ABSTRACT

Title of Dissertation: Development, characterization and optimization of a novel mammalian protein expression system

Darryl B. Sampey, Doctor of Philosophy, 2017

Dissertation directed by: Professor William E. Bentley
The Fischell Department of Bioengineering

Biopharmaceutical manufacturing plays a critical role in global healthcare systems. Methods and techniques for protein expression in upstream bioprocesses can have the highest impact on product quality and safety and, ultimately, on delivering effective means of treating and curing life threatening diseases. In addition to novel products, development and regulatory approval of biosimilars require precise matching of the quality attributes between lots of reference innovator drug and those of the biosimilar candidate. With continuing pressure to reduce the cost of these lifesaving medicines, increased bioprocess yields are also key to reducing cost of manufacturing and allowing for lower prices at the pharmacy. With these goals in mind, a novel biomanufacturing platform was developed that harnesses the commercially established murine NS0 myeloma host cell in new ways to create stable, highly productive manufacturing cell lines. Cholesterol metabolic markers were screened to identify the optimal enzyme, Hsd17β7, to enable stable cell line selection and eliminate the need for cholesterol addition to the bioprocess. Enhancement of the expression vector promoters, isolation and cloning of highly efficient hybridoma-derived heavy and light chain signal peptides and optimization of coding sequences increased monoclonal antibody expression by 10-fold. The development of novel multiplex selection strategies that combines antibiotic selection with cholesterol and glutamine metabolic selection both sequentially and in parallel allowed for the rapid and consistent generation of commercial grade cell lines with greater than 1 g/L yield potential. The platform was demonstrated to be highly valuable in developing the most complex of next generation biologics and commercially significant for the production of biosimilar monoclonal antibodies whose reference drugs are currently manufactured in murine host cells.

DEVELOPMENT, CHARACTERIZATION AND OPTIMIZATION OF A NOVEL MAMMALIAN PROTEIN EXPRESSION SYSTEM

by

Darryl B. Sampey

Dissertation submitted to the Faculty of the Graduate School of the
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Advisory Committee:
Professor William E. Bentley, Chair
Associate Professor Nam Sun Wang
Professor and Associate Dean Peter Kofinas
Professor David M. Mosser
Assistant Professor Christopher Jewell

Preface

Biopharmaceutical manufacturing plays a critical role in global healthcare systems. Methods and techniques for protein expression in so called upstream bioprocesses can have the highest impact on product quality and safety[1] and, ultimately, on delivering an effective means of treating or even curing many life threatening diseases. Over the past 20 years, biopharma drugs have significantly expanded to meet a broad range of indications. Starting with protein therapeutics such the now "simple" recombinant insulin[2] made in bacterial cells, biopharmaceuticals are becoming ever more complex as this class of drug is called upon to solve more complicated health conditions. New products such as enzymes and monoclonal antibodies are now precisely designed to be more effective at treating diseases, yet cause less off-target and adverse effects. This precision in design requires protein expression technology that is equally able to produce these new drugs with the required critical quality attributes consistently and at large scale. In addition to novel products, development and regulatory approval of follow-on biologics or biosimilars require precise matching of the quality attributes between lots of reference innovator drug and those of the biosimilar candidate[3-14]. This focus on product quality has become an engine for novel, high-resolution protein analytics that have in turn raised the bar on both biosimilar and novel biologic characterization. The objectives of biopharmaceutical manufacturing science have expanded beyond simply selecting the cell line with the highest productivity and best growth characteristics. Commercial cell lines that produce the most complex products such as multimeric glycoproteins must be productive and express a product with the desired post-translational modifications. With continuing pressure to reduce the cost of these lifesaving medicines, increased bioprocess

yields are also key to reducing cost of manufacturing and allowing for lower prices at the pharmacy especially in the new age of biosimilars.

With these goals in mind, a novel biomanufacturing platform has been developed that harnesses the commercially established murine NS0 myeloma host cell in new ways to create stable, highly productive manufacturing cell lines with characteristics meeting the current demands of global healthcare systems. The use of a cholesterol metabolic selection marker and strategy eliminates the need for cholesterol addition to the bioprocess which presented a challenge during the stainless steel era and is now a road block in the age of single-use technology[15-16]. Optimization of the transcriptional, translational and translocational signaling were incrementally implemented resulting in over 10-fold increases in product yield. Media and supplement development led to elimination of serum from the entire cell line development process including single cell cloning steps significantly increasing regulatory compliance. A novel multiplex selection strategy was further introduced nearly tripling cell line specific productivity and volumetric yields. The StableFast Biomanufacturing Platform was demonstrated to be highly valuable in developing the most complex of next generation biologics and commercially significant for the production of biosimilar monoclonal antibodies whose reference drugs are currently manufactured in murine host cells.

Dedication

I am dedicating this dissertation and its successful defense to my beautiful wife, strongest supporter and best friend Cheryl Sampey.

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List of Abbreviations

3-KSR	3-ketosteroid reductase
α–	anti-
a.a.	amino acid
AGC	automatic gain control
ATCC	American Type Culture Collection
b2M	β2-microglobulin
BGH	bovine growth hormone
bp	base pair
BSA	bovine serum albumin
c	chimeric
CD	chemically defined
cDNA	complementary deoxyribonucleic acid
CDR	complementarity-determining regions
CEX	cation exchange
CH1	constant heavy chain domain 1
CH3	constant heavy chain domain 3
CH3	constant heavy chain domain 2
CHO	Chinese hamster ovary
CK	constant Kappa chain domain
CMV-MIE	cytomegalovirus major immediate-early
CV	column volume
CVCT	cumulative viable cell time
CY2	cysteine
dhfr	dihydrofolate reductase
DMEM	Dulbecco’s Modified Eagle medium
DMSO	dimethyl sulfoxide
DO	dissolved oxygen
DPBS	Dulbecco's phosphate buffered saline
ECACC	European Collection of Authenticated Cell Cultures
EDTA	ethylenediaminetetraacetic acid
ELISA	enzyme-linked immunosorbent assay
Fab	fragment antigen-binding
FAF	fatty acid-free
FBS	fetal bovine serum
Fc	fragment crystallizable
g	gravity
GLN	glutamine
GLU	glutamic acid

GS	glutamine synthetase
H	hinge domain
h	human
HC	heavy chain
HCD	higher-energy collisional dissociation
HEK	human endothelial kidney
HILIC	hydrophilic interaction liquid chromatography
HLA	human leukocyte antigen
HPLC	high performance liquid chromatography
HRP	horseradish peroxidase
Hsd17β7b5	17β-hydroxysteroid dehydrogenase type 7
Hsd3β5b5	3β-hydroxysteroid dehydrogenase type 5
ICA	integrated cell area
Ig	immunoglobulin
IgG	immunoglobulin Gamma
ILE	isoleucine
IND	Investigational New Drug
Kb	kilobase
kDa	kiloDalton
L	leader
LC	light chain
LDC	limiting dilution cloning
LEU	leucine
m	murine
M	molar
mAb	monoclonal antibody
µFd	microFaraday
µg	microgram
mg	milligram
µL	microliter
mL	milliliter
mM	millimolar
MRC	Medical Research Council
mRNA	messenger ribonucleic acid
ms	microsecond
MS	mass spectrometry
MSX	methionine sulphoximine
Multi-Select	multiplex selection
MWM	molecular weight marker
NaCl	sodium chloride

NEAA	non-essential amino acids
NeoR	neomycin resistance gene
NS0	non-secreting null murine myeloma cell line
NS0null	NS0 null cell line
NS0par	NS0 parental cell line
NZ	New Zealand
OD	optical density
opt	optimized
PBS	phosphate buffered saline
PCR	polymerase chain reaction
PEI	polyethylenimine
poly(A)	polyadenylation sequence or tail
PVDF	polyvinylidene difluoride
Qp	specific productivity rate
RCT	Research Corporation Technologies, Inc.
RNA	ribonucleic acid
RSV	respiratory syncytial virus
RT	room temperature
RT-PCR	reverse transcription-polymerase chain reaction
SDS-PAGE	sodium dodecyl sulfate polyacrylamide gel electrophoresis
SEC	size exclusion chromatography
SF-NS0	StableFast-NS0
SFM	serum-free medium
SP	signal peptide
SV40	simian virus 40
THR	threonine
TMB	3,3′,5,5′-tetramethylbenzidine
TSE	transmissible spongiform encephalopathy
UIRF	University of Iowa Research Foundation
USAMRIID	U.S. Army Medical Research Institute of Infectious Diseases
UTR	untranslated region
V	volt
VACV	vaccinia virus
VCD	viable cell density
VH	variable heavy
VK	variable Kappa
WB	western blot

Chapter 1:
Selection and screening of cholesterol selection marker genes

Abstract

Most biopharmaceutical manufacturing cell lines that have been constructed for the expression of monoclonal antibodies (mAbs) have employed the dihydrofolate reductase (DHFR) or glutamine synthetase (GS) metabolic selection markers. While these have worked, there are other approaches for stable integration and clonal selection that have advantages over these established technologies. In this Chapter 1, we develop a novel cholesterol selection methodology that for the first time exploits the widely documented cholesterol auxotrophy of the NS0 murine myeloma cell line[17-19]. An overview of the cholesterol biosynthetic pathway is shown in Figure 1. In Figure 2, details of the steps between lanosterol and cholesterol are shown identifying four reactions requiring 3-ketosteroid reductase (3-KSR) activity. This figure also indicates that lanosterol does not support NS0 growth, and that only the final two products do support growth. A survey of studies to identify and characterize murine enzymes exhibiting 3-KSR activity including their abilities to rescue the lesions in the NS0 cholesterol pathway led us to evaluate two enzymes: 3β-hydroxysteroid dehydrogenase type 5 or Hsd3β5[20] and 17β-hydroxysteroid dehydrogenase type 7 or Hsd17β7.[21–23] During initial studies with the Hsd3β5 enzyme, publications were noted that further identified murine Hsd17β7, as the gene responsible for the 3-ketosteroid reductase activity lacking in NS0 cells.[24-25] This work identified cholesterol auxotrophy in NS0 cells at the level of methylation of a CpG-rich region upstream of the Hsd17β7 transcriptional start site. Treatment of NS0 cells with the demethylating drug 5-

azacytidine reversed cholesterol auxotrophy in this cell line. Both murine 3-ksr genes Hsd3β5 and Hsd17β7 were amplified from the same cDNA library source generated from adult male BALB/c kidneys to compare each enzyme's ability to rescue cholesterol auxotrophy in NS0 cells. The selection marker expression cassette contained in the constructs used to transfect NS0 cells utilizes a non-occluded or methylated transcriptional region, the simian virus 40 (SV40) enhancer and early promoter to enable active transcription. The cholesterol auxotrophy of the host NS0 cells were clearly demonstrated in the supplemented selection medium. Selection studies were performed indicating that cells transfected with Hsd17β7 produce cholesterol-independent transfectants.

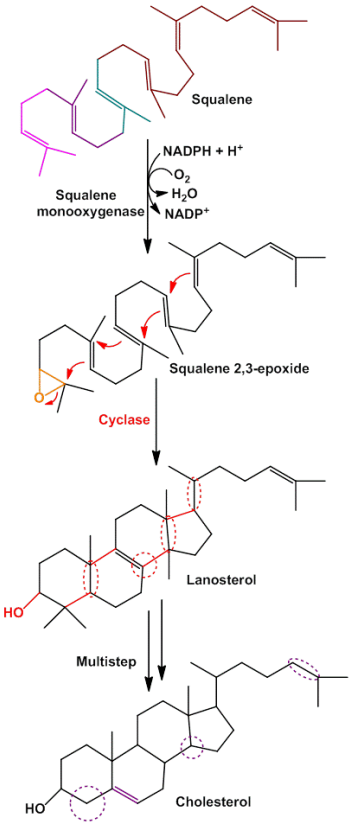


Figure 1. Overview of cholesterol biosynthetic pathway.
Image from Biosynthesis and Regulation of Cholesterol (with Animation), pharmaxchange.info, Sweety Mehta, September 16, 2013.

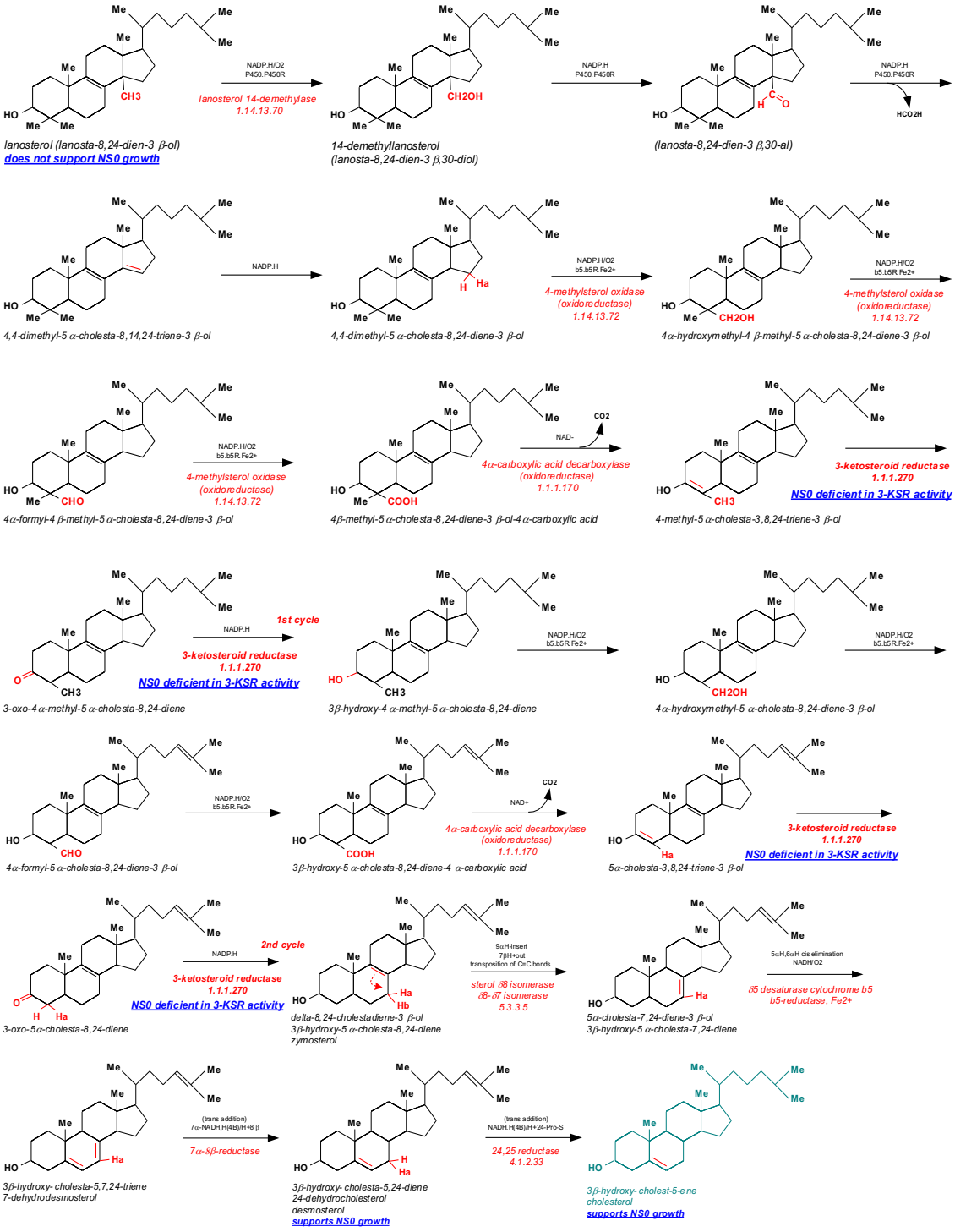


Figure 2. Pathway details of cholesterol biosynthetic pathway: Lanosterol to cholesterol. The four reactions requiring 3-ketosteroid reductase (3-KSR) activity are indicated. This figure also indicates that lanosterol does not support NS0 growth, and that only the final two products do support growth.

Results and Discussion

Construction of cholesterol selection marker plasmids: p3KSR.1 and p3KSR.2

After PCR amplification of the cDNA library source (adult male BALB/c kidney tissue) using flanking oligonucleotides specific for the published sequences of Hsd3β5 and Hsd17β7, amplicons were purified by agarose gel electrophoresis. The bands were isolated and cloned into pCR-Blunt II-TOPO vector (Invitrogen), and subsequently recloned into the pcDNA3.1(+) backbone (Invitrogen) generating the p3KSR plasmids. The coding regions (CDRs) of Hsd3β5 and Hsd17β7 in the p3KSR.1 and p3KSR.2 constructs, respectively, were confirmed to be correct by DNA sequencing and by comparison of those sequenced CDRs to the published Hsd3b5 sequence[26] and Hsd17β7 sequence[27]. Figure 3 shows the plasmid map for both p3KSR constructs.

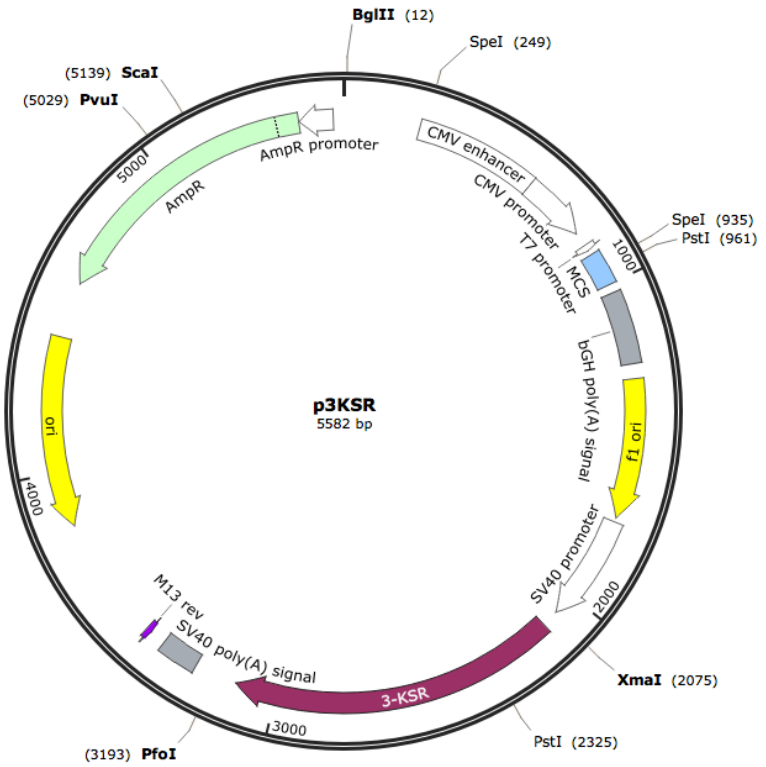


Figure 3. Plasmid map of p3KSR. Key segments and restriction sites are indicated.

Generation of NS0 Serum-Free Parental Cell Bank

A vial of serum-free medium adapted NS0 cell line (NS0-SF) was purchased from ECACC (Cat No. 03061601, Lot No. 06D023, See Appendix 2). Upon receipt, the vial was rapidly thawed at 37°C and cells were transferred to a conical vial. Ten milliliters of fresh NS0 growth medium formulated as follows;

NS0 Growth Medium:
CD Hybridoma (Gibco, Cat. No. 11279-023)
2mM GlutaMAX (Gibco, Cat. No. 35050-061)
1x Non-Essential Amino Acids (NEAA) (Gibco, Cat. No. 11140-050)
1x Cholesterol Lipid Concentrate (Gibco, Cat. No. 12531-018)

was added to the vial prior to centrifugation at 200×*g* for 5 minutes. Cells were resuspended in 5 mL fresh growth medium and were incubated at 37°C, 5% CO_2, and 90% Rh. Over the course of seven days, the NS0 cells were passaged four times until reaching a healthy 95% viability in log-phase of growth. Subsequently, the cells were cryopreserved by centrifugation at 200×*g* for 5 minutes and resuspension in 1 mL aliquots of NS0 Growth Medium containing 10% dimethyl sulfoxide (DMSO) at a cell density of 1×10^7 cells/mL. The cryopreserved vials were transferred to -80°C storage in a controlled freeze unit before transfer to the vapor phase of a liquid nitrogen dewer.

Demonstration of Cholesterol Auxotrophy in NS0 cells in Defined Medium

In preliminary experiments, we observed that p3KSR.1(Hsd3β5)-transfected cells that were selectively depleted of cholesterol containing medium did not survive selection. This may have been due to overgrowth observed in this culture, which may have generated cell numbers beyond levels supported by the culture conditions. In

subsequent experiments, viable cell density was maintained by feeding and expansion. Cells transfected with p3KSR.1(Hsd3β5) and immediately selected in cholesterol free medium produced significant numbers of live cells after a 3-week selection period. However, mock transfected NS0 host cells grown in the same selective conditions also produced significant numbers of live cells. This observation was in direct contrast to earlier data demonstrating that NS0 cultures die within 3 days of being cultured in cholesterol-free medium. The difference between the two conditions was the presence of StableFast supplement in the transfection experiment. StableFast contains bovine serum albumin (BSA) as a carrier and stabilizer, and it is likely that this albumin contains a lipid fraction, including cholesterol. We obtained delipidated BSA to be used as a component of StableFast in the next round of experiments with 3KSR selection in NS0. We anticipate this delipidated version of BSA will support carrier and stabilizing functions in NS0, while lacking a lipid fraction potentially carrying cholesterol.

An experiment was performed using parental NS0 cells comparing cell growth in chemically defined (CD) serum-free medium (SFM) Growth Medium (with cholesterol), CD SFM Basal Selection Medium (without cholesterol), Supplemented Selection Medium (including 1% standard BSA), and Delipidated Supplemented Selection Medium (containing 1% fatty acid free BSA). A bank of NS0 host cells that were adapted to chemically-defined medium were thawed into the following growth medium:

NS0 Growth Medium:
CD Hybridoma (Gibco, Cat. No. 11279-023)
2mM GlutaMAX (Gibco, Cat. No. 35050-061)
1x Non-Essential Amino Acids (NEAA) (Gibco, Cat. No. 11140-050)

1x Cholesterol Lipid Concentrate (Gibco, Cat. No. 12531-018)

The following selection media were tested:

Basal Selection Medium:
CD Hybridoma
2mM GlutaMAX
1x NEAA

Supplemented Selection Medium:
CD Hybridoma
2mM GlutaMAX
1x NEAA
1% Bovine Albumin Fraction V (Invitrogen, Cat. No. 15260-037)
0.5 ng/mL human recombinant IL-6 (Sigma, Cat. No. I1395-10UG)
1x ITS Supplement (Sigma, Cat. No. I3146-5ML)

Delipidated Supplemented Selection Medium:
CD Hybridoma
2mM GlutaMAX
1x NEAA
1% Albumin, Bovine Serum, Fraction V, Fatty Acid-Free (CalBioChem, Cat. No. 126609)
0.5 ng/mL human recombinant IL-6
1x ITS Supplement

NS0 cells were passaged six times in NS0 Growth Medium (P6: 1.85e6 cells/mL, 97% viable). Cells were aliquoted into four tubes, gently centrifuged, rinsed with 10 mL sterile phosphate buffered saline (PBS), gently centrifuged, and each resuspended in 12 mL of the four media above at a starting density of 3e5 cells/mL. Cultures were counted for 4 days.

Figure 4 shows the growth curves and viability for each of the experimental media. Solid lines indicate viable cell density (VCD) and dashed lines indicate culture viability. The figure legend indicates the various media formulations used by color. Parental NS0 cells grown in cholesterol supplemented growth medium

exhibited classic lag, exponential growth, and stationary phases maintaining viability above 90% during the entire culture. All other cholesterol reduced (Supp Select) or cholesterol free media showed low to no growth with final viabilities less than 10%. At Day 4, the selection medium supplemented with delipidated (fatty acid-free) BSA (BSA FAF) showed less than 2% viability while the medium supplemented with standard BSA showed a viability of 7%. This experiment demonstrates rapid metabolic cell death due to cholesterol withdrawal and confirmed the use of the updated StableFast supplement including BSA FAF.

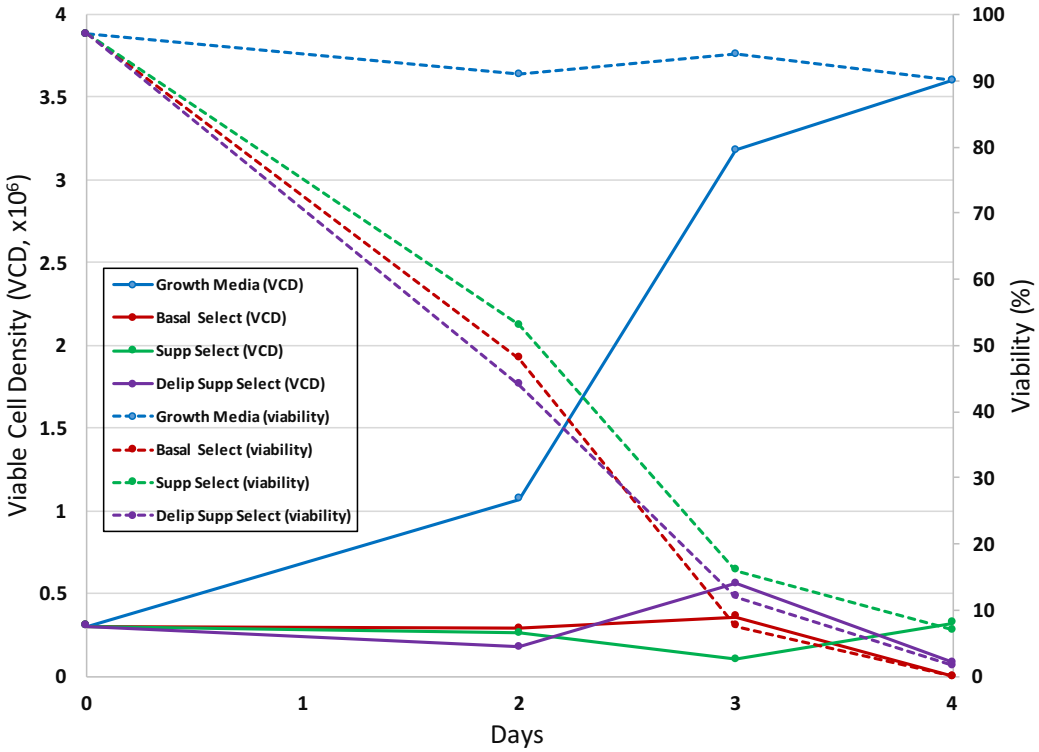


Figure 4. Growth curves of parental NS0 cells in growth vs selective media. Solid lines indicate viable cell density (VCD) and dashed lines indicate culture viability. The figure legend indicates the various media formulations used by color.

Failure of Hsd3β5 Marker to Rescue Cholesterol Auxotrophy

Parental NS0 cells were expanded and passaged 7 times in cholesterol-supplemented growth medium. The plasmid pBFKSR.1 containing the Hsd3β5 gene under constitutive SV40 promoter control was linearized and used to transfect the parental NS0 by electroporation (50μg DNA, 250V, 400μFd, single pulse, 10e6 cells). A mock transfection was performed using ultra-pure water (no DNA). After transfection, cells were cultured in Delipidated Supplemented Selective Medium (cholesterol-free). At 9 days post-transfection, only 2 live cells were visible in the entire hemacytometer chamber for the mock transection and only 1 was found for the Hsd3β5 transfected culture.

Comparison of Hsd3β5 to Hsd17β7 Markers

Parental NS0 cells were transfected with plasmids containing either the Hsd3β5 (pBFKSR.1) or Hsd17β7 (pBFKSR.2) genes under constitutive SV40 promoter control. The plasmids were linearized and NS0 cells were transfected by electroporation (50μg DNA, 250V, 400μFd, single pulse, 10e6 cells). A mock transfection was performed using ultra-pure water (no DNA). Immediately after electroporation, cells were resuspended in Growth Medium. Twenty-four (24) hours after transfection, half of each of the cells were cultured in Delipidated Supplemented Selective Medium (cholesterol-free). Forty-eight (48) hours after transfection, the other half of each of the cells were cultured in Delipidated Supplemented Selective Medium (cholesterol-free). Cultures were counted at intervals. At Day 27 post-transfection, all cultures that were incubated in Growth Medium for 48 hours post-

transfection were dead and discontinued. The remaining cultures were monitored for growth and viability over 41 days (see Figure 5). At Day 37, the Hsd3β5 (pBFKSR.1) transfected culture was completely dead and discontinued. On Day 41 both the mock and Hsd17β7 (pBFKSR.2) transfected cultures were banked. Due to the selection performance, Hsd17β7 was continued forward as the selection marker of choice for further studies. The fact that the mock transfection also survived in cholesterol-free conditions may indicate potential reversion to cholesterol independence due to epigenic changes in the endogenous 3-KSR gene[28].

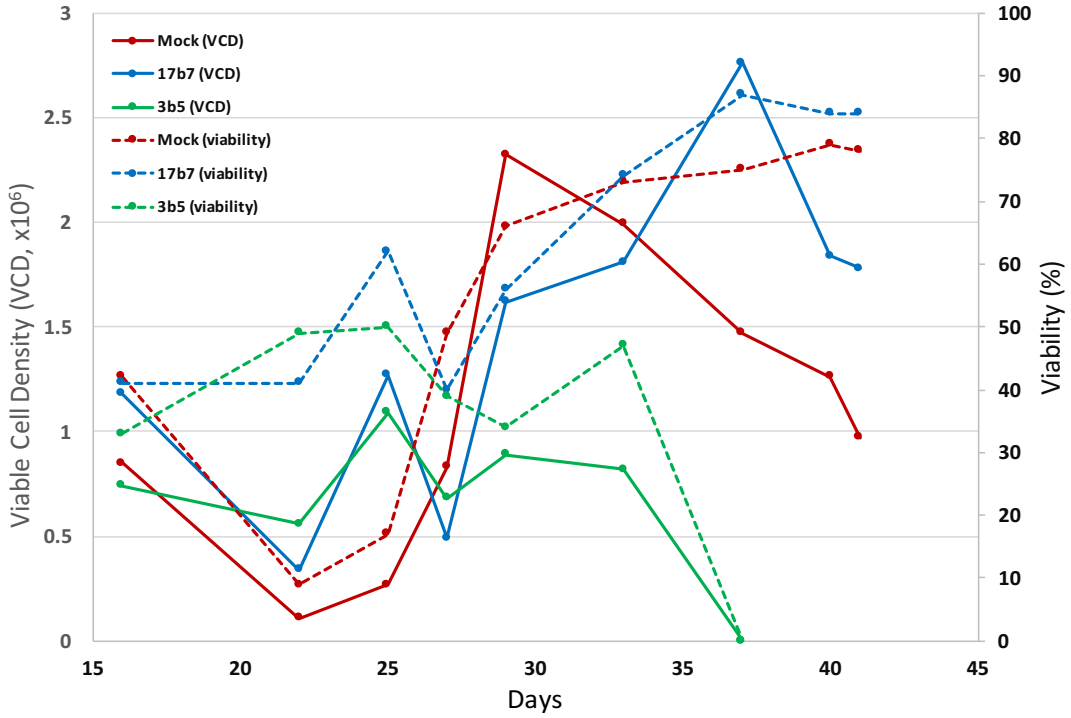


Figure 5. Growth curves of parental NS0 cells in Hsd3 5 vs Hsd17 7 selection. Solid lines indicate viable cell density (VCD) and dashed lines indicate culture viability. The figure legend indicates the various transfections by color.

We are investigating the possibility that bulk transfections are not the optimal setting for development of this system, as dying cells may provide a source of cholesterol, which coupled to the carrier protein BSA may supply transfected and

untransfected cells alike with this essential lipid. In follow-up studies, a plating approach will be employed by distributing the transfected cell pool into forty 96-well flat bottom plates. This method provides for better control in terms of spent medium exchange, thus allowing for less leaching of cholesterol into the medium by dying cells. Additionally, expression vectors will be constructed with both the Hsd17β7 selection marker and genes that code for a full-length human IgG1 antibody light and heavy chain. This vectors will be used in transfections to show expression and secretion of a recombinant antibody product in the p3KSR system.

Conclusions

In these studies, two different genes (Hsd3β5 and Hsd17β7) that are known to have 3-ketosteriod reductase (3-KSR) activity were cloned into separate expression plasmids regulated with SV40 promoter and poly-adenylation sequences, upstream and downstream, respectively. Over the course of two weeks, cells transfected with Hsd17β7 resulted in clear cholesterol-independent transfectants. Those cells transfected with Hsd3β5 did not survive the selection process. Therefore, all further development was based on the Hsd17β7 marker.

Chapter 2:
Expression vector optimization

Abstract

The productivity of a stable mammalian cell line is controlled by many gates. Several critical gates are affected by the expression vector design. In this Chapter 2, novel single chain and dual chain expression cassettes were optimized to facilitate a modular expression vector with increased expression levels to provide higher specific productivity of monoclonal antibodies (mAbs) in the NS0 platform. Initial observations of low productivity of a set of human mAbs with the parental pcDNA vector in CHO cells led to the investigation and addition of the intron A segment of the human cytomegalovirus major immediate-early (CMV-MIE) promoter that drives expression of both mAb chains. A murine IgG1 expressed by a clonal hybridoma cell line (designated mAb 2477) was selected for initial construction of the antibody expression acceptor cassettes. In addition, the variable domains of mAb 2477 were used to construct an irrelevant mouse/human chimeric IgG1 negative control for the upcoming *in vivo* studies. The mAb 2477 sequences were selected due to high expression levels in the corresponding hybridoma cell line (~200-300 μg/ml). Expression elements, including the 5′ untranslated region (UTR), signal peptide (SP) sequences, and 3′ UTR native to the 2477 cell line were evaluated during the acceptor cassette construction. This resulted in a 10-fold increase in expression of a chimeric mAb as compared with the mAb's native SPs. Heavy and light chain genes used to generate stable cell lines were optimized and synthesized for three different human chimeric mAbs in the NS0 platform leading to multi-fold increases in expression as compared with the original un-optimized genes for all three constructs.

Results and Discussion

Development of Optimized CMV-MEI Promoter with Intron A

The observation that stable CHO DG44 cell lines expressing fully human mAbs did not generate the levels of secreted material expected from a human CMV-MIE driver promoter system led to further investigation of this phenomenon. Each antibody light and heavy chains are individually expressed from a CMV-MIE promoter without an intron. It has been reported in the literature that introns often increase expression levels of recombinant genes in mammalian cells.[29] Thus, we first designed a set of oligos to amplify a large portion of the CMV-MIE promoter that included the first intron, or intron A. These oligos amplified a fragment starting at the unique *Nde*I site in the 5' region of the CMV promoter, and ending just beyond the *Pst*I site that delineates the end of intron A. Thus, the *Nde*I-*Nhe*I fragment was directionally cloned in the existing light and heavy chain constructs of mAbs 14 and 21, using the *Nde*I-*Nhe*I sites, thus replacing the intronless CMV promoter with one containing intron-A. Subsequently, expression of mAb14 and 21 light and heavy chain constructs, with or without intron A, were compared in transiently transfected 293T cells. After a 3-day transfection, supernatants were collected and assayed for human IgG titer by enzyme-linked immunosorbent assay (ELISA). Titers from intron-A containing construct were significantly higher than those of the intronless construct. Therefore, the addition of CMV intron A was included in both the light and heavy chain cassettes.

Construction of Optimized Heavy and Light Chain Expression Cassettes and Vectors

Total RNA was prepared from a 2477 hybridoma TRIzol suspension and cDNA was generated using Invitrogen's SuperScript III First-Strand Synthesis System for RT-PCR. Since the entire 2477 sequence was previously unknown, the oligo(dT) primed cDNA was PCR amplified using Finnzymes' Phusion High-Fidelity DNA polymerase with a 5' oligonucleotide "original set" mix outlined in Barbas, *et. al.*[30] that contains consensus mouse variable heavy chain (VH) and variable Kappa chain (VK) sequences at the N-terminus of the variable domains. The 5′ VH and 5′ VK primer mixes were paired separately with 3′ oligonucleotides (designated MsIgG1 and MsKappa) that anneal ~50bp downstream of the start of the heavy chain 1 or Kappa constant domains, CH1 or CK, respectively. The ~300bp VH and VK PCR products were subsequently cloned into pCR-BluntII-TOPO (Invitrogen) prior to sequencing. Analysis of several VH and VK clones revealed the entire (including all framework and complementarity determining regions [CDRs]) variable domain sequence with the exception of the N-terminal ~10 amino acids (a.a.) that differed due to the 5′ primer mix utilized for initial amplification.

To obtain additional sequence data, including the native 5′ UTR, Kozak, signal peptide, and 3′ UTR nucleotides, Clontech's SMARTer RACE cDNA Amplification Kit was used. Briefly, total RNA from 2477 was reverse transcribed using either the standard oligo(dT) primer or the modified oligo(dT) primer coupled with the "SMARTer II A" oligonucleotide. Upon reaching the end of the RNA template, the "SMARTScribe RT" adds several non-templated bases to the 3′ end of the cDNA. The "SMARTer II A" primer contains complementary bases allowing the primer to

anneal to the first strand cDNA template. The "SMARTScribe RT" will template switch from the mRNA to the oligonucleotide and complete the generation of a full-length cDNA copy of the original mRNA with an additional "SMARTer" sequence at the end. Once a full-length cDNA template is generated, gene specific primers coupled with the "universal primer mix" PCR amplify either the 5′ fragment or 3′ fragment of the cDNA. Oligonucleotides comprising the universal primer mix anneal to the bases generated upstream of the cDNA by the SMARTer II A primer. This permits amplification of the cDNA as specified by the gene specific primers for only full-length cDNA templates that contain the "SMARTer" sequence. PCR amplification of 5′ and 3′ portions of 2477 heavy chain (*e.g.*, 5′ HC race, 3′ HC race) and the 5′ and 3′ portions of 2477 light chain (*e.g.*, 5′ LC race, 3′ LC race) was performed with Clontech's Advantage 2 PCR kit. The subsequent PCR products containing TA overhangs were subcloned into pCR8/GW/TOPO TA (Invitrogen). Sequencing the 5′ HC race, 3′ HC race, 5′ LC race, and 3′ LC race inserts permitted the elucidation of the 5′ regions upstream of the VH or VK domain. These regions contain the aforementioned elements that influence the expression levels of 2477, and potentially will translate to stable cell line based-manufacturing systems for high expression levels of other VH and VK sequences when substituted for the 2477 VH and VK domains.

The creation of a full-length heavy chain IgG1 acceptor cassette began with a fully murine 2477 HC antibody sequence. The 5′ oligonucleotide contains *Pme*I and *Hind*III restriction sites upstream to the start of the 5′ UTR while the 3′ oligonucleotide contains a *Kpn*I site and *Pme*I site after the stop codon at the C-

terminus of the murine CH3 constant domain. The 2477 HC was amplified from the cDNA generated from the initial TRIzol preparation using Phusion with the above primers and contains flanking *Pme*I restriction sites. The 2477 LC was similarly amplified except the 5′ oligonucleotide contains *Pme*I and *Not*I restriction sites while the 3′ oligonucleotide contains *Xba*I and *Pme*I restriction sites. Figure 6 illustrates successful amplification of the full-length heavy and light chain domains. The HC and LC domains were independently subcloned into pCR-BluntII-TOPO. Clones containing the HC and LC were restricted with *Pme*I then ligated into the similarly restricted mammalian expression backbone pBFksr, as depicted in Figure 7 and Figure 8, respectively.

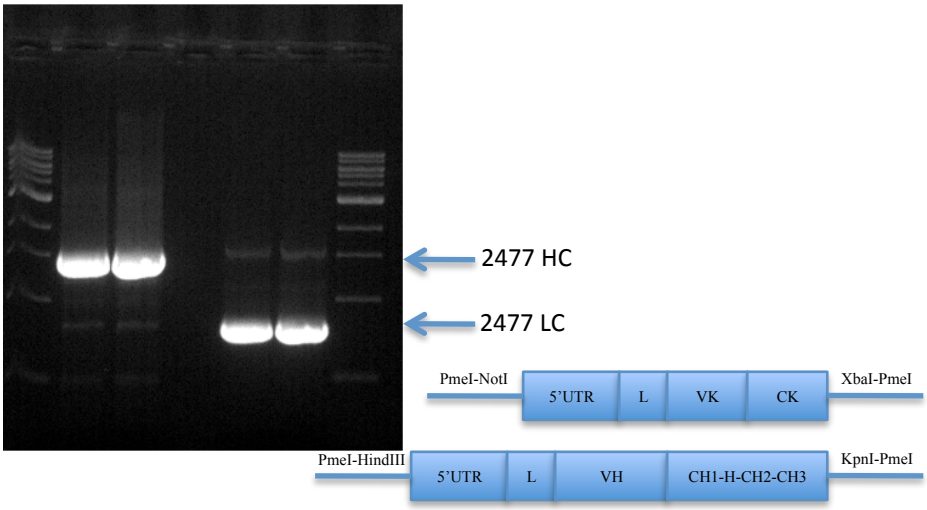


Figure 6. PCR amplification of 2477 HC and 2477LC from 2477 cDNA. Lanes 1 and 7 are 1kb MWM. Lanes 2 and 3 are 2477 HC. Lanes 5 and 6 are 2477 LC. Arrows indicate successful amplification of 2477 HC and LC bands. Graphical representation of the amplified PCR products is shown to the lower right.

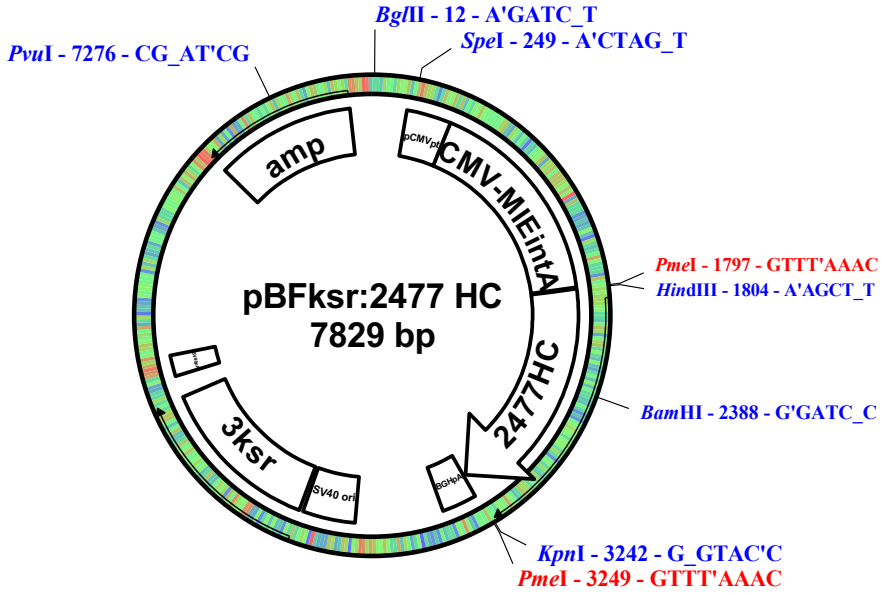


Figure 7. Plasmid map of pBFksr:2477 HC. Key segments and restriction sites are indicated.

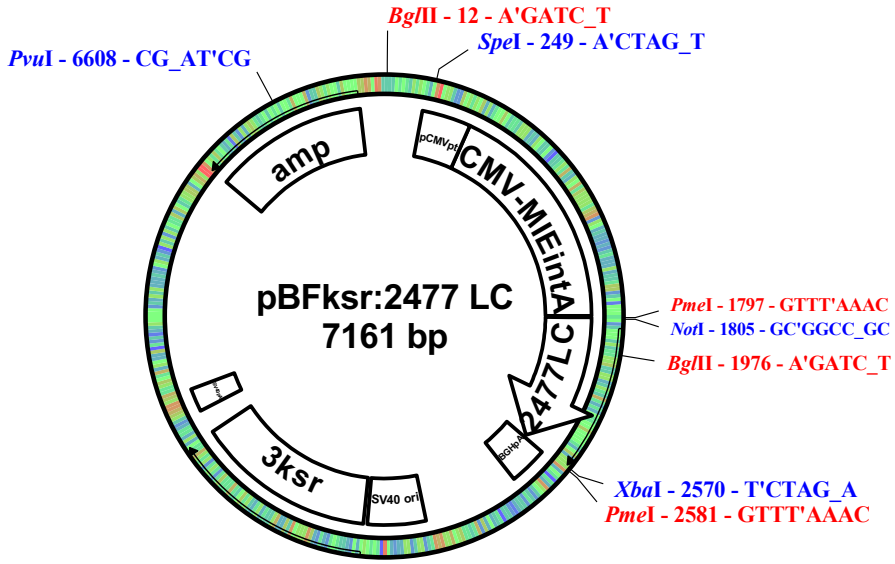


Figure 8. Plasmid map of pBFksr:2477 LC. Key segments and restriction sites are indicated.

To construct a dual chain expression vector containing both the 2477 mHC and 2477 mLC under separate transcriptional elements, the CMV promotor, mLC, and

BGH polyadenylation signal sequences were PCR amplified as a single fragment with oligonucleotides containing flanking *Bam*HI sites. The ~2.8Kb PCR product (mLC cassette) insert was restricted with *Bam*HI prior to ligation with the *Bgl*II linearized pBFksr:m2477HC construct. Upon transformation, emerging clones were analyzed by restriction analysis to determine the presence and orientation of the mLC cassette. Figure 9 illustrates that pBFksr:dual m2477 clones #2, #4, and #5 have the mLC cassette in the correct orientation. A plasmid map of the dual vector is shown in Figure 10.

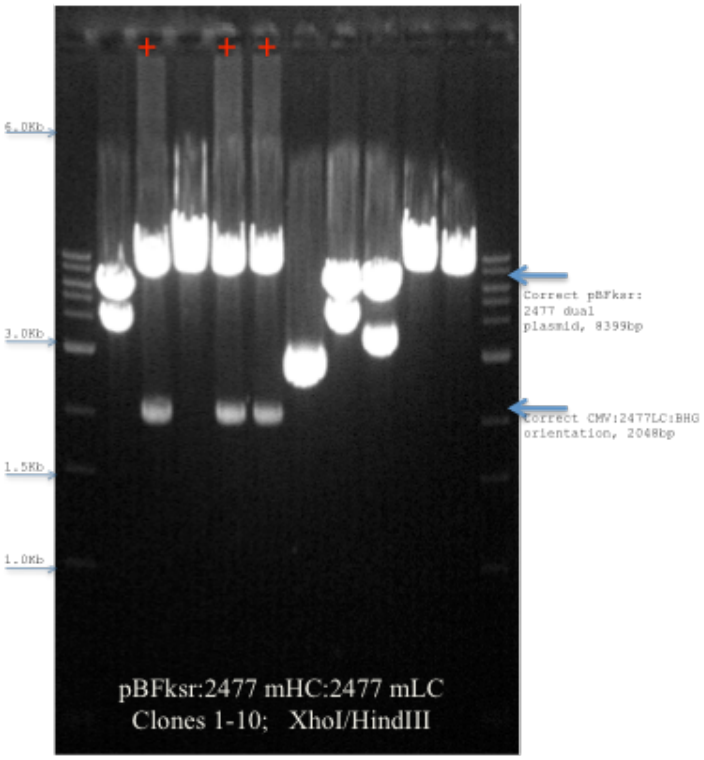


Figure 9. *Xho*I/*Hind*III restriction analysis of pBFksr:dual m2477 clones. Arrows indicate the bands expected from digest of the correctly oriented mLC cassette in the pBFksr:dual m2477 plasmid. Clones #2, #4, and #5 indicated by the plus signs are correctly oriented.

