

Purification of His-Sumo tagged RNaseR and its functional analysis

by

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A thesis submitted to the Faculty of the University of Delaware in partial fulfillment
of the requirements for the degree of Honors Degree in Applied Molecular Biology
and Biotechnology with Distinction


Spring 2022

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Purification of His-Sumo tagged RNaseR and its functional analysis

by

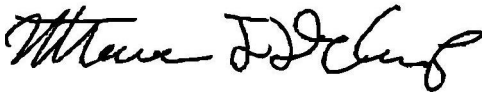
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ABSTRACT

RNaseR is an exonuclease belonging to the RNase II family that degrades linear RNA in the 3' to 5' direction and does not have a cleaving effect on circular RNA. The objective of this thesis is to overexpress and purify His-tagged RNaseR from recombinant *E. coli* cells that is free of other RNases and contaminants through growing cultures, employing purification techniques such as nickel column affinity chromatography, butyl column chromatography, and Gel Filtration Chromatography (GFC). Since RNaseR is very expensive and is produced by only one biotechnology company- Lucigen Inc, which unfortunately produces inconsistent results in multiple cases, it is crucial to produce pure RNaseR in-house that is functional and free of other contaminants that could cause circular RNA degradation. Although functional RNaseR was successfully purified in one of the purification runs, it had low reproducibility and could not be replicated using the same method and reaction conditions. Multiple optimizations were made that helped to get increased expression of RNaseR and helped in understanding the process of RNaseR purification and how the protein reacts in different reaction conditions.

Chapter 1

INTRODUCTION

1.1 CIRCULAR RNAs

Circular RNAs are essentially single stranded RNAs that close on themselves via covalent bonding of the 5' and 3' ends. These are formed because of the process of back splicing that can happen due to base pairing between flanking introns. Circular RNAs can be comprised of single or multiple exons or include exon and intron or made of only introns. These usually have longer than average length of exons and introns⁴. Since circular RNAs are closely looped structures, they do not have 5' and 3' ends or poly-A tails and 5' caps. Therefore, they are resistant to degradation by exonucleases such as RNaseR. Their closed loop formation also results in intriguing properties such as protein complex scaffolding, parental gene modulation, RNA-protein interactions, and microRNA (miRNA) sponge⁶. Since circular RNAs are not easily degraded by exonucleases, they have longer half-lives as compared to linear RNAs and are enriched in extracellular vesicles⁷. Therefore, these can be used as biomarkers for diagnosis of various cancers such as Gastric Cancer, Hepatocellular Carcinoma, and osteosarcoma⁸. Since circular RNAs are specific, cannot be degraded by RNaseR action, and hold great value as biomarkers and sources for biological therapeutic tools, enzymes such as RNaseR are desirable for circularRNA studies.

1.2 EXONUCLEASE RNASER

RNaseR is an exoribonuclease in the RNase II family that primarily participates in RNA decay. RNaseR breaks down duplex RNA with a 3' overhang and unwinds them prior to degradation¹. Messenger RNAs are reacted with pyrophosphohydrolase RppH to remove the 5' end pyrophosphate and then cleaved by exoribonucleases such as RNaseE⁹ and RNaseIII¹⁰. The broken down RNAs are then further degraded by exoribonucleases such as RNaseR. RNase R consists of a helix-turn (HTH) domain, RNB exoribonuclease domain, two cold shock domains (CSD1 and CSD2), an S1 domain and a K/R-rich domain. The RNB domain and the HTH domain is hypothesized to play a primary role in RNA unwinding and the remaining auxiliary domains are involved in RNA binding. Duplex RNA unwinds on reaching a “wedge” region in the RNB domain, and the unwound 3' end of the RNA strand is pulled into the RNB domain and broken down in the active site. Moreover, structural analyses show that it contains two open RNA-binding channels and a tri-helix region in the RNB domain. Both open channels in RNaseR, on top and in the side contain positively charged residues that act as RNA-binding sites. The top channel attaches duplex RNA, and the side channel attaches single stranded RNA. The auxiliary domains are flexible and the width changes on RNA binding. RNaseR degrades RNA in a processive manner, and one molecule is able to unwind over 500 bp of a structured substrate. Based on research, there are two possible RNA unwinding and degradation models for RNaseR:

A. According to the first model, the CSD1 and S1 domains in the top channel bind the duplex region of the RNA in the top channel and on reaching the tri-helix region, the duplex is unwound. RNA unwinding is driven by RNA hydrolysis, and the RNA is continuously degraded in a processive way and pulled into the catalytic active site of the RNB domain. The unwound 3' overhang is pulled into the active site to be broken down, and the 5' non-scissile strand leaves via the side channel.

B. The second model proposes that the single-stranded overhang of the duplex RNA enters the active site via the side channel and the duplex binds between the CSD1 and RNB domains. When the duplex region encounters the tri-helix region, the RNA is unwound and the 3' overhang is guided into the RNB active site for cleavage and the 5' end leaves via the top channel.

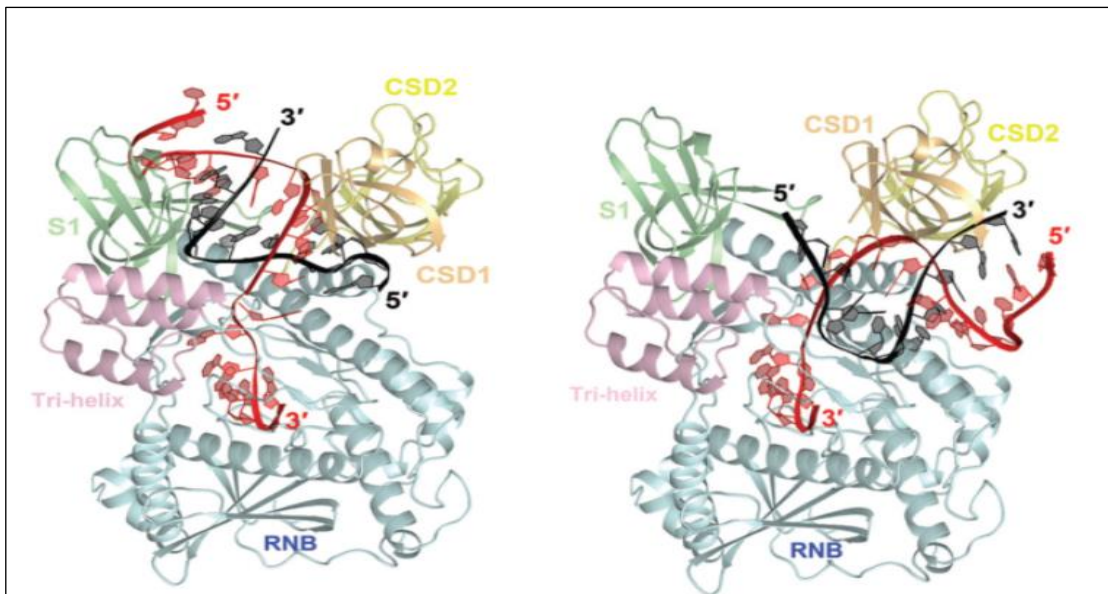


Figure 1: Two possible working models for RNaseR were built based on the crystal structure of RNase II bound with RNA and Rrp44 bound with RNA. These crystal models support both models for RNA degradation by RNaseR respectively.

Note. From Structural insights into RNA unwinding and degradation by RNaseR, by Chu LY et al. *Nucleic Acids Res.* 2017 Oct 3. doi: 10.1093/nar/gkx880. PMID:[29036353](https://pubmed.ncbi.nlm.nih.gov/29036353/). Copyright by Chu LY et al.

Of the two proposed models, the first model is more probable as it is consistent with other data that shows that the RNB domain by itself can degrade duplex RNA irrespective of the presence or absence of a 3' overhang because the tri-helix region is on the top side of the RNB domain, making it possible to be exposed to RNAs.

1.3 NICKEL COLUMN AFFINITY CHROMATOGRAPHY

Affinity chromatography is a protein purification/separation method based on a particular binding relationship between an immobilized ligand and its binding partner. It is very effective as it allows for separation and purification of specific products.

In case of proteins with an attached polyhistidine tag (a set of 6 histidine residues), histidine residues in the His tag bind to open positions in the coordination sphere of the immobilized nickel ions with high specificity and affinity. Therefore, when the lysed cell solution is loaded, the stationary column containing immobilized nickel ions bind to the attached His-tag while other proteins without the His tag that do not bind to the column will pass through the matrix. The column can then be washed and eluted from the column in native or denaturing conditions to obtain the required protein. The Ni-NTA (nickel-nitrilotriacetic acid) resin for proteins with an attached His tag is very selective and allows one-step purification of almost any His-tagged protein from any expression system. NTA binds nickel ions more strongly than metal-chelating purification systems with just three sites available for interaction with metal ions because it has four chelation sites. The additional chelation site inhibits nickel-ion

leaching, resulting in higher binding capacity and purity protein preparations than existing metal-chelating purification techniques¹¹.

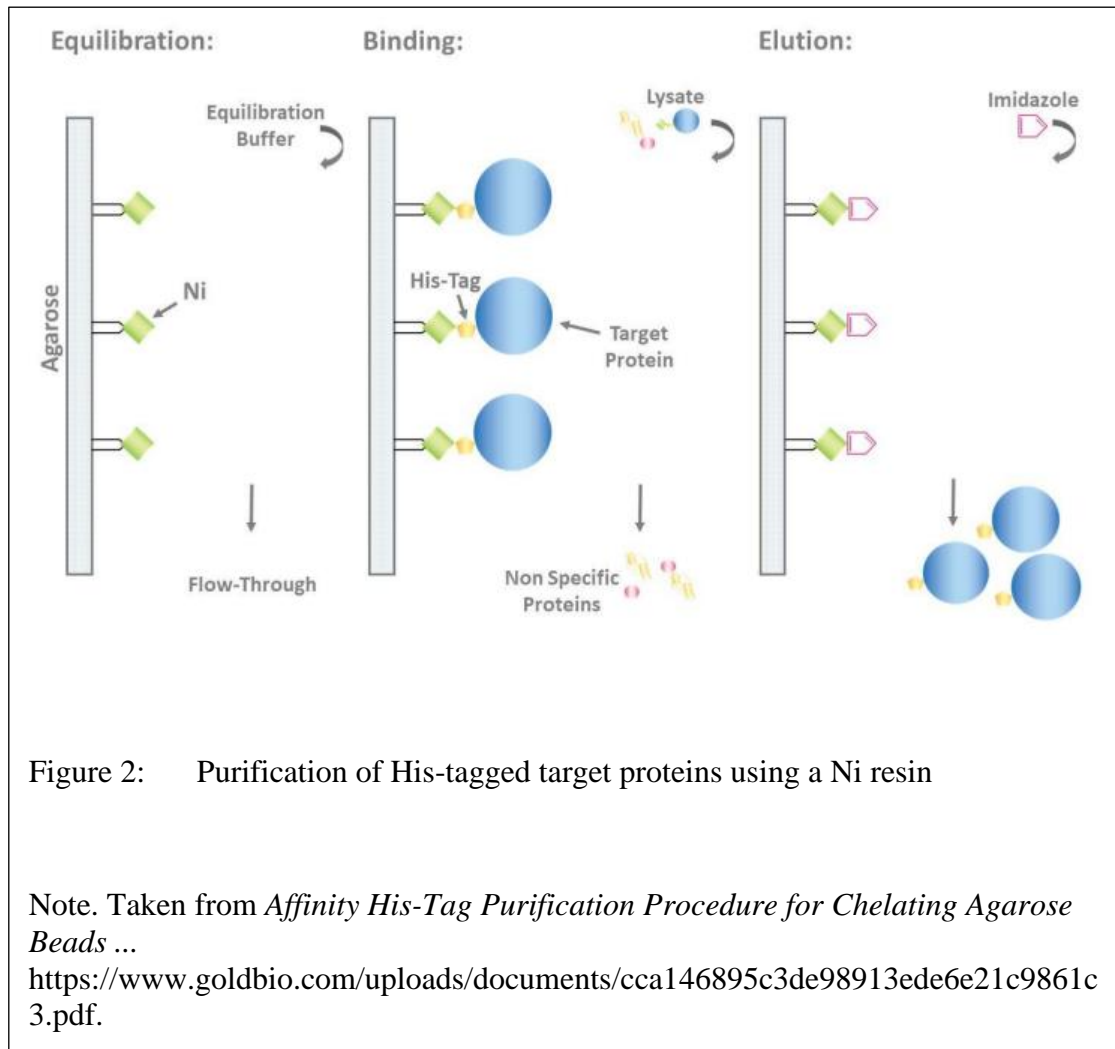


Figure 2: Purification of His-tagged target proteins using a Ni resin

Note. Taken from *Affinity His-Tag Purification Procedure for Chelating Agarose Beads ...*

<https://www.goldbio.com/uploads/documents/cca146895c3de98913ede6e21c9861c3.pdf>.

