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Converting Conventional Antibodies into ScFv and ScFv-Fc Fusion Fragments to Improve Expression and Function

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A Thesis in the Field of Biotechnology

for the Degree of Master of Liberal Arts in Extension Studies

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Abstract

Antibodies are used in many diagnostic, therapeutic, and research applications around the world. Traditionally, conventional full-length antibodies have been used for these applications, however, with the development of antibody engineering the use of antibody fragments has become more appealing.

Conventional antibodies contain an Fc region which can be beneficial as well as detrimental. In regard to therapeutics, the Fc region can elicit a heightened immune response which can be damaging to a human's health. The large size of conventional antibodies also does not allow the antibody to easily penetrate tissues and tumors. This can be challenging when it comes to drug development. The presence of the Fc region in conventional antibodies can also contribute to non-specific binding in research applications (Johnson, 2013).

To combat these issues, antibody fragments can be made to reduce immune cell activation and antibody effector functions. Antibody fragments often have a smaller size, which allows for better nuclear and tissue penetration. They can also be linked to each other to create multimeric forms or to other molecules to better target specific cells or tumors (Chames, Van Regenmortel, Weiss, & Baty, 2009). These features may be beneficial for therapeutic purposes as well as research assays. Lastly, antibody fragments can sometimes be more rapidly and less costly manufactured than conventional antibodies. This is due to the ability to be expressed in different less expensive systems (Jørgensen *et al.*, 2014)..

This study aimed to compare different antibody formats to see how expression and functional levels were affected. Conventional full-length antibodies were selected that were considered high value and low expressing from Cell Signaling Technologies™ mouse monoclonal antibody catalog. As well as the commercially available trastuzumab as a control antibody. ScFv and scFv-Fc fusion antibodies were constructed in either the variable heavy (V_H) followed by variable light (V_L) or variable light (V_L) followed by variable heavy (V_H) orientation. Newly constructed antibodies were expressed using the mammalian ExpiCHO™ system. Antibodies were then purified and quantified before being subjected to western blot and flow cytometry analysis.

It was determined that full-length antibodies had higher expression levels in the ExpiCHO™ system compared to the previously made antibodies expressed in the HEK293 systems. It was also determined that the V_L-V_H orientation seemed to be preferred over the V_H-V_L orientation based on expression levels. However, orientation preference seemed to be target specific. Furthermore, all antibody formats behaved functionally similar in western blot and flow cytometry analysis. This indicated that removing portions of the constant region did not affect functionality. Results from this study laid the groundwork for future experiments using the ExpiCHO™ system as well as different antibody formats to improve expression and functional levels of other high value targets.

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Chapter I.

Introduction

In 2018 monoclonal antibodies had a global revenue of $115 billion USD and in 2026 the revenue is predicted to increase to $148 billion USD (MarketWatch, 2020). This increase in revenue is due to a need for therapeutic and diagnostic tools to combat an increase in cancer and other chronic diseases. Currently, there are around 80 monoclonal antibodies approved by the FDA for the treatment of different diseases (Bates & Power, 2019). Monoclonal antibodies allow for the ability to devise personalized medicine for use in treatments such as immunotherapy. This increase in demand for antibodies is also present in the research world. Antibodies are used in several research applications such as western blot analyses, enzyme-linked immunosorbent assays (ELISA), immunohistochemistry, immunofluorescence, and flow cytometry to detect and characterize proteins.

Antibody Structure

Antibodies are glycoproteins that belong to the immunoglobulin superfamily. They are produced by B-cells in response to foreign antigens (Johnson, 2013). Antibodies are either secreted by the cell or membrane-bound in the form of B-cell receptors (BCR). Antibodies are Y-shaped and consist of four polypeptide chains, two identical heavy and light chains, connected by disulfide bonds. The heavy chain consists of five isotypes: IgG, IgM, IgA, IgD, and IgE. The most common isotype found in humans is IgG. IgG

can be divided into four subclasses, IgG1, IgG2, IgG3, and IgG4. These subclasses differ mostly in their hinge regions and constant domains (Vidarsson, Dekkers, & Rispens, 2014). There are also two types of light chains in humans, kappa (κ) and lambda (λ). On average, conventional antibodies have a molecular weight of about 150 kDa.

Antibodies can be further divided into a fragment antigen-binding region (Fab region) and a fragment crystallizable region (Fc region). The Fab region is comprised of one constant and one variable domain from each heavy and light chain. This region contains the variable domain which consists of three complementary determining regions (CDR) that are responsible for binding to the antigen. The Fc region is comprised of two identical protein fragments from the constant domains CH2 and CH3 of the antibody's two heavy chains. The Fc region provides increased stability, reduced aggregation, and decreased clearance rate from the body (Chiu, Goulet, Teplyakov, & Gilliland, 2019).

The Fc region also binds to Fc receptors on effector cells and is responsible for several effector functions. One function is antibody-dependent cell-mediated cytotoxicity (ADCC) in which natural killer cells, macrophages, and neutrophils are recruited to lyse the antigen. Another function is antibody-dependent cellular phagocytosis (ADCP) where antigens are marked for phagocytosis by an opsonin. Fc regions can also elicit antibody-mediated complement activation which can lead to pathogen elimination via complement-dependent cytotoxicity (CDC) (Erp, Luytjes, Ferwerda, & Kasteren, 2019).

Types of Antibodies

Antibodies also come in the form of polyclonal or monoclonal. Polyclonal antibodies are heterogeneous because they are made from different immune cells that recognize multiple epitopes on the same antigen. These antibodies can be used for the

identification of specific proteins within a complex environment. However, they are not ideal to use in *in vivo* experiments or therapeutics due to their inconsistency and increased background levels. As a result, polyclonal antibodies have largely been replaced by monoclonal antibodies. Monoclonal antibodies are made from identical immune cells that are all clones of a specific parent cell. The conventional way to produce monoclonal antibodies is by using Hybridoma technology (Lipman, Jackson, Trudel, & Weis-Garcia, 2005).

Hybridoma Technology

Hybridoma technology was invented by Köhler and Milstein in 1975. This technology involves five major processes. The first is the development of an immunogenic antigen. The next is to immunize a host animal with the antigen to elicit an immune response. Then B-cells are harvested and fused with immortalized myeloma cells to produce a hybridoma cell line. Hybridoma cells are then screened to determine which clones are best suitable for an intended application. This technology is beneficial because it is robust and stable. However, it also requires a long production process and potential loss of the intended target protein overtime (Zhang, 2012).

Recombinant Antibodies

As a result, recombinant technology has become the more dominant method. The key to recombinant antibodies is the molecular cloning of the unique heavy and light chain sequences into plasmids. This technology allows for the sequence of the antibody to be defined and optimized. Antibody sequences can be humanized to reduced immunogenicity and tags can be added for detection and purification. There are several

methods to produce recombinant antibodies. Phage display is the most frequently used technology. In phage display target antibodies are produced on the surface of bacteriophages. This method commonly uses *Escherichia coli* which allows for rapid and cost-effective production. Hybridoma technology can also be used to generate antibodies that can then be made recombinant using different cloning methods (Bazan, Całkosiński, & Gamian, 2012).

Antibody Fragments

Recombinant technology also allows for the production of antibody fragments. There are several types of antibody fragments that can be produced. One type of antibody fragment is antigen-binding fragments (Fabs). Fabs are typically 50 kDa and consist of the variable region and CH1 region of the heavy chain linked to the natural light chain by a disulfide bond.

Another type of fragment is a single-chain variable fragment (scFv). ScFvs are usually around 25 kDa and consist of the variable region of the heavy chain linked to the variable region of the light chain. The linker usually consists of four glycines and one serine repeated three times (GGGGS × 3). Glycine is used for flexibility while serine is used to increase solubility (Ahmad *et al.*, 2012). Affinity tags such as Myc, FLAG, HIS, HA, and GST can be added to fragments in order to assist with purification and detection methods (Bates & Power, 2019). The small size of scFvs allows for better tissue penetration as well as use in different expression systems such as *E.coli*. This is because they do not require N-linked glycosylation due to fact that they lack the CH2 domain.

Furthermore, Fc-fusion proteins are another type of antibody fragment. Fc-fusions are typically anywhere between 75-150 kDa depending on the size of the protein they are

linked to. Fc-fusions consist of a protein (single-domain antibody (sdAb), scFv, or Fab) linked to the Hinge, CH2, and CH3 regions of the Fc domain. Fc-fusions are beneficial because they prolong serum half-life as well as can improve the stability and solubility of some proteins. They also allow for the use of protein A or G purification as well as commonly used secondary antibodies (Jørgensen *et al.*, 2014).

Expression Systems

There are several prokaryotic and eukaryotic systems that can be used to express antibodies. Each system has its pros and cons. An expression system is usually chosen based on economic cost, protein yield, and desired protein function (Frenzel, Hust, & Schirrmann, 2013).

One type of expression system is bacteria i.e., *Escherichia coli*. This system is best used for the production of antigens and antibody fragments. This system can produce high yields of antibody in a short amount of time. It is also relatively inexpensive due to the use of low-cost media. However, bacterial expression also has some drawbacks. It results in a lack of glycosylation which can cause a loss of effector function. The presence of endotoxins can also result in an increase of inflammation (Vendel *et al.*, 2012). In order to combat these problems New England Biolabs® has constructed a strain of *E.coli* called SHuffle® that allows for formation of disulfide bonds which allows for proper folding and increased yields. So far there have been several Fabs and scFvs that have been successfully produced using this system (Robinson *et al.*, 2015).

Yeast is another system that can be used to express antibodies. Two common types of yeast strains that are used are *Saccharomyces cerevisiae* or *Pichia pastoris*. Yeast, similar to bacteria, is also relatively inexpensive and can produce high yields in a

short amount of time. However, Yeast produces proteins that differ from human glycosylation structures (Vieira Gomes, Souza Carmo, Silva Carvalho, Mendonça Bahia, & Parachin, 2018). One method to combat this issue is GlycoSwitch™ technology that uses glycoengineering to modify glycans in *Pichia pastoris* to produce proteins that are more human-like (Jacobs, Geysens, Vervecken, Contreras, & Callewaert, 2008).

Transgenic plants have become another way to produce antibodies. Most antibodies are produced in *Nicotiana benthamiana* (tobacco). However, other transgenic plants such as *Lemna minor* (duckweed) and *Medicago sativa* (alfalfa) have also been used. Transgenic plants are made using viral expression vectors and *Agrobacterium tumefaciens* to transfer genetic material. Some advantages to using transgenic plants are that they allow for large-scale production and increased safety due to use of animal free material. However, the use of plants can also be expensive and result in low yields (Oluwayelu & Adebiyi, 2016). One example of an antibody produced in transgenic plants is ZMapp™. ZMapp™ was created for the treatment of Ebola virus infection. It is comprised of three chimeric monoclonal antibodies made in *Nicotiana benthamiana* (Chen & Davis, 2016).

Transgenic animals are another source for antibody production. Rodents such as mice and rats are often used for small-scale production while cattle can be used for large scale production (Maksimenko, Deykin, Khodarovich, & Georgiev, 2013). Human antibodies have also been expressed in the milk of transgenic mice, rabbits, and goats as well as in the eggs of transgenic chickens. One example, of an antibody produced from mouse milk is a chimeric mouse/human Anti-CD20 monoclonal antibody (Tang *et al.*, 2008).

Mammalian cell lines are the most commonly used expression system for the production of antibodies. The two main cell lines used are human embryonic kidney (HEK293) and Chinese hamster ovary (CHO) cell lines. In mammalian expression systems, plasmid-based vectors are used to transfect genes into mammalian cell cultures. This system is beneficial because it creates post-translational modifications that are the most similar to humans (Holliger & Hudson, 2015). Some disadvantages to this system are slow growth and expensive media/transfection reagents. However, high protein yields can be obtained (Vazquez-Lombardi *et al.*, 2017). A well-honed system reported greater than 12 g/L without perfusion on other sophisticated methods (Huang *et al.*, 2010).

Antibody Purification

After choosing an expression system, antibodies must then be purified in order to isolate them for specificity and remove excess contaminants. One common method used to purify antibodies is affinity chromatography. This technique often uses agarose resin columns containing an immobilized ligand such as protein A or protein G. Protein A and protein G bind to the Fc region of IgG antibodies. However, fragments often lack Fc regions so other purification methods must be used. One option is to use protein L which binds to the kappa light chain. Another option is to use immobilized metal ion chromatography (IMAC). This method can be used if the antibody contains an affinity tag such as HIS. HIS-tagged antibodies can be purified using columns containing nickel, copper, zinc, or cobalt due to the tags high-affinity for metal ions (Ma & O'Kennedy, 2015).

Overall, antibodies have come a long way over the past 50 years from the discovery of Hybridoma cells to the creation of recombinant antibodies. As technology

continuously improves so does the ability to change the structure and function of antibodies. This advancement allows for increased specificity and reduced immunogenicity. It also allows for expression system optimization in order to better produce and purify antibodies. This is critical if advances in research, diagnosis, and treatment of diseases is to be achieved. This study intends to determine if certain antibody formats perform better than others in terms of expression and functional assays.

Chapter II.

Materials and Methods

This section describes the techniques that were used throughout this study to answer the research question: "Can conventional antibody expression levels and performance in functional assays be improved by removing some portions of the antibody?" The main focus of this study was to convert conventional full-length antibodies into scFv and scFv-Fc fusion antibody fragments and assess their expression and functional performance levels.

Construct Selection and Design

The first aim of this proposal was to choose several antibodies that were currently low expressing but considered top selling products and convert them into scFvs and scFv-Fc fusions. Low-expressing antibodies were considered to be those that yielded less than 50 mg of purified antibody per liter of culture. In addition to the proprietary antibodies, the publicly available sequence for Herceptin® (trastuzumab) was also used as a control as well to compensate for the sequence propriety of the other antibodies that were used. The trastuzumab sequence was obtained from DrugBank (https://www.drugbank.ca/drugs/DB00072).

The variable domains of each antibody were subjected to analysis by the publicly available software IgBlast which is based on data from IMGT (https://www.ncbi.nlm.nih.gov/igblast/). The CDR and framework sequences were identified to confirm that the antibodies were suitable for conversion. For scFvs, the variable domains of the heavy and light sequences were constructed in both the V_H-V_L

and V_L-V_H orientation and separated by a traditional Glycine-Serine-type linker (GGGGS×3). A 6x-His-Tag was added at the C-terminus for purification and detection methods followed by a stop codon. scFv-Fc fusion antibodies were then designed using the existing V_H-V_L and V_L-V_H scFvs. The 6x-His-Tag and stop codon were removed using Gibson Assembly and primers were designed to express the Fc domain.

Codon optimization was preformed according to *Homo sapiens* usage for efficient mammalian expression. This was done using the Codon Optimization tool from Integrated DNA Technologies™ website. *Bsp*QI sites were added to both the 5' and 3' ends in order to allow for the ability to clone into a vector. Internal *Bsp*QI sites and well as splice sites were accounted for using (http://www.fruitfly.org/seq_tools/splice.html).

ScFv Gene Synthesis and Golden Gate Assembly Cloning

The next step was to order synthetic gBlock® fragments of each scFv (Integrated DNA Technologies™) and clone the new fragments into a pTT5 vector. The pTT5 vector used included the Kozak sequence GCCACCATGG, a signal peptide sequence METGLRWLLLVAVLKGVQC, followed by two *Bsp*Q1 cloning sites and a mouse IgG1 constant region.

