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Extraction of plasmid DNA from *Escherichia coli* cell lysate in a thermoseparating aqueous two-phase system☆

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Abstract

The primary purification of a 6.1 kilo base pair (kbp) plasmid from a desalted alkaline lysate has been accomplished by a thermoseparating aqueous two-phase system [(50% ethylene oxide–50% propylene oxide)–Dextran T 500]. The partitioning of the different nucleic acids (plasmid DNA, RNA, genomic DNA) in the thermoseparating aqueous two-phase system was followed both qualitatively by agarose gel electrophoresis and quantitatively by analytical chromatography (size exclusion- and anion-exchange mode) and PicoGreen fluorescence analysis. The experimental results showed a complete recovery of the plasmid DNA to the top phase, while 80% of total RNA and 58% of total protein was discarded to the bottom phase. Moreover, a 3.8-fold volume reduction of the plasmid DNA solution was achieved. By using a final thermoseparating step, the $EO_{50}PO_{50}$ polymer could be efficiently recycled, resulting in plasmid solution containing less than 1% polymer. The developed thermoseparating aqueous two-phase system shows great potential for the large-scale processing of plasmid DNA.
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1. Introduction

DNA vaccination and gene therapy are expected to be the fastest growing therapeutic areas in the next decade. In DNA vaccination, nucleic acids are administered to the patient with the intent of initiating an immune response to the antigen or protein encoded by the DNA. In gene therapy, nucleic acids are introduced to either restore, cancel or enhance an imperfect gene function. The increased use of such therapies result in a need for large quantities of gene vectors such as plasmid DNA. To produce large quantities of plasmid DNA an efficient large-scale purification process, which can meet specifications in purity, needs to be developed. At present, many methods are available for the purification of small molecules, i.e. proteins (5–10 nm in size). However, these methods are often poorly suited for larger molecules such as plasmid DNA (>100 nm in size). Thus, there is a need for development of better-suited purification methods for plasmid DNA. In general, the production of plasmid DNA often involves fermentation of *E. coli* harboring the plasmid. This is followed by a cell lysis method (e.g. alkaline lysis) to release the plasmid and ends with the purification process (which can be quite complex). Many methods have been published around the use of chromatography as the main purification step [1–3]. However, a primary purification step is often considered necessary to reduce the process stream volume and to partly remove contaminants such as RNA and proteins before the chromatographic steps. The aqueous two-phase system is a gentle approach that has strong potential as a primary recovery step for plasmid purification. In an earlier publication [4], it has been shown that plasmids can be isolated in aqueous two-phase systems consisting of poly(ethylene glycol) (PEG) and salt. PEG is a linear polymer of ethylene oxide groups. The polymer is soluble in water and at a certain salt concentration a two-phase system consisting of PEG and salt is formed. In fact, salting out the PEG polymer creates the PEG–salt systems. By changing the molecular weight of the PEG polymer, the plasmid DNA can be partitioned in either phase. A drawback with this system is that the plasmid DNA is finally collected in either

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a polymer containing phase or a phase with high salt concentration, both creating specific problems for subsequent steps, e.g. chromatography.

In this work, a polymer–polymer system has been developed to purify plasmid DNA from a desalted alkaline lysate. Different factors that influence the partitioning of plasmid DNA such as salts and polymer concentration were investigated. The system is composed of the thermoseparating polymer $EO_{50}PO_{50}$ and the bottom phase forming polymer Dextran T 500. The EO–PO copolymer consists of 50% (w/w) ethylene oxide groups and 50% (w/w) propylene oxide groups that are randomly distributed within the polymer chain. The EO–PO copolymer has thermoseparating properties, i.e. when heated over its cloud point (CP) temperature the solution becomes cloudy and separates into one water phase and one polymer phase. The cloud point of a 10% solution of this polymer is 50 °C and can easily be lowered by addition of salts. The advantages of this system are that the plasmid can be exclusively recovered in a water phase and the polymer separated and recycled. This make the system very suitable for a large-scale application and also facilitates subsequent high-resolution steps, e.g. chromatography.

Partitioning of proteins in these systems has earlier been described [5–7] and recently, scale-up of thermoseparating systems for recombinant protein isolation has been accomplished [8].

2. Experimental

2.1. Instruments, columns and chromatography media

All chromatography experiments were performed using ÄKTAexplorer 10 controlled by UNICORN 4.11 software (Amersham Biosciences, Uppsala, Sweden). Agarose gel electrophoresis was performed on a Hoefer HE 33 Mini horizontal submarine unit powered by an EPS 301 supply (Amersham Biosciences). PicoGreen fluorescence was measured on a Spex Fluoromax-2 fluorimeter (Jobin Yvon Hroiba, Paris, France). MiniQ column (PE 4.6/50), XK 16/20 and XK 50/30 columns, Sephadex G-25 media (85–260 μm) and Sephacryl S-500 HR media were obtained from Amersham Biosciences.

2.2. Chemicals

The polymer Breox PAG 50 A 1000 ($EO_{50}PO_{50}$, M_r = 3900) was obtained from International Specialty Chemicals (Southampton, UK). Dextran T 500 (weight-average molecular mass, M_w = 500,000) was purchased from Amersham Biosciences. Na_2HPO_4 (pro analysis) was obtained from Merck Eurolab (Stockholm, Sweden). BCA protein assay kit was acquired from Pierce (Rockford, IL, USA). Carbencillin and RNase were purchased from Sigma (St. Louis, MO, USA). Peptone and yeast extract were from BD Microbiology systems (Sparks, MD, USA). The 1 kilo base pair (kbp) DNA ladder was obtained from Fermentas (Vilnius, Lithuania) and the Supercoiled DNA ladder (2–16 kbp) from Invitrogen (Groningen, The Netherlands). PicoGreen double-stranded (ds) DNA binding reagent was purchased from Molecular Probes (Leiden, The Netherlands). *Eco*RI, *Bam*HI and Mung Bean Nuclease were obtained from Promega (Madison, WI, USA).

