Electronic Supplementary Information on the Analytical Methods publication

Two Fluorescence Quenching Immunochromatographic Assays Based on Carbon Dot and Quantum Dot as donor probes for the Determination of Enrofloxacin

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Methods

Verification of signal probes

For the verification of donor signal probes, CDs and CDs-OVA (QDs and QDs-OVA) were diluted to the same fluorescence intensity, and 2 μL of each solution were added to the NC membrane respectively and dried at 37 ° C for 12 h. After assembly in the same manner as in the assembly FLFIAs, cut to the appropriate width, appropriate amount of PBS buffer was added and flowed to the absorbent pad by the action of capillary, results were observed under ultraviolet light after drying.

For the verification of acceptor signal probes, similar to the test procedure of FLFIAs, PBS buffer containing the same concentration of AgNPs and AgNP-Ab was added to the prepared FLFIA strips, respectively, results of AgNP-LFIA were observed within 10 min by naked eyes.

Preparation of enrofloxacin coating antigen (ENR-OVA)

2.1 mg ENR dissolved in 0.3 mL of anhydrous DMF in an ice bath, 2 μL of tri-n-butylamine and 1.5 μL of isobutyl chloroformate were added drop by drop stirring. The mixture was stirred for 1 h at 4 °C in the dark. Next, product was dropped into 10 mg OVA dissolved in 1 mL carbonate buffer (0.05 mol L^{-1}, pH 9.6) and incubation for 12 h at 4 °C. The resulting coating antigen solution was dialyzed against PBS buffer (0.01 mol L^{-1}, pH 7.4) for 3 days at 4 °C.

Synthesis of carbon dots

2.1 g of citric acid and 1mL of ethylenediamine was dissolved into 20 mL ultrapure water, the mixture was transferred in a Teflon-lined autoclave and heated for 5 h at 200

℃. Then the reactant was dialyzed for 3 days to remove unreacted regent and cryodesiccated for future use.

Synthesis of Ag nanoparticles

10 mL of 1.75 mg mL^{-1} tannic acid solution was heated in 60℃ water bath, and 250 μL 0.1 mol L^{-1} of Na_2CO_3 solution was added. After stirring for 10 min, 1 mL of 0.125 mol L^{-1} $AgNO_3$ solution was added into above solution drop by drop, and continue heating for 30 min. The product was centrifuged at 2000 rpm for 15 min and the supernatant was collected and stored with airtight and light-free at 4℃ for future use.

Synthesis of Au nanoparticles

100 mL of 0.01% $HAuCl_4$ solution (w/v) was boiled with magnetic stirring, and 2.25 mL of 1% $Na_3C_6H_5O_7$ solution (w/v) was added into the above solution under strong stirring. After boiling for 15 min, the rose red solution was cooled to room temperature under stirring and stored with airtight and light-free at 4℃.

Preparation of the donor signal probes

For the CD-OVA donor signal probe, 10 mg CD, 9 mg EDC, 5 mg NHS were added into 1 mL PBS and stirred for 15 min at 37℃. Then, 100 μL 10 mg mL^{-1} OVA solution was added into above solution, after incubating at room temperature for 4 h, the mixture was washed with pH 7.4 PBS buffer (0.01 mol L^{-1}) for 3 times by an ultrafiltration centrifuge tube to remove the unreacted CD. The CD-OVA donor probe was concentrated to 500 μL and stored at 4°C in the dark.

For the QD-Ab donor signal probe. 25 μL of 8 μM QD solution, 15 μL 10 mg mL^{-1} OVA solution, and 11.5 μL 5 mg mL^{-1} EDC solution were mixed in a 2 mL tube, and the

mixture was brought to 200 μL with pH 7.4 borate-buffered saline (BBS). The mixture was incubated at 25 ℃ for 3 h with shaking and then concentrated to 100 μL by ultrafiltration. The QD-Ab signal probe was stored at 4 ℃ in the dark.

