



# Cleavable molecular beacon-based loop-mediated isothermal amplification assay for the detection of adulterated chicken in meat

Song Yan<sup>1,2</sup> · Hangzhen Lan<sup>1,2</sup> · Zhen Wu<sup>1,2</sup> · Yangying Sun<sup>1,2</sup> · Maolin Tu<sup>1,2</sup> · Daodong Pan<sup>1,2,3</sup>

Received: 29 July 2022 / Revised: 8 September 2022 / Accepted: 9 September 2022 / Published online: 24 September 2022  
© Springer-Verlag GmbH Germany, part of Springer Nature 2022

## Abstract

A simple, sensitive, specific and fast method based on the loop-mediated isothermal amplification (LAMP) technique and cleavable molecular beacon (CMB) was developed for chicken authentication detection. LAMP and CMB were used for DNA amplification and amplicon analysis, respectively. Targeting the mitochondrial cytochrome b gene of chickens, five primers and one CMB probe were designed, and their specificity was validated against nine other animal species. The structure of CMB and concentrations of dNTPs, MgSO<sub>4</sub>, betaine, RNase H2, primers and CMB were optimized. The CMB-LAMP assay was completed within 17 min, and its limit of detection for chicken DNA was 1.5 pg μL<sup>-1</sup>. Chicken adulteration as low as 0.5% was detected in beef, and no cross-reactivity was observed. Finally, this assay was successfully applied to 20 commercial meat products. When combined with our developed DNA extraction method (the extraction time was 1 min: lysis for 10 s, washing for 20 s and elution for 30 s), the entire process (from DNA extraction to results analysis) was able to be completed within 20 min, which is at least 10 min shorter than other LAMP-based methods. Our method showed great potential for the on-site detection of chicken adulteration in meat.

**Keywords** Meat authenticity identification · Loop-mediated isothermal amplification · Cleavable molecular beacon · Quantitative detection · Chicken

## Introduction

In recent years, meat consumption has shown an increasing trend due to the increasing average individual incomes and the growing population [1, 2]. With the increasing demand for meat, meat adulteration has become a general concern in the food industry and market. A widespread phenomenon is the substitution of higher-value meat with inferior and inexpensive meat to improve profitability and/or to gain an unfair

competitive advantage [3]. Because chicken is generally less expensive than red meat, it is often used to blend into other high-value meats. Chicken adulteration is classified into three categories, namely, adulteration, substitution and mislabeling, and occurs frequently in developing countries [4]. Therefore, the development of a fast, simple and suitable identification technique for point-of-care testing (POCT) of chicken authentication to protect consumer interests, ensure fair market competition and achieve a harmonious religious environment is a critical need [5–7].

To date, meat authentication detection technologies include spectroscopic, mass spectrometric, chromatographic, nucleic acid-based molecular biology and protein-based immunoassay techniques [8–11]. Among them, nucleic acid-based molecular biology and protein-based immunoassay techniques are the most commonly used. However, protein-based detection methods are greatly affected by the protein structure, and the target protein is susceptible to denaturation and/or inactivation due to physical and chemical conditions such as heat, salt, acid and alkali during food processing, which cause it to be undetectable [5, 12]. Compared with protein, DNA not only has higher stability but is also more

✉ Hangzhen Lan  
lanhangzhen@nbu.edu.cn

✉ Daodong Pan  
daodongpan@163.com

<sup>1</sup> State Key Laboratory for Quality and Safety of Agro-products, Ningbo University, Ningbo 315211, China

<sup>2</sup> Key Laboratory of Animal Protein Deep Processing Technology of Zhejiang Province and College of Food and Pharmaceutical Sciences, Ningbo University, Ningbo 315800, China

<sup>3</sup> National R&D Center for Freshwater Fish Processing, Jiangxi Normal University, Nanchang 330022, Jiangxi, China

resistant to food processing methods. DNA exists in most tissue cells, and reliable identification information can be obtained even if the animal tissues are different, making DNA-based molecular biology techniques the mainstream method for animal-derived component identification [13, 14].

The conventional polymerase chain reaction (PCR) technique is considered the gold standard for nucleic acid detection [15]. PCR-based technologies have high sensitivity and have been applied for species identification, pathogen detection and parasite detection in meat products [16–18]. Nevertheless, in addition to its time-consuming and costly nature, PCR requires complex instruments, such as thermal cyclers, electrophoresis units, and gel imaging and documentation systems, which limits its application in resource-poor settings [11, 19]. Therefore, the rapid extraction and amplification of DNA and identification of amplicons without the use of complex instruments are current difficulties and bottlenecks that need to be solved in POCT for meat authenticity identification.

In recent years, the development of nucleic acid isothermal amplification techniques has provided a new option for POCT of food authenticity. Currently, the main isothermal amplification techniques are loop-mediated isothermal amplification (LAMP), cross-priming amplification (CPA), strand displacement amplification (SDA), rolling circle amplification (RCA), recombinase polymerase amplification (RPA), nucleic acid sequence-based amplification (NASBA), helicase-dependent isothermal DNA amplification (HAD), denaturation bubble-mediated strand exchange amplification (SEA) and clustered regularly interspaced short palindromic repeats (CRISPR). LAMP developed by Notomi and colleagues [20] amplifies target DNA using 4–6 specific primers and a DNA polymerase enzyme with strand displacement ability. LAMP amplification can be completed at a constant temperature (60–66 °C) for 20–60 min without expensive and high-precision instruments [21]. Owing to its advantages of high specificity and sensitivity, low cost and time savings, LAMP is considered to be the most promising technique for food certification, infectious disease diagnosis and microbial detection [22]. Consequently, the LAMP technique is gradually replacing PCR-based techniques and has been widely used in the food industry [23, 24]. Electrophoresis, chemical precipitation methods, colorimetry and lateral flow strip assays are commonly used to analyze LAMP amplification results. Except for electrophoresis, these methods generally do not require complex instruments. However, they all have the following disadvantages: (a) aerosol contamination can easily occur after opening the reaction tube, and (b) the LAMP reaction involves a greater number of primers and they are more likely to form primer dimers or hairpin structures that can also combine with fluorescent dyes, resulting in false-positives. Current traditional

detection methods cannot distinguish such false-positives from reaction results.

The molecular beacon (MB), which was developed by Tyagi [25], is a hairpin structure probe. Unlike common fluorescent dyes, the fluorescent signal generated by MB is specific: the loop region forms a hybrid to the target sequence, so the hairpin structure opens to generate a specific fluorescent signal. Fluorophores on the hybrids can usually be detected by lateral flow test strips [26], real-time fluorescence detectors [27] and visual detection [28]. Moreover, MBs with different fluorophores can be designed to achieve detection of multiples [29]. Based on these advantages, MB has been applied in microbial detection and disease diagnosis.

In this study, for targeting the corresponding region of the dumbbell structure and on the basis of MB, we designed a new probe, the cleavable molecular beacon (CMB), for the specific and rapid detection of chicken DNA. Contrary to common MBs, the CMB has four consecutive ribonucleotides that are in the loop region near the 3' end [30, 31]. Once CMB forms a hybrid to the target, the fluorophore at the 5' end of the CMB will emit strong fluorescent signals, and subsequently, phosphodiester bonds between the four RNAs in the hybrid will be cleaved by RNase H2 [32]. Due to the specific fluorescent signal, our method overcomes the challenge of false-positives. Moreover, compared with ordinary probes, our probe plays an important role in accelerating amplification. When combined with our developed rapid DNA extraction method, the entire reaction process (from DNA extraction to the end of amplification) takes less than 20 min, and the whole process does not require the use of complex instruments. Therefore, the CMB-LAMP assay established in our study has great potential for POCT of meat adulteration.

