ABSTRACT

ZHANG, KA. Transient and Stable Expression of Single-chain Antibody Fragment *scFv13R4* in Chinese Hamster Ovary Cells. (Under the direction of Gisele Passador-Gurgel).

Monoclonal antibodies are some of the highest earning recombinant protein therapeutics on the market. There is great interest in advancing the technology for expression and production of these proteins. Due to the requirements for protein folding and post-translational modifications, Chinese Hamster Ovary (CHO) cells have separated themselves from the rest of the pack to become the workhorse in industry for producing such therapeutics.

Here a model protein *scFv13R4*, a single-chain antibody fragment against β-galactosidase, is expressed in CHO-S (transient expression) and CHO-K1 (stable expression) cells. Using the Gateway™ Technology from Life Technologies, the *scFv13R4* gene is cloned into suitable expression plasmids and transfected. CHO-S cells are transiently transfected, with cells in suspension taking up the expression plasmid without selection and screening host genome integration. The protein is successfully expressed intracellularly, and cells are lysed and the antibody is purified using immobilized metal affinity chromatography via a hexahistidine tag. CHO-K1 adherent cells are used for stable expression, and the selection process for the best producing cell line is currently ongoing.

Transient and Stable Expression of Single-chain Antibody Fragment *scFv13R4* in Chinese Hamster Ovary Cells

by
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BIOGRAPHY

Ka (Carl) Zhang was born in Sichuan, China, in the city of Chengdu. After leaving China in 1995, his family lived in Danville, California for 8 years before moving to Richmond Hill, Canada. He attended high school at Richmond Hill High School, and soon came back to the United States for his Bachelor of Science degree in Biomedical Engineering at Duke University in Durham, North Carolina. At the Pratt School of Engineering, he conducted research at Ravin Advanced Imaging Labs in optimization of Intensity Modulated Radiation Therapy.

His interest in the biological sciences took him to the Biomanufacturing Master of Science program at North Carolina State University. His studies there exposed him to valuable information and skills valued by the pharmaceutical industry, and at the same time allowed him to conduct research in the lab of Dr. Gisele Passador-Gurgel. His research interests involve optimizing expression systems to facilitate process development for the production of biologics.

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I am grateful to have parents that will support my academic endeavors no matter where it takes me. Knowing I can count on them gives me the power to fight through tough times.

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1. Objectives

The work presented in this thesis aims to find the parameters and conditions that will give successful expression of the single-chain antibody fragment in a mammalian expression host. The goal is to achieve both transient and stable intracellular expression of the *scFv13R4* antibody fragment in CHO-S and CHO-K1 cells respectively. The expression levels should be detectable and quantifiable by conventional methods such as western blot and ELISA. Once this protocol for expression is established, further work can continue on optimization of protein expression.

A secondary objective is to successfully implement the Gateway™ Technology from Life Technologies in the interest of developing a teaching tool that covers protein expression in bacteria, yeast, insect, and mammalian expression systems. The Gateway™ Technology is a fast and efficient method of cloning a gene of interest between many different expression systems. This is beneficial for a semester long class to quickly transition from one expression host to the next, gaining experience with each host as well as seeing a comparison of the major systems used in the biopharmaceutical industry. Prior work has been completed for *Escherichia coli, Saccharomyces cerevisiae,* and *Sf*21 and Hi-5 insect cells. A protocol for a mammalian expression host would give comprehensive coverage of widely used systems in industry.

The broad goals of the project not fully covered by the work presented in this thesis include:

- Optimization of transient protein expression to achieve higher titers
- Optimization of stable protein expression by finding the best producing clone
- Elimination of His and *c-myc* tags on the end of the antibody fragment
- Engineer the expression cassette to allow for extracellular protein secretion
- Scale up and optimization of protein expression up to disposable rocker bag bioreactors

2. Introduction

2.1 Motivation

Heterologous protein production is an important facet of modern biotechnology. Produced using recombinant DNA technology, heterologous proteins are used in a wide array of applications, and one of the most predominant and market-driven uses is protein therapeutics (RNCOS, 2012). Due to the high time and cost demands of taking a drug candidate through development and regulatory approval, much pressure has been put on process development to shorten time and costs to bring a product to the market. The efficiency of this process heavily influences a company's ability to be competitive.

In heterologous protein therapeutics, the expression host plays a vital role in determining the structure of the manufacturing process. Many therapeutics, such as monoclonal antibodies, are only produced in mammalian cells due to constraints in drug safety and efficacy concerns involved with factors such as the presence of endotoxins and post-translational modifications. Chinese Hamster Ovary (CHO) cells are most frequently used in industry when mammalian cells are needed for protein expression.

In order to achieve production of the protein of interest, the expression host must transcribe and translate recombinant DNA coding for the protein. This requires the DNA to be introduced to the cell via plasmid, also known as transfection. This can be either transient, where the recombinant DNA is not integrated into the cell's chromosomes and thus has a

finite lifespan for protein expression, or stable, where the recombinant DNA is integrated into the chromosome and protein will be produced as long as the cell lives as well as by its progeny.

The optimization of protein expression will have a dramatic impact on the yield of the manufacturing process. The selection of the cell type, expression cassette construction, transfection process, and stable selection methods are all areas where optimization can occur. Current technologies have brought the protein production to several grams per liter titers. The continued study of heterologous protein expression will further improve efficiency, lowering the costs of production on both manufacturers and patients.

2.2 Expression Host Selection

The choice of expression host in biopharmaceutical production is based on a variety of factors. First and foremost, protein production is subject to different folding capabilities and post-translational modifications in different expression hosts. Some hosts like *E.coli* cannot correctly fold the protein, although efforts are ongoing to solve that problem (Guglielmi and Martineau, 2009). Also, some protein therapeutics require specific post-translational modifications to ensure safety and efficacy (Raju, 2003; Gary and Jefferis, 2006). Well known is the need of glycoproteins such as monoclonal antibodies for specific glycoforms in order to be effective and avoid clearance *in vivo*. Many functions, such as receptor binding, are associated with specific glycoforms (Jefferis *et al.*, 1998). Foreign glycoforms are also

subject to immune response and fast clearance, reducing the time a therapeutic can act in the body (Singh, 2011). Some common expression hosts like *E. coli* lack the ability to glycosylate at all, and require engineering for functional glycosylation (Wacker *et al.*, 2005). Even then, bacteria have very simple glycoforms and yeast glycoforms are high in mannose (Lua and Chuan, 2012). As seen in Figure 2.2.1 human glycoforms are very different from the glycoforms of those expression hosts. Using mammalian cells to produce the heterologous protein will generate glycoforms closer to those generated in humans, and therefore improve efficacy and reduce immunogenicity.

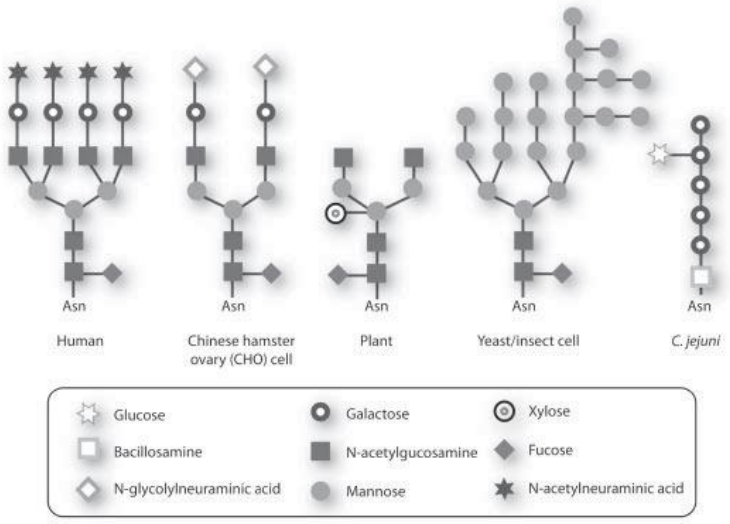

Figure 2.2.1 Comparison of glycoforms for different expression hosts (Lua and Chuan, 2012).

Other factors may not completely rule out a certain expression host, but due to economic reasons certain expression hosts will not be used. For example, bacterial hosts produce lipopolysaccharides (LPS), which are toxic and must be removed in purification. Bacterial hosts like *E. coli* are also prone to inclusion body formation, which are high concentration

insoluble protein aggregates. Recovering the product from inclusion bodies adds considerably more cost to a manufacturing process. Whether a host can express and secrete the protein of interest outside the cell is also a consideration, as cell lysis steps will introduce host cell proteins to the harvest. As the final product must be pure and free of foreign material, host cell proteins mixed with the protein of interest create problems for purification. Considerable efforts and costs are required to remove them from the final product (Wang *et al.*, 2009).

Due to these restraints, mammalian cells are predominantly used as expression hosts for heterologous protein therapeutics despite being more difficult and costlier to maintain compared to bacteria or yeast. About 60 – 70% of all heterologous protein therapeutics are produced in mammalian cells (Wurm, 2004). Several cell lines derived from mammalian tissue are used, such as mouse myeloma (NS0), human retinal (PER.C6), baby hamster kidney (BHK), monkey kidney epithelial (Vero), and human embryonic kidney (HEK293). The most commonly used mammalian expression host is the Chinese Hamster Ovary (CHO) cell. Its ability to be adapted to growth in suspension, grow in defined media, relative hardiness, and ease of genetic manipulation for heterologous protein expression has made it the favored mammalian expression host in industry (Jayapal *et al.*, 2007). For these reasons CHO was the chosen expression host for this project, in particular for its ability to grow in both adherent and suspension culture, which allows for both transient and stable expressions needed for this project.

There are two variants used in this project, CHO-S and CHO-K1. The lineages of these cell lines can be seen in Figure 2.2.2. The CHO-S cell line is a proprietary cell line owned by Life Technologies. It has been adapted to grow in suspension and can grow in defined media. It is not dihydrofolate reductase (DHFR) deficient, which is an auxotrophic marker used in conjunction with media for selective pressure in gene transfer (Jayapal *et al.*, 2007). In DHFR deficient cell lines, the gene for DHFR is knocked out. Without this enzyme, which catalyzes formation of tetrahydrofolate from folic acid, synthesis of essential compounds such as glycine, purine, and thymidine is not possible. DHFR deficient cell lines require hypoxanthine and thymidine to be supplemented in the media for growth. CHO-S does not require hypoxanthine and thymidine supplementation in the media as does other common CHO variants such as CHO-DG44. CHO-K1 is an adherent cell line that can readily be adapted to growth in suspension. This factor is beneficial in the selection for the process of generating a stable clone. Like CHO-S, it is not DHFR deficient.

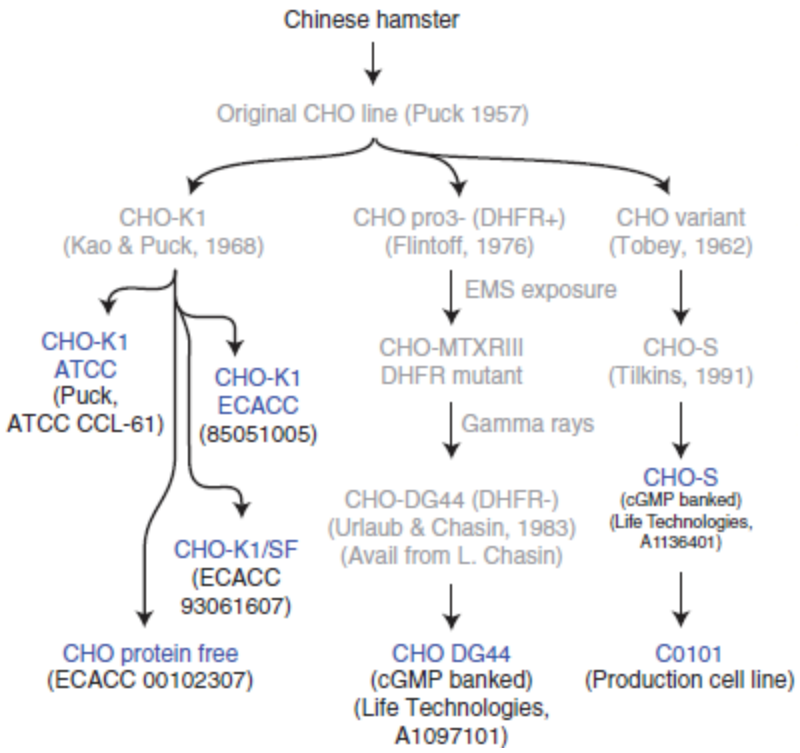

Figure 2.2.2 CHO cell lineage map (Lewis et al., 2013).

2.3 Protein of Interest

The protein of interest being expressed is scFv13R4, a single-chain antibody fragment against β-galactosidase (Sibler *et al.*, 2003). Although it is not a full antibody, it retains the antigen-binding characteristics of a full antibody by having the variable region (F_v) of the heavy and light chains. It is 272 amino acids long and is approximately 28 – 30 kDa depending on the expression host. A *c-myc* tag (recognized by the 9E10 monoclonal antibody for ELISA) and a hexahistidine tag (for immobilized metal ion affinity chromatography and western blotting) are attached on the end of the protein (Figure 2.3).

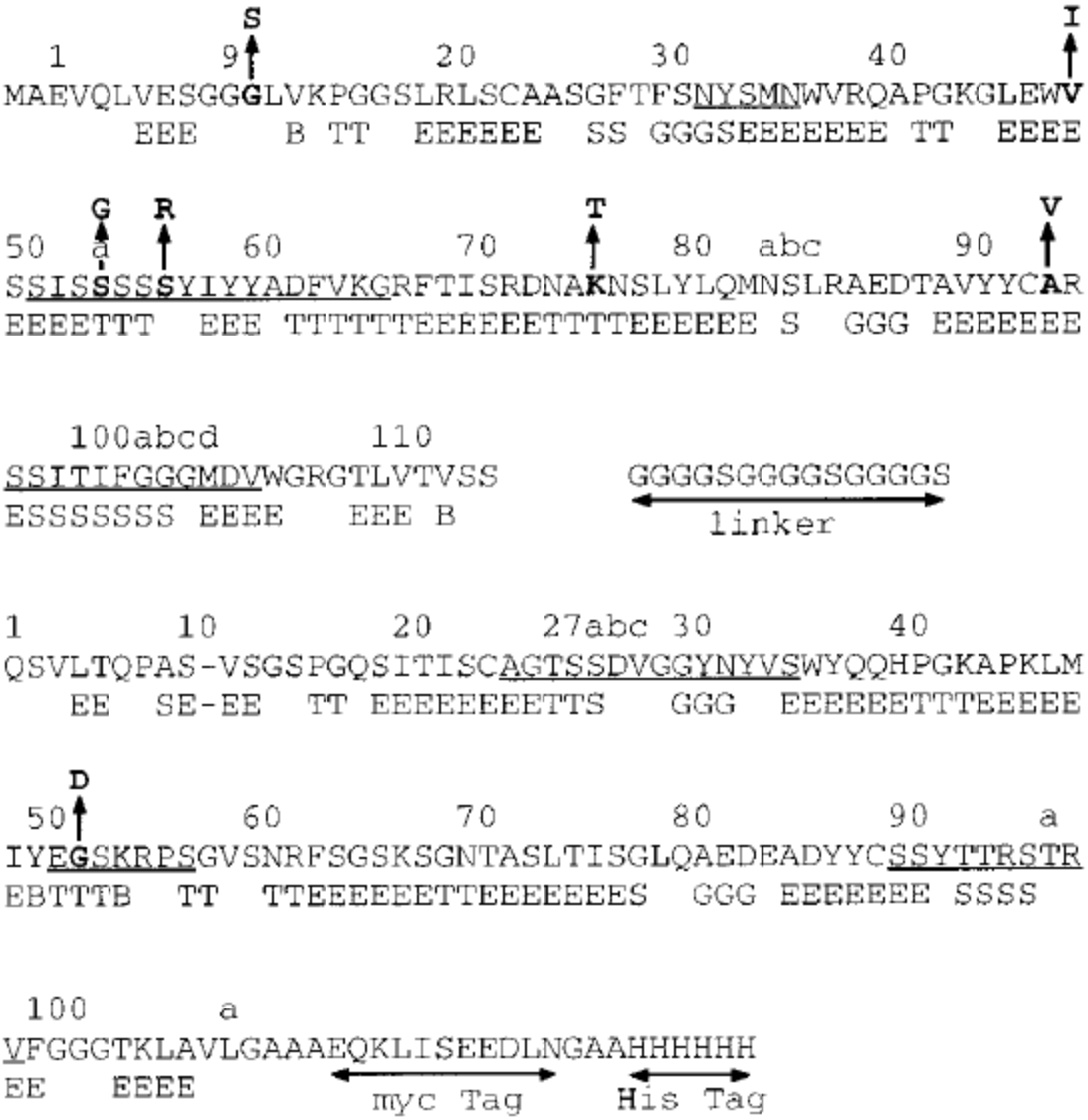

Figure 2.2.3 Amino acid sequence for *scFv13R4* (Martineau *et al.*, 1998).

There are many applications for single-chain antibody fragments that make this protein a good choice for expression. Antibody fragments have pharmacokinetic properties that allow it to be an imaging tool when radiolabeled (Bird *et al.*, 1988; Harwood *et al.*, 1999) or used in biosensor applications. The lack of the F_c region gives it the advantage of background noise reduction in many clinical diagnostic applications. Antibody fragments also have a

wide variety of therapeutic uses as well. They are often linked with compounds of therapeutic effect and with their antigen-specific binding capabilities they can deliver the compound to targeted areas (Holliger and Hudson, 2005). Shown in Table 2.3 is a list of therapeutic antibody fragments currently approved by the FDA or in development.

Table 2.3 Therapeutic antibody fragments approved and in development (Holliger and Hudson 2005).

