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# On-point detection of GM rice in 20 minutes with pullulan as CPA acceleration additive†

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1% (w/v) concentration of pullulan as a cross-priming isothermal amplification (CPA) reaction additive has a great acceleration effect on nucleic acid amplification. Using this property of pullulan, on-point specific detection of nucleic acid by lateral-flow dipstick (LFD) within 20 minutes was achieved with initial DNA template of 750 copies.

Nucleic acid testing (NAT) has been an extremely important method in fields ranging from disease diagnosis to food safety inspection. Currently, detection methods such as traditional PCR-based assays and isothermal amplification entail long processing time and bulky optical detection devices. Compared with PCR, isothermal amplification methods have been developed to make NAT easier for implementation and miniaturization. But their drawback of taking a long time for DNA detection has largely never been solved. In terms of loop-mediated isothermal amplification (LAMP), it requires relatively less time, but an hour. Therefore, some researchers have attempted to combine DNA amplification with many other non-optical detection means such as colorimetric detection, turbidity and electrochemical methods.[1–14] However, many of these methods lack detection specificity. Moreover, for the purpose of improving PCR product yields and primer binding specificity, compatible solutes are usually added to the amplification system, such as sugars like trehalose, amino acids and more obscure compounds.[15–20] But it has not been mentioned in the literature on further study of DNA amplification if additive agents have similar enhancement function on multi-primer isothermal amplification, whose principle is different from PCR.

Here we report the application of pullulan as a potent enhancer for cross-priming isothermal amplification (CPA). Then, a specific on-point detection of GM rice was carried out by lateral-flow dipstick (LFD) with the acceleration property of pullulan within 20 minutes.

As is known, pullulan is a water-soluble, extracellular neutral polysaccharide produced by growing fungus-like yeasts, *Aureobasidium pullulans* or *Pullularia pullulans*. Its structure is a linear flexible chain of 1,6-linked maltotriose units (see the ESI, Fig. S1†).[21–24]

Pullulan has outstanding thermostability. As is reported in the literature, pullulan begins to degrade thermally at approximately 250 °C without a clear appearance of a glass transition temperature ($T_g$) or melting temperature ($T_m$) upon heating.[21] Besides this, the plasticity and biodegradability of pullulan were most researched.[23–28] But until now, no reports on the use of pullulan to enhance nucleic acid amplification have been found in the literature. Sana Jahanshahi-Anbuhi *et al.* reported that pullulan provides outstanding protection for entrapped biomolecules against thermal denaturation and chemical modification.[28] We speculate that the addition of pullulan should make thermostabilization of the *Taq* polymerase and increase the enzymatic activity during nucleic acid amplification.

Firstly, the performance of pullulan (Aladdin Industrial Co., Shanghai, China.) was investigated by executing real-time fluorescent CPA at 63 °C with SYTO 9 as nucleic acid stain (previous work in our group, conducted on a Biorad MyiQ 2 Real Time PCR Detection System).[29,30] The fluorescence was collected every 60 seconds, for which time consumed is respected as cycle number. The practical sample of genetically modified rice (Huahui 1) at a mixing level of 0.5% was employed as a model target. A set of primers were designed to target *Agrobacterium tumefaciens* nopaline synthase terminator (T-*Nos*) gene in GM rice (see the ESI, Fig. S2†). Real-time CPAs were conducted with reagents as described (see the ESI†) but adding various concentrations of pullulan in a total volume of 25 μL. Five concentration gradients of pullulan were determined in this assay with a pullulan-free system as the control. The assay was carried out in three repeats.

