Electronic Supplementary Information

Single-color multiplexing by the integration of high-resolution melting pattern recognition with loop-mediated isothermal amplification

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Experimental section

Materials and apparatus

The LAMP reagents including Bst 2.0 WarmStart DNA polymerase (8 U/μL), dNTPs (10 mM), $MgSO_4$ (100 mM), and 10× isothermal amplification buffer (20 mM Tris-HCl, 50 mM KCl, 10 mM $(NH_4)_2SO_4$, 2 mM $MgSO_4$, 0.1% Tween-20) were obtained from New England Biolabs (Ipswich, MA, U.S.A.). Betaine and human serum were purchased from Sigma (St. Louis, MO, U.S.A.), and Evagreen at 20× was obtained from Biotium (Hayward, CA, U.S.A.). SYBR Gold was purchased from Invitrogen (California, CA, U.S.A.). All other reagents were of analytical

grade and used without further purification. The LAMP primers (Table S1) were synthesized and HPLC purified by Sangon Biotechnology (Shanghai, China). The degenerate bases R (A/G) and Y (C/T) were incorporated in the primers for *Staphylococcus aureus* to achieve full coverage of the diverse pathogen bacteria of *Staphylococcus aureus*. Foodborne pathogens of *Staphylococcus aureus*, *Listeria monocytogenes*, *Salmonella spp* and *Cronobacter sakazakii* were purchased from Guangdong Huankai Microbial Sci. & Tech. Co., Ltd (Guangdong, China). All LAMP amplification and melting experiments were performed in a MyGo real-time PCR system (IT-IS Life Science Ltd, U.K.). The gel electrophoresis and imaging were carried out on a Bio-Rad apparatus (Hercules, CA, U.S.A.). The ultrapure water was prepared by a Millipore filtration system (Millipore, Milfore, MA, U.S.A.).

Sequences of pathogenic DNAs

The amplification sequences used for LAMP detection of pathogenic genes including *Staphylococcus aureus* ATCC6538, *Listeria monocytogenes* ATCC19115, *Salmonella spp* CMCC50071 and *Cronobacter sakazakii* ATCC29544 were shown in Table S2. The genomic DNA was extracted by using TIAN amp Bacteria DNA kit (Tiangen Biotech, Beijing, China) following the manufacturer's instructions. The target DNA obtained from PCR amplicon and amplified by the outer primers (F3/B3) was purified by universal DNA purification kit (Tiangen Biotech, Beijing, China). The absences of primer-dimers and nonspecific PCR products were verified by agarose gel electrophoresis, and the accuracy of DNA sequences was verified by Sanger sequencing. Each target DNA was quantified by using NanoDrop 2000 (Thermo Scientific, Melbourne, Australia) at 1×10^9 copies/μL and stored at -20°C for future use.

LAMP-HRM assay

The LAMP was performed under the optimized conditions in 10 μL of reaction solution containing 1× isothermal amplification buffer, 0.2 μM F_3 and 0.2 μM B_3, 1.6 μM FIP and 1.6 μM BIP, 1.4 mM dNTP, 3.2 U of Bst 2.0 WarmStart DNA polymerase, 0.8 M betaine, 5.0 mM $MgSO_4$, 0.25 μL of EvaGreen (20×), and 1.0 μL of DNA (10^6 copies/μL). The standard LAMP reaction was performed on MyGo Pro Real-Time PCR instrument at 67 °C for 1 h. The HRM profiles of LAMP products were collected at 0.05 °C/s ramping from 75 °C to 95 °C, and then analyzed by using MyGo software. Six independent experiments for each sample were performed. For gel characterization, the LAMP products were prestained with SYBR Green gold and then electrophoresed on 2% agarose gel at a 110 V constant voltage for 60 min at room temperature. The gels were analyzed by Bio-Rad ChemiDoc MP Imaging System.

For the unknown sample analysis, 54 single source samples and mixture samples were prepared randomly for blind test. These blind samples were analyzed by LAMP-HRM under the identical conditions described above, and then compared their melting curves with those of standard references to verify the identity of samples. For complex sample analysis, the pathogenic DNAs were spiked in either fetal bovine serum (10%) or milk (10%), followed by LAMP-HRM assay using the same experimental procedure for the detection of DNAs in buffer.

