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DOKUZ EYLUL UNIVERSITY
IZMIR INTERNATIONAL
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ENGINEERING AND SYSTEMATIC COMPARISON OF CONSTITUTIVE PROMOTERS IN VARIOUS LINES OF CHINESE HAMSTER OVARY CELLS

YAGMUR TOKTAY

MOLECULAR BIOLOGY AND GENETICS

MASTER DISSERTATION

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YAGMUR TOKTAY

Assoc. Prof. Serif Senturk

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İZMİR ULUSLARARASI BİYOTIP VE GENOM ENSTİTÜSÜ MÜDÜRLÜĞÜ

YÜKSEK LİSANS TEZ ONAY FORMU

Dokuz Eylül Üniversitesi İzmir Uluslararası Biyotıp ve Genom Enstitüsü Genom Bilimleri ve Moleküler Biyoteknoloji Anabilim Dalı, Moleküler Biyoloji ve Genetik Yüksek Lisans Programı öğrencisi 2016850002 numaralı Yağmur TOKTAY, **"ENGINEERING AND SYSTEMATIC COMPARISON OF CONSTITUTIVE PROMOTERS IN VARIOUS LINES OF CHINESE HAMSTER OVARY CELLS"** konulu Yüksek Lisans tezini 27.03.2019 tarihinde başarılı olarak tamamlamıştır.

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TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

ABBREVIATIONS

CAG: CMV early enhancer/chicken β actin promoter

CHEF1a: Chinese hamster elongation factor-1α promoter

CHO: Chinese hamster ovary

CHO-WT: Chinese hamster ovary wild type cell line

CMV: Cytomegalovirus promoter

DHFR: Dihydrofolate reductase

EF1a: Elongation factor-1α promoter

EFS: Elongation factor-1α short promoter

EGFP: Enhanced green fluorescent protein

Fluc: Firefly luciferase

HSV TK: Herpes simplex virus thymidine kinase promoter

IRES: Internal ribosome entry site

PGK: Phosphoglycerate kinase promoter

rDNA: Recombinant DNA

Rluc: Renilla luciferase

SV40: Simian virus 40 promoter

UBC: Ubiquitin C promoter

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March 2019 Yagmur TOKTAY

ENGINEERING AND SYSTEMATIC COMPARISON OF CONSTITUTIVE PROMOTERS IN VARIOUS LINES OF CHINESE HAMSTER OVARY CELLS

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ABSTRACT

Following the developments in molecular biotechnology over the past two decades, the production of recombinant biological drugs has increased significantly. Chinese Hamster Ovary (CHO) cells are essentially the most preferred mammalian cell expression system for industrial manufacturing of biotherapeutics because of easy adaptation to grow in suspension, human-like post translational modifications and well-characterized transfection and gene amplification systems. In the present study, using recombinant DNA technology, we engineered a novel all-in-one dual-promoter reporter system to systematically compare the strength of natural viral, mammalian and endogenous promoters for high level of protein expression in various lines of CHO cells. We firstly studied a large panel of candidate promoters (CMV, SV40, HSV TK, PGK, EFS, EF1a, UBC, CAG and CHEF1a) in CHO-WT, CHO-DG44 and CHO-DG44 suspension cell lines for transient gene expression. Of nine promoters, luciferase assay revealed that CMV achieved the highest reporter activity. We supported our luciferase assay results via repeating the comparison of three strong promoters and one weak promoter with flow cytometry. Then, five strongest promoters were selected and placed on the backbone having mouse DHFR coding sequence to test the promoter strength in stably transfected cells. The recovery of stable cells is still in progress. So, after recovery, we will be able to compare these five strong promoters with our dual promoter system in long term culture. Conclusively, the dual-promoter reporter system eliminated the problems in co-transfection assays and proved to be a useful system to identify strong regulatory elements ensuring high levels of expression in CHO cells.

Key Words: CHO, recombinant DNA technology, promoter strength, dual promoter system

KONSTİTÜTİF PROMOTÖRLERİN MÜHENDİSLİĞİ VE ÇEŞİTLİ ÇİN HAMSTERİ YUMURTALIK HÜCRE HATLARINDA SİSTEMATİK KARŞILAŞTIRMASI

Yağmur Toktay, Dokuz Eylül Üniversitesi İzmir Uluslararası Biyotıp ve Genom Enstitüsü, yagmur.toktay@msfr.ibg.edu.tr

ÖZET

Son yirmi yılda, moleküler biyoteknolojideki gelişmelerin ardından, rekombinant biyoteknolojik ilaçların üretimi önemli ölçüde artmıştır. Çin hamsteri yumurtalık hücreleri endüstriyel biyoterapötiklerin üretiminde en çok tercih edilen memeli hücre ekspresyon sistemidir. Bu hücrenin avantajları süspansiyonda büyüyebilmesi, insan benzeri translasyon sonrası modifikasyonlar yapabilmesi ve iyi karakterize edilmiş transfeksiyon ve gen amplifikasyon sistemlerine sahip olmasıdır. Bu çalışmayı gerçekleştirmek amacıyla, rekombinant DNA teknolojisini kullanarak, yeni bir çift promotör raportör sistemi tasarladık. Bu sistem ile çeşitli CHO hücre hatlarında doğal viral, memeli ve endojen promotörleri en yüksek seviyede protein ekspresyonu için sistematik olarak karşılaştırıldı. İlk olarak, CHO-WT, CHO-DG44 ve CHO-DG44 süspanse hücre hatlarında promotör aday panelimizi (CMV, SV40, HSV TK, PGK, EFS, EF1a, UBC, CAG ve CHEF1a) test ettik. Lusiferaz analizi sonucunda, en yüksek raportör aktivitesini CMV promotöründe tespit ettik. Lusiferaz sonuçlarımızı, seçili dört promotörün akış sitometrisinde analiz edilmesi ile destekledik. Sonrasında, seçtiğimiz beş promotörü stabil hücre hatlarında karşılaştırmak amacıyla, DHFR içeren vektöre yerleştirdik. Stabil hücre hatlarının elde edilmesi halen devam etmektedir. Tamamlandığında, çift promotörlü sistemimiz ile güçlü beş promotörü uzun süreli hücre kültürü için karşılaştırabileceğiz. Sonuç olarak, çift promotör raportör sistemi, tipik olarak iki vektörün beraber verilmesinde görülen problemleri ortadan kaldırdı ve CHO hücrelerinde yüksek ekspresyon seviyeleri sağlayan güçlü düzenleyici elementleri tanımlamak için faydalı bir sistem olduğu kanıtlandı.

Anahtar Kelimeler: CHO, rekombinant DNA teknolojisi, promotör gücü, çift promotör sistemi

1. INTRODUCTION

1.1. Recombinant DNA Technology

Recombinant DNA (rDNA) is a molecule of DNA which is constructed by joining of DNA fragments from two different sources and then is introduced into a host cell (Carroll, 2013). The construction of rDNA is accomplished by artificial means which are collectively known as recombinant DNA technology. In other words, recombinant DNA technology is a manipulation of genetic materials in a desired way. It was firstly begun, in 1970, with the discovery of restriction enzymes and of their utilization in molecular genetics by Werner Arber, Daniel Nathans and Hamilton O. Smith who were all awarded with the Nobel Prize in Physiology or Medicine 1978 (Roberts, 2005). In 1972, a technique joining DNA molecules from different types of organisms, which was founded by Paul Berg receiving the Nobel Prize in Chemistry 1980, opened the door to the development of recombinant DNA technology (Jackson, D.A., Symons, R.H., Berg, P., 1972). Thanks to the collaboration of the laboratories of Stanley Cohen and Herbert Boyer, rDNA was made by joining parts of two different bacterial plasmids and then was inserted in the bacteria *Escherichia coli* to replicate itself, demonstrating the potential effect of recombinant DNA technology on biotechnology industry (Cohen, Chang, Boyer, & Helling, 1973). Thereafter, it brought along innovations such as that Genentech, first genetic-engineering company, marketed the first recombinant DNA drug, human insulin, in 1982 (Nielsen et al., 2014).

Recombinant DNA basically composes of a vector and an insert. Inserts are any short or long DNA pieces, which can be genomic DNA fragments, complementary DNAs, synthetic oligonucleotides or polymerase chain reaction (PCR) products (Carroll, 2013). On the other hand, vectors are small, circular, autonomously replicating DNA molecules and carriers. They can be plasmids, viruses or artificial chromosomes (Kurnaz, 2015). Most preferred vectors in gene cloning are plasmids which are circular, double stranded DNA molecules, acting as extra-chromosomal DNA in cells. They are easy to transfer from cell to cell and to isolate from a host cell. Three important features are required in plasmids – an origin of replication, a selectable marker gene and multiple cloning site (MCS) (Kurnaz, 2015). An origin of replication is necessary to initiate

replication of plasmids for the reproduction of itself. Providing a survival advantage, a selectable marker is required for the maintenance and the identification of the plasmid in the host. The most common selectable marker used in gene cloning is antibiotic-resistance gene encoding an enzyme that inhibits the effect of antibiotic. In the presence of antibiotic, a host cell taking the plasmid will be resistant and hence will survive whereas a host cell without the plasmid will die in a solid media containing antibiotic (Clark & Pazdernik, 2015a). The other feature, MCS, is a short segment and contains several unique restriction enzyme recognition sites. It enables the insertion of DNA avoiding a disruption on any of essential features of the plasmid (Kurnaz, 2015).

In generally, recombinant DNA technology has five steps (Figure 1.1). As a first step, a piece of DNA to be inserted into a vector is extracted from a donor genome. Then, both the vector and the insert are cut with restriction enzymes which create compatible sticky single stranded ends or blunt double stranded ends. DNA ligase enzyme fixes the interest of gene in vector by joining the fragments through catalyzing the formation of a phosphodiester bond between the 3'-OH of one strand and the 5'-monophosphate of the other DNA strand. The resulting chimeric molecule is next introduced into a host cell by transformation. In this step, prior to transformation, bacterial cells are exposed to some chemical or electrical treatments to make them permeable, ''competent'', to take up recombinant DNA from their environment. According to competent cell type, transformation can be done by either electroporation in which holes in the membrane are created by the application of short pulses of electric shock or Ca^{+2} treatment of cells followed by heat shock in which holes are generated in a solution containing divalent cations depolarizing cell membrane. Finally, the desired product is extracted from cells after the replication of the chimeric molecule autonomously or after it has become integrated into cell's chromosome.

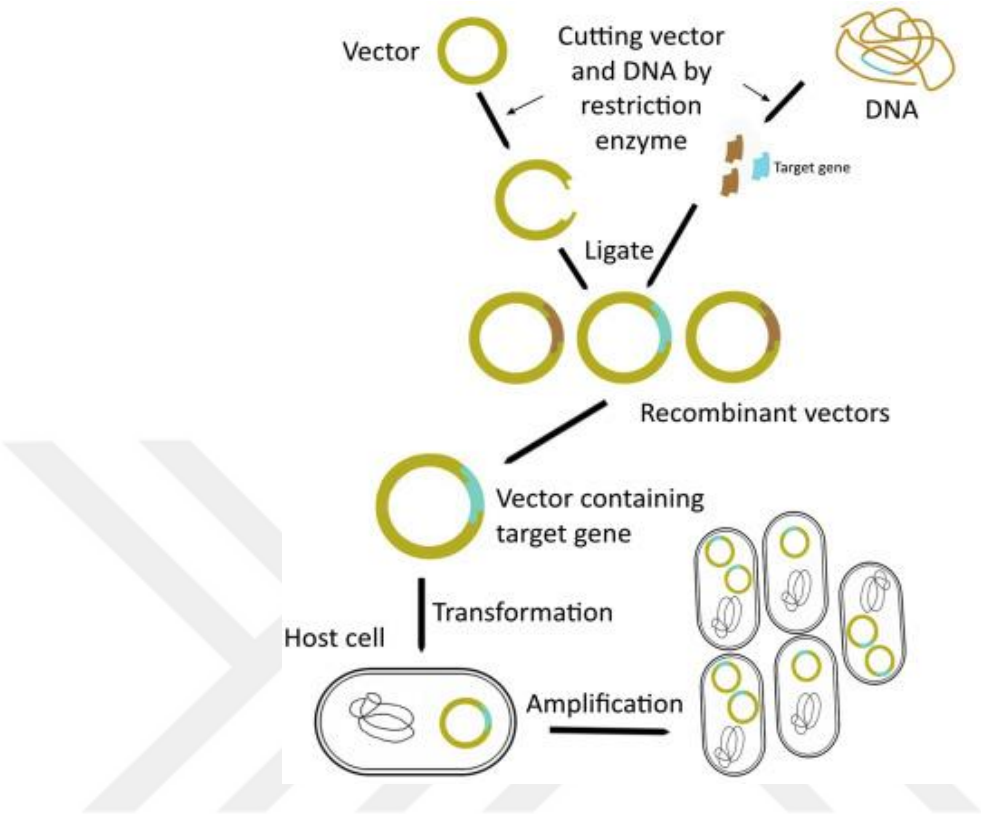

Figure 1.1: The summarization of recombinant DNA technology steps.1) Target gene is amplified from a donor DNA. 2) Both vector and target gene are cut by restriction enzymes. 3) They are joined together by ligation. 4) Recombinant vectors are transformed into a host cell. 5) The desired recombinant products are extracted from the cells. (Pham, 2017)

Recombinant DNA technology has been the basis of a broad range of applications with the acceleration gained in line with its developments. They are classified to four major group which are food and agricultural industry, pharmaceutical industry, energy applications and medical studies (Khan et al., 2016). Genetically modified products are important for food and agricultural industry. One example is the development of microbial strains producing enzymes which are used both in the improvement of yield with low cost and in the inhibition of food spoiling with increasing shelf life of products (Olempska-Beer, Merker, Ditto, & DiNovi, 2006). Another example is to make plants herbicide resistant, drought resistance or tolerance of salt (Khan et al., 2016). Gene therapy, tissue engineering, stem cell therapy, drug metabolism studies, diagnosis of diseases and genome editing are leading headlines of medical studies (Khan et al., 2016; Pham, 2017). Energy applications consist of genetically engineered microorganisms for the production of eco-friendly energy sources in consideration of environmental issues (Khan et al., 2016; Savakis & Hellingwerf,

2015; Tiwari & Pandey, 2012). Lastly, recombinant DNA technology has contributed a lot to pharmaceutical industry with therapeutic products such as vaccines, growth hormones, antibodies, anticancer drugs, antibiotics and recombinant proteins (Khan et al., 2016; Pham, 2017).

1.1.1. Recombinant Protein Therapeutics

Recombinant proteins have made a major breakthrough in a broad range of areas classifying in two major groups which are medical biotechnology and biological researches. They are useful to understand the fundamental principles of organisms during the basic researches which involve molecular biology, biochemistry, biophysics, cell biology and many others. Moreover, in medicine, they have a clinic importance in the treatment of various diseases, providing hormones, monoclonal antibodies, interleukins, FC fusion proteins, growth factors, enzymes, anticoagulants and drugs (Khan et al., 2016; Pham, 2017).

Therapeutic proteins are highly specific and have less potential to cause side effects compared to small molecules (Murray, Laurieri, & Delgoda, 2017). In contrast to chemically synthesized small molecules, recombinant protein therapeutics are produced in living cells and hence less probably elicit immunogenic responses (Murray et al., 2017). However, the choice of host cell is very significant since species origin can affect biological activity, compatibility, product safety and solubility (Jankowski et al., 2017). Bacteria is considered as the first choice since it is easy to cultivate and manipulate genetically while being cost-effective. However, bacteria are mostly used for the production of non-glycosylated proteins since they have not the required enzymes that facilitate proper folding and glycosylation of larger, complex proteins which mostly result in the formation of inclusion bodies in bacteria (Dumont, Euwart, Mei, Estes, & Kshirsagar, 2016; Graumann & Premstaller, 2006). Eukaryotic expression systems are preferred for such complex proteins. Yeasts are also cost-effective to cultivate, grow rapidly and have capacity to carry out glycosylation (Clark & Pazdernik, 2015b). Nevertheless, the glycosylation of yeasts contains a higher amount of mannose residues than that of humans, which cause immunogenic responses in humans (Gemmill & Trimble, 1999; Gerngross, 2004).

In the light of these informations, the best choice is mammalian expression systems as a host cell for the production of recombinant protein therapeutics. These mammalian cell lines mostly include baby hamster kidney cells (BHK21), human embryonic kidney cells (HEK293), human retina derived cells (PerC6), human fibrosarcoma (HT1080), murine myeloma cells (NS0 and Sp2/0) and Chinese hamster ovary cells (CHO) (Dumont et al., 2016; Durocher & Butler, 2009). Even though such mammalian-based expression systems are available, over 70% of the recombinant protein therapeutics are produced by the use of CHO cells because of reasons herein below (Dumont et al., 2016; Jayapal, Wlaschin, & Hu, 2007).

1.2. Chinese Hamster Ovary Cells

Chinese hamsters, also known scientifically as *Cricetulus griseus*, are small rodents with a striped back and a short tail, which are native to North Asia, extending from Mongolia and northeastern China to North Korea's northern regions (Feeney, 2012) (Figure 1.2). Since 1919, Chinese hamsters was used as research subjects because of their several biological features. They were influenceable to a range of infectious diseases and therefore were used firstly to type pneumococci (Hsieh, 1919). Thereafter, features of being small size, short duration of pregnancy, having low chromosome number and karyotype heterogeneity made them useful models for a wide range of biological studies as from cytogenetics and metabolic analysis to toxicology experiments (Jayapal et al., 2007). However, the cell lines derived from the tissues and organs of Chinese hamster has stolen the spotlight especially in biotechnological researches and industry (Figure 1.2). In 1986, the first recombinant therapeutic protein, tissue plasminogen activator synthesized in CHO cells by Genentech company, which led to onset of the production of CHO-based recombinant therapeutics (J. Y. Kim, Kim, & Lee, 2012).

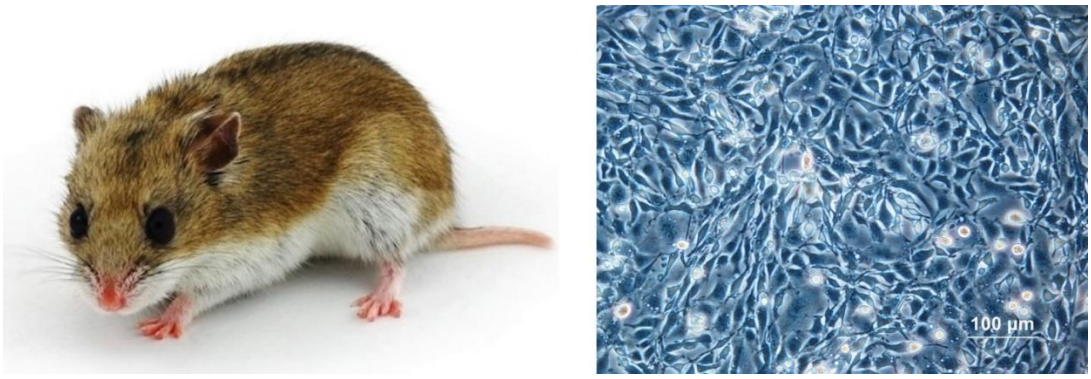

Figure 1.2: The representation of (left) Chinese hamster and (right) Chinese hamster ovary cells.

Over the past two decades, they are essentially the most preferred mammalian cell expression system for the production of biotherapeutics because of several reasons. Firstly, CHO cells are easy to maintain in cell culture and grow robustly and rapidly (Jayapal et al., 2007). Secondly, there are CHO-specific products developed and methods optimized for high transfection efficiency during the introduction of foreign DNA (Longo, Kavran, Kim, & Leahy, 2013; Reisinger, Steinfellner, Katinger, & Kunert, 2009). Thirdly, CHO cells are considered safe since they are refractory for the propagation of human viruses, minimizing biosafety risks and the requirement of viral clearance (Jayapal et al., 2007). Many biotherapeutics produced in CHO cells have been approved by the US Food and Drug Administration (FDA) (Dumont et al., 2016). Fourthly, most importantly, recombinant proteins compatible with and bioactive in humans can be produced with the use of CHO cells since they are able to perform human-like post translational modifications (J. Y. Kim et al., 2012). Fifthly, CHO cells can adapt both to grow in suspension culture which is a desired characteristic for a large scale production in industry and to grow in serum-free media which is ideal for the elimination of animal-origin products during manufacturing of biotherapeutics (Jayapal et al., 2007; J. Y. Kim et al., 2012). Lastly, there are well-characterized gene amplification systems for CHO cells, which are important to get over low titer yields and to achieve high specific productivity (Jayapal et al., 2007; J. Y. Kim et al., 2012; Kingston, Kaufman, Bebbington, & Rolfe, 2004).

1.2.1. Cell Lines

In 1957, at the University of Denver, Colorado, Theodore T. Puck isolated fibroblast cells from an ovary of a female Chinese hamster to establish the first CHO cell culture (Tjio & Puck, 1958). It is a common ancestor for the following lineages. Firstly, CHO-K1 cell line was maintained via sub-cloning of the original CHO cell line and was sequenced to be used as a primary reference resource for next cell lines while helping cell line engineering (Wurm & Hacker, 2011; Xu et al., 2011) (Figure 1.3). In 1980, a new cell line, CHO-DXB11, was generated by chemical mutagenesis of CHO-K1 cell line to generate missense mutation in a single allele of dihydrofolate reductase (DHFR) gene for rendering its activity (Urlaub & Chasin, 1980). On the other hand, maintaining a subline of original CHO cells from Theodore T. Puck, the laboratory of Dr. Siminovitch adapted cells to grow as suspension culture which is called CHO-S cell line in 1971. Then, they also generated MTX-resistant mutants, known as CHO-MTX, being suitable for DHFR deletion studies (Flintoff, Davidson, & Siminovitch, 1976). Since CHO DXB11 presents low DHFR activity, entire locus of DHFR gene was deleted through gamma radiation of other CHO cell group obtained from the Dr. Siminovitch's laboratory and CHO-DG44 cell line was generated in 1983 (Urlaub, Käs, Carothers, & Chasin, 1983).

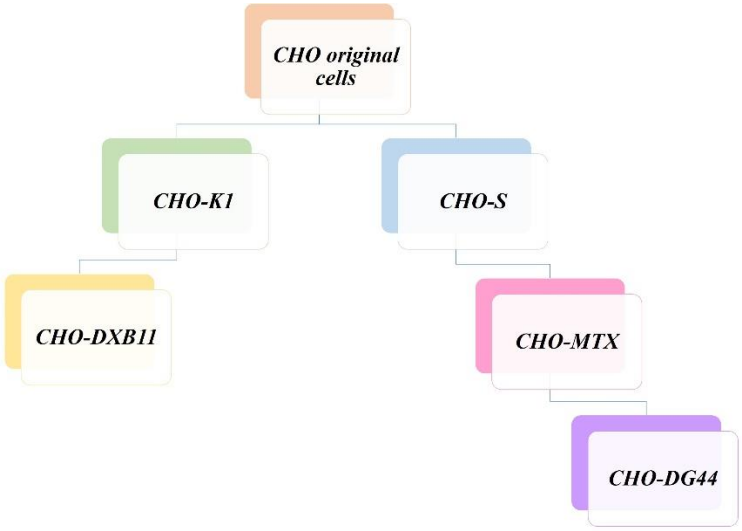

Figure 1.3: CHO cell lines. CHO-K1 cell line was obtained by sub-cloning of CHO original cells. CHO-DXB11 cell line was derived from CHO-K1 cells with nonfunctional single allele of DHFR. CHO-original cells adapted to suspension growth, named CHO-S cell line. Its MTX-resistant clones were known as CHO-MTX cell line which then was used to generate CHO-DG44 cell line with a deletion of DHFR's entire locus.

1.2.2. Gene Amplification Systems

A mutant organism, called auxotroph, is unable to synthesize a certain organic compound which the wild type can produce. Auxotroph organisms require an additional nutrient for their survival (Jayapal et al., 2007). Based on this mechanism, two well established gene amplification systems were developed to improve the protein production in CHO cells (Henriques, Elisa Rodrigues, Azeredo, Oliveira, & Rita Costa, 2009; Kingston et al., 2004; Wurm, 2004).

Dihydrofolate reductase (DHFR) is an enzyme which catalyzes the reduction of dihydrofolate to tetrahydrofolate that is a coenzyme using in the production of pyrimidine and purine (Kingston et al., 2004). Cells with DHFR deficiency only survive in the media supplemented with hypoxanthine and thymidine (HT) (Henriques et al., 2009). The media without HT provides a selection environment for cells until a functional copy of DHFR, a selection marker gene, is provided to cells with a plasmid. Methotrexate (MTX) which is an analog of dihydrofolate inhibits the activity of DHFR while increasing the selection pressure on cells (Kingston et al., 2004). When a gene of interest being linked to a functional copy of DHFR is introduced to cells in the media lacking HT, the increasing concentrations of MTX drive cells to produce more *DHFR* gene for their survival and hence more gene of interest through gene amplification (Kaufman & Sharp, 1982).

Glutamine synthetase is a housekeeping enzyme which catalyzes the reaction of glutamate and ammonia for the synthesis of glutamine (Fan, Frye, & Racher, 2013). This essential amino acid is an important nitrogen donor in the production of nucleotides. Either media supplemented with glutamine or a plasmid providing a functional copy of GS is required for the survival of glutamine auxotrophic cells. Methionine sulfoximine (MSX) is an analog of glutamate and is the inhibitor of GS enzyme (Kingston et al., 2004) (Figure 1.4). Similar to DHFR/MTX system, the use of MSX forces cells to have higher copies of GS gene and hence increased levels of gene of interest (Fan et al., 2013).

