Chapter 6

A Targeted MRM Approach for Tempo-Spatial Proteomics Analyses

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Abstract

When deciding to perform a quantitative proteomics analysis, selectivity, sensitivity, and reproducibility are important criteria to consider. The use of multiple reaction monitoring (MRM) has emerged as a powerful proteomics technique in that regard since it avoids many of the problems typically observed in discovery-based analyses. A prerequisite for such a targeted approach is that the protein targets are known, either as a result of previous global proteomics experiments or because a specific hypothesis is to be tested. When guidelines that have been established in the pharmaceutical industry many decades ago are taken into account, setting up an MRM assay is relatively straightforward. Typically, proteotypic peptides with favorable mass spectrometric properties are synthesized with a heavy isotope for each protein that is to be monitored. Retention times and calibration curves are determined using triple-quadrupole mass spectrometers. The use of iRT peptide standards is both recommended and fully integrated into the bioinformatics pipeline. Digested biological samples are mixed with the heavy and iRT standards and quantified. Here we present a generic protocol for the development of an MRM assay.

Key words MRM, Quadrupole mass spectrometry, Quantitation

1 Introduction

The ultimate goal of a quantitative proteomics experiment is to identify and quantify protein changes in time and/or space. Particularly, when monitoring the changes of specific proteins over many time and/or space points, the use of multiple reaction monitoring (MRM) has emerged as a powerful technique because of its high quantitative precision and sensitivity resulting in low detection limits [1–5]. A prerequisite for such a targeted approach is that the protein targets are known, either as a result of previous global proteomics experiments or because a specific hypothesis is to be tested.

Typically, proteotypic peptides with favorable mass spectrometric properties are selected for each protein and synthesized

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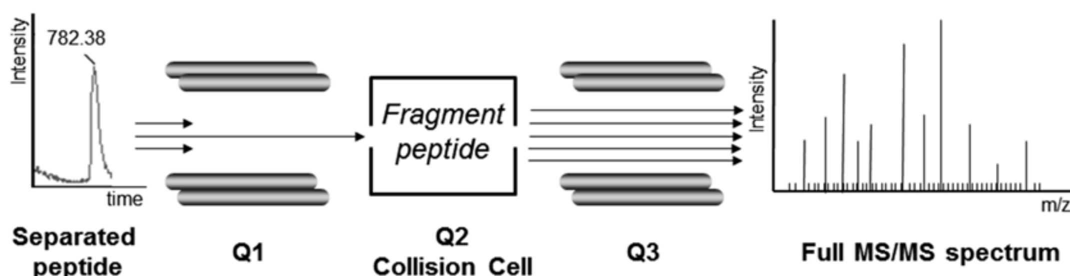

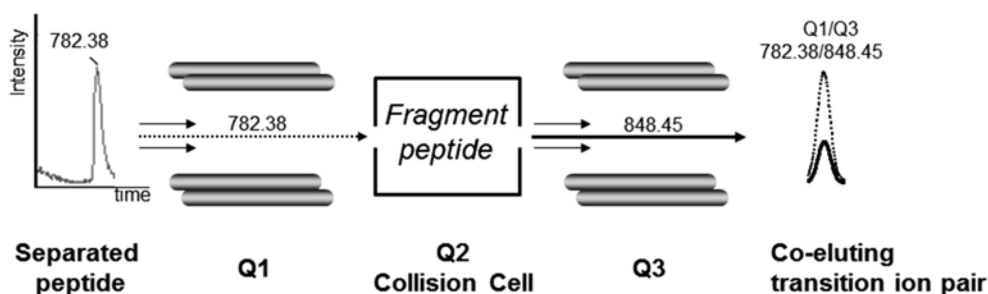

Fig. 1 The two operational modes of a QTRAP instrument. (**a**) QTRAP mode. Selected ions of separated peptides pass through Q1 and are fragmented in Q2, all fragment ions pass through Q3, and a full MS/MS spectrum is recorded. This mode is particularly useful in the early stages of assay development when identities of peptides need to be confirmed. (**b**) QQQ mode. Selected ions of separated peptides pass through Q1 and are fragmented in Q2, and all selected fragment ions pass through Q3. Co-eluting transition ion pairs are recorded. This mode is particularly useful in maximizing sensitivity of known peptides