Fragment type/format	Brand name (generic name)	Specificity/target antigen	Stage	Indication
Fab/chimeric	ReoPro (abciximab)	GpIIb/gpIIa	FDA approved	Cardiovascular disease
Fab/ovine	CroFab	Snake venom	FDA approved	Rattlesnake bite (antidote)
Fab/ovine	DigiFab	Digoxin	FDA approved	Digoxin overdose
Fab/ovine	Digibind	Digoxin	FDA approved	Digoxin overdose
Fab/mouse	CEA-scan (arcitumomab)	CEA	FDA approved	Colorectal cancer imaging
Fab/humanized	Lucentis (ranibizumab; Rhu-Fab)	VEGF	Phase 3	Macular degeneration
Fab/humanized	Thromboview	D-dimer	Phase 1	Deep vein thrombosis imaging
Fab/PEGylated humanized	CDP791	VEGF	Phase 1	Cancer (antiangiogenesis)
Fab/PEGylated humanized	CDP870	TNF-α	Phase 3	Crohn disease
Fab/bispecific humanized	MDX-H210	Her2/Neu & CD64 (ϒFcR1)	Phase 2	Breast cancer

Table 2.3 Continued

Single-chain Fv (scFv)/ humanized	Pexelizumab	Complement C5	Phase 2/3	Coronary artery bypass
$(ScFv)_4$fused to streptavidin mouse	CC49	TAG-72 Pancarcinoma antigen	Phase 1	Pretargeting radioimmunotherapy for gastrointestinal malignancies
ScFv fused to β-lactamase human	SGN-17	P97 antigen	Preclinical	Melanoma (ADEPT prodrug activation)
ScFv fused to PEG human	F5 scFv-PEG Immunoliposome	Her2	Preclinical	Breast cancer as drug targeting
Diabody $(V_H\text{-}V_L)_2$human	C6.5K-A	Her2/Neu	Preclinical	Ovarian and breast cancer
Diabody $(V_H\text{-}V_L)_2$human	L19 L19–γIFN	EDB domain of fibronectin	Preclinical	Antiangiogenesis and atherosclerotic plaque imaging
Diabody $(V_L\text{-}V_H)_2$human	T84.66	CEA	Preclinical	Colorectal cancer imaging
Minibody (scFv-$C_H3)_2$ murine-human chimera (minibody)	T84.66	CEA	Human Imaging Pilot Study	Colorectal cancer imaging pretherapy
Minibody murine-human chimera (minibody)	10H8	Her2	Preclinical	Ovarian and breast cancer
ScFv dimer Fc $(ScFv)_2$-Fc murine-human chimera (minibody)	T84.66	CEA	Preclinical	Colorectal cancer
Bispecific scFv $(V_L\text{-}V_H\text{-}V_H\text{-}V_L)$ mouse	r28M	CD28 and MAP	Preclinical	Melanoma (MAP antigen)

Table 2.3 Continued

Bispecific scFv (V_L-V_H-V_H-V_L) origin unknown	BiTE MT103	CD19 and CD3	Phase 1	B-cell tumors (non-Hodgkin lymphoma, acute and chronic lymphocytic leukemia)
Bispecific scFv (V_L-V_H-V_H-V_L) origin unknown	BiTE	Ep-CAM and CD3	Preclinical	Colorectal cancers
Bispecific tandem diabody (VH-VL- VH -VL) (mouse)	Tandab	CD19 & CD3	Preclinical	B-cell tumors (non-Hodgkin lymphoma, acute and chronic lymphocytic leukemia)
VhH–β-lactamase fusion camelid	Nanobody	CEA	Preclinical	Cancer imaging
Dab/human	Anti-TNFα dAb	TNFα	Preclinical	Rheumatoid arthritis and Crohn disease
VhH/camelid	Nanobody	TNFα	Preclinical	Rheumatoid arthritis and Crohn disease
VhH/camelid	Nanobody	Von Willebrand factor	Preclinical	Antithrombotic

Choosing this protein for expression is also useful for the secondary course development objectives of this project because it is a protein that can be expressed not only in insect and mammalian expression hosts, but also in bacterial and yeast expression hosts. Expressing this protein would allow for cross-comparison between expression hosts. Since it retains many characteristics of a full antibody, using this protein as a teaching tool would give applicable experience for a protein relevant in industry.

2.4 Expression Vector (Gateway System)

In order for the chosen expression host to express the heterologous protein the gene of interest must be introduced into the cell to be transcribed and translated. This delivery vehicle, the expression vector, requires at least a promoter to recruit the RNA polymerase and a polyadenylation sequence for efficient transcription termination and mRNA exportation (Dale, 2006). Generally enhancer elements, a selection marker, and elements such as introns or chromatin modifying sequences are included. Once taken up by the cell, this expression vector will be able to recruit the cell's native RNA polymerases for transcription of the gene of interest. The choice of promoter, polyadenylation sequence, enhancer sequence, etc., depends on the expression host used. Thus proprietary expression systems often design a product which includes cell line as well as expression vector and media designed for the cell line. Commonly used proprietary expression systems are shown in Figure 2.4.1.

Table 1: Selected mammalian expression systems for antibody production

System	Promoter	PolyA	Selection	Host Cell	Enhancer	Insulator	IRES	Citation
GS	hCMV-MIE	SV40	Glutamine synthetase	CHO, NS0	CMV	None	None	Lonza Biologics
PER.C6	hCMV-MIE	BGH	*neo* (G418)	PER.C6	CMV	None	None	(48)
CHEF-1	CHEF-1 5′	CHEF-1 3′	DHFR/*neo*	CHO	None[1]	None[1]	None	(6)
EASE	hCMV	ND[2]	DHFR/*neo*	CHO	None[3]	EASE	ECMV	(30)
UCOE	hCMV	ND[2]	*neo*/hygro	CHO	None	UCOE	None	(31)
Chick Lysozyme MAR	SV40	SV40	DHFR/*neo*	CHO	SV40	MAR	None	(29)
Ig Heavy-Chain Enhancer	MT1 Ig κ	Mouse Ig κ, Ig γ	DHFR	CHO	Mouse Ig heavy chain	None	None	(13)

[1] Sequences contained within the CHEF-1 DNA fragments may contain enhancer or chromosomal insulator activity.
[2] Element used could not be determined from available literature. [3] Sequences contained within the EASE DNA fragments may contain enhancer activity.

Figure 2.4.1 Common expression systems in industry and their components (Dale, 2006).

The expression vector used in this project is part of the Gateway™ Technology from Life Technologies (Life Technologies Co., 2003; Sasaki *et al.*, 2004). This system utilizes site-specific recombination reactions to easily move a gene of interest from an entry vector to one of many expression vectors designed for specific expression hosts.

The transfer of the gene of interest from entry vector to expression vector is made possible through recombination reactions based on the Lambda bacteriophage's reactions used for propagation. These recombination reactions occur during the lytic and lysogenic of the Lambda phage's life cycle as it propagates using a host cell. The recombination reactions are conservative, and are specific to *att* attachment sequences optimized from the original viral sequence. The Clonase™ enzymes attach at these sites to facilitate the exchange of DNA. As shown in Figure 2.4.2 the recombination reactions swap the entire DNA segment flanked by the *att* sites. A PCR construct containing the gene of interest flanked by the *att*B sites can be combined with a vector containing *att*P sites in a recombination reaction catalyzed by BP Clonase™ to obtain an entry vector that contains the gene of interest flanked by *att*L sites. This vector contains the selection markers and bacterial origin of replication necessary for

propagation in an efficient cloning organism such as *E. coli*. Once the expression host has been selected, an expression vector suitable for the intended host may be selected from the library and combined with the entry vector in a recombination reaction catalyzed by LR Clonase™ to obtain an expression vector containing the gene of interest.

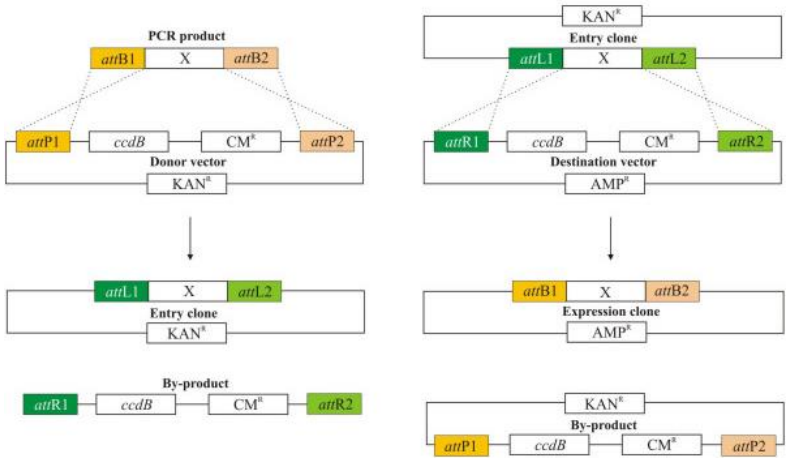

Figure 2.4.2 *att* attachment site positions and reaction scheme for the Gateway™ recombination reactions (Magnani, 2006).

These recombination reactions are simple to carry out, and in a situation where the investigator wishes to express a gene in multiple expression host organisms, the tedious tasks of collecting different expression vectors and finding corresponding useable restriction sites are not needed. Once the gene of interest is cloned into the entry vector, it is easily transferred to expression vectors designed for specific organisms.

The entry vector used in this project is pENTR11, and the expression vector used in this project is pcDNA-DEST40. To allow for propagation and storage in bacteria both of these vectors have a pUC bacterial origin of replication, as well as a selection system to ensure

culturing *E. coli* that contains the vector. The first part of the selection system is the *ccd*B gene, which codes for a protein inhibiting topoisomerase II, making it toxic for *E. coli* (Bernard and Couturier, 1992). Both pENTR11 and pcDNA-DEST40 start with the *ccd*B gene being flanked by the appropriate *att* attachment sites. When the gene of interest is cloned into the vector through the recombination reactions, the *ccd*B gene is switched out and replaced with the gene of interest. When the product of this recombination reaction is taken up by *E. coli*, the unreacted vectors and the byproducts will contain the *ccd*B gene, and the cells that take those up will die. Culturing the *E. coli* while applying selective pressure using the appropriate antibiotic, either ampicillin for pcDNA-DEST40 or kanamycin for pENTR11, will ensure the surviving cells have taken up the vector and that said vector has successfully undergone the recombination reaction.

As an added step, the second part of the selection system is a chloramphenicol resistance counter screening method. In the case of false positives where the *ccd*B gene has mutated, chloramphenicol resistance will be conferred. Thus plating the clones will show false positives if they are both ampicillin/kanamycin resistant and chloramphenicol resistant.

Unique to pcDNA-DEST40, the CHO expression vector, is the mammalian promoter/enhancer element and polyadenylation sequence. The promoter/enhancer is a human cytomegalovirus promoter. This is the most commonly used mammalian promoter in industry. The polyadenylation sequence is from bovine growth hormone (BGH). These were

chosen for their suitability for expression in a mammalian cell expression host (Life Technologies Co., 2003).

Finally, for successful expression of the heterologous protein the rare codon usage in CHO cells must be analyzed. Rare codon usage, or codon bias, is a phenomenon observed when a DNA sequence from one organism is inserted and transcribed in a different organism (Gustafsson *et al.,* 2004). All organisms have varying amounts of the aminoacyl-tRNAs, and this is reflected in its coding sequences. Since each amino acid may have multiple possible codons, different organisms will have different tendencies to use a certain codon over others depending on their inherent levels of aminoacyl-tRNA. If the sequence from one species is transcribed and expressed in another, the sequence may heavily use a codon for which the expressing species has very little aminoacyl-tRNAs. In this case expression would reach a bottleneck and often stop all together. Thus a rare codon analysis must be run to ensure codon usage in the original sequence will not trigger the use of rare codons in the expression host. Although the original sequence for *scFv13R4* is human (Martineau *et al.,* 1998), this rare codon analysis must be performed before the gene is cloned into the expression vector for CHO.

2.5 Cell Culture

2.5.1 Cell Culture Introduction

Culturing of the cells is a vital aspect of heterologous protein therapeutic production. Not only does cell culture play a vital role in cell density and thus productivity, but also in the quality of the final product. Cell culture encompasses all the equipment and techniques required to grow cells, including culture vessel type, temperature and pH conditions, media composition, and techniques for sampling, passage, and cryopreservation. The specific parameters, conditions, and protocols for techniques used will be addressed in the materials and methods section of this thesis.

2.5.2 Media

The media used is vital to the growth and protein expression of the cultured cells. In general, there are six components in cell culture media (Rae, 1997):

- Basal Salt Solution
- Amino Acids
- Energy Source
- Buffer system
- Serum/Specific growth factors (in serum-free media)
- Antibiotics

The Basal Salt Solution is designed to provide the necessary inorganic salts needed both in a metabolic capacity as well as to create an environment that mimics the osmotic pressure the cells would experience *in vivo*. For mammalian cells, essential amino acids must be supplied in the media. The energy source for cell culture is usually glucose, but for most mammalian cell cultures glutamine is the primary energy source (Zielke *et al.*, 1984; Cruz *et al.*, 1999).

As the culture grows there will be byproducts of metabolism that will alter the pH of the culture. To maintain a balanced environment mimicking physiological pH, a buffer system is needed. The most predominant form of buffering is the bicarbonate/CO_2 system, where sodium bicarbonate is provided in the balanced salt solution and CO_2 is supplied in the cell culture environment. Another common buffering system is the addition of 4-(2-Hydroxyethyl)-1-piperazineethanesulfonic Acid (HEPES) to the media (Eagle *et al.*, 1971; Shipman *et al.*, 1969). HEPES is an organic compound that achieves buffering without reliance on CO_2 levels. This is important as CO_2 is a byproduct of cellular respiration, and thus with changing CO_2 levels in the culture environment the bicarbonate/CO_2 system is not as robust.

In mammalian cell culture, growth of the cells often depends on the presence of serum. Serum contains proteins that carry hormones and/or growth factors (Barnes and Sato., 1980). The two most important of these are transferrin and insulin. Transferrin has been found to be a carrier molecule for iron, which is needed by ribonucleotide reductase in DNA synthesis (Sunstrom *et al.*, 2000). Insulin has been found to be a growth factor promoting mitosis

(Yandell *et al.*, 2004). From a safety standpoint, serum is an undefined animal product with possibility of carrying viruses capable of infecting humans. Since serum is not characterized, it is difficult to anticipate whether viral contamination will be present. There is also no guarantee of consistency from batch to batch. This is unacceptable in a regulatory environment where quality must be assured through complete control and understanding of the process and intermediate materials. Thus media development has advanced to supplement these serum components without the use of serum.

Lastly, antibiotics are prevalent in use both to keep adventitious agents from contaminating media and for selection of cells that have taken up the recombinant DNA needed to express the protein of interest. Antibiotics are generally added separately, as the specific antibiotic to be used depends on the selection marker used in the vector. The most commonly used antibiotic for prevention of media contamination is a combination of penicillin and streptomycin.

The media used in this project, CD CHO from Life Technologies and F-12K (Kaighn's modification on Ham's F-12 Nutrient Mixture) are both chemically defined and use L-glutamine as the nutrient source. They are both bicarbonate buffered, and F-12K contains phenol red as an indicator. The composition of F-12K has been made public and can be seen in Appendix A.

2.5.3 Other equipment and techniques

Critical to cell culture is the monitoring of the cell density and timely passage of cells to ensure a healthy culture. If not passaged, cells overgrow and consume all nutrients in the media. Without passage the byproducts of cellular metabolism such as lactate and ammonia will build up and have deleterious effects on growth and productivity of the culture (Butler and Spier, 1984; Ozturk *et al.*, 1992). The most basic method for determining cell density is physically counting the cells under the microscope with a hemocytometer. Dye-exclusion was introduced to differentiate between live and dead cells, as dead cells often have the same mass and general shape as live cells (Strober, 2001). Cell death is often characterized by the breakdown in integrity of the cell membrane. Thus dead cells will be infiltrated by dye molecules, and automated cell counters are programmed to recognize and discount the stained cells. Trypan Blue is most commonly used for this purpose. Counting using hemocytomers is a laborious process. It is also very inconsistent as different operators will arrive at different cell counts. For these reasons automated methods were developed. Measuring optical density using a spectrophotometer is possible, as cell density corresponds to turbidity of the culture and thus influences light scattering. However, this method is inaccurate and does not give an exact number of cells nor the viability. Many automated cell counters exist. They generally take magnified photos of a culture sample and using image processing to determine which of the image are cells. Coupled with dye-exclusion, cells are counted and live and dead cells are differentiated in the result, giving cell viability. Flow cytometry is a growing technology capable of sorting single cells by volume and other characteristics such as viability based on fluorescence (Hulett *et al.*, 1969; Darzynkiewicz *et*

al., 1992). Not only does this give information as to cell density and viability, but it can also be used to distinguish different cell lines and even cells undergoing different changes (Horan and Wheeless, 1977).

In passage of adherent cells, the cells must be detached from the surface of the flasks they are growing on. This is done by addition of enzymes such as trypsin, pronase, dispase, or collagenase with trypsin being most common. The mechanism for detachment using trypsin has been proposed to be an alteration of cell morphology that favors detachment (Revel *et al.*, 1974).

As the project is in its early stages, work has been done exclusively at the shake flask and T-flask level. Thus parameters pertinent to bioreactor scale cultures such as agitation, aeration, and bioreactor design were not explored.

2.6 Transfection

2.6.1 Transfection Introduction

The process of delivering the recombinant DNA into the expression host cell is called transfection for mammalian cells. Transfection can be done in three main ways: using a viral vector, using a physical method such as electroporation, or using chemicals such as lipids or polymers (Kim and Eberwine, 2010). Transfection efficiencies differ, and each has its own advantages and disadvantages. Physical methods such as electroporation, where the outside

charge of the cell is changed to facilitate uptake of DNA (Chu *et al*., 1987), is possible but not frequently used in industry.

Viral vectors take advantage of the natural ability of viruses to deliver its genetic material into the cell and integrate into the host cell genome. However, this integration is random. In the protein therapeutics industry, safety is of paramount importance, and the use of viral vectors provides many regulatory challenges. Viral vectors are also limited in the size of the gene it can carry, posing problems for expression of larger proteins such as full-chain monoclonal antibodies. These disadvantages greatly limit the use of viral vectors in protein therapeutic production.

Chemical methods are most commonly used for transfection in protein therapeutic production. These cationic compounds generally form a vesicle that encapsulates the anionic recombinant DNA and, when introduced to the culture, are taken up by the cell into the nucleus through endocytosis or phagocytosis. Of the cationic polymers, the most commonly used compound is polyethyleneimine (PEI). It has proven to be an efficient gene delivery tool for mammalian cell transfections (Tait *et al.*, 2004; Nimesh *et al.*, 2006). However, it has certain pH requirements for the transfection and expression media (Liu *et al.*, 2008). In this project, cationic lipid based compounds were used for the transfection of CHO-S and CHO-K1 cells. FreeStyle™ MAX Reagent, a companion product complementing the FreeStyle™ CHO-S cell line used in this project, was used for transient expression as the reagent is

optimized for the proprietary cell line. Lipofectamine™ 2000 was used for the CHO-K1 cell line in the stable expression experiments.

