

## The development of real-time polymerase chain reaction using species-specific primer targeting cytochrome-b for identification of canine meat in mackerel meatballs for halal authentication

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### Abstract

The practice of mixing food using canine meat has been widely found. This practice is motivated by the low price of canine meat in the market. The purpose of this research was to design a species-specific primer (SSP) and develop a real-time PCR method for analyzing canine meat (*Canis lupus familiaris*) in mackerel meatballs intended for halal authentication. The design of SSP was carried out in silico using NCBI-Primer BLAST and IDT sites. The primer specificity analysis was performed on DNA canine and 8 animals namely mackerel, chicken, pig, goat, frog, cow, rat, and tree shrew using real-time PCR. Method validation is determined by sensitivity test, amplification efficiency, and repeatability test. The results showed that the Cytb-78 primer (forward: CAC TAA TCT TCT CTC TGC CAT CC, reverse: GAA TCG TGT TAG GGT TGC TTT G) which is specifically designed to amplify canine mitochondrial DNA with an optimum annealing temperature of 61.8°C. A sensitivity test was performed on 8 DNA dilution series with 10 dilutions resulting in the limit of detection (LoD) value of 1 pg/μL, an amplification efficiency (E) of 96.2%, and a coefficient of determination ( $R^2$ ) of 1. The repeatability test of 6 replicate DNA isolates of fresh meat resulted in a CV value of 0.72%. Analysis of the reference meatballs showed that Cytb-78 primer could amplify canine meat mixtures up to 10% concentration. The Cytb-78 primer that has been designed and the real-time PCR method that has been developed can be used for the analysis of canine meat (*Canis lupus familiaris*) in mackerel meatballs for halal authentication.

## 1. Introduction

Halal authenticity has become a major concern in many countries. The potential global market for halal food products is not limited to the Muslim population but also non-Muslims. Halal food products represent 16% of the global food market and are predicted to grow to 20% in the future (Erwanto *et al.*, 2018). As a result of consumer awareness of the high quality of halal food products, many industries are competing to use halal labels as an attractive marketing strategy (Mahama *et al.*, 2020). Meatball is a traditional Indonesian food that can be made from beef, chicken, or fish. Mackerel meatballs are the most popular fish meatballs. Because of the high commercial value of mackerel fish, the irresponsible usage of canine meat as an addition was found to get

higher economic profits (Andhikawati and Akbarsyah, 2021).

Canine meat has become one of the targets of food-mixing in North Sulawesi, Indonesia practices. Canine meat has a great economic value and is less expensive than other meats, such as mackerel fish. Canines also have a large population and a high reproductive ability. Canines are illegal animals, and Muslims are forbidden to consume them. Canines can potentially transmit zoonotic diseases such as rabies, scabies, helminthiasis, and campylobacteriosis (Khanum, 2017; Irfan *et al.*, 2018). Consequently, a guarantee of authenticity is required as a form of consumer protection to verify that the commercial meatball is by the label.

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Several analytical methods including Fourier Transform Infrared (FTIR) spectroscopy (Kuswandi *et al.*, 2015), Nuclear Magnetic Resonance (NMR) spectroscopy (Fadzillah *et al.*, 2017), Raman spectroscopy in combination with multivariate data analysis (Mortas and Ayvaz 2022), Gas Chromatography-Mass Spectroscopy (GC-MS) (Indrasti *et al.*, 2010), and Liquid Chromatography-Mass Spectroscopy (LC-MS) (Abbas *et al.*, 2020), have been proposed and used for the identification, detection, and confirmation of non-halal meats in food product. Furthermore, certain screening methods for the rapid detection of non-halal meats, such as electronic nose technology (e-nose) (Nurjuliana *et al.*, 2011), electronic tongue technology (e-tongue) (Tian *et al.*, 2019), and Differential Scanning Calorimetry (DSC), are offered (Bertram *et al.*, 2006). All these methods necessitate advanced data processing, which entails big data analysis. Thus, methods based on specific markers, such as protein and DNA-based methods are frequently used. DNA-based analysis methods are widely used in various countries because of their high efficiency, convenience of use, and cost-effectiveness (Lo and Shaw, 2018). DNA has a higher thermal stability than protein, so it can be utilized to analyze heated processed meat items such as meatballs. Because of their specificity and sensitivity,

