Expression of the Immunoglobulin Gamma Heavy Chain for Use as a Biochemical Tool

Allison Kukuch

Indiana University
Chemistry Department
Advisor: Dr. Thomas Tolbert

Abstract

From the recent 2005 review of glycosylation of recombinant antibody therapeutics Jefferis (3) notes that production of therapeutic antibodies with a consistent human glycoform profile remains a challenge but the development of customized antibody therapies is an ultimate goal. Up to this point there have been attempts to use mammalian derived antibodies, such as from mouse cells, with unwanted side effects. This highlights the importance of further research in the area of antibodies and their possible use as tools for, at the least, therapeutic use. One goal of the Tolbert laboratory is to develop a procedure so that yeast strains can glycosylate proteins with more humanlike glycosylation. In preparation, this report focuses on the creation of a yeast strain from which the IgG Fc protein can be purified in homogeneous form. To lay the groundwork for the use of this protein for therapeutic purposes one strain will be produced with a 5' histidine tag. Besides aiding in purification this addition will test for stability of expression, indicating whether small molecule bioactive proteins may be attached in place of the histidine tag.

1. Introduction

The glycosylation of Immunoglobulin Gamma (IgG) is important to its form and function. Structural analysis done by Krapp et al. (4) found that the alteration of glycosylation of the CH2 domain generated conformational changes that reduced binding affinity. Due to this, purification of a homogeneously glycosylated IgG Fc heavy chain will be of importance in this project. So far the Tolbert lab has been able to express the IgG Fc heavy chain in yeast with the myc epitope followed by a His six tag on the C-terminus. Through the purification process it was found that the glycosylation was heterogeneous and when deglycosylated the products still showed two distinct bands when run through a gel. The hypothesis for this heterogeneity is varied proteolysis of the C-terminal end of the monomer. The current objective is to make two subclones of the IgG Fc heavy chain both without the C-terminal myc epitope or His tag and one with an N-terminal His tag. This will allow us to not only see a difference in proteolysis due to the presence/absence of a His tag but it will also show how positioning of the His tag on the N-terminus will affect expression. To test over all reduction in proteolysis a protease deficient strain of yeast will be used.

As described by Brekke and Sandlie (1) in their review of antibodies for human disease there are three mechanisms through which antibodies can be used: blocking the action of a specific molecule, targeting specific cells, or functioning as a signaling molecule. Through those three functions antibodies can be used to

treat infectious agents, anti-inflammatory diseases, and cancers to name a few. The goal here is to understand how our changes to the Fc region will affect its ability to function properly and have other molecules attached at the hinge region. From this, expression of the altered Fc region will give us a tool to explore different possible functions of this modified antibody, which points to a host of additional projects that can follow.

2. Materials and Methods

2.1 General Procedures

Growth media and agar plates used for the growth of bacteria were Low Salt LB and consisted of 2% Bacto™ Tryptone, 1% Bacto™ Yeast Extract, and 1% NaCl with 2% Bacto™ Agar when plates were made. All Bacto™ products were purchased from Becton, Dickinson, and Company. For yeast growth and agar plates YPD was used and consisted of 2% Bacto™ Tryptone, 1% Bacto™ Yeast Extract, and 2% Glucose with 2% Bacto™ Agar when plates were made. Also 1M sorbitol (Sigma) was added to media and plates of yeast cells recovering from electroporation, referred to as YPDS.

PCR was done using 10% 10x Thermo Buffer, 25% 1.25mM dNTP, 20% 10mM of both the 5' and 3' primers, and 0.1% TAQ Polymerase. These chemicals were purchased from New England Bio Labs. The program named GNT on the MyCycler™ from Bio-Rad was used for PCR. The program started at 94^{o}C

for three minutes, then 33 cycles of 94^{o}C for 45 seconds, 50^{o}C for 45 seconds, and 72^{o}C for 90 seconds. After cycling the temperature was held at 72^{o}C for 10 minutes and finally brought down to 4^{o}C at which the samples could be stored until further use.

Double digests were run with 1ul each of EcoR1 and Not1, 6ul Buffer EcoR1, 0.6ul BSA, DNA, and water for a total of a 60ul reaction. Ligations with 2ul DNA Ligase Buffer and 1ul DNA Ligase were run with varying combinations of plasmid and insert for a total of 20ul. Sequencing reactions consisted of 3ul of 5mM $MgCl_2$, 2ul of 2uM primer, 1ul of ABI Big Dye version 3.1, and DNA depending upon concentration (20-40ng) to which water was added to reach a total of 10ul. Linearization reactions used 2ul AvrII and 16ul of NEB Buffer 2 to every 150ul of DNA. Unless otherwise noted all chemicals were purchased from New England Bio Labs. Measurements of absorbance were taken with a DU Series 500 Spectrophotometer from Beckman.

