

**Purification of plasmid DNA from clarified and non-clarified lysates  
by berenil pseudo-affinity chromatography**

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## ABSTRACT

Several small molecules, like some therapeutic agents, are able to bind DNA with high specificity. These may represent a relevant alternative as ligands in affinity and pseudo-affinity chromatographic processes for plasmid DNA (pDNA) purification. In the present study, berenil (a DNA intercalator used as an anti-trypanosomal agent in veterinary applications) was tested as a ligand to specifically purify plasmids with different sizes, pVAX1-LacZ (6.05 Kbp) and pCAMBIA-1303 (12.361 Kbp) from the impurities present in *Escherichia coli* alkaline lysates. For this purpose, chromatographic experiments were set using Sepharose derivatized with berenil. The results showed that both pDNA molecules are completely purified using smaller amounts of salt in the eluent than those reported before for other pseudo-affinity and hydrophobic chromatography based processes. Total retention of all lysate components was achieved with 1.3 M ammonium sulphate in the eluent buffer and pDNA elution was obtained by decreasing the salt concentration to 0.55 M. All impurities were eluted after decreasing the concentration to 0 M. The recovery yield for the larger pDNA molecule pCAMBIA-1303 (45%) is lower than that obtained for pVAX1-LacZ (85%), presenting however a higher final purity. Furthermore, pVAX1-LacZ purification studies were also performed using non-clarified *E. coli* process streams, replacing the clarification step with a second chromatographic run on the berenil-Sepharose support. Using the same binding and elution conditions as before, a pure plasmid sample was obtained with a 33% yield. The pDNA fractions were analysed for *E. coli* host impurities, and all levels were in accordance to the requirements established by the regulatory agencies (FDA). These results suggest that this chromatographic support is a promising alternative to purify pDNA for therapeutic use.

51 **KEYWORDS:** plasmid purification; small DNA ligands; berenil; pseudo-affinity  
52 chromatography

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## 1. Introduction

Molecular therapy approaches using non-viral vectors, as plasmid DNA (pDNA) for the introduction of therapeutic genes, are becoming the chosen strategy to treat various types of diseases [1-3]. Thus, therapeutic pDNA is an emerging biotechnology product with a great potential in human and animal healthcare. Currently more than 400 gene therapy or DNA vaccines clinical trials are being conducted worldwide using these vectors (<http://www.wiley.com/legacy/wileychi/genmed/clinical/>). In addition four DNA vaccine products have already been approved for veterinary application [4-6]. Accordingly, the expected wide application of these vectors requires the large-scale production and purification of pDNA. Recent years have witnessed an increasing research effort in the development of new methods for plasmid purification that meet strict quality criteria in terms of purity, efficacy and safety, required by the Regulatory Agencies. The critical contaminants of pDNA preparations are similar in size (genomic DNA (gDNA) and endotoxins), negative charge (RNA, gDNA and endotoxins) and have similar hydrophobicity (endotoxins), which can complicate their separation [7]. The maximum levels of gDNA, host proteins and RNA in the final product should preferably be under 1% (w/w) each and the amount of endotoxin should not exceed 40 EU/mg plasmid [8]. Moreover, the purification method should not comprise the use of organic reagents, mutagenic and toxic compounds and animal derived enzymes [9].

The process of pDNA obtention includes the production in *Escherichia coli* cells by fermentation followed by a lysis step [10], usually performed using the popular alkaline lysis method [11], a concentration step with isopropanol and pre-purification/clarification with ammonium sulphate [10]. Further downstream processing aims to eliminate impurities like gDNA, low molecular weight RNA, residual proteins

and endotoxins. Chromatography is the most suitable method for this purpose [10,12], however, the poor selectivity that most of the matrices display towards pDNA is one of its major drawbacks [13]. The use of affinity ligands can be a simple and efficient approach to overcome this problem [14,15]. Affinity and pseudo-affinity chromatography use the specificity and bio-recognition properties of the ligands, based on specific and reversible interactions found in biological systems, to separate the pDNA molecules from the impurities found in cell lysates[16].

