Supporting Information For

Simultaneous Detection of Human C-terminal p53 Isoforms by Single Template Molecularly Imprinted Polymers (MIPs) Coupled with Liquid Chromatography-tandem Mass Spectrometry (LC-MS/MS)-based Targeted Proteomics

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Table 1S. Adsorption parameters of the template peptide onto the MIPs and the NIPs from the Langmuir model.

Isotherm model	Equation	Parameters	MIPs	NIPs
Langmuir	$\frac{C_e}{Q_e} = \frac{1}{Q_{max}} C_e + \frac{1}{k_L Q_{max}}$	$R^{2\,a}$	0.998	0.989
		$Q_{max}{}^{b}$	3.72	1.36
		$k_L{}^{c}$	1.88	0.168

[a] Correlation coefficient.

[b] Maximum absorption capacity.

[c] Langmuir constant.

Table 2S. Adsorption parameters of the template peptide at a concentration of 15.3 μM (20 μg/mL) toward the MIPs and the NIPs from the pseudo-second-order kinetic adsorption model.

Kinetic model	Equation	Parameters	MIPs	NIPs
Pseudo-second-order	$\frac{t}{Q} = \frac{1}{k_2 Q_e{}^2} + \frac{t}{Q_e}$	$R^{2\,a}$	0.999	0.986
		$k_2{}^{b}$	0.522	0.180
		$Q_e{}^{c}$	3.42	1.17

[a] Correlation coefficient.

[b] Rate constant of adsorption in pseudo-second-order model.

[c] Adsorption amount of the peptide template at equilibrium.

Table 3S. Comparison of the slopes of the calibrations curves using unlabeled surrogate peptides and isotope-labeled peptides as standards.

	Slope of Calibration Curve		
	Peptide-α	**Peptide-β**	**Peptide-γ**
Unlabeled Peptides	$(4.22 \pm 0.19)\times10^{-2}$	$(4.01 \pm 0.22)\times10^{-2}$	$(4.12 \pm 0.17)\times10^{-2}$
Isotope-labeled Peptides	$(4.10 \pm 0.25)\times10^{-2}$	$(3.98 \pm 0.25)\times10^{-2}$	$(4.05 \pm 0.26)\times10^{-2}$
p value*	0.684	0.934	0.794

*$p < 0.05$ is considered statistically different.

Table 4S. Comparison of the slopes of the calibrations curves measured *w* and *w/o* other surrogate peptides at high concentration.

	Slope of Calibration Curve		
	Peptide-α	**Peptide-β**	**Peptide-γ**
***w/o* Other Surrogate Peptides**	$(4.22 \pm 0.19)\times10^{-2}$	$(4.01 \pm 0.22)\times10^{-2}$	$(4.12 \pm 0.17)\times10^{-2}$
***w* Other Surrogate Peptides**	$(4.12 \pm 0.25)\times10^{-2}$	$(3.98 \pm 0.27)\times10^{-2}$	$(4.00 \pm 0.29)\times10^{-2}$
p value*	0.731	0.928	0.682

*$p < 0.05$ is considered statistically different.

Table 5S. Accuracy and precision for the QC samples.

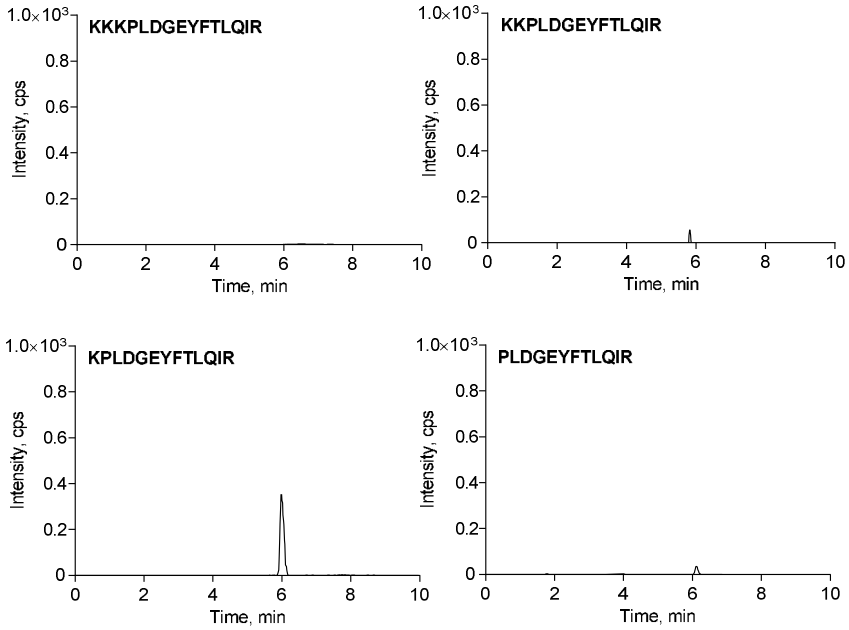
Nominal Concentration (peptide-α)	5 nM	15 nM	50 nM	400 nM
Mean	5.42	14.1	53.6	437
%Bias	8.5	-6.3	7.1	9.1
Intra-day Precision (%CV)	2.8	2.4	1.4	1.9
Inter-day Precision (%CV)	6.5	9.2	6.1	10.3
n	18	18	18	18
Number of Runs	3	3	3	3

