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1 **Nanobody-based immunomagnetic separation platform for**
2 **rapid isolation and detection of *Salmonella Enteritidis* in**
3 **food samples**

4
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14 **Abstract**

15 Rapid separation and identification of *Salmonella enteritidis* (*S. enteritidis*) in
16 food is of great importance to prevent outbreaks of foodborne diseases. Herein, by using
17 O and H antigens as targets, an epitope-based bio-panning strategy was applied to
18 isolate specific nanobodies towards *S. enteritidis*. This method constitutes an efficient
19 way to obtain specific antibody fragments and test pairwise nanobodies. On this basis,
20 a sandwich enzyme-linked immunosorbent assay (ELISA) coupled with
21 immunomagnetic separation (IMS) was developed to rapid enrich and detect *S.*
22 *enteritidis* in food. The detection limit of the IMS-ELISA was 2.4×10^3 CFU/mL,
23 which was 3 times more sensitive compared with sandwich ELISA without IMS, and
24 the incubation time was shortened by 2 h after the enrichment by IMBs. The IMS-

25 ELISA strategy which could avoid matrix interference and shorten the enrichment
26 culture time, has great potential for application in monitoring bacterial food
27 contamination.

28 Keywords: Single domain antibody; *Salmonella*; ELISA; Immunomagnetic separation;
29 Rapid detection

30

31 **1. Introduction**

32 As a ubiquitous foodborne pathogen, *Salmonella* is widely distributed in various
33 food, including meat, eggs, milk, and vegetables (Ilhan, Tayyarcın, Caglayan, Boyacı,
34 Saglam, & Tamer, 2021; Liu, Yan, Mao, Wang, & Deng, 2016; Sezer, Tayyarcın, &
35 Boyacı, 2022). Every year, large numbers of food poisoning incidents caused by
36 *Salmonella* have occurred worldwide, causing a severe threat to public health (Forshell
37 & Wierup, 2006; Kirk et al., 2015). Thus, it is necessary to establish a sensitive and
38 reliable detection method for *Salmonella* to prevent its contamination and spread.
39 Immunoassays have attracted widespread attention in *Salmonella* monitoring, owing to
40 their inherent advantages of high specificity, excellent sensitivity, ease of operation and
41 rapid readout (Liang et al., 2022).

42 One of the main bottlenecks in current immunoassays for bacterium detection is
43 the quantification at very low concentration level in complex food matrix.
44 Immunomagnetic separation (Skjerve & Olsvik, 1991; Srisa-Art, Boehle, Geiss, &
45 Henry, 2018; Wang, Cai, Gao, Yuan, & Yue, 2020) (IMS) is an important technique
46 which can effectively avoid the interference of food substrate, reduce enrichment period,
47 and improve assay's sensitivity. IMS has been developed and employed in combination
48 with several techniques, such as enzyme-linked immunosorbent assay (ELISA) (Cudjoe,
49 Hagtvedt, & Dainty, 1995; Mansfield & Forsythe, 2001; Wang, Yue, Yuan, Cai, Niu,
50 & Guo, 2013), lateral flow immunoassay (LFIA) (Cui, Xiong, Xiong, Shan, & Lai,
51 2013; Jiang et al., 2020), and polymerase chain reaction (PCR) (Chen, Li, Tao, Bie, Lu,
52 & Lu, 2017; Hyeon & Deng, 2017; Jeníková, Pazlarová, & Demnerová, 2000), etc.
53 Awarded as the most excellent approach for isolating target bacteria, IMS technology
54 was widely applied in detecting pathogenic bacteria (Kuang et al., 2013; Tatavarthy et
55 al., 2009; Zeng et al., 2014).

56 Based on antibody-antigen specific recognition, both IMS and immunoassay have
57 an exacting requirement on the affinity and specificity of the antibody. Nevertheless,
58 owing to the unstable quality of polyclonal antibody (pAb) and significant batch-to-

59 batch variation of monoclonal antibodies (Bruce & McNaughton, 2017) (mAb), there
60 is a strong demand for superior alternatives with high specificity, ease of clone storage
61 and production and batch-to-batch consistency. Recently, a miniaturized antibody
62 named nanobody (Nb) was derived from heavy-chain antibodies in camelids or
63 cartilaginous fish serum (Greenberg, Avila, Hughes, Hughes, McKinney, & Flajnik,
64 1995; Hamers-Casterman et al., 1993). Owing to its superiority in solubility, thermal
65 stability, expression, and production (Y. Wang, P. Li, et al., 2013; Y. Wang, H. Wang,
66 et al., 2013), Nb has become a promising alternative to the conventional antibody in
67 detection of pathogenic bacteria (He et al., 2020; Ren et al., 2022; Zhang et al., 2022).
68 Furthermore, without the fragment crystallizable (Fc) region, Nb-based immunoassays
69 could avoid the non-specific interaction with *Staphylococcus aureus*, resulting in better
70 selectivity in comparison with immunoassays based on conventional antibody (Ji et al.,
71 2020).

72 Specific Nbs have been developed towards foodborne pathogens including
73 *Salmonella* (He et al., 2020; Ren et al., 2022; Zhang et al., 2022), *Listeria*
74 *monocytogenes* (Tu et al., 2016), and *Staphylococcus aureus* (Hu et al., 2021). In the
75 bio-panning of specific nanobody, inactivated whole bacteria are commonly used as
76 immobilized antigen. In this situation, the recognition sites of obtained nanobodies are
77 unclear, thus, pairwise selection of Nbs is mainly based on trial-and-error. This problem
78 can be overcome by using epitope-based bio-panning strategy, in which a particular
79 antigen epitope is used as immobilized target. This strategy is beneficial for defining
80 nanobodies' recognition sites, improving the specificity, and fast realizing the pairwise
81 selection.

82 In our previous study, a polyclonal/nanobody sandwich ELISA for *S. enteritidis*
83 detection was developed, using a commercial polyclonal antibody as capture antibody
84 and a VHH as detection antibody selected from a camelid immune nanobody library.
85 In the present work, by using the same library, specific nanobodies that recognize the
86 two main antigenic epitopes on the surface of *S. enteritidis*, lipopolysaccharide (LPS,

87 O antigen) and flagellin (H antigen), respectively, were obtained by performing an
88 epitope-based bio-panning strategy, which allowed the fast and rational selection of
89 pairwise Nbs. In addition, IMS was developed by coupling Nb F18 with magnetic
90 nanoparticles and used as a tool for bacteria isolation and amplification. The protocols
91 for the nanobody-based IMS-ELISA was established under the optimized conditions.
92 As illustrated in Scheme 1, target bacteria from food sample were isolated by the
93 specific nanobody-based immunomagnetic beads. Then, the enriched *S. enteritidis* was
94 eluted and detected by double-nanobody sandwich ELISA. The proposed IMS-ELISA
95 was successfully applied in real sample analysis and compared with the previous
96 polyclonal/Nb ELISA in terms of sensitivity and detection period.

