Supporting Information for

A Photocleavable Peptide-Tagged Mass Probe for Chemical Mapping of Epidermal Growth Factor Receptor 2 (HER2) in Human Cancer Cells

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Supplementary Tables

Table S1. Accuracy and precision for QC samples.

Nominal Concentration	25.0 pM	75.0 pM	500 pM	800 pM
Mean	26.8	79.3	473	845
%Bias	7.2	5.7	-5.3	5.6
Intra-day Precision (%CV)	8.6	5.9	4.3	3.9
Inter-day Precision (%CV)	9.7	6.3	5.8	6.7
n	18	18	18	18
Number of Runs	3	3	3	3

Supplementary Figures

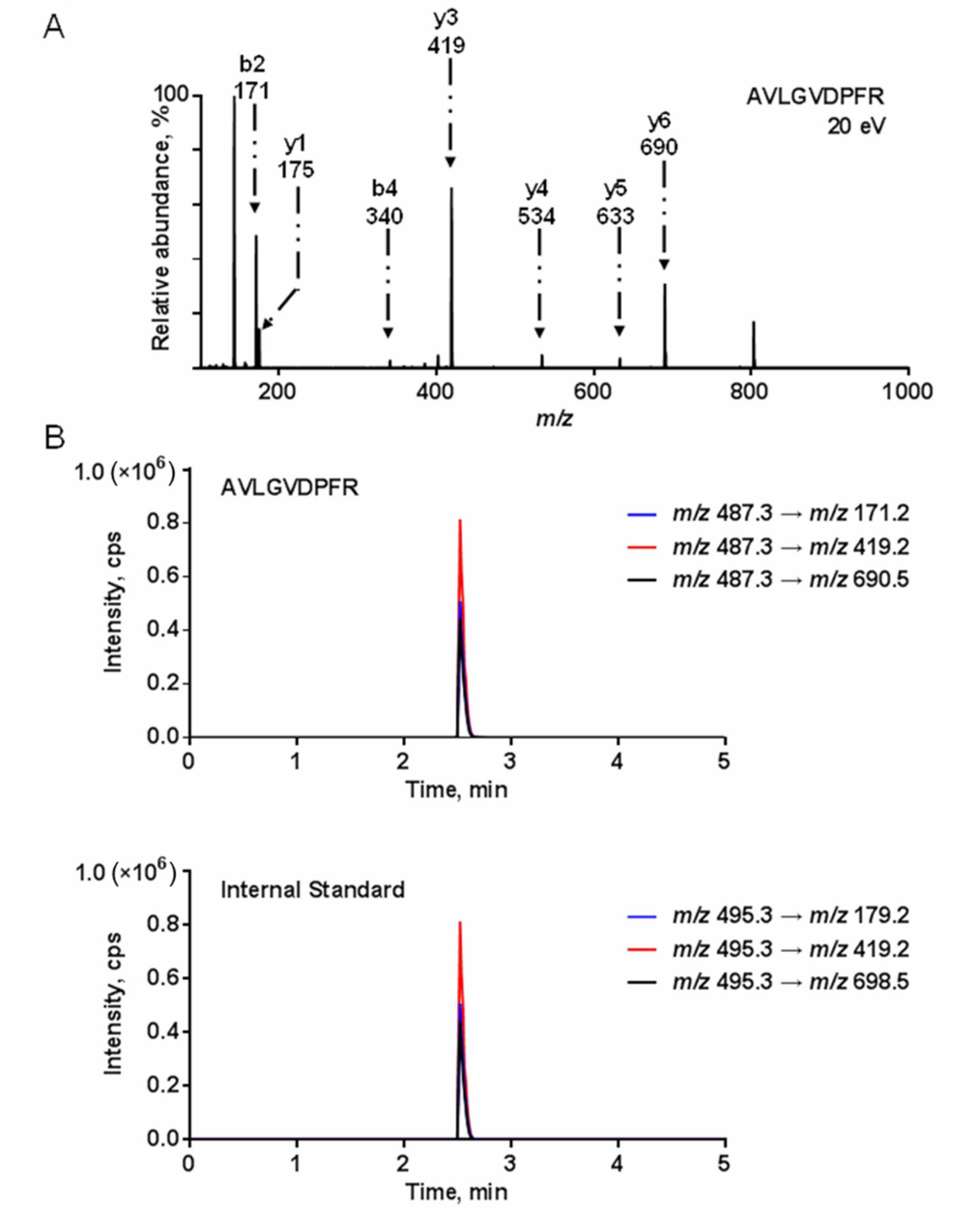

Figure S1. (A) Product ion spectrum of AVLGVDPFR and (B) LC-MS/MS chromatograms of AVLGVDPFR and its corresponding isotope-labeled internal standard. The MRM transitions of *m/z* 487.3 → *m/z* 419.2, *m/z* 487.3 → *m/z* 171.2 and *m/z* 487.3 → *m/z* 690.5 for AVLGVDPFR and *m/z* 495.3 → *m/z* 419.2, *m/z* 495.3 → *m/z* 179.2 and *m/z* 495.3 → *m/z* 698.5 for internal standard were used.

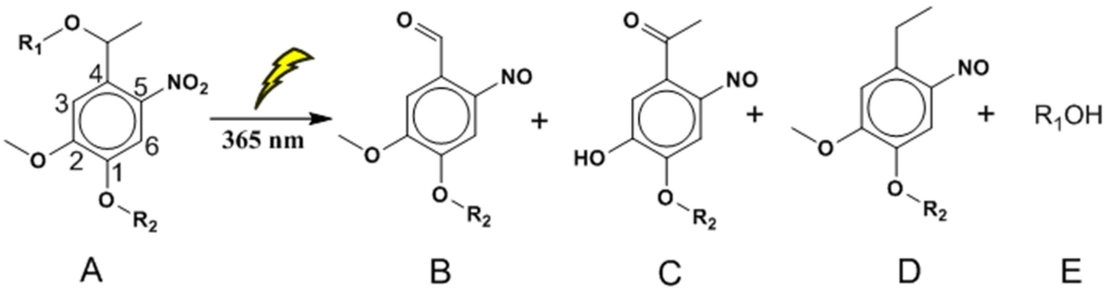

Figure S2. Photocleavable *o*-nitrobenzyl derivative linker and its photolysis products.

