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Performance improved fluorescence polarization for easy and accurate authentication of chicken adulteration

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ABSTRACT

Rapid, accurate, and effective authentication of unlabeled meat components in commercial meat products is of great significance for guaranteeing food safety and human health. In this research, we have constructed a single-strand binding protein (SSB)-assisted performance improved fluorescence polarization (FP) protocol for authentication of chicken component in meat products. The SSB-assisted FP strategy involves the use of fluorescein isothiocyanate (FITC)-labeled primers to perform polymerase chain reaction (PCR). In addition, FP enhancement was accomplished by mixing the amplified product with SSB. Based on the designed mechanism, the free primers in the final amplified product can be tightly bound by SSB, which greatly restricts the rotation of FITC, thereby resulting a high FP signal. In the presence of chicken DNA, the transformation of single-stranded primers to double-stranded amplicons greatly hinders the combination of SSB and primers, leading to the decrease of FP signal. This method well couples the advantages of both PCR and FP technology. Through this new strategy, as low as 0.035% (wt. %) of chicken adulteration in meat samples can be well authenticated. Moreover, this method is also available for commercial meat products. We expect this SSB-assisted FP strategy will provide new insights into the construction of versatile analysis tools in the field of food safety detection.

1. Introduction

With the rapid growth of global logistics systems, large-scale food adulterations such as horsemeat scandal reported in 2013 have attracted more and more international attention (Ballin, 2010; Montiel et al., 2017; O'Mahony, 2013). Meat adulteration has become a serious problem that causes huge economic losses to consumers, endangers public health, contravenes religious beliefs, and leads to unfair competition in the meat market (El Sheikha, 2019; Vlachos, Arvanitoyannis, & Tserkezou, 2016). More and more food enterprises, governmental regulators, and common consumers have realized the importance of meat adulteration detection.

To dispel consumers' concerns about the authenticity of meat products, various methods taking proteins or nucleic acids as target analytes have been proposed for rapid authentication of meat adulteration. Due to the excellent thermal stability and wide distribution of DNA, molecular biology techniques can well enable the reliable authentication of adulterated components even in processed meat products (El Sheikha et al., 2017; Khairil Mokhtar, El Sheikha, Azmi, & Mustafa, 2020a, 2020b; Lopez-Oceja, Nuñez, Baeta, Gamarra, & de Pancorbo, 2017). Typically, polymerase chain reaction (PCR) and its alternatives have been the most popular technologies for meat adulteration identification because of exponential amplification (El Sheikha, 2018; Hou et al., 2015; Montielsosa et al., 2000). However, these technologies are limited by the tedious post-analysis processes such as electrophoresis and probe hybridization. Although real-time quantitative PCR (qPCR) can solve these problems well, it still requires professional and expensive equipment, which is scarce for resource-limited laboratories (Mohamad, El Sheikha, Mustafa, & Mokhtar, 2013a, 2013b; Perestam, Fujisaki, Nava, & Hellberg, 2017; Ruijter, Lorenz, Tuomi,

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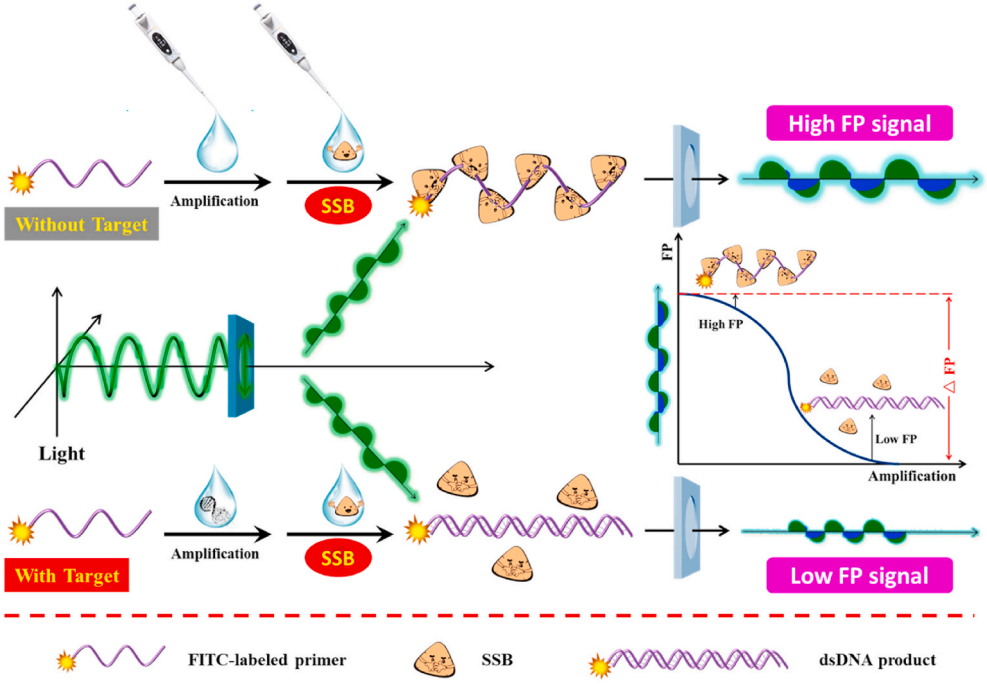

