

Degree project



Development of a qPCR method for detection and quantification of *Ustilago nuda* and *COX1* gene in Barley seeds

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Abstract

Barley loose smut caused by the fungal pathogen *Ustilago nuda* is a global concern with detrimental effects on barley production. Early detection of this infection is vital for effective disease supervision. However, current seed health testing protocols suffer from limitations in terms of time and efficiency. The present research work aimed to produce a method using qPCR for simultaneous screening of barley and *U. nuda*. A set of primers, 1F and 1R, was employed for the detection of rRNA operon internal transcribed spacer 1 sequence from *U. nuda* and a part of the *COX1* gene, present in barley seeds, was selected as an internal control for comparison with *U. nuda*. A specific 79 bp target amplicon from a part of the *COX1* gene was successfully amplified using *COX1* F and *COX1* R primers, and cloned into a vector for standard curve generation. However, attempts to replicate the previously published qPCR method by bachelor researcher for *U. nuda* internal transcribed spacer 1 sequence detection using 1F and 1R primers were unsuccessful. Several efforts were made to reproduce the results, but amplification was not observed. Further optimization, including literature review, primers and probe optimization is required to improve this method. The successful amplification of a part of the *COX1* gene in both normal and infected samples underscore its potential as a reliable internal control. However, further research is necessary to refine the detection of *U. nuda*. This study underscores the need for continuous advancements in disease screening methodologies to meet global market demands.

Popular scientific summery

Barley, an ancient and versatile grain, has been facing global challenges in production over the past two decades due to various fungal diseases. However, recent research has uncovered exciting possibilities for this grain, particularly in terms of its health benefits and potential as an alternative to commonly used cereal grains. To tap into its full potential, researchers have been focusing on understanding and managing fungal diseases that affect barley crop. Among the numerous diseases, the most detrimental pathogen is *Ustilago nuda* (*U. nuda*), which causes loose smut infection. Detecting and managing this pathogen is crucial for maintaining healthy barley crops and ensuring high-quality seed production. The present research work has made a great effort in the development of an efficient and precise method for loose smut detection.

Cytochrome oxidase gene (*COX1*) was selected as an internal control, providing a reliable reference for comparison and it exists in both natural and infected barley seeds. A technique called qPCR (quantitative polymerase chain reaction) was utilized to amplify the specific *COX1* gene sequence and accurately measure its presence. The target part of the *COX1* gene was successfully amplified using specific *COX1* F and *COX1* R primers, and a standard curve was generated to determine its abundance. This curve facilitated the quantification of *COX1* gene levels in both healthy and infected barley seeds. The successful amplification of the part of *COX1* gene in both healthy and infected samples highlights its potential as a reliable internal control.

However, replicating a previously used qPCR method for the *U. nuda* detection by a bachelor researcher proved unsuccessful. The same set of primers, namely 1F and 1R, were subsequently employed to detect internal transcribed spacer 1 sequence from *U. nuda* within the barley seeds that were infected. Optimizing the primer pair (1F and 1R) for *U. nuda* detection or refining the qPCR reaction parameters, including concentrations and temperatures, is crucial for enhancing accuracy and reliability. Meticulous method development and optimization are crucial when dealing with complex samples like fungal infections in crops. Although challenges were encountered in detecting *U. nuda*, this research provides valuable insights for further method optimization. By refining the primers and probe used in the qPCR assay for the detection of *U. nuda* and conducting a thorough literature review, the accuracy and sensitivity of the detection method can be enhanced. By improving disease detection and seed quality, farmers can enhance their crop yields and reduce losses due to fungal infections. Additionally, consumers can benefit from the increased availability of high-quality barley products, rich in essential nutrients and known for their potential to reduce cholesterol levels and manage blood sugar levels.

By implementing advanced molecular techniques and innovative seed testing methods, we are moving closer to realizing the full benefits of this ancient grain. With ongoing efforts to enhance disease detection and seed quality, barley has the potential to play a more prominent role in our diets and contribute to a healthier and more sustainable future.

Abbreviations

COX1	Cytochrome oxidase gene
ELISA	Enzyme Linked Immunosorbent Assay
<i>E. coli</i>	<i>Escherichia coli</i>
LB	Luria-Bertani
LAMP	Loop-mediated isothermal amplification
mtCOXI	Mitochondrion cytochrome oxidase subunit I
NTC	Non-template control reaction
NGS	Next-generation sequencing
NEB	New England Biolabs
NCBI	The national center for biotechnology information
PCR	Polymerase Chain Reaction
qPCR	Quantitative Polymerase Chain Reaction
RE	Restriction enzyme
R²	Coefficient correlation
ToCV	Tomato chlorosis virus
TICV	Tomato infectious chlorosis virus
T_m	Melting temperature
<i>U. nuda</i>	<i>Ustilago nuda</i>
6-FAM	6-carboxy-fluorescein

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Introduction

After corn, rice and wheat, barley (*Hordium vulgare*) stands as the fourth key grain crop in the world. It is an ancient grain that belongs to the plant family Poaceae (Ullrich, 2010). Barley is still a chief staple food and it is significant in some civilizations all over the world, predominantly in Africa and Asia. The awareness for benefits in the barley food has been amplified all over the world because of its dietary worth (Baik & Ullrich, 2008; Grando & Macpherson, 2005).

In Eurasia, more than 10,000 years ago, barley was the first cultivated cereal grain. It was consumed by Romans and other Europeans for making bread (Baik & Ullrich, 2008). It still has a significant role as a key food all over the world and is being utilized for human and animal feeding as well as for beer production. On a worldwide basis, it is used in the malt production (second largest use). Malt is known for its precious significance in alcoholic beverages (Schwarz & Li, 2011).

In European history, barley grain is frequently entitled as "corn". It has almost the same nutritional worth as corn maize. Barley is rich in carbohydrates, essential vitamins and minerals. It contains calcium, phosphorus and some amounts of protein. It is naturally enriched with vitamin B (Baik & Ullrich, 2008; Gupta et al., 2010).

Pearl barley is consumed by humans as a soup thickener and as an important component in dressings. It is used to make flour for porridge, baby foods and injera. Recently in some EU regions, barley worth has been extensively increased due to its utilization in the ethanol production (Zhou, 2009). In the human nutrition, the increase in the utilization of others cereals (e.g wheat, maize and rice) results in the extensive decrease in the cultivation and use of barley. In the past two decades, there has been a decline in global barley production. On average, the annual worldwide barley yield is over 140 million tons (Mt) and it was acquired from approximately 50 million hectares (Mha) (Tricase et al., 2018; Zhou, 2009).

Although barley is a crop with valuable qualities for processing and food product development, its potential has not been fully realized due to a lack of knowledge. There has been increased awareness about the importance of barley as a food crop, with a focus on the potential health benefits of its β -glucan content, which has been shown to help reduce blood cholesterol levels and lower the glycemic index (Östman et al., 2006). Whole barley foods also demonstrate to be connected with increased weight loss and in lowering the plasma cholesterol level. It also has higher fiber content than wheat and multiple other major grains. It holds immense potential for being used as an alternative, either partially or fully, to commonly used cereal grains such as wheat (*Triticum aestivum*), oat (*Avena sativa*), rice (*Oryza sativa*), and maize (*Zea mays*). Its versatile nature allows for its utilization in a wide range of products (Bhatty, 1999; Izydorczyk & Dexter, 2008).

Economically, Barley has many uses. In the United States (US), approximately 50 % of the barley is utilized for livestock feed, 25 % is used for the malting (about 80 % consumed in beer production), 14 % alcohol making and about 6 % is used for other purposes e.g. malted milk, breakfast foods and malt syrup (Jacomet, 2006; Langridge, 2018).

The production of barley products is difficult and compromised because of

elevated protein level, thin grains and tiresome procedure in food processing. There are different reasons that can affect or limit the barley production. These include infertility of soil, deficiency of nutrients, less soil yield potential, and unpredictability of yield due to weeds and plant diseases caused by pathogens and animal pests (Gupta et al., 2010; Keressie & Goitom, 1993).

Barley production is vulnerable to various fungal diseases, including Net-blotch (*Pyrenophora teres*), Spot-blotch (*Bipolaris sorokiniana*), Stagono spora Leaf-blotch (*Stagonospora avenae*), Loose smut barley disease (*Ustilago nuda*), Covered smut (*Ustilago hordei*), false loose smut (*Ustilago avanae*), Speckled leaf Blotch (*Septoria passerinii*), Stem Rust (*Puccinia graminis*) and Powdery Mildew (*Blumeria graminis* f. sp. *hordei*) (Neate & McMullen, 2005). These diseases can significantly impact barley crops, leading to yield losses and reduced quality. Implementing effective disease management strategies is crucial to minimize the economic and agronomic impact of these fungal pathogens on barley production.

Production of barley has been restrained by various abiotic and biotic factors that decrease the crop yield, of which loose smut by *Ustilago nuda* (*U. nuda*) is the chief multinational and harmful seed borne pathogen around the world (Gangwar et al., 2018). The loose smut disease by *U. nuda* is seed-borne and remains unseen within the seed embryo. As the infected seed plants, the fungus begins to grow without showing any noticeable signs in the seed. This may produce a smutted head where the grain has been substituted with a cluster of brown to black powdery spores of *U. nuda*. The infected heads usually come out before the healthy heads. The presence of contaminated seed batches can be detected at the heading stage of the crop, as the fungal teliospores are released from the infected plants at that time (Kumar et al., 2022). The fragile pericarp membrane that carries the spores ultimately breaks due to wind-blown. This results in the spreading of fungal teliospores to flowering heads of healthy crops. The whole process is catalyzed by wet climate, humidity and temperature ranges from 16 °C to 22 °C. Loose smut infection rate on barley seeds is directly proportional to the yield percentage losses (Jevtić et al., 2022). The lifecycle of the loose smut infection by *U. nuda* is shown in Figure 1.

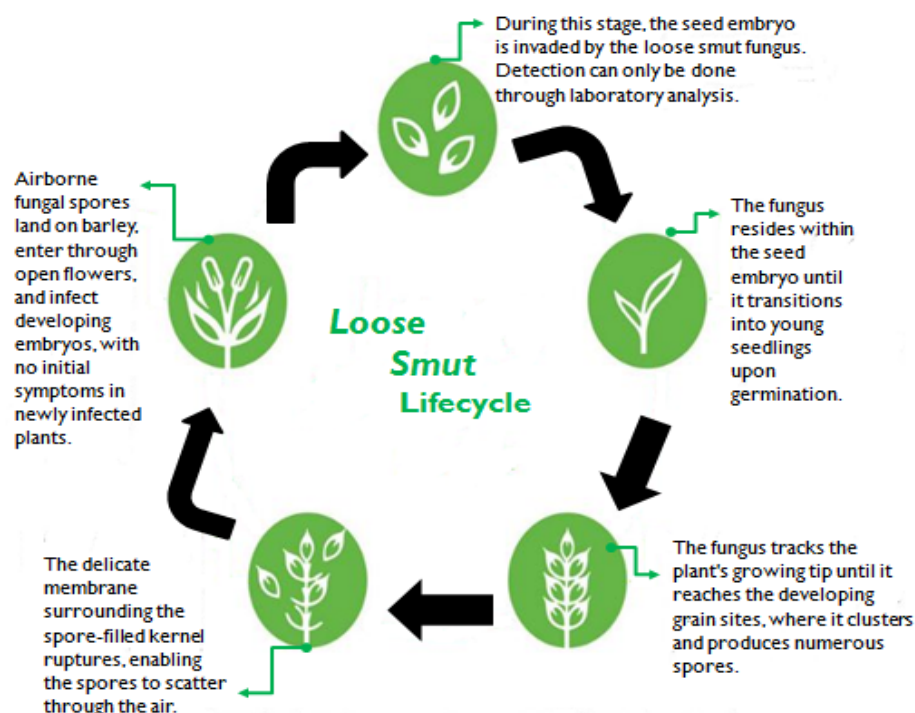


Figure 1. A comprehensive flow chart detailing the infection cycle of Loose smut fungus by *U. nuda*.