Electroporation was done using a MicroPulser^{TM} from Bio-Rad. For bacteria transformations the program used was Ec1 with a voltage of 1.8 kV for one pulse. After electroporation 1ml low salt LB was added and a one-hour recovery phase in 37^{o}C shake was allowed before plating the cells. For yeast the program used was Sc2 with 1.5 kV for one pulse. After electroporation 1ml of sorbitol was added and a one-hour recovery at 30^{o}C was followed by the addition of 2ml of YPD and an additional two-hour growth with shake at 30^{o}C.

Agarose gels used for DNA procedures were made from 1% SeaKem LE Agarose in 60ml of TAE then micro waved for 2.5 minutes after which 2.5ul of ethidium bromide was added. Model B1 Class II gel boxes from Owl Separation Systems Inc. powered by the Power Pac Basic from Bio-Rad Laboratories were used to run agarose gels. A UV Transilluminator Model M-15 by UVP was used to visualize the agarose gels. Acrylamide gels for protein procedures were made with a 15% Resolving Gel base topped off with a 5% Stacking Gel and run in Mini Protean 3 Cell boxes powered by the Power Pac Basic. Resolving Gels consisted of 15% dilution of 30% Acrylamide/Bis Solution, 1.5 M Tris at pH 8.8, and 10% SDS. At the time of making the gels 10% ammonium persulfate and 5% TEMED were added. For the Stacking Gel a 5% dilution of 30% Acrylamide/Bis Solution, 1.0 M Tris at pH 6.8, and 10% SDS were combined to which 10% ammonium persulfate and 5% TEMED were added when the gel was made. All chemicals for gel procedures were obtained from Bio-Rad Labs. After acrylamide gels were run the following stain and de-stain procedure was used to view the protein bands. Enough quick stain (25% isopropanol, 10% acetic acid, and 0.05% comassie blue) was used to cover the gel, which was micro waved for one minute on high and then removed. Next enough quick de-stain (10% acetic acid and 10% isopropanol) was used to cover the gel, which was again micro waved for one minute and removed. Finally enough slow de-stain (10% acetic acid and 30% methanol) to cover the gel was added and left at room temperature on a rotator plate until

sufficiently de-stained. Gels were dried using DryEase Mini Cellophane and Gel-Dry Drying solution from Invitrogen in a drying rack.

Protease deficient yeast cells (SMD1168H) were made competent for electroporation by the following procedure adapted from Shixuan Wu and Geoffrey Lechworth (5). A 2ml culture from frozen stock was grown in YPD overnight from which 50ul and 10ul samples were used to inoculate two 50ml cultures of YPD in baffled flasks in a 30^{o}C shake. These cultures were allowed to grow until the O.D. 600 was 1.5. The cells were then spun at 2.4krpm for 10 minutes. The pellet was resuspended in 8ml of resuspension solution containing 100mM LiAc, 0.6M sorbitol, 10% 1M DTT, and 0.7% 1.5M TrisHCl. Incubation at room temperature for 30 min was done before the cells were pelleted again. Three washes with 1.5ml ice-cold sorbitol were done for a final volume of 1.5ml of sorbitol. The cells were stored on ice before use within the same day.

The Bust n' Grab technique from Harju *et al*. (2) was adapted here to extract DNA from yeast cells. Cells picked from plates were suspended in 100ul lysis buffer (2% Triton X-100, 1% SDS, and 100mM Tris-HCl pH 8.0). The cells were frozen at -80^{o}C for 10 minutes and transferred to a 95^{o}C water bath for one minute. The freeze-thaw cycle was repeated after which the cells were vortexed for 30 seconds. 100ul of chloroform was added and the cells were vortexed for two minutes. Centrifugation was done for three minutes and the upper aqueous phase was transferred to a new tube containing 400ul of cold ethanol with gentle

mixing. The solution was then incubated at -20^{o}C for at least five minutes. After five minutes of centrifugation the ethanol was removed and the pellet was allowed to dry. The DNA was resuspended using 50ul of EB solution from the QIA kits.

