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Non-GMP plasmid production for transient transfection in bioreactors

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Abstract

We describe a generic plasmid purification process for producing DNA for larger-scale transient transfection. Data on plasmid quality with regard to residual protein, endotoxin content and presence of different plasmid forms is given. The effects of contaminants and plasmid forms on expression levels of TNFRp55 and SEAP are discussed. Transient transfection of serum-free suspension grown mammalian cells represents a suitable approach to provide research quantities of proteins (50–100 mg) within 1–2 weeks.

Introduction

As a consequence of the multitude of potential drug targets that originate from the recent progress in genomics, there is the need to produce several 10–100 mg amounts of proteins for biochemical characterization, high-throughput screening assays, crystallization, and structure determination by x-ray analysis or NMR techniques. Several eukaryotic expression systems including stable recombinant cell lines, the baculovirus-insect cell, Semliki Forest virus and Drosophila expression systems, and transient transfection using plasmid DNA are available to accomplish this task, each having its individual advantages and disadvantages. The speed of operation is probably the most important factor. We developed a large-scale transient transfection method useful for producing recombinant proteins from HEK293 or other animal cells upon contacting them with cationic polymer/DNA complexes (Schlaeger and Christensen, 1999; Schlaeger et al., 1999). A generic plasmid purification protocol was necessary to ensure an adequate plasmid supply for the application of this novel expression system at the bioreactor scale.

The numerous protocols on chromatography-based plasmid purification described in the literature (e.g. Chandra et al., 1992; Hines et al., 1992; McClung and Gonzales, 1989; Merion and Warren, 1989; Raymond et al., 1988; Wils et al., 1997; Colpan and Riesner, 1984) served as a starting point of our approach. However, while several groups have reported on large-scale processes and GMP-requirements for plasmid DNA purification for gene therapy and DNA vaccine application (e.g. Horn et al., 1995; Ferreira et al., 2000; Prazeres et al., 1999), only limited information on generic purification procedures and quality requirements for plasmid preparations used in transient transfection protocols at the bioreactor scale are available. In the following we present some of our results in this field.

Materials and methods

Chemicals and expression vectors

The extracellular domain of the human tumour necrosis factor receptor (TNF receptor p55) (Loetscher et al., 1990) was inserted into the EBV-based pREP7 expression vector (Invitrogen, Carlsbad, CA) and used as a reporter gene (ca. 10 kb). The pCMV/SEAP plasmid (7.58 kb) encoding the human placental alkaline phosphatase was obtained from Tropix (Bedford, MA) (Berger et al., 1988). Various *E. coli* hosts e.g. DH5α, XL2, JM109, HB101, 803, XL1Blue were originally obtained from different suppliers (e.g. Invitrogen) and

culture collections (ATCC, DSM) and stored in the Roche cell depository. pUC18 2.7 kb, pBR322 4.3 kb and DRIgest III (AP Biotech, Uppsala, Sweden) were used as control samples. The transfection reagent polyethylenimine (PEI) 25 kDa was purchased from Aldrich (Buchs, Switzerland). A sterile PEI stock solution was prepared as described earlier (Boussif et al., 1995). The stock solution can be stored at room temperature for several months.

Plasmid production

Several *E. coli* strains, as W3110, XL1Blue, DH5α, 294, JM109, AB1157, HB101 and 803, were evaluated as hosts for optimal plasmid production using various pCMV, pREP4 and pREP7 based expression vectors. After transformation, 5 single colonies were picked from each LB-Ampicillin plate and amplified in 5 ml cultures of the same medium overnight. Plasmids from OD3 biomass pellets were purified and quantitated by Miniprep Plasmid Kits (Bio-Rad, Richmond, CA) and further analyzed by agarose gel electrophoresis.

