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Electronic Supplementary Information

Using a ubiquitous pH meter combined with loop mediated isothermal amplification method for facile and sensitive detection of *Nosema bombycis* genomic DNA PTP1

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Experimental section

Reagents and apparatus. Betaine was purchased from Sigma (St. Louis, MO). Bst polymerase large fragments, $MgSO_4$ and the deoxynucleotide triphosphates (dNTPs) were purchased from New England Biolabs Ltd. (Beijing, China). All HPLC-purified DNA oligonucleotides including FIP, BIP, B3, F3 primers and the target gene DNA were purchased from Sangon Biotech Co., Ltd. (Shanghai, China).

The pH measurements were finished with a pH meter (PHS-3C, Shanghai INESA & Scientific Instrument C., Ltd., China). The pH electrode used in the pH meter was PHR-146 micro combination pH electrode (LAZAR Research Laboratories, Inc. Los Angeles, CA). The incubation of LAMP reaction was finished in a heating incubator (Shanghai Boxun Industry & Commerce Co., Ltd, China).

Design of LAMP primers. Nucleotide sequence of PTP1 gene was obtained from State Key Laboratory of Silkworm Genome Biology of China Southwest University. Six distinct genomic regions: F1-F2-F3 and B1c-B2c-B3c were designated on the targeted PTP1 gene from *N. bombycis* (**Fig. S1**). A set of 4 primers, outer primers: forward outer primer (F3) and backward outer primer (B3), and inner primers: forward inner primer (FIP) and backward inner primer (BIP), capable of

hybridizing to the six regions, were designed using the Primer Explorer Version 3.0 software (http://primerexplorer.jp/e/). The BIP for the PTP1 gene consisted of B1c (22 nt), a TTTT linker, and B2 (20 nt); the FIP consisted of F1c (20 nt), a TTTT linker, and a complementary sequence of F2c (18 nt). The details of the sequence are shown in **Table S1**.

3'-GATGACCTAGTCCAGAAGGAACATAAGGTCATAGAAGTTGGCCACCGGTAG
5'-CTACTGGATCAGGTGTTCCTTGTATTCCAGTATCTTCAACCGGTGGCCATC
F3

3'-GTTCATTAGTGCTTGGCGGACCAAAGGGTTCTAATGCACAAGAGTGAGTTC
5'-CAAGTAATCTCGTTGGCGGTGGTTTCCCAAGTAATGCACAAGTCACTCAAG
F2

3'-GGGGTACACAGGGTAGTGTTCGAGTAGTAGGTCATCGTTCACAAGGTCATT
5'-CCCCATGTGTCCCATCACAAGCTCATCATCCAGTAGCAAGTGTTCCAGTAA
F1

3'-GCTCACAGGGTCATTTACGACAAGGTTACTGGTCTCGGGGACAATGTGGTG
5'-CGAGTGTCCCAGTAAATGCTGTTCCAATGACCAGTGCCCCTGTTACACCAC
B1c

3'-AACCGGGAAGAATAATGCCTAGAAGTTCGGAAGGTGGTAGTCCGAGAGTAG
5'-TTGGCCCTTCTTATTACGGATCTTCAAGCCTTCCACCATCAGGCTCTCATC
B2c B3c

3'-GATGACGAGGAACAACA
5'-CTACTGCTCCTTGTTGT

Fig. S1 Nucleotide sequence of partial PTP1 gene used to design inner and outer primers for LAMP. The nucleotide sequences and the positions used to design the primers are represented by using different color highlighters.

Table S1 Primer set for PTP1 genomic DNA amplification by LAMP-pH meter assay.

Primer	Primer sequence (5' to 3')
F3	CAGTATCTTCAACCGGTGGC
B3	AGGAGCAGTAGGATGAGAGC
FIP (F1c-F2)	TGTGATGGGACACATGGGGC**TTTT**CTCGTTGGCGGTGGTTTC
BIP (B1c-B2)	CTGTTCCAATGACCAGTGCCCC**TTTT**GTGGAAGGCTTGAAGATCCG

The T-linker is indicated in bold letter.