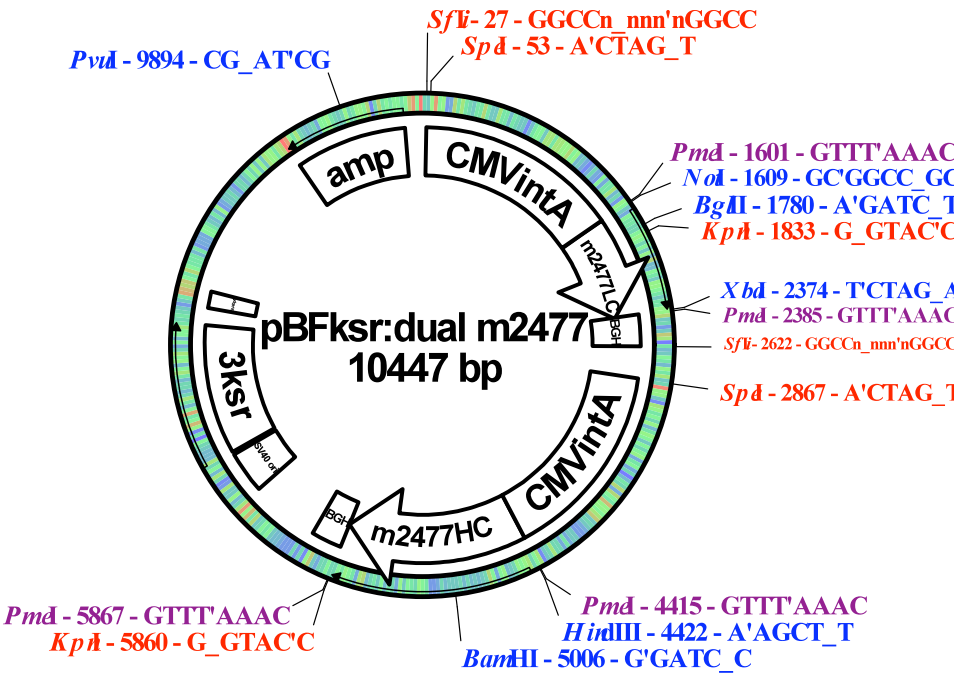


Figure 10. Plasmid map of pBFksr:dual m2477. Key segments and restriction sites are indicated.

Transient transfections of the above single and dual chain m2477 constructs were performed to determine the functionality of the expression vectors. Briefly, two different transient systems were employed: The CHO-S serum-free suspension systems and the HEK 293T/17 adherent system. CHO-S cells were thawed and expanded in FreeStyle CHO medium (Invitrogen) in 30 mL of shaker culture. After several passages, cells were seeded at 1×10^6 cells/mL and transfected with 18.75 µg each of heavy and light chain expression plasmids (~equimolar) or 37.5 µg of the dual chain construct. Cells were allowed to incubate with shaking for 3 days for mAb expression. Supernatants from each transfection were clarified by centrifugation on Day 3 and an anti-mouse IgG capture ELISA was used to determine the presence and

relative concentration of secreted mAb. OD_{450} was approximately 0.8 for all transfections (1:100 dilution of supernatant) compared to negative control background (empty plasmid transfections) of 0.09, confirming expression and secretion of a mouse IgG. Concurrent with CHO-S transients, human endothelial kidney (HEK) 293T/17 cells were transfected in 10% serum medium in 6-well plate format (2 mL each) with various concentrations and ratios of the individual heavy and light chain expression plasmids. Total DNA concentrations per transfection evaluated were 4 and 8 μg and the following ratios of heavy:light were evaluated for each of the two concentrations: 1:1, 1:2, and 2:1. As with the CHO-S experiments, transfections were allowed to proceed for 3 days and mouse IgG was analyzed in supernatants by the same ELISA method. OD_{450} was 1.34 (average of two) and 1.48 (average of two) for the 4 and 8 μg 1:1 transfections, respectively, nearly two-fold above that of the CHO-S transfections. ODs of the 1:2 H:L showed about a 30% decrease and ODs of the 2:1 H:L showed about a 9% increase at the 4 μg transfections. ODs of the 1:2 H:L showed about a 28% decrease and ODs of the 2:1 H:L showed about a 14% increase at the 8 μg transfections. In conclusion, all transient transfections of the single and dual chain m2477 constructs produced secreted mouse IgG. The 2:1 H:L ratio of the single chain constructs produced 9-14% increases in productivity in the HEK 293T/17 system.

To generate an appropriate negative control mAb for inclusion in upcoming pre-clinical studies, the variable regions of the irrelevant murine mAb 2477 (mVH, mVK) were cloned with the corresponding human constant domains (hCH, hCK) into the above acceptor expression vectors, generating single and dual chain mouse/human

chimeric IgG1 mAb constructs. The cloning strategy to engineer recombinant chimeric 2477 mAb constructs is outlined in Figure 11 and detailed below.

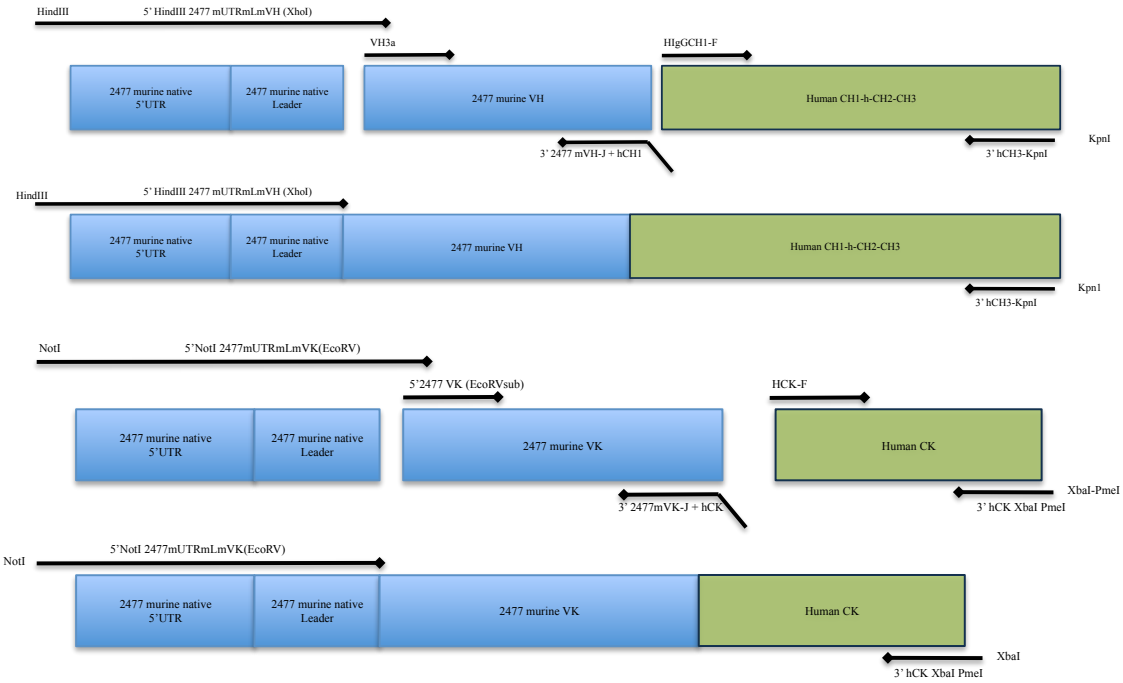


Figure 11. Chimeric 2477 HC and LC construction cloning scheme. Key segments, primers and restriction sites are shown.

Construction of the chimeric 2477 HC (cHC) utilized a series of PCR reactions where the various antibody domains are amplified individually, then linked together through a series of overlap PCR assembly reactions. Briefly, the mVH was amplified with a 5′ primer containing several bp mismatches to engineer an *Xho*I site ~5 a.a. internal to the start of the mVH and a 3′ primer that anneals to the C-terminal mVH a.a. with an overhang of 7 a.a. to the N-terminus of the human constant domain. The hCH domain was amplified with a 5′ primer and 3′ primer containing a *Kpn*I site immediately downstream of the stop codon. The two domains (mVH, hCH) were co-amplified with the 3′ primer above and a 5′ primer that extends the N-terminus by adding a *Hind*III site, the 5′ native mVH UTR, and the native mVH leader sequence

prior to annealing with the start of the mVH containing the *Xho*I site addition. The entire chimeric 2477 HC was subsequently cloned via flanking *Hind*III/*Kpn*I sites into the similarly restricted pBFksr:m2477HC, thus replacing the mHC with cHC.

Construction of the chimeric 2477 LC (cLC) was performed using the same cloning scheme with a few modifications. Briefly, *Not*I and *Xba*I were selected as the 5′ and 3′ flanking restriction sites, respectively. The 2 a.a. at the start of the mVK were altered to engineer an *Eco*RV site immediately downstream of the leader sequence cleavage site. The entire chimeric 2477 LC was cloned via *Not*I/*Xba*I into the similarly restricted pBFksr:m2477LC, thus replacing the mLC with cLC. Figure 12 illustrates successful PCR assembly of the full 2477 cHC and cLC in addition to a graphical representation of the unique engineered restriction sites contained within the chimeric antibody sequences.

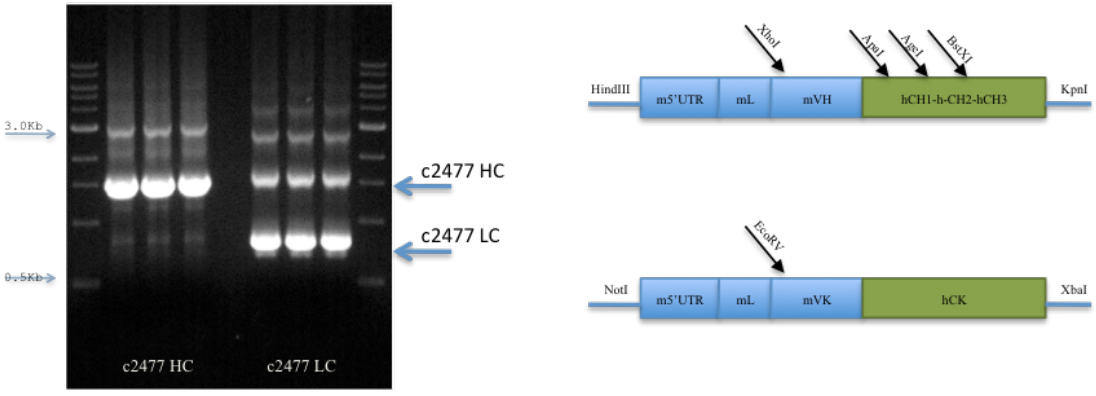


Figure 12. Agarose gel of PCR Phusion products c2477 HC and c2477 LC. Graphical representation of the individual domains and unique restriction sites engineered within each separate chimeric antibody chain (right).

To construct the chimeric 2477 dual chain mAb expression vector containing both the c2477HC and c2477LC under separate transcriptional elements, the CMV promotor, c2477LC, and bovine growth hormone (BGH) polyadenylation signal

sequences were PCR amplified as a single fragment with oligonucleotides containing flanking *Bam*HI restriction sites. The ~2.8Kb PCR product (c2477LC cassette) insert was restricted with *Bam*HI prior to ligation with the *Bgl*II linearized, antarctic phophatase treated pBFksr:c2477HC construct. Upon transformation, emerging colonies were analyzed first by PCR and subsequently by restriction analysis to determine the presence (via *Sfi*I) and orientation (via *Xba*I/*Hind*III) of the cLC cassette. Figure 13 illustrates that pBFksr:dual c2477 clone #35 clearly possesses a LC insert as indicated by the ~700bp band. Also, bands corresponding to 7870bp and 2595bp after *Sfi*I restriction indicate the presence of the light chain cassette. After *Hind*III/*Xba*I restriction, bands at the 8417 bp and 2048 bp mark indicate the two chains are in tandem alignment whereas bands at the 6318 bp and 4147 bp would indicate an opposing alignment.

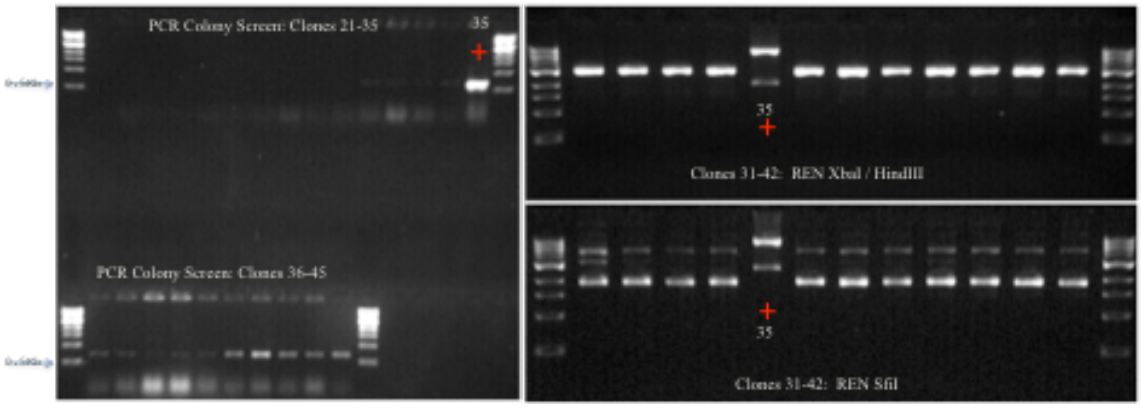


Figure 13. Analysis of pBFksr:c2477 dual chain colonies by PCR colony screen (Left) and subsequent restriction analysis by *Sfi*I (Bottom right) and *Xba*I/*Hind*III (Top right). Based on the restriction patterns, Clone #35 (indicated by the plus mark) has the correct insert with the tandem chain orientation.

Notably, the completion of the chimeric 2477 mAb single and dual chain expression vectors facilitates a two-step procedure to construct future chimeric or humanized mAbs. VH domains can be conveniently cloned via the engineered *Xho*I

site (at the 5′ end of the VH) and the *Apa*I, *Age*I or *Bst*XI sites internal to the hCH domain into the pBFksr:cHC vector. Likewise, other VK or VL domains can be cloned via the engineered *Eco*RV site and the *Xba*I site into the pBFksr:cLC vector.[31]

Construction of Chimeric α-Vaccinia Virus (VACV) L1 mAb Expression Vectors

To demonstrate the utility of the new optimized modular cassette and expression vector system, a clonal hybridoma cell line expressing the murine anti-vaccinia virus L1 mAb 7D11 was obtained and expanded in culture. Total RNA was prepared from one 7D11 hybridoma TRIzol (Invitrogen) suspension as per the manufacturer's suggested protocol. To generate cDNA from the 7D11 total RNA and elucidate the unknown sequence from the HC and LC, Clontech's SMARTer RACE cDNA Amplification Kit was used. PCR amplification of the 5′ portion of 7D11 heavy chain (*i.e.*, 5′ HC race) and the 5′ portion of 7D11 light chain (*i.e.*, 5′ LC race) was performed with Clontech's Advantage 2 PCR kit. The subsequent PCR products containing TA overhangs were subcloned into pCR8/GW/TOPO TA (Invitrogen). DNA from ten 5′ HC race clones and thirty 5′ LC race clones were restricted with *Eco*RI to determine successful ligation of the insert since *Eco*RI sites flank the insertion site within the TOPO vector itself. In total, three positive clones for the HC and LC inserts were sequenced to determine a consensus sequence for both chains. This sequence data for the heavy and light chains was also verified against independent sequence data generated at the U.S. Army Medical Research Institute of Infectious Diseases (USAMRIID), the source of the 7D11 hybridoma.

The overall approach to generate 7D11 chimeric expression plasmids was to construct both single and dual chain vectors retaining either the native leader

sequence or an alternate leader sequence from the previously described hybridoma cell line 2477. Two variants of light chain single vectors were produced that include: 1) Native, murine 7D11 5′UTR, leader, and variable domains ligated to the human constant kappa domain (designated LC-L) or 2) The 7D11 variable domain only ligated to the human constant kappa domain (designated LC-S) which was cloned downstream, in frame of the 2477 LC 5′UTR and leader sequence in the pBFksr:cLC vector. Similarly, two variants of heavy chain single vectors were produced that include: 1) native, murine 7D11 5′UTR, leader, and variable domains ligated to the human CH1 domain (designated HC-L) or 2) the 7D11 variable domain only ligated to the human CH1 domain (designated HC-S) which was cloned downstream, in frame of the 2477 HC 5′UTR and leader sequence in the pBFksr:cHC vector. Additionally, the HC-L and LC-L chains, which retain the native 7D11 leader sequences, were cloned into the dual chain expression plasmid. A separate dual plasmid containing HC-S and LC-S where the signal sequences are derived from the 2477 antibody was also generated. The 7D11 VK and VH were amplified with or without the native 5′ UTR and leader sequence and the cloning strategy is detailed for each chain in Figure 14 and Figure 15, respectively.

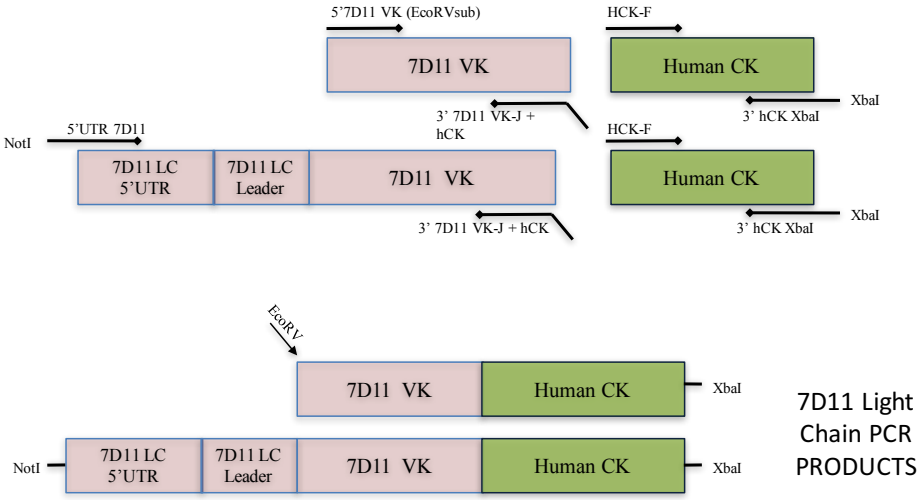


Figure 14. Chimeric 7D11 LC cloning scheme and associated PCR products. Key segments, primers and restriction sites are shown.

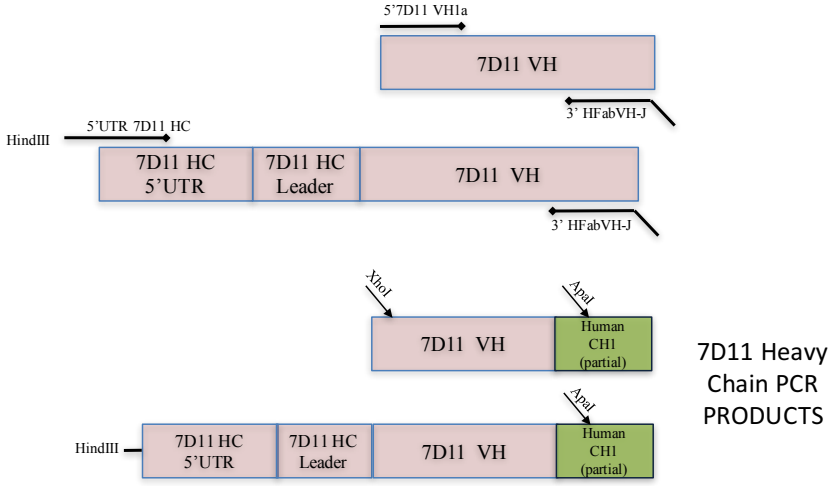


Figure 15. Chimeric 7D11 HC cloning scheme and associated PCR products. Key segments, primers and restriction sites are shown.

Generation of the chimeric 7D11 heavy chain began with the amplification of the 7D11 murine VH (HC-S) or the 7D11 murine 5′UTR, Leader, and VH (HC-L) from the pTOPO T/A plasmids outlined above. Briefly, the HC-S containing only the 7D11 VH was amplified with a 5′ primer containing several base pair mismatches to engineer an *Xho*I site ~5 a.a. internal to the start of the VH and a 3′ primer that anneals to the C-terminal VH sequence with an overhang of 7 a.a. to the N-terminus

of the human constant domain. The HC-L was amplified using a separate 5′ oligonucleotide that anneals at the beginning of the 5′UTR region containing an upstream engineered *Hind*III site. Figure 16 illustrates successful amplification of the heavy chain PCR product variants. Both HC-S and HC-L PCR products were subcloned into the pTOPO (Invitrogen) vector prior to restriction with *Xho*I/*Apa*I or *Hind*III/*Apa*I, respectively, and ligation into the similarly restricted pBFksr:cHC acceptor cassette.

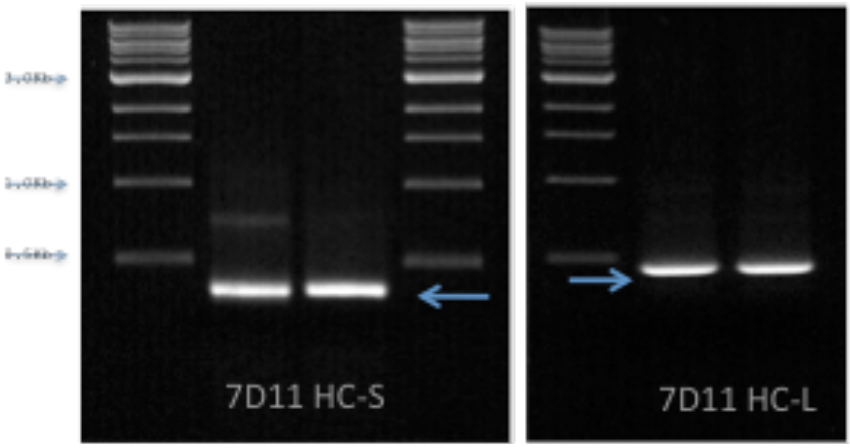


Figure 16. Amplification of 7D11 HC-S and HC-L PCR products. Central lanes containing the PCR products indicated with the blue arrow are flanked with 1kb MWM standards.

Construction of the chimeric 7D11 LC variants (LC-S and LC-L) utilized a series of PCR reactions where the various antibody domains are amplified individually, then linked together through a series of overlap PCR assembly reactions. Briefly, the LC-S containing only the 7D11 VK domain was amplified with a 5′ oligonucleotide containing a two a.a. mutation at the start of the VK to engineer an *Eco*RV site immediately downstream of the leader sequence cleavage site. The LC-L was amplified with a 5′ oligonucleotide that anneals to the native 5′UTR region with an engineered *Not*I restriction site immediately upstream of this sequence. The 3′

oligonucleotide used in amplification of both the LC-L and LC-S anneals to the C-terminal VK sequence with an overhang of 7 a.a. corresponding to the N-terminus of the human constant kappa domain. Separately, the human constant kappa (CK) domain was amplified with a 3′ primer generating an *Xba*I restriction site modification immediately downstream of the CK stop codon. All three PCR products were gel purified prior to co-amplification with the 5′ oligonucleotides used during amplification of the VK domain and a 3′ oligonucleotide used during amplification of the human CK domain. Figure 17 illustrates the successful amplification and PCR ligation of the LC-S and LC-L products. The LC-S and LC-L fragments were subcloned into the pTOPO vector, restricted with *Eco*RV/*Xba*I and *Not*I/*Xba*I, respectively, and ligated into the similarly restricted pBFksr:cLC expression vector.

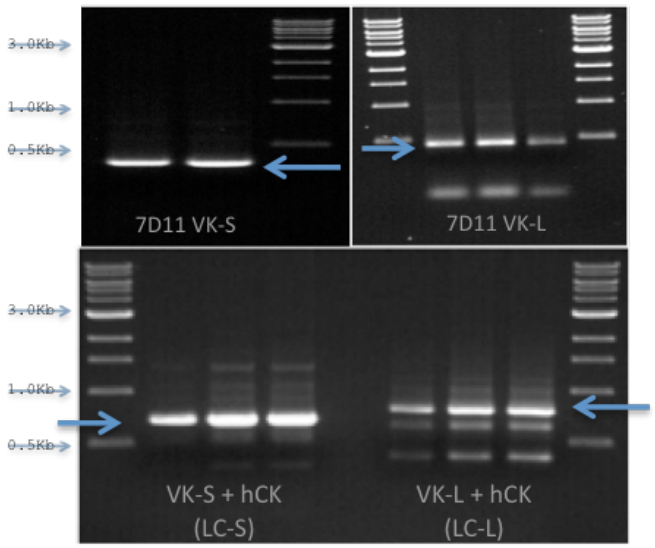


Figure 17. Amplification of 7D11 LC-S and LC-L PCR products. The ~350bp VK-S (top left) or ~450bp VK-L (top right) products were co-amplified with the ~350bp human constant kappa domain (not shown). The final PCR assembled LC domains including the attached human constant domain (700-750bp) are indicated by arrows (bottom).

To construct the chimeric 7D11 dual chain mAb expression vector containing both the c7D11 HC and c7D11 LC under separate transcriptional elements, the individual chains outlined above were restricted with their respective engineered restriction sites prior to ligation in the similarly restricted pBFksr:dual c2477 vector, effectively replacing the 2477 domains with c7D11 domains. The plasmid constructs with different leader sequences allow a direct comparison between 7D11 expression levels using either a native (7D11) or alternate (2477) leader sequence. All single and dual chain expression plasmids were confirmed for accuracy by sequencing (Macrogen, Rockville MD) prior to large-scale plasmid DNA preparation and transient expression experiments.

Transient Expression of Single and Dual Chain Chimeric 7D11 (c7D11) Constructs

Demonstration of chimeric mAb expression by single and dual c7D11 constructs was performed by transient transfection of CHO-S cells in serum-free suspension culture. For the single chain constructs in CHO-S cells, 25 μg total plasmid DNA (12.5 μg heavy chain construct and 12.5 μg light chain construct) was combined with 25 μL of FreeStyle MAX Reagent (Invitrogen) in 800 μL of OptiPRO SFM medium (Invitrogen). Lipid/DNA complexes were allowed to form during a 15 min incubation period and the transfection mix was added to 20 mL of suspension CHO-S cells at 1×10^6 vc/mL in a 125-mL shaker flask. For dual chain c7D11 constucts, 25 μg total plasmid DNA was used for each transfection.

In addition to the FreeStyle MAX Reagent (Invitrogen), two additional transfections were performed using polyethylenimine (PEI), a 25 kD linear polymer from Polysciences with the dual constructs. For the PEI transfections, 25 μg of each

dual c7D11 construct was diluted in 3.33 mL OptiPRO SFM medium. One hundred twenty five (125) microliters of 1 μg/μL PEI was added and the mixture was incubated for 15 minutes at room temperature. The transfection mix was added to 20 mL of suspension CHO-S cells at 1×10^6 vc/mL in a 125-mL shaker flask as above.

At 3 days post-transfection, cultures were counted and 1 mL samples were clarified for ELISA analysis. Briefly, an anti-human Fab specific reagent was coated to capture any human IgG. Dilutions of 1:5, 1:10, and 1:100 of clarified supernatant samples were added to the plate at 100 μL per well. HRP-labeled anti-human IgG (Fc-specific) was used as the detection reagent. Plate was developed with 3,3′,5,5′-tetramethylbenzidine (TMB) followed by H_2SO_4 to stop the reaction. In all transfections, ELISA analysis of clarified supernatants indicated significant expression of VACV L1-specific human/mouse chimeric mAb (see Figure 18). In addition, the constructs utilizing the alternate leader sequence from the previously described hybridoma cell line 2477 (denoted 'S') demonstrated a ~10-fold increase in transient mAb expression as indicated by the anti-hu Fab ELISA.

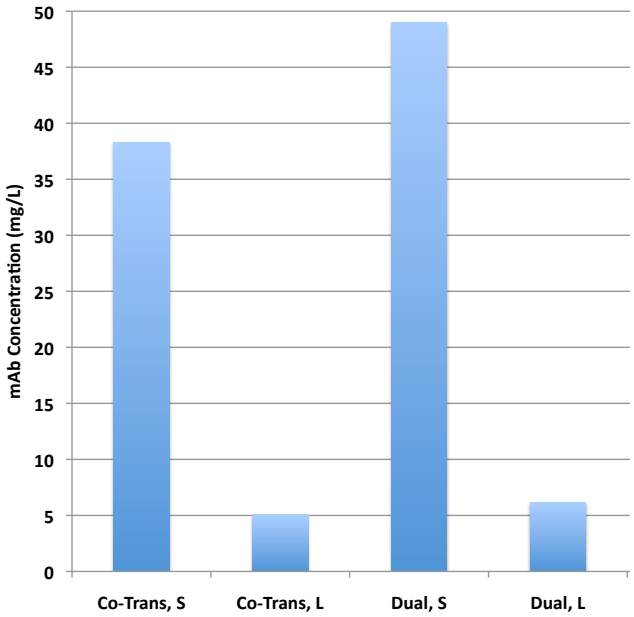


Figure 18. ELISA analyses of transient transfections by c7D11 constructs.

Construction of Optimized VACV α-B5, α-A33, and α-L1 mAb Expression Vectors

To further increase the expression levels of three different human chimeric mAbs (c7D11, c8A and c6C)[32-24] in the NS0 platform, all heavy and light chain genes used to generate stable cell lines were optimized and synthesized by GeneArt. The optimization process removes negative cis-acting sites when possible (*i.e.*, splice sites, poly(A) signals, TATA-boxes), corrects GC content to promote increased mRNA half life, and adjusts codon usage specifically for enhanced expression in a specified mammalian cell line such as the murine NS0 cell. In addition, an optimized consensus Kozak sequence (GCCACC) was inserted immediately upstream of the start codon (ATG) for each antibody chain gene construct. To facilitate cloning, flanking *Pme*I restriction sites were added to the 5′ and 3′ ends of each gene.

The overall dual chain vector construction approach is to restrict the GeneArt plasmids containing the optimized genes with *Pme*I prior to insertion and ligation in a

similarly restricted pBFksr plasmid which contains functional signal sequences native to the 2477 negative control mouse/human chimeric mAb demonstrated to increase expression by up to 10-fold. Each construct is analyzed by restriction digestion to determine the orientation of each inserted gene. Clones found to have the correct orientation were sequenced to confirm the antibody gene integrity and accuracy. The dual chain plasmids were constructed by first amplifying the CMV promoter-light chain gene-BGH poly(A) sequence from a light chain containing vector. The primers used for this PCR amplification contain flanking *Bam*HI restriction sites which were used to ligate the light chain PCR products in a *Bgl*II restricted, linearized heavy chain vector, thus generating the dual chain plasmids with the 3-*ksr* selection markers for antibody expression in NS0 cells.

The synthesized light chain and heavy chain genes, designated 6CLopt, 8ALopt, 7D11Lopt, 6CHopt, 8AHopt, and 7D11Hopt, were subcloned by GeneArt into the vector backbone pMK. The six lyophilized plasmids were resuspended and transformed into chemically competent One Shot TOP10 *E.coli* cells. Three colonies per transformation reaction were analyzed by enzyme restriction analysis prior to additional subcloning experiments.

Plasmid DNA isolated from the pMK:8AHopt transformant in addition to the pBFksr empty vector backbone were first restricted with *Pme*I. The pBFksr *Pme*I restricted plasmid reactions were heat inactivated prior to additional treatment with Antarctic Phosphatase to remove the vector 5' phosphate groups, thus decreasing the probability of vector self-ligation. All *Pme*I restricted components were gel purified prior to insertion, ligation, and subsequent transformation of the 8AHopt insert in the

pBFksr vector at a 3:1 insert to vector ratio. Since *Pme*I results in blunt-ended fragments, the transformants were analyzed for the presence and correct orientation of the 8AHopt gene. Figure 19 illustrates the *Stu*I restriction analysis for pBFksr8AHopt. The correct orientation restriction pattern results in 2379 bp and ~5.3 Kb bands, as indicated in clones 1, 4, 5, 6, and 8. An incorrect orientation restriction pattern results in 1144 bp and ~6.5 Kb bands. Similarly, plasmid DNA from pMK:8ALopt was restricted with *Pme*I, then inserted, ligated with *Pme*I restricted pBFdhfr vector backbone, and transformed into TOP10 *E.coli* as outlined above. Resulting transformants were analyzed by restriction analysis for the presence and correct orientation of the 8ALopt gene. Figure 20 outlines the *Dra*III restriction analysis patterns for pBFdhfr8ALopt clones. Although six of eight clones analyzed contained the desired insert, only clone 4 had the light chain correctly oriented.

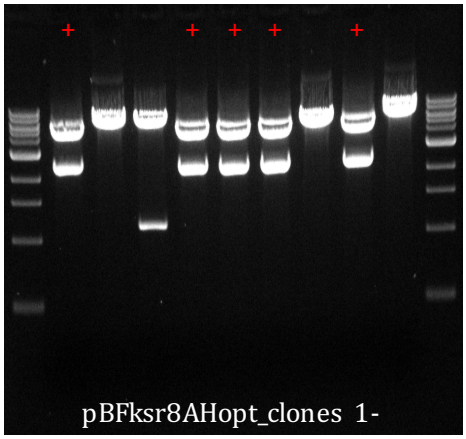


Figure 19. Restriction analysis of pBFksr8AHopt clones 1-8 restricted with *Stu*I. Lane 1, 11: 1Kb MWM; Lane 2-9: Clones 1-8; Lane 10: pBFksr empty vector. Clones with the correctly oriented insert are indicated by +.

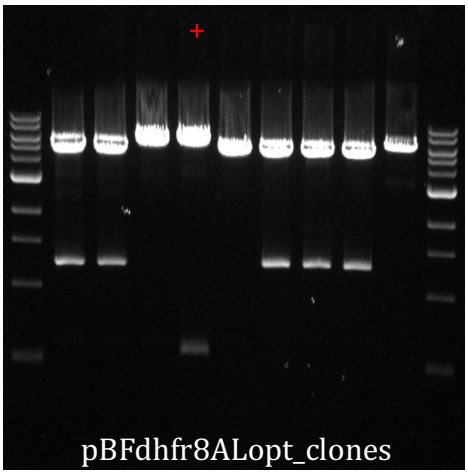


Figure 20. Restriction analysis of pBFdhfr8ALopt clones 1-8 restricted with *Dra*III. Lane 1, 11: 1Kb MWM; Lane 2-9: Clones 1-8; Lane 10: pBFdhfr empty vector. Clone with the correctly oriented insert is indicated by +**.**

To facilitate generation of the c8A dual chain construct, a primer set was designed to PCR amplify the entire region spanning the CMV promoter, light chain gene (8ALopt), and BGH poly(A) with flanking *Bam*HI restriction sites. The PCR amplification of the light chain cassette, performed with Phusion High-Fidelity DNA Polymerase (Finnzymes), generated a ~2.5Kb PCR product that was subcloned in pCR-Blunt II-TOPO (Invitrogen), designated pTOPO:CMV:8ALopt:BGH. Resulting transformants were analyzed by restriction analysis prior to restriction with *Bam*HI to facilitate cloning of the light chain cassette in the *Bgl*II linearized, Antarctic Phosphatase treated, gel purified pBFksr8AHopt plasmid. Since *Bgl*II and *Bam*HI restriction sites contain compatible cohesive ends, the 8ALopt cassette fragment containing flanking *Bam*HI restriction sites was ligated into the *Bgl*II linearized 8AHopt-containing vectors at a 3:1 light chain insert to vector ratio, transformed, and analyzed by both PCR and restriction analysis for the presence and orientation of the 8AL cassette. Figure 21 illustrates the initial PCR screen of the 8A dual chain

transformants for the light chain. Ten of ten pBFksr8A dual clones were positive for the presence of the light chain cassette. To determine the orientation of the light chain cassette insert in the pBFksr8A dual clones, clones 1-6 were restricted with *Sac*II which results in banding patterns of 2752 bp and 7165 bp or 1585 bp and 8332 bp if the insert is correctly or incorrectly oriented, respectively. Figure 22 demonstrates the correctly oriented 8ALopt cassette insert in the pBFksr8A dual construct in clones 1, 2, and 6 whereas clones 3-5 are incorrectly oriented.

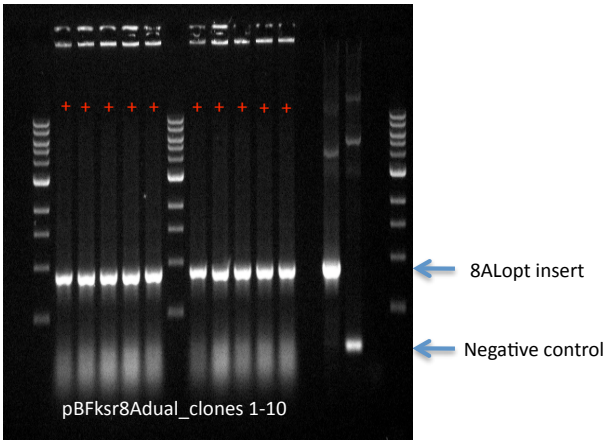


Figure 21. PCR analysis of pBFksr8Adual clones for 8ALopt insert. Lane 1,7,17: 1Kb MWM; Lane 2-6, 8-12: Clones 1-10; Lane 14: Light chain positive control from pBFdhfr8ALopt clone 4; Lane 15: pBFdhfr8AHopt clone 1 negative control. Clones positive for the 8AL gene insert are indicated by +.

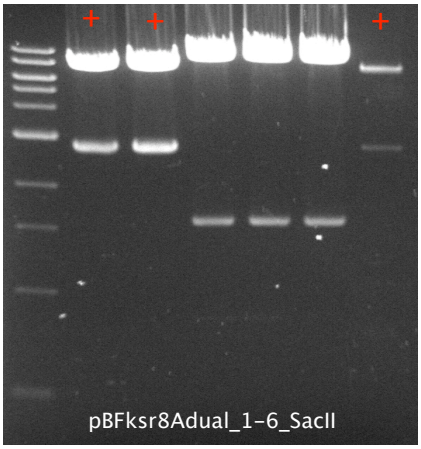


Figure 22. Restriction analysis of pBFksr8Adual clones. pBFksr8Adual clones 1-6 restricted with *Sac*II. Lane 1: 1Kb MWM; Lane 2-7: Clones 1-6. Clones with the correctly oriented insert are indicated by +.

Dual chain, optimized vectors for expression of 6C and 7D11 were similarly constructed following the approach outlined above.

Characterization of Optimized StableFast-NS0 (SF-NS0) mAb Expression Vectors

Expression vectors were optimized and constructed for production of the three anti-vaccinia virus mAbs in the StableFast-NS0 system as described above. These vectors were analyzed for mAb expression and compared with the previous un-optimized SF-NS0 expression vectors in CHO-S transient cultures (30 mL working volumes). Each of the optimized and previous generation expression vectors were used to transiently transfect shaker cultures as described above. Cultures were allowed to incubate with shaking for four days post-transfection. Clarified supernatants from each culture were analyzed for human IgG by capture ELISA as described above. Results are shown in Figure 23. Expression of each mAb was significantly enhanced for each optimized vector as compared to the previous generation vector. Production of mAbs c7D11, c8A, and c6C were increased by 4.5-,

7.4-, and 3.7-fold, respectively. These increases in transient expression may be predictive of increases in productivity of stable NS0 cells lines for each of the mAbs.

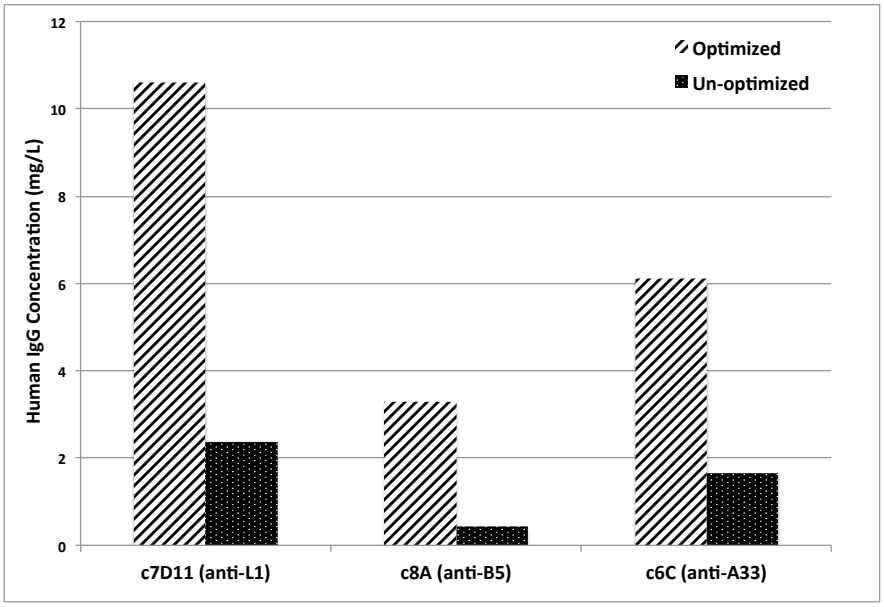


Figure 23. Concentration of human IgG in transient CHO-S cultures: Optimized vs un-optimized SF-NS0 expression vectors.

Conclusions

The initial p3KSR expression vector containing the Hsd17β7 selection marker was further optimized for the expression of IgGs. The addition of the intron A segment of the CMV-MIE promoter that drives expression of both the light and heavy mAb chains significantly increased expression of the recombinant antibody. A high expressing murine hybridoma cell line 2477 was exploited by sequencing and transferring its 5′ UTR and leader or signal peptide (SP) to the p3KSR vector. This resulted in a 10-fold increase in expression of a chimeric mAb as compared with the mAb's native SPs. To further increase expression levels, heavy and light chain genes used to generate stable cell lines were optimized and synthesized for three different human chimeric mAbs in the NS0 platform. This included codon optimization for the murine background, screening and

elimination of cryptic splice sequences, correction of GC content to promote increased mRNA half life, and the addition of an optimized consensus kozak sequence prior to each open reading frame. This *in silico* optimization led to multi-fold increases in expression as compared with the original un-optimized genes for all three constructs.

Chapter 3.
Transfection, selection and single-cell cloning optimization

Abstract

In addition to productivity, a cell line platform must be built on a foundation of robust transfection, selection and cloning protocols as well as reproducible characterization methods. In this Chapter 3, a number of studies were performed to optimize both bulk pool and miniwell approaches to stable cell line development. Methods were developed at several different scales to characterize cell line specific productivity rate or Qp to enable the ranking of emerging isolates and clones based on growth and expression. In an early demonstration of scalability and cell line characterization, a miniwell selection approach was applied to the product mAb 21, a monoclonal directed at a vaccinia virus (VACV) subunit protein, generating the best performing isolate #7 that was rapidly scaled to the 5-L bioreactor for production and purification with volumetric yields in the 18-28 mg/L range. This same early cell line was evaluated for expression stability at 52 generations. These cell line generation methods were applied and refined for three additional mAb products including the addition of a rapid 24-hour productivity assay for early screening of high numbers of isolates and clones resulting in yields nearly twice that of Isolate #7. In addition, combinations of defined and animal-free supplements were evaluated during both selection and limiting dilution cloning (LDC) processes in order to eliminate animal serum from the entire cell line development workflow ultimately allowing for the elimination of all serum from the cell line development process with the production of conditioned medium using a null NS0 cell line.

Results and Discussion

StableFast-NS0 (SF-NS0) mAb 21 Cell Line Generation

A mammalian expression vector containing monoclonal antibody (mAb) 21 was constructed for experiments using Hsd17β7 as the selection marker in NS0 cells, the platform now called StableFast-NS0 (SF-NS0). An expression vector pCdhfr:intA:mAb21 dual opt was built from pBF*dhfr*.2 which is a *dhfr*-based vector that contains a CMV promoter with the CMV intron A sequence and has a modified multiple cloning site for optimized cloning of recombinant eukaryotic genes in *dhfr*(-) CHO cells as well as optimized (opt) mAb 21 heavy and light chain sequences. To generate a mAb21 vector containing Hsd17β7, the pCdhfr:intA:mAb21 dual opt construct was digested with *Pml*I, treated with Antarctic Phosphatase (New England Biolabs) and gel purified to remove the *dhfr* (~600bp) fragment. The Hsd17β7 gel purified insert was subsequently ligated into the above vector generating pC17β7:intA:mAb21 dual opt. Because *Pml*I restriction sites are blunt-ended, pC17β7:intA:mAb21 clones were screened for the correct orientation of the 17β7 insert with the *Nhe*I restriction site present at the 5' end internal to the 17β7 gene. The *Nhe*I restriction site facilitates not only screening the directional orientation of 17β7 but also distinguishes *dhfr* from 17β7 containing constructs since there is no *Nhe*I site internal to the *dhfr* gene (see Figure 24).

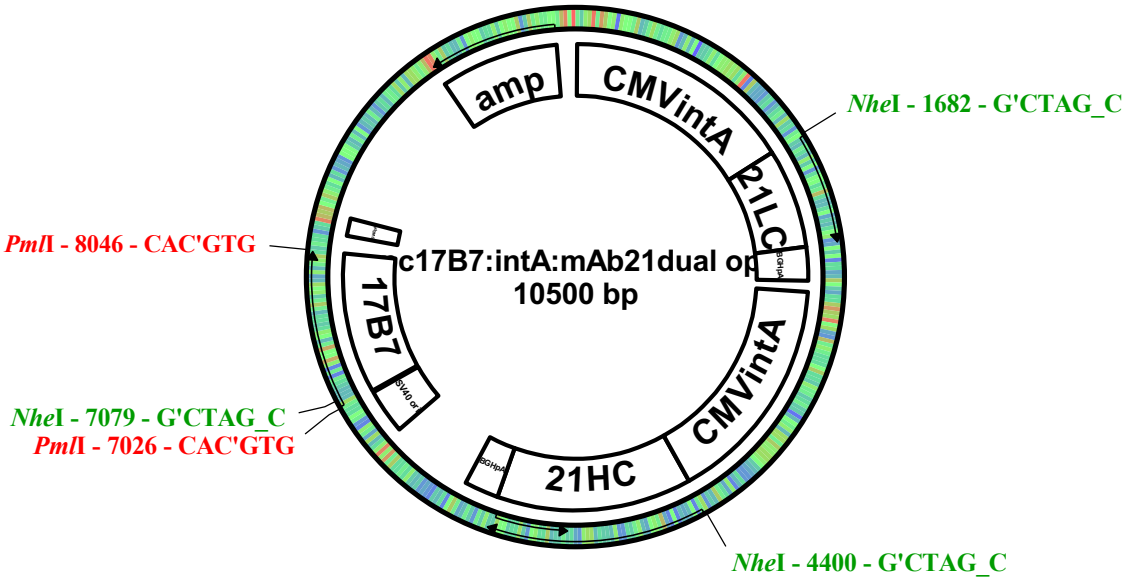


Figure 24. Plasmid map of pc17β7:intA:mAb21 dual opt. Key segments and restriction sites are indicated.

