Supporting Information for

N-terminal dimerization of unstructured peptides to form N-gemini peptides provides protease resistance in cytosolic environment

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Peptide synthesis and purification. Peptides were synthesized using manual or automated standard solid phase peptide synthesis (Thuramed Peptide Synthesizer, CEM Liberty 1 Microwave Peptide Synthesizer) using Fmoc protected amino acids on 0.057-0.25 mmol of RINK Amide resin. Four equivalents of standard amino acids were used for each peptide coupling while 1-4 equivalents of orthogonally protected lysine, dicarboxylic acid linkers, and fluorophores were used. The sidechains of orthogonally protected lysine were protected with 1-(4,4-dimethyl-2,6-dioxocyclohex-1-ylidene-3-methylbutyl (N-ivDde). The removal of ivDde from the sidechain of lysine was accomplished by treating the peptide resin 3 x 2 mins with 20 mL of 2% hydrazine monohydrate in DMF bubbling with N2 followed by washing with DMF and DCM. (5)-FAM or 5(6)-FAM was coupled to the ε-NH of lysine or the N-terminus using 4 equivalents of FAM, 5 equivalents of PyBOP/HOBt and 8 equivalents of DIPEA in DMF and allowed to bubble with N2 overnight. Peptides were cleaved from the resin in 9.5:2.5:2.5 trifluoroacetic acid (TFA), TIPS and water respectively for 3-4 hours. The TFA was evaporated and the cleaved peptides were precipitated using cold ethyl ether and extracted with water. Extracted peptides were lyophilized and then purified using semi-preparative RP-HPLC on a Vydac C18 semipreparative column with a gradient from '0 to 100% B' in 45-120 minutes. Solvent A was 95% water, 5% acetonitrile and 0.1% TFA and Solvent B was made of 95% acetonitrile, 5% water and 0.1% TFA. Purified peptides were lyophilized and their purity confirmed by Analytical LC/MS on an Agilent Rapid Resolution LC-MSD system, equipped with an online degasser, binary pump, autosampler, heated column compartment, and diode array detector.

Peptide Dimer Synthesis. To make the dimers, monomers were synthesized using standard Fmoc solid-phase peptide synthesis, and dimerized on resin using a dicarboxylic acid linker using standard peptide coupling conditions adapted from Kier and Andersen.[38] Dimerization proceeded efficiently, and the dimer was the major product of the reaction, according to LC/MS peak integration. Some succinic acid coupled to only one peptide was seen as well, and no peptide was seen that remained uncoupled. For the purposes of monitoring degradation, peptides were synthesized using 5(6)-FAM and a PEG_3 spacer. Despite the reported purity of the purchased carboxy-PEG_3-amine (reported to be 99.5% pure), significant amounts of dimers with differing lengths of PEG chains were observed by ESI-MS, even after multiple rounds of purification by RP-HPLC (Figure S2). We predicted that a longer PEG chain may make dimerization more favorable. We thus synthesized peptide 4 with a PEG_4 linker. As we predicted, no dimers other than the desired one were observed. In the case of previously synthesized peptides 5 & 6, since the PEG_3 dimer remained the major product and the 1-2 unit change in the length of the PEG chain was not expected to have a significant effect on stability, we continued our studies with the mixtures.

Lysate degradation assays. A HeLa cell pellet was washed and resuspended in PBS buffer. Cells were lysed by incubating in liquid nitrogen for 1 minute followed by thawing at 37°C, repeated for a total of 3 freeze-thaw cycles. The mixture was centrifuged at 14,000 x g at 4 °C for 15 minutes. The supernatant was reserved and kept on ice until use. Total protein in the lysate was determined by Bradford assay.

Assessment of peptide degradation was performed by incubating peptide (10 μM) with the HeLa cell lysate (3 mg/mL total cell protein) at 37°C. Aliquots were removed over time and quenched by addition of HCl to a final concentration of 100 mM. Samples were diluted 20-50x in electrophoretic buffer prior to analysis by CE-LIF.

Abl kinase assay. Abl Kinase assays were performed by incubating peptide (2.5 μM peptide **1***/1.25 μM peptide **4***) with Abl-1 (0.75 ng/μL) in assay buffer [50 mM Tris (pH 7.5), 1 mM $MnCl_2$, 5 mM $MgCl_2$, 2 mM DTT, and 1 mM ATP] at 30°C. Aliquots were removed over time and quenched by heating to 95°C for 4 minutes. Samples were diluted 20-50x in electrophoretic buffer prior to analysis by CE-LIF.

PKB kinase assay. PKB Kinase assays were performed as described above using 0.75 μM peptide **2***/0.375 μM peptide **5*** and 0.125 ng/μL PKB-α in assay buffer [8 mM MOPS (pH 7.5), 0.2 mM EDTA, 4 mM $MgCl_2$, and 1 mM ATP].

PKC kinase assay. PKC Kinase assays were performed as described above using 2.5 μM peptide **3***/1.25 μM peptide **6*** and 5 ng/μL PKC-α in assay buffer [20 mM MOPS (pH 7.2), 1 mM DTT, 1 mM $CaCl_2$, 10 mM $MgCl_2$, lipid activator, and 1 mM ATP].

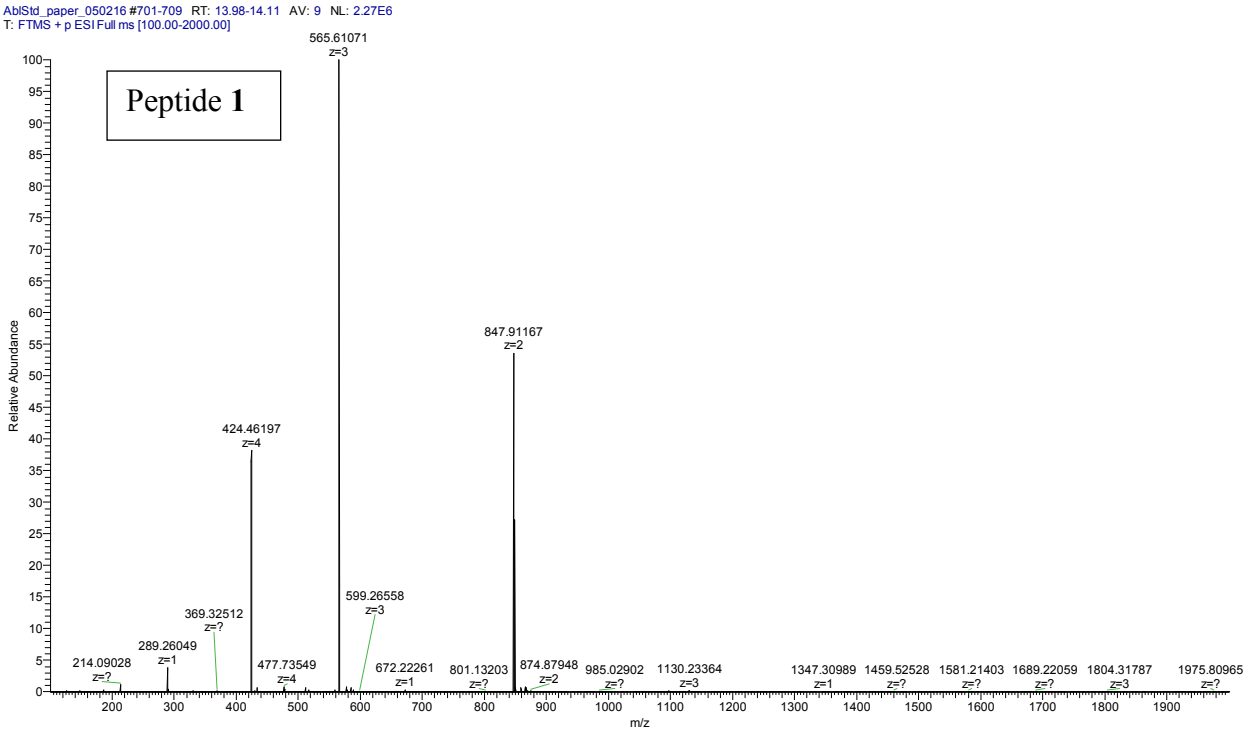
Lysate phosphorylation assay. A Baf/BCR-Abl pellet was washed 2x with PBS buffer. The pellet was resuspended with Mammalian Protein Extraction Reagent (M-PER) with 1 mM sodium pervanadate and 1X Complete EDTA-Free Mini-TAB protease inhibitor cocktail (Roche) and vortexed for 10 minutes. The mixture was then centrifuged at 14,000 x g at 4°C for 15 minutes, and the supernatant was reserved and kept on ice until use. Total protein concentration was determined by Bradford Assay.
Assessment of peptide phosphorylation was performed by mixing peptide (5 μM for peptide **1***, 2.5 μM for peptide **4***) with Baf/BCR-Abl cell lysate (3 mg/mL total cell protein) in assay buffer [50 mM Tris (pH 7.5), 1 mM $MnCl_2$, 5 mM $MgCl_2$, 2 mM DTT, and 1 mM ATP], and incubating at 30°C. Aliquots were removed and quenched by addition of HCl to a final concentration of 100 mM. Samples were diluted 20-100x in electrophoretic buffer prior to analysis by CE-LIF.

Intracellular phosphorylation assay. Approximately 10 million cells were isolated in a 1.5 mL tube and loaded with peptide **1*** or peptide **4*** by pinocytosis. The cells were incubated for 10 minutes at 37°C with hypertonic loading solution (Influx, Life Technologies) containing 24 μM peptide and 1 mM sodium pervanadate, followed by pinosome lysis in hypotonic media to release the peptide into the cytosol and initiate the phosphorylation assay. The time at which the hypotonic media was applied was used at t=0 minutes. The cells were then pelleted and resuspended and incubated in full serum media containing 1 mM sodium pervanadate for 10 minutes at 37°C. After the 10 minutes, the cells were pelleted and washed twice and then resuspended with PBS buffer. The cells were lysed and intracellular activity terminated by heat treatment at 95° for 5 minutes. The resulting lysate was centrifuged at 14000 rcf for 10 minutes at 4°C, and the supernatant was collected and analyzed via CE-LIF without any further dilution.

Capillary electrophoresis. CE-LIF (488 nm) was performed using a Proteome-Lab PA 800 (Beckman Coulter, Fullerton, CA) equipped with 30 cm fused silica capillaries of 30 μM inner diameter, 360 μM outer diameter (Polymicro Technologies, Phoenix, Az). Electrophoretic buffer for all degradation assays was 100 mM borate, 100 mM sodium dodecyl sulfate (SDS), pH 7.7.Electrophoretic buffers for in vitro phosphorylation assays for peptides 1*, 2*, and 4* was 100 mM tris-tricine, 5 mM SDS, pH 8.1; for peptide 3* was 400 mM borate, pH 9.5; for peptide 5* was 100 mM borate, 15 mM SDS, pH 11.3; for peptide 6* was 500 mM borate, pH 9.6. For all lysate and intracellular assays, the electrophoretic buffer was 100 mM tris-tricine, 5 mM SDS, pH 8.1. In the case of phosphorylation of peptide 4* in cell lysates, the peaks were de-convoluted by using a Lorentzian fit in Origin 9.0 (OriginLab Corporation, Northampton, MA) as described previously.[35]

Table S1*. Masses of peptides* ***1-3****.*

Peptide #	***Sequence***	***Expected mass***	***Observed mass***
1	**FAM -EAIYAAPFAKKK-NH_2**	1693.92	$M+2H^+$ 847.91167 $M+3H^+$ 565.61071 $M+4H^+$ 424.46197
2	**FAM-GRPRAATFAEG-NH_2**	1488.64	$M+2H^+$ 745.32693 $M+3H^+$ 497.22194
3	**FAM -QKRPSQRSKYL-NH_2**	1747.94	$M+2H^+$ 874.93024 $M+3H^+$ 583.62123 $M+4H^+$ 437.96821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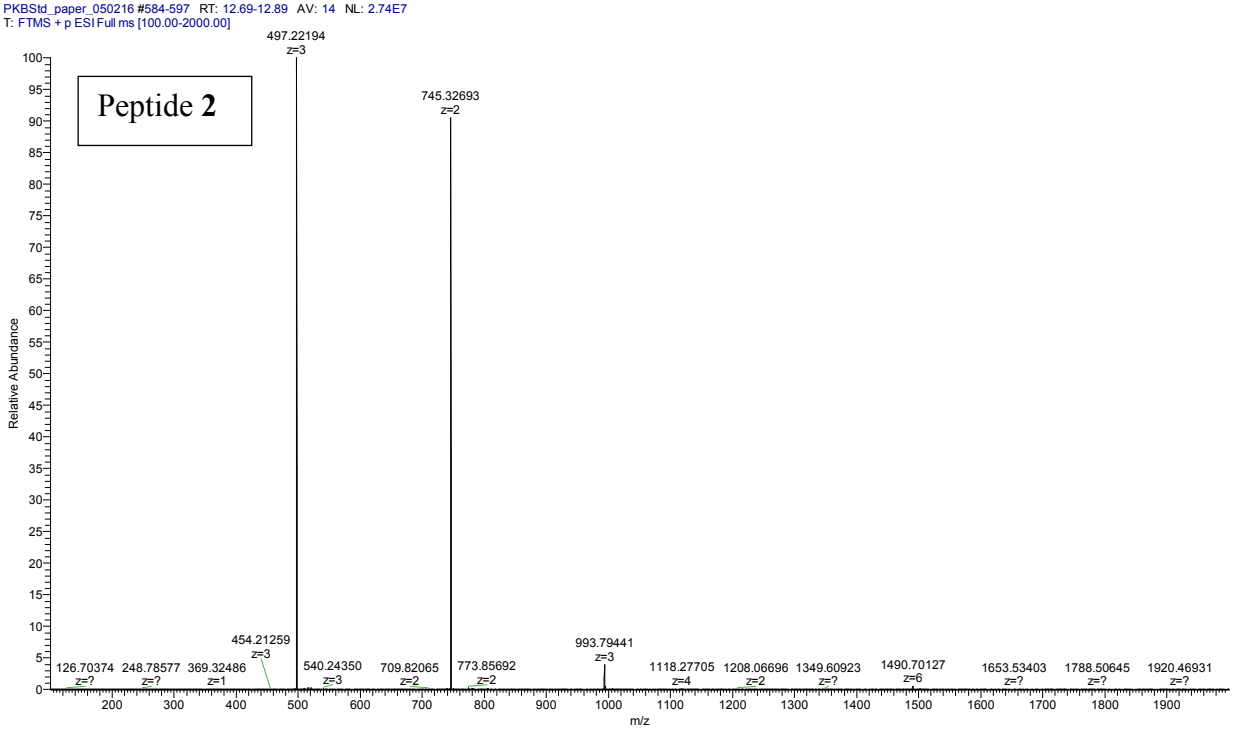
T: FTMS + p ESI Full ms [100.00-2000.00]
Peptide 1
565.61071 z=3
847.91167 z=2
424.46197 z=4
599.26558 z=3
369.32512 z=?
289.26049 z=1
214.09028 z=?
477.73549 z=4
672.22261 z=1
801.13203 z=?
874.87948 z=2
985.02902 z=?
1130.23364 z=3
1347.30989 z=1
1459.52528 z=?
1581.21403 z=?
1689.22059 z=?
1804.31787 z=3
1975.80965 z=?
Relative Abundance
m/z


PKBStd_paper_050216 #584-597 RT: 12.69-12.89 AV: 14 NL: 2.74E7
T: FTMS + p ESI Full ms [100.00-2000.00]
Peptide 2
497.22194 z=3
745.32693 z=2
454.21259 z=3
993.79441 z=3
126.70374 z=?
248.78577 z=?
369.32486 z=1
540.24350 z=3
709.82065 z=2
773.85692 z=2
1118.27705 z=4
1208.06696 z=2
1349.60923 z=?
1490.70127 z=6
1653.53403 z=?
1788.50645 z=?
1920.46931 z=?
Relative Abundance
m/z