Gel Electrophoresis. Following incubation at 65 °C for 60 min and at 90 °C for 2 min, 10 μL aliquot of LAMP amplified products were transformed into our freshly prepared 16 % non-denaturing polyacrylamide gels and performed in 1× TBE buffer (pH 8.3) at a 100 V constant voltage for 150 min for gel electrophoresis separation. Finally, gels were stained with ethidium bromide and photographed with a digital camera under the UV light illumination.

The conventional PCR for PTP1 genomic DNA detection. A conventional PCR assay targeting PTP1 genomic DNA was also performed for comparison by utilizing the outer primer sets (F3/B3). 25 μL of PCR reaction mixture consisting of 12.5 μL Premix Taq (TaKaRa Taq Version 2.0), 2 μL (0.4 mM) each of F3 and B3, 3 μL of target DNA samples and 5.5 μL ddH_2O. The PCR process was started with an initial denaturization at 94 °C for 5 min, followed by performing 94 °C for 30 s, 56 °C for 30 s, 72 °C for 30 s and an additional extension step at 72 °C for 7 min. Finally, the PCR products can be visualized on 2% agarose gels stained with ethidium bromide followed by image acquisition utilizing a Molecular Imager Gel Doc™ XR+ with Image Lab™ software (Bio-RAD Manufactured in Canada). The products were analyzed by electrophoresis at 100V for 50 minutes, and visualized in ADVANCE Mupid-One (TaKaBa made in Japan).

Quantitative real-time PCR amplifications. Quantitative real-time PCR (RT-qPCR) was carried out using a CFX96™ Real-Time PCR Detection System (Bio-Rad, USA) using fluorescent probe-based technology. The RT-qPCR primers were designed using primer express software Primer Premier 5.0. The genomic DNA PTP1 forward primers used was 5'-TGTAACAGGGGCACTGGTCAT-3', and the reverse primer was 5'-ATCTCGTTGGCGGTGGTTT-3'. Real-time PCRs on samples,

calibration curve standards, and controls were carried out in 50 μL that contained variable amounts of 20 μL DEPC water, 1 μL forward primer, 1 μL reverse primer, and 25 μL SYBR Select Master Mix (Life Technologies, China) Sample genomic DNA or diluted standard genomic DNA was added to PCR reactions in 3 μL volumes. And 15 μL of the above mixture solution was used for three parallel measurements. The reactions in 96-well plates were performed with CFX96 real-time system-C1000 Thermal Cycler. The thermal cycle conditions for SYBR Green assay were as follows: 30 s at 95 °C, followed by 40 cycles at 95 °C for 5 s, and 60 °C for 30 s.

Results and discussion

Working principle of the LAMP amplification reaction based on pH meter.