2.6.2 Transient vs. Stable expression

In this project both transient and stable expression were attempted. As the name suggests, transient expression relies on temporary expression without selection of cells for genomic integration of the gene of interest (Geisse and Fux, 2009). While there could be integration events, these cells are not isolated, and no time is spent in cultivating a clonal population with the gene of interest integrated. The protein of interest is expressed mainly from transcription of the expression vector inside the nucleus of the host cell. This vector may or may not be propagated when the cell divides, and over time the expression of the heterologous protein is lost. Transient expression is very beneficial for the research and development phase of a protein therapeutic. Protein expression can be seen in just days without the need for long and tedious cell selection. In the trial phase of products where a large quantity of protein is not yet needed and fast results are desired, it is advantageous to use transient expression. When research and development ends and the gene needs to be expressed and large scale production is needed, transient expression becomes inefficient. Without integration into the genome and selection for high producing clones, most of the cells being cultured at enormous expense will not be expressing protein at optimum levels. On a manufacturing scale, this is very wasteful and not a feasible means of production.

Once a gene encoding a protein with clinical efficacy is deemed ready for large scale protein production, stable expression is the preferred method of transfecting and expressing the gene of interest. Chances for correct integration that allows for protein expression are low. This means many cells must be screened for those that have integrated the gene of interest and also those that are high producers. This process is labor and time intensive. Different pools of adherent cells are transfected in well plates. Once transfected, several rounds of selective pressure is applied, usually in the form antibiotic supplementation to the medium. The pools of transfectants are adapted to suspension, a process that takes several weeks. Pools producing protein are then subjected to limited dilution cloning, where one cell is put into each well of a 96-well plate. Growth is monitored, and each cell is tested for protein production. Once a high producing cell line has been isolated, it can be banked and saved to be cultured to express at high levels the protein of interest at any time without further need of transfection.

3. Materials and Methods

3. 1 The Gene *scFv13R4*

Bacterial stock PM12 containing the gene *scFv13R4* was kindly provided by Dr. Pierre Martineau (Martineau *et al.,* 1998). PM12 is composed of *E. coli* strain BL21(DE3)lacZ::Tn5 pLysS transformed with the plasmid pET163R4. Plasmid pET163R4 was constructed by cloning the gene *scFv13R4*, a 819bp *Nco*I-*Eco*RI fragment from plasmid pPM163R4, in the *Nco*I and *Eco*RI sites of the expression vector pET-23d(+) from Novagen, Inc. After purification of the plasmid pET163R4 and subsequent digestion with enzymes *Nco*I and *Eco*RI, the gene *scFv13R4* was cloned into the entry vector pENTR11 (Life Technologies) generating the entry clone pENTR11-*scFv13R4*. This clone is the entry vector for all subsequent experimental projects in the lab.

The nucleotide sequence is shown below 5' to 3':

atggccgaggtgcagctggtggagtctgggggaggcctggtcaagcctgggggtccctg
agactctcctgtgcagcctctggattcaccttcagtaactatagcatgaactgggtccgc
caggctccagggaaggggctggagtgggtctcatccattagtagtagtagttacata
tactacgcagacttcgtgaagggccgattcaccatctccagagacaacgccaagaactca
ctgtatctgcaaatgaacagcctgagagccgaggacacggctgtttattactgtgcgaga
tccagtattacgatttttggtggcggtatggacgtctggggcagaggcaccctggtcacc
gtctcctcaggtggaggcggttcaggcggaggtggcagcggcggtggcggatcgcagtct
gtgctgactcagcctgcctccgtgtctgggtctcctggacagtcgatcaccatctcctgc
gctggaaccagcagtgacgttggtggttataactatgtctcctggtaccaacaacaccca
ggcaaagcccccaaactcatgatttatgagggcagtaagcggccctcaggggtttctaat
cgcttctctggctccaagtctggcaacacggcctccctgacaatctctgggctccaggct
gaggacgaggctgattattactgcagctcatatacaaccaggagcactcgagttttcggc
ggagggaccaagctggccgtcctaggt**gcggccgc**agaacaaaaactcatctcagaagag
gatctgaatgggccgcacatcaccatcatcaccattaa

The sequence in purple codes for the heavy chain variable region, while the sequence in brown codes for the light chain variable region of the antibody fragment. The portion in green is the *c-myc* tag and the portion in red is the His-tag. The *Nco*I site is on the 5' end, and the *Eco*RI site is on the 3' end.

3.2 Rare codon analysis

The GenScript online tool for rare codon analysis was used (http://www.genscript.com/cgi-bin/tools/rare_codon_analysis) to check for codon bias. The protein sequence was input into the webpage with *Cricetulus griseus* (CHO) selected as the expression host. After clicking "analysis", the results were recorded.

3.3 Entry and expression vectors

3.3.1 Propagation of Plasmids pENTR11-*scFv13R4* and pcDNA-DEST40

The entry vector (pENTR11) and the mammalian expression vector (pcDNA-DEST40) were both obtained from Life Technologies. Their vector maps are shown below in Figure 3.3.1. The gene *scFv13R4* was cloned into the entry vector pENTR11 as a result of previous work in the lab group. No modifications based on codon bias were made on the sequence for *scFv13R4*.

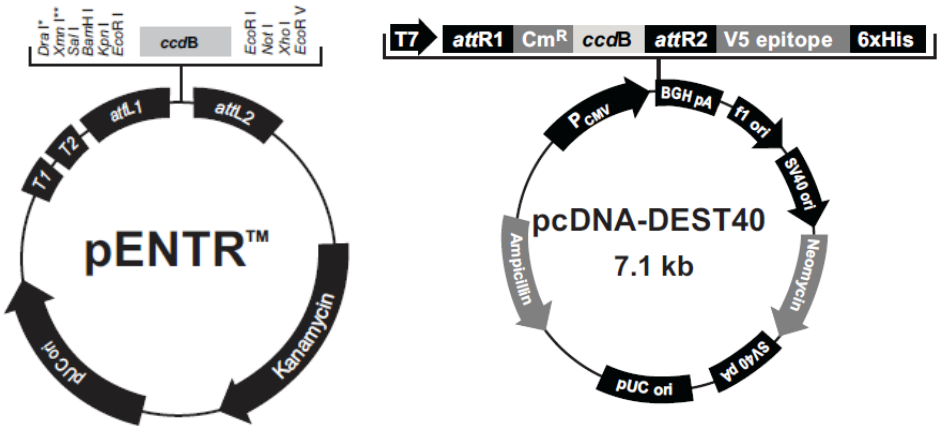

Figure 3.3.1 The vector maps for pENTR11 and pcDNA-DEST40.

To prepare for recombination reactions, pcDNA-DEST40 was transformed into chemically competent One Shot® *ccd*B Survival™ 2 T1^{R} *E. coli* cells from Life Technologies. The detailed transformation protocol can be found in Appendix B. pcDNA-DEST40 at 150 ng/µL was diluted using ultra-pure water to 10 ng/µL, and 1 µL (1 ng) was used for the transformation. Transformed cells (60 µL) were plated on LB + Ampicillin (100mg/mL antibiotic final concentration) and incubated overnight at 37^{o}C. A single colony was picked using a pipette tip and cultured in 15 mL snap cap tubes (4 mL LB + 100 mg/mL Ampicillin), incubated overnight at 37^{o}C with vigorous shaking at 250 rpm. Cells were then pelleted at 4500 g for 5 minutes.

The pENTR11-*scFv13R4* was already banked on DB3.1 pENTR11-*scFv13R4* clone #3 from the Gurgel lab group. They were cultured in four 15 mL snap cap tubes (4 mL LB + 50mg/mL Kanamycin) by dipping a pipette tip in the stock culture and dispensing the tip into

the snap cap tube. The tubes were incubated overnight at 37^{o}C with vigorous shaking at 250 rpm. Cells were then pelleted at 4500 g for 5 minutes.

DNA purification was done using the Miniprep plasmid purification kit from QIAGen according to the manufacturer's instructions. Measurement of plasmid concentration and purity for the purified pcDNA-DEST40 and pENTR11-*scFv13R4* plasmids was carried out using the NanoDrop 1000 spectrophotometer from Thermo Scientific. Purity is given in a ratio of wavelengths 260/280 nm, with values close to 1.8 being most pure (NanoDrop Technologies, Inc, 2007).

3.3.2 LR Recombination Reaction and *E. coli* transformation

The LR Recombination reaction, part of the Gateway™ Technology, was performed on pENTR11-*scFv13R4* and pcDNA-DEST40 using the LR Clonase™ II enzyme from Life Technologies according to the protocol in Appendix C. 2 µL of enzyme diluted in 6 µL of TE buffer was used. 1 µL each of the purified pENTR11-*scFv13R4* (156.4 ng/µL) and stock pcDNA-DEST40 (150 ng/µL) were used for the reaction.

The resulting pcDNA-DEST40-*scFv13R4* from the LR recombination reaction was then transformed into chemically competent *E. coli* DH5-α using 50 µL of stock cells and 1 µL of pcDNA-DEST40-*scFv13R4.* The cells and DNA were combined and incubated on ice for 30 minutes. The cells were then heat shocked at 42^{o}C for 30 seconds, and then added to 250 µL

of S.O.C. medium in a 15 mL snap cap tube. The tube was then incubated at 37^{o}C with vigorous shaking at 225 rpm for 1 hour to allow for recovery of heat shocked cells. The culture was then plated on plates with LB + 100 mg/mL Ampicillin and LB + 34 mg/mL Chloramphenicol once using 20 µL of culture and once with 100 µL of culture.

The recombination reaction was repeated using the combination of plasmids shown in Table 3.3.2, using the same protocol.

Table 3.3.2 The combinations of different plasmid amounts for the LR Recombination reactions.

Plate ID	Entry	Expression	Volume plated	Notes
1, 2, 3	pENTR11-*scFv13R4* 150 ng	pcDNA-DEST40	100 µL	
4, 5	pENTR11-*scFv13R4* 150 ng	pcDNA-DEST40 *	100 µL	
6, 7, 8	pENTR11-*scFv13R4* 150 ng	pcDNA-DEST40	100 µL	
9, 10, 11	pENTR11-*scFv13R4* 150 ng	pcDNA-DEST40	20 µL	
12, 13, 14	pENTR11-*scFv13R4* 100 ng	pcDNA-DEST40	100 µL	
15, 16	pENTR11-*scFv13R4* 100 ng	pcDNA-DEST40	20 µL	
17, 18	pENTR11-*scFv13R4* 50 ng	pcDNA-DEST40	100 µL	
19, 20	pENTR11-*scFv13R4* 50 ng	pcDNA-DEST40	20 µL	
21	pENTR11-*scFv13R4* 150 ng	pYES-DEST52 (150 ng/µL)	100 µL	
22	pENTR11-*scFv13R4* 150 ng	pYES-DEST52 (150 ng/µL)	20 µL	
23, 24	N/A	pcDNA/GW-40/lacZ (500 ng/µL)	20 µL	No recombination performed, transfection control

* The amount of pcDNA-DEST40-*scFv13R4* used for the transformation was 5 µL instead of 1 µL

The transformation was repeated using the same protocol with the results of the recombination reactions. A lacZ transfection positive control was also transformed, using 1 µL of pcDNA/GW-40/lacZ (500 ng/µL).

For confirmation of gene presence and size, a colony PCR was performed using *scFv13R4* forward and reverse primers from IDT. The plasmid pYES-DEST52-*scFv13R4* (1 µL at 70 ng/µL) was used as the positive control. The subsequent DNA gel was performed using 0.5 µg/mL Ethidium Bromide. The detailed protocol for the colony PCR and DNA gel can be found in Appendix D and E respectively.

3.4 CHO-S Cell Culture

The FreeStyle™ CHO-S cells were obtained from Life Technologies. The media used was CD CHO from Life Technologies supplemented with 4 mM L-glutamine. CHO-S cells (1 x 10^7 cells/mL) were removed from liquid nitrogen storage and thawed in a water bath at 37^{o}C. The contents were then transferred to a 125 mL baffled shake flask containing 30 mL of pre-warmed supplemented media. Cells were incubated with shaking at 135 rpm at 37^{o}C and 5% CO_2. The cells are allowed to grow to 2 – 5 x 10^6 cells/mL before being passaged down to 1 – 2 x 10^5 cells/mL.

Sampling for cell density was done by aseptically removing 1 mL of the culture into a 1.5 mL Eppendorf tube. Using a Bio-Rad TC20™ automated cell counter, CHO-S cells were counted by mixing 20 µL of culture with 20 µL of Trypan blue and pipetting 10 µL of mixture into the specialized TC20 counting slides. The device provides the viable cell count and viability as cells/mL and percentage, respectively.

3.5 Transient Transfection

Previously transformed DH5-α pcDNA-DEST40-*scFv13R4* clones 8, 13, and 16 were cultured in 15-mL snap cap tubes (4 mL LB + 100 mg/mL Ampicillin) for 8 hours at 37^{o}C with shaking at 250 rpm. The entire contents were each transferred to 250-mL shake flasks (50 mL LB + 100 mg/mL Ampicillin) and grown at 37^{o}C with shaking at 250 rpm overnight. The four flasks were harvested and cells centrifuged at 4500 g for 20 minutes. The plasmids were purified using a QIAGen Midiprep DNA purification kit according to the manufacturer's instructions. The only deviations are in the isopropanol and ethanol steps, where due to equipment limitations the centrifugation was done at 4500 g for 60 minutes in each of the steps. Clone #8 was selected for use, and in subsequent transfections this process was repeated to obtain more plasmid DNA (clone #8).

Twenty-four hours prior to transfection, CHO-S cell cultures were passaged to 5 x 10^5 cells/mL. On the day of the transfection, the culture was passaged to 1 x 10^6 cells/mL if necessary. Transfection of CHO-S cells was attempted using two transfection reagents: Lipofectamine™ 2000 and FreeStyle™ MAX Reagent, both from Life Technologies.

For transfection using Lipofectamine™, 10 µL of Lipofectamine™ 2000 reagent and 40 µL of Opti-MEM media were combined and incubated at room temperature for 5 minutes. This mixture was combined with 50 µL of pcDNA-DEST40-*scFv13R4* clone #8 and incubated for 15 minutes. The entire mixture was then added to a 50 mL CHO-S culture. After 72 hours

cells were harvested by centrifugation at 1000 g for 10 minutes. Cell pellet was frozen at -20^{o}C until cell lysis.

Transfections using FreeStyle™ MAX Reagent (per 30 mL culture) was done by first combining 37.5 µg of pcDNA-DEST40-*scFv13R4* clone #8 (at 1.051 µg/µL) and 560 µL of Opti-MEM media. FreeStyle™ MAX Reagent (37.5 µL) was combined with 562.5 µL of Opti-MEM media and incubated for 5 minutes at room temperature. The reagent and DNA mixtures were then mixed together and incubated for 20 minutes. The entire mixture was then added to each flask. For the initial time course experiment, flasks were sampled each day by aseptically removing 1 mL from the culture. The cell density was measured and the sample was centrifuged at 1000 g for 5 minutes. Cell pellets were stored at -20^{o}C until cell lysis. After the optimal expression time was determined, subsequent transfections were harvested by centrifugation (1000 g for 5 minutes) 48 hours after start of transfection without intermittent sampling. Cell pellets were stored at -20^{o}C until cell lysis.

In the case of purification the cell pellets were resuspended in 4 mL of 1X Native buffer (composition shown in Appendix F) containing 0.5 µg/mL leupeptin and 100 µg/mL PMSF. Cells were lysed through two freeze-thaw cycles from liquid nitrogen to a water bath at 42^{o}C. Host cell DNA was sheared by passing the cell lysate through a 20 gauge needle four times. Lysate was then centrifuged at 3000 g for 15 minutes, and supernatant was transferred to a fresh tube and stored at -20^{o}C until purification and detection.

For the initial time course experiment cell pellets were resuspended in varying amounts of 1X Native buffer depending on cell density to standardize the cell concentration in each sample. No purification was performed.

3.6 Protein Purification and Detection

Cell lysate were purified using Imobilized Metal Ion Chromatography (IMAC). The full protocol can be found in Appendix G. Western blot were performed with both anti-his C terminal HRP antibody and anti-*c-myc*:HRP antibody, and the full protocol can be found in Appendix H. ELISA was done with anti-*c-myc*:HRP antibody, and the full protocol can be found in Appendix I.

Under non reduced conditions, the SDS-PAGE portion of the western blot protocol was modified to not include boiling at 95^{o}C and no β-mercaptoethanol was added to the loading buffer.

3.7 CHO-K1 Cell Culture

CHO-K1 cells (1 x 10^{7} cells/mL) were thawed from liquid nitrogen in a water bath at 37^{o}C. The contents were transferred to a T-75 flask with 12 mL of pre-warmed F-12K media supplemented with 10% Fetal Bovine Serum (FBS). Cells were incubated at at 37^{o}C and 5% CO_2. The cells were checked daily for growth and passaged when confluent. For passaging, the media was eliminated and 5 mL of trypsin-EDTA was added. Two 10 minute incubations

at 37^{o}C and 5% CO_2 with gentle movement of the flask in between were used to dislodge cells, and the resulting 5 mL of culture was split evenly between five new T-75 flasks, each with 12 mL of pre-warmed F-12K media with 10% FBS.

3.8 Stable Transfection

Linearization of pcDNA-DEST40-*scFv13R4* clone #8 was done through restriction digestion using *Pvu*I-HF® from New England Biolabs. Twenty µg of pcDNA-DEST40-*scFv13R4* clone #8 (645.1 ng/µL) was combined with 139 µL dH_2O, 25 µL CutSmart™ buffer, and 5 µL of *Pvu*I-HF® in that order. Mixture was incubated at 37^{o}C for 1 hour. DNA was purified using the Ultraclean® 15 DNA purification kit from MO-BIO according to the manufacturer's instructions.

