POSTER

Effect of DNA Contaminants on Transfection Efficiency and Mutation Rate in CHO Cells

M. El-Mogy[1], Y. Haj-Ahmad[1,2]. [1]Brock University, St. Catharines, ON, CANADA. [2]Norgen Biotek Corp., Thorold, ON, CANADA

Abstract

Plasmid DNA is widely used to deliver genes into mammalian cells for the construction of new cell lines, gene therapy and gene expression studies. There are different methods to isolate and purify plasmid DNA, which include: cesium chloride banding, ethanol precipitation and the use of commercial kits. Various contaminants are introduced together with the isolated DNA and cause the reduction of the efficiency of delivery and create mutations that decrease the expression level of the delivered genes. We evaluated the effect of different plasmid DNA contaminants on transfection efficiency in Chinese Hamster Ovary (CHO) cells. pCMVβ was prepared using an endotoxin-free commercial kit. This plasmid was transfected into CHO cells after spiking with different contaminants. Five conditions of varying concentrations were carried out, with the clean plasmid being used as a control. These conditions are: ethanol, endotoxin, cesium chloride, ethidium bromide and the latter two contaminants together in different ratios. The transfection efficiency as well as mutation rate were determined through X-Gal staining and counting of the transfected cells and quantitative PCR (qPCR). The reversibility of the interaction of the contaminants with DNA was examined through cleaning of the spiked DNA followed by transfection into CHO cells. In general, most of the examined contaminants appear to have a negative effect on the transfection efficiency. Furthermore, these contaminants cause a significant increase in transfected plasmid mutations.

Introduction

- Plasmid DNA is widely used to deliver genes into mammalian cells for the construction of new cell lines, for gene therapy and for gene expression studies. Various contaminants are introduced into plasmid DNA during different preparation methods. These contaminants include ethidium bromide (EtBr), cesium chloride (CsCl), endotoxin and ethanol (EtOH).
- Ethidium bromide is a DNA intercalating agent that positions itself between the nitrogen bases and fluoresces under UV light (1, 2 and 3). It is used to stain DNA during its preparation with cesium chloride banding. The latter is another DNA contaminant that is used in the same process of DNA preparation.
- A high concentration of 70% EtOH is used to precipitate plasmid DNA during the alakaline lysis procedure, 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 (4).
- Endotoxins are lipopolysaccharides produced by Gram-negative bacteria such as Escherichia coli (E. coli). Endotoxins are toxic to humans. The use of E. coli in protein production, especially therapeutic proteins, requires additional steps for endotoxin removal (5 and 6).
- The aim of this study is to investigate the effect of these contaminants on plasmid DNA transfection efficiency and mutation rate in cultured Chinese hamster ovary (CHO) cells.

Methods

1- The plasmid pCMVβ was prepared using Norgen's Plasmid DNA MaxiPrep Kit (endotoxin-free). This kit was used because it does not include any ethidium bromide or cesium chloride in its steps. Furthermore, the prepared plasmid is endotoxin-free, which is suitable for the endotoxin part of this study.

2- pCMVβ was spiked with the contaminants in five different conditions: ethidium bromide, cesium chloride, ethanol, endotoxin and a combination of ethidium bromide/cesium chloride (different concentrations for each). The DNA was incubated for 10 minutes at room temperature with subsequent transfection into CHO cells using calcium phosphate in a 24-well plate and incubating the plates at 37ºC and 5% CO_2. Two groups were used: one for staining and the second for DNA isolation. In each group, triplicate wells were used for each concentration. The concentrations of each contaminant are shown in Table 1.

3- 21 hours post-transfection, the first group was stained with X-gal. Blue cells were counted 4 hours post-staining to determine the efficiency of transfection.

4- DNA was isolated from the second group, using Norgen's RNA/DNA/Protein Purification Kit.

5- Transfection efficiency was evaluated using qPCR with specific pCMVβ primers.

6- Spiking, cleaning and transfecting CHO cells with the highest contaminant concentrations was used to evaluate the reversibility of the contaminant action. Cleaning was done using Norgen's CleanAll DNA/RNA Clean-up and Concentration Kit after spiking and incubating the DNA at room temperature for 10 min. The control DNA was incubated at room temperature and processed using the CleanAll kit as well, with no contaminants added. Transfection was performed as described in step 2, using triplicates in two groups for staining and DNA isolation. The transfection efficiency was also determined using qPCR.

7- Isolated DNA was digested with HindIII and then used in qPCR reactions to determine the mutation rate inside the HindIII recognition site (AAGCTT). The presence of any mutation within the site will prevent the digestion and subsequently increase the amplification.

