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In-silico primer designing and validation of *Bos taurus* and *Sus scrofa* SET proto-oncogene for non-halal detection

Fatimah Mustafawi Muhammadi[1*], Jumailatus Solihah[2], Anti Damayanti Hamdani[2]
[1]*Department of Food Nanotechnology, Politeknik AKA Bogor, Jl. Pangeran Sogiri 283, Tanah Baru, Bogor Utara, Bogor, West Java, 16154, Indonesia*
[2]*Department of Biology, Faculty of Science and Technology, Universitas Islam Negeri Sunan Kalijaga, Jl. Timoho 153, Papringan, Caturtunggal, Depok, Sleman, Special Region of Yogyakarta, 55281, Indonesia*
**Corresponding author: fatimahmustafawi@kemenperin.go.id*

ABSTRACT
The SET gene is part of the proto-oncogene family. Proto-oncogenes families are expressed in cell growth regulation, notably during embryogenesis, wound healing, liver regeneration, and mitotic stimulation by growth factors. While proto-oncogenes have been extensively studied for their role in cancer detection, their application in halal certification assessments remains minimal. Polymer Chain Reaction (PCR) has recently been an alternative method in species authentication due to its high accuracy, with primers being a critical component in determining the amplification process's success. Consequently, primers must be carefully and specifically designed. This study uses a bioinformatics approach to explore the potential of the SET proto-oncogene as a candidate primer for non-halal detection. SET proto-oncogene sequences from pig (*Sus scrofa*) and cattle (*Bos taurus*) were retrieved from the National Center for Biotechnology Information (NCBI) database and aligned using MEGA6 software to identify conserved regions suitable for primer design. The designed primers were tested through in-silico PCR and subsequently validated using conventional PCR. The results demonstrated that two primer pairs successfully produced amplicons with lengths of 1068 bp and 1231 bp in cattle and 1275 bp and 1222 bp in pigs, consistent with in-silico predictions.

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INTRODUCTION

Muslims in Indonesia have reached 87.2% of its population (BPS Kota Samarinda, 2024). Muslim diet is regulated in the Holy Qur'an, and they can only consume halal foods (Aziz et al., 2023). Halal food refers to food products prepared according to Islamic law (Prachugsorn et al., 2022). The Indonesian government made strict rules for food industries to accommodate the Muslims' needs for halal-labeled products. The food products must be recognized as halal, and halal certification issued by Majelis Ulama Indonesia (MUI) must be obtained through halal testing (Nur, 2021). Nonetheless, the authenticity of halal-labeled products has also come into question due to the cases of mislabeling and fraudulent practices in the market of halal-labeled products (Serrano, 2020). Hence, the significance of accurate and reliable methods for non-halal detection is required. One of the most precise and sensitive techniques to detect DNA contamination in a food product is Polymerase Chain Reaction (PCR) (Akter et al., 2021; Cahyadi et al., 2020; Raharjo et al., 2019; Rosyid et al., 2023).

The PCR method is based on the specific hybridization of oligonucleotides, enabling exponential amplification within minutes (Meiser et al., 2022). The main components of this method are a DNA template, primer pair, DNA polymerase enzyme, and nucleotides (deoxynucleoside triphosphate).

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Primer is a short single-strand DNA complementary with the DNA target (Purwakasih & Achyar, 2021). The crucial factor of a successful PCR assay is the oligonucleotide primer design. Carefully designed primer pairs will ensure the amplification result specificity and the targeted amplicon high yield (Delghandi et al., 2022). Designing primer pairs can be performed through software and evaluated using in-silico PCR. This pre-experiment step provides information about the real PCR assay and minimizes failures, leading to cost efficiency.

Proto-oncogenes are essential regulatory genes that control cell growth and differentiation. These genes are typically expressed during key biological processes such as embryogenesis, wound healing, liver regeneration, and in response to mitotic stimulation by growth factors. Their function in these contexts is tightly regulated to ensure proper cell cycle progression and tissue repair (Kontomanolis et al., 2020). One notable characteristic of proto-oncogenes is the presence of highly conserved regions essential for their functional roles across species. These conserved regions allow for the identification of orthologous genes, which are genes in different species that share a common ancestral gene. Despite these similarities, orthologous genes can also possess variable regions, leading to differences in gene sequences across species. These variable regions make orthologous genes valuable as potential interspecies biomarkers, as they can help detect specific biological processes or diseases in a wide range of organisms (Brubaker et al., 2020).

