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Comparison of Alkaline Lysis with Electroextraction and Optimization of Electric Pulses to Extract Plasmid DNA from *Escherichia coli*

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Abstract The use of plasmid DNA (pDNA) as a pharmaceutical tool has increased since it represents a safer vector for gene transfer compared to viral vectors. Different pDNA extraction methods have been described; among them is alkaline lysis, currently the most commonly used. Although alkaline lysis represents an established method for isolation of pDNA, some drawbacks are recognized, such as entrapment of pDNA in cell debris, leading to lower pDNA recovery; the time-consuming process; and increase of the volume due to the buffers used, all leading to increased cost of production. We compared the concentration of extracted pDNA when two methods for extracting pDNA from *Escherichia coli* were used: alkaline lysis and a method based on membrane electroporation, electroextraction. At the same time, we also studied the effect of different pulse protocols on bacterial inactivation. The concentration of pDNA was assayed with anion exchange chromatography. When alkaline lysis was used, two incubations of lysis time (5 and 10 min) were compared in terms of the amount of isolated pDNA. We did not observe any difference in pDNA concentration regardless of incubation time used. In electroextraction, different pulse protocols were used in order to exceed the pDNA concentration obtained by alkaline lysis. We show that electroextraction gives a higher concentration of extracted pDNA than alkaline lysis, suggesting the use of electroporation as a potentially superior method for extracting pDNA from *E. coli*. In addition, electroextraction represents a quicker alternative to alkaline lysis for extracting pDNA.

Keywords Alkaline lysis · Electroextraction · Plasmid DNA · *Escherichia coli*

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Introduction

The first plasmid DNA (pDNA) was identified by Lederberg (1952). By the end of the 1960s, the genetic understanding of pDNA had reached a level of detail which allowed massive exploitation of these genetic elements. Today, the importance of pDNA as a pharmaceutical substance has increased considerably due to its use in gene therapy, cell therapy and DNA vaccination (Saade and Petrovsky 2012). The concept of using pDNA for vaccines has gained particular recognition due to its apparent safety and simplicity compared to live-attenuated viral vaccines (Atkinson and Chalmers 2010). pDNA poses zero threat for being virulent, and there is no large variability in lot-to-lot potency (Schleef et al. 2010). Although today there are many methods known for pDNA isolation (such as alkaline lysis), their low efficiency and/or high cost still represents a challenge. Therefore, the economic perspective of this fast-developing field underlines the need for development of an industrial-scale method for the production of pDNA in an adequate quantity and quality (Schleef et al. 2010).

Escherichia coli (Tatum and Lederberg 1947) is the most widely used bacteria for pDNA production. Bacterial cells carrying pDNA are propagated in shaker flask or fermenters and then harvested by centrifugation. Cells in sediment are resuspended in buffer solution or distilled water (dH_2O). In order to obtain pDNA from bacteria, the

cells need to be lysed. One of the most commonly used methods for lysing bacterial cells is alkaline lysis. Cells are lysed with NaOH/SDS, leading to release of the cell content. After neutralization with acidic potassium acetate, pDNA remains in solution, while denatured proteins, genomic DNA (gDNA) and cellular debris are coprecipitated in insoluble salt–detergent complexes. Precipitated debris is removed by either centrifugation or filtration, producing cleared lysates, from which pDNA is purified (Naglak et al. 1990). Alkaline lysis in large-scale production has several drawbacks such as increase of the volume, preparation of different solutions and entrapment of pDNA in cell debris with subsequent lower recovery of pDNA, which then requires additional extraction steps. Consequently, the cost of pDNA manufacturing remains quite high, and the need to develop new pDNA isolation tools in order to lower production costs is evident.