1.3.Dual Promoter System

Proteins often require a partner either for their stability, function and folding or for being tracked during biological researches. A vector containing two different genes of interest under a single promoter control is called bicistronic vector which enables researchers to study two proteins at the same time. Although it is certain that cells with one gene have definitely the another gene, the major problem of bicistronic vectors is the low expression levels of the second gene as compared with that of the upstream one (Ishii-Watabe, Xu, Uchida, Hayakawa, & Mizuguchi, 2002). The introduction of two separate vectors at once can be a solution for the co-expression of two different target genes. However, it may give rise to obtain cells having only one of genes to be studied and hence result in a requirement to screen cells for each gene of interest (Assur, Hendrickson, & Mancia, 2012). Recently, dual promoter systems which mimic bicistronic vectors with one additional promoter upstream of the second gene are preferred to overcome both issues aforementioned (Jeong et al., 2004).

1.4. Aim of The Study

The aim of the project was to engineer a dual promoter system which would be used to make a systematic comparison of regulatory elements for high expression levels in various lines of CHO cells. Promoters are leading regulatory elements determining expression in cells. Choosing a promoter with appropriate strength for desired gene expression levels is an important step in the vector engineering. In addition, CHO cells are the most favored mammalian cell line for the production of biotherapeutics due to the reasons aforementioned. Therefore, many studies have focused on cell line engineering to improve the productivity of CHO cells. In literature, there are examples for comparison of different constitutive or synthetic promoters in CHO cells (Brown, Sweeney, Mainwaring, & James, 2014; Ebadat et al., 2017; RunningDeer & Allison, 2004). However, a large panel of candidate promoters were compared in this project with the engineered dual promoter reporter system which overcame the issues related to co-transfection while also being cost- and time-effective tool. To achieve this goal, completed vector constructs were transfected to three lines of CHO cells which were then analyzed by luciferase assay. Secondly,

flow cytometry analysis was conducted as an alternative biological method to support the results. Lastly, select promoters were transfected to suspension cells to perform a comparison in long term culture. The experimental design is summarized in Figure 1.4.

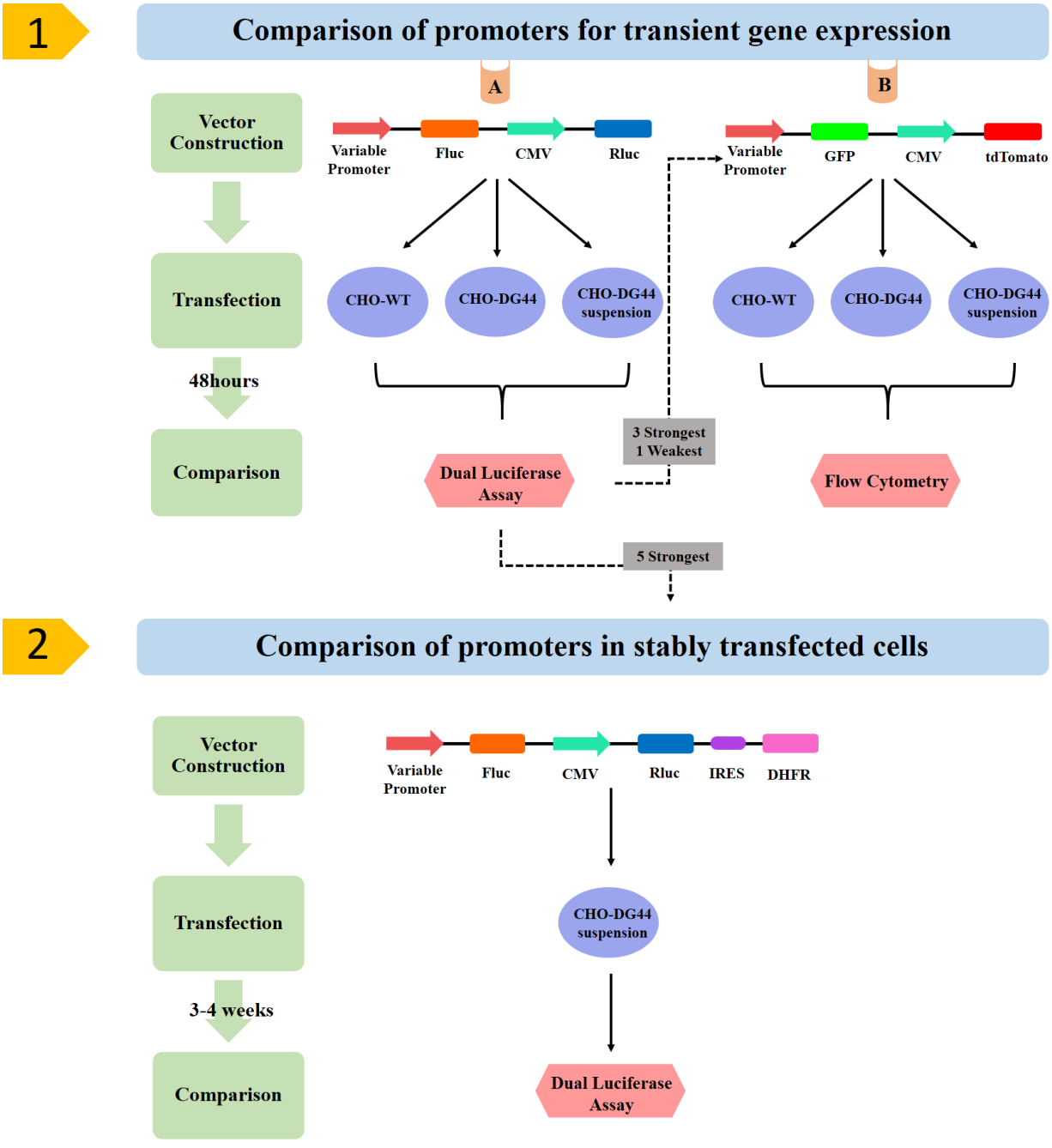

Figure 1.4: Experimental design of the project.

2. MATERIALS AND METHODS

2.1. Materials

2.1.1. Bacterial Strains

The bacterial strain used in the study was *Escherichia coli* DH10b (C3019I, New England Biolabs, USA).

2.1.2. Reagents Used in Bacteria Experiments

Luria-Bertani (LB) media, super optimal broth (SOB) media and antibiotic stocks were used for bacterial growth and cloning experiments. Their preparation was listed in Table 2.1.

Table 2.1: Reagents Used in Bacteria Experiments

Reagents	Preparation
LB Media	10g tryptone, 5g yeast extract and 10g NaCl were dissolved in 1 L ddH_2O. Autoclaved.
SOB Media	20g bactotryptone, 5g yeast extract, 0.58g NaCl, 10ml of 1M $MgCl_2$ and 10ml of 1M $MgSO_4$ were dissolved in 600-700ml ddH_2O. The volume of the homogenous mixture was finalized to 1L with ddH_2O. Autoclaved.
Ampicillin Stock (100mg/ml)	1000mg of ampicillin was dissolved in 10ml ddH_2O. Filter sterilized and stored at -20°C.
Kanamycin Stock (50mg/ml)	500mg of kanamycin was dissolved in 10ml ddH_2O. Filter sterilized and stored at -20°C.

2.1.3. Nucleic Acids

pcDNA3.1(+)myc-HisA, pLentiCRISPRv2.1 EFS-Cas9-P2A-Ven, pENTR5/EF1ap verA, TTi-GFP, pCAG ERT2CreERT2, pLV hUbC VP64 dCas9 VP64-T2A-GFP, pRL-TK, pRL-SV40, pEGFP-N1, pENTR4/tdTomato, pUC19 AEC01 plasmids were used in this study. The main backbone used to construct dual promoter system was pSF-CMV-Fluc-CMV-BCL2-Sbf1 (OG4071) purchased from Oxford Genetics.

2.1.4. Oligonucleotides

Oligonucleotides used in this study were synthesized by Sentegen (Ankara, Turkey) and Macrogen (Ankara, Turkey). The complete list of oligonucleotides is given in Table 2.2.

Table 2.2: Primer List

Primer ID	**Sequence (5'→3')**
SV40-F (**BglII**)	GGCGAC**AGATCT**CTGTGGAATGTGTGTCAGTT
SV40-R (**NotI**)	TAATAT**GCGGCCGC**CGAAAATGGATATACAAGCT
EFS-F (**BglII**)	GGCGCG**AGATCT**TAGGTCTTGAAAGGAGTGGG
EFS-R (**NotI**)	TAATAA**GCGGCCGC**CCTGTGTTCTGGCGGCAAAC
EF1a-F (**BglII**)	TTAAT**AGATCT**CCCGTCAGTGGGCAGAGCGC
EF1a-R (**NotI**)	TGGCA**GCGGCCGC**TATTAGTACCAAGCTAATTC
PGK-F (**BglII**)	TAATAA**AGATCT**GGGTAGGGGAGGCGCTTTTC
PGK-R (**HindIII**)	TATTAT**AAGCTT**CGAAAGGCCCGGAGATGAGG
TK-F (**BglII**)	GGCGCG**AGATCT**AATGAGTCTTCGGACCTCGC
TK-R (**NotI**)	TAATAA**GCGGCCGC**TTAAGCGGGTCGCTGCAGGG
UBC-F (**BglII**)	TATTAT**AGATCT**GGCCTCCGCGCCGGGTTTTG
UBC-R (**NotI**)	CAGTAT**GCGGCCGC**TCGTCTAACAAAAAAGCCAA
CHEF1a-F (**BglII**)	CATTAT**AGATCT**GGATGGCGGGGCTGACGTCG
CHEF1a-R (**EcoRI**)	CAGGAC**GAATTC**GTTGGATTTGAATTAGCGGT
Rluc-F (**SalI**)	TATTAA**GTCGAC**GCCACCATGACTTCGAAAGTTTATGA

Rluc-R (**SpeI**)	GCAGAC**ACTAGT**TTATTGTTCATTTTTGAGAA
EGFP-F **(NcoI)**	TAATATAGATCTGCCA**CCATGG**TGAGCAAGGGCGAGGA
EGFP-R (**XbaI**)	GGCGCG**TCTAGA**TTACTTGTACAGCTCGTCCA
tdTomato-F (**SalI**)	TATTAA**GTCGAC**GCCACCATGGTGAGCAAGGGCGAGGA
tdTomato-R (**SpeI**)	GGGCGG**ACTAGT**TTACTTGTACAGCTCGTCCA
IRES-F (**XbaI**)	TAGTGT**TCTAGA**TTCCGCCCCCCCCCCTAAC
IRES-R	GTTCAATGGTCGAACCATGGTGGC/ATCGTGTTTTTCAAA GGA
DHFR-F	TCCTTTGAAAAACACGAT/GCCACCATGGTTCGACCATTG AAC
DHFR-R (**BamHI**)	TCGGCG**GGATCC**TTAGTCTTTCTTCTCGTAGA
OG4071-Seq-F	TCAATCGTTGCGTTACACAC
OG4071-Seq-R	AGAATGGCGCTGGGCCTTTC

2.1.5. Restriction Endonucleases

All restriction enzymes, polymerases and T4 DNA ligase was listed in Table 2.3.

Table 2.3: Restriction Enzymes List

Enzyme Name	Catalog Number	Vendor
BclI	R0160S	New England Biolabs
BglII	R0144S	New England Biolabs
EcoRI	R3101S	New England Biolabs
HindIII	R3104S	New England Biolabs
NcoI	R0193S	New England Biolabs
NotI	R0189S	New England Biolabs
SalI	R3138S	New England Biolabs
SpeI	R0133S	New England Biolabs
XbaI	R0145S	New England Biolabs

XhoI	R0146S	New England Biolabs
Phusion polymerase	M0530S	New England Biolabs
Taq polymerase	EP1701	Thermo Fisher Scientific
T4 DNA Ligase	M0202S	New England Biolabs

2.1.6. Cell Culture Materials and Reagents

Tissue culture flasks and dishes, plates, cryovials and serological pipettes were general materials used in cell culture experiments and were obtained from Sarstedt (Nümbrecht, Germany). Penicillin/streptomycin solution, TrypLE express enzyme, dimethyl sulfoxide (DMSO), phosphate buffered saline (PBS) and fetal bovine serum (FBS) were used for the maintenance of all cell lines. Opti-MEM reduced serum media and Lipofectamine™ 3000 Transfection Reagent were used for the transfection of all cell lines. 4D-Nucleofector™ X Unit – Transfection was only used for the transfection of CHO-DG44 suspension cell line. Dulbecco's modified Eagle's medium/nutrient mixture F-12 (DMEM/F-12), Minimum essential medium – alpha (MEM-Alpha) and chemically defined DG44 medium (CD-DG44) was used to grow cell lines. L-glutamine and Pluronic™ F-68 Non-ionic Surfactant (100X) were used as supplementations of CD-DG44 media. All materials were listed in Table 2.4.

Table 2.4: Materials Used in Cell Culture

Materials	**Catalog Numbers**	**Vendors**
Penicillin-Streptomycin	15140122	Gibco
TrypLE Express Enzyme	12604013	Gibco
DMSO	472301-100ML	Sigma
PBS	70011036	Gibco
FBS	10500064	Gibco
Opi-MEM	31985062	Gibco
Lipofectamine™ 3000 Transfection Reagent	L3000015	Invitrogen
4D-Nucleofector™ X Unit – Transfection	V4XC- 3024	Lonza
DMEM/F-12	11320-033	Gibco

MEM-Alpha	BE12-169F	Lonza
CD-DG44	12610-010	Gibco
L-Glutamine (200mM)	A2916801	Gibco
Pluronic™ F-68 Non-ionic Surfactant	24040032	Gibco

2.1.7. Chemicals and Commercial Kits

Chemicals and commercial kits used in this study was listed in Table 2.5.

Table 2.5: Chemicals and Commercial Kits

Materials	Catalog Numbers	Vendors
Acetic acid	27225-2.5L-R	Sigma
Agarose	A9539-100G	Sigma
Ampicillin	A0166	Sigma
EDTA	E5134-500G	Sigma
Ethanol	920.026.2500	Isolab
Gel Loading Dye, Purple (6X)	B7024S	New England Biolabs
Isopropanol	24137-2.5L-R	Sigma
Kanamycin	11815024	Thermo Fisher Scientific
LB Broth with Agar	L2897-1KG	Sigma
NaCl	M106404.1000	Merck
Tris	T1503-1KG	Sigma
Tryptone	1553.0500	AppliChem
Yeast Extract	MB16401	Nzytech
1kb DNA Ladder	N3232S	New England Biolabs
100bp DNA Ladder	N3231S	New England Biolabs
Deoxynucleotide (dNTP) Solution Mix	N0447S	New England Biolabs
Dual-Glo® Luciferase Assay System	E2920	Promega
Mix and Go! E.coli Transformation Kit	T3002	Zymo Research

NucleoSpin® Plasmid	740588.50	Macherey Nagel
NucleoBond® Xtra Midi / Maxi	740412.10	Macherey Nagel
NucleoSpin® Gel and PCR Clean-up	740609.50	Macherey Nagel

2.1.8. Machines

Machines used in this study was listed in Table 2.6.

Table 2.6: Machines List

Machines	**Vendors**
Basic Power Supply	Biorad
MySpin^{TM} 6 Mini Centrifuge	Thermo Scientific
Water Bath and Lid	Nüve
pH Meter	Hanna
Microwave Oven	Beko
SimpliAmp Thermal Cycler	Applied Biosystems
Vortex Mixer	Thermo Scientific
Electrophoresis Gel System	Biorad
Centrifuge 5810R	Eppendorf
Centrifuge MicroCL 17R	Thermo Scientific
Incubator MaxQ 4000	Thermo Scientific
Heracell CO_2 Incubator	Thermo Scientific
Nanodrop 2000	Thermo Scientific
GelDoc XR^{+} with Image Lab Software	Biorad
Axio Vert.A1 Inverted Microscope	ZEISS
Centro XS^{3} LB 960 Microplate Luminometer	Berthold Technologies

2.2. METHODS

2.2.1. Chemically Competent Cell Preparation

A scratch of stock bacteria was taken using pipette tip which was then left into 13ml conical tube containing 5ml LB media. The tube was incubated at 37ºC at 225rpm for 16-18 hours. 1.1 ml of bacteria from the starter culture was added into 500ml erlen flask containing 110ml SOB media. It was incubated at 18ºC at 225rpm until getting the OD value of 0.4 – 0.6. Then, the culture was divided into two 50ml falcon tubes which were then put on ice for 10min. During this 10min, 10ml of 1X wash buffer and 10 ml of 1X competent buffer was prepared by adding an equal amount of dilution buffer onto them. Prepared buffers were put on ice. In the meantime, the tubes containing culture were centrifuged at 4ºC at 3200rpm for 10min. The supernatant was discarded. Cells were re-suspended gently in 5ml ice cold 1X wash buffer. Then, the tubes were centrifuged again at 4ºC at 3200rpm for 10min. The supernatant was discarded. Cells were re-suspended gently in 5ml ice cold 1X competent buffer. Finally, they were aliquoted on ice as 100µl per 1.5ml microcentrifuge tubes. Aliquots were stored at -80ºC.

2.2.2. Ligation

The volume of vector DNA and insert DNA used in the ligation reaction should be determined before combining the reagents. The vector mass was used as 50ng. Following the formulation below, the insert mass was also calculated. Depending on their concentrations, their volumes were determined for the ligation reaction. Then, all reagents were combined in a 1.5ml microcentrifuge tube. There were also two control samples. After combining reagents in amounts showed in Table 2.7., the reaction was incubated at room temperature for 2h.

$$insert\ mass = 3\ x\ \left(\frac{insert\ size}{vector\ size}\ x\ (vector\ mass)\right)$$

Table 2.7: Ligation Reaction Set up

Reagents	Cloning Sample	1st Control	2nd Control
T4 DNA Ligase Buffer 10X	1µl	1µl	1µl
Vector DNA	50ng	50ng	50ng
Insert DNA	x	-	-
Nuclease Free Water	Up to 10µl	Up to 10µl	Up to 10µl
T4 DNA Ligase	1µl	-	1µl

2.2.3. Transformation

100ng of plasmid was added into 50µl of competent bacteria near flame. It was placed on ice for 30min. Then, it was incubated in 42^{o}C water bath for 45-90 seconds. Next, it was again put on ice for 2min. 1ml of LB media was added onto it which was then incubated at 37^{o}C for 1hour. At the end of 1 hour, it was centrifuged at full speed for 1min. The supernatant was mostly discarded. The pellet cells were dissolved with left over and spread on LB-agar plates containing antibiotic. The plates were incubated at 37^{o}C for 16-18 hours.

2.2.4. Plasmid Isolation

Plasmid isolation was performed by following three techniques. Plasmids to be used in diagnostic digestion was isolated by kit-free isolation. Plasmids which were to be sequenced or to be used for transfection were isolated by following the manufacturer's instructions of either NucleoSpin® Plasmid or NucleoBond® Xtra Midi / Maxi kits.

2.2.4.1. Column-free Isolation

Column-free isolation was performed with the remaining buffer solutions of NucleoBond® Xtra Midi / Maxi plasmid isolation kit. Therefore, P1, P2 and P3 buffers were named to refer Resuspension, Lysis and Neutralization buffers, respectively.

A single colony of bacteria was picked up with a pipette tip. It was left into 5ml LB media containing 5µl ampicillin/kanamycin in 13 ml conical tube. Then, it was incubated at 37ºC at 225rpm for overnight. After 16-18h, it was transferred into a falcon tube and centrifuged at 4ºC at 3900rpm for 10min. The supernatant was discarded. 250µl buffer P1 was added onto it. The cells were re-suspended by pipetting and transferred to a 1.5ml microcentrifuge tube. 250µl buffer P2 was added onto it and the tube was inverted 8-12times. Then, it was incubated at room temperature for 5min. 300µl buffer P3 was added onto it and the tube was inverted 8-12times until blue samples turn colorless completely. Next, it was centrifuged at room temperature at full speed for 15min. The supernatant was brought to a new 1.5ml microcentrifuge tube. 0.8 times volume of cold (4ºC) isopropanol was added onto the supernatant. It was vortexed thoroughly and centrifuged at 4ºC at full speed for 15min. The supernatant was discarded and 700µl cold (4ºC) 70% ethanol was added onto the pellet. It was vortexed and centrifuged at full speed for 5min. The supernatant was discarded and the pellet was left to dry. After that, the pellet was re-suspended with 100µl TE buffer.

2.2.4.2. NucleoSpin® Plasmid Isolation

A single colony of bacteria was picked up with a pipette tip. It was left into 5ml LB media containing 5µl ampicillin/kanamycin in 13 ml conical tube. Then, it was incubated at 37ºC at 225rpm for overnight. After 16-18h, it was transferred into a falcon tube and centrifuged at 4ºC at 3900rpm for 10min. The supernatant was discarded. 250µl Buffer A1 was added onto the pellet for the re-suspension by pipetting up and down. It was transferred to a microcentrifuge tube. 250µl Buffer A2 was added onto it. The tube was inverted gently 6-8times and incubated at room temperature for 5min. 300µl Buffer A3 was added and mixed thoroughly by inverting the tube 6-8times until blue samples turn colorless completely. Then, the tube was centrifuged for 5min at 11000 x g at room temperature. A column was placed in a collection tube and 700µl of the supernatant was loaded onto the column. It was centrifuged for 1min at 11000 x g. The supernatant was discarded and the column was placed back into the collection tube. 500ul AW was pipetted onto the column which was then centrifuged for 1min at 11000 x g. 600µl Buffer A4 was added onto the column and then it was centrifuged for 1min at 11000 x g. The flow-through was discarded and the column was placed back into the collection tube. It was centrifuged for 2min at 11000 x g.

The collection tube was discarded and the column was placed in a 1.5ml microcentrifuge tube. 50µl Buffer AE was added onto the column. It was incubated for 1min at room temperature and then centrifuged for 1min at 11000x g.

2.2.4.3. NucleoBond® Xtra Midi / Maxi Plasmid Isolation

A 5ml starter culture of LB medium with a single colony was incubated at 37ºC at 225 rpm for 16-18h. The overnight culture was transferred into 500ml erlen flask containing 150ml LB medium with 150µl kanamycin/ampicillin. The culture was grown at 37ºC at 225rpm for 16-18hours. Then, it was centrifuged at 4ºC at 3900 rpm for 15min. The supernatant was discarded and the pellet was re-suspended completely in 8ml of Resuspension Buffer by pipetting up and down. 8ml of Lysis Buffer was added to the suspension and mixed gently by inverting the tube 5times. The mixture was incubated at room temperature for 5min. A column together with the inserted column filter was equilibrated with 12ml of Equilibration Buffer to wet the entire filter. The column was allowed to empty by gravity flow. 8ml of Neutralization Buffer was added to the suspension and mixed gently by inverting the tube until blue samples turns colorless completely. The lysate was loaded onto the column. It was again allowed to empty by gravity flow. Then, 5ml of Equilibration Buffer was used to wash the column with the filter. Next, the filter was pulled out and the column was washed with 8ml of Wash Buffer. 3.5ml of cold (4ºC) isopropanol was added to a new falcon tube and the plasmid DNA was eluted onto it with the application of 5ml Elution Buffer to the column. The tube was vortexed thoroughly and centrifuged at 4ºC at 3900rpm for 1hour. The supernatant was discarded and 2ml of cold (4ºC) 70% ethanol was added to the pellet. It was centrifuged at 4ºC at 3900rpm for 5min. Ethanol was removed carefully from the tube. The pellet was allowed to dry at room temperature. The DNA pellet is dissolved in 500µl of Tris buffer.

2.2.5. Restriction Enzyme Digestion of DNA

For cloning restriction digestion, 1-3µg template, 0.5µl enzyme, 5µl buffer and up to 50µl ddH_2O were combined in a PCR tube. Then, it was incubated at 37ºC for 8hours.

For diagnostic digestion, 1-5µg template, 0.25µl enzyme, 2.5µl buffer and up to 25µl ddH_2O were combined in a PCR tube. Then, it was incubated at 37ºC for 2hours.

2.2.6. Polymerase Chain Reaction

Following Table 2.8., appropriate amounts of ddH_2O, 5X buffer HF, 10mM dNTPs, 10µM forward primer, 10µM reverse primer, template DNA, DMSO and Phusion DNA polymerase were combined in a 0.2ml PCR tube. 5X Buffer GC was also supplied by the company since it was necessary for GC-rich templates. Then, PCR conditions in Table 2.9. were followed.

Table 2.8: PCR Reaction Set up with *Phusion polymerase*

Components	Amount
5X Buffer HF / GC	5µl
10mM dNTPs	0.5µl
10µM Forward Primer	1.25µl
10µM Reverse Primer	1.25µl
Template DNA	50ng
DMSO	0.75µl
Phusion DNA Polymerase	0.25µl
ddH_2O	Up to 25µl

Table 2.9: PCR Conditions

Cycle Step	Temperature	Time	# Cycles
Initial Denaturation	98ºC	30 sec	1
Denaturation	98ºC	10 sec	30
Annealing	60ºC	20 sec	
Extension	72ºC	20 sec	
Final Extension	72ºC	10 min	1
	4ºC	Hold	

Following Table 2.10., appropriate amounts of ddH_2O, 10X Dream Taq Buffer, 10mM dNTPs, 10µM forward primer, 10µM reverse primer, template DNA, DMSO and Taq DNA polymerase were combined in a 0.2ml PCR tube. Then, PCR conditions in Table 2.11. were followed.

Table 2.10: PCR Reaction Set up with *Taq polymerase*

Components	Amount
10X Dream Taq Buffer	2µl
10mM dNTPs	0.4µl
10µM Forward Primer	0.8µl
10µM Reverse Primer	0.8µl
Template DNA	50ng
DMSO	1µl
Taq DNA Polymerase	0.2µl
ddH_2O	Up to 20µl

Table 2.11: PCR Conditions

Cycle Step	Temperature	Time	# Cycles
Initial Denaturation	95°C	1 min	1
Denaturation Annealing Extension	95°C 60°C 72°C	30 sec 30 sec 1 min	35
Final Extension	72°C 4°C	10 min Hold	1

2.2.7. Colony PCR

All reaction components in required amounts showed in Table 2.12. were combined in a 0.2ml PCR tube. As a template, a single colony was picked with a pipette tip. The tip was swirled a few times in the tube and then was left into a 5ml of LB media to be grown according to the result of the experiment. PCR conditions in Table 2.13. were followed for the tube in where all reagents were combined.

