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OPTIMIZATION OF ANNEALING TEMPERATURE AND PRIMER CONCENTRATION OF CYTOCHROME B (CYT B) GENE FOR PIG DNA DETECTION WITH REAL-TIME PCR METHOD

OPTIMASI SUHU ANNEALING DAN KONSENTRASI PRIMER GEN SITOKROM B (CYT B) UNTUK DETEKSI DNA BABI DENGAN METODE REAL-TIME PCR

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Penelitian

A B S T R A C T

Background: *One company in Indonesia has developed a pig DNA detection kit by designing primers with the real-time Polymerase Chain Reaction (PCR) method using the cytochrome b (cyt b) gene. It is necessary to optimize the PCR process to optimize pig DNA detection, including annealing temperature and primer concentration, which can increase sensitivity, specificity, and precision.* ***Purpose:*** *This research aims to determine the optimum annealing temperature and primer concentration for the detection of pig DNA using cyt b gene.* ***Method:*** *In this research, the extracted sample isolates were subjected to 12 treatments with 2 repetitions. Optimal data analysis was based on the lowest Cycle Threshold (CT) value in the amplification curve.* ***Result:*** *Out of a total of 24 samples, an increase in the CT value was observed at annealing temperatures of 57 °C, 59 °C, and 60 °C compared to 58 °C, across various primer concentrations. The primer concentrations with the lowest CT values were successively found to be 0.4 µM, 0.3 µM, and 0.2 µM.* ***Conclusion:*** *The results of the research that has been conducted indicate that the optimal annealing temperature for detecting pig DNA using the cyt b gene in this research is 58 °C, and the optimal concentrations of forward and reverse primers are 0.4 µM.*

A B S T R A K

Latar belakang: Salah satu perusahaan di Indonesia telah mengembangkan kit deteksi DNA babi dengan metode *real-time Polymerase Chain Reaction* (PCR) dengan merancangkan primer menggunakan gen sitokrom b (*cyt* b). Perlu dilakukan optimasi proses PCR untuk mengoptimalkan deteksi DNA babi, termasuk suhu *annealing* dan konsentrasi primer, sehingga dapat meningkatkan sensitivitas, spesifisitas, dan presisi. **Tujuan:** Penelitian ini bertujuan untuk menentukan suhu *annealing* dan konsentrasi primer yang optimum untuk mendeteksi DNA babi menggunakan gen *cyt* b. **Metode:** Pada penelitian ini, isolat sampel hasil ekstraksi dilakukan 12 perlakuan dengan 2 kali pengulangan. Analisis data optimal dilihat berdasarkan nilai *Cycle Treshold* (CT) terendah pada kurva amplifikasi. **Hasil:** Pada total 24 sampel, terjadi peningkatan nilai CT pada suhu *annealing* 57 °C, 59 °C, dan 60 °C dibandingkan suhu 58 °C pada berbagai konsentrasi primer. Konsentrasi primer dengan nilai CT terendah secara berturut-turut sebesar 0.4 µM, 0.3 µM, dan 0.2 µM. **Kesimpulan:** Hasil penelitian yang telah dilakukan menunjukkan bahwa suhu *annealing* yang optimal untuk mendeteksi DNA babi menggunakan gen *cyt* b pada penelitian ini adalah 58 °C, dan konsentrasi primer *forward* dan *reverse* yang optimal adalah 0.4 µM.

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INTRODUCTION

Indonesia is a country with a majority Muslim population, which significantly influence lifestyles, especially based on halal product selection. Therefore, applications for halal certification in Indonesia have increased. This is also reinforced by the obligation to certify halal based on the Law on Halal Product Guarantee issued by the government to provide protection and guarantees regarding the halal products consumed and used by the public (Ministry of Religion, 2014; Republika, 2021).

One of the critical aspects of halal products is that they do not contain pork and its derivatives. Efforts to examine the pork content in a product can be carried out using the *real-time Polymerase Chain Reaction* (real-time PCR) method. Real-time PCR is a method for doubling target DNA, with readings taken at each cycle. This method is known for its high sensitivity and specificity (Arya *et al*., 2005; Janudin *et al*., 2022).

