Electronic Supplementary Material (ESI) for Analyst.

Real-time quantitative nicking endonuclease-mediated isothermal amplification with small molecular beacons

Wentao Xu[a, b, 1], Chenguang Wang[a, 1], Pengyu Zhu[a], Tianxiao Guo[a], Yuancong Xu[a, b], Kunlun Huang[a, b], and Yunbo Luo[a, b, *]

[a]Lab of Food Safety, College of Food Science and Nutritional Engineering, China Agricultural University, Beijing 100083, China；

[b]The Supervision, Inspection & Testing Center of Genetically Modified Food Safety, Ministry of Agriculture, Beijing 100083, China

[1]The two authors contribute equally

*To whom correspondence should be addressed. Tel/Fax: +86 010 62736479; Email: lyb@cau.edu.cn

Supplementary Information

Multistate model to predict Tm and free energy of molecular beacon

In multistate model, molecular beacon exists three status:

MBT→MBfold+T→MBcoil+T,

where MBT is molecular beacon-template duplex, MBfold is the beacon that forms stem-loop structure, and MBcoil is the beacon in the form of random coil.

The sum of fluorescence value (RFU) = $\alpha\frac{[MBT]}{[MB]}+\beta\frac{[MBfold]}{[MB]}+\gamma\frac{[MBcoil]}{[MB]}$, (1)

where α、β、γ are the RFU that molecular beacon binds to the template, folded molecular beacon and random coil molecular beacon, and

[MB]=[MBT]+[MBfold]+[MBcoil].

Then, the equilibrium constant (K) of each state is decided by the equation

$K_{12}=\frac{[MBclosed][T]}{[MBT]}$, (2)

$K_{23}=\frac{[MBcoil]}{[MBclosed]}$, (3)

where K_{12} is the equilibrium constant of unfolding of molecular beacon-template duplex, and K_{23} is the equilibrium constant of unfolding of molecular beacon.

Substituting these calculations to equation 1, RFU can be described by another equation

$RFU=\frac{\alpha T+\beta K_{12}+\gamma K_{12}K_{23}}{T+K_{12}+K_{12}K_{23}}$. (4)

In the absence of template, namely T=0,

$RFU=\frac{\beta K_{12}+\gamma K_{12}K_{23}}{K_{12}+K_{12}K_{23}}$, (5)

$K_{23}=\frac{RFU-\beta}{\gamma-RFU}$. (6)

Based on the equation of Gibbs free energy and Van 't Hoff equation, unfolding of molecular beacon is decided by the queation

△G=△G_0+Rθln$K$=△H-θ△S, (7)

where △G_0 is △G at absolute temperature, R is the gas constant, and θ is the temperature in Kelvin. Equation could be transformed to a straight line having the equation

$R\ln\left(\frac{RFU-\beta}{\gamma-RFU}\right)=-\frac{1}{\theta}$△H+△S, (8)

where $\ln\left(\frac{RFU-\beta}{\gamma-RFU}\right)$ is in linear relationship with $\frac{1}{\theta}$, △H and △S are the slope and y-intercept of the line, respectively.

In the presence of template, equation 4 can be transformed to equation 9

$$K_{12}=\frac{(\alpha - RFU)T}{(RFU-\beta)+(RFU-\gamma)K_{23}}, \quad (9)$$

Where K_{23} can be calculated by the data acquired from equation 8, α、β、γ and RFU is screened from the experimental data. Thus K_{12} at any temperature could be calculated, melting temperature (Tm) is the temperature where K_{12} equals T-0.5B.

Overall, melting temperature, free energy and other thermodynamic parameters were all obtained from this multistate model algorithms.

