# Primer Design and Optimization of PCR Methods for Detecting Mixed Rat Meat in Food Samples

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**Abstract.** Food safety is very important to support the halal tourism program. One of the problems faced by Muslim consumers is the possibility of mixing non-halal meat such as rats in meat-based food products. Molecularly we can detect the presence of rat meat in meat-based products using the PCR technique. It is necessary to design a PCR primer based on the type of rodentia that is often found in landfills, so that the detection results are more accurate. This study aims to design rodents-specific primers and optimize PCR method for detecting mixed rat meat in food samples. Primers were designed using Geneious Prime and Primer BLAST on NCBI (National Center for Biotechnology Information). The specific primers were designed on the mitochondrial region called ND5 gene (NADH dehydrogenase subunit 5), which are forward primer 5′-GCAGTTCTCTTCATGATAYATAC-3′ and reverse primer 5′-GTTTCAGGCGTTGGTGTT-3′ with a PCR product size of 919 bp. The optimization steps carried out in the PCR process include optimization of annealing temperature and primer concentration. The optimization results show that the optimum annealing temperature was 48 °C and the optimum primer concentration was 0.4 μM because it produces a thick and clear specific band.

**Keywords:** Primer Design · PCR Optimization · Rodent ND5 Gene · Halal Detection

## 1 Introduction

Halal tourism is part of the tourism industry aimed at Muslim tourists. Halal tourism includes understanding Halal meaning of all aspects of tourist activities. Halal tourism services provided to tourists refer to the rules Islam. One example of Islamic service in halal tourism is providing halal food services [1].

Halal food is food that can be eaten according to Islamic teachings, which must be in accordance with what is ordered in the Qur'an and Hadith. Food can be said to be halal if there are no elements or ingredients that are prohibited for consumption by Muslims and the processing does not conflict with Islamic law [2]. One of the food ingredients that are not halal in Islamic teachings besides pigs and dogs are rats. When viewed from

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a religious perspective, the presence of rats as food is a serious concern for followers of the Islamic religion.

Food safety is very important to support the halal tourism program. One of the problems faced by Muslim consumers is the possibility of mixing non-halal meat such as rats in meat-based food products. This counterfeiting action was taken to reduce production costs because rats were easy to obtain [3]. Based on an investigation conducted by liputan6.com in 2011 it was found that the perpetrators of forgeries hunted rats in landfills.

Molecularly mixing rat meat in meat-based products can be detected using the PCR technique. The PCR technique can detect genes quickly and accurately. PCR also has high sensitivity and specificity [3]. The PCR method has often been used for testing related to DNA, including detecting the presence of mixing rat meat in food products [4–6]. However, the PCR method used in this study generally used DNA sequences from *Rattus novergicus* in the PCR primer design because this type of rat is commonly used as experimental animals in the laboratory. In fact, based on research conducted by [7], the types of rodents that are generally found in landfills are house shrews *(Suncus murinus)*, riol rats *(Riol novergicus)*, house mice *(Mus musculus)*, house mice *(Rattus rattus)*, wirok rat *(Bandicota indica)*, and rice field rat *(Rattus argentiventer)*. However, the PCR primer for these various rat species has not available yet, therefore, it is necessary to design a PCR primer based on the type of rodentia that is often found in landfills, so that the detection process can cover all rat species and can be more accurate.

Prior to PCR with the sample to be tested, optimization needs to be carried out in order to obtain the appropriate PCR composition and conditions so as to obtain optimal PCR results. Optimization of the PCR method needs to be done for time efficiency and material use so that the detection process can be carried out quickly and precisely. PCR optimization can be done by varying the primer concentration and *annealing* temperature.

## 2 Materials and Methods

### 2.1 Materials

The materials used in this study were complete mitochondrial DNA sequences of *Rattus rattus* (NC_012374.1), *Mus musculus* (KY018919.1), *Bandicota indica* (NC_028335.1), *Suncus murinus* (NC_024604.1), and *Rattus novergicus* (KM_820832.1). Downloaded from GenBank NCBI (http://www.ncbi.nlm.nih.gov). Mouse DNA isolate, 2x my Taq HS Red Mix, designed forward and reverse primers, *nuclease-free water* 1.5% agarose gel, 1x TAE buffer, 100 bp DNA ladder, loading dye, and GelRed.