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As shown in Fig. 1, the amplification efficiency was improved significantly in a concentration-dependent fashion by pullulan up to a concentration of 1% (w/v). But above 4% (w/v), a decrease of reaction efficiency was observed because of the viscosity of a high pullulan concentration. Combined with agarose gel electrophoresis (AGE) analysis, the CPA product yield of the sample with 1% (w/v) pullulan addition was higher in comparison with pullulan-free condition. Nonspecific amplification did not appear with pullulan as a CPA additive. The specific amplification evidence is as follows: (a) according to the image of gel electrophoresis shown in Fig. 1a, the electrophoretic band locations of both CPA reaction systems were exactly the same. (b) As shown in Fig. 1b, for every DNA amplification system, there was only one melting curve peak, which revealed the uniqueness of CPA product sequences. (c) CPA amplicons were confirmed by LFD afterwards. As shown in Fig. 4a, strong signals apparent as red bands at the test line were clearly visible for T-*Nos*. Hence, the improved product yield with 1% (w/v) pullulan addition is not at the price of amplification specificity.

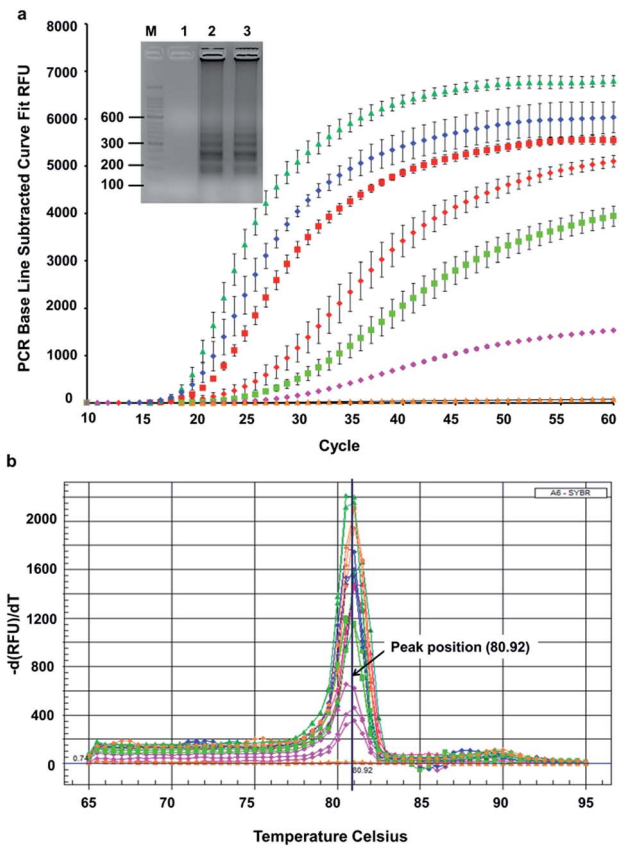


Fig. 1 Real-time fluorescent CPA with different concentrations of pullulan. Pullulan concentrations: green triangle, 1% (w/v); blue diamond, 0.5% (w/v); red square, 2% (w/v); red diamond, pullulan-free (control group); green square, 4% (w/v); pink diamond, 5% (w/v); brown triangle, no template control. (a) Amplification curves of T-*Nos* transcript. Error bars, SD. Inset shows gel electrophoresis for CPA products after amplification for one hour. M is DNA marker (50 bp), lane 1 is no template control, lane 2 is 1% (w/v) concentration of pullulan system and lane 3 is pullulan-free condition. (b) Melt curves of different amplification system with pullulan-free condition as control.

A series of real-time florescent PCR assays with plant reference gene, sucrose phosphate synthase (Sps) were carried out before this assay. The result showed that 1% (w/v) concentration of pullulan also has an acceleration effect on the PCR reaction (data not shown). Compared with the pullulan-free system, the average threshold cycle (Ct) value of the specific concentration was decreased by 2.5 cycles, which was roughly 4 minutes if converted to time. Therefore, a 1% (w/v) concentration of pullulan as a reaction additive has universal applicability to accelerate DNA amplification for different reaction systems.

