Published in final edited form as:

Methods Mol Biol. 2013 ; 1077: 121–131. doi:10.1007/978-1-62703-637-5_8.

Targeted Quantitation of Acetylated Lysine Peptides by Selected Reaction Monitoring Mass Spectrometry

Matthew J. Rardin, Jason M. Held, and Bradford W. Gibson

Abstract

Mass spectrometry (MS) allows for the large-scale identification of multiple peptide analytes in complex mixtures. However, the low abundance of acetylated peptides in the overall mixture requires an enrichment step. After enrichment, the resulting acetylated peptides of interest can be quantitated using selected reaction monitoring (SRM)-MS with stable isotope dilution. Here, we describe the enrichment of lysine acetylated peptides from trypsin digested mouse liver mitochondria, and the targeted quantitation of a known lysine acetylation site in succinate dehydrogenase A using SRM-MS on a triple quadrupole instrument.

Keywords

Selected reaction monitoring; Targeted quantitation; Peptide immunoprecipitation; Acetylation; Sirtuin

1 Introduction

Large-scale affinity enrichment of posttranslational modifications (PTMs) from sirtuin knockout models, in combination with advancements in mass spectrometry, is rapidly identifying a large number of potential sirtuin substrates [1, 2]. However, quantitative data from discovery-based shotgun proteomic data sets can be limited by instrument sensitivity and sampling efficiency. Selected reaction monitoring mass spectrometry (SRM-MS) with stable isotope dilution (SID) has emerged as one of the most sensitive techniques for the precise quantitative analysis of predetermined peptide analytes from multiple samples [3-5]. SRM takes advantage of unique features available in a triple quadrupole mass spectrometer. As shown in Fig. 1, in SRM the first quadrupole is set to pass a peptide analyte of a known mass-to-charge ratio (m/z) into the second quadrupole (Q2). In Q2, the peptide precursor(s) (P) contained in the mass selection window, typically ± 1 m/z , undergo collision-activated dissociation (CAD) generating a set of fragment ions ($F_{1, 2, \text{etc.}}$). One or more of these fragments ions can then be selected by the third quadrupole to pass onto the detector thus generating a chromatographic trace for each $P \rightarrow F_n$ transition. These traces are then used to determine the relative abundance of peptide analytes among samples. In practice, one often targets several peptides at a time in parallel acquisitions of SRM transitions, although this multiplexing approach comes at a cost of reduced sensitivity.

In SID, a synthetic peptide containing an isotopically labeled amino acid such as arginine ($^{13}\text{C}_6$, $^{15}\text{N}_4$ -Arg) or lysine ($^{13}\text{C}_6$, $^{15}\text{N}_2$ -Lys) is first used to optimize the $P > F$ transitions and can then be used to generate a standard curve for determining the limit of detection (LOD) and quantitation (LOQ). This standard is then subsequently spiked into the peptide mixture at a known concentration and later used for normalization before quantitation.

Here we demonstrate a method for the targeted quantitation of a previously identified lysine acetylated peptide ($_{172}\text{AFGGQSLK}^{\text{Ac}}\text{FGK}_{182}$) in wild-type (WT) and SIRT3 knockout (KO) animals on the mitochondrial protein succinate dehydrogenase A (SDHA) [2]. To control for biological variability we started with mitochondrial samples from five WT and five KO animals. The steps for proteolysis, solid phase extraction, peptide enrichment, and quantitation by LC-SRM using a heavy labeled synthetic peptide ($_{172}\text{AFGGQSLK}^{\text{Ac}}\text{FGK}[+8]_{182}$) are described. Using this technique we demonstrate a 6.0-fold increase (p value— $2\text{E}-9$) in acetylation at lysine-179 on SDHA in the SIRT3 KO animals.

2 Materials

2.1 Protein Digestion

1. Prepare crude or gradient purified mitochondria according to Rardin et al. [6] in the presence of deacetylase inhibitors: 10 mM nicotinamide and $0.5\text{ }\mu\text{M}$ trichostatin A.
2. 0.1 M Triethylammonium bicarbonate solution (TEAB), pH 8.5, in water.
3. $1\text{ }\%$ (w/v) n -Dodecyl- β -D-maltoside in 0.1 M TEAB, store at $-20\text{ }^{\circ}\text{C}$ (*see Note* ¹).
4. 10 M urea prepared fresh in 0.1 M TEAB.
5. Reducing reagent: 0.5 M Tris (2-carboxyethyl) phosphine Bond Breaker solution (Thermo Scientific, Rockford, IL, USA).
6. Alkylating reagent: 0.21 M iodoacetamide prepared fresh in 0.1 M TEAB.
7. Proteolysis: Sequencing grade modified trypsin.
8. Formic acid (FA) (*see Note* ²).
9. Low range pH strips.

