Supplementary Material for

AviTag-nanobody based enzyme immunoassays for sensitive determination of aflatoxin B_1 in cereal

Ting He[a,b,1,*], Tingting Yan[a,c,1], Jiang Zhu[a,c], Ying Li[a,c], Xin Zhou[a,c,d], Yunhuang Yang[a,c,d,**], Maili Liu[a,c,d]

[a] State Key Laboratory of Magnetic Resonance and Atomic Molecular Physics, Key Laboratory of Magnetic Resonance in Biological Systems, National Center for Magnetic Resonance in Wuhan, Wuhan National Laboratory for Optoelectronics, Wuhan Institute of Physics and Mathematics, Innovation Academy for Precision Measurement Science and Technology, Chinese Academy of Sciences, Wuhan 430071, China

[b] Wuhan Academy of Agricultural Sciences, Wuhan 430072, China

[c] University of Chinese Academy of Sciences, Beijing 100049, China

[d] Optics Valley Laboratory, Hubei 430074, China

* Corresponding author. Innovation Academy for Precision Measurement Science and Technology, Chinese Academy of Sciences, West No. 30 Xiao Hong Shan, Wuhan 430071, China.

** Corresponding author. Innovation Academy for Precision Measurement Science and Technology, Chinese Academy of Sciences, West No. 30 Xiao Hong Shan, Wuhan 430071, China.

Email addresses: ht210@apm.ac.cn (T. He), yang_yh@apm.ac.cn (Y. Yang).

[1] These authors contributed equally to this work.

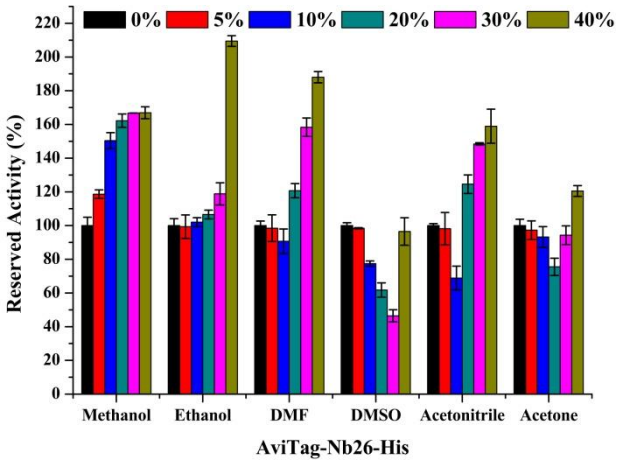


Fig. S1. Identification of organic tolerance. PBS buffers containing each organic solvent at different concentrations (0%, 10%, 20%, 40%, 60%, and 80%) were equally mixed with the biotinylated AviTag-Nb26-His, and then 100 μL of the mixture was added into wells. The bound of biotinylated AviTag-Nb26-His fusion protein was detected by 100 μL SA-HRP (1/10000 dilution in PBST). The error bar represents the standard deviation of triplicate tests.

