Development of an Absolute Quantitation Assay for Simultaneous Measurement of 9 Proteins From Cell Lysates by MRM

Stéphane Parent[1], Michael Schirm[1], Anne Jang[1], Chunyan Li[1], Simon Bourque[1], Rudolf Guilbaud[1], Nolwen Guigal-Stephan[2], Brian Lockhart[2] and Lorella Di Donato[1]

[1]Caprion Proteomics Inc.; [2]Servier

Purpose

We present a multiplexed multiple reaction monitoring (MRM) assay for the measurement of 9 cancer-related proteins in HeLa cell lysates. For each protein, unique tryptic peptides were identified (at least 5 peptides/protein). To compensate for any analytical variability, tryptic heavy-labeled winged peptides were added to all samples at a fixed concentration during processing. Recombinant proteins were used for the preparation of calibration standards and Quality Control (QC) samples. Samples were digested with trypsin (25:1 ratio) and desalted (C18). Samples were analyzed by LC-MS using a NanoAcquity UPLC system (Waters) coupled to a QTRAP 6500 mass spectrometer (AB Sciex). The multiplexed MRM assay monitors 55 unique peptides, 2 transitions per peptide. Two transitions per peptide were measured. The MRM assay development includes assessment of assay sensitivity, specificity, identification of surrogate matrix for preparation of calibration standards, measurement of endogenous levels of proteins in HeLa cell lysates for the preparation of QCs at 3 different levels, as well as establishing the curve fit, concentration of heavy-labeled peptides, curve range, and the evaluation of matrix effect, carry-over and precision and accuracy. Results obtained allow for the absolute quantification of the 9 proteins in cell lysates with the ultimate goal of developing a predictive model of drug efficacy.

Materials and Methods

Recombinant proteins

The 9 recombinant proteins were purchased from commercial suppliers, based on the following criteria: purity ≥ 90%, isoform of interest, full-length if possible, absence of carrier protein, and buffer compatible with mass spectrometry (e.g. absence of detergent).

Heavy-labeled peptides

A bioinformatics analysis of the 9 proteins of interest was performed using an in-house developed peptide selection software. Crude heavy-labeled winged peptides (w-IS) were synthesized by JPT (Berlin, Germany). The heavy-label (^{13}C and ^{15}N) is incorporated at the C-terminus on Lys or Arg (exposed after the trypsin cleavage site). The winged peptides contain 3 natural amino acids at the adjacent flanking N- and C-terminal sequences of the endogenous peptide to provide trypsin cleavage sites such that they will be submitted to the same digestion step as the protein of interest.

Naïve Matrix

The HeLa cell line was selected as a naïve matrix for the preparation of Quality Control (QC) samples. The HeLa cell pellet was processed to provide a cell lysate to be used for the preparation of QC samples (spiked with recombinant proteins and not spiked to determine endogenous levels). Briefly, the cell pellet was resuspended in 0.1% w/v RapiGest SF (Waters) and heated at 95°C for 10 minutes. The sample was then sonicated on ice using a probe sonicator and the protein content was determined using a micro-BCA protein assay. For QC and study samples, 30 µg of total protein was used.

Sample processing and general procedure

The stock of the 9 recombinant proteins was serially diluted in a buffer containing 250 µg/mL bovine serum albumin (BSA) and 0.1% w/v RapiGest SF in 50 mM ammonium bicarbonate, to obtain the desired concentrations for the preparation of calibration standards.

The final assay conditions were the following: Twenty (20) µL of sample (calibration standards, blank and QC samples) was aliquoted in 1.5 mL tubes. Forty (40) µL of heavy-labeled winged peptides was added to all samples with the exception of the blank samples. All samples were heated at 95°C for 10 minutes. Eighty (80) µL of trifluoroethanol (TFE) was added to all samples and incubated at room temperature for 30 minutes. The samples were then diluted 5-fold with ammonium bicarbonate (final 10% TFE), digested with trypsin (1:25 (w:w) enzyme:protein ratio, Promega Corporation) at 37°C with shaking overnight. The digestion was stopped by the addition of trifluoroacetic acid to a final concentration of 1% (v/v). Samples were desalted on C18 96-well plates (3M Bioanalytical technologies). The eluate was divided equally into two 96-well plates, one for MS injection and one as a back-up plate. All plates containing the C18 eluate were vacuum-evaporated to dryness and stored at -20°C until analysis by MRM.

