Supplementary Information for

A Rapid Abiotic/Biotic Hybrid Sandwich Detection System for Trace Pork Adulteration in Halal Meat Extract

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1. Reagents and materials

Human immunoglobulin G (IgG), human serum albumin (HSA), porcine serum albumin (PSA),
bovine serum albumin (BSA), goat serum albumin (GSA), cheep serum albumin (CSA), rabbit
serum albumin (RSA), Lysozyme (Lyz), Papain, 11-mercaptoundecanoic acid (MUA), N-
hydroxysuccinimide (NHS), ethanolamine, sodium dodecyl sulphate (SDS), DEAE–Sephadex A-
50, and Hitrap Protein A HP were purchased from Sigma–Aldrich (MO, USA). L-Cysteine,
ethylenediamine tetra-acetate (EDTA), N-Isopropyl acryl- amide (NIPAm) and N,N’-
methylenebisacrylamide (MBAA) were purchased from Nacalai Tesque Co. (Kyoto, Japan).
Sephadex G-100 was purchased from GE Healthcare (Tokyo, Japan). Ethanol (EtOH), Dimethyl
sulfoxide (DMSO), 2,2’-azobis (2-methylpropionamidine) dihydrochloride (V-50), and potassium
Eu-encapsulated Preyssler-type phosphotungstate were purchased from Wako Pure Chemical
Industries, Ltd. (Osaka, Japan). 2-Methacryloyloxyethylphosphorylcho- line (MPC) was
purchased from NOF Corporation (Tokyo, Japan). 1- (3-Dimethylaminopropyl)-3-
ethylcarbodiimide hydrochloride (EDC) was purchased from Tokyo Chemical Industries (Tokyo,
Japan). The Protein–Free (PBS) blocking buffer and Methacryloxyethyl Thiocarbamoyl
Rhodamine B (MTRB) were purchased from Thermo Fisher Scientific (MA, USA). ATTO 647N
NHS-ester was purchased from ATTO-TEC GmbH (Siegen, Germany). Bio-safe Coomassie G-
250 was purchased from Bio-Rad Laboratories, Inc. (Japan). PNGase F PRIME glycosidase was purchased
from Funakoshi Co., Ltd. (Japan). The Pig Albumin ELISA Kit (E101-110) and polyclonal
antibody of pig serum albumin (Anti-PSA) was purchased from Bethyl Laboratories, Inc.
(Montgomery, USA). The HiTrap™ Protein A HP column, 1 ml from GE Healthcare, GmbH,
Germany). PSA-MIP-NGs capable of PSA recognition were prepared using a previously reported
procedure.[1] 4-(2-methacrylamidoethylaminomethyl) phenylboronic acid (MAPBA) as a
functional monomer were synthesized using a previously reported procedure.[2]

7987.
2. Instrumentation

The particle size distributions and the zeta potentials were measured using a Dynamic Light Scattering (DLS) system Zetasizer Nano ZS (Malvern Instruments Ltd., Malvern, U.K.). The protein concentration was measured using NanoDrop™ One UV-Vis Spectrophotometer (Thermo Fisher Scientific, Inc., USA). The fluorescence spectra were obtained using an F-2500 fluorescence spectrophotometer (Hitachi High-Technologies, Tokyo, Japan). The fluorescence intensities were carried out by custom-made liquid handling robot equipped with a fluorescence microscope (System Instruments Co. Ltd., Tokyo, Japan). SDS-PAGE was performed using AE-6500 (ATTO, Tokyo, Japan) as electrophoresis tank with PowerPac Basic Power Suply (Bio-Rad, California, USA) with e-PAGEL (ATTO, Tokyo, Japan) as polyacrylamide gel. The absorbance at 450 nm of ELISA assay were measured using a Perkin Elmer Wallac Envision 2100 Multilabel Microplate Reader (PerkinElmer, Inc., Waltham, MA, USA). Transmission electron microscopy (TEM) observation was conducted using JEM-2000FEX II (accelerating voltage: 200 kV, JEOL Ltd., Tokyo, Japan).