At present, there are many traditional methods (e.g visual test and embryo test) which are in use for the detection of *U. nuda*. Seed health testing measures are also taken (e.g physical and biochemical test) but they are time-taking (Asaad et al., 2014). Therefore, it is imperative to develop a seed testing method that is rapid, precise and accurate for the detection and identification of all pathogens e.g bacteria, fungi and viruses.

A precise, accurate and inexpensive laboratory method was developed for the *U. nuda* detection in barley seeds. The investigation was conducted at the health laboratory of The International Centre for Agricultural Research in the Dry Areas (ICARDA). The produced method just needs 5 hours to complete the process. This can be done by heating the barley seeds and sodium hydroxide (NaOH) solution at 40°C for 3.5 hours (h). Sodium chloride (NaCl) with a mixture of water and glycerol (1:1) are used for the separation of embryos which are later examined under the microscope. Hence, the infected embryos are checked and counted under the microscope and the loose smut infection by *U. nuda* can be detected (Asaad et al., 2014). It could, therefore, be accomplished that loose smut infection by *U. nuda* is very vital that critically reduces the production and productivity globally, however effective control of smut diseases in barley can be achieved through various methods, such as using certified smut-free seeds, planting host-resistant varieties, treating seeds with hot water or solar heat, applying systemic fungicidal seed treatment, or a combination of these methods (Woldemichael, 2019).

Molecular methods encompass a variety of techniques for the detection and identification of *U. nuda* and other pathogens. These include polymerase chain reaction (PCR), loop-mediated isothermal amplification (LAMP), DNA microarrays, next-generation sequencing (NGS), and quantitative polymerase chain reaction (qPCR). Among these, qPCR has emerged as a powerful tool for pathogen detection

and quantification. It combines the principles of PCR with fluorescent probe technology to enable real-time monitoring of the amplification process. qPCR offers high sensitivity, specificity, and the ability to quantify target DNA, making it ideal for *U. nuda* detection. It allows for rapid, accurate, and quantitative assessment of pathogen presence in samples, facilitating early detection, disease monitoring, and effective management strategies in agriculture (Smith & Osborn, 2009).

Fluorescence microscopy has also been used to identify the early growth of the loose smut pathogen in wheat and barley (*U. tritici* and *U. nuda*, respectively) (Wunderle et al., 2012). A PCR test for the detection of loose smut fungus was introduced where DNA primer sequences for *U. nuda* have been utilized to amplify the loose smut DNA. This method was only suitable for samples highly infected with *U. nuda* (Bates et al., 2001). Real time Quantitative Polymerase Chain Reaction (real time-qPCR) technique has been developed for the rapid and precise finding of as small as 43 replicas of *U. nuda* DNA genome. The developed method can be optimized for increased sensitivity and specificity by conducting studies with larger sample sizes and field samples, improving the monitoring of *U. nuda* infection in barley seeds and enhancing current microscopic detection methods (Setu, 2021).

In the present study, the selection of a part of the cytochrome oxidase gene (*COX1*) as an internal control was motivated by its presence in barley seeds. *COX1* represents a mitochondrial terminal complex and is widely employed as a conventional marker for internal control due to its extensive taxonomic coverage (Deagle et al., 2014). The *COX1* gene exhibits essential characteristics and demonstrates the ability for PCR amplification, which aligns with the objectives of this research. *COX1* is primarily situated in the inner mitochondrial membrane, and its 5'-end region is commonly utilized by the DNA barcoding consortium, underscoring its significance in research endeavors (Hajibabaei et al., 2007).

A triplex reverse transcription polymerase chain reaction (RT-PCR) was developed for the potato virus identification. *COX1* mRNA was employed as an internal control, highlighting its significance in identifying false negatives during analysis. The utilization of this marker gene also contributes to maintaining quality control (He et al., 2006). In a separate research, the *COX1* gene was employed as an internal control. They developed a one step, reverse transcription TaqMan® polymerase chain reaction (RT-PCR) approach for the rapid, cost-effective detection of tomato chlorosis virus (ToCV) and tomato infectious chlorosis virus (TICV) (Papayiannis et al., 2011).

In this study, DNA isolated from various barley seeds was examined, with a particular focus on the presence of a part of *COX1* gene. The aim of this project was to develop a probe-based qPCR technique for the accurate detection and quantification of *U. nuda* in naturally infected barley seeds. In a prior study conducted by Setu, a primer pair labeled as 1F and 1R was employed for the detection of *U. nuda*. The same primers were subsequently utilized to identify *U. nuda* within the infected barley seeds. In the present research, a part of the *COX1* gene, which is naturally present in barley seeds, was utilized as an internal control due to its favorable characteristics and its suitability for PCR amplification.

The project had three main objectives. The first objective was to create samples that could be used to build a standard curve for qPCR, facilitating the

quantification of the part of *COX1* gene. This involved isolating DNA from barley seeds, performing PCR amplification of a part of the *COX1* gene, cloning it into a vector, transforming it into *Escherichia coli* (*E. coli*), isolating plasmids, digesting them using restriction enzymes, and sequencing the fragments. The digested DNA served as the template for constructing the standard curve for a specific part of the *COX1* gene that was used as an internal control. The second objective focused on using the developed material from the previous objective in an absolute qPCR method to detect and quantify a part of the *COX1* gene in barley seeds. By running qPCR reactions with the standardized samples, the abundance of a part of the *COX1* gene within the seeds could be determined. The choice of using the *COX1* gene as an internal control was based on its presence in both healthy and infected barley seeds. The final objective was to utilize the previously employed primers (1F and 1R) and probe to detect and quantify the internal transcribed spacer 1 sequence from *U. nuda* in the infected barley seeds while comparing the quantitative values obtained for a part of *COX1* gene with those for *U. nuda*. The ultimate aim of the present research was to establish a probe-based qPCR method capable of detecting and quantifying *U. nuda* and a part of the *COX1* gene in naturally infected barley seeds.

Materials and Methods

Seed Sample Selection

Five different dried barley seed samples were included in the analysis. The first sample served as the 'control,' devoid of any *U. nuda* fungus. The remaining four samples exhibited varying *U. nuda* fungus percentages: 1.2 %, 1.5 %, 2.1 %, and 2.9 %. The assessment of infected seeds was carried out by the company "Frökontrollen," employing a manual method that involved microscopic examination of the embryos to quantify the proportion of seeds affected by loose smut. The flow chart of the entire process is shown in Figure 2.

Extraction of DNA from barley seed

The liquid nitrogen technique was employed to effectively crush the dried barley seeds for subsequent analysis. Each dry sample, weighing up to 100 g, was placed in a mortar and pestle and subjected to grinding in the presence of liquid nitrogen. Following the grinding process, approximately 30 g of each sample was transferred into individual 1.5 ml micro centrifuge tubes, ensuring an equal distribution of the crushed material. Immediate storage of the tubes on a bed of dry ice was implemented to maintain sample integrity. For long-term preservation, one control sample and four infected samples with different *U. nuda* fungus percentages (1.2 %, 1.5 %, 2.1 %, and 2.9 %) were stored at -80 °C. This storage arrangement facilitated the potential reuse of the samples in subsequent analyses. DNA was extracted from crushed barley seeds, including one control sample (a sample that contain no loose smut infection) and four infected samples with varying percentages of *U. nuda* fungal infection (e.g., 1.2 %, 1.5 %, 2.1 %, and 2.9 %), using the E.Z.N.A. SP Plant DNA Kit (OmegaBiotek) according to the manufacturer's instructions. DNA extraction was carried out twice for each of five samples. The concentration of the extracted DNA samples were measured using the Qubit® 4.0 dsDNA BR Assay Kit (Life

Technologies).