Two days prior to transfection CHO-K1 cultures were trypsinized and counted. Twelve well plates were seeded with 1 mL of 2 x 10^5 cells/mL per well (total of 10 wells). Transfection was done using FreeStyle™ MAX Reagent using the linearized pcDNA-DEST40-*scFv13R4* clone #8 (545.7 ng/µL). For each well, 1.6 µg of DNA was combined with 96 µL of Opti-MEM media. The transfection reagent (4 µL) was then combined with 96 µL of Opti-MEM media and incubated at room temperature for 5 minutes. The two mixtures were then combined and incubated for 20 minutes at room temperature. The complete contents were then added to the well.

3.9 Stable selection

The following procedure is described for each well. One day after transfection the media was eliminated and fresh media (F-12K supplemented with 10% FBS) was added. Three days post transfection the media was eliminated and cells were detached (300 µL of trypsin-EDTA). One mL of fresh medium was added before centrifuging at 300 g for 5 minutes. After aspirating the supernatant, the cell pellet was gently resuspended in 1 mL of fresh media and transferred to a T-25 flask containing 4 mL of pre-warmed fresh media. Once T-25 flasks reached confluency, each was passaged to three T-75 flasks as per CHO-K1 cell culture methods previously described. Once all T-75 flasks were confluent, media were aspirated and 12 mL of fresh media supplemented with G418 (300 µg/mL) was added to each flask. After 72 hours media were aspirated and 12 mL of fresh media supplemented with G418 (300 µg/mL) were again added to each flask. After another 72 hours cells were banked in liquid nitrogen (1×10^7 cells/mL in F-12K + 10% FBS and 5% DMSO .

Part of the flask culture was set aside to be adapted to suspension in 125-mL baffled shake flasks. For adaptation to suspension, each pool's flask was sampled every three days and 10 mL of fresh media (F-12K + 10% FBS) were added. Starting with a 30 mL culture, cells were passaged each time the volume of the culture reached 60 mL. For passaging, cells were centrifuged (200 g for 5 minutes) and resuspended in 30 mL of fresh media. Cell viability was observed and this process continued until cell viability stabilized above 95%. A western blot was run on 2 mL culture samples to see which pool had the highest production. These

cells were banked again as described previously and the pool with the highest production was selected for limited dilution cloning. The full protocol can be found in Appendix J.

4. Results and Discussion

Key for DNA Gels and western blots:

L: Ladder
-: negative control
+: positive control
Lys: cell lysate
S: supernatant from purification
W: wash
E: elution

4.1 Rare Codon Analysis

The results for the rare codon analysis on the GenScript website are shown below in Figure 4.1. The results show the overall score based on a GenScript optimized algorithm, the GC content, and the percentage distribution of codons according to usage. The overall score is 0.8, which is at the borderline of acceptable as 1.0 is ideal and > 0.8 will give good expression. The GC content is 56%, and 3 codons are considered rare and may affect expression.

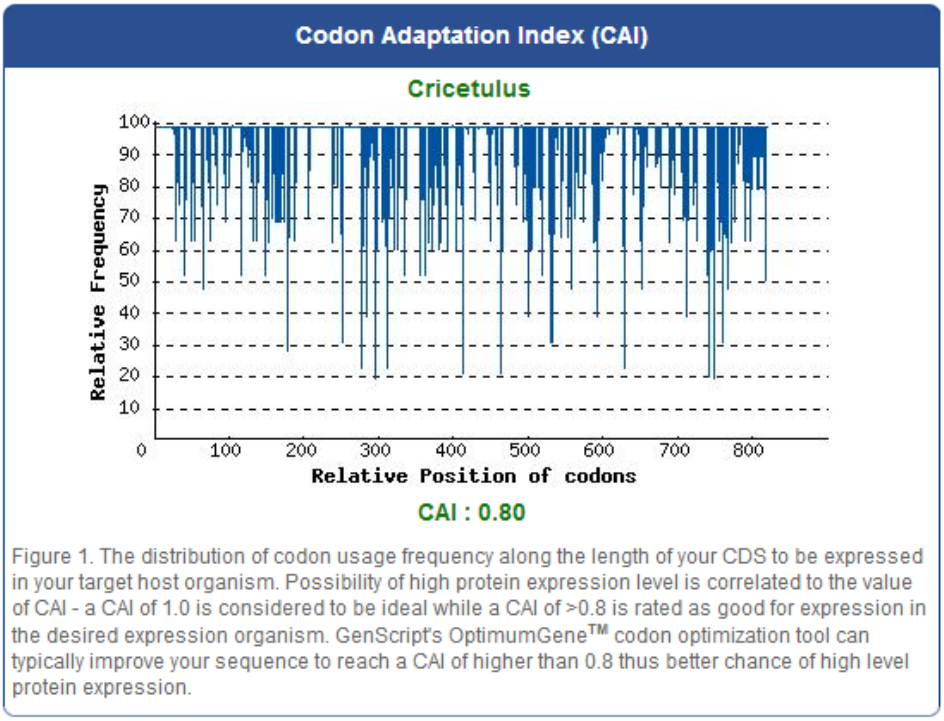

Figure 4.1(a) Overall score of rare codon analysis.

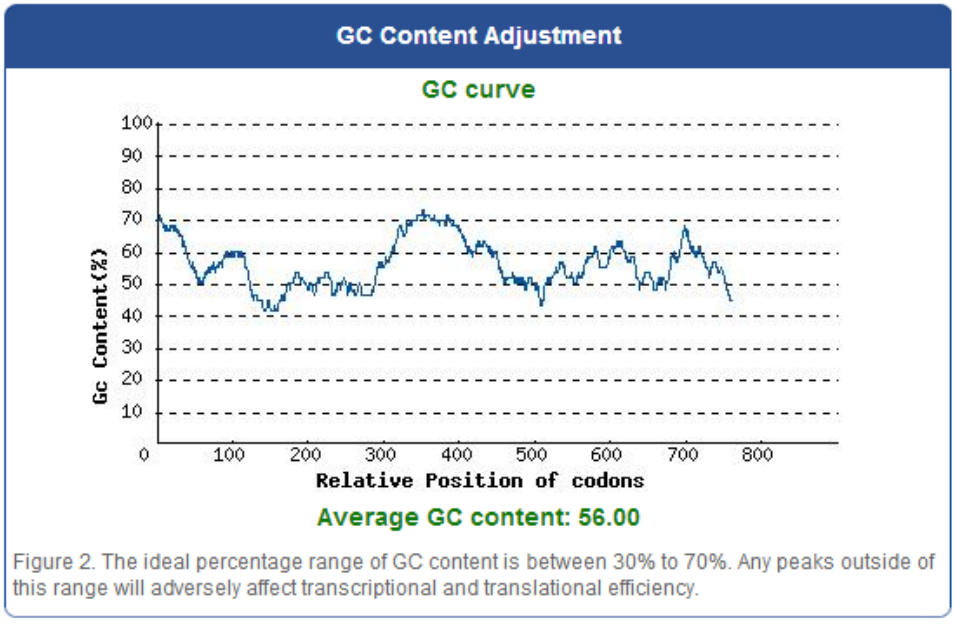

Figure 4.1(b) GC content from rare codon analysis.

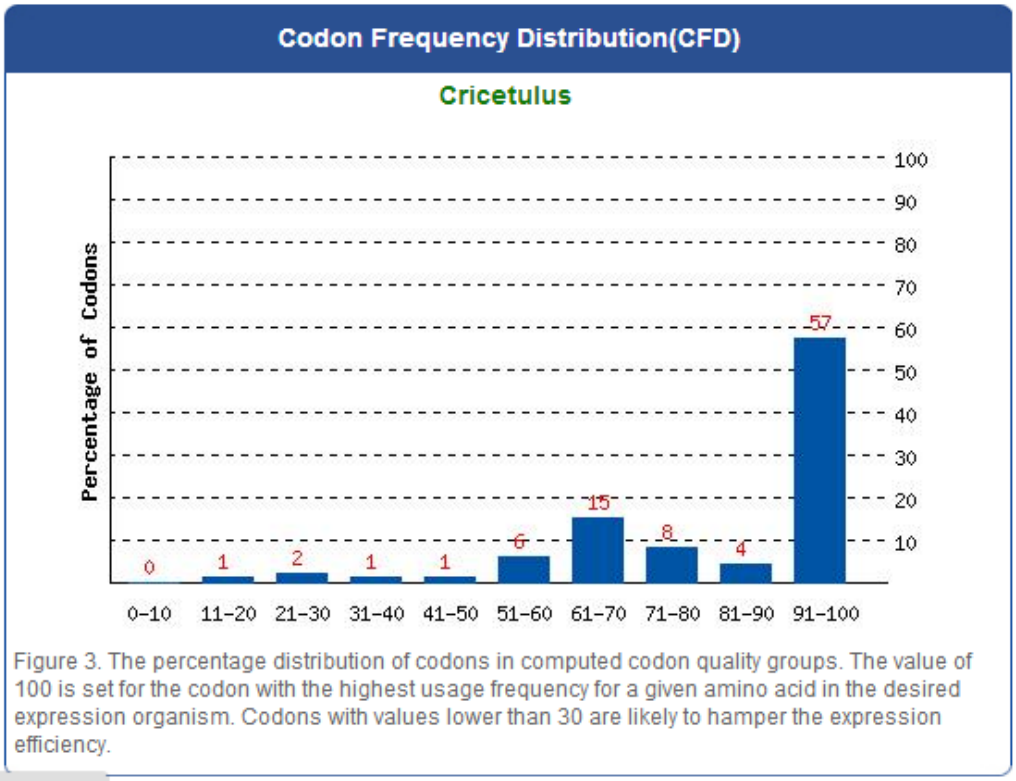

Figure 4.1(c) Distribution of codons from rare codon analysis.

As the sequence for *scFv13R4* is from a human antibody and thus mammalian in origin, it was expected that effects of codon bias would not hinder expression of the protein. From the results generated by the online tool, the original sequence is good enough for expression in CHO. Its GC content is 56%, which is within the recommended 30 – 70%. Lower or higher than that range would make DNA too hard or too easy to unzip for transcription and thus protein expression. This may not give optimal levels of expression, but expression should not be hindered by the effects of codon bias.

4.2 Propagation of Plasmids pENTR11-*scFv13R4* and pcDNA-DEST40

After DNA purification, the concentration and purity was measured using the NanoDrop 1000 spectrophotometer. For pENTR11-*scFv13R4* the concentration was 43.9 ng/μL and the 260/280 value was 2.05. For pcDNA-DEST40 the concentration was 109.7 ng/μL and the 260/280 value was 1.96 The concentrations obtained were too low to work with. Thus for the subsequent LR recombination reaction stock pcDNA-DEST40 from Life Technologies was used.

4.2.1 *E. coli* DH5-α Transformation

The initial LR recombination reaction or transformation was unsuccessful, as no growth was observed on any of the plates. After adjusting amounts of pENTR11-*scFv13R4* as described the recombination reactions were repeated. Different volumes of pcDNA-DEST40-*scFv13R4* were also tested to see if the transformation would be affected. Once again DH5-α was transformed and plated according to the scheme in Table 3.3.2. The results of plating are shown in Figure 4.2.1.1. It is clear that all of the samples grew in Ampicillin and not in Chloramphenicol as expected, as Ampicillin resistance is conferred from the pcDNA-DEST40 vector, and chloramphenicol resistance will be swapped out once if successful recombination has occurred.

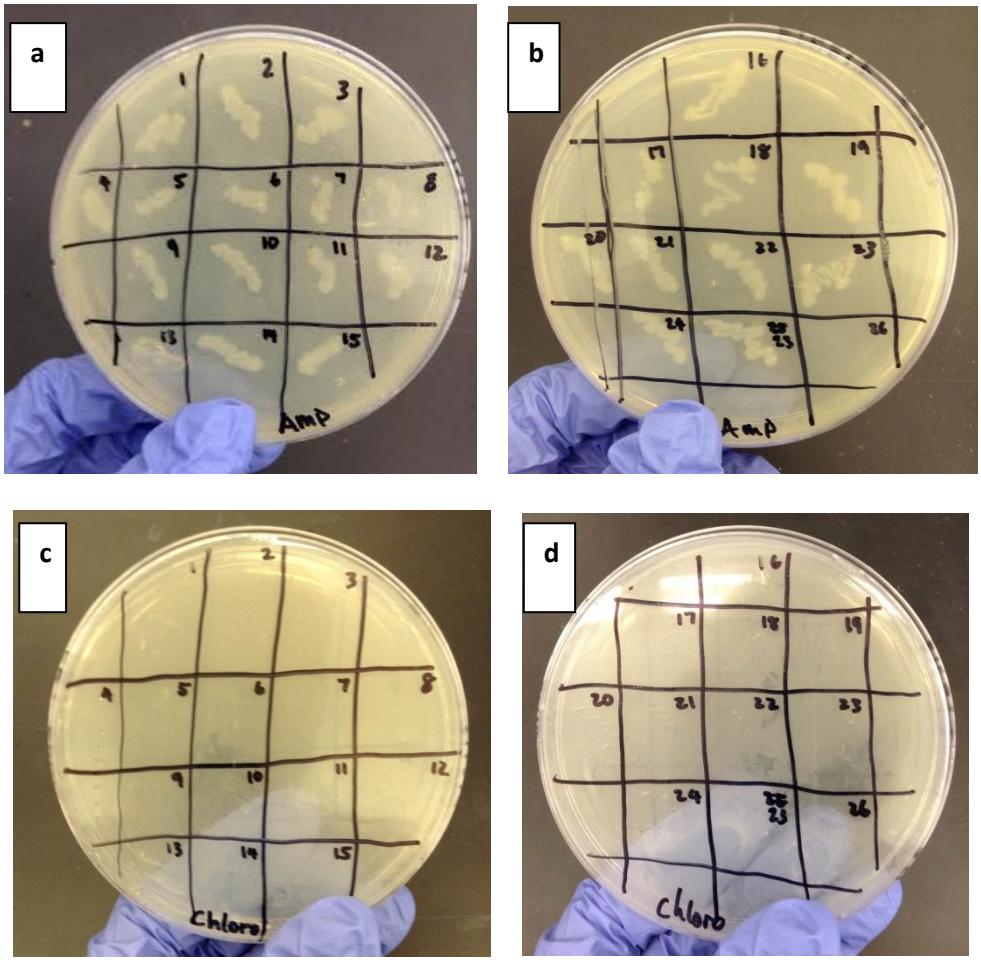

Figure 4.2.1.1 LB + Ampicillin plates (a and b) and the LB + Chloramphenicol plates (c and d) with clones streaked according to the grid.

Colony PCR, shown in Figure 4.2.1.2, confirms the correct size for the gene that has been transferred to pcDNA-DEST40 in all clones except for clone #18. The negative control (-) shows trace of DNA. This could have been due to contamination of the work area, as PCR is very sensitive to contamination. A new PCR biosafety cabinet was installed and the colony PCR was repeated (results shown in Figure 4.2.1.3) and the negative control came out as expected. Clones #8, #13, and #16 had the brightest PCR bands, and were chosen to proceed with purification for transfection.

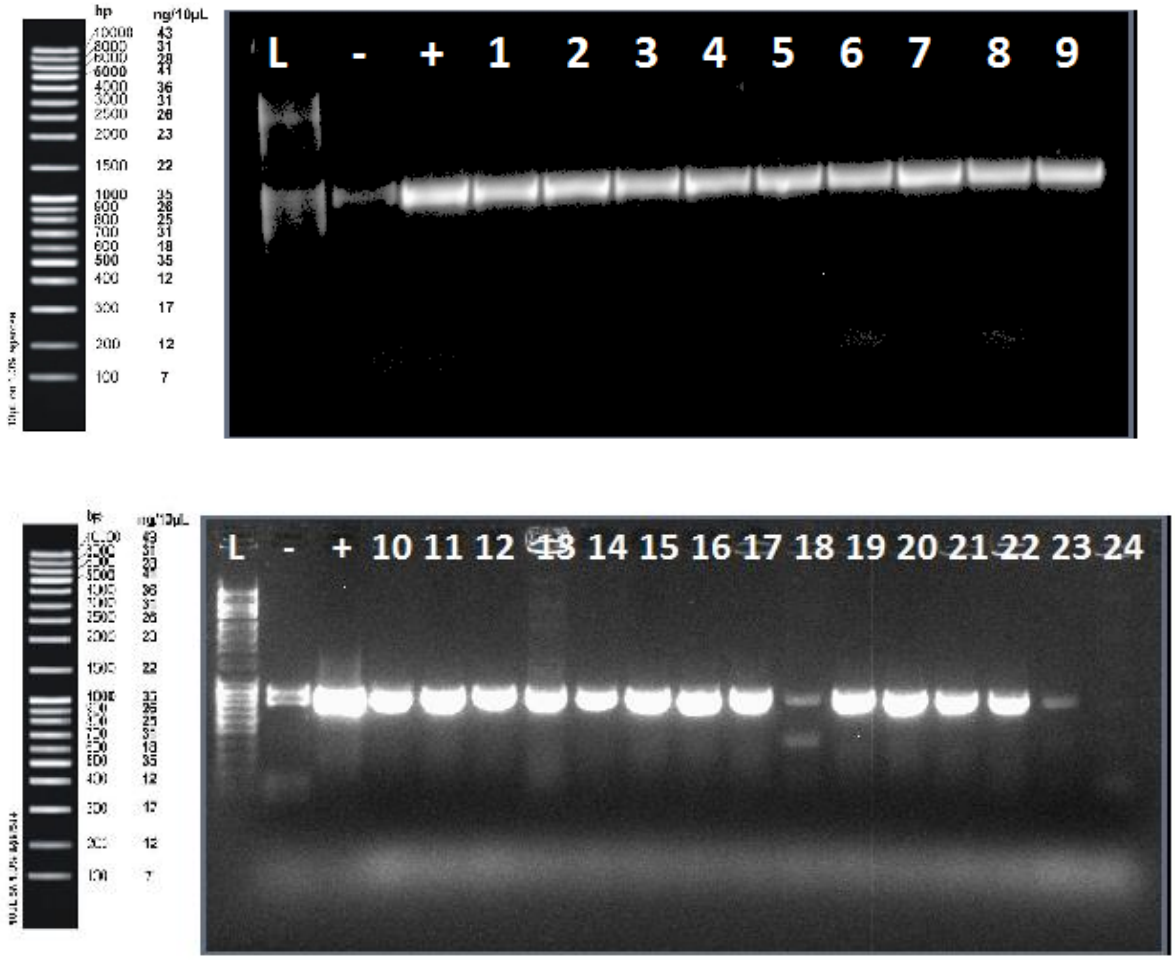

Figure 4.2.1.2 DNA gels for colony PCR of all clones (1 – 24) from the transformation. Ladder is Fisher Bioreagent *ex*ACT*Gene* 1 Kb ladder. Positive control is pYES-DEST52-*scFv13R4*.

