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Research Paper

Development of a Gelatin-Based Genomic Reference Material for Halal Authentication Using Real-Time PCR

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Abstract
Indonesia, home to over 270 million people, has the largest Muslim population globally, with approximately 87.18% adhering to Islam, driving significant demand for halal products, particularly in the food and pharmaceutical sectors. Gelatin, commonly used in medicinal capsules, often originates from porcine sources, necessitating precise halal authentication methods. This study presents the development of a novel genomic DNA-based Reference Material (RM) for gelatin, specifically for porcine DNA detection, employing Real-Time Polymerase Chain Reaction (qPCR) techniques. The methodology encompassed in-silico primer design, sample extraction optimization, DNA quality and quantity analysis, linearity assessment, limit of detection (LoD) and quantification (LoQ) determination, and RM characterization. Results indicated that the designed primers could reliably and efficiently detect porcine DNA, with optimal annealing at 58°C and primer concentration at 500 nM, achieving a PCR efficiency of 96.74%. The LoD and LoQ for pork meat samples were determined to be 0.02 pg/μL and 0.004 pg/μL, respectively, while the LoD for porcine gelatin was 0.27 ng/μL. The RMs exhibited robust homogeneity (Sig. 0.052), significant intergroup differences (Sig. 0.000), and low variation (CV 0.96%). Short-term storage at -80°C and -20°C preserved Ct value stability and consistency. Conclusively, this study successfully developed a novel gelatin-based genomic DNA RM for halal authentication, offering a scientifically validated tool that strengthens the halal assurance system, addressing Indonesian consumers' demand for porcine-free products. These findings hold substantial implications for regulatory authorities, especially in Indonesia, and could inform the development of standardized qPCR RMs for porcine DNA detection in halal compliance testing.

Keywords
Halal Authentication, Reference Material (RM), Genomic DNA, Gelatin, qPCR, Pharmaceutical Products

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1. INTRODUCTION

Indonesia, the world's fourth most populous nation with over 270 million inhabitants, has approximately 87.18% of its population adhering to Islam (Ananta et al., 2015). This demographic creates a substantial demand for halal products, particularly in the food and pharmaceutical sectors. Recent estimates from the State of the Global Islamic Economy Report (2022) suggest that halal product consumption in Indonesia alone has reached $146.7 billion. In response to these needs, the Indonesian government enacted Law Number 33 of 2014 on Halal Product Assurance (UUJPH), underscoring the significance of halal compliance within the Muslim community (Purwanto et al., 2021). As Muslim consumers increasingly seek assurance regarding the authenticity and integrity of halal products, the demand for precise identification methods and verification of halal-certified goods has grown (Cheman, 2005). Despite regulatory frameworks and the recognition of Indonesian halal certification in multiple countries, standardized methods for porcine DNA testing within the national context remain undeveloped.

In the pharmaceutical industry, gelatin plays a crucial role as a key ingredient in the production of soft and hard capsule shells, tablet coatings, and microencapsulation processes (Al-Nimry et al., 2021). Derived from animal collagen through partial hydrolysis, gelatin is widely used due to its ability to prevent oxidation, enhance drug acceptability, and improve palatability (Hassan et al., 2018; Nikzad et al., 2017; Nurilmala et al., 2021). The primary sources of gelatin include porcine, bovine, avian, and fish species (Corliss et al., 1977; Jones, 2004), with porcine-derived gelatin often being preferred for its cost-effectiveness (Sultana et al., 2020). Given the significant halal concerns surrounding porcine gelatin, its

detection in pharmaceutical products remains a critical area of research and regulation.

The detection of porcine DNA in gelatin products, especially those used in drug capsules, can be achieved through nucleic acid amplification techniques such as Polymerase Chain Reaction (PCR) (Al-Kahtani et al., 2017; Cai et al., 2012; Erwanto, 2018; Kim et al., 2016; Cai et al., 2012; Perestam et al., 2017; Soares et al., 2013; Vallejo et al., 2021). Among these, the Real-Time PCR (qPCR) method offers significant advantages over conventional PCR (cPCR), notably in terms of detection accuracy during the exponential phase of each cycle, unlike cPCR, which relies on the less precise plateau phase for detection (Karimi et al., 2020). Furthermore, qPCR integrates a thermal cycler with a fluorescent detector for target quantification (Raso and Biassoni, 2014), making it particularly effective for detecting porcine DNA in purified gelatin and capsule products (Xie et al., 2022). DNA-based detection methods offer superior specificity and sensitivity compared to protein-based approaches, as DNA is more stable and allows for the precise identification of species using specific primers (Chen et al., 2004; Lanzilao et al., 2005; Sentandreu and Sentandreu, 2014). In this study, qPCR analysis focuses on mitochondrial DNA, which, due to its high copy number, is ideal for analyses involving limited or degraded DNA, as commonly seen in gelatin products (Kesmen et al., 2009; Ni'Mah et al., 2016).

To ensure accurate test results, address potential systematic errors, and validate the qPCR-based detection of porcine DNA, the availability of Reference Materials (RMs) is essential. RMs serve to maintain measurement consistency, validate analytical methods, and confirm the metrological reliability of instrumentation (International Organization for Standardization (ISO), 2017; Vallejo et al., 2021). Despite the critical role of RMs, there is a noticeable gap in the availability of gelatin genomic DNA-based RMs, particularly in halal authentication contexts. Previous research has predominantly focused on RMs related to the quantification of Genetically Modified Organisms (GMOs) using transgenic homozygous plant materials (Li et al., 2020, 2024).

This study is pioneering in its development of gelatin genomic DNA-based RMs for the halal authentication of porcine DNA in pharmaceutical capsules using qPCR. By establishing these RMs, this research addresses a critical gap and provides a reliable internal control for accurate and trustworthy halal testing through nucleic acid amplification methods. The successful implementation of such RMs could enhance the halal assurance system and provide a valuable scientific basis for regulatory authorities to adopt standardized qPCR RMs in porcine DNA detection.

2. EXPERIMENTAL SECTION

2.1 Materials

The materials used in this study include gelatin powder (porcine, bovine) (Sigma Aldrich®, USA), meat (porcine, chicken, bovine, unbranded), gSYNC™ DNA Extraction Kit (Geneaid, China), 6× DNA gel loading dye (Geneaid, China), Agarose Gel (Vivantis, Malaysia), THUNDERBIRD Next SYBR® qPCR Mix 1 × 1 mL Master Mix (Toyobo, Japan), Cytochrome Beta Forward and Reverse primers (IDT, USA), GelRed (Biotium, USA), 1kb DNA ladder (Geneaid, China), TE buffer (Himedia, India), Nuclease-Free Water (NFW) (Himedia, India), TAE buffer (Himedia, India), Kimwivest (Kimtech, USA), and other consumables (microtubes, micropipette tips, parafilm, tissue).

2.2 Procedures

2.2.1 *In-Silico* Primer Analysis

Primer Design

The primers were designed in silico using the SnapGene software (Dotmatics, USA) (Nguyen et al., 2023). The mitochondrial DNA reference for Sus scrofa used is GenBank accession number: ON060839.1 (Kang et al., 2018).

Primer Quality Analysis

The specificity of the primers was analyzed using the NCBI BLAST (Basic Local Alignment Tool) software accessible at (https://www.premierbiosoft.com/netprimer/). The analysis includes the evaluation of primer length, melting temperature, GC%, and hairpin and cross-dimer structures. The primers were designed to meet the desired quality standards (Rodríguez et al., 2015).

Primer Specificity Analysis

The specificity of the primers was analyzed using the NCBI BLAST (Basic Local Alignment Tool) software accessible at https://www.ncbi.nlm.nih.gov/. MSA was conducted using Mega 11 software, accessible at https://www.megasoftware.net/. The primer specificity analysis ensures that the primers bind to and amplify only the target DNA sequences, avoiding non-specific amplification (Sabina and Leamon, 2015).

2.2.2 DNA Extraction from Samples

DNA extraction was performed following the standard protocol of the gSYNC™ DNA Extraction Kit (Geneaid, China) with modifications to sample weight and kit fluid volume. The concentration and purity of the extracted DNA were measured using a Nanodrop EPOCH2 (BioTek, USA). Readings were taken in duplicate using the elution buffer from the gSYNC™ DNA Extraction Kit (Geneaid, China) as a blank (Zehra and Kaur, 2023).

2.2.3 Primer Annealing Optimization

Primer annealing temperature optimization was performed using the CFX Deep Well Real-Time PCR System (BioRad, USA). The designed primers, PF_Gematin and PR_Gematin, were used as markers. A total of 16 reactions with 2 replicates for on-target samples (pork meat) at each gradient temperature, duplicates for out-of-target samples (chicken and beef), and 2 reactions of Non-Template Control (NTC) without DNA were tested. The gradient temperatures tested were 54°C, 57.9°C, 62.3°C, 63.5°C, and 64°C using a concentration of 500 nM (Tan et al., 2020; Tasrip et al., 2021). The amplification procedure began with an initial pre-denaturation phase at 95°C

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for 30 seconds. Subsequently, 40 cycles were performed, each consisting of denaturation at 95°C for 5 seconds and gradient temperatures. This was immediately followed by a melting curve analysis. Amplification and melt curve data were processed and interpreted using the Bio-Rad CFX Maestro V2.0 software (Bio-Rad, USA).

2.2.4 Primer Concentration Optimization

Primer concentration optimization was conducted using the CFX Deep Well Real-Time PCR System (BioRad, USA) with primers PF_Gematin and PR_Gematin as markers. A total of 20 reactions were carried out with 2 duplicates at each tested concentration (100 nM, 250 nM, 500 nM, 700 nM, and 900 nM), and NTC (No Template Control) also in duplicates without DNA addition (Guo et al., 2020). Each of concentration was tested in triplicate, totaling 18 reactions, and 2 reactions were conducted for the NTC. The amplification procedure began with an initial pre-denaturation phase at 95°C for 30 seconds. Subsequently, 40 cycles were performed, each consisting of denaturation at 95°C for 5 seconds and annealing/extension at 58°C for 5 seconds. This was immediately followed by a melting curve analysis. Amplification and melt curve data were processed and interpreted using the Bio-Rad CFX Maestro V2.0 software (Bio-Rad, USA).

