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Optimization of Authentication Methods for Processed Chicken Meat Products Based on *ND5* Gene qPCR

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ABSTRACT

Consumers have the right to correct information about the products they consume. One example of a food product that is vulnerable to counterfeiting is chicken nuggets. To overcome this problem, qPCR can be an effective solution. Previous research has designed primers targeting the chicken *ND5* gene. However, the PCR conditions for this primer pair have never been optimized. This study aims to optimize qPCR conditions and conduct trials to authenticate chicken nuggets. This research is a descriptive study using a molecular approach. Genomic DNA was isolated from five weight variations of chicken meat samples as a qPCR standard curve and three chicken nugget samples. Optimizing the annealing temperature with gradient PCR gave the best results at a temperature of 57.6°C. The results of the authentication test indicated that the chicken meat content in TD nuggets was 54.7%, C nuggets 43.8%, and D nuggets 37.3%. This value is still following the Indonesian National Standard (SNI).

Keywords: *qPCR, Authentication, Gallus, ND5 Gene, Chicken Nuggets.*

1 Introduction

Food safety is a crucial aspect of the current global food system. Consumer Protection Law Number 8 of 1999 emphasizes consumers' rights to obtain food products that are safe, comfortable, and have clear information. Consumers have the right to protection against security risks and the right to correct information about the products they consume. This places a huge responsibility not only on manufacturers to ensure product safety but also on consumers to choose quality and guaranteed products.

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One potential threat to food safety is the contamination of food by pathogenic bacteria such as *Listeria monocytogenes* [1]. These bacteria can cause serious infections particularly in pregnant women, infants, and older adults [2]. In this context, lubricants in the food industry play an important role as heat transfer media and lubricants in food production equipment. However, the presence of *L. monocytogenes* in this lubricant can threaten not only the safety of the product but also its halal status [3].

One example of a food product that is very popular and vulnerable to counterfeiting is chicken nuggets. Chicken nuggets are popular among people because of their practicality. However, cases of cheating such as dilution (the activity of mixing high-quality materials with low-quality materials), substitution (the activity of replacing nutrients, ingredients, food, or food parts with lower-quality products), concealment (the activity of hiding low-quality ingredients from a portion of food or product), mislabeling (wrong claims or distortion of information on packaging labels), unapproved enhancement (the process of adding ingredients that are not permitted to improve product quality), *counterfeiting* (activities of imitating brand names, packaging concepts, recipes, and processing methods), and grey market production/theft/diversion (unreported sales of excess products) can threaten consumer safety[1]. For example, the case of chicken nuggets in the United States containing milk allergens without appropriate labeling shows how important strict monitoring and regulation of food products is [2].

According to the standards set by the Food, Drug, and Cosmetic Testing Institute of the Indonesian Ulema Council (LPPOM MUI), quality chicken nuggets should contain at least 80% chicken meat, with the rest being other additives. However, research shows that some nugget products tested had much lower meat content than advertised, with more fat, epithelium, bone, nerve, and connective tissue content than expected [3].

To overcome this problem, authentication technology such as real-time Polymerase Chain Reaction (qPCR) is a solution that is considered effective. This method allows molecular species identification based on DNA, with a high level of specificity and sensitivity [4]. The use of the gene encoding NADH dehydrogenase subunit 5 (*ND5)* as an authentication target for chicken nuggets is also the focus of this research. Gen *ND5*, which is located in the mitochondria and has more copies, makes it easier to detect contamination or adulteration in food products.

Previous research [5] has designed primers targeting the *ND5* gene of several chicken species, including *Gallus gallus, Gallus gallus spadiceus, Gallus lafayetii, Gallus varius,* and *Gallus sonneratii*. However, the PCR conditions for this primer pair have never been optimized. Optimization of the PCR process is critical to ensure accurate and consistent results in this authentication analysis. Variables such as annealing temperature, primer concentration, and DNA template concentration must be optimized according to the conditions faced so that the PCR technique can provide optimal and reliable results in detecting food product contamination [6].

Thus, this research aims to optimize the *ND5* qPCR conditions and carry out chicken nugget authentication trials with optimum qPCR conditions. It is hoped that the results of this research can be applied to authenticate processed chicken meat products so as to provide

better protection for consumers and increase confidence in the safety and quality of the food products consumed.