1.4 GEL FILTRATION CHROMATOGRAPHY

Gel-filtration chromatography (GFC) is a type of partition chromatography that is used to separate molecules of varying sizes. It is a versatile method that allows effective separation of biological molecules in high yield⁵. The fundamental principle of gel filtration is simple. As a function of their respective sizes, molecules are partitioned between a mobile phase and a stationary phase (comprising a porous matrix with prescribed porosity). The column is commonly in bead form and has two quantifiable volumes- the external volume, which is the liquid between the beads, and the internal volume, which is the liquid within the beads. The external volume is referred to as the void volume (V_0) and the sum of the external and internal volumes is the total volume (V_t). Following sample application, molecules larger than the pores of the stationary phase matrix will be excluded from the internal volume within the beads and thus migrate quickly through the column, emerging at V_0 , whereas molecules smaller than the matrix pores, as well as those intermediate in size, will equilibrate with both the external and internal liquid volumes, causing them to migrate much more slowly and emerge at a volume (V_e) greater than V_0 . As a result, molecules are eluted in decreasing order of molecular size.

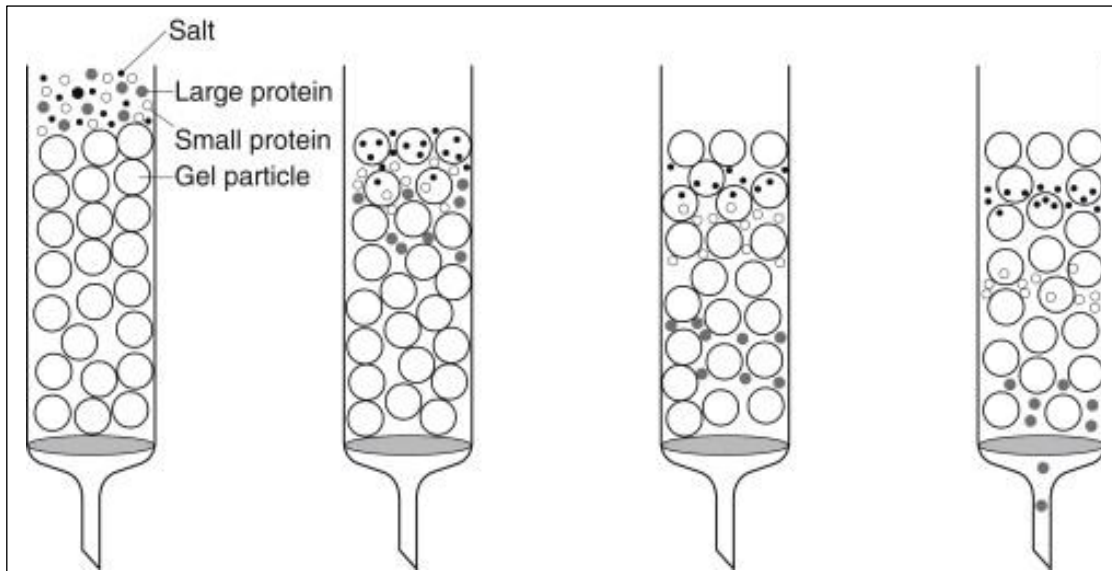


Figure 3: Progressive separation of molecules of different sizes by gel filtration

Note. Taken from *Fermentation (Industrial)/Recovery of Metabolites*, by S.G.

Prapulla & N.G. Karanth. *Nucleic Acids Res.* 14 April 2014.

<https://doi.org/10.1016/B978-0-12-384730-0.00109-9>. Copyright by S.G. Prapulla

Many matrices are available to be used during GFC such as Agarose-Based Support Media, Silica-Based Support Media, Hydroxylated Meth-Acrylic Polymer Resins, and many more. The choice of matrix depends on the molecules being separated in order to avoid partial adsorption of molecules to the matrix, which slows their flow through the column.

Gel Filtration also comes with its disadvantages because the target protein becomes an abundant substrate for proteases present in the mixture over time and there

is a higher chance of the target protein getting proteolyzed. Moreover, the requirement for large volumes for the eluent significantly increases the running costs for the method which inherently has a lower resolution compared to other chromatographic techniques. Despite these drawbacks, gel-filtration chromatography continues to play an important role in biomolecule separation because to its ease of use, dependability, adaptability, and scale-up.

Chapter 2

RATIONALE

Characterization of circular RNAs is integral as it allows researchers to gain insight into the possible functions of circular RNAs and decode their meanings. In addition to roles such as binding to RNA-binding proteins, sponging miRNAs, and regulating mRNA synthesis, they are also associated with and can act as potential biomarkers in case of many neurological, cardiovascular, autoimmune diseases, and various cancers¹⁵. The objective of this thesis is to purify RNaseR with a His-Sumo tag and test its activity on linear and circular RNA.

Based on research, cell lines such as C41DE3 and C41DE3/pLysS can be used to grow cells with the pSAT-RNR plasmid that contains the gene for the enzyme RNaseR with a polyhistidine tag. The pSAT-RNR plasmid contains Ampicillin resistance gene. The *E coli* cells are transformed with this plasmid and the obtained transformants are then grown in primary and secondary cultures in presence of ampicillin to ensure successful transformation or insertion of plasmid in cells¹⁴. The cells are

lysed or ruptured, and the enzyme can be retrieved. After the tagged RNaseR has been extracted, chromatography processes such as Nickel Column Chromatography can be used to purify RNaseR because His-tag proteins have higher affinity for nickel immobilized column beads compared to native proteins. Urea wash and a high salt wash can also be added during Ni-NTA chromatography as RNaseR is shown to be active at high urea concentrations and high salt environments. This step will further help us degrade contaminants because most contaminants break down in the presence of high concentrations of Urea, allowing us to obtain pure RNaseR¹³. Next, the tag can be cleaved, and Ni-NTA chromatography can be run again. At this point of the experiment, if the protein is not pure, the protein can be concentrated, and gel filtration chromatography can be run to separate the constituents of the fraction based on size. After obtaining pure RNaseR, an activity assay can be run to test if RNaseR is functional. This can be achieved by running a real-time quantitative polymerase chain reaction (RT-qPCR) using linear and circular primers to check levels of linear and circular RNA in real time after treatment with purified RNaseR. Controls such as buffer, no template control (blank), and positive control are required to validate the results. The purified RNaseR can then be used

in experiments such as RT-PCR, Single molecule fluorescence in situ hybridization (smFISH) to determine the level and distribution of circular RNAs in various biological contexts¹²

MATERIALS AND METHODS

2.1 CELL LINES

C41(DE) cells were transformed with the pSAT-RNR plasmid containing the *rnr* gene fused with His₆-tag required for the production of RNaseR. The cell line was then grown on a plate containing ampicillin (100 µg/ml) to identify colonies that were successfully transformed. A single colony from the ampicillin plate was inoculated in 100 ml of primary cultures were then obtained in Lennox Broth (LB) or auto-induction (AI) media. The secondary cultures were grown in a 37°C incubator shaker for 16 hours at 200 rpm. 15ml of the primary culture was transferred to a 2-liter LB media along with 2 ml Ampicillin. The secondary cultures were grown in a 37°C incubator for 4 hours rotating at 200 rpm. The cultures were then induced with 0.05mMIPTG and then grown in a 20°C incubator and then shaken at 180 rpm.

LB Broth Recipe

LB Broth was prepared by dissolving 40 grams of LB Lennox Media in 2 liters of Deionization (DI) water.

Auto Induction Media Recipe

The Auto Induction Media Recipe is as follows:

Table 1: 20X Stock (see Table 2) Composition

Component	Weight per Volume
1M Na ₂ HPO ₄	141.96g per liter
1M KH ₂ PO ₄	136.086g per liter
0.5M (NH ₄) ₂ SO ₄	66.07g per liter

Table 2: Auto-Induction Medium composition

Component	Volume
Base Media (20g of N-Zamine and 10g of Yeast Extract in 2l of DI Water)	2l
20X Stock	100ml
10% α -lactose monohydrate (24g per 240ml of Base Media)	40ml
1M MgSO ₄ (6.018g per liter of Base Media)	4ml

1X Trace Metals	400ul
50% Glycerol	20ml
Ampicillin	2ml
Overnight culture	15ml

2.2 BUFFER PREPARATION

Buffers were prepared for lysis, nickel column chromatography, and gel filtration chromatography. The buffer compositions were as follows:

Ni-NTA Buffers

Table 3: Buffer A (low salt) composition

Component	Volume or Weight
25mM HEPES	11.8g
500mM NaCl	58.4g (250ml in 4H stock)
20mM Imidazole	2.8g
10% Glycerol	200ml

Water	Fill it up to 2l
-------	------------------

Check pH and make sure it is 7.0

Table 4: Buffer A (high salt) composition

Component	Volume or Weight
25mM HEPES	6g
1M NaCl	58.4g (250ml in 4H stock)
20mM Imidazole	1.36g
10% Glycerol	100ml
20mM BME	1.4ml
Water	Fill it up to 1l

Check pH and make sure it is 7.0

Table 5: Urea Wash Buffer composition

Component	Volume or Weight

25mM HEPES	6g
500mM NaCl	29.2g
20mM Imidazole	1.36g
10% Glycerol	100ml
20mM BME	1.4ml
1M Urea	60g
Water	Fill it up to 1l

Check pH and make sure it is 7.0

Table 6: Buffer B composition

Component	Volume or Weight
25mM HEPES	3g
500mM NaCl	14.6g
250mM Imidazole	17.1g
10% Glycerol	100ml
2120mM BME	700ul

Water	Fill it up to 500ml
-------	---------------------

Check pH and make sure it is 7.0

Table 7: Gel Filtration Chromatography (GFC) Buffer Composition

Component	Volume or Weight
50mM Tris pH 8.0	50ml
100mM NaCl	25ml
0.1mM EDTA	200ul
5% Glycerol	50ml
1mM DTT	0.16g
0.1% TritonX	1ml
Water	Fill it up to 1l

Check pH and make sure it is 7.0

2.3 HARVESTING CELLS AND LYSIS

The secondary cultures were harvested to obtain the cells as follows. The cultures were poured into centrifuge bottles and balanced on a scale. They were centrifuged at 5000rpm for 20 minutes at 4°C. The supernatant was discarded and pellets were scooped and stored at -20°C. For lysis, the pellet from 4L culture (about 15 grams) was then taken out, thawed on an ice bucket, and transferred to a beaker. 100 ml of high salt buffer A was then added into the beaker for lysis and the pellet was allowed to dissolve in a cold room. After the pellet had dissolved, the cells were lysed using a sonicator using eight cycles of 40 seconds ON and 1 minute 30 seconds OFF. The crude lysate was then transferred to a pre-chilled 100ml bottle and centrifuged at 13,000 rpm for 45 minutes. The supernatant was then transferred to a pre-chilled 250ml Erlenmeyer flask.

2.4 Ni-NTA CHROMATOGRAPHY

The nickel column chromatography involved packing a column with nickel beads that bind to His-tagged proteins as in case of RNaseR. The AKTA Pure machine was cleaned using 0.5M NaOH and Guanidinium Hydrochloride. The desired fractions were then run on an 8% or a 12% SDS-Gel as follows. 10ul of the fraction and 10ul of 10X loading dye were mixed in an Eppendorf tube and incubated at 100°C for 2 min. 10ul of the solution was loaded onto the gel, and an appropriate amount of Ladder or molecular weight standard was loaded (typically 10ul). The ladder used was Precision Plus Protein™ Standards Unstained ladder by BIO-RAD.

2.5 Gel Filtration (GFC) CHROMATOGRAPHY

The desired fractions were concentrated by pouring them into a concentrator and centrifuging at 5000rcf for 30 minutes at 4°C. The pure fractions that did not need to be purified further were saved at -80°C and the fractions that needed to be purified were pooled together and gel filtration chromatography was run to separate the constituents based on different sizes and shapes. The desired fractions were subjected to SDS-PAGE as described above.

2.6 ACTIVITY ASSAY

The concentration of GFC fractions and the pure concentrated fractions after Ni-NTA chromatography was analyzed using Bradford Assay and a spectrophotometer. According to the Lucigen RNaseR manual, one unit or 50ng of RNaseR can convert 1ul of RNA into acid-soluble nucleotides in 10 minutes at 37°C². The standard concentration of RNaseR used in the laboratory was 1.5 units. An activity assay was then run to confirm the function of RNaseR enzyme. First, 1ug of HS-1 RNA was pipetted into a tube with an appropriate amount of RNaseR, 1ul of RNaseR reaction buffer, and volume was adjusted to 10ul with DI water. This tube was incubated at 37°C for 1 hour. This tube was either labeled using the fraction number or “Buffer + Enzyme”. A blank control or NTC tube was also prepared in some cases where no RNA was added to the cDNA preparation. This control showed whether contamination was present in the PCR tubes, or a false positive result was produced due to primer-dimer formation.

