Video Article

Peptide and Protein Quantification Using Automated Immuno-MALDI (iMALDI)

Huiyan Li¹, Robert Popp¹, Bjorn Frohlich¹, Michael X. Chen², Christoph H. Borchers^{1,3,4,5}

¹University of Victoria-Genome BC Proteomics Centre

²Jewish General Hospital, McGill University

³Dept of Biochemistry and Microbiology, University of Victoria

⁴Proteomics Centre, Segal Cancer Centre, Lady Davis Institute, Jewish General Hospital, McGill University

⁵Gerald Bronfman Department of Oncology, Jewish General Hospital

Correspondence to: Christoph H. Borchers at christoph@proteincentre.com

URL: <https://www.jove.com/video/55933>

DOI: [doi:10.3791/55933](https://doi.org/10.3791/55933)

Keywords: Chemistry, Issue 126, Protein quantification, peptides, mass spectrometry, immuno-MALDI, iMALDI, immuno-enrichment, automation

Date Published: 8/18/2017

Citation: Li, H., Popp, R., Frohlich, B., Chen, M.X., Borchers, C.H. Peptide and Protein Quantification Using Automated Immuno-MALDI (iMALDI). *J. Vis. Exp.* (126), e55933, doi:10.3791/55933 (2017).

Abstract

Mass spectrometry (MS) is one of the most commonly used technologies for quantifying proteins in complex samples, with excellent assay specificity as a result of the direct detection of the mass-to-charge ratio of each target molecule. However, MS-based proteomics, like most other analytical techniques, has a bias towards measuring high-abundance analytes, so it is challenging to achieve detection limits of low ng/mL or pg/mL in complex samples, and this is the concentration range for many disease-relevant proteins in biofluids such as human plasma. To assist in the detection of low-abundance analytes, immuno-enrichment has been integrated into the assay to concentrate and purify the analyte before MS measurement, significantly improving assay sensitivity. In this work, the immuno- Matrix-Assisted Laser Desorption/Ionization (iMALDI) technology is presented for the quantification of proteins and peptides in biofluids, based on immuno-enrichment on beads, followed by MALDI-MS measurement without prior elution. The anti-peptide antibodies are functionalized on magnetic beads, and incubated with samples. After washing, the beads are directly transferred onto a MALDI target plate, and the signals are measured by a MALDI-Time of Flight (MALDI-TOF) instrument after the matrix solution has been applied to the beads. The sample preparation procedure is simplified compared to other immuno-MS assays, and the MALDI measurement is fast. The whole sample preparation is automated with a liquid handling system, with improved assay reproducibility and higher throughput. In this article, the iMALDI assay is used for determining the peptide angiotensin I (Ang I) concentration in plasma, which is used clinically as readout of plasma renin activity for the screening of primary aldosteronism (PA).

Video Link

The video component of this article can be found at <https://www.jove.com/video/55933/>

Introduction

Mass spectrometry has become an indispensable tool in quantitative proteomics. Mass spectrometry can determine the masses of target proteins or peptides, therefore the obtained analyte signals can be highly specific compared to immunoassays. Two ionization methods, electrospray and MALDI, are most commonly used for detecting proteins and peptides^{1,2,3,4}. A major challenge in MS-based protein quantification lies in the detection of low-abundance proteins in complex samples at ng/mL or pg/mL concentrations in the presence of high-abundance proteins, and many candidate protein biomarkers found in human plasma are within this range⁵. This problem is largely caused by the inherently wide dynamic range and complexity of the human proteome⁶.