2.2.5 qPCR Efficiency Test

The efficiency test for primer reactions was conducted using a serial dilution of samples (Svec et al., 2015), with concentrations of 2×10^2; 2×10^1; 2×10^0; 2×10^{-1}; 2×10^{-2}; 2×10^{-3} ng/μl. Dilutions were prepared using 1× TE Buffer (Mokhtar et al., 2024). Each dilution point was tested in triplicate, totaling 18 reactions, and 2 reactions were conducted for the NTC. The six DNA dilutions were then used as DNA templates with the optimized Real-Time PCR protocol (Svec et al., 2015). After running, the data were analyzed to obtain a standard curve with the regression equation $y = ax + b$, where 'y' is the Ct value, 'x' is the log10 of the copy number of the sample, and 'b' is the intercept of the regression line (Oh and Jang, 2020). Linear regression analysis was performed using Microsoft Excel (Microsoft Corp, USA). With the slope of the regression line, the efficiency of the Real-Time PCR assay was calculated using the formula: $Efficiencyreaction(E) = 10^{-\frac{1}{slope}} - 1$. The slope of the regression curve should be between -3.9 and -2.9 for an efficiency of 90%-110%, and the regression coefficient R^2 should be ≥ 0.98 to indicate good efficiency (Broeders et al., 2014).

2.2.6 Evaluation of Limit of Detection and Limit of Quantification (LoD and LoQ)

LoD and LoQ tests were performed with meat and gelatin samples (Masiri et al., 2016). For the meat samples, 10× dilutions were made at 5 points and 5× at 1 point, resulting in sample concentrations of 2×10^2; 2×10^1; 2×10^0; 2×10^{-1}; 2×10^{-1}; 2×10^{-3}; 2×10^{-4}; and 4×10^{-5} ng/μL. For gelatin samples, 10×, 5×, and 2× dilutions were made, resulting in concentrations of 27; 27×10^{-1}; 54×10^{-2}; and 27×10^{-2} ng/μL. The tests were conducted using the same equipment and under the same conditions as the meat samples. The tests were conducted with the CFX Deep Well Real-Time PCR System (BioRad, USA) in triplicate for each concentration and duplicate NTCs as negative controls (K-), using the optimized amplification protocol. Data were analyzed using CFX Maestro v2 software.

2.2.7 RMs Characterization Tests

Homogeneity Test

The DNA extracted from porcine gelatin samples was homogenized, and the DNA concentration was checked using NanoDrop EPOCH2 (BioTek, USA). Samples of 50 μl were placed into 10 random 1.5 ml tubes and tested according to Hikmah et al. (2024), International Organization for Standardization (ISO) (2017), and Khayyira et al. (2018). Homogeneity was tested using Real-Time PCR with the CFX96 Touch (Bio-Rad, USA), following the optimized and validated protocol. Data analysis was based on Ct (Threshold Cycle) values and the coefficient of variation. Samples were considered homogeneous if the statistical confidence level was > 95% (Noguchi et al., 2016; Romano et al., 2017).

Short-term Storage Stability Test The stability test was conducted following to International Organization for Standardization (ISO) (2017) and Niu et al. (2022). DNA extracted from porcine gelatin samples was stored at 37°C, 4°C, -20°C, and -80°C for short-term stability evaluation over two weeks, with sampling on days 1, 3, 7, and 13 International Organization for Standardization (ISO) (2017). DNA stability was tested using the Real-Time PCR CFX96 Touch (Bio-Rad, USA), focusing on short-term stability as per previous studies.

2.2.8 Data Analysis

Data analysis was performed using CFX Maestro 2.2 (Bio-Rad, USA) for running qPCR, including amplification curve analysis, melting curve analysis, and Ct values. Microsoft Excel (Microsoft Corp, USA) was used for linearity and reaction efficiency tests. Sample homogeneity was tested with IBM SPSS Statistics v20 (IBM, USA) using Levene's Test and One-way ANOVA. Short-term stability tests were analyzed with GraphPad Prism 9 (GraphPad Software, Inc., USA) for evaluating various storage temperatures over two weeks.

3. RESULT AND DISCUSSION

3.1 *In-Silico* Primer Design and Analysis

The design and in-silico analysis of primers aim to ensure that the primers employed can specifically and efficiently recognize and amplify the target DNA sequences (Kumar and Chordia, 2015). This process encompasses several key steps: the design of primers using SnapGene software, the analysis of primer quality with NetPrimer, and the assessment of primer specificity using Nucleotide-BLAST from NCBI. Each of these steps plays a crucial role in ensuring that the designed primers

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not only align with the target sequence but also meet the necessary quality and specificity standards required for experimental validity (Bustin and Huggett, 2017).

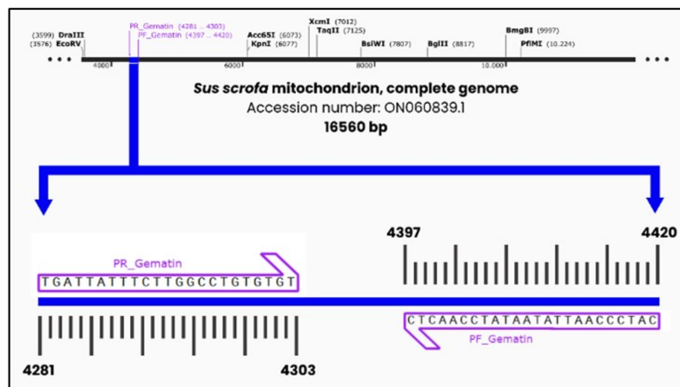

Figure 1. Annealing of Forward and Reverse Primers with a 140 bp Amplicon on *Sus scrofa* Mitochondrion

The primer design was conducted *in-silico* using SnapGene software, referencing the mitochondrial genome sequence of Sus scrofa (GenBank accession number: ON060839.1). The forward primer (PF_Gematin) anneals at positions 4397-4420, while the reverse primer (PR_Gematin) anneals at positions 4281-4303, producing an amplicon of 140 bp. This design demonstrates that the primers exhibit high specificity and accurately align with the target sequence (Nguyen et al., 2023). The mapping of the forward primer (PF_Gematin) and reverse primer (PR_Gematin) binding sites on the mitochondrial DNA reference sequence of Sus scrofa is presented in Figure 1. The forward primer anneals at positions 4397-4420, while the reverse primer anneals at positions 4281-4303.

The primer sequences are presented in Table 1. The results indicate that the forward primer is 24 bases long, while the reverse primer is 23 bases long. The amplicon length produced by these primers is 140 base pairs. This information is crucial to ensure that the designed primers have appropriate lengths for efficient and specific amplification.

Primer Quality Analysis

The quality of the primers was evaluated using NetPrimer software, accessible at `https://www.premierbiosoft.com/netprimer/`. This analysis aimed to assess the quality of the designed primers, examine their properties, and evaluate their binding efficiency to the DNA template (Rodríguez et al., 2015).

The evaluation of primer quality in genetic and molecular biology research focuses on a series of critical parameters that influence the efficacy and specificity of Polymerase Chain Reaction (PCR) reactions. These parameters include primer length, GC content, overall primer quality rating, melting temperature (Tm), Tm variation between primers, annealing temperature (Ta), Ta variation, as well as the potential formation of hairpins, self-dimers, and cross-dimers. The requirements for these parameters are detailed in Table 2.

The primer design results obtained in this study show conformity with the ideal primer length criteria, which ranges between 18-24 nucleotides, as suggested by Hung and Weng (2016) The forward and reverse primers have lengths of 24 and 23 nucleotides, respectively, which fall within the recommended range, thereby supporting efficient binding at the annealing temperature (Borah, 2015). Additionally, the Tm values for both primers, PF_Gematin (55.16°C) and PR_Gematin (59.15°C), are consistent with a Tm variation not exceeding 5°C, aligning with the standards established by Asif et al. (2021). Although the GC content of PF_Gematin (33.33%) and PR_Gematin (39.19%) is lower than the ideal range of 40-60% (Green and Sambrook, 2020), this can still be optimized through adjustments to the PCR parameters in the laboratory. Additionally, the designed primers do not exhibit significant hairpin or self-dimer formation, which could otherwise interfere with PCR efficiency (Chen et al., 2017; Nazarenko, 2002). The cross-dimer analysis also indicated a value of -6.97 kcal/mol, meeting the criteria established by Zhao (2014), thereby suggesting that it is unlikely to impede the amplification process. Overall, this primer design demonstrates significant potential for use in efficient and specific PCR reactions.

Primer Specificity Analysis

The primer specificity test aims to identify potential nonspecific amplification, ensuring accurate amplification of the target to avoid false results that could compromise the validity of the research (Sabina and Leamon, 2015). The types of target and non-target samples are presented in Table 3. The results of the *in-silico* specificity analysis via Multiple Sequence Alignment (MSA) are shown in Figure 2.

The MSA results of the specificity test demonstrate the primer PF_Gematin's specificity across various species, including pig (*Sus scrofa* OQ689020), rat (*Rattus norvegicus* NC 001665.2), rabbit (*Oryctolagus cuniculus* NC 001913.1), cow (*Bos taurus* NC 006853.1), catfish (*Clarias batrachus* NC_023923 .1), pangasius (*Pangasianodon hypophthalmus* NC_021752.1), chicken (*Gallus gallus* NC_053523.1), duck (*Anas platyrhynchos* NC_009684.1), and wolf (*Canis lupus familiaris* NC 008092.1). The PF_Gematin primer exhibits high specificity towards pig (*Sus scrofa*), with no significant matches to other species (Figure 2). Similar results were observed for the PR_Gematin primer, consistent with previous studies conducted by Ali et al. (2015) and Shuhaimi and Sam-on (2022) (Figure 2). Pork and its derivatives are often mixed with gelatin or other adulterants in food products or halal goods, making accurate detection crucial Zia et al. (2020). These results confirm that the *in-silico* primer design successfully yielded a primer specific for amplifying the target gene in pig species (*Sus scrofa*) (Sabina and Leamon, 2015).

3.2 Genomic DNA Extraction

The optimization of the sample extraction protocol was performed to ensure that the obtained DNA is of high quality and sufficient for analysis. This process involves optimizing sample weight to yield DNA with optimal purity and concentration, as well as adjusting the extraction protocol to align with the

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Table 1. Oligonucleotide Primers

Primer Name	Oligonucleotide Sequence (5' → 3')	Primer Length (bp)	Amplicon Size (bp)
PF_Gematin	GAGTTGGATATTATAATTGGGATG	24	140
PR_Gematin	TGATTATTTCTTGGCCTGTGTGT	23	

Table 2. Primer Quality Analysis Results Using the NetPrimer website, Including Primer Length, GC%, Melting Temperature (Tm), Tm Difference, and Secondary Structure Analysis.

