Effect of DNA Contaminants on Calcium Phosphate-Based DNA Delivery and Gene Expression

Mohamed A. El-Mogy* and Yousef Haj-Ahmad

Department of Biological Sciences, Brock University, St. Catharines, ON, Canada.

Plasmid DNA is widely used to deliver genes into mammalian cells for the construction of new cell lines, gene therapy and gene expression studies. During DNA preparation, various contaminants can be introduced and reduce its delivery efficiency and create mutations that decrease the expression level of the delivered genes. We evaluated the effect of different plasmid DNA contaminants on calcium phosphate-based transfection efficiency as well as gene expression in Chinese hamster ovary cells. pCMVβ was transfected into the cells after spiking with five different contaminants conditions: ethanol, endotoxin, cesium chloride, ethidium bromide and the combination of the latter two contaminants. Transfection efficiencies were determined through the counting of the Lac-Z-expressing cells as well as quantitative PCR. The reversibility of the contaminant interaction with DNA was examined through cleaning of the spiked DNA followed by transfection. As concluded from our results, qPCR offers accurate measurement of transfection efficiency than the conventional Lac-Z activity assay. In general, all of the examined contaminants, with the exception of ethanol, have a significant negative effect on gene expression. While this effect is resulted from the reduced delivery in most of the used contaminants, ethidium bromide showed no significant decrease in delivery, indicating alternative mechanism for its negative effect on gene expression.

*Corresponding author: Mohamed El-Mogy, Department of Biological Sciences, Brock University, St. Catharines, ON, L2S 3A1, Canada. Phone: +1-905-360-0440. Fax: +1-905-688-1855. E-mail: melmogy@hotmail.com

Financial support: This work was supported by the Egyptian Government and the Egyptian Cultural & Educational Bureau in Canada.

Introduction

Contamination is a major problem that is encountered when using different methods of DNA preparation. The purity of a DNA molecule is important for its biological function as well as its stability. Since DNA preparation involves many different steps, the risk of having DNA contamination is high. Moreover, human therapeutic protein produced from any expression system should meet strict requirements of contaminant elimination [1]. Several contaminants are being introduced into plasmid DNA during different preparation methods. These contaminants include ethidium bromide (EtBr), cesium chloride (CsCl), endotoxin, and ethanol (EtOH). They are introduced into DNA preparations in different amounts depending on the method used and the handling techniques. Furthermore, EtBr and CsCl are mixed together with DNA during the CsCl gradient preparation.

Ethidium bromide intercalates preferably around DNA molecules through a non-covalent reversible manner [2-4]. EtBr binds to DNA with an unknown effect of salts, divalent cations or DNA-interacting proteins on this interaction [5]. Cesium chloride is the most commonly used application of CsCl in molecular biology is in density gradient ultracentrifugation for DNA purification (isopycnic centrifugation) and using EtBr as a dye to visualize the DNA band [6, 7].

An endotoxin is a lipopolysaccharide produced by gram-negative bacteria such as *Escherichia coli* (*E. coli*) and is located in the cell wall [8]. Endotoxins are toxic to humans and therefore,

the use of *E. coli* in protein production, especially therapeutic proteins, requires additional steps for endotoxin removal [9, 10, 11]. A high concentration of 70% EtOH is used to precipitate plasmid DNA during alkaline lysis procedures, which can contaminate the final DNA preparation. In guinea pig gastric mucosal cells, 7.5% EtOH caused apoptotic DNA fragmentation after treatment for 8 hours [12]. In contrast, EtOH was found to increase DNA uptake by primary cortical neurons using a lipid-based transfection method [13]. However, the effect of ethanol on calcium phosphate transfection of mammalian cells has not yet been studied.

Graham and van der Eb introduced the calcium phosphate transfection early in 1973 [14] and since that time, the method has been used in the transfection of various cell lines [15, 16, 17], either adherent or in suspension [18, 19, 20, 21, 22]. It has been used as a transfection tool in the expression of different recombinant proteins in mammalian cells including human anti-Rhesus-D immunoglobulin G (IgG) [23] and human IgG [24] as well as plasmids delivery for the mammalian imaging real-time gene expression system [25].

To determine the effect of these contaminants on transfection efficiency and gene expression in mammalian cells, we designed a set of experiments using endotoxin-free pCMVβ as a model plasmid. Experiments were carried out in *Chinese hamster* ovary (CHO) cells through calcium phosphate transfection of DNA, after spiking with five different conditions of varying concentrations of contaminants.

Materials and methods

Endotoxin-free plasmid DNA preparation

Endotoxin-free plasmid DNA preparation was carried out using the Endotoxin-free DNA MaxiPrep Kit (Norgen Biotek Corp.), according to the manufacturer's instructions.

Cell line and maintenance

The used *Chinese hamster* ovary (CHO) cells subclone K1 (ATCC CCL-61) derived from the parental cell line [26]. Cells were maintained as a monolayer in Petri cell culture dishes and cultured in advanced Dulbecco's Modified Eagle Medium (Advanced D-MEM: Invitrogen Corp., Gibco), containing 5% (v/v) fetal bovine serum (FBS, PAA Laboratories Inc.), 1% (v/v) penicillin/streptomycin (Invitrogen Corp., Gibco) and 1% (v/v) glutamine (Invitrogen Corp., Gibco). Growing cells were incubated in a water-jacketed incubator (Fisher Scientific, Pittsburgh, PA) at 37°C with 96% relative humidity and 5% CO_2.

