Journal of Engineering and Natural Sciences
Mühendislik ve Fen Bilimleri Dergisi

Sigma 28, 9-14, 2010

Research Article / Araştırma Makalesi

USING AQUEOUS TWO-PHASE SYSTEM COMBINED WITH ISOPROPANOL PRECIPITATION FOR ISOLATION OF PLASMID DNA FROM ALKALINE LYSATE

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Received/Geliş: 30.09.2009 Revised/Düzeltme: 08.01.2010 Accepted/Kabul: 18.02.2010

ABSTRACT

This work presents a study of the partitioning of a plasmid from alkaline lysate in polyethylene glycol (PEG) / ammonium sulfate aqueous two-phase system (ATPS). The aqueous two-phase extraction system was prepared with a 35 % w/w PEG 600, 6 % w/w ammonium sulfate and 20 % w/w of lysate load. The performance of this process was determined by qualitative assays. Qualitative analysis was realized by using 1 % of agarose gel electrophoresis. PEG 600 (35% w/w) - $(NH_4)_2SO_4$ (6% w/w) system recovered total amount of plasmid with small amount of RNA in bottom phase. In addition, the bottom phase of the system was integrated with 2-propanol precipitation step. Plasmid yield and RNA profile were observed for the use of different 2-propanol concentrations. Finally, maximized plasmid yield was obtained at 0,7 (v/v) 2-propanol concentration. In higher concentrations than 2-propanol of 0,7 (v/v) plasmid yields were decreased, and in this case plasmid was removed together with RNA in supernatant.
Keywords: Plasmid DNA, plasmid isolation, aqueous two phase systems, gene therapy, 2-propanol precipitation.

PLASMİD DNA'NIN ALKALİ LİZAT'TAN İZOLE EDİLMESİNDE İKİ FAZLI SULU SİSTEM İLE İSOPROPANOL ÇÖKTÜRME YÖNTEMİNİN BİRLEŞTİRİLEREK KULLANIMININ İNCELENMESİ

ÖZET

Bu çalışmada plazmid DNA'nın alkali lizat örneğinden polietilen glikol 600 ve amonyum sülfat bileşiminden oluşan iki fazlı sulu sistemde ayrılması ve bu ortamdan plazmid DNA'nın daha saf olarak elde edilmesi için 2- propanol ile çöktürme yönteminin kullanılması incelendi. Seçilen iki fazlı sulu sistemin bileşimi % 35 w/w PEG 600– % 6 w/w amonyum sülfattır. Kullanılan iki fazlı sulu sisteme uygulanan lizat örneği kütlece % 20 dir. Elde edilen sonuçlar %1'lik agarose gel elektroforezinden alınan jel örnekleri ile kalitatif olarak incelendi. PEG 600 (%35 w/w) - $(NH_4)_2SO_4$ (%6 w/w) bileşiminden oluşan iki fazlı sulu sistemin alt fazından plazmid DNA'nın yanısıra safsızlık olarak RNA gözlemlendi. Plazmid DNA'nın RNA'dan uzaklaştırılarak daha saf olarak elde edilmesi için farklı konsantrasyonlarda 2-propropanol ile çöktürme işlemi yapıldı
Sonuç olarak plazmidin veriminin en yüksek olduğu 2-propanol konsantrasyonu 0,7 (v/v) olarak belirlendi. 0,7 (v/v) den daha yüksek 2-propanol konsantrasyonları için plazmid veriminin düştüğü gözlendi.
Anahtar Sözcükler: Plazmid DNA, plazmid izolasyonu, iki fazlı sulu sistemler, gen tedavisi, 2-propanol ile çöktürme.

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1. INTRODUCTION

Future developments in molecular therapies such as gene therapy and DNA vaccination have increased to develop new methods for plasmid purification in the healthcare fields.[1] Gene therapy and DNA vaccination are performed by introducing nucleic acids into the human cell. Both viral and non-viral vectors have been used as vehicles to transport genetic materials into the target cells. However, viral vectors because of toxicity and immunogenicity are regarded less acceptable. Although non-viral vectors based on plasmid DNA are less effective for transecting target cells, these agents are better alternatives cause of their safety perspective and easier produce. Gene therapy and plasmid based vaccines demand methods to produce and purify this molecule at large scale and pharmaceutical grade [2- 4].

Plasmid production always begins with the culture of transformed *Escherichia coli* bacteria followed by an alkaline lysate method to release plasmid from the *Escherichia coli* cell. RNAse free alkaline lysis method efficiently removes most of the cell walls, organelles, proteins and genomic DNA of the bacteria. RNA is leaving as main contaminant together with target plasmid [5, 6].

