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Milligram scale parallel purification of plasmid DNA using anion-exchange membrane capsules and a multi-channel peristaltic pump

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Abstract

A parallel chromatographic procedure for the purification of milligram amounts of plasmid DNA was developed. Initial studies showed that ion-exchange membrane capsules displayed high capacity for plasmid DNA. Interestingly, a weak anion exchanger (DEAE) proved to be superior to the strong quarternary ammonium group with respect to elution and regeneration properties and the 75 cm^2 Sartobind D membrane capsule (MA75D, Sartorius) was selected for further studies. A method for reducing endotoxin levels by using CTAB as a precipitant was optimised. By introducing this step into the protocol, endotoxin levels could be reduced approximately 100-fold to ≤5 EU/mg plasmid. The parallel procedure was set up on a multi-channel peristaltic pump and evaluated with four different vectors (2.7–11.5 kbp). Starting with 5–10 g of *E. coli* cell paste (wet weight) generally saturated the membrane adsorber, resulting in plasmid DNA yields close to 10 mg.

Keywords: Purification; Plasmid DNA; Membranes; Multi-parallel

1. Introduction

The molecular biology science of today demands large quantities of plasmid DNA (pDNA) for cloning, large-scale protein production and DNA vaccine/gene therapy applications. Plasmid DNA is generally prepared from *E. coli* cultures using the alkaline lysis protocol developed by Birnboim and Doly [1] followed by preferably an ion-exchange step. Many commercially available kits are based on this principle and with a maxiprep kit it is possible to obtain yields of 1 mg plasmid DNA in about 3 h of work (e.g. Wizard® *Plus* Maxiprep DNA Purification System, Promega Corporation). Even larger capacity kits (sometimes referred to as gigaprep) can be used to obtain quantities of plasmid DNA up to 10 mg (Qiagen, Macherey-Nagel, PhoenIX, Invitrogen). Protocols for small-scale pDNA purification can be readily automated. However, at the mega- and gigaprep scales, commercial kits often require the use of vacuum and/or manually handled gravity-flow devices, which makes the process less suitable for parallel purifications.

The quality demands on the purified product depend on its usage. For gene therapy, the restrictions are significant, whereas the quality requirements for normal molecular biology applications are less strict [2]. Important parameters regarding the quality of the pDNA are the conformation (e.g. fraction of supercoiled pDNA), amount of impurities such as protein, RNA and chromosomal DNA (chDNA), as well as endotoxin levels. Most of the proteins and chDNA are denatured and precipitated during the lysis procedure. RNase treatment can be used to reduce RNA levels but still, efficient chromatographic steps are needed in order to achieve pure pDNA. In addition to anion-exchange chromatography, there are several other chromatographic steps that can be used in pDNA purification processes e.g. gel filtration, hydrophobic interaction chromatography, reverse phase and thiophilic interaction chromatography [3]. Moreover, either pDNA or the above mentioned contaminants can be selectively

Abbreviations: chDNA, chromosomal DNA; CTAB, cetyltrimethylammonium bromide; CV, column volumes; MWCO, molecular weight cut-off; pDNA, plasmid DNA

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precipitated using e.g. isopropanol [4], cetyltrimethylammonium bromide (CTAB) [5], polyethylene glycol [6], LiCl [7], CsCl [8] or spermidine [9]. Since pDNA molecules are very large (supercoiled plasmids have a size of several thousand Å in length) [2], the small pores of many traditional chromatographic media are inaccessible for the plasmids, which thus drastically reduces the dynamic binding surface area. Therefore, it is appropriate to use either media with very large pores, resins substituted with "tentacle" like extensions, or membrane based chromatography in order to increase the binding capacity for pDNA [2].

The present study describes the development of a multi-parallel purification method for pDNA at "gigaprep" scale. The chromatographic steps, i.e. anion exchange and buffer change by gel filtration, as well as different filtration steps are driven by a conventional peristaltic pump equipped with a 12-channel pump head. An optional step to reduce the levels of endotoxin to meet the demands for pharmaceutical grade pDNA is also described. With this procedure it is possible to produce 5–10 mg of pDNA from a dozen constructs on a daily basis.

2. Materials and methods

2.1. Chemicals and reagents

All chemicals, reagents and enzymes were purchased from Sigma–Aldrich.

