Protocol

Proteomics Phospho Kit

Collection of 24 non-naturally occurring phosphopeptides for standardization of phosphoproteomics workflows and retention times in HPLC-MS based phosphoproteomics experiments

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1 Introduction

Phosphoproteomics has matured into a strong technology for the simultaneous detection of phosphopeptides in complex samples. However, phosphoproteomics workflows consist of several steps that all need to be controlled carefully (Urban, J., *Analytica Chimica Acta* **2021**, 338857). If phosphoproteomics is performed in a targeted fashion, HPLC retention time (RT) windows for the selected proteotypic phosphopeptides need to be established carefully, as RTs are not easily transferable between instruments.
The best method to control phosphoproteomics workflows and to define RT windows is through phosphopeptide standard substances.

For the generation of an optimized set of phosphopeptide RT standards, suitable candidate peptides from the PROCAL set of RT calibration peptides (Zolg, D.P. et al. *Proteomics* **2017**, pmic.201700263) were converted to their respective pY peptides *in silico*. 96 peptides were synthesized and analyzed by LC-MS/MS on an Orbitrap Fusion Lumos (Thermo) instrument. After careful examination of each peptide and selection of the best suited candidates, a set of 24 peptides was defined. Aliquots of 10 or 100 pmol/peptide were pooled into the new Proteomics Phospho Kit.
Figure 1 shows a typical LC-MS chromatogram of the peptide kit. The peptides displayed high Andromeda scores, overall good LC-MS characteristics (relatively sharp peak shapes), and a high stability of RTs over multiple injections.

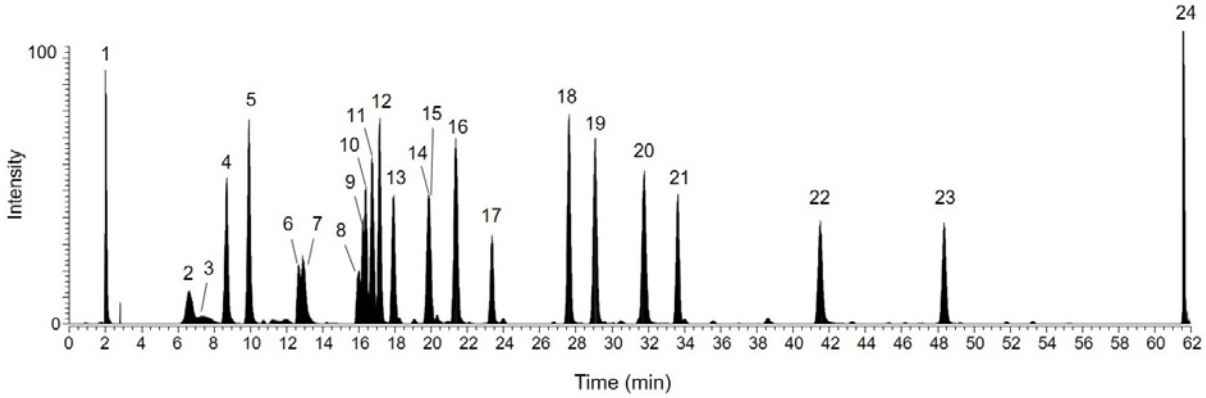

Figure 1: Typical LC-MS chromatogram of the Proteomics Phospho Kit using the following LC-MS conditions: A Dionex UltiMate 3000 RSLCnano System equipped with a Vanquish pump module and coupled to a Fusion Lumos Tribrid mass spectrometer (Thermo Fisher Scientific) was operated under micro-flow conditions. Peptides were dissolved in 1% FA (formic acid) in water, and 500 fmol of peptides were injected directly onto a commercially available Acclaim PepMap 100 C18 LC column (2 µm particle size, 1 mm ID × 150 mm; Thermo Fisher Scientific). Peptides were separated at a flow rate of 50 µl/min and using a 60 min linear gradient of 1 to 15% micro-flow solvent B (0.1% formic acid and 3% DMSO in ACN) in micro-flow solvent A (0.1% formic acid and 3% DMSO in H_2O).