2.3. Cultivation

Control *E. coli* cells TG1 and *E. coli* cells TG1 harboring the plasmid pUC 19 (2.686 kbp) having an insert of the *dmg*A and *dem*A genes from a *Streptococcus dysgalactiae* strain (AJ243529, 3.433 kbp) [9] were provided by Dr. Jozsef Vasi (Amersham Biosciences). The plasmid is referred to as pJV4 later on in the text. The bacterial cells were grown overnight (37 °C, 250 rpm) in 2 l baffled shake flasks containing 500 ml 2 × YT medium (16 g/l peptone, 5 g/l NaCl, 10 g/l yeast extract and 100 mg/l carbencillin).

2.4. Alkaline lysis

A modified alkaline lysis method was used [10]. A 500 ml overnight cell culture was harvested by centrifugation at 9000 rpm in a Sorvall SLA 3000 rotor for 10 min (4 °C). The supernatant was carefully removed and 5 g of the bacterial pellet was resuspended by gentle vortexing in 36 ml suspension buffer (61 mM glucose, 50 mM EDTA, 10 mM Tris–HCl, pH 8). After the cells were completely resuspended, 78 ml of lysis buffer [0.2 M NaOH, 1% sodium dodecylsulfate (SDS)] was added while stirring gently with a magnetic stirrer. To assure a complete mixing, the gentle stirring was continued for 10 min at room temperature. A volume of 59 ml cold (4 °C) neutralization buffer (3 M potassium acetate, pH 5.5) was added to the lysate. The solution was kept on an ice-bath under magnetic stirring for 20 min. A white precipitate was formed containing SDS, genomic DNA, proteins and cell debris. The precipitate was then removed by centrifugation in an SS-34-rotor at 4 °C (30 min at 10000 rpm). The supernatant was then carefully transferred to a fresh tube and stored at 4 °C for short time until further processing.

2.5. Desalting of clarified alkaline lysate

The clarified alkaline lysate was desalted on Sephadex G-25 matrix. More specifically, the beads were packed in a XK 50/30 column, giving a total bed volume of 225 ml. The column was coupled to an ÄKTAexplorer 10 system and equilibrated with the mobile phase (5 mM sodium phosphate buffer, pH 7). Samples to be desalted (50–100 ml) were applied to the column by a 150 ml superloop at a flow rate of 5 ml/min. The eluate from the column was monitored by UV absorbance at 260 and 280 nm and conductivity, allowing accurate control over the buffer exchange of the lysate.

2.6. Aqueous two-phase systems

The optimized system, containing 4.5% (w/w) Dextran T 500 and 4.5% (w/w) $EO_{50}PO_{50}$ were made up by weighing appropriate amounts of a 25% stock solution of dextran and a 100% $EO_{50}PO_{50}$ stock solution in a 10 ml graded test tube. Fifty millimolar Na_2HPO_4 was used as the buffer salt, and added to the system from a 1 M stock solution. The clarified and desalted alkaline lysate was added to a final mass of 10 g. The system was mixed carefully until all polymers were dissolved and phase separation was accomplished by centrifugation (1600 × g, 10 min) at room temperature (primary system). The volumes of the top and bottom phase were determined and the phases were isolated in separate containers. The top phase was placed in a water bath at 55 °C for 3 min, followed by 2 min centrifugation to obtain a water phase and a concentrated polymer phase (thermoseparated system). The volumes of the thermoseparated top and bottom phase were determined and the phases were isolated in separate containers.

Systems containing different polymer concentrations were prepared in a similar way, e.g. 4% (w/w) $EO_{50}PO_{50}$–6% (w/w) Dextran T 500, 3% (w/w) $EO_{50}PO_{50}$–7% (w/w) Dextran T 500, 3% (w/w) $EO_{50}PO_{50}$–8% (w/w) Dextran T 500 and 2.5% (w/w) $EO_{50}PO_{50}$–9% (w/w) Dextran T 500. The Na_2HPO_4 concentration in these systems was kept constant at 50 mM.

The partitioning of a protein in a two-phase system is described by the partition coefficient K, and defined as $K = C_T/C_B$ where C_T and C_B are the concentrations of the protein in the top and bottom phases, respectively.

2.7. Agarose gel electrophoresis

The top and bottom phases of the primary system and the thermoseparated system were analyzed with agarose gel electrophoresis. The agarose gel (0.8%, w/v, Duchefa, The Netherlands) containing 15 μg/ml ethidium bromide (Quantum Biotechnologies, USA) was run at 90 V for 30 min. The agarose gel was photographed and analyzed using the gel documentation software AlphaImager 2200 v5.5 from Alpha Innotech Corporation (San Leandro, CA, USA).

2.8. Protein measurements

The top and bottom phases of the primary system and the thermoseparated system were analyzed for protein content with the Bicinchoninic Acid (BCA) Protein Assay according to the manufacturer's instructions. Each sample was compensated for the presence of interfering substances by using samples from reference systems devoid of proteins. These reference systems were prepared by replacing the desalted alkaline lysate with 5 mM sodium phosphate buffer, pH 7 in the aqueous two-phase system process.