The scFv fragments were cloned into the pTT5 vector using traditional Golden Gate assembly methods. First the gBlock® fragments were digested and ligated to the vector. A 20 µl solution was made for each fragment that included 2 µl T4 DNA Ligase Buffer (#B0202S, New England Biolabs®), 1 µl T4 DNA Ligase (#M0202S, New England Biolabs®), 1 µl *Bsp*QI (#R0712S, New England Biolabs®), 75 µl Vector (75ng/µl), 12.25 µl water, and 3 µl DNA fragment (10ng/µl). The solution was placed in a thermal cycler and run at 37ºC for 40 minutes followed by 60ºC for 5 minutes.

Next a transformation was performed. 1 µl of the ligated solution was added to 10µl of NEB® 5-alpha Competent *E. coli* (High Efficiency) cells (#C2987I, New England Biolabs®) and placed on ice for 20 minutes. The mixture was then heat-shocked at 42ºC for 30 seconds then placed on ice for 5 minutes. 100 µl of SOC was added and the mixture was placed at 37ºC for 20 minutes. 100 µl of the solution was spread onto a 10cm Agarose plate containing 100 µg/ml of Ampicillin using glass beads. The plates were incubated at 37ºC overnight.

A single colony was then picked and put into 125 ml of LB broth containing Ampicillin. The cultures were incubated at 37ºC and shaken overnight. Cultures were then centrifuged, and the supernatant was removed from the pellet. The DNA was then purified using a Qiagen Plasmid Plus Maxi Kit (#12963, Qiagen®). 8 ml of P1 Resuspension buffer containing RNAse A and LyseBlue was added to the pellet and mixed until the solution appeared homogenous. Then 8 ml of P2 Lysis buffer was added and mixed for 5 minutes followed by the addition of 8 ml of S3 Neutralization buffer.

The remaining solution was poured into a filter and remained for 10 minutes before being filtered through the column using a plunger. 5 ml of Binding buffer was added, and the solution was poured onto a column. The solution was filtered through the column using a vacuum manifold. 1 ml of Endotoxin Removal buffer was added and filtered through followed by 1 ml of PE buffer. The column was removed from the manifold and placed in a tabletop centrifuge and spun at 16,000 RCF for 1.5 minutes. 300µl of Elution buffer was added to the column and spun at 10,000 RCF for 1 minute. The column was discarded, and the remaining DNA was quantified using the QIAxpert®

A_{260} dsDNA method. The DNA concentration was then normalized to 1µg/µl using Elution Buffer.

ScFv-Fc Fusion Gibson Assembly Cloning

scFv antibodies were then converted into scFv-Fc fusions using PCR amplification from the scFv plasmids and then Gibson Assembly into the Fc vector. A forward PCR primer was designed around the 3' end of the scFv variable region and a reverse primer was designed around the hinge region. In addition, forward and reverse vector primers were designed after the Ampicillin resistance gene. All primers were designed and ordered through Integrated DNA Technologies™. For trastuzumab scFv-Fc fusion V_H-V_L the following sequences were used:

Table 1. Trastuzumab scFv-Fc Fusion Primers

Primer Name	**Primer Sequence (5'- 3')**
Her1_fwd	GTGGAAATTAAGGGCGTGCCCAGGGATTGTGGTTGTAAGC
Her1_rev	ATCCCTGGGCACGCCCTTAATTTCCACCTTCGTTCCTTGACCAAACG
Vector_fwd	GCATTGGTAACTGTCAGACCAAG
Vector_rev	GTAAACTTGGTCTGACAGTTAC

Primer names and sequences used for trastuzumab scFv-Fc fusion V_H-V_L Gibson Assembly PCR.

For the first fragment, 20 µl of template scFv DNA (0.5ng/ 10µl) was combined with 10 µl of Her1_fwd primer (1µM) , 10µl of Vector_rev primer (1µM), and 40 µl of Q5® Hot Start High-Fidelity 2x Master Mix (#M0494S, New England Biolabs®). For the second fragment PCR reaction, 20 µl of template scFv DNA (0.5ng/ 10µl) was combined

with 10 µl of Her1_rev primer (1µM) , 10 µl of Vector_fwd primer (1µM), and 40 µl of Q5® Hot Start High-Fidelity 2x Master Mix. The reaction was run on a thermal cycler at 98˚C for 30 seconds, then cycled 25 times at 98ºC for 10 seconds, 60ºC for 20 seconds, and 72ºC for 1 minute and 30 seconds. Then set to 72ºC for 2 minutes before being ramped down to 4ºC. For fragments that needed to be repeated 30 cycles were used.

5 µl of each PCR fragment was combined with 2 µl of Gel Loading Dye, Purple (6X) (#B7025S, New England Biolabs®). Each sample as well as 5 µl of 1 kb DNA ladder (#N3232L, New England Biolabs®) were run on a 0.8% Agarose gel containing SYBR™ Safe DNA Gel Stain (#S33102, Thermo Fischer Scientific™) at 150V for 60 minutes. The gel was imaged using the Bio-Rad ChemiDoc™ system and labeled with ImageLab Software.

75 µl of each PCR fragment was then combined with 1 µl of *Dpn*I (#R0176S, New England Biolabs®) and 9 µl of CutSmart® buffer (#B7204S, New England Biolabs®) for 30 minutes at 37ºC. Afterwards, 160 µl of AMPure XP beads (#A63881, Beckman Coulter®) were added and mixed 10 times into 80 µl of sample and then placed on a magnet for 2 minutes. The supernatant was removed and 200 µl of 70% ethanol was added and then discarded. Another 200 µl of 70% ethanol was added and then discarded. 40 µl of elution buffer (1M Tris-Cl pH 8.0/ .5mM EDTA) was added and mixed. The samples were then placed on the magnet for 2 minutes before the DNA was removed. The QIAxpert® was then used to measure the A_{260} concentrations.

The PCR fragments were then ligated using NEBuilder® HiFi DNA Assembly Master Mix (#E2621S, New England Biolabs®). 10 µl of the combined two fragments were added to 10 µL of NEBuilder® HiFi DNA Assembly Master Mix and incubated at

50ºC for 30 minutes. 2 µl of the ligated product was added to 50 µl of NEB® 5-alpha Competent *E. coli* (High Efficiency) and incubated on ice for 30 minutes. The mixture was then heat-shocked at 42ºC for 30 seconds then placed on ice for 2 minutes. 950 µl of SOC (#B9020S, New England Biolabs®) was added and the mixture was placed at 37ºC, 250 RPM for 1 hour. 100 µl of the solution was spread onto a 10 cm Agarose plate containing 100µg/ml of Ampicillin using glass beads. The plates were incubated at 37ºC overnight. The following day a single colony was picked, cultured, and purified in the same way as the scFv procedure above.

Sequence Verification

Sequence specific primers were designed, and Sanger sequencing was performed to verify that the correct insert sequences were cloned. Primer sequences that bound to 5' and 3' regions on the vector flanking the insert sequence were used as well as other internally designed primers. All primers were designed and ordered through Integrated DNA Technologies™.

Table 2. Sanger Sequencing Primers

Primer Name	Primer Sequence (5'-3')
pTTfwd	TTGAGTGACAATGACATCCAC
trastuzumab Heavy 1	GGATCTGCTGCCCAAACTAA
trastuzumab Heavy 2	GAGCAGTTCAACAGCACTTTC
pTTrev	TGTCCTTCCGAGTGAGAGACACAA
mIgG1_rev	GTTGCGTCTGAGCTGTGTGCA
28410_rev	TTAGTTTGGGCAGCAGATCC

List of Sanger sequencing primers used to sequence full-length, scFv, and scFv-Fc fusion inserts.

For trastuzumab full-length heavy chain, the primers pTTfwd, trastuzumab Heavy 1, and trastuzumab Heavy 2 were used to sequence the insert. For trastuzumab full-length light chain, the primers pTTfwd and pTTrev were used. For the full-length Anti-Chk1, Anti-ApoE4, and Anti-GAPDH heavy chains, the primers pTTfwd, pTTrev, and mIgG1rev were used. For the full-length Anti-Chk1, Anti-ApoE4, and Anti-GAPDH light chains, the primers pTTfwd and pTTrev were used. For all of the scFvs, the primers pTTfwd and 28410_rev were used. For all of the scFv-Fc fusions, pTTfwd, pTTrev, and mIgG1rev were used.

Sanger sequencing was preformed using a 20 µl reaction consisting of 0.5 µl of BigDye™ Terminator v3.1 Ready Reaction Mix (#4337455, Thermo Fischer Scientific™), 7.5 µl 5X Sequencing Buffer (#4337455, Thermo Fischer Scientific™), 1 µl DNA template (300ng), 2 µl primer (1.6µM), and 9 µl water. The reaction was then run on a thermal cycler at 96ºC for 1 minute, then cycled 25 times at 96ºC for 10 seconds, 50ºC for 5 seconds, and 60ºC for 4 minutes. Then ramped down to 4 ºC. After cycling, the sequencing reaction was cleaned up using CleanSEQ® magnetic beads (#A29154, Beckman Coulter®). 10 µl of CleanSEQ® magnetic beads were added to the reaction followed by 62 µl of 85% ethanol. The solution was mixed and placed on a magnet for 3 minutes, the supernatant was then discarded, and 100 µl of 85% ethanol was added. After 30 seconds the ethanol was discarded, and the plate was left to dry for 10 minutes. 60 µl of nuclease-free water was added and the plate sat for 2 minutes before it was placed back onto the magnet for 3 minutes. 20 µl of the cleared solution was transferred to a MicroAmp® Optical 96-Well Reaction Plate (#N8010560, Thermo Fischer Scientific™)

and loaded onto the 3730xl Genetic Analyzer™. Sequencing analysis was preformed using Sequencher® .

Transfection

The second aim was to express the scFvs and scFv-Fc fusions in a mammalian system. This was done using the ExpiCHO™ Expression System (#A29133, Invitrogen™). ExpiCHO-S™ cells were counted using Beckman Coulter's Vi-Cell XR® cell viability analyzer. They were then diluted to 6 x 10^6 cells/ml into a 250 ml flask containing 50 ml of ExpiCHO™ Expression Medium. 40 µg of DNA was added to 3.9 ml of OptiPro™ SFM Complexation Medium. 160 µl of Expifectamine™ CHO Reagent was added. The solution was mixed and added to the 50 ml flasks and placed in an orbital shaker in a 37ºC incubator with 8% CO_2 overnight.

After 18-22 hours post-transfection, 300 µl of Expifectamine™ CHO Enhancer and 8 ml of ExpiCHO™ Feed was added to each flask and placed in an orbital shaker in a 32ºC incubator with 5% CO_2. On day 5 post-transfection another 8 ml of ExpiCHO™ Feed was added to the flask and returned to the orbital shaker in the 32ºC incubator. Day 12 post-transfection, Beckman Coulter's Vi-Cell XR® cell viability analyzer was used to determine cell viability. The cell viability was determined to be >75%. The samples were then harvested and centrifuged at 3500 RCF for 30 minutes. The supernatant was removed and put into a new tube.

Octet® Concentration

ForteBio's Octet® RED96 system was used to determine the concentrations of the full-length antibodies and scFv-Fc fusions using protein A biosensors. A standard curve

was calculated using eight increments of mouse IgG1 ranging from 500 µg/ml to 3.91 µg/ml. The antibody samples were diluted 10-fold with 1X PBS. Glycine pH 1.8 was used for regeneration and 1X PBS was used for neutralization. The samples were run at 400 RPM at 30 ºC.

Protein A Purification

Purification of the full-length antibodies and scFv-Fc fusions was done using protein A. 1-3 ml of the MabSelect™ SuRe™ protein A medium (#GE17-5438-01, MilliporeSigma®) was added to the column. The column was washed with 20 ml of 1X PBS and allowed to flow through. Binding of antibody to the protein A resin was performed in solution. 1 ml of 1X PBS was then added to the resin and the solution was put into a 50 ml tube containing the supernatant. The tube was placed in an orbital shaker overnight at 4ºC.

The next day the supernatant/protein A agarose mixture was poured into a column. The flow through was collected and stored at 4ºC. The sample was washed with 20 ml of 1X PBS (pH 7.4 / 0.1% Triton X114) and allowed to flow through the column. The sample was then washed with 20 ml 1X PBS and allowed to flow through. 360 µl of neutralization buffer (1M Tris pH 8.5) was added to a new 50 ml tube and placed under the column. 3 ml of elution buffer (.1M Glycine pH 3.5) was added to the column, mixed, and incubated for 2 minutes. The flow through was then collected into the 50 mL tube. Another 3 ml of elution buffer was added to the column, mixed, and incubated for 2 minutes before being collected into another 50 mL tube. This process was repeated several times. The concentration was then determined using the QIAxpert®: 2 µl of reference buffer (360 µl of neutralization buffer / 3 ml of elution buffer) as well as 2 µl of

each sample were loaded onto a slide. The A_{280} concentrations were taken using the extinction coefficient for each antibody. The extinction coefficients were determined using Expasy's ProtParam tool.

The samples were then pooled and subjected to dialysis. Slide-A-Lyzer® MINI Dialysis Devices 20K MWCO (#69590, Thermo Fischer Scientific™) were used. The conical tubes were filled with 14 ml of 1X PBS pH 7.4. The membranes were wet with 1ml of 1X PBS pH 7.4 and then filled with 0.5 ml of sample. The dialysis tubes were then placed on an orbital shaker for 2 hours at 4ºC. The tubes were then filled with 14ml of new 1X PBS pH 7.4 and shaken overnight at 4 ºC. The QIAxpert® was then used to determine the A_{280} concentrations.. 2 µl of reference buffer (1X PBS pH7.4 from the overnight dialysis) and 2 µl of each sample were loaded onto a slide. The A_{280} concentrations were taken using the extinction coefficient for each antibody.

Nickel Purification

Purification of the scFv antibodies was done using a nickel column by binding to the 6x-His-Tag. 20 ml of Ni Sepharose™ 6 Fast Flow medium (# GE17-5318-01, MilliporeSigma®) was added to a column and washed 3 times with 10 ml of 1X PBS pH8. The supernatant was added to the column and the flow through was collected. The flow through was then filtered through the column, collected, and stored at 4 ºC. The column was then washed with 20 ml of nickel wash buffer pH 8 (1X PBS pH 7.4/ 30mM Imidazole). This process was repeated four more times. The wash was collected and stored at 4ºC. 20 ml of elution buffer pH 8 (1X PBS pH 7.4/ 400 mM Imidazole/ 300mM NaCl) was added and sat for 2 minutes before several fractions were collected. The concentrations were then determined using the QIAxpert®. 2 µl of reference buffer

(elution buffer) as well as 2 µl of each sample were loaded onto a slide. The A_{280} concentrations were taken using the extinction coefficient for each antibody.

The fractions were pooled and concentrated to 1 mg/ml using Amicon® Ultra-15 Centrifugal Filter Units 10K MWCO (#UFC901024, MilliporeSigma®) and then subjected to dialysis. Slide-A-Lyzer® MINI Dialysis Devices 3.5K MWCO (#69550, Thermo Fischer Scientific™) were used. The conical tubes were filled with 44.5 ml of 1X PBS pH 7.4. The membranes were wet with 4 ml of 1X PBS pH 7.4 and then filled with 2 ml of sample. The dialysis tubes were then placed on an orbital shaker for 2 hours at 4ºC. The tubes were then filled with 44.5 ml of new 1X PBS pH 7.4 and shaken overnight at 4ºC. The QIAxpert® was then used to determine the A_{280} concentrations.. 2 µl of reference buffer (1X PBS pH7.4 from overnight dialysis) and 2 µl of each sample were loaded onto a slide. The A_{280} concentrations were taken using the extinction coefficient for each antibody.

