Generation of Stable Pentraxin ExpiCHO Cell Lines

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Abstract

Transfection – the introduction of foreign genetic material into eukaryotic cells – is used to study gene expression and protein production. There are two types of transfection: transient and stable. Transient transfection involves the foreign DNA being delivered into the cells' nucleus but degraded as the cells divide. Transient needs to be performed repeatedly to maintain the finite expression, which is inefficient for long-term research. Stable transfection, where the foreign DNA integrates into the cells' genome, is expressed for months, with the gene of interest being replicated through generations. Transient transfection is completed within three weeks, while stable needs months due to the selection screening. Along with these trade-offs of stable and transient transfection, new technologies and protocols are consistently developed, making it difficult to determine the most efficient and replicable transfection method. This project will determine this by comparing the duration and protein yield of stable and transient transfection. The procedure involves preparing the plasmids for transfection; transfecting the plasmids stably with antibiotics and cloning; transfecting the plasmids transiently; and testing for protein in the cultures. The results show production of Pentraxin I in the Zeocin transient culture was a total of 1.91 mg. Production of Pentraxin I in the Geneticin and Zeocin stable cultures was scarce and requires cloning for a substantial yield. The antibiotic selection phases are complete, with Zeocin finishing in 38 days and Geneticin in 64 days. The next steps are diluting and cloning the stable cultures; testing for protein; and comparing its yield to transient transfection.

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Introduction

Transfection is a lab procedure used to introduce foreign genetic material into the nucleus of eukaryotic cells. A powerful tool used to artificially modify cells' genetic content, transfection can enhance or inhibit gene expression. It is used to study the function and regulation of genes and gene products. Specifically, transfection allows for more insight on cellular processes and the mechanisms of diseases to help understand how to diagnose and prognose diseases. By producing recombinant proteins in mammalian cells, it allows for a therapeutic strategy to treat diseases. Inherited, genetic diseases could also be treated by employing transfection gene therapy (Kim & Eberwine, 2010).

Depending on the nature of the genetic material (DNA, RNA, siRNA, etc.), it can be transfected transiently or stably into mammalian cells. Transient transfection, used for short-term expression, entails the foreign DNA being delivered into the nucleus and mRNA is delivered into the cytosol. The genetic material is transfected as a plasmid and doesn't require integration into the host cells' genome. This results in the DNA eventually degrading by mitosis or environmental factors. Transient's short-term expression can be used to study the effects of gene knock-in (the substitution of a DNA sequence) or knock-down (the deletion of a gene) (Chong et al., 2021).

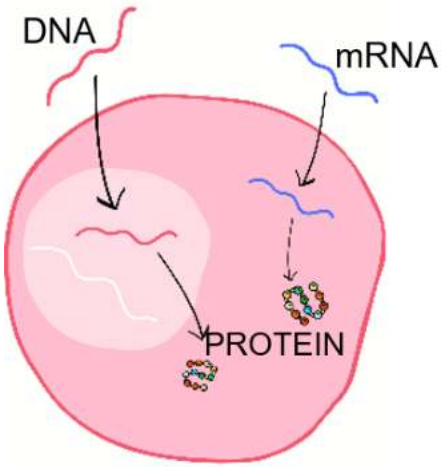


Figure 1. Transient

Stable transfection involves foreign DNA being integrated into the cells' genome and expressed even after the cells divide. In stable, the plasmid is either linearized to help integrate or maintained as an episomal vector, which can replicate autonomously without integration. Primarily used for large-scale protein production, stable transfection results in long-term gene expression and a stable cell line (Chong et al., 2021).

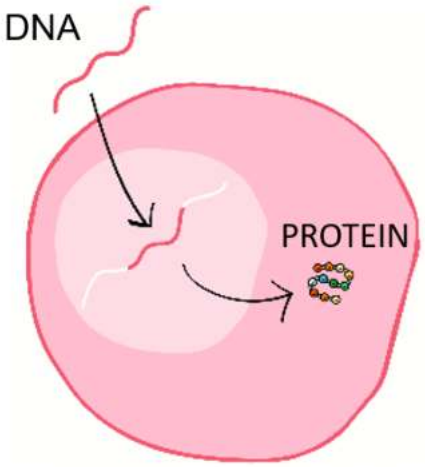


Figure 2. Stable transfection (student created)

But the process to develop a stable cell line is strenuous and harder to achieve than transient due to the selective screening. The introduced genetic material usually co-expresses a selection marker gene, which confers resistance to an antibiotic. The cells are then selected and only survive if they contain the plasmid with the selection marker. Stable has lower efficiency since a low number of cells integrate the foreign DNA, resulting in low levels of expression. Transient is the opposite, with a high number of transfected cells and high expression levels (Fus-Kujawa et al., 2021). Attaining homogeneity among the transfected cells takes months and makes stable transfection the less favored method, since transient only requires a few weeks to develop (Gray, 1997).

There are many methods available for transfection, depending on the type of cells and the form of the foreign nucleic acid being used. The foreign material could be physically, chemically, or biologically introduced into the cells. The carrier of the nucleic acid can also vary from viruses to plasmids, broadening the possibilities for transfection.

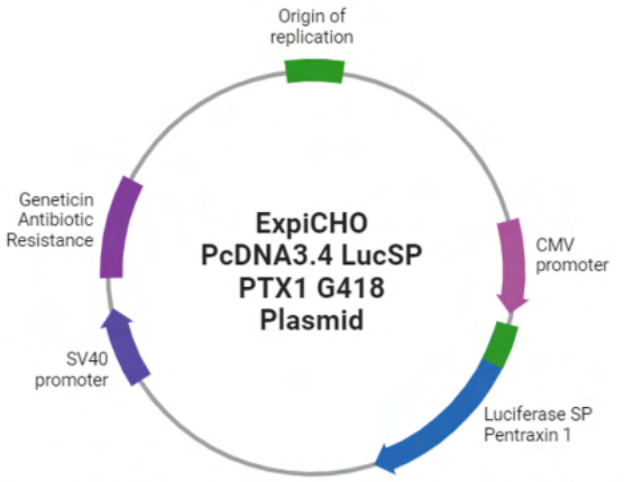


Figure 3. Geneticin resistance plasmid (student created)

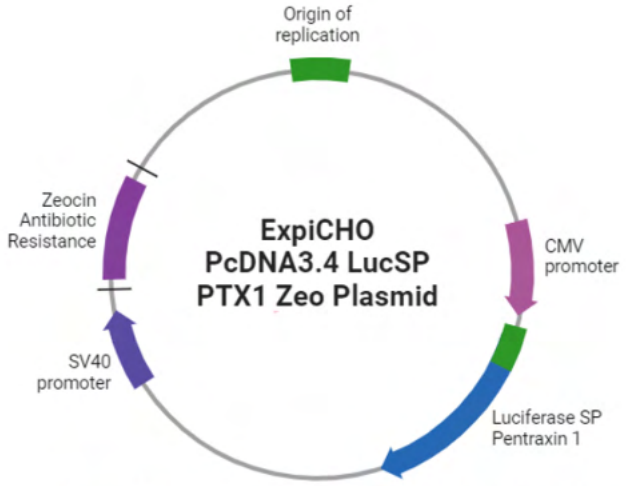


Figure 4. Zeocin resistance plasmid (student created)

In this project, the plasmids (Figure 3 & 4) were transfected stably and transiently. The plasmids were transfected into CHO (Chinese hamster ovary) cells. The plasmids produce a protein called Pentraxin 1 (PTX1), which regulates inflammatory response in liver. The plasmids contain the Luciferase signal peptide, which directs the protein to be secreted from the cell. The plasmid in Figure 3 was already prepared for transfection. It confers resistance to an antibiotic called Geneticin (G418). The second plasmid was constructed during the project and confers resistance to an antibiotic called Zeocin (Zeo).

Purpose

With many technologies and protocols of transfection being developed, transfection is a constantly evolving procedure. It is difficult to pick a reliable and consistent method when so many exist. This project with determine the optimal method of transfection to make it an attainable and efficient tool.

Hypothesis

If stable transfection efficiently expresses the gene of interest, better than transient, then it can be determined as the optimal transfection method.

Research Goals

1. Which method of transfection has a higher yield of protein?
2. Which antibiotic was more effective when creating a stable cell line?
3. How long did the overall procedure for transient and stable transfection take to perform?

