

PLS-Annotation	Theoretical MH	Experimental MH	ppm
R.KVLGSSQNSSphosG.S	1143.50471	1143.504016	0.61
S.SQNSSphosGSEASETPVKR.R	1743.75506	1743.755114	-0.03
S.SQNSSphosGSEASETPVKR.R	1743.75506	1743.75487	0.11
S.SQNSSphosGSEASETPVKR.R	1743.75506	1743.754003	0.61
S.SQNSSphosGSEASETPVKR.R	1743.75506	1743.754382	0.39
G.SSQNSSphosGSEASETPVKR.R	1830.78709	1830.785876	0.66
K.VLGSSQNSSphosSphosGSEASETPVKR.R	2179.92736	2179.92821	-0.39
S.SQNSSphosGSEASETPVKRR.K	1899.85617	1899.851965	2.21
S.SQNSSphosGSEASETPVKRR.K	1899.85617	1899.853796	1.25
S.SQNSSphosGSEASETPVKRR.K	1899.85617	1899.852514	1.92
R.KVLGSSQNSSphosSphosGSEASETPVKR.R	2308.02232	2308.020788	0.66
S.SQNSSGphosEASETPVKRR.K	1899.85617	1899.85924	-1.62
R.KVLGSSQNSSphosSphosGSEASETPVKR.R	2308.02232	2308.025915	-1.56
R.KVLGSSQNSSphosSGSphosEASETPVKR.R	2308.02232	2308.025134	-1.22
R.KVLGSSQNSSphosGSEA.S	1430.61644	1430.616687	-0.17
R.KVLGSSQNSSphosGSEA.S	1430.61644	1430.616809	-0.26
R.KVLGSSQNSSphosGSEASETPVK.R	2071.95488	2071.957751	-1.39
S.VESphosDRYDSphosQDEDFVDNA.S	2163.74331	2163.74071	1.2
V.ELSVESphosDRYDSphosQDEDFV.D	2192.79501	2192.792712	1.05
V.LGSSQNSSphosGSEASETPV.K	1716.69654	1716.699695	-1.84
R.KVLGSSQNSSphosGSEA.S	1430.61644	1430.616809	-0.26
R.KVLGSSQNSSphosGSEA.S	1430.61644	1430.617785	-0.94
R.KVLGSSQNSSphosGSEA.S	1430.61644	1430.617297	-0.6
R.KVLGSSQNSSphosGSEA.S	1430.61644	1430.617419	-0.68
R.KVLGSSQNSSphosGSEA.S	1430.61644	1430.615466	0.68
R.KVLGSSQNSSphosGSEA.S	1430.61644	1430.615588	0.6
V.LGSSQNSSphosGSEASETPV.K	1716.69654	1716.697009	-0.27
R.KVLGSSQNSSphosGSEA.S	1430.61644	1430.616931	-0.34
R.KVLGSSQNSSphosGSEA.S	1430.61644	1430.616931	-0.34
R.KVLGSSQNSSphosGSEA.S	1430.61644	1430.617297	-0.6
R.KVLGSSQNSSphosGSEA.S	1430.61644	1430.615832	0.42
R.KVLGSSQNSSphosGSEA.S	1430.61644	1430.615466	0.68
R.KVLGSSQNSSphosGSEA.S	1430.61644	1430.616198	0.17
R.KVLGSSQNSSphosGSEA.S	1430.61644	1430.617419	-0.68
R.KVLGSSQNSSphosGSEA.S	1430.61644	1430.617663	-0.85
V.LGSSQNSSphosGSEASETPV.K	1716.69654	1716.698596	-1.2
V.LGSSQNSSphosGSEASETPV.K	1716.69654	1716.697986	-0.84
V.LGSSQNSSphosGSEASETPV.K	1716.69654	1716.697863	-0.77
S.SQNSSphosGSEASETPVKR.R	1743.75506	1743.756213	-0.66
R.KVLGSSQNSSphosGSEA.S	1430.61644	1430.616687	-0.17
R.KVLGSSQNSSphosGSEAS.E	1517.64847	1517.649157	-0.45
R.KVLGSSQNSSphosGSEA.S	1430.61644	1430.616198	0.17
S.SQNSSphosGSEASETPVKR.R	1743.75506	1743.753271	1.03
S.SQNSSphosGSEASETPVKR.R	1743.75506	1743.755114	-0.03
R.KVLGSSQNSSphosGSEASETPV.K	1943.85992	1943.859363	0.29
R.KVLGSSQNSSphosGSEASETPV.K	1943.85992	1943.860949	-0.53
R.KVLGSSQNSSphosGSEASETPV.K	1943.85992	1943.862292	-1.22
R.KVLGSSQNSSphosGSEASETPV.K	1943.85992	1943.862903	-1.53
R.KVLGSSQNSSphosGSEASETPV.K	1943.85992	1943.85935	0.29
R.KVLGSSQNSSphosGSEASETPV.K	1943.85992	1943.859729	0.1
R.KVLGSSQNSSphosGSEASETPV.K	1943.85992	1943.860827	-0.47

