Materials and methods

Materials

The Bst DNA 2.0 polymerase large fragment was purchased from New England Biolabs and the PfAgo protein from JiaoHong Biotech (Shanghai, China). Deoxyribonucleotide triphosphates (dNTPs) were obtained from Sangon Biotech (Shanghai, China). The 2× Universal SYBR Green Fast qPCR Mix (RK21203) was provided by ABclonal Technology Co., Ltd., and the DNA extraction kit was acquired from Chengdu Foregene Biotechnology Co., Ltd.

Design of TLAMP primers and guide DNA

In our study, TLAMP primers were designed based on the mitochondrial genes of the pig Sus scrofa (GenBank: NC_000845.1, cytochrome *b* gene) and cattle *Bos taurus* (GenBank: NC_006853.1, 16S rRNA gene) retrieved from the NCBI database. These regions exhibit high interspecific variation and high intraspecific conservation, avoiding cross-pairing with non-target species (e.g., chicken, rabbit, and duck) by BLAST alignment. The TLAMP primers were generated using the online primer design software of LAMP (https://lamp.neb.com). Similar to the six-primer LAMP, the TLAMP primer design includes two outer primers (F3 and B3), two inner primers (FIP and BIP), and two loop primers. The loop primers (TLPs) in TLAMP are modified to include an extended 5' region that corresponds to the 5' region (F1C or B1C) of the inner primers. This adjustment to the loop primers makes use of the sequences in traditional LAMP primers, without requiring new sequences, thereby offering a simple approach to TLP design. For guide DNA design, the principles were as follows: (i) the guide DNAs (15 nt in length, 5'-phosphorylated) target the core conserved regions of TLAMP amplicons to ensure efficient complementary pairing with target amplicons; (ii) overlap with primer sequences was avoided to prevent interference with the formation of guide DNA-PfAgo complexes. All the sequences detailed in Table S3 were synthesized by Sangon Biotech (Shanghai, China).

Sample preparation

All meat products, including pork, beef, chicken, rabbit, and duck, were sourced from the local food market (Chengdu, China), ensuring a comprehensive representation of commonly consumed meats. The genomic DNA of each sample was extracted using a DNA extraction kit (Foregene, China) under the guidance of the manufacturer's manual. Subsequently, the DNA concentration was precisely measured using a Microvolume UV-Vis Spectrophotometer ND5000 (Biotek, China). To test the purity of the collected sample, the A260/A280 ratio of DNA was measured using a spectrophotometer. All samples had a ratio between 1.8–2.0, meeting nucleic acid purity standards (free of

protein or phenol contaminants). For integrity testing, we did not check every sample, but representative 1% agarose electrophoretic gels showed that the extracted DNA presented a single, clear band (no smearing or degraded fragments), confirming that the extracted genomic DNA had good integrity and was suitable for TLAMP amplification (data not shown). The extracted DNA solutions, crucial for our experiments, were carefully preserved at −20 °C to maintain their integrity for future use.

Establishment of the one-pot TLAMP-PfAgo (OTPA) system

The OTPA system was performed in a single reaction tube with a total volume of 26 μL, including a 20-μL TLAMP reaction system and a 6-μL PfAgo reporting reaction. Specifically, the 6-μL PfAgo reporting reaction was sealed at the bottom of the reaction tube by a microcrystalline wax with a melting temperature of 80 °C. The sealing procedure was as follows: firstly, the PfAgo reporting reaction was added to the bottom of the tube; then about 8 mg of microcrystalline wax was added to the surface of the reaction; next, the tube was heated to 80 °C for 1 min to melt the microcrystalline wax and evenly cover the surface of the PfAgo reaction solution; finally, the tube was cooled to room temperature and the 20-μL TLAMP reaction was added on the top of resolidified microcrystalline wax. The TLAMP reaction system consisted of 19 μL reagent mixture and 1 μL template. The reagent mixture consisted of 2.4 U Bst DNA polymerase, 3 μL betaine (5 mol/L), 1.2 μL dNTPs (10 mmol/L), 1.5 μL 10× buffer (200 mmol/L Tris-HCl (pH 8.8), 100 mmol/L $(NH_4)_2SO_4$, 1% Tween 20, 50 mmol/L $MgSO_4$, and 600 mmol/L KCl), 0.48 μL of each inner primer (FIP and BIP, 50 μmol/L), 0.24 μL of each turn-back loop primer (TLF and TLB, 50 μmol/L), and 0.12 μL of each outer primer (F3 and B3, 50 μmol/L). The PfAgo reporting reaction consisted of 0.5 μL PfAgo (5 μmol/L), 0.9 μL Mn^{2+} (40 mmol/L), 0.24 μL of each guide DNA (40 μmol/L), 1.6 μL of 10× PfAgo reaction buffer, and 0.8 μL FAM-labeled reporting probe (10 μmol/L). In the OTPA detection system, the amplification reaction of TLAMP and the cleavage of PfAgo protein occur successively. The temperature was first set at 60 °C for 20 min, and then raised to 95 °C for 10 min. The fluorescence intensity of the reaction was recorded using a real-time fluorescence thermal cycler (Foregene Biotech, China).