3. Preparation of the template Fc domain and deglycosylated Fc domain

Papain digestion of antibodies was carried out using a previously described method\textsuperscript{[3]} with certain modifications. Human IgG was digested with papain at 37 °C for 24 h to obtain the Fc domain. After digestion, the solution was filtered through an Amicon ultracentrifugal filter (10 kDa cut-off, 7500 ×g, three times at 25 °C for 20 min) for desalting, followed by the buffer exchange with 20 mM phosphate buffer at pH 7.0. The obtained IgG fragments were first purified by ultrafiltration (100 kDa cut-off, 7500 ×g, three times at 25 °C for 20 min) to separate the Fc domain (~50 kDa) from the whole IgG (~150 kDa). The collected solution was purified using HiTrap Protein A HP (1 mL). For the evaluation of the purified Fc domain, sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) was carried out. The purified Fc domain were freeze-dried for further use.

For preparing the deglycosylated Fc domain,\textsuperscript{[4]} the purified Fc domain step was incubated with 20 µL of 2 mg/mL PNGase F PRIME glycosidase at 37 °C for 30 min. The solution was then purified and analyzed in the same manner as the Fc domain.
4. Preparation of F-Fc-MIP-NGs and NIP-NGs

The Fc domain (2.5 mg, 50 nmol) as a template molecule, MAPBA (15.7 mg, 0.06 mmol) as a functional monomer, MTRB (0.42 mg, 0.63 μmol) as a fluorescence monomer, MPC (3.7 mg, 0.012 mmol) and NIPAm (102 mg, 0.9 mmol) as a comonomers, MBAA (7.71 mg, 0.05 mmol) as a cross-linker, and V-50 (54.2 mg, 0.2 mmol) as an initiator were dissolved in 10 mM carbonate buffer containing of 2% dimethyl sulfoxide (pH 9.2, 25 mL). Then, the nanogel was synthesized by emulsifier-free precipitation polymerization at 50 °C for 12 h. After polymerization, the solvent was exchanged with 10 mM phosphate buffer saline, (PBS; 140 mM of NaCl, pH 7.4) using ultrafiltration with 10 kDa cut off (7500 × g, three times at 25 °C for 20 min), and the collected nanogels were incubated with SDS aqueous solution (40 mg/mL, 1 mL) for 5 min at 25 °C. The template (Fc domain) removal was performed with size-exclusion chromatography followed by anion-exchange chromatography, as reported previously.[1,2] Finally, further purification to remove SDS was performed using a PD-10 column (Desalt column). The collected fractions (2.5 mL) were applied to a desalt column (PD-10) and eluted with 10 mM PBS (3.5 mL) for removing SDS. To collect the nanogel fractions, fluorescence from the MTRB residues were used as a marker at λem of 575 nm (λex: 548 nm). Non-imprinted polymer nanogels (NIP-NGs) were prepared using the same procedure as Fc-MIP-NGs without adding the Fc domain. The particle size distributions of the obtained MIP-NGs and NIP-NGs, both before and after purification, were determined by dynamic light scattering (DLS) measurements.

F-Fc-MIP-NGs were prepared by the in-cavity post-imprinting modification (PIM) with ATTO 647N NHS ester as the fluorescence reporter. The obtained Fc-MIP-NGs (500 μg/mL, 1000 μL) were incubated with 5 μL of 10 mg/mL ATTO 647N NHS-ester in DMSO at 25 °C for 2 h. The unreacted fluorescent dye was then removed using an Amicon ultracentrifugal filter (10 kDa cut-off, 7500 × g, three times at 25 °C for 20 min) with PBS. To confirm the successful conjugation of fluorescent dyes in Fc-MIP-NGs, the fluorescence intensity of ATTO 647N both before and
after PIM was measured using fluorescence spectroscopy ($\lambda_{\text{ex}}$: 646 nm and $\lambda_{\text{em}}$: 664 nm). F-NIP-NGs were prepared in the same manner as F-Fc-MIP-NGs.