R.KVLGSSQNSSphosGSEASETPV.K	1943.85992	1943.863513	-1.85
R.KVLGSSQNSSphosGSEASETPV.K	1943.85992	1943.862536	-1.35
R.KVLGSSQNSSphosGSEASETPV.K	1943.85992	1943.860827	-0.47
R.KVLGSSQNSSphosGSEASETPV.K	1943.85992	1943.861804	-0.97
R.KVLGSSQNSSphosGSEASETPV.K	1943.85992	1943.861316	-0.72
R.KVLGSSQNSSphosGSEASETPV.K	1943.85992	1943.863513	-1.85
R.KVLGSSQNSSphosGSEASETPV.K	1943.85992	1943.86217	-1.16
R.KVLGSSQNSSphosGSEASETPV.K	1943.85992	1943.863147	-1.66
R.KVLGSSQNSSphosGSEASETPV.K	1943.85992	1943.863513	-1.85
R.KVLGSSQNSSphosGSEASETPV.K	1943.85992	1943.863025	-1.6
R.KVLGSSQNSSphosGSEASETPV.K	1943.85992	1943.86217	-1.16
R.KVLGSSQNSSphosGSEASETPV.K	1943.85992	1943.861316	-0.72
R.KVLGSSQNSSphosGSEASETPV.K	1943.85992	1943.85924	0.35
R.KVLGSSQNSSphosGSEASETPV.K	1943.85992	1943.861071	-0.59
R.KVLGSSQNSSphosGSEASETPV.K	1943.85992	1943.861926	-1.03
R.KVLGSSQNSSphosGSEASETPV.K	1943.85992	1943.861926	-1.03
R.KVLGSSQNSSphosGSEASETPV.K	1943.85992	1943.861926	-1.03
R.KVLGSSQNSSphosGSEASETPV.K	1943.85992	1943.86217	-1.16
R.KVLGSSQNSSphosGSEASETPV.K	1943.85992	1943.861194	-0.66
R.KVLGSSQNSSphosGSEASETPV.K	1943.85992	1943.86156	-0.84
R.KVLGSSQNSSphosGSEASETPV.K	1943.85992	1943.861804	-0.97
R.KVLGSSQNSSphosGSEASETPV.K	1943.85992	1943.862536	-1.35
L.GSSQNSSphosGSEASETPVK.R	1731.70744	1731.709094	-0.96
R.KVLGSSQNSSphosGSEA.S	1430.61644	1430.614856	1.11
S.SQNSSphosGSEASETPVKR.R	1743.75506	1743.752355	1.55
K.VLGSSQNSSphosGSEASETPVK.R	1943.85992	1943.858996	0.48
S.SQNSSphosGSEASETPVKR.R	1743.75506	1743.753271	1.03
S.SQNSSphosGSEASETPVKR.R	1743.75506	1743.755481	-0.24
L.SVESphosDRY.D	935.35117	935.34978	1.49
R.KVLGSSQNSSphosGSEASET.P	1747.73874	1747.740344	-0.92
R.KVLGSSQNSSphosGSEASET.P	1747.73874	1747.738025	0.41
R.KVLGSSQNSSphosGSEA.S	1430.61644	1430.614123	1.62
R.KVLGSSQNSSphosGSEA.S	1430.61644	1430.613757	1.88
R.KVLGSSQNSSphosGSEASET.P	1747.73874	1747.740344	-0.92
R.KVLGSSQNSSphosGSEASETPV.K	1943.85992	1943.860827	-0.47