	Standard	Forward		Reverse		Reference
Sequence	-	GAGTTGGAT ATTATAATT GGGATG		TGATTAT TTCTTGGC CTGTGTGT		-
Primer length	18-24 base pairs	24 base	✓	23 base	✓	(Hung and Weng, 2016)
GC%	45-65%	33.33%	×	39.13%	×	(Browne et al., 2020)
Temperature Melting/Tm	42-65°C	55.16°C	✓	59.15°C	✓	(Masnaini et al., 2023)
Tm Difference	≤5°C	3.99	✓	3.99	✓	(Asif et al., 2021)
Hairpins	$\Delta G \geq -3$ kcal/mol	-	✓	-	✓	(Zhao, 2014)
Sa	$\Delta G \leq -6$ kcal/mol	-10.23 kcal/mol	×	-9.28 kcal/mol	×	(Sasmito et al., 2014)
Cross dimer	$\Delta G \leq -6$ kcal/mol	- 5.85 kcal/mol			✓	(Zhao, 2014)

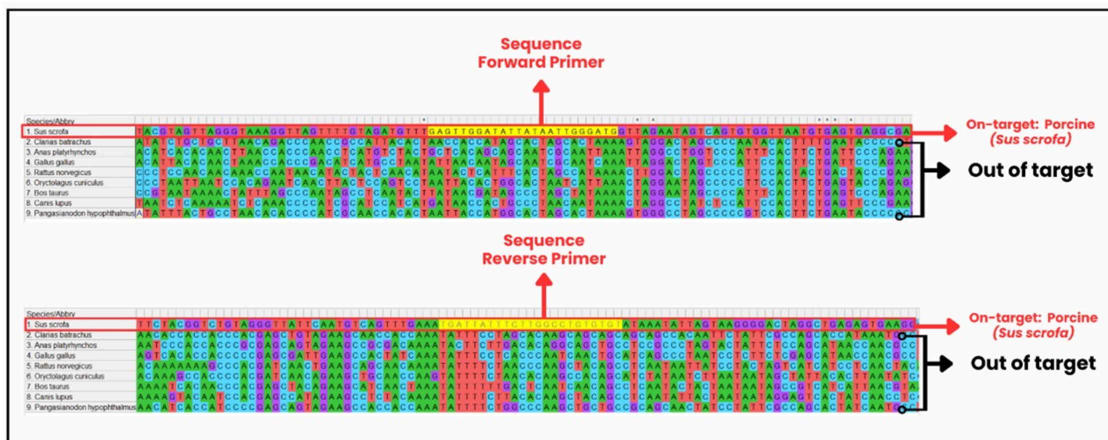

Figure 2. Specificity Analysis Results of PF & PR_Gematin Using BLAST Nucleotide Multiple Sequence Alignment (MSA) on Mega11

standards of the used kit. These steps ensure efficient DNA extraction and consistent results across various sample types (Nikzad et al., 2017).

Optimizing sample weight during extraction is a crucial step in ensuring the efficiency and accuracy of DNA analysis. The results indicate that the optimal sample weight for meat is 50 mg (Table 4). Although a 100 mg sample yields a higher DNA concentration (56.860 ng/μl), the DNA purity at 50 mg (191.81 ng/μl with a purity ratio of 2.117) better meets the desired purity standard of 1.8–2.0, as outlined by Green and Sambrook (2020). This high purity is crucial for ensuring DNA quality without contamination from proteins or other substances, which is essential for accurate analysis (Manen et al., 2005; Rohland and Hofreiter, 2007).

For the gelatin samples, the optimal sample weight is 100 mg. A sample weight of 200 mg yields a higher DNA concentration (37.375 ng/μl) compared to a sample weight of 100 mg (22.532 ng/μl). However, a sample weight of 100 mg was chosen because it approaches the desired purity standard (purity of 1.671) while providing a sufficient concentration for adequate DNA analysis (Tan and Yiap, 2009). This sample weight selection also optimizes the efficiency of material usage and extraction time (Tretiakov et al., 2022).

DNA purity is a critical factor in ensuring valid results in molecular analysis. Pure DNA, free from protein contamination or other compounds, will yield more accurate and reproducible results in various applications, including biomolecular PCR applications (Rohland and Hofreiter, 2007). Therefore,

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Table 3. Types of Samples Used in the Primer Specificity Test

Common name	Scientific name
Pig	*Sus scrofa*
Beef	*Bos taurus*
Red snapper	*Lutjanus sebae*
Tuna	*Thunnus albacares*
Catfish	*Clarias batrachus*
Patin fish	*Pangasianodon hypophthalmus*
Chicken	*Gallus gallus*
Duck	*Anas platyrhynchos*
Wolf	*Canis lupus*
Rat	*Rattus norvegicus*
Rabbit	*Oryctolagus cuniculus*

Table 4. Sample Weights from Optimization Results

Meat Sample	Weight	Gelatin Sample	Weight
Pig	50 mg	Porcine gelatin	100 mg
Beef	50 mg	Bovine Gelatin	100 mg
Chicken	50 mg	Chicken gelatin	100 mg
Pig	100 mg	Porcine gelatin	200 mg
Beef	100 mg	Bovine Gelatin	200 mg
Chicken	100 mg	Chicken gelatin	200 mg

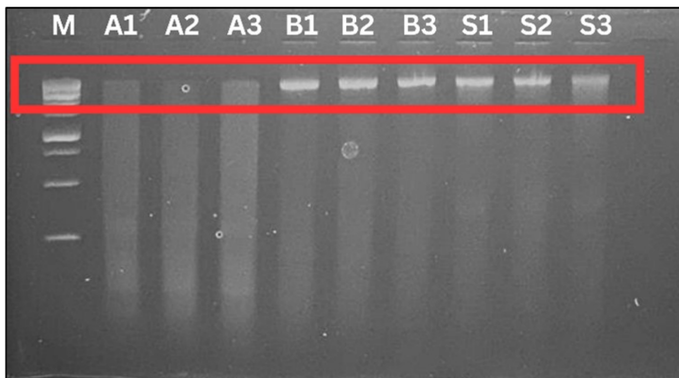

Figure 3. Electrophoresis Results of Meat Samples. Legend: M: Marker (1 kb); Chicken: A1; A2; A3; Beef: S1; S2; S3; Pig: B1; B2; B3

although higher DNA concentrations can be obtained by using larger sample weights, selecting a sample weight that achieves optimal purity is more important to ensure the reliability of the analysis results (Vyhnánek et al., 2015).

The analysis of DNA presence in extraction results is performed both qualitatively and quantitatively. Quantitative testing employs the NanoDrop EPOCH2 to measure DNA concentration and purity, while qualitative testing is conducted using agarose gel electrophoresis to assess DNA quality. DNA purity is determined by the absorbance ratio A260/A280 using the NanoDrop instrument. This analysis serves as a primary indicator of extraction success. A purity ratio between 1.8 and

Table 5. Concentration and Purity Results from the Optimization of Chicken, Beef, and Pork Samples Using the EPOCH Microplate Reader Nanodrop

Sample Type	Concentration (ng/μL)	Purity (A260/A280)
Chicken Meat Sample 50 mg	304.761	2.127
Beef Meat Sample 50 mg	167.304	2.107
Porcine Meat Sample 50 mg	191.81	2.117
Chicken Meat Sample 100 mg	566.811	2.153
Beef Meat Sample 100 mg	37.432	1.980
Porcine Meat Sample 100 mg	56.860	1.990

Table 6. Concentration and purity results from the optimization of chicken, beef, and pork gelatin samples using the EPOCH Microplate Reader Nanodrop.

Sample Type	Concentration (ng/μL)	Purity (A260/A280)
Chicken Gelatin Sample 100 mg	5.505	1.023
Bovine Gelatin Sample 100 mg	1.621	1.281
Porcine Gelatin Sample 100 mg	22.536	1.671
Bovine Gelatin Sample 200 mg	37.375	2.057
Porcine Gelatin Sample 200 mg	15.100	1.853
Chicken Gelatin Sample 200 mg	7.827	2.423

2.0 is considered optimal for DNA, indicating that the sample is relatively free from protein contamination and other compounds that could interfere with further analysis (Karunakaran et al., 2020). In this study, various sample weights were tested to determine the optimal conditions that yield DNA with concentrations and purity levels that meet the desired standards.

The Nanodrop analysis results in Table 5 indicate that although a 100 mg sample weight for pork yields a higher DNA concentration (56.860 ng/μl), the DNA purity at a 50 mg sample weight (191.81 ng/μl, purity 2.117) is more in line with the desired purity standards (1.8-2.0). High purity is crucial to ensure DNA quality without protein or other contaminants, which is essential for accurate analysis (Dragon et al., 2023).

Meanwhile, for gelatin samples in Table 6, a 200 mg sample weight provides a higher DNA concentration (37.375 ng/μl) compared to a 100 mg sample weight (22.532 ng/μl). However,

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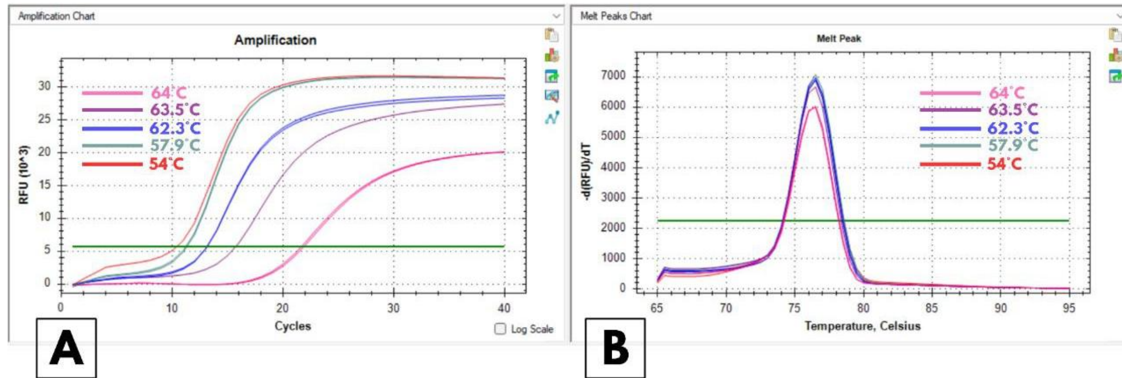

Figure 4. A) Amplification Curve of the Confirmation Test B) Melt Peak Curve of Annealing Temperature Optimization

