

**Perceval: A Genetically Encoded Fluorescent Reporter of ATP:ADP
Ratio and its Potential for Application in Plant Cells**

Chancellor's Honors Thesis

Emma Cappella

Abstract

This project explored the potential of using Perceval, a genetically encoded fluorescent protein sensor, to monitor ATP:ADP ratios in plant cells. Because plant cells contain chlorophyll, which has strong natural fluorescence, a major challenge was determining whether Perceval's signal could still be detected in that background. To begin, the Perceval plasmid was successfully purified using both commercial kits and an in-lab protocol, and its identity was confirmed through restriction enzyme digestion and DNA gel electrophoresis. Several rounds of bacterial transformation were attempted, and ultimately, Perceval was successfully introduced into *E. coli* cells and induced to express protein. Preliminary protein expression was observed on an SDS-PAGE gel, though confirmation of successful protein expression remains a step for future work. While this study did not reach the point of fluorescence testing in the presence of chlorophyll, it established reliable protocols and identified key troubleshooting steps for plasmid prep, transformation, and expression. The methods and progress described here provide a solid foundation for future research aimed at applying Perceval as a real-time, in vivo reporter of energy dynamics in photosynthetic systems

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I. Introduction

One of the fundamental aspects of all living organisms is the production and maintenance of energy, including ATP, the primary energy source for cells (Metallo & Vander Heiden, 2013). Consequently, accurate measurements of ATP levels within cells are essential for understanding cellular metabolism (Imamura et al., 2009). Real time measurements of the ATP:ADP ratio, especially under dynamic conditions like stress, growth, or photosynthesis formulate a strategy in which energy homeostasis can be depicted in vivo (Tantama et al., 2013). Genetically encoded fluorescent biosensors offer a non-invasive method for monitoring such metabolic indicators in living cells. One such sensor, Perceval, has been shown to report changes in the ATP:ADP ratio through ratiometric fluorescence in mammalian cells (Berg et al., 2009). Perceval's use of competitive binding to report on the ATP:ADP ratio makes it a more accurate metabolic reporter as opposed to other proteins, like luciferase. As such, it is necessary to consider if it is capable of effectively reporting the ATP:ADP ratio in other cell types, such as chloroplast-containing cells. Being able to monitor the ratio in chloroplast-containing cells allows for direct insight into photosynthetic efficiency, with the potential to better understand how light energy is harnessed in organisms like plants (Farquhar & Sharkey, 1982). However, plants containing chloroplasts also contain chlorophyll, a naturally fluorescent molecule that may interfere with sensor performance (Heber & Kraus, 1971). Such possibilities resulted in this study, which aims to investigate whether Perceval can function as a reliable ATP:ADP sensor in plant cells, particularly in the presence of chlorophyll. To do so, Perceval was cloned into *E.coli* for protein expression with the intent of attempting to express it into plant cells for in vivo characterization in future experiments to answer the question: can Perceval be used to detect and report on the ATP:ADP ratio in the presence of chlorophyll?

II. Background

IIa. Genetically Encoded Biosensors compared to other ATP Assessment Methods

Understanding how cells manage their energy supply is a central goal of cell biology and metabolic research. Across all types of life, energy levels are tightly regulated and present immense variations depending on cellular processes and environmental conditions (Metallo & Vander Heiden, 2013). Traditional methods to assess ATP, such as luciferase-based bioluminescence assays or fluorescent dye-based probes, have limitations that do not apply to genetically encoded fluorescent biosensors. Although the approaches are accurate, they may fail to capture real-time fluctuations in living cells.

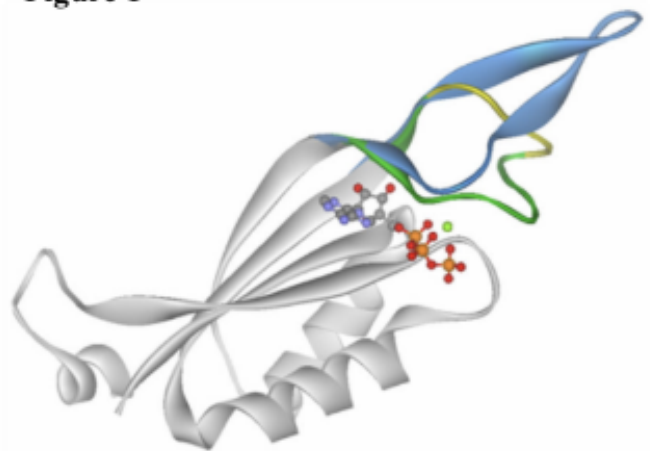
In the case of Luciferase-based bioluminescence assays, luciferase catalyzes a reaction with ATP and luciferin, producing light which can then be quantified to provide a very sensitive depiction of ATP levels. However, it is limited in the fact that the ATP hydrolysis reaction required to produce light can impact the energy balance (Ainscow EK et al., 2002). Additionally, Luciferase-based bioluminescence assays as well as methods such as mass spectrometry require cell lysis and do not provide real time data (Yamada S et al., 2006). Another method is the use of fluorescent dye-based probes such as magnesium green, which indirectly measures ATP by binding to Magnesium ions. Although this method can be used in live cells, it's indirect nature makes it less specific than genetically encoded sensors (Rutter GA et al., 1993). Furthermore, other methods such as NMR spectroscopy can be used to detect phosphate groups in ATP and other nucleotides but has low ATP sensitivity, poor spatial resolution, and is very expensive (Koretsky AP et al., 1990).

Genetically encoded fluorescent biosensors address these limitations. These sensors are proteins that can be expressed inside living cells and respond to specific molecular targets with measurable changes in fluorescence. Their genetically encoded nature makes them ideal for targeting subcellular compartments and good temporal and spatial detection of changes in energy. Perceval is a genetically encoded fluorescent biosensor developed by Berg et al. in 2009, and is specifically designed to report on the ratio of ATP to ADP in real time in living cells. It has a much stronger fluorescence signal than those produced by the use of other methods like Luciferase, and its ability to report via competitive binding prevents impact on energy balance. As a result, it is capable of creating a very accurate and clear depiction of the variation of cellular metabolism in animal cells, and potentially in other cell types as well.

IIb. Mechanism and Validation of Perceval

Perceval is a fusion protein composed of a yellow circularly permuted fluorescent protein, circularly permuted monomeric Venus, inserted into the T loop of the bacterial regulatory protein GlnK1. **Figure 1** depicts a model of GlnK1 and its T loop, which is in blue. GlnK1 is a member of the PII family of proteins, which regulate metabolism and bind adenine nucleotides with high specificity. In fact, the mechanism of PII proteins exists to maintain homeostasis (Yildiz et al., 2007). The binding of either ATP or ADP to GlnK1 triggers a large conformational change in the protein. The T loop of GlnK1 is typically a

Figure 1



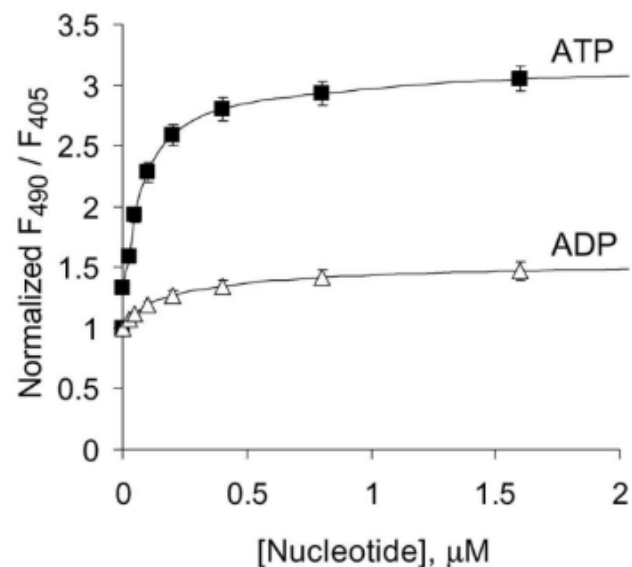
Berg et al., 2009

loose and disordered structure, but when Mg-ATP binds GlnK1, it becomes more compact and ordered. Due to this localized structural change occurring upon ATP binding, the impact on the domain containing the fluorescent protein is impacted, altering its fluorescent properties. Thus, GlnK1 was selected as an optimal backbone for the fluorescent ATP sensor and made the ATP:ADP ratio observable.