Preparation of the acceptor signal probes

For AgNP-Ab signal probe, First, 5 μL 0.2 mol L^{-1} K_2CO_3 was added into 1 mL AgNP solution to adjust the pH, then 0, 5, 10, 15, 20, 25, and 30 μL 0.65 mg mL^{-1} anti-ENR Ab was added into the solution, incubating at room temperature for 1 h. Next, 20 μL 20% bovine serum albumin (BSA) aqueous solution (w/v) and 10 μL 20% PEG-20000 aqueous solution (w/v) were added into the solution, after incubating at room temperature for 30 min, the mixture was centrifuged at 2,000 rpm for 15 min at 4 ℃, and then the supernatant was taken out and centrifuged at 10,000 rpm for 30 min at 4 ℃. Throwing away the supernatant, the AgNP-Ab signal probe was resuspended in 250 μL of pH 7 working buffer. The preparation of AuNP-Ab signal probe was similar to the AgNP-Ab signal probe and just adjust the dosage of 0.2 mol L^{-1} K_2CO_3 and ENR-Ab. After centrifuging at 10,000 rpm for 30 min at 4 ℃, the AuNP-Ab signal probe was re-suspended in 250 μL of pH 9 working buffer.

Preparation of immunochromatographic strip

The NC membrane was pasted onto the middle of the PVC sheet and overlapped 1 mm by a conjugate pad in left side and by an absorbent pad in right side. For “Turn On” pattern FLFIAs, the CD-OVA (QD-OVA) was immobilized at 0.75 μL cm^{-1} on the NC membrane as the control line (C line), ENR-OVA and CD-OVA (QD-OVA) was mixed and immobilized at 0.75 μL cm^{-1} at a distance of 0.5 cm below the C line

as the test line (T line); For “Turn Off” pattern LFIAs, the goat anti-rabbit IgG was immobilized at 0.75 μL cm^{-1}on the NC membrane as the control line (C line), and ENR-OVA was immobilized at 0.75 μL cm^{-1} at a distance of 0.5 cm to the C line as the test line (T line). Then, the membrane was dried at 37℃ overnight in incubator. Finally, all pads were pasted onto a PVC sheet and cut into strips with widths of 0.37 cm.

Results

Verification of signal probes

The successful coupling of label materials and protein was verified by chromatographic experiments, and results are shown in Fig.S5.

For donor signal probe (Fig.S5a and b), after PBS buffer flowed through the CDs (QDs) coupled to protein (OVA), the CDs (QDs) were still bound to the NC membrane, and there was concentrated and obvious fluorescence under the irradiation of the UV lamp (Fig.S5 a and b (1')), which was basically the same as before the dropwise addition of PBS buffer (Fig.S5a and b (1)). Conversely, CDs (QDs) not coupled to OVA could not bind to the NC membrane. After the PBS buffer flowed through the CDs (QDs) by capillary action, the CDs (QDs) was washed away, and there was no obvious and concentrated fluorescence (Fig.S5 a and b (2')).

For acceptor signal probe (Fig.S5c and d), AgNPs (AuNPs) successfully coupled to the Ab were captured by the coating antigen immobilized on the NC membrane during chromatography, showing a distinct yellow (red) band (Fig.S5c and d left), while the unconjugated AgNPs (AuNPs) were not captured by the coating antigen, so that no yellow (red) band will appear (Fig.S5c and d right). These results demonstrate that only the labeling material and the antibody are successfully coupled to function as a label.

Optimization of donor signal probes

For donor signal probes, the coupling ratio of CD (QD) and OVA was optimized. For C-FLFIA, CD and OVA with a series of mass ratio (1:0.02 to 1:0.2) were coupled by active ether method to synthesize the donor signal probe, and donor probes with same

concentration of OVA were sprayed onto NC membranes at the volume of 0.75 μL cm^{-1} and dried for 12 h. After adding 150 μL of PBS buffer to the strip, CD that did not combined with OVA was removed from the C line and T line. The fluorescence intensity gradually enhanced with the coupling ratio increasing and was stable when the coupling ratio reached 1:0.1. So, the CD-OVA with coupling ratio of 1:0.1 was chosen as the optimum conditions. For Q-FLFIA, the QD-OVA was optimized by the similar way and the coupling mole ratio ultimately was fixed at 1:8.