A “Buffer” tube was also made in all PCR runs which included all reagents except RNaseR enzyme. This acted as a control that checked for the effectiveness of the PCR to amplify cDNA. This tube A positive control tube with commercial Lucigen RNaseR was also prepared in some runs. The required tubes converted to cDNA by taking 5ul out of tubes and adding 2ul of iScript mastermix and 3ul of Nuclease-Free (NF) H₂O and performing the following cDNA protocol:

Table 8: BIORAD cDNA synthesis reaction protocol

Temperature	Time
25°C	5 minutes
46°C	20 minutes
95°C	1 minute

After cDNA preparation, PCR tubes were prepared by adding 5ul of iTaq in each, 3ul of NF H₂O, 1ul of forward and reverse primers. Two set of primers-linearZNF609, and circularZNF609, or linearZBTB44 and circular ZBTB44 were used in the assay to test the presence of linear and circular DNA in the sample. The PCR was then set up and the amplification protocol used was as follows:

Table 9: iTaq 2-step PCR reaction protocol

Temperature	Time
--------------------	-------------

95°C	5 minutes
95°C	30 seconds
60°C	1 minute
65°C-95°C	5 seconds each, in increments of 0.5°C

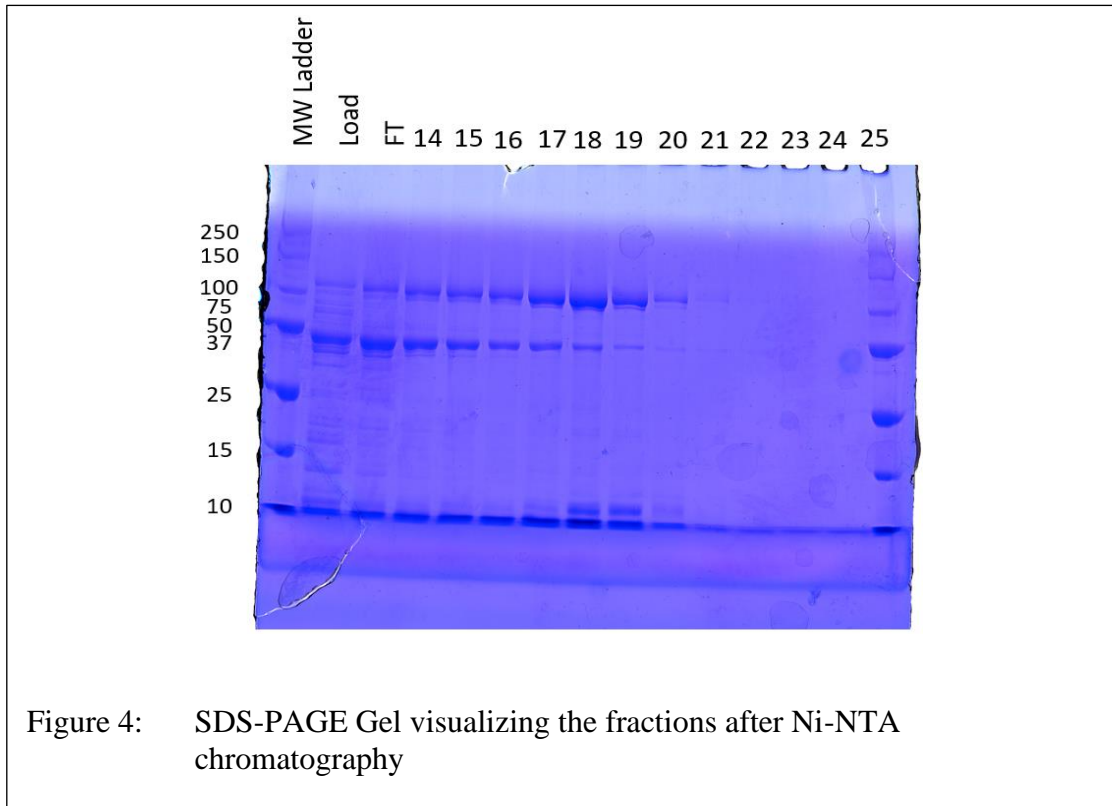
Finally, an agarose gel was run in some cases to confirm the presence or absence of linear and circular DNA in the sample.

Chapter 3

EXPERIMENTAL RESULTS

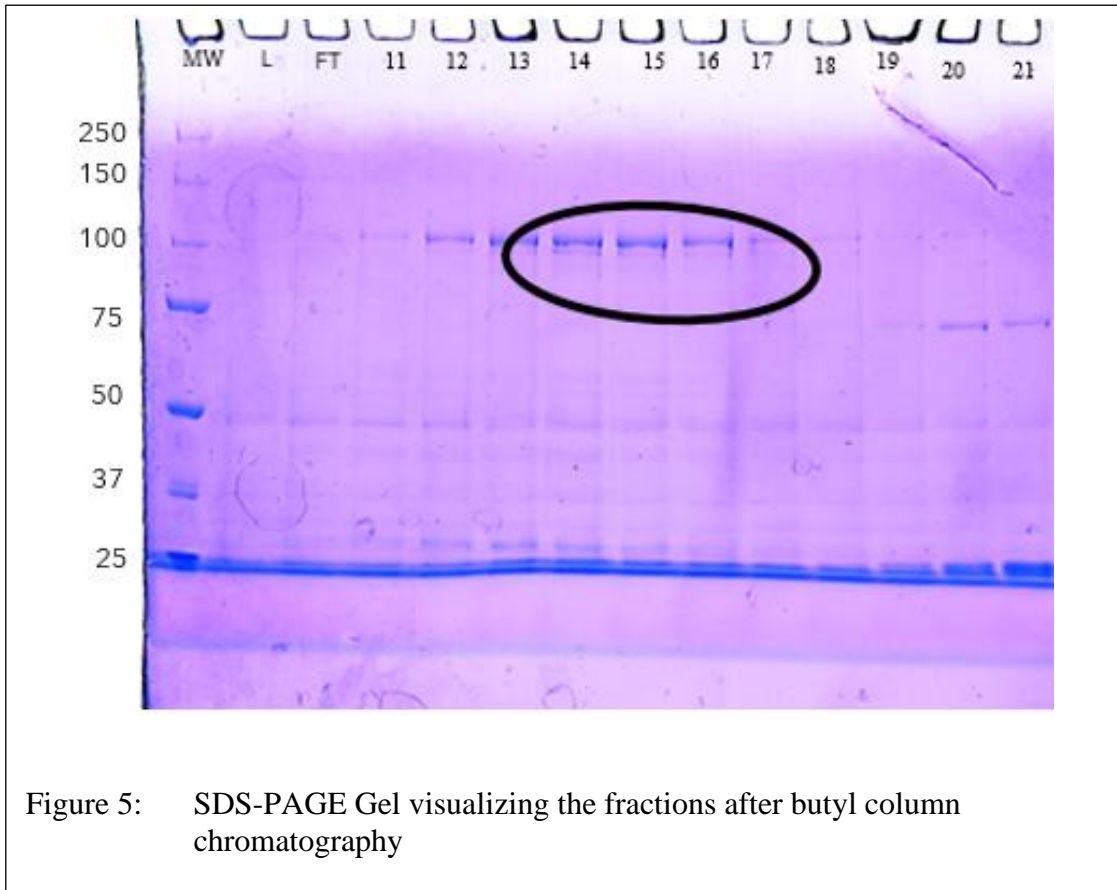
Twelve different runs were attempted to purify and isolate RNaseR. Each run included growing a culture of recombinant cells producing RNaseR, harvesting and lysing cell samples, and performing purification techniques such as nickel column affinity chromatography, butyl column chromatography, and gel filtration chromatography to separate components based on the affinity of the tag, hydrophobicity, and size respectively. Chapter 3 (Experimental Results) expands on four runs that were significant and had or are hypothesized to have a considerable impact on the purification procedure of RNaseR.

3.1 RNASER PURIFICATION RUN 1

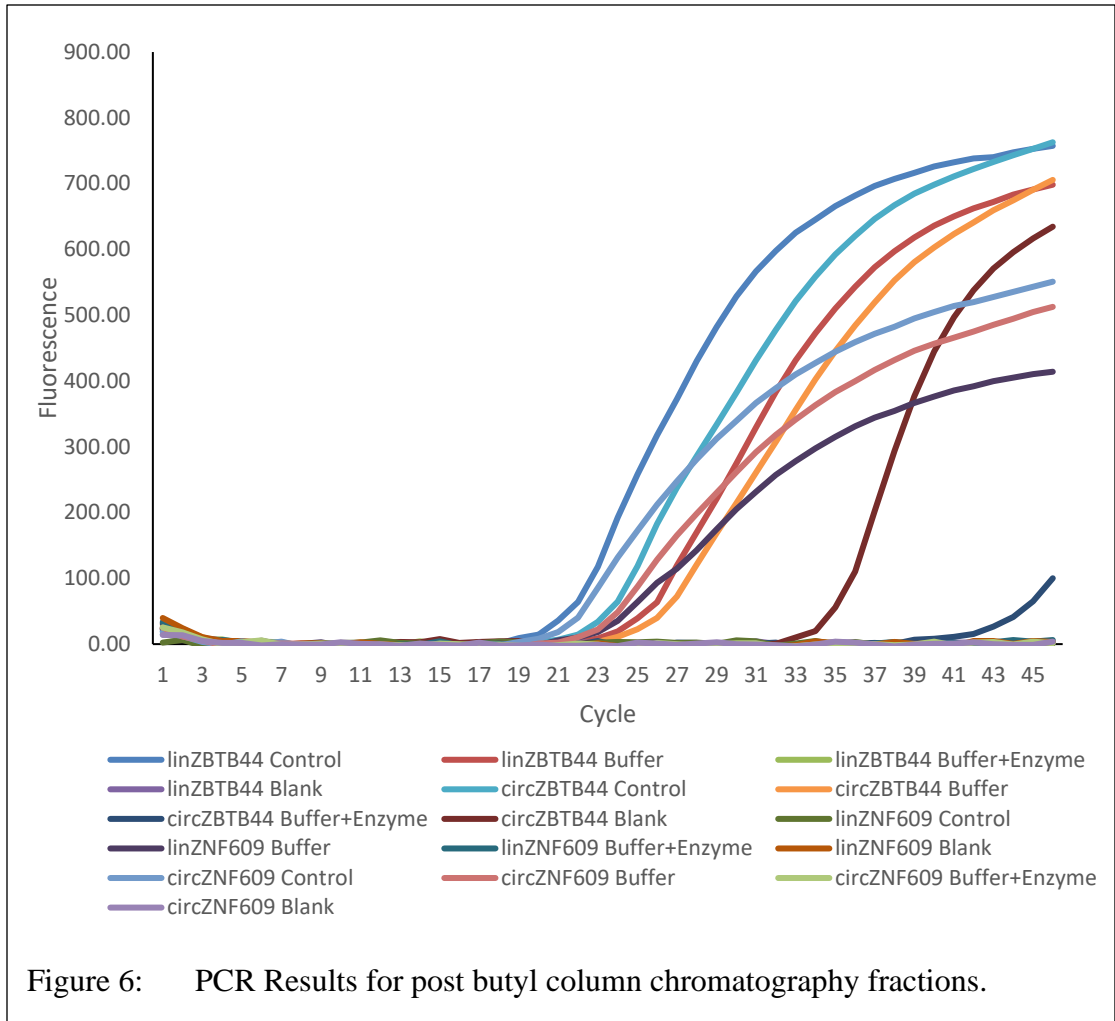


The expression of the protein was high, and the sample was not excessively contaminated. The fractions 17, 18, and 19 were pooled together as they showed good RNaseR expression and were loaded on a butyl column to separate the components based on hydrophobicity.

After the SDS-PAGE Gel, these fractions were run through a butyl column that separated the protein based on its hydrophobicity.



Fractions 14-16 were pooled together, and PCR was run to check the activity of RNaseR.

