Supporting Information

Comparative Proteomic Profiling of Mammalian Cell Lysates Using Phosphopeptide Microarrays

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1. Materials and Methods.

1.1 Materials.

All chemicals were purchased at the highest grade and used without further purification, unless otherwise noted. Fmoc-amino acids, HOBT, HBTU, TIS, TFA were from GL Biochem (China). Piperidine was from Merck (USA). HPLC profiles and ESI mass spectra were acquired in the positive or negative mode by using a Shimadzu IT-TOF. Analytical and semi-preparative RP-HPLC separations were performed on Phenomex C_{18} analytical (150 x 3.0 mm) and semi-preparative (250 x 21.2 mm) columns, respectively, using a Shimadzu Prominence HPLC system equiped with a Shimadzu SPD-20A detector. Eluents A (0.1 % TFA/acetonitrile) and B (0.1 % TFA/water) were used as the mobile phases. SH2 domain proteins were expressed and purified in house. Plain glass slides were purchased from Sigma Aldrich, and modified to generate the corresponding avidin-coated surface as previously described.[1] Pro-Q™ Diamond dye was bought from Invitrogen. All the SH2 domains bacterial constructs are commercially available (Open Biosystems, Thermo Scientific).[2]

1.2 Peptide synthesis.

Peptide synthesis was performed by using standard Fmoc Strategy combined with IRORI™ directed sorting technology, as previously described.[1] Rink amide resin was used as the solid support. Standard HOBT/HBTU/DIEA coupling method was used throughput the whole process. Each microreactor contains around 40 mg of rink amide resin and a unique R_f tag for sorting application. The resin was swelled in HPLC-grade DMF for 1 h at room temperature. Subsequently the Fmoc group was deprotected by treatment of 20% piperidine for 1 h at room temperature. Following removal of the piperidine, the resin was washed extensively with DMF and DCM. The microreactors were then dried thoroughly under the high vaccum. Next, the microreactors were sorted and distributed into several reaction vessels, and each containing a unique Fmoc amino acid (4.0 eq), preactivated with HBTU/HOBt/DIEA (1/1/2 ratio relative to the amino

acid). The resins were swelled in DMF for 30 min before coupling. The coupling reactions were carried out for 8 h at room temperature with shaking. At the end, the microreactors were collected and washed thoroughly with DMF and DCM. Any unreacted resin residue was capped with a solution of Ac_2O (10 eq), DIEA (20 eq) in DCM (200 mL), and the reaction mixture was allowed to react for 2 h at room temperature, followed by extensive wash with DCM and DMF. Subsequently the resin was deprotected with 20% piperidine again and ready for next coupling cycle. The above cycle was repeated until the last amino acid has been coupled. (Biotin)-GG was attached at the N-terminus of peptides for microarray immobilization. After the whole coupling process was finished, the microreactors were collected, washed thoroughly and dried under high vacuum for 2 h at RT. The microreactors were then decoded and cleaved under 95% TFA, 2.5% TIS, 2.5% H_2O for 4 h at room temperature. For those peptides containing cysteine or methionine residue, the peptides were cleaved from the resin by using 94%TFA, 2.5% EDT, 2.5% H_2O and 1% TIS. Following prolonged concentration until >80% of cleavage cocktail was removed, cold ether (chilled to -20 °C) was added to the liquid residue to precipitate the peptides. The peptides were allowed to precipitate at -20 °C for overnight. The ether layer was then decanted and the precipitates were dried thoroughly *in vacuo*. This process was repeated for a couple more times. The resulting peptide solids were dissolved in 1 mL DMSO and stored at -20 °C. LCMS was performed, to ensure the peptides were of correct mass and sufficient purity for subsequent microarray experiments.
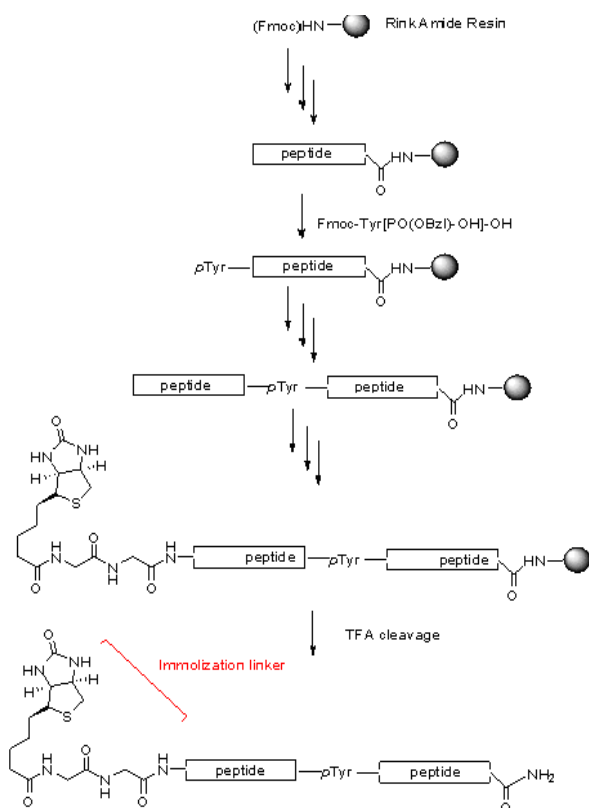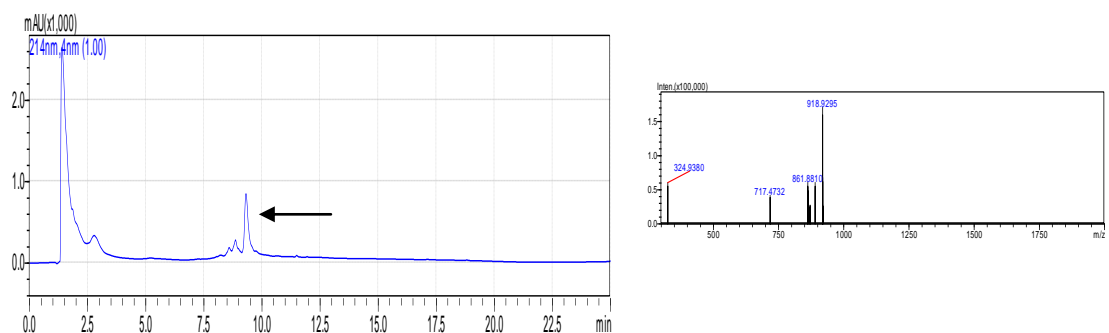

