Electronic Supplementary Material (ESI) for ChemComm.

Electronic Supplementary Information (ESI)

Combinatorial Immunoassay for Multiple Biomarkers by Stable Isotope tagging Strategy

Gongwei Sun,[a] Biao Huang,[b] Yi Zhang,[b] Yuqing Zhang,[a] Zhi Xing,[a] Sichun Zhang*[a] and Xinrong Zhang[a]

[a] Beijing Key Laboratory for Microanalytical Methods and Instrumentation, Department of Chemistry, Tsinghua University, Beijing 100084, China.
[b] Key Laboratory of Nuclear Medicine, Ministry of Health, Jiangsu Key Laboratory of Molecular Nuclear Medicine, Jiangsu Institute of Nuclear Medicine, Wuxi, Jiangsu 214063, China.

Corresponding Author: E-mail: sczhang@mail.tsinghua.edu.cn

Experimental Section

1. Reagents and Materials. Streptavidin-functional magnetic microparticles (Dynabeads M-280 Streptavidin) and separation magnets (DynaMag-2) were commercially available from Invitrogen Co. (Shanghai, China). S-2-(4-Isothiocyanatobenzyl)-diethylenetriamine pentaacetic acid (NCS-DTPA) was purchased from Macrocyclics (Dallas TX). Biotinamidohexanoyl-6-aminohexanoic acid N-hydroxysuccinimide ester (biotin-LC-LC-NHS) and Albumin bovine serum (BSA) were obtained from Sigma Aldrich. Sm_2O_3, Ho_2O_3, $PrCl_3$, $GdCl_3{\cdot}6H_2O$, $TmCl_3{\cdot}6H_2O$, and $LuCl_3{\cdot}6H_2O$ (purity, 99.9%~99.99%) were purchased from Aladdin Chemistry Co. Lat. ^{151}Eu-, ^{153}Eu-enriched Eu_2O_3 (=97.7% ^{151}Eu, 99.8% ^{153}Eu), ^{163}Dy-enriched Dy_2O_3 (=94.6% ^{163}Dy) and ^{166}Er-, ^{168}Er-, ^{170}Er-enriched Er_2O_3 (=98.1% ^{166}Er, 98.2% ^{168}Er, 97.7% ^{170}Er) were from Cambridge Isotope Laboratories Inc. The deionized water (18.2 $M\Omega cm^{-1}$) from a Milli-Q water purification system (Millipore Milford, MA, USA) was used throughout the experiments. All of the antigens, monoclonal antibodies and the REEs-labelled antibodies (Table S1) were purchased from the Jiangsu Institute of Nuclear Medicine (Wuxi, China). All the buffers used in the experiment were purchased from the Jiangsu Institute of Nuclear Medicine except PBS (1x, pH 7.4) from coring and NH_4Ac (0.5M, pH 5.8) from Beijing Dingguochangsheng Biotechnology Co. Ltd. All Chemicals and reagents used were at least of analytical or higher grader.

Table S1. List of antibodies against antigens tagged with different REEs.

Antigens	Tagged REEs
Ferritin (FER)	Pr
Human epididymis protein 4 (HE4)	^{151}Eu
Beta-2-microglobulin (β2-MG)	Sm
Alpha-fetal protein (AFP)	^{153}Eu
Pepsinogens II (PGII)	Gd
β-human chorionic gonadotropin (βhCG)	^{163}Dy

Prostate specific antigen (PSA)	Ho
Carbohydrate antigen 242 (CA242)	^{166}Er
Carcino-embryonic antigen (CEA)	^{168}Er
Carbohydrate antigen 153 (CA153)	Tm
Human epidermal growth factor receptor 2 (HER2)	^{170}Er
Pepsinogens I (PGI)	Lu

2. Instrumentations. An iCAP Q ICP-MS (Thermo Fisher Scientific GmnH, Germany) was used for the experiments. The instrument settings were optimized (Table S2) prior to analysis by using tuning solution according to the recommendation from manufacturer. The chromatographic separation system included a Model EP-1 Econo Pump and a Model EM-1 Econo UV monitor (Bio-Rad). The nanoESI capillary was pulled using a P-2000 laser puller (Sutter Instrument Co., Novato.CA, USA). A voltage of 1.5 kV was used for nanoESI-MS analysis.