At 90% confluencey, cells were passaged by using EDTA-trypsin solution (0.53 mM tetrasodium EDTA, 0.05% (v/v) Trypsin). The used volume is determined by the size of the cell culture plate. For a 150 mm plate, a 4 mL volume was used. After aspirating the media, cells were washed with 6 mL phosphate buffered saline (PBS), pH 7.4. Upon removal of the wash by aspiration, EDTA-trypsin was added to the monolayer for two minutes, and then cells were lifted by gentle tapping of the side of the dish. Six millilitres of the culture medium was then added to the cell suspension with pipetting to split the cells and 4 mL of the cell suspension was added to a new culture plate. The volume was completed to 20 mL with new culture medium and the cells were incubated at 37°C.

Endotoxin preparation and quantification

A freshly prepared *E. coli* culture, grown overnight, was centrifuged at 3000 x g in a Beckman GPR centrifuge for 30 minutes. The supernatant was then filtered through a sterile Acrodisc® 25mm syringe filter with 0.2 µm HT Tuffryn® membrane (PALL, Life Sciences) to eliminate any bacteria left from the centrifugation step and the collected supernatant was used as a source of endotoxin. The level of endotoxin in the supernatant was quantified by measuring the endotoxin units (EU) per mL using the Limulus Amebocyte

Lysate (LAL) Endochrome Kit (Charles River Laboratories), according to the manufacturer's instructions.

Spiking conditions

pCMVβ (Clontech, GenBank Accession No. U02451) was spiked with the following five contaminant conditions: EtBr, CsCl, a combination of EtBr/CsCl (different concentrations for each), endotoxin and EtOH. The range of EtBr and CsCl spiking concentrations were determined based on the start concentrations used in plasmid DNA purification protocols by CsCl/EtBr gradient [27] with more than 99.90% and 99.99% residual contaminant removal of EtBr and CsCl, respectively. Endotoxin was used to spike DNA to concentrations within 10 to 1000 fold from the basic endotoxin levels of the prepared endotoxin-free plasmid. The used EtOH concentrations were based on the start concentration used in EtOH precipitation of DNA [27] with more than 98.5% residual EtOH removal. The concentrations used from each contaminant are shown in Table 1.

After spiking, DNA was incubated for 10 minutes at room temperature (23°C) with the subsequent transfection into CHO cells using calcium phosphate in 6-well plates. Samples were done in two groups as one was used for staining and the second for DNA isolation. In each group, triplicate wells were used for each concentration. Another set of DNA was prepared in 6 groups, each group was spiked with the highest concentration from each contaminant condition mentioned above in addition to the control (no spiking). DNA was incubated for 10 minutes at room temperature with the contaminant and then was cleaned and transfected into CHO cells. Triplicates were used in two groups, one group for staining and the second for DNA isolation.

Calcium phosphate transfection

Calcium phosphate transfection was carried out according to the optimized protocol done by Jordan *et al.* [15]. On the day prior to transfection, cells were split in a 6-well plate and allowed to attach overnight. The amount of cells used was optimized so that the confluencey would be 60-70% at the transfection time. Two hours before transfection, the medium was replaced with 2 mL fresh medium. Transfection solutions were warmed up to room temperature prior to use. The transfection mixture was made by diluting 5 µg plasmid DNA into 90 µL TE buffer (pH 7.6). A 10 µL volume of 2.5 M $CaCl_2$ (filter sterilized) was added to the DNA-TE slowly with swirling followed by gentle vortexing for complete mixing of components. Next, 2X HEPES (140 mM NaCl, 1.5 mM Na_2HPO_4, 50 mM HEPES, pH 7.05, filter sterilized) was placed in a 1.5 mL Eppendorf tube and vigorously shaken on a vortex while adding the DNA-TE-$CaCl_2$ slowly and dropwise. This mixture was let stand for 30 seconds and then mixed well by pipetting to split the aggregates. The solution was added evenly and dropwise on the medium surface. Cells were then incubated at 37°C and the medium changed 6 hours post-transfection to reduce cytotoxicity. The transfection efficiency was tested 21 hours post-transfection.

LacZ activity assay

Positive LacZ transfected cells were stained and visualized under a light microscope. The medium was aspirated 21 hours post-transfection from the 6-well plate followed by 2 washes with 1 mL PBS. A 1 mL volume of 4% paraformaldehyde (pH 7.2) was added to each plate well and incubated at 37°C for 2 minutes. The cells were then washed twice with 1 mL PBS. A 2 mL volume of X-gal stain (35 mM $K_3Fe(CN)_6$, 35 mM $K_4Fe(CN)_6$, 1 mM $MgCl_2$, in PBS; pH 7.4, supplemented with 1 mg/mL of X-gal) was added and the cells were incubated overnight at 37°C with 96% relative humidity. The next day, the number of blue cells was counted to determine the transfection efficiency.

DNA isolation and cleaning

DNA was isolated by using the RNA/DNA/ Protein Purification Kit (Norgen Biotek Corp.).

