ENHANCING THE qPCR DETECTION OF *CANDIDATUS* LIBERIBACTER ASIATICUS

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ABSTRACT

Huanglongbing (HLB), also known as citrus greening disease, is transmitted by the Asian citrus psyllid and infects citrus trees, leading to leaf mottling, poorly colored and bitter-tasting fruit, and ultimately, tree death. The causative agent for HLB is Candidatus Liberibacter, a gram-negative, alpha-proteobacterium. While no treatment for HLB currently exists, the quantitative Polymerase Chain Reaction (qPCR) serves as a gold standard for detecting target genes. The qPCR system identifies the presence of our target gene through simultaneous thermocycling and detection. This study aimed to answer the question: Can we reduce the cycle threshold (Ct) value in qPCR runs without increasing the initial concentration of nucleic acid? We proposed two methods to lower Ct values. The first method involved using ribonucleic acid (RNA) instead of deoxyribonucleic acid (DNA), with reverse transcription converting RNA to complementary DNA (cDNA). The second method utilized protein additives such as T4 Gene 32 Protein, RecA, and ET-SSB. Dialysis of the protein sample was performed to test the difference between the stock solution and the dialyzed counterpart. The substitution of DNA with RNA resulted in an enhancement in signal detection, yielding a difference of approximately 1.5 in the cycle threshold at high template concentrations and about 4.9 at low template concentrations. However, the effects of proteins did not meet our expectations due to various factors that could potentially influence the interpretation of the collected data. This unexpected outcome opens a new avenue for experimentation with different factors to validate these results. The goal of this project is to improve the detection of signal at the minimal amount of genetic material, thereby enabling the detection of the presence of infected plant hosts before they spread to healthy host plants.

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TABLE OF CONTENTS

Abstract……2

Acknowledgments……3

1. Introduction……6

2. Material and Methods……8

Quantitative Polymerase Chain Reaction

Huanglongbing (HLB)

Reverse Transcriptase Reaction

Extraction of RNA and DNA

Quantitative PCR of Extracted Nucleic Acid Sample Assay

Protein Additive Sample

Protein Additive Assay

Titration Series with Protein Additives

Glyercol Assay

Micro-Dialysis of Protein Storage Buffer

Titration Series with Dialyzed Protein Additives

Testing the Limits of Detection with Low Concentration of Template

3. Results……..18

Detection of Signal Between RNA and DNA

Enhancing Detection of Signal with Proteins

4. Discussion…….…….…….…….…….…….…….…….30

Analysis of Result in Detection of Signal Between RNA and DNA

Analysis of Result in Detection of Signal Between Protein Additives

5. Conclusion…….…….…….…….…….…….…….……32

6. References…….…….…….…….…….…….…….…….34

INTRODUCTION

The bacterium *Candidatus* Liberibacter asiaticus, a gram-negative, phloem-limited alpha proteobacterium, is classified under the genus *Candidatus* Liberibacter. This bacterium is recognized as the causative agent of Huanglongbing (HLB), also known as citrus greening disease. The genus *Candidatus* Liberibacter currently comprises three identified species: *Candidatus* Liberibacter asiaticus, *Candidatus* Liberibacter africanus, and *Candidatus* Liberibacter americanus. Originating from Asia and Africa, these bacteria have proliferated globally over the past decade, leading to a significant decrease in citrus fruit production. The primary vector for this disease is the Asian citrus psyllid, Diaphorina citri [1,9,10].

Infection in citrus plants manifests in various stages. The initial stage is typically characterized by blotchy, mottled leaves. The second stage results in a reduction in fruit size and quality, while the final stage culminates in the death of the host plant [1,3,9].

Currently, there is no known cure for HLB. The culturing of the disease has proven to be a challenging and slow process. One method that has been established as the gold standard for detecting target genes is the quantitative polymerase chain reaction (qPCR) [9]. This laboratory technique enables the visualization of the reaction by using a hydrolysis probe to measure the quantity of available nucleic acids. Similar to the polymerase chain reaction (PCR), qPCR is also utilized to amplify the gene of interest in DNA. However, qPCR differs in that it allows for the monitoring of the amplification process and quantitatively determines the initial concentration of the sample. This is achieved by adding a hydrolysis probe to the reaction. The hydrolysis probe is an oligonucleotide sequence containing a fluorescence reporter dye (fluorophore) and a quencher dye [14]. In proximity, the fluorophore does not emit fluorescence. Only after the sequence is hydrolyzed by the DNA polymerase's 5' to 3' exonuclease activity does the

fluorophore separate from the quencher. Once separated, the free fluorophore can fluoresce. Eventually, a sufficient number of free fluorophores will be present in the solution for the Opus Real-Time PCR system to detect a signal.

The application of this technique raises the question of whether it is feasible to augment the detection of the signal without a corresponding increase in the initial concentration. Enhancing the signal detection from a given sample enables the production of a signal even at the lowest nucleic acid concentrations. Given the low concentration of DNA molecules at high cycle threshold (Ct) values, it becomes challenging to ascertain the usability of a result. However, the employment of methods to enhance the detection by the PCR system attempts to facilitate a more accurate reading of the sample. This, in turn, leads to greater consistency in signal production at low concentrations. The implications of this enhanced detection are significant, as it could potentially improve the reliability and reproducibility of experimental results. Further research is warranted to explore these possibilities in greater depth.