DNA-based methods using polymerase chain reaction are now considered the gold standard for identifying and confirming non-halal meats in food products (Yusop and Bakar, 2020; Yörük, 2021 Sampurna *et al.*, 2024). Numerous PCR methods have been reported for identifying non-halal meats in food products, such as real-time PCR and conventional PCR using species-specific primers (SSP), single-plex or multiplex PCR, restriction fragment length polymorphisms (RFLP), and random amplified polymorphic DNA (RAPD) (Salihah *et al.*, 2016; Rohman *et al.*, 2020; Muflihah *et al.*, 2023). Real-time PCR using SSP targeting on mitochondrial DNA has been used successfully to analyze non-halal meat-based products such as analysis of pork in meatballs (Orbayinah *et al.*, 2019), analysis of canine meat in meatballs (Rohman *et al.*, 2021), and analysis of porcine gelatin in soft candy (Salamah *et al.*, 2021). Real-time PCR targeting on Cytb has been used for analysis of canine meat in meatball formulation using primers targeting on 96 bp amplicon length (Rohman *et al.*, 2020). However, the usage of different primers is essential to be explored for better results.

Therefore, the present study was intended to design an SSP targeting distinct Cytb primers based on prior research and to develop a real-time PCR method using a designed primer for identifying canine meat in mackerel

meatballs intended for halal authentication.

## 2. Materials and methods

### 2.1 Study period and location

The study was conducted from January 2023 to March 2023 at Halal Center Gadjah Mada University, Yogyakarta, Indonesia.

### 2.2 Materials

The meats including canine, mackerel, chicken, pork, goat, frog, beef, tree shrew, and rat were obtained from the traditional markets and slaughterhouses in Yogyakarta, Indonesia. Meatball samples were prepared and formulated in the laboratory. The designed SSP for real-time PCR analysis were purchased from Genetika Science Company, Jakarta, Indonesia.

### 2.3 Primer designing

A pair of SSP targeting Cytochrome-b (Cytb) was designed using PrimerQuest software from Integrated DNA Technologies (IDT). The DNA sequence of *Canis lupus familiaris* mitochondrion complete genome with an accession number MW549038.1 was retrieved from NCBI GenBank.

The primer specificity was checked in silico using Basic Local Alignment Search Tool Nucleotide (BLASTn) from NCBI by comparing canine species with mackerel, chicken, pig, goat, frog, cow, tree shrew, and rat. Furthermore, the presence of predicted secondary structure was considered using the OligoAnalyzer Tool from IDT.

### 2.4 Preparation of reference meatballs

The reference meatballs were formulated by mixing 90% ground meat with 10% components consisting of tapioca starch, garlic, salt, and other spices. The composition of canine-mackerel meat used in the reference meatballs was 100–0%; 90–10%; 80–20%; 70–30%; 60–40%; 50–50%; 40–60%; 30–70%; 20–80%; 10–90%; and 0–100%. The mixture was homogenized and made into a ball shape, then boiled for 15 mins. The binary reference meatballs were stored at -20°C immediately after preparation until being used for DNA isolation.

### 2.5 DNA isolation

The DNA isolation from raw meats and meatballs was carried out using FavorPrep™ Tissue Genomic DNA Extraction Mini Kit (Favorgen, Taiwan) according to the manufacturer's instructions. There are several stages, consisting of cell lysis, DNA binding, contaminant washing, and DNA elution.

## 2.6 Quantification and purity assessment of DNA

The DNA isolated from raw meats and meatballs was analyzed using NANO-Quant SPARK TECAN (Switzerland) at wavelengths of 230 nm, 260 nm, and 280 nm to obtain DNA concentration and DNA purity. The purity of the DNA isolated was determined by the purity index ( $A_{260}/A_{280}$  ratio) with the range of 1.8–2.0.

## 2.7 Electrophoresis gel agarose for DNA qualitative analyzed

The DNA isolated from raw meats was analyzed qualitatively using 1% agarose gel electrophoresis for 45 mins at 100 volts with SYBR<sup>TM</sup> Safe DNA Gel Stain (Invitrogen, US), and visualized under UV light.

## 2.8 Optimizing the annealing temperature of the primer

The optimization of the annealing temperature of SSP targeting Cytb DNA mitochondrion *Canis lupus familiaris* was carried out in the temperature range from 52.30°C to 62.30°C based on the prediction of melting temperature of the designed primer. The temperature which was used for optimization was 52.3; 53.0; 54.3; 56.2; 58.7; 60.6; 61.8; and 62.3°C.

