**Validation of a high-throughput immunobead array technique for multiplex detection
of three foodborne pathogens in chicken products**

Ratthaphol Charlormroj¹, Manlika Makornwattana¹, Irene R. Grant², Christopher T. Elliott²,
Nitsara Karoonuthaisiri^{1*}

¹ National Center for Genetic Engineering and Biotechnology (BIOTEC),
National Science and Technology Development Agency (NSTDA), Pathum Thani,
THAILAND

² Institute for Global Food Security, School of Biological Sciences, Queen's University
Belfast, Belfast, UNITED KINGDOM

Corresponding Author: Nitsara Karoonuthaisiri, Ph.D.

¹Biosensing Technology Research Unit, National Center for Genetic Engineering and
Biotechnology (BIOTEC), Pathum Thani, 12120, Thailand.

Email: nitsara.kar@biotec.or.th

Tel.: +66-2-5646700, ext. 3255 Fax: +66-2-5646707

Abstract

This study rigorously evaluated a previously developed immunobead array method to simultaneously detect three important foodborne pathogens, *Campylobacter jejuni*, *Listeria monocytogenes*, and *Salmonella* spp., for its actual application in routine food testing. Due to the limitation of the detection limit of the developed method, an enrichment step was included in this study by using Campylobacter Enrichment Broth for *C. jejuni* and Universal Pre-enrichment Broth for *L. monocytogenes* and *Salmonella* spp.. The findings show that the immunobead array method was capable of detecting as low as 1 CFU of the pathogens spiked in the culture media after being cultured for 24 hours for all three pathogens. The immunobead array method was further evaluated for its pathogen detection capabilities in ready-to-eat (RTE) and ready-to-cook (RTC) chicken samples and proven to be able to detect as low as 1 CFU of the pathogens spiked in the food samples after being cultured for 24 hours in the case of *Salmonella* spp., and *L. monocytogenes* and 48 hours in the case of *C. jejuni*. The method was subsequently validated with three types of chicken products (RTE, n=30; RTC, n=20; raw chicken, n=20) and was found to give the same results as the conventional plating method. Our findings demonstrated that the previously developed immunobead array method could be used for actual food testing with minimal enrichment period of only 52 hours, whereas the conventional ISO protocols for the same pathogens take 90-144 hours. The immunobead array was therefore an inexpensive, rapid and simple method for the food testing.

Keywords: Immunobead array; *Campylobacter jejuni*; *Listeria monocytogenes*; *Salmonella* spp.; Multiplex detection

1. Introduction

Foodborne pathogens are a serious public health problem causing millions of infections annually (United States Center for Disease Control and Prevention, 2011) and adversely affecting food industries worldwide (Scallan et al., 2011). Of the foodborne pathogens, *Campylobacter* spp., *Listeria monocytogenes*, and *Salmonella* spp. are of global concern due to their high prevalence and severity (Centers for Disease Control and Prevention, 2013; Crim et al., 2015; Hara-Kudo et al., 2012; Korsak et al., 2015; Torso et al., 2015). To detect these important pathogens in food samples, conventional methods based on culturing are available (ISO 10272:2006 for *C. jejuni*, ISO 11290:1996 for *L. monocytogenes*, and ISO 6579:2002 for *Salmonella* spp.). However, these conventional culture methods are time-consuming and laborious, which can present a risk from a delay in protecting consumers from pathogens contamination in food. Therefore, there is an urgent need to develop multiplex detection for these pathogens.

The suspension bead array has been used as a multiplex detection method in many applications such as allergy testing (Focke-Tejkl et al., 2014), tumor makers detection (Kim et al., 2012; Wang et al., 2012), and plant diseases detection (Bergervoet et al., 2008; Charlermroj et al., 2013). This technology employs paramagnetic microsphere sets which are fluorescently barcoded by unique ratios between red and infrared dyes inside. Each set of microsphere can be covalently linked with a specific antibody through its carboxyl surface via carbodiimide reaction. Different antibody-linked microsphere sets can be employed to simultaneously capture different analytes in a sample before a mixture of R-phycoerythrin (RPE)-linked secondary antibodies specific to the analytes was added to report the signals. To obtain the signal for each analyte, two lasers (red and green) are used. The red laser is used to detect the fluorescent barcode within each microsphere which, in turn, can be used to identify the specific antibody linked with that microsphere. The green laser is used to measure RPE

73 signals and report whether the particular microsphere set has captured the analyte (Dunbar et
74 al., 2003).