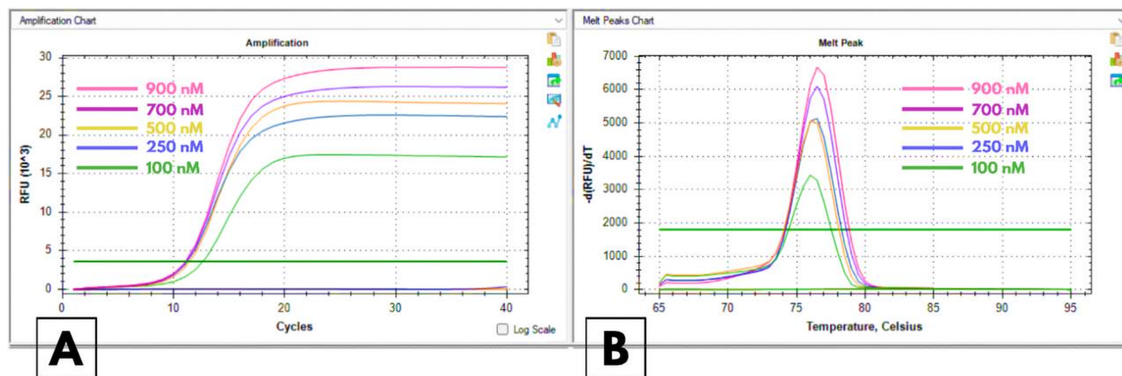

Figure 5. A) Amplification Curve of the Confirmation Test; (B) Melt Peak Curve of Primer Concentration Optimization. Notes: Concentrations of 100 nM; 250 nM; 500 nM; 700 nM; and 900 nM.

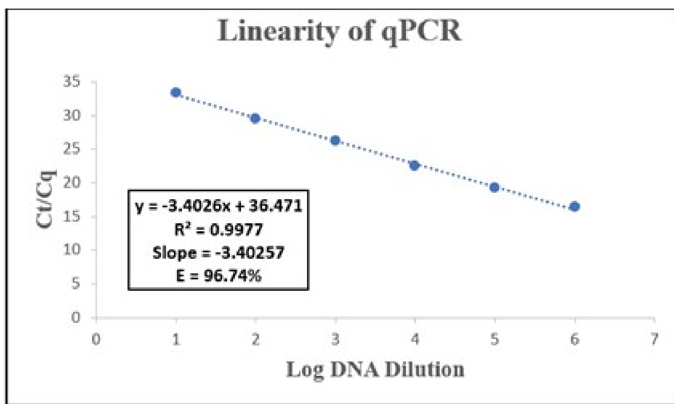

Figure 6. Regression Equation of Linearity Test Using Porcine DNA

the 100 mg sample weight was chosen as it approaches the desired purity standards (1.8-2.0, purity 1.671) while providing a sufficient concentration for adequate DNA analysis (Dragon et al., 2023). This sample weight selection also optimizes the efficiency of material usage and extraction time (Bridgeman et al., 2021). For gelatin, no samples met the criteria of a concentration > 100 ng/μl, although some samples exhibited purity close to the standard. These results are consistent with BSN's research on 200 mg pork gelatin, which reported concentrations of 4-9 ng/μl and purity ranging from 1.25 to 2.00. This indicates that while the concentration of gelatin remains low, some samples possess the appropriate purity (Dewantoro et al., 2023).

Qualitative analysis of genomic DNA extraction results is performed using electrophoresis. Electrophoresis is a technique used to separate DNA fragments based on their size (molecular weight) and physical structure (Bridgeman et al., 2021). High-quality genomic DNA is characterized by several key factors: fragments larger than 1000 bp indicate that the DNA is not degraded; clear and sharp bands at the top of the electrophoresis gel signify intact large fragments; bands that do not spread suggest that the DNA is free from contamination; and a sufficiently high DNA concentration ensures that there is adequate genetic material for further analysis (Levin et al., 2018). These results indicate successful extraction, as evidenced by the presence of DNA bands on the gel. Although the chicken DNA appears smeared, data from the NanoDrop con-

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Table 7. LoD and LoQ Values for Porcine Meat Samples

DNA Concentration (ng/μL)	Dilution Factor	Ct Value 1	2	3	Average Ct/Cq ± SD	Remarks
200	1×	14.9	14.79	14.62	14.77 ± 0.11	D (LoD 100%)
20	10×	17.21	17.24	17.15	17.20 ± 0.03	D (LoD 100%)
2	10×	20.59	20.64	20.62	20.61 ± 0.02	D (LoD 100%)
0.2	10×	24.43	24.59	24.45	24.49 ± 0.07	D (LoD 100%)
0.02	10×	28.06	28.26	28.16	28.16 ± 0.08	D (LoD 100%)
0.002	10×	32.39	32.12	32.53	32.34 ± 0.2	D (LoD 100%)
0.0002	10×	35.17	37.46	34.71	35.78 ± 0.9	D (LoD 100%)
0.00004	5×	N/A	N/A	38.16	12.72 ± 17.98	ND (LoD 33.34%)
ddH_2O	-	N/A	N/A	N/A	N/A	ND

Remarks:
D = Detected
ND = Not Detected

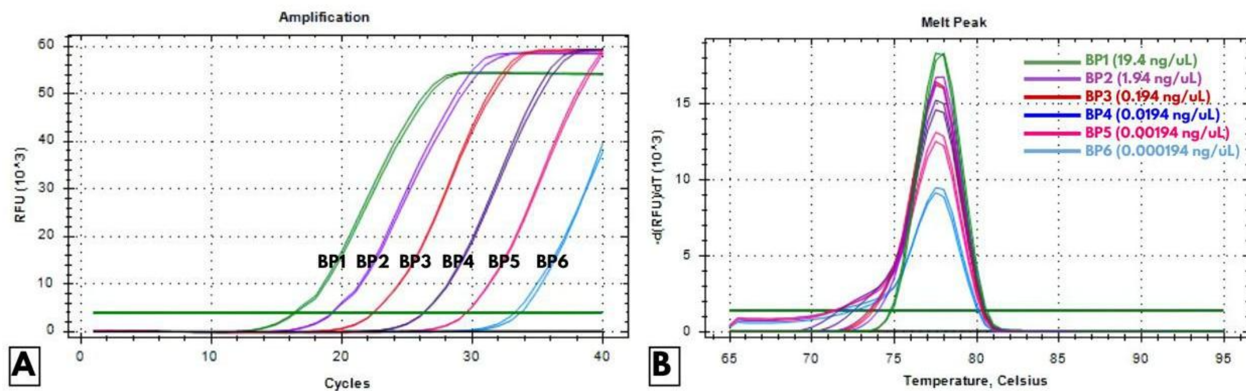

Figure 7. A) Linearity Test Amplification Curve B) Linearity Test Melt Peak Curve; Notes: BP1 (19.4 ng/μL); BP2 (1.94 ng/μL); BP3 (0.194 ng/μL); BP4 (0.0194 ng/μL); BP5 (0.00194 ng/μL); and BP6 (0.000194 ng/μL)

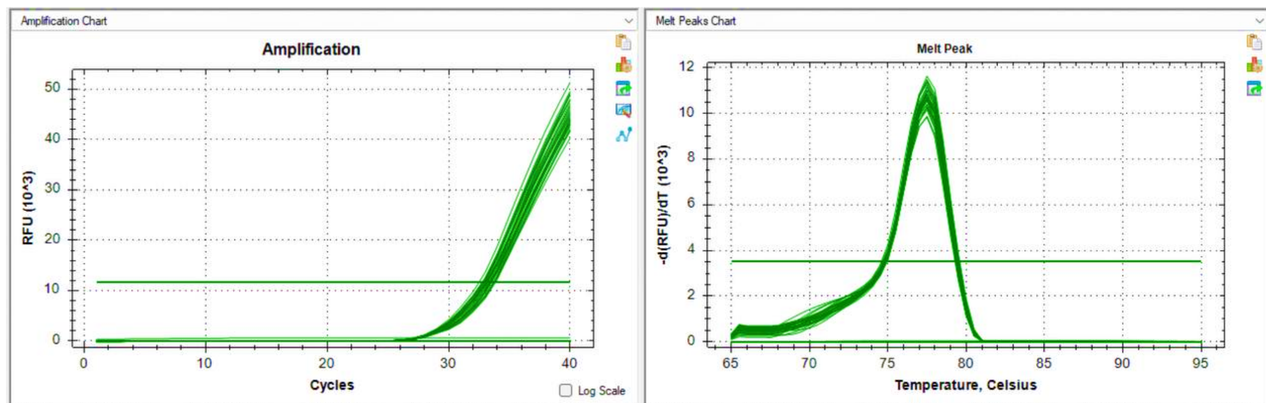

Figure 8. Amplification and Melt Peak Curves of the Homogeneity Test

firm the presence of DNA (Prayitno and Nuryandani, 1970) as cited in states that a smear at the bottom of genomic DNA bands indicates the presence of molecules with varying weights due to DNA degradation, although intact and fragmented genomic DNA is still present. This is not problematic because the genomic DNA contains a vast number of base pairs, while the PCR amplicon is only 140 bp (see Figure 3).

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Table 8. LoD and LoQ Values for Porcine Gelatin Samples

DNA Concentration (ng/μL)	Dilution Factor	Ct Value 1	Ct Value 2	Ct Value 3	Average Ct/Cq ± SD	Remarks
27	1×	29.41	29.4	29.22	29.34 ± 0.08	D (LoD 100%)
2.7	10×	32.22	32.58	32.36	32.38 ± 0.14	D (LoD 100%)
0.54	5×	36.27	37.07	36.75	36.69 ± 0.4	D (LoD 100%)
0.27	2×	N/A	38.36	N/A	12.78 ± 18.08	D (LoD 33.34%)
Positive control	-	13.56	13.5	13.53	13.53	-
Negative control	-	N/A	N/A	N/A	N/A	ND

Remarks:
D = Detected
ND = Not Detected

Table 9. Variance Homogeneity Test for Threshold Cycle Values

Levene Statistic	df1	df2	Sig.
1,620	9	20	0.176

3.3 Optimization of Primer Annealing Temperature

Optimization of the annealing temperature aims to determine the most effective temperature at which primers can function effectively with the DNA template (Yuniarti et al., 2020). This process is carried out using gradient PCR to test primer pairs and obtain the ideal annealing temperature (Okanti et al., 2020). The annealing temperature was tested using porcine gelatin samples at gradient temperatures of 54°C, 57.9°C, 62.3°C, 63.5°C, and 64°C (see Figure 4) to determine the temperature that enhances the efficiency and specificity of PCR amplification (Tan et al., 2020; Tasrip et al., 2021). The results indicate that 57.9°C is the optimal temperature for the Gelatin Primer Set, based on the sigmoid amplification curve, which demonstrates high efficiency and specificity (Ruijter et al., 2021). The annealing temperature optimization profile should exhibit a consistent melt curve and melt peak, forming a single peak, which indicates target specificity (Nagy et al., 2016). The presence of a double peak indicates two targets within a single cDNA, suggesting that the primers do not specifically recognize the template (Rodríguez et al., 2015).