Several molecules, like certain antibiotics and anticancer agents, bind DNA with high specificity [17] and due to the complex structure of double-helical DNA, they can do so by different binding modes. Besides covalent binding there are several classes of specific and unspecific noncovalent binding modes, like minor groove binding, intercalation between base pairs, bisintercalation, major groove binding and a combination of the above [18]. Berenil (1,3-bis(4-phenylamidinium) triazene), is used as an anti-trypanosomal agent in veterinary applications [19] and is a member of the aromatic diamidine class of DNA binding agents, which reversibly and preferentially binds to the DNA minor groove at central AATT sequence [20,21]. Berenil was recently applied as ligand for chromatographic separation of the supercoiled (sc) plasmid isoform from the less active open circular (oc) isoform [15], showing a great affinity for pDNA, especially for the sc isoform. The DNA binding affinity of this ligand has been attributed to several factors: electrostatic interactions with the AT sequences, hydrophobic contacts between the phenyl rings and the hydrophobic regions of the DNA backbone, the triazene group neighbouring the polar phosphodiester groups and hydrogen bonds between the amidines and thymine and/or adenine acceptor groups of the bases at the floor of the groove [15].

105           This study reports a pseudo-affinity chromatographic technique to purify pDNA  
106 directly from clarified and non-clarified *E. coli* lysate solutions that show some  
107 improvements over existing methods in terms of using smaller amounts of salt in the  
108 overall purification process. This approach was tested for the purification of pDNA  
109 molecules with different sizes (pVAX1-LacZ and pCAMBIA-1303, with 6.05 Kbp and  
110 12.361 Kbp, respectively).

## 2. Materials and Methods

### 2.1. Materials

Sepharose CL-6B was obtained from Amersham Biosciences (Uppsala, Sweden). Berenil and 1,4-butanediol diglycidyl ether were purchased from Sigma-Aldrich (St. Louis, MO, USA). All salts used were of analytical grade.

### 2.2. Bacterial Culture

*Escherichia coli* DH5 $\alpha$  strain harbouring 6.05 Kbp plasmid pVAX1-LacZ (Invitrogen, Carlsband, CA, USA) and *Escherichia coli* XL1blue strain harbouring 12.361 Kbp plasmid pCAMBIA-1303 (Cambia, Brisbane, Australia), were cultured overnight in Luria Bertani agar (Lennox) medium (Laboratorios Conda, Madrid, Spain), supplemented with 30  $\mu$ g/mL of kanamycin at 37°C. *E.coli* DH5 $\alpha$  strain, cell growth was carried out at same temperature in shaken flasks with Terrific Broth medium (20 g/L tryptone, 24 g/L yeast extract, 4 mL/L glycerol, 0.017 M KH<sub>2</sub>PO<sub>4</sub>, 0.072 M K<sub>2</sub>HPO<sub>4</sub>) supplemented with 30  $\mu$ g/mL kanamycin. The XL1blue strain cells were grown in similar conditions using Luria Bertani medium (5 g/L yeast extract, 10 g/L tryptone, 10 g/L NaCl, pH 7.0). Both cell strains were harvested by centrifugation at the end of the exponential growth phase and stored at -20°C until needed. Plasmid-free *E. coli* cells were also grown under the same conditions, as described before, but with no antibiotic present.

### 2.3. Lysis and Primary Isolation

Cells harbouring the plasmid were lysed using a modification of the alkaline method proposed by Sambrook *et al.* [22]. 250 mL of the cell broth was centrifuged at

