"Starvation Day" Optimization of a Multi-Day Process for Antibody Production in *Saccharomyces cerevisiae*

A THESIS

Presented to

The Faculty of the Department of Molecular Biology

The Colorado College

In Partial Fulfillment of the Requirements for the Degree

Bachelor of Arts

By

Delaney A. McCann

Adimab, LLC

May, 2022

Abstract

The method of displaying recombinant proteins on the surface of *Saccharomyces cerevisiae* via genetic fusion, a technology known as yeast surface display (YSD), has become a valuable protein-engineering tool for a broad spectrum of biotechnology and biomedical applications. For example, YSD can be used to display monoclonal immunoglobulin Gs (IgGs) for drug development. Other display systems, such as phage or mammalian display, can produce specific IgGs, but cannot match the high throughput advantages of YSD. Yeast display technologies facilitate expedited production and optimization of full-length human IgGs. YSD allows for facile production of relatively large quantities of purified protein that can then be screened for affinity, quality, polyspecificity, and interactions. This process is extremely valuable in the development of antibody-based drugs. The timely design, development, and trial of these drugs can result in thousands of lives saved. Adimab LLC, the antibody engineering biotechnology company where this study was carried out, uses YSD to develop antigen-specific leads for the antibody-based drug development pipeline. The current multi-day process for producing and harvesting purified IgG involves multiple feed days in which the yeast cells are provided with nutrient media. The goal of this study was to optimize the feed day conditions for an increased and higher quality protein yield. A control condition followed the standard protocol while an experimental condition, containing the same clone samples, underwent the altered protocol featuring fewer feed days. The samples that received one fewer feed day were left in shakers for a day known as "Starvation Day", rather than receiving feed. Aliquots were taken from both conditions to compare optical densities of the cultures, concentrations of final protein yields were compared, and pH changes were monitored throughout. The optimization of this process, involving the removal of one of the feed days, resulted in higher cell densities in the samples, higher final protein yields, and protein products of higher quality. This resulted in a change in the standard process. Moving forward, the process can continue to be optimized by evaluating components of the medias, while taking into account changes in culture volume and pH. This optimization can provide larger protein yields for critical analytical assays and decrease the labor involved in the multi-day process.

Disclosure

The study discussed in this paper has been incorporated into the protocol for antibody protein production at Adimab, LLC. Due to the nature of this process and its importance to the continued profit of the company, several proprietary details pertaining to the cell lines, materials, methods, and data analysis have been excluded.

Introduction

Monoclonal Antibodies as Functional Therapeutics

Science and medicine are progressing to develop more therapies that mimic naturally occurring immune responses. At the forefront of this movement is the application of monoclonal antibodies. Monoclonal antibodies are laboratory-generated antibodies that often are produced at an industrial scale in mammalian cell lines; they are designed to recognize a specific epitope on a disease-relevant antigen. Like naturally existing IgGs, monoclonal antibodies can interact with other components in the immune system to elicit beneficial responses. Targeting antigens or erroneous self-cells for cell-mediated death is one way monoclonal antibodies can be harnessed in assisting the innate immune system.[1] These aforementioned functions of monoclonal antibodies make them desirable as pharmaceutical drugs.

Monoclonal antibodies are utilized in the treatment of numerous diseases and ailments, for example, those rooted in auto immune disorders and cancers. In recent decades, immunotherapies involving these molecules have been approved for the treatment of some cancers. The drug rituximab is used in the treatment of several types of blood cancers. It targets cells with the CD20 marker on their surface, a marker expressed in most B cell malignancies. In some cases, the monoclonal antibody drugs (mAbs) are designed to interact with the microenvironments of the cancerous tumors.[2] They can target the action of a chemical or receptor that is involved in the development of the condition.[1] This can either be direct, such as targeting a tumor for immune system recognition, or indirect, such as inhibiting a protein or receptor that may blind the immune system to the presence of the tumor.[1]

Conversely, monoclonal antibodies can also be used to attenuate immune responses. This is the most common mechanism for targeting auto-immune disorders. In auto-immune disorders, the body's immune system incorrectly identifies self-tissue as foreign.[2] Antibody therapy can be used to suppress these reactions so as to minimize the symptoms experienced as a result of the body's overreaction. Asthma, arthritis, MS, and transplant rejection are common examples of conditions that may benefit from treatment with specialized monoclonal antibodies. Adalimumab, commonly known as HUMIRA, is an example of an FDA-approved mAb drug that treats ulcerative colitis, inflammation, and ulcers in the bowels.[2] Adalimumab is designed to bind to an inflammatory protein, inhibit its ability to elicit inflammation, and decrease the symptoms of ulcerative colitis.[2]

Finally, and especially pertinent today, is the use of monoclonal antibodies in the fight against viruses. Monoclonal antibodies developed to recognize a specific antigen can be introduced into an infected patient and elicit subsequent immune responses to clear the virus upon antigen recognition.[3] This can save the patient valuable time, the time it would take for their own immune systems to randomly generate antibodies that might recognize the specific virus. For this reason, these molecules are being developed for broad coverage of strains of influenza and SARs-CoV-2. The unique and desirable specificity of monoclonal antibodies allows them to target the foreign or unhealthy cells without harming the healthy cells.[3] This is an advantage over other therapies such as chemotherapy or radiation treatment, which do not distinguish between cellular targets. This characteristic lead to the coupling of IgGs with other components such as drugs, enzymes, chemicals, or dyes for targeted treatment delivery, tests, or recognition/diagnosis. Monoclonal antibodies possess the potential to revolutionize several disease treatment plans. The efficiency with which we can develop, test, and select these molecules is important for the overall progression of their application in medicine.

Monoclonal Antibody Protein Production Mechanisms

Originally, monoclonal antibodies were developed in hybridoma cells.[1] These cells came from the fusion of spleen cells of an immunized mammal and an immortal pluripotent cell line. The hybridoma cell line could then be used to produce antibodies for the desired antigen. However, this process was generally not efficient, and the generated antibody sequences were host specific, not human. Next, phage display became a common platform.[1] In phage display, the sequence for the desired antibody is introduced into a phage plasmid. The phage then infects a host, such as *Escherichia coli*, resulting in the incorporation of the DNA and the subsequent production of the desired protein.[4] However, because this takes place in a non-eukaryotic host, the post translational processing, folding, and protein modifications are not accurate representations of what would occur in antibodies produced in mammalian cells.[5] Since IgGs are composed of heavy chains and light chains connected by disulfide bridges, it became advantageous

to only produce the parts of the antibody that possessed the specificity and recognition necessary to target antigens. This platform allowed for several different IgG subspecies structures and combinations to be synthesized. This resulted in smaller protein fragments, so-called single chain fragment variables (ScFvs), that could either perform the necessary functions while infiltrating smaller microenvironments or that could be fused with the remaining parts of the IgG[6].

