Supporting Information:

Mass Barcode Signal Amplification for Multiplex Allergy Diagnosis by MALDI-MS

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SI-1. Materials and reagents.

Pierce protein A/G@MBs (1 μm diameter) were purchased from Thermo Fisher Scientific (Reinach, Switzerland). Estapor tosyl-activated MBs (1.29 µm diameter) were kindly offered by Merck Chimie (France). Three kinds of mass barcodes, $[S(CH_2)_{11}(OCH_2CH_2)_xOH]_2$), x=3 for Mbc1 (670 Da), x=4 for Mbc2 (758 Da), x=6 for Mbc3 (934 Da), were bought from Sigma-Aldrich (Buchs, Switzerland). Protein linker 1 (PL1, 98%), HS-$(CH_2)_{10}$-COOH, was purchased from Sigma-Aldrich (Buchs, Switzerland). PL2 (HS-$(PEG)_n$-CH_2CH_2-COOH, MW 2000) and PL3 (HS-$(PEG)_n$-NHS, MW 2000) were bought from Nanocs (NY, USA). PL4 (HS-$(CH_2)_{11}(OCH_2CH_2)_6$-OCH_2COOH) was obtained from SensoPath technologies (MT, USA). *N*-hydroxysuccinimide (NHS, 98%), 1-ethyl-3(3-dimethylaminopropyl) carbodiimide hydrochloride (EDC, >98%), 2-(N-morpholino)ethanesulfonic acid (MES), gold(III) chloride trihydrate (>99.9%), sodium citrate dehydrate (>99%), sodium phosphate dibasic dehydrate (>99%), sodium phosphate monobasic dehydrate (>99%) and polyoxyethylene-sorbitan monolaurate (Tween 20) were obtained from Sigma-Aldrich (Buchs, S witzerland). Acetic acid (99.5%), acetonitrile (99.8%), trifluoroacetic acid (TFA, >99%), sinapinic acid (≥ 99%), and 2,5-dihydroxybenzoic acid (DHB) (>99%) were purchased from Fluka (Buchs, Switzerland). Purified bovine milk proteins, including β-lactoglobulin B (β-lac B), bovine serum albumin (BSA), lactoferrin, α-casein, β-casein, and κ-casein were obtained from Sigma-Aldrich (Buchs, Switzerland). Polyclonal anti-bovine β-lac B antibodies (Abs) and monoclonal anti-human IgE Abs were purchased from AbD Serotec (Oxford, U.K.). Blood serum of patient allergic to cow's milk was purchased from Bioreclamation LLC (Westbury, NY, USA). Deionized water (18.2 MΩ cm) was purified by an alpha Q Millipore system (Zug, Switzerland) and used in all aqueous solutions.

SI-2. Synthesis and characterization of gold nanoparticles (AuNPs).

The citrate stabilized AuNPs were synthesized following a protocol reported previously.[1] Briefly, 250 mL of 1 mM $HAuCl_4$ in water was brought to the boil with vigorous stirring, and then rapidly mixed with 25 mL of 38.8 mM sodium citrate. Boiling was continued for 10 min. The heating source was then removed and stirring was continued for another 15 min.

The mean diameter of the synthesized AuNPs was defined as ~12 nm by the transmission electron microscopy (TEM) image recorded by a FEI CM12 (Phillips, Eindhoven, Netherlands) transmission electron microscope, operating with a LaB6 electron source at 120 kV, as shown in Figure S1(a). An alternative method to determine the mean diameter and approximate concentration of the colloidal AuNPs solution is UV-Vis spectroscopy analysis on a PerkinElmer Lambda XLS+ spectrophotometer (Waltham, MA, USA) with a 10 mm polystyrene cell, as detailed by Haiss *et al*.[2] Based on this method, the concentration was calculated as 4.5×10^{12} particles/mL, and the mean diameter was defined as 13 nm, in Figure S1(b). Considering that the AuNPs solutions were diluted twice for UV-Vis absorption measurement, the original concentration of AuNPs was 9×10^{12} particles/mL (~15 nM).