## Material and methods

### Materials and reagents

Chicken (*Gallus gallus*), duck (*Anas platyrhynchos*), pig (*Sus scrofa*), cow (*Bos taurus*), horse (*Equus caballus*), goat (*Capra hircus*), rabbit (*Oryctolagus cuniculus*), ostrich (*Struthio camelus*), camel (*Camelus* Linnaeus) and goose (*Anser cygnoides orientalis*) meat were collected from local markets in Ningbo, China. Twenty kinds of commercial meat products were from online shops. All the meat samples were cut into small pieces, labeled and immediately stored at  $-20\text{ }^{\circ}\text{C}$  until use. All chemicals used in this research were of analytical grade. Bst 2.0 WarmStart DNA polymerase ( $8\text{ U }\mu\text{L}^{-1}$ ),  $\text{MgSO}_4$  (100 mM), 10× isothermal amplification buffer [200 mM Tris-HCl, 500 mM KCl, 100 mM  $(\text{NH}_4)_2\text{SO}_4$ , and 20 mM  $\text{MgSO}_4$ , 1.0% Tween 20 and

pH 8.8 at 25 °C] and the ribonuclease RNase H2 (5 U  $\mu\text{L}^{-1}$ ) were from New England Biolabs (Ipswich, MA, USA). Deoxynucleotide (dNTP) solution mix (25 mM), betaine, sterilized double-distilled water, primers and cleavable molecular beacons were from Sangon Biotech (Shanghai, China). SYBR Green I (PCR grade, 10,000 $\times$ ) was purchased from Meilun Biotech (Dalian, China). A TIANamp Genomic DNA kit was purchased from Tiangen Biotech (Beijing, China). Sodium dodecyl sulfate, sodium chloride, tris(hydroxymethyl)aminomethane, ethylenediaminetetraacetic acid (EDTA), *N,N*-dimethylformamide, zirconium oxychloride, terephthalic acid, 2-aminoterephthalic acid, glacial acetic acid, ethanol, polyethyleneimine (molecular weight = 600), polyvinylpyrrolidone (average molecular weight approximately 1,300,000), methanol and agarose were obtained from Macklin Biochemical Technology Co. (Shanghai, China). The 2 $\times$  PCR Master mix, loading buffer and 2K DNA marker were from TransGen Biotech (Beijing, China).

### DNA extraction

DNA from meat samples was extracted using two methods: the DNA kit method and the method developed by our laboratory. When using the TIANamp Genomic DNA Kit to extract DNA, the procedure strictly followed the operating manual. In our method, UIO-66-NH<sub>2</sub>, which was used as an adsorbent for DNA extraction and purification, was coated on a nitrocellulose membrane. This modified membrane was adhered to a stainless-steel stick, and this simple device was called an “extraction stick” (see Electronic Supplementary Material Fig. S1). First, chicken and other meats were minced, weighed to 30 mg and then transferred into 1.5 mL centrifuge tubes. After that, 300  $\mu\text{L}$  of lysis buffer (100 mM Tris-HCl [pH 8.0], 5 mM EDTA, 200 mM NaCl, 1% SDS) was added to the centrifuge tubes, and the extraction

sticks were immersed for 10 s to capture nucleic acids. Then, the extraction sticks were put into sterilized double-distilled water for 20 s, and finally, they were placed into 300  $\mu\text{L}$  of eluate (10 mM Tris, pH 7.0) for 30 s to remove sample matrices. The purity and concentration of DNA were quantified by measuring ultraviolet (UV) absorption using an ultraviolet-visible spectrophotometer (Nano-300, Allsheng, Hangzhou, China). DNA with a 260/280 nm ratio between 1.8 and 2.0 was used as template DNA. Detailed steps of DNA isolation are described in the supplementary material.

### Design of LAMP primers and CMB probes

The mitochondrial DNA cytochrome b (CYTB) gene sequences of chicken and other common animals were downloaded from the National Biotechnology Information Center (NCBI) (<https://www.ncbi.nlm.nih.gov/>). The sequences were aligned using the Clustal Omega program (<https://www.ebi.ac.uk/Tools/msa/clustalo/>) to select a chicken-specific region. LAMP primers, including outer primers (F3, B3), inner primers (FIP, BIP) and loop primers (LF, LB), were designed according to the published sequences of the CYTB gene (GenBank accession NC\_053523.1) using Primer Explorer version 5 (<http://primerexplorer.jp/>). The CMB probes were designed using the online OligoAnalyzer Tool (<https://sg.idtdna.com/calc/analyzer>) and the NUPACK platform (<http://www.nupack.org/>). The sequences of the LAMP primers and CMB probes used in this study are shown in Table 1.

### SYBR Green I-LAMP assay

The SYBR Green I-LAMP reaction mixture (25  $\mu\text{L}$ ) contained 10 $\times$  isothermal amplification buffer, 4 mM MgSO<sub>4</sub>, 1.6 mM each of dNTPs, 0.8 M betaine, 0.2  $\mu\text{M}$  F3, 0.2  $\mu\text{M}$  B3, 1.6  $\mu\text{M}$  FIP, 1.6  $\mu\text{M}$  BIP, 0.8  $\mu\text{M}$  LF, 0.8  $\mu\text{M}$  LB, 0.32

**Table 1** The sequences of the primers and probes used in this study

Primer/probe	Description	Sequence (5'–3')
F3	Forward outer primer	GCCCCATCCAACATCTCTG
B3	Backward outer primer	CGTTTGCGTGAGATTCCG
FIP	Forward inner primer	ATGGCTAGTAGTAGGCCGGTGATTCCGGCTCCCTATTAGCAGT
BIP	Backward inner primer	CACAGCAGACACATCCCTAGCCTCAGCCGTATTGTACGTTCC
LF	Forward loop primer	GGATTGGGTCATGAGGCAG
LB	Backward loop primer	TTCTCCTCCGTAGCCCACA
CMB	4 bases in stem complementary to the target sequence	FAM-CCGCTTCTCCTCCGTAGCCCACAAGAAGCGG-DABCYL
CMB1	0 bases in stem complementary to the target sequence	FAM-TTCTCCTCCGTAGCCCACATGCAGAGGAGAA-DABCYL
CMB2	2 bases in stem complementary to the target sequence	FAM-ACTTCTCCTCCGTAGCCCACAGGAGAAGT-DABCYL
CMB3	6 bases in stem complementary to the target sequence	FAM-ACGGCATTCTCCTCCGTAGCCCACAAATGCCGT-DABCYL
CMB4	8 bases in stem complementary to the target sequence	FAM-GCGGCATATTCTCCTCCGTAGCCCACATATGCCGT-DABCYL

U  $\mu\text{L}^{-1}$  Bst 2.0 WarmStart DNA polymerase, 1  $\mu\text{L}$  SYBR Green I and 2  $\mu\text{L}$  of target DNA. The reactions were incubated at 62 °C for 50 min in a LightCycler 96 real-time PCR system (Roche Diagnostics, Basel, Switzerland).

