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Extraction of plasmid DNA using reactor scale alkaline lysis and selective precipitation for scalable transient transfection

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Abstract

DNA extracted and purified for vaccination, gene therapy or transfection of cultured cells has to meet different criteria. We describe herein, a scalable process for the primary extraction of plasmid DNA suitable for transient expression of recombinant protein. We focus on the scale up of alkaline lysis for the extraction of plasmid DNA from *Escherichia coli*, and use a simple stirred tank reactor system to achieve this. By adding a series of three precipitations (including a selective precipitation step with ammonium acetate) we enrich very quickly the plasmid DNA content in the extract. The process has been thus far used to extract up to 100 mg of plasmid from 1.5 l of clarified lysate, corresponding to an *E.coli* bioreactor fermentation of 3 l.

Introduction

The consistent production of large amounts of plasmid DNA requires approaches, which combine techniques from the repertoire of molecular biology protocols, (Sambrook et al., 1989; Ausubel et al., 1994) and bioprocessing (Levy et al., 2000).

The most widely used method to extract plasmid DNA from *E. coli* is the procedure of alkaline lysis (Birnboim and Doly, 1979). Resuspended cells, lysis solution and neutralising salt solution are mixed typically in a 1: 1: 1 ratio. Lysis is carried out under strongly alkaline conditions (typically pH 12 – 12.5), resulting in the irreversible denaturation of high molecular weight chromosomal DNA (Birnboim and Doly, 1979). The lysate is neutralised subsequently through the addition of acidic high molar salt solution. This results in the formation of an insoluble floc phase, consisting of cell debris, protein – SDS complexes and aggregated chromosomal DNA. Plasmid DNA remaining in solution can be easily separated by centrifugation or filtration.

Previous scale up work on alkaline lysis has largely concentrated on the development of processes that attempt to reproduce the gentle mixing conditions during lysis and neutralisation which are present in existing small-scale protocols. Harsh mixing of the components of the alkaline lysis reaction may deleteriously affect the quality of the final DNA preparation. This concerns in particular contamination by DNA fragments of both plasmid and genomic DNA origin, resulting from shear induced fragmentation (Levy et al., 1999). Such contamination is difficult to remove further downstream, as the majority of extraction / purification methods rely on differences in molecular weight or chemical properties between plasmid DNA and other host cell contaminants. However, gentle and effective mixing with large volumes during alkaline lysis is difficult to achieve, particularly with respect to the changing rheological properties of the lysate during the process (Ciccolini et al., 1999). For this reason we evaluated and now routinely use a stirred tank reactor in which the components of the reaction are added gradually. Contrary to expectations, we obtain intact, largely supercoiled plasmid DNA preparations.

A number of downstream process operations are routinely used in order to achieve a final DNA product. Tangential flow filtration (Kahn et al., 2000) and precipitation (Horn et al., 1995) are techniques, which are typically used to enrich the plasmid DNA frac-

tion. Various forms of chromatography: size exclusion (Raymond et al., 1988), anion exchange (Prazeres et al., 1998), hydroxyapatite (Bachvarov et al., 1983), reversed phase (Green et al., 1997) and affinity (Wills et al., 1997) are also applied in order to purify plasmid DNA from host cell contaminants. The majority of large-scale methods applied in industry are multi-step operations designed to meet the purity requirements for gene therapy.

In our efforts to easily produce considerable quantities of plasmid DNA for large-scale transient transfection, (Jordan et al., 1998), we have devised a rather simple, robust and fast method to extract plasmid DNA. This produces transfectable DNA from clarified lysate using a series of precipitations only. In three steps we extract, at the current scale, up to 100 mg of a crude plasmid DNA preparation in less than 3 hours.

Materials and methods

E. coli fermentation

DH5α containing pEGFPN1 (Clontech, Palo Alto, CA), a plasmid of 4.7 kb, were cultivated in a semi defined glycerol medium containing 0.01% antifoam 204 (Sigma, Buchs, Switzerland) in the presence of the antibiotic kanamycin at a concentration of 50 mg/l. The pH of the medium was corrected to 7.0 before use.