Materials
- PPE
- Fume hood
- P1000 pipette
- P10 pipette
- P100 pipette
- P200 pipette
- P2.5 pipette
- Deionized water
- -80° and -20° freezer
- 1.5 mL Eppendorf Tube
- Ethanol
- Bleach
- 50mL centrifuge tube

Litigation Protocol with T4 DNA Ligase
- 10X T4 DNA Ligase Buffer:
  - 50 mM tris-HCl
  - 10 mM MgCl2
  - 10 mM Dithiothreitol 1 mM ATP
  - pH 7.5 @ 25°C

Plasmid Prep Protocol
- LiCl
- Table top centrifuge
- Polyethylene glycol (PEG)
- Solution I
  - 50 mM Sucrose or Glucose
  - 25 mM Tris-HCl, pH 8.0
  - 10 mM EDTA, pH 8.0
- Solution II
  - 0.2 M NaOH
  - 1% (w/v) Sodium dodecyl sulfate
- Solution III
  - 60.0 mL 5 M KAcOH
  - 11.5 mL Glacial Acetic acid
  - 28.5 mL H_2O
- TE buffer
  - 10 mM Tris, pH 8.0
  - 1 mM EDTA, pH 8.0

Protein Gel
- Laemmeli Buffer
  - Tris-HCl
  - Glycerol
  - SDS
  - Bromophenol Blue
  - DTT

Restriction Enzyme Digestion Protocol
- 10X NEBuffer:
  - 50 mM NaCl
  - 10 mM Tris-HCl
  - 10 mM MgCl2
  - 1 mM DTT
  - pH 7.9@25°C

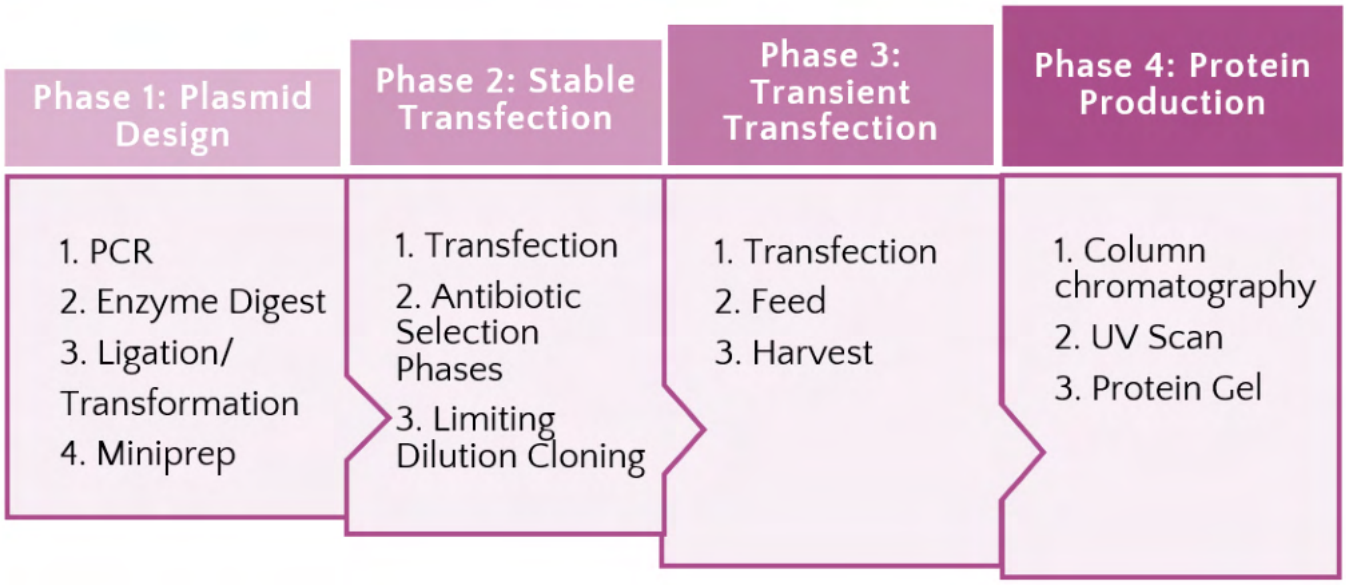
DNA Gel Electrophoresis
- DNA electrophoresis system
- UV transilluminator
- 100 mL beaker
- Stir bar
- Agarose
- 1X TAE Buffer:
  - 40 mM Tris
  - 20 mM acetic acid
  - 1mM EDTA
- Ethidium bromide
- 6X Gel Loading dye

Stable Transfection Protocol
- Automated cell counter
  - Trypan blue dye
- Incubator
- 125mL shake flask
- Electronic mL pipette
- ExpiCHO Expression Medium/Stable Expression Medium
- OptioPRO^{SFM}, Expifectamine reagent
- Zeocin/Geneticin

E. coli Transformation Protocol
- SOC medium
- NanoDrop Lite Spectrophotometer
- LB agar plate

PCR Protocol
- PCR machine
- HF Buffer
  - Ammonium fluoride
  - Hydroflouric acid
- Sectrophotometer

Methodology



Phase 1

Before transfection, a plasmid needs to be constructed to contain the genes of interest. The plasmid containing the Geneticin resistance gene was ordered, so it did not need any further preparation. But to confer resistance to the antibiotic Zeocin, the plasmid needed to be constructed to contain the resistance gene. The following protocols were used for this phase:

Polymerase Chain Reaction (PCR) (See Protocol #3 in Addendum)

PCR is used to amplify DNA, making copies of a specific segment. A sample of the plasmid containing the gene of interest is collected in a test tube and placed into the PCR machine. The DNA goes through repetitive cycles of varying temperatures to produce copies of the gene of interest.

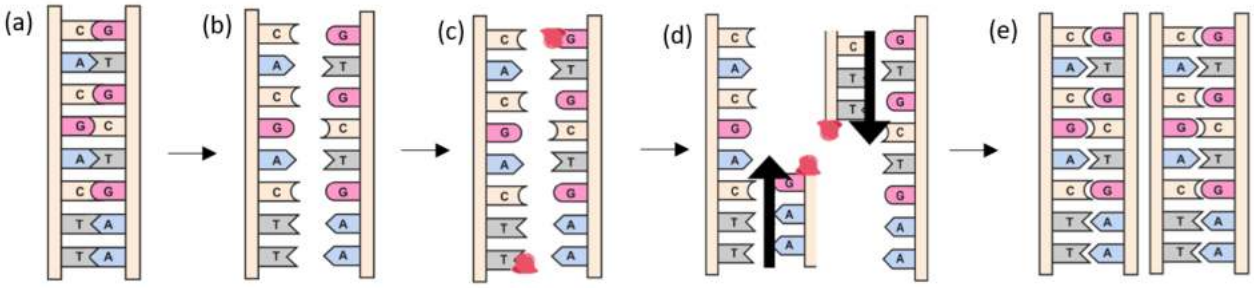


Figure 5. PCR Steps (a) DNA before PCR (b) Denaturation – The DNA is heated to an extreme temperature where the complemetary base pairs are separated (c) Annealing – The DNA is cooled to a temperatue where the DNA polymerase enzyme can bind to the separated DNA strands (d) Extension – The polymerase synthesizes in a 3' to 5' direction, producing strands identical to the template strands. (e) The resulting copies of DNA after one cycle (student created)

Restriction Enzyme Digestion (See Protocol #1 in Addendum)

In a digest, DNA segments can be cut out or linearized using the specific restriction sites. The restriction enzyme cuts the DNA sequence at these sites, leaving blunt or sticky ends. Blunt ends are where the enzyme cuts straight through, while sticky ends have a strand hanging over the other. To make a recombinant plasmid, a vector and an insert need to be digested. In this project, the Zeo insert and its vector needed to be digested. The two components were cut to have sticky ends because the overhangs assist when putting the two together. They were cut with the same enzymes to have the ends compatible.

2 Enzyme Digestion

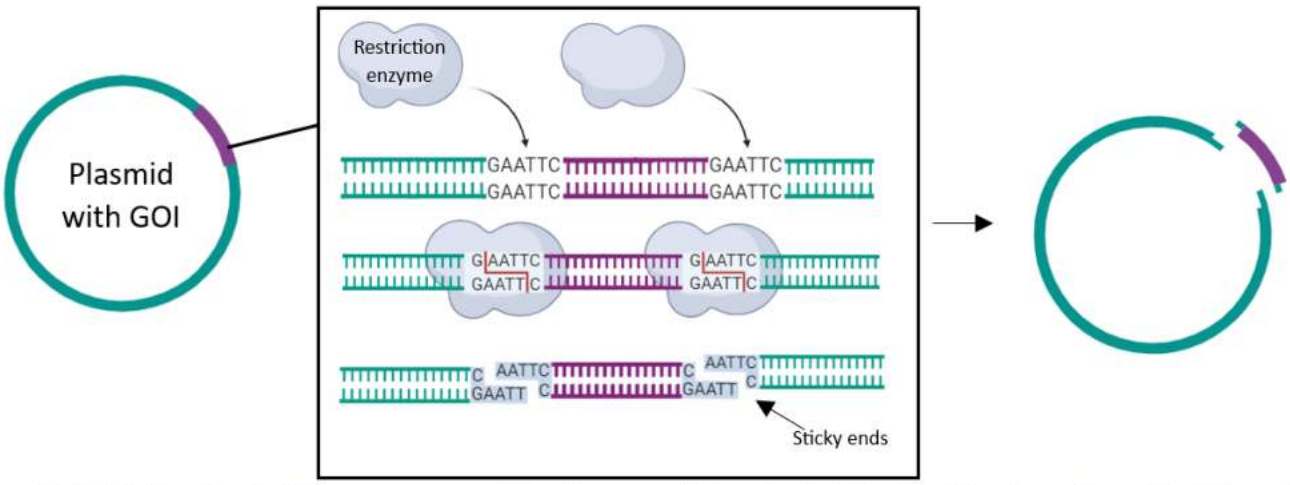


Figure 6. Restriction digest with two enzymes, cutting the gene of interest out of its plasmid and resulting with sticky ends (student created via Biorender)

1 Enzyme Digestion

Once the recombinant plasmid is recovered from plasmid prep, it needs to be linearized. The linearization cuts the plasmid to have sticky ends, which is efficient for stable transfection.