RNase—ribonuclease A from bovine pancreas Type III-A, ≥85% (SDS-PAGE), 91 Kunitz units/mg protein (R5125).

CTAB—cetyltrimethylammonium bromide for molecular biology, ∼99%. (H6269).

2.2. Cells and plasmids

Four different plasmid backbones with various inserts were used in this study. The constructs (denoted A–D) were: A, pUC 18 (Invitrogen) – 2.7 kb; B, pCMV/myc/ER (Invitrogen) – 7.6 kb; C, peak10 (Edge Biosystems) – 8.9 kb; D, pCEP4 (Invitrogen) – 11.5 kb.

All constructs carried the *bla* gene providing ampicillin resistance. The vectors were transformed into *E. coli* XL1 Blue (Stratagene) $CaCl_2$ competent cells using standard protocols. Clones were picked from single colonies and stored as glycerol stocks at −80 °C. Material for pDNA purification was then prepared either from over night shake flask cultures in LB medium (37 °C, 140 rpm) or bioreactor cultivations in minimal medium. The concentration of carbenicillin was 100 μg/mL. Cells were harvested by centrifugation at 7000 × *g* for 20 min at 4 °C and the resulting cell paste was stored at −80 °C.

2.3. Cell lysis protocol

The desired amount of frozen cell paste (typically 5–7 g wet weight) was thawed on ice in 250 mL centrifuge bottles. Cell lysis was performed using a slightly modified Birnboim and Doly protocol [1]. The cells were resuspended in 40 mL suspension buffer (50 mM Tris–HCl, 10 mM EDTA, pH 8.0) using a Turrax T25 Basic (Rose Scientific) at a speed of 8000 min^{-1}. RNase was added to a final concentration of 100 μg/mL. Cell lysis was performed with 80 mL lysis solution (0.145 M NaOH, 1% SDS). The mixture was gently stirred with a spoon for 5 min. Neutralisation was achieved by adding 40 mL of neutralisation solution (3 M KAc, 10 mM EDTA, pH 5.5) and by gentle mixing with a spoon for 5 min. Samples were then clarified by centrifugation at 38,000 × *g*, 4 °C for 45 min in a Beckman Avanti J-20 (Beckman Coulter). Immediately following centrifugation, the supernatants were poured through a piece of Miracloth (Calbiochem) with a pore size of 22–25 μm. Typically, 155–165 mL clarified lysate was obtained in this way.

2.4. Precipitation of plasmid DNA

Precipitation experiments with CTAB were conducted. The aim was to find the optimal concentration of CTAB that reduces the level of contaminants (RNA and/or endotoxins) while giving high yields of plasmid DNA. Precipitation with a final concentration of 0.1–4 g/L of CTAB was performed by adding an appropriate volume of a CTAB stock solution (2 g/L or 10 g/L CTAB in 50 mM NaCl) to a clarified lysate. After an incubation time of typically 20 min the pellet was recovered by centrifugation (38,000 × *g* for 20 min at 20 °C), and was washed twice with 70% ethanol prior to dissolving over night in buffer (0.60 M NaCl, 25 mM Tris–HCl, 1 mM EDTA, pH 7.4) at 6 °C with continuous gentle agitation. Precipitation with isopropanol, where 0.7 volumes were added to a clarified lysate, was used for comparison.

2.5. Pre-chromatographic steps and purification of pDNA

Method development was performed on an ÄKTA Purifier (GE Healthcare Bio-Sciences) equipped with additional eight-way valves for column scouting, a fraction collector and autosampler. Several different anion exchange chromatography resins were tested, i.e. Q sepharose HP, Q sepharose FF, Q sepharose XL (agarose-based beads with additional functionalised dextrane chains) and DEAE sepharose FF (1 mL pre-packed HiTrap columns, GE Healthcare); EMD TMAE Fractogel (Merck) ∼1 mL packed in HR 10/10 columns (GE Healthcare), MA 5 Q and D (5 cm^2 membrane area, 0.14 mL strong- and weak anion-exchange membrane capsules, respectively, Sartorius). Using a purified reference plasmid (B) the dynamic binding capacities of the different columns were tested in breakthrough experiments (i.e. the capacity is the amount pDNA loaded to achieve 10% breakthrough). Binding and elution parameters were optimised by scouting different NaCl concentrations (0–2 M) in DNA binding buffer (25 mM Tris–HCl, 1 mM EDTA, pH 7.4).