Table S1. Sequences of LAMP Primers

pathogen	primer sequences (5'-3')	
Staphylococcus aureus (*S.aur*)	F3	TTTAACAGCTAAAGAGTTTGGT
	B3[a]	TTTTCATAATCRATCACTGGAC
	FIP	[c](5'-FAM)CCTTCAGCAAGCTTTAACTCATAGTTTTTCAGATAGCATGC CATACAGTC
	BIP[b]	ACAATAATAACGAGGTYATTGCAGCTTTTCTTGAACACTTTCATAAC AGGTAC
Listeria monocytogenes (*L.mon*)	F3	GTCTTTTAAGTGGAGTAAACCTT
	B3	ACAAGACTTCACCAATCCA
	FIP	[d](5'-TAMRA)CCTGTGCCAAAGCATTTTTACATTTTTTAGGCAAGTCAT CTTGTTCG
	BIP	TAAGTCTCTTTGCAATTGACCGACTTTTACGTGTACACAGAAAAGC G
Salmonella spp (*S.*spp)	F3	CGGCCCGATTTTCTCTGG
	B3	CGGCAATAGCGTCACCTT
	FIP	GCGCGGCATCCGCATCAATATGCCCGGTAAACAGATGAGT
	BIP	GCGAACGGCGAAGCGTACTGTCGCACCGTCAAAGGAAC
Cronobacter sakazakii (*C.sak*)	F3	TCCGCAGGAGTTGAAGAGG
	B3	CAGCAGCGTGTCTGTTTCA
	FIP	TATGCGGGATCGAACCGCAGATTTTGGCTATAGCTCAGCTGGGA
	BIP	GCTCCACCATCACTTCGGAGTGTTTTTTCAGCTTGTTCCGGATTGT

[a]R and [b]Y mean the degenerate bases, and R is a mixture of bases A and G; Y is a mixture of bases C and T. [c,d] indicate the FAM- and TAMRA-labeled primers for gel electrophoresis.

Table S2. Sequences and characteristics of target genes

pathogen	target sequences (5'-3')	size (bp)	GC (%)
S.aur	TTTAACAGCTAAAGAGTTTGGTGCCTTTACAGATAGCATGCC ATACAGTCATTTCACGCAAACTGTTGGCCACTATGAGTTAAA GCTTGCTGAAGGTTATGAAACACATTTAGTGGGAATAAAGA ACAATAATAACGAGGTCATTGCAGCTTGCTTACTTACTGCTG TACCTGTTATGAAAGTGTTCAAGTATTTTTATTCAAATCGCG GTCCAGTGATCGATTATGAAAA	231	37
L.mon	GTCTTTTAAGTGGAGTAAACCTTTTTGAACGTGGATAGGCA AGTCATCTTGTTCGATTAATATATAATTAGCCGTTTTGGTTTTA TAATGTAAAAATGCTTTGGCACAGGCTAGTTTTAAGTCTCTT TGCAATTGACCGACGTTCGCACTTGCATGATAAAGTAAAAA AGCAATCAGCGCTTTTCTGTGTACACGTATGGATTGGTGAA GTCTTGT	216	37
*S.*spp	CGGCCCGATTTTCTCTGGATGGTATGCCCGGTAAACAGATGA GTATTGATGCCGATTTGAAGGCCGGTATTATTGATGCGGATG CCGCGCGCGAACGGCGAAGCGTACTGGAAAGGGAAAGCCA GCTTTACGGTTCCTTTGACGGTGCGATGAAGTTTATCAAAG GTGACGCTATTGCCG	180	52
C.sak	CAGCAGCGTGTCTGTTTCAATTTTCAGCTTGTTCCGGATTGT TAAAGAGCAAATACTTCGCAGTATACTCACTGAGTACACTCT GAAGTGATGGTGGAGCTATGCGGGATCGAACCGCAGACCTC CTGCGTGCAAAGCAGGCGCTCTCCCAGCTGAGCTATAGCCC CATCGTAGTTAAACCTCTTCAACTCCTGCGGA	198	51

Table S3. PCA factors obtained with HRM profiles against 54 unknown pathogenic DNA samples.