### 2.2 Methods

In this study, *Muridae*-specific primer design was carried out using primer3 which was integrated into the Geneious Prime software. The specific primer designed on the mitochondrial region is the *ND5* gene (NADH dehydrogenase subunit 5). The initial stage of primary design is to determine the target gene at the *Gene Bank National Center for Biotechnology Information* (NCBI). The primer designed must meet the criteria for

an ideal primer with a nucleotide length of 18–25 bp, the difference in Tm *(melting temperature)* between the forward and reverse primers is not more than 5 °C, and does not form a secondary structure *(hairpin or self-dimer).*

Primary specificity was checked in silico using NCBI BLAST primer. After that, check again using the Geneious Prime software. The purpose of this check is to ensure that the primer that has been designed will adhere well to the template DNA sequence. The design primers that met the criteria were then synthesized at IDT, Singapore.

Optimization was carried out using gradient PCR with the annealing temperature setting used calculated based on (Tm- 5) to (Tm + 5). The PCR conditions used were: initial denaturation at 95 °C, followed by 35 cycles consisting of denaturation at 95 °C for 15 s, annealing using a temperature range of ± 5 °C from the Primary Tm value for 15 s, and elongation at 72 °C for 10 s. The PCR process ended with a final elongation step at 72 °C for 5 min. The PCR products were visualized by electrophoresis of 1.5% agarose gel. Then the results of the electrophoresis were seen using a UV transilluminator. Furthermore, optimization of the primary concentration was carried out starting from a low concentration of 0.2 μM; 0.3 μM; 0.4 μM to 0.5 μM. The PCR program was set using the optimum annealing temperature.

## 3 Result and Discussion

Primer design is the first step that determines the success of DNA amplification by the PCR method. The design to obtain a primer that meets the criteria for a good primer for amplification is carried out in silico, namely designing the primer with the help of a computer program. In this study, the primary design was carried out using the ND5 gene sequences *Rattus rattus, Rattus novergicus, Suncus murinus, Mus musculus,* and *Bandicot indicota,* which were aligned using multiple alignment tools on Geneious Prime (Fig. 1). The goal is that the designed primer can amplify all rodentia that are commonly found in landfills as rat hunting locations.

In general, the ideal primer is between 18 and 30 oligonucleotides in length. A good primer is a primer that meets the criteria for primary parameters. These parameters include: melting temperature (Tm), percentage of the amount of G and C (%GC), hairpin and self-dimer. The primary criteria can be viewed using the Genious Prime bioinformatics software to ascertain whether the primers that have been designed have met the criteria (Table 1).

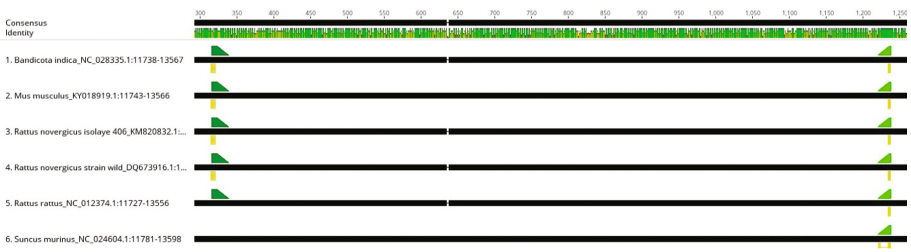
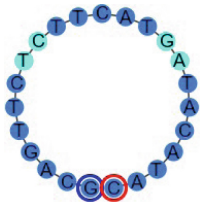
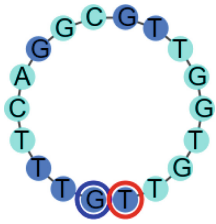



**Fig. 1.** Muridae-specific primary design.

**Table 1.** Primary Characteristics of Design Results

| No | Karakteristik Primer | DNA Fold |
|---|---|---|
| 1 | Name: Mur_MA_Fwd<br>Type: Primer Bind (primer_bind) (Created by primer3)<br>Length: 23<br>Interval: 316 -> 338<br>%GC: 36, 4<br>Hairpin Tm: None *Self-Dimer*: None Tm: 52, 4 – 55, 0<br>Sequence: GCAGTTCTCTTCATGATAYATAC<br>Product size: 919 |  |
| 2 | Name: Mur_MA_Rev<br>Type: Primer Bind (primer_bind) (Created by primer3)<br>Length: 18<br>Interval: 1237 -> 1220<br>%GC: 50,0<br>Hairpin Tm: None Self-Dimer: None Tm: 56, 1<br>Sequence: GTTTCAGGCGTTGGTGTT<br>Product size: 919 |  |

The results (Table 1) obtained one universal forward primer for rodents which has a length of 23 bases with a sequence of 5′-GCAGTTCTCTTCATGATAYATAC-3′ and a reverse primer has a length of 18 bases with a sequence of 5′-GTTTCAGGCGTTGGTGTT-3′ with a PCR product size of 919 bp. This result is in accordance with the required number of nucleotide bases, which ranges from 18 to 30 oligonucleotides. If the primer is too short it can reduce the specificity of the primer. Primers that have a length of more than 30 base pairs will cause primer attachment to be non-specific [8].

