OPTIMIZING THE TRANSFECTION CONDITIONS FOR THE GENERATION OF STABLE TRANSGENIC CELL LINES

A Thesis Submitted to
the Graduate School of Engineering and Sciences of
İzmir Institute of Technology
in Partial Fulfillment of the Requirements for the Degree of

MASTER OF SCIENCE

in Biotechnology

by

Karin KREEKMAN

July 2020

İZMİR

ACKNOWLEDGEMENTS

I would like to thank Assoc. Prof. Engin ÖZÇİVİCİ for the great help, guidance and patience throughout my graduate studies, especially while writing this thesis.

I am forever grateful to Dr. Aziz ÇAYLI for the enormous support and trust that have created a big impact on my life. I truly appreciate the opportunity to conduct the experiments of this study in the laboratories of Florabio. The constructive discussions about the project were also a great help.

I have had a real pleasure of working with all the current and former colleagues at Florabio. My work would never be the same without the pleasant and supportive atmosphere. Special thanks to my dear friends Mine and Neşve.

Last but not least, I am thankful for the emotional support of my partner Fırat who has taken the best possible care of my sleeping schedule and is always ready to listen to my rant about the trivial daily problems.

ABSTRACT

OPTIMIZING THE TRANSFECTION CONDITIONS FOR THE GENERATION OF STABLE TRANSGENIC CELL LINES

Transgenic cell lines that produce biopharmaceutical proteins are widely utilized in the biotech industry and the demand is not predicted to decline in the near future. For obtaining an industrially usable cell line, process components like expression vector, host cell line and transfection method need to be carefully selected. Due to all practical reasons, the industry prefers to use the most conventional Chinese hamster ovary (CHO) cell line. The generation of recombinant cell lines is known to be time-consuming, labor-intensive and expensive. Therefore, the several steps of this process are under constant development. One of the first work packages is transfection, where genes encoding for the therapeutic protein are taken into mammalian host cells.

In this study, we aimed to generate a more cost-effective transfection procedure using the electroporation based technology of nucleofection. This method is favored by the researchers for its high and reproducible transfection efficiency, but also known for the high cost and lack of public information on its components and related consumables. As a result of this study, a novel nucleofection solution was developed for the transfection of CHO-DG44 cells, showing comparable if not better performance over the commercial Lonza's solution in terms of transient and stable expression of recombinant proteins. The transfection was further improved by selecting a more effective nucleofection program and by linearizing the plasmid prior to transfection. These enhancements, optimized on the basis of the biotherapeutic protein production, are potentially advantageous for any research requiring a large number of efficient transfection experiments.

ÖZET

STABİL HÜCRE HATLARININ GELİŞTRİLMESİ İÇİN TRANSFEKSİYON KOŞULLARININ OPTİMİZE EDİLMESİ

Biyofarmasötik proteinler üreten rekombinant hücre hatları biyoteknoloji endüstrisinde yaygın olarak kullanılmaktadır ve bunlara olan talebin yakın gelecekte azalması beklenmemektedir. Endüstriyel olarak kullanılabilir bir hücre hattı oluşturmak için, ekspresyon vektörü, konak hücre hattı ve transfeksiyon yöntemi olarak bileşenlerin dikkatle seçilmesi gerekmektedir. Pratik nedenlerden dolayı, endüstride en geleneksel Çin hamsteri yumurtalık (CHO) hücre hattını kullanmak tercih edilir. Rekombinant hücre hatlarının oluşturulması zaman alıcı, zahmetli ve pahalıdır, bu nedenle daha fazla geliştirilmeye ihtiyaç duyar. Çalışma paketlerinden biri, terapötik proteini kodlayan genlerin konak hücrelere alındığı transfeksiyondur.

Bu projede amaç, nükleofeksiyonun elektroporasyon tabanlı teknolojisinin kullanıldığı, uygun maliyetli bir transfeksiyon prosedürü oluşturmaktır. Araştırmacılar, yüksek ve tekrarlanabilir transfeksiyon verimliliği nedeniyle bu yöntemi tercih ediyorlar, ancak bileşenleri ve ilgili sarf malzemeleri hakkında yüksek maliyet ve kamuya açık bilgi eksikliği sorunu da var. Bu çalışmanın bir sonucu olarak, CHO-DG44 hücrelerinin transfeksiyonu için yeni bir nükleofeksiyon çözeltisi geliştirilmiştir. Bu çözelti, rekombinant proteinlerin geçici ve stabil ekspresyonu açısından ticari Lonza'nın çözeltisi üzerinde daha iyi olmasa bile karşılaştırılabilir bir performans gösterir. Daha etkili bir nükleofeksiyon programı seçerek ve plazmidi transfeksiyondan önce doğrusal hale getirerek transfeksiyonu daha da geliştirdik. Biyoterapötik protein üretimine göre optimize edilen bu geliştirmeler, birçok verimli transfeksiyon deneyi gerektiren herhangi bir araştırma için avantajlı olabilir.

TABLE OF CONTENTS

LIST OF FIGURES

Figure **Page**

LIST OF TABLES

CHAPTER 1

INTRODUCTION

Transfection is a procedure of taking foreign nucleic acid into cells to obtain genetically modified cells and usually the subsequent expression from the inserted sequence [1]. Transfection is most often used to study or modify the function and regulation of any gene or its translation product [2–6], and to produce recombinant proteins [7–9]. In this study, the transfection of CHO-DG44 cells is performed with the aim to obtain stably expressing recombinant cell lines that would produce biopharmaceutical proteins.

1.1. Production of Biopharmaceuticals in Recombinant Cell Lines

Biopharmaceuticals are therapeutic products that are created through genetic manipulation of living cells or organisms, and generally include large molecules like proteins, peptides and nucleic acids [10]. The market of biotherapeutics continues to grow dynamically and is predicted to reach 208 billion USD by the end of 2020 [11]. The largest, most profitable and rapidly developing group of biopharmaceuticals is monoclonal antibodies (mAbs), as they contributed to about 66% of the 163 billion USD sales of biopharmaceuticals (excluding vaccines) in 2016 [10,11].

In addition to novel therapeutics, the forthcoming expiration of patent protection periods for many previously registered important biopharmaceuticals has prompted development studies for the biosimilar drugs [12,13]. Biosimilars, or follow-on biologics, contain an active ingredient that is a very close version of a previously registered biotherapeutical reference product [12]. Thanks to shortened development time, biosimilars bring lower therapy costs with better availability of therapies [11].

The production of mAbs needs to be cost-effective, meet all regulatory demands, yield a product with assured clinical efficacy, sufficient titer and consistent quality

(post-translational modification pattern and correct folding are the main concerns) [14]. These parameters have made the cellular recombinant expression systems to be the most common production system for protein therapeutics [14,15]. Mammalian cells are preferred to other cellular systems thanks to the ability of mammalian cells to produce and secrete large, complex proteins with human-like modifications [16]. The most common mammalian expression systems use non-human cell lines of CHO (Chinese hamster ovary), BHK (baby hamster kidney), NS0 and Sp2/0 (murine myeloma cells), or human cell lines HEK293 (human embryo kidney 293), HT-1080 (fibrosarcoma cells), PER.C6 (retinal cells) [16,17].

In 1986, CHO epithelial cell line was used for the production of the first approved mammalian recombinant biotherapeutic protein, tissue plasminogen activator, t-PA [14,15]. Since then and even 35 years later, CHO remains to be the cell line of choice for 70% of industrially produced biotherapeutics [15,18]. Thanks to the long time period of popularity, CHO is the most well-characterized cell line that is used for the production of biotherapeutics [19,20]. Both, the biotech companies and also regulatory agencies are familiar with the cell line, which results in more predictable processes with shorter timelines [18,20]. A great variety of serum-free culture media [21,22], strategies for gene integration and selection [23], strong expression systems [24] and other important parts of production cell line development have been established for CHO cell line [20,25]. CHO cells are easily adapted to grow in suspension culture and they can process complex, human-like post-translational modifications [16]. Additionally, CHO is noted to be tolerant to changing conditions during the manufacturing process and less susceptible to being contaminated with most of the human viruses [16,18].

The CHO-DG44, the cell line that was used in this study, is a derivative of CHO cells with both alleles of dihydrofolate reductase (DHFR) gene deleted [26]. The missing *DHFR* makes cell growth of CHO-DG44 cells dependent on the supplementation of thymidine, hypoxanthine and glycine [20,26]. DHFR selection marker in combination with its deletion mutant CHO-DG44 cell line have become one of the standard systems used for generating cell lines for recombinant protein production [27].

1.2. Transfection for Generation of Recombinant Cell Line

The gene of interest (GOI) is transfected to the cells as part of an expression vector. Such exogenous plasmid DNA in mammalian cells is either lost during the cell division or it gets stably integrated into the genomic DNA [1]. In a transient expression system, the protein production phase directly follows the transfection, as cells are harvested after a relatively short cultivation time. However, with the need to produce large amounts of the target protein with consistent quality at every production run, stable expression systems are widely utilized [28]. Stable cell line generation (Figure 1.1) is more complex, time-consuming and labor-intensive and it usually requires multiple transfection experiments to obtain a stable cell line with high productivity [15,28].

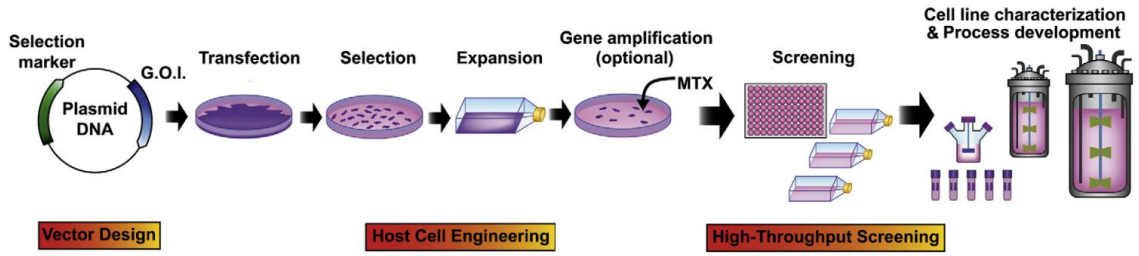

Figure 1.1. Workflow of stable cell line development.

Plasmid with the gene of interest and a selection marker gene is transfected to the cells. Selective pressure is then used to continue only with cells that have stably integrated the genes into their chromosomes. Surviving cells can be further subjected to gene amplification with an inhibitor of the selection protein (MTX with DHFR selection). The resulting heterogeneous cells are screened for clones with desired growth and productivity. The best candidate clones are tested for an extended time period and also in production-like settings. (Source: adapted from "Recombinant therapeutic protein production in cultivated mammalian cells: current status and future prospects" by M. Matasci, et al, 2008, *Drug Discovery Today: Technologies*, 5, p. 37-42. [29])

For assuring the integration of foreign DNA to the genome, the GOI is transferred to the cells together with a selective marker gene, which is usually *DHFR* gene in the transfection of CHO-DG44 cell line [15,30]. Cells with sufficient activity of integrated *DHFR* gene can survive in the selective culture medium that lacks thymidine, hypoxanthine and glycine [27,28]. The expression of GOI can be enhanced by treating the cells with increasing concentrations of methotrexate (MTX), an inhibitor of DHFR [27,29]. To overcome the inhibition, surviving cells need to increase the copy number of *DHFR* by gene amplification. The amplified region of the genome includes the surrounding DNA; therefore, the neighboring GOI also gets amplified [29].

