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

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

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Abstract

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

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

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

1. Introduction

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

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

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

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

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

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

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

Materials and Methods

2.1. Materials and reagents

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

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

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

2.2. Epitope-based bio-panning

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

2.3. Expression and purification of nanobody

The phagemid DNA of five positive clones was transformed into *E. coli* Top10F′ competent cells by heat shock. A single colony carrying nanobody expression plasmid was cultivated in 100 mL of SB-ampicillin medium (50 μg/mL) at 37°C with shaking at 250 rpm. When OD_{600} value reached 0.6−0.8, IPTG was added with the final concentration of 0.2 mM. Subsequently, bacteria cells were further incubated for 10 h at 28 ℃ under shaking. The cells were pelleted by centrifugation and the proteins were extracted by B-PER reagent. Then the extract was purified by the Ni-NTA Superflow

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

2.4. Preparation of immunomagnetic beads (IMBs)

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

2.5. Immunomagnetic separation (IMS) procedure

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

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

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

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

Nanobody and phage displayed nanobody were applied as capture antibody and detection antibody, respectively, to elect the best matched pair of the sandwich ELISA. In brief, 100 μL per well of Nbs were coated overnight at 4°C in a microplate plate and then blocked with 300 μL of 3% skimmed milk powder in PBS at 37°C for 1 h. After three times washing with PBST, 100 μL of *S. enteritidis* was added to the wells and incubated for 1 h. The plate was washed three times, after which 100 μL per well of phage displayed Nb was added and incubated for 1 h at 37 °C. Then, 100 μL of HRP-conjugated anti-M13 antibodies was added to each well. After 1 h of incubation and six-times washing, peroxidase substrate TMB solution was added and incubated for 15 min. The reaction was terminated by 50 μL of 2 M H_2SO_4 solution, and the optical density (OD) was measured at 450 nm by a microplate reader.

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

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

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

2.8. Cross-reactivity.

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

2.9. Real sample analysis

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

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

2. Results and discussion

3.1. Epitope-based bio-panning

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

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

3.2. Expression and identification of nanobody

The plasmids of five positive clones were transformed into E. coli Top10F′. After purification by Ni-affinity chromatography columns, the purity of five nanobodies were 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℃) for 5 min
254 (Fig. S3). The nanobodies maintained 60% of their binding activity even after
255 incubating at 90℃ 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℃. 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**