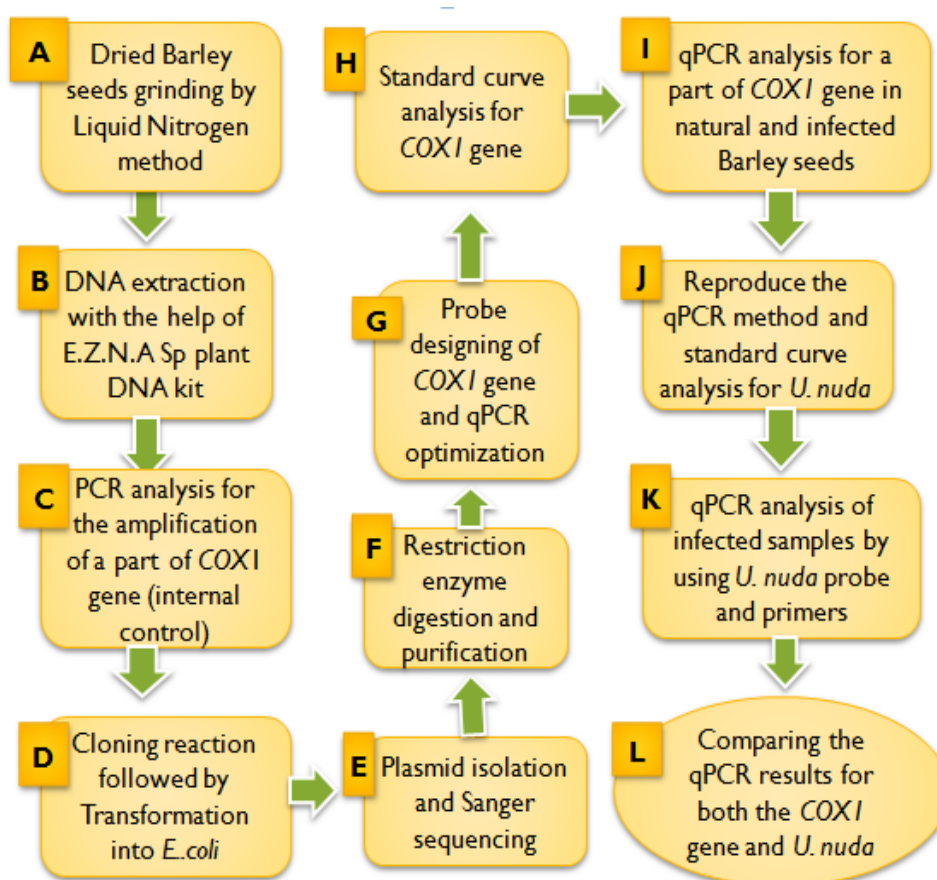


Figure 2. A comprehensive flow chart detailing the entire process, commencing from the crushing of dried barley seeds and culminating with qPCR analysis. **A)** Grinding of dry barley seeds with the help of Liquid nitrogen. **B)** Isolation of DNA from the crushed barley seeds. **C)** PCR amplification of a part of the *COX1* gene as an internal control due to its presence in the barley seeds. **D)** TOPO cloning of the amplified part of the *COX1* gene followed by its transformation into *E. coli*. **E)** Analysis of Positive Clones, Plasmid Isolation, and Sanger Sequencing. **F)** Restriction enzyme digestion of the cloned part of *COX1* gene followed by its purification. **G)** Probe designing and qPCR optimization for a part of the *COX1* gene. **H)** Standard curve analysis for a part of the *COX1* internal control gene where 10-fold serial dilutions up to 8 data points were prepared. **I)** qPCR analysis of a part of *COX1* gene in natural and infected barley seeds by using *COX1* primers (*COX1* F and *COX1* R) and specifically designed probe. **J)** Reproduce the qPCR method for the standard curve analysis of *U. nuda* in the infected barley seeds by using the same *U. nuda* primers (1F and 1R) and probe as used by bachelor researcher. **K)** qPCR study of infected barley seeds sample by using the *U. nuda* primers (1F and 1R) and probe. **L)** qPCR results comparison between the obtained value of *COX1* gene and for the *U. nuda*.

Primers

The amplification of a part of *COX1* gene was performed using a pair of previously published primers (Zabaleta Vanegas et al., 2008). The forward primer, *COX1*F: 5'-CGT CGT ATT CCA GAT TAT CCA-3' (Sigma), and the reverse primer, *COX1* R: 5'-CAA CTA CGG ATA TAT AAG AAC CGA AAC TG-3' (Sigma), were employed in the standard PCR assay. These primers were specifically designed for targeting and amplifying a

part of the *COX1* gene region of interest. The *COX1* primers with all required characteristics are given in Table 1.

Table 1: *COX1* primers for standard PCR analysis

Primers	Sequence(5'→3')	Length	GC%	Tm	Dimer
<i>COX1 F</i>	CGTCGTATTCCAGATTATCCA	21	42.8	61.3	No
<i>COX1 R</i>	CAACTACGGATATATAAGAACCGAAACTG	29	37.9	65.1	No

Standard PCR Assay

DNA isolated from control barley seeds was used to perform PCR amplification of the target part of the *COX1* gene using *COX1 F* and *COX1 R* primers. A PTC-200 Bio-Rad PCR system was utilized for the standard PCR assay, and OneTaq DNA Polymerase from Life Technologies was employed to facilitate DNA replication. The standard PCR reaction was performed in a 25 µL reaction volume, and the setup is detailed in Table 2.

Table 2: Standard PCR reaction setup for 25 µl reaction

PCR components	25 µL (volume)	Final concentration
10 X PCR buffer	2.5 µL	1X
50 mM MgCl₂	0.75 µL	1.5 mM
100 mM dNTP Mix	0.5 µL	0.2 mM
10 µM <i>COX1 F</i> primer	1.25 µL	0.5 µM
10 µM <i>COX1 R</i> primer	1.25 µL	0.5 µM
DNA template (1 ng/ µL)	1 µL	1-500 ng
Taq Polymerase (5 U/µL)	0.1 µL	0.5 U/rxn
ddH₂O	17.65 µL	-
Total	25 µl	

Annealing temperature optimization for the standard PCR reaction

To optimize the annealing temperature for standard PCR, five gradient PCR reactions were performed using the *COX1 F* and *COX1 R* primers. The optimal annealing temperature was determined by testing five different temperatures: 54 °C, 55 °C, 56 °C, 57 °C, and 58 °C. The objective of this optimization was to identify the best annealing temperature that would yield the most efficient amplification. The PCR was then performed using the Taq DNA polymerase PCR reaction program (Life technologies). The cycling parameters for the standard PCR reaction are outlined in Table 3.

PCR product purification

Five different annealing temperatures were tested. After amplification by standard PCR, the resulting five products were purified using the QIAquick PCR Purification Kit (Qiagen) according to the recommended protocol. The concentrations of the purified products were measured using the Qubit® 4.0 dsDNA BR Assay Kit (Life Technologies), and their purity (A 260/280 and A 260/230) was confirmed using the Denovix DS-11+ Spectrophotometer (Hindash & Hindash, 2022).

Following the purification step, the samples underwent analysis through gel electrophoresis. 1 % agarose gel was prepared by mixing of 100 ml of 1X TAE buffer with 1 g of agarose. Gel visualization was achieved by adding 10 µl of gel red stain (10,000 X) (Biotium) to enhance the visualization of the PCR products. Subsequently, 10 µl of each PCR sample was mixed with 2 µl of 6 X gel loading dye (NEB), and 10 µl from this blend was carefully loaded into the gel wells. To serve as size markers, a low molecular weight ladder (100 bp, NEB) was loaded in the first and last gel wells. The electrophoresis was achieved at 85 V for 30 minutes. Following electrophoresis, the gel was examined under UV light.

Table 3: Cycling parameters for standard PCR reaction (Life technologies)

	Step	Temperature (°C)	Time
	Initial Denaturation	94	3 minutes
30 PCR Cycles	Denature	94	45 seconds
	Anneal	54-58	30 seconds
	Extend	72	90 seconds
	Fina extension	72	10 minutes
	Hold	4	Indefinitely

Cloning reaction

The PCR sample with the most amplified band was selected for cloning reaction. The "amplified band" is the resulting DNA fragment, which can be well visualized as a band on an agarose gel after electrophoresis. To clone the PCR amplified product, the pCR™4-TOPO™ TA vector was used, which is a 3956 bp vector that includes a Kanamycin resistant gene site (Alvarez-Quinto et al., 2017). For the cloning process, the TOPO® TA Cloning® Kit (Life Technologies) for sequencing was utilized. Since freshly amplified PCR product already possesses the essential 3' adenine required for TA cloning, additional A tailing is unnecessary. Following the cloning reaction, the cloned PCR product was further processed for transformation into One Shot® TOP10 chemically competent *E. coli* cells (Life Technologies).

Transformation of cloned PCR product into *E. coli*

Into a vial containing One Shot® TOP10 *E. coli* competent cells (Life Technologies), 2 µl of the cloning reaction mixture was transferred as per the protocol. Four LB Agar plates with 50 µg/ml kanamycin (Sigma) were prepared. In three of these plates, 50 µl of the transformation reaction was evenly distributed, while in the fourth plate, 100 µl of the transformation reaction was carefully spread. The plates were then incubated overnight at 37 °C.

Analysis of Positive Clones, Plasmid Isolation, and Sanger Sequencing

Nine distinct colonies were selected from the four LB agar plates and marked on the back side. LB media with 50 µg/ml kanamycin (Sigma) was prepared and warmed to 37 °C. Subsequently, each individual colony was carefully transferred into separate 5 ml tubes containing 3 ml of LB medium supplemented with 50 µg/ml kanamycin (Sigma). These tubes were then positioned in an incubator for an overnight

incubation at 37 °C while being agitated at a speed of 200 rpm.

Plasmids were isolated from each tube using the PureYield™ Plasmid Miniprep System (Promega). An alternative protocol suitable for large culture volumes was employed for plasmid isolation. The isolated plasmids were analyzed using the Qubit® 4.0 dsDNA BR Assay Kit (Life Technologies) for quantification. The purity of the isolated plasmids was checked using the Denovix DS-11 + Spectrophotometer, and then stored at -20 °C.

The 9 isolated plasmid samples were sent to KIGene Karolinska Institutet, Stockholm for Sanger DNA sequencing. For sequencing, the M-13 forward primer [-20] (5'GTAAAACGACGGCCAG-3') from the TOPO® TA Cloning® Kit (Life Technologies) was used. The sequencing data obtained from the 9 samples were analyzed using Chromas lite software (Lite, 1998) and manually compared to the TOPO vector sequence (Appendix 2).

Restriction enzyme digestion and Purification

The plasmid containing the amplified and cloned part of the *COX1* gene was subjected to digestion using the restriction enzyme (RE) NotI (NEB). A 100 µl reaction was prepared following the NEB protocol. Specifically, 2 µg of plasmid DNA (10.3 µl from a stock solution with a concentration of 194 ng/µl) was mixed with 10 µl of 1 X NEB buffer, 2 µl of NotI enzyme (10 units), and nuclease free water to a final volume of 100 µl. The same NotI RE digestion procedure (NEB) was carried out for another sample containing a cloned part of *COX1* gene, but for a 50 µl reaction volume. The reaction tubes were placed in a water bath at 37 °C and incubated for one hour. After the incubation, the NotI enzyme was heat inactivated by incubating the reaction tubes at 65 °C for 20 minutes. The digested plasmids were purified using the QIAquick PCR purification kit (QIAGEN) as per the protocol. Buffer PB was used without indicator, and the DNA was eluted in nuclease free water. The purified samples were quantified using the Qubit® 4.0 dsDNA BR Assay Kit (Life Technologies). To assess the linearization of the plasmids, a 1 % agarose gel was prepared, and 10 µl of 1 kb DNA ladder (NEB) was loaded into the first well. 10 µl of each digested and undigested samples were taken and combined with 2 µl of 6 X gel loading dye (NEB). Then, 10 µl from both the digested and undigested samples were loaded into separate gel wells. Gel electrophoresis was performed at 90 V for 1 hour.

Probe designing and Comparative qPCR reaction optimization

A specific *COX1* probe with the sequence 5'-TGCTTACGCCGGATGGAATGCTCT-3', consisting of 24 base pairs, was designed for targeted part of the *COX1* gene. Two mismatches present in the published probe were rectified to align with a short part of the *COX1* gene that was being amplified (Appendix 1). The probe was tagged with a 6-carboxy-fluorescein (6-FAM) diagnostic dye. To ensure the probe was accurately designed, its validation was confirmed using the primer blast software (NCBI).