For the acceleration mechanism of pullulan, the melting curve peak position of the pullulan system did not move in comparison with the control (shown in Fig. 1b), which was different from commercial PCR additive trehalose. As is known, pullulan has the property of resistance to high temperature and viscosity. It also has certain protective effects on the enzyme conformation. Thereby, it can enhance the amplification reaction by increasing the enzyme activity and thermal stability. But for the trehalose reaction system, the peak position of the melt curve moved forward in comparison with standard amplification system. In other words, trehalose can promote the yield by lowering the melting temperature of DNA. In conclusion, the enhancing mechanism of pullulan is different from other additives judging from the melting curve peak position.

Furthermore, the sensitivity and reliability of the pullulan reaction system were tested with initial template of 750 copies. The initial DNA template was estimated by spectrophotometry (see the ESI, Fig. S3†). Our purpose was to evaluate pullulan as a CPA reaction additive quantitatively and the result was regarded as a basis for field testing within 20 minutes.

As shown in Fig. 2, with the increasing concentration of pullulan as isothermal amplification additive, the average Ct values decreased at the beginning and increased steadily afterwards. The minimum Ct value was obtained at 1% (w/v) pullulan concentration, which was approximately seven minutes lower than pullulan-free condition. Exactly, the average Ct value of 1% (w/v) pullulan reaction system was 18.72 (SD = 0.59), which was less than 20 minutes. More importantly, if defining the time of fluorescence values beginning to rise as the total detection duration, the pullulan optimization condition was accelerated to almost one third of the time of traditional CPA system. The relative standard deviation data was less than 0.05, which displays the reliability of results. Based on the data, it was proved that the acceleration function of pullulan has good repeatability and reliability.

To maximize the potential of pullulan acceleration and execute on-point testing of nucleic acid amplification within 20 minutes, LFD was chosen in this assay. Here, the authenticity of CPA products was confirmed and detected by LFD semi-quantitatively (see the ESI†). As we know, LFD has high sensitivity and specificity because of its special modification and detection principle (see the ESI, Fig. S4†).[31–38] Thereby, it can greatly simplify and reduce the total time for the CPA-based assay.

LFD is widely used for endpoint detection. In this assay, the LFD method was employed to detect CPA products every five

a

| Number | Average Ct values | Ctstd.dev | SD | RSD |
|---|---|---|---|---|
| 1 | 20.81 | 0.824 | 0.83 | 0.04 |
| 2 | 18.72 | 0.588 | 0.59 | 0.03 |
| 3 | 24.01 | 0.426 | 0.42 | 0.02 |
| 4 | 28.87 | 0.725 | 0.72 | 0.03 |
| 5 | 34.44 | 0.245 | 0.24 | 0.01 |
| 6 | 25.94 | 1.316 | 1.32 | 0.05 |

b

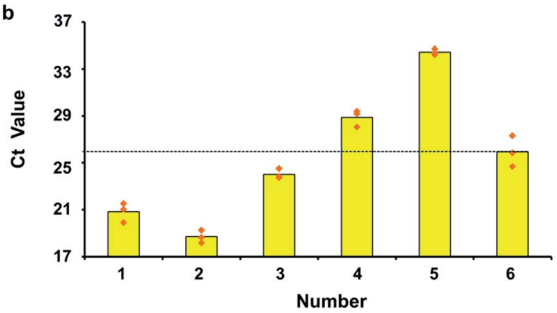


Fig. 2 Optimization of the CPA assay for T-*Nos* gene detection. (a) Average Ct values and evaluation with different concentrations of pullulan as CPA additive. Numbers 1–5: pullulan concentrations of 0.5%, 1%, 2%, 4%, 5% (w/v), respectively. Number 6: pullulan-free condition as control. (b) Histogram of initial data and corresponding average Ct values. The horizontal line is the control value.

minutes during amplification, so as to achieve real-time simulation of the CPA reaction. Here, the 1% (w/v) concentration pullulan reaction system was chosen with pullulan-free conditions as the control. A batch of 25 μL reaction mixtures were arranged to amplify simultaneously in a thermal block. Then, a group of three replicates were taken out every five minutes and cooled simultaneously.