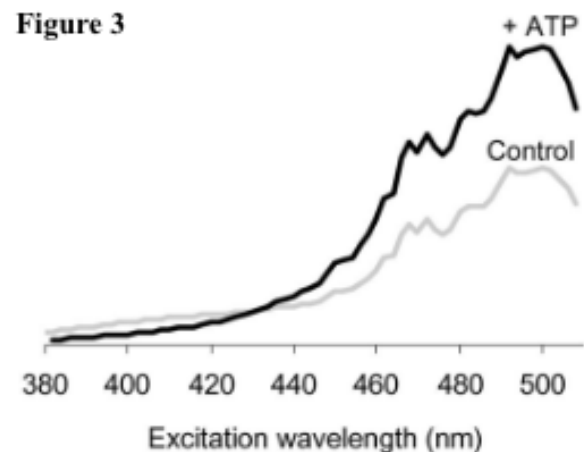
Unlike sensors that respond only to ATP concentration, Perceval is designed to respond to the ratio of ATP to ADP, which is a more accurate reflection of cellular energy status (Tantama et al., 2013). The competition between ATP and ADP binding at the T loop is what allows the sensor to act as a reporter of the ratio, and allows for the actual free energy of ATP hydrolysis available for cellular reactions to be calculated. The protein shows a five times higher affinity for ATP than ADP, with the affinity for ATP binding being 0.04 μM while the affinity for ADP binding is only 0.2 μM (Berg et al., 2009). The fluorescence of Perceval increases from 0 to 0.5 μM ATP or ADP then levels out, making 0 to 1 μM of ATP a good range to observe the

fluorescence. **Figure 2** shows the ratio of fluorescence intensities when exciting at 490 nm divided by 405 nm with application of ATP and ADP. The fluorescence emission of Perceval can be monitored at two excitation wavelengths (~ 405 nm and ~ 490 nm), and the ratio of these emissions is used as a ratiometric signal. Saturation with ATP enhances the peak at 490 nanometers, and diminishes

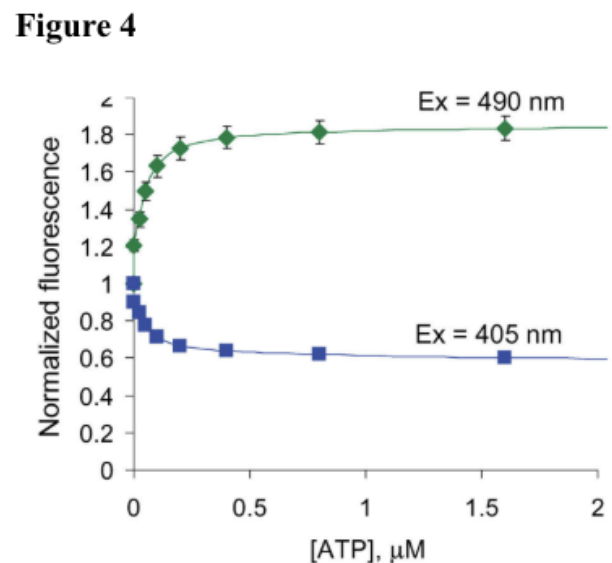
Figure 2



the peak at 405 nanometers which can be seen in **Figure 3**, which compares the intensity of excitation wavelengths in a control without ATP (grey line), and when in the presence of ATP (black line). Furthermore, **Figure 4** demonstrates the normalized fluorescence at each wavelength with a clear depiction of the decreases in fluorescence at the 405 peak and increased fluorescence at the 490 peak when the ATP is increased from 0 to 0.5 μM .



Berg et al., 2009



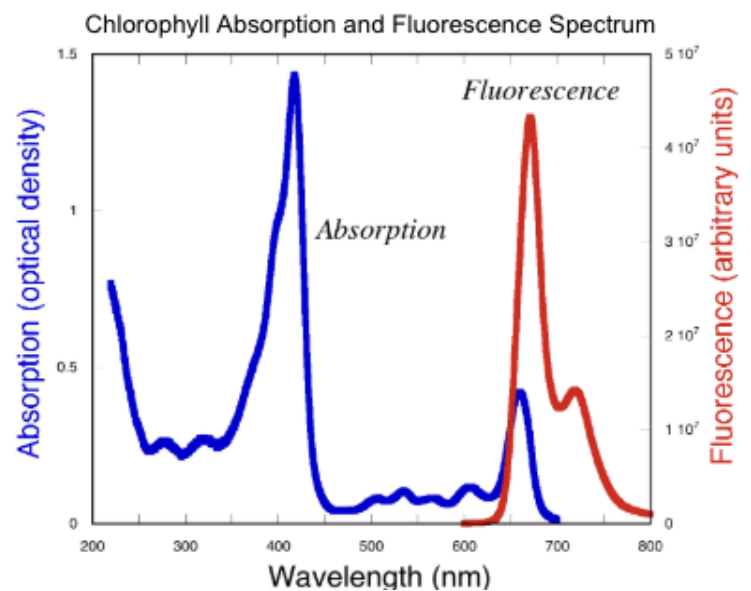
Berg et al., 2009

In the study by Berg et al., Perceval was expressed in *E. coli*, yeast, and mammalian cells to validate its functionality. In the bacteria, cells exhibited rapid and reversible changes in Perceval fluorescence in response to changes in metabolic state, such as glucose availability. In mammalian cells, metabolic perturbations like the inhibition of glycolysis or oxidative phosphorylation also resulted in clear, measurable changes in ATP:ADP ratios indicated by the Perceval sensor. The sensor demonstrated high sensitivity, reversibility, and specificity for the ATP:ADP ratio. Its ratiometric nature allowed for corrections in signal fluctuations due to photobleaching or uneven expression. The success of Perceval in different model organisms indicates its potential for broader application in other systems, such as plant cells.

IIc. Challenges in Using Perceval in Plant Cells

While Perceval has shown effectiveness in bacterial and animal systems, expressing it in plant cells introduces unique challenges. One such issue is the autofluorescence generated by chlorophyll. Chlorophyll, found in the chloroplasts of green plant cells, autofluoresces between 600-750 nanometers at a very high intensity. **Figure 5** depicts the chlorophyll absorbance and fluorescence spectrum. Thus, it strongly absorbs and emits light in the same spectral regions as many fluorescent proteins, including the yellow fluorescent protein used to make Perceval. Perceval fluoresces at 405 and 490 nanometers at a much lower intensity than chlorophyll. This overlap could interfere with signal detection and data interpretation. Therefore, testing the sensor's performance in a chlorophyll-rich environment is crucial to determine its utility in plant biology.