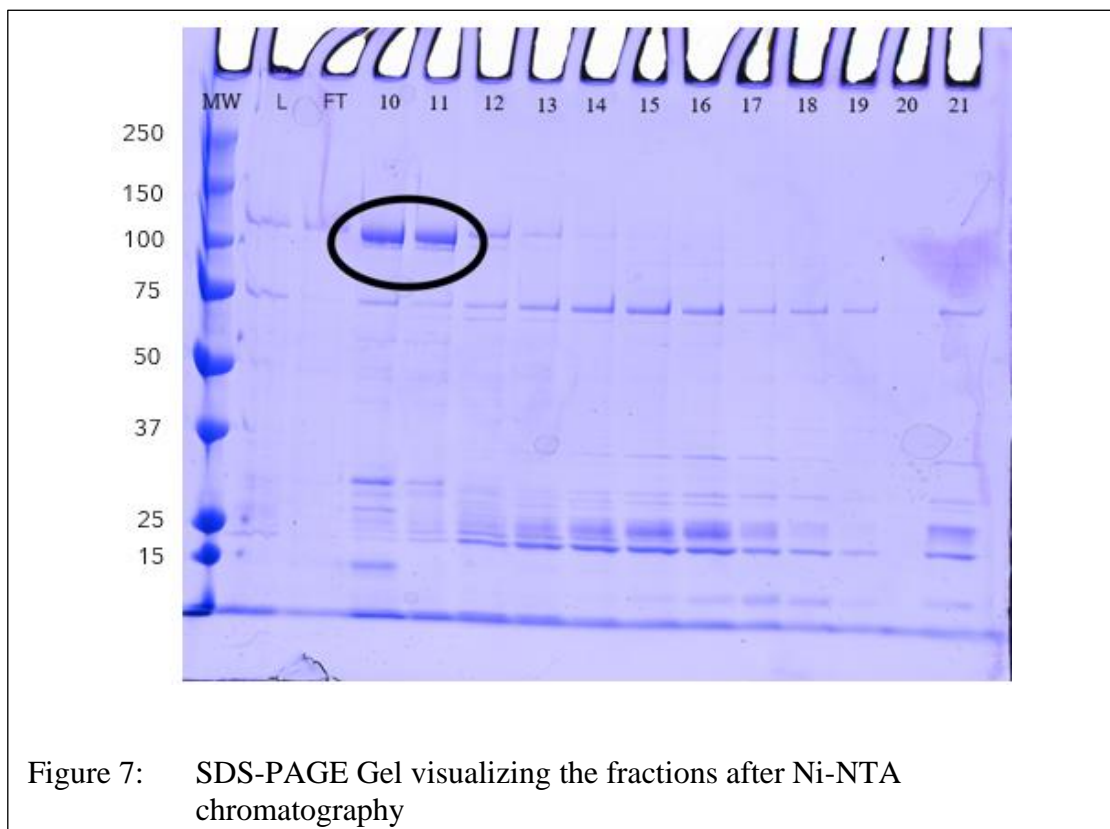


Amplification was observed for linZBTB44, circZBTB44, and circZNF609 controls, linZBT44, circZBT44, linZNF609, circZNF609 buffer samples, and circZBTB44 Buffer+Enzyme samples. The linZBTB44 samples show expected results. However, no amplification for circZNF609 Buffer + Enzyme sample shows that the enzyme degraded

circRNA as well. Moreover, amplification for circZBT44 blank, and no amplification for linZNF609 control implies that the PCR results are not completely reliable.

Next, Gel Filtration Chromatography was performed. However, no proteins were observed in the fractions; The proteins were lost due to backpressure.

3.2 RNASER PURIFICATION RUN 2



Fractions 10 and 11 were pooled together as they looked relatively pure and had good expression of our desired protein, RNaseR. The pooled fraction was then loaded onto a butyl column that would separate the components based on hydrophobicity.

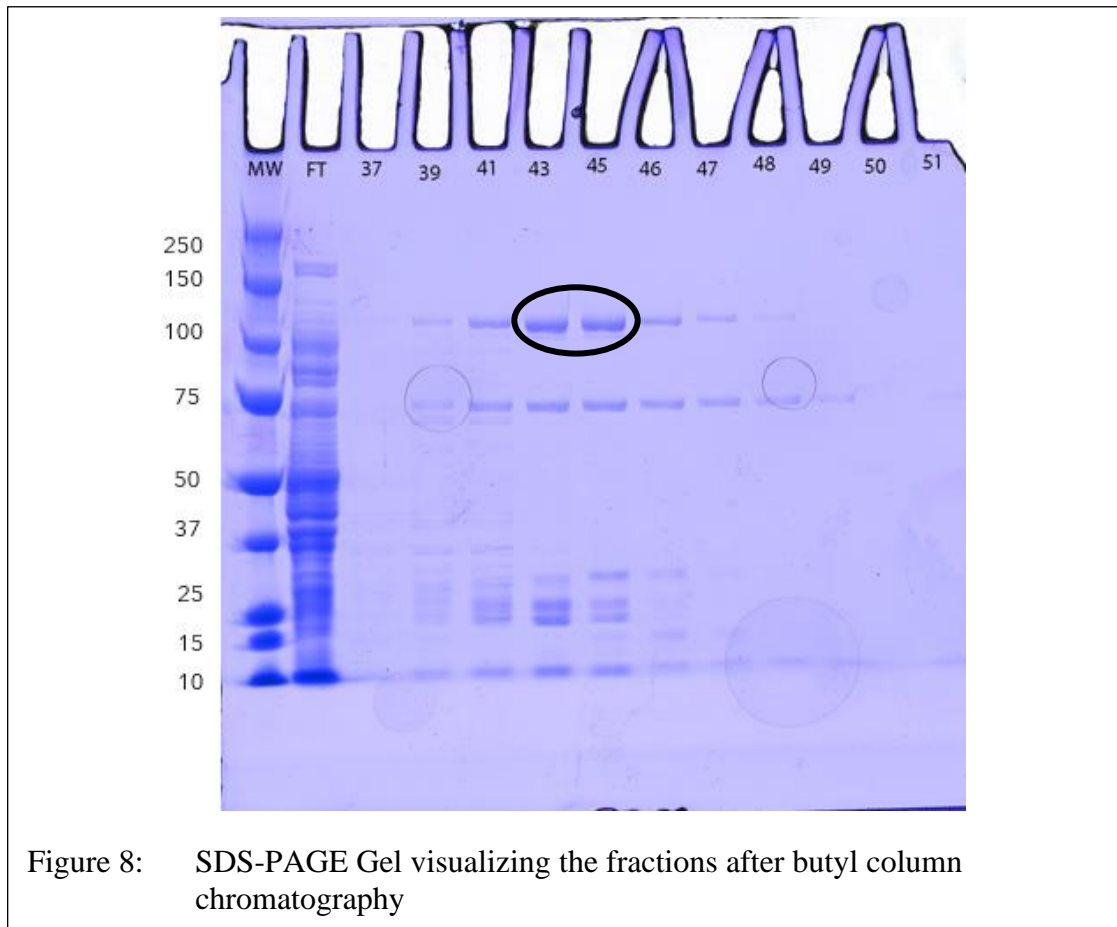
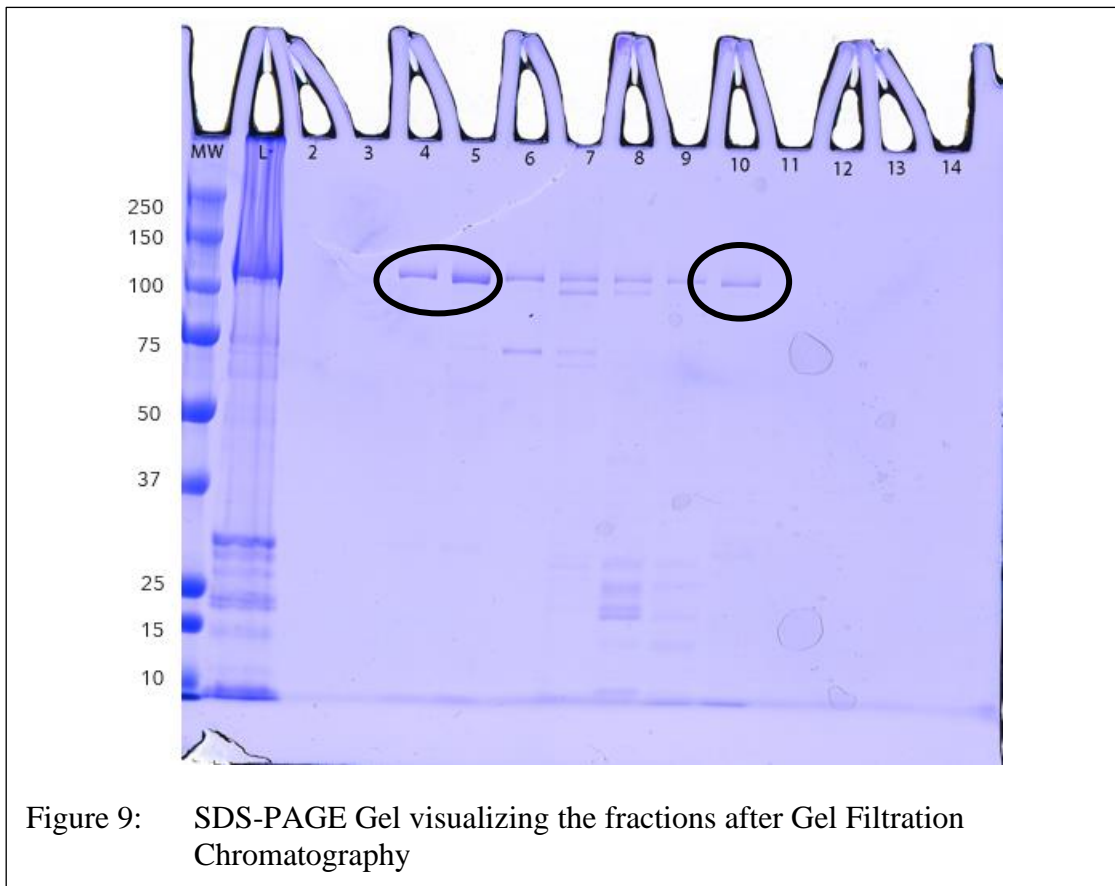


Figure 8: SDS-PAGE Gel visualizing the fractions after butyl column chromatography



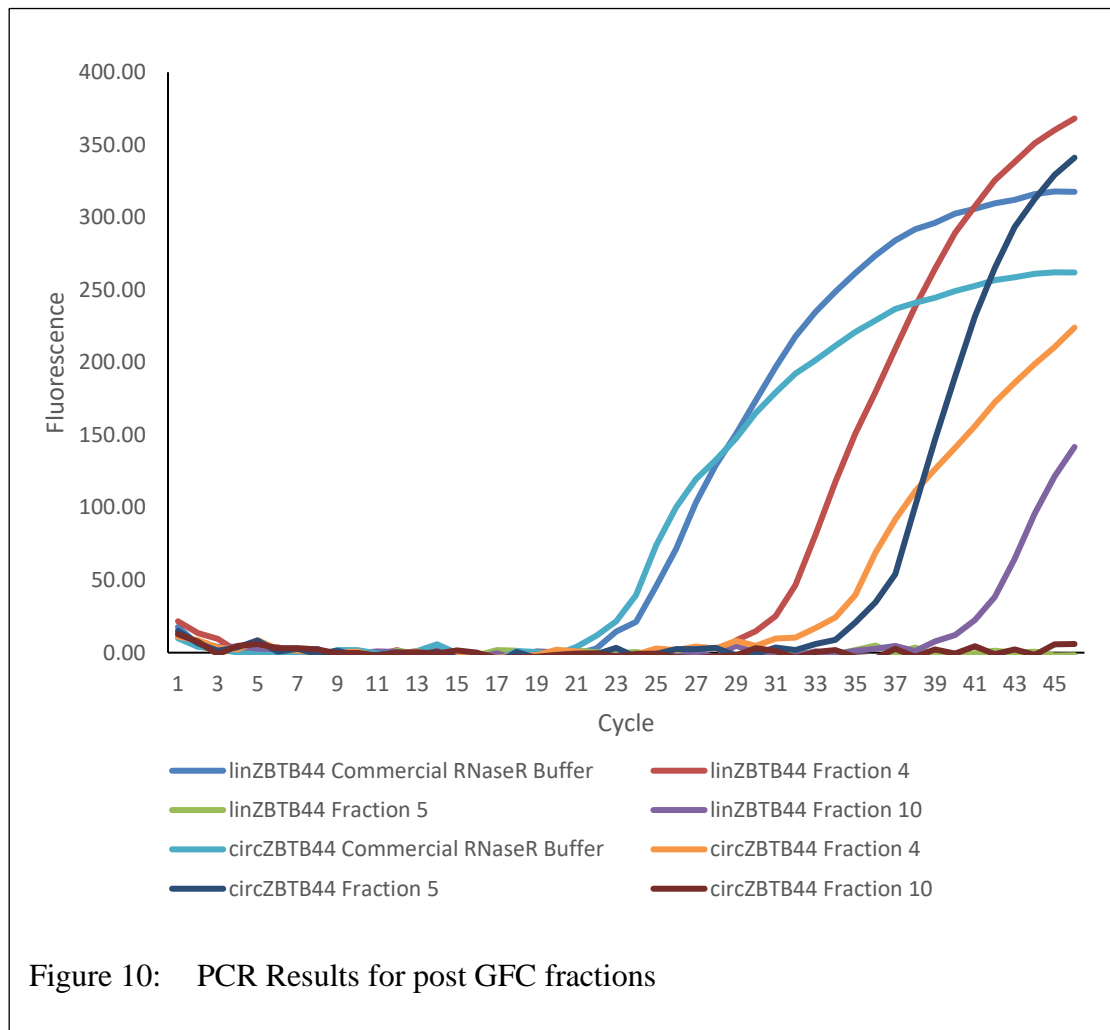
Fractions 4, 5, and 10 were chosen for the PCR assay since they looked pure and had relatively low contaminants. Fractions were not pooled together, and assay was done as follows:

RNA -> Buffer, Fraction 4, Fraction 5, Fraction 10. 1ug of RNA (0.75ul), 1ul buffer, 1ul fraction, 7.25ul water (8.25ul for buffer since fraction was not added). Each tube had a total volume of 10ul.