A pre culture of 1.5 l (OD600 6–8) was concentrated in 50 ml and used as the inoculum for a 12 l volume cultivated in a 20 l Bioreactor (ISF200, INFORS, Bottmingen, Switzerland). The reactor was maintained under air saturation at an air flow rate of 10 l/min and a stirrer speed of 600 rpm. Additionally temperature was maintained at 37 °C and pH setpoint controlled at 7.0. Offline measurement of optical density at 600 nm was used to monitor the growth of the culture. Bacteria were harvested in late log phase, typically at OD600's between 20 and 25 units, reached after 9–10 hours cultivation time. Cells were harvested by recovering the fermentation broth from the reactor and transferring to 750 ml beakers, which were subsequently centrifuged at 4,000 g for 10 minutes at 4°C. Cell pellets from 3 l of fermentation broth (OD600 20–25) were resuspended in 200 ml of resuspension buffer (50 mM Glucose, 25 mM Tris, 10 mM EDTA, pH 8.0) and frozen immediately at –20 °C.

Material for plasmid DNA productivity estimation was harvested at various time points by centrifuging 1 ml volumes of the culture at 13,000 rpm for 5 minutes at 4 °C. Cell pellets were typically stored at –20 °C for subsequent extractions.

DNA extraction

200 ml of centrifuged and resuspended cells from 3 l of fermentation broth were thawed and diluted further to reach a final volume of 500 ml with chilled resuspension buffer. Alkaline lysis was carried out in a 2 l stirred tank reactor fitted with two rushton turbines (Applikon, Dietikon, Switzerland), in which pH and temperature were monitored but not controlled (Figure 1). 500 ml of resuspended cell suspension and 500 ml of lysis solution (1% SDS, 0.2 M NaOH) were gradually pumped through two tubes at flowrates of 70 ml/min over a Y connecting piece into a single tube leading to the bottom of the reactor. During this period of addition of the mixed fluid streams, the stirrer speed was set to 500 rpm.

After complete addition of the lysate stream to the reactor (duration 7 minutes), agitation at 500 rpm was continued for a further 5 minutes. Thereafter, 500 ml of chilled neutralising salt solution (3 M Potassium Acetate / 11.5% acetic acid) was added to the reactor via the sparger at a flowrate of 70 ml min^{-1}, whilst the reactor was continually stirred at a rate of 500 rpm.

The contents of the reactor were split into two 750 ml centrifuge beakers, which were then centrifuged at 4,000 g for 30 minutes at 4 °C. The supernatant was clarified further by a single passage over a coarse folded filter (Macherey – Nagel, Oensingen, Switzerland). Liquid recovery was between 1.2 and 1.3 l from 1.5 l of starting materials. Plasmid DNA was precipitated from clarified liquor by the addition of 0.6 volumes isopropanol. After mixing and incubation for 10 minutes at room temperature, the precipitate was recovered by centrifugation at 4,000 g for 20 minutes.

After removing the supernatant, crude DNA pellet was resuspended in a total volume of 200 ml TE, pH 8.0 (10 mM Tris, 1 mM EDTA). Solid ammonium acetate was added to this volume to reach a concentration of 2.5 M. This was mixed rapidly and incubated on ice for a period of 5 minutes, after which it was centrifuged at 4,000 g for 5 minutes at 4 °C. The DNA containing supernatant was precipitated directly with 0.6 volumes of isopropanol at room temperature (incubation and centrifugation conditions as previously). The resulting pellet was washed in 70% ethanol and centrifuged at 4,000 g for 10 minutes; this was followed by careful removal of the supernatant by pipette. Further excess liquid was removed and the pellet air-