To overcome these detection challenges, immuno-MS methods have been developed to enrich the target proteins or peptides from the sample solutions onto a solid surface, followed by elution of the analytes and MS measurement^{7,8,9,10}. Through immuno-enrichment, the analytes are purified from complex samples and therefore the ion-suppression effects from other molecules are minimized. Among various solid supports, magnetic beads are currently most widely used as they have the advantages of high antibody binding capacity and ease of handling. Magnetic beads with different functionalizations and sizes have been developed and commercialized for immunoprecipitation experiments. To date, immuno-enrichment on beads has been interfaced with both electrospray ionization (ESI) and MALDI-MS for protein and peptide measurement. In stable isotope standards and capture by anti-peptide antibodies (SISCAPA) technology, proteins in the samples are digested, followed by incubation with antibody-coated beads for immuno-enrichment. In "classical" SISCAPA, the captured proteotypic peptides are eluted from the beads, and measured by Liquid Chromatography-ESI-MS (LC-MS), or by direct infusion ESI-Multiple Reaction Monitoring-MS (ESI-MRM-MS)^{11,12}. Immuno-enrichment improved the MRM assay sensitivity by 3-4 orders of magnitude, reaching the low ng/mL range¹³.

Compared to electrospray-MS, MALDI-MS is faster, and does not involve the cleaning and re-equilibration of LC columns so there are no carryover and contamination issues, making it more suitable for high-throughput studies¹⁴. Immuno-MALDI technology has been developed in our laboratory to combine immuno-enrichment with MALDI-MS for sensitive and specific quantification of peptides and proteins (based on quantitation of proteotypic peptides)^{15,16,17}. After immuno-enrichment, the beads are deposited on a MALDI target plate, the matrix solution is added to beads, and the plate is ready for analysis by a MALDI-TOF-MS after drying. Elution of the peptides from the beads is not performed

as a separate step, but affinity-bound analytes are eluted by the MALDI matrix solution when it is added to the bead spots, thereby simplifying the sample preparation and minimizing sample loss. The iMALDI technology has been applied in a variety of applications^{18,19}, and recently has been automated and used for measuring Angiotensin I (Ang I) for determining plasma renin activity (PRA)²⁰. This protocol will demonstrate the procedure used to perform an automated iMALDI assay. Taking the PRA assay as an example, inter-day CVs of less than 10% have been achieved through automation, with the capability of measuring 744 samples per day²⁰.

The iMALDI PRA assay demonstrated in this manuscript does not require protein digestion, as the target molecule (Ang I) is a peptide with a molecular weight of 1295.7 Da. In other assays where protein digestion is necessary and a peptide is used as the surrogate for the intact protein, the selected peptide for iMALDI should be unique and specific to the target protein, with a mass over 800 Da so that it can be easily distinguished from the chemical noise from the MALDI matrix solution. Anti-peptide antibodies are required for the immuno-enrichment of the peptides. The protocol for an iMALDI assay measuring PRA consists of four steps: 1) generation of Ang I in human plasma; 2) immuno-enrichment of Ang I using antibody-coated beads; 3) transfer of beads to a MALDI target plate and adding matrix solution; and 4) signal acquisition by a MALDI-TOF-MS and data analysis²⁰.

Protocol

The amounts of the reagents described below are based on the measurement of 20 patient plasma samples. The protocol presented below follows the guidelines of the University of Victoria's human research ethics committee.

1. Generation of Ang I in Human Plasma

1. Thaw plasma samples (≥ 200 μL) in a room temperature water bath for 5 min, and then put the samples on ice until completely thawed.
2. Transfer 200 μL of each plasma sample manually to separate wells of a 1.1 mL deep-well plate (sample plate), and centrifuge the plate in a centrifuge for 10 min at 2 °C and 1278 x g.
3. Use an automated liquid handling system to serially dilute a 500 fmol/ μL Ang I NAT standard solution to prepare six calibrator solutions with chicken egg white albumin (0.1, 0.2, 0.6, 1.9, 5.7, 17.2 fmol/ μL).
4. Pipette 200 μL of each calibrator to a well, and 125 μL of generation buffer to sample plate.
NOTE: The CEWA in phosphate buffered saline (PBS) buffer needs to be freshly prepared on the day of the experiment.
5. Using an automated liquid handling system, in a new plate mix 125 μL of plasma supernatant or 125 μL of CEWA in PBS with 25 μL of Ang I generation buffer, which contains 1 M Tris, 0.2 mM ethylenediaminetetraacetic acid (EDTA), and 1 mM phenylmethylsulfonyl fluoride (PMSF).
NOTE: Prepare the Ang I generation buffer by mixing a 1 M Tris/0.2 mM EDTA aqueous buffer (adjust to pH 5.5 using acetic acid) and a PMSF solution (100 mM in methanol). Both solutions can be stored up to 1 month at 4 °C. Mix the two solutions on the day of the experiment.
6. Automatically transfer the solutions into a 96-well plate, 3 replicates per solution, 34 μL per well. Incubate the plate at 37 °C for 3 h.