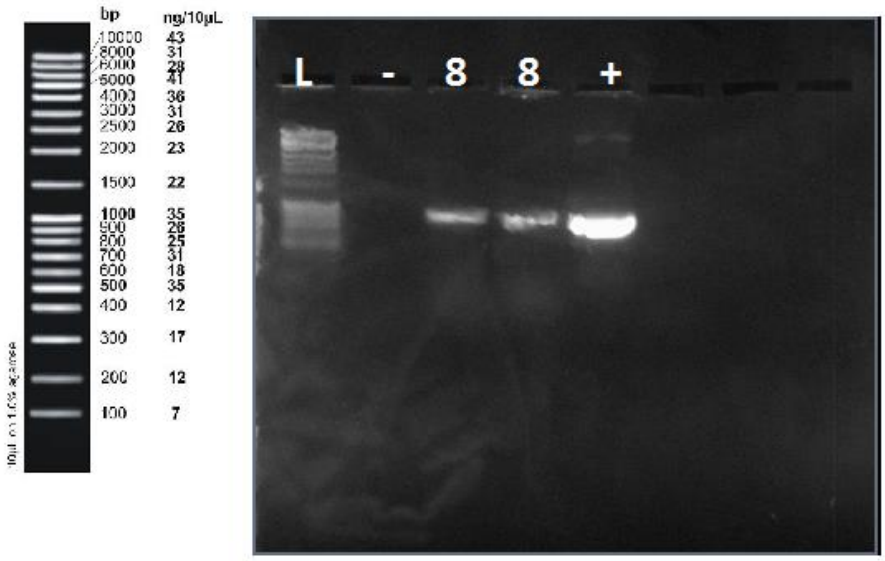

Figure 4.2.1.3 Repeated DNA gel using clone #8 showing no negative control contamination. Ladder is Fisher Bioreagent *ex*ACT*Gene* 1 Kb ladder. Positive control is pYES-DEST52-*scFv13R4*.

4.2.2 Plasmid Purification of Clones #8, #13, and #16

The results of the Midiprep purification procedure can be seen in Table 4.2.2.

Table 4.2.2 Concentration and purity of the Midiprep purification.

Clone	Concentration (ng/µL)	Purity (260/280 nm)
8	521	1.84
13	323.2	1.85
16	520	1.88

Clone #8 was selected for transfection as it had the highest concentration (and highest amount, as all samples were resuspended in equal volumes) and the highest purity (closest to 1.8).

4.3 Transient Expression

The first transfection was done using the Lipofectamine™ 2000 transfection reagent. After cell lysis, a western blot was performed and yielded no detectable protein. After purification using IMAC the western blot was repeated but again showed no protein. This can be seen in Figure 4.3.1. The lack of expression can be attributed to the choice of transfection reagent. Lipofectamine™ 2000 is designed for adherent cell lines, and is designed for transfection in well plates where there is no agitation. It has not been optimized for the CHO-S cell line or transfection in a shake flask culture. This is likely why expression was minimal to none, and no protein was detected.

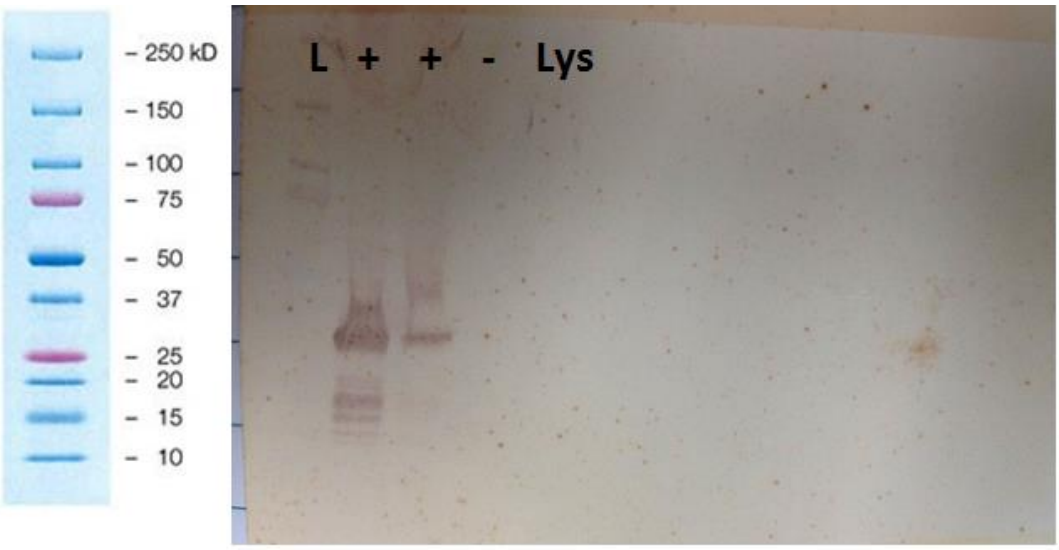

Figure 4.3.1 Western blot for cell lysate (top) and post purification with IMAC (bottom). Ladder is BioRad Precision Plus Protein Dual Color Standard. Positive control is scFv13R4 purified from *E. coli*.

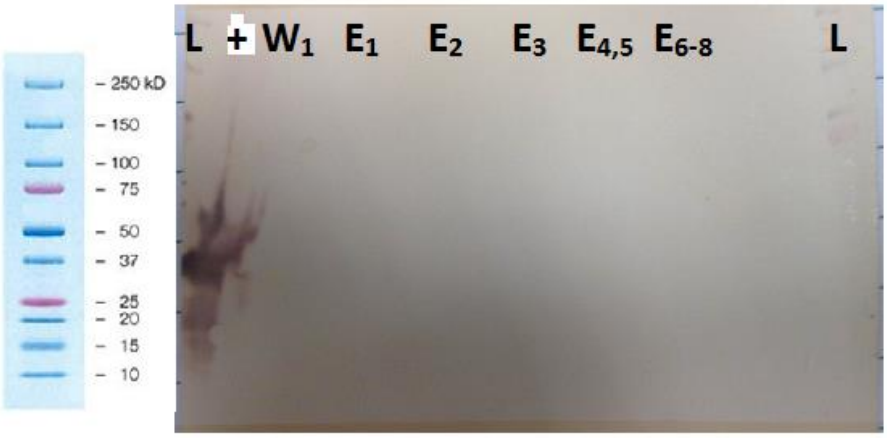

Figure 4.3.1 Continued

The proprietary transfection reagent for CHO-S cells, FreeStyle™ MAX Reagent, specifically designed for CHO-S and shake flask cultures, was then tested. Since this was the first time this reagent has been used, a time course experiment was set up to determine the optimal harvest time after transfection. The recorded cell densities and viabilities are shown in Table 4.3.1, and the subsequent western blot is shown in Figure 4.3.2.

Table 4.3.1 Time course experiment data showing cell density and viability along the days samples were taken.

Time after transfection (days)	**Flask 1**		**Flask 2**	
	Cell density (cells/mL)	**Viability**	**Cell density (cells/mL)**	**Viability**
0	7.55×10^5	97%	8.82×10^5	96%
1	1.44×10^6	97%	1.79×10^6	97%
2	2.49×10^6	93%	2.89×10^6	95%
3	3.02×10^6	96%	3.90×10^6	88%
4	3.11×10^6	85%	3.53×10^6	90%
5	3.62×10^6	80%	3.92×10^6	62%
6	2.36×10^6	29%	2.27×10^6	27%
7	1.10×10^6	10%	6.56×10^5	7%

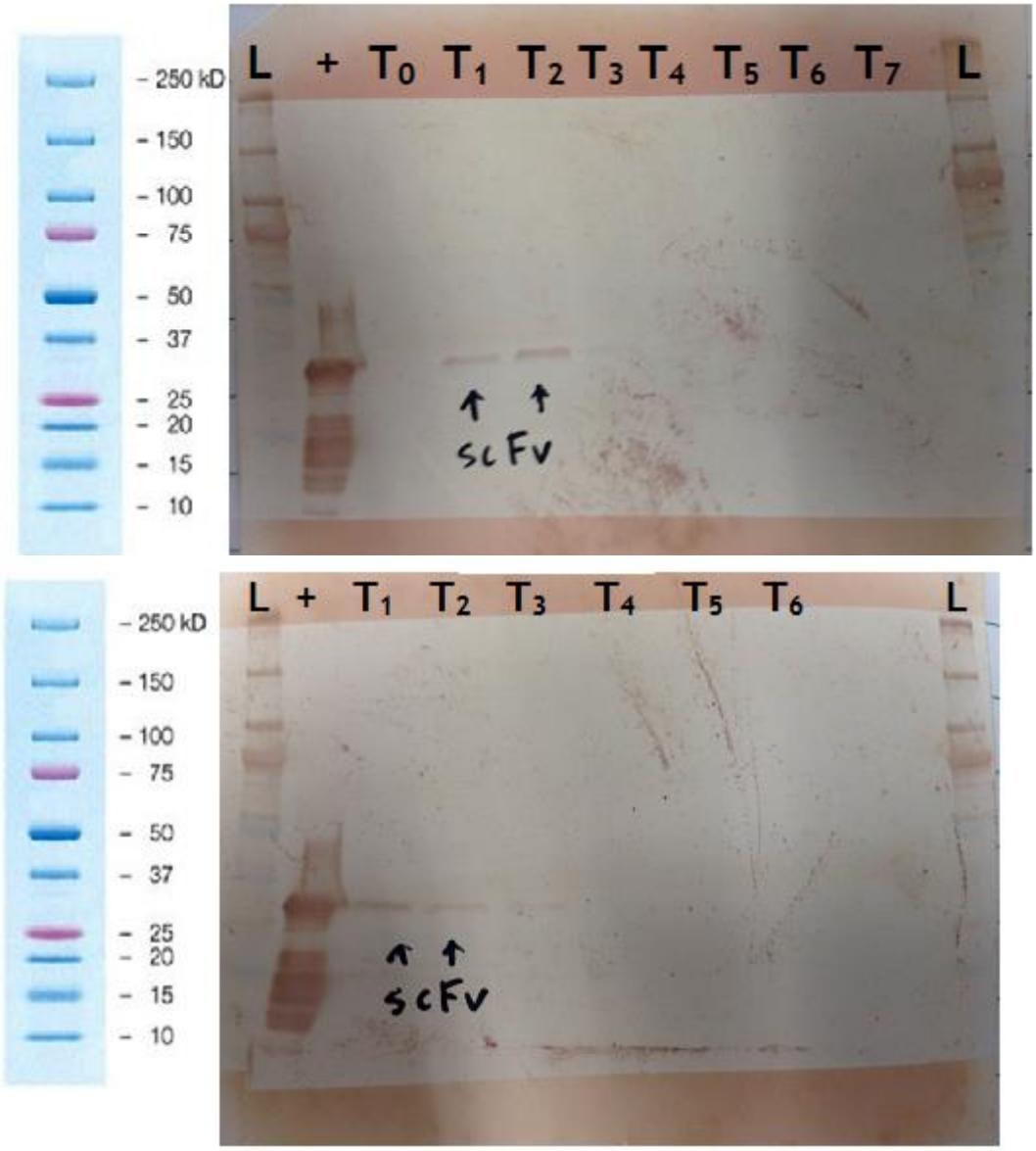

Figure 4.3.2 Western blot for Flask 1 (top) and Flask 2 (bottom) of the time course experiment. Ladder is BioRad Precision Plus Protein Dual Color Standard. Positive control is scFv13R4 purified from *E. coli*. T_o corresponds to 0 hours after transfection, T_1 corresponds to 24 hours after transfection, etc.

From the time course experiment it can be seen that approximately 48 hours after transfection is the optimal time for harvesting protein. The cell density data suggests that expression

levels are low, as cells that are overproducing protein will show a decrease in growth. The cells double as they normally do, about 24 hours, without slowing down. The only decrease in cell density comes at the end of the time course when viability of the culture starts to drop. This is not due to protein production, but rather the buildup of waste products and the lack of nutrients available in the media. If protein expression was high, it would consume energy such that growth must slow down, and this is not seen from the cell density data.

Subsequent transfections were harvested after 48 hours, and protein was purified using IMAC. The resulting western blot showed clear bands for elution 1 and elution 2 having the correct protein mass of approximately 28 kDa, but another band about 56 kDa (two times the weight of the protein) also appears clearly. This can be seen in Figure 4.3.3 for elutions 1 and 2 samples. At first binding of other His-tagged proteins was suspected. Accounting for the possibility of host cell proteins having a stretch rich in Histidine residues, the *c-myc* tag was used as the antibody binding tag in the next western blot. However, the same unknown band appeared, as shown in Figure 4.3.4. Since denaturing conditions (boiling and addition of β-mercaptoethanol) were used, it is unlikely that the unknown band is from a high molecular weight aggregate. Nevertheless, conditions of the SDS-PAGE portion of the western blot were altered to weed out any possible causes. The purified samples were not boiled and the SDS-PAGE portion of the western was run under non-reducing conditions (no β-mercaptoethanol addition to the loading buffer). Even after these modifications, the unknown band still appears (Figure 4.3.5). The possibility of host cell proteins with Nickel ion affinity was tested by western blot of cell lysate of cells not transfected to see if the same band

appears. The results are shown in Figure 4.3.6, and the protein from the unknown band does not show up in the not transfected cell lysate. From these results, it is suspected that the IMAC purification is contributing to the appearance of the artifact. The western blot of the time course experiments, which did not include IMAC purification, did not show the unknown band. To investigate this issue, a western blot should be run for concentrated unpurified cell lysate, and afterwards the lysate should be purified to see if the unknown band reappears. Also, different purification methods such as ion exchange can be used for purification to see if the unknown band is still present.

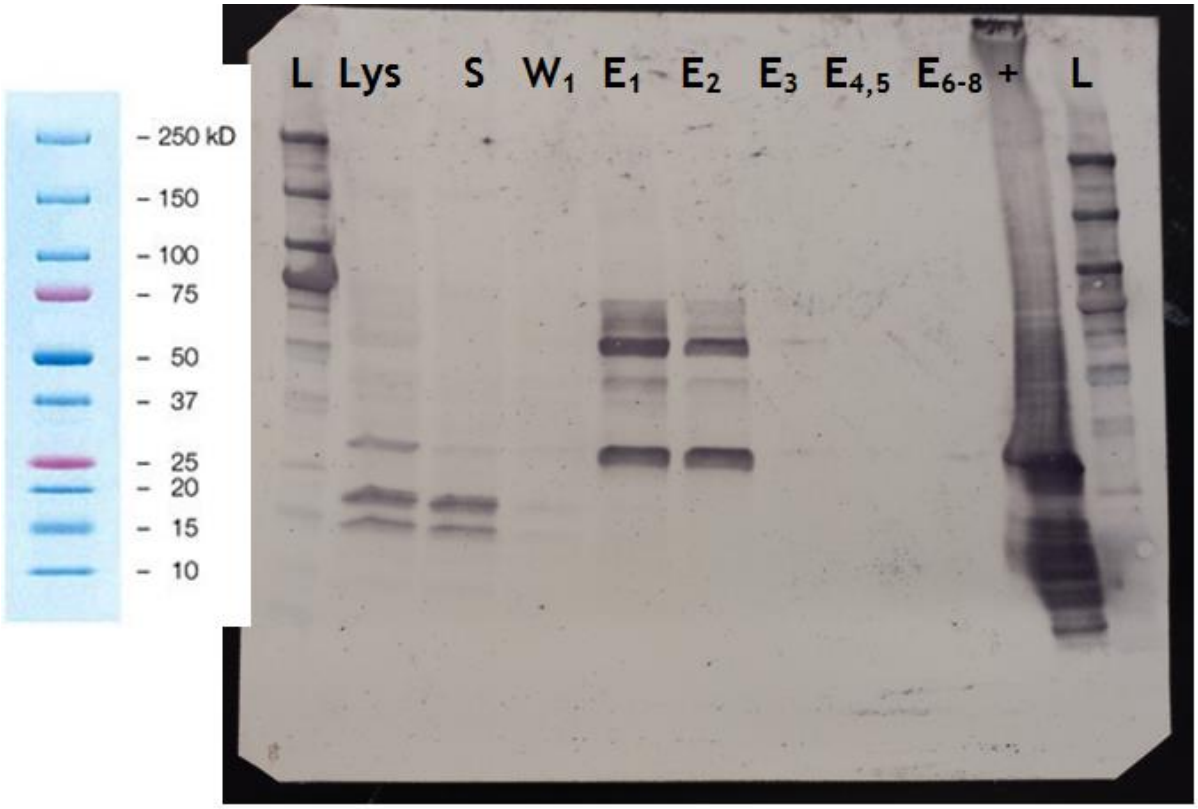

Figure 4.3.3 Western blot showing unknown band after purification of protein. Ladder is BioRad Precision Plus Protein Dual Color Standard. Positive control is scFv13R4 purified from *E. coli*.