Optimization of acceptor signal probes

For acceptor signal probes, the pH and the amount of Ab were optimized to get the optimum coupling conditions of AgNP-Ab (AuNP-Ab) and the higher sensitivity of C-FLFIA (Q-FLFIA). For AgNP-Ab, 1, 2, 3, 4, and 5 μL 0.2 mol L^{-1} of K_2CO_3 aqueous solution were added into 1mL of nanoparticle solution to adjust the pH of the coupling system; then, 0, 5, 10, 15, 20, 25, and 30 μL 0.65 mg mL^{-1} Ab were added into 1mL of AgNP solution and mixed for 1 h at room temperature to optimize the feasibility and sensitivity of proposed assay. The yellow color of T line became shallow with the amount of K_2CO_3 aqueous solution increasing and disappeared when the amount of K_2CO_3 aqueous solution reached to 3 μL. So, the AgNP-Ab probe was coupled without K_2CO_3. With the increase of Ab, the yellow color on T line became deeper, and when the amount of Ab increased to 5 μL, the blue fluorescence on T line was completely quenched and higher sensitivity was gotten. Thus, 5 μL Ab were used to prepare AgNP-Ab acceptor signal probe. Similarly, for AuNP-Ab, 5 μL of K_2CO_3 aqueous solution and 10 μL of Ab

was optimized to get a clear red color line on the T line as well as higher sensitivity of Q-FLFIA.

Dilution of donor signal probe and coating antigen for FLFIA

For C-FLFIA, the concentration of the donor signal probe and coating antigen have great influence on the stability and sensitivity of the method. When the concentration of the donor probe is too low, the fluorescent band on the C/T line is not clear, affecting the interpretation of the result. When the concentration of the probe is too high, a large number of receptor probes are required to quench the fluorescence of the T-line and reduce the sensitivity of the method. The optimal amount of CD-OVA on C and T lines (by OVA) was 1.75 μg cm^{-1}. After optimizing the probe amount, the concentration of the coating antigen was optimized for optimum sensitivity, and the optimal amount of coating antigen on T line was 0.24 μg cm^{-1} for C-FLFIA. For Q-FLFIA, the optimal amount of QD-OVA on C and T lines (by OVA) was 1.125 μg cm^{-1}, and the optimal amount of coating antigen on T line was 0.3 μg cm^{-1} for Q-FLFIA.

Dilution of goat anti-mouse IgG for LFIAs

For Ag-LFIA,in order to ensure that the color of the C line is consistent with that of the T line, the goat anti-mouse IgG was diluted as 0.375 μg cm^{-1}. And for Au-LFIA, the goat anti-mouse IgG was diluted as 0.25 μg cm^{-1}. The spray volume of both coating antigen and the goat anti-mouse IgG were 0.75 μL cm^{-1}.