Scheme S1. Schematic representation of the solid phase peptide synthesis procedure applied.
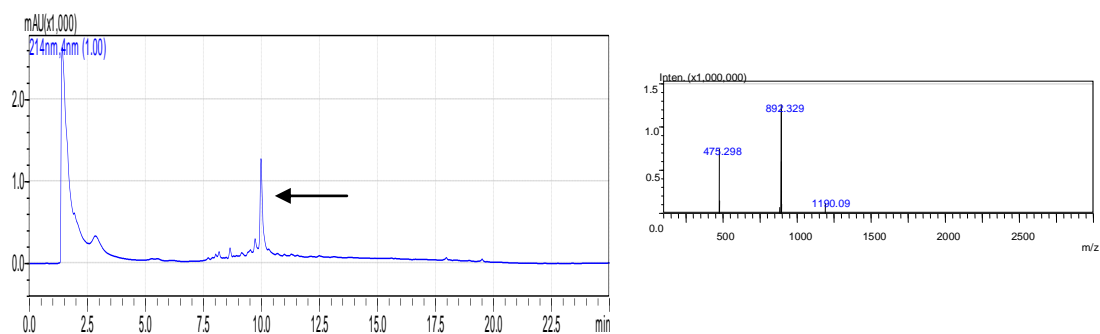
1. Peptide A01(AEKPF-pY-VNVEF): Expected MW(M) 1761.7; Observed MW (M^{2+}) 881.8
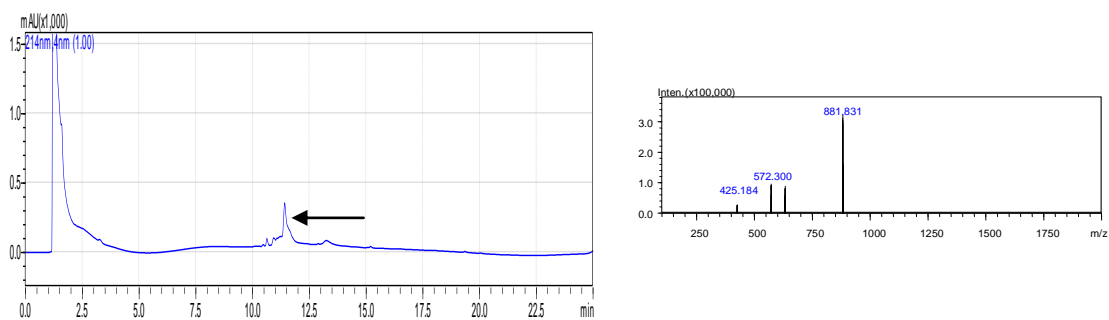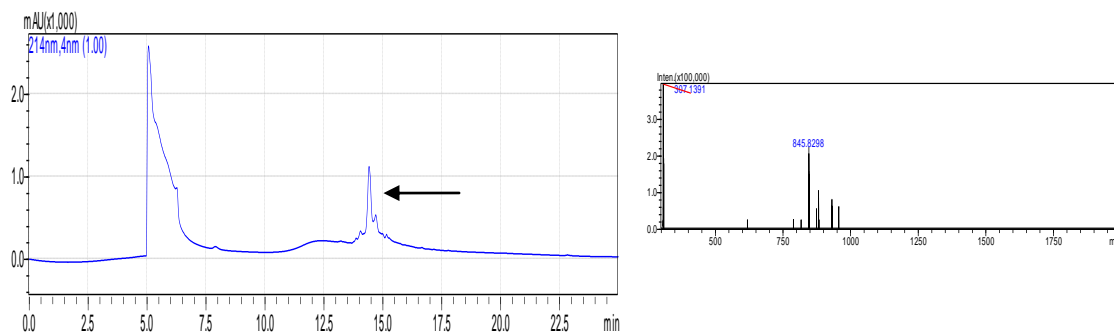

2. Peptide B17 (IRYHR-pY-HGRSA): Expected MW(M) 1834.8; Observed MW (M^{2+}) 918.9

3. Peptide B18 (IYETD-pY-YRKGG): Expected MW(M) 1783.7; Observed MW (M^{2+}) 892.3

4. Peptide D08 (SETDD-*p*Y-AEIID): Expected MW(M) 1689.6; Observed MW (M^{2+}) 845.8

5. Peptide D09 (SPPAL-*p*Y-AEPLD): Expected MW(M) 1591.6; Observed MW (M^{2+}) 796.3
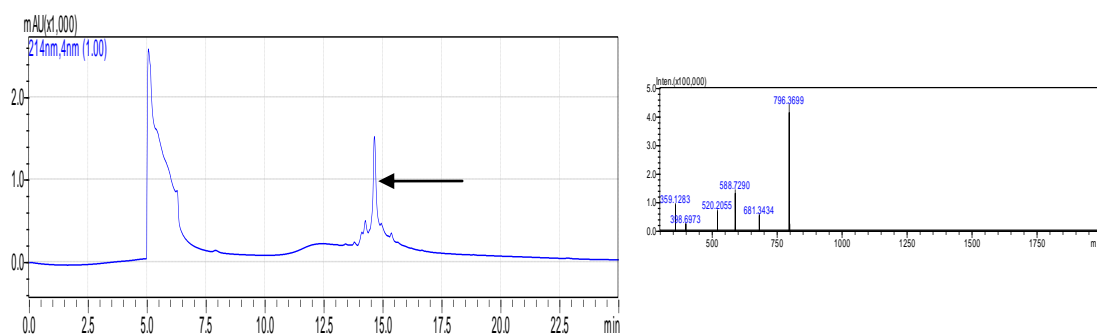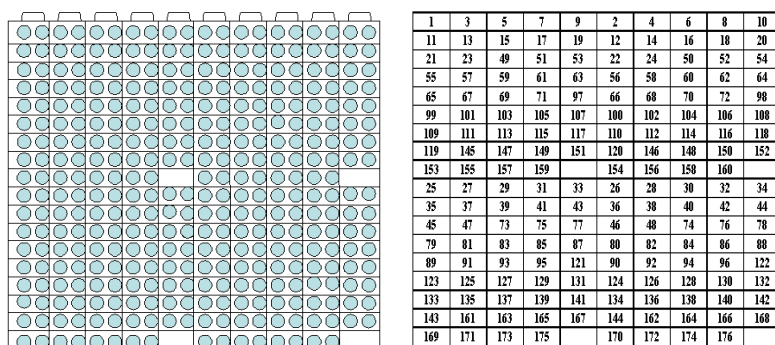

Fig. S1. LC-MS results of representative library members.

1.3 Microarray preparation.

All phosphopeptide stocks (176-member phosphopeptides library) were prepared and diluted to final spotting concentration around 1mM in DMSO/PBS (1:1) spotting solution, and were distributed in 384-well plates. All phosphopeptides were shown to be completely soluble in this spotting solution. Avidin slides were spotted with our 176-member phosphopeptides library on an ESI SMA arrayer (Ontario, Canada) with the print-head installed with 4 Stealth SMP8B Microspotting pins (Telechem, U.S.A.). Spots generated were of approximately 350 μm diameter and were printed with a spot-spot spacing of 450 μm. The pins were rinsed in between samples using two cycles of wash (for 10 s) and sonication (for 10 s) in reservoirs containing 70 % ethanol followed by drying under reduced pressure (for 10 s). The slides were allowed to stand for 2 h on the printer platform and stored at 4 ℃ until needed for use. Before incubation with the labelled protein, the slides were rinsed with deionised H$_2$O (DI H$_2$O) for 10 minutes and blocked with TBS-containing 1 % BSA for 0.5 h. For the studies with the 176-member library, all phosphopeptides were spotted on the same slide in duplicate.

1.4 Microarray spotting formats.

1	3	5	7	9	2	4	6	8	10
11	13	15	17	19	12	14	16	18	20
21	23	49	51	53	22	24	50	52	54
55	57	59	61	63	56	58	60	62	64
65	67	69	71	97	66	68	70	72	98
99	101	103	105	107	100	102	104	106	108
109	111	113	115	117	110	112	114	116	118
119	145	147	149	151	120	146	148	150	152
153	155	157	159		154	156	158	160	
25	27	29	31	33	26	28	30	32	34
35	37	39	41	43	36	38	40	42	44
45	47	73	75	77	46	48	74	76	78
79	81	83	85	87	80	82	84	86	88
89	91	93	95	121	90	92	94	96	122
123	125	127	129	131	124	126	128	130	132
133	135	137	139	141	134	136	138	140	142
143	161	163	165	167	144	162	164	166	168
169	171	173	175		170	172	174	176	

Fig. S2. Grid layout with 176 phosphopeptides spotted in duplicate. (see **Table S1** for peptide sequences, ID and source proteins.

