



## Identification of pig DNA using loop-mediated isothermal amplification

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### Abstract

Loop-mediated isothermal amplification (LAMP) assay and LAMP primers were developed in this study to detect mitochondrial DNA (mtDNA) of pig. The specific LAMP primers were designed from cytochrome *b* (*cyt b*) mtDNA of pig that composed of two inner primers (FIP, BIP) and outer primers (F3, B3). The FIP and BIP primers are tandem primers used for loop amplification. The FIP primer set is composed of the forward primer (F2) and the reverse F1c primer linked by additional 4 nucleotide bases, TTTT. The BIP primer set is composed of the reverse primer (B2) and the forward B1c primer linked by TTTT. All LAMP primers recognized six separate regions within target DNA. The *cyt b* mtDNA was amplified at a range of temperature 57-60°C using *Bst* DNA polymerase for 60 min with 1 ng of DNA from meat samples. The specificity of LAMP primers was performed using DNA from four meat species (pork, cattle, chicken and duck). The LAMP product was successfully amplified only from *cyt b* gene of pig whereas no amplified genes was detected from other species. These suggest that the LAMP assay studied was suitable for identifying pig DNA in meat samples.

**Keywords:** LAMP, pig DNA identification, mitochondrial DNA, cytochrome *b* gene

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## 1. Introduction

Identifications of meat and meat products for *Halal* authentication are major concern for Muslim consumers since Islamic law forbids Muslims from eating or using any products derived from pigs [1]. The analytical methods currently used to detect pork adulteration rely on either protein or DNA analysis [2]. More recently DNA based methods have been applied for meat identification with advantage that the DNA is much more stable molecule. Mitochondrial DNA (mtDNA) has been widely used for species identification, as it is undamaged by processing at cooking temperatures [3], this allows analysis of processed and heat treated products [4]. The mtDNA exists in multiple copies (approximately 1,000 copies) per cell [5] and relatively tolerant of environmental stresses such as heat, salt and pressure. In addition, its rate of evolution facilitates identification between closely related species [6]. The mitochondrial cytochrome *b* (*cyt b*) gene are numerous sequences available in the DNA bank databases, this work used mtDNA of *cyt b* gene as target for detection of pig DNA in meat samples.

Loop-mediated isothermal amplification (LAMP) is one-step amplification reaction that amplifies a target DNA sequence with high sensitivity, specificity and rapidity under isothermal condition [7]. LAMP is based on the principle of autocycling strand displacement DNA synthesis activity using *Bacillus stearothermophilus* (*Bst*) DNA polymerase and a set of four to six primers

that recognize six to eight regions within target DNA, making them highly specific [8]. The LAMP method has many characteristics that makes it suitable for the rapid and simple detection of nucleic acid sequences in samples, it has been used to detect bacteria [9-11], viruses [12-16], parasites [17-19], genetically modified organisms [20-23] and species origin of meat [1]. In this study, LAMP primers and LAMP assay condition were developed to detect pig DNA in meat sources using *cyt b* gene of mtDNA.

## 2. Materials and Experiment

### 2.1 Sample preparation and DNA extraction

Meat samples of pork, cattle, chicken and duck were used. Approximately 25 mg of ground meat was subjected for DNA extraction. DNA was extracted from the meat samples using Dneasy Tissue Kit (Qiagen, USA) according to the manufacturer's manual. The DNA solution was stored at 4°C and used as a template. DNA concentration was measured by UV absorption spectrophotometry.

### 2.2 The design of LAMP primers

The pork specific LAMP primers were designed using the *cyt b* sequences. The pig (*Sus scrofa*) sequences were collected from the database of NCBI (GenBank accession number KT279759.1) and multiple sequence alignment was performed using ClustalW in BioEdit program in order to develop a consensus sequence. Moreover, the MEGA7 program was employed to obtain phylogenetic trees. LAMP primers were designed using the Primer Explorer version 5.0 and synthesized by Pacific Science Co., Ltd.