Small-scale expression trials were performed in 12 well plates kept at 30^{o}C with shaking. Each well contained 2ml of YPD and zeocin at 100ug/ml (except for the SMD control well). Glucose (1%) was added each day and to prevent evaporation sterile water was added as needed to keep all wells at the same level.

2.2 Cloning

The IgG Fc DNA from MGC12857 was grown over night in low salt LB with 35ug/ml chloramphenicol to ensure selection of the vector. The plasmid was then purified from the bacterial cells using QIA prep Spin Mini Prep Kit. Two PCR reactions with a 50ul volume each were run with two different primers on program GNT. Primers for the cell line now called 'His' are as followed

5'-EcoR1-His6-IgG

gcgccgGAATTCcatcatcatcatcatacatgcccaccgtgcccagca

3'-IgG-STOP-NOT1

gggcccgcgGCGGCCGCttatttacccggagacagggagg

Primers for the cell line 'No' are as follows

5'-IgGFc-EcoR1-PP

gcgccgGAATTCacatgcccaccgtgcccagca

3’-IgG-STOP-NOT1

gggcccgcgGCGGCCGCttatttacccggagacagggagg

PCR products were run on an argarose gel and purified with the QIA Quick Gel Extraction Kit.

The plasmid pGAPZαA from Top10F’ was grown over night in low salt LB with 25ug/ml zeocin and then collected via the QIA Mini Prep kit. The plasmid was purified through argarose gel and then the QIA Gel Extraction kit. Both PCR products and the pGAP plasmid, 45ul, were digested with EcoR1 and Not1 in a double digest for 2 hours at 37^{o}C. For one additional hour 1ul CIP was added to the plasmid digest only to prevent recircularization. QIA Quick PCR Purification Kit was used to purify the digests. Ligations were done at room temperature for one hour with 7ul of both plasmid and insert along with a control that contained only plasmid.

2.3 Electroporation

Electroporation using Top 10F’ bacterial cells was done for each of the three ligations. For this procedure 1ul of the ligation was added to 80ul of cells then the cells were shocked, after which 800ul low salt LB was added and the cells were allowed to recover for one hour at 37^{o}C. 100ul and 200ul of cells each for His and No were plated with zeocin. 100ul of the control was plated with zeocin and all plates were grown overnight at 37^{o}C.

Cells that grew were picked for sequencing reactions to check for correct insertion of the DNA and to make sure that there were no mutations. First overnight growths of each colony were grown in low salt LB and zeocin. Next plasmid mini preps were done and a gel was run to check that the plasmids were of the correct size. These plasmids were then PCR checked to ensure insertion of the IgG DNA. Cells that passed both tests were sent to the Indiana Molecular Biology Institute for sequencing from the 5'α mating factor and 3' AOX. Frozen stocks were made from colonies that were correctly sequenced.

2.4 Yeast Transformation

Over night one His and one No colony were grown in low salt LB and the purified plasmids were linearized with Avr II. QIA PCR Clean Kit was used to purify the linear DNA and the absorbance was taken to determine the concentration of DNA. Yeast cells SMD1168H were made competent as described above. For transformation 1-3ug of DNA was added to 80ul of cells. After electroporating the cells 1ml of sorbitol was added for a one-hour recovery at 30^{o}C. Then 2ml YPD was added and the cells were allowed to shake at 30^{o}C for two hours. The cells were plated on YPDS plates with 100ug/ml zeocin. For both His and No the following plates were made: two 50ml, two 100ml, and one 250ml. A control plate with zeocin of SMD cells that did not undergo transformation was plated with 100ul of cells. Also plates without zeocin were

made with 100ul for both His and No. All of these plates were allowed to grow for four days at 30^{o}C. Colonies that grew under the 100ug/ml selection were plated on 500ug/ml zeocin and the colonies that were able to grow were used for small-scale expression.

