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Synthesis of coating antigens

An immunogen was prepared by conjugating DAN with BSA (Tochi et al., 2015). Briefly, a mixture consisting of 8.9 mg of DAN, 15.1 mg of EDC, and 11.1 mg of NHS was dissolved in 1mL of DMF solution and stirred for 8 h at room temperature. The mixture was added dropwise into 3 mL of 0.01 M CB solution with BSA (5.56 mg/mL). After stirring overnight, the reactants were dialyzed for three days in 0.01 M PBS to obtain the immunogen (DAN-BSA).

Traditional ELISA method for detecting DAN

First, 100 μL of the diluted solution was added into ELISA wells and incubated at 37 °C for 2 h. The ELISA wells were washed thrice with PBST (0.01 M PBS containing 0.05% Tween 20, v/v). The ELISA wells were washed with PBST (0.01 M PBS containing 0.05% Tween 20, v/v) three times. The ELISA wells were blocked with 260 μL of blocking buffer (0.01 M CBS containing 0.2% gelatin) and then incubated at 4 °C overnight. After the ELISA wells were washed with PBST three times, 50 μL of standard solution and 50 μL of mAb were sequentially added into each well. After incubation at 37 °C for 30 min, the ELISA wells were washed with PBST four times. HRP-conjugated goat anti-mouse secondary antibody at 100 μL was added and incubation was continued for 30 min. the ELISA wells were washed with PBST four times, and then read at 450 nm by microplate reader.

A standard curve of OD_{450} values was generated for DAN concentrations (0, 0.5, 1, 2.5, 5, 10, 25, 50, and 100 ng/mL) in a traditional ELISA. Fig. S1 shows a good linear, $Y = -0.986 \lg X + 2.299$ ($R^2 = 0.993$). The LOD was1.767 ng/mL.

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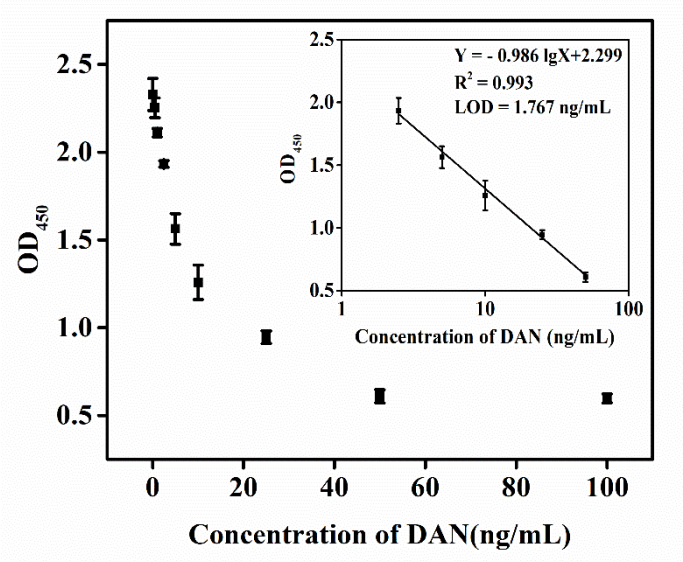

Supplemental Figure S1. Calibration curve for traditional ELISA. The inset shows that with PBS as a negative control, the DAN concentration ranges from 0 ng/mL to 100 ng/mL. As DAN concentration increases, the OD_{450} decreases linearly with a linear range of 2.5 ng/mL to 50 ng/mL. Error bars represent the standard deviation of the three measurements.

Linear relationship and LOD based on colorimetric signal in the proposed ELISA

The standard curves of proposed ELISA based on colorimetric signal in PBS were established (Fig. S2). The calibration curve was constructed by plotting the OD_{487} versus the logarithm of DAN concentration (0-100 ng/mL). The linear equation of the proposed method for quantitative detection of DAN can be described as Y = -0.603 lgX + 0.786 (R^2 = 0.993) with a good linear detection range from 1 ng/mL to 10 ng/mL. The LOD was 0.684 ng/mL.

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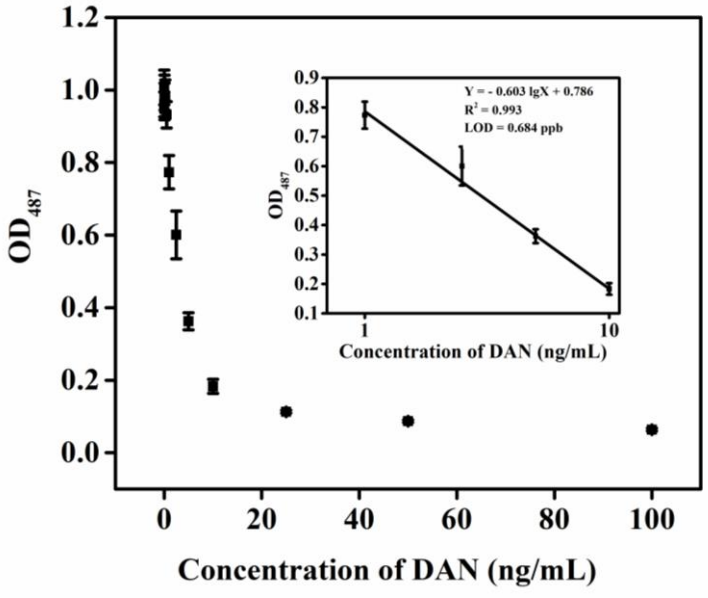

Supplemental Figure S2. Calibration curve of proposed colorimetric ELISA. The DAN concentration ranges from 0 ng/mL to 100 ng/mL. The inset shows a linear range of 1 ng/mL to 10 ng/mL. Error bars = SD.