The optimization of the qPCR method was conducted using an Agilent Aria MX PCR system (AH Diagnostics). TaqMan Universal PCR Master Mix kit by Applied Biosystems was employed for this assay. A pair of *COX1* primers, *COX1* forward: 5'-CGT CGT ATT CCA GAT TAT CCA-3' (Sigma) and *COX1* reverse: 5'-CAA CTA CGG ATA TAT AAG AAC CGA AAC TG-3' (Sigma), were utilized along with a

6FAM-labeled *COX1* probe 5'-TGC TTA CGC CGG ATG GAA TGC TCT-3' (Thermo Fisher Scientific). To achieve optimal results, different concentrations of probes and primers were tested. For the primers, concentrations of 100 nM, 150 nM, 300 nM, 600 nM, and 900 nM were evaluated to determine the best amplification. The optimal concentration of the *COX1* probe was assessed at 100 nM, 125 nM, 150 nM, 200 nM, and 250 nM, respectively. All reactions were performed in triplicate on a 96-well plate (Applied Biosystems). The reaction was conducted in a 20 µl volume according to the protocol, using different concentrations of template DNA (ranging from 1 to 10 ng). The control and infected samples with various *U. nuda* fungus percentages (e.g., 1.2 %, 1.5 %, 2.1 %, and 2.9 %, respectively) were also analyzed using *COX1* primers and probe. To maintain uniformity, all samples were standardized to the same concentration prior to their utilization in the qPCR analysis. A non-template control reaction (NTC) was included, which consisted of nuclease-free water without template DNA. The cycling parameters remained consistent throughout the optimization process, including UNG incubation at 50 °C for 2 minutes, polymerase activation at 95 °C for 10 minutes, 40 cycles of denaturalization at 95 °C for 15 seconds, followed by annealing and extension at 60 °C for 1 minute.

Standard curve

To generate a standard curve, 8 data points were used with copy numbers ranging from 9×10^8 to 90 copies. A series of 10-fold serial dilutions was prepared, starting from 4.0×10^{-7} ng to 4 ng of linearized plasmid DNA. All dilutions were made using nuclease-free water. 1X Taqman Universal PCR Master Mix kit (Applied Biosystems) was utilized, along with *COX1* primers (0.3 µM each of forward and reverse, Sigma) and the *COX1* probe (0.125 µM, Thermo Fisher Scientific), in a total reaction volume of 20 µl. All standard reactions were performed in triplicates. The standard curve was constructed by plotting the threshold (C_q) values against the logarithm of the DNA copy number. This curve allowed for the determination of the unknown sample's concentration by comparing it to the standard samples of known concentrations.

DNA copy number

The gene copy number, which represents the number of copies of a specific gene present in an individual's genome, can be calculated using the following formula (Bignell et al., 2004; Lucito et al., 2003).

$\text{Copy number} = \frac{\text{Amount of DNA (ng)} \times \text{Avogadro's number}}{\text{Length of DNA (bp)} \times 1 \times 10^9 \times 660}$
--

In this method, a conversion factor of 1×10^9 is used to convert the gene copy number into nanograms. The Avogadro's constant is 6.023×10^{23} . Additionally, the average mass of 1 bp of dsDNA is 660 whereas, 4032 is DNA length in base pairs (Vector with DNA insert).

Amplification efficiency (E)

The term used to describe the fold increase in amplicon per PCR cycle is "amplification efficiency". This value can be determined manually by calculating the standard curve slope, or it can be determined automatically by software using the fluorescence data generated during the qPCR reaction. In the current study, the amplification efficiency (E) was automatically calculated by Agilent Aria MX PCR system (AH Diagnostics).

U. nuda primers and probe

In order to create the standard curve for the *U. nuda*, a previously published 6-FAM labeled MGB *U. nuda* probe (5'-ATA GGC AAG ACG GAC GAA AGC TCG-3') (Thermo Fisher Scientific) was utilized along with the primer pair 1F (5'-ATC GTG GCT CCC TTG AAA TAG-3') (Sigma) and 1R (5'-CCT CTC CGA AGT CCT GAT AGT A-3') (Sigma) (Setu, 2021). The primer pair (1F and 1R) and 6-FAM labeled MGB *U. nuda* probe were specifically used for the qPCR amplification of internal transcribed spacer 1 sequence from *U. nuda* in the infected barley seeds.

Standard curve for the *U. nuda*

To generate a standard curve for the *U. nuda*, a specific 6-FAM labeled MGB *U. nuda* probe and primer pair (1F and 1R) were employed for the amplification of linearized *U. nuda* plasmid DNA (obtained from a previous master's degree student). The qPCR reactions were performed in triplicates with a total reaction volume of 20 µl, including 1X TaqMan Environmental Mastermix (Applied Biosystems), 0.2 µM probe (Thermo Fisher Scientific), 0.9 µM of each primer (Sigma), and nuclease-free water. The concentrations of the primers and probe remained consistent with those employed by the bachelor researcher for *U. nuda* detection (Setu, 2021). A series of 10-fold serial dilutions ranging from 4.0 x 10⁻⁷ ng to 4 ng of linearized *U. nuda* plasmid DNA were prepared for the standard curve.

In addition to reproduce the standard curve for *U. nuda*, the infected samples with various *U. nuda* fungus percentages (e.g., 1.2 %, 1.5 %, 2.1 %, and 2.9 %, respectively) were also analyzed using *U. nuda* specific primers and probe. Agilent Aria MX PCR system (AH Diagnostics) was used for this activity. To ensure consistency, all the samples were diluted to the same concentration before being used in the qPCR reaction.

The Qubit results initially exhibited significant concentration fluctuations during the preparation of dilutions for the qPCR standard curve. Despite changing the Qubit® 4.0 dsDNA BR Assay Kit twice, the issue persisted. It was eventually mitigated by transitioning to the Qubit® 4.0 dsDNA High Sensitivity Assay Kit, resulting in more stable values.

Results

In an attempt to develop a probe-based qPCR technique for detecting and quantifying *U. nuda* in naturally infected barley seeds, a part of the *COX1* gene was selected as an internal control due to its presence in barley seeds. The objective was to create a standard curve for qPCR in order to quantify a part of the *COX1* gene from the barley

seeds. To achieve this, DNA was extracted from the crushed barley seeds and then a part of the *COX1* gene was amplified using a thermal cycler PTC-200 Bio-Rad PCR system. The amplified part of the *COX1* gene was then cloned into a TOPO®TA cloning vector and effectively transformed into *E. coli*, after which plasmid isolation was performed. Sanger sequencing was carried out to verify the presence of a part of the *COX1* gene, and the plasmid DNA was subsequently digested using the RE NotI. An absolute qPCR method was developed to detect and quantify a part of the *COX1* gene in both control and infected samples with different fungal infection (e.g., 1.2 %, 1.5 %, 2.1 %, and 2.9 %, respectively). Another objective of the present research was to detect and quantify *U. nuda* in infected barley seeds and to compare the value of a part of *COX1* gene (as an internal control) with the *U. nuda*. Further details about the achieved results are presented below.

Isolation of DNA from barley seeds

The E.Z.N.A. SP Plant DNA Kit was utilized for the DNA isolation process, which was conducted twice for each sample. The concentrations of the isolated DNA samples were measured with the Qubit® 4.0 dsDNA BR Assay Kit while their purity was checked with the Denovix DS-11 + spectrophotometer (Hindash & Hindash, 2022), and the results are presented in Table 4.

Table 4: Spectrophotometer and Qubit® 4.0 analysis of DNA samples extracted from one control and four infected samples with different *U. nuda* fungus percentages (1.2 %, 1.5 %, 2.1 %, and 2.9 %)

Samples	Nanodrop Conc (ng/μl)	A260/230	A260/280	Qubit conc (ng/μl)
C1	35.02	1.45	1.87	6.88
C2	20.90	1.61	1.88	8.20
1.2 a	107.91	1.18	1.81	36.20
1.2b	242.20	1.05	1.82	25.01
1.5a	39.21	1.49	1.84	9.72
1.5b	79.48	1.40	1.94	18.01
2.1a	40.16	1.67	1.83	20.25
2.1b	72.40	1.29	1.79	16.50
2.9a	39.39	0.99	1.76	15.96
2.9b	45.03	1.33	1.94	12.20

“C 1= Control barley DNA sample (containing no infection) no1, C2=control barley DNA sample (containing no infection) no 2, 1.2a= Barley DNA sample a with 1.2% *U. nuda*, 1.2b=Barley DNA sample b with 1.2% *U. nuda* and so on.”

The purity (A260/280) values obtained ranged from 1.76 to 1.94. Additionally, the A260/230 ratio fell within the range of 0.99 to 1.67. The Qubit concentration measurements exhibited a range of 6.88 ng/μl to 36.20 ng/μl, indicating varying concentrations across the samples.

Standard PCR assay

The DNA extracted from the barley seeds was utilized to amplify a specific region of the *COX1* gene, with a specific length of 79 bp. This amplification was achieved by employing the *COX1* F and *COX1* R primers. In order to identify optimal annealing temperature, five different annealing temperatures (ranging from 54 °C-58 °C) were tested using a gradient thermal cycler PTC-200 Bio-Rad PCR system. After the PCR reaction, the five samples were purified and then analyzed using 1 % gel electrophoresis, as depicted in Figure 3.

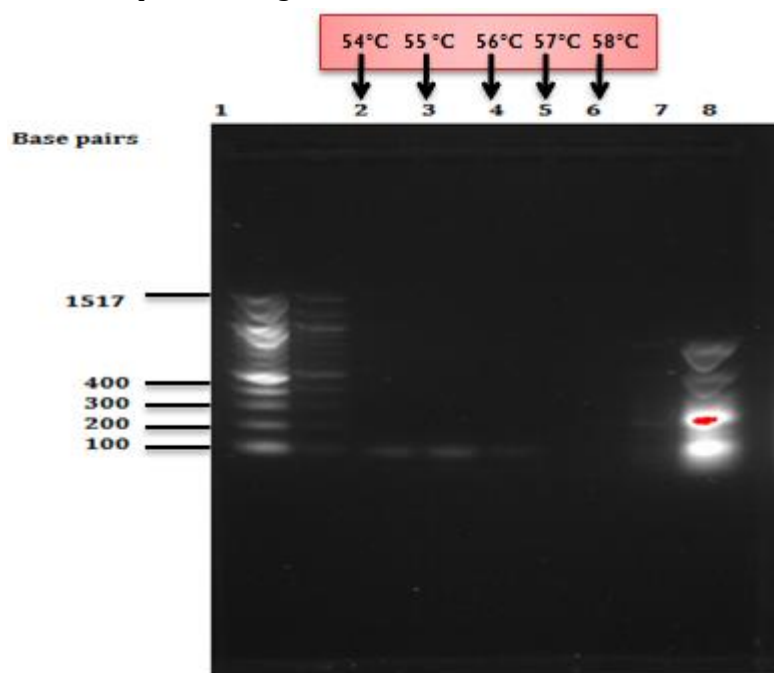


Figure 3. Gel electrophoresis showing optimization of annealing temperature by checking five different annealing temperatures. Lane 1 contains 100 bp DNA ladder (NEB) whereas Lane 8 contains low molecular weight DNA ladder (NEB), Lanes 2 to 6 correspond to the five PCR samples, with each sample annealing at a different temperature ranging from 54 °C to 58 °C.

According to Figure 3, successful amplification of the targeted part of the *COX1* gene was observed in lanes 2, 3, and 4, corresponding to annealing temperatures of 54 °C, 55 °C, and 56 °C, respectively. However, no amplification was detected in lanes 5 and 6, indicating that the annealing temperatures of 57 °C and 58 °C were not optimal for amplifying the desired *COX1* gene fragment. These results suggest that the best amplification of the *COX1* gene fragment was achieved at temperatures of 54 °C-56 °C. The amplified PCR products were purified and subsequently quantified using the Denovix DS-11+ Spectrophotometer and their concentrations were measured using the Qubit® 4.0 dsDNA BR Assay Kit as shown in Table 5.