SDS-PAGE

SDS-PAGE was performed on supernatant before and after purification. 20 µl of sample was mixed with 20 µl of 2x SDS/DTT (#7723S, Cell Signaling Technology®) at a 1:1 ratio and boiled for 5 minutes on a heat block set to 95ºC. 10 µl of each sample mixture as well as 10 µl of Mark 12™ Unstained Standard (#LC5677, Thermo Fischer Scientific™) was loaded into a NuPAGE™ Novex™ 4-12% Bis-Tris Protein Gel (#NP0323BOX, Thermo Fischer Scientific™) in 1X MES running buffer. The gels were run at 180V for 35 minutes. The gels were stained with Simply Blue™ Safe Stain (#LC6060, Thermo Fischer Scientific™) and shaken for 1 hour. The gels were de-stained

with diH_2O for 1 hour and then imaged using the Bio-Rad ChemiDoc™ system and labeled using ImageLab Software.

Western Blot

The third aim was to functionally test the conventional full length, scFv, and scFv-Fc fusion antibodies using western blot and flow cytometry assays. Overexpression lysates were obtained of 293T cells transfected with full-length human constructs expressing Her2, Anti-Chk1, Anti-ApoE4, and Anti-GAPDH containing C-term MycDDK tags. Endogenous lysates for K562 (ATCC®CCL-243™), SKBR3 (ATCC® HTB-30™), A172 (ATCC®CRL-1620™), HeLa (ATCC®CCL-2™), 293T (ATCC® CRL-11268™), and HepG2 (ATCC® HB-8065™) cell lines were also obtained. 2 µl of overexpression lysates and 5 µl of endogenous lysates were loaded onto 4–20% Mini-PROTEAN® TGX™ Precast Protein Gels (#4561096, Bio-Rad) along with 7.5 µl of a biotinylated protein ladder (#7727, Cell Signaling Technology®).

Gels were run for 30 minutes at 200 V. The proteins were transferred onto Midi PVDF Membranes (#1704157, Bio-Rad) using a Trans-Blot® Turbo™ Transfer System (#1704150, Bio-Rad). Membranes were incubated in blocking buffer containing 5% nonfat dry milk and 1X Tris Buffered Saline with Tween 20 (TBST) for 1 hour. The membranes were then incubated in 5% nonfat dry milk and 1X TBST overnight at 4°C with the appropriate primary antibodies. Control antibodies Myc-Tag (9B11) Mouse mAb (#2276, Cell Signaling Technology®) and β-Actin (E4D9Z) Mouse mAb (#58169, Cell Signaling Technology®) were also used.

The next day the membranes were washed three times for 5 minutes each with 1X TBST. They were then incubated for 1 hour at room temperature in 5% nonfat dry milk

and 1X TBST containing the appropriate secondary antibodies. The secondary antibodies that were used were Anti-biotin, HRP-linked Antibody (#7075, Cell Signaling Technology®) for the protein ladder, Anti-mouse IgG, HRP-linked Antibody (#7076, Cell Signaling Technology®) for the full-length and scFv-Fc fusion antibodies, and His-Tag (D3I1O) XP® Rabbit mAb (HRP Conjugate) (#12688, Cell Signaling Technology®) for the scFv antibodies. After, the membranes were washed three times for 5 minutes each with 1X TBST. They were then incubated with a 1:1 solution of 20X LumiGLO® Reagent and 20X Peroxide (#7003, Cell Signaling Technology®) for 1 minute. The membranes were imaged using the Bio-Rad ChemiDoc™ system and labeled using ImageLab Software.

Flow Cytometry

Antibodies were then subjected to flow cytometry assays to further determine functionally. K562, SKBR3, A172, HeLa, 293T, and HepG2 methanol permeabilized cells were obtained. They were resuspended with 3 ml of 1X PBS, centrifuged, supernatant discarded, and the cells were resuspended with Dilution Buffer (0.5% BSA in 1X PBS). Primary antibodies for the full-length, scFv, and scFv-Fc fusions were diluted at 5.12 µg/ml, 1.28 µg/ml, and .32 µg/ml concentrations using Dilution Buffer.

50 µl of Dilution Buffer was added into each sample well along with 50 µl of the appropriate cell types ($1x10^5$ cells) and 50 µl of the appropriate primary antibody dilution. The samples were left at room temperature for 1 hour. Samples were then washed with 100 µl of Dilution Buffer, centrifuged, and the supernatant was discarded. Control samples were also made containing each cell type with no primary antibody as well as each cell type with secondary antibodies only.

Secondary antibody stocks were made using Anti-mouse IgG (H+L), F(ab')2 Fragment (Alexa Fluor® 488 Conjugate) (#4408, Cell Signaling Technology®) at a 1:1000 dilution for the full-length and scFv-Fc fusion antibodies and His-Tag (D3I1O) XP® Rabbit mAb (Alexa Fluor® 488 Conjugate) (#14930, Cell Signaling Technology®) at a 1:50 dilution for the scFv antibodies. 100 µl of the appropriate secondary antibody stocks were added to each sample. Samples were incubated in the dark at room temperature for 30 minutes. Samples were washed with 100 µl of Dilution Buffer, centrifuged, and the supernatant was discarded. 200 µl of Dilution Buffer was added to each sample before being run on the Attune NxT Flow Cytometer™ and subsequently analyzed using FlowJo software.

Chapter III.

Results

This section describes the results of the work described in the previous section. The first aim was to select, design, and construct the antibody fragments. The second aim was to express, purify, and quantify the antibodies. The third and final aim was to functionally test the antibodies using western blot and flow cytometry assays.

Construct Selection and Design

The following full-length conventional antibodies were chosen Anti-Chk1 Mouse mAb, Anti-ApoE4 Mouse mAb, and Anti-GAPDH Mouse mAb. These were previously expressed in HEK293 cells and yielded 41.4 mg/L, 36 mg/L, and 19.8 mg/L respectively. For conversion into scFv and scFv-Fc fusions the variable and constant regions were determined to be suitable based on IMGT. Codon optimization was successful and no internal splice sites or *Bsp*QI sites were found. The following amino acid and nucleotide sequences were used for trastuzumab. The other sequences cannot be displayed due to the proprietary rights of Cell Signaling Technology® but were constructed in the same format as trastuzumab. Figure 1 displays the variable region amino acid sequences of trastuzumab heavy chain and light chain. There CDR's are highlighted in yellow (CDR1), green (CDR2), and red (CDR3).

Trastuzumab Heavy Chain:

EVQLVESGGGLVQPGGSLRLSCAASGFNIKDTYIHWVRQAPGKGLEWVARIYPTNGYTRYADSV
KGRFTISADTSKNTAYLQMNSLRAEDTAVYYCSRWGGDGFYAMDYWGQGTLVTVSS

Trastuzumab Light Chain:

DIQMTQSPSSLSASVGDRVTITCRASQDVNTAVAWYQQKPGKAPKLLIYSASFLYSGVPSRFSGSR
SGTDFTLTISSLQPEDFATYYCQQHYTTPPTFGQGTKVEIK

Figure 1. Trastuzumab Heavy and Light Chain Amino Acid Sequences

Trastuzumab heavy and light chain variable region amino acid sequences. CDR1 is indicated in yellow, CDR2 in green, and CDR3 in red.

Figure 2 displays the amino acid sequences for trastuzumab in the scFv format in the variable region heavy chain (V_H) followed by variable region light chain (V_L) orientation as well as the variable region light chain (V_L) followed by variable region heavy chain orientation (V_H) orientation. Each scFv is connected by a GGGGS × 3 linker and has a C-term 6x-His-Tag followed by a stop codon. Figure 3 displays the same sequences that were used for the scFvs in the V_H-V_L and V_L-V_H orientations except in the nucleotide format with the 3' and 5' *Bsp*QI sites added.

Trastuzumab scFv V_H-V_L:

EVQLVESGGGLVQPGGSLRLSCAASGFNIKDTYIHWVRQAPGKGLEWVARIYPTNGYTRYADSV
KGRFTISADTSKNTAYLQMNSLRAEDTAVYYCSRWGGDGFYAMDYWGQGTLVTVSSGGGGSGG
GGSGGGGSDIQMTQSPSSLSASVGDRVTITCRASQDVNTAVAWYQQKPGKAPKLLIYSASFLYSG
VPSRFSGSRSGTDFTLTISSLQPEDFATYYCQQHYTTPPTFGQGTKVEIKHHHHHH*

Trastuzumab scFv V_L-V_H:

DIQMTQSPSSLSASVGDRVTITCRASQDVNTAVAWYQQKPGKAPKLLIYSASFLYSGVPSRFSGSR
SGTDFTLTISSLQPEDFATYYCQQHYTTPPTFGQGTKVEIKGGGGSGGGGSGGGGSEVQLVESGGG
LVQPGGSLRLSCAASGFNIKDTYIHWVRQAPGKGLEWVARIYPTNGYTRYADSVKGRFTISADTS
KNTAYLQMNSLRAEDTAVYYCSRWGGDGFYAMDYWGQGTLVTVSSHHHHHH*

Figure 2. Trastuzumab scFv Amino Acid Sequences

Trastuzumab scFv amino acids sequences in V_H-V_L and V_L-V_H orientations. V_H is indicated in blue, GGGGS × 3 linker in yellow, V_L in purple, 6x-His-Tag is gray, followed by a stop codon in red.

Trastuzumab scFv V_H-V_L:

gcatgtcgctcttcatgtGAGGTTCAGCTCGTCGAGTCCGGAGGCGGCCTTGTACAGCCAGGTGGTAGTT
TGCGGTTGAGTTGTGCAGCATCTGGGTTCAACATCAAGGACACCTATATCCACTGGGTTAGGC
AAGCCCCAGGGAAGGGCCTCGAATGGGTCGCTCGGATCTACCCGACGAACGGATATACTCGA
TATGCCGATAGCGTAAAAGGGAGGTTCACCATCTCCGCAGATACCAGCAAGAACACAGCCTA
TCTGCAAATGAATAGCCTGAGGGCAGAAGATACTGCCGTCTACTACTGTTCTAGGTGGGGTGG
GGACGGCTTCTACGCTATGGATTATTGGGGCCAAGGGACCTTGGTCACTGTCAGTAGTGGAGG
GGGAGGCTCTGGCGGCGGAGGCTCCGGTGGTGGCGGCAGTGATATTCAAATGACTCAGTCAC
CCAGTAGTCTGTCTGCCTCAGTTGGCGACCGGGTCACTATCACCTGCCGCGCCTCTCAGGACG
TCAACACAGCTGTCGCTTGGTACCAACAAAAGCCTGGGAAAGCTCCTAAGTTGCTTATATATT
CCGCGTCTTTTTTGTATTCTGGAGTCCCTAGCAGATTCTCAGGGTCTCGCTCCGGTACTGATTT
CACCCTGACCATATCTTCTTTGCAGCCTGAAGATTTTGCCACATATTATTGTCAGCAACATTAC
ACAACCCCCCCTACGTTTGGTCAAGGAACGAAGGTGGAAATTAAGCATCATCACCATCATCAC
TGAgcctgaagagctgtacag

Trastuzumab scFv V_L-V_H:

gcatgtcgctcttcatgtGATATTCAAATGACACAAAGTCCATCATCCCTCTCCGCCAGTGTTGGCGACAG
AGTCACGATCACCTGCCGCGCATCCCAAGATGTTAACACTGCAGTAGCATGGTATCAGCAAAA
ACCCGGAAAAGCCCCTAAGCTGCTCATATATAGCGCGTCCTTTCTTTATTCTGGCGTACCTTCC
AGATTCAGCGGATCACGCTCTGGGACGGATTTCACCTTGACAATTTCCAGTCTGCAACCTGAA
GATTTCGCAACATACTACTGTCAACAACATTACACTACACCGCCCACTTTTGGGCAAGGGACG
AAAGTAGAAATAAAAGGAGGAGGAGGGAGTGGCGGCGGAGGCTCCGGAGGTGGAGGGTCTG
AAGTGCAATTGGTTGAGTCCGGAGGGGGCCTCGTGCAACCCGGCGGCTCATTGCGGCTTTCTT
GCGCCGCGAGCGGATTTAATATAAAGGACACATATATCCATTGGGTAAGGCAGGCACCGGGT
AAAGGACTCGAGTGGGTCGCCAGAATATACCCGACTAACGGGTACACCCGCTACGCTGACTC
AGTCAAGGGCAGATTCACCATCTCCGCTGACACCAGTAAAAATACTGCTTATCTTCAGATGAA
TTCTCTTCGGGCCGAGGACACTGCGGTTTACTACTGCTCCCGGTGGGGAGGCGACGGATTTTA
TGCTATGGATTATTGGGGCCAGGGGACTCTCGTGACTGTAAGTAGCCATCATCACCATCATCA
CTAGgcctgaagagctgtacag

Figure 3. Trastuzumab scFv Nucleotide Sequences

Trastuzumab scFv nucleotide sequences in V_H-V_L and V_L-V_H orientations. V_H is indicated in blue, GGGGS × 3 linker in yellow, V_L in purple, 6x-His-Tag is gray, followed by a stop codon in red. The BspQI sequences are indicated in black on the 5' and 3' ends.

Figure 4 displays the amino acid sequences for trastuzumab in the scFv-Fc fusion format in the V_H-V_L and V_L-V_H orientations. Each scFv-Fc fusion is connected by a GGGGS × 3 linker. In addition, there is another glycine linker that connects the end of the variable region to the hinge region followed by the CH2 domain, CH3 domain, and two stop codons. Figure 5 displays the same sequences that were used for the scFv-Fc fusions in the V_H-V_L and V_L-V_H orientations except in the nucleotide format.

Trastuzumab scFv-Fc Fusion V_H-V_L:

EVQLVESGGGLVQPGGSLRLSCAASGFNIKDTYIHWVRQAPGKGLEWVARIYPTNGYTRYADSV
KGRFTISADTSKNTAYLQMNSLRAEDTAVYYCSRWGGDGFYAMDYWGQGTLVTVSSGGGGSGG
GGSGGGGSDIQMTQSPSSLSASVGDRVTITCRASQDVNTAVAWYQQKPGKAPKLLIYSASFLYSG
VPSRFSGSRSGTDFTLTISSLQPEDFATYYCQQHYTTPPTFGQGTKVEIKGVPRDCGCKPCICTVPEV
SSVFIFPPKPKDVLTITLTPKVTCVVVDISKDDPEVQFSWFVDDVEVHTAQTQPREEQFNSTFRSVS
ELPIMHQDWLNGKEFKCRVNSAAFPAPIEKTISKTKGRPKAPQVYTIPPPKEQMAKDKVSLTCMIT
DFFPEDITVEWQWNGQPAENYKNTQPIMDTDGSYFVYSKLNVQKSNWEAGNTFTCSVLHEGLHN
HHTEKSLSHSPGK**

Trastuzumab scFv-Fc Fusion V_L-V_H:

DIQMTQSPSSLSASVGDRVTITCRASQDVNTAVAWYQQKPGKAPKLLIYSASFLYSGVPSRFSGSR
SGTDFTLTISSLQPEDFATYYCQQHYTTPPTFGQGTKVEIKGGGGSGGGGSGGGGSEVQLVESGGG
LVQPGGSLRLSCAASGFNIKDTYIHWVRQAPGKGLEWVARIYPTNGYTRYADSVKGRFTISADTS
KNTAYLQMNSLRAEDTAVYYCSRWGGDGFYAMDYWGQGTLVTVSSGVPRDCGCKPCICTVPEV
SSVFIFPPKPKDVLTITLTPKVTCVVVDISKDDPEVQFSWFVDDVEVHTAQTQPREEQFNSTFRSVS
ELPIMHQDWLNGKEFKCRVNSAAFPAPIEKTISKTKGRPKAPQVYTIPPPKEQMAKDKVSLTCMIT
DFFPEDITVEWQWNGQPAENYKNTQPIMDTDGSYFVYSKLNVQKSNWEAGNTFTCSVLHEGLHN
HHTEKSLSHSPGK**

Figure 4. Trastuzumab scFv-Fc Fusion Amino Acid Sequences

Trastuzumab scFv- Fc fusion amino acid sequences in V_H-V_L and V_L-V_H orientations. V_H is indicated in blue, GGGGS × 3 linker in yellow, V_L in purple, Glycine linker in gray, followed by the hinge region in orange, CH2 domain in green, CH3 domain in black and 2 stop codons in red.

