Evaluation of a General System Suitability Workflow for Peptide Quantitation Applications by LC-SRM-MS

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Overview

Purpose: Determination of LC-MS system performance is critical for peptide quantitation experiments. Here we develop and evaluate a system suitability sample and method for use on nanoflow-based triple quadrupole systems for peptide quantitation applications.

Methods: Isotopically-labeled and light peptides were spiked into HeLa cell lysate digest to generate a quantitative evaluation of system performance. Samples were analyzed on several TSQ Quantiva triple quadrupole mass spectrometers using nanoflow HPLCs and data were analyzed using Skyline software.

Results: A workflow for system suitability evaluation of nanoflow LC-MS triple quadrupole systems was developed and tested to provide a useful tool to create a system performance baseline and benchmark for peptide quantitation applications.

Introduction

With increased interest in targeted peptide quantitation by LC-MS/MS, additional focus has been given to the robustness and reproducibility of such assays. System suitability, which evaluates the overall performance of an LC-MS platform, is a standard practice in many commercial and GMP/GLP-regulated environments, but has not been standardized in most basic research laboratories. Here we demonstrate a simple system suitability protocol (SSP) to evaluate the robustness, reproducibility and sensitivity of LC-SRM-MS systems used for peptide quantitation applications. A mixture of commercially available reagents, a simple LC gradient and SRM transition list along with processing software were tested at different LC flow rates to evaluate the ability of the SSP to diagnose problems and track performance over time.

Methods

Sample Preparation

Heavy, C15/N15-labeled peptides PRTC Retention Time Standards (Pierce) and light synthetic versions were spiked into a HeLa cell lysate digest (Pierce) to generate a fixed system suitability standard (10 fmol/uL heavy PRTC peptides, 300 amol/uL light peptides in 500 ng/uL HeLa Lysate digest) as well as a standard curve in which the heavy peptides were at a fixed concentration (5 fmol/uL) and the light concentrations varied from 0.5 amol/uL to 10 fmol/uL in a sample background of 500 ng/uL HeLa lysate digest. Samples were prepared in 2% acetonitrile/0.2% formic acid.

Liquid Chromatography (or more generically Separations)

Samples were evaluated on different LC-MS hardware set-ups:

Thermo Scientific™ Easy nano™ LC with Thermo Scientific™ TSQ Quantiva™ Mass Spectrometer

NanoAcquity (Waters Corp) with TSQ Quantiva MS

The general LC gradient was as follows:

2-8% B over 1 minute, 8-35% B over 17 minutes, 35-95% B over 1 minute, hold at 95% B for 4 minutes, 95-2% B over 1 minutes and re-equilibration at 2% B for 6 minutes.

Injection volume was 1 uL. In the case of Easy nano LC, the sample was loaded for 3 uL volume at variable flow rates (300 nL/min to >1 uL/min, pressure dependent, max pressure of 800 bar). For the nanoAcquity system, which employed a trap column, samples were loaded for 1 minute at 5 uL/min.

Mass Spectrometry

The TSQ Quantiva MS was the only triple quadrupole MS evaluated in this study. A list (Q1/Q3) of 468 transitions were monitored, untimed, with a cycle time of 1 sec. ESI was 1800-2200 V with an ITT temperature of 325. Collision gas pressure was set to 1.5 mTorr, and variable collision energies were used, based on the following charge-state related equations:

2+: CE = m/z (0.0339) + 2.3597

3+: CE = m/z (0.0295) + 1.5123

Data Analysis

Data were imported into Skyline (University of Washington) and Thermo Scientific™ TraceFinder™ Software. Extracted ion chromatograms generated peak areas and peak area ratios for determination of regression versus concentration and %RSD for transitions.