2 Materials and methods

2.1 Materials

The materials needed for optimization were fresh chicken meats. The materials used for authentication testing of processed chicken meat products were chicken nuggets. Genomic DNA was extracted using the QIAamp® DNA Mini Kit (QIAGEN), which includes a 1.5 ml microtube, buffer ATL, Proteinase K, buffer AL, absolute ethanol (96-100%), buffer AW1, buffer AW2, and buffer AE. DNA amplification involved a master mix of 2x SensiFAST™ SYBR® No-ROX mix (Bioline), forward and reverse primer (Table 1), tips, nuclease-free water, and PCR tube. For electrophoresis, agarose, buffer TAE 1x, a 100 bp DNA ladder, loading dye, and GelRed were used.

Table 1. Primers for chicken *ND5* gene amplification

Primer Name	Primer Sequence	Tm Primer	Amplicon Size	Reference
Gallus-F	5'-TCACCCACCCAAACCAAACA-3'	57.3°C	189 bp	[5]
Gallus-R	5'-GGTGGGTGCGGATGAGTAAA-3'	57.4°C		

2.2 Methods

This research is a descriptive study using a molecular approach by optimizing the molecular-based *ND5* Gallus (chicken) gene authentication method for processed chicken meat product samples.

Genomic DNA Isolation

The DNA isolation process followed the QIAamp DNA Mini Kit protocol. Chicken meat was isolated in five sample weight variations as a qPCR standard curve: 10 mg, 20 mg, 30 mg, 40 mg, and 50 mg, while chicken nuggets were isolated from 50 mg samples. Each one was put in a sterile 1.5 mL microtube. Buffer 180 µL of ATL and 20 µL of Proteinase K were added, then vortexed and incubated at 56°C until complete lysis (1–3 hours), with vortexing occasionally. Next, 200 µL of buffer AL was added, vortexed for 15 seconds, and incubated at 70°C for 10 minutes. After that, 200 µL of absolute ethanol was added, vortexed, and spun down briefly. The mixture is pipetted in the QIAamp Mini spin column and centrifuged at 8000 rpm for 5 minutes. Flow-through is discarded, and the QIAamp Mini Spin column is repeated in buffers AW1 and AW2 by centrifuging respectively at 8000 rpm for 5 minutes and 13,000 rpm for 5 minutes. After dry centrifugation, the spin column was placed in a new tube, and 50 µL buffer AE was added, incubated, and centrifuged at 8000 rpm for 1 minute to elute DNA. DNA quality and quantity were measured using a NanoPhotometer. To ensure DNA purity (A260/280 1.8-2.0), dilution is carried out if necessary. If the results of the first

elution do not meet the criteria, a second elution is carried out with an additional 50 µL buffer AE.

Optimization of PCR Annealing Temperature

To get the optimum annealing temperature, gradient PCR was carried out with the following reaction composition: 10 µL total volume including 5 µL 2x GoTaq Green MM, 1 µL chicken DNA as template, 0.5 µL primer *Gallus*-F 10 µM, and 0.5 µL primer *Gallus*-R 10 µM. All solutions were homogenized by a brief vortex. PCR was carried out according to the protocol of the 2x GoTaq Green MM program with initial denaturation at 95°C for 2 minutes, followed by 35 cycles of denaturation at 95°C for 30 seconds, annealing using a temperature gradient range (52°C, 53.3°C, 55.3°C, 56.4°C, 57.6°C, 58.7°C, 61.5°C) for 30 seconds, and elongation at 72°C for 10 seconds. The final elongation stage was carried out at 72°C for 5 minutes. PCR products were visualized using 1.5% agarose gel electrophoresis with running buffer TAE 1X at 100 volts for 30 minutes, and the results were observed using GelDoc to determine the optimum temperature annealing based on the single and thickest DNA band and appropriate amplicon size.