Scheme 1. Schematic illustration for the detection of chicken ingredient in processed meat products by SSB enhanced FP strategy.

Hecker, & van den Hoff, 2014; Schmittgen & Livak, 2008).

Uniquely, fluorescence polarization (FP) is a self-reference technology that is independent on the dye concentration and is mainly determined by the parallel and perpendicular fluorescent intensities excited by the vertical-polarized light (Chen, Levine, & Kwok, 1999; Josephine, Sandrine, Corinne, Jennifer, & Eric, 2009; Qiao et al., 2018; Smith & Eremin, 2008). The features of FP such as simplicity, sensitivity, and low cost make FP-based methods more captivating than traditional fluorescence-based methods (Zhang et al., 2017). In the past few years, many FP-based sensing protocols have been established for qualitative and quantitative detection of molecules, proteins, and cells (Gao, Xu, Li, & Jin, 2016; Jia, Fu, Huang, Yang, & Jia, 2015; Jiang, Tian, Hu, Zhao, & Zhao, 2014; Liang, He, Tian, Zhao, & Zhao, 2018; Qiang, Qin, & Hailin, 2014; Smith & Eremin, 2008; Wang et al., 2016). Of note, only a few methods are available for the detection of genomic DNA in complex biological samples. Studies have proven that changes of molecular weight caused by amplification will affect the FP signal of fluorescein labeled on primers (Qiao et al., 2018). Unfortunately, due to the inherent characteristics of double-stranded DNA (dsDNA) in terms of molecular weight and structure, this method still has some limitations such as poor sensitivity and weak signal. To overcome this bottleneck, we tried to enhance FP signal from the interaction between DNA and protein molecules.

Recent studies have found that some materials including gold nanoparticles (AuNPs) (Gao et al., 2016), polystyrene microsphere (PS) (Huang et al., 2015a), graphene oxide (Huang et al., 2015b), carbon nanotube (Huang et al., 2014), and single-strand binding protein (SSB) (Zhu et al., 2012) are effective for improving the performance of DNA-based FP technologies. Among of them, SSB is one of the important proteins for stabilizing and controlling single-strand DNA (ssDNA) in cells and participates in several processes by interacting with DNA-handling enzymes (Cadman & Peter, 2004; Genschel, Curth, & Urbanke, 2000; Gregor, Claus, & Ute, 2003; Meyer & Laine, 1990). Importantly, SSB can be tightly combined with ssDNA, which will greatly limit the flexibility of ssDNA (Kunzelmann, Morris, Chavda, Eccleston, & Webb, 2010).

Inspired by the selective binding of SSB to ssDNA, in this research, we developed a novel SSB-assisted FP strategy for easy and rapid identification of chicken adulteration in meat products. In the presence of chicken DNA, pre-amplification induces the transformation of fluorophore-labeled single-stranded primers to double-stranded amplicons, which hinders the combination of primers and subsequent SSB addition, resulting a corresponding change in FP signal. This strategy skillfully integrates the high efficiency of PCR and the simplicity of FP analysis, which obviously shortens the detection time and improves the detection sensitivity. Through this novel method, adulteration with chicken in meat products can be easily and rapidly identified with satisfactory results. We envision this SSB-assisted FP platform holds considerable promise for food safety rapid and multiple monitoring.