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Results

Peptide Parameters for Monitoring by SRM-MS for System Suitability

The goal of this work was to generate a system suitability method that could be rapidly executed (< 1 hour per injection), and that would stress-test the LC and MS systems in manners similar to what is expected in peptide quantitation experiments. We generated a transition list for system suitability monitoring of 30 peptides in a sample containing HeLa lysate digest and 15 exogenous peptides (Pierce Retention Time Standards), spiked in as standards in both 13C/15N-labeled and unlabeled forms. The transition list monitored ≥ 5 transitions per spiked peptide (251 total), and an additional 217 transitions from the HeLa peptides. We chose to monitor multiple precursor charge states (2+ and 3+) for the HeLa peptides so that we had over 400 transitions in the unscheduled SRM list. The method is unscheduled so that dramatic retention time shifts could be monitored and the transition list is large so that we could ensure ~500 SRMs/sec cycle time (~ 1-2 msec dwell per transition). HeLa was used at 500 ng/uL (500 ng on-column) to mimic sample load in quantitative experiments and to monitor for system performance of both peak area ratios (15 L/H peptide pairs) and raw peak areas. Not all transitions are used in data analysis, just the 5 most intense. The list of spiked peptides and transitions used for data analysis is show in Table 1.

TABLE 2. List of Pierce Retention Time Peptides and Transitions used for Data Analysis in the System Suitability Method.

Peptide Sequence	Precursor m/z	Transitions				
		1	2	3	4	5
SSAAPPPPR	488.76	369.22	466.28	563.33	660.38	731.42
GISNEGQNASIK	609.31	532.31	717.39	846.43	960.47	1047.51
HVLTSIGEK	492.28	446.26*	533.29	634.34	747.42	846.49
DIPVPKPK	447.28	244.17	372.26	469.31	665.43	778.52
IGDYAGIK	418.73	317.22	388.26	551.32	666.35	723.36
TASEFDSAIAQDK	691.83	574.32	645.36	732.39	847.42	994.48
SAAGAFGPELSR	581.80	601.33	658.35	805.42	876.46	933.48
ELGQSGVDTYLQTK	769.89	652.37	753.41	868.44	1024.53	1111.56
GLILVGGYGTR	552.32	496.25*	610.29	709.36	822.45	935.53
GILFVGSGVSGGEEGAR	796.41	675.31	762.34	918.43	1062.48	1161.55
SFANQPLEVVYSK	741.39	595.35**	724.39**	934.52	1176.63	1247.66
LTILEELR	493.80	288.20	417.25	546.29	659.37	873.50
ELASGLSFPVGFK	676.37	547.32	694.39	781.42	1038.56	1109.60
LSSEAPALFQFDLK	783.41	522.29	650.35	797.42	1078.59	1149.63
NGFILDGFPR	568.30	476.26	591.29	817.46	964.53	1021.55

FIGURE 1. Representative Chromatogram from System Suitability Method in XCalibur. The extracted ion chromatogram represents all monitored transitions, including those of HeLa lysate digest.

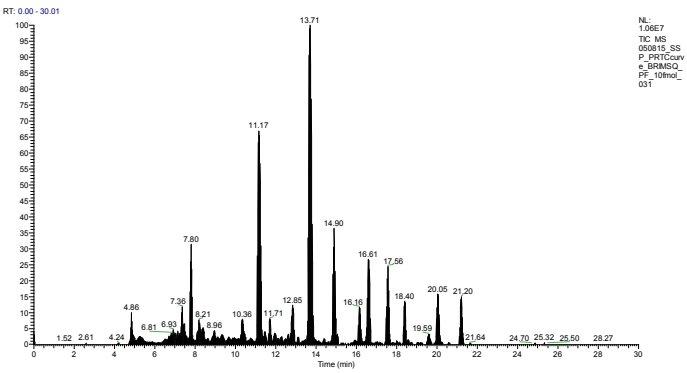


Figure 2. Extracted Ion Chromtogram for all peptides monitored in Skyline.