3.4 Optimization of Primer Concentration

Primer concentration optimization aims to determine the concentration level that achieves efficient and optimal amplification (Masnaini et al., 2023). For standard PCR applications, the recommended primer concentration typically ranges from 0.1 μM to 1 μM per primer (Ruiz-Villalba et al., 2017). If the primer concentration exceeds this optimal range, there is a risk of increased non-specific product formation and primer-dimer formation. Primer-dimers are short oligonucleotides that form when primers bind to each other (Xie et al., 2022). Primer concentration testing (see Figure 5) was conduc(Guo et al., 2020) of 100 nM, 250 nM, 500 nM, 700 nM, and 900 nM (Guo et al., 2020). The optimal concentration was found to be 500 nM for the Gelatin primer set, which produced a sigmoid amplification curve with a steep slope and stable plateau. Melt curve analysis revealed a sharp single peak without primer-dimer formation or by-products, indicating specific amplification (Nagy et al., 2016).

3.5 qPCR Reaction Efficiency

The qPCR efficiency test results (Figure 6) demonstrate good amplification linearity of the target nucleic acid sequences in the samples, which is crucial for assessing amplification performance, matrix interference, and sample dilution linearity (Hays et al., 2024). Efficiency testing was conducted using a serial dilution of the sample ($2 \times 10^2; 2 \times 10^1; 2 \times 10^0; 2 \times 10^{-1}; 2 \times 10^{-2}; 2 \times 10^{-3}$ ng/μl) with 1× TE Buffer as the solvent. These six DNA dilutions were employed as templates in an optimized Real-Time PCR protocol. The results indicated a qPCR efficiency of 96.74%, with a regression equation of $y = -3.4026x + 36.471$, an R^2 value of 0.9977, and a slope of -3.40257. This efficiency approaches the theoretical maximum of 100%, demonstrating successful amplification of the DNA template with no interference or inhibition (Ruijter et al., 2021). An R^2 value close to 1 and an efficiency within the range of 90%-110% indicate that the optimized qPCR method provides consistent and accurate results. The slope of the regression curve should fall between -3.9 and -2.9, with a regression coefficient $R^2 \geq 0.98$, to demonstrate good efficiency (Broeders et al., 2014). Figure 7A illustrates the amplification curves demonstrating significant linearity across a range of DNA concentrations (from 19.4 ng/μL to 0.000194 ng/μL), indicating effective and consistent amplification without significant interference(VanInsberghe et al., 2018). The melt peak curve shown in Figure 7B reveals sharp and consistent melting points for each DNA sample, indicating specific PCR amplification products without the presence of primer dimers or other nonspecific products (Liang et al., 2016). These results confirm that the serial dilution does not interfere with the PCR reaction, demonstrating high specificity and efficiency of the assay (Svec et al., 2015).

Table 10. Analysis of Variance (ANOVA) for Threshold Cycle Values

Difference	Sum of Squares	Stdev	Df	MS	p-value	Mean Total	CV%
Between Groups	3.016	0.35061	9	0.335	0.000	33.2440	1.055%
Within Groups	0.549		20	0.027			

3.6 Evaluation of Limit of Detection and Limit of Quantification (LoD and LoQ)

The Limit of Detection (LoD) is defined as the lowest concentration of an analyte at which ≥ 95% of positive replications are detected, thereby ensuring less than 5% false negative results (Klymus et al., 2020). Meanwhile, the Limit of Quantification (LOQ) is defined as the lowest amount of an analyte in a sample that can be reliably quantified with an acceptable level of precision and accuracy (Sanchez, 2020).

In this study, the LoD and LOQ tests were conducted on porcine meat and porcine gelatin samples using qPCR. The aim of these tests was to evaluate the detection capability of the primers used against the DNA of these two sample types and to determine the detection and quantification limits of the primers (Erwanto, 2018). The LoD and LOQ tests using qPCR were conducted on porcine meat samples, employing porcine meat as a positive control and a serial dilution initially diluted 10×, followed by a 5× dilution. The DNA concentrations obtained from this testing varied, but the amplification curves remained detectable. Based on the results shown in Table 7, the primers used were able to detect porcine meat DNA down to a concentration of 0.0002 ng/μL. At this concentration, the average Ct/Cq value obtained was 35.98 with 100% detection (LoD 100%). However, at a lower concentration of 0.00004 ng/μL, the Ct/Cq value could not be fully detected by qPCR, indicating that at this concentration, DNA detection did not reach 100% (ND or Not Detected) (Klymus et al., 2020).

Subsequently, the LoD and LOQ tests were also conducted on porcine gelatin samples using a serial dilution initially diluted 10×, followed by 5× and 2× dilutions. The DNA concentrations obtained showed varying Ct/Cq values, yet the amplification curves remained detectable. Based on the results shown in Table 8, the primers used for the porcine gelatin samples were able to detect down to a concentration of 0.54 ng/μL, with an average Ct/Cq value of 36.69 and 100% detection (LoD 100%). Although detection still occurred at a concentration of 0.27 ng/μL, it was only at 33.34%, indicating that at this concentration, detection did not reach LoD 100%. Based on the detectable concentrations, RMs with a concentration of 2 ng/μL were still detected effectively.

From the obtained results, there are several differences between the LoD and LOQ tests on porcine meat and porcine gelatin samples. In porcine meat samples, the primers were able to detect DNA at very low concentrations, down to 0.0002 ng/μL with 100% LoD. In contrast, for porcine gelatin samples, the primers only achieved detection up to a concentration of 0.54 ng/μL for 100% LoD. At a lower concentration of 0.27 ng/μL, detection still occurred but was not complete, reaching only 33.34%.

This difference indicates that the primer detection efficiency is higher in porcine meat samples compared to porcine gelatin samples (Shabani et al., 2015). This may be due to differences in the matrix or composition of each sample, which can affect the efficiency of DNA amplification and detection by qPCR (Sultana et al., 2020).

3.7 Characterization Analysis of Reference Materials (RMs)

Reference Materials characterization testing ensures that the produced reference materials meet high-quality standards (Niu et al., 2022). This process includes testing for homogeneity to ensure uniformity of reference material units through random sampling and statistical analysis, as well as short-term storage stability testing to assess the durability and quality of reference materials at various temperatures, according to International Organization for Standardization (ISO) (2017) and Niu et al. (2022). These steps ensure that the produced reference materials are homogeneous, stable, and reliable for various analytical applications.

Homogeneity Testing

A primary requirement for the production of Reference Materials (RMs) is homogeneity or consistency among the various units produced, as outlined in International Organization for Standardization (ISO) (2017), which mandates that RM producers measure variation between units. Therefore, homogeneity testing is a crucial step. This test is conducted according to International Organization for Standardization (ISO) (2017) by evaluating Ct (Threshold Cycle) values, analyzed using one-way ANOVA statistical methods. One-way ANOVA is performed using IBM SPSS Statistics v20 (IBM, New York, USA). An effective homogeneity test involves taking a minimal sample volume, ideally 1 μL (Liang et al., 2016). Additionally, a good sample distribution analysis is assessed through the Coefficient of Variation (CV) value. The CV value represents the ratio of the standard deviation to the median value, expressed as a percentage (Forootan et al., 2017). The standard coefficient of variation (CV) for a reliable Real-Time PCR assay should be less than 5% (Pavsic et al., 2016). A sample is considered homogeneous if the coefficient of variation (CV) is less than 5% (Arachchige et al., 2022).

Based on the results of the variance homogeneity test conducted using Levene's Test for the Threshold Cycle (Ct value), a significance level of 0.052 was obtained (see Table 9). According to León et al. (2017), data is considered homogeneous if the significance value is greater than 0.05. Therefore, the results

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indicate that the analyzed Threshold Cycle (Ct value) data is homogeneous. Subsequently, an analysis of variance (ANOVA) was conducted to assess the differences among the tested groups (Mishra et al., 2019). The results indicate significant differences between the groups, as shown by the significance value of 0.000 (Table 10). A significance value below 0.05 indicates meaningful differences among the groups, suggesting the treatment has a statistically significant effect (Di Leo and Sardanelli, 2020). Despite these differences, the prior homogeneity test confirms uniform variation within each group, indicating the results are due to the treatment rather than random variation (McShane et al., 2019).

The amplification and melt peak curves demonstrate that all samples exhibit consistent amplification and melt peak patterns (Figure 8). This pattern supports the results of the homogeneity test, as it indicates that the threshold cycle (Ct value) data exhibit minimal and consistent variation across all tested samples (Aminfar et al., 2019).

The sample distribution analysis is further supported by the Coefficient of Variation (CV%) values. The CV is the ratio of the standard deviation to the median, expressed as a percentage. For the threshold cycle (Ct value) data, the CV is 0.96%, which falls within the acceptable standard for Real-Time PCR tests, indicating a CV of less than 5% (Arachchige et al., 2022; Pavsic et al., 2016). This indicates that the variation among samples is very low, supporting the conclusion that the data are homogeneous.

Short-Term Storage Stability Test

Storage stability and temperature testing are crucial processes to ensure the quality and safety of gelatin-based genomic DNA products. This testing is conducted to determine the resilience of genomic DNA to different temperatures compared to the ideal storage temperature as outlined in International Organization for Standardization (ISO) (2017) and Niu et al. (2022). Testing was conducted under four temperature conditions: -80°C, -20°C, 4°C, and 37°C, over a two-week storage period, with sampling on days 1, 3, 7, and 13. The selection of these four temperatures is well-founded. The temperature of -80°C was chosen because it is a common reference temperature used for long-term storage of biological materials, providing insight into the stability of genomic DNA under optimal extreme storage conditions Bento et al. (2022). The temperature of -20°C was selected because it is the standard storage temperature in many laboratories and is frequently used for medium-term storage (Xu and Kasprzyk-Hordern, 2023). The temperature of 4°C is commonly used for short-term storage in laboratories and represents typical refrigeration conditions encountered in everyday storage (Lear et al., 2018). The temperature of 37°C was chosen to simulate higher or worst-case temperature conditions that might be encountered during shipping or temporary storage, aiding in the evaluation of genomic DNA stability under non-ideal conditions (McDevitt et al., 2014).