Table S2. Operating parameters of ICP-MS

Parameter	Value
cool gas flow (L/min)	14
auxiliary gas flow (L/min)	0.8
nebulizer gas flow (L/min)	0.92
sample uptake (s)	40
dwell time (ms)	0.02
channel	3
number of repeats per sample	3
PC detector voltage (V)	1275
RF power (W)	1548.61
analogue detector voltage (V)	-1950

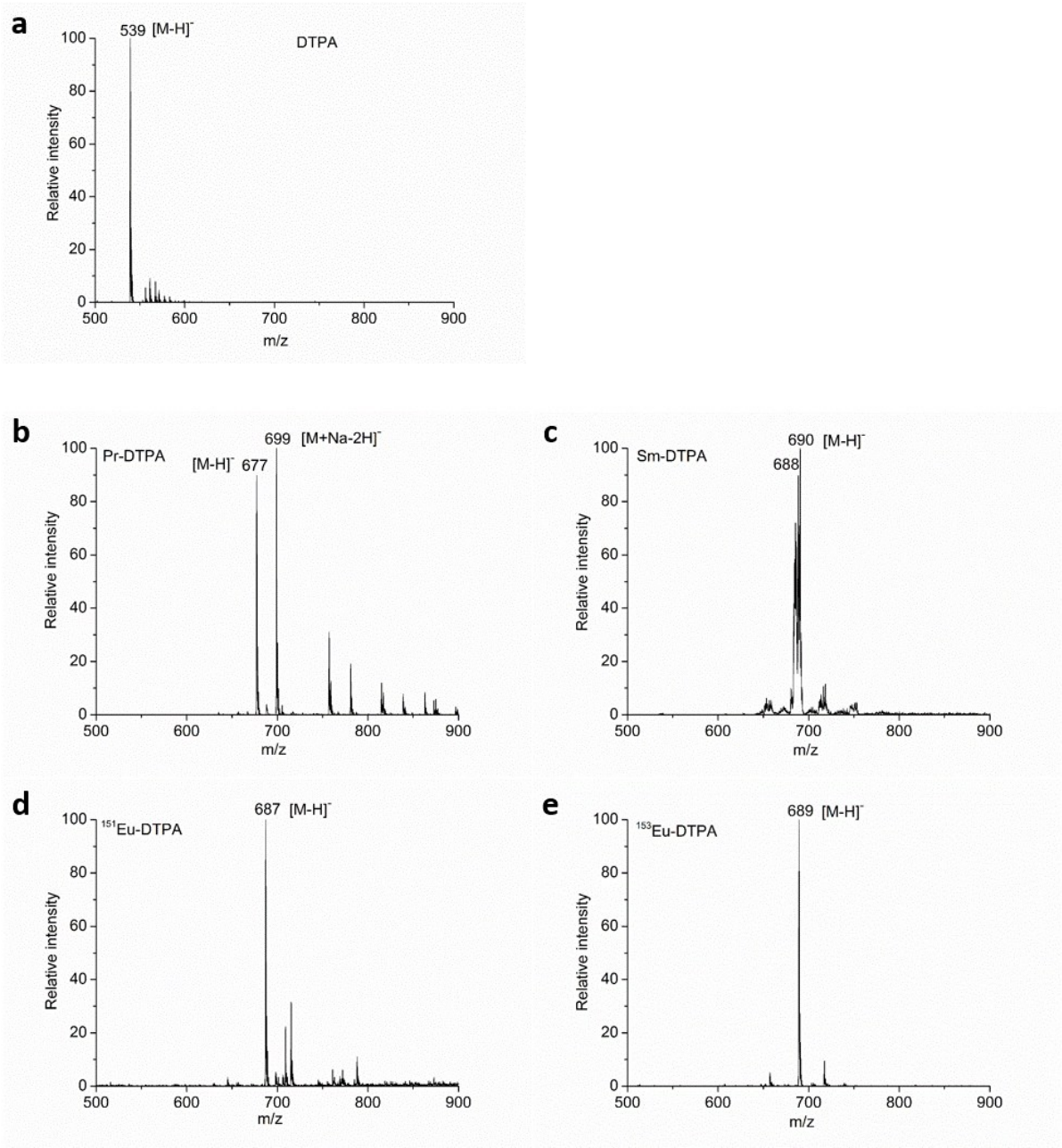
3. Experimental procedures

3.1 Antibody Labeling

Labeling Antibody with Biotin-LC-LC-NHS. The labeling antibodies were prepared according to the method reported previously.[1] Briefly, 0.05 mg biotin-LC-LC-NHS in 0.05 M PBS buffer (pH 7.2) was reacted with 0.25 mg monoclonal antibodies (McAbs) for 1 h at 30℃. Then, the reaction mixture was dialyzed against PBS buffer (3 times) for 24 h to remove the excess of reactants.

Labeling Antibody with REEs. In the first step of chelating elemental ions: $PrCl_3$, $GdCl_3 \cdot 6H_2O$, $TmCl_3 \cdot 6H_2O$, and $LuCl_3 \cdot 6H_2O$ were directly dissolved to 100 mM by NH_4Ac buffer (pH 5.8, 0.5 M); Isotope oxides or rare earth oxide were dissolved with high-purity HNO_3, and also diluted to 100 mM by NH_4Ac buffer as elemental stock solutions. Then, each of 2.08 mg NCS-DTPA was dissolved to 120 μl by NH_4Ac buffer, and 40 μl elemental stock solution were added to the solution, respectively. The reaction was allowed to proceed for 1h with gentle shaking at 37℃. In the next step of labeling antibody: The labeling procedure have been described previously.[2] Briefly, 0.5 mg antibodies in sodium bicarbonate buffer (pH 8.5, 50 mM) were reacted with 50 μl rare earth ion chelating agent. The reaction between the NCS group of NCS-DTPA and NH_2 group of antibodies was allowed to process for 18h with gentle shaking at room temperature. The labeling antibodies were purified through gel filtration on a column of Sepharose CL-6B (1 cm x 40 cm) with an elution buffer of 50mM Tris-HCl (pH 7.8).

NanoESI-MS results of REEs-DTPA chelates (Figure S1)



