# The N-terminal loop of IRAK-4 death domain regulates ordered assembly of the Myddosome signalling scaffold

**Anthony C.G. Dossang[*, †, 1], Precious G. Motshwene[*, ‡, 1], Yang Yang*[1], Martyn F. Symmons*, Clare E. Bryant[§], Satty Borman[†], Julie George[||], Alexander N. R. Weber[¶] and Nicholas J. Gay*[2]**

## SUPPLEMENTARY METHODS - PHOSPHOSITE MAPPING.

Phosphosite mapping analysis was then carried out on the isolated IRAK-4 death domain. The band corresponding to IRAK-4 DD extracted from the reducing SDS-PAGE gel was digested using Trypsin, Chymotrypsin and AspN endoproteinases. The analysis was carried out using MALDI-TOF MS on an Ultraflex mass spectrometer and by LC-ESI MS on an Agilent HPLC coupled to a Bruker Amazon ion-trap and an Orbitrap mass spectrophotometer where appropriate. One hundred and twenty nine peptides were identified by LC/MS-MS and 44 peptides were identified by MALDI-TOF MS covering 100 % of the IRAK-4 DD (-7) to 107 (Supplementary data tables 1 to 6). The stoichiometry of both species was calculated from the relevant ion intensities of corresponding ion chromatograms. The stoichiometry of the singly phosphorylated form was determined to be equal at 53 % with an error as high as ± 10 % (as it depends on the relative ionisation efficiency of each form; phosphorylated and non-phosphorylated). In total 16 peptide string masses identified confirmed single phosphorylation of Serine 8 in IRAK-4 DD (Supplementary data tables 1 to 6). Phosphorylation at Serine 8 was further confirmed by the fragmentation of the peptide GAMDPEFMNKPITPSTYVR by MS/MS as shown in supplementary tables 7 and 8.

**Table 1:** a) List 1 of 2 of peptides identified by LC / MS – MS after digestion of IRAK-4 DD using Chymotrypsin. b) List 2 of 2 of peptides identified by LC / MS – MS after digestion of IRAK-4 DD (Section 3.2.4) using Chymotrypsin.

(a)

| Acc. # | Protein ID | Pep. # | % Cov. | Score |
|---|---|---|---|---|
| 9947 | IRAK4 DEATH DOMAIN | 57 | 94% | *1182* |

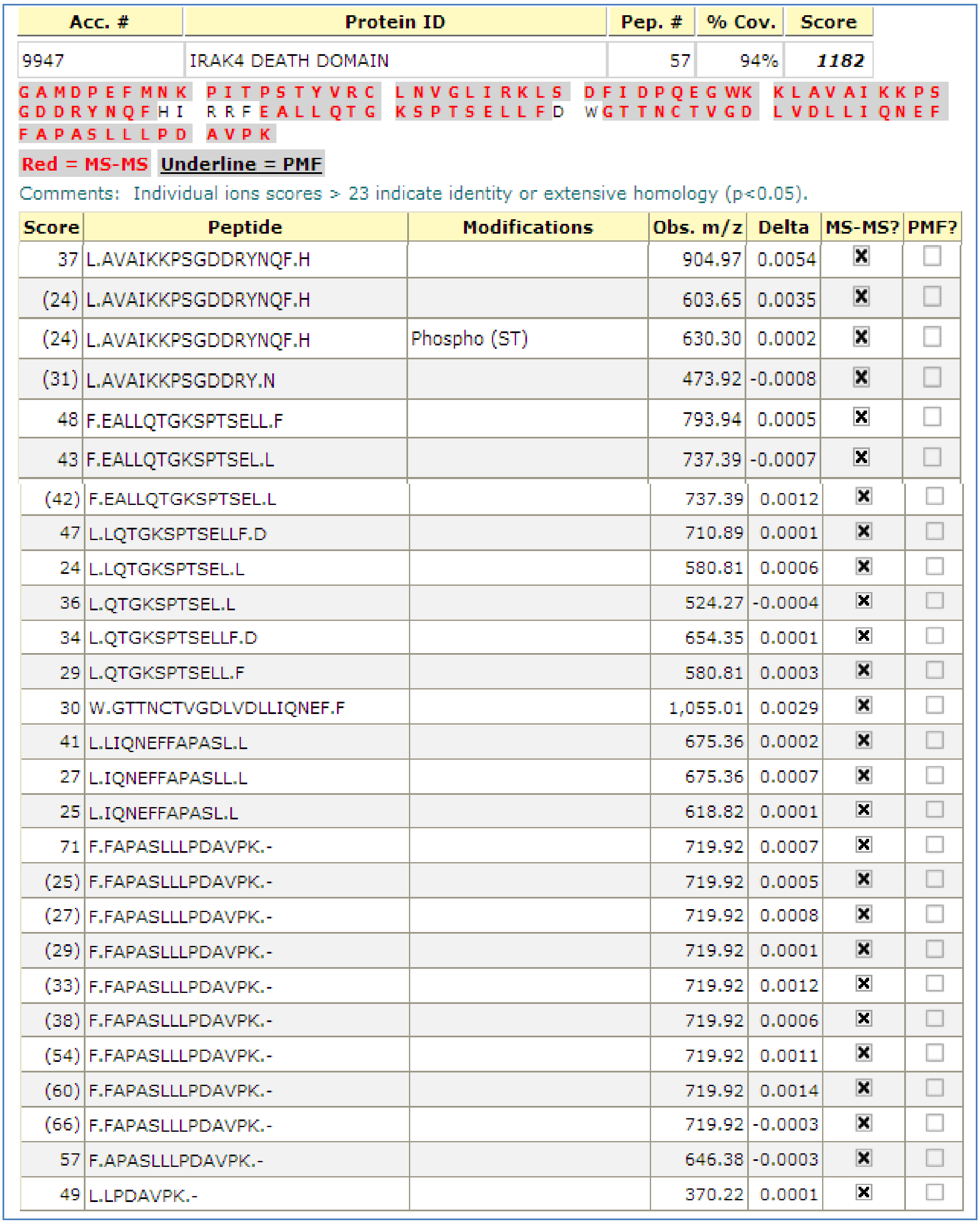
GAMDPEFMNK PITPSTYVRC LNVGLIRKLS DFIDPQEGWK KLAVAIKKPS
GDDRYNQFHI RRFEALLQTG KSPTSELLFD WGTTNCTVGD LVDLLIQNEF
FAPASLLLPD AVPK

Red = MS-MS **Underline = PMF**

Comments: Individual ions scores > 23 indicate identity or extensive homology (p<0.05).

| Score | Peptide | Modifications | Obs. m/z | Delta | MS-MS? | PMF? |
|---|---|---|---|---|---|---|
| 66 | -.GAMDPEFMNKPITPSTYVRCL.N | Phospho (Y); 2 Oxidation (M) | 847.04 | 0.0022 | ☒ | ☐ |
| 40 | -.GAMDPEFMNKPITPSTY.V | 2 Oxidation (M) | 965.93 | 0.0031 | ☒ | ☐ |
| 33 | -.GAMDPEFMNKPITPSTYVRCLNVGL.I | Phospho (ST); 2 Oxidation (M) | 974.78 | 0.0029 | ☒ | ☐ |
| (23) | -.GAMDPEFMNKPITPSTY.V | Oxidation (M) | 957.94 | 0.0031 | ☒ | ☐ |
| (30) | -.GAMDPEFMNKPITPSTY.V | 2 Oxidation (M) | 965.93 | 0.0000 | ☒ | ☐ |
| (30) | -.GAMDPEFMNKPITPSTYVRCL.N | Phospho (ST); Oxidation (M) | 1,262.06 | 0.0040 | ☒ | ☐ |
| (32) | -.GAMDPEFMNKPITPSTY.V | Phospho (Y); 2 Oxidation (M) | 1,005.92 | 0.0006 | ☒ | ☐ |
| (34) | -.GAMDPEFMNKPITPSTY.V | Oxidation (M) | 957.93 | -0.0002 | ☒ | ☐ |
| (35) | -.GAMDPEFMNKPITPSTY.V |  | 949.94 | 0.0001 | ☒ | ☐ |
| (49) | -.GAMDPEFMNKPITPSTYVRCL.N | Phospho (ST); Oxidation (M) | 841.71 | 0.0019 | ☒ | ☐ |
| (56) | -.GAMDPEFMNKPITPSTYVRCL.N | Phospho (ST) | 836.38 | 0.0029 | ☒ | ☐ |
| 56 | F.MNKPITPSTY.V | Oxidation (M) | 584.29 | 0.0002 | ☒ | ☐ |
| (32) | F.MNKPITPSTY.V | Phospho (ST); Oxidation (M) | 624.27 | 0.0012 | ☒ | ☐ |
| (50) | F.MNKPITPSTY.V |  | 576.29 | 0.0030 | ☒ | ☐ |
| 42 | Y.VRCLNVGL.I |  | 465.76 | -0.0006 | ☒ | ☐ |
| 46 | L.NVGLIRKLSDFIDPQEGW.K |  | 696.37 | 0.0009 | ☒ | ☐ |
| 43 | L.NVGLIRKLSDF.I |  | 631.37 | 0.0004 | ☒ | ☐ |
| 30 | L.NVGLIRKL.S |  | 456.80 | -0.0005 | ☒ | ☐ |
| (30) | L.NVGLIRKLSDFIDPQEGW.K |  | 696.37 | 0.0016 | ☒ | ☐ |
| (32) | L.NVGLIRKLSDFIDPQEGW.K |  | 696.37 | 0.0000 | ☒ | ☐ |
| (34) | L.NVGLIRKLSDFIDPQEGW.K |  | 696.37 | -0.0011 | ☒ | ☐ |
| 34 | L.IRKLSDFIDPQEGW.K |  | 568.63 | -0.0002 | ☒ | ☐ |
| 28 | L.IRKLSDFIDPQEGWKKL.A |  | 519.04 | -0.0017 | ☒ | ☐ |
| (23) | L.IRKLSDFIDPQEGW.K |  | 852.44 | -0.0006 | ☒ | ☐ |
| 35 | L.SDFIDPQEGWKKL.A |  | 781.90 | 0.0000 | ☒ | ☐ |
| 31 | L.SDFIDPQEGW.K |  | 597.26 | -0.0010 | ☒ | ☐ |
| (27) | L.SDFIDPQEGWKKL.A |  | 521.60 | -0.0012 | ☒ | ☐ |
| 33 | W.KKLAVAIKKPSGDDRY.N |  | 448.01 | -0.0005 | ☒ | ☐ |
| (26) | W.KKLAVAIKKPSGDDRY.N |  | 448.01 | 0.0001 | ☒ | ☐ |
| 67 | L.AVAIKKPSGDDRY.N |  | 710.38 | 0.0005 | ☒ | ☐ |
| 37 | L.AVAIKKPSGDDRYNQF.H |  | 904.97 | 0.0054 | ☒ | ☐ |

(b)



