Chapter 13

Immuno-Mass Spectrometry: Quantification of Low-Abundance Proteins in Biological Fluids

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Abstract

Mass spectrometry is emerging as one of the most promising analytical techniques to examine simultaneously hundreds of analytes quickly, precisely, and accurately, using minute sample volumes. Currently, a major bottleneck in the verification phase of putative biomarkers is the lack of methods/reagents to quantify low levels of analytes in biological fluids. A major objective is to establish a high-throughput multiple reaction monitoring (MRM) assay capable of quantifying low-abundance proteins or peptides in biological fluids (low μg/L range) using mass spectrometry. The experimental procedure we propose, called immuno-mass spectrometry, consists of immuno-capturing analytes of interest from relevant biological fluids in 96-well microtiter plates and performing in-well tryptic digestion, with subsequent MRM of digested peptides on a triple quadrupole mass spectrometer. With such a strategy, limits of detection of 0.1–1 μg/L proteins in serum with a coefficient of variation of <20% can be obtained. This methodology could be adapted quickly and easily to potential candidates of interest, thus providing a much needed technology to bridge the gap between discovery and validation platforms.

Key words: Biomarkers, Multiple reaction monitoring, Quantification, Targeted proteomics, Verification phase, Immuno-mass spectrometry

1. Introduction

There is an emerging interest to quantify proteins and peptides in biological fluids using mass spectrometry (MS) since it provides a rapid and specific assay platform for analyte quantification. The premise is that a peptide that is cleaved from a protein can serve as a representation of the concentration of that intact protein (1). This peptide, referred to as a signature or proteotypic peptide, must be unique to the protein of interest (2). Classically, these experiments are performed on a triple quadrupole mass spectrometer

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in multiple reaction monitoring (MRM) mode. In this approach, the discriminating power of mass analyzers to select a specific analyte is utilized to transmit only ions of a specific mass-to-charge ratio (m/z), which are fragmented and some of the resulting product ions monitored in a two-stage mass selection process. To achieve absolute protein quantification, an internal standard peptide is required to spike into a sample so that the ratio between the synthetic and endogenous peptide can be determined (3–5).

However, the sensitivity of MS-based serum protein quantification assays is limited by the dynamic range of most mass spectrometers (6). Therefore, an enrichment strategy involving immunodepletion, gel-based, or chromatographic techniques is often coupled to MRM assays to increase the sensitivity of detection. The studies published thus far utilizing mass spectrometry as a quantification tool have successfully achieved detection limits of 300–500 µg/L for proteins in serum with minimal or no purification steps (7, 8). However, for an assay to be clinically relevant, the detection limits should be in the low µg/L range.

To achieve this level of sensitivity with MS, a purification/enrichment step is essential. But it is also equally important for these MRM assays to be relatively simple to perform, robust, and high-throughput. Notably, antibody capture of the analyte is one of the most sensitive purification methodologies (9). Combining antibody immunoextraction with MRM assays may achieve low µg/L sensitivity. In a recent study, using an immuno-MS (immunoextraction in combination with mass spectrometry) approach, with minimal prefractionation steps and potentially preparing up to 96 samples simultaneously on a microtiter plate, we were able to demonstrate that MRM can be performed on an ion-trap mass analyzer using a purified recombinant protein digest to achieve a limit of detection (LOD) of 10 attomoles with a coefficient of variation (CV) of <20% (10). Furthermore, we successfully quantified the protein of interest in serum down to less than 1 µg/L, with a detection limit 0.1 µg/L.

2. Materials

2.1. Recombinant Proteins and Antibodies

1. If needed, reconstitute the carrier-free human recombinant protein of interest according to the manufacturer's package insert, ideally in a solvent that is compatible with chromatography/mass spectrometry.
2. If needed, reconstitute the monoclonal or polyclonal antibody of interest according to the manufacturer's package insert.

2.2. Reagents for Immunoextraction on 96-Well Microtiter Plate (See Note 1)

1. 200 mM ammonium bicarbonate (Fisher Scientific, #A643-500).
2. Reagent-grade methanol and deionized water.
3. 20 mM dithiothreitol (DTT) (Sigma–Aldrich, #D9779).
4. 100 mM iodoacetamide (Sigma–Aldrich, #I1149).
5. Sequencing-grade modified trypsin (Promega, #V5111). Dissolve 20 µg of trypsin in 160 µL of 200 mM ammonium bicarbonate (stock concentration of 0.125 µg/µL trypsin solution stored at −20°C). Check the pH of the freshly made solution and adjust accordingly to pH 8.
6. 96-Well white polysterene microplate; high binding, flat bottom (Greiner Bio One, #655074).
7. Omix pipette C18 tip (Varian, Inc. #A57003MB).
8. Coating buffer: 50 mM Tris, pH 7.8.
9. Washing solution: 0.9% NaCl (Fisher Scientific, #S271).
10. Automated 96-well plate washer (Bio-Tek Instruments, ELx405).
11. C18 extraction (Omix pipette tip):
    (a) Buffer A: 0.1% formic acid in water.
    (b) Buffer B: 65% ACN and 0.1% formic acid in water.