97 **Materials and Methods**

98 **2.1. Materials and reagents**

99 Helper phage M13KO7 was purchased from Renyu Biotechnology (Chengdu,
100 China). HRP-Anti-M13 phage monoclonal antibody (M13-HRP) was acquired from
101 Sino Biological Inc. (Beijing, China). Thermo Fisher Scientific Inc. (Carlsbad, USA)
102 provided the 96-well microplates, B-PER™ Bacterial Protein Extraction Reagent and
103 HisPur™ Ni-NTA Resin. Carboxyl magnetic beads (0.5 μm) was obtained from Sangon
104 Biotech (Shanghai, China) while the magnetic separator stand 2/15 was obtained from
105 Beaver (Suzhou, China). N-Hydroxy succinimide (NHS), 1-ethyl-3(3-
106 dimethylaminopropyl) carbodiimide (EDC) and 2-(4-Morpholino) ethanesulfonic acid
107 (MES) were bought from Sigma (St. Louis, MO). All the other organic reagents used
108 were of analytical grade.

109 All the bacterial strains were preserved in our laboratory. The species of bacteria
110 used in this work are as follows: *Salmonella enteritidis* (*S. enteritidis*), *Salmonella*
111 *typhimurium* (*S. typhimurium*), *Salmonella paratyphi B* (*S. paratyphiB*), *Escherichia*
112 *coli* (*E. coli*) (ATCC25922), *Staphylococcus aureus* (*S. aureus*) (ATCC29213), *Listeria*
113 *monocytogenes* (*L. monocytogenes*) (CMCC54004), *Candida albicans* (*C. albicans*)

114 (ATCC10231), *Campylobacter coli* (*C. coli*) (ATCC29428), *Enterobacter sakazakii* (*E.*
115 *sakazakii*) (ATCC29544) and *Shigella flexneri* (*S. flexneri*) (CICC 21534).

116 **2.2. Epitope-based bio-panning**

117 A phage displayed-nanobody library has been constructed and stored in our
118 laboratory (He et al., 2020). The epitope-based bio-panning procedures were proceeded
119 with O and H antigens of *S. enteritidis*, respectively. For the first round of bio-panning,
120 100 μ L of O (100 μ g/mL) and H antigen (500 μ g/mL) were coated in the microtiter
121 plate overnight at 4°C, respectively. After blocking the remaining protein binding sites
122 with 3% nonfat milk powder, 100 μ L of the constructed phage-displayed nanobody
123 library was added to each well and incubated at 37°C for 1 h. Afterwards, unbound
124 phages were removed through washings for 10 times. The bound phages were eluted
125 with 100 μ L of 0.1 M Glycine-HCl (pH 2.2) after incubation for 15 min at 37°C. The
126 eluate was immediately neutralized with 4 μ L of 1 M Tris base (pH 9), and the eluted
127 phage were amplified for the next round of panning. In the bio-panning procedures, the
128 number of input phages remained the same, whereas concentrations of coated flagella
129 and lipopolysaccharide gradually decreased. After four rounds of panning, 25 clones
130 were selected from the eluted phages titer plate of O and H antigen, respectively. The
131 phages were amplified, and the supernatant was characterized by phage ELISA.
132 Subsequently, the positive colonies were sent for sequencing.

133 **2.3. Expression and purification of nanobody**

134 The phagemid DNA of five positive clones was transformed into *E. coli* Top10F'
135 competent cells by heat shock. A single colony carrying nanobody expression plasmid
136 was cultivated in 100 mL of SB-ampicillin medium (50 μ g/mL) at 37°C with shaking
137 at 250 rpm. When OD₆₀₀ value reached 0.6–0.8, IPTG was added with the final
138 concentration of 0.2 mM. Subsequently, bacteria cells were further incubated for 10 h
139 at 28 °C under shaking. The cells were pelleted by centrifugation and the proteins were
140 extracted by B-PER reagent. Then the extract was purified by the Ni-NTA Superflow

141 Agarose column. The soluble nanobody with 6 × His tag was eluted by 200 mM
142 imidazole buffer and dialyzed in 0.01 M PBS for 2-3 days. The size and purity of Nb
143 were verified by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-
144 PAGE) and the concentration of the Nb was detected by Nanodrop 2000. Eventually,
145 the specificity and thermal stability of five nanobodies were determined by indirect
146 ELISA.

147 **2.4. Preparation of immunomagnetic beads (IMBs)**

148 Briefly, 0.5 mg Carboxylic MBs were added in 2 mL centrifuge tubes and washed
149 with MES buffer for three times. After magnetic separation, 200 µL of freshly prepared
150 EDC (5 mg/mL) and NHS (5 mg/mL) were added to IMBs and shaken at 37°C for 30
151 min to activate the beads. Subsequently, the activated IMBs were mixed with various
152 amounts of nanobody (25, 50, 100, 150, and 200 µg) at 37°C for 2 h. To block the
153 unconjugated sites of IMBs, the complexes were incubated in PBST containing 1%
154 bovine serum albumin for 30 min at 37°C with shaking. After washing with PBST for
155 three times, the IMBs were resuspended in 1 mL PBS buffer and stored as 4°C. The
156 coupling rate of nanobody was calculated by the following equation: Coupling rate (%)
157 = $(1 - C_2/C_1) \times 100$ (%), where C_1 and C_2 are the concentration of Nb before and after
158 coupling, respectively. The experiment was repeated three times to obtain the mean and
159 the standard deviation of the measured values.

160 **2.5. Immunomagnetic separation (IMS) procedure**

161 The medium of *S. enteritidis* was centrifuged and resuspended in PBS buffer.
162 Conjugated IMBs were incubated with 1 mL of *S. enteritidis* at different concentration.
163 After the antigen-nanobody binding reaction was finished, the IMBs- bacteria
164 complexes were separated by the magnetic separator. Afterwards, the supernatant and
165 IMBs-bacteria complexes were diluted to an appropriate concentration, and cultured on
166 LB agar plates at 37°C for 12 h. Each concentration was coated with three parallel
167 plates. To obtain the best performance, IMBs dose, concentration of *S. enteritidis*,

168 incubation time and IMS time were optimized. The capture efficiency (CE) of the IMBs
169 was calculated by the equation: $CE (\%) = (1 - N_2/N_1) \times 100\%$, where N_1 and N_2 are the
170 Colony Forming Units of *S. enteritidis* cells in the control and supernatant, respectively
171 (Wu, Tu, Huang, He, Fu, & Li, 2019).