The application of the real-time PCR method requires a commercial kit specifically designed to detect pig DNA. Currently, Indonesia does not have a domestically produced pig DNA detection kit using the real-time PCR method. Therefore, Bio Farma, a company in Indonesia, has developed a pig DNA detection kit by designing primers using the *cytochrome b* (cyt b) gene in pig *mitochondrial DNA* (mtDNA). The cyt b gene can be used as a genetic marker because, based on its position, it has a more conserved region, ensuring species identification without causing ambiguity (Farias *et al*., 2001). The selection of the mtDNA cyt b gene is based on its abundance in cells and its nucleotide sequence, which remains relatively undamaged during processing and heating (Dooley *et al*., 2004; Kim *et al*., 2018).

The designed primer produces an amplification curve and a *Cycle Threshold* (CT) value. However, it is necessary to optimize the PCR process for effective pig DNA detection. Optimization is essential to enhance the DNA amplification process and achieve the desired product. This optimization is carried out by varying the conditions used in the PCR process, including the annealing temperature and primer concentration, which can increase the sensitivity, specificity, and precision (Handoyo and Rudiretna, 2000; Tania *et al*., 2014).

The annealing temperature represents a crucial step in PCR step, determining the specificity of the reaction (BIO-RAD Laboratories, 2022). The annealing temperature is usually not more than 5 °C below the *Melting Temperature* (TM) of the primer selected for DNA amplification. The recommended ideal TM for determining the annealing temperature based on primer design is 62 °C (IDT, 2022). The application of an annealing temperature that is too high causes the bond between the primer and the DNA template to be ineffective, resulting in low or even non-existent PCR products. Conversely, if the temperature is too low, nonspecific bonding can occur (BIO-RAD Laboratories, 2006).

Optimization of real-time PCR conditions against primer concentrations is also a crucial aspect. The final primer concentration in the reaction mix used for the DNA binding dye method is usually lower than that for the fluorescence probe method, ranging from 0.2 µM to 0.4 µM (Hudson, 2008). Therefore, we choose a primer concentration within the range of 0.2 to 0.4 µM, considering the use of the binding dye method (SYBRGreen) in this research. The use of an excessively high primer concentration will result in dimer primers and will not increase DNA amplification. If the concentration is too low, non-specific primer binding will occur which can increase the risk of false-negative results and reduce the sensitivity of the PCR reaction (Gunson *et al*., 2003). Based on this background, the authors are interested in conducting research on the "Optimization of Annealing Temperature and Primer Concentration of the Cytochrome b (cyt b) Gene for Pig DNA Detection with Real-Time PCR Method."

MATERIAL AND METHOD

Quasi-experimental design

This research was conducted by determining the optimal annealing temperature and primer concentration for pig DNA detection using real-time PCR. In this research, the annealing temperature was determined by varying the temperature at 57 °C, 58 °C, 59 °C, and 60 °C. Additionally, the primer concentration was determined over a range of concentration variations, namely 0.2 µM, 0.3 µM, and 0.4 µM. Then the variations of annealing temperature and primer concentration were examined by real-time PCR.

Collection of samples

This research has obtained research approval from the code of ethics with the number 59/KEPK/EC/III/2023.The population consisted of pork obtained from supermarkets in Bandung City. The sample used was a sample of pork as much as 50 grams. There were 12 treatments, derived from three variations of primer concentration multiplied by four variations of annealing temperature, with two repetitions for each temperature variation. Additionally, a negative control test *Non-Template Control* (NTC) was conducted.

DNA extraction

The DNA extraction process used the Wizard® Genomic DNA Purification Kit (Cat. No. A1120). Tissues were ground in liquid nitrogen using a mortar and pestle that had been prechilled in liquid nitrogen. After grinding, the liquid nitrogen was allowed to