2.9. PicoGreen fluorescence analysis

Plasmid DNA was quantified using the dsDNA-binding reagent PicoGreen. Calibration curves were made using samples from the aqueous two-phase system containing the TG1 lysate devoid of plasmid (blank system). The phases from the two-phase systems and the TG1 lysate were diluted 1000 times in sterile TE buffer (10 mM Tris–HCl, 1 mM EDTA, pH 7.5). A concentration of 0–100 ng/ml of pure pJV4 plasmid DNA was added to the top phase, thermoseparated top phase and lysate whereas a concentration of 0–50 ng/ml was used for the bottom phase and the thermoseparated bottom phase. Next, a volume of 500 μl PicoGreen reagent was added to 500 μl of these reference samples followed by 2 min incubation. Fluorescence was measured using an excitation wavelength of 480 nm and emission was set to 520 nm. The spectral bandwidth was set to 2 nm in both the emission and excitation pathways. For the quantitation of plasmid DNA in the phases (top and bottom phases of the primary system and the thermoseparated system), the samples (500 μl) were added to 500 μl of the PicoGreen reagent and incubated and measured as above, after which the plasmid DNA concentration was calculated from the standard curves.

2.10. Restriction enzyme digestion

Plasmid identity was confirmed by linearization with *Eco*RI and *Bam*HI. A volume of 4 μl of the desalted lysate and the thermoseparated top phase were treated with 20 units of restriction endonucleases *Bam*HI and *Eco*RI respectively for 1 h, at 37 °C (total reaction volume was 20 μl). The cleavage pattern of the pJV4 plasmid DNA was then visualized by agarose gel electrophoresis.

The presence of denatured plasmid DNA was investigated by treatment with Mung Bean Nuclease. A total of 10 units of Mung Bean Nuclease was added to the samples (desalted alkaline lysate and thermoseparated top phase) and incubated at 37 °C (total reaction volume was 25 μl). Four microlitres samples were withdrawn every second minute and analyzed by agarose gel electrophoresis.

2.11. Chromatography analysis

The recovery of supercoiled plasmid DNA after the two-phase system process was determined by ion-exchange chromatography analysis on a MiniQ column. The MiniQ column was coupled to an ÄKTAexplorer 10 system and equilibrated at 0.4 ml/min with 0.5 M NaCl, 25 mM Tris–HCl, pH 8. One hundred microlitres samples were applied on the column. Applying a gradient from 0.5 to 0.8 M NaCl in 18 column volumes could then elute the different nucleic acids adsorbed to the column. The chromatography run was monitored by UV absorbance at 260 nm.

The plasmid content in the thermoseparated top phase and the desalted alkaline lysate were determined by incubating

the samples with RNase (≈100 μg/ml) for 15 min prior to the chromatography run. After the run, the area under the plasmid peak was calculated. The plasmid content was then calculated by using the relationship 50 μg plasmid/ml = 1 AU [11], taking into account the volume of the phase and dilutions.

The MiniQ analysis was also used to quantitate the RNA in the samples. This was performed by a second run of the sample without addition of RNase. In this case the relationship 40 μg RNA/ml = 1 AU was used [11].

A complementary analysis method was also performed on the samples, size exclusion chromatography on a Sephacryl S-500 HR column (group separation [12]). Sephacryl S-500 HR beads were packed in a XK 16/20 column to a bed height of 12 cm, giving a total bed volume of 24 ml. The column was integrated to the ÄKTAexplorer 10 system and equilibrated at 1 ml/min with 2 M ammonium sulfate, 25 mM Tris–HCl, pH 8. A 1 ml sample was injected on the column and the eluted nucleic acids were monitored by UV absorbance at 260 nm.

3. Results and discussion

3.1. Partitioning in $EO_{50}PO_{50}$/dextran system: the effect of polymer concentration and salt composition

In this work, the partitioning of a recombinant 6.1 kbp plasmid from a desalted alkaline lysate has been studied in a thermoseparating aqueous two-phase system. The goal was to obtain a one-sided partitioning of the plasmid to the $EO_{50}PO_{50}$ top phase in the primary system. The partitioning of the plasmid DNA in an aqueous two-phase system can be influenced by different factors such as polymer concentration and salt composition. The influence of these factors was hence studied. Qualitative analysis by agarose gel electrophoresis showed that by decreasing the polymer concentration in both phases a more extreme partitioning of the plasmid DNA to the $EO_{50}PO_{50}$ phase could be achieved (systems 1, 7 and 10 in Table 1). The explanation for this could be that the entropic effect of the $EO_{50}PO_{50}$ polymer is dominating in a system containing high $EO_{50}PO_{50}$ polymer concentration [13], i.e. the dextran phase will in this case be the most favorable phase for the plasmid DNA. In a system containing low $EO_{50}PO_{50}$ polymer concentration the entropic effect is not the dominating factor for determining the partitioning. The results in Table 1 show that the system is very sensitive to small changes in polymer concentrations. By decreasing the total polymer concentration in the top phase from 5 to 4.5% (w/w) the plasmid DNA can be exclusively partitioned to the $EO_{50}PO_{50}$ phase (systems 7 and 10 in Table 1).

Thus, a system close to the critical point is the most suitable system for partitioning of plasmid DNA to the $EO_{50}PO_{50}$ phase.

It has earlier been described in the literature [14] that DNA can effectively be transferred between phases by addition of a suitable salt. To achieve a dominating effect of the salt in a two-phase system, the concentration of the salt must be at least 10 times higher than the buffer [14,15]. The addition of a salt to a two-phase system forces the anion and the cation to partition together between the two different polymer phases and this will generate an electrical potential difference between the phases (Donnan potential) [14–17]. The HPO_4^{2-} anion has affinity for the dextran phase. This will create an electrochemical driving force in the system. If a negatively charged substance, e.g. plasmid DNA is added to this system, the DNA will partition to the top phase. Due to this reason, different concentrations of sodium phosphate were tested at different polymer concentrations (Table 1, systems 2, 3, 8, 9, 11 and 12). As can be seen from Table 1 (systems 2 and 3) the DNA

Table 1
Partitioning of plasmid DNA and total RNA in the primary $EO_{50}PO_{50}$–Dextran T 500 two-phase system

