Primers and Probes Design of Multiplex qPCR For Food Authentication Detection

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Abstract. Indonesia continues to confront difficulties and barriers in obtaining halal certification because some unresponsible individuals continue to adulterate halal food by combining it with pork or fanged animals in order to increase their profits. Therefore, it is necessary for Halal Examining Institutions (Lembaga Pemeriksa Halal/LPH) to create techniques that can identify different types of non-halal meat in a product. The solution is multiplex quantitative real-time PCR (qPCR), which can quantitatively and concurrently detect the non-halal content of numerous animal species. The most important component that determines the optimal performance of multiplex qPCR analysis is the design of the primers and probes. In order to detect and amplify DNA from swine, rat, and bovine, this study attempts to build primers and probes that satisfy the multiplex qPCR criteria for food authentication. Using Geneious Prime software, the ND5 sequences of Sus scrofa, Rattus rattus, and Bos taurus were aligned. In contrast to designing a probe using a species-specific sequence, primers for these species were created using a conserved area. These bioinformatic analyses led to the development of two conserved primers with 166 bp amplicons and Sus, Rattus, and Bos species-specific probes.

Keywords: Food authentication detection · Multiplex qPCR · ND5 · Porcine · Rat

1 Introduction

Halal cuisine is currently a concern for several Muslim-majority and Muslim-minority nations, including Thailand, Vietnam, South Korea, China, and European nations. It is difficult to compete in the industrial halal market. Due to the presumption that every product produced in Indonesia is halal, halal certification for that product is not necessary, Indonesia still has difficulties and barriers with regard to halal certification. However, it came out that irresponsible individuals had mixed halal food with pork or fanged animals in order to increase their profits. Therefore, it is necessary for Halal Examining Institutions (Lembaga Pemeriksa Halal/LPH) to create techniques for identifying non-halal components in products.

LPH is assisting Halal Product Guarantee Agency (Badan Penyelenggara Jaminan Produk Halal/BPJPH) in examining and / or testing the halalness of a product. LPPOM

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MUI is one LPH owned by MUI (Majelis Ulama Indonesia) that facilitates halal certification in Indonesia and is equipped with a halal testing laboratory [1–4]. Various analysis methods are offered by the LPPOM MUI Laboratory on its official website www.halalmui.org including porcine specific protein with rapid tests, identification of species (porcine, chicken, bovine, sheep, horse) using real time PCR, and so on. However, pork is not the only haram content found in food products. The case of mixing beef with rat meat in meatball products must be proven through testing in a halal laboratory [5, 6].

Multiplex real time PCR technique can be used as a solution in detecting the contamination of porcine and rat simultaneously. Multiplex real time PCR is a variant of the PCR method that can detect, amplify and quantify several target genes at once in real time in one PCR reaction. This technique offers effectiveness and efficiency in halal testing because it only requires small amounts of samples and reagents and saves processing time [7–11].

To get accurate results, one of the keys to the success of multiplex real time PCR lies in the primers and probes design that will be used [12]. Thus, this study aims to design a pair of universal primers and species-specific probes that meet the multiplex qPCR criteria to detect and amplify DNA from porcine, rat and bovine.

2 Material and Method

2.1 Primer and Probe Design

The target gene to be amplified was mitochondrial DNA, namely the ND5 gene which encodes NADH dehydrogenase subunit 5 in Sus scrofa, Rattus rattus and Bos taurus. Genes in mitochondrial DNA are often used in species identification because of the high sequence variation between species and more copies per cell than nuclear DNA [13].

ND5 sequence templates of B. taurus (NC_006853.1), S. scrofa (NC_000845.1) and R. rattus (NC_012374.1) came from NCBI downloads. The sequences were aligned using multiple alignment tools of Geneious Prime bioinformatic software (https://www.geneious.com). According to the conserved region of these three species, a pair of primers was created. Meanwhile, the specific region of each species was used as probes. The primers and probes specificity were analyzed using NCBI's Primer BLAST tools.