172 To evaluate the specificity of IMBs, nine types of foodborne pathogens including
173 *S. typhimurium*, *S. paratyphiB*, *E. coli*, *S. aureus*, *L. monocytogenes*, *C. albicans*, *C.*
174 *coli*, *E. sakazalii*, and *S. flexneri* were enriched by immunomagnetic separation.
175 Meanwhile, *S. enteritidis* positive control and blank control was set up. The IMS
176 procedure and capture efficiency were determined as the steps above.

177 **2.6. Nanobody-based sandwich ELISA for the detection of *S. enteritidis***

178 Nanobody and phage displayed nanobody were applied as capture antibody and
179 detection antibody, respectively, to elect the best matched pair of the sandwich ELISA.
180 In brief, 100 μ L per well of Nbs were coated overnight at 4°C in a microplate plate and
181 then blocked with 300 μ L of 3% skimmed milk powder in PBS at 37°C for 1 h. After
182 three times washing with PBST, 100 μ L of *S. enteritidis* was added to the wells and
183 incubated for 1 h. The plate was washed three times, after which 100 μ L per well of
184 phage displayed Nb was added and incubated for 1 h at 37 °C. Then, 100 μ L of HRP-
185 conjugated anti-M13 antibodies was added to each well. After 1 h of incubation and
186 six-times washing, peroxidase substrate TMB solution was added and incubated for 15
187 min. The reaction was terminated by 50 μ L of 2 M H₂SO₄ solution, and the optical
188 density (OD) was measured at 450 nm by a microplate reader.

189 **2.7. Detection of *S. enteritidis* by IMS-ELISA**

190 To improve the sensitivity, the sandwich ELISA was combined with
191 immunomagnetic separation. Briefly, 1 mL of *S. enteritidis* was incubated with 100 μ g
192 IMBs for 45 min, after which the bacteria were enriched by magnetic separation for 5
193 min. Then, eluted by 100 μ L of 0.2 M phosphate-citrate buffer (pH 2.6) for 15 minutes,
194 the bacteria were released from IMBs-bacteria complexes. The eluent was obtained

195 after magnetic separation and the pH of eluent was neutralized with 1.0 M Tris-HCl
196 (pH 9). Finally, the eluted bacteria were applied to sandwich ELISA.

197 **2.8. Cross-reactivity.**

198 The cross-reactivity of IMS-ELISA was evaluated towards ten types of
199 foodborne pathogens including three Salmonella serotypes, *S. enteritidis*, *S.*
200 *typhimurium*, *S. paratyphi*, and seven non-Salmonella strains, *E. coli*, *S. aureus*, *L.*
201 *monocytogenes*, *C. albicans*, *C. coli*, *E. sakazalii* and *S. flexneri*. After enriched by
202 IMBs, the bacteria were eluted and applied to sandwich ELISA. All the procedures
203 were performed according to the steps above.

204 **2.9. Real sample analysis**

205 To evaluate the applicability of the developed method, various amounts of *S.*
206 *enteritidis* were spiked into food samples to a final concentration of 1×10^7 , 1×10^6
207 and 1×10^5 CFU/mL, respectively. All the food samples, including chicken meat,
208 cabbage, tomato, apple Juice, were bought from a local supermarket in Yangling, China,
209 and confirmed to be free of *S. enteritidis* by the plate counting method. After washing
210 with PBS twice, the samples were applied to the established method to determine the
211 recovery.

212 As for the immunomagnetic enrichment of bacteria, food sample spiked with *S.*
213 *enteritidis* was inoculated in LB liquid medium to ensure the final bacterial
214 concentration reached 1 CFU/mL. The mixture was cultured at 37°C with shaking and
215 1 mL of that was collected after 3, 4, 5, 6 and 7 h, respectively. Then the culture was
216 centrifuged at 8000g for 10 min and resuspended in 1 mL of PBS, which was used for
217 immunomagnetic enrichment. Furthermore, the eluted bacteria were analyzed by the
218 sandwich ELISA. The non-spiked sample was also analyzed and considered as the
219 negative control.

220 **2. Results and discussion**

221 **3.1. Epitope-based bio-panning**

222 In the development of sandwich ELISA, it is required to have two paired
223 antibodies that recognize different epitopes of the antigen. In the traditional bio-panning
224 of nanobodies, whole bacteria are usually used as immobilized antigen. Thus, the
225 binding sites of nanobodies are unknown, and the selection of pairwise antibodies is
226 based on trial-and-error, which is time-consuming. To simplify the pairwise selection
227 procedure, an epitope-based bio-panning strategy was applied in this work. There are
228 two main antigenic epitopes on the surface of *Salmonella*, i.e. lipopolysaccharide (LPS,
229 O antigen) and flagellin (H antigen). Thus, to shorten the pairwise selection steps,
230 specific nanobodies with the binding ability towards *Salmonella* O and H antigens were
231 selected in this work.

232 From Figure 2A and B, the titer of output phage of bio-panning towards O and H
233 antigens was increased after each round, which indicated effective enrichment of
234 specific phage clones. Afterwards, 25 phage clones were selected from titer plate of O
235 and H antigens, respectively, and analyzed by phage ELISA. Among these, 19 phage
236 clones were identified to specifically bind to O antigen, while 18 phage clones bound
237 to H antigen (Fig. 2C, D). The positive clones were sequenced and categorized into
238 five types based on the diversity in the CDR region (Fig. 2E), among which three
239 sequences (Nb-F14, Nb-F18 and Nb-F23) were corresponding to the previous sequence
240 (Nb-316, Nb-413, Nb-422) obtained by whole bacteria-based bio-panning, and two
241 novel sequences were obtained named Nb-F5 and Nb-F6. Moreover, four nanobodies
242 (Nb-F5, Nb-F6, Nb-F14, and Nb-F18) can specifically identify H antigen, whereas Nb-
243 F23 recognizes both H and O antigen.

244 **3.2. Expression and identification of nanobody**

245 The plasmids of five positive clones were transformed into *E. coli* Top10F'. After
246 purification by Ni-affinity chromatography columns, the purity of five nanobodies were
247 analyzed by SDS-PAGE. The sizes of those nanobodies are approximately 15 kDa,

248 which is consistent with the theoretical values (Fig. S1). Afterwards, the specificity and
249 thermal stability of the five nanobodies were determined by indirect ELISA. As shown
250 in Fig. S2, Nb-F23 is a broad-spectrum antibody, which can combine with *Salmonella*
251 *spp.*, while the other nanobodies showed great specificity to *S. enteritidis*.