### CMB-LAMP assay

The CMB-LAMP reaction mixture (25  $\mu\text{L}$ ) contained 10 $\times$  isothermal amplification buffer, 4 mM  $\text{MgSO}_4$ , 1.6 mM each of dNTPs, 0.8 M betaine, 0.2  $\mu\text{M}$  F3, 0.2  $\mu\text{M}$  B3, 1.6  $\mu\text{M}$  FIP, 1.6  $\mu\text{M}$  BIP, 0.8  $\mu\text{M}$  LF, 0.8  $\mu\text{M}$  CMB, 0.32 U  $\mu\text{L}^{-1}$  Bst 2.0 WarmStart DNA polymerase, 5 U  $\mu\text{L}^{-1}$  of ribonuclease RNase H2 and 2  $\mu\text{L}$  of target DNA. The reaction was incubated at 62 °C for 50 min in the LightCycler 96 real-time PCR system.

### Specificity and sensitivity of the CMB-LAMP assay

The specificity of the CMB-LAMP assay was assessed using DNA extracted from chicken, beef, pork, duck, mutton, rabbit, goose, ostrich, camel and horse meat at a concentration of 15 ng  $\mu\text{L}^{-1}$  under the same reaction conditions in parallel with the SYBR Green I-LAMP assay. Chicken DNA samples were serially diluted (1:10) ranging from 15 to 15 $\times 10^{-6}$  ng  $\mu\text{L}^{-1}$  for the sensitivity tests. All assays were conducted with four replicates.

To test whether the CMB-LAMP assay could detect chicken components in mixed meat, six different species of meat (chicken, pork, duck, horse, mutton and beef) were minced and then randomly combined in a 1:1:1:1:1:95 ratio. DNAs extracted from these samples using our method were used as template. CMB-LAMP and SYBR Green I-LAMP assays were conducted at 62 °C for 50 min. The results of SYBR Green I-LAMP assays were analyzed and compared with those of CMB-LAMP assays.

Chicken and beef were minced and mixed in various mass ratios to obtain a series of spiked samples (100, 50, 25, 10, 0.5 and 0.1% w/w chicken). CMB-LAMP assays were performed using DNA extracted from these samples, and sterilized double-distilled water was used as a no-template control (NTC), with four replicate measurements.

### Application of the CMB-LAMP assay

Twenty kinds of commercial meat products were analyzed to verify the applicability of the CMB-LAMP assay in parallel with the SYBR Green I-LAMP assay. DNAs extracted from these samples using DNA extraction methods developed by our group were used as templates for each reaction with four replicates on different days.

## Results and discussion

### Working principle of the CMB-LAMP assay

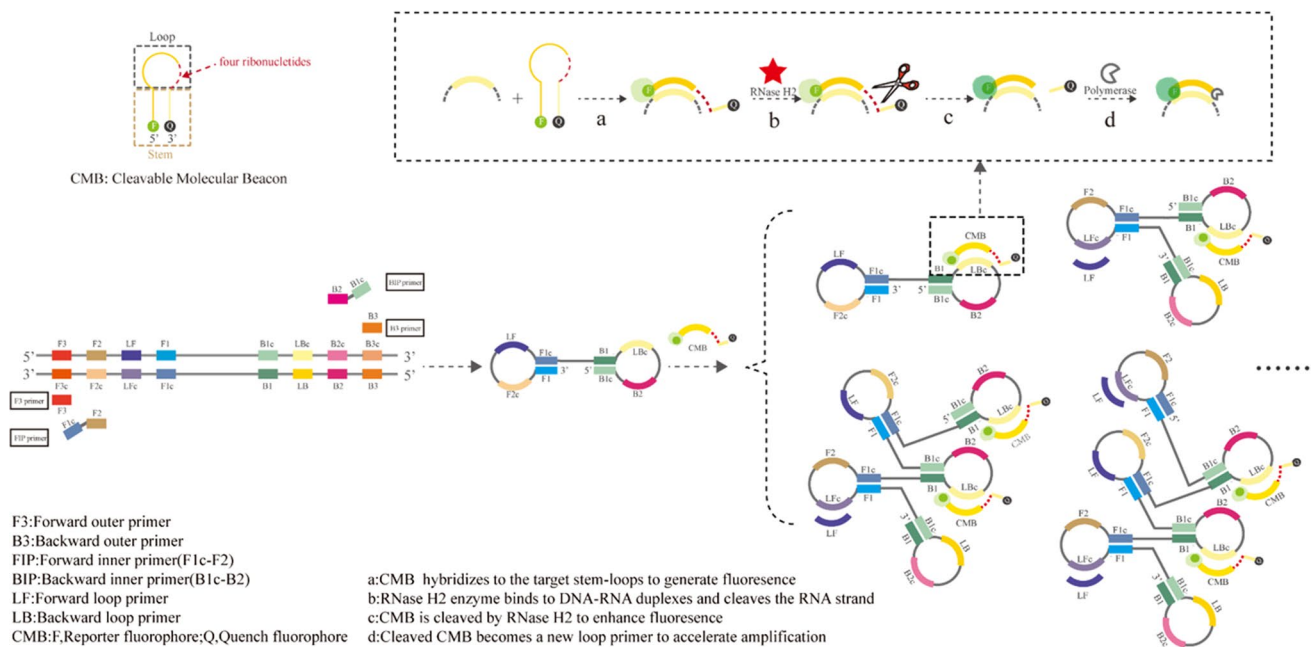
Molecular beacon (MB) technology is an analytical technology based on the phenomenon of fluorescence resonance energy transfer and the principle of complementary base pairing [33]. The principle of the CMB-LAMP assay is depicted in Fig. 1.

MB is an oligonucleotide probe that has a fluorophore at the 5'-end and a quencher at the 3'-end and can form a stem-loop hairpin structure [34]. MB maintains its hairpin structure in the absence of the target sequence so that the fluorophore and the quencher are relatively close. Since fluorescence resonance energy transfer occurs, the fluorescence emitted by the fluorophore is absorbed by the quencher, and therefore, no fluorescent signal can be detected. The hairpin structure opens when the target sequence is present. Moreover, the distance between the fluorophore and the quencher increases, and the fluorescence of the MB recovers. Based on MB, CMB has four consecutive ribonucleotides at the end of its 3'-end stems. In our study, CMB was designed based on the sequence of the loop primer LB. Initially, outer primers (F3 and B3) and inner primers (FIP and BIP) were utilized to produce dumbbell-structure DNA amplicons. Then, the loop primer (LF) and CMB probe were hybridized to the complementary region on the dumbbell structure, which caused the CMB probe to open the hairpin structure and emit a detectable fluorescent signal. After that, RNase H2 could recognize and cleave the phosphodiester bonds of the RNA strand in the dumbbell-structure-CMB hybrid. At the same time, the remaining DNA strand remained intact and could be used as a loop primer (LB) to participate in the subsequent autocycling reactions. As the reaction proceeded, massive amplification products with multiple single-stranded loop structures were formed.

### The influence of the structure of CMB

To study whether the structure of the CMB influences the assay, five different CMBs were designed, and the sequences of those CMBs are shown in Table 1. All CMBs contained a target-specific probe sequence complementary to the LBc region on the dumbbell structure, a 6-FAM fluorophore at the 5'-end, and a DABCYL quencher at the 3'-end. CMB1, CMB2, CMB, CMB3 and CMB4 differed in the number of bases that were in the 5'-end stem structure and complementary to the target sequence (0, 2, 4, 6 and 8). The same template was tested separately under the same conditions but with different CMBs. The





**Fig. 1** Schematic illustration of the CMB-LAMP assay

fluorescence intensity increased and then decreased as the number of bases increased (Fig. 2A). The fluorescence intensity reached a maximum when the number of bases was four.