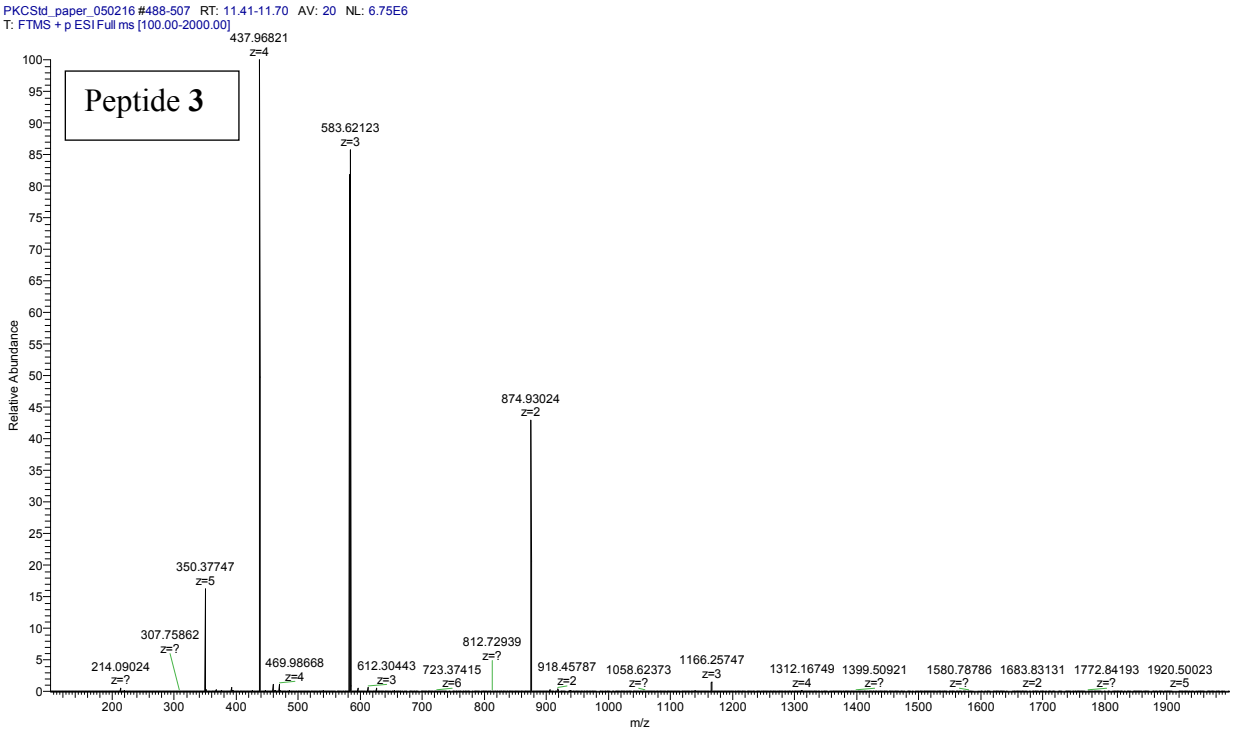
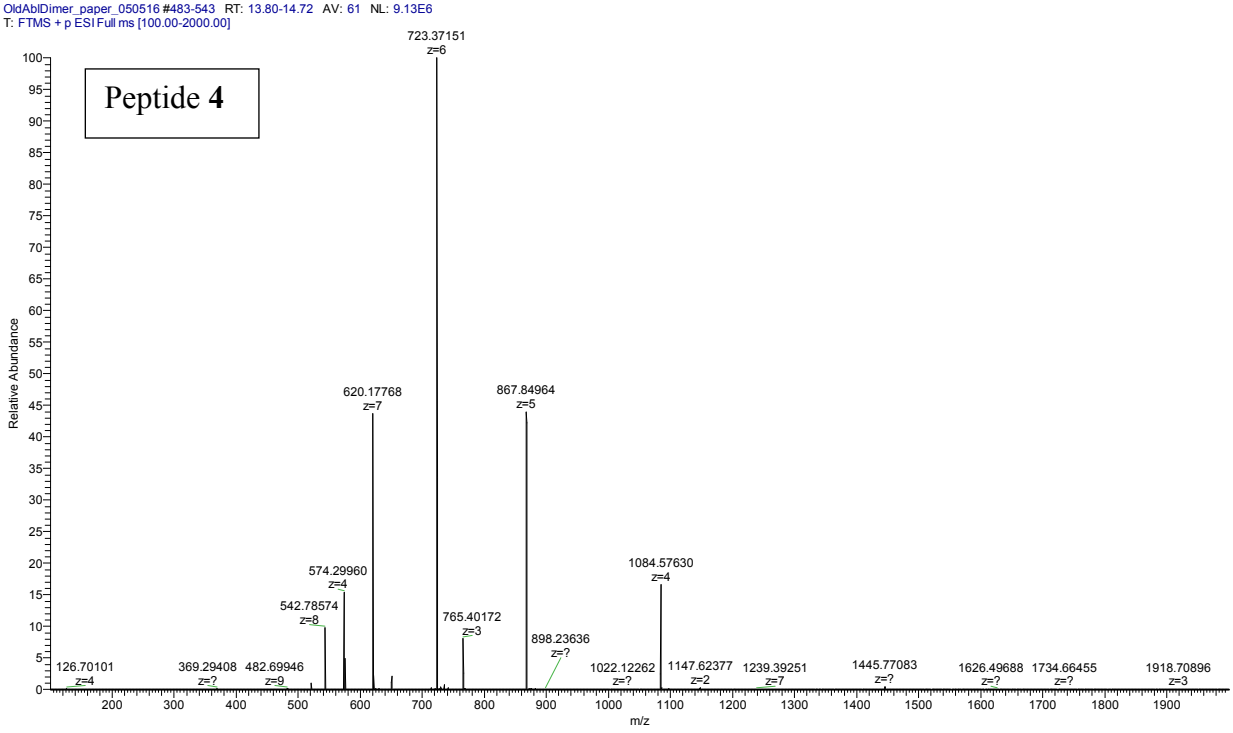


Figure S1. *HR ESI-MS spectra of peptide standards* ***1-3***.

***Table S2**. Masses of peptides **4-8**.*

Peptide #	***Sequence***	***Expected mass***	***Observed mass***
4	**Succinic(GK(FAM)-PEG_4-EAIYAAPFAKKK-NH_2)$_2$**	4334.23	$M+4H^+$ 1084.57630 $M+5H^+$ 867.84964 $M+6H^+$ 723.37151 $M+7H^+$ 620.17768 $M+8H^+$ 542.78574
5	**Succinic(GK(FAM)-PEG_3-GRPRAATFAEG-NH_2)$_2$**	3838.13	$M+3H^+$ 1280.29573 $M+4H^+$ 960.45916 $M+5H^+$ 768.56257
6	**Succinic(GK(FAM)-PEG_3-QKRPSQRSKYL-NH_2)$_2$**	4354.86	$M+5H^+$ 871.84982 $M+6H^+$ 726.70534 $M+7H^+$ 623.03407 $M+8H^+$ 545.28260
7	**Isophthalic(GK(FAM)-PEG_3-GRPRAATFAEG-NH_2)$_2$**	3886.17	$M+3H^+$ 1295.96030 $M+4H^+$ 972.45975 $M+5H^+$ 778.16266 $M+6H^+$ 648.64176
8	**Terephthalic(GK(FAM)-PEG_3-GRPRAATFAEG-NH_2)$_2$**	3886.17	$M+3H^+$ 1295.96030 $M+4H^+$ 972.45975 $M+5H^+$ 778.16266 $M+6H^+$ 648.64176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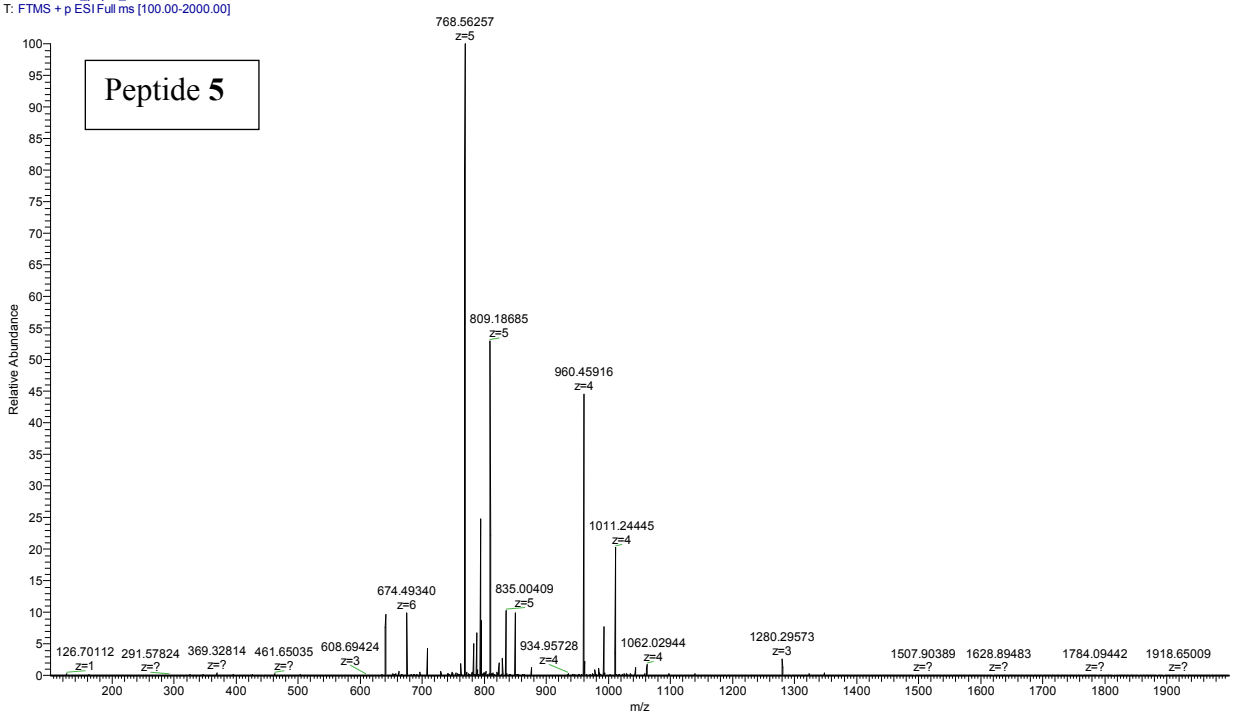
T: FTMS + p ESI Full ms [100.00-2000.00]
Peptide 4
723.37151 z=6
620.17768 z=7
867.84964 z=5
574.29960 z=4
542.78574 z=8
765.40172 z=3
898.23636 z=?
1084.57630 z=4
126.70101 z=4
369.29408 z=?
482.69946 z=9
1022.12262 z=?
1147.62377 z=2
1239.39251 z=7
1445.77083 z=?
1626.49688 z=?
1734.66455 z=?
1918.70896 z=3
Relative Abundance
m/z


OldPKBDimer_paper_050516 #498-545 RT: 13.39-14.10 AV: 48 NL: 7.07E6
T: FTMS + p ESI Full ms [100.00-2000.00]
Peptide 5
768.56257 z=5
809.18685 z=5
960.45916 z=4
1011.24445 z=4
674.49340 z=6
835.00409 z=5
126.70112 z=1
291.57824 z=?
369.32814 z=?
461.65035 z=?
608.69424 z=3
934.95728 z=4
1062.02944 z=4
1280.29573 z=3
1507.90389 z=?
1628.89483 z=?
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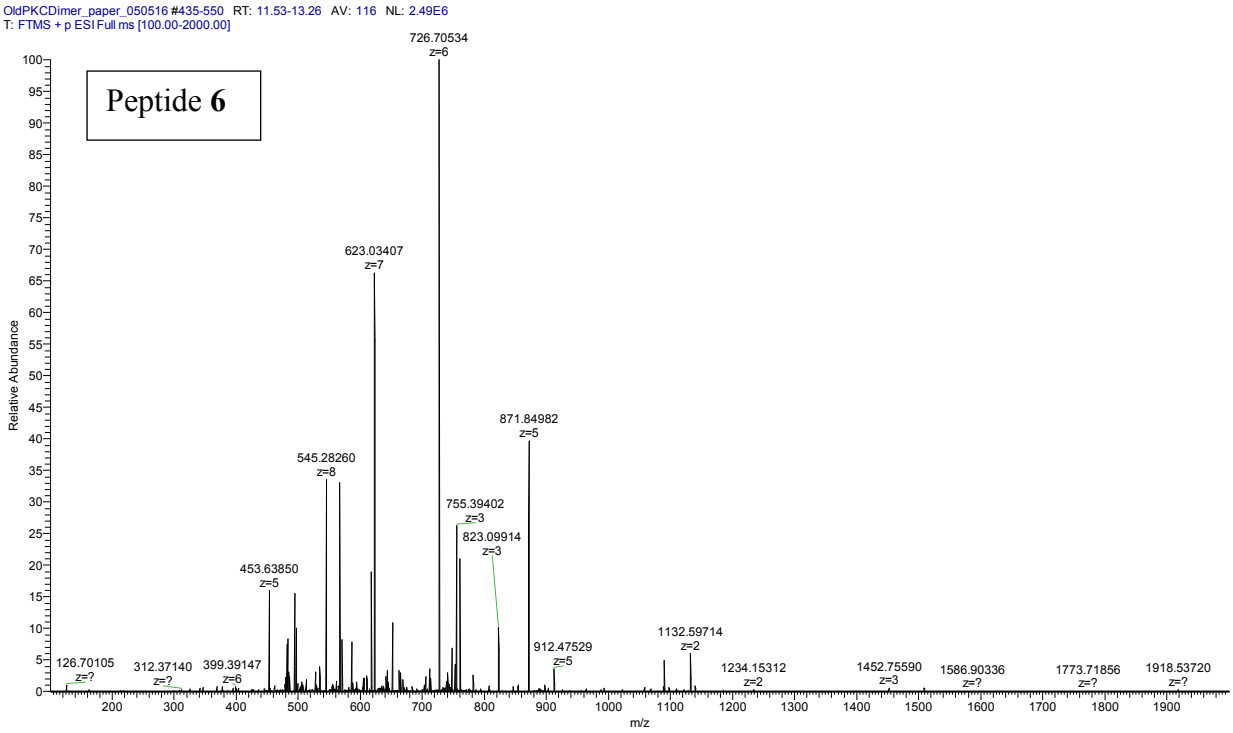
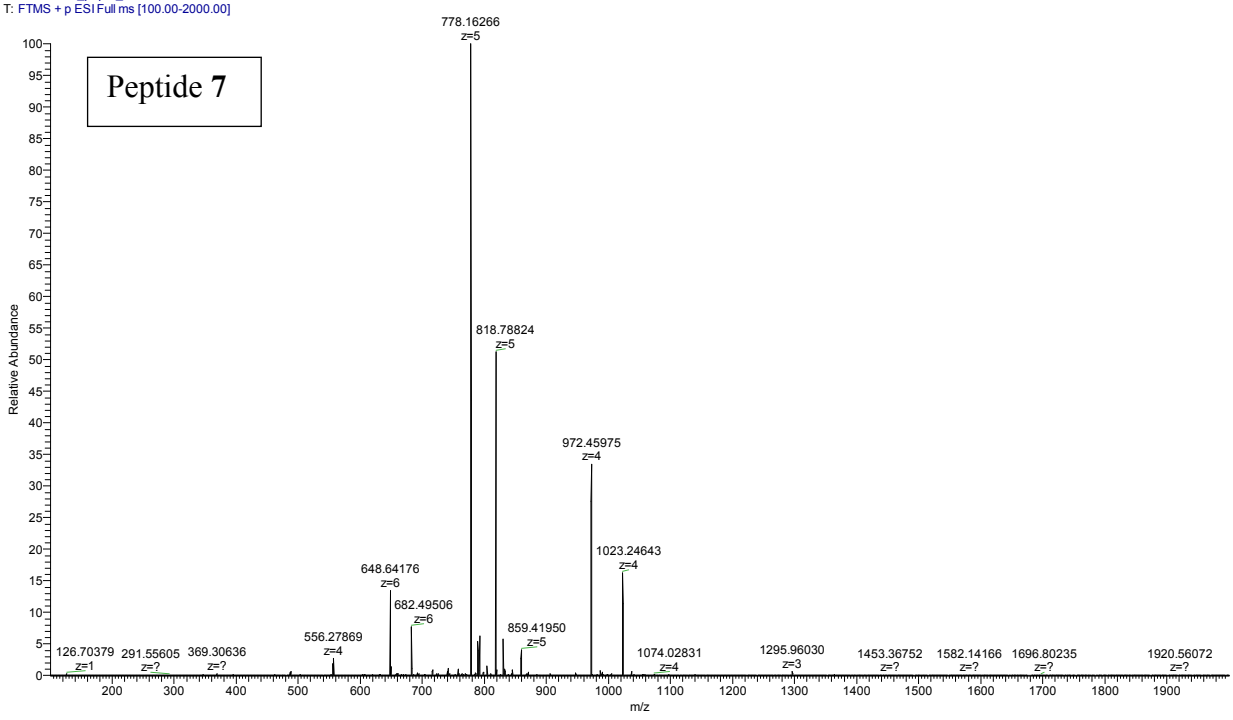
1918.65009 z=?
Relative Abundance
m/z


OldPKCDimer_paper_050516 #435-550 RT: 11.53-13.26 AV: 116 NL: 2.49E6
T: FTMS + p ESI Full ms [100.00-2000.00]
Peptide 6
Relative Abundance
m/z
726.70534 z=6
623.03407 z=7
871.84982 z=5
545.28260 z=8
755.39402 z=3
823.09914 z=3
453.63850 z=5
912.47529 z=5
1132.59714 z=2
126.70105 z=?
312.37140 z=?
399.39147 z=6
1234.15312 z=2
1452.75590 z=3
1586.90336 z=?
1773.71856 z=?
1918.53720 z=?