with a heavy isotope. Retention times and calibration curves are determined using predominantly triple-quadrupole mass spectrometers (Fig. 1), although quadrupole time-of-flight and Orbitrap mass spectrometers can also be used. We preferentially use the QTRAP in the trap mode (Fig. 1a) during the initial stages of assay development, to establish retention time of the peptides, select the best suited three to five fragment ions, and optimize scheduling. Once this is accomplished, scheduled analysis is performed in the more sensitive QQQ mode (Fig. 1b). To determine realistic limits of detection (LOD) and quantitation (LOQ), it is recommended to analyze the heavy standards in a complex biological background. This prevents unwanted absorption of the peptides and helps to identify interferences of the peptides with the background at an early stage of the method development. Thus, digested biological samples are mixed with the heavy and retention time iRT standards and quantified over a dynamic range [6].

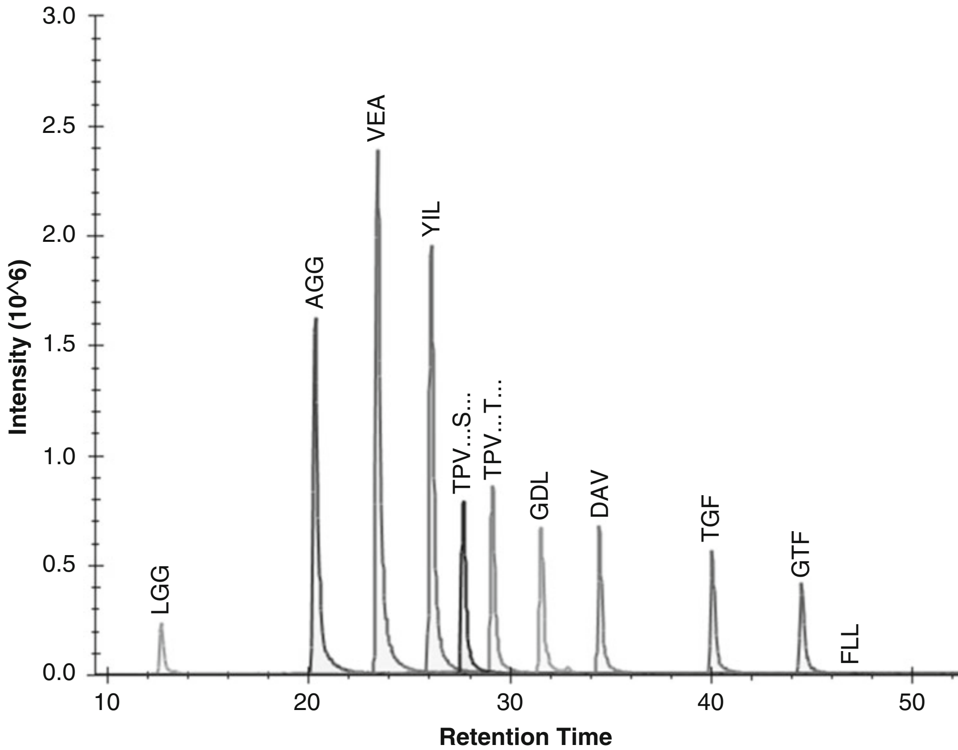

Fig. 2 Elution profile of 11 iRT peptides in a 45-min gradient. Peptides are LGGNETQVR (LGG), AGGSSEPVTGLADK (AGG), VEATFGVDESANK (VEA), YILAGVESNK (YIL), TPVISGGPYYER (TPV…S...), TPVITGAPYYER (TPV…T...), GDLDAASYYAPVR (GLD), DAVTPADFSEWSK (DAV), TGFIIDPGGVIR (TGF), GTFIIDPAAIVR (GTF), FLLQFGAQGSPLFK (FLL)