Almost four decades ago a physical method (electroporation) for extraction of molecules by use of electric pulses was described (Neumann and Rosenheck 1972). Electroporation results in a significant increase of the cell plasma membrane permeability for molecules that are otherwise deprived of transport mechanisms. Namely, when an electric field is applied to a biological cell, transmembrane voltage is induced. When the induced transmembrane voltage exceeds a certain value, structural rearrangements of lipids in the membrane bilayer occur (Teissie and Rols 1993; Kotnik et al. 2010). As a consequence, the membrane becomes permeable and small or large molecules that otherwise cannot pass the cell membrane can be introduced into or extracted from the cells. The method is already successfully used in different biomedical applications, including electrochemotherapy (Edhemovic et al. 2011; Mali et al. 2013), DNA vaccination (Sardesai and Weiner 2011), gene therapy (Neumann et al. 1982; Daud et al. 2008), transdermal drug delivery (Wong et al. 2011) and cell electrofusion (Foung et al. 1990; Usaj et al. 2010). On the other hand, application of a sufficiently high electric field causes irreversible membrane damage, which results in destruction of cells (Rubinsky 2007). Such an application is already used in irreversible tissue ablation (Neal et al. 2011) and is showing great promise also in water and food treatment for inactivation of different microorganisms (Morales-de la Peña et al. 2011; Zgalin et al. 2012). With the destruction of cells with the electric field also the opportunity arose for extraction of cellular components, electroextraction (Sack et al. 2010; Haberl et al. 2013).

Here, a comparison of the standard extraction method for pDNA (alkaline lysis) and electroextraction is presented. We show that electroextraction yields a higher pDNA concentration when compared to alkaline lysis and that an even higher yield of extracted pDNA can be obtained with further electric pulse protocol optimization.

Materials and Methods

Preparation of Bacterial Culture

Escherichia coli K12 TOP10 strain carrying plasmid pEGFP-N1 (Clontech Laboratories, Mountain View, CA; cat. no. E4102S) encoding kanamycin resistance was inoculated in 200 ml of Luria broth (Sigma-Aldrich Chemie, Deisenhofen, Germany) in an Erlenmeyer flask. To ensure bacterial selectivity, 200 µl of antibiotic kanamycin sulfate (Carl Roth, Essen, Germany) was added at a concentration 50 µg/ml. *Escherichia coli* cells were incubated for 17 h with continuous shaking at 37 °C. *Escherichia coli* cells were harvested by centrifugation (4,700 rpm, 30 min) at 4 °C. Supernatant of the culture medium was removed, and cells were resuspended in 10 ml of distilled water so that cell density was around 10^9 cells/ml. To determine cell density in 10 ml of distilled water, a plate count method was used: cells were serially diluted with distilled water, then 0.1 ml of the dilution was plated into Luria broth agar medium. Plates were incubated at 37 °C for 24 h in the incubator, and bacterial colonies were counted manually. For the experiment we used a 10^{-1} dilution of prepared bacterial cells.

Alkaline Lysis

Bacteria were lysed using a modification of the alkaline method described by Birnboim and Doly (1979) at room temperature (22 °C). Briefly, cells from bacterial culture were harvested by centrifugation (4,700 rpm, 30 min) at 4 °C and resuspended in 50 mM Tris buffer (Sigma-Aldrich Chemie), pH 8.0, containing 10 mM EDTA (10 ml of buffer per 1 g of biomass). The suspension was treated with an equal volume of cell lysis buffer containing 0.2 M NaOH (Sigma-Aldrich Chemie, cat. no. 30620) and 1 % SDS (Sigma-Aldrich Chemie, cat. no. L4390), followed by an equal volume of neutralizing solution (3 M potassium acetate, adjusted to pH 5.0 with glacial acetic acid, chilled to 4 °C; Sigma-Aldrich Chemie, cat. no. 25059). We tested 5- and 10-min lysis incubation times, which means that lysis with NaOH after 5 or 10 min was stopped with neutralization. Precipitated material was removed by centrifugation (5,000 rpm, 10 min, 4 °C).