1.5 Pro-Q™ staining and detection.

Pro-Q™ staining was routinely carried out on spotted slides to ensure the consistency and quality of the slides. The slide was washed with H_2O and stained with Pro-Q™ Diamond dye for 1 h at room temperature. Subsequently the slide was destained with a solution of 20 % acetonitrile in sodium acetate (pH=4) for 0.5 h and visualized with the Tecan Launch LS Reloaded Microarray Scanner (Tecan Trading AG, Switzerland)under the Cy3 laser channel (λ_{Ex} = 532 nm; λ_{Em} = 575 nm).

1.6 SH2 domain proteins expression and purification.

Overnight expression host bacterial cultures were diluted 1:100 in LB media supplemented with 50 μg/mL of kanamycin and grown at 37 °C. At OD_{600} reaching 0.6-0.8, expression was induced by addition of IPTG (isopropyl-β-D-1-thiogalactopyranoside) with final concentration at 0.1mM, and cultures were grown further at 15°C overnight for 18h. Successful overexpression of the proteins was verified by Coomassie blue staining and immunoblot analysis with anti-His antibody (Amersham Biosciences). After cell harvest and lysis at PBS buffer by sonication (6 times pulses of 15 s each at half maximal power, on ice), the solution was clarified by centrifugation at 13.2k rpm in an Eppendorf centrifuge, for 30 min at 4°C and affinity purified using vendor's protocols. Fractions containing the desired fusion protein were pooled and dialysed into a suitable buffer, and stored at -20 °C. Protein concentration was determined using the Bradford protein assay (Bio-Rad). Protein purity was determined by separation on a 15 % SDS-PAGE gel.

1.7 Protein/mammalian cell lysates proteomes labeling and screening on the phosphopeptide microarray.

50 μg protein/lysate proteomes were minimally labelled with either 1.0 μL Cy5/Cy3-NHS ester, together with 10 μl $NaHCO_3$ (0.5M, pH 9.0) in 50μl total solution (Monofunctional Cy5/Cy3 dye: Amersham, G.E. Healthcare) for 1 h on ice, following the manufacturer's protocols and our previously published procedures [3]. The unreacted dye was quenched with a 10-fold molar excess of hydroxylamine for a further 1 h. The unreacted dye was further removed by buffer exchange in a final TBS buffer solution with a Microcon® centrifugal filter units (M3 Millipore, USA). The labelled proteomes

was reconstituted in a final buffer volume of 100 μL TBS (pH 7.4). In a standard lysates microarray experiment, the labelled lysates (0.5 mg/mL; 40 μL) were applied under coverslip to the arrays (around 10μg lysates/grid). For the dual colour reciprocal microarray screening experiments, an equal amount of Cy3 labelled SH2 domain proteins (for SH2 domain screening) or a mixture of Cy3/Cy5-labelled proteomes A and a Cy5/Cy3-labelled proteomes B (for dual colour reciprocal proteomic profiling) was applied together to the spotted microarray slides. The samples were incubated with the array in a humidified chamber for 1 h at room temperature, before repeating rinses with TBST (TBS containing 0.05% Tween® 20), typically 3×10 min washes with gentle shaking. Slides were scanned using a Tecan Launch LS Reloaded microarray scanner installed with the 2 relevant lasers (Cy3: $\lambda_{ex/em}$= 532/575 nm; Cy5: $\lambda_{ex/em}$= 633/692 nm).

1.8 Data extraction and analysis.

Microarray data was extracted using the Array-Pro-Analyzer software. Data from duplicated points was background subtracted and averaged. The dataset was presented as coloured heatmaps, using the Treeview software (http://rana.lbl.gov/EisenSoftware.htm). Other data analysis was carried out with Microsoft Excel.

1.9 Cell culture.

HeLa (human cervical cancer line), MCF7(non-invasive breast cancer line), SW480 (early stage of colon cancer line), SW620 (late stage of colon cancer line) and normal human cell line HEK293T (human embryonic kidney line) were cultured in growth medium (DMEM supplemented with 10% fetal bovine serum(FBS), 100 mg/L streptomycin, and 100 IU/mL penicillin); MDA-MB231(invasive breast cancer cell) was cultured in RPMI 1640 growth medium. All cells were maintained in a humidified atmosphere of 5% CO_2 at 37 °C, and grown to ~90% confluence. Cells were detached by trypsin digestion, washed by PBS for three times, centrifuged, and stored at -20 °C until required for use.

1.10 Preparation of cellular lysates proteomes.

Cell pellets were thawed on wet ice with PBS buffer added (one T75 cell pellet was resuspended with 200-400 μL PBS buffer). Pellets were resuspended, incubated on ice for 15 min. Lysis was performed by 30-50 passages through a 22G needle using a 1ml syringe, then samples were centrifuged at 13.2k rpm at 4°C for 30 min. Cleared supernatant was transferred to chilled Eppendorf tubes. Protein concentration was determined by Bradford assays (Bio-Rad).

1.11 Lysates proteomes pull down assay.

NeutrAvidin™ agarose beads (Pierce, USA) were pre-incubated with selected biotinylated peptide probes (final concentration 10μM) at room temperature for 1 h, then washed 3 times with TBS buffer to remove the excess unbound probes. After wash, beads were added into cellular lysate (2.5 mg) in TBS buffer containing phosphatase inhibitor (final concentration 2mM Na_3VO_4), and allowed to incubate at room

temperature for 1 h with rocking. NeutrAvidin™ agarose beads were centrifuged for 5 min at 4000×g at room temperature and washed with TBST (0.05% Tween®20 in TBS buffer) 5-10 times. A negative control (same pull down procedures but without the probes, with DMSO as the negative control as phosphopeptides stocks were dissolved in DMSO solvent). Suitable amounts of the beads were taken, resuspended in 1×SDS loading dye buffer, and analyzed by Western blotting as described below.

1.12 Western blotting (WB).

Primary antibody GRB2 (Cat.#:ab-28164) was bought from Abcam, and others such as LDH-B (Cat.#:sc-100775), PRDX1(Cat.#:sc-7381), PHB (Cat.#:sc-18198), GAPDH-HRP (Cat.#:sc-25778), c-SRC (Cat.#:sc-130074), and secondary antibody donkey anti-goat IgG-HRP (Cat.#: sc-2020), goat anti-rabbit IgG-HRP (Cat.#:sc-2004) were bought from Santa Cruz. Secondary antibody goat anti-mouse HRP (Cat.#:1858112) was obtained from Pierce. Briefly, suitable amount of proteomes or proteins (the exact amount of proteins/lysates proteomes will be specified in each parallel group of the experiments) were loaded and separated by SDS-PAGE and transferred onto PVDF membrane, blocked for 1 h at room temperature or overnight at 4 °C in blocking buffer (4% BSA in TBS containing 0.1% Tween®20). After that, membranes were incubated with primary antibody for 1 h at room temperature in primary antibody blocking buffer (3% BSA in TBS containing 0.1% Tween®20). Following 3 washes (5-10 minutes each) in TBST (TBS containing 0.1% Tween®20), the blot was then incubated with HRP-conjugated secondary antibody for 1 h at room temperature in the buffer same as primary antibody blocking buffer. At the end of this period, it was again washed three times with 10-15 min each in TBST (TBS containing 0.1% Tween®20) and detected by chemiluminescence using the Super Signal West Pico/Dura Kit (Pierce, Thermo Scientific).