5445 g for 30 min at 4°C with a Sigma 3-18K centrifuge. The supernatants were discarded and the bacterial pellets were resuspended in 20 mL of 50 mM glucose, 25 mM Tris-HCl, 10 mM ethylenediamine tetraacetic acid (EDTA) (pH 8.0). Lysis was performed by adding 20 mL of a 200 mM NaOH, 1% (w/v) sodium dodecylsulphate solution. After 5 min of incubation at room temperature, cellular debris, gDNA and proteins were precipitated by gently adding and mixing 16 mL of prechilled 3 M potassium acetate (pH 5.0). The precipitate was removed by a double centrifugation at 20 000 g for 30 min at 4°C with a Beckman Allegra 25 R centrifuge. The plasmid in the supernatant was precipitated after addition of 0.7 volumes of isopropanol and a 30 min incubation period on ice. The pDNA was recovered by centrifugation at 16 000 g for 30 min at 4°C. The pellets were then redissolved in 1 mL of 10 mM Tris-HCl buffer (pH 8.0). A fraction of this solution was subjected to a clarification step. For this purpose, and after optimization studies, solid ammonium sulphate was dissolved in the pDNA solutions up to a final concentration of 2.0 M, for the pVAX1-LacZ solution, and 2.5 M for the pCAMBIA-1303 solution, followed by a 15 min incubation period on ice. Precipitated proteins and RNA were then removed by centrifugation at 10 000 g for 20 min at 4°C. The supernatant was recovered and its nucleic acid concentration quantified by measuring the absorbance at 260 nm.

#### *2.4. Preparation of Berenil-Sepharose support*

Sepharose CL-6B was epoxi-activated according to the method described by Sundberg and Porath [23] and coupled to berenil as described before [15]. The orange derivatized gel so obtained was stored at 4°C in deionised water.

#### *2.5. Preparative Chromatography*



Chromatographic studies were performed in a Fast Protein Liquid Chromatography (FPLC) system (Amersham Biosciences, Uppsala, Sweden) at room temperature. A 10 cm x 10 mm column (Amersham Biosciences, Uppsala, Sweden) was packed with 2 mL berenil-derivatized (Fig. 1) gel and initially tested with different ammonium sulphate concentrations (0.2 M to 1.5 M) in the mobile phase. Prior to the sample application, and after achieving the optimal conditions of binding and elution, the column was equilibrated with 1.3 M ammonium sulphate in 10 mM Tris-HCl buffer (pH 8.0) at a flow rate of 1 mL/min.

#### *2.5.1. Injection of clarified samples*

Clarified samples (25  $\mu$ L) were loaded onto the column in equilibration buffer at a flow rate of 1 mL/min. To promote the selective elution of bonded species, the salt concentration was first decreased to 0.55 M ammonium sulphate in 10 mM Tris-HCl buffer (pH 8.0) and then to 0 M. The absorbance was continuously monitored at 280 nm. Fractions were pooled according to the chromatograms obtained, concentrated and desalted using Vivaspin concentrators (Vivaproducts, Littleton, MA, USA) and kept for further analysis as described below.

#### *2.5.2. Injection of non-clarified samples*

Non-clarified samples (25  $\mu$ L) with a nucleic acid concentration around 600  $\mu$ g/mL in equilibration buffer were loaded onto the column at 1 mL/min flow rate and the elution was performed as described above for clarified samples. A second chromatographic step was then performed in the same binding and elution conditions, injecting the pDNA fraction, pooled after the first run and concentrated to an approximate nucleic acid concentration of 600  $\mu$ g/mL,. Fractions were pooled according

to the chromatograms obtained, concentrated and desalted using Vivaspin concentrators (Vivaproducts, Littleton, MA, USA) and kept for further analysis as described below. After the chromatographic runs, the column was washed with at least 5 bed volumes of deionised water.

## 2.6. Agarose gel electrophoresis

Pooled fractions were analysed by horizontal electrophoresis (100V for 40 min) using 1 % and 0.8% agarose gel in TAE buffer (40 mM Tris base, 20 mM acetic acid, and 1 mM EDTA, pH 8.0) in the presence of 0.5 µg/mL ethidium bromide. The gels were visualized in a UVITEC Cambridge system (UVITEC Limited, Cambridge, UK).