IsoPKBDimer_paper_050216 #613-646 RT: 13.66-14.16 AV: 34 NL: 3.87E6
T: FTMS + p ESI Full ms [100.00-2000.00]
Peptide 7
Relative Abundance
m/z
778.16266 z=5
818.78824 z=5
972.45975 z=4
1023.24643 z=4
648.64176 z=6
682.49506 z=6
859.41950 z=5
556.27869 z=4
126.70379 z=1
291.55605 z=?
369.30636 z=?
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1920.56072 z=?

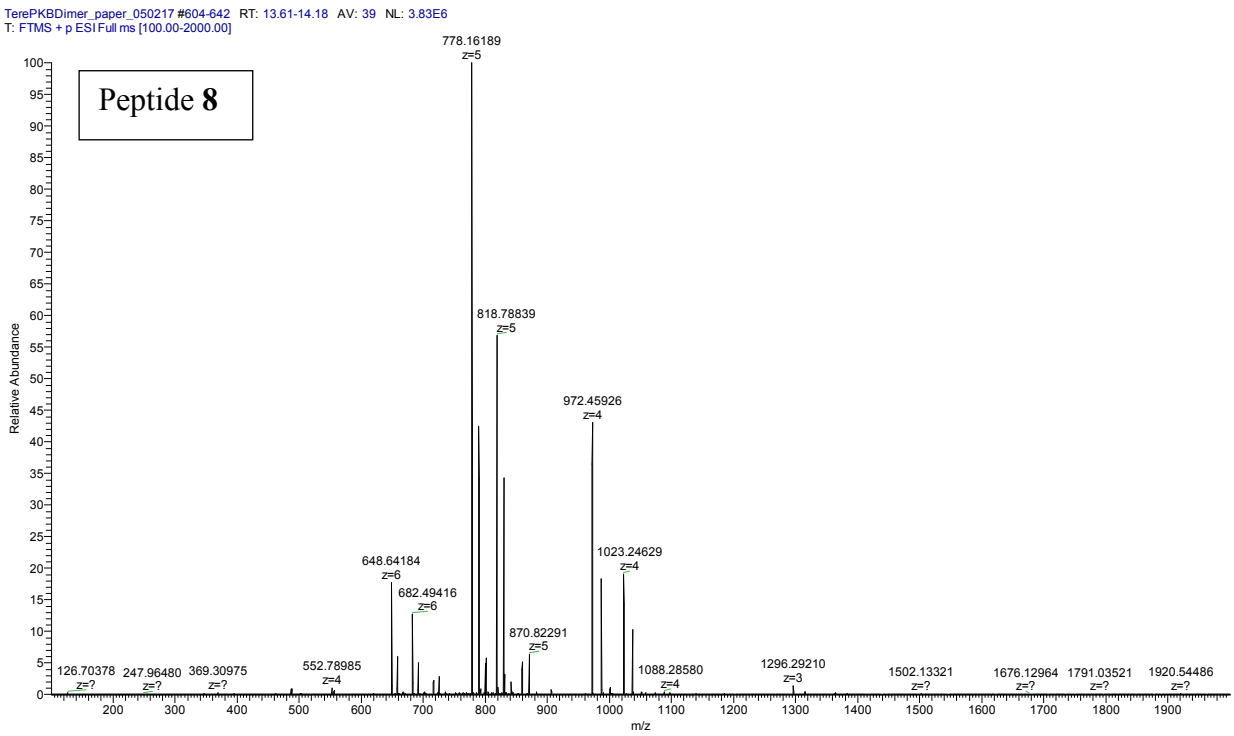
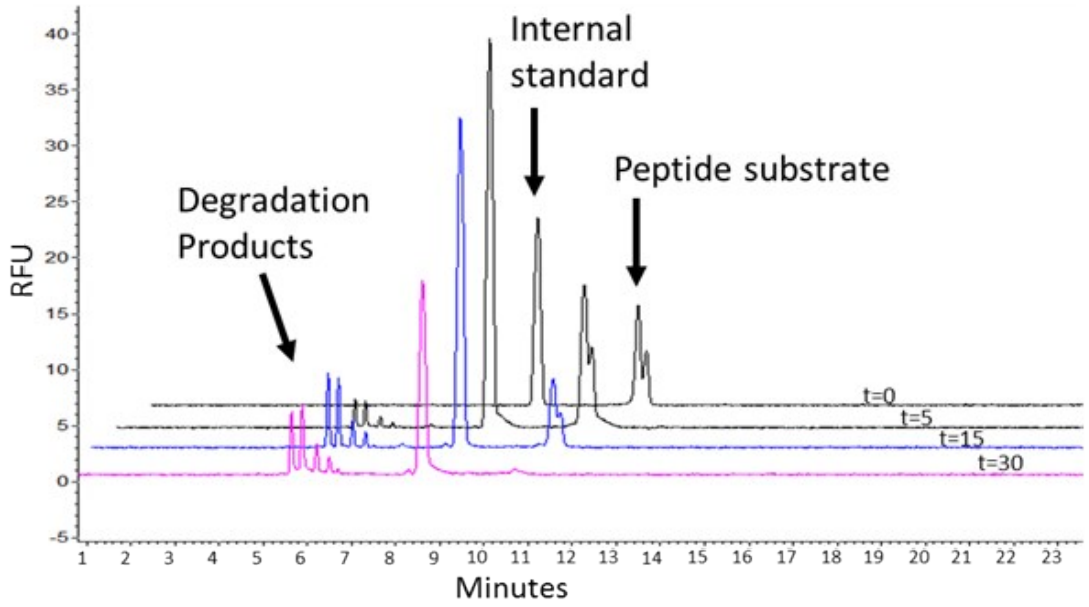
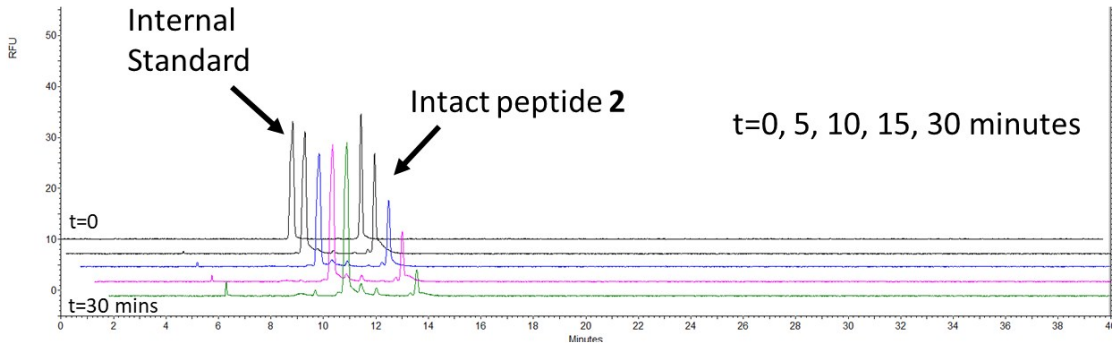
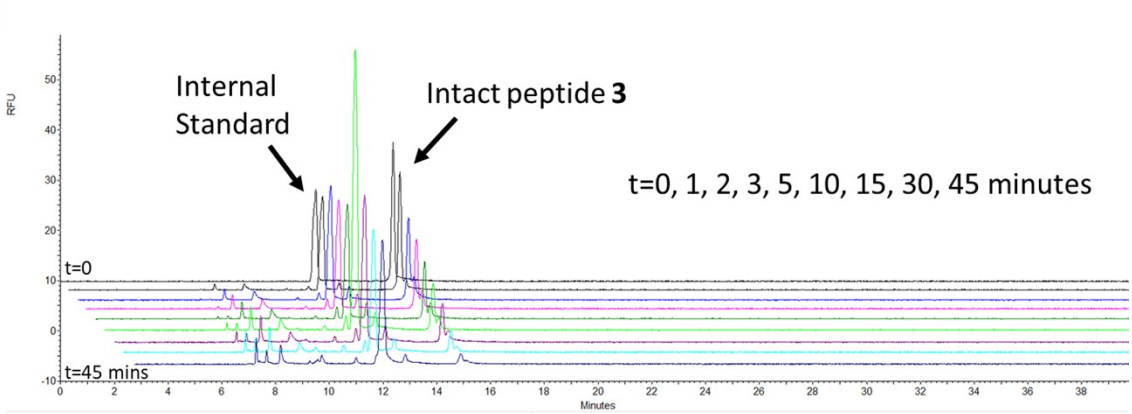
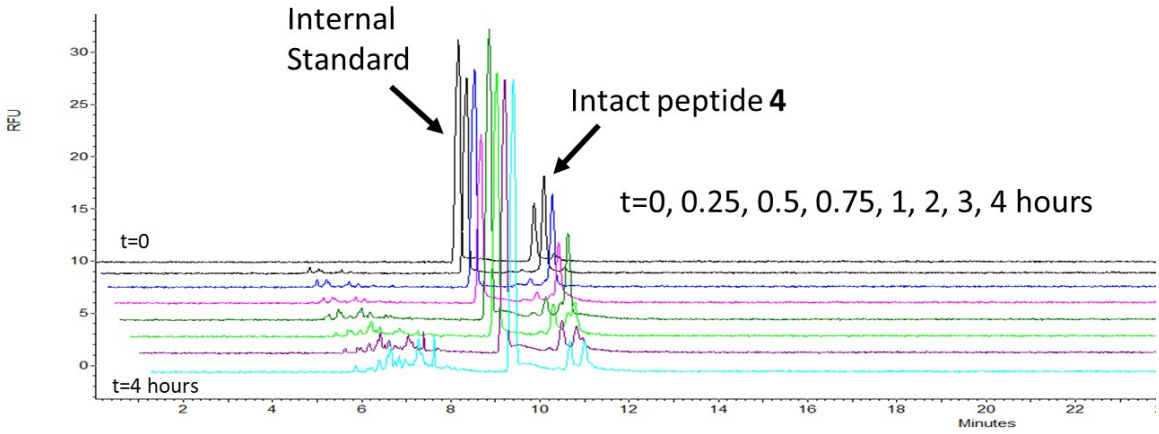


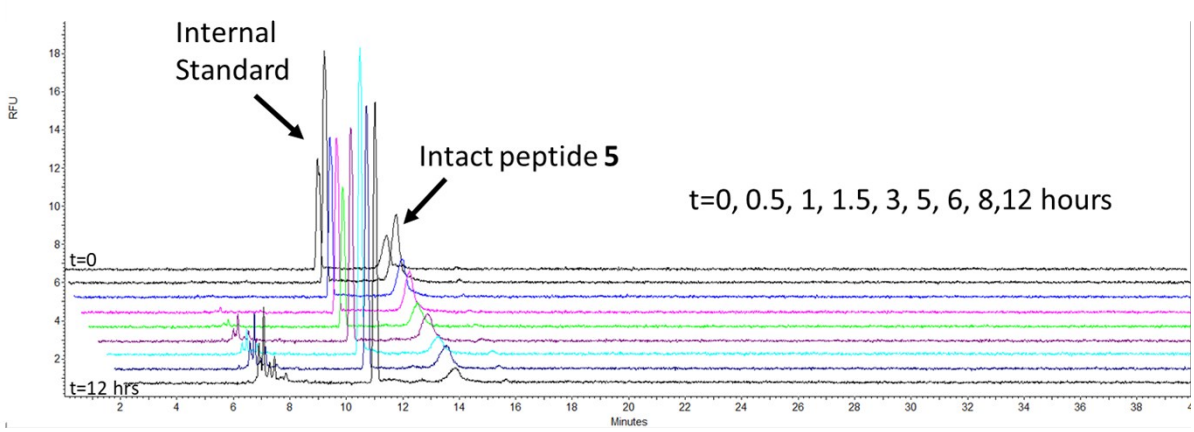
Figure S2. *HR ESI-MS spectra of peptide dimers* ***4-8***. *Minimal amounts of different lengths of PEG chains are seen.*

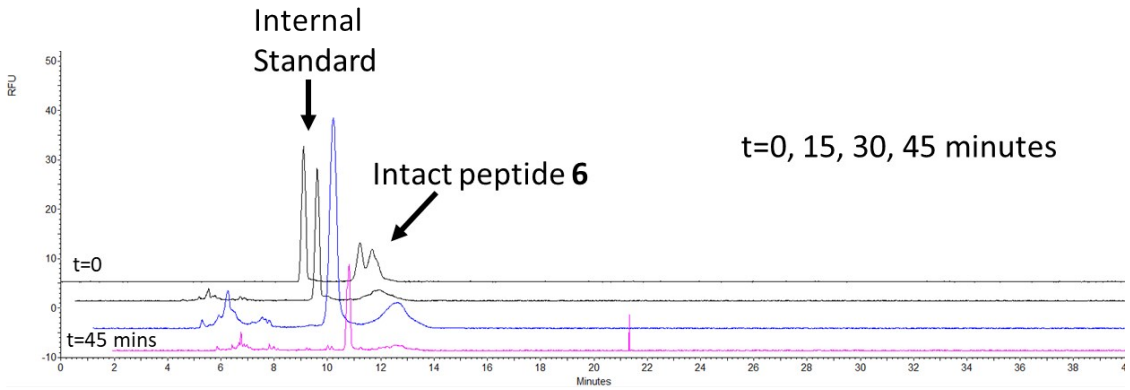



Internal
Standard
Intact peptide **2**
t=0, 5, 10, 15, 30 minutes
t=0
t=30 mins
RFU
Minutes


Internal
Standard
Intact peptide **3**
t=0, 1, 2, 3, 5, 10, 15, 30, 45 minutes
t=0
t=45 mins
RFU
Minutes


Internal
Standard
Intact peptide **4**
t=0, 0.25, 0.5, 0.75, 1, 2, 3, 4 hours
t=0
t=4 hours
RFU
Minutes


Internal
Standard
Intact peptide 5
t=0, 0.5, 1, 1.5, 3, 5, 6, 8,12 hours
t=0
t=12 hrs
RFU
Minutes


Internal
Standard
Intact peptide 6
t=0, 15, 30, 45 minutes
t=0
t=45 mins
RFU
Minutes