2.3. HPLC and Mobile Phases

1. HPLC instrument: Nano-LC system (Proxeon Easy-nLC or equivalent) (capillary liquid chromatography).
2. Columns: 2.5-cm C18 trap column trimmed from a 5-cm IntegraFrit C18 column (inner diameter 150 µm, 300 Å pore size, 5 µm, New Objective #IF150-50-50 H002) and a 5-cm analytical PicoFrit C18 column (inner diameter 75 µm, 90 Å pore size, 2.7 µm, New Objective #PF7515-H035) with a 15-µm tip.
3. Mobile phases: Phase A: 0.1% (v/v) formic acid in an aqueous water solution,and phase B: 0.1% (v/v) formic acid in ACN.

2.4. Mass Spectrometer and Related Items

1. Mass spectrometer: A triple quadrupole mass analyzer (Quantum Ultra, Thermo Fisher).
2. Ionization source: Nano-electrospray ionization.
3. Database search software (Mascot, Matrix Science).

2.5. Synthetic Peptides

1. Heavy-labeled (^{13}C or ^{15}N) synthetic peptides of interest [internal standard, Thermo Scientific, Custom HeavyPeptides AQUA Kit (BSD-10137166, http://www.thermo.com/heavypeptides)].
2. Synthetic peptides (for transition optimization).

3. Methods

For simplicity, the protocol described below will be for one protein of interest (Protein X). However, this method can be expanded to include simultaneous quantification of multiple proteins (3, 4). The experimental procedure requires having one antibody towards Protein (3,4). The choice of a monoclonal antibody or polyclonal antibody will vary depending on the analyte of interest. The overall objective is to establish a high-throughput MRM assay capable of quantifying low-abundance proteins in biological fluids (low ng/L–μg/L range) using mass spectrometry.

3.1. Proteotypic Peptide Selection

The objective of this step is to select two to three proteotypic peptides that display the most intense signal to represent Protein X for quantification purposes using mass spectrometry. We recommend that steps 2–5 be performed on an ion trap mass analyzer, rather than on a triple quadrupole mass analyzer. This information can be obtained by in silico analysis or empirically derived (see Notes 2–5). Below is a detailed protocol for empirically derived proteotypic peptide selection.

1. Digest 10 μg of purified human recombinant Protein X:
   (a) To a microcentrifuge tube containing 10 μg of purified recombinant protein, add 48 μL of freshly made ammonium bicarbonate (200 mM).
   (b) Add 40 μL water, 10 μL methanol, and 2 μL DTT (20 mM).
   (c) Incubate the sample at room temperature for 30 min with shaking.
   (d) Add 2 μL iodoacetamide (100 mM) and incubate at room temperature for 1 h, shaking in the dark (cover with aluminum foil).
   (e) Add 0.25 μg trypsin and incubate the sample at 37°C overnight (~16 h).
   (f) Stop the trypsin digestion process by the addition of 5 μL of 10% (v/v) aqueous formic acid.
2. Inject the digested peptide solution (quantity or volume) into the LC–MS system, operating in data-dependent mode. Refer to Subheading 3.3 for LC gradient and instrument settings.
3. Search the raw data generated with a search algorithm (Mascot, Matrix Science; version 2.1.03) containing appropriate search parameters and database.
   (a) Analyze the resulting Mascot Generic Files with a forward and reverse concatenated International Protein

Index human database (version 3.62; 1,67,894 entries forward and reverse combined).

(b) Select trypsin as the digestion enzyme.

(c) Perform search with fixed carbamidomethylation of cysteines and variable oxidation of methionine residues selected.

(d) Indicate a fragment tolerance of 0.4 Da and a precursor tolerance of 7 ppm.

(e) Parameters for DTA creation: minimum mass, 300 Da; maximum mass, 4,000 Da; automatic precursor charge selection; minimum peaks, ten per MS/MS scan for acquisition; and minimum scans per group, 1.