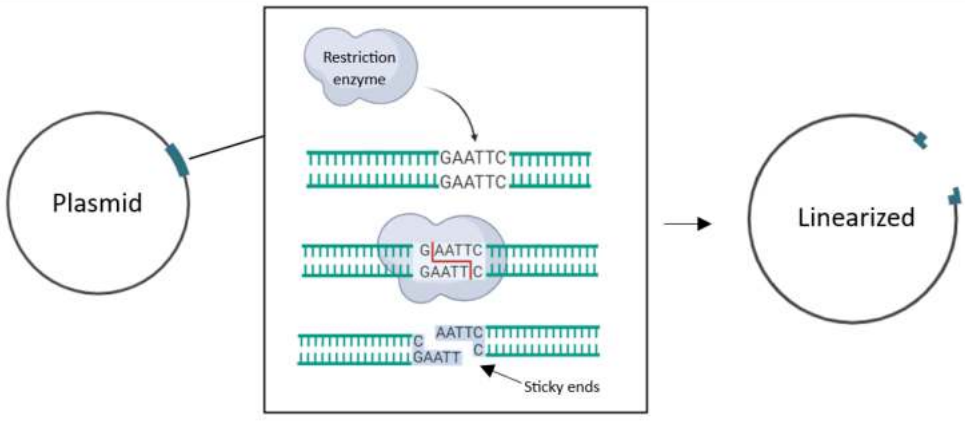


Figure 7. Restriction digest with one enzyme, cutting open the plasmid to have sticky ends (student created via Biorender)

Ligation (See Protocol #4 in Addendum)

The vector plasmid and the Zeo insert are put together, or ligated, using T4 DNA ligase.

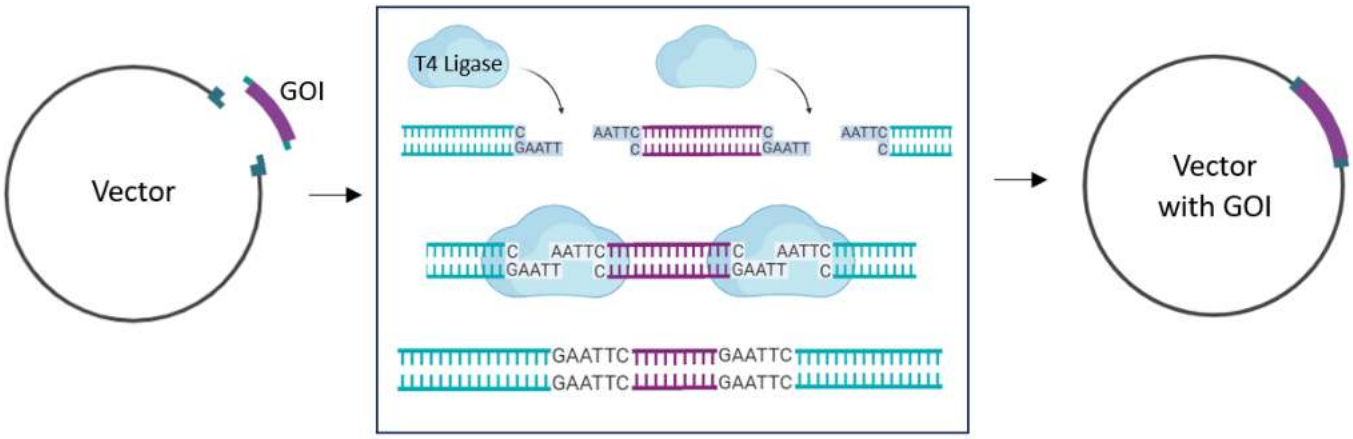


Figure 8. Ligation of gene of interest and vector plasmid (student created)

E. coli Transformation and Selection (See Protocol #6 in Addendum)

To check if the ligation occurred successfully, the plasmid was inserted into E. coli cells. This was done through the heat shock method. The cells are cooled and then placed on a heat block. The sudden change in temperature causes a pressure difference from the inside and outside of the cells. This induces pores to form in the cells' membrane, making it permeable for the plasmid to enter. The bacteria were streaked onto two Luria broth (LB agar) plates, one with antibiotic and one without. The goal was to have more colonies grown on the plate with antibiotic because it means the cells have the plasmid and were able to resist the antibiotic. The colonies were then picked and inoculated into culture medium.

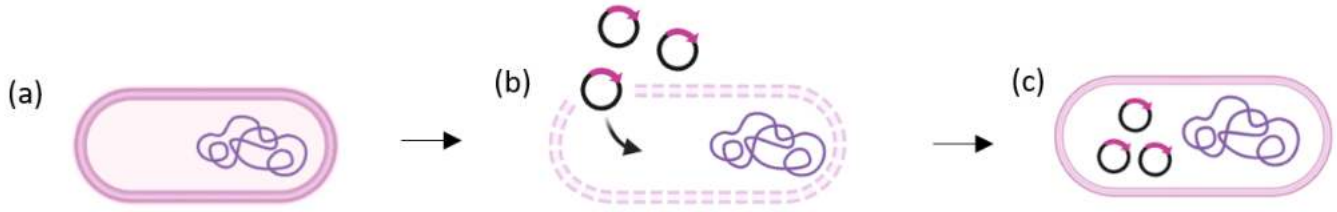


Figure 9. Heat shock transformation (a) E. coli cell with a solid membrane (b) Heat is suddenly applied to the cells to make the membrane permeable, so the plasmids can enter (c) Cells are cooled again to solidify the membrane (student created)

Plasmid Miniprep (See Protocol #7 in Addendum)

To extract the recombinant plasmid from the E. coli cells and clean out any contamination, plasmid prep is needed. The culture from the transformation went through a cycle of centrifugation and resuspension to purify the DNA. The following methods were used:

- Alkaline Lysis Method: Separated the plasmid DNA from the cell debris
- LiCl Precipitation: Removed unwanted molecules
- PEG Precipitation: Precipitated the protein to separate it from DNA

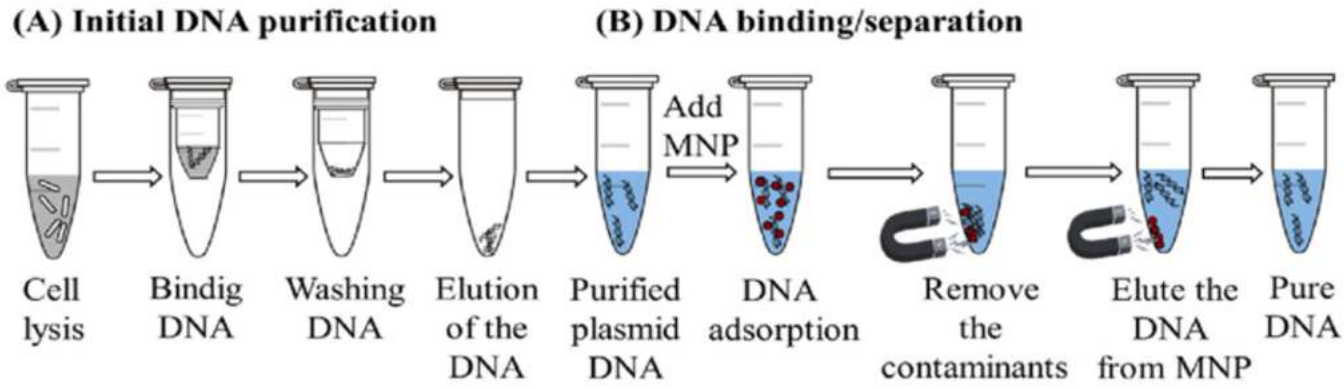


Figure 10. Plasmid prep steps where DNA is washed and eluted (Vanyorek et al., 2019)

Phase 2

The plasmids prepared in Phase 1 were transfected stably into two ExpiCHO cultures. After the initial transfection, the cells needed to be maintained by passaging and applying antibiotics. Once the cells recovered, a single cell with high productivity is selected, and a homogenous cell line is grown from the one cell. The following protocol was used in this phase:

Creation and Scale up of a Stable Cell Line using ExpiCHO™ Products (See Protocol #5 in Addendum)

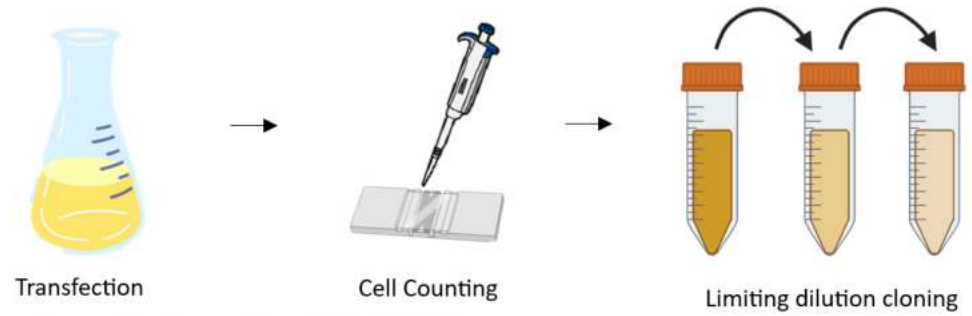


Figure 11. Steps for stable transfection (student created)

Phase 3

The same plasmids from Phase 1 were transfected transiently as a control variable. The cells were fed with ExpiCHO Feed and glucose to help with the protein production and cell growth. Once the culture grew and eventually crashed, it was harvested. The following protocol was used:

ExpiCHO™ Expression System User Guide (See Protocol #5 in Addendum)

Phase 4

The supernatant of the cells, the fluid that remained after the cells were centrifuged, was used to check to protein. The fractions collected from the column were put into a well plate and into a spectrophotometer. The instrument measured the absorbance of the fractions at a range of wavelengths to see the presence of protein. For protein, a peak in absorbance is expected at 280 nanometers. The following protocol was used:

Protein Purification (See Protocol #8 in Addendum)

To ensure only the protein of interest is collected, a column chromatography is needed to filter out anything else the supernatant may contain. Pentraxin 1, the protein of interest, is known to have calcium-dependent binding. The supernatant is conditioned with calcium chloride, which allows this binding to occur.

