Supporting Information

Submitted to *Analytical Chemistry*

A Boosting to Amplify Signal with Isobaric Labeling (BASIL) Strategy for Comprehensive Quantitative Phosphoproteomic Characterization of Small Populations of Cells

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Running title: Signal boosting strategy for sensitive phosphoproteomics

Keywords: phosphorylation, phosphoproteomics, pancreatic islets, small populations of cells.

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SI Methods:

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Cell culture and treatment

Breast cancer cell line MCF-7 was obtained from the American Type Culture Collection and were grown as previously described[20]. Briefly, MCF-7 cells were maintained in 15-cm dishes in Dulbelcco's Modified Eagle Medium (Thermo Fisher Scientific) supplemented with 10% heat-inactivated fetal bovine serum (Thermo Fisher Scientific), 100 U/mL penicillin, and 100 µg/mL streptomycin (Thermo Fisher Scientific). Cells were seeded into 15-cm culture plates and grown until near confluence at 37 ^{o}C with 5% CO_2. MCF-7 cells were rinsed twice with ice cold PBS and harvested in 1 mL ice-cold PBS containing 1% phosphatase inhibitor (Thermo Fisher Scientific).

For the AML cells, MOLM-14 and K562 cells were maintained in RPMI-1640 medium supplemented with 10% fetal bovine serum (FBS); CMK cells were maintained in RPMI-1640 medium supplemented with 20% FBS. Culture media were also supplemented with 2 mM L-glutamine, 100 U/mL penicillin, and 100 µg/mL streptomycin. All cell lines were cultured at 37 °C in 5% CO_2. Cell lines used in this study were not cultured for longer than 6 months from initial purchase or characterization. Indicated number of cells were washed with PBS, pelleted, and stored at -80 ^{o}C for phosphoprotein analyses.

Human islets from eight non-diabetic cadaveric donors were obtained from the Integrative Islet Distribution Program (IIDP). Donor islets were required to have a minimum of 85% purity and 90% viability. ~150 Islets per condition were cultured in 2 mL Standard Islet Medium (Prodo) supplemented with human AB serum (Prodo), Ciprofloxacin (Fisher), and glutamine and glutathione (Prodo) at 37 ºC, under 100% humidity and 5% CO_2. Islet cultures were allowed to

acclimate overnight and then were either treated with cytokines IL-1β and IFN- γ by adding fresh medium containing 50 U/mL and 1000 U/mL of IL-1β and IFN- γ, respectively, or left untreated by adding fresh medium without cytokines for 24 h. Islets were then collected into micro-centrifuge tubes and washed twice with PBS containing 1% phosphatase inhibitor cocktail (Thermo Scientific) and 10 mM NaF (Sigma Aldrich). Islet pellets were flash frozen with liquid N_2 and stored at -70 ºC until further analyses.

EndoC-βH2 cells, a conditionally immortalized human β-cell line[21], were cultured in Matrigel-fibronectin–coated (100 μg/ml and 2 μg/ml, respectively)[22] 150 mm culture dishes in Dulbecco's modified Eagle's medium, low glucose (1 g/L), containing 2% bovine serum albumin (fraction V), 10 mM nicotinamide, 5.5 μg/mL transferrin, 6.7 ng/mL sodium selenite, 50 μM β-mercaptoethanol, 100 U/mL penicillin, and 100 μg/mL streptomycin. To create a reference sample with enhanced phosphorylation content, cells were stimulated at the same cytokine concentration for 30 min. After stimulation, cells were washed twice with ice-cold PBS containing 1% HALT phosphatase inhibitor cocktail (Thermo), 1% Protease Inhibitor and 10mM NaF (Sigma Aldrich), and immediately scaping-harvested in ice-cold PBS containing 1% HALT phosphatase inhibitor cocktail (Thermo) and 10mM NaF (Sigma Aldrich), centrifuged at 1000 rpm for 2 min. The resulting cell pellets were washed twice with PBS containing 1% phosphatase inhibitor cocktail (Thermo Scientific) and 10 mM NaF (Sigma Aldrich). Cell pellets were flash frozen with liquid N_2 and stored at -80 ºC until further analyses.

