

# Loop-Mediated Isothermal Amplification Assays for the Rapid Identification of Duck-Derived Ingredients in Adulterated Meat

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**Abstract** Meat adulteration is a growing concern in the marketplace today. To protect consumer rights and prevent unfair competition, it is essential to use an efficient assay to identify rapidly the species of meat being sold. In this context, a loop-mediated isothermal amplification (LAMP) assay coupled with a lateral flow dipstick (LFD) or hydroxynaphthol blue dye (HNB) was developed for the detection of duck genes in meat products. The LAMP-LFD and LAMP-HNB assays were performed at 65 °C for 30 min, with no cross-reactivity against four other species of meat. Sensitivity evaluation showed that the two assays can detect 3 pg of duck DNA per reaction, which is 10 times higher than that of the real-time quantitative polymerase chain reaction (qPCR). Through testing the experimental adulteration models, which were prepared by mixing duck meat with beef at different concentrations (0.01 to 10%), the detection limits of the two assays were confirmed as 0.1% duck meat. Combining the simple DNA extraction assay and the LAMP-LFD or LAMP-HNB assay, adulteration with only 1 mg duck meat can be identified within 40 min. The LAMP-LFD and LAMP-HNB assays are simple and highly sensitive, which provide valuable tools for the

identification of duck gene from adulterated meat. The simple DNA extraction assay further enables our LAMP assays to be applicable in the field.

**Keywords** Loop-mediated isothermal amplification (LAMP) · Duck meat · Adulteration · Lateral flow dipstick (LFD) · Hydroxynaphthol blue dye (HNB) · On-site detection

## Introduction

Meat adulteration, namely mixing valuable meat with a cheaper or objectionable species, has recently become an issue of increasing public concern (Ali et al. 2012). In addition to posing a health risk to consumers with metabolic disorders or allergies, meat adulteration has triggered many economic and religious problems (Wang et al. 2015; Barakat et al. 2014). However, meat adulteration is still a general phenomenon in worldwide markets. In an investigation performed on 100 types of meat products, meat from undeclared species was found in 22.0% of cases, primarily with poultry substituting for beef (Ayaz et al. 2006). Duck meat is one of the eligible candidates for meat adulteration due to its low cost and ready availability. To maximize the profit, some retailers always adulterate beef and mutton with cheaper alternatives and fraudulently label the true meat species (Boyaci et al. 2014). Hence, identification of meat species is vital to ensure food quality and protect human health (Rohman et al. 2011).

Various methods have been developed for the detection and identification of meat species (Stamoulisa et al. 2010; Ali et al. 2014; Fajardo et al. 2010; Kesmen et al. 2012), of which the conventional polymerase chain reaction (PCR) and real-time quantitative PCR (qPCR) have been widely used. qPCR was recommended as a standard method by the General Administration of Quality Supervision, Inspection and the

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Quarantine of the People's Republic of China (AQSIQ) to identify duck genes. Although qPCR is characterized by a higher sensitivity, specificity, and accuracy, it involves steps with temperature increments and decrements, and requires an expensive qPCR system, which makes the assay only practically available in a specialist analytical laboratory.

Another molecular diagnostic technique, loop-mediated isothermal amplification (LAMP), is a novel assay that amplifies DNA using *Bst* DNA polymerase with a strand displacement activity, as well as utilizing a set of primers designed to target six to eight distinct regions of a sequence (Notomi et al. 2000; Uematsu et al. 2015). As this technique can be performed under isothermal conditions, LAMP assay is simpler than PCR and qPCR. Although the LAMP assay has been applied in many research studies of food analysis, including the detection of allergens (Sun et al. 2015), genetically modified organisms (GMOs) (Guan et al. 2010; Cheng et al. 2014), food-borne pathogens (D'Agostino et al. 2015; Kokkinos et al. 2014), and meat species identification (Zahradnik et al. 2015; Abdulmawjood et al. 2014), no research group has developed a LAMP assay specifically for the detection of duck-derived ingredients in order to identify cases of meat adulteration.