MQScore	PhLocScore	File	Scan	PValue	PhosphoSite
3.504	18.4	010C_123	374	0.0337706	Ser95
3.359	18.4	010C_123	717	1.00E-05	Ser95
3.502	18.4	010C_123	729	0.0027716	Ser95
1.897	18.4	010C_123	800	0.0019685	Ser95
3.499	18.4	010C_123	829	0.000651	Ser95
3.514	18.4	010C_123	838	0.0032086	Ser95
2.737	47.2	010C_123	903	0.0027716	Ser94-Ser95
1.083	18.0	020C_123	633	0.0127737	Ser95
1.094	22.0	020C_123	721	0.0026339	Ser95
1.405	22.0	020C_123	726	0.0018536	Ser95
1.323	36.0	020C_123	845	0.0286487	Ser94-Ser95
2.485	30.6	040C_123	767	0.0649909	Ser97
1.302	38.7	040C_123	814	0.0060403	Ser94-Ser95
1.916	29.3	040C_123	815	0.0251673	Ser94-Ser97
3.197	25.5	100C_123	772	1.00E-05	Ser95
3.134	18.4	100C_123	775	0.0027716	Ser95
2.584	18.0	100C_123	814	0.0130197	Ser95
3.166	25.5	load001_123	1396	1.00E-05	Ser43-Ser48
2.38	32.9	load001_123	1811	0.0168628	Ser43-Ser48
3.804	18.4	load001_123	20	1.00E-05	Ser95
3.312	18.0	load001_123	3343	1.00E-05	Ser95
3.248	22.0	load001_123	3458	0.0006916	Ser95
3.467	18.4	load001_123	3596	0.0032086	Ser95
3.37	18.4	load001_123	3600	0.0137652	Ser95
3.412	18.4	load001_123	3702	1.00E-05	Ser95
3.457	22.0	load001_123	3710	0.0006154	Ser95
3.725	18.4	load001_123	4	1.00E-05	Ser95
3.478	18.4	load001_123	4051	1.00E-05	Ser95
3.314	18.4	load001_123	4163	0.0032086	Ser95
3.437	22.0	load001_123	4183	1.00E-05	Ser95
3.387	18.0	load001_123	4274	0.0027716	Ser95
3.468	18.4	load001_123	4412	1.00E-05	Ser95
3.154	22.0	load001_123	4524	1.00E-05	Ser95
3.337	18.4	load001_123	4664	1.00E-05	Ser95
3.059	22.0	load001_123	4933	0.000651	Ser95
2.574	18.4	load001_123	532	0.000651	Ser95
3.539	18.0	load001_123	727	1.00E-05	Ser95
3.656	18.0	load001_123	732	1.00E-05	Ser95
3.189	18.4	load001_123	746	1.00E-05	Ser95
3.486	18.4	load001_123	750	0.0017401	Ser95
3.053	18.4	load001_123	756	1.00E-05	Ser95
3.267	18.4	load001_123	761	0.0017401	Ser95
2.009	18.4	load001_123	791	0.0127737	Ser95
3.159	18.4	load001_123	793	0.0045965	Ser95
3.361	32.9	load002_123	1092	1.00E-05	Ser95
3.464	32.9	load002_123	1194	0.0006154	Ser95
3.476	32.9	load002_123	1278	1.00E-05	Ser95
3.405	25.5	load002_123	1284	1.00E-05	Ser95
1.184	22.0	load002_123	1311	0.0060403	Ser95
3.381	36.8	load002_123	1374	1.00E-05	Ser95
3.35	25.5	load002_123	1455	0.0027716	Ser95

3.477	36.8	load002_123	1537	1.00E-05	Ser95
3.467	32.9	load002_123	1543	1.00E-05	Ser95
3.441	32.9	load002_123	1627	1.00E-05	Ser95
3.384	36.8	load002_123	1705	1.00E-05	Ser95
3.47	32.9	load002_123	1711	0.000651	Ser95
3.439	36.8	load002_123	1783	1.00E-05	Ser95
3.327	36.8	load002_123	1789	0.0052506	Ser95
3.281	32.9	load002_123	1867	1.00E-05	Ser95
3.417	36.8	load002_123	1873	1.00E-05	Ser95
3.534	32.9	load002_123	1956	1.00E-05	Ser95
3.441	36.8	load002_123	2124	1.00E-05	Ser95
3.422	32.9	load002_123	2203	0.0006154	Ser95
3.444	36.8	load002_123	2209	1.00E-05	Ser95
3.484	36.8	load002_123	2292	1.00E-05	Ser95
3.471	36.8	load002_123	2444	1.00E-05	Ser95
3.328	36.8	load002_123	2448	1.00E-05	Ser95
3.485	32.9	load002_123	2527	1.00E-05	Ser95
3.441	36.8	load002_123	2532	0.0045965	Ser95
3.295	36.8	load002_123	2610	1.00E-05	Ser95
3.322	32.9	load002_123	2616	1.00E-05	Ser95
3.294	36.8	load002_123	2689	0.0052506	Ser95
3.405	32.9	load002_123	2773	1.00E-05	Ser95
3.662	18.4	load002_123	761	1.00E-05	Ser95
3.36	18.4	load002_123	765	1.00E-05	Ser95
1.244	18.0	load002_123	789	0.0286487	Ser95
3.638	18.4	load002_123	790	0.0137652	Ser95
1.219	18.0	load002_123	795	0.0242157	Ser95
3.402	18.4	load002_123	807	1.00E-05	Ser95
2.702	55.2	load002_123	808	0.0478853	Ser43
2.671	36.8	load002_123	827	0.0052506	Ser95
3.169	18.4	load002_123	831	1.00E-05	Ser95
3.404	18.4	load002_123	843	0.0052506	Ser95
3.496	18.4	load002_123	850	1.00E-05	Ser95
3.463	22.0	load002_123	911	1.00E-05	Ser95
3.222	36.8	load002_123	994	0.0032086	Ser95

