



MOLECULAR DETECTION OF *Sus scrofa* DNA USING *IGF2* GENE PRIMERS AS PROOF OF CONCEPT FOR SYBR GREEN-BASED QUALITATIVE REAL-TIME PCR

DETEKSI MOLEKULER DNA *Sus scrofa* DENGAN PRIMER GEN *IGF2* SEBAGAI KONSEP PENDUKUNG REAL-TIME PCR KUALITATIF BERBASIS SYBR GREEN

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Abstract

Detection of porcine (*Sus scrofa*) DNA is essential for halal meat authentication and requires highly specific and reproducible molecular assays. This study evaluated *insulin-like growth factor 2 (IGF2)* gene primers as a proof of concept for qualitative detection using SYBR Green-based real-time PCR. The *IGF2* locus was selected due to its interspecies sequence divergence and reported specificity to *Sus scrofa*. Qualitative analysis included *Sus scrofa* DNA, *Bos taurus* DNA as a non-target control, and a no-template control (NTC). Conventional PCR identified 58 °C as the optimal annealing temperature. In real-time PCR, *Sus scrofa* DNA was consistently detected with a mean cycle threshold (Ct) of 29.22 ± 0.11 and a low coefficient of variation (CV) of 0.38% across three technical replicates, indicating high intra-assay precision. Melting curve analysis yielded a single, well-defined peak with a melting temperature (T_m) of 80 °C, supporting the amplicon's specificity. These findings supported the development of feasible *IGF2* primers for the qualitative detection of *Sus scrofa* DNA using real-time PCR with shorter amplicons, which served as a foundation for further validation of the qPCR assay.

Keywords: Halal meat authentication; *IGF2*; Qualitative detection; Real-time PCR; *Sus scrofa*; SYBR Green

Abstrak

Deteksi DNA babi (*Sus scrofa*) merupakan aspek penting dalam autentikasi halal daging, sehingga diperlukan metode analisis molekuler yang spesifik dan andal. Penelitian ini bertujuan untuk mengevaluasi kelayakan primer gen *insulin-like growth factor 2 (IGF2)* sebagai konsep pendukung penerapannya dalam teknik real-time PCR berbasis SYBR Green. Gen *IGF2* dipilih berdasarkan variasi sekuens antarspesies yang memadai, salinan genomik rendah, dan spesifisitas tinggi terhadap DNA *Sus scrofa*. Penelitian ini menggunakan pendekatan kualitatif, dengan sampel berupa DNA *Sus scrofa* dan DNA *Bos taurus*, serta kontrol negatif. DNA diekstraksi dari daging segar dan hasil optimasi PCR menunjukkan amplifikasi paling spesifik pada suhu annealing 58 °C. Pada real-time PCR, amplifikasi DNA *Sus scrofa* terdeteksi dengan nilai cycle threshold (Ct) rata-rata $29,22 \pm 0,11$ dan koefisien variasi sebesar 0,38% pada tiga ulangan teknis, yang mengindikasikan presisi intra-assay yang tinggi. Analisis melting curve menunjukkan satu puncak leleh tunggal pada T_m 80 °C, yang mengonfirmasi spesifisitas amplifikasi. Temuan ini memungkinkan primer *IGF2* layak dikembangkan dengan ukuran amplicon yang lebih pendek untuk deteksi kualitatif DNA *Sus scrofa* melalui real-time PCR, serta dapat menjadi dasar pengembangan lebih lanjut untuk pengujian dengan teknik qPCR yang teroptimasi.

Kata Kunci: Autentikasi halal daging; Deteksi kualitatif; *IGF2*; Real-time PCR; *Sus scrofa*; SYBR Green

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INTRODUCTION

The growth of the global Muslim population has elevated halal authentication to an issue of international regulatory and commercial significance. Strengthened certification systems and increasing consumer scrutiny require analytically robust methods to ensure product integrity (Harati & Farzaneh, 2024). Among prohibited materials, porcine-derived components from *Sus scrofa* represent a persistent challenge due to their economic attractiveness as low-cost protein substitutes (Muflihah et al., 2023). In processed foods, protein degradation during thermal and mechanical treatment limits the reliability of protein-based assays, thereby positioning DNA-based detection as the preferred analytical strategy for species authentication (Cai et al., 2020; Muflihah et al., 2023). Detection of DNA from *Sus scrofa* loads using molecular approaches commonly employs the Polymerase Chain Reaction (PCR). Conventional PCR assays target species-specific genetic markers, including the *insulin-like growth factor 2 (IGF2)* gene, for qualitative identification (Hamdani et al., 2018).