Transient expression studies were performed in human endothelial kidney (HEK)-293T/17 cells (ATCC CRL 11268), which were maintained in complete high glucose Dulbecco's Modified Eagle medium (DMEM) supplemented with 2 mM of L-glutamine, 1x NEAA, and 10% fetal bovine serum (FBS). Antibody expression was analyzed in HEK-293T/17 cells transiently transfected with the above mammalian expression vectors, which were prepared using the QIAgen plasmid filter miniprep spin kit (QIAgen). Briefly, $1x10^6$ cells were seeded per well of a poly-D-Lysine-coated 6-well plate in 2 mL of cDMEM. After overnight incubation at 37°C, 5% CO_2, cells were transfected with unrestricted recombinant plasmid DNAs using the cationic lipid reagent Lipofectamine™ 2000 (Invitrogen) according to the manufacturer's instructions. Transfections were incubated for 72 h at 37°C, 5% CO_2, and subsequently, cell culture supernatants were collected, clarified by centrifugation, and analyzed by ELISA specific for human IgG1. Results indicated comparable

expression levels of mAb 21 in pC17β7:intA:mAb21 dual opt and the original *dhfr*-based vector, pCdhfr:intA:mAb21 dual opt.

Parental NS0 cultures with a 22-24 hour doubling rate, greater than 95% viable, without morphological aberrations, and without significant cell debris present in the culture medium were used for the stable transfection. Fifty micrograms of highly pure, *Pvu*I or *Ahd*I linearized plasmid DNA were mixed in an electroporation cuvette with 12 million CD-SFM-NS0 cells and gently mixed. A single pulse of 250 volts (V), 400 micro-Faraday (μFd) was delivered to the cuvette. The time constants were consistently under 8 microseconds (ms) to ensure proper delivery of DNA and cell viability. Cells were subsequently resuspended in 12 mL of NS0 Growth Medium and were seeded in a T-75 flask overnight to allow for recovery. The following day cells were gently pelleted by centrifugation at 200×*g* for 5 minutes at room temperature to remove growth medium with cholesterol. The cell pellet was resuspended in 1 mL of selective medium and the entire cell suspension was transferred to 800 mL of Delipidated Supplemented Selection Medium (without cholesterol) and plated on 40 flat bottom 96-well culture plates, with 200 μL of cell suspension dispensed per well. Approximately 2,500 cells were plated per well using this dilution strategy. On average, only 5-7 wells per plate will contain colonies following a 2-3 week incubation under selective growth conditions. The expected transfection and selectable efficiency is, therefore, in the order of 3×10^{-5}. Plates were incubated at 37^{o}C, 5% CO_2, 90% Rh for 2-3 weeks, at which time the transfection and selection efficiencies were scored and supernatants assayed for presence of secreted IgG by ELISA.

Three weeks post-transfection, 33 wells had significant, visible cell growth and the cell culture supernatants were subsequently screened by human IgG1-specific ELISA. Fourteen of the 33 colonies (designations 2, 4, 5, 7, 13, 17, 21, 24, 26, 27, 28, 29, 31, and 33) exceeded 43 ng/mL of antibody in the respective supernatants. The 14 clones producing mAb21 in SF-NS0 cells were scaled up to T-75 flasks in selective medium prior initiation of Specific Productivity Rate (Qp) analysis. Briefly, each isolate was seeded at a density of 3×10^5 viable cells/mL and allowed to grow for 3 days. At the end of the growth period, cells were counted and supernatants were analyzed for mAb 21 via ELISA. These growth and expression results are used to calculate the specific productivity rate in pg mAb 21 per cell per day. The value for Qp was determined based on the calculation of cumulative viable cell time (CVCT) or cumulative volumetric cell-hours.[35-37] The integrated cell area (ICA) associated with any time points was calculated as follows:

$$Integrated\ Cell\ Area\ (ICA) = \frac{x_1 - x_0}{\ln\left(\frac{x_1}{x_0}\right)} \times t_1 - t_0$$

where x_0 is the viable cell density at t_0; x_1 is the viable cell density at t_1; t_0 is the elapsed time in days at first sample and t_1 is the elapsed time at second sample. Specific productivity rate (Qp) was calculated using the equation:

$$Specific\ Productivity\ Rate\ \left(Q_p\right) = \frac{[mAb]_1 - [mAb]_0}{\text{ICA}}$$

where [mAb] is the concentration of monoclonal antibody product at time 1 and time 0.

As shown in Table 1, isolates #7 and #27 highlighted in green had the highest Qp values and were scaled up to shaker and spinner suspension culture. Further

productivity analysis indicated that isolate #7 was the superior cell line and was, therefore, utilized for process development and bioreactor studies.

Table 1. SF-NS0 isolates expressing mAb 21 – Specific Productivity Rate (Qp).

Isolate Number	Seed Density (vc/mL)	Final Density (vc/mL)	mAb 21 (ug/mL)	Doubling Time (h)	Productivity (pg/cell/day)
5	3.0E+05	2.40E+06	0.242	23.3	0.060
7	3.0E+05	2.10E+06	1.583	24.9	0.440
17	3.0E+05	3.56E+06	0.591	19.6	0.102
21	3.0E+05	1.70E+06	0.093	28.0	0.031
26	3.0E+05	1.56E+06	0.379	29.4	0.136
27	3.0E+05	1.97E+06	1.613	25.8	0.474
28	3.0E+05	1.71E+06	0.237	27.9	0.079
31	3.0E+05	2.03E+06	0.153	25.4	0.044
33	3.0E+05	2.95E+06	0.238	21.2	0.049

SF-NS0 mAb 21 Cell Culture Process Development and Scale Up

An initial process optimization study for cell line isolate #7 expressing mAb 21 focused on the analysis of fresh versus spent medium for consumption rates of essential metabolites, such as amino acids and primary carbon sources, by a high performance liquid chromatography (HPLC)-based methodology. These initial studies identified specific rate limiting metabolites that are rapidly depleted in high density cultures in the stirred tank bioreactor. The HPLC data was used to determine both the timing and formulation of a bolus feed during a fed-batch bioreactor run at the 5-L scale to demonstrate system scalability including growth and recombinant antibody production.

To serve as a predictor for the bioreactor culture performance, a 7-day time course was performed in a 250-mL spinner flask with a 120 mL culture volume. Fresh batch medium was seeded to an initial density of approximately 5×10^5 cells/mL. The culture was grown in controlled conditions with samples taken each day for cell

counts and spent media analysis. The batch medium formulation was CD Hybridoma with 4 mM GlutaMAX-I (Invitrogen, Cat. No. 35050-061) and 1×NEAA. Culture conditions were 37°C, 5% CO_2, 95% relative humidity, and 40 rpm agitation. The HPLC methodology was used to identify several key amino acids that were preferentially consumed during the course of the culture growth. As the culture proceeded through Days 4-6, we observed preferential depletions of the following amino acids: Lysine, asparagine, phenylalanine, tyrosine, and glutamine. As a basis for the Day 5 amino acid feed, we calculated the amounts of each amino acid depleted at Day 5, multiplied that number by 2, and again multiplied by the culture volume. A special case was glutamine which has been historically regarded as a critical nutrient source for NS0 cells and was shown to rapidly deplete over the first 2-3 days in the time course culture. For glutamine, we added a quantity to the first feed on Day 4 to result in a final concentration of 8 mM, a widely used concentration for batch culture.[38] Finally, sodium butyrate was added as a third feed on Day 6 as the cell density and viability were beginning to decline. Sodium butyrate has been shown to increase expression levels of monoclonal antibodies in NS0 cultures.[39] Compositions and timing for feeds are shown in Table 2.

Table 2. Composition of feeds for SF-NS0 mAb 21 bioreactor (080417R1).

Component	Feed 1 Day 4 (mM)	Feed 2 Day 5 (mM)	Feed 3 Day 6 (mM)
Glutamine	8.00		
Lysine		0.552	
Asparagine		1.07	
Phenylalanine		0.183	
Tyrosine		0.384	
Sodium Butyrate			2.50

Isolate #7 expressing mAb 21 was scaled up to the 5-L bioreactor. The T-flask culture emerging from the screening process was transferred to shaker flask culture for a single passage and then immediately to spinner flask culture for scale up. Cells remained highly viable throughout expansion. As each seed stage reached the appropriate density range (~1.0-2.2×10^6 viable cells/mL), the culture was scaled up to the next vessel (see Figure 25).

SF-NS0 bioreactor #1 (080417R1) was inoculated with 1250 mL Stage 3 seed culture for an initial density of 4.30×10^5 viable cells/mL. The bioreactor proceeded for 7 days and was fed the glutamine (Feed 1), amino acid (Feed 2), and sodium butyrate (Feed 3) described above. On Day 7, the bioreactor was stopped and supernatant was harvested by centrifugation and filtered at 0.22 μm for further analysis and purification (see Figure 26).

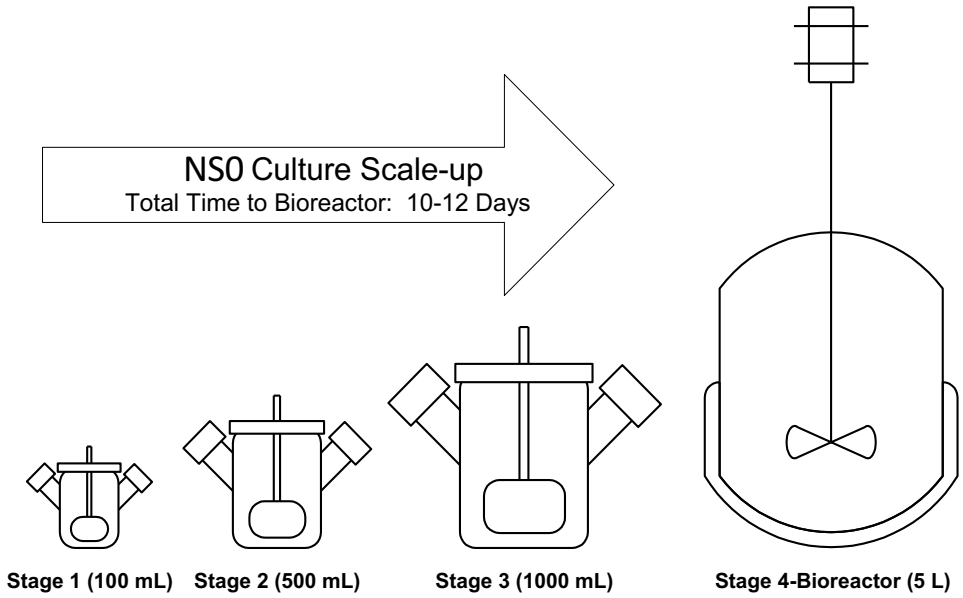


Figure 25. Scale-up strategy for 5-L bioreactor. Graphic indicates each seed stage volume and overall scale-up timing for a 5-L bioreactor.

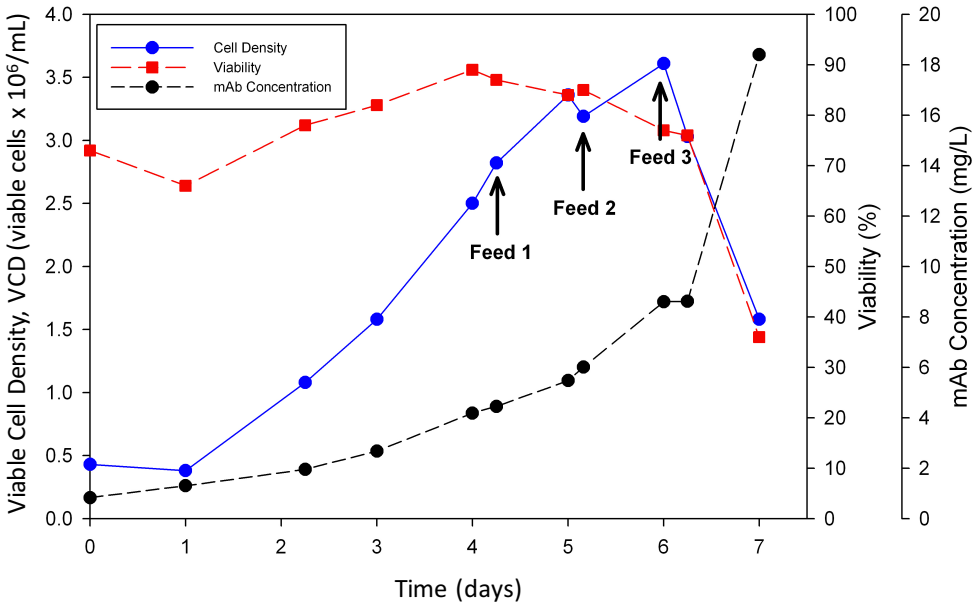


Figure 26. Growth and productivity curve for SF-NS0 mAb 21 bioreactor (080417R1). Viable cell density is indicated by the solid blue curve, culture viability is indicated by the dashed red curve and mAb concentration is indicated by the dashed black curve. Glutamine (Feed 1), amino acid (Feed 2), and sodium butyrate (Feed 3) additions are indicated by the black arrows.

In a parallel experiment, three shaker flasks using aliquots of the bioreactor backup cell culture were grown over 4 days. To evaluate the addition of sodium butyrate to the SF-NS0 culture, the following feeds were administered on Day 2: Shake 1-0.0 mM, Shake 2-1.0 mM, Shake 3-0.5 mM. The negative control shaker (Shake 1) achieved the highest cell density (5.40×10^6 viable cells/mL) on Day 3 while the 1.0 mM (Shake 2) and 0.5 mM (Shake 3) cultures achieved 3.29×10^6 viable cells/mL and 3.74×10^6 viable cells/mL, respectively. On Day 4 cultures were harvested and clarified supernatants were analyzed via ELISA for mAb 21 concentration. All cultures produced mAb 21 and similar volumetric levels at about 30 mg/L.

mAb21 SF-NS0 shaker flask (Shake 1, 0.0 mM Sodium Butyrate) supernatant (25 mL) was centrifuged and then clarified by consecutive filtration through 0.45 µm and

0.2 μm filters. The supernatant was diluted with an equal volume of PBS buffer and then loaded onto a 6.6×44 mm Prosep vA high Capacity column (Millipore) at 90 cm/hr. The resin was then washed with 10 column volumes (CVs) PBS buffer, followed by 5 CVs 50% PBS & 50% 2M NaCl, and 5 CV 0.2M Glycine, pH 5.5. mAb21 was eluted with 0.2M Glycine, pH 3.5. The column was regenerated with 5CV 0.2M phosphoric acid, pH 1.5 and re-equilibrated with 10 CVs PBS. The pH of the mAb21 eluate was immediately neutralized by adding 1/10th volume 1M TrisHCl, pH 9.0. A 50 mL aliquot of mAb21 SF-NS0 cell culture supernatant from bioreactor run 080417R1 was also purified as above.

As seen in previous purifications of human IgG mAbs using Millipore's ProsepVa High Capacity resin, final antibody preparations exceeded 90% purity (see Figure 27). As seen in Table 3, the SF-NS0 shaker culture yielded a slightly higher volumetric yield than the SF-NS0 culture from the bioreactor run, consistent with the results obtained by ELISA. The average volumetric culture yield obtained with these two SF-NS0 cultures (28 mg/L) were the same as the average yield obtained in previous purifications of mAb21 from CHO cell line cultures using CD-CHO and CHO-S-SFM media (Table 3).

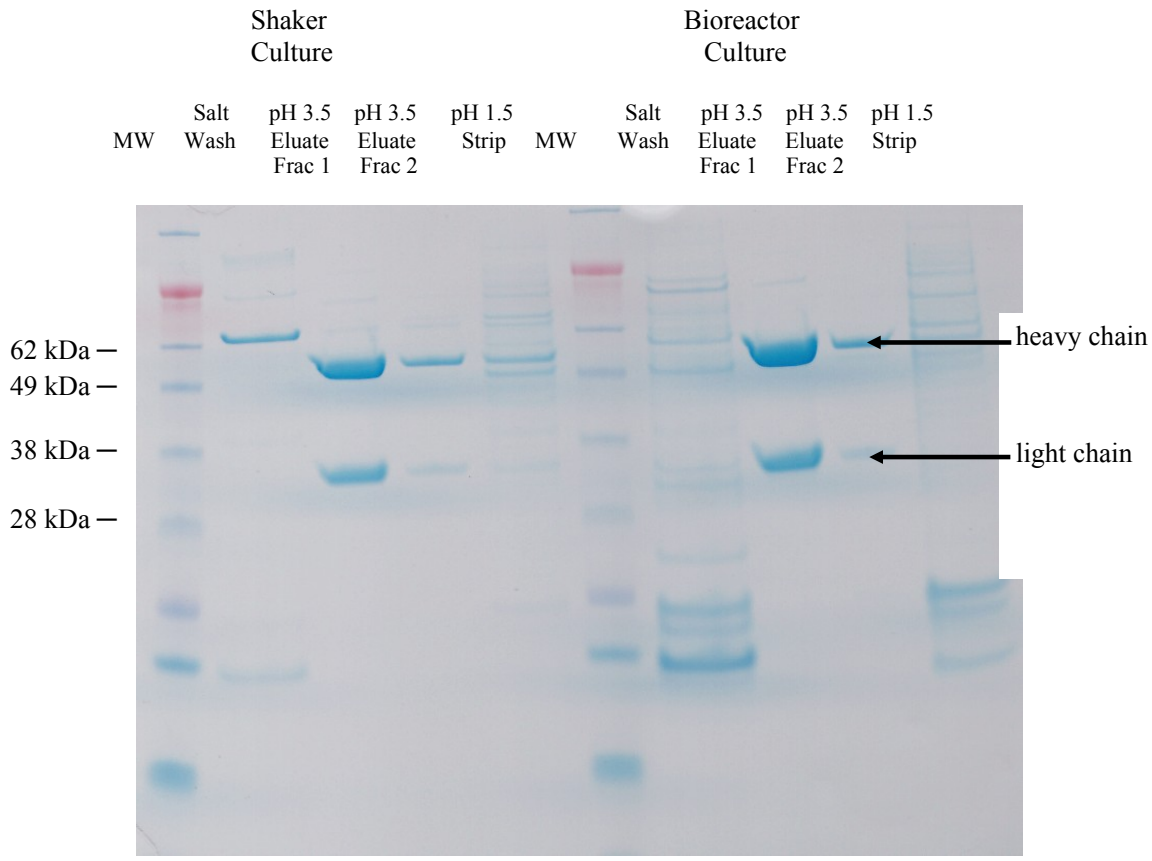


Figure 26. SDS-PAGE of capture of mAb21 with Prosep Va High Capacity resin. Molecular weights and heavy and light chain bands are indicated.

Table 3. mAb 21 purification yields.

Culture	Purification Yield *	Volumetric Culture Yield (mg/L)	Average Yield (mg/L)
SF-NS0 Shaker	0.91 mg/25 mL	33	28
SF-NS0 Bioreactor	1.31 mg/50mL	23	
CHO – CD medium	7.63 mg/500 mL	15	28
CHO – SFM	39.15 mg/960 mL	41	

** Based on theoretical extinction coefficient of 1.4 and 90% purity by SDS-PAGE*

SF-NS0 mAb 21 Cell Culture Stability Study

Cell line stability is a critical factor for process scale up. For large-scale commercial manufacturing, cell lines routinely grow for over 25 generations to achieve high-density culture in the 10,000-20,000 liter production bioreactor. A preliminary study to determine cell line stability was performed for the SF-NS0 mAb 21 isolate #7. In the previous study, this cell line was scaled up to the 5-L bioreactor in fed-batch mode with a final purified mAb volumetric yield of 23 mg/L of culture supernatant. This initial bioreactor run was started at 11 generations after suspension adaptation. To examine long-term stability of the cell line, a portion of the seed culture at generation 11 was propagated in spinner culture until generation 52 was reached (about 7 weeks). This culture was used to inoculate a 5-L fed-batch bioreactor.

As seen in Figure 27, the 52-generation culture grew better than the initial bioreactor with a peak density of about 7×10^6 viable cells/mL and better early viability (data not shown). Both cultures proceeded for 7 days and each had 3 feeds. The volumetric yield of the stability culture was only about 7 mg/L as compared to the volumetric yield of over 18 mg/L for the initial culture. This loss of over half of the productivity accompanied by an increase in growth performance may indicate cell line instability. Another possibility of the observed decrease in productivity and increase in growth may be due to the non-clonal nature of Isolate #7 (*e.g.*, better growing non-producers proliferated during long-term propagation). Further studies are warranted and include interim testing during long-term propagation and limiting dilution cloning (LDC) of Isolate #7 to ensure clonality.

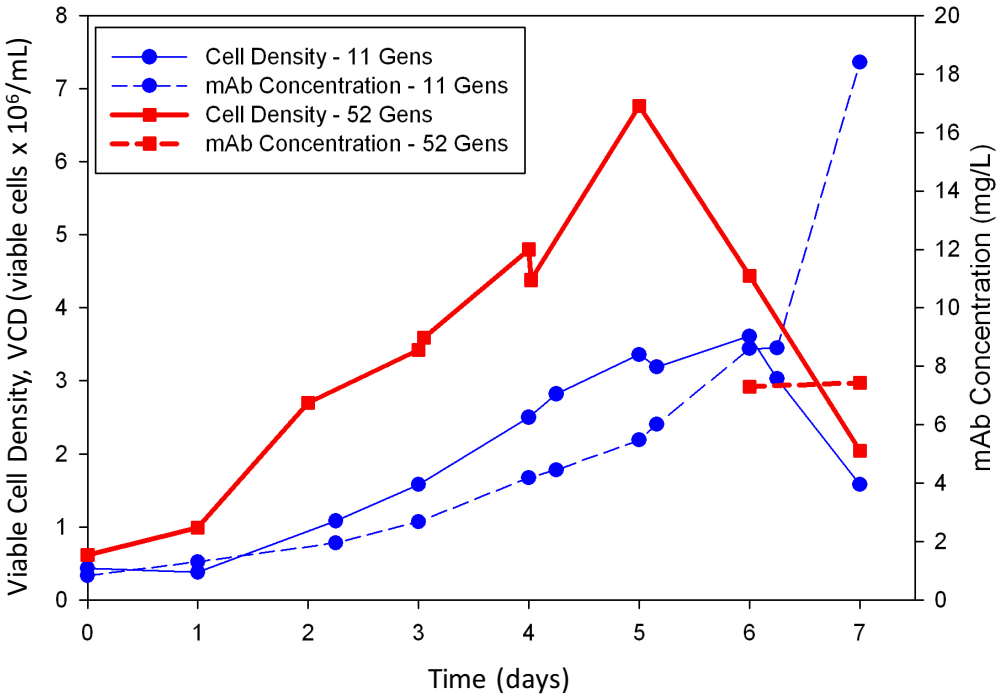


Figure 27. Growth and productivity curves for SF-NS0 mAb 21 (Isolate #7) stability study. Viable cell density is indicated by the solid curves and mAb concentration is indicated by the dashed curves. Blue curves with circle markers indicate the 11 generation run and red curves with square markers indicate the 52 generation run.

Adaptation and Scale Up of SF-NS0 Non-Optimized mAb 14 Cell Lines

SF-NS0 cell lines expressing the original, non-optimized version mAb 14 (α-VACV D8), but with the CMV intron-A sequence added to each expression cassette were generated. Upon selection in miniwell format (40×96-well plates), 23 isolates were selected to scale up for Qp analysis (see Table 4). Overall, best performing isolates surpassed the previously generated cell lines expressing mAb 14 by over four-fold. Isolate #171 was selected for suspension adaptation. The cells adapted to spinner culture rapidly, maintained high expression levels, and were banked.

Table 4. Qp analysis of SF-NS0 isolates expressing mAb 14—Highest producers highlighted.

Isolate Number	Seed Density (vc/mL)	Final Density (vc/mL)	mAb 21 (ug/mL)	Doubling Time (h)	Productivity (pg/cell/day)
3	3.0E+05	1.96E+06	4.31	26.6	1.271
6	3.0E+05	7.50E+05	10.98	54.5	6.971
14	3.0E+05	1.90E+06	2.79	27.0	0.845
22	3.0E+05	1.66E+06	1.06	29.2	0.361
25	3.0E+05	1.32E+06	5.3	33.7	2.181
45	3.0E+05	2.00E+06	3.39	26.3	0.983
58	3.0E+05	1.82E+06	0	27.7	0.000
96	3.0E+05	1.56E+06	2.95	30.3	1.057
131	3.0E+05	3.26E+06	7.36	20.9	1.378
305	3.0E+05	1.76E+06	8.08	28.2	2.615
147	3.0E+05	4.28E+06	0.462	18.8	0.067
164	3.0E+05	2.40E+06	5.66	24.0	1.398
171	3.0E+05	1.44E+06	8.53	31.8	3.268
173	3.0E+05	2.38E+06	2.14	24.1	0.532
226	3.0E+05	4.16E+06	2.17	19.0	0.324
256	3.0E+05	1.40E+06	2.98	32.4	1.169
275	3.0E+05	1.82E+06	4.2	27.7	1.321
314	3.0E+05	2.86E+06	8.93	22.1	1.884
349	3.0E+05	1.64E+06	1.47	29.4	0.505
378	3.0E+05	3.90E+06	2.2	19.5	0.349
396	3.0E+05	2.64E+06	2.45	22.9	0.556
6 (2^{nd} SPR)	3.0E+05	2.32E+06	23.6	31.8	4.504
171 (2^{nd} SPR)	3.0E+05	2.77E+06	28.8	29.3	4.691
305 (2^{nd} SPR)	3.0E+05	2.58E+06	8.56	30.3	1.486
314 (2^{nd} SPR)	3.0E+05	2.24E+06	23.6	32.4	4.646
415	3.0E+05	2.90E+06	0.325	22.0	0.068
419	3.0E+05	2.20E+06	9.31	25.0	2.483

A cell bank vial of SF-NSO mAb 14 #171 was thawed and resuspended directly into 50 mL spinner flask culture. A growth and productivity time course was performed at the 120-mL spinner flask scale. As seen in Figure 28, the volumetric yield of the second SF-NS0 cell line (SF-NS0 mAb 14 Isolate #171) is twice that of the first prototype cell line (SF-NS0 mAb 21 Isolate #7) in spinner flask culture.

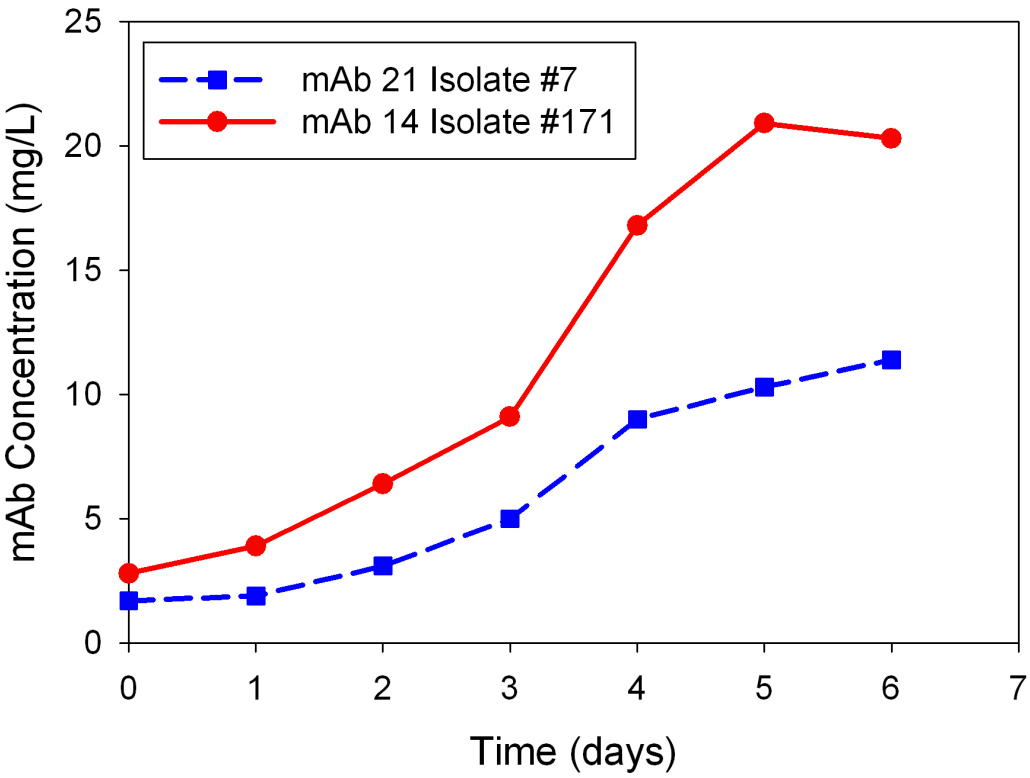


Figure 28. Volumetric yield curves for SF-NS0 mAb 21 Isolate #7 and mAb 14 Isolate #171. Blue dashed curve with square markers indicates the volumetric productivity for Isolate #7 and red solid curve with round markers indicates the volumetric productivity for Isolate #171.

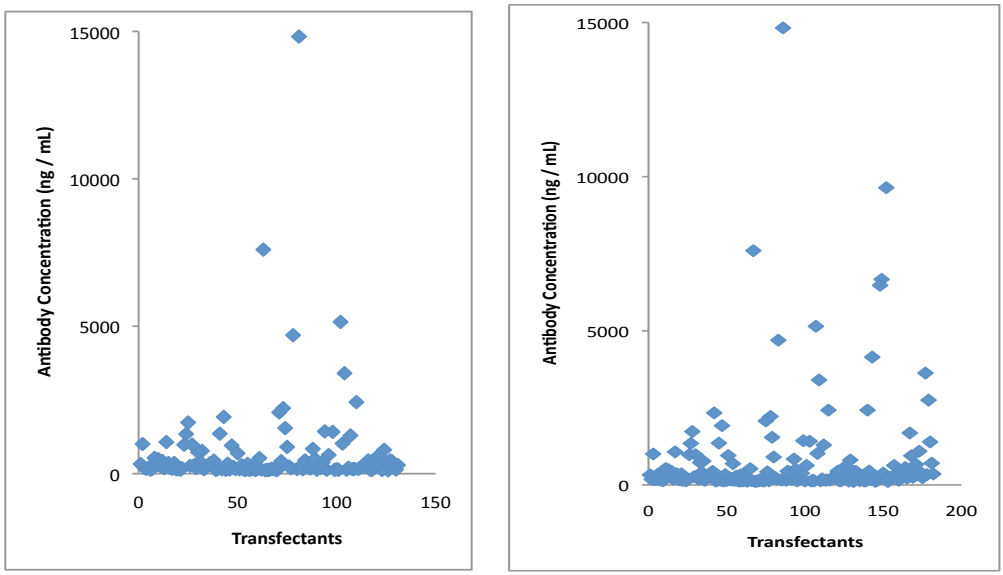
SF-NS0 Stable Cell Line Generation Using the pBFksr:c7D11dualS#1 Vector

Using the StableFast-NS0 system, stable cell lines expressing the anti-L1 chimeric mouse/human mAb c7D11 were generated using the SF-NS0 parental cell bank. Two identical transfections were performed to increase the resulting number of transfectants and the likelihood of isolating a high-expressing cell line. For each transfection, forty five (45) µg of highly pure, *Pvu*I linearized plasmid DNA (pBFksr:c7D11dualS#1) was mixed in an electroporation cuvette with 12×10^6 NS0 cells that had been previously serially passaged three times. A single pulse of 250 V, 400 µFd was delivered to each cuvette. The time constant was 5.4 and 5.6 ms for

tranfection one and two, respectively. Cells were subsequently resuspended in 12 mL of NS0 Growth Medium and were seeded in a T-75 flask overnight to allow for recovery. The following day cells were gently pelleted by centrifugation at 200×*g* for 5 minutes at room temperature to remove growth medium with cholesterol. Each of the two cell pellets were resuspended in 800 mL of Delipidated Supplemented Selection Medium (without cholesterol) and plated on 40 flat bottom 96-well culture plates at 200 μL of cell suspension dispensed per well. Approximately 2,500 cells were plated per well using this dilution strategy in a total of ~80 96-well plates. Plates were incubated at 37°C, 5% CO_2, 90% Rh for 2–3 weeks, with weekly 1:1 medium replacement feeds, at which time supernatants of actively growing wells were assayed for presence of secreted IgG by ELISA. Two and a half weeks post-transfection, 2265 wells had significant, visible cell growth and the cell culture supernatants were subsequently screened by a human IgG-specific capture ELISA.

One hundred ninety one (191) of the 2265 colonies exhibited significant IgG expression (over twice the background signal) and were scaled from 96-well plates to 12-well plates, and eventually 6-well plates in selective medium. After reaching confluency in the 6-well plates, the transfectants were further evaluated for antibody production over a 24-hour period. Briefly, the cell density of each isolate was determined and 0.5×10^6 cells were centrifuged and resuspended in 0.5 mL fresh supplemented selection CD-SFM medium. These aliquots were placed in 24-well plates corresponding to a seeding density of 1×10^6 cells/mL. After a 24-hour incubation period, supernatants were harvested and evaluated by the human IgG capture ELISA. Figure 29 illustrates the resulting antibody concentrations produced

by each isolate in two separate screening rounds. Sixty-five of the best performing isolates of the 182 isolates assayed were further evaluated to determine the specific productivity rate at small scale.



Round #1 Round #2

Figure 29. SF-NS0 isolates expressing chimeric 7D11 ─ 24-hour assay. Scatter plot indicates mAb concentrations achieved by isolates over a 24-hour period. Two screens, Round #1 and Round #2, were performed.

Since the 24-hour antibody production assay does not evaluate specific antibody production per cell or cellular growth rate, 21 of the 30 expanded isolates were further evaluated by Qp analysis in 6-well format. Briefly, the cell densities of the thirty isolates in log-phase growth were determined. For each isolate, two aliquots of 4×10^5 cells were centrifuged and resuspended in 1.5 mL fresh supplemented selection CD-SFM medium and 1.5 mL non-supplemented selection medium, respectively. These aliquots were placed in 6-well plates corresponding to a seeding density of 2.67×10^5 cells/mL. After approximately 72 hours, the cell densities for each isolate

pair was determined and supernatants were harvested and evaluated by the human IgG capture ELISA as described above.

Table 5 outlines the antibody production and corresponding growth rate resulting in a specific productivity rate or Qp (pg/cell/day) for each isolate in either supplemented or non-supplemented selection medium. As shown in Table 5, the six isolates of the 21 total analyzed highlighted in green had the highest SPR values and will be further analyzed for productivity at larger scale. Since the supplemented versus non-supplemented medium Qp values are comparable, further expansion and analysis of the given six cell lines will be conducted in the non-supplemented selection medium utilized for large-scale generation of mAb.

Upon a further set of 6-well Qp analysis, a total of 13 cell lines were scaled up and evaluated in T-75 flasks for Qp. Briefly, after the cell densities of the isolates in log-phase growth were determined, an aliquot of 3.60×10^6 cells was centrifuged and resuspended in 12mL fresh supplemented selection CD-SFM medium. The aliquots were placed in T-75 flasks corresponding to a seeding density of 3×10^5 cells/mL. After approximately 72 hours, the cell densities for each isolate was determined and supernatants were harvested and evaluated by the human IgG capture. Table 6 outlines the antibody production and corresponding growth rate resulting in a Qp for each isolate at a larger scale.

Table 5. SF-NS0 isolates expressing chimeric 7D11 – 3-day Qp, 6-well format.

SPR #1 (20NOV09 - 23NOV09)
NO SUPPLEMENTS

Isolate Number	Seed Density (vc/mL)	Final Density (vc/mL)	mAb (ug/mL)	Doubling Time (h)	Productivity (pg/cell/day)
37	2.7E+05	1.28E+06	4.769	31.0	2.114
75	2.7E+05	7.90E+05	0.9036	44.7	0.586
82	2.7E+05	1.06E+06	0.8397	35.2	0.434
102	2.7E+05	1.22E+06	4.61	31.9	2.126
167	2.7E+05	3.26E+06	2.06	19.4	0.400
193	2.7E+05	2.88E+06	4.42	20.4	0.963
195	2.7E+05	2.66E+06	11.49	21.1	2.691
225	2.7E+05	2.38E+06	6.82	22.2	1.767
285	2.7E+05	3.54E+06	0.513	18.8	0.092
397	2.7E+05	4.30E+06	4.27	17.5	0.641
399	2.7E+05	6.48E+06	15.35	15.2	1.560
423	2.7E+05	2.48E+06	3.038	21.8	0.758
487	2.7E+05	2.68E+06	12.05	21.0	2.803
520	2.7E+05	3.12E+06	8.245	19.7	1.669
558	2.7E+05	1.70E+06	7.159	26.2	2.495
561	2.7E+05	2.50E+06	8.686	21.7	2.152
563	2.7E+05	2.90E+06	2.393	20.3	0.518
589	2.7E+05	1.90E+06	11.07	24.7	3.503
591	2.7E+05	3.40E+06	19.08	19.2	3.550
603	2.7E+05	1.92E+06	10.73	24.7	3.348
666	2.7E+05	4.00E+06	8.473	18.0	1.355

SPR #1 (20NOV09 - 23NOV09)
WITH SUPPLEMENTS

Isolate Number	Seed Density (vc/mL)	Final Density (vc/mL)	mAb (ug/mL)	Doubling Time (h)	Productivity (pg/cell/day)
37	2.7E+05	1.06E+06	4.72	35.2	2.439
75	2.7E+05	1.30E+06	1.129	30.6	0.494
82	2.7E+05	1.88E+06	0.9235	24.9	0.295
102	2.7E+05	2.12E+06	5.132	23.4	1.474
167	2.7E+05	1.98E+06	3.067	24.2	0.936
193	2.7E+05	2.58E+06	4.657	21.4	1.122
195	2.7E+05	2.38E+06	8.884	22.2	2.301
225	2.7E+05	2.10E+06	6.312	23.5	1.828
285	2.7E+05	2.20E+06	0.5356	23.0	0.149
397	2.7E+05	3.44E+06	4.271	19.0	0.790
399	2.7E+05	4.40E+06	16.81	17.3	2.470
423	2.7E+05	3.68E+06	3.84	18.5	0.667
487	2.7E+05	2.84E+06	11.1	20.5	2.449
520	2.7E+05	2.64E+06	7.252	21.2	1.710
558	2.7E+05	2.56E+06	8.838	21.5	2.143
561	2.7E+05	3.56E+06	9.809	18.7	1.757
563	2.7E+05	2.78E+06	3.607	20.7	0.812
589	2.7E+05	1.68E+06	9.789	26.4	3.447
591	2.7E+05	1.21E+06	15.43	32.3	7.129
603	2.7E+05	1.40E+06	10.07	29.4	4.122
666	2.7E+05	3.28E+06	7.971	19.4	1.533

Table 6. SF-NS0 isolates expressing chimeric 7D11 – 3-day Qp, T-75 format.

Isolate Number	Seed Density (vc/mL)	Final Density (vc/mL)	mAb (ug/mL)	Doubling Time (h)	Productivity (pg/cell/day)
195	3.0E+05	1.80E+06	6.354	27.4	2.031
369	3.0E+05	1.52E+06	3.67	30.2	1.368
399	3.0E+05	1.36E+06	8.398	32.8	3.396
487	3.0E+05	1.76E+06	7.406	28.0	2.414
589	3.0E+05	1.11E+06	6.618	37.9	3.151
591	3.0E+05	1.85E+06	11.09	27.2	3.463
603	3.0E+05	2.56E+06	11.27	23.1	2.646
752	3.0E+05	1.59E+06	9.215	29.4	3.273
1044	3.0E+05	1.29E+06	4.995	33.6	2.109
1303	3.0E+05	1.56E+06	18.316	37.9	5.238
1351	3.0E+05	1.34E+06	11.208	41.8	3.635
1407	3.0E+05	1.08E+06	15.324	48.8	5.907
2153	3.0E+05	1.96E+06	8.976	33.3	2.113

After all productivity assays were completed, the top four c7D11-expressing isolates that consistently outperformed all other isolates, as outlined in green above, were selected to undergo limiting dilution cloning (LDC) to generate clonal cell lines that will likely have a improved Qp values. Briefly, the cell densities of the isolates in log-phase growth were determined. Each culture was diluted in selective medium supplemented with 10% Charcoal-Stripped Fetal Bovine Serum to 15 cells/mL, in a total volume of 40 mL. Two hundred µL was dispensed per well in one flat bottom 96-well plate, effectively delivering approximately 3 cells per well. The remaining 20 mL of culture from the first dilution was further diluted 1:3 in selective medium, and plated as described above. The resulting two plates were seeded at approximately 1 cell/well. The remaining 20 mL of culture from the second dilution was further diluted 1:3 in selective medium, and plated as described above. The resulting two plates were seeded at approximately 0.3 cells/well. All five plates per isolate were incubated at growth conditions for approximately 2-3 weeks with weekly 1:1 medium exchanges, or until clonal colonies emerge.

In total, three clones from isolate #591, one clone from isolate #752, 32 clones from isolate #1303, and zero clones from #1407 were expanded from the 96-well seeding plates to a T-75 flask volume. The clones were evaluated for antibody production levels by the 72-hour productivity assay, 6-well scale Qp assay, and the T-75 scale Qp assay. Table 7 outlines the antibody production and corresponding growth rate resulting in a Qp for each clone. As illustrated in three independent assays, clone #591-2 consistently outperformed all other c7D11 clones. In addition, clone #591-2 had a significant increase in antibody yield over the parental isolate, as highlighted in Table 7, and will remain the focus of further analysis as the cell lines advance to suspension culture time-course evaluation, medium supplementation, and feed formulation experiments.

Table 7. Qp rates for top-performing NS0:c7D11 clones (T-75 scale).

Isolate Number	Clone Number	Seed Density (vc/mL)	Final Density (vc/mL)	mAb (ug/mL)	Doubling Time (h)	Productivity (pg/cell/day)
591	**Parental Isolate**	**3.0E+05**	**1.70E+06**	**8.735**	**28.5**	**2.949**
591	1	3.0E+05	3.26E+06	14.625	19.9	2.879
591	2	3.0E+05	1.78E+06	20.203	26.7	6.807
591	3	3.0E+05	1.02E+06	6.224	38.8	3.304
752	**Parental Isolate**	**3.0E+05**	**1.94E+06**	**12.447**	**28.5**	**3.985**
752	1	3.0E+05	7.40E+05	11.877	52.6	8.003
1303	**Parental Isolate**	**3.0E+05**	**1.23E+06**	**10.183**	**39.9**	**5.097**
1303	2	3.0E+05	2.24E+06	9.963	23.6	2.749
1303	6	3.0E+05	2.30E+06	5.714	24.5	1.465
1303	10	3.0E+05	6.00E+05	3.829	72.0	2.836
1303	14	3.0E+05	4.80E+05	5.625	101.0	5.054
1303	15	3.0E+05	1.59E+06	7.127	29.9	2.514
1303	28	3.0E+05	1.73E+06	6.902	27.1	2.383
1303	29	3.0E+05	1.04E+06	9.197	38.2	4.810
1303	30	3.0E+05	2.88E+06	12.396	22.1	2.599
1303	31	3.0E+05	2.12E+06	11.86	25.5	3.267
1303	32	3.0E+05	1.83E+06	6.688	27.6	2.093

SF-NS0 Stable Cell Line Generation Using the α-A33 pBFksr:6Cdual#1 Vector

Utilizing the stable cell line generation techniques outlined for the mouse/human chimeric 7D11 mAb above, stable cell lines were also generated for the chimpanzee/human chimeric α-A33 mAb, c6C. One NS0 parental cell line pool was

transfected with linearized c6C plasmid and dispensed into 96-well plates. After three weeks of 1:1 medium replacements, the transfection resulted in only two emerging isolates. The two isolates were expanded and evaluated in the same fashion as the c7D11-expressing isolates. The two isolates exhibited significant IgG expression (over twice the background signal) and were scaled from 96-well plates to 12-well plates, and eventually 6-well plates in supplemented selective CD-SFM medium. After reaching confluency in the 6-well plates, the transfectants were further evaluated for antibody production over a 24-hour period. Overall, the antibody production for the two isolates over a 24-hour period has been determined and was repeated three independent times. In addition, the isolates were further evaluated to assess Qp at 6-well scale. The isolates were further analyzed for specific productivity at the 12 mL/T-75 scale, as previously described. Table 8 outlines the antibody production and corresponding growth rate, if appropriate, for the 24 hour assay, the 6-well scale SPR assay, and the T-75 scale SPR assay resulting in a specific productivity rate (pg/cell/day) for each isolate. The 6-well and T-75 SPR assays were conducted on two separate occasions. Overall, the NS0:c6C#1 isolate consistently outperformed the NS0:c6C#2 isolate and will be further evaluated.

An additional cell line expressing the α-VACV protein B5 was developed as previously described.

Table 8. SF-NS0 isolates expressing chimeric 6C.

SF-NSO c6C Isolates: 24-hour Productivity Assay (24 well plate)

Isolate Number	Date	mAb (ug/mL)
1	2009DEC31	2.438
1	2010JAN13	3.704
1	2010JAN15	2.575
2	2009DEC31	2.536
2	2010JAN13	3.104
2	2010JAN15	2.594

SF-NSO c6C Isolates: 6-well SPR (With SUPPLEMENTS)

Isolate Number	Date	Seed Density (vc/mL)	Final Density (vc/mL)	mAb (ug/mL)	Doubling Time (h)	Productivity (pg/cell/day)
1	2009DEC31	2.7E+05	2.20E+05	2.589	-241.6	3.780
1	2010JAN15	2.7E+05	4.50E+05	6.581	94.9	6.162
2	2009DEC31	2.7E+05	1.40E+05	2.307	-72.5	4.031
2	2010JAN15	2.7E+05	2.00E+05	3.180	-171.5	4.572

SF-NSO c6C Isolates: T-75 SPR (With SUPPLEMENTS)

Isolate Number	Date	Seed Density (vc/mL)	Final Density (vc/mL)	mAb (ug/mL)	Doubling Time (h)	Productivity (pg/cell/day)
1	2009DEC31	3.0E+05	6.20E+05	2.710	64.4	2.095
1	2010JAN15	3.0E+05	9.20E+05	6.395	44.2	3.519
2	2009DEC31	3.0E+05	3.40E+05	2.252	373.7	2.502
2	2010JAN15	3.0E+05	4.80E+05	3.422	105.4	2.945

Development of a Rapid SF-NS0 Stable Pool Generation Method

The miniwell method of screening transfected cells involves diluting the 24 hr pool in 800 ml of selective medium and dispensing 200 µl per well into forty 96-well plates. Obviously, screening 40 plates per transfection was cumbersome and time consuming. To develop a streamlined method of screening transfected pools, three methods were tested in the following order. Firstly, after 24 hr incubation in T-75 flask discussed above, cells were transferred to 30 ml selective medium in a 125 ml shake flask and incubated at 135 rpm. All cells were dead within 5 days using this method. Secondly, selection in 30 ml medium in a shake flask was tested but shaking speed was reduced to 80 rpm. A pool of surviving cells was generated within 12 days. Thirdly, selection was performed in T-75 flasks by substituting growth medium with selection medium. Cells were incubated without shaking. This method generated a

pool of surviving cells within 12 days that was transferred to 30 ml selective medium in 125 ml shake flasks and incubated with shaking at 135 rpm. A culture derived from this stably transfected pool was maintained for one week in shaker and stirred tank environments. Supernatants were analyzed for productivity by ELISA. Volumetric yields of both cultures were over 20 mg/L—A 10-fold increase over previous SF-NS0 stable pools.