252 Moreover, we confirmed the thermostability of the five nanobodies via indirect
253 ELISA after incubation at various temperatures (37, 40, 60, 70, 80, and 90°C) for 5 min
254 (Fig. S3). The nanobodies maintained 60% of their binding activity even after
255 incubating at 90°C for 5 min. In contrast, the avidity of anti-*S. enteritidis* monoclonal
256 antibodies faded away as the temperature increased, and nearly no binding activity was
257 observed at 80°C. Based on the above results, the thermostability of the four nanobodies
258 exhibits excellent characteristics and could be applied in the detection of *S. enteritidis*
259 in food.

260 **3.3. Pairwise selection**

261 There are two major advantages of the epitope-based bio-panning method. On one
262 hand, nanobodies that recognize specific antigenic epitopes of bacteria are easily
263 obtained. On the other, it facilitates the pairwise selection for sandwich immunoassay
264 development. In this nanobody-based sandwich ELISA, five Nbs (Nb-F5, Nb-F6, Nb-
265 F14, Nb-F18, and Nb-F23) were respectively matched with their phage-display Nbs to
266 select the best Nb pair. Every possible Nb pair was detected within a checkerboard
267 procedure, leading to 25 combinations. It can be clearly seen in Fig. 2 that Nb-F23
268 (Phage-23), which recognizes O antigen, could form a sandwich format with Nb
269 binding to *Salmonella* H antigen (Nb-F5, Nb-F6, Nb-F14, and Nb-F18). Among them,
270 the Nb-F18/Phage-F23 pair resulted in the highest ratio of positive to negative
271 absorbance (P/N) value. Thus, Nb-F18 was used as capture antibody, while phage
272 displaying nanobody F23 (Phage-F23) as detection antibody.

273 **3.4. Optimization and performance of the IMS**

274 Firstly, the optimal coupling rate between nanobody and magnetic beads was
275 determined. 0.5 mg magnetic beads were prepared by coupling with different antibody
276 amounts. As shown in Fig. 3A, the coupling efficiency increased with the nanobody
277 dose, and reached the maximum values when the amount of nanobody was 100 µg.
278 Given the active groups on the surface of magnetic beads are limited, the coupling
279 efficiency decreased with higher amount of nanobody. Thus, 100 µg nanobody was
280 employed in the synthesis of IMBs. To achieve the best capture efficiency (CE) of IMS,
281 the amount of IMBs used for bacterial separation was optimized, as well. From Fig. 3B,
282 the CE of IMBs increased with the amount of magnetic beads and reached a plateau at
283 0.5 mg. Therefore, 0.5 mg was selected as the optimal amount of IMBs. Furthermore,
284 we determined the optimal incubation and separation time by observing the CE of IMBs.
285 After incubating with bacterial suspension for 45 min, the CE reached the maximum
286 value and tended to be stable (Fig. 3C). Thus, the optimal incubation time was 45 min.
287 As for separation time, the CE of IMBs gradually increased with the increase of
288 separation time, and tended to be stable after 5 min. Hence, 5 min was chosen as the
289 ideal separation time.

290 Based on the optimal experimental conditions above, the CE of IMBs was
291 calculated at the concentration of *S. enteritidis* ranging from 10^2 to 10^7 CFU/mL. As
292 shown in Fig. 3E, the CE of IMBs remained almost 80% for the concentrations from
293 10^2 to 10^5 CFU/mL; however, it decreased with the further increase of *S. enteritidis*
294 concentration. The result indicated that the maximum catch of 0.5 mg IMBs was about
295 10^5 CFU. Afterwards, IMBs were used to capture nine types of foodborne pathogens.
296 The CE for *S. enteritidis* was 91.4%, while those for the other bacteria were below 25%,
297 which indicated that the prepared IMBs had good specificity and could resist
298 disturbances of other pathogens (Fig. 3F).

299 **3.5. Development and optimization of sandwich ELISA**

300 To improve the sensitivity of the sandwich ELISA, the optimal working conditions
301 was confirmed by a checkerboard titration. Initially, the sandwich ELISA had been

302 performed at various concentrations of capture antibody (2.5 to 20 $\mu\text{g/mL}$) and
303 detection antibody (10^{11} to 10^{12} pfu/mL). As illustrated in Fig. 5A and B, the
304 immunoassay achieved the highest sensitivity, with lowest EC_{50} value, when the Nb-
305 F18 and phage-F23 were employed at concentrations of 10 $\mu\text{g/mL}$ and 7.2×10^{10}
306 pfu/mL, respectively.

307 Furthermore, the standard curve of the sandwich ELISA was established to detect
308 *S. enteritidis* under the optimal experimental conditions. Fig. 5C has shown that the
309 calibration curve with a linear range of 3.9×10^4 – 6.3×10^5 CFU/mL, demonstrating
310 that the proposed method could quantitatively detect *S. enteritidis*, while the calibration
311 curve equation was expressed as $y=1.264 \lg(x)-5.56$ ($R^2=0.979$). The limit of detection
312 (LOD) of the sandwich ELISA was determined as 7.3×10^3 CFU/mL, which was
313 calculated as the value of blank samples plus threefold standard deviations(Li et al.,
314 2020).

315 **3.6. Cross-reactivity analysis**

316 To evaluate the specificity of the IMS-ELISA, ten pathogens, including *S.*
317 *enteritidis*, *S. typhimurium*, *S. paratyphi B*, *E. coli*, *S. aureus*, *L. monocytogenes*, *C.*
318 *albicans*, *C. coli*, *E. sakazalii* and *S. flexneri* at the concentration of 1×10^5 CFU/mL
319 were tested. As illustrated in Fig. 5D, this method has excellent selectivity with no
320 cross-reactivity with other pathogens.

321 **3.7. Matrix effect and sample analysis**

322 The practicability of the IMS-ELISA was further demonstrated by spiking various
323 concentrations (1×10^5 , 1×10^6 and 1×10^7 CFU/mL) of *S. enteritidis* in food samples,
324 including chicken meat, cabbage, tomato, and apple Juice. As shown in Table 1, the
325 average recoveries of the spiked food ranged from 82.7% to 1117%, demonstrating
326 satisfying application potential and credibility of the immunoassay for *S. enteritidis*
327 detection in food samples.

328 To further improve the proposed assay's sensitivity, a pre-enrichment step was
329 employed prior to the IMS-ELISA analysis. As shown in Table 2, less than 1 CFU/mL
330 *S. enteritidis* in various food samples can be detected by ELISA after 6h cultivation,
331 whereas it can be confirmed after just 4 h with the combination of IMS. In general, the
332 estimated concentration of *S. enteritidis* increased about 3 times after IMS.