LAMP products were originally detected by gel electrophoresis (Chandrasekar et al. 2015), but the procedure is not only time-consuming but also can cause contamination easily, which greatly reduces the probability for field application of the LAMP assay. Recently, to simplify and speed up the LAMP assay, a lateral flow dipstick (LFD) device and a hydroxynaphthol blue (HNB) dye-based colorimetric determination assay were introduced to detect the amplicons (Ding et al. 2010; Deng et al. 2015; Niu et al. 2015; Duan et al. 2014; Luo et al. 2014). The amplicons detected with LFD were labeled by FITC and biotin. The dual labeled LAMP products can be detected by a gold-labeled anti-FITC antibody following chromatography on an LFD (Thongkao et al. 2015). HNB is a magnesium-chelating dye. Massive amplification produces a pyrophosphate by-product that precipitates with magnesium ions. HNB loses its bound magnesium and the color of the reaction mixture changes from purple to blue (Ghosh et al. 2015). Since the results can be visualized directly without the need of any special equipment, these two assays were considered to be more suitable for on-site detection.

Here, we developed the LAMP-LFD and LAMP-HNB assays for the rapid identification of duck genes from meat products. Specificity, sensitivity and the detection limits of the assays were evaluated and validated against beef in a series of mock adulteration experiments and also evaluated with qPCR. The assays feature a high sensitivity, rapidity, and simplicity for detecting duck-derived ingredients. In addition, the simple DNA extraction assay introduced in the study enables the LAMP assays to be more suitable for the on-site detection of meat adulteration.

## Material and Methods

### Samples

The five species of meat used in the study were pork, beef, mutton, chicken, and duck and were purchased from a supermarket in Hangzhou, China. All the meat samples were transported under ice-chilled conditions (4 °C) and stored at −40 °C for future work and DNA extraction.

### DNA Extraction

DNA was extracted from 50 mg of raw meat samples using DNeasy tissue Isolation Kit (Qiagen, Darmstadt, Germany) according to the manufacturer's manual. The quality and concentration of the final extractions were measured using a NanoDrop 1000 UV spectrophotometer (NanoDrop Technologies Inc., Wilmington, DE, USA) by scanning at OD<sub>260</sub>/OD<sub>280</sub> and OD<sub>260</sub>/OD<sub>230</sub>.

Meanwhile, a simple DNA extraction assay was introduced. Briefly, 1, 50, and 100 mg of meat samples were cut into small pieces and mixed with 500 µL of ddH<sub>2</sub>O, respectively. The suspension was then incubated at 95 °C for 5 min. After incubation, the mixture was briefly centrifuged, and the supernatant was applied as the template for the LAMP-LFD or LAMP-HNB assay.

### Conventional LAMP Assay

Complete sequences of *Anas* mitochondrial DNA were downloaded from GenBank and blasted using the "Megalign" program (DNASar, Inc., USA). LAMP primers targeting within the D-loop region (nt430-nt628) of mitochondrial DNA were designed using Primer Explorer V3 software with standard settings (Eiken Chemical Co., Ltd., Tokyo, Japan). The sequences and locations of the primers are shown in Table 1. The primers were synthesized by Sangon Biotech Co., Ltd. (Shanghai, China). The LAMP assay was performed in a 25-µL reaction mixture. Each reaction contained 1 × *Bst* DNA polymerase buffer (New England Biolabs Ltd., Ipswich, MA, USA), 0.4 µM of FIP (inner forward primer, F1c + F2) and BIP (inner reverse primer, B1c + B2), 0.2 µM of LF and LB, 0.1 µM of F3 and B3, 1.4 µM of dNTP (Sangon Biotech Co., Ltd., Shanghai, China), 8 U *Bst* DNA polymerase (New England Biolabs Ltd. Ipswich, MA, USA), 8 mM MgSO<sub>4</sub>, and 3 µL of extracted DNA. To find the optimal reaction condition, the amplification were carried out in DNA Engine Thermal cycler (Bio-Rad, Foster, USA) at 65 °C for 15, 20, 30, and 40 min, respectively. Then, the mixtures were heated at 80 °C for 5 min to terminate the reaction. Three microliters of the amplicons were detected using 1.5% agarose gel electrophoresis.