2. Materials and methods

2.1. Chemicals and apparatus

The pre-amplification procedures were performed in a thermal cycler S1000 (BIO-RAD). The FP signals were measured using a portable Sentry 201 for on-site requirements (Milwaukee, USA). SSB, Taq DNA polymerase (5 U/μL), dNTP mix (25 mM), agarose and proteinase K solution (20 mg/mL) were from Sangon Biotech (Shanghai, China). Other common chemical reagents used in this study were purchased from Sinopharm Chemical Reagent Co., Ltd. (Wuhan, China) of analytical grade and used directly without further purifications. Specific primers for mitochondrial 12S rRNA gene of chicken were used based on previously published sequences (Hou et al., 2015) and verified by the BLAST program. All primer sequences were synthesized by General Biosystems (Anhui, China) and the detailed sequences were shown in Table S1. Ultrapure deionized water (>18 MΩ) was used throughout the research.

2.2. Samples preparation

Fresh and raw meat samples were purchased from the local supermarkets. Meat samples of duck, quail, pig, bovine, sheep, goat, horse, donkey, dog and rabbit were treated as the negative controls. All raw samples were cut into small pieces (ca. 2 × 10 × 10 mm), dried at 70 °C, and ground into powders for subsequent use (Qin et al., 2019). To validate this method, adulterated samples were prepared by blending

chicken into beef with a series of mass proportion (wt. %): 100% (pure chicken), 70%, 50%, 30%, 20%, 10%, 5%, 2%, 1%, 0.5%, 0.2%, 0.1%, 0.05%, 0.02%, 0.01% and 0% (pure beef). For testing of real commercial products, twenty-nine meat samples that labeled as chicken-free were purchased from the local markets. For each brand of products, three samples acquired in different markets to guarantee that they were from different production batches. All meat samples were immediately stored at −20 °C after preparation until DNA extraction.

2.3. DNA extraction from meat samples

DNA was extracted by using carboxylated magnetic nanoparticles (MNPs) as the separation substrates (Huang et al., 2015; Qin et al., 2021). Briefly, 50 mg of meat samples were mixed with 700 μL of extraction buffer (10 mM Tris-HCl, 200 mM NaCl, 5 mM EDTA, 1% SDS, pH 8.0) and 30 μL Proteinase K (20 mg/mL). After incubation at 65 °C for 1 h, the mixture was centrifuged at 10000 g at 4 °C for 5 min, and the supernatant was transferred to a new 1.5 mL sterile tube which contained 350 μL of PEG/NaCl solution and 20 μg MNPs. After incubation at room temperature for 5 min, the MNPs were collected with the applied magnetic field and washed twice with 70% ethanol. The DNA templates were finally diluted and dispersed in 100 μL TE buffer (10 mM Tris-HCl, 1 mM EDTA, pH 8.0). The purity of DNA templates was analyzed by Nanodrop 2000, and then diluted with TE buffer to 50 ng/μL for subsequent use.

2.4. FP authentication of chicken adulteration in meat samples

The amplification was carried out in a thermal cycler S1000 with a total volume of 50 μL, containing 1 × PCR buffer, 1.5 mM $MgCl_2$, 0.2 mM of each dNTP, 60 nM of each primer, 3 U Taq DNA polymerase and 100 ng of DNA template. The amplification was performed under the following conditions: denaturation at 95 °C for 5 min, followed by 30 cycles of 94 °C for 20 s, 58 °C for 20 s, and 72 °C for 20 s. A final extension was performed at 72 °C for 3 min. For FP measurement, 10 μL of the amplified product was mixed with 0.9 μM SSB at room temperature for 3.5 min and then the FP signal was recorded using a portable Sentry 201. For control, the amplified product was also analyzed by 2% agarose gel electrophoresis (150 V, 30 min).

3. Results and discussion

3.1. Mechanism for determination of chicken adulteration

Usually, for the FP-based detections, there are two major facts determining the FP signal: (1) the molecular weight variations of the fluorescent probes; (2) the rotation freedom of the fluorophore. For this research, the protocol we designed for the rapid and sensitive identification of adulterated chicken in meat products is simply depicted in Scheme 1. DNA templates from different meat samples were first extracted and separated using the MNP-based method. Then, the obtained DNA templates were amplified using fluorescein isothiocyanate (FITC)-labeled forward primers (FITC-CF) and common reverse primers (CR). According to the mechanism, the free FITC-CF in amplified product will be tightly bound by SSB that added after amplification, which greatly restricts the rotation freedom of FITC, resulting in a high FP signal. In the presence of chicken DNA, the transformation of single-stranded primers to double-stranded amplicons will exhaust the prime set and hinder the binding between SSB and double stranded amplicons, leading to the decrease of final FP signal. Moreover, the intensity of FP signal is inversely proportional to the ratio of chicken in meat samples, which enables the rapid identification of chicken adulteration in meat products within 2.5 h.