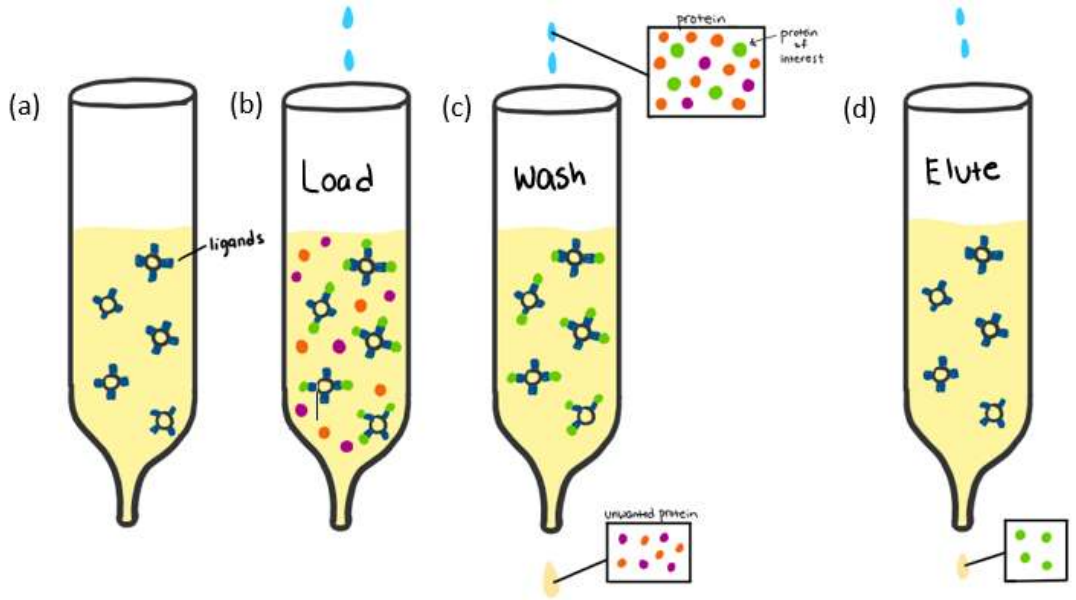


Figure 12. Protein purification steps (a) The resin in the coumn, Phosphoethanolamine (PE), has immobilized ligands with calcium-binding properties that can "catch" the protein. (b) The fluid is loaded into the column. (c) The wash buffer is run through to discard any other contaminants, while the Pentraxin attaches to the ligands. (d) EDTA, which attracts the calicum more strongly, pulls the calcium away from the Pentraxin. The bind between the Pentraxin and ligands is broken, and the protein is released through the column. The fractions are collected in this step. (student created)

Data/Analysis

Phase 1

Zeo Insert PCR and Digest

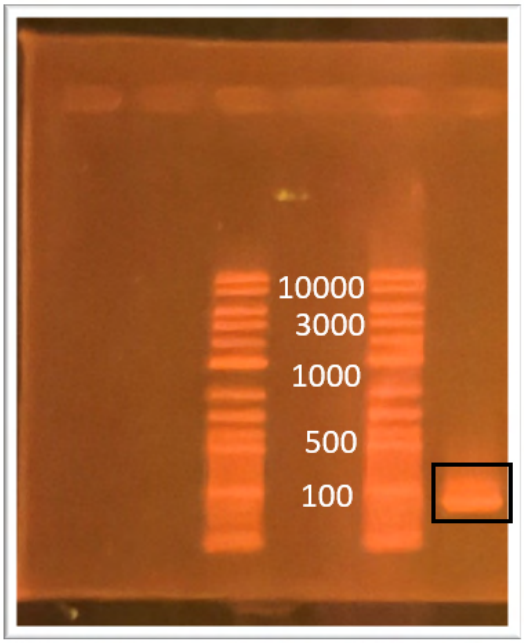


Figure 13. Agarose gel of Zeo gene after PCR

Loading Order	Observations
DNA ladder	Expected to see about 383 base pairs after PCR. Band is just below 500 base pairs.
DNA ladder	
Zeo after PCR	

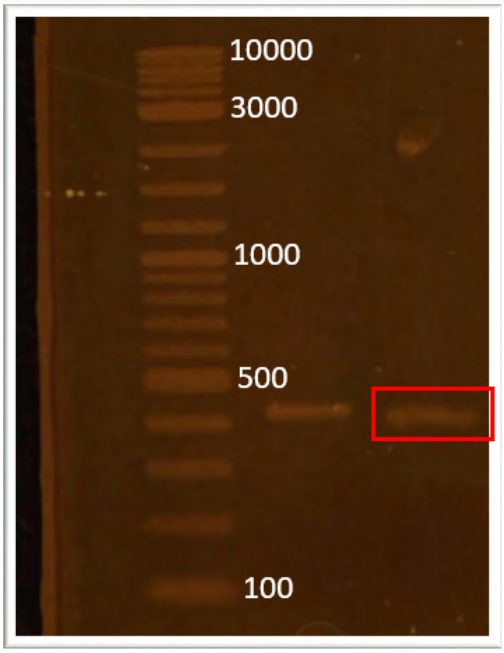


Figure 14. Agarose gel of Zeo gene after restriction digest

Loading Order	Observations
DNA ladder	Cut band is just below the uncut, which is expected, since digested DNA can run through the gel faster.
Uncut Zeo after PCR	
Cut Zeo after PCR	

Digested Vector Plasmid

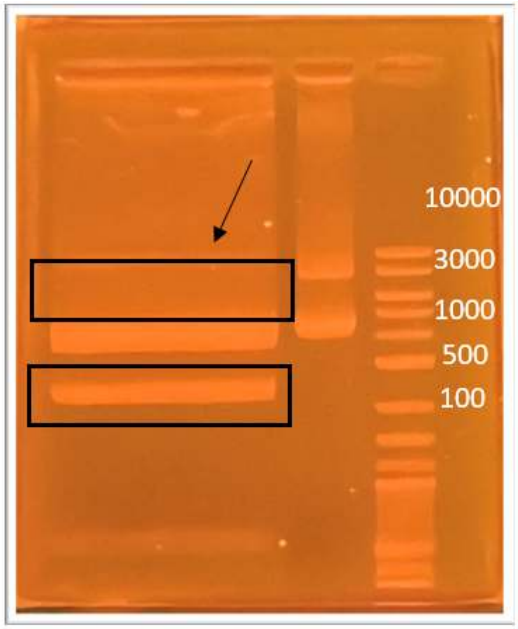


Figure 15. Agarose gel of vector plasmid after restriction digest

Loading Order	Observations
Digested vector plasmid	A faint band is seen at the bottom (the lower box), which is the part cut out. The upper box is the digested vector. The band with the arrow is plasmid that failed to be digested.
Undigested vector plasmid	
DNA ladder	

Recombinant Zeo Plasmid Linearization

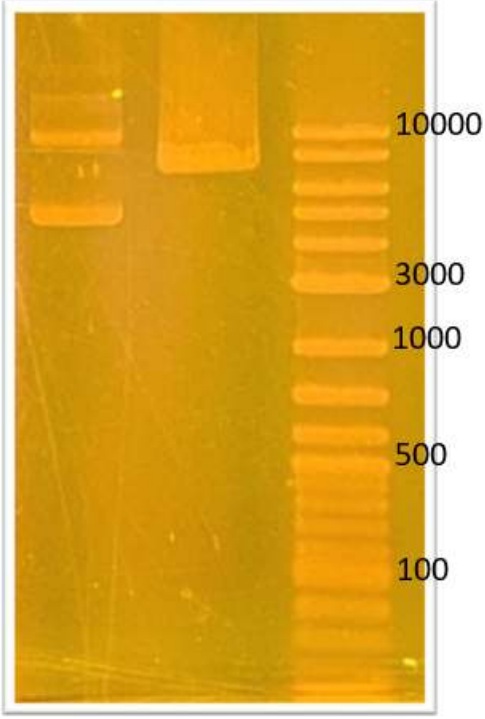


Figure 16. Agarose gel of Zeo recombinant plasmid after restriction digest

Loading Order:	Observations
Uncut plasmid	Linearized plasmid expected to be 6679 base pairs. It is seen to be under the 10,000 base pairs band
Linearized plasmid	
DNA ladder	