Figure 5



Another potential challenge is that Perceval is sensitive to changes in pH, which can alter its fluorescence (Berg et al. 2009). Since the chloroplast is a metabolically active organelle, there is a potential for the pH in the chloroplast impacting the fluorescence and altering the outcome. Berg et al. mediated this issue in animal cells by using the pH dye indicator SNARF-5F, which fluoresces red and allowed the researchers to adjust the curve based on that. However, this is not a possible solution for application in plants because chlorophyll, similar to the pH dye indicator,

also fluoresces red. There is a possibility that taking measurements in the stroma of cells mediates the effect of the pH, ensuring that Perceval is unaffected.

IId. Potential Significance of This Research

If successful, the adaptation of Perceval for use in plant systems would broaden the tools available to plant biologists. Unlike traditional biochemical assays which require cell lysis and do not provide real time cellular metabolism, Perceval offers a non-invasive, ratiometric, real-time presentation of the ATP:ADP ratio in living cells (Berg et al., 2009). In the context of plant physiology, this would allow for dynamic monitoring of cellular energy status during photosynthesis, respiration, and stress responses, all of which are processes that are variable and regulated in time and space. The ATP:ADP ratio is a fundamental indicator of a cell's energetic state and directly influences numerous metabolic pathways, including those governing carbon fixation, nutrient assimilation, and signaling (Tantama et al., 2013; Metallo & Vander Heiden, 2013). Having the ability to monitor this ratio within chloroplasts would yield unique insights into how plants balance ATP production via photophosphorylation with consumption in biosynthetic pathways. Such measurements are very valuable under fluctuating environmental conditions like changes in light intensity, drought, or nutrient limitation, where energy management is critical to survival.

Moreover, the successful implementation of Perceval in chlorophyll-rich environments would address a longstanding technical barrier. Chlorophyll's autofluorescence between 600-750 nanometers interferes with many standard fluorescent standards, but Perceval's excitation at 405 nm and 490 nm lies outside this emission range. This suggests it may be suited for use in chloroplast-containing cells. Validating its ability to function and detectability in the presence of

chlorophyll would pave the way for in vivo imaging intact tissues, rather than just isolated cells. As a result, it could potentially provide subcellular resolution of energy dynamics in photosynthetic tissues.

On a larger scale, Perceval could be used as a tool in synthetic biology and crop improvement strategies. Genetically modified plants that have altered photosynthetic pathways could be more accurately evaluated using Perceval through directly visualizing energy status in the target plant tissues. In doing so, researchers could identify the most metabolically favorable modifications to make in plants, optimizing crops as a whole. Macroscopically, this could have implications for food production and accessibility, improved resilience to climate change, more efficient use of water and nutrients, and even enhanced biofuel development.

In summary, studying Perceval in plants has the potential to validate a useful method for energy sensing in plants, expanding the field of live-cell metabolic imaging in photosynthetic organisms. It addresses an existing gap in current methods by providing real time, non invasive ATP sensing compatible with chlorophyll, as well as a biological necessity: to understand how plant cells manage energy at the cellular and subcellular levels in environments that are always changing.

III. Methods

IIIa. Experimental Plan

Due to time constraints, the initial goal of this project was to complete a mock experiment. Essentially, this entailed acquiring the Perceval plasmid, doing a plasmid prep to make sufficient plasmid amounts, then clarifying the plasmid was produced via restriction

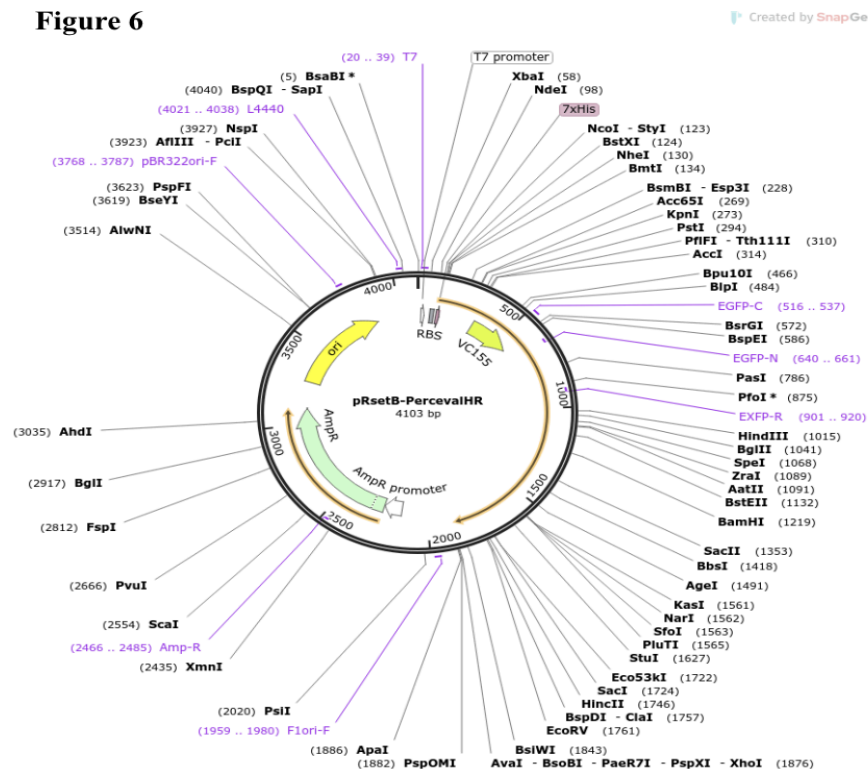
enzyme digestion and running a DNA gel. The next step was to transform the plasmid into a competent line of e.coli cells, then grow them and induce to produce the protein. Following this, I aimed to run the protein on an SDS page to confirm Perceval production, and then move forward to purify the protein using immobilized metal-affinity chromatography. Assuming the success of these experiments, the penultimate part of the mock experiment was to perform fluorescence measurements and dual excitation techniques, adding ATP and other nucleotides to analyze the response of Perceval. Finally, the ideal end of the mock experiment was to add chlorophyll and monitor the protein for fluorescence and see if the Perceval fluorescence was visible.

Also implied within the workload of the mock experiment is taking the time to learn and optimize several microbiology techniques, including media and reagent preparation, plasmid purification, and bacterial transformation with several different competent cell line types. Additionally, optimization of restriction enzyme selection and utilization, as well as the DNA gel procedure were time consuming and difficult protocols to perfect due to limited knowledge within the Bruce lab. However, through the use of meticulous repetition and problem solving, each experiment was cultivated to provide the most accurate results for future researchers to use in the continuation of this experiment.

Due to several challenges with the transformation process and time limitations when it came to performing trial and error, the experiment only progressed to the point of running an SDS page to confirm Perceval production. The roadblocks which resulted in the delay in project success as well as future directions for others who pursue this research question will be further discussed throughout the methods, discussion, and limitation section.

IIIb. Obtaining Perceval Plasmid

Perceval Plasmid pRsetB-PercevalHR was ordered from addgene, where it is listed as plasmid #49081. It was sent in bacteria as an agar stab, and contained an N-terminal His Tag as well as a gene for ampicillin resistance. The growth strain was DH5a cells. The plasmid is 4,103 base pairs long. **Figure 6** shows the sequence map for pRsetB-PercevalHR provided by addgene.