2.2 Desalting

1. Solid phase extraction (SPE) cartridges: Oasis HLB 1 cc (30 mg) Extraction Cartridges (Waters, Milford, MA, USA).
2. Vacuum extraction manifold.
3. Acetonitrile (ACN).
4. Speedvac concentrator.

¹Maltoside precipitates at $4\text{ }^{\circ}\text{C}$. Therefore it is necessary to warm it prior to use.

²When working with concentrated formic acid (FA), work in a chemical fume hood or use a mask as it can be severely irritating to skin and mucous membranes.

2.3 Immunoprecipitation

1. Immunoprecipitation buffer: 50 mM Tris-HCl, pH 8.0, 100 mM NaCl, 1 mM EDTA (NET) (*see* Note ³). Store at 4 °C.
2. Protein G agarose beads.
3. 1.5 mL polypropylene siliconized micro centrifuge tubes (*see* Note ⁴).
4. Anti-acetyl lysine antibody (*see* Note ⁵).
5. Peptide elution buffer: 40 % (v/v) ACN, 1 % (v/v) trifluoroacetic acid (TFA) in HPLC grade water.
6. Flat gel loading pipet tips.
7. ZipTipC18 pipette tips (Millipore, Billerica, MA, USA).

2.4 Mass Spectrometry

1. HPLC buffers: Buffer A (0.1 % formic acid) and Buffer B (0.1 % formic acid, 90 % acetonitrile).
2. Reversed-phase HPLC column: Eksigent Nano cHiPLC ChromXP C18 column, 75 µM inner diameter, 15 cm length, 3 µM particle size, designed for use with the Eksigent cHiPLC-Nanoflex System (*see* Note ⁶).
3. HPLC: Eksigent NanoLC-Ultra 2Dplus (*see* Note ⁶).
4. Triple quadrupole mass spectrometer: 5500QTRAP, with Analyst data acquisition software.
5. Skyline: <http://proteome.gs.washington.edu/software/skyline>

3 Methods

3.1 Denaturation, Reduction, Alkylation, and Digestion of Mitochondrial Protein

1. Centrifuge 1 mg of mitochondrial protein per sample for 10 min at $15,000 \times g$ in a 1.5 mL microfuge tube. Aspirate off the supernatant and resuspend the pellet in 100 µL of 1 % maltoside. Vortex the tube as necessary until the lysate becomes clear. Add 100 µL of 10 M urea and mix by pipetting up and down 5–10 times.

³Small amount of nonionic detergents such as NP-40 may be used during the immunoprecipitation step, but may cause ion suppression if not properly removed during the washing step. Therefore, we chose to leave out detergents from our sample preparation and did not observe a significant difference in peptide enrichment.

⁴Siliconized tubes provide less friction than more commonly used microfuge tubes and allow for adequate mixing of the beads in the absence of detergent.

⁵The amount of antibody needed is often greater than the normal aliquot sold by various vendors and may require contacting the vendor to inquire about availability.

⁶This protocol for SRM quantitation of acetylated peptides uses a nanoflow HPLC (nanoliters per minute flow rate) since this approach maximizes assay sensitivity. The specific system described in this method uses an Eksigent Nanoflex as part of the LC system; however any C18 column and HPLC system compatible with flow rates from 250 to 500 nL/min can be used.

