

## Purification of Plasmid (pVaxLacZ) by Hydrophobic Interaction Chromatography

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### ABSTRACT

*This paper describes a method for the plasmid DNA purification, which includes an ammonium sulphate precipitation, followed by hydrophobic interaction chromatography (HIC) using Phenyl Sepharose 6 Fast Flow (low sub). The use of HIC took advantage of the more hydrophobic character of single stranded nucleic acid impurities as compared with double-stranded plasmid DNA.*

**Key words:** Gene therapy, hydrophobic interaction chromatography, plasmid, purification

### INTRODUCTION

The use of the recombinant DNA with progress of the gene therapy or nucleic acid vaccination DNA in the cure of diseases such as cancer and AIDS, has increased the need to obtain pure plasmid of DNA (Diogo et al., 2000). In some research applications it is possible to use crude cell extracts of varying degrees of purity. However, plasmid DNA used for non-viral therapeutic gene transfer or nucleic acid vaccination has to be highly purified and free of contaminating components such as bacterial proteins, toxins, genomic DNA (gDNA) or RNA (Prazeres et al., 1999). It is now clear that the demand for large amounts of plasmid DNA will be enormous in view of the potential number of users and the prospect of applying DNA vaccines in veterinary diseases (Little-van

den Hurk et al., 2000). Product recovery costs become critical in the overall economics of modern biotechnology processes, and the need to have a process complying with the guidelines issued by regulatory agencies increases the interest in developing methods for the downstream processing of plasmids (Ribeiro et al., 2002). A process for the production of plasmid DNA generally follows the steps of fermentation, primary isolations, and purification (Prazeres et al., 1999). Large-scale purifications require scalable methods such as column chromatography (Prazeres et al., 1998). Hydrophobic interaction techniques have been used in the purification of therapeutic proteins, but so far there are no reports on the application of HIC for plasmid purification (Diogo et al., 2000). The use of HIC has be advantage of the different hydrophobic character

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of double-stranded plasmid DNA and other nucleic acids impurities with high content in single strands, such as RNA and denatured gDNA. The aim of this work was to study the possibility of purifying plasmid DNA using HIC as the final operation in a simple purification process.

## MATERIALS AND METHODS

### *Materials*

Phenyl Sepharose 6 Fast Flow (low sub) was obtained from Pharmacia (Uppsala, Sweden). RNase-DNase free was from Boehringer (Mannheim, Germany), Luria broth (LB) was from Sigma (St. Louis, MO), and agarose was from FMC (Rockland, ME). All salts used were of analytical grade.

### *Bacterial culture*

*Escherichia coli* DH5 $\alpha$  harboring pVax-LacZ (Invitrogen) was grown overnight at 37°C in 100 ml shake flasks containing 25 ml of LB medium with 30  $\mu$ g/ml kanamycin at 200 rpm. Larger culture volumes (250 ml) were inoculated with the appropriate amount of overnight culture and incubated under the same conditions. *E. coli* DH5 $\alpha$  without plasmid was also grown under same conditions as described before, but with no kanamycin present.

### *Lysis and primary isolation*

A modified alkaline method was used for cell lysis (Sambrook et al. 1989). After the alkaline lysis method the plasmid in the supernatant was precipitated after addition of 0.6 volumes of isopropanol and a 45 min incubation period at -20°C. The plasmid was separated by centrifugation at 10 000 g during 20 min. The pellets were then redissolved in TE buffer (10 mM Tris-HCl, 1 mM EDTA, pH 8.0). Next, solid ammonium sulphate was dissolved in the plasmid solution the concentration interval among 2.0-3.0 M, followed by 15 min incubation on ice. Precipitated proteins were removed by centrifugation at 10 000 g during 20 min at 4°C. The supernatant was then diluted and loaded directly on the HIC column. Total plasmid was quantified by anion-exchange high-performance liquid chromatography (HPLC). The same isolation procedure was applied to DH5 $\alpha$  cells without plasmids to check the behaviour of cell impurities in the HIC column.

### *Preparative chromatography*

Chromatography was performed in a Pharmacia fast protein liquid chromatography (FPLC) system (P 500 pump, LCC 500 chromatography controller). An XK16/20 (20cm x 1.6cm) column (Pharmacia) was packed with 28 ml of the HIC gel. Partially purified pVax-LacZ plasmid from the ammonium sulphate precipitation was loaded onto the column and isocratic elution was carried out with 1.5 M ammonium sulphate in 10mM Tris-HCl (pH 8.0) at a flow rate of 1 ml/min. One-milliliter fractions were collected. The absorbance of the eluate was measured continuously at 254 nm. The samples analysed by 0.8% agarose gel electrophoresis, stained with ethidium bromide (0.5  $\mu$ g ml<sup>-1</sup>).

### *Plasmid standards*

Plasmid standards were prepared using a plasmid mini-prep kit (Qiagen, Germany) according to the instructions of the manufacturer.