Yet other IgG production systems have been developed such as the use of yeast cells in a process known as yeast surface display (YSD).[5] The production of monoclonal IgGs and other protein species in a yeast platform is the method utilized by the institution where this study was carried out, Adimab LLC. Specifically, this study was carried out in an engineered strain of *Saccharomyces cerevisiae*. This strain has been genetically modified to allow for display and soluble expression of full length IgGs. Large antibody libraries can be screened in yeast due to their high throughput quality compared to mammalian cells and *S. cerevisiae* undergoes efficient homologous recombination, facilitating plasmid construction within the cell. YSD is favorable to phage display in *E. coli* because yeast have a secretory system, with the protein of interest being secreted from yeast cells into the surrounding supernatant.[5] This helps to ensure a less complicated harvest of the desired protein product. The yeast can be placed under selective pressure to facilitate proper selection of clones. When it is desirable to produce intact, full-length IgGs, an auxotrophic selection approach can be used to ensure only those yeast cells that successfully express both the heavy and light chains thrive. In standard YSD, exogenous genes can be fused with genes that encode for proteins associated with the cell wall. This results in an anchored IgG product.[5] Yeast cells with antibodies displayed on their surface are then subjected to multiple rounds of screening with fluorescently labeled antigens.[6] The detectable fluorescence on antigens, bound to certain cells, makes it possible to screen for positive antibody interaction. The yeast platform is extremely high throughput. *S. cerevisiae* cells are much easier to maintain than live animals, have a quick replication period, and are easily manipulated. The protocol followed in this study can yield multiple milliliters of supernatant with concentrations of protein of up to 1 mg/mL in under 10 days. The proteins produced in yeast are invaluable for immediate applications in assays and preliminary antibody quality screening. The yeast-produced antibodies are of high enough quality that they can often be used in studies employing animal models, like rodents. This quality refers to the purity of the antibodies, as well as the antibodies possessing characteristics close enough to their human orthologs.[5] Once the ideal antibody sequence is determined in yeast, it can be implemented and expressed in a mammalian cell platform with relative ease. For final application in human trials, the IgGs will need to be produced at a higher quality, in mammalian cells, like Chinese Hamster Ovary (CHO) cells.[1,7] However, the ability to quickly screen IgGs in YSD is priceless in the process of developing human-approved drugs. The high throughput quality of the yeast display platform means that hundreds of antibody candidates can be screened and processed prior to further development. This allows for an overall expedited development timeline which, down the line, can mean providing life changing treatment to thousands of patients in a timely manner.

IgG Production Protocol Optimization Efforts

Since the proteins produced in this platform are essential to numerous screening tests and assays, the higher the protein yield, the more samples can be assessed in a fixed time frame and within a given production capacity. By optimizing the titer obtained from our standard process, we may be able to decrease the scale required to produce the amount of IgG needed for the standard assays. If this scale is decreased, the saved capacity allows for more submissions of additional samples to be incorporated into

the timeline. Overall, this can result in more samples expressed and screened, and subsequently, a broader range of monoclonal IgGs can be developed and successful clones can be selected for. With the optimization of our standard protocol as the goal, experimental conditions were developed and implemented into the process. The standard protocol utilized by the company follows a several day timeline in which the antibody-producing yeast are inoculated in plate cultures, divided into additional plates to allow for culture expansion, supplemented with media to induce desired antibody production, fed over a number of days, and harvested through purification (Figure 1A). The process used uses multi-well plates with different samples in each well. In a process run, prior to the start of my study, low levels of cross-contamination between neighboring wells were observed in downstream assays. In an effort to decrease any occurrence of cross-contamination, plates were placed in an experimental condition in which they received one less day of feed than the usual protocol. By removing an addition of feed media and substituting it with a day in which the plates were left untouched in the shakers to process, called Starvation Day, the final volumes of the wells would be lower (Figure 1B). It was hypothesized that the lower well volumes would decrease the likelihood of cross contamination during the harvest process. Upon reviewing the results of this preliminary study, the Starvation Day plates were found to yield higher titers in more than half the samples. These results, obtained from a less labor-intensive experimental workflow, were enough to spark interest in a more comprehensive study. This secondary study would include a more diverse range of IgGs to test that the experimental condition was beneficial across the platform, include more precise downstream quality analysis, and include an additional control protocol (Figure 1C) to evaluate the necessity of Starvation Day.

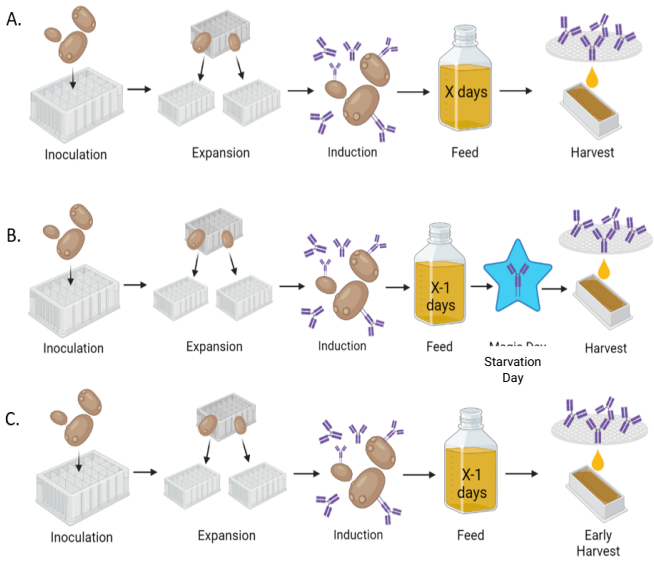

Figure 1. Control and experimental workflow conditions for obtaining purified protein products from a yeast platform. A. The control workflow begins with the inoculation of a plate with the desired protein-producing *S. cerevisiae* clones. Samples are expanded to additional plates to provide more space for growth and increase final product yields. Samples are induced to begin producing the target protein and are fed a number of days to maintain nutrient availability and encourage continued protein production, processing, and secretion. Resultant proteins are then harvested from the supernatant. B. The experimental workflow follows the same principle as the control but forgoes a day of feed for a day without intervention prior to harvest, called Starvation Day. C. The secondary experimental workflow, designed to confirm the necessity of a Starvation Day, also forgoes a day of feed, but is harvested a day early.