Table 2.12: Colony PCR Reaction Set up

Components	Amounts
10X *Taq* Buffer	2µl
10mM dNTPs	0.4µl
10µM Forward Primer	0.8µl
10µM Reverse Primer	0.8µl
Taq Polymerase	0.2µl
Nuclease free water	15.8µl
Template	Colony

Table 2.13: Colony PCR Conditions

Cycle Step	Temperature	Time	# Cycles
Initial Denaturation	95°C	5min	1
Denaturation Annealing Extension	95°C 60°C 72°C	30 sec 30 sec 1 min	30
Final Extension	72°C 4°C	5 min Hold	1

2.2.8. Agarose Gel Electrophoresis

An appropriate amount of agarose, following the Table 2.14., was weighed and mixed with 50ml TAE buffer. The mixture was microwaved for 1-3min until the agarose is completely dissolved. The homogenized mixture was cooled to 50ºC and 2µl Safe View was added to it. Then, it was poured into a gel tray with the well comb placed. Waiting at room temperature for 20-30min, it was completely solidified. Then, the well comb was removed and the agarose gel was placed into the gel box which was filled with 1X TAE buffer until the gel is covered. A molecular weight DNA ladder was loaded into the first lane of the gel. 6X loading dye was added to the samples. They were vortexed and loaded into the additional wells of the gel. It was run at 100V for 45min. The gel was carefully removed from the gel box and samples were visualized using a device that has UV light.

Table 2.14: Agarose Gel Concentration to Resolve DNA Fragments

Percent Agarose Gel (w/v)	DNA Size Resolution
0.8%	800bp to 12kb
1.0%	500bp to 10kb
1.2%	400bp to 7kb
1.5%	200bp to 3kb
2.0%	50bp to 2kb

2.2.9. Gel Purification and PCR Clean Up

Inserts and cut vectors were gel-purified or PCR-cleaned by following the manufacturer's instructions of NucleoSpin® Gel and PCR Clean-up kit.

2.2.9.1. Gel Purification

The weight of an empty 1.5ml microcentrifuge tube was measured. The DNA fragment of interest was excised from an agarose gel and transferred to the tube. The weight of the tube was re-measured to determine the weight of the gel slice. For each 100 mg of gel slice, 200 µl Buffer NT1

was added into the tube. It was incubated for 5-10min at 50ºC while vortexing every 2-3min until the gel slice is completely dissolved. A column was placed into a collection tube and up to 700µl sample was loaded into the column. It was centrifuged for 30s at 11000 x g. The flow through was discarded and the column was placed back into the collection tube. 700µl Buffer NT3 was added to the column to wash the sample. It was centrifuged for 30s at 11000 x g. The flow through was discarded and the column was placed back into the collection tube. It was centrifuged for 1min at 11000 x g to remove Buffer NT3 completely. Then, the column was placed into a new 1.5 ml microcentrifuge tube. 30µl Buffer NE was added onto the column. It was incubated at room temperature for 1 min and then centrifuged for 1min at 11000 x g. The column was discarded and the eluted sample was stored at -20ºC.

2.2.9.2. PCR Clean-up

1 volume of sample was mixed with 2 volume of Buffer NT1. A column was placed into a collection tube and up to 700µl sample was loaded into the column. It was centrifuged for 30s at 11000 x g. The flow through was discarded and the column back was placed back into the collection tube. 700µl Buffer NT3 was added to the column. It was centrifuged for 30s at 11000 x g. The flow through was discarded and the column back was placed back into the collection tube. It was centrifuged for 1min at 11000 x g to remove Buffer NT3 completely. Then, the column was placed into a new 1.5 ml microcentrifuge tube. 30µl Buffer NE was added onto the column. It was incubated at room temperature for 1 min and then centrifuged for 1min at 11000 x g. The column was discarded and the eluted sample was stored at -20ºC.

2.2.10. Cell Culture Methods

2.2.10.1. Maintenance of CHO Cells

CHO-wild type (CHO-WT) cell line was kindly provided by Assoc. Prof. Gunes Ozhan from Dokuz Eylul University Izmir International Biomedicine and Genome Institute. The cells were grown in DMEM/F-12 medium (supplemented with 10% FBS and 1% Penicillin/Streptomycin) at 37ºC and 5% CO_2 conditions in incubator. CHO-DG44 cell line was provided from Lawrence

Chasin. The cells were grown in MEM-alpha medium (supplemented with 10% FBS and 1% Penicillin/Streptomycin) at 37ºC and 5% CO_2 conditions in incubator. CHO-DG44 cells were adapted to suspension growth by Ayca Zeybek Kuyucu and Umut Ekin in Dokuz Eylul University Izmir International Biomedicine and Genome Institute. The cells were grown in CD-DG44 medium (supplemented with L-glutamine as 8mM final concentration and Pluronic^{TM} F-68 as 18ml/L) at 37ºC and 8% CO_2 conditions in incubator.

2.2.10.2. Cell Thawing

The cryovial containing the frozen cells was removed from liquid nitrogen storage or -80˚C and immediately placed into a 37°C water bath. The cells were thawed in the water bath until there was just a small bit of ice left in the vial. Then, it was transferred into a laminar flow hood. The cells were re-suspended with 1ml of pre-warmed complete growth medium and transferred into 15ml centrifuge tube containing 9ml of complete growth medium. The cell suspension was centrifuged at 1200-1500rpm for 3-5min. The supernatant was discarded and the cell pellet was gently re-suspended in 8-10ml of complete growth medium. The cell suspension was transferred into 100mm cell culture dish.

2.2.10.2. Sub-culturing Cells

The cell culture media was discarded. The cells were washed with PBS which was then removed from the dish. TrypLE was added enough to cover the cell layer. The dish was gently rocked and incubated at 37ºC for 3-5 min. The detachment of cells was observed under the microscope and the equivalent of 2 volumes of pre-warmed complete growth medium was added. The cells were transferred to a 15ml centrifuge tube. 10µl sample was separated for cell counting with hemocytometer. According to the required cell number for the experiment, the cells were seeded into multi-well plates, flasks or dishes.

2.2.10.3. Cryopreservation

When cells reached to roughly 90% confluency, the cell culture media was discarded. The cells were washed with PBS which was then removed from the dish. TrypLE was added enough to

cover the cell layer. The dish was gently rocked and incubated at 37°C for 3-5 min. Cells harvested with complete growth medium were transferred into a falcon tube to be centrifuged at 1200-1500rpm for 3-5min. The supernatant was discarded and the cell pellet was gently re-suspended in in cold freezing medium (containing 72% medium, 20% FBS and 8% DMSO) at the concentration of 10^6 cells/vial. The vial was held at -20°C for 1 hour and then transferred to -80°C. The vials were placed into liquid nitrogen tank for long term storage.

2.2.11. Transfection

In the first part of the project, CHO-WT, CHO-DG44 and CHO-DG44 suspension cell lines were all transfected by following the manufacturer's instructions of Lipofectamine™ 3000 Transfection Reagent. On the other hand, CHO-DG44 suspension cell line was transfected by following the manufacturer's instructions of 4D-Nucleofector™ X Unit – Transfection to obtain stable cells for the second part of the project.

2.2.11.1. Lipofectamine™ 3000 Transfection Reagent

Two separate 1.5ml microcentrifuge tubes were labeled for OptiMEM plus Lipofectamine 3000 and OptiMEM plus P3000 reagent. For 48-well plate, OptiMEM medium was used 12.5µl per well while Lipofectamine 3000 was 0.75µl/well and P3000 reagent was 0.5µl/well for 0.25µg DNA. To prepare a master mix, the amount of reagents was multiplied by the number of total samples with an extra. Then, reagents were combined in the previously labeled tubes. The mixture of OptiMEM and P3000 was equally divided onto plasmids to dilute DNA. The combination of OptiMEM and Lipofectamine 3000 was also equally added into them. The tubes were vortexed and incubated at room temperature for 15min. Finally, 25µl of each DNA-lipid complex was added to the cultured cells. After 48h, the transfection procedure was terminated, and the cells were analyzed.

2.2.11.2. 4D-Nucleofector™ X Unit – Transfection

Cells collected from the flask were centrifuged at 100 x g for 10 minutes. The supernatant was discarded and the pellet was re-suspended with appropriate amount of Nucleofector solution

according to the number of total samples. Then, it was distributed to 1.5ml microcentrifuge tubes as $1x10^6$ cells/100ul. DNAs were added onto them as being 3ug/ul concentration. Master mixes containing cells and DNAs were transferred to Nucleocuvette vessels for Nucleofection process (recommended program of FF-137). After run completion, cells were mixed with pre-warmed media by gently pipetting up and down with the use of supplied sterile Pasteur pipettes. Then, they were cultured into pre-prepared wells containing 1.4ml media.

2.2.12. Dual Luciferase Reporter Assay

The cell culture media was discarded from the wells and the cells were washed with PBS twice for CHO-WT and CHO-DG44 cells. For CHO-DG44 suspension culture, the cell culture media was collected to 1.5ml microcentrifuge tubes and was centrifuged at 1.5rpm for 5min. They were also washed with PBS twice, repeating centrifugation step. 25µl/well PBS and 25µl/well Luciferase Reagent were combined in a tube. The mixture was equally divided into the wells to lyse the cells and start firefly luciferase activity. After 10minutes, lysed cells were collected and transferred into 96-well white plate. The firefly luminescence was measured using the luminometer. Then, Dual-Glo Stop&Glo Reagent was prepared with buffer in 1:100 ratio according to the number of total samples. 25µl/well from this mixture was added onto the wells. The Renilla luminescence was measured using the luminometer. The ratio of luminescence from the experimental reporter (Fluc) to luminescence from the control reporter (Rluc) was calculated to plot the graph.

2.2.13. Flow Cytometry

Adherent cells were firstly trypsinized and collected with complete medium while cell culture media was directly collected for suspension culture. Then, collected cells were transferred to a 15ml falcon tube and were centrifuged at 1.5rpm for 2min. The supernatant was discarded and the pellet was re-suspended with 200ul DAPI-PBS in the dark. It was incubated on ice for 2min. Then, it was centrifuged at 1.5rpm for 2min. The supernatant was discarded. The pellet was re-suspended with 100ul FACS buffer and was transferred to FACS tubes. Finally, it was left to flow cytometry facility. Reads were analyzed by FlowJo V10 software.

3. RESULTS

3.1. Promoters and Reporters Were Obtained from Related Plasmids

Molecular cloning studies were begun with the extraction of promoters and reporter genes from the related plasmids. pcDNA3.1 (+) / myc-HisA, pLentiCRISPRv2.1 EFS-Cas9-P2A-Ven, pRL-TK, TTi-GFP and pRL-SV40 plasmids were PCR-amplified with corresponding primers (Table 2.2) to obtain SV40 promoter, EFS promoter, HSV TK promoter, PGK promoter and Renilla luciferase reporter gene, respectively (Figure 3.1 and 3.2).

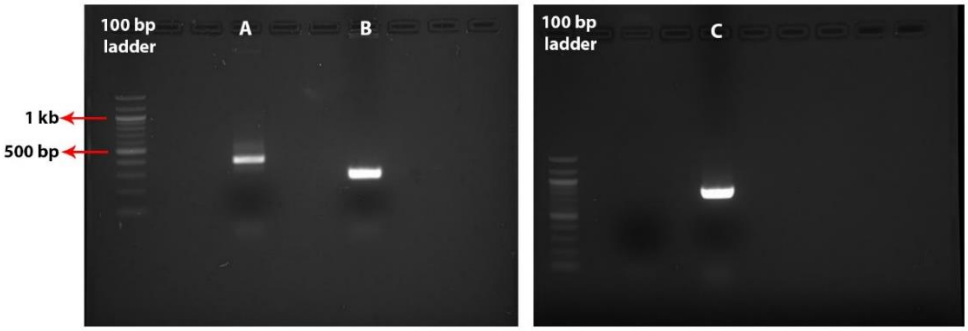

Figure 3.1: The amplification of SV40, EFS and HSV TK promoters. pcDNA3.1(+)/myc-HisA (A), pLentiCRISPRv2.1 EFS-Cas9-P2A-Ven (B) and pRL-TK (C) were PCR-amplified to obtain SV40 promoter (371bp), EFS promoter (256bp) and TK promoter (752bp), respectively. Gel electrophoresis was performed on 2% agarose gel.

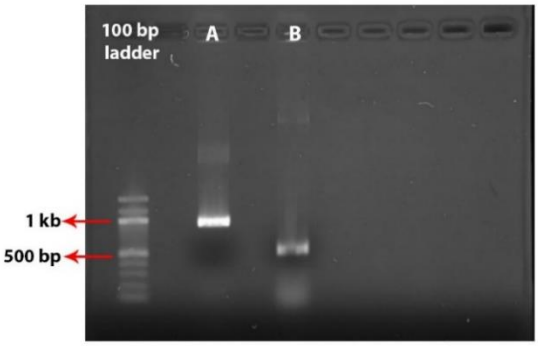

Figure 3.2: The amplification of Rluc reporter and PGK promoter. pRL-SV40 (A) and TTi-GFP (B) were PCR-amplified to obtain Rluc reporter gene (936bp) and PGK promoter (500bp), respectively. Gel electrophoresis was performed on 1.8% agarose gel.

pENTR5-EF1a VerA plasmid was used as a donor template to obtain EF1a promoter (Figure 3.3). As EF1a promoter contains BglII restriction site within its sequence, sequential digestion method was applied to it. For that, in four separated tubes, EF1a promoter was firstly

incubated with NotI restriction enzyme at 37ºC for 8 hours. Then, with the addition of BglII restriction enzyme, time-dependent incubation was set up as holding the tubes at 37ºC for 10min, 20min, 30min and 1hour (Figure 3.3). To extract the EF1a promoter from the gel, it was cut under UV in the time zones (10 min, 20 min and 30 min) where the single band (1190 bp) was more intense than the double bands (628 bp and 556 bp).

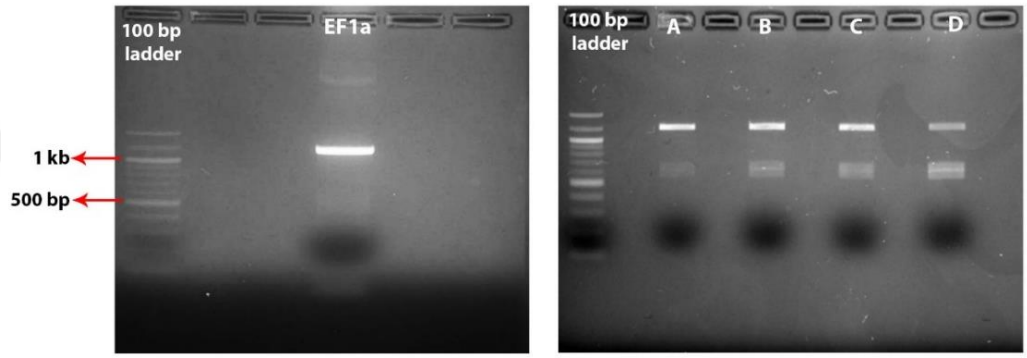

Figure 3.3: Cloning preparation of EF1a promoter. (Left) pENTR5/EF1ap VerA was PCR-amplified to obtain EF1a promoter (1190bp). (Right) The representation of time-dependent restriction digestion incubation of the EF1a promoter – expected band sizes were 1190bp, 628bp and 556bp. Time-zones were A) 10min, B) 20min, C) 30min and D) 1h. Both gel electrophoresis experiments were performed on 1.6% agarose gel.

pLV hUbC VP64 dCas9 VP64-T2A-GFP and pEGFP-N1 plasmids were used to obtain UBC promoter and EGFP reporter gene, respectively (Figure 3.4).

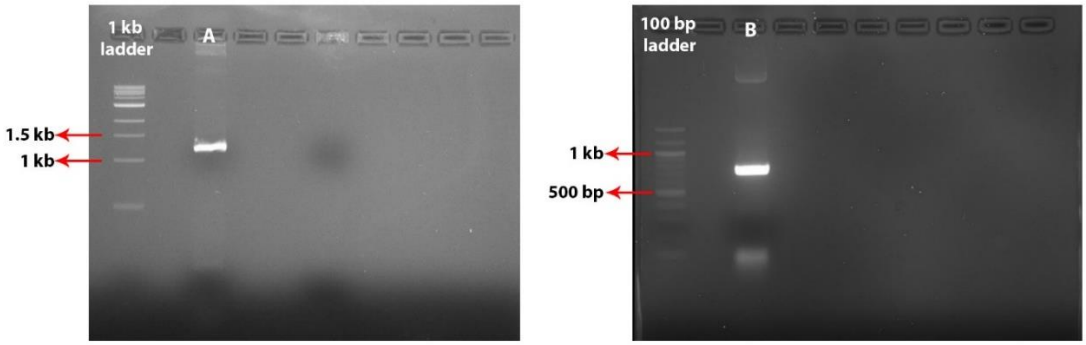

Figure 3.4: The amplification of UBC promoter and EGFP reporter gene. pLV hUbC VP64 dCas9 VP64-T2A-GFP (A) and pEGFP-N1 (B) plasmids were PCR-amplified to obtain UBC promoter (1211bp) and EGFP reporter gene (720bp), respectively. Both gel electrophoresis experiments were performed on 1.6% agarose gel.

tdTomato gene has a dimer orientation. For this reason, the cut-and-paste method was applied with the help of restriction enzymes instead of being obtained with primers. After cloning of the EGFP reporter was completed, CMV-GFP-CMV-Bcl2 (CGCB) vector was digested with SalI and

SpeI enzymes while pENTR4-tdTomato was incubated with SalI and XbaI restriction enzymes. SpeI and XbaI enzymes produced compatible cohesive ends for ligation (Figure 3.5). Both cut vector (5795bp) and tdTomato reporter gene (1476bp) were extracted from the gel by performing the gel purification kit.

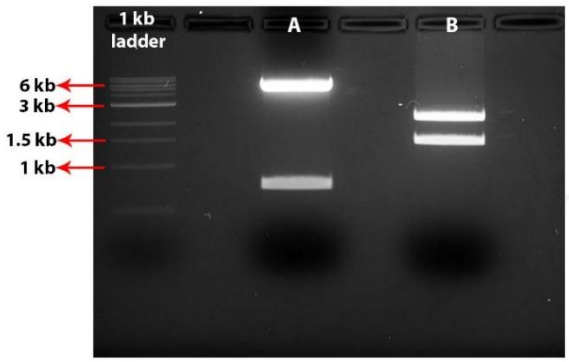

Figure 3.5: Cloning preparation of CGCB vector and tdTomato reporter gene. A) CGCB vector was digested with SalI and SpeI restriction enzymes – expected band sizes were 5795bp and 741bp. B) pENTR4-tdTomato plasmid was digested with SalI and XbaI restriction enzymes – expected band sizes were 2254bp and 1476bp. Gel electrophoresis was performed on 1.4% agarose gel.

The genomic DNA of CHO-WT DNA was PCR-amplified with corresponding primers to obtain CHEF1a promoter. Since it had high GC content, gradient PCR was established by increasing the temperature values (60^{o}C, 63^{o}C, 66^{o}C, 69^{o}C, 72^{o}C and 75^{o}C) (Figure 3.6).

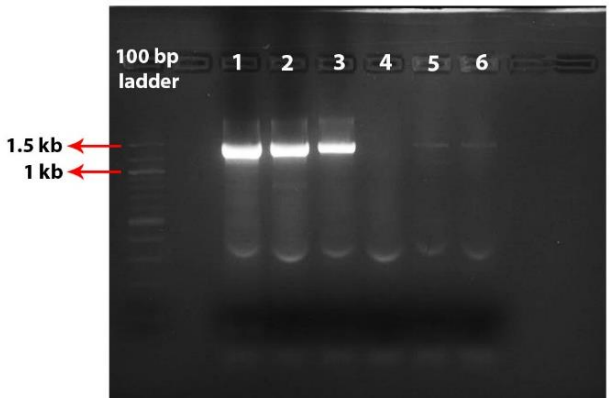

Figure 3.6: The amplification of CHEF1a promoter. The genomic DNA of CHO-WT was PCR-amplified to obtain CHEF1a promoter (1473bp). Gradient PCR conditions were 60^{o}C (1), 63^{o}C (2), 66^{o}C (3), 69^{o}C (4), 72^{o}C (5) and 75^{o}C (6). Gel electrophoresis was performed on 1.4% agarose gel.

TTi-GFP vector had intermediary role in the cloning of CAG promoter. Firstly, it was obtained from pCAG ERT2CreERT2 plasmid with SalI and EcoRI restriction enzymes. Then, it

was cloned into TTi-GFP vector which was simultaneously restricted by XhoI and EcoRI enzymes. Following ligation and transformation steps, 8 colonies were chosen to isolate TTi-GFP vector containing CAG promoter. They were exposed to diagnostic digestion with SpeI restriction enzyme (Figure 3.7).

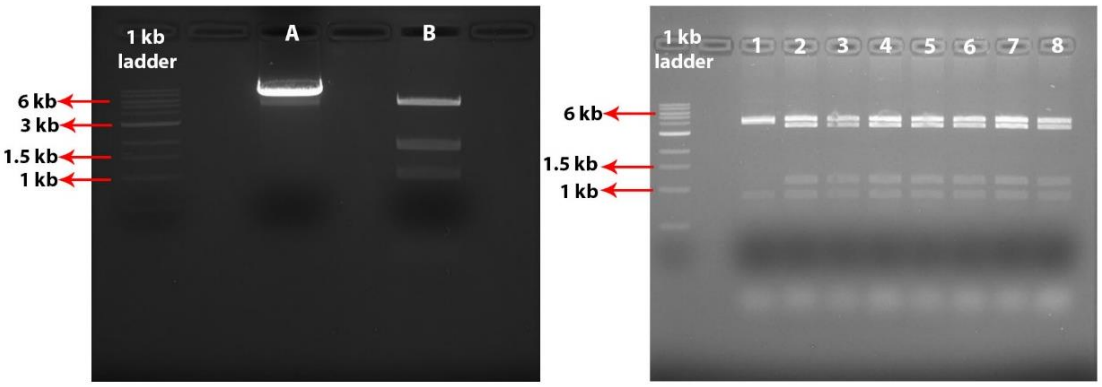

Figure 3.7: The cloning of CAG promoter into TTi-GFP vector. A) TTiGFP plasmid was digested with XhoI and EcoRI restriction enzymes – expected band sizes were 8100bp and 17bp. B) pCAG ERT2CreERT2 plasmid was digested with SalI and EcoRI restriction enzymes – expected band sizes were 5047bp, 1718bp (CAG promoter) and 965bp. (1-8) TTi-GFP plasmids containing CAG promoter were digested with SpeI restriction enzyme – expected band sizes were 4296bp, 3493bp, 1146bp and 883bp. Both gel electrophoresis experiments were performed on 1% agarose gel.

All colonies except first one were exhibited the expected band sizes and therefore a successful cloning. One of them was exposed to restriction reaction with BclI and EcoRI enzymes to obtain CAG promoter (Figure 3.8). CAG promoter (expected band length in 1725bp) was purified from agarose gel by performing gel purification kit.

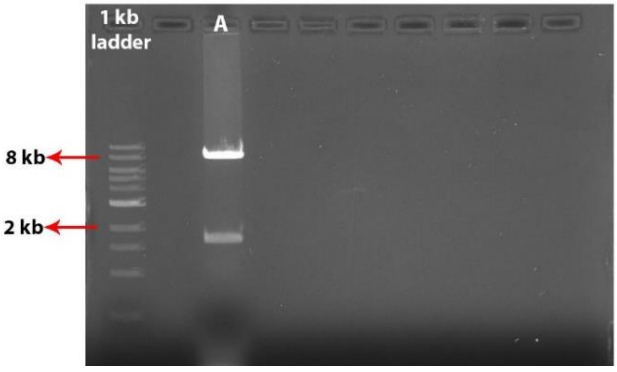

Figure 3.8: The obtainment of CAG promoter. A) TTi-GFP vector containing CAG promoter was restricted with BclI and EcoRI enzymes – expected band lengths were 8094bp and 1725bp – to obtain CAG promoter (1725bp). Gel electrophoresis was performed on 0.8% agarose gel.

3.2. Promoters Were Cloned into the Backbone Containing Fluc/Rluc Reporters

It was sufficient to clone Rluc reporter into the gene region downstream of the second promoter since OG4071 vector already contains Fluc reporter. For this reason, OG4071 vector was left for incubation with SalI and SpeI enzymes which were also used to cut Rluc reporter gene (Figure 3.9). The band of 6721bp to be used for cloning was purified from the gel with the help of gel purification kit.

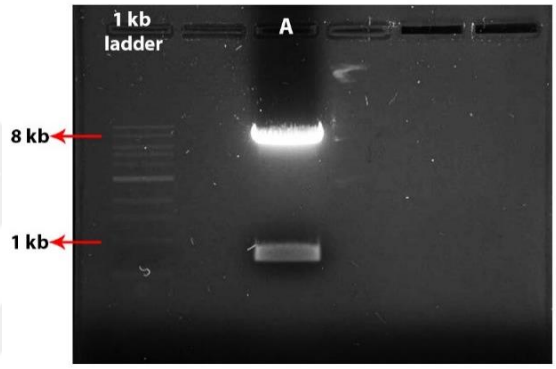

Figure 3.9: The preparation of OG4071 plasmid for cloning of Rluc. A) The representation of OG4071 plasmid digested with SalI and SpeI restriction enzymes – expected band sizes were 6721bp and 741bp. Gel electrophoresis was performed on 1.2% agarose gel.