Trastuzumab scFv-Fc Fusion V_H-V_L:

GAGGTTCAGCTCGTCGAGTCCGGAGGCGGCCTTGTACAGCCAGGTGGTAGTTTGCGGTTGAGT
TGTGCAGCATCTGGGTTCAACATCAAGGACACCTATATCCACTGGGTTAGGCAAGCCCCAGGG
AAGGGCCTCGAATGGGTCGCTCGGATCTACCCGACGAACGGATATACTCGATATGCCGATAG
CGTAAAAGGGAGGTTCACCATCTCCGCAGATACCAGCAAGAACACAGCCTATCTGCAAATGA
ATAGCCTGAGGGCAGAAGATACTGCCGTCTACTACTGTTCTAGGTGGGGTGGGGACGGCTTCT
ACGCTATGGATTATTGGGGCCAAGGGACCTTGGTCACTGTCAGTAGTGGAGGGGGAGGCTCT
GGCGGCGGAGGCTCCGGTGGTGGCGGCAGTGATATTCAAATGACTCAGTCACCCAGTAGTCT
GTCTGCCTCAGTTGGCGACCGGGTCACTATCACCTGCCGCGCCTCTCAGGACGTCAACACAGC
TGTCGCTTGGTACCAACAAAAGCCTGGGAAAGCTCCTAAGTTGCTTATATATTCCGCGTCTTTT
TTGTATTCTGGAGTCCCTAGCAGATTCTCAGGGTCTCGCTCCGGTACTGATTTCACCCTGACCA
TATCTTCTTTGCAGCCTGAAGATTTTGCCACATATTATTGTCAGCAACATTACACAACCCCCCC
TACGTTTGGTCAAGGAACGAAGGTGGAAATTAAGGGCGTGCCCAGGGATTGTGGTTGTAAGC
CTTGCATATGTACAGTCCCAGAAGTATCATCTGTCTTCATCTTCCCCCCAAAGCCCAAGGATGT
GCTCACCATTACTCTGACTCCTAAGGTCACGTGTGTTGTGGTAGACATCAGCAAGGATGATCC
CGAGGTCCAGTTCAGCTGGTTTGTAGATGATGTGGAGGTGCACACAGCTCAGACGCAACCCCG
GGAGGAGCAGTTCAACAGCACTTTCCGCTCAGTCAGTGAACTTCCCATCATGCACCAGGACTG
GCTCAATGGCAAGGAGTTCAAATGCAGGGTCAACAGTGCAGCTTTCCCTGCCCCCATCGAGAA
AACCATCTCCAAAACCAAAGGCAGACCGAAGGCTCCACAGGTGTACACCATTCCACCTCCCA
AGGAGCAGATGGCCAAGGATAAAGTCAGTCTGACCTGCATGATAACAGACTTCTTCCCTGAA
GACATTACTGTGGAGTGGCAGTGGAATGGGCAGCCAGCGGAGAACTACAAGAACACTCAGCC
CATCATGGACACAGATGGCTCTTACTTCGTCTACAGCAAGCTCAATGTGCAAAAGAGCAACTG
GGAGGCAGGAAATACTTTCACCTGCTCTGTGTTACATGAGGGCCTGCACAACCACCATACTGA
GAAGAGTCTCTCCCACTCTCCTGGTAAATGATAG

Trastuzumab scFv-Fc Fusion V_L-V_H:

GATATTCAAATGACACAAAGTCCATCATCCCTCTCCGCCAGTGTTGGCGACAGAGTCACGATC
ACCTGCCGCGCATCCCAAGATGTTAACACTGCAGTAGCATGGTATCAGCAAAAACCCGGAAA
AGCCCCTAAGCTGCTCATATATAGCGCGTCCTTTCTTTATTCTGGCGTACCTTCCAGATTCAGC
GGATCACGCTCTGGGACGGATTTCACCTTGACAATTTCCAGTCTGCAACCTGAAGATTTCGCA
ACATACTACTGTCAACAACATTACACTACACCGCCCACTTTTGGGCAAGGGACGAAAGTAGA
AATAAAAGGAGGAGGAGGGAGTGGCGGCGGAGGCTCCGGAGGTGGAGGGTCTGAAGTGCAA
TTGGTTGAGTCCGGAGGGGGCCTCGTGCAACCCGGCGGCTCATTGCGGCTTTCTTGCGCCGCG
AGCGGATTTAATATAAAGGACACATATATCCATTGGGTAAGGCAGGCACCGGGTAAAGGACT
CGAGTGGGTCGCCAGAATATACCCGACTAACGGGTACACCCGCTACGCTGACTCAGTCAAGG
GCAGATTCACCATCTCCGCTGACACCAGTAAAAATACTGCTTATCTTCAGATGAATTCTCTTCG
GGCCGAGGACACTGCGGTTTACTACTGCTCCCGGTGGGGAGGCGACGGATTTTATGCTATGGA
TTATTGGGGCCAGGGGACTCTCGTGACTGTAAGTAGCGGCGTGCCCAGGGATTGTGGTTGTAA
GCCTTGCATATGTACAGTCCCAGAAGTATCATCTGTCTTCATCTTCCCCCCAAAGCCCAAGGAT
GTGCTCACCATTACTCTGACTCCTAAGGTCACGTGTGTTGTGGTAGACATCAGCAAGGATGAT
CCCGAGGTCCAGTTCAGCTGGTTTGTAGATGATGTGGAGGTGCACACAGCTCAGACGCAACCC
CGGGAGGAGCAGTTCAACAGCACTTTCCGCTCAGTCAGTGAACTTCCCATCATGCACCAGGAC
TGGCTCAATGGCAAGGAGTTCAAATGCAGGGTCAACAGTGCAGCTTTCCCTGCCCCCATCGAG
AAAACCATCTCCAAAACCAAAGGCAGACCGAAGGCTCCACAGGTGTACACCATTCCACCTCC
CAAGGAGCAGATGGCCAAGGATAAAGTCAGTCTGACCTGCATGATAACAGACTTCTTCCCTG
AAGACATTACTGTGGAGTGGCAGTGGAATGGGCAGCCAGCGGAGAACTACAAGAACACTCAG
CCCATCATGGACACAGATGGCTCTTACTTCGTCTACAGCAAGCTCAATGTGCAAAAGAGCAAC
TGGGAGGCAGGAAATACTTTCACCTGCTCTGTGTTACATGAGGGCCTGCACAACCACCATACT
GAGAAGAGTCTCTCCCACTCTCCTGGTAAATGATAG

Figure 5. Trastuzumab scFv-Fc Fusion Nucleotide Sequences

Trastuzumab scFv-Fc fusion nucleotide sequences scFv-Fc fusion in V_H-V_L and V_L-V_H orientations. V_H is indicated in blue, GGGGS × 3 linker in yellow, V_L in purple, Glycine linker in gray, followed by the hinge region in orange, CH2 domain in green, CH3 domain in black and 2 stop codons in red.

scFv Gene Synthesis and Golden Gate Assembly Cloning

ScFv gBlock® fragments were cloned into pTT5 vectors using Golden Gate Assembly. All scFvs were considered to be successfully cloned upon Sanger sequence verification and yield quantification via the QIAxpert®. Vector maps of full-length trastuzumab heavy chain (Figure 6A) and light chain (Figure 6B) cloned into the pTT5 vector are displayed below. Vector maps of trastuzumab in the scFv format in either the V_H-V_L orientation (Figure 7A) or the V_L-V_H orientation (Figure 7B) are also displayed below. Anti-Chk1, Anti-ApoE4, and Anti-GAPDH antibodies were constructed with the same design formats.

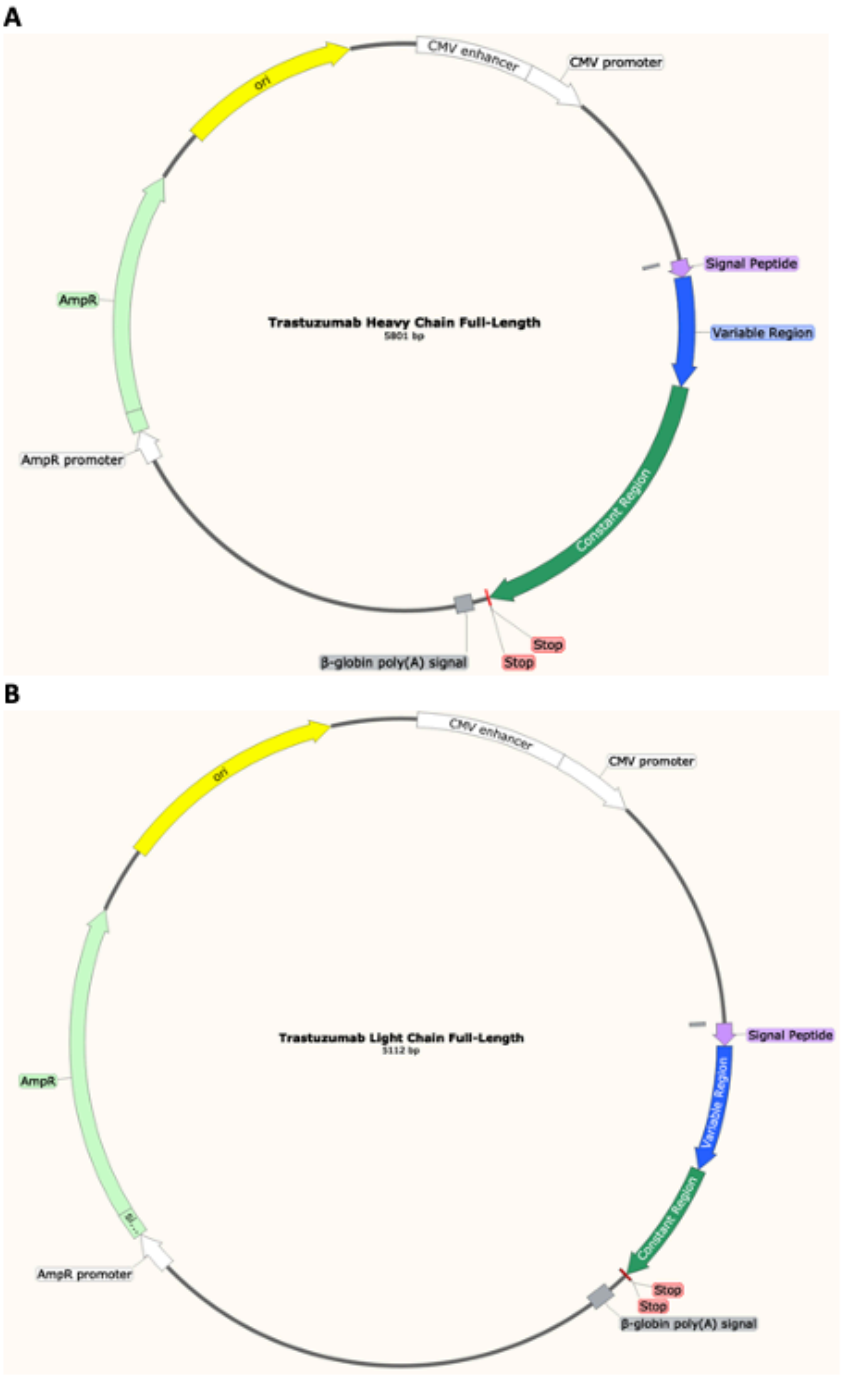


Figure 6. Vector Maps of Trastuzumab Full-Length Heavy and Light Chain

(A) Full-length trastuzumab heavy chain and (B) Full-length trastuzumab light chain cloned into pTT5 vectors containing a Kozak sequence, signal peptide sequence, and mouse IgG1 constant region.

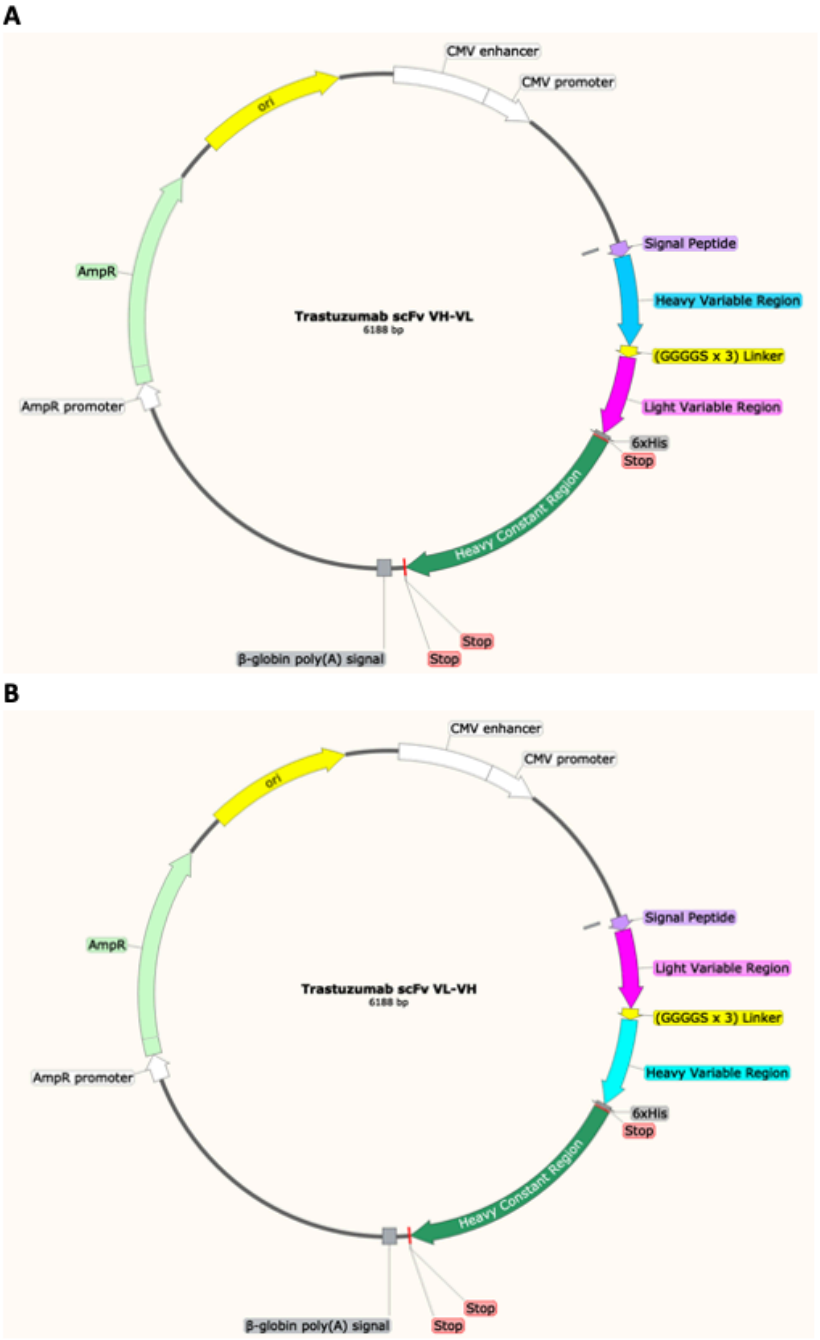


Figure 7. Vector Maps of Trastuzumab scFv V_H-V_L and V_L- V_H.