Based on the test results analyzed with GraphPad Prism, samples stored at -80°C, -20°C, 4°C, and 37°C from day 1 to day 13 remained stable and did not exhibit significant changes

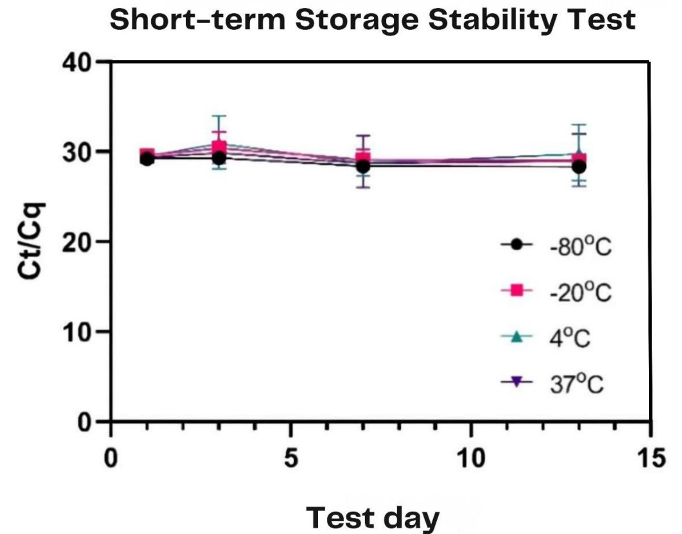

Figure 9. Short-Term Storage Stability Test (Geometric Mean with 95% CI)

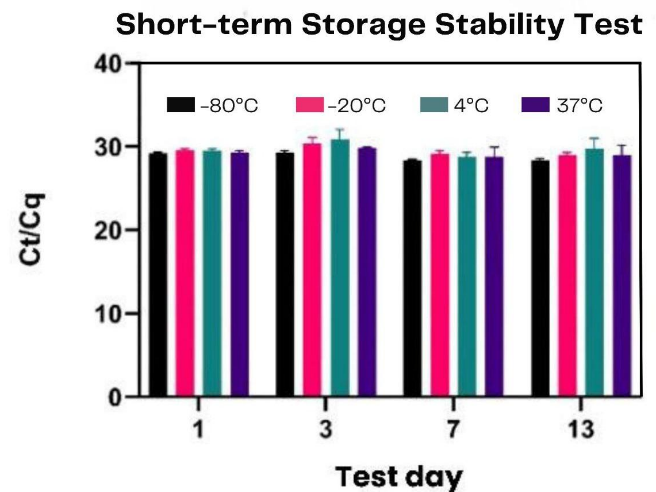

Figure 10. Short-Term Storage Stability Test (Mean with SD)

in Ct/Cq values (Figures 9 and 10). The Ct/Cq values tended to remain stable across all storage temperatures, with only slight variation (Figure 9). These findings are further supported by results showing that the Ct/Cq values remained relatively constant for all storage temperatures, with no significant differences in Ct/Cq values across the different temperatures on each testing day (Figure 10). The small standard deviations in both graphs indicate low data variation and high consistency across replicates (Schurch et al., 2016).

Based on the test results, -80°C and -20°C are the optimal choices for the short-term storage of gelatin-based genomic reference materials. The stable and consistent Ct/Cq values, along

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with the small standard deviations, indicate high reproducibility across replicates at these temperatures. In contrast, 4°C and 37°C exhibited greater variation and less consistency, making them less optimal compared to -80°C and -20°C. Therefore, for short-term storage, -80°C and -20°C are recommended to maintain the integrity of gelatin-based genomic DNA.

4. CONCLUSIONS

This study successfully developed genomic DNA-based reference materials (RMs) for the authentication of halal pharmaceutical products using a biomolecular PCR approach. In-silico primer design and wet lab validation demonstrated high specificity and good performance for detecting porcine DNA in samples relative to the target sequence. Homogeneity variance testing and ANOVA revealed homogeneous threshold cycle (Ct value) and low variation among samples. Storage stability tests indicated -80°C and -20°C as the optimal choices for short-term storage of gelatin-based genomic reference materials, due to their stable and consistent Ct/Cq values. In contrast, 4°C and 37°C exhibited greater variation and less consistency. Overall, RMs remained stable across all storage temperatures, but -80°C and -20°C yielded the most optimal results. This development is highly significant for halal authentication testing using biomolecular methods. These RMs can be used by halal testing institutions to assess testing accuracy and are also valuable for developing standardization methods for porcine DNA detection using PCR, supporting the establishment of regulations and halal authentication standards in Indonesia.

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REFERENCES

Al-Kahtani, H. A., E. A. Ismail, and M. Asif Ahmed (2017). Pork Detection in Binary Meat Mixtures and Some Commercial Food Products Using Conventional and Real-Time PCR Techniques. *Food Chemistry*, **219**; 54–60

Al-Nimry, S., A. A. Dayah, I. Hasan, and R. Daghmash (2021). Cosmetic, Biomedical and Pharmaceutical Applications of Fish Gelatin/Hydrolysates. *Marine Drugs*, **19**(3); 145

Ali, M. E., M. A. Razzak, S. B. A. Hamid, M. M. Rahman, M. A. Amin, N. R. A. Rashid, and Asing (2015). Multiplex PCR Assay for the Detection of Five Meat Species Forbidden in Islamic Foods. *Food Chemistry*, **177**; 214–224

Aminfar, Z., B. Rabiei, M. Tohidfar, and M. H. Mirjalili (2019). Selection and Validation of Reference Genes for Quantitative Real-Time PCR in *Rosmarinus Officinalis* L. In Various Tissues and under Elicitation. *Biocatalysis and Agricultural Biotechnology*, **20**; 101246

Ananta, A., E. Nurvidya Arifin, and M. Sairi Hasbullah (2015). *Demography of Indonesia's Ethnicity*. ISEAS Publishing

Arachchige, C. N. P. G., L. A. Prendergast, and R. G. Staudte (2022). Robust Analogs to the Coefficient of Variation. *Journal of Applied Statistics*, **49**(2); 268–290

Asif, M., D. Zhiyong, A. Iram, and M. Nisar (2021). Linguistic Analysis of Neologism Related to Coronavirus (COVID-19). *Social Sciences & Humanities Open*, **4**(1); 100201

Bento, C. S. A., S. Alarico, N. Empadinhas, H. C. de Sousa, and M. E. M. Braga (2022). Sequential ScCO_2 Drying and Sterilisation of Alginate-Gelatine Aerogels for Biomedical Applications. *The Journal of Supercritical Fluids*, **184**; 105570

Borah, J. C. (2015). Shikimic Acid: A Highly Prospective Molecule in Pharmaceutical Industry. *Current Science*, **109**(9); 1672–1679

Bridgeman, S., G. Ellison, P. Newsholme, and C. Mamotte (2021). The HDAC Inhibitor Butyrate Impairs B Cell Function and Activates the Disallowed Gene Hexokinase I. *International Journal of Molecular Sciences*, **22**(24); 13330

Broeders, S., I. Huber, L. Grohmann, G. Berben, I. Taverniers, M. Mazzara, N. Roosens, and D. Morisset (2014). Guidelines for Validation of Qualitative Real-Time PCR Methods. *Trends in Food Science & Technology*, **37**(2); 115–126

Browne, P. D., T. K. Nielsen, W. Kot, A. Aggerholm, M. T. P. Gilbert, L. Puetz, M. Rasmussen, A. Zervas, and L. H. Hansen (2020). GC Bias Affects Genomic And Metagenomic Reconstructions, Underrepresenting GC-Poor Organisms. *GigaScience*, **9**(2)

Bustin, S. and J. Huggett (2017). qPCR Primer Design Revisited. *Biomolecular Detection and Quantification*, **14**; 19–28

Cai, H., X. Gu, M. S. Scanlan, D. H. Ramatlapeng, and C. R. Lively (2012). Real-Time PCR Assays for Detection and Quantitation of Porcine and Bovine DNA in Gelatin Mixtures and Gelatin Capsules. *Journal of Food Composition and Analysis*, **25**(1); 83–87

Cheman, Y. (2005). Analysis of Potential Lard Adulteration in Chocolate and Chocolate Products Using Fourier Transform Infrared Spectroscopy. *Food Chemistry*, **90**(4); 815–819

Chen, F., Y. P. Hsieh, and R. C. Bridgman (2004). Monoclonal Antibody–Based Sandwich Enzyme-Linked Immunosorbent Assay for Sensitive Detection of Prohibited Ruminant Proteins in Feedstuffs. *Journal of Food Protection*, **67**(3); 544–549

Chen, K., Y. Zhou, K. Li, and J. Xiao (2017). Multiplex PCR with the Blunt Hairpin Primers for Next Generation Sequencing. *Biotechnology and Bioprocess Engineering*, **22**(3); 347–351

Corliss, J. O., M. Aikawa, and C. R. Sterling (1977). *The Science and Technology of Gelatin*. Academic Press, London (UK)

Dewantoro, A., A. Safarrida, D. Rahmasari, U. Nuraeni, R. D. Susmiarni, R. Damacena, and M. M. Amin (2023). Verification of Porcine Detection Method Based on SNI ISO/TS 20224-3:2020 on the Gelatin Matrix. *Jurnal Standardisasi*,

© 2025 The Authors.