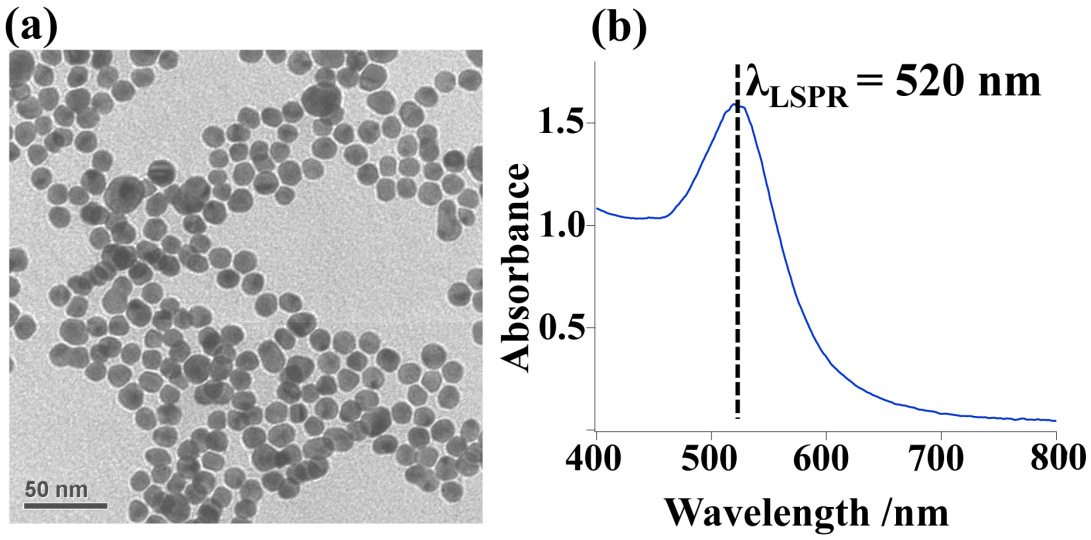

Figure S1. (a) TEM image of synthesized AuNPs. **(b)** UV-Vis spectrum of the synthesized AuNPs.

SI-3. Preparation of mass-barcoded AuNPs probes.

To prepare the AuNPs probes, the obtained AuNPs were firstly derivatized with mass barcodes and protein linkers through the formation of stable Au-S bonds on particle surfaces. The saturation amount of thiol-terminated oligomers that can replace citrates and fully wrap the AuNPs in a brush shape was estimated to be around 15 nmol for 500 μL of AuNPs by considering that each thiol-PEG molecule occupied a footprint area of 0.35 nm^2 on the AuNPs surface.[3] It is necessary to ensure complete surface functionalization for efficient protection of AuNPs from the salt-induced aggregation and nonspecific adsorption during the following protein conjugation and immunobinding steps. Therefore, in a typical experiment, five times higher amount (75 nmol) of thiol-containing oligomer mixture of mass barcodes (64 nmol) and protein linkers (11 nmol) in ethanol was mixed with 500 μL of AuNPs in water and incubated for 12 h with moderate vortexing. Excessive reagents were removed by centrifugation for 20 min at 13000 rpm. The precipitate was washed with pure water (1 mL each) for three times by successive centrifugation and redispersion. For semi-quantification purposes, similar protocol was applied for the preparation of internal standard conjugated AuNPs (IS@AuNPs), just by replacing the polymer mixture with pure IS (75 nmol), which was Mbc2, when Mbc1 was used for AuNPs probe in the analysis of a model analyte, anti-bovine β-lac B Abs. The successful modification of AuNPs by the mass barcode, *e.g.* Mbc1, and the chosen protein linker PL4 was demonstrated by MALDI-MS analysis in Figure S2.