Phase 2

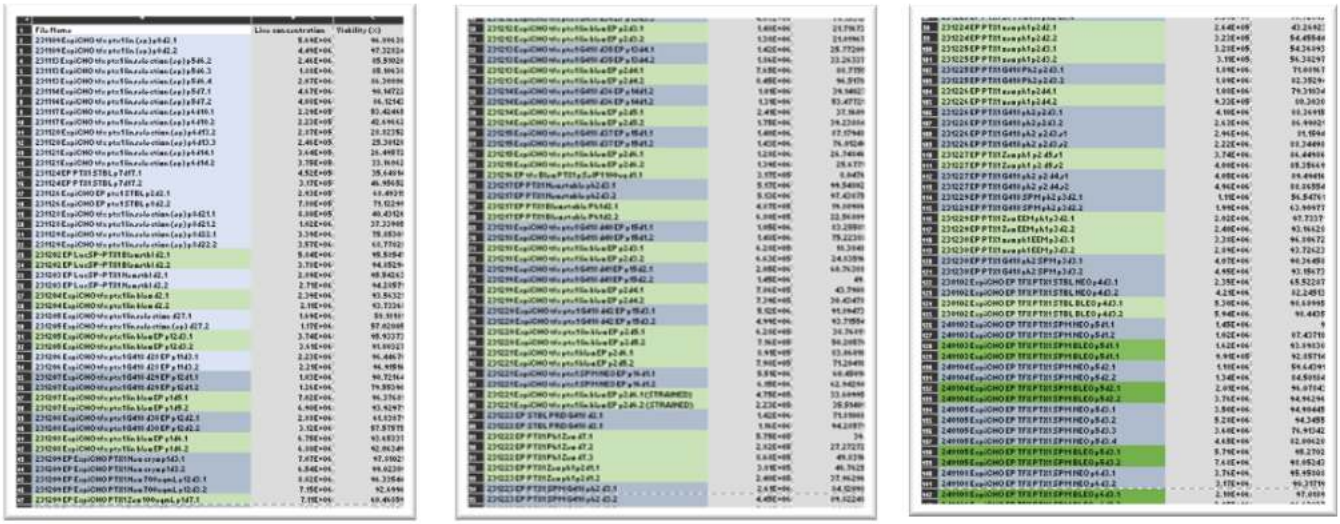


Figure 17. All data of stably transfected cultures collected from cell counter in a spreadsheet

	G418 Culture Selection Phase 1
	G418 Culture Selection Phase 2
	Zeo Culture Selection Phase 1
	Zeo Culture Selection Phase 2