System	$EO_{50}PO_{50}$ (%, w/w)	Dextran T 500 (%, w/w)	Salt	Partitioning of plasmid DNA	Partitioning of RNA
1	7	7	5 mM sodium phosphate buffer, pH 7	BP	BP/TP
2	7	7	50 mM Na_2HPO_4	BP	BP/TP
3	7	7	100 mM Na_2HPO_4	BP	BP/TP
4	7	7	100 mM LiCl	BP	BP/TP
5	7	7	200 mM LiCl	BP	BP/TP
6	5	5	100 mM LiCl	BP	BP/TP
7	5	5	5 mM sodium phosphate buffer, pH 7	TP/BP	TP + BP
8	5	5	50 mM Na_2HPO_4	TP	TP + BP
9	5	5	100 mM Na_2HPO_4	BP	TP + BP
10	4.5	4.5	5 mM sodium phosphate buffer, pH 7	TP	TP/BP
11	4.5	4.5	50 mM Na_2HPO_4	TP	TP + BP
12	4.5	4.5	100 mM Na_2HPO_4	TP	TP + BP
13	4.5	4.5	50 mM Tris–borate	TP/BP	TP + BP
14	4.5	4.5	100 mM Tris–borate	TP/BP	TP + BP

Different polymer concentrations and salt composition have been investigated. TP/BP: TP and some in BP; BP/TP: BP and some in TP; TP + BP: equal partitioning between top and bottom phase. TP: top ($EO_{50}PO_{50}$) phase in primary system. BP: bottom (Dextran T 500) phase in primary system.

is partitioned to the dextran bottom phase in systems containing high polymer concentrations with 50 and 100 mM sodium phosphate. In systems containing low polymer concentrations (systems 10, 11 and 12) and salt, the DNA is exclusively partitioned to the $EO_{50}PO_{50}$ top phase already at a concentration of 5 mM sodium phosphate. In the cases where sodium phosphate were added to the system, more RNA could be discarded to the dextran phase. A Tris–borate buffer (Table 1, systems 13 and 14) was also used as a salt to try to enhance partitioning of the plasmid DNA to the $EO_{50}PO_{50}$ top phase. The borate ion has the same effect as the HPO_4^{2-} ion, i.e. it creates a negatively charged bottom phase [18]. Despite this, some of the plasmid DNA is partitioned to the bottom phase in a system containing borate salt. In the case of LiCl (Table 1, systems 4, 5 and 6) it has earlier been described that it can be used for increased partitioning of plasmid DNA to the top phase in a system composed of PEG and dextran [14]. However, LiCl had no effect in the systems studied. Thus, the phosphate was considered to be the most suitable salt for plasmid extraction. The optimized two-phase system regarding plasmid DNA partitioning was therefore considered to be 4.5% (w/w) Dextran T 500, 4.5% (w/w) $EO_{50}PO_{50}$ with 50 mM Na_2HPO_4. Based on these studies, it could be concluded that the entropic effect is dominating for plasmid partitioning in a system containing high polymer concentrations even in the presence of salt, whereas the electrochemical force is dominating in the systems containing low polymer concentrations close to the critical point.

3.2. Partitioning of plasmid DNA in the optimized aqueous two-phase system

Desalted alkaline lysate was partitioned in systems containing 4.5% (w/w) Dextran T 500, 4.5% (w/w) $EO_{50}PO_{50}$ and 50 mM Na_2HPO_4 (prepared according to Section 2.6). The systems were mixed and after phase separation (primary system) the phases were isolated in separate containers. The $EO_{50}PO_{50}$ phase was put in a water bath at 55 °C, which separated into two new phases, one water phase and one dense polymer phase (thermoseparated system). The thermoseparating step was utilized for the recovery of the $EO_{50}PO_{50}$ polymer, and for the isolation of the plasmid in a water phase with low polymer concentration (<1%, determined with refractive index measurements [19]). The phases were analyzed by electrophoresis on agarose gels stained with ethidium bromide (Fig. 1). From the agarose gel electrophoresis, it can be seen that all forms of plasmid DNA are exclusively partitioned to the $EO_{50}PO_{50}$ phase in the primary system and to the water phase in the thermoseparated system respectively. The explanation for the latter can be found in the dominating role of excluded volume (entropic) effects. This is due to the high polymer concentration in the thermoseparated $EO_{50}PO_{50}$ phase (≈40%) which leads to partitioning of high molecular weight compounds to the water phase [5–7,13,20,21].

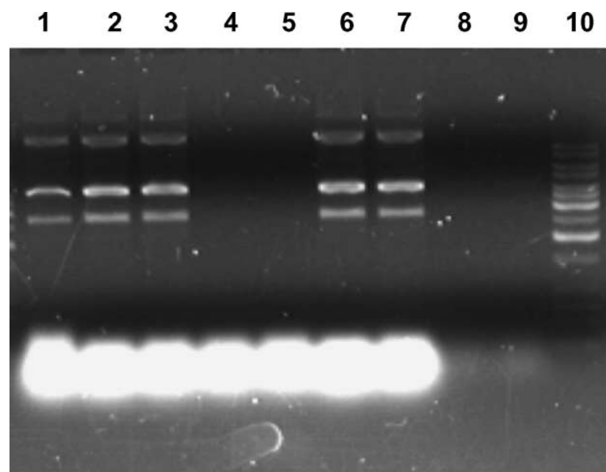

Fig. 1. The partitioning of plasmid DNA and RNA in a system composed of 4.5% $EO_{50}PO_{50}$–4.5% Dextran T 500, 50 mM Na_2HPO_4 as visualized by agarose gel electrophoresis. Duplicate systems were made. Lane 1: Desalted lysate diluted four times; Lane 2, 3: $EO_{50}PO_{50}$ phase 1 and 2; Lane 4, 5: Dextran phase 1 and 2; Lane 6, 7: Thermoseparated water phase 1 and 2; Lane 8, 9: Thermoseparated $EO_{50}PO_{50}$ phase 1 and 2. Lane 10: 1 kbp DNA ladder (50 ng). The plasmid band corresponds to linear, open circular, supercoiled and denatured plasmid DNA (from top to bottom).