Methods

96-well, protein-producing, *S. cerevisiae* sample plate construction and culture refresh

Yeast clones were chosen for broad representation of antibody lines from the company library. Well-known clones were selected for use in the study. Cells come from frozen strains and already contain the desired protein-encoding DNA sequence. The samples were then configured in groupings in the 96 well plate such that IgG sample repeats were grouped within the same column. A volume of the cells was moved to a new plate and a volume of selective growth media was added to each well. A mixing program on an automated lab bench assistant ensured the cultures and media were properly mixed. This refreshed plate was placed in a high-speed shaker overnight to allow for culture proliferation.

Inoculating seed plates with samples

A volume of a combination of a selective growth media and penicillin-streptomycin was added to each well in an empty "seed" plate. A volume of cells from each well in the refreshed source plate was transferred to separate larger wells in the seed plates. The plates were placed in lower speed shakers for a period of time to allow for sample culture expansion.

Expansion of the sample cultures into more plates

A number of empty "expansion" plates were prepared for receiving an aliquot of the seed plates. A volume of a selective growth media combined with a volume of penicillin-streptomycin was added to the expansion plate wells to serve as a supportive media for the sample and as a deterrent for unwanted growth. In order to expand the sample cultures and acquire optimum cell growth, a volume of the samples in the seed plates was transferred to the respective wells in the expansion plates using a robotic liquid handler. The expansion plates were placed in the lower speed shakers for a period of time to further encourage cell proliferation.

Inducing the production of desired protein products in the cell samples

A selective expression media formulated to induce the secretion of the IgGs, was added to each of the wells of the expansion plate. The induced expansion plates were returned to the lower speed shakers for a period of time to allow for the production of proteins to commence.

Encouraging continued cell growth and protein production

A selective "feed" media containing the appropriate carbon and nitrogen sources was prepared. To replenish the nutrients necessary for cells to continue to proliferate and produce proteins, the samples were supplemented with a volume of the feed media over X number of days. After each feed, the plates were returned to the lower speed shakers. In order to test the condition of Starvation Day, the experimental condition plates were supplemented with a volume of feed media over X-1 days. On the day of the last feed occurrence for the control condition plates, the experimental plates were left untouched and unfed in the lower speed shakers.

Purification of the desired protein products from the cell samples

In order to harvest the protein products in the supernatant of the samples with ease, identical expansion plates were combined into one "combine" plate. To adjust the samples to a pH desirable for pH-dependent protein-resin binding, a volume of a specific base was added to the wells of the combine

plates prior to the addition of the samples. Resin plates specialized for IgG fragment binding were prepared by loading a volume of a specific resin with constant region protein-binding capabilities into a fritted filter plate. The buffered combine plates were then reconfigured to ensure one sample was loaded onto one well in a corresponding resin plate. “Collection” plates were prepared by adding a volume of an alkaline solution to each well. To collect the desired protein that was bound to the resin, the filter plates were placed on top of a collection plate and a volume of acidic buffer solution was washed over the resin. The pH-dependent binding capabilities of the resin were interrupted by the sudden change in pH and the desired proteins were eluted into the collection plate. The alkaline solution in the collection plate wells served as a neutralizing agent to the acidic elution buffer as it entered the collection plate. This served to restore pH conditions more conducive to storing the desired protein eluate. Final purified protein products then underwent quality control and analytical analysis.

Measuring the optical density (OD) of cells in the plates

In order to track the cell growth in the control and experimental conditions throughout the production process, aliquots were taken from the plates at predetermined points in the workflow for comparison. A fresh, 96-well plate was filled with a volume of autoclaved water. A volume of yeast cells was taken from an expansion plate representative of each condition and added to the fresh plate. An automated lab bench assistant mixed the autoclaved water and the culture sample several times in each well. The plate was spun at high rpm for several minutes to form a cell pellet at the bottom of the wells. Additional supernatant was flicked out of the plate. A volume of autoclaved water was added to each of the wells and the solution was mixed by an automated lab bench assistant to ensure uniform cell distribution. The plate was spun a second time at high rpm for several minutes to form a cell pellet. The additional supernatant was flicked out, leaving only the cells in the wells. A volume of autoclaved water and diluted cell sample were added to the wells of a UV star plate. To determine A600 readings for the samples, the UV star plate was analyzed for A600 absorbance in a plate reader. The raw A600 reads were used to calculate the optical densities by taking into account the dilution factors. The OD measurements for identical samples under experimental and control conditions were compared to record differences in cell growth.

Measuring final protein titers

To compare the final protein yields of the control and experimental samples, an aliquot was taken from the final purified protein products, for both conditions and was added to a fresh 96-well UV star plate. To acquire A280 readings for protein absorbance and A260 readings for nucleic acid absorbance in the samples, the UV star plate was read in the plate reader. The raw A280, A260, and A280/260 ratios, in addition to the known sample volumes, were used to calculate the protein concentrations for the products. These calculations were used to determine which conditions produced higher protein titers.

Size Exclusion Chromatography (SEC) determines the quality of purified protein products

SEC was run as a quality control assay to compare the proteins produced in the control and experimental conditions. An aliquot was taken from the final purified protein products and added to a fresh QC plate. The QC plate was loaded onto an Agilent 1260 Infinity II size exclusion chromatography system. A fresh size exclusion column, packed with polymer beads, was loaded into the machine and the lines were flushed with mobile phase buffer. A needle took up sample from each well, one at a time, and the proteins were allowed to flow through the system, exiting the column at different times based on

their sizes and interactions with the column matrix. As the molecules exited the column, measurements were recorded at 215 and 280 nm wavelengths to observe absorption of the peptide bonds and aromatic side chains, respectively. These measurements were used to identify characteristic peaks and species. The quality of the samples was evaluated based on the percent major peak, with samples having higher percentages representing more uniform products. Resultant peak schemes were compared between control and experimental conditions to identify any significant difference that implied discrepancies in quality. Additionally, retention times were evaluated for undesired protein species, longer retention times representing smaller species, and shorter retention times unwanted protein aggregates. Together, these measurements were used to determine the qualities of the purified protein products.