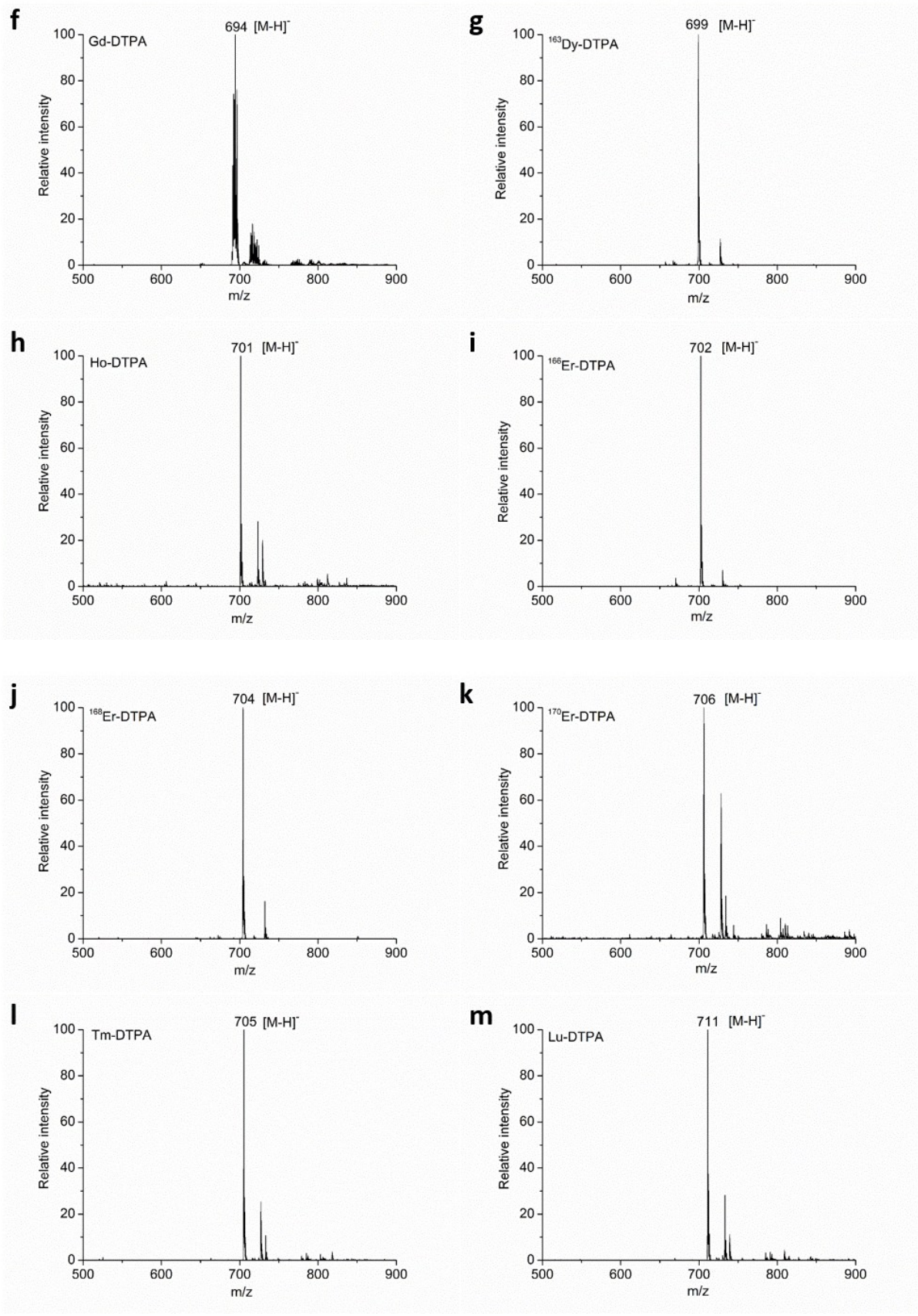


Figure S1. NanoESI-MS results of REEs-DTPA chelates and DTPA (from a to m), respectively.

3.2 Assay Protocol

The multiplex immunoassay was performed in a single analysis. Firstly, the suspension of magnetic microparticles (MMPs, 10 mg/ml) were reacted with biotinylated monoclonal antibodies (1 mg/ml) in PBS (1x, pH 7.4) for 30min at 25℃ with gentle shaking, respectively. The antibody-conjugated MMPs were washed with washing buffers (Tris-HCl, 0.05 M; pH, 7.4) for 3 times and selectively mixed according to the measuring requirements. Then, 50 μl of series dilution of standards or serums with assay buffers (Tris-HCl, 0.05M; pH, 7.8) were added and the mixture were shaken for 1 hours at 25℃. After washing 3 times by washing buffer, 200 μl of REEs-labelled antibodies or antigens were added and incubated for 1h with continuous shaking at 25℃ and then washed six times with washing buffer to thoroughly remove the extra labeling antibodies. Then, adding 100 μl of HNO_3 solution (1%, v/v) to dissociate labeling elemental ions from MMPs. After 10min of continuous shaking at room temperature, the solutions were introduced into ICP-MS for subsequently detection.

3.3 TRFIA Procedures

Human serum samples (AFP, CEA, FER, PGI, PGII and β2-MG) were measured with a commercial TRFIA kit (Jiangsu Institute of Nuclear Medicine) according to the manufacturer's instructions. Briefly, 50 μl of diluted standards or serum samples were added to microtiter well and incubated for 1h at 25℃. Then, the microtiter plate was washed for 3 times. 200 μl of diluted europium-labelled antibodies were added to microtiter well and incubated for 1h at 25℃. After washing six times, enhancement solution was added. The microtiter plate was incubated for 5 min and then read in time-resolved fluorescence instrument.