Recently, proto-oncogenes have been increasingly studied for their potential applications in cancer detection and as therapeutic targets. For example, the overexpression or mutation of proto-oncogenes can lead to uncontrolled cell proliferation, a hallmark of cancer. Research has shown that monitoring the expression or mutations of proto-oncogenes can help in early cancer detection and in developing targeted therapies (Dornburg et al., 2021; Grible et al., 2021).

However, using proto-oncogenes for applications such as halal certification remains limited. Halal certification ensures that products meet specific standards, often detecting animal-derived ingredients. The analysis of proto-oncogenes for halal detection is an emerging field, primarily performed using in-silico PCR. This computational approach simulates the polymerase chain reaction (PCR) process, predicting the presence or absence of specific genes in a given sample. By using in-silico PCR, researchers can minimize laboratory testing costs while efficiently screening for gene sequences, making it a promising tool for halal certification (Cordaro et al., 2021).

RESEARCH METHOD

Materials

The materials used in this research were cow and pig liver, DreamTaq Green PCR Master Mix #K1081 (Promega, Madison, USA), DNA ladder 1 Kbp (Microzone Ltd.), Tris-HCl, NaCl, EDTA, SDS, chloroform, isoamyl alcohol, and agarose. SET genes of *Bos taurus* (AC_000168.1) and *Sus scrofa* (NC_010443.4) were downloaded from the NCBI (National Center for Biotechnology Information) database. The devices utilized in this research were microcentrifuge Heraeus Pico 17 (Thermo Fisher, Massachusetts, USA), thermocycler My cycler (BIO-RAD, California, USA), UV-Transilluminator Gel Doc XR system (BIO-RAD, California, USA), and electrophoresis apparatus Power Pac™ Basic (BIO-RAD, California, USA).

Methods

1. Primer Design

   The SET genes of *Bos taurus* (AC_000168.1) and *Sus scrofa* (NC_010443.4) were used to design the primer and retrieved in FASTA format from the NCBI database. The primer design was performed using MEGA6 software to select the conserved regions among the target species through the Alignment by Muscle option. The obtained primer candidates were evaluated using in silico PCR at UCSC Genome Browser Tools (http://genome.ucsc.edu/cgi-bin/hgPcr).
2. DNA Extraction

   DNA extraction was performed according to Nasif (2024) with modification. Approximately 0.5 g of the minced liver was lysed in an extraction buffer solution (pH 7.8) containing Tris-HCl, NaCl, EDTA, and SDS, with concentration shown in Table 1. A total of 20

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µL of proteinase K (20 mg/mL) was added. The mixture was incubated for 90 minutes at 55°C and once again at room temperature overnight.

Table 1. Composition of the extraction buffer.

Reagent	Concentration
Tris-HCL	100 mM
NaCl	150 mM
EDTA	100 mM
SDS	2.5%

The mixture was centrifuged, and the supernatant was transferred to a new tube. Phenol and chloroform: isoamyl alcohol was added sequentially, followed by centrifugation to remove impurities. For DNA precipitation, sodium acetate and cold alcohol were added, and the mixture was incubated at 4°C. After centrifugation, the pellets were washed with 70% ethanol, air-dried, resuspended in 200 µL of 1X TE buffer, and stored at 4°C for further analysis.

3. DNA Quantification

Concentration and purity assessments were performed according to Lucena-Aguilar et al. (2016) with slight modifications. DNA concentration was quantified by UV light absorbance at 260 nm, and the purity was assessed using the ratio of 260/280 nm absorbance. Samples were diluted to reach the required concentration based on Table 2, and absorbance measurements were taken using a spectrophotometer.

4. PCR Amplification

An oligonucleotide primer set (synthesized by Integrated DNA Technologies, Singapore) derived from cattle and pig chromosomal DNA SET gene sequences was used for PCR amplification. The PCR amplification was performed in a 25 µL total volume with component composition based on the instructions of DreamTaq Green PCR Master Mix (2X) #K1081, as stated in Table 2.

The mixture tubes were placed in a thermocycler. The cycling parameters were initial denaturation at 95°C for 3 min, 25 cycles of denaturation at 95°C for 30 s, annealing at 45°C and 50°C for 30 s, followed by elongation at 72°C for 2 min, and final elongation at 72°C for 10 min. The PCR products were analyzed utilizing an electrophoresis apparatus. The electrophoresis was performed on 1% gel agarose containing ethidium bromide at constant 100 V for 39 min in TAE buffer 1X (Tris-HCl 40 mM, sodium acetate 20 mM, and EDTA 1 mM; pH 7.6). Fifty µL of PCR products and 1 Kbp DNA ladder marker were loaded into the gel agarose wells. The gel was finally visualized using a UV-transilluminator Gel Doc.