Table 1. Spiking concentrations of the different contaminants

Contaminant	Unit	Used concentrations			
		1	2	3	4
EtBr	nM	0	2.5	25	250
CsCl	µM	0	0.56	5.6	56
EtBr/CsCl	nM/µM	0	2.2/ 0.51	22/ 5.1	220/51
Endotoxin	EU/µg DNA	0.32	4	40	400
EtOH	% (v/v)	0	0.1	0.5	1

The cleaning of spiked DNA (for studying interaction reversibility) was carried out by using the CleanAll Kit (Norgen Biotek Corp.).

Quantitative polymerase chain reaction

Quantitative polymerase chain reaction (qPCR) was performed on a known concentration of template DNA (4 to 10 ng), using the Bio-Rad iCycler thermal cycler. Two specific primers for pCMVβ were used, designated pCMVβ-F (5'-TCGCTACCAT TACCAGTTGG TC -3') and pCMVβ-R (5'- GAGTTAGCTC ACTCATTAGG CACC -3'), of amplicon size of 677 bp. The reaction mixture contained 10 µL of 2X SYBR GREEN master mix (Bio-Rad), and 1.2 µL of each primer (5 mM stock). The total volume of the reaction was completed with dH_2O to 20 µL. A 15 minutes heating at 95°C was used to activate the hotstart enzyme. Forty amplification cycles were performed as follow: 15 seconds at 95°C, 30 second at 59°C and 1 minute at 72°C. The reaction was kept at 57°C for 1 minute before starting a melting curve analysis by a 0.5°C increment every 10 sec over 80 rounds. A standard curve of known plasmid concentration (10 fg to 1 ng) was used to determine the initial concentration of plasmid in each sample.

Results

LacZ expression

LacZ activity was used to measure the number of cells positively expressing the LacZ gene, which is a part of pCMVβ. Transfected cells that are expressing the gene developed a blue color due to the X-gal staining. Cells with positive expression were counted under a light microscope and the average cell count of the triplicate transfections was calculated. LacZ expressing cells were calculated as a percentage from the control value that was set at 100%. The only deviation from this calculation was the endotoxin concentration, since the amount of endotoxin in the used non-spiked plasmid was 0.32 EU/µg DNA. This concentration was considered 100% in the case of endotoxin spiking. All of the contaminant conditions showed a reduction in the number of cells expressing the gene, but with different trends (Figure 1).

All the results were scrutinised by a one-way ANOVA using Tukey's test at a significance level of 0.05. The used EtBr spiking concentrations were 0, 2.5, 25, and 250 nM. At 0 and 2.5 nM, no statistical significant reduction in number of expressing cells was noticed. However, with the other EtBr concentrations (2.5, 25, and 250 nM), a significant decrease was obtained (63.7±13.8%, 34.9±6.6%, and 18.9±5.6%, respectively) (Figure 1-A). CsCl showed no significant reduction when increasing the concentrations from 0 µM to 0.56 µM (100.0±17.3% and 72.7±12.9%, respectively). On the other hand, significant reduction in the number of expressing cells was found with the higher concentrations of 5.6 µM and 56 µM (52.8±8.8% and 42.3±1.7%, respectively) (Figure 1-B).

Figure 1-C shows the outcome of combining EtBr and CsCl. Another trend of reduction occurs and includes a steady state between two