Specificity and sensitivity assay

Genomic DNAs of pork and beef were successively diluted 10-fold (range from 3×10^{-3} to 3×10^{-6} ng/μL) to be used as templates to evaluate the sensitivity of the OTPA, with ddH_2O as a negative control. To assess the specificity of OTPA, genomic DNA from other animal-derived meat was tested using the same method.

Establishment of duplex OTPA detection system

The duplex OTPA detection system is similar to singleplex detection with subtle modifications. The specific TLAMP primers and guide DNAs targeting beef and pork were added to the TLAMP reaction (20 μL) and PfAgo reaction (6 μL). The TLAMP reaction consisted of 2.4 U Bst2.0 DNA polymerase enzyme, 3 μL betaine (5 mol/L),

1.2 μL dNTP (10 mmol/L), 1.5 μL 10× buffer, 1.68 μL duplex primer mixture (0.48 μL of each inner primer (FIP and BIP (50 μmol/L)), 0.24 μL of each turn-back loop primer (TLF and TLB (50 μmol/L)), and 0.12 μL of each outer primer (F3 and B3 (50 μmol/L)). The PfAgo reaction system contained 1 μmol/L PfAgo, 0.9 μL Mn^{2+} (40 mmol/L), 0.24 μL of each guide DNA (40 μmol/L), 1.6 μL 10× PfAgo reaction buffer, 0.8 μL FAM-labeled reporting probe (10 μmol/L), and 0.8 μL ROX-labeled reporting probe (10 μmol/L). The reaction was carried out at 60 °C for 20 min, and at 95 °C for 10 min. The fluorescence intensity of the reaction was recorded using a real-time fluorescence thermal cycler (Foregene Biotech, China).

Table S1 Optimized detection conditions for OTPA

Content	Concentration
FIP	1.6 μmol/L
BIP	1.6 μmol/L
TLF	0.8 μmol/L
TLB	0.8 μmol/L
F3	0.4 μmol/L
B3	0.4 μmol/L
KCl	60 mmol/L
$MgSO_4$	5.0 mmol/L
Hot-strat *Bst*2.0 DNA polymerase	0.4 U/μL
PfAgo	0.3 μmol/L
$MnCl_2$	1.4 mmol/L
gDNA	0.4 μmol/L

The total volume of the OTPA reaction was 25 μL with 8 mg of microcrystalline wax.

Table S2 Comparison of the analytical performance of some reported PfAgo-based methods

Method	Reporting	Target	Sensitivity	Time	One-pot test?	Ref.
Recombinase-aided amplification and restriction endonuclease-assisted *Pyrococcus furiosus* argonaute (PfAgo) detection	Fluorescence or lateral flow strip	African swine fever virus (ASFV)	10 copies	65 min	No	Zhao et al., 2024
RPA-PfAgo	Fluorescence	SARS-CoV-2 key mutation L452R	1 copy	90 min	No	Zhao et al., 2022
PCR/HDA-PfAgo	Fluorescence	Tumor genomic DNA and HPV	1.6 amol/L (about single-copy level)	For PCR, >90 min; for HAD, >60 min	No	He et al., 2019
LCR-PfAgo	Fluorescence	SARS-CoV-2 and HPV	10 amol/L HPV11 DNA or SARS-CoV-2 RNA	70 min	No	Wang et al., 2021
LAMP-PfAgo	Fluorescence	SARS-CoV-2	17.6 copies	35 min	Yes	Xun et al., 2021
OTPA	**Fluorescence or visualized detection**	**Pork and beef**	**3×10^{-4} ng/μL of pork DNA and 2×10^{-4} ng/μL of beef DNA**	**30 min**	**Yes**	**This work**

