

# Specific Primer Design for Detection of gene Cyt b for Shark Species *Prionace glauca* and gene COI for *Carcharhinus* spp. Using Real-Time PCR Method

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**Abstract.** International market demand for sharks is increasing, thus increasing the number of catches of several shark species that are included in the International Union for Conservation of Nature's (IUCN) Red List and the Convention International Trade of Endangered Species (CITES) Appendix II. Authentication using biomoleculars by utilizing DNA is necessary. This study was aimed to design specific primers based on *cytochrome c oxidase I* and *cytochrome b* marker genes for endangered sharks (*Prionace glauca* and *Carcharhinus* spp.) and to apply them in vitro to identify fishery products using real-time PCR techniques. This research begins with designing target species-specific primers. Designing a specific primer using BioEdit software. The Oligo Evaluator and NCBI's Blast (Basic Local Alignment Search Tool) web tool for performing primary specification validation. The next stage is the sample preparation, DNA isolation, DNA amplification, DNA quality and quantity testing, and real-time PCR analysis. Primer's design of target species *Prionace glauca* using *cyt b* gene and degenerate primer for genus *Carcharhinus* spp. COI gene markers were successfully carried out in silico. Efficient real-time PCR conditions of *Prionace glauca* and *Carcharhinus* spp. complies with testing standards using the real-time PCR method.

## 1 Introduction

Sharks are part of cartilaginous fish (*Elasmobranchii*) with an important ecological role in the waters by balancing the structure of the organisms in the marine community food web [1]. Almost entire shark's body can be used as a commodity for export. Shark meat can be used as highly nutritious food, dried shark fins (hisit) for soup, and the skin can be processed into high-quality leather craft industry materials, and shark liver oil as a pharmaceutical raw material. Additionally, teeth, bile, guts, bones, gills, and others can still be processed for various purposes, including glue, ornaments, animal feed, medicinal materials, and other uses [2]. Along with the growth in demand for sharks on the world market, more shark species are

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being caught. Overfishing of sharks may result in a substantial population collapse since sharks have a conservative life cycle with slow growth and limited fecundity [3].

According to the International Union for Conservation of Nature's (IUCN) Red List, Indonesia is inhabited by approximately 118 species of sharks from 200 species of sharks worldwide. Indonesia is also reported as the country with the most enormous contribution of shark fins globally, with production figures of 60,000–100,000 tons/year [4]. The number of shark and ray catches in Indonesia was recorded at 12.31%, which is equivalent to 88,730 tons/year. The high catch and utilization of sharks in Indonesia has caused a drastic decrease in shark population levels, reaching 70% [5]. High shark fishing exploitation has been an international problem since 2013, when several shark species have been classified as CITES Appendix II. Excessive use of sharks as raw materials for processed fishery products can also lead to reduced shark populations, which makes them vulnerable to extinction [6].

Shark species are protected in many of the world's oceans, and international conventions have defined and documented the rules governing their movement. The Convention on International Trade of Endangered Species (CITES) notes that there are 12 endangered shark species included in Appendix II, including *R. typus*, *Cetorhinus maximus*, *Carcharodon carcharias*, *C. longimanus*, *Lamna nasus*, *S. lewini*, *S. mokaran*, *S. zyanea*, *C. falciformis*, *A. superciliosus*, *A. pelagicus*, and *A. vulpinus* [7]. This number will continue to increase along with the high exploitation of shark catches both as target and side catches. *Prionace glauca* is one of the shark species caught bycatch in pelagic fisheries, generally in tuna swamp fisheries [8]. According to research by Fahmi and Dharmadi [9], the population of *Prionace glauca* in nature is unknown, but it is suspected that there has been a decrease in catches in the southern waters of Java. *Prionace glauca* has also been caught being used for fins as soup since the middle of the 20th century [10]. *Prionace glauca* is classified as NT (Near Threatened) according to data from the International Union for Conservation of Nature (IUCN) Red List. The DNA barcoding technique was used in research to locate frozen fish fillets without labels in supermarkets in Bogor [11]. The identification results in the study showed that the four frozen fish file samples were identified as *Prionace glauca*. The identification of processed fishery products in Izdiharini's research [12], including shredded, salted fish, empal, filet, and smoke sold online was also identified as *Prionace glauca*. This suggests the free capture and sale of *Prionace glauca* species is still found. *Prionace glauca* is one of the shark species that is widely caught, eaten, and exported to several nations, including Portugal, Spain, and Italy, in addition to Indonesia [13]. *Prionace glauca*'s existence could become more and more threatened if it is continuously captured.

The latest data from the CITES uploaded in February 2023, the status of *Carcharhinus* spp. has been included in the CITES Appendix II category, previously only *C. falciformis* and *C. longimanus* were included in this list. The CITES Appendix II includes species that are not threatened with extinction, but whose existence could be threatened with extinction if their capture and trade continue without control and enforcement [14]. *Carcharhinus* spp. are classified as EN (Endangered), VU (Vulnerable), NT (Near Threatened), CR (Critically Endangered), and LC (Least Concern) on the IUCN Red List based on their conservation status. Haya [15] research identified *Carcharhinus* spp. shark species include *C. tjujot*, *C. sealei*, and *C. limbatus* in processed smoked fish samples obtained from the North Coast of Java (Pantura). This shows that Indonesian people, especially those around the North Coast of Java, still consume processed shark products.

Shark species listed in Appendix II by CITES require a special export permit in international trade for each part of their body. Enforcement of these regulations can be applied by identifying species on protected shark products to prevent illegal trade that can hinder the sustainability of the shark species [16]. Identification of species of complete sharks can be done taxonomically with a morphological approach. However, identification in this way will be difficult for shark products in the form of body pieces and processed products.

Nucleotide bases or Deoxyribonucleic Acid (DNA) can be used for biomolecular authentication to identify *elasmobranchii* whose morphological markers are challenging to distinguish [17]. DNA barcoding is a taxonomic technique based on amplifying brief DNA fragments in the genome's mitochondria. It uses short genetic markers from some areas of the standard DNA genome [18]. These DNA markers can be done using several genes, such as COI, *cyt b* or others. A genetic marker is a trait that can be inherited by offspring and aims to detect or monitor variations in DNA composition within a species [19]. DNA gene markers used as targets in real-time PCR bind directly to primers designed to allow specific DNA amplification of the desired gene target. In their study, Abdullah et al. [20] used mini-barcode DNA analysis (COI and cytochrome b) to verify commercial shark-processed items.