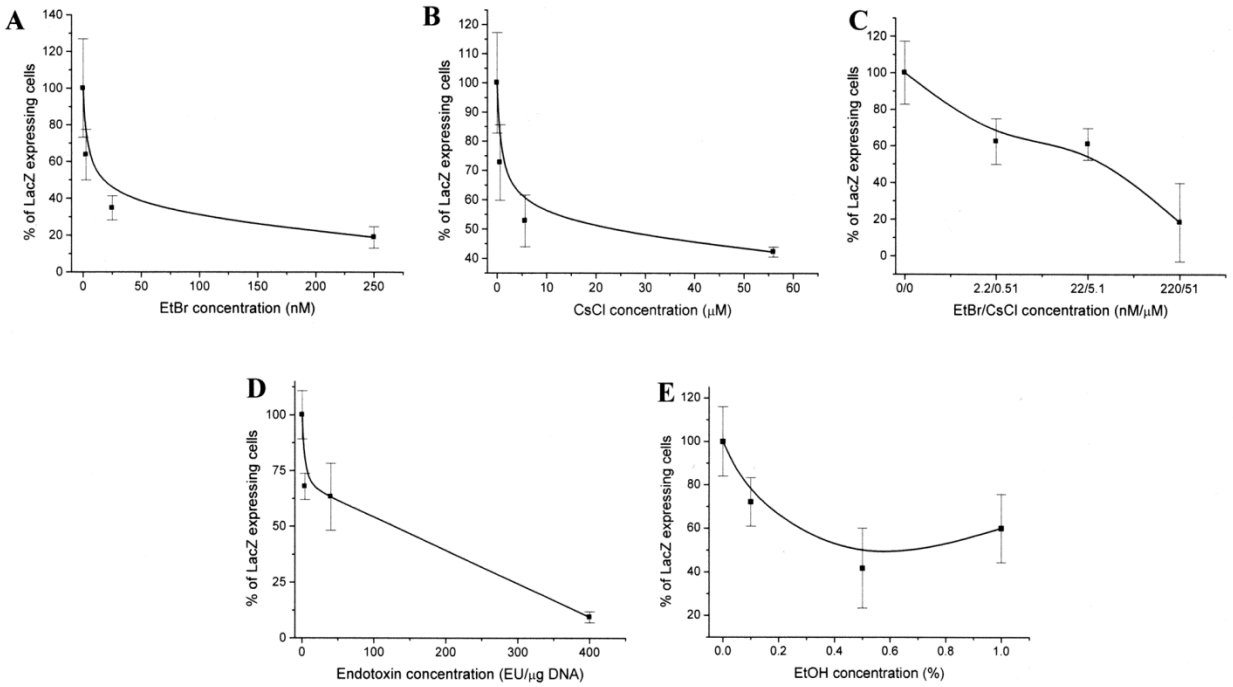

Figure 1. Effect of contaminants on the number of cells expressing LacZ. Plasmid DNA was spiked with the different contaminant conditions and transfected into CHO cells. 21 hours post-transfection, cells were stained by X-gal and the number of cells which developed the blue color were counted. Records were calculated as a percentage from the control value. [A] EtBr (0, 2.5, 25 & 250 nM), [B] CsCl (0, 0.56, 5.6 & 56 μM), [C] EtBr/CsCl (0/0, 2.2/0.51, 22/5.1 & 220/51 nM/μM), [D] Endotoxin (0.32, 4, 40 & 400 EU/μg DNA) and [E] EtOH (0, 0.1, 0.5 & 1%).

concentrations (2.2 nM/0.51 μM and 22 nM/5.1 μM: 62.4±12.5% and 60.9±8.7%, respectively). The difference between these concentrations and the lower concentration (0 nM/0 μM: 100.0±17.3%) was not significant. However the reduction between any of the above three concentrations and the highest one (220 nM/51 μM: 18.4±21.4%) is significant. A similar pattern was observed with endotoxin spiking. Two concentrations (4 and 40 EU/μg DNA) showed similar cell numbers (68.0±5.9% and 63.0±15.0%, respectively) that is significantly lower than the baseline concentration (0.32 EU/μg DNA: 100.0±10.8%) and higher than the highest used concentration (400 EU/μg DNA: 9.2±2.4%) (Figure 1-D). The last used contaminant was EtOH, with percentage spiking (v/v) concentrations of 0, 0.1, 0.5 and 1%. A reduction in LacZ-expressing cells was observed between the 0% (100.0±16.0%) and the last three concentrations. However, no significant difference was seen between the three positive spiking concentrations (0.1, 0.5 and 1%: 72.2±11.2%, 41.8±18.4% and 60.2±15.7%, respectively) (Figure 1-E).

Transfection efficiency

qPCR was performed to determine the exact transfection efficiency by using the isolated DNA on 21 hours post-transfected cells. Duplicate measurements were carried out for each of the triplicate samples for every spiking concentration within the different conditions. Since the amount of DNA per CHO cell is 3.1 pg [28], and 10 ng of DNA was used per reaction, it was possible to calculate the average plasmid copy number per cell in each spiking condition (Figure 2). The results were attested for their statistical significance by a one-way ANOVA using Tukey's test at $P<0.05$.

No significant effects on the amount of delivered plasmids were observed for two of the contaminants used to spike the DNA. First,