5. Fluorescence measurements of the Fc domain

The glass substrates ($4.3 \times 9.8$ mm) coated with Au (165 nm thickness) with a binder of Ti (5 nm thickness) were washed with pure water and ethanol. The substrates were then dried under N$_2$ and cleaned using UV-O$_3$ treatment for 20 min. The substrates were incubated in ethanol containing 11-mercaptoundecanoic acid (MUA; 1.0 mM) for 24 h at 25 °C to form a carboxylated self-assembled monolayer (SAM) on the surface. After washing the substrates with ethanol and pure water, 100 μL of an aqueous solution containing 0.05 M N-hydroxysuccinimide (NHS) and 0.2 M 1-(3-(dimethylamino) propyl)-3-ethylcarbodiimide·HCl (EDC) was dropped onto the substrates and incubated for 120 min at 25 °C. After rinsing with water, 100 μL PBS containing F-Fc-MIP-NGs or F-NIP-NGs (100 μg/mL) was dropped onto the modified substrates and incubated for 60 min at 25 °C. After the immobilization of NGs, the blocking of unreacted NHS esters was performed by adding 1 M ethanolamine aqueous solution (pH 8.5, 100 μL) for 30 min at 25 °C, followed by the addition of a protein-free (PBS) blocking buffer solution for 30 min (100 μL).

Binding behavior of the Fc domain to the immobilized F-Fc-MIP-NGs and F-NIP-NGs was investigated using a custom-made liquid handling robot equipped with a fluorescence microscope (Figure S2). The NG-immobilized substrate was inserted into a designed flat-type pipette tip and the fluorescence on the substrate was measured as follows. First, the flat-type pipette tips were placed on the tip rack, which was being captured using a robot arm. Next, PBS containing the Fc domain (0, 10, 50, 100, 200, 400, 800 and 1,600 nM) was aspirated into the flat-type pipette tip (150 μL) and incubated for 2 min at 25 °C. After discharging the solution, PBS (150 μL) was aspirated, and the robot arm was moved into the detection port to capture a surface image of the substrate and measure a fluorescence intensity using a Zyla 5.5 sCMOS camera (Andor Technology Ltd, Belfast, UK) equipped with a fluorescence turret (BX3-URA, Olympus, Tokyo, Japan). The experiments were performed in triplicate, and six different regions of interest (ROIs) were selected from each substrate (5× objective lens; LMPLFLN5X, Olympus, Tokyo, Japan); exposure time, 0.1 sec; light source, mercury lamp (HGLGPS-SET, Olympus, Tokyo, Japan), bandpass filters (Cy5), 604–644 nm for excitation and 672–712 nm for emission).
Fluorescence intensities of an initial surface ($F_0$=immobilized NGs–Fonly substrate) on a substrate and a sample-applied surface ($F=F_{incubated~PSA–Fonly~substrate}$) were measured, and relative fluorescence intensity was then calculated from $(F – F_0)/F_0$. To evaluate the selectivity of F-Fc-MIP-NGs and F-NIP-NGs for the Fc domain, 100 nM of structurally related reference proteins, including whole IgG, the deglycosylated Fc domain, and PSA in PBS were used.

6. Preparation of immobilized PSA-MIP-NGs on the gold-coated substrate

The surface activation of the substrate was conducted in the same manner as described in the section 5. PSA-MIP-NGs (100 µg/mL) were immobilized on the activated substrate for 60 min. After washing with pure water, the blocking process was performed by dropping 1 M ethanolamine aqueous solution (pH 8.5, 100 µL) for 30 min, followed by dropping various blocking reagents (PBS, Protein free PBS-blocking buffer, 0.1% and 0.5% (w/v) BSA, and 0.5% (w/v) skimmed milk) for 60 min (100 µL). Finally, the gold-coated substrate with immobilized PSA-MIP-NGs was washed with pure water to evaluate the optimization of the detection conditions.