Addgene plasmid #49081

IIIc. Media Preparation

All culture media and buffers used in the molecular procedures were prepared under sterile conditions. Luria-Bertani Agar Miller Broth was used to make agar plates, with a ratio of 40 grams for one liter of media. Plates were poured at several different times throughout the

semester to prevent contamination and ensure there were no confounding variables when experiments had to be repeated. As such, typically only 100 mL of agar was prepared each time. After adding correct amounts of LB Agar Miller Broth, the media was autoclaved. Once the autoclave cycle ended, the agar media was allowed to cool and then ampicillin was added to achieve the desired concentration of 100 $\mu\text{g/mL}$. It was then poured to make plates under a sterile fume hood next to a flame. After the plates solidified, they were labeled, wrapped in tinfoil, and stored upside down in the four degree fridge for later use.

Typically, the agar media was autoclaved alongside 5 mL, 50 mL, and 1 L liquid media to allow for sequential growth of e.coli cells after their initial transformation. To make the liquid media, the desired ratio of 25 grams of LB Miller suspended in 1 L of water was followed. The media was then sufficiently covered and sealed. After autoclaving, the liquid media was labeled and stored at room temperature, and was continuously monitored throughout the week to ensure no unwanted growth had occurred before culture was grown. The ampicillin was not added until right before the culture was grown, but once again met the desired concentration of 100 $\mu\text{g/mL}$.

IIIId. Plasmid Preparation

Following obtaining the Perceval plasmid as an agar stab, it was streaked on agar plates and allowed to grow overnight. Next, colonies were selected and grown in liquid media, then turned into glycerol stocks and stored in -80 degrees for future use and plasmid purification. The first attempt at perceval plasmid purification was performed using the QIA Miniprep kit. The glycerol stocks were thawed and grown in 5 mL liquid media overnight. Then, the QIA Miniprep kit procedure was followed and the reagents included in the kit were used. The plasmid concentration was measured and then was stored in -80 degrees for later use in transformation.

A second plasmid purification was performed using buffers and materials made in the lab rather than a commercially available kit. Following the addgene protocol for “Kit-free Alkaline Lysis Plasmid Miniprep,” a resuspension buffer, a denaturing solution, and a renaturing solution were prepared. Next, another glycerol stock of the Perceval was streaked on agar media plates with ampicillin and left to grow overnight in the 37 degree Celsius incubator. Colonies were selected and added to 2 mL liquid media with ampicillin and cultures and incubated at 37 degrees for 12-18 hours in a shaking incubator. The addgene protocol was followed, the concentration of the plasmid was measured, and then stored in -80 degrees celsius for future use in transformation.

IIIe. Restriction Enzyme Digestion

To confirm the presence of perceval plasmid, a restriction enzyme digestion was performed, and expected bands were identified. Due to the circular nature of the plasmid, a dual-digestion was necessary and thus two restriction enzymes were selected from those available in the Bruce lab. The plasmid sequence was cross referenced with HF (high fidelity) restriction enzymes listed in the Bruce lab inventory to identify those which were compatible with cut sites on the plasmid. Using HF enzymes was necessary to ensure both enzymes used in the double digestion were equally active in the buffer. According to New England Biolabs, HF enzymes are 100% active in Cutsmart buffers (New England Biolabs, n.d.). Several combinations were identified alongside their proposed cuts and resulting fragment lengths which would be visible on an ethidium bromide DNA gel. It is necessary to note that all restriction enzymes readily available only made one cut on the plasmid at one location. It is necessary to note that the majority of the enzymes in the lab were aged, with the newest having expired in 2018. That said, the enzymes were stored in -20 degrees celsius for optimal preservation and

future use. **Table 1** lists the restriction enzymes that were selected for use, where they cut in the DNA, and their expiration dates.

Table 1		
Restriction Enzyme	Cut Site	Expiration Date
BstEII	1132	09/2015
PstI	294	09/2013
SacI	1724	11/2015
HindIII	1015	01/2015

It was also necessary to include a single restriction enzyme digestion condition in order to visualize a fragment at 4,103 base pairs, representative of the actual plasmid size. One cut would simply make the plasmid linear rather than circular, allowing for a band corresponding to the linear size of the plasmid to be seen on an ethidium bromide gel. Furthermore, another plasmid coding for an eGFP (pET29b+) also underwent restriction enzyme digestion to provide a control. **Table 2** lists the different conditions that were performed for the first digestion, including the restriction enzyme combinations (whether single or double), and the expected size of the fragments.

Table 2		
Plasmid Type	Restriction Enzymes	Expected Fragment Sizes
Perceval	None	Unknown
Perceval	HindIII	4,103 bp
Perceval	HindIII, SacI	709 bp and 3,394 bp
pET29b+	None	Unknown
pET29b+	HindIII	5,370 bp

The initial restriction enzyme digestion was limited in the amount of plasmid which was available for use, but after a second plasmid purification, the restriction enzyme digestion was repeated with different combinations of restriction enzymes and different concentrations of plasmid. The concentrations of plasmid were variable in order to see if different concentrations would be more visible on a DNA gel. The second digestion did not include the eGFP as a control, rather just included the perceval plasmid without any restriction enzyme added. Note that the Plasmid alone is listed with a fragment length of 4103 base pairs, but may not show up on a gel at that location due to its circular form. **Table 3** lists the different conditions that were performed for the second digestion, including the restriction enzyme combinations (whether single or double), the plasmid concentration, and the expected size of the fragments.

Table 3			
Plasmid Type	Restriction Enzymes	Plasmid Concentration	Expected Fragment Sizes
Perceval	None	1X	Unknown
Perceval	HindIII	1X	4,103 bp
Perceval	HindIII	2X	4,103 bp
Perceval	HindIII + SacI	1X	709 bp and 3,394 bp
Perceval	HindIII + SacI	2X	709 bp and 3,394 bp
Perceval	BstEII + PstI	1X	838 bp and 3,265 bp
Perceval	BstEII + PstI	2X	838 bp and 3,265 bp

Using the selected restriction enzymes, the protocol for both the first and second digestion attempts was the same. The plasmid DNA was combined with the restriction enzyme(s) and cutsmart buffer, incubated at 37 degrees celsius for one hour, then the digestion was stopped with heat inactivation by putting the samples in a 65 degree celsius water bath for 15 minutes. Afterwards, they were run in a DNA agarose gel and analyzed with UV light.

III f. DNA Gel Electrophoresis

For analyzing the restriction enzyme digest samples, a 1% agarose gel was cast with 8 wells for samples. The TAE buffer was also prepared to be used as a running buffer. Using the initial plasmid concentration following the plasmid purification, the volume of the digested sample containing 100 ng of DNA was calculated and then a 6X loading buffer was added. Then, the samples as well as the DNA ladder were added to the wells and run at 80V for one hour. After one hour, the gel was soaked in a running buffer and EtBr, then the DNA was visualized under a UV transilluminator and imaged. Then, the results were analyzed and based on the fragment position on the gel, it was determined that perceval plasmid was present and moving on with the transformation was appropriate.

III g. Transformation and Growing in Liquid Media

Throughout the course of this experiment, 8 different attempts to transform perceval plasmid into 8 different competent cell lines were performed. 7 of the competent cell lines were BL21 cells, and 1 was DH5a. 6 of the BL21 cell lines and the DH5a cell line were obtained from the -80 degree freezer, and their origin date was unknown. The final BL21 cell line that was used was obtained from genessee scientific and was stored in -80 degrees until use 3 days later. For each cell line, the same transformation protocol was used, except for one small difference with the BL21 cells obtained from genessee scientific, where instead of pelleting cells and resuspending before plating, the cells and SOC media were not pelleted and instead were plated right out of the media.