## 2.7. Analytical Chromatography

pDNA concentration and purity of feed samples injected onto the berenil column and the fractions pooled after the chromatographic runs was assessed by high-performance liquid chromatography (HPLC), according to the method described by Diogo *et al.* [24]. A 4.6/100 mm HIC (Hydrophobic Interaction Chromatography) Source 15 PHE PE column (Amersham Biosciences, Uppsala, Sweden) was connected to a Waters HPLC system (Waters Corporation, Milford, MA, USA) and equilibrated with 1.5 M ammonium sulphate in 10 mM Tris-HCl buffer (pH 8.0). Samples (20 µl) were injected and eluted at a flow rate of 1 mL/min. After injection, the elution occurred with the equilibration buffer for 3 min, and then the elution buffer was instantly changed to Tris-HCl 10 mM (pH 8.0) without ammonium sulphate. This condition was maintained for 6 min in order to elute bound species. The column was then re-equilibrated for 7 min with the equilibration buffer to prepare the column for the next run. The absorbance of the eluate was continuously recorded at 254 nm. The

concentration of pDNA in each sample was calculated using a calibration curve constructed with pDNA standards (2.5 - 400 µg/mL), purified using the Qiagen plasmid midi kit (Hilden, Germany), according to the manufacturer's instructions. Plasmid quantification was achieved by measuring the absorbance at 260 nm and assuming that a solution of 50 µg/mL has an absorbance of 1.0. The purity degree was defined as the percentage of the pDNA peak area in relation to the total area of all chromatographic peaks.

## 2.8. Protein Analysis

Protein concentration of feed samples and of pDNA chromatographic fractions was measured using the micro-bicinchoninic acid (BCA) assay from Pierce (Rockford, IL, USA), according to the manufacturer's instructions. 50 µL of each sample were added to 200 µL of BCA reagent in a microplate and incubated for 30 min at 60°C. Absorbance was measured at 595 nm in a microplate reader. The calibration curve was prepared using bovine serum albumin standards (0.025-1 mg/mL).

## 2.9. Endotoxin Analysis

Analysis of endotoxin contamination on both feed samples and pDNA fractions pooled after chromatography was performed using the ToxinSensor™ Chromogenic LAL Endotoxin Assay kit from GenScript (GenScript USA Inc., Piscataway, NJ, USA). The detection level of the method was 0.005 EU/mL.

## 2.10. Genomic DNA analysis

Genomic DNA contamination in purified plasmid solutions and in feed samples was assessed using real-time polymerase chain reaction (PCR) in a iQ5 Multicolor

Real-Time PCR Detection System (Bio-Rad, Hercules, CA, USA), according to the method described by Martins *et al.* [25]. The feed sample was diluted 1000 fold. Sense (5'-ACACGGTCCAGAACTCCTACG-3') and antisense (5'-GCCGGTGCTTCTTCTGCGGGTAACGTCA-3') primers were used to amplify a 181-bp fragment of the 16S rRNA gene. PCR amplicons were quantified by following the change in fluorescence of the DNA binding dye Syber Green (Bio-Rad, Hercules, CA, USA). *E. coli* genomic DNA was purified with the Wizard gDNA purification kit (Promega, Madison, WI, USA) and used to generate a standard curve ranging from 0.005–50 µg/mL. Negative controls, with no template, were run at the same time as the standards.

### 3. Results and Discussion

#### 3.1. Clarification of pDNA lysates by salt precipitation

RNA is the critical contaminant of non-clarified cell lysates, and since it is structurally similar to pDNA [7], its separation can represent a true challenge. One common procedure for RNA removal is the precipitation with high salt concentrations, using ammonium sulphate as the clarifying agent. This precipitation step is very effective in reducing the amounts of high molecular weight RNA, proteins and endotoxins, as well as in improving intermediate pDNA recovery, thus representing an excellent complement to any further purification step [26].