Nominal Concentration (peptide-β)	5 nM	15 nM	50 nM	400 nM
Mean	5.32	15.6	47.6	442
%Bias	6.4	4.1	-4.7	10.4
Intra-day Precision (%CV)	2.3	2.9	2.4	2.7
Inter-day Precision (%CV)	7.1	8.0	7.1	8.1
n	18	18	18	18
Number of Runs	3	3	3	3

Nominal Concentration (peptide-γ)	5 nM	15 nM	50 nM	400 nM
Mean	5.59	16.1	45.7	382
%Bias	11.9	7.1	-8.6	-4.5
Intra-day Precision (%CV)	3.2	2.6	2.7	2.1
Inter-day Precision (%CV)	8.9	8.8	7.6	9.8
n	18	18	18	18
Number of Runs	3	3	3	3

Supplementary Figures

Figure 1S. Aligned amino acid sequences of p53 α, β and γ using Blast. The sequence difference is highlighted in blue.



Tryptic Products of KKKPLDGEYFTLQIRGR	Stoichiometry (%)[a]
KKKPLDGEYFTLQIR	1.9 ± 0.4
KKPLDGEYFTLQIR	2.6 ± 0.5
KPLDGEYFTLQIR	93.9 ± 0.8
PLDGEYFTLQIR	1.3 ± 0.3

[a]Stoichiometry is defined as the ratio of the moles of tryptic product and the initial moles of substrate peptide.

Figure 2S. LC-MS/MS chromatograms and stoichiometries of tryptic products of KKKPLDGEYFTLQIRGR, substrate peptide of KPLDGEYFTLQIR (peptide-α).

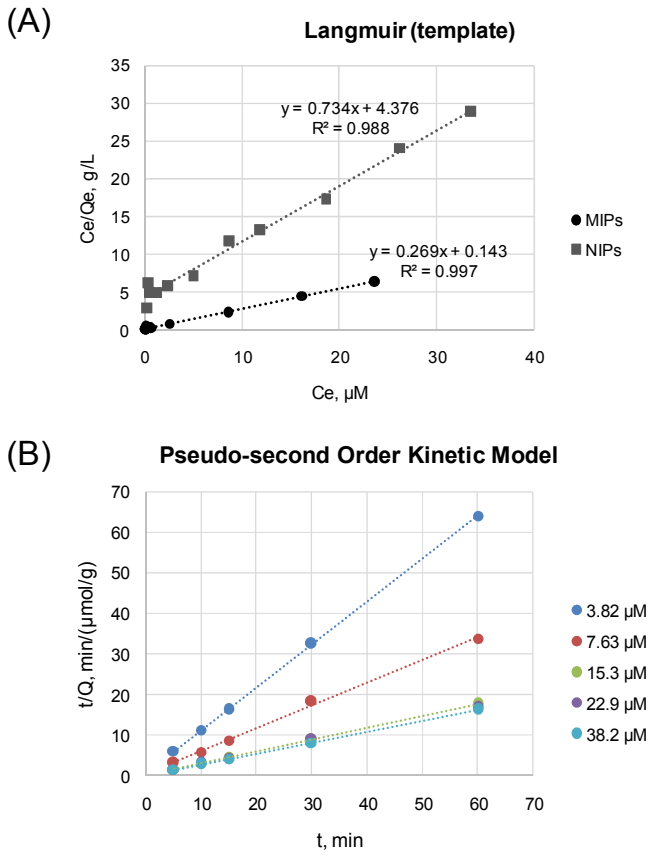


Figure 3S. The best fit adsorption isotherm model and kinetic model. (A) Langmuir isotherm adsorption of the template peptide. (B) Pseudo-second-order kinetic model of the template peptide to the MIPs at different concentrations (3.82, 7.63, 15.3, 22.9, 38.2 μM).

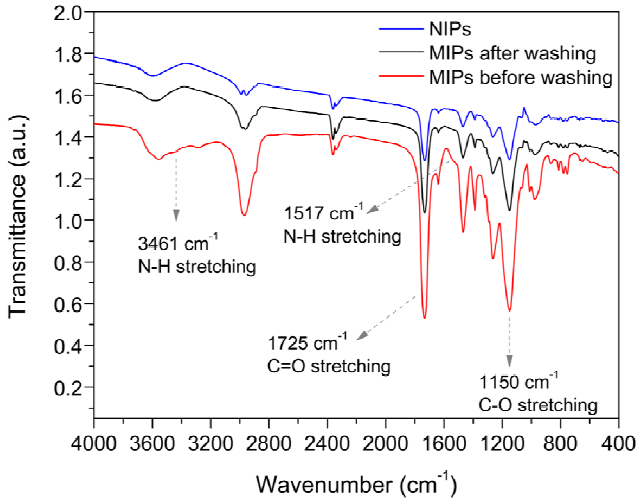


Figure 4S. FT-IR spectra of the prepared MIPs before and after removal of the template peptide, and the NIPs. The strong adsorption peaks at 1725 cm^{-1} and 1150 cm^{-1} suggest the existence of TRIM in all the studied polymers. The bands present at 3461 cm^{-1} and 1517 cm^{-1} disappeared after removal of the template from the MIPs, confirming the formation of imprinted polymers.