2. Immuno-enrichment of Ang I Using Antibody-coated Beads

1. Conjugation of antibody onto magnetic Protein G beads

NOTE: Conjugation of the antibody with the beads is performed manually during the 3 h Ang I generation period on the day of the experiment. For other analytes, the conjugated beads might be able to be stored in PBS buffer containing 0.015% CHAPS (PBSC) at 4 °C for three months or longer, depending on the properties and stability of the antibody.

1. Transfer 110 μL of bead slurry (enough for measuring 20 samples and making a 6-point standard curve) into a 1.5 mL tube. Wash the beads seven times with 1 mL of 25% acetonitrile/PBSC, and three times with 1 mL of PBSC. Use a magnetic stand to pellet the beads between each washing step. Remove the wash buffer after the last wash.
NOTE: Washing 7 times with 25% acetonitrile/PBSC is critical for removing the MS-incompatible additives in the bead slurry, such as Tween-20. If there are no such additives in the selected beads, this extensive washing step might not be necessary.
2. Resuspend the beads in 110 μL of PBSC, and add 110 μL of anti-Ang I antibody (final antibody concentration: 100 $\mu\text{g}/\text{mL}$). Mix the beads and solution by pipetting, and then incubate them at room temperature for 1 h, rotating at 8 rpm.
3. Wash the beads 3 times with 1 mL of PBSC, and resuspend in 1100 μL of PBSC.
NOTE: If any bead solution has flowed into the cap of the tube during incubation, spin down the solution using a benchtop centrifuge at 2680 x g.
4. Transfer the bead solution manually to a 96-well plate (bead standard plate). Use the automated liquid handling system to aliquot the beads to the same 96-well plate, 120 μL per well.

2. Immuno-enrichment on the beads

1. After the 3 h Ang I generation period, place the incubation plate on ice for 10 min to terminate the generation of Ang I.
2. Automatically dilute the SIS peptide stock solution (10 pmol/ μL) 100-fold with PBS buffer, and further automatically aliquot the stable isotope standard peptide dilution to a 96-well PCR plate. Transfer 1.5 μL of a stable isotope standards (SIS) peptide solution (containing 100 fmol) into each well of the incubation plate and mix it with the plasma samples or the CEWA in PBS buffers.
3. Automatically transfer the contents of the incubation plate to the bead solution, 10 mL per well, mix.
4. Incubate the plate at 4 °C for 1 h while rotating at 8 rpm.
5. Wash the beads three times automatically with 5 mM ammonium bicarbonate (AmBic) solution, 100 μL per well per wash. After the last wash, resuspend the beads in 7 μL of 5 mM AmBic solution in each well. Use a magnet to pull the beads to the bottom after the last wash.

3. Transfer of Beads onto a MALDI Target Plate and Adding Matrix Solution

1. Transfer 7 μL of the bead slurry automatically onto a MALDI target plate with a spot size of 2,600 μm . Let the beads dry out.

NOTE: A small USB-powered fan can be used to accelerate the bead drying process.

- Automatically add 2 μL of α -cyano-4-hydroxycinnamic acid (HCCA)-matrix solution (containing 3 mg/mL HCCA, 1.8 mg/mL ammonium citrate, 70% acetonitrile, and 0.1% trifluoroacetic acid) from the matrix well onto each sample spot on the target plate.