Geneticin Selection Phase 1

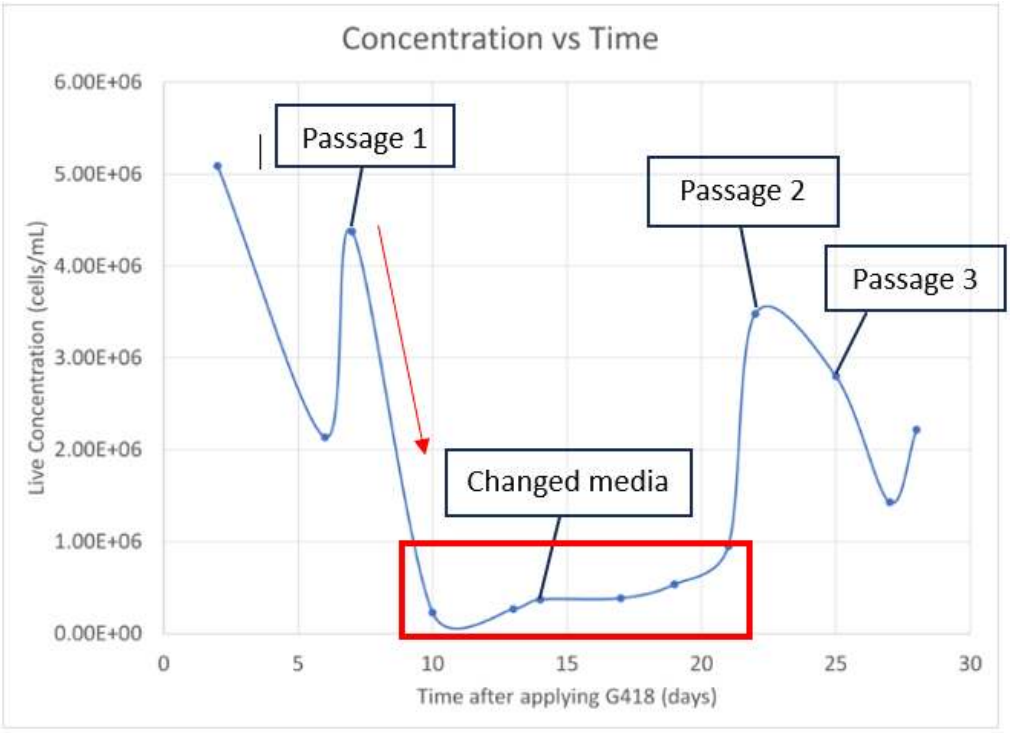


Figure 18. Graph of G418 culture's concentration during selection phase 1

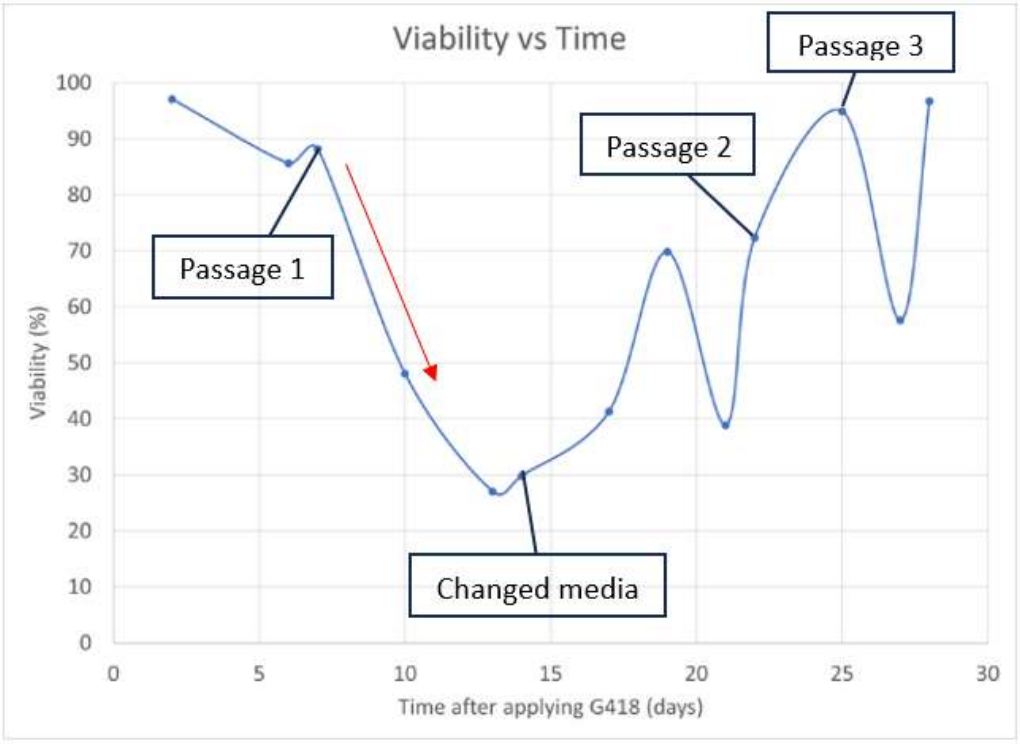


Figure 19. Graph of G418 culture's viability during selection phase 1

Geneticin Selection Phase 2

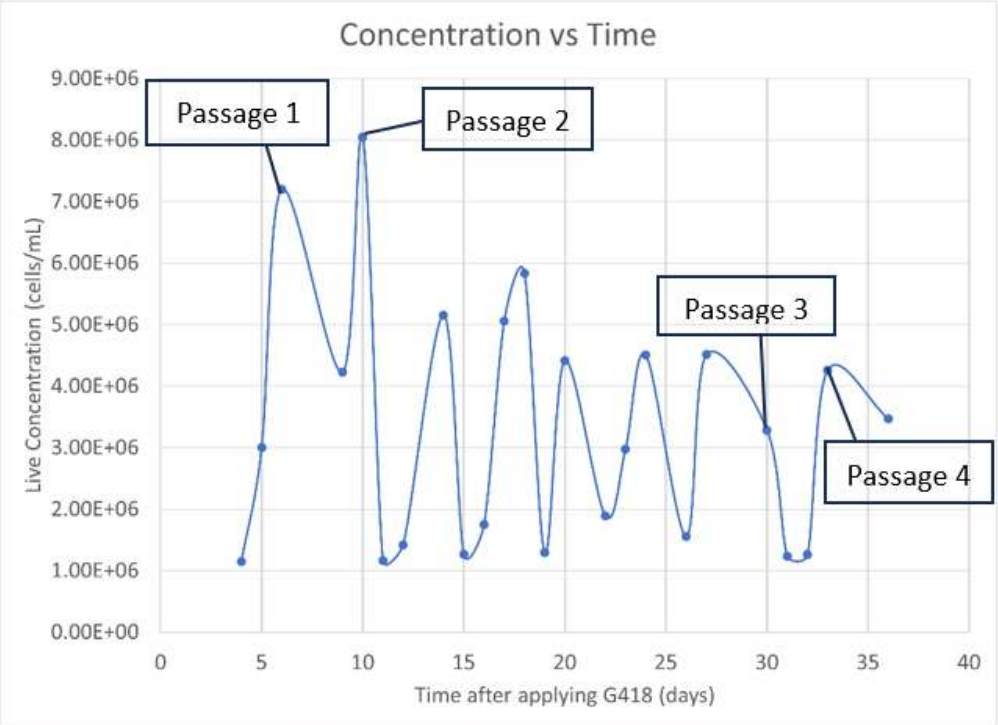


Figure 20. Graph of G418 culture's concentration during selection phase 2

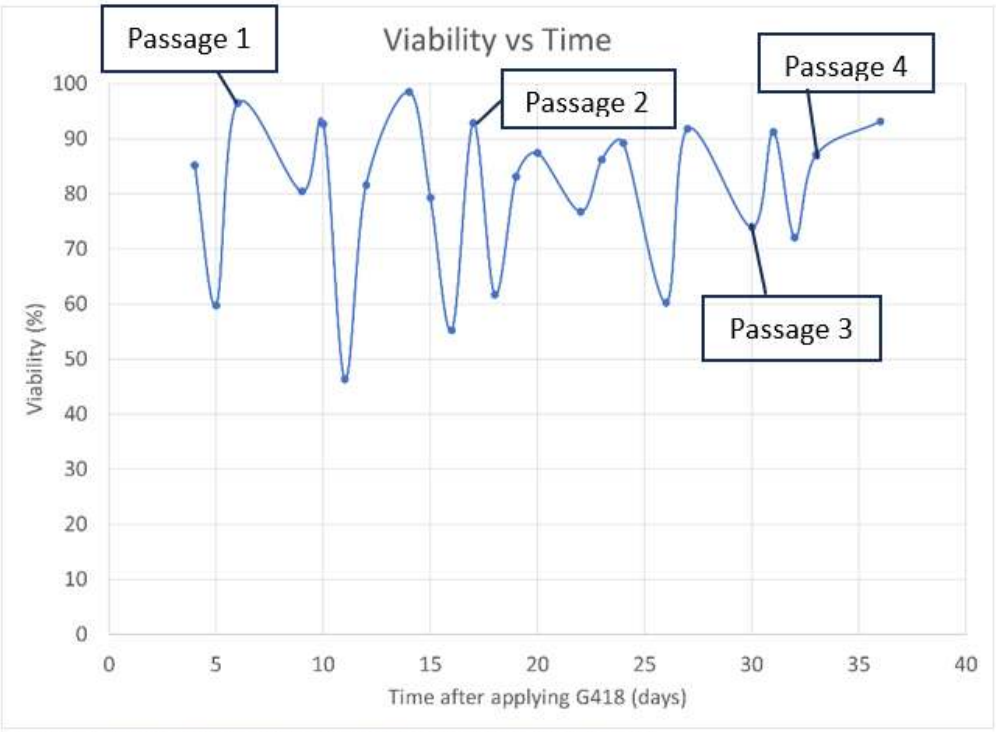


Figure 21. Graph of G418 culture's viability during selection phase 2

Zeocin Selection Phase 1

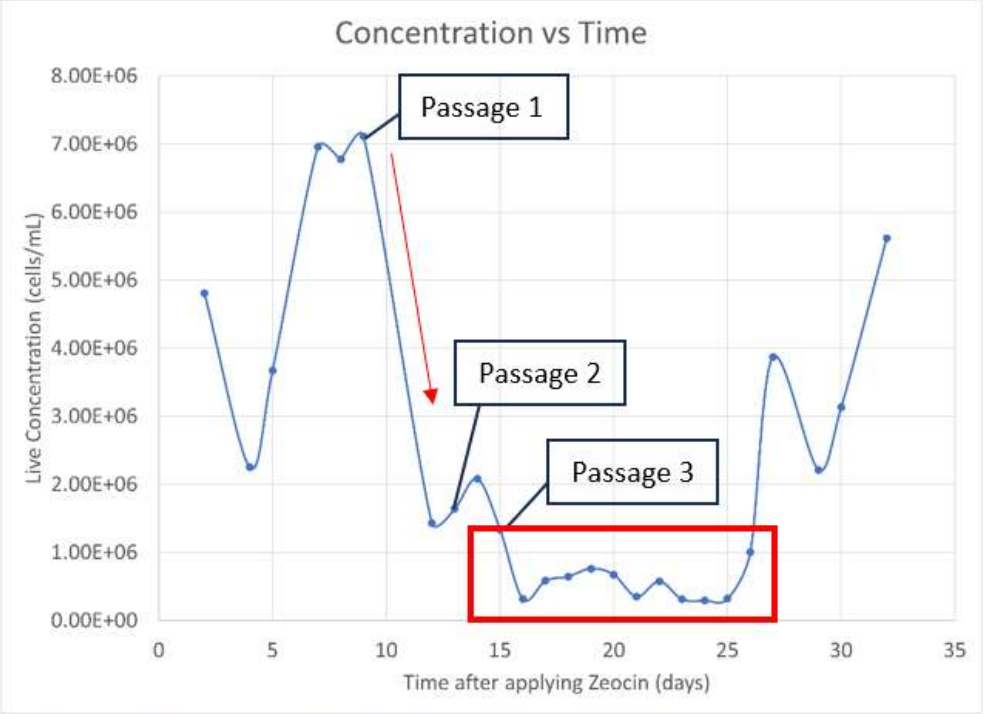


Figure 22. Graph of Zeo culture's concentratoin during selection phase 1

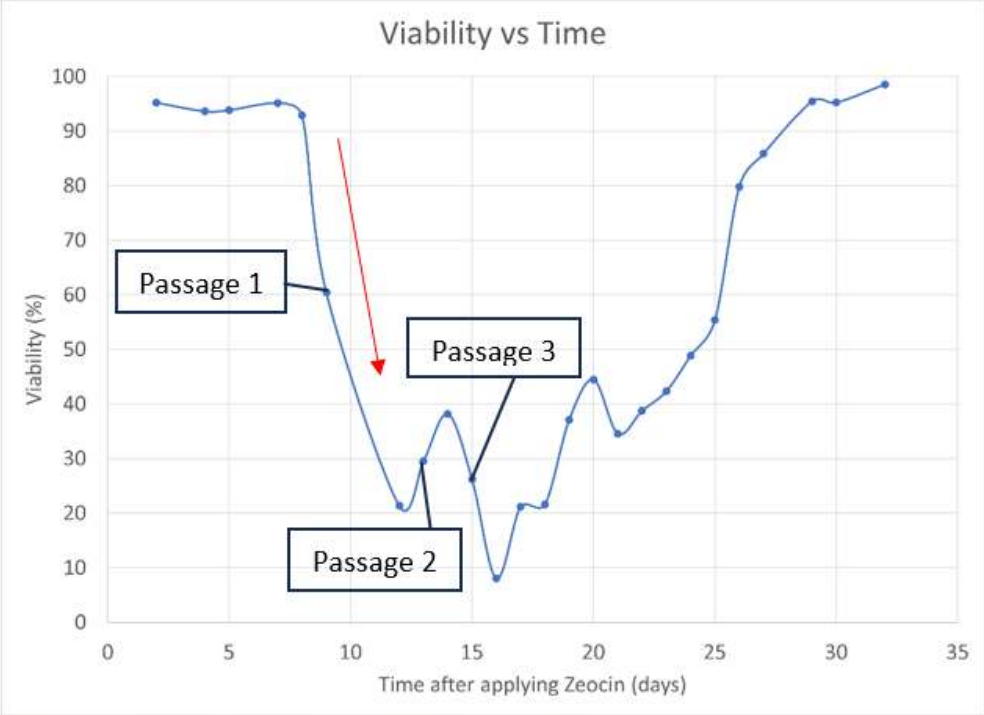


Figure 23. Graph of Zeo culture's viability during selection phase 1

Zeocin Selection Phase 2

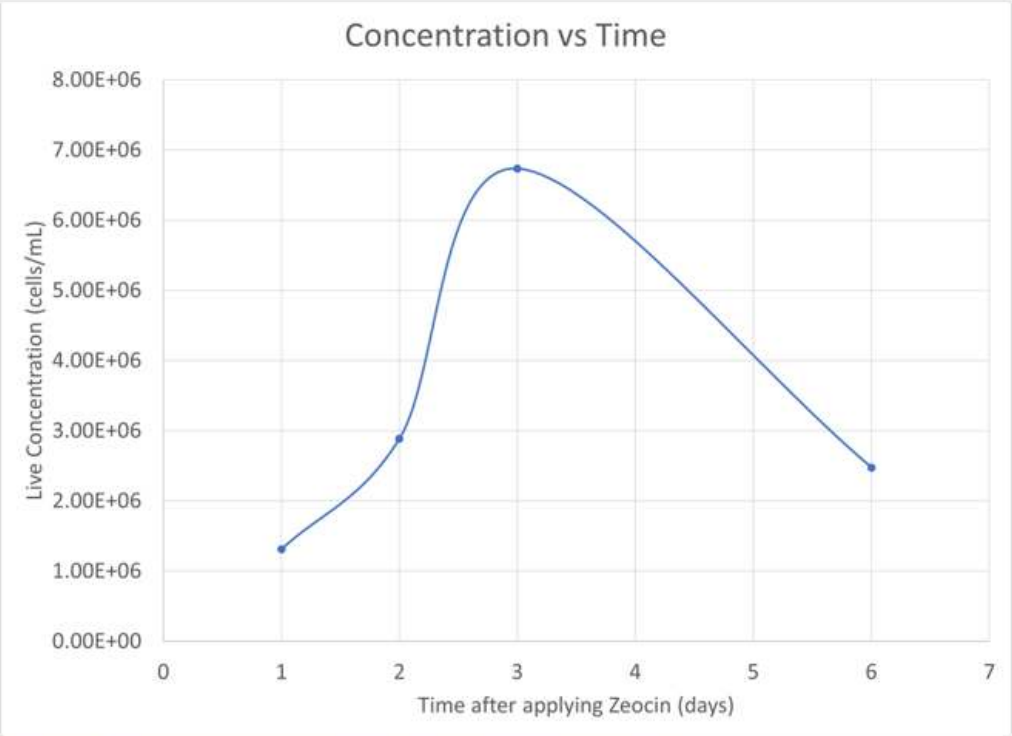