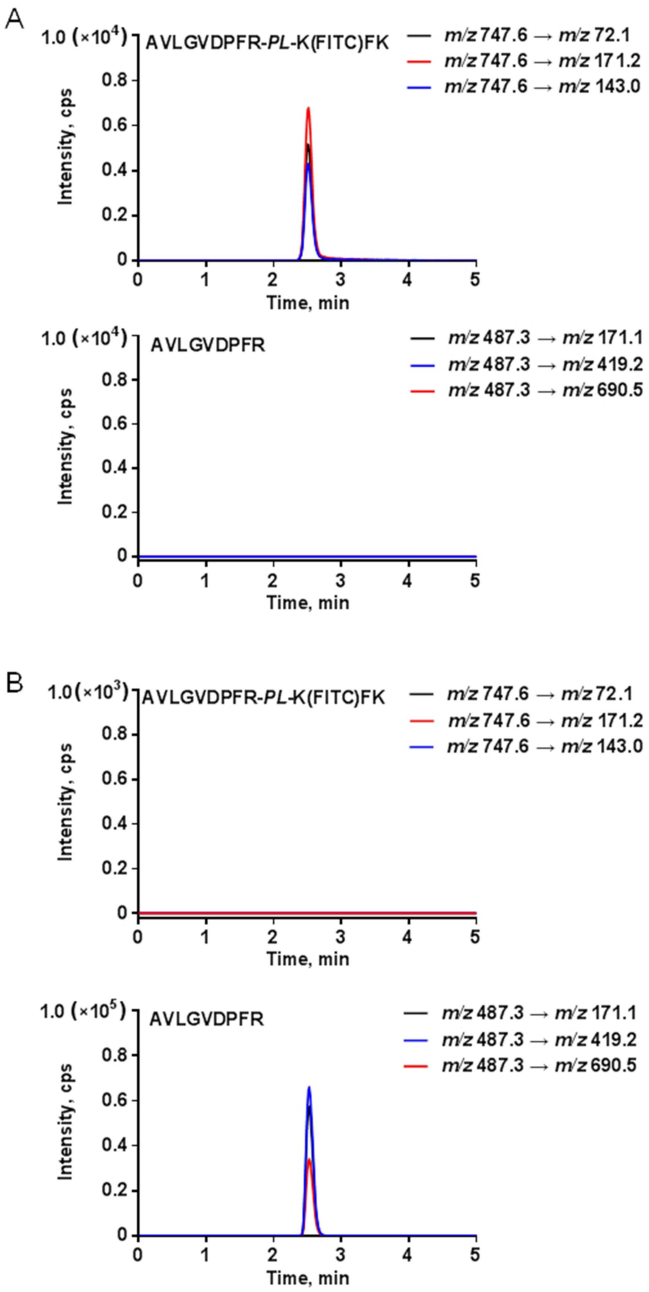

Figure S3. LC-MS/MS chromatograms of AVLGVDPFR-*PL*-K(FITC)FK and the reporter peptide AVLGVDPFR (A) before and (B) after photolysis. The results indicated that the starting photolytic peptide has been consumed and the presence of the reporter peptide. The photolysis efficiency was calculated by comparing the response ratio of the reporter peptide after digestion to that of an equimolar amount of synthetic peptide standard in the digestion.

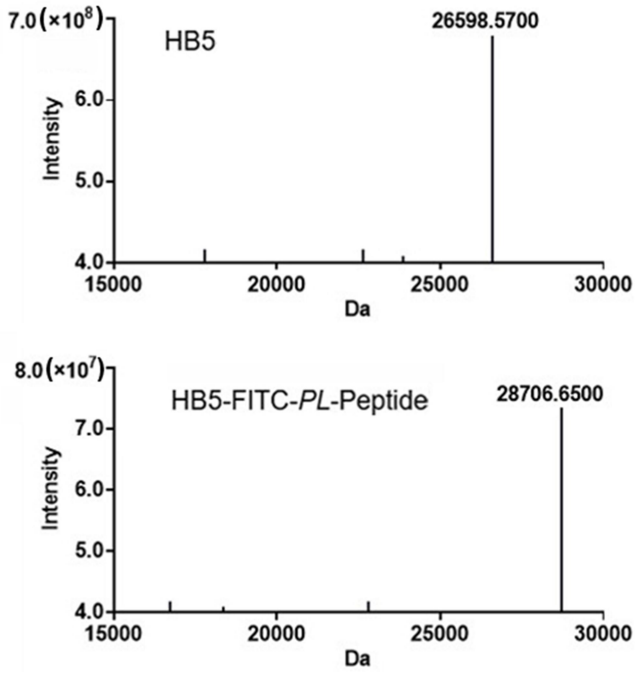

Figure S4. Mass spectra of HB5 and HB5-FITC-*PL*-Peptide probe. Mass spectrometry detection was performed using an AB Sciex 5800 MALDI-TOF/TOF, which was operated in the positive mode.