The *IGF2* gene is a key regulator of somatic growth and cellular differentiation, particularly in muscle tissue, making it a biologically relevant and conserved genomic locus (Hamdani et al., 2018). Beyond its physiological significance, *IGF2* contains interspecies sequence polymorphisms that provide discriminatory genetic signatures suitable for species authentication. These conserved yet sufficiently divergent regions enable the design of primers that selectively amplify *Sus scrofa* DNA while minimizing cross-reactivity with phylogenetically related livestock species (Baral & Rotwein, 2019). The presence of stable, species-specific sequence variation within *IGF2* supports its utility as a molecular marker in DNA-based halal authentication systems (Amaniyah et al., 2023; Ampaporn et al., 2023). By targeting conserved coding regions flanked by discriminatory nucleotide differences, *IGF2*-based assays can achieve high analytical specificity while maintaining amplification reliability. This balance between evolutionary conservation and interspecies divergence underpins the rationale for selecting *IGF2* as a target locus for *Sus scrofa* detection in meat products.

Conventional PCR-based molecular assays are well-established for species identification due to their high specificity and analytical robustness (Yang et al., 2022). However, conventional PCR relies on post-amplification analysis, typically through gel electrophoresis, which limits throughput, delays result interpretation, and increases the risk of contamination during post-PCR handling (Emiru et al., 2019). These limitations constrain its suitability for high-integrity diagnostic applications. Real-time PCR (quantitative PCR, qPCR) overcomes these drawbacks by enabling continuous monitoring of DNA amplification through fluorescence-based detection, commonly using intercalating dyes (Artika et al., 2022). The closed-tube format reduces contamination risk, shortens turnaround time, and allows quantitative assessment of amplification kinetics (Ahmadi et al., 2025). Accordingly, qPCR has emerged as the preferred platform for sensitive, reproducible, and diagnostically reliable DNA-based species authentication.

Real-time PCR data were analyzed using the cycle threshold (Ct), also referred to as the quantification cycle (Cq), in accordance with MIQE terminology, and the coefficient of variation (CV). The Cq value is defined as the PCR cycle at which the fluorescence signal surpasses a predefined threshold above background noise and is inversely proportional to the initial quantity of target nucleic acid (Adams, 2020). Amplification kinetics progress through lag, exponential, and plateau phases; quantitative interpretation is derived from the exponential phase, during which amplification efficiency approaches 100%, and the target DNA theoretically doubles with each cycle (Ruiz-Villalba et al., 2017). Assay precision was assessed by calculating the CV, defined as the ratio of the standard deviation (SD) to the mean Ct value across technical replicates (Forootan et al., 2017). Lower CV values indicate higher repeatability and technical consistency and are commonly used to evaluate intra-assay and inter-assay variability in quantitative real-time PCR workflows (Vishnuraj et al., 2023).

Accordingly, this study evaluates the analytical performance of an *IGF2*-targeted assay on a real-time PCR platform using a 547 bp amplicon validated in conventional PCR. By assessing the feasibility of qualitative detection of *Sus scrofa* DNA, this study provides proof of concept for adapting conventional PCR targets to real-time systems. This study addresses a preliminary

framework for the development of validated qPCR assays for halal authentication, with emphasis on specificity, reproducibility, and analytical transparency.

MATERIAL AND METHODS

Primer Preparation

The primers used in this study were based on the design reported by Hamdani et al. (2018), targeting the mRNA sequence of the *IGF2* gene of *Sus scrofa*. Reference sequences for *Sus scrofa* and *Bos taurus* were retrieved from the National Center for Biotechnology Information (NCBI) database. Transcript structure analysis and multiple sequence alignment were performed using SnapGene (GSL Biotech LLC, USA) and Clustal Omega to identify species-specific polymorphic regions within the coding sequence (CDS). Regions exhibiting interspecies variation were selected as candidate targets for primer binding to enhance species discrimination.

Primer pairs were designed using Primer3 software, following standard parameters for primer length, GC content, melting temperature, and amplicon size. Primer specificity was first evaluated by silico PCR using the UCSC Genome Browser against the *Sus scrofa* and *Bos taurus* genomes. Further validation was conducted using Primer-BLAST (NCBI) to assess potential off-target amplification against non-target species. Primer sets predicted to amplify the *Sus scrofa* genome exclusively were selected as candidate PCR-based DNA markers for halal authentication. Primers were synthesized by Integrated DNA Technologies (IDT), Singapore. The sequences of the forward and reverse primers are provided in Table 1.