Researchers have extensively explored MB structural dynamics and composition [35–37], but the number of bases in the 5'-end stem structure and complementary to the target sequence has not been reported. Based on the existing relevant literature, we can briefly speculate on the mechanism: the fewer bases that were in the 5'-end stem structure and complementary to the target sequence, the greater the end of the stem exposed, so the CMB-dumbbell-structure hybrids were more likely to form trimers with other complementary stem sequences, resulting in a decrease in fluorescence intensity. When the base number increased, the stem dissociation ratio decreased, causing the hybridization efficiency to be reduced and the fluorescence intensity to decrease.

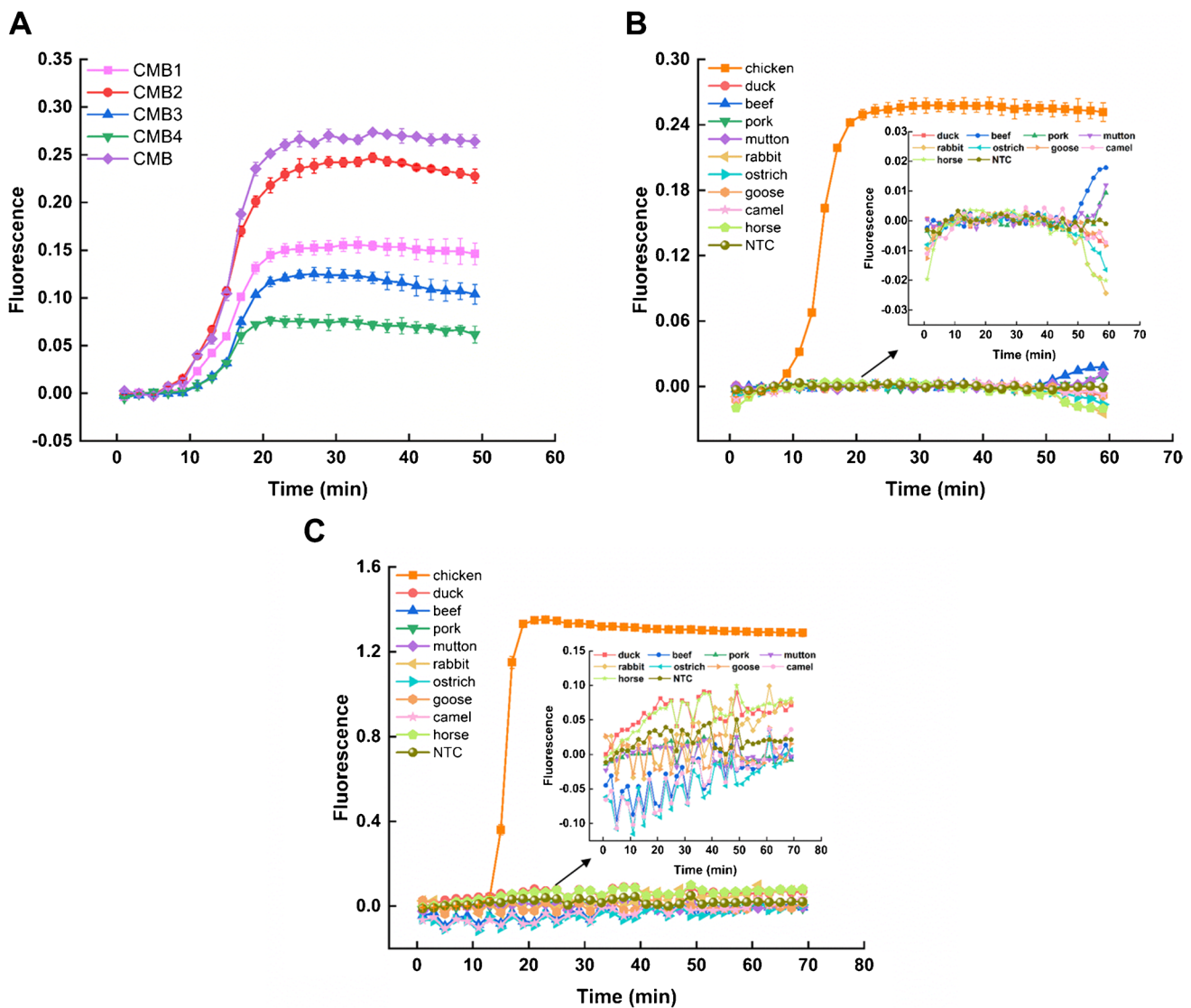
As shown in Fig. 2A, the fluorescent signal in the plateau significantly decreased due to the so-called hook effect, which refers to the competition between the amplicon strands and the hybridization probe CMB [38]. The hook effect that occurred in the late period of amplification is because of the relatively high concentration of amplicons, which self-annealed faster than the amplicons hybridized to CMB. However, the hook effect does not affect the amplification efficiency, specificity or target detection [39]. In addition, the hook effect can be reduced by adjusting the concentration of the DNA template,  $Mg^{2+}$  and probe, and the amplification time. Therefore, the amplification time in CMB-LAMP should be controlled within 30 min.

## Optimization of the SYBR Green I-LAMP and CMB-LAMP assays

To achieve the highest sensitivity of the CMB-LAMP assay for the detection of chicken DNA in meat samples, the experimental conditions were optimized. First, the SYBR Green I-LAMP reaction system was used to optimize the conventional LAMP reaction conditions, such as the reaction temperature and concentrations of primers, dNTPs,  $MgSO_4$  and betaine. The optimal reaction temperature was 64 °C, and the optimal reaction mixture contained 1.2  $\mu M$  inner primers, 0.6  $\mu M$  outer primers, 0.4  $\mu M$  loop primers, 1.2 mM dNTPs, 4 mM  $MgSO_4$  and 0.6 M betaine (see Electronic Supplementary Material Figs. S2–4). Then, the influence of the CMB probe and RNase H2 on the CMB-LAMP reaction system was investigated. The reaction speed and fluorescence intensity of CMB-LAMP assays were significantly affected by the concentration of CMB and RNase H2 (see Electronic Supplementary Material Fig. S5). The optimal concentrations of CMB and RNase H2 were 0.6  $\mu M$  and 2.0 U  $mL^{-1}$ , respectively. Subsequent studies were based on these optimal conditions.

