In-house validation and calibration of pork detection using duplex SYBR-Green I real-time PCR approach

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Abstract

An effective detection method for identifying pork in processed food is urgently needed as there is a possibility that the food is adulterated with other materials. The aim of the present work was to validate duplex detection and quantification of pork and 18S rRNA using real-time PCR SYBR Green I approach and its quality control development. The validation parameters which consisted of specificity, detection limit (sensitivity), precision, linearity, PCR efficiency, and robustness were investigated. All these activities were done after obtaining the best formulation for the duplex. Quality control for pork detection was developed to avoid dubious things. The optimum primer concentration by duplex method of pork and 18S rRNA were 0.75 pmol (pork) and 0.1 pmol (18S rRNA), respectively. The detection limit of DNA template concentration was 0.78125 ng, which could be more sensitive since PCR result still obtained thick bands. The high precision and linearity marked by the % CV and R^2 were between 0.145 – 0.898 and 0.9813, respectively. In addition, the efficiency of PCR was 95.584%. All processed food products containing pork were amplified and showed bands as targeted for pork and 19S rRNA genes, while non-pork processed food products did not show amplification of pork gene (18S rRNA only). Standard curve as calibration for pork quantification was developed successfully with good efficiency (E = 90%).

Introduction

Food industries which produce processed food products have a great market share and the potential to develop. There is a high possibility that processed food products are adulterated with other materials which are not in accordance with the information on the packaging label. Soares *et al.* (2013) described that species identification in food products is important for increasing consumer awareness of the composition and verifying labelling system of food products. This problem must be addressed as it is closely related to the halalness of processed food products consumed by Indonesians who are mostly Muslim. Therefore, detection methods for the identification of pork in processed food products are urgently required.

Detection methods in the molecular field have been developed and widely used to facilitate the identification of meat species (Girish *et al.*, 2005). Detection method done based on protein and DNA analysis is one of the recommended methods. Galimberti *et al.* (2013) also explained that validation of food composition originality mostly depends on protein or DNA analysis. The technique of protein analysis has some advantages when applied to raw meat because it has high sensitivity (Martin *et al.*, 2009). However, it has a limitation when it is applied to processed food products, because protein can be denatured by some processing steps (Rasmussen and Morrissey, 2008). Meanwhile, detection method of DNA analysis has resistance to high temperature, high pressure, and its presence in every cell that makes DNA usable for the detection of species in processed foods (Mafra *et al.*, 2008). Hellberg and Morrisey (2011) also described that DNA can be easily isolated from food matrices.

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Mitochondrial DNA (mtDNA) is one of the most widely used population genetic markers (Nabholz *et al.*, 2008). The genetic marker can be used to identify individuals or species of genetic differentiation among populations. Markers of mtDNA used for the identification were cytochrome b (cyt b) (Tanabe *et al.*, 2007; Soares *et al.*, 2013; Hossain *et al.*, 2017), cytochrome c oxidase I (COI) gene (Haider *et al.*, 2012; Dai *et al.*, 2015), and the 12S RNA and 16S rRNA genes (Martin *et al.*, 2009; Mitani *et al.*, 2009; Cawthorn *et al.*, 2012). COII, D-Loop, ATPase 6, and ATPase 8 can be used for the identification of meat species (Cho *et al.*, 2014). Fajardo *et al.* (2010) also explained that cyt b, 12S RNA, 16S RNA, and displacement loop region (D-loops) are the genes that are often used as markers of mitochondrial DNA. The most studied genes for phylogeny and molecular identification used cyt b as genetic marker in mitochondrial DNA (Amaral *et al.*, 2014). This highly sustainable area of the locus can be used as a primary-binding site, while mutations in variable regions make the gene usable to distinguish species (Balitzki-Korte *et al.*, 2005).