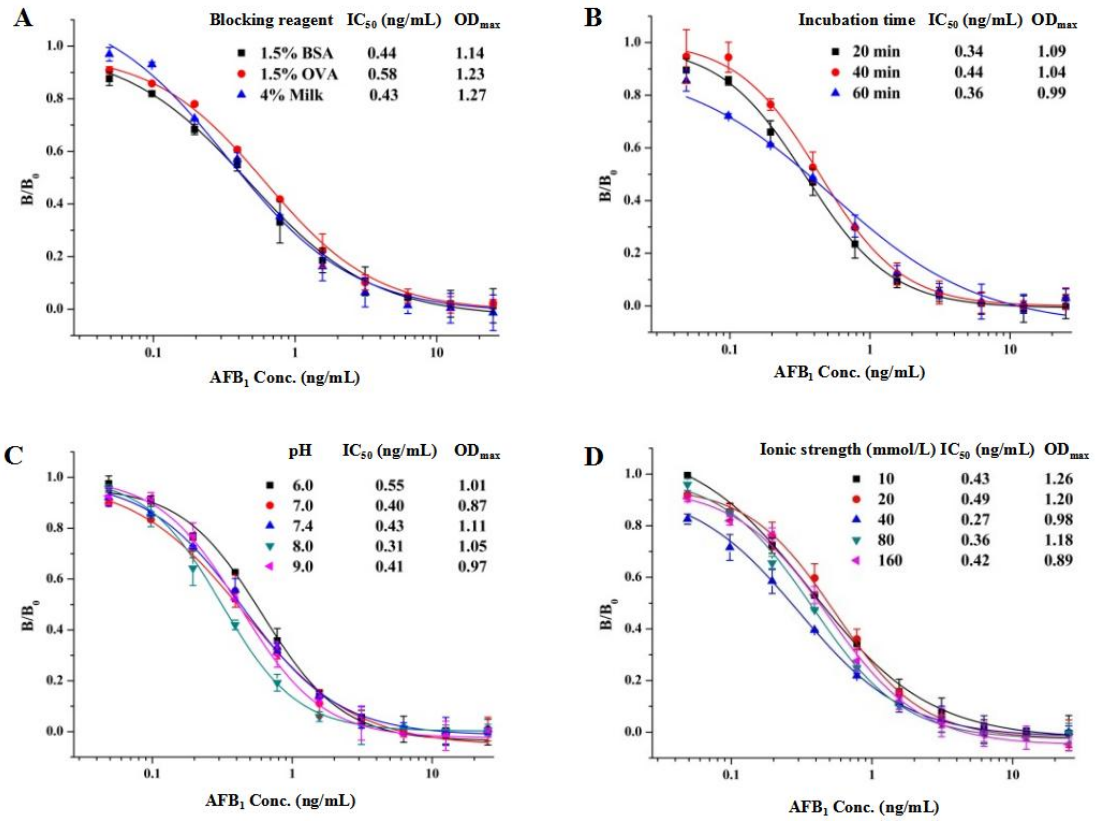


Fig. S2. Influence of different parameters on the performance of BA-ELISA. (A) Blocking reagent. (B) Incubation time of SA-HRP. (C) pH of buffer. (D) Ionic strength in PBS buffer. Each point represents the mean of three replicates.

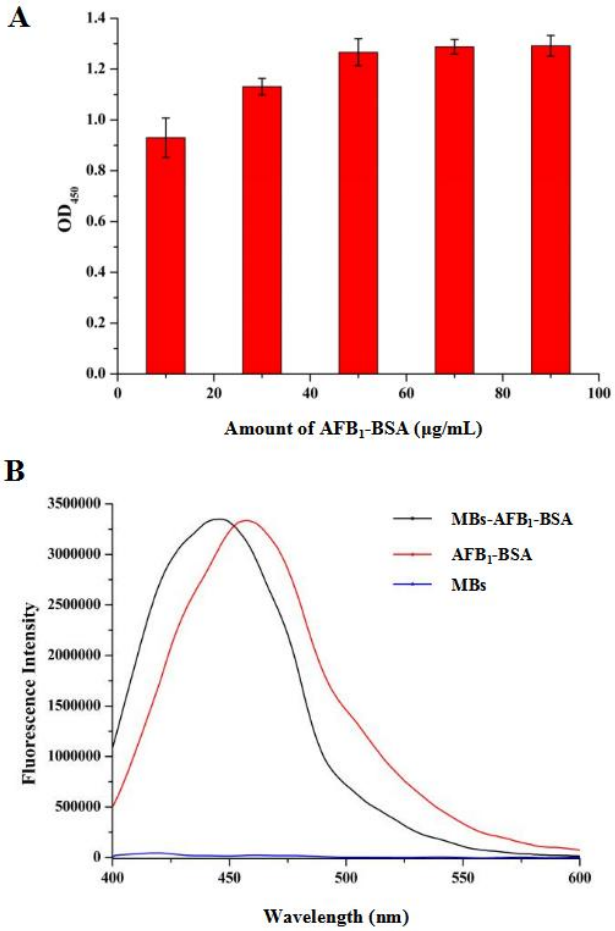


Fig. S3. Optimization and characteristic of AFB_1-BSA conjugated MBs. (A) Optimization of the amount of AFB_1-BSA coupling to MBs. (B) Fluorescence emission spectra (λex = 365 nm) of MBs, AFB_1-BSA, and MBs-AFB_1-BSA. The solution (100 μL) was measured at 365nm excitation wavelength. MBs: 5 mg/mL; AFB_1-BSA: 20 μg/mL; MBs-AFB_1-BSA: 15 mg/mL.