The general procedure followed in the transformation began with combining 50 microliters of competent cells with 1 microliter of plasmid (or however much corresponded to 10

ng of plasmid) and then incubated on ice for 30 minutes. Then, samples were transferred to a 42 degree water bath for 45 seconds and placed back on ice for 2 minutes. Afterwards, 1 mL of SOC media was added and tubes were incubated at 37 degrees and shaken for one hour. For 6 of the BL21 cell lines, the next step was centrifuging to get a pellet, then resuspending the pellet in 25 microliters of SOC media. In the case of the Genessee Scientific BL21 cells, there was no pelleting. Then, underneath a sterile fume hood, 25 microliters are added to agar plates containing ampicillin and a spreader is used to ensure good colony distance. Following this, the plates are incubated at 37 degrees celsius overnight (12-16 hours) before colony selection.

Assuming colony growth occurs, colonies are then selected from the plates and transferred to 5 mL media containing ampicillin, then incubated at 37 degrees celsius on the shaker and allowed to grow for 12-16 hours. 5 mL cultures are then either stored as glycerol stocks in -80 degrees or poured into 50 mL media containing ampicillin, following the same growth protocol as the 5 mL culture. The next step is to transfer into a 1 L culture with ampicillin. The E.coli is allowed to grow in the 1 L culture for 2 hours shaking in 37 degree incubator, and then for the third hour the optical density is monitored. Once the optical density reaches 0.4-0.6, the cells are induced using IPTG and allowed to grow for the next 3-4 hours shaking in 37 degrees. In the case of DH5a cells, induction with IPTG is not possible due to the lack of a promoter. However, in BL21 cells, 1 mL samples are removed beginning before induction, and then every 30 minutes after induction. Those samples are pelleted and stored for running on an SDS gel. After the 4 hours of growth ends, the 1L culture is pelleted, weighed, and stored for experiments with the protein.

IIIh. Protein Identification

Following the induction and growth of E.coli cells which produce Perceval protein, it was necessary to confirm the protein was being produced. So, the samples taken every 30 minutes after the induction were prepared by lysing the cells and spinning them down to obtain the supernatant, which contains the Perceval protein. The protein in the supernatant was then loaded onto an SDS page gel. The gel was then stained with Coomassie blue, and imaged.

IV. Results

IVa. Plasmid Prep and Purification Results

The first plasmid preparation which was done with the QIA miniprep, resulted in an average DNA concentration of 24.4 ng/ μ L, notably lower than the ideal 50 ng/ μ L. **Table 4** details the concentration of each of the 6 plasmid samples, as well as their A260/A280 ratios. The average A260/A280 ratio is 1.858. An A260/A280 ratio of 1.8 is generally considered to be pure for dsDNA (Thermo Fisher Scientific, 2012).

Table 4		
Sample #	Concentration (ng/ μ L)	A260/A280
1	25.3	1.81
2	24.8	1.83
3	27.8	1.83
4	21.9	1.90
5	25.0	1.85
6	21.7	1.93

The second plasmid purification which was done using buffers made in the lab rather than the commercially provided ones in the QIA miniprep kit resulted in an average

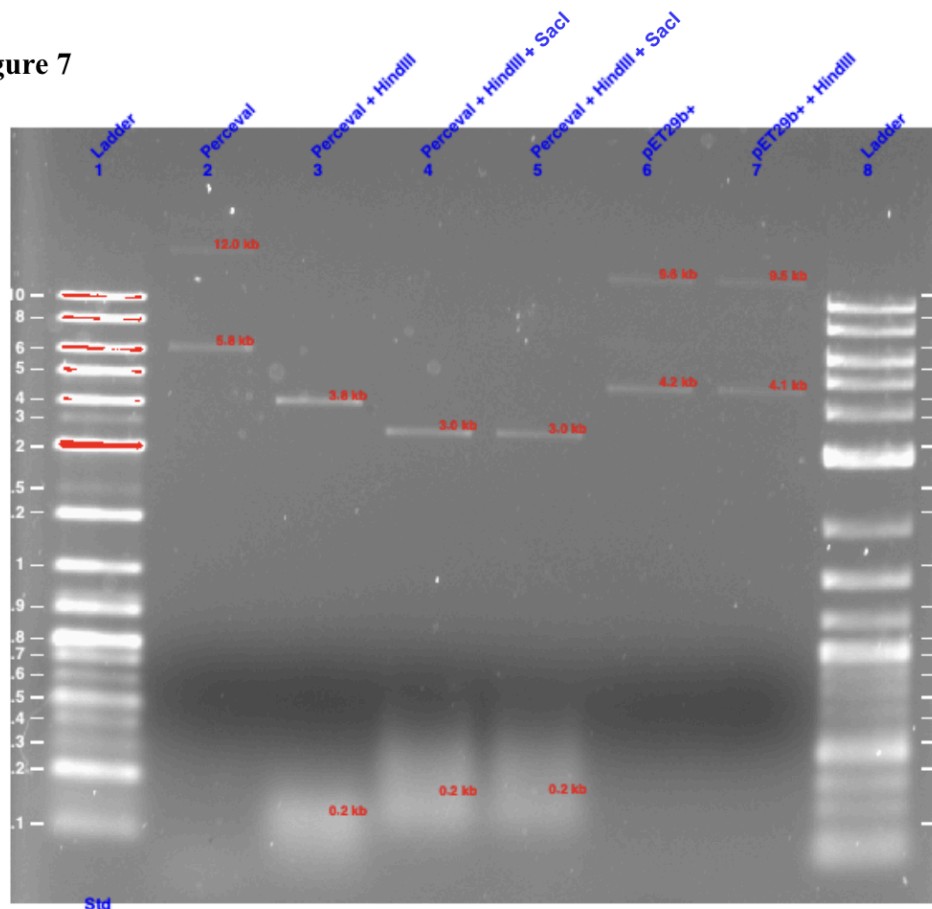
concentration of 1,183 ng/μL, notably higher than the desired 50 ng/μL. The average A260/A280 ratio was 2.13. **Table 5** details the concentration of each of the 5 plasmid samples, as well as their A260/A280 ratios.

Table 5		
Sample #	Concentration (ng/μL)	A260/A280
1	1317.6	2.10
2	992.1	2.15
3	1077.2	2.14
4	1166.8	2.12
5	1359.4	2.12

IVb. Restriction Enzyme Digestion & DNA Gel

The gel which resulted from the first restriction enzyme digestion (and use of the plasmid purification which yielded an average concentration of only 24.4 ng/μL) can be seen in **Figure 7**.