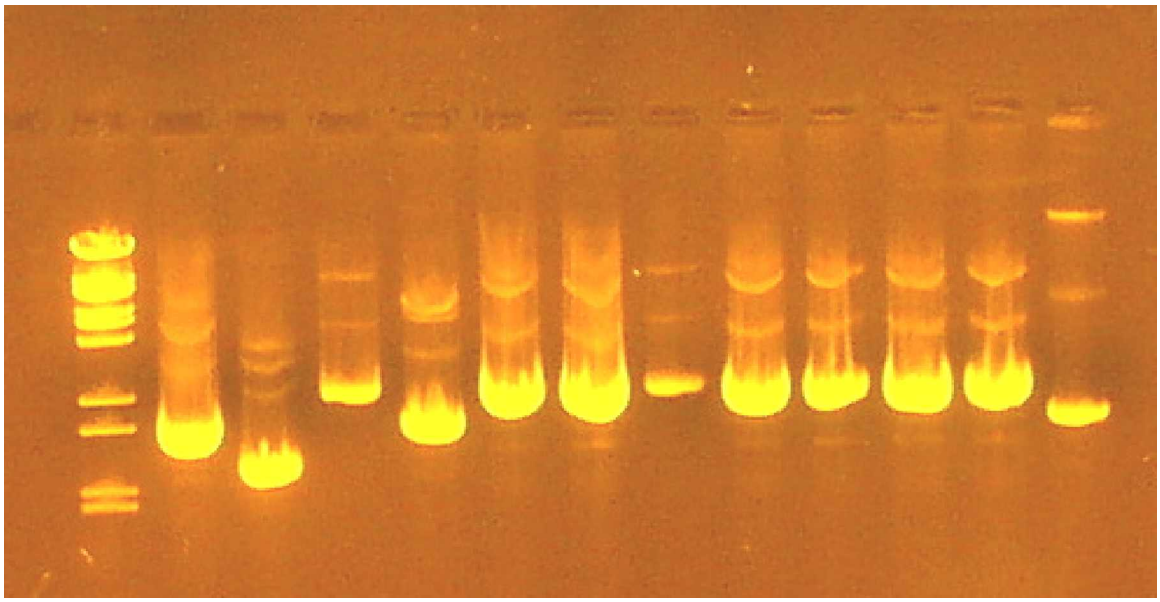
2.5 Yeast Expression

Small-scale yeast expression was done with the addition of 1% glucose every 24 hours. Samples of 50ul were taken out at 48, 72, 96, and 120 hours. These samples were spun at 6krpm for 5 minutes from which 40ul of the supernatant was taken. 10ul of 4x protein dye was added and vortexed. After 5 minutes at 95^{o}C the samples were run on 15% agarose gel along with the size marker Mark 12 MW Standard. Gels were then stained, de-stained, and dried so that the protein bands could be seen.

3. Results and Discussion

PCR amplification of the IgG insert DNA was successful but digestion and ligation posed a few problems. After ensuring that both enzymes were able to do a single digestion the double digestion was done with differing amounts of plasmid versus insert. Finally it was found that using 8ul of each produces many colonies. This is due to the fact that the plasmid is many-fold larger than the insert so an

equal volume will ensure that there will be more copies of insert for every plasmid.



λ Bste II N6 N5 N3 N2 N1 H6 H5 H4 H3 H2 H1 pGAP

Figure 1 Agarose gel of plasmid size check for inclusion of insert into the plasmid

Plates that were grown from the ligations showed there were 50% background colonies. Due to this, size exclusion via agarose gel was done as shown in figure 1. Only the colonies that showed a larger size than the original pGAP plasmid were tested further (N3, N1, H6, H5, H4, H3, H2, H1).