Table S3 All nucleic acid sequences used in this work

Type	Name	Sequence (5'–3')
TLAMP primers	T-P-F3	ATTCATTGACCTCCCAGC
	T-P-B3	GTTTGCATGTAGATAGCGAAT
	T-P-FIP	GTTAGGATTTGCAAGATTAGGCAGACAAACATCTCAT CATGATGAAACT
	T-P-BIP	AGCAATACATTACACATCAGACACA ATCCGTAATTTACGTCTCGA
	T-P-TLF	GTTAGGATTTGCAAGATTAGGCAGATGCCTAAGAGG GAACCGA
	T-P-TLB	AGCAATACATTACACATCAGACACAACAGCTTTCTC ATCAGTTACACACA
	T-B-F3	ACTAATGGCTAATCAGCCCAT
	T-B-B3	CCACGCTTATCATTATGCTG
	T-B-FIP	GCTGAGTCCAAGCATCCCC GCTCACACATAACTGTGCT
	T-B-BIP	TATGGCCGTCAAAGGCCCTGGTGCTCAAGATGCAGT TAAGTC
	T-B-TLF	GCTGAGTCCAAGCATCCCCCAAAATAAAAAAATACC AAATGTATGAC
	T-B-TLB	TATGGCCGTCAAAGGCCCTGACCCGGAGCATCTATT GTAGCTG
PfAgo gDNA and reporting probe	g-P-1	AGCAATACATTACACATCAGACACA
	g-P-2	CTTGCAAATCCTAAC
	g-P-3	GGCCTGTTCTTAGCA
	g-B-1	TCAAAGGCCCTGACCC
	g-B-2	GGAGCATCTATTGTAG
	FQ-P-FAM	ATCCTAACAGGCCTGTTCTT
	FQ-B-ROX	CTGACCCGGAGCATCTATTG
PCR primers	P-P-F1	GCCATATACTCTCCTTGGTGACA
	P-P-R1	ATGAAAGAGGCAAATAGATTTTCG
	P-B-F1	GCCATATACTCTCCTTGGTGACA
	P-B-R1	GTAGGCTTGGGAATAGTACGA

“P” in the middle of the sequence name indicates the sequences for prok and “B” in the middle of the sequence name indicates the sequences for beef. F1-F3: forward outer primers; B1-B3: backward outer primers; FIP/BIP: forward/backward inner primer; TLF/TLB: turn-back loop forward/backward primer; LF: loop forward; LB: loop backward; FQ: fluorophore and quencher.

Table S4 Comparison of the performance of duplex OTPA and singleplex OTPA

Category	Duplex OTPA	Singleplex OTPA
Detection efficiency	Simultaneous detection of two targets in one tube (30 min total)	One independent tubes required (30 min/tube, 60 min total)
Reagent/sample saving	25 μL reaction volume (8 mg microcrystalline wax) for two targets	50 μL total (2×25 μL) for two targets
Contamination risk	Fully closed-tube operation (no tube opening between reactions)	Multiple tube openings (for two reactions), increasing amplicon contamination risk
Internal control	The target signal of one species (e.g., beef ROX) can act as an internal control for reaction validity	No internal control, failed reactions (e.g., reagent degradation) may cause false negatives
Practical applicability	Directly detects mixed adulteration (e.g., pork in beef sausages) in one step	Requires two separate tests to confirm mixed species, delaying results

Table S5 Cost per test of the OTPA assay

Regents	Cost (CNY)
PfAgo	1.225
Primer sets	0.057
Guide DNAs	0.112
Hot-strat Bst2.0 DNA polymerase	0.9
Buffer (including metal ions)	0.55
Probe	0.176
dNTP	0.016
Total	3.036

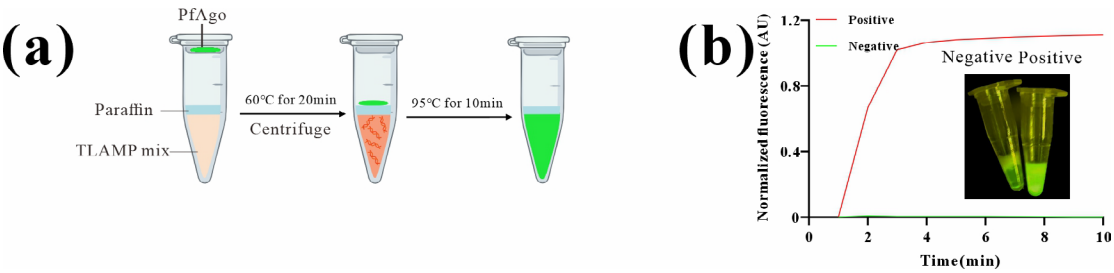


Fig. S1 One-pot two-step detection based on TLAMP and PfAgo. (a) The scheme of the two-step detection procedure. (b) The real-time fluorescence curve and the endpoint image under blue light of the two-step detection.

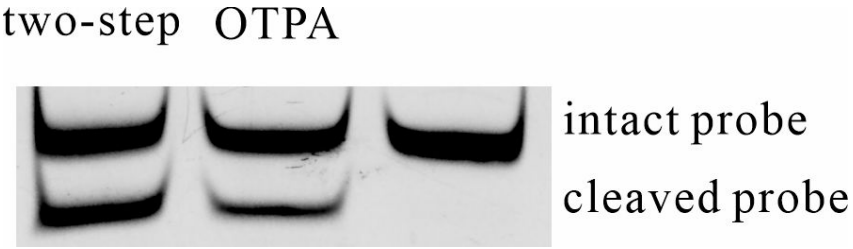


Fig. S2 Denaturing PAGE analysis of the probe cleavage of the two-step reaction and OTPA.