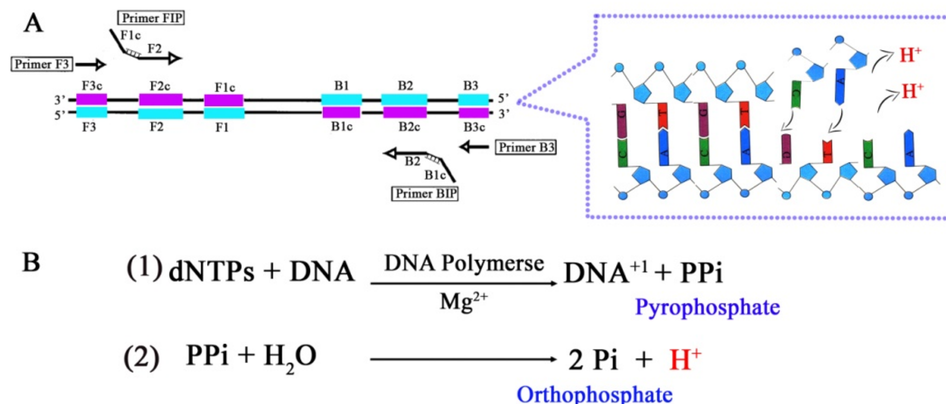

Fig. S2 The working principle of detecting a change in pH for amplification of nucleic acid. (A) The schematic diagram the released hydrogen ions during the LAMP amplification procedure; (B) The equation of the nucleotide incorporation reaction: Where DNA represented the target template DNA. The hydrolysis and incorporation of a nucleotide resulted in the extension of DNA by a single base, represented as DNA^{+1}. In addition, the pyrophosphate hydrolyzed with the generation of the hydrogen ions. It must be noted that this reaction is in a unique dynamic equilibrium.

Optimized conditions for LAMP reactions. In order to maximize the efficiency and sensitivity of the system, the optimization of the experimental conditions including the reaction temperature and time of the LAMP were

investigated. The standardized PTP1 genomic DNA with a concentration of 0.5 ng/μL was used during the LAMP reaction. **Fig. S3A** has shown the LAMP reactions at different temperatures of 50 °C, 55 °C, 60 °C, 65 °C and 70 °C for the detection of PTP1 genomic DNA. All the LAMP processes in the optimization of the reaction temperature were performed for 60 min. From the experimental results, the nucleic acid amplification could be accomplished between the temperatures from 60 °C to 65 °C, when the temperature was up to 70 °C, a sharp decline in the value of pH changes was observed. Considering most references reported previously chosen 65 °C as the reaction temperature, an optimum amplification temperature of 65 °C was adopted in our LAMP reaction.

Similarly, the LAMP with different reaction times of 10, 20, 30, 40, 50, 60, 70, 80 and 90 min for the detection of PTP1 genomic DNA was investigated. As shown in **Fig. S3B**, the values of pH change increased with the increasing of reaction time and then leveled off after 60 min, indicating that 60 min was enough for LAMP amplification. Thus, 60 min was employed throughout the LAMP process.

The buffer solution is a vital factor to retain the amplification efficiency and specificity of the reaction system. And maintaining low buffering condition in LAMP is of great significance to our experiment. In an alkaline environment, the solution containing proper concentration of NH_4Cl will form the NH_3-NH_4Cl buffer solution, which can meet our requirement of low buffering condition. A low concentration of NH_4Cl is not enough to form a buffer system, whereas if the concentration of NH_4Cl is too much, the hydrolysis of NH_4^+ can reduce the pH value of the solution and further interference experiment results. To investigate the effect of NH_4^+ concentration on the -ΔpH, 0.5 ng/μL PTP1 genomic DNA was detected by using

different concentration of NH_4^+ in the LAMP reaction. As shown in Fig.S3C, with increasing of the NH_4^+ concentration from 1 to 4 mM, the -ΔpH response was increased slowly, indicating too low concentration of NH_4^+ to retain the amplification efficiency. However, the increase of the response was enlarged obviously when the NH_4^+ concentration was reached at 5 mM. And when the concentration increased to 6 mM, the response value increased continuously, however, avoiding the hydrolysis of the excess of NH_4^+ to interference the experiment results, a concentration of 5 mM NH_4Cl was selected for the LAMP reactions. Since it could not only provide adequate buffering condition but also maintain the amplification efficiency and specificity of the reaction system.