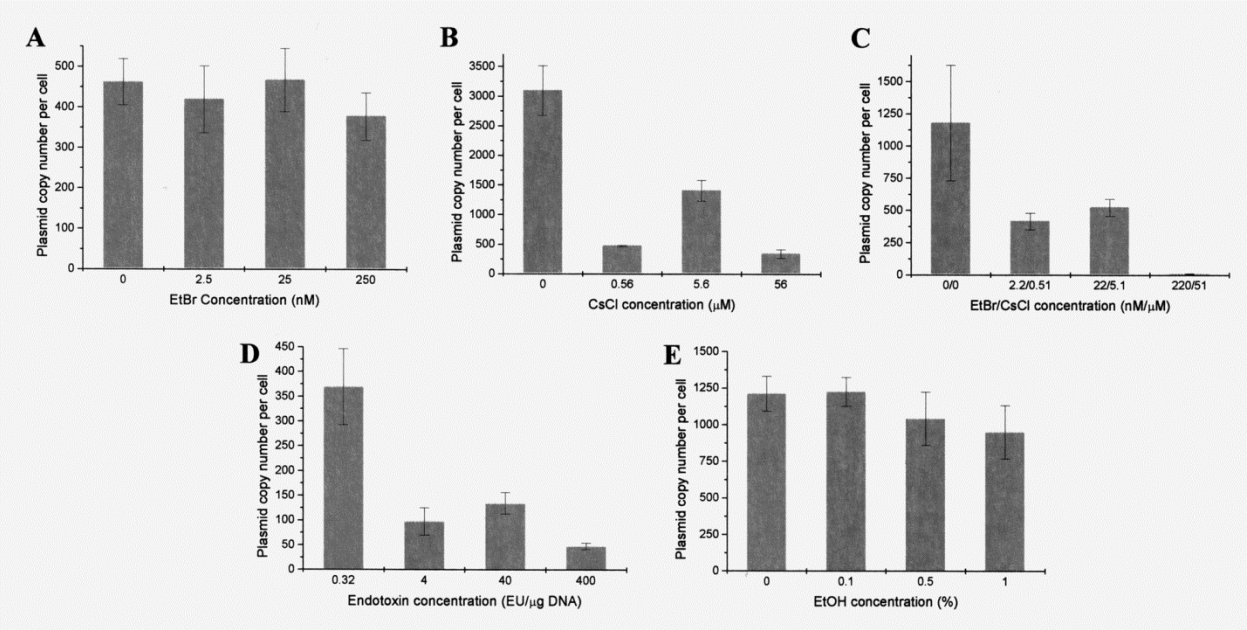

Figure 2. Effect of contaminants on transfection efficiency. Plasmid copy number per cell was measured by qPCR on DNA isolated from CHO cells 21 hours post-transfection with pCMVβ. Plasmid DNA was spiked with the different contaminant conditions before transfection into CHO cells. [A] EtBr (0, 2.5, 25 & 250 nM), [B] CsCl (0, 0.56, 5.6 & 56 μM), [C] EtBr/CsCl (0/0, 2.2/0.51, 22/5.1 & 220/51 nM/μM), [D] Endotoxin (0.32, 4, 40 & 400 EU/μg DNA) and [E] EtOH (0, 0.1, 0.5 & 1%).

spiking with increasing EtBr concentrations (0, 2.5, 25, and 250 nM) did not affect delivery of the plasmid. This can be inferred from statistically similar plasmid copy number per cell of the control sample (462.3±56.9) and the spiked samples (419.3±82.3, 467.3±77.6 and 377.9±58.4, respectively) (Figure 2-A). The second condition (Figure 2-E) that produced a similar trend was EtOH spiking (at concentrations of 0, 0.1, 0.5 and 1%), where no major change in plasmid copy number per cell was observed for any of the concentrations tested, including the control (1211.3±120.0, 1225.2±98.8, 1042.0±182.0 and 949.4±182.5, respectively).

In the other three spiking conditions (CsCl, EtBr/CsCl and endotoxin), significant differences were observed between the plasmid copy number per cell of the control and the spiked samples. CsCl spiking showed a significant decrease in plasmid copy number per cell between the 0 μM (3094.0±419.3) and all the higher CsCl concentrations (Figure 2-B). The 5.6 μM concentration (1405.3±174.6) shows higher plasmid copy number per cell over the 0.56 and 56 μM concentration. The later two showed no significant difference between them (470.9±11.4 and 342.8±72.8, respectively). When EtBr/CsCl was used, the plasmid copy number of 1178.1±447.8 was obtained with the 0 nM/0 μM concentration. However, the value is significantly higher than all of the spiked concentrations. When comparing plasmid copy number per cell with the other three spiked concentrations (2.2 nM/0.51 μM, 22 nM/5.1 μM and 220 nM/51 μM), no significant changes were obtained between any of them (414.8±64.0, 522.8±65.2 and 7.85±2.4, respectively) (Figure 2-C). Similarly to the previous condition, endotoxin spiking has a significantly higher plasmid copy number per cell when using 0.32 EU/μg DNA (369.2±76.9) over the other three higher spiking conditions (Figure 2-D). Concentrations of 4, 40 and 400 EU/μg DNA showed insignificant differences between their values (97.4±27.8, 134.4±21.9 and 47.7±6.7, respectively).

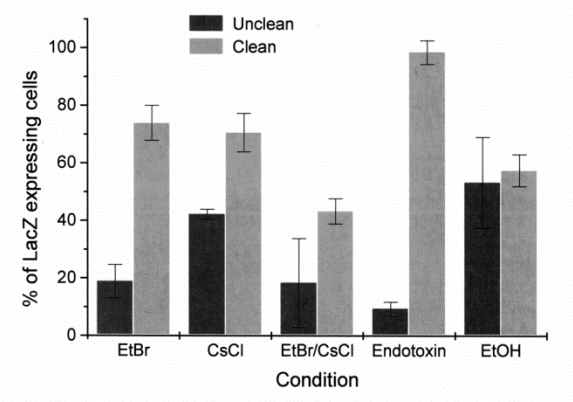

Figure 3. Effect of cleaning on the number of cells expressing LacZ. Plasmid DNA was spiked with the highest concentration of each contaminant and transfected with or without cleaning and then transfected into CHO cells. 21 hours post-transfection, cells were stained by X-gal and the number of cells which developed the blue color was counted. Numbers were calculated as a percentage from the control value. The used contaminant concentrations were: 250 nM (EtBr), 56 µM (CsCl), 220 nM/51 mM (EtBr/CsCl), 400 EU/µg DNA (endotoxin) and 1% (EtOH).

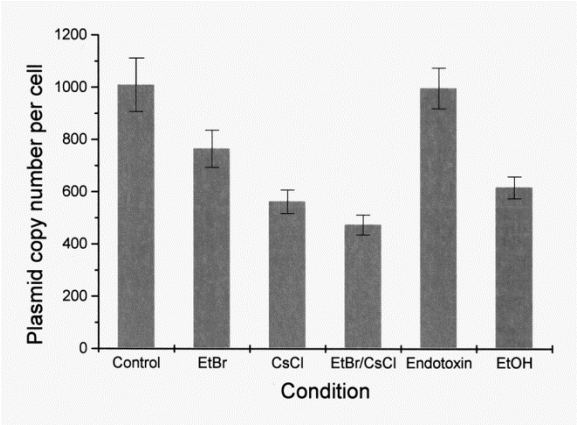

Figure 4. Effect of cleaning on transfection efficiency. Plasmid copy number per cell was measured by qPCR on DNA isolated from CHO cells 21 hours post-transfection with pCMVβ. Plasmid DNA was spiked with the highest concentration from each contaminant and subsequently cleaned and transfected into CHO cells. The used contaminant concentrations are: 250 nM (EtBr), 56 µM (CsCl), 220 nM/51 mM (EtBr/CsCl), 400 EU/µg DNA (endotoxin) and 1% (EtOH). The used control is a non-spiked plasmid that was cleaned.

