Supplementary Information

A Multiplexed Immuno-MALDI Mass Spectrometry Assay for Simultaneous and Precise Quantitation of PTEN and p110α in Cell Lines and Tumor Tissues

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Table of Contents

Materials and Methods

Evaluation of the impact of reduction and alkylation on PTEN and p110α iMALDI assays

MDA-MB 231 lysate (10 µg of total protein per replicate in 100 µL) was digested using trypsin (1:10 protein:trypsin) either with (+RA) or without prior reduction of disulfide bonds and alkylation of free Cys residues (-RA), using five replicates per condition. Reduction and alkylation were performed by adding 10 µL 0.74 mM iodoacetamide, followed by incubation for 30 min at 37 °C in the dark, and quenching using 10 µL 1.48 mM dithiothreitol. Tryptic digestion was performed the same way for both conditions, as described in the main method section. To each replicate, 2.5 fmol PTEN SIS and p110α SIS were added as internal standards. iMALDI assays were conducted as described in the main method section. Two-sided t-tests with a confidence level of 0.99 were performed to compare the END:dSIS ratios and SIS:dSIS ratios for PTEN and p110α between samples digested with and without reduction and alkylation.

iMALDI method validation – linearity

MDA-MB 231 lysate was used as matrix and 10 µg total protein per replicate was digested as described in the main method section. Mixed calibration stock solutions of PTEN+p110α dSIS (2.5, 1.75, 1, 0.75, 0.5, 0.375, 0.25, 0.125, 0.06, 0.03, 0.016, 0 fmol/µL, each) were prepared in PBSC using an automated calibration curve-preparation protocol.

Calibration standards were prepared by adding 20 µL of calibration stock solution to 10 µg MDA-MB 231 digest, resulting in 50, 35, 20, 15, 10, 7.5, 5, 2.5, 1.25, 0.6, 0.3, 0 fmol dSIS per standard. Three replicates were prepared for each standard. 2.5 fmol PTEN SIS and p110α SIS (PTEN+p110α SIS) were added to

each replicate as internal standards. Peptides were analysed using the developed iMALDI assay as described in the main method section.

The peak intensities ratios of PTEN dSIS and p110α dSIS (PTEN+p110α dSIS) to the respective SIS peptides were calculated and calibration curves were generated. 1/x^2 weighted regressions were calculated for each both target peptides in linear mode and 1/x weighted regressions in reflectron mode. To assess the linearity, the ratios of the residuals divided by the calculated fitted values (in percent) were calculated.

iMALDI method validation – accuracy

MDA-MB 231 digest was used as matrix. Ten µg of total protein per replicate was digested as described in the main method section, and three accuracy test solutions were prepared. Acc #1: MDA-MB 231 digest was spiked with PTEN dSIS and p110α dSIS, both to a final concentration of 1.8 fmol/µg total protein (18 fmol total PTEN/ p110α dSIS per replicate). Acc #2: Acc #1 was diluted with MDA-MB 231 digest to a final concentration of 1.0 fmol/µg total protein for both dSIS (10 fmol total PTEN/ p110α dSIS per replicate). Acc #3: Acc #1 was diluted as above to a final concentration of 0.2 fmol/µg total protein (2 fmol total PTEN/ p110α dSIS per replicate). Four replicates were prepared for each accuracy test solution.

For external calibration, BSA digest (10 µg total protein per replicate) was spiked with increasing amounts of PTEN+p110α dSIS, ranging from 0 to 20 fmol.

Peptide enrichment, bead washing and spotting, matrix spotting, and spot washing were performed as described in the main method section. To each accuracy sample and calibration standard, 2.5 fmol PTEN+p110α SIS were added as internal standards (normalizer).

To assess accuracy, PTEN+p110α dSIS concentrations in the Acc #1-#3 samples were determined using the calibration curve generated above.

iMALDI method validation – interference

Recombinant PTEN and p110α/p85α proteins were each spiked into MDA-MB 231 lysate to a concentration of 1.5 fmol recombinant protein/1 µg total lysate protein (15 fmol protein per protein and 10-µg replicate). The sample was then diluted either 2-, 4-, or 8-fold using PBSC and digested as described in the main method section. To each digest, 2.5 fmol PTEN+p110α dSIS were spiked in as internal standards. Four replicates were prepared per dilution. Assay preparation, bead washing and spotting, matrix spotting and spot washing were performed as described in the main method section. For external calibration, constant PTEN+p110α dSIS (2.5 fmol per replicate) were spiked in together with variable amounts of PTEN and p110α NAT (ranging from 0 to 20 fmol) and 10 µg total protein BSA digest as surrogate matrix. Three replicates were prepared for each calibration standard. Concentrations of recombinant PTEN and p110α were determined based on the external calibration.

iMALDI method validation – precision

To assess the five-day precision across the working range of the multiplexed PTEN+p110α assay, three different pools of MDA-MB 231 cell lysate (labeled 'low', 'medium', 'high') were prepared by adding PTEN+p110α dSIS to achieve concentrations of 2, 10, and 18 fmol /10 µg of total lysate protein, respectively. The pools were then diluted to a total protein concentration of 0.1 µg/µL, and each pool was split into five aliquots and stored at -80 °C until used.

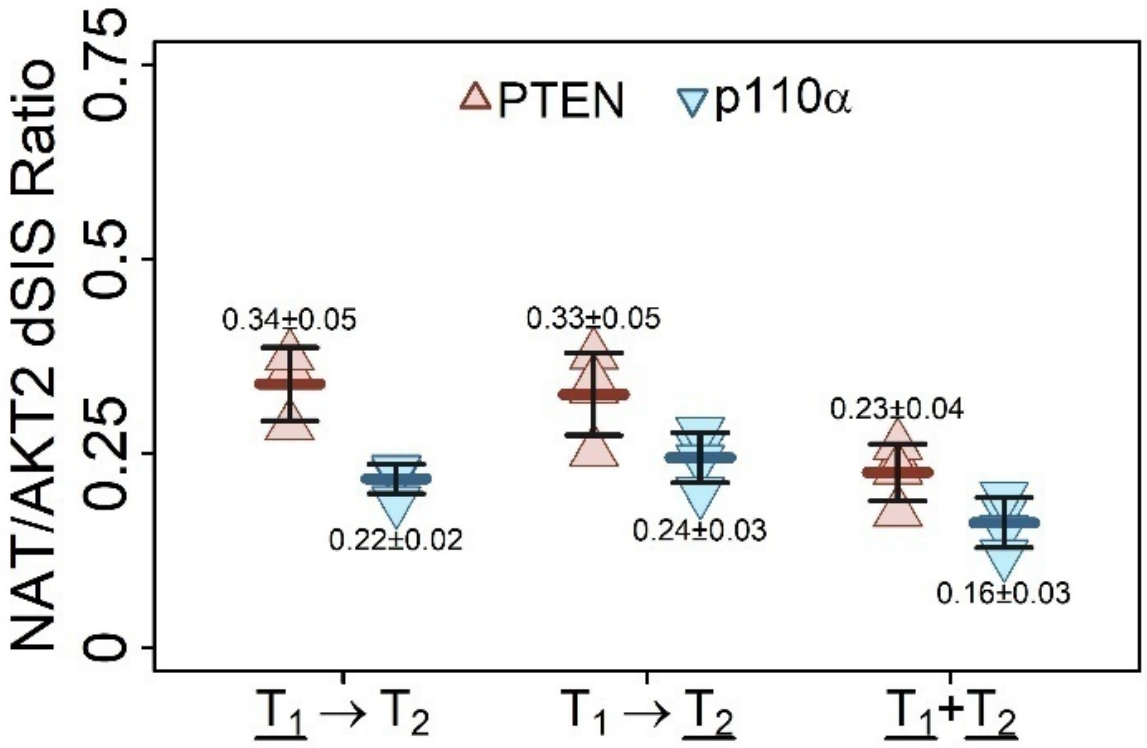
For five consecutive days, one aliquot each of the 'low', 'medium', and 'high' pool was analyzed in three technical replicates. Ten µg of total protein was analyzed per replicate, resulting in PTEN+p110α dSIS amounts of 2, 10, and 18 fmol. An external calibration curve with varying concentrations of PTEN+p110α dSIS (0-20 fmol per replicate) in BSA digest (10 µg total protein per replicate) was generated fresh, daily. Constant amounts of 2.5 fmol PTEN+p110α SIS were added as internal standards

to each sample and calibration standard. Antibody-coupled magnetic beads were prepared fresh daily and stored while rotating at 4 °C until use. Assay preparation, bead washing and spotting, matrix spotting, and spot washing were performed as described in the main method section. Spiked-in PTEN+p110α dSIS concentrations were determined using the calibration curves prepared on the same day.