Membrane chromatography was selected as the purification method of choice and protocols for the purification of milligram amounts of pDNA were then optimised for MA 75 D and Q membrane capsules (75 cm^2 membrane area, 2.1 mL); starting with either clarified lysate or CTAB precipitated plasmid. Binding conditions were optimised by adding different volumes from

a 5 M NaCl stock solution to clarified lysate. For MA 75Q, final concentrations of NaCl in the range of 0.27–0.50 M were used. The corresponding interval for MA75 D membranes was 0.20–0.275 M NaCl. The conductivity of CTAB precipitated samples was optimised by varying the salt contents in the buffer for DNA solubilisation.

The samples were thoroughly mixed and subsequently filtered through 0.22 μm CA bottle top filters (Corning) or 0.45 μm GD/X capsule filters (glass microfiber, Whatman) to remove particulate matter. Due to the fact that some loss of plasmid DNA occurs during CTAB precipitation, duplicate lysates (2 × 5 g cell paste) for each construct were prepared, pooled and loaded onto the membrane capsule. The membrane was typically equilibrated with more than 10 column volumes (CV) of elution buffer (1 M NaCl, 25 mM Tris–HCl, 1 mM EDTA, pH 7.4) followed by the appropriate equilibration solution (0.55 M NaCl, 25 mM Tris–HCl, 1 mM EDTA, pH 7.4 for clarified lysate and a 0.60 M NaCl buffer for CTAB precipitated pDNA). Flows of 3 mL/min were used for elution/sample loading. After a wash step of at least 5 CV of equilibration buffer, the pDNA was eluted by adding buffer containing 1.5 and 1 M NaCl for the Q and D membranes, respectively.

2.6. Parallel purification using a multi-channel peristaltic pump

All parallel purification experiments were run with an MCP multi-channel peristaltic pump (Ismatec). The peristaltic pump has a 12-cassette pump head (CA 12) with 1.02 mm i.d. Tygon tubing. The outlets from the peristaltic pump were connected to a parallel chromatography system (BioOptiX 10, Teledyne Isco) via Luer fittings. The membrane capsules were also connected to the system via their Luer fittings. The BioOptix system was only used for fraction collection (Foxy 200) and monitoring of the absorbance at 260 nm. The whole purification process can be performed without online monitoring solely using a multi-channel peristaltic pump. Prior to equilibration of the system, the tubing and membranes were washed with 1 M NaOH to remove contaminants. All buffers were sterile filtered (0.2 μm) before use.

Two protocols were set up, one standard method with capture of pDNA directly from the lysate, and one that includes the CTAB precipitation step for preparation of pDNA with low endotoxin content. Clarified lysates were prepared from 5–7 g of *E. coli* cell paste as described above. For the standard procedure, conductivity was adjusted by adding 0.20 M NaCl to the lysates from a 5 M stock solution. Filtration using GD/X capsule filters was performed in parallel on the peristaltic pump using a flow rate of 10 mL/min. To prepare low endotoxin pDNA, 2 g/L of CTAB was added to duplicate tubes of clarified lysate. After centrifugation and wash steps, the recovered pellets were dissolved in 60 mL of 0.60 M NaCl binding buffer by gentle mixing over night. The duplicate samples were then pooled and filtered as above. The flow rates used during ion exchange were 3–5 mL/min.

After anion-exchange chromatography, fractions containing DNA were pooled and buffer changed with gel filtration to 1 × TE (10 mM Tris–HCl, 1 mM EDTA, pH 8) or 1× PBS (137 mM NaCl, 2.7 mM KCl, 4.3 mM Na_2HPO_4, 1.4 mM KH_2PO_4, pH 7.4) using Sephadex G-25 columns (HiPrep Desalt 26/10, GE Healthcare). Ideally, 15 mL eluate is collected, which is the volume used for buffer change. The desalting procedure was also run in parallel with the peristaltic pump. Columns were equilibrated with ≥75 mL TE or PBS buffer, after which 15 mL sample was loaded at 5 mL/min, the subsequent 20 mL was then collected and contained the DNA. Purified pDNA was finally concentrated by ultrafiltration in centrifugal cells (10,000 MWCO), sterile filtered and stored in the freezer.