Spiking reversibility

To study the reversibility of contaminant interaction with DNA, a spiking followed by DNA cleaning and transfection was carried out. The highest concentration of each contaminant was used to spike the same amount of DNA. Following the incubation time at room temperature, spiked DNA as well as the control DNA was cleaned. DNA was then quantified to be consistent with the amount of transfected DNA. 21 hours post-transfection, the LacZ activity assay and DNA isolation were performed (triplicate samples for each).

The light microscope was used to count cells with positive LacZ expression (blue color) and the percentage of cells expressing the gene was calculated from the control value that was set at 100%. Three of the conditions (EtBr, CsCl and endotoxin) had values over 70% (73.9±6.1%, 70.6±6.7% and 98.5±4.1%, respectively). The two other conditions (EtBr/CsCl and EtOH) had a lower value (43.3±4.4% and 57.5±5.5%, respectively) of expressing cells. The effect of cleaning was compared to direct spiking (no cleaning), with respect to the number of cells expressing LacZ (Figure 3). The data was analysed statistically by a Student's t-test at a significance level of 0.05. Significant improvement (at $P<0.05$) in the expression of LacZ was noted in three of the cleaned conditions (EtBr, CsCl and endotoxin). The percentage of cells expressing the LacZ gene increased from 18.9±5.8% to 73.9±6.1% with EtBr and 42.3±1.7% to 70.6±6.7% with CsCl. In the case of the endotoxin contaminant, the improvement was dramatic, increasing from 9.4±2.4% to 98.5±4.1%. Insignificant improvement was obtained with EtBr/CsCl (from 18.4±15.4% to 43.3±4.4%) and EtOH (53.3±15.7% to 57.5±5.5%).

qPCR on the isolated DNA showed almost the same plasmid copy number per cell for both the control and the endotoxin (1009.4±102.5 and 996.6±77.6, respectively). Lower numbers were obtained with the other conditions; EtBr (764.7±70.8), CsCl (562.5±44.8), EtBr/CsCl (474.0±37.8) and EtOH (616.6±41.6) (Figure 4). In order to provide a better understanding of the success of the cleaning step, the comparison between the percentage of transfection efficiency of cleaned and uncleaned conditions, relative to their controls, is examined (Figure 5). Statistical analysis was

performed by a Student's t-test at a significance level of 0.05. No significant change in transfection efficiency was obtained in both EtBr and EtOH. On the other hand, considerable improvement in transfection efficiency of the cleaned CsCl, EtBr/CsCl and endotoxin was achieved. Transfection efficiency improved from the uncleaned to the cleaned of these three conditions by 5, 67 and 7.7 folds, respectively.

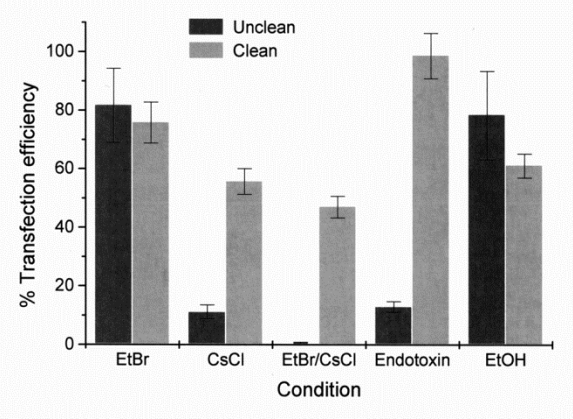

Figure 5. Transfection efficiencies of uncleaned and cleaned DNA. Transfection efficiency was measured by qPCR on the DNA isolated from CHO cells 21 hours post-transfection with pCMVβ and normalized for both uncleaned and cleaned conditions using independent controls. Plasmid DNA was spiked with the highest concentration of each contaminant and transfected with or without cleaning into CHO cells. The used contaminant concentrations were: 250 nM (EtBr), 56 µM (CsCl), 220 nM/51 mM (EtBr/CsCl), 400 EU/µg DNA (endotoxin) and 1% (EtOH).

Discussion

The LacZ activity assay is widely used to determine the transfection efficiency of most cell culture transfection methods. However, this assay is only an indicator, rather than a direct measurement of transfection efficiency. To expand, LacZ activity is a measure of transgene (LacZ) expression efficiency, rather than the efficiency of transgene uptake by the cells. As such, the accuracy of this assay is subject to mutations within the coding sequence of the LacZ gene that may disrupt its expression or function but does not affect transgene uptake negatively. Although most of the used contaminants have significant negative effect of the number of cells expressing Lac-Z, this can be accounted for either the reduced transfection efficiency or gene expression.

The use of qPCR allowed for a precise determination of the plasmid copy number per cell. This is an accurate measurement of transfection efficiency, as qPCR measures the physical presence of the DNA molecule and not the expression of the genes encoded in the plasmid. The stability of the DNA molecule is also directly measured by this method, in contrast to the LacZ assay which measures an enzyme that is a downstream product of the transfected DNA, which differs in stability relative to the transfected DNA itself.