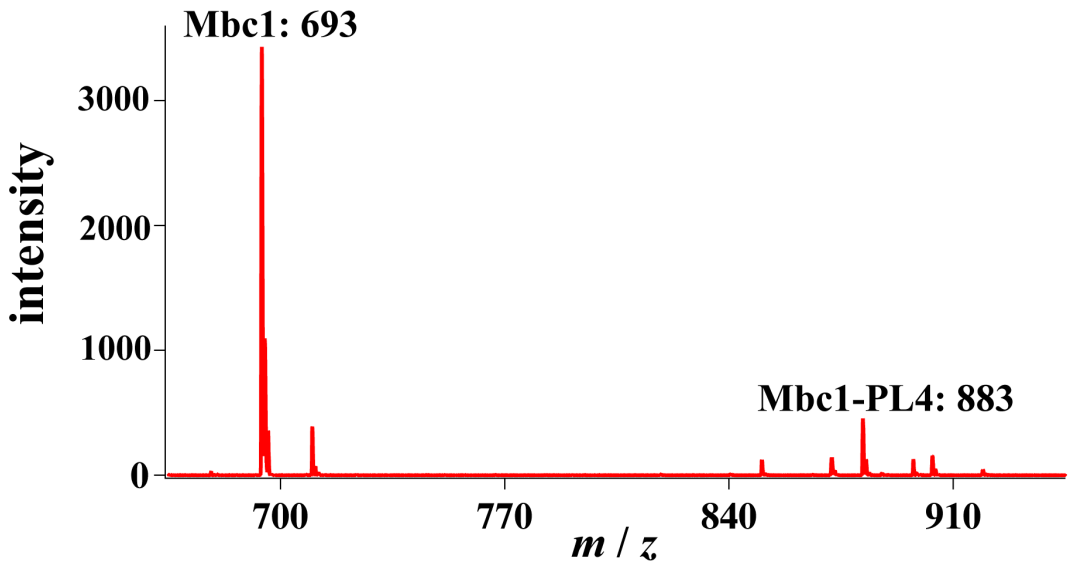

Figure S2. MALDI-MS analysis of mass barcode Mbc1 and chosen protein linker PL4 present on

modified AuNPs. The Mbc1 corresponded to a peak at *m/z* 693 $(M+Na)^+$. The PL4 formed disulfide bonds with Mbc1 ($(HO(OCH_2CH_2)_3(CH_2)_{11}S\text{-}S(CH_2)_{11}(OCH_2CH_2)_6\text{-}OCH_2COOH$) and presented as a major peak at *m/z* 883 $(M+Na)^+$, indicating the successful immobilization of PL4 to the AuNPs. PL: protein linker.

In a second step, proteins were immobilized on the mass-barcoded AuNPs *via* EDC-NHS coupling protocol. Before protein conjugation, 500 μL of AuNPs probes were concentrated to 10 μL by centrifugation. Then, they were activated with 50 μL of freshly prepared EDC (15 mg/mL) and NHS (15 mg/mL) in 15 mM MES buffer (pH 5) for 30 min at room temperature (RT). After washing with MES buffer for 3 times (1 mL each), the NHS-activated AuNPs were immediately incubated with sufficient amount of appropriate allergenic proteins (30 μL, 1 mg/mL) in 10 mM phosphate buffer (PB, pH 7.4) for 3h at RT. Then, after washing with 10 mM PB for 3 times to remove unbound proteins, obtained proteins@AuNPs were finally resuspended in 500 μL of 10 mM PB to a final concentration of 15 nM and stored at 4°C. The successful immobilization of proteins, *e.g.* β-lac B, was also demonstrated by MALDI-MS analysis in Figure S3, providing β-lac B@AuNPs.