Electroextraction

Electroextraction was performed at room temperature (22 °C). We used a cuvette with integrated aluminum electrodes (Eppendorf, Hamburg, Germany). The volume of *E. coli* cells placed in the cuvette was 90 µl (for all electric pulse parameters), and samples were exposed to square wave electric pulses using an electric pulse

generator (HVP-VG; IGEA, Carpi, Italy). We repeated the electroextraction procedure 10 times in order to obtain a sample volume large enough ($\sim$1 ml) for further analysis. To permeabilize the cell membrane and to release pDNA from cells, three electric pulse protocols were used: (1) a train of eight rectangular pulses with a duration of 100 μs, with an applied voltage of 3,000 V, resulting in an electric field (E) of 30 kV/cm and a pulse repetition frequency of 1 Hz; (2) a train of 24 rectangular pulses with a duration of 100 μs, with an applied voltage of 3,000 V, resulting in $E = 30$ kV/cm and a pulse repetition frequency of 1 Hz; (3) one rectangular pulse with a duration of 1 ms, with an applied voltage 1,250 V, resulting in $E = 12.5$ kV/cm. Applied electric field (E) is approximated by $E = U/d$, where U denotes applied voltage and d, electrode distance ($d = 1$ mm).

After electric pulse application, 50 μl of the sample was taken in order to determine the influence of electric pulses on *E. coli* viability. The rest of the sample ($\sim$850 μl) was filtered through a 0.22-μm filter (Millex-GV; Millipore Corporation, Billerica, MA, USA) and analyzed for pDNA. Also, agarose gel electrophoresis was performed in order to confirm the presence of pDNA only.

Viability

To determine the influence of electric pulses on bacterial viability, a plate count method was used (Reasoner 2004). Treated and untreated samples (90 μl of *E. coli* cells placed in a cuvette, with no electric pulse application) were serially diluted with distilled water, then 0.1 ml of the dilution was plated into Luria broth agar medium (Sigma-Aldrich Chemie) for *E. coli*. Plates were incubated at 37 °C for 24 h in the incubator (Kambič, Semič, Slovenia) prior to counting. All counts were done manually and expressed as log (N/N_0), where N represents the number of colony-forming units per milliliter in treated sample and N_0, the number of colony-forming units per milliliter in untreated sample, i.e., control.

Determination and Quantification of Extracted pDNA

pDNA in samples (from alkaline lysis and electroextraction) was quantified by anion exchange chromatography. The Agilent 1200 system (Agilent Technologies, Santa Clara, CA) in combination with CIMac pDNA anion exchange column (BIASeparations, Ajdovščina, Slovenia) was used for separation of pDNA from impurities. pDNA samples were diluted (1:3) in mobile phase A (200 mM Tris, pH 8.0), filtered through a 0.45-μm filter and injected to a CIMac pDNA column. Injection volume was 20 μl, and flow rate was 1 ml/min. pDNA was separated in a linear gradient from 60 to 70 % of mobile phase B (200 mM Tris, 1 M NaCl, pH 8.0). pDNA was detected with UV at 260 nm. Each peak on the chromatogram is specific for a specific molecule (sheared gDNA, RNA, aromatic amino acids, etc). We determined the surface of the peak specific for pDNA and calculated the pDNA concentration from the calibration curve, which was obtained from pEGFP-N1 isolated with a HiSpeed Plasmid Maxi Kit (Qiagen, Hilden, Germany).

Electrotransformation of Naive *E. coli* with Electroextracted pDNA

To determine that electroextracted pDNA was functional, we performed electrotransformation of electrocompetent naive *E. coli* ER1821. Naive bacterial cells (200 μl) and 100 μl of electroextracted pDNA were placed in a chilled cuvette with integrated aluminum electrodes ($d = 2$ mm). The sample was thoroughly mixed with a pipette and incubated on ice for 2 min. One rectangular pulse with a duration of 1 ms and an applied voltage of 2,500 V, resulting in $E = 12.5$ kV/cm, was used in order to electrotransform naive *E. coli* cells. After electroporation, the entire volume was removed from the cuvette and placed into the tube with 700 μl of Luria broth. Cells were incubated for 1 h in the incubator (37 °C). Samples were serially diluted with distilled water, then 0.1 ml of the dilution was plated into Luria broth agar medium with antibiotic kanamycin sulfate at a concentration of 50 μg/ml. In the control, the electric pulse was omitted. Plates were incubated at 37 °C for 24 h in the incubator prior to counting. Values reported in Table 1 are the mean (±SD) number of transformants per microgram of pDNA obtained from triplicate trials.