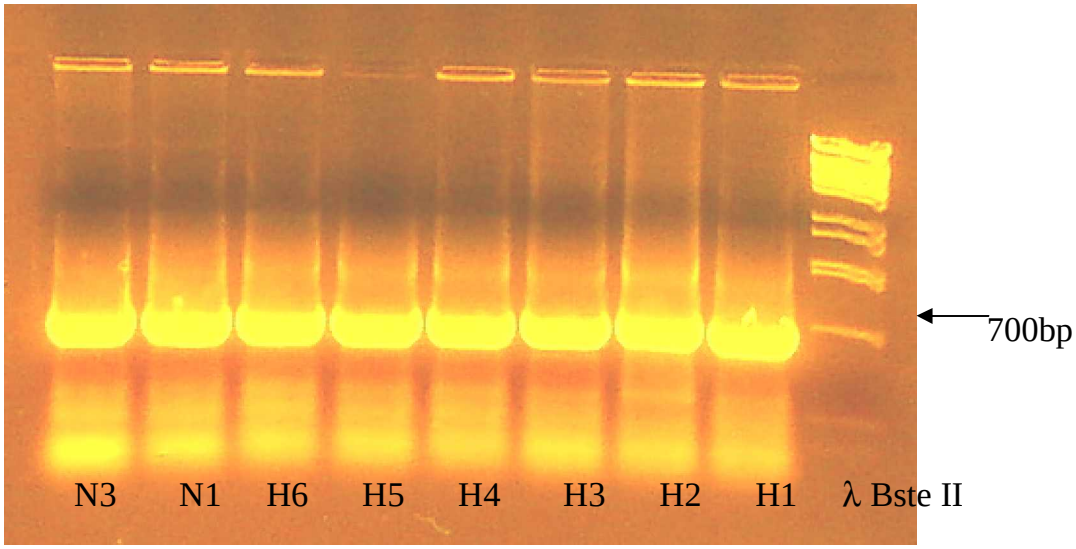
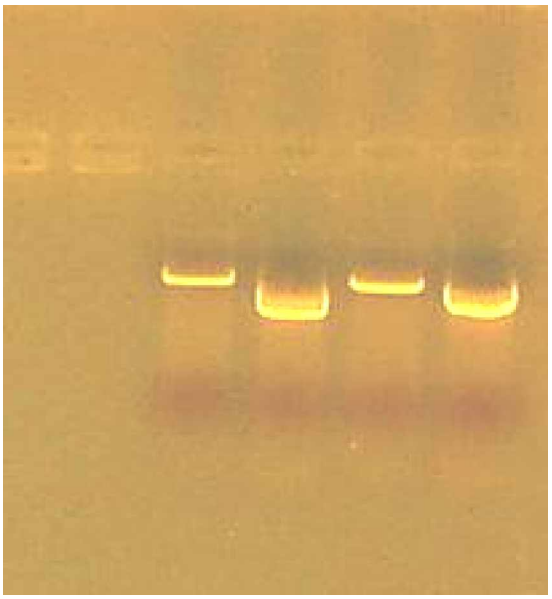


Figure 2 Agarose gel of PCR products to check for insert

To ensure that the chosen colonies contained the insert PCR was done on each colony. As seen in figure 2 all colonies checked by size were confirmed to have the IgG insert. From these colonies two for each construct were chosen for sequencing (H1, H2, N1, and N3). The first sequencing produced a 900bp read through from both directions for H1 while the others were not sufficiently sequenced. The samples were sent back with an increase in DNA concentration slightly over the amount suggested in the online protocol from the Indiana University Molecular Biology DNA Sequencing Facility. The second set of sequences produced a complete read through of 800bp from both directions for N1. Frozen stocks of these two colonies were made for future use.



His plasmid | His linear | No plasmid | No linear

Figure 3 Linearization of both the His and No plasmids

After a few trials it was found that linearization using Avr II was optimal after an overnight digestion as seen in figure 3. Electroporation of the SMD cells proved to be challenging. The first attempt had significant contamination. Then after two attempts, which produced few colonies, a transformation that produced 100 to 300 colonies per plate was achieved. Again there was a 50% background but 28 colonies (with three controls) were selected and checked for insertion of the IgG DNA. While the insert was confirmed and a small-scale expression was done, contamination was found so the procedure was repeated.

In the subsequent transformations, small numbers of colonies were again produced; therefore before moving to small-scale expression a selection on higher zeocin concentrations were performed. This was done to identify multiple

integrants. A cell line with more than one copy of the IgG gene should be able to produce higher yields of the protein and is therefore preferred over single integrant strains. Due to the low number of colonies produced in the transformations thus far additional transformations will be performed. With the high colony number that will be expected in new transformations the cells can be selected for multiple integrants in liquid cultures instead of on plates. Through this process cells can be selected in up to 2000ug/ml zeocin.