2.7. Sanitisation of the chromatographic media/membranes

The MA75D membrane capsules were generally washed with 1 M NaOH for at least 10 CV and stored in 1 M KCl in 20% ethanol. A more thorough procedure with three wash steps: i.e. 1 M NaOH, 50% isopropanol and 1 M phosphoric acid, can also be applied. The Q-membranes were more difficult to regenerate (1 M NaOH, isopropanol and 1 M phosphoric acid was not sufficient). An incubation procedure with a solution containing 1 mg/mL DNase I, 50 mM Tris–HCl, 10 mM $MgCl_2$, 250 mM NaCl, pH 7.6 at 37 °C over night was tested with some success. After incubation, the membranes were re-equilibrated with 1 M KCl in 20% ethanol.

2.8. DNA/protein concentration determination and agarose gels

Electrophoresis equipment and precast 0.8% agarose gels with 8 or 12 wells were used (BioRad). The Ready-Load 1 kb plus (Invitrogen) was used as marker. Runtimes up to 100 min were used with the parameters: 80 V, 400 mA. Typically, the gel was analysed after 20 min for the presence of RNA. The relative distribution of different forms of plasmid DNA (supercoiled versus relaxed and linear, respectively) was estimated by measurements using the Kodak electrophoresis documentation and analysis system (Kodak EDAS290 camera and Kodak 1D Image Analysis Software).

All DNA concentration determinations were made spectrophotometrically on an ND-1000 (NanoDrop Technologies). One unit of OD 260 nm in a 10 mm cuvette is assumed to correspond to 50 μg/mL ds DNA.

Protein concentration was estimated according to the Bradford procedure [10]. Typically, 200 μL Bradford reagent (BioRad Laboratories, Inc.) was mixed with 800 μL sample. The standard curve was prepared with BSA as reference. A Smart Spec 3000 spectrophotometer (BioRad) was used to quantify the protein content (using a wavelength of 595 nm).

2.9. Endotoxin content

The amount of endotoxin was quantified using an Endosafe PTS (Charles River Laboratories) portable system. Chips with a sensitivity of 0.01–1.0 EU/mL were used. The sample was prepared by appropriate dilution (10–10^4 depending on the sample) in endotoxin free LAL reagent (Charles River Laboratories).

3. Results

3.1. Comparison of pDNA binding properties of different chromatographic matrices

The capacity for pDNA was determined for different ion-exchange media as described in Section 2. Standard agarose-based beads, resins with functionalised surface extensions and membrane adsorbers were evaluated. The lowest capacity, approximately 0.2 mg/mL matrix, was seen for the sepharose fast flow resins (Q and DEAE), which indicates that only the surface of the beads is accessible for pDNA (Fig. 1). Q sepharose high performance has a smaller bead size than the fast flow resin and, in accordance, display higher capacity, about 0.5 mg/mL. Even higher capacity was detected for the resins with "tentacles", approximately 0.8 mg/mL for EMD TMAE fractogel and approximately 1 mg/mL for Q sepharose XL (data not shown). The membrane ion-exchange adsorbers displayed the highest capacity for pDNA, approximately 4 mg/mL or 0.1 mg/cm^2.

Plasmid DNA binds very strongly to all tested chromatographic media. At pH 7.4, complete adsorption of pDNA could be obtained at 700 mM NaCl for all Q resins and at 500 mM for DEAE sepharose. For Sartobind D membranes, 600 mM NaCl could be used in the binding buffer without any loss of plasmid DNA in the flow through. The strong electrostatic binding of DNA to the media can also be problematic which is seen during elution where complete desorption is very hard to achieve, particularly for the strong Q ion-exchange group (data not shown).