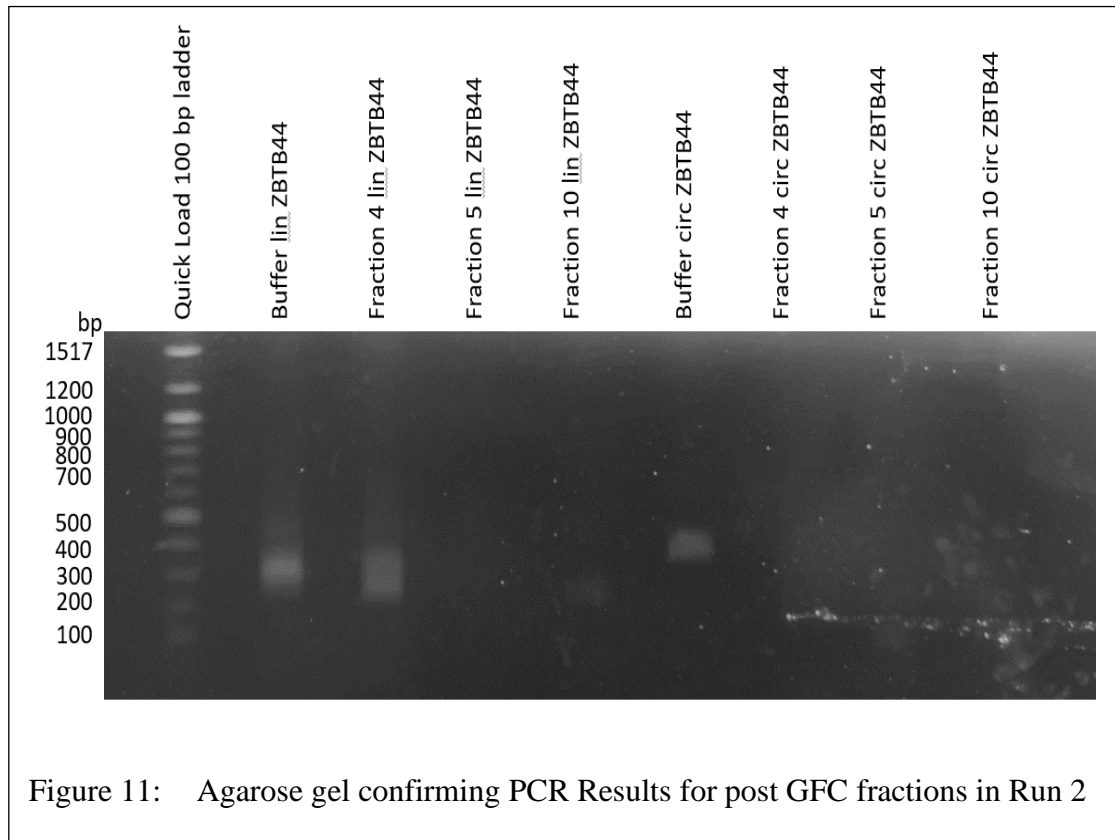
Tubes were allowed to incubate at 37°C for 1 hour and then cDNA was made. 4ul master mix (standard value) and 6ul water was added to each tube to make the volume of each tube 20ul in total.

PCR was ran in order:

Buffer linZBTB44, Fraction 4 linZBTB44, Fraction 5 linZBTB44, Fraction 10 linZBTB44, Buffer circZBTB44, Fraction 4 circZBTB44, Fraction 5 circ ZBTB44, Fraction 10 circ ZBTB44. 4ul cDNA, 22.5ul MM, 4.5ul primers, 13.5ul water was added to every tube.



Commercial RNaseR buffer sample signal for both circZBTB44 and linZBTB44 were as expected. Fraction 4 showed fluorescence for linZBTB44 and circZBTB44. Fraction 10 seemed to have degraded everything both linear and circular RNA. This might have been caused due to contamination of other RNases. Fraction 5 seemed to work and only degraded linearZBTB44 but showed fluorescence for circZBTB44. However, Cq value for circRNA was pretty high. Thus, an agarose gel was run to confirm the PCR results.



Ladder ran fine. Bands were observed for buffer linZBTB44, Fraction 4 linZBTB44. Faint band observed for fraction 10 linZBTB44 and a band was observed for buffer circZBTB44. However, no circRNA bands were observed for any fractions. Thus, fraction 5 did not work.

Since the bands produced during this run were relatively pure and free of contaminants, an SDS-Gel was ran to visualize the fractions at different concentrations and PCR was run again.

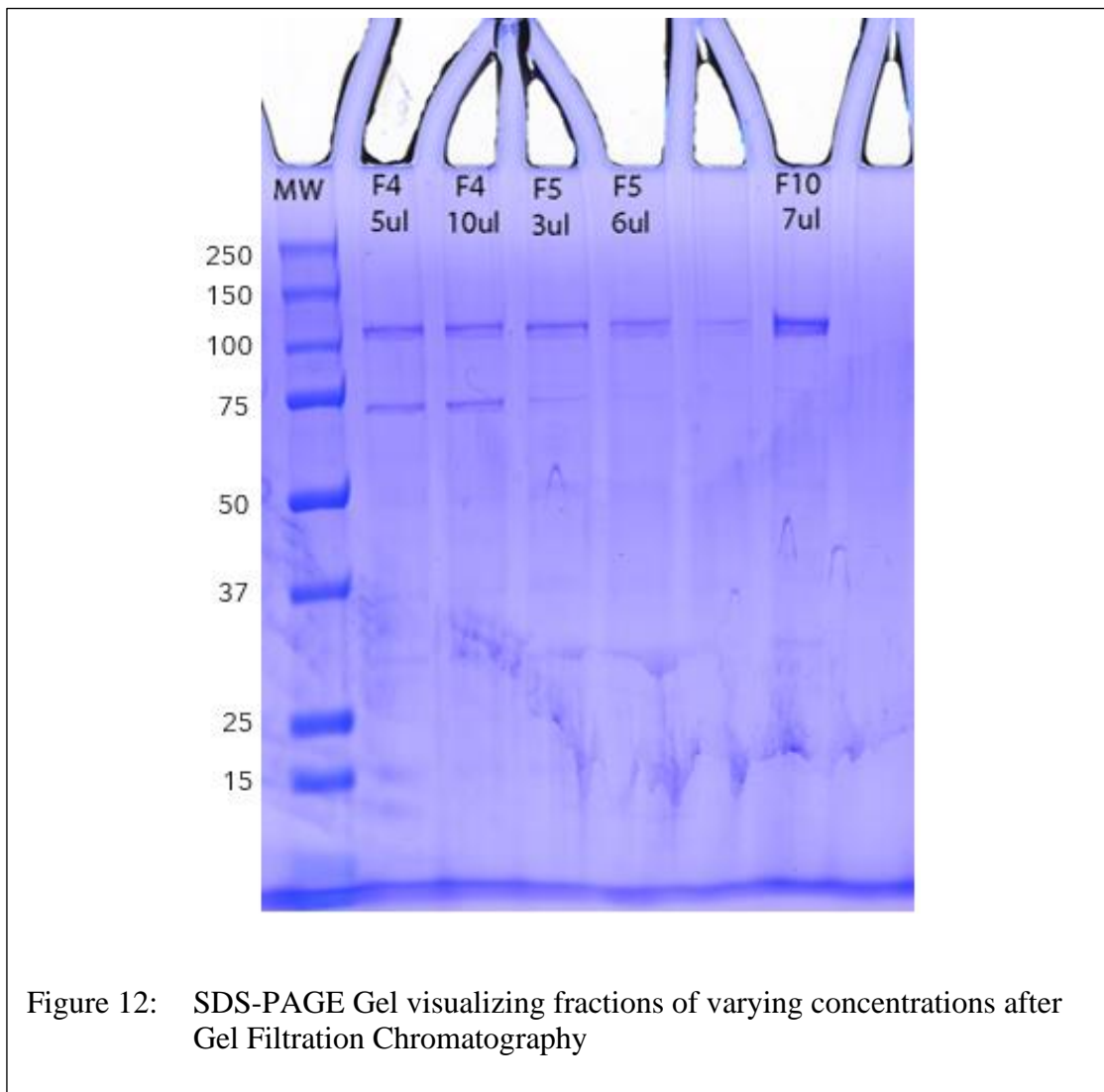
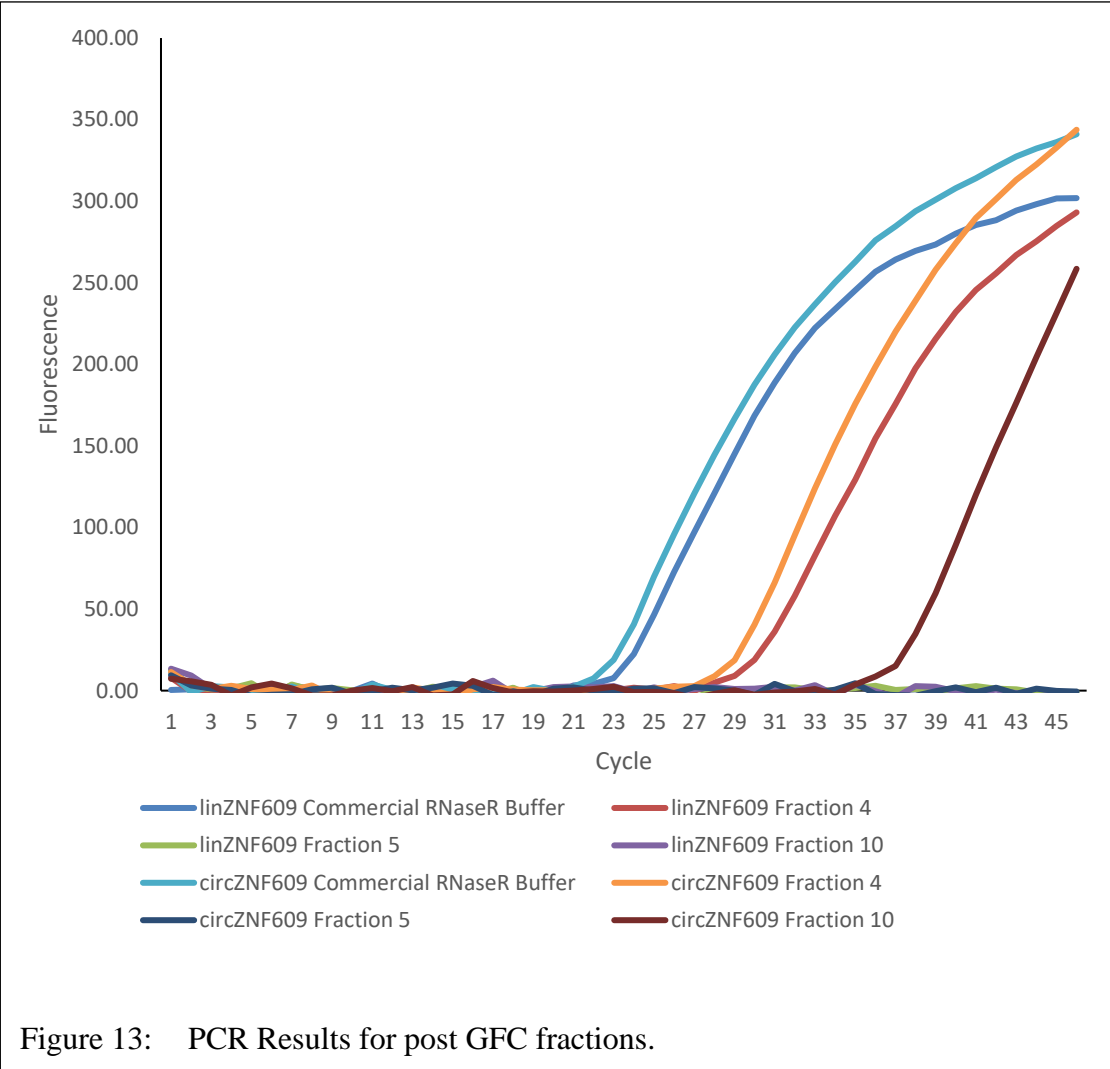
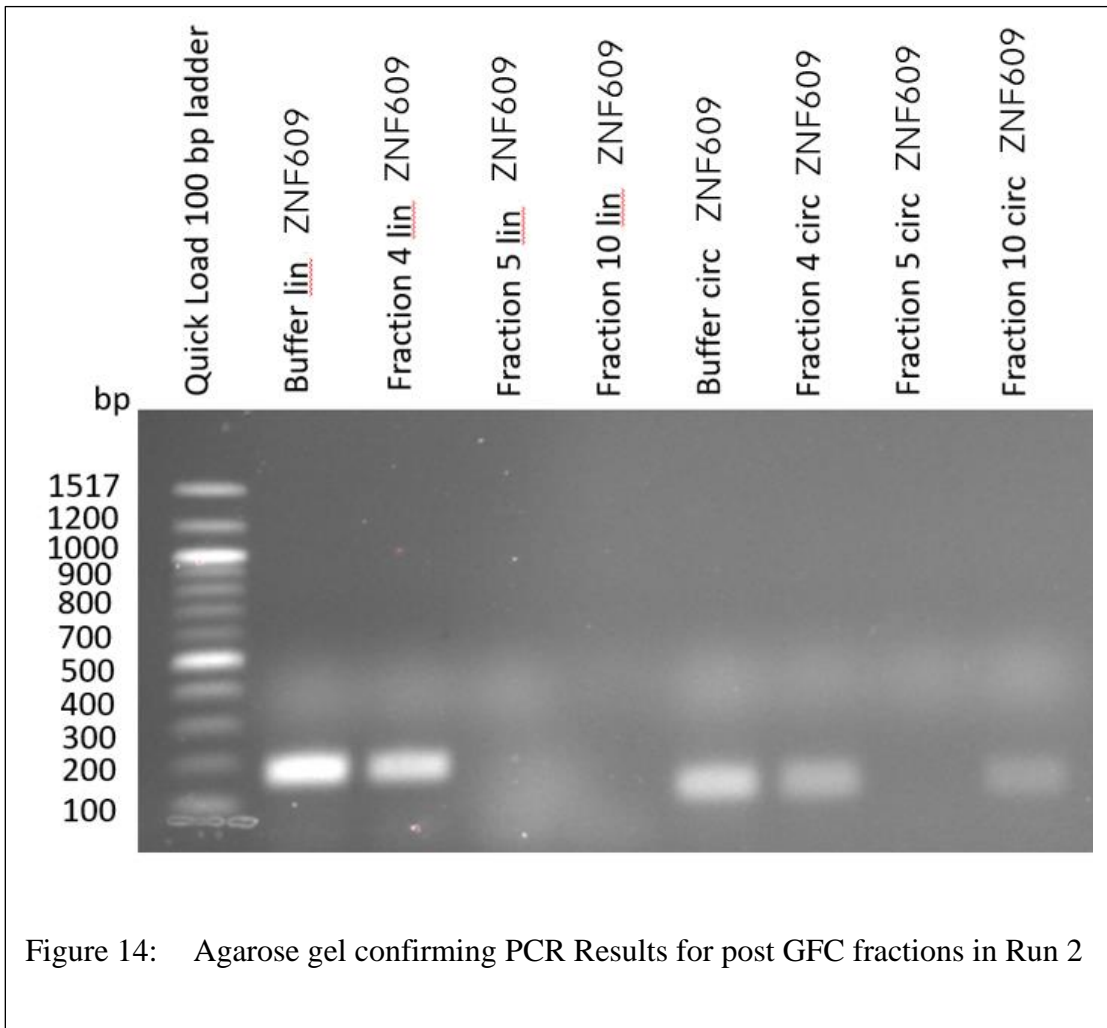