1.13 Antibody detection on the microarray platform using the Cy3 labelled antibody to prove the existence of proteins-phosphopeptides interactions.

All the antibodies used for the detection on the microarray platform were firstly labelled with Cy3 monofunctional dye (Amersham, G.E. Healthcare). The labelling procedure was as follows: 40 μL antibodies solution about 8 μg (only 4 μg of anti-LDH-B was used) were mixed with 0.3 μL Cy3 dye and 10 μL NaHCO$_3$ (0.5M, pH = 9.0). After reaction for 1 h in ice, the unreacted dye was quenched with 5μL hydroxylamine solution (0.21 g hydroxylamine (Cat.#:25558-0, Sigma Aldrich) dissolved in 1ml 2.5M NaOH) for another 1 h. After that, the mixture was separated with G25 column (Cat.#: 27-5325-01, G.E. Healthcare) to remove unreacted dyes. Next the slides were washed for 10 min with H$_2$O and then blocked with 1% BSA/TBS for 30 min to maximally reduce non-specific bindings in next experiment. After blocking, the slides were incubated with 30μg MCF7 cell lysates or SW620 cell lysates (only for anti-PHB detection) in TBS buffer per subgrid for 1 h, and then briefly washed with H$_2$O. Slides were incubated with labelled antibodies for another 1 h, and then washed with H$_2$O for 2-3 times, for 2 min each time. Finally, slides were dried and scanned using the microarray scanner.

1.14 Mass spectrometric analysis.

The LTQ-FT ultra (Thermo Electron, Bremen, Germany) was coupled with an online Shimadzu UFLC systems utilizing nanospray ionization. Peptides were first enriched with a Zorbax 300SB C_{18} column (5 mm × 0.3 mm, Agilent Technologies, Santa Clara, CA) followed by elution into an integrated nanopore column (75 μm × 100 mm, New Objective, Woburn, MA) packed with C_{18} material (5 μm particle size, 300 Å pore size, Macho BioResources, Inc.). Mobile phase A (0.1% formic acid or TFA, H_2O) and mobile phase B (0.1% formic acid or TFA, acetonitrile) were used to establish the 90-min gradient comprised of 3 min of 0-5 % B, then 52 min of 5 - 30% B, followed by 12 min of 30 - 60% B; maintained at 80% B for 8 min, before re-equilibrating at 5% B for 15 min. Sample was injected into the MS with an electrospray potential of 1.8 kV without sheath and auxiliary gas flow, ion transfer tube temperature of 180 °C, and collision gas pressure of 0.85 mTorr. A full survey MS scan (350-2000 m/z range) was acquired in the 7-T FT-ICR cell at a resolution of 100,000 and a maximum ion accumulation time of 1s. Precursor ion charge state screening was activated. The linear ion trap was used to collect peptides where 10 most intense ions were selected for collision induced dissociation (CID) in MS^2, which were performed concurrently with a maximum ion accumulation time of 200 ms. Dynamic exclusion was activated for this process, with a repeat count of 1 and exclusion duration of 30 s. For CID, the activation Q was set at 0.25, isolation width (m/z) 2.0, activation time 30 ms, and normalized collision energy of 35%.[4] The Extract_Msn (version 4.0) program found in Bioworks Browser 3.3 (Thermo Electron, Bremen, Germany) was used to extract tandem MS spectra in the dta format from the raw data of LTQ-FT ultra. These data files were then converted into MASCOT generic file format using an in-house program. Intensity values and fragment ion m/z ratios were not manipulated. This data was used to obtain protein identities by searching against the corresponding database by means of an in-house MASCOT server (version 2.2.03) (Matrix Science, Boston, MA). Mass tolerances of 10 ppm for peptide precursors; and 0.8 Da mass tolerance for fragment ions. Only proteins with a MOWSE score higher than 39, corresponding to $p < 0.05$ were considered significant. The peptide/protein list(s) obtained was exported to html file.

2. Supplementary Figures
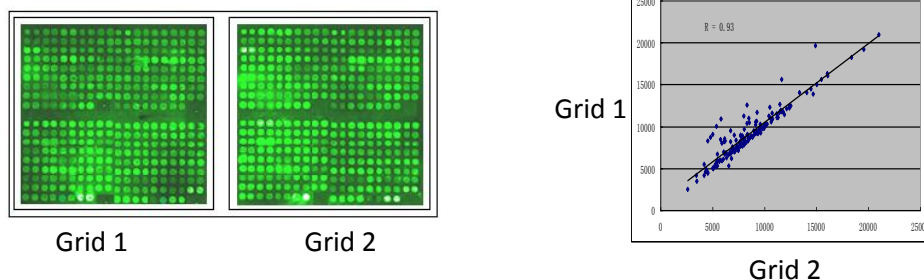
Fig. S3. The Pro-Q™ images of the 176-member pY peptide library spotted on 2 separate subgrids, indicating a high degree of grid-to-grid reproducibility. Pearson correlation, r, between grids was found to be 0.93.

Native GRB2   Denatured GRB2   BSA   Renin (Asp protease)
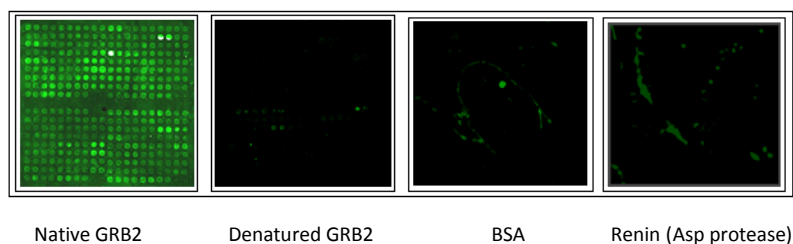
Fig. S4. Fingerprintings of the native, denatured and non-pY binding proteins (negative control) on the microarray. The denatured and non-pY binding proteins microarray experiments showed almost no binding (all proteins labelled with Cy-3 and applied at concentration at 0.5μM). Slides were scanned at the same intensity: PMT setting - 140.

2.1. Validation of phosphopeptide protein interaction via pure protein pull-down.

To validate the interactions between selective phosphopeptides and the SH2 domains identified from the pure SH2 domain microarray screening, pure protein pull down experiments were carried out. 20μL of NeutrAvidin™ agarose beads (coated with D04 for GRB2, H01 for SRC1) was incubated with 20 μg of pure proteins. The detailed procedure is listed in 1.11.

A

Lanes	1	2	3	4	5	6	7	8	9
Beads with D04		-	+	+	+	+	-	-	-
Labelled Proteins	Ladder	GRB2	GRB2	BSA	SRC1	SRC1 & GRB2	GRB2	SRC1	BSA

B

Lanes	1	2	3	4	5	6	7	8	9
Beads with H01		-	+	+	+	+	-	-	-
Labelled Proteins	Ladder	SRC1	SRC1	BSA	GRB2	SRC1 & GRB2	SRC1	GRB2	BSA

Fig. S5. Fluorescent gel results of the pure SH2 domain protein pull down assay. (A) Fluorescent gel for probe D04 applied to pull down GRB2. (B) Fluorescent gel for probe H01 applied to pull down SRC1. Proteins were labelled with Cy3. Lanes 2-6 represent pull downs conducted, lanes 7-9 were just labelled proteins run alongside as controls.