### 2.3 LAMP reaction

The LAMP reactions were performed in a 25  $\mu$ l of total reaction mixture containing 0.008  $\mu$ M of each outer primer (F3, B3) 0.032  $\mu$ M of each inner primer (FIP, BIP), 1x reaction buffer, 1 M betaine, 2 mM MgSO<sub>4</sub>, 0.4 mM dNTPs, 8 units of *Bst* DNA polymerase (New England Biolabs, USA) and 0.4-2 ng of DNA template. Amplification reactions were carried out at 57-63°C for 30-90 min using C1000 Thermal Cycler (Bio-Rad Laboratories, Inc., USA). LAMP products were analyzed by 1% agarose gel electrophoresis. DNA ladder 100 bp and 1000 bp (GeneON, Germany) were used as size reference marker. LAMP products were visualized by the Bio-Rad Gel Doc XR+ system.

### 2.4 Specificity of LAMP assay

Specificity test was evaluated using DNA samples that were extracted from four meat species: pork (*Sus scrofa*), cattle (*Bos sp.*), chicken (*Gallus gallus*) and duck (*Anus sp.*). 1 ng DNA was used as the template in each LAMP assay, with equimolar primer amount. Nucleotide-free distilled water was used as a negative control.

## 3. Results and Discussion

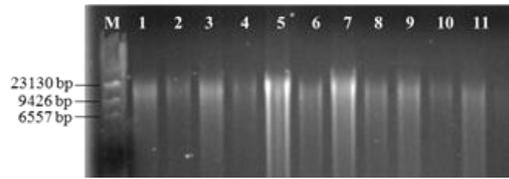
Genomic DNA was used as a template in LAMP assay. DNA samples from four species (pork, cattle, chicken and duck) were extracted using Dneasy Tissue Kit as the electrophoresis patterns of genomic DNA shown in Figure 1. A region of cytochrome *b* was selected for LAMP assay. The result from phylogenetic tree showed that nucleotide sequence of *cyt b* gene from pig was

distinguished from other species (cattle, chicken and duck) so it was suitable to be a target region for pig specific LAMP primer design (Figure 2). The Primer Explorer (version 5.0) software was used to design LAMP primers. F3 and B3 primers are forward and backward outer primer, respectively. The forward inner primer (FIP) and backward inner primer (BIP) are tandem primers used for loop amplification. The FIP primer set is composed of the forward primer (F2) and the reverse F1c primer linked by additional 4 nucleotide bases, TTTT. The BIP primer set is composed of the reverse primer (B2) and the forward B1c primer linked by TTTT (Table1). Nucleotide sequences of target regions are shaded over the *cyt b* gene corresponding to F3, F2, F1c, B1c, B2 and B3 primers (Figure 3).

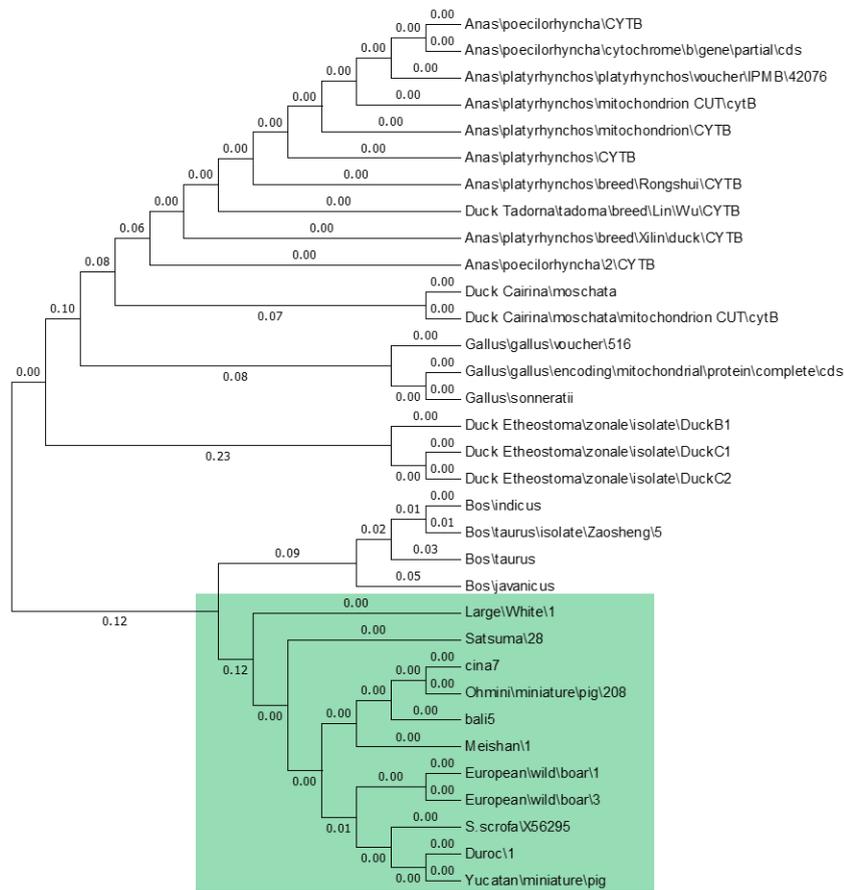
To determine the optimal condition for the LAMP assay, the reaction mixture was performed over a range of 57 to 63°C for 30-90 min. LAMP products were observed at 57 and 60°C as ladder-like pattern on gel electrophoresis from the reaction of 60 min with 1 ng of DNA template (Figure 4). The amplification efficiency appeared to decrease at 63°C (data not shown), as higher temperatures generally produce more stringent conditions for primer binding [24]. The result from Figure 4 indicated that LAMP primers were specific to pig DNA.