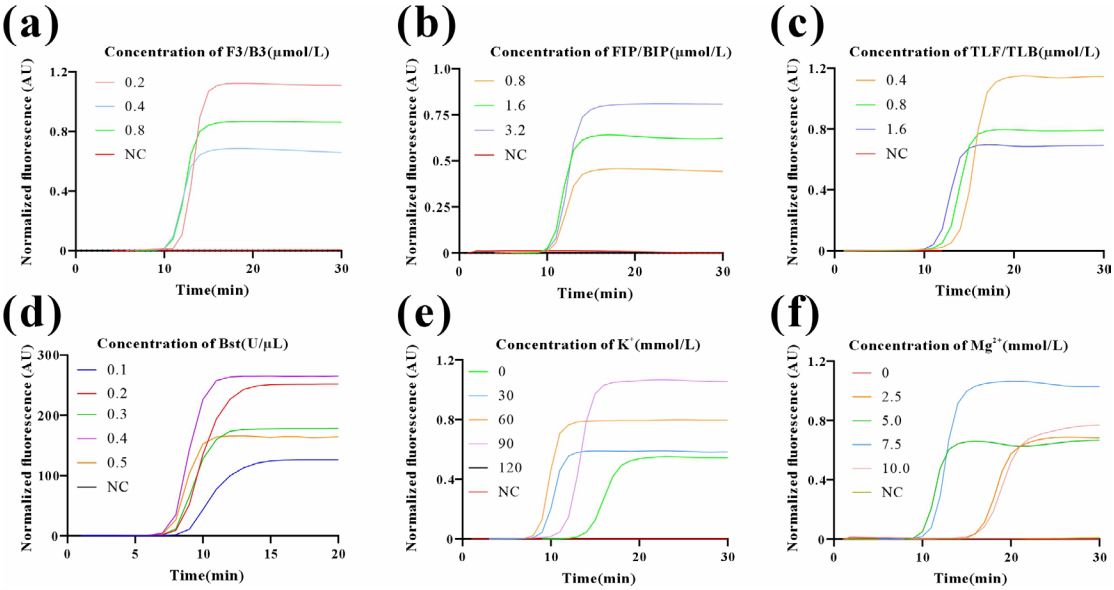


Fig. S3 Optimization of the TLAMP reaction in the OTPA assay using pork as the target. (a) Optimization of the concentration of outer primer. (b) Optimization of the concentration of inner primer. (c) Optimization of the concentration of turn-back loop primer. (d) Optimization of the concentration of DNA polymerase. (e) Optimization of the concentration of K^+. (f) Optimization of the concentration of Mg^{2+}.