Table S1. Scheme of other oligonucleotides

Sequence name	Sequence(5'-3')	Reference
Op-B_1	CAGCCGCCGCGAGC	44
Op-B_2	AGGGTTAGCCACAC	
Op-S_1-BspQI	CGATTCCGCTCCAGACTTGCTCTTCCGATGGCGAACTCA	
Op-S_2-BspQI	ACCGCATCGAATGCATGTGCTCTTCGGGCACCGTAAACA	
Op-S_1-BstNBI	CGATTCCGCTCCAGACTTGAGTCCGATGGCGAACTCA	
Op-S_2-BstNBI	ACCGCATCGAATGCATGTGAGTCGGGCACCGTAAACA	
beacon-21	CTCGAGCGTGAGTGATGAAGGCTTTCTCGAG	This study
beacon-19	CTCGACGTGAGTGATGAAGGCTTTTCGAG	
beacon-17	CTCGAGTGAGTGATGAAGGCTTTCGAG	
beacon-15	CTCGATGAGTGATGAAGGCTTCGAG	
beacon-13	CTCGAGAGTGATGAAGGCTCGAG	
beacon-11	CTCGAAGTGATGAAGGTCGAG	
beacon-9	CTCGAGTGATGAAGTCGAG	
beacon complementary	ACGACCCGAA AGCCTTCATC ACTCACGCGG	
B_1 complementary	TTCCCTACTGCTGCCTCCCGTAGGAGTCTGGGCCGTGTCT	
B_2 complementary	AGGATTAGATACCCTGGTAGTCCACGCCGTAAACGATGAG	
S_1 complementary	CTCCGTCAGACTTTCGTCCATTGCGGAAGATTCCC	
S_2 complementary	GCCGCGGTAATACGTAGGTGGCAAGCGTTATCCGG	
B_1 M1	ACAGACTCCTACGGGAGGCAGCAGT [a]	
B_1 M2	CCAGACTCCTACGGGAGGCAGCAGG	
B_2 M1	ATTTACGGCGTGGACTACCAGGGTA	
B_2 M2	GTTTACGGCGTGGACTACCAGGGTG	
S_1 M1	ATGAATAGTCGGTTACTTGAGTCTCCGCAATGGACGAAAGTCG	
S_2 M1	ATGAATAGTCGGTTACTTGAGTCCTTGCCACCTACGTATTACA	

[a] mismatched positions are underlined, the following are the same.

Table S2 Practical samples test

Samples	SMB-NEMA(ng/μL)[a]	qPCR(ng/μL)[a]
Polluted water 1	2.74±0.02	2.74±0.05
Polluted water 2	15.2±0.42	14.97±0.5
Spring water	0	0
Soybean powder	0	0
Rice	0	0
Milk out of shelf life 1	5.72±0.42	5.67±0.27
Milk out of shelf life 2	14.28±0.77	14.32±0.75
Milk on sale	0	0
Chinese tofu[b]	12.7±0.5	12.9±0.36
Pork	0	0

[a] all tests were performed in triplicate.

[b] tofu was stored at room temperature (28℃) for 10 days.

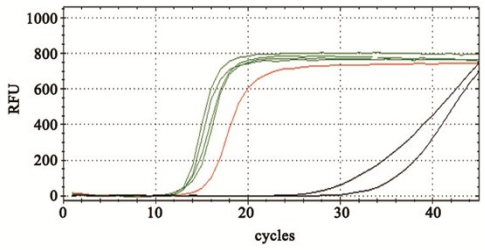

Fig. S1 Simulation of de novo synthesis by real-time fluorescence amplification detection. Normal NEMA amplification (green) and negative control (red) were detected using SYBR Green I. *De novo synthesis* simulation was detected using SYBR Green I (black) without addition of primer and template.

