFH promoter lacks some sequences that are important for neuronal-specific expression.

Consistent with the known capability of HSV-1 to be retrogradely transported through axons, we also observed X-gal positive cells in specific areas of neocortex (not shown). Cell counts showed that these 3 rats contained an average of 688 ± 130 (mean ± SD) X-gal positive striatal cells. The gene transfer efficiency was 4.7% [(100 × mean X-gal positive cells)/([infectious vector particles/μL] × μL injected) = (100 × 688)/(2.4 × 10^3 × 6)], and this gene transfer efficiency is similar to that obtained using smaller vectors such as pHSVlac (3) or pNFHlac (20). Larger numbers of transduced cells await improvements to the packaging system, and the importance of long-term expression indicates improvements in the promoter and vector system.

The strategy used to construct this 51 kb vector is a general one that may support the routine construction of large HSV-1 vectors. Potential uses of large HSV-1 vectors include the construction of libraries, the characterization of large promoter fragments or large genomic clones and the construction of vectors that co-express multiple genes. For example, co-expression of tyrosine hydroxylase, GTP cyclohyrolase, aromatic amino acid decarboxylase and a vesicular monoamine transporter might support the efficient production and regulated release of dopamine. Thus, co-expression of these four genes might enable an efficacious gene therapy approach to Parkinson's disease.

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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 lipopolysaccarides (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