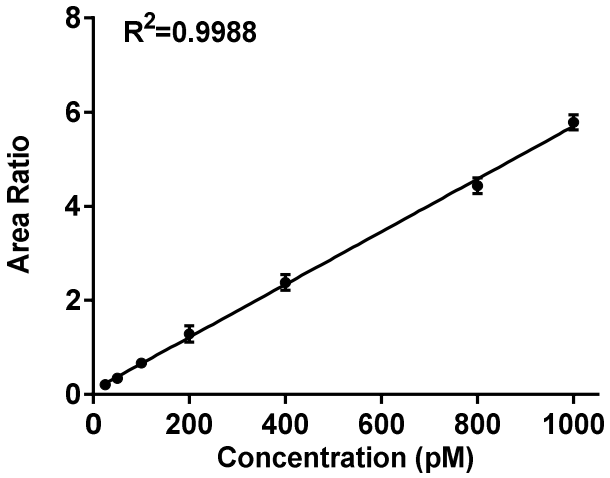

Figure S5. Representative calibration curve (25 pM to 1 nM) of the reporter peptide mass response with the concentration of HER2 epitope peptide. The relative peak area ratio of the reporter peptide and the stable isotope-labeled internal standard with the same sequence was plotted against the concentration of the HER2 epitope peptide standard.

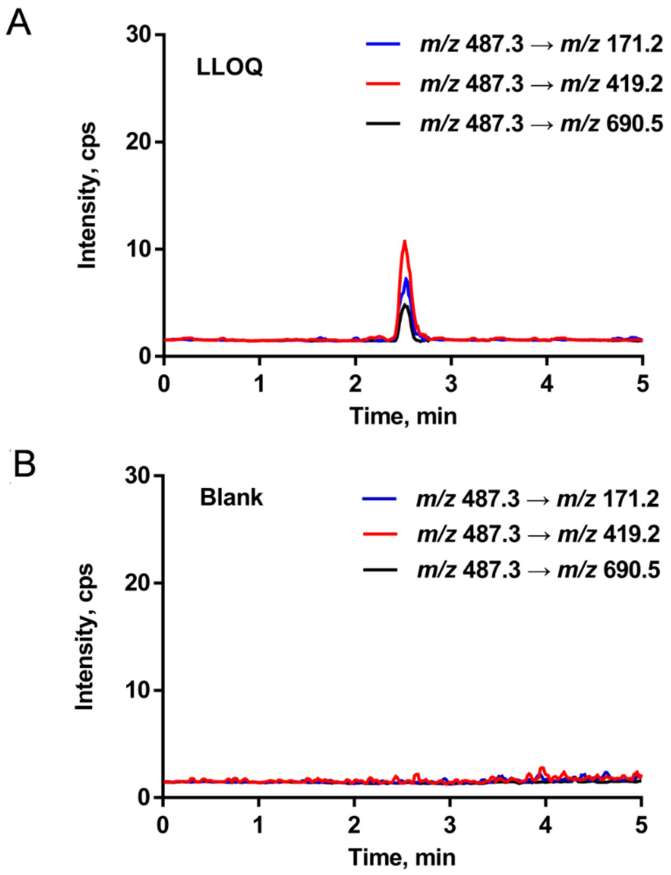

Figure S6. The LC-MS/MS chromatograms for (A) the LLOQ (25 pM) of the reporter peptide and (B) matrix blank. The internal standard is omitted for clarity.