333 **3. Conclusions**

334 In summary, nanobodies specific towards *Salmonella* O antigen and H antigen
335 were obtained through an epitope-based bio-panning strategy, which could benefit for
336 defining the recognition site and realizing the efficient pairwise matching of nanobody.
337 Further, a double nanobody sandwich ELISA combined with IMS was established to
338 detect *S. enteritidis*. The LOD of sandwich ELISA can reach 7.3×10^3 CFU/mL. The
339 combination with nanobody-based IMS enabled the reduction of the pre-enrichment
340 time by 2 h and the improvement of the sensitivity of the sandwich ELISA by about 3
341 times. More importantly, the IMS-ELISA could provide a rapid, reliable, low cost, and
342 simple detection strategy while enriching the target pathogen and avoiding the
343 interference of food substrate. Thus, this method has great potential application in
344 monitoring bacterial food contamination.

345

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490

Table 1. Recovery of *S. enteritidis* from spiked food samples.

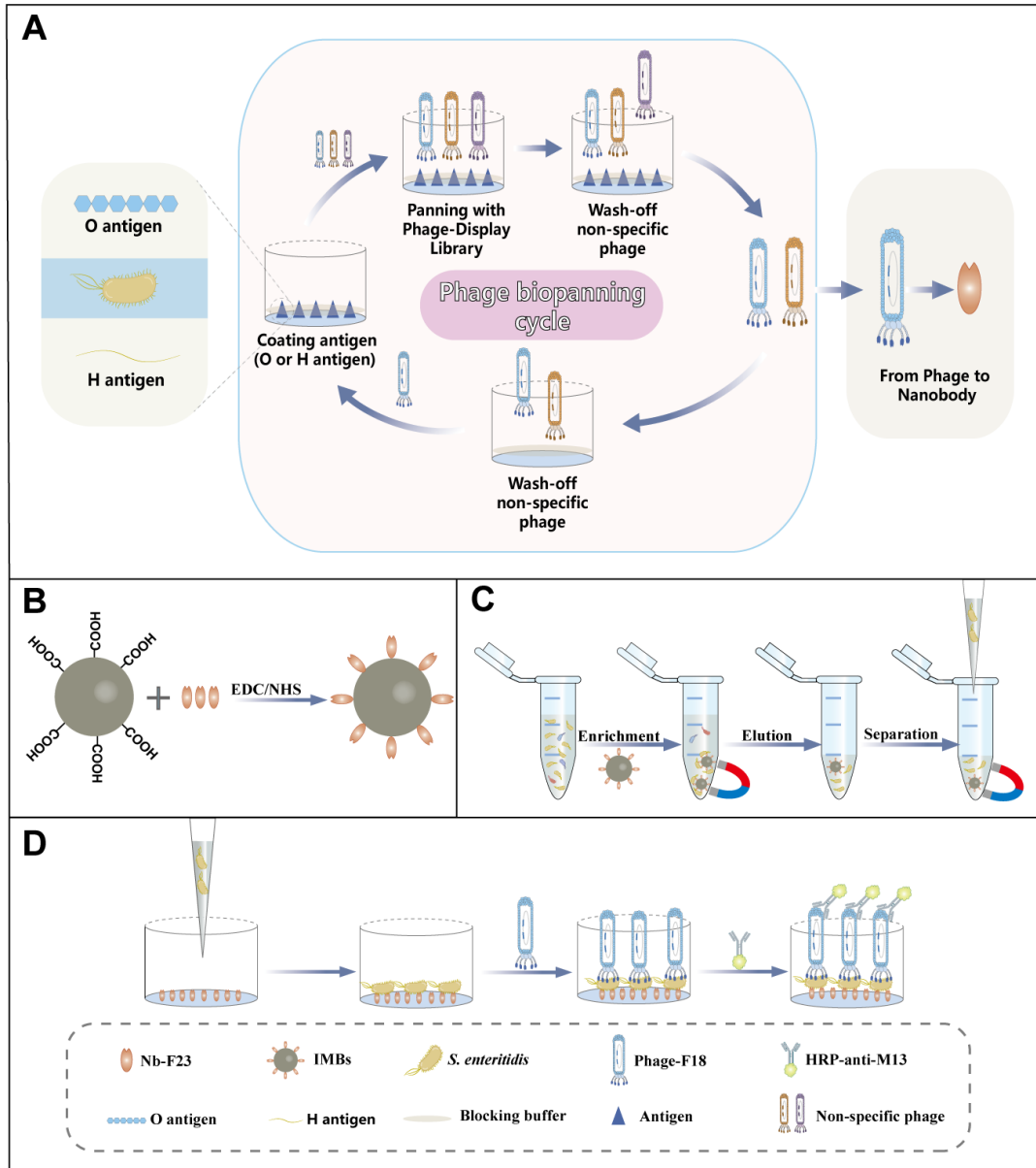
Sample	Spiked level (CFU/mL)	Detected level (CFU/mL)	Recovery (%)	CV (%), n=3
Chicken	1.0×10^5	8.74×10^4	87.4	2.7
	1.0×10^6	9.08×10^5	90.8	6.9
	1.0×10^7	9.62×10^6	96.2	7.3
Cabbage	1.0×10^5	8.27×10^4	82.7	4.7
	1.0×10^6	9.32×10^5	93.2	3.5
	1.0×10^7	1.17×10^7	117	5.8
Tomato	1.0×10^5	9.75×10^4	97.5	2.6
	1.0×10^6	9.64×10^5	96.4	5.3
	1.0×10^7	8.82×10^6	88.2	6.1
Apple Juice	1.0×10^5	1.16×10^5	116	8.3
	1.0×10^6	1.08×10^6	108	7.2
	1.0×10^7	9.34×10^6	93.4	6.3

492

493 **Table 2.** Detection of *S. enteritidis* in food sample before and after IMBs enrichment and as a
494 function of the duration of the pre-enrichment step. Food samples were spiked with 10 CFU/mL
495 and cultivated for different times, then analyzed by the ELISA or the IMB-ELISA.