Table 2. Concentration of PCR Components.

Component	Initial Concentration	Final Concentration	Initial Volume (µL)	Final Volume (µL)
Master mix	-	-	12.5	25
Reverse primer	10 µM	1	1	
Forward primer	10 µM	1	1	
DNA template	196 ng/µL (*Sus scrofa*) and 100 ng/µL (*Bos taurus*)	-	1	
ddH_2O	-	-	9.5	

Data analysis

The in-silico designed primers must be validated through conventional PCR and visualized via gel electrophoresis using extracted DNA from the target species, *Bos taurus* and *Sus scrofa*. The observed sizes of the amplified products on the gel are determined by comparing it with a DNA marker.

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The in-silico predicted amplicon with experimental outcomes (e.g., size, specificity, and intensity) were compared. This process confirms whether the in-silico PCR predictions align with the results of conventional PCR, thereby verifying the effectiveness of the primer design based on the SET proto-oncogene as the template.

RESULT AND DISCUSSION

This study used the SET proto-oncogene to design primer sets targeting cattle and pig DNA sequences. A DNA biomarker using a proto-oncogene as a target was selected due to its highly conserved regions across species, alongside its polymorphism. This allows the design of primer sets applicable to multiple target species.

Effective primer hybridization to the target region significantly influences the amplification result. Qu & Zhang (2015) reported that mismatches between the primer and template DNA can impact the stability of primer hybridization. Instability in attachment, particularly in the 3' region, can lead to the formation of nonspecific amplicons. Therefore, in-silico primer design and PCR are required to optimize experimental protocols cost-effectively (Rahmasari et al., 2024). Sequence alignment was performed using Alignment by MUSCLE in MEGA 6 software for primer design. MUSCLE alignment was chosen for its higher accuracy than other algorithms, such as Clustal-Omega and MAFFT (Edgar, 2022). The alignment generated three primer candidates, as seen in Table 3.

Table 3. Characteristics of the primers designed.

No	Primer Chacateristics		Amplicon Size	
	Forward	**Reverse**	***B. taurus***	***S. scrofa***
1	Sequence (5' to 3'): TTT TGG GTA ACA ACA TTT GTT AAC CA Length: 26 %GC: 30.8% Tm: 62.5°C Hairpin **T_m**: 63.4°C, 65.7°C, 61°C, and 44°C	Sequence (5' to 3'): CTG TCT CTT CCT GCT GGC TTT ATT CT Length: 26 %GC: 46.2% T_m: 64.4°C Hairpin T_m: 49.5°C	1300 bp and 306 bp	1498 bp
2	Sequence (5’ to 3’): GTA CAA AAT GAA ATA GAC AGG TAA AG Length: 26 %GC: 30.8% T_m: 54.9°C Hairpin T_m: 25.7°C, -25.5°C, 11.6°C, and 9.2°C	Sequence (5' to 3'): ACT TAC AAA ATC TAT TCT GTA ACC TG Length: 26 %GC: 30.8% T_m: 54.6°C Hairpin T_m: 34.2°C, 30.9°C, and 28.9°C	1231 bp	1275 bp
3	Sequence (5' to 3'): CAA AGA ATT TCA TCT GAA TGA G Length: 22 %GC: 31.8% T_m: 54.2°C Hairpin T_m: 41.2°C, 37.2°C, 29.8°C, and 32.7°C	Sequence (5' to 3'): CAA ATC TTT TTA CCT CTC CTT C Length: 22 %GC: 36.4% T_m: 54.1°C Hairpin T_m: 23.2°C, -3.3°C, -3.6°C, -9.6°C and -9.4°C	1068 bp	1222 bp

The primer candidates were within the ideal range (18-30 bp). A less than 18 bp length leads to mispriming, allowing the primer to bind non-target regions and produce undesirable amplicons (Masnaini et al., 2023). A primer longer than 30 bp causes a slower hybridization rate and a lower likelihood of annealing to the targeted sequence. Furthermore, long primers tend to yield fewer amplicons due to the accumulation of by-products and the depletion of essential components for DNA synthesis in the PCR amplification process (Mubarak et al., 2020).

In addition to primer length, the melting temperature (T_m) also plays a pivotal role in PCR. The T_m indicates that primers have annealed to 50% of the target sequences at that temperature, leaving the

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other 50% unbound. The primer set should ideally have a similar T_m with a maximum difference of 2°C to achieve efficient amplification (Mubarak et al., 2020). According to Table 3, primer candidates 1, 2, and 3 have met the criteria (1.9°C, 0.3°C, and 0.1°C respectively).