Legend for Table

The PLS-Annotation column describes the identified phosphopeptide sequence with localized phosphorylation site(s). The next three columns are related: theoretical mass (MH) and experimental determined mass (MH) of each phosphopeptide as well as their relative difference in parts-per-million (ppm). Phosphopeptide identifications were accepted with a mass accuracy of 5ppm or less. The majority of identifications were 1-2ppm from theoretical mass (MH). Columns MQScore and PValue evaluate the quality of peptide-spectrum matching (MQScore) and its significance (PValue) derived after InsPecT searches against a concatenated database. PhLocScore column is the output PLS algorithm for each Scan from each file of the experiment. Following FASTA alignment, we assign the position of each site within the protein, as described on the last column.

Identification of protein phosphorylation sites.

We used an established an experimental routine for identification of phosphosites from protein complexes. Our implementation of MudPIT includes a repeated loading step before application of salt to facilitate separation of phosphopeptides from complex peptide mixtures derived from multiple proteases (Shimogawa et al., 2006). Our methodology identifies multiple phosphopeptides describing the same phosphorylation site and covers of protein sequence in excess of 80%.

Protein digestion and MudPIT

Precipitated protein complexes were solubilized in 50mM TEAB, 0.2% ProteaseMax (Promega), reduced with DTT and alkylated with iodoacetamide. Samples were split in three equal aliquots and digested with trypsin, elastase and subtilisin (MacCoss et al., 2002). Digestion was stopped by acidifying the samples. Pooled samples were loaded on a triphasic MudPIT precolumn RP(Polaris C18-A)/Poly SULFOETHYL /RP(Polaris C18-A).

We used a 2D Vented Column Setup with a Proxeon nano-flow HPLC pump (Taylor et al., 2009). The buffer solutions were 5% acetonitrile/0.1% formic acid (buffer A) and 90% acetonitrile/0.1% formic acid (buffer B) and 5%acetonitrile/0.1% formic/500mM ammonium acetate (buffer C). The triphasic column was connected to an analytical column of a 100- μ m i.d. capillary with a 5- μ m pulled tip and packed with 15cm of 3- μ m Aqua C18 material (Phenomenex, Ventura, CA). Phosphopeptides usually display low solution charge (Beausoleil et al., 2004). After the loading step, one more reversed phase gradient to was run to separate low solution charge phosphopeptides. A total of 5 salt steps delivered by autosampler were applied: 10%, 20%, 40% and 100% C. The last step included 90% C/ 10% B for complete elution of strong cation exchange resin (Motoyama et al., 2006). The flow rate was at 300nL/min (splitless). Peptides eluted from the microcapillary fritless column were directly electrosprayed into a linear ion trap-orbitrap (LTQ Orbitrap XL) mass spectrometer with the application of a distal 2.5 kV spray voltage. A cycle of one full-scan mass spectrum (400-1400 m/z) acquired followed by 5 data-dependent MS/MS spectra at 30% normalized collision energy was repeated

continuously throughout each step of the multidimensional separation. Full scan MS was acquired in orbitrap analyzer at resolution 60,000. Tandem MS2 were acquired in the linear ion trap analyzer.

Data-dependent MS2 files were extracted to mzXML using TPP v4.3 and searched using InsPecT (Tanner et al., 2005). We used the following database: UniprotKB E. Coli K12 supplement with BPV E1 helicase FASTA sequence. The forward database was concatenated to its decoy, and target-decoy database (Elias and Gygi, 2007) was searched with InsPecT.

Ph-report - software package

In order to characterize phosphorylation sites, we implemented a software solution referred to as Ph-report. Ph-report provides the minimal information required to localize a phosphorylation site. We started with peptide-spectrum matches provided by InsPecT (Tanner et al., 2005). The next steps are detailed as follows: i) estimate the significance of results (p-value) of peptide-spectrum identification using PValue.py from InsPecT package; ii) calculate theoretical mass for each significant peptide match using *ipc* (<http://isotopatcalc.sourceforge.net/index.php>) and compare with experimental derived mass; iii) calculate PLS score (Phosphate Localization Score) for each significant peptide match (Payne et al., 2008); iv) align the localized phosphorylation site using *fasta36* (<http://faculty.virginia.edu/wrpearson/fasta/fasta36/>, Mackey et al., 2002).

Ph-report provides several levels of filtering when analyzing phosphopeptides: a) mass accuracy of precursor ion (ppm value), b) quality of peptide-spectrum match score (MQ score) derived from InsPecT, c) PLS score derived from PhosphateLocalization score. Lastly, the identified spectrum is displayed with annotated b and y sequencing ions as well phosphate neutral losses from precursor.

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