**Table 1** List of LAMP primers used for experiments in this study and their sequences targeting the D-loop gene from the duck

Primer name	Sequence 5' → 3'	Position
FIP (inner forward primer, F1c + F2)	GCCCTGACCGAGGAACCAGAGCCCAT ACGTTCCCCCTA	nt519–500, nt452–469
BIP (inner reverse primer, B1c + B2)	TTCACTCACCTCTCCTTGCCCTGCCGCGATTA CGCATTGA	nt552–531, nt586–603
F3 (outer forward primer)	TGTCGGACGTGACTAGCT	nt430–447
B3 (outer reverse primer)	CAGAGGCGCCAAAAAGCT	nt628–611
LF (loop forward primer)	GGCGCAAAAATGTGAGGAGG	nt499–480
LB (loop reverse primer)	AAAGTGGCATCTGTGGAATACTTC	nt555–578

### LAMP-LFD Assay

The LAMP-LFD assay was developed based on the conventional LAMP assay with many modifications. Although the reaction mixture and the amplification conditions of the LAMP-LFD was the same as used in the conventional LAMP, the LF, and LB primers used here were labeled with biotin and FITC at the 5' end, respectively. After incubation, the product was detected by an LFD device (Ustar Biotech Co., Ltd., Hangzhou, China). Test line and control line can be visualized in LFD when a positive sample was tested, while only the control line can be observed when a negative sample was tested.

### LAMP-HNB Assay

Another modified assay, LAMP-HNB, was carried out by adding 120  $\mu$ M HNB dye into the reaction mixture of the conventional LAMP. Briefly, reactions were performed at 65 °C in 25  $\mu$ L solution containing 1  $\times$  *Bst* DNA polymerase buffer, 0.4  $\mu$ M of FIP and BIP, 0.2  $\mu$ M of LF and LB, 0.1  $\mu$ M of F3 and B3, 1.4  $\mu$ M of dNTP, 8 U *Bst* DNA polymerase, 8 mM MgSO<sub>4</sub>, 120  $\mu$ M HNB, and 3  $\mu$ L of target DNA for 30 min. Then the mixtures were heated at 80 °C for 5 min to terminate the reaction. The results can be determined by directly visualizing the color change with the naked eye. A positive LAMP reaction was indicated by a sky blue color; whereas it remained violet for negative results.

### Real-Time Quantitative PCR Assay

Real-time quantitative PCR was performed with a *premix Ex Taq*<sup>TM</sup> Kit (TaKaRa biotechnology Co., Ltd., Dalian, China) using the primers and probe recommend by AQSIIQ. Sequences of the forward and reverse primers were 5'-AAGCCTTCCTCTAGCTCAGC-3' (nt2177-nt2196), and 5'-AGAAAATGCTTTAGTTAAGTC-3' (nt2256-nt2236), respectively. Sequence of the probe was 5'-FAM-CTCAGCCGCTTAAACAACGC-3'-TAMRA (nt2191-nt2210). Each 25  $\mu$ L reaction mixture included 1  $\times$  *Premix Ex Taq*<sup>TM</sup>, 0.2  $\mu$ M of each primer, 0.1  $\mu$ M of

probe, and 3  $\mu$ L of template DNA. The amplification conditions were conducted as follows: 95 °C for 5 min for activation, followed by 40 cycles of amplification at 94 °C for 10 s, 55 °C for 30 s, and 72 °C for 30 s.

### Specificity and Sensitivity Evaluation of the LAMP-LFD and LAMP-HNB Assays

Specificity evaluations of the LAMP-LFD and LAMP-HNB assays were conducted using DNA samples (30 ng/reaction) extracted from duck, beef, mutton, chicken, and pork. The sensitivity of each assay was assessed by testing tenfold serial dilutions of DNA isolated from pure duck meat. The concentrations of the duck DNA ranged from 10<sup>1</sup> to 10<sup>-4</sup> ng/ $\mu$ L.

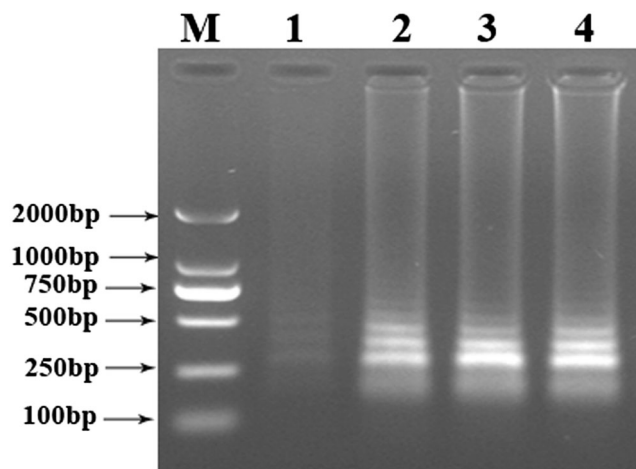
### Detection Limits of the LAMP-LFD and LAMP-HNB Assays

To determine the detection limits of the developed assays, a model of meat adulteration was developed. First, duck and beef meat were homogenized. The beef was adulteration with different quantities of duck meat, achieving a series of samples, which contained 10, 5, 1, 0.1, and 0.01% w/w duck meat, respectively. DNA extracted from the spiked samples was tested using the LAMP-LFD in parallel with the LAMP-HNB with five replicates for each sample. Pure beef meat was used as the negative control, while pure duck meat was used as the positive control.