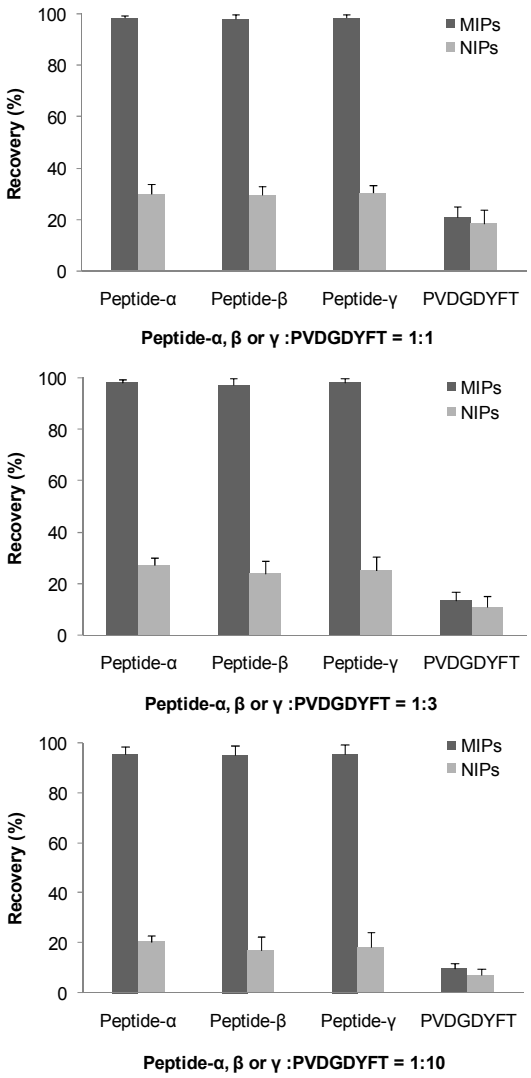


Figure 5S. Selectivity of the MIPs at different concentration ratios of the isoform-specific surrogate peptides and the competing peptide PVDGDYFT.

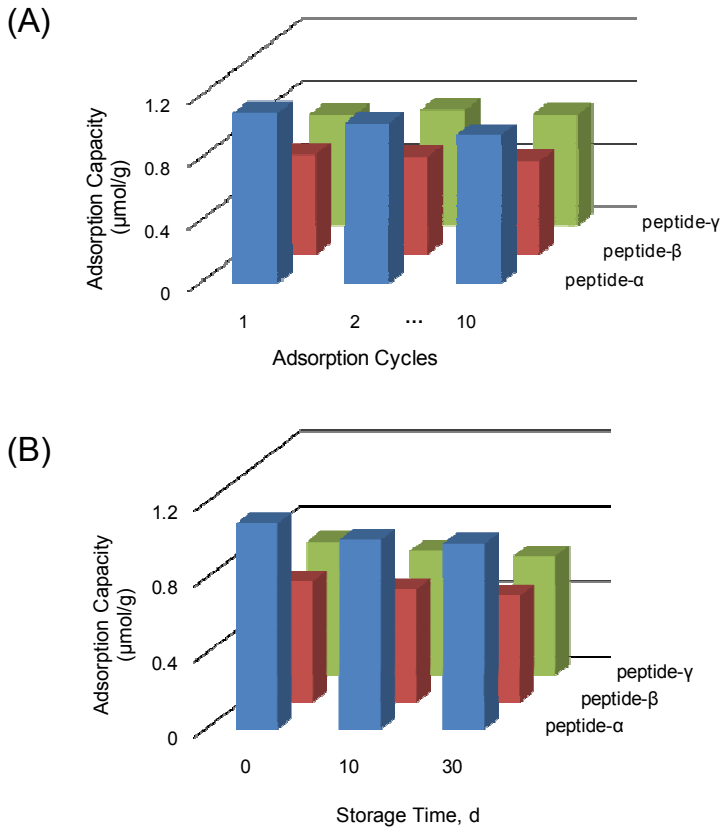


Figure 6S. Assessment of (A) regeneration/reuse and (B) stability of the MIPs.