Figure 7



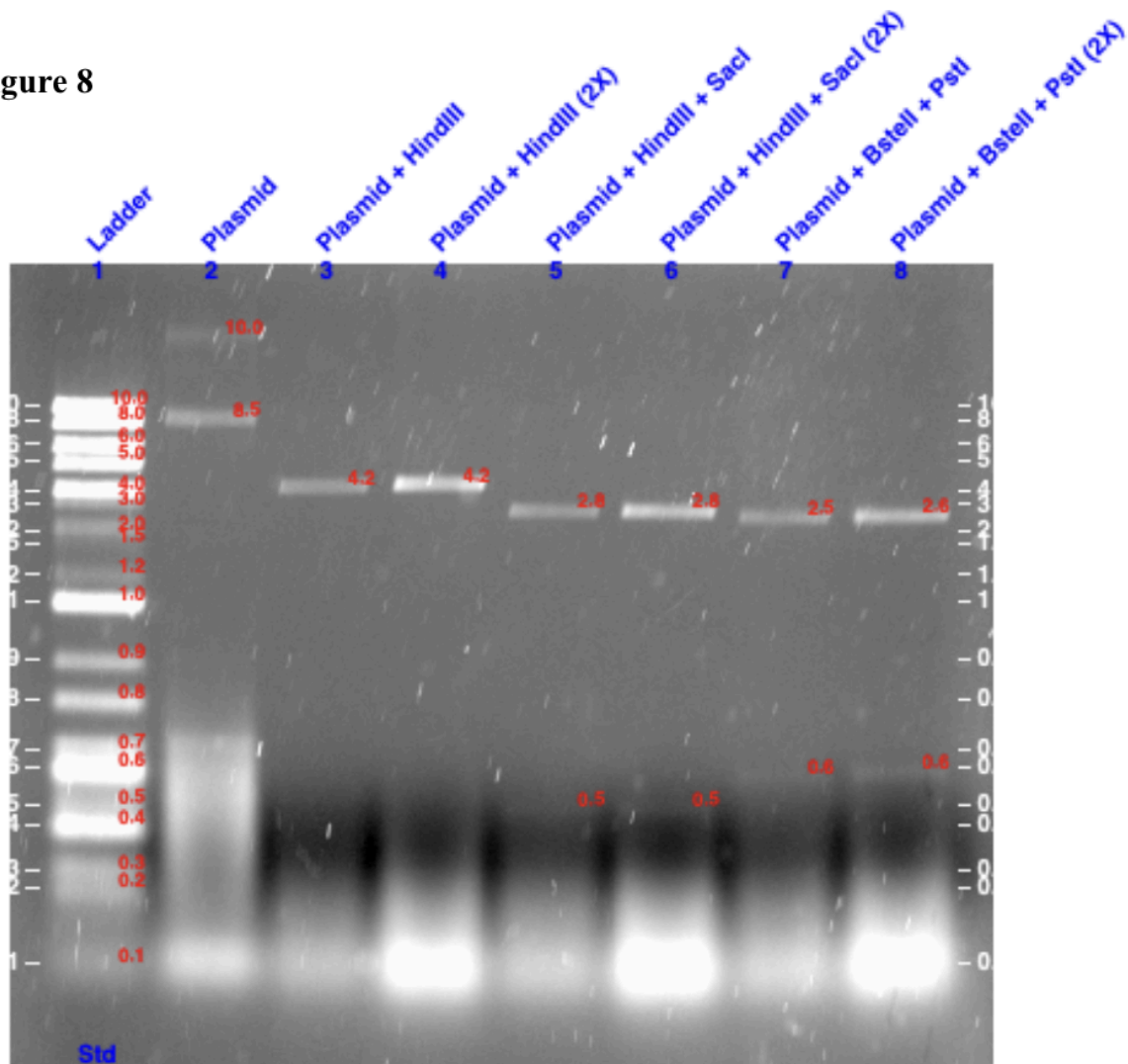
The DNA gel resulting from performing a restriction enzyme digest on the first plasmid purification resulted in bands at 12.0 kb and 5.8 kb for the control perceval plasmid with no restriction enzymes. For the perceval plasmid with one single restriction enzyme, there was a band at 3.8 kb and a smear at 0.2 kb. For the perceval plasmid with two restriction enzymes, notably HindIII and SacI, there were bands at 3.0 kb and smears at around 0.2 kb. For the eGFP, pET29b+, the bands for both the condition with and without a restriction enzyme were the same, located at 9.5 kb and 4.2 kb. **Table 6** lists the actual bands of each condition as well as the expected bands.

Table 6		
Condition	Actual Bands (bp)	Expected Bands (bp)
Perceval Plasmid	12,000 and 5,800	Unknown
Perceval Plasmid + HindIII	3,800 and 200	4,103
Perceval Plasmid + HindIII + SacI	3,000 and 200	709 and 3,394
pET29b+	9,500 and 4,200	Unknown
pET29b+ + HindIII	9,500 and 4,200	5,370

Based on the results of this gel and the lack of correspondence with expected bands, the second plasmid purification was performed. After a high concentration was observed (1,183 nanograms per microliter), a second restriction enzyme digestion was performed with hopes of seeing more concise fragments in locations that correspond with the perceval plasmid and its length. **Figure 8** is the DNA gel resulting from the second plasmid prep. In this gel, the smears are not labeled as they were in the previous one. Notably, the plasmid by itself had bands at 10 kb and 8.5 kb, along with a smear ranging from 0.7 to 0.1 kb. The plasmid with HindIII had a band at 4.2 kb and a smear around 0.1 kb. The plasmid with HindIII but double the DNA concentration is brighter and maintains bands at 4.2 kb and a smear at 0.1 kb. The condition with

Perceval, HindIII, and SacI (both 1X and 2X) has bands at 2.8 kb and 0.5 kb, although the one at 0.5 kb is faint. The condition containing Perceval, BstEII, and PstI (both 1X and 2X) has bands at 2.5 kb and 0.6 kb.

Figure 8



As mentioned, the difference in the visibility of 1X DNA vs 2X DNA is notable, with the 2X DNA samples being much brighter on the gel especially on the bands which are larger in size. The smaller bands, such as those detected in the double digestions around 0.5 and 0.6 kb,

are less noticeable and the difference between the 1X vs 2X DNA is less obvious. **Table 7** lists the actual bands of each condition as well as the expected bands.

Table 7		
Condition	Actual Bands (bp)	Expected Bands (bp)
Perceval Plasmid	10,000 and 8,500	Unknown
Perceval Plasmid + HindIII (1X and 2X)	4,200	4,103
Perceval Plasmid + HindIII + SacI (1X and 2X)	2,800 and 500	3,394 and 709
Perceval Plasmid + BstEII + PstI (1X and 2X)	2,500 and 600	3,265 and 838

Due to the presence of 2 bands in the double digestions and their relative proximity to the expected bands, another restriction enzyme digest was not necessary, and the plasmid generated in the second plasmid purification was used in the transformations that were performed.

IVc. Transformation

Several transformations were performed, and of the 8 that were done, only two resulted in colonies growing. It is necessary to note that 4 of the transformations that were done were performed using the plasmid from the QIA prep and B121 cells, none of which were successful or yielded colonies. The 5th and 6th transformations were with the plasmid produced in the second plasmid purification and used 2 different B121 competent cell lines which were stored in the -80 freezer, thawed, and then used. Much like the 4 with the plasmid from the first plasmid prep, They resulted in minimal cell pellets which indicated poor growth even in the SOC media. Regardless, they were plated and did not yield colonies. The 7th transformation was performed with DH5a competent cells, and did yield colonies which were eventually turned into glycerol

stocks for future use. The 8th and final transformation was by far the most successful and resulted in abundant colonies and used new BL21 competent cells.

The colonies from this transformation were grown, induced, and samples were taken every 30 minutes (a step the other transformations never reached). These samples were what was used to see if the protein was being produced on the SDS gel. It is necessary to note that these cells grew slowly and were induced after 3.5 hours of growth in 1L culture despite only having an optical density of 0.3 rather than the ideal 0.4. After 4 hours of being induced, the pellet was spun down and weighed to be 1.2 grams, notably smaller than expected. The pellet was then stored for future use.

IVd. Protein Gel

The samples taken at each 30 minute interval were run on an SDS page gel, and results indicated that although there was protein present, it was unclear whether it was the target protein or not. The most fluorescent bands on the gel were evident at about 32 kD, 40 kD, and 70 kD.