## Specificity of the CMB-LAMP assay

To verify the specificity of the CMB-LAMP method, different DNA templates extracted from 10 different animal species, including chicken, beef, pork, goat, mutton, rabbit, horse, ostrich, camel and goose, were studied. Typical chicken DNA amplification curves were obtained from



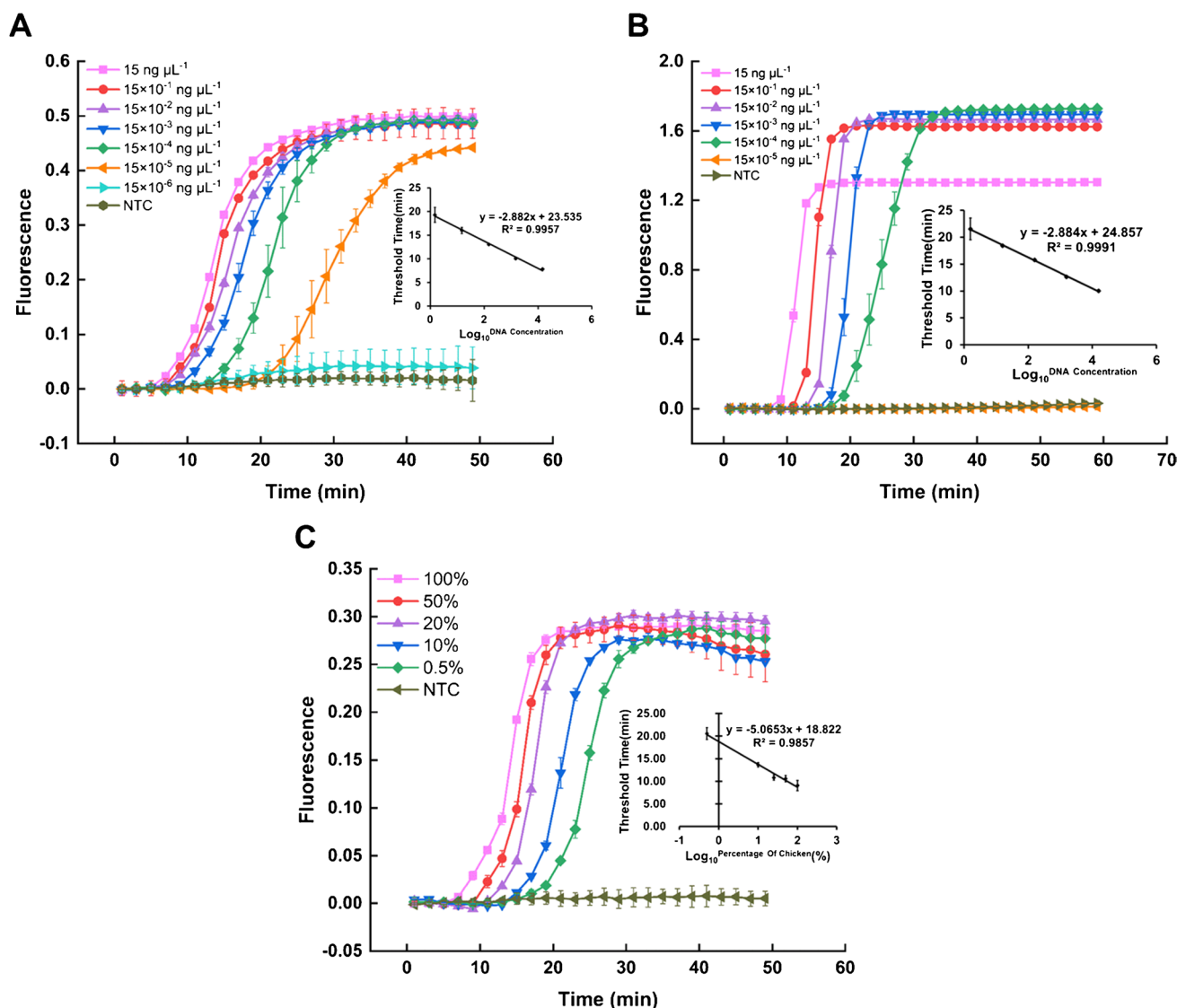
**Fig. 2** Real-time fluorescence curves of different structures of CMB in the CMB-LAMP assay (A). CMB1, CMB2, CMB, CMB3 and CMB4 differ in the number of bases that are in the stem structure and complementary to the target sequence (0, 2, 4, 6 and 8, respectively).

Specificity of CMB-LAMP assays (B) and SYBR Green I-LAMP assays (C) with total DNA of chicken meat and nine other kinds of meat as targets

CMB-LAMP reactions, while no interference signals were detected from other templates (Fig. 2B), which indicated that the CMB-LAMP method has high specificity for chicken components. The same results were obtained from the SYBR Green I-LAMP assay (Fig. 2C), demonstrating that both methods are highly specific for detecting chicken DNA. The cycle threshold (Ct) value in the CMB-LAMP method, which refers to the time needed for a sample to amplify and cross a threshold (cutoff) to be considered positive, was 7.9 min and is much shorter than the Ct value in the SYBR Green I-LAMP method (12.8 min), which indicated that the CMB-LAMP method is much more time-efficient than the SYBR Green I-LAMP method.

### Limit of detection of the CMB-LAMP assay

To assess the sensitivity of the CMB-LAMP assay, 10-fold serial dilutions of chicken DNA ranging from 15 to  $15 \times 10^{-6}$  ng  $\mu\text{L}^{-1}$  were used as templates, and sterilized double-distilled water was the NTC in parallel with the SYBR Green I-LAMP assay (Fig. 3A, B). Both methods obtained typical amplification curves with chicken DNA concentrations of 15 ng  $\mu\text{L}^{-1}$ ,  $15 \times 10^{-1}$  ng  $\mu\text{L}^{-1}$ ,  $15 \times 10^{-2}$  ng  $\mu\text{L}^{-1}$ ,  $15 \times 10^{-3}$  ng  $\mu\text{L}^{-1}$  and  $15 \times 10^{-4}$  ng  $\mu\text{L}^{-1}$ . Nevertheless, no positive signals were found in NTC and the reactions that contained a chicken DNA concentration of  $15 \times 10^{-5}$  ng  $\mu\text{L}^{-1}$ , illustrating that the sensitivity of the CMB-LAMP method was similar



**Fig. 3** Comparison of sensitivity between CMB-LAMP assays and SYBR Green I-LAMP assays with chicken DNA concentrations of  $15 \text{ ng } \mu\text{L}^{-1}$ ,  $15 \times 10^{-1} \text{ ng } \mu\text{L}^{-1}$ ,  $15 \times 10^{-2} \text{ ng } \mu\text{L}^{-1}$ ,  $15 \times 10^{-3} \text{ ng } \mu\text{L}^{-1}$ ,  $15 \times 10^{-4} \text{ ng } \mu\text{L}^{-1}$ ,  $15 \times 10^{-5} \text{ ng } \mu\text{L}^{-1}$  and  $15 \times 10^{-6} \text{ ng } \mu\text{L}^{-1}$ ; sterilized

double-distilled water was used as the NTC. The real-time fluorescence amplification curves produced from CMB-LAMP assays (**A**) and SYBR Green I-LAMP assays (**B**). The CMB-LAMP assay detects 100%, 50%, 25%, 10% and 0.5% chicken adulteration (**C**)

to that of the SYBR Green I-LAMP method, both reaching  $15 \times 10^{-4} \text{ ng } \mu\text{L}^{-1}$ . Moreover, the template DNA concentration and the Ct value of both methods showed good linear relationships.

In addition, when detecting the same DNA concentration, the amplification curves showed that the Ct value in the CMB-LAMP assay was at least 2 min less than that in the SYBR Green I-LAMP assay. In addition, this CMB-LAMP method was coupled with a rapid DNA extraction method to achieve fast adulteration detection. The entire process (from DNA extraction to CMB-LAMP results analysis) was able to be finished within 20 min, which is at least 10 min shorter than the previously reported LAMP-lateral flow dipstick

method and 10 times as sensitive as that of real-time PCR (RT-PCR) assays [40].