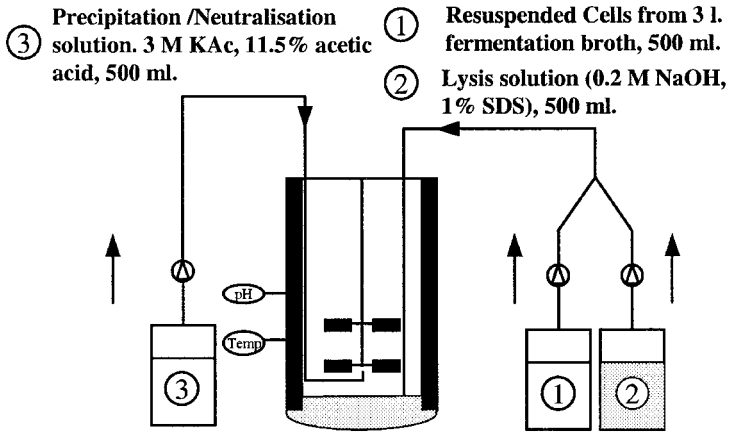

Figure 1. Schematic diagram of alkaline lysis at the 1.5 l scale in a stirred tank reactor. Resuspened cells from 3 l fermentation broth (1) and lysis solution (2) are mixed online by pumping the two streams at an equal flowrate (70 ml/min) into a single stream leading to the reactor, mixing is continued by the agitator (500 rpm). After complete addition of lysis components and incubation for 5 minutes in the reactor, solution 3 is added at the same flowrate (70 ml/min), the precipitate is mixed for a further 10 minutes after complete addition. Plasmid containing supernatant is harvested by cenrifugation and filtration.

dried for 30 minutes, before resuspension in 20 ml of TE. All isopropanol/ammonium acetate precipitation was performed in 250 ml polypropylene conical base centrifuge tubes (Corning, NY).

Small scale extractions were performed on bacterial pellets corresponding to 1 ml of culture volume by taking the pellet up in 300 μl of chilled resuspension buffer (as previously with the addition of RNase) and adding 300 μl of lysis solution. This mixture was incubated at room temperature for 5 minutes before the addition of 300 μl chilled neutralising salt solution and incubation on ice for 10 minutes. The precipitate was then centrifuged at 13,000 rpm for 20 minutes at 4 °C. The resulting supernatant was then transferred carefully to a fresh tube containing 600 μl of isopropanol, after a 5 minute incubation time this was centrifuged at 13,000 rpm for 10 minutes at room temperature. The resulting pellet was washed in 70% Ethanol, after aspiration the pellet was dried and resuspended in TE buffer pH 8.0, before analysis.

DNA/RNA analysis

The presence, purity and integrity of plasmid was validated first by electrophoresis on a 1% agarose gel in the presence of ethidium bromide. DNA was quantified using the Picogreen reagent (Molecular Probes, Leiden, Netherlands) in microtiter plate format using a lambda DNA standard (Molecular Probes). Before quantification, pEGFPN1 preparations were digested with RNase A (Sigma), followed by digestion with a single site restriction enzyme either Hind III or Eco RI, (Gibco, Paisley, Scotland). Preparation's were diluted for storage to a concentration of 1 μg μl^{-1}. Similarly, RNA concentrations were determined using the Ribogreen reagent (Molecular Probes), after digestion with DNase RQ1 (Promega/Catalysis AG, Wallisellen, Switzerland).

Analysis of Lipopolysaccharide (LPS)

LPS was quantified using a commercial chromogenic LAL assay, endochrome, (Endotell AG, Allschwill, Switzerland). Standard solutions were prepared ranging from 5 to 0.005 EU/ml in pyrogen free borosilicate tubes using pyrogen free 'Endosafe' water supplied with the kit. Samples were accordingly pre-diluted in order to be in the range of the standard curve. The assay was perfomed in microtiter plate format in a kinetic mode. Onset absorbance was set to 0.2 units, the time required to reach this value was the value used to analyse the samples relative to the standard solutions. Based on adequate controls, a significant background noise can be excluded for DNA, RNA and TE buffer under the assay conditions.