evaporate and approximately 10 – 20 mg of the ground tissue transferred to 600 µL of nuclei lysis solution in a 1.5 mL microcentrifuge tube. The lysate was incubated at 65 °C for 15 – 30 minutes then 3 µL of RNase solution was added to the nuclear lysate and mixed the sample by inverting the tube 2 – 5 times. The mixture was incubated for 15 – 30 minutes at 37°C and the sample allowed to cool to room temperature for 5 minutes before proceeding. To the room temperature sample, 200 µL of protein precipitation solution was added and vortexed vigorously at high speed for 20 seconds. The sample was chilled on ice for 5 minutes then centrifuged for 4 minutes at 13.000 – 16.000 × g. The supernatant containing the DNA (leaving the protein pellet behind) was removed and transferred to a clean 1.5 mL microcentrifuge tube containing 600 µL of room temperature isopropanol. The solution was gently mixed by inversion until the white thread-like strands of DNA formed a visible mass. It was centrifuged for 1 minute at 13.000 – 16.000 × g at room temperature then the supernatant was carefully decanted and then was added 600 µL of room temperature 70% ethanol, and the tube gently inverted several times to wash the DNA. It was then centrifuged for 1 minute at 13.000 – 16.000 × g at room temperature. The ethanol was carefully aspirated using either a drawn pasteur pipette or a sequencing pipette tip. The DNA pellet was very loose at this point, and care was needed to avoid aspirating the pellet into the pipette. The tube was inverted on clean absorbent paper, and the pellet air-dried for 10 – 15 minutes. Then 100 µL of DNA rehydration solution was added and the DNA rehydrated by incubating at 65 °C for 1 hour. The solution was periodically mixed by gently tapping the tube. Alternatively, the DNA was rehydrated by incubating the solution overnight at room temperature or at 4 °C. The DNA was stored at 2 – 8 °C and 1 µL of extracted DNA was used for PCR amplification.

PCR procedure

The PCR was set up in a PCR strip tube. After adding 10 µL of the reaction mix containing the HotShot Diamond™ Mastermix (Cat. No. HS002), forward and reverse primers (IDT), Nuclease-Free Water Promega (Cat. No. P1193), EvaGreen® Dye, 20X in Water (Cat. No. 31000), and extracted pork DNA, the setup was complete. The master mix contained Taq polymerase, anti-Taq polymerase monoclonal antibodies, buffer, $MgCl_2$, dNTPs, and stabilizer. The primers used were as follows: primer forward 5' TCGGAACAGACCTCGTAGAA 3' and primer reverse 5' ATGACGAAGGGCAGGATAAAG 3'.

The PCR vial tube was placed in the PCR machine (Bio-Rad CFX96 Touch™ Real-Time PCR Detection System) and it was subjected to initial denaturation at 95 °C for 5 minutes, followed by 40 cycles of denaturation at 95 °C for 10 seconds, annealing at 57 °C - 60 °C (configured using the gradient feature) for 30 seconds, and extension at 72 °C for 20 seconds. A final extension step was carried out at 72 °C for 2 minutes.

RESULT

Based on Table 1, it is indicated that there is an increase in CT values at annealing temperatures of 57 °C and at temperatures of 59 °C and 60 °C compared to 58 °C. The annealing temperature that has the lowest CT value at various primary concentrations used is 58 °C. In the Table 1, it can be seen that there was an increase in CT values at primer concentrations successively of 0.4 µM, 0.3 µM, and 0.2 µM. Thus, in this research, the lowest CT value is observed at primer concentrations of 0.4 µM.

Optimization of annealing temperature at 57 °C with primer concentrations of 0.2 µM, 0.3 µM, and 0.4 µM

The results in Figure 1 show that the formation of a curve indicates the amplification of pig DNA using forward and reverse primers, with NTC remaining negative. The use of primers with concentrations of 0.2 µM, 0.3 µM, and 0.4 µM resulted in average CT values of 28.91, 27.77, and 26.95, respectively. The concentration of 0.4 µM is the primer concentration that has the lowest CT value. Therefore, the primer concentration of 0.4 µM is the most optimal at an annealing temperature of 57 °C.