Standard ammonium sulphate precipitation uses 2.5 M of salt concentration to clarify crude cell lysates, which has a great environmental impact due to its high eutrophication potential [26]. However, previous studies performed by Freitas *et al*, [27] conclude that precipitation with high salt concentration is not always needed. Based on these conclusions, optimization studies were performed in the precipitation step to select the best conditions for high pDNA recovery and purity by analysing the influence of ammonium sulphate concentration on RNA removal. Ammonium sulphate was added to the non-clarified pDNA containing suspensions in a concentration range from 1.3 M up to 2.5 M and the precipitated proteins and RNA were then removed by centrifugation. The obtained samples were injected onto the berenil-Sepharose column (with the optimized conditions of binding and elution) and the purity of eluted pDNA fractions in terms of RNA contamination was evaluated by HPLC analysis.

The data obtained showed that for the lower size pDNA molecule, pVAX1-LacZ (6.05 Kbp), a concentration of 2.0 M ammonium sulphate in the precipitation step was enough to achieve a pure sample after the chromatographic step using berenil as ligand. Precipitation with a salt concentration of 1.9 M and lower, led to a similar recovery

yield, but resulted in impure pDNA samples. The pDNA purity started from approximately 66% for the clarification with 1.3 M of ammonium sulphate, gradually increasing to 80% for the precipitation with 1.9 M of salt. On the other hand, when the clarifying step was performed with ammonium sulphate concentrations above 2.0 M, all the pDNA samples were 100% pure, with recovery yield similar to that obtained for 2.0 M. Therefore, this salt concentration was chosen since the clarification followed by the berenil-Sepharose chromatographic step delivered a pure pDNA sample, with a good yield and using a smaller amount of salt in the process.

In contrast, for pCAMBIA-1303 (12.361 Kbp) a higher ammonium sulphate concentration (2.5 M) was needed to obtain a 100% HPLC pure sample after the same chromatographic process. Using a salt concentration of 2.0M in the clarification step, the purity of pDNA samples was set around 20% increasing to more than 40% with 2.25M of ammonium sulphate. It is known that pDNA size can influence the recovery by alkaline lysis, and since bigger plasmids are more susceptible to shear forces, smaller pDNA concentrations are normally obtained [28]. So, for pCAMBIA-1303, the pDNA/RNA ratio in non-clarified solutions is smaller and possibly because of that, higher ammonium sulphate concentrations are required to maximize plasmid purification and recovery from the highly RNA contaminated solutions.

### 3.2. Berenil – Sepharose Pseudo-Affinity Chromatography

#### 3.2.1. Injection of clarified pDNA solutions

Recently, berenil-Sepharose pseudo-affinity support (Fig. 1) was successfully used for separation of sc plasmid isoform from the less active oc isoform, showing a great affinity for pDNA [15]. Therefore, the ability of this support to purify pDNA directly from clarified *E. coli* cell lysates was exploited in the present study. Moreover,

the applicability of this process to purify pDNA molecules with different sizes was also tested. For this purpose, solutions of pVAX1-LacZ (6.05 Kbp) and pCAMBIA-1303 (12.361 Kbp), obtained separately after clarification with ammonium sulphate, were used as feedstock for the pseudo-affinity chromatography studies. Several binding-elution experiments were performed to achieve optimal buffer conditions to separate pDNA from the lysate impurities, namely RNA. These experiments showed that the chromatographic conditions are the same for both plasmids: total retention of all lysate components was achieved using 1.3 M of ammonium sulphate in the binding buffer and elution of pDNA was obtained by a simple decrease of salt concentration to 0.55 M. The more hydrophobic impurities, like RNA, were eluted only when the concentration was decreased to 0 M. Fig. 2 shows the chromatograms after injection of pVAX1-LacZ and pCAMBIA-1303 samples onto the berenil-Sepharose column. Both plasmids showed a comparable separation performance represented by similar chromatograms, which demonstrates the reproducibility of the chromatographic process for plasmid molecules of different sizes. The chromatograms are characterized by a first small system peak, followed by a sharp higher peak of pDNA and a smaller one of strongly retained species such as RNA. As shown by agarose gel electrophoresis (Fig.2) and confirmed by HPLC, RNA was completely separated from pDNA molecules (electrophoresis lane 1 in Fig. 2 for both plasmids) and eluted in the peak 2. By the observation of Fig. 2, it can be concluded that all cell impurities that absorb at 280 nm are retained longer in the column when compared with pDNA. Plasmid molecules did not interact with the column as strongly as RNA. In those double-stranded molecules, the hydrophobic bases are packed and shielded inside the helix and thus interaction with the support ligands is smaller, eluting first. On the other hand, RNA molecules are

inherently single-stranded with the hydrophobic bases largely exposed, and thus elute after the plasmid [29].