In this research endeavor, the detection of the minutest quantity of DNA molecules and the precision of such detection are of paramount importance. This is because the results will ascertain whether the signal emanating from the sample is of sufficient significance. This, in turn, impacts the reproducibility of the experiment. This research paper proposes two methods for enhancing the detection capabilities of qPCR.

The first method involves the use of ribonucleic acid (RNA) as opposed to deoxyribonucleic acid (DNA). Given that a cell contains more copies of ribosomal RNA (rRNA) molecules than genomic DNA molecules, the process of reverse transcription enables these rRNA molecules to be converted into complementary DNA (cDNA). The resultant cDNA can then serve as a template for DNA replication by DNA polymerase enzymes [4,13]. The presence

of multiple cDNA copies facilitates amplification through repeated rounds of replication, leading to a more rapid amplification of DNA copies compared to replication from the original single copy of the DNA template.

The second method entails the use of additives to assist in the replication of our gene of interest [6]. Especially protein additives since they are known to aid in the replication of DNA within a cell. Numerous proteins are involved in various processes, including increasing processivity, stabilizing, synthesizing, proofreading and repairing, regulating, and terminating. The three proteins that were experimented with in this study were T4 gene 32 protein, Rec A protein, and extreme-thermostable single-stranded DNA binding (ET-SSB) protein [8,12]. Proteins such as T4 Gene 32 protein and ET-SSB are known to increase the processivity of DNA polymerases, enabling it to replicate longer on a single strand of template. This leads to higher yields and superior quality of the amplified DNA products. Proteins such as Rec A are known to excise and repair damaged DNA in SOS response. Rec A is also known to enhance the primer-template binding during quantitative-PCR. These methods, if successful, could significantly enhance the detection capabilities of qPCR, thereby improving the accuracy and reliability of experimental results.

METHODS

Quantitative Polymerase Chain Reaction

Quantitative Polymerase Chain Reaction or qPCR reactions were conducted on the with a CFX Opus Real-Time PCR system (BioRad cat# 12011319). The protocol for thermos cycling for all qPCR reactions was 50 °C for 2 min and 95 °C for 10 min, followed by 50 cycles of 95 °C

for 15 s and 60 °C for 1 min, with fluorescence data collection at the last step. Results of the amplification curve was analyzed using the Bio-Rad CFX Maestro software (BioRad cat# 12013758, v. 2.3)

Huanglongbing (HLB)

Disease caused by the bacterium *Candidatus* Liberibacter asiaticus (CLas) was identified using its 16S rDNA gene for detection. Plant tissue infected with CLas. was used as the DNA template for the assays. Reactions contained 0.1 µM of each primer and probe in 1× iTaq (Bio-Rad, cat# 1725131) consisting of a reaction volume of 20 µl (Table 1). The sequence of the forward primer is as follows: 5' GTCGAGCCTATGCAA TACG 3' and the reverse primer is as follows: 5' TACCTTTTTCTACGGATAACGCA 3'. Hydrolysis probe with 5' labeled 2' -chloro-7' -phenyl -1, 4 -dichloro-6-carboxyflyrorescin (VIC) and 3' labeled MG-NFQ (Thermo Fisher Scientific, cat# 4316034) [14]. The sequence is as follows: 5' AGACGGTGAGTAACCGT 3'Reactions were carried out in two different colored plates. Bio-Rad Multiplate™ 96-well PCR Plates, low profile, unskirted clear (cat# MLL9601), Bio-Rad Multiplate™ 96-well PCR Plates, low profile, unskirted clear (cat# MLL9651).

Table 1: Reaction scheme for 1x qPCR reaction

Components	1x Reaction
H_2O	7.8 µl
iTaq Universal Probe Supermix	10 µl
Forward Primer	0.48 µl
Reverse Primer	0.48 µl
Hydrolysis Probe	0.24 µl
Template	1 µl

Reverse Transcriptase Reaction

Samples were extracted from infected leaf tissue using a mortar and pestle and purified using Qiagen DNeasy Plant Mini Kit (cat# 69104). Both samples used forward primers, reverse primers, and hydrolysis probe for HLB. The sample containing RNase used 1x iTaq Universal probe supermix, while the sample without RNase used iScript™ Reverse Transcription Supermix (Biorad cat# 1708840). Samples were allowed to run for 50 cycles and afterwards analyzed on BioRad CFX Maestro software (BioRad cat# 12013758, v. 2.3).

Table 2. Reaction Scheme for Total Nucleic Acid Assay

Components	1x Reaction
H_2O	5.3 µl
Reverse Transcriptase	2.5 µl
iScript™ Reverse Transcription Supermix	10 µl
Forward Primer	0.48 µl
Reverse Primer	0.48 µl
Hydrolysis Probe	0.24 µl
Template	1 µl

Extraction of RNA and DNA

Nucleic acid samples were extracted in accordance with the protocol outlined by the Qiagen DNeasy Plant Mini Kit. The process commenced with the addition of 100% ethanol to buffers AW1 and AW2. Utilizing a pre-chilled mortar and pestle, spatula, and two 1.5 ml centrifuge tubes (USA Scientific cat# 4036-3212) filled with liquid nitrogen, infected leaf tissue was placed into the mortar and submerged under liquid nitrogen. The tissue was subsequently ground into a fine powder using the pestle, with periodic re-submersion in liquid nitrogen to facilitate the grinding process.