## 2.9 Analysis of DNA using real-time PCR

The DNA isolated was analyzed using PCR CFX96 (Bio-Rad, USA) with a total volume of 10  $\mu$ L, containing 5  $\mu$ L of 2 $\times$  SensiFAST<sup>TM</sup> SYBR<sup>®</sup> No-Rox Kit (Meridian Bioscience, USA), 0.4  $\mu$ L of each forward and reverse primer (final concentration 0.4  $\mu$ M), 1  $\mu$ L DNA template with concentration of 25 ng/ $\mu$ L, and 3.2  $\mu$ L nuclease-free water (NFW). The thermal cycler program which was used was as follows: pre-denaturation at 95°C for 3 mins, followed by 30 cycles of denaturation at 95°C for 5 s, annealing at 61.8°C for 10 s, and extension at 72°C for 20 s. For the melting curve analysis, the temperature was performed at 65°C–95°C with a slope of 0.5°C/5 s.

## 2.10 Validation of real-time PCR method

Real-time PCR using designed SSP was subjected to validation by determining several parameters, namely specificity, sensitivity, amplification efficiency, and repeatability. The validated real-time PCR method was employed for the analysis of canine DNA in reference meatballs. The specificity test was performed by amplifying 25 ng/ $\mu$ L of DNAs isolated from raw meats (canine, mackerel, chicken, pig, goat, frog, cow, rat, and tree shrew) and no template control (NTC). The sensitivity of SSP was determined by the limit of detection (LoD) based on the amplification of DNA isolated from canine meat with serial dilution to obtain a concentration of DNA at levels of 0.001; 0.01; 0.1; 1; 10;

100; 1,000; and 10,000 pg/ $\mu$ L.

The LoD value was the lowest concentration of DNA which still could be amplified with a reproducible cycle threshold (Ct) using real-time PCR. The amplification efficiency (E), slope, and coefficient of determination ( $R^2$ ) were carried out by standard curve by plotting log concentration of DNA (x-axis) and Ct value (y-axis). E-value was aimed to know the capability of the real-time PCR method in amplifying the DNA targets. The recommended value of E is 90–110%, out of this range is unacceptable and indicates that further optimization needs to be carried out (Sharma, 2021). The repeatability of the real-time PCR method for precision evaluation was evaluated based on the coefficient of variation (CV) Ct from six replications of DNA amplification.

## 2.11 Application of real-time PCR for identification of reference meatballs

The real-time PCR method that has been validated using SSP was further applied for the analysis of canine DNA in reference meatballs at the optimum annealing temperature.

## 3. Results and discussion

The purpose of this research was to develop a primer pair arrangement that will specifically amplify the Cytb fragment of *Canis lupus familiaris* mitochondrial DNA (mtDNA).

This fragment was chosen as the targeted mtDNA because of the degree of intraspecies and interspecies variability. This gene also produces many copies per cell, which improves the sensitivity of real-time PCR methods and contributes to the survival of a few copies of DNA when tissue is subjected to harsh processing conditions, such as boiling and sterilizing processes (Orbayinah et al., 2019). Cytb-78 (forward: CAC TAA TCT TCT CTC TGC CAT CC; reverse: GAA TCG TGT TAG GGT TGC TTT G) was utilized as the target primer.

The primer was chosen based on numerous primer design parameters, including amplicon length of 75–150 bp, primer length of 18–30 bp, GC content of 40–60%, and primer melting temperature ( $T_m$ ) of 50–65°C (Sharma, 2021). Table 1 shows the primer parameters of the developed Cytb-78 primer pair.

The results of an in-silico specificity test using BLASTn revealed that Cytb-78 only attached to *Canis lupus familiaris* and did not attach to mackerel, chicken, pig, goat, frog, cow, rat, and tree shrew. Furthermore, analysis is performed on primer secondary structures, which may result in false positives and overly high

amplification efficiency values. Secondary structures are more stable than DNA templates, which can cause primer failure to attach and a significant decrease in amplification. The OligoAnalyzer Tool on the IDT was used to analyze the primer secondary structures. Table 2 shows the results of Cytb-78 secondary structures.