7. Fluorescence measurements by the developed sandwich detection system for PSA

Fluorescence measurements to analyze the binding and selectivity of the abiotic/biotic sandwich detection system for PSA were performed using a custom-made liquid handling robot equipped with a fluorescence microscope. The abiotic/biotic sandwich detection was performed using the following procedure: (1) the immobilization of PSA-MIP-NGs and the blocking process described in the section 6 (PBS, Protein free PBS-blocking buffer, 0.1% and 0.5% (w/v) BSA, and 0.5% (w/v) skimmed milk) were performed; (2) the cocktail solution was prepared by mixing equal volumes of PBS containing Anti-PSA (0.01, 0.1, 1, 5, and 10 µg/mL) with various concentrations of F-Fc-MIP-NGs (1, 10, 50, 100, and 200 µg/mL), followed by incubation for 30 min. Various concentrations of PSA (0-100 nM) was added to the reaction mixture; (3) the premixed cocktail solution was dropped onto the PSA-MIP-NGs-immobilized substrate and was incubated for 1, 5, 10, 20, 30, and 60 min. (4) After washing with pure water ($3 \times 500$ µL) and PBS ($3 \times 500$ µL), the substrate was inserted into a designed flat-type pipette tip, followed by aspirating 150 µL PBS, and the fluorescence intensity was measured by the custom-made liquid-handling robot equipped with a fluorescence microscope under the conditions described in the section 5. The relative
fluorescence intensity of the binding experiments was calculated using an equation of \((F - F_0)/F_0\), where \(F_0\) and \(F\) are the fluorescence intensities before and after incubation, respectively. To investigate the selectivity of the developed detection system for PSA, 10 nM of reference proteins (BSA, GSA, SSA, and RSA) dissolved in PBS was added to the cocktail solution.

8. Stability of MIP-NGs immobilized on the gold-coated glass substrate

To evaluate the stability of the developed detection system, the PSA-MIP-NGs-immobilized substrates were stored at 4 °C until use. The individual fluorescence detections of 1 nM PSA for the stored substrates on days 1, 3, 7, 15, and 30 were measured under the optimized conditions (the blocking agent: 0.5% w/v BSA, and the cocktail solution prepared by mixing equal volumes of PBS containing 0.1 μg/mL of Anti-PSA with 100 μg/mL of F-Fc-MIP-NGs). The stability was evaluated using the relative fluorescence intensity compared to that on the first day of the preparation.

9. Preparation of meat extract samples

Meat extract samples were prepared from three different raw meat samples (beef and lamb as halal meat, and pork as non-halal meat) purchased from a local Japanese supermarket. Raw meat samples were chopped separately. A portion of 1 g was weighed and mixed with 5 mL of the extraction buffer (PBS), then homogenized using the benchtop homogenizer Polytron PT 1600 E (Kinematica AG, Luzern, Switzerland) for 2 min (10,000 rpm), followed by centrifugation for 30 min at 4 °C (3 \(\times\) 16,000 \(\times\)g). The clear supernatant was collected and filtered through a 0.2 μm PTFE filter (DISMIC-13HP, Toyo Roshi Kaisha Ltd., Tokyo, Japan) three times. The filtered meat extract samples were then used to measure the total protein concentrations using a NanoDrop One UV/Vis Spectrophotometer (absorbance at 280 nm (A280) was measured). The meat extract samples were then kept at –20 °C until use.

10. Recovery of the developed detection system

To investigate the recovery of the developed detection system in real meat extract samples, the beef extracts at different dilutions (1-, 10-, 100-, and 500-folds) appropriated for the PSA binding experiments were spiked with 1 nM PSA dissolved in PBS, and the binding experiment of the
spiked sample was performed for a PSA concentration range of 0–100 nM using the optimized conditions (the blocking agent: 0.5% w/v BSA, and the cocktail solution prepared by mixing equal volumes of PBS containing 0.1 μg/mL of Anti-PSA with 100 μg/mL of F-Fc-MIP-NGs), and the recovery rates were calculated using the following equation:

\[
\text{Recovery rates(\%) = \frac{\text{(Relative fluorescence intensity)}_{\text{spiked sample}}}{\text{(Relative fluorescence intensity)}_{\text{buffer}}} \times 100}
\]

where, \(\text{(relative fluorescence intensity)}_{\text{spiked sample}}\) and \(\text{(relative fluorescence intensity)}_{\text{buffer}}\) values were obtained from the PSA binding experiments in the spiked sample and buffer solution, respectively.