The binding between berenil-Sepharose support and the lysate components results not only from hydrophobic interactions between the phenyl rings of berenil and the hydrophobic regions of the backbone of the molecules, but also from other more specific interactions that are responsible for the great affinity that the support shows for those molecules [15].

An epoxy activated Sepharose gel obtained using the same experimental conditions but without berenil ligand was used for the control experiments (results not shown). A distinct pattern from the observed with the berenil-Sepharose support was obtained: total retention was not observed at 1.3 M of ammonium sulphate and the lysate components were not separated by decreasing the salt concentration. Accordingly, these experiments unequivocally identified berenil bonded onto the activated Sepharose as the ligand responsible for the retention and separation of pDNA molecules. Moreover, the column cleaning and the high number of chromatographic runs did not cause any change in the chromatographic performance of the derivatized gel.

### *3.2.2. Injection of non-clarified pVAX1-LacZ solution*

The ability of the berenil-Sepharose pseudo-affinity chromatographic matrix to separate and purify pDNA directly from non-clarified *E. coli* process streams was also tested. This was accomplished without the clarification step with a high ammonium sulphate concentration. In a first run, the feedstock sample was injected onto the berenil-Sepharose column with 1.3M of ammonium sulphate in the eluent. The chromatographic profile obtained is shown in Fig. 3A, as well as the agarose gel



electrophoresis analysis of the pDNA eluted fractions. The electrophoresis shows that the pDNA fraction still contains a slight RNA contamination, even though the great majority of this contaminant clearly elutes in the second peak when the ammonium sulphate concentration is decreased to zero. In the second run, the pDNA fraction obtained after the first chromatographic step was concentrated and injected onto the berenil-Sepharose column using the same buffer conditions. The obtained chromatogram (Fig. 3B) showed two well defined peaks, the first one corresponding to the elution of pDNA after decreasing the salt concentration to 0.55M and a second peak corresponding to the all contaminants with higher hydrophobicity, eluted after decreasing the salt concentration to 0M.

### *3.3. Plasmid Quality and Purity assessment*

The performance of pDNA purification for both pVAX1-LacZ and pCAMBIA-1303, as well as for the non-clarified pVAX1-LacZ after two runs with the berenil-Sepharose support, was examined by the determination of process parameters such as yield and purity (Table 1) by HPLC analysis. Besides HPLC analysis of RNA contamination, the purity of the recovered plasmid fractions was also determined by quantification of proteins (BCA assay), endotoxins (Chromogenic Limulus amoebocyte lysate Endotoxin assay) and gDNA (real-time PCR) (Table 2).

For the clarified samples, agarose gel electrophoresis revealed that the plasmid pools were RNA free (Fig. 2) and this was also confirmed by HPLC analysis (Fig. 4). The analytical chromatogram shown in Fig. 4A represents the clarified lysate injected onto the berenil-Sepharose column. Clearly a great percentage of the sample was constituted by impurities, namely RNA. In this feed sample, the HPLC pDNA purity was 23% (Table 1) for pVAX1-LacZ and 7% for pCAMBIA-1303, however after the