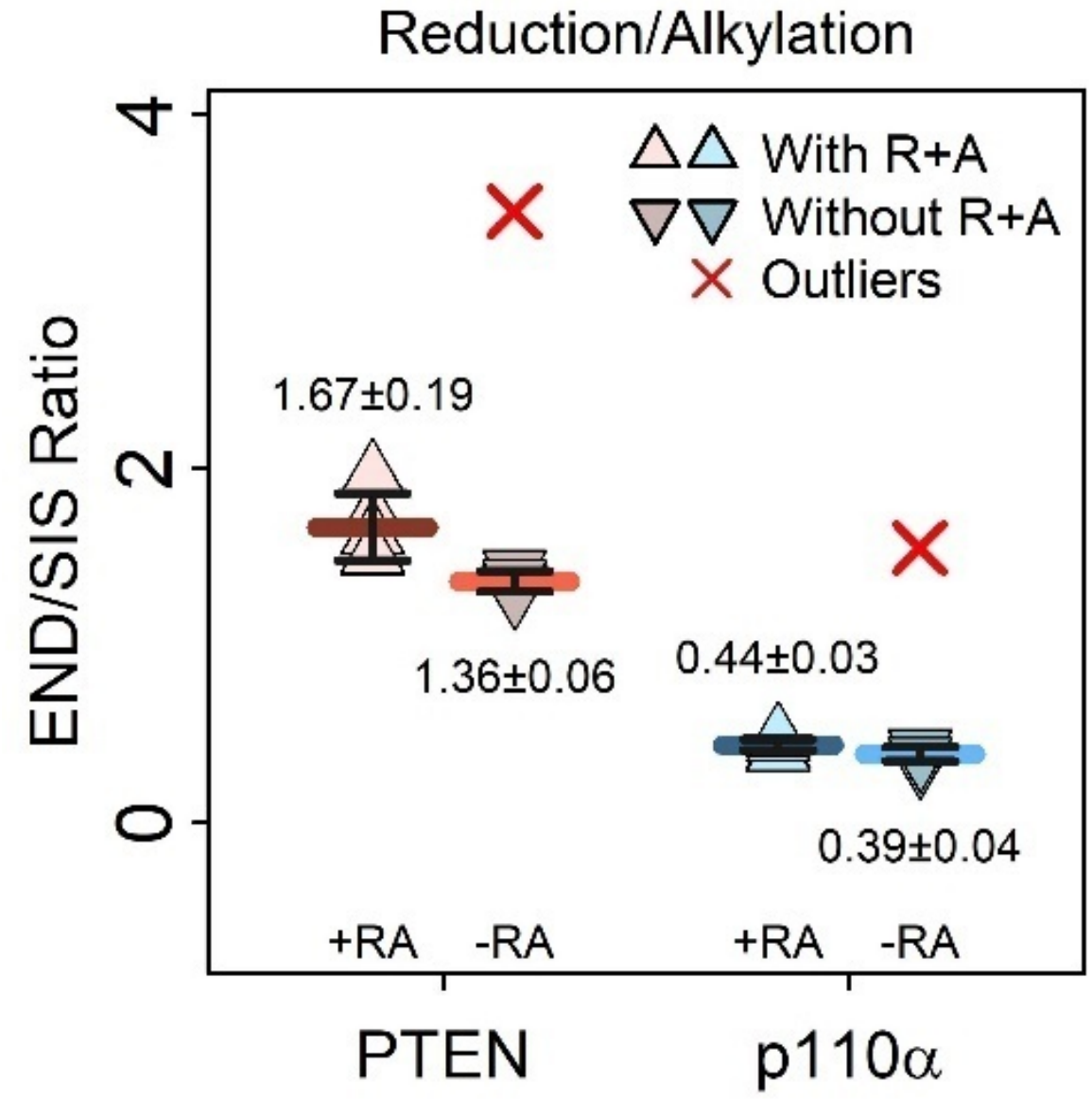
Overall CVs were calculated based on the average Intra-day CVs and the inter-day CVs (**Equation. 1**).[1]