25(2); 79

Di Leo, G. and F. Sardanelli (2020). Statistical Significance: p Value, 0.05 Threshold, and Applications to Radiomics—Reasons for a Conservative Approach. *European Radiology Experimental*, **4**(1); 18

Dragon, A. H., C. J. Rowe, A. M. Rhodes, O. L. Pak, T. A. Davis, and E. Ronzier (2023). Systematic Identification of the Optimal Housekeeping Genes for Accurate Transcriptomic and Proteomic Profiling of Tissues Following Complex Traumatic Injury. *Methods and Protocols*, **6**(2); 22

Erwanto, Y. (2018). *Molecular Based Method Using PCR Technology on Porcine Derivative Detection for Halal Authentication*. IntechOpen

Forootan, A., R. Sjöback, J. Björkman, B. Sjögreen, L. Linz, and M. Kubista (2017). Methods to Determine Limit of Detection and Limit of Quantification in Quantitative Real-Time PCR (qPCR). *Biomolecular Detection and Quantification*, **12**; 1–6

Green, M. R. and J. Sambrook (2020). Cloning in Plasmid Vectors: Directional Cloning. *Cold Spring Harbor Protocols*, **2020**(11); 485–488

Guo, Y., Q. Cao, Z. Hong, Y. Tan, S. Chen, H. Jin, K. Tan, D. Wang, and Y. Yan (2020). The Origin, Transmission and Clinical Therapies on Coronavirus Disease 2019 (COVID-19) Outbreak an Update on the Status. *Military Medical Research*, **7**(1); 11

Hassan, N., T. Ahmad, and N. M. Zain (2018). Chemical and Chemometric Methods for Halal Authentication of Gelatin: An Overview. *Journal of Food Science*, **83**(12); 2903–2911

Hays, A., M. Wissel, K. Colletti, R. Soon, M. Azadeh, J. Smith, R. Doddareddy, M. Chalfant, W. Adamowicz, S. S. Ramaswamy, et al. (2024). Recommendations for Method Development and Validation of qPCR and dPCR Assays in Support of Cell and Gene Therapy Drug Development. *The AAPS Journal*, **26**(1); 24

Hikmah, N. N., A. Guntarti, and N. Salamah (2024). Concentration of Isolated DNA Face Masks Made of Gelatin for Halal Authentication. *Journal of Halal Science and Research*, **5**(1); 1–5

Hung, J. and Z. Weng (2016). Designing Polymerase Chain Reaction Primers Using Primer3Plus. *Cold Spring Harbor Protocols*, **2016**(9); 093096

International Organization for Standardization (ISO) (2017). *Reference Materials - Guidance for Characterization and Assessment of Homogeneity and Stability*. ISO Guide:35

Jones, R. T. (2004). *Manufacture and Physio-Chemical Properties*. Pharmaceutical Press, second edition edition

Kang, S. S. N., H. G. Lee, and H. Kim (2018). Development and Comparison of a Porcine Gelatin Detection System Targeting Mitochondrial Markers for Halal Authentication. *LWT*, **97**; 697–702

Karimi, K., M. Arzanlou, and I. Pertot (2020). Development of Novel Species-Specific Primers for the Specific Identification of *Colletotrichum nymphaeae* Based on Conventional PCR and LAMP Techniques. *European Journal of Plant Pathology*, **156**(2); 463–475

Karunakaran, D., A. W. Turner, A. Duchez, S. Soubeyrand, A. Rasheed, D. Smyth, D. P. Cook, M. Nikpay, J. W. Kandiah, C. Pan, et al. (2020). RIPK1 Gene Variants Associate with Obesity in Humans and Can Be Therapeutically Silenced to Reduce Obesity in Mice. *Nature Metabolism*, **2**(10); 1113–1125

Kesmen, Z., A. Gulluce, F. Sahin, and H. Yetim (2009). Identification of Meat Species by Taqman-Based Real-Time PCR Assay. *Meat Science*, **82**(4); 444–449

Khayyira, A. S., V. M. Estepane, and A. Malik (2018). Rapid PCR-Based Detection Optimization of Porcine DNA in Gelatin Capsule Shell. *International Journal of Applied Pharmaceutics*, **10**(6); 217

Kim, M., I. Yoo, S.-Y. Lee, Y. Hong, and H.-Y. Kim (2016). Quantitative Detection of Pork in Commercial Meat Products by Taqman® Real-Time PCR Assay Targeting the Mitochondrial D-Loop Region. *Food Chemistry*, **210**; 102–106

Klymus, K. E., C. M. Merkes, M. J. Allison, C. S. Goldberg, C. C. Helbing, M. E. Hunter, C. A. Jackson, R. F. Lance, A. M. Mangan, E. M. Monroe, A. J. Piaggio, J. P. Stokdyk, C. C. Wilson, and C. A. Richter (2020). Reporting the Limits of Detection and Quantification for Environmental DNA Assays. *Environmental DNA*, **2**(3); 271–282

Kumar, A. and N. Chordia (2015). *In Silico* PCR Primer Designing and Validation. PCR Primer Design, pages 143–151

Lanzilao, I., F. Burgalassi, S. Fancelli, M. Settimelli, and R. Fani (2005). Polymerase Chain Reaction-Restriction Fragment Length Polymorphism Analysis of Mitochondrial Cytb Gene from Species of Dairy Interest. *Journal of AOAC International*, **88**(1); 128–135

Lear, G., I. Dickie, J. Banks, S. Boyer, H. L. Buckley, T. R. Buckley, R. Cruickshank, A. Dopheide, K. M. Handley, S. Hermans, J. Kamke, C. K. Lee, R. Macdiarmid, S. E. Morales, D. A. Orlovich, R. Smissen, J. Wood, and R. Holdaway (2018). Methods for the Extraction, Storage, Amplification and Sequencing of DNA from Environmental Samples. *New Zealand Journal of Ecology*, **42**(1)

León, C. M., M. Munoz, C. Hernández, M. S. Ayala, C. Flórez, A. Teherán, J. R. Cubides, and J. D. Ramírez (2017). Analytical Performance of Four Polymerase Chain Reaction (PCR) and Real Time PCR (qPCR) Assays for the Detection of Six Leishmania Species DNA in Colombia. *Frontiers in Microbiology*, **8**

Levin, R. E., F.-G. C. Ekezie, and D.-W. Sun (2018). DNA-Based Technique: Polymerase Chain Reaction (PCR). In *Modern Techniques for Food Authentication*. Elsevier, pages 527–616

Li, J., H. Gao, Y. Li, S. Zhai, F. Xiao, G. Wu, and Y. Wu (2024). The Development of a Series of Genomic DNA Reference Materials with Specific Copy Number Ratios for The Detection of Genetically Modified Maize DBN9936. *Foods*, **13**(5); 747

Li, J., L. Zhang, L. Li, X. Li, X. Zhang, S. Zhai, H. Gao, Y. Li, G. Wu, and Y. Wu (2020). Development of Genomic DNA

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Certified Reference Materials for Genetically Modified Rice Kefeng 6. *ACS Omega*, **5**(34); 21602–21609

Liang, W., L. Xu, Z. Sui, Y. Li, L. Li, Y. Wen, C. Li, S. Ren, and G. Liu (2016). Quantification of Plasmid DNA Reference Materials for Shiga Toxin-Producing Escherichia Coli Based on UV, HR-ICP-MS and Digital PCR. *Chemistry Central Journal*, **10**(1); 55

Manen, J., O. Sinitsyna, L. Aeschbach, A. V. Markov, and A. Sinitsyn (2005). A Fully Automatable Enzymatic Method for DNA Extraction from Plant Tissues. *BMC Plant Biology*, **5**(1); 23

Masiri, J., L. Benoit, B. Barrios-Lopez, C. Thienes, M. Meshgi, A. Agapov, A. Dobritsa, C. Nadala, and M. Samadpour (2016). Development and Validation of a Rapid Test System for Detection of Pork Meat and Collagen Residues. *Meat Science*, **121**; 397–402

Masnaini, M., A. Achyar, M. Chatri, D. H. Putri, Y. Ahda, and Irdawati (2023). Primer Design and Optimization of PCR Methods for Detecting Mixed Rat Meat in Food Samples. In *Proceedings of The International Conference on Natural Resources and Technology*. pages 282–289

McDevitt, S. L., M. E. Hogan, D. J. Pappas, L. Y. Wong, and J. A. Noble (2014). DNA Storage Under High Temperature Conditions Does Not Affect Performance in Human Leukocyte Antigen Genotyping via Next-Generation Sequencing (DNA Integrity Maintained in Extreme Conditions). *Biopreservation and Biobanking*, **12**(6); 402–408

McShane, B. B., D. Gal, A. Gelman, C. Robert, and J. L. Tackett (2019). Abandon Statistical Significance. *The American Statistician*, **73**(sup1); 235–245

Mishra, P., U. Singh, C. Pandey, P. Mishra, and G. Pandey (2019). Application of Student's t-Test, Analysis of Variance, and Covariance. *Annals of Cardiac Anaesthesia*, **22**(4); 407

Mokhtar, N. F. K., S. N. Aziz, N. H. Yusoff, R. M. H. Raja Nhari, N. A. Mohamad, and A. Mohd Hashim (2024). Porcine Detection in Commercial Edible Bird Nest Beverages Using Real Time PCR Targeting Chromosomally Encoded Multiple Porcine Repetitive Elements. *Journal of Food Composition and Analysis*, **131**; 106211

Nagy, A., L. Cerníková, E. Vitásková, V. Krivda, A. Dán, Z. Dirbáková, H. Jirincová, B. Procházka, K. Sedlák, and M. Havlícková (2016). MeltMan: Optimization, Evaluation, and Universal Application of a qPCR System Integrating the TaqMan qPCR and Melting Analysis into a Single Assay. *PLoS ONE*, **11**(3); e0151204

Nazarenko, I. (2002). Multiplex Quantitative PCR Using Self-Quenched Primers Labeled with a Single Fluorophore. *Nucleic Acids Research*, **30**(9); 37e–37

Nguyen, P. T. D., A. Giovanni, S. Maekawa, T. H. Pham, P. Wang, and S.-C. Chen (2023). An Integrated In Silico and *In Vivo* Study of Nucleic Acid Vaccine Against *Nocardia seriolae* Infection in Orange-Spotted Grouper Epinephelus Coioides. *Fish & Shellfish Immunology*, **143**; 109202

Nikzad, J., S. Shahhosseini, M. Tabarzad, N. Nafissi-Varcheh, and M. Torshabi (2017). Simultaneous Detection of Bovine and Porcine DNA in Pharmaceutical Gelatin Capsules by Duplex PCR Assay for Halal Authentication. *DARU Journal of Pharmaceutical Sciences*, **25**(1); 3

Niu, Q., X. Su, L. Lian, J. Huang, S. Xue, W. Zhou, H. Zhao, X. Lu, S. Cui, J. Chen, and B. Yang (2022). Developing Qualitative Plasmid DNA Reference Materials to Detect Mechanisms of Quinolone and Fluoroquinolone Resistance in Foodborne Pathogens. *Foods*, **11**(2); 154

Ni'Mah, A., Y. Kartikasari, A. D. Pratama, L. R. Kartikasari, B. S. Hertanto, and M. Cahyadi (2016). Detection of Pork Contamination in Fresh and Cooked Beef Using Genetic Marker Mitochondrial-DNA Cytochrome b by Duplex-PCR. *Journal of the Indonesian Tropical Animal Agriculture*, **41**(1); 7–12