Following ligation and transformation steps, the purified Rluc reporter gene was cloned into the purified OG4071 plasmid cut in the gene region downstream of the second promoter. Five colonies were chosen for colony PCR to confirm the cloning experiment (Figure 3.10). Two successful colonies were sequenced for validation. Completed backbone design was shown in Figure 3.11.

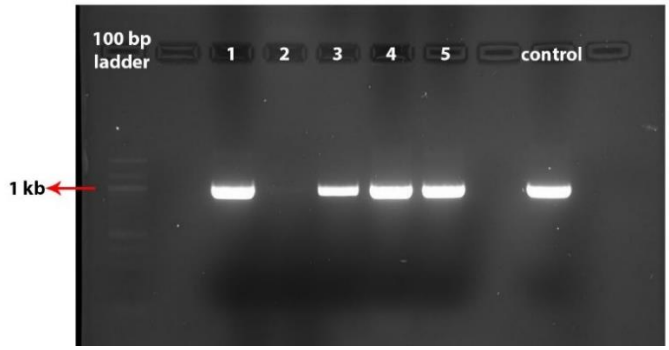

Figure 3.10: The validation of Rluc reporter gene. (1-5) The reaction of the five selected colonies for colony PCR with Rluc primers resulted in the expected 936bp band images. (Control) The control sample was established with the use of purified Rluc gene as a template – expected band size was 936bp. Gel electrophoresis was performed on 1.8% agarose gel.

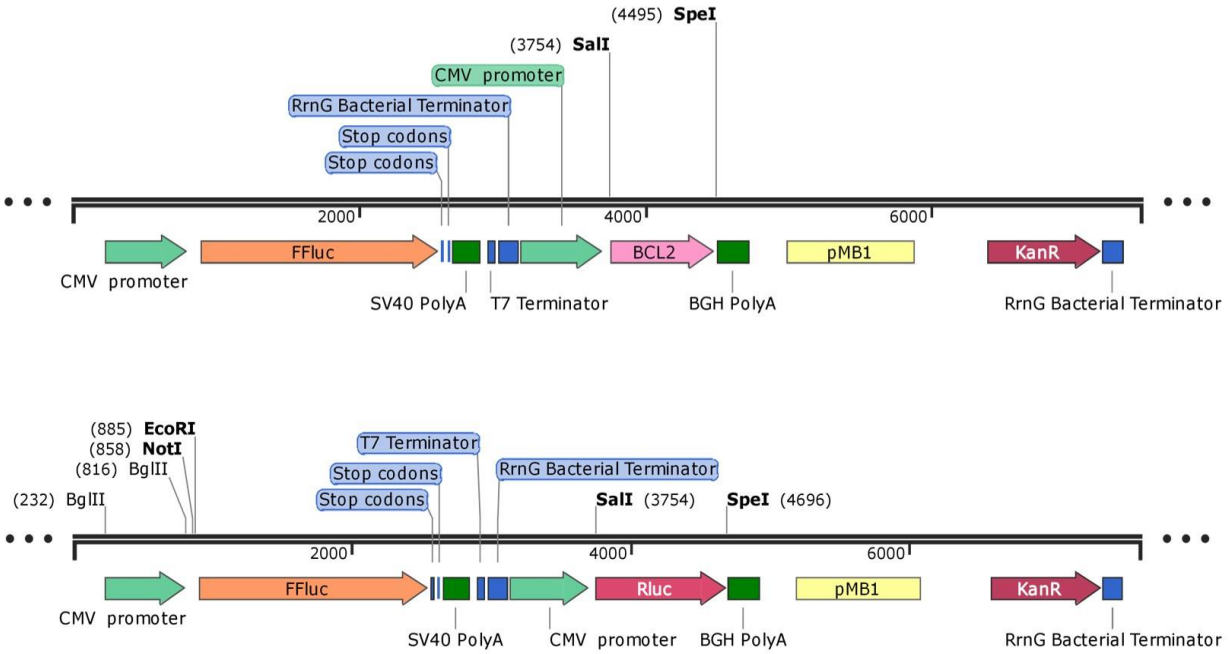

Figure 3.11: Vector design of CFCR backbone. (Up) The original vector map of OG4071 which was restricted with SalI and SpeI enzymes to remove BCL2 gene. (Bottom) The map of CFCR vector which was constructed with Rluc reporter replaced for BCL2 gene. Vector maps were created by SnapGene software.

In the light of our cloning strategies, most purified promoters which were SV40, TK, EFS, EF1a, PGK and UBC were left for incubation with BglII and NotI restriction enzymes while cloning of CAG and CHEF1a promoters was done with BglII and EcoRI restriction enzymes. Therefore, our first backbone, CMV-Fluc-CMV-Rluc (CFCR), was also restricted separately with two groups of these enzymes (Figure 3.12).

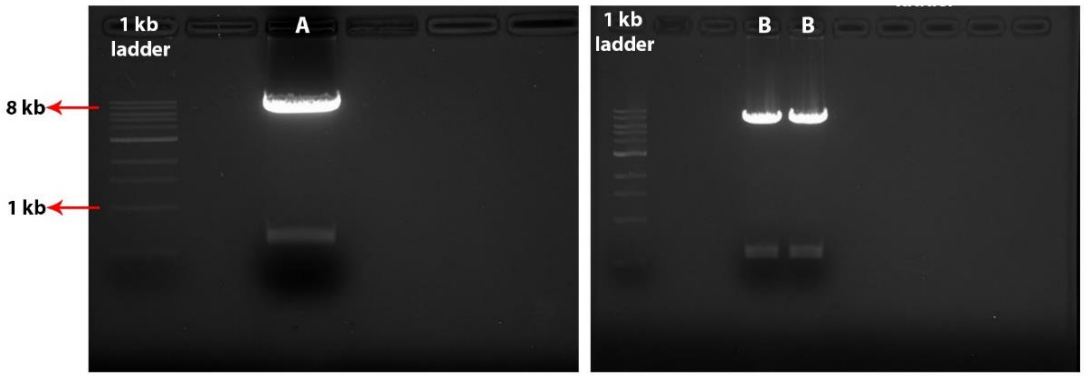

Figure 3.12: The preparation of CFCR plasmid for promoter cloning. A) CFCR plasmid was digested with BglII and NotI restriction enzymes – expected band sizes were 7037bp, 584bp and 42bp. B) CFCR plasmid was digested with BglII and EcoRI restriction enzymes – expected band sizes were 7010bp, 584bp and 42bp. Gel electrophoresis was performed on 0.8% agarose gel.

The purified, cut CFRCR plasmid was used for ligation reactions with the previously purified SV40, EFS, HSV TK and PGK promoters. After transformation, 4 colonies for each were chosen for colony PCR to validate the promoter cloning experiment (Figure 3.13). For SV40 and EFS promoters, two colonies could be chosen per each group. However, for HSV TK and PGK promoters, only one colony was successful per each group according to the results of colony PCR. To carry on our cloning experiments in a controlled manner, it was decided that two samples per vectors should have been chosen to sequence and validate the cloning as one being back-up sample. Therefore, colony PCR was repeated for HSV TK and PGK promoters (Figure 3.14).

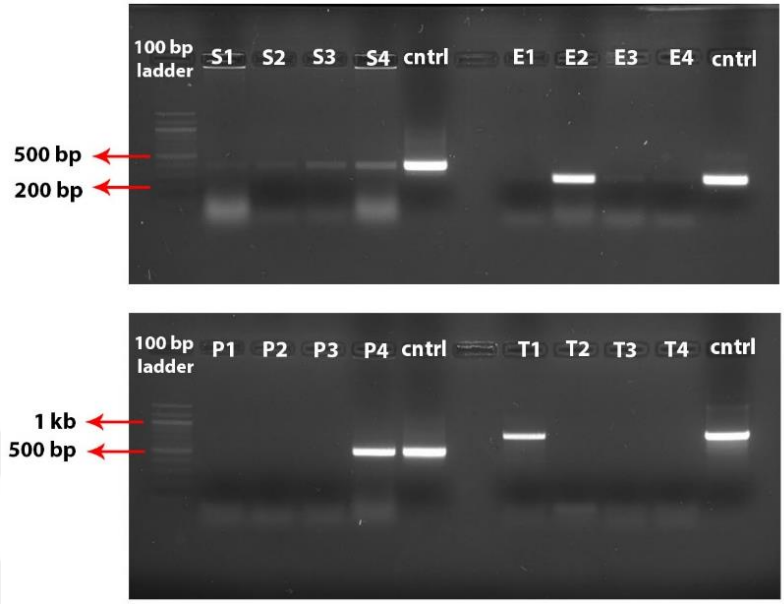

Figure 3.13: The validation of cloning of SV40, EFS, PGK and HSV TK promoter. Four colonies for each promoter cloning were used for colony PCR with the associated primers – expected band sizes were 371bp for (S1-S4), 256bp for (E1-E4), 500bp for (P1-P4) and 752bp for (T1-T4). The control samples were established with the use of the purified promoters as a template – expected band sizes were 371bp for SV40 (S) promoter, 256bp for EFS (E) promoter, 500bp for PGK (P) promoter and 752bp for HSV TK (T) promoter. Gel electrophoresis was performed on 2% agarose gel.

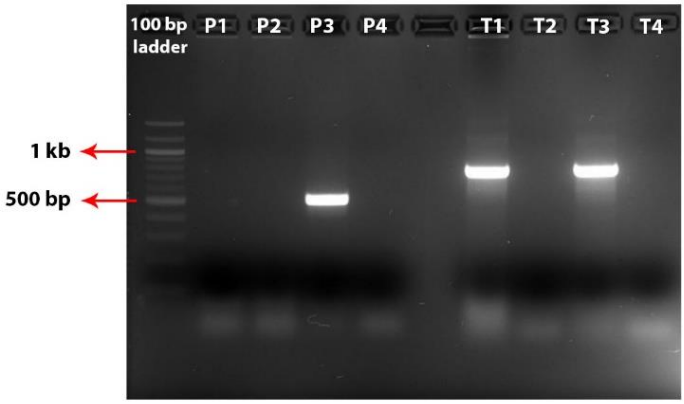

Figure 3.14: The validation of cloning of PGK and HSV TK promoters. Four colonies for each promoter cloning were used for colony PCR with the associated primers – expected band sizes were 500bp for PGK promoter (P1-P4) and 752bp for HSV TK promoter (T1-T4). Gel electrophoresis was performed on 2% agarose gel.

According to the expected band sizes, two colonies per each promoter were isolated by kit-free isolation. All isolated plasmids were subjected to diagnostic digestion with BglII and NotI enzymes (Figure 3.15).

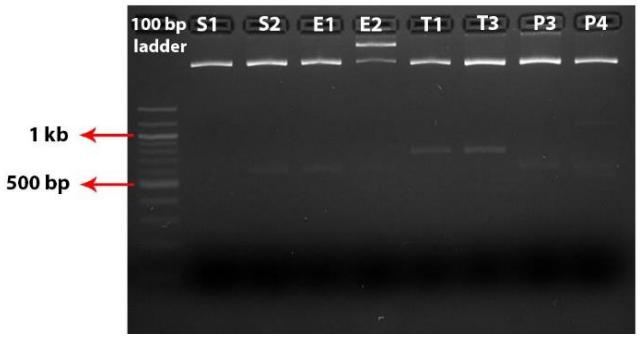

Figure 3.15: The validation of the promoter cloning experiments. Selected and isolated plasmids were digested with BglII and NotI restriction enzymes – expected band sizes were promoter-specific; 371bp for SV40 (S) promoter, 256bp for EFS (E) promoter, 752bp for HSV TK (T) promoter and 500bp for PGK (P) promoter. Gel electrophoresis was performed on 2% agarose gel.

Only HSV TK promoter cloning exhibited successful colonies with the right orientation of HSV TK. Therefore, cloning experiments were repeated for SV40, EFS and PGK promoters (Figure 3.16). According to the expected band sizes, selected plasmids were isolated by kit-free isolation and then were used in diagnostic digestion to analyze the success of colonies (Figure 3.17).

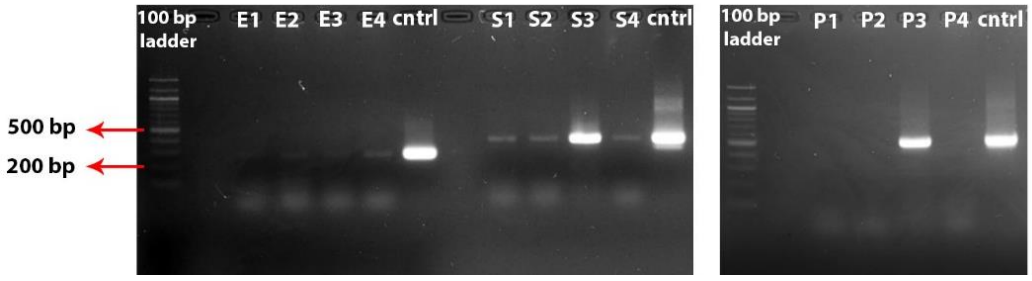

Figure 3.16: The validation of cloning of EFS, SV40 and PGK promoters. Four colonies for each promoter cloning were used for colony PCR with the associated primers – expected band sizes were 256bp for (E1-E4), 371bp for (S1-S4) and 500bp for (P1-P4). The control samples were established with the use of the purified promoters as a template – expected band sizes were 256bp for EFS (E) promoter, 371bp for SV40 (S) promoter and 500bp for PGK (P) promoter. Gel electrophoresis was performed on 2% agarose gel.

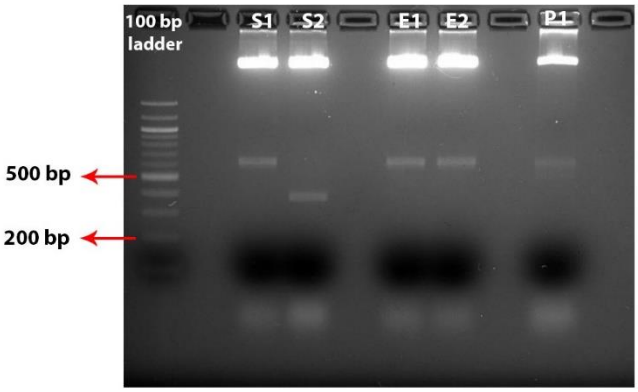

Figure 3.17: The validation of the promoter cloning experiments. Selected and isolated plasmids were digested with BglII and NotI restriction enzymes – expected band sizes were promoter-specific; 256bp for EFS promoter, 371bp for SV40 promoter and 752bp for TK promoter. Gel electrophoresis was performed on 2% agarose gel.

One colony for SV40 promoter showed success for cloning. Therefore, the cloning procedure was repeated to get one more successful colony (Figure 3.18). Except colony 3, all were isolated by kit-free isolation protocol. Diagnostic digestion was then performed (Figure 3.19) and one more successful colony was obtained.

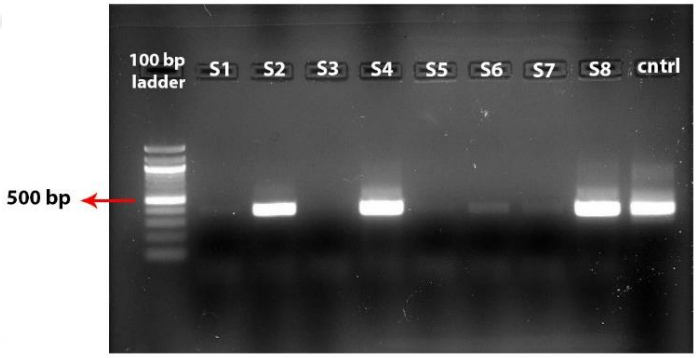

Figure 3.18: The validation of cloning of SV40 promoter. (S1-S8) Eight selected colonies for colony PCR with SV40 primers - the expected band size was 371bp. (Cntrl) The control sample was established with the use of the purified SV40 promoter as a template – expected band size was 371bp. Gel electrophoresis was performed n 2% agarose gel.

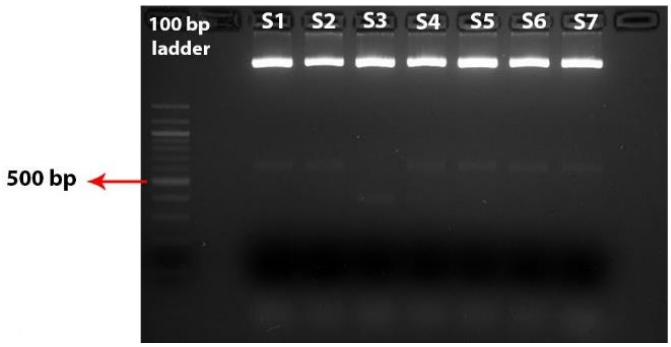

Figure 3.19: The validation of SV40 promoter cloning experiments. (1-7) Selected and isolated plasmids were digested with BglII and NotI restriction enzymes – expected band size was 371bp. Gel electrophoresis was performed on 2% agarose gel.

As a result of the cloning studies of SV40 and HSV TK promoters, two successful samples were obtained per each group. On the other hand, the cloning experiments was repeated for EFS and PGK promoters. Eight colonies per promoters were chosen for colony PCR reaction (Figure 3.20). According to the success rate of colony PCR, they were isolated by kit-free isolation procedure. Finally, successful samples of all four promoters (SV40, HSV TK, EFS and PGK) in Fluc/Rluc backbone were sequenced for a final validation. Their maps were shown in Figure 3.21.

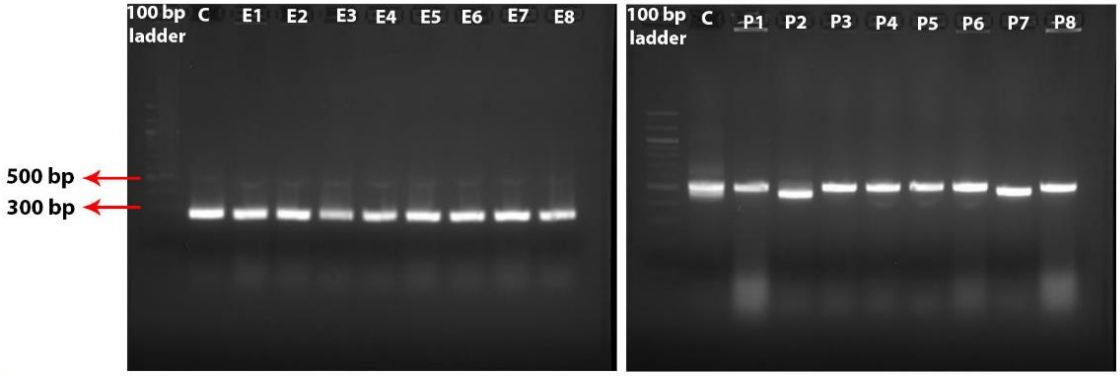

Figure 3.20: The validation of cloning of EFS and PGK promoters. Eight colonies for each promoter cloning were used for colony PCR with the associated primers – expected band sizes were 256bp for (E1-E4) and 500bp for (P1-P4). The control samples were established with the use of the purified promoters as a template – expected band sizes were 256bp for EFS (E) promoter and 500bp for PGK (P) promoter. Gel electrophoresis was performed on 2% agarose gel.

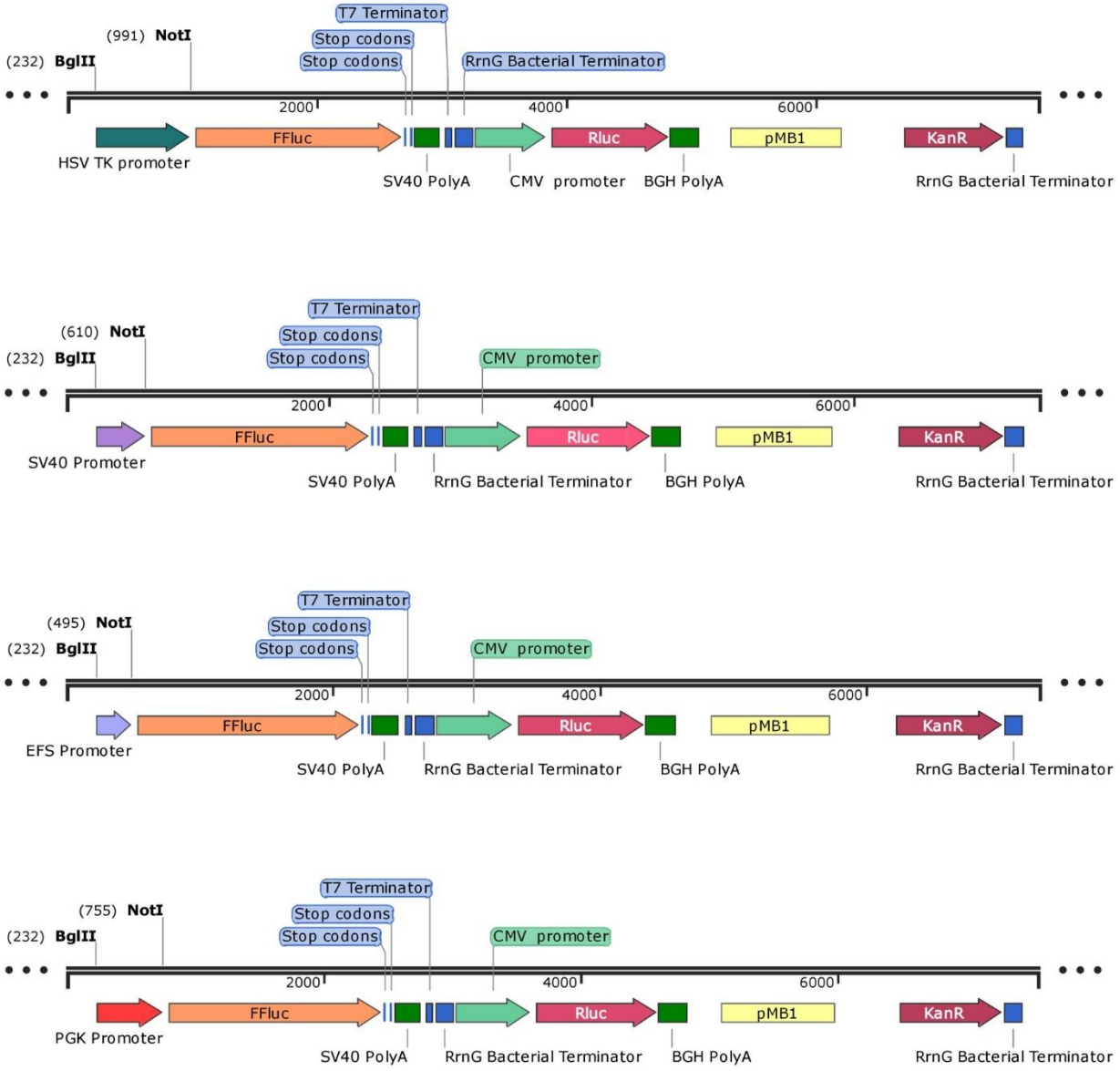

Figure 3.21: The maps of completed vectors. From top to bottom, the maps of HSV TK-Fluc-CMV-Rluc (TFCR), SV40-Fluc-CMV-Rluc (SFCR), EFS-Fluc-CMV-Rluc (EfsFCR) and PGK-Fluc-CMV-Rluc (PFCR) were respectively shown. Vector maps were created by SnapGene software.

The remaining promoters for Fluc/Rluc backbone were EF1a, UBC, CHEF1a and CAG. Firstly, EF1a and UBC promoters were studied since both would be cloned into the backbone with BglII and NotI restriction enzymes which were also used in the cloning of SV40, HSV TK, EFS and PGK promoters. Following ligation and transformation steps, the purified EF1a promoter was cloned into Fluc/Rluc template cut in the region of the first promoter – variable site. Then, 8 colonies were chosen for colony PCR to validate the promoter cloning experiment (Figure 3.22).

One was exposed to diagnostic digestion with BglII and NotI restriction enzymes (Figure 3.23). According to the expected band sizes, it was also sequenced and validated.

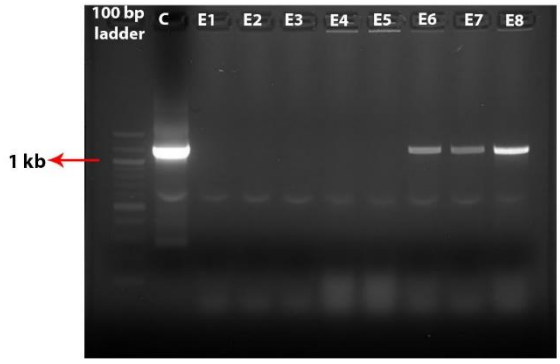

Figure 3.22: The validation of cloning of EF1a promoter. **(**E1-E8) Eight colonies were used for colony PCR with EF1a primers – expected band sizes were 1190bp. (C) The control sample was established with the use of the purified EF1a promoter as a template – expected band size was 1190bp. Gel electrophoresis was performed on 1.6% agarose gel.

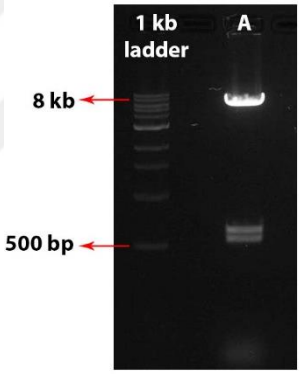

Figure 3.23: The validation of EF1a promoter cloning. A) One sample isolated from the selected colony was restricted with BglII and NotI enzymes for diagnostic digestion – expected band sizes were 7037bp, 635bp and 562bp. Gel electrophoresis was performed on 1.2% agarose gel.

Cutting both with BglII and NotI, the purified UBC promoter and the purified Fluc/Rluc backbone was ligated and transformed. Since colony number was only three, all were directly grown for kit-free isolation. Then, plasmids were used in a diagnostic digestion to validate the cloning experiment (Figure 3.24). Only one colony exhibited the expected band sizes and was successfully sequenced. The maps of completed EF1a-Fluc-CMV-Rluc (EF1aFCR) and UBC-Fluc-CMV-Rluc (UFCR) vectors were shown in Figure 3.25.

