Supporting Information

SIMPATIQCO: A Server-Based Software Suite Which Facilitates Monitoring the Time Course of LC-MS Performance Metrics on Orbitrap Instruments

Peter Pichler[1,2,*,+], Michael Mazanek[3,*], Frederico Dusberger[1], Lisa Weilnböck[4], Christian G. Huber[4], Christoph Stingl[5], Theo M. Luider[5], Werner L. Straube[1], Thomas Köcher[1], Karl Mechtler[1,3,+]

[1]Research Institute of Molecular Pathology, Vienna, Austria

[2]Christian Doppler Laboratory for Proteome Analysis, University of Vienna, Austria

[3]Institute of Molecular Biotechnology, Vienna, Austria

[4]Department of Molecular Biology, University of Salzburg, Austria

[5]Department of Neurology, Erasmus MC, Rotterdam, The Netherlands

[*] equally contributing first authors

[+] corresponding authors

Peter Pichler

Research Institute of Molecular Pathology

Dr. Bohr-Gasse 7, 1030 Vienna, Austria

Email: peter.pichler@imp.ac.at

Phone: +43-1-79044-4293

Fax: +43-1-79044-110

Karl Mechtler

Research Institute of Molecular Pathology

Dr. Bohr-Gasse 7, 1030 Vienna, Austria

Email: karl.mechtler@imp.ac.at

Phone: +43-1-79044-4280

Fax: +43-1-79044-110

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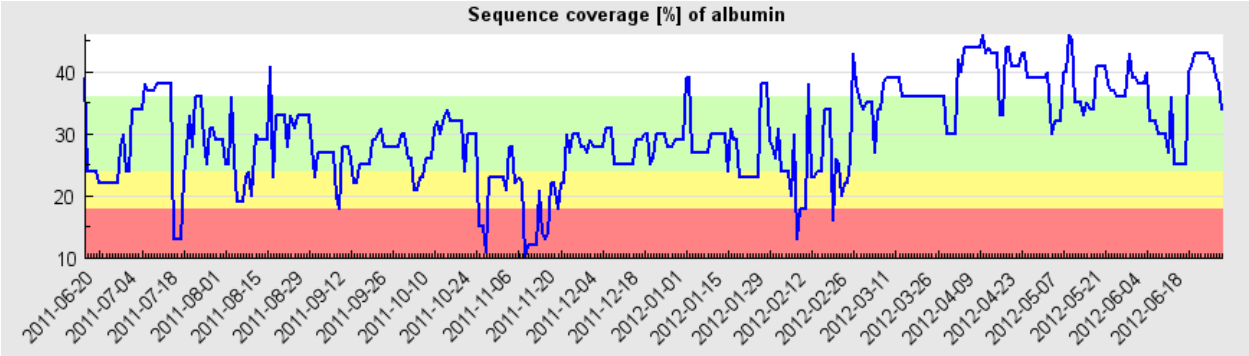
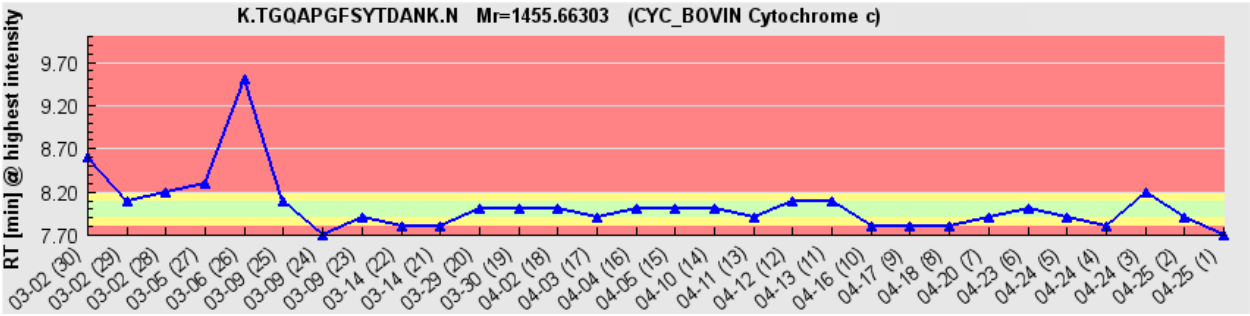
Laboratory A

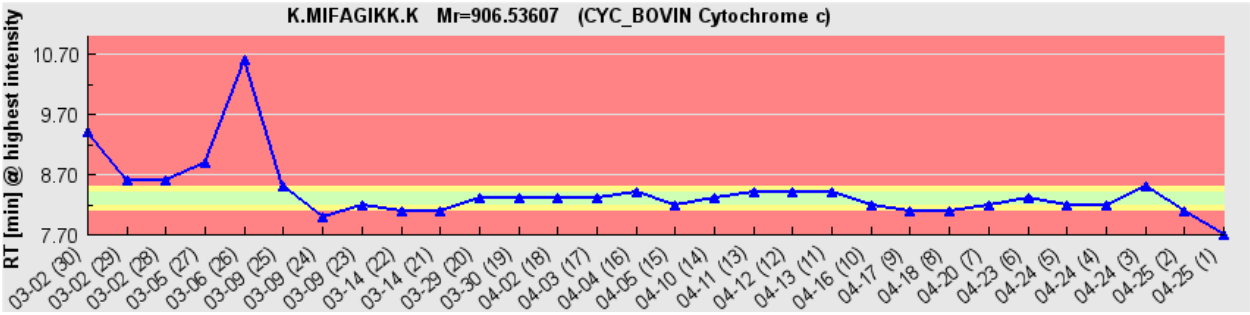
Instrument 1: Orbitrap XL ETD

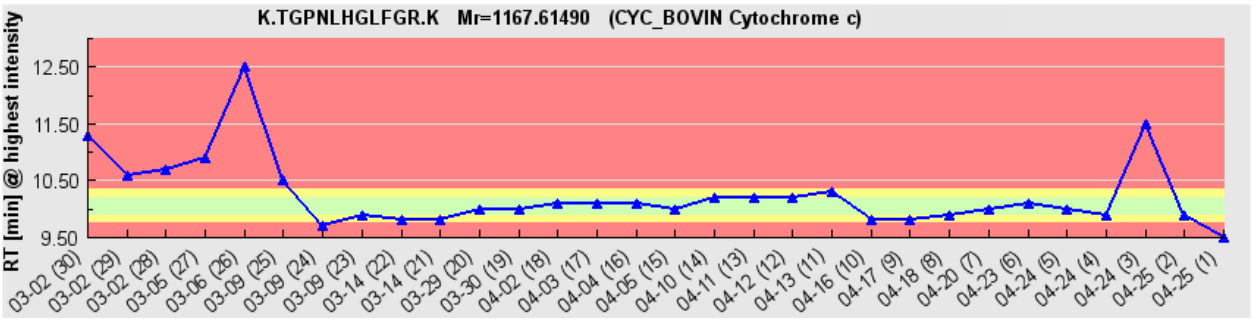
QC 1 sample: BSA (2.5 fmol) sequence coverage

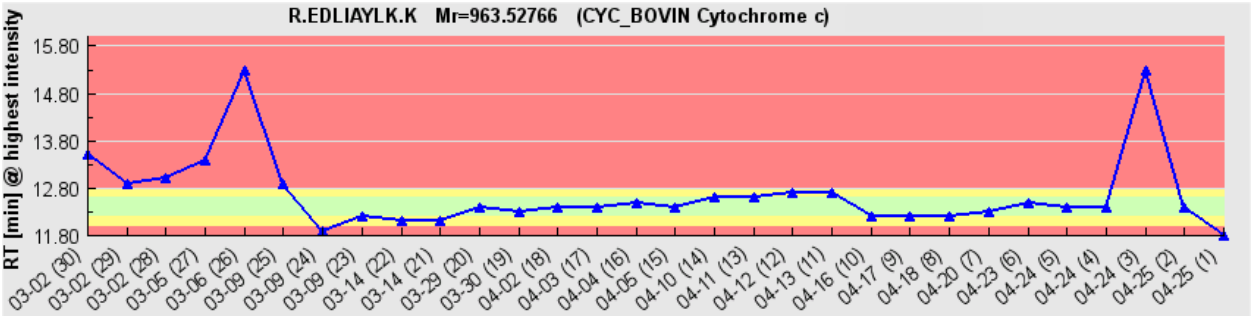


QC 1 sample: retention times of cytochrome c peptides (500 fmol)