To test the specificity of the LAMP assay, DNA samples were isolated from four meat species (pork, cattle, chicken and duck). In all species, the LAMP product was successfully amplified only from *cyt b* gene of pig mtDNA. No amplification

was detected from other species or the negative control (Figure 5). The result showed that the LAMP primers were specific to the corresponding target specie. The data obtained from this study suggest that the LAMP assays have specificity, indicating that it is suitable for identifying pig DNA.



**Figure 1** Total genomic DNA extracted from pork (Lanes 1-3), cattle (Lanes 4-6), chicken (Lanes 7-8) and duck (Lanes 9-11). M:  $\lambda$ /HindIII DNA marker.



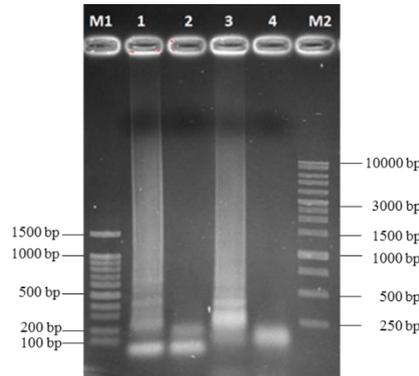
**Figure 2** *Cyt b* phylogenetic tree of pig, cattle, chicken and duck. *Anas* and *Gallus* : refer to genus of duck (*Anus* sp.) and chicken (*Gallus gallus*) respectively, whereas *Bos* refer to genus of cattle (*Bos* sp.). Target regions are shaded over the *cyt b* gene of pig.

**Table 1** Nucleotide sequences of the primers for LAMP

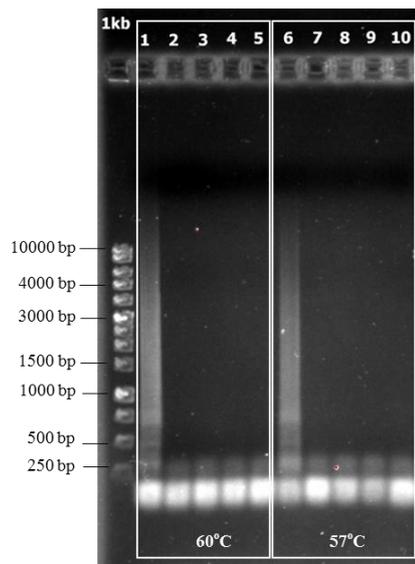
Primer	Sequences (5' to 3')
F3	ATTCATTGACCTCCCAGC
B3	TGTAGGTAGCGAATAACTCAT
FIP	GTTAGGATTTGCAAGATTAGGCAGATTTTCAAACATCTCATCATGATGAAACT
BIP	AGCAATACATTACACATCAGACACATTTTCGTAATTTACATCTCGACAGAT
F2	CAAACATCTCATCATGATGAAACT
F1c	GTTAGGATTTGCAAGATTAGGCAGA
B2	CGTAATTTACATCTCGACAGAT
B1c	AGCAATACATTACACATCAGACACA