$$(Eq.1) CV_{Total} = \sqrt{mean(Intra - Day\ CV)^2 + Inter - Day\ CV^2}$$

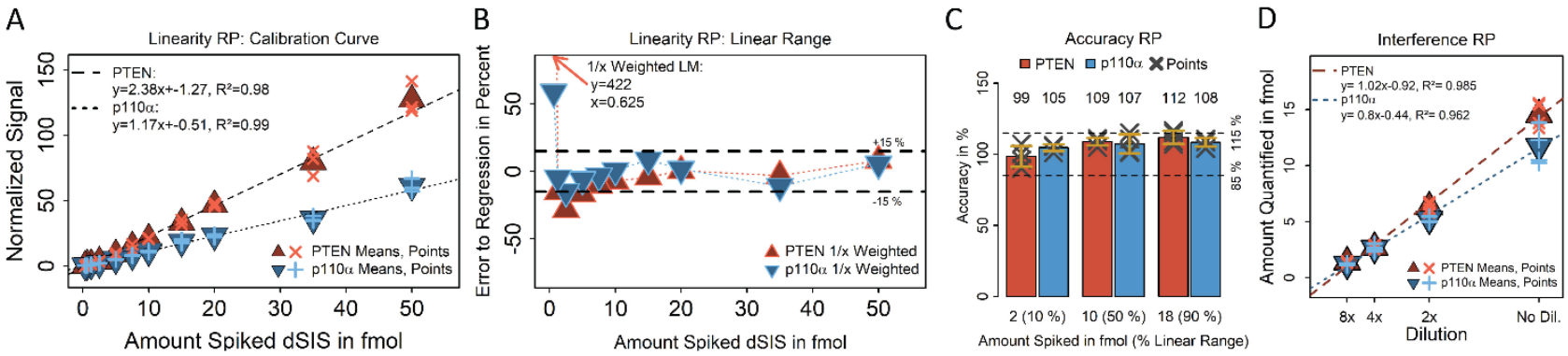
Supplementary Information -- Figures



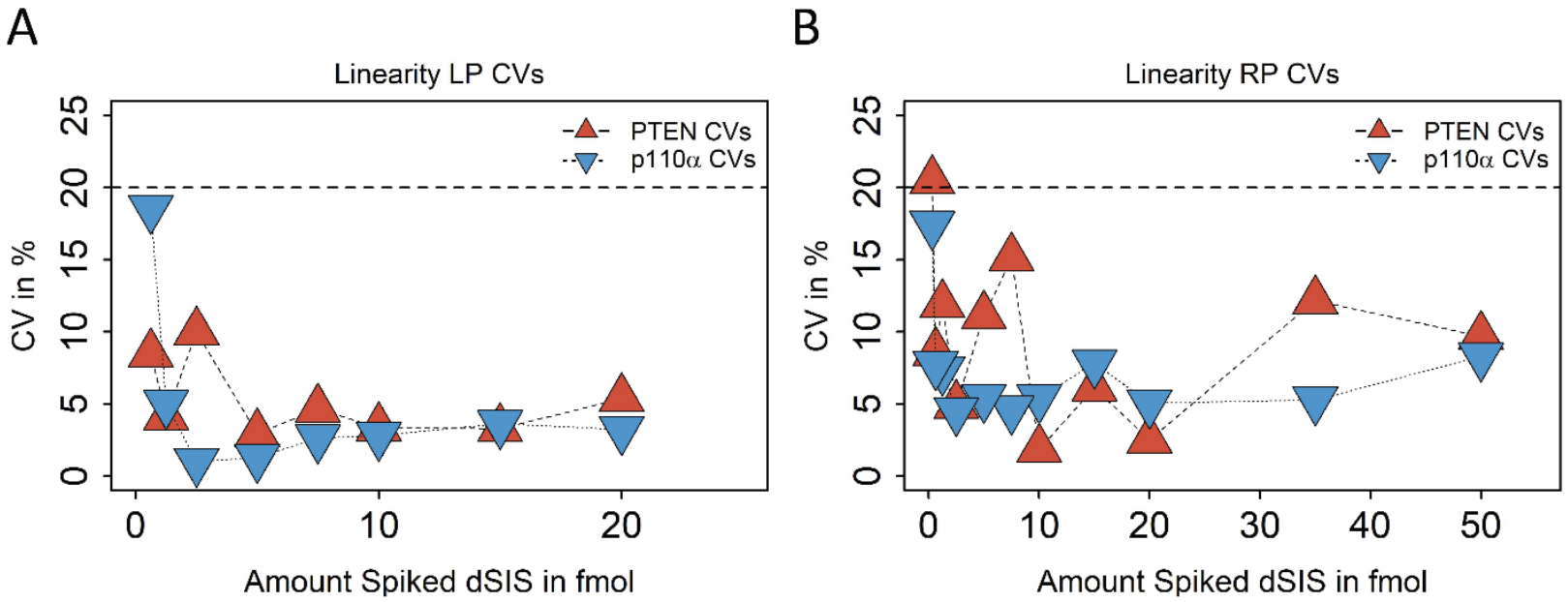
Supplementary Information Figure S1. Comparison of sequential ($\underline{T_1}$→T_2 or T_1→$\underline{T_2}$) and simultaneous enrichment of PTEN and p110α NAT peptides ($\underline{T_1}$+$\underline{T_2}$). Peptide intensities were normalized using 1 fmol AKT2 dSIS, which was spiked into the MALDI matrix. A slightly lower recovery was detected for simultaneous enrichment, but was not significant (p>0.01).



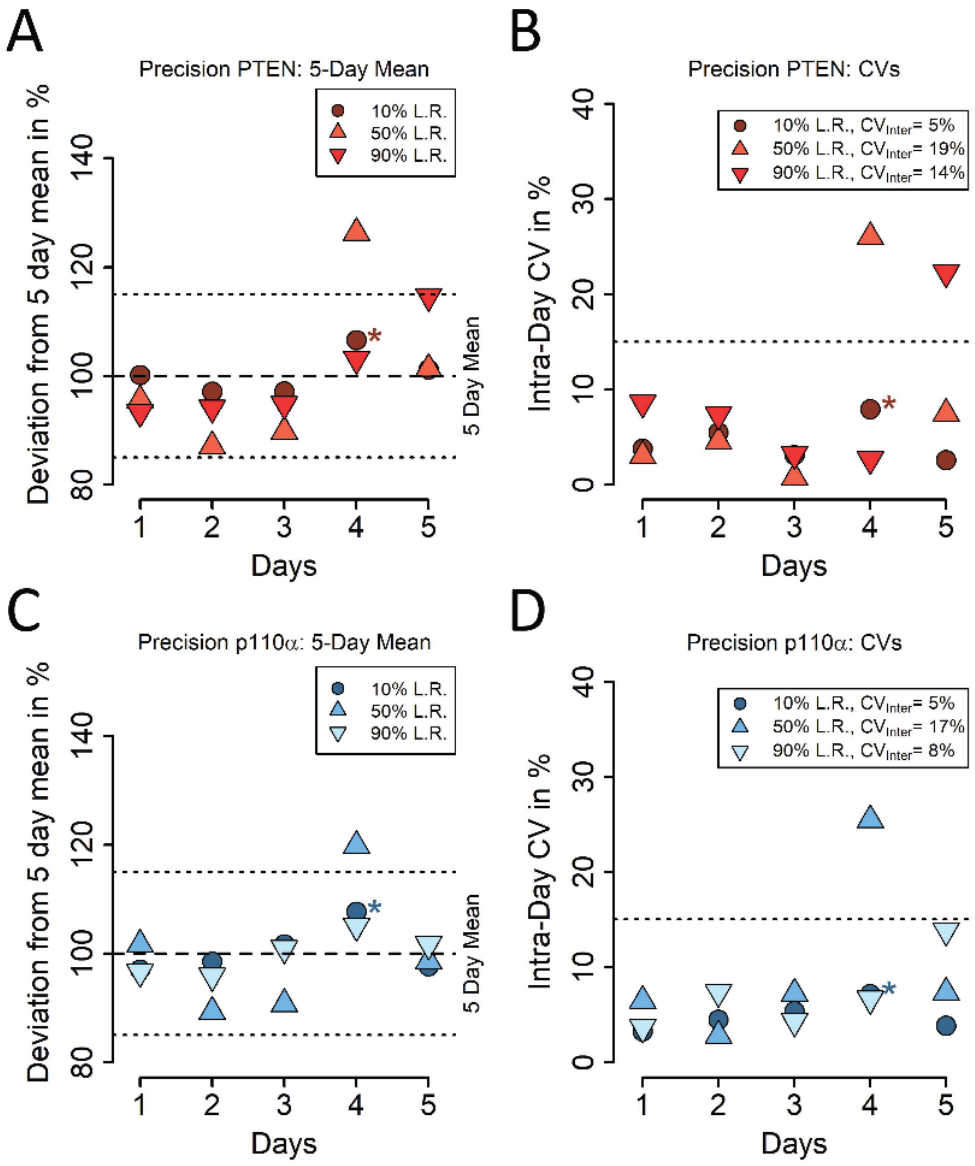
Supplementary Information Figure S2. Reduction and alkylation (+RA) of disulfide bonds prior to tryptic digest does not significantly alter PTEN and p110α recovery. Ten µg of MDA-MB-231 lysate (total protein) with (+RA) and without reduction and alkylation (-RA) were analyzed by multiplexed iMALDI (n=5). Error bars represent absolute standard deviations; horizontal bars represent the means. ** An outlier (END/SIS Ratio > 3rd quartile+3x Interquartile Range) was excluded due to a potential contamination observed in the MALDI-TOF spectrum.*



