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Development of an efficient detection method for monoclonal protein using MALDI-TOF mass spectrometry

매트릭스 보조 레이저 탈착 이온화 비행시간형 질량분석기를 이용한 단클론성 단백의 효율적인 검출법 개발

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Department of Medicine (Laboratory Medicine)
Seoul National University College of Medicine

Jikyo Lee

의학석사 학위논문

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지도 교수 송 상 훈

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서울대학교 대학원
의학과 검사의학 전공

이 지 교

Development of an efficient detection method for monoclonal protein using MALDI-TOF mass spectrometry

Jikyo Lee
(Directed by Sang Hoon Song)

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Approved by Thesis Committee:

Professor _____ Chairman
Professor _____ Vice Chairman
Professor _____

Abstract

Introduction: Monoclonal protein (M-protein) is an abnormal increase of monoclonal protein found in the bloodstream that is produced by one or more clonal cells in patients with plasma cell disorders (PCDs) such as multiple myeloma (MM), monoclonal gammopathy of undetermined significance (MGUS), and solitary plasmacytoma. Protein electrophoresis (PEP), immunoelectrophoresis (IEP), and immunofixation electrophoresis (IFE) are usually performed to evaluate the presence of M-protein for diagnosis and monitoring disease status. Recently, detection methods based on mass spectrometry (MS) are emerging as highly sensitive for detecting M-protein in smaller amounts. The aim of this study is to develop and evaluate efficient method for screening and detecting monoclonal protein using mass spectrometry in a clinical laboratory.

Methods: Residual samples were used after performing routine tests at Seoul National University Hospital (SNUH). Based on IFE results, normal serum and abnormal serum samples including different subtypes of M protein were selected. Different sample preparation techniques were performed for optimizing the method. Nanobody affinity beads (NB), Melon IgG purification spin kit with C4 ZIPTIP

and Magnetic beads (MB) consisting of IgG were used for separation of immunoglobulins. Matrix-assisted laser desorption/ionization time-of-flight (MALDI-TOF) MS (Bruker Daltonics, Germany) was performed to detect and analyze the samples. High sensitive resolution MS, liquid chromatography combined with Synapt G2 quadrupole time-of-flight (qTOF) mass spectrometer MS (Waters, U.K.), was performed and compared to confirm the m/z difference occurs in MALDI-TOF MS. For analytical performance, precision of m/z values and limit of detection (LoD) was evaluated. MALDI-TOF MS combined with NB serum preparation (NB-MALDI-TOF) was performed for twenty-five normal and twenty-five abnormal IFE samples.

Results: Reduced light chains were observable as a peak at a certain mass-to-charge ratio (m/z ratio) range in the mass spectrum. A single charged light chain peak at 22,000 to 24,500 Da, while double charged light chain was observed at 11,000 to 12,500 Da. A single charged heavy chain peak was observed at 50,000 Da, and a double charged heavy chain peak was observed at 25,000 Da. Monoclonal peaks were observed in abnormal samples, while polyclonal peak was observed in normal ones. Comparing three methods for IgG purification, NB showed the lowest LoD value at 0.1 g/dL. In MALDI-TOF MS combined with NB serum preparation (NB-

MALDI-TOF) method, results showed 92% concordance with IFE results among fifty samples. NB-MALDI-TOF showed 92% sensitivity and 92% specificity.

Conclusions: In conclusion, NB-MALDI-TOF method was sensitive and useful as a qualitative method to detect M-protein in addition to IFE. NB-MALDI-TOF might be helpful to identify M-protein in false negative IFE or confirm ambiguous result of IFE.

Keyword: Multiple myeloma, Paraproteinemias, M-protein, Matrix assisted laser desorption ionization mass spectrometry

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List of Abbreviations and Symbols

CHCA	α−Cyano−4−hydroxycinnamic acid
CV	coefficient of variation
DTT	Dithiothreitol
DW	Distilled water
FLC	Free light chain
HC	Heavy chain
IAA	Iodoacetamide
IEP	Immunoelectrophoresis
IFE	Immunofixation electrophoresis
LC	Light chain
LC−MS	Liquid chromatography−mass spectrometry
LoD	Limit of detection
m/z ratio	mass to charge ratio
MADLI−TOF	Matrix assisted laser desorption/ionization−time of flight
MB	Magnetic beads
MM	Multiple myeloma
M−protein	Monoclonal protein
MRD	Minimal residual disease
MS	Mass spectrometry

NB	Nanobody beads
PBS	Phosphate buffered saline
PCDs	Plasma cell disorders.
PEP	Protein electrophoresis
QE Orbitrap	Q-Exactive quadrupole orbitrap
qTOF	Quadrupole time of flight
s/o	Suspicious of
TCEP	Tris-2-Carboxyethlphosphine Hydrochloride
t-mAbs	Therapy-related monoclonal antibodies

INTRODUCTION

Monoclonal protein (M-protein) is produced by increased plasma cells indicating an abnormal increase of clonal cells [1]. M-protein is a characteristic feature of plasma cell disorders (PCDs), including multiple myeloma (MM), smoldering multiple myeloma, non-IgM monoclonal gammopathy of undetermined significance (MGUS), IgM MGUS, light chain MGUS, solitary plasmacytoma, solitary plasmacytoma with minimal marrow involvement, and systemic AL amyloidosis [2].