### *Analytical chromatography*

A 4.6 x 10cm polyether ether ketone (PEEK) column packed with Poros 20 PI strong anion-exchange media from Perseptive Biosystems was connected to a Merck-Hitachi HPLC system and equilibrated with 0.7M NaCl in TE buffer. Samples (100  $\mu$ l) were injected and eluted at 2 ml/min. These samples were incubated with RNase-DNase free for 60 min at 37°C before HPLC analysis. Plasmid was quantified through a calibration curve, which was constructed using pVax-LacZ standards (2-40  $\mu$ g ml<sup>-1</sup>).

### *Protein analysis*

The protein concentration was measured by modifying micro bicinchoninic acid (BCA) assay from Pierce (Rockford, IL, USA) (Rhoderick et al. 1989). A calibration curve was made with bovine serum albumin (BSA) standards. Absorbance was measured at 595 nm in a micro plate reader.

### *Genomic DNA analysis*

Genomic DNA (gDNA) was analysed by PCR. A 361 bp sequence of the ribosomal RNA gene from *E. coli* DH5 $\alpha$  was chosen for amplification in the PCR analysis (forward primer 5'-ACA CGG TCC AGA CTC CTA CG-3'; reverse primer 5'-ACA ACC TGG AAT TCT ACC CC-3'). The volumes of the samples to analyse were 0.35  $\mu$ l and the final volume of the mixture of used PCR was of 50  $\mu$ l

(Taq DNA 1x buffer; 200  $\mu\text{M}$  dNTP's mixture, 1.5  $\mu\text{M}$  of  $\text{MgCl}_2$  and 2.5 U Taq polymerase, all from Promega (Madison, WI) and 0.75  $\mu\text{M}$  of each primer from Interactiva (Ulm, Germany). For each sample analysed, a calibration curve was made using 0.01-100 ng de standard gDNA. PCR was performed with a first denaturation step at 94°C for 3 min followed by 40 cycles of amplification (60°C, 1 min; 74°C, 1.5 min; 94°C, 1 min) and a final extension step (60°C, 1 min; 74°C, 10 min). Amplified samples (20  $\mu\text{l}$ ) were visualized by 2% agarose gel analysis stained with ethidium bromide (0.5  $\mu\text{g ml}^{-1}$ ).

## RESULTS AND DISCUSSION

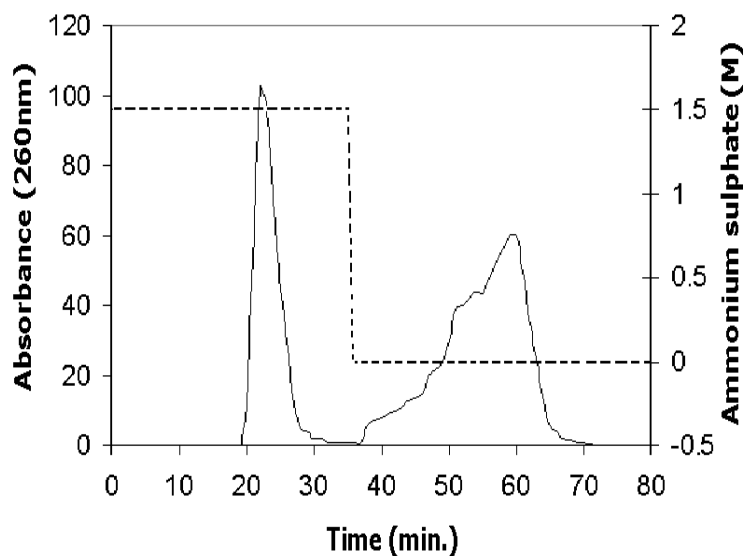
The results of the purification for the plasmid DNA are summarized in Table 1. Along the purification process, there was decrease in the plasmid mass. In the stage of alkaline lysis, it was 8716  $\mu\text{g}$ , while is after HIC it was 1230  $\mu\text{g}$ . There was 5.7 fold increase in the purity (lysis solution and after HIC it was in 100%, in all the concentrations) which used ammonium sulphate. The best purification factor was obtained after HIC, using the precipitation with 2.5M ammonium sulphate, 51% yield. Studies accomplished by Diogo et al. (2000) working in the purification of plasmid using hydrophobic interaction chromatography obtained similar results. Fig. 1 shows the analysis of plasmid solution in HIC after the precipitation with 2.5M ammonium sulphate which has two peaks. The first peak (20 to 29 fractions) corresponded the unretained fraction plasmid DNA. The fractions were collected and analysed by HPLC. The chromatogram (results not shown) obtained no