Limiting Dilution Cloning (LDC) of the Rapid SF-NS0 Stable Pool

The pool of stable cells was cloned by limiting dilution to single cell densities in 96-well plates using 10% FBS supplemented selection medium. Growing wells from the stable pool were analyzed for expression by ELISA. Out of 101 emerging clonal cell lines, 51 clones were expressing the mAb product (50%). These 51 clones were scaled to 6-well plates. Out of the original 51 clones, 37 clones demonstrated favorable growth characteristics (*e.g.*, doubling time at ~24 hours) and were analyzed for Qp. From these data, the doubling time and Qp rate were calculated (see Table 9). From these clones, the five top performers were scaled up to 30 mL shaker cultures and further evaluated for Qp in suspension culture for two days. As seen in Table 10, clone E12-2 reached a mAb concentration of nearly 100 mg/L in 48 hours.

Table 9. Specific Productivity Rate (Qp) analysis of NS0::c7D11 clones (top five performers are highlighted in green).

Isolate Number	Seed Density (vc/mL)	Final Density (vc/mL)	mAb (ug/mL)	Doubling Time (h)	Productivity (pg/cell/day)
H7	2.7E+05	2.20E+06	52.23	23.7	14.114
H11	2.7E+05	2.20E+06	46.187	23.7	12.481
E12-2	2.7E+05	2.00E+06	41.693	24.8	12.261
B3	2.7E+05	2.20E+06	45.179	23.7	12.209
D3	2.7E+05	2.50E+06	42.837	22.3	10.321
F10-0.3	2.7E+05	1.80E+06	30.156	26.1	9.726
C2-2	2.7E+05	2.00E+06	33.034	24.8	9.714
E9	2.7E+05	1.70E+06	27.525	27.0	9.329
F2-2	2.7E+05	1.10E+06	18.165	35.2	8.859
E8-1	2.7E+05	2.50E+06	34.78	22.3	8.380
A5	2.7E+05	2.40E+06	33.199	22.7	8.299
C4	2.7E+05	1.50E+05	4.911	-86.5	7.851
E7	2.7E+05	1.90E+06	25.305	25.4	7.785
E10	2.7E+05	2.50E+06	27.704	22.3	6.675
B4-2	2.7E+05	1.80E+06	20.545	26.1	6.626
B6	2.7E+05	1.60E+06	17.92	27.9	6.399
C3	2.7E+05	3.00E+06	29.446	20.6	6.009
A11	2.7E+05	2.40E+06	23.999	22.7	5.999
B3-2	2.7E+05	2.40E+06	23.946	22.7	5.986
D5-2	2.7E+05	2.20E+06	20.383	23.7	5.508
A4-2	2.7E+05	2.00E+06	17.199	24.8	5.058
E5	2.7E+05	1.60E+06	13.28	27.9	4.742
F10-1	2.7E+05	2.20E+06	16.384	23.7	4.428
B5	2.7E+05	2.20E+06	11.556	23.7	3.123
E2-2	2.7E+05	2.00E+06	7.393	24.8	2.174
H4	2.7E+05	1.70E+06	6.168	27.0	2.090
A9-2	2.7E+05	1.70E+06	4.627	27.0	1.568
E1	2.7E+05	1.40E+06	1.8	30.1	0.720
D10-2	2.7E+05	2.50E+06	2.259	22.3	0.544
B4	2.7E+05	2.40E+06	1.93	22.7	0.482
F5	2.7E+05	1.90E+06	1.192	25.4	0.367
D8	2.7E+05	1.40E+06	0.773	30.1	0.309
E2	2.7E+05	2.30E+06	0.917	23.2	0.238
C12	2.7E+05	1.00E+06	0.313	37.8	0.165
B12	2.7E+05	2.20E+06	0.567	23.7	0.153
E8-0.3	2.7E+05	2.50E+06	0.399	22.3	0.096
H9	2.7E+05	2.10E+06	0.191	24.2	0.054

Table 10. Specific Productivity Rate (Qp) analysis of top five NS0::c7D11 clones in suspension culture (30 mL shaker flask).

Isolate Number	Seed Density (vc/mL)	Final Density (vc/mL)	mAb (ug/mL)	Doubling Time (h)	Productivity (pg/cell/day)
E12-2	7.3E+05	2.32E+06	93.7	28.9	30.688
H11	5.3E+05	2.34E+06	70.2	22.5	24.432
D3	7.3E+05	2.33E+06	70.7	28.8	23.079
B3	5.3E+05	1.16E+06	63	42.8	37.205
H7	6.7E+05	1.85E+06	57.9	32.6	23.007

Characterization of New Zealand Fetal Bovine Serum-Supplemented SF-NS0 Clones

A cloning study was performed to replace the U.S.-sourced charcoal-stripped fetal bovine serum (FBS) with a fully characterized and TSE-free New Zealand (NZ) sourced FBS to allay safety and regulatory concerns.

Two SF-NS0 stable pools, Pool #2 and Pool #3, were cloned by LDC in 96-well plates at densities of 0.3 cell/well and 1 cell/well to isolate clonal cell lines. The medium used in the LDC process was supplemented with 10% qualified FBS (Hyclone FBS from New Zealand, and gamma irradiated). A total of 1,181 wells were screened. Based on IgG concentration, 570 cell lines were scaled up to 24- well and then 12-well plates.

A 24-hour productivity assay was used to select best expressers for further analysis. A total of 504 clones were scored for 24-hour IgG productivity. Based on an IgG concentration cutoff of ≥ 1 mg/L, a total of 190 cell lines were scaled up to 6-well plates for Qp analysis.

A 6-well Qp analysis was used to select best expressers for further scale-up and analysis. A total of 184 of our initially scaled up 190 clones were analyzed and the best performing 49 clones were scaled to T-75 flasks for large-scale Qp analysis.

A T-75 Qp analysis was used to select best expressers for suspension adaptation and further analysis. A total of 49 clones were analyzed and the best performing 20 clones were scaled to 30-mL shaker flasks for suspension Qp analysis (see Table 11).

A 30-mL shaker flask Qp analysis was used to select best performers for mAb production. Briefly, cells were counted, centrifuged, and seeded in 30 mL of fresh

medium at an initial density of 5×10^5 in 125-mL disposable shaker flasks and incubated for three (3) days. At the end of this growth period, the cells were counted and supernatants were analyzed by ELISA. From these data, the average doubling time and Qp in pg/cell/day were calculated. A total of 18 clones were analyzed and the best performing 11 clones were scaled to 200-mL culture volume in shaker flasks for mAb production (see Table 12). The best performing 11 clones were scaled to 200-mL culture volume in shaker flasks for mAb production (see Table 13).

Table 11. Best performing NZ FBS clones—Qp in T-75 flasks.

Dilution (cells/well)	Clone Number	Count (e6/mL)	Vol Req for 4.2e6 cells (uL)	Time In	Time out	Seed Density (vc/mL)	Final Density (vc/mL)	mAb (ug/mL)	Doubling Time (h)	Productivity (pg/cell/day)
1	3-261a	1.1	3818	900	950	3.5E+05	1.90E+06	67.6	29.5	20.030
1	3-330	0.87	4828	900	950	3.5E+05	1.90E+06	59.7	29.5	17.689
1	3-503	0.94	4468	1145	1145	3.5E+05	2.40E+06	42.5	25.9	10.303
1	3-261b	0.85	4941	900	950	3.5E+05	1.80E+06	32	30.5	9.922
1	3-66	1.5	2800	900	930	3.5E+05	2.50E+06	32	25.4	7.485
1	2-280	1.1	3818	1145	1145	3.5E+05	2.10E+06	29.7	27.8	8.082
1	2-294	1.6	2625	1145	1145	3.5E+05	1.60E+06	20.2	32.8	6.906
0.3	3-388	1.4	3000	900	930	3.5E+05	2.00E+06	19.6	28.6	5.560
1	3-324	0.82	5122	900	950	3.5E+05	1.50E+06	19	34.3	6.847
1	3-5	0.99	4242	900	930	3.5E+05	2.00E+06	19	28.6	5.390
1	3-269	1.2	3500	1015	1005	3.5E+05	2.10E+06	19	27.8	5.170
1	3-37	1.2	3500	900	950	3.5E+05	1.90E+06	18	29.5	5.333
1	2-441	0.47	8936	1000	1000	3.5E+05	1.40E+06	18	36.0	6.857
1	3-93	1.4	3000	1015	1005	3.5E+05	2.40E+06	16	25.9	3.879
1	2-336	1.1	3818	1145	1145	3.5E+05	1.50E+06	15.6	34.3	5.622
1	3-351	1.3	3231	1015	920	3.5E+05	1.50E+06	15	34.3	5.405
0.3	3-448	1	4200	900	950	3.5E+05	1.90E+06	15	29.5	4.444
1	3-88	1.3	3231	900	930	3.5E+05	1.90E+06	15	29.5	4.444
1	3-115	1.1	3818	900	930	3.5E+05	2.90E+06	15	23.6	3.077
1	3-497	1.1	3818	1000	1000	3.5E+05	1.20E+06	15	40.5	6.452
1	2-304	1.2	3500	1000	1000	3.5E+05	3.00E+05	15	-323.7	15.385
0.3	2-328	1.4	3000	1145	1145	3.5E+05	1.60E+06	13.8	32.8	4.718
1	3-375	0.91	4615	1015	920	3.5E+05	3.00E+06	13	23.2	2.587
1	2-296	1.2	3500	1230	1215	3.5E+05	1.70E+06	11.5	31.6	3.740
1	3-363	1.5	2800	1015	1005	3.5E+05	1.90E+06	11	29.5	3.259
1	3-344	1.4	3000	900	930	3.5E+05	2.50E+06	11	25.4	2.573
1	2-359	0.41	10244	1000	1000	3.5E+05	8.20E+05	10.5	58.6	5.983
0.3	3-401	1.8	2333	1015	920	3.5E+05	1.20E+06	10	40.5	4.301
1	3-352	1.5	2800	1015	1005	3.5E+05	1.90E+06	10	29.5	2.963
1	3-306	0.82	5122	1230	1215	3.5E+05	2.50E+06	9.49	25.4	2.220
1	2-478	0.81	5185	1230	1215	3.5E+05	2.00E+06	9.04	28.6	2.565
1	3-356	1.7	2471	1015	1005	3.5E+05	1.80E+06	9	30.5	2.791
0.3	2-337	1	4200	1145	1145	3.5E+05	2.00E+06	8.78	28.6	2.491
0.3	2-395	1.3	3231	1230	1215	3.5E+05	1.80E+06	7.5	30.5	2.326
1	3-310	1.5	2800	1015	1005	3.5E+05	1.90E+06	7	29.5	2.074
0.3	3-421	1.2	3500	1015	920	3.5E+05	2.20E+06	7	27.1	1.830
1	3-303	1.3	3231	1015	950	3.5E+05	2.30E+06	7	26.5	1.761
3	3-64	1.3	3231	1015	920	3.5E+05	2.40E+06	7	25.9	1.697
0.3	2-78	0.83	5060	1145	1145	3.5E+05	1.90E+06	6.82	29.5	2.021
1	2-390	1.6	2625	1145	1145	3.5E+05	2.20E+06	5.25	27.1	1.373
1	2-375	1.3	3231	1230	1215	3.5E+05	1.80E+06	4.38	30.5	1.358
1	2-435	1.3	3231	1230	1215	3.5E+05	1.90E+06	3.89	29.5	1.153
1	3-373	0.81	5185	1015	920	3.5E+05	1.90E+06	3	29.5	0.889
1	2-450	1.3	3231	1230	1215	3.5E+05	1.50E+06	2.82	34.3	1.016
1	2-318	1.3	3231	1230	1215	3.5E+05	1.70E+06	2.12	31.6	0.689
0.3	2-403	1.5	2800	1230	1215	3.5E+05	1.80E+06	1.73	30.5	0.536
0.3	2-396	0.87	4828	1230	1215	3.5E+05	2.20E+06	1.7	27.1	0.444
1	2-431	1.5	2800	1230	1215	3.5E+05	1.10E+06	1.54	43.6	0.708
1	2-333	1.2	3500	1230	1215	3.5E+05	2.10E+06	1.5	27.8	0.408

Table 12. Best performing NZ FBS clones—Qp in 30-mL shaker flasks.

Dilution (cells/well)	Clone Number	Count (e6/mL)	Vol Req for 15e6 cells (uL)	Time In	Time out	Seed Density (vc/mL)	Final Density (vc/mL)	mAb (ug/mL)	Doubling Time (h)	Productivity (pg/cell/day)
1	3-261a	0.93	16.13	230	215	4.9E+05	2.63E+06	78.3	29.7	16.731
1	3-330	1	15.00	230	215	4.9E+05	2.89E+06	64	28.1	12.623
1	3-261b	0.98	15.31	230	215	5.0E+05	2.58E+06	53.1	30.4	11.494
0.3	3-388	0.92	16.30	230	215	4.6E+05	3.14E+06	50.1	26.0	9.278
1	3-324	1.2	12.50	230	215	4.1E+05	4.13E+06	37.5	21.6	5.507
1	3-503	1.37	10.95	1145	1145	4.1E+05	2.60E+06	31.5	27.0	6.977
1	3-351	1.1	13.64	230	215	4.7E+05	3.69E+06	28	24.2	4.487
1	3-269	1	15.00	230	215	3.6E+05	3.44E+06	28	22.1	4.912
1	3-37	1.3	11.54	230	215	7.2E+05	3.90E+06	24	29.5	3.463
1	2-390	0.8	18.75	1045	1035	6.3E+05	2.35E+06	20	37.7	4.482
1	3-497	1.96	7.65	1145	1145	6.2E+05	1.97E+06	20	43.2	5.148
0.3	2-328	1.2	12.50	1045	1035	4.2E+05	1.98E+06	18	32.2	5.000
1	3-66	0.61	24.59	230	215	4.9E+05	1.62E+06	16.5	41.7	5.213
1	2-280	1.26	11.90	1100	1100	4.1E+05	2.86E+06	14	25.5	2.859
1	3-37	2.36	6.36	1145	1145	3.9E+05	3.66E+06	7.5	22.3	1.235
1	2-336	1.07	14.02	1100	1100	4.7E+05	2.19E+06	7	32.4	1.754
1	3-93	0.94	15.96	230	215	4.5E+05	1.16E+06	6.3	52.7	2.609
1	2-294	0.96	15.63	1045	1035	5.1E+05	3.69E+06	4.5	25.2	0.714

Table 13. Best performing NZ FBS clones—200 mL production summary.

Clone	Cell Bank Condition	Purified Concentration (A280, mg/mL)	Purified Volume (mL)	Total Purified (mg)	Purified Yield (mg/L culture)	Final Titer in Supernatant (ELISA, mg/L)
3-324	T-flask	2.18	4.0	8.72	43.6	66
3-388	Shaker	2.41	4.4	10.6	53	48
3-261a	Shaker	5.01	5.0	25.05	125.3	278
3-261b	Shaker	2.35	13.0	30.55	152.8	287
3-330	Shaker	5.53	5.0	27.65	138.3	268
3-503	Shaker	2.00	7.5	15.00	75.0	215
3-351	Shaker	3.01	7.0	21.07	105.4	sample not taken
3-269	Shaker	4.73	4.5	21.29	106.4	sample not taken
3-497	Shaker	1.87	9.0	16.83	84.15	177
2-280	T-flask	0.79	7.0	5.53	27.65	sample not taken
3-37	Shaker	1.46	4.8	7.01	35.04	60

Eight clones were isolated with 3-day volumetric productivities at 28 mg/L or greater in suspension conditions with a total of 11 clones (green highlighted in Table 12) advanced to mAb production at 200-mL shaker scale for production. The top three clones attained volumetric yields of 0.27-0.29 g/L in simple shaker flask culture over 6 days. These yields suggest volumetric productivities of greater than 1 g/L in

bioreactor culture with appropriate process development.

FBS-Free Conditioned Medium Study to Support LDC

A study was performed to explore the full replacement of fetal bovine serum (FBS) with various ratios of conditioned FBS-free medium to support single cell density growth of StableFast-NS0 during limiting dilution cloning (LDC).

For all experiments, the following medium was utilized:

Pre-warm 1-L CD Hybridoma Medium for 20 minutes in a 37°C water bath. Wipe down all bottles with 70% IPA and place in BSC. Weigh out 10 g of Albumin Bovine Serum, Fatty Acid Free, Low Endotoxin (Sigma, Cat. No. A8806-5G). Add to a new, pre-warmed 1 L bottle of CD Hybridoma, Chemically Defined Medium (Invitrogen, Cat. No. 11279-023). Invert bottle gently to fully dissolve. Using aseptic techniques, add the following components to the 1-L CD Hybridoma Medium bottle:

10 mL of GlutaMAX-1, 100X (Invitrogen, Cat. No. 35050-061)
10 mL of MEM NEAA, MEM Non-Essential Amino Acids, 100X Solution (Invitrogen, Cat. No. 11140-050)
10 mL of ITSE Liquid Media Supplement, 100X Solution (Invitria, Cat. No. 777ITS032)
50 µL of Human Recombinant IL-6 10 µg/mL (Biosource-Invitrogen, Cat. No. PHC0064)

Mix and pour into funnel of 1-L Stericup. Filter into receiver bottle using vacuum pump system. Remove funnel and cap with sterile bottle top. Label bottle with components and preparation date. Record components and lot numbers in laboratory notebook. Store at 4°C. Medium expires 1 month after preparation date.

Conditioned medium was generated by collecting three-day batch medium from a null StableFast-NS0 stable pool created by the transfection of parental NS0 cells with

the empty pBFksr vector. Batch was started at 0.34e6 and harvested at 2e6, 93% viability. Conditioned medium production runs were performed in T-150 flasks at 30 mL culture per flask.

For the conditioned medium experiment, a stable pool of transfected NS0 cells were plated at 1 cell per well in 96 well plates at 200 uL per well. A total of 480 wells were seeded in 10% FBS-supplemented selective medium, and additional 192 wells were seeded for each conditioned media parameter (Selective Media + 10%/25%/50% Conditioned Media). Plates were fed 90µL out / 110µL in of their respective media formulations on days 7 and 11 post plating.

Cloning efficiency was calculated using the following equation:

$$Cloning\ Efficiency = \frac{Number\ of\ wells\ with\ positive\ growth}{Total\ number\ of\ wells\ seeded \times Dilution\ factor}$$

The FBS-supplemented wells had a 42.5% cloning efficiency. The 50% conditioned media wells had a 32.8% efficiency. The 25% conditioned media wells had a 16.1% efficiency. No wells grew under the 10% conditioned media parameter. Therefore, due to the similar cloning efficiency, FBS was eliminated from the cloning method and replaced with 50% conditioned medium.

Conclusions

Cell line generation techniques and methods of analysis were developed for the cholesterol selection based NS0 biomanufacturing platform now called StableFast-NS0. A miniwell selection approach was applied to the product mAb 21, a monoclonal directed at a vaccinia virus (VACV) subunit protein, generating the best performing isolate #7 that was rapidly scaled to the 5-L bioreactor for production and purification with volumetric yields in the 18-28 mg/L range. This isolate was subjected to a 52 generation stability

study that showed a marked decrease in expression with a final bioreactor yield of 7 mg/L with significantly higher viable cell density (VCD) indicating a probable lack of clonality with low/non-producers overtaking cells with higher productivity over the generational timecourse. With refined screening methods, isolate #121 for the second product, mAb 14, was developed that demonstrated yields nearly twice that of mAb 21 Isolate #7. Armed with improved vectors coming from the efforts in promoter, SP and gene optimization, cell lines for two new mAbs, c7D11 and c6C, were generated. Efforts for the c7D11 studies included a new rapid pool generation method followed by the single cell cloning and isolation of E12-2 expressing mAb c7D11 at a rate of over 30 pcd, the highest to date and well into the commercially acceptable Qp range. In an effort to improve the platform's regulatory compliance, a study demonstrated that our original U.S.-sourced charcoal-stripped FBS could be effectively replaced with a New Zealand-sourced FBS thereby lowering concerns over the introduction of transmissible spongiform encephalopathy (TSE) prions, a growing concern of the regulators. A further study allowed for the elimination of all serum from the cell line development process with the production of conditioned medium using a null NS0 cell line generated by transfecting the parental NS0 with a vector containing only the Hsd17β7 selection marker and empty heavy and light chain cassettes. By plating and feeding the cells with the conditioned medium at a 50:50 ratio with fresh selective medium, cloning efficiency was 77% of that of the FBS-supplemented LDC process.

Chapter 4:
Initial process scale-up and medium optimization

Abstract

Following an efficient cell line development process, the best performing pools, isolates and clones need to be scalable for rapid production of pre-clinical materials and to set the stage for advanced process and product development. In Chapter 4, processes for the expression of mAb products in the novel NS0 platform were developed and scaled up to 5-L bioreactors for in-depth characterization.[40-43] Initial studies were performed to screen for best principle carbon source batching and feeding as well as evaluation of complex supplements. These experiments indicated that higher batch concentration of glutamine, the SF-NS0 cell line's preferred principal carbon source, led to higher growth and mAb yields. Further details of preferential amino acid consumption were revealed by HPLC analysis of spent media indicating that several critical amino acids were being preferentially consumed. An assessment of ammonia, a key inhibiting metabolite, was used to better balance glucose and glutamine concentrations in the medium for optimal growth and yield. Controlled feeding to maintain both glutamine and glucose at lower levels, 2mM and 2 g/L targets, respectively, resulted in significant increases in both densities and yields as compared with batch controls. Custom supplements and feeds were developed to increase cell density and extend culture viability with the goal of increasing protein yields. These strategies were scaled to the 5-L benchtop bioreactor system resulting in a yield of nearly 250 mg/L, a 5-fold increase over the earlier clonal cell lines and processes. Processes established in the classical bioreactor were then adapted and demonstrated in single-use systems at the 3.5-L and 10-L scales with yields ranging from 150 mg/L to nearly 400 mg/L.

Results and Discussion

SF-NS0 Stable Cell Line c7D11 (α-L1) 591-2 Medium Optimization

SF-NS0 stable cell lines expressing the anti-L1 chimeric mouse/human mAb c7D11 were generated, cloned, and evaluated for specific productivity as outlined above. Clone #591-2 consistently outperformed all other c7D11 clones in three independent assays and had a significant increase in antibody yield over the parental isolate. To begin scaling up of this cell line for eventual bioreactor process development, commercially available medium supplements were evaluated in small shaker culture (40 mL working volume). Several complex and defined supplements are available from commercial suppliers. Table 14 shows the initial experimental design of batch supplement screening. In addition to the two hydrolysates, two initial concentrations of glutamine (GlutaMAX, Invitrogen) were evaluated. Glutamine is a significant carbon source for mammalian cells including the NS0 cell line.

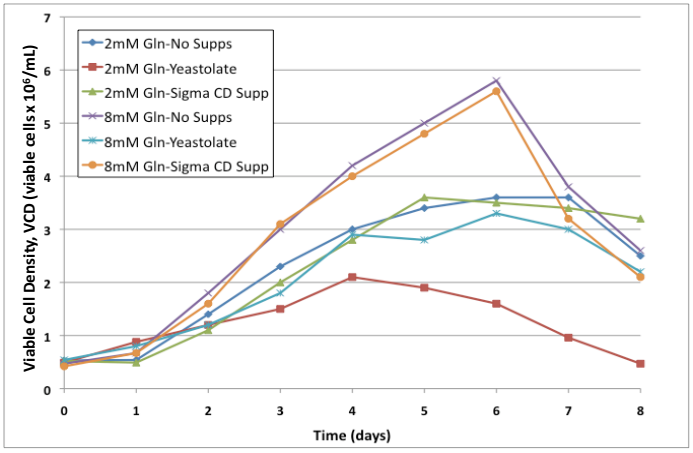
Table 14. Batch supplement screening—Experimental design.

Flask	GlutaMAX (mM)	Invitrogen Yeastolate (ml/L)	Sigma EX-CELL CD Hydrolysate (mL/L)
1	2	0	0
2	2	20	0
3	2	0	50
4	8	0	0
5	8	20	0
6	8	0	50

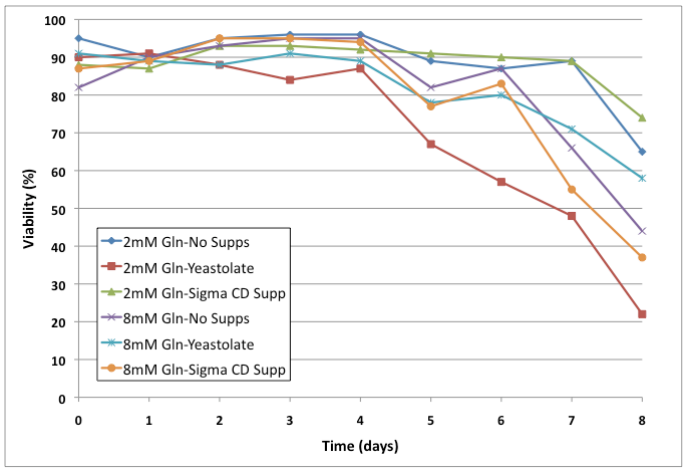
As seen in Figures 30A-C, cultures with an 8mM initial concentration of glutamine grew to a significantly higher density when compared with those starting at 4mM. Exceptions were the cultures with Yeastolate (Invitrogen) supplement, which had significantly lower growth and viability as compared with other cultures with the

same initial glutamine concentration. All 8mM glutamine cultures had better mAb expression than the respective 4mM cultures. Interestingly, the 8mM glutamine/Yeastolate culture had significantly lower density, but similar productivity as compared to the non-supplemented and Sigma CD supplemented cultures. These results led to another round of experiments in shakers to include nutrient feeding and spent medium analysis for amino acids.

A.



B.



C.

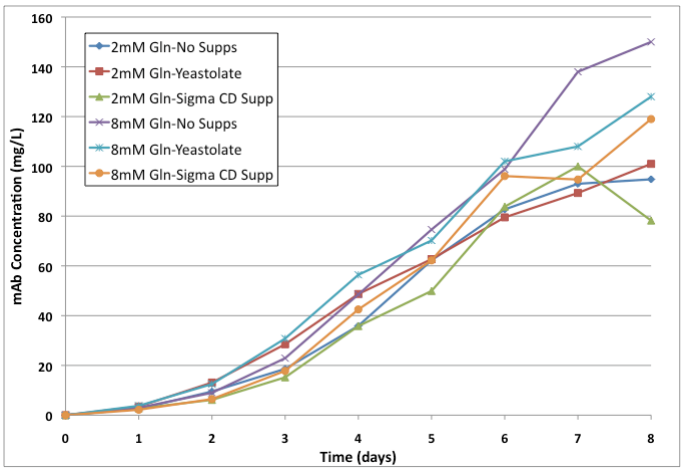


Figure 30. Batch supplement screening—40 mL shaker flask cultures.
A: Viable cell density; B: Cell viability; C: mAb concentration.

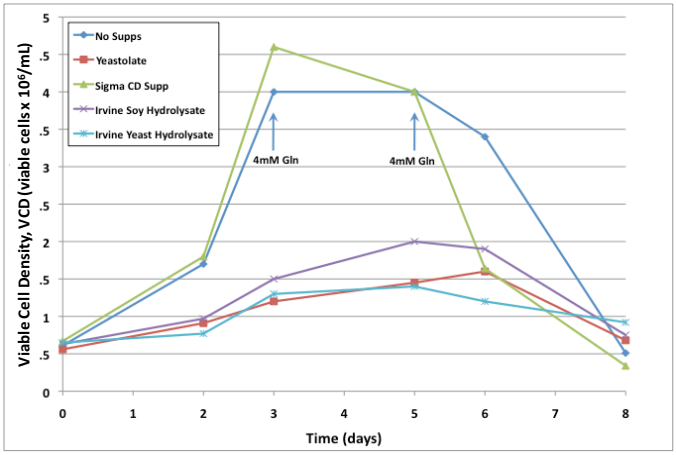
Experiments were performed to evaluate the impact of two additional complex medium supplements, soy and yeast hydrolysate (Irvine Scientific), and the effect of a glutamine feed on cell growth and mAb productivity. In addition, analysis of amino acid consumption in spent medium was performed. Supplementation and feeding of key amino acids has been consistently demonstrated to improve volumetric productivity of NS0 cell lines expressing IgGs. Table 15 shows the initial experimental design of batch supplement screening with glutamine feed.

Table 15. Batch supplement screening with glutamine feed—Experimental design.

Flask	L-Glutamine (mM)	Invitrogen Yeastolate (mL/L)	Sigma EX-CELL CD Hydrolysate (mL/L)	Irvine Soy Hydrolysate (g/L)	Irvine Yeast Hydrolysate (g/L)
1	8	0	0	0	0
2	8	20	0	0	0
3	8	0	50	0	0
4	8	0	0	6	0
5	8	0	0	0	6

As seen in Figures 31A-C, cultures without complex supplement or with chemically defined supplement (Sigma EX-CELL CD Hydrolysate) achieved about a two-fold greater cell density than cultures supplemented with complex hydrolysates. Four millimolar (4mM) glutamine feeds were added to the high-density cultures (Flasks 1 and 3, no supplement and Sigma CD supplement, respectively). As observed during the previous experiment, the complex hydrolysate supplemented cultures had significantly lower densities, but similar productivities as compared to the non-supplemented and Sigma CD supplemented cultures. In a future effort, the SF-NS0 clone 591-2 cell line will be adapted to complex hydrolysate supplemented medium prior to further shake flasks experiments. Figure 32 shows the results of spent media analysis for amino acid concentration. This analysis was performed

A.



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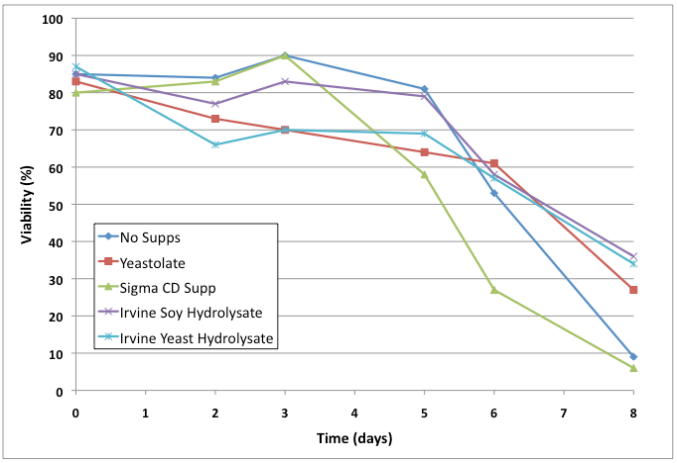


C.

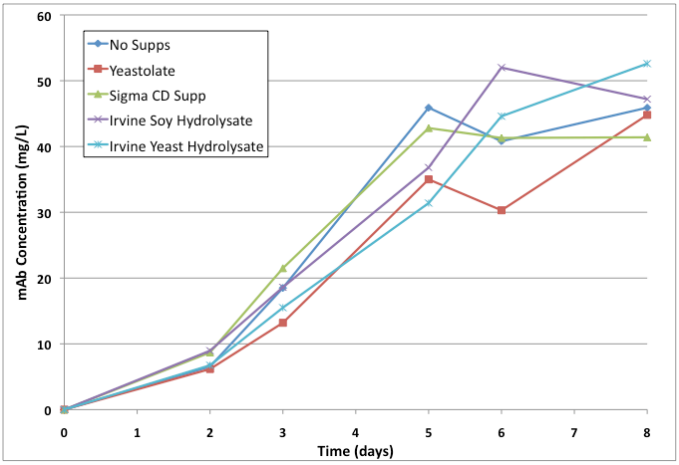


Figure 31. Batch supplement screening with glutamine feeds—40 mL shaker flask cultures. A: Viable cell density; B: Cell viability; C: mAb concentration.

using a newly developed HPLC method. From this analysis, uptake rate of a range of amino acids can be determined. The most significantly depleted amino acids include glutamic acid (GLU), glutamine (GLN), cysteine (CY2), isoleucine (ILE), leucine (LEU) and threonine (THR). In addition, the three amino acids glycine, alanine, and proline were produced over the course of the culture (data not shown). This amino acid analysis will be used to formulate a feed for the next experiments in spinner flask and 5-liter bioreactor cultures.

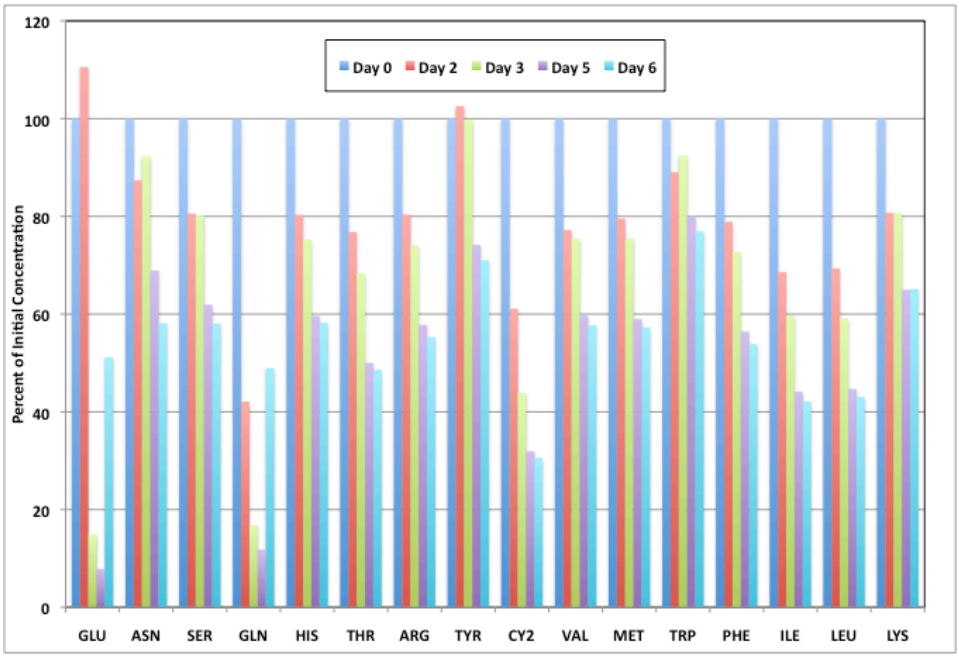


Figure 32. Amino acid analysis of culture #3 (Sigma EX-CELL CD Hydrolysate supplemented). Amino acid levels were evaluated at days 0, 2, 3, 5 and 6.

StableFast-NS0 cell line c7D11 (α-L1) 591-2 expressing mAb c7D11 was scaled up to the 5-L bioreactor as described in Chapter 3 (see Figure 25). A frozen seed vial of the cell line was thawed and expanded through T-flask culture to spinner cultures up to 1 L volume. A New Brunswick Scientific Celligen Plus bioreactor was used for the study described in this report. The bioreactor was rigged with pH and dissolved

oxygen (DO) probes, batched with 2 L of PBS buffer, and autoclaved at 121°C for 55 minutes. Upon removal from the autoclave, the bioreactor was connected to the control tower and sterile air was delivered to both the headspace (overlay) and via sparge. The PBS was pumped out and replaced with 3 L of fresh batch medium. The bioreactor was inoculated with 1 L Stage 3 seed culture for an initial density of 5.5×10^5 viable cells/mL. On Day 4, a single feed added the following nutrients: 3 mM glutamine, 1 g/L glucose, and 0.3 mM asparagine. The bioreactor run was ended on Day 7 and harvested by centrifugation followed by 0.22 mm filtration and storage at 4°C.

The growth parameters are shown in Figure 33 of both the 5-L bioreactor and a 120-mL pilot spinner culture used as a predictor for the bioreactor run. Maximum cell density was 3.97×10^6 and 4.40×10^6 viable cells/mL for the bioreactor and pilot spinner, respectively. Both culture profiles were similar including a rapid growth phase and rapid decline and death phase with no stationary phase at high density. Also, analysis of ammonia indicated a steady accumulation to about 8 mM for each culture. Final antibody titers of both cultures were about 50 mg/L by ELISA analysis. During the follow-up study, a scale down experiment will be conducted in 40 mL shaker flasks to determine the effect of reducing glutamine to 2 mM and evaluating the addition of cholesterol and minimal glucose and glutamine feeds. Minimizing ammonia accumulation through limited glutamine supplementation may result in higher and sustained viable cell density and higher mAb titer.

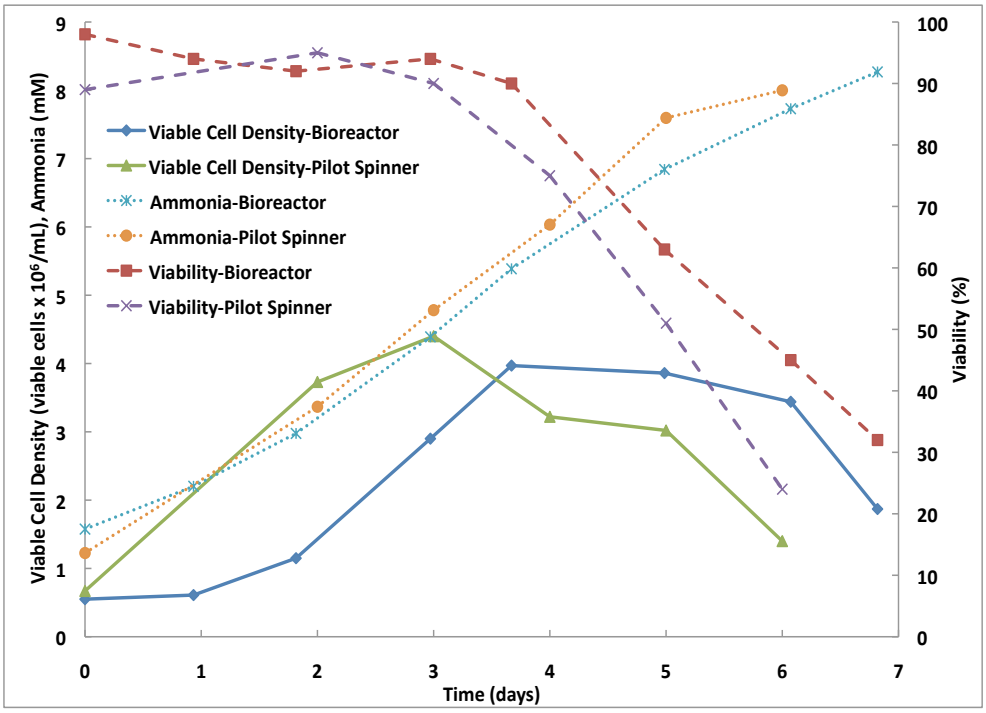


Figure 33. Growth and ammonia curves for 5-L bioreactor and pilot spinner. Viable cell densities are indicated by solid curves, ammonia data are indicated by dotted curves, and culture viability data are indicated by dashed curves.

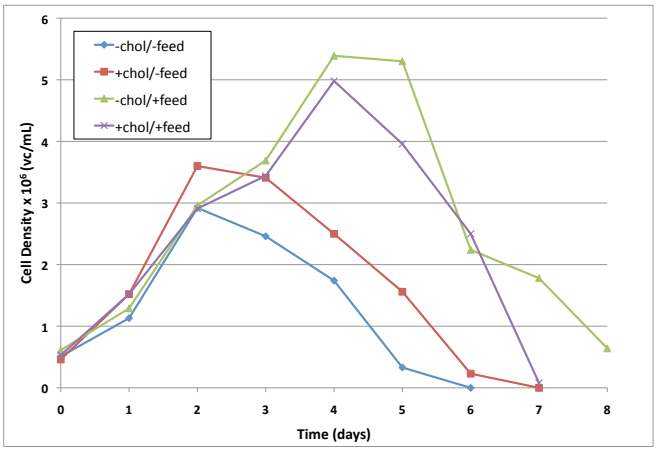
StableFast-NS0 cell line c7D11 (α-L1) 591-2 expressing mAb c7D11 was thawed and scaled for experiments to determine the effect of reducing glutamine to 2 mM and evaluating the addition of cholesterol and minimal glucose and glutamine feeds. Minimizing ammonia accumulation through limited glutamine supplementation may result in higher and sustained viable cell density and higher mAb titer. Table 16 shows the experimental design to evaluate the effects of cholesterol supplementation and feeding of low levels of glutamine and glucose.

Table 16. Cholesterol supplementation and low-level feed—Experimental design.

Flask	Starting L-Gln (mM)	Cholesterol Lipid Concentrate (mL/L)	Feed
1	2	0	NONE
2	2	4	NONE
3	2	0	Maintain glucose and glutamine at or below 2 g/L and 2 mM, respectively
4	2	4	Maintain glucose and glutamine at or below 2 g/L and 2 mM, respectively

As seen in Figures 34A-C, there were no significant differences between cultures with cholesterol supplementation and those without. However, both cultures that were fed glutamine and glucose to maintain levels at or below 2 mM and 2 g/L, respectively, performed significantly better than cultures that were not fed. Peak cell density of the fed-batch cultures was about 5×10^6 compared to maximum densities of about 3×10^6 for the batch cultures. Significantly, mAb yields were nearly 6-fold higher in the fed-batch cultures compared to the yields of the batch cultures. This feeding strategy will be scaled to the 5-L bioreactor and applied to other cell lines to increase volumetric productivity of mAbs c7D11, c8A, and c6C.

A.



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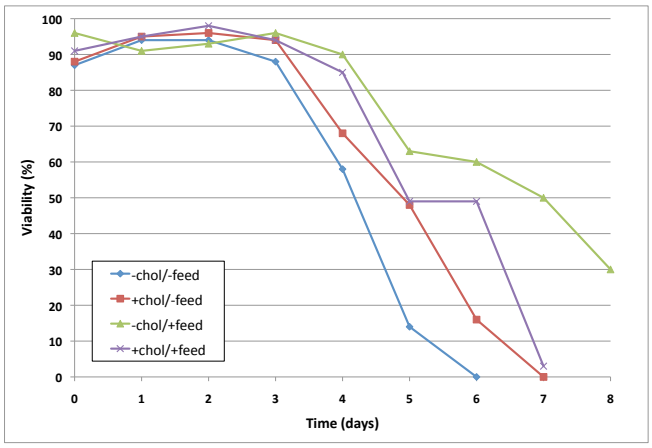

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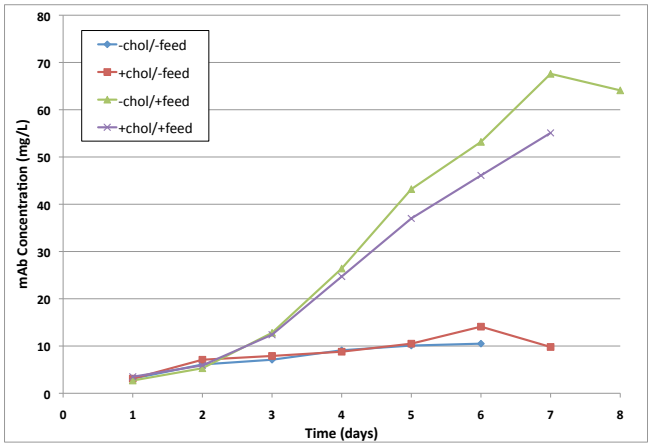

Figure 34. Cholesterol supplementation and low-level feed—40 mL shaker flask cultures. A: Viable cell density; B: Cell viability; C: mAb concentration.

Based on shaker studies, StableFast-NS0 cell line c7D11 (α-L1) 591-2 expressing mAb c7D11 was thawed and scaled to the 5-L bioreactor to evaluate the effect of a low glutamine fed batch strategy. The bioreactor was batched with 3 liters of basal medium and the run was started with the following initial parameters:

Temperature:	37°C
pH:	7.00
Dissolved O_2:	40%
Agitation:	40 rpm
Gas Flow:	5 mL/min

The culture was started with the inoculation of the reactor with 1 liter of spinner flask culture (2.68×10^6 viable cells/mL viable cell density [VCD]) yielding an initial VCD of 0.65×10^6. pH was maintained at 7.00 with the automatic addition of CO_2 and dissolved O_2 was maintained at 40% with the automatic addition of pure O_2 and by increasing overall gas flow rate. Figure 35 shows the growth, productivity, and metabolic activity of the culture during the 7-day run. A total of 3 glutamine feeds were added with a target of 2 mM.

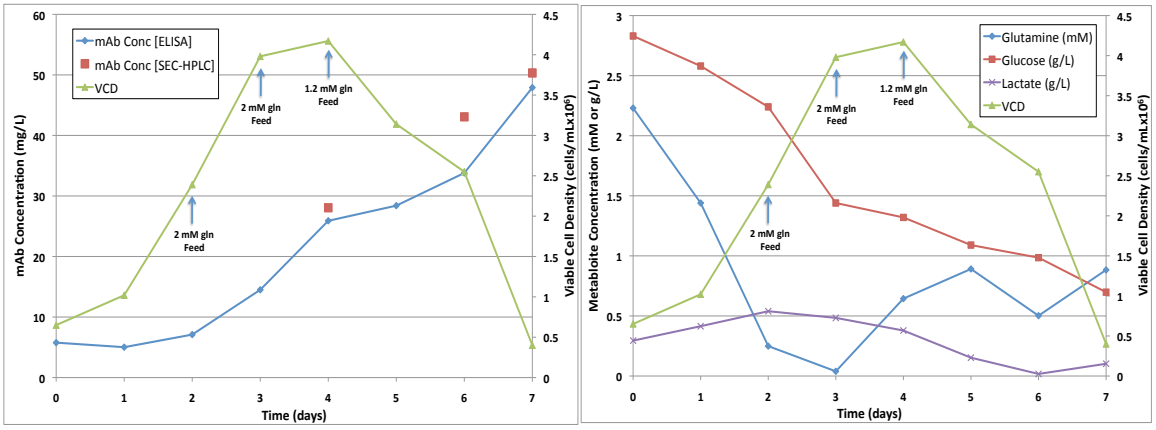

Figure 35. Time course of 5-L bioreactor 100915-R1. Viable cell density is indicated both on the left and right panes with a green solid curve and triangle markers. The left pane shows mAb concentration by ELISA (blue solid curve/diamond markers) and by SEC-HPLC (red square markers). The right pane shows the nutrient (glutamine and glucose, blue curve/diamond markers and red curve square markers, respectively) and lactate metabolite (purple curve/cross markers) concentrations.

As seen in Figure 35, peak cell density of the fed-batch culture was about 4×10^6 compared to a maximum density of about 5×10^6 for the best performing shaker culture. On Day 3, the glutamine was completely exhausted leading to rapid decline of viability and density. A follow up bioreactor run will be performed with a target of 3 mM glutamine to prevent early depletion of this critical nutrient. mAb levels were comparable to that of the shaker culture with a final volumetric yield of about 50 mg/L.