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FAPASLLLPD AVPK

**Red = MS-MS** **Underline = PMF**

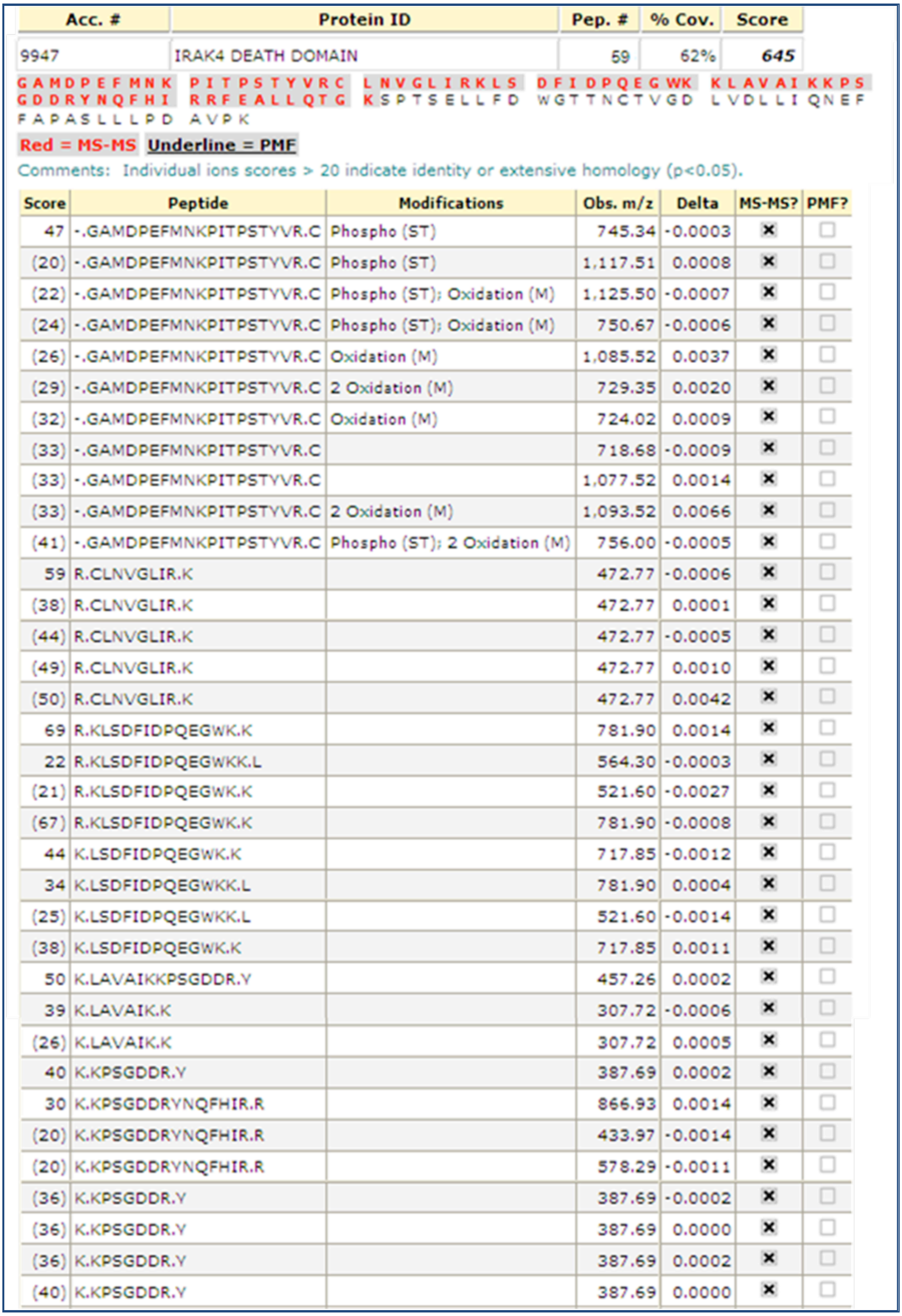
Comments: Individual ions scores > 23 indicate identity or extensive homology (p<0.05).

| Score | Peptide | Modifications | Obs. m/z | Delta | MS-MS? | PMF? |
|---|---|---|---|---|---|---|
| 37 | L.AVAIKKPSGDDRYNQF.H |  | 904.97 | 0.0054 | ☒ | ☐ |
| (24) | L.AVAIKKPSGDDRYNQF.H |  | 603.65 | 0.0035 | ☒ | ☐ |
| (24) | L.AVAIKKPSGDDRYNQF.H | Phospho (ST) | 630.30 | 0.0002 | ☒ | ☐ |
| (31) | L.AVAIKKPSGDDRY.N |  | 473.92 | -0.0008 | ☒ | ☐ |
| 48 | F.EALLQTGKSPTSELL.F |  | 793.94 | 0.0005 | ☒ | ☐ |
| 43 | F.EALLQTGKSPTSEL.L |  | 737.39 | -0.0007 | ☒ | ☐ |
| (42) | F.EALLQTGKSPTSEL.L |  | 737.39 | 0.0012 | ☒ | ☐ |
| 47 | L.LQTGKSPTSELLF.D |  | 710.89 | 0.0001 | ☒ | ☐ |
| 24 | L.LQTGKSPTSEL.L |  | 580.81 | 0.0006 | ☒ | ☐ |
| 36 | L.QTGKSPTSEL.L |  | 524.27 | -0.0004 | ☒ | ☐ |
| 34 | L.QTGKSPTSELLF.D |  | 654.35 | 0.0001 | ☒ | ☐ |
| 29 | L.QTGKSPTSELL.F |  | 580.81 | 0.0003 | ☒ | ☐ |
| 30 | W.GTTNCTVGDLVDLLIQNEF.F |  | 1,055.01 | 0.0029 | ☒ | ☐ |
| 41 | L.LIQNEFFAPASL.L |  | 675.36 | 0.0002 | ☒ | ☐ |
| 27 | L.IQNEFFAPASLL.L |  | 675.36 | 0.0007 | ☒ | ☐ |
| 25 | L.IQNEFFAPASL.L |  | 618.82 | 0.0001 | ☒ | ☐ |
| 71 | F.FAPASLLLPDAVPK.- |  | 719.92 | 0.0007 | ☒ | ☐ |
| (25) | F.FAPASLLLPDAVPK.- |  | 719.92 | 0.0005 | ☒ | ☐ |
| (27) | F.FAPASLLLPDAVPK.- |  | 719.92 | 0.0008 | ☒ | ☐ |
| (29) | F.FAPASLLLPDAVPK.- |  | 719.92 | 0.0001 | ☒ | ☐ |
| (33) | F.FAPASLLLPDAVPK.- |  | 719.92 | 0.0012 | ☒ | ☐ |
| (38) | F.FAPASLLLPDAVPK.- |  | 719.92 | 0.0006 | ☒ | ☐ |
| (54) | F.FAPASLLLPDAVPK.- |  | 719.92 | 0.0011 | ☒ | ☐ |
| (60) | F.FAPASLLLPDAVPK.- |  | 719.92 | 0.0014 | ☒ | ☐ |
| (66) | F.FAPASLLLPDAVPK.- |  | 719.92 | -0.0003 | ☒ | ☐ |
| 57 | F.APASLLLPDAVPK.- |  | 646.38 | -0.0003 | ☒ | ☐ |
| 49 | L.LPDAVPK.- |  | 370.22 | 0.0001 | ☒ | ☐ |

**Table 2:** a) List 1 of 2 of peptides identified by LC / MS – MS after digestion of IRAK-4 using Trypsin.

b) Part 2 of 2 of peptides identified by LC / MS – MS after digestion of IRAK-4 DD using Trypsin.

(a)



| Acc. # | Protein ID | Pep. # | % Cov. | Score |
|---|---|---|---|---|
| 9947 | IRAK4 DEATH DOMAIN | 59 | 62% | ***645*** |

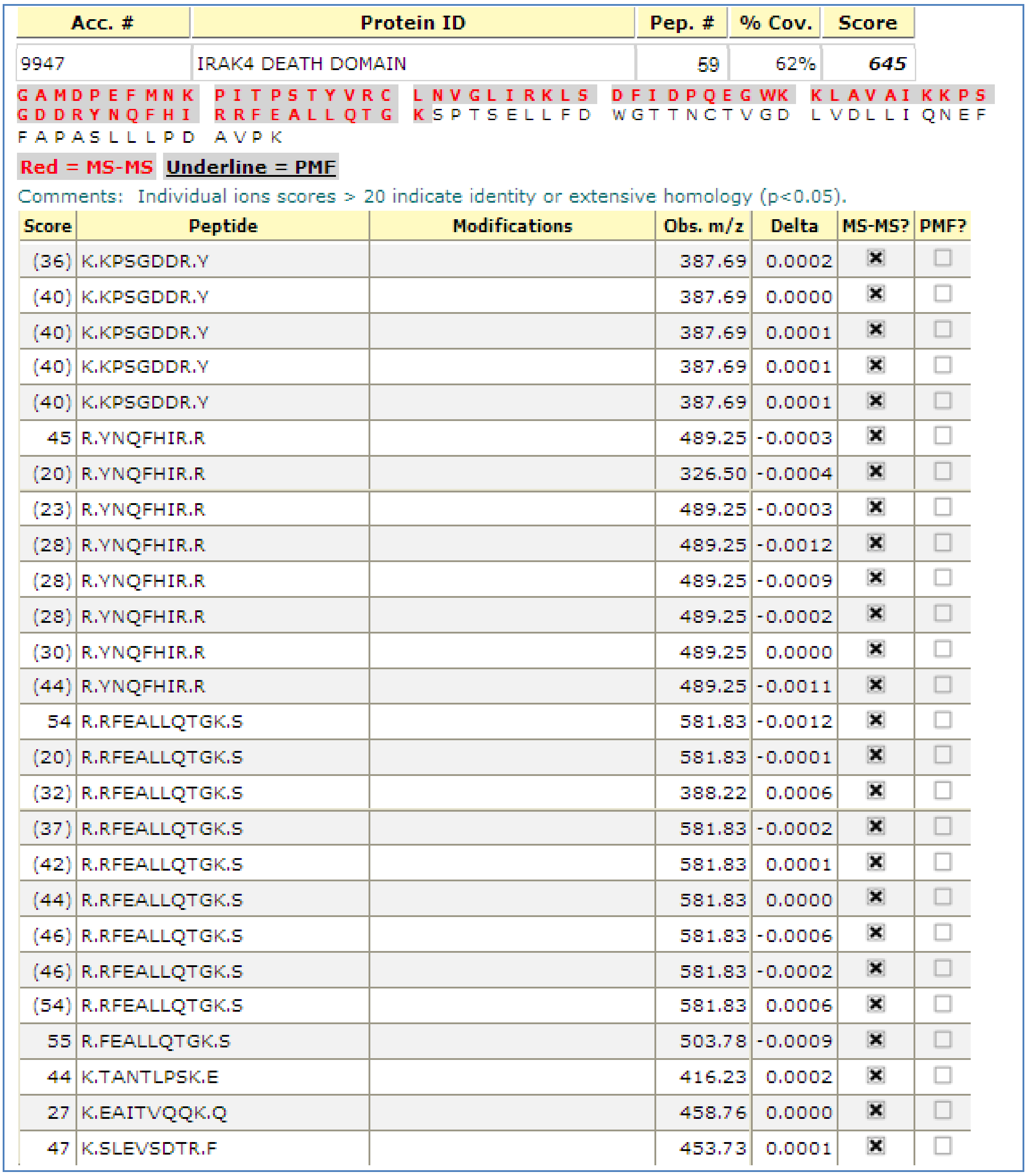
GAMDPEFMNK PITPSTYVRC LNVGLIRKLS DFIDPQEGWK KLAVAIKKPS
GDDRYNQFHI RRFEALLQTG KSPTSELLFD WGTTNCTVGD LVDLLIQNEF
FAPASLLLPD AVPK

**Red = MS-MS** **Underline = PMF**

Comments: Individual ions scores > 20 indicate identity or extensive homology (p<0.05).