The PCR sample that annealed at 54 °C exhibited a higher Qubit concentration of 22.0 ng/μl compared to the other samples. The purity values (A260/280) for all samples ranged from 1.75 to 1.97. Furthermore, the A260/230 ratio for the samples ranged from 1.19 to 1.85, indicating the presence of minimal contamination.

Cloning and Sanger sequencing

The purified PCR product with the best amplification, as confirmed by gel electrophoresis, was cloned into the pCR™4-TOPO™ TA vector. The resulting cloned construct was then successfully transformed into chemically competent *E. coli* cells. Following the transformation, the transformed reaction mixtures were plated onto LB agar plates. After an overnight incubation, distinct colonies were observed on the plates. A total of 9 different colonies were selected and individually grown. An alternative protocol from the PureYield™ Plasmid Miniprep System was followed for the plasmid isolation. The relevant 9 isolated plasmids were quantified with Denovix DS-11 + Spectrophotometer to find out the purity of DNA samples. Additionally, each of the isolated plasmid was analyzed using the Qubit® 4.0 dsDNA BR Assay Kit (Table 6).

The 9 isolated plasmids exhibited Qubit concentrations, ranging from 104.01 ng/μl to 194.02 ng/μl, indicating variations in the concentrations. The purity values (A260/280) obtained for all plasmids ranged from 1.69 to 1.98. Additionally, the A260/230 ratio for the plasmids fell within the range of 1.75 to 2.25.

Table 5: Spectrophotometer analysis of five purified PCR samples which were annealed at 54 °C -57 °C along with Qubit® 4.0 analysis

Samples	Nanodrop Conc (ng/μl)	A260/230	A260/280	Qubit conc (ng/μl)
S 1	36.01	1.19	1.93	22.0
S 2	13.60	1.81	1.75	2.62
S 3	14.11	1.85	1.95	3.62
S 4	16.51	1.36	1.88	3.81
S 5	17.52	1.49	1.97	4.50

Table 6: Qubit® 4.0 BR Assay Kit and Spectrophotometer analysis of isolated plasmids

Samples	Nanodrop Conc (ng/μl)	A260/230	A260/280	Qubit conc (ng/μl)
P1	390.51	1.75	1.97	178.01
P2	198.65	2.24	1.91	190.04
P3	210.43	2.18	1.93	170.21
P4	118.35	2.17	1.95	104.01
P5	163.79	2.18	1.95	161.60
P6	181.62	2.23	1.87	137.02
P7	114.55	2.25	1.91	138.03
P8	167.93	2.24	1.98	135.05
P9	117.94	2.21	1.69	194.02

After the plasmid isolation, the samples were sent for Sanger sequencing. The results of the sequencing were deemed satisfactory for samples number 3, 4 and 9 as shown in Appendix 3. The successful cloning of a part of *COX1* sequence of interest into the pCR™4-TOPO™ TA vector was confirmed through sequencing. This information is important because it suggests that any downstream applications that rely on the presence of a part of the *COX1* gene should focus on samples 3, 4, and 9 (Appendix 3). The sequencing data from sample number 9 was aligned with *COX1* gene sequence of interest from Genbank accession number MN127982.1 and was found to be 100 % aligned (Appendix 4).

Cloned plasmid samples number 9 and 4 were treated with the RE NotI following the protocol provided by NEB. The resulting linearized plasmids were then purified by QIAquick PCR purification kit and then analyzed using 1 % gel electrophoresis, as depicted in Figure 4.

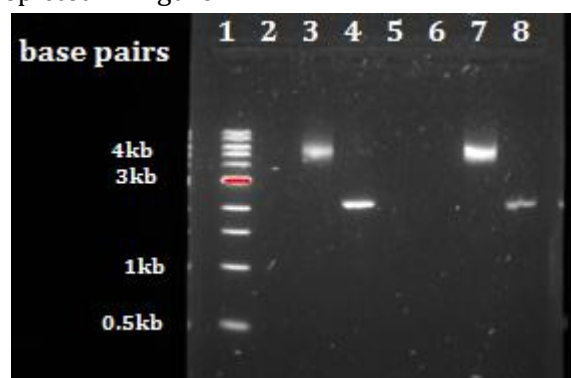


Figure 4. Agarose gel electrophoresis showing RE digestion with enzyme NotI (NEB). Lane 1 contains 1 kb DNA ladder (NEB). Lane 3 corresponds to the digested sample number 9 with expected size of 4032 bp (vector 3956+ insert 79), while lane 4 corresponds to the undigested sample number 9. Similarly, lane 7 represents the digested sample number 4, and lane 8 represents the undigested sample number 4.

Figure 4 shows clear differences between the digested and undigested samples. In Lane 4, the undigested plasmid sample no 9 migrates faster as compared to the digested sample no 9 in Lane 3. Similarly, there is a noticeable distinction in the migration pattern between the digested sample no 4 in Lane 7, which moves slowly, and the undigested sample in Lane 8, which moves faster, indicating successful digestion with the RE NotI.

The digested samples were purified using the QIAquick PCR Purification Kit, the concentrations of the digested samples were determined using the Qubit® 4.0 dsDNA BR Assay Kit. The concentration of the digested sample no 9 was found to be 4.08 ng/μl, while the second digested sample no 4 had a concentration of 0.506 ng/μl.

Real-time qPCR reaction

The digested plasmid, which contained the desired segment of the *COX1* gene, served as the DNA template for the subsequent qPCR analysis. During the qPCR reaction, the optimization process included assessing different concentrations of both the probe and primers. For the amplification of the *COX1* gene fragment, primer concentrations of 150 nM, 300 nM, 600 nM, and 900 nM were tested. Additionally, probe

concentrations of 125 nM, 250 nM, and 300 nM were evaluated to determine the optimal amplification. Based on the C_q values obtained, it was found that *COX1* primers at 300 nM and *COX1* probe at 125 nM provided the best results (data not shown).

Standard curve generation

In this research, a standard curve was generated for the *COX1* gene of interest. Serial dilutions were prepared, ranging from 4.0×10^{-7} ng to 4 ng, resulting in eight data points. The copy number range spanned from 90 to 9×10^8 copies. Negative control reactions (NTC) were performed using nuclease-free water as a substitute for DNA. As expected, the NTCs showed no amplification, indicating the absence of target DNA in the control reactions. Using Microsoft Excel, a linear correlation was established by plotting the threshold C_q values against the logarithm of DNA copy number on the x-axis, as depicted in Figure 5. The calculated coefficient of correlation (R²) was 0.992, indicating a strong correlation, and the slope of the curve was -3.532. The amplification efficiency (E), representing the fold increase in amplicon per cycle, was calculated to be 91.92 %.

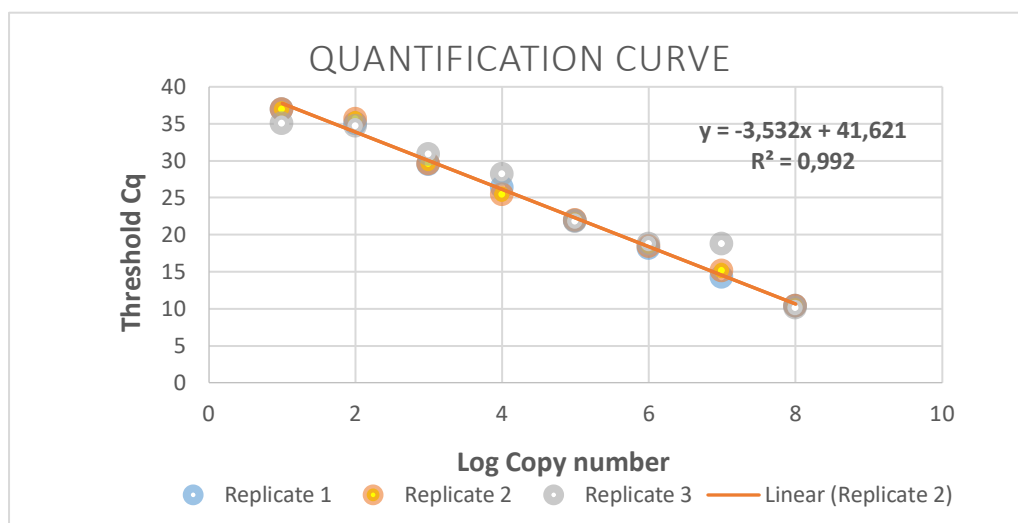


Figure 5. A standard curve generated by performing a 10-fold serial dilution, resulting in 8 data points, using linearized plasmid DNA containing a 79 bp target sequence from the genomic DNA of the *COX1* gene. To establish the relationship between the threshold C_q values obtained from the qPCR assay and the logarithm of the total DNA copies, a linear regression analysis was conducted. The graph represents replicates of the experiment as distinct points.

qPCR for infected seed samples

The standard curve for *U. nuda* using primer pair 1F and 1R was attempted to be reproduced following a previously published method but was unsuccessful. Despite trying various dilutions of primers and probes, no success was achieved. The same *U. nuda* primers (0.9 μM) and probe concentration (0.2 μM) were applied to the samples infected with *U. nuda* at different percentages (1.2 %, 1.5 %, 2.1 %, 2.9 %), but no amplification signal was observed after the qPCR reaction. In an effort to troubleshoot the issue, new *U. nuda* primers were ordered and tested, but the results remained unsatisfactory (data not shown).

Consequently, the targeted region of the *COX1* gene was successfully identified in both the control and infected seeds, although notable variations were observed in the obtained Cq values. Despite employing multiple measures to mitigate variability, fluctuations in the observed values persisted (data not shown).

Discussion

qPCR is an extremely effective and widely recognized technique developed for the quantification of nucleic acids in various applications. One of the major advancement in PCR-based applications was the concept of monitoring DNA amplification in real-time, using fluorescent DNA-specific probes or dyes. This advancement has significantly enhanced the analysis and understanding of nucleic acid amplification, allowing researchers to watch the progress of PCR reactions in real time, and obtain valuable insights into the kinetics and efficiency of the amplification process (Arya et al., 2005; Higuchi et al., 1992).

The inclusion of an internal control is essential in experimental and diagnostic assays as it plays a crucial role in ensuring the accuracy and reliability of the obtained results. By incorporating an internal control, researchers can effectively monitor and account for various factors that may influence the performance of the assay (Jain et al., 2006). Thus, the utilization of a part of the *COX1* gene as an internal control served as an important quality control measure, contributing to the confidence and reliability of the obtained results.

The aim of the present study was to establish a probe-based qPCR process for the concurrent detection of a part of the *COX1* gene and *U. nuda* in infected barley seeds. The initial step involved extracting DNA from barley seeds, which was successfully accomplished using the E.Z.N.A SP plant DNA kit. This particular kit is widely preferred by researchers due to its superior precision in isolating plant DNA (Wallenhammar et al., 2012; Zhu et al., 2006).