Authentication Test

DNA molecules were extracted using the QIAamp DNA Mini Kit method from nuggets from three different brands used as template DNA in the process of amplification for authentication testing. Composition of qPCR reactions *ND5* consists of 5 µL of 2x SensiFAST™ SYBR® No-ROX mix (Bioline), 0.5 µL of primer *Gallus-F* 10 µM dan 0.5 µL of primer *Gallus-R* 10 µM, 1 µL of DNA template, and nuclease-free water to bring the reaction volume to 10 µL. This was also done for No-Template Control (NTC). The qPCR reaction was carried out according to the following program: enzyme activation at 95°C for 3 minutes, followed by 40 cycles consisting of denaturation at temperature 95°C for 5 seconds and annealing-extension at the optimum annealing temperature for 30 seconds. The qPCR stage ends with the melting curve analysis by raising the temperature by 1°C of 65°C gradually up to 95°C and holding it for 1 minute. Data from the authentication test were in the form of Ct values, which will be converted into chicken meat mass based on a standard curve with the linear regression equation obtained. From this data, the percentage composition of chicken meat in chicken nuggets can be calculated with the following formula:

$$\%\ \text{Chicken meat on nugget} = \frac{\text{Chicken meat concentration } (\frac{\text{mg}}{\text{mL}})}{\frac{\text{Mass of chicken nugget (mg)}}{\text{Volume of elution buffer (mL)}}} x100\%$$

3 Results and discussion

3.1 Results

Genomic DNA Isolation

The results of measuring the quality and quantity of DNA isolation using the QIAamp DNA Mini Kit isolation method can be seen in Table 2. In this method, all samples have purity values close to good standards.

Table 2. Results of DNA Quantity and Quality Measurements

Sample	Concentration (ng/µl)	Purity (A260/A280)
Chicken meat 10 mg	192.65	2.15
Chicken meat 20 mg	105.55	2.11
Chicken meat 30 mg	291.45	2.15
Chicken meat 40 mg	437.20	2.12
Chicken meat 50 mg	243.35	2.12
Nugget C	88.700	2.05
Nugget TD	41.050	2.02
Nugget D	59.500	2.07

Optimization of PCR Annealing Temperature

Primer synthesis results from IDT, Singapore, show that the Tm of primer *Gallus*-F is 57.3°C and the Tm of primer *Gallus*-R is 57.4°C. With this data then the gradient temperature used was in the range of 52°C – 61.5°C. Based on the results of gradient PCR product electrophoresis (Figure 1), the optimum annealing temperature was 57.6°C. This is based on the thickest band and closest to the amplicon size, namely ±189 bp.

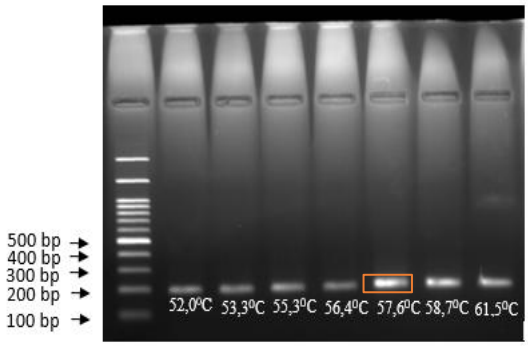


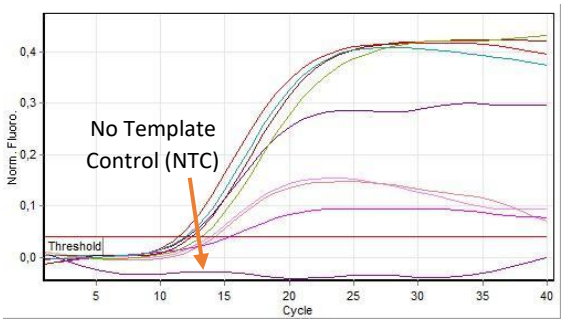
Fig. 1 Electropherogram of chicken DNA amplification results with variations in annealing temperature

Authentication Test

Based on the results of DNA sample isolation on chicken nuggets carried out using the QIAamp DNA Mini Kit method, DNA measurement results were obtained with good concentration and purity (Table 2). Followed by amplification using the optimum real-time PCR method and carrying out an authentication test using processed chicken meat food samples in the form of nuggets.