A typical elution profile of the iRT peptides is shown in Fig. 2. The use of iRT peptide standards is recommended to monitor and ensure consistent chromatographic performance throughout the assay [6]. Additionally, the iRT peptide standards can be fully integrated into the bioinformatics pipeline using Skyline [7]. Figure 3 shows an example of a calibration curve of standard peptides and their transition ion responses from 30 to 500,000 attomol, measured in a complex background, e.g., HeLa lysate if a HeLa cell culture is to be investigated. The calibration curves of the heavy standards can be used to determine targeted peptide amounts. As shown in Fig. 3, a response of 100,000 AU for a targeted peptide could be associated with an amount of 550 amol, in comparison to the calibration curve of the heavy standard shown (*see* **Note 1**). The most accurate quantitative results are usually achieved when the heavy standards are in the same range as the expected light samples. For this, it might be necessary to do a preliminary analysis first, followed by a full analysis with heavy peptides spiked in according to the preliminary results. Here, we provide a generic guideline for the development of an MRM assay.

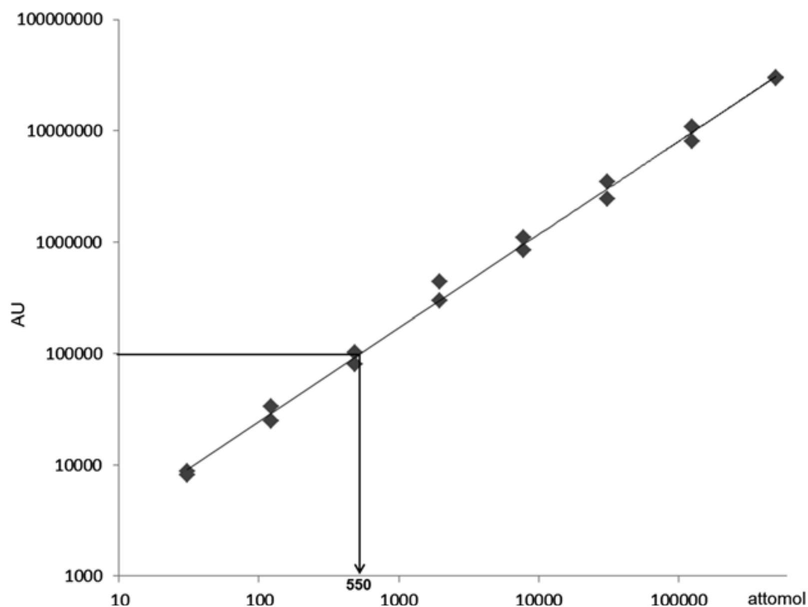

Fig. 3 Example of a calibration curve for a standard peptide covering a dynamic range from 30 to 500,000 attomol. This curve can be used to quantify unknown samples. If an unknown sample has a response of 100,000 AU, the sample contains 550 attomol of that particular peptide

2 Materials

2.1 Chemicals

2.1.1 LC and LC Samples

1. Solvent A: 0.2 % Formic acid. Add 2 mL of formic acid (for mass spectrometry; 98 %) to 998 mL of water (HPLC gradient grade quality).
2. Solvent B: Acetonitrile containing 0.2 % formic acid. Add 2 mL of formic acid (for mass spectrometry; 98 %) to 998 mL of acetonitrile (HPLC gradient grade quality).
3. Synthetic heavy standard peptides (either prepared in-house or commercially).
4. Synthetic iRT peptides (either prepared in-house or commercially).
5. Standard solvent (30 % acetonitrile, 70 % of 0.2 % formic acid). Add 30 mL of acetonitrile to a 69.8 mL of water and 0.2 mL of formic acid.
6. All reagents should be of the highest purity available.