4. Inspect the list of identified peptides for peptides containing methionine and cysteine and for miscleavages, and remove these peptides from further consideration (see Note 3 for exceptions to this rule). Additional information regarding guidelines for selection of proteotypic peptides can be found in the literature (11–13).
5. Generate an extracted ion chromatogram (XIC) for each of the peptides identified to inspect which peptides have the highest signal intensity.
6. On the Quantum Ultra (or an equivalent triple quadrupole mass analyzer), perform a selected ion monitoring (SIM) scan on five to seven highest intensity peptides observed for Protein X. It should be noted that some proteins may yield fewer overall peptides and after removal of low-intensity proteotypic peptides, you may be left with a much smaller pool of peptides to work with.
7. From the SIM analysis, select the top two to three proteotypic peptides with the highest signal intensity and chromatographic peak shape for further analysis (12).

3.2. Transitions Selection

1. Use the digested recombinant protein (or alternatively, use the two to three light-labeled synthetic proteotypic peptides) for this step.
2. Using a static spray tip, directly infuse the peptide mixture into the mass spectrometer (triple quadrupole).
3. In tune mode, tune on the peptide of interest in MS mode (optimize capillary temperature, tube lens offset, and spray voltage):

   (a) If the final assay will measure more than one peptide at a time, a specific temperature that will satisfy all peptides must be selected.

4. In MS/MS mode, optimize at least five product ions (transitions) of highest relative intensity (define optimal collision

energies for each). Select product ions with m/z values greater than the m/z values of the precursor ion (see Notes 6–8):

(a) After this is completed, the optimized products must correspond to b- or y-type ions.

5. After the transitions have been identified, create a method that contains the transition information.

(a) Run the method.

(b) Select the top three to four transitions with the highest signal intensity (good signal-to-noise ratio) for each proteotypic peptide. If all transitions have similar intensity, then select those with m/z values greater than precursor m/z values.

(c) Note that all the transitions should have the same retention time for each proteotypic peptide.

At the end of this step, two to three proteotypic peptides to represent Protein X should have been selected and three to four product ions should have been identified for each peptide. Select only the top performing proteotypic peptide (consistently identified, intense signal, reproducible fragmentation, etc.) for further MRM method development.

3.3. MRM Method Development and Verification Studies

The objective of this step is to develop an MRM method, including defining HPLC conditions for the proteotypic peptide and its associated three to four product ions selected from above to monitor Protein X. Once the method is set up, verification studies are needed to characterize the performance of the method using synthetic peptides:

1. Create an MRM method in the Xcalibur software method editor for the Quantum Ultra (or equivalent software method for another triple quadrupole mass analyzer). Some of the typical MS settings are as follows:

(a) Scan width (m/z): 0.002.

(b) Scan time (s): 0.1.

(c) Resolution (peak width at full-width half-maximum): Q1: 0.2 and Q3: 0.7.

(d) Microscans: 1.

(e) Skimmer offset (V): 7.

(f) Data type: Centroid.

(g) Polarity: Positive.

2. Nano-LC conditions: A 40-min dual column (precolumn and analytical column) run consisting of a 7.5-min sample load, 23-min discontinuous gradient, and 5-min analytical and 4.5-min precolumn equilibration step. The flow rate should be

8 μL/min for sample loading/precolumn equilibration, 0.8 μL/min for analytical column equilibration, and 0.4 μL/min for the gradient.

3. Linearity study:
   (a) Using synthetic light-labeled peptides, inject increasing concentrations of the purified peptide (or amounts on column). This should be performed in triplicate. For example, one can inject 0, 0.1, 0.5, 1, 10, and 100 fmol on a column, or equivalent concentrations in μg/L.
4. Determining the LOD and limit of quantification (LOQ):
   (a) LOD is defined as the lowest concentration of analyte that can be discriminated from zero with 95% confidence (analyte concentration at signal of background plus two standard deviations). The background signal can be obtained via injection of the mobile buffers as unknown specimens or by immuno-purification of a complex matrix which does not contain Protein X. The "background" can be determined by calculating the level of noise on both sides of the chromatographic peak. The Xcalibur software will determine the background and integrate the peaks.
   (b) LOQ is defined as the concentration of analyte that can be measured with <20% CV.
5. Precision study:
   (a) Within-day precision analysis: measure ten replicate analysis of two different levels of peptide mixture spanning the dynamic range of the method (as established in the linearity study). For example, inject 0.5 and 10 fmol of peptide mixture.
   (b) Between-day precision analysis: measure the two peptide levels two times per day for 5 days.
   (c) Calculate the imprecision of the method at the two levels.