Upon achieving a fine powder consistency, 20 mg of tissue was allocated into each of the pre-chilled 1.5 ml centrifuge tubes. Once both samples contained approximately 20 mg of tissue, within a 10% margin of each other, AP1 buffer was introduced to both samples. RNase was also

added at this stage, but only to one of the samples, resulting in one sample containing pure DNA, while the other sample contained the total amount of nucleic acids (both RNA and DNA). Both samples were then incubated at 65 °C for 10 minutes to enable the RNase and buffer to eliminate unwanted components from our purified sample.

Following incubation, buffer P3 was added to neutralize the pH of the AP1 buffer and renature the nucleic acid back into solution, while all other components precipitated out. Both samples were centrifuged, and the lysate was pipetted into a QIAshredder spin column to remove any residual cell debris present in the lysate. The flow-through was collected from both samples and transferred to a new 1.5 ml centrifuge tube.

The samples then proceeded to the wash step, which involved the addition of AW1 buffer, followed by mixing and transferring to a DNeasy spin column for centrifugation. This process was repeated until all the samples were loaded onto the column, with the flow-through being discarded after each centrifugation. Subsequently, the column was placed in a new 2 ml collection tube, and buffer AW2 was added to the column, followed by centrifugation. This process was performed twice.

Finally, the column was placed in a new 1.5 ml centrifuge tube, and the addition of buffer AE and centrifugation allowed the sample to be eluted from the column into the tube. This process was also performed twice to ensure the complete transfer of the sample into the tube. Ultimately, two 1.5 ml centrifuge tubes were obtained, one containing pure DNA, and the other containing total nucleic acids (both RNA and DNA). This comprehensive extraction process provides a robust foundation for subsequent analyses.

Quantitative PCR of Extracted Nucleic Acid Sample Assay

Both extracted samples were concurrently assayed using a thermocycling protocol that entailed an initial 10-minute incubation at 50 °C to activate the reverse transcriptase, followed by a 3-minute incubation at 95 °C. This was succeeded by 50 cycles of 10-second incubation at 95 °C and 30-second incubation at 58 °C, with fluorescence data collection occurring at the final step. The amplification results were subsequently analyzed using the BioRad CFX Maestro software.

Protein Additive Samples

Three protein additives, namely T4 Gene 32 Protein (NEB cat# M0300S), RecA (NEB cat# M0249S), and Extreme Thermostable Single-Stranded DNA Binding (ET-SSB) Protein (NEB cat# M2401S), were employed to assess their effects on cycle threshold values.

Protein Additive Assay

The quantitative PCR reaction was conducted using stock solutions of T4 Gene 32 Protein, RecA, and ET-SSB. The reaction mixture comprised these protein additives, iTaq Universal probe polymerase, primers, a hydrolysis probe, and the template (as detailed in Table 3). The reaction was assayed using a thermocycling protocol that involved a 2-minute incubation at 50 °C and a 10-minute incubation at 95 °C, followed by 50 cycles of 15-second incubation at 95 °C and 1-minute incubation at 60 °C, with fluorescence data collection at the final step. The amplification results were analyzed using the Bio-Rad CFX Maestro software. This comprehensive approach facilitated a thorough evaluation of the effects of these protein additives on the cycle threshold values.

Table 3. Reaction scheme for protein additive

Components	1x Reaction
Protein additive	7.8 µl
ITaq Universal Probe Supermix	10 µl
Forward Primer	0.48 µl
Reverse Primer	0.48 µl
Hydrolysis Probe	0.24 µl
Template	1 µl

Titration Series with Protein Additives

A titration series was performed using stock protein additives to investigate the impact of varying protein concentrations on signal detection. The T4 Gene 32 Protein was titrated to concentrations of 5.4 µg/µl, 1.11 µg/µl, 0.54 µg/µl, and 0.054 µg/µl. RecA was titrated to concentrations of 0.6 µg/µl, 0.06 µg/µl, and 0.006 µg/µl. Lastly, ET-SSB was titrated to concentrations of 0.248 µg/µl, 0.124 µg/µl, and 0.0248 µg/µl.

Utilizing the reaction scheme outlined in Table 3, the titrated protein concentrations were tested on the qPCR system under a thermocycling protocol of 50 °C for 2 minutes and 95 °C for 10 minutes. This was followed by 50 cycles of 95 °C for 15 seconds and 60 °C for 1 minute, with fluorescence data collection occurring at the final step. The amplification results were subsequently analyzed using the Bio-Rad CFX Maestro software. This comprehensive approach facilitated a thorough evaluation of the effects of varying protein concentrations on signal detection.

Glycerol Assay

An experiment was conducted to assess the impact of glycerol on the efficiency of the quantitative PCR reaction. This involved testing various glycerol concentrations, including 30%, 20%, 10%, and 5%. These concentrations were prepared using 100% glycerin and distilled water, and were diluted to the desired concentrations, each with a total volume of 50 ml.

These different glycerol concentrations were then compared with a control reaction mixture that contained 0% glycerol, in order to evaluate any potential effects on detection. The reactions were executed under a thermocycling protocol that consisted of a 2-minute incubation at 50 °C and a 10-minute incubation at 95 °C. This was followed by 50 cycles of a 15-second incubation at 95 °C and a 1-minute incubation at 60 °C, with fluorescence data being collected at the final step.