Table 1. The *insulin-like growth factor 2 (IGF2)* primers

Identity	Sequences	Gene ID	Size
Forward	AAGTCCGAGAGGGACGTGT	396916 (<i>Sus scrofa</i>)	547 bp
Reverse	AATTTGGCTCACTTCCGATG		

DNA Extraction

Genomic DNA was extracted from pork (*Sus scrofa*) and beef (*Bos taurus*) samples using the Quick-DNA™ Miniprep Plus Kit (Zymo Research, USA) according to the manufacturer's protocol. Meat samples were purchased separately from Ciputat Market, South Tangerang, Banten, Indonesia, and processed independently to minimize cross-contamination. *Sus scrofa* DNA used as a positive control was obtained from the collection of the Faculty of Medicine, UIN Syarif Hidayatullah Jakarta. DNA quantification method for concentration and purity was determined using a UV-Vis spectrophotometer (GeneQuant Pro, UK) by measuring its absorbance. Briefly, 10 µL of the DNA sample was diluted with nuclease-free water to a final volume of 350 µL and transferred into a microvolume cuvette. Absorbance was measured at 260 and 280 nm. Extracted DNA was stored at -20 °C until analysis.

DNA Amplification (Conventional PCR)

PCR amplification was performed using GoTaq® Green Master Mix (Promega, USA) in a total reaction volume of 25 µL, containing 12.5 µL master mix, 0.5 µL each of forward and reverse primers (10 pmol/µL), 1 µL DNA template, and 10.5 µL nuclease-free water (NFW). All reactions included a positive control (*Sus scrofa* DNA), a non-target control (*Bos taurus* DNA), and a no-template control (NTC) to monitor contamination and non-specific amplification. PCR reactions were carried out in a SimpliAmp™ Thermal Cycler (Applied Biosystems, Thermo Fisher Scientific, USA). Annealing temperatures were optimized at 51, 52, 53, 54, 57, and 58 °C. The thermal cycling conditions consisted of an initial denaturation at 95 °C for 2 min; 35 cycles of denaturation at 95 °C for 30 s, annealing at the respective temperature (51–58 °C) for 30 s, and extension at 72 °C for 30 s; followed by a final extension at 72 °C for 5 min and a hold at 4 °C. PCR products were stored at 4 °C before electrophoretic analysis. All amplification experiments were performed in triplicate to assess reproducibility.

Agarose Gel Electrophoresis

PCR products (amplicons of *Sus scrofa* DNA) were analyzed by 1.5% (w/v) agarose gel electrophoresis prepared in 1× TAE buffer. Agarose (1.5 g) was dissolved in 100 mL of 1× TAE buffer, heated until fully dissolved, cooled to 50–60 °C, and stained with 1 µL of Hi-SyBr Safe Gel Stain (Himedia, India). The gel was cast with a comb and allowed to solidify at room temperature. Electrophoresis was performed in 1× TAE buffer at 100 V for 40 min. PCR products mixed with loading dye were loaded into the wells, and a 1 kb DNA ladder (GeneRuler, Thermo Scientific, USA) was used as a molecular size marker. DNA bands were visualized under UV illumination and documented using a gel documentation system. Fragment sizes were estimated by comparison with the DNA ladder.

Real-time PCR (qPCR)

Real-time PCR was performed using InnuMIX® qPCR DSGreen Standard (IST Innuscreen GmbH, Germany) in a total reaction volume of 20 µL. Each reaction consisted of 10 µL InnuMIX qPCR DSGreen Standard, 2 µL forward primer [10 µM], 2 µL reverse primer (10 µM), 5 µL DNA template, and 1 µL NFW. Reactions were homogenized by vortexing, briefly centrifuged, and run on an RD-96 Real-Time PCR System (ReiGed-Diagnostic, Numedika, Netherlands). The cycling conditions included an initial denaturation at 95 °C for 120 s, followed by 40 cycles of denaturation at 95 °C for 20 s and annealing at 58 °C for 50 s, with fluorescence acquisition during the annealing step. Each sample (*Sus scrofa* DNA) was analyzed in three technical replicates. Positive (*Sus scrofa* DNA), non-target (*Bos taurus* DNA), and NTC were included in every run. A melting curve analysis was performed immediately after amplification by gradually increasing the temperature from 65 °C to 95 °C to verify amplification specificity. Samples were considered positive when the Ct value was <30 and the melting temperature (T_m) was 80 ± 1.0 °C with a single, well-defined peak, indicating the absence of primer-dimer formation or non-specific products.