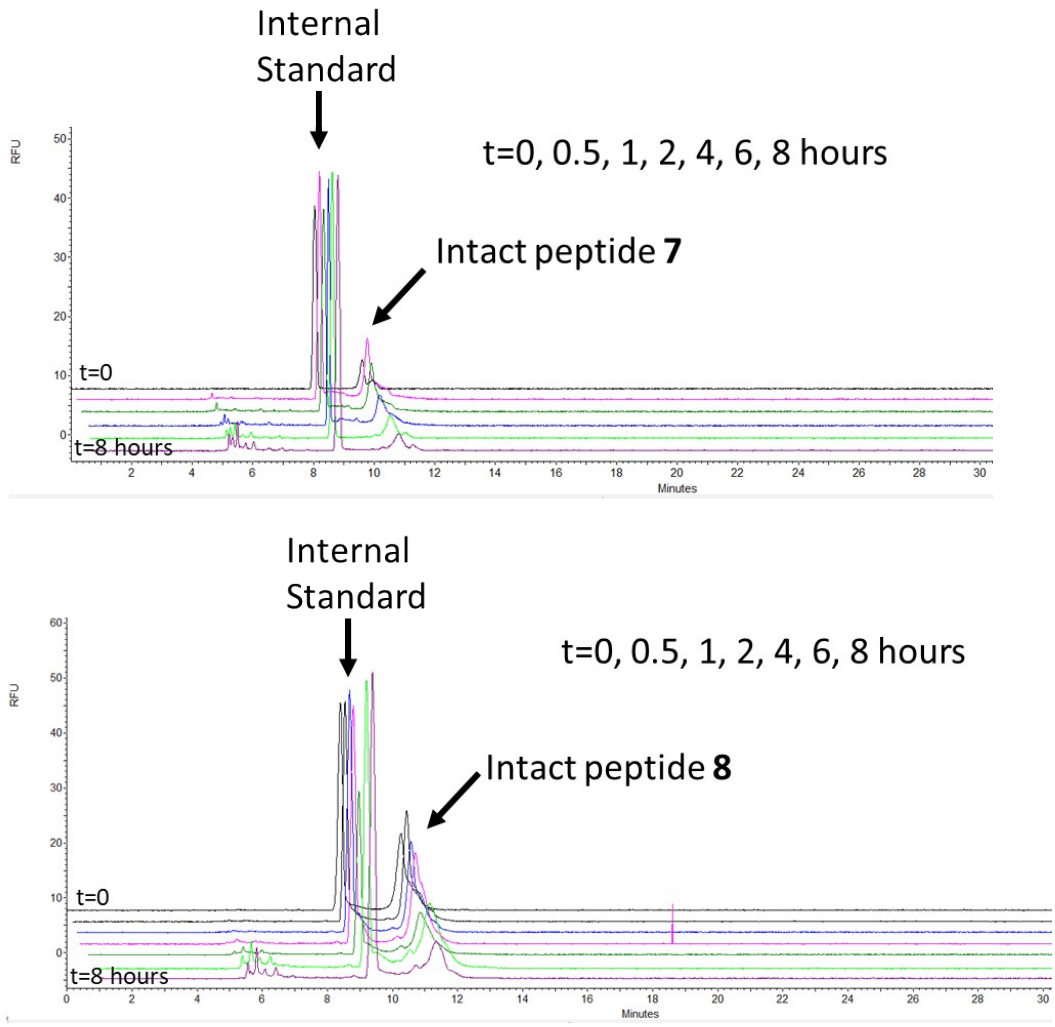
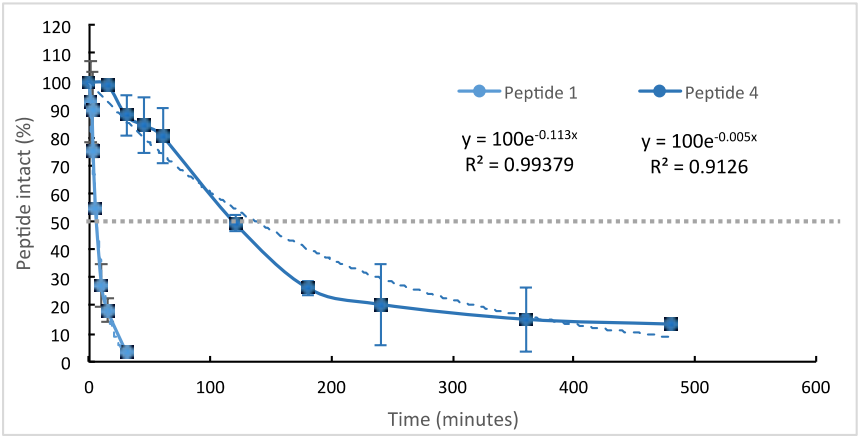
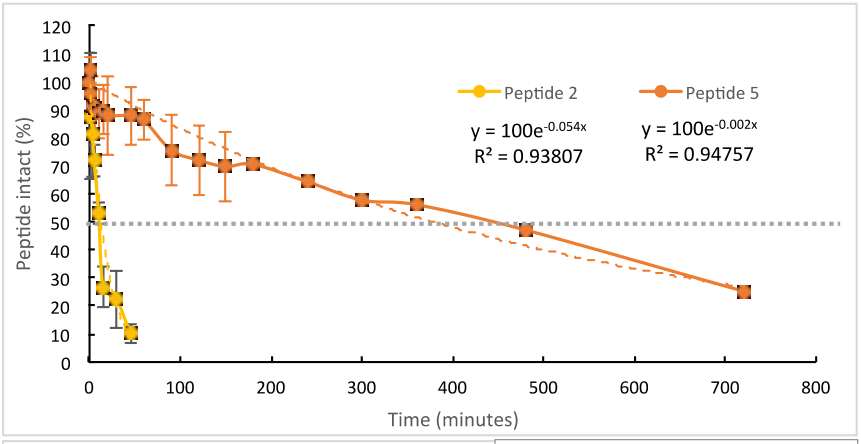


Figure S3. *Electropherograms following the degradation of peptides* ***1-8*** *in HeLa cytosolic lysates. Intact peptides may show up as two peaks or with a shoulder due to the separation of the 5-FAM and 6-FAM labeled forms.*

A.



B.



C.

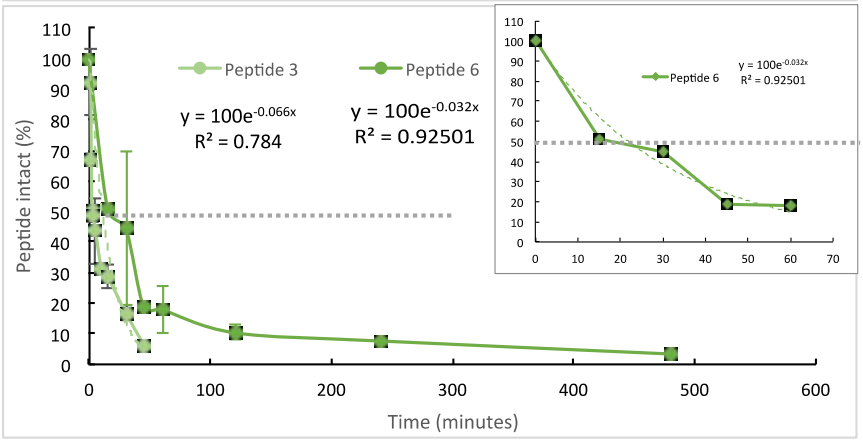


Figure S4. *Degradation of peptides in HeLa cytosolic lysate. Error bars represent the standard deviation of three runs. Solid line represents trendline highlighting the data. Dashed colored line represents exponential fit to the data (equations are shown in legend). The gray horizontal dashed line shows 50% degradation. (a) Peptides 1 and 3; (b) Peptides 2 and 4; (c) Peptides 3 and 6 (inset shows fit to first 60 min of degradation of peptide 6).*

Table S3. *Half-lives of peptides* ***1-6*** *in HeLa cytosolic lysates from experimental data and exponential fit.*[a]

Peptide	$t_{1/2}$ (minutes)	Exponential Fit
1	5±1	6
2	15±1	13
3	2±0.2	11[b]
4	120±5	139
5	420±24	347
6	15±3	722

[a] Error was determined based on the standard deviation from three runs. [b]The exponential fit was poor for this peptide.

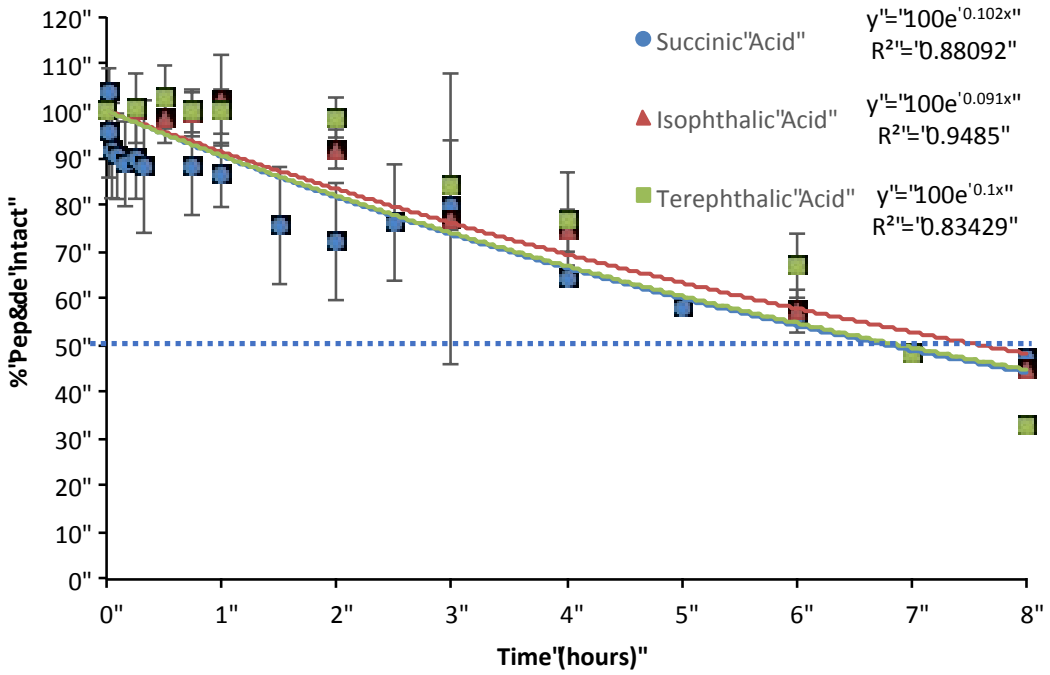


Figure S5*. Degradation of peptides with different linkers in HeLa cell lysates analyzed by CE-LIF: Peptide* ***5*** *(succinic acid linker),* ***7*** *(isophthalic acid linker), and* ***8*** *(terephthalic linker). The horizontal dashed line shows 50% degradation.*

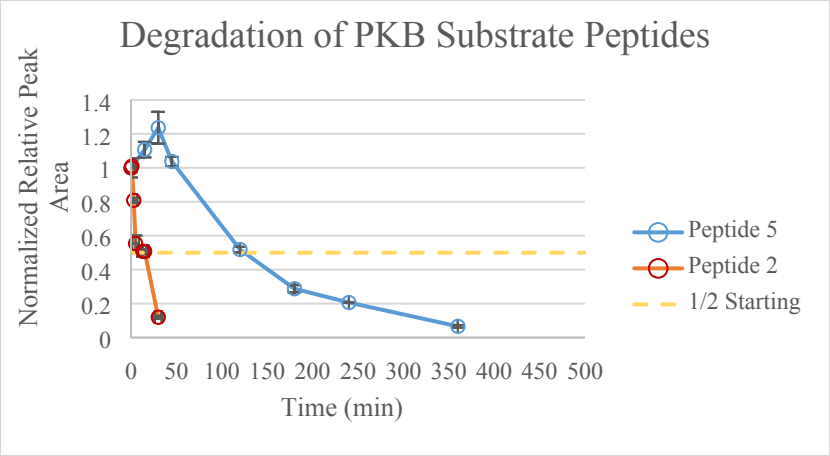


Figure S6. *Degradation of peptides **2** & **5** in HeLa cell lysates analyzed by CE-ESI-MS. The fold increase in half-life seen here matches that seen by CE-LIF.*

Degradation of peptides 2 & 5 in HeLa cell lysates analyzed by CE-ESI-MS.

Materials and Reagents. Deionized water was generated with a Nanopure Diamond water purifier (Barnstead International, Dubuque IA). HPLC grade methanol and formic acid (99.9%) were obtained from Fisher Scientific (Fairlawn, NJ). APDIPES ((2-Aminopropyl)di-isopropylethoxysilane) was obtained from Gelest (Morrisville, PA). The background electrolyte (BGE) for all experiments was 212.5 mM ammonium acetate in 50% methanol, 48% water, and 2% formic acid (v/v/v). For analysis, samples were diluted 5x in BGE with Glu-1-Fibrinopeptide B as an internal standard.

Microfluidic Device Design and Preparation. The design of CE-ESI devices has been described in detail previously.[40,41] Briefly, the device consists of four reservoirs, an injection cross, a 10 cm serpentine separation channel, a pumping channel, and an ESI orifice at the corner of the device. Devices were fabricated in-house by photolithography and wet etching from 0.5 mm thick B270 glass (Perkin Elmer, Waltham, MA). Channels were etched to a depth of 10 µm and a full width of 70 µm. The integrated ESI emitter was created by dicing the corner of the device at 90° using a dicer from Disco Technologies (Santa Clara, CA) and polishing the edges with 3 µm cerium oxide abrasive lapping paper on a lapping wheel (Ultra Tec, Santa Ana, CA). Reservoirs were formed by attaching glass cylinders with 6 mm inner diameter to the surface of the device with epoxy (Loctite E-120HP, Henkel Corporation, Germany).

The devices were coated with APDIPES via chemical vapor deposition. All channels were then coated with NHS-PEG_{450} reagent (NanoCS, Boston, MA) in 10 mM phosphate buffer. After coating the channels, 3.5 µL of trichloro-(1*H*, 1*H*, 2*H*, 2*H*-perflurorooctyl)-silane was applied the ESI emitter (at the corner of the device) to increase the hydrophobicity of the corner and facilitate electrospray.

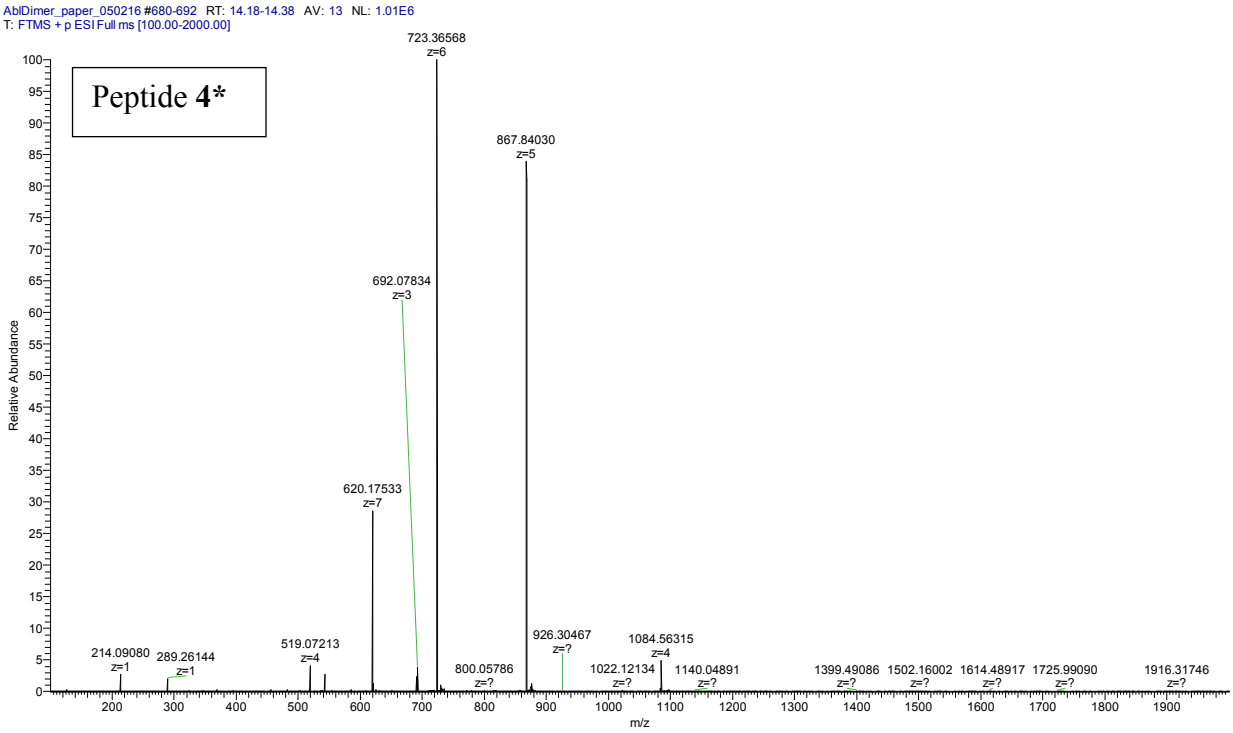
CE-ESI-MS Operation. CE-ESI devices were operated by application of voltages to the solutions in the reservoirs via platinum wire electrodes. For this analysis, +15 kV and +1.8 kV were

