Supplementary Materials

Ultrasensitive strips for the quadruple detection of nitrofuran metabolite residues

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1. Experimental

1.1 Materials and reagents

Nitrofuran parent compounds, metabolite residue were from Aladdin Industrial Inc. Oligonucleotides were purchased from Shanghai Sangon Biological Engineering Technology (Shanghai, China). Vent (exo-) DNA polymerase, Nt.BbvCI, Taq DNApolymerase, deoxynucleoside triphosphates (dNTPs) were purchased from New England Biolabs (New England, USA). HAuCl4 was from Sigma-Aldrich (Steinheim, Germany). Bovine serum albumin (BSA) was obtained from Dingguo Biotech. Co. (Beijing, China). Nitrocellulose membrane was purchased from Sartorius (Goettingen, Germany). Fiberglass and absorbent paper were purchased from Shanghai Kinbio (Shanghai, China). All buffer solutions used in this study were prepared in our lab. Other chemicals were purchased from standard commercial sources and were of analytical grade purity.

1.2 Primer Design and Multiplex SDA

The oligonucleotides in SDA assay were designed using primer software PREMIER 5.0 (**Table S1**). All primers were analyzed for hairpin structures and hybrids using the Integrated DNA Technologies design tools. All of the tag oligonucleotides were modified with carboxyl group to conjugate with amino groups in specific antibodies for the four metabolites. The tag sequences included four parts, the blue sequences in 3'-end providing the site for primer annealing, the red sequences for nicking enzyme *Nt.BbvCI* recognition, the green highlighted sequences complementary to

the probes on T-line, and brown highlighted sequences complementary to the probes on AuNPs.

Table S1. Nucleotides used in this study.

Items	Sequence (5'-3')	Length (nt)
Primer	AGCCGTTGTTCTTCAT	16
Probe on AuNP	SH-C_6-TCAAACTGCATAGGTAGT	18
C-line	ACTACCTATGCAGTTTGA	18
AMOZ-Tag	COOH-TCAAACTGCATAGGTAGTCCAGAAGCCGAACAAGCTGAGGATGAAGAACAACGGCT	56
AMOZ-T1	CCAGAAGCCGAACAAGCT	18
AOZ-Tag	COOH-TCAAACTGCATAGGTAGTGAAGCTAGTGCTCCTAGAGCTGAGGATGAAGAACAACGGCT	59
AOZ-T2	GAAGCTAGTGCTCCTAGAGCT	18
AHD-Tag	COOH-TCAAACTGCATAGGTAGTAGACCATCAAGGTCTGCAGCTGAGGATGAAGAACAACGGCT	59
AHD-T3	AGACCATCAAGGTCTGCAGCT	18
SEM-Tag	COOH-TCAAACTGCATAGGTAGTTCAGCTAATGTCGATTCAGCTGAGGATGAAGAACAACGGCT	59
SEM-T4	TCAGCTAATGTCGATTCAGCT	18

Note: [a]Red characters indicate the recognition sequence of *Nt.BbvCI*; [b]Blue characters indicate the sites for primer annealing; [c] Characters highlighted in brown indicate the sites binding to AuNPs; [d]Characters highlighted in green indicate the sites binding to oligonucleotides in T lines (AMOZ-T1, AOZ-T2, AHD-T3 and SEM-T4) .

The biotin-derivatives were incubated with four types of antibodies modified with DNA tag probes to form De-Ab complex in a 500 µl of PBS supplementary with 0.1 mg/ml salmon sperm DNA. The mixture was stayed at room temperature for 10 min. To enrich the target complexes, 10 µl of MBs coated with streptavidin were then

added to the mixture. The mixture was intermittently shook at room temperature for 10 min. Then, a magnet separator was used to enrich the MBs through the interaction of biotin modified De-Ab complexes and streptavidin in the MBs. The MBs were washed three times with PBST (PBS supplementary with 0.05% Tween-20). The collected MBs were suspended with 10 µl of ddH_2O as template in SDA reactions.

SDA reactions were carried out in 25 µl amplification mixtures as previous studies . Briefly, each reaction contained 5 µl template of enriched DNA tags by MBs, 0.4 µM of each primer, 1.25 µl of *Nt.BbvCI* nicking enzyme (5 U), 2 µl of Klenow DNA polymerase (10 U), 50 µM dNTPs and 1×reaction mix (Klenow fragment exo reaction buffer). The mixture was incubated at 50 ℃ for 30 min.

SDA products were analyzed by LFB, and 10 µl of amplicons were diluted in 50 µl of 4×saline-sodium citrate (SSC) buffer. It was loaded onto the sample pad in the biosensor. Visible red lines (T line; C line) should be observed in positive reactions, and only the control lines were visual in negative and blank controls.