3.4. Immunoextraction Coupled to MRM (Immuno-MS Approach)

1. Add 100 ng/well of antibody in coating buffer to a 96-well microplate (200 μL), seal the microplate tightly so that no evaporation occurs, and leave the plate overnight at room temperature (see Notes 9–12) (Fig. 1).
2. Wash the plate five times with 0.9% NaCl washing solution (200 μL each); this can be performed using the automated washing machine or manually (see Note 13). Gently pat down the plate on a paper cloth to ensure that residual salt solution is removed.
3. Load 100 μL of serum/plasma/standard to each well (it is recommended to perform all sample analyses in triplicate). To generate a standard curve, use the recombinant protein to

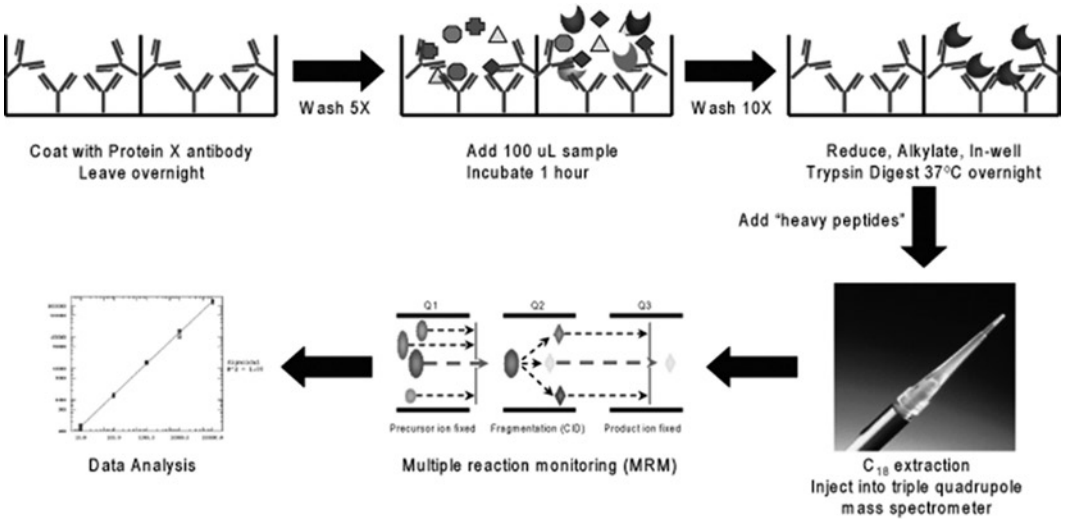

Fig. 1. Strategy for immunoextraction coupled-MRM.

spike into a complex matrix such as 6% bovine serum albumin (BSA) of increasing concentration. Load the unknown samples after the standards (see Notes 9 and 14).

4. Incubate the plate for 1 h at room temperature, with shaking.
5. Wash the plate ten times with 0.9% NaCl washing solution and gently pat down the plate after the last wash.
6. Add 48 µL ammonium bicarbonate (200 mM), 10 µL methanol, 40 µL water, and 2 µL DTT (20 mM) to each well and incubate at room temperature for 30 min on a shaking device.
7. Add 2 µL iodoacetamide (100 mM) and incubate in the dark for 1 h at room temperature.
8. Add 0.25 µg of trypsin to each well and incubate the plate in a 37°C incubator (sealed) overnight (~16 h).
9. Transfer the contents from each well to microcentrifuge tubes.
10. Spike in appropriate concentration of synthetic heavy-labeled peptide (fixed concentration) to serve as the internal standard (e.g., 10 fmol of a heavy-labeled peptide mixture).

3.5. Sample Analysis

1. Acidify the samples by adding 5 µL of 10% formic acid (if not already performed).
2. Perform a C18 extraction using the Omix pipette tips:
   (a) Condition the tip in Buffer B (aspirate and dispense five times).
   (b) Wash the tip in Buffer A.
   (c) Aspirate and dispense the sample ten times.

(d) Wash the tip in Buffer A.

(e) Elute the peptides in 5 µL of Buffer B.

(f) Add 80 µL of Buffer A.

3. Deposit the sample into a 96-well plate.
4. Run the developed LC–MRM method (Subheading 3.3).
5. Run a quality control (QC) sample before and after the analytical run to ensure that the retention time and peak area have not changed. Refer to Note 15 for more detail.