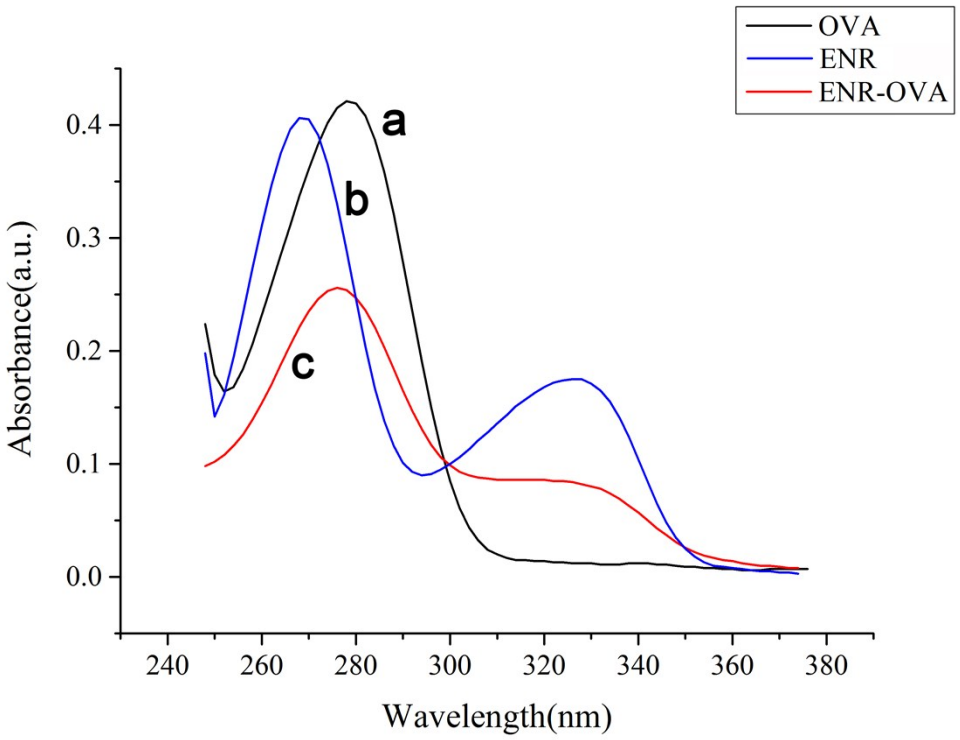

Fig. S1. Characterization results of ENR-OVA.

UV-Vis spectra of a: OVA, b: ENR, and c: ENR-OVA

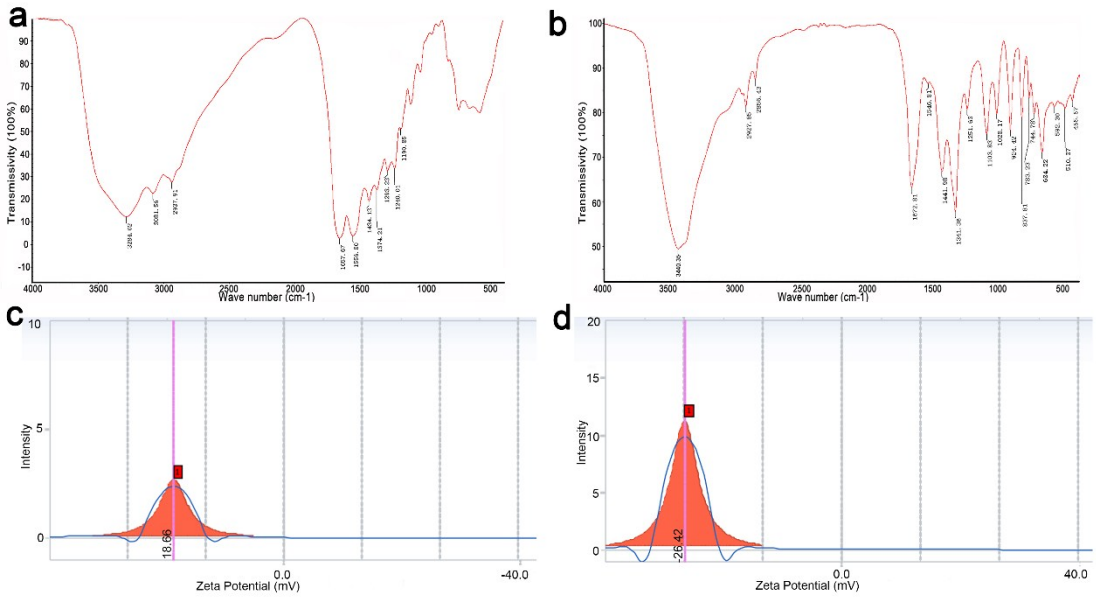

Fig. S2. Fourier transform infrared spectrometer (FT-IR) spectra of a: CD and b: QD and Zeta potential of c: CD and d: QD.