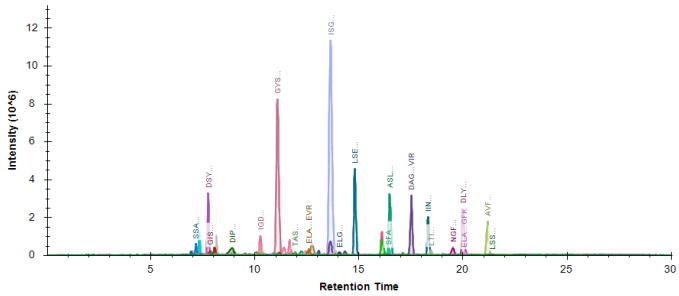
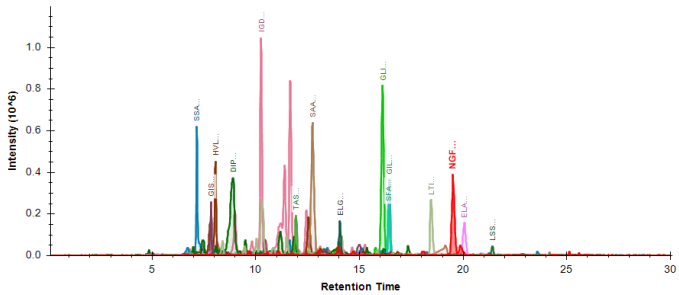


Figure 3. Extracted Ion Chromtogram for PRTC peptides in Skyline.



Response Curve and Development of System Suitability Sample

Heavy PRTC peptides were spiked into 500 ng/uL HeLa lysate digest at 9 increasing concentrations (0.5, 1, 5, 25, 100, 500, 1000, 2500, 10000 amol/uL). Samples were analyzed to determine the approximate LOD on a TSQ Quantiva with a nanoAcquity using a trapping column.

TABLE 1. Limits of Detection for PRTC Peptides in System Suitability Method

Peptide Sequence	LOD (amol/uL)
SSAAPPPPR	ND*
GISNEGQNASIK	25
HVLTSIGEK	ND*
DIPVPKPK	ND**
IGDYAGIK	1000
TASEFDSAIAQDK	500
SAAGAFGPELSR	100
ELGQSGVDTYLQTK	100
GLILVGGYGTR	100
GILFVGSGVSGGEEGAR	100
SFANQPLEVVYSK	100
LTILEELR	25
ELASGLSFPVGFK	25
LSSEAPALFQFDLK	25
NGFILDGFPR	100

*: Not detected due to early eluting peptides not binding to trap column
**: This peptide wasn't detected due to degradation of the standard in the sample

Reproducibility of System Suitability Method on Multiple Quantiva MS Instruments

Reproducibility of peptide peak area and retention time were monitored on multiple TSQ Quantiva MS to determine the "normal" variability (Figure 4). An additional example is shown on a TSQ Quantiva MS that was exhibiting peak area inconsistency (Figure 5).

FIGURE 4. Reproducibility of Peak Areas and Retention Times on 2 Separate TSQ Quantiva Instruments. Quantiva A was equipped with a nanoAcquity pump and a trap column, while Quantiva B was equipped with an EASYnano LC. Both columns were PicoFrit, A was packed with Reprosil C18 AQ, B was packed with PepMap C18. Slight retention time order changes were noticed for some peptides, but all other data were equal.

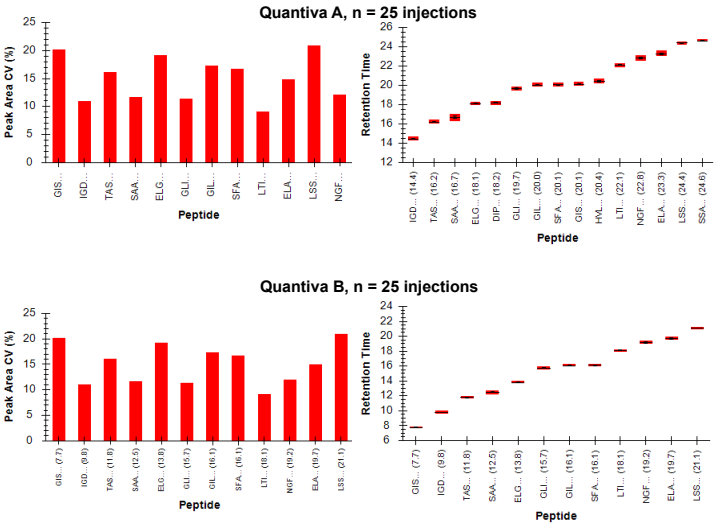
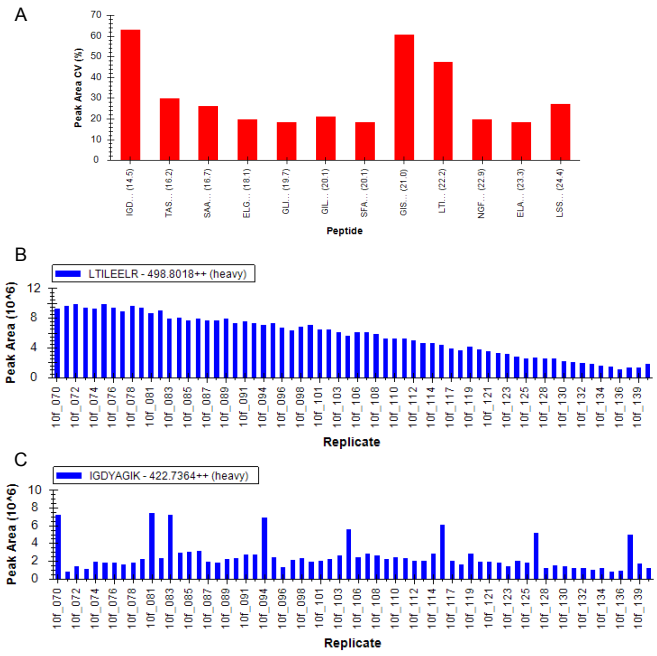