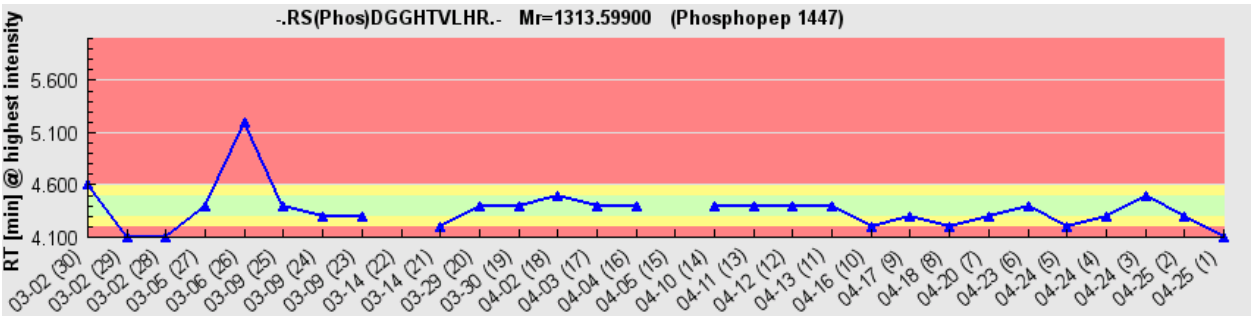


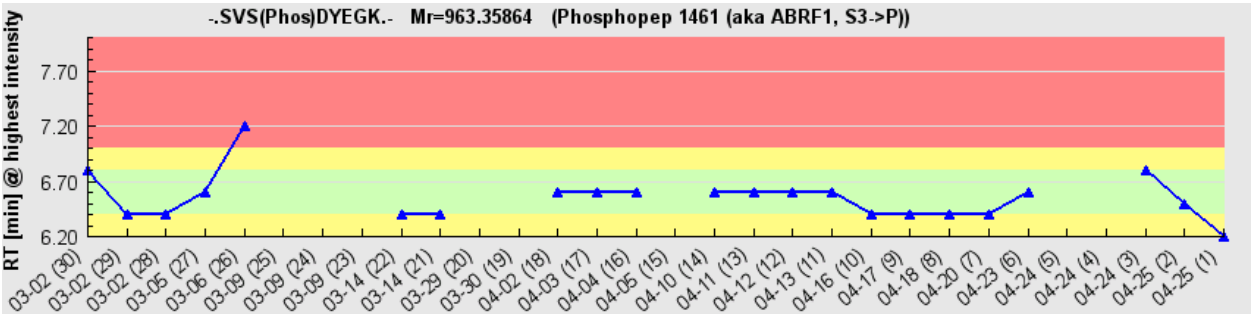


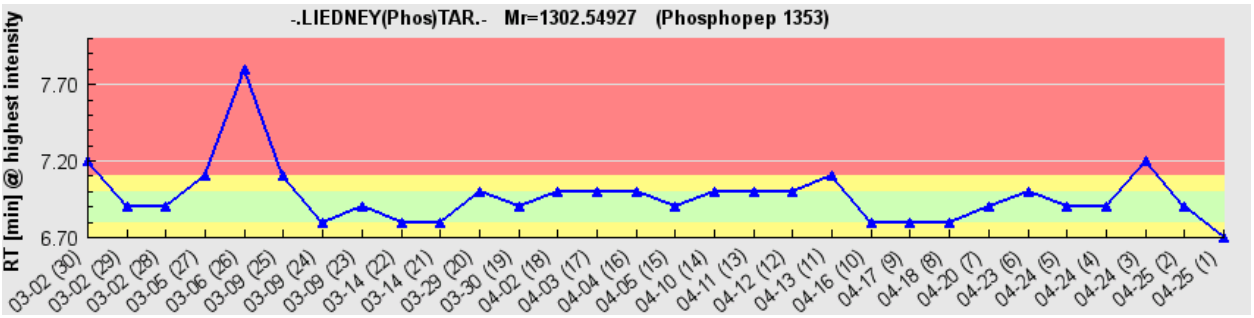


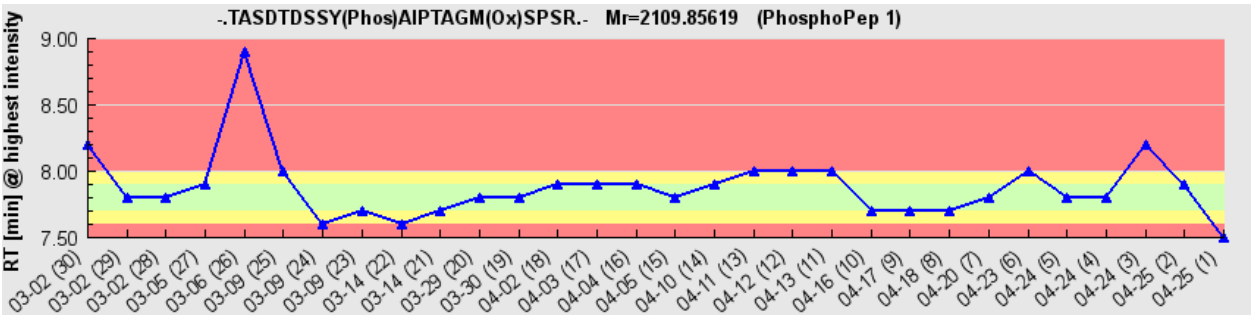


QC 1 sample: retention times of phosphopeptides (2.5 fmol)






-.LIEDNEY(Phos)TAR.- Mr=1302.54927 (Phosphopep 1353)
RT [min] @ highest intensity
7.70
7.20
6.70
03-02 (30)
03-02 (29)
03-02 (28)
03-05 (27)
03-06 (26)
03-09 (25)
03-09 (24)
03-09 (23)
03-14 (22)
03-14 (21)
03-29 (20)
03-30 (19)
04-02 (18)
04-03 (17)
04-04 (16)
04-05 (15)
04-10 (14)
04-11 (13)
04-12 (12)
04-13 (11)
04-16 (10)
04-17 (9)
04-18 (8)
04-20 (7)
04-23 (6)
04-24 (5)
04-24 (4)
04-24 (3)
04-25 (2)
04-25 (1)


-.TASDTDSSY(Phos)AIPTAGM(Ox)SPSR.- Mr=2109.85619 (PhosphoPep 1)
RT [min] @ highest intensity
9.00
8.50
8.00
7.50
03-02 (30)
03-02 (29)
03-02 (28)
03-05 (27)
03-06 (26)
03-09 (25)
03-09 (24)
03-09 (23)
03-14 (22)
03-14 (21)
03-29 (20)
03-30 (19)
04-02 (18)
04-03 (17)
04-04 (16)
04-05 (15)
04-10 (14)
04-11 (13)
04-12 (12)
04-13 (11)
04-16 (10)
04-17 (9)
04-18 (8)
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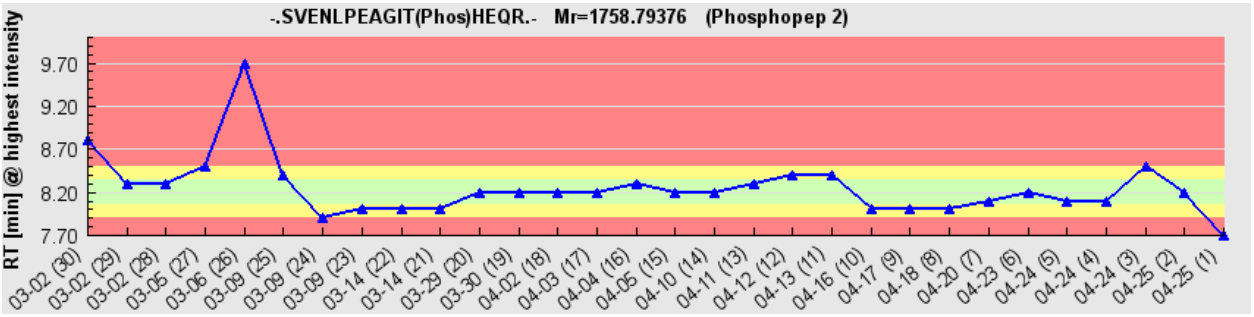
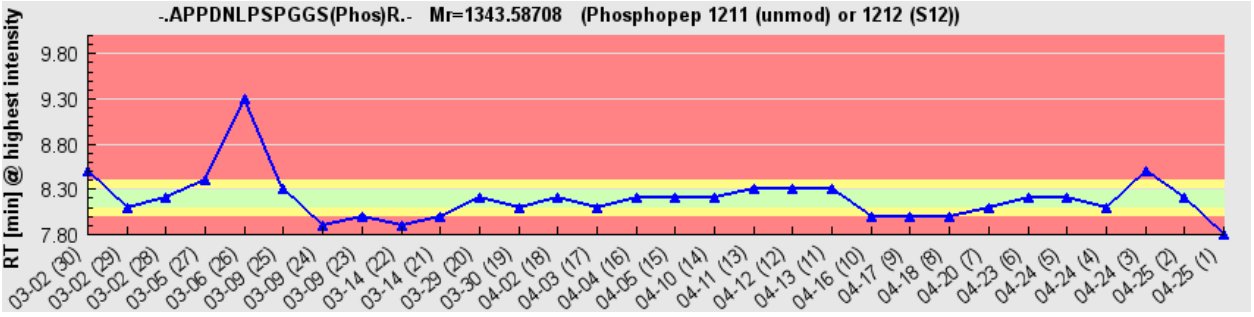
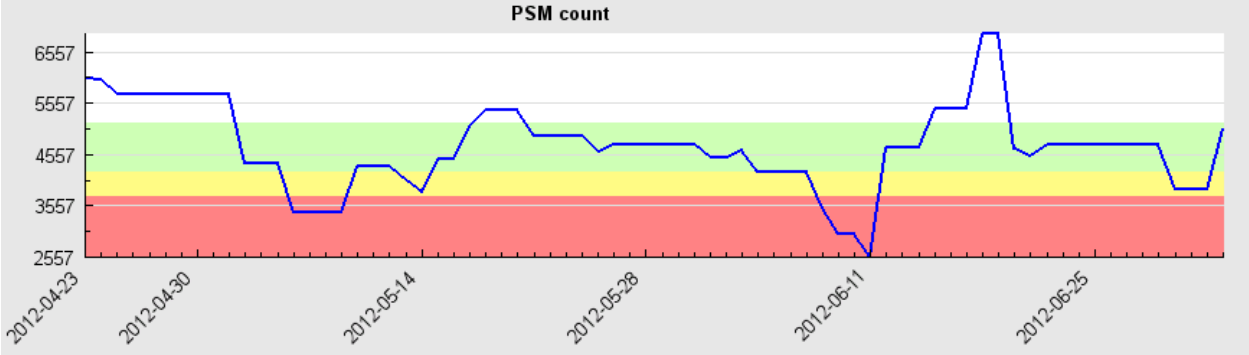
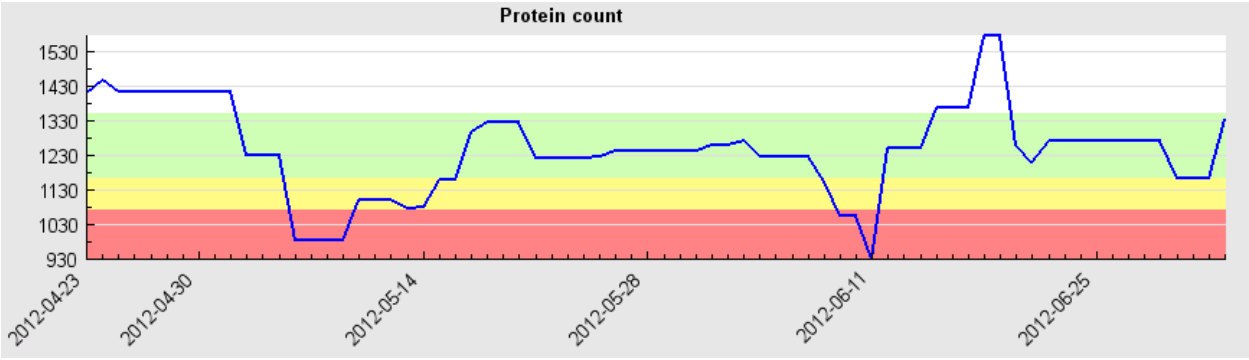
04-24 (4)
04-24 (3)
04-25 (2)
04-25 (1)