In the present work, amplification of the cyt b gene was performed on duplex by real-time PCR. Sakalar and Kaynak (2016) conducted research on the detection of pork on poultry and beef using real-time PCR duplex. Real-time PCR is one of the methods for detecting specific DNA in processed food products. The use of fluorescence dyes in real-time PCR, such as SYBR Green I will give some advantages such as low cost since probe is not used and easy for handling. SYBR Green I is commonly used in real-time PCR reactions, that intercalate with double strand of DNA.

In-house validation of duplex detection of pork and 18S rRNA and its quality control development were conducted using real-time PCR SYBR Green I approach. The parameters such as specificity, detection limit (sensitivity), precision, accuracy, PCR efficiency, linearity, and robustness were reported. Furthermore, in the present work, the quality control of duplex detection methods was also developed. The controls consisted of positive, negative and internal (18S rRNA) controls. Positive control was used to confirm that the reagents were working well. The negative control was used to determine the presence of target DNA contamination, while the internal control was used to ensure that there was no inhibitor of template DNA. In addition, real-time PCR method allowed the quantification of the sample. With all those developed activities, the performance of the duplex developed methods could be analysed.

Materials and methods

Samples

Raw meat samples (pork and beef) were purchased from local markets. The samples were used as positive controls. For targeted sample detection, we used processed food products already confirmed as halal food, as shown on the pack (abon/meat floss, corned, rolade). We also used processed pork (sausage, ham, corned, and pasta). All those samples were used to ensure the success of pork detection methods. The collection of processed food products was conducted based on survey results widely used by the people in Indonesia. The samples were also selected in different shapes/forms, such as meat fibre, texture, and mixture of dough. The samplings of raw meats and processed food products were carried using sterile scalpels to avoid cross-contamination. The samplings were repeated at least three replicates in each sample.

DNA extraction

The raw meat and processed food products were extracted using Genomic DNA Mini Kit (Invitrogen, Carlsbad, USA) and DNeasymericon Food Kit (QIAGEN GmbH, Hilden, Germany), respectively. The extracted DNA was analysed by agarose gel electrophoresis to ensure the quality of DNA in 1% agarose gel in 1× TAE buffer (Thermo Scientific, Carlsbad, USA) for 25 min at 100 V and stained with ethidium bromide (Invitrogen, Carlsbad, USA) for 10 min. The agarose gel was visualised under UV light.

Primer design

Primers were used in amplification of DNA fragment in raw meat and processed food product. The target site for the specific detection of a 300 bp fragment was the cyt b gene in pork DNA. The primer design was referred to Desriani and Widyowati (2017). The target site of 18S rRNA consisted of 99 bp fragments and was referred to Martin *et al.* (2009). 18S rRNA was used as an endogenous control for eukaryotic DNA detection (Leonard *et al.*, 2016).

Optimisation of primer formulations

The optimisation of primer formulations (pork and 18S rRNA) was required to know the optimum primer concentrations on each reaction by using duplex qualitative PCR assay. There were six formulations in 20 μL total reaction volume containing SYBR Green I Thunderbird (TOYOBO, Osaka, Japan), 1× TE buffer, 1 μL (20 ng) of DNA extract, and primer concentrations of pork in 0.75, 1, 1.25, 1.5, 1.75, and 2 pmol. In addition, the same

primer concentration (0.1 pmol) of 18S rRNA was used in each formulation. Positive and negative (NTC) controls were used in assay for each reaction. The PCR reactions were examined in Kyratec SuperCycler using the following program conditions: pre-denaturation at 95°C for 3 min; 35 cycles at 95°C for 15 s, 55°C for 30 s, and 72°C for 1 min. PCR products were analysed using 2% agarose gel for electrophoresis in 1× TAE buffer (Thermo Scientific, Carlsbad, USA) for 25 min at 110 V. Then, it was stained with ethidium bromide (Invitrogen, Carlsbad, USA) for 10 min. The agarose gel was visualised under UV light.