Protein concentration estimations

The BioRad assay was performed in microtiter plate format according to the manufacturers (BioRad, Hercules, CA) instructions. To generate the standard curve BSA concentrations between 0.5 mg ml^{-1} and 0.05 mg ml^{-1} were used. The detection limit of this

assay was around 50 μg ml^{-1}. Additionally DNA samples were run on 10% SDS PAGE gels, followed by coomasie and silver staining. All DNA samples were treated with DNase and RNase before each assay, as both RNA and DNA produce a significant background signal when analysed particularly with the BioRad assay and SDS PAGE after silver staining. The concentrations of these enzymes (RNase and DNase were too small to be detected alone by BioRad assay or on an SDS PAGE gel after silver staining.

Stirred tank reactor transfection

Transfections of suspension adapted HEK293–EBNA cells (Invitrogen, Carlsbad, CA) were carried out in a controlled 2 l-stirred tank bioreactor (Applikon) applying a modified version of the calcium phosphate protocol described by Meissner et al. (2001). Briefly, 60 ml precipitate were added to 600 ml medium in the reactor containing cells at a density of 5×10^5 cells/ml. Precipitate was formed by mixing 30 ml of 2 $\times$ $CaCl_2$ solution, containing 1.5 mg plasmid DNA, with 30 ml 2$\times$ Hepes Phosphate buffer. Precipitate was allowed to form for 3 minutes before addition to cells. 400 ml of medium was added to the reactor, 4 hours post transfection, in order to dissolve the precipitate. GFP was measured after cell lysis (Triton X – 100) in a 12 well plate (3 measurements per reactor) using a fluorescence plate reader (Cytofluor 4000, Perseptive Biosystems, Framingham, MA), gain 80, Excitation filter: 488 nm, bandwidth 20 nm, emission filter: 520 nm, bandwidth 25 nm.

Results and discussion

E. coli fermentation

The productivity of an *E. coli* culture for plasmid DNA was evaluated by routine miniprep purification followed by quantification using the dsDNA specific picogreen reagent. Applying a similar approach to that used by Noites et al. (1999) we monitored plasmid DNA production during fermentation over time. This allowed predicting the best time at which to perform bioreactor harvests. The drawback of this method is that data are only available when the entire fermentation process has been executed, as a result of the time required to process the DNA miniprep and the assay. DNA replication is growth related. As culture density increases the plasmid DNA amount increases (Figure 2). The plateau of plasmid production is reached in stationary phase. Using a semi defined glycerol medium, we harvested bacteria 9–10 hours post innoculation at densities equivalent to OD600 20–30 in a 12 l volume. Using the strain DH5α containing pEGFPN1, this corresponds to approximately 350 mg of extractable plasmid per reactor.

Alkaline lysis

The major concerns for plasmid recovery were the exclusion of chromosomal DNA and chromosomal DNA fragments from the floc precipitate and a risk of plasmid DNA degradation by shear forces in the reactor. If a significant genomic DNA contamination remained in the liquid phase after precipitation, it would be difficult to separate it at a later stage from plasmid DNA, due to the similar physico-chemical properties of the two molecules. Likewise, shearing or degradation of plasmid would result in a reduction in the yield of DNA obtained. For these reasons, most alkaline lysis processes advocate gentle mixing of the resuspended cells, lysis solution and neutralising salt solution. At small-scale this is achieved by the addition of the individual components stepwise to a small vessel and mixing by gentle inversion, Birnboim and Doly (1979). At large scale (multiple litre extraction) this is more problematic. Theodossiou et al. (1997) performed alkaline lysis at medium scale by gentle swirling of cells and lysis solution. Thatcher et al. (1999) at a scale of several litres used a spoon to manually distribute cells and lysis solution. Marquet et al. (1995) propose a stirred tank using a low shear barred impellor, which covers the height and width of the reactor. Wan et al. (1998) describe the use of static mixers in order to ensure good mixing and minimal shear induced damage of bacterial genomic DNA.