75 For food safety applications, the suspension immunobead array method has been
76 developed to detect pathogens and toxins in food products. For instance, the suspension bead
77 array was used to simultaneously detect four foodborne pathogens (*Salmonella* spp.,
78 *Escherichia coli*, *Listeria* spp., and *Campylobacter* spp.) and staphylococcal enterotoxin toxin
79 using artificial spiked inactivated bacterial cells and enterotoxin in different types of food
80 (Kim et al., 2010). However, these previous studies did not validate their systems using
81 enriched food samples. Another group used the suspension bead array to detect
82 contaminations of *L. monocytogenes* and *L. ivanovii* in baby milk powder and vegetables
83 using macrophage enrichment (Day and Basavanna, 2015). However, no one has ever
84 reported the use of this immunobead array method with enriched food samples for detection
85 of *L. monocytogenes*, *Salmonella* spp. and *C. jejuni*.

86 Previously, our group successfully developed an immunobead array technique to
87 simultaneously detect *L. monocytogenes*, *Salmonella* spp. and *C. jejuni* in different types of
88 food samples without pre-enrichment (Karoornuthaisiri et al., 2015). The sensitivities of
89 detection for *Listeria monocytogenes*, *Campylobacter jejuni*, and *Salmonella* spp. were 2.8
90 $\times 10^7$ CFU/mL, 7.2×10^6 CFU/mL, and 4.4×10^5 CFU/mL, respectively (Karoornuthaisiri et al.,
91 2015). While the sensitivities of detection of the developed immunobead array technique
92 were better than a sandwich ELISA method when using the same sets of antibodies, the limits
93 of detection were not low enough to detect the pathogens at the concentrations required by
94 food safety regulations (e.g. United States Food and Drug Administration and European
95 Commission).

96 To make this developed technique applicable for food safety testing, enrichment steps
97 to increase bacterial numbers are required and validation with actual food samples must be

undertaken. Therefore, this study evaluated commercial enrichment culture media to ensure sufficient numbers of pathogens were present at time of testing, and validated the previously developed proof-of-concept suspension immunobead array by testing three chicken products.

2. Materials and methods

2.1 Chemicals and culture media

Sulfo-N-hydroxysulfosuccinimide (sulfo-NHS, #24510) and 1-ethyl-3-[3-dimethylaminopropyl] carbodiimidehydrochloride (EDC, #22980) were purchased from Thermo Fisher Scientific, USA. Luria-Bertani (LB) broth was prepared from tryptone (10 g), yeast extract (5 g) and sodium chloride (10 g) in one liter of distilled water. *Campylobacter* Enrichment Broth (CEB, #LAB135; supplemented with 20 mg/L cefoperazone, 20 mg/L vancomycin, 20 mg/L trimethoprim, and 25 mg/L natamycin, #X132) and Harlequin *Listeria* Chromogenic Agar (#HAL010; supplemented with 10 mg/L polymyxin B and 20 mg/L ceftazidime, #X072, and 50 mg/L cycloheximide, 20 mg/L nalidixic acid, and 600 mg/L phosphatidyl inositol, #X010) were purchased from LAB M, UK. Xylose Lysine Deoxycholate (XLD, #CM0469) used as a selective agar for *Salmonella* spp. was purchased from Oxoid, UK. The Simplate® for *Campylobacter* (C-CI, #66006) and the Simplate® for total plate count (TPC-CI, #66002) were purchased from Biocontrol, USA. The Universal Pre-enrichment Broth (UPB, #91366) was purchased from Sigma-Aldrich, USA.