Optimisation of annealing temperatures by using real-time PCR

The optimisation of annealing temperatures for duplex was required to determine the optimum temperature for each reaction in the real-time PCR assay. The variation of annealing temperature on duplex (pork and 18S rRNA) was used at 45 – 55°C. The PCR reaction contained SYBR Green I Thunderbird (TOYOBO, Osaka, Japan), 1× TAE buffer, 1 µL of DNA extract (20 ng), and each primer. Primer concentration for duplex was the optimum candidate of primer concentration (1.75 pmol of pork and 0.1 pmol of 18S rRNA). The real-time PCR assays were performed on CFX96 Touch™ Thermal Cycler Real-Time PCR Detection System (Bio-Rad Laboratories, Hercules, CA, USA) using the following program conditions: pre-denaturation at 95°C for 3 min; 35 cycles at 95°C for 15 s, variation of annealing temperatures for 60 s, and 72°C for 1 min. The collection of fluorescence signal was carried out at the end of each cycle. The temperature of melting curve data was increased by 0.2°C from 65°C to 95°C. PCR products were collected, processed, and analysed by electrophoresis in 2% agarose gel in 1× TAE buffer (Thermo Scientific, Carlsbad, USA) for 25 min at 110 V. It was stained with ethidium bromide (Invitrogen, Carlsbad, USA) for 10 min. The agarose gel was visualised under UV light.

Detection limit of duplex

To determine the detection limit of DNA concentration for duplex real-time PCR assay, the template DNA of pork were diluted 8-fold (100, 50, 25, 12.5, 6.25, 3.125, 1.5625, 0.78125 ng) to each reaction.

Processed food products assay

Processed food product samples assay, positive, and negative control were analysed using the optimum formulation reaction by real-time PCR SYBR Green I for duplex (pork and 18S rRNA).

Standard curve of processed food

Standard curve was needed to quantify pork in processed food as calibration. It was determined by using real-time PCR. DNA template on 20 ng by percentage of pork in beef (µL/µL) containing 50%, 25%, 10% 5%, 1% in total volume of 2 µL to pork and 18S rRNA reaction real-time PCR, respectively. Ct of pork and 18S rRNA can be calculated by using the following equation (Soares *et al.*, 2013):

$$\Delta\Delta Ct = Ct_{pork} - Ct_{18S\ rRNA}$$

Where Ct_{pork} and $Ct_{18S\ rRNA}$ = cycle threshold of amplification curve in real-time PCR. It can be plotted in regression equation. In this assay, the PCR reaction was modified by removing extension step that has been proven to have the same result (data not shown).

Data analysis

Statistical analysis was employed to calculate the PCR efficiency. It was calculated using the following equation (Druml *et al.*, 2015):

$$E(\%) = \left[10^{\left(-\frac{1}{slope}\right)} - 1\right] \times 100$$

The PCR efficiency was accepted if it was between 90 and 110%, with regression value of slope between -3.1 and -3.6. In addition, the *R²* value of ≥ 0.98 (Iwobi *et al.*, 2015).

Results

The optimum of primer concentrations and annealing temperatures

Optimisation of duplex (pork and 18SrRNA) method using primer concentration formulations were successfully examined. From six formulations (data not shown), two candidates for optimum formulation were obtained, i.e. 1st formulation (0.75 pmol of pork; 0.1 pmol of 18S rRNA) and 5th formulation (1.75 pmol of pork; 0.1 pmol of 18S rRNA). Each of the candidate formulations had an advantage and disadvantage. The 1st formulations showed clear and clean bands with different proportion of pork and 18S rRNA bands, while the 5th formulation showed thick proportion of bands but primer-dimer.

Based on the optimisation of annealing temperatures, the optimum temperature for annealing by duplex method of 5th formulation was 55°C. Real-time PCR showed high and sharp peak of DNA

amplification and melting curves. The result of electrophoresis also indicated two thick and clear bands of pork and 18S rRNA. However, it still had primer-dimer bands. In the next analysis, the 1st formulation with annealing temperature at 55°C was used. This temperature was chosen because pork was not successfully amplified at more than 55°C (data not shown).