The amplification results were subsequently analyzed using the BioRad CFX Maestro software. This comprehensive approach facilitated a thorough evaluation of the effects of varying glycerol concentrations on the efficiency of the quantitative PCR reaction. This could provide valuable insights for optimizing reaction conditions in future experiments.

Micro-Dialysis of Protein Storage Buffer

It was found that the stock solution of the protein additive's storage buffer contained 50% glycerol, a measure implemented to prevent freezing at -20 °C. The glycerol in the storage buffer was removed by conducting a micro-dialysis using a Millipore 0.025 µm filter paper (Millipore cat# VSWP02500) [5]. The exchange buffer used was a mixture of tris buffered saline and water in a 2:3 ratio.

The dialysis was carried out in a Styrofoam box filled halfway with ice. Aluminum weigh boats, containing the 2:3 ratio of tris buffered saline and water mixture, were placed atop the ice in the Styrofoam box for cooling. A nanodrop reading was conducted on the protein both before and after dialysis to record any changes in concentration.

Once the buffer was sufficiently chilled, a filter paper was positioned above the buffer in the aluminum weigh boat. Subsequently, 25 µl of protein was pipetted onto the filter paper and allowed to dialyze for 50 minutes. Following this, the dialyzed protein was carefully pipetted into a new test tube.

Titration Series with Dialyzed Protein Additives

The dialyzed proteins underwent the same procedure as the titration series conducted for the stock protein. The concentrations of T4 Gene 32 Protein, RecA, and ET-SSB were adjusted to be identical in order to investigate whether dialysis could enhance the protein's ability to detect the signal. The reaction scheme and thermocycling conditions remained consistent with those previously used. This approach allowed for a direct comparison of the effects of dialysis on the performance of the proteins.

Testing the Limits of Detection with Low Concentration of Template

This experiment aimed to evaluate whether the utilization of RNA and protein methods could enhance the detection of signals at low template concentrations. For the RNA method, the experiment was conducted using DNA. Both templates were diluted to an identical concentration of 3.3 µg/µl and subjected to a reverse transcriptase protocol consisting of a 10-minute incubation at 50 °C and a 3-minute incubation at 95 °C. This was followed by 50 cycles of a 10-second incubation at 95 °C and a 30-second incubation at 58 °C, with fluorescence data

collection occurring at the final step. The amplification results were subsequently analyzed using the Bio-Rad CFX Maestro software.

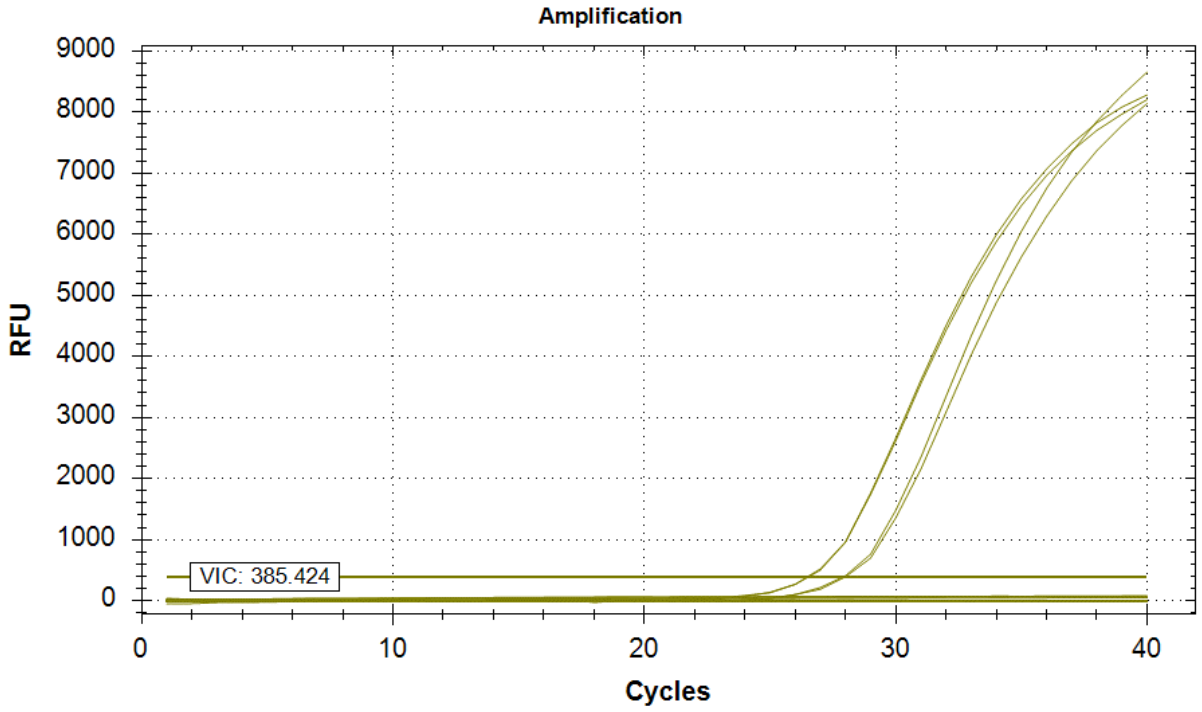
The protein method involved testing the same template but with varying concentrations of dialyzed protein additives. The reaction mixture scheme was consistent with that outlined in Table 3. The thermocycling protocol entailed a 2-minute incubation at 50 °C and a 10-minute incubation at 95 °C, followed by 50 cycles of a 15-second incubation at 95 °C and a 1-minute incubation at 60 °C, with fluorescence data collection at the final step. The amplification results were analyzed using the Bio-Rad CFX Maestro software. This comprehensive approach facilitated a thorough evaluation of the potential of RNA and protein methods to enhance signal detection at low template concentrations.