4. Signal Acquisition by a MALDI-TOF-MS and Data Analysis

- Analyze the sample spots with a MALDI-TOF instrument using positive reflector mode. Perform internal calibration, data smoothing, and baseline subtraction automatically with vendor-specific software.
NOTE: The mode (positive/negative, linear/reflector) selected for MALDI-TOF measurement depends on the target peptides or proteins.
- Calculate the relative response ratio (Nat/SIS intensity ratio) and compare it to the standard curve to determine of the Ang I concentration in each sample. Calculate PRA using Equation (1), where $\Delta t_{\text{Ang I generation}}$ represents the time used for the generation of Ang I.
$$\text{PRA} = [\text{Ang I}] / \Delta t_{\text{Ang I generation}} \quad (1)$$

Representative Results

An automated iMALDI procedure for measuring Ang I is shown in **Figure 1**. Target peptides (either endogenous peptides or peptides from digested proteins) are enriched on anti-peptide magnetic beads, and then the beads are transferred to a target plate for MALDI measurement. The whole procedure is simplified compared to other immuno-MS technologies that require additional peptide elution steps. Automation of the iMALDI assay allows for high-throughput analysis of a large number of samples with an inter-day coefficient of variation (CV) below 10%²⁰. Representative spectra obtained by measuring Ang I in human plasma samples are shown in **Figure 2**. The SIS peptides function as an internal standard for peptide quantitation. Correlation of PRA values from 188 patient samples obtained with the automated iMALDI assay with PRA values obtained by using a clinical LC-MS/MS procedure¹⁷ is shown in **Figure 3**. The two methods have a correlation coefficient of 0.98; the difference between the slopes might be caused by the use of different internal standards in the two methods, or by the use of different antibodies in iMALDI procedure²⁰. The linear range of the assay and the assay precision are shown in **Figure 4** and **Figure 5**.

Figure 1: Process flow schematic of an automated iMALDI PRA assay. Adapted from reference²⁰, with permission. [Please click here to view a larger version of this figure.](#)

Figure 2: Representative spectra of the NAT and SIS Ang I peptides measured from a human plasma sample.

Figure 3: Correlation of PRA values measured by iMALDI and by LC-MS/MS from 188 patient samples. Adapted from reference²⁰, with permission.

Figure 4: Linear ranges of the iMALDI PRA assay in both (A) reflector and (B) linear mode. Adapted from reference²⁰, with permission. [Please click here to view a larger version of this figure.](#)

Figure 5: Intraday precision (A) and interday precision (B) of iMALDI PRA assays on plasma pools with low, medium, and high PRA values. Adapted from reference²⁰, with permission. [Please click here to view a larger version of this figure.](#)

Discussion

Compared to conventional MS-based protein quantification, iMALDI uses antibodies to enrich the analytes and purify them from complex samples, therefore making it possible to quantify proteins or peptides at low concentrations. A critical step in the iMALDI protocol is the immuno-enrichment of the target peptides. For this purpose, antibodies with high specificity and affinity should be selected. In SISCAPA, it has been reported that antibody affinities at 10^{-9} M or better would be needed to achieve high sensitivity at low ng/mL²¹. In addition, beads with high antibody binding capacity are preferred for optimal enrichment.

It is also important to generate relatively "clean" spectra for the target molecules with minimal background levels. High signal-to-noise ratios can be achieved through extensive bead washing after the immuno-enrichment step. Also, we have found that washing the bead spots on the target plate after adding and drying the matrix solution can significantly reduce the noise signals and thus improve the signal-to-noise ratios.

The iMALDI PRA assay demonstrated in this work does not require the proteolysis steps, as the target molecule Ang I is a peptide. To quantify protein molecules in a sample, the protein is typically digested into peptides before immuno-enrichment, although for epitope-containing peptides, the digestion can be performed on the captured protein^{22,23}. In this case, an existing protein digestion protocol can be easily inserted into the iMALDI workflow. Depending on the digestion buffer, a desalting step might be needed before capture of the peptides on beads, to avoid interference with the antibody-peptide binding.