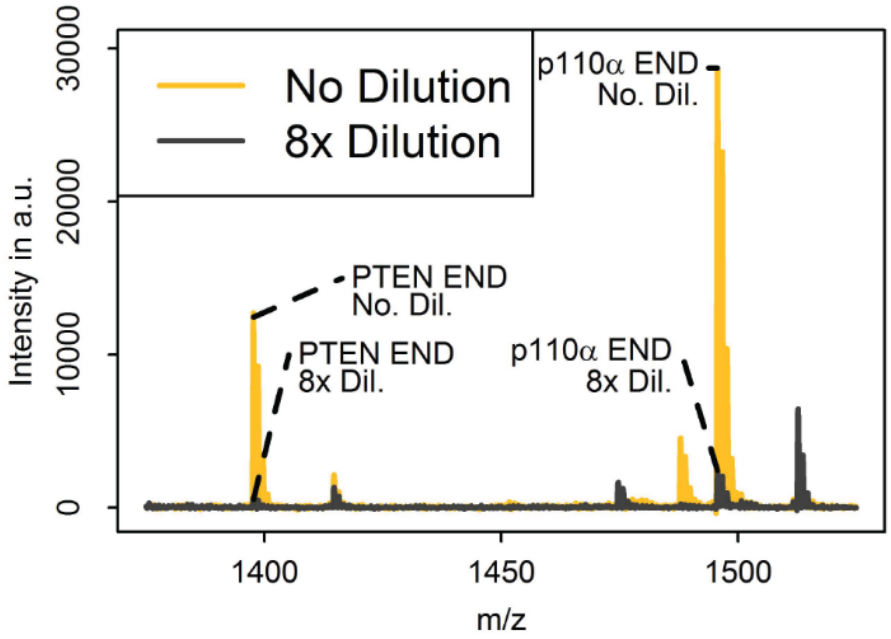
Supplementary Information Figure S3. Method validation of the multiplexed PTEN+p110α iMALDI assay in Reflectron mode (RP). All data shown was recorded in RP mode. **(A+B) Linearity of the multiplexed PTEN+p110α iMALDI assay.** 10 µg of MDA-MB-231 digest were spiked with 0-50 fmol PTEN+p110α dSIS peptides and 2.5 fmol PTEN and p110α SIS. dSIS amounts were calculated based on dSIS/SIS ratios and plotted against the known dSIS spike-ins (n=3). **(A)** The linear range is from 1-50 fmol. **(B)** All means were within a ±15% error margin of the regression and thus were considered linear**. (C) Accuracy of the multiplexed PTEN/p110α iMALDI assay.** 10 µg of MDA-MB-231 lysate were spiked with 2, 10, and 18 fmol of PTEN+p110α dSIS (10%, 50 %, and 90% of the linear range in LP). PTEN+p110α dSIS amounts were determined and compared to the theoretical spike-ins to assess accuracy in %. Error bars represent absolute standard deviations. N=4. High accuracies well within ±15 % are obtained across the linear range. **(D)** Interference testing for PTEN and p110α iMALDI assays. 10 µg MDA-MB-231 lysate (total protein) was spiked with 15 fmol of recombinant PTEN and p110α/p85α, respectively, and diluted 2-, 4-, and 8-fold prior to tryptic digestion. Dilutional linearity was determined.



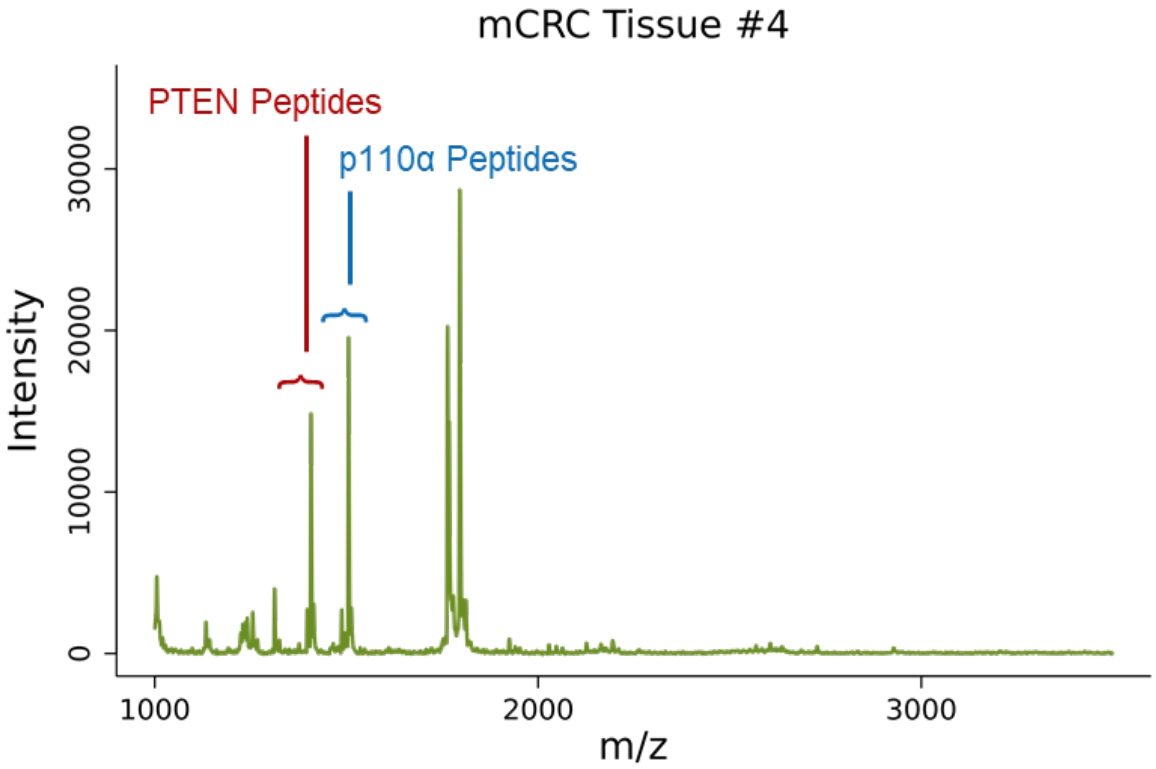
Supplementary Information Figure S4. CVs of calibration curves presented in figure 2B and Supplementary Figure S1A, and which were used to assess linearity. Ten µg of MDA-MB 231 digest per replicate were spiked with 0-50 fmol PTEN+p110α dSIS peptides and 2.5 fmol PTEN and p110α SIS. The CVs of the normalized signals for each tested standard are shown. N=3 per concentration. Data recorded in (A) linear and (B) reflectron mode, showing that the CVs were consistently below 20%.



Supplementary Information Figure S5. Inter- and intra-day precision of the multiplexed PTEN/p110α iMALDI assay. Three pools of MDA-MB-231 lysate were spiked with 2, 10, or 18 fmol of PTEN+p110α dSIS, corresponding to 10%, 50%, and 90% of the assay's linear range (low, medium, and high levels), respectively. One fresh aliquot from each pool was analyzed each day for five consecutive days in triplicate. Data was recorded in the RP mode. **(A+C)** The deviation from the 5-day mean for low, medium, and high samples is given for **(A)** PTEN and **(C)** p110α. **(B+D)** Intra-day CVs and 5-day inter-day CVs for low, medium, and high-level samples**.** *An outlier (amount quantified > 3rd quartile$_{5\text{-day 'low' results}}$+3x Interquartile Range$_{5\text{-day 'low' results}}$) was excluded from the 'low' sample on Day 4.*



Supplementary Information Figure S6. Mass spectra of endogenous PTEN and p110α peptides quantified in 10 µg MDA-MB 231 cell lysate spiked with 15 fmol of PTEN and p110α recombinant protein (shown in gold), as well as in an 8x diluted samples (shown in black). Spectra were recorded in the reflecton mode.



Supplementary Information Figure S7. MALDI Mass Spectrum of endogenous PTEN and p110α peptides quantified in 10 µg mCRC tumor tissue sample #4, recorded in linear mode, showing the high specificity of the antibody enrichment of analytes from complex matrices.

Supplementary Information -Tables

Supplementary Information Table S1. NAT/SIS peptide intensity ratios of the multiplexing experiments presented in Figure 1 are shown. *E.coli* lysate digest (10 µg per replicate) was spiked with 1 fmol ('low') or 10 fmol ('high') PTEN+p110α NAT. Peptides were enriched using either singleplex, sequential (i.e. PTEN-p110α or p110α-PTEN, referred to as $\underline{T_1}\rightarrow T_2$ or $T_1\rightarrow\underline{T_2}$), or simultaneous enrichment ($\underline{T_1}+\underline{T_2}$). Mean NAT/SIS ratios and corresponding standard deviations in fmol are displayed, as well as the corresponding CVs in %. Additionally, the ratio of the mean NAT/SIS ratios using multiplexed assays and the corresponding mean NAT/SIS ratio of the singleplex assay are displayed (e.g. mean NAT/SIS $PTEN_{\underline{T1}\rightarrow T2\ High}$ divided by mean NAT/SIS $PTEN_{Singleplex\ High}$). Data was recorded in the linear mode. Both multiplexing methods showed similar performance.