Increasing number of patients with MM are treated with therapy-related monoclonal antibodies (t-mAbs) in combination with chemotherapy with or without peripheral blood stem cell transplant (PBSCT) [3]. Minimal residual disease (MRD) refers to the presence of residual cancer cells after treatment that can lead to relapse, which may not be detected by traditional method [4]. As a result, clinical need for detecting small amounts of M-protein after treatment is growing.

In laboratory, protein electrophoresis (PEP), immune-electrophoresis (IEP), and immunofixation electrophoresis (IFE) have been used to detect and evaluate the presence of M-protein. IFE is considered as the most sensitive method [5]. To address the

need for more sensitive MRD detection, mass spectrometry (MS) has emerged as a promising area [6]. It has undergone significant development and refinement past 10 years [5].

Matrix-Assisted Laser Desorption/Ionization Time-of-Flight (MALDI-TOF) MS is a widely-used method in microbiology for identifying bacteria rapidly [7]. It has also been used for M-protein detection in several studies, including MASS-FIX [8], which uses nanobody affinity beads (NB) to prepare samples and was developed and validated at the Mayo Clinic. This MALDI-TOF based method has been reported to be more sensitive than IFE and could be an effective method for detecting low levels of M-protein [5].

This study was aimed to develop an efficient and sensitive method for detecting M-protein using MALDI-TOF MS, which potentially offers increased sensitivity and accuracy in detecting low levels of M-protein.

MATERIALS AND METHODS

1. Samples and materials

Resdiual daratumumab and cetuximab were provided by Seoul National University Hospital (Seoul, Korea). Residual serum samples after routine IFE tests were used. This study was eligible for IRB exemption as a method evaluation study at SNUH. The serum samples were stored in the ultra-low temperature freezer (-80℃) and transferred to a regular freezer (-15℃~-23℃) the day before use. Samples stored in aliquots and multiple freeze-thaw cycles were avoided.

Dithiothreitol (DTT), Tris-2-carboxyethlphosphine hydrochloride (TCEP) and formic acid (FA) were purchased from Sigma-Aldrich (Steinheim, Germany). Acetonitrile (ACN) was purchased from Burdick and Jackson (Muskegon, MI, USA). HPLC-grade deionized water was from the MilliQ® device (Millipore, MA, USA). Dulbecco's phosphate-buffered saline (DPBS) was obtained from Welgene (WelGENE Inc., Daegu, Korea).

2. Sample preparation methods

IgG was purified from serum using a Melon Gel Spin Kit (Thermo Fisher Scientific Inc., MA, USA) according to the manufacturer's instructions. A 20 μL serum was diluted at a ratio of 1:10 with purification buffer and added to the spin column containing 200 μL of settled gel resin. For reduction, 20 μL of purified IgG was incubated with 20 μL of 40 mM TCEP in 0.2 % trifluoroacetic acid (TFA) for 15 min. Reduced IgG was then desalted using C4 ZipTip (Millipore, MA, USA).

For bead-based immunoglobulin enrichment, Dynabeads™ magnetic beads (Thermo Fisher Scientific Inc., MA, USA) were used for IgG purification and CaptureSelect™ nanobody affinity beads (Thermo Fisher Scientific Inc., MA, USA) were used for purification of IgG, IgA, IgM, kappa and lambda.

For 2 μL of serum, 20 μL of nanobody affinity beads (NB) and 50 μL of magnetic beads (MB) were applied. Storage buffer was removed and replaced by PBS before use. After mixing the beads with sera by tapping, the samples were incubated for 30 minutes at room temperature on a shaker. Magnetic bar was used to remove MB. The samples for NB were washed three times with DW and two

times with PBS. The elution of immunoglobulins from the beads was performed simultaneously with reduction by adding 200mM of DTT 0.1% TFA or 20 mM of TCEP in 0.1% TFA.

3. High resolution MS analysis

To identify the m/z difference of MALDI-TOF, high resolution tests were performed using t-mAb drugs, daratumumab and cetuximab, which have known amino acid sequences and molecular weights. The average molecular mass of daratumumab and cetuximab was calculated from the known amino acid sequence of the kappa light chain. In addition, a normal and an abnormal serum, which were reported to have daratumumab interference, were requested. High resolution MS analysis was requested to the Korea Basic Science Institute of the Republic of Korea (KBSI) after immunoglobulin enrichment using Melon kit with C4 ZIPTIP.

A 50 μL sample was reduced by adding 10 μL of 100 mM DTT at 55℃ for 30 min. All samples were buffer-exchanged to MS-grade water to native MS measurements. Samples were then acidified with FA prior to injection. For IAA treatment, each protein stock solution (5 mg/ml) was diluted with 6 M urea at a ratio of 1/5. This will result in a concentration of 1 mg/ml. Next, 5 μL of 200 mM DTT (50 mM Tris-HCl, pH 8.0) was added to 100 μL of the solution and allowed to undergo reduction reaction at room temperature for 1 hour. Then, the solution was treated with 20 μL of 200 mM IAA (50 mM Tris-HCl, pH 8.0), followed by alkylation

reaction. Then, 20 μL of 200 mM DTT was added and allowed to undergo reduction in the dark for 1 hour.