Figure 12: SDS-PAGE Gel visualizing fractions of varying concentrations after Gel Filtration Chromatography



Buffer fluorescence was as expected. Fraction 4 showed fluorescence for both linear and circRNA. Fraction 5 seemed to have degraded both linear and circular RNA. Fraction 10 however seemed to have worked and shows amplification for circZNF609 but not for linZNF609.



Ladder ran fine. Bands were observed for buffer linZNF609, Fraction 4 linZNF609, Buffer circZNF609, Fraction 4 circZNF609, and fraction 10 circZNF609. This implies that fraction 10 had proper functionality.

Since Fraction 10 seemed to be working according to the other runs, three concentrations, low, optimum, and high, (0.5 ug/ul, 1ug/ul, and 1.5 ug/ul) were used in a PCR. Concentration of Fraction 10 was determined using Bradford assay. PCR results were as follows:

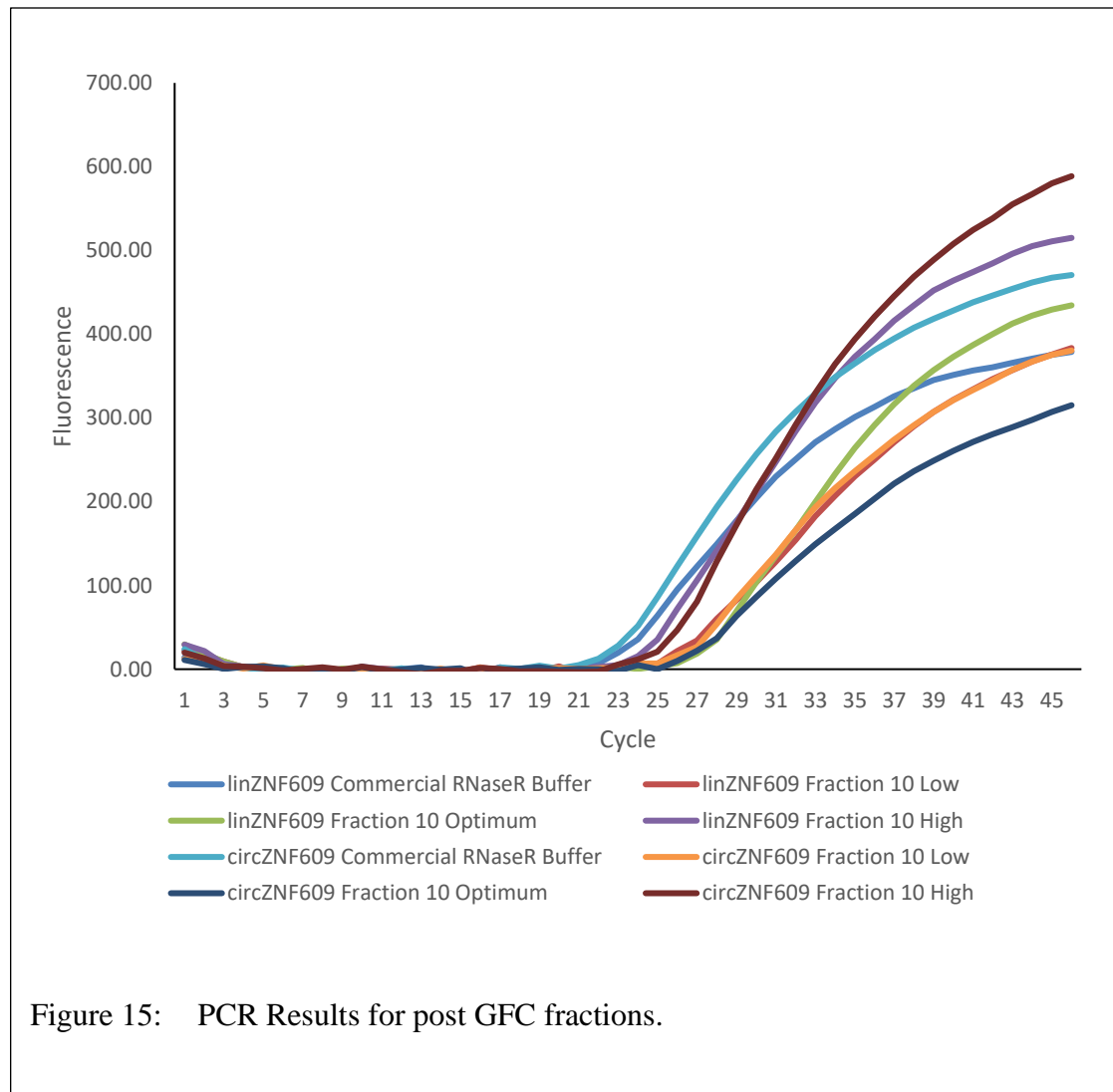
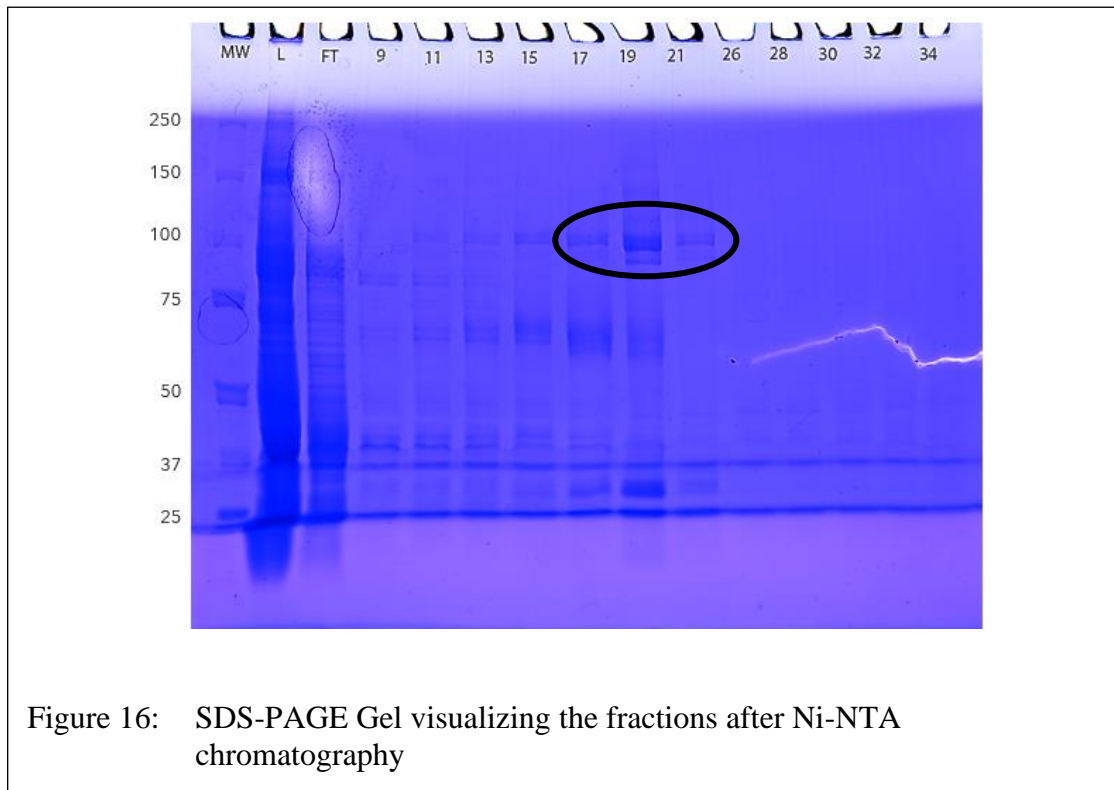


Figure 15: PCR Results for post GFC fractions.

No degradation was observed because not enough RNaseR was added to the PCR reaction tubes. There was a miscommunication and stocks were diluted ten-fold. Since 5ul of Fraction 10 was left, a new run was started to obtain more RNaseR.

3.3 RNASER PURIFICATION RUN 3



Fractions 17-21 were pooled together as they had relatively high expression of RNaseR and were more pure than other fractions that were collected after Ni-NTA column affinity chromatography.