Similar to other immuno-MS methods, production of anti-peptide antibodies is a major challenge in the development of iMALDI assays. Recently, Triple X Proteomics technology has been developed to produce affinity binders that can target multiple peptides per binder²⁴. This may reduce the cost of anti-peptide antibodies, and would facilitate the development of simultaneous multiplexed assays using a single incubation. Also, antibody fragments²⁵ or synthetic aptamers²⁶ could be alternatives to the use of intact antibodies and would have the advantage of easier production. Synthetic aptamers have been reported as showing lower background levels than intact antibodies when used for affinity-enrichment²⁶.

iMALDI technology combines the advantages of immunoassays with MALDI mass spectrometry, enabling rapid, high-throughput protein/peptide analysis in a large number of complex samples, with high sensitivity and specificity. Unlike other immuno-MS methods, after immuno-capture, the target peptides are not eluted in the tube, but the beads carrying analytes are transferred onto the MALDI target plate for MS-analysis, thus avoiding the loss of peptides due to their adsorption to plastic tubes. Compared to widely used proteomic tools such as western blot or ELISA, only one antibody is required in iMALDI, significantly reducing the assay development time and cost. The lower limit of detection we

have obtained in the PRA assay is comparable to conventional ELISA, and well within the physiological range. The antibody and the mass-to-charge ratio provide a double selection process, which ensures the high specificity of the assay. Applications of iMALDI is not limited to PRA assays, but can be applied for quantitation of any protein expression and even quantification of protein modification in complex samples. Notably, the automated high-throughput iMALDI could be a powerful clinical tool for the discovery and validation of protein biomarkers in a variety of diseases, due to the presence of benchtop MALDI instruments currently used in clinics for bacterial identification.

Disclosures

C.H.B holds the patent on the iMALDI technology.

Acknowledgements

We thank the financial support from Genome Canada and Genome British Columbia for operations (204PRO) and technology development (214PRO) through the Genome Innovations Network (GIN). We thank the Drug Discovery Platform at the Research Institute of the McGill University Health Center for the use of the MALDI-TOF instrument for filming. H.L. is grateful for support from a postdoctoral fellowship from the National Science and Engineering Research Council of Canada (NSERC). C.H.B is grateful for support from the Leading Edge Endowment Fund (LEEF). C.H.B. is grateful for support from the Segal McGill Chair in Molecular Oncology at McGill University (Montreal, Quebec, Canada). M.X.C. and C.H.B. are grateful for support from the Warren Y. Soper Charitable Trust and the Alvin Segal Family Foundation to the Jewish General Hospital (Montreal, Quebec, Canada).