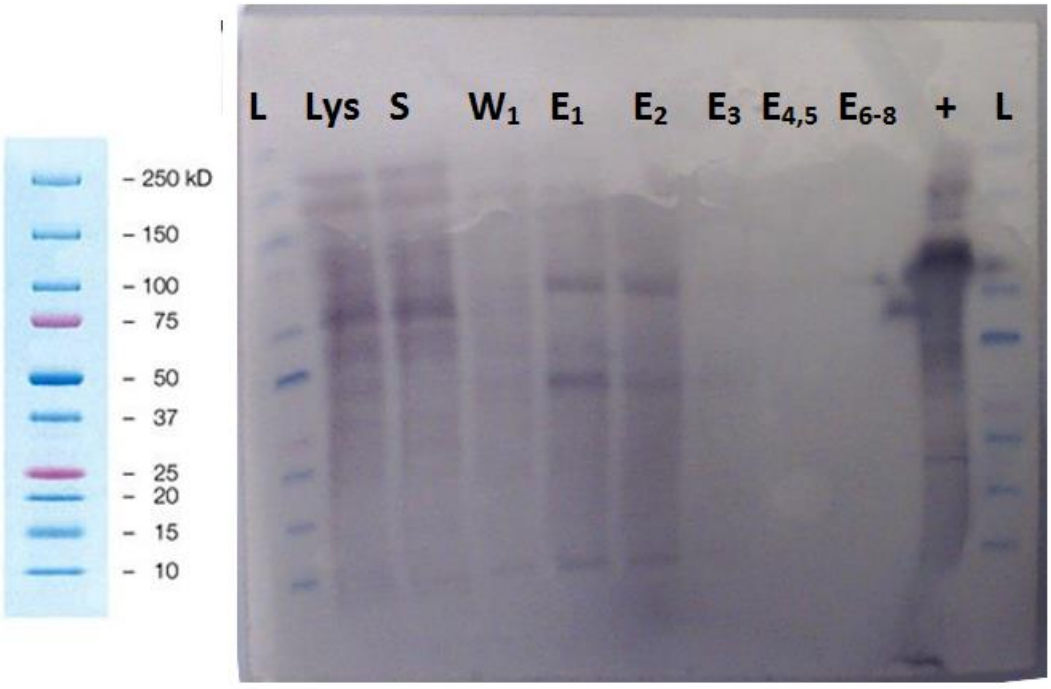

Figure 4.3.4 Western blot using the *c-myc* tag for antibody binding. Ladder is BioRad Precision Plus Protein Dual Color Standard. Positive control is scFv13R4 purified from *E. coli*.

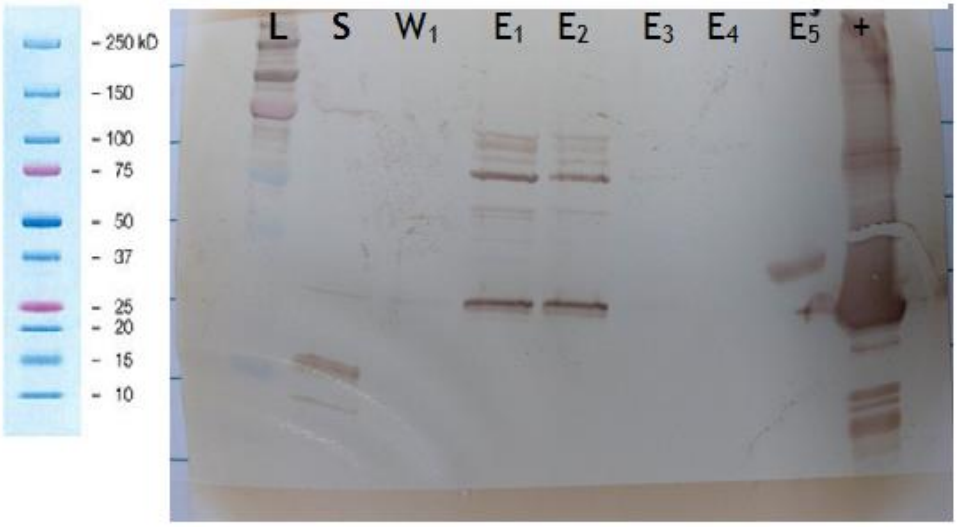

Figure 4.3.5 Western blot run under non-reducing conditions and without boiling samples. Ladder is BioRad Precision Plus Protein Dual Color Standard. Positive control is scFv13R4 purified from *E. coli*.

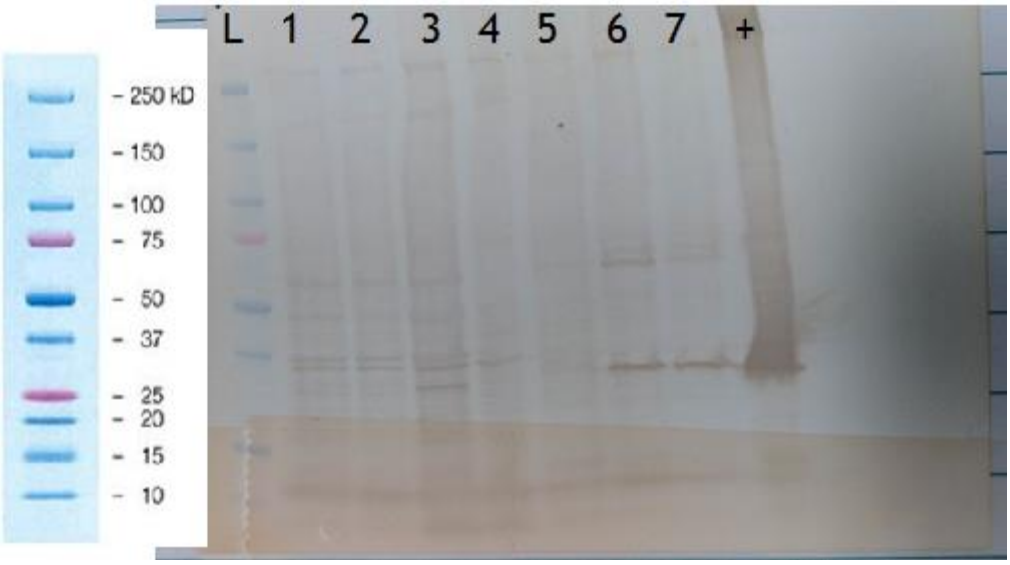

Figure 4.3.6 Western blot with cell lysate from cells not transfected compared with transfected cells. Ladder is BioRad Precision Plus Protein Dual Color Standard. Positive control is scFv13R4 purified from *E. coli*. 1 – Untransfected cell lysate (reduced); 2 – Untrasfected cell lysate (non-reduced); 3 – Transfected cell lysate; 4 – Supernatant from purification; 5 – Wash 1 of purification; 6 – Elution 1 of purification; 7 – Elution 2 of purification.

ELISA was performed using the purified protein. The first ELISA was performed with the purified protein (elutions 1 and 2) being diluted 1:1000, 1:2000, 1:4000, and 1:8000. The standard curve is shown in Figure 4.3.7 and the calculated values of protein concentrations based on optical densities are shown in Table 4.3.2. It is clear that the optical density signals detected for the protein is not within the linear range of the standard curve for any of the dilutions, making the calculated concentration values clearly unreliable. This is likely due to the dilution of the protein for the assay. The protein must not be expressed at high enough levels to be detected through ELISA after dilutions of such magnitude. Lower dilutions should be used.

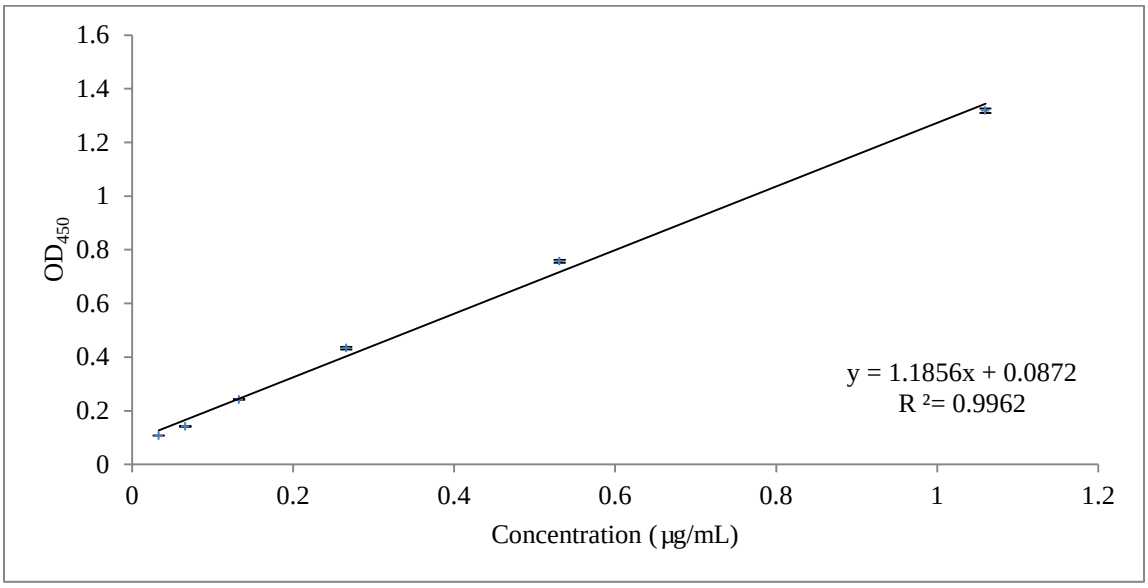

Figure 4.3.7 Standard curve for ELISA (samples diluted 1000, 2000, 4000, and 8000 fold).

Table 4.3.2 The measured OD_{450} values and the concentrations calculated using the equation obtained from the standard curve for the first ELISA.

Dilutions	Elution 1 OD	Elution 2 OD	Elution 1 concentration (µg/mL)	Elution 2 concentration (µg/mL)
1:1000	0.087	0.09	-0.169	2.362
1:2000	0.0825	0.0835	-7.928	-6.242
1:4000	0.081	0.0835	-20.918	-12.483
1:8000	0.081	0.087	-41.835	-1.350

The ELISA was repeated with lower dilution values (1:100, 1:200, 1:500, and 1:1000). The results are shown in Figure 4.3.8 and Table 4.3.3. This time the measured OD_{450} values for the 1:100 and 1:200 dilutions do fall within the linear range of the standard curve. The calculated values for protein concentration for the two different dilutions are very similar for both elution 1 and elution 2. Since both dilutions fall within the linear range and the values

are in agreement, the concentration values are averaged, giving elution 1 a concentration of 134.635 µg/mL and elution 2 a concentration of 102.603 µg/mL. Averaging the concentration across the two elutions where the protein predominantly appears, the production titer would be 118.619 µg/mL. This titer is considerably lower than the titer of the same *scFv13R4* antibody fragment harvested from *E. coli* (6.12 mg/mL). This is expected, as *E. coli* is well known for its high levels of protein production. However, this concentration is low even compared to other mammalian systems, as industry standards have CHO expressing at the few g/L level (Wurm, 2004). With units converted, the concentration obtained would be 118.619 mg/L. This shows that there is much room for optimization of expression. It is worthy to note that, as previously discussed, transient expression will never reach the expression levels obtained from isolating a very high producer and entering production with only that clonal population.

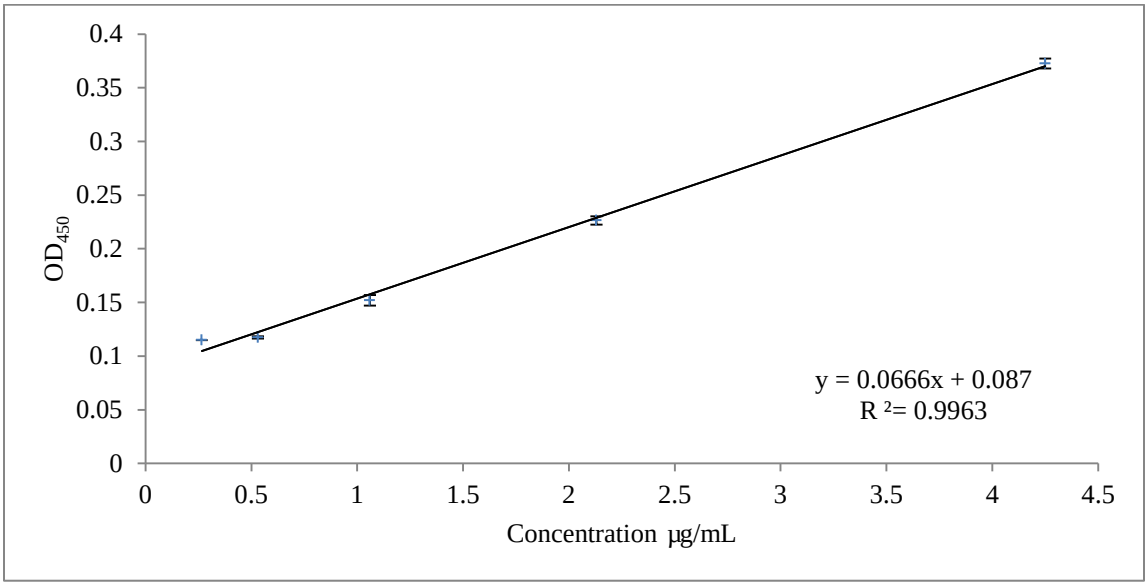

Figure 4.3.8 Standard curve for ELISA (samples diluted 100, 200, 500, and 1000 fold).

Table 4.3.3 The measured OD_{450} values and the concentrations calculated using the equation obtained from the standard curve for the second ELISA.

Dilutions	Elution 1 OD	Elution 2 OD	Elution 1 concentration (µg/mL)	Elution 2 concentration (µg/mL)
1:100	0.176	0.155	134.134	102.102
1:200	0.132	0.121	135.135	103.103
1:500	0.105	0.097	135.135	72.573
1:1000	0.100	0.094	195.195	105.105

4.4 Stable Expression

After linearization of pcDNA-DEST40-*scFv13R4* the DNA was purified and concentrated using the UltraClean® 15 system. The purification and concentration yielded a concentration of 545.7 ng/µL and a purity of 1.88 (260/280 nm). Next PCR was run to confirm the success of the restriction digestion. Figure 4.4.1 shows the resulting DNA gel confirming the correct gene has been linearized.

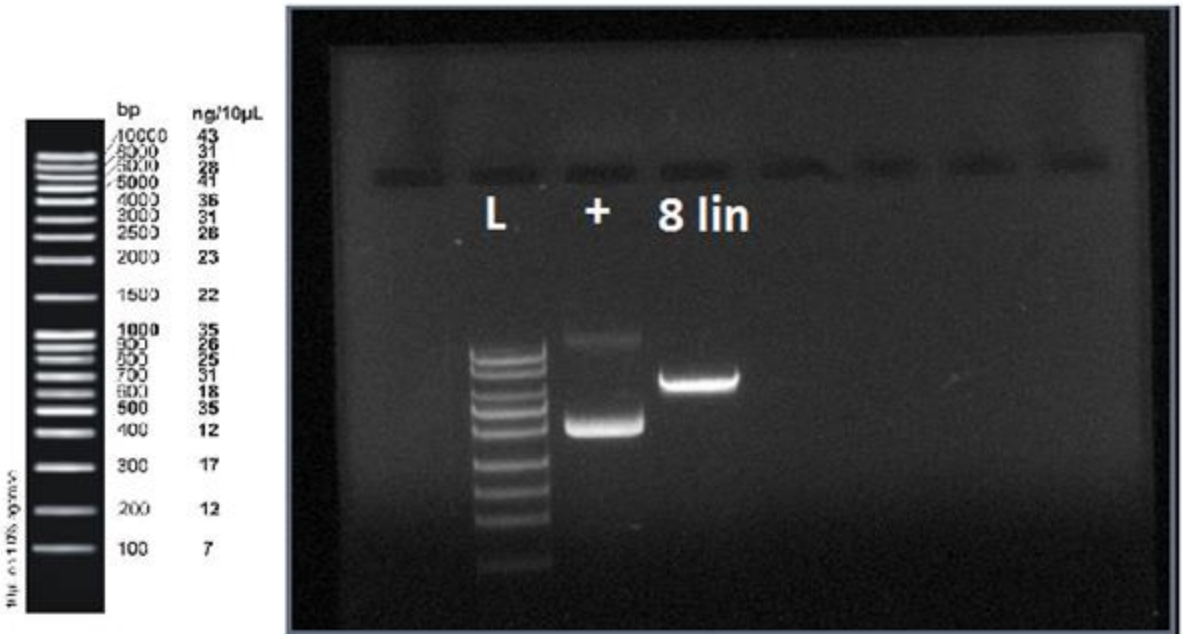

Figure 4.4.1 DNA Gel showing size of linearized plasmid for pcDNA-DEST40-*scFv13R4* clone #8 (8 lin) and non-linearized positive control. Ladder is Fisher Bioreagent *ex*ACT*Gene* 1 Kb ladder. Positive control is circular pcDNA-DEST40-*scFv13R4* clone #8.

After going through the selection process, adaptation to suspension took several weeks before the cultures started to regain viability (Figure 4.4.2). Along the process pools 2, 5, and 7 completely died out. This is expected, but could possibly be optimized through the use of a different cell passage strategy. When collecting cells for passage, culture should be taken from the top of the flask, as suspension adapted cells will float more than the not adapted dead cells at the bottom.

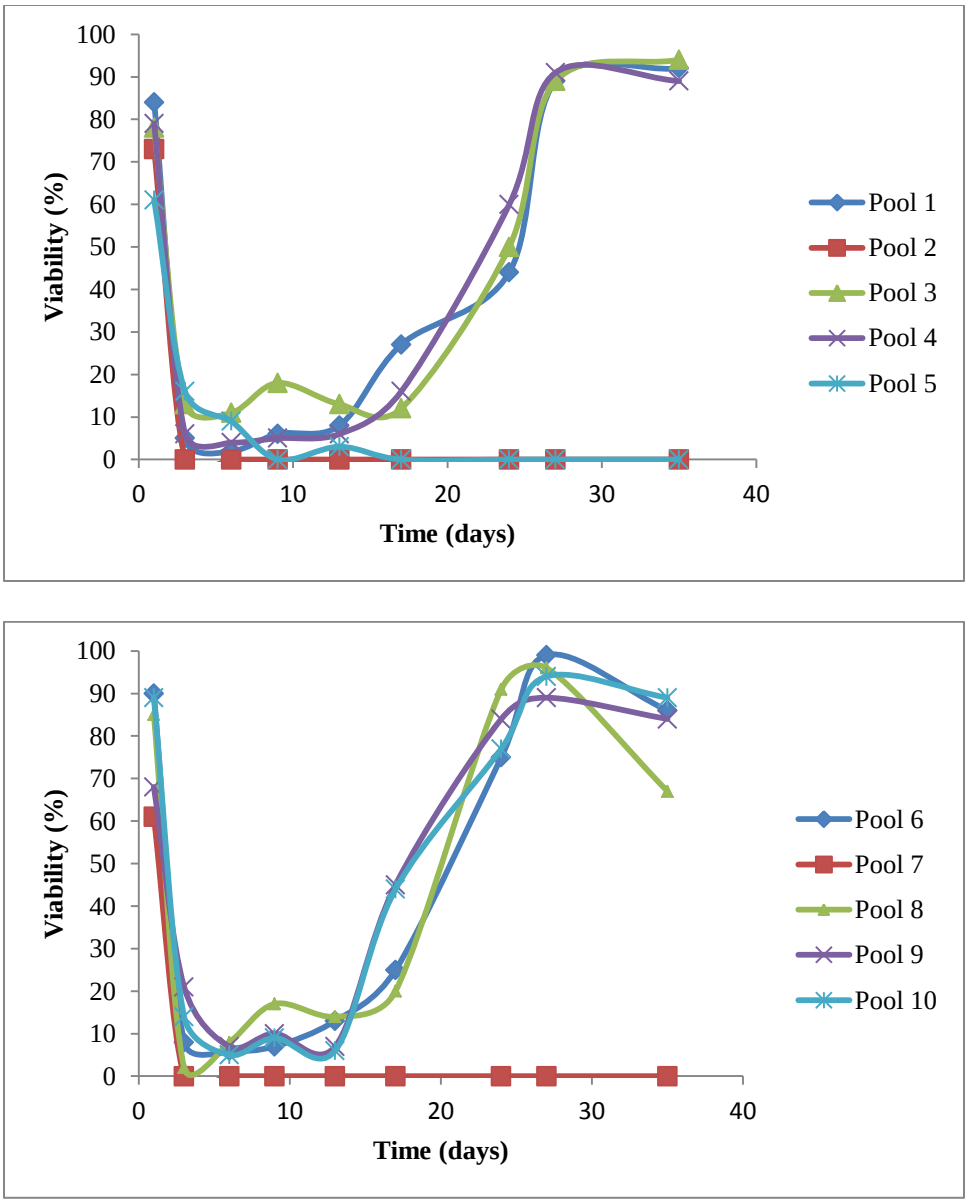

Figure 4.4.2 Cell viability over the process of adaptation to suspension.