2. Bring the volume up to ~990 μL using 0.1 M TEAB and add 9 μL of the TCEP reducing agent for a working concentration of 4.5 mM (*see Note* ⁷). Briefly vortex the sample to mix, then incubate the sample at 37 °C for 1 h.
3. Remove the sample from the incubator and briefly centrifuge to ensure the sample is at the bottom of the tube to prevent sample loss when opening the lid. Add 50 μL of the alkylating reagent for a final concentration of 10 mM. Briefly vortex the sample to mix, then incubate the sample in the dark at room temperature for 30 min.
4. Remove the sample from the dark and add 20 μg of trypsin resuspended in 50 μL of 0.1 M TEAB for a working trypsin to protein ratio of 1:50. Incubate the sample overnight at 37 °C.
5. Following proteolysis, remove the sample from the incubator and add concentrated FA in a stepwise fashion (2–4 μL at a time) (*see Note* ⁸) until the pH < 3 (*see Note* ⁹).

3.2 Desalting and Solid Phase Extraction (SPE)

1. Place SPE column into the vacuum manifold and add 3×400 μL of 0.1 % FA/80 % ACN in water to condition the column (*see Note* ¹⁰).
2. Turn on the vacuum pump and adjust the flow rate to a flow rate of 0.5 drops per second.
3. Add 4 × 400 μL of 0.1 % FA in water to equilibrate the column.
4. Add the acidified peptides to the column.
5. Add 4 × 400 μL of 0.1 % FA in water to wash the column. Turn off the vacuum pump when the wash solution is 0.5 cm from the top of the column. Remove the lid and place a 1.5 mL microfuge tube into the manifold to collect the sample during elution.
6. Add 3 × 400 μL of 0.1 % FA/80 % ACN in water and turn the vacuum pump on to elute peptides from the column into the 1.5 mL microfuge tube. Turn off the vacuum pump prior to removal of the column (*see Note* ¹¹).
7. Place the sample into the Speedvac concentrator and evaporate the sample to near dryness.

3.3 Lysine Acetylated Peptide Enrichment

1. Peptides are resuspended in 500 μL of NET buffer.

⁷Concentrated denaturants, such as urea and maltoside, can inhibit trypsin activity during proteolysis. Therefore it is necessary to dilute out the sample to 1 M and 0.1 % (w/v) respectively, prior to the addition of trypsin.

⁸The acidification of TEAB results in the production of CO₂ that is released following the addition of FA. This should be allowed to bleed off for several minutes at room temperature prior to moving on to the solid phase extraction step.

⁹Low range pH strips provide the most efficient method for monitoring changes in pH while minimizing sample loss.

¹⁰To maximize binding efficiency the column should not be allowed to dry out between changes in solutions once it has been wetted. Once the solution reaches ~0.5 cm above the column the next solution may be added.

¹¹To prevent sample loss following the elution of peptides from the column, turn off the vacuum pump prior to removal of the column. Allow pressure to equilibrate using the pressure release tab and *not* by removing the column above the eluate.

2. Place 40 μL of protein G agarose slurry in a separate siliconized microfuge tube and wash the agarose beads $3 \times 500 \mu\text{L}$ with NET buffer. Beads are then resuspended in 500 μL of NET buffer and the resuspended peptides from step 1.
3. Add 80 μg of anti-acetyl lysine antibody to the sample and incubate overnight at 4 $^{\circ}\text{C}$ with gentle rocking to mix the sample.
4. Samples are washed $3 \times 1 \text{ mL}$ with NET buffer for 5 min at 4 $^{\circ}\text{C}$ with gentle rocking (*see Note* ¹²).
5. Aspirate off the supernatant of the final wash of the NET buffer and add 200 μL of the peptide elution buffer.
6. Incubate the sample at 30 $^{\circ}\text{C}$ with gentle agitation for 5 min. Pellet the beads by centrifugation at $4,000 \times g$ for 2 min, and remove 100 μL of the supernatant. Save the supernatant containing the eluted peptides in a separate microfuge tube. Add 100 μL of the peptide elution buffer to the beads and incubate the sample at 30 $^{\circ}\text{C}$ with gentle agitation for 5 min.
7. Repeat step 6.
8. Centrifuge the final elution at $3,000 \times g$ for 2 min and collect the remaining supernatant of $\sim 200 \mu\text{L}$ and combine with the previous elutions.
9. Speedvac the solution containing the eluted peptides to near dryness and resuspend in 30 μL of 0.1 % FA and 1 % ACN in HPLC grade water (*see Note* ¹³).
10. Desalt peptides two times using a C18 ZipTip (*see Note* ¹⁴).
11. Speedvac desalted peptides to dryness and resuspend samples in 20 μL of 0.1 % FA, 1 % ACN, in HPLC grade water (*see Note* ¹⁵).