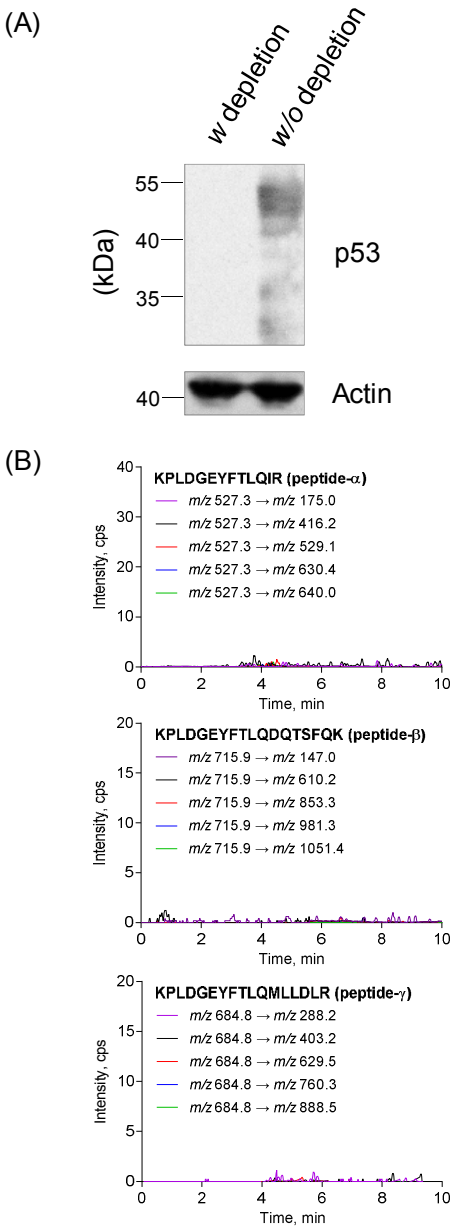


Figure 7S. (A) Western blotting image and (B) LC-MS/MS chromatograms of p53-depleted MCF-7 cell extract. In the Western blotting image, the large intense spot at 53 kDa corresponds to wild-type p53. The small MW forms include C-terminal p53 isoforms and their N-terminal truncated forms in cellular process.

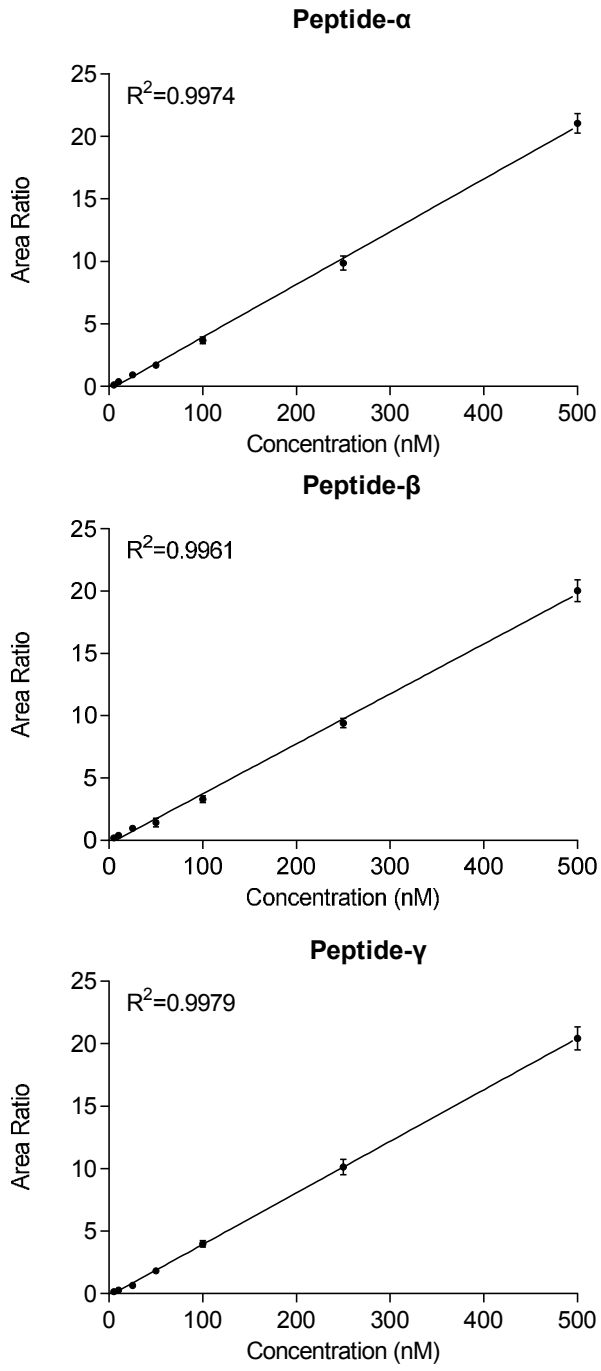


Figure 8S. Calibration curves of the isoform-specific surrogate peptides as standards.