The obtained purity values (A260/280) ranging from 1.76 to 1.94 indicate a consistent level of purity, although slightly lower than the expected range of 1.8-2.0. While these values are still within an acceptable range, it suggests the presence of some contaminants, potentially including proteins, in the samples. The lower A260/280 ratios could be due to residual protein contamination during the extraction process (Dilhari et al., 2017). The A260/230 ratio falling within the range of 0.99 to 1.67 is lower than the expected range of 2.0 or above (Table 4). This indicate the presence of contaminants such as seeds and kit contamination or poorly cleaned lab equipment, which may interfere with downstream applications. Variations in concentration can stem from ethanol residues too, leading to interference in concentration measurements (Arseneau et al., 2017).

To perform standard PCR analysis, a part of the *COX1* gene amplification was conducted using a gradient PCR. A short part of the *COX1* gene (79 bp) amplification was achieved using a pair of specific published primers, *COX1* F and *COX1* R (Appendix 1), which were selected for their suitability in targeting the *COX1* gene region of interest (Zabaleta Vanegas et al., 2008). A 79 bp target part of the *COX1* gene was successfully amplified by PCR, and the amplification was verified through 1 % gel electrophoresis (Figure 3). However, 1 % agarose gel percentage was not ideal for this small fragment size, as higher gel percentages are typically recommended for

better results. Still the reason of using 1 % is because it balances the migration speed and separation of fragments well. The strategic use of 1 % agarose gel, despite its conventional association with larger DNA fragments, underscores a deliberate approach aiming to optimize the visualization and analysis of DNA fragments (Armstrong & Schulz, 2015). Consequently, the gel's resolution suffered because the smaller DNA fragments migrated too quickly through the gel. Higher percentages, like 2 %, offer better resolution for smaller DNA fragments, while lower percentages are better for larger fragments. Low-percentage gels have larger pores, suitable for larger DNA, but provide lower resolution for smaller fragments due to quicker migration (Lee et al., 2012; Lee & Bahaman, 2012).

In the current study, OneTaq DNA Polymerase was used, which not only aids in amplification but also facilitates cloning. The enzyme adds a single dA nucleotide at the 3' end of amplified PCR product, allowing for ligation with dT overhang vectors commonly used in TA cloning. Hence, it is recommended to use fresh PCR product for successful TA cloning, as this enzyme enables efficient ligation (D'Arpa, 2009; Green & Sambrook, 2021). A part of the *COX1* gene was successfully cloned into the pCR™4-TOPO™ TA vector using the TA cloning technique. The cloning reaction was then transferred into One Shot® TOP10 *E. coli* competent cells followed by plasmid isolation and sequencing.

The sequencing data was analyzed using Chromas lite software (Lite, 1998), which revealed that successful cloning had only occurred in sample numbers 3, 4, and 9 (Appendix 3). The main purpose of sequencing step was to confirm that a part of the *COX1* gene sequence of interest had been properly inserted into the vector. However, in sample 1, random nucleotide sequences were observed, indicating that the cloning was unsuccessful in this sample. Sample 6 only showed the presence of the TOPO vector sequence (Appendix 2), suggesting that no part of the *COX1* gene sequence was cloned. Sample numbers 2, 7, and 8 showed some parts of the forward and reverse primer sequences, indicating that the PCR product was amplified but not cloned successfully into the TOPO vector. Overall, the results suggest that successful cloning of a part of the *COX1* gene sequence was achieved only in a few samples, and further optimization may be required to increase the success rate of cloning (Appendix 3). If forward primer binds to its complementary strand and becomes reverse, it may be due to the presence of secondary structures in the DNA template that can interfere with the binding of the primer (Bustin & Nolan, 2004).

One of the three samples that were successfully cloned, namely sample 9, showed a perfect alignment (100 %) with a part of the *COX1* genomic DNA from NCBI database when analyzed using the Clustal omega multiple sequence alignment tool (Appendix 4). Plasmid samples with desired part of the *COX1* gene (sample numbers 9 and 4) were subjected to digestion with the RE NotI. The linearized plasmids were analysed through 1 % gel electrophoresis (Figure 4).

qPCR requires linear DNA templates to work properly and provide accurate results. The linearized plasmid DNA can then be used to create a standard curve for qPCR, which is essential for accurate quantification of gene expression levels (Hou et al., 2010). In this case, the sample was likely a mixture of plasmid DNA, which can be circular or linear, before and after digestion with a RE NotI that cuts the plasmid at a specific site. Based on the results, it appears that after digestion, a visible band of about 4 kb - 4.5 kb length was observed. This band likely corresponds to the

linearized pCR™4-TOPO® vector with the insert, which has a total length of 4032 bp. This is because the RE cut the plasmid at a specific site, resulting in a linearized vector of a specific size, to which the insert was added (Figure 3). Overall, the clear band observed after digestion confirms that the plasmid was successfully linearized and the insert was successfully added.

Standard curve

To generate a standard curve, a range of dilutions for linearized plasmid DNA was prepared. The dilution series consisted of 8 data points (10 fold dilutions), which were prepared by starting with an initial concentration of 4 ng (9×10^8 copies) and gradually diluting it until a concentration of 4.0×10^{-7} (90 copies) was reached. The effectiveness of qPCR is greatly influenced by the design of the oligonucleotides, which takes into account the specificity of the primer and probe sequences, as well as their respective concentrations. If the oligonucleotide design or concentrations are not optimal, it can have a significant impact on the efficiency of qPCR. The term "oligonucleotide design" refers to the strategic planning and structuring of short DNA or RNA sequences, known as oligonucleotides, for use as primers or probes in qPCR (Bustin & Huggett, 2017; Raso et al., 2011).

When the experiment was conducted with a lower copy number of 45 copies, it was observed that the spectrum of the standard curve was not fully covered. The triplicate did not show any Cq values, indicating that the amplification did not reach a detectable threshold level. An adjustment was made to the experimental approach by initiating the standard curve with a higher initial concentration of 4 ng (9×10^8 copies) and subsequently diluting it until a concentration of 4.0×10^{-7} (90 copies) was achieved. The fluctuation in Cq values observed during the experiment could be attributed to several factors, including pipetting errors, variations in reaction conditions, or sample degradation. To minimize these fluctuations, it is crucial to optimize the experimental conditions, use precise pipetting techniques, use sterile filter tips and include appropriate controls. In summary, the results obtained when going down to 45 copies showed limitations in sensitivity, leading to undetectable Cq values. However, by adjusting the experimental approach to start with a higher initial concentration, a more reliable and accurate standard curve was achieved (Figure 5).

The Qubit results showed that there were high fluctuations in the concentrations during the preparation of dilutions for the qPCR standard curve. Despite taking multiple measurements, there was no success in obtaining consistent concentrations from the same sample, which suggests that the issue was not with the sample itself. It is hypothesized that the double-stranded DNA may have been ruptured during the vortex phase, leading to the high variability in the concentration measurements. This process results in the generation of additional DNA fragments, which can interfere with the Qubit kit's ability to accurately quantify intact double-stranded DNA. The kit relies on the binding of a fluorescent dye to intact DNA, and the presence of ruptured DNA fragments can disrupt this binding interaction, leading to variability in the concentration measurements. The impact of DNA rupture during the vortex phase introduces a source of inconsistency in the Qubit assay, affecting its reliability in providing precise and accurate DNA concentration values (Gong et al., 2019; Wang et al., 2016). Furthermore, changing the Qubit® 4.0 dsDNA BR Assay Kit

twice did not resolve the problem.

The problem was ultimately minimized by switching to the Qubit® 4.0 dsDNA high sensitivity Assay Kit, which produced more sustained values. This indicates that the use of a more sensitive kit may be necessary for obtaining consistent and reliable measurements, especially in cases where there may be issues with sample quality or preparation (Li et al., 2014). Several factors, such as DNA quality, purity, and sample preparation, can affect the accuracy of the assay results. Additionally, the presence of contaminants in the assay kit or dye can lead to inaccurate readings (Davis et al., 2018; Schipor et al., 2016). To avoid such issues, it is recommended to use a high-quality DNA extraction method and ensure the integrity of the DNA sample. It is also suggested to check the kit's expiry date and storage conditions and avoid cross-contamination during sample handling (Albano et al., 2020; Tomás-Callejas et al., 2012).

The qPCR optimization results showed that the primer and probe concentrations are crucial parameters that need to be optimized to obtain accurate results. Among the different primer concentrations tested, *COX1* primers at 300 nM resulted in the best amplification efficiency. Similarly, among the different probe concentrations tested, *COX1* probe at 125 nM resulted in the best amplification efficiency. These results suggest that a lower probe concentration is more favorable for a part of the *COX1* gene amplification. Higher probe concentrations can lead to background noise, non-specific binding, and decreased reaction efficiency, while lower concentrations can lead to decreased signal and sensitivity. Therefore, it is important to optimize the concentration of probes to achieve the best amplification results (Al Sulaiman et al., 2017; Rocha et al., 2016).

The correlation coefficient (R^2) of the standard curve generated in this study was found to be 0.992, indicating a linear correlation between the threshold C_q values and the log DNA copy number. This high R^2 value suggests that the standard curve was accurate and reliable for quantifying a part of the *COX1* gene. To put this correlation coefficient value into perspective, a standard value of 0.98 is often considered acceptable for a qPCR standard curve (Nolan et al., 2006).

In the present study, the amplification efficiency of a part of the *COX1* gene was calculated to be 91.92 %. This result falls within the range of reported amplification efficiencies for other studies (Mas'ud et al., 2021) that have amplified the *COX1* gene using qPCR, which suggests that the present assay is comparable to other established methods. In another study, qPCR was used to measure the expression of COX-2 mRNA in colorectal cancer (CRC) tissues and cell lines. They reported an amplification efficiency of 97.9 % for their qPCR assay, which is within the acceptable range for qPCR assays (Leung, 2009).

Successful qPCR analysis was conducted on a part of the *COX1* DNA isolated from control and infected barley seeds with varying percentages of fungal infection (e.g., 1.2 %, 1.5 %, 2.1 %, and 2.9 %). However, significant variations were observed in the obtained C_q values, indicating potential differences in the amplification efficiency (data not shown). Despite the observed variations, a part of the *COX1* gene was successfully amplified in both control and infected seeds, confirming the presence of the target gene in the samples. Further investigation is warranted to understand the factors contributing to the variations in C_q values and their impact

on the qPCR results.

Standard curve was prepared for a part of the *COX1* gene, which was used as an internal control. The intention was to compare the quantitative value of a part of the *COX1* gene with that of *U. nuda*. The qPCR method developed by a bachelor researcher for the detection of *U. nuda* was tried to reproduce but was not successful. The same *U. nuda* primers and probe concentration were used, which included 0.2 μ M probe and 0.9 μ M of each primer (Setu, 2021). However, no amplification was seen in infected samples, nor in samples used for standard curve (plasmid DNA containing target from *U. nuda*). After several attempts, it was concluded that there might be several reasons for the lack of amplification, such as contamination in the *U. nuda* sample, probe, or primers. Additionally, the *U. nuda* probe used was not sufficient for multiple qPCR reactions so both the material and time was running out. Further optimization is required for the method developed, including literature review and primers and probe optimization for the *U. nuda* sample. In summary, a part of the *COX1* gene was successfully amplified for the standard curve, but the *U. nuda* detection method needs further optimization for successful amplification of the *U. nuda* samples.