3.6. Data Analysis

1. Manually verify that all transitions for a given peptide elute at the same time and at the appropriate retention time for the QC sample and check for acceptability.
2. Refer to the precision analysis study to determine the variability in retention time for each peptide:
   (a) Peaks should only be considered "real" if they are within the determined ± retention time, and if the signal-to-noise (S/N) ratio is ≥3:1 (14, 15).
3. Export the integrated peak areas for light and heavy proteotypic peptides.
4. Calculate the ratio of the light to heavy peptide peak area for each sample (standard and unknowns):
   (a) Use the peak area of one transition for quantification purposes. The remaining transitions for the proteotypic peptide are used for confirmation of the identification of the peptide.
5. Generate a calibration curve using the standard's light:heavy peptide ratio and create a "line of best fit" with a $y = mx + b$ equation:
   (a) Ensure that it is linear and passes through all points on the curve.
6. Apply the unknown sample's ratios (y) to the equation and solve for x (concentration).

4. Notes

1. All reagents for sample preparation should be made on the day of the experiment.
2. As mentioned, proteotypic peptide selection can be performed using in silico analysis or taken from previously performed experimental data:
   (a) Search the literature for studied proteotypic peptide and transitions for Protein X.

(b) Check various online open-source databases such as GPMDB (http://gpmdb.thegpm.org) or MRM Atlas (http://www.mrmatlas.org/) to see if there is any MS/MS information for Protein X.

(c) Even if information is available, it is recommended to digest the recombinant protein and perform empirical analysis via LC–MS on Protein X since different instruments may yield different fragmentation patterns.

(d) Selecting transitions and/or peptides via in silico means may not always yield the "correct answers" as there is no way to predict ionization efficiency, making it difficult to predict which peptide will yield the best signal intensity.

3. While we do not recommend the use of peptides containing modified amino acids, sometimes modified amino acids are acceptable and may be the peptide for that protein with excellent *S*:*N* ratio (>3). Trial and error are required at this point to select the proteotypic peptide.

4. In the selection of proteotypic peptides, small peptides (six to seven amino acids) are not ideal candidates since they tend to be nonspecific (11).

5. Generally, selecting more transitions per peptide can increase selectivity but potentially at the risk of decreased sensitivity:

   (a) If adding the peak areas for the three transitions gives better signal-to-noise ratio, then it is recommended to use this consistently for all samples analyzed.

6. It is important to ensure that adequate scans across a peak is performed. For example, a good rule of thumb is to have more than ten scans per peak. A method quantifying many proteins requires monitoring a lot of transitions in a single experiment. In such a case, the dwell time of each individual transition is reduced and hence will vary with the number of peptides monitored. Practical dwell-time settings range between 10 ms for good sensitivity and 100 ms for excellent sensitivity. To ensure precise LC–MS quantification, at least ten data points should be acquired across the chromatographic elution profile of a peptide (12).

7. As a cautionary note, some peptides are not stable for long periods of time. For example, hydrophobic peptides can adhere to plastic, while aspartic and glutamic acid can undergo deamidation to asparagine and glutamine. In addition, methionine can become oxidized over time. To deal with this problem, perform the following: The stock solutions made on day

1 should be run on the mass spectrometer and the resulting file kept as a "reference file." With each analytical run or day, the stock solutions can be run on the mass spectrometer and the resulting file checked against the initial "reference file." A decrease in signal intensity should flag the need to make a new stock solution.

8. It is recommended to analyze samples on the MS immediately after sample preparation to avoid sample degeneration over time.
9. If specimen volume is an issue, one can load 50 µL of specimen and 50 µL of water. It is important to ensure that the final specimen volume does not exceed 150 µL.
10. The amount of capture antibody coated per well may vary for each protein. Test 100, 200, and 500 ng of antibody per well to see which yields the greatest capture. Note that the actual binding capacity of the wells is ~20–50 ng.
11. Do not exceed the stated coating solution volume as the plates are high protein/peptide binding and will mop up any peptides that adhere to it. For example, if 200 µL of coating antibody is used, do not exceed 150 µL of sample volume or digestion volume.
12. It is not recommended to block the wells with agents such as BSA as this will introduce unnecessary peptides into the final sample. In general, we have not experienced any issues with peptides originating from the antibody interfering with our MRM assay.
13. Do not let the plate wells dry up in the process of washing the wells. It is recommended to wash using an automatic plate washer or if performing the washing manually, dispense and discard the solution one strip of wells at a time. The plate can be gently tapped (face down) on a paper cloth to remove excess salt/buffer.
14. It is recommended to have a variety of negative controls in the run. For example, coat the well with the antibody but do not add the sample. Alternatively, coat the well with a different antibody (not against Protein X), or do not coat the well with the antibody but add the 100 µL of sample.
15. A QC sample should be assayed with each analytical run. The purpose of the QC is to ensure that the instrument is performing within the required specifications (accuracy and precision). The QC sample can be BSA, commercially purchased (Michrom Bioresources, Inc., #PTD-00001-15).

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