Peptide Fractionation by Basic Reversed-phase Liquid Chromatography (bRPLC)

The peptides were fractionated using a reversed-phase Waters XBridge C18 column (250 mm × 4.6 mm column packed with 3.5-µm particles) on Agilent 1200 HPLC System operating at flow rate of 1 mL/min with solvent A (5 mM ammonium formate, pH 10, 2% ACN) and solvent B (5 mM ammonium formate, pH 10, 90% ACN). Peptides were separated by a gradient mixture from 0% B to 16% B in 6 min, 40% B in 60 min, 44% B in 4 min and ramped to 60% B in 5 min. The 60% B mixture was kept for 14 min. Fractions were collected from at 2 min to 95 min during the fractionation run and a total of 96 fractions was collected at equal time intervals. These 96 fractions were subsequently concatenated into 6 fractions. Each pooled fraction was dried down by vacuum centrifugation and subjected to IMAC for phosphopeptide enrichment.

IMAC enrichment

For IMAC, peptides were reconstituted at 1 µg/µL in 80% ACN/0.1%TFA prior to enrichment. The Fe^{3+}-NTA agarose beads were prepared by replacing the Ni^{2+} ion on the Ni-NTA beads with Fe^{3+} through buffer exchange. Phosphopeptide enrichment was performed as previously described[20]. Briefly, peptide samples were incubated with 10 µL 50% bead slurry at room temperature for 30 min with shaking and the supernatant was discarded. The beads were resuspended in 100 µL 80%ACN/0.1%TFA and loaded on Empore C18 silica-packed Stage Tips for washing and desalting. Before sample loading, the Stage Tips were washed with 100 µL methanol twice, 50 µL 50% ACN/0.1% FA once, and 100 µL 1% FA twice. After washing the beads with 50 µL 80%ACN/0.1%TFA twice and 50 µL 1% FA once, the phosphopeptides were eluted from the IMAC beads to the C18 membrane with 70 µL 500 mM phosphate buffer, pH 7.0 three times and washed with 100 µL 1%FA once before being eluted from the C18 membrane with

60 µL 50%ACN/0.1%FA. Eluted phosphopeptides were dried down and stored at -80°C until LC-MS/MS analysis.

LC-MS/MS analysis

Lyophilized phosphopeptides were reconstituted in 12 µL 0.1%FA with 2% ACN and a 5 µL sample was injected directly into a nanoACQUITY UPLC system (Waters Corp., Milford, MA). Peptides were separated on an in-house packed analytical column (75 µm i.d. × 20 cm) containing 1.9-µm ReproSil C18 resin with a column heater set at 50 °C. The mobile phases consisted of (A) 0.1% FA with 3%ACN and (B) 0.1% FA in 90% ACN. The gradient setting for peptides separation was : 2-6% solvent B in 1 min, 6-30% solvent B in 84 min, 30-60% solvent B in 9 min, 60-90% solvent B in 1 min, and finally 90% solvent B for 5 min. The flow rate was controlled at 200 nL/min. The LC system was coupled online with an Orbitrap Fusion Lumos Tribrid mass spectrometer (Thermo Scientific, San Jose, CA). Data were acquired in a data-dependent mode with a full MS scan from m/z 350-1800 at a resolution of 60,000 at m/z 400 with AGC setting set to 4×10^5 and maximum ion injection period set to 50 ms. The MS/MS isolation window was set at 0.7 m/z and HCD fragmentation was performed at a normalized collision energy of 30% with AGC set as 1×10^5 and a maximum ion injection time of 300 ms. The MS/MS spectra was acquired at a resolution of 50,000. The cycle time was 2 seconds and dynamic exclusion time was set at 45 s.