2.2 Bacterial strains and growth conditions

The bacterial strains used in this study were purchased from American Type Culture Collection (ATCC, VA) and Department of Medical Sciences Thailand (DMST, Thailand). For bacterial inoculum preparation, a single colony of *L. monocytogenes* (ATCC 19115), and *Salmonella* Enteritidis (ATCC 13076) from LB agar were inoculated into 10 mL LB broth for

18 h at 37°C on an orbital shaker at 200 RPM. In the case of *C. jejuni* (DMST 15190), one cryobead of cell suspension was inoculated into 5 mL CEB and incubated without shaking for 48 h at 41.5°C under microaerobic conditions (5% CO₂ and 10% O₂). Concentration of each bacteria culture was calculated from numbers of colonies on corresponding selective agars for pathogens, and by measuring absorbance at 600 nm using a spectrophotometer (Cintra 404, USA). The bacteria were diluted in phosphate buffered saline (PBS) pH 7.4 (137 mM NaCl, 3 mM KCl, 10 mM Na₂HPO₄ and 2 mM KH₂PO₄) to 10² CFU/mL and used as a starting stock. The concentration of the stock was also enumerated by a plate count technique on selective agar.

2.3 Bacterial enrichment

An enrichment step was required to increase the number of bacteria in food products to a detectable level. For *L. monocytogenes* and *Salmonella* spp., the enrichment protocol was performed as previously described (Bailey and Cox, 1992). Briefly, 25 g of food sample were added to 225 mL UPB and blended for 1 min using a stomacher (BagMixer 400P Lab Blenders, Interscience USA). The blended samples were cultured at 37°C for 24-48 h without shaking. For *C. jejuni*, the enrichment was performed following the previously reported protocol (Sails et al., 2002). Briefly, 1 g of food sample was placed in 9 mL CEB and blended for 1 min. The blended samples were cultured at 41.5°C under microaerobic conditions for 24-48 h without shaking.

2.4 Suspension immunobead array assays

The suspension immunobead array assay employed the sandwich ELISA principle to detect the pathogens. In this study, three sets of fluorescently barcoded microspheres were used. Each barcoded microsphere set was linked with a capture antibody specific to a

pathogen while a detecting antibody which was able to pair with the capture antibody was linked with R-phycoerythrin (RPE). The detector identified the barcoded microspheres as well as their corresponding signals obtained from RPE molecule on the detecting antibody (Figure 1C). Specific antibody sets used for multiplex detection of the three pathogens are shown in Table 1. The detailed protocol to conjugate antibodies with the MagPlex beads sets (Luminex, TX, USA) was previously described (Karoonthaisiri et al., 2015). Briefly, 10^6 beads were activated by sulfo-NHS (10 μ L, 50 mg/mL) and EDC (10 μ L, 50 mg/mL) before being conjugated with 5 μ g of a designated antibody. To conjugate fluorescent RPE to the detecting antibodies, a Lightning-LinkTM R-phycoerythrin conjugation kit (703-0010, Innova Biosciences, UK) was used according to manufacturer's protocols and kept at 4°C in the dark until use.

In each well of a microplate (Greiner, #650101), enriched samples from the UPB (for *Salmonella* and *Listeria*) and CEB (for *Campylobacter*) media (50 μ L each) were mixed together before antibody-coated beads (50 μ L total, 1×10^5 beads for each antibody) were added and incubated for 1 h on a shaker at room temperature in the dark. The unbound samples were removed by an automated microplate washer (Bio-Plex Pro, BIO-RAD USA) with PBS containing 0.05% Tween 20 (PBST) three times. To detect the signal, a mixture of RPE-labeled antibodies (100 μ L total, C818-RPE and rPAb-RPE at 2 μ g/mL, and SalAb-RPE at 1 μ g/mL) was added and incubated on a shaker for 1 h in the dark. A washing step was performed as before, and the beads were resuspended in 100 μ L PBST prior to signal detection using a MAGPIX® detector (Luminex, TX, USA). The median fluorescent intensity (MFI) values were recorded. Each test was repeated at least twice, and culture media without spiked pathogens were used as negative control. Results were considered positive when the intensity values were greater than three times the background MFI (Dunbar et al., 2003).

2.5 Simultaneous detection of three foodborne pathogens after enrichment

To test multiplex detection using a suspension immunobead array technique, the designated amounts of bacteria (0 and 1 CFU) were spiked in culture media, ready-to-eat chicken product (RTE, n=6), and ready-to-cook chicken product (RTC, n=6). To spike 1 CFU of bacteria, 10 µL of a 10² CFU/mL starting bacterial stock diluted in phosphate buffered saline (PBS) pH 7.4 (137 mM NaCl, 3 mM KCl, 10 mM Na₂HPO₄ and 2 mM KH₂PO₄) was used. The concentration of the starting stock was also enumerated by a plate count technique on selective agar. In addition, to ensure those chicken products were not contamination with the pathogens of interest, the chicken samples were tested for *Campylobacter*, *Listeria monocytogenes*, and *Salmonella* according to the corresponding ISO protocols which basically were cultured and plated on selective agar for *Campylobacter*, *Listeria monocytogenes*, and *Salmonella* detection.