Figure 3.24: The validation of UBC promoter cloning experiments. U1-U3) Three UFCR plasmids were isolated from the selected colonies and were digested with SalI and SpeI restriction enzymes – expected band sizes were 4365bp, 2948bp and 942bp. Gel electrophoresis was performed on 1% agarose gel.

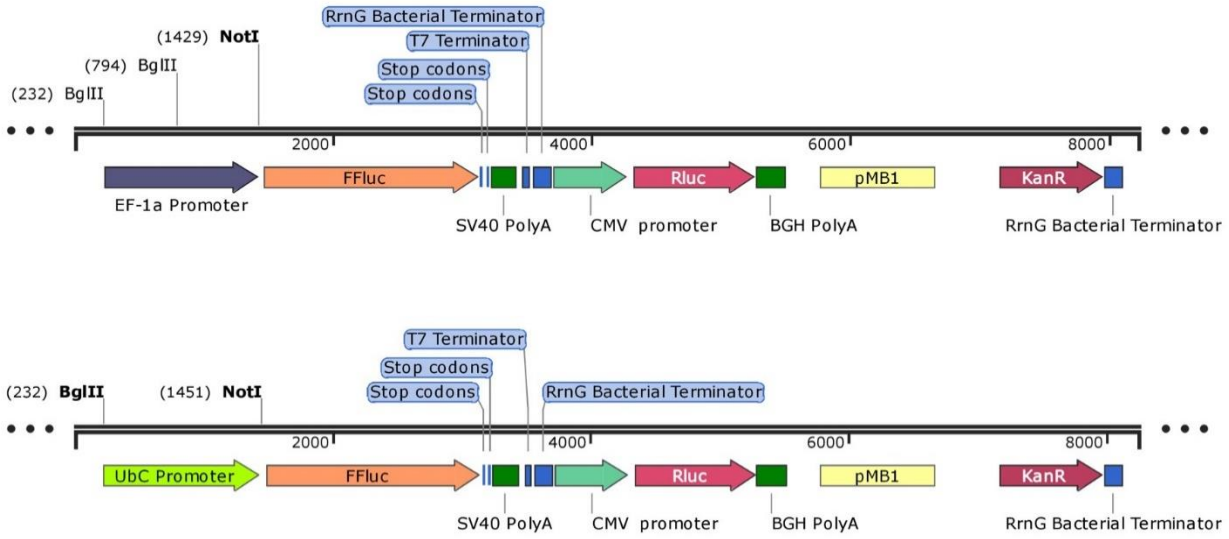

Figure 3.25: The maps of completed vectors. From top to bottom, the maps of EF1aFCR and UFCR were respectively shown. Vector maps were created by SnapGene software.

As mentioned in the part of '*obtaining promoters and reporters*', TTi-GFP plasmid would be used as an intermediary vector for CAG promoter cloning. Using the compatibility of BclI and BglII restriction enzymes, CAG promoter was obtained from TTi-GFP plasmid with BclI and EcoRI enzymes while Fluc/Rluc backbone was restricted with BglII and EcoRI enzymes showed in Figure 3.12. Following ligation and transformation steps, eight colonies were chosen to isolate plasmids which were then exposed to diagnostic digestion to see the success of cloning. Two samples exhibited the expected band sizes. However, the longer band of one of them was a little

lower than that of other. Therefore, two more diagnostic digestion was set up with different enzymes to see which one had accurate cloning (Figure 3.26). The second sample was the expected one that's why it was sequenced and validated.

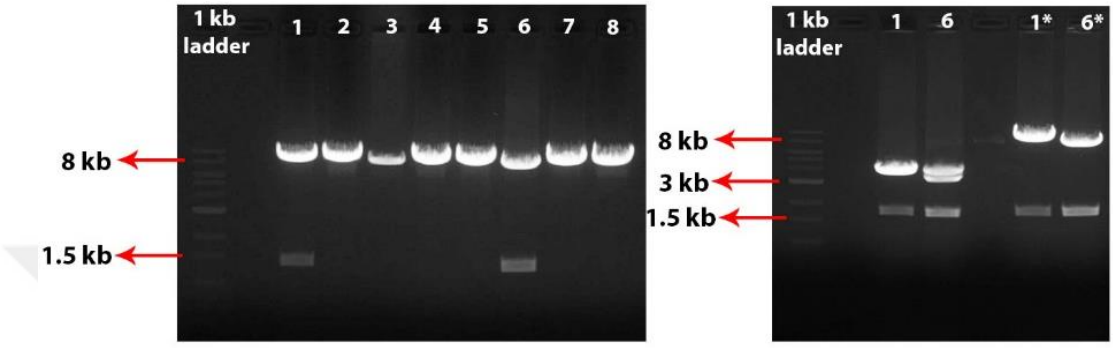

Figure 3.26: The validation of CAG promoter cloning experiments. (1-8) CAG-Fluc-CMV-Rluc (CagFCR) vectors were digested with NcoI and EcoRI restriction enzymes – expected band sizes were 7374bp, 1341bp and 20bp. (1 and 6) 1st and 6th samples of CagFCR vectors were digested with SpeI and EcoRI restriction enzymes – expected band sizes were 3811bp, 3222bp and 1702bp. (1^* and 6^*) Same 1st and 6th CagFCR vectors were also restricted with XbaI enzyme – expected band sizes were 6968bp and 1767bp. Gel electrophoresis was performed on 0.8% agarose gel.

CHEF1a promoter was cloned into Fluc/Rluc backbone with BglII and EcoRI enzymes as used in the cloning of CAG promoter. Eight colonies were chosen to isolate plasmids by kit-free isolation. Then, all were exposed to diagnostic digestion to see whether the cloning procedure did work well (Figure 3.27). Since it seemed that all colonies were successful, two of them were sequenced and validated. The maps of CagFCR and CHEF1a-Fluc-CMV-Rluc (CHEF1aFCR) were shown in Figure 3.28.

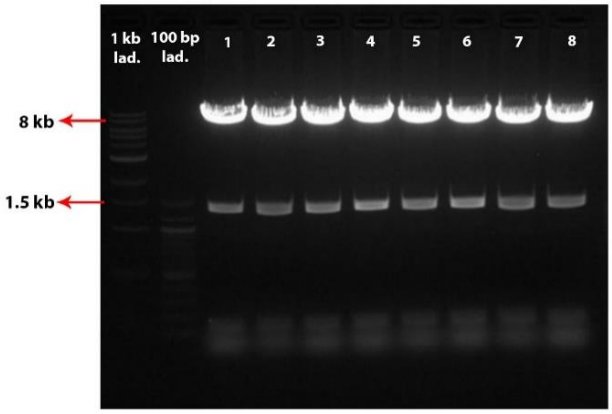

Figure 3.27: The validation of CHEF1a promoter cloning experiments. (1-8) Chef1aFCR plasmids were digested with NcoI and BglII restriction enzymes – expected band sizes were 6990bp, 1341bp and 158bp. Gel electrophoresis was performed on 1% agarose gel.

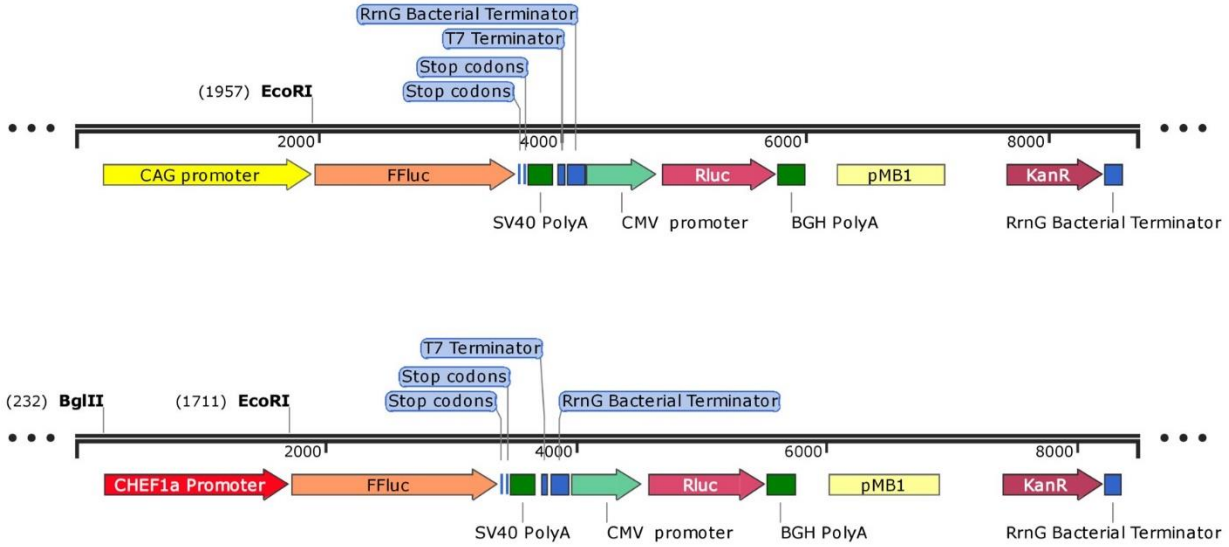

Figure 3.28: The maps of completed vectors. From top to bottom, the maps of EF1aFCR and UFCR were respectively shown. Vector maps were created by SnapGene software.

One additional plasmid without promoter upstream of Fluc gene was obtained in line with the requirement of a baseline for gene expression levels. For that, CFCR plasmid was digested with BglII restriction enzyme at the start and at the end of CMV promoter (Figure 3.29). After the removal of CMV promoter, following 1 hour ligation and transformation steps, two colonies were chosen to isolate FCR plasmid, which were then subjected to diagnostic digestion with SalI and SpeI enzymes (Figure 3.29).

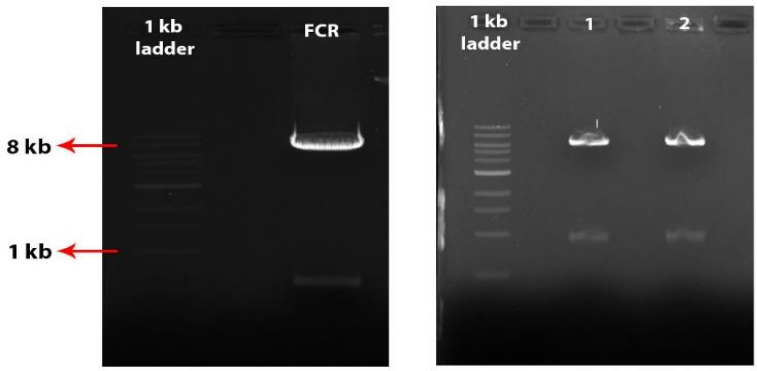

Figure 3.29: The preparation of FCR vector. (Left) CFCR plasmid was digested with BglII restriction enzyme – expected band sizes were 7079bp and 584bp. (Right) Two FCR samples were digested with SalI and SpeI restriction enzymes – expected band sizes were 6154bp and 936bp. Both gel electrophoresis experiments were performed on 0.8% agarose gel.

3.3. CMV Promoter Showed the Highest Expression Levels in All CHO Cell Lines According to Luciferase Assay

Transfection protocol was firstly optimized for CHO cells. For this optimization, pEGFP-N1 vector was used to compare EGFP expression levels by fluorescence microscope. Following manufacturer's instructions, the ratio of Lipofectamine 3000 reagent to DNA was studied as 1:1, 2:1 and 3:1. According to observations (Figure 3.30 – 3.35), the ratio 3:1 was determined to use for maximum transfection efficiency.

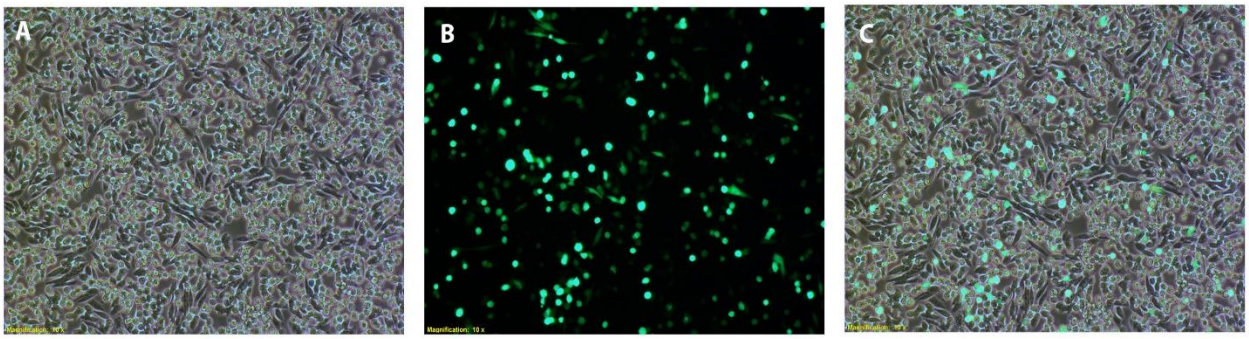

Figure 3.30: Fluorescence microscopy images of CHO-DG44 cells transfected with pEGFP-N1 vector in 1:1 Lipofectamine:DNA. Representative phase contrast images of negative control group not transfected with pEGFP-N1 (A), the EGFP-enriched cells after the transfection with pEGFP-N1 (B) and the overlap between negative control and EGFP-enriched cells (C). Images were taken at 10X magnification.

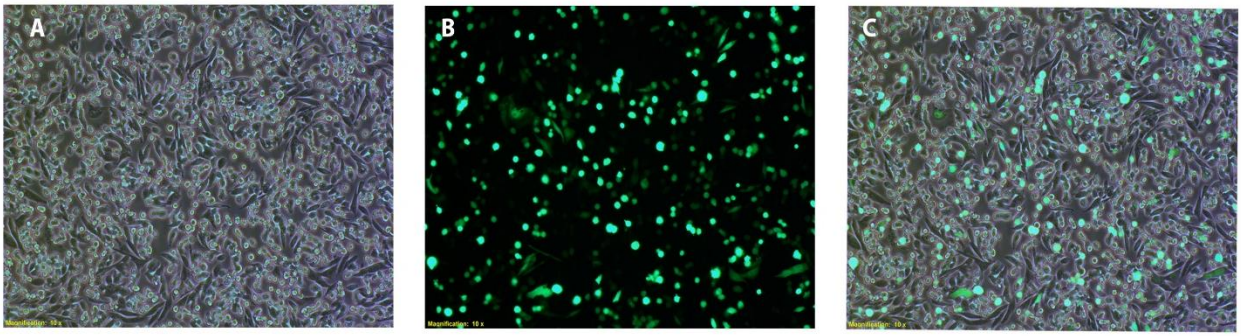

Figure 3.31: Fluorescence microscopy images of CHO-DG44 cells transfected with pEGFP-N1 vector in 1:2 Lipofectamine:DNA. Representative phase contrast images of negative control group not transfected with pEGFP-N1 (A), the EGFP-enriched cells after the transfection with pEGFP-N1 (B) and the overlap between negative control and EGFP-enriched cells (C). Images were taken at 10X magnification.

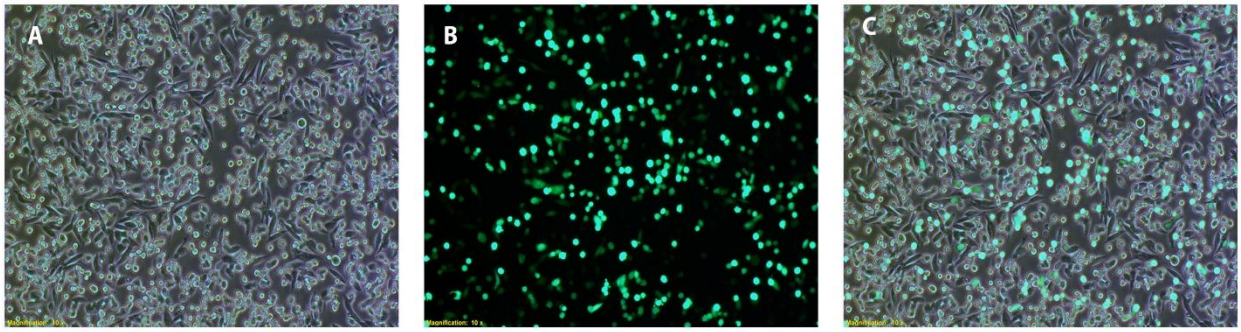

Figure 3.32: Fluorescence microscopy images of CHO-DG44 cells transfected with pEGFP-N1 vector in 1:3 Lipofectamine:DNA. Representative phase contrast images of negative control group not transfected with pEGFP-N1 (A), the EGFP-enriched cells after the transfection with pEGFP-N1 (B) and the overlap between negative control and EGFP-enriched cells (C). Images were taken at 10X magnification.

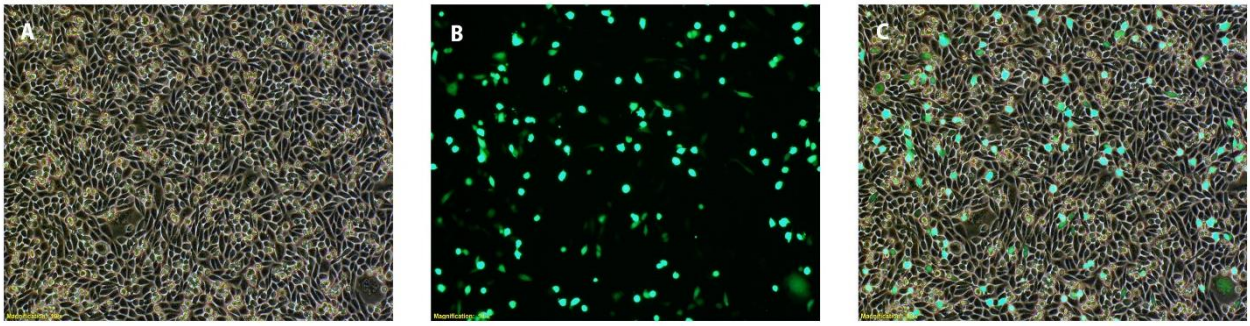

Figure 3.33: Fluorescence microscopy images of CHO-WT cells transfected with pEGFP-N1 vector in 1:1 Lipofectamine:DNA. Representative phase contrast images of negative control group not transfected with pEGFP-N1 (A), the EGFP-enriched cells after the transfection with pEGFP-N1 (B) and the overlap between negative control and EGFP-enriched cells (C). Images were taken at 10X magnification.

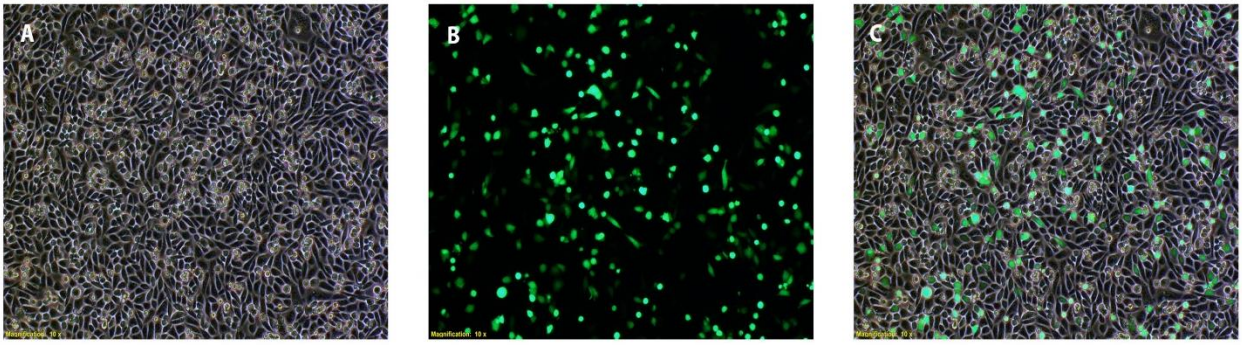

Figure 3.34: Fluorescence microscopy images of CHO-WT cells transfected with pEGFP-N1 vector in 1:2 Lipofectamine:DNA. Representative phase contrast images of negative control group not transfected with pEGFP-N1 (A), the EGFP-enriched cells after the transfection with pEGFP-N1 (B) and the overlap between negative control and EGFP-enriched cells (C). Images were taken at 10X magnification.

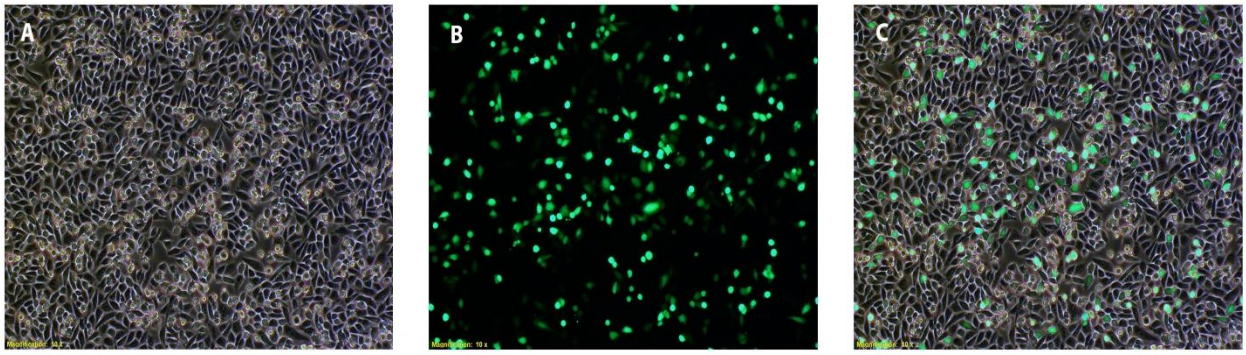

Figure 3.35: Fluorescence microscopy images of CHO-WT cells transfected with pEGFP-N1 vector in 1:3 Lipofectamine:DNA. Representative phase contrast images of negative control group not transfected with pEGFP-N1 (A), the EGFP-enriched cells after the transfection with pEGFP-N1 (B) and the overlap between negative control and EGFP-enriched cells (C). Images were taken at 10X magnification.

Following the optimized 3:1 transfection protocol's instructions, successfully designed, sequenced and validated vector constructs were transfected to three CHO cell lines – CHO-WT, CHO-DG44 and CHO-DG44 suspension – by Lipofectamine 3000 Transfection Reagent. Dual luciferase assay was performed at 48h post-transfection and the results were analyzed based on the ratio of experimental/control reporter activity (in this case, Fluc/Rluc ratio) (Figure 3.36, 3.37 and 3.38).

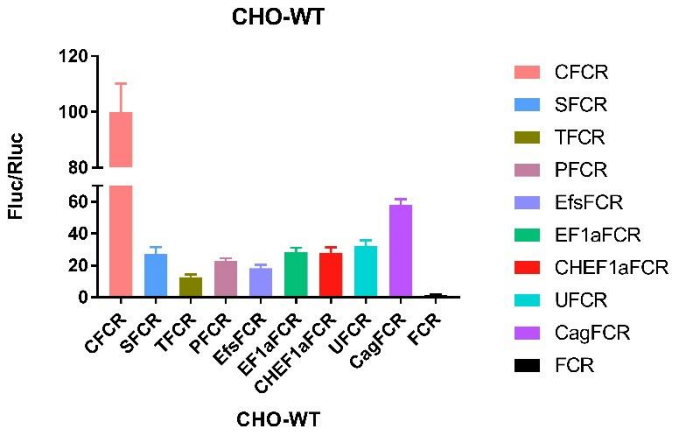

Figure 3.36: Effect of different promoters on the expression levels in CHO-WT cells. The vectors containing CMV, SV40, HSV TK, PGK, EFS, EF1a, CHEF1a, UBC and CAG promoters were transfected into **CHO-WT** cells, and the cells were analyzed at 48h post-transfection using DLR assay. CFCR, CMV-Fluc-CMV-Rluc; SFCR, SV40-Fluc-CMV-Rluc; TFCR, HSV TK-Fluc-CMV-Rluc; PFCR, PGK-Fluc-CMV-Rluc; EfsFCR, EFS-Fluc-CMV-Rluc; EF1aFCR, EF1a-Fluc-CMV-Rluc; CHEF1aFCR, CHEF1a-Fluc-CMV-Rluc; UFCR, UBC-Fluc-CMV-Rluc; CagFCR, CAG-Fluc-CMV-Rluc; -FCR, -Fluc-CMV-Rluc.

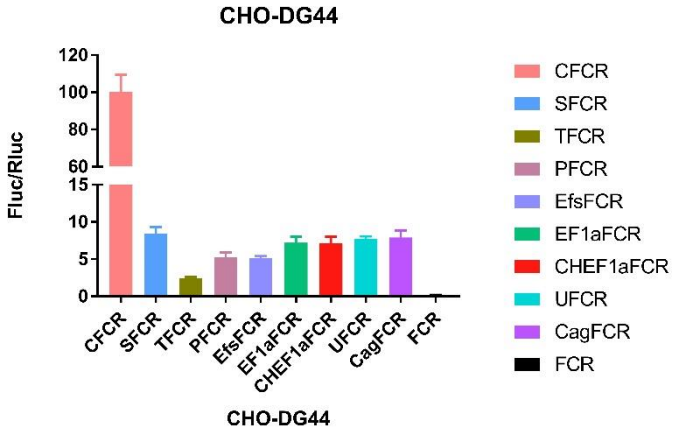

Figure 3.37: Effect of different promoters on the expression levels in CHO-DG44 cells. The vectors containing CMV, SV40, HSV TK, PGK, EFS, EF1a, CHEF1a, UBC and CAG promoters were transfected into **CHO-DG44** cells, and the cells were analyzed at 48h post-transfection using DLR assay. CFCR, CMV-Fluc-CMV-Rluc; SFCR, SV40-Fluc-CMV-Rluc; TFCR, HSV TK-Fluc-CMV-Rluc; PFCR, PGK-Fluc-CMV-Rluc; EfsFCR, EFS-Fluc-CMV-Rluc; EF1aFCR, EF1a-Fluc-CMV-Rluc; CHEF1aFCR, CHEF1a-Fluc-CMV-Rluc; UFCR, UBC-Fluc-CMV-Rluc; CagFCR, CAG-Fluc-CMV-Rluc; -FCR, -Fluc-CMV-Rluc.