2.1.2 FASP

1. *Lysis buffer*. Add 5 g sodium dodecyl sulfate (SDS), 1.58 g (100 mM) Tris/HCl (pH 7.6; adjust with HCl as necessary), 1.54 g (100 mM) D,L-dithiothreitol (DTT), supplemented with 1× protease inhibitor cocktail (complete, EDTA free, Roche) and 17.42 mg (1 mM) phenylmethanesulfonyl fluoride (PMSF) brought up to 100 mL with ddH_2O.

2. *Urea A (UA) buffer*. Add 48.05 g (8 M) urea and 1.58 g (100 mM) Tris/HCl (pH 8.5, adjust with HCl as necessary) to a total volume of 100 mL of ddH_2O; prepare fresh on the day of the digestion.
3. *Urea B (UB) buffer*. Add 48.05 g (8 M) urea and 1.58 g (100 mM) Tris/HCl (pH 8.0, adjust with HCl as necessary) to a total volume of 100 mL of ddH_2O; prepare fresh on the day of the digestion.
4. *27 mM Tris(2-carboxyethyl)phosphine hydrochloride (TCEP) solution*. Add 14.33 g (500 mM) TCEP to 1.58 g (100 mM) Tris/HCl (pH 8.5, adjust with HCl as necessary) in a total volume of 100 mL of ddH_2O and store at –20 °C. Right before reduction step, thaw aliquot and dilute 5.4 µL of 500 mM TCEP by adding 94.6 µL of UA buffer.
5. *Alkylating solution*. Prepare fresh using 0.925 mg (50 mM) iodoacetamide (IAA) in 1 mL of UA buffer.
6. *50 mM Ammonium bicarbonate (NH4HCO3) solution*. Add 0.40 g of NH_4HCO_3 to 100 mL of H_2O.
7. *100 mM Calcium chloride (CaCl2) solution*. Add 14.69 mg of calcium chloride dehydrate to 1 mL of water.

2.1.3 Preparation of Heavy Standard Peptide Solutions

1. Heavy standard proteotypic peptides of target proteins are synthesized, either commercially or if available in-house (*see* **Note 2**).
2. Prepare solutions of peptide standards at a nominal concentration of 1 pmol/µL using 30 % acetonitrile and 70 % of 0.2 % formic acid (*see* **Note 3**).
3. Eppendorf tubes; pipettes.

2.1.4 Preparation of iRT Peptide Solutions

1. Eleven iRT peptides (LGGNETQVR (LGG), AGGSSEPVT GLADK (AGG), VEATFGVDESANK (VEA), YILAGVESNK (YIL), TPVISGGPYYER(TPV…S), TPVITGAPYYER(TPV…T), GDLDAASYYAPVR (GLD)) are synthesized, either commercially or if available in-house (*see* **Note 4**).
2. Prepare a solution containing all iRT peptide standards at a nominal concentration of 1 pmol/µL using 30 % acetonitrile and 70 % of 0.2 % formic acid.
3. Eppendorf tubes; pipettes.

2.2 Liquid Chromatography-Mass Spectrometry (LC-MS/MS)

1. QTRAP 6500 (AB Sciex, Framingham, MA) with an Eksigent ekspert nanoLC 425 pump, ekspert nanoLC400 autosampler, ekspert cHiPLC, and Analyst software. The LC system is coupled to a NanoSpray III Source and Heated Interface (AB/Sciex).
2. CHiPLC Chrom XP C18-CL 3 µm trap column, 120 Å (200 µm × 0.5 mm).

3. CHiPLC Chrom XP C18-CL 3 μm column, 120 Å (75 μm × 150 mm).
4. Sonicate solvent A and B prior to use (*see* **Note 5**).

2.3 Data Analysis

Skyline software (*see* **Note 6**).

3 Methods

3.1 Preparation of HeLa Tryptic Peptide Solutions Using a Modified Filter-Assisted Sample Preparation (FASP) Procedure [8]

3.1.1 Lysate Preparation

1. Resuspend cells or tissue in a 1:10 sample-to-buffer ratio and incubate at 95 °C for 5 min.
2. Lyse cells or homogenize tissue using standard methods. Clarify lysate by centrifugation at 16,000 × *g* for 5 min at 20 °C and determine protein concentration (*see* **Note** 7).