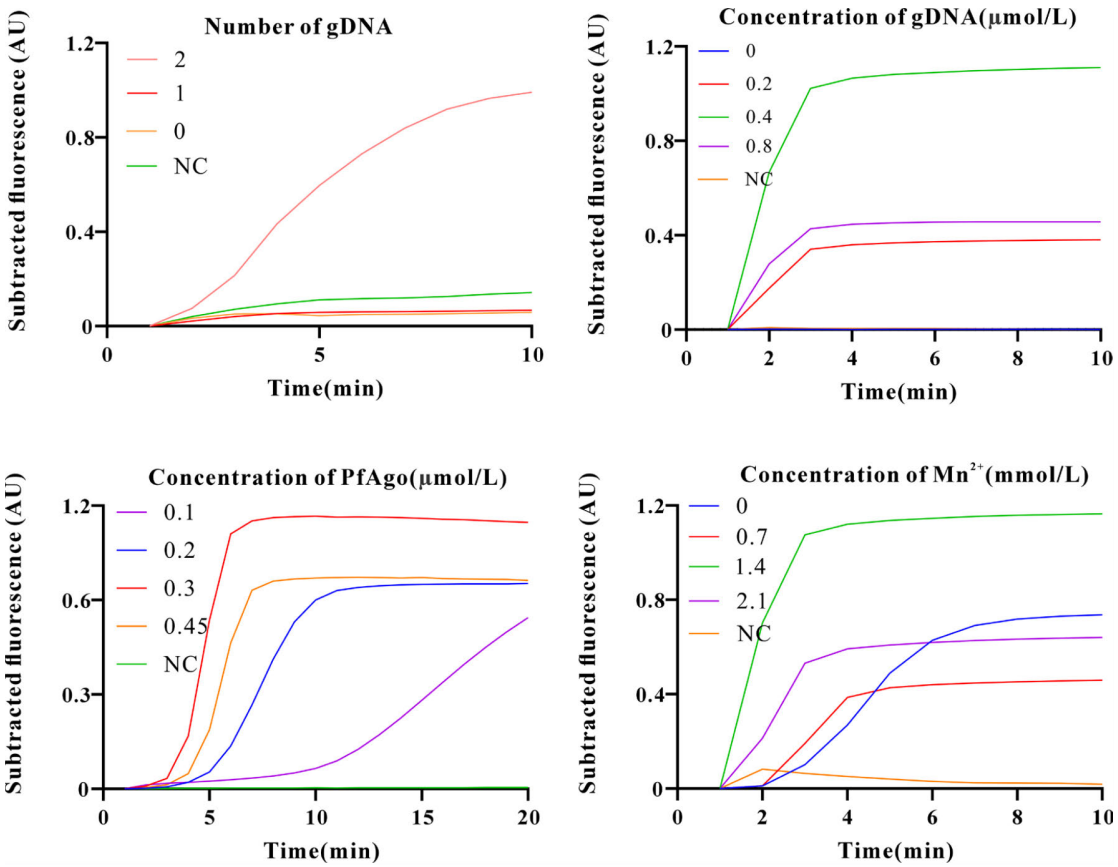


Fig. S4 Optimization of PfAgo-based cleavage in OTPA.

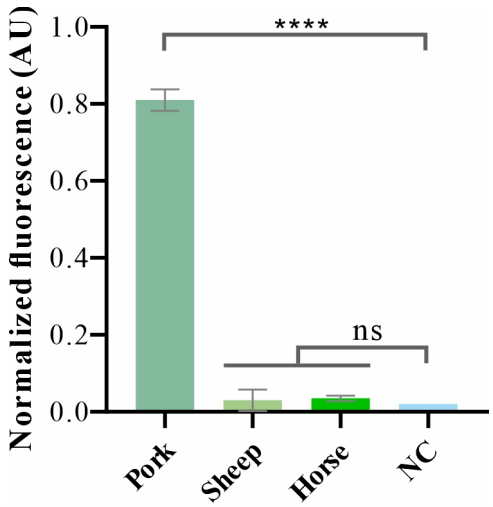


Fig. S5 Additional specificity evaluation of the OTPA assay against sheep and horse genomic DNA. The assay was tested using genomic DNA extracted from pure pork, sheep, and horse meat. NC: negative control (ddH_2O). Data are presented as mean±SD from three independent replicates (n=3). Statistical significance was determined by one-way ANOVA. Asterisks indicate significant differences compared to the 0.01% group and NC (** P<0.0001); ns indicates no significant difference (P>0.05).**

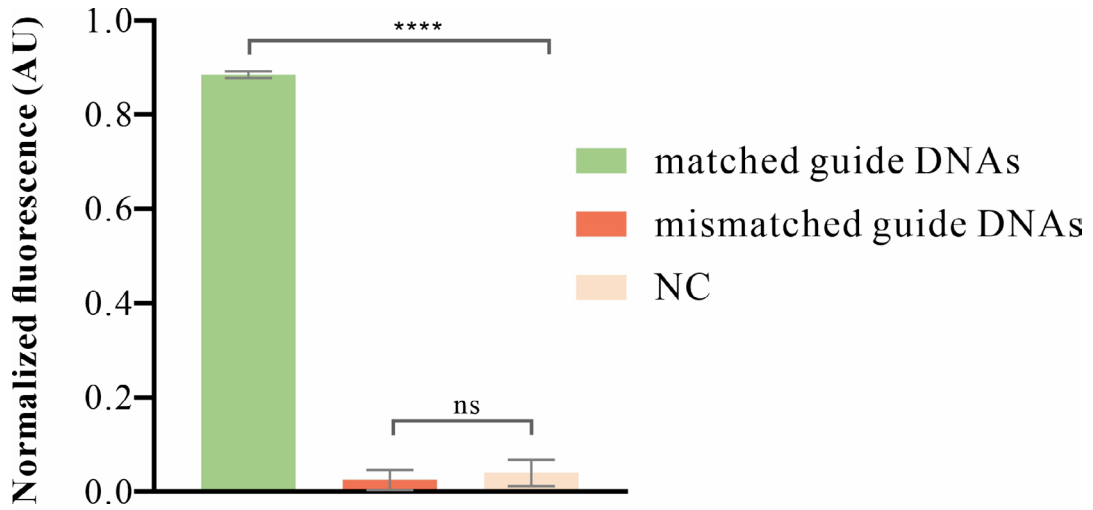


Fig. S6 Validation of PfAgo specificity using non-complementary guide DNAs. The fluorescence intensity was measured using specific guide DNA versus irrelevant (non-complementary) guide DNAs in the presence of target amplicons.