	Peptide	Singleplex High	Singleplex Low	$\underline{T_1}\rightarrow T_2$ High	$\underline{T_1}\rightarrow T_2$ Low	$T_1\rightarrow\underline{T_2}$ High	$T_1\rightarrow\underline{T_2}$ Low	$\underline{T_1}\rightarrow\underline{T_2}$ High	$\underline{T_1}\rightarrow\underline{T_2}$ Low
NAT in fmol	-	**10**	**1**	**10**	**1**	**10**	**1**	**10**	**1**
1	PTEN	2	0.3	2.1	-	1.9	0.3	1.9	0.3
2	PTEN	1.5	0.3	1.9	0.3	1.8	0.3	1.8	0.2
3	PTEN	1.9	0.3	2	0.3	2	0.3	1.9	0.3
4	PTEN	1.8	0.3	2	0.3	2	0.2	1.8	0.2
Mean	PTEN	1.8	0.3	2	0.3	1.9	0.3	1.9	0.3
Abs. SD	PTEN	0.2	0.02	0.1	0.01	0.1	0.02	0.1	0.01
CV	PTEN	11.4	8.1	3.5	1.8	4	8.8	3.1	4.4
$\frac{NAT}{SIS}_{Multiplex}/\frac{NAT}{SIS}_{Singleplex}$	PTEN	-	-	1.09	1.07	1.05	1.04	1.02	0.93
1	p110α	4.3	0.5	4.4	0.4	4.5	0.4	4.5	0.4
2	p110α	4.4	0.4	4.1	0.4	5.1	0.4	4.2	0.4
3	p110α	4.5	0.4	4.2	0.4	4.8	0.4	4.4	0.4
4	p110α	4.6	0.4	4.2	0.4	4.8	0.4	4.4	0.4
Mean	p110α	4.5	0.4	4.2	0.4	4.8	0.4	4.4	0.4
Abs. SD	p110α	0.1	0.1	0.2	0.02	0.2	0.03	0.1	0.02
CV	p110α	3	12.9	3.6	5.7	4.9	8	2.7	4.1
$\frac{NAT}{SIS}_{Multiplex}/\frac{NAT}{SIS}_{Singleplex}$	p110α	-	-	0.95	0.93	1.08	0.95	0.99	0.88

Supplementary Information Table S2. NAT/AKT dSIS peptide intensity ratios of the multiplexing experiments presented in Supplementary Figure S1 are displayed. *E.coli* lysate digest (10 µg per replicate) were spiked with 1 fmol ('low') or 10 fmol ('high') PTEN+p110α NAT. Peptides were enriched using either sequential enrichment (PTEN→p110α or p110α→PTEN, referred to as $\underline{T_1}\rightarrow T_2$ or $T_1\rightarrow\underline{T_2}$) or simultaneous enrichment ($\underline{T_1}+\underline{T_2}$). A constant amount of AKT2 dSIS was spiked into the MALDI matrix (1 fmol per spot). Data was recorded in the linear ion mode. Both multiplexing methods showed similar performance.

	Peptide	$\underline{T_1}\rightarrow T_2$ High	$\underline{T_1}\rightarrow T_2$ Low	$T_1\rightarrow\underline{T_2}$ High	$T_1\rightarrow\underline{T_2}$ Low	$\underline{T_1}+\underline{T_2}$ High	$\underline{T_1}+\underline{T_2}$ Low
NAT in fmol	-	10	1	10	1	10	1
1	PTEN	2.8		3.3	0.3	2.1	0.2
2	PTEN	3.1	0.4	3.5	0.4	2.4	0.3
3	PTEN	3.3	0.4	4.2	0.3	3	0.2
4	PTEN	2.5	0.3	3.5	0.3	2.2	0.2
Mean	PTEN	2.9	0.3	3.6	0.3	2.4	0.2
Abs. SD	PTEN	0.4	0.05	0.4	0.05	0.4	0.04
CV	PTEN	12	14	11	16	16	16
1	p110α	2.9		3.1	0.3	2	0.2
2	P110α	2.9	0.2	3.4	0.3	2.3	0.2
3	p110α	2.8	0.2	3.5	0.2	2.5	0.2
4	p110α	2.7	0.2	3.3	0.2	1.9	0.1
Mean	p110α	2.8	0.2	3.3	0.2	2.2	0.2
Abs. SD	p110α	0.1	0.03	0.1	0.03	0.3	0.03
CV	p110α	4	11	4	13	12	20

Supplementary Information Table S3. END/SIS peptide intensity ratios of the reduction and alkylation experiments shown in Supplementary Information Figure S2 are shown. MDA-MB 231 cell lysate (10 µg total protein per replicate) with or without reduction and alkylation prior to digestion was analysed. PTEN+p110α SIS peptides (2.5 fmol per replicate) were added to each sample. Data was recorded in the linear mode. No significant difference ($p<0.01$) was found between samples with or without reduction and alkylation prior to digestion.

		Replicate							
Protein	**Reduced+ Alkylated**	**1**	**2**	**3**	**4**	**5**	**Mean**	**Abs. SD**	**CV**
PTEN	+	1.74	1.96	1.51	1.62	1.52	1.67	0.19	11
PTEN	-	1.35	1.43	1.38	1.29	3.45	1.36	0.06	4
p110α	+	0.42	0.45	0.4	0.45	0.48	0.44	0.03	7
p110α	-	0.42	0.42	0.34	0.38	1.55	0.39	0.04	10

Supplementary Information Table S4. Linearity values of the calibration curves presented in Figure 2 B are displayed. MDA-MB 231 lysate digest (10 µg per replicate) was spiked with varying amounts of PTEN+p110α dSIS and constant amounts of corresponding SIS standards (2.5 fmol). dSIS/SIS ratios (normalized by multiplying dSIS/SIS ratios by the amount of spiked-in SIS) are displayed for each replicate. Corresponding means, standard deviation, and relative standard deviation (Abs. SD, CV) are shown for each tested dSIS amount. Data presented were collected in the linear mode. The linear range of the calibration curve is from 0.6 to 20 fmol. N.d.=Not detectable.

	PTEN				p110α			
Spiked dSIS in fmol	**dSIS/SIS (normalized)**	**Mean**	**Abs. SD**	**CV**	**dSIS/SIS (normalized)**	**Mean**	**Abs. SD**	**CV**
50	46.9				33.5			
50	43.9	47.4	3.8	8	29.2	32.3	2.7	8
50	51.5				34.2			
35	35.2				23.1			
35	35.4	36.1	1.4	4	25.0	24.7	1.4	6
35	37.7				26.0			
20	25.7				17.0			
20	23.2	24.2	1.3	5	16.0	16.7	0.5	3
20	23.6				17.0			
15	19.1				13.3			
15	20.0	19.8	0.7	3	13.5	13.1	0.5	4
15	20.3				12.6			
10	13.6				8.7			
10	14.4	14.1	0.5	3	9.2	9.0	0.3	3
10	14.3				9.1			
7.5	10.9				6.7			
7.5	10.4	10.9	0.5	5	6.9	6.9	0.2	3
7.5	11.5				7.1			
5	6.5				4.7			
5	6.1	6.4	0.2	3	4.6	4.7	0.1	1
5	6.4				4.7			
2.5	2.8				2.3			
2.5	3.3	3.1	0.3	10	2.4	2.4	0.0	1
2.5	3.2				2.4			
1.25	1.6				1.4			
1.25	1.5	1.6	0.1	4	1.3	1.3	0.1	5
1.25	1.7				1.3			
0.62	0.9				1.0			
0.62	0.9	0.9	0.1	8	0.7	0.8	0.2	19
0.62	0.8				0.7			
0.31	n.d.				0.6			
0.31	n.d.	-	-	-	0.5	0.5	0.1	9
0.31	n.d.				0.5			

Supplementary Information Table S5. Linearity values of the calibration curves presented in Supplementary Figure S2 B are displayed. MDA-MB 231 lysate digest (10 µg per replicate) were spiked with varying amounts of PTEN+p110α dSIS and constant amounts of corresponding SIS standards (2.5 fmol). dSIS/SIS ratios (normalized by multiplying dSIS/SIS ratios by the amount of spiked-in SIS) are shown for each replicate. Corresponding means, standard deviation and relative standard deviation (Abs. SD, CV) are shown for each tested dSIS amount. Data presented were collected in the reflectron mode. The linear range of the calibration curve was from 0.6 to 20 fmol. *N.d.*=Not detectable.