Growth and plasmid production of soluble human TNFRp55 in pREP7 using *E. coli* DH5α as host were evaluated in overnight shake flask cultures (50 ml volume, 16 h, 37 °C, 200 rpm) inoculated from liquid nitrogen ampoules. Four different media were compared: Luria broth LB: tryptone 10 g l^{-1}, yeast extract 5 g l^{-1}, sodium chloride 5 g l^{-1}; Super broth: tryptone 25 g l^{-1}, yeast extract 15 g l^{-1}, sodium chloride 5 g l^{-1}; Terrific broth TB-phosphate: 12 g l^{-1}l tryptone, yeast extract 24 g l^{-1}, glycerol 4 g l^{-1}, K2HPO4 9.4 g l^{-1}, KH2PO4 2.2 g l^{-1}; CIRCLEGROW (BIO101, Vista, CA). All fermentations were performed in 10 l Chemap FZ2000 stirred bioreactors using TB medium supplemented with 100 μg ml^{-1} ampicillin. The fermentation temperature was 37 °C. No pH control was implemented. The dissolved oxygen was maintained at 10% DO levels controlled via stirrer speed (600–1200 rpm), aeration (2–6 l min^{-1}) and oxygen enrichment of the incoming air flow. The biomass was collected with a continuous bowl centrifuge (Varifuge 20RS, Heraeus SEPATECH, Osterode, Germany) and used immediately for plasmid purification or frozen at –20 °C. Plasmid from OD3 biomass samples was quantitated and analyzed as described in the previous paragraph.

Plasmid purification

Alkaline lysis and 2-propanol precipitation.

150 g frozen biomass was resuspended in 0.8 l S1-buffer overnight (4 °C, 50 rpm). 0.4 l S1-buffer (50 °C) was added for 1 hr (RT, polypropylene container, stirrer diameter 12 cm, 100 rpm). Fresh biomass was resuspended in 1.2 l S1-buffer for 3 hr at room temperature. Lysis occurred after the addition of 1.2 l S2-buffer (RT) followed by incubation for 5 min. Cellular debris was precipitated with the addition of 1.2 l S3-buffer (–20 °C) followed by incubation on ice for 30 min. Any particulate matter in the cleared lysate was removed by centrifugation (5000g, 60 min, 4 °C). Plasmid DNA was precipitated from the lysate by the addition of 2.5 l 2-propanol (4 °C). DNA was collected by centrifugation (5000 g, 60 min, 4 °C, N2 overlay for safety reasons). The pelleted DNA was resuspended in 150–250 ml buffer A using a 25 ml pipette and kept at 4 °C overnight prior to chromatographic purification. In the small scale lysis experiment the plasmid content was determined by Miniprep analysis after addition of S3-buffer.

Fast size-exclusion chromatography (SEC)

Chromatography was performed on Sephacryl S-500 HR using a FPLC system (AP Biotech). A XK 2.6 $\times$ 40 column was eluted at room temperature using buffer A (bed volume ca. 200 ml). A typical sample volume of 50 ml with a DNA concentration of 1–2 mg ml^{-1} was loaded onto the column and the linear flow rate was set at 30 cm h^{-1}. Eluted fractions were analyzed by agarose gel electrophoresis. The appropriate fractions without visible RNA contamination were pooled, the DNA precipitated in 70% (v/v) ethanol and filter-sterilized through 0.22 μm polyethersulfone (PES) membranes (Millipore Corp., Bedford, MA). Aliquots of plasmid (1 or 2 mg ml^{-1}) were frozen at –20 °C.

Anion-exchange (Source 30Q) and high-resolution size-exclusion chromatography (Sephacryl S-1000 SF) were applied to increase the total yield of RNA-free plasmid by rechromatographing fractions after SEC and to isolate/enrich supercoiled and relaxed plasmid forms, respectively.

The composition of lysis buffers S1, S2 and S3 and chromatography buffers A and B was as follows: S1-Buffer: 50 mM Tris/HCL, 10 mM EDTA, pH 8.0; S2-Buffer: 200 mM NaOH, 1% (w/v) SDS; S3-Buffer: 2.8 M Potassium acetate, pH 5.1; Buffer A: 20 mM Tris/HCL pH 8.3; 1 mM EDTA; 0.5 M NaCl; 0.02%

Triton X-100; 10% (v/v) EtOH; Buffer B (anion-exchanger only): 20 mM Tris/HCL pH 8.3; 1 mM EDTA; 1 M NaCl; 0.02% Triton X-100; 10% (v/v) EtOH.