The free Gibbs energy difference ( $\Delta G$ ) detected in

Table 1. Parameter of Cytb-78 primer.

| F/R     | Primer length (bp) | % GC | Tm (°C) | Amplicon length (bp) |
|---------|--------------------|------|---------|----------------------|
| Forward | 23                 | 47.8 | 62      | 97                   |
| Reverse | 22                 | 45.5 | 62      |                      |

Table 2. Secondary structures of Cytb-78 primer.

| Secondary structure    | Forward | Reverse | Acceptance of $\Delta G$ |
|------------------------|---------|---------|--------------------------|
| Hairpin (kcal/mol)     | 2.75    | 0.15    | More than -3             |
| Self-dimer (kcal/mol)  | -3.14   | -3.61   | More than -6             |
| Heterodimer (kcal/mol) | -4.50   |         | More than -6             |

secondary structure analysis describes the stability of the primer secondary structure. The secondary structure can be broken down when the primer has an  $\Delta G$  value greater than -3 kcal/mol on the hairpin and greater than -6 kcal/mol on the self-dimer and heterodimer (Yen *et al.*, 2013).

This indicates the secondary structures produced on the Cytb-78 primer were easy to destroy, so the Cytb-78 primer was chosen as an SSP that can amplify canine DNA.

To produce sufficient DNA templates, the DNA was extracted using a commercial kit with silica-based techniques. Analysis of DNA concentration and purity was carried out spectroscopically at wavelengths of 260 nm and 280 nm. At a wavelength of 260 nm, the nucleic acid contained in DNA can absorb the most ultraviolet (UV) photons, so it can be utilized to determine DNA concentrations (Sharma, 2021). At a wavelength of 280 nm, the presence of proteins, phenols, and carbohydrates may be determined, so it can be used to obtain DNA purity. DNA was expressed as pure when the  $A_{260}/A_{280}$  ratio was 1.8–2.0 (Salamah *et al.*, 2021).

As shown in Table 3, the purity of the DNA isolated was in the range of 1.8–2.0, indicating that the DNA was pure enough to be used as a DNA template for PCR amplification.

The DNA extracted from raw meats was examined qualitatively using 1% agarose gel electrophoresis and visualized using UV light. Figure 1 shows that DNA was successfully extracted without degradation.

The optimum annealing temperature ( $T_a$ ) is associated with primer attachment in the DNA target. The higher  $T_a$  may cause the primer not to attach to the targeted DNA. While the lower  $T_a$  may cause the primer to not specifically attach to the targeted DNA (Salihah *et al.*, 2016).  $T_a$  was generally 5°C lower than  $T_m$ .  $T_a$  optimization was performed at 52.3; 53.0; 54.3; 56.2; 58.7; 60.6; 61.8; and 62.3°C by the PCR's touchdown method. Due to the instrument's functional limits, these temperatures cannot be programmed at a linear distance from each other.

The optimum  $T_a$  for the Cytb-78 primer was 61.8°C

Table 3. The concentration and purity of DNA isolated from raw meat.

| Samples         | Concentration of DNA (ng/ $\mu$ L) | Purity index ( $A_{280}/A_{260}$ ) |
|-----------------|------------------------------------|------------------------------------|
| Canine meat     | 309.77                             | 1.85                               |
| Mackerel meat   | 211.51                             | 1.94                               |
| Chicken meat    | 698.80                             | 1.92                               |
| Pork            | 134.19                             | 1.81                               |
| Goat meat       | 273.86                             | 1.85                               |
| Frog meat       | 339.91                             | 1.99                               |
| Beef            | 217.01                             | 1.82                               |
| Rat meat        | 132.41                             | 1.81                               |
| Tree shrew meat | 188.98                             | 1.80                               |

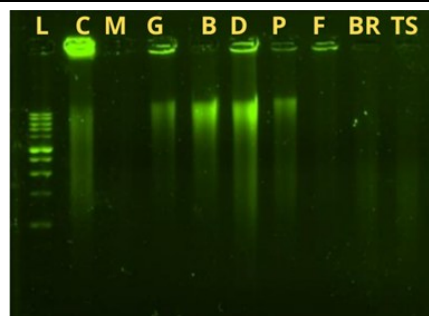


Figure 1. Agarose gel electrophoresis of DNA isolated from raw meat. L: ladder (1 kb DNA ladder), C: chicken, M: mackerel, G: goat, B: beef, D: canine, P: pork, BR: bengal rat, TS: tree shrew.

because it has a lower  $C_t$  value (Figure 2a), which indicates the primer may be detected in an earlier cycle. According to Melting Curve Analysis (MCA), the amplification of Cytb-78 primer at an optimum  $T_a$  of 61.8°C produced a single peak (Figure 2b), confirming that no primer dimer or non-specific products were identified during PCR amplification, meaning that the optimum condition was achieved.