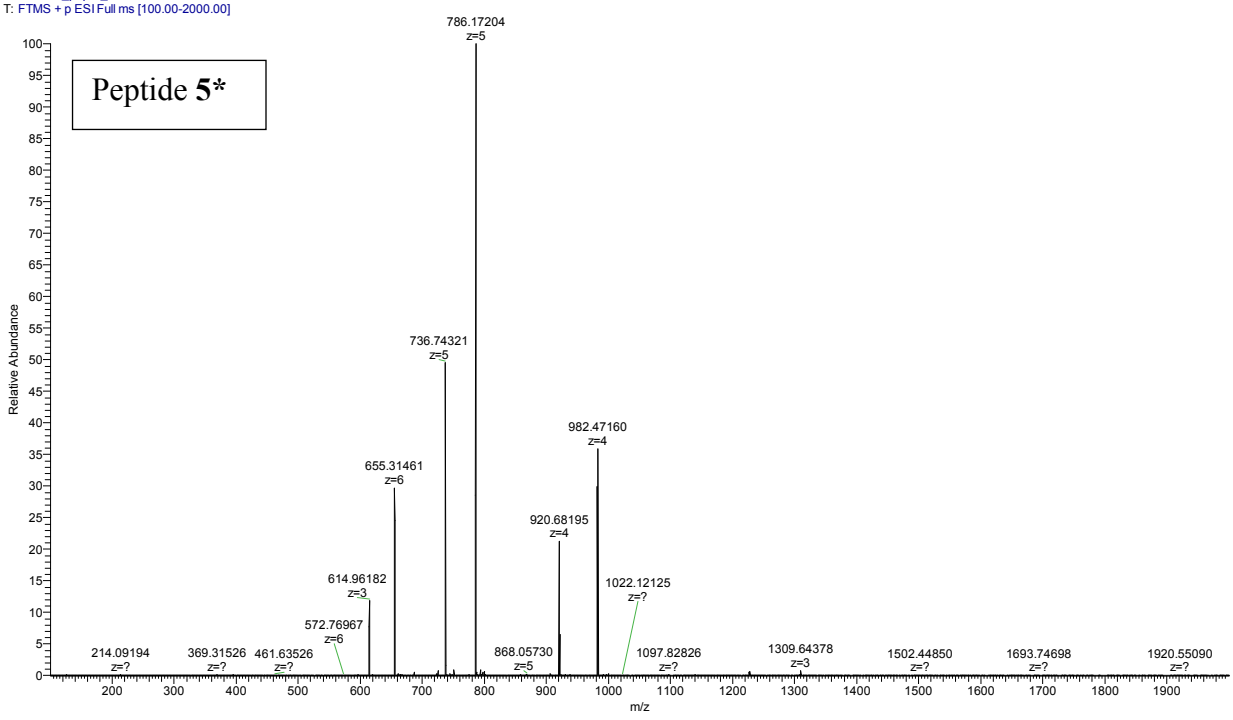
applied to the reservoir at the top of the injection cross and the pumping channel reservoir, respectively. The electrodes were powered with an in-house power supply and controlled using a custom LabVIEW (National Instruments, Austin, TX) program. A system of valves (Clippard, Cincinnati, OH) controlled with the same LabVIEW program was used to control pressures to perform hydrodynamic injections (2 psi) and provide pressure to the pumping channel for ESI (2 psi). All injections were 20 s.

The CE-ESI devices were positioned in front of the mass spectrometer using a custom x-y-z stage. MS analysis was performed using a quadrupole time-of-flight mass spectrometer (Waters Synapt G2, Waters Corporation, Milford, MA). The mass range was set to 300-1600 *m/z* with a 0.09 s scan time. Extracted ion electropherograms were generated for the analyte of interest and internal standard with a 0.15 *m/z* window. Peak areas in the EI electropherograms were calculated using the integration function in the MassLynx software.

Table S4. *Masses of peptides* ***4*-6****.*

Peptide #	***Sequence***	***Expected mass***	***Observed mass***
4*	**Succinic(GK(FAM)-PEG$_4$-EAIYAAPFAKKK-NH$_2$)$_2$**	4334.94	M+4H$^+$ 1084.57630 M+5H$^+$ 867.84964 M+6H$^+$ 723.37151 M+7H$^+$ 620.17768
5*	**Succinic(GK(FAM)-PEG$_4$-GRPRAATFAEG-NH$_2$)$_2$**	3926.23	M+3H$^+$ 1309.64378 M+4H$^+$ 982.47160 M+5H$^+$ 786.17204 M+6H$^+$ 655.31461
6*	**Succinic(GK(FAM)-PEG$_4$-QKRPSQRSKYL-NH$_2$)$_2$**	4442.96	M+3H$^+$ 1481.77576 M+4H$^+$ 1111.58443 M+5H$^+$ 889.45801 M+6H$^+$ 741.37922 M+7H$^+$ 635.61162 M+8H$^+$ 556.28779 M+9H$^+$ 494.59194 M+10H$^+$ 445.23262


AblDimer_paper_050216 #680-692 RT: 14.18-14.38 AV: 13 NL: 1.01E6
T: FTMS + p ESI Full ms [100.00-2000.00]
Peptide 4*
723.36568 z=6
867.84030 z=5
692.07834 z=3
620.17533 z=7
926.30467 z=?
1084.56315 z=4
519.07213 z=4
214.09080 z=1
289.26144 z=1
800.05786 z=?
1022.12134 z=?
1140.04891 z=?
1399.49086 z=?
1502.16002 z=?
1614.48917 z=?
1725.99090 z=?
1916.31746 z=?
Relative Abundance
m/z


PKBDimer_paper_050216 #617-634 RT: 13.71-13.97 AV: 18 NL: 4.45E6
T: FTMS + p ESI Full ms [100.00-2000.00]
Peptide 5*
786.17204 z=5
736.74321 z=5
982.47160 z=4
655.31461 z=6
920.68195 z=4
614.96182 z=3
1022.12125 z=?
572.76967 z=6
214.09194 z=?
369.31526 z=?
461.63526 z=?
868.05730 z=5
1097.82826 z=?
1309.64378 z=3
1502.44850 z=?
1693.74698 z=?
1920.55090 z=?
Relative Abundance
m/z

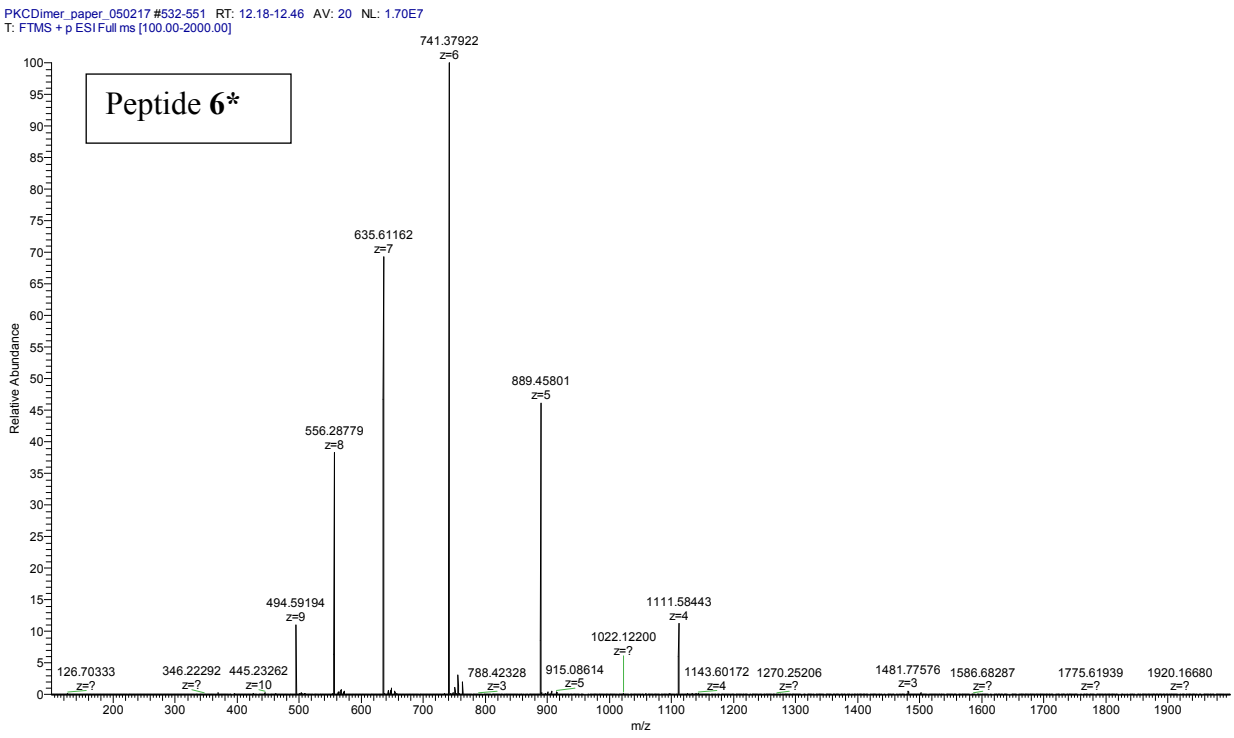
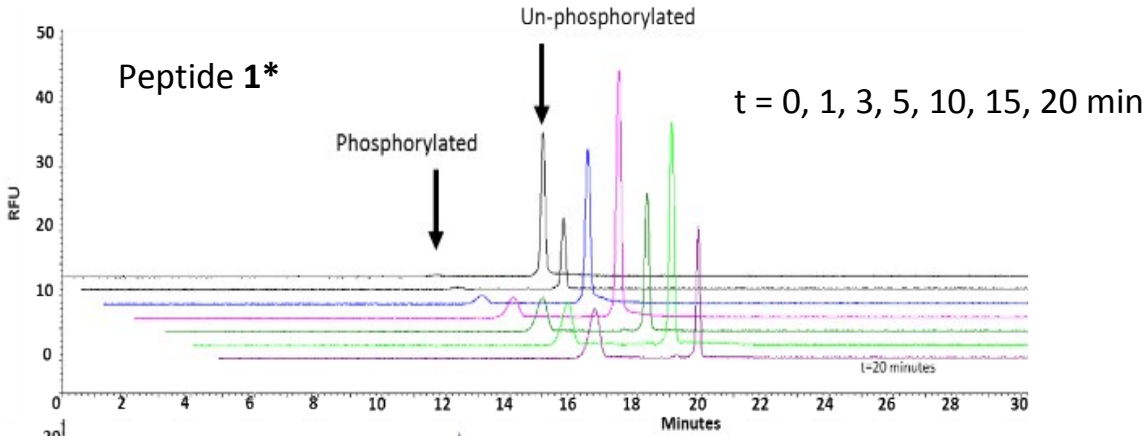
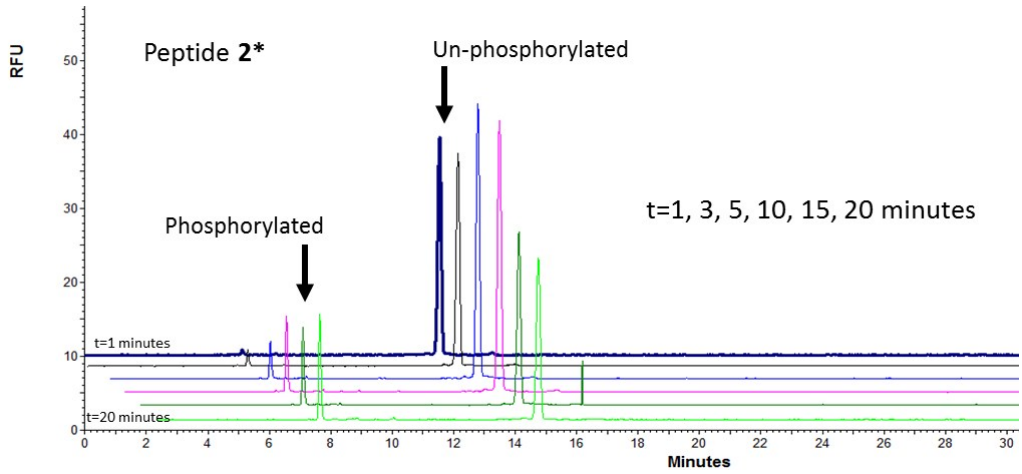
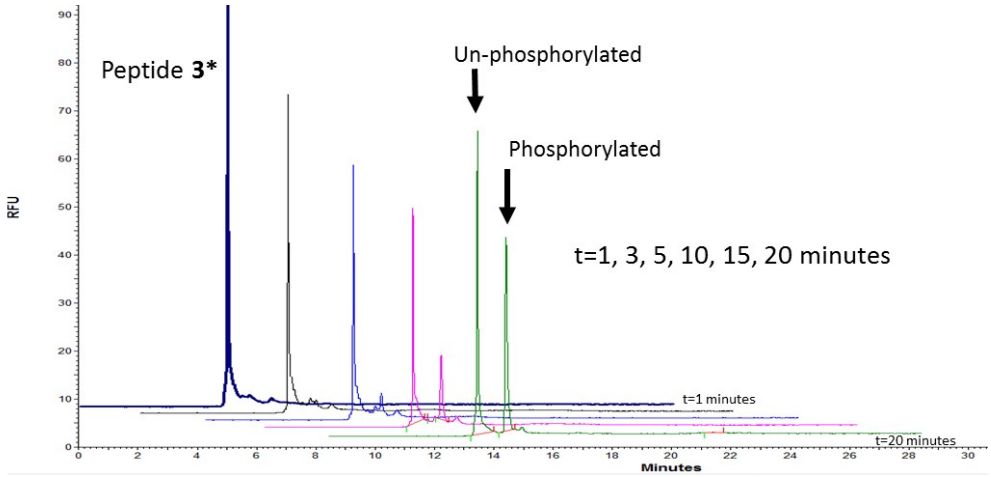
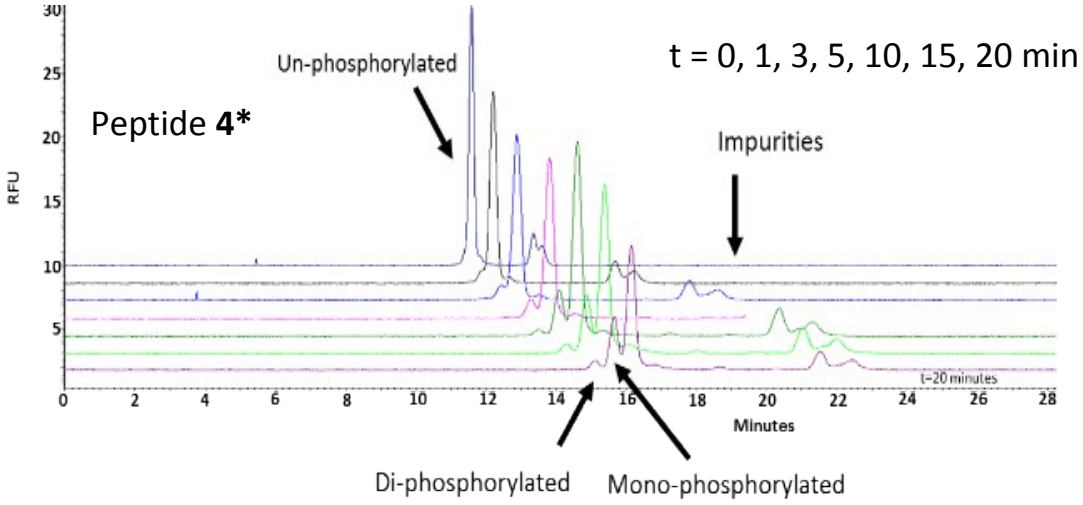


Figure S7. HR ESI-MS spectra of peptide dimers **4*-6***. Peptide **5*** contains an un-identified impurity that could not be separated by RP-HPLC.