A

Lanes	1	2	3	4	5	6
Beads with D04	-	+	+	+	+	-
Proteins	GRB2	GRB2	BSA	SRC1	GRB2 & SRC1	GRB2
Anti-GRB2:						

B

Lanes	1	2	3	4	5	6
Beads with H01	-	+	+	+	+	-
Proteins	SRC1	SRC1	BSA	GRB2	SRC1 & GRB2	SRC1
Anti-SRC1:						
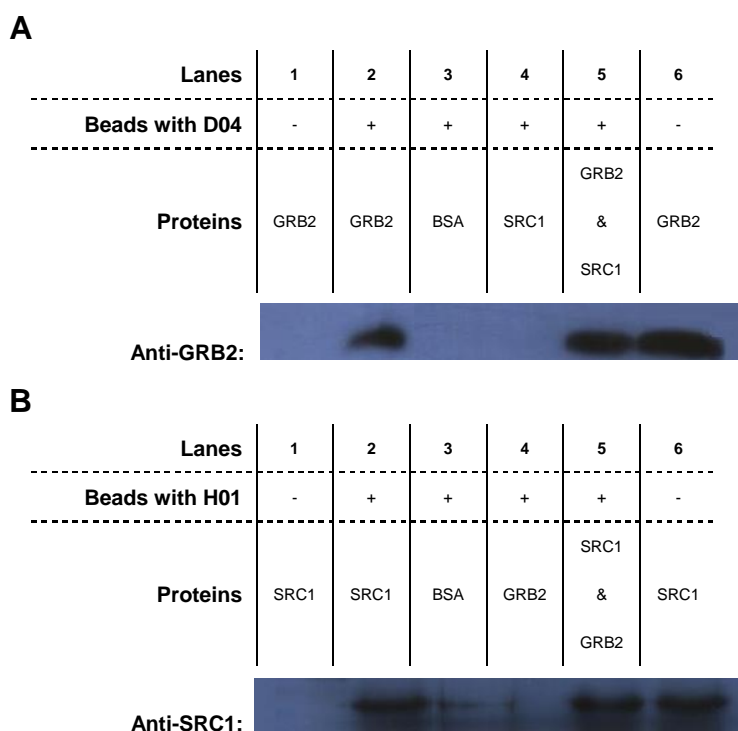
Fig. S6. 2.2. Western blotting results of the pure proteins pulled down. (A) Western blotting with peptide D04 selectively pulling down GRB2, as detected with anti-GRB2 antibodies. Lanes 1-5 represent pull downs with pure proteins, GRB2 was loaded in lane 6, as a positive control. (B) Western blotting with peptide H01 selectively pulling down SRC1, as detected with anti-SRC antibodies. Lanes 1-5 represent pull downs with pure proteins, SRC1 was loaded in lane 6, as a positive control.
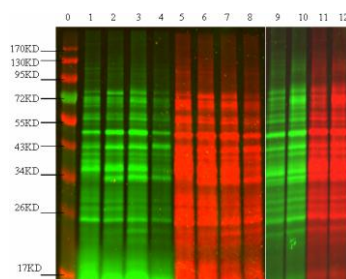
2.2. Validation of phosphopeptide-protein interactions pair via pull-down with MCF7 cell lysates.

A   D04   Uncoated beads   **B**   H01   Uncoated beads

Anti-GRB2 **Anti-SRC:**

Fig. S7. Western blotting results of the lysate pulled down with selective probes identified. (A) D04 for GRB2 and (B) H01 for SRC.

2.3. Cy3 and Cy5 labelling of cell lysates.

Fig. S8. Cy3 and Cy5 labelled mammalian cell lysates proteomes: 0.Protein ladder; 1. Cy3-MCF7, 2. Cy3-MDAMB231, 3. Cy3-HeLa, 4. Cy3-HEK293T, 5. Cy5-MCF7, 6. Cy5-MDAMB231, 7. Cy5-HeLa, 8.Cy5-HEK293T, 9.Cy3-SW480, 10. Cy3-SW620, 11. Cy5-SW480, 12.Cy5-SW620. Approximately 2 µg of samples were loaded per lane. (Note that lanes 9-12 were run on a separate gel but are shown alongside).

## 2.4. Testing the dual colour system for any bias.

To make sure that the dual colour system worked in a good condition, we tested the same lysate (MCF7) labelled with both Cy3 and Cy5 dye.
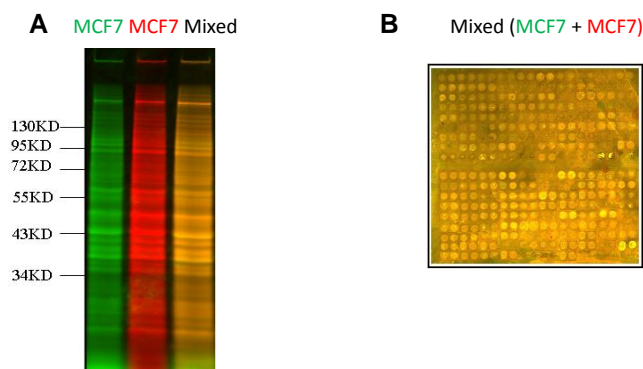

Fig. S9. Testing the dual colour labelling strategy with MCF7 lysates labelled in Cy3 and Cy5. (A) Fluorescent gel image. (B) Microarray image.
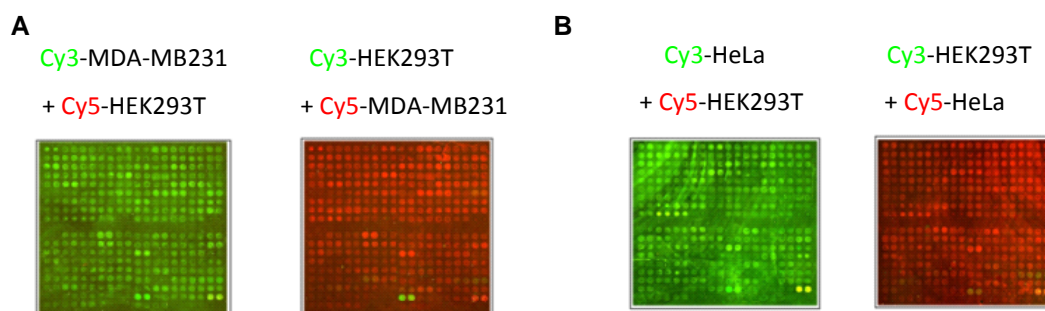
2.5. Experiments using dephosphorylated microarrays.
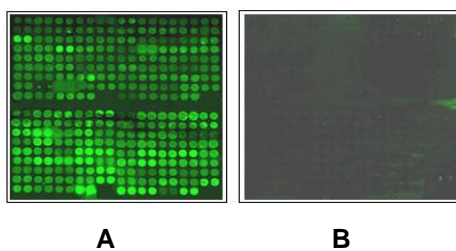
Fig. S10. Pro-Q^{TM} Diamond stains of the phosphopeptide microarray. (A) Without treatment of alkaline phosphatase. (B) Following treatment with alkaline phosphatase.

Cy3-MCF7   Cy3-HeLa   Cy3-SW480   Cy3-MDA-MB231
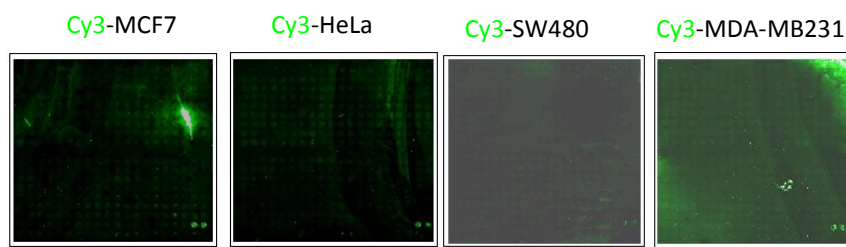
Fig. S11. Cy3 labelled cell lysates tested with dephosphorylated microarrays, showing negligible binding. Slides were scanned at a PMT of 160.