The specificity test of the primer was proposed to ensure that the designed primer would produce specific results that only amplify canine DNA. The primer specificity of Cytb-78 was tested by amplifying 25 ng/ $\mu$ L of canine DNA isolated from raw meat and 8 other animals, namely mackerel, chicken, pig, goat, frog, cow,

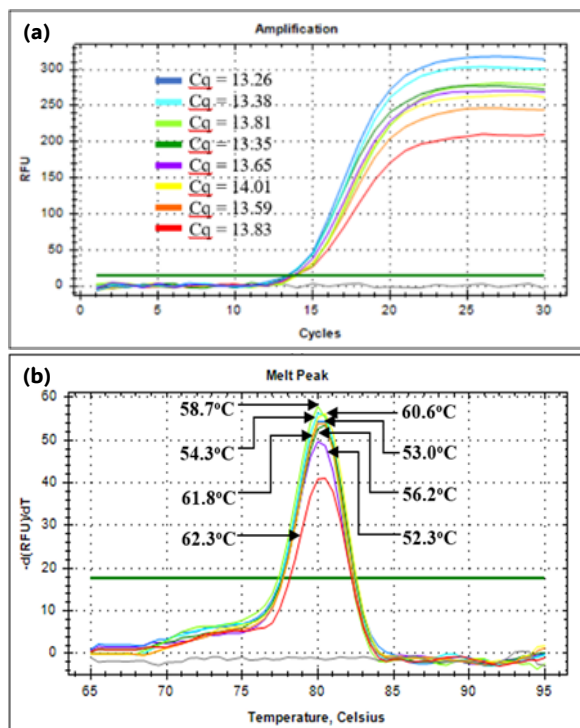


Figure 2. Annealing temperature optimization of Cytb-78 primer. (a) Amplification curve for optimization of Cytb-78 primer targeting mitochondrial DNA for *Canis lupus familiaris* at 8 temperature levels; (b) The melt peak curve produces a single peak of denaturation at  $T_m$  80.00°C and 80.50°C.

rat, and tree shrew, at an optimum  $T_a$  of 61.8°C for 30 cycles. The amplification curve (Figure 3a), shows that the Cytb-78 primer provides the highest amplification response to canine DNA with a  $C_q$  value of 13.59 and an RFU value of 245. Increased fluorescence intensity also occurred in beef DNA ( $C_q = 28.04$ ; RFU = 19.0); rat DNA ( $C_q = 29.06$ ; RFU = 13.0), and pork DNA ( $C_q = 29.65$ ; RFU = 3.90). On the assumption that there is no cross-contamination, the Cytb-78 primer was not specific to canine DNA.

However, the Cytb-78 primer can still be used for canine DNA analysis with consideration of  $C_q$  and RFU value that has a quiet distance from the DNA of beef, rat, and pork.

In addition, a single denaturation peak of canine DNA was formed above the threshold line on the MCA (Figure 3b). The denaturation peak of beef, rat, and pork was formed below the threshold line, which means that the denaturing peak is regarded as worthless. The sensitivity test of the real-time PCR method was evaluated by determining the limit of detection (LoD) value of DNA isolated from canine meat. The LoD was aimed as the smallest concentration of DNA that can still be amplified. The LoD value was determined by the amplification of 10-fold serial dilutions of canine meat, namely 0.001; 0.01; 0.1; 1; 10; 100; 1,000; and 10,000  $\mu\text{g}/\mu\text{L}$  at an optimum temperature of 61.8°C.