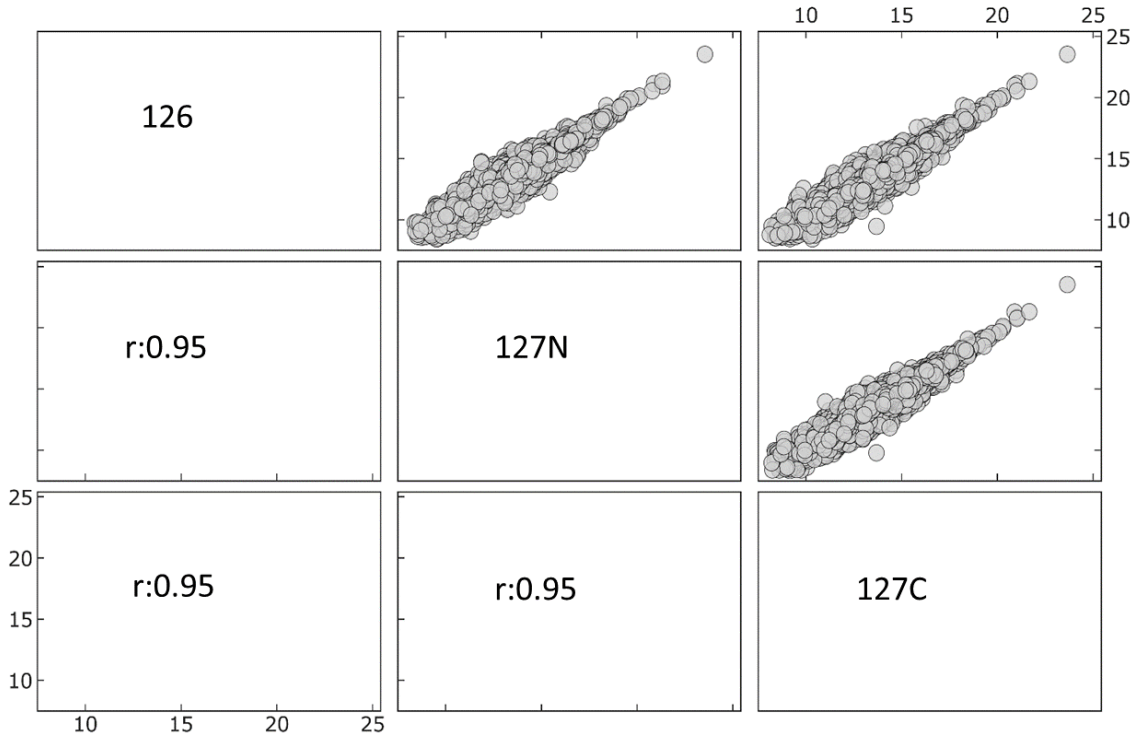

Figure S1. Correlation of the TMT reporter ion intensities (log2 scale) between channel 126, 127N and 127C, each with 10 µg of tryptic peptides from the MCF-7 cells.

(a)

Channel	Without boosting		With boosting	
	Samples	input amount (μg)	Samples	input amount (μg)
126	MOLM14	10	MOLM14	10
127N	K562	10	K562	10
127C	MOLM14	10	MOLM14	10
128N	K562	10	K562	10
128C	CMK	10	CMK	10
129N	MOLM14	10	MOLM14	10
129C	CMK	10	CMK	10
130N	Reference	10	Reference	10
130C	K562	10	K562	10
131	Empty	0	Boosting	300

(b)

	Without boosting	With boosting	
LC-MS/MS	1D-LC	1D-LC	2D-LC
Phosphopeptides	3991	7480	23006
Phosphorylation sites	3673	6795	18868
Phosphorylation sites (Class 1)	2833	5424	15083
S	2569	4941	13468
T	250	464	1531
Y	14	19	84

Figure S2. Summary of quantitative phosphoproteome analysis of 3 AML cell lines (MOLM14, K562 and CMK) with and without adding boosting simples. (a) The TMT-10 channel assignment. Reference and boosting samples: the mix of all three cell line samples. (b) The summary of identified phosphopeptides and phosphorylation sites.