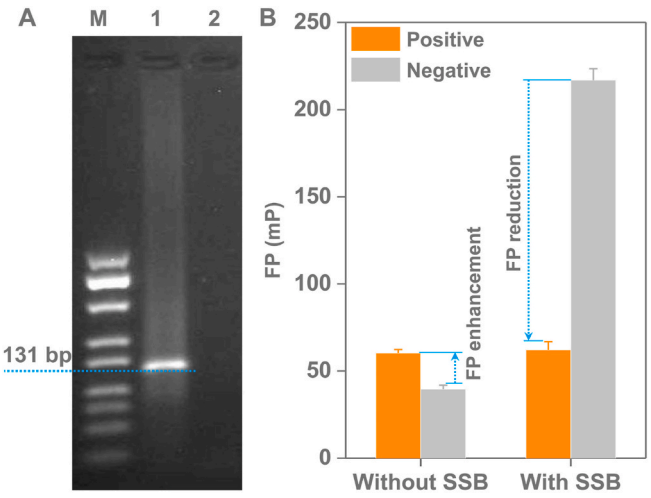

Fig. 1. Feasibility confirmation results of SSB-assisted FP strategy by gel electrophoresis (A) and FP analysis (B). Lane M, DNA marker; Lane 1, positive control that amplified with chicken DNA; Lane 2, negative control that amplified with beef DNA. Error bars represent the standard deviation of three parallel experiments.

3.2. Feasibility confirmation of the SSB-assisted FP strategy

To validate the feasibility of the designed method, the amplification was firstly confirmed in Fig. 1A. As expected, the presence of chicken DNA led to the appearance of target band in lane 1 (positive control) while the presence of bovine DNA resulted in no band in lane 2 (negative control). Considering that no non-specific bands are observed in lane 1–2, the amplification system is available for amplifying chicken DNA. Furthermore, Fig. 1B shows the change in FP signal of amplification products before and after mixing with SSB. One can find that before the addition of SSB, the FP signal of positive control is higher than that of negative control. After the addition of SSB, the FP signal of negative control increases significantly, while the FP signal of positive control changes slightly. There are two reasons for this phenomenon. Firstly, a large number of FITC-labeled primer in the positive control are amplified and converted into double-stranded amplicons during amplification process, resulting in a decreased FP signal by the molecular weight increase; Secondly, the molecular weight (MW) of primer is about 7280 while the MW of the amplicon is about 85000. The MW of SSB is about 76000. The binding number of primers to the SSB is dynamic from 1 to 4 and the MW of the composite is about from 83000 to 99000, which isn't dramatic different from the MW of the amplified products. Results shown in Fig. 1B strongly demonstrated the small variations between these groups. Therefore, the second reason that the exhaustion of FITC-labeled primer will definitely decrease the amount of SSB-primer composites in the detection system, which alleviate the restriction of rotation freedom of FITC and also decrease the FP values. All these results strongly demonstrated that with this SSB-assisted FP method, detection of chicken adulteration in meat products can be well achieved.

3.3. Optimization of conditions for SSB-assisted FP authentication of chicken adulteration

To achieve the best assay performance, several critical parameters were investigated. The net decrease of FP signal ($\triangle FP = FP_0 - FP$, FP_0 and FP mean the FP signal in the absence and presence of target DNA, respectively.) is used as the criterion. It should be pointed out that the FP signal is determined by the number of amplicons and free primers in the amplified product. In the same amplification system, the more amplicons, the fewer residual single-stranded primers, which means a big amount ratio of amplicon and primer. In the case of a fixed FP_0, the