longer showed the presence on the impurity peak, indicating an improvement in HPLC purity (to 100%). The second peak corresponded the host impurities such as RNA, gDNA, denatured plasmid DNA and proteins eluted after the plasmid. Agarose gel electrophoresis also analyzed the complete removal of RNA (results not shown) and the absence of gDNA (Fig. 2) for all the concentrations of ammonium sulphate. Genomic DNA from *E. coli* is double-stranded, but becomes mostly single-stranded during alkaline lysis. During this process, the complementary strands of gDNA are completely separated and partially cleaved. The resulting DNA molecules show a high exposure of the hydrophobic bases and can thus interact with the HIC ligands (Diogo et al. 2000). According to the specifications, gDNA contamination should be plasmid  $<0.1 \text{ ng } \mu\text{g}^{-1}$ . Plasmid molecules did not interact with the HIC column, eluting in the flow through. The reason for this behaviour was that in double-stranded plasmid molecules, the hydrophobic bases were packed and shielded inside the helix and thus hydrophobic interaction with the support ligands was minimal. Diogo et al. (1999) with the same HIC gel also separated denatured plasmid from native plasmid. Because denatured forms contain large stretches of single-stranded DNA, there is more exposure of hydrophobic bases and, consequently, hydrophobic interaction is greater and retention time is longer. In fact, HIC support removed denatured plasmid variants that were usually produced with alkaline lysis of plasmid isolation (Diogo et al., 2000). Our results indicate the ability of the HIC support to also separate proteins and genomic DNA from plasmids.

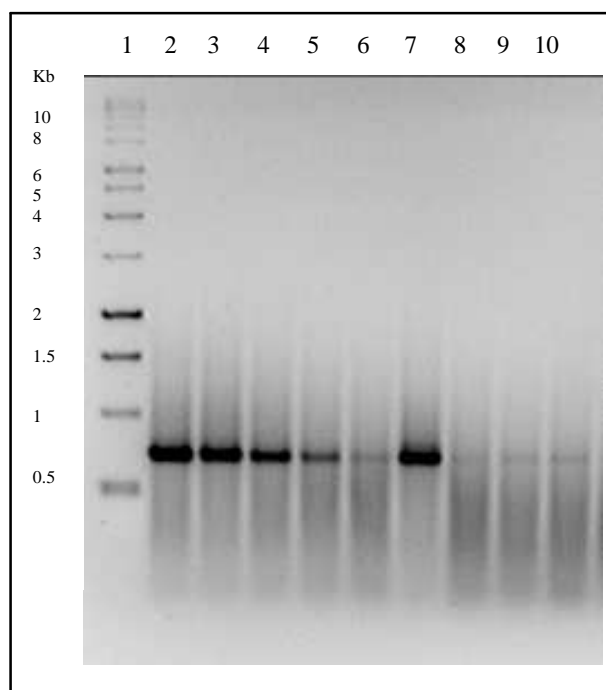
**Table 1** - Purification of pVax-LacZ plasmid

Process steps	Plasmid Mass ( $\mu\text{g}$ )	Purity (%)	Purification Factor
Lysis	8716	5.7	1.0
Isopropanol precipitation	4219	6.7	1.7
$(\text{NH}_4)_2\text{SO}_4$ precipitation 2.0M	1837	26	3.1
$(\text{NH}_4)_2\text{SO}_4$ precipitation 2.5M	1490	32	2.4
$(\text{NH}_4)_2\text{SO}_4$ precipitation 3.0M	1388	37	2.2
HIC 2.0M	1202	100	3.2
HIC 2.5M	1229	100	3.3
HIC 3.0M	1184	100	3.1

Results reported are the average of two independents experiments and errors were judged 5% of the mean value



**Figure 1** - Hydrophobic interaction chromatography performed on plasmid solutions after 2.5M ammonium sulphate precipitation. Elution was isocratic with 1.5M ammonium sulphate in 10 mM Tris-HCl (pH 8.0) at a low rate of  $1\text{ mL min}^{-1}$ .



**Figure 2** - Analysis of gDNA by agarose gel electrophoresis: molecular weight markers (lane 1). Calibration curve (lane 2-6). Lysis solution (lane 7). Samples after HIC 2.0M, 2.5M and 3.0M precipitation with sulphate of ammonium (lane 8-10).

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## RESUMO

O DNA plasmidial tem sua aplicação na terapia gênica, tendo que apresentar elevado grau de pureza. Um método de purificação foi descrito neste trabalho, que inclui precipitação com sulfato de amônio seguida da cromatografia de interação hidrofóbica, usando a resina phenyl sepharose 6 fast flow. Ocorreu aumento da pureza das amostras analisadas, em torno de 30% após a precipitação com sulfato de amônio. Houve significativa redução dos contaminantes após a eluição das amostras na cromatografia de interação. O maior rendimento obtido foi 53% e fator de purificação 3,1 vezes após a eluição na cromatografia de interação hidrofóbica. A vantagem deste método é a possibilidade de explorar o caráter hidrofóbico, constituído de material genético de fita simples, tais como RNA e DNA genômico e proteínas, que podem interferir na liberação deste material para possíveis aplicações em terapia gênica.

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