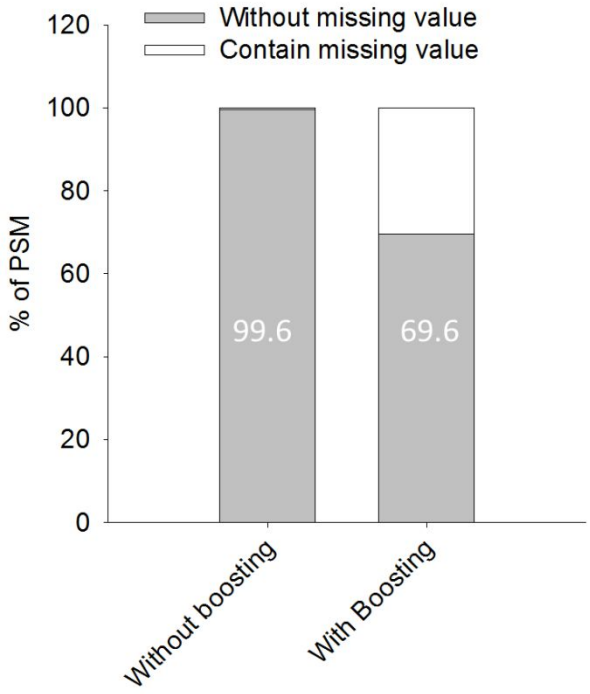

Figure S3. The percentage of missing value of quantifiable PSMs with and without using the boosting sample.

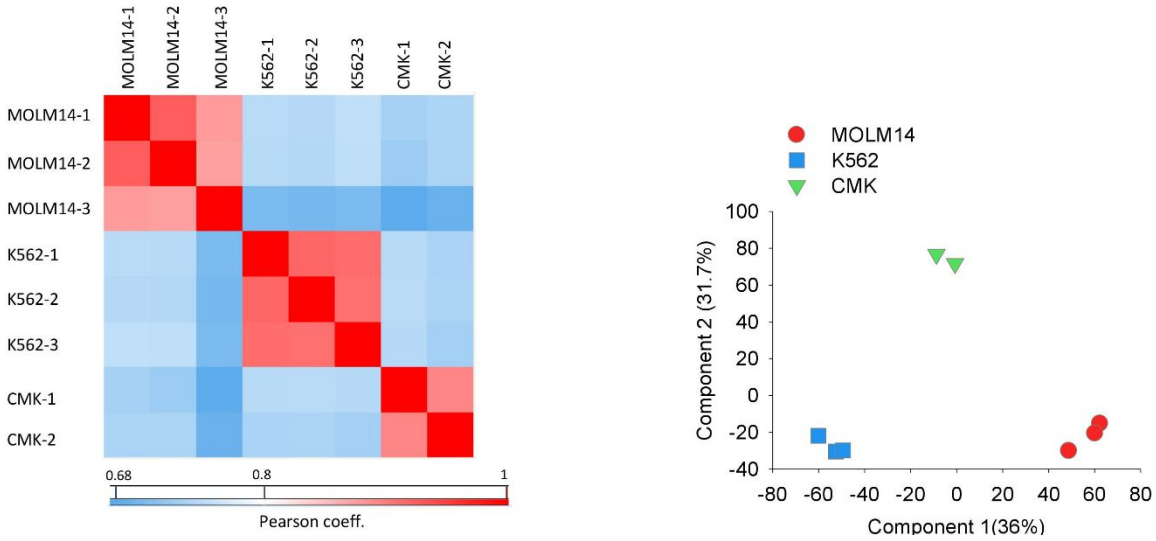

Figure S4. Summary of quantitative phosphoproteome analysis of 3 AML cell lines (MOLM14, K562 and CMK). Left, the Pearson correlation of the study samples. Right, the PCA result of the quantitative phosphoproteomic analysis of the AML cell lines.