Authentication of processed fishery products can be done by the Polymerase Chain Reaction (PCR) method. PCR is one of the widely studied and widely used methods to quickly double the number of DNA molecules [21]. DNA amplification is carried out by analyzing new DNA that complements the target DNA through enzymes and oligonucleotides as primers [22]. PCR methods can be modified into several types, for example, PCR sequencing and real-time PCR. PCR sequencing requires more DNA, making genetic detection more difficult. The process has low sensitivity, more significant cost, and requires post-PCR procedures, so it takes a long time [23]. Real-time PCR is an alternative method that is fast, sensitive, simple, and reliable [24]. Real-time PCR has various benefits over other PCR techniques, including the ability to identify species using small samples, a low risk of contamination, a lack of visual inspection, and the ability to simultaneously identify numerous samples [25]. The optimal amplicon length for conventional PCR is 100-500 bp [26], while the amplicon length for real-time PCR is 50- 150 bp [27]. Authentication of processed products of several shark species using the real-time PCR method has been carried out by Aldina [28] and Patmawati [29] for species of *Rhincodon typus*, *Sphyrna lewini*, *Sphyrna mokarran*, *Sphyrna zygaena*, *Carcharhinus longimanus*, *Carcharhinus falciformis*, *Alopias pelagicus*, *Alopias superciliosus*, and *Rhynchobatus australiae*.

To be able to detect shark species specifically and precisely, shark species-specific primers are needed. Specific primers avoid erroneous detection of non-target shark species. It is important to design shark species-specific primers. The use of specific primers in qPCR reactions can produce the desired target amplicon. This strategy can save costs [30]. In addition, the use of specific primers provides more accurate, specific, and reliable quantification results from qPCR reactions [31]. Designing appropriate target-specific primers avoids primer matches with non-target species and allows undesired amplification. This process will influence the success of detecting shark species correctly.

Authentication and identification of processed fishery products made from shark raw materials are critical to anticipate and monitor the exploitation of protected sharks. Such measures can be used as a preventive measure against the extinction of certain shark species due to overexploitation. Therefore, research on the design of specific DNA barcoding primers of endangered sharks using real-time PCR methods needs to be carried out as its application to detect raw material species in several types of fishery products. This study was aimed to design specific primers based on *cytochrome c oxidase I* and *cytochrome b* marker genes for endangered sharks (*Prionace glauca* and *Carcharhinus* spp.) and to apply them in vitro to identify fishery products using real-time PCR techniques.

## 2 Materials and methods

### 2.1 Materials

The tools used in this study include 1.5 mL microcentrifuge tube, 2 mL collection tube, scissors, aluminum foil, pipette tips, measuring cup, Erlenmeyer flask, freezer, microwave, water bath, vortex (BIOSAN V-32 Multi-Vortex), centrifugation (SORVALL LEGEND MICRO17 Centrifuge Thermo SCIENTIFIC and Thermo SCIENTIFIC mySPIN 12), Thermo-Shaker (TS 100, bioSan), UV tray, computer, Image Lab software, Bio-RAD Gel Doc™ EZ Imager, digital scales (KERN, ABJ-NM/ABS-N), horizontal electrophoresis (Mupid-exu Submarine electrophoresis system), micropipette 0.5-10µL (volac), 20-200µL (Thermo Scientific), and 100-1,000µL (Thermo Scientific), DNA plate, ultra clear sealing film, and real-time PCR (Applied Biosystems™, StepOne™ Real-Time PCR System).

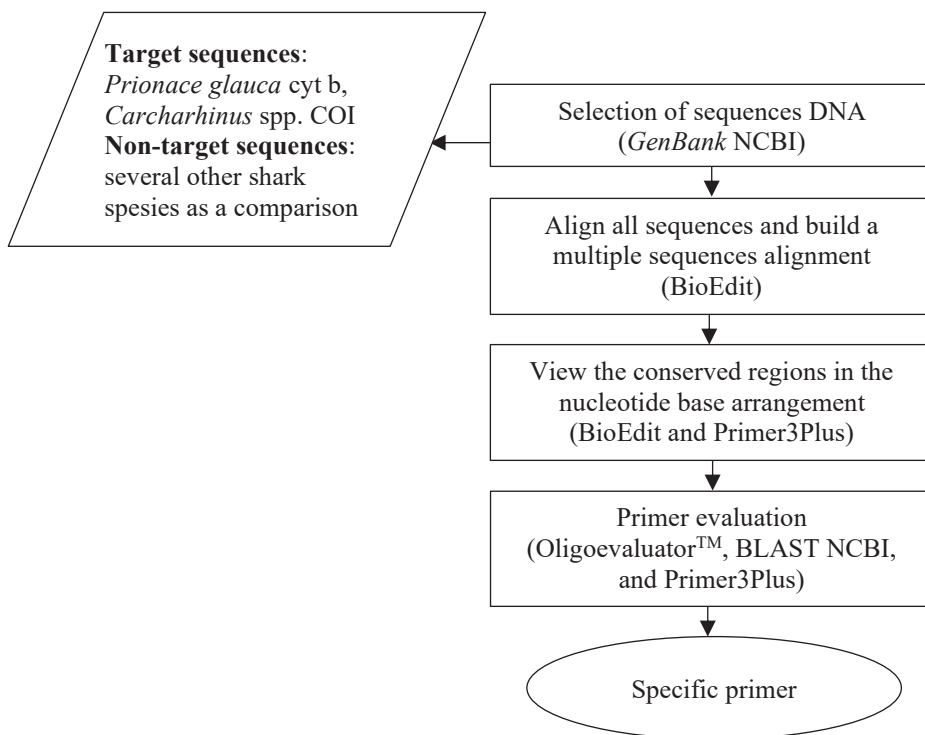
The materials used in this study were shark filet samples, DNA isolation KIT (Geneaid Genomic DNA Mini Tissue Kit), *Prionace glauca* isolate (filet, shredded, smoke shark, salted shark) [12], *Carcharhinus falciformis* isolate [29], isolate (*C. limbatus*, *C. tjtjot*, and *C. sealei*) [15], specific primer, DNA marker (Quick-Load® 100 bp DNA Ladder, New England BioLabs), DNA template, DNA loading dye, ethanol, agarose LE (iNtRON BIOTECHNOLOGY), GelRed Nucleic Acid (BIOTIUM), ddH<sub>2</sub>O, master mix real-time PCR (sample DNA isolate, H<sub>2</sub>O, Taq polymerase, ROX, forward primer, and reverse primer), AE buffer, and TBE buffer.

### 2.2 Methods

There are various steps to the work process in research. The initial stage before testing in the laboratory is primer design in silico. The design of the primer is carried out to obtain specific primers used in the qPCR amplification process. The next stage is sample preparation of 0.20-0.25 grams and continues with DNA isolation using the spin-column method. The isolated DNA is then quantified for concentration and purity, then visualized by electrophoresis, sequencing, and DNA amplification, and then electrophoresis is carried out again to obtain qPCR products.