Mass spectrometry conditions

The MRM assay was developed using trypsin digested recombinant proteins. Recombinant proteins were digested with trypsin and diluted to a final concentration of 200 pmol/mL. The peptide mix was used to develop the MRM assay on a NanoAcquity UPLC (Waters) coupled to a QTRAP 6500 mass spectrometer (AB Sciex). The optimal 2 transitions (combination of peptide precursor and fragment ion mass-to-charge ratio that are monitored by the mass spectrometer) per peptide, were determined using selected reaction monitoring (SRM)-triggered MS/MS. The two most intense fragment ions (b- or y-fragment ions only) in the MS/MS spectrum and the corresponding elution time were determined for each acquired peptide. After, the collision energy (CE) was optimized for each of the chosen transitions. The developed MRM assay was then used to analyze the study samples.

Data analysis

Baseline integration was performed using MultiQuant software (v2.1.1296.0, AB Sciex). Standard curves for all peptides were generated using MultiQuant with 1/X linear weighting as curve fit, except for one peptide, which used a 1/X2 linear weighting for both transitions. The concentration for the QC samples was automatically back-calculated from the calibration curve using the MultiQuant software. The LOD is defined as the first concentration detected with both replicates with a signal to noise ratio of 3:1. The LLOQ and ULOQ are defined respectively as the lowest and highest concentration with a precision < 30% and accuracy within 70-130%.

Process flow

Calibration curve range for each peptide is presented in Table 4.

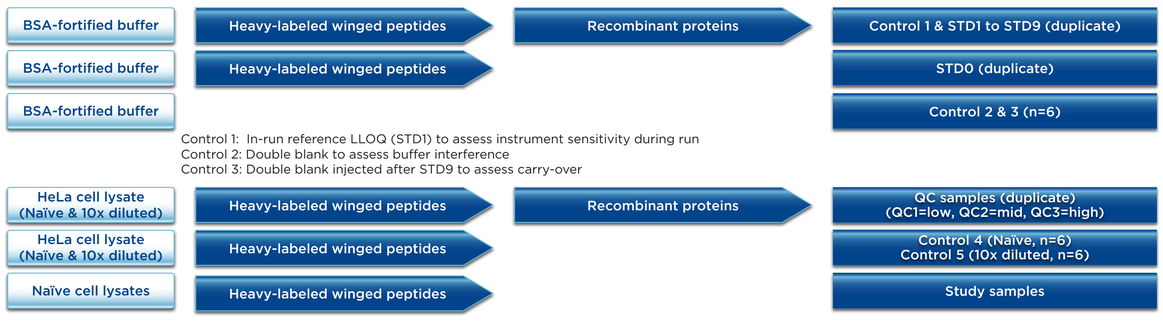

Figure 1. Preparation of Calibration Standards, QC samples, Controls and Study samples using heavy-labeled winged peptides.

Figure 2. Process flow for Calibration Standards, QC samples, controls and Study samples using heavy-labeled winged peptides.

Results

Typical calibration curve

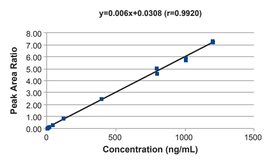

Figure 3. A representative calibration curve. Protein A, peptide 1, transition 1, Light/Heavy peak area ratio in function of standard concentration of recombinant protein. A 1/X weighting factor was used for the curve fit.

The importance of appropriate controls and thorough data analysis

Example 1: Ionization suppression / enhancement in surrogate buffer & matrix for each peptide

Table 1. Internal standard (w-IS) response in buffer (STDs) versus matrix (QC samples, naïve and 10x diluted). For peptide F1, the difference in the mean peak areas of the internal standard in naïve QC samples and 10x diluted QC samples were 18.2% and -9.8% respectively when compared to the mean peak area of calibration standards. Although no formal criteria were established, this is deemed a reasonable difference. On the other hand, for peptide F2, the internal standard is significantly affected by matrix since the mean QC peak area displays 48.0% and -35.8% bias from the mean STD peak area. This assessment is routinely performed for all peptides. Peptide F1 was selected over peptide F2.