preparative chromatographic step, both plasmid samples were 100% pure (Table 1) and free of RNA. Fig. 4B represents the analytical chromatogram for the obtained pDNA, and show a single plasmid peak, confirming the agarose gel electrophoresis results. The obtained HPLC purification factors (Table 1) are higher than those described for similar chromatographic procedures (1.9 and 1.5) [30,31], particularly for pCAMBRIA-1303 which has an impressive value of 14.3. HPLC analysis also showed that it was possible to recover 85% of the loaded pVAX1-LacZ (Table 1). This value is similar to the 84% obtained with an affinity support by Sousa and co-workers [31] but slightly higher than the 70% yield obtained with a pure hydrophobic chromatographic matrix [29]. Nevertheless for pCAMBRIA-1303, the recovery yield was much lower than for the smaller pDNA molecule (Table 1). This may be due to the fact that bigger plasmid molecules are more unstable and degrade more easily during the extraction and purification procedures than smaller ones, since they are more sensible to shear forces [28] and more susceptible to losses during the chromatographic step. The quantitative analysis of impurities in injected feed and in pDNA fractions is shown in table 2. The BCA protein assay indicated that both plasmid pool solutions have undetectable levels of proteins. Despite the feed solution of pVAX1-LacZ contained a small amount of this impurity (66 µg/mL), the berenil-Sepharose gel was capable of separating the plasmid molecules from the protein contaminants present in cell lysates. No proteins were detected in pCAMBRIA-1303 feed solution, possibly because the clarification step was accomplished with 2.5 M of ammonium sulphate. Real-time PCR analysis of pooled plasmid fractions showed a great reduction of gDNA content after the berenil-Sepharose chromatographic step. A notable gDNA decrease of 3653 fold was achieved for pVAX1-LacZ and an impressive 554472 fold reduction was achieved for pCAMBRIA-1303 (Table 2). Berenil-Sepharose support is then extremely efficient to eliminate

gDNA from pDNA solutions, even in cases where they are heavily contaminated. 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 gDNA molecules show a high exposure of their hydrophobic bases [28] and can thus interact more strongly with berenil, eluting largely in the second peak (Fig.2). The chromatographic process here described was also very effective in reducing the endotoxin levels from plasmid solutions. A reduction of 20 fold was obtained for both types of plasmid molecules (Table 2) demonstrating that endotoxins bind to the berenil support more strongly than pDNA. The quality analysis of pDNA showed that pVAX1-LacZ and pCAMBIA-1303, purified with the berenil-Sepharose support, meet the specifications of the regulatory agencies, namely FDA (Table 3) [8]. Both RNA and proteins (preferably <1%) are undetectable in the final plasmid solutions and the endotoxin levels are ten times lower than the maximum required by FDA (40 EU/mg pDNA). Genomic DNA content was the only parameter that greatly varies among pDNA feed solutions and plasmid pools, being much higher for pVAX1-LacZ. Nevertheless, both small and higher size plasmid pools are under FDA specifications (preferably <1%) (Table 3).

Starting from a highly contaminated non-clarified lysate (Table 1) and after two consecutive chromatographic runs on the berenil-Sepharose column, the pVAX1-LacZ fraction became 100% free from RNA, with a purification factor of almost 23. Nevertheless, the yield (33%) is lower than expected. As before, the BCA protein assay showed that the pDNA pool had undetectable levels of proteins (Table 2), despite the feed solution being highly contaminated (1170 µg/mL), demonstrating that the berenil-Sepharose gel is able to completely remove this impurity. Real-time PCR analysis of both feed and plasmid fraction showed a remarkable 302792 fold decrease in gDNA

contamination (Table 2), which is in accordance with the values discussed before. In regard to endotoxin contamination reduction, the chromatographic runs are very efficient. After two runs, and starting from a highly contaminated solution, a 21 fold reduction was achieved, a value analogous to that obtained with only one run through the berenil-Sepharose column. Table 3 shows the comparison between the pDNA sample composition, collected after the second chromatographic run, and FDA specifications. All values are in accordance to the requirements for molecular therapy products.

In an overall analysis, the berenil-Sepharose chromatographic process meets all the requirements to be used as a pDNA purification step. This new chromatographic support enables pDNA purification using a lower salt concentration, compared with other processes that use hydrophobic or pseudo-affinity ligands [16,29], showing a better purification performance and yield for a plasmid with similar size.