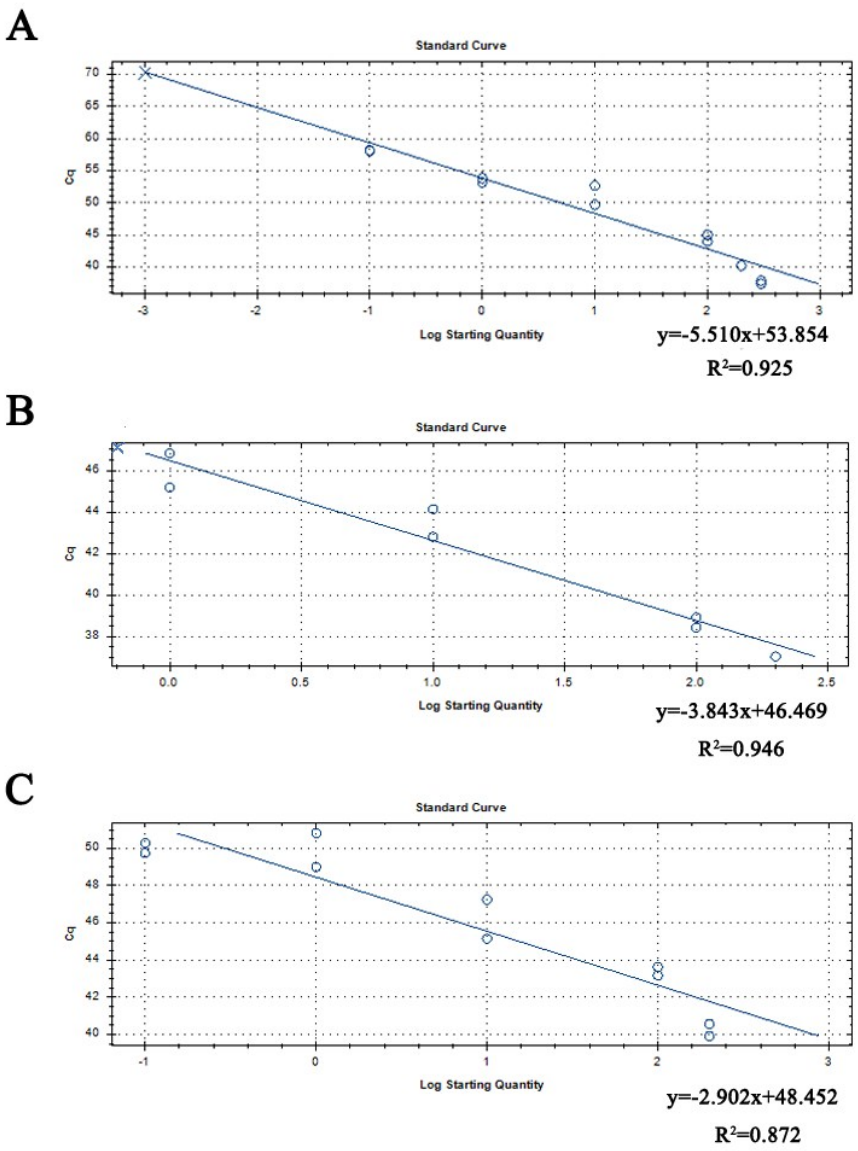

Fig. S2 Standard curve of NEMA quantitation with molecular beacons that possess different Tm. Letters A-C represents NEMA quantitation with Molecular beacons that possess Tm of 60℃, 55℃ and 50℃. All experiments were conducted in ten-fold serial dilution. Except 60℃ Tm group, the other two standard curve were established without several improper plot. (A) 60 ℃ Tm group standard curve from 200 ng/μL to 0.1 ng/μL. (B) 55℃ Tm group standard curve from 200 ng/μL to 1 ng/μL. (C) 50℃ Tm group standard curve from 300 ng/μL to 0.1 ng/μL. Cross (×) in the figure stand for the negative control. Standard equation and linear correlation coefficient (R^2) were listed at bottom right.

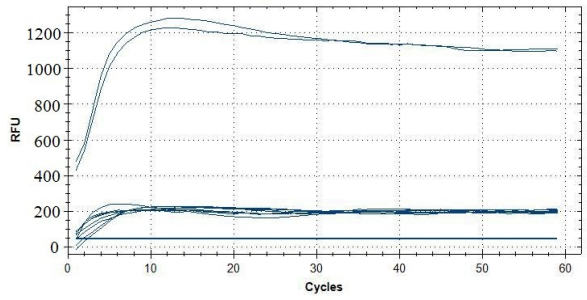

Fig. S3 Specificity improvement in SMB-NEMA. Positive amplifications are the 5' mismatched B_1/B_2 primers conditions and positive control. The others are 3' mismatched primers conditions, mismatched molecular beacon conditions and negative control.