Currently used traditional chromatographic process such as anion-exchange and size-exclusion, have been reported to be very efficient in removing RNA. However, limiting process in large size molecule, time consuming and costly cleaning procedure have increased the interest to use new simple low cost alternative methods [6- 8]. As non chromatographic process, aqueous two-phase systems especially in biotechnology application, is preferred, because high proportion of water (80-95%) is provided nontoxic environment. Lower viscosity of the phases and high interfacial contact between phases cause more mass transfer of biological molecule between phases in a short time. Binodial curve describes the composition and concentration of phase where phase separation occurs. The system is composed polyethylene glycol enriched top phase / a second polymer or a salt enriched bottom phase [7, 9].

Here we describe the partitioning of a 6.1 kbp plasmid DNA vector, present in an *Escherichia coli* alkaline lysate, in the PEG 600 / ammonium sulfate polymer-salt aqueous two phase system combined with 2-propanol precipitation for more pure pDNA without RNA contamination.

1.1. Materials and Methods

Chemicals

PEG 600 was obtained from Sigma. Ammonium sulfate and 2-propanol were from Merck. The 6050 bp (base pair) ColE1-type plasmid pVAX1/*lacZ*, designed by Invitrogen (Carlsbad, CA, USA) for the development of DNA vaccines, was used as a model plasmid. This vector contains the human cytomegalovirus (CMV) immediate early promoter; the BGH polyadenylation sequences, a kanamycine resistance gene, a pMB1 origin (pUC-derived), a multiple cloning site, a T7 promoter/priming site and a reporter (β-galactosidase) gene. *Escherichia coli DH5α* from invitrogen was used as the host strain.

Standard Plasmid DNA

The pDNA used as a control was purified using the Qiagen Mega Kit (Qiagen, Hilden, Germany) and resuspended in TE buffer (10 mMTris-HCl, 1mM EDTA, pH:8) pDNA was quantitated by UV spectrophotometer and purity was assessed by the ratio 260/280 (Jasco Model Spectrophotometer, 7850). Purified DNA preparations were pooled and stored a +4 ^{0}C until used in gel electrophoresis as control sample.

Plasmid Production

Escherichia coli cells harboring plasmid pVAX1/lacZ was cultivated overnight (up to a optical density of approximately 3) in 1000 ml shake flasks containing 250 mL of Luria Bertani medium (10g/L tryptone, 5 gr/L yeast extract, 5 g/L NaCl) supplemented with 30 µg /mL of

kanamycine at 37 ^{0}C and 200 rpm. Growth was suspended at late log phase. Cells were stored at -20 ^{0}C until further processing with alkaline lysis as described below.

Alkaline Lysis

Cells (250 mL) were harvested by centrifugation at 15.000x g (20 min. 4 ^{0}C) and the pellets suspended in 8 ml of 50 mM glucose, 25 mM Tris-HCl, 10 mM EDTA, pH: 8 (buffer 1) The cells were lysed by adding and gently mixing (10 min in ice) 8ml of 200 mM NaOH, 1% (w/v) sodium dodecyl sulfate (buffer 2). The lysate was neutralized with 8 ml of a solution of 3M potassium acetate, 11.5 % (v/v) glacial acetic acid (10 min on ice) (buffer 3). All buffers were previously chilled. After neutralization, cell debris, protein and gDNA precipitates were removed by centrifugation (15.000 x g, 20 min. 4 ^{0}C). The second centrifugation at 15.000 g, 20 min. at the same temperature was performed to further clarify the supernatant. The plasmid containing lysate was kept at -20 ^{0}C until used in ATPS described below [10, 11].

Aqueous two - phase Extraction

ATPS were prepared in 15 ml graduated tube with conical tips by adding distilled water, ammonium sulphate, PEG 600, *Escherichia coli* lysate and, up to a total weight of 5 g. The amount of lysate loaded was 1 g [12]. The components were added in the following order; PEG, salt, water, lysate. After thoroughly mixing, the mixture was centrifuged for 30 min.15000 g at 4^{0}C. Top and bottom phase were isolated and stored at 4^{0}C for analysis. Each phase was analyzed by gel electrophoresis in 1 % agarose gel run with TAE (Tris-Acetate-EDTA) buffer in the presence of 0.5 μg/mL ethidium bromide.