#### 2.2.1 Primer design

Primer design was carried out in silico by downloading sequences from shark species *Prionace glauca* and *Carcharhinus* spp. as well as several other shark species as a comparison on the NCBI (National Center for Biotechnology Information) website to see conserved areas in nucleotide base arrangements. COI and cyt b genes are examples of markers. The downloaded sequence is then imported into the Bioedit application to view the conserved regions in the nucleotide base arrangement. Multiple alignments on the acquired target and non-target sequences were performed with the ClustalW option in the Bioedit application [32]. Pieces of 150-300 bp nucleotide bases are then entered into the Primer3Plus website to get some recommendations or candidates for forward and reverse primer pairs to be used. The best primer pair can be determined through criteria based on %GC, T<sub>m</sub> value, secondary structure, and the possibility of hairpin, primer dimer, and mispriming. The OligoEvaluator™ and NCBI's BLAST (Basic Local Alignment Search Tool) web tool are the finest options for performing primer specification validation. The web tool is used to analyze the potential for a primer, dimer, and hairpin contained in the designed primer [33]. Illustration of primer design can be seen in Figure 1.



**Fig. 1.** Illustration of primer design

### 2.2.2 DNA isolation

The frozen shark file raw materials are stored in a freezer at  $-20^{\circ}\text{C}$ . Each sample weighed 0.20-0.25 grams, then mashed and put into a 1.5 ml microcentrifuge tube. The sample genetic materials is then isolated or stored in  $-20^{\circ}\text{C}$  if not used immediately. DNA isolation is carried out using the Geneaid Genomic DNA Mini Tissue kit based on the kit protocol.

### 2.2.3 DNA amplification

DNA amplification is performed using StepOne Real-Time PCR Systems (Thermo Fisher Scientific, Waltham, Massachusetts, U.S.). The total qPCR for *Prionace glauca* used was  $25\mu\text{L}$ , consisting of  $12.5\mu\text{L}$  Taq,  $5.5\mu\text{L}$  H<sub>2</sub>O,  $1\mu\text{L}$  forward primer,  $1\mu\text{L}$  reverse primer, and  $5\mu\text{L}$  sample (Bioline, Taiwan). As for *Carcharhinus* spp. the total qPCR used is  $20\mu\text{L}$ , consisting of  $10\mu\text{L}$  Taq,  $7.5\mu\text{L}$  H<sub>2</sub>O,  $0.5\mu\text{L}$  ROX,  $1\mu\text{L}$  forward primer,  $1\mu\text{L}$  reverse primer, and  $5\mu\text{L}$  sample. The mixture is put into a PCR microtube and placed in a PCR well. The amplification process consists of pre-denaturation, denaturation, annealing, extension, and post-extension stages. Pre-denaturation stage conditions at  $95^{\circ}\text{C}$  for 2 minutes, the denaturation stage at  $95^{\circ}\text{C}$  for 40 seconds, the annealing stage at  $50^{\circ}\text{C}$  for 40 seconds, the extension stage at  $72^{\circ}\text{C}$  for 40 seconds, and one final extension cycle at  $72^{\circ}\text{C}$  for 1 minute. The denaturation, annealing, and extension stages in the amplification process were carried out in as many as 40 cycles followed by melt-curve analysis.

### 2.3 Data analysis

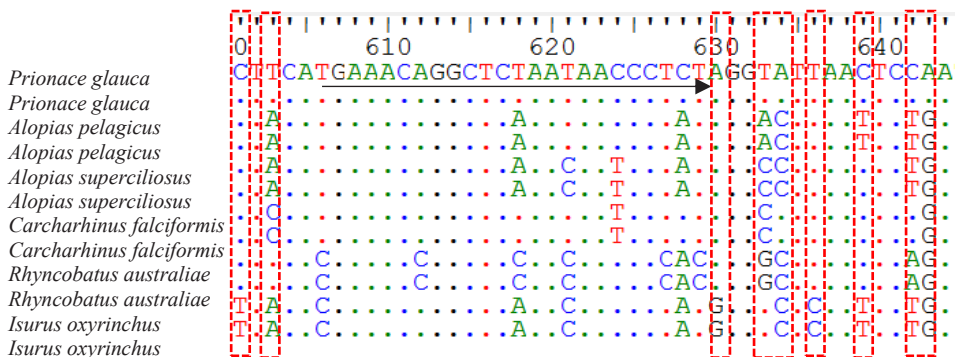
The amplification data and primer sequences were visually analyzed on the amplification results and bioinformatically using the BLAST (Basic Local Alignment Search Tool) algorithm contained in the NCBI database in the primer sequence. The BLAST technique can be used to align sequences and find sequences with common areas across many databases. Data analysis is also utilized to assess if the primers employed were successful or unsuccessful. The program displays each alignment's expectation values (E-values) as indicators to find match opportunities and assess potential biological relationships between sequences.

## 3 Result and discussion

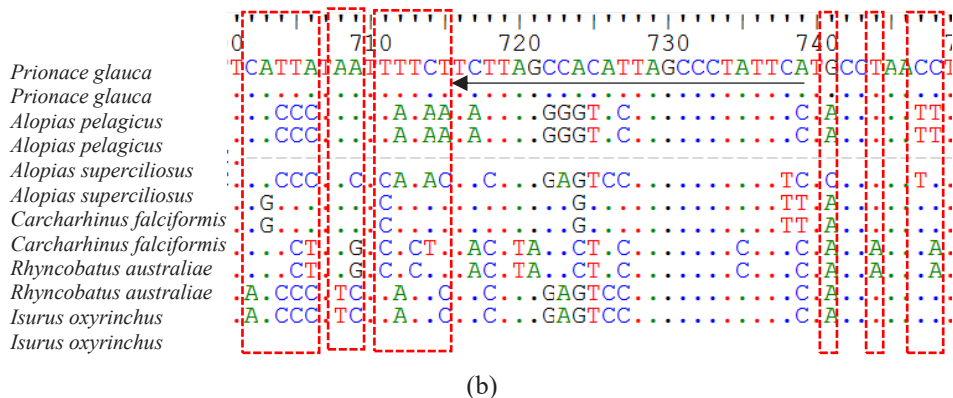
### 3.1 *Prionace glauca* primer profile

Primer design is carried out to obtain primers that can be used in DNA amplification by the Real-Time Polymerase Chain Reaction (qPCR) method [34]. The primer design in this study was carried out on shark species and genera that are included in the list of protected shark species in Indonesia, one of which is the species *Prionace glauca*. *Prionace glauca* is classified as NT (Near Threatened) according to data from the IUCN Red List. Several gene markers have been tested using *Prionace glauca* primers, including *cyt b*, COI, and NADH2. Based on the primer design results of some of these gene markers, *cyt b* was chosen because the results were considered better to order and use later. Cytochrome b is a gene marker widely used in DNA analysis to identify raw material species in fishery products [35].

It is possible to create a primer in silico that satisfies the requirements for amplification. According to Saraswati *et al.* [36], in silico analysis is a crucial computational prediction in primer design. Bioinformatics software is used to complete the initial design. However, further laboratory testing is required [21]. Figure 2 showed the *Prionace glauca* primer pair obtained from various sequence alignments performed using the Bioedit program.