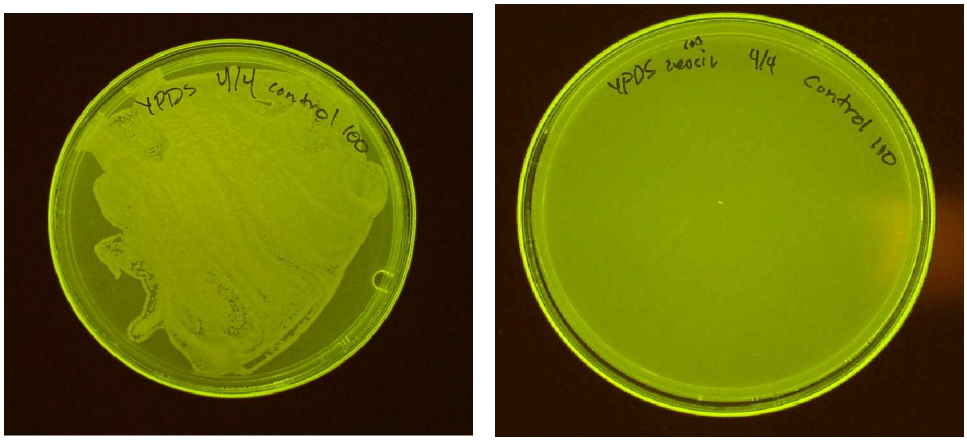


Figure 4 Control plates for transformation

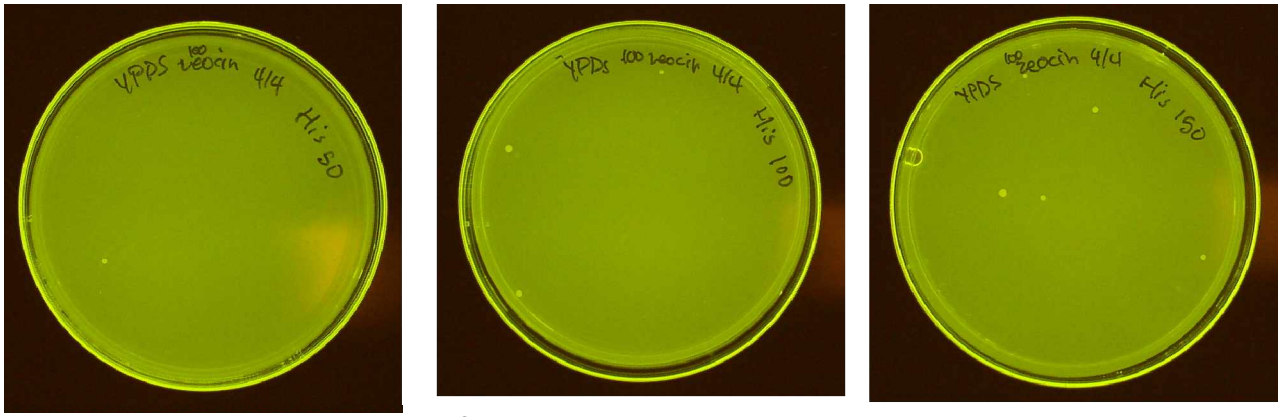


Figure 5 Plates with transformed IgG with histidine tagged colonies

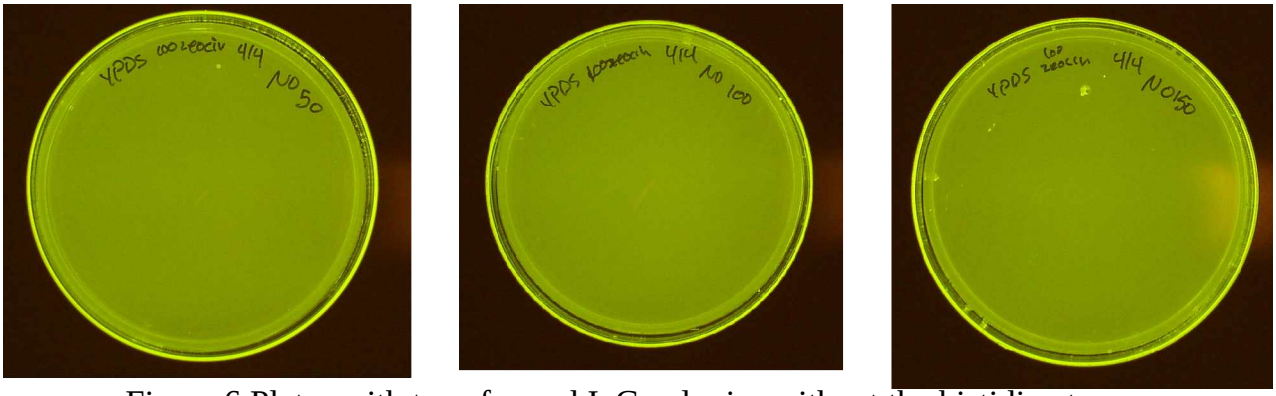


Figure 6 Plates with transformed IgG colonies without the histidine tag

While the colony count continues to be low, figure 4 shows that the contamination problem has been corrected with the non-zeocin plate showing a lawn of colonies while the zeocin containing plate did not allow cells that were not transformed to grow.