Peptide **2***
Un-phosphorylated
Phosphorylated
t=1, 3, 5, 10, 15, 20 minutes
t=1 minutes
t=20 minutes
RFU
Minutes


Peptide **3***
Un-phosphorylated
Phosphorylated
t=1, 3, 5, 10, 15, 20 minutes
t=1 minutes
t=20 minutes
RFU
Minutes


Peptide **4***
Un-phosphorylated
t = 0, 1, 3, 5, 10, 15, 20 min
Impurities
t=20 minutes
RFU
Minutes
Di-phosphorylated
Mono-phosphorylated

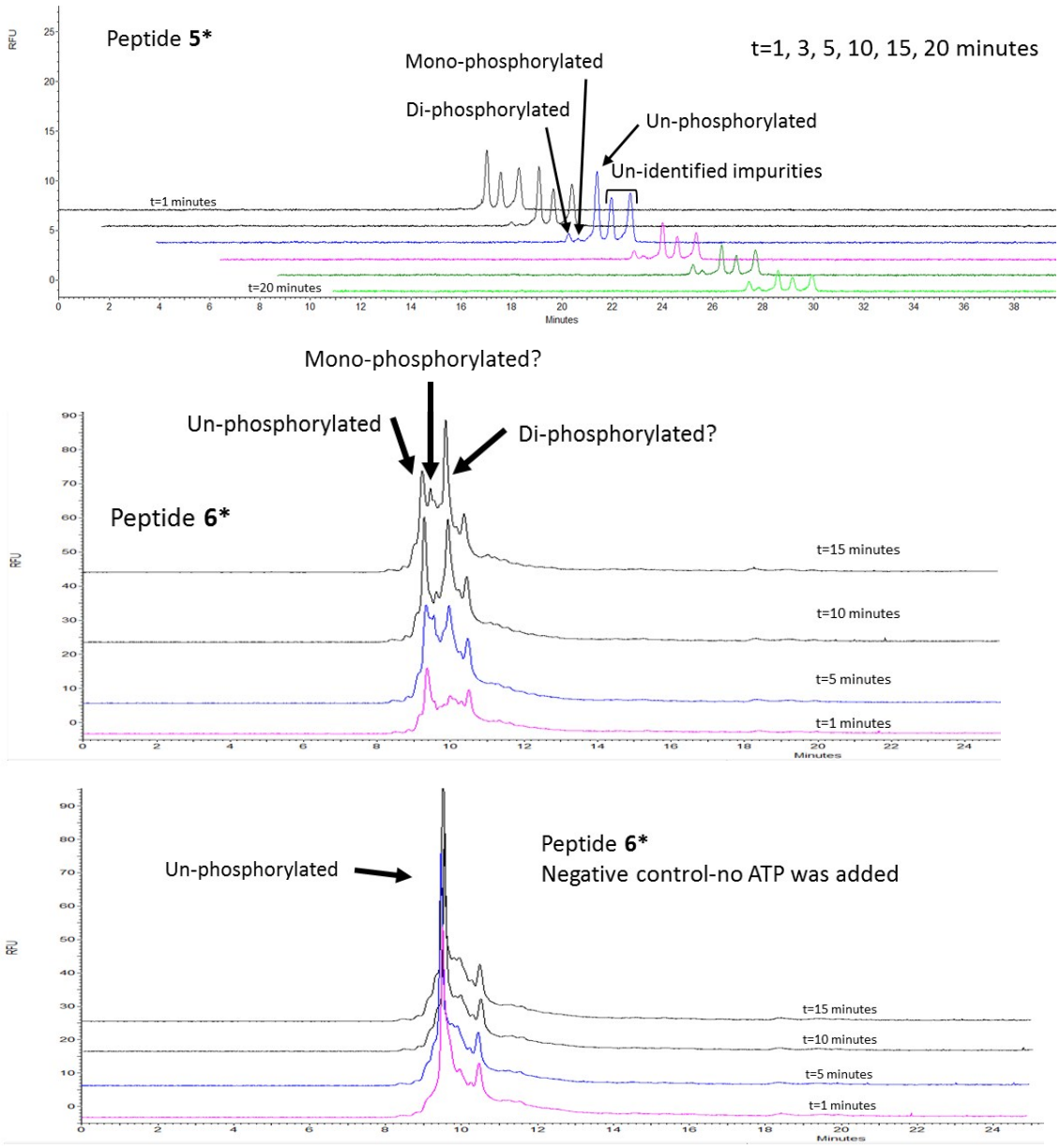
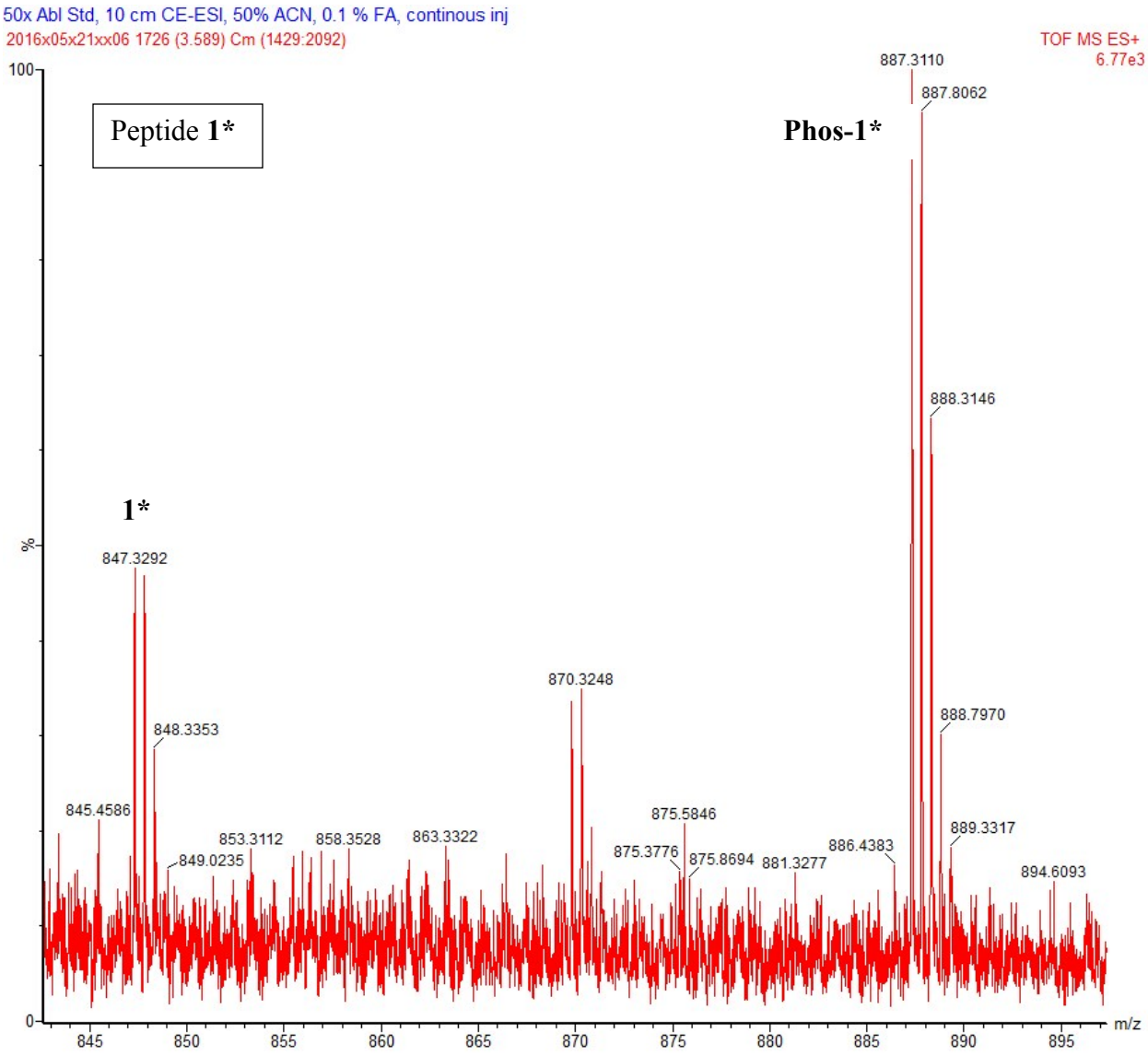


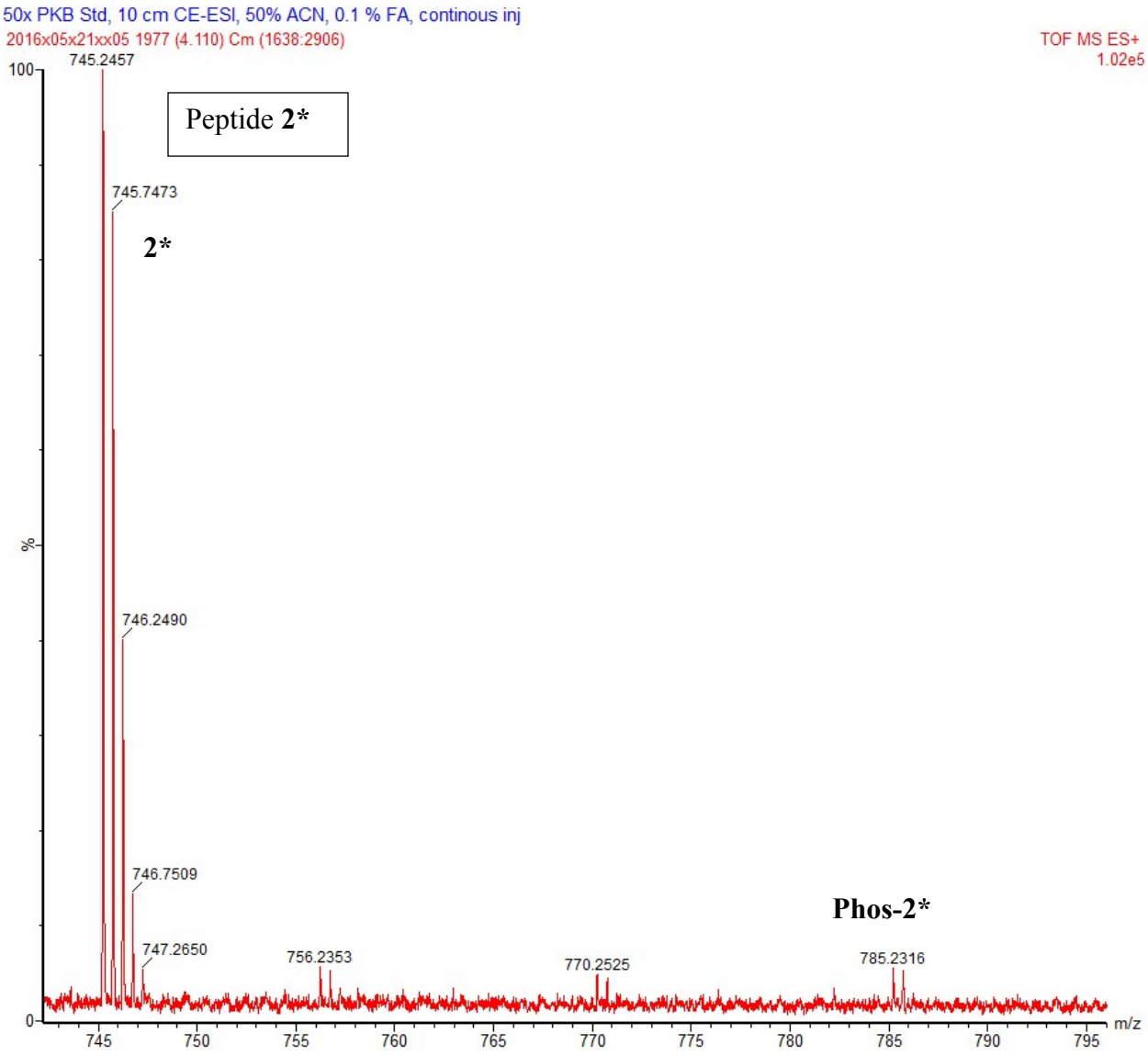
Figure S8. *Electropherograms following in vitro phosphorylation of peptides by their respective kinase. The identification of the un-phosphorylated peptide **5*** was based on it being the only peak with changes in integration over time coupled with evidence of phosphorylation by HR ESI-MS (see figure S10). Full separation was not achieved with peptide **6***. New peaks were seen growing (compared to the negative control) and HR ESI-MS evidence confirms phosphorylation was taking place (see figure S10).*

Table S5. *HR Masses of peptides **1*-3*** and their phosphorylated forms.*

Peptide #	***Sequence***	***Expected mass***	***Observed mass***

1*	**FAM-EAIYAAPFAKKK-NH_2**	1693.92	$M+2H^+$ 847.3292
Phos-1*	**FAM-EAIY(PO_3H_2)AAPFAKKK-NH_2**	1773.90	$M+2H^+$ 887.3110
2*	**FAM-GRPRAATFAEG-NH_2**	1488.64	$M+2H^+$ 745.2457
Phos-2*	**FAM-GRPRAAT(PO_3H_2)FAEG-NH_2**	1569.55	$M+2H^+$ 785.2316
3*	**FAM-QKRPSQRSKYL-NH_2**	1747.94	$M+4H^+$ 437.9072
Phos-3*	**FAM-QKRPS(PO_3H_2)QRSKYL-NH_2**	1827.91	$M+4H^+$ 477.8910


50x Abl Std, 10 cm CE-ESI, 50% ACN, 0.1 % FA, continous inj
2016x05x21xx06 1726 (3.589) Cm (1429:2092)
TOF MS ES+
6.77e3
Peptide 1*
Phos-1*
1*
887.3110
887.8062
888.3146
847.3292
848.3353
870.3248
888.7970
845.4586
875.5846
889.3317
853.3112
858.3528
863.3322
849.0235
875.3776
875.8694
881.3277
886.4383
894.6093
100
%
0
845
850
855
860
865
870
875
880
885
890
895
m/z


50x PKB Std, 10 cm CE-ESI, 50% ACN, 0.1 % FA, continous inj
2016x05x21xx05 1977 (4.110) Cm (1638:2906)
TOF MS ES+
1.02e5
100
%
0
745.2457
Peptide 2*
745.7473
2*
746.2490
746.7509
747.2650
756.2353
770.2525
785.2316
Phos-2*
745
750
755
760
765
770
775
780
785
790
795
m/z