Recombinant cell pools that are able to recover from the selective pressure and gene amplification, are screened for high cell specific productivity (usually at least 20 pg/cell/day of the desired bioproduct) and fast growth that show potential for scalability to manufacturing processes [10,31]. To obtain reproducible and stable production that meets regulatory requirements, a monoclonal cell line needs to be isolated from the heterogeneous cell pool [32].

A successful transfection experiment has high gene transfer efficiency, good cell survival with minimal effects on normal physiology, and is easily performed and reproduced [1]. The preferred method of transfection is chosen based on application and cell type [33]. The transfection techniques can be divided into categories of biological, chemical and physical. Biological transfection refers mostly to transduction, which is virus-mediated gene delivery. Despite its high efficiency, viral transfection is often avoided for therapeutic protein production to avoid the possible immunogenicity and additional safety requirements [1,34,35]. Chemical methods are usually recruiting the complex formation of the nucleic acid with a cationic polymer, a cationic lipid, or calcium phosphate [33]. Chemical transfections continue to be regularly used, especially for fast large scale transfections [36,37]. The most widely used physical transfection method is electroporation, while direct microinjection, biolistic particle delivery or laser irradiation have also been described for gene transfer [1,33].

The method description of DNA electrotransfer into cells was first published in 1982 by Neumann [38]. Due to the high and reproducible transfection efficiencies of > 80%, electroporation is now considered to be the standard for the development of recombinant CHO cells [32]. During electroporation, through a rather poorly understood multistep mechanism, short high voltage electric pulses cause the plasma membrane to become transiently permeable, which enables the exogenous nucleic acid to pass the

membrane barrier (Figure 1.2) [39–42]. The main drawbacks of electroporation are the need for specialized equipment and the frequent cell death that is commonly observed after the electroporation process [33].

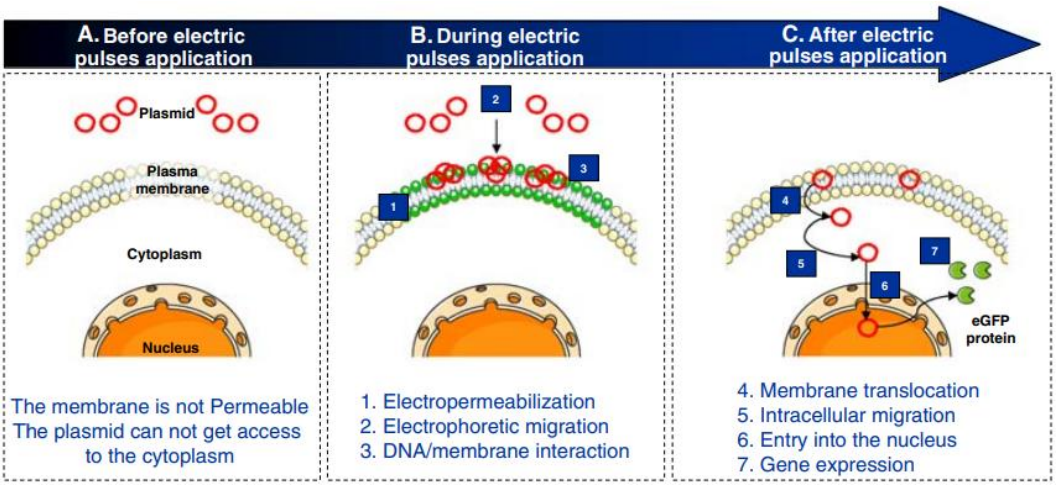

Figure 1.2. The multistep process of electroporation.

(A) Large hydrophilic molecules, like DNA, are unable to pass the cell membrane under normal conditions. (B) During electroporation the sufficiently large electric field intensity causes the membrane to become permeabilized. The negatively charged DNA molecule migrates with the help of electrophoretic force and interacts with particular domains of the permeabilized cell membrane. (C) After the electric field is turned off, DNA needs to translocate across the membrane, migrate through the crowded cytoplasm and pass the nuclear envelope, as the transfected genes can be expressed only after entering the nucleus. (Source: reprinted from "What is (Still not) Known of the Mechanism by Which Electroporation Mediates Gene Transfer and Expression in Cells and Tissues," by J. Escoffre, T. Portet, A. Luc et al., 2009, *Mol Biotechnol*, 41, 286-295. Copyright 2008 by the Humana Press. [42])

1.3. Nucleofector Technology

An improved version of electroporation, nucleofection, was developed in 2004 by the company of Amaxa [43]. The greatest value of the nucleofection technology is seen

for the gene transfer into a range of primary and hard-to-transfect cell types as it enables the transfer of molecules directly to the nucleus, independently of cell division [44,45]. The strengths of nucleofection are made possible by using the combination of specific electrical parameters and cell type specific buffers. When the cell type specific optimized conditions are used, very high transfection efficiencies up to 95% can be reached with much lower cell toxicity compared to conventional electroporation [46]. Pulses with very high voltage (field strength up to 2-10 kV/cm) are used to enable the transfer of extracellular macromolecules directly to the nucleus, while the extent of cell death is reduced by using extremely short pulses (10-200 μs) [47]. The necessary equipment for nucleofection can be seen in Figure 1.3.

Figure1.3. Device and kit of 4D-Nucleofector. (Photograph by Author, 07.05.2020).

The high cost of using Nucleofector is the main limitation and reason for researchers, especially in academic laboratories, to continue using the preceding and less efficient transfection methods. The expenditure is not limited only to the Nucleofector device but the user also needs to rely on the supply of consumables. The compatible Nucleofector kit that must be purchased for the desired efficiency of the system includes conductive polymer cuvettes and the cell type specific proprietary Nucleofector buffers. Features of all these components are unknown to the public which

makes them difficult to be replaced or improved and results in a high level of dependence. The price for one kit is approximately 400 USD and it includes materials for 24 reactions. Cell line development research, taking an example from the company of Florabio, might need more than 500 transfections within a year based on the type and the scale of the application [48].

Therefore, the aim of this thesis is to develop a more economical alternative to the commercial nucleofection buffer that could be used with CHO-DG44 cells for producing stable recombinant cell lines without significantly compromising in the transfection quality. The performance of the developed buffer will be compared to the reference material, both, in transient and stable level of expression. Additionally, other parameters of CHO-DG44 nucleofection protocol are to be optimized for yielding more efficient transfections.

CHAPTER 2

MATERIALS AND METHODS

2.1. Buffer Preparation

Before the decision on buffer components, some main parameters of the Lonza's reference buffer were measured. Radiometer ABL90 FLEX blood gas analyzer was used to determine the concentration of electrolytes Ca^{2+}, Cl^{-}, K^{+}, Na^{+} and the pH. Sample of buffer was diluted in water if the concentration was higher than the measurable range of the ions. Gonotec Osmomat 3000 freezing point osmometer was used for the measurement of osmolality. The same equipment was used for characterization and quality control after preparation of the experimental buffers. Thanks to the bigger available volume of in-house buffers, pH could be determined more accurately by Mettler Toledo SevenMulti pH probe. The ionic strength I of the solution was calculated using the following formula, where C_i is the concentration of ion i in mol/L and Z_i is the charge of ion i.

$$I = \frac{1}{2}\sum_i C_i Z_i^2 \tag{2.1}$$

All the used chemicals were ordered from Sigma-Aldrich. Prepared solutions were sterilized by Minisart syringe filter with a pore size of 0.2 μm. The detailed preparation protocol of the final developed buffer TB4 is given in Appendix A.

2.2. Expression Plasmids

In this study the initial optimization was done with the help of green fluorescent protein (GFP) expression from the simple pmaxGFP plasmid that is included in Lonza's Nucleofector kit. To assess the transient and stable expression of biopharmaceutical recombinant proteins, the coding sequences of an Fc fusion protein or an IgG1 monoclonal antibody were cloned into the Florabio's expression plasmids. Signal peptides were used to direct the secretion of the proteins. *DHFR* gene was included in the plasmids to enable the selection for transfected cells. For transfection, plasmids were isolated with an endotoxin-free midiprep kit (MN, NucleoBond Xtra Midi EF) according to the manual of the manufacturer. Concentration and purity of DNA were determined with NanoDrop 1000 spectrophotometer from Thermo Scientific. Pure DNA had an OD260/OD280 ratio 1.80-2.00 and OD260/OD230 ratio > 2.00.

For obtaining linear form of the DNA constructs for the transfection, 40 μg endotoxin-free plasmid DNA was restricted with 0.5 μL PvuI-HF, BglII-HF and/or XhoI-HF at 37°C for 16 hours. All enzymes were obtained from New England Biolabs. To remove the reaction buffer and enzyme from the samples, the restriction reaction was mixed with 5x of its volume Qiagen PB buffer (Cat.No.: 19066), and DNA was bound to QIAprep 2.0 Spin Miniprep Columns (Qiagen, Cat.No.: 27115) that were then washed with the wash buffer PE (Qiagen, Cat.No.: 19065). The DNA was eluted with 30 μL buffer EB (Qiagen, Cat.No.: 19086). Due to the limited binding capacity, up to 50 μg of DNA was purified from one spin column. Concentration and quality of the DNA preparation were determined with NanoDrop 1000 spectrophotometer from Thermo Scientific. Pure DNA had an OD260/OD280 ratio 1.80-2.00 and OD260/OD230 ratio > 2.00. Complete linearization was confirmed on agarose gel.

2.3. Cell Culture

CHO-DG44 cells were obtained from the establisher of the cell line, Prof. Lawrence Chasin, at Columbia University [26]. The cells were initially adherent and

dependent on serum-containing media. At Florabio, the cells were gradually adapted to anchorage-independent growth in chemically defined serum-free media. The fully adapted cell line was passaged every 3 days as a regular stock culture, inoculated with 3×10^5 cells/mL into Florabio's proprietary cell culture media PROM29. Cells for transfections were taken from day 2 culture, with passage number up to 30. All cells in this study were cultured in a humid atmosphere at 37°C, 7% pCO_2, 110 rpm linear shaking (Thermo Heraeus HERAcell 240 CO_2 incubator; Edmund Bühler KS-15B shaker). Non-treated polystyrene culture dishes from Thermo Scientific Nunc were used for cell cultivation.

The viable cell concentration and viability of all cell cultures were measured with CASY Cell Counter and Analyzer (OMNI Life Science, TTC-2EC-1102) that quantifies cells based on the resistance signal, distinguishing live and dead cells with different size and conductivity [49]. For measurement, the cell suspension sample in an Eppendorf tube was mixed and 10-50 µL aliquot (depending on the expected cell concentration) was combined with 5 mL of specific in-house isotonic measurement buffer inside a CASYcup. Measurement and analysis were performed according to the instruction manual of CASY [49]. For all measurements, the evaluation cursor (that separates live and dead cells) was set to 10.50 µm and the normalization cursor (that separates cell debris and dead cells) was set to 7.23 µm. The CASY analyzer calculates the percentage of viability by dividing the number of viable cells by the total number of cells (sum of dead and viable cells) [49].