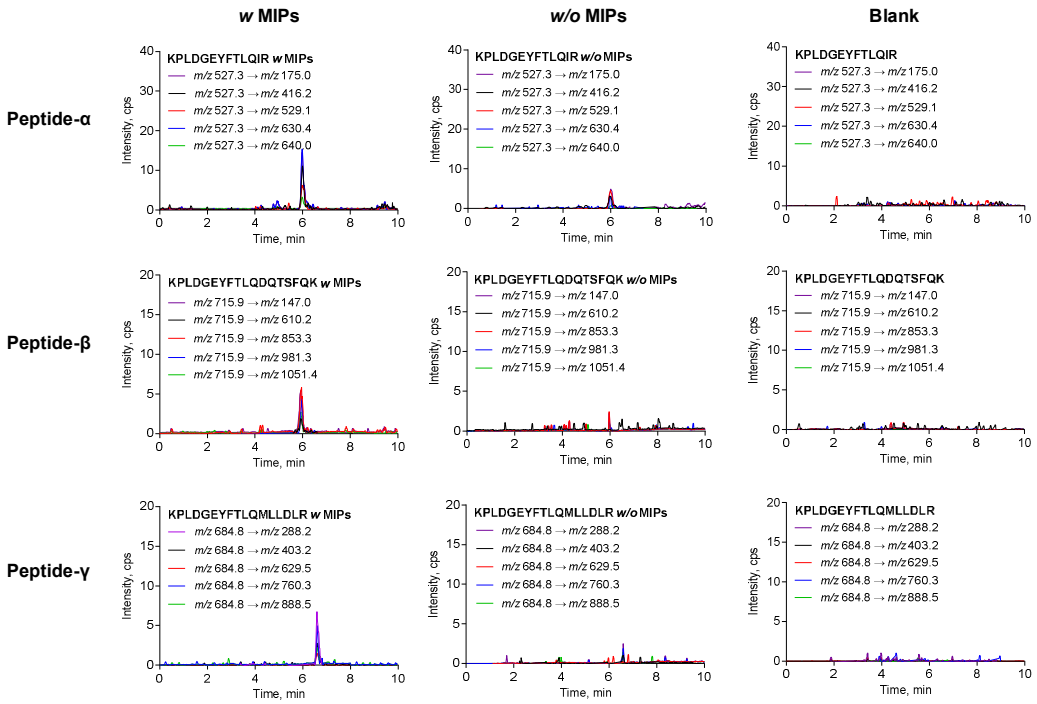


Figure 9S. Chromatograms of 5 nM peptide-α, β and γ (i.e., LLOQ in this study) *w* and *w/o* MIPs, and the corresponding blanks.

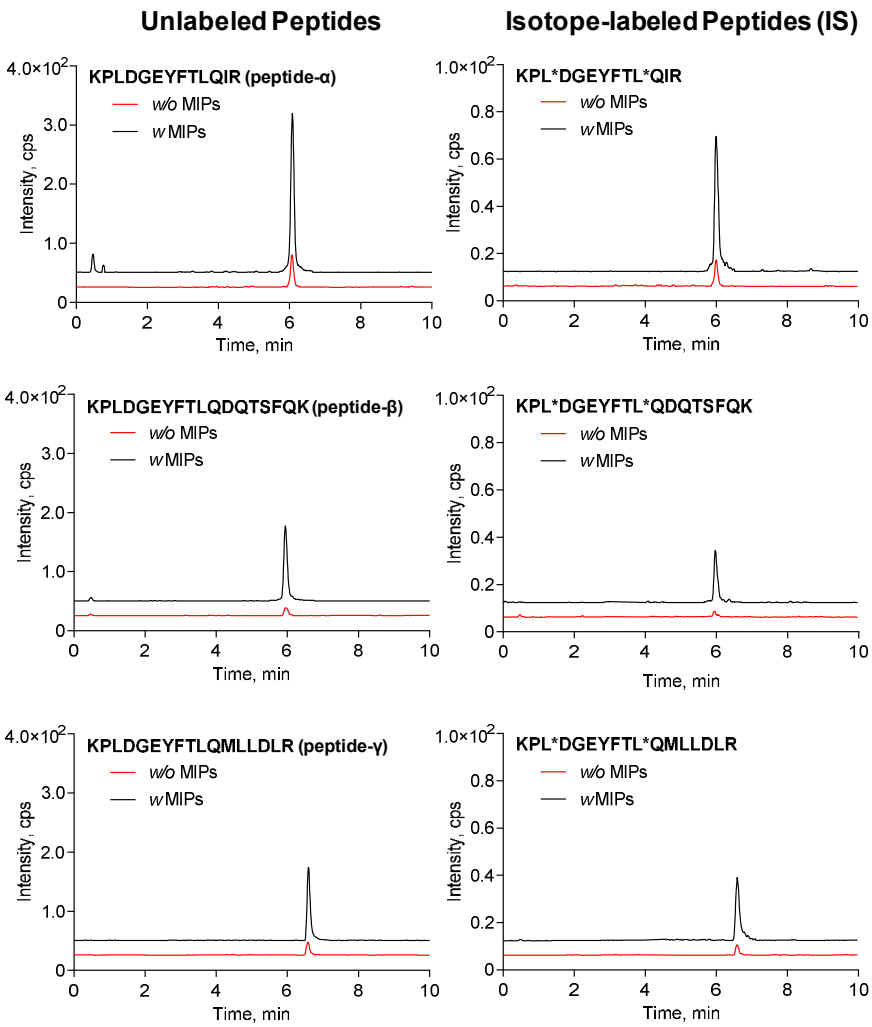


Figure 10S. Representative LC-MS/MS chromatograms of the unlabeled and isotope-labeled surrogate peptides *w* and *w/o* MIPs. Only one MRM transition for each surrogate peptide is presented for clarity.