We used a 2-l stirred tank for the initial scale-up of the alkaline lysis method. Our process uses a standard baffled laboratory bioreactor fitted with two rushton turbines. At the 50–100 mg scale, lysis is performed in a volume of 1.5 l using a stirrer speed of 500 rpm. Cell lysis is initiated by pumping resuspended cells and lysis solution into a single stream, after which the lysate is further agitated in the reactor. The neutralising salt solution is added gradually under agitation to the reactor. As the cells and lysis solution are mixed initially by pumping the two fluid streams together and into the reactor gradually there is a varying exposure time to lysis solution within the total population of cells, as well as a varying time of plasmid DNA ex-

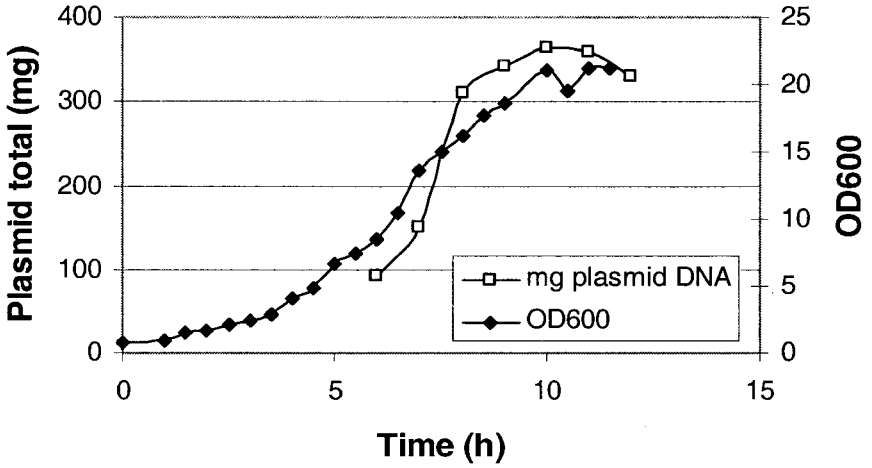

Figure 2. Fermentation of DH5α containing pEGFPN1 at the 12 l scale. Samples taken each hour from the bioreactor, DNA extracted from 1 ml culture volume by miniprep (alkaline lysis and isopropanol precipitation), followed by measurement with picogreen reagent, after DNA linearisation. Fermentation parameters: Temp 37 °C, Stirrer speed 600 rpm, Air flow rate 10 l/min.

posure to strongly alkaline conditions. The mode of addition of cells and lysis solution, however, ensures that the two remain in a 1: 1 ratio throughout lysis and that local pH fluctuations are minimised.

In exploratory experiments (using a smaller quantity of cell mass), viscosities of the lysis phase and neutralisation phase were evaluated. Viscosity measurements in a coaxial cylinder (Viscotester VT550, Haake, Karlsruhe) in samples from the endpoints of lysis and neutralisation, showed a decrease in viscosity from 3.94 $\pm$ 0.08 mPas (lysis) to 2.23 $\pm$ 0.14 mPas (neutralisation). This corresponds to a decrease in viscosity equivalent to a factor of 1.8.

Figure 3 provides a visual guide to the appearance of the liquid at various phases in the process. Figure 3a, a photograph taken during the lysis period, shows that the stirred fluid climbs the stirrer shaft. The addition of neutralising salt solution leads to a dramatic transition in the liquid properties of the lysate and the formation of the floc precipitate. Initially the floc appears as a large globular mass (Figure 3b), its appearance is similar to that generated in bench-scale laboratory protocols in which components are added batch wise and mixing is achieved by gentle inversion. Complete neutralisation and precipitation takes place in the reaction volume upon addition of sufficient acidic potassium acetate (Figure 3c). Eventually, the floc appearance changes to become much smaller in size, corresponding to a homogenous particle in water suspension. This transition coincides with a rapid change in pH to a consistently observed endpoint of pH 5.0 $\pm$ 0.1. After centrifugation and simple 'folded filter' filtration a clear plasmid DNA containing supernatant is obtained (Figure 3d). Theodossiou et al. (1999) showed floc formation after alkaline lysis and used the buoyant nature of the floc as a means to separate it from the plasmid containing liquor. However, the authors provided no specific information on how alkaline lysis was performed, except that the three components were mixed in a 1 : 1 : 1 ratio. Based on the observations that the floc obtained in our extractions in the stirred tank settles within 30 minutes under non agitated conditions (not shown), we conclude that it's properties are somewhat different from the published one.