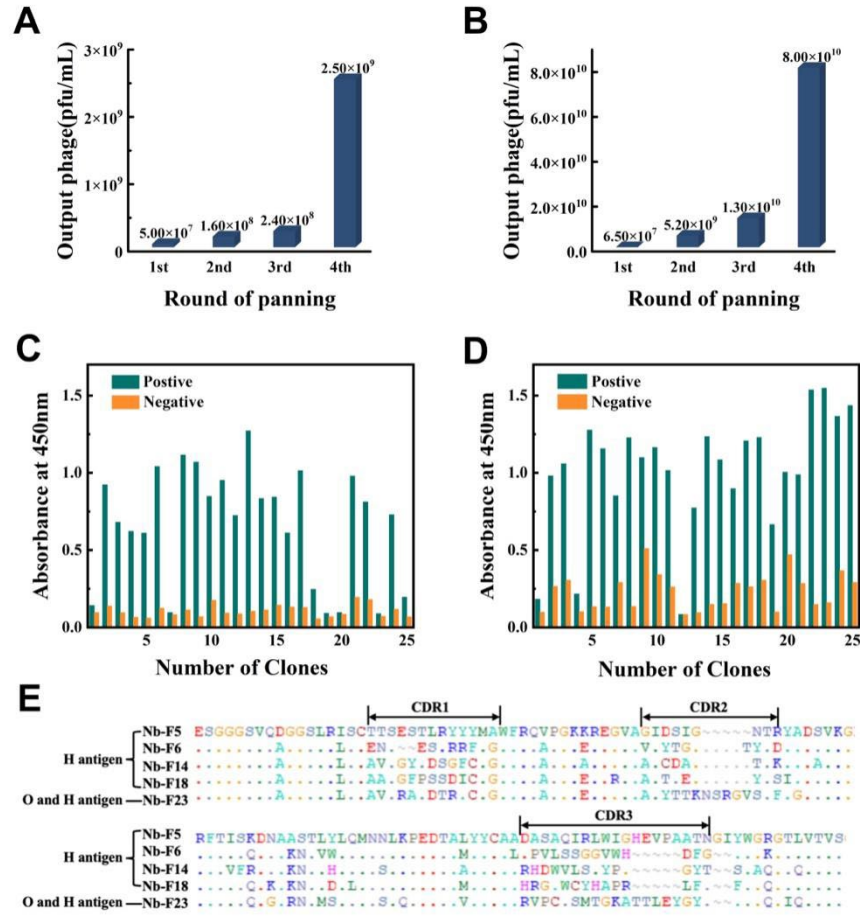
Sample	Control	Enrichment by IMBs	Pre-enrichment period (h)				
			3	4	5	6	7
Chicken	ND ^a	No enrichment	ND	ND	ND	4.42×10^4	1.12×10^5
		Enrichment	ND	3.57×10^4	5.68×10^4	1.26×10^5	2.96×10^5
Cabbage	ND	No enrichment	ND	ND	ND	5.09×10^4	1.05×10^5
		Enrichment	ND	3.32×10^4	6.33×10^4	1.58×10^5	3.36×10^5
Tomato	ND	No enrichment	ND	ND	ND	4.75×10^4	1.22×10^5
		Enrichment	ND	3.48×10^4	6.71×10^4	1.41×10^5	3.05×10^5
Apple Juice	ND	No enrichment	ND	ND	ND	4.79×10^4	1.09×10^5
		Enrichment	ND	3.80×10^4	6.09×10^4	1.34×10^5	2.99×10^5

496 ^a Not Detectable



497

498 **Fig. 1.** Overall detecting process of this method for *S. enteritidis* (A); Synthesis of the IMB (B);
 499 Procedure of IMS and elution (C); and Sandwich ELISA for *S. enteritidis* detection after enrichment
 500 (D).

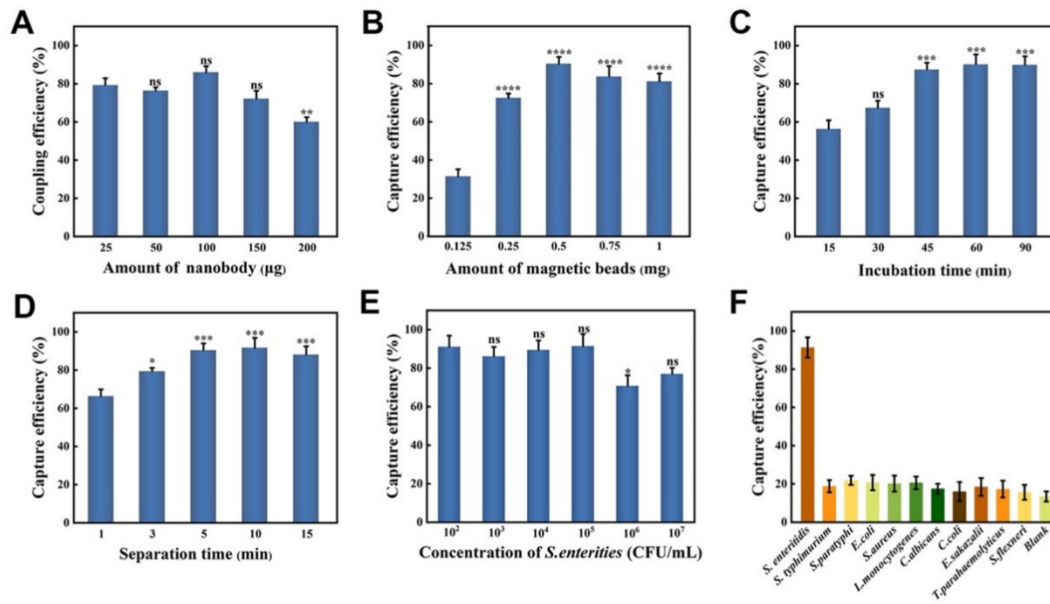


501

502 **Fig. 2.** Bio-panning of nanobody against flagella and LPS. Number of output phage after bio-

503 panning of (A) flagella and (B) LPS. Identification of 25 clones of (C) flagella and (D) LPS via

504 phage ELISA. (E) The alignment of the five different amino acid sequences.