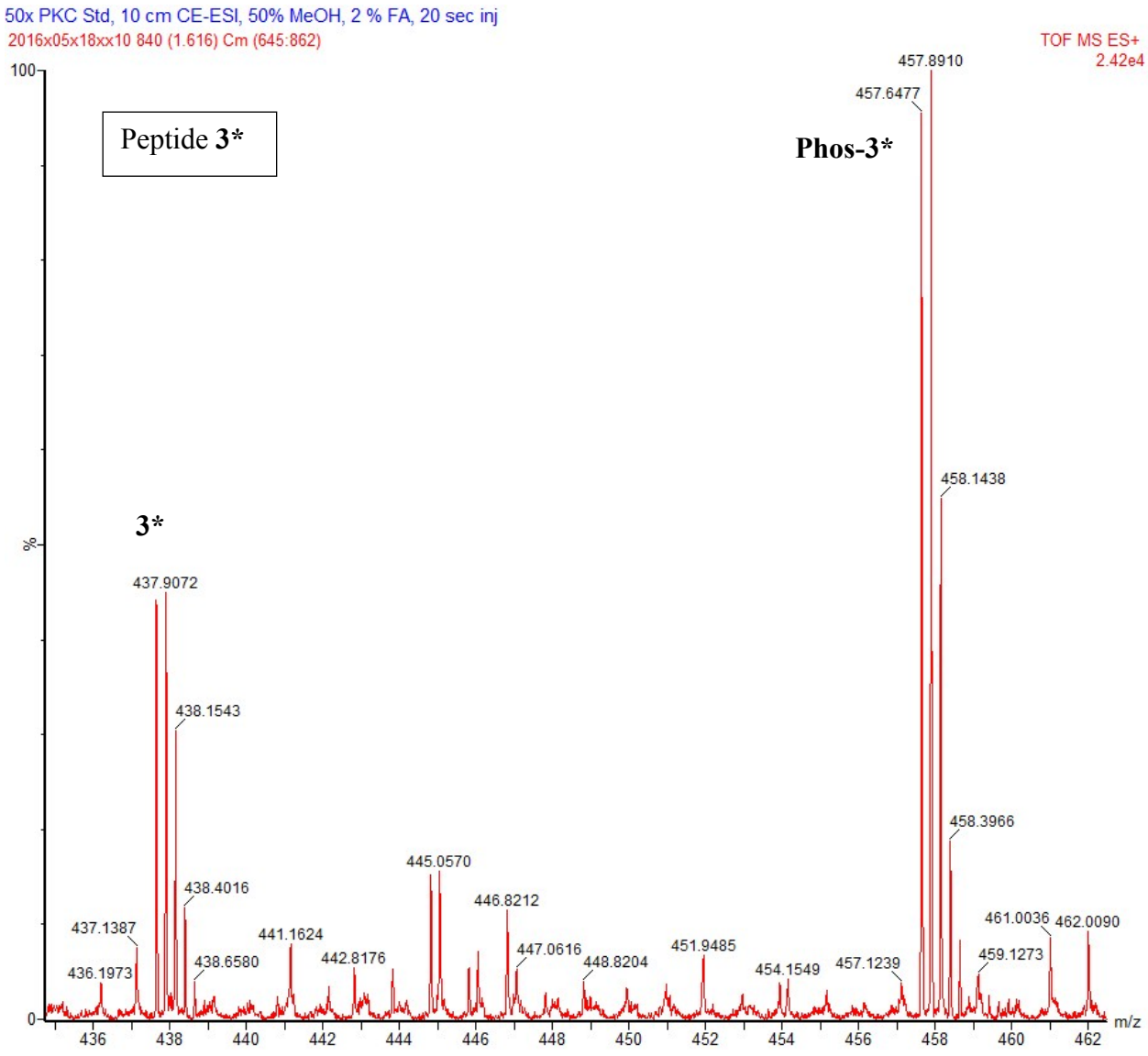
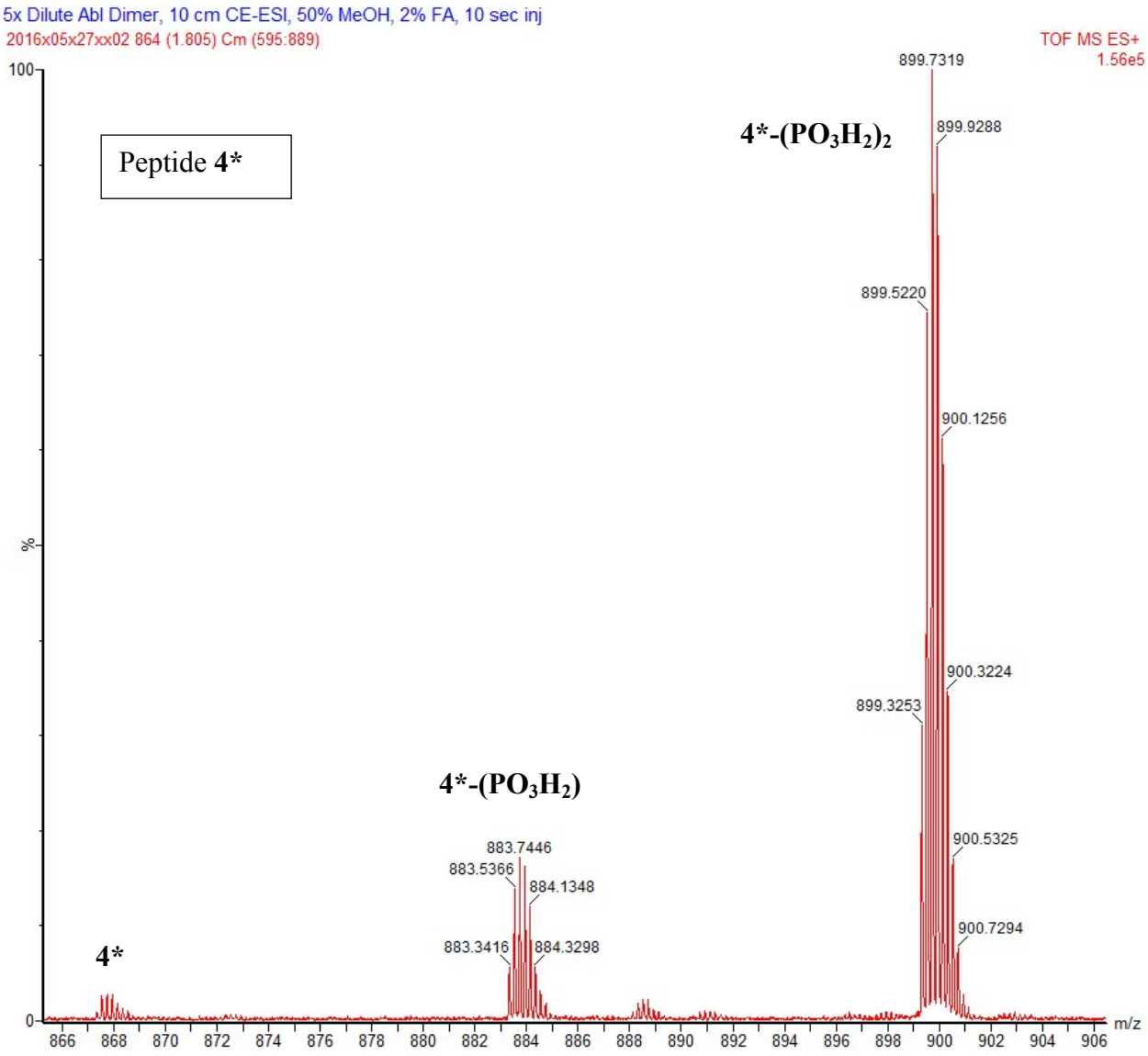
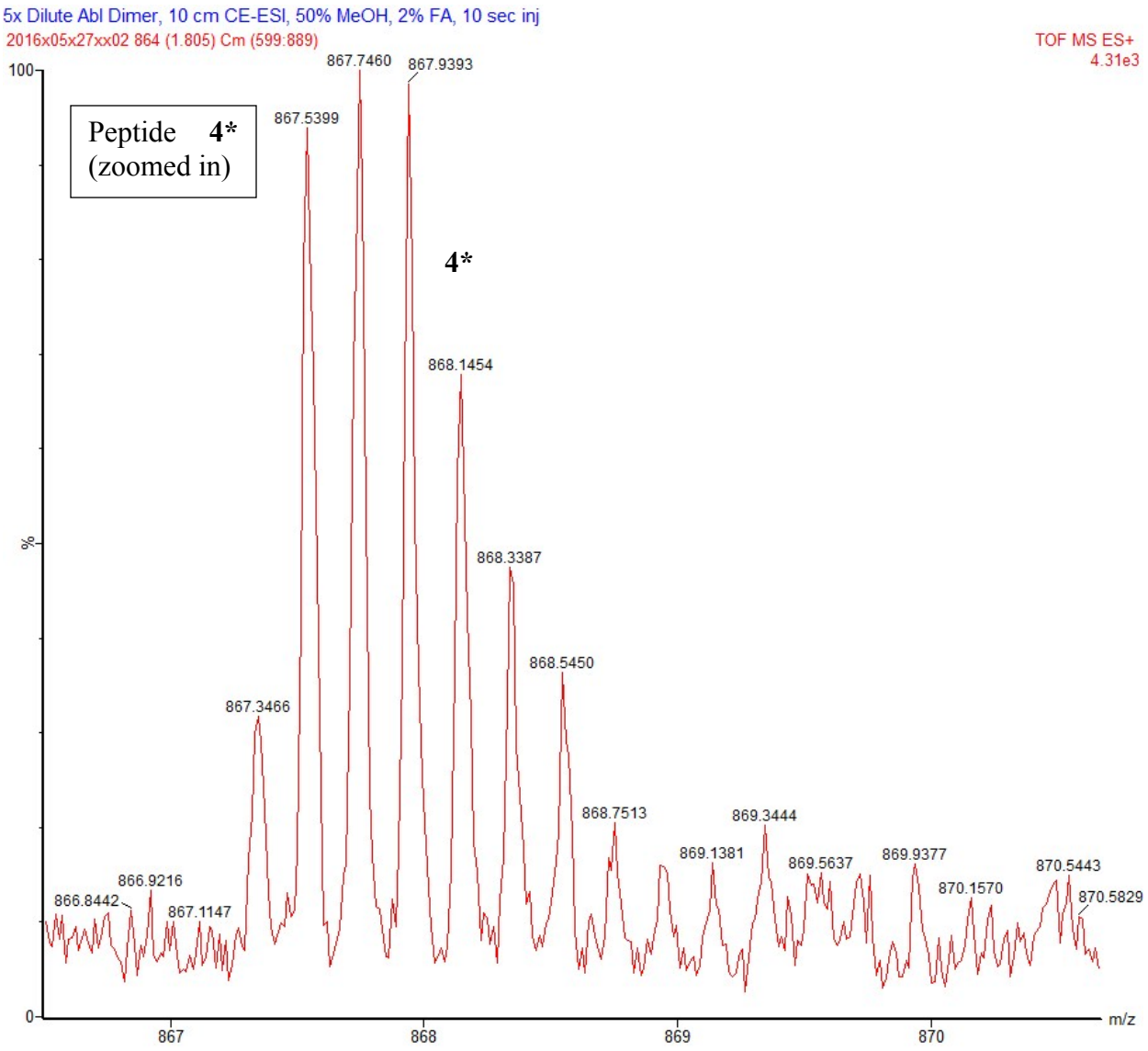


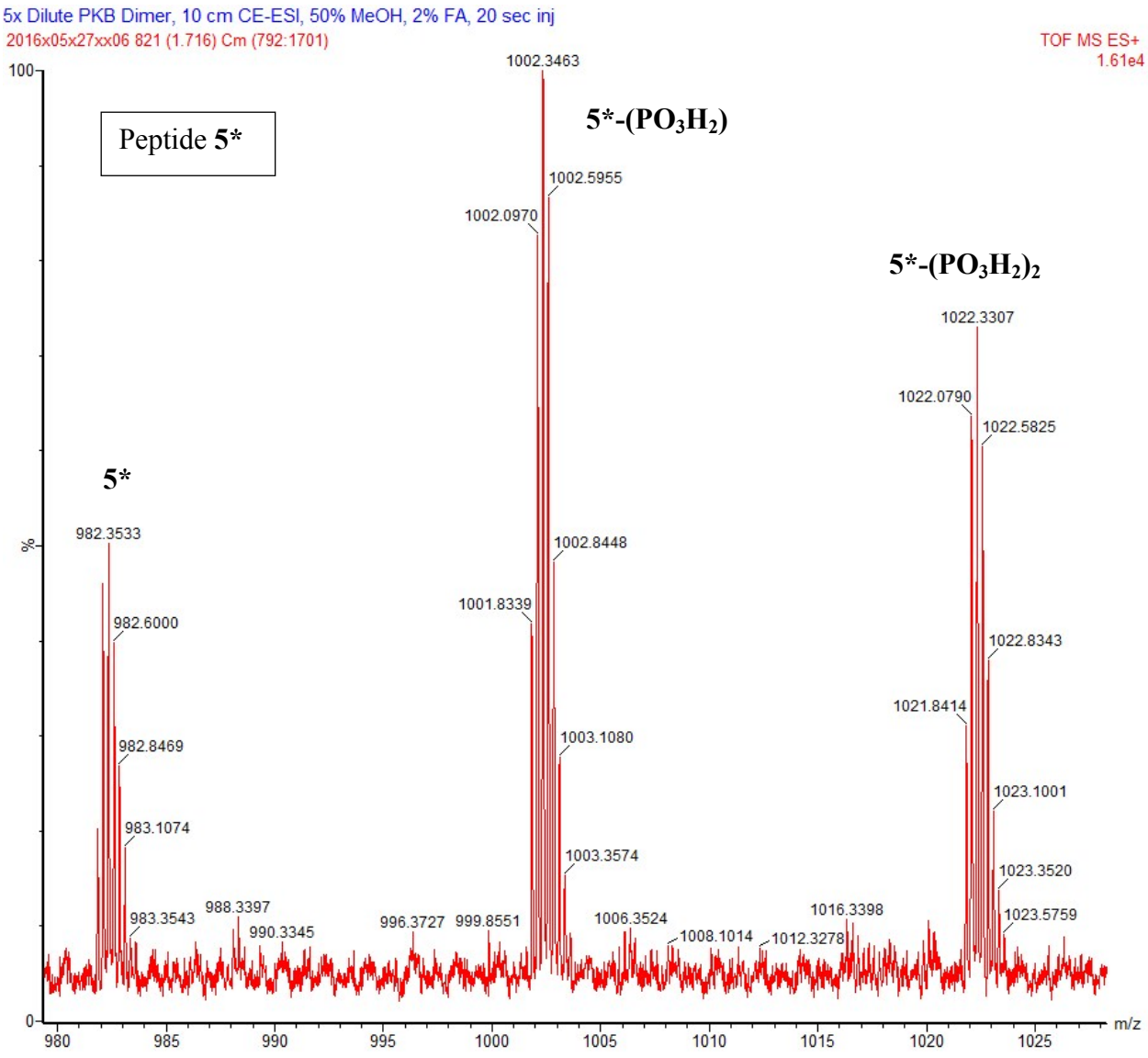
Figure S9. *HR ESI-MS of the 20 minute time point from in vitro phosphorylation assays of peptide standards **1*-3***.*

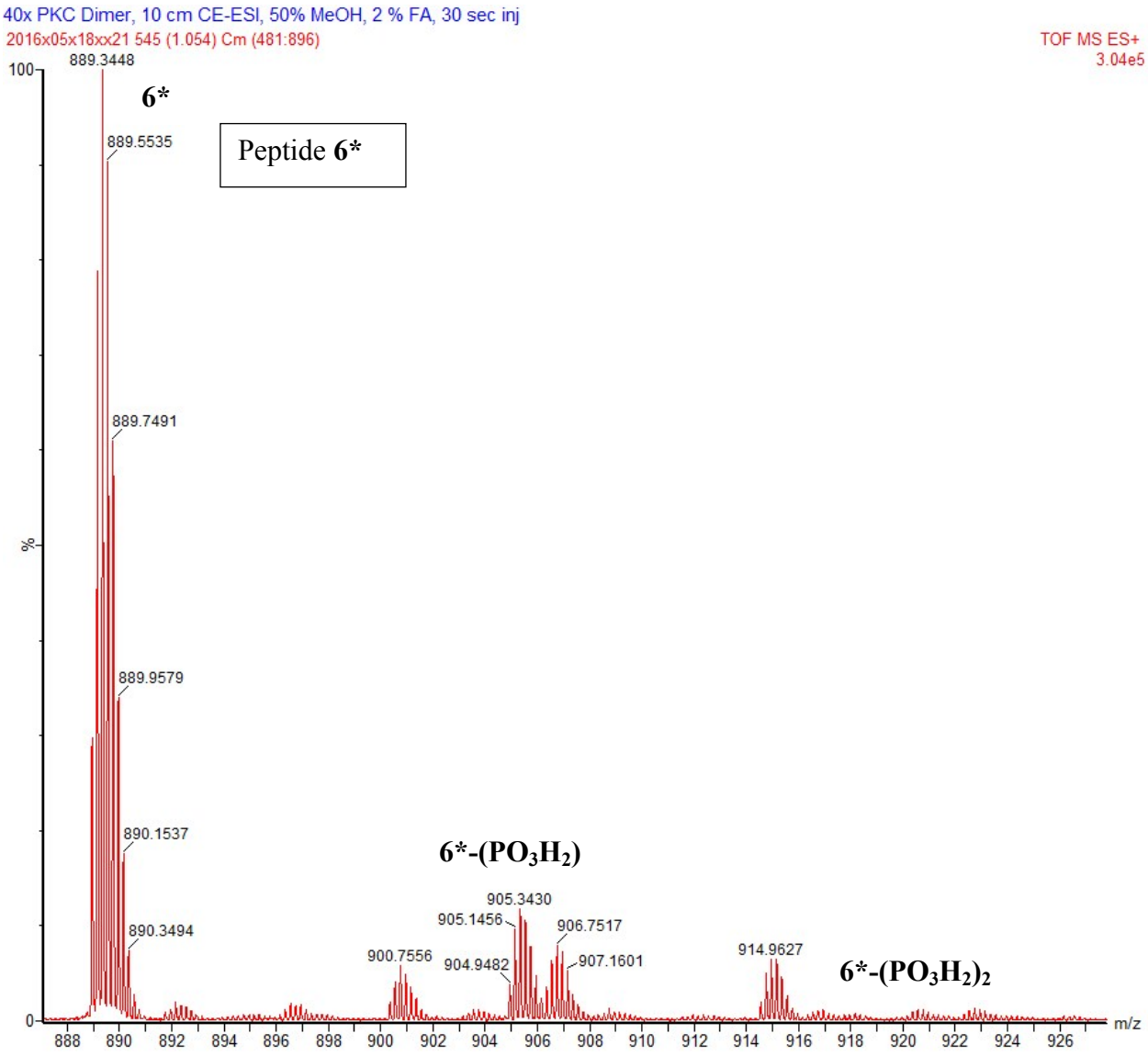
Table S6. *HR Masses of peptides* ***4*-6*** *and their mono- and di-phosphorylated forms.*