RESULTS

The spectrophotometric measurement of *Sus scrofa* DNA extracted from meat samples showed a concentration of 88 ng/µL with an A₂₆₀/A₂₈₀ purity ratio of 1.895, indicating acceptable DNA quality for downstream PCR analysis. Visualization of *IGF2* amplicons by 1.5% agarose gel electrophoresis revealed distinct DNA bands in both the molecular marker (M) and the amplified samples. The GeneRuler 1 kb DNA Ladder showed clear separation of fragments ranging from 250 bp to 10,000 bp (Figure 1a). A specific DNA band corresponding to the expected *IGF2* amplicon size (547 bp) was detected at annealing temperatures of 53; 54; 57; and 58 °C. Among these, the band obtained at 58 °C exhibited the highest clarity and specificity compared to other annealing temperatures. No amplification was observed in the negative control (K-), confirming the absence of contamination (Figure 1b).

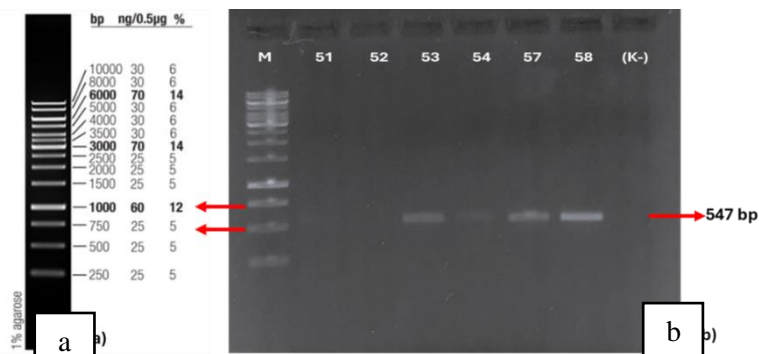


Figure 1. GeneRuler 1 kb DNA ladder (a) and PCR products separated on 1.5% agarose gel (100 V, 40 min) (b). M= DNA marker; 53–58= annealing temperatures (°C); K-= negative control. The *IGF2* amplicon (547 bp) is located between the 500 bp and 750 bp bands (red arrow, right panel)

Real-time PCR analysis of *Sus scrofa* DNA (88 ng/μL), performed in three technical replicates, yielded a mean Ct value of 29.30 ± 0.11 (SD) (Ct value of 29.22 in Figure 2). The coefficient of variation (CV) was 0.38%, calculated using the formula $CV (\%) = (SD/\text{mean}) \times 100$, indicating high intra-assay precision. According to the RD-96 PCR system criteria, the sample was classified as positive for *Sus scrofa* DNA. In contrast, beef (*Bos taurus*) samples and the NTC showed no amplification and were reported as negative. Melting curve analysis demonstrated a single peak with a melting temperature (T_m) of 80 °C (Figure 3), supporting the specificity of the amplified product.

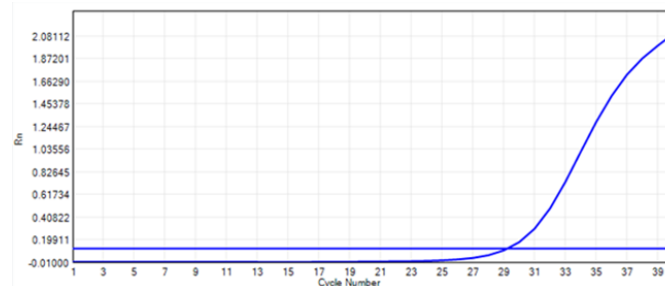


Figure 2. Real-time PCR amplification using *IGF2* primers on samples containing *Sus scrofa* DNA showed a Ct value of 29.22, as detected by the RD-96 real-time PCR instrument

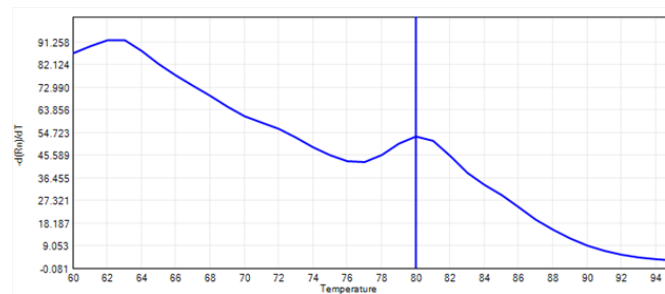


Figure 3. Real-time PCR melt-curve analysis using *IGF2* primers on samples containing *Sus scrofa* DNA demonstrated a melting temperature (T_m) peak at 80 °C, as detected by the RD-96 PCR system