	PTEN				p110α			
Spiked dSIS in fmol	**dSIS/SIS (normalized)**	**Mean**	**Abs. SD**	**CV**	**dSIS/SIS (normalized)**	**Mean**	**Abs. SD**	**CV**
50	141.4				66.9			
50	121.4	127.2	12.3	10	57.1	61.2	5.1	8
50	118.8				59.7			
35	68.8				38.1			
35	82.0	79.5	9.7	12	34.6	36.0	1.9	5
35	87.7				35.2			
20	46.4				24.0			
20	47.9	46.6	1.2	3	23.9	23.3	1.2	5
20	45.6				21.9			
15	35.3				18.1			
15	31.6	33.0	2.0	6	20.1	18.5	1.5	8
15	32.1				17.3			
10	21.1				10.6			
10	20.5	20.9	0.4	2	11.6	11.3	0.6	5
10	21.2				11.6			
7.5	16.1				8.2			
7.5	12.3	14.9	2.3	15	7.6	8.0	0.4	5
7.5	16.3				8.2			
5	9.7				4.7			
5	9.0	8.8	1.0	11	5.2	5.0	0.3	5
5	7.8				5.2			
2.5	3.5				2.1			
2.5	3.4	3.4	0.2	5	2.0	2.1	0.1	5
2.5	3.2				2.1			
1.25	1.2				0.9			
1.25	1.5	1.4	0.2	12	1.0	0.9	0.1	7
1.25	1.6				0.8			
0.62	1.1				0.4			
0.62	1.0	1.1	0.1	9	0.3	0.4	0.0	8
0.62	1.2				0.3			
0.31	n.d.				0.3			
0.31	n.d.	-	-	-	0.2	0.2	0.0	17
0.31	n.d.				0.3			

Supplementary Information Table S6. Accuracy results shown in Figure 2C are displayed. MDA-MB 231 cell lysate (10 µg total protein per replicate) was spiked with 2, 10 or 18 fmol PTEN+p110α dSIS peptides. PTEN+p110α SIS peptides (2.5 fmol per replicate) were added to each sample as internal standard. Recovered dSIS peptide amounts and accuracy (recovered amount/theoretical spike-in amount) were calculated. Data was recorded in linear mode. High accuracies were found across the working range of the assay.

		Absolute Peptide Amounts Quantified in fmol			Accuracy in %		
	Peptide	2 fmol spike-in	10 fmol spike-in	18 fmol spike-in	2 fmol spike-in	10 fmol spike-in	18 fmol spike-in
Replicate 1	PTEN	2.1	10.9	15.6	104	109	87
Replicate 2	PTEN	2	9.5	17	101	95	95
Replicate 3	PTEN	2	9.9	16.9	100	99	94
Replicate 4	PTEN	2.1	9.7	15.2	104	97	84
Mean	PTEN	2	10	16.2	102	100	90
Abs. SD	PTEN	0	0.6	0.9	2	6	5
CV (%)	PTEN	2	6	6	2	6	6
Replicate 1	p110α	2.2	11	17.2	111	110	95
Replicate 2	p110α	2.2	10.3	17.8	110	104	99
Replicate 3	p110α	2.3	10.2	17.9	114	102	99
Replicate 4	p110α	2.2	10.2	16.6	112	102	92
Mean	p110α	2.2	10.5	17.4	112	105	97
Abs. SD	p110α	0	0.4	0.6	2	4	3
CV (%)	p110α	2	4	4	2	4	4

Supplementary Information Table S7. Accuracy results shown in Supplementary Figure 2C are shown. MDA-MB 231 cell lysate (10 µg total protein per replicate) was spiked with 2, 10, or 18 fmol PTEN+p110α dSIS peptides. PTEN+p110α SIS peptides (2.5 fmol per replicate) were added to each sample as internal standard. Recovered dSIS peptide amounts and accuracy (recovered amount/theoretical spike-in amount) were calculated. Data was recorded in the reflectron mode. High accuracies were found across the working range of the assay.

		Absolute Peptide Amounts Quantified in fmol			Accuracy in %		
	Peptide	2 fmol spike-in	10 fmol spike-in	18 fmol spike-in	2 fmol spike-in	10 fmol spike-in	18 fmol spike-in
Replicate 1	PTEN	2	11	19.6	101	110	109
Replicate 2	PTEN	2.1	10.5	21	107	105	117
Replicate 3	PTEN	1.8	11.1	19.2	90	111	107
Replicate 4	PTEN	1.9	10.9	20.7	96	109	115
Mean	PTEN	2	10.9	20.1	99	109	112
Abs. SD	PTEN	0.1	0.3	0.8	7	3	5
CV (%)	PTEN	7	2	4	7	2	4
Replicate 1	p110α	2	11.5	19.9	101	115	110
Replicate 2	p110α	2.1	11.1	19.6	105	111	109
Replicate 3	p110α	2.1	10.2	18.7	106	102	104
Replicate 4	p110α	2.1	10.1	19.9	106	101	111
Mean	p110α	2.1	10.7	19.5	105	107	109
Abs. SD	p110α	0	0.7	0.5	3	7	3
CV (%)	p110α	2	6	3	2	6	3

Supplementary Information Table S8. Interference results shown in Figure 2D are given here. MDA-MB 231 cell lysate (10 µg total protein per replicate) was spiked with recombinant PTEN and p110α/p85α protein (approximately 15 fmol per replicate). Samples were serially diluted by a factor of 2, 4, and 8 using PBSC. PTEN+p110α dSIS peptides (2.5 fmol per replicate) were added to each sample as internal standard. Endogenous PTEN and p110α amounts (in fmol) were calculated for each sample. Data was recorded in the linear mode. Dilutional linearity was demonstrated.

		Dilution			
	Protein	**No dilution**	**2x**	**4x**	**8x**
Replicate 1	PTEN	13.4	6.8	3.4	1.9
Replicate 2	PTEN	14.4	7.5	3	1.8
Replicate 3	PTEN	14.1	6.9	3.4	1.7
Replicate 4	PTEN	13.8	7.2	3.5	1.8
Mean	PTEN	13.9	7.1	3.3	1.8
Abs. SD.	PTEN	0.4	0.3	0.2	0.1
CV (%)	PTEN	3	5	7	6
Replicate 1	p110α	10.8	5.6	3.2	1.4
Replicate 2	p110α	11.8	5.5	2.7	1.3
Replicate 3	p110α	11.2	5.5	2.9	1.3
Replicate 4	p110α	12.0	5.8	2.8	1.3
Mean	p110α	11.4	5.6	2.9	1.3
Abs. SD.	p110α	0.5	0.1	0.2	0
CV (%)	p110α	5	2	7	4

Supplementary Information Table S9. Interference results shown in Supplementary Figure 2D are given here. MDA-MB 231 cell lysate (10 µg total protein per replicate) was spiked with recombinant PTEN and p110α/p85α protein (approximately 15 fmol per replicate). Samples were serially diluted by a factor of 2, 4, and 8 using PBSC. PTEN+p110α dSIS peptides (2.5 fmol per replicate) were added to each sample as internal standard. Endogenous PTEN and p110α amounts (in fmol) were calculated for each sample. Data was recorded in the reflectron mode. Dilutional linearity was demonstrated.