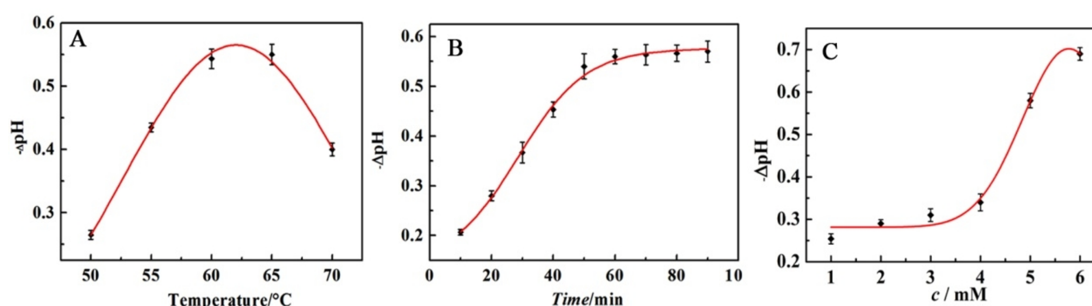

Fig. S3 The optimal conditions of LAMP-pH meter assay. (A) Reaction temperature optimization, the temperature at 65 °C was selected. (B) Reaction time optimization; the reactions were carried out at 60 min. (C) NH_4Cl concentration optimization, the concentration of 5 mM NH_4Cl was selected.

Comparison of sensitivity between LAMP-pH and conventional PCR assays. From Fig. S4 we could see that the PCR was just able to detect template DNA at 5 pg/μL. Therefore, the LAMP-pH assay had higher sensitivity compared to standard PCR method (See Table S2). Moreover, the time required for the pH-LAMP method was about 1 hour. By contrast, 3-4 h was required for the PCR reaction followed by gel electrophoresis. In addition, the proposed method was simple and cost-effective and may be of particular benefit to the farmer in underdeveloped countries.

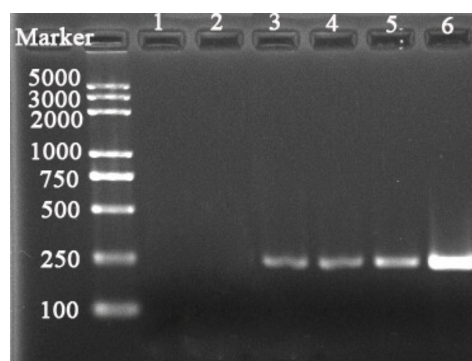

Fig. S4 Gel electrophoresis for specific amplification by PCR. Lane 1, Black control (dd H_2O_2); Lane 2, 0.5 pg/μL; Lanes 3-6 contain, 5 pg/μL, 0.05 ng/μL, 0.5 ng/μL and PTP1 5 ng/μL genomic DNA, respectively.

Table S2 Comparison of the LAMP-pH platform and PCR

Concentration of PTP1 genomic DNA	LAMP-pH	PCR
0.5 pg/μL	+	-
5 pg/μL	+	+
0.05 ng/μL	+	+
0.5 ng/μL	+	+
5 ng/μL	+	+

\+ is positive result; - is negetive result

Specificity of the LAMP reaction.

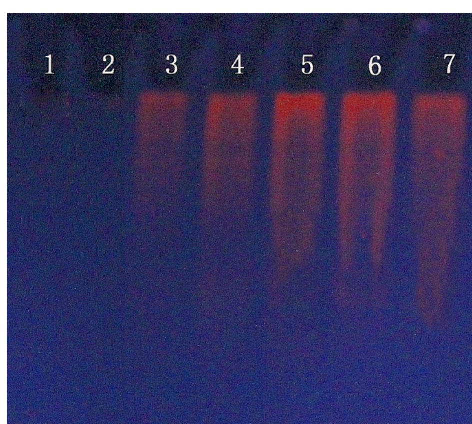

Fig. S5 Gel electrophoresis for specific amplification by loop mediated isothermal amplification. Lane 1, Black control (dd H_2O); Lane 2, Non-template control; Lanes 3-6 contain 0.5 pg/μL, 5 pg/μL, 0.05 ng/μL and 0.5 ng/μL PTP1 genomic DNA, respectively.

Quantification of PTP1 genomic DNA using the quantitative real-time-PCR. For the quantification of PTP1 genomic DNA, a standard curve (5, 1, 0.5, 0.1, 0.05, 0.005 ng/μL of PTP1 genomic DNA) was prepared including three replicate reactions for each DNA quantity (Fig. S6). According to the obtained linear equation Ct = 14.77 - 6.306 lg*c*, we could calculate the concentration of the PTP1 genomic DNA in four different samples.