The remaining pools were sampled and cell lysates were tested for protein production. Figure 4.4.3 shows the western blot results. Pools 1, 3, 4, 9, and 10 showed signs of protein production. Pool 9 was chosen to move forward to limited dilution cloning, where one cell was transferred to each well of a 96-well plate. Alternatively, flow cytometry could be used

to isolate single cells into individual wells. A technique called Fluorescence Activated Cell Sorting (FACS) can be used, as it can sort individual cells by viability (Herzenberg *et al.*, 2002). Efforts are currently ongoing to isolate a high-producing clonal population. In an industry setting, many more pools would undergo limited dilution cloning, since the more candidates available the more likely the best-producing cell line would be found. With limited resources and manpower, the selection of candidates in this project had to be kept to a smaller scale.

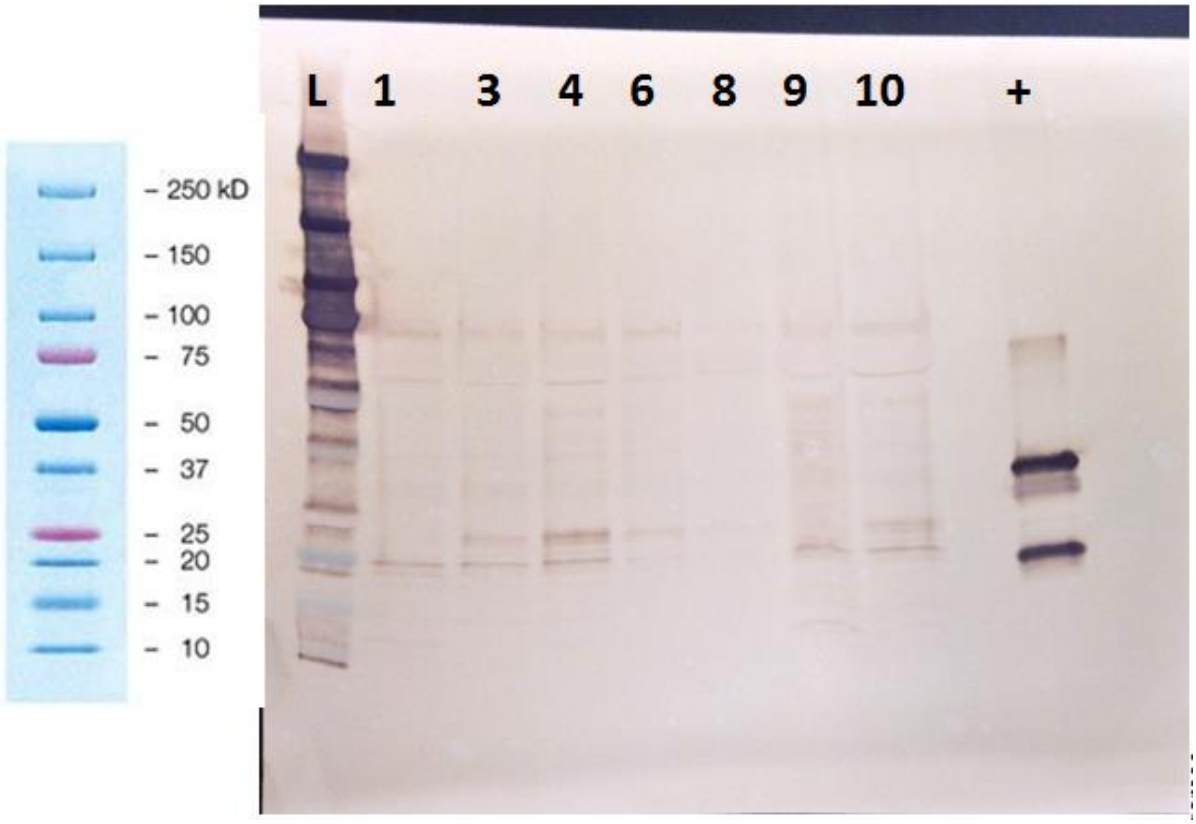

Figure 4.4.3 Western blot showing cell lysate of transfectant pools. Ladder is BioRad Precision Plus Protein Dual Color Standard. Positive control is scFv13R4 purified from *E. coli*.

5. Conclusion

The goal of this thesis project was to express a single-chain antibody fragment *scFv13R4* in a mammalian cell expression host under both transient and stable conditions. In the transient expression experiments the gene of interest *scFv13R4* was successfully cloned from pENTR11-*scFv13R4* to pcDNA-DEST40, giving the expression vector pcDNA-DEST40-*scFv13R4* specifically designed for expression in mammalian cells. The transfection was initially unsuccessful using Lipofectamine™ 2000, as it was designed for adherent cultures. When FreeStyle™ MAX Reagent was used, *scFv13R4* expression was observed. From the time course experiment it was found that the optimal time for harvest is 48 hours after transfection. Subsequent transfections utilized IMAC to purify the protein. Western blots on purified protein showed an unknown band approximately twice the size of protein scFv13R4. Further experimentation suggested this was an artifact of the purification process, and efforts are ongoing to verify this explanation as well as purify the protein using other methods.

The stable transfection was successful in generating pools of transfectants that showed protein production. Due to the lengthy processes of adaptation to suspension and limited dilution cloning, a single high-producing clone has not yet been isolated. Work is continuing to establish a stable cell line expressing *scFv13R4*.

6. Future work

Several aspects of this project can be further explored:

6.1 Protein Purification

It is likely that the unknown band seen in transient expression is an artifact of IMAC purification, because other explanations such as high molecular weight aggregate and Histidine-rich host cell proteins have been tested and ruled out. New methods should be investigated for the purification of *scFv13R4*, such as ion exchange. With a new purification method, reliance on the His tag can be eliminated once an antibody for western blot and ELISA is made. His and *c-myc* tags can then be eliminated from the sequence, further mimicking an industry product.

6.2 Isolation of Best Producer

Due to the time constraints of this project and the lengthy time requirements to isolate a high producing clonal population, first and foremost the stable cell line selection process must be taken to completion.

6.3 Protein Sequence Optimization

Although the rare codon analysis showed the sequence is good enough for expression, the sequence could yet be optimized to avoid any effects of codon bias.

6.4 Expression Cassette Engineering

One area of interest in future development is engineering the expression vector itself. As discussed in the introduction and seen in the experimental results, isolating a clonal population of cells producing the protein of interest stably is a long and laborious task. Much of it is left to chance, and is determined by how much resources and time can be devoted to screening as many pools and cells as possible. The main problem is integration of the recombinant DNA into the host genome (Dale, 2006). In order for the heterologous protein to be expressed, the DNA must be integrated into the euchromatin, which is loosely coiled and transcriptionally active. If integrated into the tightly coiled and rarely transcribed heterochromatin, there will be little or no expression. This is shown in Figure 6.4. There are ways to engineer the expression vector to contain sequences that help guide this integration. Some of these sequences include matrix attachment regions (MARs) which recruit proteins that guide and bind to specific sequences known to flank euchromatin (Spiker and Thompson, 1996). Another sequence found to increase stable expression is the ubiquitous chromosome opening element (UCOE), which is already an established system by Millipore (Millipore Co., 2010). By increasing the chance of proper chromosomal integration, fewer pools will need to be screened and higher producers are more likely. By developing a vector with features just described, future work on expression of other proteins will be made much more efficient.

Another area for improvement in the expression vector is the addition of a secretion tag. Currently *scFv13R4* is expressed intracellularly, meaning cells must be lysed to harvest the proteins. This lysis releases many host cell proteins that can both damage the antibody and

cause problems for purification further down the manufacturing process. Having a secretion tag will allow for the protein to be expressed and then exported outside of the cell, so the cells can be pelleted and the protein of interest may be collected from the media.

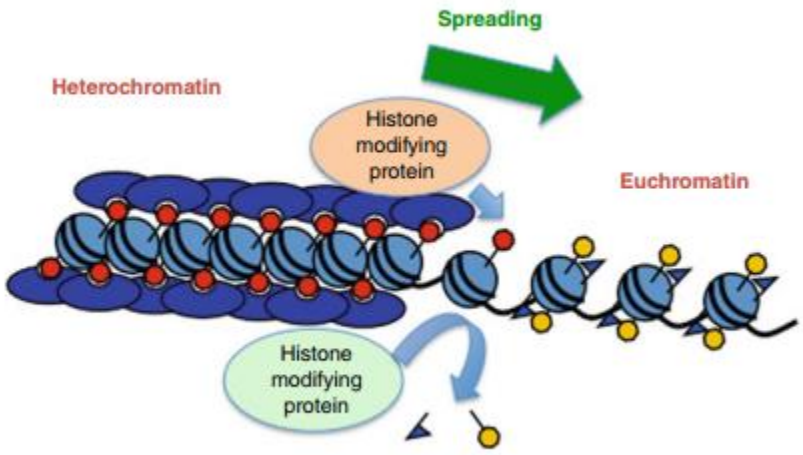

Figure 6.4 Euchromatin (transcriptionally active) vs. heterochromatin (transcriptionally inactive) (Murakami, 2013).

6.5 Scale up

The experiments were done at the shake flask scale. For production this process must be scaled up to the bioreactor scale. Once a stable cell line is isolated, bioreactor runs in rocker bag disposable bioreactors is the next step. Process development for cell culture parameters such as rocking speed, feeding strategy, can be done to optimize expression. This can be tied to other upstream manufacturing classes at BTEC.

6.6 Media optimization

The media used is also important to the expression levels of heterologous proteins. The same media was used throughout the experiments for this project. For optimization, first there could be tests on using different media when culturing vs. post transfection and awaiting expression. There are proprietary media formulations that target the protein expression phase, such as CD FortiCHO™ from Life Technologies. Building upon this, spent media analysis can be employed to see which nutrients are lacking during the culture process, as each transfected cell line will behave slightly differently. These lacking nutrients can then be supplemented to ensure optimal growth.

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APPENDICES

Appendix A – F-12K Media Formulation (ATCC Co., 2004)

Formulation for F-12K Medium ATCC® 30-2004

Inorganic Salts (g/liter)

$CaCl_2 \cdot 2H_2O$	0.13524
$CuSO_4 \cdot 5H_2O$	0.0000025
$FeSO_4 \cdot 7H_2O$	0.000834
$MgCl_2 \cdot 6H_2O$	0.10572
$MgSO_4$ (anhydrous)	0.19264
KCl	0.28329
KH_2PO_4 (anhydrous)	0.05852
$NaHCO_3$	1.50000
Na_2HPO_4 (anhydrous)	0.11502
NaCl	7.59720
$ZnSO_4 \cdot 7H_2O$	0.000144

Amino Acids (g/liter)

L-Arginine (free base)	0.42140
L-Alanine	0.01782
L-Asparagine·H_2O	0.03020
L-Aspartic Acid	0.02662
L-Cysteine·HCl·H_2O	0.07024
L-Glutamic Acid	0.02942
L-Glutamine	0.29220
Glycine	0.01501
L-Histidine·HCl·H_2O	0.04192
L-Isoleucine	0.00782
L-Leucine	0.02624
L-Lysine·HCl	0.07304
L-Methionine	0.00895
L-Phenylalanine	0.00991
L-Proline	0.06906
L-Serine	0.02102
L-Threonine	0.02382
L-Tryptophan	0.00408
L-Tyrosine (free base)	0.01087
L-Valine	0.02342

Vitamins (g/liter)

D-Biotin	0.0000733
Choline Chloride	0.01396
Folic Acid	0.00132
Hypoxanthine	0.00408
myo-Inositol	0.01802
Nicotinamide	0.0000366
D-Pantothenic Acid (hemicalcium)	0.000477
Putrescine·2HCl	0.000322
Pyridoxine·HCl	0.0000617
Riboflavin	0.0000376
Thiamine·HCl	0.000337
Thymidine	0.000727
Vitamin B-12	0.001355

Other (g/liter)

D-Glucose	1.26000
Phenol Red, Sodium Salt	0.00332
Sodium Pyruvate	0.22000
Lipoic Acid	0.00021

Appendix B – One Shot® ccdB Survival™ 2 T1R Transformation Protocol (Life Technologies Co., 2013)

Transformation Procedure

Use this procedure to transform One Shot® ccdB Survival™ 2 T1R chemically competent cells. Include the pUC19 control plasmid DNA to verify the transformation efficiency. Do not use these cells for electroporation.

1. Thaw one vial of One Shot® cells on ice for each transformation.
2. Add 1–5 µL of DNA (10 pg to 100 ng) into a vial of One Shot® cells and mix gently. Do not mix by pipetting up and down. For the pUC19 control, add 1 µL (10 pg) of DNA into a separate vial of One Shot® cells and mix gently.
3. Incubate the vials on ice for 30 minutes.
4. Heat-shock the cells for 30 seconds at 42 ℃ without shaking.
5. Remove the vials from the 42 ℃ bath and place them on ice for 2 minutes.
6. Add 250 µL of pre-warmed S.O.C. Medium to each vial.
7. Cap the vials tightly and shake horizontally at 37 ℃ for 1 hour at 225 rpm in a shaking incubator.*
8. Spread 25–100 µL from each transformation on a pre-warmed selective plate. Plate two different volumes to ensure that at least one plate will have well-spaced colonies. For the pUC19 control, dilute the transformation mix 1:10 into LB Medium and plate 25–100 µL.
9. Store the remaining transformation mix at 4 ℃. Additional cells may be plated out the next day, if desired.
10. Incubate plates overnight at 37 ℃.

Appendix C – LR Recombination Reaction Protocol (Life Technologies Co., 2004)

LR Reaction

LR Clonase™ II enzyme mix is supplied as a 5X solution. If you wish to scale the reaction volume, make sure the LR Clonase™ II enzyme mix is at a final concentration of 1X. For a positive control, use 100 ng (2 µl) of pENTR™-gus.

1. Add the following components to a 1.5 ml microcentrifuge tube at room temperature and mix:

Entry clone (50-150 ng) 1-7 µl
Destination vector (150 ng/µl) 1 µl
TE buffer, pH 8.0 to 8 µl

2. Thaw on ice the LR Clonase™ II enzyme mix for about 2 minutes. Vortex the LR Clonase™ II enzyme mix briefly twice (2 seconds each time).

3. To each sample (Step 1, above), add 2 µl of LR Clonase™ II enzyme mix to the reaction and mix well by vortexing briefly twice. Microcentrifuge briefly.

4. Return LR Clonase™ II enzyme mix to -20 ℃ or -80 ℃ storage.

5. Incubate reactions at 25 ℃ for 1 hour.

6. Add 1 µl of the Proteinase K solution to each sample to terminate the reaction. Vortex briefly. Incubate samples at 37 ℃ for 10 minutes.

Transformation

1. Transform 1 µl of each LR reaction into 50 µl of One Shot® OmniMAX™ 2 T1 Phage-Resistant Cells (Catalog no. C8540-03). Incubate on ice for 30 minutes. Heat-shock cells by incubating at 42°C for 30 seconds. Add 250 µl of S.O.C. Medium and incubate at 37°C for 1 hour with shaking. Plate 20 µl and 100 µl of each transformation onto selective plates. **Note:** Any competent cells with a transformation efficiency of $>1.0 \times 10^8$ transformants/µg may be used.

2. Transform 1 µl of pUC19 DNA (10 ng/ml) into 50 µl of One Shot® OmniMAX™ 2 T1 Phage-Resistant Cells as described above. Plate 20 µl and 100 µl on LB plates containing 100 µg/ml ampicillin.

Appendix D – Colony PCR Protocol

1. Heathly colonies are selected and marked.

2. Prepare a master mix on ice for 10 reactions (6 colonies, 1 negative control, 1 positive control and 2 extra reactions, see above for details). Mix the PCR components in a 1.5ml Eppendorf tube in the following order:

Component	Master Mix (for 1 reaction)	Master Mix (for 10 reactions)
10X *Ex Taq* PCR Buffer (with 20mM MgCl2)	2µl	20µl
dNTP Mixture (2.5 mM each dNTP)	2µl	20µl
T7 Promoter Primer (10 µM stock)	1µl	10µl
T7 Terminator Primer (10 µM stock)	1µl	10µl
Sterile Water	13.8µl	138µl
TaKaRa *Ex Taq* DNA polymerase (5 units/µl)	0.2µl	2µl
Total Volume	20µl	200µl

3. Dispense 20µl aliquots into 0.5ml PCR tubes on ice. Label the PCR tubes with the number of the colony you will be testing, - and + control, accordingly.