2.4. Transfection

As preparation for transfection, 2.5 mL cell culture media (Florabio, PROM29) was prepared to a nontreated 6-well plate (Thermo Scientific Nunc), and 1 µg of GFP plasmid or 5 µg of Florabio's expression plasmid was combined with 100 µL of transfection buffer. In each experiment the reference transfection buffer from Lonza's recommended kit (Lonza, Amaxa SG Cell Line 4D-Nucleofector X Kit L, cat.no.: V4XC-30254) was used as the positive control for the experimental buffers. From the stock culture that was passaged 2 days before transfection, 2×10^6 cells were

centrifuged at 1000 rpm, 5 min, RT° (Eppendorf 5804, Eppendorf A-4-44 rotor). The supernatant was carefully aspirated and the pellet resuspended in the DNA-buffer mixture; the resulting cell suspension was pipetted to the Nucleofector cuvette without causing any air bubbles. Cells were then transfected with 4D-Nucleofector (Lonza, 4D-Nucleofector X Unit AAF-1002X, and 4D-Nucleofector Core Unit AAF-1002B), using program FF-137 (unless stated differently in the text). Subsequently, 500 µL pre-warmed culture media was added to the cuvettes and incubated at 37°C for 15 minutes. Cells were then transferred to the 6-well plate and incubated under standard conditions.

The consumables of Nucleofector are only sold as a complete kit that includes both, the cuvettes and the buffer – cuvettes cannot be purchased separately. Therefore, to use the experimental transfection buffers and perform more transfections than enabled by one kit, the cuvettes must be reused. After each transfection, the remaining cell debris and other residues in the cuvette were removed by immediately resuspending with 3x 1 mL autoclave-sterilized ultrapure water. Cuvettes were kept sterile and stored at +4°C.

For the generation of stable cell lines, transfectants had to be selected for successful integration of the *DHFR* gene. After 24-48 hours of recovery step in the normal growth media (Florabio, PROM29), selective media (Florabio, SELM5) that contains no thymidine, hypoxanthine and glycine was used to culture the cell pools. In approximately 3 weeks, the cells with sufficiently active *DHFR* gene were able to recover from selective pressure and were thereafter treated with increasing concentration of MTX to induce the gene amplification. Recombinant protein concentration was measured from the cell pools as soon as they started growing after gene amplification.

2.5. Measurement of Expressed Products

GFP that was originally derived from a jellyfish, is commonly used as a reporter gene, as the expressed protein emits green light which is detectable with the standard fluorescence measurements [50]. To quantify the expression of GFP in transfected samples, 100 µL of cell suspension was taken to a 96-well plate and fluorescence was

measured with Tecan Ultra 384 Multimode Microplate Reader at λexc 485 nm and λem 535 nm wavelengths.

The enzyme-linked immunosorbent assay (ELISA) was used to determine the concentration of expressed and secreted biotherapeutical protein in the supernatant of transfected cells. A customized sandwich ELISA protocol was established and used. Wells of 96-well plates (Thermo Maxisorp NC-442404) were coated with Fc specific anti-human IgG capture antibody (Sigma, I2136), then blocked with 2% solution of bovine serum albumin (BSA, Sigma, A2153) and washed between steps with wash buffer (1x DPBS supplemented with 0.5% Tween 20). Assay buffer (1x DPBS supplemented with 1% BSA, 0.5% Tween 20) was used for diluting the samples. Captured protein from samples was detected with Fc specific anti-human IgG–peroxidase detection antibody (Sigma, A0170) and TMB (3,3′,5,5′-Tetramethylbenzidine, Sigma T0440) was used as a substrate. Stopping the reaction with 2N sulfuric acid solution resulted in the formation of a yellow reaction product. Absorbance could then be measured with Tecan Ultra 384 Multimode Microplate Reader at 450 nm. Product concentration in each sample was calculated based on the standard curve that was generated with the original assayed protein or with human IgG (Sigma, I2511).

2.6. Data Analysis

The data of viable cell concentration, viability, product concentration and transient cell specific productivity were presented as mean ± standard deviation (SD). For all experiments at least 3 replicates were performed and analyzed. To determine the statistical difference, unpaired t-test (two-tail) or one-way ANOVA followed by Dunnett's multiple comparisons test to the control group were performed. $P < 0.05$ was considered as statistically significant. Significance on the graphs was indicated with *, where $p < 0.05$; **, where $p < 0.01$; ***, where $p < 0.001$; ****, where $p < 0.0001$. Non-significant correlations ($p > 0.05$) were not indicated on the graphs. Data were analyzed and figures were plotted with GraphPad Prism 8.0.1 software.

The cell specific productivity qP [pg/cell/day] of transfectants was calculated based on the following equation [7,23,51]

$$qP = \frac{(P_1 - P_0) * \mu}{(X_1 - X_0)} \quad (2.2)$$

X [10^5 cells/mL] represents the concentration of viable cells and P [mg/L] the concentration of the secreted product. Growth rate μ [1/day] is expressed with the equation below.

$$\mu = \frac{ln\left(\frac{X_1}{X_0}\right)}{(t_1 - t_0)} \quad (2.3)$$

CHAPTER 3

RESULTS AND DISCUSSION

3.1. Development of Transfection Buffer

The reference transfection buffer Lonza SG Cell Line Solution is a proprietary product that is included as part of the Nucleofector kit. There is no publicly available information about the content of this particular buffer. However, several publications include in-house buffers for nucleofection of different cell lines [52–55], none of which is developed and tested for CHO cells. The inventors of nucleofection hold a patent that includes general, not cell line specific, description of their transfection buffers [47]. Amaxa's patent claims a buffer solution with high buffer capacity (at least 20 mmol/pH) and high ionic strength (at least 200 mmol/L) that contains Na_2HPO_4/NaH_2PO_4 and/or HEPES and/or Tris/HCl and/or K_2HPO_4/KH_2PO_4, at least 10 mM Mg^{2+}, 2-6 mM K^+, 100-150 mM Na^+, and possible additional components, such as NaCl, Na-succinate, mannitol, glucose, Na-lactobionate and/or peptides [47]. Examples of advantageous buffer compositions that are given in the patent are shown at Table 3.1.

Knowledge from these materials was combined to get an initial prototype for the transfection buffer. Following the criteria that are given in the patent, Sol 1 was prepared in two versions that differ from each other in the value of osmolality. The compositions of prepared buffers are given in Table 3.2. The second prototype of the nucleofection buffer was prepared considering the direct measurement of reference buffer SG Cell Line Solution with available devices that included blood gas analyzer and osmometer. Surprisingly the concentration of sodium ions was determined to be lower than the detection limit of 40 mM. The detected concentration of potassium ions was 40 mM, which also significantly differs from the patented value. The relatively high osmolality of 390 mOsm/kg H_2O and neutral pH 7.0 were measured. Inspired by this collected data, potassium phosphate buffering system was used instead of sodium phosphate. To keep the K^+ concentration in the range of reference material, osmolality

had to be increased by different compounds other than the potassium phosphate buffer itself. As one idea, sodium phosphate buffer was used to increase osmolality; secondly, sugar alcohol D-mannitol was used in the concentration that was sufficient for obtaining required osmolality value. The resulting concentration of D-mannitol in Sol 2-A, 250 mM, differs significantly from the concentration of mannitol that was mentioned in the patent of Lonza as a possible supplement (1-100 mM) [47]. Mannitol has been also used previously as an osmotic agent [56] and as electroporation medium for plant cells [57].

Table 3.1. Exemplary buffer compositions from the patent of Lonza [47].

Buffer #	**Components**
1	4-6 mM KCl, 10-20 mM $MgCl_2$, and 120-160 mM Na_2HPO_4/NaH_2PO_4 (pH 7.2)
2	4-6 mM KCl, 10-20 mM $MgCl_2$, 5-25 mM HEPES and 120-160 mM Na_2HPO_4/NaH_2PO_4 (pH 7.2)
3	4-6 mM KCl, 10-20 mM $MgCl_2$, 50-160 mM Na_2HPO_4/NaH_2PO_4 (pH 7.2) and 5-100 mM sodium lactobionate or 5-100 mM mannitol or 5-100 mM sodium succinate or 5-100 mM sodium chloride
4	4-6 mM KCl, 10-20 mM $MgCl_2$, 5-25 mM HEPES, 50-160 mM Na_2HPO_4/NaH_2PO_4 (pH 7.2) and 5-100 mM sodium lactobionate or 5-100 mM mannitol or 5-100 mM sodium succinate or 5-100 mM sodium chloride
5	4-6 mM KCl, 10-20 mM $MgCl_2$, 80-100 mM NaCl, 8-12 mM glucose, 0.3-0.5 mM $Ca(NO_3)_2$, 20-25 mM HEPES and 50-100 mM tris/HC1 or 30-50 mM Na_2HPO_4/NaH_2PO_4, (pH 7.2)
6	0.1-3.0 mM $MgCl_2$, 50-200 mM K_2HPO_4/KH_2PO_4 (pH 6.5) and/or 1-50 mM mannitol and/or 1-50 mM sodium succinate

The resulting transfection buffers were characterized with the two most important aspects – the expression level of GOI and cell survival after transfection. The expression level of transfected gene indicates indirectly the transfection efficiency – how well cells have taken up the transfected DNA construct. Recovery of the cells after electroporation is important to obtain viable cell pool for further selection procedure. A large percentage of dead cells means that next work steps need to be done on unhealthy

cell population where the previously optimized procedure for selection might be too harsh or it might take longer time to obtain a stably producing recovered cell pool.

Table 3.2. The composition of prepared prototypes of transfection buffer.

Buffer	**Components**
Sol 1-A	120 mM Na_2HPO_4/NaH_2PO_4 (pH 7.2) + 5 mM KCl + 15 mM $MgCl_2$. Osmolality: 290 mOsm/kg H_2O. Ionic strength: 530 mmol/L
Sol 1-B	160 mM Na_2HPO_4/NaH_2PO_4 (pH 7.2) + 5 mM KCl + 15 mM $MgCl_2$. Osmolality: 380 mOsm/kg H_2O. Ionic strength: 690 mmol/L
Sol 2-A	30 mM K_2HPO_4/KH_2PO_4 (pH 7.2) + 15 mM $MgCl_2$ + 250 mM D-mannitol. Osmolality: 348 mOsm/kg H_2O. Ionic strength: 165 mmol/L
Sol 2-B	30 mM K_2HPO_4/KH_2PO_4 (pH 7.2) + 15 mM $MgCl_2$ + 130 mM Na_2HPO_4/NaH_2PO_4 (pH 7.0). Osmolality: 348 mOsm/kg H_2O. Ionic strength: 685 mmol/L

Cells were transfected with plasmid that encodes for GFP and were assessed 48 hours after transfection by counting viable cells and quantifying GFP signal. Results are illustrated in Figure 3.1. Sol 2 buffer prototypes significantly reduced cell survival (Figure 3.1A). The lowest viability resulted from Sol 2-A, with mean value of 81% (Figure 3.1B), which can still be considered as a relatively healthy cell population. The effect of Sol 1 prototypes on both, cell survival and GFP productivity was non-significant compared to the Lonza's control buffer. Transfection with buffer Sol 2-A resulted in 2-fold increase ($p < 0.0001$) of GFP expression as compared to control (Figure 3.1C). Due to weaker cell recovery, the improvement of productivity was even sharper when expressed as cell specific productivity (Figure 3.1D).