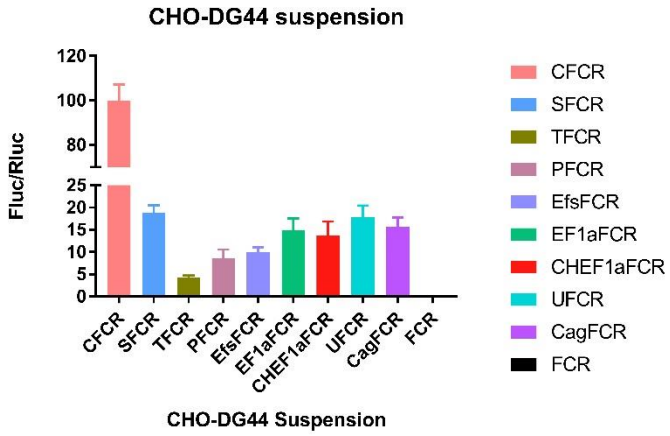

Figure 3.38: Effect of different promoters on the expression levels in CHO-DG44 suspension cells. The vectors containing CMV, SV40, HSV TK, PGK, EFS, EF1a, CHEF1a, UBC and CAG promoters were transfected into **CHO-DG44** suspension cells, and the cells were analyzed at 48h post-transfection using DLR assay. CFCR, CMV-Fluc-CMV-Rluc; SFCR, SV40-Fluc-CMV-Rluc; TFCR, HSV TK-Fluc-CMV-Rluc; PFCR, PGK-Fluc-CMV-Rluc; EfsFCR, EFS-Fluc-CMV-Rluc; EF1aFCR, EF1a-Fluc-CMV-Rluc; CHEF1aFCR, CHEF1a-Fluc-CMV-Rluc; UFCR, UBC-Fluc-CMV-Rluc; CagFCR, CAG-Fluc-CMV-Rluc; -FCR, -Fluc-CMV-Rluc.

Of the nine promoters, CMV promoter yielded the highest reporter expression levels, followed by SV40, CAG, UBC, CHEF1a and EF1a promoters. On the other hand, HSV TK promoter had the weakest strength on gene expression levels, followed by PGK and EFS promoters.

3.4. Chosen Promoters Were Cloned into the Backbone Containing EGFP/tdTomato Reporters

An eight hour incubation with NcoI and XbaI enzymes was first set up to remove the Fluc gene from the OG4071 vector for the constitution of the second reporter system (Figure 3.39). After removal of the Fluc gene (1650bp), the remaining 5812bp vector was gel purified with gel purification kit. At the same time, for cloning, the previously purified EGFP gene was also subjected to eight hour restriction incubation with the same enzymes. Following ligation and transformation steps, six colonies were chosen for the validation of cloning with colony PCR (Figure 3.39). Since all colonies showed successful cloning, two of them was sequenced and validated. The map of CMV-EGFP-CMV-BCL2 (CGCB) was shown in Figure 3.40.

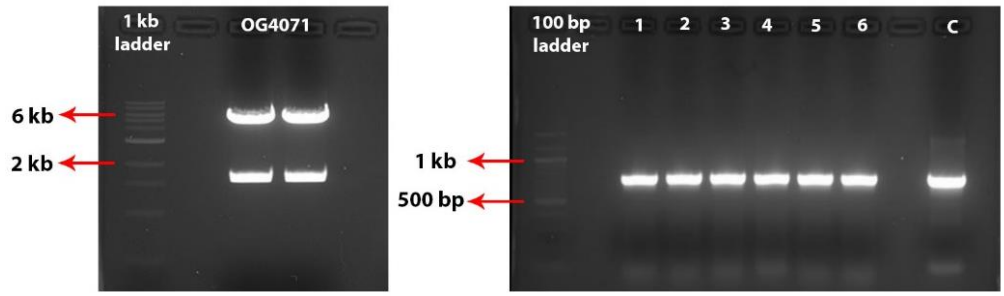

Figure 3.39: The validation of EGFP reporter gene cloning. OG4071) OG4071 plasmid was digested with NcoI and XbaI restriction enzymes – expected band sizes were 5812bp and 1650bp. (1-6) Six selected colonies for the colony PCR with the EGFP primers resulted in the expected 720bp band size. (C) The control sample established with the use of EGFP gene as a template – expected band size was 720bp. Gel electrophoresis was performed on 0.8% agarose gel for OG4071 restriction and on 1.8% agarose gel for colony PCR.

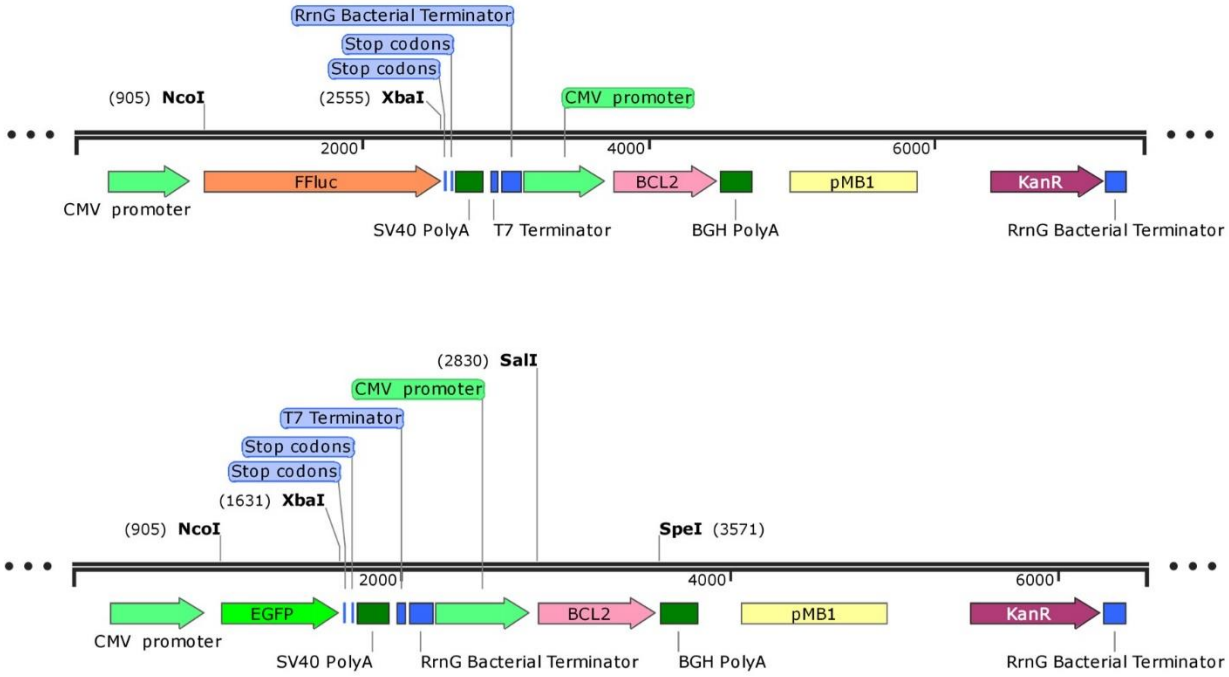

Figure 3.40: Vector design of CFCR backbone. (Up) The original vector map of OG4071 which was restricted with SalI and SpeI enzymes to remove BCL2 gene. (Bottom) The map of CGCB vector which was constructed with EGFP reporter replaced for Fluc reporter. Vector maps were created by SnapGene software.

tdTomato reporter gene has the recognition site of NotI enzyme which was chosen for the cloning of promoters. Therefore, without tdTomato reporter gene, CGCB backbone was cut with BglII and NotI enzymes to place promoters in the second reporter system (Figure 3.41).

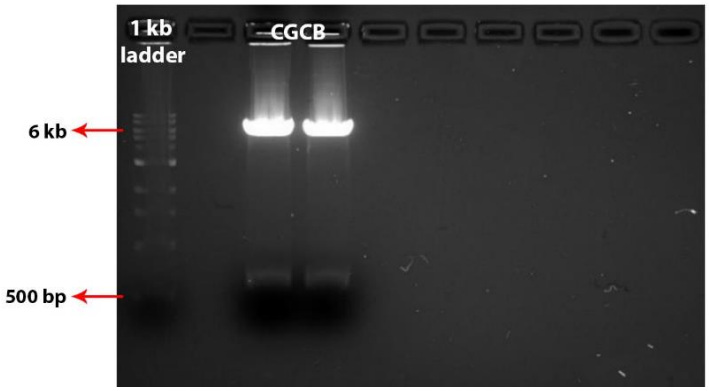

Figure 3.41: The preparation of the second reporter system for cloning of promoters. CGCB) The backbone CGCB was digested with BglII and NotI enzymes for cloning of promoters SV40 and HSV TK – expected band sizes were 5912bp, 584bp and 42bp. Gel electrophoresis experiments were performed on 1.2% agarose gel.

Following ligation and transformation steps for the cloning of SV40 and HSV TK promoters, eight colonies for each were used to validate the cloning with colony PCR. According to the expected band sizes, successful colonies were grown for kit-free isolation. Then, all were exposed to diagnostic digestion with BglII and NotI enzymes (Figure 3.42 and 3.43).

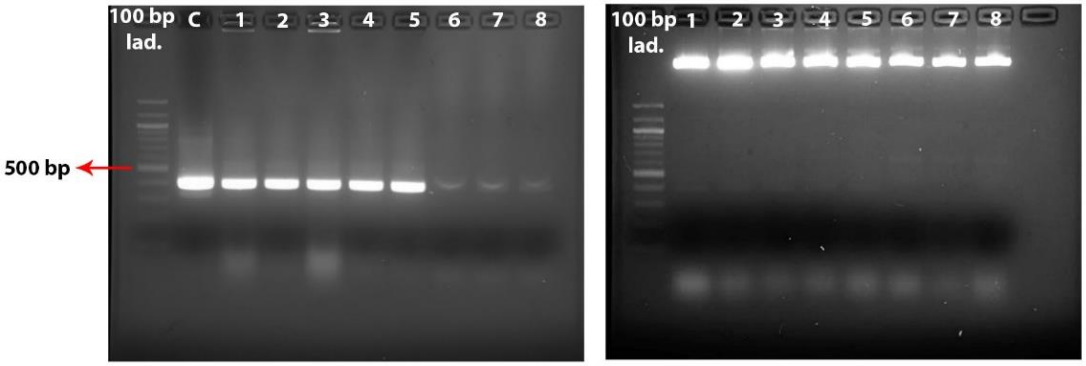

Figure 3.42: The validation of SV40 promoter cloning. (Left) Eight colonies (1-8) were used for colony PCR with SV40 primers – expected band size was 371bp. The control sample (C) was established with the use of the purified SV40 promoter as a template – expected band size was 371bp. (Right) All were digested with BglII and NotI restriction enzymes – expected band size was 371bp. Gel electrophoresis was performed on 1.8% agarose gel.

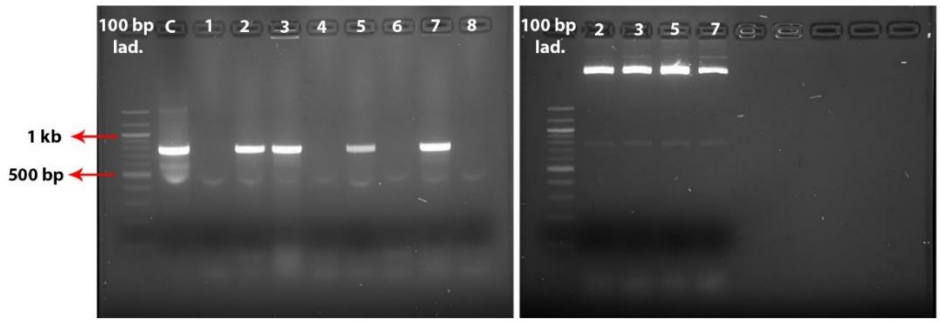

Figure 3.43: The validation of HSV TK promoter cloning. (Left) Eight colonies (1-8) were used for colony PCR with HSV TK primers – expected band size was 752bp. The control sample (C) was established with the use of the purified HSV TK promoter as a template – expected band size was 752bp. (Right) Four successful colonies were digested with BglII and NotI restriction enzymes – expected band size was 752bp. Gel electrophoresis was performed on 1.8% agarose gel.

Achieving successful cloning of SV40 and HSV TK promoters, SGCB and TGCB plasmids were cut with SalI and SpeI restriction enzymes to complete the second reporter system (Figure 3.44). After ligation and transformation steps, two colonies for each template with different promoter were chosen and colony PCR was performed for the validation (Figure 3.44). Due to the dimer structure of tdTomato gene, bands were expected to show its total length (1431bp) and the length of its monomers (around 700 bp). Cloning was confirmed one more time by subjecting the isolated plasmids from colonies giving expected band sizes to a diagnostic digestion with EcoRI and SalI enzymes (Figure 3.44). According to their success, two SGCT plasmids and one TGCT plasmid were sequenced and validated.

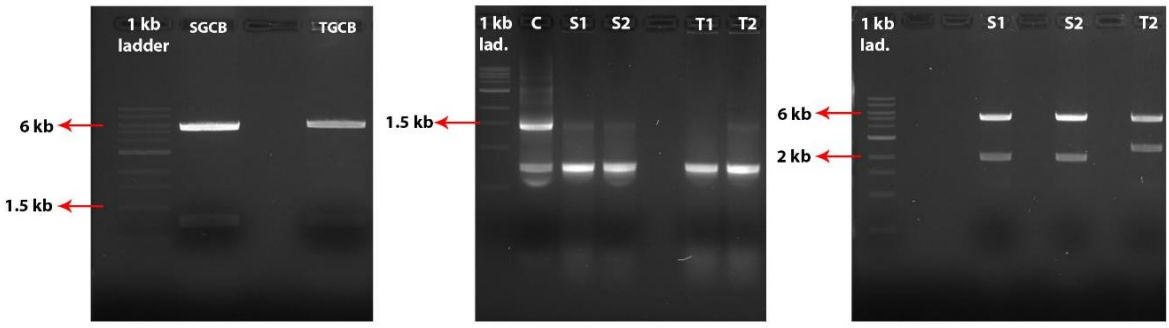

Figure 3.44: The completion of the second reporter system for SV40 and HSV TK promoters. (Left) SGCB and TGCB plasmids were digested with SalI and SpeI restriction enzymes – expected band sizes were around 5500bp and 720bp. (Middle) Two colonies for each promoter were used for colony PCR with tdTomato primers – expected band sizes were 1431bp and around 700bp. The control sample (C) was established with the use of the purified tdTomato reporters as a template – expected band sizes were 1431bp and around 700bp. (Right) Successful colonies were digested with EcoRI and SalI restriction enzymes – expected band size were 5004bp and 1995bp for SGCT (S1 and S2) while 4903bp and 2345bp for TGCT (T1). Gel electrophoresis was performed on 1% agarose gel for the first and third experiments and was performed on 1.6% agarose gel for the second experiment.

CHEF1a promoter was cloned into CGCB backbone with BglII and EcoRI enzymes. The promoter cut with these enzymes was already prepared during the first reporter system construction, hence only the backbone was digested with BglII and EcoRI enzymes in this part (Figure 3.45). Following ligation and transformation steps, eight colonies were chosen to validate the cloning with diagnostic digestion. Firstly, all were digested with NcoI and BglII restriction enzymes (Figure 3.45). Then, according to the expected band sizes, two successful colonies were exposed to second diagnostic digestion with NotI and XbaI enzymes (Figure 3.45).

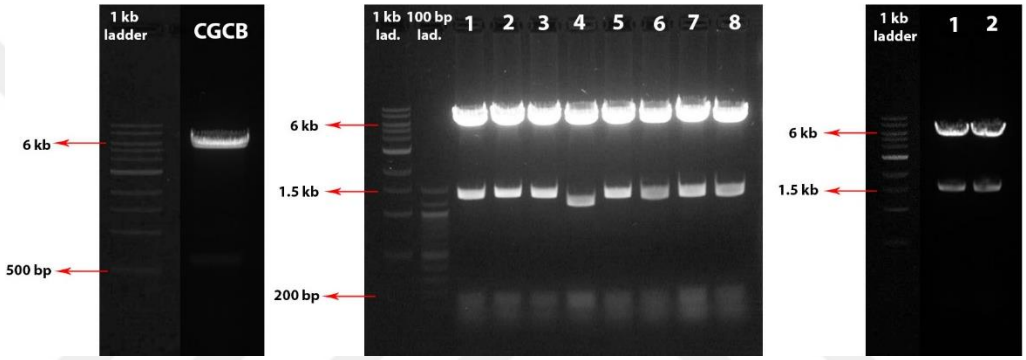

Figure 3.45: The validation of CHEF1a promoter cloning. (Left) CGCB backbone was digested with BglII and EcoRI enzymes for cloning of CHEF1a promoter – expected band sizes were 5883bp, 584bp and 69bp. (Middle) Eight colonies (1-8) were digested with NcoI and BglII restriction enzymes – expected band sizes were 5865bp, 1341bp and 158bp. (Right) First two successful colonies were additionally digested with NotI and XbaI restriction enzymes – expected band sizes were 5861bp and 1503bp. All gel electrophoresis experiments were performed on 0.8% agarose gel.

One of the two successful colonies showed above was cut with SalI and SpeI restriction enzymes to place tdTomato reporter gene (Figure 3.46). After ligation and transformation steps, two colonies were chosen and colony PCR was performed for the validation (Figure 3.46). Due to the dimer structure of tdTomato gene, bands were expected to show its total length (1431bp) and the length of its monomers (around 700 bp). Even both were successful, cloning was confirmed one more time by the isolation of plasmids and then by diagnostic digestion with EcoRI and SalI restriction enzymes (Figure 3.46). According to the expected band sizes, the second reporter system was completed for CHEF1a promoter.

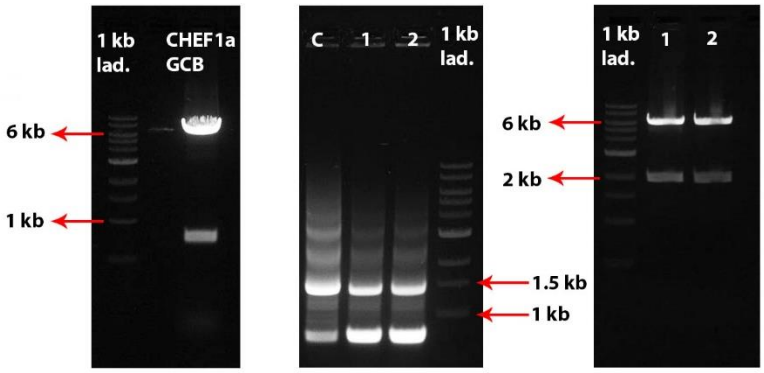

Figure 3.46: The completion of the second reporter system for CHEF1a promoter. (Left) CHEF1aGCB was digested with SalI and SpeI restriction enzymes – expected band sizes were 6623bp and 741bp. (Middle) Two colonies were used for colony PCR with tdTomato primers – expected band sizes were 1431bp and around 700bp. The control sample (Ctrl) was established with the use of the purified tdTomato reporter as a template – expected band sizes were 1431bp and around 700bp. (Right) Successful colonies were digested with EcoRI and SalI restriction enzymes – expected band size were 6135bp and 1945bp. All gel electrophoresis experiments were performed on 0.8% agarose gel.

Our original backbone has already consist of CMV promoter. That's why the work of tdTomato reporter gene cloning was started without changing the promoter site. The preparation and purification of tdTomato and CMV-GFP-CMV-Bcl2 (CGCB) vector were performed as in Figure 3.5. To complete the constitution of the second reporter system for CMV promoter, tdTomato reporter gene was cloned into CGCB by following ligation and transformation steps. For the validation of cloning, the colony PCR reaction was set up with 4 colonies chosen after transformation incubation (Figure 3.47). Due to the dimer structure of tdTomato gene, bands were expected to show its total length (1431 bp) and the length of its monomers (around 700 bp). Additionally, cloning was confirmed one more time by firstly the isolation of plasmids from 4 selected colonies and then by the restriction digestion incubation of the isolated plasmids with HindIII and SalI enzymes (Figure 3.47). The maps of CMV-EGFP-CMV-tdTomato (CGCT), SV40-EGFP-CMV-tdTomato (SGCT), HSV TK-EGFP-CMV-tdTomato (TGCT) and CHEF1a-EGFP-CMV-tdTomato (CHEF1aGCT) were shown in Figure 3.48.

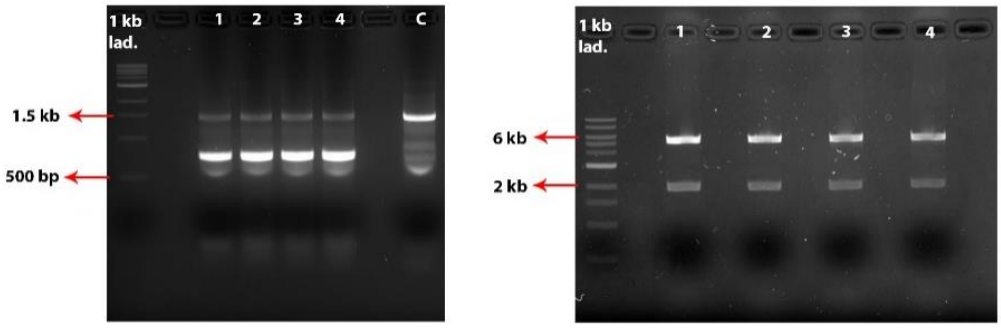

Figure 3.47: The validation of CGCT plasmid. (Left) The reaction of the 4 selected colonies for colony PCR with tdTomato primers resulted in the expected 1431bp and 700bp band sizes. The control sample was established with the use of tdTomato gene as a template – expected band size was 1431bp and around 700bp. (Right) Plasmids that were isolated from the 4 selected colonies were digested with HindIII and SalI restriction enzymes – expected band sizes were 5288bp and 1959bp. Gel electrophoresis was performed on 1.6% agarose gel for left part and on 1.2% agarose gel for right part.

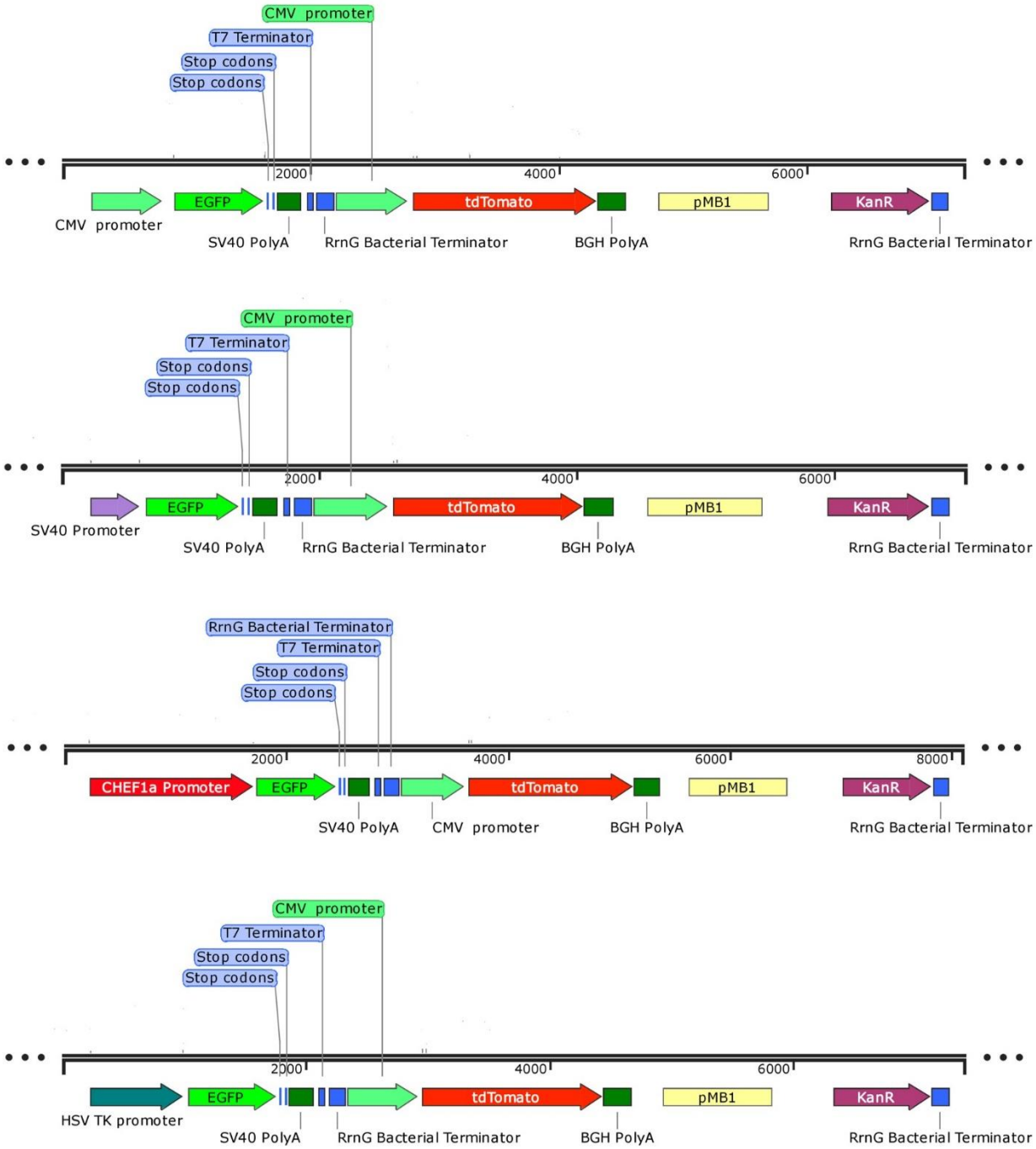

Figure 3.48: The maps of completed vectors. From top to bottom, the maps of CGCT, SGCT, CHEF1aGCT and TGCT were respectively shown. Vector maps were created by SnapGene software.