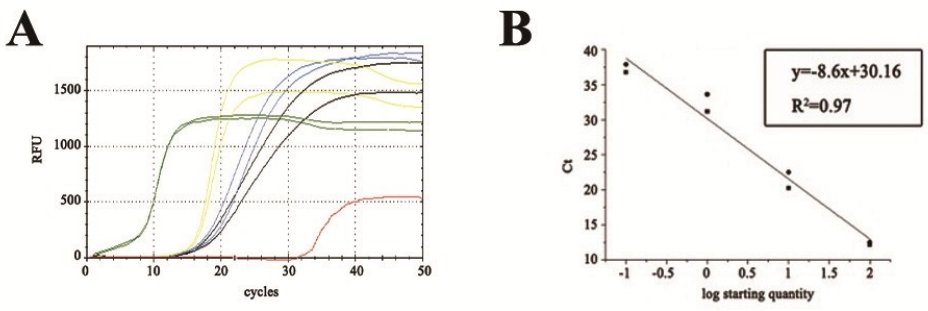

Fig. S4 Amplification and Linear regression curve of NEMA using SYBR Green I. (A) Ten-fold dilution template was added to the reaction. 100 ng/μL (green), 10 ng/μL (yellow), 1 ng/μL (blue), 0.1 ng/μL (red) and negative control (black) are indicated. (B) The standard equation is y=-8.6x+30.16 with R^2=0.97.

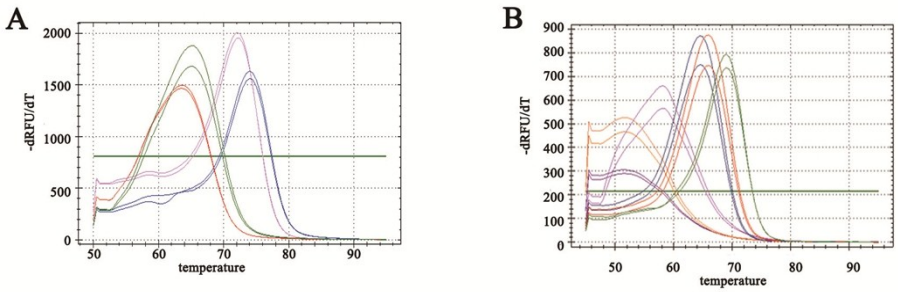

Fig. S5 Melting temperature determination of different small molecular beacons and primers. (A) Melting temperature of primers. S_1 (green), S_2 (red), B_1 (pink) and B_2 (blue) are shown in the melt peak figure, respectively. (B) Comparison about melting temperature estimated and experimental results. Experimental melting temperature of estimated 75℃ (green), 70℃ (red), 65℃ (blue), 60℃ (pink), 55℃ (orange), 50℃ (purple) Tm from software are shown as solid line in the figure, respectively.