3.2. Preparation of pDNA by alkaline lysis followed by membrane ion-exchange chromatography

A cell pellet of *E. coli* carrying the B model plasmid was used to optimise a plasmid preparation protocol using alkaline lysis followed by anion-exchange chromatography on 75 cm^2 Sartobind Q and D membrane adsorber capsules. After lysis and clarification (see Section 2 for details) NaCl was added to adjust the conductivity for optimal binding of pDNA to the ion-exchanger. It is critical that the conditions allow the pDNA to be efficiently adsorbed whereas the remaining RNA is not. A concentration of NaCl that is sufficiently high is expected to give a good separation of DNA and RNA, since the smaller, less charged RNA fragments should be recovered in the flow through. For Sartobind D membranes, we found that addition of 0.2 M NaCl to the lysate (from a 5 M stock solution) assured that most RNA was found in the flow through fraction while pDNA remained bound to the D-membrane. The yield was 6.4 mg (1.1 mg pDNA/g cell paste) under these conditions with an A_{260}/A_{280} ratio of 1.9. DNA binding decreased with increasing concentrations of NaCl added to the lysate. For the Q-membranes, the yield remained the same in the interval 0.27–0.45 M NaCl (around 1.5 mg pDNA/g cell paste with an A_{260}/A_{280} ratio of 1.9). A drastic decrease in pDNA adsorption was observed when using 0.50 M NaCl in the feedstock (0.2 mg pDNA/g cell paste). Due to incomplete desorption of pDNA from the Q-membrane, which was also seen during regeneration of membranes, we found that the D-membranes were the most suitable for pDNA purification.

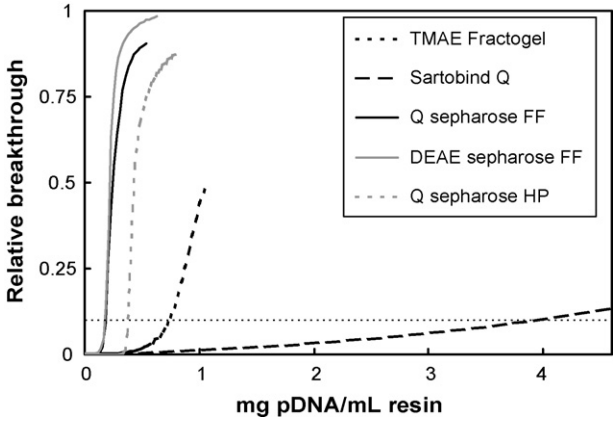

Fig. 1. Dynamic loading capacity of a selection of anion-exchange resins. The capacity for pDNA was determined for several different ion-exchange chromatographic media. The purified 7619 bp B plasmid was used to determine the 10% breakthrough value (dotted line) using an Äkta purifier system at 1 mL/min. One milliliter pre-packed HiTrap columns of Q sepharose HP, Q sepharose FF and DEAE sepharose FF were used (GE Healthcare). 0.84 mL EMD TMAE Fractogel was packed in a 5 mm diameter HR column (GE Healthcare) and for Sartobind Q the MA 5 capsule was used.

3.3. Precipitation of DNA with CTAB

Precipitation with the ionic detergent CTAB was assessed in order to achieve reduction of RNA- and/or endotoxin levels. For selective precipitation of DNA from RNA, we attempted to use relatively low CTAB concentrations, i.e. 0.1, 0.2 and 0.4 g/L. The same cell pellet as in the previous experiments was used (plasmid B), but RNase treatment was omitted from the lysis protocol in order to fully evaluate the potential for RNA removal. Due to incomplete precipitation of DNA as well as some co-precipitation of RNA at all concentrations tested, further efforts to reduce RNA by CTAB precipitation were abandoned (data not shown). Instead endotoxin removal was investigated in another set of experiments. Precipitations were performed at 10-fold higher CTAB concentrations, thus ensuring that all DNA is precipitated, and a downstream membrane anion-exchange chromatography step was optimised as described in Section 2. Endotoxin measurements on purified pDNA fractions showed significant reduction of endotoxins as compared to a reference preparation without the CTAB step (Table 1). Endotoxin

Table 1
Endotoxin levels after precipitation at different concentrations of CTAB and purification on an MA 75 Q membrane

Sample[a]	Endotoxin level[b] (EU/mg plasmid)
1 g/L CTAB	57
2 g/L CTAB	15
4 g/L CTAB	10
Reference[c]	1400

[a] The B plasmid was used in the experiment and 1 M NaOH was used to wash the chromatographic system to remove endotoxins.
[b] Determined with the Endosafe PTS.
[c] The reference was the same cell paste with the precipitation step omitted.

levels in the precipitate decreased with increasing concentrations of CTAB indicating that the endotoxins were efficiently solubilised by this detergent. For further experiments, 2 g/L was selected as the CTAB concentration of choice.