Based on the results of the authentication test amplification curve contained in Figure 2, it can be seen that chicken DNA with a mass of 10 mg (SB10) produces a Ct value of 11.38; chicken DNA with a mass of 20 mg (SB20) produces a Ct value of 12.08; on chicken DNA with a mass of 30 mg (SB30), it produces a Ct value of 13.82; on chicken DNA with a mass of 40 mg (SB40), it produces a Ct value of 14.15; and on chicken DNA with a mass of 50 mg (SB50), it produces a Ct value of 15.31. For authentication tests, on sample nugget C, it produces a Ct value of 12.48; on sample nugget TD, it produces a Ct value of 13.21; and on sample

nugget D, it produces a Ct value of 11.94. The amplification results are also presented in the form of a standard curve in Figure 3.



No.	Color	Name	Type	Ct	Given Conc (mg/ml)	Calc Conc (mg/ml)
4		SB20	Standard	12,08	4,00E+02	3,89E+02
5		SB30	Standard	13,82	6,00E+02	6,58E+02
8		SB40	Standard	14,15	8,00E+02	7,27E+02
10		SB50	Standard	15,31	1,00E+03	1,03E+03
20		C	Unknown	12,48		4,38E+02
22		TD	Unknown	13,21		5,47E+02
23		D	Unknown	11,94		3,73E+02
26		NTC	NTC			

Fig. 2 Authentication Test Amplification Curve on Chicken Nugget Samples

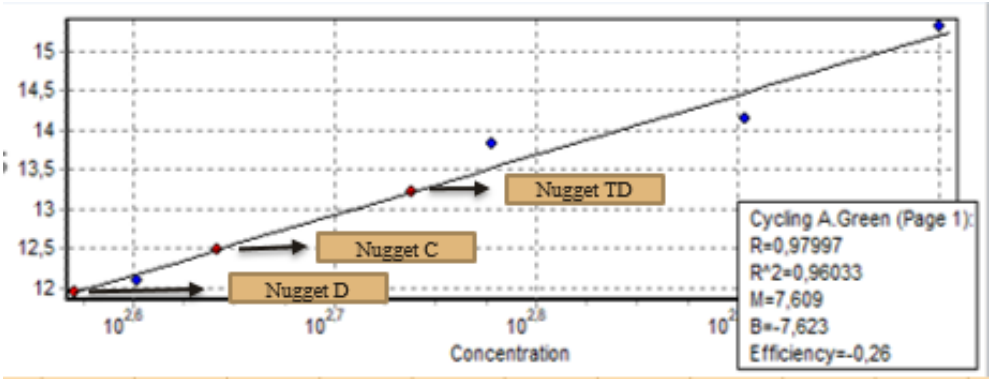


Fig. 3 Standard Curve of Authentication Test Results on Nugget Chicken

Based on the standard curve (Fig. 3), there are two components: the red dot indicates the DNA of the nugget sample (unknown) and the blue dots indicate chicken DNA (standard). The standard curve has an R^2 value equal to 0.96033, with the line equation y = 7.609x - 7.623 (y: Ct value, x: log of sample concentration). Based on the linear regression equation, the starting material can be calculated in units of mg/ml (Fig. 2). This value can then be used to determine the chicken meat content in chicken nuggets using the % chicken meat formula above; it is known that the mass of the nugget used for DNA isolation was 50 mg and eluted with 50 µl buffer AE. The chicken meat content in nugget C is 43.8%, nugget TD 54.7%, and nugget D 37.3%. TD nugget samples had the highest percentage of chicken meat content (Table 3).

Table 3. Calculation results of chicken meat content

No	Sample	Chicken meat concentration (mg/ml)	% Chicken meat	
			Authentication Test	Information on Nugget Packaging
1	DNA *Nugget* C	438	43.8%	35.26%
2	DNA *Nugget* TD	547	54.7%	No information
3	DNA *Nugget* D	373	37.3%	No information

3.2 Discussion

The optimum annealing temperature for the Gallus-F and Gallus-R primer pair was successfully obtained, namely 57.6°C (Figure 1). Although other annealing temperature variations also produce specific DNA bands with appropriate sizes (±186 bp), the DNA band at a temperature of 57.6°C looks thicker. The thicker band intensity means that more template DNA is amplified [7]. Optimizing the PCR conditions of annealing temperature is essential because it is related to the specificity and sensitivity of PCR [8]. High annealing temperatures can make it difficult for primers to bind to the DNA template, resulting in PCR products that are thin or even invisible. If the annealing temperature is too low, the primer will bind to the DNA in non-specific regions [9].