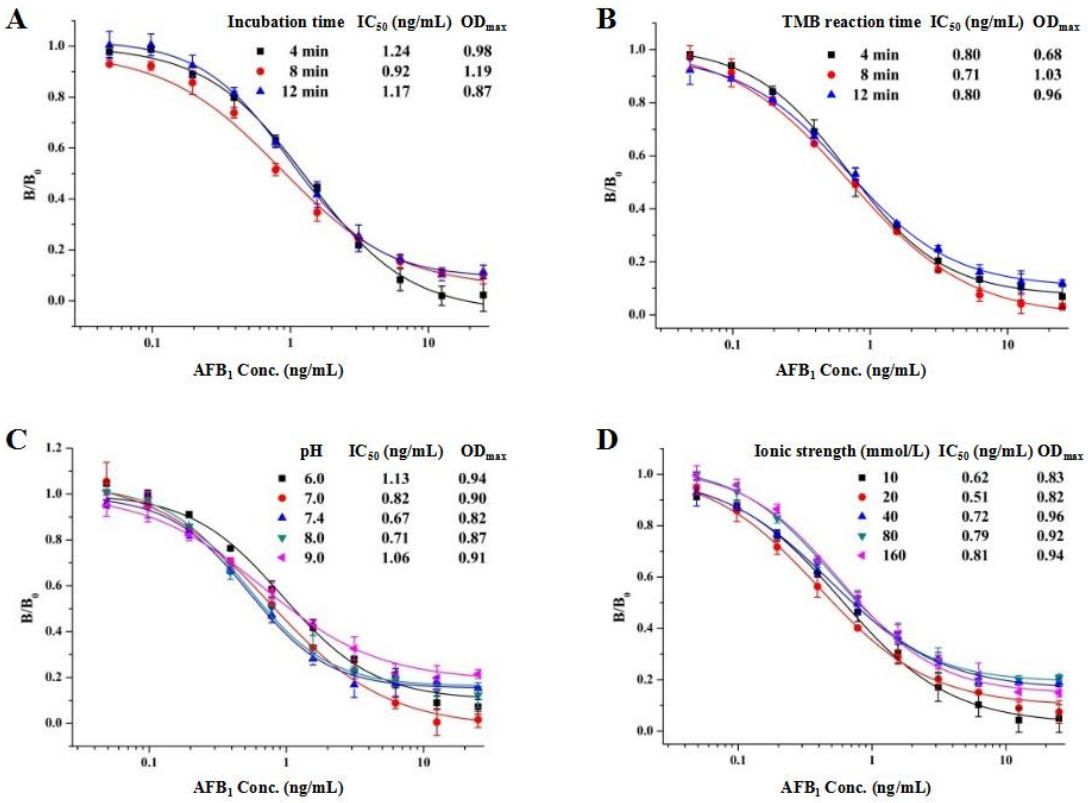


Fig. S4. Influence of different parameters on the performance of MB-ELISA. (A) Incubation time of SA-HRP. (B) TMB reaction time. (C) pH of buffer. (D) Ionic strength in PBS buffer. Each point represents the mean of three replicates.

