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Dual-color blending based visual LAMP for food allergen detection: A strategy with enlarged color variation range and contrast

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ABSTRACT

Food allergy has been a serious public health problem around the world. Its prevention relies heavily on the effective avoidance of any contaminated food, making clear and accurate detection very important. LAMP is one of the most potent methods for allergen rapid detection. However, its current colorimetric readouts usually have low color contrast and narrow color variation range. Thus, here we proposed a strategy based on color evolution to enlarge the variation range as well as the contrast to improve its suitability for naked-eye observation. By simply blending two commonly used color change processes during amplification, a wider color variation window, and a near contrast color change, purple-to-green with a hues difference of 10 were obtained. Three important allergens (walnuts, hazelnuts, and peanuts) were tested with a comparable sensitivity towards fluorescent real-time LAMP. Its feasibility for practical use has also been studied. This simple but effective strategy provides a new idea for the colorimetric detection of LAMP amplicons and can be applied to various fields.

1. Introduction

Food allergy is a public health problem around the world, especially the pediatric one can easily cause serious consequence. According to statistics, food allergies affect nearly 6% of the adult (Lyons et al., 2019) and 10% of children (Sicherer & Sampson, 2018). For some allergic individuals, exposure to specific food allergens can even cause life-threatening reactions (anaphylactic shock). Currently, the only effective way to prevent such reactions is avoidance, making proper labeling quite essential. Many legislations on food allergen labeling have been put into place in different countries or organizations. However, besides knowingly and deliberately introduced allergenic ingredients, some "hidden allergens" contaminated unintentionally during the products transportation, storage, and/or processing, can also pose a threat to the health of allergic individuals, which has further challenged the tracing of allergens more accurately, rapidly, and conveniently.

Enzyme linked immunosorbent assay (ELISA) is currently the most commonly used immunoassay method for the determination of allergens in the food industry and official food control agencies (Alves, Barroso, Gonzalez-Garcia, Oliveira, & Delerue-Matos, 2016). However, such immunology-based analysis methods rely on the specific binding between antigen and antibody, while the protein is susceptible to the processing processes, resulting in the change of specific binding site and thus false negative. At the same time, complex food matrixes may contain other components that can bind to antibodies, which are prone to cause false positives (van Hengel, 2007). High performance liquid chromatography (HPLC) is another popular method for allergen detection. It can quantitatively detect several allergenic components in complex food matrix in one operation with high confidence, high sensitivity and high specificity. With it, Guillem Campmajo et al classified 10 types of nuts, and quantitatively detected the adulteration level of peanut or hazelnut in almond products (Campmajo, Saez-Vigo, Saurina, & Nunez, 2020). However, this method requires tedious sample preparation processes as well as the expensive equipment and trained personnel. Different from the direct detection of allergen proteins, the nucleic acid-based analysis is detecting genes encoding the allergen proteins. Given the higher stability and anti-matrix interference ability of DNA comparing to protein, DNA-based assays can be a good

Abbreviations: LAMP, loop-mediated isothermal amplification.

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alternative for allergens detection (Holzhauser, 2018; Stephan & Vieths, 2004).

Among those DNA-based assays, the most traditional one, polymerase chain reaction (PCR) is of great importance. However, it is not perfect for the in-the-field detection in resource limited areas, as its need of accurate temperature control and specific instrument for readouts monitoring. Aiming at this problem, isothermal amplification emerged and has been recognized as a valuable alternative technology. Loop-mediated isothermal amplification (LAMP) is one of such potential technologies to amplify nucleic acid at a constant temperature in vitro efficiently, rapidly and cost-effectively (Kim & Kim, 2018; Notomi et al., 2000; Randhawa, Singh, Morisset, Sood, & Žel, 2013; Zhang, Wu, Wu, Ping, & Wu, 2019). It is processed with a DNA polymerase of strand-displacement activity and 4–6 rationally designed primers. Besides its inherent isothermal merit, LAMP has much higher amplification efficiency generating two orders of magnitude more amplicons and thus can be detected directly with naked eyes through several methods like turbidity (Mori, Nagamine, Tomita, & Notomi, 2001) or by-products based on colorimetric detection (Zhang, Wang, Wang, Wu, & Ying, 2014a; Zhang, Wang, Wang, Ying, & Wu, 2015; Zhang, Wu, Wang, Wang, & Ying, 2014b). Those rapid, easy, and simple detection methods have better leveraged the accessibility of this amplification technique in resource-limited areas. Given that LAMP also has high specificity and sensitivity, it has been extended into several formats (Cai, Jung, Bhadra, & Ellington, 2018; Chen et al., 2020; Du et al., 2017; Jiang, Li, Milligan, Bhadra, & Ellington, 2013; Li, Ellington, & Chen, 2011) and widely used in the fields ranging from food safety to medical diagnosis (Li et al., 2020; Pang et al., 2017; Wu et al., 2020).