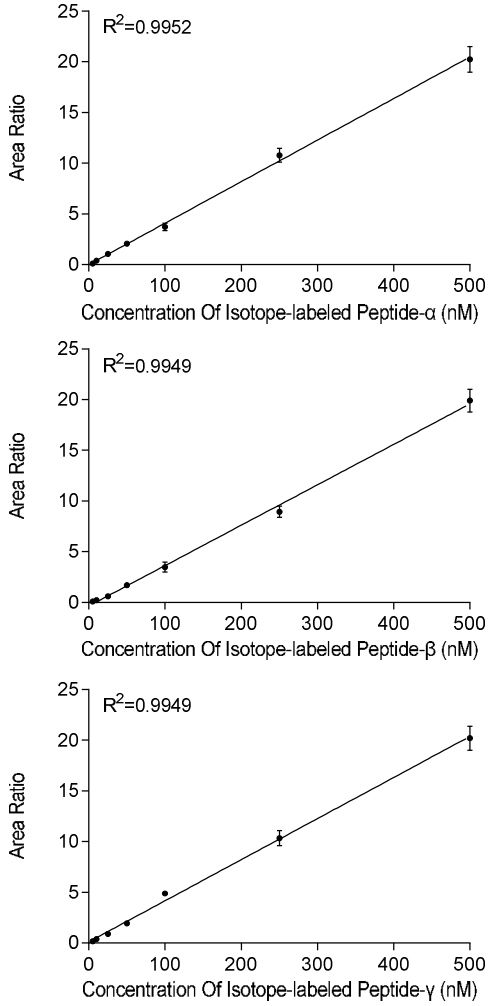


Figure 11S. Calibration curves of the isotope-labeled surrogate peptides as standards in p53-depleted cell extract.

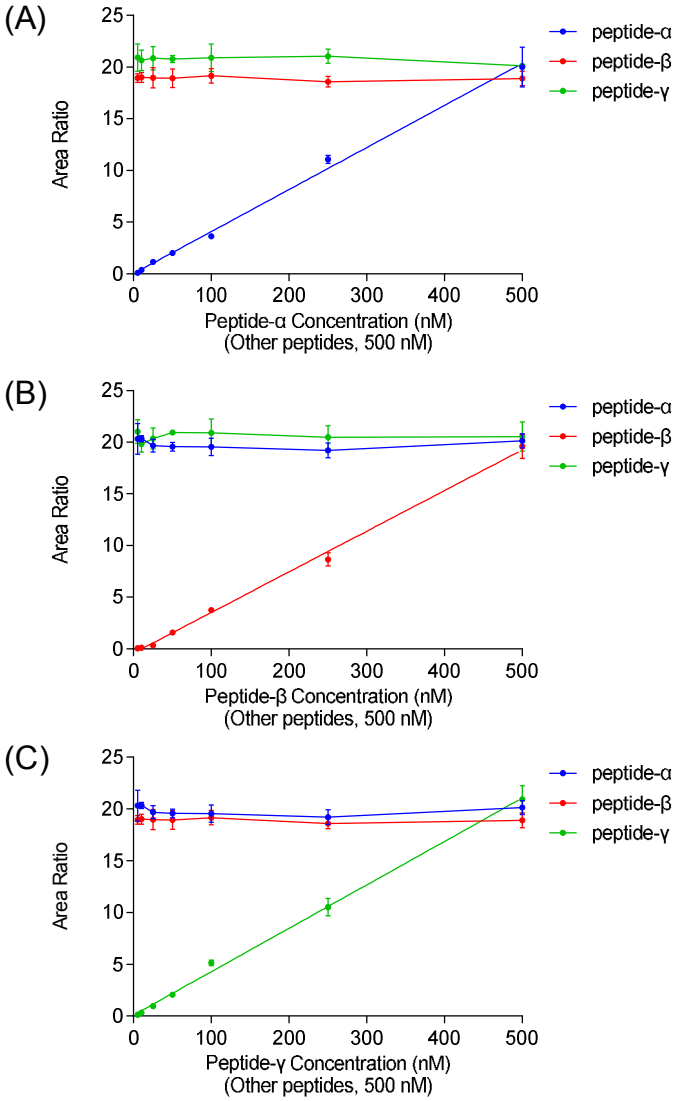
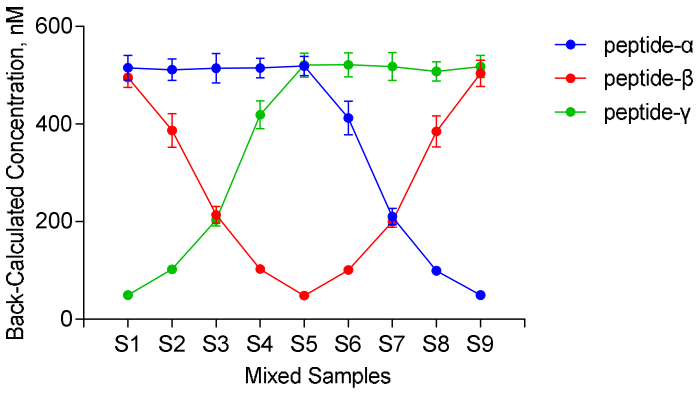


Figure 12S. Calibration curves contain all three surrogate peptides, with one peptide with increasing concentration ((A) peptide-α, (B) peptide-β and (C) peptide-γ) and the other two with a constant concentration of 500 nM each.