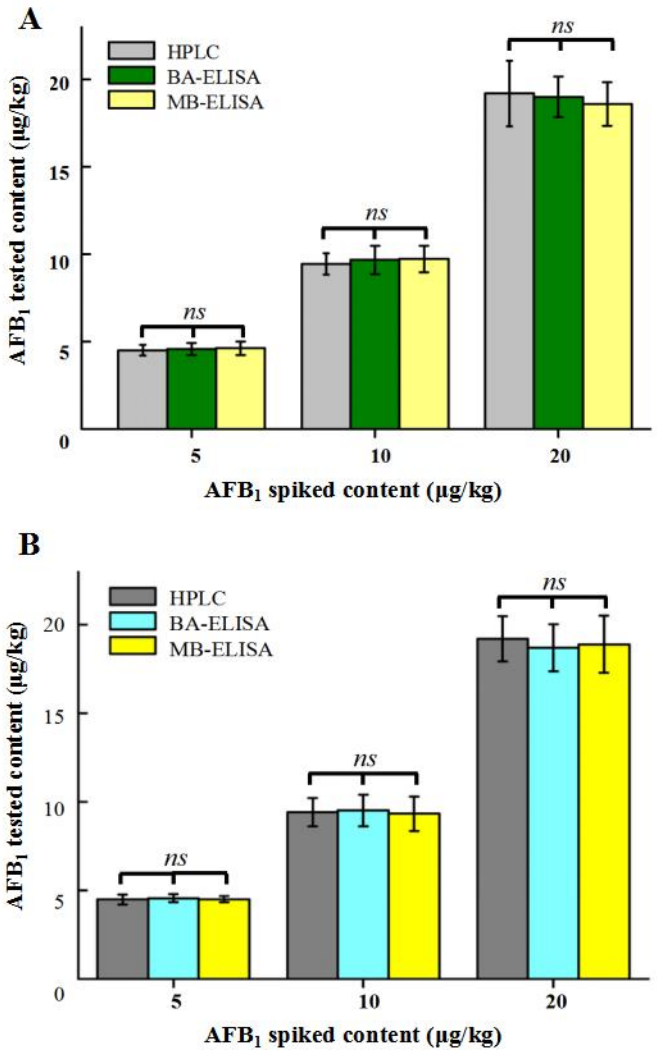


Fig. S5. Comparison of AFB_1 content in spiked wheat (A) and corn (B) by BA-ELISA, MB-ELISA and HPLC methods. Note: *ns* means no significant difference by ANOVA, $P > 0.05$.

Table S1. Primers used for AviTag-Nb fusions and BirA genes amplification.

Proteins	Primers	Sequences (5'-3')
AviTag-Nb26-His	AF-1	TATG**GGCCTGAACGACATCTTCGAGGCCCAGAAGATCGAGTGGCACGAG**G (*Nde* I)
	AR-1	GATCC**CTCGTGCCACTCGATCTTCTGGGCCTCGAAGATGTCGTTCAGGCC**CA (*Bam*H I)
	NF-1	CGGGATCCGGCGGAGGCGGAAGTCAGTTGCAGCTCGTGGAG (*Bam*H I)
	NR-1	TCCTCTCGAGTGAGGAGACGGTGACCTGG (*Xho* I)
Nb26-AviTag-His	NF-2	GATATACATATGCAGTTGCAGCTCGTGGAG (*Nde* I)
	NR-2	CGGGATCCGCCTCCGCCTGAGGAGACGGTGACCTG (*Bam*H I)
	AF-2	GATCC**GGCCTGAACGACATCTTCGAGGCCCAGAAGATCGAGTGGCACGAG**C (*Bam*H I)
	AR-2	TCGAG**CTCGTGCCACTCGATCTTCTGGGCCTCGAAGATGTCGTTCAGGCC**G (*Xho* I)
Nb26-His-AviTag	NF-2	GATATACATATGCAGTTGCAGCTCGTGGAG (*Nde* I)
	NR-3	CGGGATCCGCCTCCGCCGTGGTGGTGGTGGTGGTG (*Bam*H I)
	AF-3	GATC**GGCCTGAACGACATCTTCGAGGCCCAGAAGATCGAGTGGCACGAGTGA**C (*Bam*H I)
	AR-3	TCGAG**TCACTCGTGCCACTCGATCTTCTGGGCCTCGAAGATGTCGTTCAGGCC**G (*Xho* I)
BirA	BF	GATATACCATGGTGAAGGATAACACCGTGCCACTG (*Nco* I)
	BR	CGGTCGACTCAAAGCTTACCTTTTTCTGCACTACG (*Sal* I)

Note: The restriction sites are underlined. The AviTag gene coding sequences are in bold.