-.SVENLPEAGIT(Phos)HEQR.- Mr=1758.79376 (Phosphopep 2)
RT [min] @ highest intensity
9.70
9.20
8.70
8.20
7.70
03-02 (30)
03-02 (29)
03-02 (28)
03-05 (27)
03-06 (26)
03-09 (25)
03-09 (24)
03-09 (23)
03-14 (22)
03-14 (21)
03-29 (20)
03-30 (19)
04-02 (18)
04-03 (17)
04-04 (16)
04-05 (15)
04-10 (14)
04-11 (13)
04-12 (12)
04-13 (11)
04-16 (10)
04-17 (9)
04-18 (8)
04-20 (7)
04-23 (6)
04-24 (5)
04-24 (4)
04-24 (3)
04-25 (2)
04-25 (1)

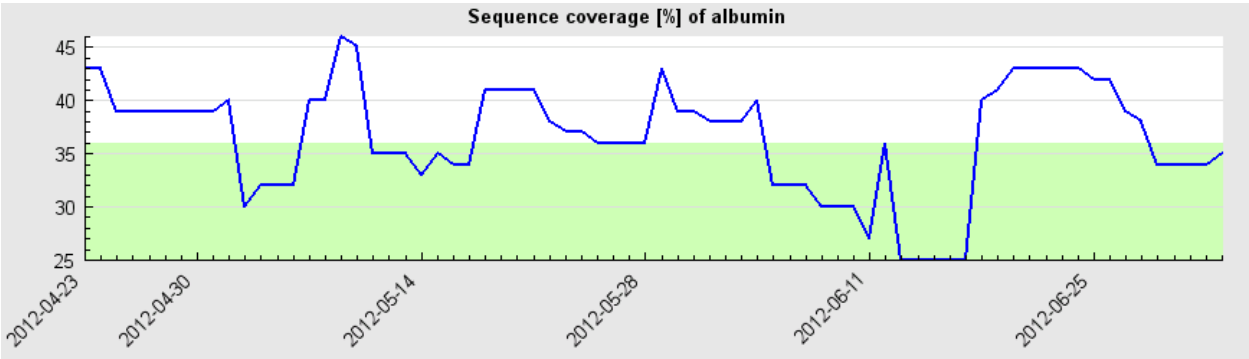

-.APPDNLPSPGGS(Phos)R.- Mr=1343.58708 (Phosphopep 1211 (unmod) or 1212 (S12))
RT [min] @ highest intensity
9.80
9.30
8.80
8.30
7.80
03-02 (30)
03-02 (29)
03-02 (28)
03-05 (27)
03-06 (26)
03-09 (25)
03-09 (24)
03-09 (23)
03-14 (22)
03-14 (21)
03-29 (20)
03-30 (19)
04-02 (18)
04-03 (17)
04-04 (16)
04-05 (15)
04-10 (14)
04-11 (13)
04-12 (12)
04-13 (11)
04-16 (10)
04-17 (9)
04-18 (8)
04-20 (7)
04-23 (6)
04-24 (5)
04-24 (4)
04-24 (3)
04-25 (2)
04-25 (1)

QC2 sample: peptide-spectrum matches and proteins HeLa (0.1 µg)



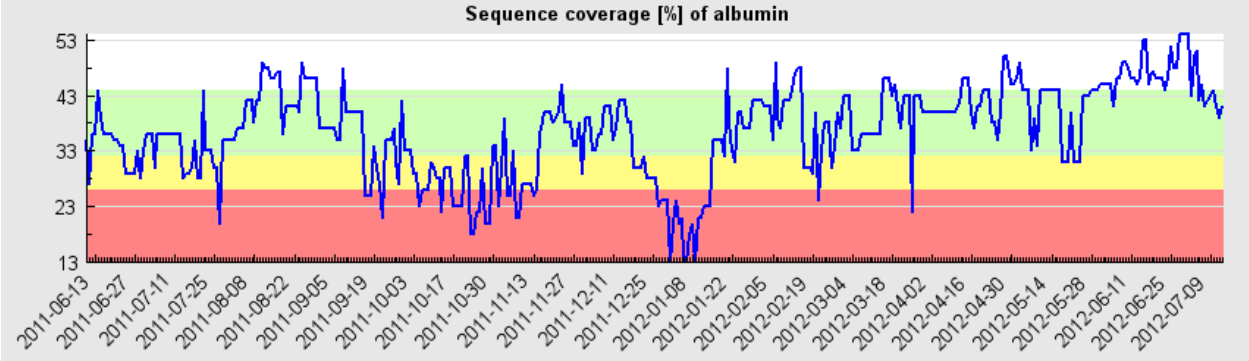


For comparison, same time period, QC 1 sample: BSA (2.5 fmol) sequence coverage

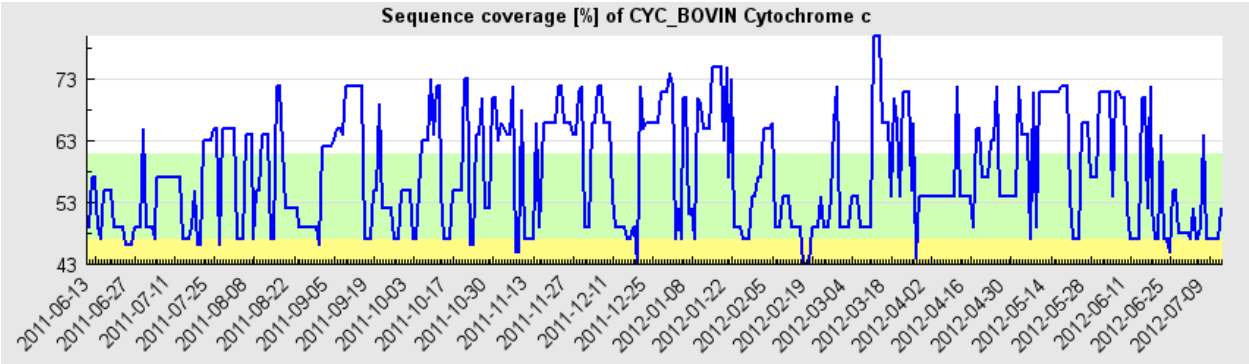


Instrument 2: Velos Orbitrap ETD

QC 1 sample: BSA (1 fmol) sequence coverage

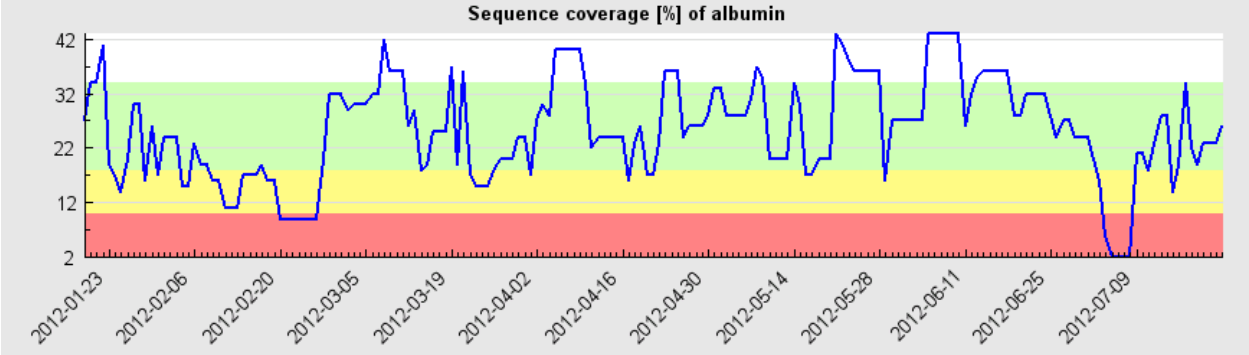


For comparison, same time period, QC 1 sample: cytochrome c (500 fmol) sequence coverage