sample	factor 1	factor 2	identification	verification	judgment
1	1.00	-1.00	*S.aur*	*S.aur*	√
2	0.88	0.02	*S.aur*+*L.mon*	*S.aur*+*L.mon*	√
3	-0.55	-0.36	*S.aur*+*S.*spp	*S.aur*+*S.*spp	√
4	0.98	-0.57	*S.aur*	*S.aur*	√
5	0.86	-0.23	*S.aur*+*L.mon*	*S.aur*+*L.mon*	√
6	-0.83	-0.49	*S.*spp	*S.*spp	√
7	0.98	-0.99	*S.aur*	*S.aur*	√
8	-0.49	-0.42	*S.aur*+*S.*spp	*S.aur*+*S.*spp	√
9	0.81	0.45	*L.mon*	*L.mon*	√
10	-0.82	-0.34	*S.*spp	*S.*spp	√
11	0.77	0.48	*L.mon*	*L.mon*	√
12	0.43	1.00	*S.aur*+*L.mon*+*S.*spp	*S.aur*+*L.mon*+*S.*spp	√
13	-0.74	-0.57	*S.*spp	*S.*spp	√
14	0.48	0.43	*S.aur*+*L.mon*+*S.*spp	*S.aur*+*L.mon*+*S.*spp	√
15	-0.77	0.33	*L.mon*+*S.*spp	*L.mon*+*S.*spp	√
16	-0.96	-0.33	*S.*spp	*S.*spp	√
17	0.93	-0.26	*S.aur*+*L.mon*	*S.aur*+*L.mon*	√
18	-0.90	-0.33	*S.*spp	*S.*spp	√
19	0.80	0.45	*L.mon*	*L.mon*	√
20	-0.47	-0.59	*S.aur*+*S.*spp	*S.aur*+*S.*spp	√
21	0.34	0.21	*S.aur*+*L.mon*+*S.*spp	*S.aur*+*L.mon*+*S.*spp	√
22	0.80	0.54	*L.mon*	*L.mon*	√
23	0.12	-0.04	*S.aur*+*L.mon*+*S.*spp	*S.aur*+*L.mon*+*S.*spp	√
24	0.95	-0.94	*S.aur*	*S.aur*	√
25	-1.00	-0.20	*S.*spp	*S.*spp	√
26	-0.58	0.07	*L.mon*+*S.*spp	*L.mon*+*S.spp*	√
27	0.82	0.38	*L.mon*	*L.mon*	√
28	-0.83	0.31	*L.mon*+*S.*spp	*L.mon*+*S.*spp	√
29	0.97	-0.70	*S.aur*	*S.aur*	√
30	0.05	-0.20	*S.aur*+*L.mon*+*S.*spp	*S.aur*+*L.mon*+*S.*spp	√
31	0.97	-0.83	*S.aur*	*S.aur*	√
32	0.34	-0.44	*S.aur*+*S.*spp	*S.aur*+*S.*spp	√
33	0.33	0.33	*L.mon*+*S.*spp	*L.mon*+*S.*spp	√
34	0.34	0.01	*S.aur*+*L.mon*+*S.*spp	*S.aur*+*L.mon*+*S.*spp	√
35	-0.96	-1.00	*S.aur*	*S.aur*	√
36	-0.87	-0.24	*S.aur*+*L.mon*	*S.aur*+*L.mon*	√
37	-0.81	-0.27	*S.aur*+*L.mon*	*S.aur*+*L.mon*	√