Investigating pH levels in samples under both conditions

In order to ensure that pH levels remained within the desirable ranges in the experimental condition, pH measurements were taken in experimental and control samples at predetermined points throughout the process. A pH probe was used to measure the pH of four wells from the same plates throughout the process. These measurements were taken in both conditions for comparison. Most importantly, pH was taken before and after the presence or absence of feeds to make sure pH was not dropping too low when the samples missed a day of feeding. Finally, the pH was closely observed before harvesting the protein, to determine if the experimental condition would have effects on the pH that may inhibit the samples' ability to bind to the pH-dependent resin.

Results

Higher cell densities are recorded for samples undergoing the experimental workflow.

In order to determine if the experimental workflow was detrimental to the proliferation of the *S. cerevisiae* cells, aliquots were obtained from both the experimental and control conditions at predetermined points in the process. The aliquots were used to obtain an A600 absorbance reading in a plate reader and the optical densities were calculated using the known volumes and dilution factors unique to each condition. The ODs were tracked throughout the workflow (Figure 2). Aliquot number 4 marks the first aliquot taken after the conditions begin to vary, with the control receiving an additional day of feed and the experimental skipping the last feed. Surprisingly, the Starvation Day condition revealed higher ODs, with averages around 65 at timepoint 4 and 128 at timepoint 5. Comparatively, the control samples yielded ODs with an average of 46 at point 4 and 48 at point 5. Throughout aliquots 1-3 the conditions maintain comparable OD trends; this is consistent with the knowledge that experimental variance in treatment began at aliquot number 4. The last aliquot, number 5, is representative of the cell densities just prior to

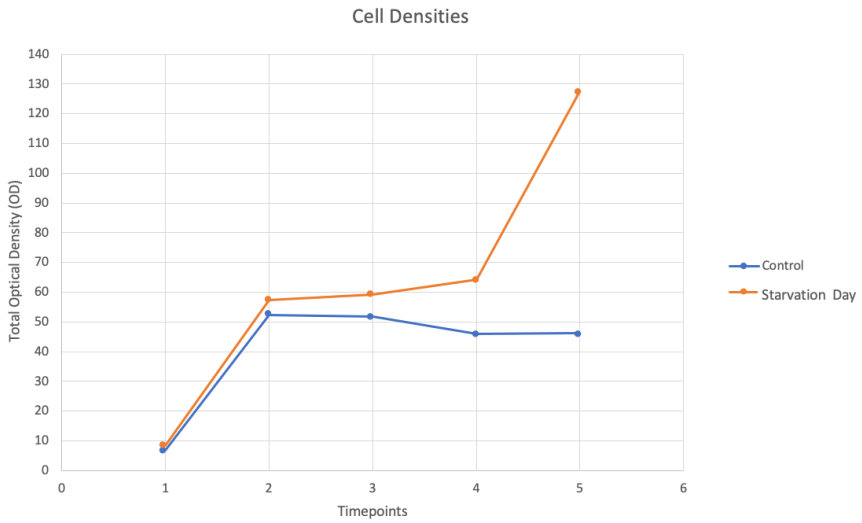

Figure 2. Optical Density (OD) reads from samples undergoing experimental workflow surpass control sample reads. The points represent total OD values taken at predetermined timepoints in the process, ending with one immediately prior to harvest (x=5). The values were calculated from A600 absorbances and the known dilution factors of the aliquots. Experimental samples possessed more than double the cell density measurements that were observed in the control, indicating the presence of more protein-producing cells.

harvest. The difference in values between the experimental and control conditions is representative of the differences in the presence of potential protein-producing cells leading into harvest. This indicates that there was reason to suspect a potential increase in protein yield with this experimental condition. These data were unexpected and inspired the further investigation of Starvation Day as a process optimization.

Comparable protein concentration yields observed for experimental conditions.

To test if protein yield would be significantly altered in the samples under Starvation Day conditions, aliquots of purified protein were taken from both experimental and control conditions for comparison. The aliquots were used to obtain A280 reads and calculations based on the known volumes of the samples provided the concentration of purified protein in the samples. The specific concentrations measured for each sample under experimental conditions, in mg/mL, were plotted against the respective sample's measured concentration under control conditions (Figure 3). The dots represent the points plotted for experimental and control concentrations. The points depicted above the y=x line are representative of samples that produced higher protein concentrations under the experimental conditions. Alternatively, those below the line represent samples that

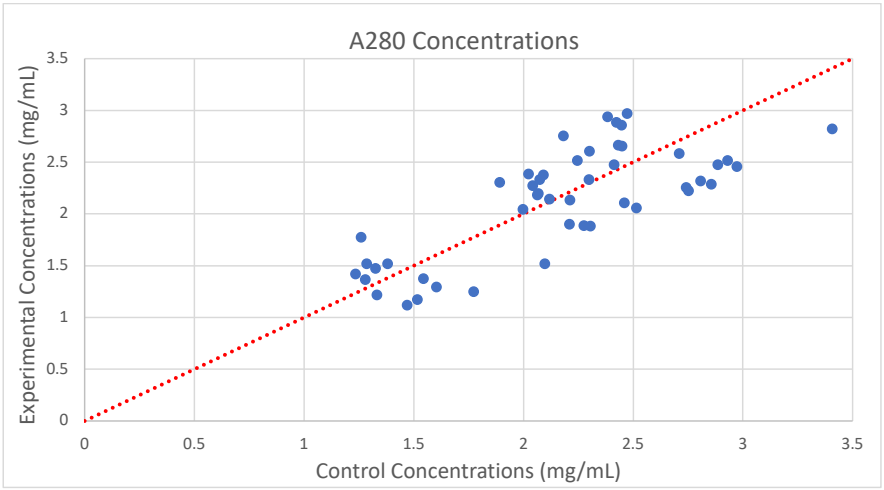

Figure 3. Experimental A280 concentrations normalized for control concentrations. The points are each representative of a calculated concentration of protein in the final samples in experimental vs control conditions of the same IgG-producing yeast type. The measurements were obtained from purified protein eluates using A280 absorbance. The points depicted below the y=x line represent samples that produced higher protein concentrations in the control conditions. The points above the line represent samples that performed better in the experimental, Starvation Day, conditions.

performed better in control conditions. The comparable, and in some cases, higher concentrations observed in the experimental conditions provided another incentive to further investigate Starvation Day. The potential of increased titers is always exciting in yeast platform optimization. The more protein produced in a given period of time, the more efficiently and quickly the screening and analytical workflow can commence.

Higher cell densities observed for a comprehensive range of samples under experimental conditions.