Instrument 3: Q-Exactive

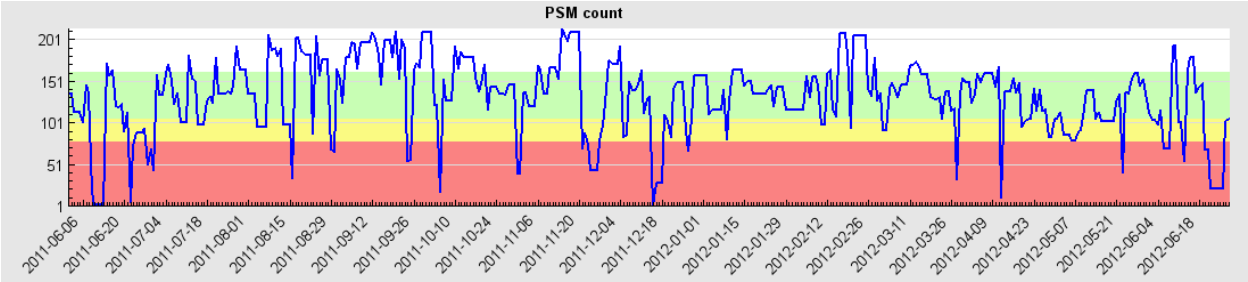
QC 1 sample: BSA (500 amol) sequence coverage



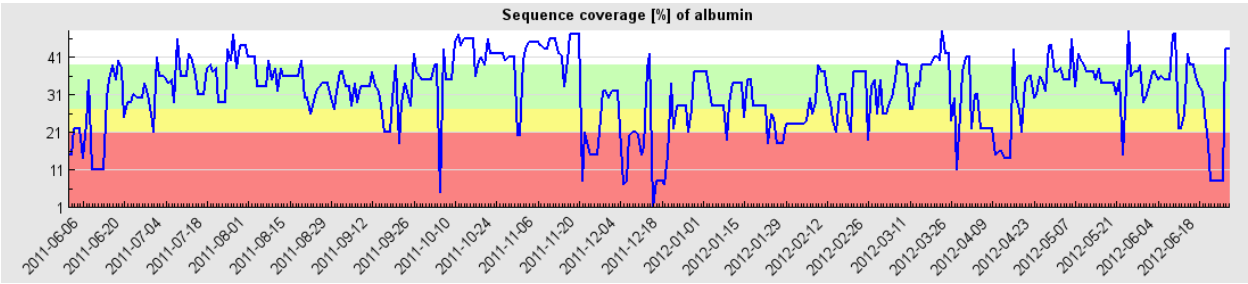
Laboratory B

Instrument 4: Velos Orbitrap ETD

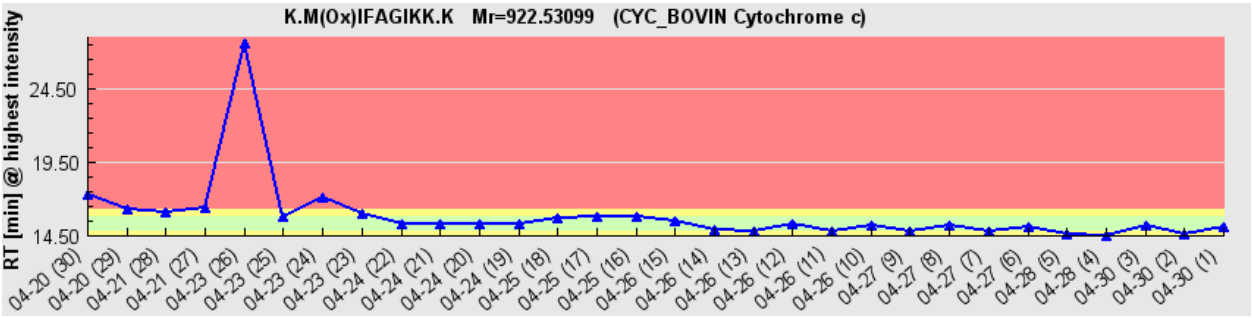
QC sample: peptide-spectrum matches (500 fmol cytochrome c, 1 fmol BSA)

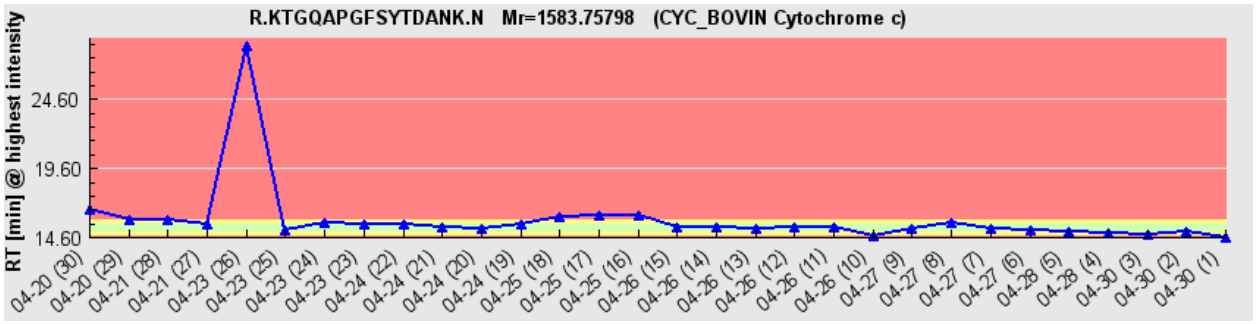


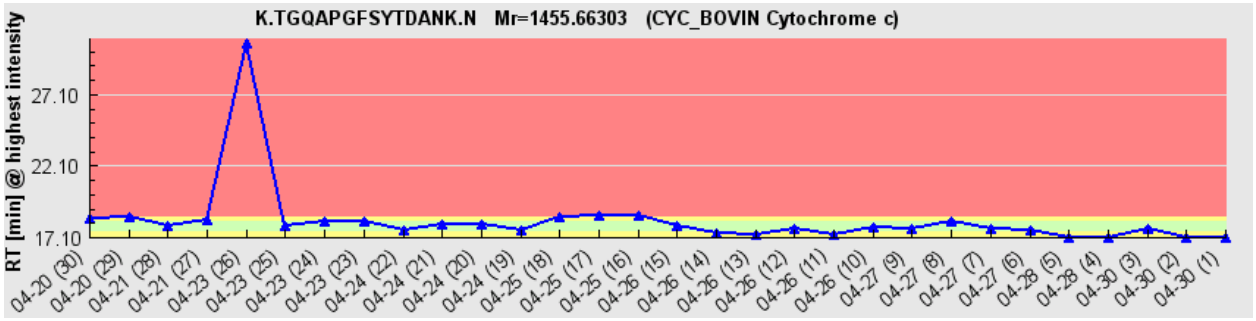
QC sample: BSA (1 fmol) sequence coverage

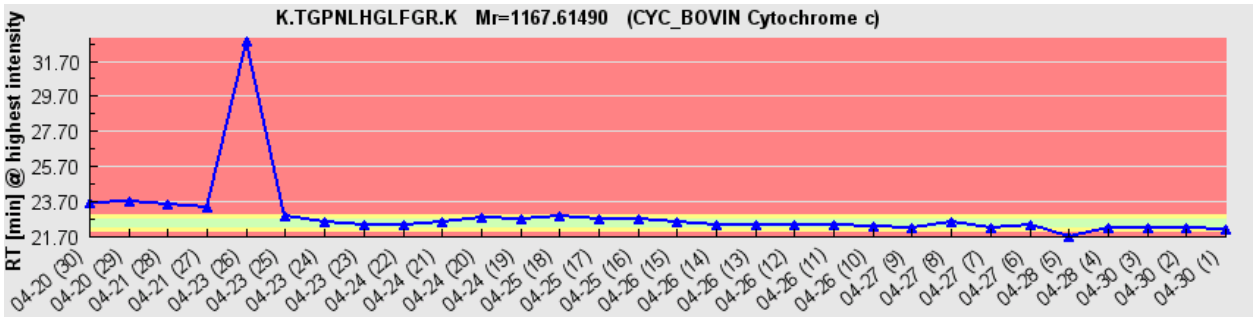


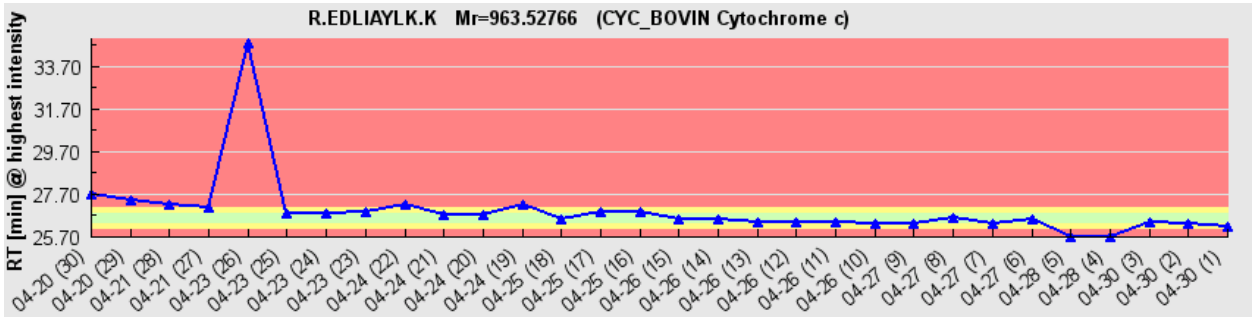
QC sample: retention times of cytochrome c peptides (500 fmol)




R.KTGQAPGFSYTDANK.N Mr=1583.75798 (CYC_BOVIN Cytochrome c)
RT [min] @ highest intensity
24.60
19.60
14.60


K.TGQAPGFSYTDANK.N Mr=1455.66303 (CYC_BOVIN Cytochrome c)
RT [min] @ highest intensity
27.10
22.10
17.10