| Score | Peptide | Modifications | Obs. m/z | Delta | MS-MS? | PMF? |
|---|---|---|---|---|---|---|
| 47 | -.GAMDPEFMNKPITPSTYVR.C | Phospho (ST) | 745.34 | -0.0003 | ☒ | ☐ |
| (20) | -.GAMDPEFMNKPITPSTYVR.C | Phospho (ST) | 1,117.51 | 0.0008 | ☒ | ☐ |
| (22) | -.GAMDPEFMNKPITPSTYVR.C | Phospho (ST); Oxidation (M) | 1,125.50 | -0.0007 | ☒ | ☐ |
| (24) | -.GAMDPEFMNKPITPSTYVR.C | Phospho (ST); Oxidation (M) | 750.67 | -0.0006 | ☒ | ☐ |
| (26) | -.GAMDPEFMNKPITPSTYVR.C | Oxidation (M) | 1,085.52 | 0.0037 | ☒ | ☐ |
| (29) | -.GAMDPEFMNKPITPSTYVR.C | 2 Oxidation (M) | 729.35 | 0.0020 | ☒ | ☐ |
| (32) | -.GAMDPEFMNKPITPSTYVR.C | Oxidation (M) | 724.02 | 0.0009 | ☒ | ☐ |
| (33) | -.GAMDPEFMNKPITPSTYVR.C |  | 718.68 | -0.0009 | ☒ | ☐ |
| (33) | -.GAMDPEFMNKPITPSTYVR.C |  | 1,077.52 | 0.0014 | ☒ | ☐ |
| (33) | -.GAMDPEFMNKPITPSTYVR.C | 2 Oxidation (M) | 1,093.52 | 0.0066 | ☒ | ☐ |
| (41) | -.GAMDPEFMNKPITPSTYVR.C | Phospho (ST); 2 Oxidation (M) | 756.00 | -0.0005 | ☒ | ☐ |
| 59 | R.CLNVGLIR.K |  | 472.77 | -0.0006 | ☒ | ☐ |
| (38) | R.CLNVGLIR.K |  | 472.77 | 0.0001 | ☒ | ☐ |
| (44) | R.CLNVGLIR.K |  | 472.77 | -0.0005 | ☒ | ☐ |
| (49) | R.CLNVGLIR.K |  | 472.77 | 0.0010 | ☒ | ☐ |
| (50) | R.CLNVGLIR.K |  | 472.77 | 0.0042 | ☒ | ☐ |
| 69 | R.KLSDFIDPQEGWK.K |  | 781.90 | 0.0014 | ☒ | ☐ |
| 22 | R.KLSDFIDPQEGWKK.L |  | 564.30 | -0.0003 | ☒ | ☐ |
| (21) | R.KLSDFIDPQEGWK.K |  | 521.60 | -0.0027 | ☒ | ☐ |
| (67) | R.KLSDFIDPQEGWK.K |  | 781.90 | -0.0008 | ☒ | ☐ |
| 44 | K.LSDFIDPQEGWK.K |  | 717.85 | -0.0012 | ☒ | ☐ |
| 34 | K.LSDFIDPQEGWKK.L |  | 781.90 | 0.0004 | ☒ | ☐ |
| (25) | K.LSDFIDPQEGWKK.L |  | 521.60 | -0.0014 | ☒ | ☐ |
| (38) | K.LSDFIDPQEGWK.K |  | 717.85 | 0.0011 | ☒ | ☐ |
| 50 | K.LAVAIKKPSGDDR.Y |  | 457.26 | 0.0002 | ☒ | ☐ |
| 39 | K.LAVAIK.K |  | 307.72 | -0.0006 | ☒ | ☐ |
| (26) | K.LAVAIK.K |  | 307.72 | 0.0005 | ☒ | ☐ |
| 40 | K.KPSGDDR.Y |  | 387.69 | 0.0002 | ☒ | ☐ |
| 30 | K.KPSGDDRYNQFHIR.R |  | 866.93 | 0.0014 | ☒ | ☐ |
| (20) | K.KPSGDDRYNQFHIR.R |  | 433.97 | -0.0014 | ☒ | ☐ |
| (20) | K.KPSGDDRYNQFHIR.R |  | 578.29 | -0.0011 | ☒ | ☐ |
| (36) | K.KPSGDDR.Y |  | 387.69 | -0.0002 | ☒ | ☐ |
| (36) | K.KPSGDDR.Y |  | 387.69 | 0.0000 | ☒ | ☐ |
| (36) | K.KPSGDDR.Y |  | 387.69 | 0.0002 | ☒ | ☐ |
| (40) | K.KPSGDDR.Y |  | 387.69 | 0.0000 | ☒ | ☐ |

(b)



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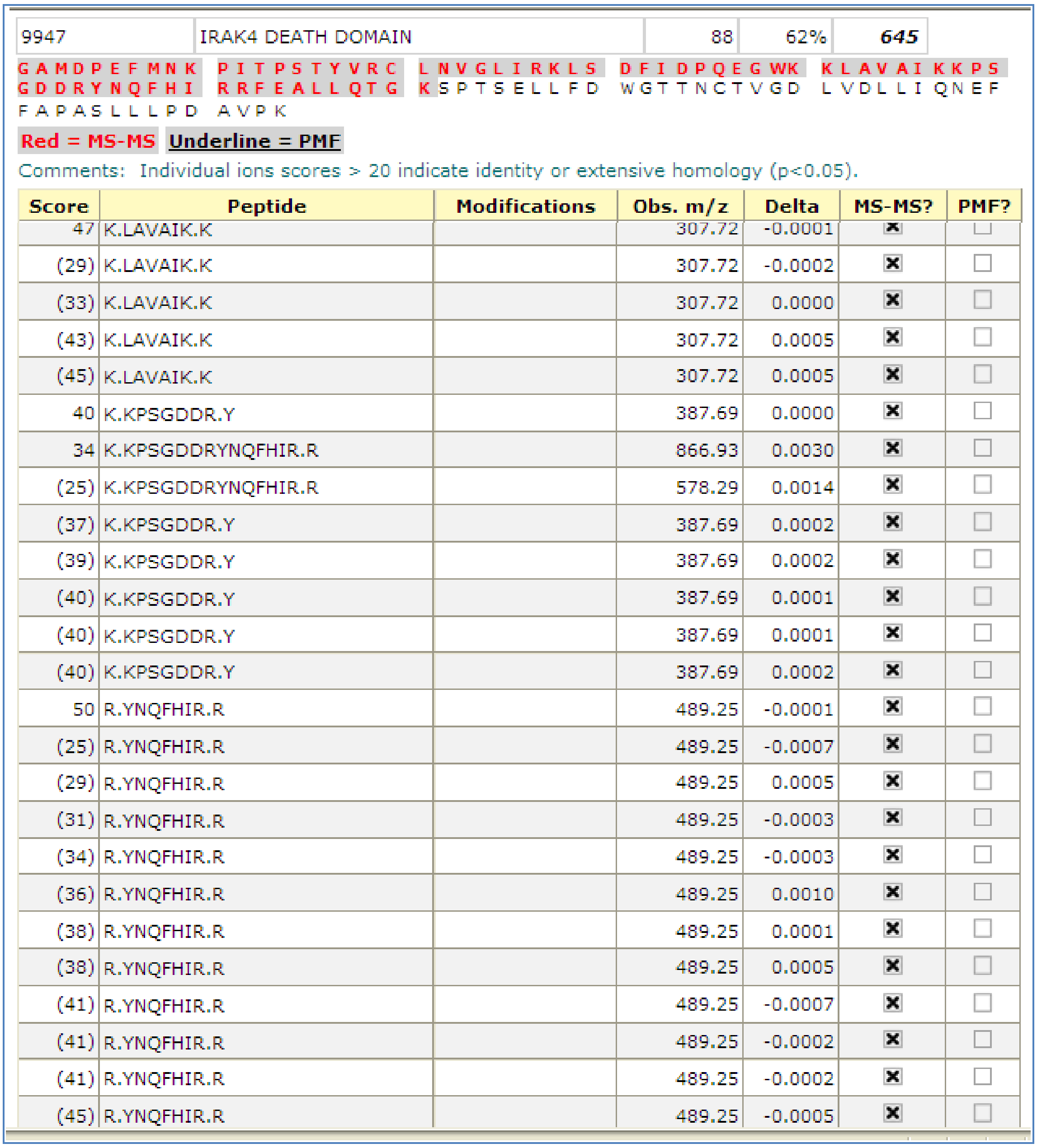
GDDRYNQFHI RRFEALLQTG KSPTSELLFD WGTTNCTVGD LVDLLIQNEF
FAPASLLLPD AVPK

**Red = MS-MS** **Underline = PMF**

Comments: Individual ions scores > 20 indicate identity or extensive homology (p<0.05).

| Score | Peptide | Modifications | Obs. m/z | Delta | MS-MS? | PMF? |
|---|---|---|---|---|---|---|
| (36) | K.KPSGDDR.Y | | 387.69 | 0.0002 | ☒ | ☐ |
| (40) | K.KPSGDDR.Y | | 387.69 | 0.0000 | ☒ | ☐ |
| (40) | K.KPSGDDR.Y | | 387.69 | 0.0001 | ☒ | ☐ |
| (40) | K.KPSGDDR.Y | | 387.69 | 0.0001 | ☒ | ☐ |
| (40) | K.KPSGDDR.Y | | 387.69 | 0.0001 | ☒ | ☐ |
| 45 | R.YNQFHIR.R | | 489.25 | -0.0003 | ☒ | ☐ |
| (20) | R.YNQFHIR.R | | 326.50 | -0.0004 | ☒ | ☐ |
| (23) | R.YNQFHIR.R | | 489.25 | -0.0003 | ☒ | ☐ |
| (28) | R.YNQFHIR.R | | 489.25 | -0.0012 | ☒ | ☐ |
| (28) | R.YNQFHIR.R | | 489.25 | -0.0009 | ☒ | ☐ |
| (28) | R.YNQFHIR.R | | 489.25 | -0.0002 | ☒ | ☐ |
| (30) | R.YNQFHIR.R | | 489.25 | 0.0000 | ☒ | ☐ |
| (44) | R.YNQFHIR.R | | 489.25 | -0.0011 | ☒ | ☐ |
| 54 | R.RFEALLQTGK.S | | 581.83 | -0.0012 | ☒ | ☐ |
| (20) | R.RFEALLQTGK.S | | 581.83 | -0.0001 | ☒ | ☐ |
| (32) | R.RFEALLQTGK.S | | 388.22 | 0.0006 | ☒ | ☐ |
| (37) | R.RFEALLQTGK.S | | 581.83 | -0.0002 | ☒ | ☐ |
| (42) | R.RFEALLQTGK.S | | 581.83 | 0.0001 | ☒ | ☐ |
| (44) | R.RFEALLQTGK.S | | 581.83 | 0.0000 | ☒ | ☐ |
| (46) | R.RFEALLQTGK.S | | 581.83 | -0.0006 | ☒ | ☐ |
| (46) | R.RFEALLQTGK.S | | 581.83 | -0.0002 | ☒ | ☐ |
| (54) | R.RFEALLQTGK.S | | 581.83 | 0.0006 | ☒ | ☐ |
| 55 | R.FEALLQTGK.S | | 503.78 | -0.0009 | ☒ | ☐ |
| 44 | K.TANTLPSK.E | | 416.23 | 0.0002 | ☒ | ☐ |
| 27 | K.EAITVQQK.Q | | 458.76 | 0.0000 | ☒ | ☐ |
| 47 | K.SLEVSDTR.F | | 453.73 | 0.0001 | ☒ | ☐ |

(c)