In summary, an optimized set of reference peptides for phosphoproteomics was developed that features several advantages:

- Non-naturally occurring peptides (allowing application in biological samples)
- Non-isotopically labeled peptides (facilitating inclusion into database searches)
- Broad LC gradient coverage
- Stable RTs across multiple injections
- Use for column performance evaluation
- Use for optimization of LC-MS gradients

2 List of Components

Polypropylene vial with a pool of the following peptides:

No.	Sequence	M(monoiso)	[M+H]+	[M+2H]2+	[M+3H]3+	Score*	Observed RT**
1	ySAHEEHHYDK	1494.55651	1495.56	748.29	499.19	195.51	9.29
2	YFGyTSDTFGK	1364.53259	1365.54	683.27	455.85	121.73	26.91
3	LSSGyDGTSYK	1256.49621	1257.50	629.26	419.84	42.34	12.54
4	yGIFHDEGGGK	1258.50194	1259.51	630.26	420.51	166.09	18.86
5	yGETTTSSELK	1294.53299	1295.54	648.27	432.52	245.50	13.19
6	yDLHHSTDEIK	1436.59731	1437.61	719.31	479.87	197.79	12.11
7	yDFSTHEDHDK	1472.52454	1473.53	737.27	491.85	223.36	10.60
8	FyEDTGEDGLK	1352.51732	1353.53	677.27	451.85	202.44	19.55
9	HyTEELLSTVK	1398.64320	1399.65	700.33	467.22	236.32	25.42
10	HAyEGSFDVGK	1288.51251	1289.52	645.26	430.51	190.02	16.71
11	AGySEELHDDK	1342.50782	1343.52	672.26	448.51	254.50	10.83
12	VVyDEDLHDDK	1426.56533	1427.57	714.29	476.53	195.36	13.04
13	HTAySDFLSDK	1362.54930	1363.56	682.28	455.19	163.38	22.74
14	HFSyEGHSVDK	1384.54488	1385.55	693.28	462.52	138.76	10.52
15	FDEyVADVHSK	1388.56494	1389.57	695.29	463.86	265.31	18.03
16	TLDSyVSGHEK	1314.54930	1315.56	658.28	439.19	221.68	15.01
17	VDSGTyFSESK	1298.50677	1299.51	650.26	433.84	250.92	15.79
18	GGGEDyHLFDK	1316.50742	1317.52	659.26	439.84	166.09	21.11
19	TGEDLyDDHTK	1372.51839	1373.53	687.27	458.51	270.06	11.03
20	HFGADySHLEK	1382.56561	1383.57	692.29	461.86	157.66	14.39
21	IHDFSyTSDTK	1392.55987	1393.57	697.29	465.19	97.45	14.47
22	SGGFSHyDLDK	1304.50743	1305.52	653.26	435.84	200.71	17.53
23	VITGTEyGFFK	1340.60535	1341.61	671.31	447.88	157.99	33.69
24	TSEEEHyVHIK	1450.61296	1451.62	726.31	484.55	153.24	13.27

* Andromeda score. ** Observed retention time (average from 6 replicate injections) using the following LC-MS conditions: 60 min gradient, 4 to 42% elution solvent (solvent A: 1 % formic acid in H2O, elution solvent B: 5 % DMSO in MeCN).

3 Storage

- The Retention Time Standardization Kit should be stored at -20°C. Once constituted, it should be stored at 4 °C for short term storage and stored at -20 °C for longer term storage. The constituted kit should be used up within 3 months.

PLEASE READ THE ENTIRE PROTOCOL BEFORE STARTING YOUR EXPERIMENTS! PLEASE CONTACT JPT PEPTIDE TECHNOLOGIES´ TECHNICAL SERVICES FOR ASSISTANCE IF NECESSARY.

4 Experimental Protocol

1. Kit reconstitution (dissolution): Add 100 µL of 1% FA (formic acid) in water for dissolution of the kit (use this volume for both available sizes – the 100 pmol/peptide kit and the 10 pmol/peptide kit).

2. After addition of the solvent, promote the solubilization process by vortexing the vial for 60 seconds followed by sonication for 30 seconds. This furnishes a 1 pmol/µL/peptide stock solution (for the 100 pmol/peptide kit) or a 100 fmol/µL/peptide stock solution (for the 10 pmol/peptide kit) that can be further aliquoted and diluted. It is not recommended to further dilute the stock solutions for storage due to the possibility of unspecific binding of peptides to glass and plastic material when stored at low concentrations.

3. For the 100 pmol/peptide kit (stock solution concentration 1 pmol/µL/peptide) dilute an aliquot of the solution (5 µL) with 1% FA in water (45 µL) to obtain a ready to use solution with a concentration of 100 fmol/µL/peptide.
   For the 10 pmol/peptide kit (stock solution concentration 100 fmol/µL/peptide) the stock solution is already ready to use for LCMS.

4. Optionally add the solution from step 3 to a sample followed by sample cleanup using established protocols like Stage Tip based reverse phase C18 desalting.

5. Inject the solution from step 3 or 4 into the LC-MS instrument. The optimal amount of peptide to be injected depends on the sensitivity of the used LC-MS instrument. When using a highly sensitive Orbitrap instrument, good signal to noise ratios are obtained with injected amounts of 100-500 fmol/peptide.

6. Use suitable database search software (e.g. Mascot, MaxQuant, Sequest) for data analysis by adding the sequences to your target database or using the fasta file that is provided upon request.