Table 1. Spiking Concentrations of the Different Contaminants

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

Results

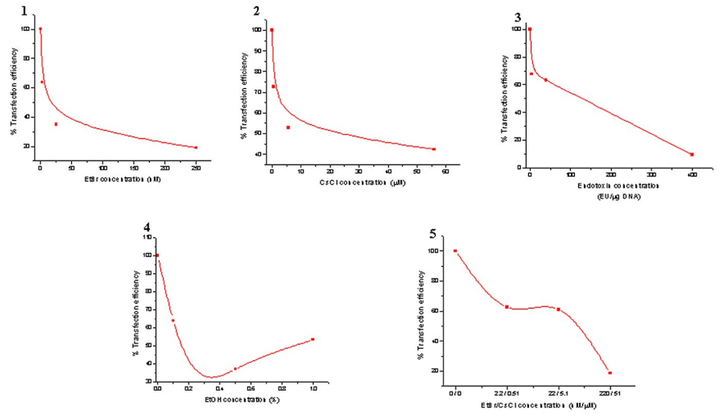

Figure 1. Transfection efficiency with the different contaminants, as determined through 21 hrs post-transfection X-gal staining and counting of transfected cells. [1] EtBr (0, 2.5, 25 & 250 nM), [2] CsCl (0, 0.56, 5.6 & 56 µM), [3] Endotoxin (0.32, 4, 40 & 400 EU/µg DNA), [4] EtOH (0, 0.1, 0.5 & 1%) and [5] EtBr/CsCl (0/0, 2.2/0.51, 22/5.1 & 220/51 nM/µM).

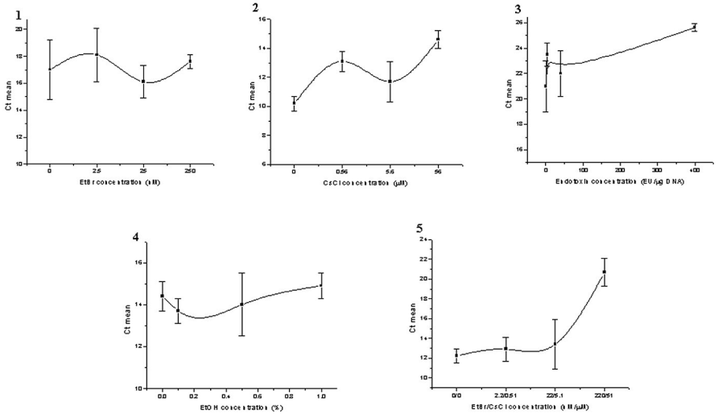

Figure 2: qPCR concentration threshold (Ct) value with the different contaminants. [1] EtBr (0, 2.5, 25 & 250 nM), [2] CsCl (0, 0.56, 5.6 & 56 µM), [3] Endotoxin (0.32, 4, 40 & 400 EU/µg DNA), [4] EtOH (0, 0.1, 0.5 & 1%) and [5] EtBr/CsCl (0/0, 2.2/0.51, 22/5.1 & 220/51 nM/µM).

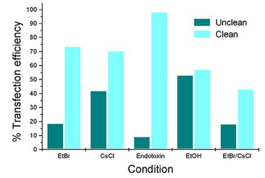

Figure 3: Transfection efficiency with uncleaned and cleaned conditions. The highest concentrations of different contaminants were used. Transfection efficiency was determined through 21 hrs post-transfection X-gal staining and counting of transfected cells. Used concentrations: EtBr (250 nM), CsCl (56 µM), endotoxin (400 EU/µg DNA), EtOH (1%) and EtBr/CsCl (220/51 nM/µM).

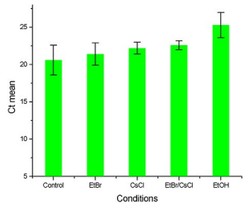

Figure 4: qPCR Ct values with the highest concentrations of different contaminants, after spiking and cleaning with Norgen's CleanAll DNA/RNA Clean-up and Concentration Kit. Concentrations used: EtBr (250 nM), CsCl (56 µM), EtBr/CsCl (220/51 nM/µM) and EtOH (1%).

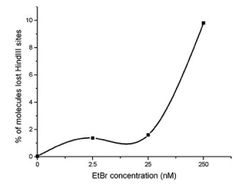

Figure 5. Percentage of pCMVβ molecules that lost HindIII sites, as determined through qPCR of EtBr-spiked DNA (concentrations: 0, 2.5, 25 & 250 nM), after HindIII digestion

Conclusions

- All five contaminant conditions affect the transfection efficiency (Figure 1), however EtBr and EtOH do not have significant effect on the delivery process as determined in Figure 2.
- The interaction between the DNA and all the contaminants is reversible. The contaminants can be cleaned from the DNA, and the degree of reversibility varies with the different contaminants (Figures 3 and 4).
- EtBr shows a significant effect on gene expression by increasing the mutation rate within the transfected DNA (Figure 5).

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Brock University

NORGEN BIOTEK CORPORATION

Contact Information:
3430 Schmon Parkway
Thorold, ON CANADA
L2V 4Y6
Phone: (905) 227-8848
Fax: (905) 227-1061
yousef@brocku.ca