38	0.29	-0.40	*S.aur*+*S.*spp	*S.aur*+*S.*spp	√
39	0.98	-0.33	*S.*spp	*S.*spp	√
40	0.28	0.41	*L.mon*+*S.*spp	*L.mon*+*S.*spp	√
41	0.38	-0.39	*S.aur*+*S.*spp	*S.aur*+*S.*spp	√
42	0.43	-0.04	*S.aur*+*L.mon*+*S.*spp	*S.aur*+*L.mon*+*S.*spp	√
43	0.99	-0.35	*S.*spp	*S.*spp	√
44	-0.69	0.89	*L.mon*	*L.mon*	√
45	0.38	-0.43	*S.aur*+*S.*spp	*S.aur*+*S.*spp	√
46	-1.00	-1.00	*S.aur*	*S.aur*	√
47	0.39	0.02	*S.aur*+*L.mon*+*S.*spp	*S.aur*+*L.mon*+*S.*spp	√
48	0.96	-0.35	*S.*spp	*S.*spp	√
49	-0.81	-0.24	*S.aur*+*L.mon*	*S.aur*+*L.mon*	√
50	0.40	0.01	*S.aur*+*L.mon*+*S.*spp	*S.aur*+*L.mon*+*S.*spp	√
51	1.00	-0.34	*S.*spp	*S.*spp	√
52	-0.97	-0.28	*S.aur*+*L.mon*	*S.aur*+*L.mon*	√
53	-0.67	0.90	*L.mon*	*L.mon*	√
54	-0.97	-0.93	*S.aur*	*S.aur*	√