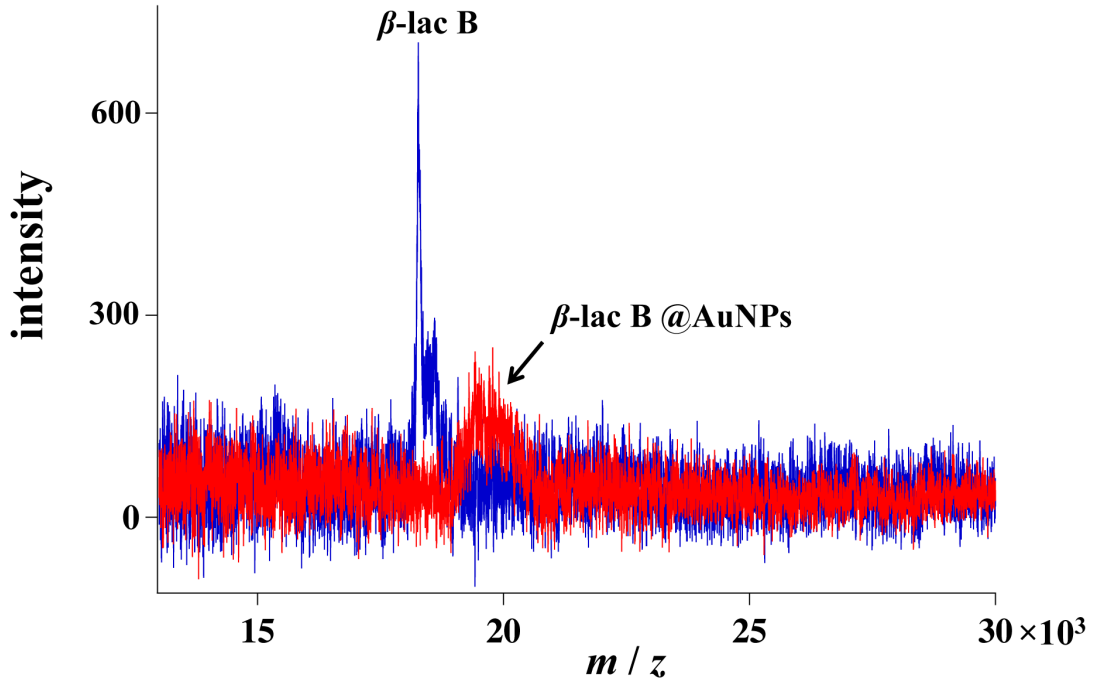

Figure S3. MALDI-MS analysis of β-lac B (1 μg/mL, blue spectrum) and β-lac B@AuNPs (15 nM, red spectrum) with 5 mg/mL sinapinic acid as the matrix. There is a mass shift of around 900 Da for β-lac B covalently conjugated on Mbc1-coded AuNPs, which may origin from the Mbc1-PL4 modification (+860 Da) to β-lac B.

SI-4. Semi-quantification of anti-bovine *β*-lac B Abs.

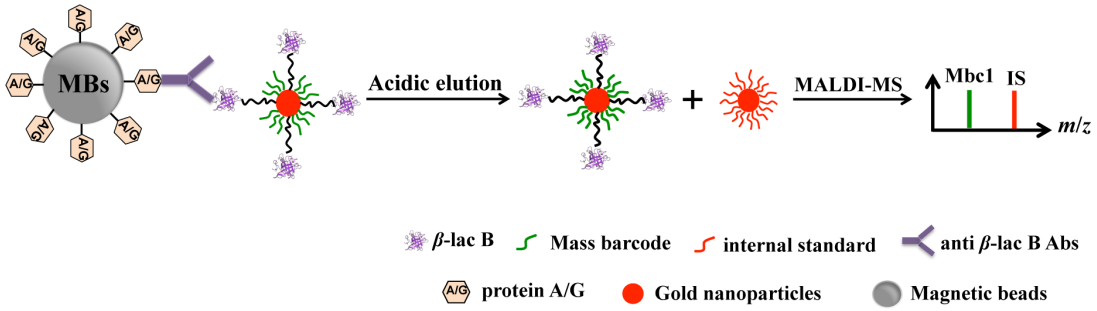

Figure S4. Schematic representation of *β*-lac B Abs semi-quantification procedure with mass barcoded AuNPs and MBs. MALDI-MS: matrix–assisted laser desorption/ionization mass spectrometry; MBs: magnetic beads; Mbc: mass barcode; IS: internal standard; Abs: antibodies.

300 μL of anti-bovine *β*-lac B Abs at different concentrations in 10 mM PB were incubated with 10 μL of 10 mg/mL protein A/G@MBs for 30 min at RT. After magnetic separation, the captured anti-bovine *β*-lac B Abs on the MBs were mixed with 300 μL of *β*-lac B@AuNPs (1 nM AuNPs)[4] for another 30 min. After rinsing with 10 mM PB for 3 times (300 μL each), the *β*-lac B@AuNPs captured on MBs surface were eluted with 5 μL of 10% acetic acid and then concentrated to ≈1 μL with a weak stream of nitrogen. The concentrated sample was deposited together with 0.5 μL of 10 mg/mL DHB (dissolved in 70% acetonitrile, 29.9% water, 0.1% TFA) and 0.5 μL of IS@AuNPs (0.1 nM AuNPs) on a MALDI target plate for MS detection by a Microflex LRF MALDI TOF instrument (Brüker Daltonics, Bremen, Germany). The instrument was operated in a positive reflectron mode. An average spectrum from 800 laser shots at different positions was collected for each sample spot. Instrumental parameters were optimized to achieve highest sensitivity and resolution for the detection of mass barcode molecules.

SI-5. Choice of MBs with immunoaffinity coating.