## Results

### Optimization of the Conventional LAMP Assay

The 30 ng of duck DNA was incubated for 15, 20, 30, and 40 min at 65 °C, respectively to determine the optimal incubation time of the conventional LAMP assay. From the gel picture (Fig. 1), positive results, the typical ladder-like pattern, was obtained after amplification for 20, 30, and 40 min. However, the bands generated after 30 and 40 min were stronger and clearer than that of 20 min. No significant difference



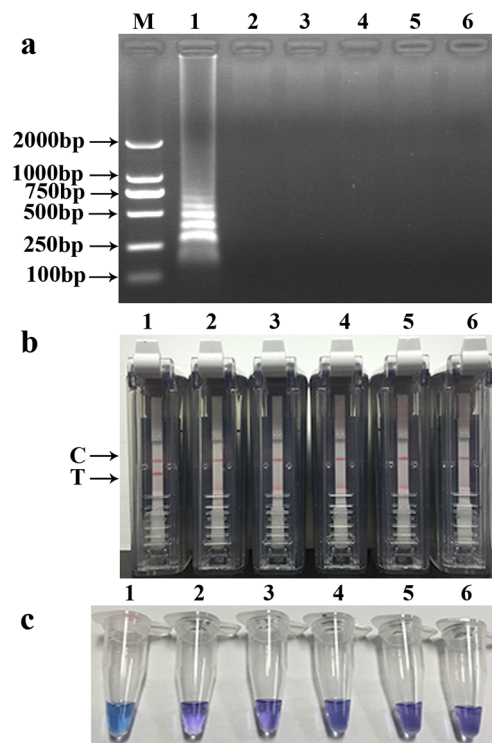
**Fig. 1** Different reaction times of the conventional LAMP assay. *M* DL 2000 marker (Takara); 1–4 the reaction times of 15, 20, 30, and 40 min

was observed on the results between 30 and 40 min. On the basis of rapidity and simplicity, the incubation time of the conventional LAMP was identified as 30 min.

#### Evaluation of the Conventional LAMP, LAMP-LFD, and LAMP-HNB Assays

Specificities of the conventional LAMP, LAMP-LFD, and LAMP-HNB assays were evaluated by testing DNA samples extracted from pork, beef, mutton, chicken, and duck meats (Fig. 2). The results showed that only DNA from duck meat was detected as positive by the three assays, displaying a ladder-like pattern in the gel picture (the conventional LAMP), two lines in LFD (LAMP-LFD), and a blue solution after incubation with HNB (LAMP-HNB). DNA samples originating from the other four species of meat gave negative results with the three LAMP assays.

The sensitivities were assessed by testing tenfold serial dilutions of duck DNA (10 ng/μL to 0.1 pg/μL) using the conventional LAMP, LAMP-LFD, LAMP-HNB assays as well as the qPCR assay (Fig. 3). The results revealed that the conventional LAMP, LAMP-LFD, and LAMP-HNB assays achieved the same sensitivity of 3 pg/reaction duck DNA (Fig. 3a–c), while the qPCR only detected 30 pg/reaction of duck DNA (Fig. 3d). To estimate the detection limits of the developed LAMP assays, beef sample adulteration with various additives of duck meat (10, 5, 1, 0.1, and 0.01%) were tested using the LAMP-LFD in parallel with the LAMP-HNB with five replicates for each concentration (Table 2). Five replicates of each sample with 0.1% of duck meat adulteration were detected by the LAMP-LFD and LAMP-HNB, while none were detected at a concentration of 0.01%. In addition, the conventional LAMP needs about 1 h to accomplish each test, but



**Fig. 2** The specificity of the LAMP assays. **a** Products were determined by agarose gels electrophoresis. **b** Detection by LFD. **c** Visual observation by adding HNB dye to the reaction tubes. A positive reaction is indicated by a color change from purple to blue. *M* DL2000 marker, *C* control line, *T* test line, 1–5 duck, chicken, pork, beef, and mutton, 6 NTC