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

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

3.5. Development and optimization of sandwich ELISA

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

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

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

3.6. Cross-reactivity analysis

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

3.7. Matrix effect and sample analysis

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

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

3. Conclusions

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

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

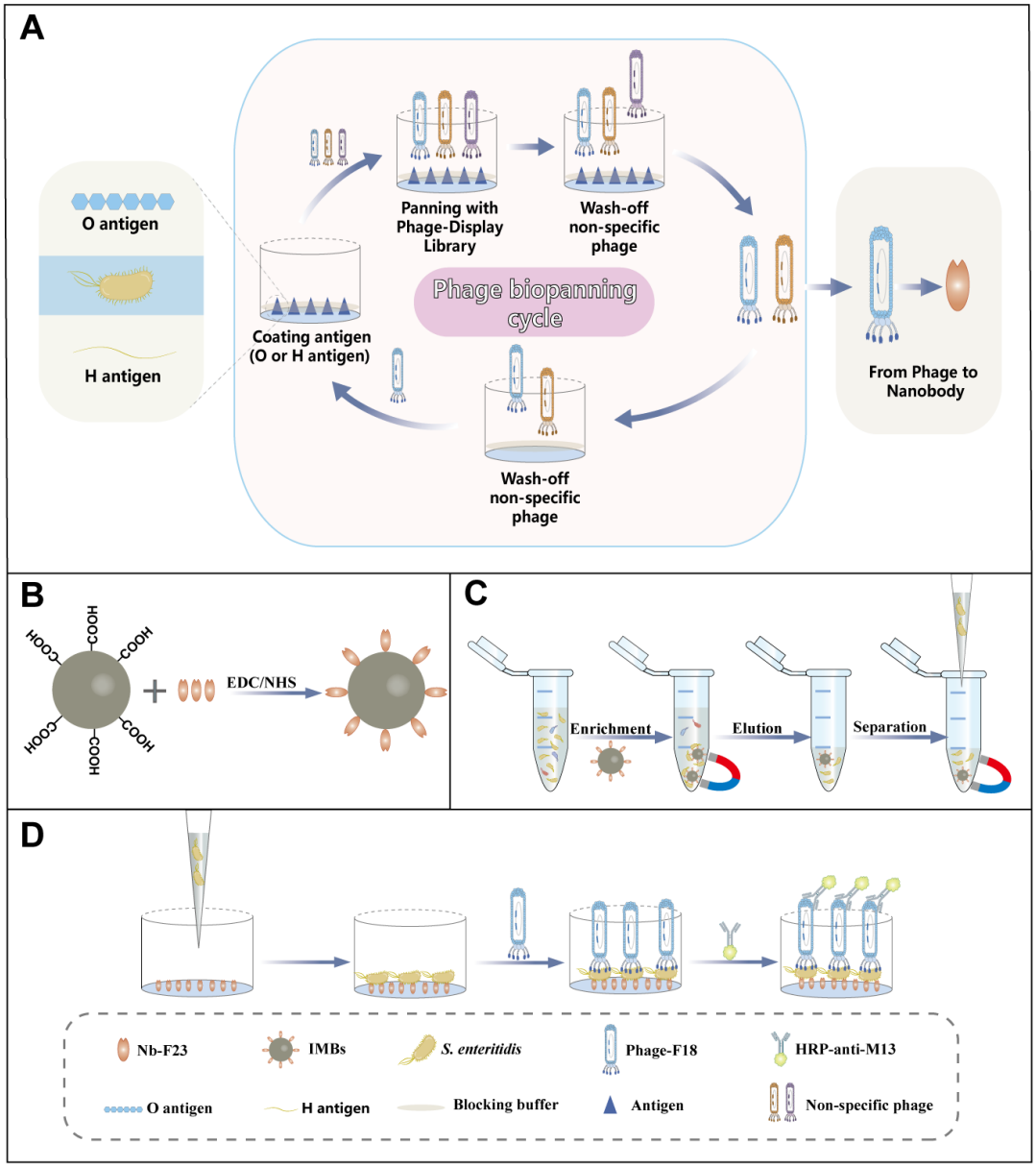
Sample	Spiked level (CFU/mL)	Detected level (CFU/mL)	Recovery (%)	CV (%), n=3
Chicken	1.0×10^5	8.74×10^4	87.4	2.7
	1.0×10^6	9.08×10^5	90.8	6.9
	1.0×10^7	9.62×10^6	96.2	7.3
Cabbage	1.0×10^5	8.27×10^4	82.7	4.7
	1.0×10^6	9.32×10^5	93.2	3.5
	1.0×10^7	1.17×10^7	117	5.8
Tomato	1.0×10^5	9.75×10^4	97.5	2.6
	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).