In the preliminary feasibility test, two kinds of MBs, tosyl-activated MBs and protein A/G@MBs, were compared. The immunoaffinity reaction between *β*-lac B Abs captured on MBs and *β*-lac B on AuNPs was studied as the model system. To simplify the process, 2.5 μg of *β*-lac B were nonspecifically adsorbed onto 500 μL of 15 nM AuNPs that were further blocked with 1 μL of Tween 20 prior to the washing step. The *β*-lac B-coated AuNPs through nonspecific adsorption (*β*-lac B_NA@AuNPs) were finally dissolved in 500 μL of 10 mM PB containing 0.1% Tween 20.

Anti-bovine *β*-lac B Abs can be covalently immobilized onto tosyl-activated MBs following a commercially available protocol.[5] 20 μL of the formed *β*-lac B Abs@MBs (5 mg/mL) were then treated with 20 μL of *β*-lac B_NA@AuNPs for 30 min at RT in 300 μL 10 mM PB. No obvious red color was observed in the supernatant after acidic elution and MALDI-MS analysis of the supernatant gave weak signal of *β*-lac B. The low immunobinding efficiency of *β*-lac B Abs@MBs with *β*-lac B_NA@AuNPs may come from the steric hindrance between the two kinds of particles and the random orientation of the Abs and antigens on particles.

Instead, we adopted commercial recombinant protein A/G@MBs, where each protein A/G holds four Fc binding domains toward anti-bovine *β*-lac B Abs and thus can capture more Abs on MBs. Moreover, the Abs are highly oriented on MBs, exposing Fab-binding domains outside properly for efficient binding with antigens. A high concentration of anti-bovine *β*-lac B Abs (10 nM in 300 μL of 10 mM PB) were analyzed with 10 μL protein A/G@MBs (10 mg/mL) and 20 μL *β*-lac B_NA@AuNPs (15 nM). The collected and concentrated supernatant had a red color and gave a strong peak of *β*-lac B in MALDI mass spectra (data not shown). Therefore, protein A/G@MBs were employed as the immunomagnetic support in this work.

SI-6. Choice of protein linker.

Four protein linkers (PLs) were compared for the protein conjugation on AuNPs. Their structures were as shown below:

PL1: HS-$(CH_2)_{10}$-COOH, MW 218 Da;

PL2: HS-$(PEG)_n$-CH_2CH_2-COOH, MW 2000 Da;

PL3: HS-$(PEG)_n$-NHS, MW 2000 Da;

PL4: HS-$(CH_2)_{11}(OCH_2CH_2)_6$-OCH_2COOH, MW 526 Da.

The carboxyl-based PLs, including PL1, PL2 and PL4, were modified onto AuNPs together with Mbc1 at an amount ratio of 10:90. The total amount of the oligomer mixture was 75 nmol per 500 μL of AuNPs. Then, three kinds of β-lac B@AuNPs probes were prepared *via* EDC-NHS reaction with the three PLs. *K*-casein@AuNPs were also prepared with the PLs and used for control experiments. These AuNPs probes were utilized with protein A/G@MBs to detect 10 nM of anti-bovine β-lac B Abs in 300 μL 10 mM PB. For PL1, serious nonspecific adsorption was observed from control experiments, which may come from the strong hydrophobicity of the PL1. For PL2, the obtained AuNPs could not be stabilized in salt-rich solution during the NHS activation-based protein conjugation process.

To prepare proteins@AuNPs *via* PL3, 50 μL of protein solution (1 mg/mL) in 10 mM PB firstly reacted with excess amount of PL3 (3 μL, 10 mg/mL) for 3h at RT. Then, the formed protein-PL3 complex was purified by ultrafiltration with Amicon Ultra-2mL centrifugal filter to remove excess PL3. The purification procedure was repeated for 3 times. 2.5 μg of the protein-PL3 complex was added into 500 μL of 15 nM AuNPs solution for 20 min reaction. The protein-conjugated AuNPs were further treated with excess amount of Mbc1 (75 nmol) for 12h at RT. The obtained β-lac B@AuNPs probes were then used with protein A/G@MBs to detect 10 nM anti-bovine β-lac B Abs in 300 μL 10 mM PB. However, the captured AuNPs gave only weak MS signal of Mbc1.