DNA extraction by precipitation

Three subsequent precipitations are applied in order to yield a 'partially purified' DNA preparation from clarified alkaline lysate. Isopropanol precipitation is used to recover and concentrate DNA from the clarified lysate. During this step a significant quantity of RNA is co-precipitated.

The majority of RNA can be selectively precipitated with ammonium acetate and separated by centrifugation, from plasmid DNA which remains soluble. RNA removal was demonstrated in the course of extracting a 10.3 kb plasmid (Figure 4). Comparing soluble DNA/RNA after isopropanol precipitation (Figure 4, lane 1) with what remains after ammonium acetate treatment (Figure 4, lane 3), as well as the composition of the ammonium acetate precipitate

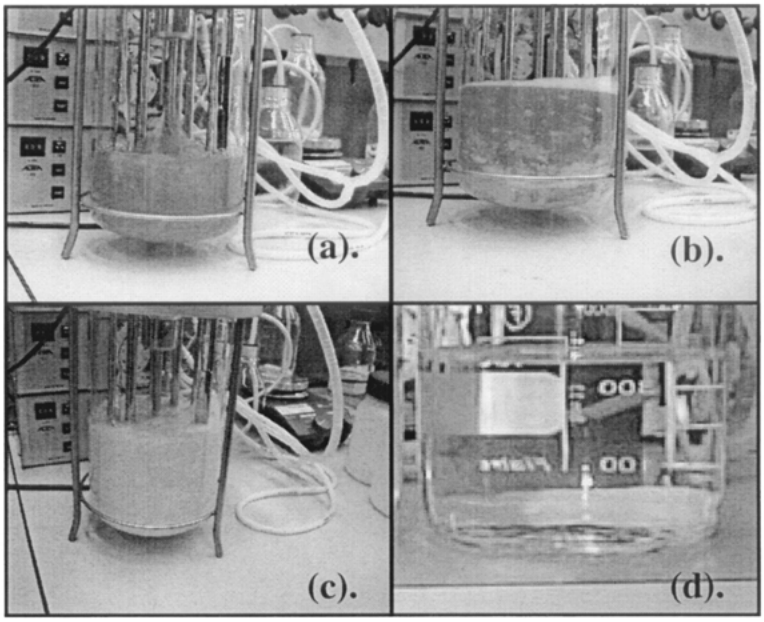

Figure 3. (a) Lysis, (b) Addition of 3 M KAc (start), (c) Addition of KAc (final), (d) Clarified lysate after centrifugation and filtration.

(Figure 4, lane 2), it is evident that a drastic reduction in RNA content occurs. The third and final precipitation with isopropanol and the subsequent wash with 70% ethanol, yields the final DNA preparation (Figure 4, lane 4).

On the current scale up to 100 mg of plasmid DNA can be extracted using this method in less than 3 hours. Three similar extractions of the plasmid pEGFPN1 (4.7 kb) performed with cells derived from 3 l of fermentation broth, yielded on average 77.2 ± 14.7 mg plasmid DNA (the average RNA content of these preparations was 101.4 ± 21.8 mg) since the picogreen assay would not distinguish between different types of DNA this value may include genomic DNA. However, based on qualitative estimations through agarose gel electrophoresis we consider such a contamination to represent a minor fraction of the total DNA. Plasmid DNA yields correspond closely to values obtained when using standard miniprep extractions from 1 ml culture aliquots.