Statistical Analysis

All experiments were performed in triplicate on 1 day and repeated on 3 different days to check for reproducibility of results within a given set of experiments. Since cell density was always (on all 3 days of the experiment) approximately 10^9 cells/ml (with minimal deviations), we believe that variations in bacterial cell density were not large enough to influence our results. Results were analyzed using an unpaired t test analysis (SigmaPlot 11.0; Systat Software, Richmond, CA) and were considered statistically different at $p < 0.05$.

Results and Discussion

In our study we extracted pDNA from *E. coli* bacteria using two different extraction methods: alkaline lysis and electroextraction based on electroporation of the cell

membrane. We determined the concentration of pDNA in both samples. Figure 1 shows the pDNA concentration for the different protocols used to extract pDNA from *E. coli*. In the alkaline lysis method (Birnboim and Doly 1979), we stopped lysis with neutralization after 5 or 10 min and successfully extracted around 0.300 μg/ml of pDNA. For both neutralization times (5 and 10 min) in the alkaline lysis method, the volume of the sample was always the same. We did not observe any difference in pDNA concentration when the 5- or 10-min lysis time was used; therefore, all bacterial cells were already disrupted after 5 min of lysis. Namely, during lysis the bacterial membrane is degraded and gDNA and pDNA are denatured (Birnboim and Doly 1979).

In the next part of our study we electroextracted pDNA from *E. coli*. Electroporation can release intracellular material through the permeabilized cell membrane (Sack et al. 2010; Brodelius et al. 1988; Ganeva et al. 2004; Suga and Hatakeyama 2009; Zakhartsev et al. 2007). Also, rapid electroporation-mediated plasmid transfer between microorganisms was reported previously (Ward and Jarvis 1991; Kilbane and Bielaga 1991; Baulard et al. 1992; Gunn and Nickoloff 1995). In a few reports also the amount of electroextracted pDNA and other cell components from microorganisms was evaluated (Suga and Hatakeyama 2009; Zakhartsev et al. 2007; Moser et al. 1995; Takayuki et al. 1999). The release of intracellular material using electric pulses was also patent-protected (Martin et al. 2002, 2004; Wu et al. 2007). In order to determine the concentration of electroextracted pDNA from *E. coli*, bacterial cells were harvested by centrifugation and resuspended in dH_2O. Afterward three different pulse protocols were used to permeabilize the cell membrane and extract pDNA from cells: (1) 8 × 100 μs, 30 kV/cm, 1 Hz; (2) 24 × 100 μs, 30 kV/cm, 1 Hz; and (3) 1 × 1 ms, 12.5 kV/cm. Efficient pDNA electroextraction requires optimization of electric parameters (Zakhartsev et al. 2007; Takayuki et al. 1999), and different electric pulse parameters seem to regulate the degree of membrane permeabilization and control the release of intracellular content (proteins, pDNA, etc.) (Shiina et al. 2007).

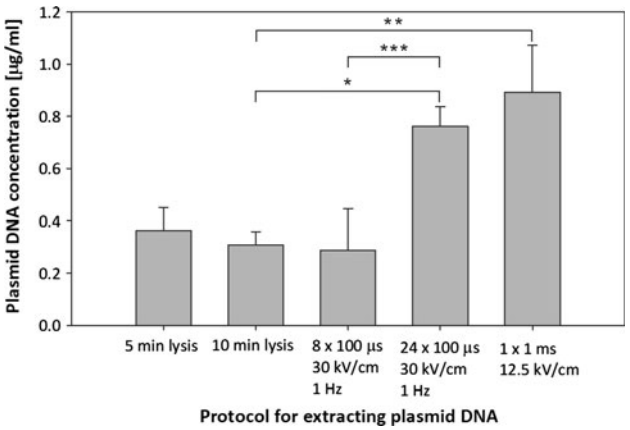

Fig. 1 Effect of different protocols on extracted pDNA concentration. Experiments were completed at room temperature (22 °C). Values represent means ± standard deviation. *$p < 0.001$, **$p = 0.006$, ***$p = 0.009$