(a)



**Fig. 2.** Results of multiple sequence alignment using bioedit application: (a) *Prionace glauca* forward primer, (b) *Prionace glauca* reverse primer

The results shown in Figure 2 show the alignment of the DNA sequence of *Prionace glauca* using a bioedit application. The results showed conserved regions marked with red squares and nucleotide base sequences as primers marked with black arrows. The image is the result of the alignment of the target DNA sequence of *Prionace glauca* with the DNA sequence of other sharks, including *Alopias pelagicus*, *Alopias superciliosus*, *Carcharhinus falciformis*, *Rhyncobatus australiae*, and *Isurus oxyrinchus*. According to BLAST data, the forward and reverse primer designs contained 100 *Prionace glauca* sequences. With a product length of 134 bp, the primer was also demonstrated to adhere to the *Prionace glauca* MG516052.1 sequence code. According to Pradnyaniti et al. [37], these criteria include the melting temperature (Tm) value, the percentage of G and C (% GC), the 3' dimer, stability, repetitions, hairpins, runs, and false priming.

The primer design criteria, according to Purwakasih and Achyar [38] have nucleotide lengths ranging from 18-30 bp, have a percentage of GC base composition of 40-60%, have TM values between forward and reverse primers that do not differ by more than 5°C, and do not form hairpin or self dimer secondary structures. Table 1 shows the conclusions of the analysis of a particular primer design.

**Table 1.** Results of primary design evaluation of *Prionace glauca*

Sequence	Base count (bp)	TM (°C)	GC%	Primer dimer	Secondary structure	Product length (bp)
Prionace glauca Forward						
TGAAACAGGCTCTA	24	63.0	41.7	No	Weak	134
ATAACCCCTCT						
Reverse						
TGAATAGGGCTAAT	24	64.5	41.7	No	Very weak	
GTGGCTAAGA						

The results in Table 1 show that the primer forward and reverse *Prionace glauca* designed on each parameter have almost the same value. The evaluation results are by several criteria, including the ideal primer length, which ranges from 18-30 bp. Less than 18 base primers may cause the primer to stick in unintended areas [39]. The recommended Tm value is between 50 and 65°C, with a maximum Tm differential of 5°C between the forward and reverse primers. The efficiency of the amplification process may diminish if there is a temperature differential of more than 5°C [40]. The percentage of the amount of GC will affect the bonds between DNA strands, so it is recommended at 40-60% vulnerable. GC

values below 40% can reduce efficiency in the PCR process because primers cannot compete effectively to stick to the template. While too high GC values can form too strong a bond between the primer and the target DNA, this can cause the resulting PCR product to be too low [41].

### 3.2 DNA isolate of *Prionace glauca*

Isolation of *Prionace glauca* samples was carried out using the Geneaid kit. DNA isolation is generally a technique carried out to obtain pure DNA without proteins and RNA from a cell in a tissue. Good DNA quality can be used in various activities, including molecular markers, genome libraries, and sequencing [42, 43]. The lysis process is an initial process that plays an important role in determining the success of the DNA isolation process [44].

The concentration and purity of the isolated DNA are then assessed using quantitative and qualitative testing. The test can be carried out quantitatively using the spectrophotometer's absorbance reading or qualitatively utilizing electrophoresis. At wavelengths with a 260/280 ratio, the purity level of DNA isolates can be observed. This ratio is generally used to determine the presence of proteins or phenols in isolated nucleic acid samples [45]. The wavelength of 260 nm is the maximum absorption for nucleic acids, while 280 nm is the maximum absorption for proteins [46]. A value of more than 20 ng/L indicates a high level of DNA concentration [47]. The purity value at the A260/280 wavelength is also said to be good if it is at a vulnerable ratio of 1.8-2.0. The results of the quantitative concentration and purity test of DNA isolates can be seen in Table 2.

**Table 2.** DNA isolate concentration and purity test results

Sample code	Species	A260/A280	Concentration (ng/μL)
PG 282 A	<i>Prionace glauca</i>	2.1	150.4
PG 282 B	<i>Prionace glauca</i>	1.9	78.0
PG 290	<i>Prionace glauca</i>	2.0	60.5
PG 240	<i>Prionace glauca</i>	1.9	32.8
M1 [12]	<i>Prionace glauca</i>	1.9	22.2
M2 [12]	<i>Prionace glauca</i>	2.1	104.5
M3 [12]	<i>Prionace glauca</i>	1.9	62.7
M4 [12]	<i>Prionace glauca</i>	0.9	23.5
I8	<i>Lamna nasus</i>	2.0	139.2
I10	<i>Isurus oxyrinchus</i>	2.0	63.4
APL1	<i>Alopias pelagicus</i>	2.1	150.4
1-SL	<i>Sphyrna lewini</i>	1.9	78.0
1-RAS	<i>Rhynchobatus australiae</i>	2.0	60.5
4-CF	<i>Carcharhinus falciformis</i>	1.9	32.8

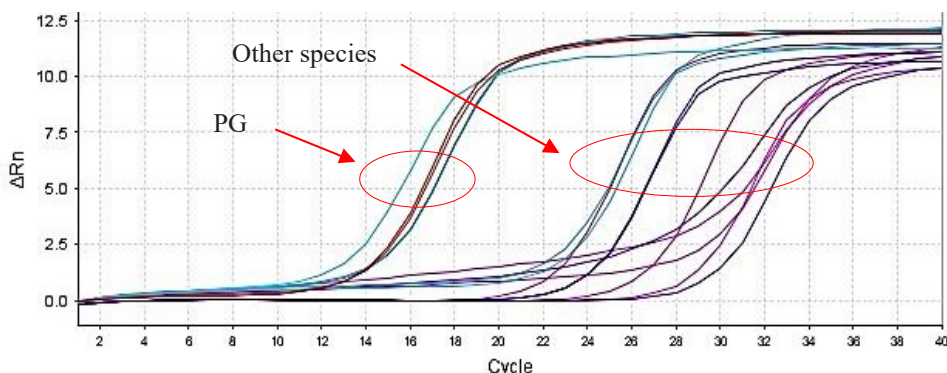
The concentration and purity values of *Prionace glauca* DNA isolate results, and several comparison sample codes tested produced different values. The sample concentration values and comparisons in the table above range from 22.2 to 150.4 ng/μL. The measured DNA concentration is diluted to obtain a uniform concentration in PCR analysis. Concentration values that are too small cannot be used in the PCR process. Tested samples of *Prionace glauca* and comparative samples had DNA purity values that ranged from 1.8 to 2.1. According to Sophian [48] research, DNA purity is considered good if the susceptible ratio is between 1.7 and 2.1. High impurity components, such as proteins, RNA, lipids, and detectable polysaccharides, can contribute to low DNA purity levels. The incomplete degradation of these proteins during the DNA extraction procedure can result in impurity contamination.