| | | | | |
|---|---|---|---|---|
| 9947 | IRAK4 DEATH DOMAIN | 88 | 62% | ***645*** |

GAMDPEFMNK PITPSTYVRC LNVGLIRKLS DFIDPQEGWK KLAVAIKKPS
GDDRYNQFHI RRFEALLQTG KSPTSELLFD WGTTNCTVGD LVDLLIQNEF
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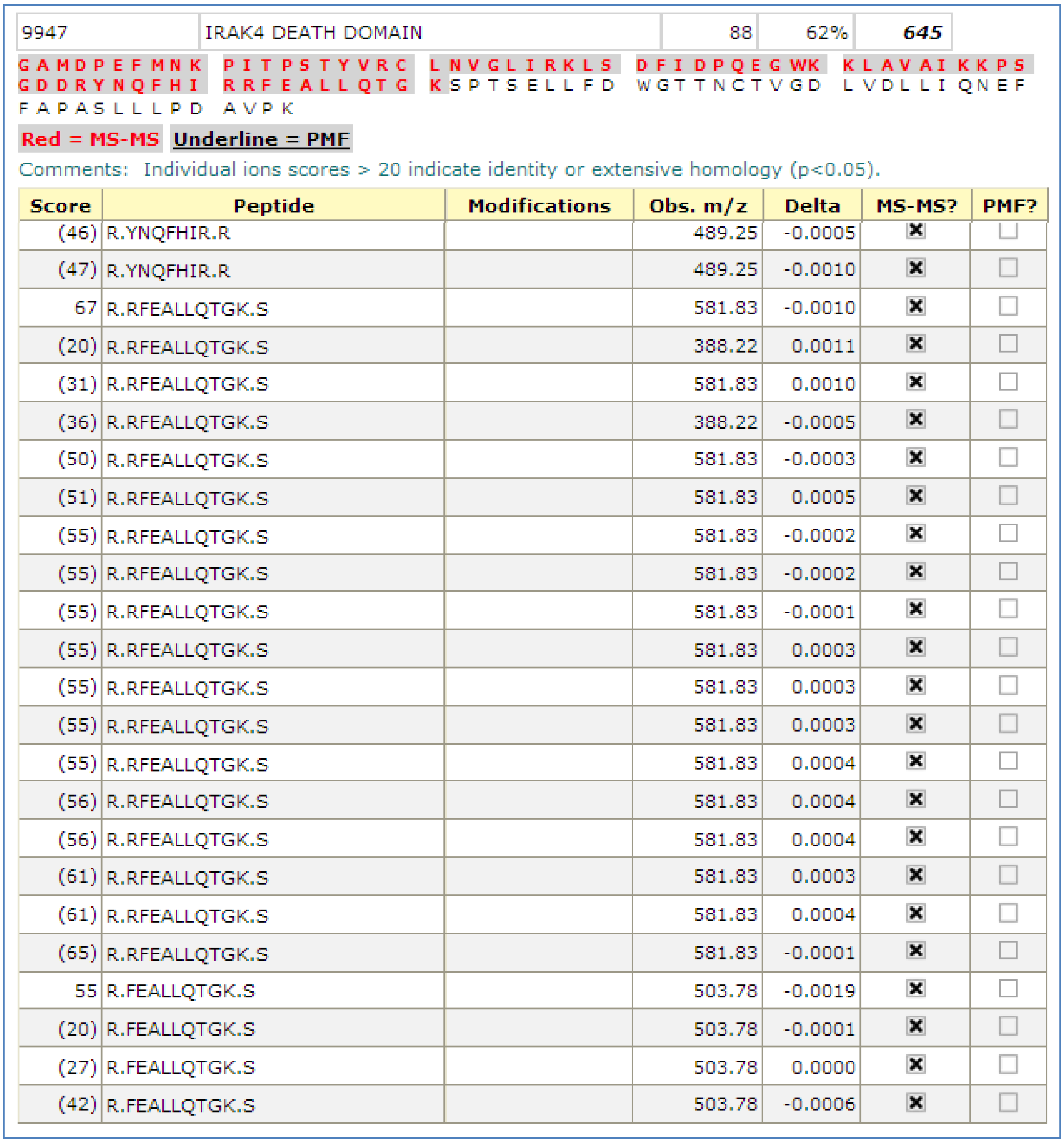
| Score | Peptide | Modifications | Obs. m/z | Delta | MS-MS? | PMF? |
|---|---|---|---|---|---|---|
| 47 | K.LAVAIK.K | | 307.72 | -0.0001 | ☒ | ☐ |
| (29) | K.LAVAIK.K | | 307.72 | -0.0002 | ☒ | ☐ |
| (33) | K.LAVAIK.K | | 307.72 | 0.0000 | ☒ | ☐ |
| (43) | K.LAVAIK.K | | 307.72 | 0.0005 | ☒ | ☐ |
| (45) | K.LAVAIK.K | | 307.72 | 0.0005 | ☒ | ☐ |
| 40 | K.KPSGDDR.Y | | 387.69 | 0.0000 | ☒ | ☐ |
| 34 | K.KPSGDDRYNQFHIR.R | | 866.93 | 0.0030 | ☒ | ☐ |
| (25) | K.KPSGDDRYNQFHIR.R | | 578.29 | 0.0014 | ☒ | ☐ |
| (37) | K.KPSGDDR.Y | | 387.69 | 0.0002 | ☒ | ☐ |
| (39) | K.KPSGDDR.Y | | 387.69 | 0.0002 | ☒ | ☐ |
| (40) | K.KPSGDDR.Y | | 387.69 | 0.0001 | ☒ | ☐ |
| (40) | K.KPSGDDR.Y | | 387.69 | 0.0001 | ☒ | ☐ |
| (40) | K.KPSGDDR.Y | | 387.69 | 0.0002 | ☒ | ☐ |
| 50 | R.YNQFHIR.R | | 489.25 | -0.0001 | ☒ | ☐ |
| (25) | R.YNQFHIR.R | | 489.25 | -0.0007 | ☒ | ☐ |
| (29) | R.YNQFHIR.R | | 489.25 | 0.0005 | ☒ | ☐ |
| (31) | R.YNQFHIR.R | | 489.25 | -0.0003 | ☒ | ☐ |
| (34) | R.YNQFHIR.R | | 489.25 | -0.0003 | ☒ | ☐ |
| (36) | R.YNQFHIR.R | | 489.25 | 0.0010 | ☒ | ☐ |
| (38) | R.YNQFHIR.R | | 489.25 | 0.0001 | ☒ | ☐ |
| (38) | R.YNQFHIR.R | | 489.25 | 0.0005 | ☒ | ☐ |
| (41) | R.YNQFHIR.R | | 489.25 | -0.0007 | ☒ | ☐ |
| (41) | R.YNQFHIR.R | | 489.25 | -0.0002 | ☒ | ☐ |
| (41) | R.YNQFHIR.R | | 489.25 | -0.0002 | ☒ | ☐ |
| (45) | R.YNQFHIR.R | | 489.25 | -0.0005 | ☒ | ☐ |

(d)



| | | | | |
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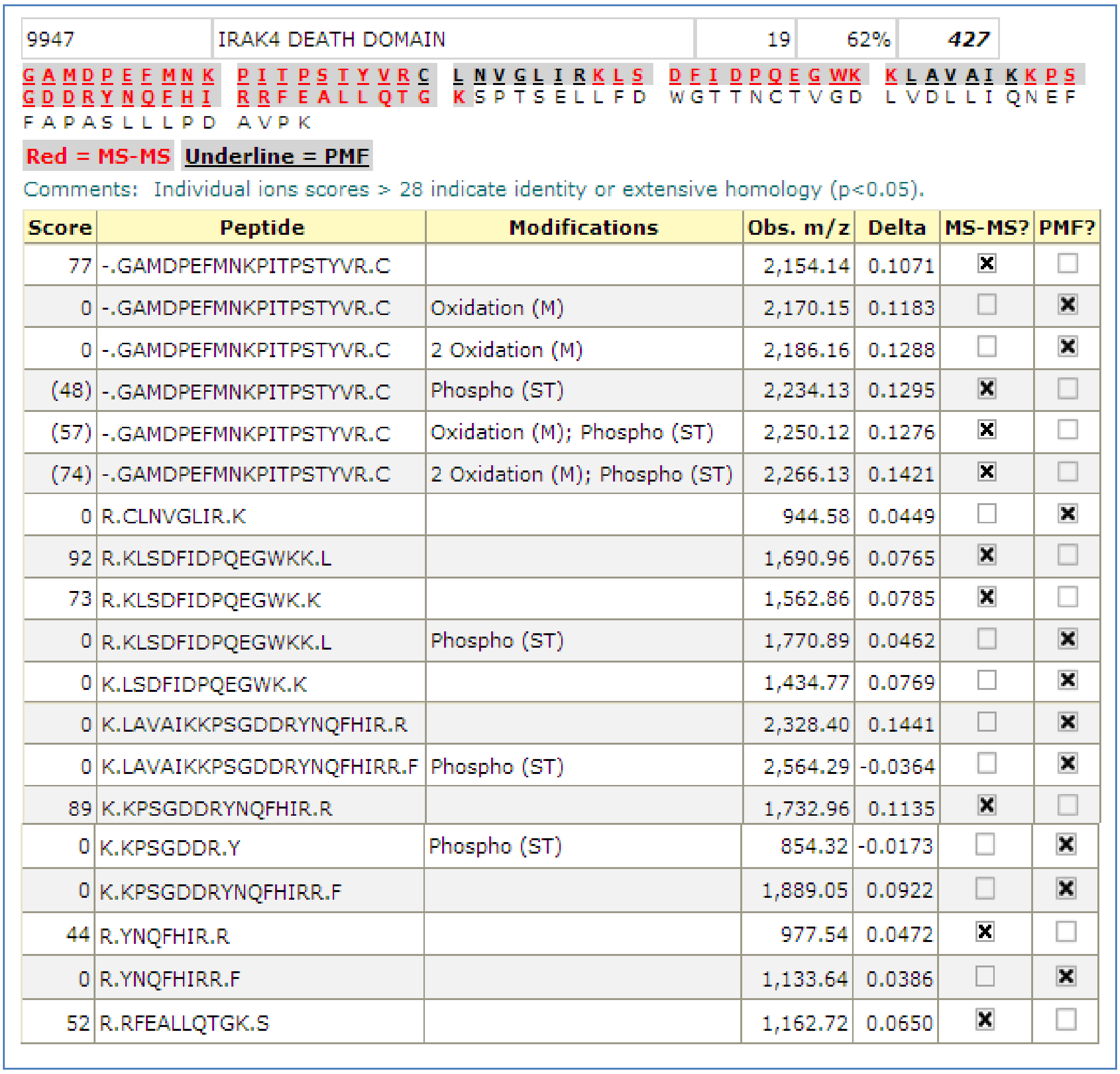
| (20) | R.RFEALLQTGK.S | | 388.22 | 0.0011 | ☒ | ☐ |
| (31) | R.RFEALLQTGK.S | | 581.83 | 0.0010 | ☒ | ☐ |
| (36) | R.RFEALLQTGK.S | | 388.22 | -0.0005 | ☒ | ☐ |
| (50) | R.RFEALLQTGK.S | | 581.83 | -0.0003 | ☒ | ☐ |
| (51) | R.RFEALLQTGK.S | | 581.83 | 0.0005 | ☒ | ☐ |
| (55) | R.RFEALLQTGK.S | | 581.83 | -0.0002 | ☒ | ☐ |
| (55) | R.RFEALLQTGK.S | | 581.83 | -0.0002 | ☒ | ☐ |
| (55) | R.RFEALLQTGK.S | | 581.83 | -0.0001 | ☒ | ☐ |
| (55) | R.RFEALLQTGK.S | | 581.83 | 0.0003 | ☒ | ☐ |
| (55) | R.RFEALLQTGK.S | | 581.83 | 0.0003 | ☒ | ☐ |
| (55) | R.RFEALLQTGK.S | | 581.83 | 0.0003 | ☒ | ☐ |
| (55) | R.RFEALLQTGK.S | | 581.83 | 0.0004 | ☒ | ☐ |
| (56) | R.RFEALLQTGK.S | | 581.83 | 0.0004 | ☒ | ☐ |
| (56) | R.RFEALLQTGK.S | | 581.83 | 0.0004 | ☒ | ☐ |
| (61) | R.RFEALLQTGK.S | | 581.83 | 0.0003 | ☒ | ☐ |
| (61) | R.RFEALLQTGK.S | | 581.83 | 0.0004 | ☒ | ☐ |
| (65) | R.RFEALLQTGK.S | | 581.83 | -0.0001 | ☒ | ☐ |
| 55 | R.FEALLQTGK.S | | 503.78 | -0.0019 | ☒ | ☐ |
| (20) | R.FEALLQTGK.S | | 503.78 | -0.0001 | ☒ | ☐ |
| (27) | R.FEALLQTGK.S | | 503.78 | 0.0000 | ☒ | ☐ |
| (42) | R.FEALLQTGK.S | | 503.78 | -0.0006 | ☒ | ☐ |