Although much efforts have been put into the visual detection of LAMP amplicons (Goto, Honda, Ogura, Nomoto, & Hanaki, 2009; Poole et al., 2017; Wang et al., 2017; Zhang et al., 2015, 2020), there are still needs can not be satisfied in practical applications. The turbidity method is direct and simple but the results are hard to tell with naked eyes especially when there are limited targets. So, certain instruments (such as turbidimeters) or technical expertise are still needed. As for the methods based on the chelating of fluorescent DNA-binding dyes, the result can be easily distinguished by untrained personnel, however, most dyes (such as SYBR Green I) must be added to the solution after amplification with the need of opening the lids (Girish et al., 2020; Qian et al., 2017; Singh, Pal, Soo, & Randhawa, 2019; Zhang, Lowe, & Gooding, 2014c), greatly increasing the risk of cross-contamination. To this end, visual colorimetric dyes is attractive if it can be pre-added into the LAMP reaction mixtures, without inhibiting the amplification reactions (Goto et al., 2009; Li et al., 2016).

Cresol red (CR) is one of such dyes and sensitive towards the pH change. When pH is lower than 7.2, it would be yellow; When pH goes greater than 8.8, it becomes purple. During the amplification of nucleic acids, as the nucleictides incorporated into the growing stands, protons released, showing a decrease in pH with a color change from purplish red to yellow (Xiong, Huang, Xu, & Huang, 2020; Zhang et al., 2014b). Hydroxynaphthol blue (HNB) is a metal indicator, which has also been used for LAMP colorimetric detection (Goto et al., 2009). During the LAMP reaction, the concentration of Mg^{2+} ions in the solution decrease significantly, causing the HNB loaded essay to change from purple to blue (Goto et al., 2009). Based on the different action modes of these dyes and the color evolution principle, we hypothesized that CR and HNB could be used in LAMP reactions at the same time and get enhanced colorimetric results. One of the key benefits of this method is if we blend the two processes of colorimetric, a much wider visual color variation window can be expected to make the results easier to identify, which is rarely reported in colorimetric detection. What's more, the reaction is conducted in one tube and no lid-opening was needed that reduced the risk of cross-contamination, make this method easy to operate. Therefore, in this study, a visual detection method based on dual-color blending was developed. The feasibility of the method in practical use was determined by testing the rice flour spiked with walnut, hazelnut, and peanut respectively. This proposed strategy based on the blending of two colorimetric dyes can also be used in a wider range of applications, such as detection of pathogenic bacteria, environmental contaminants, and disease diagnosis related biomarkers.

2. Materials and methods

2.1. Material and DNA separation

Walnuts, hazelnuts, peanuts, rice, and other materials were purchased from local retail stores. Allergen spiked samples were prepared with rice powder as the matrix containing 0.0001% ~ 10% w/w of different allergens. Food mixer was used for homogenization. The Plant Genomic DNA Kit (Tiangen, China) was used as described by the manufacturer to extract and purify the genomic DNAs. The quality and quantity of extracted DNA were analyzed using a Nano-Drop ND-1000 spectrophotometer (Saveen Werner, United States) and stored at −20 °C before use.