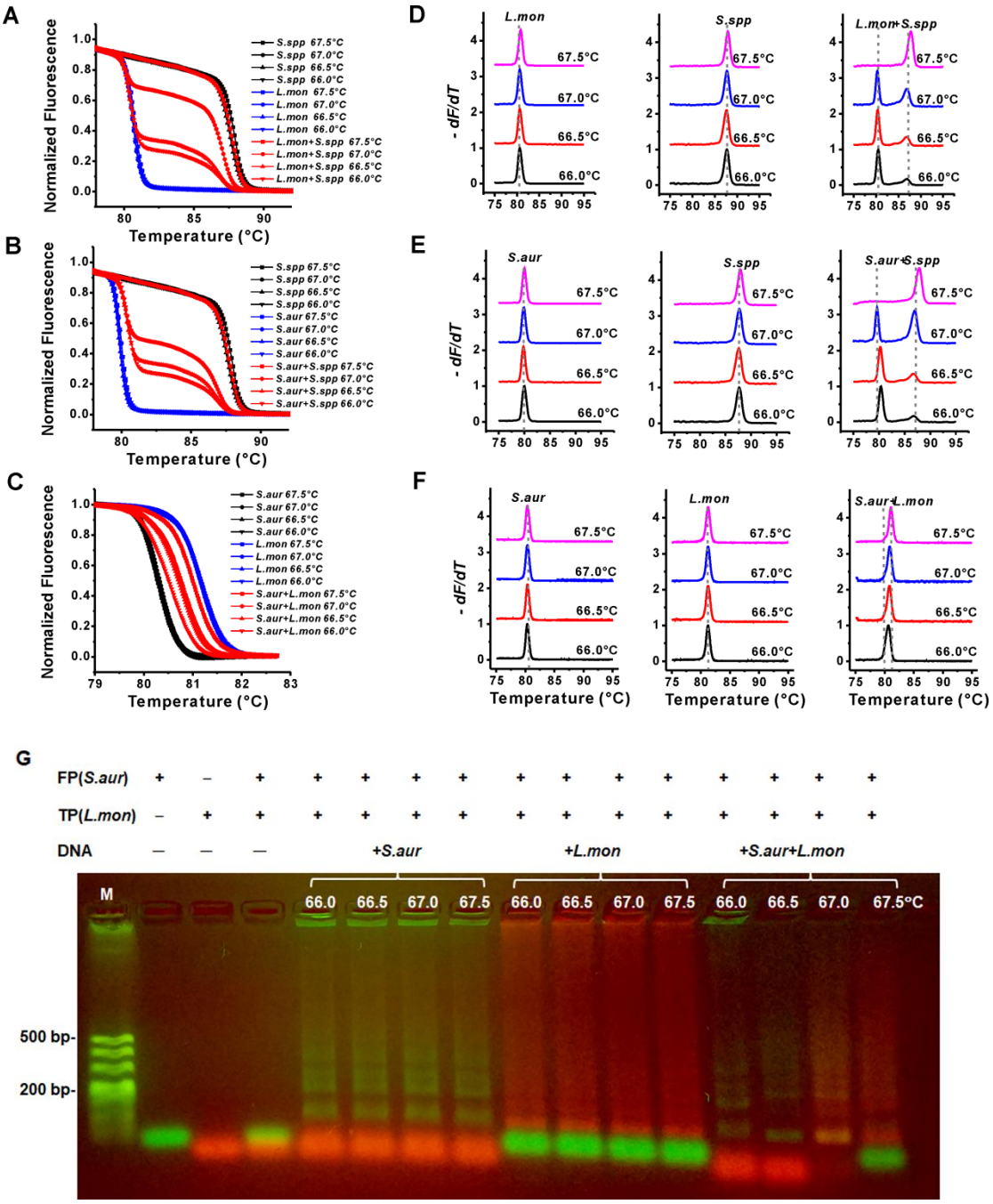

Fig. S1 Effect of reaction temperature upon the duplex assay using two sets of primers for *S.* spp + *L. mon* (A, D), *S.* spp + *S. aur* (B, E), and *L. mon* + *S. aur* (C, F, G), respectively. The results are shown in HRM melting curves (A, B and C), the corresponding melting peaks (D, E and F), and gel images (G). FP and TP indicate the FAM- and TAMRA-labeled primers, respectively (Table S1). The LAMP reactions were carried out at 66.0 °C, 66.5 °C, 67.0 °C, and 67.5 °C, respectively. The results indicate that the reaction temperature does affect the amplification of single-pathogen

but obviously affect the amplification of duplex-pathogen, which is further confirmed by the gel imaging of the LAMP amplified DNA sequences using two sets of labeled primers under different reaction temperature (G). This may be ascribed to the variable LAMP amplification efficiency for the duplex-pathogen sequences. The similar amplification efficiencies were obtained at 67.0 °C. Thus, the reaction temperature of 67.0 °C was used in subsequent research.

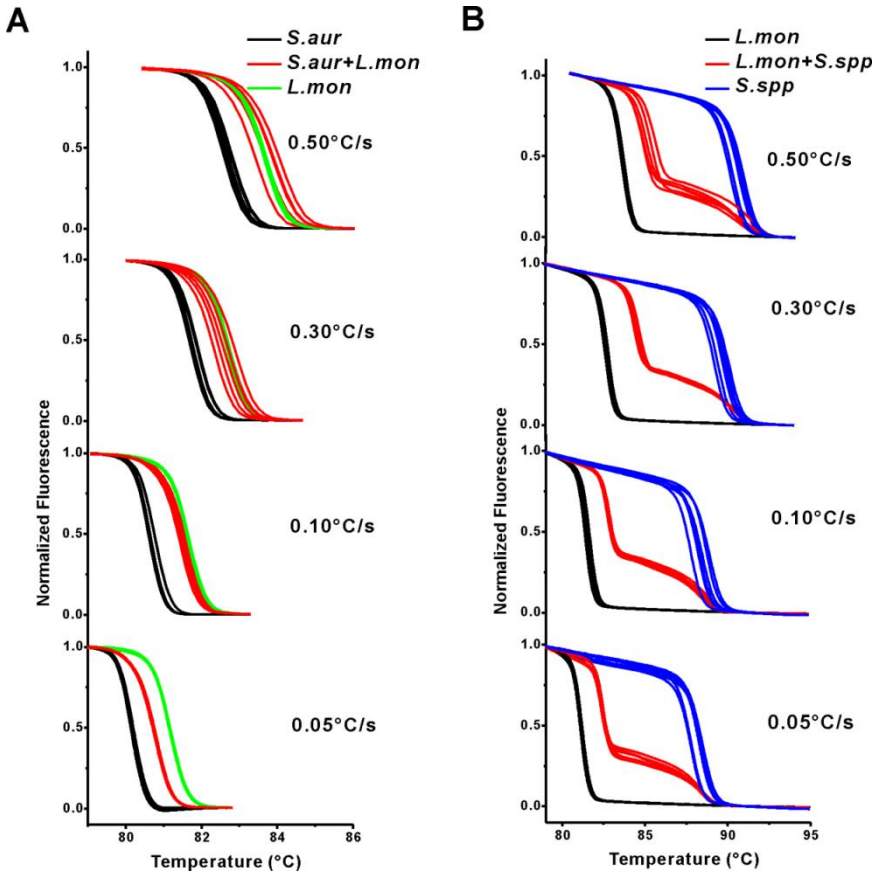

Fig. S2 Effect of melting rate on the LAMP-HRM duplex assay using two sets of primers for *S. aur* + *L. mon* (A) and *L. mon* + *S.* spp (B). The melting rate was varied from 0.05 °C/s to 0.50 °C/s. The apparent melting temperature increases with the increase of melting rate for all cases, and a slow melting rate results in the improved discrimination of the melting curves between singleplex target and duplex targets, especially for the duplex-pathogen *S. aur* + *L. mon* with a small T_m difference (~1.29 °C).