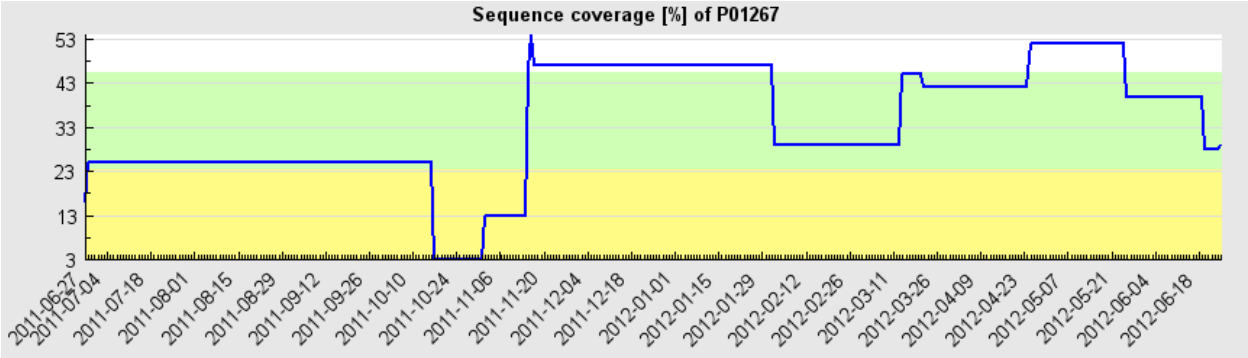

K.TGPNLHGLFGR.K Mr=1167.61490 (CYC_BOVIN Cytochrome c)
RT [min] @ highest intensity
31.70
29.70
27.70
25.70
23.70
21.70


R.EDLIAYLK.K Mr=963.52766 (CYC_BOVIN Cytochrome c)
RT [min] @ highest intensity
33.70
31.70
29.70
27.70
25.70

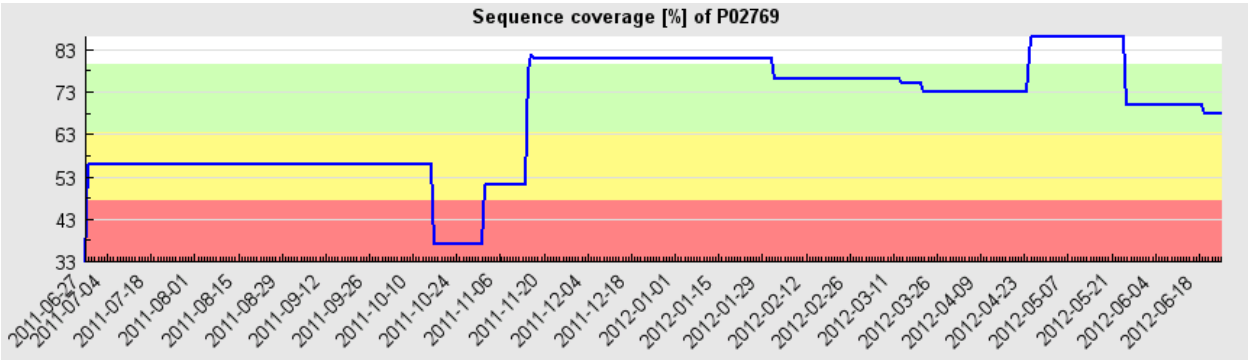
Laboratory C

Instrument 5: Orbitrap XL

QC sample: thyroglobulin (313 ng) sequence coverage



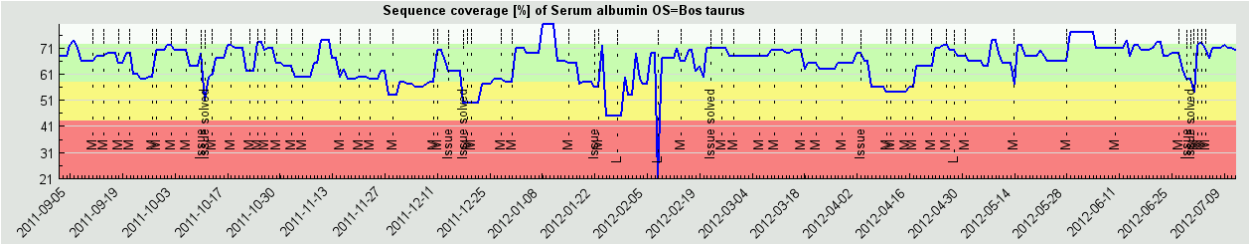
QC sample: BSA (313 ng) sequence coverage



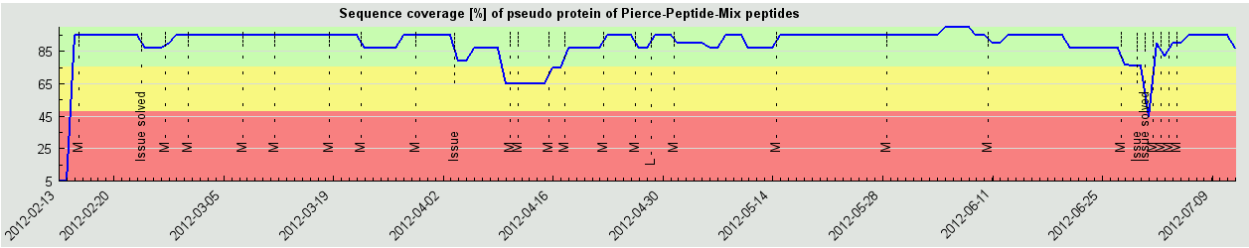
Laboratory D

Instrument 6: Orbitrap XL

QC sample: BSA (100 fmol) sequence coverage



QC sample: peptide mix (2.5 fmol) “pseudo-protein” sequence coverage



Supplementary Figure S-1. Longitudinal monitoring of the performance of six LC-MS systems in four laboratories (A-D). All data was acquired using CID fragmentation except Q-Exactive data which was generated using HCD fragmentation. Note that LC-MS system metrics such as "sequence coverage" reflect the combined performance of all system parts from autosampler and LC to electrospray and MS, so that a malfunctioning of any part may contribute to the observed variation.

(A) Instrument 1 Orbitrap XL ETD: Sequence coverage of 2.5 fmol BSA monitored over a period of one year, supplemented with plots of the retention times of cytochrome c peptides and synthetic phosphopeptides. The retention time shift observed on March 6, 2012 was associated with very high trap column pressure, whereas the delay on April 24, 2012 was only identified post-hoc (prolonged retention time delay at higher concentrations of running solution B). Note that for some phosphopeptides, several data-points are missing as would be expected for a "sensitivity test" sample only modestly above the limit of detection. A comparison between BSA sequence coverage as determined with the QC 1 sample and the numbers of proteins and peptide-spectrum matches (PSMs) obtained with the QC2 sample (HeLa) illustrates that the two QC samples provide to some extent complementary information.

Instrument 2 Velos Orbitrap ETD: A low amount of sample such as 1 fmol BSA leads to a more frequent detection of "impaired" performance (as indicated by red background color) than a large amount of sample such as 500 fmol cytochrome C (displayed time period: one year).

Instrument 3 Q-Exactive: Sequence coverage of 500 amol BSA monitored over 6 months.

(B) Instrument 4 Velos Orbitrap ETD: Both the number of PSMs identified in the QC-sample runs, and the sequence coverage obtained with 1 fmol of BSA provide information on system

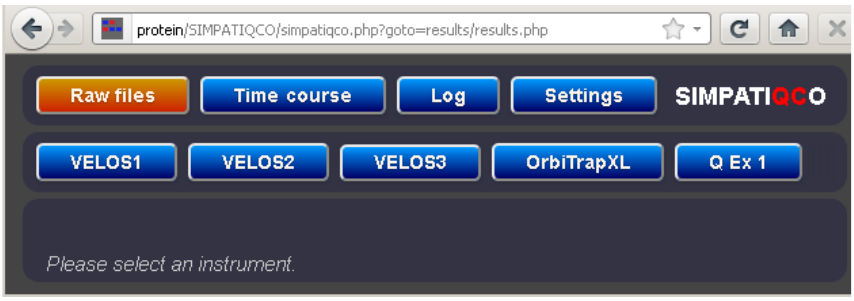
performance monitored over a period of one year. Retention time plots illustrate that deviant LC performance was detected as a substantial delay in peptide retention times on April 23, 2012 (column pressure lower than normal, following LC system maintenance with exchange of LC column).

(C) Instrument 5 Orbitrap XL: Monitoring of the sequence coverage obtained with a QC sample containing 313 ng of a tryptic digest of thyroglobulin and 313 ng of a tryptic digest of BSA provide similar information over a period of one year.