RESULTS

Detection of Signal Between RNA and DNA

The collection of data results of using total nucleic acid as compared with only using DNA sample were obtained in the form of the amplification curve and the calculation for the averages, standard deviation, and T-test for significance of the cycle threshold values were done using Excel.

Figure 1. Amplification Curve for Total Nucleic Acid and DNA



The amplification curve above shows the presence of signal produced from both samples of total nucleic acid and DNA. The cycle threshold values were taken from both samples and inputted into excel, where the average and standard deviation were calculated for each data set from both total nucleic acid and DNA.

Table 4. Average and Standard Deviation of Total Nucleic Acid and DNA

Sample	Cycle Threshold (Ct) Value		Average	Standard Deviation
Total Nucleic Acid	26.46	26.51	26.49	0.0353553
DNA	27.98	27.89	27.94	0.0636396

The average cycle threshold value for total nucleic acid falls around 26.49 ± 0.035 while the average cycle threshold value for DNA was around 27.94 ± 0.064. To analyze if these results were significant, a statical t-test was performed on the data set using the t-test function on Excel.

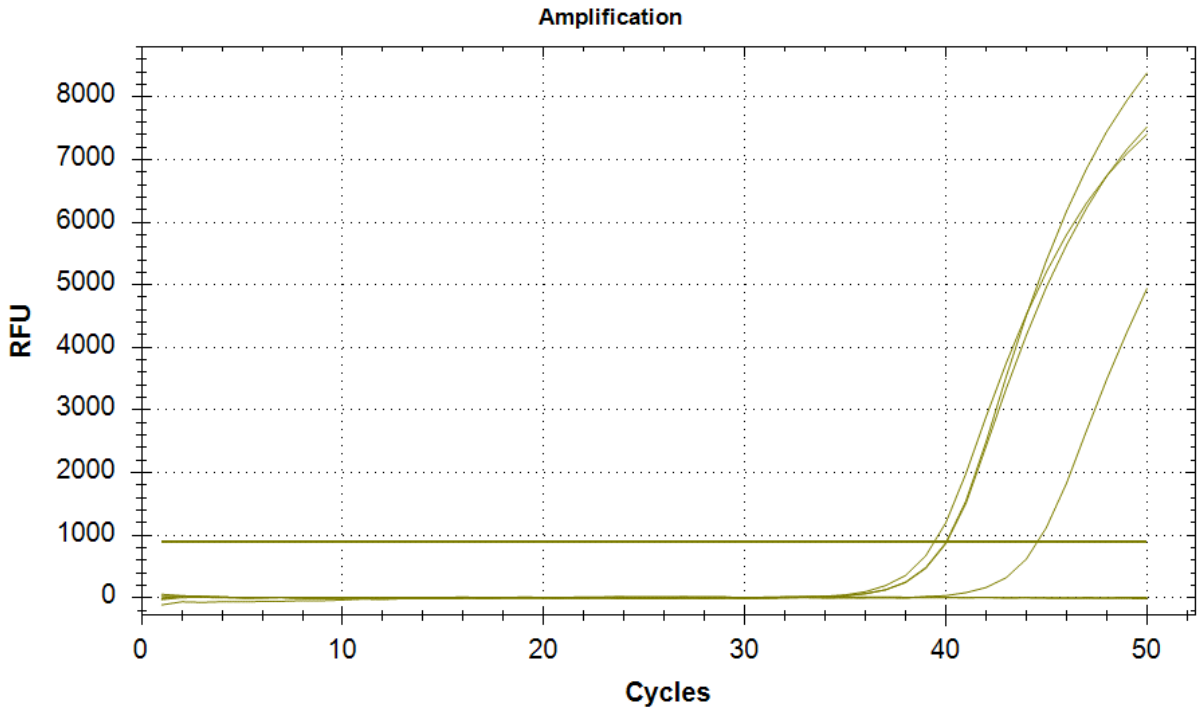
Table 5. T-test Results

Statistics	Variable 1 (Total Nucleic Acid)	Variable 2 (DNA)
Mean	26.5	27.95
Variance	0	0.005
Pooled Variance	0.0025	
Hypothesize Mean Difference	1.4	
Degree of Freedom	2	
t stat	-57	
P(T<=t)	0.000308	
T Critical two tail	4.302653	

Results of the T-test reveal that the probability of our t stat being larger than our T critical values is 0.03% which is lower than our alpha value of 0.05. Which means that our results are significant and therefore reject the null hypothesis that these two samples are not significantly different from each other.

The next results demonstrate the performance of signal detection using low concentrations of RNA and DNA.