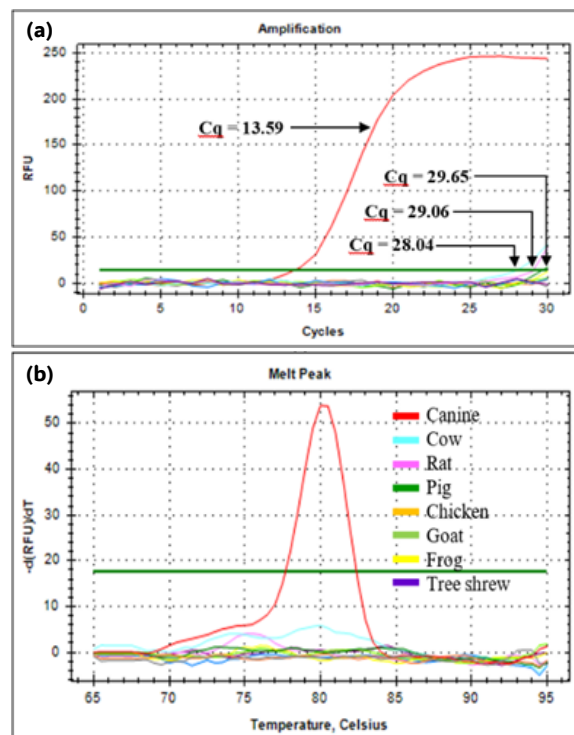


Figure 3. The specificity test of Cytb-78 primer at an optimum  $T_a$  of 61.8°C. (a) The amplification curve of the specificity test of Cytb-78 primer for canine and 8 animals shows that there is an increase in fluorescence intensity in beef, rat, and pork DNA; (b) The melt peak curve produces a denaturation peak which is above the threshold so the designed primer is specific for canine DNA at an optimum  $T_a$  of 61.8°C.

At a concentration of 1  $\mu\text{g}/\mu\text{L}$ , canine DNA was still amplified with a  $C_t$  value of 29.72 (Figure 4a). Below the concentration of 1  $\mu\text{g}/\mu\text{L}$ , the amplification curve was not detected. As a result, the LoD value for canine DNA analysis using real-time PCR was 1  $\mu\text{g}/\mu\text{L}$ . The resulting repeatability test for accuracy evaluation was performed using six replicates of the  $C_q$  DNA isolated from canine meat.  $C_q$  values were in the range of 16.89–17.17 with a CV of 0.72%. According to the European network of GMO laboratories, the acceptance criteria of CV is  $\leq 25\%$  (Salamah *et al.*, 2021). The results reveal that the CV value achieved met the acceptance criteria, and the devised real-time PCR method was precise and applicable for PCR assay.

The analysis begins with the DNA isolation using a commercial kit, namely FavorPrep™ Tissue Genomic DNA Extraction Mini Kit with a manufacturing isolation protocol. Table 4 shows that the entire meatball samples met the purity index requirement, i.e.,  $A_{260}/A_{280}$  is in the range of 1.8–2.0. The data is random and does not stagnate as the concentration of the mixture increases.

TA = the mixture of canine-mackerel meat used in the reference meatballs was 100–0% (TA1<sup>(+)</sup>); 90–10% (TA2); 80–20% (TA3); 70–30% (TA4); 60–40% (TA5); 50–50% (TA6); 40–60% (TA7); 30–70% (TA8); 20–80% (TA9); 10–90% (TA10); and 0–100% (TA11<sup>(-)</sup>). A

validated real-time PCR method was used to amplify the reference meatball samples, i.e., at optimum Ta of 61.8°C for 30 cycles.

The validated real-time PCR method can amplify the entire mixture of canine meat in the reference meatballs up to a concentration of 10% (Figure 5a). Based on MCA, a single peak of denaturation was found (Figure 5b), indicating that the Cytb-78 primer only amplified the canine DNA.