4. With a sterile toothpick or micropipette tip, pick a single colony from the LB/Amp/Cam plate. Use a fresh LB/Amp/Cam plate to preserve the clones you are testing; you may use the plates you have poured in Lab Session 3, Laboratory Activity 1.1. For example, if you are testing 6 colonies for the presence of the insert, draw 6 squares on the bottom of the LB/Amp/Cam plate using a sharpie and number them from 1 to 6 (Figure 3.B). For each colony you pick, streak or touch the fresh LB/Amp/Cam plate on its corresponding number, then touch the labeled PCR tube, swirling and pipetting up and down to mix the colony with the PCR mix. Close the tube tightly. *If desired, you can also use this tip to start an overnight liquid culture.*

5. For the + control, add 1µl of 100ng/µl pET23d(+)-*scFv13R4*, previously purified by the instructors, to the PCR tube labeled "+ control".

6. For the – control, do not add DNA to the PCR tube labeled "- control".

7. Place the PCR tubes into the tube holder of the Eppendorf PCR machine located in room 105. Your instructors will demonstrate how to use the machine.

8. Amplify using the following cycling parameters:

Step	Time	Temperature	Cycles
Initial Denaturation	7 minutes	95 ℃	1X
Denaturation	1 minute	95 ℃	
Annealing	1 minute	54 ℃	25X
Extension	1 minute	72 ℃	
Final Extension	7 minutes	72 ℃	1X
Refrigeration	∞	4 ℃	1X

Appendix E – DNA Gel Electrophoresis Protocol

1. If needed, prepare 1X TAE buffer by mixing 20ml 50X TAE buffer with 980ml water.
2. Prepare a 0.8% agarose gel by adding 0.8g of agarose to 100ml of 1X TAE buffer. Microwave for 30 seconds with the cap on loosely. Swirl and repeat until agarose is completely in solution. If reusing the agarose solution from last week, microwave the bottle for 30 seconds with the cap on loosely, swirl and repeat until agarose is completely melted. *Remember to use rubber "hot hands" or autoclave gloves to handle the hot bottle.*

3. Allow the agarose solution to cool slightly before pouring the gel on the tray because hot agarose can distort the gel apparatus.

4. Before pouring the gel, add 5µl of EtBr to 100ml agarose solution (0.5µg/ml final concentration).

5. Pour 20-40ml of agarose solution onto your gel apparatus with a 15-well comb. Allow gel to solidify for about 10-15 minutes.

6. While the gel is cooling down, prepare your PCR samples for loading on the gel by adding 4µl of 6X Orange loading buffer to each 20µl PCR reaction.

7. Load 10µl of each sample into separate wells of the 1% agarose ge. Load 10µl of Fisher BioReagent *ex*ACTG*ene* 1 Kb Plus DNA Ladder to each of the wells 1 and 10 for confirmation of the fragment size.

8. After loading all samples, make sure the 1X TAE buffer covers the gel, place the electrophoresis cover on and connect the leads to the power supply. Make sure the direction of migration is set from the – pole to + pole. Set the migration voltage to 70V.

9. Check the migration after 5 minutes to see if the blue dye is migrating from the – pole to + pole.

10. After ~30 minutes, stop the run and examine the agarose gel in an imaging system.

Appendix F – 5X Native Buffer Composition (Life Technologies Co., 2006)

5X Native
Purification Buffer

250 mM NaH_2PO_4, pH 8.0
2.5 M NaCl
Prepare 200 ml solution as follows:
1. To 180 ml deionized water, add
Sodium phosphate, monobasic 7 g
NaCl 29.2 g
2. Mix well and adjust the pH with NaOH to pH 8.0.
3. Bring the final volume to 200 ml with water.
4. Store buffer at room temperature.

Appendix G – Ni-NTA Purification System Protocol

I. Preparation of Native Buffers: In the following procedure, use the Native Binding, Wash, and Elution Buffers, columns, and cell lysate prepared under native conditions for purification. Be sure to check the pH of your buffers before starting. All buffers for purification under native conditions are prepared from the 5X Native Purification Buffer supplied with the system.

A. Dilute and adjust the pH of the 5X Native Purification Buffer to create 1X Native Purification Buffer. For both lysates, prepare 200ml of 1X Native Purification Buffer, by combining:
160ml of sterile distilled water
40ml of 5X Native Purification Buffer (supplied with the system)
Mix well and adjust pH to 8.0 with NaOH or HCl.

B. From this, prepare the following buffers:
Native Binding Buffer: Use 60ml of the 1X Native Purification Buffer for use as the Native Binding Buffer (used for column preparation, cell lysis and binding).
Native Wash Buffer: Prepare 100ml of Native Wash Buffer with 20mM imidazole, by combining:
100ml of 1X Native Purification Buffer
670µl of 3M Imidazole, pH 6.0
Mix well and adjust pH to 8.0 with NaOH or HCl.
Native Elution Buffer: Prepare 30ml of Native Elution Buffer with 250mM imidazole, by combining:
27.5ml of 1X Native Purification Buffer
2.5ml of 3M Imidazole, pH 6.0
Mix well and adjust pH to 8.0 with NaOH or HCl.
Note: Imidazole is included in the Native Wash and Elution buffers to minimize the binding of untagged, contaminating proteins and increase the purity of the target protein with fewer wash steps.

II. Preparation of Ni-NTA Column: Before preparing a column, remove the snap-off cap at the bottom of the column and replace with the yellow cap.

1. Resuspend the Ni-NTA Agarose in its bottle by inverting and gently tapping the bottle repeatedly.

2. Pipet or pour 1.5ml of the resin into each 10-ml Purification Column. Allow the resin to gently pellet it by low-speed centrifugation (1,900rpm for 1 minute). Gently aspirate the supernatants.

3. Add 6ml of sterile, distilled water to each column and resuspend the resin by alternately inverting and gently tapping the columns.

4. Allow the resin to settle using centrifugation as described in Step 2, and gently aspirate the supernatants.

5. Add 6ml of Native Binding Buffer to each column.

6. Resuspend the resin by alternately inverting and gently tapping the columns.

7. Allow the resin to settle using centrifugation as described in Step 2, and gently aspirate the supernatants.

8. Repeat Steps 5 through 7.

III. Purification Under Native Conditions:

7. Add 8ml of the CHO lysate prepared under native conditions (from Laboratory Activity 2.2) to a prepared purification column.

2. Bind for 30–60 minutes using gentle agitation to keep the resin suspended in the lysate solution.

3. Settle the resin by low speed centrifugation (1,900rpm for 1 minute), and carefully aspirate the supernatants. Save supernatants at 4 ℃ for Bradford assay and SDS-PAGE analysis (sample **S**).

4. Wash with 8ml of Native Wash Buffer. Settle the resin by low speed centrifugation (1,900rpm for 1 minute), and carefully aspirate the supernatant. Save the supernatant at 4 ℃ for Bradford assay and SDS-PAGE analysis (sample $\mathbf{W_1}$).

5. Repeat Step 4 three more times. Save supernatants at 4 ℃ for Bradford assay and SDS-PAGE analysis (samples $\mathbf{W_2}$-$\mathbf{W_4}$).

6. Clamp each column in a vertical position and remove the yellow cap on the lower end. Elute the protein from each column with a total of 8ml of Native Elution Buffer according to the following: add 1ml of Native Elution Buffer, collect the 1ml elution fraction in a 15ml Falcon tube (labeled $\mathbf{E_1}$ for elution fraction 1) and only then move to the next fraction ($\mathbf{E_2}$) and so on.

7. Save the elution fractions at 4 ℃ for Bradford assay and SDS-PAGE analysis (samples $\mathbf{E_1}$-$\mathbf{E_8}$).

Appendix H – Western Blot Protocol

SDS-PAGE

I Sample Prep

1. Set heat block to 95 ℃.

2. For samples **L**, **S**, **W1**, **W2**, **E1** and **E2**, mix 18μL of each sample with 6μL of 4X loading sample buffer into separate Eppendorf tubes.

3. Combine 9μL of **E3** and 9μL of **E4** and add 6μL of 4X loading sample buffer to the combined sample. Follow the same procedure for elution fractions **E5/6** and **E7/8**.

4. Close the tubes and use a cap lock to lock the lids in place; this will prevent the tubes from opening during the incubation at high temperature. Incubate at 95 ℃ for 5 minutes.

5. Also, incubate an Eppendorf tube containing 20μLof pre-mixed (-) control (non-transfected) and loading sample buffer at 95 ℃ for 5 minutes.

6. Centrifuge tubes briefly to collect samples to the bottom of the tubes. Keep the samples on ice until loading on the gel. *Samples not used for loading will be stored at -20 ℃ for later use.*

7. As shown in Figure 3, load 10μL of Precision Plus Protein Dual Color Standard (MWM), 10μL of (-) control sample and 20μL of the other samples to individual wells.

II Gel Electrophoresis

1. Slide two gels into holders, facing inward. *If only one gel is used, fill other side with a buffer dam.*

2. Using ~350mL of 1X TGS running buffer, fill the compartment between the gels with liquid. If no leaks are evident after ~1 minute, fill the outside compartment to ~2 inches, above gel bottom. If inner compartment leaks, fill outside to keep inner compartment full.

3. After the samples have been loaded, attach the electrodes to the power supply. Run the gels at 300V constant voltage. Run the gels until the bromophenol blue tracking dye reaches the bottom of the gel.

4. Turn off the power supply and detach the leads from the power supply.

5. Remove your gels from the apparatus, pour the buffer into the sink and disassemble the gels from the plates as demonstrated by your instructors.

6. After removing the gels from the apparatus, pull apart the two pieces of plastic gently from each gel.

7. Hold the plate with the gel stuck to it over the staining tray and squirt a stream of distilled water under the gel to loosen it. The gel should drop into the staining tray.

8. Rinse the gel two times with distilled water.

9. Place the gel onto the UV trans-illuminator, turn on the UV light and expose the gel for 2-3 minutes. The protein bands should appear clearly after the UV exposure. Photograph your gel and analyze the results. Proceed with Western Blot.

Western Blot

I Gel Transfer

1. After visualization under UV light, place the completed gels containing protein samples to be tested into separate containers. Two gels will be transferred at the same time.

2. Open a midi PVDF Transfer Pack from BIORAD. The anode stack and membrane are located on top; use the "Bottom (+)" finger tab to remove the transfer pack from the packaging. *To prevent the membrane from drying out, use transfer packs immediately after opening.*

3. Assemble the blotting sandwich according to the instructions below.

4. Place bottom stack and blotting membrane on the cassette base. Make sure you place the stack in the center of the cassette.

5. Place gels on top of the membrane. Make sure you place the foot of the gels towards the center of the midi membrane, facing each other. Cut a small corner piece of the membrane to indicate the first well on each side. Remove air bubbles with blot roller. *Do not press too hard!*

6. Place the "Top (-)" stack on top of the gel. Remove air bubbles with blot roller. *Do not press too hard!*

7. Close and lock cassette lid. Insert the cassette into the Trans-Blot® Turbo™ Transfer System and begin the transfer. Use the "Turbo program" for midi size gels (7 minutes transfer). Your instructors will show how to select the right program.

8. After transfer, remove cassette from the instrument. *The stacks will be warm after transfer and part of the stack may be stuck to the lid.*

9. Remove the membrane from the cassette. With a pair of scissors, cut the membrane in half, each half corresponding to one gel.

10. Place each half-membrane into a container filled halfway (~30ml) with the wash buffer PBS-T (Phosphate Buffered Saline/0.1% Tween-20), making sure the transferred side is facing up. Label each container with your group number. Cover the container with a lid and proceed immediately to membrane development.

II Membrane Development

1. Place the container with the membrane and PBS-T on a rotator and wash for 5 minutes.

2. During the wash, prepare the blocking buffer by adding 1.5g of BSA to 50mL of PBS-T (to a final concentration of 3% BSA). *Half of this solution will be used to block the membrane and half as antibody solution.*

3. Pour the wash buffer down the sink and then add 25mL of the blocking buffer to the tray. Let incubate while rocking for 45 minutes.

4. Towards the end of the incubation period, prepare the antibody dilution by adding 5µl of anti-His C Terminal HRP antibody to 25ml of blocking buffer (to a final antibody dilution of 1:5,000). *Do not prepare the antibody solution in advance, only when needed!*

5. Pour the blocking buffer down the sink and add 25ml of antibody dilution to the tray. Cover the tray, place on rotator and incubate for 45 minutes.
6. Wash the membrane with PBS-T for 5 minutes for a total of 4 washes.
7. Pour the washing solution down the sink.
8. Prepare the developer solution by combining 18ml of high purity water and 2ml of BIORAD Opti-4CN™ Diluent in a conical tube and then adding 400µl of development reagent concentrate. Vortex the solution.
9. Pour 20ml of developer solution over the membrane and place on rotator for 5-15 minutes.
10. Once sample lanes are clearly visible, pour off any extra developer solution, wash with tap water and place staining tray upright allowing membrane to dry.

Appendix I – ELISA Protocol

1. Determine number of streptavidin-coated 8-well strips to be used in assay and remove them from refrigeration.

2. Place the strips into the plate rack and place provided blank well strips into empty columns to ensure that each assay plate contains a full 96 wells. Allow them to equilibrate to room temperature. Place unneeded strips back at 4 ℃.

3. Wash the assay plate using pre-programmed method number 06, ELISA_WASH on the Biotek ELx405 plate washer.

4. Prepare a 1:500 dilution of biotinylated β-galactosidase in sample diluent by mixing 20µl of biotinylated β-galactosidase stock solution and 9980ml of sample diluent for a total of 10ml.

5. Load 100µl of the diluted biotinylated β-galactosidase solution into each well.

6. Cover the assay plate and incubate 30 minutes at room temperature on a shaker.

7. Meanwhile, prepare dilutions for standard curve. In a 2-ml tube, prepare a 1:200 dilution of the 690µg/ml purified scFv13R4 standard stock to make a final concentration of 3.45µg/ml by adding 10µl of standard to 1.99ml of sample diluent. Close lid on tube and vortex to mix.

8. Perform seven additional 2-fold serial dilutions of the 3.45µg/ml standard (STD 1) in Eppendorf tubes. Make 600µl of each dilution by mixing 300µl of the previously diluted sample and 300µl of sample diluent. For example, mix 300µl of STD 1 with 300µl of sample diluent to make STD 2. Then, mix 300µl of STD 2 with 300µl of sample diluent to make STD 3 and so on.

9. Perform dilutions as necessary.

10. After coating incubation, wash the assay plate using pre-programmed method number 06, ELISA_WASH on the Biotek ELx405 plate washer.

12. Load 100µl of samples and standards into the wells of the assay plate according the plate layout.

13. Cover the assay plate and incubate on a shaker at room temperature for 30 minutes.

14. After sample/standard incubation, wash assay plate using pre-programmed method number 06, ELISA_WASH on the Biotek ELx405 plate washer.

15. Prepare a 1:20,000 dilution of anti-*c-myc*:HRP to make the antibody solution by mixing 1µl of the antibody into 20ml sample diluent.

16. Add 100µl of diluted antibody to each well of the assay plate.

17. Cover the assay plate and incubate for 30 minutes at room temperature on a shaker.

18. After antibody incubation, wash the assay plate using pre-programmed method number 06, ELISA_WASH on the Biotek ELx405 plate washer.

19. Add 100µl of TMB substrate to each well of the assay plate. Gently swirl the plate to ensure even coating.

20. Incubate on a flat surface at room temperature for 15 minutes. Do not cover the plate.

21. Stop reaction by adding 50µl of Stop Solution to each well. Gently shake plate to mix until all blue has turned yellow.
22. Load the plate onto the plate reader.
23. Open the Gen5 software and select “New Experiment”.
24. Select the “ELISA_450” protocol to read absorbance at 450 nm and click the “Read Plate” icon.
25. Answer “Yes” to read the plate.

Appendix J – Dilution Cloning Protocol

PURPOSE:

Produce a clonal cell line through limited dilution cloning.

SCOPE:

This document applies to experiments being executed in BTEC.

DEFINITIONS:

N/A

MATERIALS AND EQUPMENT

CO2 Incubator

- Operated at 37°C and 5% CO2 with relative humidity 80-85%.

96-well round bottom sterile plates Corning #7007

Sterile Pipets

Multi-channel pipettor (100-300 l volume)

Hyclone CDM4CHO basal media with 6 mM glutamine and 10% FBS or Sigma EX-CELL CHO Cloning media (C6366) with 6 mM glutamine

EXPERIMENTAL DESIGN

Pre-warm in the incubator for 1 hour.

Add 200 ul of sterile media or PBS to the outside perimeter wells of nine 96-well plates. You should have 60 wells in the center left for cell plating.

Take a viable cell density and viability measurement. Cell viability must be > 70% to initial limited dilution cloning.

Inoculum:

VCD (x1e6 cells/mL)	Viablity

Dilute cells to 1000 cells/mL to achieve a total volume of 5 mL. This can be done in several different ways but the easiest would be to first dilute the cells to 1e5 cells/mL and then do a 1:10 dilution of this suspension to obtain 1e4 cells/mL and then another 1:10 dilution to obtain 1e3 cells/mL (1000 cells/mL) for a 5 mL volume.

Take 200 uL of the well-mixed cell suspension and add to 40 ml of pre-warmed media in a sterile 50-mL conical tube. Close the tube and gently invert several times to ensure adequate mixing and re-suspension of the cells. This brings the cell density to 5 cells/mL.

Using a sterile reservoir aliquot 3 mL to begin plating.

Aliquot 200 uL of the cell suspension into the 60 empty wells of three 96-well plates.

- It is critical to ensure that your reservoir is well mixed prior to starting a new plate. Work quickly and efficiently to ensure no cell settling. Mix by pipetting prior to transferring additional cell suspension to the reservoir.
- After you have completed all three plates, place them in the incubator as soon as possible.

Repeat steps 5.4 through 5.6 to create an additional 6 plates for a total of 9 plates.

Observe the plates once a week and after 3-4 weeks, passage the successful wells that showed cell growth.

- Passage the successful clones that showed growth in 48-well or 24-well plates. Assume a 1:2 to 1:5 dilution rate to transfer the cells into the next set of plates.

Determine cloning efficiency by calculating the fraction of wells that achieved growth in the 96-well plate.

Once cells are in the 24-well or 48-well plate, allow them to grow for 5-7 days and take a 100 uL sample of IgG ELISA. At least 20 of the clones should be passaged further into 6-well plates and then into shake flasks.