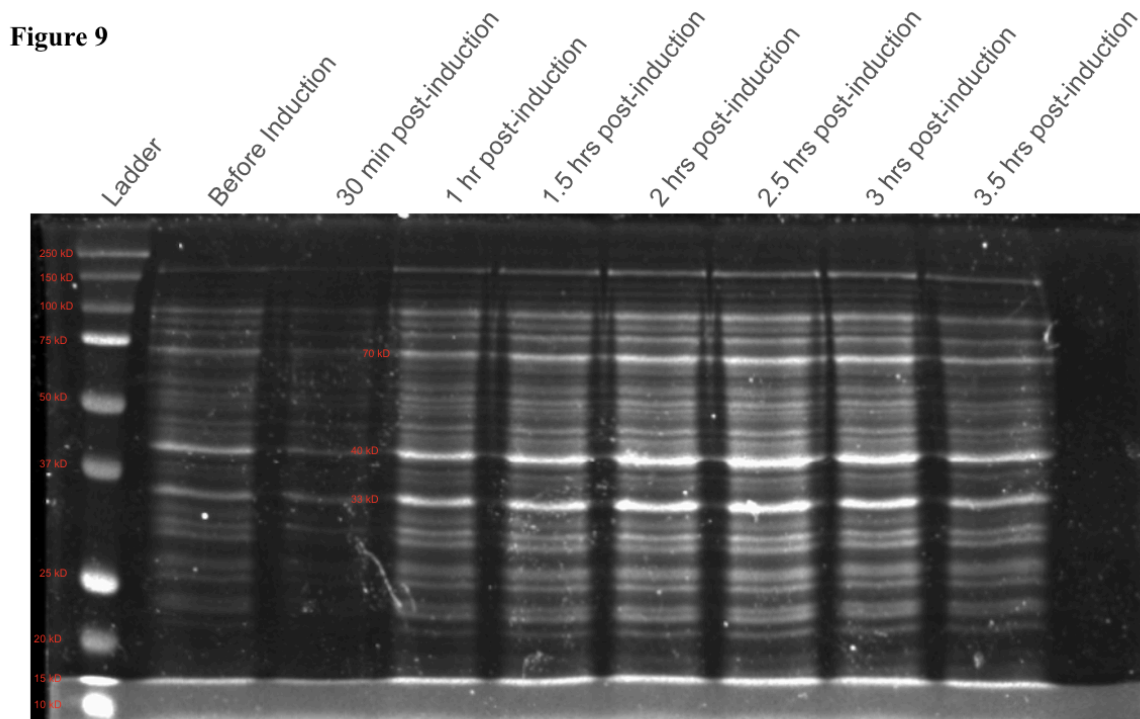


Figure 9 is the SDS gel which was run with the samples. As is evident, there are many different proteins present on the gel, with bands ranging from 15 kD to 150 kD. It is necessary to note that the expected weight of the Perceval protein is 65.7 kD. So, the closest to that weight would be the band at around 70 kD, which is not the brightest band and therefore not the most dense.

V. Discussion & Implications

Va. Plasmid Purification, Restriction Enzyme Digest, and DNA Gel Discussion

The initial plasmid purification yielding a concentration of 24.4 ng/ μ L is likely a result of contamination and insufficiency of the materials used. The QIA miniprep kit that was used did not have any sort of dates on it that indicated when the reagents had been opened, how many times they had been used, and how old the kit was. Thus, using the kit and the commercially made buffers had the potential to result in such a low concentration. That said, the purity of the plasmid is notably high with the A260/A280 ratio being 1.86 with purity being defined as 1.8.

Going forward with the restriction enzyme digest with such a low plasmid concentration posed some challenges. For starters, more volume of the plasmid had to be used in the digest, resulting in the dilution of the plasmid when it was run on the gel. Additionally, when considering the fact that there were many failed transformation attempts using this plasmid, it is reasonable to assume that there was little to no plasmid at all, and thus the bacteria was unable to form colonies because it never attained the ampicillin resistant gene that was in the plasmid. That said, it is necessary to note that the DNA gel could possibly be interpreted as having a plasmid that had been affected by restriction enzyme digestion and perhaps the ladder was slightly off. Although the numbers themselves did not align (see **table 6**) there are some indications that there

was plasmid and it was cut by the restriction enzymes. For instance, although the condition with the perceval plasmid and HindIII should have shown up at 4,103 base pairs, its appearance at 3,800 is not too far off when considering that the double digestion was also about 300 base pairs off from the expected fragment length. Also, the 200 base pair band that was present in the single digest can possibly be explained, like some of the other smearing on the initial gel, as a result of Star activity. The intended band at 709 base pairs may not be visible at all when considering how dilute the plasmid was, as well as the possibility that the restriction enzymes that were used ranged from 10-12 years past their expiration date. It is possible that they were not as efficient as they were when they were first bought, resulting in a line around the 709 base pair mark that was undetectable since it is already expected to be almost 5x smaller in size than the one at 3,394. The two bands present in the eGFP (pET29b+) could potentially be a result of contamination, as could the other unexplainable aspects of the initial gel, like the perceval plasmid appearing so high up on the gel and having two bands at 12,000 and 5800.

The second plasmid purification resulting in such a high concentration of plasmid (1,183 ng/ μ L) can be attributed to the production and use of fresh reagents which were free of contamination and had never been used before. Furthermore, a new protocol from addgene, the source of the Perceval plasmid that was purchased commercially, was used, which potentially aligned with the best possible course of action to reproduce that specific plasmid. Since the plasmid concentration was so high, it was unsurprising that the purity was not as pure as the first plasmid purification with an A260/A280 ratio of 2.13, much farther from the typical pure value of 1.8. Regardless, the concentrated plasmid allowed for the restriction enzyme digest to be repeated with more restriction enzymes and with different concentrations, and overall showed that the Perceval Plasmid was present. Although the numbers did not match exactly, their relative

proximity to expected values and the two bands shown with the presence of two restriction enzymes and the singular band with one restriction enzyme indicated that the Perceval plasmid was there.

Again in the second DNA gel, smearing is observed at the bottom of each lane. Like with the first DNA gel, this is a plausible result of star activity. However, there are clearly pronounced bands at 2 distinct locations on the DNA gel for the double digestion, which was to be expected, and they were relatively similar to the expected cuts. Thus, from the second DNA gel, it was concluded that Perceval Plasmid had been produced and could be used in the transformation process.

Vb. Transformations & Growing the Cells

It is necessary to acknowledge two possible causes of the number of failed transformations: first, it is possible that the BL21 cells that were used were never competent enough to complete a transformation. This is entirely possible due to the small cell pellet and the complete lack of colonies with all BL21 cell lines except for the new BL21 cell line which was obtained from Genessee scientific. The literature states that BL21 cells are very sensitive to temperature and become less competent if not in ideal conditions and as time passes. In several of the transformations which were performed, the researcher was unable to determine the age of the competent cells and how they had been made. Thus, knowing if they were actually competent was a challenge. Second, there is a possibility that no perceval plasmid was produced in the first plasmid purification, and that could have resulted in the cells not growing due to not possessing the ampicillin resistance gene. However, the growth of the Genessee Scientific cells on plates and in media with ampicillin indicated a successful transformation with the plasmid from the

second plasmid purification and that there were BL21 cells containing perceval plasmid. This further validated the hypothesis that Perceval plasmid was present on the DNA gel.

The Genessee scientific cells which were grown and then induced grew very slowly, only reaching 0.3 optical density after 3.5 hours of growth in 1L media. They were induced regardless due to the protocol for typical protein production in BL21 cells, and eventual pelleting confirmed that the cells were growing very slowly. The pellet weight at the end was only 1.2 grams, while typical pellets after this amount of time range from 2-4 grams (Takara Bio, 2016). So, it is reasonable to assume that due to the slow growth of the bacteria, very little protein was produced and if it can be seen on the SDS gel, it will be very minimal.