Peptide #	***Sequence***	***Expected mass***	***Observed mass***
4*	**Succinic(GK(FAM)-PEG_4-EAIYAAPFAKKK-NH_2)$_2$**	4334.94	$M+5H^+$ 867.7460
4*-(PO_3H_2)		4414.92	$M+5H^+$ 883.7446
4*-(PO_3H_2)$_2$		4494.90	$M+5H^+$ 899.7319
5*	**Succinic(GK(FAM)-PEG_4-GRPRAATFAEG-NH_2)$_2$**	3926.23	$M+4H^+$ 982.3533
5*-(PO_3H_2)		4006.21	$M+4H^+$ 1002.3463
5*-(PO_3H_2)$_2$		4086.19	$M+4H^+$ 1022.3307
6*	**Succinic(GK(FAM)-PEG_4-QKRPSQRSKYL-NH_2)$_2$**	4442.96	$M+5H^+$ 889.3448
6*-(PO_3H_2)		4522.94	$M+5H^+$ 905.3430
6*-(PO_3H_2)$_2$		4602.92	$M+5H^+$ 920.7670


5x Dilute Abl Dimer, 10 cm CE-ESI, 50% MeOH, 2% FA, 10 sec inj
2016x05x27xx02 864 (1.805) Cm (595:889)
TOF MS ES+
1.56e5
Peptide 4*
4*-(PO3H2)2
4*-(PO3H2)
4*
899.7319
899.9288
899.5220
900.1256
900.3224
899.3253
900.5325
900.7294
883.7446
883.5366
884.1348
883.3416
884.3298
100
%
0
m/z
866
868
870
872
874
876
878
880
882
884
886
888
890
892
894
896
898
900
902
904
906


5x Dilute Abl Dimer, 10 cm CE-ESI, 50% MeOH, 2% FA, 10 sec inj
2016x05x27xx02 864 (1.805) Cm (599:889)
TOF MS ES+
4.31e3
Peptide 4*
(zoomed in)
4*
100
%
0
867.7460
867.9393
867.5399
868.1454
868.3387
868.5450
867.3466
868.7513
869.3444
869.1381
869.5637
869.9377
870.1570
870.5443
870.5829
866.9216
866.8442
867.1147
867
868
869
870
m/z


5x Dilute PKB Dimer, 10 cm CE-ESI, 50% MeOH, 2% FA, 20 sec inj
2016x05x27xx06 821 (1.716) Cm (792:1701)
TOF MS ES+
1.61e4
Peptide 5*
5*
5*-(PO3H2)
5*-(PO3H2)2
982.3533
982.6000
982.8469
983.1074
983.3543
988.3397
990.3345
996.3727
999.8551
1001.8339
1002.0970
1002.3463
1002.5955
1002.8448
1003.1080
1003.3574
1006.3524
1008.1014
1012.3278
1016.3398
1021.8414
1022.0790
1022.3307
1022.5825
1022.8343
1023.1001
1023.3520
1023.5759
%
m/z
100
0
980
985
990
995
1000
1005
1010
1015
1020
1025


40x PKC Dimer, 10 cm CE-ESI, 50% MeOH, 2 % FA, 30 sec inj
2016x05x18xx21 545 (1.054) Cm (481:896)
TOF MS ES+
3.04e5
100
%
0
889.3448
6*
889.5535
Peptide 6*
889.7491
889.9579
890.1537
890.3494
6*-(PO3H2)
905.3430
905.1456
906.7517
900.7556
904.9482
907.1601
914.9627
6*-(PO3H2)2
m/z
888
890
892
894
896
898
900
902
904
906
908
910
912
914
916
918
920
922
924
926

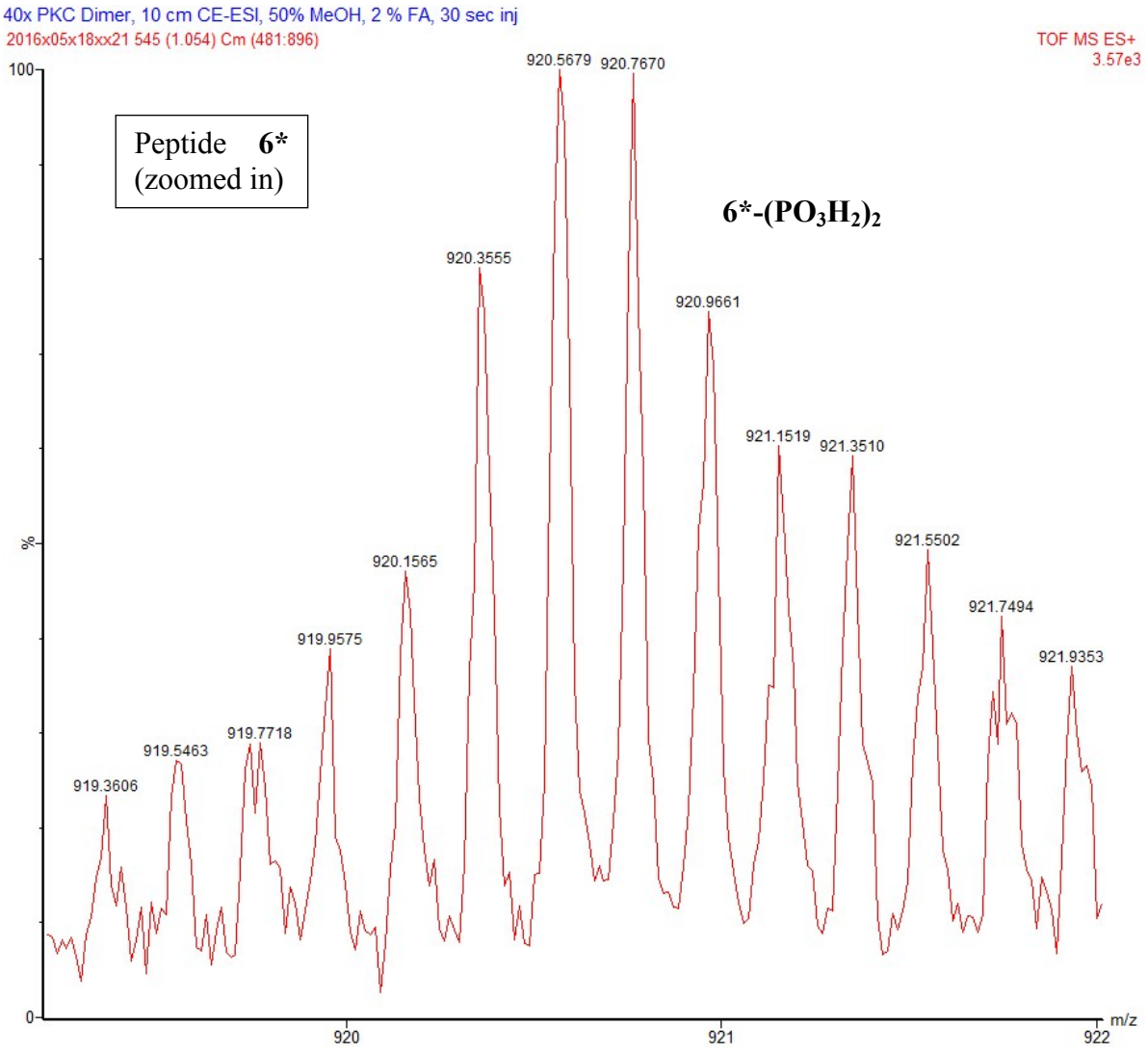
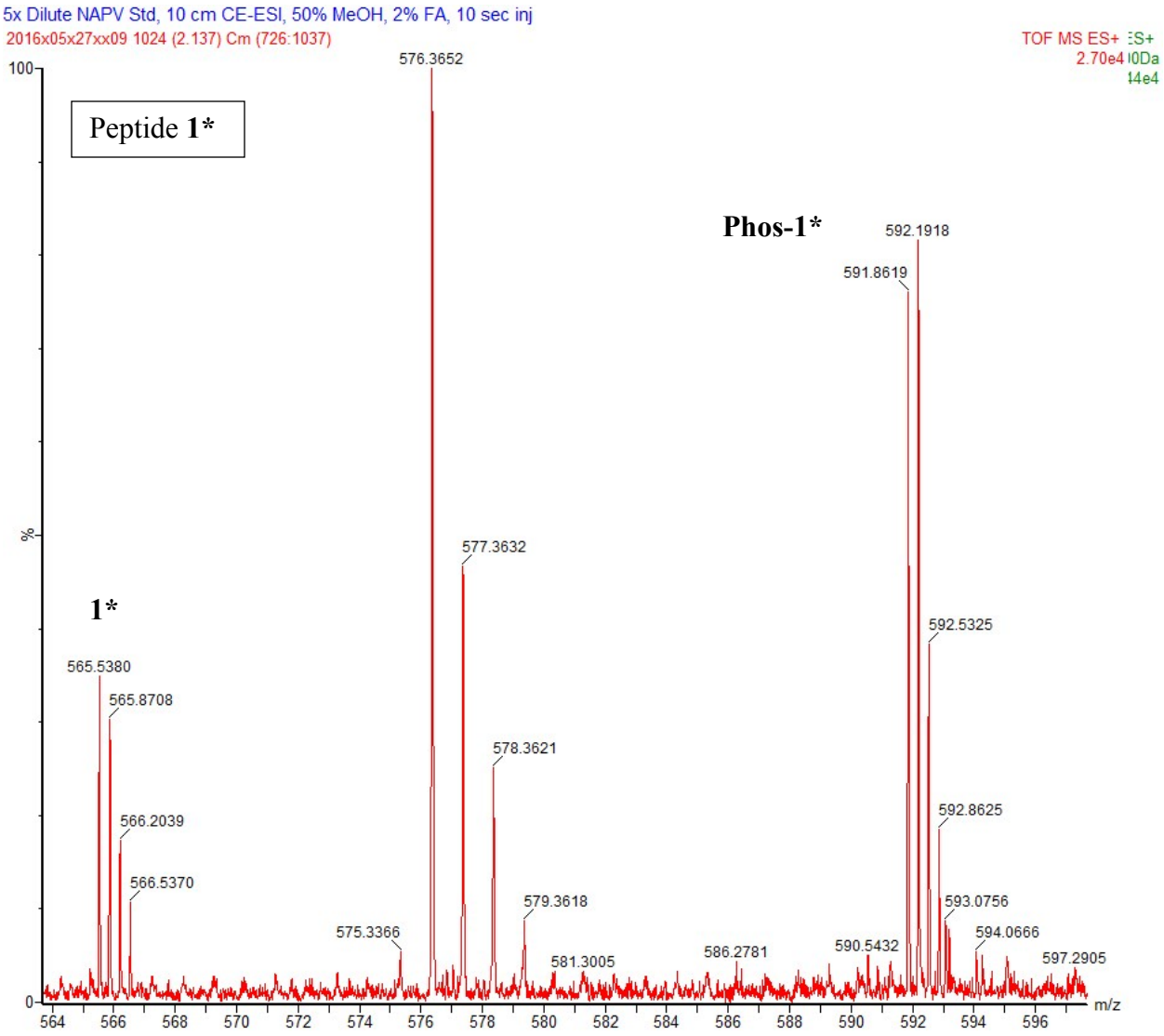


Figure S10. *HR ESI-MS spectra of the 20 minute time point from in vitro phosphorylation of peptide dimers* ***4*-6****.*

Table S7*. Masses of peptides* ***1**** *&* ***4**** *and their mono- and di-phosphorylated forms.*

Peptide #	***Sequence***	***Expected mass***	***Observed mass***
1*	**FAM-EAIYAAPFAKKK-NH_2**	1693.92	$M+3H^+$ 565.5380
Phos-1*	**FAM-EAIY(PO_3H_2)AAPFAKKK-NH_2**	1773.90	$M+3H^+$ 592.1918
4*	**Succinic(GK(FAM)-PEG_4-EAIYAAPFAKKK-NH_2)$_2$**	4334.94	$M+5H^+$ 867.7460
4*-(PO_3H_2)		4414.92	$M+5H^+$ 883.9397
4*-(PO_3H_2)$_2$		4494.90	$M+5H^+$ 899.7319


5x Dilute NAPV Std, 10 cm CE-ESI, 50% MeOH, 2% FA, 10 sec inj
2016x05x27xx09 1024 (2.137) Cm (726:1037)
TOF MS ES+
Peptide 1*
Phos-1*
1*
576.3652
592.1918
591.8619
577.3632
592.5325
565.5380
565.8708
578.3621
592.8625
566.2039
566.5370
579.3618
593.0756
575.3366
594.0666
581.3005
586.2781
590.5432
597.2905
100
%
0
m/z
564
566
568
570
572
574
576
578
580
582
584
586
588
590
592
594
596

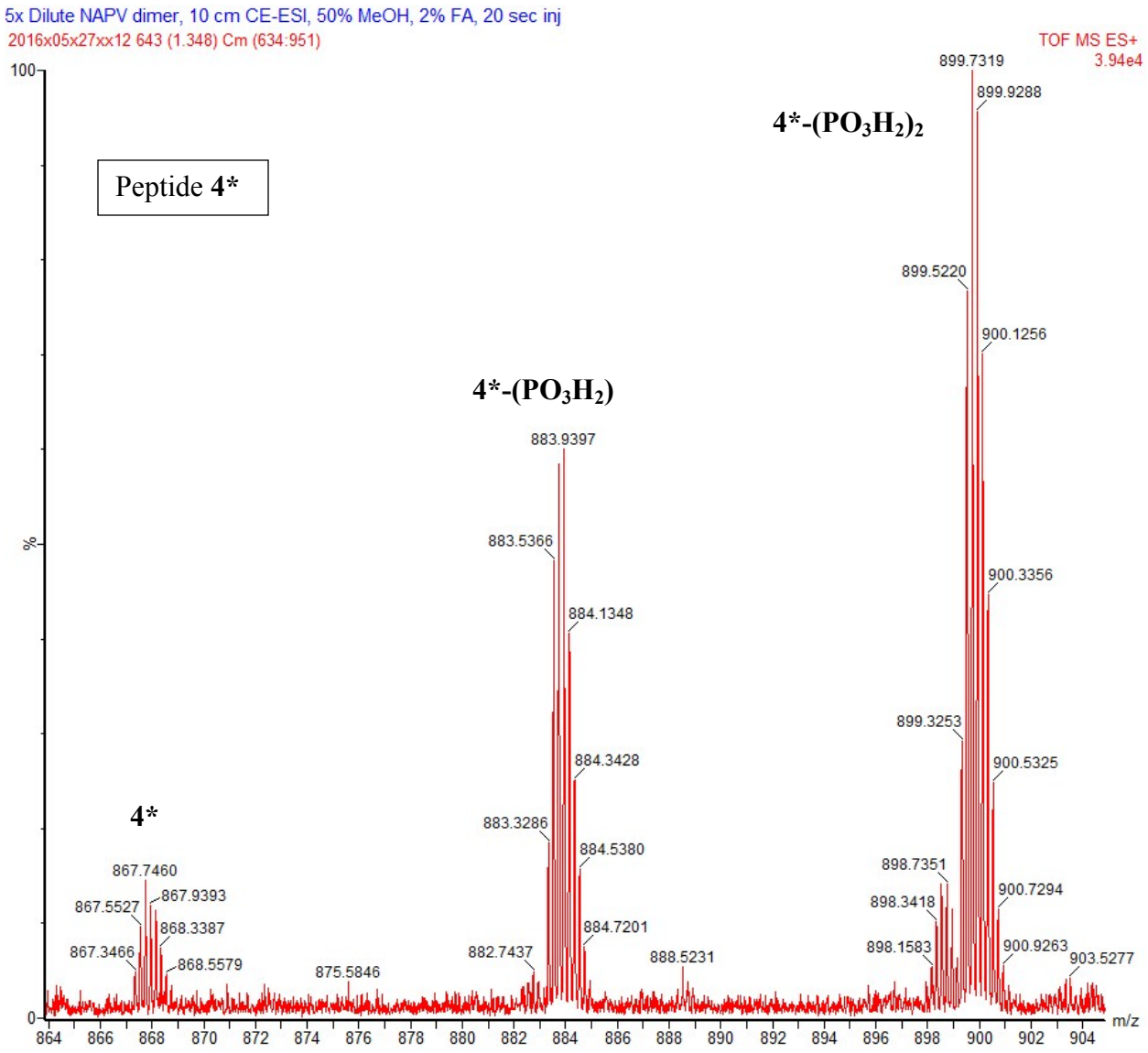


Figure S11. *HR ESI-MS spectra of the 2 hours time point from lysate phosphorylation assays of peptides **1*** & **4***.*