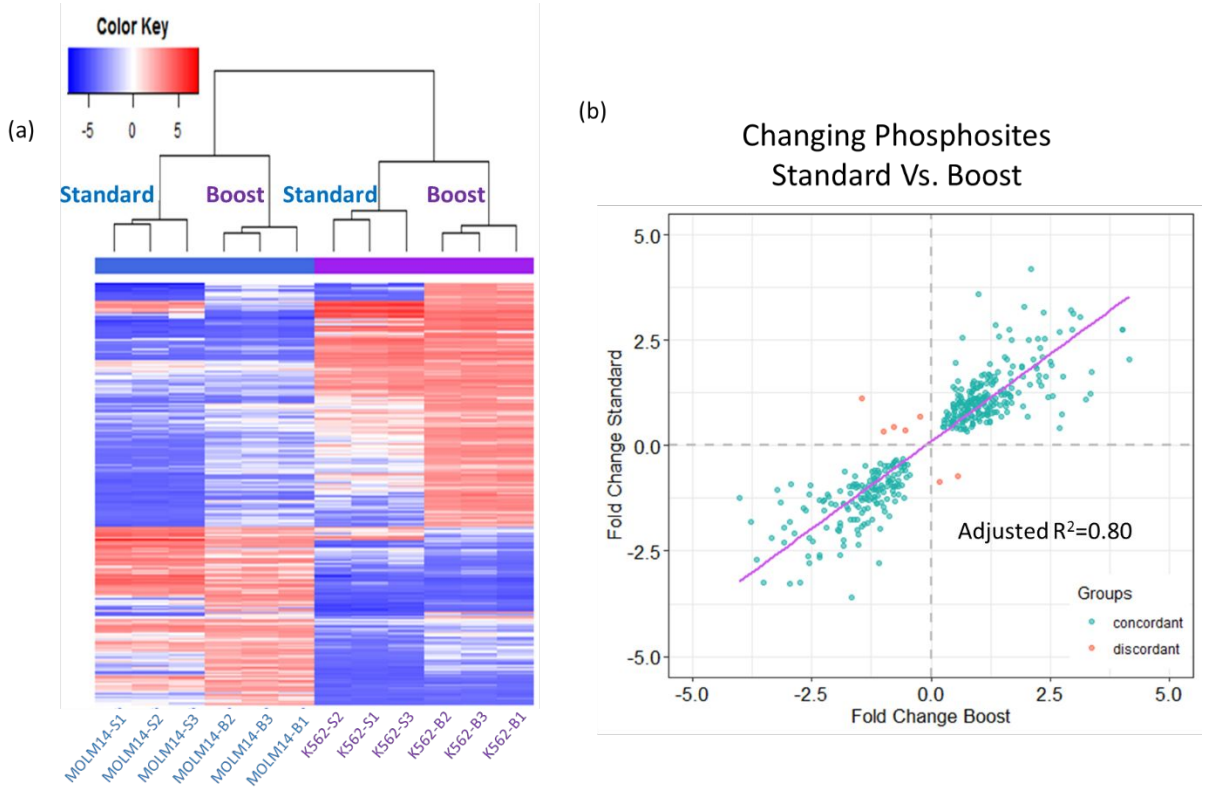

Figure S5. The correlation of quantification results between the standard TMT-based phosphoproteomics workflow and the boosting (BASIL) approach. (a) Unsupervised clustering of significantly changing phosphorylation sites from both approaches. (b) Correlation of fold changes for differentially expressed phosphosites from both the standard and "boost" approaches. A T-test with Benjamini-Hochberg correction was applied.