3.3. Quantitative analysis of plasmid DNA by chromatography

The total amount of DNA (genomic and plasmid DNA) in the lysate and in the thermoseparated top phase were analyzed by size exclusion chromatography on a Sephacryl S-500 HR column (group separation analysis). The method has previously been described and is able to separate DNA from RNA. This is accomplished by compaction of RNA in the presence of 2 M ammonium sulfate [12]. The DNA is eluted in the void volume, whereas smaller substances such as RNA and proteins are delayed in the column and elute later. The DNA peak consists of all isoforms of plasmid DNA (e.g. supercoiled, denatured, open circular, concatamers, etc.) and remaining contaminating genomic DNA. Since most of the genomic DNA is precipitated or degraded in the alkaline lysis step [1,3], the DNA peak exists almost entirely of plasmid DNA. The genomic DNA level was measured by performing group separation analysis on desalted alkaline lysates using cells devoid of plasmid DNA. The genomic DNA level were found to be in the range 0.3–1 μg/ml.

To quantify the different isoforms of plasmid DNA, analytical anion-exchange chromatography on a MiniQ column was used. The method is based on adsorption of the different nucleic acids species to the non-porous matrix and elution is accomplished by applying an increasing salt gradient to the column. The different nucleic acid species are eluted in order of increasing length and conformational differences (difference in charge density). The supercoiling of plasmid DNA leads to local compression of charges causing the supercoiled plasmid DNA to bind harder and to elute later than, e.g. the open circular form (Fig. 2). This is in accordance

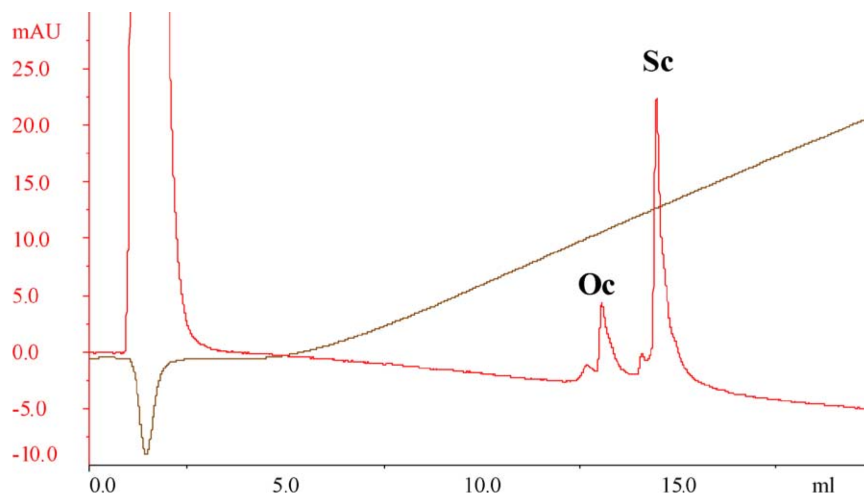

Fig. 2. Analytical anion-exchange chromatography of a thermoseparated water phase from the optimized system [4.5% (w/w) Dextran T 500–4.5% (w/w) $EO_{50}PO_{50}$–50 mM Na_2HPO_4]. Sc: supercoiled plasmid DNA; OC: open circular plasmid DNA.

with earlier published results [2]. To facilitate the plasmid DNA quantification, RNA was degraded by incubation of the samples with RNase prior to the chromatography runs. The results from the group separation chromatography and the MiniQ chromatography analysis of the starting material and the thermoseparated top phase are presented in Table 2. As can be seen from the table, the two chromatographic methods give similar high yields for the plasmid DNA after thermoseparation (93–100%). Because of the concentrating effect of the two-phase system, the concentration of plasmid DNA in the thermoseparated water phase is higher than in the desalted lysate. The higher concentration values detected using the group separation method are due to the incomplete separation of genomic DNA and plasmid DNA [12]. This leads to a higher value of plasmid DNA compared to the MiniQ method that can specifically detect plasmid DNA. Hence, both methods can be used for quantifying plasmid DNA in the lysate and the thermoseparated water phase. However, if the group separation method is to be used, the genomic DNA contamination must be kept low by optimizing the experimental conditions for alkaline lysis.

3.4. PicoGreen fluorescence analysis

Because of the interaction of the polymers with the matrix surface, leading to smeared chromatograms, are the above described methods not useful for quantitation of the amount of plasmid DNA in the phases of the primary system (data not shown). The fluorescent probe PicoGreen however, is an ultrasensitive dye that specifically bind to dsDNA, thus allowing the determination of the plasmid DNA concentration even in the primary system. The use of this method has been extensively investigated [22–24], also in connection to aqueous two-phase extractions [25]. As mentioned in Section 2.9, TG1 lysate devoid of plasmid was partitioned in the primary system, followed by the thermoseparation step. After addition of pure pJV4 to the different phases, fluorescence calibration curves were made for each phase. The partitioning of TG1 lysate without plasmid was performed to overcome the contribution of fluorescence from interfering substances such as genomic DNA, SDS and single-stranded (ss) DNA. Only minimal contribution to the fluorescence signal in the lysate should come from these contaminants at a 1000-fold dilution [23]. Despite the dilution, a higher fluorescence signal was obtained for the desalted lysate compared to the phases of the two-phase system. The higher fluorescence signal in the lysate is in this case probably due to a dilution effect of the interfering substances in the two-phase system. The result from the PicoGreen measurements is presented in Table 2 and shows a 90% yield of plasmid DNA in the thermoseparated water phase. This result correlates well with the results of the two chromatographic methods. Thus, the