A follow up 5-L bioreactor run was performed with a target of 3-5 mM glutamine to prevent early nutrient depletion. Based on shaker studies during the previous reporting period, StableFast-NS0 cell line c7D11 (α-L1) 591-2 expressing mAb c7D11 was thawed and scaled to the 5-L bioreactor. The bioreactor was batched with 3 liters of basal medium and the run was started with the following initial parameters:

Temperature:	37°C
pH:	7.00
Dissolved O_2:	40%
Agitation:	40 rpm
Gas Flow:	10 mL/min

The culture was started with the inoculation of the reactor with 1 liter of spinner flask culture (2.23×10^6 viable cells/mL VCD) yielding an initial VCD of 0.55×10^6. pH was maintained at 7.00 with the automatic addition of CO_2 and dissolved O_2 was maintained at 40% with the automatic addition of pure O_2 and by increasing overall gas flow rate. Figure 36 shows the growth, productivity, and metabolic activity of the culture during the 7-day run. A total of 2 glutamine feeds were added with a target of 3-5 mM.

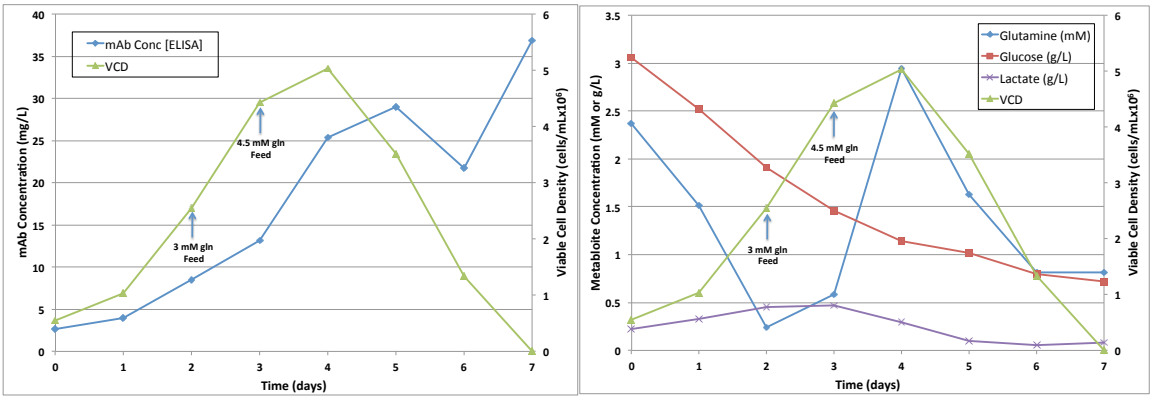

Figure 36. Time course of 5-L bioreactor 100923-R1. Viable cell density is indicated both on the left and right panes with a green solid curve and triangle markers. The left pane shows mAb concentration by ELISA (blue solid curve/diamond markers). The right pane shows the nutrient (glutamine and glucose, blue curve/diamond markers and red curve square markers, respectively) and lactate metabolite (purple curve/cross markers) concentrations.

As seen in Figure 36, peak cell density of the fed-batch culture was about 5×10^6 which is 1×10^6 greater than the previous bioreactor and comparable to the best performing shaker culture. In this run, glutamine was never fully exhausted with a minimum of about 0.24 mM on Day 2. Although cell density was 25% higher than that of the previous run, final volumetric mAb titer was lower at about 37 mg/L as compared to nearly 50 mg/L from the previous run. One possible limiting nutrient is glucose which, although not fully consumed, steadily falls to a value below 1 g/L. This low glucose also affects the lactate concentration which may have an effect on cell growth and productivity.

A follow up study to the second 5-L bioreactor was performed to determine the response of the StableFast-NS0 cell line c7D11 (α-L1) 591-2 expressing mAb c7D11 to varying batch and feed glucose in 40-mL shaker culture. Cells were seeded into four (4) 40-mL shaker cultures with a starting viable cell density of 5×10^6. Table 17

describes the experimental design including batch and feed targets of glucose and glutamine.

Table 17. Glucose-glutamine feed—Experimental design.

Flask	L-Glutamine (mM)	Batch Glucose (g/L)	Feed
1	2	3.4	Gln→0.5-3.0 mM Gluc→No Feed
2	2	5	Gln→0.5-3.0 mM Gluc→No Feed
3	2	10	Gln→0.5-3.0 mM Gluc→No Feed
4	2	3.4	Gln→0.5-3.0 mM Gluc→2-4 g/L

Flasks start at 40 mL each at 6e5 vc/mL

As seen in Figure 37, all four cultures exhibited similar growth patterns and expression levels. The glucose uptake rates were also quite similar with inflection points at Day 4 when growth and viability significantly dropped for all cultures. All cultures exhibited similar lactate profiles (data not shown) with maximum concentrations of about 1 g/L. Since the high batch glucose of 5-10 g/L had no ill effects on growth and productivity, future studies will include 10 g/L of glucose in the batch medium.

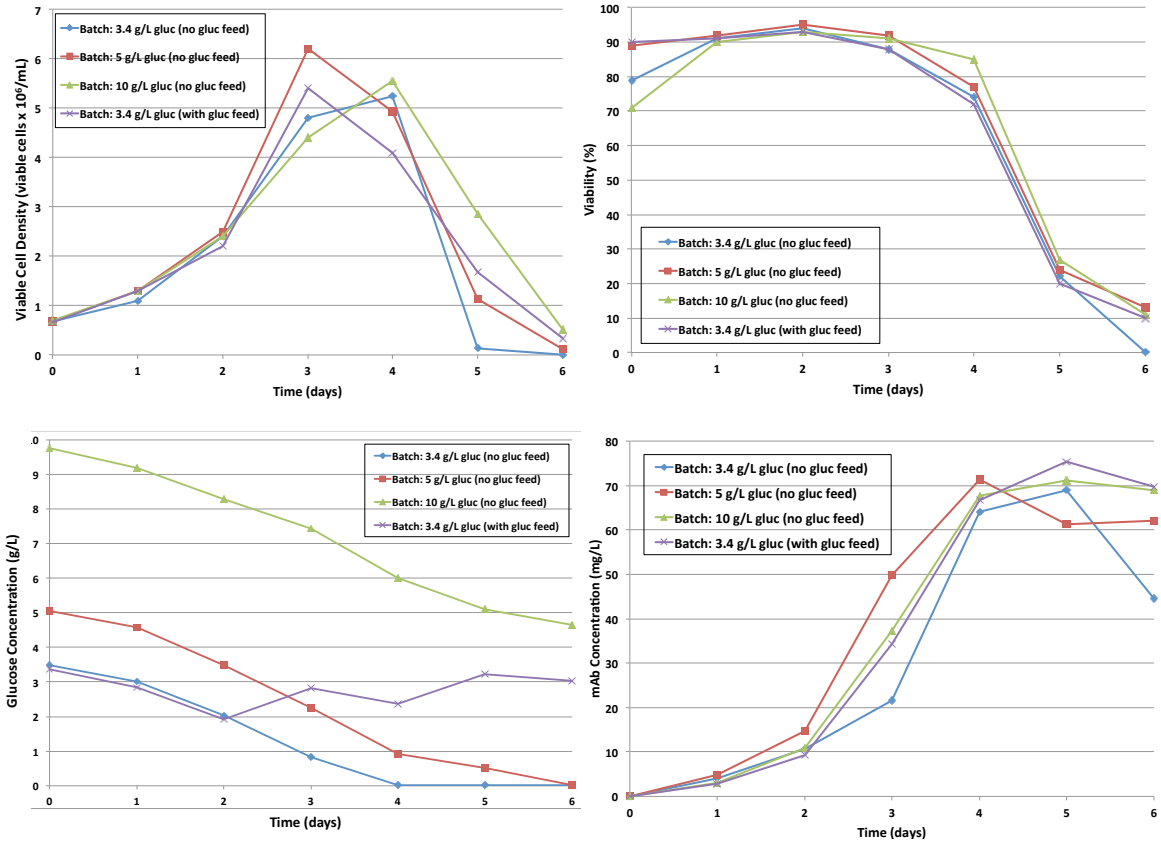

Figure 37. Time course of glucose-glutamine feed shake flasks. Top left panel indicates viable cell density; top right panel indicates culture viability; bottom left panel indicates culture glucose concentration; and bottom right panel indicates mAb titer.

A stably transfected pool of SF-NS0 cells was scaled up directly to a 5-L bioreactor. This stable pool was generated using the rapid method described at the end of Chapter 3. The total time from transfection to bioreactor was 6 weeks. Briefly, cells were scaled from a T-75 flask (12 mL) through small shaker culture (30 mL) and up through spinner flask stages to an inoculum of 1 liter. The bioreactor was batched with chemically-defined production medium (CD Hybridoma, Life Technologies) supplemented with 10 g/L glucose, 4 mM glutamine, and 1× Non-Essential Amino Acids (NEAA, Life Technologies). Bioreactor setpoints included: pH=7.00, Dissolved Oxygen (DO)=40%, Temperature=37°C, and Agitation=40 RPM. The bioreactor was fed four times during

the run to maintain glutamine concentration at 2-5 mM. At peak cell density, temperature was decreased to 34°C to prolong stationary period and mAb expression. Bioreactor was harvested and analyzed at Day 10. Growth and productivity are shown in Figure 38. Final product concentration was 139 mg/L using HPLC analysis. Total production was approximately 700 mg for the 5-L bioreactor. A pilot experiment in fed-batch spinner flask was run 2 days ahead of the bioreactor schedule (see gray dashed) and the final yield (Day 7) was 132 mg/L.

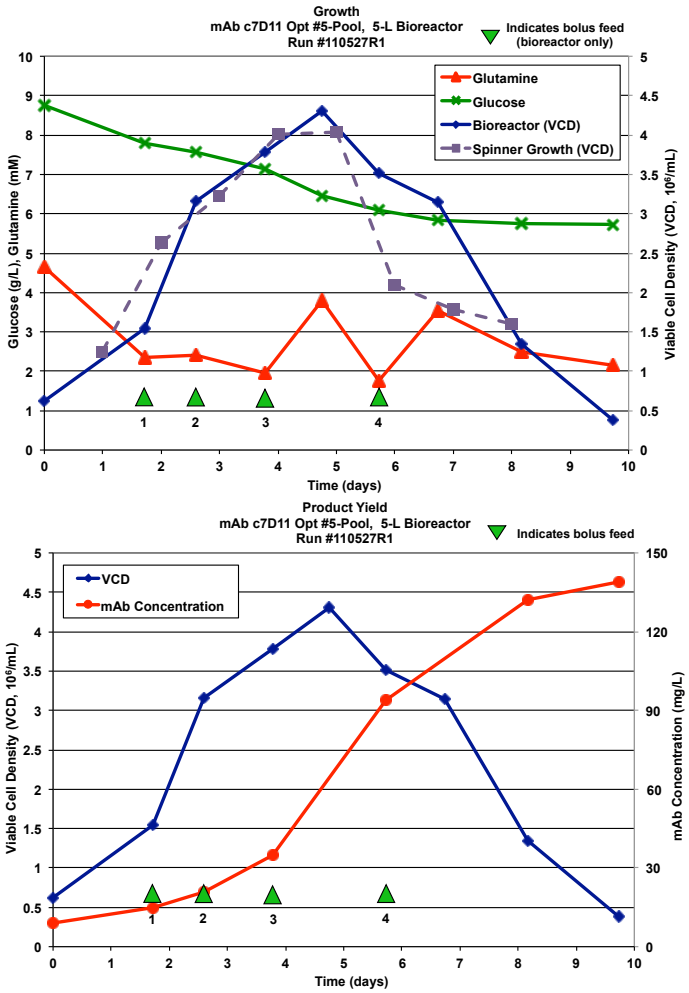

Figure 38. Growth and productivity of a stably transfected pool of SF-NS0 in a 5-L bioreactor. Top panel indicates growth (VCD) and culture nutrient concentrations (glucose and glutamine). Dashed curve in top panel indicates growth of pilot fed-batch spinner flask. Bottom panel indicates mAb product concentration (red curve) and growth (blue curve). Numbered triangles indicate glutamine bolus feeds.

Clone E12-2 was scaled up for a 5-L bioreactor run. The process was run as before with 10 g/L glucose in the batch medium and feeding glutamine to maintain concentration at 1-5 mM. The culture reached a maximum density of 6.25×10^6 viable cells per mL at Day 4. The run was harvested at 9.5 days and final product concentration was 241 mg/L. Total production was approximately 1.2 grams for the five-liter bioreactor. This concentration was determined using an HPLC size exclusion chromatography (SEC) method that has been show to be robust, reproducible, and accurate. Figure 39 shows the historic bioreactor runs starting with the clone 591-2 using unoptimized genetic sequences. As seen, the optimized pool yielded a 3-fold increase in productivity and the selected clone E12-2 produced about five times as much monoclonal antibody product as the unoptimized clone 591-2.

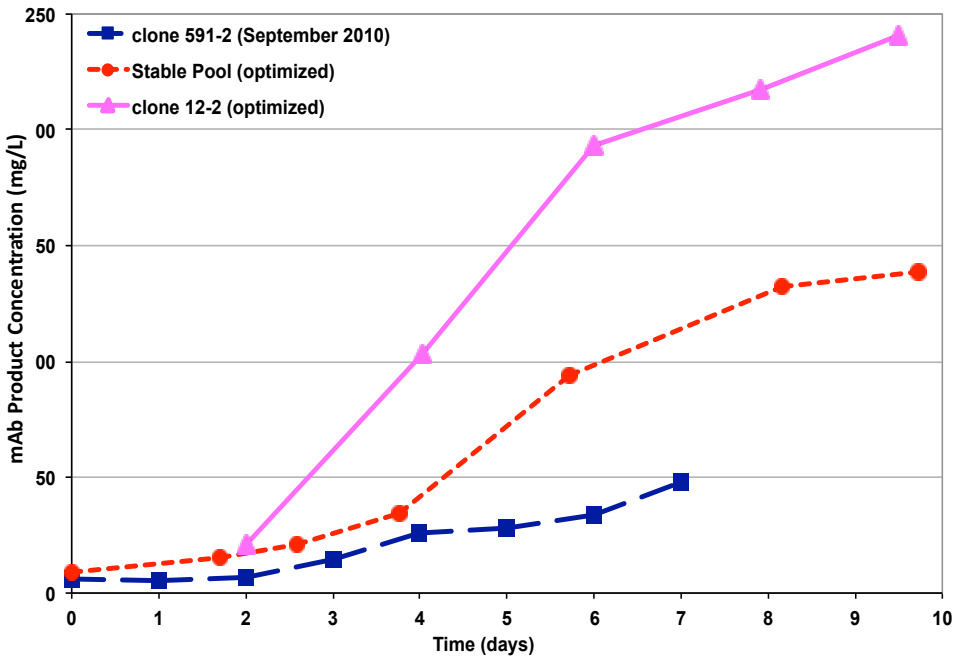

Figure 39. Productivity in 5-L bioreactor culture—An historic perspective. A comparison of cell line performance in bioreactors. Clone 591-2 (blue dashed curve with square markers) was generated with un-optimized heavy and light chain genes. Optimized stable pool is indicated by the dashed red curve with circle markers. The high performing clone 12-2 was isolated from the optimized pool and is indicated by the solid pink curve with triangle markers.

Bioprocess Scale-Up and Consistency Demonstration in Single-Use Bioreactors

Three clonal SF-NS0 cell lines producing three different human-mouse chimeric IgG1 monoclonal antibodies targeting the vaccinia virus (VACV) subunit proteins L1, B5, and A33 were thawed and scaled-up for production in 3.5-L and 10-L working volume single-use bioreactors (Eppendorf/New Brunswick Scientific, CelliGen BioBLU). Bioreactors were run in fed-batch mode using off-the-shelf media and feeds with no process development. Over the course of six (6) runs, the process was modified to include a temperature shift from 37°C in growth phase to 34°C during production phase. Cultures were harvested, purified by a single-step Protein A chromatography method, and dialyzed into PBS, pH7.4 (downstream process data not shown).

Cell Lines

c7D11 (α-VACV L1), Clone E12-2, 2e7, 1 mL, 29JUN11
c6C (α-VACV A33), Clone #53, P4, 1e7, 1 mL, 22JUL11
c8A (α-VACV B5), Clone B10, 1e7, 1 mL, 24JUN11

Expansion Medium

CD Hybridoma Medium (Gibco, Cat No 11279-023)
2 mM l-glutamine (Gibco, Cat No 25030-081)
1x MEM Non-Essential Amino Acids (Gibco, Cat No 11140-050)

Bioreactor Batch Medium

CD Hybridoma Medium (Gibco, Cat No 11279-023)
4 mM l-glutamine, pre-seed concentration (Gibco, Cat No 25030-081)
1x MEM Non-Essential Amino Acids, pre-seed concentration (Gibco, Cat No 11140-050)

8.75 g/L dextrose (d-glucose), post-seed concentration (JT Baker, Cat No 1916-01)

Bioreactor Feed Components

200 mM l-glutamine (Gibco, Cat No 25030-081)
100x MEM Non-Essential Amino Acids (Gibco, Cat No 11140-050)

Thaw, Expansion, Spinner Flask Cultures

SF-NS0 clones were thawed and expanded in T-flask and then spinner flask cultures in expansion medium maintaining VCD in the 0.5-2.5x10^6 cells per mL range. Seed cultures were scaled-up to 1 L in 3-L spinner flasks to seed the bioreactors. Expansion conditions were 37°C, 5% CO_2, >90% relative humidity (rh), 40-45 rpm agitation rate. See Figure 40 for the E12-2 clone population double level curve.

Bioreactor Cultures

The bioreactor runs were carried out in 3.5-L (BioBLU 5c) or 10-L (BioBLU 14c) working volume Eppendorf/New Brunswick Scientific CelliGen BioBLU single-use bioreactor system. The cultures were seeded in Bioreactor Batch Medium (see above) at ~1:4 dilution. The target inoculation density for the cultures was 0.25-0.5×10^6 VCD. The bioreactors were controlled at the following set points: 37ºC (initial growth phase), 40% DO_2, pH 7.10, agitation rates of 40-70 rpm, and gas sparge rates of 0.02 – 0.10 vvm. DO_2 and pH were maintained by cascaded gas mix control (air, O_2, CO_2). The cultures were monitored daily for glutamine and cell concentrations. Cultures were fed with 200 mM glutamine solution to maintain a target glutamine concentration of 2-4 mM. A single feed of 100× NEAA was added

in combination with the first glutamine feed to supplement the culture to 0.5× NEAA at the feed #1 timepoint. The cultures were maintained until the viability decreased to below 40%. At that point, the bioreactor run was harvested and purified.

Analytical Methods

Cell concentration and viability were determined by the Trypan Blue Dye Exclusion Method. An aseptic sample was removed from the spinner flask or bioreactor. 50µl of sample was added to 50µl of trypan blue stain and mixed gently. Approximately 10µl of stained sample was loaded onto the hemacytomer. Using the microscope, the viable and non-viable cells from the four corners of the hemacytometer grid were counted. The cell concentration was determined as follows:

$$Viable\ Cell\ Density\ (VCD) = \frac{Cell\ number\ counted}{Number\ of\ squares\ counted} \times Dilution\ factor \times 10^4$$

Glutamine and glucose concentrations in the bioreactors were measured with a YSI 2700 Select Biochemistry Analyzer.

Antibody product concentration was determined by a human IgG ELISA method.

Bioreactor process times ranged from 6-9 days although the single 9-day process (c7D11, clone E12-2) was supplemented with a media addition apparently extending the process time by 2 days. This c7D11, clone E12-2 was also the best performing bioreactor run with a final titer of nearly 400 mg/L (see Figure 41).

An initial run for clone 6C is shown in Figure 42 and was performed as per the E12-2 run without the media addition, but at 37°C for the entire run. A second c6C,

clone #53 run shown in Figure 43 included a temperature drop from 37°C in growth phase to 34°C at peak cell density (Day 4) resulting in a notable titer improvement (180 to 215 mg/L, 19% increase). This temperature drop was included on all subsequent runs.

The first c8A, clone B10 run was performed at 37°C for the entire process with a final yield of about 150 mg/L (Figure 44). A second c8A, clone B10 run was scaled further to a 10-L working volume (Figure 45). Although peak cell density was lower than that of the 3.5-L run by $1x10^6$, volumetric yield increased by 17% to 175 mg/L. This may be due to prolongation of culture density due to the temperature decrease.

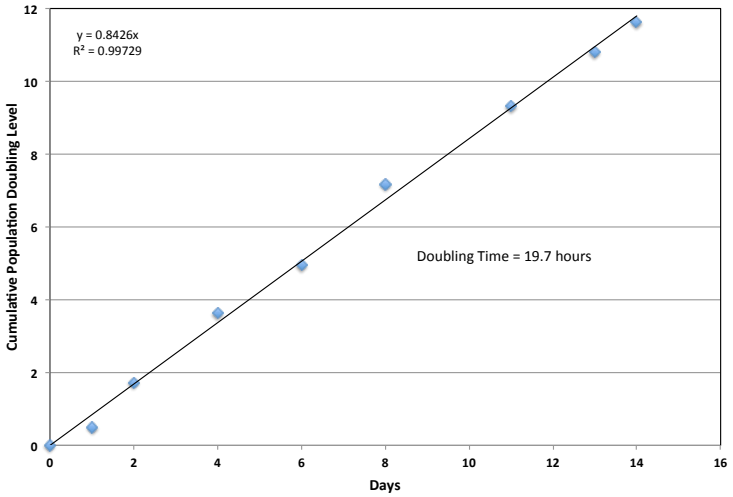

Figure 40. Population doubling levels during thaw and expansion of SF-NS0 clone E12-2. Curve indicates stable growth rate with a doubling time of 19.7 hours.

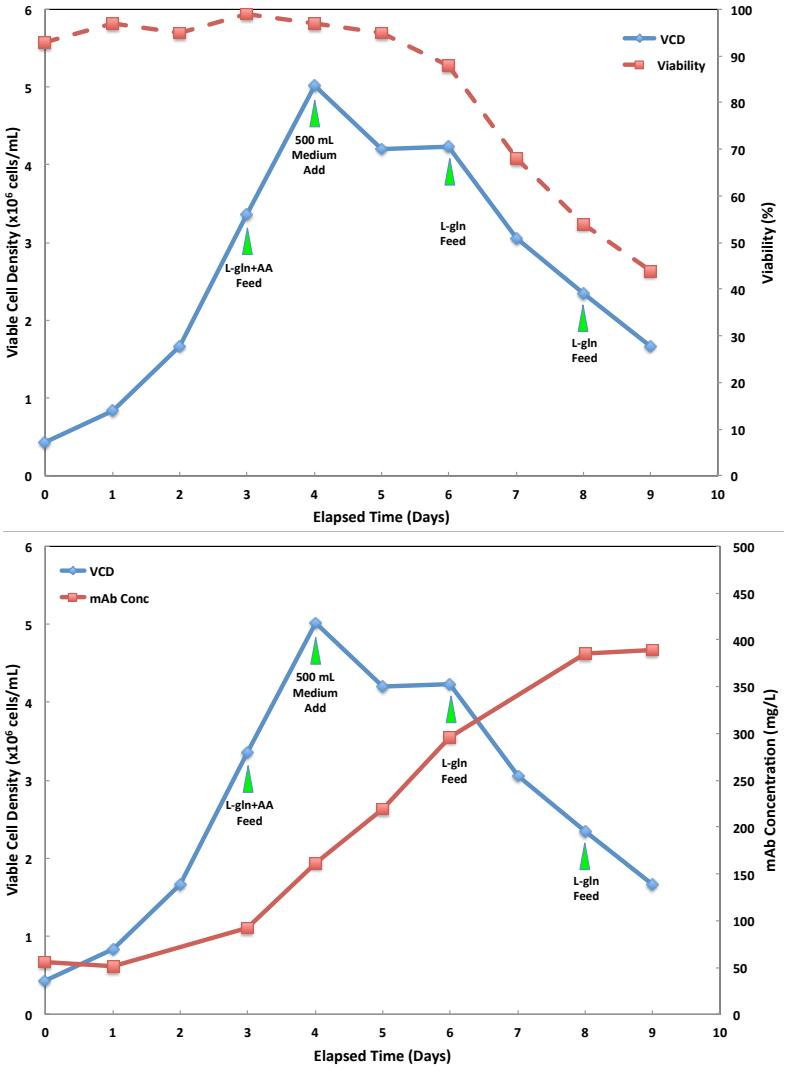

Figure 41. Growth and titer of SF-NS0 clone E12-2 3-L bioreactor. Top panel indicates growth (VCD) and culture viability. Bottom panel indicates mAb concentration and growth (VCD). Green arrows indicate bolus feeds.

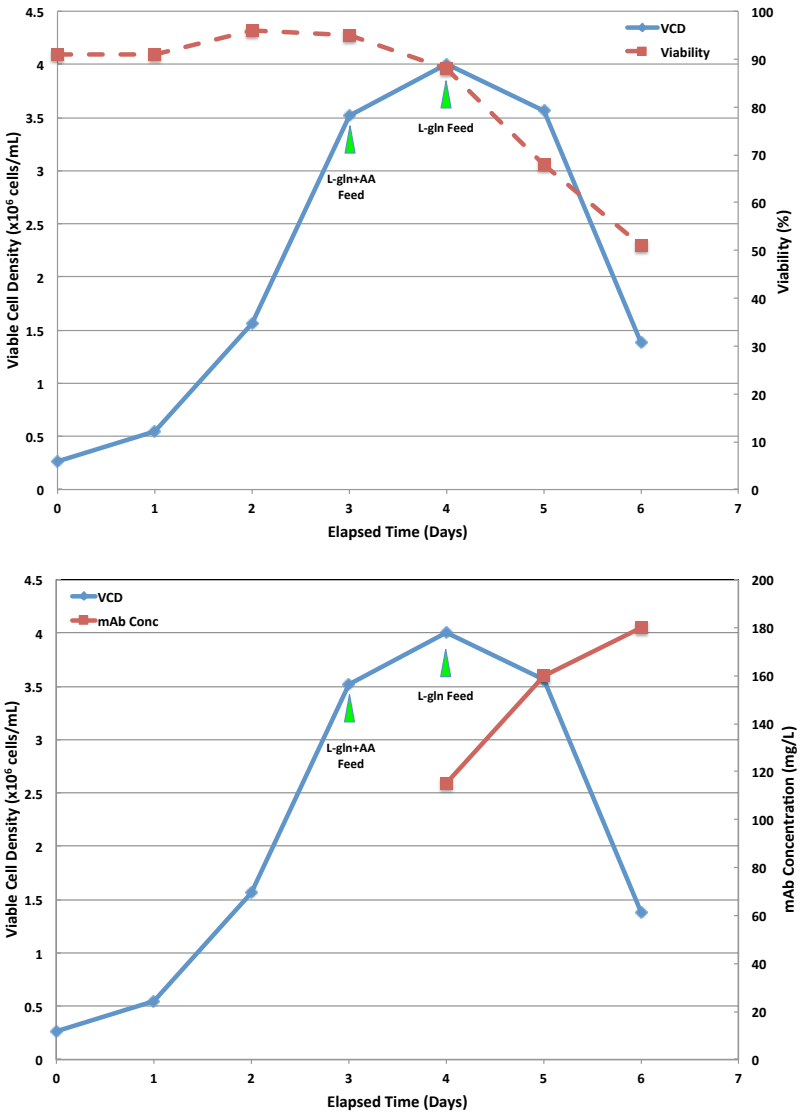

Figure 42. c6C, clone #53 3.5L Bioreactor Run #1 (37°C). Top panel indicates growth (VCD) and culture viability. Bottom panel indicates mAb concentration and growth (VCD). Green arrows indicate bolus feeds.

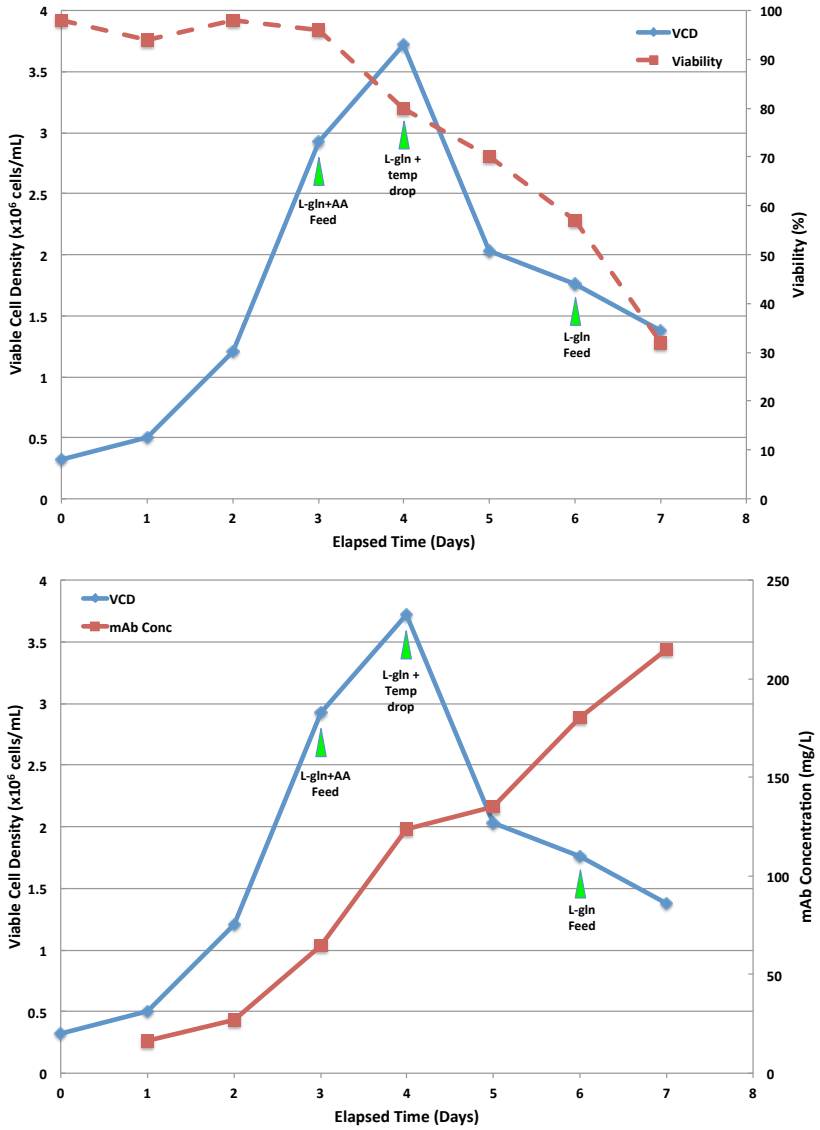

Figure 43. c6C, clone #53 3.5L Bioreactor Run #2 (Temp drop to 34°C on Day 4). Top panel indicates growth (VCD) and culture viability. Bottom panel indicates mAb concentration and growth (VCD). Green arrows indicate bolus feeds.

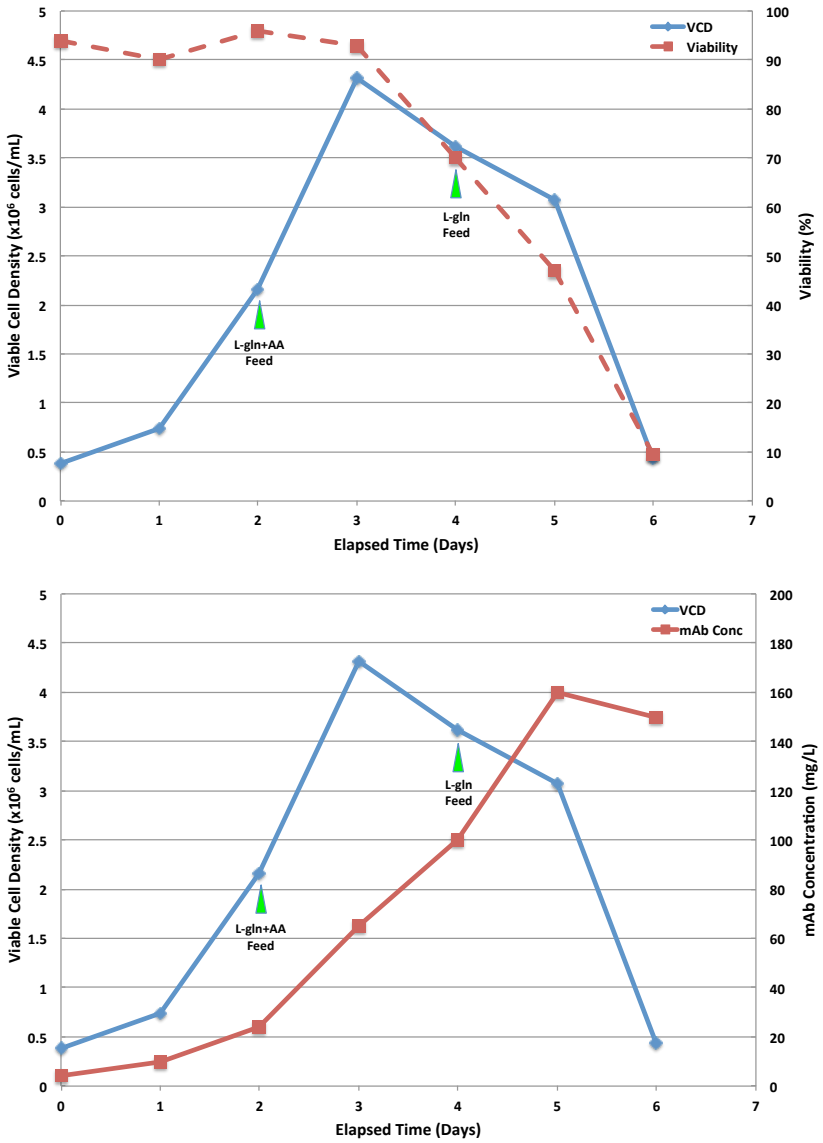

Figure 44. c8A, clone B10 3.5L Bioreactor Run #1 (37°C). Top panel indicates growth (VCD) and culture viability. Bottom panel indicates mAb concentration and growth (VCD). Green arrows indicate bolus feeds.

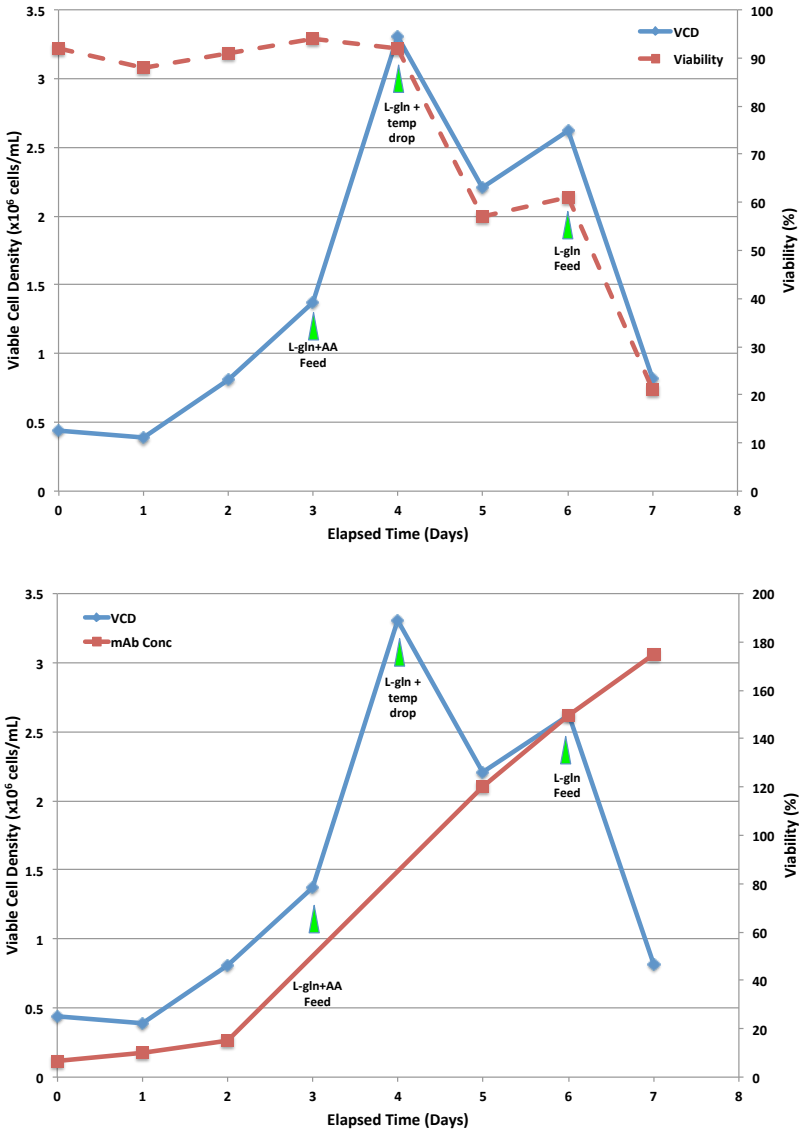

Figure 45. c8A, clone B10 10L Bioreactor Run #2 (Temp drop to 34°C on Day 4). Top panel indicates growth (VCD) and culture viability. Bottom panel indicates mAb concentration and growth (VCD). Green arrows indicate bolus feeds.

Conclusions

Initial experiments in shaker flasks indicated that the addition of complex supplements such as yeast extracts did not improve either viable cell density or yield. This may be revisited with cell lines adapted to such supplements. These initial experiments also indicated that higher batch concetration of glutamine, the SF-NS0 cell line's preferred principal carbon source, led to higher growth and mAb yields. Further studies using HPLC analysis, determined that several critical amino acids were being preferentially consumed. The process was scaled to the 5-L bioreactor and an evaluation of ammonia generation showed steady increases to about 8mM prompting another round of glutamine supplementation experiments in shaker flasks. Controlled feeding to maintain both glutamine and glucose at lower levels, 2mM and 2 g/L targets, respectively, resulted in significant increases in both densities and yields as compared with batch controls. A supplementation of cholesterol had no effect. Follow up experiments in both 5-L bioreactors and shaker flasks led to a high-yielding process with high batch glucose (10 g/L) and controlled glutamine feeds to maintain a concentration of 2-5mM. This process was applied to a rapid generation stable pool at the 5-L scale resulting in a yield of 135 mg/L at six weeks post-transfection. The best performing clone E12-2 was subsequently scaled to the 5-L bioreactor at eight weeks post-transfection resulting in a yield of nearly 250 mg/L, a 5-fold increase over the earlier clonal cell lines and processes. This process was adapted to a single-use bioreactor platform and clonal cell lines expressing three different mAb products were scaled up to 3.5-L and 10-L working volumes. During these production campaigns, a temperature drop from 37°C to 34°C was incorporated that served to extend bioprocess time at high

cell densities resulting in higher accumulation of secreted mAb product. Performance of the StableFast-NS0 platform was consistent with yields ranging from 150 mg/L to nearly 400 mg/L.

Chapter 5:
Multiplex Selection for Increased Specific Productivity

Abstract

During the development of the StableFast-NS0 platform, it was noted that the cholesterol metabolic selection approach may result in low specific productivity and low protein titers due to the difficulty of amplification in this cell line. In other NS0 host cell systems, the glutamine auxotrophy is exploited by incorporating a glutamine synthetase (GS) gene into the vector also encoding the protein of interest, and stably transfected cells are selected for by removing glutamine from the culture media. In addition, the GS inhibitor methionine sulphoximine (MSX) can be added to the culture media to drive selection towards the incorporation multiple copies of the GS gene and concomitantly the gene encoding the protein of interest.[44-48] Each system has its benefits and limitations. In Chapter 5, studies were performed to develop and implement multiple or multiplexed selection strategies with the goal of consistently generating cell lines with 1 g/L productivity potential. Initial studies indicated that the increasing selection stringency/stress to the NS0 host cell is NeoR/G418 → 17β7(KSR)/cholesterol → GS/glutamine. Sequential selection of co-transfected pools resulted in clear recoveries of populations resistant to the up to all three selection pressures. These results were used to inform the generation of stable miniwells. Cell lines generated by multiplex selection demonstrated Qp values of nearly triple than that of single cholesterol selection generated clones.

Results and Discussion

Expression Vector Construction

Plasmids used are listed in Table 18. Briefly, plasmid pBF-1 containing independent expression cassettes for an IgG heavy and light chain both driven by independent identical CMV promoter sequences and also containing a third independent expression cassette for the murine 3-ketosteroid reductase (3-ksr) Hsd17β7 metabolic selection marker driven by an SV40 promoter was modified for the project by replacing the existing signal peptides (SPs) with those more appropriate for biopharmaceutical development. SP replacement was accomplished by Gibson assembly[49-50] of the restricted plasmid backbone, PCR modified amplicons and synthetic gene fragments generating plasmid pBF-SP-V2. Gibson assembly was further used to replace the 3-ksr selection marker in pBF-SP-V2 with GS while simultaneously engineering unique restriction sites flanking the selection marker for convenience generating the pBF-GS plasmid. The NeoR selection marker in pCR2.1-TOPO was amplified by PCR appending flanking *Pml*I and *Eco*RI restriction sites complementary to those sites engineered into pBF-GS. Restriction and ligation of pBF-GS and the NeoR PCR product by *Pml*I and *Eco*RI generated the pBF-NEO plasmid. All plasmids were propagated in One Shot TOP10 Chemically Competent *E. coli* (ThermoFisher Scientific, MA). Oligonucleotide primers and gene fragments were obtained from Integrated DNA Technologies (Coralville, IA) and are listed in Table 19.

Table 18. List of plasmids.

Plasmid Name	Selection Marker	Notes
pBF-1	3-ksr	Original plasmid containing independent CMV driven IgG light and heavy chain expression cassettes with SV40 driven cholesterol metabolic selection marker
pBF-SP-V2	3-ksr	Modification of pBF-1 with new signal peptides cloned in frame with each light and heavy chain ORF
pBF-GS	Glutamine Synthetase (GS)	Modification of pBF- SP-V2 with GS selection marker replacing 3-ksr marker
pCR2.1-TOPO	NeoR	Backbone plasmid used to amplify Neomycin/G418 resistance gene for subcloning into pBF-SP-V2 plasmid
pBF-NEO	NeoR	Modification of pBF-SP-V2 with NeoR selection marker replacing 3-ksr marker

Table 19. List of primers and gene fragments.

Primer Name(s)	Sequence (5′ → 3′)
LC _SP_fr1.FOR (Fragment 1.FOR)	TTCTGCAGTCACCGTCCGCTAGCTTAATTAAACCGGTAGCGCTGCCGCCACC
LC.REV (Fragment 1.REV)	GCGGGTTTAAACGGCGCGCCTCAGCACTCGCCTCTGTTGAAG
PROM.FOR (Fragment 2.FOR)	TCAACAGAGGCGAGTGCTGAGGCGCGCCGTTTAAACC
PROM.REV (Fragment 2.REV)	CCCATGGTGGCGGCAAGCTTAAGTTTAAACGCTAGCGGACGG
SP_HC_A (Fragment 3_HC_A)	GTCCGC . . . ACAAGA
SP_HC_B (Fragment 3_2_HC_B)	GTAACG . . . GCCTTC
GS_A (GS_mouse_optimize_gibson_A)	TTTCGG . . . CCCCAA
GS_B (GS_mouse_optimize_gibson_B)	TGTGCG . . . TAGAGC
NEO_PmlI5'	TTTCGCCACGTGCCACCATGATTGAACAAGATGGATTGCAC
NEO_EcoRI_3'	TTTTTGAATTCTTCAGAAGAACTCGTCAAGAAGG

Transfection

For each transfection, 12×10^6 NS0 Parental cells were pelleted and washed. The cells were resuspended in 700µL of basal (not supplemented) CD Hybridoma Media (Gibco) and mixed with 45µg total of linearized plasmid DNA. The mixture was transferred to an electroporation cuvette. Two 250V, 400µFd pulses were

administered with a one minute rest period in between the pulses using a Gene Pulser II electroporator (Bio-Rad). The transfected cells were added to a T-75 flask containing 12mL of growth media and set in an appropriate incubator.

Productivity Measurements

Initial screening of IgG product concentration in cell culture supernatant was determined by an Enzyme Linked Immunosorbent Assay (ELISA). Briefly, flat bottom 96-well ELISA plates were coated with an anti-human IgG—Fab Specific Ab (Sigma) overnight at 4°C. Plates were washed and samples and standards were added to appropriate wells. After incubation at room temperature (RT), plates were washed and a secondary HRP-labeled anti-human IgG—Fc Specific Ab (Sigma) was added to all wells. After incubation at RT, plates were washed and developed with TMB substrate (KPL). Developing reaction was stopped with 1N H_2SO_4 and plates were read at 450nm absorbance. For larger scale production studies, IgG product concentration was determined by HPLC using a MAbPac Protein A analytical column and the manufacturer's protocol (Thermo).

Specific Productivity Rate (Qp)

The specific productivity rate (Qp) assay measures the growth and production rates of each stable cell line to determine the amount of secreted protein product per cell per day. The Qp number is an indicator of the expected level of performance of each cell line in scale-up conditions. Although the Qp can vary during scale-up, and upon medium and cell culture process optimization, it is commonly a relatively stable reference point. Briefly, cells were seeded at an initial density of 3.5×10^5 in fresh medium and incubated for three days. At the end of this growth period, the cells were

counted and the supernatants were analyzed by ELISA or Protein A HPLC. From these data, the doubling time and Qp in pg/cell/day (pcd) were calculated.

Limiting Dilution Cloning (LDC)

Selected stably transfected pools and miniwells were cloned by limiting dilution cloning (LDC). Briefly, cultures were counted and diluted to 3 (positive control), 1, and 0.3 cells per well in 200μL in 96-well plates. Plates were fed weekly (90μL removed, 110μL added) and examined for colony formation. Upon growth of ≥30% confluence, wells were screened for protein product concentration by ELISA. Positive expressing clones were scaled up for further characterization.

Studies 1 and 2

The goal of the first two studies was to compare a number of initial conditions and strategies in bulk selection to determine the best performing conditions for sequential and parallel multiplex selection experiments. In Study 1, initial conditions were evaluated in T-25 (5mL) cultures such as recovery time (24 vs 48 hours post-transfection) and frequency of feeding during selection (data not shown). From this preliminary study, it was determined that a recovery time of 24 hours and feeding of cultures every 48 hours is required for successful selection recovery.

In Study 2, a number of selection strategies and conditions were evaluated in 6-well plate format with single vector transfections. These transfections included direct comparison under cholesterol selection of the original signal peptide (SP) containing expression plasmids for the IgG with the new SP containing constructs that were designed to produce a more appropriate biopharmaceutical. The cholesterol selection marker was then replaced with a GS metabolic selection marker and this expression

plasmid was evaluated in a number of glutamine selection strategies and conditions. Finally, the selection marker was replaced with the neomycin resistance gene (NeoR) and evaluated under two different concentrations of Geneticin/G418 (Gibco). Additionally, the GS and NeoR vectors were evaluated in the Null Parental NS0 cell line (NS0Null). The NS0Null cell lines was created by stably transfected with a plasmid containing only the 3-ketosteriod reductase (KSR) selection marker and with empty light and heavy chain expression cassettes. The NS0null cell line is cholesterol independent and does not secrete a recombinant protein product.