Table 1. *Cycle Threshold* (CT) values of each annealing temperature and primer concentration

		Annealing temperature											
		57°C			58°C			59°C			60°C		
		R1	R2	A	R1	R2	A	R1	R2	A	R1	R2	A
Primer concentration (µM)	0.2	28.68	29.14	28.91	28.55	28.68	28.62	28.70	28.98	28.84	29.53	29.24	29.39
	0.3	27.79	27.75	27.77	27.45	27.28	27.37	27.65	27.27	27.46	28.01	27.87	27.94
	0.4	26.85	27.05	26.95	26.83	26.68	26.76	26.97	26.90	26.94	27.99	27.38	27.69
NTC	N/A												

Note: R1 (Repetitions 1), R2 (Repetitions 2), A(Average)

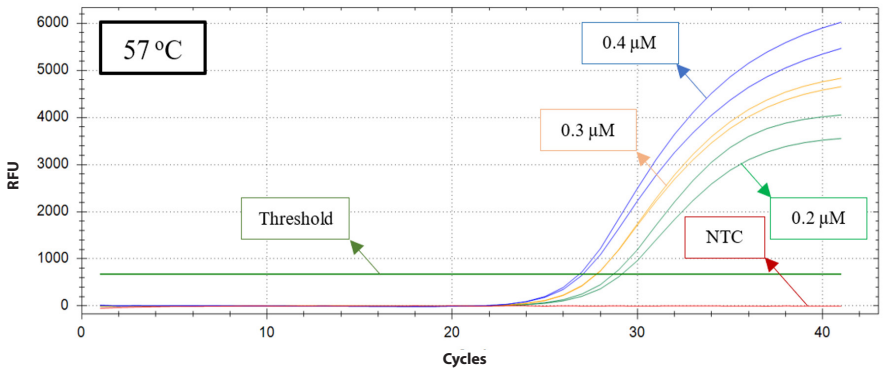

Figure 1. Amplification curve at annealing temperature of 57 °C

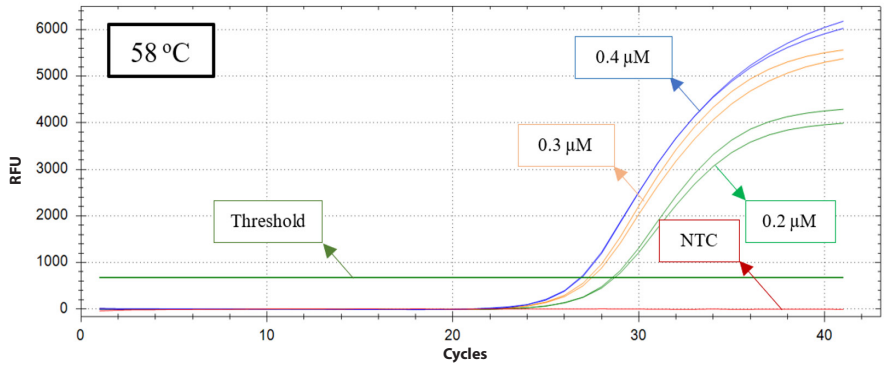

Figure 2. Amplification curve at annealing temperature of 58 °C

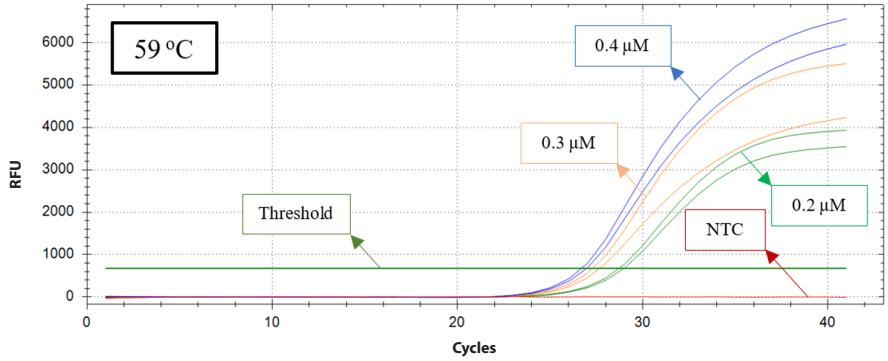

Figure 3. Amplification curve at annealing temperature of 59 °C

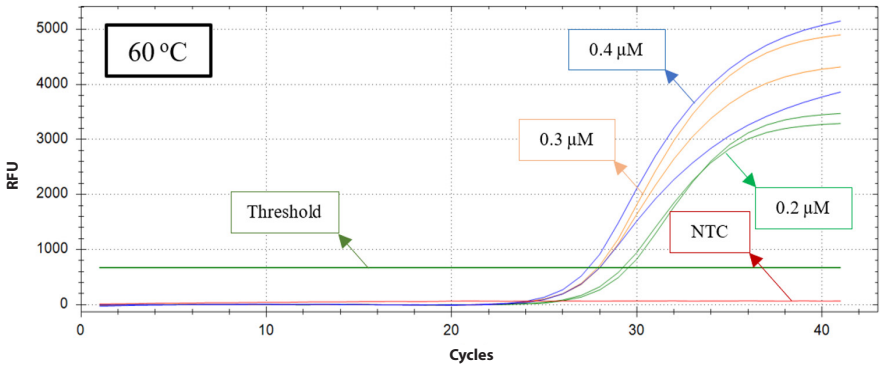

Figure 4. Amplification curve at annealing temperature of 60 °C

Optimization of annealing temperature at 58 °C with primer concentrations of 0.2 µM, 0.3 µM, and 0.4 µM

The results in Figure 2 show a real-time PCR amplification curve at an annealing temperature of 58 °C. The NTC in the test did not exhibit an increase in fluorescence. The average CT values for each primer concentration of 0.2 µM, 0.3 µM, and 0.4 µM were 28.62, 27.37, and 26.76, respectively. The concentration of 0.4 µM is the primer concentration that has the lowest CT value. So, the primer concentration of 0.4 µM is the most optimal at an annealing temperature of 58 °C.

Optimization of annealing temperature at 59 °C with primer concentrations of 0.2 µM, 0.3 µM, and 0.4 µM

The results in Figure 3 show an increase in fluorescence at all primer concentrations used and a negative NTC without the formation of a curve. The primer concentration of 0.2 µM had an average CT value of 28.84, the 0.3 µM primer concentration had an average CT value of 27.46, and the 0.4 µM primer concentration had an average CT value of 26.94. The concentration of 0.4 µM is the primer concentration that has the lowest CT value. So, the primer concentration of 0.4 µM is the most optimal at an annealing temperature of 59 °C.

Optimization of annealing temperature at 60 °C with primer concentrations of 0.2 µM, 0.3 µM, and 0.4 µM