Protein F (peptide)	Peak Area Internal Standard (w-IS) (mean)		
	STDs	QC Naïve	QC 10x diluted
Peptide F1	196520	232325	177265
%diff		18.2	-9.8
Peptide F2	460556	681592	295577
%diff		48.0	-35.8

Example 2: Matrix interference

Table 2. Assessment of matrix interference by measuring the ratio of 2 transitions. For all peptides, 2 transitions are monitored. Although the ratio between the 2 transitions will vary from peptide to peptide, it should remain stable between the buffer and the matrix. For peptide H1, the ratio is stable across sample types, with 2.47% and -13.62% bias in QC samples and naïve matrix compared with the STDs, over transitions ratio of STDs. On the other hand, for peptides H2 and H3, positive (32.99% and 56.29% for peptide H2) or negative (-32.08% in Naïve matrix for peptide H3) bias are observed for QC samples and Naïve matrix, indicating some matrix interference. Although no formal criteria were established, these are considered important differences (>30%). If the source of the interference can be clearly assigned to one transition, the results of the other transition can still be used. This assessment is routinely performed for all peptides. Peptide H1 was preferred over peptides H2 or H3.

Protein H (peptide)	Ratio T1/T2		
	STDs	QCs	Naïve matrix samples
Peptide H1	2.21	2.27	1.91
%diff		2.47	-13.62
Peptide H2	1.90	2.52	2.96
%diff		32.99	56.29
Peptide H3	10.46	9.46	7.10
%diff		-9.51	-32.08

Example 3: Looking at internal standard mean peak area precision

Table 3. Internal standard peak area precision in STDs, QC samples and naïve matrix. The precision (%CV) of mean internal standard peak area is monitored for all sample types and all transitions. For peptides E1 and E2, these %CV values are between 7.6% and 15.3% across sample types and transitions. For peptide E3, the %CV are higher in STD and QC samples (19.9%-22.6%) and the %CV is > 60% for both transitions in naïve matrix, indicating poor digestion reproducibility or poor stability of this peptide in naïve matrix.

Protein E (peptide, transition)	STDs Precision (%CV)	QC Samples Precision (%CV)	Naïve Matrix (Control 4) Precision (%CV)
Peptide E1, transition 1	15.3	14.7	7.6
Peptide E1, transition 2	14.7	12.8	10.1
Peptide E2, transition 1	10.2	12.7	7.7
Peptide E2, transition 2	10.6	12.9	10.9
Peptide E3, transition 1	21.8	19.9	63.0
Peptide E3, transition 2	20.1	22.6	62.6

Curve Range: LOD/LLOQ/ULOQ

- For 8 out of 9 proteins, LOD and LLOQ are below 10 ng/mL.
- Excellent dynamic range (>250-fold) for 10 peptides. Good dynamic range (>100-fold) for peptide A1 and I1. Acceptable dynamic range (20-30-fold) for peptides D1, E1 and E3.

Table 4. Final assay. Summary at the peptide and protein levels.

Protein	Peptide	LOD (ng/mL)	LLOQ (ng/mL)	ULOQ (ng/mL)	Best Transition
Protein A	Peptide A1	4	8	1 200	T1
Protein B	Peptide B1	2	2	600	T1
	Peptide B2	2	2	600	T1
Protein C	Peptide C1	4	4	1 200	T1
	Peptide C2	4	4	1 200	T2
Protein D	Peptide D1	33	33	1 000	T1
Protein E	Peptide E1	5	5	100	T1
	Peptide E2	1	1	250	T1
	Peptide E3	1	1	30	T1
Protein F	Peptide F1	10	10	3 000	T1
Protein G	Peptide G1	4	4	1 200	T1
	Peptide G2	4	4	1 200	T1
Protein H	Peptide H1	10	10	3 000	T1
	Peptide H2	10	10	3 000	T1
Protein I	Peptide I1	10	10	1 000	T1

Calibration Standards: Assay Precision and Accuracy

- Good precision (%CV) across the calibration range for all peptides.