11. Detection of pork contamination in halal meat extract samples

To evaluate the feasibility of detecting pork contamination in halal meat extracts (beef and lamb meat extract samples) using the developed detection system, the meat extracts were prepared by mixing pork and halal meat extracts at concentrations of 0.001, 0.01, 0.1, 1, and 10 wt%. Contaminated halal meat extract samples (0.001, 0.01, 0.1, 1, and 10 wt%), 100 wt% halal beef and lamb extracts as a negative control, and 100 wt% pork extract as a positive control were added to the cocktail of anti-PSA and F-Fc-MIP-NGs, followed by dropping them on the capture MIP-NG-immobilized gold-coated substrate under the optimized conditions (the blocking agent: 0.5% w/v BSA, and the cocktail solution prepared by mixing equal volumes of PBS containing 0.1 μg/mL of Anti-PSA with 100 μg/mL of F-Fc-MIP-NGs). The fluorescence intensities were then measured, and the relative fluorescence intensity was calculated using the conditions described above.

12. ELISA for the detection of pork contamination in halal meat extract samples

The Pig Albumin ELISA Kit (E101-110; Bethyl Laboratories), based on a sandwich ELISA assay and colorimetric detection with a 3.3 h analysis time, was used. The halal meat extract samples for detecting pork contamination by the ELISA kit were prepared in the same manner as described previously. After stopping the reaction, the absorbance of the yellow product was measured at 450 nm using a microplate reader.
13. Preparation of F-Fc-MIP-NGs

**Figure S1.** Schematic illustration of the preparation of F-Fc-MIP-NGs via emulsifier-free precipitation polymerization and PIM
14. Preparation of the Fc domain and the deglycosylated Fc domain from human IgG

**Figure S2.** Schematic representation of (a) preparation of Fc domain using papain digestion and purification through Hitrap Protein A HP 1 mL, and (b) preparation of deglycosylated Fc domain using PNGase F PRIME glycosidase.
Figure S3. A typical chromatogram on the purification of the Fc domain with Hitrap Protein A HP column. Absorbance at 280 nm was measured by Nanodrop under the condition as follows; elution buffer as 0.1 M sodium citrate, pH 3.5, approximate flow rate as 1 mL/min.
Figure S4. SDS-PAGE analysis of purified Fc domain. Lane 1: Protein marker (Bio-Rad), Lane 2-3: IgG, Lane 4-5: Digested IgG, Lane 6-7: 1st purified Fc domain, Lane 8-9: 2nd purified Fc domain and Lane 10-11: purified deglycosylated Fc domain.

The sample buffer was prepared as follows; tris(hydroxymethyl)aminomethane 3 g, glycine 14.4 g, and SDS 1 g were dissolved in 1 L of pure water. The elution buffer was prepared as follows; bromophenol blue (BPB) 1 mg, SDS 400 mg, and glycerol 2 mL were dissolved in 0.5 M tris-HCl buffer (pH 6.8) (2.5 mL), and the solution was then diluted by 5.5 mL of pure water.

To evaluate the protein size using SDS-PAGE, 50 µL of the sample solution (100 µg/mL) and 50 µL of the sample buffer were mixed together and incubated for 5 min at 85 °C. After the incubation, the mixed sample was injected to 12.5% cross-linked polyacrylamide gel for SDS-PAGE, and then the SDS-PAGE was carried out by applying 20 mA for 70 min followed by washing with pure water triplicate on shaker and staining using the bio-safe Coomassie G-250 stain solution for 1 h at room temperature with slowly shaking.
15. Circular dichroism spectra of Fc domain

Circular dichroism spectra were measured for PSA (13.2 mg/mL) dissolved in 10 mM PBS (pH 7.4, 140 mM, 100 mL) before and after the incubation at 50 °C for 12 h (the polymerization condition) at 25 °C by J-725K spectrometer (JASCO, Tokyo, Japan). The CD spectra between 200-255 nm were collected.