(A) ScFv trastuzumab V_H- (GGGGS x 3) -V_L- 6x-His-Tag and (B) ScFv trastuzumab V_L- (GGGGS x 3) -V_H – 6X His-Tag cloned into a pTT5 vectors containing a Kozak sequence, signal peptide sequence, and mouse IgG1 constant region.

scFv-Fc Fusion Gibson Assembly Cloning

ScFv-Fc fusion fragments were sub-cloned from the scFv vectors using Gibson Assembly. Vector maps of trastuzumab scFv-Fc fusion in the V_H-V_L orientation (Figure 8A) well as the V_L-V_H orientation (Figure 8B) are displayed below. Anti-Chk1, Anti-ApoE4, and Anti-GAPDH were constructed with the same design format. PCR images of the scFv-Fc fusion fragments that were generated are shown below in Figure 9A. Several fragments were repeated (in red) by increasing PCR cycling from 25 to 30 cycles to increase yield (Figure 9B). All scFvs were considered to be successfully cloned upon Sanger sequence verification and yield quantification via the QIAxpert®.

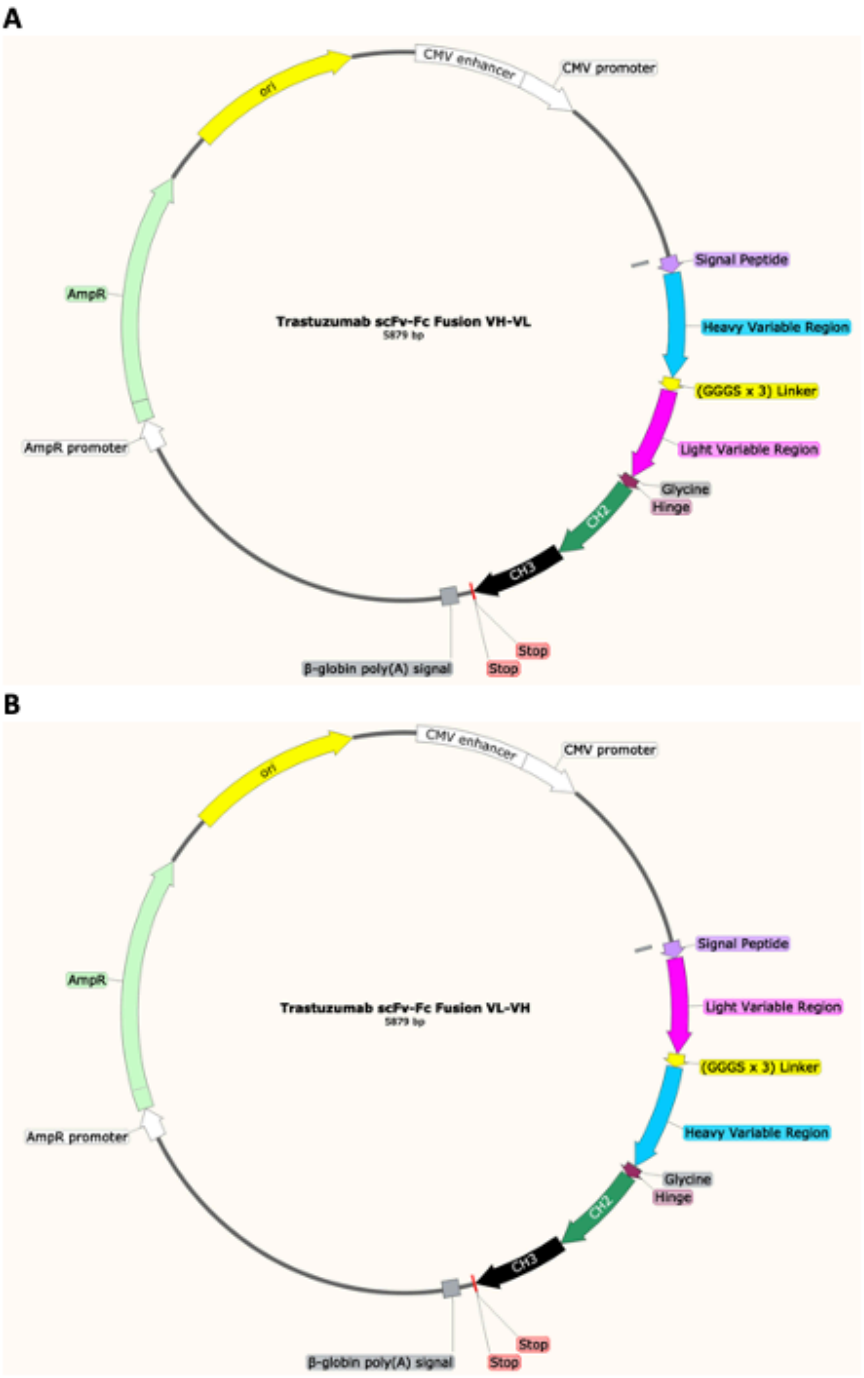


Figure 8. Vector Maps of Trastuzumab scFv-Fc Fusion V_H-V_L and V_L- V_H.

(A) ScFv-Fc fusion trastuzumab V_H- (GGGGS x 3) -V_L and (B) ScFv-Fc fusion trastuzumab V_L- (GGGGS x 3)-V_H cloned into pTT5 vectors containing a Kozak sequence, signal peptide sequence, and mouse IgG1 constant region.

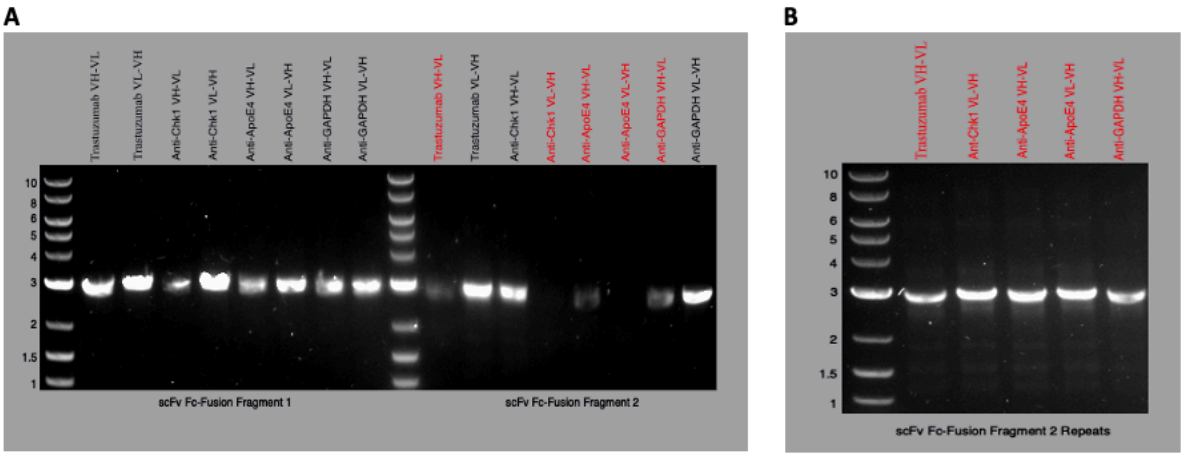


Figure 9. PCR Images of scFv-Fc Fusion Fragments

(A) PCR image of scFv-Fc fusion fragment 1 and fragment 2. In red are the ones that were repeated. (B) PCR of scFv-Fc fusion fragment 2's (in red) that were repeated using 30x cycles.

Transfection

All full-length, scFv, and scFv-Fc fusion constructs for trastuzumab, Anti-Chk1, Anti-ApoE4, and Anti-GAPDH were successfully transfected into ExpiCHO™ cells. They were harvested and concentrations (µg/ml) were determined using ForteBio's Octet® RED96 System (Table 3). Quantifications were obtained for full-length and scFv-Fc fusion antibodies. However, scFv antibodies were not quantified due to unavailability of a Ni-NTA (NTA) biosensor. Mouse IgG1was used as the standard for both full-length and scFv-Fc fusions due to unavailability of an Fc fusion standard. Antibodies seemed to express better in a certain scFv-Fc fusion orientation compared to their full-length versions.

Table 3. ForteBio's Octet® RED96 System Protein A Concentrations

Antibody	Format	Concentration (µg/ml)
Trastuzumab	Full-Length ExpiCHO™	183.5
	scFv-Fc fusion V_H-V_L	>1000
	scFv-Fc fusion V_L-V_H	629.9
Anti-Chk1	Full-Length ExpiCHO™	70.8
	scFv-Fc fusion V_H-V_L	142.4
	scFv-Fc fusion V_L-V_H	144.1
Anti-ApoE4	Full-Length ExpiCHO™	31.5
	scFv-Fc fusion V_H-V_L	304.3
	scFv-Fc fusion V_L-V_H	13.8
Anti-GAPDH	Full-Length ExpiCHO™	57.8
	scFv-Fc fusion V_H-V_L	14.3
	scFv-Fc fusion V_L-V_H	103.6

Concentrations of full-length and scFv-Fc fusion antibodies determined by ForteBio's Octet® RED96 system using a protein A biosensor.

Purification

Full-length, scFv, and scFv-fc fusion antibodies for trastuzumab, Anti-Chk1, Anti-ApoE4, and Anti-GAPDH were then purified. A sample of crude supernatant (Sup), flow through (FT), wash (W), and well as elution fractions (E1-E7) were saved for each antibody. An SDS-PAGE gel was then run for each sample (Figure 10A and 10B).

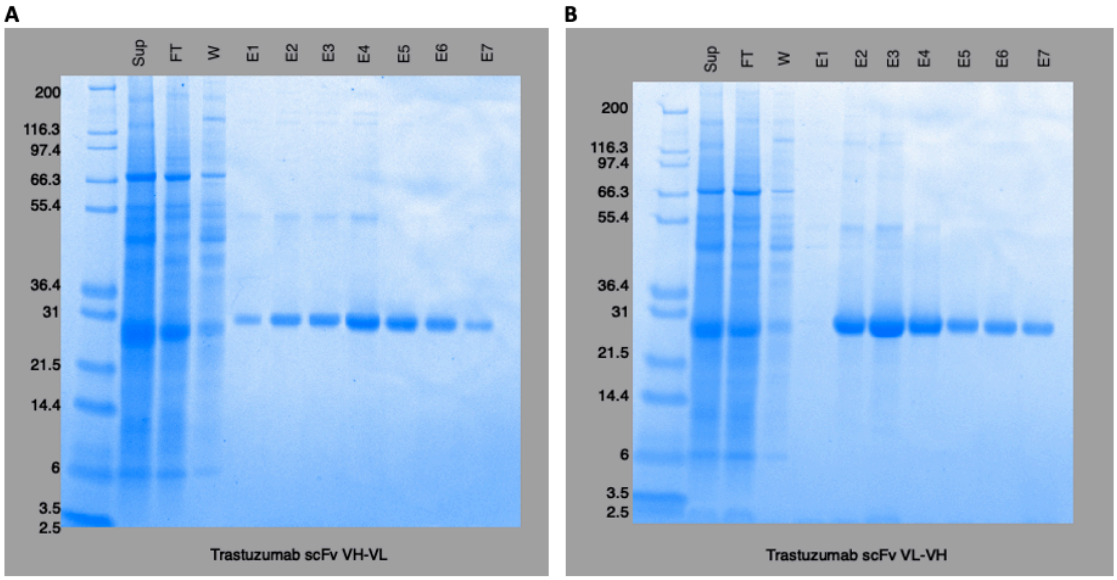


Figure 10. SDS-PAGE of Nickel Purified Trastuzumab scFv V_H-V_L and V_L-V_H

(A) Trastuzumab scFv V_H-V_L and (B) Trastuzumab scFv V_L-V_H. The first lane of both images is the crude supernatant (Sup) followed by the flow through (FT), wash (W), and 7 elution fractions (E1-E7).

Full-length antibodies for trastuzumab, Anti-Chk1, Anti-ApoE4, and Anti-GAPDH were purified using protein A. Figure 11A is a reducing SDS-PAGE gel showing the full-length heavy chains are approximately 50 kDa and the light chains are approximately 25 kDa. scFv-Fc fusion antibodies were also purified using protein A. The SDS-PAGE gel shows the molecular weight of each is approximately 54 kDa (Figure 11B). The Anti-ApoE4 V_H-V_L and Anti-GAPDH V_L-V_H formats each have two distinct bands indicating that there may have been a cleavage of the scFv from the Fc region in these formats. Lastly, scFv antibodies were purified using Nickel columns. SDS-PAGE gels indicate that the molecular weight of each is approximately 30 kDa (Figure 11C).

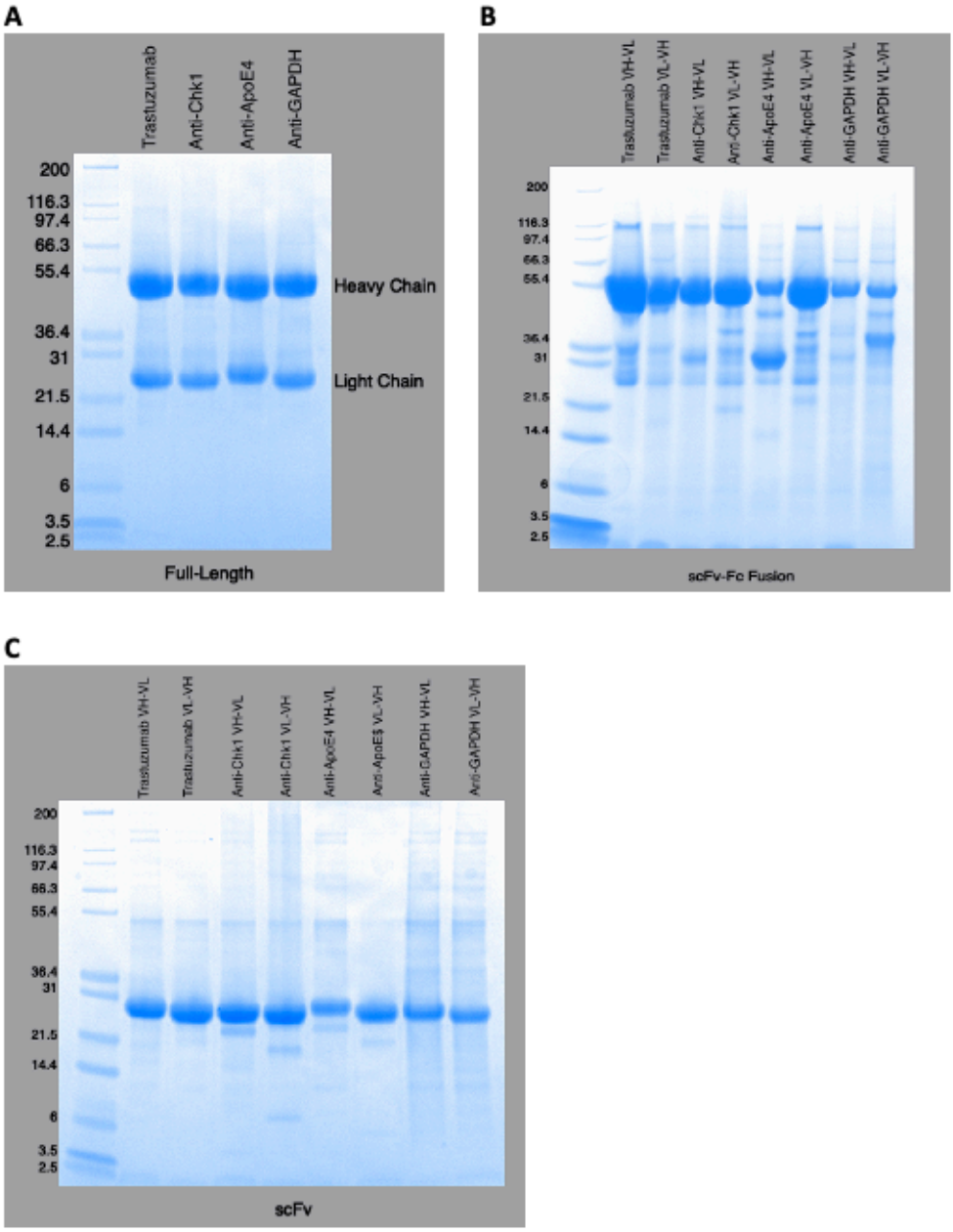


Figure 11. SDS-PAGE of Dialyzed Full-Length, scFv-Fc Fusion, and scFvs.