Noguchi, A., K. Nakamura, K. Sakata, N. Sato-Fukuda, T. Ishigaki, J. Mano, R. Takabatake, K. Kitta, R. Teshima, K. Kondo, and T. Nishimaki-Mogami (2016). Development and Interlaboratory Validation of a Simple Screening Method for Genetically Modified Maize Using a ΔΔ Cq-Based Multiplex Real-Time PCR Assay. *Analytical Chemistry*, **88**(8); 4285–4293

Nurilmala, M., N. Darmawan, E. A. W. Putri, A. M. Jacoeb, and T. T. Irawadi (2021). Pangasius Fish Skin and Swim Bladder as Gelatin Sources for Hard Capsule Material. *International Journal of Biomaterials*, **2021**; 1–6

Oh, S. H. and C. S. Jang (2020). Development and Validation of a Real-Time PCR Based Assay to Detect Adulteration with Corn in Commercial Turmeric Powder Products. *Foods*, **9**(7); 882

Okanti, J. R., D. H. Putri, and A. Fathoni (2020). Analysis of ZDS and LCYb Enzyme Coding Gene Related to Beta Carotene Biosynthesis in Cassava (Manihot Esculenta Crantz) Using Reverse Transcription PCR (RT-PCR). *Bioscience*, **4**(1); 01

Pavsic, J., J. Zel, and M. Milavec (2016). Assessment of the Real-Time PCR and Different Digital PCR Platforms for DNA Quantification. *Analytical and Bioanalytical Chemistry*, **408**(1); 107–121

Perestam, A. T., K. K. Fujisaki, O. Nava, and R. S. Hellberg (2017). Comparison of Real-Time PCR and ELISA-Based Methods for the Detection of Beef and Pork in Processed Meat Products. *Food Control*, **71**; 346–352

Prayitno, E. and E. Nuryandani (1970). Optimization of DNA Extraction of Physic Nut (*Jatropha curcas*) by Selecting the Appropriate Leaf. *Nusantara Bioscience*, **3**(1)

Purwanto, H., S. Jati, and A. Rofiq (2021). Policy Analysis of Enforcement of Halal Product Guarantee Regulations Through the Regulatory Impact Analysis (RIA) Approach. *Journal of Digital Marketing and Halal Industry*, **3**(2); 149–164

Raso, A. and R. Biassoni (2014). Twenty Years of qPCR: A Mature Technology? In *Twenty Years of qPCR: A Mature Technology?* pages 1–3

Rodríguez, A., M. Rodríguez, J. J. Córdoba, and M. J. Andrade (2015). Design of Primers and Probes for Quantitative Real-Time PCR Methods. In *Design of Primers and Probes for*

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Quantitative Real-Time PCR Methods. pages 31–56

Rohland, N. and M. Hofreiter (2007). Comparison and Optimization of Ancient DNA Extraction. *BioTechniques*, **42**(3); 343–352

Romano, S., A. Brandao, J. Gumpinger, M. Gschweitl, and S. Beretta (2017). Qualification of AM Parts: Extreme Value Statistics Applied to Tomographic Measurements. *Materials & Design*, **131**; 32–48

Ruijter, J. M., R. J. Barnewall, I. B. Marsh, A. N. Szentirmay, J. C. Quinn, R. van Houdt, Q. D. Gunst, and M. J. B. van den Hoff (2021). Efficiency Correction Is Required for Accurate Quantitative PCR Analysis and Reporting. *Clinical Chemistry*, **67**(6); 829–842

Ruiz-Villalba, A., E. van Pelt-Verkuil, Q. D. Gunst, J. M. Ruijter, and M. J. van den Hoff (2017). Amplification of Nonspecific Products in Quantitative Polymerase Chain Reactions (qPCR). *Biomolecular Detection and Quantification*, **14**; 7–18

Sabina, J. and J. H. Leamon (2015). Bias in Whole Genome Amplification: Causes and Considerations. In *Whole Genome Amplification: Methods and Protocols*. pages 15–41

Sanchez, J. M. (2020). Linear Calibrations in Chromatography: The Incorrect Use of Ordinary Least Squares for Determinations at Low Levels, and the Need to Redefine the Limit of Quantification with This Regression Model. *Journal of Separation Science*, **43**(13); 2708–2717

Sasmito, D. E. K., R. Kurniawan, and I. Muhimmah (2014). Karakteristik Primer Pada Polymerase Chain Reaction (PCR) Untuk Sekuensing DNA: Mini Review. In *Seminar Informatika Medis 2014*. pages 93–102 (in Indonesia)

Schurch, N. J., P. Schofield, M. Gierlinski, C. Cole, A. Sherstnev, V. Singh, N. Wrobel, K. Gharbi, G. G. Simpson, T. Owen-Hughes, M. Blaxter, and G. J. Barton (2016). How Many Biological Replicates Are Needed in an RNA-Seq Experiment and Which Differential Expression Tool Should You Use? *RNA*, **22**(6); 839–851

Sentandreu, M. A. and E. Sentandreu (2014). Authenticity of Meat Products: Tools against Fraud. *Food Research International*, **60**; 19–29

Shabani, H., M. Mehdizadeh, S. M. Mousavi, E. A. Dezfouli, T. Solgi, M. Khodaverdi, M. Rabiei, H. Rastegar, and M. Alebouyeh (2015). Halal Authenticity of Gelatin Using Species-Specific PCR. *Food Chemistry*, **184**; 203–206

Shuhaimi, M. and M. F. S. Sam-on (2022). Rapid DNA-Based Analytical Techniques to Verify the Authenticity of Halal Food. *Journal of Halal Studies*, **4**(1); 1–23

Soares, S., J. S. Amaral, M. B. P. P. Oliveira, and I. Mafra (2013). A SYBR Green Real-Time PCR Assay to Detect and Quantify Pork Meat in Processed Poultry Meat Products. *Meat Science*, **94**(1); 115–120

Sultana, S., M. A. M. Hossain, A. Azlan, M. R. Johan, Z. Z. Chowdhury, and M. E. Ali (2020). TaqMan Probe Based Multiplex Quantitative PCR Assay for Determination of Bovine, Porcine and Fish Dna in Gelatin Admixture, Food Products and Dietary Supplements. *Food Chemistry*, **325**; 126756

Svec, D., A. Tichopad, V. Novosadova, M. W. Pfaffl, and M. Kubista (2015). How Good Is a PCR Efficiency Estimate: Recommendations for Precise and Robust qPCR Efficiency Assessments. *Biomolecular Detection and Quantification*, **3**; 9–16

Tan, L. L., S. A. Ahmed, S. K. Ng, M. Citartan, C. A. Raabe, T. S. Rozhdestvensky, and T. H. Tang (2020). Rapid Detection of Porcine DNA in Processed Food Samples Using a Streamlined DNA Extraction Method Combined with the Sybr Green Real-Time PCR Assay. *Food Chemistry*, **309**; 125654

Tan, S. C. and B. C. Yiap (2009). DNA, RNA, and Protein Extraction: The Past and The Present. *BioMed Research International*, **2009**; 1–10

Tasrip, N. A., M. N. Mohd Desa, N. F. Khairil Mokhtar, N. Sajali, A. Mohd Hashim, M. E. Ali, and C. Y. Kqueen (2021). Rapid Porcine Detection in Gelatin-Based Highly Processed Products Using Loop Mediated Isothermal Amplification. *Journal of Food Science and Technology*, **58**(12); 4504–4513

Tretiakov, M. Y., V. K. Tokhtar, A. E. Ivleva, J. N. Kurkina, I. V. Batlutskaya, and I. V. Lyashenko (2022). Optimizing the Dry Plant Sample Preparation Method for Efficient DNA Extraction in *Syringa vulgaris*. *International Journal of Ecosystems and Ecology Science (IJEES)*, **12**(1); 201–206

Vallejo, C. V., C. P. Tere, M. N. Calderon, M. M. Arias, and J. E. Leguizamon (2021). Development of a Genomic DNA Reference Material for *Salmonella enteritidis* Detection Using Polymerase Chain Reaction. *Molecular and Cellular Probes*, **55**; 101690

VanInsberghe, M., H. Zahn, A. K. White, O. I. Petriv, and C. L. Hansen (2018). Highly Multiplexed Single-Cell Quantitative PCR. *PLoS ONE*, **13**(1); e0191601

Vyhnánek, T., V. Trojan, K. Štiasna, M. Presinszká, E. Mrkvicová, L. Hrivna, and L. Havel (2015). Testing of Dna Isolation for the Identification of Hemp. *Potravinarstvo Slovak Journal of Food Sciences*, **9**(1); 393–397

Xie, N. G., M. X. Wang, P. Song, S. Mao, Y. Wang, Y. Yang, J. Luo, S. Ren, and D. Y. Zhang (2022). Designing Highly Multiplex PCR Primer Sets with Simulated Annealing Design Using Dimer Likelihood Estimation (SADDLE). *Nature Communications*, **13**(1); 1–12

Xu, L. and B. Kasprzyk-Hordern (2023). Assessment of the Stability of Antimicrobials and Resistance Genes during Short- and Long-Term Storage Condition: Accounting for Uncertainties in Bioanalytical Workflows. *Analytical and Bioanalytical Chemistry*, **415**(24); 6027–6038

Yuniarti, H., A. Rinanti, and S. Bambang Cholis (2020). Optimization of Primary Annealing Temperature with BigDye Reagent in Sequencing Reaction. *International Journal of Scientific and Technology Research*, **9**(3); 1431–1434

Zehra, A. and S. Kaur (2023). Virus Concentration and Taqman Real-time Polymerase Chain Reaction (qPCR) Quantification of Adenoviral DNA for Detection of Sewage and Farm Wastewater-Associated Adenoviral Markers: Optimization and Validation of Virus Concentration Methods.

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Journal of Soil Salinity and Water Quality, **15**(1); 115–126

Zhao, Z. (2014). Optimal GC Content for PCRiPrimers Designed for the Detection of Bacterial and Viral Pathogens. *Journal of Microbiological Methods*, **98**(3); 289–295

Zia, Q., M. Alawami, N. F. K. Mokhtar, R. M. H. R. Nhari, and I. Hanish (2020). Current Analytical Methods for Porcine Identification in Meat and Meat Products. *Food Chemistry*, **324**; 126664

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