3.1.2 FASP (Double Digestion)

1. Combine up to 200 μg of lysate with 200 μL of UA buffer in a 30 K microcon filter unit and centrifuge at 14,000 × *g* for 15 min.
2. Discard flow-through from the collection tube, add an additional 200 μL of UA buffer to the filter unit, and centrifuge at 14,000 × *g* for 15 min.
3. Repeat **step 2** at least two additional times (*see* **Note 8**).
4. Discard the flow-through, add 100 μL of UA buffer containing 27 mM TCEP to the filter unit, and mix at 600 rpm in a thermo-mixer for 1 min. Then incubate for 20 min at room temperature without shaking (*see* **Note 9**).
5. After the incubation with TCEP is complete, centrifuge the filter unit at 14,000 × *g* for 10 min.
6. Discard the flow-through, add an additional 100 μL of UA buffer to the filter unit, and then centrifuge at 14,000 × *g* for 15 min.
7. Discard the flow-through, add 100 μL of alkylating solution, and mix at 600 rpm in a thermo-mixer for 1 min. Then incubate for 20 min at room temperature without shaking, in the dark (*see* **Note 10**).
8. After the incubation with the alkylation solution is complete, centrifuge the filter unit at 14,000 × *g* for 10 min.
9. Discard the flow-through, add 100 μL of UB buffer to the filter unit, and centrifuge at 14,000 × *g* for 15 min.
10. Repeat **step 9** two additional times.
11. Transfer the filter unit to a new collection tube, add 40 μL of UB buffer containing Lys-C (enzyme-to-protein ratio 1:50),

and mix at 600 rpm for 1 min in the thermo-mixer, followed by a 4-h incubation at room temperature without shaking in the dark.

12. Then add 120 μL of trypsin dissolved in 50 mM NH_4HCO_3 (enzyme-to-protein ratio 1:100), along with 1.6 μL of 100 mM $CaCl_2$ and mix for 1 min at 600 rpm in a thermo-mixer (*see* **Note 11**).
13. Incubate the units at room temperature for 14 h, in the dark.
14. Centrifuge the filter unit at 14,000 × *g* for 15 min (*see* **Note 12**).
15. To increase recovery of peptides, add 100 μL of 50 mM NH_4HCO_3 to the filter unit and centrifuge at 14,000 × *g* for 15 min (*see* **Note 12**).
16. Repeat **step 15** two additional times and combine all flow-through (*see* **Note 12**).
17. Add enough formic acid to bring up the concentration to 5 %.
18. Lyophilize the peptide solution.
19. Resuspend the lyophilized peptides in 100 μL of 0.2 % formic acid and desalt by HPLC, e.g., with a C8 peptide macrotrap (3 × 8 mm) (200 μg maximum capacity) (*see* **Note 13**).
20. Lyophilize the desalted peptides and store at −20 °C until ready for mass spec analysis (*see* **Note 14**).
21. Prior to analysis, resuspend HeLa digest in 0.2 % formic acid at a nominal concentration of 4 μg/μL.