1.3 Preparation of gold-nanoparticle (AuNP)-DNA conjugates

AuNPs with an average diameter of 15 nm were prepared according to our previously reported method (Fang et al., 2010). Briefly, 4 mL of 1% trisodium citrate was added into 100 mL of a rapidly stirred and boiling $HAuCl_4$ solution (0.01%) in a 500-mL round bottom flask. After turning red, the solution was boiled for additional 10 minutes. It was cooled to room temperature with gentle stirring. The AuNPs were collected and concentrated 4 times by centrifugation (12×10^3 rpm, 20 min). The resulting AuNPs solution was stored at 4 °C until use.

To prepare AuNP-DNA conjugate, 100 µL of ultra-pure water (18.2 MΩ/cm, Millipore, Billerica, MA, USA) containing 1 OD of thiolated DNA was added to 1 mL of the AuNPs solution (4 times concentrated) and shaken gently overnight at 4°C. The DNA coated AuNPs (1.1 mL) were subjected to “aging” by adding 110 µL100 mM phosphate buffer (pH 7.0) with 1% sodium dodecyl sulfate and 1.5 M NaCl. The solution was kept at 4 °C for 12 hours. Particles were centrifuged (12×10^3 rpm, 20 min) and rinsed three times with rinsing buffer (20 mM Na_3PO_4, 5% BSA, 0.25% Tween-20 and 10% sucrose) to remove any unbound DNA. The red pellet was re-suspended in 150 µL of rinsing buffer and then stored in a refrigerator at 4 °C until use. The AuNP-DNA conjugate solution was dispensed onto fiberglass as conjugate pad.

1.4 Construction of lateral flow biosensor

Thirty microliters of 100 μM DNA probes (test line probe and control line probe) were dispensed onto the nitrocellulose membrane simultaneously with a lateral flow dispenser (Shanghai Kinbio, Shanghai, China).The membrane was then dried at room temperature for 12 hours. Fiberglass was used as sample pads after being soaked in sample pad buffer (0.5% Triton, 1% BSA, 2% sucrose, 50 mM boric acid, pH 8.0). It was dried and stored in low-humidity at room temperature. Sample pad, conjugate pad, nitrocellulose membrane and absorbent pad were attached along the long axis of an adhesive plate with an overlap of 1-2 mm, and cut into 4-mm-wide strips using a paper cutter.

1.5 Limit of detection analysis

For quantatitive analysis, the optical intensities of the test and the control lines were recorded simultaneously by the strip reader, which automatically located the red bands in a fixed reaction area and then measured strip parameters such as peak height and area integral. The optical intensity of the test line and control line could be recorded simultaneously by using the Shanghai Kinbio strip reader software. To define a positive result, we used the signal/noise (S/N) ratio (S/N≥3) as reference. Signal was the optical intensity of the test line with detected sample, and noise was the intensity of the test line with PBS.

1.6 Nitrofuran metabolite residues detection in real sample

Meat samples (pork meat) were obtained from a local supermarket. Before spiking, meat samples were minced and homogenized. All samples were spiked with SEM, AHD, AOZ, and AMOZ at different concentrations (shown in **Table S2**). The analytes were subsequently derived to Biotin–analytes (Bio-AOZ, Bio-SEM, Bio-MOZ, and Bio-AHD) and extracted. Briefly, 2 g homogenized fish muscle was placed into a 50-mL centrifuge tube. Next, 4 mL of H_2O, 0.5 mL of 1 M HCl, and 100 μL of 40 mM Biotin in DMSO were added successively to the homogenized samples. Each sample was thoroughly mixed and incubated for 3 h in a water bath at 55 °C. After cooling to RT, 5 mL of 0.1 M K_2HPO_4, 0.4 mL of 1 M NaOH, and 6 mL of ethyl acetate were added to the sample, shaken vigorously (30 s), and centrifuged at 3500×g for 10 min at RT. The upper ethyl acetate layer (3 mL) was transferred to glass tubes and evaporated by heating to dryness at 45 °C under nitrogen. The resulting residues were dissolved in 2 mL of a 1:1 (v/v) mixture of hexane and 0.1 M PBS at pH 7.4. The buffer phase, containing the derivative, was separated by centrifugation at 3000×g for 10 min and collected for LFB determination.