Considering the obtained productivity results, further experiments were conducted with the prototype buffer Sol 2-A. The chosen prototype possesses high osmolality but notably lower ionic strength as compared to other prototypes and the Lonza's patented solution. This could be achieved by using a relatively high concentration of D-mannitol. Mannitol gets deprotonated only in strongly basic solutions, and therefore, does not increase the ionic strength of the neutral buffer [58]. With no sodium present in the Sol 2-A, the Na^+/K^+ ratio matches better with the

intracellular conditions than other buffer prototypes, which contain a relatively high concentration of sodium. It has also been published earlier that electroporation of cells could be more efficient and with improved cell survival when buffer's ionic composition resembles the intracellular environment [59].

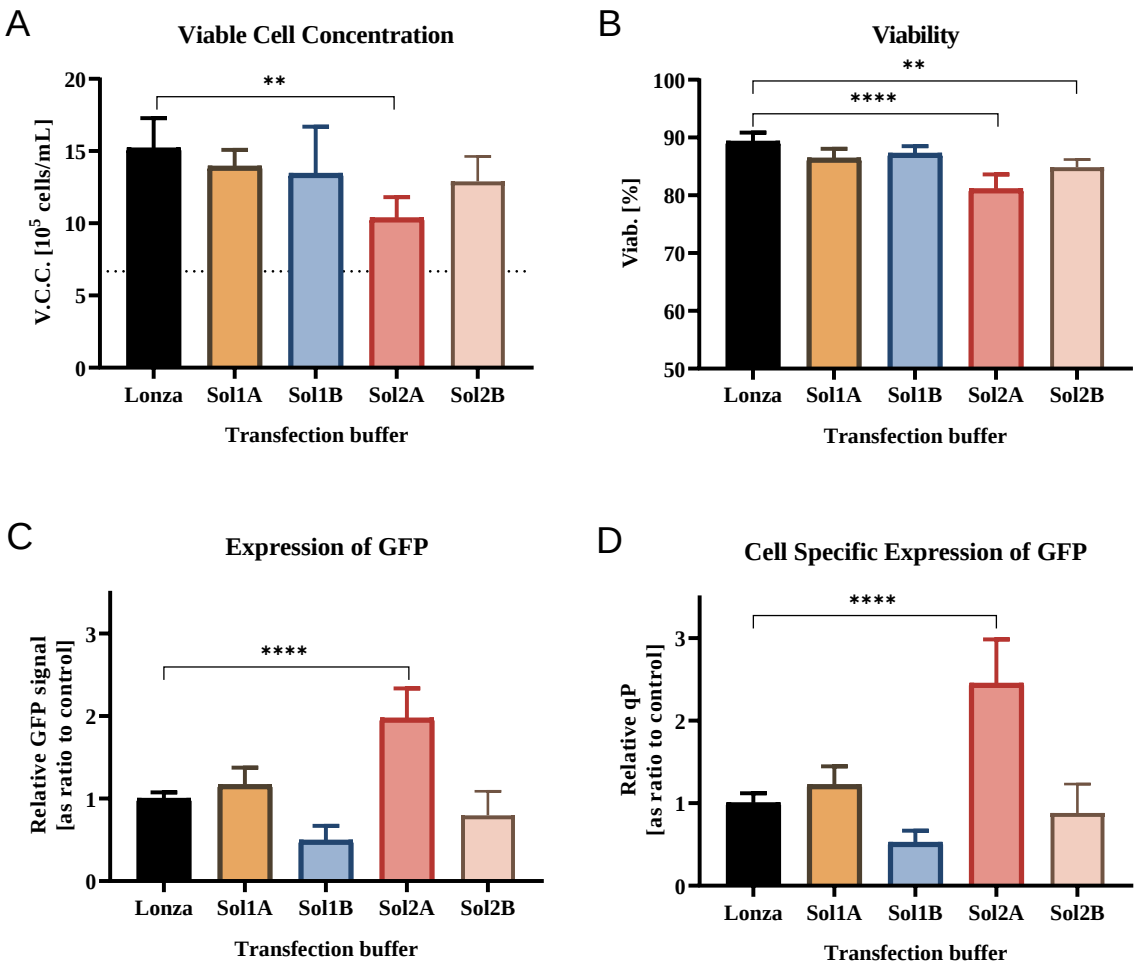

Figure 3.1. Transfection data of tested buffer prototypes.

Contents of each buffer are given in Table 3.2 Data was obtained 72 hours after transfection. Results are presented as mean ± SD. **: $p < 0.01$; ****: $p < 0.0001$. (A) Viable cell concentration. The inoculation cell concentration (6.7×10^5 cells/mL) is indicated with a dotted line. (B) Viability percentage. (C) GFP fluorescence signal expressed as the ratio to Lonza's control solution. (D) Calculated cell specific GFP productivity, expressed as the ratio to Lonza's control solution.

It was aimed to further improve the performance of Sol 2-A by testing the available chemicals that could have a beneficial effect on the transfection. Supplementation of basic buffer formulas with Ficoll and polyethylene glycol (PEG) among other tested chemicals has been proven to yield more efficient transfection and better survival [55]. Addition of membrane sealing agents was expected to enhance transfection efficiency, as they help to ensure that the permeability of cell membrane is generated only transiently [41]. Glycerol was tested for the ability to aid in the membrane fusion and therefore to recover the membrane after permeabilization [60]. Tween 80 (also known as Polysorbate 80), similarly to Ficoll and PEG [55] was expected to have membrane sealing properties. Calcium nitrate was tested for the known fusion triggering properties of enhanced calcium ions in the electroporation buffer [55]. Pluronic F68 (also known as poloxamer P188 or Kolliphor P188) triblock copolymer as an amphiphilic molecule is shown to significantly inhibit apoptosis and necrosis as it interacts with the lipid bilayer and restores its integrity [41,55]. Additionally, pluronic-block copolymers have also been shown to increase transfection efficiency [54,55]. Succinic acid as an anti-oxidant was tested to protect cells from the oxidative stress of reactive oxygen species that are produced during electroporation [55,61]. The supplemented transfection buffers were prepared as given in Table 3.3.

Table 3.3. Prepared supplemented transfection solutions.

Buffer	**Components**
TB1	Sol 2-A + 0.005% Pluronic F68
TB2	Sol 2-A + 5 mM succinic acid
TB3	Sol 2-A + 0.05% Tween 80
TB4	Sol 2-A + 0.4 mM calcium nitrate tetrahydrate, $Ca(NO_3)_2 * 4H_20$
TB5	Sol 2-A + 0.5% glycerol

Cells were transfected with pmaxGFP plasmid using the supplemented transfection solutions, and Sol 2-A as an unsupplemented control solution. Obtained data about cell survival and productivity is illustrated in Figure 3.2. The only

statistically significant desired effect resulted from supplementation with calcium nitrate, which improved the recovery of cells ($p < 0.005$) (Figure 3.2A). With the addition of Ca^{2+}-ions to Sol 2-A, the achieved viable cell concentration 3 days after transfection ($14.9 \pm 0.6 \times 10^5$ cells/mL) was highly comparable to the respective result with Lonza's reference buffer ($15.2 \pm 2.1 \times 10^5$ cells/mL, Figure 3.1A). Calcium nitrate had no notable effect on the measured GFP signal.

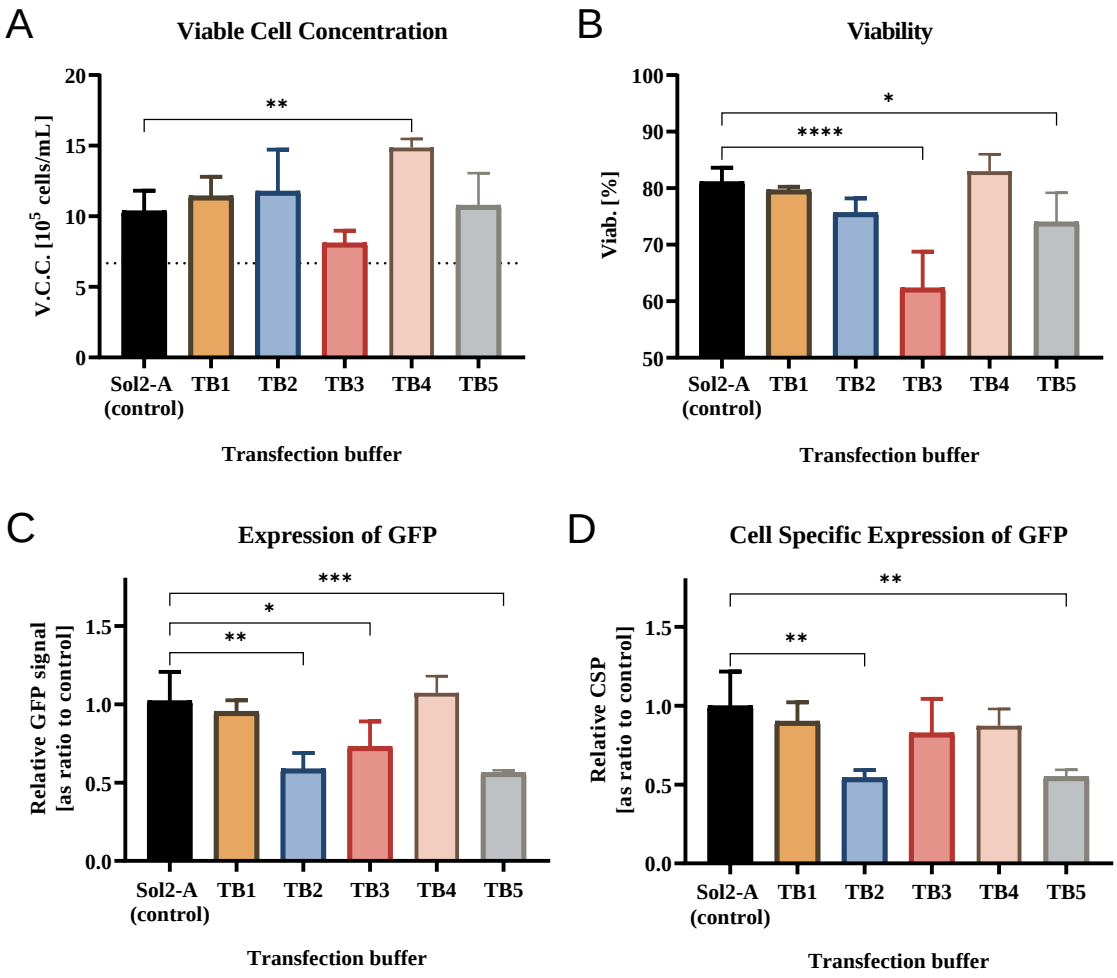

Figure 3.2. Transfection data of supplemented Sol 2-A.