Ethical aspects and impact on the society

Barley is important for the human and animal feeding. Efforts are being made to increase the yield of barley but there are pathogens that can cause diseases and reduces the yield. A loose smut disease by *U. nuda* causes the reduction in the barley production (Woldemichael, 2019). It is important to know that this disease will not spread. It is needed good, fast and reliable method in order to detect this disease in the seeds. In the pursuit of developing a precise and sensitive qPCR method for the simultaneous detection of a part of the *COX1* gene and the *U. nuda* in barley seeds, several critical ethical considerations come to the forefront. Scientific integrity is paramount, necessitating honesty, transparency, and rigorous adherence to ethical research practices. The responsible management of data, especially sensitive information, is crucial, ensuring privacy and security. The societal impact of this development is two-fold. On one hand, it has the potential to bolster food security by aiding in the early detection of pathogens and diseases in barley seeds, contributing to higher crop yields and more reliable food supplies. However, it's crucial to address the equitable distribution of these benefits. Researchers must consider how the technology could impact the livelihoods of farmers and communities. Moreover, the cost-effectiveness of the method should be taken into account to ensure that it doesn't create financial barriers for those who need it most. If animal or human subjects are involved, ethical standards governing their treatment and welfare must be rigorously followed, encompassing the well-being of animals and informed consent for human participants. Environmental ethics require researchers to assess and mitigate potential harm, complying with regulations and adopting sustainable practices. Community engagement, respecting local knowledge and concerns, is vital, as is transparently addressing conflicts of interest. Ethical publication practices, including journal submission and transparency, are essential. Lastly, a commitment to ongoing learning and adaptation to evolving ethical standards ensures the research's integrity and contributes to the broader ethical framework of scientific

inquiry. Furthermore, this study did not necessitate additional ethical approval since it did not engage patient samples, human samples, or animal models. Additionally, neither the student nor the supervisors employed any viral techniques. Prioritizing risk assessment, strict adherence to lab protocols was maintained. Moreover, no conflicts of interest were identified during the study, and all data analysis tools and software utilized were openly accessible.

Future perspective

The current study successfully developed a standard curve for the amplification of a part of the *COX1* gene, which can serve as an internal control for the detection and quantification of *U. nuda* in naturally infected barley seeds. In addition, the *COX1* method needs to further develop. For an internal control to work well it needs to be quantified without too much variation. The inability to reproduce the previously developed *U. nuda* detection method is a significant outcome of this study (Setu, 2021). While this might appear as a challenge, it presents an important opportunity to further refine and enhance the method for the specific detection of *U. nuda* in barley seeds. This process begins with an in-depth literature review to better understand the nuances and complexities of *U. nuda* detection. Exploring existing research and methodologies offers the opportunity to identify potential areas for improvement and fine-tuning. One critical aspect to address is the optimization of primers and probes specifically designed for *U. nuda*. This involves meticulous adjustments to the molecular components used in the qPCR assay, including primers and probes that target the unique genetic markers of the fungus. It's essential to consider factors such as primer design, probe specificity, and concentration to ensure the most accurate and reliable detection possible. This comprehensive approach will contribute to enhanced detection and management of *U. nuda* in barley seeds. Further research and development in this area can have a significant impact on the agricultural industry and food security.

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“Indeed, my Lord is near and responsive”. **Al-Quran**

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Appendices

Appendix 1

Published *COX1* primers and specific designed *COX1* probe

Accession no: MN127982.1

COX1 F: 5'CGTCGTATTCCAGATTATCCA3'

*COX1*R: 5'CAACTACGGATATATAAGAACCGAAACTG-3'

Reverse compliment (R. primer): CAGTTTCGGTTCTTATATATCCGTAGTTG

Probe: 5'TGCTTACGCCGGATGGAATGCTCT-3'

A part of the *COX1* Gene (79 bp)

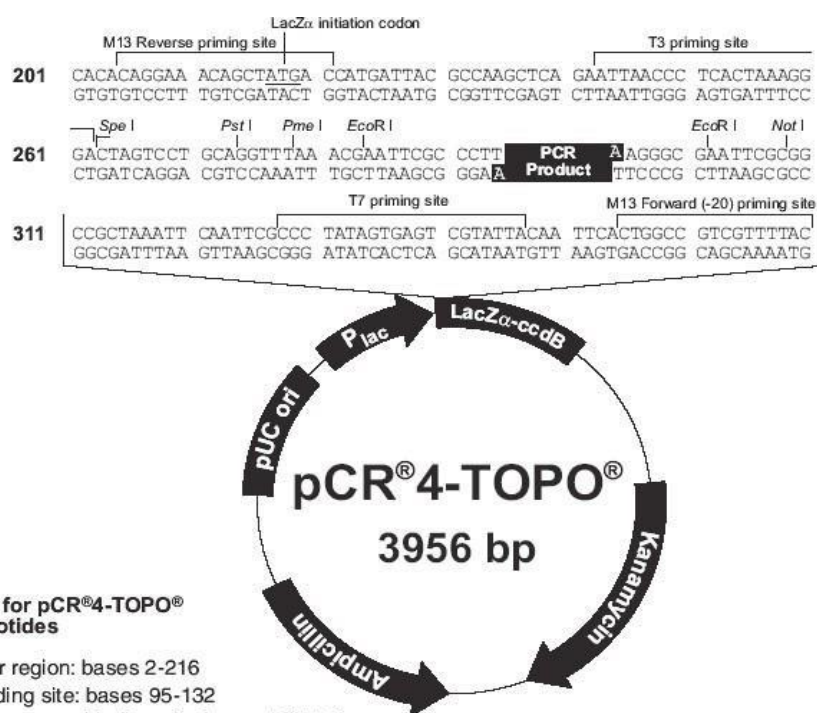
CGTCGTATTCCAGATTATCCAGATGCTTACGCCGGATGGAATGCTCTGAGCAGTTTCGGTTCTTATATA
TCCGTAGTTG

*yellow primers *Red probe *Green corrected mismatched

Appendix 2

pCR™4-TOPO™ TA vectorMap (Life technologies)

TOPOvector map (3956bp in size) showing both the cloning as well as restriction sites



Comments for pCR®4-TOPO® 3956 nucleotides

lac promoter region: bases 2-216
 CAP binding site: bases 95-132
 RNA polymerase binding site: bases 133-178
 Lac repressor binding site: bases 179-199
 Start of transcription: base 179
 M13 Reverse priming site: bases 205-221
 LacZα-*ccdB* gene fusion: bases 217-810
 LacZα portion of fusion: bases 217-497
ccdB portion of fusion: bases 508-810
 T3 priming site: bases 243-262
 TOPO® Cloning site: bases 294-295
 T7 priming site: bases 328-347
 M13 Forward (-20) priming site: bases 355-370
 Kanamycin promoter: bases 1021-1070
 Kanamycin resistance gene: bases 1159-1953
 Ampicillin (*bla*) resistance gene: bases 2203-3063 (c)
 Ampicillin (*bla*) promoter: bases 3064-3160 (c)
 pUC origin: bases 3161-3834
 (c) = complementary strand

Appendix 3

Sanger sequencing analysis

CGTCGTATTCCAGATTATCCAGATGCTTACGCCGGATGGAATGCTCTGAG**CAGTTTCGGTTCTTATATCCGTAGTTG**

***Red** primers ***Yellow** Sequence of interest from COX1 gene

***Turquoise** Part of Topo Vector

Sample no: 9

ATNNNATNNNGATTGATTTAGCGGCCGCGATTGCCCCTT**CGTCGTATTCCAGATTATC**
CAGATGCTTACGCCGGATGGAATGCTCTGAGCAGTTTCGGTTCTTATATATCCGTAGTT
GAAGGGCGAATTCGTTTAAACCTGCAGGACTAGTCCCTTTAGTGAGGGTTAATTCTGAG
CTTGGCGTAATCATGGTCATAGCTGTTTCCTGTGTGAAATTGTTATCCGCTCACAATTCC
ACACAACATACGAGCCGGAAGCATAAAGTGTAAGCCTGGGGTGCCTAATGAGTGAGC
TAACTCACATTAATTGCGTTGCGCTCACTGCCCGCTTTCCAGTCGGGAAACCTGTCGTG
CCAGCTGCATTAATGAATCGGCCAACGCGCGGGGAGAGGCGGTTTGCGTATTGGGCG
CTCTTCCGCTTCCTCGCTCACTGACTCGCTGCGCTCGGGACGTTCCGGCTGCGGCGAGC
GGTATCAGCTCNCTCATAGGTGCGTAATACGGGTATCCACAGAATCATGGAT

Sample no: 3

NTNNNTNGGCGATTGATTTAGCGGCCGCGATTGCCCCTT**CGTCGTATTCCAGATTATC**
CAGATGCTTACGCCGGATGGAATGCTCTGAGCAGTTTCGGTTCTTATATATCCGTAGTT
GAAGGGCGAATTCGTTTAAACCTGCAGGACTAGTCCCTTTAGTGAGGGTTAATTCTGAG
CTTGGCGTAATCATGGTCATAGCTGTTTCCTGTGTGAAATTGTTATCCGCTCACAATTCC
ACACAACATACGAGCCGGAAGCATAAAGTGTAAGCCTGGGGTGCCTAATGAGTGAGC
TAACTCACATTAATTGCGTTGCGCTCACTGCCCGCTTTCCAGTCGGGAAACCTGTCGTG
CCAGCTGCATTAATGAATCGGCCAACGCGCGGGGAGAGGCGGTTTGCGTATTGGGCG
CTCTTCCGCTTCCTCGCTCACTGACTCGCTGCGCTCGGTCGTTCCGGCTGCGGCGAACG
GGTATCAGCTCCACTCAAAGGCGGTATTACGGTTATGCTCTAAATCATN

Sample no: 4

GANNATAGGCGATTGATTTAGCGGCCGCGATTGCCCCTT**CGTCGTATTCCAGATTATCC**
AGATGCTTACGCCGGATGGAATGCTCTGAGCAGTTTCGGTTCTTATATATCCGTAGTTG
AAAGGGCGAATTCGTTTAAACCTGCAGGACTAGTCCCTTTAGTGAGGGTTAATTCTGAGC
TTGGCGTAATCATGGTCATAGCTGTTTCCTGTGTGAAATTGTTATCCGCTCACAATTCCA
CACAACATACGAGCCGGAAGCATAAAGTGTAAGCCTGGGGTGCCTAATGAGTGAGCT
AACTCACATTAATTGCGTTGCGCTCACTGCCCGCTTTCCAGTCGGGAAACCTGTCGTG
CAGCTGCATTAATGAATCGGCCAACGCGCGGGGAGAGGCGGTTTGCGTATTGGGCGCT
CTTCCGCTTCCTCGCTCACTGACTCGCTGCGCTCGGTCGTTCCGGCTGCGGCGAGCGGT
ATCAGCTCACTCAAAGGCGGTAAATACGGTTATCCACAGAATCAGGGGATAACGCAGGAA
AGAACATGTGAGCAAAAGGCCAGCAAAAGGCCAGGAACCGTAAAAAGGCCGCGTTGCT
GGCGTTTTTCCATAGGCTCCGCCCCCTGACGAGCATCACAAAAATCGACGCTCANTCA
GAGGTGGCGAAACCGACAGGGACTATAAGGATACCAAGCGTTTCCCCTGAAGCTCCCT
CGTGCGCTCTCCTGTTCCGACCATGCCGTTTACCGGAATACCT