Analytical methods

The accumulation of the soluble human TNFRp55 in the culture medium was analyzed by ELISA as described earlier (Godfried et al., 1994). The SEAP activity was determined by using a commercially available multiwell chemiluminescence assay following the protocol of the supplier (Roche Molecular Biochemicals, Lucerne, Switzerland). The results were expressed as relative light units per milliliter culture medium (RLU ml^{-1}). Both recombinant reporter proteins are relatively stable and the culture supernatant could be stored at –20 °C before analysis.

Protein was measured by Bradford assay (BSA as standard). Endotoxin determinations were performed with a chromogenic assay according to the directions of the manufacturer (BioWhittaker, Walkersville, MD). Agarose gel electrophoresis was performed with 1% agarose gels using ethidium bromide staining. Plasmid Miniprep Kits were obtained from BioRad or Qiagen (Hilden, Germany). The plasmid concentrations were determined spectrophotometrically and estimated by (1%) agarose gel electrophoresis 'calibrated' with pUC18 DNA.

Cell culture and transient transfection

The subclone 293EBNA from the human embryonic kidney cell line HEK293 (Invitrogen) were adapted to serum-free growth in a Primatone RL fortified HL medium (Schumpp and Schlaeger, 1990). The low-calcium HL medium is a mixture of fortified DHI (calcium-free) and RPMI 1640 (calcium-free) media (2:1 w/w) and contains heparin at a concentration of 30 U ml^{-1} (Sigma) as described recently (Schlaeger and Christensen, 1999). Calcium-free DHI and calcium-free RPMI 1640 were purchased from Life Technologies (Basel, Switzerland). The cells were routinely cultured in spinner flasks using stirrer speeds of 95–105 rpm, 37 °C, and 70% of the recommended working volume (Bellco).

HEK293EBNA cells were routinely grown in the presence of heparin to avoid cell aggregation. For transfection experiments exponentially growing cells were centrifuged and resuspended in heparin-free HL medium as described (Schlaeger and Christensen, 1999). The cell concentration was adjusted to 5–6 × 10E5 cells ml^{-1} and the diluted culture was incubated for at least 6 hr before transfection took place. After addition of transfection complexes, the cells were incubated for 1–5 days without medium replacement. Two days post-transfection, the cultures were generally fed once a day with a nutrient mixture (2 g l^{-1} glucose, 2 mM glutamine, 1 × essential and non-essential amino acids (Invitrogen).

Transfection complexes were prepared separately in 1/10 of the culture volume using heparin-free fresh medium. 0.2 μg DNA was diluted into 0.1 ml fresh medium. Two minutes later 1 μl PEI solution (stock solution 0.9 mg ml^{-1}) was added and the solution gently mixed. This corresponds to a PEI nitrogen to DNA phosphate (N : P) ratio of 33 : 1. After 15 min at room temperature the DNA complexes were given to 1 ml cell suspension.

Results and discussion

Plasmid amplification in E. coli DH5α

Various plasmids were compared in different *E. coli* host strains with respect to plasmid yields and the observed plasmid forms. Figure 1 shows the plasmid quality as judged by the amount of supercoiled monomeric DNA for a TNFRp55 plasmid from overnight cultures of 5 colonies each picked for *E. coli* hosts DH5α, JM109, HB101, and 803. The plasmid yields and quality clearly is a function of host strains (Schorr et al., 1997). It was also found to be different for the same host when using different expression vectors and genes of interest (data not shown). When comparing different plasmids, DH5α gave in most cases a high yield of monomeric supercoiled plasmid DNA.