Detection limit of duplex

The detection limit for DNA concentration can still be detected using the duplex method up to 0.78125 ng. It means that the duplex method can be amplified to detect pork and 18S rRNA. The CV value was between 0.145 – 0.898%, while regression coefficient (R^2) was about 0.9813. The PCR efficiency was high, which was 95.584%.

Assay of PCR robustness

The samples of processed food products containing pork and non-pork were analysed and confirmed by electrophoresis. Four samples of food products containing pork and three samples of non-pork products were used. Based on the results, the pork samples showed two bands for pork and 18SrRNA, while non-pork samples did not show any pork bands, but only the 18S rRNA.

Standard curve of processed food

A good efficiency value was obtained from the construction of standard curve in processed food products. It was 90% with the slope and R^2 values of -3.6 and 0.98, respectively.

Discussion

Mikeska and Dobrovic (2009) explained that primer concentration optimisation can increase PCR performance. From the optimisation of pork and 18S rRNA primer formulations, two candidates of optimum formulations were obtained, i e. primer concentrations in 1st and 5th formulations. The 1st formulation showed clean and clear bands but the weakness of the formulation was the different thickness proportion of pork and 18S rRNA bands. Meanwhile, the 5th formulation had the same proportion of pork and 18S rRNA bands but the weakness was a primer-dimer. Confirmation for duplex detection should be done by using agarose gel for electrophoresis, since the melting temperature for each DNA target were very close making it difficult to differentiate each other. The melting temperature was 81.1°C (pork) and 81.5°C (18S rRNA). This was the limitation encountered in the present work

that needs to be improved in future works. In real-time PCR, primer design, GC content, amplicon length, and others are some parameters that need to be considered.

We tried to get the best duplex real-time PCR with clear and clean band and had an almost equal proportion between pork and 18SrRNA bands. For that purpose, the annealing temperature especially for 5th formulation showing an equal proportion for each target but with primer-dimer was optimised. Temperature and annealing time are two of the most important parameters for optimisation with PCR multiplex (Butler, 2012). Annealing temperature optimisation is a critical process in PCR (Rychlik *et al.*, 1990; Prezioso and Jahns, 2000). The optimisation of annealing temperature started from 45°C - 55°C for duplex detection with 5th formulation. All the temperatures still showed primer dimer (data not shown). Annealing temperature optimisation did not resolve primer dimer problem for the 5th formulation. Ct values for DNA template 20 ng were 12.58 – 16.05 for each temperature, respectively (data not shown). Based on the above result, the 1st formulation shown as clear and clean band was chosen for further work, with annealing temperature of 55°C.

The novelty of the present work was the use of in-house validation of pork detection. In-house validation is an examination method of examining in the laboratory which produces right results; it is useful and cost-effective (Boque *et al.*, 2002). In the present work, in-house validation on real-time PCR used some parameters, such as specificity, detection limit (sensitivity), precision, linearity, PCR efficiency, and robustness. Target (pork) and non-target animal species (such as beef, chicken, duck, goat, and fish) were performed for pork specificity assay. The result in PCR acquired only the pork band (300 bp) in agarose gel (data not shown).

The Ct and Tm values of detection limit were 14.890 – 21.957 (Tm = 80.30°C ± 10°C) (Figure 1). Clear and clean bands also appeared in DNA electrophoresis. The smaller the concentration of sample, the thinner the band on the sample was. In the present work, the detection limit of minimum target DNA concentration could detect the concentration of 0.78125 ng of the sample. It could even be more sensitive since that concentration obtained thick bands.