TB1: Pluronic F68; TB2: succinic acid; TB3: Tween 80; TB4: calcium nitrate tetrahydrate; TB5: glycerol. Data was obtained 72 hours after transfection. Results are presented as mean ± SD. *: $p < 0.05$; **: $p < 0.01$; ***: $p < 0.001$; ****: $p < 0.0001$. (A) Viable cell concentration. The inoculation cell concentration (6.7×10^5 cells/mL) is indicated with a dotted line. (B) Viability percentage. (C) GFP fluorescence signal expressed as the ratio to Sol 2-A control solution. (D) Calculated cell specific GFP productivity, expressed as the ratio to Sol 2-A control solution.

A significant negative effect on productivity (expressed as a ratio to control in Figure 3.2C, Figure 3.2D) was seen from supplementation with succinic acid, Tween 80 and glycerol. The latter two also significantly reduced cell survival after transfection (Figure 3.2B). The dramatic decrease of viability with the addition of Tween 80 could be related to its surfactant characteristics which might bring about increased permeability [62] that is too harsh in combination with electropermeabilization.

3.2. Characterization of TB4 for Generating Recombinant Cell Lines

For the further experiments, Sol 2-A with the supplementation of 0.4 mM calcium nitrate tetrahydrate was used as the developed in-house transfection buffer and the solution was documented as TB4. After GFP had been used to optimize the buffer, experiments were conducted with the aim of expressing a biopharmaceutical protein. Here, expression plasmids that were used for transfection had significantly bigger size as they include also selective marker genes and other genetic elements that support the stable and high expression of transfected GOIs. The expression data for these experiments was measured with ELISA.

Viable cell concentration, percentage of viable cells and productivity were assessed 48 hours after transfection. The analyzed and illustrated data was taken from 4 independent experiments, whereas at least 3 data points are included from each experiment. The four used experiments were performed at different times, with modifications in parameters related to cell culture, media and plasmid. Direct comparison of these experimental data would result in too great error bars. Therefore, for comparison of transfection buffers, each data point was divided to the mean value of the corresponding control in the same experiment. The obtained ratios could be then analyzed independently from the irrelevant experimental differences.

Here, as opposed to the previous optimization experiments, the developed transfection buffer TB4 very strongly reduced the cell growth and survival within 2 days after transfection (Figure 3.3A, Figure 3.3B). Also, the improvement in productivity with TB4 was smaller compared to the respective GFP data (Figure 3.3C). Difference from the optimization experiments might stem from the properties of the used DNA. The expression plasmids were larger (7…9 kb) than the previously used

pmaxGFP plasmid (~3.5 kb) and higher amount of DNA was transfected (respectively, 5 μg and 1 μg). The smaller size of plasmid is commonly known to facilitate the uptake of foreign DNA [63,64]. However, the relative concentrations of expressed and secreted protein from transfectants with Lonza (ratio 0.95 ± 0.15) and TB4 (ratio 0.94 ± 0.16) solutions were highly comparable. Therefore, with lower surviving cell number but the same total concentration of expressed product, significantly higher ($p = 0.0004$) cell specific productivity was still seen with transfection buffer TB4 (Figure 3.3D).

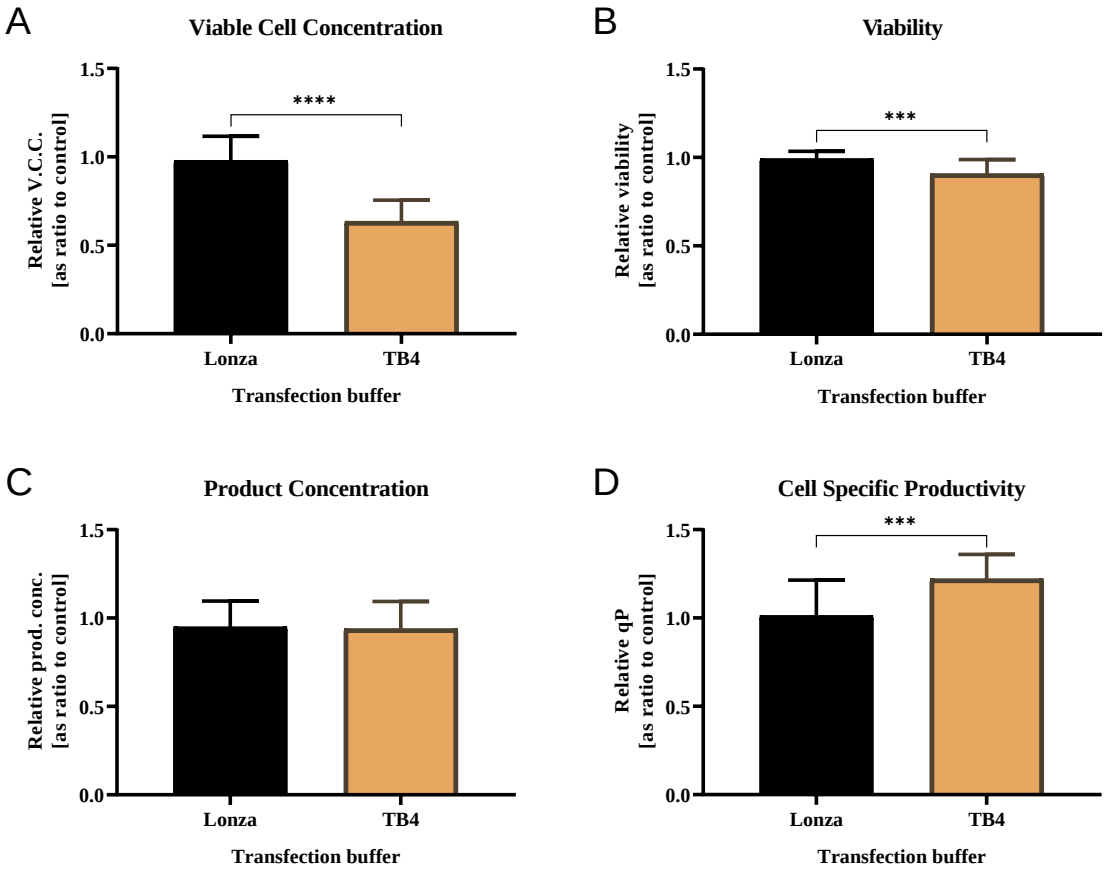

Figure 3.3. Data of testing TB4 for transient transfection.

Data was obtained 48 hours after transfection and normalized to the values of the control group to make the independent experiments comparable. Results are presented as mean ± SD. ***: $p < 0.001$; ****: $p < 0.0001$. (A) Viable cell concentration. (B) Viability. (C) Concentration of secreted recombinant Fc fusion protein or IgG mAb. (D) Cell specific productivity of secreted recombinant Fc fusion protein or IgG mAb.

As the main goal of expression cell line generation is to obtain a cell line that could produce the recombinant protein with high titer, it can be stated that improved product concentration is here the most important outcome. However, the characterized transiently transfected cell populations need to go through a selection procedure, which might be unsuccessful if the selection is too harsh for a cell pool with compromised viability. Lower concentration of viable cells directly after transfection also means a decrease in the diversity of starting material which will be used for selection. Therefore, it was important to show whether the drawbacks from significantly reduced cell survival of TB4-mediated transfections have vital effect on the results of stable cell line generation or is the isolation of high producers still achievable.

The transfected cell pools were kept in culture and selective pressure to the *DHFR* expression was applied through the use of media that lacks thymidine, hypoxanthine and glycine. The selection was performed through the use of metabolic selection marker with the aim to find cells that have integrated the transfected DNA to their genome. As soon as the cell pools recovered from the amplification steps with MTX, which takes up to 2 months, the supernatants were assayed in ELISA to determine their productivity and usability for production process. Here, the outcome is strongly influenced by the exact steps of selection process in order to eliminate cells that have managed to survive but not express the GOI [22]. Another important effect comes from the sequence of used DNA, which needs to ensure efficient integration, prevent silencing and promote the co-amplification of GOI together with *DHFR* gene [22]. Nevertheless, successful transfection in the very beginning of the experiment is prerequisite for starting with healthy cell population where the majority of cells would have successfully taken up the foreign DNA.

A large set of cell specific productivity data was used for analysis from cell pools that recovered from selection pressure after being transfected with either Lonza's SG Cell Line solution (82 data points from 11 independent experiments) or TB4 solution (66 data points from 10 independent experiments). A great percentage (56% of control transfectants, 44% of TB4 transfectants) of assayed cell pools were non-producers, that is, cell specific productivity was equal to 0 pg/cell/day (Figure 3.4A). Although the highest producing cell pool (31.8 pg/cell/day) was obtained from the transfection with Lonza's buffer, the 90th percentile value 7.6 pg/cell/day of control pools is more than 2 times lower than the 90th percentile value 17.83 pg/cell/day of pools from TB4-mediated transfections. The distribution of single data points is

illustrated in Figure 3.4B, while only values starting from 5 pg/cell/day are plotted. It can be observed that transfections with TB4 did result in greater number of pools with cell specific productivity more than 20 pg/cell/day. Cells with these productivity values can be considered as potential cell lines for the industrially scalable production of recombinant protein that they express [15]. Most confidently it can be concluded that using the novel TB4 solution instead of Lonza's SG Cell Line solution does not compromise the efficacy of stable cell line generation.

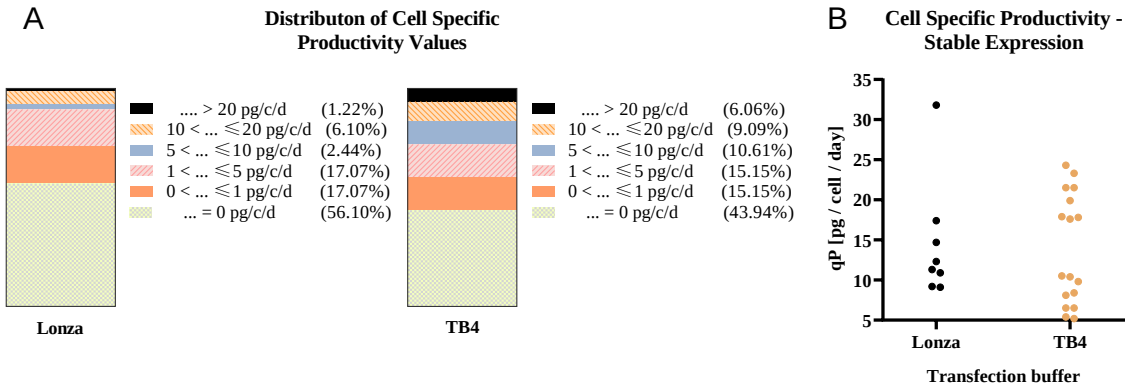

Figure 3.4. Productivity data of stable expression cell lines.

Cell specific productivity of secreted recombinant Fc fusion protein or IgG mAb. (A) Productivity values are depicted as percentage distribution of the number of cell lines whose productivity values fall into the indicated range. (B) Single data points of productivity values starting from 5 pg/cell/day are plotted.

3.3. Enhanced Results with an Alternative Nucleofection Program

The protocol of nucleofection has cell-type specific recommendation for default Nucleofector program to be used. Each program defines the specific unique electrical parameters which are not known to the user of the device. While there is no protocol precisely for CHO-DG44 cell line, the program FF-137 is recommended for CHO-S cells [65]. The technical support of Lonza encourages users to fine-tune the transfection process for every specific cell line.