3.5. CMV Promoter Showed the Highest Expression Levels in All CHO Cell Lines According to Flow Cytometry

Completed and validated plasmids in the second reporter system were transfected to all three cell lines – CHO-WT, CHO-DG44 and CHO-DG44 suspension – by Lipofectamine 3000 Transfection Reagent. The expression levels of both EGFP and tdTomato reporter genes were measured by using flow cytometry (Figure 49, 50 and 51). Since tdTomato reporter gene was used as an internal control placed downstream of invariable promoter part, the ratio of EGFP/tdTomato was used to compare promoter strength in all three cell lines (Table 3.1, 3.2 and 3.3). As expected, the cells transfected with CMV promoter-containing vector exhibited the highest expression levels, followed by those containing CHEF1a and SV40 whereas HSV TK promoter resulted in the lowest expression in the cells, supporting our luciferase assay results.

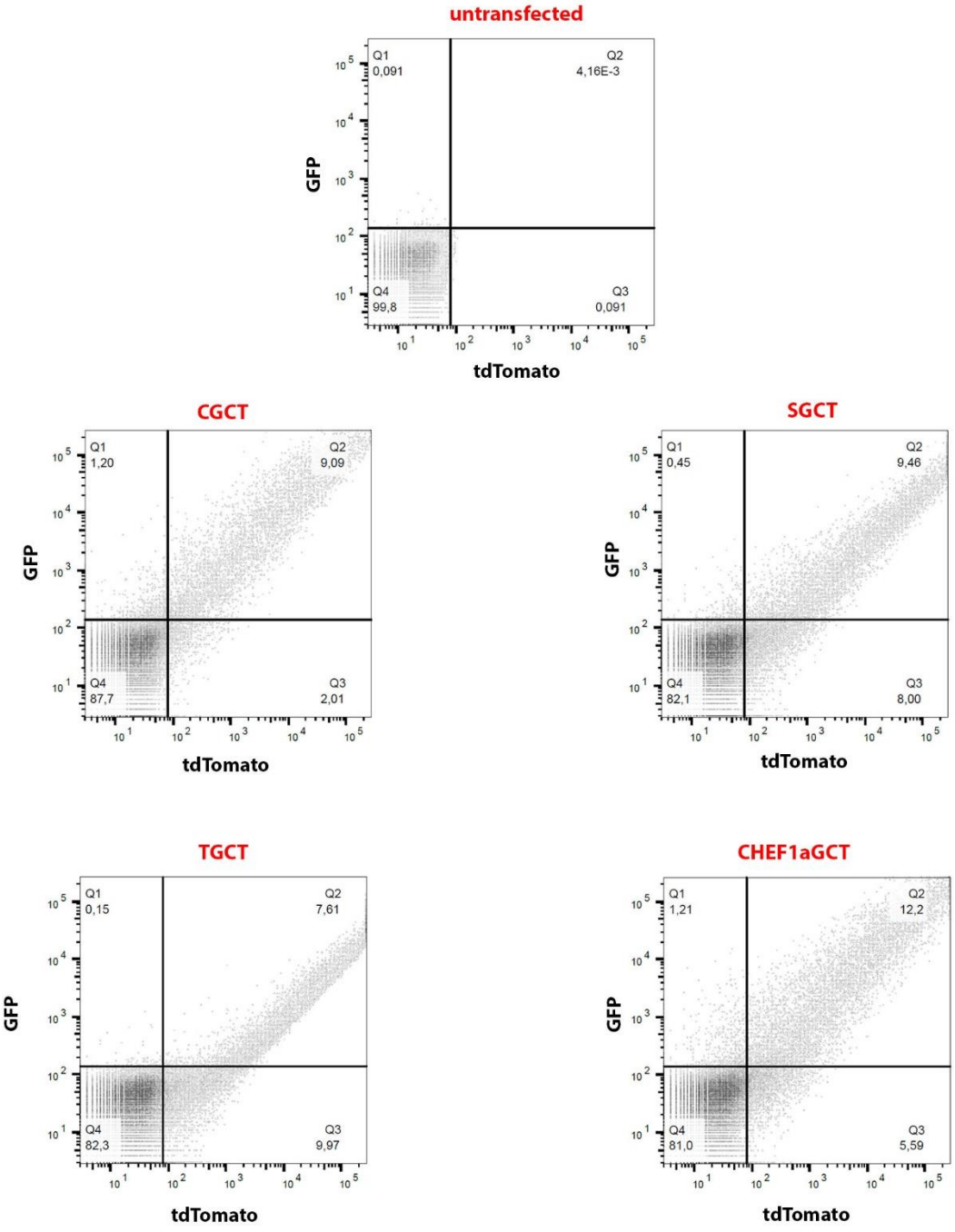

Figure 3.49: Comparison of promoter activities in CHO-WT cells. (Top) The analysis of untransfected cells were shown. (Middle) From left to right, the analysis of CGCT-transfected and SGCT-transfected cells were shown. (Bottom) From left to right, the analysis TGCT-transfected and CHEF1aGCT-transfected cells were shown.

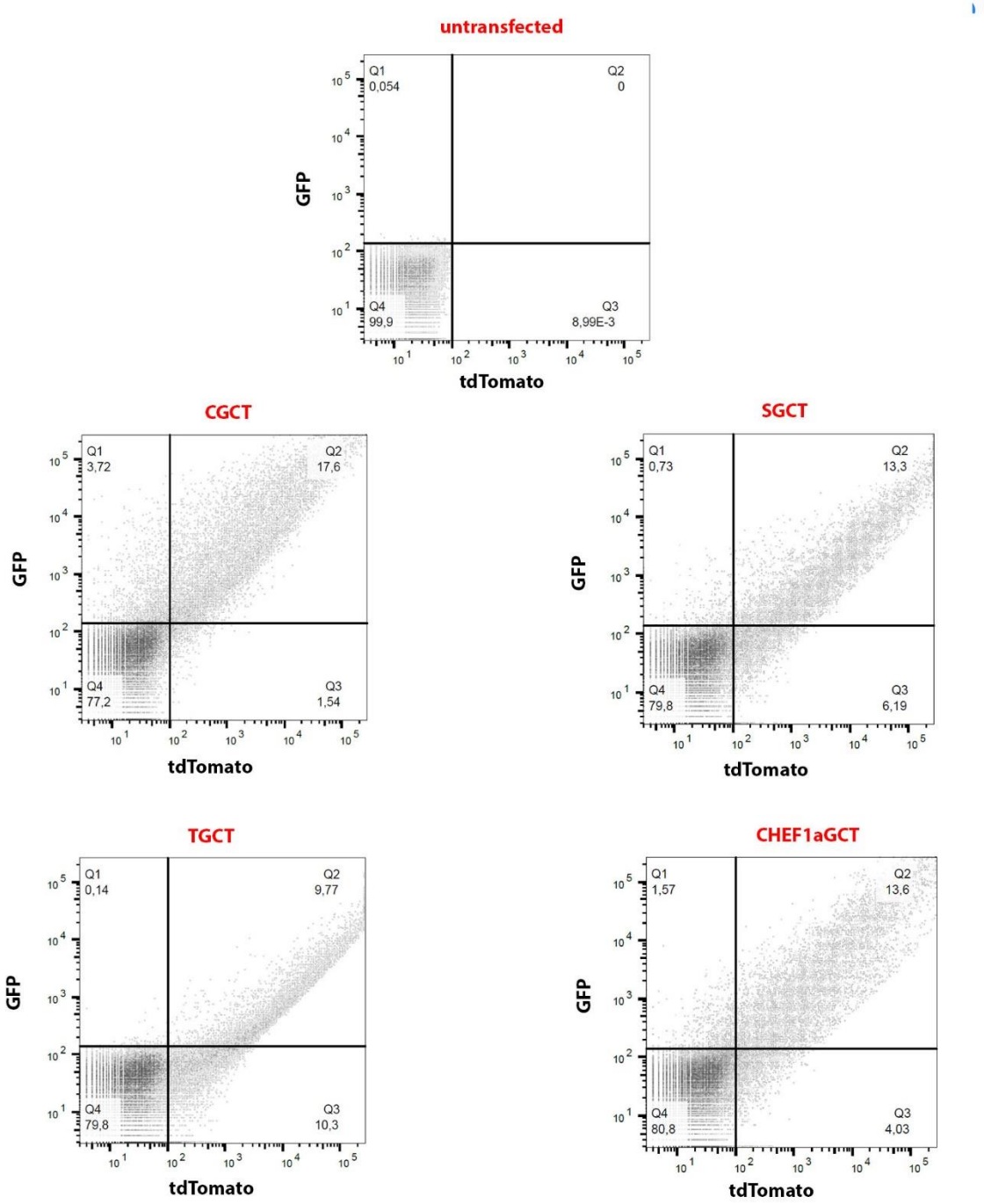

Figure 3.50: Comparison of promoter activities in CHO-DG44 cells. (Top) The analysis of untransfected cells were shown. (Middle) From left to right, the analysis of CGCT-transfected and SGCT-transfected cells were shown. (Bottom) From left to right, the analysis TGCT-transfected and CHEF1aGCT-transfected cells were shown.

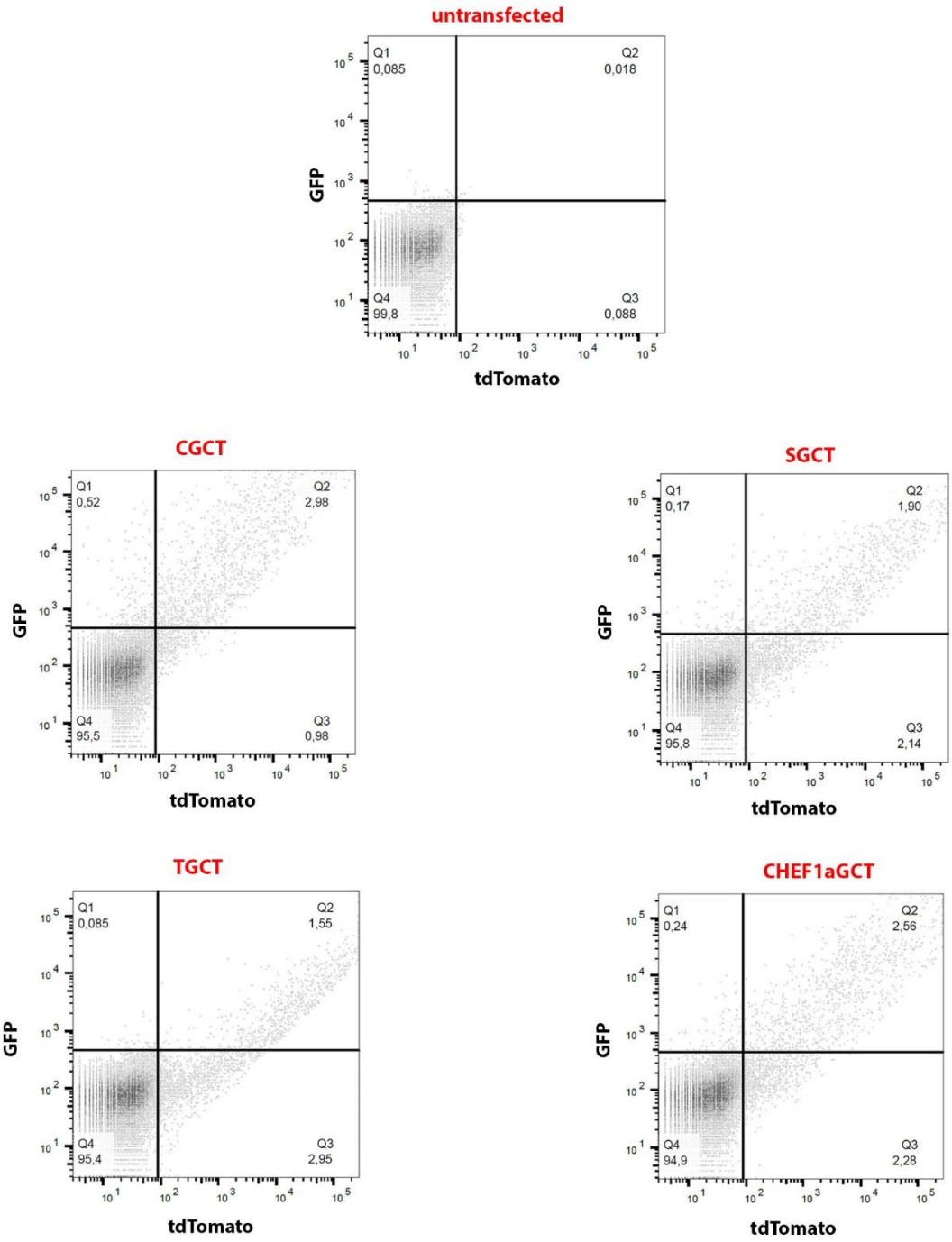

Figure 3.51: Comparison of promoter activities in CHO-DG44 suspension cells. (Top) The analysis of untransfected cells were shown. (Middle) From left to right, the analysis of CGCT-transfected and SGCT-transfected cells were shown. (Bottom) From left to right, the analysis TGCT-transfected and CHEF1aGCT-transfected cells were shown.

Table 3.1: Comparison of promoter activities with GFP/tdTomato ratio in CHO-WT cells

Plasmid Name	GFP Percentage	tdTomato Percentage	Ratio
CGCT	10,29	11,10	0,93
SGCT	9,91	17,46	0,6
TGCT	7,76	17,58	0,45
CHEF1aGCT	13,41	17,79	0,8

Table 3.2: Comparison of promoter activities with GFP/tdTomato ratio in CHO-DG44 cells

Plasmid Name	GFP Percentage	tdTomato Percentage	Ratio
CGCT	21,49	19,05	1,13
SGCT	14,18	19,48	0,73
TGCT	10,06	19,90	0,5
CHEF1aGCT	15,34	17,60	0,87

Table 3.3: Comparison of promoter activities with GFP/tdTomato ratio in CHO-DG44 suspension cells

Plasmid Name	GFP Percentage	tdTomato Percentage	Ratio
CGCT	3,21	3,55	0,90
SGCT	1,91	3,49	0,55
TGCT	1,49	3,82	0,40
CHEF1aGCT	2,58	4,21	0,60

3.6. Chosen Promoters Were Cloned into the Backbone Containing Fluc/Rluc Reporters with IRES-DHFR

It is important to see whether the pattern of promoter strength on transient gene expression is applicable to long-term cell culture. That's why five promoters giving the highest expression levels selected from the first comparison would be analyzed in stable cells. Selection marker, DHFR gene, was used to generate stable cell lines, connecting to Rluc reporter gene by IRES sequence. For that, TTi-GFP and pUCl9AEC01 plasmids were PCR-amplified with corresponding primers (Table 2.2.) to obtain IRES and DHFR genes, respectively (Figure 3.52). Then, these two

amplified genes were used as templates to assemble IRES and DHFR genes by two-step assembly PCR (Figure 3.52).

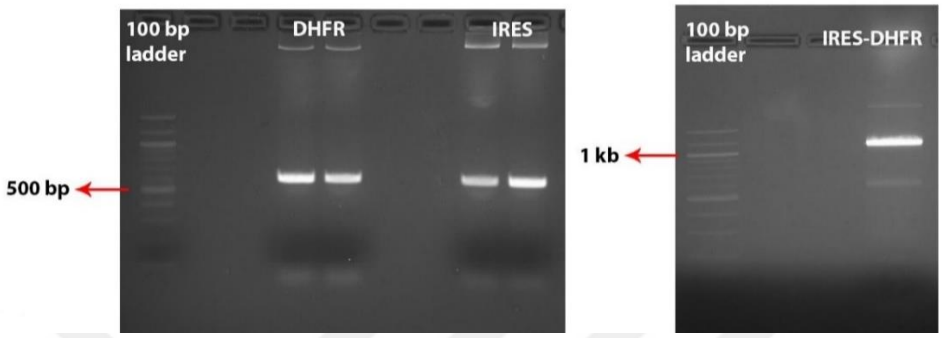

Figure 3.52: The assembly of IRES and DHFR. (Left) pUC19AEC01 and TTi-GFP were PCR-amplified to obtain DHFR gene (570 bp) and IRES (568 bp), respectively. (Right) The purified IRES and DHFR genes were used as templates to assemble them (1138bp). Both gel electrophoresis experiments were performed on 1.6% agarose gel.

Our cloning strategy was comprised of two step. Firstly, we cloned IRES-DHFR sequence downstream of Fluc reporter gene with XbaI and BamHI restriction enzymes. The backbone CFCR was digested with these two enzymes (Figure 3.53). Following ligation and transformation steps, the purified IRES-DHFR sequence was cloned into the purified CFCR plasmid. Four colonies were used to validate the cloning procedure with colony PCR (3.53). According to the expected band sizes, two colonies were isolated and then subjected to two diagnostic digestion reactions in which one was with BamHI and HindIII enzymes and other was with NcoI (Figure 3.53).

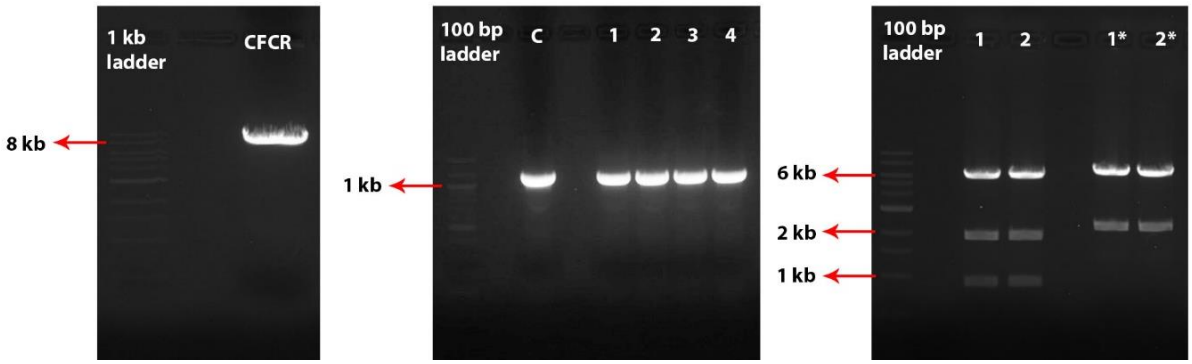

Figure 3.53: Cloning of IRES-DHFR to CFCR backbone. (Left) The backbone CFCR was restricted with XbaI and BamHI enzymes – expected band sizes were 7614bp and 49bp. (Middle) Four selected colonies were used for the colony PCR with IRES forward primer and DHFR reverse primer resulted in the expected 1138bp band lengths. The control sample (C) was established with the use of IRES-DHFR gene cassette as a template – expected band size was 1138bp. (Right) Plasmids (1 and 2) were isolated from the selected two colonies and were digested with BamHI and HindIII restriction enzymes – expected band sizes were 5928bp, 1911bp and 914bp. Same plasmids (1* and 2*) were also restricted with NcoI enzyme – expected band sizes were 6530bp and 2223bp. The first and third gel electrophoresis experiments were performed on 0.8% agarose gel while the second one was performed on 1.6% agarose gel.

Achieving success in the cloning of IRES-DHFR sequence into the backbone CFCR, one sample was sequenced and validated. Then, it was used in the second step of our cloning strategy. The plasmid CMV-Fluc-IRES-DHFR-CMV-Rluc (CFIDCR) was restricted with XbaI and NheI enzymes in order to obtain IRES-DHFR while the backbone CFCR was cut with SpeI enzyme to place IRES-DHFR sequence downstream of Rluc reporter gene (Figure 3.54). Avoiding from the loss of sample, CFCR backbone cut with single enzyme was only PCR-cleaned, however, 5µl of sample was run on agarose gel to be ensure that plasmid was linear as a result of restriction (3.54). Following ligation and transformation steps, only five colonies were formed, therefore all were used to isolate plasmids and then to validate the cloning by diagnostic digestion with BamHI restriction enzyme (Figure 3.54). Expected band sizes were seen in samples 3 and 5. The maps of CFIDCR and CMV-Fluc-CMV-Rluc-IRES-DHFR (CFCRID) vectors were shown in Figure 3.55.

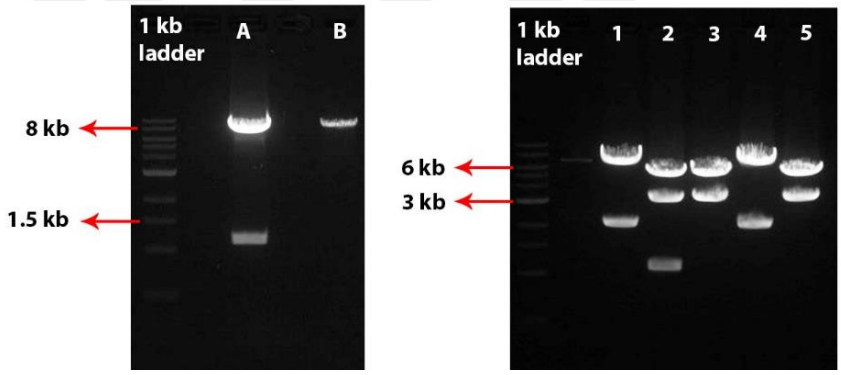

Figure 3.54: The validation of IRES-DHFR cloning into the backbone CFCR. (Left) The plasmid CFIDCR (A) was restricted with XbaI and NheI enzymes in order to obtain IRES-DHFR sequence (1138bp) – expected band sizes were 7598bp and 1155bp. Additionally, the backbone CFCR (B) was run on agarose gel to be ensure that it was linear as a result of restriction with SpeI enzyme. (Right) Plasmids isolated from five colonies were digested with BamHI enzyme – expected band sizes were 5587bp and 3231bp. Both gel electrophoresis experiments were performed on 0.8% agarose gel.

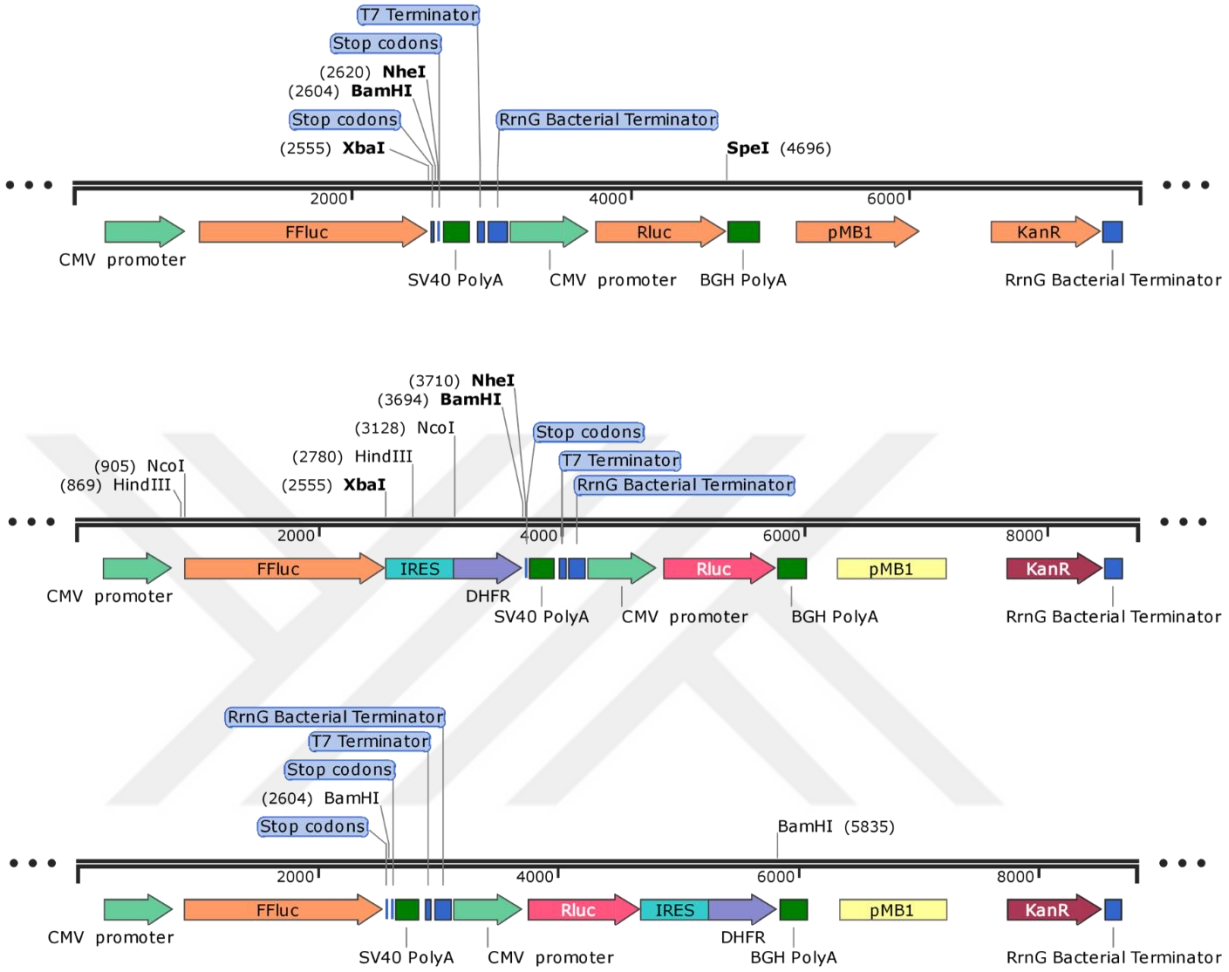

Figure 3.55: The vector design of CFCRID. (Up) The vector map of CFCR which was restricted with XbaI and BamHI enzymes for the cloning of IRES-DHFR. (Middle) The map of CFIDCR vector which was restricted with XbaI and NheI enzymes to extract IRES-DHFR. (Bottom) The map of CFCRID vector. Vector maps were created by SnapGene software.