2.6. Two-colour microarray application

A

Cy3-MDA-MB231   Cy3-HEK293T

+ Cy5-HEK293T   + Cy5-MDA-MB231

B

Cy3-HeLa   Cy3-HEK293T

+ Cy5-HEK293T   + Cy5-HeLa

Fig. S12 Dual colour labelling and application. (A) MDA-MB231 lysates controlled with HEK293T and (B) HeLa lysates controlled with HEK293T. Binding intensity patterns were reproducible across channels.

2.7. Western blots to confirm the presence of the proteins in the pull down with cellular lysates.

A   D24   Uncoated
Agarose   Beads

LDH-B
37kD

B   D24   Uncoated
Agarose   Beads

PRDX1
22kD

C   D07   Uncoated
Agarose   Beads

PHB
29kD

S12
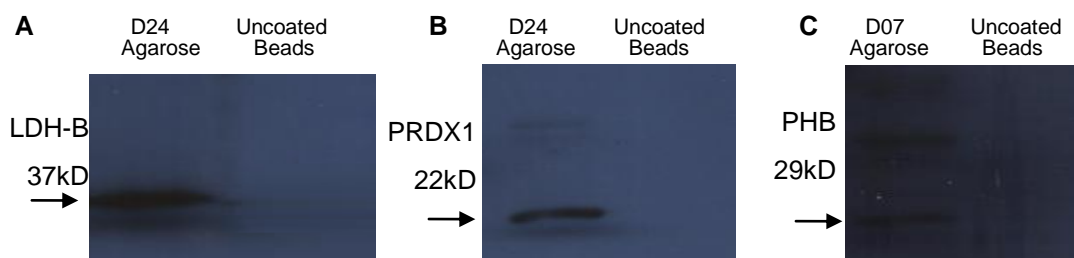
Fig. S13. Western blots to confirm the presence of the 3 biomarker candidate proteins (PRDX1, LDH-B, PHB) pu lled down from the corresponding cancer cell lysates. (A) and (B) from MCF7 lysates and (C) from SW620 lysates.

2.8. Fluorescently labelled antibodies applied to microarrays pre-incubated with cancer cell lysates.
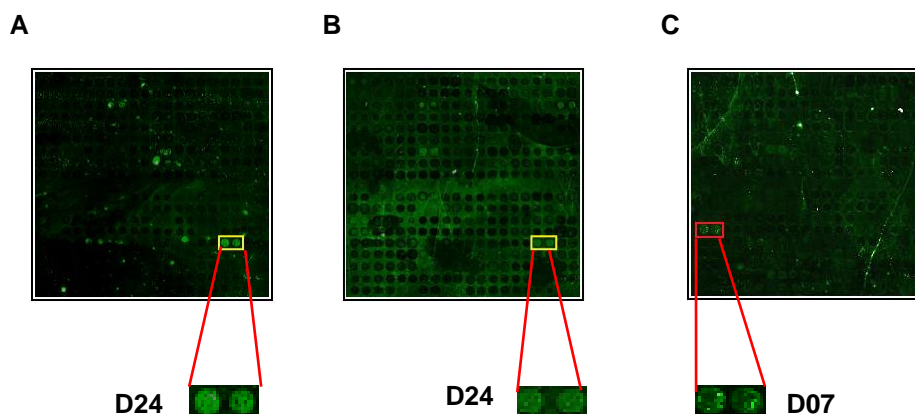
Fig. S14 Antibody validation for the interactions between peptide probes, D24 (YFMTE-*p*Y- VATRW), and corresponding proteins (A) LDH-B and (B) PRDX1 with MCF7 lysates on the microarrays; (C) peptide probes, D07 (SESVV-*p*Y-ADIRK), and corresponding protein PHB with SW620 lysates. D24 boxed in Yellow, D07 boxed in Red.

Table S1 Peptide sequences, molecular weight (MW) and original protein sources of 176 phosphopeptides used in this study.

NO.	384 well plate	Peptide Sequence	MW^a	Original protein source(pYsite)
1	Plate1_A01	AEKPF-*p*Y-VNVEF	1761.7	BCR(177)
2	Plate1_A02	AEMTG-*p*Y-VVTRW	1731.7	Mitogen kinase
3	Plate1_A03	AENAE-*p*Y-LRVAP	1651.7	EGF receptor(1197)
4	Plate1_A04	DEELH-*p*Y-ASLNF	1756.6	CD33(340)
5	Plate1_A05	DEGIH-*p*Y-SELIQ	1722.6	SIGLEC2(822)
6	Plate1_A06	DEKVD-*p*Y-VQVDK	1756.7	Gab2(643)
7	Plate1_A07	DERVD-*p*Y-VVVDQ	1755.7	Gab1(689)
8	Plate1_A08	DESVD-*p*Y-VPMLD	1701.6	PDGF(751)
9	Plate1_A09	DKQVE-*p*Y-LDLDL	1769.7	GAB1(657)
10	Plate1_A10	DSGGF-*p*Y-ITSRT	1622.6	Src-1
11	Plate1_A11	DTETV-*p*Y-SEVRK	1745.6	CD31(713)
12	Plate1_A12	EANSH-*p*Y-GHNDD	1677.5	CD31(663)
13	Plate1_A13	EDEDY-*p*Y-KASVT	1738.6	Type 12
14	Plate1_A14	EDGGV-*p*Y-SSSGL	1489.5	Photo-oncogene FER kinase
15	Plate1_A15	EDGIS-*p*Y-TTLRF	1720.7	SIGLEC2(762)
16	Plate1_A16	EDSTY-*p*Y-KASKG	1667.6	Fak chick kinase