The detection limits of the developed method are precision, linearity, and PCR efficiency. The developed methods showed high precision, linearity, and PCR efficiency. The CV values of the method were 0.145 – 0.898% (Table 1). Broeders *et al.* (2014) explained that RSD or CV by real-time PCR
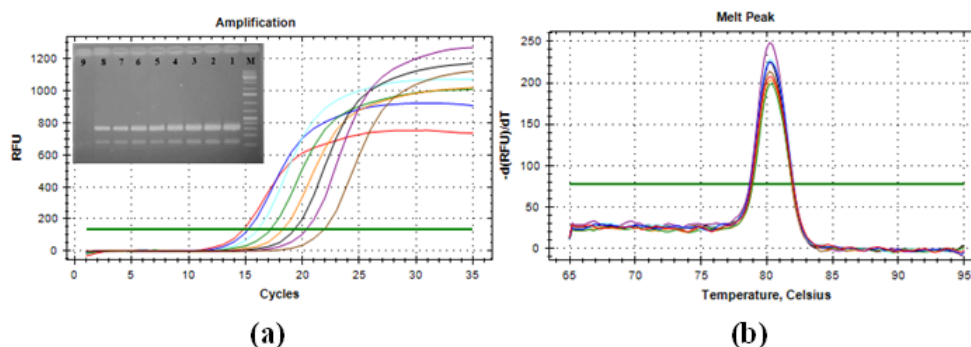
Figure 1. The amplification (a) and melting (b) curves of detection limit (red: 100 ng; blue: 50 ng; light green: 25 ng; green: 12.5 ng; orange: 6.25 ng; black: 3.125 ng, purple: 1.625 ng; brown: 0.78125). Agarose gel by electrophoresis (a) on lanes (M: marker, 1: 100 ng; 2: 50 ng; 3: 25 ng; 4: 12.5 ng; 5: 6.25 ng; 6: 3.125 ng, 7: 1.625 ng, 8: 0.78125 ng, dan 9: NTC).

Table 1. %CV values of precision parameter.

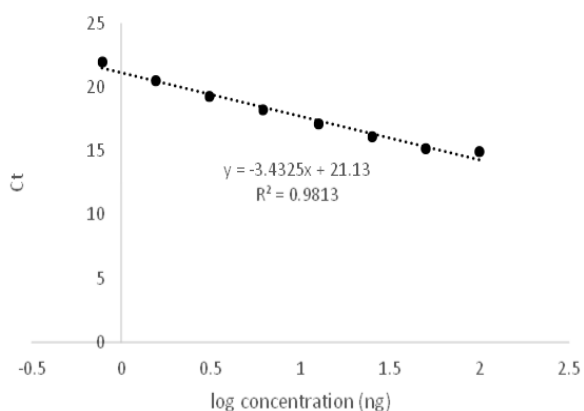| Concentration (ng)	Ct (Replicates)			CV (%)
	1	2	3	
100	14.88	14.87	14.92	0.145
50	15.27	14.95	15.19	0.898
25	16.06	16.02	16.14	0.310
12.5	16.98	17.03	17.23	0.632
6.25	18.31	18.27	18	0.757
3.125	19.28	19.11	19.33	0.489
1.5625	20.52	20.38	20.54	0.348
0.78125	21.99	21.84	22.04	0.387

Figure 2. The slope and R^2 values of duplex detection.

method can be accepted when it does not exceed 25%. The slope and regression coefficient (R^2) were -3.4325 and 0.9813, respectively (Figure 2). These values of slope and regression coefficient were also recommended for values of efficiency (E= 90-110%) (Iwobi *et al.*, 2015). The efficiency of PCR value was 95.584%, which explained that inhibitor did not appear in the reaction.

Applications on processed food products were carried out on samples containing pork and non-pork (shown by the labels) using duplex real-time PCR. In addition, using positive and negative controls were required to know that the method could detect duplex (pork and 18s rRNA) and the absence of contamination. Melting curves on processed food product which contained pork and non-pork showed that there was no significant difference of –d(RFU)/dT value. Based on agarose gel by using electrophoresis, all pork processed food products (Figure 3) showed pork and 18S rRNA bands, while non-pork processed food products did not show pork band (18S rRNA only).