3.2 Preparation of Serial Dilutions of Heavy Standard Peptide and iRT Peptide Solutions in Complex Background

1. To 1.5 μL of trypsin-digested HeLa lysate (4 μg/μL), add 3 μL of heavy standard solution (1 pmol/μL), 0.6 μL of iRT peptide solution (1 pmol/μL), and 0.9 μL of 0.2 % formic acid: solution A, 500 fmol/μL of heavy standard.
2. Keep 1 μL of solution A (500 fmol/μL) for injection (*see* **Note 15**).
3. Dilute 1.5 μL of solution A (500 fmol/μL) in 1.125 μL trypsin-digested HeLa lysate, 0.45 μL iRT peptide solution (1 pmol/μL), and 2.925 μL of 0.2 % formic acid: solution B, 125 fmol/μL of heavy standard.
4. Keep 1 μL of solution B (125 fmol/μL) for injection.
5. Dilute 1.5 μL of solution B (125 fmol/μL) in 1.125 μL trypsin-digested HeLa lysate, 0.45 μL iRT peptide solution (1 pmol/μL), and 2.925 μL of 0.2 % formic acid: solution C, 31.3 fmol/μL of heavy standard.
6. Keep 1 μL of solution C (31.3 fmol/μL) for injection.
7. Dilute 1.5 μL of solution C (31.3 fmol/μL) in 1.125 μL trypsin-digested HeLa lysate, 0.45 μL iRT peptide solution (1 pmol/μL), and 2.925 μL of 0.2 % formic acid: solution D, 7.8 fmol/μL of heavy standard.

8. Keep 1 μL of solution D (7.8 fmol/μL) for injection.
9. Dilute 1.5 μL of solution D (7.8 fmol/μL) in 1.125 μL trypsin-digested HeLa lysate, 0.45 μL iRT peptide solution (1 pmol/μL), and 2.925 μL of 0.2 % formic acid: solution E, 1.95 fmol/μL of heavy standard.
10. Keep 1 μL of solution E (1.95 fmol/μL) for injection.
11. Dilute 1.5 μL of solution E (1.95 fmol/μL) in 1.125 μL trypsin-digested HeLa lysate, 0.45 μL iRT peptide solution (1 pmol/μL), and 2.925 μL of 0.2 % formic acid: solution F, 488 amol/μL of heavy standard.
12. Keep 1 μL of solution F (488 amol/μL) for injection.
13. Dilute 1.5 μL of solution F (488 amol/μL) in 1.125 μL trypsin-digested HeLa lysate, 0.45 μL iRT peptide solution (1 pmol/μL), and 2.925 μL of 0.2 % formic acid: solution G, 122 amol/μL of heavy standard.
14. Keep 1 μL of solution G (122 amol/μL) for injection.
15. Dilute 1.5 μL of solution G (122 amol/μL) in 1.125 μL trypsin-digested HeLa lysate, 0.45 μL iRT peptide solution (1 pmol/μL), and 2.925 μL of 0.2 % formic acid: solution H, 30.5 amol/μL of heavy standard.
16. Keep 1 μL of solution H (30.5 amol/μL) for injection.
17. Dilute 1.5 μL of solution H (30.5 amol/μL) in 1.125 μL trypsin-digested HeLa lysate, 0.45 μL iRT peptide solution (1 pmol/μL), and 2.925 μL of 0.2 % formic acid: solution I, 7.6 amol/μL of heavy standard.
18. Keep 1 μL of solution I (7.6 amol/μL) for injection (*see* **Note 16**).
19. Repeat **steps 1–17** two additional times so that three samples are created for each solution.
20. Calculate 2–3 transitions per iRT, heavy standard, and light sample peptide.

3.3 LC-MRM of Heavy Proteotypic Standard Peptide Solutions

1. For the separation and analysis of the samples, a nanoLC QTRAP 6500 (AB Sciex, Framingham, MA) can be used (*see* **Note 5**).
2. The peptides are separated using a CHiPLC Chrom XP C18-CL 3 μm column, 120 Å (75 μm × 150 mm) equipped with a CHiPLC Chrom XP C18-CL 3 μm trap column, 120 Å (200 μm × 0.5 mm) at a column temperature of 45 °C and a flow rate of 300 nL/min. Solvent A is 0.2 % formic acid and solvent B is 98.8 % acetonitrile containing 0.2 % formic acid. Linear gradients from 5 to 30 % B are applied within 45 min, 30–90 % B in 2 min, followed by 100 % B for 10 min. Preliminary mass spectra are recorded in positive ion mode acquiring data from the transition lists, initially in the QTRAP

mode (Fig. 1a) to confirm correct peak identification. Optimization of declustering potentials and collision energy is done automatically in Skyline. Subsequent analyses are performed using MRM scheduling in QQQ mode (Fig. 1b) (*see* **Note 17**).