2.2. LAMP reaction

The oligonucleotide primers used were listed in the supporting information (Table 1) and synthesized by Sangon Biotech (China). The reactions were carried out in 20 μL mixture containing 1.6 μM each of primers FIP and BIP, 0.2 μM each of F3 and B3, 0.8 μM each of FLP and BLP, 10 mM $(NH4)_2SO_4$, 8 mM MgSO4, 1.4 mM of each dNTP, 0.1% v/v Tween-20, 10 mM KCl, 8 U DNA polymerase (New England Biolabs, USA), and 1 μL of templates of food allergen with 10-fold serial dilution from 10^7 to 10^2 fg/μL. 1 M KOH was used to adjust the initial pH of the reaction mixture to 8.8–9.0, as measured by pH-indicator strips (Merck, Germany).

When the reactions were carried out for colorimetric detection, metal bath heater (Thermo Fisher, USA) was used, CR and HNB were added with certain concentrations as needed. Reactions were incubated at 65 °C for 1 h and then inactivated at 95 °C for 5 min. Reactions were performed with a total volume of 20 μL containing either template DNA or H_2O for non-template controls (NTC). The color of the mixture after the reaction was photographed under natural light by a smartphone (Huawei, China) with white paper as the background. Photoshop image processing software was used to measure the Lab value to quantify the intensity of color change. To compare the sensitivity of the colorimetric method proposed in this work and the traditional real-time LAMP, SYTO9 was employed, and the fluorescence changes were recorded every 30 s for 1 h (Table S4).

2.3. Chromatic aberration calculation

The Lab value of each reaction tube was measured by Photoshop image processing software. The chromatic aberration value (color difference value) between negative and positive samples was obtained through CIEDE2000 formula published by the International Lighting Commission:

$$\Delta E_{00} = \sqrt{\left(\frac{\Delta L'}{K_L S_L}\right)^2 + \left(\frac{\Delta C'}{K_C S_C}\right)^2 + \left(\frac{\Delta H'}{K_H S_H}\right)^2 + R_T \frac{\Delta C'}{K_C S_C}\frac{\Delta H'}{K_H S_H}}$$

$K_L = K_C = K_H = 1$ was used here and a more detailed description and calculation process were explained in the supporting information (Luo, Cui, & Rigg, 2001).

3. Results and discussion

3.1. Colorimetric detection

CR is a common acid-base indicator. In a specific LAMP reaction solution, the pH decreases as the amplification progresses (Yuan, Kong,

Table 1
Primers used in LAMP reaction.

Target	Primers	Sequence (5′–3′)	Ref.
Walnuts	WL-F3	CACTGCCAGGACTACCTGAGGCA	(Administration, 2016a)
	WL-B3	CTGCACCATCTCCTCCATTTCCTC	
	WL-FIP	TCCATCTGACTCAGCTGCTGGCAGGTCAGGGGGTTATGATGAAGAC	
	WL-BIP	GAGCAGTGCCAGTGTGAGGGGTCCCGGATACCCTCCTSCTGCTG	
	WL-FLP	GCCTGAAATGCTGCCGCTGGTT	
	WL-BLP	GTTGAGGCAGGTGGTGAGG	
Hazelnuts	HZ-F3	CAACCAGTGCCAGCGGTACATGAG	(Administration, 2016b)
	HZ-B3	GGCAAGTCCCTAGCAGTCTCCATAACC	
	HZ-FIP	CCGCTCGTCCATCTGCCTCAGCTCAGTACGGGAGTTACGATGGCAGCAA	
	HZ-BIP	GCCGATGTGAGGGGTTGAGGCACCCTCATTTCCTCACCTCGCATCTC	
	HZ-FLP	TGGCAGCACTGCTCAAGTTCCTG	
	HZ-BLP	AGGCGGTAATGCAGCAGCAG	
Peanuts	PEA-F3	CGCGGAAAGCGCCAA	(Sheu, Tsou, Lien, & Lee, 2018)
	PEA-B3	GCGTTCAAAGACTCGATGGT	
	PEA-FIP	TGGTCACTCGTCGCCCCGAGGAAGCCAAACGTTTCTGCT	
	PEA-BIP	TCGGCAACGGATATCTCGGCTACGGGATTCTGCAATTCACA	