Analysis

Three identically prepared extracts of pEGFPN1 are shown at a DNA concentration of 1 μg μl^{-1} on a 1% agarose gel (Figure 5) in comparison to pure DNA (purified using the column kit method, AX10,000, Macherey-Nagel). Two further bands appear in crude extracts of pEGFPN1 on the agarose gel in addition to the characteristic pattern of pure plasmid DNA which is largely supercoiled pDNA. The band migrating at the bottom of the gel is bacterial RNA. Above the pDNA bands (MW > 10,000 bp), an as yet unidentified band is observed, it could be genomic DNA or a further form of plasmid DNA, however, further analysis is required in order to clarify this. Traces of bacterial genomic DNA may be detected using more sensitive methods (a requirement for gene therapy products), such as southern blotting (Marquet et al., 1997). The upper band represents only a minor fraction of the total DNA in the extract, therefore we believe it to have little effect on the transfectability of the DNA preparation (a small amount of salmon sperm DNA is used in numerous transfection protocols as 'carrier DNA').

Table 1. Principal contaminants of crude DNA extracts prepared by alkaline lysis and 3 step precipitation. All DNA preparations were diluted to 1 mg/ml based on picogreen quantification

	RNA (mg/ml)	LPS (EU/ml)	Protein (mg/ml)
Extract 1	1.15	194,200	< 0.05
Extract 2	1.19	500,200	< 0.05
Extract 3	1.63	3,000	< 0.05

The 3 separate batches of pEGFPN1 were tested for other potential contaminants. Values describing RNA, LPS and protein are summarised in Table 1. The

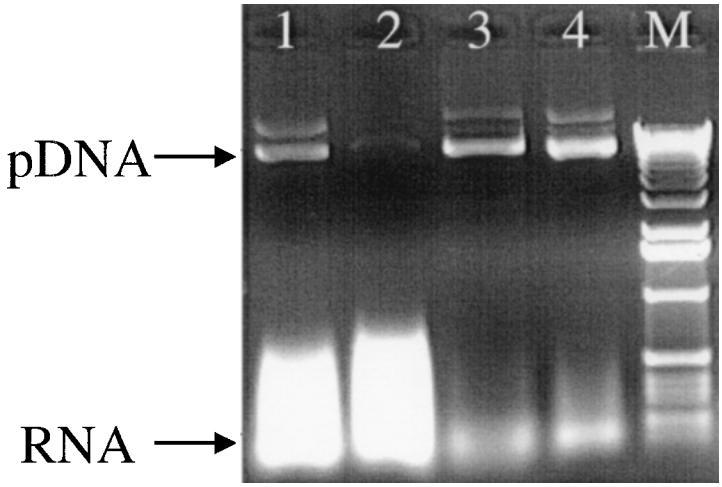

Figure 4. Removal of bacterial RNA by ammonium acetate precipitation, analysis on 1% agarose gel. Starting material-isopropanol precipitated plasmid pEAK8 (10.3 kb) from cleared alkaline lysate. Wells 1, 3 and 4 contain 0.16 μg plasmid DNA.

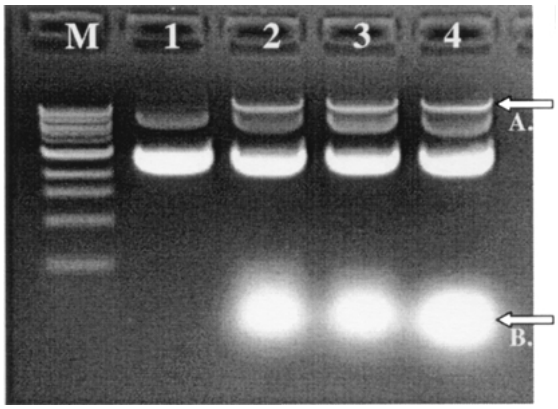

Figure 5. Comparison of DNA extracted by the described method and DNA purified using a column kit protocol. All pEGFPN1 (4.7 kb), at 1 μg/μl. 1% Agarose gel + EtBr. Pure DNA (AX 10,000, Macherey-Nagel).

three batches contained respectively 1.15, 1.19 and 1.63 μg μl^{-1} RNA at an estimated DNA concentration of 1 μg/μl. LPS concentrations were more variable ranging from 3,000 (prep 3) to circa 5 $\times$ 10^5 EU ml^{-1} (prep 2). SDS PAGE gel electrophoresis yields no detectable protein bands in either the pure or crude DNA extracts by coomasie or silver staining (RNase and DNase treatments were used before silver staining in order to remove non specific background staining of nucleic acids). Likewise, upon nucleic acid digestion, protein was undetectable using a standard Biorad protein assay (<50 μg ml^{-1}).