3. Inject 1 μL of solution I through A (*see* **Note 18**).
4. Inject 1 μL of 0.2 % formic acid solution (blank).
5. Repeat **steps 5** and **6** two more times.
6. After an initial data analysis, spike in the heavy standard concentration to your sample that is most appropriate, i.e., at roughly the same concentration range.
7. Inject 1 μL of this adjusted solution.
8. Repeat **step 7** two additional times for triplicate measurements.

3.4 Data Analysis

Import the wiff (or appropriate raw) files of all measurements into Skyline software (*see* **Note 19**).

4 Notes

1. In addition to the calibration curves and dynamic range, analytical validation is achieved by testing repeatability (technical replicates, injecting the same sample ten times), reproducibility (biological replicates, injecting ten samples that have been prepared the same way ten times), limit of detection (best approximated by using ca. three times noise), and limit of quantitation (best approximated by using ca. ten times noise), similar to common practice in the pharmaceutical field [2, 9–12].
2. Generally, it is sufficient to have one heavy-labeled amino acid such as [$^{13}C_6$]Lys or [$^{13}C_6$]Arg at the C-terminal side of the tryptic peptide. It may be necessary to purify and/or desalt the peptides after synthesis. Peptides containing Cys, Met, His, N-terminal Glu or Gln, glycosylation site motifs (NXS/T), or Pro following Lys should be avoided if possible.
3. If the stock solutions are to be stored in the freezer, we recommend using higher concentrations (nmol/μL) for storage to avoid sample adsorption to the storage vials.
4. iRT peptides are used to assess chromatographic reproducibility. They also enable users to schedule their analysis in smaller windows and help verify transitions in case of interference. Finally, they aid in transferring methods from one lab to another lab.
5. The use of this setup is not mandatory. Other QQQ mass spectrometers and quadrupole-based mass spectrometers such as

Q-TOF or Q-Exactive from other vendors with nanoLC setup are equally suited for this purpose.

6. This software tool has been developed by the MacCoss lab and is freely available at proteome.gs.washington.edu/software/skyline/. Extensive documentation is provided on the Skyline website for data analysis and interpretation. Commercial packages may serve the same purpose.
7. Depending on the cell or tissue type used lysis methods will vary.
8. The purpose of **steps 1** and **2** is to wash away the SDS; since proteins are extended and denatured they will remain in the filter unit. It may be necessary to repeat this wash step multiple times. We have found five washes to be sufficient for most samples.
9. The final concentration of TCEP once the 100 μL is added to the 30 μL of filtrate that remains in the filter unit will be 20 mM. The purpose of this step is to ensure complete reduction of all disulfide bridges.
10. The purpose of this step is to alkylate the cysteine residues to prevent disulfide bridges from reforming.
11. Adding $CaCl_2$ will enhance the activity of trypsin.
12. The flow-through contains the trypsin-digested peptides. Keep!
13. The FASP procedure is optimized for the recovery of purified tryptic peptides from intact cells or tissue. Loss of sample will be greater than 50 %; therefore adjust the amount of starting material accordingly. A total of 100 μg will be sufficient for a comprehensive assay development.
14. Determine peptide concentration, e.g., through UV response during the desalting step.
15. The resulting 1 μL contains 500 fmol of each heavy standard and 100 fmol of each iRT peptide.
16. The serial dilutions are prepared to determine linearity, dynamic range, and LOD/LOQ values.
17. While theoretically all samples can be analyzed without MRM scheduling, it is highly recommended to take advantage of the MRM scheduling. Once retention times have been recorded in a preliminary analysis, transitions in the following analysis according to the predetermined retention times should be scheduled. This ensures higher sensitivity and more data points across the peaks for smoother peak shapes and better accuracy in quantification.
18. Start with the lowest concentration to avoid carryover.

19. The files are imported and show target masses, individual transitions per peptide, peak areas, and retention times over the multiple analysis. Considerable manual intervention is needed to confirm assignment of peaks.

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