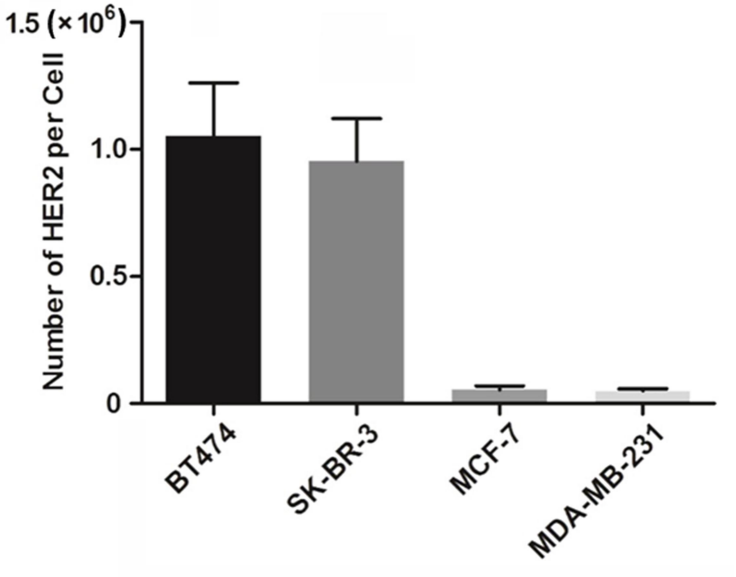

Figure S7. The level of HER2 in BT474, SK-BR-3, MCF-7 and MDA-MB-231 cells using our assay. The concentrations of HER2 were correlatively quantified to be (10.5 ± 2.11) × 10^5/cell in BT474 cells, (9.53 ± 1.68) × 10^5/cell in SK-BR-3 cells, (5.40 ± 1.56) × 10^4/cell in MCF-7, and (5.10 ± 0.70) × 10^4/cell in MDA-MB-231 cells.

Supplementary Material

S1. Chemicals and Reagents

Peptides including maleimide modified photocleavable substrate peptide, reporter peptide, and stable isotope-labeled internal standard peptide were synthetized by ChinaPeptides Co., Ltd. (Shanghai, China). Photocleavable substrate peptide has the following sequence: AVLGVDPFR-*PL*-K(FITC)FK-maleimide. Purity of the peptides was also operated by the manufacturer. The stable isotope-labeled amino acid was supplied by Cambridge Isotope Laboratories, Inc. (Andover, MA, USA). Human epidermal growth factor 2 (HER2) aptamer HB5 with a disulfide modification at the 5′ end was obtained from Sangon Biotechnology Co., Ltd. (Shanghai, China). Fluorescein isothiocyanate-labeled HB5 was synthesized and purified by Genscript (Nanjing, China). Aptamer HB5 has the following sequence: 5'-thiol-C6-AACCG CCCAA ATCCC TAAGA GTCTG CACTT GTCAT TTTGT ATATG TATTT GGTTT TTGGC TCTCA CAGAC ACACT ACACA CGCAC A-3'. A peptide from the extracelluar juxtamembrane region of HER2 with the sequence of INCTHSCVDLDDKGCPAEQR and N-terminal biotinylation was synthesized by Synpeptide Co., Ltd. (Shanghai, China). Sequencing grade modified trypsin was obtained from Promega (Madison, WI, USA). Ammonium bicarbonate (NH_4HCO_3) was obtained from Qiangshun Chemical Reagent Co., Ltd. (Shanghai, China). Ethylenediaminetetraacetic acid disodium salt (EDTA-2Na) was obtained from Sinopharm Chemical Reagent Company (Shanghai, China). Ammonium hydroxide was obtained from Haotian technology Co., Ltd. (Shanghai, China). Phosphate

buffered saline (PBS) was purchased from the Beyotime Institute of Biotechnology (Jiangsu, China). Acetonitrile (ACN) and methanol were obtained from Tedia Company, Inc. (Fairfield, OH, USA). Trifluoroacetic acid (TFA) and formic acid (FA) were provided by Aladdin Chemistry Co., Ltd. (Shanghai, China) and Xilong Chemical Industrial Factory Co., Ltd. (Shantou, China), respectively. Fetal bovine serum (FBS), Dulbecco's modified eagle medium (DMEM), Roswell Park Memorial Institute (RPMI) 1640 medium, Leibovitz's L-15 medium and penicillin-streptomycin solution were obtained from Thermo Scientific HyClone (Logan, UT, USA). McCoy's 5A medium and bovine serum albumin (BSA) were purchased from Sigma-Aldrich (St. Louis, MO, USA). Trypan blue was obtained from Generay Biotech Co., Ltd. (Shanghai, China). Water was purified and deionized with a Milli-Q system manufactured by Millipore (Bedford, MA, USA). All the solutions were prepared in DEPC-treated water (Beyotime Biotechnology, Haimen, China).