When a train of eight electric pulses (8 × 100 μs, 30 kV/cm, 1 Hz) was used, the concentration of pDNA extracted was approximately the same (0.289 μg/ml) as obtained with the alkaline lysis method (Fig. 1). There was no statistically significant difference between 8 × 100-μs pulses and 5-min lysis ($p = 0.512$) or 10-min lysis ($p = 0.849$). However, when a higher number of pulses (24 × 100 μs, 30 kV/cm, 1 Hz) was used, an approximately 2.5-fold higher concentration of extracted pDNA was obtained compared to 10-min alkaline lysis ($p < 0.001$) and an ~2.5-fold higher concentration of pDNA compared to eight pulses ($p = 0.009$). Our results are in agreement with those of Rols and Teissie (1998), who showed that a higher number of pulses enables greater permeabilization of mammalian cell membrane. Since it was shown that a strong electric field intensity is not needed to introduce pDNA into bacterial cells (Xie and Tsong 1992) and that longer pulses with lower field strength are more advantageous (Kubiniec et al. 1988), we also applied one 1-ms pulse with a lower electric field strength (12.5 kV/cm) to electroextract pDNA from *E. coli*. Under this condition (1 × 1 ms, 12.5 kV/cm), we extracted a threefold higher concentration of pDNA (0.893 μg/ml) compared to 10-min alkaline lysis ($p = 0.006$), in contrast to the study of Moser et al. (1995), where electroextraction gave a comparable yield of pDNA to that obtained with alkaline lysis. Our data therefore suggest that electroporation could provide a way to extract pDNA from *E. coli* and that pulsed parameters used for electroporation determine the amount and success of pDNA extraction.

The data in Fig. 2 show the inactivation of *E. coli* as a function of different electroporation conditions in logarithmic scale. The inactivation increased with applied number of pulses, with a constant electric field (30 kV/cm) and pulse duration (100-μs pulse). Namely, when a train of 24 pulses was applied, the inactivation level decreased approximately 2.5 times compared to a train of eight pulses, which is in agreement with previously published results (Gusbeth et al. 2009; Zgalin et al. 2012). When only one 1-ms pulse with a lower electric field (1 × 1 ms, 12.5 kV/cm) was applied, the inactivation level of *E. coli* was approximately threefold lower compared to the eight electric pulses applied (8 × 100 μs, 30 kV/cm, 1 Hz) ($p = 0.008$) and approximately 7.5-fold lower compared to the second protocol used (24 × 100 μs, 30 kV/cm, 1 Hz) ($p = 0.019$). However, at these conditions (1 × 1 ms, 12.5 kV/cm) we obtained the highest pDNA extraction (see Fig. 1).

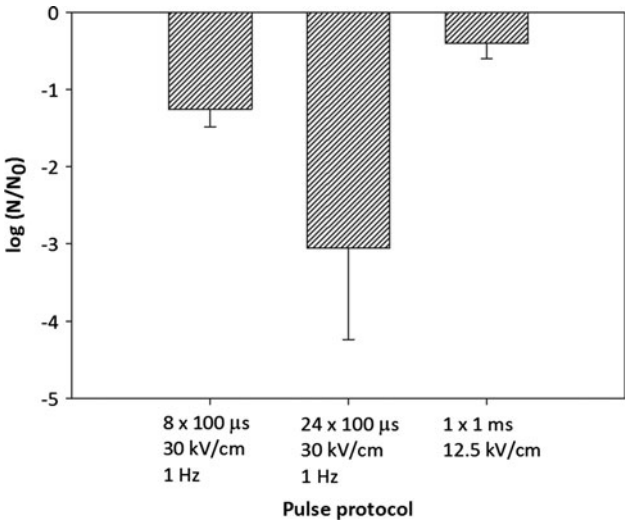

Fig. 2 Inactivation of *E. coli* after three different electric pulse protocols. Pulses were applied at room temperature (22 °C). Values represent means ± standard deviation