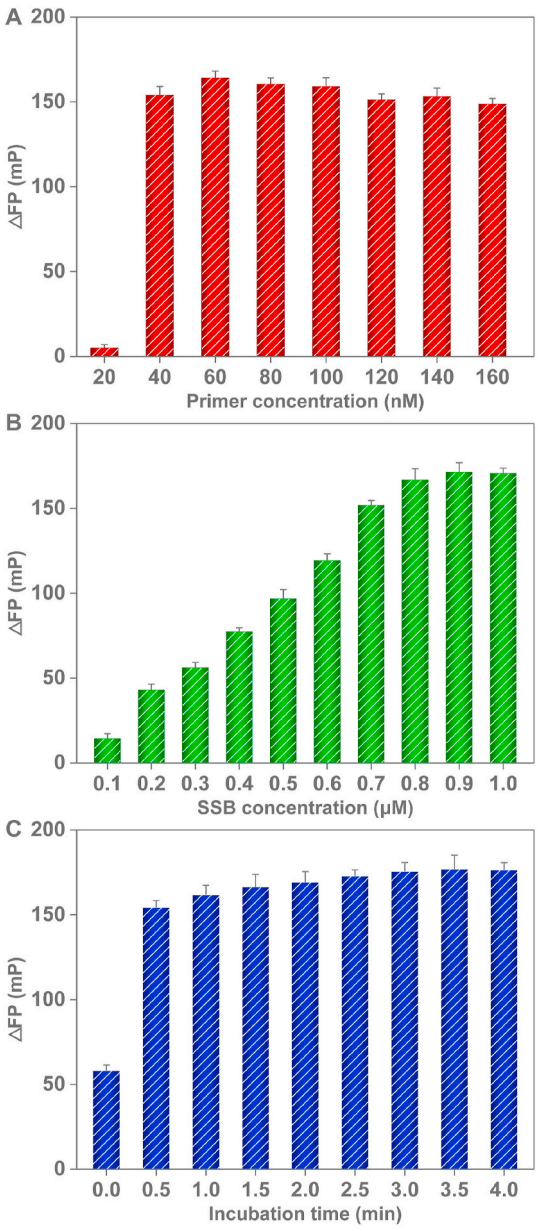

Fig. 2. Optimization results of primer concentration (A), SSB concentration (B), and the incubation time of SSB and amplified product (C). Error bars represent the standard deviation of three parallel experiments.

lower the measured FP signal, the larger the calculated △FP. Combined with the results in Fig. 1B, the predominant FP signal will be determined by the SSB assisted restriction of rotation freedom rather than the final amplicons. Therefore, the decreased final FP signal can be attributed to both the amplification induced molecular weight increase of dsDNA products and the amplification induced exhaustion of primer, which further influence the concentration of SSB-primer composites. Since primer concentration is directly related to the amplification efficiency as well as the bind between SSB and primer, we first optimized the concentration of primers. The effect of different primer concentrations on the detection performance is shown in Fig. 2A. We can find that when the primer concentration was employed at 60 nM, the value of △FP reaches the maximum. It is reasonable because lower or higher primer concentration will lead to a decrease in amount ratio of amplicon and primer of the amplified product. Therefore, we used 60 nM as the optimal primer concentration. Fig. 2B gathered the change tendency of △FP with the increase of SSB. It can be found that increasing the concentration of SSB from 0.1 μM to 0.9 μM can promote the improvement of △FP to a plateau level, indicating the optimal concentration at which FITC-CF can be fully bound by SSB. So, we adopted 0.9 μM as the optimal concentration of SSB. In addition, the incubation time of SSB and amplified product was also studied. As shown in Fig. 2C, an incubation time of 3.5 min is sufficient for binding SSB and FICT-CF, and that was selected as the optimal incubation time.

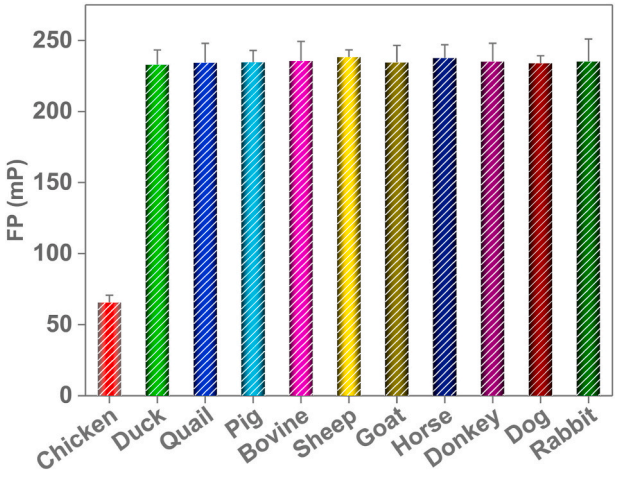

Fig. 4. Specificity results of SSB-assisted FP strategy. Error bars represent the standard deviation of three parallel experiments.