Table 5. Precision (%CV) of calibration STD in the final assay. Values above 30% are reported in red.

Transition	STD9	STD8	STD7	STD6	STD5	STD4	STD3	STD2	STD1
Peptide A1	1.3%	2.0%	6.1%	1.9%	1.9%	1.5%	2.1%	12.5%	56.6%
Peptide B1	3.7%	3.6%	1.6%	1.1%	0.0%	3.9%	1.8%	1.1%	7.2%
Peptide B2	0.4%	0.1%	2.1%	1.7%	0.7%	7.6%	3.0%	7.5%	2.7%
Peptide C1	0.6%	0.9%	5.0%	4.4%	5.5%	7.7%	4.0%	5.4%	5.5%
Peptide C2	26.8%	15.7%	4.1%	8.1%	1.9%	13.4%	0.3%	12.2%	9.8%
Peptide D1	0.5%	0.4%	1.4%	9.0%	4.9%	1.8%	3.9%	3.0%	4.9%
Peptide E1	4.1%	2.6%	2.5%	8.6%	1.7%	8.2%	2.8%	---	---
Peptide E2	2.2%	1.1%	2.0%	1.4%	5.2%	0.8%	8.5%	1.6%	8.5%
Peptide E3	10.9%	2.0%	6.4%	16.6%	2.4%	17.3%	14.1%	5.2%	17.6%
Peptide F1	1.9%	1.8%	14.6%	0.8%	3.8%	0.3%	1.3%	0.7%	0.4%
Peptide G1	3.9%	1.5%	1.5%	0.5%	6.0%	12.8%	7.0%	8.4%	22.0%
Peptide G2	2.2%	4.8%	1.1%	1.0%	7.2%	0.9%	6.7%	4.4%	6.7%
Peptide H1	3.7%	2.1%	2.8%	3.9%	8.3%	2.9%	6.3%	3.2%	13.3%
Peptide H2	1.8%	1.8%	2.8%	3.5%	3.7%	0.2%	5.7%	5.9%	4.9%
Peptide I1	4.7%	1.0%	31.2%	11.1%	2.4%	7.1%	6.3%	1.5%	9.1%

- Good accuracy (% of nominal) across the calibration range for most peptides.

Table 6. Accuracy (% of nominal) of calibration STD in the final assay. Values outside the 70-130% range are reported in red.

Transition	STD9	STD8	STD7	STD6	STD5	STD4	STD3	STD2	STD1
Peptide A1	100.8	96.3	100.6	103.0	110.7	98.2	95.3	95.2	23.4
Peptide B1	103.9	97.6	98.2	97.2	102.0	99.0	101.0	109.9	91.2
Peptide B2	102.0	98.9	99.2	96.6	106.5	100.0	98.0	98.4	100.5
Peptide C1	100.3	100.0	100.2	97.9	104.2	96.2	98.5	99.7	102.9
Peptide C2	119.9	98.6	100.9	107.3	94.6	93.8	121.9	91.9	92.4
Peptide D1	60.4	52.4	56.7	77.7	101.0	99.1	95.0	100.2	104.3
Peptide E1	153.2	135.7	134.8	101.8	93.9	100.1	101.7	---	---
Peptide E2	140.1	122.7	125.9	105.9	84.2	88.6	90.4	115.1	115.9
Peptide E3	107.9	95.6	95.6	141.9	90.0	93.4	91.7	117.6	108.4
Peptide F1	87.1	85.0	97.3	101.9	112.2	108.3	107.9	106.1	94.2
Peptide G1	96.5	76.1	80.7	108.1	113.1	92.5	86.4	94.3	109.1
Peptide G2	100.4	95.9	103.1	103.4	97.3	98.6	110.3	93.9	97.0
Peptide H1	102.2	99.0	97.0	101.5	103.1	93.8	99.9	104.8	98.7
Peptide H2	98.7	95.6	105.5	104.0	100.1	101.6	101.0	96.7	96.9
Peptide I1	65.2	54.4	56.7	82.0	110.0	99.1	104.0	100.1	89.8

QC Samples: Assay Precision and Accuracy

- Percentage CV below 20% for all QC samples (low, mid and high). Peptide E3 has a CV of 38.7% for QC1 (low).
- Good accuracy (within 70-130% of nominal) for matrix QC samples for most peptides.