**Table 3:** Peptides identified by MALDI-TOF MS after digestion of IRAK-4 DD using Trypsin.



| 9947 | IRAK4 DEATH DOMAIN | 19 | 62% | ***427*** |
|---|---|---|---|---|

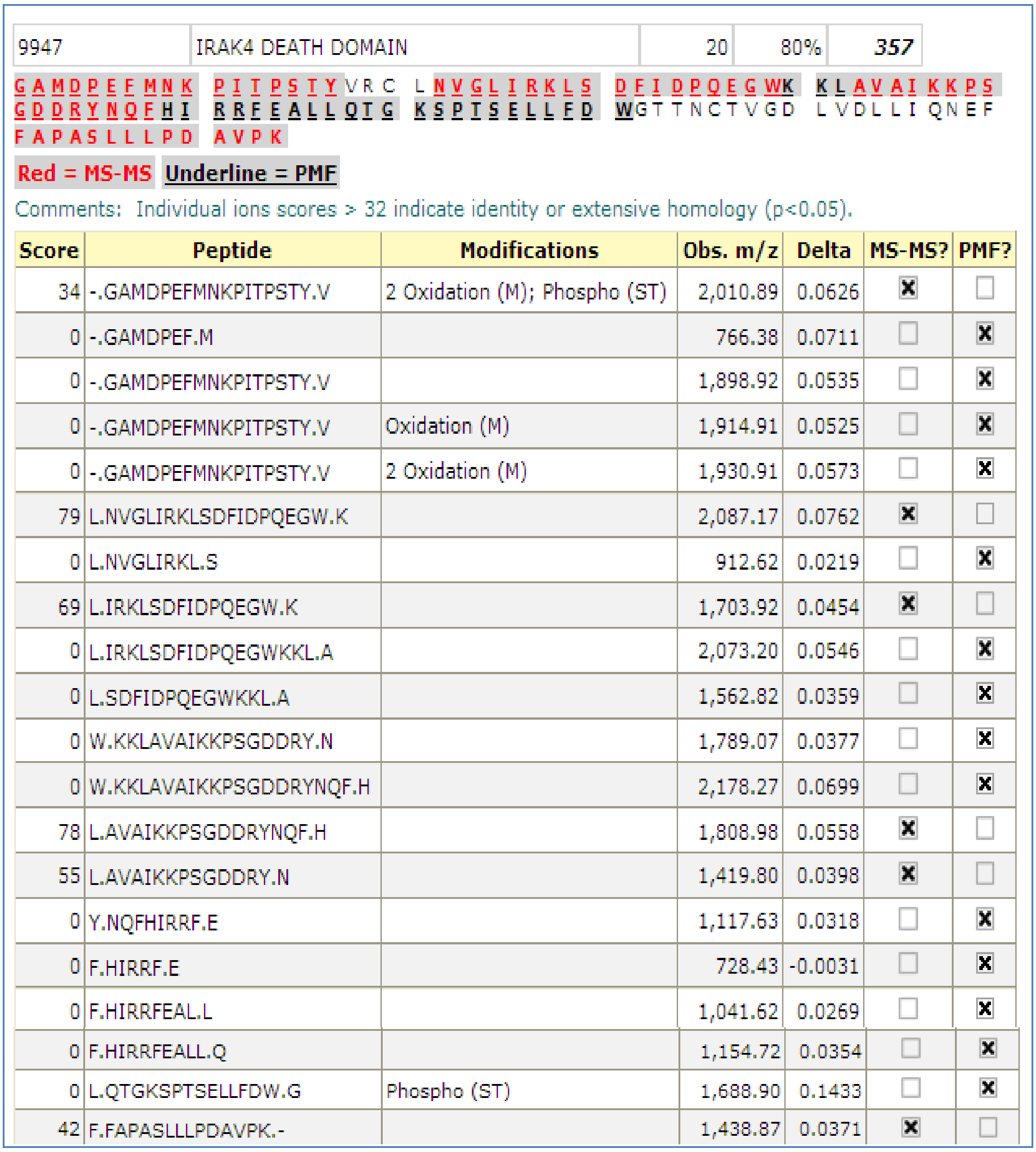
GAMDPEFMNK PITPSTYVRC LNVGLIRKLS DFIDPQEGWK KLAVAIKKPS
GDDRYNQFHI RRFEALLQTG KSPTSELLFD WGTTNCTVGD LVDLLIQNEF
FAPASLLLPD AVPK

**Red = MS-MS** **Underline = PMF**

Comments: Individual ions scores > 28 indicate identity or extensive homology (p<0.05).

| Score | Peptide | Modifications | Obs. m/z | Delta | MS-MS? | PMF? |
|---|---|---|---|---|---|---|
| 77 | -.GAMDPEFMNKPITPSTYVR.C | | 2,154.14 | 0.1071 | ☒ | ☐ |
| 0 | -.GAMDPEFMNKPITPSTYVR.C | Oxidation (M) | 2,170.15 | 0.1183 | ☐ | ☒ |
| 0 | -.GAMDPEFMNKPITPSTYVR.C | 2 Oxidation (M) | 2,186.16 | 0.1288 | ☐ | ☒ |
| (48) | -.GAMDPEFMNKPITPSTYVR.C | Phospho (ST) | 2,234.13 | 0.1295 | ☒ | ☐ |
| (57) | -.GAMDPEFMNKPITPSTYVR.C | Oxidation (M); Phospho (ST) | 2,250.12 | 0.1276 | ☒ | ☐ |
| (74) | -.GAMDPEFMNKPITPSTYVR.C | 2 Oxidation (M); Phospho (ST) | 2,266.13 | 0.1421 | ☒ | ☐ |
| 0 | R.CLNVGLIR.K | | 944.58 | 0.0449 | ☐ | ☒ |
| 92 | R.KLSDFIDPQEGWKK.L | | 1,690.96 | 0.0765 | ☒ | ☐ |
| 73 | R.KLSDFIDPQEGWK.K | | 1,562.86 | 0.0785 | ☒ | ☐ |
| 0 | R.KLSDFIDPQEGWKK.L | Phospho (ST) | 1,770.89 | 0.0462 | ☐ | ☒ |
| 0 | K.LSDFIDPQEGWK.K | | 1,434.77 | 0.0769 | ☐ | ☒ |
| 0 | K.LAVAIKKPSGDDRYNQFHIR.R | | 2,328.40 | 0.1441 | ☐ | ☒ |
| 0 | K.LAVAIKKPSGDDRYNQFHIRR.F | Phospho (ST) | 2,564.29 | -0.0364 | ☐ | ☒ |
| 89 | K.KPSGDDRYNQFHIR.R | | 1,732.96 | 0.1135 | ☒ | ☐ |
| 0 | K.KPSGDDR.Y | Phospho (ST) | 854.32 | -0.0173 | ☐ | ☒ |
| 0 | K.KPSGDDRYNQFHIRR.F | | 1,889.05 | 0.0922 | ☐ | ☒ |
| 44 | R.YNQFHIR.R | | 977.54 | 0.0472 | ☒ | ☐ |
| 0 | R.YNQFHIRR.F | | 1,133.64 | 0.0386 | ☐ | ☒ |
| 52 | R.RFEALLQTGK.S | | 1,162.72 | 0.0650 | ☒ | ☐ |

**Table 4:** Peptides identified by MALDI-TOF MS after digestion of IRAK-4 DD using Chymotrypsin.



| | | | | |
|---|---|---|---|---|
| 9947 | IRAK4 DEATH DOMAIN | 20 | 80% | ***357*** |

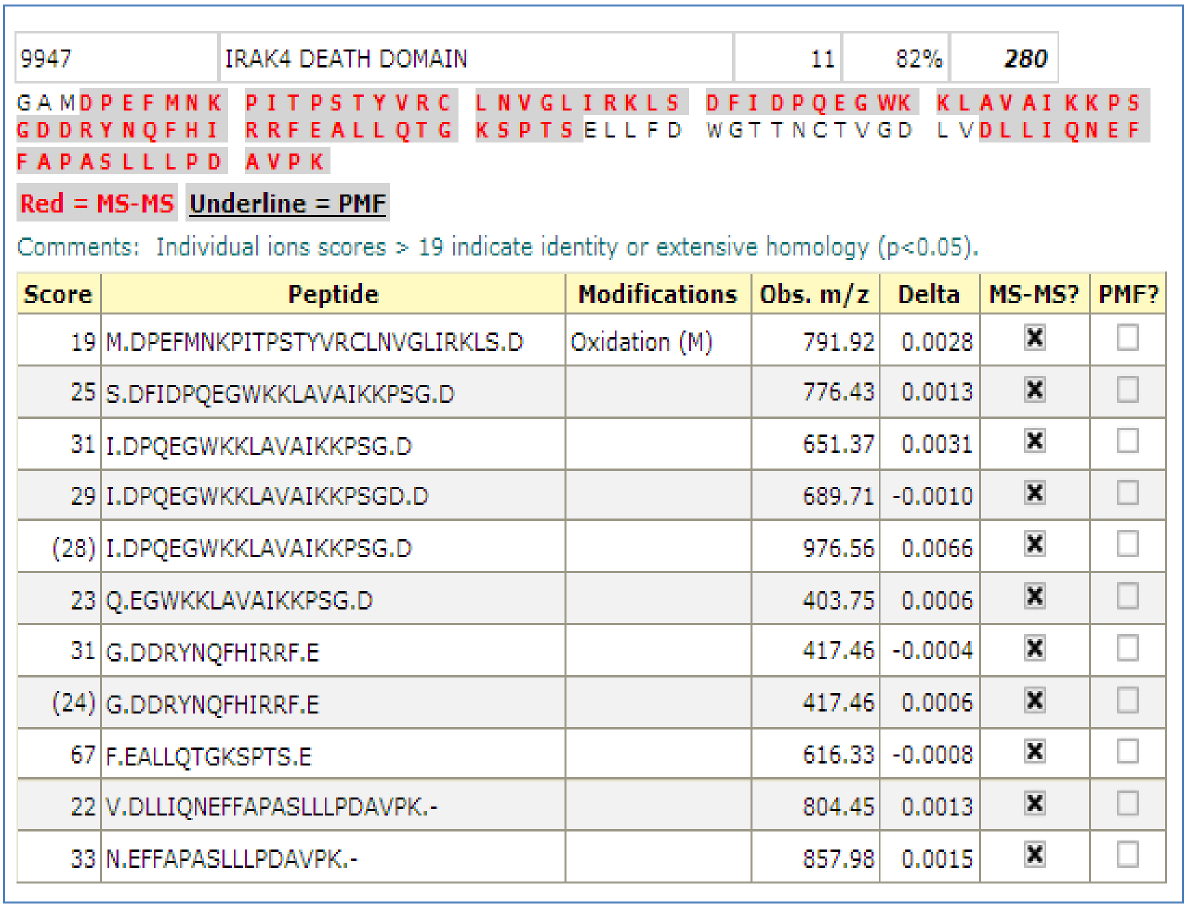
GAMDPEFMNK PITPSTYVRC LNVGLIRKLS DFIDPQEGWK KLAVAIKKPS
GDDRYNQFHI RRFEALLQTG KSPTSELLFD WGTTNCTVGD LVDLLIQNEF
FAPASLLLPD AVPK

**Red = MS-MS** **Underline = PMF**

Comments: Individual ions scores > 32 indicate identity or extensive homology (p<0.05).

| Score | Peptide | Modifications | Obs. m/z | Delta | MS-MS? | PMF? |
|---|---|---|---|---|---|---|
| 34 | -.GAMDPEFMNKPITPSTY.V | 2 Oxidation (M); Phospho (ST) | 2,010.89 | 0.0626 | ☒ | ☐ |
| 0 | -.GAMDPEF.M | | 766.38 | 0.0711 | ☐ | ☒ |
| 0 | -.GAMDPEFMNKPITPSTY.V | | 1,898.92 | 0.0535 | ☐ | ☒ |
| 0 | -.GAMDPEFMNKPITPSTY.V | Oxidation (M) | 1,914.91 | 0.0525 | ☐ | ☒ |
| 0 | -.GAMDPEFMNKPITPSTY.V | 2 Oxidation (M) | 1,930.91 | 0.0573 | ☐ | ☒ |
| 79 | L.NVGLIRKLSDFIDPQEGW.K | | 2,087.17 | 0.0762 | ☒ | ☐ |
| 0 | L.NVGLIRKL.S | | 912.62 | 0.0219 | ☐ | ☒ |
| 69 | L.IRKLSDFIDPQEGW.K | | 1,703.92 | 0.0454 | ☒ | ☐ |
| 0 | L.IRKLSDFIDPQEGWKKL.A | | 2,073.20 | 0.0546 | ☐ | ☒ |
| 0 | L.SDFIDPQEGWKKL.A | | 1,562.82 | 0.0359 | ☐ | ☒ |
| 0 | W.KKLAVAIKKPSGDDRY.N | | 1,789.07 | 0.0377 | ☐ | ☒ |
| 0 | W.KKLAVAIKKPSGDDRYNQF.H | | 2,178.27 | 0.0699 | ☐ | ☒ |
| 78 | L.AVAIKKPSGDDRYNQF.H | | 1,808.98 | 0.0558 | ☒ | ☐ |
| 55 | L.AVAIKKPSGDDRY.N | | 1,419.80 | 0.0398 | ☒ | ☐ |
| 0 | Y.NQFHIRRF.E | | 1,117.63 | 0.0318 | ☐ | ☒ |
| 0 | F.HIRRF.E | | 728.43 | -0.0031 | ☐ | ☒ |
| 0 | F.HIRRFEAL.L | | 1,041.62 | 0.0269 | ☐ | ☒ |
| 0 | F.HIRRFEALL.Q | | 1,154.72 | 0.0354 | ☐ | ☒ |
| 0 | L.QTGKSPTSELLFDW.G | Phospho (ST) | 1,688.90 | 0.1433 | ☐ | ☒ |
| 42 | F.FAPASLLLPDAVPK.- | | 1,438.87 | 0.0371 | ☒ | ☐ |