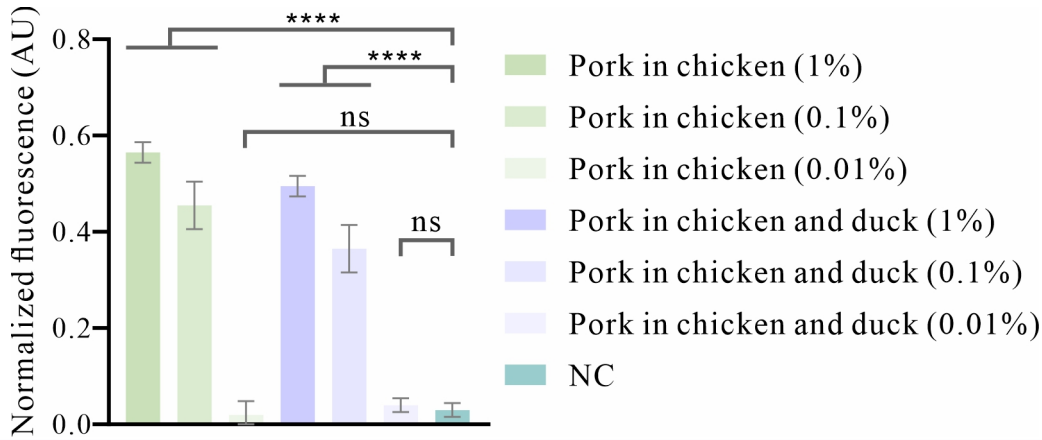


Fig. S7 Evaluation of the OTPA assay for detecting pork adulteration in complex meat matrices. The normalized endpoint fluorescence intensity was measured using DNA templates extracted from minced chicken and a mixture of chicken and duck (purple bars) spiked with varying percentages of pork (1%, 0.1%, and 0.01%, w/w). NC: negative control (pure chicken/duck DNA without pork). Data are presented as mean±SD from three independent replicates (n=3). Statistical significance was determined by one-way ANOVA. Asterisks indicate significant differences compared to the NC (** P<0.0001); ns indicates no significant difference (P>0.05).**

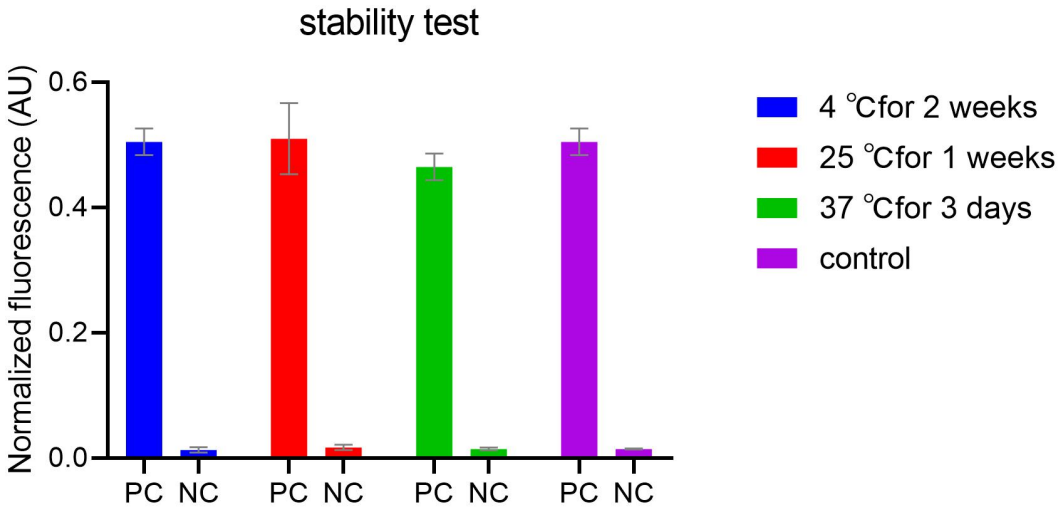


Fig. S8 Stability test of OTPA. Prefabricated reaction tubes containing microcrystalline wax were stored at 4 °C (two weeks), room temperature (25 °C, one week), and 37 °C (3 d). Besides, the freshly prepared reaction tubes served as controls to detect pork DNA (3×10^{-4} ng/μL).