**Figure S5.** CD spectra of the Fc domain (100 nM in 10 mM phosphate buffer, pH 7.4) before (red line) and after (blue line) incubation at 50 °C for 12 h.
16. Purification of PSA-MIP-NGs

Figure S6. A typical size exclusion chromatogram of PSA-MIP-NGs with the fluorescence detection ($\lambda_{\text{ex}}$: 548 nm at $\lambda_{\text{em}}$: 570 nm). Column size: 10 cm x 1.5 cm I.D. (column bed: 7cm); Eluent: PBS.
Figure S7. Fluorescence spectra at $\lambda_{\text{ex}}$: 280 nm of tryptophan residues in MIP-NGs for Fc domain before and after purification by two-steps purification. Fluorescence intensities of tryptophan residues of Fc domain at $\lambda_{\text{em}}$: 350 nm before and after purification were 218 and 41, respectively. The concentration of obtained MIP-NGs before and after purification was 500 $\mu$g/mL.

To estimate the removal rate of PSA from PSA-MIP-NGs, the fluorescence intensities of tryptophan residues of PSA in MIP-NGs at $\lambda_{\text{em}}$: 350 nm ($\lambda_{\text{ex}}$: 280 nm) before and after purification were measured under equal concentration of MIP-NGs (500 $\mu$g/mL) and then calculated from the following equation.

\[
\text{Removal rate (\%)} = \frac{\lambda_{\text{em before}} - \lambda_{\text{em after}}}{\lambda_{\text{em before}}} \times 100
\]
17. Particle size distribution of MIP-NGs and NIP-NGs

(a) MIP-NGs

Before purification

- Z-average: 28.42 nm
- Zeta potential: 4.40 mV

After purification

- Z-average: 21.12 nm
- Zeta potential: 19.70 mV

(b) NIP-NGs

Before purification

- Z-average: 18.79 nm
- Zeta potential: 6.75 mV

After purification

- Z-average: 17.80 nm
- Zeta potential: 2.93 mV

Figure S8. Particle size distributions of MIP-NGs and NIP-NGs after purification by size exclusion chromatography and ion-exchange chromatography determined by DLS. (a): MIP-NGs before and after the purification; Z-average particle size: from 28.42 to 21.12 nm; zeta potential: from 4.4 to 19.70 mV. (b): NIP-NGs before and after the purification; Z-average particle size: from 18.79 to 17.80 nm; zeta potential: from 6.75 to 2.93 mV.
18. Transmission electron microscopy images of NGs

Figure S9. Transmission electron microscopy images of NGs

The dispersions containing MIP-NGs and NIP-NGs were dropped on the carbon-coated copper grid (STEM Cu100P Grid, Okenshoji Co. Ltd., Tokyo, Japan). After drying the copper grid in vacuo, 1 wt% potassium Eu-encapsulated Preyssler-type phosphotungstate (Wako Pure Chemical Industries, Osaka, Japan) aqueous solution were put on the grid, then the grid was dried again.
19. Post-imprinting modification of MIP-NGs and NIP-NGs

**Figure S10.** Fluorescence intensity of the ATTO 647N residues in F-Fc-MIP-NGs and F-NIP-NGs after PIM treatment.
20. Fluorescent detection of Fc domain on the immobilized F-Fc-MIP-NGs

Figure S11. Schematic illustration of the immobilisation of F-Fc-MIP-NGs on the gold-coated substrate.

The fluorescence measurement were performed using a custom-made liquid handling robot equipped with a fluorescence microscope.[5]

21. Immobilization of F-Fc-MIP-NGs on the gold-coated substrate

The immobilization procedure of F-Fc-MIP-NGs was performed on the gold-coated substrate.

**Figure S12.** Fluorescence intensity of the gold-coated glass substrate before (red) and after (blue) the immobilization of F-Fc-MIP-NGs.
22. Curve fitting of binding isotherms

The affinity constant for PSA was estimated by curve fitting using DeltaGraph 5.4.5v. The fitting equation (2) is shown below.

\[ Y = \frac{[(1 + KG + KH) - \sqrt{(1 + KG + KH) - 4K^2HG}] \times D}{2KG} \quad (2) \]

The equation is used with the affinity constant for 1:1 complex formation, where \( Y \) is Adsorption \( (\Delta F_{\text{binding}}/\Delta F_{\text{immobilization}}) \), \( K \) is an affinity constant, \( H \) is found by fitting raw data to a theoretical curve, \( G \) is a PSA concentration, and \( D \) is the maximum bound amount of PSA.