The duplex methods were effective and efficient for detection pork and 18S rRNA, and reagents were used because PCR reagents were fed into single tube in a PCR reaction. The present work could be further modified and improved in future works. The prediction of the Tm values of pork (300 bp) and 18S rRNA (99 bp) amplicons showed that the peak was not significantly different in melting curve. Therefore, it is necessary to do further research which includes electrophoresis to confirm the result obtained.

Standard curve is also known as calibration curve. It is required to quantify the unknown sample using real time PCR based on the ΔΔCt method. Before determining the quantification of pork in processed foods, it is necessary to construct a standard curve with the percentage of pork in beef. The approach is based on the difference in using sample and 18sRNA Ct values (Soares *et al.*, 2013; Amaral *et al.*, 2017). Based on the results, the value of the standard curve efficiency fulfilled the theory requirements. The efficiency value was 90% with the criteria of slope value of -3.6 and R^2 was 0.98 (Figure 4). The extension step of real-time PCR condition did not affect the results of analysis. It was shown by the amplification and melting peak values. In addition, confirmation by electrophoresis was also performed. It showed pork and 18S rRNA bands (data not shown), respectively.

(a)
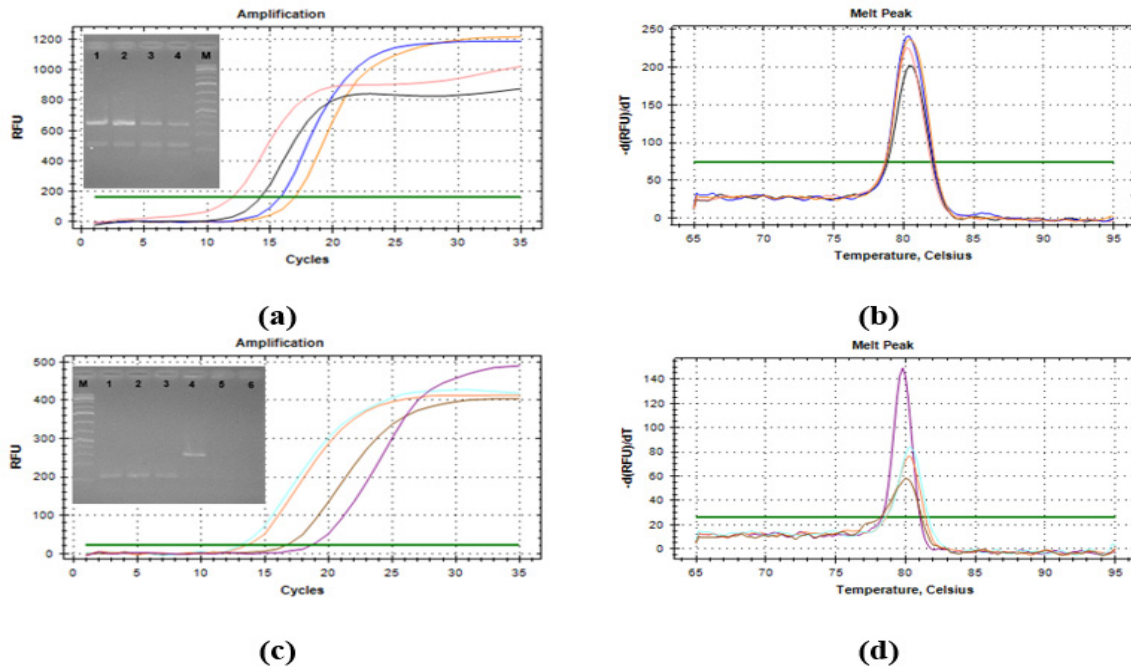

(b)

(c)

(d)

Figure 3. The amplification and melting curves of pork a-b (red: sausage, blue: ham, orange: pasta, black: corned) and non-pork c-d (brown: abon, light green: corned, peach: rolade) on processed food product, positive control (purple: positive control of pork). Agarose gel by electrophoresis (a) on lanes (M: Marker, 1: sausage, 2: ham, 3: corned, 4: pasta) and (c) (M: Marker, 1: abon, 2: corned, 3: rolade, 4: positive control of pork, 5-6: NTC).