Figure 2. Amplification Curve for RNA and DNA at Ct Values



The amplification curve above shows the presence of all replicates of RNA while the presence of DNA only had the presence of one signal. The cycle threshold values were noted down and the average and standard deviation were calculated using excel. The result of the calculation is as follows:

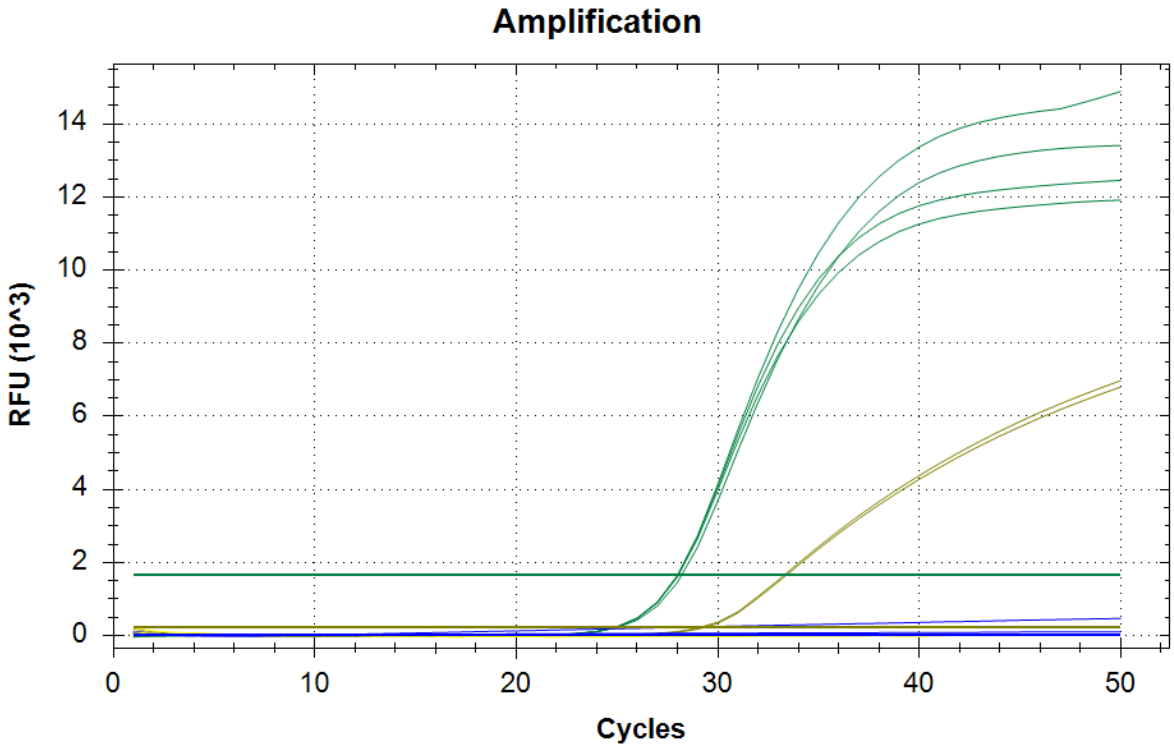
Table 6. Average and Standard Deviation of RNA and DNA at High Ct values

Sample	Cycle Threshold (Ct) Value			Average	Standard Deviation
RNA	40.06	40.03	39.44	39.735	0.417119
DNA	44.57	N/A	N/A	44.57	N/A

The result from the table shows that all three replicates from the RNA sample had the presence of a signal whereas the DNA sample only detected signal in one out of the three replicates. The averages cycle threshold values for RNA and DNA were roughly a 4.8 difference. There was no standard deviation in DNA sample because there was only detection of one signal.

Enhancing Detection of Signal with Proteins

Figure 3. Amplification Curve of Stock Protein Additives



The amplification curve shows the result of performing the qPCR reaction with the stock protein additive solution. From the amplification curve, we see no detection in signal from T4 Gene 32 Protein and extreme thermostable single stranded binding protein. However, there was signal detected from RecA Protein (greenish yellow) and the control group of water and buffer samples (turquoise).

To observe the detection of signal over a range of concentrations to find the optimal concentration of protein for enhancing signal detection, a titration series was performed on the three protein samples.

Table 7. Ct values, Averages, and Standard Deviation for Titration of Different Concentration of Stock Protein Sample

Protein Additive Experiment	Ct Values		Average	Standard Deviation
Control (H2O)	25.05	25.05	25.05	0
T4 Gene 32 Protein Conc. (ug/uL)	Ct Values			
5.4	28.08	27.71	27.895	0.26163
1.11	27.51	27.52	27.515	0.007071
0.54	26.24	26.28	26.26	0.028284
0.05	25.03	25.02	25.025	0.007071
RecA conc. (ug/ul)	Ct Values			
0.6	26.08	26	26.04	0.056569
0.06	25.14	25.19	25.165	0.035355
0.006	25.29	25.10	25.195	0.13435
ET-SSB conc. (ug/ul)	Ct Values			
1.28	40.24	N/A	40.24	N/A
0.128	N/A	N/A	N/A	N/A
0.0248	25.15	25.10	25.125	0.035355

All proteins were compared against water and buffer control and averages and standard deviation were calculated for each sample. The average cycle threshold values are shown to be much higher at higher concentrations of protein samples. As the concentration decreases, we see a decrease in cycle threshold values similar to the values of water and buffer control.

A glycerol test was performed with different concentrations of glycerol and DNA sample and the results of the amplification were collected and analyzed. The averages and standard deviation were taken for each concentration of glycerol's cycle threshold value.