The amplification curve of the optimization results at an annealing temperature of 60 °C in Figure 4 shows that at each primer concentration, there is an amplification curve with an average CT value at each primer concentration of 0.2 µM, 0.3 µM, and 0.4 µM, being 29.39, 27.94, and 27.69, respectively. The NTC does not show an increase in fluorescence or is negative. The concentration of 0.4 µM is the primer concentration that has the lowest CT value. Therefore, the primer concentration of 0.4 µM is the most optimal at an annealing temperature of 60 °C.

DISCUSSION

Based on the results of the optimization conducted, an annealing temperature of 58 °C resulted in the lowest CT value at various concentrations of primers, as compared to other annealing temperatures (Table 1). These results indicate that the annealing temperature of 58 °C is optimal for forward and reverse primers with a melting temperature of 62 °C to obtain large amounts of amplification results and has high specificity. Amplification at annealing temperatures below (57 °C) and above (59 °C and 60 °C) the optimal 58 °C is still able to produce CT values that are relatively close together, but higher than CT values with annealing temperature 58 °C. This shows that the annealing temperature below and above of 58° C experienced a slower amplification process because the primer attachment process to the DNA fragment was not optimal. The use of annealing temperature which is low causes non-specific amplification and temperature annealing that is too high can affect the efficiency of the reaction, resulting in a lack of amplification so that the resulting CT value is high (Bustin *et al*., 2009). This is also in accordance with Borah (2011) who stated that annealing temperature which exceeds the optimum temperature can inhibit the attachment of the primer with the template, so that fewer PCR products are produced and can affect the efficiency of the reaction causing higher CT values with low reproducibility.

Other research states that annealing temperature should not exceed the dissociation temperature of the primer. This causes the primer to not stick and results in the polymerase enzyme not being able to catalyze the installation of complementary nitrogenous bases present in the reagent into the DNA template which in the end will not form new DNA because the polymerase enzyme will function to synthesize complementary DNA from the template DNA if there is a primer that has started the polymerase (Hsu *et al*., 1997).