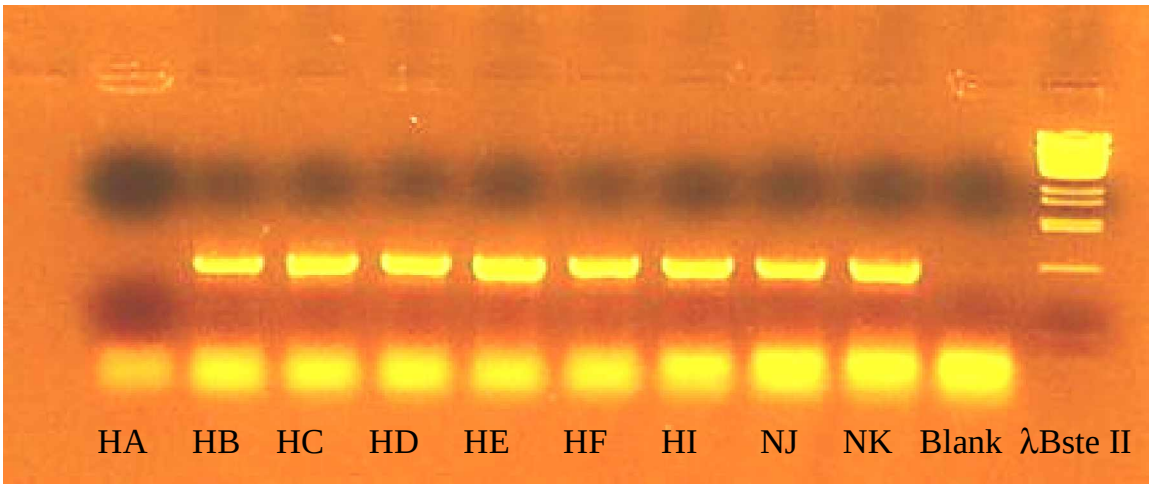
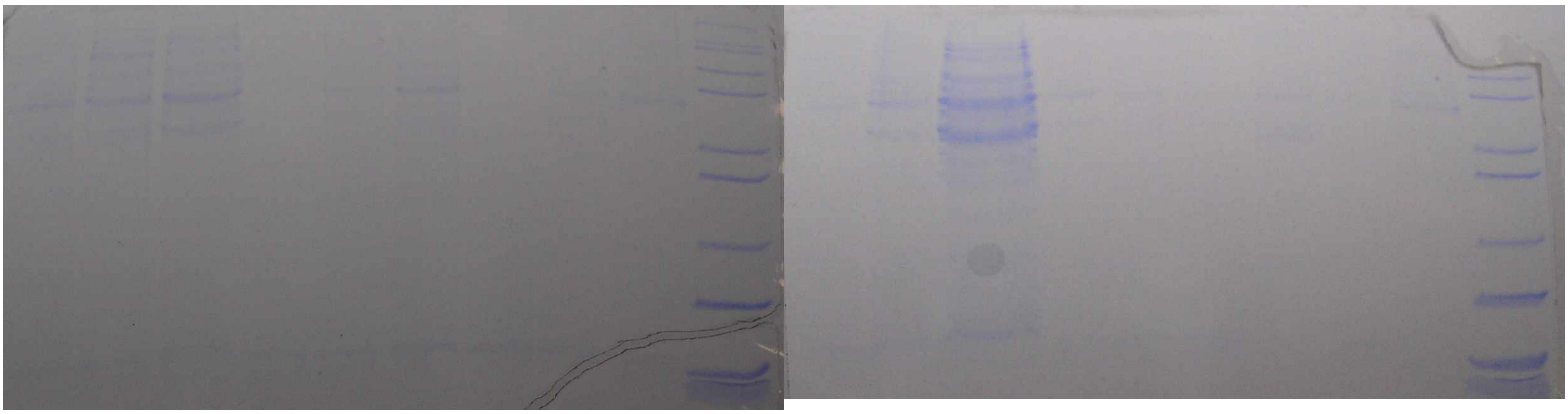