In order to determine if the Starvation Day experimental condition resulted in increases of protein production across a wider variety of samples, the experimental conditions were repeated with a broad range of samples that produce IgGs representative of the library composition. Once again, aliquots were taken from the experimental and control conditions at the same predetermined points in the workflow. The aliquots were used to measure A600 absorbance and the known volumes and dilution factors were used to calculate the optical density of *S. cerevisiae* cells in the samples. Across all of the distinct sample types, Starvation Day and control ODs followed a similar trend (Figure 4). The quantities are comparable for points 1-3, but Starvation Day begins to outperform the control at point 4, the first point after conditions diverge. On average, experimental conditions possessed nearly 2.5 times more cells leading up to harvest than their control counter parts. The presence of a larger population of cells could mean higher potential for subsequent protein production. Observing higher OD reads consistently across all

sample types in the experimental conditions reaffirmed the preliminary study's suspicion that Starvation Day may be incorporated as an optimized protocol.

Samples under Starvation Day conditions maintained pH levels conducive to *S. cerevisiae* growth and reached the pH range necessary for purification upon harvest.

In order to confirm that Starvation Day conditions still maintained sample pHs within the range necessary for *S. cerevisiae* cell growth and survival, the pH of random samples was recorded at predetermined timepoints throughout the protocol. The pH was compared to pH data obtained from the control condition at the same timepoints. Starvation Day and control sample pHs followed the same rise and fall trend until about timepoint 4 (Figure 5), which marks the first measurement taken after the conditions diverge. The fluctuation observed between alternating points is consistent with the accumulation of byproducts of fermentation/respiration and media buffering. During *S. cerevisiae* fermentation, the cells generate acidic byproducts. These byproducts subsequently lower the pH of the sample. In the workflow, samples are routinely supplemented with media containing an alkaline component to buffer the acidic byproducts and maintain a pH conducive to cell life. Between timepoints 4 and 7 the Starvation Day sample pHs are consistently lower than the control samples. This is representative of the lack of buffering the Starvation Day samples have when they do not receive the final feed. The increasing acidity of the samples is due to the continued fermentation/respiration of the

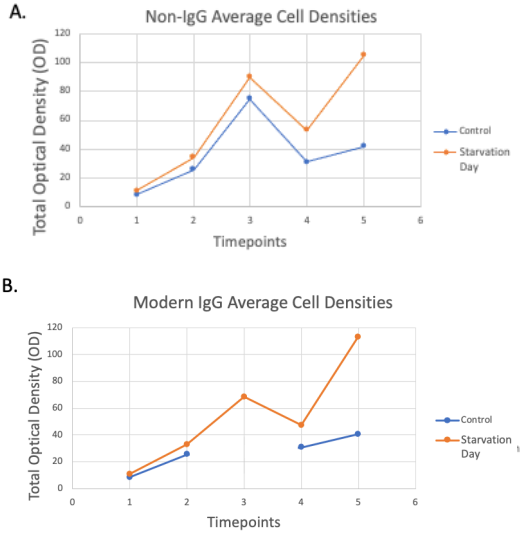

Figure 4. Experimental and control OD comparisons reconfirm the general trend observed in the preliminary study across a wider range of samples. The OD comparison from the preliminary study (Figure 2) demonstrates that IgG samples under both conditions follow the same general trend until timepoint 4. At timepoint 4, the conditions diverge and a change in ODs is observed. Starvation Day conditions yield an OD more than double that of the control conditions. A. An averaged OD comparison of alternative antibody formats in the secondary study demonstrates that, once again, samples under both conditions follow the same trend until aliquot 4. This data demonstrates that Starvation Day conditions increase cell densities when compared to control conditions in a diverse range of samples. B. An average OD comparison of modern IgG samples, containing the current standard strain modification, in control and experimental conditions demonstrates that Starvation Day outperforms control conditions in yet another sample type. This data set is missing a data point in the control condition at aliquot number 3. The trend similarity observed between aliquot numbers 1 and 2, as well as the trends observed in the other sample conditions, allow us to comfortably assume that the missing data point would have yielded a trend similar to that of the experimental condition. Finally, the trend divergence at aliquot number 4 and the increased cell densities under Starvation Day conditions match the patterns observed in other sample types. This lends itself to reconfirming the assumed trend of the missing point.

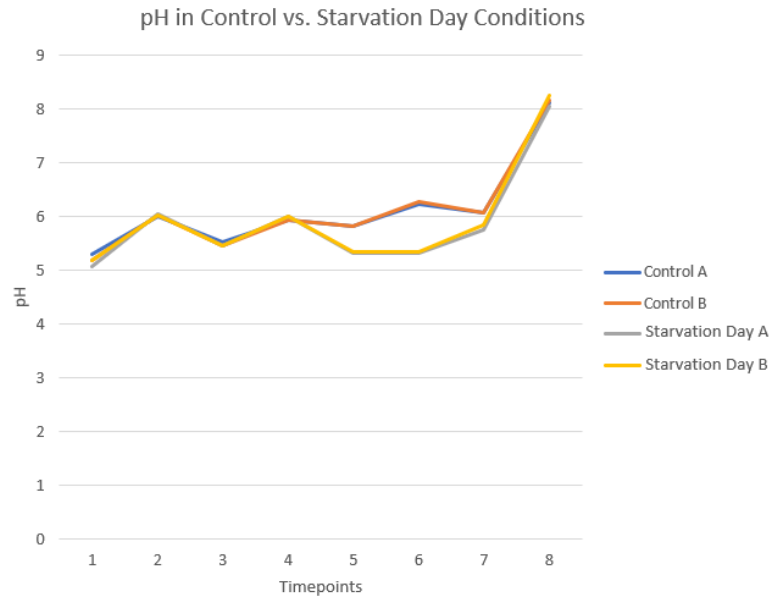

Figure 5. Samples generated under experimental production conditions maintain pH levels conducive for yeast growth and protein production. pH was monitored to make sure that without the additional feed buffering step, the culture media did not get too acidic and therefore inhibit yeast growth or health. The pH of random sample wells from each condition was measured and recorded to ensure pH did not drop too low around the day they would typically receive their last feed. The final pH was measured after identical samples had been recombined and were buffered. The pH of the experimental condition was within the pH-dependent binding range for the specific purification resin.

cells, but the pH remains within the acceptable range. At timepoint 7, the samples from both conditions return to comparable pHs, this is indicative of the point in the protocol where samples are buffered in preparation for purification. This buffering brings the pH of the samples to a range that is compatible with the pH-dependent binding resin used for protein purification. All samples across the conditions reached this pH range. This demonstrates that Starvation Day conditions are not detrimental to the pH maintenance of the samples. Even without the feed and subsequent buffering, the pH remains in the desired range and does not require any more additional buffering in preparation for purification than the control.