In addition to optimization of annealing temperature, optimization of the primer concentration is also important, because it improves the performance and optimum condition of the real-time PCR test polymerization reaction (Roux, 2009). The use of optimum primer concentration has a major influence on DNA amplification and increases the quality of PCR product (Joko *et al*., 2011; Markoulatos *et al*., 2002). So, this test serves to determine the sensitivity of the method using different primer concentrations and to obtain optimal primer concentrations for detection of pig DNA targeting the cytochrome b gene using real-time PCR. Concentrations of forward and reverse primers are considered optimal when the amplification produces a low CT value and a negative NTC because the CT value correlates with the quantity of the DNA template (Pestana *et al*., 2010).

In this research, the primer concentration according to the reference was used, namely 0.2 - 0.4 µM (Hudson, 2008). So, the primer concentration forward and reverse used were 0.2 µM, 0.3 µM, and 0.4 µM. In Table 1, it can be seen that the three concentrations of primers tested at various annealing temperatures can amplify products with various CT values. CT < 31 is a strong positive reaction and indicates large amounts of target DNA. Meanwhile, CT > 31 shows a weak reaction or indicates a false positive result (Caraguel *et al*., 2011).

Based on the optimization results of the primer concentrations tested at various annealing temperatures, all primer concentrations used showed an increase in fluorescence with CT values that were still far from the end of the cycle. This shows that the lowest primer concentration of 0.2 µM can still amplify the product well. However, from the CT formed, it can be seen that the

concentration is 0.4 µM at all annealing temperature variations used has the lowest CT value compared to other primer concentrations. So, in this research the optimal primer concentration for detection of pig DNA targeting using the cytochrome b gene at various annealing temperatures used is 0.4 µM.

In this research, primer concentrations were used in the dilution range of 0.2 – 0.4 µM, but this is a limitation because it has not shown the optimum and efficient primer concentration, where this research aims to obtain a test with the most sensitive and efficient results. The use of primer concentrations that are too high or more than 0.4 µM, according to this research, in order to obtain a lower CT value or earlier amplification, might increase the chance of mispriming or primer-dimer which means it will produce non-specific products or false positives will occur (Pestana *et al.*, 2010). Dimer primers, an incidental outcome of the PCR process, result from primer molecules adhering to each other. The existence of dimer primers can be detected and assessed through techniques such as analyzing the melting curve or conducting electrophoresis on the PCR product (Life Technologies, 2014; Harshitha and Arunraj, 2021).

As for the NTC test in this research, it showed no formation of CT values and amplification curves at each variation of temperature annealing and primer concentration. This indicates that there is no increase in fluorescence or there is no contamination from DNA (Life Technologies, 2014). However, if there is increased fluorescence in the NTC, then the reading is considered unreliable and needs to be retested (Hilscher *et al*., 2005).

Indicator in determination of the optimum of temperature annealing and primer concentration apart from analyzing the amplification curve, primer specificity testing was also carried out by analyzing the melting peak (melt peak curve) by looking at the melting point (melt peak) and the *Melting Temperature* (TM) value generated. In addition, this research used samples derived from isolated pork. So that the test validation was not carried out because the exact concentration and purity of the sample used were not known. In order to conduct a sensitivity test effectively, it is imperative to have knowledge of the sample's concentration. Utilizing samples with well-established concentrations and adequate purity is anticipated to yield precise results in both validation and sensitivity testing processes (Amaral *et al*., 2017). Identifying species through PCR analysis in meat samples can pose challenges due to the presence of inhibitory substances in products with a complex composition. The risk of encountering amplification is higher when a large amount of DNA (>50 ng) is tested (Dooley *et al*., 2004).

CONCLUSION

The optimal annealing temperature for forward and reverse primers, using the cytochrome b gene for pig DNA detection by real-time PCR method, is 58 °C. Meanwhile, the optimal concentration in this research is 0.4 µM. For further research, the optimization of primer concentration was carried out using a wider range. An analysis was carried out on the melting curve to determine the specificity of the reaction. Next, testing was carried out using samples of positive control from synthetic DNA fragments for validation tests, and also testing using samples from processed products.

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