3.4 Define SRM Transitions for the Peptide AFGGQSLK^AcFGK Using Skyline

1. In the Transition settings menu, Prediction tab, choose the default collision energy equation for the ABI QTRAP 5500. The declustering potential used for all SRM transitions on the QTRAP 5500 is 100 and the collision cell exit potential is 40.
2. In the Transition settings menu, Filter tab, choose ions from $m/z >$ precursor to last ion (*see Note* ¹⁶).
3. In the Transition settings menu, Library tab, pick four product ions (*see Note* ¹⁷).

¹²We find the use of a flat gel loading pipet tip minimizes the amount of agarose beads that are lost during removal of the supernatant.

¹³If there is large carry over of beads from extraction of the supernatant, samples can be centrifuged for 5 min at $15,000 \times g$ and the supernatant transferred to a new microfuge tube prior to desalting with the C18 ZipTips.

¹⁴It is beneficial to perform two ZipTips to maximize the efficiency of peptide recovery. In addition, peptides are eluted from the ZipTip into the same elution buffer prior to concentrating.

¹⁵It is essential to resuspend all samples with equal volumes of buffer to ensure the same amount of sample is loaded onto the mass spectrometer for quantitation.

¹⁶Fragment ions with an m/z larger than the precursor are optimal for SRM transitions since they decrease interferences from singly charged precursor ions.

¹⁷Three SRM transitions are typically used per peptide in the final assay. Therefore it is best to begin assay development with four or more transitions per peptide in case there are interferences from the matrix.

4. For peptide AFGGQSLK^{Ac}FGK, chose SRM transitions corresponding to fragment ions (F) y9, y8, y6, and y4 (*see Note* ¹⁸).
5. A stable isotope-modified version of the AFGGQSLK^{Ac}FGK peptide synthesized with a ¹³C₆ ¹⁵N₂-Lys at the C terminus is spiked into all samples. To make SRM transitions for this stable isotope-labeled peptide with an 8 Da mass shift, AFGGQSLK^{Ac}FGK[+8], add a heavy isotope label modification in Skyline and include this peptide in the peptide list. Use the same collision energy, declustering potential, and transitions as the light (endogenous) AFGGQSLK^{Ac}FGK peptide.

3.5 Optimize and Validate LC-SRM Assay

1. A typical LC-MS instrument configuration for targeted assays is shown in Fig. 1 (*see Note* ¹⁹).
2. Use Skyline to optimize the collision energy and/or declustering potential for each transition using a synthetic peptide (*see Note* ²⁰). The retention time of the peptide is used for retention time scheduling in the final assay.
3. The final SRM mass spectrometry parameters for AFGGQSLK^{Ac}FGK are:

Q1	Q3	Retention time	Peptide	Fragment ion	Declustering potential	Collision energy	Collision cell exit potential
591.3	963.5	31.76	AFGGQSLK ^{Ac} FGK	y9	78.1	32.1	40
591.3	906.5	31.76	AFGGQSLK ^{Ac} FGK	y8	88.1	30.1	40
591.3	721.4	31.76	AFGGQSLK ^{Ac} FGK	y6	88.1	28.1	40
591.3	521.3	31.76	AFGGQSLK ^{Ac} FGK	y4	98.1	36.1	40
595.3	971.5	31.76	AFGGQSLK ^{Ac} FGK [+8]	y9	78.1	32.1	40
595.3	914.5	31.76	AFGGQSLK ^{Ac} FGK [+8]	y8	88.1	30.1	40
595.3	729.4	31.76	AFGGQSLK ^{Ac} FGK [+8]	y6	88.1	28.1	40
595.3	529.3	31.76	AFGGQSLK ^{Ac} FGK [+8]	y4	98.1	36.1	40

4. Import the transition list from Skyline into Analyst for data acquisition on the 5500 QTRAP. The final assay uses retention time scheduling based on the peptide retention determined in the collision energy and declustering potential optimization analyses (*see Note* ²¹).

¹⁸These fragment ions have the highest intensity in the MS/MS spectra of the peptide, although the highest abundance fragments do not always generate the P>F transitions with the highest selectivity and lowest coefficient of variation.