Stirred tank reactor transfection

Despite the presence of high concentrations of RNA and variable levels of LPS we have found such preparations to be highly effective in transfecting HEK293 cells by the calcium phosphate co-precipitation method. We have seen no difference in transient expression level for two different recombinant proteins, GFP and a human recombinant antibody (anti RhD IgG) in small-scale transfection systems (adherent and suspension adapted HEK293 cells) when comparing pure plasmid DNA and plasmid extracted as described here (data not shown). Figure 6 shows expression profiles in stirred tank reactors transfected in parallel comparing DNA containing impurities (prepared using the extraction method described in this paper) with 'pure' DNA (Macherey – Nagel, AX 10,000). In the one particular experiment shown in Figure 6, transient GFP expression is higher in the reactor transfected with crude DNA than the corresponding control transfection using pure DNA.

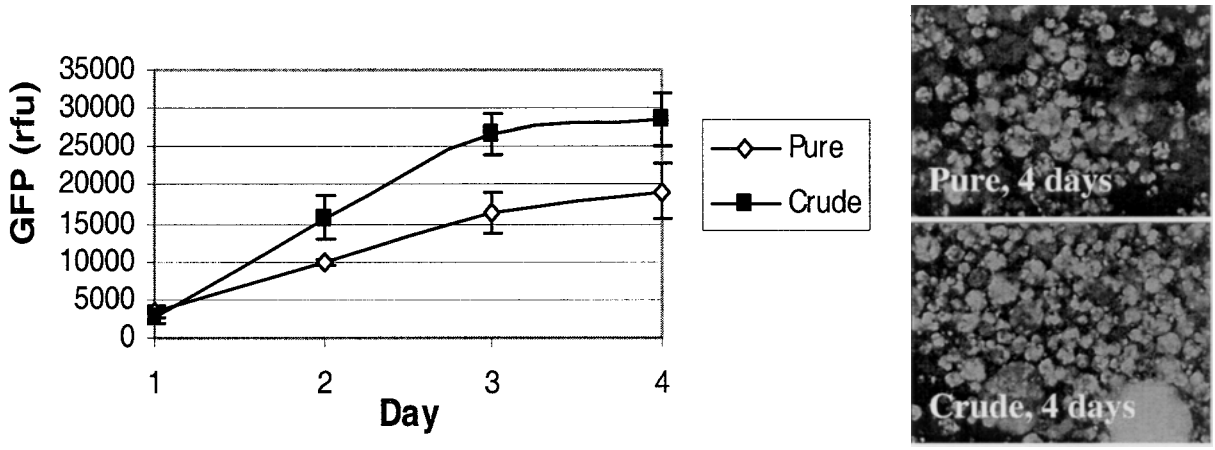

Figure 6. Transient GFP expression 3 days post transfection from HEK293–EBNA cells in stirred tank reactor upon transfection of different preparations of pEGFPN1 (1.5 mg DNA per reactor), by the calcium phosphate co-precipitation method. 'Pure' refers to DNA preparations purified by a kit method (Macherey-Nagel, AX 10,000), 'crude' refers to partially purified DNA prepared as described. Bars represent the mean of 3 measurements from samples from each reactor after lysis. Pictures right show reactor samples 4 days post transfection, before lysis.

Transfections at scale are limited today by the cost and effort required to produce large quantities of plasmid DNA. Transfectable DNA prepared by a simple and cost effective method, even though containing considerable impurities, should prove useful for large-scale transient expression systems.

Conclusion

An approach to plasmid production has been applied to produce transfectable plasmid DNA at the 100 mg scale, suitable for use in large-scale transient expression applications. The procedure takes less than 3 hours from frozen cell mass.

We conceive our method of scalable DNA production to meet the needs of cell based production processes, specifically large-scale transfection, in which the cost and effort required to purify DNA is a key bottleneck.

Acknowledgements

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