### 3.3 Real-Time PCR efficiency of *Prionace glauca* based *cyt b* gene.

#### 3.3.1 Optimal primer concentration

qPCR has the principle of breaking a double-stranded chain into a single-stranded chain, which is influenced by several factors, including temperature and time [49]. DNA molecule duplication in the qPCR method is carried out directly using optical light technology to make quantification easier [50]. Real-time PCR analysis is very sensitive, quick, and specific [51]. The qPCR method still has several drawbacks, including expensive equipment and reagents and the knowledge of precise and proper methodologies needed to produce accurate test findings [52]. DNA amplification process by qPCR in research using species-specific primer candidate *Prionace glauca cyt b* gene marker. qPCR results are presented in the form of amplification curves and melting curves. Figure 3 demonstrates the *Prionace glauca cyt b* primer amplification curve.



**Fig. 3.** Amplification curve of *Prionace glauca cyt b*

DNA amplification analysis with qPCR was carried out by looking at the increase in the graph on the amplification curve and assessed based on the cycle threshold (Ct) value. Figure 3 above shows the amplification process in *Prionace glauca* samples first, while other shark samples tested include *Alopias pelagicus*, *Rhynchobatus australiae*, *Sphyrna lewini*, *Carcharhinus falciformis*, and *Isurus oxyrinchus* species late to amplification. This shows that *Prionace glauca* samples with *cyt b* gene markers that have been designed successfully amplify target species characterized by differences in the number of Ct values with non-target species. Research conducted by Nasution and Andryas [53] states that the lower the Ct value, the higher the target DNA count, on the other hand, the higher the Ct value, the lower the target DNA count. The Ct value indicates the beginning of the exponential growth phase, as seen from the number of sample cycles that begin to be read.

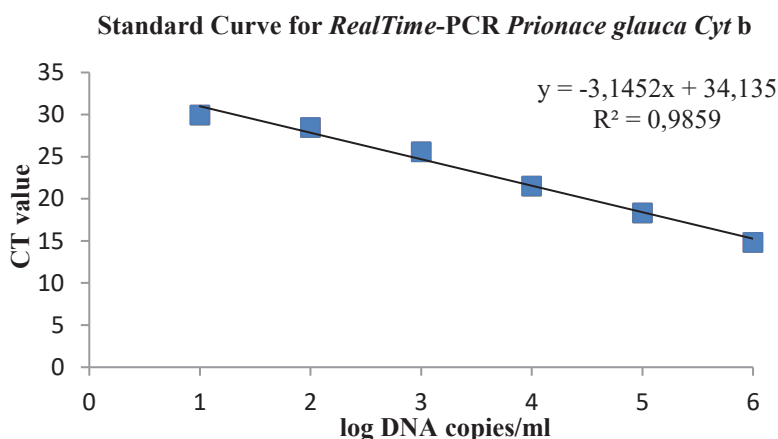
#### 3.3.2 Efficiency of qPCR conditions

The effectiveness of qPCR was evaluated using diluted samples of *cyt b* gene isolates from *Prionace glauca*. Dilution is carried out in stages ranging from  $10^0$  ng/ $\mu$ L to the smallest dilution of  $10^{-5}$  ng/ $\mu$ L. The efficiency results are presented in two forms of curves, namely the amplification and the melting curve. According to Bio-Rad [54], qPCR quality between 90 and 105% is excellent and ideal.

The amplification and melt curves represent the qPCR efficiency. On the amplification curve, staged dilution samples ranging from  $10^0$  ng/L to  $10^{-5}$  ng/L effectively amplified one after the other. This indicates that *Prionace glauca* primers may be detected at the lowest

dilution or  $10^{-5}$  ng/L. The following amplification result is in melt curve analysis needed to ensure the specificity of real-time PCR amplification [50]. The melting curve is a derivative of the temperature and fluorescence relationship curve. The current temperature of 50% is the  $T_m$  temperature at which double-stranded DNA amplicons separate into single strands. The  $T_m$  value is a sign of temperature when peaks appear on the  $T_m$  curve. Melt curve analysis with qPCR method in Maulani et al. [35] research. Identification of non-specific or primer-dimer amplification is also advised. The dilution curve was successfully detected until dilution of  $10^{-5}$  with a melting curve temperature of  $77.55^{\circ}\text{C}$ .

Multilevel dilution samples range from  $10^{-1}$  ng/ $\mu\text{L}$  to the smallest dilution of  $10^{-5}$  ng/ $\mu\text{L}$ . The validity of an qPCR reaction can be measured from several parameters, including linear regression value [ $y = mx + b$ ], real-time PCR efficiency value (E), and determinant coefficient (R<sup>2</sup>). The value can be determined using a standard curve. The standard curve is the relationship between the log copy number value on the x-axis to the Ct value on the y-axis resulting from several dilutions carried out in stages [55]. Figure 4 depicts the standard curve for the *Prionace glauca* sample using the Microsoft Excel program.



**Fig. 4.** Standard curve for real-time PCR *Prionace glauca* cyt b

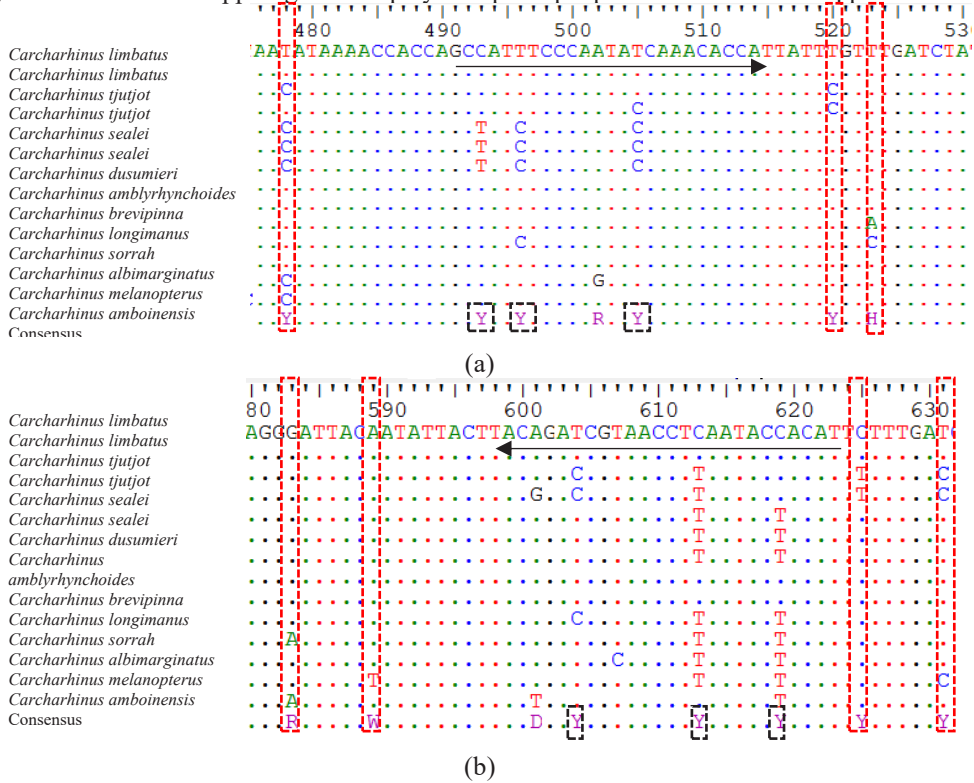
The Ct value is used as a reference to create a standard curve by plotting a log copy number against the Ct value. The value of the coefficient of determination (R<sup>2</sup>) and the value of efficiency (E) are obtained from the linear regression plot formed and can be used to evaluate and validate real-time PCR data. The slope and R<sup>2</sup> values of the standard curve in Figure 4 are -3.1452 and 0.9859, respectively. A real-time PCR efficiency value of 107% was obtained. According to Bio-Rad [54], a correlation coefficient (R<sup>2</sup>) value of >0.980 and an efficiency percentage value (%E) that is between 90 and 105% indicate the best real-time PCR quality. This shows that the results obtained have met these standards. Efficiency percentage values that are slightly larger than the standard are still acceptable. The difference in value can occur due to error factors in the pipetting process [55].