Based on the advice of Lonza's technical support (personal communication, February, 2019), four other programs were tested for the transfection of used CHO-DG44 suspension cell line. These programs had no official data from Lonza but had been reported by other users to deliver good performance. Programs EH-158, DS-158, DU-158, DG-208 were tested against FF-137. Cells were transfected in triplicates with a Florabio's plasmid that encodes for a recombinant biotherapeutic protein. Lonza's transfection buffer was used in combination with the 5 different transfection programs.

The only statistically significant effect on cell recovery was seen from program EH-158, where 48 hours after transfection the number of viable cells was 3 times reduced ($p < 0.01$) compared to control program FF-137 (Figure 3.5A). The other tested programs resulted only in slight, non-significant changes in the viability of transfectants (Figure 3.5B). Transient expression productivity (Figure 3.5C), however, was improved by using the alternative programs, as pools from DU-158 (4.4 ± 0.9 mg/L) and DG-208 (4.7 ± 0.2 mg/L) secreted significantly more ($p < 0.01$) recombinant protein than the control pools (2.7 ± 0.2 mg/L). The best cell specific productivity was calculated from the pools transfected with EH-158, as the cell populations with 3 times fewer cells could produce and secrete the protein in the amount that was highly comparable to pools in the other groups (Figure 3.5D).

All the transfected cell populations were cultured further to apply selection pressure and generate stable production cell pools. The cell specific productivity values of each pool that recovered from selection were plotted in Figure 3.5E. For the experimental groups that have less than 3 data points plotted, no more data could be obtained as the rest of the cell pools never began to grow after the applied selection. From cell pools that were transfected with EH-158 that had the highest specific transient productivity, only 1 out of 3 pools recovered which can be explained by the poorest survival of these cell pools directly after transfection. Interestingly, despite the highly similar day 2 performances of programs DU-158 and DG-208, the cell specific productivity of stable pools from DG-208 was notably improved as compared to DU-158 and to other tested programs. Therefore, DG-208 was selected to be the most optimal nucleofection program in the described transfection system.

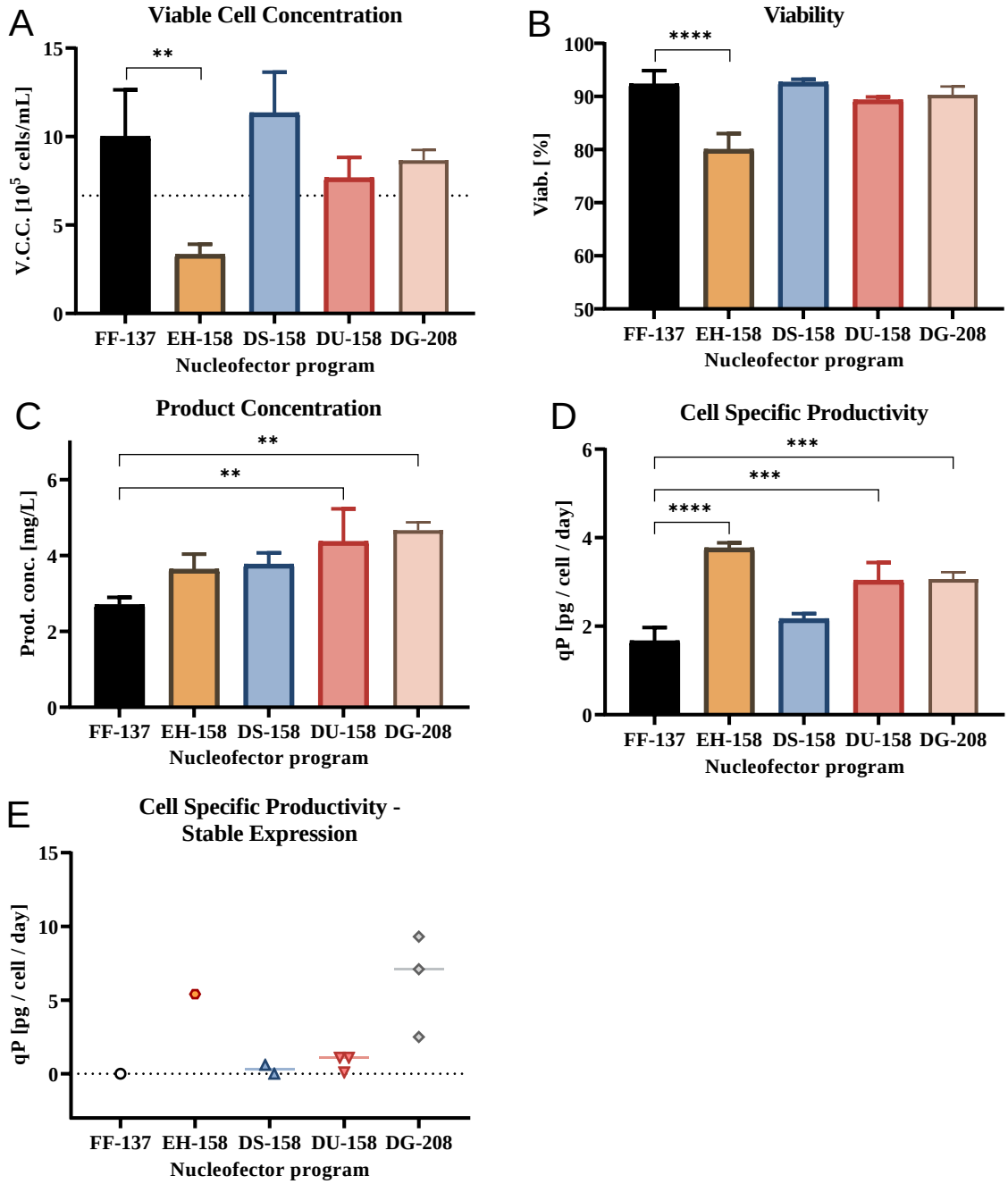

Figure 3.5. Transfection data of alternative Nucleofector programs.

Data for graphs A-D was obtained 48 hours after transfection. Presented as mean ± SD. **: $p < 0.01$; ***: $p < 0.001$; ****: $p < 0.0001$. (A) Viable cell concentration. The inoculation cell concentration (6.7×10^5 cells/mL) is indicated with a dotted line. (B) Viability percentage. (C) Concentration of secreted recombinant Fc fusion protein. (D) Cell specific productivity of the Fc fusion protein. (E) Stable cell specific productivity of the Fc fusion protein. Measured from the cell pools after selection step (40-60 days after transfection). The graph shows data points and their median.

3.4. Higher Expression from Transfection of Linearized Plasmid

DNA that is used in transfection is produced in prokaryotic E. coli cells and is in the form of circular plasmid for easy replication in the bacteria. Plasmid DNA is not replicated in eukaryotic cells and therefore needs to be integrated into the genome in order to keep the transfected genes expressed for extended time period. After transfection of circular supercoiled plasmid, the extrachromosomal DNA is converted to nicked circular form and partially to linear DNA molecules [66]. The linear fragments ligate into concatamers [67] and can then integrate to the genome through nonhomologous recombination [15]. Linearization site of the plasmid inside the cells cannot be predicted or directed. As a result, the restriction of DNA might happen in the important coding or regulatory sequence and therefore negatively affect the expression level of the GOI [67].

In order to make the DNA uptake and integration more controlled, the plasmid can be linearized by restriction enzyme digestion prior to transfection. Linear DNA is presumably more difficult to be taken up by the cells; decreased transfection efficiency has been reported compared to supercoiled or open circular plasmid [68,69]. Contradictory results have been published whether the linear topology of DNA improves or worsens the integration of transfected gene to the genome [38,70,71]. It has also been shown that the restriction site used for linearization greatly affects the outcome, resulting either in decreased or increased efficiency at both, transient transfection and stable integration [72].

The backbone of used plasmid was examined for unique restriction sites that were located in the part of bacterial genes and not interfering with the sequences related to our genes of interest. Linearization was tested in 3 different ways; used restriction sites are shown in Figure 3.6. BglII restricts the plasmid just before the eukaryotic sequences. BglII and XhoI double-digestion divides the plasmid into 2 fragments, separating the eukaryotic sequence from the prokaryotic part. For transfection, the fragments were not purified from gel, but used as a mixture directly from the restriction reaction. PvuI restriction site linearizes the plasmid from the middle of prokaryotic sequence, destructing the ampicillin resistance gene. Plasmid was linearized and purified as described in chapter 2.2. Transfection of linearized plasmid directly from the mixture of restriction reaction or from agarose gel purification were also tested, but no difference between purification methods was detected (data not shown).

Cells were transfected with Florabio's plasmids that encode for a recombinant biotherapeutic protein, and Lonza's transfection buffer was used. Two independent experiments were performed with triplicate transfections for each experimental group. Regarding the cell recovery after transfection, the viable cell concentration and viability from transfections with circular plasmid yielded only very slightly better results that were not statistically significant ($p > 0.05$) (Figure 3.7A, Figure 3.7B). However, almost 3-fold ($p < 0.0001$) increase of transient productivity was seen when plasmid was transfected in any of the 3 linearized forms (Figure 3.7C, Figure 3.7D). Some significant differences can also be detected when comparing the productivity results of differently linearized plasmids – BglII restricted plasmid yielded the product concentration values that were significantly lower than that of PvuI linearization ($p = 0.0001$) and BglII + XhoI restriction ($p < 0.05$). PvuI linearization of plasmid resulted overall in the best results, and using a single enzyme instead of double restriction was preferred, so linearization of the plasmids with PvuI was set as the standard.

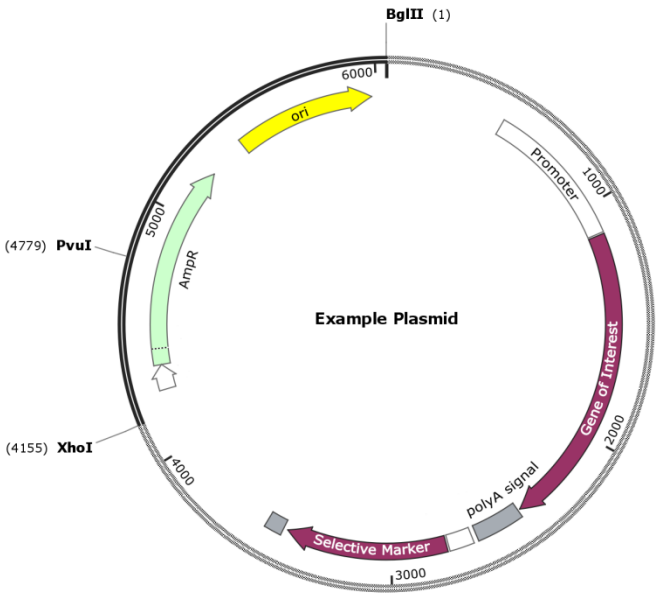

Figure 3.6. Illustration of restriction sites used for linearization of the plasmid.

An exemplary standard expression plasmid is given to demonstrate the separation of eukaryotic and prokaryotic DNA elements. Source: created with SnapGene Viewer version 5.1.3.1 (GSL Biotech, Chicago, IL) [73].