The limit of detection of the CMB-LAMP assay was studied by analyzing the chicken–beef mixed samples. The chicken content in the mixture ranged from 0.1 to 100% (w/w), and sterilized double-distilled water was used as the NTC. All reactions were performed with four replicates. Amplification curves were obtained from the samples that contained 0.5% to 100% (w/w) chicken, while no positive signal was detected with lower chicken content and the NTC sample (Fig. 3C). It has been established that there is no economic benefit of adulteration when it is below 1% [41]. Therefore, this CMB-LAMP method is sufficient and

practical for the detection of chicken adulteration. Twenty percent chicken content was used to investigate the reproducibility of CMB-LAMP. Experiments were performed on different days under optimal conditions, and the reproducibility was less than 2.62% (RSD,  $N=3$ ), which demonstrated the excellent reproducibility of this method.

### Method validation

The applicability of the CMB-LAMP method was further studied. Six different animal meats (chicken, duck, beef, pork, mutton, horse) were minced and mixed in a ratio of 1:1:1:1:1:95 (w/w). To achieve fast on-site detection, our developed DNA extraction method was combined with the CMB-LAMP and SYBR Green I-LAMP methods in this experiment. As shown in Table 2, the CMB-LAMP method can accurately detect 1% (w/w) adulterated chicken in the mixed samples within 15 min. The SYBR Green I-LAMP method showed similar sensitivity to the CMB-LAMP method but nearly 9 min slower in amplification. The amplification curves were placed in the supplementary material (see Electronic Supplementary Material Fig. S6).

### Application of the CMB-LAMP assay

To evaluate the feasibility of the CMB-LAMP assay, DNA extracted from 20 kinds of commercial meat samples, with a positive control and an NTC, was used. All reactions were performed with four replicates. As shown in Table 3, one beef ball and mutton shashlik displayed positive results. The

CMB-LAMP method and SYBR Green I-LAMP method showed excellent consistency in the amplification results. The amplification curves are shown in the supplementary material (see Electronic Supplementary Material Fig. S6). Based on the calibration curve of the CMB-LAMP method, the chicken content in the mutton shashlik and beef ball were  $1.6 \text{ pg } \mu\text{L}^{-1}$  and  $7.8 \times 10^{-1} \text{ ng } \mu\text{L}^{-1}$ , respectively.

### Method comparison

In summary, a CMB-LAMP assay to detect chicken adulteration was established in this study and compared with other nucleic acid-based molecular biology methods (Table 4). Compared to RT-PCR, daPCR (direct asymmetric PCR), SEA, RPA and other reported LAMP methods, the CMB-LAMP method has the advantages of faster amplification speed, higher sensitivity, higher multiplexing potential and relatively low cost. In particular, the combination of CMB-LAMP and the rapid DNA extraction method developed by our laboratory provides a highly efficient on-site detection method for meat adulteration.

### Conclusion

A specific, sensitive and quantitative CMB-LAMP method was developed in this study to detect chicken components in adulterated meat. This method has several advantages: (a) it is timesaving—by combining with a simple DNA extraction method, the whole process from DNA

**Table 2** The Ct values of the CMB-LAMP assay and SYBR Green I-LAMP in mixed meat

Sample (w/w)	CMB-LAMP Ct value (min)	SYBR Green I-LAMP Ct value (min)
Beef/mutton/pork/duck/horse/chicken = 95:1:1:1:1:1	12.71±0.38	24.13±0.28
Beef/mutton/pork/duck/horse/chicken = 1:95:1:1:1:1	13.56±0.13	23.55±0.09
Beef/mutton/pork/duck/horse/chicken = 1:1:95:1:1:1	12.72±0.15	22.72±1.01
Beef/mutton/pork/duck/horse/chicken = 1:1:1:95:1:1	13.35±0.25	23.61±0.08
Beef/mutton/pork/duck/horse/chicken = 1:1:1:1:95:1	13.45±0.21	24.46±0.57
Beef/mutton/pork/duck/horse/chicken = 1:1:1:1:1:95	10.54±0.16	19.03±0.26

**Table 3** Analysis results of commercial products using the CMB-LAMP and SYBR Green I-LAMP assays

Meat products	Number of samples	CMB-LAMP		SYBR Green I-LAMP	
		Adulterant number	Adulteration percentage	Adulterant number	Adulteration percentage
Beef roll	3	0	0%	0	0%
Beef ball	3	1	33.3%	1	33.3%
Pork shashlik	4	0	0%	0	0%
Mutton shashlik	5	1	20%	1	20%
Beef shashlik	5	0	0%	0	0%



**Table 4** Comparison of the CMB-LAMP method to other nucleic acid-based methods

Methods	Species detected	Target gene	Number of primers	Number of enzymes	Amplification conditions	Limit of detection	Multi-plexing potential	References
RT-PCR	Chicken	Cytb	2	1	95 °C, 5 min 40 cycles: 95 °C, 5 s and 60 °C, 30 s	5×10 <sup>-2</sup> ng chicken DNA	High	[40]
LAMP-lateral flow dipstick	Chicken	Cytb	6	1	60 °C, 30–40 min	5 pg chicken DNA 0.1% chicken	High	[40]
RPA-lateral flow dipstick	Chicken	ND4L	2	3	39 °C, 20 min	20 pg μL <sup>-1</sup> chicken DNA 1% chicken	High	[42]
Fluorescent biosensor	Pork	Cytb	2	1	98 °C, 30 s; 50 cycles: 98 °C, 35 s; 59 °C, 5 s; 72 °C, 10 s and 72 °C, 1 min	0.01 ng porcine DNA 0.1% pork	High	[43]
SEA	Duck	Mitochondrial DNA	2	1	62 °C, 1 h	10 pg μL <sup>-1</sup> duck DNA 0.1% duck	No	[44]
CMB-LAMP	Chicken	Cytb	5	2	64 °C, 17 min	1.5 pg μL <sup>-1</sup> chicken DNA 0.5% chicken	High	This work
SYBR-LAMP	Chicken	Cytb	6	1	64 °C, 20 min	1.5 pg μL <sup>-1</sup> chicken DNA	No	This work

extraction to results analysis can be finished in approximately 20 min; (b) the method can quantitatively detect chicken DNA at concentrations as low as 1.5 pg μL<sup>-1</sup>; (c) the developed method is suitable for mixed meat samples; and (d) it can avoid false-positives. However, the specific mechanism of CMB needs to be further explored in future research to improve the sensitivity. Moreover, this method is not suitable for quantification in resource-limited areas where real-time fluorescence systems are not available. In this case, a constant-temperature water bath and a handheld fluorescence detector can replace the real-time fluorescence system to achieve rapid on-site and qualitative detection of chicken. Overall, our study provides a reliable and effective method for the detection of poultry adulteration in meat in the form of POCT.

**Supplementary Information** The online version contains supplementary material available at <https://doi.org/10.1007/s00216-022-04342-7>.

**Acknowledgments** The National Natural Science Foundation of China (No. 32102060), Key R&D Program of Zhejiang (Nos. 2021C02059-4 and 2022C02028), Natural Science Foundation of Zhejiang Province (No. LQ21B050003), Science and Technology Programs of Ningbo (Nos. 202003N4127 and 20211ZDYF020179) and State Key Laboratory for Managing Biotic and Chemical Threats to the Quality and Safety of Agro-products (No. 2021DG700024-KF202112) are gratefully acknowledged for financial support.