3.4. Parallel Purification

For the parallel purifications, MA 75D membranes were used since the strong ion-exchanger was found to be difficult to regenerate and DNA bound irreversibly to the matrix to a significant extent (data not shown). Four constructs (A–D) of different sizes and in different vector backbones were used to evaluate the parallel process. Flow through fractions and desalted eluates from the parallel purifications were analysed by gel electrophoresis and absorbance measurements (Figs. 2 and 3, Table 2). For comparison, plasmid DNA was also prepared from all constructs using a commercial midiprep kit (Qiagen Tip 100). The yields are summarised in Table 2. For the standard purification, good yields of plasmid DNA (around 7–8 mg) were obtained for all constructs except for D where 4 mg of DNA was obtained, which probably

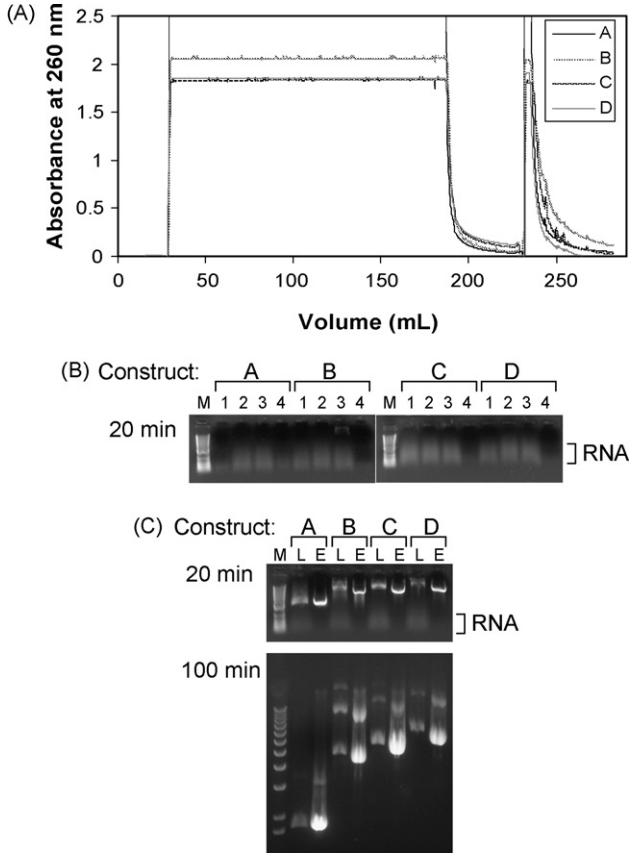

Fig. 2. Parallel purification of four different plasmids. (A) A characteristic parallel UV (260 nm) trace recorded by the BioOptix system during the ion-exchange chromatography step on the peristaltic pump. A 160 mL sample loading phase (high A_{260}) is followed by a 20 CV wash step with binding buffer and elution with high conductivity buffer. (B) Agarose gel electrophoresis of four flow through fractions collected during sample load and wash. RNA is clearly visible in the flow through whereas pDNA, in small amounts, only is detected in the plasmid B run. (C) Agarose gel electrophoresis of lysate (L) and eluate (E) fractions during pDNA purification. The gel is shown after 20 min in order to display RNA and after 100 min to show separated plasmids.