After transfection and recovery, 3mL of each transfection was centrifuged for each experimental condition outlined in Table 20. Pelleted cells were resuspended in 3mL of respective selective media and split between 2 wells of a 6-well plate (1.5mL per well, each condition in duplicate). Negative controls used 1.5mL of mock transfection recovered cells, pelleted and resuspended in 1.5mL of appropriate selection media in one well of a 6-well plate. Feeding was performed every 48 hours by the replacement of 750µL of supernatant with 775µL of fresh selection media. The GS transfections that were gradually depleted of GlutaMAX were sequentially weaned as shown in Table 21.

Table 20. Stable pool selection conditions for bulk screen in 6-well plates.

Plasmid	Par Cell	SP	Cholesterol	Glutamax	G418 (μg/mL)
pBF-1 (KSR only)	Std	old	-	2mM	-
pBF-SP-V2 (KSR only)	Std	new	-	2mM	-
Negative KSR (H_2O)	Std	N/A	-	2mM	-
pBF-1 (KSR only)	Std	old	-	0.5mM 1 wk/0mM 2 wk	-
pBF-GS	Std	new	+	2mM 1 wk/0mM 3 wk	-
pBF-GS	Std	new	+	0.5mM 1 wk/0mM 2 wk	-
pBF-GS	Std	new	+	-	-
Negative GS (H_2O)	Std	N/A	+	-	-
pBF-NEO	Std	new	+	2 mM	100
pBF-NEO	Std	new	+	2 mM	200
Negative Neo (H_2O)	Std	N/A	+	2 mM	100
Negative Neo (H_2O)	Std	N/A	+	2 mM	200
pBF-GS	Null	new	-	0.5mM 1 wk/0mM 2 wk	-
pBF-GS	Null	new	-	-	-
Negative GS (H_2O)	Null	N/A	-	-	-
pBF-NEO	Null	new	-	2 mM	100
pBF-NEO	Null	new	-	2 mM	200
Negative Neo (H_2O)	Null	N/A	-	2 mM	100
Negative Neo (H_2O)	Null	N/A	-	2 mM	200

Table 21. GlutaMAX depletion schedules.

0.5mM→0mM over 1 week:

Day	GlutaMAX (mM)
0	0.5
2	0.375
4	0.25
6	0.125
8	0

2mM→0mM over 2 weeks:

Day	GlutaMAX (mM)
0	2
2	1.75
4	1.5
6	1.25
8	1
10	0.75
12	0.5
14	0.25
16	0

Figure 46 shows the viable cell density and cell viability of each cell line at each feeding. Counts prior to day 10 were performed using a Countess (Invitrogen). Data for days 10 and 12 were acquired manually using a hemocytometer. Only cells transfected with Neomycin resistance showed clear recovery from selection. GS transfections in NS0par cells showed a depletion strategy dependent response with a trend to recovery.

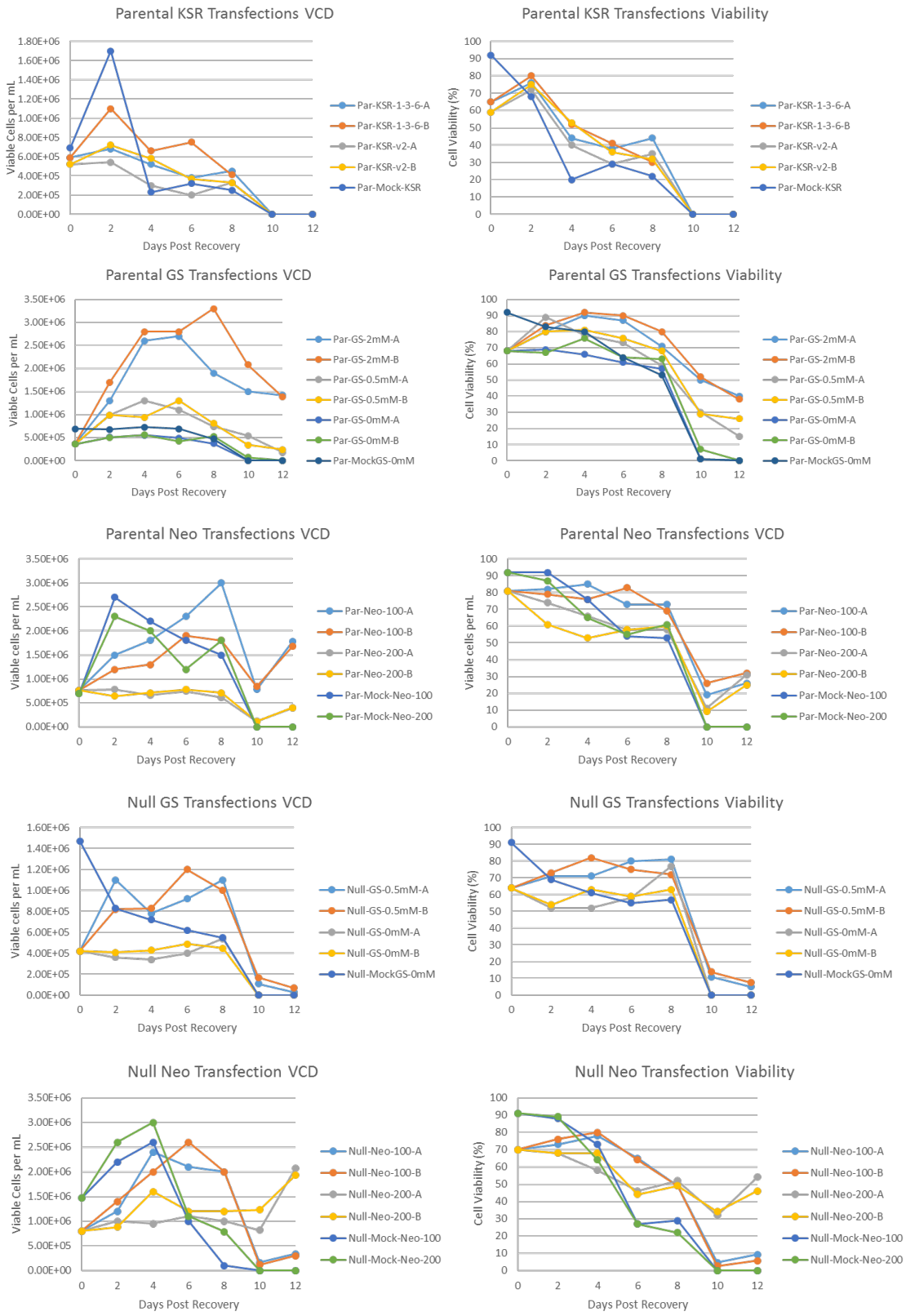

Figure 46. Study 2 Growth and Viability. Left panels indicate VCD and right panels indicate culture viability.

On day 12 post-recovery, supernatants for each culture were tested undiluted for antibody production by ELISA (see Figure 47). Results below 0.06 µg/mL titer fell below the linear range of the standard curve.

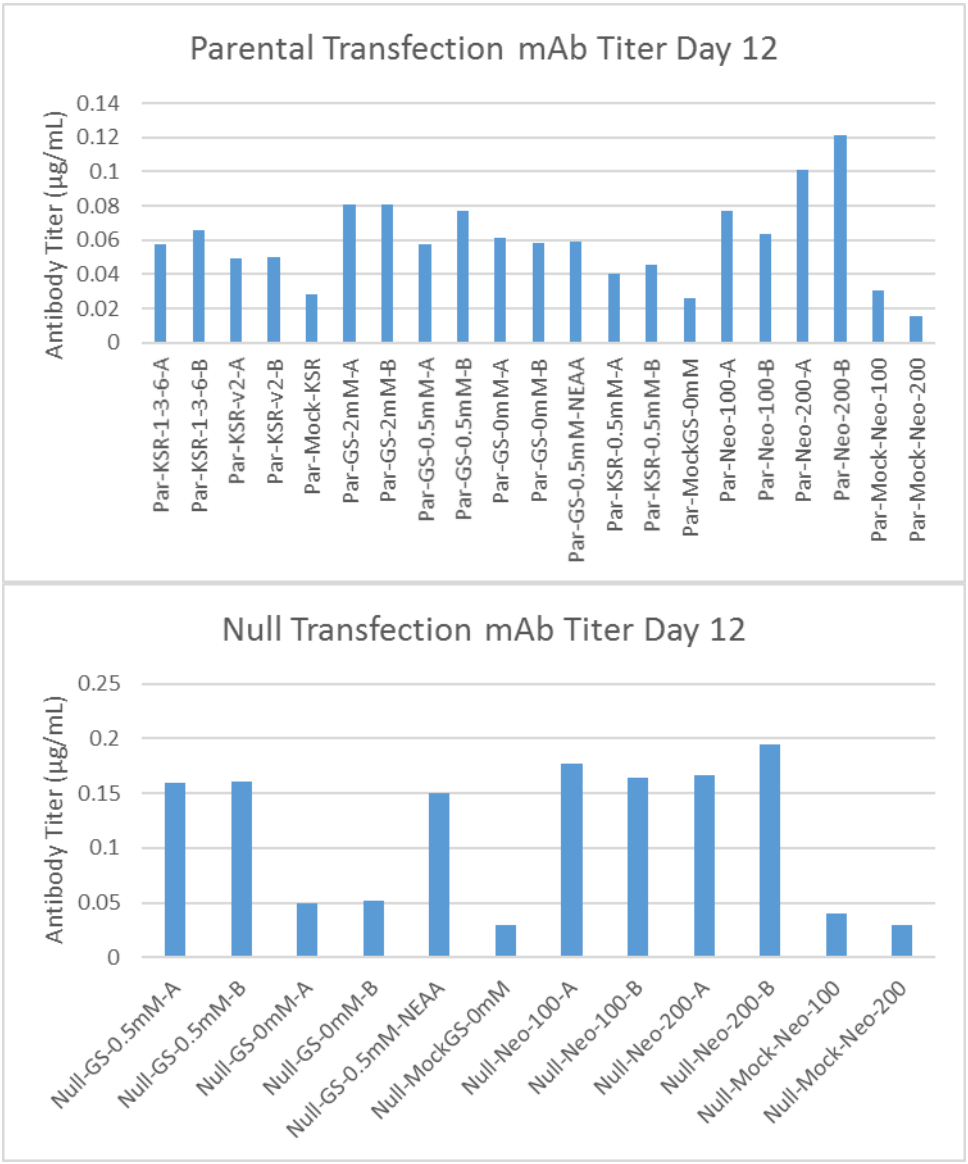

Figure 47. Antibody Titer Analysis of Cell Culture Supernatant. Top panel shows mAb titer of the standard parental NS0 transfections and the lower panel indicates mAb titer of the Null parental NS0 transfections.

Study 3

In Study 3, the best performing conditions in Study 2 were combined to explore the effects of sequential multiplexed selection at the T-75 flask scale. Single selection positive control transfections using the established cholesterol selection strategy were performed using original and new SP vectors. Multiplexed selection was conducted by linearizing each of the neomycin (Neo), cholesterol (KSR) and glutamine synthetase (GS) selection marker containing biosimilar IgG expression vectors and co-transfecting aliquots of 12e6 parental NS0 cells with 15µg of each DNA construct by electroporation. Cultures were allowed to recover for 24 hours after which a single selection pressure was applied to the bulk transfected culture. Upon recovery from the initial selection a second selection pressure was applied and, in some cases, upon the second recovery a third selection pressure was applied. All experimental conditions were performed in duplicate.

After electroporation and recovery, each transfection was centrifuged for each experimental condition outlined in Table 22. Pelleted cells were resuspended in 12mL of respective selective media. Feeding was performed every 48 hours by the centrifugation of the cell culture, and resuspension of the cell pellet in 6mL of supernatant and 6mL of fresh selection media. As cultures transfected with multiple selection vectors approached 75% viability, an additional selection pressure was added. GlutaMAX depletion over one week was applied as per Table 23 for GS selection strategies.

Table 22. Stable pool selection conditions for bulk screen in T-75 Flasks.

Condition Number	Plasmid(s)	Par Cell	SP	Selection #1	Selection #2 at 75% viability	Selection #3 at 75% viability
1	pBF-1 KSR only)	Std	original	Cholesterol	-	-
2	pBF-SP-V2 (KSR only)	Std	new	Cholesterol	-	-
3	Neo-KSR-GS (triple transfection)	Std	new	G418-200ug/mL	(-) Cholesterol, 0.5mM GlutaMAX	(-) GlutaMAX
4	Neo-KSR-GS (triple transfection)	Std	new	G418-200ug/mL	(-) Cholesterol, 2mM GlutaMAX	-
5	Neo-KSR-GS (triple transfection)	Std	new	G418-200ug/mL	0.5mM GlutaMAX to zero over 1 week	-
6	Neo-KSR-GS (triple transfection)	Std	new	(-) Cholesterol, 2mM GlutaMAX	0.5mM GlutaMAX to zero over 1 week	-

Table 23. GlutaMAX depletion schedule.

0.5mM→0mM over 1 week:

Day	GlutaMAX (mM)
0	0.5
2	0.375
4	0.25
6	0.125
8	0

Figure 48 shows the viable cell density and cell viability percentage of the KSR control conditions 1 and 2. All KSR control transfections with the single cholesterol selection strategy recovered albeit somewhat slower than historic data; 20-30 days vs 15 days, respectively.

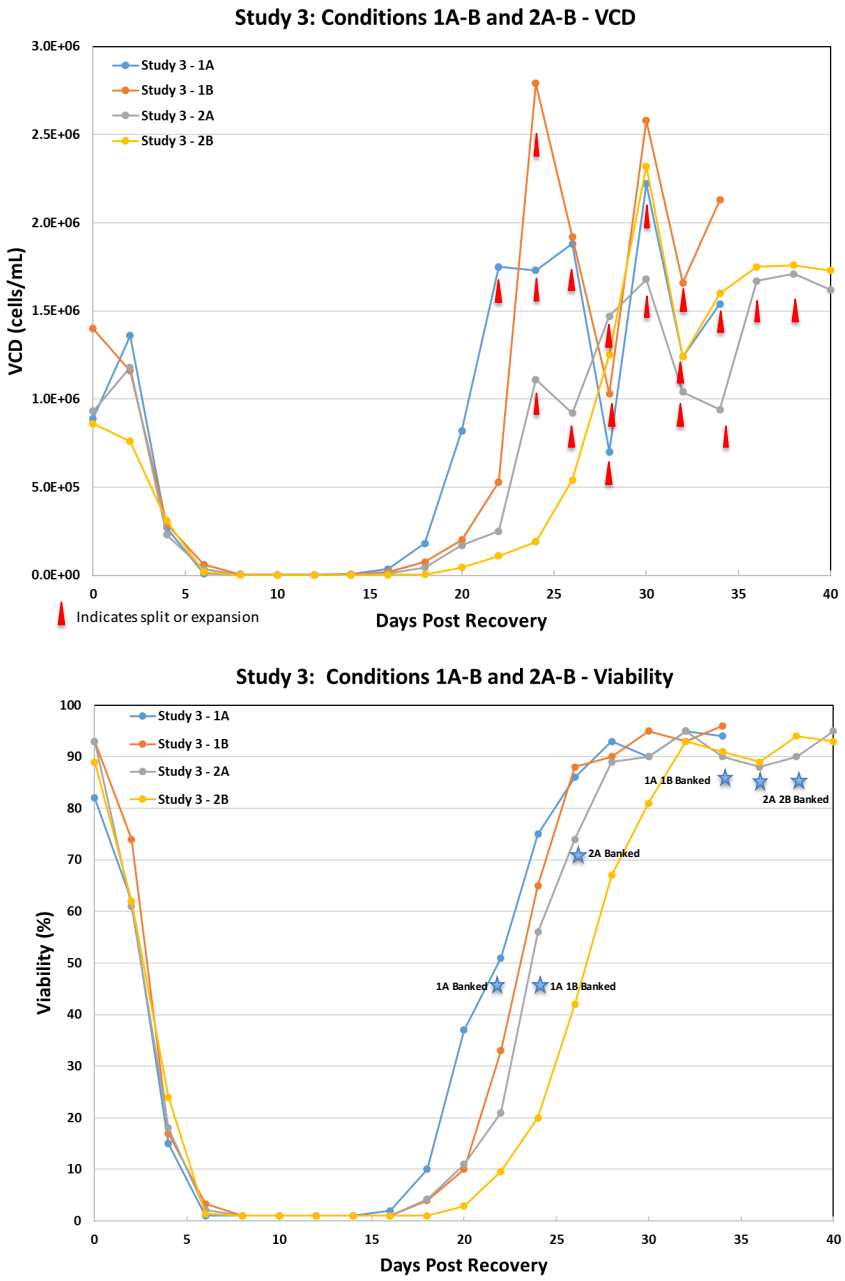

Figure 48. Study 3, Conditions 1 and 2—KSR Control: Growth and Viability. Top panel shows culture growth (VCD) and the lower panel indicates viability. Red arrows indicate splits or expansions. Blue stars indicated when cultures were banked.

Figure 49 shows the viable cell density and cell viability percentage of the Condition 3 replicates. These cells were transfected with all three plasmids and subjected to initial selection pressure of 200µg/mL G418. Both recovered rapidly as seen in earlier experiments and were then subjected to cholesterol selection with

reduction of GlutaMAX to 0.5mM. Replicate 3B did not recover from this second selection. Replicate 3A did recover, but with persistent reduced viability. At 2 weeks after recovery to sustained 40-50% viability under Neo and KSR selection, glutamine was reduced applying the third GS selection pressure. Cells responded favorably rebounding to over 80% viability, were analyzed for 6-day fed batch productivity and were banked.

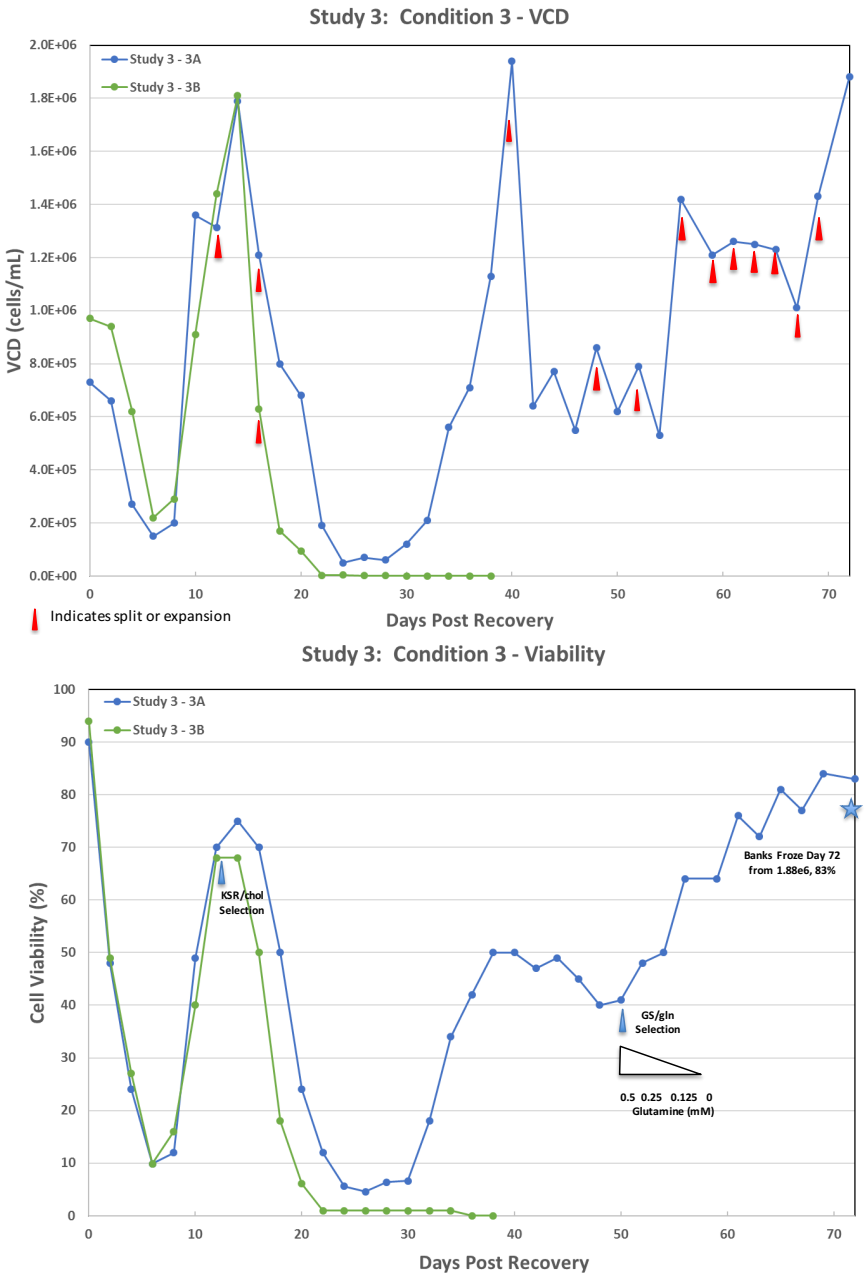

Figure 49. Study 3, Condition 3—Neo→KSR→GS: Growth and Viability. Top panel shows culture growth (VCD) and the lower panel indicates viability. Red arrows indicate splits or expansions. Blue arrows indicate initiation of selective pressures. Blue stars indicated when cultures were banked. Triangle graphic indicates timing of glutamine withdrawal.

Figure 50 shows the viable cell density and cell viability percentage of the Condition 4 replicates. These cells were transfected with all three plasmids and subjected to initial 200μg /mL G418. Both recovered rapidly as expected and were then subjected to cholesterol selection with full 2mM GlutaMAX supplementation. Both replicates recovered rapidly (12 days vs 20-30 days for the KSR controls). GlutaMAX was reduced applying the third GS selection pressure. Replicate A recovered from this third selection in less than 6 days, however replicate B took approximately 30 days to recover.

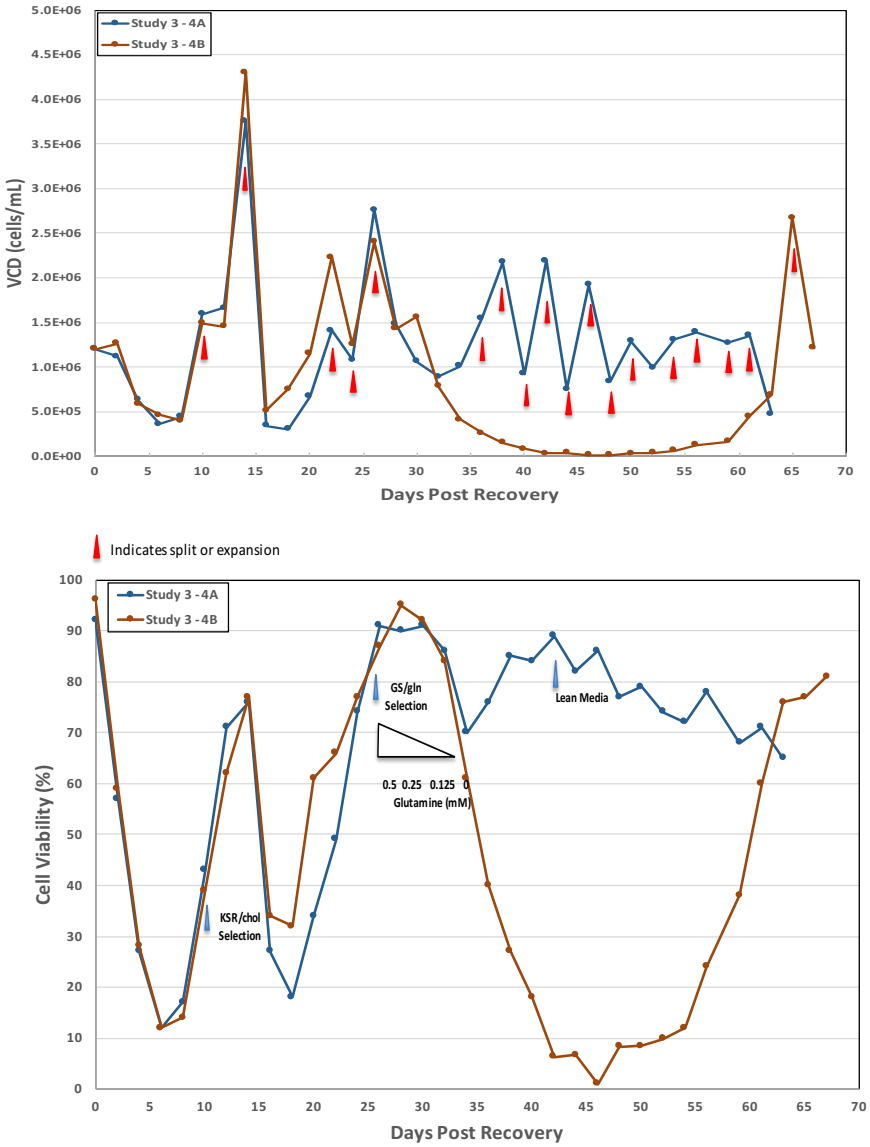

Figure 50. Study 3, Condition 4—Neo→KSR→GS: Growth and Viability. Top panel shows culture growth (VCD) and the lower panel indicates viability. Red arrows indicate splits or expansions. Blue arrows indicate initiation of selective pressures or media addition. Blue stars indicated when cultures were banked. Triangle graphic indicates timing of glutamine withdrawal.

Figure 51 shows the viable cell density and cell viability percentage of the Condition 5 replicates. These cells were transfected with all three plasmids and subjected to initial 200µg /mL G418. Both recovered rapidly as expected and were

then subjected to GlutaMAX reduction to implement GS selection. Both replicates recovered slowly but completely to >90% viability in about 30 days.

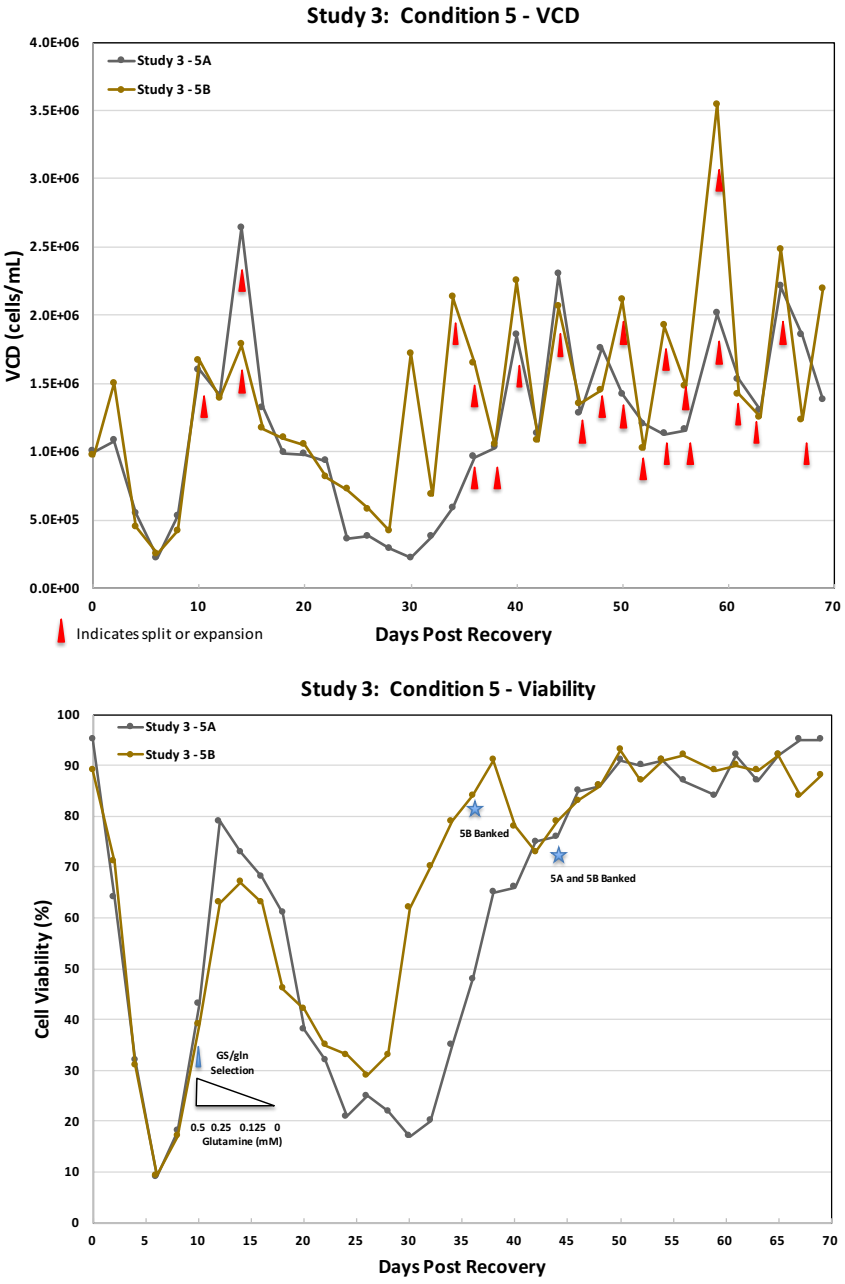

Figure 51. Study 3, Condition 5—Neo→GS: Growth and Viability. Top panel shows culture growth (VCD) and the lower panel indicates viability. Red arrows indicate splits or expansions. Blue arrows indicate initiation of selective pressures or media addition. Blue stars indicated when cultures were banked. Triangle graphic indicates timing of glutamine withdrawal.

Figure 52 shows the viable cell density and cell viability percentage of the Condition 6 replicates. These cells were transfected with all three plasmids and subjected to initial cholesterol selection. Replicate B did not recover. Replicate A did recover, but with persistent reduced viability. At 30 days after recovery to sustained 40-70% viability KSR selection, glutamine was reduced implementing the third GS selection. Viability dropped to below 10%, but then recovered to over 70% prior to banking.

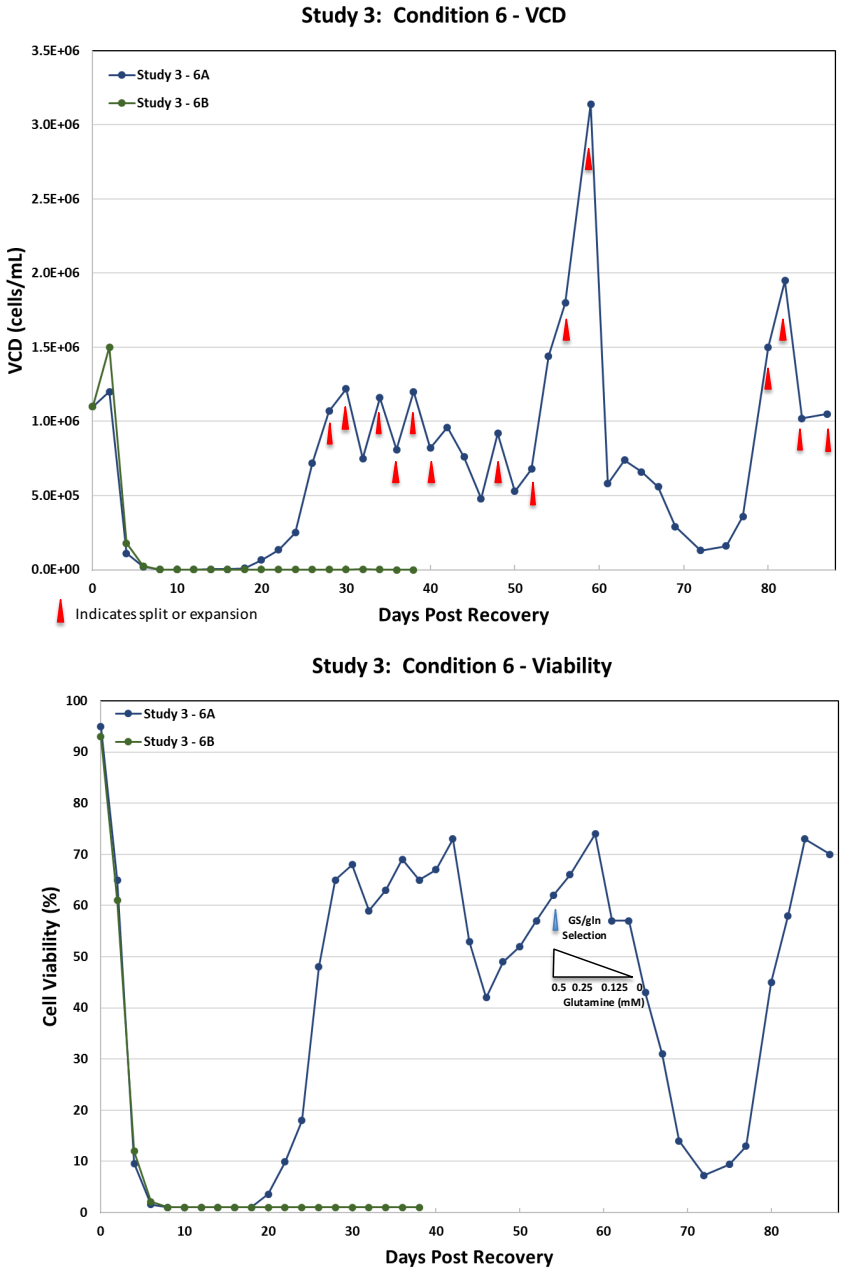

Figure 52. Study 3, Condition 6—KSR→GS: Growth and Viability. Top panel shows culture growth (VCD) and the lower panel indicates viability. Red arrows indicate splits or expansions. Blue arrows indicate initiation of selective pressures or media addition. Blue stars indicated when cultures were banked. Triangle graphic indicates timing of glutamine withdrawal.

Table 24 shows the results of 6-day productivity analysis of the recovered pools at various selection stages including several from Study 4. For each of these runs, pools

were seeded at $0.35x10^6$ VCD in 12 mL fresh medium in a T-75 flask. In the cases of non-GS selection, 4mM of GlutaMAX was fed on Day 3. In the cases of GS selection, 2 g/L glucose was fed on Day 3. Supernatants were assayed for IgG titer by Protein A HPLC.

Table 25 shows the limiting dilution cloning (LDC) plates that were seeded at either the recovery of dual Neo+KSR selected Condition 4A and 4B pools or at the recovery of the triple Neo+KSR+GS selected Condition 4A pool. Best producers were scaled up for further analysis.

Efficiencies and performances of the various LDC conditions are shown in Table 26. Preliminary 3-day specific productivity rate (Qp) analysis are shown for early emerging clones in Table 27. The green highlighted clones were selected for scale-up and further characterization. Additional Qp results for the scaled-up and other Study 3 clones were combined with miniwell Qp studies and are described in the Study 4 section that follows.

Table 24. Stable pool T75 6-day productivity runs.

Condition Number	Plasmid(s)	Par Cell	SP	Selection	Titer A (mg/L)	Titer B (mg/L)
Study 3-1	KSR 1-3-6	Std	old	Cholesterol	70	60
Study 3-2	KSR_v2	Std	new	Cholesterol	65	68
Study 3-3	Neo-KSR-GS	Std	new	G418	-	55
Study 3-3	Neo-KSR-GS	Std	new	G418+chol+gln	26	-
Study 3-4	Neo-KSR-GS	Std	new	G418	62	-
Study 3-4	Neo-KSR-GS	Std	new	G418+chol	65	44
Study 3-4	Neo-KSR-GS	Std	new	G418+chol+gln	29	-
Study 3-5	Neo-KSR-GS	Std	new	G418	59	-
Study 3-5	Neo-KSR-GS	Std	new	G418+gln	-	54
Study 4-2	Neo-KSR	Std	new	G418	21	-
Study 4-3	Neo-KSR-GS	Std	new	G418	58	-
Study 4-3	Neo-KSR-GS	Std	new	G418+chol	55	-

Table 25. Limited Dilution Cloning (LDC) Plates.

Condition Number	Plasmid(s)	Selection	3 cell/well plates	1 cell/well plates	0.3 cells/well plates
Study 3-4A	Neo-KSR-GS	Neo-KSR	1	2	2
Study 3-4B	Neo-KSR-GS	Neo-KSR	1	2	2
Study 3-4A	Neo-KSR-GS	Neo-KSR-GS	1	5	4

Table 26. LDC Efficiencies.

4A Dual	# of wells	+ for growth	Growth Efficiency	+ production	Production Efficiency of Total	Production % of Growers	Scaled to 24w	%Scaled Total	%Scaled Growers	%Scaled of Producers
3 c/w	96	67	69.8%	49	51.0%	73.1%	11	11.5%	16.4%	22.4%
1 c/w	192	64	33.3%	44	22.9%	68.8%	15	7.8%	23.4%	34.1%
0.3 c/w	192	20	10.4%	13	20.3%	65.0%	3	4.7%	15.0%	23.1%
TOTALS	***480***	***151***	***42.9%***	***106***	***30.1%***	***70.2%***	***29***	***8.2%***	***19.2%***	***27.4%***

4B Dual	# of wells	+ for growth	Growth Efficiency	+ production	Production Efficiency of Total	Production % of Growers	Scaled to 24w	%Scaled Efficiency of Total	%Scaled Growers	%Scaled of Producers
3 c/w	96	74	77.1%	42	43.8%	56.8%	21	21.9%	28.4%	50.0%
1 c/w	192	76	39.6%	36	18.8%	47.4%	20	10.4%	26.3%	55.6%
0.3 c/w	192	40	20.8%	12	18.8%	30.0%	7	10.9%	17.5%	58.3%
TOTALS	***480***	***190***	***54.0%***	***90***	***25.6%***	***47.4%***	***48***	***13.6%***	***25.3%***	***53.3%***

4A Triple	# of wells	+ for growth	Growth Efficiency	+ production	Production Efficiency of Total	Production % of Growers	Scaled to 24w	%Scaled Total	%Scaled Growers	%Scaled of Producers
3 c/w	96	60	62.5%	60	62.5%	100.0%	6	6.3%	10.0%	10.0%
1 c/w	480	174	36.3%	171	35.6%	98.3%	9	1.9%	5.2%	5.3%
0.3 c/w	384	64	16.7%	63	49.2%	98.4%	9	14.1%	14.1%	14.3%
TOTALS	***960***	***298***	***42.3%***	***294***	***41.8%***	***98.7%***	***24***	***6.8%***	***8.1%***	***8.2%***

Table 27. Preliminary 3-day specific productivity rate analysis (Qp)—6-well scale.

Cell Line ID	Source	Scale	3D SPR Concentration (g/L)	Q_p (By 3D SPR) (pg/cell/day)
A1	S3-C4A (double) 3c/w	6-well	**31.7**	**13.4**
A28	S3-C4A (double) 3c/w	6-well	**1.1**	**0.4**
A33	S3-C4A (double) 3c/w	6-well	**1**	**0.4**
A37	S3-C4A (double) 3c/w	6-well	**12.8**	**3.5**
A56	S3-C4A (double) 1c/w	6-well	**1**	**0.5**
A92	S3-C4A (double) 0.3c/w	6-well	**29**	**12.8**
B1	S3-C4B (double) 3c/w	6-well	**2.4**	**0.9**
B14	S3-C4B (double) 3c/w	6-well	**0.8**	**0.3**
B23	S3-C4B (double) 3c/w	6-well	**1.7**	**0.5**
B30	S3-C4B (double) 3c/w	6-well	**0.4**	**0.1**
B34	S3-C4B (double) 3c/w	6-well	**2.1**	**0.6**
B38	S3-C4B (double) 3c/w	6-well	**0.7**	**0.3**
B42	S3-C4B (double) 3c/w	6-well	**6.7**	**2.6**
B56	S3-C4B (double) 1c/w	6-well	**0**	**0.0**
B60	S3-C4B (double) 1c/w	6-well	**0**	**0.0**
B74	S3-C4B (double) 1c/w	6-well	**1.7**	**0.6**
B89	S3-C4B (double) 0.3c/w	6-well	**0.7**	**0.3**
B91	S3-C4B (double) 0.3c/w	6-well	**0.4**	**0.2**
B100	S3-C4B (double) 0.3c/w	6-well	**0.4**	**0.1**
B111	S3-C4B (double) 0.3c/w	6-well	**0.4**	**0.1**
B116	S3-C4B (double) 3c/w	6-well	**2.4**	**0.6**
B122	S3-C4B (double) 3c/w	6-well	**0.4**	**0.1**
B134	S3-C4B (double) 3c/w	6-well	**1**	**0.3**
B138	S3-C4B (double) 1c/w	6-well	**4.5**	**1.6**
B148	S3-C4B (double) 1c/w	6-well	**91.2**	**37.0**
B151	S3-C4B (double) 1c/w	6-well	**0.5**	**0.2**
B156	S3-C4B (double) 1c/w	6-well	**0.6**	**0.2**
B160	S3-C4B (double) 1c/w	6-well	**20**	**8.8**

Study 4

In Study 4, the best performing multiplex conditions in Study 3 were evaluated in miniwell transfection format. Miniwell pools were created by segregating a limited number of cells in 96-well plate format 24 hours post-transfection for multiple selection pressures immediately applied in a parallel strategy or upon recovery from the penultimate selection pressure in a sequential strategy. With an appropriate miniwell plating density, this segregation assists with early isolation of rare high producer isolates and reduces overgrowth of low/non-producer transfectants. Three miniwell transfection and selection strategies were evaluated: Parallel and sequential dual Neo→KSR and sequential triple Neo→KSR→GS. In the parallel dual selection strategy, cells were plated 24 hours post-transfection and both G418 drug and cholesterol metabolic selection pressures were applied simultaneously. Sequential strategies were performed as per the previous study, *i.e.*, each sequential pressure was applied upon the bulk 12mL T75 culture upon recovery with the final selection pressure applied after 96-well plating at a range of viable cell densities per well. Minipool selection conditions are shown in Table 28.

Condition 1—Neo-KSR, Parallel Selection

Upon 24 hours recovery, cells were centrifuged and resuspended in 400 mL of Neo-KSR Selective Medium. The resuspended culture was distributed across twenty (20) 96-well plates at 200µL per well at approximately 5,000 cells per well. This is twice the plating density of the original cholesterol single selection miniwells to compensate for expected increased killing due to the addition of G418. Plates were

every two days. Upon recovery, wells were screened for productivity by ELISA and scaled up as appropriate for further characterization and banking.

Condition 2—Neo-KSR, Sequential Selection

Upon 24 hours recovery, cells were centrifuged and resuspended in 12 mL of Neo Selective Medium in a T75 flask. Flask were fed every two days by centrifuging and resuspending with 6mL conditioned medium and 6mL of fresh Neo Selective Medium. Upon recovery to 75% viability (approximately 12 days), cells were centrifuged and resuspended in the Neo-KSR Selective Medium (dual selection). Cells were distributed into 96-well plates at the following densities: 2,500; 1,250; 600; and 313 cells per well in 200μL per well. Plates were fed every two days. Upon recovery, wells will be screened for productivity by ELISA and scaled up as appropriate for further characterization and banking.

Condition 3—Neo-KSR-GS, Sequential Selection

Upon 24 hours recovery, cells were centrifuged and resuspended in 12 mL of Neo Selective Medium in a T75 flask. Flask were fed as per Condition 2 above. Upon recovery to 75% viability (approximately 12 days), cells were centrifuged and resuspended in 12 mL Neo-KSR Selective Medium (dual selection) in a T75 flask. Flask were fed as per Condition 2 above. Upon recovery to 75% viability (approximately 12 days), cells were centrifuged and resuspended in Neo-KSR-GS Selective Medium. Cells were distributed into 96-well plates at the following densities: 2,400; 1,200; 600; and 300 cells per well in 200μL per well. The GS selection process gradually depleted GlutaMAX as per Table 28 and then miniwells

were fed twice per week. Upon recovery, wells were screened for productivity by ELISA and scaled up as appropriate for further characterization and banking.

Table 28. Stable minipool selection conditions.

Plasmids	Selection #1	Sel #1 vessel	Selection #2 at 75% viability	Sel #2 vessel	Selection #3 at 75% viability	Sel #3 vessel
Neo-KSR	G418-200ug/mL + (-) Chol	20x96-well 5,000 cells/well	-	-	-	-
Neo-KSR	G418-200ug/mL	T-75 Bulk Selection	(-) Chol	96-well 2,500 cells/well 1,250 cells/well 625 cells/well 313 cells/well	-	-
Neo-KSR-GS	G418-200ug/mL	T-75 Bulk Selection	(-) Chol	T-75 Bulk Selection	0.5mM GlutaMAX to zero over 1 week	96-well 2,500 cells/well 1,250 cells/well 625 cells/well 313 cells/well

Specific productivity rate (Qp) analysis was performed in 6-well format for over 60 Study 4 miniwells and Study 3 clones. Table 29 shows the confirmatory 3-day Qp analysis for the best performers. All were evaluated in T-75 flask culture. The four top expressors were above 30 pcd with two at approximately 60 p/c/d. As shown in Figure 53, the top multiplexed clone Qp rates (Top 5: Avg. Qp=45 pcd, Best Qp=61 pcd) exceeded the original SF-NS0 clones with single cholesterol selection strategy (Top 5: Avg. Qp=16 pcd, Best Qp=25 pcd) by 2.8-fold on average. All cell lines were adapted to shaker flask and lean production medium. Several lines did not adapt and were discontinued. Once fully adapted, each cell line was evaluated in fed-batch mode in 30-mL working volume shaker flask culture for up to 8 days. Non-triple selected cell lines were fed with 2mM GlutaMAX. Triple selected cell lines were fed with 2 g/L glucose. Final results for the top 10 performers is shown in Table 30. A comparison of previous best performers using only cholesterol selection is shown in Figure 8.8 indicating a nearly 3-fold increase in Qp for the best performers.

Table 29. Confirmatory T-75 three-day specific productivity rate analysis.