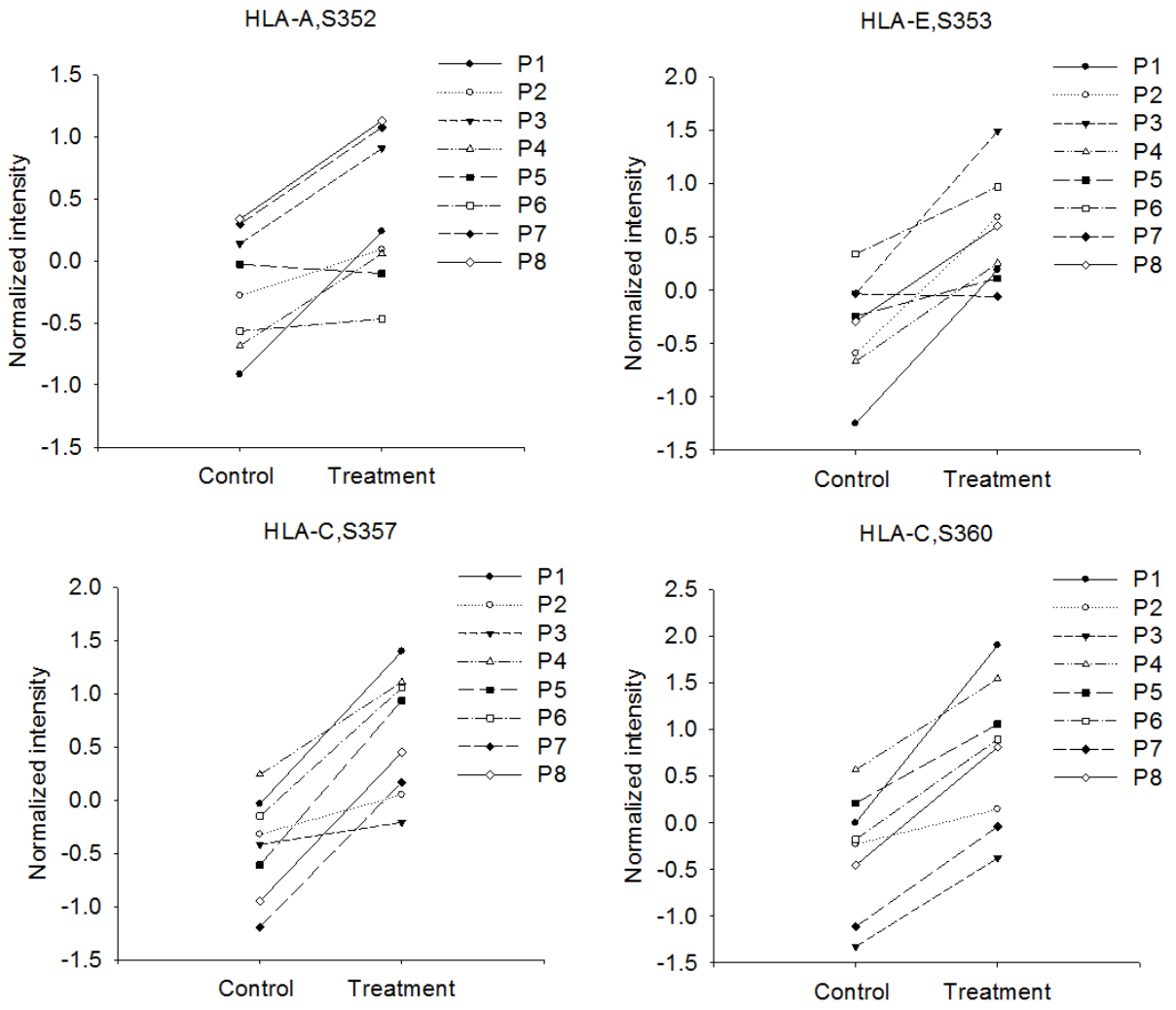

Figure S6. Distribution of abundance of phosphorylation sites of HLA family in human islets samples before and after cytokine treatment.