Protocol

Retention Time Standardization Kit

Collection of 40 non-naturally occurring peptides for standardization of retention times in HPLC-MS based proteomics experiments

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Product Use & Liability

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1 Introduction

Targeted proteomics (e.g. the development of SRM/MRM assays) is dependent on the definition of HPLC retention time (RT) windows for the selected proteotypic peptides.[1] RT window definition is however hampered by the fact that RTs are not easily transferable between different labs and can also considerably vary within laboratories (e.g. on different HPLC-MS instruments).

The best method to define RT windows is through calibration of RTs by standard substances. Although a number of retention time standards have been described, many of them display limitations. Those range from narrow RT windows (meaning that the difference between the RT of the most hydrophilic and of the most hydrophobic peptide is comparably low), limited peptide numbers and restricted usability for column performance evaluation, up to the fact that they often contain naturally occurring sequences prohibiting spike-in into biological samples.

For the generation of an optimized set of peptide RT standards, suitable candidate peptides were identified through an iterative selection process. The process started from 10,000 *in silico* generated non-naturally occurring peptide sequences followed by iterative steps of synthesis and experimental examination by LC-MS/MS on an Orbitrap Fusion Lumos (Thermo) instrument. As result of the comprehensive examination of each peptide, a set of 40 peptides was defined. The peptides were pooled in aliquots of 10 or 100 pmol/peptide to furnish the new Retention Time Standardization Kit.

Figure 1a and 1b show typical LC-MS chromatograms of the peptide kit for two different gradients. The peptides were compiled to yield a broad and even coverage of RTs. In addition, the Retention Time Standardization Kit proved to have good detectability (high Andromeda scores) of each peptide, good LC-MS characteristics (sharp peak shapes), and a high stability of RTs over multiple injections (Figure 1c). Beside its application to RT normalization, the peptide set can also be used for the evaluation of HPLC column performance. This is exemplified in Figure 1d where two pairs of peptides could not be separated on a used column (upper trace) but were separable by using a fresh column (lower trace).