Further, different bacterial growth media were investigated. While E. coli cells grown on Super broth and CIRCLEGROW reached the highest cell densities in shake flask cultures, the specific plasmid production was maximal with Terrific broth (data not shown). Plasmid production kinetics in a 10 l bioreactor are presented in Figure 2. An OD_{600} value of 20 was reached without nutrient feeding when cultivating pREP7-TNFRp55 bearing DH5α in Terrific broth supplemented with ampicillin. The specific plasmid content per cell was highest at 8 hr fermentation time. On average, biomass yields of 250–450 g wet biomass were obtained from 10 l bioreactor runs. Crude plasmid yields were in the range of 10–25 mg per liter reactor volume. This is comparable to values reported

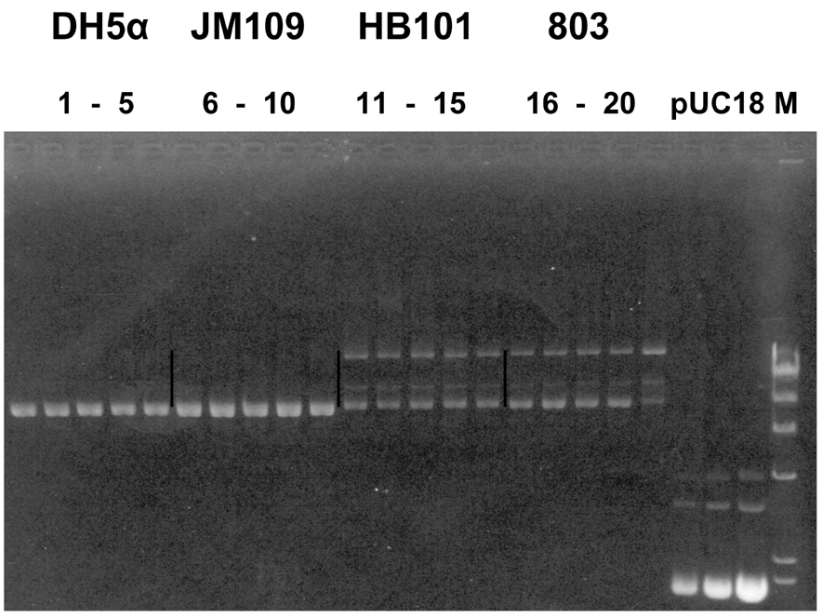

Figure 1. Plasmid quality and quantity as a function of E. coli host. Plasmid products from 5 colonies each for different E. coli host strains were analyzed by agarose gel electrophoresis. pREP7-TNFRp55 was used as an examplary plasmid.

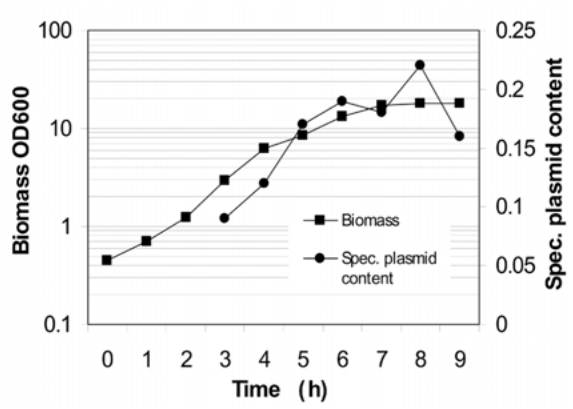

Figure 2. Optical density and specific plasmid content for E. coli plasmid production in a 10 l bioreactor. Product: pREP7-TNFRp55 plasmid; medium: TB-phosphate plus ampicillin; fermentation conditions: 37 °C; 10% dissolved oxygen; no pH control. The specific plasmid content was measured from OD3 samples analyzed by Miniprep (BioRad).

in the literature for batch fermentations (Lahijani et al., 1996).

Generic non-GMP plasmid purification procedure

Figure 3 outlines the described plasmid purification process suitable for producing DNA for large-scale transient transfection.

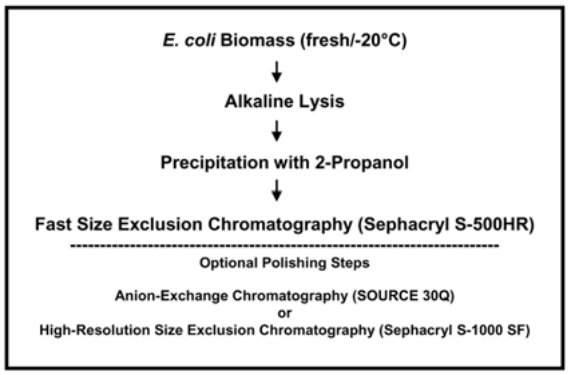

Figure 3. Process outline for the pilot-scale purification of plasmid DNA.