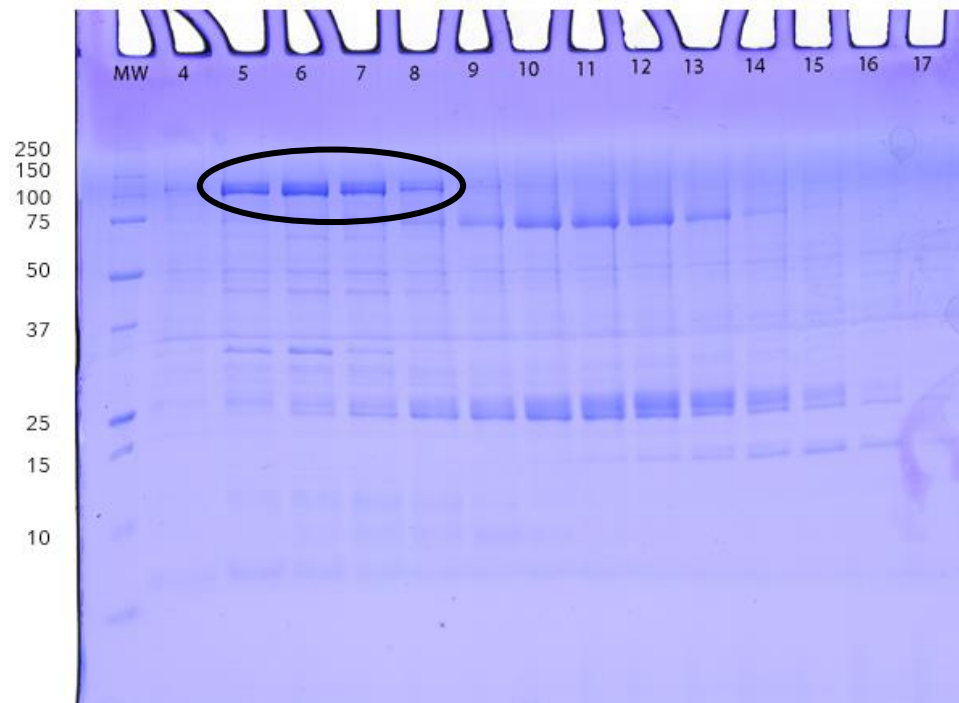
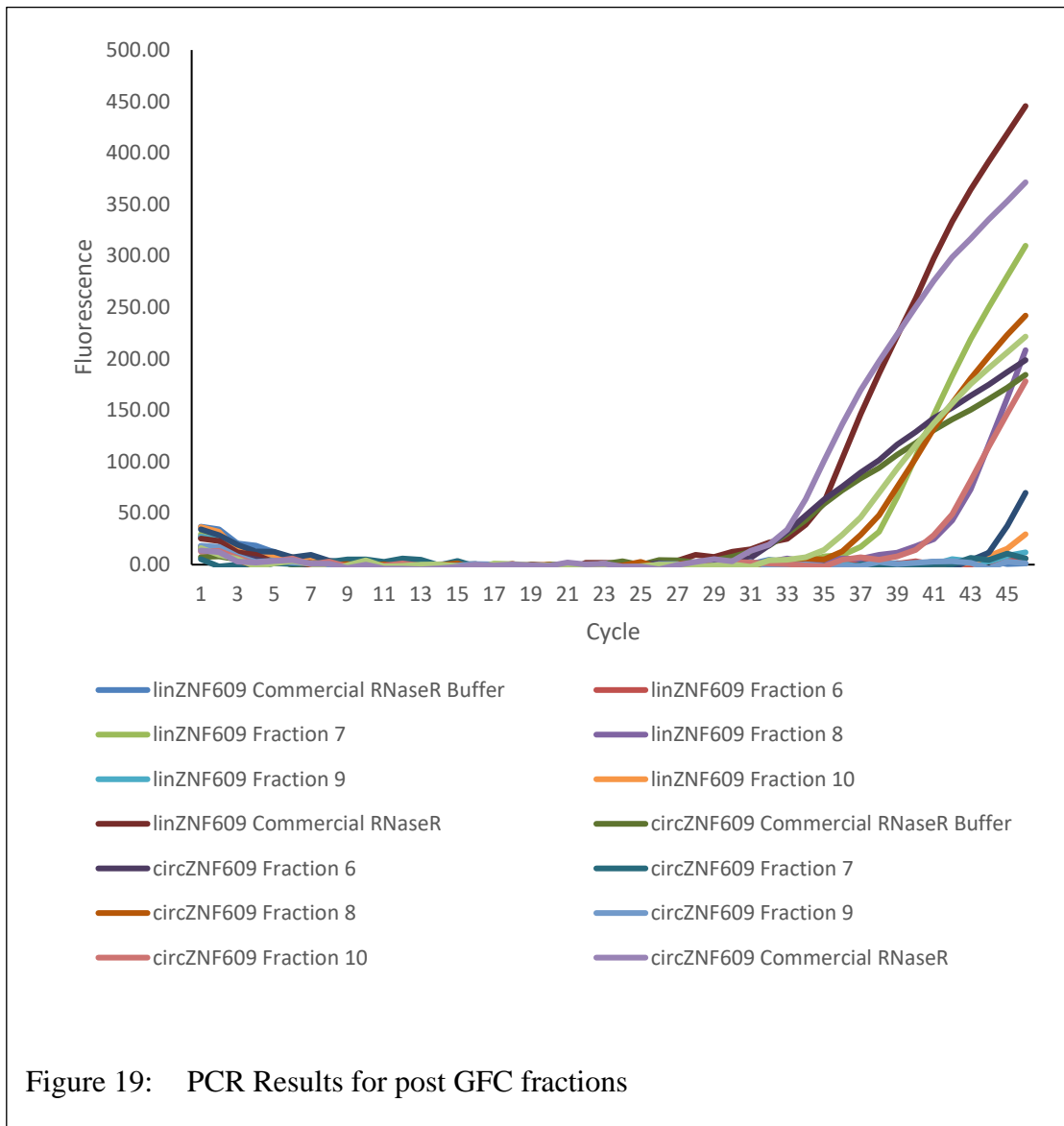


Figure 17: SDS-PAGE Gel visualizing the fractions after butyl column chromatography



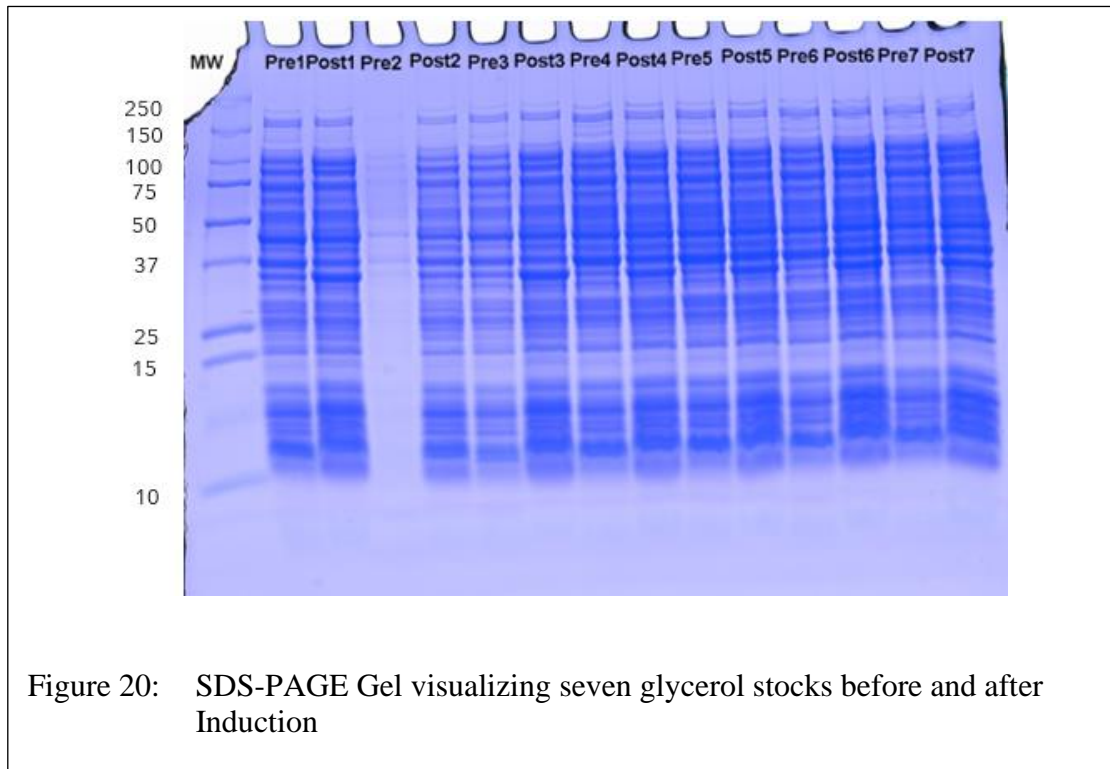
Figure 18: SDS-PAGE Gel visualizing the fractions after GFC



Fractions 6,7,8,9,10 were chosen for PCR assay. In retrospect, only 7,8, and 9 should have been chosen. Next, PCR was run for the above-mentioned fractions. PCR results were erroneous as no amplification was observed for linZNF609 Commercial RNaseR buffer, and commercial RNaseR showed no activity.

3.4 INDUCTION TEST

Since expression of our desired protein was relatively low and did not overexpress after IPTG induction, the glycerol stocks of the recombinant plasmid -containing expression strains were grown in small 15ml conical tubes and were visualized on an SDS-PAGE Gel.



The pSAT-RNR plasmid was then purified from the glycerol stock sent from Johns Hopkins University (JHU), four different BL21 cell lines were transformed microcultures were grown and were visualized alongside the JHU stock post induction with IPTG.

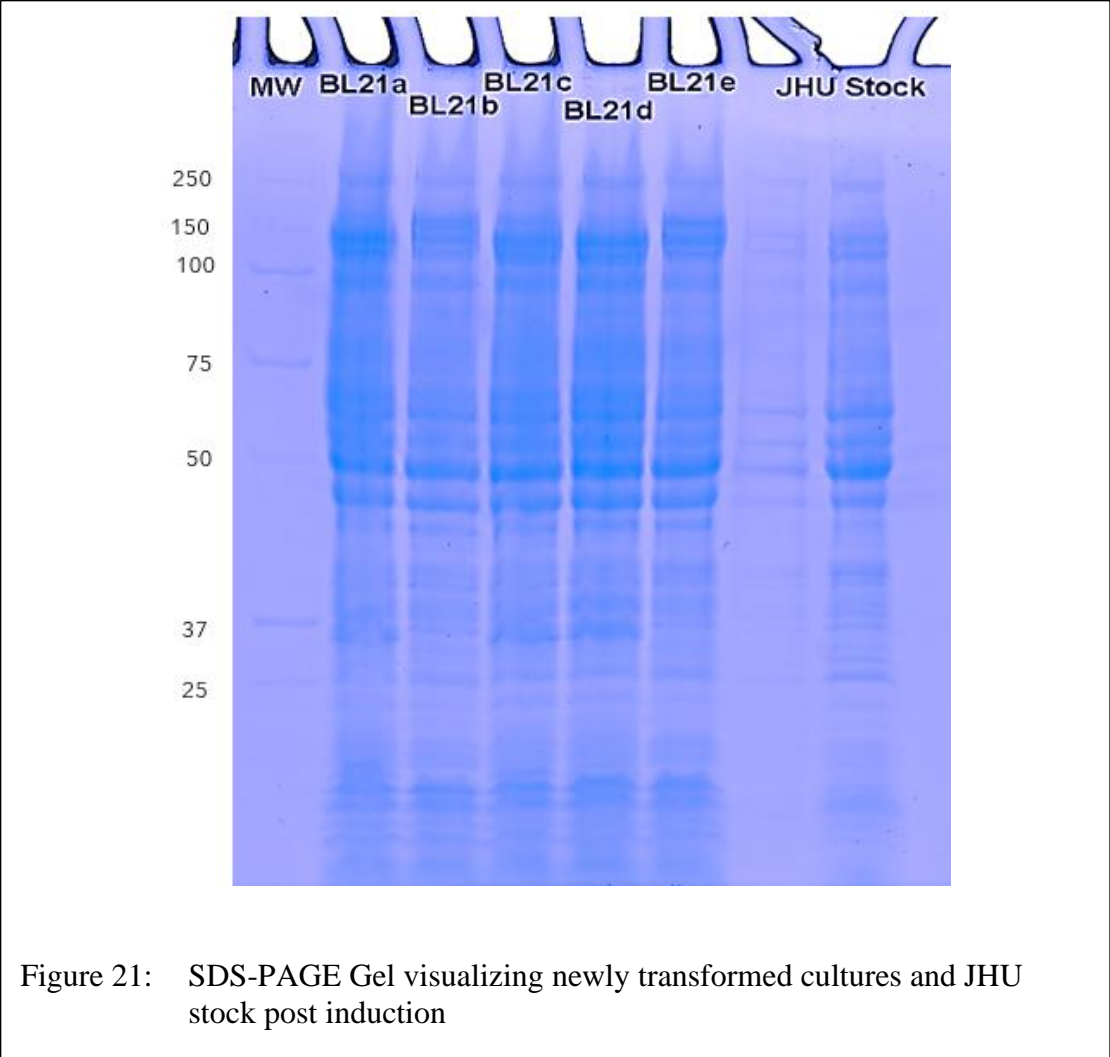


Figure 21: SDS-PAGE Gel visualizing newly transformed cultures and JHU stock post induction

3.5 RNASER PURIFICATION RUN 4 (AI Media)

Since expression of our desired protein, RNaseR was low, and overexpression was not observed post-induction, Auto Induction (AI) Media was used.