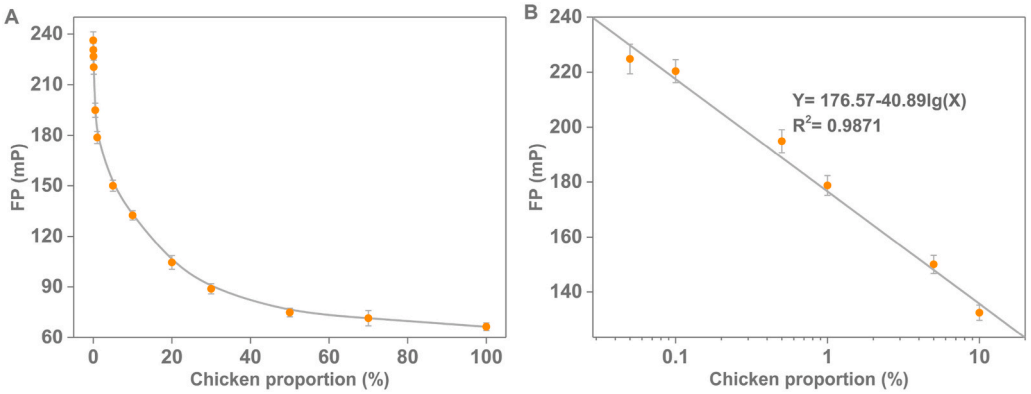

Fig. 3. (A) FP response of a series of raw chicken adulterated beef samples in the range from 0% (pure beef) to 100% (pure beef). (B) Corresponding linear relationship between FP value and adulteration ratio of chicken (0.05%–10%). Error bars represent the standard deviation of three parallel experiments.