We used the calcium phosphate transfection method which performs better than some cationic polymer such as PEI in some applications. Chenuet *et al.* [29] studied recombinant CHO DG44 cell lines construction and compared between PEI transfection and calcium phosphate transfection. The specific protein productivity was higher with the latter transfection reagent. Calcium phosphate transfection relies on the co-precipitates of plasmid DNA (pDNA) with calcium phosphate. Jordan *et al.* [15] tested the effect of phosphate and calcium concentrations on DNA transfections and showed the importance of these parameters on this process. The high salt concentration, which results from spiking with CsCl, may be the main reason for the decreased transfection efficiency.

Cesium chloride reduced the transfection efficiency by interfering with the interaction of plasmid DNA (pDNA) and calcium phosphate. Cesium is very reactive and is the highest electropositive metal, even more positive than sodium or potassium [30]. The presence of cesium in the transfection reaction may lead to a side reaction that reduces the amount of precipitated DNA or disrupts the optimal transfection pH. CsCl may also reduce DNA transfection efficiency by directly interacting with the DNA and thus protecting it against the mutagenic effect of EtBr when a combination of

EtBr and CsCl was used for spiking DNA. CsCl shielded the DNA molecule from interacting with the mutagen EtBr.

The similar molecular weight as well as charge makes it difficult to separate the endotoxin from pDNA [31]. Therefore, we could not have a completely endotoxin-free DNA, as our control contained 0.32 EU/µg DNA. It is generally accepted that transfection efficiency is negatively affected by the presence of an endotoxin in the transfection mixture [27]. This was confirmed when we spiked the DNA with different concentrations of endotoxin. We cannot refer to any cellular effect that might directly cause such a reduction since mammalian cells are not sensitive to endotoxin [32]. Endotoxin interaction with DNA and/or the cell membrane may reduce transfection. The presence of endotoxin in the transfection mixture may decrease DNA precipitation which in turn will decrease transfection efficiency. Also, the lipopolysaccharide nature of an endotoxin may increase the cellular resistance to transfection by interacting with the cellular membrane.

In general, the interactions of the contaminants used in this study with pDNA are reversible, as demonstrated by the DNA cleaning procedures that restored transfection efficiencies and transgene expression rates that were comparable to that of non-spiked controls. Cleaning of pDNA spiked with CsCl, EtBr/CsCl and endotoxin improved transfection efficiency (increased copy number), verifying that these contaminants acted to lower the efficiency of transgene delivery to the cells. The cleaning of EtBr spiked samples did not have an effect on the efficiency of transfection, nevertheless, an improved transgene (LacZ) expression was observed. This indicated that EtBr affects the DNA structure and function inside the cell and does not interfere with the efficiency of transgene delivery.

In conclusion, at the used very low residual CsCl, EtBr, and endotoxin, there is still an interference with gene expression which reflects the need to assess the concentrations of these contaminants in DNA preparations before carrying on with the different applications that requires delivery and/or expression. All of the investigated contaminants, except EtOH, had reversible, negative effects on gene expression. CsCl and endotoxins affected the efficiency of DNA transfection by calcium phosphate whereas EtBr does not affect the transfection efficiency which indicates a different mode of action. Most likely, EtBr exerts its negative effect on gene expression through mutagenesis. More studies are needed to understand the nature and the mechanisms of the interactions between CsCl and the transfection process or endotoxin with the DNA and the cell membrane.

Acknowledgments

The authors would like to thank the Egyptian Government and the Egyptian Cultural & Educational Bureau in Canada for their financial support of this project. The authors would also like to thank the staff of Norgen Biotek for their technical support.

Reference

1. Rozkov A, Larsson B, Gillstrom S, Bjornestedt R, Schmidt SR. 2008. Large-scale production of endotoxin-free plasmids for transient expression in mammalian cell culture. Biotechnology and Bioengineering. 99:557-566.
2. Reinhardt CG, Krugh TR. 1978. A comparative study of ethidium bromide complexes with dinucleotides and DNA: direct evidence for intercalation and nucleic acid sequence preferences. Biochemistry. 17:4845-4854.
3. Garland F, Graves DE, Yielding LW, Cheung HC. 1980. Comparative studies of the binding of ethidium bromide and its photoreactive analogues to nucleic acids by fluorescence and rapid kinetics. Biochemistry. 19:3221-3226.
4. Graves DE, Watkins CL, Yielding LW. 1981. Ethidium bromide and its photoreactive analogues: spectroscopic analysis of deoxyribonucleic acid binding properties. Biochemistry. 20:1887-1892.
5. Hardwick JM, von Sprecken RS, Yielding KL, Yielding LW. 1984. Ethidium binding sites on plasmid DNA determined by photoaffinity labelling. J. Biol. Chem. 259:11090-11097.