#### **4. Conclusions**

In this study, a new chromatographic process using berenil as ligand has been developed and applied for purification of pDNA molecules with different sizes: pVAX1-LacZ (6.05 Kbp) and pCAMBIA-1303 (12.361 Kbp). Both types of plasmids were successfully separated from host impurities, according to FDA specifications, showing that the method can be applied to small plasmids as well as to large plasmids.

An optimization of the clarification step was also performed, showing that for pVAX1-LacZ, precipitation with 2.0M is enough to achieve a high pDNA recovery and purity, a value lower than the 2.5M usually used. However pCAMBIA-1303 was only completely purified (HPLC purity of 100%) using 2.5 M of salt perhaps due to its higher size. Additionally, a high recovery yield of 85% was achieved when purifying the smaller pDNA molecule. The purification of pCAMBIA-1303 showed a lower yield (45%) however its purification quality is superior to that obtained with pVAX1-LacZ, which could be interesting since future requirements for multigene vectors, including extensive control regions, may require the production of larger plasmids.

Despite the loss in yield, the replace of the clarification step with ammonium sulphate by a second chromatographic run showed advantages such as a gained in time, reduction in salt usage and procedure steps. Moreover, the purification achieved after two runs using berenil-Sepharose column is similar in terms of proteins and RNA decontamination. For gDNA, the purification is more efficient after two runs however for endotoxins the association of a clarification step with a chromatographic run with berenil-Sepharose gel is more effective (considering the relation contaminant mass/pDNA mass rather than the concentration values *per se*).

The pseudo-affinity chromatographic method developed has shown to be very effective in purifying plasmid DNA from *E. coli* cell lysate impurities, using smaller

amounts of salt in the overall process. The essential separation mechanism seems to involve not only differential hydrophobic interactions between the ligand, pDNA molecules and host impurities, but also other important and specific contributions of the affinity binding. Therefore with this pseudo-affinity support, the purification method uses the characteristics of both affinity and hydrophobic processes, is more environmentally friendly and less costly, and it is ready to be used as a main pDNA purification process.

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## Figure legends:

Fig. 1. Schematic representation of the berenil-Sepharose support. Berenil is covalently bonded to the epoxy arm. Oxygen atoms are represented in red, nitrogen atoms in blue and carbon in grey. (Generated with Avogadro: an open-source molecular builder and visualization tool. Version 1.0.3).

Fig. 2. Chromatographic separation of clarified feed solution (25  $\mu$ L) of pVAX1-LacZ (6.05 Kbp) and pCAMBIA-1303 (12.361 Kbp) from the host cell impurities on berenil-Sepharose support. Agarose gel electrophoresis analysis of the pDNA fractions. Peak 1 and electrophoresis lane1: pDNA fractions collected after elution with 0.55 M  $(\text{NH}_4)_2\text{SO}_4$ ; Peak 2: impurities eluted with 0 M  $(\text{NH}_4)_2\text{SO}_4$ . The clarified lysate was also run in the agarose gel for comparative purposes (lane feed).

Figure 3. Chromatographic separation of non-clarified pVAX1-LacZ samples on berenil-Sepharose column: (A) After one run through the support and agarose gel electrophoresis analysis of the eluted fractions: Peak 1 and electrophoresis lane1 - pDNA fraction eluted with 0.55M  $(\text{NH}_4)_2\text{SO}_4$ ; peak 2 and electrophoresis lane 2 - RNA eluted with 0M  $(\text{NH}_4)_2\text{SO}_4$ ; (B) After the second run through the berenil support.

Fig. 4. HPLC analysis of different pVAX1-LacZ samples: A – *E.coli* clarified lysate diluted 1:20; B – pDNA fraction collected after the chromatographic run; C – 400  $\mu$ g/mL pVAX1-LacZ standard for comparative purposes. The chromatograms from HPLC analysis of pCAMBIA-1303 samples were similar to those here represented.