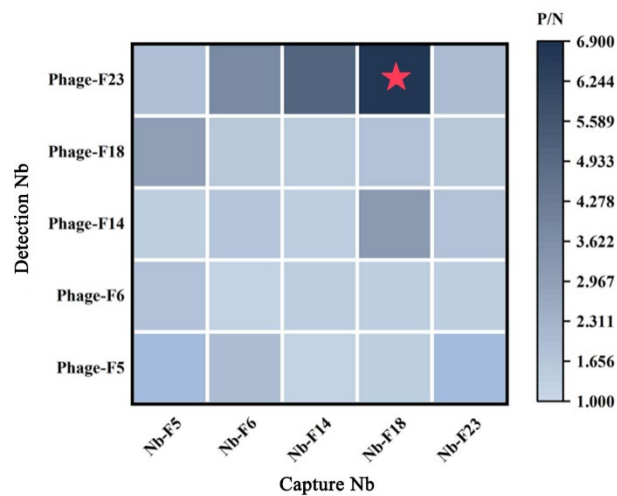


505

506 **Fig. 3.** The effects of (A) antibody dose, (B) IMB dose, (C) Incubation time, (D) IMS time on

507 Immunomagnetic separation procedure. The sensitivity (E) and specificity (F) of the IMBs. The

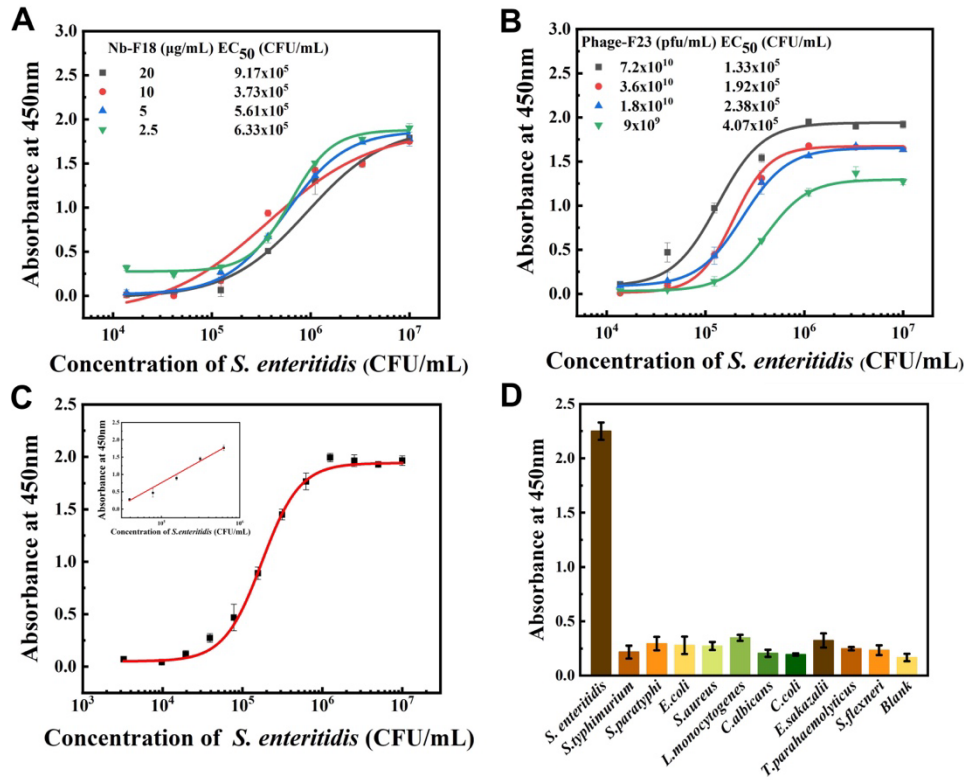
508 error bars represent the error value of five parallel.



509

510 **Fig. 4.** Heatmap of the sandwich ELISA pairing assay performed with the five Nbs against *S.*

511 *enteritidis*. P/N represents ratio of positive to negative absorbance value.



512

513 **Fig. 5.** Nanobody-based sandwich ELISA to detect *S. enteritidis*. Optimization of (A) Nb-F18

514 concentration, and (B) Phage-F23 concentration of the sandwich ELISA towards *S. enteritidis*. (C)

515 The standard curve of the sandwich ELISA. The inset shows the linear standard curves of IMS-

516 ELISA from 1.4×10^4 – 5.9×10^5 CFU/mL. (D) Specificity of IMS-ELISA towards 10 different

517 types of foodborne pathogens applied at the concentration of 1×10^8 CFU/mL. Error bars show

518 standard deviations from three independent experiments.

519

520 **Nanobody-based immunomagnetic separation platform for**
521 **rapid isolation and detection of *Salmonella enteritidis* in**
522 **food samples**

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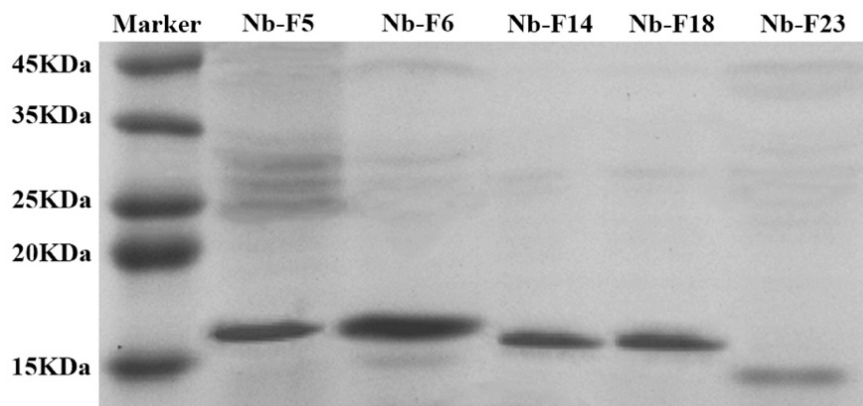
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533 1:The first two authors contribute equally to this work.