3.4 Sample Collection

The human serum samples were collected at the Jiangyuan Hospital (Wuxi, China), after the patients gave informed consent. Twenty one serum samples collected were used in the group of FER, AFP and CEA. Twenty serum samples collected were

used in the group of PGI, PGII and PSA. Twenty five serum samples collected were used in the group of FER, AFP and β2-MG. These serum samples were stored at -20℃ until they were assayed.

3.5 Optimization of experimental condition for combinatorial immunoassay

The conditions affecting the combinatorial immunoassay were optimized to achieve the best analytical performance, including the diluted ratio of the biotinylated antibodies, REEs-labelled antibodies/antigens and MMPs. Besides, the time that the streptavidin-functional MMPs captured the biotinylated antibodies were also optimized for the competitive and non-competitive immunoassay. Fig. S2 represented the optimization of biotinylated antibodies, REEs-labelled antibodies, and MMPs for FER, AFP and CEA. A diluted ratio of 1:300 for biotinylated anti-FER, AFP and CEA antibodies was chosen. A diluted ratio of 1:200 for REEs-labelled FER, AFP and CEA antibodies was chosen. A diluted ratio of 1:30 for MMPs of FER, AFP and CEA was chosen for further studies. Fig. S3 represented the optimization of biotinylated antibodies, REEs-labelled antibodies, and MMPs for PGI, PGII and PSA. A diluted ratio of 1:300 for biotinylated anti-PGI, anti-PGII and anti-PSA was chosen. A diluted ratio of 1:200 for REEs-labelled PGI, PGII and PSA was chosen. A diluted ratio of 1:30 for MMPs of PGI, PGII and PSA was chosen for further studies. Fig. S4 represented the optimization of REEs-labelled antibodies and MMPs for β2-MG. A diluted ratio of 1:80 for Sm-labelled β2-MG antigens and 1:80 for MMPs of β2-MG were chosen for the further studies. The time that the streptavidin-functional MMPs captured the biotinylated anti-FER, anti-AFP and anti-β2-MG was 30 min.

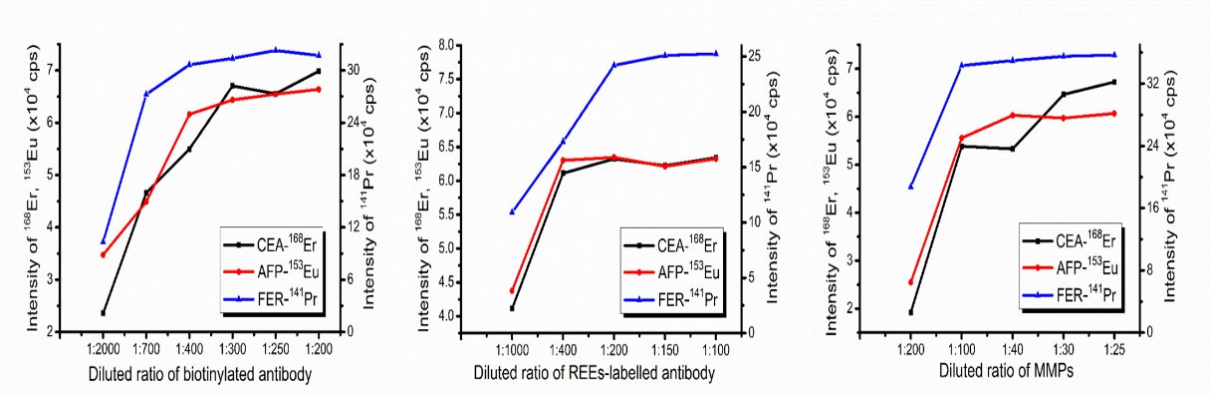


Figure S2. Optimization of biotinylated antibodies, REEs-labelled antibodies, and MMPs for FER, AFP and CEA.