Mixed Samples	Concentrations (nM)		
	peptide-α	peptide-β	peptide-γ
S1	500	500	50
S2	500	400	100
S3	500	200	200
S4	500	100	400
S5	500	50	500
S6	400	100	500
S7	200	200	500
S8	100	400	500
S9	50	500	500

Figure 13S. Simultaneous quantification of the isoform-specific surrogate peptides at varying concentrations. Nine samples (S1 to S9) containing peptide-α, β and γ with concentrations between 50 nM and 500 nM were measured and the calculated concentrations are shown as filled diamond and bar.

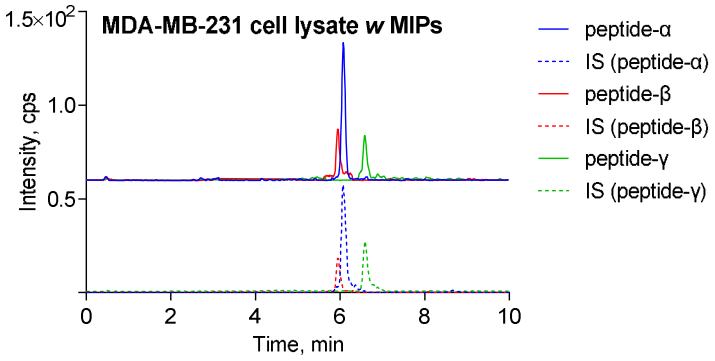


Figure 14S. The LC-MS/MS chromatogram of cell lysate sample. Only one MRM transition for each surrogate peptide is presented for clarity.

Supplementary Material

S1. Chemicals and reagents

Peptides including surrogate peptides, substrate peptides and internal standards containing stable-isotope labeled amino acid were developed from ChinaPeptides Co., Ltd. (Shanghai, China). Purity of the peptides is over 95%. The stable isotope-labeled amino acid used in internal standard synthesis was supplied by Cambridge Isotope Laboratories, Inc. (Andover, MA, USA). Peptide modification with fluorescein isothiocyanate (FITC) was carried out by Synpeptide Co., Ltd. (Shanghai, China). The monomers methyl methacrylate (MAA) and N-tert-butylacrylamide (TBAm), the crosslinkers ethylene glycol dimethacrylate (EGDMA), trimethylolpropanetrimethacrylate (TRIM) and divinylbenzene (DVB), and initiator 2, 2'-azobis(2-methylpropionitrile) (ABMP) were all purchased from Aladdin Chemistry Co., Ltd. (Shanghai, China). Ammonium bicarbonate (NH_4HCO_3) was provided by Qiangshun Chemical Reagent Co., Ltd. (Shanghai, China). Sequencing grade modified trypsin was purchased from Promega (Madison, WI, USA). DL-dithiothreitol (DTT), iodoacetamide (IAA), Tris-HCl, trifluoroacetic acid (TFA) and formic acid (FA) were all obtained from Sigma-Aldrich (St. Louis, MO, USA). Nuclear and cytoplasmic protein extraction kit was supplied by Keygen Biotech (Nanjing, China). BCA protein assay kit was obtained from Pierce Biotechnology, Inc. (Rockford, IL, USA). Mouse anti-p53 antibody DO-11, which recognizes an epitope within the central region of p53 between amino acids 181-190, was purchased from Bio-Rad Laboratories (Hercules, CA, USA). Protein A/G-agarose was purchased from

Abmart (Shanghai, China). Phosphate buffered saline (PBS) was purchased from the Beyotime Institute of Biotechnology (Haimen, China). Dichloromethane (DCM) was from Sinopharm Chemical Reagent Co., Ltd. (Shanghai, China). HPLC-grade acetonitrile (ACN) and methanol were obtained from Tedia Company, Inc. (Fairfield, OH, USA). Water was purified and deionized with a Milli-Q system manufactured by Millipore (Bedford, MA, USA). Fetal bovine serum, Roswell Park Memorial Institute (RPMI) 1640 medium, Leibovitz's L-15 medium, Dulbecco's modified Eagle media (DMEM) and penicillin-streptomycin solution were obtained from Thermo Scientific HyClone (Logan, UT, USA). MEGM mammary epithelial cell growth medium was obtained from LONZA (Basel, Switzerland). Trypan blue and sodium dodecyl sulfate (SDS) were obtained from Generay Biotech Co., Ltd. (Shanghai, China).