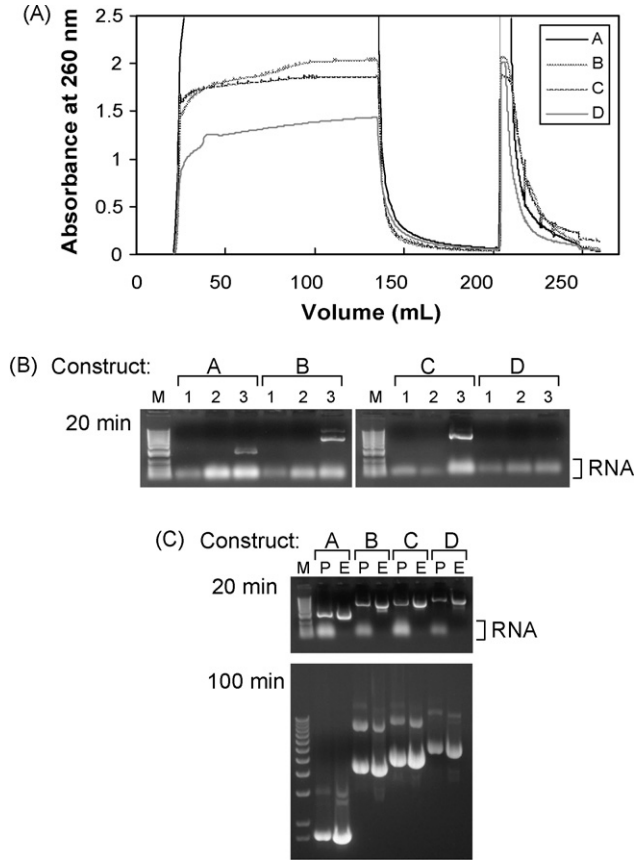

Fig. 3. Parallel purification of CTAB precipitated samples of the four sample constructs. (A) UV (260 nm) trace recorded by the BioOptix system during the ion-exchange chromatography step on the peristaltic pump. A 120 mL sample loading phase (high A_{260}) is followed by a 50 mL wash step with binding buffer and elution with high conductivity buffer. (B) Agarose gel electrophoresis of three flow through fractions collected during sample load. RNA is clearly visible in the flow throughs. The pDNA detected at the end of the sample load phase of plasmids A–C indicates saturation of the membrane capsules. (C) Agarose gel electrophoresis of precipitated lysate after solubilisation in binding buffer (P) and eluate (E). The gel is shown after 20 min in order to display RNA and after 100 min to show separated plasmids.

is a result of lower copy number in the *E. coli* cells (Table 2). The gels in Fig. 2 show that no RNA is detected in the final, purified product and that the supercoiled form is enriched in the eluate as compared to the lysate. The fraction of supercoiled pDNA ranged from 70 to above 90%, as deduced by densitometry measurements of the agarose gels. It should be noted that the fraction of supercoiled pDNA mainly depends on factors such as plasmid size, host strain and fermentation procedures, storage conditions and shear [2]. The flow through fractions were essentially free of pDNA. By using the CTAB method, around 7–8 mg of pDNA was obtained for all constructs, except for D again (4 mg). The yield was approximately 50% as compared to purification without precipitation (Table 2). Agarose gel electrophoresis gave very similar results as for purification without precipitation. Analysis of the flow through showed that a lot of RNA passed the matrix without binding (Fig. 3). Some pDNA was seen in the flow through at the end of sample load for constructs A, B and C, which indicates saturation of the matrix. The parallel purification method has comparable or slightly higher

Table 2
Data from parallel pDNA purification experiments

Purification method[a]	Construct	Yield pDNA[b] (mg)	Yield per gram cell paste[c] (mg/g)	A_{260}/A_{280}	Protein content[d] (mg/mg)	Endotoxin content[e] (EU/mg)
Standard protocol	A	7.4	1.4	1.85	0.12	160
	B	7.3	1.3	1.82	0.11	ND[f]
	C	8.4	1.5	1.82	0.11	160
	D	3.8	0.7	1.77	0.13	ND
Low endotoxin protocol (CTAB)	A	8.4	0.8	1.93	0.10	0.8
	B	6.5	0.6	1.91	0.13	ND
	C	7.5	0.7	1.91	0.06	3.4
	D	4.1	0.4	1.90	0.04	5.2
Midiprep[g]	A	0.06	0.8	1.93	0.11	ND
	B	0.08	1.1	1.92	0.10	ND
	C	0.08	0.9	1.92	0.08	770
	D	0.06	0.7	1.90	0.10	650

[a] pDNA was prepared according to the protocols described in Section 2. The Tip-100 midiprep kit from Qiagen was used as a reference.
[b] Amount of pDNA purified from one MA75D capsule or Tip 100.
[c] Lysate was prepared from approximately 5 g wet weight of bacterial cell paste. After CTAB precipitation, duplicate preparations were pooled before chromatography. Between 0.05 and 0.09 g cell pellet was used for the midiprep procedure.
[d] Estimated with the BioRad protein assay. Expressed as mg protein per mg pDNA.
[e] Determined with the Endosafe PTS.
[f] Not determined.
[g] Prepared using the Qiagen 100 tip according to manufacturer's procedure.

yields than a midiprep preparation. A reference purification of the B-construct on EMD TMAE Fractogel gave a yield of 1.0 mg plasmid DNA/g cell paste (data not shown).