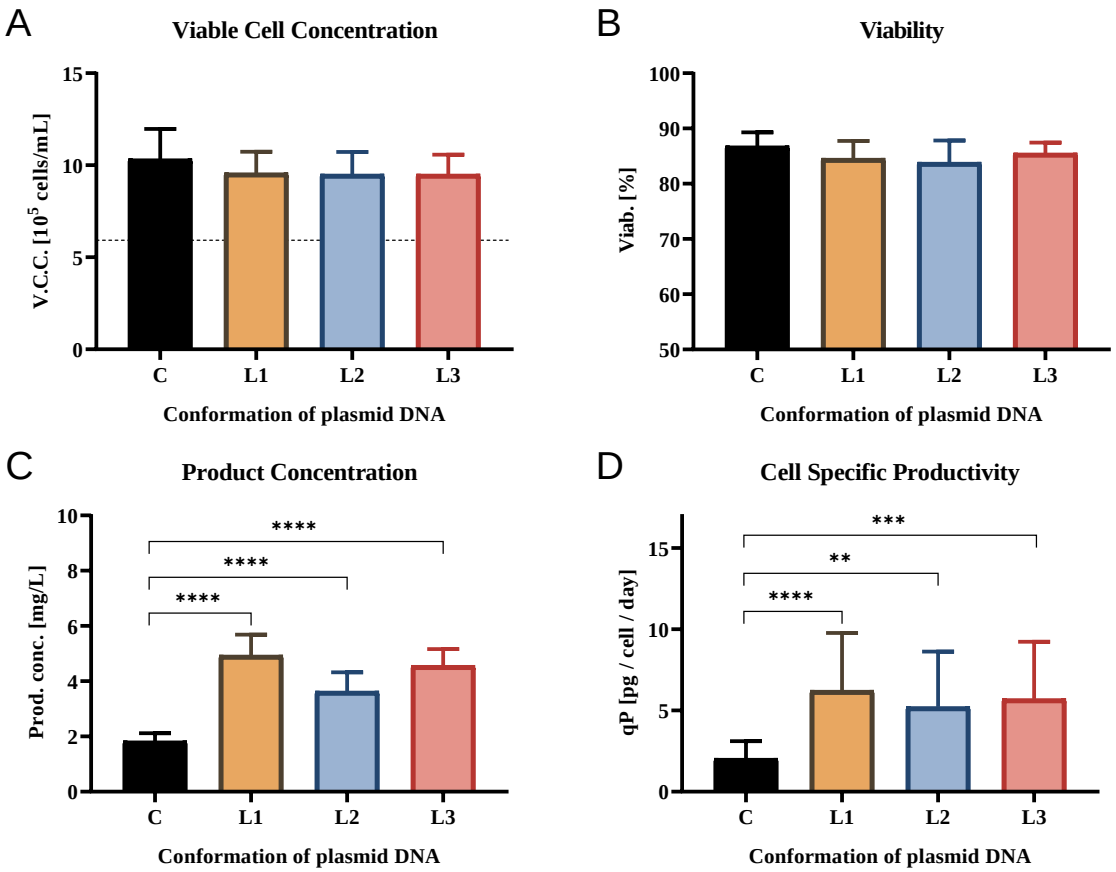

Figure 3.7. Transfection data of linearized plasmids.

C: circular plasmid DNA; L1: plasmid linearized with PvuI; L2: plasmid linearized with BglII; L3: plasmid restricted with BglII+ XhoI. Data was obtained 48 hours after transfection. Results are presented as mean ± SD. **: $p < 0.01$; ***: $p < 0.001$; ****: $p < 0.0001$. (A) Viable cell concentration. The inoculation cell concentration (6.7×10^5 cells/mL) is indicated with a dotted line. (B) Viability percentage. (C) Concentration of secreted recombinant Fc fusion protein. (D) Cell specific productivity of secreted recombinant Fc fusion protein.

CHAPTER 4

CONCLUSION

In the first part of the thesis, GFP expression was used to develop a nucleofection buffer for transfecting CHO-DG44 cell line. Based on the knowledge from literature and measurements of the commercial reference solution, several buffer compositions were tested. The best results were obtained with TB4, which slightly reduced cell viability after transfection, but doubled the resulting expression level of GFP. In the second part of the study, the developed solution TB4 was used to generate recombinant cell lines for production of biotherapeutic proteins. Although the performance of the buffer was weakened, likely due to the qualities of the transfected plasmid, TB4 could still significantly increase the transient cell specific productivity as compared to the control solution. Furthermore, it was proved possible to generate stable expression cell lines from TB4 mediated transfectants. In the final parts of the study, the overall nucleofection procedure of CHO-DG44 cells was enhanced by observing that the transient expression level is significantly improved when using an alternative nucleofection program, DG-208, and linearizing the plasmid prior to transfection. All the results of this study are optimized based on the cell line that is an industrial standard for production of therapeutic proteins. However, these enhancements could potentially be useful for any research that requires a large number of efficient transfection experiments. In order to achieve complete independence from the commercial nucleofection consumables, the compatible electroporation cuvettes would need to be developed.

REFERENCES

1. Kim, Tae Kyung & Eberwine, James H. Mammalian cell transfection: the present and the future. *Anal. Bioanal. Chem.* 397, 3173–3178 (2010).

2. Crea, Francesco, Sarti, Donatella, Falciani, Francesco & Al-Rubeai, Mohamed. Over-expression of hTERT in CHO K1 results in decreased apoptosis and reduced serum dependency. *J. Biotechnol.* 121, 109–123 (2006).

3. Kaneda, Megan M. *et al.* PI3Kγ is a molecular switch that controls immune suppression. *Nature* 539, 437–442 (2016).

4. Aypek, Hande, Bay, Veysel & Meşe, Gülistan. Altered cellular localization and hemichannel activities of KID syndrome associated connexin26 I30N and D50Y mutations. *BMC Cell Biol.* 17, 1–8 (2016).

5. Bruzauskaite, Ieva *et al.* Relevance of HCN2-expressing human mesenchymal stem cells for the generation of biological pacemakers. *Stem Cell Res. Ther.* 7, 67 (2016).

6. Anil-Inevi, Muge *et al.* Biofabrication of in situ Self Assembled 3D Cell Cultures in a Weightlessness Environment Generated using Magnetic Levitation. *Sci. Rep.* 8, 1–10 (2018).

7. Zboray, Katalin *et al.* Heterologous protein production using euchromatin-containing expression vectors in mammalian cells. *Nucleic Acids Res.* 43, 102 (2015).

8. Zahn-Zabal, Monique *et al.* Development of stable cell lines for production or regulated expression using matrix attachment regions. *J. Biotechnol.* 87, 29–42 (2001).

9. Maccani, Andreas *et al. Pichia pastoris* secretes recombinant proteins less efficiently than Chinese hamster ovary cells but allows higher space-time yields for less complex proteins. *Biotechnol. J.* 9, 526–537 (2014).

10. Zhang, Jinyou. Mammalian Cell Culture for Biopharmaceutical Production. in *Manual of Industrial Microbiology and Biotechnology, Third Edition* 157–178 (American Society of Microbiology, 2010). doi:10.1128/9781555816827.ch12.

11. Kesik-Brodacka, Malgorzata. Progress in biopharmaceutical development. *Biotechnol. Appl. Biochem.* 65, 306–322 (2018).

12. Kayser, Oliver & Warzecha, Heribert. *Manufacturing of Pharmaceutical Proteins Post-translational Modifi cation of Protein Biopharmaceuticals Handbook of Pharmaceutical Biotechnology Medical Product Regulatory Affairs. Wiley-Blackwell* (2012).

13. Moorkens, Evelien *et al.* The market of biopharmaceutical medicines: A snapshot

of a diverse industrial landscape. *Front. Pharmacol.* 8, (2017).

14. Kunert, Renate & Reinhart, David. Advances in recombinant antibody manufacturing. *Appl. Microbiol. Biotechnol.* 100, 3451–3461 (2016).

15. Wurm, Florian M. Production of recombinant protein therapeutics in cultivated mammalian cells. *Nat. Biotechnol.* 22, 1393–1398 (2004).

16. Dumont, Jennifer, Euwart, Don, Mei, Baisong, Estes, Scott & Kshirsagar, Rashmi. Human cell lines for biopharmaceutical manufacturing: history, status, and future perspectives. *Crit. Rev. Biotechnol.* 36, 1110–1122 (2016).

17. O'Flaherty, Róisín *et al.* Mammalian cell culture for production of recombinant proteins: A review of the critical steps in their biomanufacturing. *Biotechnol. Adv.* 107552 (2020) doi:10.1016/j.biotechadv.2020.107552.

18. Jayapal, Karthik P., Wlaschin, Katie F., Hu, Wei Shou & Yap, Miranda G. S. Recombinant protein therapeutics from CHO Cells - 20 years and counting. *Chem. Eng. Prog.* 103, 40–47 (2007).

19. Dahodwala, Hussain & Sharfstein, Susan T. The 'Omics Revolution in CHO Biology: Roadmap to Improved CHO Productivity. in *Heterologous Protein Production in CHO Cells. Methods in Molecular Biology* vol. 1603 153–168 (Humana Press Inc., 2017).

20. Hacker, David L., Chenuet, Sébastien & Wurm, Florian M. Chinese Hamster Ovary Cells, Recombinant Protein Production. in *Encyclopedia of Industrial Biotechnology: Bioprocess, Bioseparation, and Cell Technology* 1–8 (John Wiley & Sons, Inc., 2010). doi:10.1002/9780470054581.eib214.

21. Ritacco, Frank V., Wu, Yongqi & Khetan, Anurag. Cell culture media for recombinant protein expression in Chinese hamster ovary (CHO) cells: History, key components, and optimization strategies. *Biotechnol. Prog.* 34, 1407–1426 (2018).

22. Hunter, Molly, Yuan, Ping, Vavilala, Divya & Fox, Mark. Optimization of Protein Expression in Mammalian Cells. *Curr. Protoc. Protein Sci.* 95, e77 (2018).

23. Clarke, Colin *et al.* Predicting cell-specific productivity from CHO gene expression. *J. Biotechnol.* 151, 159–165 (2011).

24. Yeo, Jessna H. M. *et al.* Optimized Selection Marker and CHO Host Cell Combinations for Generating High Monoclonal Antibody Producing Cell Lines. *Biotechnol. J.* 12, (2017).

25. Alves, Christina S. & Dobrowsky, Terrence M. *Strategies and Considerations for Improving Expression of "Difficult to Express" Proteins in CHO Cells*. *Methods in Molecular Biology* vol. 1603 (2017).

26. Urlaub, Gail, Käs, Emmanuel, Carothers, Adelaide M. & Chasin, Lawerence A. *Deletion of the Diploid Dihydrofolate Reductase Locus from Cultured*

Mammalian Cells. *Cell* vol. 33 (1983).

27. De Jesus, Maria J. & Wurm, Florian M. Mammalian Cells in Biotech Production. in *Pharmaceutical Biotechnology: Drug Discovery and Clinical Applications, Second Edition* (eds. Kayser, O. & Warzecha, H.) 43–57 (Wiley-VCH Verlag GmbH & Co. KGaA, 2012). doi:10.1002/9783527632909.ch3.