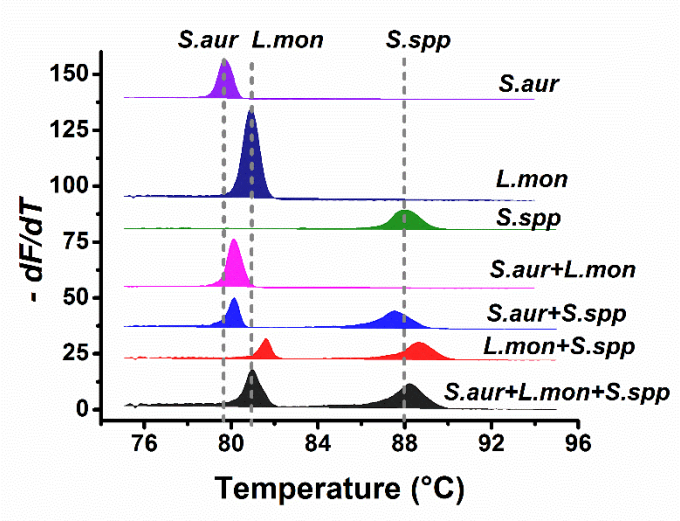

Fig. S3 Triplex LAMP-HRM assay using three sets of primers for *S.aur*, *L.mon* and *S.*spp. The HRM profiles are displayed with the melting peaks of singleplex, duplex and triplex LAMP products. It is a challenge to identify the duplex-pathogen *S.aur* + *L.mon*, and triplex-pathogen *S.aur* + *L.mon* + *S.spp* with T_m value alone, because only a single melting peak is observed for *S.aur* + *L.mon*, and double peaks are observed for *S.aur* + *L.mon* + *S.*spp.