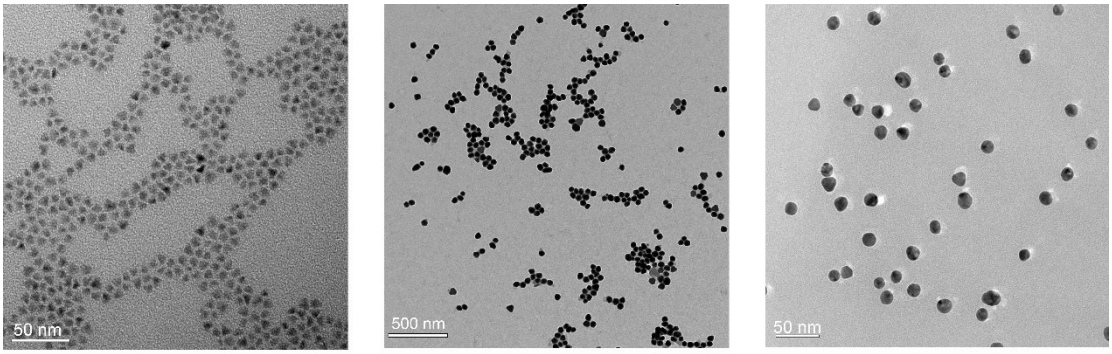

Fig. S3. Transmission electron microscopy (TEM) images of a: QDs, b: AgNP and c: AuNP