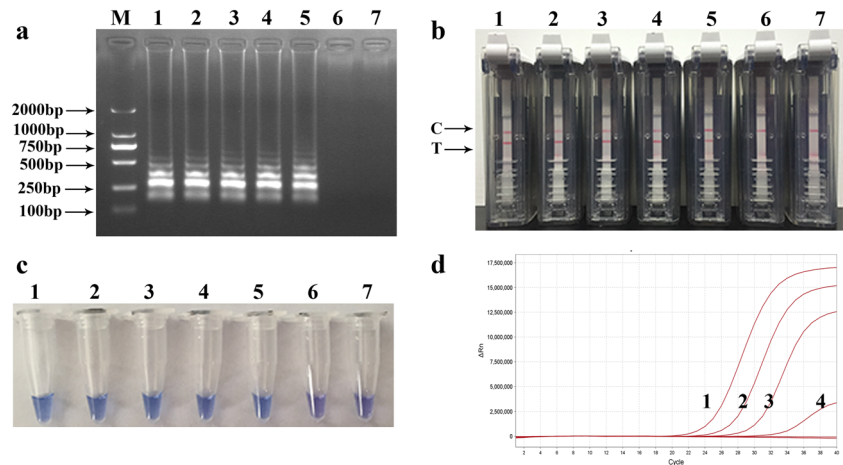
the LAMP-LFD and LAMP-HNB only needs 35 and 30 min for each test.

#### LAMP-LFD and LAMP-HNB Combined with Simple DNA Extraction

To achieve on-site detection, a simple DNA extraction assay coupled with the LAMP-LFD or LAMP-HNB was also validated in this study. The whole process for DNA extraction takes approximately 8 min. DNA extracted from five species of meat (1 to 100 mg) was used to evaluate the simple DNA extraction assay. Positive amplifications were only observed with duck DNA.

To assess the efficiency of the simple DNA extraction assay, DNA was extracted from 1, 50, and 100 mg samples of duck meat using a simple DNA extraction assay in parallel with the DNA extraction kit. The extracted DNA was tested using the LAMP-LFD and LAMP-HNB, and the results are shown in Table 3. When the incubation time was set at 30 min, the LAMP-LFD and LAMP-HNB were capable of detecting DNA originating from 1 mg duck meat using both a simple DNA extraction assay and a DNA extraction kit. However, when the incubation time decreased to 20 min, the detection limit of the two LAMP

**Fig. 3** Comparison of sensitivity between the LAMP assays and qPCR. **a–c** The sensitivity of conventional LAMP assay, LAMP-LFD, and LAMP-HNB. **d** The sensitivity of qPCR. *M* DL2000 marker, *C* control line, *T* test line, *1–6* 30, 3, and 0.3 ng/tube and 30, 3, and 0.3 pg/tube, *7* NTC



assays increased to 100 mg duck meat using the simple DNA extraction assay (Table 3).

## Discussion

The conventional methods for the identification of animal species in meat or meat products mostly rely on protein analyses, such as enzyme-linked immunosorbent assays (ELISAs) and chromatographic assays (Ballin et al. 2009). These protein-based approaches have serious limitations as the structure and species-specific proteins will be destroyed by salt, heat, or pressure during cooking process (Fajardo et al. 2010). In this case, nucleotide amplification based technology can provide a better alternative (Fajardo et al. 2010). Therefore, three LAMP assays were developed for rapid identification of duck-derived ingredients from meat in this study.

First of all, a conventional LAMP assay was developed. For the sake of the best amplification efficacy, the amplification temperature was first evaluated. Although the results indicated that our assay can be performed between 60 and 65 °C without significant differences (results not shown), the highest temperature (65 °C) was adopted since the higher temperature can reduce an unspecific amplification or primers cross

**Table 2** Detection limits of the LAMP-LFD and LAMP-HNB assays tested using an experimental adulteration model

Concentration of duck meat in the model (%)	Positive reactions (positive no/total reaction)	
	LAMP/LFD	LAMP/HNB
10	5/5	5/5
5	5/5	5/5
1	5/5	5/5
0.1	5/5	5/5
0.01	0/5	0/5

annealing theoretically (Ding et al. 2010). Another vital factor, the reaction time, was also optimized, and the optimal result (30 min) was finally confirmed considering the sensitivity combining with rapidity. Under the standard condition, the LAMP assays were proven as specific and more sensitive than the qPCR, no matter which detection assay after amplification. Results of the detection limits (0.1% duck meat in adulteration models) also indicated that the duck gene can be detected by the developed assays even if only 1 g duck meat was mixed into 1 kg of meat product. Although the previous study showed that the highest detection limit for the identification of meat adulteration can reach 0.01% (Barakat et al. 2015), the sensitivity of our LAMP assays are considered to be sufficient for detection of duck genes from adulterated meat, because it was generally considered as an accidental contamination if the amount of undeclared species is less than 0.1%, without economic benefit (Kesmen et al. 2012).