### 3.4 *Carcharhinus* spp. primer profile

The primer design was then carried out on the genus *Carcharhinus* spp., which is included in the list of protected shark species in Indonesia. The genus has been listed in the CITES Appendix II category since February 2023, with only *C. falciformis* and *C. longimanus* species previously included in this list. Primer design of *Carcharhinus* spp. was tried for two gene markers, *cyt b*, and COI. COI is chosen to be ordered and employed subsequently based on the outcomes of the two gene markers' principal design. Compared to the few

*Carcharhinus* species *cyt b* gene markers, the developed COI gene markers were evaluated better and had more diversified sequence data available on the NCBI website.

COI gene markers are mitochondrial genes generally used for diversity studies using DNA barcodes. The COI gene has many advantages in studying genetic characteristics, including fewer deletions and insertions in its sequences and many conserved parts so that they can be used as DNA barcodes in most species. The COI gene is also known to have an amino acid arrangement and protein arrangement that rarely undergoes substitution so that it can be stable [54]. Similar to the primer design of the species *Prionace glauca*, which is utilized in silico analysis, computational prediction was applied in the primer design of the genus *Carcharhinus* spp. Figure 5 displays the principal pair of *Carcharhinus* spp.



**Fig. 5.** Results of multiple sequence alignment using bioedit: (a) Forward primer *Carcharhinus* spp., (b) Reverse primer *Carcharhinus* spp.

The results of aligning the DNA sequence of *Carcharhinus* species using a bioedit tool and producing primers with degenerated designs are shown in Figure 5. Several species of *Carcharhinus*, including *C. limbatus*, *C. tjtjtjt*, *C. sealei*, *C. dusumieri*, *C. amblyrhynchoides*, *C. brevipinna*, *C. longimanus*, *C. sorrah*, *C. albimarginatus*, *C. melanopterus*, and *C. amboinensis*, are represented in the target DNA sequence alignment. A red box denotes the conserved regions in the picture, while a black arrow designates the primer's nucleotide base sequence. Degenerate primer is the design of primers that recognize more than one nucleotide base and is generally designed for universal primers [55]. Differences in nucleotide bases in forward primers in sequences 493, 496, and 505 and reverse primers in sequences 604, 613, and 619 are shown. The difference in bases is that T->C becomes Y, and C->T becomes Y. The best main specifications are then used to check

the primer design outcomes using the BLAST-NCBI online tool and the Primer3Plus website's primer pasting checks.

BLAST results in the design forward primers showed that there were 111 vertebrate sequences, including 91 species of sharks and rays consisting of 28 species of *Carcharhinus*. *Carcharhinus* species contained in the BLAST results consist of *C. amboinensis*, *C. leucas*, *C. limbatus*, *C. albimarginatus*, *C. acronatus*, *C. falciformis*, *C. brevipinna*, *C. longimanus*, and *C. brachurus*. Non-target species still attached to the primerBLAST results of the design are caused by improper selection of sustainable areas in primers. BLAST results in the designed reverse primers showed 162 vertebrate sequences, including 155 sharks and rays *Carcharhinus* spp. *Carcharhinus limbatus*, *C. tilstoni*, *C. brevipinna*, *C. amblyrhynchoides*, and *C. leiodon* are among the species found in the BLAST results. The diverse number of *Carcharhinus* species contained in the forward and reverse primer BLAST results can already represent the genus *Carcharhinus* spp. The primer was also shown to attach to *Carcharhinus limbatus* sample sequences with a product length of 132 bp. Table 3 displays the findings of the analysis of specific primer designs of COI gene markers from *Carcharhinus* spp.

**Table 3.** Results of primer design evaluation *Carcharhinus* spp.

Sequence	Base count (bp)	TM (°C)	GC%	Primer dimer	Secondary structure	Product length (bp)
Carcharhinus spp. Forward (degenerate) GCYATYTCCCAATA YCAAACACCA	24	64.5	37.5	No	No	132
Reverse (degenerate) TGTGGYATTGAGGT YACGATYTGT	24	56.2	37.5	No	No	

The results in Table 3 show the evaluation of the primer design of *Carcharhinus* spp. analyzed using the Oligo Evaluator web tool, including primer length, melting temperature (TM), % GC, primer dimer, secondary structure, and product length. The forward and reverse primer *Carcharhinus* spp. designed in each parameter have almost the same value. The primer length in both primers has met the ideal length, ranging from 18-30 bp [39]. The TM value obtained in the forward primer is 64.5°C and in the reverse primer is 56.2°C. According to research by Safitri et al. [40], the ideal TM value is between 50°C and 65°C, and the difference between the TM values of the two primers should not be greater than 5°C. If it exceeds this value, a decrease in the efficiency of the amplification process may occur.

The G and C or % GC percentage in both primers has the same value of 37.5%. The percentage of GC is the percentage between the number of guanine bases and cytosine present in a primer. The ideal primer criterion is to have a % GC value of 40-60% [58]. Hairpins and dimers, for example, are secondary structures that should be avoided in primers. This can result in not attaching the primer to the DNA template. Hairpins have similarities with dimers; the difference is in the complementary primer ends [39]. The length of the amplified product or amplicon in the primer is 132 bp. The length of the product is by the ideal amplicon product for real-time PCR, which is 50-150 bp [27].