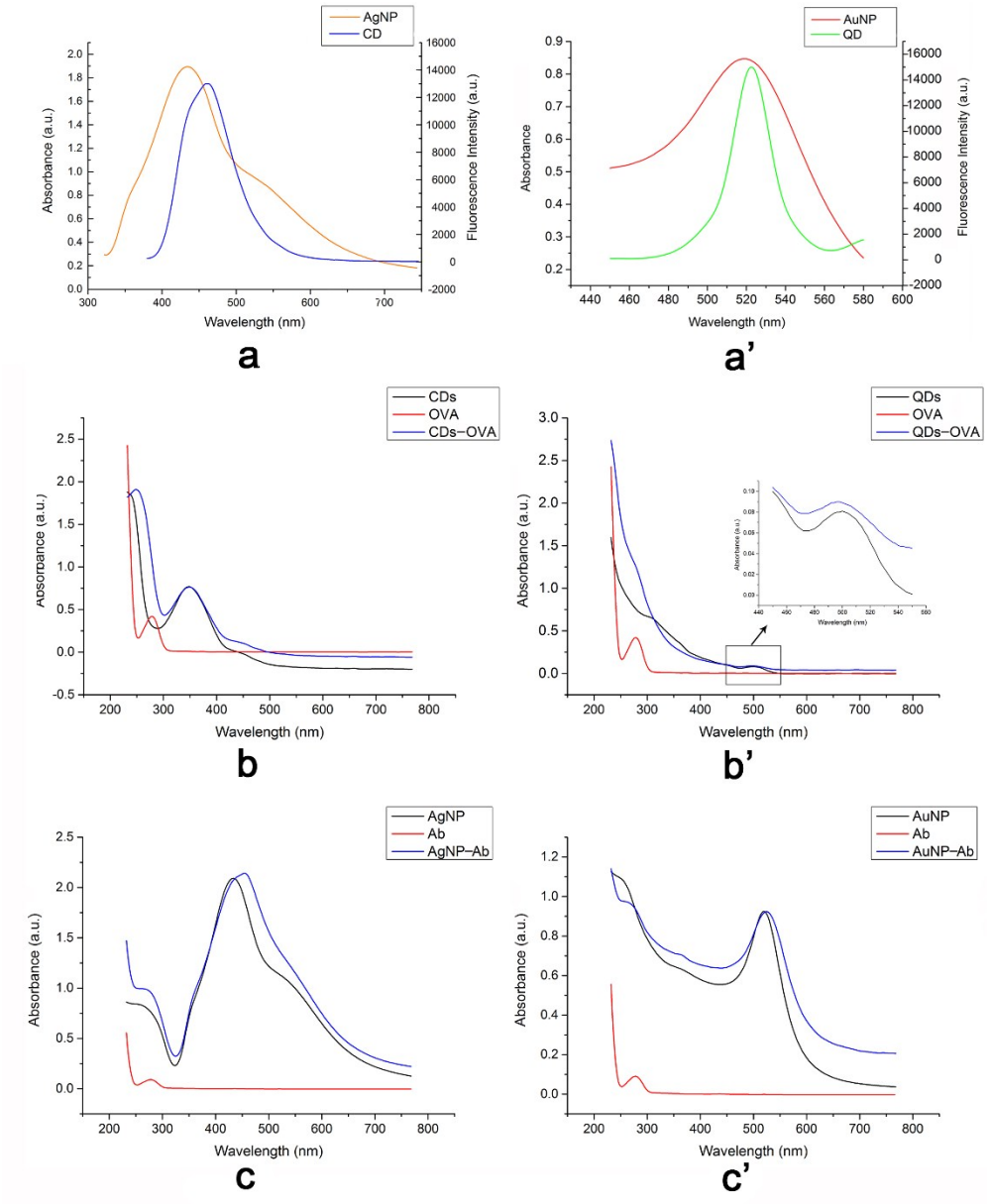

Fig. S4. Characterization results of signal conjugate.

A: Fluorescence spectra of CD under an excitation of 356.5 nm and UV-Vis absorption spectrum of AgNP. A': Fluorescence spectra of QD under an excitation of 310 nm and UV-Vis absorption spectrum of AuNP. B: UV-Vis absorption spectrum of CD, OVA, and CD-OVA. B': UV-Vis absorption spectrum of QD, OVA, and QD-

OVA. C: UV-Vis absorption spectrum of AgNP, Ab, and AgNP-Ab. C': UV-Vis absorption spectrum of AuNP, Ab, and AuNP-Ab.

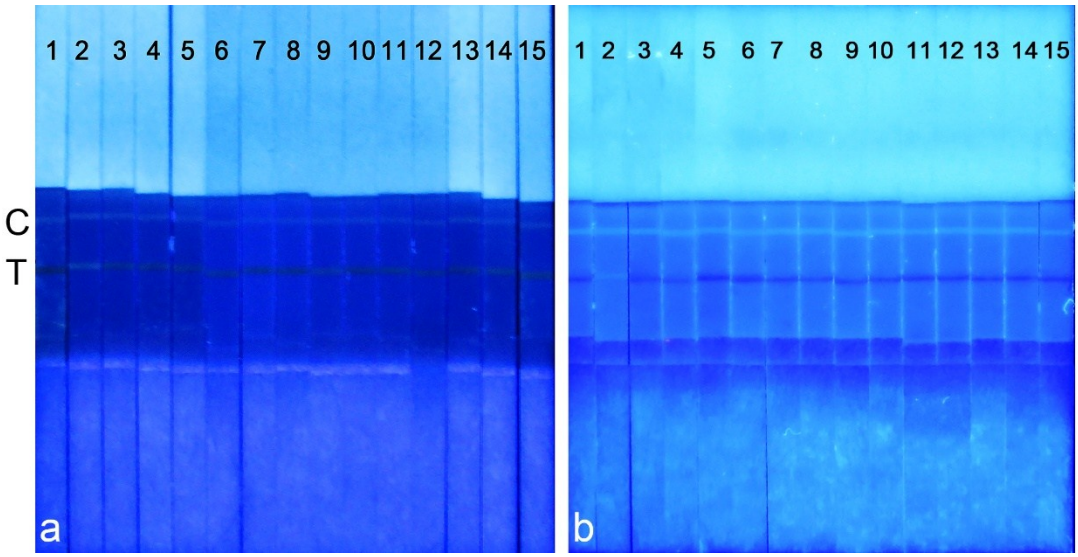

Fig. S5. Specificity analysis of the CD-FLFIA and QD-FLFIA strips.

From left to right: 1 PBS, 2 enrofloxacin, 3 enoxacin; 4 flumequine, 5 danofloxacin, 6 marbofloxacin, 7 sparfloxacin, 8 gatifloxacin, 9 fleroxacin, 10 lomefloxacin, 11 difloxacin, 12 sarafloxacin, 13 ciprofloxacin, 14 norfloxacin, 15 ofloxacin. a: C-FLFIA (λ_{ex}=365 nm). The concentration of ENR was 0.1 μg L^{-1}, and the concentration of other 13 quinolones was 10 μg L^{-1}. b: Q-FLFIA (λ_{ex}=310 nm). The concentration of ENR was 0.25 μg L^{-1}, and the concentration of other 13 quinolones was 25 μg L^{-1}.