2.2 In Silico Multiplex PCR

The bioinformatics tool Geneious Prime was also used to simulate multiplex PCR or do in silico PCR. To produce a PCR product, the system was given the ND5 sequence template, primer, and probe sequences. Using the style palette present in the template, styles can be applied.

3 Result and Discussion

3.1 Primer and Probe Design

While the probes were created specifically for each species, the primers were created in regions with conserved sequences for each species so that they may amplify simultaneously in a single PCR. Using primer design tools on Geneious Prime, the potential primer or probe sequence was examined. Table 1 displays the outcomes of the primer and probe configurations.

Primer for multiplex real-time PCR still had to meet the standard of ideal criteria of a primer that is 18–30 bases in length, 40–60% GC content, Tm does not have a secondary structure like a hairpin or primer dimer, and the temperature difference between forward and reverse is less than 3 °C [14, 15]. In addition, primers and probes that use multiplex real-time PCR do not form dimers between primers and probes [16]. The data in Table 1 showed that the designed primers and probes were meet the ideal parameter.

The number of target genes that need to be amplified in multiplex real-time PCR is another factor that must be taken into account because it depends on the real-time PCR thermal cycler employed. The number of channels real-time PCR devices have available to detect fluorescent probes is what restricts the number of target genes [17].

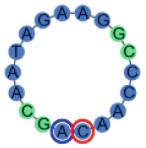
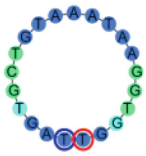
Using the Primer-BLAST tools on the NCBI website, the specificity of the constructed primers and probes was reviewed [18, 19]. Table 2 provides an overview of the NCBI Primer-BLAST outcomes. The results showed that BoSusRat_F and BoSusRat_R primer mostly annealed to all species members of the Bos, Sus, and Rattus. Meanwhile, each probe was specifically annealed only to the member of its genus. The probe Bos detected the species of the Bos genus; the probe Sus detected the species of the Sus genus, and the probe Rat detected the species of the Rat genus.

3.2 In Silico Multiplex PCR

Using geneious prime, in silico PCR was carried out concurrently for the nd5 sequences of B. Taurus (nc 006853.1), S. Scrofa (nc 000845.1), and R. Rattus (nc 012374.1). Figure 1 depicts the silico PCR outcome. The silico PCR result was consistent with the primer blast result in that the probes specifically recognized the genus whereas the primer universally annealed to the b. Taurus, s. Scrofa, and r. Rattus nd5 sequence.

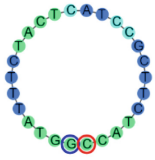
PCR is also known as electronic PCR or virtual PCR in silico since it is computationally amplified with the use of bioinformatic tools. Prior to performing laboratory studies, it is crucial to perform in silico PCR to check for faults in primer design and ensure that the primer's anneal is solely unique to the target gene [20, 21].

Table 1. Designed primer or probe's generous prime output characteristic

No.	Primer/Probe	
	Characteristic	*DNAFold*
1.	Name: BoSusRat_F Type: bind primer (bind primer) (Created by primer3) Length: 18 Interval: 720 -> 737 (705 -> 722) Mismatches: 0 %GC: 50.0 Hairpin Tm: None Self-Dimer Tm: None Tm: 54.5 Pair Dimer Tm: None Sequence: AGCAATAGAAGGCCCAAC Product Size: 166	
2.	Name: BoSusRat_R Type: Primer Bind (primer bind) (Created by primer3) Length: 21 Interval: 885 -> 865 (870 -> 850) Mismatches: 0 %GC: 38.1 Hairpin Tm: None Self-Dimer Tm: None Tm: 54.3 Pair Dimer Tm: None Sequence: TAGTGCTGTAAATAAGGTGGT Product Size: 166	

(*continued*)

Table 1. (*continued*)