The induced electric field leads to an increase in the membrane potential difference (Pucihar et al. 2009). After a critical value is reached, membrane permeabilization occurs, where pulse duration and number of pulses control the size and the number of induced membrane pores, through which transport of molecules occurs (Pucihar et al. 2011). Pulse duration represents a particularly critical parameter for membrane permeabilization (Rols and Teissie 1998). For bacterial cells, it has also been shown with kinetic studies that the most effective permeabilization of the bacterial membrane occurs, when a bacterial cell is parallel to the field and the pulse duration is longer (in the millisecond range) (Eynard et al. 1998). Furthermore, pulses in the millisecond time range are permeabilizing and electrophoretic and, therefore, can drag larger and charged molecules (such as DNA) through the permeabilized membrane (Sukharev et al. 1992; Wolf et al. 1994). In addition, longer pulses with low electric field strength (such as our 1-ms pulse with 12.5 kV/cm) preserve cell viability, as was already shown for mammalian cells (Rols and Teissie 1998). Therefore, we stipulate that electrophoretic force dragged pDNA from bacterial cells without killing the bacteria.

gDNA is an undesirable molecule in pDNA extract. With the alkaline lysis method in the first step (treating bacteria with NaOH/SDS) viscous liquid is formed with degraded gDNA and other compounds. Subsequent treatment with neutralizing solution generates viscoelastic gDNA fragments, which are difficult to remove from the lysate with a simple filtration procedure. Thus, additional high-speed centrifugation is needed, which could due to the shear force on the precipitated gDNA represent a contamination risk of gDNA fragments in the sample

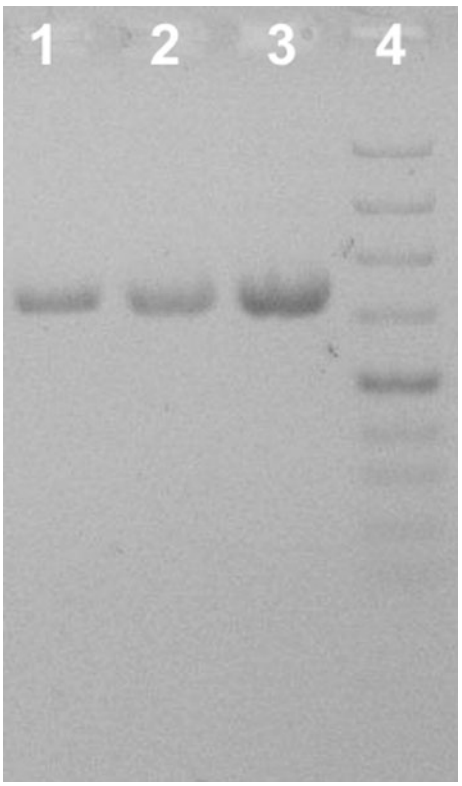

Fig. 3 Syber green–stained agarose gel depicts pDNA electroextracted from *E. coli*. No gDNA was observed in samples. *Lane 1* pDNA extracted with pulses 8 × 100 µs, 30 kV/cm, 1 Hz; *lane 2* pDNA extracted with pulses 24 × 100 µs, 30 kV/cm, 1 Hz; *lane 3* pDNA extracted with pulses 1 × 1 ms, 12.5 kV/cm; *lane 4* ZipRuler^{TM} Express, DNA Ladder 2

(Tschapalda et al. 2009). The proposed electroextraction method could therefore be beneficial since we show that most bacterial cells survive (since bacterial cell survives only if gDNA remains inside the bacteria) when treated with 1-ms pulse with $E = 12.5$ kV/cm. At the same time at these conditions the highest pDNA extraction was obtained. Also, no gDNA was observed on syber green–stained agarose gel (Fig. 3).