Li, Fang, & Chen, 2018), and the color of the solution changes from magenta to yellow. Here, we first tested the concentration effect of cresol red to get better visual performance. With the increase of cresol red concentration, the negative sample changed from purple to magenta, and the positive one changed from yellow to orange (Fig. 1a). Chromatic aberration was used here to quantify the color difference between samples. CIEDE2000 formula was applied, which can best match the person's vision theoretically (Luo et al., 2001). The chromatic aberration between positive and negative samples was the greatest when the concentration of CR reached 125 μM (Fig. 1b and Table S1), indicating the signal to noise of sample was the highest at this condition, which would be best for visualization with naked eyes.

HNB is another pH indicator, exists in a red form below pH 4, undergoes a red-to-blue change, and remains blue at pH 5 to 12. It has also been reported as a colorimetric indicator for Ca^{2+} and Mg^{2+}. At pH 8.8, HNB can chelate Mg^{2+} forming a magenta HNB-Mg^{2+} complex. After the addition of dNTPs, the color of HNB and MG co-existing system changes to violet (Goto et al., 2009). As the reaction progresses, Mg^{2+} is consumed in positive samples, and the color of the reaction solution changes from violet to cerulean blue. However, as the color change of HNB during LAMP amplification is resulted from a combined balance between pH, Mg^{2+}, and dNTPs concentration level change, sometimes the results are a little bit confusing. Those small differences are usually difficult to be recognized with naked eyes. According to the color theory of Johannes Itten, we can get the highest contrast when the hues changing from one to the one directly opposite to it (with a hues difference of 12) on a color wheel (Fig. 2). Higher hue contrast will be easier to be told with naked eyes. With this concept, we revisited the two colorimetric detection methods mentioned above. When we add only HNB for a typical LAMP detection, the color variation range would be from 4 to 7 crossing only 3 hues theoretically. With only CR as the

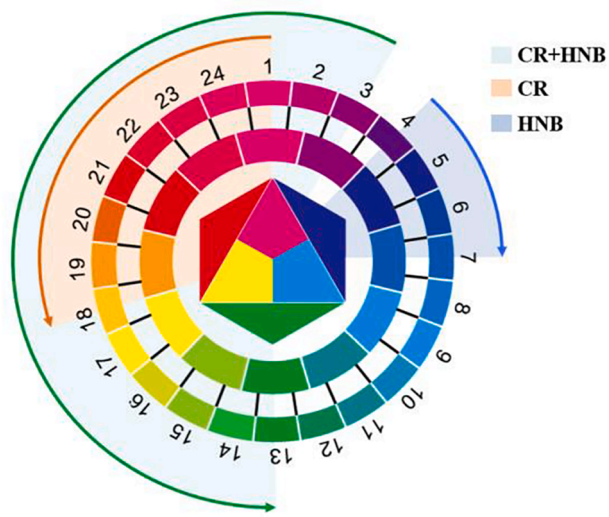

Fig. 2. The modern color wheel with 24 hues and the visual windows (color variation range) with different dyes theoretically.