The enrichment protocol was the same as that in Section 2.2. After 24 h and 48 h, the samples were analyzed using the suspension immunobead array as described in Section 2.4. The results were compared with the results of plating of the same enrichment cultures on selective agars (same as described in Section 2.2).

2.6 Validation of the suspension immunobead array for simultaneously detecting three foodborne pathogens in food products

To validate multiplex detection using the suspension immunobead array technique, ready-to-eat chicken (RTE, n=30), ready-to-cook chicken (RTC, n=20), and raw chicken (RC, n=20) obtained from local supermarkets and open-air markets were used (Table 2). The RTE products included grilled black pepper chicken (n=10), teriyaki chicken (n=10), roasted chicken BBQ with honey (n=5), and sausage chicken (n=5). The RTC products included

sesame chicken (n=10) and seasoned chicken (n=10). The RC products included raw chicken breast without skin (n=10) and raw chicken thigh (n=10). The samples were tested as described in Section 2.3 after being cultured for 24 h and 48 h. *L. monocytogenes*, *S. Enteritidis*, and *C. jejuni* (2×10^8 CFU/mL for each) were used as positive control. The culture media were used as negative control. All experiments were performed at least three times.

3. Results and Discussion

3.1 Capability of multiplex foodborne pathogens detection in culture media

Previously, we successfully developed a proof-of-concept immunobead array technique to simultaneously detect *C. jejuni*, *L. monocytogenes*, and *Salmonella* spp. in different types of food samples without pre-enrichment (Karoonthaisiri et al., 2015), but limits of detection were found to be insufficient to meet food safety criteria specified in legislation by European Commission (Regulation (EC) No. 2073/2005, and No. 776/2006). Therefore, this study examined the possibility of including an enrichment step before testing of chicken products.

First, the possibility to enrich all three bacteria in the same medium culture was evaluated. The Universal Pre-enrichment Broth (UPB) was reported to be able to enrich *E. coli* O157:H7, *L. monocytogenes*, and *Salmonella* spp. (Nam et al., 2004; Suo and Wang, 2013); however, there was no information on its ability to enrich *Campylobacter* spp. To test the ability of UPB to enrich the three pathogens of interest, 1 colony forming unit (CFU) of each pathogen (*C. jejuni*, *L. monocytogenes*, and *S. Enteritidis*) was inoculated into UPB at 37°C for 24-48 h under aerobic condition for *L. monocytogenes* and *S. Enteritidis*, and 41.5°C under microaerobic conditions for *C. jejuni*. The results showed that the UPB was suitable for culturing *L. monocytogenes* and *S. Enteritidis*, but not *C. jejuni* (Figure 2A). The numbers of *L. monocytogenes* and *S. Enteritidis* ranged between 2.4×10^8 - 4.3×10^8 CFU/mL and 2.7×10^8 -

7.0x10⁸ CFU/mL after being cultured for 24 h and 48 h, respectively. The numbers of *C. jejuni* were only 1x10³ CFU/mL and 1x10⁶ CFU/mL when cultured in UPB for 24 h and 48 h, respectively, which were lower than the detection limit (7.2x10⁶ CFU/mL) of this method previously reported for *Campylobacter* spp. detection (Karoonthaisiri et al., 2015). Therefore, for the enrichment of *C. jejuni*, Campylobacter Enrichment Broth (CEB) was used instead. With the CEB, *C. jejuni* increased to 5x10⁷ CFU/mL and 5x10⁸ CFU/mL after being cultured for 24 h and 48 h, respectively (Figure 2B). Consequently, to exploit multiplex capacity of the immunobead array in testing the samples whose bacterial contaminants were unknown, the samples were enriched with two enrichment media (UPB and CEB) before being combined and tested by the immunobead array method.