(A) SDS-PAGE gel of protein A purified trastuzumab, Anti-Chk1, Anti-ApoE4, and Anti-GAPDH full-length antibodies. (B) SDS-PAGE gel of protein A purified trastuzumab, Anti-Chk1, Anti-ApoE4, and Anti-GAPDH scFv-Fc fusion antibodies in either the V_H-V_L or V_L-V_H orientation. (C) SDS-PAGE gel of nickel purified trastuzumab, Anti-Chk1, Anti-ApoE4, and Anti-GAPDH scFv antibodies in either the V_H-V_L or V_L-V_H orientations.

Total Yield and Molar Yield Quantifications

After purification and dialysis, the QIAxpert® was used to determine the A_{280} concentrations of each antibody . The total yields were then calculated by multiplying the concentration (mg/ml) x total volume (ml) for a 50 ml culture. The total yields were then multiplied by 20 to determine the total yield for a 1L culture in order to compare to the previous HEK293 expressed antibodies (Table 4).

Table 4. QIAxpert® A_{280} Antibody Concentrations and Total Yields

Antibody	Format	Concentration (mg/ml)	Total Volume (ml)	Total Yield (mg/L)
Trastuzumab	Full-Length ExpiCHO™	3.8	5.5	421
	scFv V_H-V_L	1	2.5	48
	scFv V_L-V_H	1.4	6	167
	scFv-Fc fusion V_H-V_L	7.5	6.5	972
	scFv-Fc fusion V_L-V_H	2.03	6	244
Anti-Chk1	Full-Length HEK293	-	-	41.4
	Full-Length ExpiCHO™	2.6	4.5	229
	scFv V_H-V_L	1	3.5	72
	scFv V_L-V_H	1.4	4.5	122
	scFv-Fc fusion V_H-V_L	1.75	4	140
	scFv-Fc fusion V_L-V_H	2.87	4	230
Anti-ApoE4	Full-Length HEK293	-	-	36
	Full-Length ExpiCHO™	3.5	3.5	242
	scFv V_H-V_L	0.8	0.9	14
	scFv V_L-V_H	0.7	7	95
	scFv-Fc fusion V_H-V_L	1.17	4.5	105
	scFv-Fc fusion V_L-V_H	4.15	4	332
Anti-GAPDH	Full-Length HEK293	-	-	19.8
	Full-Length ExpiCHO™	3.1	3.5	218
	scFv V_H-V_L	1	3.5	71
	scFv V_L-V_H	1.1	1.5	34
	scFv-Fc fusion V_H-V_L	0.66	4	53
	scFv-Fc fusion V_L-V_H	1.06	4.5	95

Concentrations and total yields of full-length, scFv, and scFv-Fc fusion antibodies determined by using the QIAxpert® A_{280}.

Table 4 displays the total yields (mg/L) of trastuzumab, Anti-Chk1, Anti-ApoE4, and Anti-GAPDH in the full-length form expressed in either HEK293 or ExpiCHO™, the scFv format in either the V_H-V_L or V_L-V_H orientation, and the scFv-Fc fusion format in either the V_H-V_L or V_L-V_H orientation. As a result, all of the full-length ExpiCHO™ antibodies yielded higher than the full-length antibodies expressed in HEK293. For trastuzumab scFv formats, the V_L-V_H orientation yielded the highest (167 mg/L). On the contrary, for the scFv-Fc fusion format the V_H-V_L orientation yielded the highest (972 mg/L). The scFv-Fc fusion V_H-V_L antibody also yielded the most compared to all other antibody formats for trastuzumab.

For Anti-Chk1 in both the scFv and scFv-Fc fusion formats the V_L-V_H orientation yielded the most (122 mg/L and 230 mg/L respectively). Overall, the scFv-Fc fusion V_L-V_H orientation yielded the highest compared to all other antibody formats for Anti-Chk1. Similarly, for Anti-ApoE4 in both the scFv and scFv-Fc fusion formats the V_L-V_H orientation yielded the most (95 mg/L and 332 mg/L respectively). Overall, the scFv- Fc fusion V_L-V_H orientation yielded the highest compared to all other antibody formats for Anti-ApoE4. Lastly, for Anti-GAPDH in the scFv format the V_H-V_L orientation yielded most (71 mg/L) and for the scFv-Fc fusion format the V_L-V_H orientation yielded the highest (95 mg/L). Overall, for Anti-GAPDH the full-length construct expressed in ExpiCHO™ yielded the most (218 mg/L).

The molar yields of each antibody format were then determined in order to better compare the dimeric full-length and scfv-Fc fusion antibodies to the monomeric scFv forms. This was calculated by dividing the protein yield (g/L) by the molecular weight (Daltons) to determine the amount of nmoles (Table 5). Molecular weight of each antibody was determined using Expasy's ProtParam tool.

Table 5. Molar Yield Calculations

Antibody	Format	Protein Yield (g/L)	Molecular Weight (Daltons)	Molar Yield (nmoles)
Trastuzumab	Full-Length ExpiCHO™	0.421	144,944	2,904
	scFv V_H-V_L	0.048	26,611	1,803
	scFv V_L-V_H	0.167	26,611	6,275
	scFv-Fc fusion V_H-V_L	0.972	103,031	9,434
	scFv-Fc fusion V_L-V_H	0.244	103,031	2,368
Anti-Chk1	Full-Length HEK293	0.041	145,767	284
	Full-Length ExpiCHO™	0.231	145,767	1,577
	scFv V_H-V_L	0.072	27,023	2,664
	scFv V_L-V_H	0.122	27,023	4,514
	scFv-Fc fusion V_H-V_L	0.141	103,836	1,348
	scFv-Fc fusion V_L-V_H	0.231	103,836	2,215
Anti-ApoE4	Full-Length HEK293	0.036	143,793	250
	Full-Length ExpiCHO™	0.242	143,793	1,682
	scFv V_H-V_L	0.014	26,035	537
	scFv V_L-V_H	0.095	26,035	3,648
	scFv-Fc fusion V_H-V_L	0.105	101,862	1,030
	scFv-Fc fusion V_L-V_H	0.332	101,862	3,259
Anti-GAPDH	Full-Length HEK293	0.019	145,649	137
	Full-Length ExpiCHO™	0.218	145,649	1,496
	scFv V_H-V_L	0.071	26,964	2,633
	scFv V_L-V_H	0.034	26,964	1,260
	scFv-Fc fusion V_H-V_L	0.053	103,718	511
	scFv-Fc fusion V_L-V_H	0.095	103,718	915

Molar yield calculations of each antibody format.

Table 5 displays the molar yields (nmoles) of trastuzumab, Anti-Chk1, Anti-ApoE4, and Anti-GAPDH antibodies. As a result, all of the full-length ExpiCHO™ antibodies yielded higher than the full-length antibodies expressed in HEK293. For trastuzumab scFv formats, the V_L-V_H orientation yielded the highest (6,275 nmoles). On the contrary, for the scFv-Fc fusion format the V_H-V_L orientation yielded the highest (9,434 nmoles). The scFv-Fc fusion V_H-V_L antibody also yielded the most compared to all other antibody formats for trastuzumab.

For Anti-Chk1 in both the scFv and scFv-Fc fusion formats the V_L-V_H orientation yielded the most (4,514 nmoles and 2,215 nmoles respectively). Overall, the scFv V_L-V_H orientation yielded the highest compared to all other antibody formats for Anti-Chk1. Similarly, for Anti-ApoE4 in both the scFv and scFv-Fc fusion formats the V_L-V_H orientation yielded the most (3,648 nmoles and 3,259 nmoles respectively). Overall, the scFv V_L-V_H orientation yielded the highest compared to all other antibody formats for Anti-ApoE4. Lastly, for Anti-GAPDH in the scFv format the V_H-V_L orientation yielded the most (2,633 nmoles) and for the scFv-Fc fusion format the V_L-V_H orientation yielded the highest (915 nmoles). Overall, for Anti-GAPDH the scFv V_H-V_L orientation yielded the most.

Western Blot

Full-length antibodies for trastuzumab, Anti-Chk1, Anti-ApoE4, and Anti-GAPDH were then subjected to western blot analysis to determine functionality. Each antibody was normalized to 1 mg/ml and added at a 1:1000 dilution; approximately 0.007 nmoles were added for each full-length antibody, 0.009nmoles for each scFv-Fc fusion, and 0.04 nmoles for each scFv.

Figure 12 displays the western blot for the trastuzumab antibodies. The molecular weight of trastuzumab is predicted to be 185 kDa. Figure 12A displays transfected hHer2 (+) cell lysate and Figure 12B displays K562 (-) and SKBR3 (+) endogenous lysates with all 5 primary antibody types. Unfortunately, none of the trastuzumab antibodies worked by either overexpression or endogenous cell models. Panel 6 in Figure 12A is the control primary antibody (Myc-Tag (9B11) Mouse mAb) for the overexpression cell model. There is a band present around 185 kDa which indicates that the overexpression transfection, western blot transfer, and development were successful. Panel 6 in Figure 12B is the control primary antibody (β-Actin (E4D9Z) Mouse mAb) for the endogenous cell model. There is a band present at approximately 45 kDa (the molecular weight of β-Actin) which indicates that the endogenous cell lines were expressed.

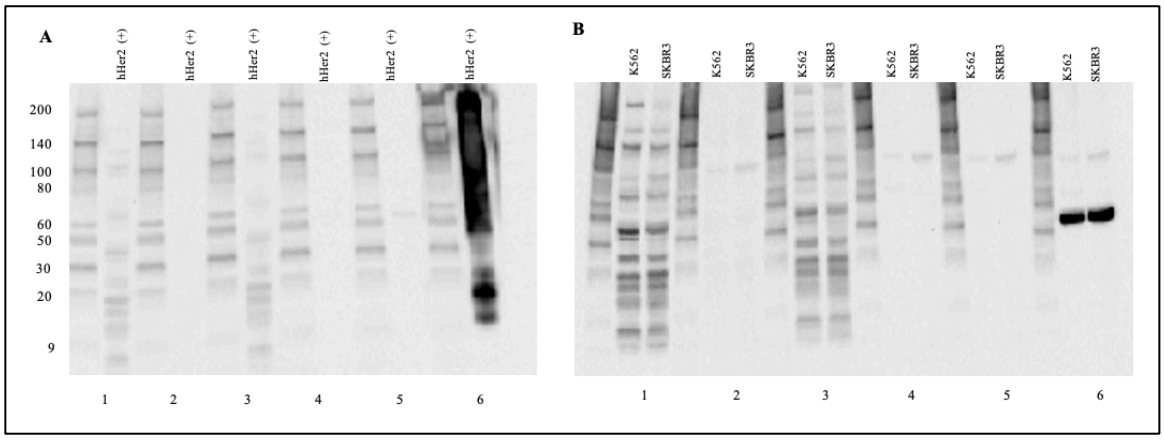


Figure 12. Western Blot Images of Trastuzumab Antibodies

(A) Transfected hHer2 (+) cell lysate and (B) K562(-) and SKBR3(+) cell lysates. Panel 1 in both is using full-length trastuzumab ExpiCHO™, Panel 2 trastuzumab scFv-Fc fusion V_H-V_L, Panel 3 trastuzumab scFv-Fc fusion V_L-V_H, Panel 4 trastuzumab scFv V_H-V_L, and Panel 5 trastuzumab scFv V_L-V_H. Panel 6 (A) Myc-Tag (9B11) Mouse mAb and Panel 6 (B) β-Actin (E4D9Z) Mouse mAb.

Figure 13 is the western blot for the Anti-Chk1 antibodies. The molecular weight of Anti-Chk1 is expected to be approximately 56 kDa. Figure 13A displays transfected hChk1 (+) cell lysate and Figure 13B displays A172 (+) and HeLa (++) endogenous lysates with all 6 primary antibody types. Anti-Chk1 antibodies worked with both overexpression and endogenous cell models. The full-length antibodies seem to be similar in intensity. However, a strong background band is present in both scFv-Fc fusion antibodies around 40 kDa. In addition, the scFv formats appear to have a weaker signal. Panel 7 in Figure 13A and B are the control primary antibodies Myc-Tag (9B11) Mouse mAb and β-Actin (E4D9Z) Mouse mAb respectively. Both have bands present at the correct molecular weights indicating that the cell lines were expressed.

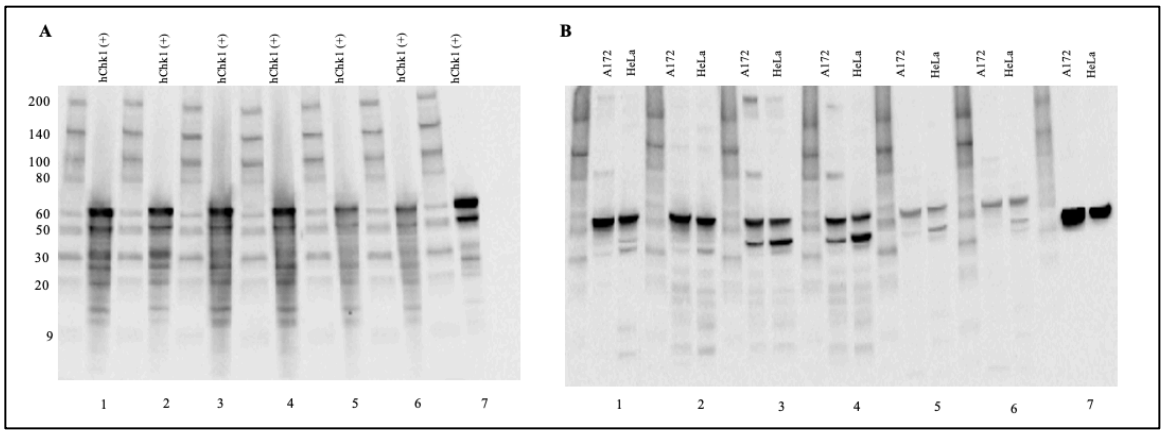


Figure 13. Western Blot Images of Anti-Chk1 Antibodies

(A) Transfected hChk1 (+) cell lysate and (B) A172(+) and HeLa (++) cell lysates. Panel 1 in both is using Anti-Chk1 full-length HEK293, Panel 2 Anti-Chk1 full-length ExpiCHO™, Panel 3 Anti-Chk1 scFv-Fc fusion V_H-V_L, Panel 4 Anti-Chk1 scFv-Fc fusion V_L-V_H, Panel 5 Anti-Chk1 scFv V_H-V_L, and Panel 6 Anti-Chk1 scFv V_L-V_H. Panel 7 (A) Myc-Tag (9B11) Mouse mAb and Panel 7 (B) β-Actin (E4D9Z) Mouse mAb .

Figure 14 is the western blot analysis for the Anti-ApoE4 antibodies. The molecular weight of Anti-ApoE4 is expected to be approximately 35 kDa. Figure 14A displays transfected hApoE4 (+) cell lysate and Figure 14B displays 293T (+) and HepG2 (++) endogenous lysates with all 6 primary antibody types. All antibodies worked with overexpression cell models. The full-length antibodies seem to be similar in intensity. However, the scFv-Fc fusion and scFv formats seem to have a stronger and cleaner signal in one orientation over the other. However, it is unclear if the antibodies worked with endogenous cell models due to high background and multiple non-specific bands. Panel 7 in Figure 14A and B are the control primary antibodies Myc-Tag (9B11) Mouse mAb and β-Actin (E4D9Z) Mouse mAb respectively. Both have bands present at the correct molecular weights indicating that the cell lines were expressed.