Experimental conditions produce higher protein titers across broad range of samples.

To confirm that the experimental condition resulted in acceptable protein yields for a diverse range of sample types, the resultant purified protein products were aliquoted for comparison with the control conditions. The protein concentrations obtained from the experimental conditions were plotted against

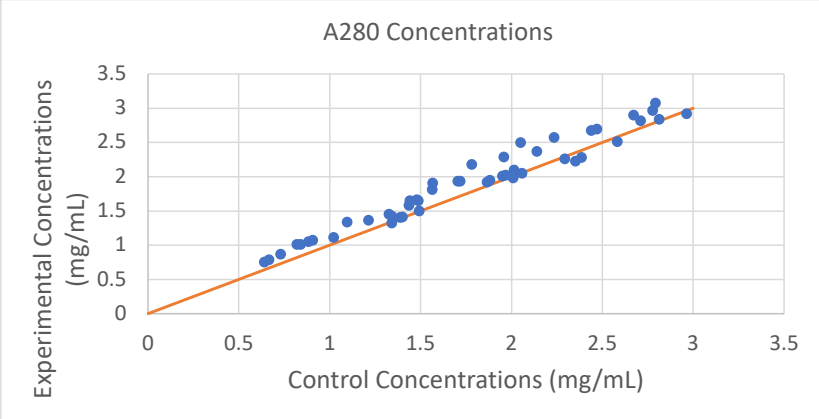

Figure 6. A280 concentration comparison for a broad range of samples demonstrates the merits of experimental conditions. The experimental concentration values of the diverse samples are plotted against their control counter parts. The blue dots above the y=x line represent samples that performed better in the experimental conditions. This demonstrates that, overall, the experimental condition produces higher protein concentrations across many IgG-producing cell lines.

the respective control condition concentrations (Figure 6). The experimental conditions outperform control conditions in nearly every sample. Those that did not outperform the control produced comparable yields. There was no correlation to sample type and superior performance in a specific condition. This indicates that the observed difference can likely be attributed to natural variation among sample runs and not to incompatibility with any one sample type and the experimental condition. A280 reads demonstrated that the protein yields were satisfactory in quantity when produced under experimental conditions, but further analysis was required to determine if the subsequent protein products met required quality standards.

Protein harvested from samples under the experimental condition met the quality parameters of proteins produced under control conditions.

Size exclusion chromatography (SEC) was performed with samples from both experimental and control conditions in order to determine sample quality. The SEC data demonstrates peaks, with height relative to abundance, at times when materials were passing through the column. A sharper peak is indicative of a more pure and higher quality sample, free of unwanted aggregates or degraded forms (Figure 7). A comparison of the largest difference between the two conditions was calculated. The calculated difference comes from differences in major peak, or the peak that included the majority of material detected in the column. The SEC data comparison revealed a maximum peak difference of only 3.7%. The similarities of the peak shapes across the conditions demonstrate that the quality of the protein product is not drastically altered by the Starvation Day conditions. The red experimental peak appears to be larger and slightly sharper than the control peak. This indicates that, at least in this condition, the

slight discrepancy observed in peak structure across the conditions can be attributed to a slightly higher quality sample from the experimental condition.

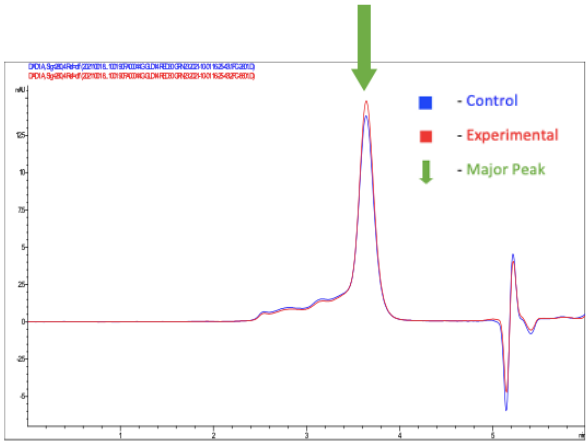

Figure 7. Size exclusion chromatography (SEC) peak comparison recorded a maximum peak difference of 3.7%. The peaks were obtained by running an SEC assay on the protein products generated under control and experimental conditions. The blue line represents the control while the red line represents the experimental condition. This peak overlay demonstrates the greatest peak discrepancy observed between any sample's control and experimental products. A peak difference of 3.7% was recorded. This peak comparison indicates that there is no difference in the general quality of the protein products obtained from the experimental condition.

Proteins produced under experimental conditions consistently had higher quality metrics than their control-produced counterparts.

The data derived from the SEC assay was used not only to conclude that experimentally produced proteins met the quality parameters set by control-produced proteins; it also demonstrated that they surpassed them (Table 1). The percent major peak reveals the uniformity of the sample. Theoretically, high quality samples should contain one species. These proteins should be nearly identical, therefore making their way through the column at the same time, producing one peak. Here, the proteins produced under experimental conditions had a higher percent major peak across all species tested (columns 2 and 3). This indicates that, even if only slightly, the experimental conditions yielded higher quality protein products. The retention time for a given protein or IgG are known and generally vary only slightly within the same sample species. Samples that have a delayed retention time are suspected of being slowed down by unwanted interactions with the column matrix and this is indicative of lower quality. SEC data depicts identical retention time averages for the conditions. This data further confirms that the experimental condition does nothing to harm the quality of the proteins produced, in fact, it demonstrates that Starvation Day results in slightly higher quality proteins.

Commented [SH1]: Move table to come after this subsection.

Table 1. Experimental conditions yield higher quality protein products in a broad range of samples.

SEC Comparison of Control and Experimentally Produced Protein Products				
Sample Type	**Average Major Peak % Control**	**Average Major Peak % Experimental**	**Average Retention Time Control (Mins)**	**Average Retention Time Experimental (Mins)**
Old IgG Clone	68.0	71.0	3.50	3.50
Modern IgG Clone with LC Type A	90.1	90.8	3.54	3.54
Modern IgG Clone with LC Type B	73.6	76.1	3.54	3.54
HCAb	94.1	94.5	3.65	3.65
sc-Fv	94.7	94.9	3.72	3.72
Old IgG Clones with Mixed LC Types	68.2	69.3	3.49	3.49

The experimentally produced and control-produced protein products, listed in column 1, underwent a quality control SEC assay. In all cases, the experimental products demonstrated higher major peak percentages, which corresponds to more uniform proteins. The experimental and control products maintained identical retention times, revealing that no major changes in quality, observed by the SEC assay, occurred between the two conditions.