## Declarations

**Conflict of interest** The authors declare no conflicts of interest.

## References

- Godfray H CJ, Aveyard P, Garnett T, Hall J W, Key T J, Lorimer J, Pierrehumbert R T, Scarborough P, Springmann M, Jebb S A. Meat consumption, health, and the environment. *Science*. 2018;361(6399). <https://doi.org/10.1126/science.aam5324>.
- Mounika T, Girish P S, Shashi Kumar M, Kumari A, Singh S, Karabasanavar N S. Identification of sheep (*Ovis aries*) meat by alkaline lysis-loop mediated isothermal amplification technique targeting mitochondrial D-loop region. *J Food Sci Technol-Mysore*. 2021;58(10):3825–34. <https://doi.org/10.1007/s13197-020-04843-2>.
- Roy S, Abd Rahman I, Santos J H, Ahmed M U. Meat species identification using DNA-redox electrostatic interactions and non-specific adsorption on graphene biochips. *Food Control*. 2016;61:70–8. <https://doi.org/10.1016/j.foodcont.2015.09.029>.
- Hossain M A M, Uddin S M K, Sultana S, Bonny S Q, Khan M F, Chowdhury Z Z, Johan M R, Ali M E. Heptaplex Polymerase Chain Reaction Assay for the Simultaneous Detection of Beef, Buffalo, Chicken, Cat, Dog, Pork, and Fish in Raw and Heat-Treated Food Products. *J Agric Food Chem*. 2019;67(29):8268–78. <https://doi.org/10.1021/acs.jafc.9b02518>.
- Abbas O, Zadavec M, Baeten V, Mikus T, Lesic T, Vulic A, Prpic J, Jemersic L, Pleadin J. Analytical methods used for the

- authentication of food of animal origin. *Food Chem.* 2018;246:6–17. <https://doi.org/10.1016/j.foodchem.2017.11.007>.
6. Hu X, Xu H, Zhang Y, Lu X, Yang Q, Zhang W. Saltatory rolling circle amplification (SRCA) for sensitive visual detection of horsemeat adulteration in beef products. *Eur Food Res Technol.* 2021;247(11):2667–76. <https://doi.org/10.1007/s00217-021-03720-2>.
  7. Zhao L, Wang K, Yan C, Xiao J, Wu H, Zhang H, Zhang X, Zhang C, Hu Y, Lu X, Zheng W. A PCR-based lateral flow assay for the detection of Turkey ingredient in food products. *Food Control.* 2020;107. <https://doi.org/10.1016/j.foodcont.2019.106774>.
  8. Sezer B, Bjelak A, Velioglu HM, Boyaci IH. Identification of meat species in processed meat products by using protein based laser induced breakdown spectroscopy assay. *Food Chem.* 2022;372. <https://doi.org/10.1016/j.foodchem.2021.131245>.
  9. Stachniuk A, Sumara A, Montowska M, Fornal E. Liquid chromatography-mass spectrometry bottom-up proteomic methods in animal species analysis of processed meat for food authentication and the detection of adulterations. *Mass Spectrom Rev.* 2021;40(1):3–30. <https://doi.org/10.1002/mas.21605>.
  10. Zhang M, Li Y, Zhang Y, Kang C, Zhao W, Ren N, Guo W, Wang S. Rapid LC-MS/MS method for the detection of seven animal species in meat products. *Food Chem.* 2022;371. <https://doi.org/10.1016/j.foodchem.2021.131075>.
  11. Zia Q, Alawami M, Mokhtar NFK, Nhari RMHR, Hanish I. Current analytical methods for porcine identification in meat and meat products. *Food Chem.* 2020;324. <https://doi.org/10.1016/j.foodchem.2020.126664>.
  12. Yin R, Sun Y, Wang K, Feng N, Zhang H, Xiao M. Development of a PCR-based lateral flow strip assay for the simple, rapid, and accurate detection of pork in meat and meat products. *Food Chem.* 2020;318. <https://doi.org/10.1016/j.foodchem.2020.126541>.
  13. Kumar Y. Isothermal amplification-based methods for assessment of microbiological safety and authenticity of meat and meat products. *Food Control.* 2021;121. <https://doi.org/10.1016/j.foodcont.2020.107679>.
  14. Kumar Y, Narsaiah K. Rapid point-of-care testing methods/devices for meat species identification: A review. *Compr Rev Food Sci Food Saf.* 2021;20(1):900–23. <https://doi.org/10.1111/1541-4337.12674>.
  15. Nguyen HA, Lee NY. Polydopamine aggregation: A novel strategy for power-free readout of loop-mediated isothermal amplification integrated into a paper device for multiplex pathogens detection. *Biosens Bioelectron.* 2021;189. <https://doi.org/10.1016/j.bios.2021.113353>.
  16. Lo Y-T, Shaw P-C. DNA-based techniques for authentication of processed food and food supplements. *Food Chem.* 2018;240:767–74. <https://doi.org/10.1016/j.foodchem.2017.08.022>.
  17. Quembo CJ, Jori F, Vosloo W, Heath L. Genetic characterization of African swine fever virus isolates from soft ticks at the wildlife/domestic interface in Mozambique and identification of a novel genotype. *Transbound Emerg Dis.* 2018;65(2):420–31. <https://doi.org/10.1111/tbed.12700>.
  18. Shehata HR, Li J, Chen S, Redda H, Cheng S, Tabujara N, Li H, Warriner K, Hanner R. Droplet digital polymerase chain reaction (ddPCR) assays integrated with an internal control for quantification of bovine, porcine, chicken and turkey species in food and feed. *PLoS One.* 2017;12(8). <https://doi.org/10.1371/journal.pone.0182872>.
  19. Girish PS, Barbudde SB, Kumari A, Rawool DB, Karabasanavar NS, Muthukumar M, Vaithiyanathan S. Rapid detection of pork using alkaline lysis- Loop Mediated Isothermal Amplification (AL-LAMP) technique. *Food Control.* 2020;110. <https://doi.org/10.1016/j.foodcont.2019.107015>.
  20. Notomi T, Okayama H, Masubuchi H, Yonekawa T, Watanabe K, Amino N, Hase T. Loop-mediated isothermal amplification of DNA. *Nucleic Acids Res.* 2000;28(12). <https://doi.org/10.1093/nar/28.12.e63>.
  21. Viet Loan Dao T, Herbst K, Boerner K, Meurer M, Kremer LPM, Kirmmaier D, Freistaedter A, Papagiannidis D, Galmozzi C, Stanifer ML, Boulant S, Klein S, Chlanda P, Khalid D, Miranda IB, Schnitzler P, Kraeusslich H-G, Knop M, Anders S. A colorimetric RT-LAMP assay and LAMP-sequencing for detecting SARS-CoV-2 RNA in clinical samples. *Sci Transl Med.* 2020;12(556). <https://doi.org/10.1126/scitranslmed.abc7075>.
  22. Notomi T, Mori Y, Tomita N, Kanda H. Loop-mediated isothermal amplification (LAMP): principle, features, and future prospects. *J Microbiol.* 2015;53(1):1–5. <https://doi.org/10.1007/s12275-015-4656-9>.
  23. Cai S, Kong F, Xu S. Detection of porcine-derived ingredients from adulterated meat based on real-time loop-mediated isothermal amplification. *Mol Cell Probes.* 2020;53. <https://doi.org/10.1016/j.mcp.2020.101609>.
  24. Shi Y, Feng Y, Xu C, Xu Z, Cheng D, Lu Y. Loop-Mediated Isothermal Amplification Assays for the Rapid Identification of Duck-Derived Ingredients in Adulterated Meat. *Food Anal Methods.* 2017;10(7):2325–31. <https://doi.org/10.1007/s12161-016-0767-0>.
  25. Tyagi S, Kramer FR. Molecular beacons: Probes that fluoresce upon hybridization. *Nat Biotechnol.* 1996;14(3):303–8. <https://doi.org/10.1038/nbt0396-303>.
  26. Varona M, Eitzmann DR, Anderson JL. Sequence-Specific Detection of ORF1a, BRAF, and ompW DNA Sequences with Loop Mediated Isothermal Amplification on Lateral Flow Immunoassay Strips Enabled by Molecular Beacons. *Anal Chem.* 2021;93(9):4149–53. <https://doi.org/10.1021/acs.analchem.0c05355>.
  27. Bakthavathsalam P, Longatte G, Jensen SO, Manefield M, Gooding JJ. Locked nucleic acid molecular beacon for multiplex detection of loop mediated isothermal amplification. *Sens Actuators B-Chem.* 2018;268:255–63. <https://doi.org/10.1016/j.snb.2018.04.081>.
  28. Varona M, Anderson JL. Visual Detection of Single-Nucleotide Polymorphisms Using Molecular Beacon Loop-Mediated Isothermal Amplification with Centrifuge-Free DNA Extraction. *Anal Chem.* 2019;91(11):6991–5. <https://doi.org/10.1021/acs.analchem.9b01762>.
  29. Han Y, Zhang F, Gong H, Cai C. Functional three helix molecular beacon fluorescent “turn-on” probe for simple and sensitive simultaneous detection of two HIV DNAs. *Sensors Actuators B Chem.* 2019;281:303–10. <https://doi.org/10.1016/j.snb.2018.10.110>.
  30. Liu W, Huang S, Liu N, Dong D, Yang Z, Tang Y, Ma W, He X, Ao D, Xu Y, Zou D, Huang L. Establishment of an accurate and fast detection method using molecular beacons in loop-mediated isothermal amplification assay. *Sci Rep.* 2017;7. <https://doi.org/10.1038/srep40125>.
  31. Ding X, Yin K, Chen J, Wang K, Liu C. A ribonuclease-dependent cleavable beacon primer triggering DNA amplification for single nucleotide mutation detection with ultrahigh sensitivity and selectivity. *Chem Commun.* 2019;55(84):12623–6. <https://doi.org/10.1039/c9cc06296c>.
  32. Varona M, Anderson JL. Advances in Mutation Detection Using Loop-Mediated Isothermal Amplification. *Acs Omega.* 2021;6(5):3463–9. <https://doi.org/10.1021/acsomega.0c06093>.
  33. Su Y, Huang S, Hong L, Zou D, Tang Y, Chao S, He X, Xu Y, Liu X, Li L, Feng L, Li W, Liu W, Ke Y, Huang L. Establishment of the molecular beacon-loop-mediated isothermal amplification method for the rapid detection of *Porphyromonas gingivalis*. *J Microbiol Methods.* 2019;160:68–72. <https://doi.org/10.1016/j.mimet.2019.01.013>.
  34. Ding X, Yin K, Li Z, Pandian V, Smyth JA, Helal Z, Liu C. Cleavable hairpin beacon-enhanced fluorescence detection of nucleic