TABLE 3. Tabular Format Showing the Metrics for Reproducibility. Data shown for "Quantiva A" in Figure 4 above.

Peptide	Precursor (m/z)	Mean Total Area	Stdev Total Area	CV Total Area	Range Retention Time (min)	Mean Retention Time (min)	Stdev Retention Time (min)	CV Retention Time
GISNEGQNASIK	613.3168	286439	57652	20.10%	0.4	7.74	0.12	1.5%
IGDYAGIK	422.7364	2063102	214090	10.40%	0.63	10.05	0.19	1.9%
TASEFDSAIAQDK	695.8324	388942	58838	15.10%	0.6	11.78	0.18	1.6%
SAAGAFGPELSR	586.8003	1746362	205828	11.80%	0.67	12.49	0.19	1.5%
ELGQSGVDTYLQTK	773.8956	298643	56344	18.90%	0.73	13.8	0.23	1.7%
GLILVGGYGTR	558.3260	2307724	253952	11%	0.87	15.72	0.28	1.8%
GILFVGSGVSGGEEGAR	801.4115	486846	82493	16.90%	0.87	16.05	0.28	1.7%
SFANQPLEVVYSK	745.3925	607356	97740	16.10%	0.77	16.09	0.24	1.5%
LTILEELR	498.8018	1095067	89617	8.20%	0.92	18.09	0.3	1.6%
ELASGLSFPVGFK	680.3736	743740	110292	14.80%	0.8	19.66	0.25	1.3%
LSSEAPALFQFDLK	787.4212	117784	23978	20.40%	0.77	21.05	0.24	1.2%
NGFILDGFPR	573.3025	1100957	128813	11.70%	0.8	19.15	0.26	1.4%

FIGURE 5. Use of the System Suitability Method to Diagnose Peak Area Variability. (A) Higher-than-normal peak area CVs led us to plot the peak areas vs time and a slight, continual drop was observed for most peptides (two examples shown in (B) and (C)). The continual drop in signal detected in the system suitability samples is indicative of contaminated element in the ion path. An automated MS diagnostics routine confirmed the site of contamination and the system was restored to normal function



Conclusions

- System Suitability evaluations of LC-MS systems are essential to benchmark system performance before quantitative evaluation of any sample.
- We developed a sample, method, and processing options for monitoring LC-MS system performance for quantitative LC-MS peptide applications using nanoflow chromatography
- The sample and method is available for incorporation into the routine peptide quantitation LC-MS workflow
- Regardless of the LC-MS hardware, the sample and method can be applied to evaluate system consistency and performance
- It is encouraged to utilize and periodically (daily) evaluate system performance over time to catch problems early and minimize system down-time
- An LC-MS-based system suitability protocol can pinpoint problems in either the LC or MS system, facilitating focused troubleshooting of the issues
- Software tools are available and in development for rapid data analysis.

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SRM-MS transition lists and additional method details are available upon request:
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