S2. Cell Culture and Tissue Collection

HER2-positive breast cancer cell line BT474 and HER2-negative breast cancer cell lines MDA-MB-231 and MCF-7 were obtained from the Cell Resource Center of Chinese Academy of Medical Sciences (Shanghai, China). HER2-positive breast cancer cell line SK-BR-3 was obtained from ATCC (Manassas, VA, USA). MCF-7 and BT474 cells were respectively cultured in DMEM and RPMI 1640. Each media was supplemented with 10% FBS and 1% penicillin/streptomycin at 37°C under a 5% CO2 atmosphere. MDA-MB-231 cells were cultured in L-15 supplemented with 10%

FBS and 1% penicillin/streptomycin at 37°C in a free gas exchange with atmospheric air, as instructed by the ATCC. SK-BR-3 cells were incubated in McCoy's 5A supplemented with 15% FBS and 1% penicillin/streptomycin at 37°C under a 5% CO2 atmosphere. Cells were split every 5-7 days by lifting cells with 0.25% trypsin and fed between splits through the addition of fresh medium. All experiments were performed in the exponential growth phase of cells. Cell viability was assessed by trypan blue (0.4%) exclusion and the number of viable cells was counted with a hemocytometer (Qiujing, Shanghai, China). Two individual counters each counted the cells three times.

Breast tissue collection in this study was approved by the Institutional Review Board of Nanjing Medical University. The methods were carried out in accordance with the approved guidelines. Forty-two pairs of breast tissue samples consisting of tumors and adjacent sections from patients with breast cancer were collected consecutively between March 2014 and January 2016 at the First Affiliated Hospital with Nanjing Medical University, Nanjing, China (mean patient age, 51.6 ± 8.1 years; age range, 41-68 years). The patients were biologically unrelated, but all belonged to the Han Chinese ethnic group from the Jiangsu province in China. Informed consent was obtained from the subjects. Tissue sections were confirmed as being normal or cancerous by hospital pathologists. Each tissue sample was fixed in 10% neutral buffered formalin for 48 h immediately, and then paraffin-embedded. Sections (4 μm) were deparaffinized routinely, rehydrated and retrieved.

S3. Immobilization of Biotinylated HER2 Epitope Peptide

The streptavidin agarose beads (200 μL) were mixed with 200 μL of fresh PBS containing 100 μM biotinylated HER2 peptide. And the mixture was incubated at 37°C for 2 h with gentle stirring. After washing three times with PBS, the streptavidin agarose beads were incubated with HB5-FITC-*PL*-Peptide probe at 37°C for 2.5 h. Then, washing three times with 500 μL PBS to remove the unbound probe. The probe-bound beads were mixed with 100 μL of DEPC-treated water.

S4. Determination of K_d of HB5 and HB5-FITC-*PL*-Peptide

The HER2 peptide-immobilized streptavidin agarose beads were incubated with different concentration of fluorescein isothiocyanate-labeled aptamer HB5 or HB5-FITC-*PL*-Peptide at 37°C for 2 h (10, 20, 40, 80, 160 and 320 nM of probe in 200 μL of binding buffer). Washing three times with 500 μL PBS to remove the unbound HB5. Then, the beads were heated at 95°C for 30 min, and gradually cooled. The supernatant was analyzed using HPLC. The HPLC system consisted of a Shimadzu LC-20AB solvent delivery pump, a Rheodyne manual valve injector, and a Shimadzu RF-20A fluorescence detector (Shimadzu Corporation, Tokyo, Japan). The sample was analyzed using an Aqua HPLC C8 column (5 μm, 4.6 mm × 150 mm; Thermo Scientific, USA) at room temperature.

S5. IHC and FISH

HER2 expression in breast cancer tissue was detected using 1:300 polyclonal

antibody 18299-1-AP (Proteintech, Wuhan, China) overnight at 4°C. Positive and negative controls were run together with the test sample. The pathological grade of tumors was defined according to the 2018 ASCO/CAP updated guidelines: IHC 3+ = complete, intense circumferential membrane staining in >10% of tumor cells; IHC 2+ = weak to moderate complete membrane staining observed in >10% of tumor cells; IHC 1+ = faint/barely perceptible incomplete membrane staining in >10% of tumor cells; IHC 0 = no staining or incomplete and faint/barely perceptible membrane staining in ≤ 10% of tumor cells.[1] In general, scores of 0 and 1+ were considered as negative for HER2 expression, 3+ as positive while 2+ were equivocal and reflex testing should be performed (same specimen using ISH) or order a new test (new specimen if available, using IHC or ISH).