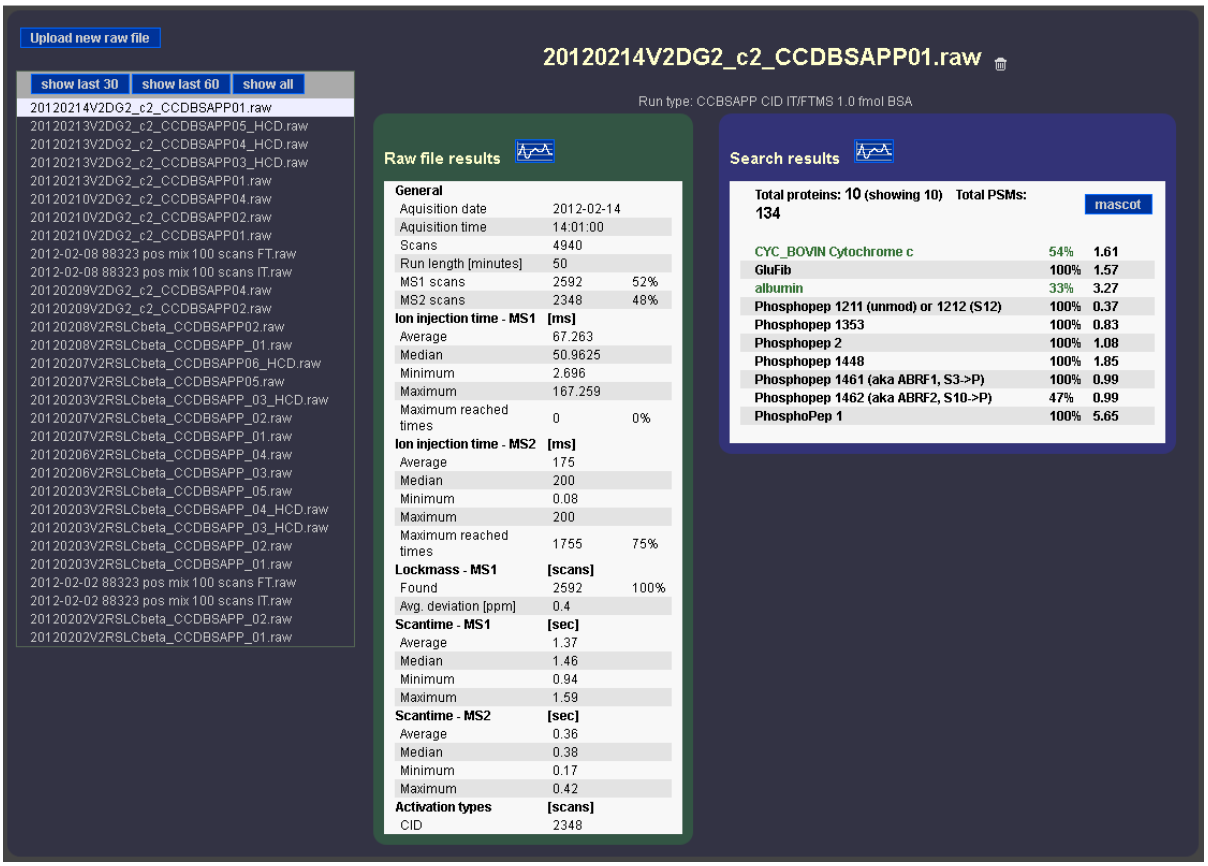
(D) Instrument 6 Orbitrap XL: Time course of the sequence coverage of 100 fmol tryptic BSA digest from September 2011 to July 2012, and sequence coverage of 2.5 fmol spiked QC-mix peptides (concatenated "pseudo-protein" database sequence) from February to July 2012. The following system maintenance events are annotated: Replacement of the LC column (“L”), mass spectrometry maintenance such as mass calibration, exchange of electrospray emitter, electron multiplier calibration and cleaning of heated capillary (“M”). “Issue” designates a malfunction or disturbance of LC or MS devices, and “Issue solved” its resolution.

Green band: within 1 x median absolute deviation (MAD). Yellow: 1–2 x MAD. Red: outside 2 x MAD.

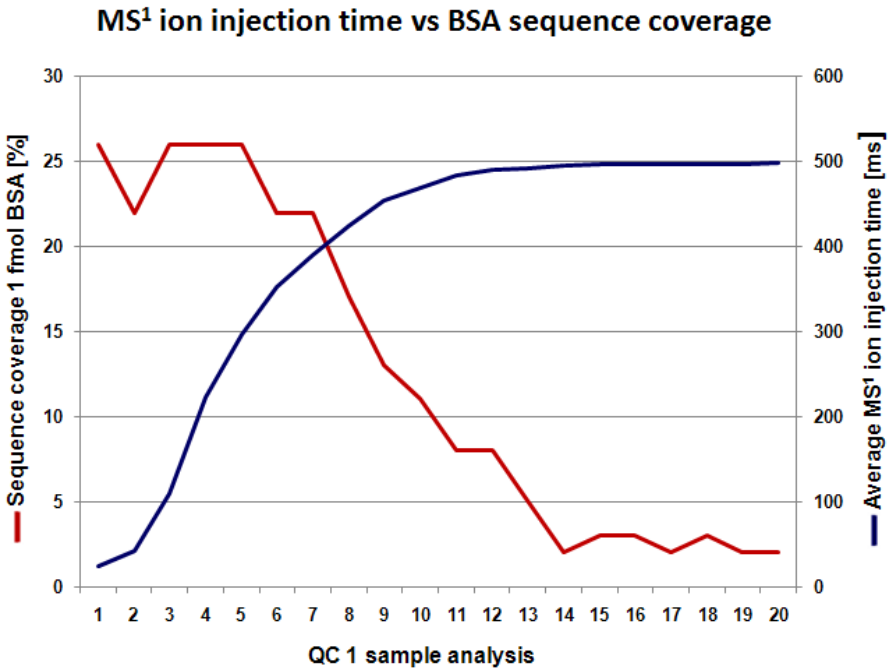
A



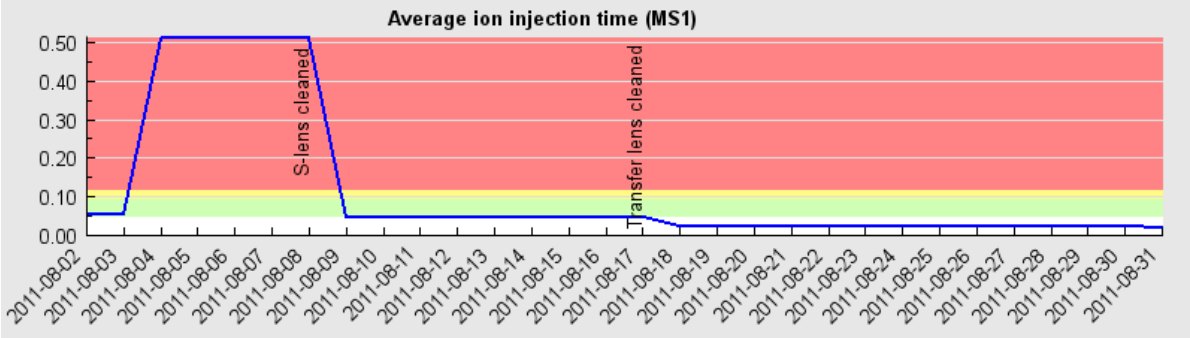
B



Supplementary Figure S-2. Access to SIMPATIQCO server via web-browser. Top-level menu **(A)** and overview of the results of an individual QC run **(B)**. Further details can be displayed by clicking on the clip art symbols adjacent to "Raw file results" or "Search results".



Supplementary Figure S-3. MS^1 ion injection time and BSA sequence coverage. Average MS^1 ion injection time increased before a drop in the sequence coverage of 1 fmol BSA became apparent.



Supplementary Figure S-4. Time course of average ion injection time of 100 MS^1 scans of a calibration mixture acquired off-line with ESI source. With this metric, a reduction is visible after cleaning of the S-lens and the exit lens, with a further improvement after cleaning of the transfer lens. Green band: within 1 x median absolute deviation (MAD). Yellow: 1–2 x MAD. Red: outside 2 x MAD.

SUPPLEMENTARY MATERIALS AND METHODS

Different QC samples and LC-MS methods were used in the four contributing laboratories. The respective sections are therefore subdivided accordingly:

Laboratory A

Preparation of a QC sample for monitoring sensitivity (QC 1)

Bovine cytochrome c tryptic digest LC/MS standard was purchased from Dionex (Thermo Scientific) and diluted in 0.1% trifluoroacetic acid (TFA) to a concentration of 1 µM. BSA (Sigma) was dissolved in a 50 mM aqueous solution of ammonium bicarbonate (ABC) at a concentration of 1 mg/mL. Disulfide bonds were reduced by incubation for 30 minutes at 56° C with 5 mM dithiotreitol (DTT), and alkylated with 25mM iodoacetamide (IAA) for 30 mins at room temperature in the dark. Trypsin was added twice in a ratio of 1:50 (w/w) at 0 hr and 4 hr and digestion was allowed to proceed over night (o/n) at 37° C. The digest was stopped by addition of triflouroacetic acid (TFA) to approximately pH 3. The solution was diluted to 1 µM concentration in 0.1% TFA, and a sample mixture was prepared so that a 10 µL injection volume of the QC sample reflected 500 fmol of cytochrome c; 2.5/1.0/0.5 fmol of BSA for Velos ion trap and Orbitrap XL- ETD, Velos Orbitrap ETD, and Q-Exactive instruments respectively; and 2.5 fmol each of 10 synthetic phosphopeptides (QC 1 sample). The sequences of the phosphopeptides were as follows: SVpSDYEGK, APPDNLPSPGGpSR, LIEDNEpYTAR, RpSDGGHTVLHR, ENIMRpSENSESQLTSK, QLGEPEKpSQDSSPVLpSELK,

QLGEPEKpSQDSpSPVLpSELK, NpSVEQGRRL, TASDTDSSpYAIPTAGMSPSR, SVENLPEAGIpTHEQR.

Preparation of a QC sample for monitoring speed of acquisition and performance (QC 2)

HeLa cell extracts were prepared as described.[1] Protein concentration was determined by a Bradford assay, and protein was purified by acetone precipitation. The pellet was redissolved in an aqueous solution of 8 M urea in 0.5 M ABC to a protein concentration of 5 µg/µL. Disulfide bonds were reduced with 5% (w/w protein) DTT for 30 min at 56° C and alkylated with 25% (w/w) IAA at room temperature in the dark. The reaction was quenched by adding 25% (w/w) DTT. The sample was diluted with 50 mM ABC to a final concentration of 6 M urea and digested with 2% (w/w) Lys-C at 30°C for 2 hrs. Next the solution was diluted to a concentration of 0.8 M urea with 50 mM ABC. Trypsin was added to a concentration of 3% (w/w) and digestion was performed at 37° C. A second aliquot of trypsin (again 3% w/w) was added after 4 hrs, and digestion was continued o/n. Digests were stopped by acidification with TFA to approximately pH 3. For QC 2 runs, 0.1 µg of the sample was injected onto the LC-MS/MS system.