		Dilution			
	Protein	**No dilution**	**2x**	**4x**	**8x**
Replicate 1	PTEN	13.4	5.8	2.9	1.3
Replicate 2	PTEN	13.8	6.1	2.6	1.4
Replicate 3	PTEN	15.6	6.7	2.5	1.4
Replicate 4	PTEN	15.4	6.6	2.8	1.5
Mean	PTEN	14.5	6.3	2.7	1.4
Abs. SD.	PTEN	1.1	0.4	0.2	0.1
CV (%)	PTEN	8	7	7	5
Replicate 1	p110α	10.5	5.0	3	1.3
Replicate 2	p110α	10.2	5.0	2.6	1.3
Replicate 3	p110α	12.3	5.5	2.5	1.2
Replicate 4	p110α	13.8	5.5	2.3	1.1
Mean	p110α	11.7	5.2	2.6	1.2
Abs. SD.	p110α	1.7	0.3	0.3	0.1
CV (%)	p110α	14	5	11	5

Supplementary Information Table S10. 5-day precision results for PTEN in LP shown in Figure 3A are given here. Three pools of MDA-MD 231 lysate were spiked with 2, 10, and 18 fmol PTEN+p110α dSIS (per 10 µg total lysate protein). Each pool (10 µg total protein per replicate) was analysed each day in triplicate. Quantified amounts of PTEN dSIS per replicate (in fmol) are shown. **An outlier (amount quantified > 3rd quartile$_{\text{5-day 'low' results}}$+3x Interquartile Range$_{\text{5-day 'low' results}}$) was excluded from the 'low' sample on Day 4.*

	2 fmol spike-in					10 fmol spike-in					18 fmol spike-in				
Day	**1**	**2**	**3**	**4**	**5**	**1**	**2**	**3**	**4**	**5**	**1**	**2**	**3**	**4**	**5**
Rep. 1	2.1	2.0	2.1	2.4	2.4	9.1	9.3	9.5	12.2	10.5	14.5	14.4	15.3	15.6	15.0
Rep. 2	2.1	2.2	2.2	3.7*	2.4	9.4	9.1	10.3	11.4	10.4	14.4	14.3	16.7	14.3	18.7
Rep. 3	2.1	2.1	2.3	2.2	2.4	9.1	9.2	9.3	9.5	11.3	14.8	14.6	15.1	15.7	18.6
Mean	2.1	2.1	2.2	2.3	2.4	9.2	9.2	9.7	11.0	10.7	14.6	14.5	15.7	15.2	17.4
Abs. SD.	0.0	0.1	0.1	0.1	0.0	0.2	0.1	0.5	1.4	0.5	0.2	0.2	0.8	0.8	2.1
CV (%)	1	3	5	6	0	2	1	6	13	5	1	1	5	5	12
Spike	2	2	2	2	2	10	10	10	10	10	18	18	18	18	18
Acc. (%)	106	105	110	113	120	92	92	97	110	107	81	80	87	84	97
Mean$_{\text{5 Day}}$	2.2	2.2	2.2	2.2	2.2	10.0	10.0	10.0	10.0	10.0	15.5	15.5	15.5	15.5	15.5
Abs. SD.$_{\text{5 Day}}$	0.1	0.1	0.1	0.1	0.1	1.0	1.0	1.0	1.0	1.0	1.4	1.4	1.4	1.4	1.4
CV$_{\text{5 Day}}$ (%)	6	6	6	6	6	10	10	10	10	10	9	9	9	9	9
Acc.$_{\text{5 Day}}$ (%)	111	111	111	111	111	100	100	100	100	100	86	86	86	86	86
CV$_{\text{Total}}$ (%)	7	7	7	7	7	11	11	11	11	11	10	10	10	10	10

Supplementary Information Table S11. Five-day precision results for p110α in LP shown in Figure 3A are given here. Three pools of MDA-MD 231 lysate were spiked with 2, 10, and 18 fmol PTEN+p110α dSIS (per 10 µg total lysate protein). Each pool (10 µg total protein per replicate) was analysed each day in triplicate. Quantified amounts of p110α dSIS per replicate (in fmol) are shown.

**An outlier (amount quantified > 3rd quartile$_{5\text{-day 'low' results}}$+3x Interquartile Range$_{5\text{-day 'low' results}}$) was excluded from the 'low' sample on Day 4.*

	2 fmol spike-in					10 fmol spike-in					18 fmol spike-in				
Day	**1**	**2**	**3**	**4**	**5**	**1**	**2**	**3**	**4**	**5**	**1**	**2**	**3**	**4**	**5**
Rep. 1	2.3	2.2	2.5	2.5	2.3	10.2	10.0	10.8	13.5	11.1	16.6	16.6	17.1	16.8	16.8
Rep. 2	2.6	2.3	2.3	4.1*	2.4	10.5	9.9	10.9	12.3	10.7	16.1	16.9	17.4	16.3	20.1
Rep. 3	2.5	2.3	2.4	2.4	2.4	10.2	10.0	9.8	10.2	12.5	16.0	16.3	16.0	17.6	18.7
Mean	2.5	2.3	2.4	2.4	2.4	10.3	10.0	10.5	12.0	11.4	16.2	16.6	16.8	16.9	18.5
Abs. SD.	0.2	0.1	0.1	0.1	0.0	0.2	0.1	0.6	1.7	1.0	0.3	0.3	0.8	0.7	1.7
CV (%)	6	2	5	4	1	2	1	6	14	8	2	2	5	4	9
Spike	2.0	2.0	2.0	2.0	2.0	10.0	10.0	10.0	10.0	10.0	18.0	18.0	18.0	18.0	18.0
Acc. (%)	123	114	121	122	118	103	100	105	120	114	90	92	93	94	103
Mean$_{5\ Day}$	2.4	2.4	2.4	2.4	2.4	10.8	10.8	10.8	10.8	10.8	17.0	17.0	17.0	17.0	17.0
Abs. SD.$_{5\ Day}$	0.1	0.1	0.1	0.1	0.1	1.1	1.1	1.1	1.1	1.1	1.1	1.1	1.1	1.1	1.1
CV$_{5\ Day}$ (%)	4	4	4	4	4	10	10	10	10	10	7	7	7	7	7
Acc.$_{5\ Day}$ (%)	119	119	119	119	119	108	108	108	108	108	94	94	94	94	94
CV$_{Total}$ (%)	6	6	6	6	6	12	12	12	12	12	8	8	8	8	8

Supplementary Information Table S12. Five-day precision results for PTEN in RP shown in Supplementary Figure 3A are given here. Three pools of MDA-MD 231 lysate were spiked with 2, 10, and 18 fmol PTEN+p110α dSIS (per 10 µg total lysate protein). Each pool (10 µg total protein per replicate) was analyzed each day in triplicate. Quantified amounts of PTEN dSIS per replicate (in fmol) are shown.