No.	Primer/Probe	
	Characteristic	*DNAFold*
3.	Name: Probe Bos Type: DNA probe bind (Created by primer3) Length: 23 Interval: 795 -> 817 (780 -> 802) Mismatches: 0 %GC: 47.8 Hairpin Tm: None Self-Dimer Tm: None Tm: 59.9 Pair Dimer Tm: None Sequence: AATCCGTTTCTATCCCCTCACAG	
4.	Name: Probe Sus Type: DNA probe bind (Created by primer3) Length: 26 Interval: 783 -> 808 (768 -> 793) Mismatches: 0 %GC: 46.2 Hairpin Tm: None Self-Dimer Tm: None Tm: 60.8 Pair Dimer Tm: None Sequence: GGTATTTCTACTCATCCGCTTCTACC	

(*continued*)

Table 1. (*continued*)

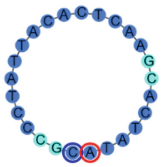
No.	Primer/Probe	
	Characteristic	*DNAFold*
5.	Name: Probe Rattus Type: DNA probe bind (Created by primer3) Length: 25 Interval: 747 -> 771 (729 -> 753) Mismatches: 0 %GC: 44.0 Hairpin Tm: None Self-Dimer Tm: None Tm: 60.3 Pair Dimer Tm: None Sequence: CGCCCTATTACACTCAAGCACTATA	

Table 2. Result of primer and probe specificity check using primer blast

Target Template	Primer	Probe		
	BoSusRat_F and BoSusRat_R	*Bos*	*Sus*	*Rat*
Bos taurus	yes	yes	no	no
Bos frontalis	yes	yes*[1]	no	no
Bos grunniens	yes	yes*[4]	no	no
Bos javanicus	yes	yes	no	no
Bos sauveli	no	no	no	no
Sus scrofa	yes	no	yes	no
Sus ahoenobarbus	no	no	no	no
Sus barbatus	yes	no	yes	no
Sus bucculentus	no	no	no	no
Sus cebifrons	yes	no	yes*[1]	no
Sus celebensis	yes	no	yes*[1]	no
Sus oliveri	no	no	no	no

(*continued*)

Table 2. (*continued*)

Target Template	Primer	Probe		
	BoSusRat_F and BoSusRat_R	*Bos*	*Sus*	*Rat*
Sus philippensis	no	no	no	no
Sus salvanius	no	no	no	no
Sus verrucosus	yes	no	yes*[1]	no
Rattus adustus	no	no	no	no
Rattus andamanensis	yes	no	no	yes*[2]
Rattus annandalei	no	no	no	no
Rattus korinchi	yes	no	no	yes*[3]
Rattus tanezumi	yes	no	no	yes
Rattus hoogerwerfi	yes	no	no	yes*[1]
Rattus leucopus	yes	no	no	yes*[2]
Rattus sordidus	yes	no	no	yes*[1]
Rattus lutreolus	yes	no	no	yes*[1]
Rattus rattus	yes	no	no	yes
Rattus exulans	yes	no	no	yes*[1]
Rattus norvegicus	no	no	no	no
Mus musculus	yes	no	no	yes*[5]

* number of mismatches between probe and target sequence.

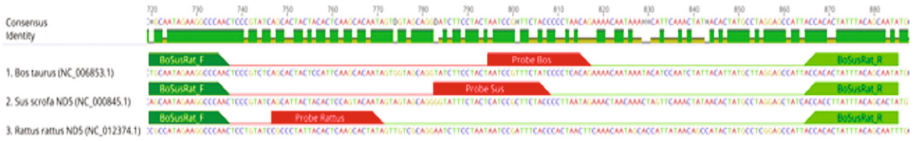


Fig. 1. In Silico PCR of Universal Primer and Species-Specific Probes on Multiple Alignment of *ND5* Sequences. Red arrows indicate probes, while green arrows indicate forward primer and reverse primer

4 Conclusion

This study produced a pair of universal primers that amplify the 166 bp segment of the mitochondrial ND5 gene in members of the Bos, Sus, and Rattus genus. The development of specific probes to identify the mitochondrial ND5 gene in B. taurus, S. scrofa, and R. rattus was also successful.

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