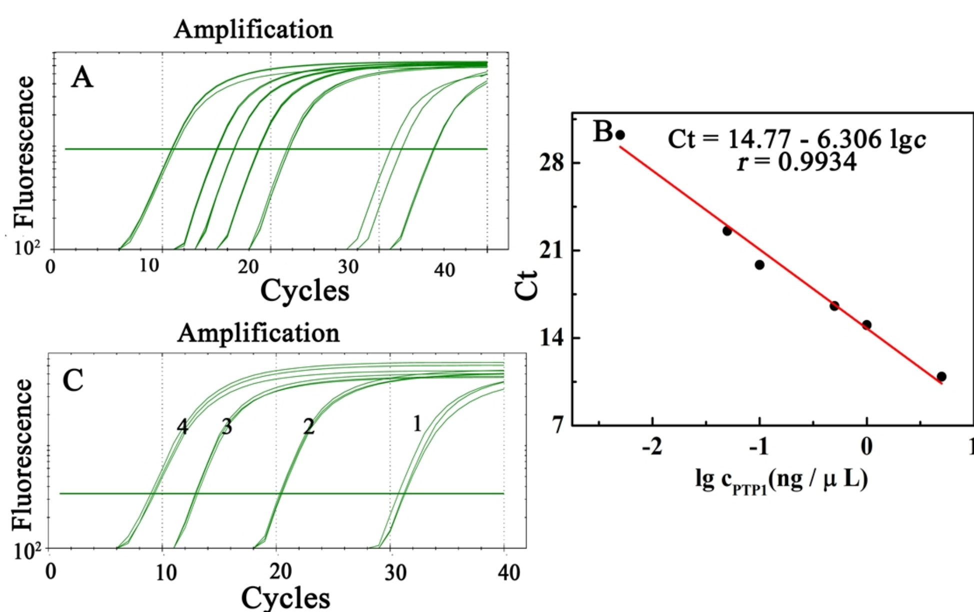

Fig. S6 (A) the fluorescence density at different concentrations of PTP1 genomic DNA: control, 0.005 ng/μL, 0.05 ng/μL, 0.1 ng/μL, 0.5 ng/μL, 1 ng/μL, and 5 ng/μL (from right to left); (B) the calibration plot of Ct *vs* lgc_{PTP1}; (C) the fluorescence density at four different samples.

The accuracy of proposed assay. Quantitative real-time-PCR is widely used for gene expression analysis due to its large dynamic range, tremendous sensitivity, high sequence specificity, and little to no postamplification processing[1]. Thus, we performed the RT-qPCR to validate the accuracy of our assay. The proposed assay was used for detection of genomic DNA PTP1 in the grinding fluid of silkworm healthy eggs, using the standard addition method. We spiked genomic DNA of different concentrations into10-fold-diluted grinding fluid of silkworm healthy eggs.

And then, the amount of genomic DNA PTP1 was examined by both the proposed assay and real-time quantitative PCR method. The results from Table S3 exhibited an excellent consistency on the detection of genomic DNA PTP1 concentrations obtained by both methods, indicating the accuracy of our method.

Table S3. Determination of genomic DNA PTP1 added in the grinding fluid of silkworm eggs (*n=3*) with the LAMP-pH platform and the real-time quantitative PCR

Sample No.	Added genomic DNA in grinding fluid (ng μL^{-1})	Detected by proposed assay (ng μL^{-1})	Detected by RT-qPCR (ng μL^{-1})	Relative error (%)
1	0.005	0.00512	0.00467	7.03
2	0.05	0.0513	0.0491	4.29
3	0.5	0.484	0.507	-4.75
4	5	5.32	5.17	2.82

References

1. H.P Cao and J M. Shockey, J. Agric. Food Chem., 2012, 60, 12296-12303.