2. Results

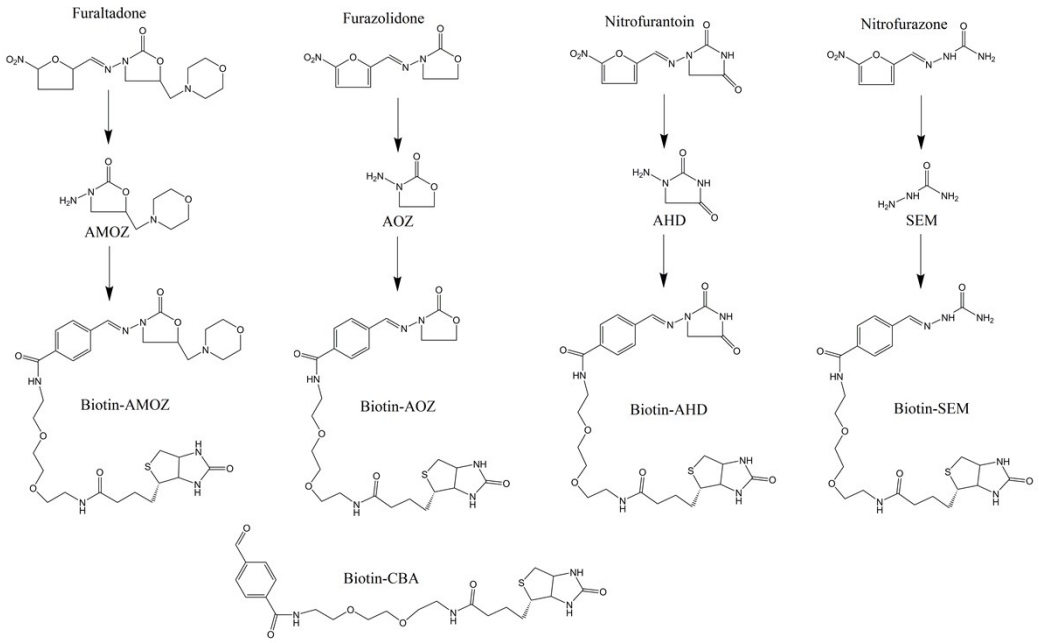

Fig. S1. Structures of nitrofuran parent compounds, metabolites and Biotin-CBA derivatives.

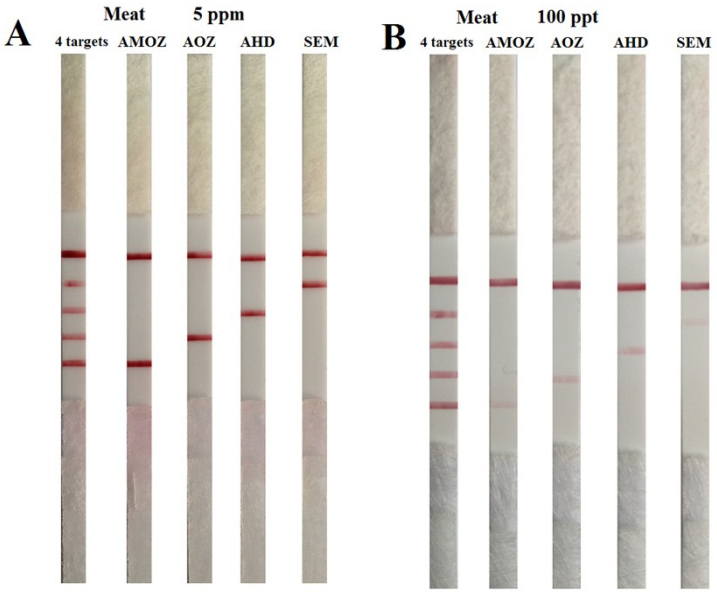

Fig. S2. Multiplex detection of nitrofuran metabolites in meat samples with (A) 5 ppm and (B) 100 ppt additive amount.

Table S2. Recoveries of nitrofuran metabolites in selected meat samples by LFB.

Sample (Meat)	Added Concentration (ppt)	Founded concentration (ppt)	Recovery (%) (n=3)
AMOZ	2.3×10^{6}	2.14×10^{6}	93.21
	2.3×10^{4}	2.11×10^{4}	91.83
	2.3×10^{3}	2.58×10^{3}	112.34
	2.3×10^{1}	2.40×10^{1}	104.27
AOZ	2.8×10^{6}	2.60×10^{6}	92.85
	2.8×10^{4}	2.89×10^{4}	103.26
	2.8×10^{3}	2.97×10^{3}	105.94
	2.8×10^{1}	2.99×10^{1}	106.77
AHD	2.1×10^{6}	2.43×10^{6}	115.93
	2.1×10^{4}	2.09×10^{4}	99.65
	2.1×10^{3}	2.58×10^{3}	122.64
	2.1×10^{1}	2.04×10^{1}	97.14
SEM	1.8×10^{6}	1.72×10^{6}	95.35
	1.8×10^{4}	1.89×10^{4}	104.76
	1.8×10^{3}	1.81×10^{3}	100.43
	1.8×10^{1}	1.84×10^{1}	102.45