¹⁹This step is optional. The benefits of optimizing the collision energy and/or declustering potential for each transition varies by mass spectrometer. For the 5500 QTRAP, collision energy and declustering potential optimization improves sensitivity for very small and very large peptides. For collision energy optimization in Skyline, use a step size of 2 and for declustering potential use a step size of 10. Use five steps for each optimization step.

²⁰For this assay the autosampler is configured in "direct injection" mode, without a trap column, where the sample is transferred from the autosampler directly to the HPLC column inline with the mass spectrometer. A 1 µL sample loop is used for the autosampler. The LC flow rate is 300 nL/min. The LC gradient is 97 % Buffer A at 0–5 min, 85 % A at 8 min, 65 % A at 42 min, 10 % A at 45 min to 49 min, 97 % A at 50 min and equilibrated for at least 30 min prior to the next analysis.

5. Perform a standard curve in triplicate by spiking in the stable isotope-labeled peptide to determine the linear range, limit of detection (LOD), and limit of quantitation (LOQ) (*see* Note ²²). Use 250 ng of crude mitochondria as a sample matrix for each concentration point. Perform three independent calibration curves using with calibration points of 0, 4, 12, 40, 110, 330, 1,000, 3,000, and 25,000 amol per injection.
6. Integrate peaks with Skyline to determine the peak area of the stable isotope-labeled AFGGQSLK^{Ac}FGK peptide where K is ¹³C₆, ¹⁵N₂-Lys.
7. Calculate the LOD and LOQ with the following equations:

$$LOD = x_b + 3s_b$$

$$LOQ = x_b + 10s_b$$

where x_b is the mean concentration of the blank and s_b is the standard deviation of the blank.

3.6 Apply LC-SRM Assay to Quantify Targeted Acetylation Sites in Samples

1. Analyze each sample with 25 fmol heavy labeled AFGGQSLK^{Ac}FGK spiked in using the validated, retention time scheduled SRM assay.
2. Perform two replicate LC-SRM analyses of each sample.
3. Integrate peak areas in Skyline. Representative results are shown in Fig. 2b.
4. Use the peak area ratio of the light to heavy peptide to determine the molar amount of the AFGGQSLK^{Ac}FGK peptide in each sample (*see* Note ²³).

Acknowledgments

This work was supported by NIH grant R24 DK085610 (B.W.G.) and the NCRR shared instrumentation grant S10 RR027953 (B.W.G.) for the 5500 QTRAP system.

References

1. Chen Y, Zhao W, Yang JS, Cheng Z, Luo H, Lu Z, Tan M, Gu W, Zhao Y. Quantitative acetylome analysis reveals the roles of SIRT1 in regulating diverse substrates and cellular pathways. *Mol Cell Proteomics*. 2012; 11(10):1048–1062.10.1074/mcp.M112.019547 [PubMed: 22826441]
2. Schilling B, Rardin MJ, MacLean BX, Zawadzka AM, Frewen BE, Cusack MP, Sorensen DJ, Bereman MS, Jing E, Wu CC, Verdin E, Kahn CR, Maccoss MJ, Gibson BW. Platform-

²¹Standard curves for peptides analysis typically begin at 1 amol and go as high as 1 pmol for nanoLC-SRM. The optimal experimental design is to spike in a stable isotope-labeled synthetic peptide into a matrix similar to the assay matrix in order to identify potential interferences. Complete each concentration curve in its entirety before acquiring additional curves. Perform at least three standard curves. There are several ways to calculate the LOD and LOQ [7], each with their own strengths and weaknesses.

²²Retention time scheduling methods acquire each SRM transition during a small window of time when the peptide of interest elutes. Since the peak width and the number of data points per peak that must be collected (7–9, typically) determine the maximum duty cycle of the assay, acquiring additional transitions decreases the dwell time per SRM transition. Therefore, retention time scheduling, rather than acquisition throughout the entire LC-MS analysis, increases the number of transitions that can be measured during a single LC-MS analysis. Retention time scheduling is optional.