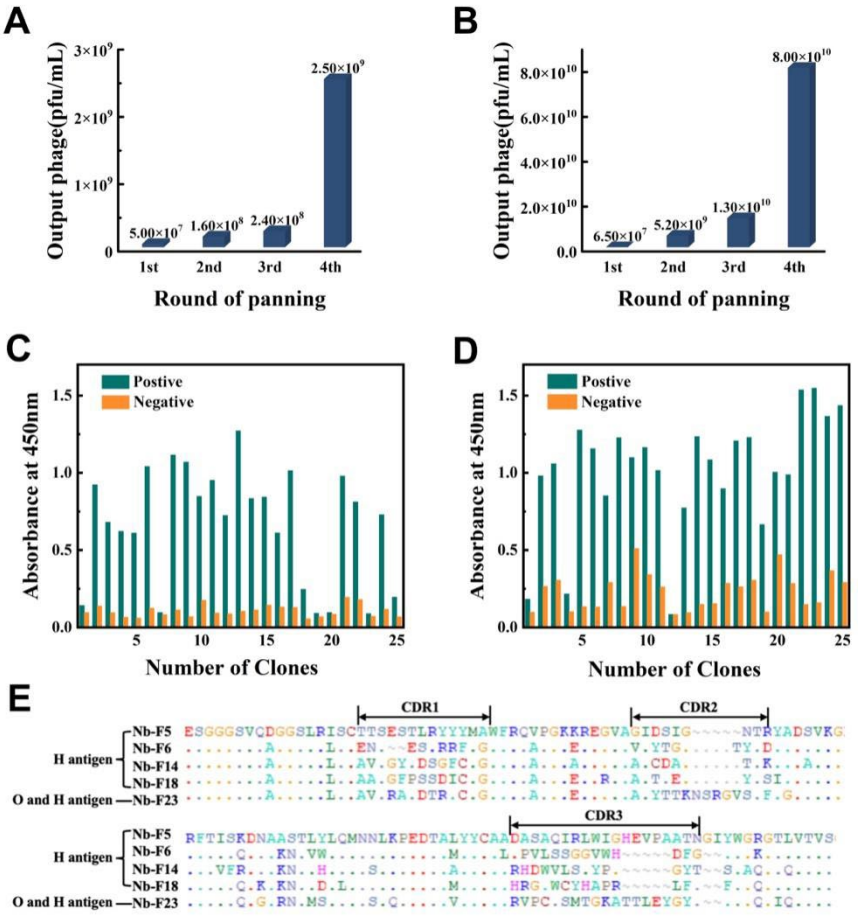
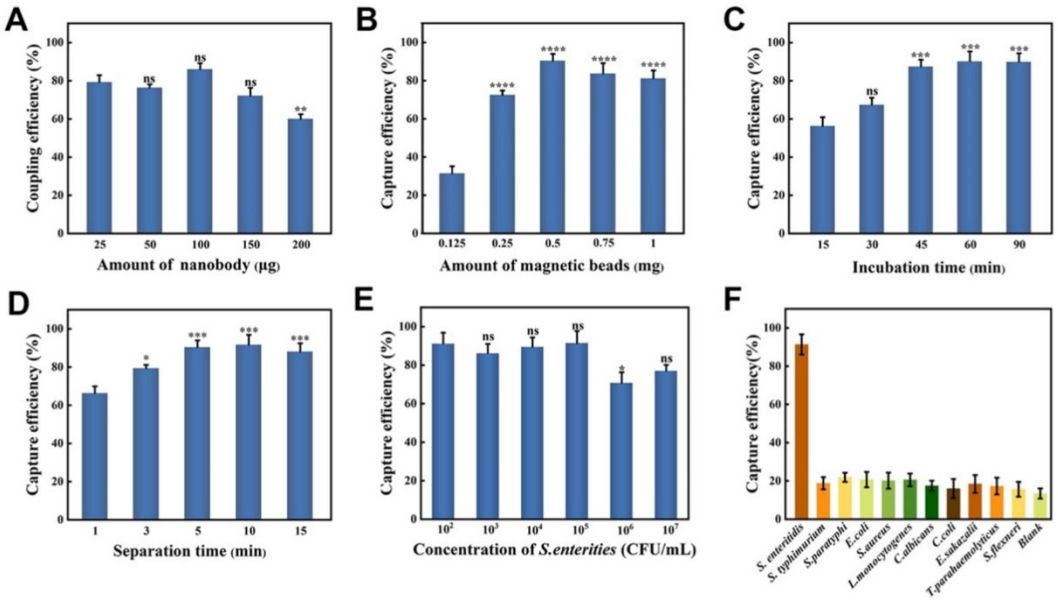
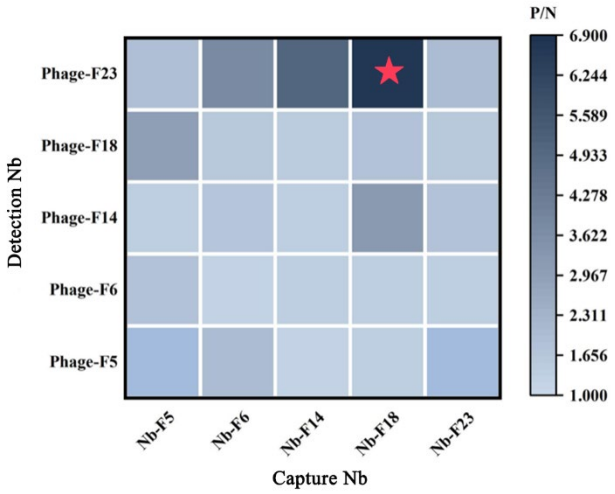