The cut-off value of commercialized ELISA kit for DAN by naked-eye detection.

The negative sample of milk confirmed by LC-MS/MS were spiked with DAN at 10, 20, 30, 40, 50, and 60 ng/mL for qualitative analysis. Fig.S3. showed that the cut-off value by naked-eye was 50 ng/mL because the color changed to colorless at 50 ng/mL.

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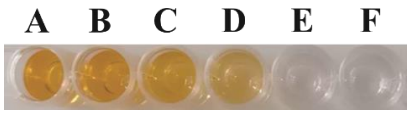

Supplemental Figure S3. The commercialized ELISA kit in milk at different DAN spiked concentration. (A) 10 ng/mL, (B) 20 ng/mL, (C) 30 ng/mL, (D) 40 ng/mL, (E) 50 ng/mL, and (F) 60 ng/mL.

LOD of the commercialized ELISA kit by adopting OPD as enzyme substrate

We have compared the LOD of the commercialized ELISA kit by adopting OPD as enzyme substrate with proposed ELISA. The LOD was 1.15 ng/mL, it's indicating that LOD of the proposed fluorescence immunoassay was 3.41-fold lower than that of commercialized ELISA kit by adopting OPD as enzyme substrate.

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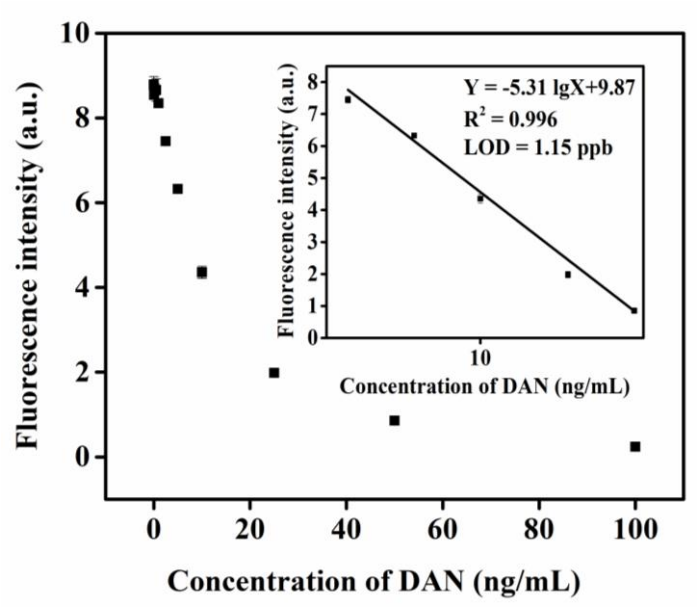

Supplemental Figure S4. Calibration curve for commercialized ELISA kit by adopting OPD as enzyme substrate. The inset shows that with PBS as a negative control, the DAN concentration ranges from 0 ng/mL to 100 ng/mL. As DAN concentration increases, the fluorescence intensity decreases linearly with a linear range of 2.5 ng/mL to 50 ng/mL. Error bars = SD.

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Supplemental Table S1. Determination of fluorescence intensity in checkerboard titration

Concentration of Bio-mAb (μg/mL)	Concentration of DAN-BSA (μg/mL)						
	2	**1.5**	**1**	**0.75**	**0.50**	**0.25**	**0.1**
2	4.887	4.847	4.931	5.012	3.274	1.514	0.8422
1.5	4.985	5.027	5.279	5.074	3.486	1.273	0.8865
1	5.239	5.267	5.341	4.782	3.227	1.544	0.8523
0.75	4.246	5.321	5.527[a]	4.648	2.835	1.113	0.5684
0.50	3.452	4.587	2.931	3.174	1.915	0.948	0.3415
0.25	1.998	2.999	1.473	1.273	1.037	0.732	0.2513
0.10	1.289	1.389	1.345	1.127	0.687	0.465	0.0927

Note: “a” indicates the optimal concentrations of DAN–BSA and Bio–mAb for the following ELISA.

Supplemental Table S2. LOD of the proposed method and others.

Methods	LOD	Sample	Ref
Fluorescence detection	0.9 ng/mL	milk	(Kuldeep et al., 2012)
Microbiological assay	63 ng/mL	milk	(Tumini et al., 2017)
Immunoaffinity column detection	4.56 ng/mL	milk	(Niu et al., 2019)
MALDI-TOF MS	10 ng/mL	water	(Tang et al., 2019)
Fluorescence ELISA	0.337 ng/mL	milk	This work

Supplemental Table S3. Detection of ENR in real milk samples using the proposed

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ELISA and HPLC-MS/MS.

Sample	The proposed ELISA	HPLC-MS/MS	Sample	The proposed ELISA	HPLC-MS/MS
1	N.D. [a]	N.D.	11	N.D.	N.D.
2	N.D.	N.D.	12	N.D.	N.D.
3	N.D.	N.D.	13	N.D.	N.D.
4	N.D.	N.D.	14	N.D.	N.D.
5	N.D.	N.D.	15	N.D.	N.D.
6	0.47ng/mL	0.52ng/mL	16	N.D.	N.D.
7	N.D.	N.D.	17	N.D.	N.D.
8	N.D.	N.D.	18	N.D.	N.D.
9	N.D.	N.D.	19	N.D.	N.D.
10	N.D.	N.D.	20	N.D.	N.D.

[a] Not Detected

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