Figure 24. Graph of Zeo culture's concentration during selection phase 2

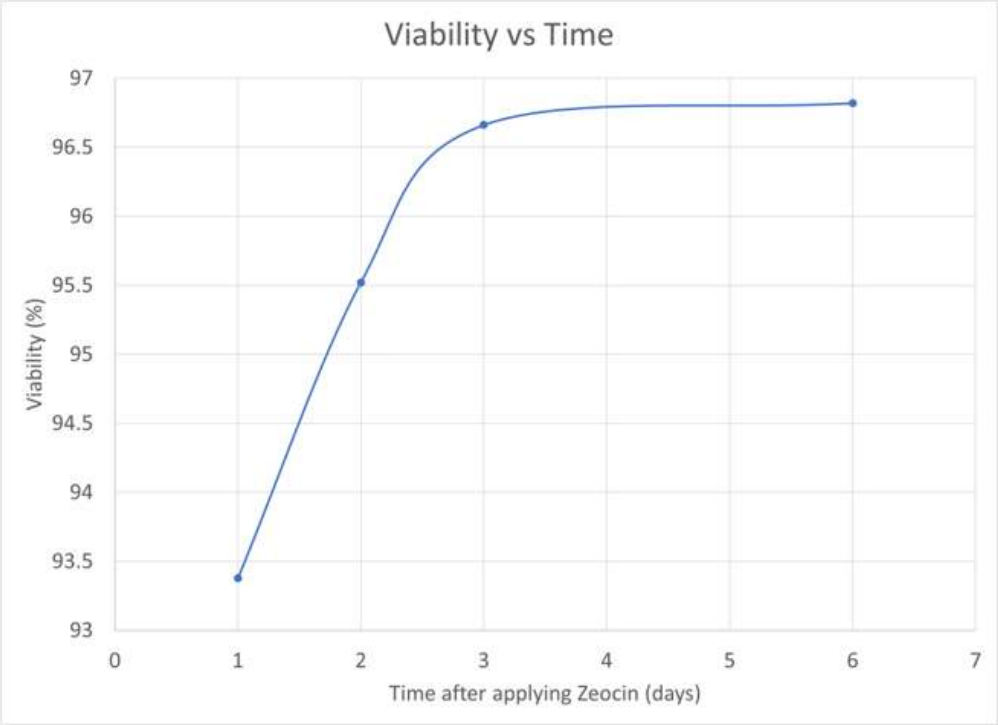


Figure 25. Graph of Zeo culture's viability during selection phase 2

Phase 4

UV Scans

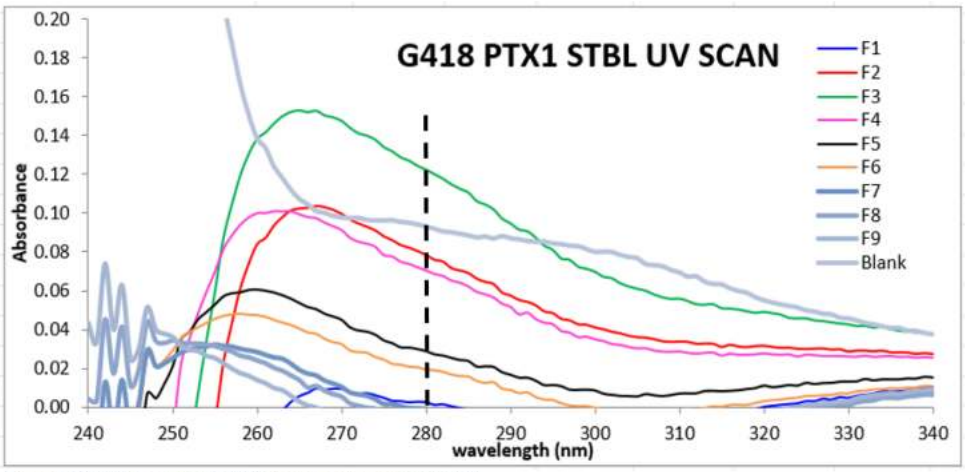


Figure 26. UV scan of G418 stable culture for PTX1

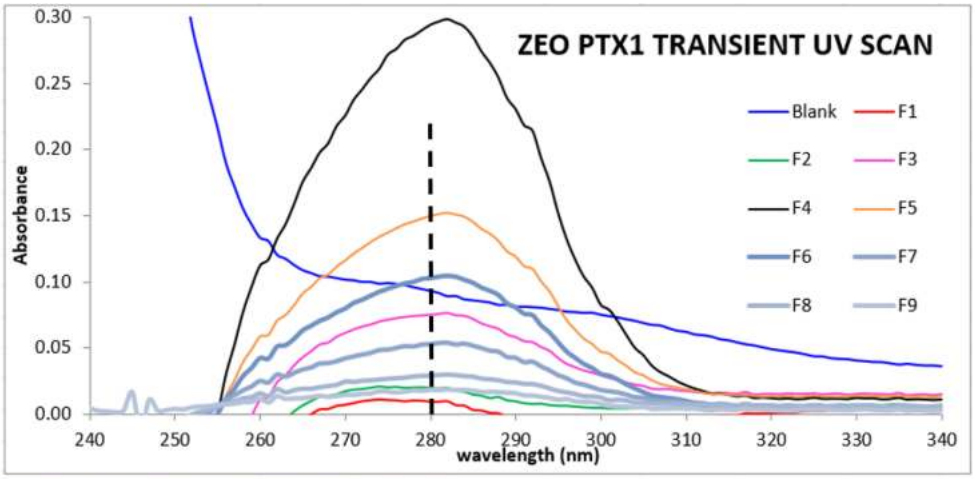


Figure 28. UV scan of Zeo transient culture for PTX1

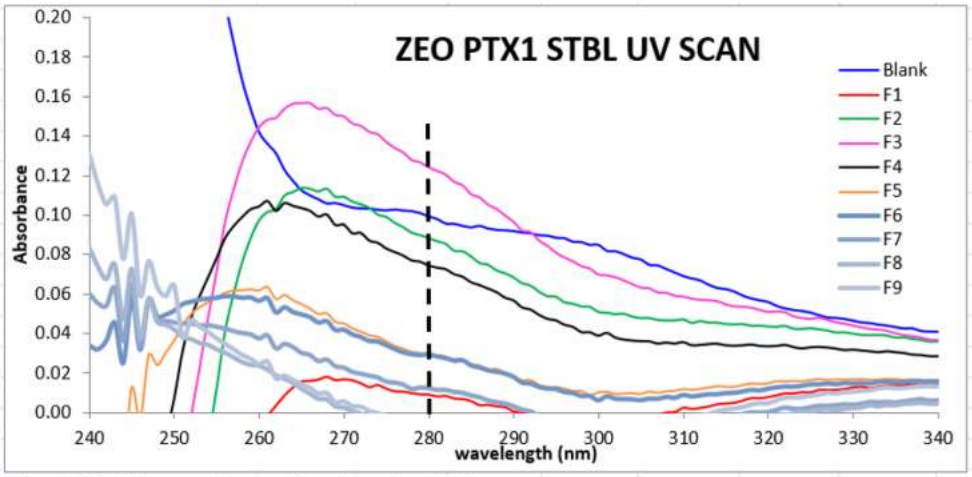


Figure 27. UV scan of Zeo stable culture for PTX1

Zeo Transient Protein Gel

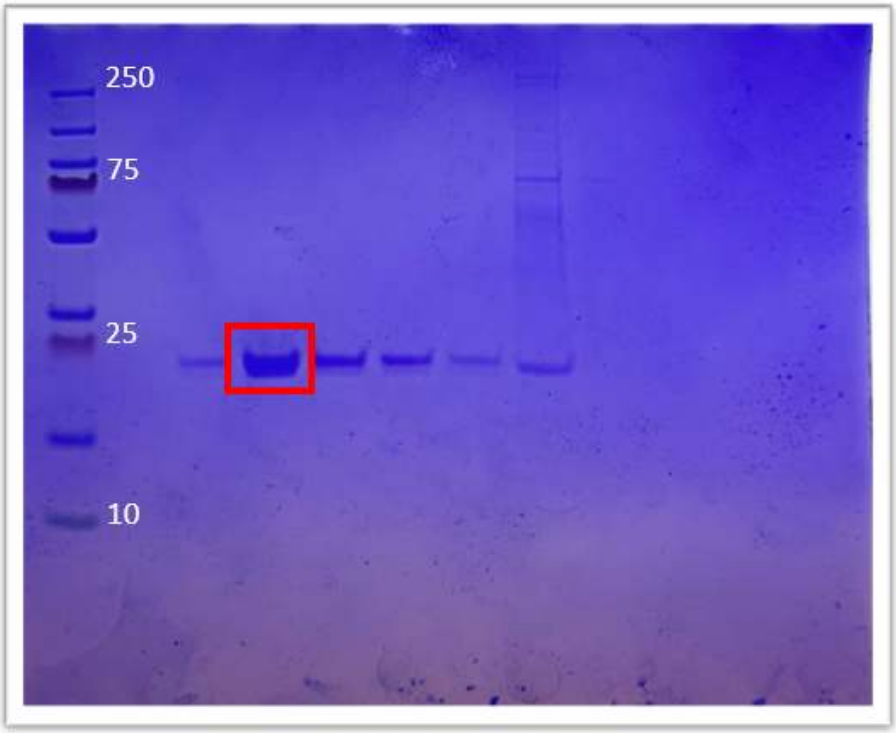


Figure 29. Protein gel of the fractions from Zeo transient culture

Sample	Protein (mg)
F2	0.041
F3	0.177
F4	0.836
F5	0.403
F6	0.286
F7	0.137
Total	1.91

Table 1. Quantitative results of protein produced from Zeo transient culture. The highest value in the table is the thickest on the gel.