Table 2
Plasmid DNA concentration in the desalted alkaline lysate and the thermoseparated water phase quantified with group separation, MiniQ and PicoGreen fluorescence analysis

Sample	Group separation		MiniQ		PicoGreeen fluorescence	
	Plasmid (μg/ml)	Yield (%)	Plasmid (μg/ml)	Yield (%)	Plasmid (μg/ml)	Yield (%)
Desalted alkaline lysate	17.9	–	14.0	–	15.2	–
Thermoseparated water phase	25.4	93	22.9	100	20.7	90

Primary system composition: 4.5% $EO_{50}PO_{50}$–4.5% Dextran T 500, 50 mM Na_2HPO_4. Thermoseparation of the $EO_{50}PO_{50}$ top phase was performed at 55 °C.

Table 3
The partitioning of proteins in a system composed of 4.5% $EO_{50}PO_{50}$–4.5% Dextran T 500, 50 mM Na_2HPO_4

Sample	Protein concentration (mg/ml)	Total protein (mg)	Yield (%)	K
Desalted alkaline lysate	0.18	1.3	–	
Primary $EO_{50}PO_{50}$ phase	0.15	0.85	65	1.8
Primary dextran phase	0.11	0.47		
Thermoseparated water phase	0.15	0.75	58	14.4
Thermoseparated $EO_{50}PO_{50}$ phase	0.08	0.05		

Thermoseparation of the $EO_{50}PO_{50}$ top phase was performed at 55 °C.

PicoGreen assay is a method that can be used for quantification of plasmid DNA in a Breox/dextran thermoseparating aqueous two-phase system.

3.5. Partitioning of major contaminants in the optimized aqueous two-phase system

In the primary purification step for plasmid isolation, the main objective is to remove the major contaminants from the plasmid DNA containing solution. When the bacteria are lysed in the alkaline lysis step, contaminants such as proteins, RNA, genomic DNA and cell debris are released. After the neutralization step, the lysate is centrifuged and most of the cell debris and genomic DNA are removed together with some RNA and proteins. However, a significant amount of RNA and proteins will still be present in the sample. Analysis of the total protein partitioning in the primary system showed a K value of 1.8 (Table 3). This gives a 65% yield of total protein in the $EO_{50}PO_{50}$ top phase. In the thermoseparation step, nearly all proteins from the top phase are transferred to the water phase [5–7] due to the excluded volume entropic effect. Thus, the optimized system is able to discard proteins to an extent of 42% after thermoseparation. Fig. 1 shows that contaminating RNA is to a high degree partitioned to the bottom phase in the primary system. Since RNA and plasmid DNA have similar charge properties, one would expect RNA to have similar partition behavior as plasmid DNA and thus partition exclusively to the top phase. However, this is not the case in this system.

Table 4
Amount of total RNA in the thermoseparated water phases relative the desalted alkaline lysate

System composition	Sample	RNA (%)
Start material	Desalted alkaline lysate	–
4.5 % $EO_{50}PO_{50}$–4.5% Dextran T 500, 50 mM Na_2HPO_4	Thermoseparated water phase	46
2.5 % $EO_{50}PO_{50}$–9.0% Dextran T 500, 50 mM Na_2HPO_4	Thermoseparated water phase	20

Thermoseparation of the $EO_{50}PO_{50}$ top phase was performed at 55 °C.

One explanation could be the smaller size of the RNA. Based on analysis of plasmid DNA partitioning (see Section 3.1) we conclude that high molecular weight nucleic acids are partitioned to the top phase in a system close to the critical point. Molecules of lower molecular weight are partitioned to the bottom phase. Analysis of RNA partitioning in the two-phase system by MiniQ chromatography showed that the 4.5% (w/w) $EO_{50}PO_{50}$–4.5% (w/w) Dextran T 500 system is able to remove 54% of the total RNA (Table 4).

3.6. Reduction of the top phase volume–concentrating the plasmid DNA sample

When a primary purification step is designed, one of the issues is to decrease the process volume of the working solution. In a two-phase system, this can be achieved by decreasing the volume of the phase containing the target molecule. By moving along a tie-line in a phase diagram the volume ratio between the two phases can be altered without changing the partitioning of the substance. If the dextran concentration is increased in the system a larger volume of the bottom phase is created with a concomitant reduction of the top phase volume. Five systems composed of different $EO_{50}PO_{50}$–Dextran T 500 ratios [4.5:4.5; 4:6; 3:7; 3:8 and 2.5:9, all (w/w)] (see Section 2.6) were studied. The obtained phase volumes for these systems are shown in Table 5. Analysis of the systems by agarose gel electrophoresis (Fig. 3) shows that the extreme partitioning of plasmid DNA can still be achieved when the top phase volume is gradually decreased. In the primary system comprising of 2.5% (w/w) $EO_{50}PO_{50}$–9% (w/w) Dextran T 500 the top to

Table 5
The volumes of the top and the bottom phases in the primary system, thermoseparated top phase and concentrating effect as a function of polymer concentrations in the primary systems

System	$EO_{50}PO_{50}$ (%, w/w)	Dextran T 500 (%, w/w)	Desalted lysate (V_0, ml)	Top phase (V_T, ml)	Bottom phase (V_B, ml)	Concentrating effect (V_0/V_T)	Thermoseparation water phase (V_{TT}, ml)	Volume ratio (V_T/V_B)	Concentrating effect (V_0/V_{TT})
1	4.5	4.5	7.2	5.5	4.3	1.3	4.2	1.3	1.7
2	4	6	6.5	4.4	5.4	1.5	3.3	0.8	2.0
3	3	7	6.1	2.4	7.3	2.5	1.8	0.3	3.3
4	3	8	5.7	2.5	7.2	2.3	1.9	0.4	3.0
5	2.5	9	5.4	1.9	7.9	2.8	1.4	0.2	3.8