Table S2. Optimization of concentrations of biotinylated AviTag-Nb26-His and AFB_1-BSA for BA-ELISA.

Biotinylated AviTag-Nb26-His concentration (ng/mL)	AFB_1-BSA concentration (μg/mL)					
	4	2	1	0.5	0.25	0.125
2000	2.73	2.37	2.40	2.38	1.78	1.24
666.67	2.24	2.24	2.32	1.98	0.90	0.53
222.22	1.94	2.27	2.16	1.62	0.33	0.22
74.07	1.74	2.00	1.43	0.71	0.11	0.13
24.69	1.14	1.07	0.62	0.30	0.10	0.09
8.23	0.31	0.44	0.29	0.15	0.10	0.09
2.74	0.13	0.12	0.10	0.13	0.11	0.07
Blank	0.10	0.14	0.12	0.09	0.10	0.09

Table S3. Optimization of concentrations of biotinylated AviTag-Nb26-His and MBs-AFB_1-BSA for MB-ELISA.

Biotinylated AviTag-Nb26-His concentration (ng/mL)	Amount of MBs-AFB_1-BSA (μg per well)				
	25	20	15	10	5
2000	2.48	2.19	1.65	1.52	1.20
666.67	1.98	1.92	1.24	1.06	0.93
222.22	1.43	1.06	0.95	0.76	0.62
74.07	1.00	0.65	0.69	0.45	0.35
24.69	0.51	0.34	0.44	0.29	0.19
8.23	0.23	0.24	0.19	0.16	0.09
Blank	0.12	0.11	0.09	0.09	0.09

Table S4. Cross-reactivity of BA-ELISA and MB-ELISA with AFB_1 structural analogs.

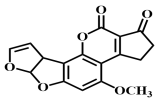
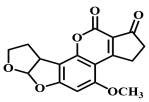
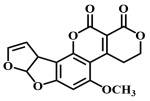
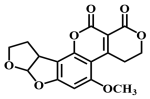
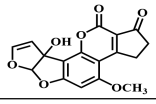
Analytes	Structure	BA-ELISA		MB-ELISA	
		IC_{50} (ng/mL)	CR (%)	IC_{50} (ng/mL)	CR (%)
AFB_1		0.28	100	0.54	100
AFB_2		12.8	2.19	21.4	2.52
AFG_1		12.0	2.33	15.1	3.58
AFG_2		33.4	0.84	85.1	0.63
AFM_1		5.86	4.78	15.9	3.40

Table S5. Concentration of AFB_1 in real wheat and corn samples by BA-ELISA, MB-ELISA and HPLC methods.

Sample	HPLC found AFB_1 (μg/kg)	BA-ELISA		MB-ELISA	
		average found (μg/kg)	RSD (%), n=6	average found (μg/kg)	RSD (%), n=6
wheat-1	3.48	3.27	7.15	3.12	6.22
wheat-2	7.05	6.65	7.30	6.52	8.19
corn-1	35.6	31.8	9.17	32.5	9.13
corn-2	76.5	69.9	8.25	66.8	10.3

Table S6. Comparison of Nb- or Nb-fusion-based enzyme immunoassays for AFB_1.

Nb/Nb-fusion	Method	IC_{50} (ng/mL)	LOD (ng/mL)	Linear range (ng/mL)	Assay time	Ref.
Nb	ELISA	0.754	-[a]	0.117−5.676	120 min	(He et al., 2014)
Nb	MB-dcELISA	0.75	0.13	0.24−2.21	35 min	(Zhao et al., 2019)
Nb-alkaline phosphatase	One-step ELISA	19.8	2.6	4.3−92	70 min	(Cao et al., 2016)
Nb-nanoluciferase	BLEIA	0.41	0.05	0.10−1.64	120 min	(Ren et al., 2019)
AviTag-Nb	BA-ELISA	0.28	0.07	0.12−0.95	50 min	This work
	MB-ELISA	0.53	0.12	0.21−1.37	26 min	This work

[a] Data not provided.

References

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