Clone Number	Source	Doubling Time (h)	ICA	Qp (pg/cell/day)	σ	Density at Banking (vc/mL)	Viability at Banking (%)
148	Study 3, Double Neo/KSR Pool Clone	12.8	12.8636	1.86	0.162	1.00E+06	83
92	Study 3, Double Neo/KSR Pool Clone	30.5	2.65631	6.40	0.095	1.79E+06	93
137	Study 3, Double Neo/KSR Pool Clone	27.1	3.01913	6.99	0.129	1.52E+06	89
219	Study 4, Double Neo/KSR Miniwell	25.9	3.19432	7.89	0.125	1.50E+06	87
23	Study 4, Double Neo/KSR Miniwell	32.8	2.46739	9.56	0.140	1.61E+06	88
146	Study 4, Double Neo/KSR Miniwell	34.3	2.37067	10.67	0.006	9.60E+05	97
244	Study 3, Triple Neo/KSR/GS Pool	32.8	2.46739	16.05	0.250	1.30E+06	82
232	Study 3, Triple Neo/KSR/GS Pool	40.5	2.06956	17.44	0.127	1.08E+06	83
153	Study 4, Double Neo/KSR Miniwell	29.5	2.74875	18.59	0.049	1.70E+06	93
20	Study 4, Double Neo/KSR Miniwell	34.3	2.37067	18.94	0.064	1.62E+06	95
141	Study 3, Double Neo/KSR Pool Clone	25.9	3.19432	19.07	0.020	1.64E+06	93
76	Study 4, Double Neo/KSR Miniwell	34.3	2.37067	20.21	0.022	1.10E+06	91
207	Study 4, Double Neo/KSR Miniwell	31.6	2.56256	21.19	0.010	1.32E+06	96
109	Study 4, Double Neo/KSR Miniwell	27.8	2.93008	21.47	0.041	1.89E+06	90
8A1	Study 4, Double Neo/KSR Miniwell	34.3	2.37067	22.78	0.051	1.50E+06	87
204	Study 4, Double Neo/KSR Miniwell	34.3	2.37067	27.42	0.022	1.10E+06	94
160	Study 3, Double Neo/KSR Pool Clone	29.5	2.74875	33.40	0.024	2.98E+06	91
101	Study 4, Double Neo/KSR Miniwell	26.5	3.10719	43.58	0.007	1.20E+06	91
190	Study 3, Double Neo/KSR Pool Clone	27.1	3.01913	59.29	0.008	1.30E+06	82
148	Study 3, Double Neo/KSR Pool Clone	27.1	3.01913	61.47	0.031	9.60E+05	94

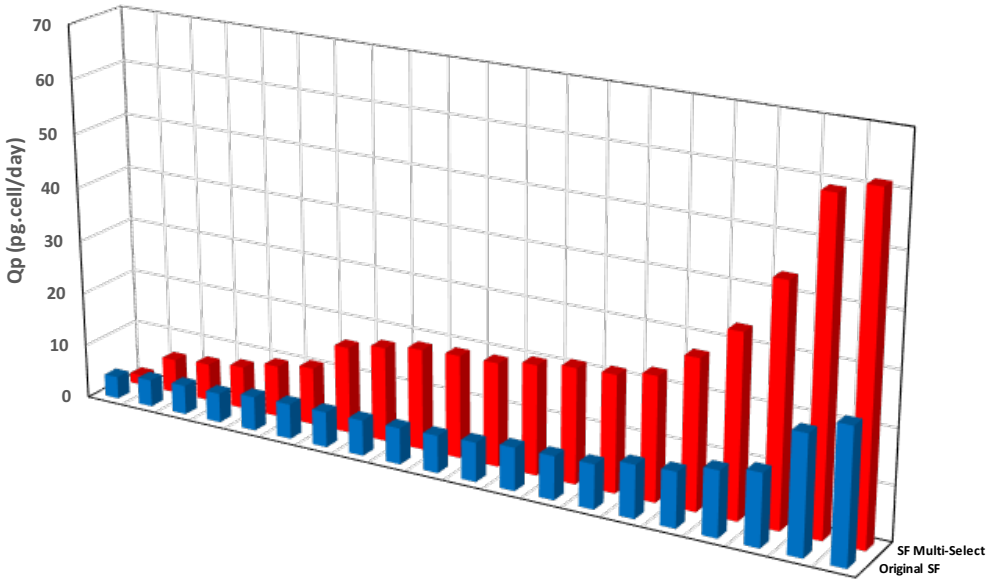

Figure 53. Comparison of specific productivity rates of best performing original StableFast (cholesterol selection only) and StableFast-Multi-Select (cholesterol plus G418 and/or glutamine selection) cell lines. Front blue columns indicate single cholesterol selection generated cell lines and rear red columns indicate multiplex selected cell lines.

Table 30. Best performing multiplex selection cell lines—Lean production media, 30mL shaker flask

Cell Line ID (C=clone, M=miniwell)	Seeding Density	Selection	Culture Volume (mL)	Harvest Time (days)	Final Titer in Supernatant (ProtA-HPLC, mg/L)
190-C	0.5e6	Neo-KSR	30	6	340.2
148-C	0.5e6	Neo-KSR	30	6	369.5
160-C	0.5e6	Neo-KSR	30	5	318.9
153-M	0.5e6	Neo-KSR	30	8	176.5
20-M	0.5e6	Neo-KSR	30	7	44.6
76-M	0.5e6	Neo-KSR	30	7	61.4
8A1-M	0.5e6	Neo-KSR	30	7	44.2
141-C	0.5e6	Neo-KSR	30	8	42.3
232-C	0.5e6	Neo-KSR-GS	30	6	120.6
244-C	0.5e6	Neo-KSR-GS	30	7	125.8

Conclusions

Initial single vector/single selection Studies 1 and 2 indicted that the increasing selection stringency/stress to the NS0 host cell is NeoR/G418 → 17β7(KSR)/cholesterol → GS/glutamine. In Study 3, co-transfections with all three vectors were performed with selection pressures applied to the bulk transfection sequentially as the cultures recovered from the previous selection process. In Conditions 3 and 4 all three pressures were sequentially applied with stable pools

recovering between each successive selection over a 60 day period. This result indicates that the co-transfected and linearized plasmids are perhaps forming multimeric concatemers prior to stable genomic integration. Each successive selection process is acting like a sieve only allowing cells with each successive selection marker to survive and expand. This dynamic was also reflected in the sequential dual selection Conditions 5 and 6, Neo → GS and KSR → GS, respectively. These results were used to inform the design of Study 4 whereby several approaches to stable miniwell generation were evaluated. A combination of clones generated from Study 3 stable pools and miniwell isolates were evaluated for specific productivity resulting in Qp values of nearly triple than that of single cholesterol selection generated clones. This significant increase in Qp may reflect increased heavy and light chain gene copy numbers due to the concatemerization and multiplex selection processes.

Chapter 6:
Application to development of novel and biosimilar drugs

Abstract

In Chapter 6, we describe the use of the novel NS0 platform to develop a complex human leukocyte antigen (HLA) IgG4 fusion protein and a biosimilar or follow-on biologic in a commercial product development setting. The fusion protein required the construction of a new vector with three independent expression cassettes plus the cholesterol selection marker cassette. This vector was initially analyzed for expression in transient transfection in both an adherent and suspension system and then used to generate a stable NS0 cell line. The use of western blot assays allowed for the selective evaluation of expression of each of the protein subunits and also revealed segregation of such subunits intracellularly and extracellularly (*i.e.*, secreted into the medium). These western blot assays also assisted with early purification development.

The StableFast-NS0 platform was used to generate a biosimilar mAb. A high expressing clonal cell line was isolated and scaled up to the 5-L bioreactor for production. Purified mAb was dialyzed into the same formulation buffer as the reference commercial product and a range of analytical methods were used to compare the biosimilar and reference products.[51] SDS-PAGE and SEC-HPLC were used to demonstrate intact and digested molecular size comparability of the biosimilar to the reference product. CEX-HPLC showed some differences in charge heterogeneity between the biosimilar to the reference product. HILIC-MS/MS analysis revealed a significant diversity in clonal glycoforms. Further studies indicated that the glycan distribution can be tuned by selective feeding of the culture.[52]

Results and Discussion

SF-NS0 Cell Line Development for a Human HLA-IgG4 Fusion Protein

The StableFast-NS0 platform was used to express a complex six-subunit HLA-IgG4 fusion protein consisting of two of each of three unique subunits: An HLA-IgG4 heavy chain fusion subunit, a light chain, and a β2-microglobulin.

Expression Vector Construction

Construction of the pBFksr::HLA-IgG4::LC3 bicistronic expression vector for StableFast-NS0 cell line generation is depicted in Figure 54 and the vector map is shown in Figure 55. An expression cassette and vector containing the human β2 microglobulin gene was created and used to create a tricistronic expression vector that encodes all three fusion protein subunits (human HLA-IgG4 heavy chain fusion, a-CD28 light chain [LC3], and human β2-microglobulin). Expression of all three genes was confirmed in transient HEK293 culture by ELISA and western blot analyses of supernatants. The strategy for tricistronic vector construction is illustrated in Figure 56. A PCR product has been generated that appends the *Sgr*DI and *Asc*I restriction sites to the 5′ and 3′ flanks of the cassette, respectively. This PCR product was subcloned into a TOPO vector and subsequently restricted with *SgrDI* and *AscI* to release the insert. This insert was ligated directionally with a similarly restricted pBFksr::HLA-IgG4::LC3 bicistronic expression vector to create the final tricistronic construct (see Figure 57).

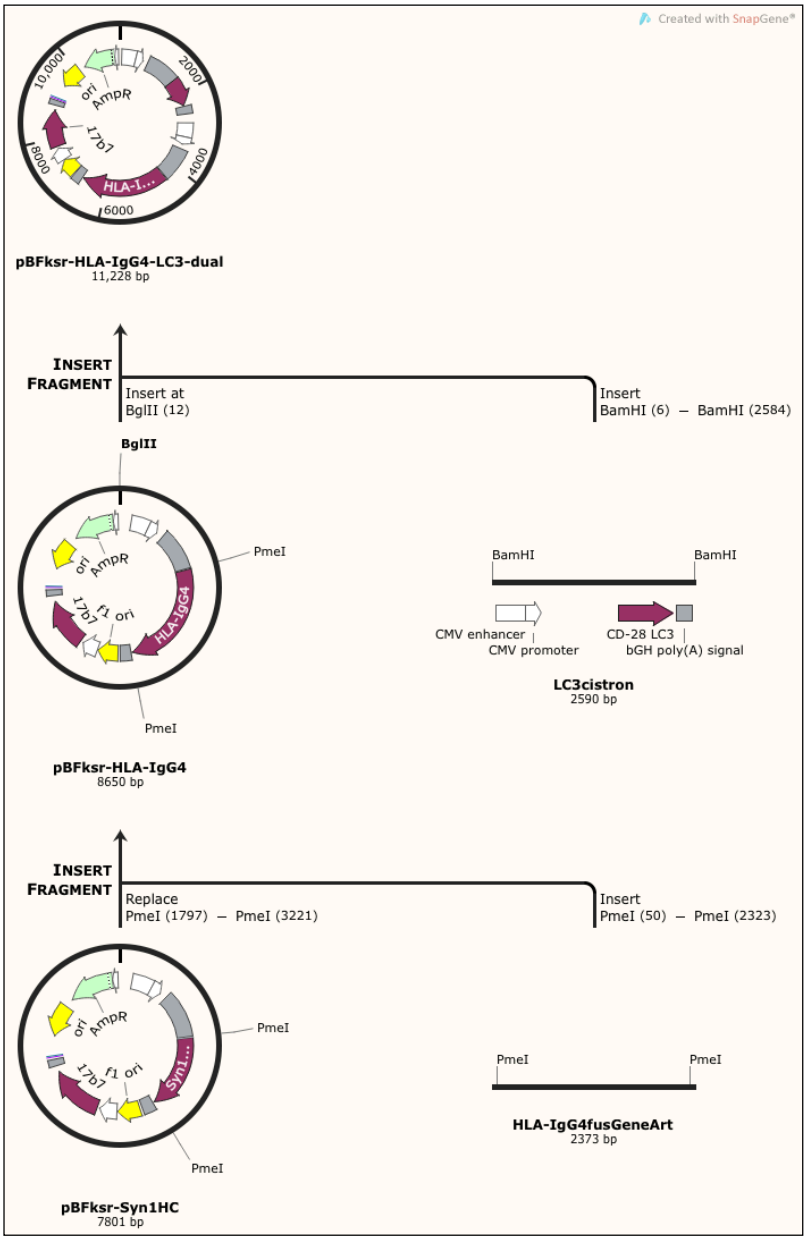

Figure 54. Vector construction history for pBFksr::HLA-IgG4::LC3. Key gene segments and restriction sites are shown.

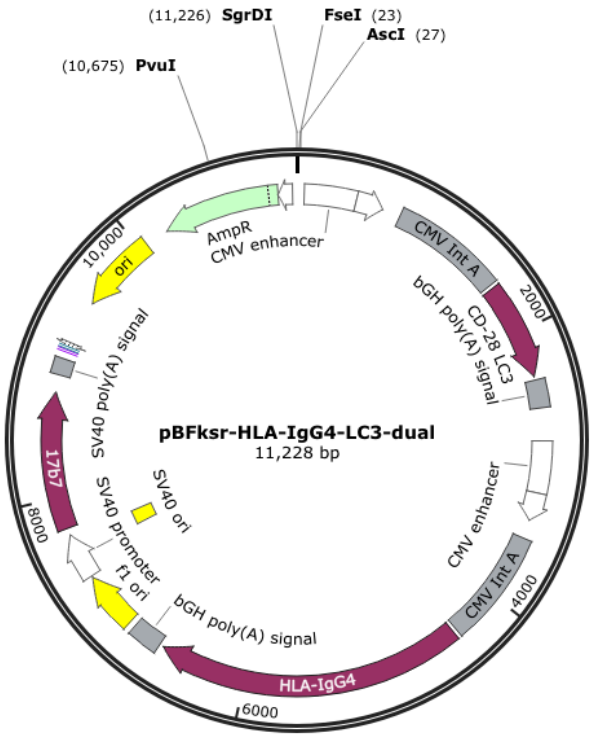

Figure 55. pBFksr::HLA-IgG4::LC3 vector map. Key gene segments and restriction sites are shown.

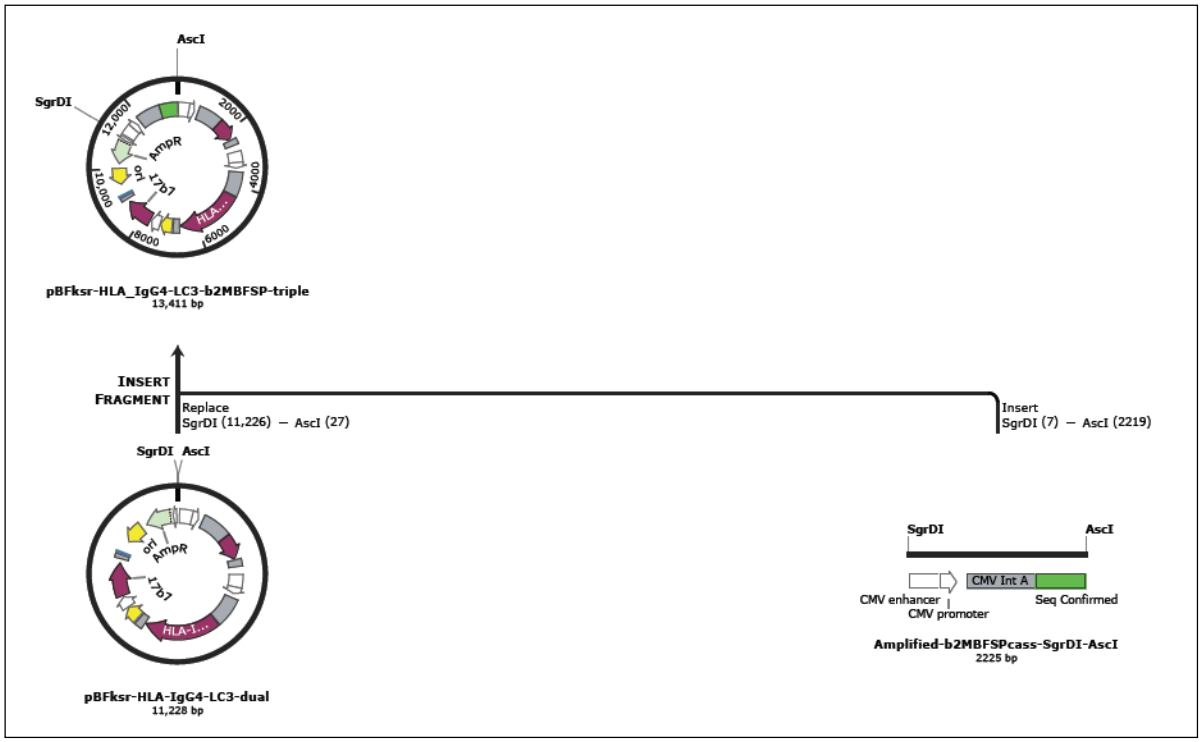

Figure 56. Vector construction strategy for pBFksr::HLA-IgG4::LC3::b2MBFSP. Key gene segments and restriction sites are shown.

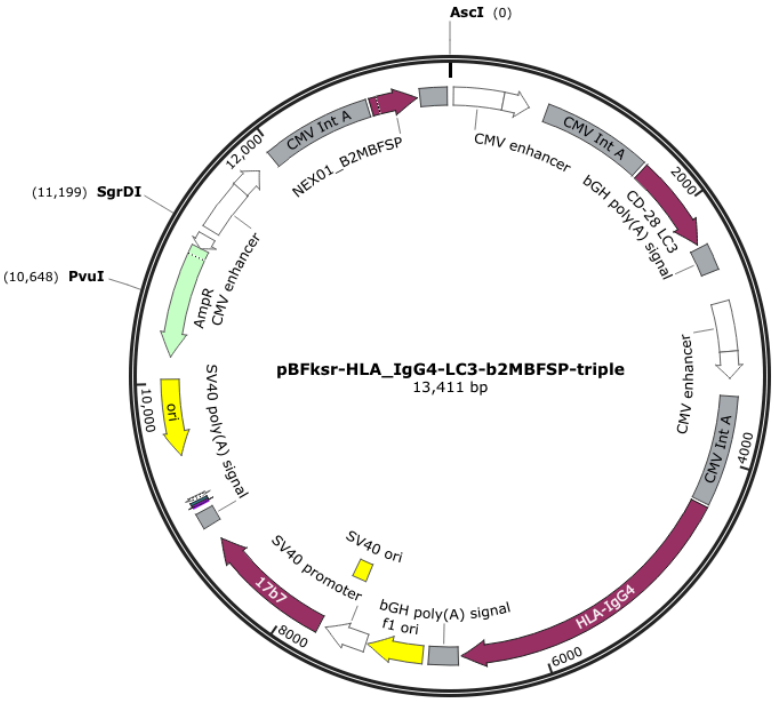

Figure 57. pBFksr::HLA-IgG4::LC3::b2MBFSP vector map. Key gene segments and restriction sites are shown.

Large-Scale Transient Transfection

In order to generate small amounts of the HLA-IgG4 fusion protein and examine initial purification conditions, a transient co-transfection of HEK293 cells was performed using the pBFksr::HLA-IgG4::LC3 bicistronic expression vector and pTOPO::b2MBFSP expression vector. Briefly, a total of fifteen (15) 150-mm cell culture dishes were seeded with adherent HEK 293 cells and allowed to grow to ~90% confluency. Equal amounts of plasmid DNAs (dual and b2M) were used with Lipofectamine 2000 reagent for transfection. Cultures were incubated for 6 days and harvested for purification. Clarified supernatant was loaded onto a 1 mL rProtein A Sepharose Fast Flow column at 0.5 CV/min. Column was washed and eluted with 0.2M glycine at pH=3.5. All remaining bound protein was further eluted at pH=2.5. Fractions were collected during both high and low pH elutions and analyzed by SDS-

PAGE and western blot. Figure 58 shows the silver stained SDS-PAGE gel of the elution fractions. Clearly, small quantities of multiple subunit proteins are eluting at both high and low pH. Western blots of those same gels shown in Figures 59-60 (minus one fraction for the WB standard in lane #2) show faint, but inconclusive staining of the bands seen in the silver stained gel. In Figure 61, the picture becomes clearer as we see concentration of the bands stained by anti-human Fc in the elution, but little capture of the kappa light chain or b2M.

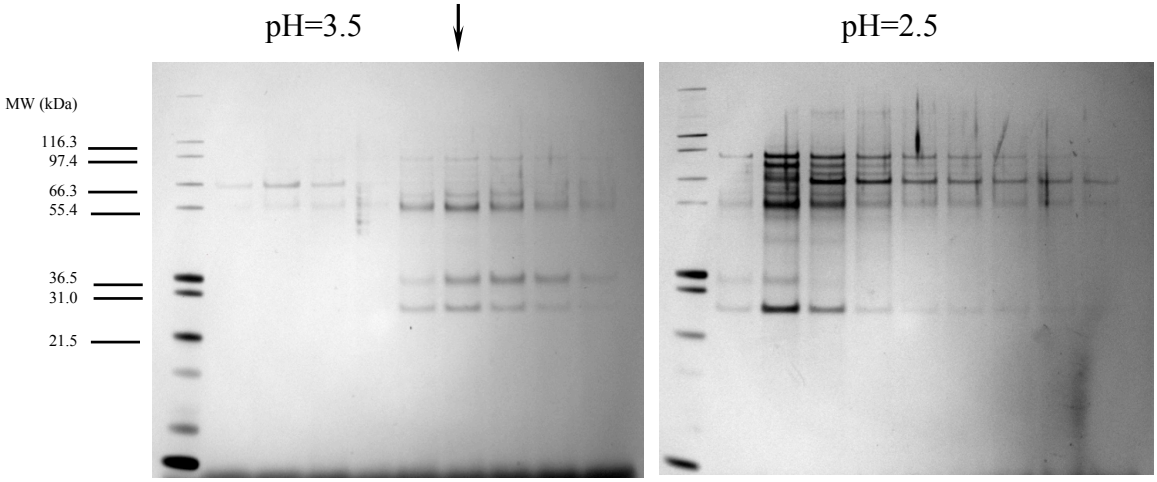

Figure 58. SDS-PAGE of elution fractions (silver stained). MW markers were loaded in lane 1 of each gel. Fraction #6 is indicated by arrow. Molecular weights in kDa are indicated on the left.

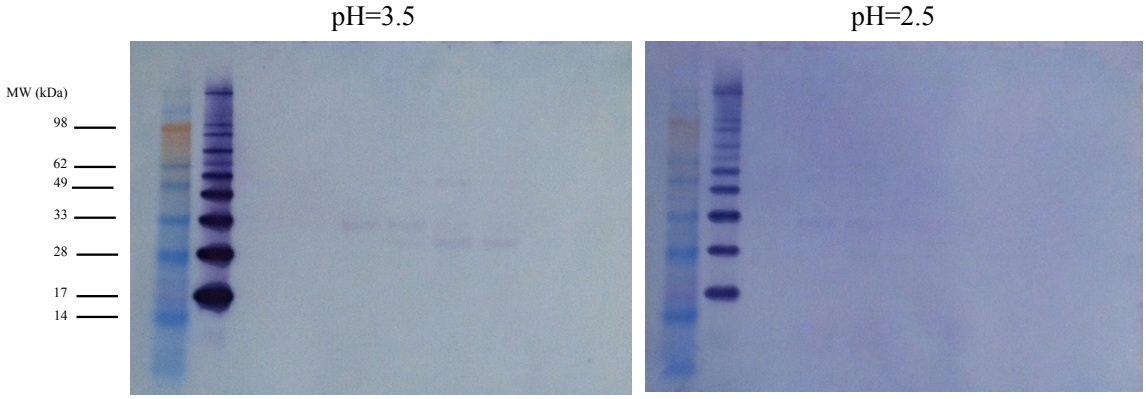

Figure 59. Western blot of elution fractions (anti-human Fc). MW markers were loaded in lane 1 and WB standards were loaded in lane 2 of each gel. Molecular weights in kDa are indicated on the left.

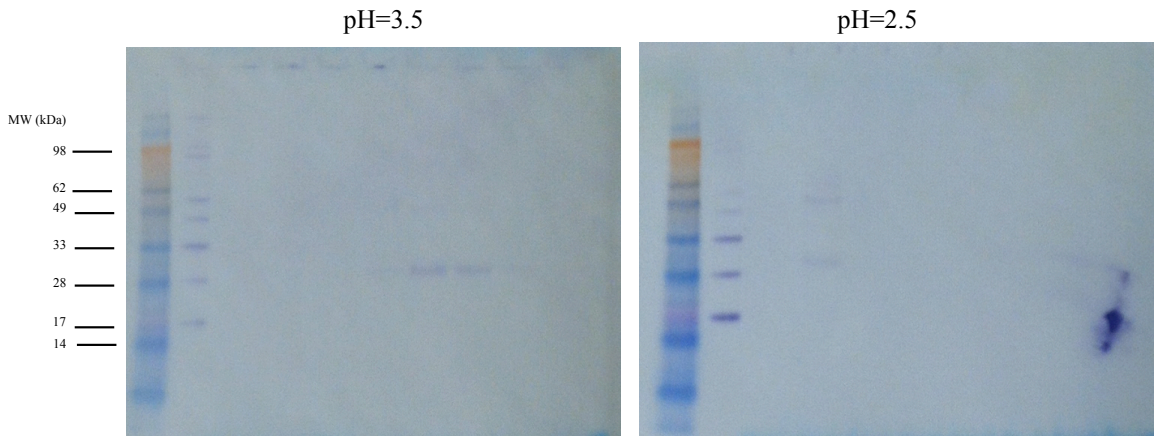

Figure 60. Western blot of elution fractions (anti-human β2M). MW markers were loaded in lane 1 and WB standards were loaded in lane 2 of each gel. Molecular weights in kDa are indicated on the left.

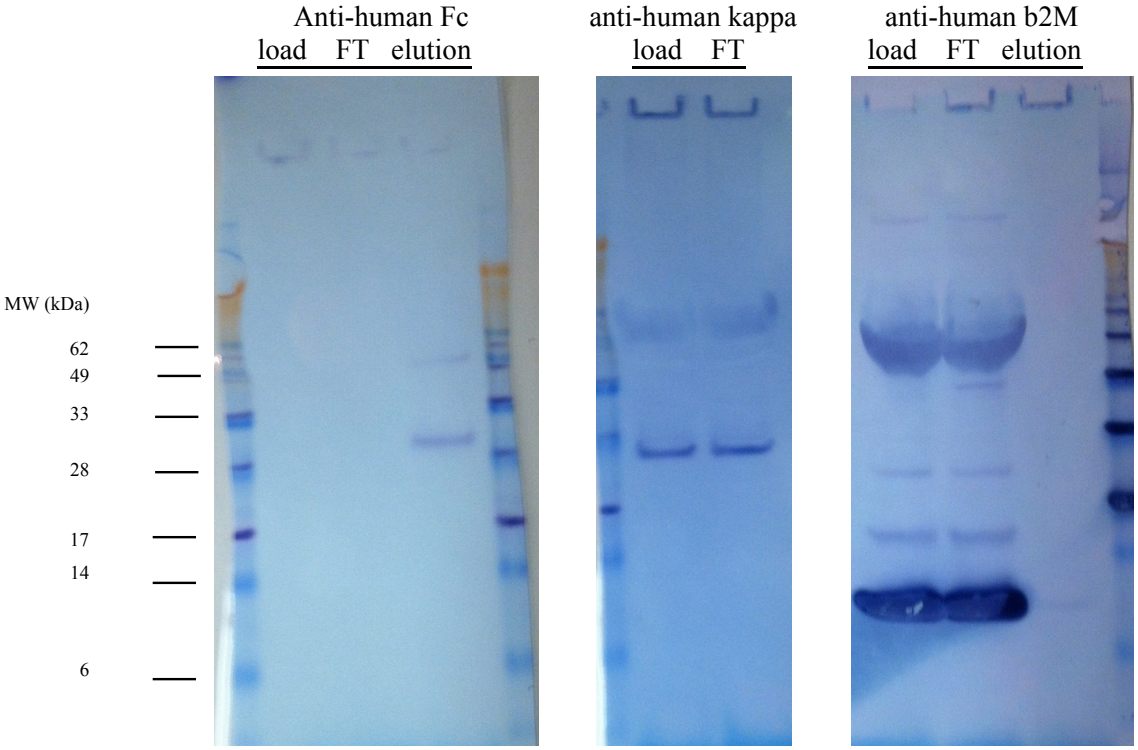

Figure 61. Western blots of load, flow-though, and elution fraction #6. MW markers were loaded in outside lanes of anti-human Fc gel, in lane 1 of anti-human kappa gel and in lane 4 of anti-human b2M gel. Molecular weights in kDa are indicated on the left.

Transient Transfection of Expi293F Cells

The Expi293F cell line was thawed and established in 30-mL shaker culture as per vendor's protocol. One day before transfection, cells were seeded at 2×10^6 per mL. The following day, cell density was $>3\times10^6$ cells/mL at 96% viability. For each reaction, a total of 7.5×10^7 cells were transfected with 30 μg plasmid DNA using the ExpiFectamine™ 293 Reagent in Opti-MEM® I medium. At 17 hours after transfection, ExpiFectamine™ 293 Transfection Enhancers were added. Culture supernatant was harvested by centrifugation 5 days post-transfection. One mL culture samples were centrifuged at 400×*g* for 10 minutes. Cell pellet samples were washed with 1 mL of DPBS, centrifuged as above, and stored at -80°C. Clarified and 0.2 mm filtered supernatant was stored at 4°C. Cell pellet and supernatant negative controls were prepared from the parental Expi293F cells (not transfected) as above. Pellets were thawed and lyzed in the following lysis buffer for 10 minutes on ice:

20 mM Tris
100 mM NaCl
1 mM EDTA
0.5% Triton X100

Lysis reactions were centrifuged at ~16,000×*g* for 10 minutes to remove debris. Lysis supernatants and cell culture supernatants from negative and positive pellets were loaded onto a 10% SDS-PAGE gel in triplicate under reducing conditions. SDS-PAGE was run and then transferred to a PVDF membrane. Membrane was divided into three independent blots with all samples represented on each. Blots were blocked in 5% non-fat dry milk buffer for 2 hours at room temperature and then

probed at 1:1000 dilution in blocking buffer for 1 hour with the following primary antibodies:

Goat anti-human IgG (Fc specific)-HRP
Mouse anti-human kappa light chain
Rabbit anti-human β2 microglobulin

Blots were washed and secondary anti-mouse IgG (H+L)-HRP and anti-rabbit IgG (H+L)-HRP were added to the mouse anti-human kappa light chain and rabbit anti-human β2 microglobulin probed blots, respectively, at 1:1000 dilution in blocking buffer for 1 hour. After final washes, blots were developed with TMB membrane substrate and stopped with dH_2O washes. Blots are shown in Figure 62.

Apparent bands of each subunit are boxed in Figure 62. Theoretical molecular weights predicted from amino acid sequence for the HLA-IgG4, kappa light chain, β2 microglobulin are 83.8 kDa, 23.9 kDa, and 11.7 kDa, respectively.

On the anti-human Fc probed blot, there is a strong signal for the HLA-IgG4 fusion subunit in the positively transfected cell pellet, but no signal in the corresponding supernatant. This indicates positive translation, but no apparent secretion.

On the anti-human kappa light chain probed blot, there is a nonspecific signal across all sample lanes at ~33 kDa. There is a strong signal for the kappa light chain in the positively transfected supernatant with a faint signal in the corresponding cell pellet. This indicates positive translation and secretion.

On the anti-human β2 microglobulin probed blot, there are strong signals in all lanes below the 20 kDa marker as the human endothelial kidney cell line is a

constitutive producer of β2 microglobulin. There is a marked increase in signal of the positively transfected culture supernatant as compared with the negative control supernatant sample. This indicates positive translation and secretion of β2 microglobulin above background.

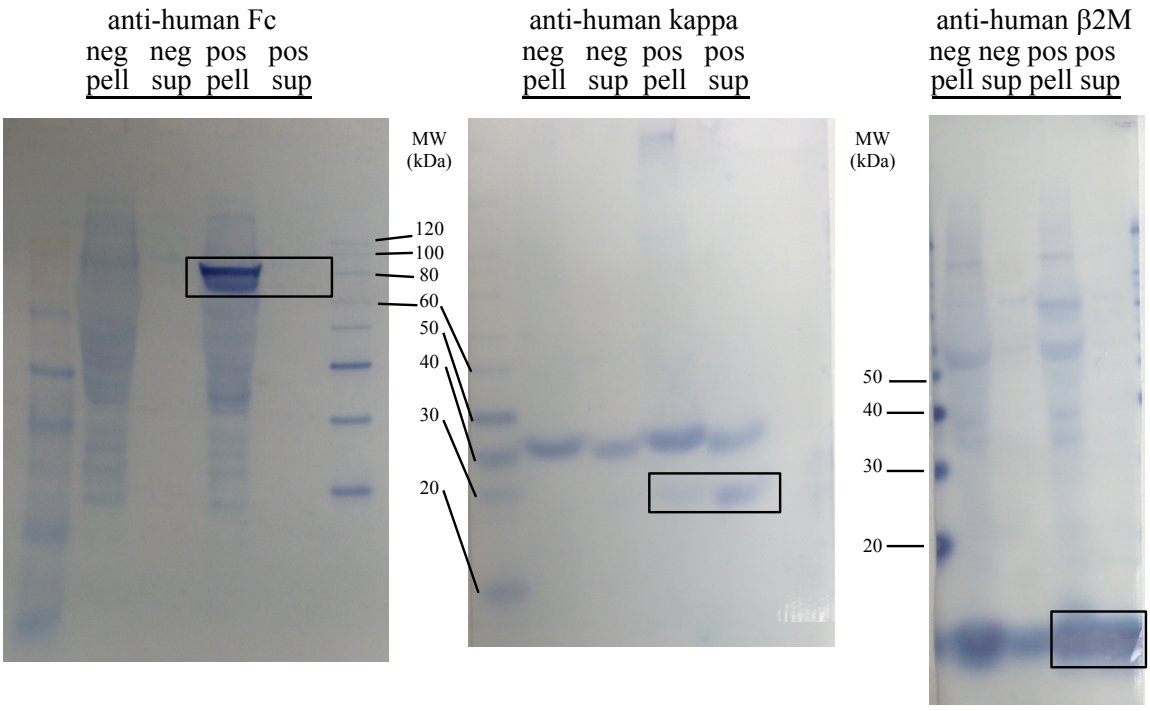

Figure 62. Western blots of pellet and supernatants for HLA-IgG4 Expi293 transient transfection. MW markers were loaded in outside lanes of anti-human Fc gel, in lane 1 of anti-human kappa gel and in lane 1 of anti-human b2M gel. Molecular weights in kDa are indicated. Boxes indicate HLA-IgG4 fusion subunit, kappa light chain and β2 microglobulin from left to right panels, respectively.

Stable Transfection of NS0 Cells

Parental NS0 cells were expanded in supplemented serum-free growth medium. Upon establishment of health culture, ten million cells (10×10^6) were transfected with 45 μg linearized (Δ*Pvu*I) expression vector DNA. Cells were allowed to recover for 24 hours in bulk in growth medium. Following recovery, cells were washed in supplemented serum-free selective medium (cholesterol$^-$), and resuspended in 12 mL

of the selective medium in a T-75 flask. The following day, culture was expanded to 30 mL in a T-150 flask to provide for culture growth during early selection phase. Viability of culture fell and then recovered within two weeks and banks of the stable pool of cells were frozen.

Culture and Western Blot Analysis of StableFast-NS0 Pool

The StableFast-NS0 pool described above was thawed and passaged in T-75 flasks in both Gibco and Sigma production media. Upon recovery from thaw, aliquots of cells were distributed to 6-well plates for production of the HLA-Ig fusion protein. On Day 4 post seeding, two (2) mL culture samples were centrifuged at 400×*g* for 10 minutes. Cell pellet samples were washed with 2 mL of DPBS, centrifuged as above, and stored at -80°C. On Day 9 post seeding, two (2) mL culture samples were centrifuged as above and clarified supernatant was stored at 4°C. Cell pellet and supernatant irrelevant controls were prepared from a StableFast-NS0 cell line producing a human IgG_1 as above. Pellets were thawed and lyzed in the following lysis buffer for 10 minutes on ice:

20 mM Tris
100 mM NaCl
1 mM EDTA
0.5% Triton X100

Lysis reactions were centrifuged at ~16,000×*g* for 10 minutes to remove debris. Lysis supernatants and cell culture supernatants from negative and positive pellets were loaded at 20 µL/lane onto a 10% SDS-PAGE gel under reducing conditions. SDS-PAGE was run and then transferred to a PVDF membrane. Blot was blocked in 5% non-fat dry milk buffer overnight at 4°C. Blot was washed and then probed at

1:1000 dilution in blocking buffer for 1 hour with goat anti-human IgG (Fc specific)-HRP. Blot was washed, developed with TMB membrane substrate, and stopped with dH_2O washes.

As seen in Figure 63, strong bands of the apparent HLA-IgG_4 subunit from pellet preps are seen running just above the 80 kDa marker (theoretical molecular weight is 83.8 kDa). A very light band of this protein is just visible in the Sigma medium supernatant. By contrast, the IgG_1 heavy chain of the control culture is significantly stronger in the supernatant fraction versus the pellet prep. This result continues to support the conclusion that the HLA-IgG_4 subunit is being actively translated, but that very little is secreted.

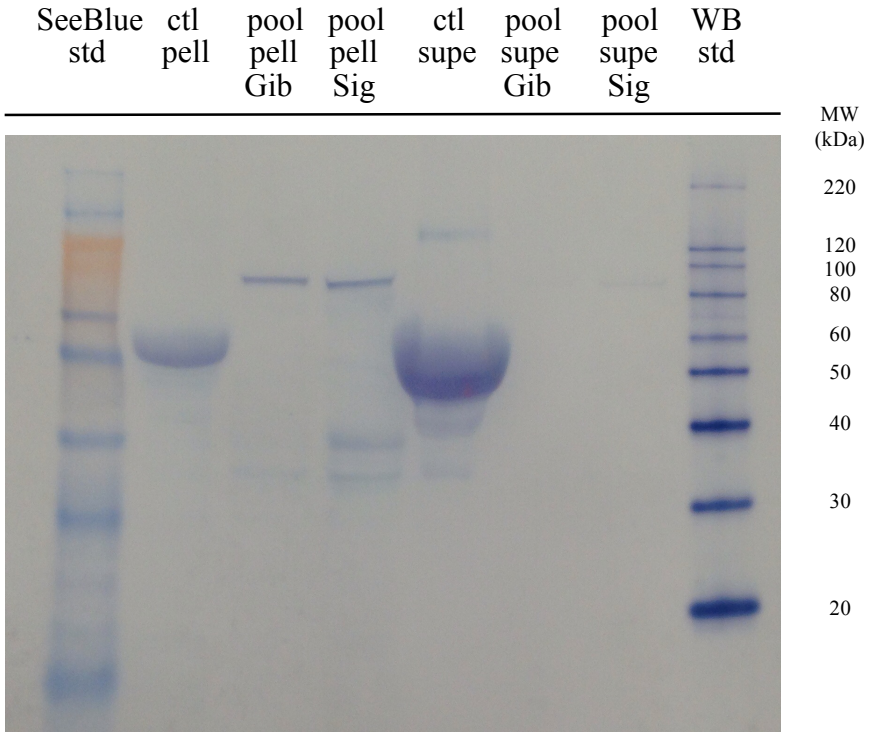

Figure 63. Western blots of pellet and supernatants for HLA-IgG4 StableFast-NS0 pool. Standards and samples are identified at the top of each lane. Molecular weights in kDa are indicated on the right.

StableFast Biosimilars Development Demonstration

To demonstrate the utility of the StableFast-NS0 platform in the biosimilar development and manufacturing arena, a StableFast-NS0 cell line was developed that produces a biosimilar of the marketed product Synagis® (palivuzumab, MedImmune).

Cell line development proceeded as described in Chapters 2 and 3. A high expressing clonal StableFast-NS0 cell line was generated by limiting dilution of a stable pool. This cell line was scaled up in suspension culture to a 5-L fed-batch bioreactor. Supernatant was harvested on day 7 and purified by single step Protein A capture chromatography. The purified mAb was dialyzed into Synagis formulation buffer (1.9 mg histidine, 0.06 mg glycine, 0.2 mg chloride). Final purified yield from the bioreactor was 140 mg/L.

The palivizumab biosimilar was compared to a single reference lot of marketed product (*Synagis*®, 50 mg/0.5 mL single dose vial, Lot 10G14-80). Both proteins were diluted to identical concentrations using absorbance at 280nm. The proteins were run on a reducing SDS-PAGE gel to compare gross purity and size of heavy and light chains (see Figure 64).

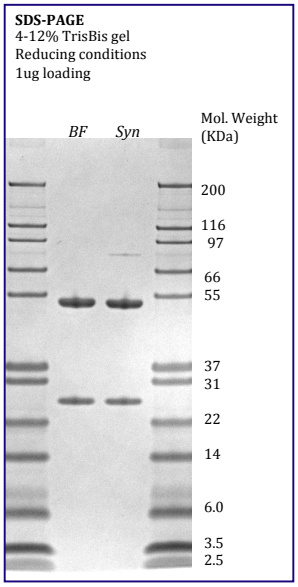

Figure 64. SDS-PAGE of biosimilar palivizumab (BF) and *Synagis* (Syn). MW markers were loaded in the outside lanes and molecular weights in kDa are indicated on the right.

Native biosimilar palivizumab and *Synagis*® were compared using size exclusion chromatography (SEC) HPLC. As shown in Figure 65, the overlaid chromatograms are nearly indistinguishable.

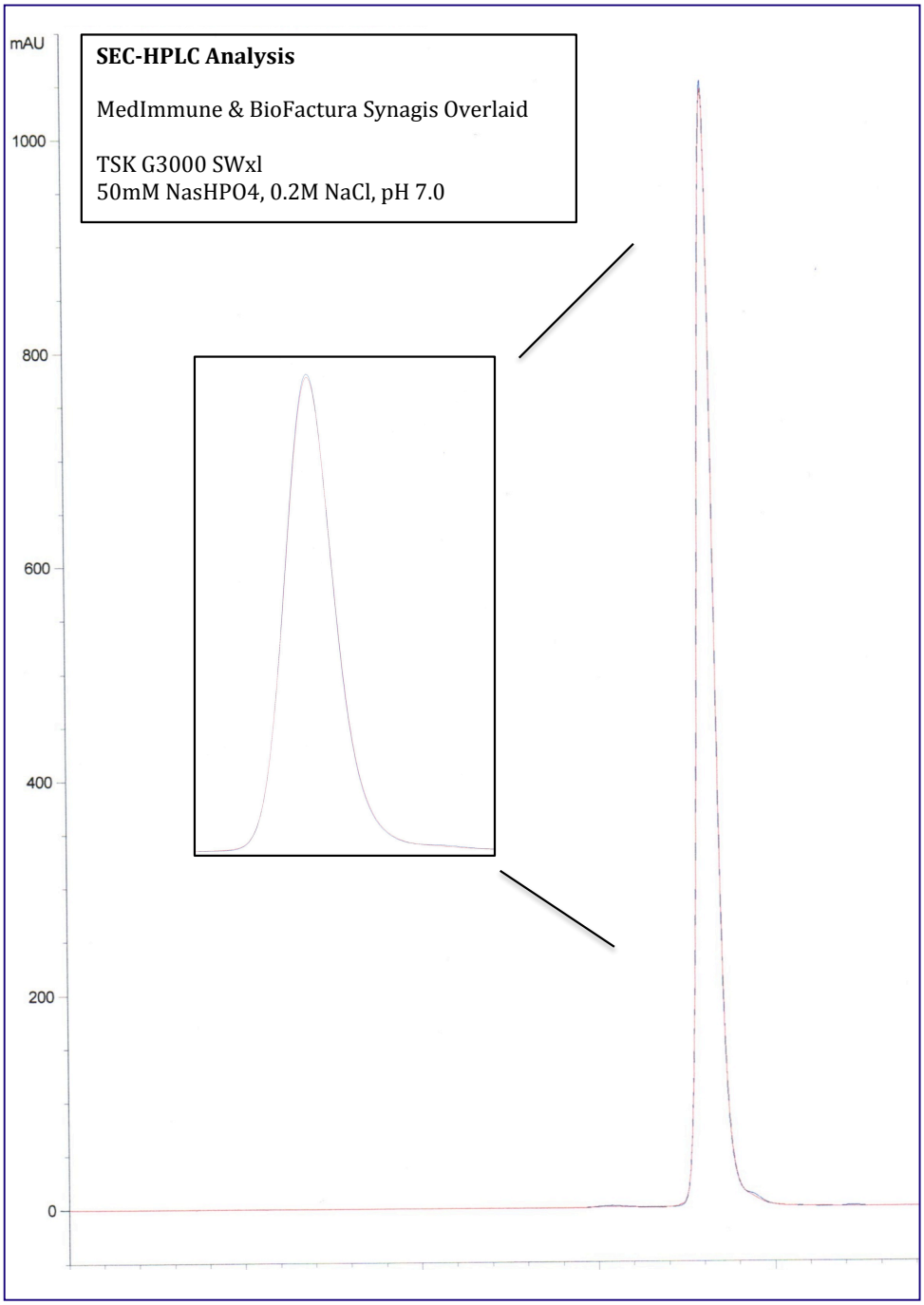

Figure 65. SEC-HPLC analysis of biosimilar palivizumab and *Synagis*. Chromatograms of biosimilar palivizumab and Synagis reference standard are overlaid.

To further characterize and compare the protein structures of the biosimilar palivizumab and *Synagis*, the mAbs were digested with papain and peptide mapping was performed using the same HPLC size exclusion chromatography. As shown in Figure 66, the retention times for each of the peptide fragments are nearly identical.

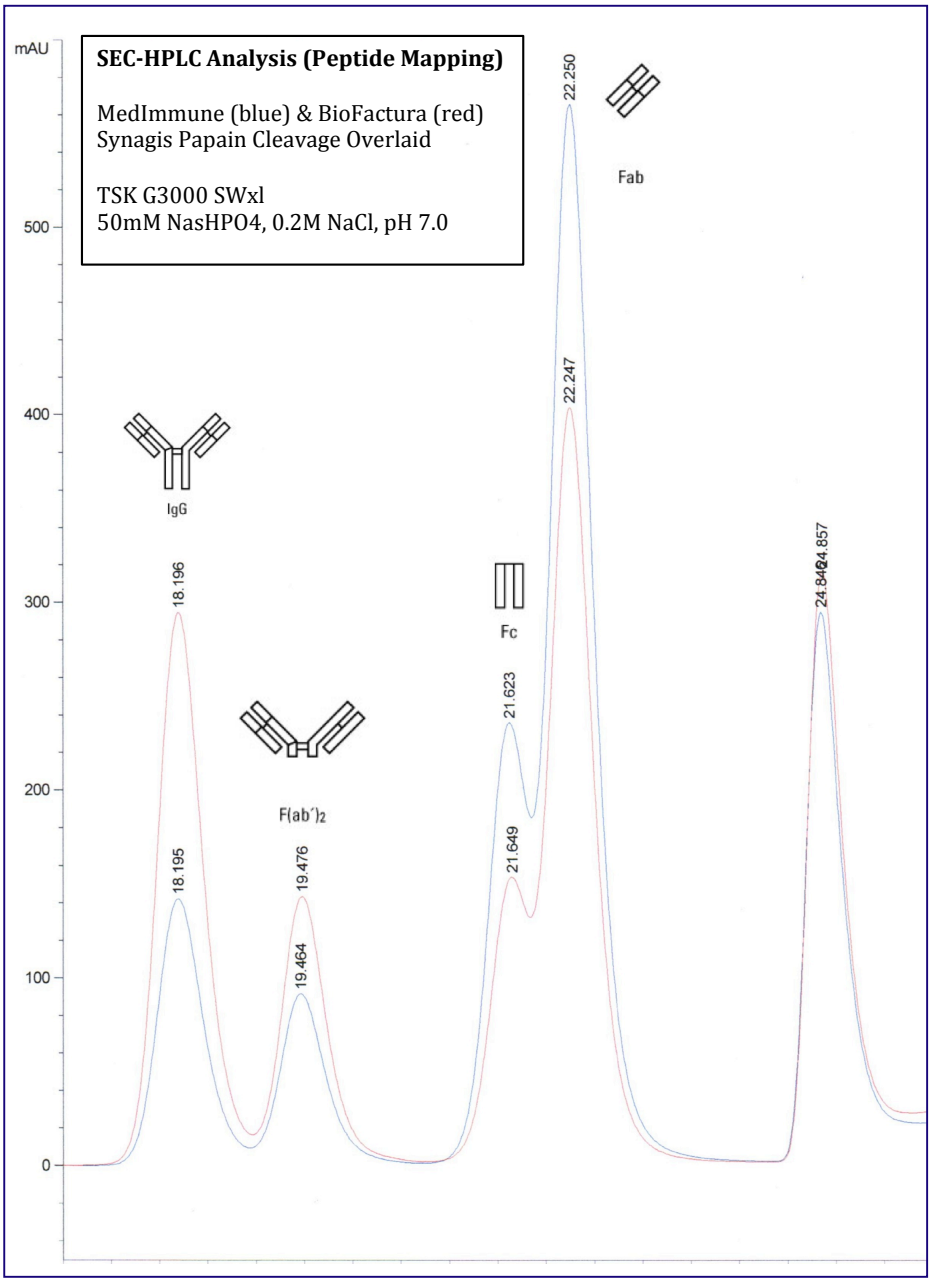

Figure 66. Peptide mapping analysis of biosimilar palivizumab and *Synagis*. Chromatograms of biosimilar palivizumab and Synagis reference standard are overlaid.