(a)
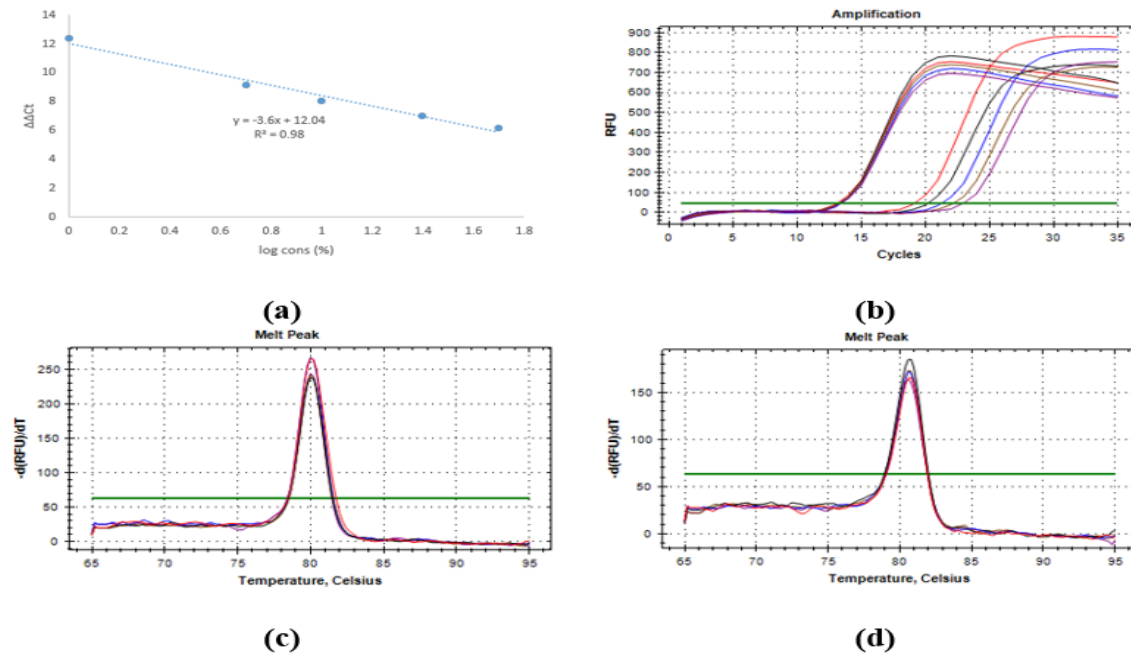

(b)

(c)

(d)

Figure 4. The standard curve (a) with the percentage of pork in beef (1 – 5% pork). Amplification curve (b) of binary mixtures (1 – 50% of pork DNA). Melting curves of pork (c) and 18S rRNA (d) in binary mixtures.

In the present work, the development of real-time PCR can be applied in various types of processed foods. The application in pork quantification of processed food products should carried out in the $\Delta\Delta Ct_{pork}$ range of between 6.2 – 12.34.

Conclusion

The present work aimed to conduct in-house validation and standard curve in quantification as calibration had been successfully examined. Duplex analysis by real-time PCR SYBR Green I method

could detect pork and 18S rRNA on processed food products. It also can be categorised as specific and sensitive methods. The detection limit of target DNA concentration was 0.78125 ng of the sample. It could be more sensitive since PCR result still obtained thick bands. CV value was between 0.104 – 1.703%. The R^2 and efficiency of PCR were 0.9813 and 95.584%, respectively. Standard curves for quantification of processed foods in pork (1 – 50%) in beef showed an efficiency value of E = 90%.

Acknowledgement

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