Starting from fresh or frozen *E. coli* biomass we performed an alkaline lysis procedure based on the method described by Birnboim, 1983. Sodium dodecylsulfate (SDS) was used as the anionic detergent leading to effective plasmid release and comparatively low initial endotoxin levels (Kahn et al., 2000). No addition of exogenous proteins like lysozyme or RNase was performed. After precipitating plasmid DNA with 2-propanol, residual RNA, protein and other low molecular mass contaminants were removed by size-exclusion chromatography on Sephacryl S-500HR (Figure 4). The chromatographic separation

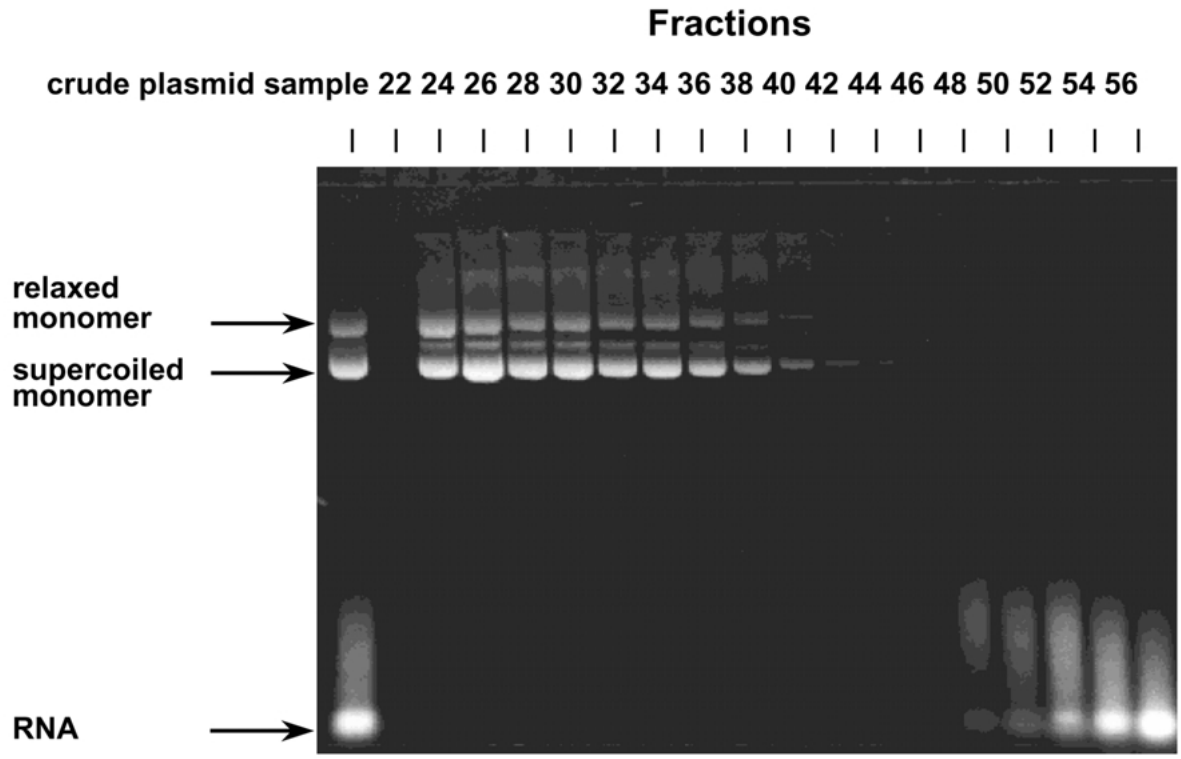

Figure 4. Fast size-exclusion chromatography chromatography of a crude TNFRp55 plasmid preparation. SEC chromatography was performed on Sephacryl S-500 HR (bed volume ca. 200 ml, ambient temp., Buffer A, sample volume 50 ml, plasmid concentration: 2 mg ml^{-1}, linear flow rate 30 cm h^{-1}).

was performed within 2 hr at high linear velocities and with large sample volumes, basically serving as a 'group fractionation' method. Transfection-grade plasmid can routinely be purified within 2 working days. Aliquots of plasmid preparations are sterilized throught 0.22 μm PES membranes and stored at –20 °C until further use. No significant differences were observed in plasmid yield and quality for fresh or frozen biomass extractions.