6. Radloff R, Bauer W, Vinograd J. 1967. A dye- buoyant density method for the detection and isolation of closed circular duplex DNA: the closed circular DNA in HeLa cells. Proc. Natl. Acad. Sci. USA. 5:1514-1521
7. Garrett RH, Grisham, CM. 2009. *Biochemistry*. 4th ed. Books/Cole, Cengage Learning, Boston, MA.
8. Manthous CA, Hall JB, Samsel, RW. 1993. Endotoxin in human disease. Part 2: Biologic effects and clinical evaluations of anti-endotoxin therapies. Chest. 104:1872-1881.
9. Raetz C. Bacterial lipopolysaccharides: A remarkable family of bioactive macroamphiphiles. In Escherichia coli and Salmonella cellular and molecular biology. 2nd edition. Edited by Neidhart FC, Ingraham JL, Low KB, Magasanik B, Schaechter M, Umberger HE. Washington D.C.: American Society for Microbiology; 1996:1035-1063.
10. Petsch D, Anspach FB. 2000. Endotoxin removal from protein solutions. J. Biotechnol. 76:97-119.
11. Chen RH, Huang CJ, Newton BS, Ritter G, Old LJ, Batt CA. (2009). Factors affecting endotoxin removal from recombinant therapeutic proteins by anion exchange chromatography. Protein Expr Purif. 64(1):76-81.
12. Mizushima T, Tsutsumi S, Rokutan K, Tsuchiya T. 1999. Suppression of ethanol-induced apoptotic DNA fragmentation by geranylgeranylacetone in cultured guinea pig gastric mucosal cells. Digestive Diseases and Sciences. 44:510-514.
13. Anji A, Shaik KA, Kumari M. 2003. Effect of *ethanol* on lipid-mediated transfection of primary cortical neurons. Annals of the New York Academy of Sciences. 993:95-102.
14. Graham FL, van der Eb AJ. 1973. A new technique for the assay of infectivity of human adenovirus 5 DNA. Virology. 52:456-467.
15. Jordan M, Schallhorn A, Wurm FW. 1996. Transfecting mammalian cells: Optimization of critical parameters affecting calcium-phosphate precipitate formation. Nucleic Acids Res. 24:596-601.
16. Urabe M, Kume A, Tobita K, Ozawa K. 2000. DNA/calcium phosphate precipitates mixed with medium are stable and maintain high transfection efficiency. Analytical Biochemistry. 278: 91-92.
17. Chowdhury EH, Kunou M, Nagaoka M, Kundu AK, Hoshiba T, Akaike T. 2004. High-efficiency gene delivery for expression in mammalian cells by nanoprecipitates of Ca-Mg phosphate. Gene. 341: 77-82.
18. Seelos C. 1997. A critical parameter determining the aging of DNAcalcium- phosphate precipitates. Anal Biochem. 245: 109-111.
19. Wilson SP, Smith LA. 1997. Addition of glycerol during DNA exposure enhances calcium phosphate transfection. Anal. Biochem. 246: 148-150.
20. Jordan M, Köhne C, Wurm FM. 1998. Calcium-phosphate mediated DNA transfer into HEK-293 cells in suspension: control of physicochemical parameters allows transfection in stirred media: Transfection and protein expression in mammalian cells. Cytotechnology. 26: 39-47.
21. Meissner P, Girard P, Kulangara A, Tsao MC, Jordan M, Wurm FM. Process development for transient gene expression in mammalian cells at the 3 l scale: 10-50 mg of r-protein in days. In Animal Cell Technology: Products from Cells, Cells as Products. Edited by Bernard A, Griffiths B, Noé W, Wurm F. Kluwer Academic Publishers, Dordrecht, 1999: 351-357.
22. Jordan M. Wurm F. 2004. Transfection of adherent and suspended cells by calcium phosphate. Methods. 33: 136-143.
23. Miescher S, Zahn-Zabal M, De Jesus M, Moudry R, Fisch I, Vogel M, Kobr M, Imboden MA, Kragten E, Bichler J, Mermod N, Stadler BM, Amstutz H, Wurm F. 2000. CHO expression of a novel human recombinant IgG1 anti-Rhd antibody isolated by phage display. Br. J. Haematol. 111: 157-166.
24. Girard P, Derouazi M, Baumgartner G, Bourgeois M, Jordan M, Jacko B, Wurm FM. 2002. 100 Liter-Transient Transfection. Cytotechnology. 38: 15-21.
25. Wells AL, Condeelis JS, Singer RH, Zenklusen D. 2007. Imaging real-time gene expression in Mammalian cells with single-transcript resolution. CSH Protoc. doi: 10.1101/pdb.prot4869.
26. Puck TT, Cieciura SJ, Robinson A. 1958. Genetics of somatic mammalian cells: iii. Long-term cultivation of euploid cells from human and animal subjects. J. Exp. Med. 108:945-956.
27. Sambrook J, Russell D. Molecular Cloning: A Laboratory Manual. 3rd edition. New York: Cold Spring Harbor; Cold Spring Harbor Laboratory Press; 2001.
28. Gregory TR. 2005. Citing online sources: Animal Genome Size Database. www.genomesize.com/result_species.php?id=4570
29. Chenuet S, Martinet D, Besuchet-Schmutz N, Wicht M, Jaccard N, Bon AC, Derouazi M, Hacker DL, Beckmann JS, Wurm FM. 2008. Calcium phosphate transfection generates mammalian recombinant cell lines with higher specific productivity than polyfection. Biotechnol. Bioeng. 101:937-945.
30. Patnaik P. Handbook of inorganic chemicals. New York: The McGraw-Hill Companies, Inc.; 2003.
31. Anspach FB. 2001. Endotoxin removal by affinity sorbents. J. Biochem. Biophys Methods. 49(1-3): 665 -681.
32. Wright JL, Jordan M, Wurm FM. 2003. Transfection of partially purified plasmid DNA for high-level transient protein expression in HEK293-EBNA cells. J. Biotechnol. 102:211-221.