Two-color FISH was performed on sections from formalin-fixed, paraffin-embedded tissue sections. Before hybridization, sections were deparaffinized, dehydrated in 100% ethanol, and air-dried. Commercially available HER2 probe and CEP17 probe were used according to the manufacturer's recommendations (GP Medical Technologies, Beijing, China). Also, all results were judged on the basis of guidelines. According to the 2018 ASCO/CAP updated guidelines,[1] HER2/CEP17 ratio ≥ 2.0 and the average HER2 copy number ≥ 4.0 signals/cells indicated positive amplification. HER2/CEP17 ratio ≥ 2.0 and the average HER2 copy number < 4 signals/cell, HER2/CEP17 ratio < 2.0 and the average HER2 copy number ≥ 6 signals/cell, or the cases HER2/CEP17 ratio < 2.0 and average HER2 copy number ≥ 4.0 and < 6 signals/cell were considered equivocal, and needed additional work to

determine. HER2/CEP17 ratio < 2.0 and average HER2 copy number < 4 signals/cell indicated negative amplification.

S6. Flow Cytometry

This step was similar with our previous work. After denaturation into secondary structure, fluorescein isothiocyanate-labeled aptamers were incubated with 1×10^6 of SK-BR-3 and MCF-7 cells in binding buffer at 37°C for 2 h respectively. Washing three times with PBS, cells were then analyzed on a FACScan flow cytometer (Becton Dickinson, Franklin Lakes, NJ, USA). The data were analyzed using the Cell Quest software (BD Biosciences, San Jose, CA).

S7. HPLC

The HPLC system consisted mainly of three parts, including a Shimadzu LC-20AB solvent delivery pump, a Rheodyne manual valve injector and a Shimadzu SPD-20A UV/VIS detector (Shimadzu Corporation, Tokyo, Japan). The samples were analyzed using an Aqua HPLC C8 column (5 μm, 4.6 mm×150 mm; Thermo scientific, USA) at room temperature. Solvent A (50 mM trimethylamine, pH 7.6) and solvent B (ACN) form the mobile phase. with a flow rate of 1 mL/min, a linear gradient was applied in the following manner (duration listed in parentheses): B 5% (0 min) → 16% (5 min) → 40% (20 min) →5% (25 min) → stop (26 min). The data were acquired and processed with Lab Solutions LC-solution Version 1.2 working station.

S8. Calibration Standards and Quality Controls

After accurately weighing the synthetic biotinylated HER2 peptide and dissolving it in diethylpyrocarbonate-treated water, we prepared a stock solution of HER2 epitope at a concentration of 100 μM. The concentrations of the calibrators were 25, 50, 100, 200, 400, 800, 1000 pM. The concentration of quality control (QC) specimens for the lower limit of the measured, low QC, mid QC, and high QC were prepared at 25, 75, 500, 800 pM. As we previously reported, lower limit of the measured is the lower limit of quantification, the concentration at which all defined performance characteristics of the measurement procedure were met. CV < 20% and recovery < 15% bias of the target concentrations of the lower limit of the measured were acceptable according to the recommended and stated precision and recovery. The selection of internal standard was consistent to our previous work, we chose the corresponding isotope-labeled synthetic peptide (200 pM) as an internal standard.

References

1 A. C. Wolff, M. E. H. Hammond, K. H. Allison, B. E. Harvey, P. B. Mangu, J. M. S. Bartlett, M. Bilous, I. O. Ellis, P. Fitzgibbons, W. Hanna, R. B. Jenkins, M. F. Press, P. A. Spears, G. H. Vance, G. Viale, L. M. McShane and M. Dowsett, *Arch. Pathol. Lab. Med.,* 2018, **142**, 1364-1382.