Before being used for testing real food samples, the developed immunobead array technique was used to detect the bacterial cells in culture media. The pathogens were individually spiked into the appropriate culture medium and after pre-enrichment for 24 h and 48 h, the enriched cultures were mixed and tested using the bead array technique. The numbers of each bacterium were enumerated on appropriate selective agars. The immunobead array could simultaneously detect the three pathogens even for the cultures that were inoculated with only 1 CFU for only 24 h (Figure 3). The samples from the 24-h enrichment of the cultures were plated on their corresponding selective agar and the numbers were found to be 3x10⁸, 6x10⁸, and 5x10⁸ CFU/mL for *C. jejuni*, *L. monocytogenes*, and *S. Enteritidis*, respectively (Figure 3B). After being cultured for 48 h, the immunobead array technique was still able to detect all three pathogens with similar signals to those obtained from the 24-h enrichment. This was not surprising because the numbers of colonies on the selective agars from the 24 h and 48 h enrichment cultures were similar (Figure 3B-C).

3.2 Multiplex detection of three foodborne pathogens in chicken products

To validate the multiplex capacity of the developed immunobead assay to detect the three pathogens in real food samples, the bacteria were spiked into two kinds of chicken products - ready-to-eat (RTE) and ready-to-cook (RTC) products - and incubated using the appropriate culture media and culture conditions for each pathogen. Pathogen-free culture media (0 CFU) were used as negative control. In all experiments, the appropriate selective agar for each pathogen was used in parallel to enumerate and identify each foodborne pathogen, and total plate counts were used to enumerate the total bacteria in food samples.

From the results in RTE food samples, although the immunobead array could detect *C. jejuni* when it was enriched for 24 h, its signal was only marginal above the cutoff value. The method, however, could give much higher signal of *C. jejuni* detection after enrichment for 48 h (Figure 4A). For *L. monocytogenes* and *S. Enteritidis* detection, the immunobead array could detect both pathogens even after enrichment culture for 24 h when spiked with 1 CFU at the beginning (Figure 4A). From the plate count method on selective agars, the numbers of *L. monocytogenes* and *S. Enteritidis* after being enriched for 24 h were 2×10^8 and 3×10^8 CFU/mL, respectively (Figure 4B). After being cultured for 48 h, the numbers of pathogens on the selective agars were of the same magnitude (1×10^8 and 4×10^8 CFU/mL for *L. monocytogenes* and *S. Enteritidis*, respectively) (Figure 4C). In contrast, the numbers of bacteria cells from the total plate count method after being cultured for 48 h dramatically increased 10-times when compared with being cultured for 24 h (from 1.2×10^9 to 1.0×10^{10} , and from 2.7×10^9 to 2.0×10^{10} CFU/mL for *L. monocytogenes* and *S. Enteritidis* cultures, respectively). When consider the signals from the immunobead array method, the detection signals of the *L. monocytogenes* and *S. Enteritidis* samples after being cultured for 48 h were lower than their corresponding signal after being cultured for 24 h (Figure 4A). The reason of decreasing signals of the immunobead array method might be from the fact that the increasing background microflora in food samples might interrupt binding between

antibodies and antigens (Wang and Salazar, 2016), resulting in lower signals obtained from the detection. This observation was also found in milk when native background flora such as *Enterococcus* and *Lactobacillus* spp. was reported to interfere the detection of *L. monocytogenes* and *S. enterica* (Suh and Knabel, 2001). Although the immnobead array signal decreased after enrichment for 48 h, this method could still correctly detect the presence of these three pathogens.

Generally, if ISO protocols were followed, after the first 24 h of enrichment, the bacterial cultures would need to be transferred to fresh media (Fraser broth for *L. monocytogenes*, and Rappaport-Vassiliadis medium with soya (RVS) and Muller-Kauffmann tetrathionate-novobiocin (MKTn) broth for *Salmonella* spp.) to provide sufficient nutrition. In this study, UPB was used as culture medium up to 48 h without having to transfer the samples to another medium, and it still allowed the cells to be tested accurately with the immunobead array. This could be because the UPB contained sodium pyruvate which helped the repair of injured bacteria cells by balancing pH changes and reactive oxygen atoms by bacterial flora in samples (Knabel and Thielen, 1995; Ray and Speck, 1973).