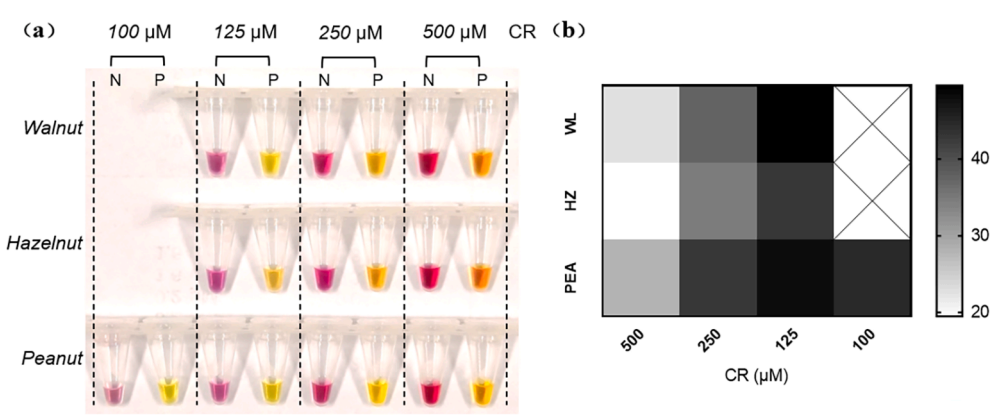

Fig. 1. (a) Performance of CR based colorimetric detection of LAMP. Three food allergen systems were tested, and CR was added with different concentrations. N represented for the no-template-control (NTC) and P represented for positive samples with 1 ng/μL templates. (b) Heat map of color differences between positive and negative samples with different concentrations of CR. PEA, HZ and WL represent for peanut, hazelnut and walnut respectively.

indicator, samples after amplification would undergo the color change for 1 to 18, that is, 7 hues differences change. However, if we blend the two processes of colorimetric change, according to the principle of color evolution, a much wider visual color variation window from 3 to 13 can be expected, with a hues difference of 10, which is almost a contrast color change.

As a proof-of-concept, we evaluated the dual-color system for LAMP detection. The final concentration of CR was fixed at 125 μM. By changing the concentration of HNB, the optimum ratio between CR and HNB was determined. When HNB concentration changed from 0 to 100 μM, the color of positive samples changed from pink to dark purple, while the color of negative samples changed from yellow to green, making the color difference between the negative and positive samples more obvious (Fig. 3a). According to the result of chromatic aberration, it would be the most noticeable for naked eyes observation when the concentration of HNB reached 50 μM (Fig. 3b and Table S2). What needs to be noted is that both CR and HNB can be added before amplification and no lid-opening was needed, making such a method still simple and easy-to-operate. Therefore, for all the three food allergens tested, the best ratio between HNB and CR would be 1:2.5, that is, 50 μM of HNB with 125 μM CR. With this condition, a near contrast change, purple-to-green, after amplification was observed as expected.

3.2. Comparison of the sensitivities of single- or dual- dye based detection

Further, performances of LAMP assays loaded with CR and/or HNB were compared with the regards to the detection of 10-fold diluted food allergen templates. Each assay was carried out in three replicates, and the results were reproducible. Under natural light, the color of CR-loaded assays changed from reddish violet to yellow, and the color of mixed-dye-loaded assays changed from purple to green, its visual sensitivity reached as low as 1 ng/μL for walnut genomic DNAs, 10 pg/μL for hazelnut or peanut, which was equivalent to the real-time LAMP results (Fig. 4a and Table S3) as well as the series of samples with only CR loaded. With a low concentration of target template (diluted plasmid DNA), the color of mixed-dye-loaded assays appeared green, avoiding misjudgment as a weakly positive sample. In contrast, under the same lighting conditions, the positive and negative results of single HNB-loaded assays were not sufficiently distinguishable (Fig. S1). We reasoned this for the collided effect of pH towards the Mg^{2+} consumption induced HNB color change. Based on this, we conferred that HNB was more like providing a blue background in this color blending strategy. After blending it to the evolution of CR caused color changes, a wider color variation window, and a higher color contrast change was obtained.

3.3. Application for the detection of allergens-spiked samples

To investigate the feasibility of dye-loaded LAMP assays in practical applications, dual-dye or single CR dye was applied to test food allergens-contaminated samples. Contaminated samples for different allergens walnut, hazelnut, or peanut were prepared with rice flour as the matrix. The sensitivity and specificity were investigated, and the results were shown in Fig. 4b. As expected, similar to the real-time LAMP (Table S4), our color blending method can detect walnuts in spiked rice samples as low as 1% w/w, hazelnuts, and peanuts to 0.01% w/w. Additionally, in these complex matrices, the specificities of dual dye-loaded LAMP assays were also excellent, and no non-specific results were observed. In the walnut-assay, two common non-target allergens, hazelnut and peanut were tested simultaneously. Similarly, in the hazelnut or peanut assay, the other two non-specific samples were tested. As is shown in Fig. 4b, after amplification, the color of the solution corresponding to the interfering allergens was the same as the NTC. They could be easily recognized by the naked eye from positive or weakly positive results colored green or purple.