Analysis of the protein content gave similar levels of protein contamination (approximately 0.1 mg protein/mg plasmid) for the three purification processes discussed (with/without CTAB precipitation and midiprep, respectively). The protein content is reduced about 50-fold during the ion-exchange step. The endotoxin content in all parallel pDNA preparations that included the CTAB precipitation step was significantly reduced. Without precipitation, the endotoxin content in the purified product was 160 EU/mg plasmid. When using the CTAB method, the endotoxin levels were $\leq$5 EU/mg plasmid, or up to 200 times lower compared to the standard purification (Table 2). The lowest measured level was 0.8 EU/mg plasmid for construct A. The corresponding endotoxin values for the commercial midiprep kit were around 700–800 EU/mg plasmid.

4. Discussion

The aim of this study was to develop a rapid parallel procedure for producing high quality plasmid DNA at milligram scale. By using a peristaltic pump, time consuming and laborious gravity-flow procedures could be avoided and, in addition, peristaltic pumps are readily equipped with multi-channel pump heads, which in contrast to most vacuum devices facilitates parallelisation.

After performing the established alkaline lysis protocol for pDNA extraction from *E. coli* [1], the major remaining contaminant is RNA. At preparative scale this is typically handled by introducing downstream chromatographic steps, predominantly using anion exchangers [2]. A known limitation of pDNA chromatography is the low capacity of many chromatographic media due to the large size of the plasmids. Even media with functionalised surface extensions or "tentacles" have a limited ability to bind pDNA. For example, in this study we found that EMD TMAE fractogel had a dynamic binding capacity of 0.8 mg/mL for a 7.6 kb model vector, which is about one hundredth of the capacity for e.g. albumin. The capacity of conventional sepharose fast flow resins (Q and DEAE) was several times lower. Higher binding capacity can be obtained by using membrane chromatography, i.e. 4 mg/mL for the Sartobind membranes tested in this study. In order to reach even higher capacities one has to consider columns based on novel advanced monolith-technology for which capacities of up to 8 mg/mL have been reported [11]. Membranes, as well as monoliths, have different properties as compared to traditional column media. A convective flow brings the biomolecules through large pores into direct contact with the functionalised groups of the resin [12–14]. Since no passive diffusion is needed for binding, there are no mass transfer limitations. As a result, high flow rates are possible and scale up is linear [12–14]. The Sartobind membranes used here features user-friendly capsules with standard Luer connections up to a scale of 15 layers (i.e. the 75 cm^2 format used in this protocol). This is particularly important since no individual packing or preparation of columns is required during parallel processing. In addition, further scale up is possible using process scale equipment.

Since nucleic acids are highly negatively charged, their interaction with the positively charged anion exchanger is very strong and it has been reported that the interaction between pDNA and strong ion exchangers often is too strong. Teeters et al. [12,13] report that the recovery of pDNA, bound to a Q-surface, might be as low as 50–80%. This is also consistent with our results for Sartobind Q membranes, which were very difficult to regenerate thus indicating irreversible binding of nucleic acids. A major distinction between a quarternary ammonium group of a strong anion exchanger (Q) with the diethylamine group of a weak anion exchanger (D) is the difference in pK_a. The diethylamine group has a lower pK_a and is thus expected to

be neutral at high pH (pH > 9). This fact is important when it comes to regenerating the membrane unit. In this study, Sartobind D units were used three times without any significant loss in capacity. It should be possible to use the capsule over and over again as long as a regeneration procedure is carried out properly. This would make the membrane chromatography procedure a highly cost-effective method as compared to other commercial kit procedures that use disposable columns. In order to reduce costs-of-goods one would have to turn to bulk material, which instead would increase the labour costs significantly due to time-consuming column packing procedures, particularly when running parallel experiments.

An additional problem during pDNA purification is endotoxins, particularly if the pDNA is to be used for gene therapy or DNA vaccine production. Endotoxin also reduces transfection efficiencies (Qiagen). Endotoxin can be removed by various chromatographic methods as well as through different extraction or precipitation procedures [15]. The cationic detergent CTAB has been shown to selectively precipitate pDNA [5,16]. By adding a CTAB precipitation step prior to anion-exchange chromatography, endotoxin levels were reduced to values below 10 EU/mg pDNA, which is 10 times lower than the limit for pharmaceutical grade pDNA [2].

Even without the CTAB step endotoxin levels are much lower than after a commercial midiprep procedure. Also, the product is recovered with the supercoiled form enriched and the protein content heavily reduced. Altogether, this gives a complete plasmid purification platform with the capacity to produce milligram amounts of high quality product using a parallel and cost efficient process.

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