The primer pair of Gallus-F and Gallus-R was chosen because their specificity had been tested in silico by Safitri and Achyar [5]. These primers amplify five Gallus species, including *Gallus gallus* (chicken), *Gallus gallus spadiceus* (red partridge), *Gallus lafayetii* (Sri Lankan wild chicken), *Gallus varius* (green partridge), and *Gallus sonneratii* (gray partridge). This primer pair does not amplify *Bos taurus* (cow), *Brachyura* (crab), *Salmo salar (salmon), Scomberomorini* (mackerel), and *Caridea* (shrimp). Thus, the primer pair Gallus-F and Gallus-R specifically amplified five Gallus species.

Martati et al. [10] also optimized the annealing temperature in detecting the expression level of the Superoxide Dismutase gene (*SOD*) in the liver tissue of Wistar rats (*Rattus novergicus*) with real-time PCR. The optimum annealing temperature obtained for the SOD target gene primer pair was 50°C. In real-time PCR, the optimum annealing temperature is very important to obtain a single peak output and the lowest possible Ct value. A single peak in the melt curve analysis indicates high primer specificity, while a low Ct value indicates higher sensitivity [10]. qPCR amplification was carried out with SYBR Green as a fluorescent dye, which binds to double-stranded DNA, producing a signal that increases with the number of cycles. Amplification and melting curve analysis were used to detect specific amplification and primer dimers [11].

The authentication test was carried out to determine the ability of the optimized qPCR method to detect the authenticity or correctness of information on the content of chicken meat in chicken nuggets. Based on the results of the authentication test, nugget C contains 43.8% chicken meat, while the packaging states that the chicken meat content is 35.26%. In addition, TD nuggets contain 54.7% and D nuggets 37.3%, but these two nuggets do not have information on the percentage of meat content on the packaging. Thus, the TD nugget sample has a higher percentage of chicken meat content.

The National Standardization Agency (BSN) has classified chicken meat nuggets into two categories: chicken meat nuggets with a minimum chicken meat content of 35% and combination chicken meat nuggets with a minimum chicken meat content of 23% [12]. Thus, the percentage of chicken meat in each of the three nugget samples was still within the Indonesian National Standard (SNI) for chicken nuggets. According to research conducted by Deshazo et al. [2], some of the nuggets under investigation only had 40–50%

chicken flesh; the remaining nuggets contained the same or more fat in addition to bone, connective tissue, nerves, and epithelium.

Research on the authenticity of chicken nuggets has revealed a variety of approaches for maintaining product integrity and consumer safety. The use of omics disciplines such as genomics, transcriptomics, proteomics, metabolomics, etc., has been stressed for identifying chicken species and breeds in processed meals, especially nuggets. These investigations emphasize the need to correctly identify chicken content and detect potential adulteration, especially with non-chicken species [13]. Several studies that developed PCR-based techniques in the application of detection and authentication of meat products using the *ND5* gene as a biomarker showed high sensitivity with a limit of detection (LOD) of 0.01-0.001 ng for buffalo and porcine [14] and 0.002 ng for cattle [15]. The chicken *ND5* gene was used by [16] to study the genetic variety between Black and White Lines of Silky Fowl (*Gallus gallus*). However, there is no study involving the chicken *ND5* gene as a tool for chicken meat authentication.
Several genes used for the chicken detection were mitochondrial *cytb* [17]; [18]; [19]; [20]; [21] ; [22]; [23]; [24]; [25], *TGF-beta-3* [15], and mitochondrial 16S rRNA gene [26]; [27]; [28]. Those studies developed chicken meat adulteration detection with a variety limit of detection.

However, there is no available PCR-based method that can quantify the content of chicken meat in chicken-processed food. This is very important to investigate processed food products circulating on the market. This study offers the advantages of using real-time PCR to quantify the starting material of chicken meat in the chicken nugget, whether it is consistent with the information specified in the packaging by the producer or not.

4 Conclusion

The optimum annealing temperature for chicken *ND5* gene qPCR is 57.6°C. Results of trials to authenticate chicken meat content in nugget products using the chicken *ND5* qPCR method show nugget TD contains 54.7%, nugget C contains 43.8%, and nugget D contains 37.3%. This study offers the advantages of using real-time PCR to quantify the starting material of chicken meat in the chicken nugget with the need for further validation.

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