When comparing the signals obtained from the culture media and from the RTE food samples, all signals from detection in RTE food samples were lower than detection signals in culture media, especially in case of *C. jejuni* and *L. monocytogenes*. In RTC food samples, the results were similar to the pathogens detection in RTE food samples (Figure 5). The immunobead array method was able to detect *C. jejuni* after enrichment for 48 h and *L. monocytogenes* and *S. Enteritidis* after enrichment for 24 h when the samples were spiked with 1 CFU from beginning. Again, the signals from pathogen detection in RTC samples were lower than those from the culture media. The reason for the lower signals obtained in the RTE and RTC samples might be from the fact that the binding sites of the antibodies might be blocked by some ingredients from the food matrix such as lipid or protein. For

instance, different ingredients and formulation of preservative in food products could affect antigen expression in different types of food (Banada and Bhunia, 2008; Lathrop et al., 2008). Moreover, in RTE food samples testing, the numbers of pathogens found on selective agar were lower than those found in the culture media testing even when the same amount of pathogens was spiked. This lower number of pathogens after enrichment might be due to growth competition with other bacteria in the microflora of food samples. The microflora diversities in chicken products depend on manufacturing conditions. For example, *Acinetobacter* spp., *Carnobacterium* spp., *Rahnella* spp., *Pseudomonas* spp., *Brochothrix* spp., and *Weissella* spp. were found in fresh chicken products during storage in a refrigerator under aerobic condition (Liang et al., 2012). Furthermore, enzymes and antimicrobial components might be released from food pretreatments such as homogenization and blending, and these components might affect foodborne detection (Bhunia, 2014). In addition, the expression of antigens under different induced-stress conditions from being cultured longer might affect binding of antibody. For example, high temperature and salts could affect antigen expression in *L. monocytogenes*, but low temperature and acid stress conditions did not affect (Geng et al., 2003). Therefore, it should not be surprising that the signals obtained from the RTE and RTC food samples were lower than those obtained from the culture media in all cases. Nevertheless, the system was capable of specifically detecting pathogens spiked into the chicken products.

3.3 Validation of the suspension immunobead array for simultaneously detecting three foodborne pathogens in food products

To validate the bead array, three different types of chicken products (ready-to-eat (RTE, n=30), ready-to-cook (RTC, n=20), and raw chicken (RC, n=20) obtained from local supermarkets and open-air markets were tested using the developed immunobead array

method. Plating on selective agars was used to confirm the results obtained with the immunobead array technique.

For all tested samples (70 samples), *C. jejuni*, *L. monocytogenes*, and *Salmonella* spp. were not found in the immunobead array and these results were confirmed by plating on selective agars (Table 3). The numbers of total bacterial cells after enrichment in all food types on non-selective agar were 1.0×10^9 - 7.0×10^9 and 1.2×10^{10} - 6.3×10^{10} CFU/mL at 24 h and 48 h, respectively.

Previously, in Thailand, between August 2010 and March 2011, high prevalence of *Salmonella* spp. and *Listeria* spp. contamination in food products collected from open-air markets and supermarkets were reported (Ananchaipattana et al., 2012). In that study, 41 of 137 meat samples were found to be contaminated with *Salmonella* spp., while 7 of 88 meat and seafood samples were found to be contaminated with *Listeria* spp.. Subsequently, there have not been any published reports of foodborne pathogen contamination in Thailand. However, trend of *Salmonella* and *Listeria* infection was decreasing. In 2013, the incidence in US was significantly decreased when it was compared during 2010-2012, (Crim et al., 2014). In addition, the prevalence of the contamination of *L. monocytogenes* depended on seasons. For instance, winter season (24 positive samples from 125 samples) had higher incidence of *L. monocytogenes* than spring season (17 positive samples from 134 samples) (Strawn et al., 2013), whereas the contaminations of *Salmonella* spp. were high in summer season (Hall et al., 2002). The fact that we could not find any contamination in food products in Thailand might therefore be from the decreasing trend of pathogen contamination incidences as well as the season of sample collection in this study.