Originally the plasmid preparation process was performed with 150 g biomass per purification batch (see Materials and methods). However, with plasmids routinely being produced at the 10 l bioreactor scale, it was desirable to work up the biomass from one reactor per purification batch. We thus investigated the effect of biomass concentration on lysis pH and measured plasmid yields from pH adjusted and pH non-adjusted plasmid batches (Figure 5). The lysis pH-value dropped to pH 10.5 for a wet biomass concentration in the S1-buffer of 400 g l^{-1} (if the pH was not adjusted) and the plasmid yield decreased considerably. When maintaining the pH-value between pH 12–12.5 the specific plasmid yield was found to be constant for biomass concentrations of up to 400 g l^{-1}.

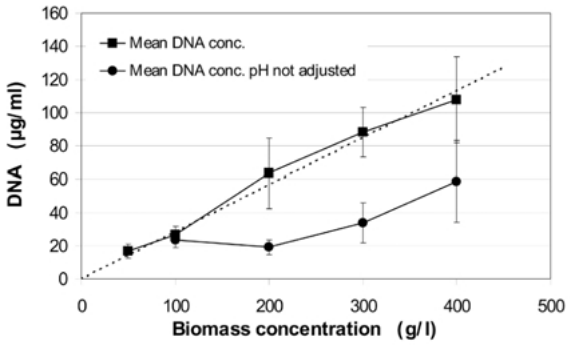

Figure 5. Effect of pH value during alkaline lysis of *E. coli* biomass. Plasmid yield as function of biomass concentration for TNFRp55 (BT29/2) with and without pH adjustment. Standard deviations are derived from 2 sets of lysis experiments each with Miniprep analyses performed in triplicate.