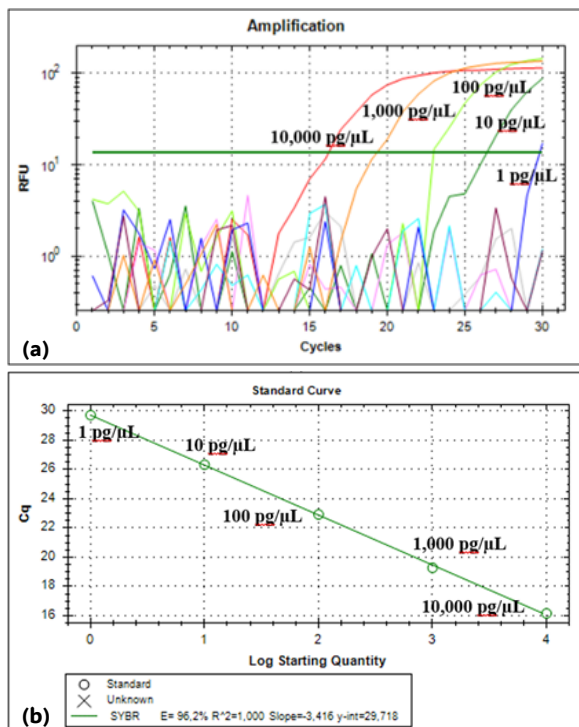


Figure 4. The sensitivity test of Cytb-78 primer at an optimum Ta of 61.8°C. (a) The amplification curves of 8 series of 10 dilution DNA concentrations (0.001; 0.01; 0.1; 1; 10; 100; 1,000; and 10,000 pg/μL) on a logarithmic scale show that the developed real-time PCR method has LoD value of 1 pg/μL; (b) The standard curve produces E-value = 96.2%,  $R^2 = 1$ , slope = -3.416, and y-intercept = 29.718 amplification was then transformed into a standard curve by plotting the log value of DNA concentration (x axis) and the Cq value (y axis), as shown in Figure 4B for evaluation of amplification efficiency (E). The E-value obtained from this curve was 96.2%, which was within the accepted E-value range (90–110%). The  $R^2$  value produced by the standard curve is 1.

#### 4. Conclusion

The SSP Cytb-78 (forward: CAC TAA TCT TCT CTC TGC CAT CC, reverse: GAA TCG TGT TAG GGT TGC TTT G) was able to specifically amplify DNA isolated from canine meat using a real-time PCR method at an optimum annealing temperature of 61.8°C. The real-time PCR method using Cytb-78 primer can amplify canine DNA to a concentration of 1 pg/μL. The coefficient of determination ( $R^2$ ) is 1 with an amplification efficiency (E) of 96.2%, and a CV value of 0.72% from raw meat. The validated real-time PCR method can be applied to reference meatball samples up

Table 4. The concentration and purity of DNA isolated from reference meatball samples.

| Samples             | Concentration of DNA (ng/ μL) | Purity index ( $A_{280}/A_{260}$ ) |
|---------------------|-------------------------------|------------------------------------|
| TA1 <sup>(+)</sup>  | 67.23                         | 1.95                               |
| TA2                 | 69.78                         | 1.95                               |
| TA3                 | 65.34                         | 1.95                               |
| TA4                 | 80.57                         | 1.95                               |
| TA5                 | 94.06                         | 1.94                               |
| TA6                 | 69.95                         | 1.95                               |
| TA7                 | 105.80                        | 1.93                               |
| TA8                 | 141.92                        | 1.89                               |
| TA9                 | 120.13                        | 1.86                               |
| TA10                | 111.03                        | 1.86                               |
| TA11 <sup>(-)</sup> | 114.04                        | 1.93                               |

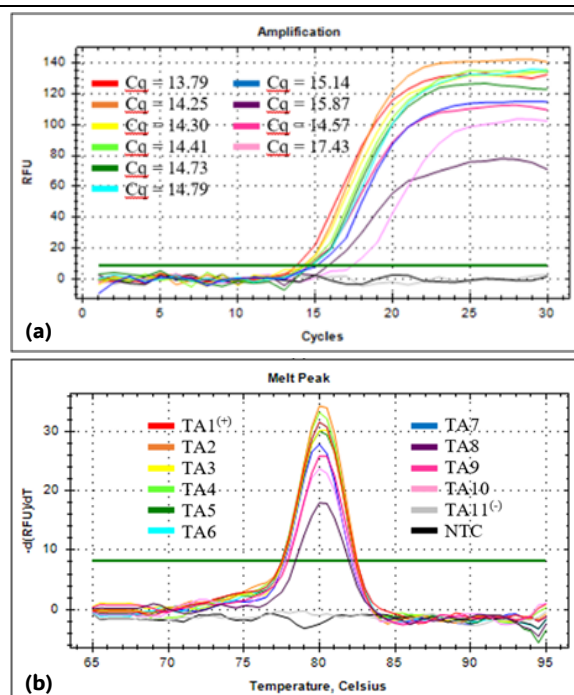


Figure 5. Application of real-time PCR using validated method for identification of reference meatballs. (a) The amplification curve of reference meatball analysis shows that the validated real-time PCR method can amplify a binary mixture of canine meat-mackerel up to a concentration of 10% canine meat; (b) The melting curve produces a denaturation peak so that the designed Cytb primer is specific to canine DNA, especially in food samples.

to a concentration of 10% canine meat in the reference meatballs. The developed real-time PCR method can be used to analyze canine meat in food products to support the development of standard methods for halal authentication.

#### Conflict of interest

The authors declare no conflict of interest.

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