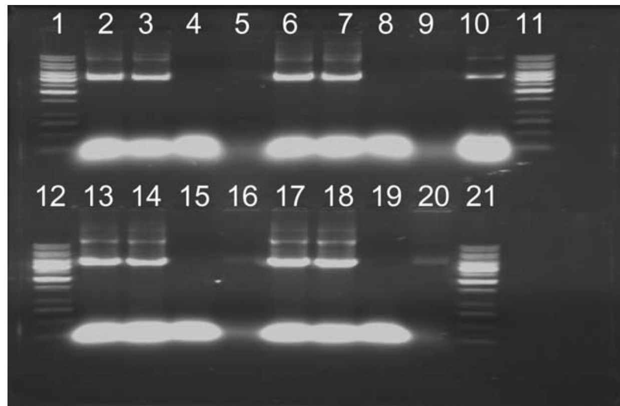

Fig. 3. The partitioning of plasmid DNA in two-phase systems with different polymer concentrations. 4:6 system = 4% $EO_{50}PO_{50}$–6.0% Dextran T 500; 3:7 system = 3.0% $EO_{50}PO_{50}$–7% Dextran T 500; 3:8 system = 3.0% $EO_{50}PO_{50}$–8.0% Dextran T 500; 2.5:9 system = 2.5% $EO_{50}PO_{50}$–9.0% Dextran T 500. All systems contained 50 mM Na_2HPO_4. $EO_{50}PO_{50}$ phase: TP; dextran phase: BP; thermoseparated water phase: TTP; thermoseparated $EO_{50}PO_{50}$ phase: TBP. Lanes—1, 11, 12, 21: 1 kbp DNA ladder (50 ng); 2: TP 4:6 system; 3: TTP 4:6 system; 4: BP 4:6 system; 5: TBP 4:6 system; 6: TP 3:7 system; 7: TTP 3:7 system; 8: BP 3:7 system; 9: TBP 3:7 system; 10: desalted alkaline lysate; 13: TP 3:8 system; 14: TTP 3:8 system; 15: BP 3:8 system; 16: TBP 3:8 system; 17: TP 2.5:9 system; 18: TTP 2.5:9 system; 19: BP 2.5:9 system; 20: TBP 2.5:9 system.

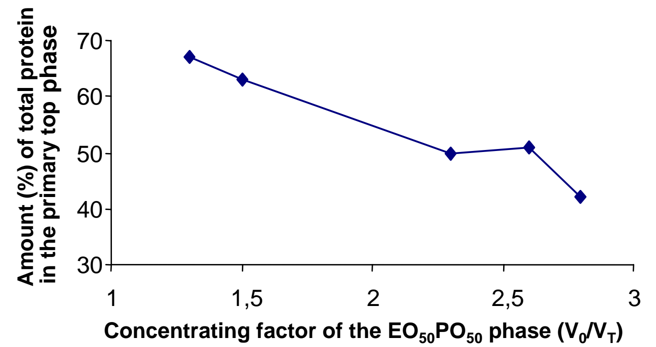

Fig. 4. Remaining total protein in the top phase of the primary system as a function of the concentrating factor of the top phase. V_0 = starting volume of the clarified alkaline lysate, V_T = volume of the $EO_{50}PO_{50}$ phase. The phase systems and phase volumes are described in Table 5.

bottom phase volume ratio (V_T/V_B) was 0.2 (Table 5). This leads to a 3.8-fold volume reduction of the thermoseparated water phase relative to the lysis solution. In all of these systems RNA is partly discarded to the bottom phase. With a reduction of the top phase volume follows an increase in the bottom phase volume, giving a more efficient removal of RNA and proteins from the top phase. Measurements of the total protein concentration in the primary top phase of the different systems showed that the amount of remaining proteins could be reduced from 65% in the original system to 42% when the top phase volume was reduced a factor of 3 in the system with lowest $EO_{50}PO_{50}$ concentration (Fig. 4).