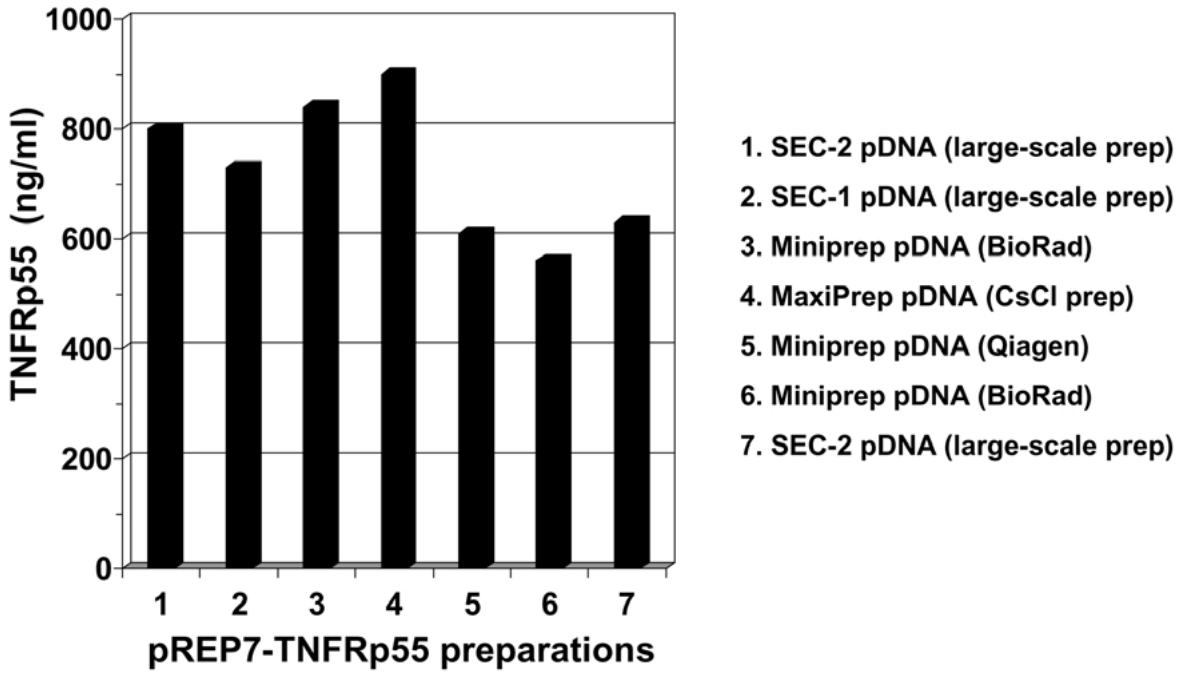

Figure 6. TNFRp55 production as function of different plasmid preparations. Transient gene expression using PEI-mediated DNA uptake (3 days), 0.2 μg ml^{-1} DNA each, 50 ml spinner flasks.

Plasmid quality and effect of contaminants and plasmid forms on transient transfection

Different batches of pREP7-TNFRp55 plasmid were generated either using the described procedure, several commercially available plasmid purification kits or the classical CsCl gradient centrifugation method. When using these preparations for transient transfection with 293EBNA cells repeatedly over a period of several months, product titers for TNFrp55 of 700 $\pm$ 100 ng ml^{-1} (Figure 6) were observed indicating the comparability of plasmid quality.

Further, we prepared endotoxin-free SEAP plasmid (20 EU mg^{-1} DNA) with Endofree Plasmid Maxi Kits (Qiagen). Exogenous endotoxin (Sigma, 4000 EU ml^{-1} Standard) was then added to the system at levels of up to 25 EU ml^{-1} either prior to the complex formation or after the addition of the plasmid formulation to the cells. We found no effect (decrease) on SEAP expression levels and near zero endotoxin concentrations were determined by the chromogenic assay (data not shown). Most likely, this is due to the fact that PEI like polymyxin B acts as a cationic scavenger for endotoxin (Montbriand and Malone, 1996; Mitzner et al., 1993). However, when we attempted to use crude plasmid (without being purified by fast SEC chromatogaphy) for transfection (Figure 7) we already observed a substantial drop in SEAP expression levels with low levels of additional RNA or other unknown components present in the system.

Anion exchange chromatagraphy on Source 30Q and high-resolution size-exclusion chromatography on Sephacryl S-1000 SF was performed to further resolve relaxed and supercoiled plasmid forms. Preparations exceeding 80–90% of either form were obtained and tested with regard to SEAP expression. No effect on gene expression was observed for the described transient expression system (data not shown).

Plasmid quality of selected preparations

Figure 8 demonstrates the quality of several plasmid preparations encoding 7 different proteins. For all plasmids analyzed so far there are usually 2 major bands visible upon agarose gel electrophoresis. The lower band representing the closed circular or supercoiled and the upper band the open circular or relaxed form of the respective plasmid. In some cases the linear form and concatemers are present (data not shown). We have never observed denatured plasmid bands (below supercoiled), verified by deliberately denaturing plasmid at pH 13 (data not shown) (Sayers