17	Plate1_A17	EDTLT-pY-ADLDM	1705.6	SIRP(470)
18	Plate1_A18	EGVAT-pY-AAAVL	1483.6	CATENIN(654)
19	Plate1_A19	EGDND-pY-IIPLP	1664.6	PDGF(1021)
20	Plate1_A20	ENGLN-pY-IDLDL	1697.7	IRS1(1179)
21	Plate1_A21	EPNVS-pY-ICSRY	1749.6	Kinase gsk3
22	Plate1_A22	ESDGS-pY-QKPSY	1679.6	SELECTIN E(603)
23	Plate1_A23	ESDGG-pY-MDMSK	1638.5	PDGF(740)
24	Plate1_A24	ETDKE-pY-YTVKD	1809.7	JANUS KINASE1(1022)
25	Plate1_B01	FEEDD-pY-ESPND	1778.5	SLP76(113)
26	Plate1_B02	FGMTR-pY-VLDDE	1764.6	Tyrosine kinase TXK
27	Plate1_B03	FLFNM-pY-LTRER	1908.8	HOAX1(343)
28	Plate1_B04	FMMTP-pY-VVTRY	1826.7	JNK-2 kinase
29	Plate1_B05	GFLTE-pY-VATRW	1761.7	ERK2(187)
30	Plate1_B06	GNNYV-pY-IDPTQ	1702.6	KIT(570)
31	Plate1_B07	GSAAP-pY-LKTKF	1601.7	STAT3(705)
32	Plate1_B08	GWMED-pY-DYVHL	1846.6	P130cas
33	Plate1_B09	GWMVH-pY-TSKDT	1743.6	protein kinase c
34	Plate1_B10	HNSAL-pY-SQVQK	1693.7	p62dok
35	Plate1_B11	HRQLN-pY-IQVDL	1817.8	FRS2(436)
36	Plate1_B12	IEDNE-pY-TAREG	1715.6	LYN(397)
37	Plate1_B13	IEDED-pY-YKASV	1750.6	Type 12
38	Plate1_B14	IEDNE-pY-TARQG	1714.6	C-SRC(419)
39	Plate1_B15	IESDI-pY-AEIPD	1683.6	Type 12
40	Plate1_B16	IESSN-pY-MAPYD	1708.6	PDGF(771)
41	Plate1_B17	IRYHR-pY-HGRSA	1834.8	Protooncogene kinase, PIM 1
42	Plate1_B18	IYETD-pY-YRKGG	1783.7	INSULIN(1189)
43	Plate1_B19	KAVDG-pY-VKPQI	1636.7	STAT5B(699)
44	Plate1_B20	KDRMS-pY-HVRSH	1834.7	Myc Zinc protein
45	Plate1_B21	KKRCP-pY-TKHQT	1808.8	HOAX10(326)
46	Plate1_B22	KVVAL-pY-DYMPM	1748.7	BTK kinase
47	Plate1_B23	LISSD-pY-ELLSD	1673.6	JAK3(785)
48	Plate1_B24	LNSDG-pY-TPEPA	1582.6	ZAP70(292)
49	Plate1_C01	LNSKG-pY-TKSID	1644.7	Erk kinase
50	Plate1_C02	LPPEG-pY-VVVVK	1618.7	PTK2B(906)
51	Plate1_C03	MDTSV-pY-ESPYS	1697.6	Tyrosine kinase Zap 70
52	Plate1_C04	MKDEE-pY-EQMVK	1848.7	type 13
53	Plate1_C05	MTGDT-pY-TAHAG	1543.5	ABL

54	Plate1_C06	NNHTE-*p*Y-ASIQT	1696.6	SIRP(453)
55	Plate1_C07	NQSSG-*p*Y-RYGTD	1666.6	Fyn kinase
56	Plate1_C08	NSDVQ-*p*Y-TEVQV	1700.6	CD31(690)
57	Plate1_C09	NSKRD-*p*Y-TGCST	1650.6	MYELIN(200)
58	Plate1_C10	NVVPL-*p*Y-DLLLE	1706.8	ESTROGEN RECEPTOR(537)
59	Plate1_C11	PEGLN-*p*Y-ACLTH	1636.6	ROS1(2334)
60	Plate1_C12	PEGHE-*p*Y-*p*Y-RVRE	1933.7	TYK2(1054)
61	Plate1_C13	PEQDE-*p*Y-DIPRH	1817.7	P130cas
62	Plate1_C14	PGSLE-*p*Y-LCLPA	1581.6	INTERLUKIN 3 RECEPTOR(628)
63	Plate1_C15	PKGTG-*p*Y-IKTEL	1625.7	STAT1(701)
64	Plate1_C16	PLDKD-*p*Y-pY-VVRE	1895.7	JAK3(981)
65	Plate1_C17	PPDHQ-*p*Y-YNDFP	1811.6	SHC(349)
66	Plate1_C18	PQDKE-*p*Y-YKVKE	1845.8	JANUS KINASE2(1007)
67	Plate1_C19	PSFSE-*p*Y-ASVQV	1632.6	SIRP(496)
68	Plate1_C20	PSTRD-*p*Y-EIQRE	1812.7	Fak kinase
69	Plate1_C21	PQDKE-*p*Y-pY-KVKE	1925.8	JAK kinase
70	Plate1_C22	QGPVI-*p*Y-AQLDH	1659.7	MYELIN(241)
71	Plate1_C23	QKQPI-*p*Y-IVMEL	1780.8	FES kinase
72	Plate1_C24	QQQEV-*p*Y-GMMPR	1785.7	SPECTRIN(1176)
73	Plate1_D01	RNEGV-*p*Y-TAIAV	1611.7	Type 12
74	Plate1_D02	REGLN-*p*Y-MVLAT	1685.7	ROS1(2274)
75	Plate1_D03	SAEPQ-*p*Y-QPGDQ	1638.6	PHotooncogene FGR kinase
76	Plate1_D04	SDDVR-*p*Y-VNAFK	1732.7	VEGF (1213)
77	Plate1_D05	SDGHE-*p*Y-IYVDP	1713.6	PDGF(579)
78	Plate1_D06	SEEPI-*p*Y-IVTEY	1761.7	C-SRC(338)
79	Plate1_D07	SESVV-*p*Y-ADIRK	1685.7	MYELIN(263)
80	Plate1_D08	SETDD-*p*Y-AEIID	1689.6	Protein tyrosine kinase 2 beta Type 12
81	Plate1_D09	SPPAL-*p*Y-AEPLD	1591.6	P62dok
82	Plate1_D10	SSNPE-*p*Y-LSASD	1588.5	INSULIN(999)
83	Plate1_D11	SSSSE-*p*Y-GSVSP	1505.5	Mitogen kinase
84	Plate1_D12	SSTVQ-*p*Y-STVVH	1626.6	INTERLUKIN 6 RECEPTOR(759)
85	Plate1_D13	STEPQ-*p*Y-QPGEN	1668.6	C-SRC(530)
86	Plate1_D14	TDKEY-*p*Y-TVKDD	1795.7	JANUS KINASE 1(1023)
87	Plate1_D15	TGGSV-*p*Y-TEDND	1576.5	Type 3
88	Plate1_D16	TNDIT-*p*Y-ADLNL	1671.6	SIRP(429)
89	Plate1_D17	TSKLI-*p*Y-DFIED	1762.7	Type 12
90	Plate1_D18	TSSVL-*p*Y-TAVQP	1584.6	PDGF(1009)