Sample no: 1

CNNNGGGGAATTATAAGAATTAATTAAGGTGGAAATAGTGAATTAGCTATAGTGAAAAA
TCAATCGAAATTTATAAAGGGAGGTGTAGCTGTTGCTATTAAGACAATGTTCTGAGCTTA
GACGGATTGATTTTTAGTAGAGGAGAGCTGTTAGTTATCTCGGGGGTTCCAGTCGTACC
TAAGAAATTAATTGATACAGGATAGAGCATGTGGAGAAACGTGGTGGT

Reverse primer: CAACTACGGATATATAAGAACCGAAA

Forward primer (R. complement): TGGATAATCTGGAATACGACG

Sample no: 2

ATNTATANGGCGATTGATTTAGCGGCCGCGATTGCGCCTTCAACTACGGATATATAAGA
ACCGAAACTGCTCAGAGCATTCCATCCGGCGTAAGCATCTGGATAATCTGGAATACGAC
GAAGGGCGAATTCGTTTAAACCTGCAGGACTAGTCCCTTTAGTGAGGGTTAATTCTGAG
CTTGGCGTAATCATGGTCATAGCTGTTTCCTGTGTGAAATTGTTATCCGCTCACAATTCC
ACACAACATACGAGCCGGAAGCATAAAGTGTAAGCCTGGGGTGCCTAATGAGTGAGC
TAACTCACATTAATTGCGTTGCGCTCACTGCCCGCTTTCCAGTCGGGAAACCTGTCGTG
CCAGCTGCATTAATGAATCGGCCAACGCGCGGGGAGAGGCGGTTTGCGTATTGGGCG
CTCTTCCGCTTCCTCGCTCACTGACTCGCTGCGCTCGGTTCAGCTGCGGCGAGCG
GAACAACCTCACTCTAAGGCGGTAATACGGTTATTGCTTAACTCC

Sample 5

CCGAACNATAGGGCGAATTGAATTTAGCGGCCGCGAATTCGCCCTTCAACTACGGATAT
ATAAGAACCGAAACTGCTCAGAGCATTCCATCCGGCGTAAGCATCTGGATAATCTGGAA
TACGACGAAGGGCGAATTCGTTTAAACCTGCAGGACTAGTCCCTTTAGTGAGGGTTAAT
TCTGAGCTTGGCGTAATCATGGTCATAGCTGTTTCCTGTGTGAAATTGTTATCCGCTCAC
AATTCCACACAACATACGAGCCGGAAGCATAAAGTGTAAGCCTGGGGTGCCTAATGAG
TGAGCTAACTCACATTAATTGCGTTGCGCTCACTGCCCGCTTTCCAGTCGGGAAACCTG
TCGTGCCAGCTGCATTAATGAATCGGCCAACGCGCGGGGAGAGGCGGTTTGCGTATTG
GGCGCTCTTCCGCTTCCTCGCTCACTGACTCGCTGCGCTCGGTTCGGCTGCGGCG
AGCGGTATCAGCTCACTCAAAGGCGGTAATACGGTTATCCACAGAATCAGGGGATAACG
CAGGAAAGAACATGTGAGCAAAAGGCCAGCAAAAGGCCAGGAACCGTAAAAAGGCCCG
GTTGCTGGCGTTTTTCCATAGGCTCCGCCCCCTGACGAGCATCACAAAAATCGACGCT
CAAGTCAGAGGTGGCGAAACCCGACAGGACTATAAAGATACCAGGCGTTTCCCCCTGG
AAGCTCCCTCGTGCGCTCTCCTGTTCCGACCCTGCCGCTTACCGGATACCTGTCCGCC
TTTCTCCCTTCGGGAAGCGTGGCGCTTTCTCATAGCTCACGCTGTAGGTATCTCAGTTC
GGGGTAGGTCGTTTCGCTCCAAGCTGGGCTGGGTGCACGAACCCCCCGTTCCANCCCGA
ACGCTGGGCCTT

Sample no: 6

AAAGTNTCTATAGGGCGAATTCGTTTAAACCTGCAGGACTAGTCCCTTTAGTGAGGGTTA
ATTCTGAGCTTGGCGTAATCATGGTCATAGCTGTTTCCTGTGTGAAATTGTTATCCGCTC
ACAATTCCACACAACATACGAGCCGGAAGCATAAAGTGTAAGCCTGGGGTGCCTAATG
AGTGAGCTAACTCACATTAATTGCGTTGCGCTCACTGCCCGCTTTCCAGTCGGGAAACC
TGTCGTGCCAGCTGCATTAATGAATCGGCCAACGCGCGGGGAGAGGCGGTTTGCGTAT
TGGGCGCTCTTCCGCTTCCTCGCTCACTGACTCGCTGCGCTCGGTTCGGCTGCGG
CGAGCGGTATCAGCTCACTCAAAGGCGGTAATACGGTTATCCACAGAATCAGGGGATAA

CGCAGGAAAGAACATGTGAGCAAAAGGCCAGCAAAAGGCCAGGAACCGTAAAAAGGCC
GCGTTGCTGGCGTTTTTCCATAGGCTCCGCCCCCTGACGAGCATCACAAAAATCGAC
GCTCAAGTCAGAGGTGGCGAAACCCGACAGGACTATAAAGATACCAGGCGTTTTCCCC
TGGAAGCTCCCTCGTGCGCTCTCCTGTTCCGACCCTGCCGCTTACCGGATACCTGTCC
GCCTTTCTCCCTTCGGGAAGCGTGGCGCTTTCTCATAGCTCACGCTGTAGGTATCTCAG
TTCGGTGTAGGTCGTTGCTCCAAGCTGGGCTGTGTGCACGAACCCCCCGTTCAGCCC
GACCGCTGCGCCTTATCCGGTAACTATCGTCTTGAGTCCACCCGGTAGACACGACTTAT
CGCCACTGGCAGCATCCACTGGTAACNGGATTAACAAATCGAGGATGTAAGCGGTGCT
ACAAAGTTCTTGAATGGGGGCCCTAACTAGGGCC

Sample no: 7

ATNNATANGGCGATTGATTTAGCGGCCGCGATTGCCCCTTCAACTACGGATATATAAGA
ACCGAAACTGCTTAGAGCATTCCATCCGGCGTAAGCATGTGGATAATCTGGAATACGAC
GAAGGGCGAATTCGTTTAAACCTGCAGGACTAGTCCCTTTAGTGAGGGTTAATTCTGAG
CTTGGCGTAATCATGGTCATAGCTGTTTCCTGTGTGAAATTGTTATCCGCTCACAATTCC
ACACAACATACGAGCCGGAAGCATAAAGTGTAAGCCTGGGGTGCCTAATGAGTGAGC
TAACTCACATTAATTGCGTTGCGCTCACTGCCCGCTTTCAGTCGGGAAACCTGTCGTG
CCAGCTGCATTAATGAATCGGCCAACGCGCGGGGAGAGGCGGTTTGCCTATTGGGCG
CTCTTCCGCTTCCTCGCTCACTGACTCGCTGCGCTCGGTGCTTCGGCTGCGGCGAGCG
GTATCAGCTCACTCAAAGGCGGTAATACGGTTATCCACAGAATCAGGGGATAACGCAGG
AAAGAACATGTGAGCAAAAGGCCAGCAAAAGGCCATGAAACCGTAAAAAAGGCCGCG
TTGCTGGCGTTTTTCCATAGGCTCCCCCCCCTCTGACCATCATCCCAAAATCGACGCT
CAAGTCATAAGTGTTAAGCACGACAAGACTATA

Sample no: 8

NNNNAACTAAGGGCGAATTGAATTTAGCGGCCGCGAATTGCCCCTTCAACTACGGATA
TATAAGAACCGAAACTGCTCAGAGCATTCCATCCGGCGTAAGCATCTGGATAATCTGGA
ATACGACGAAGGGCGAATTCGTTTAAACCTGCAGGACTAGTCCCTTTAGTGAGGGTTAA
TTCTGAGCTTGGCGTAATCATGGTCATAGCTGTTTCCTGTGTGAAATTGTTATCCGCTCA
CAATTCCACACAACATACGAGCCGGAAGCATAAAGTGTAAGCCTGGGGTGCCTAATGA
GTGAGCTAACTCACATTAATTGCGTTGCGCTCACTGCCCGCTTTCAGTCGGGAAACCT
GTCGTGCCAGCTGCTAATTAATGAATCGGCCAACGCGCGGGGAGAGGCGGTTTGCCTATT
GGGCGCTCTTCCGCTTCCTCGCTCACTGACTCGCTGCGCTCGGTGCTTCGGCTGCGGC
GAGCGGTATCAGCTCACTCAAAGGCGGTAATACGGTTATCCACAGAATCAGGGGATAAC
GCAGGAAAGAACATGTGAGCAAAAGGCCAGCAAAAGGCCAGGAACCGTAAAAAAGGCCG
CGTTGCTGGCGTTTTTCCATAGGCTCCGCCCCCTGACGAGCATCACAAAAATCGACGC
TCAAGTCAGAGGTGGCGAAACCCGACAGGACTATAAAGATACCAGGCGTTTCCCCCTG
GAAGCTCCCTCGTGCGCTCTCCTGTTCCGACCCTGCCGCTTACCGGATACCTGTCCGC
CTTTCTCCCTTCGGGAAGCGTGGCGCTTTCTCATAGCTCACGCTGTAGGTATCTCAGTT
CGGTGTAAGTCGTTCCCTCCAAGCTGGGCTGGGTGCCGAACCCCCCGTTCAGCCGAA
CGCTGGGCCTTATCCG

Appendix 4

Clustal Omega multiple sequencing alignment tool result

Sample no: 9

```
Seq      CGTCGTATTCCAGATTATCCAGATGCTTACGCCGGATGGAATGCTCTGAGCAGTTTCGGT      60
GenCGTCGTATTCCAGATTATCCAGATGCTTACGCCGGATGGAATGCTCTGAGCAGTTTCGGT      60
*****
```

```
Seq      TCTTATATATCCGTAGTTG      79
GenTCTTATATATCCGTAGTTG      79
*****
```

*Seq= sequencing sample no 9

*Gen= *COX1* gene sequence from GenbankwithAccession no: MN127982.1

*yellow= Alignment