Fig. 2. Bio-panning of nanobody against flagella and LPS. Number of output phage after bio-panning of (A) flagella and (B) LPS. Identification of 25 clones of (C) flagella and (D) LPS via phage ELISA. (E) The alignment of the five different amino acid sequences.



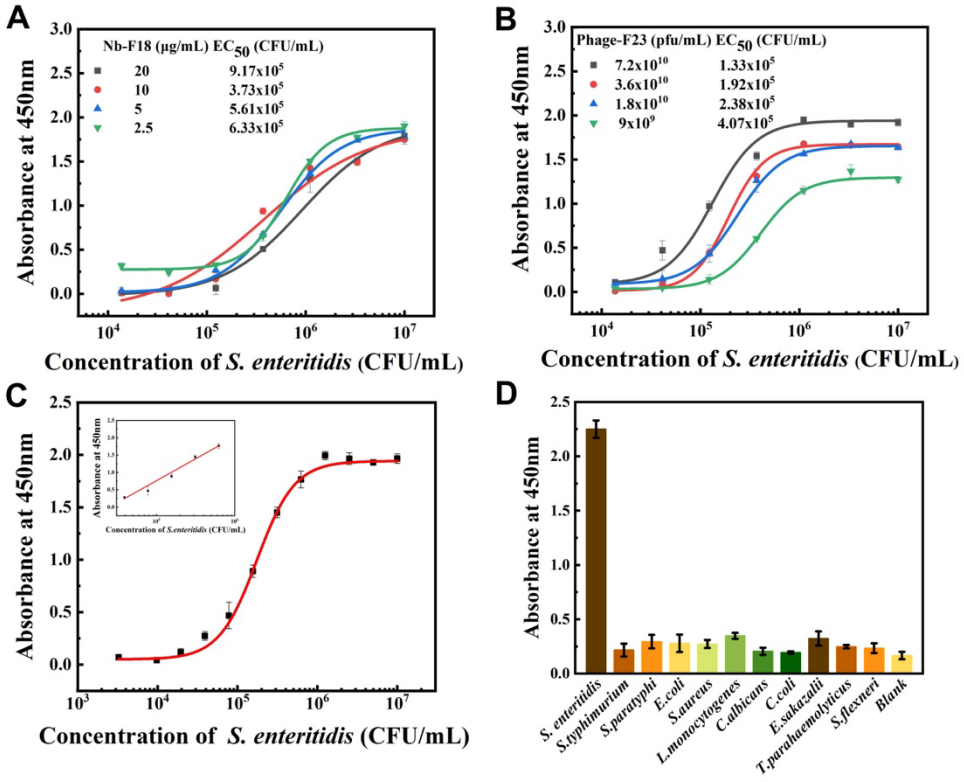
505

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. Th inset shows the linear standard curves of IMS-
516 ELISA from 1.4 × 104–5.9 × 105 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 derivations from three independent experiments.