Figure 7 PCR check of insertion of IgG DNA

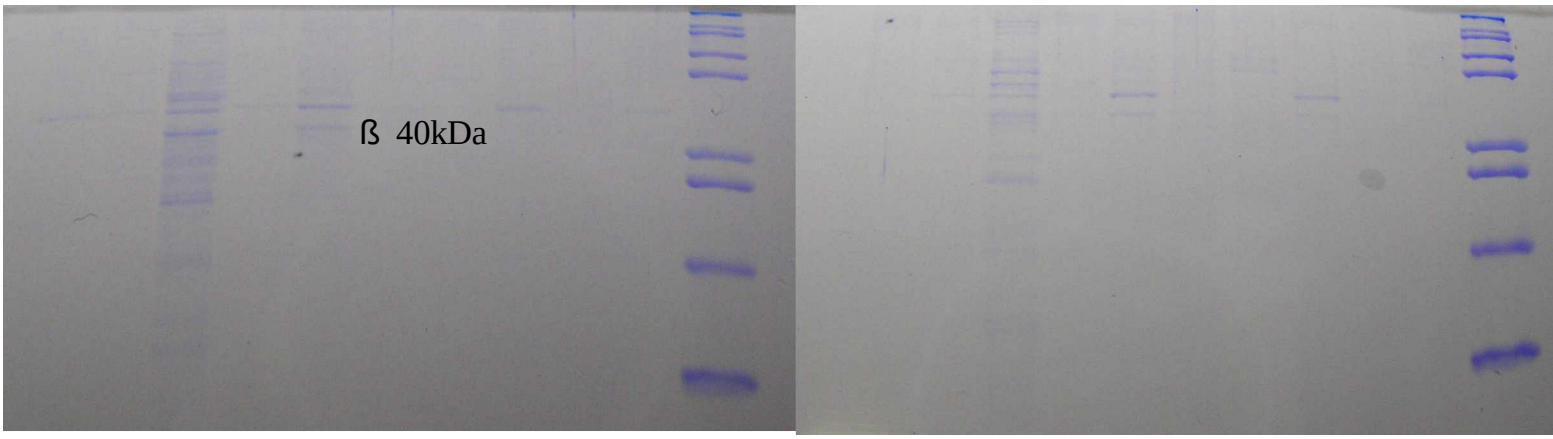
A PCR check of the insertion of IgG was done on the selected colonies showing that HA did not have the DNA insert (figure 7). Due to the fact that the

Bust ‘n Grab protocol used can sometimes not yield results HA was not excluded from further analysis.



HA HB HC HE HF HI NJ NK SMD Marker HA HB HC HE HF HI NJ NK SMD Marker

Figure 8 Days two and three of small-scale expression



HA HB HC HD HE HF HI NJ NKSMD Marker HA HB HC HD HE HF HI NJ NK SMD Marker

Figure 9 Days four and five of small-scale expression

Small-scale expression was done showing that there was a product made in the transformed cells (at 40kDa) that was not made in the control of SMD cells (poor picture quality does not show all bands present in the gel). This indicates

that IgG protein is being made. Knowing that the DNA is inserted and the protein can be made, transformations to achieve high integrant cell lines are currently being done.

Future plans include but are not limited to large-scale expression of the best protein expressers from the small-scale expression, purification of IgG, quantification of the amount being expressed, and characterization of the protein to look for proteolysis. Testing of the IgG with the histidine tag will be essential to ensure that the protein can function with additional amino acids attached. If this His protein can function correctly the attachment of other bioactive molecules in place of the histidine tag will be done. Ultimately it is this attachment of other bioactive molecules that will confirm the ability for IgG to be used as a biochemical tool.

Alpha-mannosidase II was also being expressed at the same time as the above expression of IgG. The procedures that were performed included linearization, transformation, electroporation, and small-scale expression in both the SMD1168H and GS115 yeast cells. Also a large-scale expression and purification from the GS115 cell line was done.

4. References

(1) Brekke, Ole Henrik, & Sandlie, Inger. Therapeutic Antibodies for Human Disease at the Dawn of the Twenty-First Century. *Nature*. 2003, 2, 52-62.

(2) Harju, Susanna, Fedosyuk, Halyna, and Peterson, Kennith R. Rapid isolation of yeast genomic DNA:Bust n' Grab. *BMC Biotechnology* 2004, **4**:8.

(3) Jefferis, Royston. Glycosylation of Recombinant Antibody Therapeutics, *Biotechnology*. 2005, 21, 11-16.

(4) Krapp, S.; Mimura, Y.; Jefferis, R.; Huber, R.; & Sondermann, P. Structural Analysis of Human IgG-Fc Glycofoma Reveals a Correlation Between Glycosylation and Structural Integrity. *Journal of Molecular Biology*. 2003, 325, 979-989.

(5) Wu, Shixuan, and Lechworth, Geoffrey J., High efficiency transformation by electroporation of *Pichia pastoris* pretreated with lithium acetate and dithiothreitol. *BioTechniques* 36:152-154