²³Absolute quantitation using heavy peptides have several caveats, including digestion efficiency and accurate quantitation of the synthetic peptides. In this assay, organelle enrichment and peptide immunopurification efficiency are additional variables. Therefore, the molar concentration in each sample is a “best estimate” and not a completely rigorous value.

independent and label-free quantitation of proteomic data using MS1 extracted ion chromatograms in skyline: application to protein acetylation and phosphorylation. *Mol Cell Proteomics*. 2012; 11(5):202–214.10.1074/mcp.M112.017707 [PubMed: 22454539]

3. Addona TA, Abbatiello SE, Schilling B, Skates SJ, Mani DR, Bunk DM, Spiegelman CH, Zimmerman LJ, Ham AJ, Keshishian H, Hall SC, Allen S, Blackman RK, Borchers CH, Buck C, Cardasis HL, Cusack MP, Dodder NG, Gibson BW, Held JM, Hiltke T, Jackson A, Johansen EB, Kinsinger CR, Li J, Mesri M, Neubert TA, Niles RK, Pulsipher TC, Ransohoff D, Rodriguez H, Rudnick PA, Smith D, Tabb DL, Tegeler TJ, Variyath AM, Vega-Montoto LJ, Wahlander A, Waldemarson S, Wang M, Whiteaker JR, Zhao L, Anderson NL, Fisher SJ, Liebler DC, Paulovich AG, Regnier FE, Tempst P, Carr SA. Multi-site assessment of the precision and reproducibility of multiple reaction monitoring-based measurements of proteins in plasma. *Nat Biotechnol*. 2009; 27(7):633–641.10.1038/nbt.1546 [PubMed: 19561596]
4. Keshishian H, Addona T, Burgess M, Kuhn E, Carr SA. Quantitative, multiplexed assays for low abundance proteins in plasma by targeted mass spectrometry and stable isotope dilution. *Mol Cell Proteomics*. 2007; 6(12):2212–2229.10.1074/mcp.M700354-MCP200 [PubMed: 17939991]
5. Gerber SA, Rush J, Stemman O, Kirschner MW, Gygi SP. Absolute quantification of proteins and phosphoproteins from cell lysates by tandem MS. *Proc Natl Acad Sci U S A*. 2003; 100(12):6940–6945.10.1073/pnas.0832254100 [PubMed: 12771378]
6. Rardin MJ, Taylor GS, Dixon JE. Distinguishing mitochondrial inner membrane orientation of dual specific phosphatase 18 and 21. *Methods Enzymol*. 2009; 457:275–287.10.1016/S0076-6879(09)05015-0 [PubMed: 19426873]
7. Shrivastava A, Gupta VB. Methods for the determination of limit of detection and limit of quantitation of the analytical methods. *Chron Young Sci*. 2011; 2(1):21–25.10.4103/2229-5186.79345

Fig. 1.

Schematic of LC-SRM instrumentation. Samples are introduced into the LC-MS system via the autosampler. The HPLC pumps deliver a mixture of Buffer A (0.1 % formic acid) and Solvent B (0.1 % formic acid, 80 % acetonitrile) to the system. In a typical reversed-phase LC-MS analysis, the sample binds to the C18 HPLC column in buffer and peptides are gradually eluted by increasing the percentage of the organic-containing Solvent B. A triple quadrupole mass spectrometer is connected inline with the HPLC system. A triple quadrupole isolates a specific precursor ion with a known mass-to-charge (m/z) ratio in the first quadrupole (Q1). The precursor ion is fragmented in Q2 by collision-activated dissociation (CAD) to form product ions. A product ion of a specific m/z is isolated in Q3 and allowed to pass through the detector. Each Q1/Q3 m/z combination is called an SRM transition

Fig. 2.

LC-SRM analysis of peptide AFGGQSLK^{Ac}FGK from SDHA. **(a)** Concentration curve analysis of a synthetic, stable isotope-labeled AFGGQSLK^{Ac}FGK peptide spiked into a mitochondrial lysate is used to assess the linear range, limit of detection (LOD), and limit of quantitation (LOQ). **(b)** LC-SRM data of a wild-type (WT) and SIRT3 knockout mouse liver mitochondria. Demonstrating increased endogenous AFGGQSLK^{Ac}FGK peptide relative to the spiked-in synthetic, stable isotope-labeled AFGGQSLK^{Ac}FGK peptide. **(c)** Two LC-SRM technical replicates are performed per sample. In this study, five WT and five SIRT3 KO mouse livers were analyzed. The SIRT3 knockout mice have a 6.0-fold increase in levels of the acetylated AFGGQSLK^{Ac}FGK peptide