4. Conclusions

The capability to rapidly detect multiple pathogens simultaneously is urgently required for food safety testing. In this study, we demonstrated that pre-enrichment of food samples for three pathogens permitted their detection by a previously developed immunobead array method. The whole process to simultaneously detect *Salmonella* spp., *L. monocytogenes* and *C. jejuni*, which included a short enrichment step and the immunobead array assay, took only 52 hours, whereas the conventional culture methods for the same pathogens according to the ISO protocols take 90-144 hours. Furthermore, in case that *Campylobacter* spp. detection is not required, the multiplex detection of *Salmonella* spp. and *L. monocytogenes* could be finished after enrichment in the same buffer for 24 h. In addition, detection using the immunobead array method did not require long and often two-step enrichment protocols, thus making the immunobead array method attractive in term of reducing labor required for detection of multiple pathogens in many food samples. In addition, the immunobead array could automatically detect 96 samples per round which help reduce cost of assay. Other advantages of this technology were that using small amount of antibodies, reducing interference from the food samples matrices and providing better sensitivities of detection than ELISA method. Furthermore, this method could be expanded to detect more pathogens of interest at the same time. Moreover, the immunobead array assay was about 1.7 times cheaper per one analyte than the immunobased-assay like a sandwich ELISA. The immunobead array is therefore considered to have utility as a routine screening method in the food industry with many types of food sample.

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Figure Captions

Figure 1. The multiplex detection of *Campylobacter* spp., *Listeria monocytogenes*, and *Salmonella* spp. using the immunobead array method. (A) Workflow of multiplex detection for foodborne pathogens. (B) Comparison of the total assay time between the international organization for standardization (ISO) method and the developed immunobead array method. (C) Schematic of immunobead array technique.

Figure 2. Concentration of each bacteria culture after enrichment. (A) Concentrations of the three foodborne pathogens spiked in Universal Pre-enrichment Broth after enrichment for 24 h and 48 h. (B) Concentrations of *Campylobacter jejuni* spiked in Campylobacter Enrichment Broth after enrichment for 24 h and 48 h indicated by white bars and pattern bars, respectively. Error bars represent $\pm$ standard deviation.

Figure 3. Multiplex detection of three foodborne pathogens in in culture media by the immunobead array assay. The designated amounts of bacteria (0 and 1 CFU) were spiked in RTE chicken product at the beginning of the enrichment. The samples were tested by (A) Immunobead array results (reported as a mean fluorescent intensity, MFI). The dashed line indicates a cutoff value which is three times of the signal from the negative control (0 CFU sample). The concentrations of bacteria (CFU/mL) were enumerated using a plating method on selective agars and from total plate count after enrichment for 24 h (B) and 48 h (C). Error bars represent $\pm$ standard deviation within a single sample.

Figure 4. Multiplex detection of three foodborne pathogens in ready-to-eat (RTE) chicken by the immunobead array assay. The designated amounts of bacteria (0 and 1 CFU) were spiked in RTE chicken product at the beginning of the enrichment. The samples were tested by (A)

Immunobead array results (reported as a mean fluorescent intensity, MFI). The dashed line indicates a cutoff value which is three times of the signal from the negative control (0 CFU sample). The concentrations of bacteria (CFU/mL) were enumerated using a plating method on selective agars and from total plate count after enrichment for 24 h (B) and 48 h (C). Error bars represent $\pm$ standard deviation within a single sample.

Figure 5. Multiplex detection of three foodborne pathogens in ready-to-cook (RTC) chicken by the immunobead array assay. The designated amounts of bacteria (0 and 1 CFU) were spiked in RTE chicken product at the beginning of the enrichment. The samples were tested by (A) Immunobead array results (reported as a mean fluorescent intensity, MFI). The dashed line indicates a cutoff value which is three times of the signal from the negative control (0 CFU sample). The concentrations of bacteria (CFU/mL) were enumerated using a plating method on selective agars and from total plate count after enrichment for 24 h (B) and 48 h (C). Error bars represent $\pm$ standard deviation within a single sample.