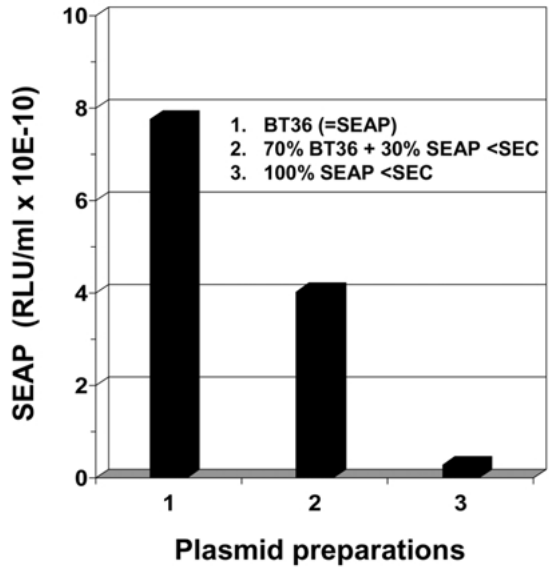

Figure 7. SEAP production as function of contaminants before fast SEC chromatography. Transient gene expression using PEI-mediated DNA uptake (4 days), 0.2 μg ml^{-1} DNA (12900 EU mg^{-1} DNA), 15 ml spinner flasks, feeding on day 2. Equivalent amounts of crude plasmid before chromatography (SEAP < SEC) substituted for purified plasmid in transfection complexes as estimated by detailed agarose gel analyses.

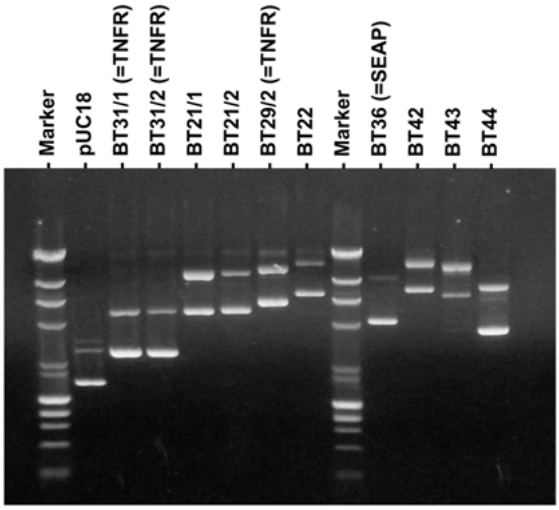

Figure 8. Plasmid quality of selected preparations. BT numbers indicate plasmid preparations for 7 different proteins. Batches of the same plasmid are compared side by side (e.g. BT31/1 and BT31/2). Plasmids encoding TNFRp55 and SEAP are labeled accordingly.

Table 1. Plasmid DNA quality criteria for transient transfection

• Identity	total size, restriction digest
• DNA homogeneity	1% agarose gels-HPLC
• RNA	nonvisualized on 1% agarose gels-HPLC
• A260–280 ratio	1.80–1.90
• Protein	50–100 μg mg^{-1} DNA, no major concern
• Endotoxin	no concern at $\sim$ 10'000 EU mg^{-1} DNA
• Sterility	membrane filtration
• Gene expression	comparable to standard preparation

et al., 1996; Qiagen Plasmid Purification Handbook, 1997). Variations in the percentage of the supercoiled and relaxed forms are visible for 2 different purifications using the same fresh E. coli biomass as starting material (e.g. BT31/1 and BT31/2 (= TNFR) or BT21/1 and BT21/2). It should be noted, however, that both these batches gave similar results when compared with regard to TNFRp55 expression. The use of XL2 hosts (BT 42, BT 43) resulted in plasmid preparations with increased levels of the relaxed form.

The mean protein content of a range of plasmid preparations is 64 $\pm$ 16 μg mg^{-1} DNA (mean value for 12 preparations). This translates into 12 ng protein ml^{-1} culture volume originating from plasmid under the given transfection conditions. Before the fast SEC chromatography step protein levels were determined to be about twice as high as for the purified preparations (data not shown). As long as any exogenous proteins are not toxic to the culture system at the observed levels no problems are expected, because any products of interest are purified to a given purity prior to any further usage. We observed a wider variation in the range of endotoxin levels (8300 $\pm$ 4000 EU mg^{-1} DNA; mean value for 10 preparations). However, with the PEI-mediated transfection system we noted no detrimental effect on product formation as described above.

Table 1 lists desirable quality criteria for plasmid preparations suitable for large-scale transient transfection.

Conclusions

We have developed a fast and robust generic process for the purification of plasmid DNA from E. coli biomass within 2 days. In connection with upstream plasmid fermentation and subsequent transient transfection of serum-free suspension grown HEK293

EBNA cells at pilot-scale we are able to provide research quantities of proteins (50–100 mg) within 1–2 weeks.

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