519

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

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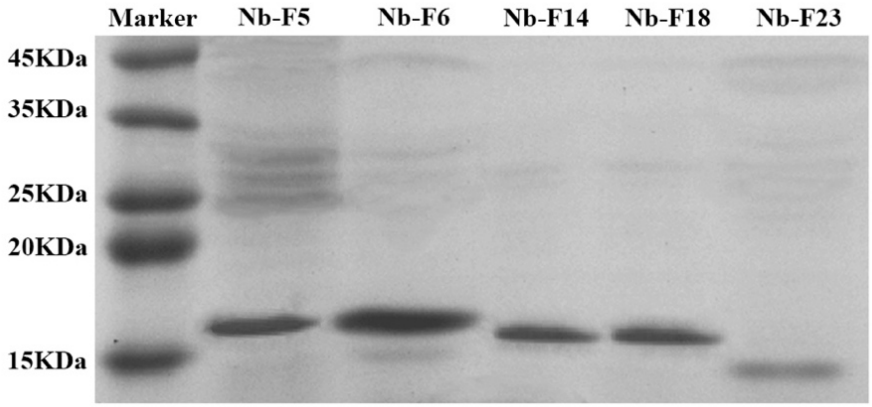


Figure S1. SDS-PAGE of five nanobodies

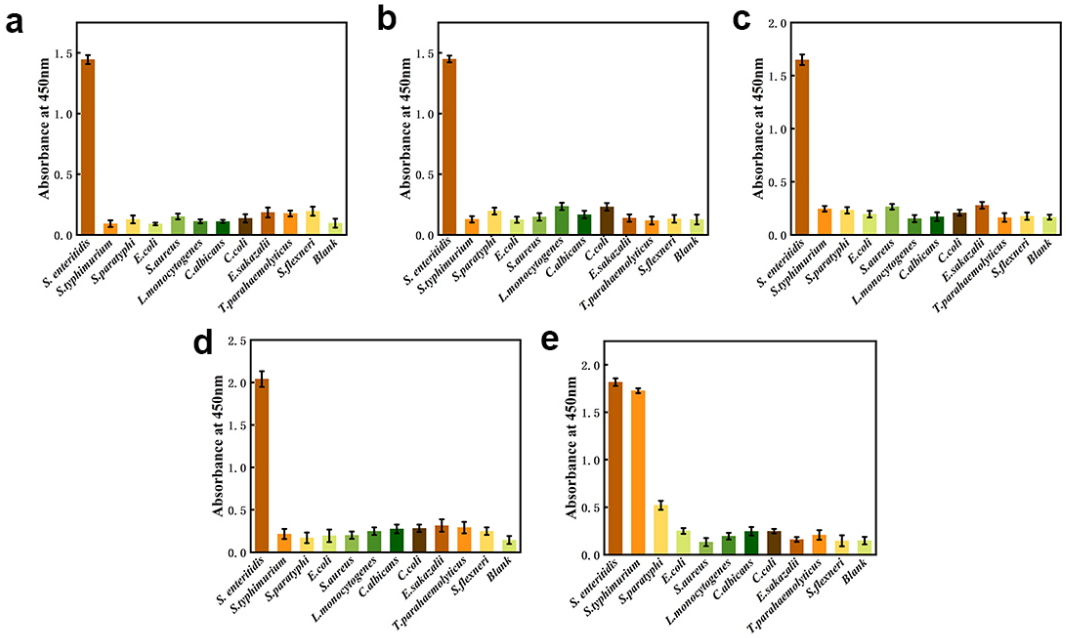


Figure S2. The Specificity of five nanobodies

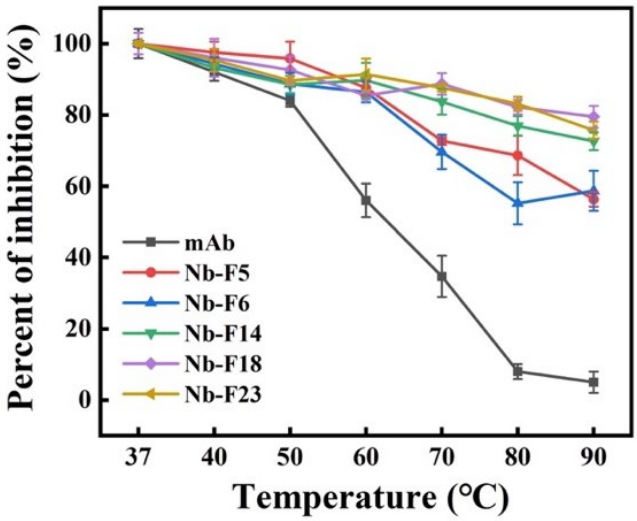
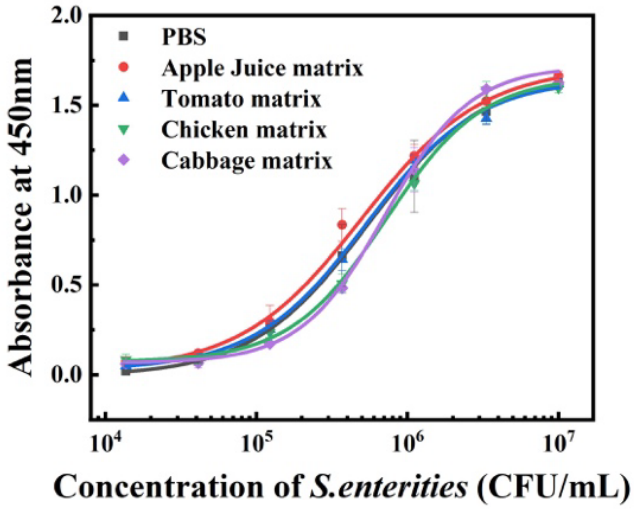


Figure S3. The thermal stability of five nanobodies



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	*S.* enteritidis	10^4	N. D.	N. D.	(Bu et al. 2018)
Nanozyme sensor based-on Ps-Pt nanosphere	*S.* typhimurium	10^2-10^3	N. D.	N. D.	(Hu et al. 2021)
Magnetic nanoparticles immunochromatographic strip	*S.* enteritidis	1.95×10^5	N. D.	N. D.	(Duan et al. 2017)
streptavidin-bridged enhanced sandwich ELISA	*Salmonella*	4.23×10^3 to 9.15×10^3	N. D.	N. D.	(Ren et al. 2022)
Small-molecule probes based chemiluminescence assay	*Salmonella*, *L.* monoctogenes	2.88×10^4, 4.88×10^3	6	10	(Michal et al. 2019)
KMO@Au Dual-readout immunochromatographic strip	*S.* typhimurium	10^3–10^4	N. D.	N. D.	(Zhang et al. 2022)
Sandwich ELISA based on Nb and pAb	*S.* enteritidis	1.40×10^5	N. D.	N. D.	(He et al. 2020)
phage-mediated double-nanobody sandwich chemiluminescent enzyme immmunoassay	*S.* typhimurium	3.63×10^3	6–8	< 10	(Zhang et al. 2022)
IMS-ELISA	*S.* enteritidis	3.2×10^3	4	<10	this work

550 [a] N. D., Not Detectable

551

552 **Table S2.** LOD_{50} and LOD_{95} calculated by the Wilrich approach for *S.* enteritidis.

Target microorganism	POD (CFU / 25 g or mL)	Chicken	Cabbage	Tomato	Apple juice	All Settings
S. enteritidis	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

Table S3. Detection of 1 CFU *S.* enteritidis in food sample after enrichment with or 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	-	-	-	-	-

[a] -: Not Detectable; +: Detectable

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

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

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