Liquid chromatography

Digested samples were separated on a U3000 RSLC nano high performance liquid chromatography (HPLC) system (Dionex, Thermo Scientific). Peptides were loaded onto a trap column (PepMap C18, 2 cm x 100 µm, 5 µm, 100 Å, Dionex, Thermo Scientific) at a flow rate of 25 µL/min using 0.1% TFA in water as a loading solvent. After desalting for 15 minutes, the trap column was switched into the column flow, and peptides were eluted from the analytical

column (Acclaim PepMap C18, 50 cm x 75 µm, 2 µm, 100 Å, Dionex, Thermo Scientific) with a flow rate of 230 nL/min for RSLC instruments coupled online to Orbitrap Velos and Q-Exactive instruments. The following gradients were applied using solvents A (0.1% formic acid, (FA)) and B (80% ACN, 10% TFE, 0.1% FA): 2% B linear increase to 40% B within 30 min for the QC 1 sensitivity sample or within 180 min for the QC 2 performance sample, followed by a 5 min gradient to 90% B which was maintained for further 5 min, then reduced to 2% B within 2 min for column equilibration for further 28 min. For the Orbitrap XL ETD system, a U3000 HPLC system was used (Dionex, Thermo Scientific). Peptides were loaded onto a trap column (PepMap C18, 5 mm x 0.3 mm, 5 µm, 100 Å, Dionex, Thermo Scientific) at a flow rate of 25 µL/min using 0.1% TFA. After 15 min the trap column was switched into the column flow. The analytical column (Acclaim PepMap C18, 25 cm x 75 µm, 3µm, 100 Å, Dionex, Thermo Scientific) was operated at a flow rate of 275 nL/min. For QC 1 runs, the following ternary gradient was used: 100% A (5% ACN, 0.1% FA) for 10 min, a linear gradient of 11 min to 100% B (30% ACN, 0.08% FA), then 5 min to 80% B and 20% C (80% ACN, 10% TFE, 0.08% FA), and 7 min to 100% C which was maintained for 13 minutes, then within 1 min back to 100%A which was maintained for further 23 min for equilibration. QC 2 runs started with 100% A for 15 min, followed by a linear gradient to 100% B within 180 min, 5 min to 85% B and 15% C, 5 min to 10% B and 90% C maintained for 5 min, then within 2 min to 100% A followed by equilibration for 28 min.

Mass spectrometry

The HPLC was coupled on-line to various types of Orbitrap mass spectrometers via an electrospray source (Thermo Scientific) using a 10 µm PicoTip emitter (New Objective). Data

acquired on a LTQ Velos ion trap mass spectrometer was also uploaded to confirm that SIMPATIQCO could generate QC data from a low-resolution instrument (data not shown). The data presented in Figure 2 and 3 and Supplementary Figure S-1 reflect only a subset of the instruments and fragmentation types that were evaluated. In general, data acquisition was started 5 min after the beginning of the linear LC gradient. Raw file data acquisition time was 50 min for QC 1 samples and 200 min for QC 2 samples. Heated capillary temperature and electrospray source voltage were set to 200 °C and 1.5 kV on Orbitrap XL ETD, 275 °C and 2.0 kV on Velos ion trap and Velos Orbitrap, and 275 °C and 1.7 kV on Q-Exactive. Lock mass was enabled using polydimethylcyclosiloxane background ions (m/z 445.120025) for internal calibration on all Orbitrap instruments. Instruments were operated in data-dependent positive ionization mode. Depending on the type of LC-run and the instrument, different acquisition methods were used:

In general, one full MS^1 scan was followed by 12 data-dependent scans. The precursor isolation window was 2 Da for Velos Orbitrap, Q-Exactive and LTQ Velos, and 3 Da for Orbitrap XL ETD.

Settings for MS^1 scans were as follows: m/z 350-2000, automatic gain control (AGC) target 1E6 ions, resolution 60,000 for Orbitrap Velos and 70,000 for Q-Exactive, maximum ion injection time 500 ms for Velos Orbitrap and 120 ms for Q-Exactive; m/z 400-1800, AGC 1E6 ions, resolution 60,000, maximum ion injection time 500 ms for Orbitrap XL ETD; m/z 350-1800, AGC target 3E4 ions and maximum ion injection time 10 ms for LTQ Velos.

Settings for MSMS scans were: CID and electron transfer dissociation (ETD) with 35% normalized collision energy (CE), AGC target 1E4 and a maximum ion injection time of 200 ms on Velos Orbitrap; the same settings were used for CID scans on LTQ Velos; HCD with AGC target 1E5, a maximum ion injection time of 250 ms and 35% CE on Velos Orbitrap, and HCD

with AGC target 1E5, 60 ms maximum injection time and 30% CE on Q-Exactive. Singly charged ions were excluded. Five data-dependent scans were acquired per MS^1 scan on Orbitrap XL ETD, further MS/MS settings were as follows: CID and ETD with 35% CE, AGC 5E4 and maximum ion injection time of 100 ms, HCD with AGC target 2E5 and a maximum ion injection time of 200 ms, exclusion of singly charged ions and ions with unassigned charge state.

On Velos ion trap, QC 1 samples were measured using a dynamic exclusion time of 5 s and an exclusion window of +/- 1.5 Da. For QC 1 samples measured on Orbitrap instruments, dynamic exclusion was switched off, a parent ion m/z list of peptides present in the QC 1 sample was imported, and the instrument was configured to fragment only precursors that matched ions on the parent list +/- 10 ppm. In addition the maximum number of dependent scans was set to 20 for QC 1 runs measured with "dynamic exclusion off". These settings led to repeated fragmentation of cytochrome c, BSA and phosphopeptides whenever a signal corresponding to a respective precursor mass was detected in the preceding MS^1 scan. This allowed calculation of LC metrics such as "Full Width at Half Maximum" intensity (FWHM) by SIMPATIQCO versions 1.0.x, as extracted ion chromatograms could be reconstructed by an analysis of the time course of the precursor intensity of all identified MS/MS spectra for a certain peptide sequence. For SIMPATIQCO versions 1.1.x, peak elution profile data such as peptide retention time, apex intensity, peak width and area are extracted directly from the MS^1 scans. Therefore QC 1 runs are now measured with a dynamic exclusion time of 30s and a dynamic exclusion window of +/- 5 ppm.

For QC 2 samples, dynamic exclusion was switched on with a dynamic exclusion window of +/- 5 ppm and an exclusion time of 60 s.

Laboratory B

Preparation of QC sample

A single type of QC sample was used by laboratory B. For QC runs, a mixture of 500 fmol of a tryptic digest of cytochrome c (Dionex, Thermo Scientific) and 1 fmol of a tryptic digest of BSA (Bruker) in 0.1% TFA was injected.

Liquid chromatography

Peptides were separated on a U3000 HPLC-system (Dionex), using a trapping column (PepMap C18, 5 mm x 0.3 mm, 5 µm, 100 Å, Dionex) for preconcentration. Sample loading time was 10 min at 25 µL/min with 0.1% TFA. The analytical column (Acclaim PepMap C18, 25 cm x 75 µm, 3µm, 100 Å, Dionex) was operated at a flow rate of 300 nL/min. A ternary gradient was used: 100% A (2.5% ACN, 0.1% FA) for 7 min, a linear gradient of 30 min duration to 100% B (40% ACN, 0.08% FA), then 5 min to 20% B and 80% C (80% ACN, 10% TFE, 0.08% FA), 5 min to 90% C and within 0.1 min to 100% A followed by 28 min column equilibration. Acquisition on the mass spectrometer was started at 15.0 min via a contact closure signal.

Mass spectrometry

An electrospray source from Thermo Scientific with a PicoTip 10 µm emitter from New Objective was used to couple the LC to a Velos Orbitrap mass spectrometer. Heated capillary temperature was 250 °C and the source voltage was set to 1.7 kV. Data acquisition time was 40 min. MS^1 scans were acquired from 400-1800 m/z at a resolution of 60,000 with an AGC of 1E6 and a maximum ion injection time of 500 ms with lock mass enabled (m/z 445.12003). The 20

most intense ions were selected for CID using an isolation window of 1.9 Da, AGC 5,000 with 100 ms maximum injection time, a collision energy of 35% and a dynamic exclusion window +/- 5 ppm of 30 s duration. Singly charged ions were excluded.

Laboratory C

Preparation of QC sample

A mixture of tryptic digests of bovine serum albumin (Sigma) and thyroglobulin (Sigma) was used as a QC sample by laboratory C. Denaturation and alkylation of the sample were as described for laboratory A. Before digestion, BSA (Sigma) and thryoglobulin (Sigma) were dialyzed against water overnight in Slyde-A-Lyzer cassettes (3.5 K MWCO, Thermo Scientific). After reduction of the sample volume to 500 µL in a vacuum concentrator, trypsin was added in a ratio of 1:50 (w/w) for overnight digestion. Finally, the sample was acidified with 1% TFA (v/v). For QC runs a mixture containing 313 ng BSA and 313 ng thyroglobulin was injected.

Liquid chromatography

Peptides were separated on a U3000 HPLC-system or a U3000 RSLC nano-HPLC system (both from Dionex, Thermo Scientific), using a monolithic poly-(styrene-divenylbenzene) separation column (20 cm x 0.2 mm, fabricated in house) after full-loop injection of 5 µL sample. The separations were performed at 45 °C at a flow rate of 1 µL/min and a linear gradient of 0 – 40% acetonitrile in 0.05% aqueous TFA in 30, 60, and 100 min, respectively, using the U3000 HPLC-system. For the RSLC HPLC-system the same linear gradients were used with a starting concentration of 1% acetonitrile.