28. Noh, Soo Min, Shin, Seunghyeon & Lee, Gyun Min. Cell Line Development for Therapeutic Protein Production. in *Cell Culture Engineering: Recombinant Protein Production* (eds. Lee, G. M. & Kildegaard, H. F.) 23–47 (Wiley-VCHVerlag GmbH& Co. KGaA, 2019). doi:10.1002/9783527811410.ch2.

29. Matasci, Mattia, Hacker, David L., Baldi, Lucia & Wurm, Florian M. Recombinant therapeutic protein production in cultivated mammalian cells: current status and future prospects. *Drug Discov. Today Technol.* 5, e37–e42 (2008).

30. Bandaranayake, Ashok D. & Almo, Steven C. Recent advances in mammalian protein production. *FEBS Lett.* 588, 253–260 (2014).

31. Ha, Tae Kwang, Lee, Jae Seong & Lee, Gyun Min. Platform Technology for Therapeutic Protein Production. in *Cell Culture Engineering* 1–22 (Wiley, 2019). doi:10.1002/9783527811410.ch1.

32. Castan, Andreas, Schulz, Patrick, Wenger, Till & Fischer, Simon. Cell Line Development. in *Biopharmaceutical Processing: Development, Design, and Implementation of Manufacturing Processes* 131–146 (Elsevier, 2018). doi:10.1016/B978-0-08-100623-8.00007-4.

33. Norton, Pamela A. & Pachuk, Catherine J. Methods for DNA introduction into mammalian cells. in *Gene Transfer and Expression in Mammalian Cells* (ed. Makrides, S. C.) vol. 38 265–277 (Elsevier Science B.V, 2003).

34. Moralli, Daniela & Monaco, Zoia L. Gene expressing human artificial chromosome vectors: Advantages and challenges for gene therapy. *Exp. Cell Res.* 390, (2020).

35. Bouard, D., Alazard-Dany, N. & Cosset, F. L. Viral vectors: from virology to transgene expression. *Br. J. Pharmacol.* 157, 153–165 (2009).

36. Gutiérrez-Granados, Sonia, Cervera, Laura, Kamen, Amine A. & Gòdia, Francesc. Advancements in mammalian cell transient gene expression (TGE) technology for accelerated production of biologics. *Crit. Rev. Biotechnol.* 38, 918–940 (2018).

37. Rajendra, Yashas, Balasubramanian, Sowmya & Hacker, David L. Large-scale transient transfection of chinese hamster ovary cells in suspension. in *Heterologous Protein Production in CHO Cells. Methods in Molecular Biology* vol. 1603 45–55 (Humana Press Inc., 2017).

38. Neumann, E., Schaefer-Ridder, M., Wang, Y. & Hofschneider, P. H. Gene transfer into mouse lyoma cells by electroporation in high electric fields. *EMBO*

J. 1, 841–845 (1982).

39. Villemejane, Julien & Mir, Lluis M. Physical methods of nucleic acid transfer: General concepts and applications. *Br. J. Pharmacol.* 157, 207–219 (2009).

40. Venslauskas, Mindaugas S. & Šatkauskas, Saulius. Mechanisms of transfer of bioactive molecules through the cell membrane by electroporation. *Eur. Biophys. J.* 44, 277–289 (2015).

41. Gissel, Hanne, Lee, Raphael C. & Gehl, Julie. Electroporation and Cellular Physiology. in *Clinical aspects of electroporation* 9–17 (Springer, 2011). doi:10.1007/978-1-4419-8363-3_2.

42. Escoffre, Jean-Michel *et al.* What is (Still not) Known of the Mechanism by Which Electroporation Mediates Gene Transfer and Expression in Cells and Tissues. *Mol. Biotechnol.* 41, 286–295 (2009).

43. Mueller-Hartmann, Herbert. Method And Circuit Arrangement For Treating Biomaterial. (2006).

44. Gresch, Oliver *et al.* New non-viral method for gene transfer into primary cells. *Methods* 33, 151–163 (2004).

45. Lonza. Nucleofector ™ Technology Stretching Transfection Dimensions [Brochure]. (2018).

46. Zeitelhofer, Manuel *et al.* High-efficiency transfection of mammalian neurons via nucleofection. *Nat. Protoc.* 2, 1692–1704 (2007).

47. Riemen, Gudula *et al.* Buffer solution for electroporation and a method comprising the use of the same. doi:10.1037/t24245-000.

48. *Florabio Teknoloji San. Ve Tic. A.Ş.* Izmir, Turkey (Retrieved from the intranet (not publicly available) of Florabio, 2019).

49. OMNI Life Science GmbH & Co. KG. CASY Cell Counter & Analyzer [Operatord guide]. (2018).

50. Patterson, George H., Knobel, Susan M., Sharif, Wallace D., Kain, Steven R. & Piston, David W. Use of the green fluorescent protein and its mutants in quantitative fluorescence microscopy. *Biophys. J.* 73, 2782–2790 (1997).

51. Meleady, Paula *et al.* Sustained productivity in recombinant Chinese Hamster Ovary (CHO) cell lines: Proteome analysis of the molecular basis for a process-related phenotype. *BMC Biotechnol.* 11, 78 (2011).

52. Li, Shulin. Optimizing Electrotransfection of Mammalian Cells In Vitro. *Cold Spring Harb. Protoc.* 2006, pdb-prot4449 (2006).

53. Flanagan, Michael *et al.* Competitive electroporation formulation for cell therapy. *Cancer Gene Ther.* 18, 579–86 (2011).

54. Flanagan, Michael *et al.* Competitive DNA transfection formulation via electroporation for human adipose stem cells and mesenchymal stem cells. *Biol. Proced. Online* 14, 1–7 (2012).

55. Alamuru-Yellapragada, Neeraja P., Kapadia, Bandish & Parsa, Kishore V. L. In-house made nucleofection buffer for efficient and cost effective transfection of RAW 264.7 macrophages. *Biochem. Biophys. Res. Commun.* 487, 247–254 (2017).

56. Sugawara, Mitsuru *et al.* Ionic strength has a greater effect than does transmembrane electric potential difference on permeation of tryptamine and indoleacetic acid across Caco-2 cells. *Biochim. Biophys. Acta* 1564, 149–155 (2002).

57. Daniel Riggs, Charles & Bates, George W. Stable transformation of tobacco by electroporation: Evidence for plasmid concatenation. *Proc. Natl. Acad. Sci.* 83, 5602–5606 (1986).

58. Gaidamauskas, Ernestas *et al.* Evidence of two-step deprotonation of D D-mannitol in aqueous solution. *Carbohydr. Res.* 340, 1553–1556 (2005).

59. Van Den Hoff, Maurice J. B., Moorman, Antoon F. M. & Lamers, Wouter H. Electroporation in 'intracellular' buffer increases cell survival. *Nucleic Acids Res.* 20, 2902 (1992).

60. Ahkong, Quet Fah, Fisher, Derek, Tampion, William & Lucy, Jack A. Mechanisms of cell fusion. *Nature* 253, 194–195 (1975).

61. Gabriel, Bruno & Teissie, Justin. Generation of reactive-oxygen species induced by electropermeabilization of Chinese hamster ovary cells and their consequence on cell viability. *Eur. J. Biochem.* 223, 25–33 (1994).

62. Basu Bhargava Subhash Kalluri Ahmet Can Sabuncu, Gaurav *et al.* Enhanced Killing Effect of Nanosecond Pulse Electric Fields on PANC1 and Jurkat Cell Lines in the Presence of Tween 80. *J. Membr. Biol.* 245, 611–616 (2012).

63. Hu, Jiemiao, Cutrera, Jeffry & Li, Shulin. The impact of non-electrical factors on electrical gene transfer. in *Electroporation Protocols* vol. 1121 47–54 (Humana Press Inc., 2014).

64. Ribeiro, Sofia *et al.* Plasmid DNA Size Does Affect Nonviral Gene Delivery Efficiency in Stem Cells. *Cell. Reprogram.* 14, 130–137 (2012).

65. Lonza. *Amaxa™ 4D-Nucleofector™ Protocol for CHO-S (Invitrogen) For 4D-Nucleofector™ X Unit–Transfection in suspension*. www.lonza.com/nucleofection-citations (2010).

66. Finn, Gregory K., Kurz, Benedikt W., Cheng, Richard Z. & Shmookler Reis, Robert J. Homologous plasmid recombination is elevated in immortally transformed cells. *Mol. Cell. Biol.* 9, 4009–4017 (1989).

67. Hartley, James L. Why proteins in mammalian cells? in *Protein Expression in*

Mammalian Cells 1–12 (Humana Press, 2012).

68. Cherng, Jong Yuh *et al.* Effect of DNA topology on the transfection efficiency of poly((2-dimethylamino)ethyl methacrylate)-plasmid complexes. *J. Control. Release* 60, 343–353 (1999).

69. Von Groll, Andrea, Levin, Yan, Barbosa, Marcia C. & Ravazzolo, Ana P. Linear DNA low efficiency transfection by liposome can be improved by the use of cationic lipid as charge neutralizer. *Biotechnol. Prog.* 22, 1220–1224 (2006).

70. Xie, Ting-Dong & Tsong, Tian Y. Study of mechanisms of electric field-induced DNA transfection. V. Effects of DNA topology on surface binding, cell uptake, expression, and integration into host chromosomes of DNA in the mammalian cell. *Biophys. J.* 65, 1684–1689 (1993).

71. Potter, Huntington, Weir, Lawrence & Leder, Philip. Enhancer-dependent expression of human kappa immunoglobulin genes introduced into mouse pre-B lymphocytes by electroporation. *Proc. Natl. Acad. Sci.* 81, 7161–7165 (1984).

72. Stuchbury, Grant & Münch, Gerald. Optimizing the generation of stable neuronal cell lines via pre-transfection restriction enzyme digestion of plasmid DNA. *Cytotechnology* 62, 189–194 (2010).

73. SnapGene Viewer version 5.1.3.1 [Computer software]. Retrieved from http://snapgene.com (2020).

APPENDIX A

PREPARATION PROTOCOL OF TB4

This protocol aims to give details about components, preparation methods and quality parameters of TB4 buffer solution that is developed for transfection of CHO-DG44 cells with nucleofection.

Name of Chemical	**Cat. Nr.**	**For 1 L**	**Unit**	**Comment**
Ultrapure water		990	mL	
KH_2PO_4	P5655, Sigma	953	mg	Stir at least 30 min
K_2HPO_4	P2222, Sigma	3832	mg	Stir at least 30 min
$MgCl_2$	M4880, Sigma	1428	mg	
D-Mannitol	M9546, Sigma	45.5	g	
$Ca(NO_3)_2 * 4H_20$	C1396, Sigma	10	mL	Add from 40 mM solution in H_2O

Measure the following parameters as quality control of the solution.

Parameter	**Target**	**Tolerance**
pH	7.1	± 0.2
K^+ [mM]	44	± 2
Ca^{2+} [mM]	0.4	± 0.2
Osmolality [mOsm/kg H_2O]	345	± 5

Sterile filter the solution within 30 minutes with 0.2 μm pore size syringe filter and store at +4°C.