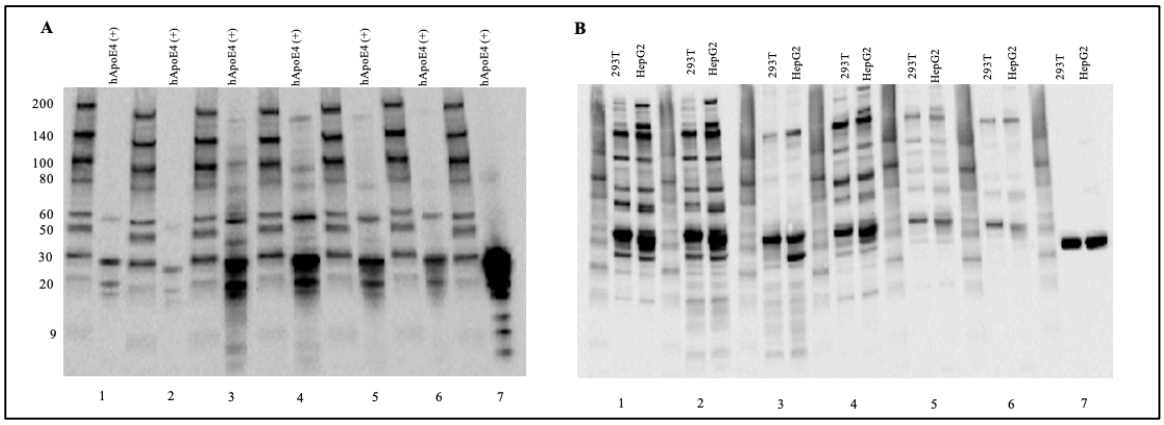


Figure 14. Western Blot Images of Anti-ApoE4 Antibodies

(A) Transfected hApoE4 (+) cell lysate and (B) 293T(+) and HepG2(++) cell lysates. Panel 1 in both is using Anti-ApoE4 full-length HEK293, Panel 2 Anti-ApoE4 full-length ExpiCHO™, Panel 3 Anti-ApoE4 scFv-Fc fusion V_H-V_L, Panel 4 Anti-ApoE4 scFv-Fc fusion V_L-V_H, Panel 5 Anti-ApoE4 scFv V_H-V_L, and Panel 6 Anti-ApoE4 scFv V_L-V_H. Panel 7 (A) Myc-Tag (9B11) Mouse mAb and Panel 7 (B) β-Actin (E4D9Z) Mouse mAb.

Figure 15 is the western blot for the Anti-GAPDH antibodies. The molecular weight of Anti-GAPDH is expected to be approximately 37 kDa. Figure 15A displays transfected hGAPDH (+) cell lysate and Figure 15B displays A172 (+) and HeLa (++) endogenous lysates with all 6 primary antibody types. Anti-GAPDH antibodies worked with both overexpression and endogenous cell models. The full-length antibodies seem to be similar in intensity. However, the scFv-Fc fusion and scFv formats seem to have a stronger and cleaner signal in one orientation over the other. Panel 7 in Figure 15A and B are the control primary antibodies Myc-Tag (9B11) Mouse mAb and β-Actin (E4D9Z) Mouse mAb respectively. Both have bands present at the correct molecular weights indicating that the cell lines were expressed.

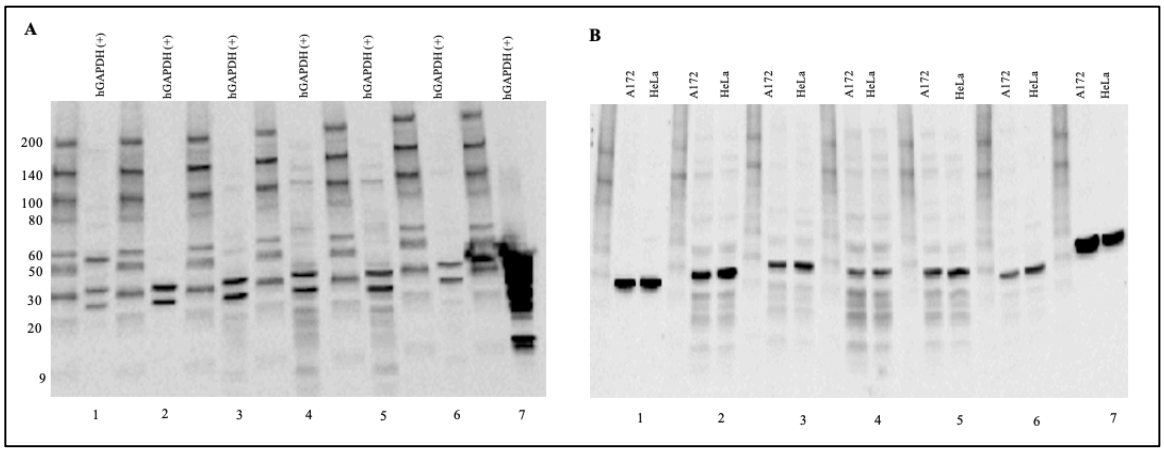


Figure 15. Western Blot Images of Anti-GAPDH Antibodies

(A) Transfected hGAPDH (+) cell lysate and (B) A172(+) and HeLa(++) cell lysates. Panel 1 in both is using Anti-GAPDH full-length HEK293, Panel 2 Anti-GAPDH full-length ExpiCHO™, Panel 3 Anti-GAPDH scFv-Fc fusion V_H-V_L, Panel 4 Anti-GAPDH scFv-Fc fusion V_L-V_H, Panel 5 Anti-GAPDH scFv V_H-V_L, and Panel 6 Anti-GAPDH scFv V_L-V_H. Panel 7 (A) Myc-Tag (9B11) Mouse mAb and Panel 7 (B) β-Actin (E4D9Z) Mouse mAb.

Flow Cytometry

Full-length antibodies for trastuzumab, Anti-Chk1, Anti-ApoE4, and Anti-GAPDH were then subjected to flow cytometry analysis to further determine functionality. Each antibody was normalized to 1 mg/ml. Three different dilutions, 0.32 µg/ml, 1.28 µg/ml, and 5.12 µg/ml were tested in order to determine optimal concentrations and molar yield amounts (pmoles) were calculated. Signal to noise ratios were determined by dividing the mean fluorescence intensity (MFI) of the positive cell line (++) by the MFI of the negative (-) or lower expressing cell line (+). A ratio above 2 is indicative of a passing sample.

Table 6 indicates the optimal concentrations (µg/ml) and pmoles for each trastuzumab antibody type as well as their signal to noise ratios. For both the scFv and scFv-Fc fusion formats the V_H-V_L orientation had the highest S/N ratios (41 and 49 respectively). The scFv-Fc fusion format V_H-V_L had the highest S/N ratio overall with the least amount of pmoles used.

Table 6. Trastuzumab Flow Cytometry Optimal Concentrations and S/N Ratios

Trastuzumab Antibody Type	Concentration (µg/ml)	pmoles	S/N Ratio
Full-Length ExpiCHO™	1.28	8.83	27
scFv V_H-V_L	1.28	48.1	41
scFv V_L-V_H	1.28	48.1	26
scFv-Fc fusion V_H-V_L	0.32	3.11	49
scFv-Fc fusion V_L-V_H	5.12	49.7	26

Trastuzumab antibody types, optimal concentrations (µg/ml), pmoles, and signal to noise (S/N) ratios.

Figure 16 displays the histogram analysis for the 5 different trastuzumab antibody formats. The red and blue peaks are unstained K562 (-) and unstained SKBR3 (+) cells.

The orange and light green peaks are K562 with the appropriate secondary antibody only and the SKBR3 with the appropriate secondary antibody only. The dark green peak is K562 with the appropriate primary and secondary antibody. The pink peak is SKBR3 with the appropriate primary and secondary antibody. Histograms for all 5 antibody types indicate a positive shift in MFI between the unstained, secondary only, and negative cell line compared to the positive cell line.

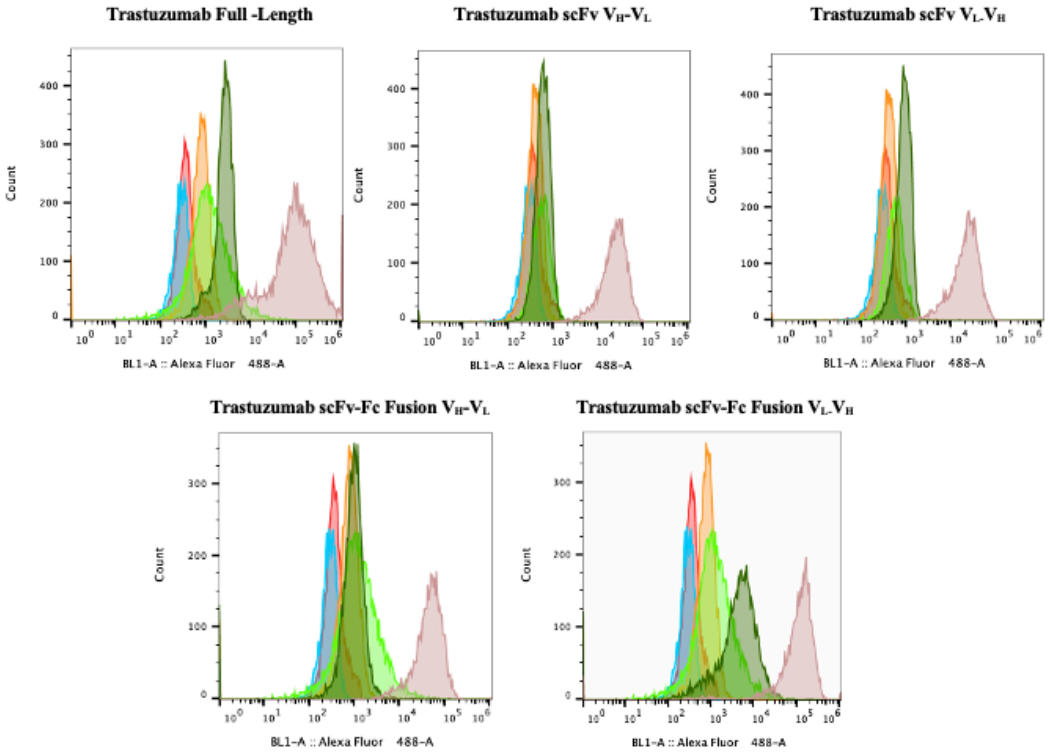


Figure 16. Flow Cytometry Histograms of Trastuzumab Antibodies

Red peak is unstained K562 (-) cells, blue peak is unstained SKBR3 (+) cells, orange peak is K562 with the appropriate secondary antibody only, light green peak is SKBR3 with the appropriate secondary antibody only, dark green peak is K562 with the appropriate primary and secondary antibody, pink peak is SKBR3 with the appropriate primary and secondary antibody.

Table 7 indicates the optimal concentrations (μg/ml) and pmoles for each Anti-Chk1 antibody type as well as their signal to noise ratios. For the full-length format the HEK293 and ExpiCHO™ had the same S/N ratios (1.2). For the scFv format the V_H-V_L orientation had the highest S/N ratio (1.31). For the scFv-Fc fusion format both the V_H-V_L and V_L-V_H had the same S/N ratios (0.96). However, no antibody formats were considered positive due to their S/N ratios being below 2.

Table 7. Anti-Chk1 Flow Cytometry Optimal Concentrations and S/N Ratios

Anti-Chk1 Antibody Type	Concentration (μg/ml)	pmoles	S/N Ratio
Full-Length HEK293	0.32	2.21	1.2
Full-Length ExpiCHO™	0.32	2.21	1.2
scFv V_H-V_L	5.12	189	1.31
scFv V_L-V_H	1.28	47.4	1.27
scFv-Fc fusion V_H-V_L	0.32	3.08	0.96
scFv-Fc fusion V_L-V_H	0.32	3.08	0.96

Anti-Chk1 antibody types, optimal concentrations (μg/ml), pmoles, and signal to noise (S/N) ratios.

Figure 17 displays the histogram analysis for the 6 different Anti-Chk1 antibody formats. The red and blue peaks are unstained A172 (+) and unstained HeLa (++) cells. The orange and light green peaks are A172 with the appropriate secondary antibody only and HeLa with the appropriate secondary antibody only. The dark green peak is A172 with the appropriate primary and secondary antibody. The pink peak is HeLa with the appropriate primary and secondary antibody. Histograms for all 6 antibody types were negative due to a lack in significant MFI shift between the unstained, secondary only, and lower expressing cell line compared to the higher expressing cell line.

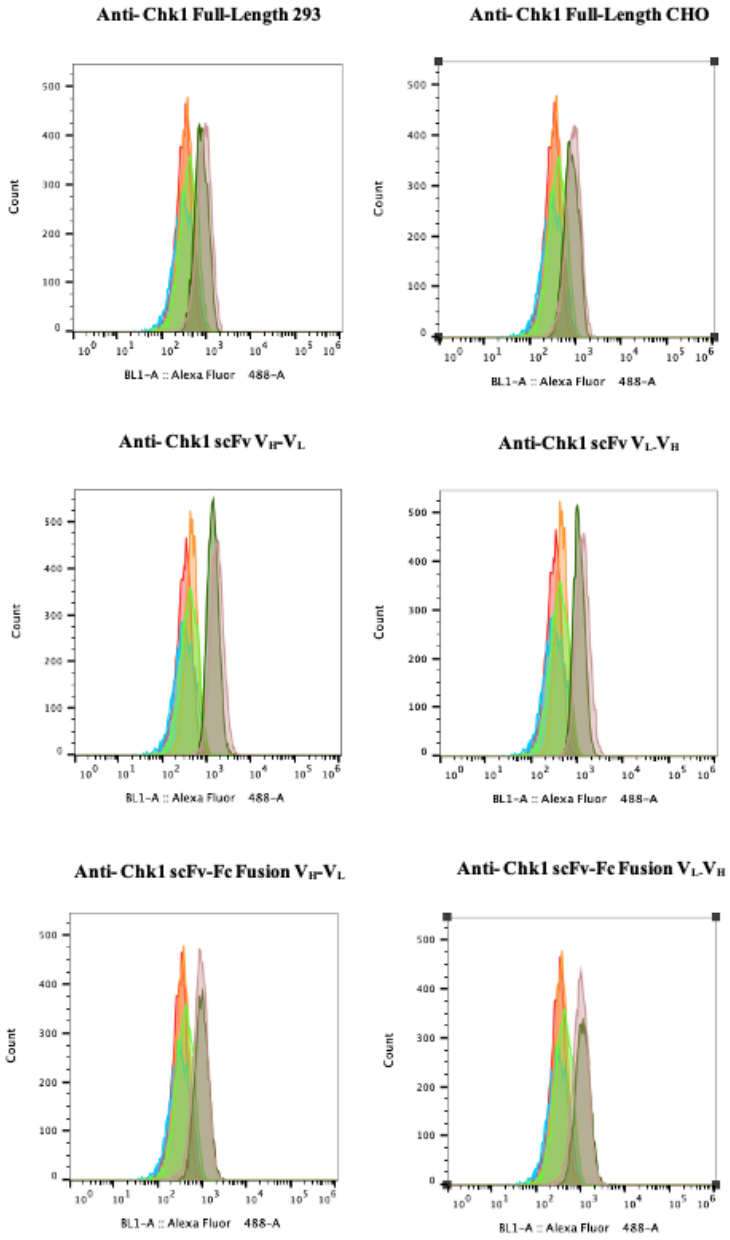


Figure 17. Flow Cytometry Histograms of Anti-Chk1 Antibodies

Red peak is unstained A172 (+) cells, blue peak is unstained HeLa (++) cells, orange peak is A172 with the appropriate secondary antibody only, light green peak is HeLa with the appropriate secondary antibody only, dark green peak is A172 with the appropriate primary and secondary antibody, pink peak is HeLa with the appropriate primary and secondary antibody.