We performed transfection of naive *E. coli* cells with electroextracted pDNA in order to show that pDNA is functional. We quantified numbers achieving antibiotic resistance (Table 1; Fig. 4). Figure 4 shows representative agar plates of naive *E. coli* transformed with electroextracted pDNA with three different pulse parameters: (1) 8 × 100 µs, 30 kV/cm, 1 Hz; (2) 24 × 100 µs, 30 kV/cm, 1 Hz; and (3) 1 × 1 ms, 12.5 kV/cm. In Table 1 transformation efficiency is presented. The highest transformation efficiency was obtained with pDNA electroextracted with one rectangular pulse with a duration of 1 ms and an applied voltage of 1,250 V, resulting in $E = 12.5$ kV/cm. At those conditions also the highest pDNA concentration was obtained. We need to note that pDNA transfection was also done with electroporation in all three cases with a

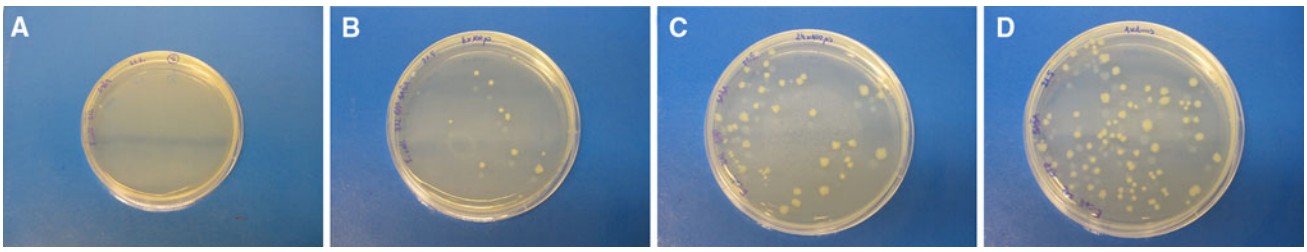

Fig. 4 Representative agar plates of naive *E. coli* transformed with electroextracted pDNA. For transformation a 1-ms pulse with $E = 12.5$ kV/cm was used: **a** control (i.e., pDNA was added, but no electric pulses were applied); **b** added pDNA was electroextracted with 8 × 100 µs, 30 kV/cm, 1 Hz; **c** added pDNA was electroextracted with 24 × 100 µs, 30 kV/cm, 1 Hz; **d** added pDNA was electroextracted with 1 × 1 ms, 12.5 kV/cm

Table 1 Electrotransformation of naive *E. coli* ER1821 with plasmid DNA electroextracted with different pulses: for transformation a 1-ms pulse with $E = 12.5$ kV/cm was used

Pulse parameters used for electroextraction of plasmid DNA	Transformation efficiency: no. of transformants per microgram of plasmid DNA
8 × 100 µs, 30 kV/cm, 1 Hz	$6.65 \times 10^3 \pm 1.68 \times 10^3$
24 × 100 µs, 30 kV/cm, 1 Hz	$1.14 \times 10^4 \pm 3.71 \times 10^3$
1 × 1 ms, 12.5 kV/cm	$2.72 \times 10^4 \pm 2.06 \times 10^3$

single 1-ms pulse of 2,500 V, i.e., 12.5 kV/cm (as described in "Materials and Methods"). Our results confirm that electroextracted pDNA is functional.

In summary we describe a relatively efficient and facile procedure for the extraction of pDNA from *E. coli*—electroextraction based on electroporation of the cell membrane. The main advantages of electroextraction compared to alkaline lysis are higher pDNA extraction, shorter process time (electroextraction, in the microsecond range; alkaline lysis, in the minute range) and reduced lysate volume. Electroextraction with pulse protocol optimization could also be used for extracting a variety of other macromolecules from microorganisms, where extracting methods have been cumbersome to date.

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