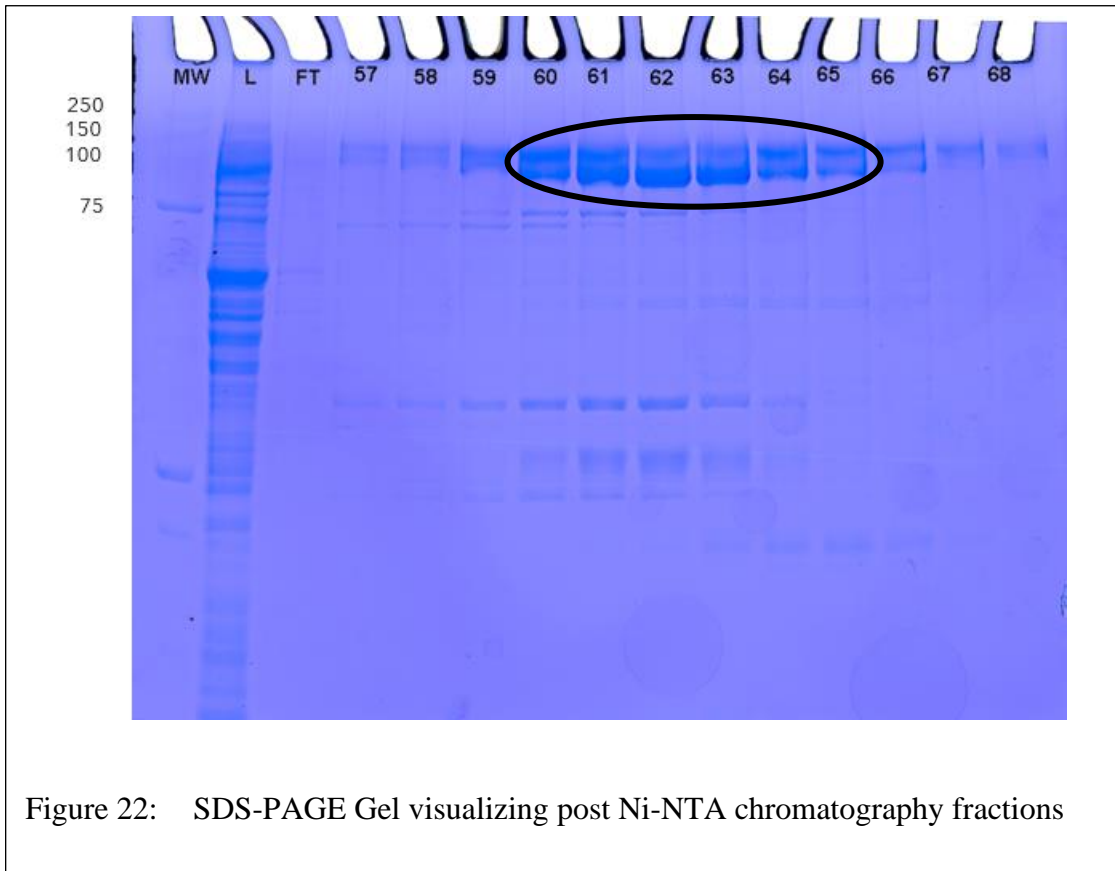
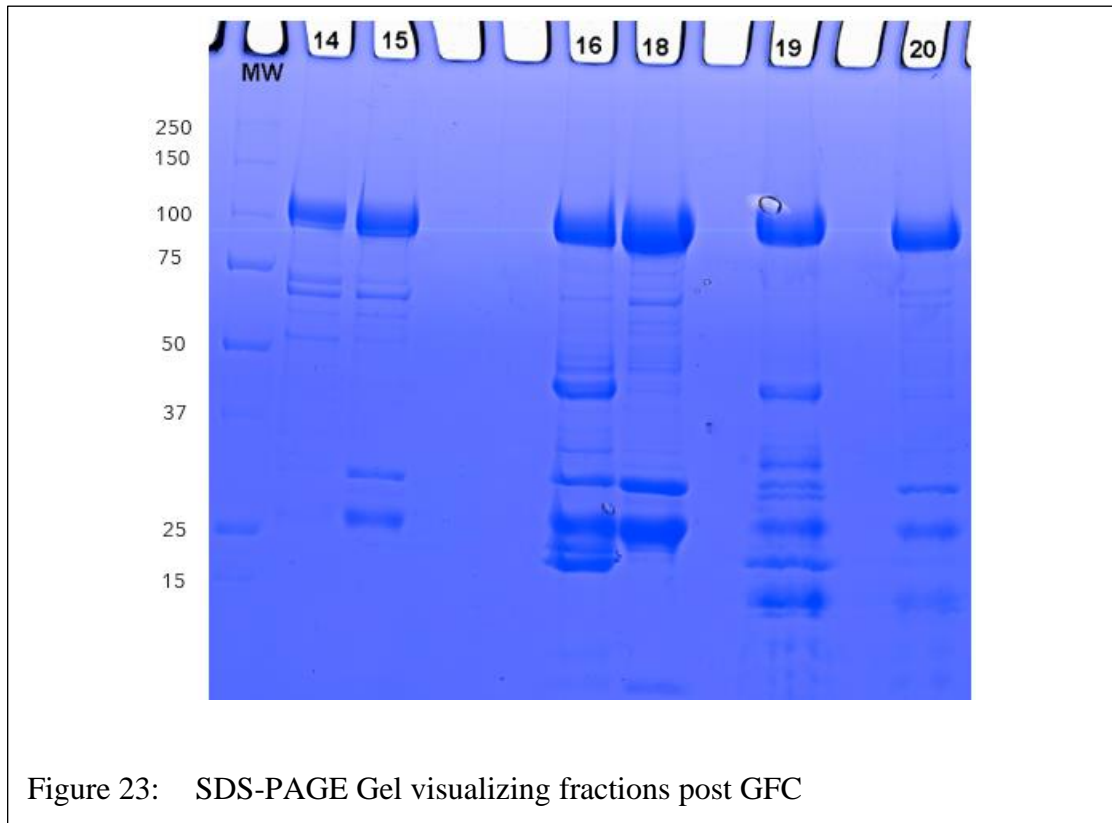


Figure 22: SDS-PAGE Gel visualizing post Ni-NTA chromatography fractions

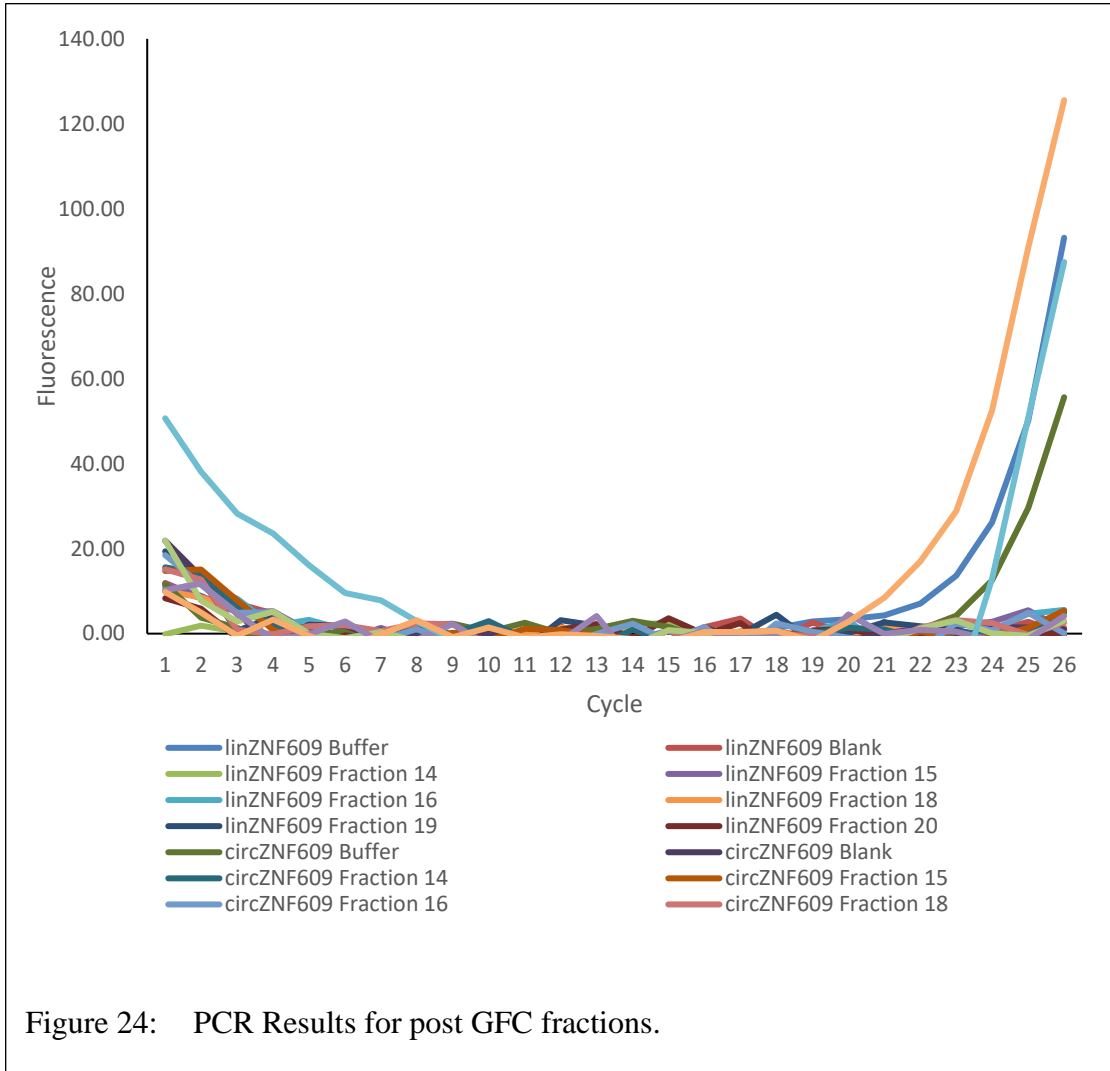
Higher expression of RNaseR was observed post Ni-NTA chromatography. However, fractions were still relatively impure and needed to be purified further using gel filtration chromatography. Fractions 60-65 were pooled together and concentrated. The concentrated fractions were then purified further using gel filtration chromatography.

Every fraction had a relatively high concentration of RNaseR but were impure as other bands were also observed alongside the RNaseR band. An activity assay using

1.5 units of RNaseR (or 1.5ug of RNaseR) was conducted to test the functionality of the produced RNaseR.



Every fraction had a relatively high concentration of RNaseR but were impure as other bands were also observed alongside the RNaseR band. An activity assay using 1.5 units of RNaseR (or 1.5ug of RNaseR) was conducted to test the functionality of the produced RNaseR.



It seems like the amount of RNaseR added was too high, the incubation time was too long, or the sample was contaminated with other RNases because no amplification is observed for any fractions. This is the most probable case because the SDS-Gel does indicate that the protein is not completely pure and free of contamination. Moreover, the PCR is inconclusive because no amplification is observed for both linear and circular RNA in case of commercial RNaseR.

Chapter 4

DISCUSSION

RNaseR Purification might seem fairly simple at first glance, but it is tough to isolate and purify because isolating the protein based on size or affinity individually does not result in a clean band, and the protein sample is vulnerable to contamination; introduction of other RNases in the sample causes the linear RNA to degrade, along with the circular RNA, resulting in an impure protein with improper functionality. Thus, multiple runs of RNaseR purification were attempted to successfully purify RNaseR.

A band around 50/60 kDa and another around 17kDa is observed in most SDS gels for the runs. A possibility for this is insufficient amount of EDTA during purification. A paper published in the year 1977³ reported RNaseR to be around 40-50kDa. This occurs because a smaller active form of RNaseR comprising the central region and C-terminal S1 RNA-binding domain of protein is observed in some cases where not enough EDTA is used during purification³. 1mM EDTA is too high for purification and enzyme activity is abolished. Purest form of RNaseR is observed at 0.5mM EDTA. Thus, 0.5mM EDTA is preferred during RNaseR purification. In most purification runs, the RNaseR band was tough to isolate, and contamination was present in spite of running Ni-NTA and GFC chromatography.

The growth in the commonly used expression medium, Lennox Broth resulted in significantly low RNaseR yield. Our optimization of RNaseR expression depicted

that growth in the Auto-Induction medium offered significantly higher RNaseR yield. Briefly, cell cultures expressing RNaseR were first harvested and lysed. RNaseR was purified using Nickel Column Chromatography and Gel Filtration Chromatography. The functionality of the purified RNaseR was analyzed using a Polymerase Chain Reaction-based activity assay that detected the presence or absence of linear and circular RNAs in each sample. The Auto Induction Media was a successful optimization as it increased the average amount of RNaseR after GFC from 3.626mg to 6.640 mg/ml.

RNaseR Purification Run 2 seemed to work as it only degraded linear RNA and not circular RNA. However, the experiment was not reproducible as the same procedure was tried multiple times after, but it resulted in contaminated bands of protein, and improper functionality of RNaseR.

Moreover, the PCR results were not reliable in most cases. This could be due to cDNA being improperly produced, and RNA being incubated for extended amounts of time with RNaseR. This can be fixed by incubating RNA for 30 minutes instead of 1 hour or decreasing RNaseR concentration from 1.5ug to 1ug per 1ug of RNA.

Chapter 5

SUMMARY AND CONCLUSIONS

RNaseR, an exoribonuclease in the RNaseII family is an important nuclease that can be used for treatment and diagnosis of innumerable diseases as it only causes degradation of linear RNA and does not affect circular RNA concentrations in a given sample. This can be very useful in the clinical world as circular RNAs are involved in various functions such as protein complex scaffolding and parental gene modulations and can act as powerful biomarkers for treatment of diseases such as Gastric Cancer and Hepatocellular Carcinoma.

The objective of this thesis to purify His-Sumo tagged RNaseR grown in C41DE3 cells was a huge step towards understanding the process of RNaseR purification and the nature of RNaseR in adverse conditions such as high urea and salt concentrations, and the possible uses of RNaseR and circRNA in the near future.

For example, circular RNA concentrations within extracellular vesicles and within the cell in general can be measured using RNaseR degradation to degrade the linear RNA and preserve the circular RNA. The path of this circular RNA can then be followed and their priority for RNA decay based on their structuredness might help researchers study causes of several diseases. Similarly, RNaseR degradation can be used in circular RNA studies around the globe to isolate circular RNAs from a mixed sample of linear and circRNA, which can then be studied in context of other diseases and cancers.

Although RNaseR purification was successful, the experiment was not reproducible. Several optimizations were made such as the addition of Urea wash and high salt wash steps during Ni-NTA chromatography, and the use of Auto-Induction Media.

If I had more time to continue the project, I would attempt using 0.5mM EDTA during GFC, keep using Auto Induction Media as it almost doubled the concentration of RNaseR in the fractions, and introduce more column chromatography steps such as butyl column chromatography, cibacron blue column chromatography, or another Ni-NTA chromatography after polyhistidine tag cleavage to attempt and obtain pure RNaseR. This should result in a cleaner band of protein, and thus hopefully desirable functionality. Additional steps such as the Urea Wash, high salt wash, and low salt wash could also be included because most proteins tend to degrade at high Urea concentrations, resulting in purer bands of our desired protein.

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