91	Plate1_D19	VDADE-pY-LIPQQ	1709.7	EGFR(1016)
92	Plate1_D20	VDTPH-pY-PRWIT	1803.7	PKC Kinase
93	Plate1_D21	VNTTL-pY-EKFTY	1797.7	TEK KINASE(1108)
94	Plate1_D22	VYESP-pY-SDPEE	1733.6	Tyrosine kinase zap-70
95	Plate1_D23	YETDY-pY-RKGGK	1798.7	Insulin (1190)
96	Plate1_D24	YFMTE-pY-VATRW	1885.7	Mitogen-activated protein kinase 7
97	Plate1_E01	AEGSA-pY-EEVPT	1571.5	PLC-r
98	Plate1_E02	AENPE-pY-LSEFS	1704.6	Erb2 receptor
99	Plate1_E03	FDNLY-pY-WDQDP	1894.6	Erb2 receptor
100	Plate1_E04	AFGTV-pY-KGIWI	1673.7	Erb2 receptor
101	Plate1_E05	AFQFS-pY-TAVFG	1656.6	CAAX Protease
102	Plate1_E06	AKIQD-pY-HILTR	1776.8	Jak2
103	Plate1_E07	APAEM-pY-DIMKT	1688.6	Stem cell growth factor
104	Plate1_E08	ASEQG-pY-EEMRA	1689.6	Erb3
105	Plate1_E09	ATVGH-pY-TAVQN	1579.6	Cyclin kinase activator
106	Plate1_E10	AYRQL-pY-LNPKG	1741.8	Chromosomal associate protein
107	Plate1_E11	CPEKV-pY-ELMRA	1757.7	Abl 1
108	Plate1_E12	DINSL-pY-DVSRM	1731.7	PLC-r
109	Plate1_E13	DRFIQ-pY-ANPAF	1760.7	Camp Phosphodiesterase
110	Plate1_E14	DVSRM-pY-VDPSE	1716.6	PLC-r
111	Plate1_E15	EDIKS-pY-YTVRQ	1820.7	PTP1B
112	Plate1_E16	EEIRF-pY-QLGEE	1831.7	Potassium gate
113	Plate1_E17	EKIQD-pY-EKMPE	1828.7	IL-1
114	Plate1_E18	ELGYE-pY-MDVGS	1681.6	Erb-3
115	Plate1_E19	AELEF-pY-MDYEA	1799.6	Alanine scanning
116	Plate1_E20	FGAKP-pY-DGIPA	1554.6	Erb2 receptor
117	Plate1_E21	GPQDI-pY-DVPPV	1618.6	P130cas
118	Plate1_E22	GQESE-pY-GNITY	1679.6	PTN6
119	Plate1_E23	GRETI-pY-PNASL	1639.7	Carcinoembroyonic antigen
120	Plate1_E24	IGEGT-pY-GTVFK	1590.6	Cell division kinase 5
121	Plate1_F01	IYIHR-pY-ENVSI	1825.8	ATP cyclase
122	Plate1_F02	KAEDE-pY-VNEPL	1725.7	ERB2 receptor
123	Plate1_F03	KPKQE-pY-LNPVE	1763.8	ERB4
124	Plate1_F04	LARDM-pY-DKEYY	1885.7	Met growth factor
125	Plate1_F05	LDSTF-pY-RSLLE	1762.7	Erb2 receptor
126	Plate1_F06	LGQRI-pY-QYIQS	1787.8	Dual specificity regulated kinase
127	Plate1_F07	LLANA-pY-IYVVQ	1685.7	Neural cell adhesion

128	Plate1_F08	LMGHE-*p*Y-MEMKN	1801.6	Cell division cycle protein 23
129	Plate1_F09	LNKQG-*p*Y-KCRQC	1759.7	Protein kinase C
130	Plate1_F10	NKPTV-*p*Y-GVSPN	1594.6	Abl
131	Plate1_F11	PPDHQ-*p*Y-YNDFP	1811.6	SHC transforming protein
132	Plate1_F12	PEDTF-*p*Y-FDPEF	1825.6	ISPK-1 Kinase
133	Plate1_F13	PEPGP-*p*Y-AQPSV	1560.6	Adaptor crk
134	Plate1_F14	RHDSG-*p*Y-EVHHQ	1783.6	Alzheimer's disease
135	Plate1_F15	RNPGF-*p*Y-VEANP	1682.6	PLC-r
136	Plate1_F16	SADHL-*p*Y-VNVSE	1652.6	Tumor necrosis factor
137	Plate1_F17	SSDPT-*p*Y-TSSLG	1533.5	Tyrosine kinase receptor
138	Plate1_F18	EEEPV-*p*Y-EAEPE	1727.7	Hematopoietic lineage cell specific protein1(HS1)
139	Plate1_F19	STKYF-*p*Y-KQNGR	1810.7	Erb4
140	Plate1_F20	TAEPD-*p*Y-GALYE	1647.6	PLC-r
141	Plate1_F21	VCAER-*p*Y-SQEVF	1749.6	Apoptosis Protein cd27
142	Plate1_F22	VDSSL-*p*Y-NLPRS	1669.7	GRB2 associated
143	Plate1_F23	VSSTH-*p*Y-YLLPE	1727.7	Activated cdc42 kinase
144	Plate1_F24	VVIAL-*p*Y-DYQTN	1717.7	T cell specific kinase
145	Plate1_G01	LV-*p*Y-LNVMELV	1610.7	Protein tyrosine kinase 2 beta
146	Plate1_G02	PE-*p*Y-VNQPDVR	1634.6	Receptor tyrosine- protein kinase erbB-2 precursor
147	Plate1_G03	NQ-*p*Y-GNVLSLP	1539.6	B-lymphocyte antigen CD19 precursor
148	Plate1_G04	PS-*p*Y-VNVQNLD	1566.6	SHC-transforming protein 1(317)
149	Plate1_G05	VR-*p*Y-VNAFKFM	1692.7	Vascular endothelial growth factor receptor 1 precursor
150	Plate1_G06	EL-*p*Y-SNALPVG	1480.6	Beta platelet-derived growth factor receptor precursor
151	Plate1_G07	NI-*p*Y-VEVEDEG	1584.6	SH2 domain protein 2A
152	Plate1_G08	VI-*p*Y-DFIEKTG	1602.7	Neural Wiskott-Aldrich syndrome protein
153	Plate1_G09	VL-*p*Y-TEVIPML	1595.7	Olfactory receptor 9Q1
154	Plate1_G10	PE-*p*Y-EEEEVAI	1625.6	Ras GTPase-activating protein 1
155	Plate1_G11	PL-*p*Y-VLVEYAA	1555.7	Fibroblast growth factor receptor 3 precursor
156	Plate1_G12	AI-*p*Y-EEIDAHQ	1606.6	TOM1-like 1 protein
157	Plate1_G13	EL-*p*Y-FLIARYL	1718.8	Bromodomain and WD repeat domain-containing protein 1
158	Plate1_G14	PP-*p*Y-RHIDPFD	1674.6	Macrophage-stimulating protein receptor precursor
159	Plate1_G15	KP-*p*Y-DGIPASE	1494.6	Epidermal growth factor receptor precursor(pY 891)
160	Plate1_G16	NP-*p*Y-CNIYLNS	1618.6	Ras GTPase-activating protein 1
161	Plate1_H01	DI-*p*Y-AEIPDET	1583.6	Protein tyrosine kinase 2 beta
162	Plate1_H02	PV-*p*Y-EEPVYEE	1671.6	Centaurin-delta 3

163	Plate1_H03	SD-pY-ENPDEHS	1610.5	B-cell linker protein
164	Plate1_H04	EV-pY-DVPPSVE	1551.6	Breast cancer anti-estrogen resistance protein 1
165	Plate1_H05	PI-pY-ELTSQFT	1616.6	Caspase-8 precursor
166	Plate1_H06	EV-pY-DTPPMAV	1539.6	Breast cancer anti-estrogen resistance protein 1
167	Plate1_H07	GW-pY-EGVCNRV	1600.6	Abl interactor 1
168	Plate1_H08	NT-pY-DVVYLKV	1631.7	Discs large homolog 4
169	Plate1_H09	PV-pY-EEVGAFP	1525.6	Centaurin-delta 3
170	Plate1_H10	AL-pY-DNVPECA	1512.5	Enhancer of filamentation 1
171	Plate1_H11	PH-pY-EKVSGDY	1612.6	Ephrin-B2 precursor
172	Plate1_H12	DS-pY-ENMDNPD	1617.5	B-lymphocyte antigen CD19 precursor
173	Plate1_H13	DV-pY-ENLHTKN	1650.6	Tyrosine-protein phosphatase non-receptor type 6
174	Plate1_H14	PI-pY-ENVNPEY	1655.6	Receptor-type tyrosine-protein phosphatase beta precursor
175	Plate1_H15	EQ-pY-ELYCEMG	1682.5	E3 ubiquitin-protein ligase CBL
176	Plate1_H16	QG-pY-EEMRAFQ	1676.6	Receptor tyrosine-protein kinase erbB-3 precursor

[a] Majority of peptides were shown to possess the expected molecular weight and have sufficient purity (>85%) as judged by LCMS analysis.

3. <u>References</u>

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