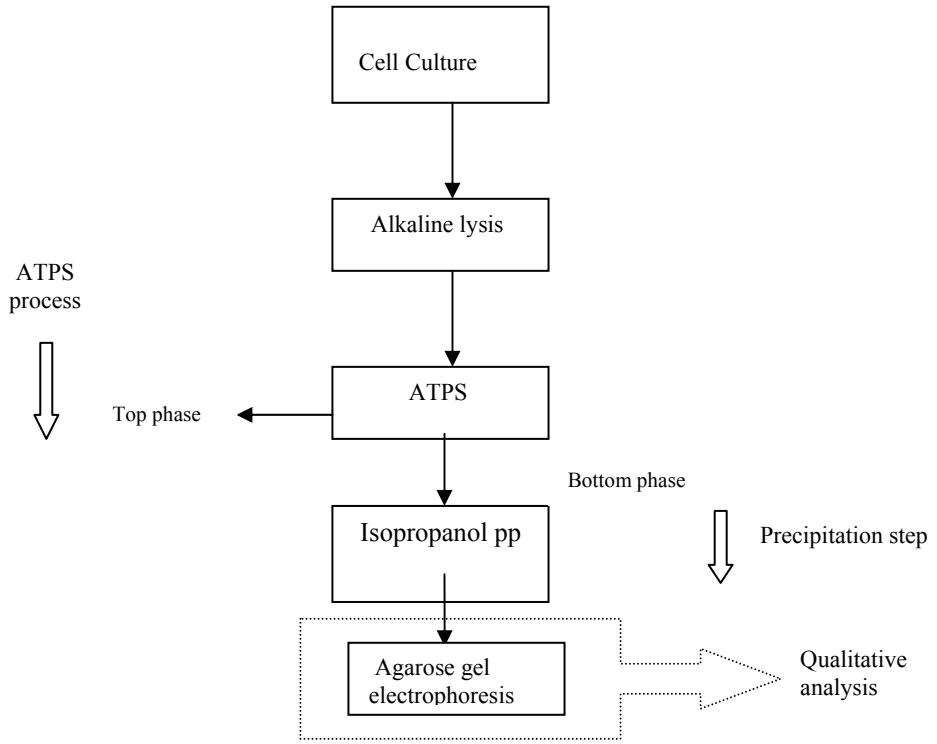

Figure 1. Schematic representation of the pDNA isolation using two step; ATPS and 2-propanol precipitation

2. RESULTS

Partitioning pDNA in PEG / $(NH_4)_2SO_4$ Systems

The partitioning and purification of a 6.1 kbp pDNA vector by using ATPS polymer-salt system was examined PEG molecular weight of 600 and ammonium sulfate. ATPS composition is the PEG 600 35 % (w/w) - $(NH_4)_2SO_4$ 6% (w/w). 20 % (w/w) lysate was load to the ATPS system. Volume of bottom phase is ~ 0.35 mL and top phase is ~ 4.2 mL. PEG 600 molecular weight was directed pDNA to the salt enriched bottom phase. Although RNA having a similar structure and physical properties as pDNA most of amount of RNA is partitioning in the top phase of the system as shown in Figure 1. The rest of the RNA together with total plasmid is in a bottom phase (Line 2, 4, 6 in Fig. 1). According to Joao Carlos and colleagues, plasmid behaves as being insoluble in the top phase, and accumulates in the salt enriched bottom phase of the system. Thus, the highest yields are achieved in the bottom phase of the ATPS process. Minimum contaminations (protein and endotoxines) in the system were obtained by 20 % (w/w) lysate load. More lysate load could increase both contaminants in pDNA and in the system that could result a decrease performance of the ATPS systems [12, 13]. The system PEG 600 / ammonium sulfate composition of 35 and 6% (w/w) respectively, is used to remove most impurities to the top phase. However it could not partition all RNA from the pDNA.

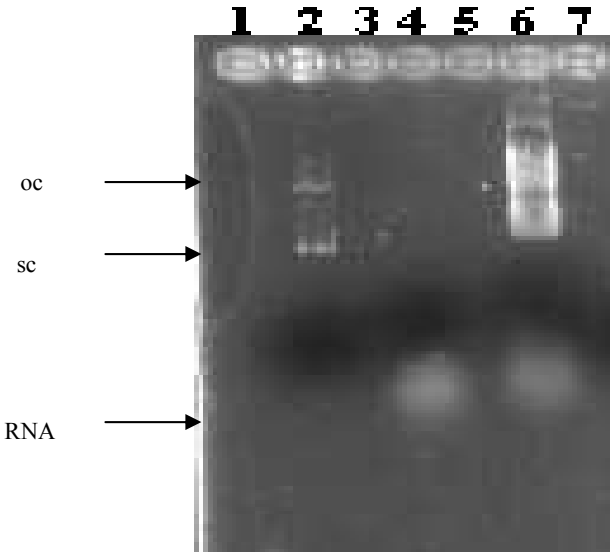

Figure 2. Agarose gel analysis of the ATPS [35% (w/w) PEG 600 / 6 %(w/w) $(NH_4)_2SO_4$]

Lane (2) pure pDNA control; lane (4) bottom phase; lane (6) top phase
oc:open circle form, sc: super coiled form