Early harvest conditions yield protein concentrations comparable to Starvation Day and control conditions.

In order to determine if Starvation Day was necessary for producing the desired protein quantities and qualities, we tested the same samples under a third condition in which the protein was harvested a day early. This condition also received one less day of feed than the control conditions, but it was not given a day for processing; it was harvested early. Aliquots of the purified protein were taken, and the protein concentrations were calculated using the known sample volumes. The three average concentrations are comparable, ranging from 2.1-2.4 mg/mL (Figure 8). Starvation Day still produced the highest titers and the early harvest produced slightly lower than control. This data indicates that the protein concentrations obtained from these three conditions are not highly variable; however, this data does not allow us to make conclusions on the quality of the protein products.

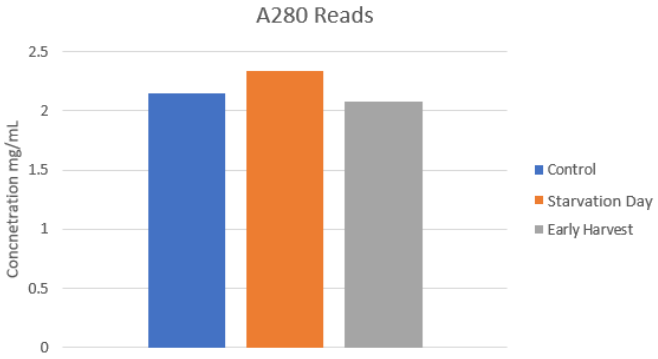

Figure 8. Early harvest compared to Control Conditions. A280 reads compare the concentrations of purified protein product generated under each condition. Early harvest yields lower concentrations, but only slightly and within the range of typical production variation. Starvation day conditions still produce the highest titers when compared to those of Early harvest and control conditions.

Proteins produced under early harvest conditions were of slightly lower quality than the Starvation Day-produced proteins.

In order to determine if Starvation Day is necessary to obtain high quality protein products, an SEC assay was performed on proteins produced under the early harvest conditions so they could be compared. The early harvest proteins consistently have a lower percent major peak than Starvation Day proteins (Figure 9). This indicates that Starvation Day may be a day of processing in which some protein products reach their final, polished form, complete with post-translational modifications. Starvation Day produces overall higher quality proteins and higher titers than both the control and the early harvest conditions.

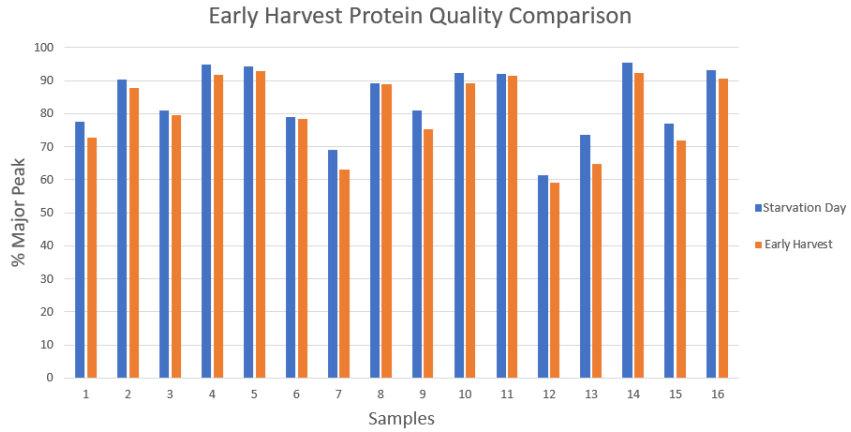

Figure 9. Proteins produced in the early harvest condition are of a lower quality than those produced from the Starvation Day conditions. The percent major peak data obtained from SEC was compared for Starvation Day and early harvest condition for each sample. In every case, the percent major peak was higher in Starvation Day conditions. While the values were comparable, as seen in the bar graph comparison, this data indicates that early harvest produced proteins are of slightly lower quality than those produced by Starvation Day conditions.

Discussion

The implementation of a day in the process that is absent of feed, Starvation Day, demonstrated more than adequate cell density, protein yield, and protein quality in the broad range of samples it was tested on. Data acquired from A600 absorbance reads confirmed that this experimental condition not only matched the cell densities observed in the control conditions but even surpassed them (Figures 2,4). The observation of higher cell densities offered reason to believe that the condition may also elicit higher protein yields. A280 data used to calculate the protein concentrations of the final products revealed that the experimental condition did, in fact, produce higher titers (Figures 3,6). Producing more protein does not inherently mean that more protein of high quality was obtained. SEC assays were used to confirm that experimentally produced proteins met the quality standards set by the control conditions (Figure 7). Measurements of percent major peak, retention time, and percent difference were obtained from SEC and then used to make an informed comparison of the protein qualities. The higher major peak percentages and uniform retention times measured via SEC revealed that the experimental condition produced proteins of slightly higher quality than the control (Table 1). The evidence that Starvation Day conditions consistently demonstrated higher cell densities, protein yields, and protein quality across a diverse range of samples and formats was enough to adopt it into the standard IgG production workflow.

While discussing the implementation of this process, the pH levels of the Starvation Day condition were determined to be important to review. With the lack of a feed, the samples were lacking the addition of an alkaline media that serves to counteract the acidic byproducts of yeast metabolism. Since much of the process is pH-dependent, both for sustaining conditions appropriate for *S. cerevisiae* cell survival and for resin binding, the removal of a buffer addition had the potential to be significant. Testing revealed that pH levels remained in the desired range throughout the process and at harvest reconfirmed the benefit of implementing Starvation Day in the standard protocol (Figure 5).