Mass spectrometry

Both HPLC systems were hyphenated to a LTQ Orbitrap XL mass spectrometer (Thermo Scientific) via an electrospray source (Thermo Scientific) and a PicoTip (10 µm I.D., New Objective) emitter. The measurements were performed in positive ionization mode. Heated capillary temperature was 250 °C and the voltage was set to 1.4 kV. Data acquisition time was 50 min for the 30 min gradient, 80 min for the 60 min gradient and 150 min for the 100 min gradient. MS^1 FTMS full scans were acquired from 450-2000 m/z at a resolution of 60,000 with an AGC of 1E6 and a maximum ion injection time of 100 ms. Peptides were identified using the three most intense precursor ions for fragmentation by CID using an isolation window of 2 Da, AGC 10,000 with 100 ms maximum injection time, a collision energy of 35% and a dynamic exclusion window +/-10 ppm of 30 s duration. Singly charged ions were excluded.

Laboratory D

Preparation of QC sample

Lyophilized cytochrome c digest (bovine) was purchased (Dionex, Thermo Scientific) and resuspended according to manufacturer's description in 0.1% TFA and 2% ACN to a concentration of 8 µM. BSA digest was purchased (Waters, MassPREP BSA Digestion Standard) and resuspended according to manufacturer's description in 0.1% TFA to a final concentration of 1 µM. Peptide mix was purchased ("Pierce Retention Time Calibration Mix", Pierce, Thermo Scientific) and resuspended according to manufacturer's description in 0.1% formic acid in water to a final concentration of 5 µM; all peptides of this mix had a heavy labeled c-terminal amino acid, either $^{13}C_6{}^{15}N_2$-lysine or $^{13}C_6{}^{15}N_4$-arginine, respectively. The peptide mix

stock solution was diluted 50-fold in 0.1% TFA (in water) to a final concentration of 100 nM. The three solutions of cytochrome c (8µM), BSA (1µM), and the peptide mix (100 nM), were mixed in a ratio of 6.25:10:2.5 resulting in a QC mixture containing 2.7 µM cytochrome c, 533 nM BSA, and 13 nM peptide mix. This QC mixture, called *CCBSAp*, was stored in 18.5 µL aliquots at -20 °C until further use. Before injection into the LC-MS system, 80 µL 0.1% TFA was added to the thawed QC mix, followed by sonification in an ultrasonic bath for 3 minutes. The mixtures finally injected had concentrations of 0.5 µM cytochrome c, 100 nM BSA, and 2.5 nM peptide mix. For each QC run, 1 µL QC mix containing 500 fmol cytochrome c digest, 100 fmol BSA digest and 2.5 fmol peptide mix (2.5 fmol of each peptide) was injected. Before February 2012, the QC mix did not contain the peptide mix, but concentrations of cytochrome c and BSA digest were identical.

Liquid chromatography

An Ultimate 3000 nano LC system (Dionex, Thermo Scientific) was used for peptide separation. Samples were loaded onto a trap column (PepMap C18, 300 µm ID x 5 mm length, 5 µm particle size, 100 Å pore size; Dionex, Thermo Scientific) and washed and desalted for 10 minutes using 0.1% TFA in water as loading solvent. Then the trap column was switched online with the analytical column (PepMap C18, 75 µm ID x 500 mm, 3 µm particle and 100 Å pore size; Dionex, Thermo Scientific) and peptides were eluted with the following binary gradient: starting with 3% solvent B to 50% solvent B in 30 min, where solvent A consisted of 0.1% formic acid in water, and solvent B consisted of 80% acetonitrile and 0.08% formic acid in water. All LC solvents were purchased from Biosolve, Valkenswaard, the Netherlands. Column flow rate was set to 250 nL/min.

Mass spectrometry

The nano LC was coupled on-line to a hybrid linear ion trap Orbitrap mass spectrometer (LTQ-Orbitrap XL, Thermo Scientific, Bremen, Germany). For electrospray ionization (ESI), metal-coated nano ESI emitters (New Objective, Woburn, MA, USA) were used and a spray voltage of 1.5 kV was applied. For peptide detection, a data-dependent acquisition method was used: a high-resolution survey scan from 400 – 1800 m/z was acquired in the Orbitrap (AGC target 1E6; resolution 30,000; lock mass 445.120025 m/z corresponding to protonated $(Si(CH_3)_2O)_6$). On the basis of this full scan the 5 most intensive ions were consecutively isolated (AGC target 1E4 ions), fragmented by CID applying 35% normalized collision energy, and detected in the ion trap. Precursor masses within a tolerance range of +/- 5 ppm that were selected once for MS/MS were excluded for further MS/MS fragmentation for 3 minutes.

Remark on the types of QC samples and workflows that can be used with SIMPATIQCO

The QC samples and LC-MS methods described above are used on a routine basis in the four laboratories. Other methods or different proteins or synthetic peptides designed for QC purpose could be used instead, as SIMPATIQCO can be configured to process QC metrics from any desired set of proteins and peptides. This holds true for both the QC 1 ("sensitivity") as well as the QC2 ("performance") sample; other QC 2 samples such as E. coli, Pyrococcus furiosus or yeast might be used and can be analyzed with SIMPATIQCO in an analogous way.[2]

SIMPATIQCO server

The SIMPATIQCO software suite was developed for deployment on a dedicated personal computer running the Windows operating system, an Apache webserver, a PostgreSQL database and PHP. Scripts that need to be installed, an installation instruction and a manual are available on the webpage http://ms.imp.ac.at/ and will be updated with every new version of the software. XRawfile2.dll distributed by Thermo Scientific as part of the freely available MSFileReader software package, is used to extract information from raw files. MS/MS spectra information, precursor intensities and retention time values are extracted using msconvert.exe from the ProteoWizard Software package.[3] In addition SIMPATIQCO requires a Mascot server to search MS/MS spectra; however the scripts may possibly be modified for other search engines. The SIMPATIQCO server runs an Apache webserver that allows easy and comfortable configuration as well as querying of the database and visualization of the time course of QC metrics through a web-browser (Mozilla Firefox recommended) from any computer in the laboratory.

The msconvert software that is used by SIMPATIQCO to extract MS/MS scan information also provides the intensity for each precursor ion selected for fragmentation, and retention time values for all scans. For SIMPATIQCO versions 1.0.x, this allows a simple reconstruction of elution time profiles without sophisticated analysis of MS^1 data from raw files provided that dynamic exclusion was switched off during data acquisition so that precursor ions were fragmented repeatedly. For each peptide sequence in a specific modification state (as reflected by the theoretical mass) all associated MS/MS spectra are sorted by the retention time. Identification of the peptide sequence via the MS/MS scan provides supplemental reliability that extracted ion

chromatograms are reconstructed correctly. In this way the most intense precursor defines the peak elution time of the peptide. Peak width at half maximum intensity is calculated by interpolation of the elution time between scans where the precursor intensity is closest below and above 50% of the peak maximum. Versions 1.0.x of SIMPATIQCO estimate chromatographic metrics such as peptide retention time, apex intensity and peak width in the above-described manner from MS/MS scan retention time and peptide sequence information. SIMPATIQCO versions 1.1.x compute LC metrics such as retention time, apex intensity, peak width and area directly from MS^1 data through the in-house developed PeakAnalyzer.exe module.

Laboratories A and B used SIMPATIQCO version 1.1.0, while SIMPATIQCO version 1.0.6 was used by laboratories C and D. Mass tolerance settings can be configured in SIMPATIQCO in accordance with the respective sample and instrument. For instance, laboratories A, B and C set a precursor ion tolerance of 15 ppm for analyses on Orbitrap instruments whereas laboratory D used 10 ppm. For Orbitrap instruments, an MS^1 tolerance setting of 7 – 15 ppm is recommended. Fragment ion tolerance settings in all laboratories were 0.5 Da for CID (and 0.5 Da for ETD and 100 mDa for HCD spectra if applicable). Carbamidomethylation of Cys was set as a fixed modification, while oxidation of Met was set as a variable modification. For QC 1 samples of laboratory A, phosphorylation of Ser, Thr and Tyr was included as a variable modification. Laboratory D configured SILAC label $^{13}C_6{}^{15}N_2$ of lysine (+7.943 u) and SILAC label $^{13}C_6{}^{15}N_4$ of arginine (+9.930 u) as additional variable modifications. QC 1 samples of laboratory A and QC samples of laboratories B-D were searched against a custom database containing the respective standard proteins and peptides. The database used by laboratories A-C also contained a list of common contaminants. For QC 2 samples of laboratory A, swissprot

human was used as a database. A cutoff score for peptide identification can also be chosen for "run-types". We used a Mascot peptide score of 25 for all searches to restrict peptide-spectrum matches to spectra of bona fide quality. Note that variable modifications are hard-coded in mascotextractor.php up to SIMPATIQCO version 1.1.0. Please refer to the online manual for an explanation how to change modifications, e.g., for "heavy" peptides.

SIMPATIQCO allows defining "proteins of interest" (POIs), for which the sequence coverage can be plotted over time. In addition, "peptide lists" can be configured, for which longitudinal QC metrics such as retention times can be displayed. For details, see Supplementary Figure S-1 and the online information on the webpage http://ms.imp.ac.at/

The metric "(%) of target value" is derived by multiplying the TIC of a scan with the respective ion injection time in seconds; this value is then expressed as a percentage of the AGC. For Orbitrap spectra, signal intensities are proportionate to charge state. Therefore TIC multiplied with the ion injection time can be regarded as an estimator of the number of charges. The same holds true for ion trap spectra, where signals can also be considered approximately proportionate to charge state. Knowing the charge of the ions would therefore be required to derive the number of ions from the charges. In addition, for MS/MS spectra, dissociation efficiency has to be taken into account, as the respective value would be expected to reflect the AGC only for the undissociated precursor. And for Orbitrap spectra, appropriate pAGC scaling is required. Due to these considerations, it should be noted that the metric "(%) of target value" represents only a coarse estimator of the underfill or overfill ratio with regard to the AGC target

value. However predictive AGC works in a similar manner suggesting that this metric provides useful information.

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