The LAMP-LFD and LAMP-HNB assays were based on the conventional LAMP with a few modifications. Compared with the LAMP combined with gel electrophoresis assay, the LAMP-LFD and LAMP-HNB assays not only avoid the pollution of carcinogens (EB) and the contamination of amplicons, but also achieve the detection of LAMP product with naked eyes, which makes the assays simpler and faster than the conventional LAMP assay. As no need with equipment, the developed assays meet the requirements of on-site detection.

Another crucial step affecting on-site detection is DNA extraction. Generally, DNA extraction has to be implemented in a laboratory since it needs an extraction kit and a high speed centrifuge, that is, it is difficult to perform the identification of meat adulteration in the field even if the LAMP-LFD and LAMP-HNB were adopted. As the great stability of the *Bst* polymerase enzyme enables the LAMP reaction to tolerate the endogenous amplification inhibitors to a greater extent than PCR (Francois et al. 2011), DNA or RNA extracted from a simplified extraction process has been utilized as a template for LAMP reaction with a

**Table 3** Comparison between the simple DNA extraction assay and the DNA extraction kit using LAMP-LFD and LAMP-HNB

Weight of duck meat (mg)	Simple DNA extraction assay		DNA extracted kit	
	20 min	30 min	20 min	30 min
1	–	+	+	+
50	–	+	+	+
100	+	+	+	+

variety of species, including lily leaves, blood, beef, and ostrich meat (Komatsu et al. 2015, Ebbinghaus et al. 2012, Wang et al. 2012, Abdulmawjood et al. 2014). The assays previously described employed a lysis buffer to increase the proportion of intracellular nucleic acids and to remove amplification inhibitor present in the template material. However, the simple DNA extraction assay introduced in this study use ddH<sub>2</sub>O as the sole reagent and omitted many steps of sample purification, which makes the assay simpler and more economic. Meanwhile, the whole procedure could be finished within 10 min using the portable equipment (water or metal bath), drastically reducing the time and achieving application in the field. Under the standard reaction condition (30 min), the amplification efficacy of the LAMP-LFD and LAMP-HNB assays was equal for DNA templates obtained from the simple extraction assay and the extraction kit (1 mg of duck meat). Furthermore, when the amount of the sample is over 100 mg, the amplification time could be slightly shortened. It is noteworthy that we do not recommend to use too much of the sample since non-specific amplification may occur when the amount of the sample is larger than 500 mg.

The developed LAMP-LFD and LAMP-HNB coupled with a simple DNA extraction assays are sensitive, rapid, and simple (no need for equipment), which are ideal for the on-site detection of duck-derived ingredient. We recommend that the food inspectors could cut appropriate sizes of meat sample first and then incubate the sample in a metal or water bath for 5 min. After that, the supernatant of the sample could be tested using the LAMP-LFD or LAMP-HNB assay and the results can be read by naked eyes. With our methods, food inspectors could perform the identification of meat adulteration within 40 min in the markets, restaurants, or retail shops.

## Conclusion

The LAMP assays developed in this study are powerful tools for identifying adulteration of duck meat within superior meat products. The LAMP assays can be performed under isothermal conditions without PCR thermal cyclers within a shorter time (30 min in this study). In combination with the LFD device or HNB dye, the amplicon can be directly detected by the naked eye instead of the conventional gel

electrophoresis. In addition, the simple DNA extraction assay further enables the LAMP-LFD and LAMP-HNB assays to be readily usable in field. This work contributes to a future approach on food analysis by meeting the demands for quick and easy-to-perform analytical methods.

## Compliance with Ethical Standards

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**Conflict of Interest** Ya Shi declares that he has no conflict of interest. Yan Feng declares that he has no conflict of interest. Changping Xu declares that he has no conflict of interest. Zhouheng Xu declares that he has no conflict of interest. Dongqing Cheng declares that he has no conflict of interest. Yiyu Lu declares that he has no conflict of interest.

**Ethical Approval** This article does not contain any studies with human participants or animals performed by any of the authors.

**Informed Consent** Not applicable.

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