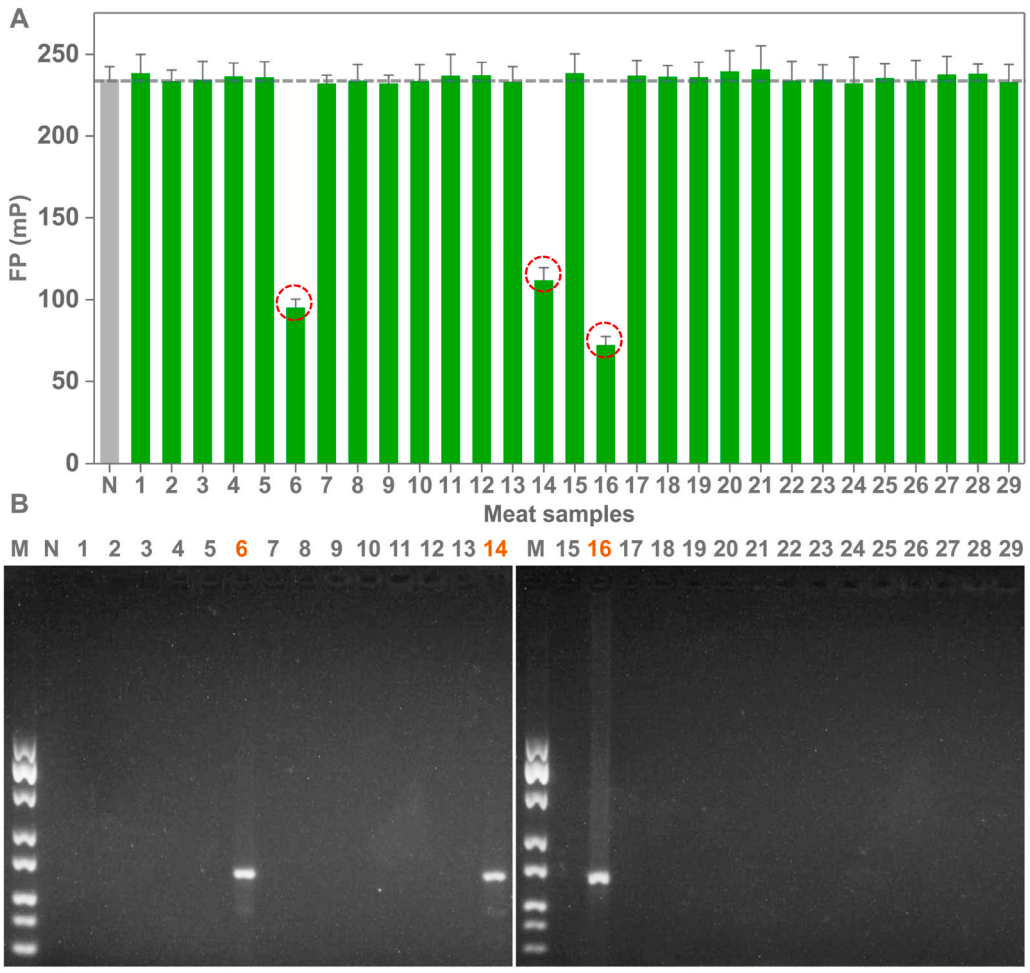

Fig. 5. Detection results of the commercial processed meat products by the designed SSB-assisted FP protocol (A) and gel electrophoresis (B). N, negative control that amplified with pure beef sample (gray); 1–29, commercial meat products (green). Error bars represent the standard deviation of three parallel experiments. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

3.4. SSB-assisted FP strategy for detection of chicken adulteration in meat samples

Under the above optimized conditions, the performance of SSB-assisted FP strategy was evaluated with gradient adulterated samples. As shown in Fig. 3A, the FP signal gradually decreases with the increase of chicken proportion from 0% (pure chicken) to 100% (pure beef). Fig. 3B plotted the FP signal as a function of the chicken proportion in the range from 0.05% to 10%. The standard curve is estimated to be: Y = 176.57–40.89lg(X) with a correlation coefficient (R^2) of 0.9871, where Y and X represent the FP value and the proportion of chicken, respectively. The limit of detection was calculated to be 0.035% on the basis of the 3σ/slope (σ, standard deviation of the blank samples). Compared to other methods, the SSB-assisted FP method realizes simple, low-cost and sensitive identification of chicken in meat products within a shorter time (Table S2).

To confirm the specificity of this SSB-assisted FP protocol for detection of chicken adulteration, eleven kinds of meats from chicken, duck, quail, pig, bovine, sheep, goat, horse, donkey, dog and rabbit were treated and analyzed with the same protocol. Results in Fig. 4 showed that the FP signal was significantly reduced only in the presence of chicken DNA, while other DNA templates cannot produce any distinguishable signal change. These results reveal that our SSB-assisted FP protocol has great specificity for detection of chicken adulteration, thus indicating the potential application of this method in real adulteration screening.

3.5. Detection of chicken adulteration in commercial meat products

Finally, the authenticity of twenty-nine commercial meat products were directly measured with this SSB-assisted FP method. As shown in Fig. 5A, we can easily find that three samples (including sample of 6, 14 and 16) of twenty-nine commercial meat products have distinct changes in FP signal compared to negative control, which indicates the existence of chicken adulteration in the corresponding meat products. Meanwhile, the accuracy of FP measurement was verified by electrophoresis analysis and the same conclusion was obtained (Fig. 5B). Simultaneously, all samples were also measured by qPCR (data not shown). The well agreement among these results strongly demonstrated the potential of the SSB-assisted strategy for detection of practical meat products.

4. Conclusions

In summary, we have developed a novel SSB-assisted FP strategy for easy and rapid detection of adulterated chicken component in meat products. This method skillfully integrated the excellent amplification efficiency of PCR and the simplicity of FP analysis using FITC-labeled primers. The capability to detect chicken component rapidly and accurately in meat products with improved sensitivity of 0.035% (wt. %) is the primary advantage over traditional PCR and other reported methods. Specifically, compared to traditional PCR methods, the designed method avoids the complex gel preparation and electrophoresis process; Compared with qPCR, this SSB-assisted FP strategy does

not require additional fluorescent dye or TaqMan probes, as well as expensive instruments for fluorescent measurements. Of great importance, this strategy is also capable of detecting commercial meat products with acceptable accuracy compared to qPCR. This method may be extended to be a universal analysis protocol for various target components by simple primer replacement. Overall, the SSB-assisted FP method can guide the development of new and improved test kits for inspection programs to enforce labelling regulation in the meat industry.

CRediT authorship contribution statement

Li Yao: Investigation, results, Writing – original draft. **Jianguo Xu:** Writing – original draft, Methodology. **Wei Qu:** Methodology, and discussion. **Dongqing Qiao:** Investigation, and validation. **Sergei A. Eremin:** Methodology, and discussion. **Jianfeng Lu:** Reproducibility study. **Wei Chen:** Supervision, Writing – review & editing. **Panzhu Qin:** Investigation, Writing – original draft.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.foodcont.2021.108604.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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