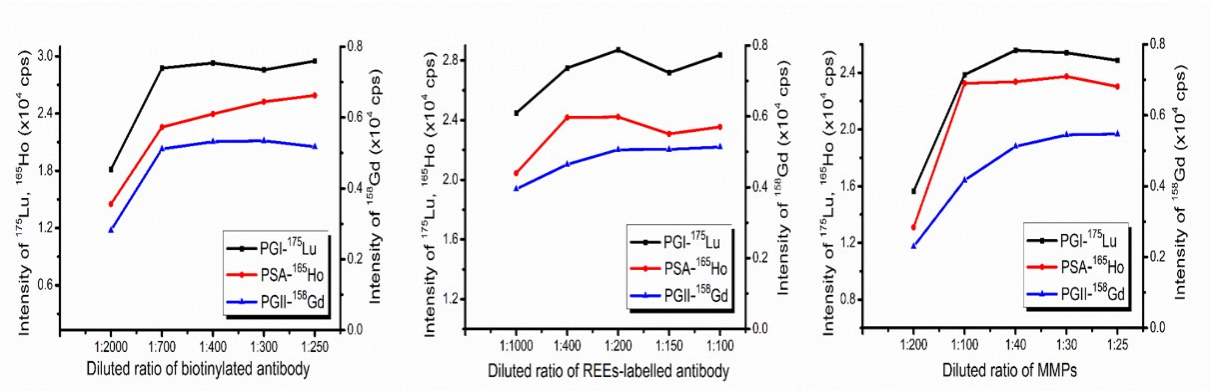


Figure S3. Optimization of biotinylated antibodies, REEs-labelled antibodies, and MMPs for PGI, PGII and PSA.

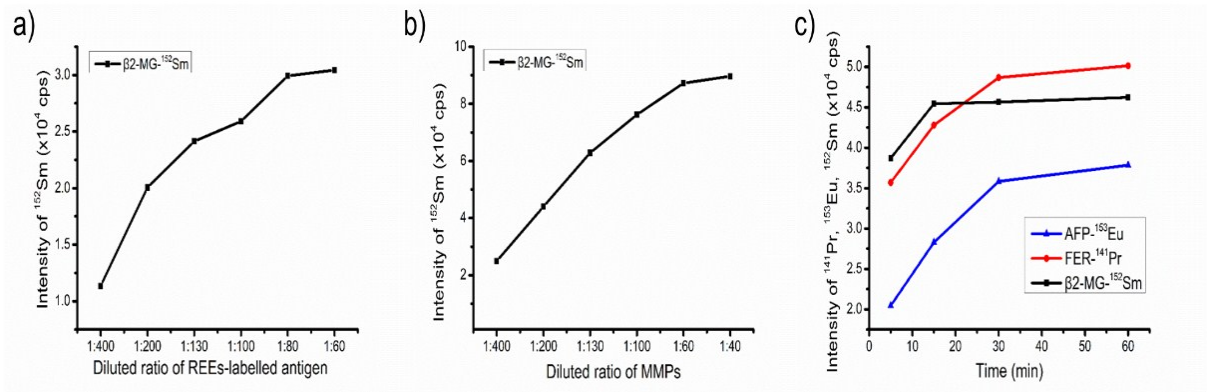


Figure S4. a) Optimization of Sm-labelled antigens for β2-MG. b) Optimization of MMPs for β2-MG. c) Optimization of time for FER, AFP and β2-MG.

The calibration curves of 12 kinds of protein targets (Figure S5)