Charge heterogeneity of both BioFactura's palivizumab and *Synagis* was determined by cation exchange HPLC. In Figure 67, some differences in distribution of charge variants were observed. These charge variants may be due to cleavage of the terminal lysine on one or both Fc domains and may be modified by process optimization.

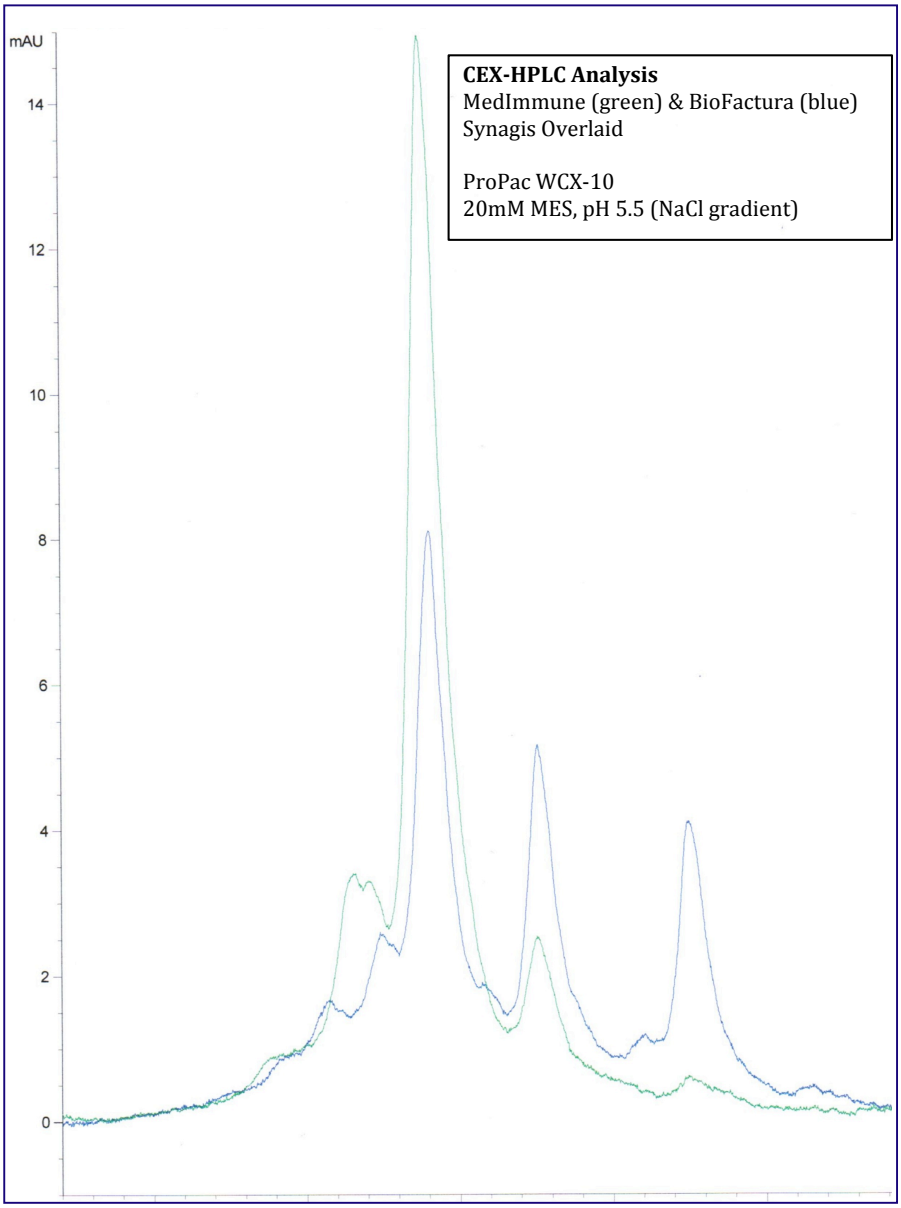

Figure 67. CEX-HPLC analysis of biosimilar palivizumab and *Synagis*. Chromatograms of biosimilar palivizumab and Synagis reference standard are overlaid.

Glycan analysis and diversity were performed using an HPLC-based hydrophilic interaction liquid chromatography (HILIC) method coupled with tandem mass spectrometry (MS/MS). The N-glycans were released from mAb by PNGase-F and labeled using RapiFluor-MS N-Glycan Kit (Waters) and characterized by nano-LC/MS/MS using a New Objective HALO Glycan nano column on a Dionex Ultimate 3000 RSLCnano system coupled with a Thermo Q-Exactive mass spectrometer. The LC/MS/MS analysis was carried out using a Thermo Scientific Q-Exactive hybrid Quadrupole-Orbitrap Mass Spectrometer and a Thermo Dionex UltiMate 3000 RSLCnano System. Labeled free glycans mixture released from mAb were loaded onto a HILIC trap cartridge at a flow rate of 5 µL/min. The trapped glycans were eluted onto a HALO Glycan 10 cm PicoFrit column (New Objective, Woburn, MA) using a linear gradient of acetonitrile (90-10%) in 0.1% formic acid. The elution duration was 60 min at a flow rate of 0.3 µL/min. Eluted glycans from the PicoFrit column were ionized and sprayed into the mass spectrometer, using a Nanospray Flex Ion Source ES071 (Thermo) under the following settings: spray voltage, 1.6 kV, Capillary temperature, 250°C.

The Q Exactive instrument was operated in the data dependent mode to automatically switch between full scan MS and MS/MS acquisition. Survey full scan MS spectra (m/z 300−2000) were acquired in the Orbitrap with 70,000 resolution (m/z 200) after accumulation of ions to a 1×10^6 target value based on predictive automatic gain control (AGC) from the previous full scan. Dynamic exclusion was set to 10 s. The 12 most intense multiply charged ions ($z \geq 2$) were sequentially isolated and fragmented in the octopole collision cell by higher-energy collisional dissociation

(HCD) using normalized HCD collision energy 28% with an AGC target 1e5 and a maxima injection time of 100 ms at 17,500 resolution.

Raw data files were analyzed using the Xcalibur software (Thermo, San Jose, CA) and SimGlycan software (PREMIER Biosoft).

The MS1 peak intensity of each glycan molecule from samples were quantified by Xcalibur software. The relative abundance of each glycan form was calculated as percentage of its intensity to total intensity of all glycan forms.

This high-resolution assay allowed for the separation and identification of key glycoforms in the biosimilar as shown in Figure 68. This enabled the evaluation of early clones and highlighted the clonal diversity of glycan profiles (Figure 69). The most comparable clone was selected for further analysis including determining the effect of a number of commercially available feeds on glycan profile (Figure 70).

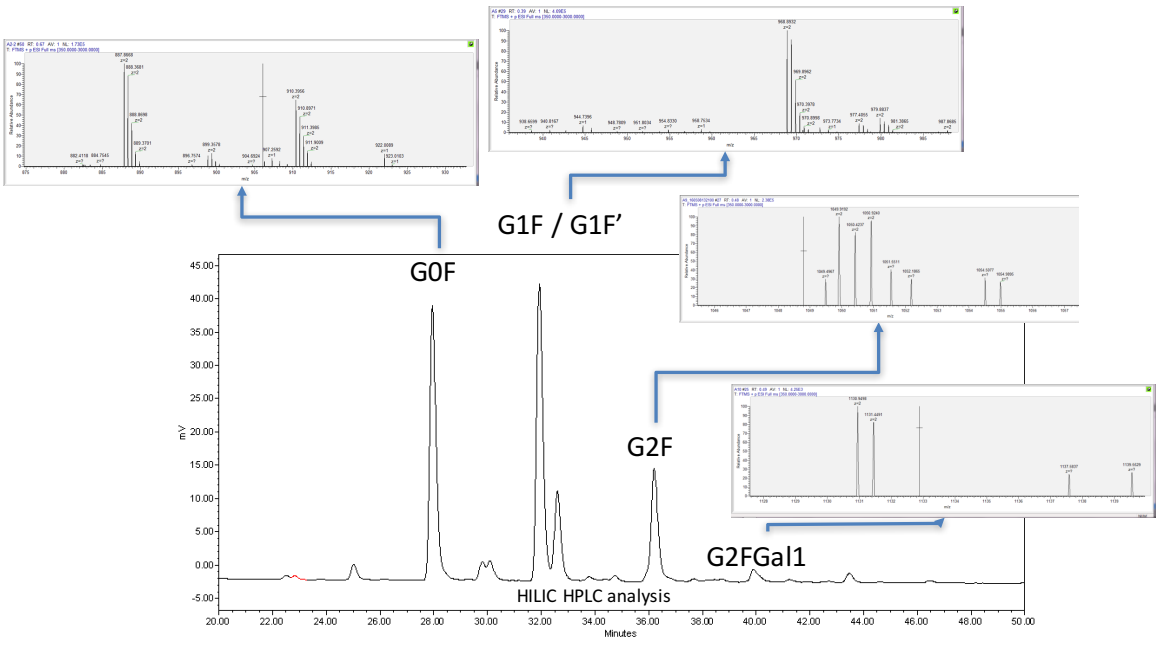

Figure 68. HILIC-MS/MS analysis of biosimilar palivizumab. Mass spectrometry analysis of major glycans are illustrated in boxes.

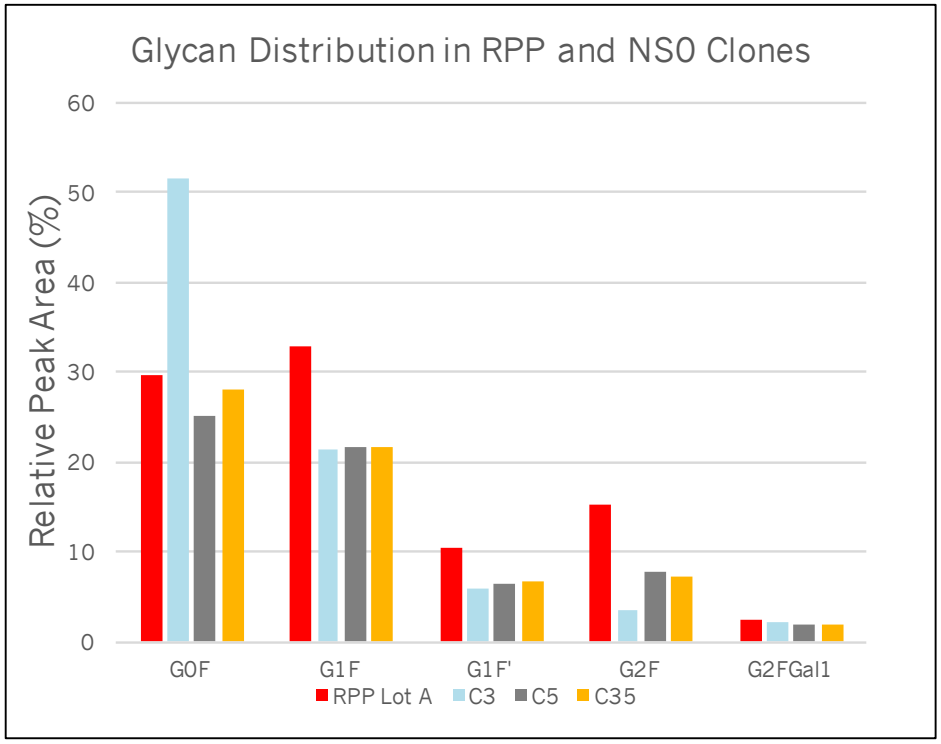

Figure 69. Comparison of reference protein product (RPP) to three clonal lots of biosimilar palivizumab. Major glycans are listed with relative peak areas indicated.

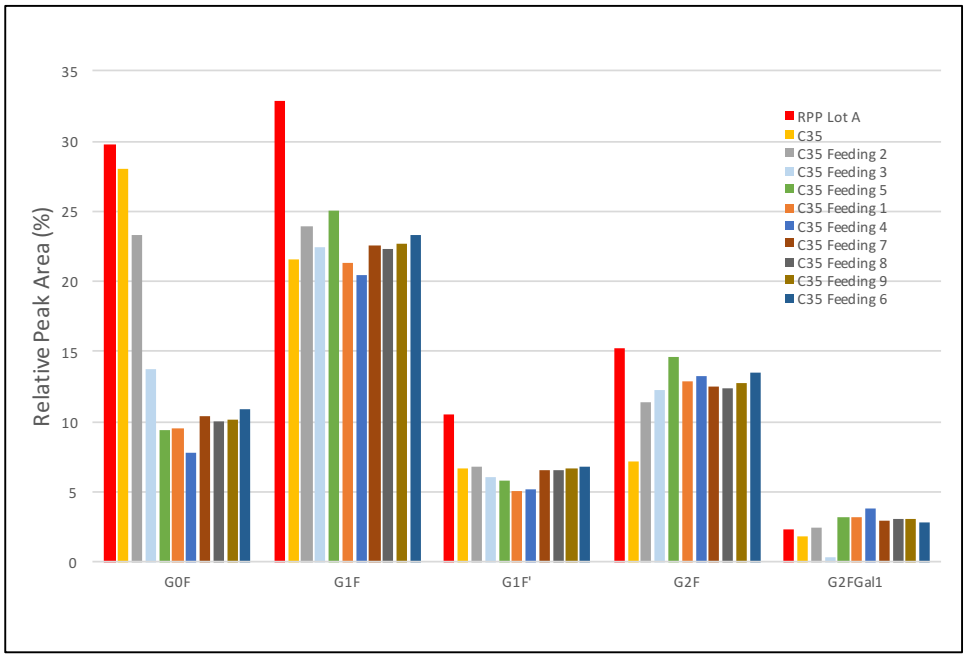

Figure 70. Effect of media feeds on glycoform distribution of a biosimilar clone. Major glycans are listed with relative peak areas indicated.

Specificity and binding kinetics of the biosimilar palivizumab and *Synagis* were evaluated by RSV F protein antigen ELISA (Figure 71) and Biacore analysis (Table 31). ELISA dilution curves and key kinetic parameters were comparable.

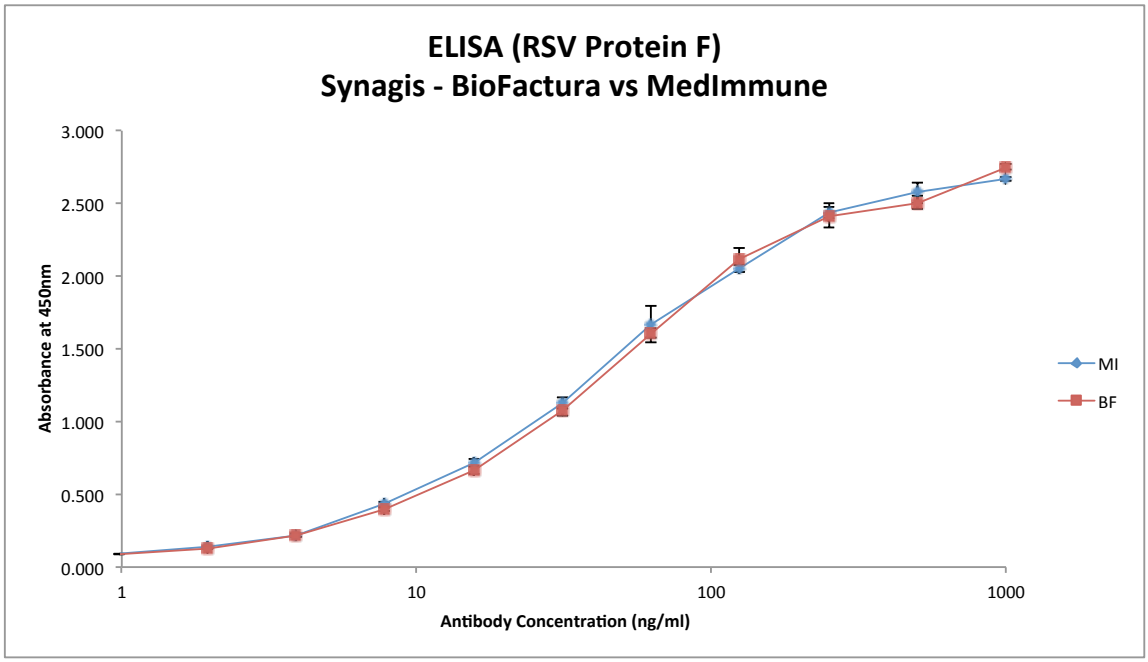

Figure 71. RSV F protein ELISA analysis of biosimilar palivizumab and *Synagis*. Absorbance curves are overlaid.

Table 31. Biacore analysis of biosimilar palivizumab and *Synagis*.

Ligand	Analyte	ka (1/Ms)	kd (1/s)	Rmax (RU)	Conc of Analyte	KA (1/M)	KD (M)	Chi2
BFSyn	RSV-F	$4.0x10^{5}$	$8.4x10^{-4}$	9.11	0-125 nM	$4.8x10^{8}$	$2.1x10^{-9}$	0.08
MISyn	RSV-F	$7.3x10^{5}$	$5.5x10^{-4}$	10.1	0-125 nM	$1.3x10^{9}$	$7.5x10^{-10}$	0.10

Conclusions

The novel StableFast-NS0 platform was used to successfully manufacture a highly complex multimeric (6 subunit) fusion glycoprotein. The cells and conditioned media were analyzed for successful expression of each subunit by western blot assay and these data were utilized for purification evaluation and development.

A high expressing cell line was generated to develop and manufacture a biosimilar mAb. SDS-PAGE and SEC-HPLC were used to demonstrate intact and digested molecular size comparability of the biosimilar to the reference product. CEX-HPLC showed some differences in charge heterogeneity between the biosimilar to the reference product that is most likely due to terminal lysine cleavage. HILIC-MS/MS analysis of several clonal cell line generated biosimilar product revealed a significant diversity in clonal glycoforms as compared to the reference product. Further studies indicated that the glycan distribution can be tuned by selective feeding of the culture.[52]

Overall, these studies demonstrate the significant value of the StableFast-NS0 platform in the development of both novel and biosimilar products.

Appendix 1:
Intellectual Property

StableFast-NS0 Cholesterol Selection—The 3-ketosteriod reductase (3-KSR) selection marker

Broad patents covering *StableFast*™ have been allowed in the following venues:

U.S. (8,076,102—See detail next page)

Australia (2006247143)

Singapore (195394)

Korea (10-1451117)

China (200680017290.4)

Europe (06770612)

StableFast-NS0 Multiplex Selection

A U.S. provisional patent application (62/153,178) was filed on April 27, 2015 that was subsequently converted to a full application under the Patent Cooperation Treaty (PCT/US2016/029472).

NS0 Serum Free Cell Line

The NS0 Serum Free Cell Line is the parental cell line used in the *StableFast*™ Biomanufacturing Platform. BioFactura obtained this cell line from the European Collection of Animal Cell Cultures (ECACC) and maintains a Commercial License with the Medical Research Council (MRC), 20 Park Crescent, London W1B 1AL, U.K. Under this license, BioFactura is permitted to use the NS0 Serum Free Cell Line for internal commercial purposes as well as provide sublicenses to *StableFast*™ customers with the pre-negotiated of a one-time upfront Research License fee of US$5,000 and a one-time upfront Commercial License fee of US$100,000 due at IND submission for novel products or US$100,000 due at first commercial approval for biosimilar products.

Human Cytomegalovirus (HCMV) Immediate-Early Promoter Regulatory DNA Sequence

The Human Cytomegalovirus (HCMV) Immediate-Early Promoter was patented by Prof. Mark F. Stinski (US Patent Numbers 5,168,062 and 5,385,839 issued December 1, 1992 and January 31, 1995, respectively) and assigned to the University of Iowa Research Foundation (UIRF). BioFactura maintained a research reagent license with UIRF until 2009 when these patents expired. Currently, this technology is off patent and in the public domain. No license is necessary.

bGH-Polyadenylation Signal

The bGH-Polyadenylation Signal was patented by Dr. Fritz M. Rottman (US Patent Number 5,122,458 issued June 16, 1992) and assigned to Research Corporation Technologies, Inc. (RCT). BioFactura maintained a nonexclusive license with RCT until 2009 when this patent expired. Currently, this technology is off patent and in the public domain. No license is necessary.

Compositions and methods for metabolic selection of transfected cells

Abstract

The present invention relates to novel selection marker vectors, and methods for using these vectors to generate stable gene expression systems in eukaryotic cells utilizing any enzyme useful in the eukaryotic sterol/cholesterol biosynthetic pathway, such as a 3-ketosteroid reductase, as a metabolic selection marker to select transfected cells. In one embodiment, the method comprises transfecting cells that are auxotrophic for cholesterol with a vector encoding 3-ketosteroid reductase and at least one heterologous protein, and selecting cells that have the ability to survive in medium lacking cholesterol and/or producing the heterologous protein in these cells in chemically defined and/or serum-free media.

Inventors: **Branco; Luis** (Rockville, MD)**, Sampey; Darryl** (Littlestown, PA)
Assignee: **BioFactura, Inc.** (Rockville, MD)
Family ID: **37432160**
Appl. No.: **11/914,725**
Filed: **May 18, 2006**
PCT Filed: **May 18, 2006**
PCT No.: **PCT/US2006/019344**
371(c)(1),(2),(4) Date: **November 16, 2007**
PCT Pub. No.: **WO2006/125126**
PCT Pub. Date: **November 23, 2006**

Parent Case Text

CROSS REFERENCE TO RELATED APPLICATIONS

This application is a national stage filing of PCT/US2006/019344, which was filed on May 18, 2006 and which claims priority to U.S. Provisional Application Ser. No. 60/681,969, filed May 18, 2005, both of which are entirely incorporated herein by reference.

Claims

What is claimed is:

1. A host cell transformed with a vector comprising a polynucleotide encoding a 3-ketosteroid reductase and a polynucleotide encoding a heterologous polypeptide, wherein the host cell is a eukaryotic cell and is auxotrophic for cholesterol.

2. The host cell of claim 1, wherein said 3-ketosteroid reductase comprises a murine 3-ketosteroid reductase.

3. The host cell of claim 1, wherein said polynucleotide encoding said reductase comprises SEQ ID

NO: 1 or SEQ ID NO:2, or encodes an amino acid sequence comprising SEQ ID NO:3 or SEQ ID NO:4.

4. The host cell of claim 1, wherein said vector is a recombinant DNA expression vector.

5. The host cell of claim 4, wherein the recombinant DNA expression vector further comprises at least a first transcription unit under control of the human cytomegalovirus promoter.

6. The host cell of claim 1, wherein said host cell is selected from the group consisting of NS-0, NS-1, and CHO-215.

7. The host cell of claim 1, wherein said host cell is an NS-0 mouse myeloma cell.

8. A kit comprising: a vector comprising a polynucleotide that encodes a 3-ketosteroid reductase; a plurality of host cells that are auxotrophic for cholesterol; chemically defined, serum-free media; growth supplements that support the growth of said plurality of host cells at low-seeding and clonal densities; and at least one protocol to utilize said kit.

9. The kit of claim 8, wherein said 3-ketosteroid reductase comprises a murine 3-ketosteroid reductase.

10. The kit of claim 8, wherein said polynucleotide encoding said reductase comprises SEQ ID NO:1 or SEQ ID NO:2, or encodes an amino acid sequence comprising SEQ ID NO:3 or SEQ ID NO:4.

11. The kit of claim 8, wherein said vector is a recombinant DNA expression vector.

12. The kit of claim 11, wherein the recombinant DNA expression vector further comprises at least a first transcription unit for a product gene under control of the human cytomegalovirus promoter.

13. The kit of claim 8, wherein said host cell is selected from the group consisting of NS-0, NS-1, and CHO-215.

14. The kit of claim 8, wherein said host cell is an NS-0 mouse myeloma cell.

15. The kit of claim 8, wherein said host cells are adapted to chemically defined, serum-free medium.

16. The kit of claim 8, wherein said host cells are adapted to chemically defined medium.

17. The kit of claim 8, wherein said growth supplements comprise at least one of fatty acid-free BSA, rhIL-6, recombinant human insulin, sodium selenite, sodium pyruvate, and ethanolamine.

18. The kit of claim 8, wherein said growth supplements comprise final concentrations in the chemically defined, serum free media of 0.1% to 5% fatty acid-free BSA, 1 ng/mL to 9 ng/mL rhIL-6, 5 mg/mL to 15 mg/L recombinant human insulin, 5 μg/L to about 8 μg/L sodium selenite, 0.01 g/L to 0.3 g/L sodium pyruvate, and 0.5 mg/L to 3.5 mg/L ethanolamine.

19. The kit of claim 8, wherein said growth supplements comprise final concentrations in the chemically defined, serum free media of 1% fatty acid-free BSA, 5 ng/mL rhIL-6, 10 mg/L recombinant human insulin, 6.7 μg/L sodium selenite, 0.11 g/L sodium pyruvate, and 2.0 mg/L ethanolamine.

20. A composition of cell culture supplements comprising 0.1% to 5% fatty acid-free BSA, 1 ng/mL to 9 ng/mL rhIL-6, 5 mg/mL to 15 mg/L recombinant human insulin, 5 μg/L to 8 μg/L sodium selenite, 0.01 g/L to 0.3 g/L sodium pyruvate, and 0.5 mg/L to 3.5 mg/L ethanolamine.

21. The composition of claim 20 comprising 1% fatty acid-free BSA, 5 ng/mL rhIL-6, 10 mg/L recombinant human insulin, 6.7 μg/L sodium selenite, 0.11 g/L sodium pyruvate, and 2.0 mg/L mg/L ethanolamine.

22. A method of making a cell that is auxotrophic for cholesterol able to survive in cholesterol-free medium, the method comprising: transfecting a eukaryotic cell that is auxotrophic for cholesterol with a vector comprising a polynucleotide that encodes a 3-ketosteroid reductase and optionally at least one polynucleotide that encodes a heterologous protein; and wherein said polynucleotide that encodes the 3-ketosteroid is expressed by the transfected cell to confer the ability to survive in cholesterol-free medium.

23. The method of claim 22, wherein said cells are selected from the group consisting of NS-0, NS-1, and CHO-215.

24. The method of claim 23, wherein said cells are NS-0 mouse myeloma cells.

25. The method of claim 22, wherein said medium is chemically defined and serum-free or chemically defined.

26. A method for obtaining cells that have the ability to survive in a medium lacking cholesterol and have the ability to express a heterologous protein comprising: transfecting eukaryotic cells that are auxotrophic for cholesterol with a vector comprising a polynucleotide encoding a 3-ketosteroid reductase, and at least one polynucleotide that encodes a heterologous protein; and selecting the cells that have the ability to survive in medium lacking cholesterol.

27. The method of claim 26, wherein said cells are selected from the group consisting of NS-0, NS-1, and CHO-215.

28. The method of claim 27, wherein said cells are NS-0 mouse myeloma cells.

29. The method of claim 26, wherein said medium is chemically defined and serum-free or chemically defined.

30. A method of expressing a heterologous protein comprising transfecting a cell that is auxotrophic for cholesterol with a vector comprising a polynucleotide encoding a 3-ketosteroid reductase, wherein the vector further comprises a polynucleotide encoding the heterologous protein; and culturing the transfected cell in a cholesterol-free medium under conditions to provide expression of said heterologous protein.

31. The method of claim 30, wherein said cells are selected from the group consisting of NS-0, NS-1, and CHO-215.

32. The method of claim 31, wherein said cells are NS-0 mouse myeloma cells.

33. The method of claim 30, wherein said medium is chemically defined and serum-free or chemically defined.

Appendix 2:
NS0 Parental Cell Line Certifications

ECACC

Health Protection Agency

NS0 (Serum Free) ECACC Number: 03061601

Cell line adapted at ECACC from the parent line NS0 (ECACC number 85110503) which was deposited by the MRC.

NSO Serum Free 03I004 Master Bank

-Adapted from Parent Line: NS0 Lot 02G017

-Adapted using: Hybridoma Medium (Sigma H4409 Lot 082K8409)
+ 8mM glutamine (Sigma G7513 Lot 43K2391)
+ 1% v/v Synthecol (Sigma S5442 Lot 013K8410)

- Frozen in: 90% culture media
10% DMSO (Sigma 154938 Lot 02957PA)

NSO Serum Free 05F022 Master Bank

Made from 03I004 master bank

- Culture media: Hybridoma Medium (Sigma H4409 Lot 082K8414)
+ 8mM glutamine (Sigma G7513 Lot 114K23661)
+ 1% v/v Synthecol (Sigma S5442 Lot 104K8406)

-- Frozen in: 45% Fresh Culture Media
45% Conditioned Culture Media
10% DMSO (Sigma 154938 Lot 07266CC)

NSO Serum Free 06D023 Working Bank

Made from 05F022 master bank

- Culture media: Hybridoma Medium (Sigma H4409 Lot 035K8403)
+ 8mM glutamine (Sigma G7513 Lot 75K2367)
+ 1% v/v Synthecol (Sigma S5442 Lot 055K8413)

- Frozen in: 45% Fresh Culture Media
45% Conditioned Culture Media
10% DMSO (Sigma 154938 Lot 02145KB)

Authorised by..... ECACC, Head of Quality.....21/6/09..... Date

Health Protection Agency Culture Collections, Centre for Emergency Preparedness and Response, Porton Down, Salisbury, SP4 0JG, United Kingdom

ECACC
European Collection of Cell Cultures

Health Protection Agency

Certificate of Analysis

Product Description: **NSO (SERUM FREE)**

Lot Number: 06/D/023

Test Description:
Cell count, viability and confluency of cells on resuscitation from frozen. SOP ECACC/049

Acceptance Criterion/Specification:
were judged acceptable if they meet two of the following criteria:
- >70% viable cells
- >2 x 10^6 total cells/amp
- Confluent within 2 days

Test Number: 36737

Test Date: 12/06/2006

Result:
9.1x10E6 68%

Test Description:
Detection of Mycoplasma by PCR using Mycoplasma-specific PCR Primers Validated by ECACC. SOP ECACC/073

Acceptance Criterion/Specification:
Positive controls yield a single 280 bp amplification product. Negative Control yields no amplified product. The criteria for a positive test result is the yield of a single 280bp PCR product.

Test Number: 36737

Test Date: 12/06/2006

Result:
Pass

Authorised by ..... **for ECACC, Head of Quality** 20/10/06 **Date**

Health Protection Agency Culture Collections, Centre for Emergency Preparedness and Response, Porton Down, Salisbury, SP4 0JG, United Kingdom

ECACC

Health Protection Agency

Certificate of Analysis

Product Description: **NSO (SERUM FREE)**

Lot Number: 06/D/023

Test Description:

Detection of Mycoplasma using a Vero indicator cell line and Hoechst 33258 fluorescent detection system. SOP ECACC/MYCO/07

Acceptance Criterion/Specification:

The Vero cells in the negative control are clearly seen as fluorescing nuclei with no cytoplasmic fluorescence. Positive control (M.hyorhiris) must show evidence of mycoplasma as fluorescing nuclei plus extra nuclear fluorescence of mycoplasma DNA. Positive test results appear as extra nuclear fluorescence of mycoplasma DNA. Negative results show no cytoplasmic fluorescence.

Test Number: 36737

Test Date: 12/06/2006

Result:

Pass

Test Description:

DNA Fingerprinting. The genome of individual cell lines has a unique set of repeated DNA sequences. These can be identified by southern blot analysis of HinfI restriction enzyme digests of genomic DNA by the DNA probe sequence 33.15. SOP ECACC/021

Acceptance Criterion/Specification:

DNA Fingerprint gels are deemed as satisfactory when the
following criteria are met: The molecular weight markers all give clear bands, the control cell lines Hela S3 and K562 give standard profiles and the control digest gives a standard profile.
The criteria for a satisfactory test is that the profile gives distinguishable bands which, if the information is available, are the same as previous analyses or appear identical to the profile from a previous batch of the source cell line run in parallel

Test Number: 36737

Test Date: 07/07/2006

Result:

Correct

Authorised by..... **ECACC, Head of Quality**..... **Date**

Health Protection Agency Culture Collections, Centre for Emergency Preparedness and Response, Porton Down, Salisbury, SP4 0JG, United Kingdom

ECACC
European Collection of Cell Cultures

Health Protection Agency

Certificate of Analysis

Product Description: NS0 (SERUM FREE)

Lot Number: 06/D/023

Test Description:
Sterility Testing of Cell Banks. SOP ECACC/048

Acceptance Criterion/Specification:
All positive controls (Bacillus subtilis and Candida albicans) show evidence of microbial growth (turbidity) and the negative controls show no evidence of microbial growth (clear).
The criterion for a positive test is turbidity in any of the test broths. All broths should be clear for a negative test result.

Test Number: 36576

Test Date: 13/06/2006

Result:
Pass

Authorised by Clare Oser **for ECACC, Head of Quality** 20/6/06 **Date**

Health Protection Agency Culture Collections, Centre for Emergency Preparedness and Response, Porton Down, Salisbury, SP4 0JG, United Kingdom

Quality Department
ECACC
Health Protection Agency Culture Collections
Centre for Emergency Preparedness and Response
Porton Down, Salisbury
Wiltshire SP4 0JG
UK

Tel +44 (0) 1980 612 594
Fax +44 (0) 1980 613 115

20 October, 2009

European Collection of Animal Cell Cultures (ECACC)

CERTIFICATE OF AUTHENTICITY

Product Number	03061601
Cell Line Name	NS0 (SERUM FREE)
Lot Number	06D023

The above consignment has been prepared at the European Collection of Cell Cultures at CEPR, Health Protection Agency, Porton Down, Salisbury, Wiltshire, UK.

This is to certify that the final product has been tested and determined that the product is authenticated as NS0 (SERUM FREE) (03061601).

Yours sincerely

pp Cathy Rowe
Quality Manager
ECACC

Enc.

Bibliography

1. Guideline on production and quality control of monoclonal antibodies and related substances. (2009) European Medicines Agency. London, United Kingdom: EMEA/CHMP/BWP/157653/2007.
2. Georgiou G and Valax P. (1996) Expression of correctly folded proteins in Escherichia coli. Curr Op in Biotech. 7(2):190-197.
3. Ahmed I, Kaspar B, and Sharma U. (2012) Biosimilars: impact of biologic product life cycle and European experience on the regulatory trajectory in the United States. Clin Ther. 34(2):400-19.
4. Gitter DM. Informed by the European Union experience: what the United States can anticipate and learn from the European Union's regulatory approach to biosimilars. (2011) Seton Hall Law Rev, 41(2): p. 559-92.
5. Guideline on similar biological medicinal products containing biotechnology derived proteins as active substance: non-clinical and clinical issues. (2006) European Medicines Agency, London, United Kingdom: EMA/CHMP/BMWP/42832.
6. Guideline on similar biological medicinal products containing biotechnology- derived proteins as active substance: Quality issues. (2006) European Medicines Agency. London, United Kingdom: CHMP/BMWP/49348.
7. Tsiftsoglou AS, Ruiz S, and Schneider CK. (2013) Development and regulation of biosimilars: current status and future challenges. BioDrugs, 27(3): p. 203-11.
8. Guideline on similar biological medicinal products containing monoclonal antibodies – non-clinical and clinical issues. (2012) European Medicines Agency, London, United Kingdom: EMA/CHMP/BMWP/403543/2010.
9. Guidelines on evaluation of Similar Biotherapeutic Products (SBPs). (2010) World Health Organisation.
10. Quality considerations in demonstrating biosimilarity of a therapeutic protein product to a reference product. (2015) US Food and Drug Administration Guidance for Industry.
11. Scientific considerations in demonstrating biosimilarity to a reference product. (2015) US Food and Drug Administration Guidance for Industry.
12. Biosimilars: Questions and answers regarding implementation of the Biologics Price Competition and Innovation Act of 2009. (2015) US Food and Drug Administration Guidance for Industry.
13. Clinical pharmacology data to support a demonstration of biosimilarity to a reference product. (2014) US Food and Drug Administration Guidance for Industry.
14. Wang J and Chow SC. (2012) On the regulatory approval pathway of biosimilar products. Pharmaceuticals (Basel), 5(4):353-68.

15. Kadarusman J, Bhatia R, McLaughlin J, and Lin WR. (2005) Growing cholesterol dependent NS0 myeloma cell line in the Wave bioreactor system: overcoming cholesterol-polymer interaction by using pretreated polymer or inert fluorinated ethylene propylene. Biotechnol. Prog. 21:1341-1346.

16. Okonkowski J, Balasubramanian U, Seamans C, Fries S, Zhang J, Salmon P, Robinson D, and Chartrain M. (2007) Cholesterol delivery to NS0 cells: challenges and solutions in disposable linear low-density polyethylene-based bioreactors. J Biosci Bioeng. 103(1):50-9.

17. Keen MJ and Steward TW. (1995) Adaptation of cholesterol-requiring NS0 mouse myeloma cells to high density growth in a fully defined protein-free and cholesterol-free culture medium. Cytotechnology 17:203-211.

18. Gorfien S, Paul B, Walowitz J, Keem R, Biddle W and Jayme D. (2000) Growth of NS0 Cells in Protein-free, Chemically Defined Medium. Biotechnol. Prog. 16: 682-687.

19. Hartman T, Sar N, Genereux K, Barritt D, He Y, Burky J, Wesson M, Tso J, Tsurushita N, Zhou W, and Sauer P. (2007) Derivation and characterization of cholesterol-independent non-GS NS0 cell lines for production of recombinant antibodies. Biotech. and Bioeng. 96(2):294-306.

20. Abbaszade IG, Clarke TR, Park CH, and Payne AH. (1995) The mouse 3 beta-hydroxysteroid dehydrogenase multigene family includes two functionally distinct groups of proteins. Mol. Endocrinol. 9(9):1214-1222.

21. Marijanovic Z, Laubner D, Möller G, Gege C, Husen B, Adamski J, and Breitling R. (2003) Closing the Gap: Identification of Human 3-Ketosteroid Reductase, the Last Unknown Enzyme of Mammalian Cholesterol Biosynthesis. Molecular Endocrinology, 17(9):1715-1725.

22. Nokelainen P, Peltoketo H, Vihko R, Vihko P. (1998) Expression cloning of a novel estrogenic mouse 17-hydroxysteroid dehydrogenase/17-ketosteroid reductase (m17HSD7), previously described as a prolactin receptor-associated protein (PRAP) in rat. Mol Endocrinol 12:1048–1059

23. Dufort I, Rheault P, Huang XF, Soucy P, and Luu-The V. (1999) Characteristics of a highly labile human type 5 17beta-hydroxysteroid dehydrogenase. Endocrinology, 140(2):568-74.

24. Seth G, Ozturk M, and Hu W-S. (2006) Reverting cholesterol auxotrophy of NS0 cells by altering epigenetic gene silencing. Biotechnol. Bioeng., 93:820–827.

25. Seth G, McIvor RS, and Hu W-S. (2006) 17Beta-hydroxysteroid dehydrogenase type 7 (Hsd17b7) reverts cholesterol auxotrophy in NS0 cells. J Biotechnol. 121(2):241-52.

26. Gen Bank: Accession No. L41519, Mus Musculus 3-Ketosteroid Reductase (HSD3b5) mRNA. (1997)

27. Gen Bank: Accession No. BC011464, Mus Musculus Hydroxysteroid (17-beta) Dehydrogenase 7, mRNA (cDNA Clone MGC:11432 Image:3966186). (2005)

28. Li J, Gu W, Edmondson DG, Lu C, Vijayasankaran N, Figueroa B, Stevenson D, Ryll T, and Li F. (2012) Generation of a Cholesterol-Independent, Non-GS NS0 Cell Line Through Chemical Treatment and Application for High Titer Antibody Production. Biotech. Bioeng. 109(7):1685-1692.

29. Chapman B, Thayer RM, Vincent KA and Haigwood NL. (1991) Effect of intron A from human cytomegalovirus (Towne) immediate-early gene on heterologous expression in mammalian cells. Nuc. Acids Res. 19(14):3979-3986.

30. Barbas III CF, Burton DR, Scott JK, and Silverman GJ. (2001) Phage Display: A Laboratory Manual. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY.

31. Guttieri MC, Sinha T, Bookwalter C, Liang M, and Schmaljohn CS. (2003) Cassette vectors for conversion of Fab fragments into full-length human IgG1 monoclonal antibodies by expression in stably transformed insect cells. Hybrid Hybridomics 22(3):135-45.

32. Chen Z, Earl P, Americo J, Damon I, Smith SK, Zhou YH, Yu F, Sebrell A, Emerson S, Cohen G, Eisenberg RJ, Svitel J, Schuck P, Satterfield W, Moss B, and Purcell R. (2006) Chimpanzee/human mAbs to vaccinia virus B5 protein neutralize vaccinia and smallpox viruses and protect mice against vaccinia virus. Proc Natl Acad Sci USA. 103(6):1882-7.

33. Chen Z, Earl P, Americo J, Damon I, Smith SK, Yu F, Sebrell A, Emerson S, Cohen G, Eisenberg RJ, Gorshkova I, Schuck P, Satterfield W, Moss B, and Purcell R. (2007) Characterization of chimpanzee/human monoclonal antibodies to vaccinia virus A33 glycoprotein and its variola virus homolog in vitro and in a vaccinia virus mouse protection model. J. Virology. 81(17):8989-8995.

34. Lustig S, Fogg C, Whitbeck JC, Eisenberg RJ, Cohen GH, and Moss B. (2005) Combinations of Polyclonal or Monoclonal Antibodies to Proteins of the Outer Membranes of the Two Infectious Forms of Vaccinia Virus Protect Mice against a Lethal Respiratory Challenge. J. Virol. 79(21):13454-13462.

35. Dutton RL, Scharer JM, and Moo-Young M. (1998) Descriptive parameter evaluation in mammalian cell culture. Cytotechnology 26:139-152.

36. Balcarcel RR and Stephanopoulos G. (2001) Rapamycin reduces hybridoma cell death and enhances monoclonal antibody production. Biotechnol Bioeng 76:1–10.

37. Sauer PW, Burky JE, Wesson MC, Sternard HD, and Qu L. (2000) A high-yielding, generic fed-batch cell culture process for production of recombinant antibodies. Biotechnol Bioeng 67:585–597.

38. Zhou W, Chen C-C, Buckland B, and Aunins J. (1997) Fed-Batch Culture Recombinant NS0 Myeloma Cells with High Monoclonal Antibody Production. Biotechnol Bioeng. 55(5):783-792.

39. Dalton AC and Barton WA. (2014) Over-expression of secreted proteins from mammalian cell lines. Protein Sci. 23(5):517–525.

40. Burky J, Wesson M, Young A, Farnsworth S, Dionne B, Zhu Y, Hartman T, Qu L, Zhou W, and Sauer P. (2007) Protein-free fed-batch culture of non-GS NS0 cell lines for production of recombinant antibodies. Biotech. and Bioeng. 96(2):281-293.

41. Ma N, Ellet J, Okediadi C, Hermes P, McCormick E, and Casnocha S. (2009) A single nutrient feed supports both chemically defined NS0 and CHO fed-batch processes: Improved productivity and lactate metabolism. Biotechnol. Prog. 25(5):1353-1363.

42. Ibarra N, Watanabe S, Bi JX, Shuttleworth J, and Al-Rubeai M. (2003) Modulation of cell cycle for enhancement of antibody production in perfusion culture of NS0 cells. Biotechnol. Prog. 19:224-228.

43. Hermes PA and Castro CD (2010) A fully defined, fed-batch, recombinant NS0 culture process for monoclonal antibody production. Biotechnol. Prog. 26:1411-1416.

44. "The glutamine synthetase (GS) gene amplification/expression system used to select and amplify transformed NS0 cells for expression is based patents issued to Celltech Biologics plc, now assigned to Lonza Group plc, subsidiary of Alusuise-Lonza Group. Related patents include U.S. 5,770,359 and 5,747,308 (coassigned to the University of Glasgow). Over 70 companies have licensed GS System technology for various uses." Web reference: www.lonzabiologics.com/biologics/en/gs

45. Bebbington CR, Renner G, Thomson S, King D, Abrams D and Yarranton GT. (1992) High level expression of recombinant antibody from myeloma cells using glutamine synthetase as an amplifiable selectable marker. Bio/technology 10:169-175.

46. Wu M-H, Dimopoulos G, Mantalaris A, and Varley J. (2004) The effect of hyperosmotic pressure on antibody production and gene expression in the GS-NS0 cell line. Biotechnol. Appl. Biochem. 40:41-46.

47. Barnes LM, Bentley CM, Moy N, and Dickson AJ (2007) Molecular analysis of successful cell line selection in transfected GS-NS0 myeloma cells. Biotechnol. Bioeng. 96:337-348.

48. Dempsey J, Ruddock S, Osborne M, Ridley A. Sturt S, and Field R (2003) Improved fermentation processes for NS0 cell lines expressing human antibodies and glutamine synthetase. Biotechnol. Prog. 19:175-178.

49. Gibson DG, Young L, Chuang RY, Venter JC, Hutchison CA 3rd, and Smith HO. (2009) Enzymatic assembly of DNA molecules up to several hundred kilobases. Nature Methods. 6(5):343-345.

50. Gibson DG. (2011) Enzymatic assembly of overlapping DNA fragments. Methods in Enzymology. 498:349-361.

51. Peakman TC, Worden J, Harris RH, Cooper H, Tite J, Page MJ, Gewert DR, Bartholemew M, Crowe JS, and Brett S (1994) Comparison of expression of a

humanized monoclonal antibody in mouse NS0 myeloma cells and Chinese hamster ovary cells. Hum. Antibod. Hybrid. 5:65-74.

52. Patel TP, Parekh RB, Moellering BJ, and Prior CP. (1992) Different culture methods lead to differences in glycosylation of a murine IgG monoclonal antibody. Biochem J. 285:839-45.