DISCUSSION

The detection of a discrete 547 bp band corresponding to the *IGF2* amplicon confirms successful amplification of *Sus scrofa* DNA and is consistent with previous reports by Hamdani et al. (2018). The absence of amplification in NTC demonstrated procedural integrity and indicated that contamination did not contribute to signal generation, as emphasized in comparable assay validation studies (Anissa et al., 2023). Thermal optimization revealed that an annealing temperature of 58 °C (Figure 1) maximized amplification stringency, as evidenced by increased band sharpness and intensity relative to lower temperatures. At lower annealing temperatures (53–54 °C), reduced band sharpness likely reflects decreased binding specificity and increased tolerance for primer-template mismatches, a phenomenon previously described in PCR thermodynamic analyses by Kahsen et al. (2024). The empirical optimum closely matched the *in silico*-predicted primer melting temperature, indicating congruence between computational design parameters and experimental performance. This thermodynamic alignment supports the structural robustness of primer-target interactions and justifies the direct transfer of optimized conditions to the real-time PCR platform.

In the real-time assay, *Sus scrofa* DNA (88 ng/μL) was detected with a mean Ct of 29.22 and an SD of 0.11 across three technical replicates ($CV = 0.38\%$), indicating high intra-assay precision and stable amplification kinetics. In molecular diagnostic contexts, CV values below 1% are widely considered indicative of strong technical reproducibility for qualitative assays (Doleschall et al., 2022). While the Ct value reflects moderate amplification efficiency (Figure 2), likely attributable to the relatively long amplicon length (547 bp), which can reduce polymerase processivity and reaction efficiency compared with shorter targets (Van Holm et al., 2021), detection remained robust and

reproducible. Crucially, amplification was absent in *Bos taurus* and NTC, supporting analytical specificity and exclusion of cross-species reactivity under the tested conditions.

Melting curve analysis yielded a single peak at T_m 80 °C (Figure 3), confirming homogeneous amplicon formation. Although *in silico* thermodynamic modeling predicted a higher theoretical T_m (94–96 °C), such deviations are expected due to differences between idealized sequence-based calculations and empirical reaction conditions, including ionic strength, dye intercalation effects, and instrument-specific calibration factors (Carvalho et al., 2024; Collatz et al., 2025). Importantly, in SYBR Green-based diagnostics, specificity is determined by the presence of a single reproducible melting transition rather than absolute concordance with theoretical T_m values. The absence of secondary peaks or shoulder formations indicates minimal primer-dimer formation and supports assay analytical specificity.

Concordance between endpoint PCR band intensity and real-time fluorescence kinetics further substantiates assay robustness. The annealing condition yielding maximal endpoint product intensity (58 °C) corresponded with stable C_t values and low inter-replicate variability in real-time amplification, demonstrating cross-platform consistency. This agreement strengthens confidence in primer performance and indicates that amplification behavior is governed by reproducible thermodynamic parameters rather than stochastic variability.

From a diagnostic systems perspective, migration from conventional PCR to a closed-tube real-time format confers substantial analytical advantages. Real-time PCR minimizes contamination risk by eliminating post-amplification handling (Sidstedt et al., 2020), enhances analytical sensitivity and dynamic detection range (Kralik & Ricchi, 2017), and enables scalable throughput compatible with high-volume screening environments (Artuvan & Aksay, 2022). These attributes are particularly relevant for food authentication workflows requiring reproducibility, specificity, and operational efficiency.

Collectively, the *IGF2* primer set demonstrated stable thermodynamic behavior, reproducible amplification kinetics, and analytical consistency across platforms. This study provided a technically validated proof of concept and a rational platform for further assay refinement, including amplicon shortening, efficiency characterization, and formal analytical validation for diagnostic-grade halal authentication applications. By developing *IGF2* primers for amplicon shortening (± 200 bp), this study can be further optimized through efficiency evaluation, extended exclusivity testing, limit of detection analysis, and interlaboratory reproducibility studies to achieve full qPCR validation.

CONCLUSION

The *IGF2* primer set (547 bp) demonstrated strong potential for adaptation to a SYBR Green-based real-time PCR platform with shortened amplicons (± 200 bp) for the qualitative detection of *Sus scrofa* DNA. This approach established a fundamental methodological framework for a more robust, optimized, and analytically validated qPCR system in molecular-based halal authentication of meat.

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