534



535

536

Figure S1. SDS-PAGE of five nanobodies

537

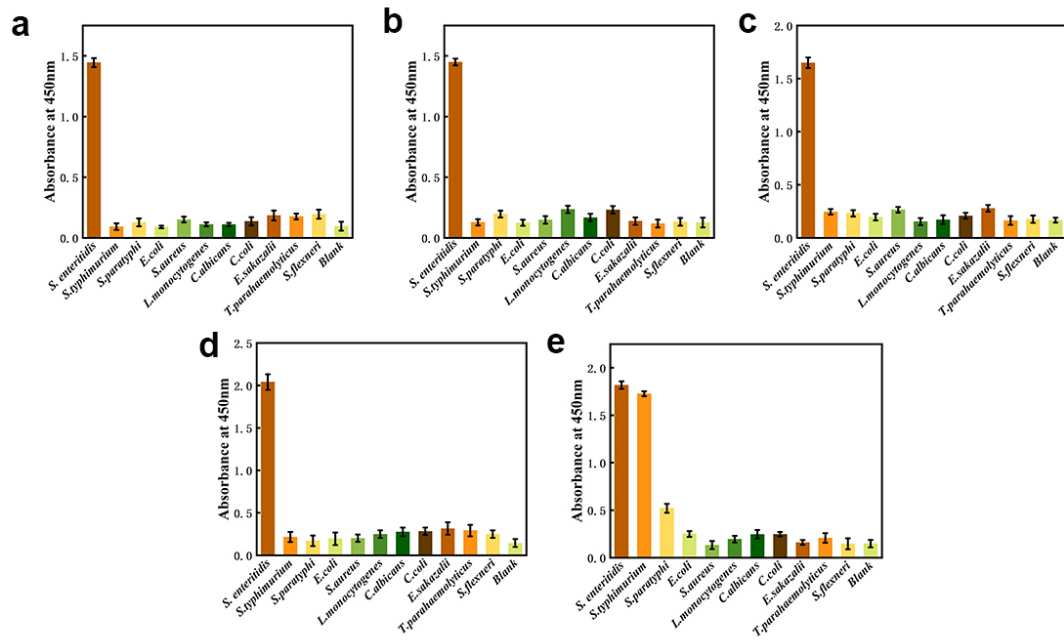
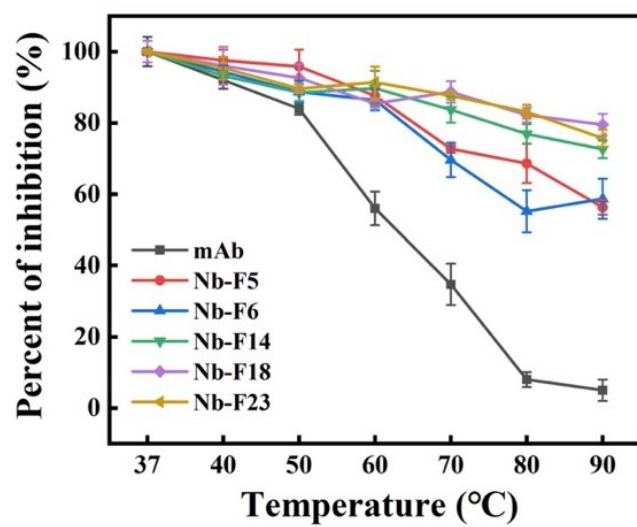


Figure S2. The Specificity of five nanobodies

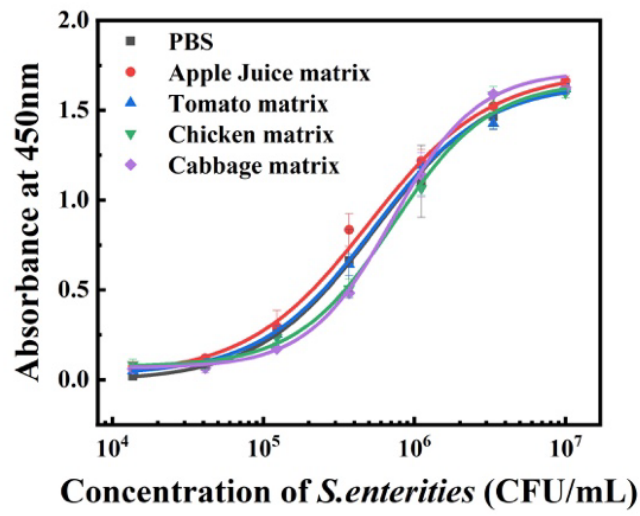


542

543

Figure S3. The thermal stability of five nanobodies

544



545

546

Figure S4. The standard curve towards *S. enteritidis* with four food samples

547

548 **Table S1.** Comparison of different previously reported immunoassays for *Salmonella*
 549 Detection.

Detection method	Foodborne pathogens	Detection limit CFU/mL	Enrichment time (h)	Detection limit CFU/mL (after enrichment)	References
Gold nanoparticles growth and accumulation immunochromatographic strip	<i>S. enteritidis</i>	10^4	N. D.	N. D.	(Bu et al. 2018)
Nanozyme sensor based-on Ps-Pt nanosphere	<i>S. typhimurium</i>	10^2 - 10^3	N. D.	N. D.	(Hu et al. 2021)
Magnetic nanoparticles immunochromatographic strip	<i>S. enteritidis</i>	1.95×10^5	N. D.	N. D.	(Duan et al. 2017)
streptavidin-bridged enhanced sandwich ELISA	<i>Salmonella</i>	4.23×10^3 to 9.15×10^3	N. D.	N. D.	(Ren et al. 2022)
Small-molecule probes based chemiluminescence assay	<i>Salmonella</i> , <i>L. monocytogenes</i>	2.88×10^4 , 4.88×10^3	6	10	(Michal et al. 2019)
KMO@Au Dual-readout immunochromatographic strip	<i>S. typhimurium</i>	10^3 - 10^4	N. D.	N. D.	(Zhang et al. 2022)
Sandwich ELISA based on Nb and pAb	<i>S. enteritidis</i>	1.40×10^5	N. D.	N. D.	(He et al. 2020)
phage-mediated double-nanobody sandwich chemiluminescent enzyme immunoassay	<i>S. typhimurium</i>	3.63×10^3	6-8	< 10	(Zhang et al. 2022)
IMS-ELISA	<i>S. enteritidis</i>	3.2×10^3	4	<10	this work

550 ^a N. D., Not Detectable

551

552 **Table S2.** LOD₅₀ and LOD₉₅ calculated by the Wilrich approach for *S. enteritidis*.

Target microorganism	POD (CFU / 25 g or mL)	Chicken	Cabbage	Tomato	Apple juice	All Settings
<i>S. enteritidis</i>	LOD ₅₀	6.5 × 10 ³	5.7 × 10 ³	8.5 × 10 ³	7.3 × 10 ³	6.9 × 10 ³
	LOD ₉₅	2.8 × 10 ⁴	2.5 × 10 ⁴	3.7 × 10 ⁴	3.2 × 10 ⁴	3.0 × 10 ⁴

553

554

555 **Table S3.** Detection of 1 CFU *S. enteritidis* in food sample after enrichment with or
 556 without IMBs enrichment.

Sample	Enrichment by IMBs	Pre-enrichment period (h) ^b				
		3	4	5	6	7
Chicken	No enrichment	- ^a	-	-	+	+
	Enrichment	-	+	+	+	+
	Negative control	- ^c	-	-	-	-
Cabbage	No enrichment	-	-	-	+	+
	Enrichment	-	-	+	+	+
	Negative control	-	-	-	-	-
Tomato	No enrichment	-	-	-	+	+
	Enrichment	-	+	+	+	+
	Negative control	-	-	-	-	-
Apple Juice	No enrichment	-	-	-	+	+
	Enrichment	-	+	+	+	+
	Negative control	-	-	-	-	-

557 ^a -: Not Detectable; +: Detectable

558 ^b Food samples were spiked with 1 CFU *S. enteritidis* and cultivated for different
 559 times, then analyzed by the IMS-ELISA with or without IMBs enrichment.

560 ^c The control group was tested simultaneously with the sample group and all the food
 561 sample were confirmed to be free of target substances.

562

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