- acid isothermal amplification and smartphone-based readout. *Sci Rep.* 2020;10(1). <https://doi.org/10.1038/s41598-020-75795-y>.
35. Aparin IO, Sergeeva OV, Mishin AS, Khaydukov EV, Korshun VA, Zatsepin TS. Excimer-FRET Cascade in Dual DNA Probes: Open Access to Large Stokes Shift, Enhanced Acceptor Light up, and Robust RNA Sensing. *Anal Chem.* 2020;92(10):7028–36. <https://doi.org/10.1021/acs.analchem.0c00270>.
  36. Varona M, Eitzmann DR, Pagariya D, Anand RK, Anderson JL. Solid-Phase Microextraction Enables Isolation of BRAF V600E Circulating Tumor DNA from Human Plasma for Detection with a Molecular Beacon Loop-Mediated Isothermal Amplification Assay. *Anal Chem.* 2020;92(4):3346–53. <https://doi.org/10.1021/acs.analchem.9b05323>.
  37. Ang YS, Yung L-YL. Rational design of hybridization chain reaction monomers for robust signal amplification. *Chem Commun.* 2016;52(22):4219–22. <https://doi.org/10.1039/c5cc08907g>.
  38. Barratt K, Mackay JF. Improving real-time PCR genotyping assays by asymmetric amplification. *J Clin Microbiol.* 2002;40(4):1571–2. <https://doi.org/10.1128/JCM.40.4.1571-1572.2002>.
  39. Burdukiewicz M, Spiess AN, Blagodatskikh KA, Lehmann W, Schierack P, Rodiger S. Algorithms for automated detection of hook effect-bearing amplification curves. *Biomol Detect Quantif.* 2018;16:1–4. <https://doi.org/10.1016/j.bdq.2018.08.001>.
  40. Wang F, Wu X, Xu D, Chen L, Ji L. Identification of Chicken-Derived Ingredients as Adulterants Using Loop-Mediated Isothermal Amplification. *J Food Prot.* 2020;83(7):1175–80. <https://doi.org/10.4315/JFP-19-542>.
  41. Kesmen Z, Yetiman AE, Sahin F, Yetim H. Detection of Chicken and Turkey Meat in Meat Mixtures by Using Real-Time PCR Assays. *J Food Sci.* 2012;77(2):C167–73. <https://doi.org/10.1111/j.1750-3841.2011.02536.x>.
  42. Lin L, Zheng Y, Huang H, Zhuang F, Chen H, Zha G, Yang P, Wang Z, Kong M, Wei H, Zou X, Lin M. A visual method to detect meat adulteration by recombinase polymerase amplification combined with lateral flow dipstick. *Food Chem.* 2021;354:129526. <https://doi.org/10.1016/j.foodchem.2021.129526>.
  43. Prachugsorn A, Thanakiatkrai P, Phooplub K, Ouiganon S, Sri-aead Y, Thavarungkul P, Kanatharana P, Buranachai C, Kitpipit T. Detection of porcine DNA in food using direct asymmetric PCR and catalyzed hairpin assembly fluorescent biosensor: A novel assay for halal food analysis. *Food Control.* 2022;139. <https://doi.org/10.1016/j.foodcont.2022.108989>.
  44. Yan C, Wang X, Zhao X, Wei M, Shi C, Ma C. Development of a direct and visual isothermal method for meat adulteration detection in low resource settings. *Food Chem.* 2020;319:126542. <https://doi.org/10.1016/j.foodchem.2020.126542>.

**Publisher's note** Springer Nature remains neutral with regard to jurisdictional claims in published maps and institutional affiliations.

Springer Nature or its licensor holds exclusive rights to this article under a publishing agreement with the author(s) or other rightsholder(s); author self-archiving of the accepted manuscript version of this article is solely governed by the terms of such publishing agreement and applicable law.