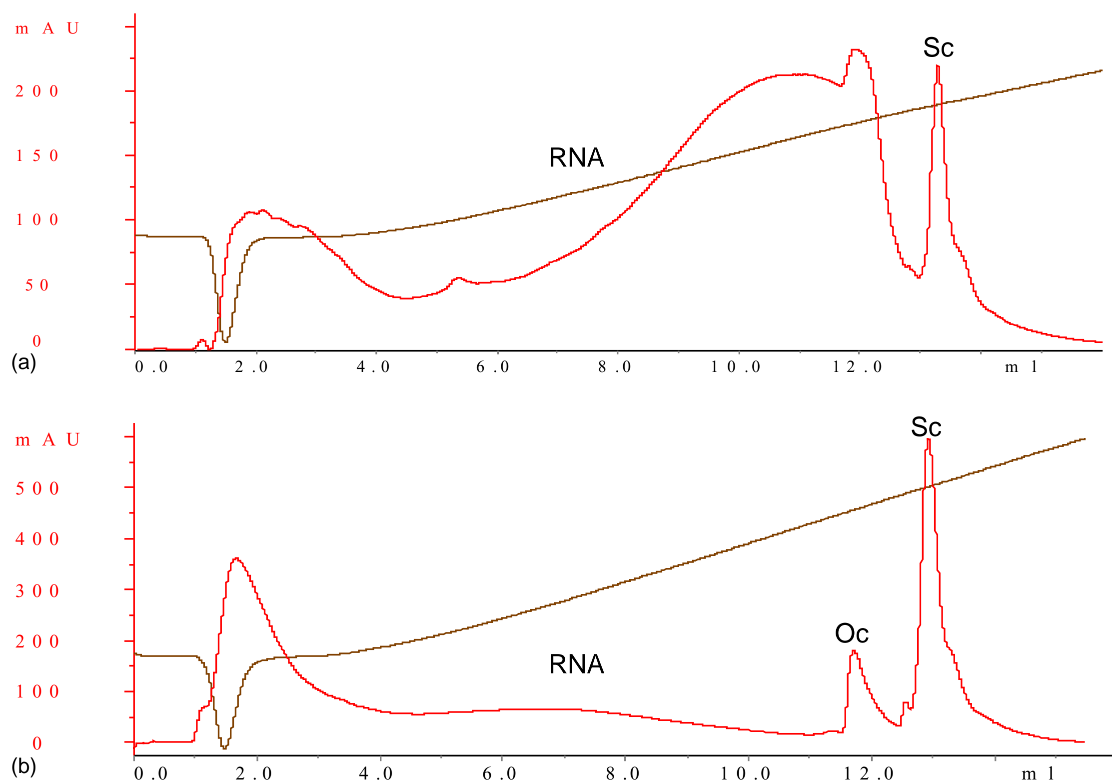

Fig. 5. Analytical anion-exchange chromatography of: (a) 100 µl desalted alkaline lysate; (b) 100 µl thermoseparated water phase from a system composed of 2.5% $EO_{50}PO_{50}$, 9% Dextran T 500 and 50 mM Na_2HPO_4. Sc: supercoiled plasmid DNA; OC: open circular plasmid DNA.

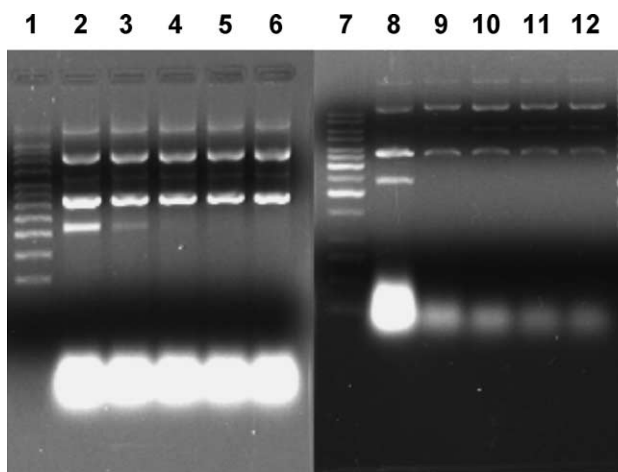

Fig. 6. Agarose gel electrophoresis of the thermoseparated water phase and desalted alkaline lysate treated with Mung Bean Nuclease. Lanes—1, 7: supercoiled DNA standard (50 ng); 2–6: thermoseparated water phase treated with Mung Bean Nuclease after 0, 2, 6, 8 and 10 min; 8–12: desalted alkaline lysate treated with Mung Bean Nuclease after 0, 2, 6, 8 and 10 min. The plasmid band corresponds to linear, open circular, supercoiled and denatured plasmid DNA (from top to bottom).

The RNA removal efficiency of the system composed of 2.5% $EO_{50}PO_{50}$, 9% Dextran T 500 and 50 mM Na_2HPO_4 was calculated from the chromatograms in Fig. 5. It was calculated that 80% of the RNA in the start material could be removed by this system. From this, it could be concluded that by moving further down the tie line in the phase diagram, the $EO_{50}PO_{50}$ phase volume could be even more reduced and the concentration of plasmid DNA further optimized, while also increasing the removal of RNA and proteins in the $EO_{50}PO_{50}$ phase.

3.7. Identification of pJV4 plasmid DNA

Plasmid identity was confirmed by treating the desalted lysate and the thermoseparated top phase with the restriction enzymes *Bam*HI and *Eco*RI. A correct cleavage with *Bam*HI should generate two bands corresponding to the pUC 19 (2686 bp) and the JV4 insert (3433 bp), while *Eco*RI should generate a single band of 6.1 kbp. The *Bam*HI cleavage pattern resulted in two bands with the correct sizes as expected. The *Eco*RI cleavage pattern resulted in one intense band with the correct size but also a faint band at approximately 3 kbp. This result suggests that some of the plasmid DNA was denatured as these molecules are resistant to endonuclease cutting enzymes. Thus, the desalted lysate and the thermoseparated water phase were treated with the single strand cutting Mung bean Nuclease [2,26]. In Fig. 6, it can be seen that in both the desalted lysate and in the thermoseparated water phase, the faster migrating plasmid band (bottom band), disappears after 2–4 min of digestion and probably becomes linearised [2]. Thus, it was concluded that a denatured form of the plasmid is present in the desalted lysate and the thermoseparated water phase. The presence of the denatured band already in the desalted lysate indicates that the denatured plasmid form is created during the alkaline lysis step due to shearing effects [26].

4. Conclusions

This work describes the use of a thermoseparating aqueous two-phase system for the primary purification of plasmid DNA from a clarified desalted alkaline lysate. Using a thermoseparating system, the plasmid DNA can be extracted to a water phase containing less than 1% polymer, with significant reduction of RNA and proteins. The composition of the sample thus obtained is beneficial for subsequent chromatography steps. Furthermore, the thermoseparating two-phase system is easy to scale up and the thermoseparating polymer can be efficiently recycled. The plasmid DNA partitioning in this system was investigated as a function of polymer concentration and salt additives. By systematically increasing the dextran concentration in the system, the top phase volume could be reduced 4-fold with no loss of plasmid DNA. Various analysis methods were compared for their compatibility with polymer–polymer two-phase systems such as chromatography analysis and fluorescence detection methods, and all of them showed to be compatible with the phase systems.

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