Before performing PCR, the concentration and purity of the extracted DNA were assessed, with the results shown in Table 4. The extracted DNA had an A260/A280 ratio of 1.978 for *B. taurus* and 1.799 for *S. scrofa*, indicating high-quality DNA suitable for PCR (Akter et al., 2021). However, the DNA concentrations were significantly higher than the recommended range of 10–100 ng/µL for optimal PCR amplification, according to Kurniasih & Erwanto (2023).

Table 4. The purity index of DNAs extracted from *B. taurus* and *S. scrofa* liver.

Sample	Concentration (ng/µL)	A260/A280
B. taurus	936.6	1.978
S. scrofa	196.6	1.799

DNA samples from both species were used as templates for PCR using three different primer sets. Primer sets 2 and 3 successfully amplified DNA from both species, demonstrating their effectiveness in detecting conserved regions, as shown in Figure 1 below. This result aligned with the predictions from in-silico PCR. Specifically, primer set 2 produced amplicons of 1231 bp for cattle and 1275 bp for pigs, while primer set 3 amplified fragments of 1068 bp for *B. taurus* and 1222 bp for *S. scrofa*. In contrast, primer set 1 failed to amplify DNA from either species, likely due to the formation of hairpin structures with high melting temperatures (T_m).

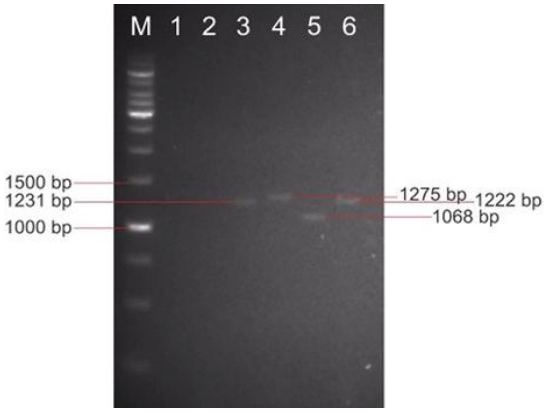

Figure 1. Electrophoresis visualization result. Lane M represents the DNA ladder. The amplification product from each primer set is shown in lanes 1 (*B. taurus*) and 2 (*S. scrofa*) by primer set 1; lanes 3 (*B. taurus*) and 4 (*S. scrofa*) by primer set 2; lane 5 (*B. taurus*) and 6 (*S. scrofa*) by primer set 3.

Nybo (2009) discussed that forming stable primer hairpin structures can significantly hinder PCR amplification. Primers with high T_m are more prone to forming such structures, preventing proper binding to the DNA template and inhibiting amplification. In this experiment, hairpin analysis using OligoAnalyzer (https://www.idtdna.com/) revealed that the forward primer in set 1 formed hairpins with dissociation temperatures of 63.4°C, 65.7°C, 61°C, and 44°C, while the reverse primer had a dissociation temperature of 49.5°C. These melting temperatures were at or above the annealing temperature (T_a), suggesting that the primers formed stable hairpin structures during annealing, preventing proper binding to the DNA template and inhibiting amplification. In contrast, primer sets 2 and 3 had hairpin melting temperatures below T_a, allowing the hairpin structures to dissociate before annealing, thereby enabling successful primer-template binding and amplification. This result aligns with Nybo's discussion on the importance of primer design in PCR efficiency. It was noted that primers should be designed to avoid stable secondary structures, such as hairpins, which can impede the amplification process.

This observation also aligns with the findings of Naqib et al. (2019), who reported that variations in primer T_m can lead to inconsistent amplification and primer failure. Their study emphasized the

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importance of adjusting primer T_m to match the optimal conditions for PCR, as mismatches in T_m can either prevent efficient binding or cause non-specific amplification. Similarly, in this experiment, the primer failure might be linked to mismatches between the primer and template DNA or an incorrect annealing temperature that led to instability in primer binding, particularly in the 3' region (Qu & Zhang, 2015). This comparison suggests carefully reevaluating primer Tm and optimizing PCR conditions are necessary to improve amplification success.

CONCLUSION

The in-silico analysis result of the SET proto-oncogene showed that this gene can distinguish pigs and other species, such as cattle, to determine halal products. The conventional PCR assay was conducted to validate the bioinformatics approach and confirmed that primer sets 2 and 3 have successfully produced different length amplicons on *B. taurus* and *S. scrofa*, proving the accuracy of T_m predictions. Thus, the developed primer pairs designed from the SET gene have the potency to analyze non-halal detection.

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