In summary, we proposed a strategy to wider the visual windows (color variation range) and increase the color change contrast by simply using two indicators, CR and HNB together in a LAMP assay. It is generally based on (1) the pH change during LAMP reaction (Keikha, 2018; Yuan et al., 2018), and (2) the color blending based evolution caused by the HNB indicated blue background. With such a method, the assays loaded with mixed dyes showed enhanced visual performance at natural light - changing from purple (negative) to green (positive). The feasibility of this method to detect food contamination samples is verified. The proposed dual-dye color blending LAMP has three main advantages: 1) LAMP reaction results can be more easily and directly recognized with the naked eyes; 2) the detection limit is equivalent to real time-LAMP, but the expensive and enormous real-time fluorescence instrument is not required. Only a portable water/metal bath is required; 3) It is a one-pot reaction, dyes can be added before the reaction, no lid-opening after amplification is entailed and thus the risk of cross-contamination is significantly reduced. Therefore, given these pros, this method proposed provides new ideas for the detection of products from isothermal amplification and can be applied to various fields from food safety and/or quality detection to medical diagnosis. The strategy to get an enlarged color variation range and contrast by simply blending two color change processes can also be applied to other kinds of application with a demand for easier naked eyes observation.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence

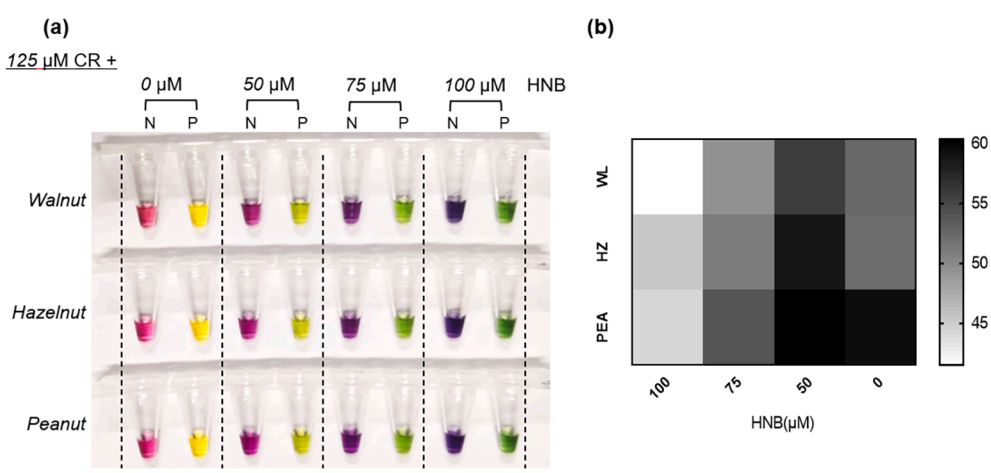

Fig. 3. (a) Performance of color blending based colorimetric detection of LAMP. Three food allergen systems were tested, and 125 μM CR was loaded together with HNB of different concentrations. N represented for the no-template-control (NTC) and P represented for positive samples with 1 ng/μL templates. (b) Heat map of color differences between positive and negative samples with different concentrations of HNB. 125 μM CR was loaded together with HNB of different concentrations before amplification. PEA, HZ and WL represent for peanut, hazelnut and walnut respectively.