S2. Cell culture and protein extraction

MCF-7 (ATCC, Manassas, VA) and MCF-7/ADR (Keygen Biotech, Nanjing, China) cells were cultured in a DMEM media supplemented with 10% fetal bovine serum and 1% penicillin/streptomycin at 37°C under a 5% CO_2 atmosphere. MCF-10A cells (ATCC, Manassas, VA) were maintained routinely in MEGM media supplemented with 100 ng/mL cholera toxin and 1% penicillin/streptomycin at 37°C under a 5% CO_2 atmosphere. MDA-MB-231 cells were obtained from the Cell Resource Center of Chinese Academy of Medical Sciences (Shanghai, China) and 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 [20]. The cells

were split every 5-7 days by lifting cells with 0.25% trypsin, and feeding between splits was accomplished through the addition of fresh medium. To maintain a highly drug-resistant cell population, MCF-7/ADR cells were periodically reselected by growing them in the presence of 1000 ng/mL DOX. Experiments were performed using the cells incubated without DOX for 48 h and cells were counted with a hemocytometer (Qiujing, Shanghai, China). Cell viability was assessed by trypan blue (0.4%) exclusion, which was completed by mixing the cell suspension, trypan blue and 1 × PBS in a ratio of 2:5:3 and counting the percentage of viable cells following a 5 min incubation at 37°C.

Cells ($5 \times 10^6 \sim 1 \times 10^7$) were washed with ice-cold PBS twice and pelleted at 500 × g for 3 min. Following the manufacturer's protocol, 200 μL of pre-cooled buffer A in the nuclear and cytoplasmic protein extraction kit was added to ~20 μL cell pellet. After the suspension was vortexed violently for 15 s and incubated on ice for 15 min, 11 μL of Buffer B was added and the mixture was vortexed violently for another 15 s. The sample was then incubated on ice for 1 min and spinned at 16,000 × g for 5 min to separate soluble (cytoplasmic protein) and insoluble nuclear materials. The precipitate was resuspended in 100 μL Buffer C. The obtained suspension was vortexed for 15 s and incubated on ice for 10 min. After repeating this operation for four times, the sample was centrifuged at 16,000 × g for 10 min at 4°C. The supernatant containing nuclear protein was collected and combined with the cytoplasmic protein fraction for the following analysis. Protein concentration was determined by BCA protein assay kit (Pierce Biotechnology, Inc., Rockford, IL,

USA).

S3. Instruments and conditions

HPLC analysis was carried out to detect the FITC-labeled template peptide. HPLC system is composed of three sections including a LC-20A pump (Shimadzu, Japan), a RF-20A fluorescence detector (Shimadzu, Japan) and 7725i manual injector with a 20-μL loop (Rheodyne, USA). An Elite C18 column (4.6 mm × 150 mm, 5 μm; Dalian, China) was employed. The mobile phase was composed of solvent A (100 mM $NH_3 \cdot H_2O$ and 100 mM NH_4Cl in water, pH = 9.1) and solvent B (ACN). A linear gradient with a flow rate of 1 mL/min was applied in the following manner: B 5% (0 min) → 15% (8 min) → 5% (10 min). The column temperature was kept at room temperature. The injection volume was 20 μL. Excitation and emission wavelengths were set at 480 nm and 526 nm, respectively.

For a simultaneous targeted analysis of three surrogate peptides, an Agilent Series 1200 HPLC system (Agilent Technologies, Waldbronn, Germany) coupled with a 6410 Triple Quad LC/MS mass spectrometer (Agilent Technologies, CA, USA) were used. Chromatographic separation of the sample was performed on a Hypersil gold column (3 μm, 20 mm × 2.1 mm; Thermo Fisher Scientific, USA) at room temperature. The mobile phase was consisted of solvent A (0.1% FA/water) and solvent B (0.1% FA/methanol). A gradient elution with a flow rate of 0.3 mL/min was performed in the following conditions: B 10% (0 min) → 10% (1 min) → 90% (4 min) → 90% (8 min) → 10% (9 min). The injection volume was 5 μL. The mass

spectrometer was equipped with an electrospray ion source and operated in the positive MRM mode. Q1 and Q3 were both set at unit resolution. The flow of the drying gas was 10 L/min, and the drying gas temperature was kept at 350°C. The electrospray capillary voltage was optimized to 4000V. The nebulizer pressure was set to 35 psi. The data were collected and processed using the Agilent MassHunter Workstation Software (version B.01.04). The peptides were detected in multiple reaction monitoring (MRM) mode. In this study, five transitions that gave the best S/N (signal/noise) and LOQ (limit of quantification) were chosen for each surrogate peptide.

S4. Preparation of p53-depleted cell extract using immuno-depletion

Cell extract complex was heated at 95°C for 8 min to denature the proteins and pre-cleaned by an addition of 10 μL Protein A/G-agarose (Abmart, Shanghai, China), followed by incubation at 4°C for 1 h on a rotator and centrifugation at 1000 ×g for 5 min. Afterward, the supernatant was mixed with 1 mL BioMagPlus Goat anti-Mouse IgG beads (Bangs Laboratories, Fisher, Indiana, USA) at a final protein concentration of 2 mg/mL, which were then treated with an anti-p53 antibody (DO-11, Bio-Rad Laboratories, Hercules, California, USA) that recognizes an epitope (aa181-190) present in all the denatured p53 isoforms. Sample was incubated at 4°C for 60 min with gentle rotation, and then placed in a magnetic field (Magnetic separator; Bangs Laboratories, Fishers, Indiana, USA) for 2 min. Supernatant was collected and subjected to the depletion protocol a second time. The resulting supernatant was then

analyzed by Western blotting using the anti-p53 antibody and LC-MS/MS (Fig. 7S).