**Table 5:** Peptides identified by LC / MS - MS after digestion of IRAK-4 DD using Asp-N.



| | | | | |
|---|---|---|---|---|
| 9947 | IRAK4 DEATH DOMAIN | 11 | 82% | ***280*** |

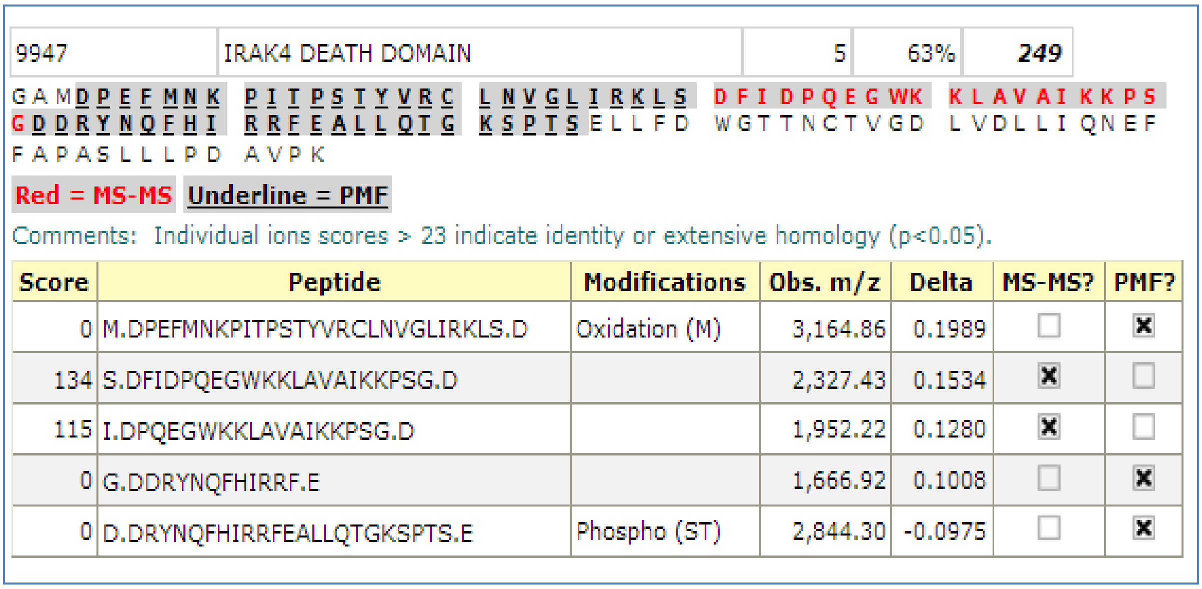
GAMDPEFMNK PITPSTYVRC LNVGLIRKLS DFIDPQEGWK KLAVAIKKPS
GDDRYNQFHI RRFEALLQTG KSPTSELLFD WGTTNCTVGD LVDLLIQNEF
FAPASLLLPD AVPK

**Red = MS-MS** **Underline = PMF**

Comments: Individual ions scores > 19 indicate identity or extensive homology (p<0.05).

| Score | Peptide | Modifications | Obs. m/z | Delta | MS-MS? | PMF? |
|---|---|---|---|---|---|---|
| 19 | M.DPEFMNKPITPSTYVRCLNVGLIRKLS.D | Oxidation (M) | 791.92 | 0.0028 | ☒ | ☐ |
| 25 | S.DFIDPQEGWKKLAVAIKKPSG.D | | 776.43 | 0.0013 | ☒ | ☐ |
| 31 | I.DPQEGWKKLAVAIKKPSG.D | | 651.37 | 0.0031 | ☒ | ☐ |
| 29 | I.DPQEGWKKLAVAIKKPSGD.D | | 689.71 | -0.0010 | ☒ | ☐ |
| (28) | I.DPQEGWKKLAVAIKKPSG.D | | 976.56 | 0.0066 | ☒ | ☐ |
| 23 | Q.EGWKKLAVAIKKPSG.D | | 403.75 | 0.0006 | ☒ | ☐ |
| 31 | G.DDRYNQFHIRRF.E | | 417.46 | -0.0004 | ☒ | ☐ |
| (24) | G.DDRYNQFHIRRF.E | | 417.46 | 0.0006 | ☒ | ☐ |
| 67 | F.EALLQTGKSPTS.E | | 616.33 | -0.0008 | ☒ | ☐ |
| 22 | V.DLLIQNEFFAPASLLLPDAVPK.- | | 804.45 | 0.0013 | ☒ | ☐ |
| 33 | N.EFFAPASLLLPDAVPK.- | | 857.98 | 0.0015 | ☒ | ☐ |

**Table 6:** Peptides identified by MALDI-TOF MS after digestion of IRAK-4 DD using Asp-N.



| | | | | |
|---|---|---|---|---|
| 9947 | IRAK4 DEATH DOMAIN | 5 | 63% | ***249*** |

GAMDPEFMNK PITPSTYVRC LNVGLIRKLS DFIDPQEGWK KLAVAIKKPS
GDDRYNQFHI RRFEALLQTG KSPTSELLFD WGTTNCTVGD LVDLLIQNEF
FAPASLLLPD AVPK

**Red = MS-MS** **Underline = PMF**

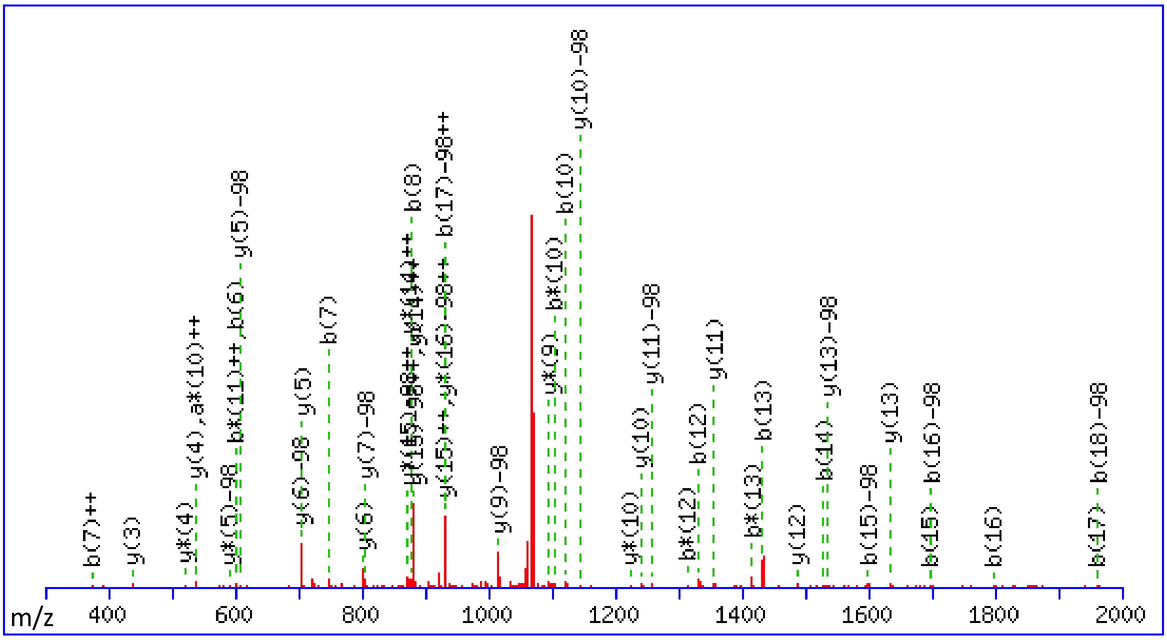
Comments: Individual ions scores > 23 indicate identity or extensive homology (p<0.05).

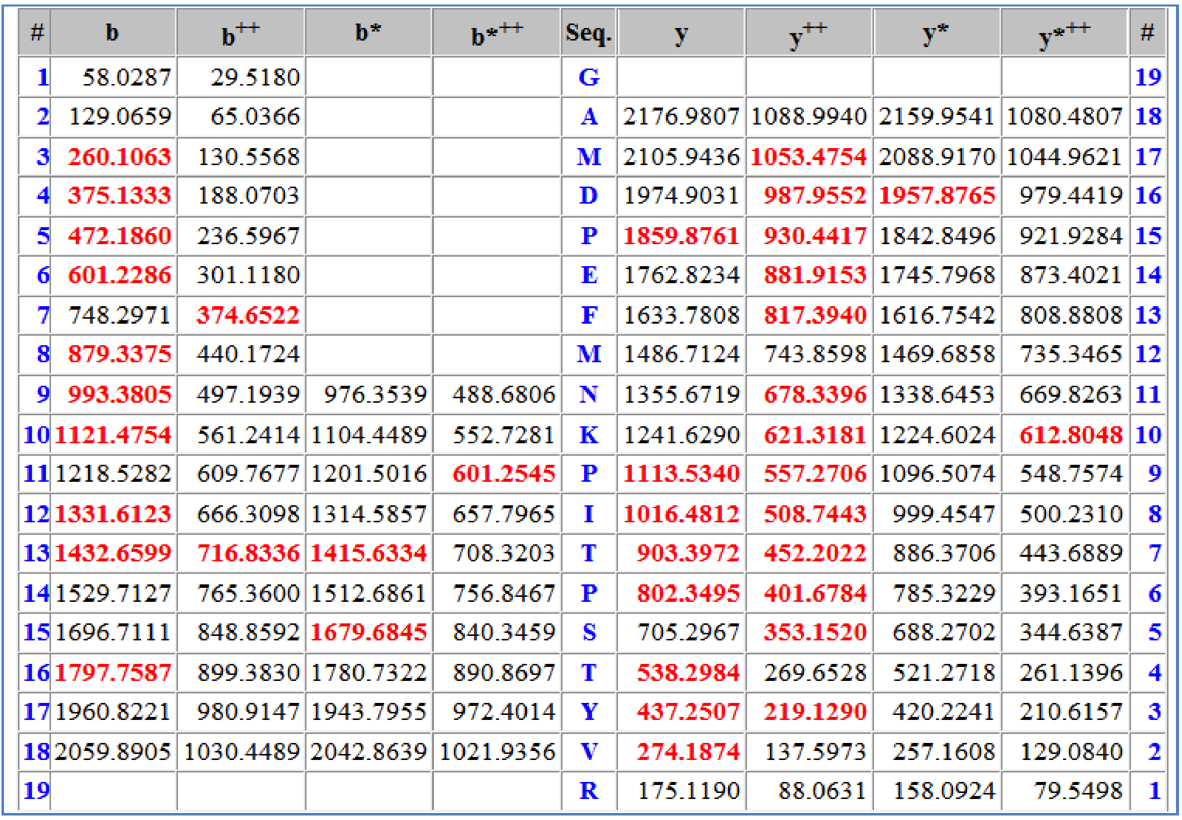
| Score | Peptide | Modifications | Obs. m/z | Delta | MS-MS? | PMF? |
|---|---|---|---|---|---|---|
| 0 | M.DPEFMNKPITPSTYVRCLNVGLIRKLS.D | Oxidation (M) | 3,164.86 | 0.1989 | ☐ | ☒ |
| 134 | S.DFIDPQEGWKKLAVAIKKPSG.D | | 2,327.43 | 0.1534 | ☒ | ☐ |
| 115 | I.DPQEGWKKLAVAIKKPSG.D | | 1,952.22 | 0.1280 | ☒ | ☐ |
| 0 | G.DDRYNQFHIRRF.E | | 1,666.92 | 0.1008 | ☐ | ☒ |
| 0 | D.DRYNQFHIRRFEALLQTGKSPTS.E | Phospho (ST) | 2,844.30 | -0.0975 | ☐ | ☒ |

**Table 7**: MS/MS Fragmentation of GAMDPEFMNKPITPSTYVR

(a) Peptide fragment spectrum of b- and y- ion series corresponding to the cleavage of the amide bond at the N-terminus and C-terminus respectively. (b) Table of the corresponding observed fragmentation masses showing matching peptide hits in Bold Red: 45/272 fragment ions using 125 most intense peaks used to give an ion score of 20 in the MASCOT database. (c) Zoomed area of peptide fragment spectrum from 1600 to 1750 m/z showing the ion b*(15) corresponding to the loss of 98 Da at Ser8. (d) Zoomed area of peptide fragment spectrum from 300 to 400 m/z showing the ion $y^{++}(5)$ corresponding to the loss of 98 Da at Ser8.