Table 7. Precision (%CV) and accuracy (% of nominal) in the final assay (Naïve matrix and 1/10 diluted matrix). Precision values above 30% are reported in red. Accuracy values outside the 70-130% range are reported in red.

Peptide	HeLa cell lysate	Precision (%CV)			Accuracy		
		QC1 (Low)	QC2 (Mid)	QC3 (High)	QC1 (Low)	QC2 (Mid)	QC3 (High)
Peptide A1	Naïve	0.7%	9.9%	3.5%	125.2	116.2	106.4
Peptide B1	Naïve	7.9%	8.5%	4.4%	117.0	105.1	111.3
Peptide B2	Naïve	2.0%	3.5%	1.5%	95.8	93.2	95.9
Peptide C1	Naïve	0.2%	4.7%	5.2%	104.2	98.1	98.7
Peptide C2	Naïve	18.6%	4.2%	6.1%	116.7	124.3	105.9
Peptide D1	Naïve	0.2%	5.1%	0.6%	115.0	85.5	72.1
Peptide E1	10x diluted	6.4%	3.3%	10.3%	107.2	103.4	116.4
Peptide E2	10x diluted	5.8%	3.4%	3.4%	124.0	125.3	140.6
Peptide E3	10x diluted	38.7%	1.9%	7.7%	150.4	158.6	28.0
Peptide F1	10x diluted	9.0%	6.0%	6.5%	110.4	114.2	67.5
Peptide G1	10x diluted	12.6%	7.3%	16.0%	102.0	101.2	67.1
Peptide G2	10x diluted	2.6%	3.6%	5.9%	116.5	111.2	89.2
Peptide H1	10x diluted	1.7%	8.3%	1.8%	116.9	113.2	108.1
Peptide H2	10x diluted	0.8%	4.0%	5.8%	114.7	104.5	90.5
Peptide I1	Naïve	0.8%	5.1%	19.4%	96.5	85.5	39.3

Endogenous Measurement

- Endogenous level detected for 7 out of 9 proteins in HeLa cells.

Table 8. Measured endogenous levels of each peptide in HeLa cell extracts. Values are average of 6 replicates, expressed in ng/mL or converted to pg/µg based on 30 µg of total protein processed.

Protein	Peptide	Level in HeLa cells (ng/mL)	Level in HeLa cells (pg/µg)
Peptide A	Peptide A1	5.2*	3.5*
Peptide B	Peptide B1	0.7*	0.4*
	Peptide B2	1.2*	0.8*
Peptide C	Peptide C1	8.9	5.9
	Peptide C2	12.4	8.3
Peptide D	Peptide D1	---	---
Peptide E	Peptide E1	17.8	11.9
	Peptide E2	20.5	13.6
	Peptide E3	(8.8)	(5.9)
Peptide F	Peptide F1	197.5	131.7
Peptide G	Peptide G1	219.5	146.4
	Peptide G2	123.1	82.1
Peptide H	Peptide H1	42.4	28.2
	Peptide H2	41.6	27.7
Peptide I	Peptide I1	---	---

*: Value is below the LLOQ level.
( ): Value obtained for a peptide not meeting the quality criteria.

Conclusions

- A sensitive LC/MRM-MS assay for the detection and quantification of proteins in cell lysates was successfully developed for 9 proteins of interest.
- Eight (8) proteins have an LOD and LLOQ < 10 ng/mL and one has an LOD and LLOQ of 33 ng/mL.
- The developed LC/MRM-MS assay met the following criteria:
  - For at least 1 peptide of each protein, precision of STD and QC samples < 30% in the curve range.
  - For at least 1 peptide of each protein, accuracy within 70-130% for > 75% of STD.
  - For at least 1 peptide of each protein, accuracy within 70-130% for > 67% of QC samples.
- Endogenous levels for 7 of the 9 proteins were detected in HeLa cell lysates (ranging from 0.4 to 146 pg/µg).