<b>Target DNA</b> (Complement) <b>CONSENSUS (*)</b>	TCAACAACGC agttggttgcg *****	<b>ATTCATTGAC</b> taagtaactg *****	<b>CTCCCAGC</b> CC gagggtcggg *****	CCT <b>CAAACAT</b> ggag <b>gtttgta</b> *****	<b>CTCATCATGA</b> gagtagtact *****
<b>Primer</b>	41	51 <b>ATTCATTGAC</b>	61 <b>CTCCCAGC</b> F3	71 <b>CAAACAT</b>	81 <b>CTCATCATGA</b> F2
<b>TGAAACTTCG</b> actttgaagc *****	GTTCCCTCTT caagggagaa *****	AGGCATCTGC tccgt <b>agacg</b> *****	CTAATCTTGC <b>gattagaacg</b> *****	AAATCCTAAC <b>ttaggattg</b> *****	AGGCCTGTTC tccggacaag *****
91 <b>TGAAACT</b>	101	111 <b>agacg</b>	121 <b>gattagaacg</b> F1C	131 <b>ttaggattg</b>	141
<b>TTAGCAATAC</b> aatcggtatg *****	<b>ATTACACATC</b> taatgtgtag *****	<b>AGACACAACA</b> tctgtgtgtg *****	ACAGCTTTCT tgtcgaaga *****	CATCAGTTAC gtagtcaatg *****	ACACATCTGT tgtg <b>tagaca</b> *****
151 <b>AGCAATAC</b>	161 <b>ATTACACATC</b> B1c	171 <b>AGACACA</b>	181	191	201 <b>tagaca</b>
<b>CGAGATGTAA</b> <b>gctctacatt</b> *****	<b>AGTTATTTCGC</b> <b>tcaataagcg</b> *****	<b>TACCTACATG</b> <b>atggatgt</b> ac *****	CAAACGGAGC gtttgctcg *****	ATCCATGTTC taggtacaag *****	TTTATTGCC aaataaacgg *****
211 <b>gctctacatt</b> B2	231 <b>tcaataagcg</b> B3	241 <b>atggatgt</b>	251	261	271
<b>TATTCATCCA</b> ataagtaggt *****	CGTAGGCCGA gcatccggct *****				
281	291				

**Figure 3** Nucleotide sequences of target *cyt b* region used for designing primers. Nucleotide sequences of *cyt b* obtained from GenBank (accession number KT279759.1) of pig (*Sus scrofa*) were used to design LAMP primers. Target regions are shaded over the sequences of *cyt b* gene corresponding to the F3, F2, F1c, B1c, B2 and B3 regions.



**Figure 4** LAMP products of pork *cyt b* gene observed by 1% agarose gel electrophoresis. M1: marker 100 bp DNA ladder, Lane 1: LAMP amplicon of *cyt b* gene from pork (sample 1), Lane 2: negative control, Lane 3: LAMP amplicon of *cyt b* gene from pork (sample 2), Lane 4: negative control, M2: marker 1000 bp DNA ladder.



**Figure 5** Specificity test of pig DNA specific primers in LAMP reaction at 60 and 57°C observed by 1% agarose gel electrophoresis with 1000 bp DNA ladder as size reference marker. Four origin of the meat species of pork (*Sus scrofa*): Lanes 1 and 6, cattle (*Bos sp.*): Lanes 2 and 7, chicken (*Gallus gallus*): Lanes 3 and 8, and duck (*Anus sp.*): Lanes 4 and 9 were used as template in each reaction, respectively, Lanes 5 and 10 was negative control in LAMP reaction for 60 and 57°C, respectively.

#### 4. Conclusions

This study, a set of LAMP primers was designed for specific amplification of mitochondrial cytochrome *b* gene of pig. The condition of the

LAMP assay was developed for pig DNA detection in meat samples. The LAMP product was successfully amplified only from *cyt b* mtDNA of pig and no amplification was detected from other

species (cattle, chicken and duck), indicating the specificity of LAMP primers. Therefore, the LAMP assay developed in this study can be applied for pork identification in meat samples. However, sensitivity and detection methods will be developed in further studies to obtain the valid methods for pig DNA identification in processed meat for *Halal* authentication.

### 5. Acknowledgement

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