Figure 4. Amplification Curve Different Glycerol Concentrations Effects on Ct Values

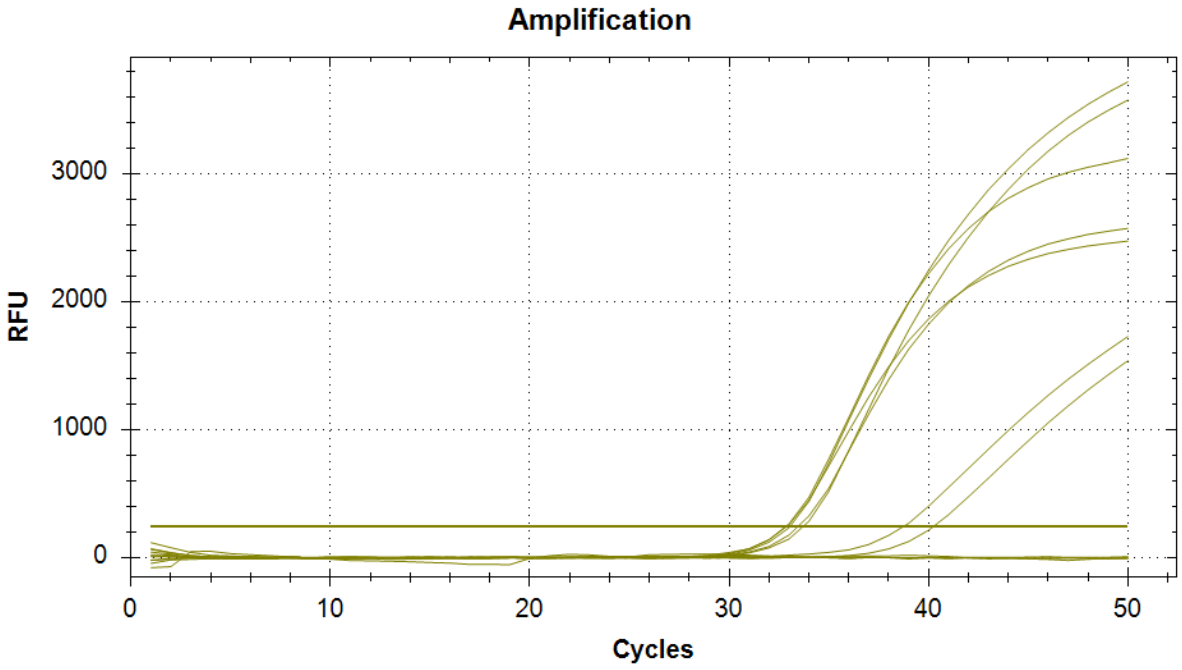


Table 8. Ct Values, Averages, and Standard Deviation for the Effects of Glycerol Concentrations

Glycerol Test	Ct Values		Average	Standard Deviation
Control (H2O)	32.81	32.86	32.835	0.035355
5% Glycerol	N/A	33.43	33.43	N/A
10% Glycerol	33.04	38.71	35.875	4.009295
20% Glycerol	33.69	40.22	36.955	4.617407
30% Glycerol	N/A	N/A	N/A	N/A

We observe that at different concentrations of glycerol, the qPCR reactions are inhibited by the higher the concentrations. As we decrease the concentration, we notice that the cycle threshold values are still much higher than the control group of water. Since we know that our proteins were stored in glycerol to prevent freezing, we had to propose a method to remove that glycerol. Resulting in the micro-dialysis of the stock protein.

The stock protein samples were then proceeded with the micro-dialysis protocol to buffer exchange with a 2:3 Tris Buffer Saline and water solution. The results were collected post amplification and analyzed.

Table 9. Ct values, Averages, and Standard Deviation of Different Titrated Concentration of Dialyzed Protein Sample

Protein Additive Experiment	Ct Values		Average	Standard Deviation
Control (H2O)	29	28.65	28.825	0.24748737
Control (Buffer)	29.02	29.12	29.07	0.07071068
T4 Gene 32 Protein Conc. (ug/uL)	Ct Values			
1.11	30.91	31.07	30.99	0.11313708
0.54	30.18	29.83	30.005	0.24748737
0.05	29.36	29.2	29.28	0.11313708
RecA conc. (ug/ul)	Ct Values			
0.6	30.31	30.38	30.345	0.04949747
0.06	29.35	29.18	29.265	0.12020815
0.006	29.35	29.24	29.295	0.07778175
ET-SSB conc. (ug/ul)	Ct Values			
1.28	29.35	29.24	29.295	0.07778175
0.128	35.64	36	35.82	0.25455844
0.0248	29.3	29.19	29.245	0.07778175

The results observed are very similar to the undialyzed protein samples where we see that all the cycle threshold values for the protein are higher than the values from the water and buffer control. The general trend with decrease in cycle threshold values as the concentration of protein was also observed with the dialyzed protein samples as well.

The dialyzed proteins effects were also tested with low concentration of template. The amplification results were obtained and analyzed. The averages and standard deviations were calculated using Excel.

Table 10. Ct Values, Averages, and Standard Deviation of Different Titrated Concentration of Dialyzed Protein Sample at Low Amounts of Nucleic Acid Template

Protein Additive Experiment	Ct Values		Average	Standard Deviation
Control (H2O)	N/A	N/A	N/A	N/A
Control (Buffer)	35.89	N/A	35.89	N/A
T4 Gene 32 Protein Conc. (ug/uL)	Ct Values			
1.11	4.99	4.37	4.68	0.438406
0.54	N/A	40.66	40.66	N/A
0.05	34.56	36.85	35.71	1.619275
RecA conc. (ug/ul)	Ct Values			
0.6	N/A	N/A	N/A	N/A
0.06	N/A	N/A	N/A	N/A
0.006	N/A	N/A	N/A	N/A
ET-SSB conc. (ug/ul)	Ct Values			
1.28	N/A	N/A	N/A	N/A
0.128	N/A	33.48	33.48	N/A
0.0248	N/A	N/A	N/A	N/A

Observations with several of the protein concentrations did not have detection in a signal. These are shown in RecA Protein and ET-SSB protein. The water and buffer controls also did not detect or had signal missing. T4 Gene 32 Protein was able to produce signals from both replicates which lead to taking further replicates to observe this trend.