Finally, PL4 was chosen as the protein linker for the allergy CRD application. The resulting AuNPs co-modified with Mbc1 and PL4 were stable throughout the NHS activation-based protein conjugation process, showed low level of nonspecific adsorption during the immunoassay, and higher signal intensity of Mbc1 than the AuNPs probes prepared with PL3.

SI-7: Mass spectra of Mbc1-coded AuNPs with and without DHB

AuNPs were previously demonstrated to facilitate MS analysis and used as desorption/ionization matrices.[6,7] In the current work, we have compared the MS analysis of mass barcodes with and without organic matrices. The results showed that mass barcode was detectable with only AuNPs-assisted LDI. However, the Mbc1 signal could be highly enhanced in the presence of DHB as a matrix, in Figure S6. It is worth to mention that DHB was used as the matrix due to its low interference to the MS signal of the chosen mass barcodes. Therefore, all the mass spectra presented in the paper were obtained with DHB matrix.

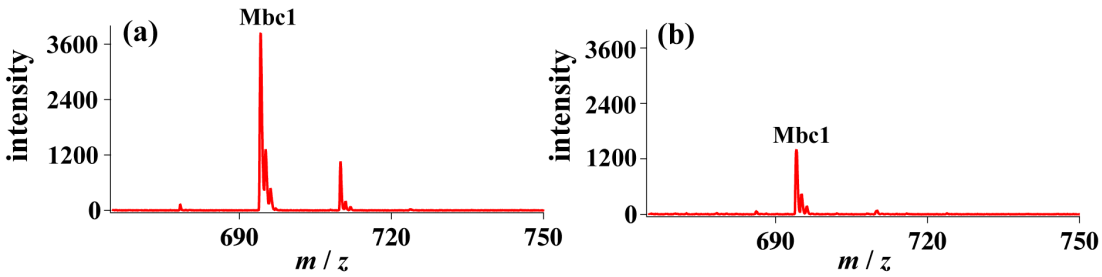

Figure S5. (a) and **(b)** MALDI mass spectra of 1 nM Mbc1-coded AuNPs with and without DHB as matrix, respectively. The spectra were acquired under positive mode with an accelerating voltage of 19 kV, a 20 Hz repetition rate, the laser intensity of ~ 40% (instrumental value) and an average of 800 shots.

SI-8. Optimization of the amount ratio between mass barcode and protein linker for AuNPs surface modification.

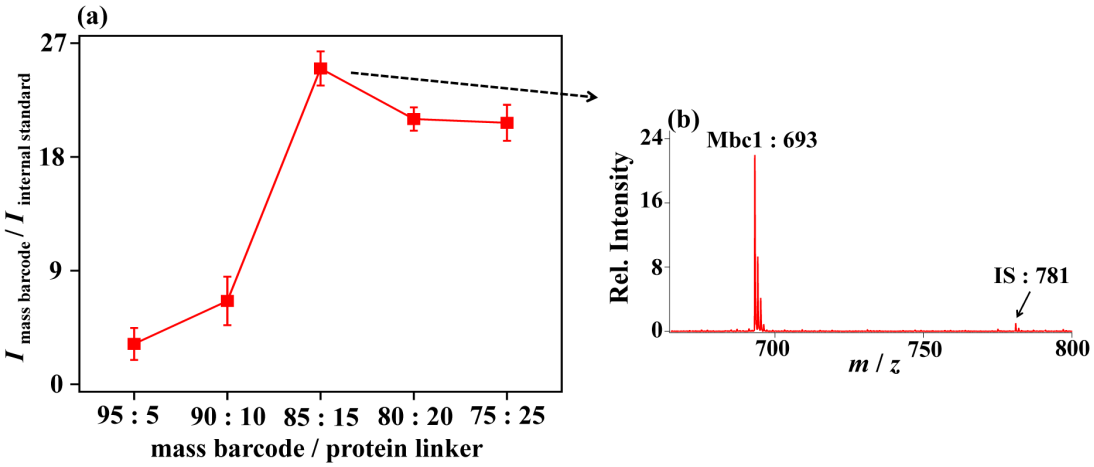

Figure S6. Detection of 1 nM anti-bovine β-lac B IgG Abs by the mass-barcoded AuNPs MALDI-MS signal amplification method combined with immunomagnetic separation. **(a)** Optimization of the amount ratio between mass barcode Mbc1 and protein linker PL4 on AuNPs. **(b)** Mass spectrum of the Mbc1 ($M + Na^+ = 693$ m/z) under the optimized condition for AuNPs functionalization. Conditions: 1 nM anti-bovine β-lac B Abs in 300 μL 10 mM PB, 100 μg of protein A/G@MBs, and 300 fmol β-lac B@AuNPs.