The implications of Starvation Day raised more questions regarding optimizing the process. Considering that the production of proteins in the yeast platform was not inhibited by instating a day of production without the addition of feed, it was questioned if that last day of production before harvest was necessary at all. Because the experimental samples did not receive any disparate intervention on the day before harvest, it was hypothesized that they could be harvested a day early, thus reducing the length of the multi-day process, along with the resources required. Identical samples representing a wide range of protein species were run side by side with the Starvation Day condition and the early harvest, experimental condition. The same measurements were taken in order to monitor cell densities at predetermined timepoints in the process and protein concentrations were calculated using data obtained from the final purified protein products. The results between the conditions were comparable. This study provided interesting information in regards to the necessity of Starvation Day. The protein yields and quality observed in the early harvest condition were slightly lower than in Starvation Day conditions. The sacrifice of product had to be weighed against the cost of continuing production an extra day. Both Starvation Day and early harvest significantly reduce the amount of resources that go into a production and, therefore, reduce the cost to complete a production. The institution where this study was carried out, Adimab, opted for continuing Starvation Day conditions. Among other changes that were made in the process in recent time, the move to early harvest could have decreased the quality of the product more than what they were willing to accept. Furthermore, the shortened production timeline was not conducive to the schedule and high throughput scaling of the institution's

workflow at this time. The adoption of early harvest into the yeast platform production is an option for other tech transfer partners, with considerations to be made regarding their own needs and requirements.

Considering the molecular mechanisms at play in the Starvation Day results raises additional questions to be answered. The absence of a feed on Starvation Day nearly allows time for *S. cerevisiae* cells in the sample wells to deplete their carbon sources before harvest. Fermentation to metabolize carbon sources is the primary energy source for *S. cerevisiae* during this stage of growth, and it results in the formation of ethanol and CO_2. These and other acidic byproducts lower the pH of the samples and ethanol serves as a source of energy in respiration, a secondary metabolic process. This shift in metabolism is known as the diauxic shift. The pre-diauxic phase is characterized by quick carbon source conversion with lower efficiency of energy generation.[9] *S. cerevisiae* metabolize the simple carbon sources through fermentation as a means of sequestering energy sources from the environment. Post-diauxic phase is characterized by decreased protein production other than proteins involved in glyoxylate metabolism, gluconeogenesis, alcohol import, and oxidative phosphorylation metabolism and diauxic growth.[9] The metabolism of compounds that cannot be fermented yields higher energy levels for the cell and results in diauxic growth, which may be represented by the higher OD measurements observed in Starvation Day conditions.

Given the resources to investigate this study further, running additional production conditions could aid in determining the mechanisms at play. Running a production with a diverse and representative range of samples under additional experimental conditions could help determine the exact number of feeds and or Starvation Days required to extract the highest amount of high-quality proteins. For example, an additional day without feed, ensuring fermentable carbon sources were fully depleted, that would result in more protein yield than just regular Starvation Day conditions would reinforce the hypothesis of the desired proteins' link to proteins responsible for alternative metabolism. Furthermore, the ability to rerun this study, as is, would result in a more complete data set. This study also introduces the possibility that the feed days, and therefore the entire protocol, could be reduced further. The cross-contamination assay, associated with the preliminary study, was found to be inadequate and therefore did not produce reliable results for determining the effect of Starvation Day conditions on cross-contamination. As this study began as a means to decrease the final volume of the wells to eliminate cross-contamination, a proper cross-contamination assay could reveal the true effect of Starvation Day on the issue. While this would not be the largest take away for the condition, this data could provide an additional benefit of the protocol.

Optimizing the culture conditions of *S. cerevisiae* to produce high-quality protein titers has obvious benefits in the industry. Once again, the less input required to obtain the desired output, the more approachable the platform. An increased efficiency in this step of the process, monoclonal antibody production for preliminary assays and characterization, leads to an increased efficiency in the entire process of drug development. The mechanisms that this study highlighted in *S. cerevisiae* could be utilized in the future for a more streamlined production timeline. Most realistically, the results of this study will allow for the institution and its partners to optimize their production protocols and accommodate more monoclonal antibody productions on a simplified timeline.

Citations

1 Liu J. K. (2014). The history of monoclonal antibody development - Progress, remaining challenges and future innovations. Annals of medicine and surgery (2012), 3(4), 113–116. https://www.ncbi.nlm.nih.gov/pmc/articles/PMC4284445/

2 Pugliese, D., Felice, C., Papa, A., Gasbarrini, A., Rapaccini, G., Guidi, L., & Armuzzi, A. (2017) Anti TNF-α therapy for ulcerative colitis: current status and prospects for the future, Expert Review of Clinical Immunology, 13:3, 223-233 https://www.tandfonline.com/doi/abs/10.1080/1744666X.2017.1243468

3 Salazar, G., Zhang, N., Fu, T. M., & An, Z. (2017). Antibody therapies for the prevention and treatment of viral infections. NPJ vaccines, 2, 19. https://doi.org/10.1038/s41541-017-0019-3 https://www.ncbi.nlm.nih.gov/pmc/articles/PMC5627241/

4 Bazan, J., Całkosiński, I., & Gamian, A. (2012). Phage display--a powerful technique for immunotherapy: 1. Introduction and potential of therapeutic applications. Human vaccines & immunotherapeutics, 8(12), 1817–1828. https://www.ncbi.nlm.nih.gov/pmc/articles/PMC3656071/

5 Gai, A., Wittrup, D. (2007) Yeast surface display for protein engineering and characterization. Current Opinion in Structural Biology: Volume 17, (4) 467-473 https://www.sciencedirect.com/science/article/pii/S0959440X07001194

6 Sun, T., Reid, F., Liu, Y., Cao, Y., Estep, P., Nauman, C., & Xu, Y. (2013) High throughput detection of antibody self-interaction by bio-layer interferometry, mAbs, 5:6, 838-841 https://www.tandfonline.com/doi/pdf/10.4161/mabs.26186?needAccess=true&

7 Emer, J. J., & Claire, W. (2009). Rituximab: a review of dermatological applications. The Journal of clinical and aesthetic dermatology, 2(5), 29–37. https://www.roche.com/products/product-details.htm?productId=b0eb216f-addf-4ed1-b01e-0b12fe0b1ef6

8 Estep, Patricia & Reid, Felicia & Nauman, Claire & Liu, Yuqi & Sun, Tingwan & Sun, Joanne & Xu, Yingda. (2013). High throughput solution-based measurement of antibody-antigen affinity and epitope binning. mAbs. 5. 270-8. https://www.researchgate.net/publication/236191786_High_throughput_solution-based_measurement_of_antibody-antigen_affinity_and_epitope_binning

9 Murphy, J. P., Stepanova, E., Everley, R. A., Paulo, J. A., & Gygi, S. P. (2015). Comprehensive Temporal Protein Dynamics during the Diauxic Shift in Saccharomyces cerevisiae. Molecular & cellular proteomics : MCP, 14(9), 2454–2465. https://www.ncbi.nlm.nih.gov/pmc/articles/PMC4563728/?tool=pmcentrez&report=abstract