### 3.5 DNA isolate of *Carcharhinus* spp.

Quantitative testing of DNA isolates of *Carcharhinus* spp. is seen based on the absorbance value on the spectrophotometer. Isolated DNA samples were measured at 230, 260, and 280

nm wavelengths. Analysis of the concentration and purity of DNA isolates contained in the sample was viewed at wavelengths with a ratio of 260/280. The wavelength of 260 nm is the maximum absorption for nucleic acids, while 280 nm is the maximum absorption for proteins [48]. An exemplary DNA purity value is at a vulnerable ratio of 1.8-2.0; if the sample used is below that vulnerable ratio, it can potentially interfere with the PCR process [59]. However, the DNA purity value mentioned in Sophian's [48] study, which is at a vulnerable ratio of 1.7 to 2.1, can still be good. Table 4 displays the outcomes of quantitative DNA isolate concentration and purity assays.

**Table 4.** DNA isolate concentration and purity test results

Sample code	Species	A260/A280	Concentration (ng/ $\mu$ L)
CC3	<i>Carcharhinus limbatus</i>	1.9	150.1
KC2	<i>Carcharhinus tjujot</i>	1.9	84.9
KC3	<i>Carcharhinus sealei</i>	1.8	62.4
Q-CAF9	<i>Carcharhinus falciformis</i>	1.9	448.1
Q-CAF10	<i>Carcharhinus falciformis</i>	1.9	112.6
CF 232 H	<i>Carcharhinus falciformis</i>	1.8	18.3
CAF 226	<i>Carcharhinus falciformis</i>	1.7	37.3
CAF 216	<i>Carcharhinus falciformis</i>	1.8	18.9
CAF 197	<i>Carcharhinus falciformis</i>	1.7	24.9
PG 240	<i>Prionace glauca</i>	1.9	32.8
PG 245	<i>Prionace glauca</i>	2.0	32.8
PG 282A	<i>Prionace glauca</i>	2.1	150.4
APL1 H	<i>Alopias pelagicus</i>	2.0	181.5
APL4 B	<i>Alopias pelagicus</i>	2.0	99.7
ASC3 H	<i>Alopias superciliosus</i>	2.0	374.5
ASC4 B	<i>Alopias superciliosus</i>	2.0	171.1
1-SL	<i>Sphyrna lewini</i>	1.9	202.6
2-SL	<i>Sphyrna lewini</i>	1.9	375.7
RA 172	<i>Rhynchobatus australiae</i>	1.9	474.3
PKL 4	<i>Rhynchobatus australiae</i>	1.9	188.8
I7	<i>Lamna nasus</i>	2.0	60.4
I8	<i>Lamna nasus</i>	2.0	139.2
I9	<i>Isurus oxyrinchus</i>	2.1	67.1
I10	<i>Isurus oxyrinchus</i>	2.0	63.4

The concentration and purity values of *Carcharhinus* spp. DNA isolate results with several comparison sample codes tested produced varying values. The sample concentration value in the table above ranges from 18.3 to 474.3 ng/ $\mu$ L, while the DNA purity value of the sample ranges from 1.7 to 2.1. The ratio value shows that the DNA of the target and non-target samples from isolation has been assessed as pure and has been by the requirements for optimal purity values, which are at a vulnerable ratio of 1.7 to 2.1 [48]. Ratio values that are too low or too high from these values can occur if the total DNA isolation process does not work well. This is caused by the presence of impurities in the form of RNA or proteins that can affect the purity of DNA. In addition, the presence of kit components and reagents, including phenol, alcohol, and chloroform, carried during the isolation process can also affect the purity of the total DNA produced.

### 3.6 Real-Time PCR efficiency of *Carcharhinus* spp. COI gene-based

#### 3.6.1 Optimal primer concentration

The optimization is related to factors that affect the components of the PCR process, including concentration, volume, environmental conditions, and the number of PCR cycles [60]. DNA amplification process by qPCR in research using species-specific primer candidate *Carcharhinus* spp. COI gene marker. qPCR results are presented in the form of amplification curves and melting curves. Results from qPCR are displayed as melting and amplification curves. Figure 6 depicts the *Carcharhinus* spp. COI's significant amplification curve.