	2 fmol spike-in					10 fmol spike-in					18 fmol spike-in				
Day	**1**	**2**	**3**	**4**	**5**	**1**	**2**	**3**	**4**	**5**	**1**	**2**	**3**	**4**	**5**
Rep. 1	2.0	1.8	1.9	2.2	2.0	10.5	10.2	10.0	16.9	10.7	20.2	17.0	19.2	20.0	17.1
Rep. 2	1.9	2.0	1.9	3.7	2.0	11.0	9.3	10.1	15.4	12.3	17.8	19.0	18.0	20.9	23.8
Rep. 3	2.0	2.0	2.0	2.0	2.1	10.5	9.7	9.9	9.9	11.0	17.2	19.6	18.9	20.1	26.9
Mean	2.0	1.9	1.9	2.1	2.0	10.7	9.7	10.0	14.1	11.3	18.4	18.6	18.7	20.3	22.6
Abs. SD.	0.1	0.1	0.1	0.2	0.1	0.3	0.4	0.1	3.7	0.8	1.6	1.4	0.6	0.5	5.0
CV (%)	4	5	3	8	3	3	5	1	26	7	9	7	3	3	22
Spike	2.0	2.0	2.0	2.0	2.0	10.0	10.0	10.0	10.0	10.0	18.0	18.0	18.0	18.0	18.0
Acc. (%)	99	96	96	106	100	107	97	100	141	113	102	103	104	113	126
$Mean_{5\ Day}$	2.0	2.0	2.0	2.0	2.0	11.2	11.2	11.2	11.2	11.2	19.7	19.7	19.7	19.7	19.7
$Abs.\ SD._{5\ Day}$	0.1	0.1	0.1	0.1	0.1	2.2	2.2	2.2	2.2	2.2	2.7	2.7	2.7	2.7	2.7
$CV_{5\ Day}$ (%)	5	5	5	5	5	19	19	19	19	19	14	14	14	14	14
$Acc._{5\ Day}$ (%)	99	99	99	99	99	111	111	111	111	111	109	109	109	109	109
CV_{Total} (%)	7	7	7	7	7	21	21	21	21	21	16	16	16	16	16

Supplementary Information Table S13. Five-day precision results for p110α in RP shown in Supplementary Figure 3A are displayed. Three pools of MDA-MD 231 lysate were spiked with 2, 10, and 18 fmol PTEN+p110α dSIS (per 10 µg total lysate protein). Each pool (10 µg total protein per replicate) was analyzed each day in triplicate. Quantified amounts of p110α dSIS per replicate (in fmol) are shown.

	2 fmol spike-in					10 fmol spike-in					18 fmol spike-in				
Day	**1**	**2**	**3**	**4**	**5**	**1**	**2**	**3**	**4**	**5**	**1**	**2**	**3**	**4**	**5**
Rep. 1	2.1	1.9	2.2	2.3	2.1	11.0	10.6	9.9	17.6	10.6	20.2	18.4	21.3	20.0	17.6
Rep. 2	2.0	2.1	2.0	4.2	2.0	12.5	10.1	11.4	13.7	11.5	20.1	19.1	19.6	22.9	23.1
Rep. 3	2.0	2.1	2.1	2.1	1.9	11.9	10.5	10.3	10.5	12.3	18.9	21.2	20.9	21.5	21.7
Mean	2.0	2.0	2.1	2.2	2.0	11.8	10.4	10.6	13.9	11.5	19.7	19.6	20.6	21.5	20.8
Abs. SD.	0.1	0.1	0.1	0.2	0.1	0.8	0.3	0.8	3.6	0.8	0.7	1.4	0.9	1.4	2.9
CV (%)	3	4	5	7	4	6	3	7	25	7	4	7	4	7	14
Spike	2.0	2.0	2.0	2.0	2.0	10.0	10.0	10.0	10.0	10.0	18.0	18.0	18.0	18.0	18.0
Acc. (%)	100	102	105	111	101	118	104	106	139	115	110	109	115	119	115
$Mean_{5\ Day}$	2.1	2.1	2.1	2.1	2.1	11.6	11.6	11.6	11.6	11.6	20.4	20.4	20.4	20.4	20.4
$Abs.\ SD._{5\ Day}$	0.1	0.1	0.1	0.1	0.1	2.0	2.0	2.0	2.0	2.0	1.6	1.6	1.6	1.6	1.6
$CV_{5\ Day}$ (%)	5	5	5	5	5	17	17	17	17	17	8	8	8	8	8
$Acc._{5\ Day}$ (%)	103	103	103	103	103	116	116	116	116	116	113	113	113	113	113
CV_{Total} (%)	7	7	7	7	7	19	19	19	19	19	11	11	11	11	

Supplementary Information Table S14. Endogenous PTEN and p110α amounts quantified in various FF tissue samples shown in Figure 4C are given here. Biological replicates were analyzed in duplicate (10 µg total protein per replicate). Quantified amounts of PTEN and p110α per replicate (in fmol) are shown. Data was recorded in the linear mode. Not detectable (n.d.) was treated as 0 for calculating means and standard deviation. *Replicates excluded due to contamination.

		Vehicle		Trastuzumab		Everolimus		Trastuzumab + Everolimus	
Biological Replicate	**Technical Replicate**	**PTEN**	**p110α**	**PTEN**	**p110α**	**PTEN**	**p110α**	**PTEN**	**p110α**
Bio. Rep. #1	1	4.1	1.0	3.6	0.8	5.3	1.1	4.8	0.8
Bio. Rep. #1	2	4.3	0.9	4.0	0.8	5.4	1.2	4.7	0.9
Bio. Rep. #2	1	0.8	0.4	3.2	0.6	5.0	0.8	3.5	0.6
Bio. Rep. #2	2	0.9	n.d.	2.9	0.8	5.2	0.8	3.9	0.8
Bio. Rep. #3	1	9.2*	2.1*	8.6*	2.0*	3.2	0.8	4.1	1.0
Bio. Rep. #3	2	n.d.	n.d.	4.2	1.1	3.3	0.9	4.3	1.1
Mean		2.0	0.4	3.6	0.8	4.6	0.9	4.2	0.9
Abs.SD		2.0	0.5	0.5	0.2	1.0	0.2	0.5	0.2
CV		99.6	105.5	14.9	24.1	22.3	17.7	11.4	17.6

Supplementary Information Table S15. Endogenous PTEN and p110α amounts quantified in various FF tissue samples shown in Figure 4A are given here. Colorectal cancer liver metastases (mCRC) and breast FF tissue samples (10 µg total protein per replicate) were analysed in triplicate. Quantified amounts of PTEN and p110α per replicate (in fmol) are shown.

		mCRC Tissues				Breast Tissue				
	Protein	**L #1**	**L #2**	**L #3**	**L #4**	**B #1**	**B #2**	**B #3**	**B #4**	**B #5**
Rep. 1	PTEN	1.0	1.9	1.6	3.9	2.6	3.3	1.3	2.2	1.1
Rep. 2	PTEN	0.9	2.1	1.5	3.9	2.1	3.1	1.5	1.7	0.0
Rep. 3	PTEN	0.0	1.7	1.3	3.7	2.3	3.5	1.4	2.7	1.2
Mean	PTEN	0.7	1.9	1.5	3.8	2.3	3.3	1.4	2.2	0.8
Abs. SD.	PTEN	0.6	0.2	0.2	0.1	0.2	0.2	0.1	0.5	0.7
CV (%)	PTEN	87	11	10	3	9	7	6	22	87
Rep. 1	p110α	0.6	0.7	1.0	1.2	0.8	1.4	0.6	0.6	1.1
Rep. 2	p110α	0.6	0.8	1.1	1.1	0.9	1.3	0.6	0.5	0.0
Rep. 3	p110α	0.6	0.7	0.9	1.2	1.0	1.2	0.6	0.8	0.9
Mean	p110α	0.6	0.7	1.0	1.2	0.9	1.3	0.6	0.6	0.7
Abs. SD.	p110α	0.0	0.1	0.1	0.1	0.1	0.1	0.0	0.1	0.6
CV (%)	p110α	7.2	7.6	6.7	7.6	12.8	6.5	7.5	17.5	87.7

References

1 R. P. Grant and A. N. Hoofnagle, *Clin. Chem.*, 2014, **60**, 941–944.