Table 1. Specific antibodies sets used in the immunobead array method for multiplex detection of three foodborne pathogens

Bacteria	Antibody conjugated bead	Detecting antibody
<i>Campylobacter jejuni</i>	C818 (USDA)	C818 (USDA)
<i>Listeria monocytogenes</i>	7G4 (BIOTEC)	rPAb (BIOTEC)
<i>Salmonella</i> Enteritidis	SalKPL (KPL)	SalAb (AbCAM)

Remarks: USDA: United State Department of Agriculture
BIOTEC: National Center for Genetic Engineering and Biotechnology, Thailand
SalKPL: Kirkegaard and Perry Laboratory Inc. (KPL), USA (Cat. #01-91-99)
SalAb: AbCam Inc., UK (Cat. #ab8273)

Table 2. Types of chicken products used in this study

Products	Sample ID	Numbers	Source
Ready-to-eat			
Grilled black pepper chicken	GBPC	10	Supermarket
Teriyaki chicken	TC	10	Supermarket
Roasted chicken BBQ with honey	BBQ	5	Supermarket
Sausage chicken	SAC	5	Supermarket
Ready-to-cook			
Sesame chicken	SEC	10	Supermarket
Seasoned chicken	SSC	10	Supermarket
Raw chicken			
Raw chicken breast without skin	RCB	10	Open-air market
Raw chicken thigh	RCT	10	Open-air market

Table 3. Validation of the immunobead array for detection three foodborne pathogens in chicken products

Samples	Results from the immunobead array						Results from plate count method			
	<i>C.jejuni</i>		<i>L.monocytogenes</i>		<i>S. Enteritidis</i>		Selective agar		Total plate count (CFU/mL)	
	24h	48h	24h	48h	24h	48h	24h	48h	24h	48h
Ready-to-eat products (RTE)										
GBPC	0/10 (-)	0/10 (-)	0/10 (-)	0/10 (-)	0/10 (-)	0/10 (-)	0/10 (-)	0/10 (-)	$3.0 \pm 1.6 \times 10^9$	$2.0 \pm 0.4 \times 10^{10}$
TC	0/10 (-)	0/10 (-)	0/10 (-)	0/10 (-)	0/10 (-)	0/10 (-)	0/10 (-)	0/10 (-)	$2.7 \pm 0.9 \times 10^9$	$2.7 \pm 0.9 \times 10^{10}$
BBQ	0/5 (-)	0/5 (-)	0/5 (-)	0/5 (-)	0/5 (-)	0/5 (-)	0/5 (-)	0/5 (-)	$2.3 \pm 0.6 \times 10^9$	$2.9 \pm 1.3 \times 10^{10}$
SAC	0/5 (-)	0/5 (-)	0/5 (-)	0/5 (-)	0/5 (-)	0/5 (-)	0/5 (-)	0/5 (-)	$2.5 \pm 1.0 \times 10^9$	$3.3 \pm 0.9 \times 10^{10}$
Ready-to-cook products (RTC)										
SEC	0/10 (-)	0/10 (-)	0/10 (-)	0/10 (-)	0/10 (-)	0/10 (-)	0/10 (-)	0/10 (-)	$4.0 \pm 1.9 \times 10^9$	$3.3 \pm 1.6 \times 10^{10}$
SSC	0/10 (-)	0/10 (-)	0/10 (-)	0/10 (-)	0/10 (-)	0/10 (-)	0/10 (-)	0/10 (-)	$3.4 \pm 1.4 \times 10^9$	$3.3 \pm 1.0 \times 10^{10}$
Raw chicken products (RC)										
RCB	0/10 (-)	0/10 (-)	0/10 (-)	0/10 (-)	0/10 (-)	0/10 (-)	0/10 (-)	0/10 (-)	$3.0 \pm 1.1 \times 10^9$	$3.9 \pm 1.3 \times 10^{10}$
RCT	0/10 (-)	0/10 (-)	0/10 (-)	0/10 (-)	0/10 (-)	0/10 (-)	0/10 (-)	0/10 (-)	$3.7 \pm 1.7 \times 10^9$	$3.7 \pm 1.3 \times 10^{10}$
Negative control	0/3 (-)	0/3 (-)	0/3 (-)	0/3 (-)	0/3 (-)	0/3 (-)	0/12 (-)	0/12 (-)	ND	ND
Positive control	3/3 (+)	3/3 (+)	3/3 (+)	3/3 (+)	3/3 (+)	3/3 (+)	12/12 (+)	12/12 (+)	ND	ND

Remarks: ND = non-detected. The symbol in parenthesis indicates whether the results are negative (-) or positive (+). The x/y indicates number of samples positive results/ total number tested

Figure 1_Revise

Figure 2

Figure 3_revise

Figure 4_revise

Figure 5_revise