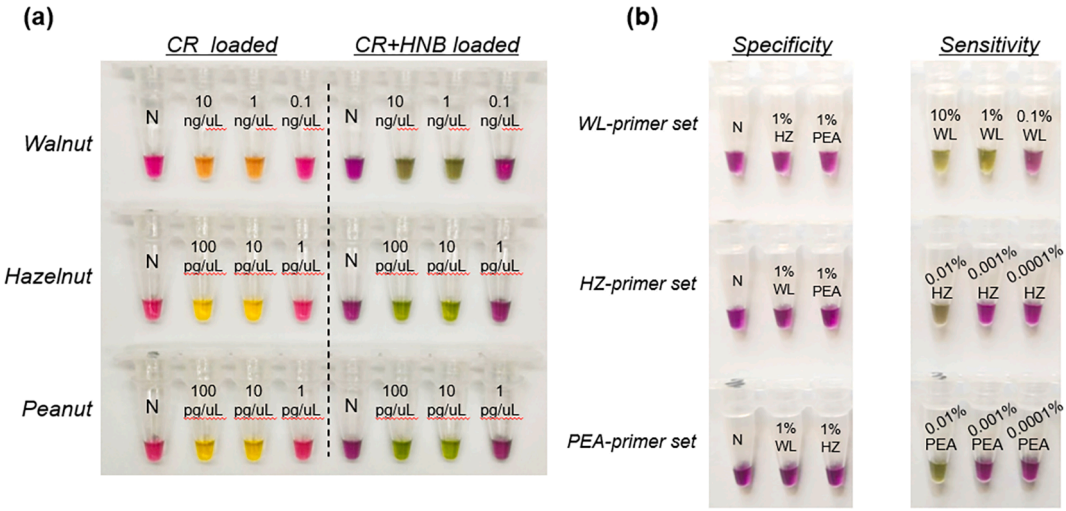

Fig. 4. (a) Sensitivities of different dye-loaded LAMP assays for the detection of serially diluted DNA. (b) Specificity and sensitivity of dual dye based colorimetric assays for aller.

the work reported in this paper.

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Appendix A. Supplementary data

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Supporting Information

Dual-color blending based visual LAMP for food allergen detection: a strategy with enlarged color variation range and contrast

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Real-time Detection of LAMP with SYTO9

To compare the sensitivity of the colorimetric method proposed and the traditional real-time LAMP, CFX96 Real-Time PCR System (Bio-Rad, USA) was used and 1×SYTO 9 (Thermo Fisher, USA) was applied. The fluorescence changes were recorded every 30 seconds at 65°C for 1h. After this, a melting curve analysis was conducted.

CIEDE2000 colour-difference Calculation

Step 1. Measuring the Lab value of each reaction tube in the same picture by Photoshop image processing software and calculate C*:

$$C^*_{ab} = \sqrt{a^{*2} + b^{*2}} \tag{1}$$

Step 2. Calculate L' , a' , C' , h':

$$L' = L^* \tag{2}$$

$$a' = (1 + G) \times a^* \tag{3}$$

$$b' = b^* \tag{4}$$

$$C' = \sqrt{a'^2 + b'^2} \tag{5}$$

$$h' = \tan^{-1}\left(\frac{b'}{a'}\right) \tag{6}$$

$$G = 0.5 \times \left(1 - \sqrt{\frac{\overline{C^*_{ab}}^7}{\overline{C^*_{ab}}^7 + 25^7}}\right) \tag{7}$$

Step 3. Calculate △L' , △C' , △H'(DL*, DC*, and DH* are the CIELAB metric lightness, chroma, and hue differences, respectively, calculated between the standard and sample in a pair):

$$\Delta L = L'_1 - L'_2 \tag{8}$$

$$\Delta C' = C'_1 - C'_2 \tag{9}$$

$$\Delta h' = h_1' - h_2' \tag{10}$$

$$\Delta H' = 2\sqrt{C_1' \times C_2'}\sin\left(\frac{\Delta h'}{2}\right) \tag{11}$$

Step 4. Calculate S_L , S_C , S_H , T and R_T(The SL, SC, and SH are the weighting functions for the lightness, chroma, and hue components, respectively.):

$$S_L = 1 + \frac{0.015\left(\bar{L}' - 50\right)^2}{\sqrt{20 + \left(\bar{L}' - 50\right)^2}} \tag{12}$$

$$S_C = 1 + 0.045\bar{C}' \tag{13}$$

$$S_H = 1 + 0.015\bar{C}'T \tag{14}$$

$$T = 1 - 0.17\cos\left(\bar{h}' - 30°\right) + 0.24\cos\left(2\bar{h}'\right) + 0.32\cos\left(3\bar{h}' + 6°\right) - 0.20\cos\left(4\bar{h}' - 63°\right) \tag{15}$$

$$R_T = -\sin\left(2\Delta\theta\right)R_C \tag{16}$$

$$\Delta\theta = 30\exp\left\{-\left[\left(\bar{h}' - 275°\right)/25\right]^2\right\} \tag{17}$$