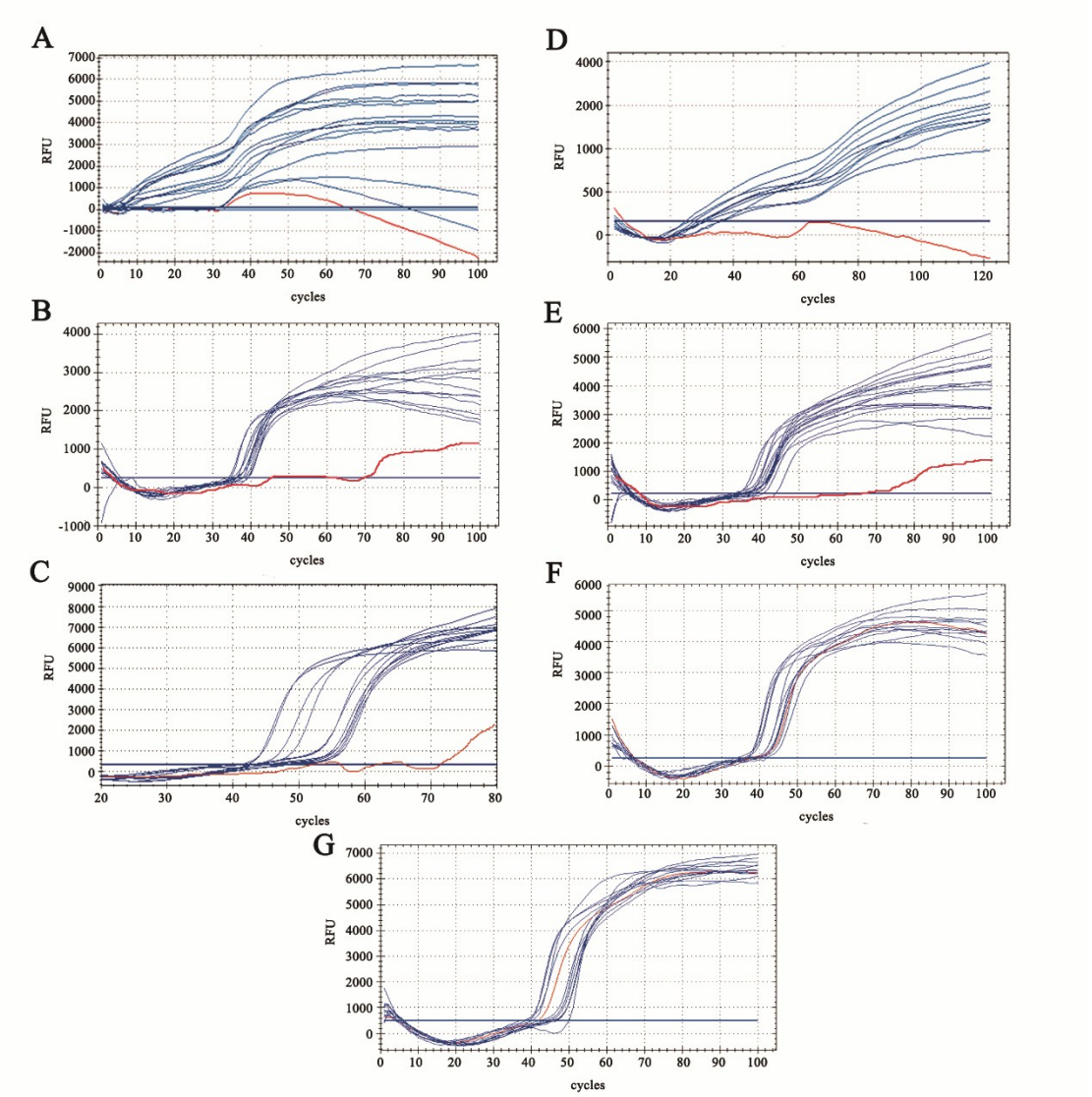

Fig. S6 Influence of number of base pairs on SMB-NEMA amplification. Letters A-G represent amplification curve with 31, 29, 27, 25, 23, 21, 19bp small molecular beacon addition, respectively. Amplification is shown in blue solid line, while negative control is shown in red solid line. Long sequence molecular beacons (31, 29bp) accomplished a continuous increase without plateau and linear amplification stage. Small molecular beacons (21, 19bp) are hard to detect ten-fold dilution template, and they are not available to distinguish negative control either. The best choice in this reaction is 23bp molecular beacon addition, which performing near 60℃ melting temperature.

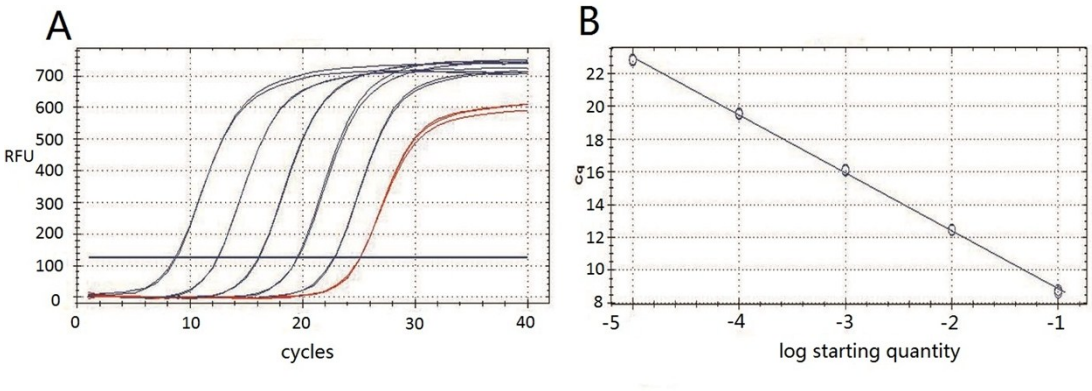

Fig. S7 Conventional qPCR amplification curve and standard curve based on bacillus cereus. A: The serial dilution are 100pg, 10pg, 1pg, 0.1pg and 0.01pg (blue curves). Negative control are presented as red curves. B: Standard curve of qPCR. The quantification equation is y=-3.529x+5.341 with coefficient of variation (R^2) = 0.999.