Table 11. Ct Values, Average, and Standard Deviation for T4 Protein at 0.05 µg/µl

Protein Additive Experiment	Ct Values					Average	Standard Deviation
Control (H2O)	N/A	N/A	39.54	N/A	N/A	**39.54**	N/A
Control (Buffer)	N/A	N/A	37.63	N/A	N/A	**37.63**	N/A
T4 Gene 32 Protein Conc. (ug/uL)	Ct Values						
0.054	37.05	N/A	38.19	N/A	N/A	**37.62**	0.806102

Observation with the results shows that most of the replicated with water, buffer, and T4 Protein did not have their signal detected. Out of the five replicates, both water and buffer control only had one signal while T4 protein had two signals out of five.

DISCUSSION

In this Capstone Project, our objective was to analyze various techniques to enhance the detection of the signal Candidatus Liberibacter asiaticus. The outcomes demonstrated that certain methods effectively improved the detection of our target pathogen, while others did not yield as successful results. Even though not all the techniques employed in this project were effective, there exists potential for adjustments and exploration of diverse parameters that could potentially enhance signal detection. This opens avenues for future research and optimization in this area.

Analysis of Result in Detection of Signal Between RNA and DNA

Our observations indicated a significant improvement in the cycle threshold value when RNA was utilized instead of DNA, as evidenced by the data obtained. The T-test results further substantiated this, demonstrating that the data was statistically significant. This was due to the probability of our t-statistic being greater than or equal to the critical T-value being 0.000308, which is less than our alpha value of 0.05. This implies that the likelihood of our t-statistic exceeding our critical T-value is approximately 0.031%.

In quantitative PCR, there is an inverse proportionality between concentration and cycle threshold value, signifying that a higher concentration corresponds to a lower cycle threshold value. This principle can be applied to assess the effects of RNA and DNA. Given that one of our samples contained the total nucleic acid sample while the other sample only comprised DNA, the total nucleic acid sample would have a larger template during initial amplification, leading to more replicates of the specific gene sequence.

This resulted in the observed outcome at the low concentrations of RNA and DNA samples. Despite the concentration being the same, the amount of template available in the RNA sample was significantly greater than in the DNA sample. Consequently, the RNA sample was able to amplify much faster than DNA, leading to the detection of signals from all replicates and significantly lower cycle threshold values compared to DNA. This finding provides a promising avenue for further research in this field.

Analysis of Result in Detection of Signal Between Protein Additives

Upon examination of the data pertaining to the protein additives, no discernible enhancement in the detection of the signal was observed in either dialyzed or undialyzed experiments. All cycle threshold values were considerably higher than those of water, and as the protein concentration decreased, the cycle threshold values became increasingly similar to those of water and buffer control. This can be attributed to the dilution of the protein, which significantly reduces the amount of protein, resulting in a solution predominantly composed of water. Consequently, a similar cycle threshold value to that of water is anticipated.

When evaluating the effects of the protein at low concentrations of the nucleic acid template, the results were inconclusive due to the inconsistent and random detection of the signal. At first glance, it appears that the T4 Gene 32 Protein at 0.054 ug/uL outperforms the control groups of water and buffer. However, upon examination of multiple replicates, it becomes evident that the detection of a signal at high cycle threshold values is likely due to random chance.

In summary, the investigation into the effects of protein additives on signal detection revealed the presence of a factor that inhibits our qPCR reaction. One speculation is that traces of glycerol in the storage buffer could still be present even after dialysis. Given that varying concentrations of glycerol are known to inhibit the qPCR reaction, this could be a contributing factor. Another potential cause for inhibition could be the presence of high salt. The buffer used for dialysis was a Tris Buffered Saline solution, and the introduction of high salt in the dialyzed protein sample could result in the inhibition of the DNA polymerase, interference with fluorescence detection, and even precipitation of the primers and probes, leading to the failure of the qPCR reaction. These factors could collectively contribute to the lack of improvement in signal detection observed in our study. This warrants further investigation to optimize the conditions for successful qPCR reactions.

CONCLUSION

In conclusion, the substitution of DNA with RNA resulted in an enhancement in signal detection, yielding a difference of approximately 1.5 in the cycle threshold at high template concentrations and about 4.9 at low template concentrations. This suggests the potential to detect less than one molecule of DNA in a single cell, thereby paving the way for experiments to determine the limit of detection achievable by the qPCR system. This could facilitate more precise and refined data collection.

However, the effects of proteins did not align with our expectations in this project due to a multitude of factors that could potentially influence the interpretation of the collected data. This unexpected outcome presents a new avenue for experimentation with different factors to validate these results. Future research in this area could provide valuable insights into the complex interplay of these factors and their impact on our experimental outcomes. This

underscores the dynamic nature of scientific inquiry, where unexpected results can lead to new questions and avenues for exploration.

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