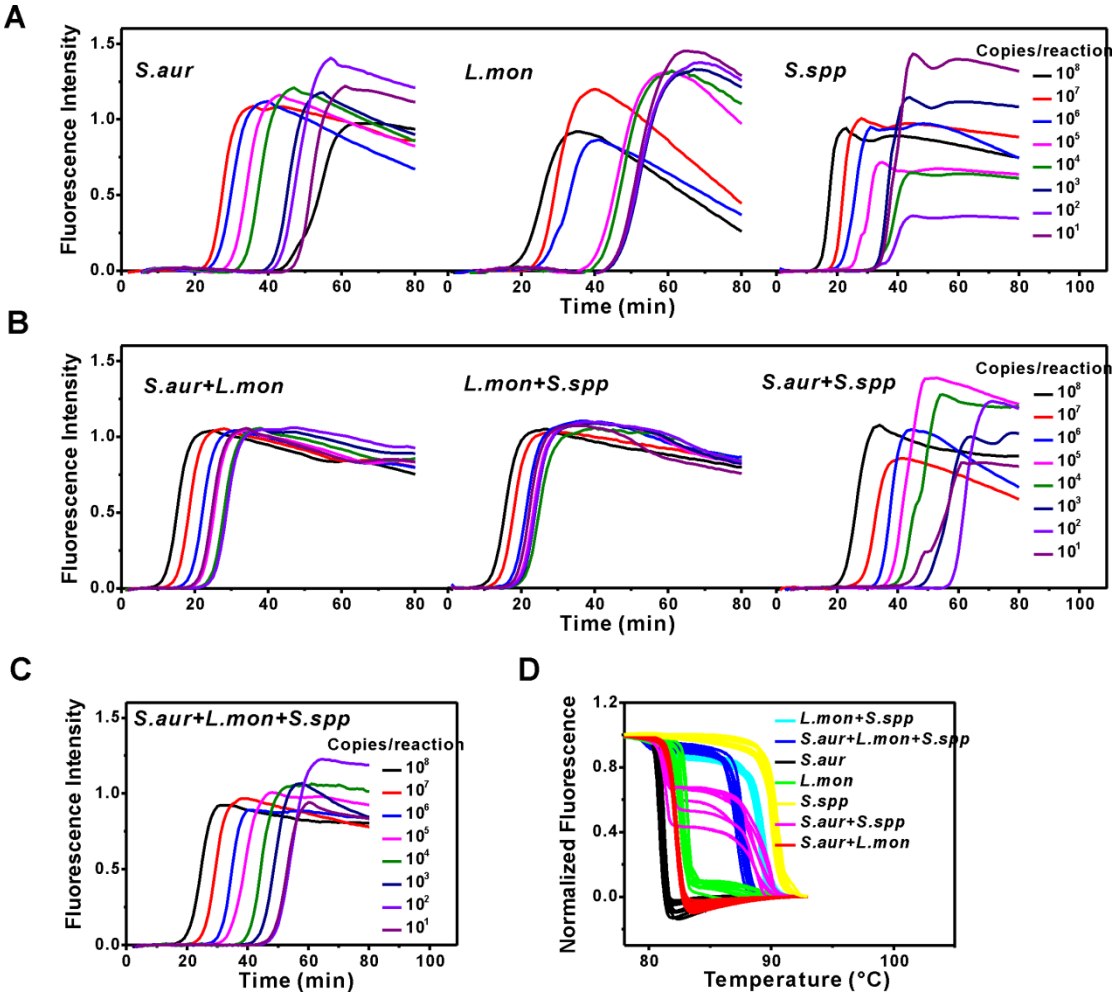

Fig. S4 Triplex LAMP-HRM assay against various DNA concentrations ranging from 10 to 10^8 copies. Real-time LAMP amplification curves for single-pathogen (A), duplex-pathogen (B), and triplex-pathogen target (C), and their corresponding HRM profiles (D).

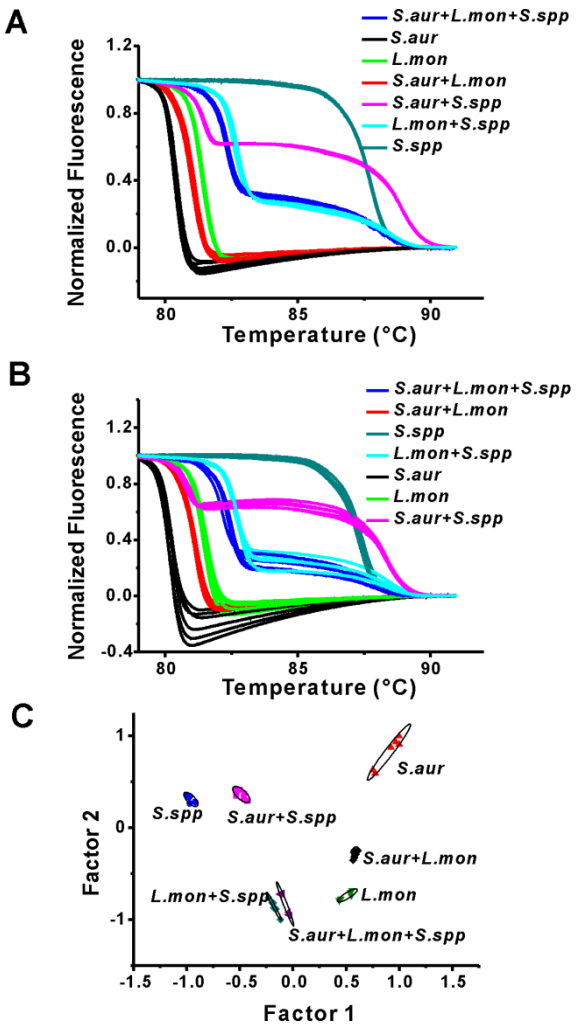

Fig. S5 Triplex LAMP-HRM profiles against seven pathogenic DNAs spiked in 10% fetal bovine serum (A) and 10% milk (B), and the corresponding PCA plot of HRM profiles obtained in 10% milk (C).