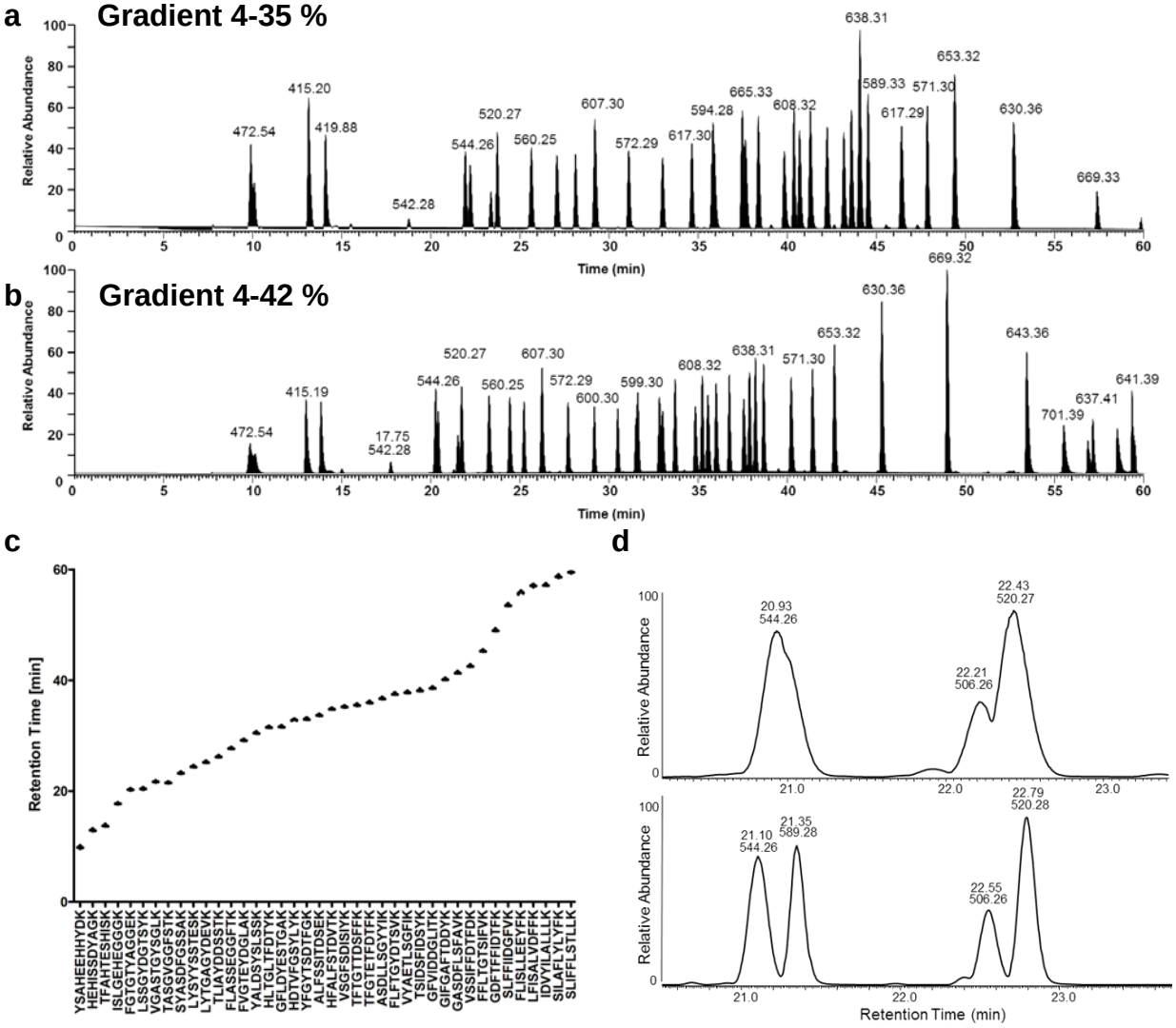

Figure 1: a) Typical LC-MS chromatogram of the Retention Time Standardization Kit using the following LC-MS conditions: 3 µm C18 LC column, column length 47 cm, 60 min gradient, 4 to 35 % elution solvent (solvent A: 1 % formic acid in H_2O, elution solvent B: 5 % DMSO in MeCN). b) Typical LC-MS chromatogram of the Retention Time Standardization Kit using the following LC-MS conditions: 60 min gradient, 4 to 42 % elution solvent (solvent A and elution solvent B as above). To ensure the detectability of the most hydrophobic peptides, the Kit was dissolved in 100 % DMSO followed by dilution to 10 % DMSO immediately before LC-MS/MS measurement. c) RT stability of all peptides of the set over 10 LC-MS injections (all data points shown). d) Representation of the elution peaks of the peptides FGTGTYAGGEK, LSSGYDGTSYK, SYASDFGSSAK and VGASTGYSGLK using a used (top) and a fresh (bottom) HPLC column.

Based on the observed favorable properties, peptides of the Retention Time Standardization Kit were spiked into every of the >1200 peptide pools generated in the course of the ProteomeTools project.[2] This allowed the calculation of RT indices for all peptides of the project thus ensuring the transferability of RTs between laboratories.

In summary, an optimized set of HPLC reference standard peptides was developed that features the following advantages:

- Non-naturally occuring peptides (allowing application in all sorts of biological samples)
- Non-isotopically labeled peptides (facilitating inclusion into database searches)
- Broader LC gradient coverage than other previously described RT calibration sets
- Higher number of peptides than other previously described RT calibration sets
- Very stable RTs across multiple injections
- Use for column performance evaluation
- Use for optimization of LC-MS gradients
- Use for transfer of RTs to other LC systems/gradients/laboratories
- Intensive use in ProteomeTools project

References

[1] Picotti, P. et al., *Nat. Methods* **2012**, *9(6)*, 555-566.

[2] Zolg, D. P. et al., *Nat. Methods* **2017**, accepted.

2 List of Components

Polypropylene vial with a pool of the following peptides:

No.	Sequence	M	$[M+H]^{+}$	$[M+2H]^{2+}$	$[M+3H]^{3+}$	Score*	HI(pred.)**	RT***
1	YSAHEEHHYDK	1414.59	1415.60	708.30	472.54	202.68	-1.95	9.93
2	HEHISSDYAGK	1242.56	1243.57	622.29	415.19	265.82	0.26	13.01
3	TFAHTESHISK	1256.61	1257.62	629.31	419.88	272.32	2.03	13.81
4	ISLGEHEGGGK	1082.54	1083.54	542.28	361.85	201.46	2.31	17.76
5	LSSGYDGTSYK	1176.53	1177.54	589.27	393.18	130.10	4.46	20.47
6	FGTGTYAGGEK	1086.50	1087.51	544.26	363.17	141.44	4.47	20.32
7	VGASTGYSGLK	1038.53	1039.54	520.27	347.19	138.76	4.89	21.76
8	TASGVGGFSTK	1010.50	1011.51	506.26	337.84	151.79	5.34	21.57
9	SYASDFGSSAK	1118.49	1119.50	560.25	373.84	128.88	5.79	23.32
10	LYSYYSSTESK	1326.60	1327.61	664.31	443.21	145.28	6.16	24.47
11	LYTGAGYDEVK	1214.58	1215.59	608.30	405.87	83.18	6.97	25.28
12	TLIAYDDSSTK	1212.59	1213.60	607.30	405.20	132.32	7.37	26.28
13	HLTGLTFDTYK	1294.66	1295.66	648.34	432.56	136.96	8.61	31.59
14	FLASSEGGFTK	1142.56	1143.57	572.29	381.86	117.52	8.80	27.76
15	GFLDYESTGAK	1186.55	1187.56	594.28	396.52	124.67	8.85	31.69
16	ALFSSITDSEK	1196.59	1197.60	599.30	399.87	134.30	9.04	33.75
17	FVGTEYDGLAK	1198.59	1199.59	600.30	400.54	147.74	9.24	29.24
18	YALDSYSLSSK	1232.59	1233.60	617.30	411.87	145.91	9.32	30.56
19	HDTVFGSYLYK	1328.64	1329.65	665.33	443.89	236.69	9.39	32.87
20	YFGYTSDTFGK	1284.57	1285.57	643.29	429.20	109.11	9.44	33.09
21	HFALFSTDVTK	1264.65	1265.65	633.33	422.56	256.96	9.73	34.89
22	TFTGTTDSFFK	1250.58	1251.59	626.30	417.87	139.31	11.41	35.60
23	VSGFSDISIYK	1214.62	1215.63	608.32	405.88	124.42	11.53	35.28
24	TFGTETFDTFK	1292.59	1293.60	647.30	431.87	102.40	11.98	36.07
25	TSIDSFIDSYK	1274.60	1275.61	638.31	425.87	72.29	12.01	38.25
26	ASDLLSGYYIK	1228.63	1229.64	615.32	410.55	137.89	12.11	36.80
27	FLFTGYDTSVK	1276.63	1277.64	639.32	426.55	83.862	12.33	37.61
28	GIFGAFTDDYK	1232.57	1233.58	617.29	411.86	115.57	12.74	40.26
29	VYAETLSGFIK	1226.65	1227.66	614.33	409.89	101.39	13.06	37.91
30	GFVIDDGLITK	1176.64	1177.65	589.33	393.22	69.72	13.26	38.68
31	GASDFLSFAVK	1140.58	1141.59	571.30	381.20	138.25	14.55	41.43
32	FFLTGTSIFVK	1258.70	1259.70	630.36	420.57	152.54	15.95	45.36
33	VSSIFFDTFDK	1304.63	1305.64	653.32	435.88	121.02	16.66	42.66
34	GDFTFFIDTFK	1336.63	1337.64	669.32	446.55	196.44	19.58	49.11
35	LFISALVDFFK	1298.73	1299.74	650.37	433.92	115.29	22.72	57.19
36	SLFFIIDGFVK	1284.71	1285.72	643.36	429.24	133.89	22.74	53.64
37	IDVYILALLLK	1272.81	1273.81	637.41	425.28	127.83	23.47	57.27
38	SILAFLYLYFK	1376.77	1377.78	689.39	459.93	134.84	23.62	58.84
39	SLIFFLSTLLK	1280.77	1281.78	641.39	427.93	129.82	23.70	59.53
40	FLISLLEEYFK	1400.76	1401.77	701.39	467.93	175.15	25.16	55.87

* Andromeda score. ** SSRCalcQ: HI (predicted) (100Å C18 column, 0.1 % Formic Acid, http://hs2.proteome.ca/SSRCalc/SSRCalcQ.html). *** Observed retention time using the following LC-MS conditions: 3 µm C18 LC column, column length 47 cm, 60 min gradient, 4 to 42 % elution solvent (solvent A: 1 % formic acid in H2O, elution solvent B: 5 % DMSO in MeCN), average retention time from 11 replicate injections.

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): In general, the kit is compatible with various LC-MS solvents:

   a) In case you do not require the six most hydrophobic peptides for your retention time calibration, you can use purely aqueous solutions for dissolution, e. g. 0.1 % formic acid in water.

   b) In case you prefer to include the six most hydrophobic peptides in your retention time calibration, it is suggested to use an organic co-solvent to resuspend the peptides. 30 % Acetonitrile in water has been successfully tested for resuspension. Alternatively, it was shown that dissolution of the kit in 100 % DMSO followed by dilution to 10 % DMSO immediately before injection is a suitable way to dissolve all peptides.

   Add 100 µL of solvent 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 to experience unspecific binding of peptides to glass and plastic material when stored in 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 your LC-MS solvent A (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. If you are using Skyline, you can use the predefined template that can also be provided upon request.