$$R_C = 2\sqrt{\frac{\bar{C}'^7}{\bar{C}'^7 + 25^7}} \tag{18}$$

Step 5. Calculate CIEDE2000 ΔE^*:

$$\Delta E_{00} = \sqrt{\left(\frac{\Delta L'}{K_L S_L}\right)^2 + \left(\frac{\Delta C'}{K_C S_C}\right)^2 + \left(\frac{\Delta H'}{K_H S_H}\right)^2 + R_T\frac{\Delta C'}{K_C S_C}\frac{\Delta H'}{K_H S_H}} \tag{19}$$

Table S1. Chromatic aberration value between positive and negative samples with different concentrations of CR.

Object	Cresol red (μM)			
	100	125	250	500
WL		49.56±0.58	37.31±0.07	22.44±0.16
HZ		43.01±0.18	34.53±0.47	19.48±0.11
PEA	44.51±0.00	48.01±0.01	42.69±0.07	27.97±0.05

Table S2. Chromatic aberration value between positive and negative samples with different ratios of CR and HNB.

Object	[HNB]/[CR]*			
	0	0.4	0.6	0.8
WL	52.49±0.61	55.62±0.18	49.43±0.07	41.53±0.48
HZ	52.18±0.19	58.73±0.65	50.97±0.03	45.26±0.00
PEA	59.28±0.06	60.37±0.11	54.08±0.24	44.45±0.59

*The final concentration of CR used was 125 μM.

Table S3. Sensitivities of real-time LAMP conducted with Real-Time PCR system.

	Sample											
	Walnut (WL)				Hazelnut (HZ)				Peanut (PEA)			
Template concentration/ pg· μL^{-1}	10^3	10^2	10^1	1	10^3	10^2	10^1	1	10^3	10^2	10^1	1
Cq/ min	30.92	/	/	/	15.275	20.315	29.705	/	19.625	22.06	26.36	/
Melt temperature/ ℃	89.5	/	/	/	89.5	89.5	89	/	89	89	89	/

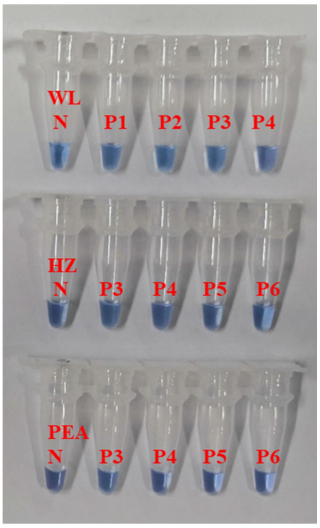

Figure S1. Comparison of the sensitivities of HNB-loaded LAMP assays for the detection of serially diluted plasmid DNA. (N) nuclease-free water; (P1) 10 ng/μL, (P2) 1 ng/μL, (P3) 100 pg/μL, (P4) 10 pg/μL, (P5) 1 pg/μL, (P6) 100 fg/μL.

Table S4. Detection limits of real-time fluorescence LAMP for allergens analysis in doped rice flour.

	Sample											
	Walnuts (WL)				Hazelnuts (HZ)				Peanuts (PEA)			
Doping amount/ w w^{-1}	NTC	10%	1%	10^{-1} %	NTC	10^{-1}%	10^{-2}%	10^{-3}%	NTC	10^{-2}%	10^{-3}%	10^{-4} %
Cq/ min		13.015	25.09			12.215	35.02			23.565		
Melt temperature/ ℃	/	89.5	89	/	/	90	89	/	/	89.5	/	/

Table S5. Chromatic aberration of dual dye loaded allergens assays for doped rice flour samples.

	Target											
	Walnuts (WL)				Hazelnuts (HZ)				Peanuts (PEA)			
Sample/ w w^{-1}	NTC	1% HZ	1% PEA	1% WL	NTC	1% WL	1% PEA	10^{-1}% HZ	NTC	1% WL	1% HZ	10^{-2}% PEA
Whether detected	-	-	-	+	-	-	-	+	-	-	-	+