The selection of the Tm of a primer is very important because the Tm of the primer will be very influential in the selection of the annealing temperature of the PCR process [9, 10]. Melting temperature (Tm) is the temperature at which 50% of the DNA double strands are separated. A good Tm requirement is in the range of 42–65 °C. Primers with too high Tm (>65 °C) will reduce the effectiveness of annealing so that the DNA amplification process does not work well. Meanwhile, Tm that is too low has a tendency to stick elsewhere and produce non-specific products. The recommended Tm difference is not more than 5 °C. Tm in this primer pair is 52.4–55.0 on the forward primer and 56.15 °C on the reverse primer with a temperature difference of 1,15–3,75 °C (Table 1).

The percentage between bases G and C also needs to be considered because the content of the number of bases G and C can affect the Tm of a primer. %GC is the percentage of guanine and cytosine in a primer, %GC should be in the range of 40–60% [9]. The designed reverse primer has %GC which is still in the range of 50%, while the forward primer is slightly below the 40–60% range, which is 36.41% (Table 1). If a primer has a low %GC value, it can reduce the efficiency of the PCR process because

the primer is not able to compete effectively for attachment to the template. Meanwhile, if the primer has a high % GC value, it can cause the formation of a bond that is too strong between the primer and the target DNA so that the resulting PCR product is low but more specific.

Another criterion that greatly influences the effectiveness of the PCR process is the presence of hairpins and dimers. The formation of loop/hairpins structures and the presence of bonds with other similar primers (self-dimer) in the primer should be avoided. Hairpin is an intramolecular interaction in the primer. Hairpins in the primer can interfere with the primer attachment process to the template in the PCR process [9]. The primer that has been designed does not have hairpin and self-dimer so that the primer meets the ideal criteria (Table 1).

In silico primer specificity test was performed by performing Primer BLAST through the NCBI database. The results of the BLAST primers obtained using the design primers (MUR_MA_Fwd and MUR_MA_Rev) are summarized in Table 2.

Primary BLAST results are gene search results that are available in the GeneBank database with an application provided by the website, in this case the NCBI website. Primer blasting on GenBank (NCBI) showed that the designed primer was able to attach correctly only to Muridae (Table 2). The use of this BLAST primer is a simulation of whether the primer pieces that have been designed are about the target gene. The results of the in silico primer specificity test indicated that the primer could be used to detect the presence of mouse DNA under optimal PCR conditions.

**Table 2.** Primary Specificity Test using BLAST Primer

| Target template | Primer Mur_MA_Fwd & Mur_MA_Rev |
|---|---|
| *Rattus novergicus* | ✓ |
| *Rattus rattus* | ✓ |
| *Rattus tanezumi* | ✓ |
| *Rattus baluensis* | ✓ |
| *Rattus exulans* | ✓ |
| *Rattus tiomanicus* | ✓ |
| *Mus musculus* | ✓ |
| *Bandicota indica* | ✓ |
| *Chiropodomys gliroides* | ✓ |
| *Leopoldamys sabanus* | ✓ |
| *Niviventer fulvescens* | ✓ |
| *Suncus murinus* | X |
| *Rattus argentiventer* | X |
| *Gallus gallus* | X |
| *Bos taurus* | X |
| *Capra aegagrus* | X |
| *Sus scrofa* | X |
| *Bubalus bubalis* | X |
| Description: (✔) Can amplify, ( ✗ ) Cannot amplify | |

Based on the results of optimization conditions for detection of the ND5 gene in mice by PCR and agarose gel electrophoresis 1.5% at annealing temperature gradient (48.0 °C; 48.5 °C; 49.3 °C; 50.2 °C; 51, 3 °C; 52.4 °C; 54.7 °C; 56.7 °C; 58.0 °C) 35 cycles, annealing time of 15 s, and a sample volume of 1 L produces a very thin band only at 48.0 °C (Fig. 2). This is probably caused by the concentration of DNA template and less annealing time. So that the volume of DNA extracted from the extraction was carried out in the PCR reaction and annealing time.