References

1. Aebersold, R., & Mann, M. Mass spectrometry-based proteomics. *Nature*. **422** (6928), 198-207 (2003).
2. Nicol, G. R. *et al.* Use of an Immunoaffinity-Mass Spectrometry-based Approach for the Quantification of Protein Biomarkers from Serum Samples of Lung Cancer Patients. *Mol. & Cell. Proteomics*. **7** (10), 1974-1982 (2008).
3. Webster, J., & Oxley, D. in *Chemical Genomics and Proteomics: Reviews and Protocols*. (ed Edward D. Zanders) 227-240, Humana Press (2012).
4. Yergey, A. L. *et al.* De novo sequencing of peptides using MALDI/TOF-TOF. *J. Am. Soc. Mass Spectrom.* **13** (7), 784-791 (2002).
5. Anderson, L. Six decades searching for meaning in the proteome. *J. Proteomics*. **107** 24-30 (2014).
6. Landegren, U. *et al.* Opportunities for Sensitive Plasma Proteome Analysis. *Anal. Chem.* **84** (4), 1824-1830 (2012).
7. Guo, A. *et al.* Immunoaffinity Enrichment and Mass Spectrometry Analysis of Protein Methylation. *Mol. & Cell. Proteomics*. **13** (1), 372-387 (2014).
8. Florentinus-Mefailoski, A., Soosaipillai, A., Dufresne, J., Diamandis, E. P., & Marshall, J. G. An enzyme-linked immuno-mass spectrometric assay with the substrate adenosine monophosphate. *Anal. Bioanal. Chem.* **407** (4), 1119-1130 (2015).
9. Fredolini, C. *et al.* Immunocapture strategies in translational proteomics. *Expert Rev Proteomics*. **13** (1), 83-98 (2016).
10. Tran, J. C. *et al.* Automated Affinity Capture and On-Tip Digestion to Accurately Quantitate in Vivo Deamidation of Therapeutic Antibodies. *Anal. Chem.* (2016).
11. Razavi, M., Leigh Anderson, N., Pope, M. E., Yip, R., & Pearson, T. W. High precision quantification of human plasma proteins using the automated SISCAPA Immuno-MS workflow. *N. Biotechnol.* **33** (5, Part A), 494-502 (2016).
12. Razavi, M. *et al.* High-Throughput SISCAPA Quantitation of Peptides from Human Plasma Digests by Ultrafast, Liquid Chromatography-Free Mass Spectrometry. *J. Proteome Res.* **11** (12), 5642-5649 (2012).
13. Anderson, N. L. *et al.* SISCAPA Peptide Enrichment on Magnetic Beads Using an In-line Bead Trap Device. *Mol. & Cell. Proteomics*. **8** (5), 995-1005 (2009).
14. Parker, C. E., Pearson, T. W., Anderson, N. L., & Borchers, C. H. Mass-spectrometry-based clinical proteomics - a review and prospective. *The Analyst*. **135** (8), 1830-1838 (2010).
15. Reid, J. D., Holmes, D. T., Mason, D. R., Shah, B., & Borchers, C. H. Towards the Development of an Immuno MALDI (iMALDI) Mass Spectrometry Assay for the Diagnosis of Hypertension. *J. Am. Soc. Mass Spectrom.* **21** (10), 1680-1686 (2010).
16. Mason, D. R., Reid, J. D., Camenzind, A. G., Holmes, D. T., & Borchers, C. H. Duplexed iMALDI for the detection of angiotensin I and angiotensin II. *Methods*. **56** (2), 213-222 (2012).
17. Camenzind, A. G., van der Gugten, J. G., Popp, R., Holmes, D. T., & Borchers, C. H. Development and evaluation of an immuno-MALDI (iMALDI) assay for angiotensin I and the diagnosis of secondary hypertension. *Clin. Proteomics*. **10** (1), 20 (2013).
18. Jiang, J. *et al.* Development of an immuno tandem mass spectrometry (iMALDI) assay for EGFR diagnosis. *Proteomics Clin Appl.* **1** (2007).
19. Jiang, J., Parker, C. E., Fuller, J. R., Kawula, T. H., & Borchers, C. H. An Immunoaffinity Tandem Mass Spectrometry (iMALDI) assay for Detection of *Francisella tularensis*. *Analytica chimica acta*. **605** (1), 70-79 (2007).
20. Popp, R. *et al.* An automated assay for the clinical measurement of plasma renin activity by immuno-MALDI (iMALDI). *Biochimica et Biophysica Acta (BBA) - Proteins and Proteomics*. **1854** (6), 547-558 (2015).
21. Schoenherr, R. M. *et al.* Automated screening of monoclonal antibodies for SISCAPA assays using a magnetic bead processor and liquid chromatography-selected reaction monitoring-mass spectrometry. *J Immunol Methods*. **353** (1-2), 49-61 (2010).
22. Borchers, C. H. *Methods of quantitation and identification of peptides and proteins*. US patent US 7846748 B2 (2010).
23. Borchers, C. H. *Methods of quantitation and identification of peptides and proteins*. Canadian patent CA 2507864C (2011).
24. Weiß, F. *et al.* Catch and measure-mass spectrometry-based immunoassays in biomarker research. *Biochimica et Biophysica Acta (BBA) - Proteins and Proteomics*. **1844** (5), 927-932 (2014).
25. Nelson, A. L. Antibody fragments: Hope and hype. *mAbs*. **2** (1), 77-83 (2010).
26. Gupta, V. *et al.* An evaluation of an aptamer for use as an affinity reagent with MS: PCSK9 as an example protein. *Bioanalysis*. **8** (15), 1557-1564 (2016).