SI-9. UV-Vis absorption measurement to check the nonspecific absorption.

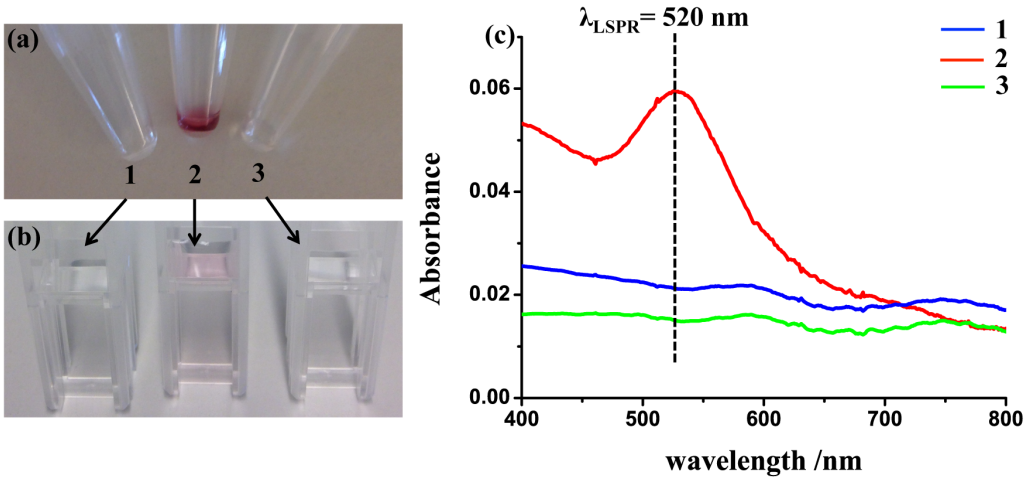

Figure S7. (a) optical image of the 5 μL supernatants eluted from 100 μg of protein A/G@MBs by the acidic solution for the analysis of **(1)** blank sample and 1 nM anti-bovine β-lac B Abs in 300 μL 10 mM PB with 300 fmol **(2)** β-lac B@AuNPs, and **(3)** κ-casein@AuNPs, respectively, under the optimized condition. **(b)** optical image of the diluted supernatants in 500 μL H_2O subjected for UV-Vis absorption measurements. **(c)** UV-Vis spectra of the diluted supernatants from the sample 1, 2 and 3.

SI-10. Detection sensitivity of the three mass barcodes by MALDI-MS.

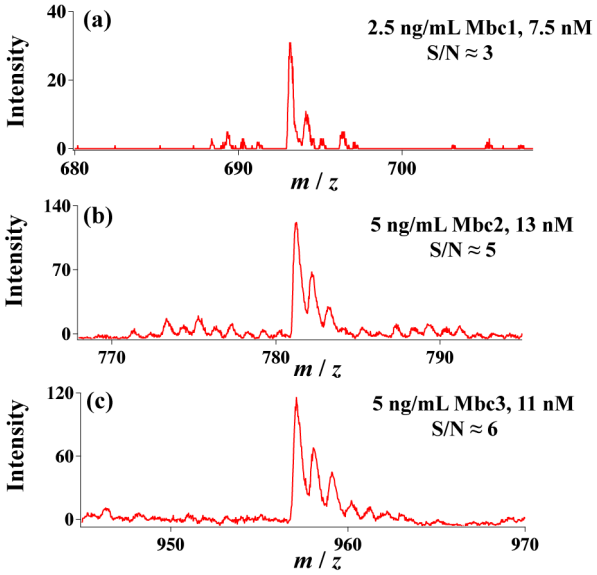

Figure S8. MALDI mass spectra of the mass barcodes: 1 *u*L of **(a)** 2.5 ng/mL Mbc1, **(b)** 5 ng/mL Mbc2, and **(c)** 5 ng/mL Mbc3. 0.5 *u*L of 10 mg/mL DHB was used as the matrix.

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