Based on the results of these optimization conditions, re-optimization was carried out at the same annealing temperature by adding the DNA sample volume to 4.2 L in the PCR formula and increasing the annealing time to 30 s. The results of the second optimization condition showed bands at all temperatures, except at 56.7 °C and 58.0 °C (Fig. 3). The presence of bands at temperatures of 48.0 °C, 48.5 °C, 49.3 °C, 50.2 °C, 51.3 °C, 52.4 °C, and 54.7 °C illustrates that the addition of DNA volume and annealing time can increase the amplification product.

Based on the results of the study, it is known that the optimal annealing temperature for the detection of mouse genes using the Mur_MA primer pair is 48 °C from each temperature tested, the best visualization results are shown at 48 °C with the thickness of the visualization results being the thickest compared to other temperatures. This temperature variation is obtained based on the forward and reverse primers used. Each


**Fig. 2.** Electropherogram of rat DNA amplification with annealing time of 15 s. Note: (A) temperature 48.0 °C, (B) temperature 48.5 °C, (C) 49.3 °C, (D) 50.2 °C, (E) 51.3 °C, (F) 52, 4 °C, (G) 54.7 °C, (H) 56.7 °C, (I) 58.0 °C.

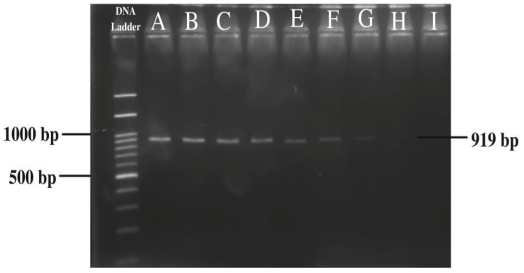



**Fig. 3.** Electropherogram of rat DNA amplification with annealing time of 30 s. Note: (A) temperature 48.0 °C, (B) temperature 48.5 °C, (C) 49.3 °C, (D) 50.2 °C, (E) 51.3 °C, (F) 52, 4 °C, (G) 54.7 °C, (H) 56.7 °C, (I) 58.0 °C.

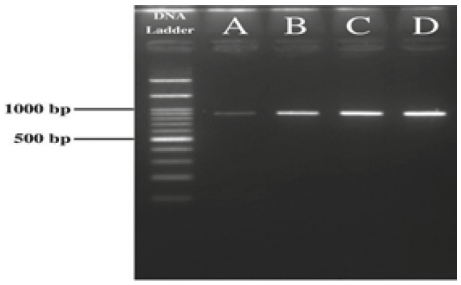



**Fig. 4.** Electropherogram of the amplification of the concentration variations of a pair of primers. Note: (A) 0.2 μM, (B) 0.3 μM, (C) 0.4 μM, (D) 0.5 μM

primer has different Tm (melting temperature) and guanine and cytosine content (GC content). The use of annealing temperature that is too high and inappropriate during the PCR process can have an impact on the sensitivity between primers and DNA templates so that the possibility of mispriming is very large so that it can affect the PCR product formed [11].

The primary concentrations were varied to 0.2 μM, 0.3 μM, 0.4 μM, and 0.5 μM. The results of PCR optimization on the four variations of primer concentration can be amplified well so that the DNA bands are clearly visible (Fig. 4). The DNA band targets are the same size but what distinguishes them from each other is the thickness and clarity of the bands.

Based on Fig. 4, the optimal forward and reverse primer concentration is 0.4 M. From the amplification results, it can be seen that the primer concentration greatly influences the resulting DNA band product. The primary concentration is usually optimal at 0.1–0.5 M. Primer concentrations that are too low or too high can result in no amplification. The higher the primary concentration can cause a thick band but sometimes followed by a non-specific band (unspecific band).

## 4 Conclusion

The sequence of nucleotide sequences from the primary design of the ND5 gene from Muridae is a forward primer 5′-GCAGTTCTCTTCATGATAYATAC-3′ and a reverse primer 5′-GTTTCAGGCGTTGGTGTT-3′ with a product length criterion of 919 bp. The design results meet the criteria well so that the primary candidate design results can be used for the PCR process. Primer blasting on GenBank (NCBI) showed that the designed primer was able to attach specifically to Muridae. The optimum condition for PCR using a pair of primers that have been designed is the annealing temperature of 48 °C. Furthermore, the optimum concentration for a pair of primers is 0.4 μM.

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