The DH5a cells were also successfully transformed due to their production of colonies and then continued growth in the 5 mL culture. That said, after they got to 5 mL culture, they were turned into glycerol stocks and were not grown in 50 mL or 1 L due to their lack of a promoter. Although they had successfully been transformed with the plasmid, and they were used in the research done by Berg et al., a method to induce them to create the protein was not inherently obvious. It would not have been effective to add IPTG, and the literature was not clear about how the DH5a cells were used to express the protein aside from stating that they were allowed to grow for a long period of time. This is a potential path that the next researcher to investigate Perceval should keep in mind; it is entirely possible no induction is necessary and simply time should be given to the cells to produce Perceval on their own.

Vc. SDS Page Protein Gel for Perceval Detection

The SDS Page protein gel which was performed to detect Perceval protein at different times after induction yielded very limited results. There is no clear indication that more proteins

were being produced, a potential result of slow growth and inducing with such a low OD. Additionally, it is not clear which band represents the Perceval protein, if at all, because although **Figure 9** shows all the protein bands, the ones at the molecular weight where it would be expected to see the Perceval protein were not evidently Perceval or exceptionally fluorescent. In most research cases, it would be appropriate to repeat this experiment and obtain a better and more clear gel, but due to the time constraints of this particular experiment, it was not possible to redo the induction and expression. That said, there are clear steps for future experiments.

VI. Limitations

This experiment was limited primarily by time constraints, and secondarily by the resources available to perform the experiment at a low cost. As is the case with many biological resources which are fit to be used in a microbiology experiment, materials such as competent cells, restriction enzymes, and plasmid prep kits are expensive. Thus, using old or expired materials had the potential to prevent sufficient results from being attained, and also required that quite a bit of trial and error was done. Many different competent cell lines were tested, and many different transformations were performed, which was quite time consuming and limited the amount of time available to move forward with the mock experiment portion of the project.

This was exacerbated by the researcher's status as a senior and the need to complete the project before the end of the semester. The plasmid was only received from addgene 3 months before the project due date, making the experiment extremely condensed even without the added pressure of invalid resources. Additionally, working in a shared laboratory environment can complicate experimental procedures, especially when it comes to maintaining a sterile environment and keeping conditions for cells the same for a long period of time when other

researchers need the space to be different. A clear example of this is when one researcher changed the temperature on the incubator without checking to see that there were cells transformed with Perceval which needed to remain at 37 degrees celsius.

A final limitation is the difference between this project involving Perceval and some of the other projects which take place in the Bruce lab. There were limited communal experiences on how to do some of the microbiology techniques used in this experiment, such as restriction enzyme digestion and running DNA gels. This limited experience also is evident in the lack of new materials to perform such experiments, including the use of restriction enzymes which ranged from 10-12 years old. Much of the protocol had to be developed from scratch using the literature and some instruction from the company which the plasmid was purchased from.

VII. Future Directions

VIIa. Broad Directions

Much of the microbiology which went into producing the plasmid and transforming it into a competent BL21 cell line was optimized with the intent of another researcher using it to continue the experiment. Ideally, the next researcher would be able to use the materials which have been created and investigate Perceval's ability to function as a reporter of the ATP:ADP ratio in the presence of chlorophyll as intended. Thus, it becomes necessary to provide instructions on how best to proceed to the end of the mock experiment and provide suggestions with what steps could be taken afterwards.

VIIb. Continuing the Mock Experiment

Given the lack of protein production, it is recommended that the researcher takes 1 of 3 paths to obtain the Perceval protein. The longest process would be to take the perceval plasmid from the second plasmid purification and transform it into the competent genessee scientific B121 cells, and then plate for overnight growth. They can then go on to select colonies and eventually grow to 1L. This can also be achieved by thawing and then growing one of the 2 mL glycerol stocks of B121 which has already been transformed with Perceval Plasmid stored in -80 degrees celsius by the current researcher. Either method will result in 1 L culture. Where the future directions differ from the previous experiment is that instead of inducing at 3.5 hours, the researcher should wait until the desired 0.4-0.6 optical density is achieved. Then, since it is evident that the cells transformed with Perceval grow quite slowly, the culture should be allowed to grow much longer once induced, and samples should be taken every 3 hours to be run on a protein gel. Only after 24 hours or so should the culture be pelleted to ensure sufficient protein production.

Another method which could be utilized to result in Perceval protein production would be to thaw one of the DH5a transformed with Perceval plasmid glycerol stocks which are in the -80 degree celsius freezer and grow into the 1 L culture. Since these do not need to be induced, they could be allowed to grow for 24 hours, potentially even longer, before they are pelleted and analyzed for the presence of the Perceval protein.

One way or another, once protein production is confirmed via an SDS page, it will be possible to move forward with the experiment. The protein can then be purified through the use of immobilized metal-affinity chromatography. Then, through the use of fluorescence measurements and dual-excitation techniques, ATP and other nucleotides may be added to analyze Perceval's response. Eventually, chlorophyll should be added and Perceval should be

monitored for fluorescence and it should be determined if Perceval is detectable in the presence of chlorophyll's autofluorescence.

VIIc. In the Event of a Successful Mock Experiment

In the event of a successful mock experiment, i.e. where Perceval's fluorescence is detectable even in the presence of chlorophyll, the next steps would be to extend this work toward the long term vision of using Perceval in plants. Future research should subclone Perceval with a transit peptide so it can be targeted specifically to the chloroplast. If properly localized, the researcher would expect to see the reporter fluoresce, indicating successful targeting. This would be a key milestone to achieve even before manipulating levels of ATP.

The modified Perceval protein could then be directed into leucoplasts, which are non-photosynthetic plant cells which do not contain chlorophyll and thus offer a fluorescence-free background. Once in these cells, the ATP concentrations would be manipulated and an assay could be performed. By imaging treated and untreated cells with a confocal microscope at the excitation wavelengths of 490 and 409 nm, the researcher could detect ratiometric changes in fluorescence, which would indicate a difference in ATP levels. If a clear difference is detected, Perceval would be validated as a functional reporter of the ATP:ADP ratio in plant cells.

Building on this, assays could then be performed in intact cells by isolating leucoplasts from root tissues. These experiments would open the door to bioenergetic analysis, including the effects of temperature and the activity of transporters like AAC, which shuttle ADP and ATP across organelle membranes. If successful, this work could have broad applications in understanding and manipulating plant cell energy metabolism.

VIII. Conclusion

This thesis intended to investigate the feasibility of using Perceval, a genetically encoded fluorescent reporter protein to monitor the ATP:ADP ratios in plant cells, especially those with chlorophyll present. Time and resource constraints limited the progress of the experiment to early stage validation steps, but substantial progress was made in the preparation and verification of the plasmid, optimizing and performing successful transformations, and initiating protein expression. Optimizing plasmid purification and restriction enzyme digestion protocols made sure that Perceval was reliably identified and can be used for future experiments. Although the protein production was inconclusive as of the SDS page, the work done to complete this thesis serves as the foundation for future research to pursue protein expression and purification, as well as fluorescence characterization. If future research indicates that Perceval is not only functional but detectable in environments containing chlorophyll, it would be considered a monumental advancement in the field of live-cell imaging and plant bioenergetics. All in all, this research has laid the groundwork for assessments of real time energy status in plant cells, and has potential implications for impact in photosynthesis research, crop improvement, and synthetic biology.

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