Table S1. Working conditions of the FLFICA.

		C-FLFICA		Q-FLFICA
Probe coupling buffer	CD-OVA	0.01 mol L^{-1} PBS, pH7.4	QDs-OVA	0.2 mol L^{-1} BBS, pH 8.3
Coupling ratio		CD: OVA=1:0.1 (mass ratio)		QD: OVA=1:8 (mole ratio)
Dilution ratio		1:6		1:11
Dilution buffer		PBS buffer, pH 7.4		PBS buffer, pH 7.4
Probe coupling buffer	AgNPs-Ab	water	AuNPs-Ab	AuNP water solution
Coupling ratio		$AgNO_3$: Ab=1:0.022 (mass ratio)		$HAuCl_4$: Ab=1:0.065 (mass ratio)
Probe volume on strip		4 μL per strip		4 μL per strip
Ab dosage on strip		0.052 μg per strip		0.104 μg per strip
Sample addictive amount		150 μL		150 μL
Coating antigen dilution ratio		1:25		1:20
Coating antigen dosage on strip		0.75 μL/cm		0.75 μL/cm
Drying temperature and time		37°C, 12 h		37°C, 12 h
NC membrane		Millipore HF135s		Millipore HF135s
Assay buffer		0.01 mol L^{-1} PBS, pH 7.4		0.01 mol L^{-1} PBS, pH 7.4
Goat-anti-rabbit Ab dilution ratio（for turn off pattern LFICAs）		80		120

Table S2. Comparison of the developed assays with a commercial ELISA test kit for the analysis of animal derived food samples spiked with ENR

Samples	Spiked conc. (μg kg^{-1})	ELISA Average ±SD (n=3) (μg kg^{-1})	C-FLFIA (n=3)	Q-FLFIA (n=3)
Crucian	0	ND[a]	-, -, -[b]	-, -, -[b]
	0.5	ND	+, +, +[c]	-, -, -
	1.25	1.35±0.09	+, +, +	+, +, +
	2.5	2.36±0.04	+, +, +	+, +, +
	5	5.03±0.19	+, +, +	+, +, +
Perch	0	ND	-, -, -	-, -, -
	0.5	ND	+, +, ±[d]	-, -, -
	1.25	1.37±0.13	+, +, +	+, +, +
	2.5	3.28±0.28	+, +, +	+, +, +
	5	5.40±0.31	+, +, +	+, +, +
Pork	0	ND	-, -, -	-, -, -
	0.5	ND	+, +, +	-, -, -
	1.25	1.44±0.11	+, +, +	+, +, +
	2.5	1.73±0.18	+, +, +	+, +, +
	5	4.15±0.07	+, +, +	+, +, +
Beef	0	ND	-, -, -	-, -, -
	0.5	ND	+, +, +	-, -, -
	1.25	1.33±0.05	+, +, +	+, +, +
	2.5	2.42±0.31	+, +, +	+, +, +
	5	4.45±0.95	+, +, +	+, +, +
Chicken	0	ND	-, -, -	-, -, -
	0.5	ND	+, ±, +	-, -, -
	1.25	1.46±0.14	+, +, +	+, +, +
	2.5	2.48±0.29	+, +, +	+, +, +
	5	4.35±0.26	+, +, +	+, +, +

Milk	0	ND	-, -, -	-, -, -
	1	1.13±0.21	+, +, +	-, -, -
	2.5	2.79±0.38	+, +, +	+, +, +
	5	4.36±0.60	+, +, +	+, +, +
	10	10.38±1.31	+, +, +	+, +, +

[a]ND: not detected. The concentration is lower than the LOD of the ELISA.

[b]-: Negative visual result.

[c]+: Positive visual result.

[d]±: Weakly positive result.