Keep working with the sample 3, four promoters were studied to clone them upstream of Fluc reporter gene since CMV promoter which resulted in the highest gene expression levels was already placed in our original backbone. Firstly, CFCRID plasmid was restricted with two enzyme groups. BglII and NotI enzymes was used for the cloning of SV40 and UBC promoter while BglII and EcoRI enzymes were used to clone CAG and CHEF1a promoters. Following ligation and transformation steps, four colonies for each promoter cloning experiments were chosen to isolate plasmids and then to validate the cloning by diagnostic digestion (Figure 3.56). The maps of UBC-Fluc-CMV-Rluc-IRES-DHFR (UFCRID), CAG-Fluc-CMV-Rluc-IRES-DHFR (CagFCRID),

CHEF1a-Fluc-CMV-Rluc-IRES-DHFR (CHEF1aFCRID) and SV40-Fluc-CMV-Rluc-IRES-DHFR (SFCRID) vectors were shown in Figure 3.57.

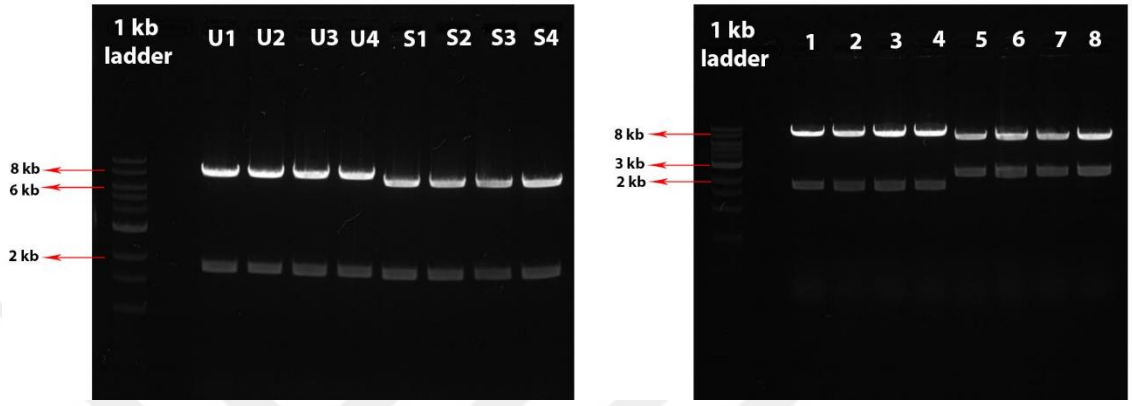

Figure 3.56: The validation of promoter cloning experiments. (Left) UFCRID vectors (U1-U4) were digested with XbaI and SpeI restriction enzymes – expected band sizes were 7662bp and 1749bp. SFCRID vectors (S1-S4) were digested with XbaI and SmaI restriction enzymes – expected band sizes were 6848bp and 1722bp. (Right) CagFCRID vectors (1-4) were digested with XbaI restriction enzyme – expected band sizes were 8123bp and 1767bp. CHEF1aFCRID vectors (5-8) were digested with XbaI and NotI restriction enzymes – expected band sizes were 7217bp and 2427bp. Gel electrophoresis was performed on 0.8% agarose gel.

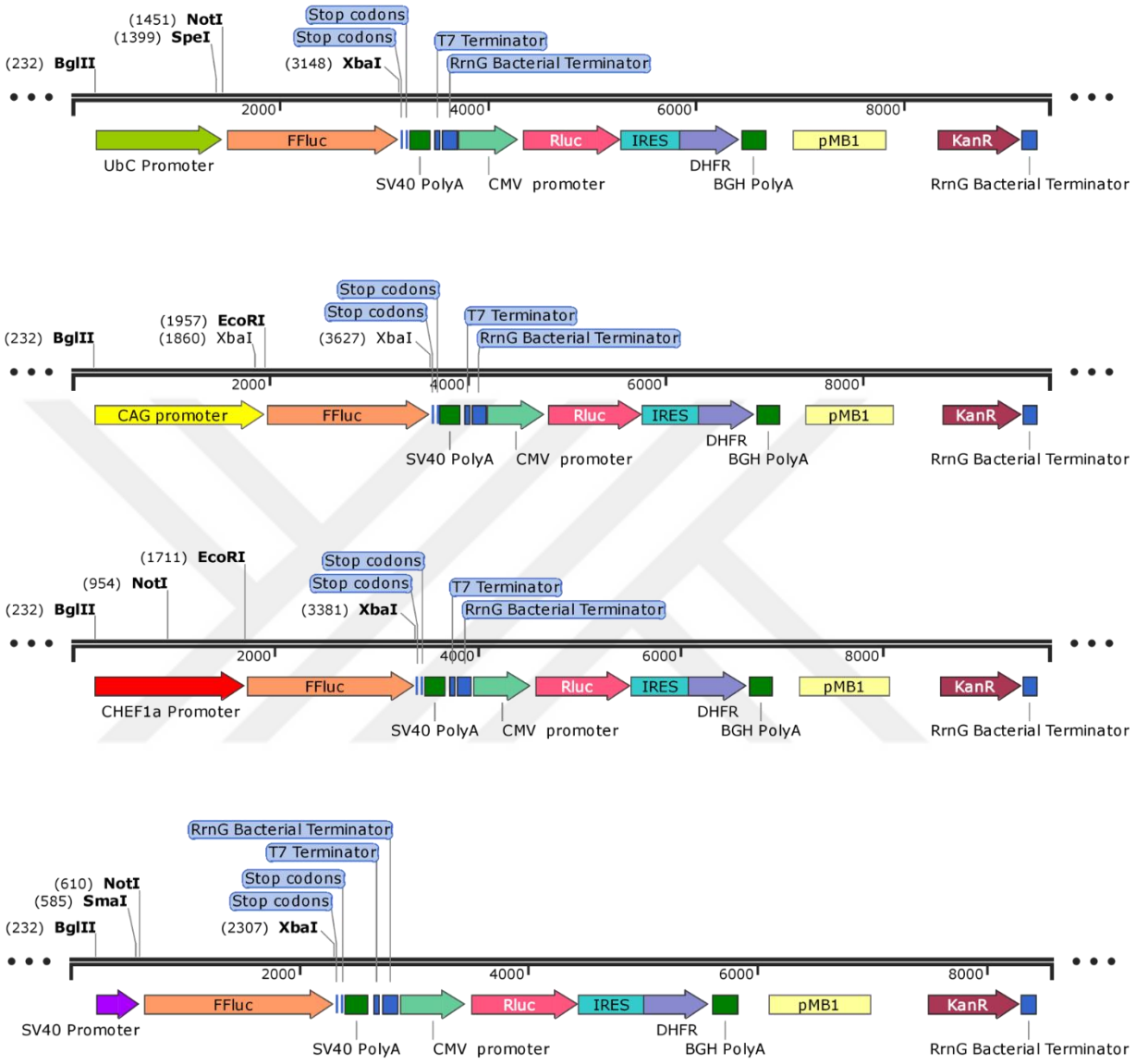

Figure 3.57: The maps of completed vectors with IRES-DHFR. From top to bottom, the maps of UFCRID, CagFCRID, CHF1aFCRID and SFCRID were respectively shown. Vector maps were created by SnapGene software.

3.7. Constructed Vectors Were Transfected into CHO-DG44 Suspension Cells with High Transfection Efficiency

Select five promoters were successfully cloned into the Fluc/Rluc backbone together with IRES-DHFR genes. Then, they were transfected to CHO-DG44 suspension cells by following the manufacturer's instruction of 4D-Nucleofector™ X Unit – Transfection. Additionally, pEGFP-N1

plasmids was transfected to cells to analyze transfection efficiency by flow cytometry. It was showed that EGFP-enriched cells were 98.4% compared to untransfected cells (Figure 3.58).

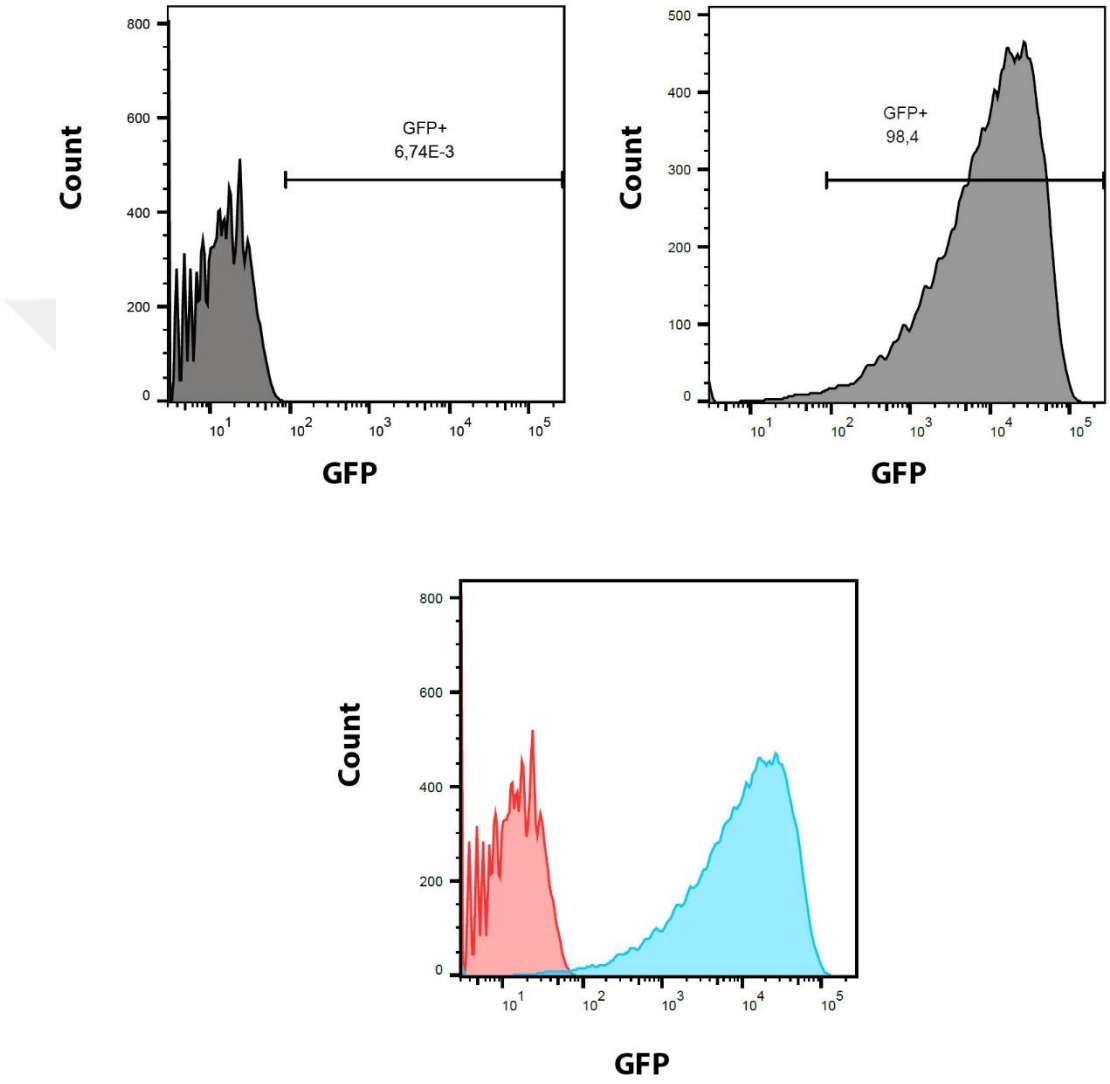

Figure 3.58: Transfection efficiency measurement of CHO-DG44 suspension cells by flow cytometry 24h post transfection. (Up-Left) Nontransfected cells. (Up-Right) Transfected cells. (Bottom) Merge of nontransfected cells' GFP measurement and transfected cells' GFP measurement.

The cell number counted after transfection was recorded as Day 0. They were transferred to selection media (-HT) at Day 2. Next, cells were counted in every three days and cell viability was also calculated according to live cell number / total cell number (Figure 3.59). It was expected to observe firstly a decreasement in the cell number and viability until reaching around 20-30% and

then an increasement to around 90%. At that point, the cells should have been sub-cultured few times to maintain stability. However, our cells could not be recovered as expected.

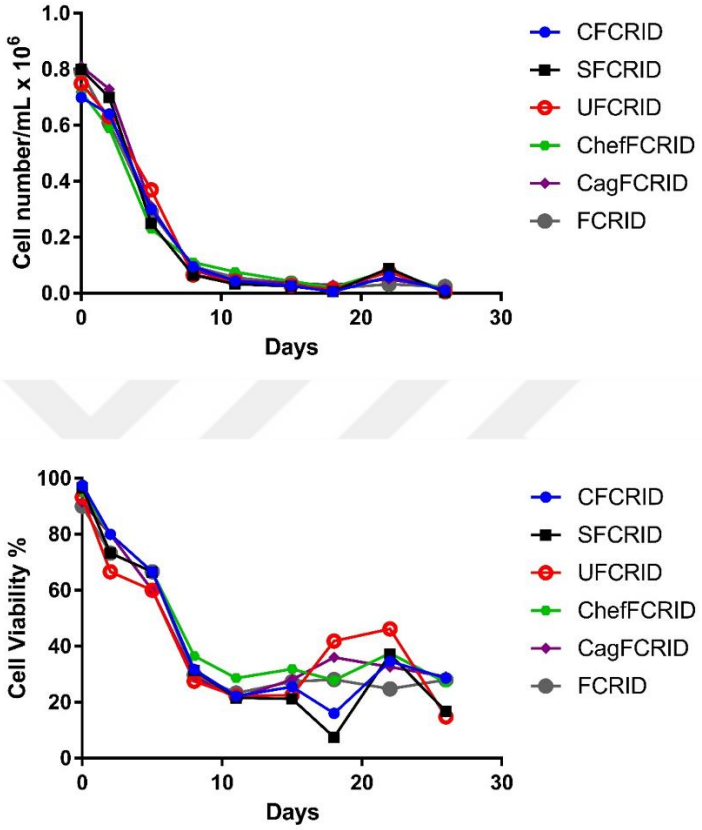

Figure 3.59: Maintaining stable cells. The cell number counting was started immediately after transfection protocol and the selection was applied to the cells at Day 2. Cell number (Top) and cell viability (Bottom) were plotted according to the values obtained in every three days.

4. DISCUSSION

Thanks to advances in recombinant DNA technology, mammalian cell lines are leading expression systems to produce recombinant protein therapeutics for past two decades. Even though there are few cell lines which are suitable for manufacturing of biotherapeutics, over 70% of them was produced in CHO cells due to their biological features such as ability for human-like post translational modifications and having well established gene amplification systems (Dumont et al., 2016; Durocher & Butler, 2009; Jayapal et al., 2007). On the other hand, another important contribution of recombinant DNA technology is to enable researchers for cell line engineering to improve protein production. They consider the development of recombinant protein production from different angles including strategies for efficient introduction of DNA constructs, apoptosis and cell cycle regulations, metabolic engineering, development of serum-free media, transcriptional activities, modifications of chromatin opening elements and improvements in post translational modifications, secretion and folding (Bahrami et al., 2018; Butler, 2005; Gupta et al., 2017; J. Y. Kim et al., 2012; S. H. Kim & Lee, 2009; Kunert & Vorauer-uhl, n.d.; Lai, Yang, & Ng, 2013; Le Fourn, Girod, Buceta, Regamey, & Mermod, 2014; Wong et al., 2010). Improvements in transcriptional level consist of gene amplification systems, chromatin opening elements, matrix attachment regions and the regulatory elements such as promoters and enhancers. Therefore, vector engineering is one of the important steps in the generation of highly productive cells. The plasmid composition has significant effect on expression levels.

In the light of all these informations, the aim of our project is to develop a system performing systematic comparison of promoters to obtain stable, high quality and yield protein production in CHO cell lines. We also aimed to minimize co-transfection related issues during the systematic comparison and therefore engineered a dual promoter system. It has two promoters and two reporters. The first promoter is variable part for the comparison while the second promoter functions as an internal control. Each promoter provides expression of a reporter gene. Firefly luciferase (Fluc) and green fluorescent protein (GFP) reporters is placed in front of the first promoter to evaluate the activity. Meanwhile, Renilla luciferase (Rluc) and tdTomato reporters is used as an internal control for them, respectively. Even though our comparison was mainly based

on luciferase assay and we studied with technical triplicates, the analysis of fluorescence reporters was a biological alternative method to support our results.

As a first step, seven common promoters (CMV, SV40, HSV TK, PGK, EFS, EF1a and UBC) and one CHO-specific promoter (CHEF1a: Chinese hamster elongation factor 1 alpha) would be compared in our dual-promoter system using two reporter organization. The backbone OG4071, which the cloning experiments were held on, had already CMV promoter and hence the remaining promoters were studied during the vector construction. We faced with problems during the cloning of EF1a, CHEF1a and CAG promoters. Considering both the sequences of promoters and reporters to be cloned and the sequences that enzymes recognize, the replacement of CMV promoter was performed with BglII and NotI enzymes for EF1a promoter while with BglII and EcoRI enzymes for CHEF1a and CAG promoters. Firstly, since EF1a promoter has BglII restriction site in its sequence, sequential digestion had been applied to it as seen in Figure 3.3. Two bands in smaller size was necessary to be ensure that restriction reaction worked, while single band showed the total length of EF1a promoter. Therefore, time zones having both single longer band and two shorter bands were used to extract the promoter. We encountered the second problem during the amplification of CHEF1a promoter. The genomic DNA of CHO-WT was used to amplify CHEF1a promoter with the use of corresponding primers. However, it could not be obtained with *Phusion polymerase* despite of trials such as either the use of GC buffer instead of HF buffer or increasing cycle number for amplification. Therefore, gradient PCR was established by increasing the temperature values (60^{o}C, 63^{o}C, 66^{o}C, 69^{o}C, 72^{o}C and 75^{o}C) with the use of *Taq polymerase*. The success in the amplification of CHEF1a promoter was achieved at first three temperature values as seen in Figure 3.6. The third difficulty was had in the cloning of CAG promoter. It could not be amplified with primers since it had high GC content. The strategy of cloning of CAG promoter was changed to cut and paste mechanism. Firstly, CAG promoter was obtained from pCAG ERT2CreERT2 plasmid with SalI and EcoRI restriction enzymes (Figure 3.7). Then, it was cloned into TTi-GFP vector which had intermediary role in the cloning of CAG promoter and was simultaneously restricted by XhoI and EcoRI enzymes (Figure 3.7). With the use of multiple cloning site of TTi-GFP vector, CAG promoter which was extracted with BclI and EcoRI was cloned into Fluc/Rluc and GFP/tdTomato templates which were restricted with BglII

and EcoRI. We used the advantage of that XhoI and SalI restriction enzymes had compatible ends while BclI was compatible with BglII. Additionally, we had to increase the incubation time for this compatibility.

In the construction of reporter systems, there were solely two problems faced during the cloning of tdTomato reporter. One was related to the dimer structure of tdTomato. It was not obtained with primers which would have resulted in monomer constructs and therefore cloned with cut-and-paste mechanism as seen in Figure 3.5. Other one was about restriction enzymes. BglII and NotI restriction enzymes were chosen for the cloning of some promoters. However, tdTomato gene contained recognition sequence of NotI restriction enzyme. Therefore, it was determined to conduct firstly promoter cloning studies and then tdTomato cloning to complete the second reporter system construction.

On the other hand, we confronted with general cloning problems. First problem was to obtain transformants with no insert. Colony PCR is normally set up to provide quick screening of colonies for a desired insert while saving time by omitting plasmid isolation step. However, some colony PCR reactions seen in the 'Results' part showed that colonies formed after transformation did not contain the desired construct. Being one of the reasons, the efficiency of enzyme activities during double digestion experiments can result in insufficient restriction enzyme digestion. Therefore, the vector backbones had to be restricted few times because of facing either with self-ligation or with uncut plasmids on the ligation control plates. Another reason could be arisen from gel purification and PCR clean up. DNA purification or PCR fragment cleaning procedures which use spin columns can result in high salt levels that inhibit enzyme activity. If one enzyme is affected from the salt inhibition, the plasmid can have compatible ends which are ligated without the need of an insert. The efficiency of enzyme activities can also be affected by its stability. We had such a misfortune in two enzymes – BglII and NotI – because of either freeze-thaw cycles or cold chain shipping conditions. Second general cloning problem in our experiments was to obtain positive colony PCR results even though the sample did not show the expected band sizes after diagnostic digestion. These false positives may be resulted from untransformed DNA carried in the ligation mix since it can be spread on the plate and adhered to colony (Agrawal & Roy, 2008).

All completed successful constructs were transfected to CHO-WT, CHO-DG44 and CHO-DG44 suspension cells for systematic comparison. It should be noted that the optimization was performed in CHO-WT and CHO-DG44 cells. However, for transient gene expression studies, suspension culture was also transfected by Lipofectamine 3000 Transfection Reagent using the ratio found in the optimization study. The reason was only arisen from receiving CHO-DG44 suspension cells afterwards. According to luciferase assay, of nine promoters, CMV promoter yielded the highest reporter expression levels in all cell lines. CAG, UBC, SV40, CHEF1a and EF1a promoters were also strong promoters following CMV. The order of these additional five promoters showed little differences between cell lines. Even though they have a common ancestor, their growth media and additional supplements are different due to their different nutritional requirements. In addition, they have undergone different mutations and selection pressures which can affect some biological features or cellular processes (Wurm & Hacker, 2011). For instance, CHO cells showed differences in their lipid metabolism and nucleotide synthesis when they are adapted to suspension growth in serum-free medium (Hackl et al., 2017). All can be reasons for little differences in the pattern of systematic comparison of promoters in cell lines.

Measuring effect of different promoters on transient transgene expression was the first step. Even we studied with triplicates, we determined that a biological alternative method would be useful to support luciferase assay results. Considering our cloning strategies, three strong promoters (CMV, SV40 and CHEF1a) and one weak promoter (HSV TK) were used in the second reporter system consisting of EGFP and tdTomato reporters. As expected, CMV promoter resulted in the highest gene expression, followed by SV40 and CHEF1a promoters while the weakest promoter was HSV TK as in the results of DLR assay.

In the last part, five promoters giving the highest expression levels would be chosen to study promoter activities in stably transfected CHO cells. For that, DHFR gene would be cloned downstream of Rluc reporter gene, linking with internal ribosome entry site (IRES). Firstly, we cloned IRES-DHFR downstream of Fluc reporter with XbaI and BamHI enzymes to prevent self-ligation of plasmid since there was only one enzyme site at the end of Rluc reporter. However, we noticed that it was better to clone IRES-DHFR downstream of Rluc reporter due to that DHFR gene should have been placed in the invariable part during obtaining stable cells. Therefore, CFCR

was restricted with SpeI enzyme while CFIDCR was restricted with XbaI and NheI enzymes to extract IRES-DHFR. All three enzymes used had compatible ends. Nevertheless, it was also a disadvantage since it could give rise to a cloning in reverse direction. BamHI was selected to determine the orientation of insert as in the map (Figure 3.55). Expected band sizes were 5587bp and 3231bp in the case of desired cloning as seen in the samples 3 and 5 (Figure 3.54). If the cloning was reverse-directed, expected band sizes were 6726bp and 2092bp as seen in the samples 1 and 4 (Figure 3.54). There was also additional outcome in which the insert was cloned twice as seen in the sample 2 (Figure 3.54).

According to the results of luciferase assay, for the reporter expression levels, CMV promoter was followed by SV40, CAG, UBC, CHEF1a and EF1a promoters. Considering both cloning strategies in the next step and observing only minimal differences between CHEF1a and EF1a promoters, CHEF1a promoter was chosen with CMV, SV40, CAG and UBC for the last part of the project. These five promoters were cloned into CFCRID vector as seen in Figure 3.56 and 3.57. Successfully completed vectors were transfected to solely CHO-DG44 suspension cells since they were mainly cell line used in the manufacturing of biotherapeutics, providing large scale industrial production. Although transfection protocol using Lipofectamine 3000 reagent was optimized during the first comparison, it was known that CHO suspension cells were hard to transfect and there was optimized highly efficient transfection method – nucleofection (Zahn, Abst, Herrmann, Schindler, & Palmen, 2007). Transfection was performed by 4D-Nucleofector^{TM} X Unit – Transfection with 98.4% transfection efficiency (Figure 3.58). Even so, we had problems at the part of cell recovery. It was expected that non-resistant cells were died and eliminated during first 10-14 days as seen in Figure 3.59. However, then, our cells could not grow even though some of them showed stable cell viability. Maintaining stable cells in suspension culture is prone to be repeated due to some optimization issues such as the DNA amount to be transfected or cell culturing procedures. We are performing our second trial.

5. CONCLUSION AND FUTURE PERSPECTIVES

We engineered a novel dual promoter reporter system providing a systematic comparison of natural viral, mammalian and endogenous promoters in various lines of CHO cells. According to luciferase results which was also supported by flow cytometry analysis, CMV promoter resulted in the highest gene expression levels in all CHO-WT, CHO-DG44 and CHO-DG44 suspension cell lines. Select five strongest promoters were also studied in stably transfected CHO-DG44 suspension cells. When the recovery of stable cells is completed, systematic comparison will be repeated to see whether CMV will still have the strongest effect on gene expression for long term culture. If not, which promoter will provide the highest gene expression in stable cells. In addition, the dual promoter reporter system eliminated the problems related to co-transfection experiments. It will be useful tool not only to identify strong regulatory elements ensuring high levels of expression but also to study the interaction of two proteins.

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