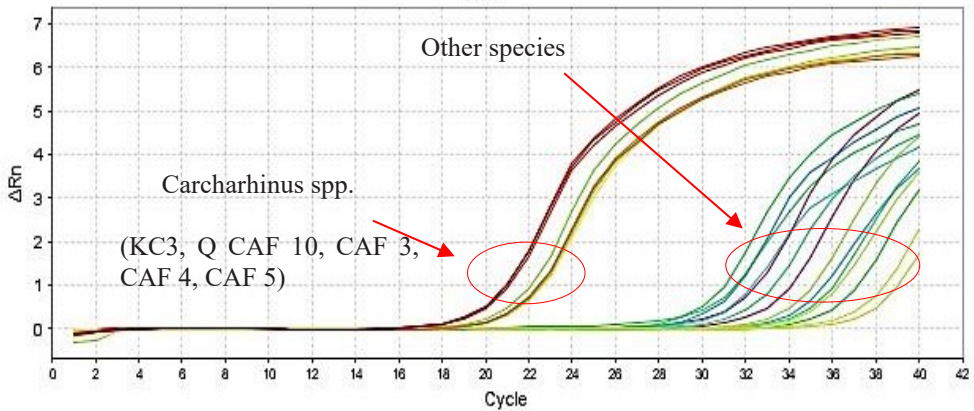


Fig. 6. Amplification curve of *Carcharhinus* spp. COI

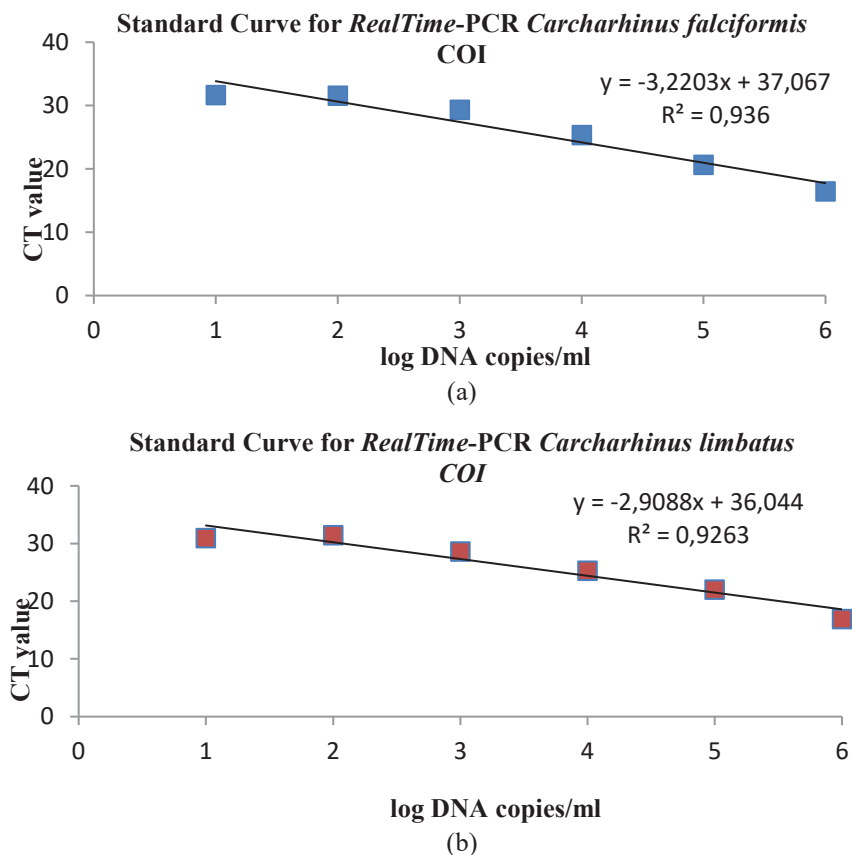
Using the qPCR method, the primer DNA amplification of *Carcharhinus* spp. COI gene markers were analyzed, and the results were evaluated based on the cycle threshold (Ct) value and the increase in graphs on the amplification curve. The amplification curve in Figure 6 above has been optimized for conditions at an annealing temperature of 63°C. The image-based amplification process occurred on *Carcharhinus* spp. first, while other shark samples tested included species of *Prionace glauca*, *Alopias pelagicus*, *Alopias superciliosus*, *Rhynchobatus australiae*, *Sphyrna lewini*, *Lamna nasus*, and *Isurus oxyrinchus* late amplified. *Carcharhinus* spp. experienced an increase in the graph at Ct values of 18-20, while other samples at Ct values of >20. The difference in the number of Ct between target and non-target species indicates that the designed *Carcharhinus* spp. COI primers successfully amplified the target species. According to Nasution and Andryas [53] research, a lower Ct value denotes a larger concentration of target DNA, whereas a higher Ct value denotes a lower concentration.

#### 3.6.2 Efficiency of qPCR conditions

qPCR efficiency testing of *Carcharhinus* spp. isolate samples of COI gene markers was the same as tests performed on samples of *Prionace glauca* isolates of previous *cyt b* gene markers. Tests were carried out on samples of *Carcharhinus* spp., namely *Carcharhinus falciformis* species with sample code Q CAF10 and *Carcharhinus limbatus* with sample code CC3 that had previously been diluted. The dilution ranges from 10<sup>1</sup> ng/L to 10<sup>-3</sup> ng/L, the lowest concentration. The amplification curve and the melting curve are the two curves used to display the efficiency findings.

qPCR efficiency is produced as an amplification curve and melts curve. Two *Carcharhinus* samples (*Carcharhinus limbatus* with sample code CC3 and *Carcharhinus falciformis* with sample code Q CAF10) were used to test efficiency. On the amplification curve, the stratified dilution samples from both sample codes were successfully amplified sequentially from a dilution of  $10^1$  ng/L to a dilution of  $10^{-3}$  ng/L. This means that *Carcharhinus* spp. primers of design can be detected down to the smallest dilution at a dilution of  $10^{-3}$  ng/ $\mu$ L. Melt curve analysis represents the following amplification result. Melt curve analysis is carried out to determine whether the test carried out is specific to the desired target sample [61]. The melting curve of the *Carcharhinus* spp. species *Carcharhinus falciformis* and *Carcharhinus limbatus*, with a melting curve temperature of 76.99°C, was found up to a dilution of  $10^{-3}$ .

From a dilution of  $10^1$  ng/L to the lowest dilution of  $10^{-3}$  ng/L, samples of multilevel dilutions were taken. The Ct value decreases at dilution  $10^1$  and increases until dilution  $10^{-3}$ . The Ct value is used as a reference for making standard curves along with the log copy number value to determine the efficiency of real-time PCR conditions. The standard curve has a line equation or linear regression value of  $[y = mx + b]$ , with y: Ct value, m: slope value, and x: condensation log value of the sample tested. Other parameters that are used for the validity of qPCR reactions are the value of real-time PCR efficiency (E) and determinant coefficient ( $R^2$ ). The value can be determined using a standard curve. Figure 7 displays the standard curve for the sample *Carcharhinus* spp. using the Microsoft Excel program.



**Fig. 7.** Standard curve for real-time PCR *Carcharhinus* spp. COI gene markers: (a) *Carcharhinus falciformis*, (b) *Carcharhinus limbatus*

The standard curve in Figure 7(a) shows the R<sup>2</sup> and slope values in the *Carcharhinus falciformis* sample, respectively, of 0.9360 and -3.2203. In contrast, the *Carcharhinus limbatus* sample has an R<sup>2</sup> value and a slope value of 0.9263 and -2.9088, respectively (Figure 7(b)). The real-time PCR efficiency value obtained in *Carcharhinus falciformis* and *Carcharhinus limbatus* samples was 104 % and 120 %, respectively. The optimal quality of real-time PCR is determined by the value of the correlation coefficient (R<sup>2</sup>) >0.980 and the efficiency percentage value (%E), which is in the range of 90% to 105% [54]. This shows that the R<sup>2</sup> value in both sample codes is still below the optimal correlation coefficient value standard. The percentage value of efficiency in the *Carcharhinus falciformis* sample is already in the vulnerable optimal value, but the *Carcharhinus limbatus* sample code is above the vulnerable value. The difference in efficiency values that occur is due to a shift in the resulting Ct value. The varying Ct value does not affect the value of the correlation coefficient and can only significantly affect the efficiency of the qPCR standard. The qPCR efficiency value that is too high is caused by the error factor in the pipetting process [55]. Another factor that can affect these results is the occurrence of non-specific or non-target amplification that can delay the emergence of Ct values [62].

For field applications, DNA extraction tests of *Carcharhinus* species included in CITES Appendix II can be performed without a high-speed centrifuge. The Chelex technique is one of the DNA extraction methods using Chelex resin, which serves to bind and remove metals in samples that can inhibit the enzymatic reactions needed in the DNA extraction process. Compared to other conventional procedures, the Chelex technique's DNA extraction process is straightforward, quick, easy to perform, and requires fewer reagents [63]. This method is quite popular because it can be done in the field or in a non-laboratory environment. Another DNA extraction method suitable for applications outside the laboratory is using a commercial QuickExtract DNA Extraction Solution kit. This extraction method eliminates some complicated stages inherent in traditional DNA extraction procedures. The QuickExtract DNA Extraction Solution kit has several advantages, including being simpler and faster, not requiring complicated laboratory equipment, and using non-toxic chemicals [64].

## 4 Conclusion

Primer's design of the target species *Prionace glauca* cyt b gene markers and *Carcharhinus* spp. degenerate primers for COI gene markers were successfully carried out with optimal amplification conditions and Ct values specific to the target species. The resulting specific primers can be used to authenticate fishery products made from blue shark species and the whole genus of *Carcharhinus* spp.

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