(a)

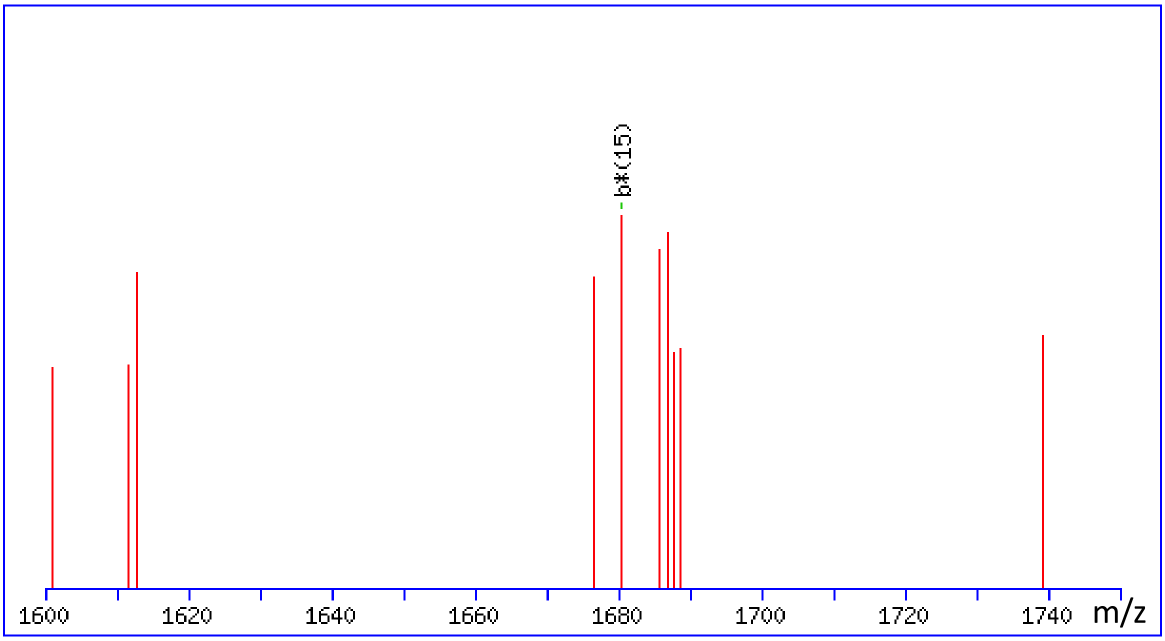




| # | b | b++ | b* | b*++ | Seq. | y | y++ | y* | y*++ | # |
|---|---|---|---|---|---|---|---|---|---|---|
| 1 | 58.0287 | 29.5180 | | | G | | | | | 19 |
| 2 | 129.0659 | 65.0366 | | | A | 2176.9807 | 1088.9940 | 2159.9541 | 1080.4807 | 18 |
| 3 | **260.1063** | 130.5568 | | | M | 2105.9436 | **1053.4754** | 2088.9170 | 1044.9621 | 17 |
| 4 | **375.1333** | 188.0703 | | | D | 1974.9031 | **987.9552** | **1957.8765** | 979.4419 | 16 |
| 5 | **472.1860** | 236.5967 | | | P | **1859.8761** | **930.4417** | 1842.8496 | 921.9284 | 15 |
| 6 | **601.2286** | 301.1180 | | | E | 1762.8234 | **881.9153** | 1745.7968 | 873.4021 | 14 |
| 7 | 748.2971 | **374.6522** | | | F | 1633.7808 | **817.3940** | 1616.7542 | 808.8808 | 13 |
| 8 | **879.3375** | 440.1724 | | | M | 1486.7124 | 743.8598 | 1469.6858 | 735.3465 | 12 |
| 9 | **993.3805** | 497.1939 | 976.3539 | 488.6806 | N | 1355.6719 | **678.3396** | 1338.6453 | 669.8263 | 11 |
| 10 | **1121.4754** | 561.2414 | 1104.4489 | 552.7281 | K | 1241.6290 | **621.3181** | 1224.6024 | **612.8048** | 10 |
| 11 | 1218.5282 | 609.7677 | 1201.5016 | **601.2545** | P | **1113.5340** | **557.2706** | 1096.5074 | 548.7574 | 9 |
| 12 | **1331.6123** | 666.3098 | 1314.5857 | 657.7965 | I | **1016.4812** | **508.7443** | 999.4547 | 500.2310 | 8 |
| 13 | **1432.6599** | **716.8336** | **1415.6334** | 708.3203 | T | **903.3972** | **452.2022** | 886.3706 | 443.6889 | 7 |
| 14 | 1529.7127 | 765.3600 | 1512.6861 | 756.8467 | P | **802.3495** | **401.6784** | 785.3229 | 393.1651 | 6 |
| 15 | 1696.7111 | 848.8592 | **1679.6845** | 840.3459 | S | 705.2967 | **353.1520** | 688.2702 | 344.6387 | 5 |
| 16 | **1797.7587** | 899.3830 | 1780.7322 | 890.8697 | T | **538.2984** | 269.6528 | 521.2718 | 261.1396 | 4 |
| 17 | 1960.8221 | 980.9147 | 1943.7955 | 972.4014 | Y | **437.2507** | **219.1290** | 420.2241 | 210.6157 | 3 |
| 18 | 2059.8905 | 1030.4489 | 2042.8639 | 1021.9356 | V | **274.1874** | 137.5973 | 257.1608 | 129.0840 | 2 |
| 19 | | | | | R | 175.1190 | 88.0631 | 158.0924 | 79.5498 | 1 |

(b)

(c)


b*(15)
1600
1620
1640
1660
1680
1700
1720
1740
m/z

(d)

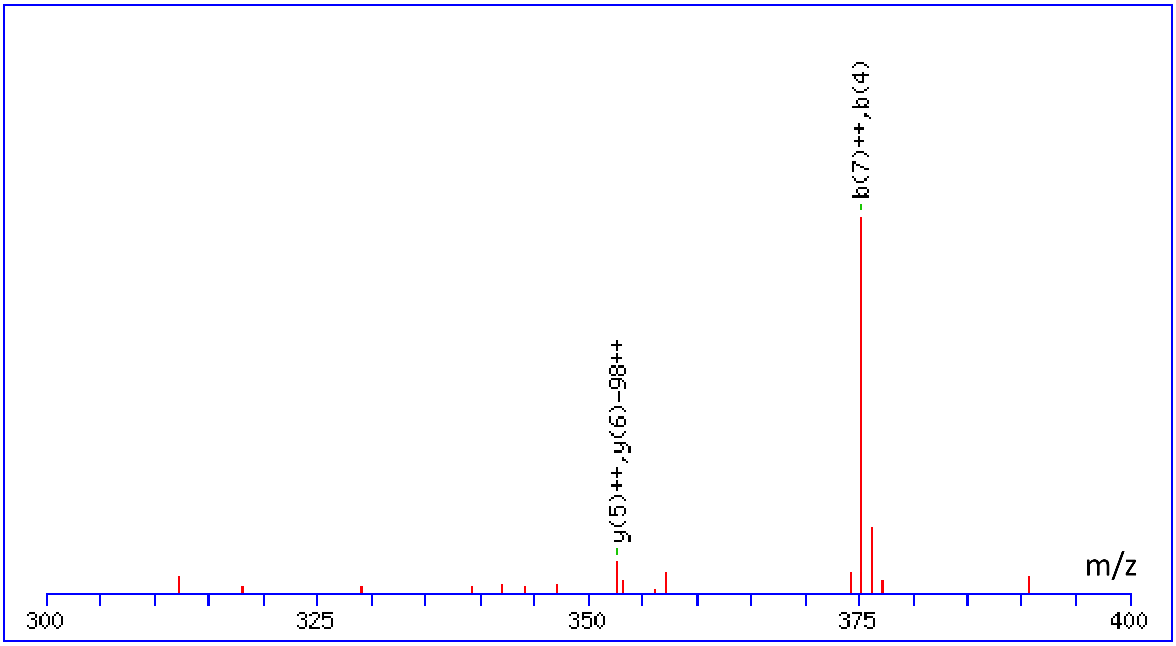
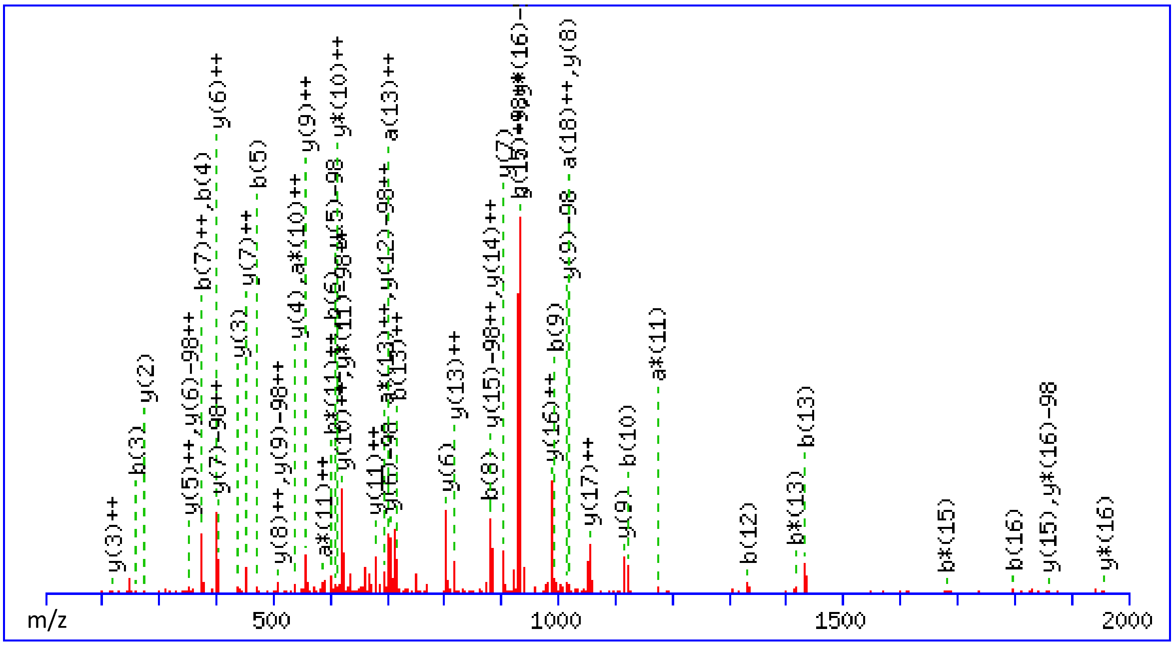