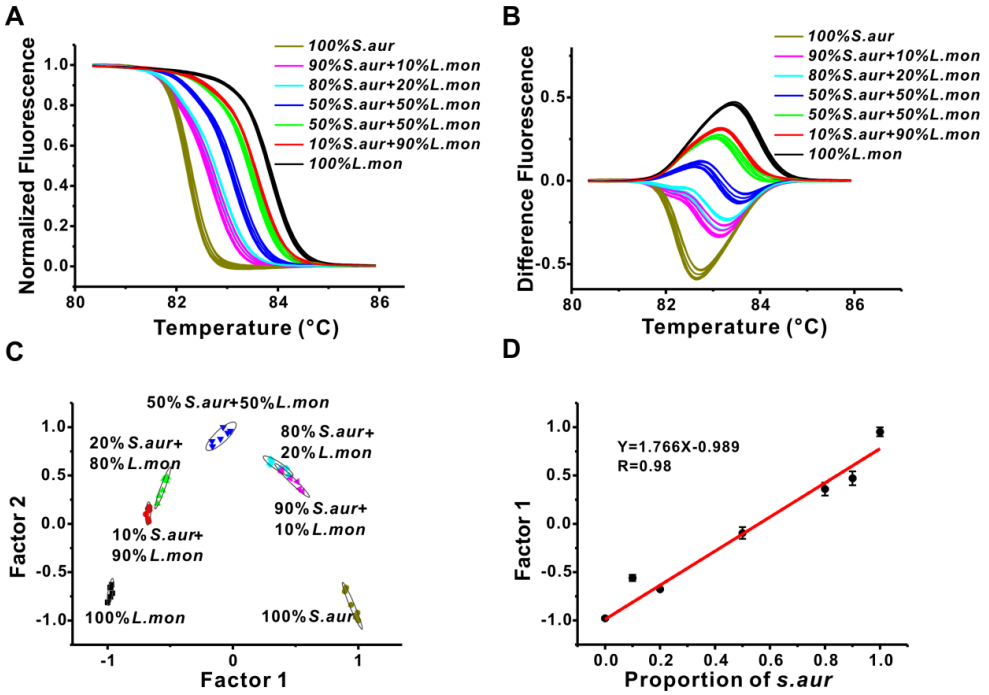

Fig. S6 Analysis of duplex-pathogen *S.aur* + *L.mon* products at different ratio. Normalized HRM melting profiles (A), difference plot of HRM profiles (B), PCA plot of HRM profiles (C), and the linear relationship between factor 1 and the proportion of *S.aur* (D).

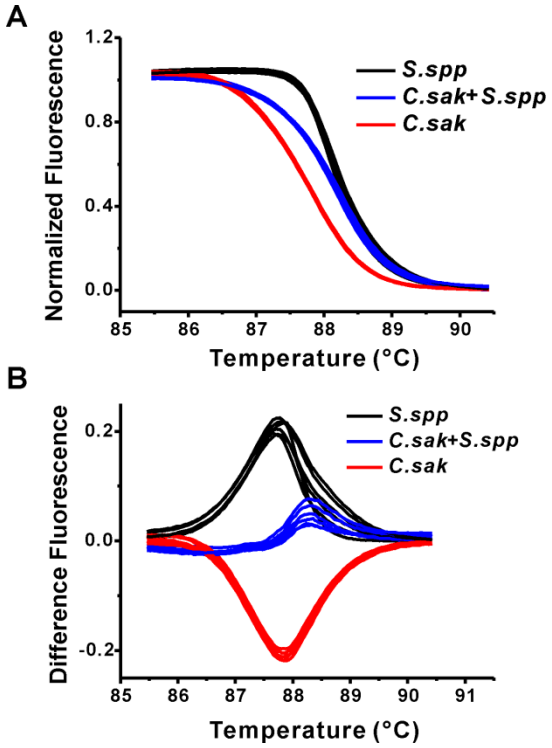

Fig. S7 Duplex assay using two sets of primers for *S.* spp and *C. sak* with a small T_m difference between their amplicons (88.05°C for *S.* spp and 87.81°C for *C. sak*). Both the distinct normalized HRM melting profiles (A) and the difference plot of HRM profiles (B) verify the generality of the proposed strategy.