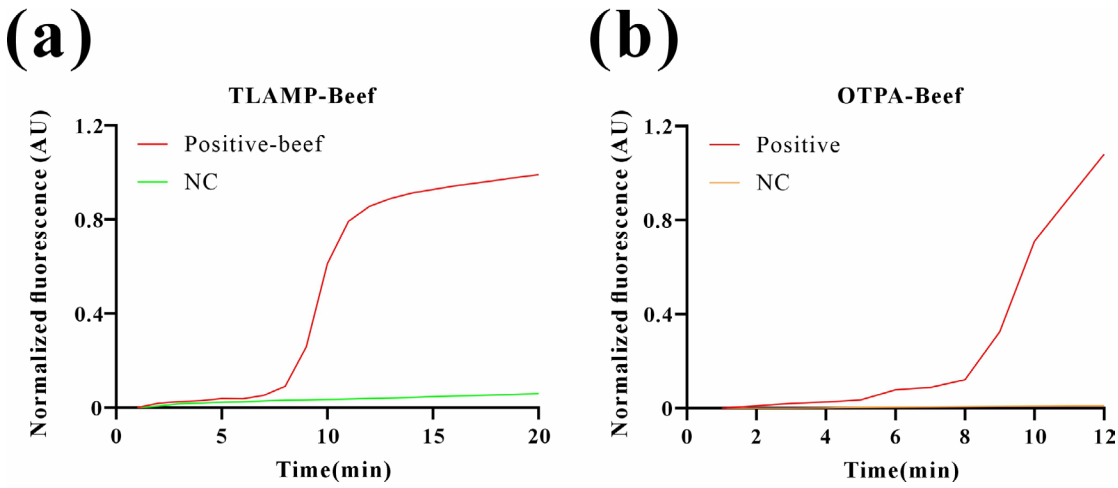


Fig. S9 Establishment of singleplex OTPA assay for beef DNA detection. (a) The TLAMP reaction for beef DNA amplification. (b) The real-time fluorescence curve of OTPA detection of beef DNA.

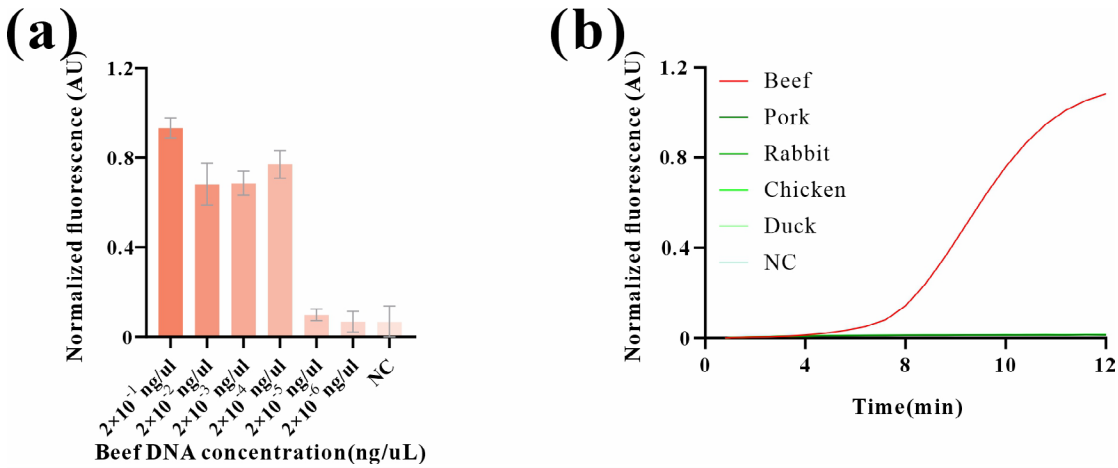


Fig. S10 Singleplex OTPA assay for beef DNA detection. (a) The sensitivity of the method. (b) The specificity of the method. The genomic DNA concentrations of pork, beef, rabbit, duck, and chicken was 0.3, 0.2, 0.5, 3, and 1.1 ng/μL, respectively.

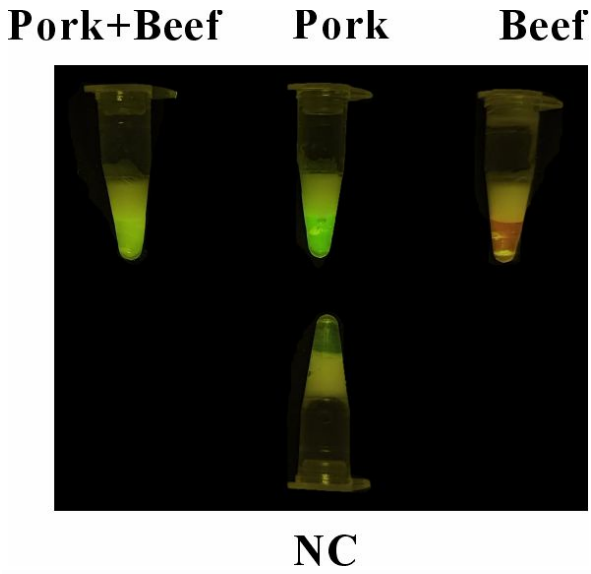


Fig. S11 Visualized duplex OTPA assay under blue light.