Effect of 2-propanol Precipitation on Removing RNA from pDNA

Plasmid DNA in bottom phase of the PEG 600 35 %(w/w) - $(NH_4)_2SO_4$ 6% (w/w) ATPS system was precipitated by using different 2-propanol concentrations. 2-propanol precipitation mechanisms are depending on reduction of free water molecules which solvate biomolecules into aqueous solution. Bottom phase of the system is salt rich phase [14]. It means that the effect of 2-propanol was observed in the presence of ammonium salt. Bottom phase was

diluted 0.4 (v/v) and was treated with 2-propanol at 4 ^{0}C for 30 min. After precipitation pDNA and RNA profile were observed on agarose gel (Line 1- 9 in Fig. 2). Various concentrations of 2-propanol were used to remove RNA. The precipitation profile obtained (Figure 2) indicated that 2-propanol concentration 0.7 (v/v) is required to maximize plasmid precipitation and also to achieve plasmid without RNA contamination. It can be seen that from Figure 2, 2-propanol has no effect on plasmid isoforms distributions. Higher than 2-isopropanol concentration of 0.7 (v/v) plasmid precipitation yields was decreased and RNA contaminants were remained at supernatant. When the 2-propanol concentration increased the amount of RNA in supernatant was increased. Plasmid DNA was found to be remove together with RNA when the 2-propanol concentrations were 0.8 (v/v), 0.9 (v/v) and 1.0 (v/v).

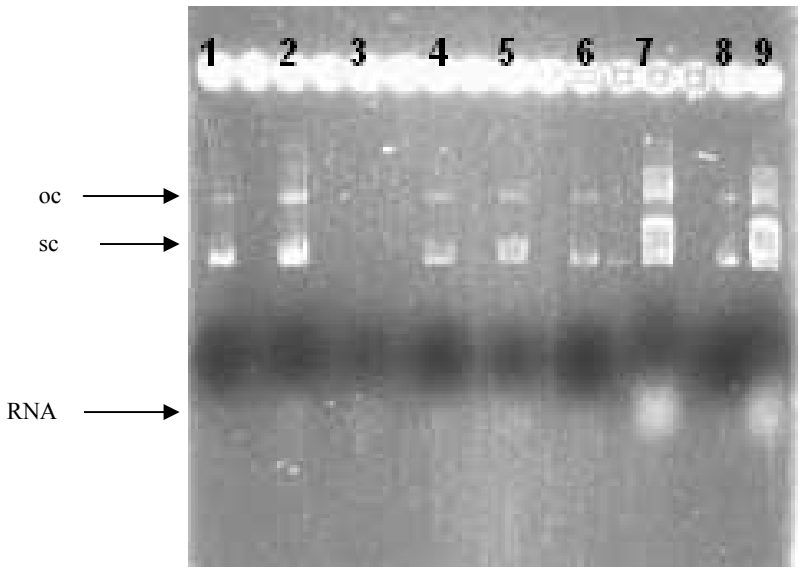

Figure 3. Agarose gel analysis of 2-propanol precipitation of 35 %(w/w) PEG 600 / 6 % (w/w) ammonium sulfate ATPS bottom phase.

Lane1, pure pDNA control; lane2–9, 2-propanol precipitation of bottom phase at 0.7 to 1.0 (v/v); lane2, precipitant sample of bottom phase after 2-propanol precipitation at 0.7 (v/v); lane3, supernatant sample of bottom phase after 2-propanol precipitation at 0.7 (v/v); lane4, lane5 respectively precipitant, supernatant sample at 0.8 (v/v); lane6, lane7 respectively precipitant, supernatant sample at 0.9 (v/v); lane8, lane9 respectively precipitant, supernatant sample at 1.0 (v/v). oc: open circle form, sc: super coiled form.

3. DISCUSSION

This work shows that it is possible to use of ATPS system combined with 2-propanol precipitation to separate RNA from plasmid. 2-propanol concentration of 0.7 (v/v) was aggregated plasmid more efficiently than RNA. Plasmid was found to be removed together with RNA in the supernatant in higher 2-propanol concentration than 0.7 (v/v). The simplicity of this method applying for plasmid DNA isolation and purification can be used as separation step to obtained pure plasmid in gene therapy and in vaccines or pre-purification step to remove large percentage of major contaminants, such as RNA.

Acknowledgment

Nese Ataci acknowledges for financial support to an Erasmus grant (222221–IC–1-2003-1-TR ERASMUS-EUC-1 TR ISTANBUL 07), and the authors also acknowledge to Assoc. Prof. Dr. Joao Carlos Marcos from the University of Minho (UM), Portugal for him educational guidance.

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