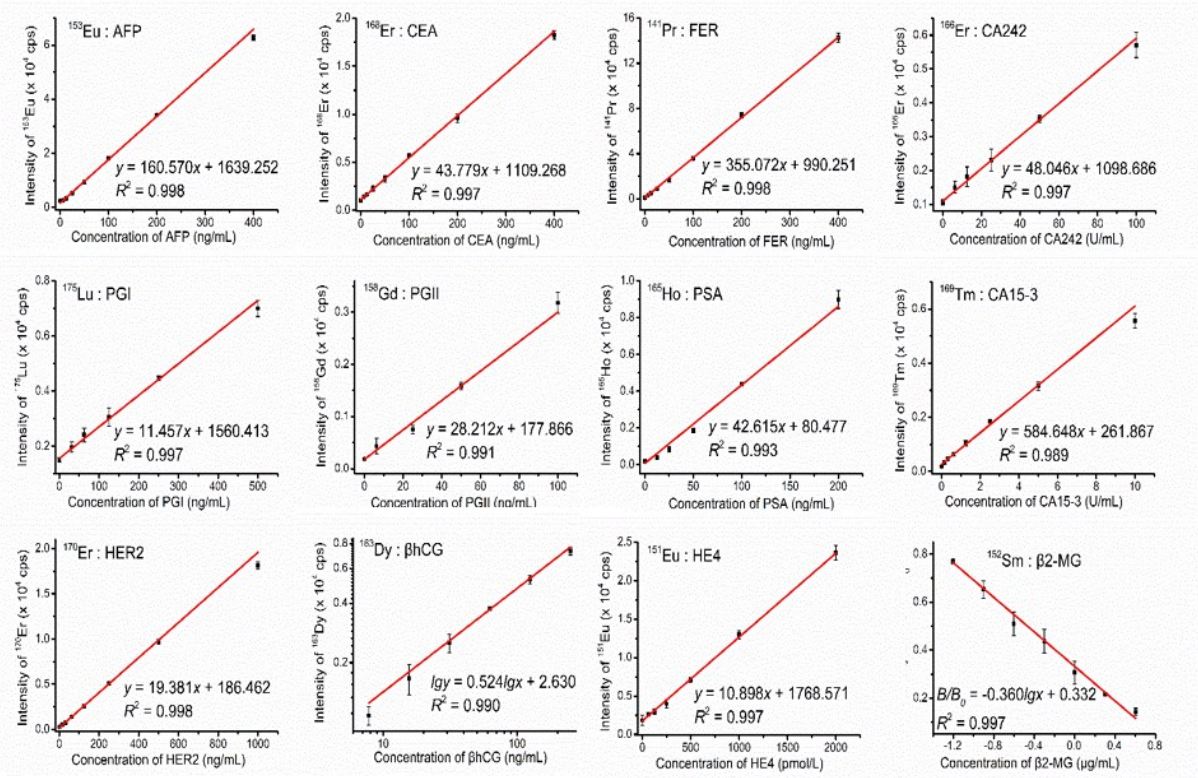


Figure S5. The calibration curves of intensities of REEs versus the concentrations of the protein targets. Error bars represent the standard deviation of triplicates.

The analytical results of serum spiking test for combinatorial immunoassay (Table S1)

Table S1. Recoveries of spiked FER, AFP and CEA (the first group); PGI, PGII and PSA (the second group); FER, AFP and β2-MG (the third group) in serum samples (mean ± s.d., n = 3).

	Added (ng/mL)	Found (ng/mL)	Recovery (%)		Added (ng/mL)	Found (ng/mL)	Recovery (%)		Added (ng/mL)	Found (ng/mL)	Recovery (%)
	10	9.64±0.342	96.4		40	38.4±0.136	96.1		20	19.2±2.45	97.1
FER	50	52.97±3.23	106	PGI	100	96.5±2.51	96.5	FER	80	92.2±5.39	115
	200	228±4.67	114		200	206±3.61	121		200	237±4.16	119
	10	10.5±0.032	105		4	4.16±0.194	104		10	9.52±0.596	95.2
AFP	50	55.9±2.99	112	PGII	10	9.22±0.182	92.2	AFP	50	60.6±2.90	121
	200	190±0.953	94.8		20	19.3±0.620	96.4		200	222±2.46	111
	10	9.82±0.434	98.2		4	3.70±0.048	92.5		0.5	0.68±0.051	136
CEA	50	55.2±1.30	110	PSA	10	9.04±0.139	90.4	β2-MG ($x10^3$)	1	1.31±0.025	131
	200	196±1.10	97.8		40	40.2±0.94	101		2	2.07±0.067	104

Reference

1. Y. Zhang, B. Huang, K. Wang, J. Zhang, X. Wang and J. Jin, *Food Sci Technol*, 2009, **34**, 311-314.
2. B. Huang, X. Zhang, L. Zhu, H. Liu, Y. Jiang and J. Jin, *Journal of Rare Earths*, 2006, **24**, 381-384.