Meanwhile, complete SYTO 9-based CPA reactions were executed as real-time controls of the LFD method. As shown in Fig. 3, with the extension of amplification time, positive results appeared at different moments. For the experimental group, a positive reaction began to emerge at the 15th minute and was

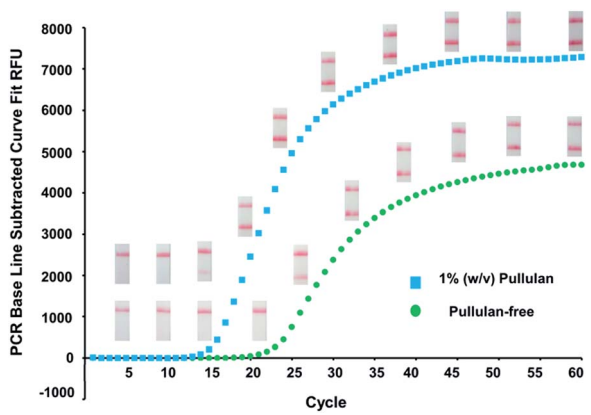


Fig. 3 Time period combination of real time fluorescent CPA and LFD assay on the 1% (w/v) concentration pullulan system (blue line) and pullulan-free conditions (green line). The CPA products were taken out for LFD detection respectively at end points of 5, 10, 15, 20, 25, 30, 35, 40, 50 and 60 minutes of DNA amplification.

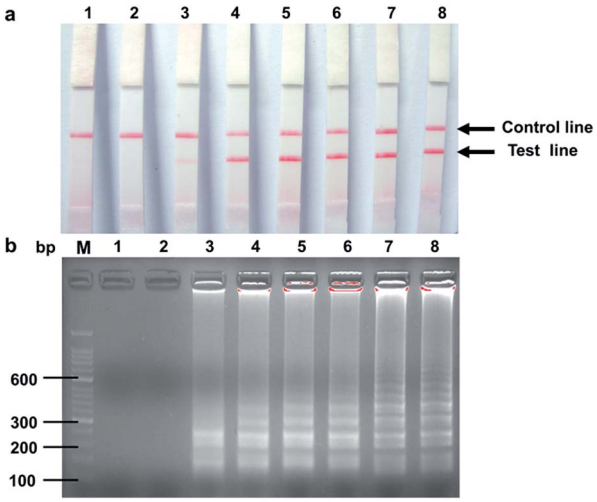


Fig. 4 Sensitivity comparisons of AGE and LFD assays for T-*Nos* of the 1% (w/v) pullulan concentration system. Lanes from left to right, DNA products amplified for 5, 10, 15, 20, 25, 30, 35 and 40 minutes respectively.

apparent at the 20th minute. However, after amplification for 20 minutes, the stripped colour shades changed very little. In terms of the control, positive bands did not appear until amplification for 25 minutes, which was nearly ten minutes later than the experimental group. In overall comparison, 1% (w/v) concentration of the pullulan reaction system showed darker colour of positive bands than the control. The results confirmed that pullulan is able to improve reaction efficiency and increase product yield (see the ESI, compare Fig. 4b with Fig. S5b† correspondingly). Most importantly, comparing the color results of LFD with the completed CPA amplification curve, it was not difficult to find that T-*Nos* gene can be detected by LFD as soon as amplification happened. From the result, it was proved that with the acceleration effect of pullulan, on-point specific detection of nucleic acid by LFD within 20 minutes was achieved with initial template of 750 copies.

To further confirm the test results of LFD, samples were processed with agarose gel electrophoresis (AGE) afterwards. As shown in Fig. 4, the sensitivity of LFD was equivalent to the limit of detection for the CPA assay followed by AGE, which both verified the acceleration property of pullulan (see the ESI†).

In conclusion, the present study has successfully proved that pullulan plays an acceleration role in the CPA reaction, which improves the amplification efficiency and raises product yield. Combining the pullulan enhancement feature with LFD, a more accelerative and specific detection of transgenic DNA can be achieved on-point within a shorter time.

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