Loading Order								
Protein Ladder	F2	F3	F4	F5	F6	F7	Load	Flowthrough

Compiled Protein Gel

Figure 30. Protein gel of fractions from different cultures

Loading Order										
Protein Ladder	Zeo Transient load	Zeo Stable load	Zeo Transient flowthrough	Zeo Transient F4	Zeo Transient F5	Zeo Transient F6	Zeo Stable F3	Zeo Stable F4	G418 Stable F2	G418 Stable F3

Discussion

Phase 1

Zeo Gene PCR (Figure 13)

The Zeo gene underwent polymerase chain reaction (PCR), where it is cycled through temperature changes to make copies of it. The cycles result in a larger quantity of DNA and new restriction sites added to the ends. The agarose gel helps show the quantity of the gene after PCR. The band looks to be under the 500 band, and 383 base pairs were expected.

Zeo Gene Restriction Digestion (Figure 14)

After PCR, the new ends were digested with the enzymes BsHII and XmaI. The digestion cut the gene in a staggered manner, allowing the gene to have sticky ends. This is done in order to make the ends of the gene align with the ends of the plasmid – like puzzle pieces. In the agarose gel, the cut PCR gene, is seen to be just underneath the band on the left, which is the uncut PCR gene. The cut is expected to be just below the uncut because digested DNA runs through a gel faster.

Vector Plasmid Restriction Digestion (Figure 15)

The agarose gel shows the digestion of the plasmid that the Zeo gene was inserted into. The plasmid was cut with the same restriction enzymes as the Zeo insert, so their ends match up. There are two long bands seen in the first column: digested plasmid and undigested plasmid. The presence of two bands means that not all of the DNA was digested. At the bottom of the gel, the part that was cut out is faintly present. The second band was used for ligation with the Zeo insert.

Zeo Recombinant Plasmid Linearization (Figure 16)

After ligation, transformation, and miniprep, the clean plasmid is linearized. For stable transfection, a linearized plasmid is essential to increase its likeliness of integrating into the cells' genome. The gel shows the recombinant Zeo plasmid, in the second column, after being cut. The DNA was expected to be about 6679 base pairs.

Phase 1 resulted in a recombinant plasmid that with a gene that confers resistance to Zeocin and one that expresses Pentraxin 1.

Phase 2

After transfection, antibiotics were applied to select and develop a stable cell culture. For each culture, there are two selection phases, each with a different concentration of antibiotics applied. The cultures were counted using a cell counter, and the data is compiled in Figure 17. The following figures show the same data as line graphs. One graph shows the live concentration of cells over time, which is how many cells were alive per milliliter. The second graph shows viability of the cells, which is the percentage of cells that are alive. The passages are marked on the graphs, which is where the cells are diluted, the medium is changed, and antibiotics are reapplied.

Geneticin Selection Phase 1 (Figure 18 & 19)

In selection phase 1 of the G418 culture, a concentration of 350 micrograms per milliliter of Geneticin was applied. The concentration (Fig. 12) and viability graph (Fig. 13) both show a steep drop in the beginning after the first passage. This is likely because Geneticin was reapplied to a diluted culture, resulting in majority of the cells being killed off. The concentration graph has a low plateau from day 10 to day 20, which shows that many of the cells didn't contain the plasmid. But the slow recovery shows that the cells with the plasmid were passing the resistance gene to the next generations. The phase ends, according to the protocol, when the cell concentration is above 6 million cells per milliliter, and the viability is greater than 90%. The phase lasted for 28 days.

Geneticin Selection Phase 2 (Figure 20 & 21)

In selection phase 2, the concentration of G418 was doubled to 700 micrograms per milliliter. The graphs show more erratic data, proving the increase in Geneticin was effective. Even though the first phase showed recovery, the second phase killed off cells again, meaning that some non-transfected cells were only detected once the concentration of Geneticin increased. Increasing the antibiotic concentration ensures that the culture only consists of transfected cells. According to the protocol, this phase ends when the viability is above 90%. This phase lasted for 36 days.

Zeocin Selection Phase 1 (Figure 22 & 23)

For selection phase 1 of the Zeocin culture, 100 micrograms per milliliter of the antibiotic was added. Selection phase 1 for the culture, which was selected with the antibiotic Zeocin, showed similar trends to Geneticin's selection phase 1. Both graphs show the overall drop and slow recovery. There is a plateau present in the concentration graph, lasting from day 15 to day 25. It is the same duration as Geneticin's plateau but delayed, meaning the effect of the antibiotics kicked in later. This phase lasted for 32 days.

Zeocin Selection Phase 2 (Figure 24 & 25)

Zeo's selection phase 2 lasted only 6 days, despite the concentration of Zeocin being tripled to 500 micrograms per milliliter. The short duration proves that the first phase was very effective, and a second phase might have not been necessary. The concentration did vary over the phase, but the viability was consistently above 90%, which is why the phase concluded so quickly.

Phase 2 resulted in stable cell cultures, which recovered from selective pressure.

Phase 3

While maintaining the stable cultures, a Zeo transient culture was grown as a control. The cells were transfected with the same Zeo plasmid but were not selected. The cells lasted for 2 weeks before they crashed, meaning their viability and live cell concentration plummeted. The culture was ready to harvest once this "crash" occurred.

Phase 3 resulted in a transient cell culture.

Phase 4

Column Chromatography

The protein column for each culture collected nine fractions, a flowthrough, and a load. The nine fractions are taken once the desired protein is eluted and should only contain Pentraxin. The flowthrough is taken while the fluid runs through the column and should have all the contaminants in it. The load should everything in it, contaminants and Pentraxin.

UV Scans (Figure 26, 27, & 28)

For the stable culture transfected with the Geneticin plasmid, a peak in absorbance is seen at about 265 nanometers but not 280, determining that no Pentraxin 1 was present. Similar to the Geneticin stable culture, the Zeocin culture lacked a peak at 280 nanometers. The control transient Zeo culture shows the peak at 280 nanometers, determining that Pentraxin 1 was produced by the transient transfection. Fractions 4,5, and 6, contained the highest concentration of protein. This was expected because as the protein is being eluted through the tube, it can take a while to reach the bottom, so the fractions collected in the middle are expected to have the most protein.

Protein Gel (Figure 29 & 30)

The fractions, load, and flowthrough from the Zeo transient culture were run through a protein gel to confirm the samples' relative abundance and mass. The fractions that contained the most protein from the UV scan were run through the gel: F2, F3, F4, F5, F6, F7. They all showed protein at almost 25 kilodaltons. The load showed a range of bands, including a band aligned with the fractions, meaning there were many things and Pentraxin present in it. The flowthrough had no band aligned with the rest of the Pentraxin band, or any bands at all. This means that none of the Pentraxin escaped through the resin before it was eluted.

In the compiled protein gel, the results contrasted what was seen in the UV scan. The transient culture did show prominent bands, and the stable cultures also showed faint bands at about 25 kilodaltons. The gel confirms that protein was produced by the stable cultures, although the quantity was small. Given the low yield, it suggests that limiting dilution cloning is needed to produce a significant amount of protein.

Phase 4 resulted in a higher yield of protein in the transient culture than the stable cultures.

Conclusion

The low yield of protein in the stable cultures led to an inconclusive hypothesis. The original goal was to prove that stable transfection would be the better method, but the results of the project have not supported this. The process for stable transfection took 3 months to perform but resulted in no protein, making transient look more favorable, since it only took 2 weeks. But the results of the antibiotic selection phases did determine Zeocin as the more effective antibiotic because its selection phases finished in half the time of Geneticin's phases.

Next Steps

To reach a conclusion about the hypothesis, more results and data need to be produced. The next goal would be to perform limiting dilution cloning on the stable cultures to produce a significant yield of protein. Once protein is produced by the stable cultures, the yield can be compared to the yield of transient. A control culture needs to be developed for the Geneticin plasmid.

In the future, different variables can be tested. The antibiotic and resistance gene can be changed to see which takes the shortest time. The gene of interest expressed or protein produced can be changed to see which is better with ExpiCHO cells. Specifically, Pentraxin II could be tested to compare its yield to Pentraxin 1.

The final goal, after studying the effect of these different variables on the transfection process, would be to find the optimal transfection method.

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