b(7)++,b(4)
y(5)++,y(6)-98++
m/z
300
325
350
375
400

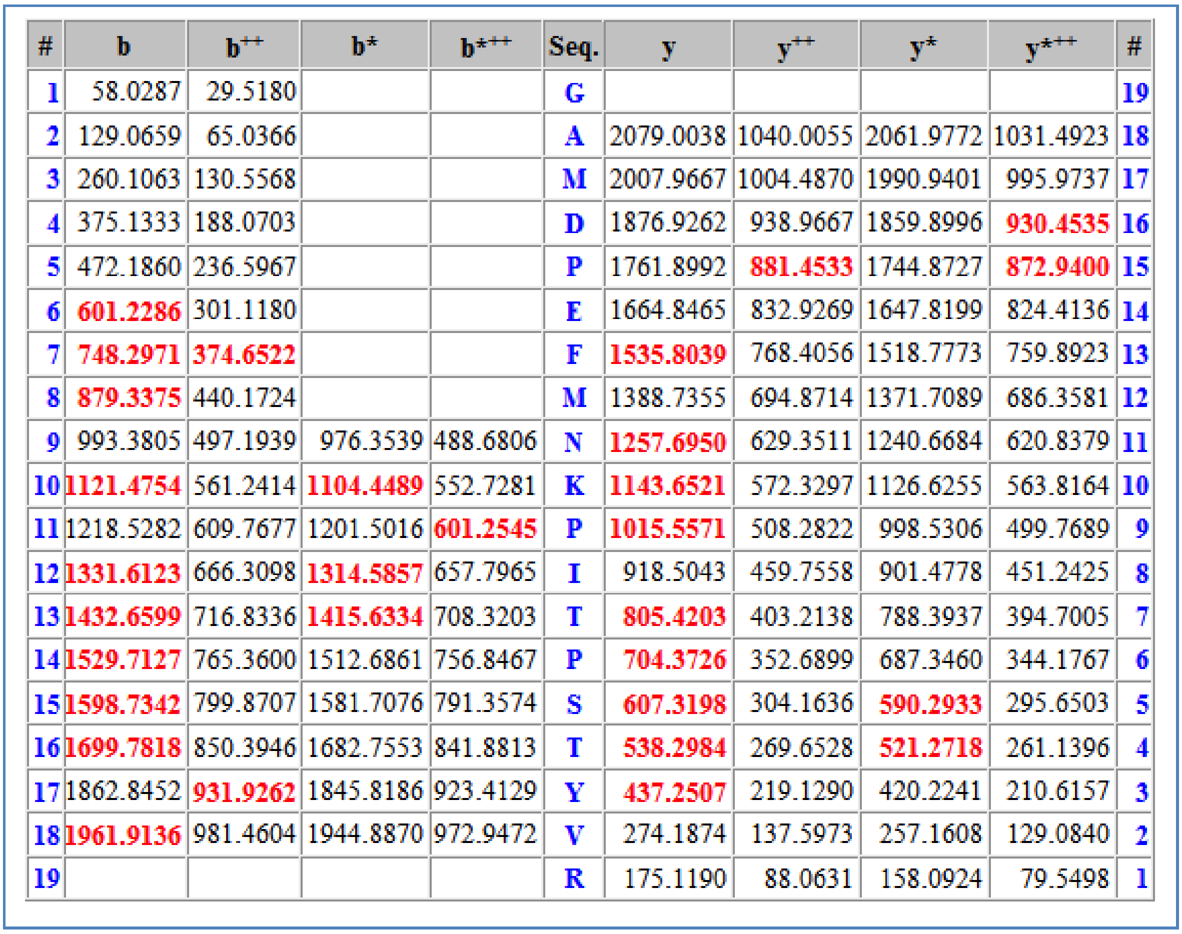
**Table 8**: MS/MS Fragmentation of GAMDPEFMNKPITPSTYVR

(a) Peptide fragment spectrum of b- and y- ion series corresponding to the cleavage of the amide bond at the N-terminus and C-terminus respectively. (b) Table of the corresponding observed fragmentation masses showing matching peptide hits in 56/272 fragment ions using 129 most intense peaks used to give an ion score of 48 in the MASCOT database. (c) Zoomed area of peptide fragment spectrum from 1550 to 1650 m/z showing the ion b(15) corresponding to the loss of 98 Da at Ser8. (d) Zoomed area of peptide fragment spectrum from 550 to 650 m/z showing the ions y(5) and y*(5) corresponding to the loss of 98 Da at Ser8.

(a)

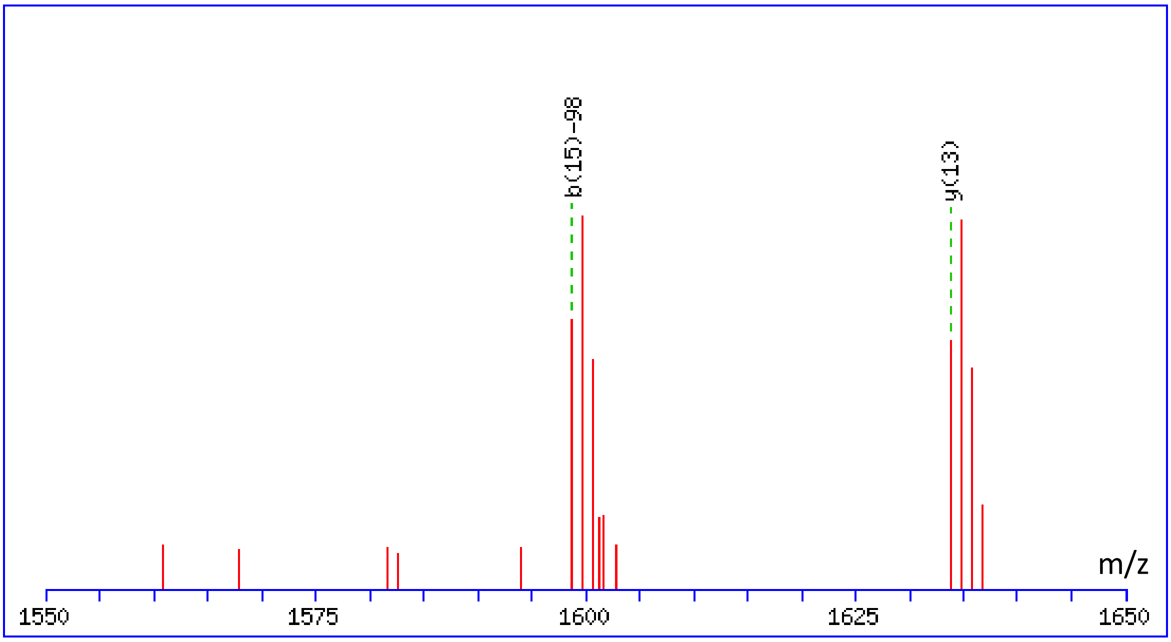


(b)

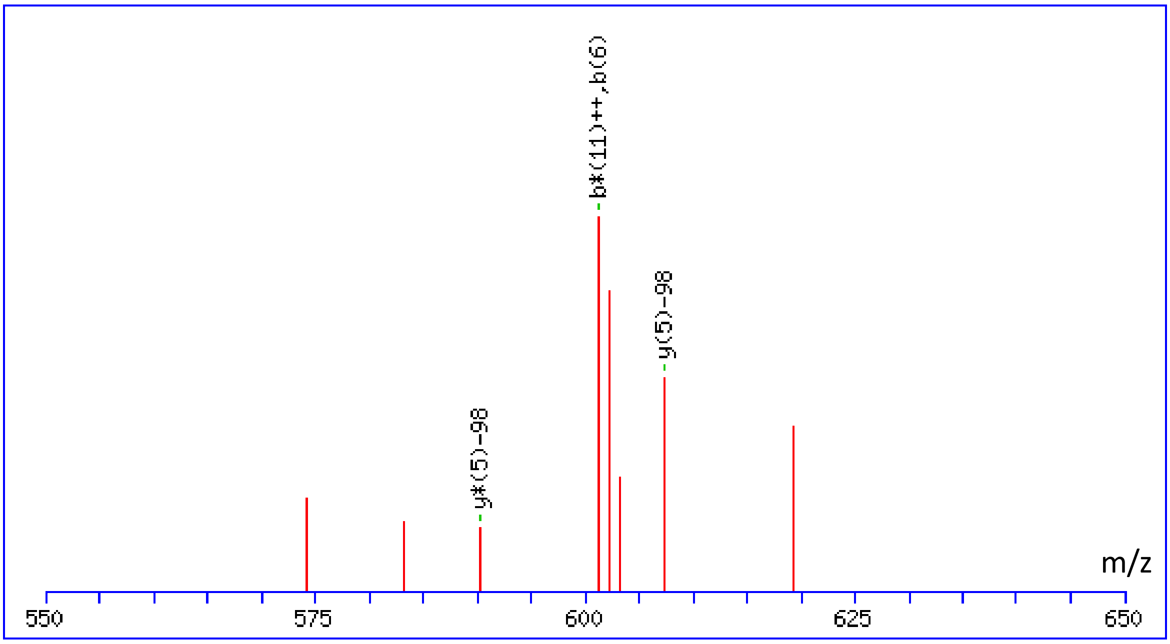


| # | b | $b^{++}$ | b* | $b^{*++}$ | Seq. | y | $y^{++}$ | y* | $y^{*++}$ | # |
|---|---|---|---|---|---|---|---|---|---|---|
| 1 | 58.0287 | 29.5180 | | | G | | | | | 19 |
| 2 | 129.0659 | 65.0366 | | | A | 2079.0038 | 1040.0055 | 2061.9772 | 1031.4923 | 18 |
| 3 | 260.1063 | 130.5568 | | | M | 2007.9667 | 1004.4870 | 1990.9401 | 995.9737 | 17 |
| 4 | 375.1333 | 188.0703 | | | D | 1876.9262 | 938.9667 | 1859.8996 | **930.4535** | 16 |
| 5 | 472.1860 | 236.5967 | | | P | 1761.8992 | **881.4533** | 1744.8727 | **872.9400** | 15 |
| 6 | **601.2286** | 301.1180 | | | E | 1664.8465 | 832.9269 | 1647.8199 | 824.4136 | 14 |
| 7 | **748.2971** | **374.6522** | | | F | **1535.8039** | 768.4056 | 1518.7773 | 759.8923 | 13 |
| 8 | **879.3375** | 440.1724 | | | M | 1388.7355 | 694.8714 | 1371.7089 | 686.3581 | 12 |
| 9 | 993.3805 | 497.1939 | 976.3539 | 488.6806 | N | **1257.6950** | 629.3511 | 1240.6684 | 620.8379 | 11 |
| 10 | **1121.4754** | 561.2414 | **1104.4489** | 552.7281 | K | **1143.6521** | 572.3297 | 1126.6255 | 563.8164 | 10 |
| 11 | 1218.5282 | 609.7677 | 1201.5016 | **601.2545** | P | **1015.5571** | 508.2822 | 998.5306 | 499.7689 | 9 |
| 12 | **1331.6123** | 666.3098 | **1314.5857** | 657.7965 | I | 918.5043 | 459.7558 | 901.4778 | 451.2425 | 8 |
| 13 | **1432.6599** | 716.8336 | **1415.6334** | 708.3203 | T | **805.4203** | 403.2138 | 788.3937 | 394.7005 | 7 |
| 14 | **1529.7127** | 765.3600 | 1512.6861 | 756.8467 | P | **704.3726** | 352.6899 | 687.3460 | 344.1767 | 6 |
| 15 | **1598.7342** | 799.8707 | 1581.7076 | 791.3574 | S | **607.3198** | 304.1636 | **590.2933** | 295.6503 | 5 |
| 16 | **1699.7818** | 850.3946 | 1682.7553 | 841.8813 | T | **538.2984** | 269.6528 | **521.2718** | 261.1396 | 4 |
| 17 | 1862.8452 | **931.9262** | 1845.8186 | 923.4129 | Y | **437.2507** | 219.1290 | 420.2241 | 210.6157 | 3 |
| 18 | **1961.9136** | 981.4604 | 1944.8870 | 972.9472 | V | 274.1874 | 137.5973 | 257.1608 | 129.0840 | 2 |
| 19 | | | | | R | 175.1190 | 88.0631 | 158.0924 | 79.5498 | 1 |

(c)



(d)



**Supplementary information - Model validation.**

After regularization in COOT, sidechain packing was optimised and total energy compared with the unphosphorylated IRAK4 structure (pdb id: 3mop, chain G) using the FoldX atomic forcefield. (FoldX version 4.0 running under Win10)[1]. Sidechain clashes were checked with 'findclash' function in UCSF-Chimera[2].

Neglecting the new stabilizing interactions of the phospho-serine residue, the helical refolding of the N-terminal 16 residues was calculated by FoldX to be comparable in energy to the crystal structure. Compared with the equivalent optimised crystal structure (minimised with the FoldX 'Repair' protocol), the refolded IRAK4 had an overall ΔΔG stabilisation of -0.2 kcal/mol (neglecting any phospho-serine contributions). This favourable change in estimated stability on helical refolding is for the IRAK4 in isolation, but no unfavourable contacts are generated when packed with other IRAK4 and IRAK2 chains to form the myddosome assembly.

The geometry and sidechain packing of the refolded IRAK4 were validated with WHATCHECK10.1 (http://swift.cmbi.ru.nl/gv/whatcheck/). Ramachandran analysis (with COOT) showed no new outliers were generated by the helix refolding: the IRAK4 crystal structure had 98 residues in preferred or allowed regions, and 7 outliers. None of the outliers is in the N-terminal region and refolding of the N-terminal region does not introduce any new Ramachandran outliers.

## References

1. Kiel, C., Serrano, L. & Herrmann, C. A detailed thermodynamic analysis of ras/effector complex interfaces. *J. Mol. Biol.* **340**, 1039-1058 (2004).

2. Yang, Z. *et al.* UCSF Chimera, MODELLER, and IMP: an integrated modeling system. *J. Struct. Biol.* **179**, 269-278 (2012).