**Figure S13.** Affinity constants (Ka) for the binding of Fc domain to F-Fc-MIP-NGs and NIP-NGs, estimated from the relative fluorescence intensity measurements data.
**Figure S14.** Affinity constants ($K_a$) for the binding of F-MIP-NGs to whole IgG and *deglycosylated Fc domain* estimated from the relative fluorescence intensity measurements data.
23. Optimization of the blocking treatments

**Figure S15.** Optimization of the blocking reagents for blank (PBS) analysis. The experiments were conducted in triplicate.

**Figure S16.** Optimization of the different concentrations of anti-PSA as a detection antibody (a). Optimization of the different concentrations of F-Fc-MIP-NGs as a fluorescent secondary antibody mimic (b). The concentration of PSA was 1 nM. The error bars were obtained from triplicate experiments.
Figure S17. Optimization of the binding times. The concentration of PSA was 10 nM. The error bars were obtained from triplicate experiments.
24. Comparison of the binding properties of PSA on the premix solution immobilization and step-by-step immobilization

**Figure S18.** The Concentration-dependent fluorescence responses of the developed detection system with step-by-step immobilization for PSA (0-100 nM) shows a linear calibration range of 0.01–10 nM ($r^2 = 0.998$).

**Figure S19.** The relative fluorescence intensity of PSA binding using the premix solution immobilization and step-by-step immobilization. The PSA concentration was 0-100 nM.
25. Linearity range, and limit of detection of the developed detection system

**Figure S20.** The Concentration-dependent fluorescence responses of the developed detection system for PSA (0-100 nM) shows a linear range of 0.01–10 nM ($r^2 = 0.969$). The error bars were obtained from triplicate experiments.
26. The Reproducibility of the developed detection system

Table S1. The relative fluorescence intensities of the developed sensor for 1 nM PSA. The relative standard deviation (%RSD) was calculated from seven triplicate experiments (n=7).

<table>
<thead>
<tr>
<th>Replicate (Time)</th>
<th>Relative fluorescence intensity $(F-F_0)/F_0$</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>0.9681</td>
</tr>
<tr>
<td>2</td>
<td>0.9250</td>
</tr>
<tr>
<td>3</td>
<td>1.0261</td>
</tr>
<tr>
<td>4</td>
<td>1.1439</td>
</tr>
<tr>
<td>5</td>
<td>1.1958</td>
</tr>
<tr>
<td>6</td>
<td>0.9697</td>
</tr>
<tr>
<td>7</td>
<td>0.9816</td>
</tr>
<tr>
<td>Average</td>
<td>1.0194</td>
</tr>
<tr>
<td>SD</td>
<td>0.076</td>
</tr>
<tr>
<td>% RSD</td>
<td>7.50</td>
</tr>
</tbody>
</table>
27. Total protein concentration of meat extract samples

Table S2. The total protein concentration of meat extracts were measured using NanoDrop One UV/Vis Spectrophotometers.

<table>
<thead>
<tr>
<th>Sample</th>
<th>Protein concentration (mg/mL)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Pork extract</td>
<td>17.17</td>
</tr>
<tr>
<td>Beef extract</td>
<td>14.21</td>
</tr>
<tr>
<td>Lamb extract</td>
<td>17.79</td>
</tr>
</tbody>
</table>

28. Preparation of dilution meat extract samples

Figure S21. The relative fluorescence intensities of spiked 1 nM of PSA into diluted beef extract samples (PBS, 1, 10, 100 and 500-fold dilutions) binding to F-MIP-NGs. Error bars were obtained from triplicate experiments.
29. Preparation of spiked samples for the recovery test

Table S3. The result of recovery rate for determination of PSA in spiked samples (n=3).

<table>
<thead>
<tr>
<th>Spiked samples (nM)</th>
<th>Measured concentration (nM)</th>
<th>Recovery (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.01</td>
<td>0.012</td>
<td>116</td>
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30. Detection of pork adulteration via commercial available ELISA

Figure S22. The absorbance at 450 (nm) of ELISA assay. (a) the calibration curve of determined for a range of PSA protein standards from 0-300 ng/mL ($r^2=0.9959$), (b) the ELISA results from various pork contamination in PBS buffer.