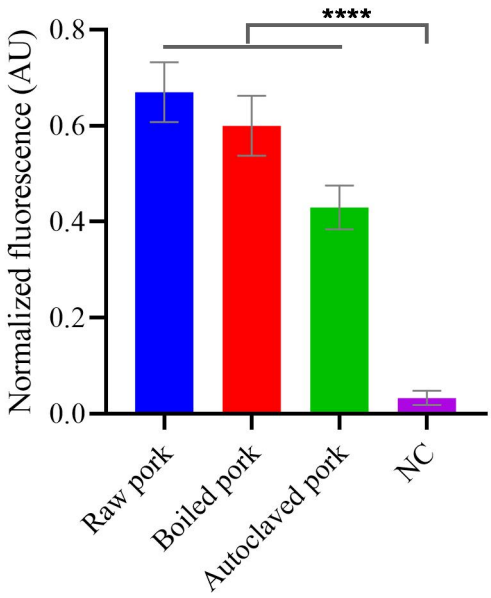


Fig. S12 Evaluation of OTPA robustness against thermal processing-induced DNA degradation. To validate the assay performance on low-integrity DNA, raw pmeat samples (pork) were subjected to different simulated processing conditions: raw meat (untreated control), boiled meat (treated at 100 °C for 60 min), and autoclaved meat (treated at 121 °C for 20 min to mimic canning sterilization). The bar chart displays the endpoint fluorescence intensity of the OTPA assay for each group. Data are presented as mean±SD (*n*=3). Statistical significance was determined using Student's *t*-test. ** indicates *P*<0.0001 vs. NC. NC: normal control without adding template.**

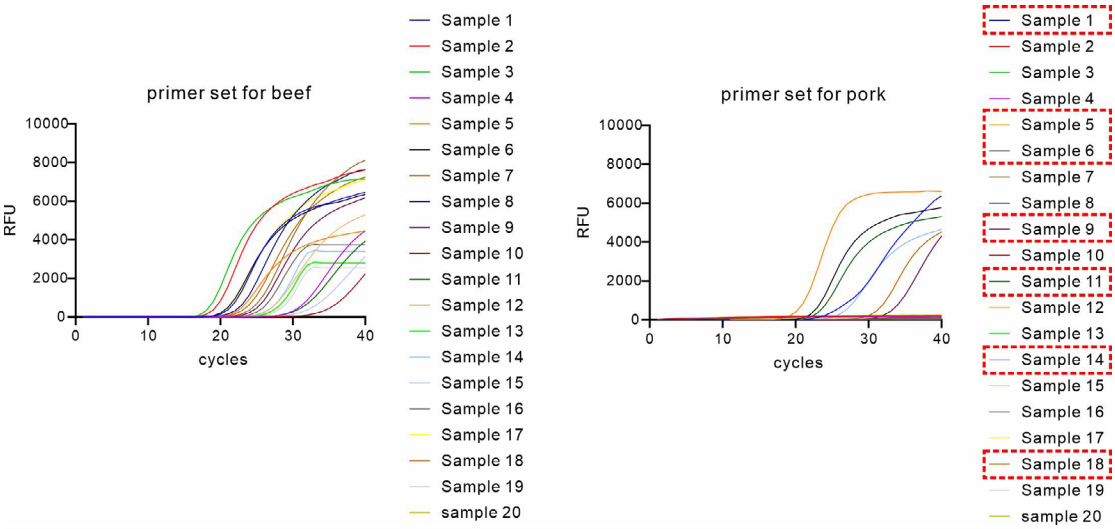


Fig. S13 Real-time PCR validation of 20 commercial beef samples was performed. Among these samples, samples 1, 5, 6, 9, 11, 14, and 18 were artificially contaminated with pork meat.

References

He RY, Wang LY, Wang F, et al., 2019. *Pyrococcus furiosus* Argonaute-mediated nucleic acid detection. *Chemical Communications*, 55(88):13219-13222. https://doi.org/10.1039/C9CC07339F

Wang LY, He RY, Lv B, et al., 2021. *Pyrococcus furiosus* Argonaute coupled with modified ligase chain reaction for detection of SARS-CoV-2 and HPV. *Talanta*, 227:122154. https://doi.org/10.1016/j.talanta.2021.122154

Xun GH, Lane ST, Petrov VA, et al., 2021. A rapid, accurate, scalable, and portable testing system for COVID-19 diagnosis. *Nature Communications*, 12:2905. https://doi.org/10.1038/s41467-021-23185-x

Zhao CJ, Yang LH, Zhang X, et al., 2022. Rapid and sensitive genotyping of SARS-CoV-2 key mutation L452R with an RPA-*PfAgo* method. *Analytical Chemistry*, 94(49):17151-17159. https://doi.org/10.1021/acs.analchem.2c03563

Zhao Y, Yang M, Zhou CY, et al., 2024. Establishment of a simple, sensitive, and specific ASFV detection method based on *Pyrococcus furiosus* Argonaute. *Biosensors and Bioelectronics*, 254:116230. https://doi.org/10.1016/j.bios.2024.116230