Table 8 indicates the optimal concentrations (μg/ml) and pmoles for each Anti-ApoE4 antibody type as well as their signal to noise ratios. For the full-length format the HEK293 and ExpiCHO™ had similar S/N ratios. For the scFv format the V_H-V_L orientation had the highest S/N ratios (2.22). For the scFv-Fc fusion format the V_L-V_H orientation had the highest S/N ratio (2.37). Overall, the scFv-Fc fusion V_L-V_H format had the highest S/N ratio.

Table 8. Anti-ApoE4 Flow Cytometry Optimal Concentrations and S/N Ratios

Anti-ApoE4 Antibody Type	Concentration (μg/ml)	pmoles	S/N Ratio
Full-Length HEK293	0.32	2.23	2.06
Full-Length ExpiCHO™	0.32	2.23	2.08
scFv V_H-V_L	5.12	197	2.22
scFv V_L-V_H	0.32	12.3	1.76
scFv-Fc fusion V_H-V_L	0.32	3.14	2.06
scFv-Fc fusion V_L-V_H	1.28	12.6	2.37

Anti-ApoE4 antibody types, optimal concentrations (μg/ml), pmoles, and signal to noise (S/N) ratios.

Figure 18 displays the histogram analysis for the 6 different Anti-ApoE4 antibody formats. The red and blue peaks are unstained 293T (+) and unstained HepG2 (++) cells. The orange and light green peaks are 293T cells with the appropriate secondary antibody only and HepG2 with the appropriate secondary antibody only. The dark green peak is 293T with the appropriate primary and secondary antibody. The pink peak is HepG2 with the appropriate primary and secondary antibody. Histograms for all 6 antibody types indicate a positive shift in MFI between the unstained and secondary only cell lines compared to the positive cell line. There is also a slight positive shift between the lower expressing cell line and the higher expressing cell line.

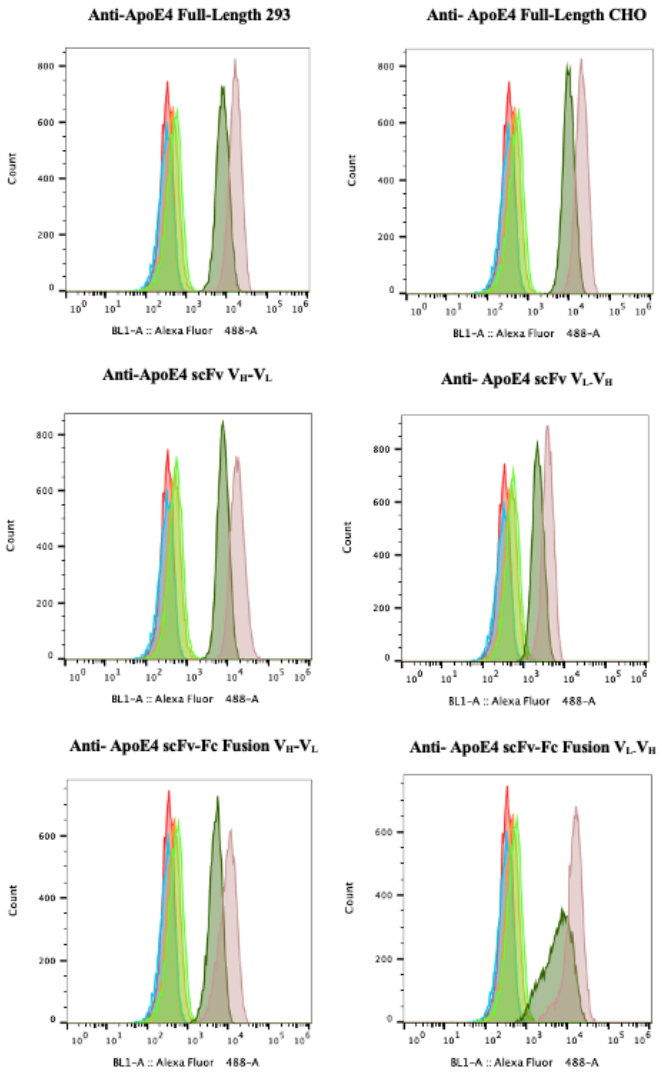


Figure 18. Flow Cytometry Histograms of Anti-ApoE4 Antibodies

Red peak is unstained 293T (+) cells, blue peak is unstained HepG2 (++) cells, orange peak is 293T with the appropriate secondary antibody only, light green peak is HepG2 with the appropriate secondary antibody only, dark green peak is 293T with the appropriate primary and secondary antibody, pink peak is HepG2 with the appropriate primary and secondary antibody.

Table 9 indicates the optimal concentrations (μg/ml) and pmoles for each Anti-GAPDH antibody type as well as their signal to noise ratios. For the full-length format the ExpiCHO™ antibody had the highest S/N ratio (1.74). For the scFv format the V_L-V_H orientation had the highest S/N ratios (2.16). For the scFv-Fc fusion format the V_H-V_L orientation had the highest S/N ratio (2.19). Overall, the scFv-Fc fusion V_H-V_L format had the highest S/N ratio.

Table 9. Anti-GAPDH Flow Cytometry Optimal Concentrations and S/N Ratios

Anti-GAPDH Antibody Type	Concentration (μg/ml)	pmoles	S/N Ratio
Full-Length HEK293	5.12	35.2	1.68
Full-Length ExpiCHO™	5.12	35.2	1.74
scFv V_H-V_L	1.28	47.5	2.08
scFv V_L-V_H	5.12	191	2.16
scFv-Fc fusion V_H-V_L	1.28	12.3	2.19
scFv-Fc fusion V_L-V_H	5.12	49.4	1.6

Anti-GAPDH antibody types, optimal concentrations (μg/ml), pmoles, and signal to noise (S/N) ratios.

Figure 19 displays the histogram analysis for the 6 different Anti-GAPDH antibody formats. The red and blue peaks are unstained A172 (+) and unstained HeLa (++) cells. The orange and light green peaks are A172 cells with the appropriate secondary antibody only and HeLa with the appropriate secondary antibody only. The dark green peak is A172 with the appropriate primary and secondary antibody. The pink peak is HeLa with the appropriate primary and secondary antibody. Histograms for all 6 antibody types indicate a positive shift in MFI between the unstained and secondary only cell lines compared to the positive cell line. There is also a slight positive shift between the lower expressing cell line and the higher expressing cell line.

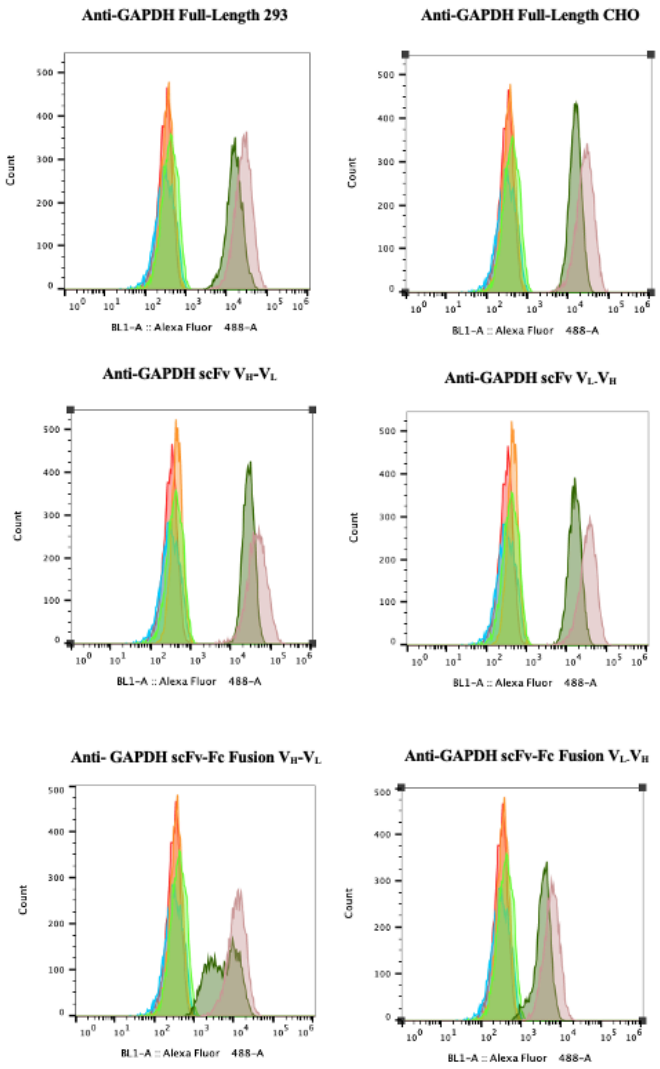


Figure 19. Flow Cytometry Histograms of Anti-GAPDH Antibodies

Red peak is unstained A172 (+) cells, blue peak is unstained HeLa (++) cells, orange peak is A172 with the appropriate secondary antibody only, light green peak is HeLa with the appropriate secondary antibody only, dark green peak is A172 with the appropriate primary and secondary antibody, pink peak is HeLa with the appropriate primary and secondary antibody.

Chapter IV.

Discussion

The main purpose of this study was to determine if expression and function could be improved by changing an antibody's structure. Three mouse monoclonal antibodies were selected from Cell Signaling Technologies™ catalog that were considered high value but low expressing (Anti-Chk1, Anti-ApoE4, and Anti-GAPDH). In addition, trastuzumab was selected as a control antibody because it has been generated by others and the DNA and protein sequences could be fully described. Due to the sequence propriety of the other antibodies those sequences are not included in this thesis. Full-length conventional antibody formats were compared to their scFv and scFv-Fc fusion forms in either the V_H-V_L or V_L-V_H orientations. The expression levels of the full-length antibodies were also compared in two separate expression systems.

The HEK293 and ExpiCHO™ systems are similar in that each is a serum-free, suspension cell culture that is amenable to standardized processes. In each of these cases, the antibody was expressed from cotransfection of two plasmids: one plasmid encoding the heavy chain gene and one plasmid encoding the light chain gene. For each antibody tested, the yields were significantly greater using the ExpiCHO™ system. Neither system was optimized for these experiments and it may simply be that the ExpiCHO™ system performed better based on previous efforts to improve culture conditions. Regardless, these data support the recommendation to convert from the HEK293 system to the ExpiCHO™ based system for increased yields.

The next comparison was made between the antibody formats. It was determined that each antibody exhibited a decrease in total yield when converted to the scFv format

(Table 4). However, on a molar mass basis, most antibodies showed an increase in yield when converted to the scFv format compared to the full-length and scFv-Fc fusion formats (Table 5). Also, regarding orientation, most scFv antibodies yielded higher in the V_L-V_H orientation with the exception of Anti-GAPDH. On the contrary, it was determined that most antibodies displayed an increase in total yield when converted to the scFv-Fc fusion format compared to full-length and scFv formats (Table 4). Similarly, on a molar mass basis, most antibodies demonstrated an increase in yield when compared to their full-length versions (Table 5). Most scFv-Fc fusion antibodies yielded highest in the V_L-V_H orientation except for trastuzumab.

The potency of these molecules varied by assay. For trastuzumab, western blot analysis indicated that all antibody formats were not functional using both transfected and endogenous cell models (Figure 12). This could be due to linearization of the protein and improper epitope binding. Based on literature it seems that the binding region for trastuzumab encompasses three loop regions of Her2 formed by residues 557–561, 570–573, and the base loop 593–603 (Cho *et al*, 2003). This protein structure indicates that the epitope may be conformational in nature. This is further proven by all antibody formats being functional by flow cytometry analysis (Figure 16). The format that had the highest S/N ratio as well as the least amount of pmoles used was the scFv-Fc fusion V_H-V_L antibody (Table 6). The absence of published western blots with trastuzumab is indirect evidence that the Her2 epitope is conformationally dependent.

For Anti-Chk1, all antibody formats worked by western blot analysis using transfected as well as endogenous cell models. However, both Fc fusions formats had a background band present around 40 kDa (Figure 13). This could be due to alternative

splicing or cleavage of the scFv from the Fc region. It appears that the scFvs were less potent than the other formats as indicated by fainter bands. Further development of binding conditions would likely improve the images. On the contrary, all antibody formats were not functional by flow cytometry analysis (Table 7 and Figure 17). This could also be due to improper epitope binding. It is unknown exactly which epitope was used to create this antibody; however, it may have been linear due to the antibodies failure to function in flow cytometry but success in western blot assays.

For Anti-ApoE4, all antibody formats worked by western blot analysis using transfected cell models. The scFv-Fc fusions and scFvs appeared to be more potent than the full-length antibodies due to stronger bands. It is unclear whether all antibody formats worked by western blot analysis using endogenous cell models due to high background and multiple non-specific bands being present (Figure 14). However, all antibody formats were functional by flow cytometry analysis (Figure 18). This difference in functional assays could be due to the fact that the antigen used to create this antibody encompasses an α-helix region (127-132) of the Anti-ApoE4 protein, according to the protein data bank (pdb.org). This protein structure indicates that the antibody may work better in a conformational assay such as flow cytometry rather than western blot. The format that had the highest S/N ratio in flow cytometry was the scFv Fc-fusion V_L-V_H antibody (Table 8). The secondary antibody in flow cytometry had differing levels of background contributing to fluorescence. It is possible that testing other sources of the detection antibody would enable the antibody and scFv variants to produce a better signal to noise value.

For Anti-GAPDH, western blot analysis indicated that all antibody formats were functional using overexpression and endogenous cell models (Figure 15). All antibodies seemed to be similar in intensity. However, each antibody format seemed to have a preference in orientation. All antibody formats were also functional by flow cytometry analysis (Figure 19) with the scFv-Fc fusion V_H-V_L format having the highest S/N ratio (Table 9). The antigen used to create this antibody encompasses a β strand and loop region (76-82) of the Anti-GAPDH protein, according to the protein data bank (pdb.org). This protein structure indicates that the antibody may work well in both western blot and flow cytometry.

Overall, both full-length antibodies seemed to function similarly in both western blot and flow cytometry analysis. Most scFv antibodies seemed to prefer one orientation over the other in both western blot and flow cytometry analysis. The stronger and cleaner western blot band also corresponded to the higher S/N ratio in the flow cytometry analysis. However, more pmoles were required for signal intensity compared to the full-length versions. Most scFv-Fc fusions antibodies also seemed to prefer one orientation over the other in both western blot and flow cytometry. The stronger and cleaner western blot band also corresponded to the higher S/N ratio in flow cytometry. On the contrary, most scFv-Fc fusions required fewer pmoles compared to their full-length and scFv versions.

In future experiments, it would be interesting to compare all antibody formats expressed using HEK293 cells in order to determine if all formats express better in ExpiCHO™ or HEK293 systems. It would also be interesting to express all antibody formats in other systems, such as Escherichia coli (*E. coli*), to see if expression levels

differ in other systems. In addition, it would be noteworthy to test trastuzumab and Anti-ApoE4 by western blot in non-denaturing conditions to determine function in their native conformation. It would also be beneficial to test these antibodies by other applications such as immunohistochemistry (IHC) or immunofluorescence (IF) to see if antibody format makes a difference when it comes to functionality in these applications. These data demonstrate that the yield of antibodies can be increased by changing the cell type. These data also confirm that at least some antibodies tolerate conversion to scFv and scFv-Fc fusion formats for similar or improved performance.

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