For analysis of native MS measurements, high resolution mass spectrometer analysis was performed on the AQUITY UPLC™ system coupled to a Synapt G2-HDMS mass spectrometer (Waters, Manchester, U.K.) and the MassLynx 4.1 software at KBSI.

Chromatographic separation was performed using ACQUITY UPLC Protein BEH C4 Van Guard Pre-column (2.1 μm, 2.1 mm × 50 mm; Waters, MA, USA) and a BioResolve RP mAb Polyphenyl analytical column (2.7 μm, 2.1 mm × 100 mm; Waters, MA, USA) with the mobile phase A and B, which consisted of water and acetonitrile with 0.1% formic acid, respectively, using a flow rate of 0.4 mL/min. The chromatographic gradient of mobile phase B was as follows: 0 min, 3%; 3 min, 3%; 14 min, 95%; 16 min 3%; 18 min, 95%; 20 min, 3%; 22 min, 95%; 24 min, 3% and 30 min, 3%. The total run time for chromatographic separation was 30 min. The extracts were analyzed in positive-ion modes of electrospray ionization (ESI) source within the acquisition mass range of m/z 500 to 4,000.

The other MS parameters were optimized as follows: capillary voltage of 2.5 kV, cone voltage of 40 V, source temperature of

140℃, and desolvation gas flow rate of 500 L/h. The acquired multiple charge profiles were deconvoluted using the MaxEnt 1 (Waters, Manchester, U.K.) algorithm. Finally, chromatogram and spectra were obtained from MassLynx 4.1 software (Waters, Manchester, U.K.).

4. MALDI-TOF analysis

For the control and calibrator preparation, a normal serum was selected as the negative control and prepared in the same way as the sample. The positive control was prepared by adding daratumumab to normal serum to achieve a final concentration of 1 g/dL.

For matrix preparation, 1 ml of TA50 was prepared by mixing ACN and 0.1% TFA in a 50:50 ratio. A 10 mg/ml α-Cyano-4-hydroxycinnamic acid (CHCA) matrix (Bruker Daltonics, Bremen, Germany) was prepared by adding 10 mg of CHCA to an EP tube, dissolving it in 1 ml of TA50, and vortexing for more than 1 minute to dissolve CHCA. The CHCA was then centrifuged at 13,000 rpm for 1 minute to pellet it. Samples were loaded on a 96 well plate with the sandwich method, where 1 μL of the sample was placed between two CHCA matrix layers.

Mass spectra were obtained using MALDI-TOF Microflex LT (Bruker Daltonics, Bremen, Germany) in linear mode. Before analysis, instrument calibration was performed with Protein II Standard (Bruker Daltonics, Bremen, Germany), containing 3 Calibrants at 22,307.0, 23,982.0, and 44,163.0, which should be set under 1,000 ppm to ensure accuracy. In our method, mass range for

detection was set from 9,000 to 60,000 and a total of 500 summed laser shots were obtained for each sample. Abnormalities in DW, ACN, and matrix were checked before proceeding to data analysis. Meaningful peaks with an S/N ratio >3−6 and intensity >1,000 were obtained.

AutoeXecute from flexContrl 3.4 (Bruker Daltonics, Bremen, Germany) software was used to automatically run mass detection. Flexanalysis (Bruker Daltonics, Bremen, Germany) software to observe the mass spectrum and analyze the results. The qualitative analysis reveals revealed the presence of a monoclonal peak for IgG, IgA, IgM, kappa, and lambda. The single charged light chain was observed in the m/z ratio range between 22,000 to 24,500 Da. Double charged light chain was observed in the m/z ratio range of 11,000 to 12,500 Da.

5. Analytical performance of MALDI-TOF

To determine precision, Melon kit with C4 ZIPTIP, MB, and NB methods combined with MALDI-TOF were performed five times a day, and repeated for consecutive five days. Normal and abnormal (IgG/kappa) serum samples were tested in three different ways. The m/z values at the highest intensity of normal and an abnormal peak were collected. Average, standard deviation, and coefficient of variation (CV) of m/z values were calculated by Microsoft Excel.

For evaluation of limit of detection (LoD), daratumumab was serially diluted in normal serum at concentrations of 0, 0.05, 0.1, 0.2, 0.5, 1.0 g/dL.

6. Analysis of patient samples using MALDI-TOF combined with NB method

To evaluate MALDI-TOF MS combined with NB serum preparation (NB-MALDI-TOF) method, twenty-five normal samples and twenty-five abnormal samples were tested.

After purifying IgG, IgA, IgM, kappa mixed lambda (kappa:lambda bead ratio = 50 : 50), kappa, and lambda by NB using 2 μL of serum, 20 μL of NB. After mixing the beads with patient's sera by tapping, the samples were incubated for 30 minutes at room temperature on a shaker. The samples were washed three times with DW and two times with PBS.

For elution and reduction, 20 μL of 20 mM of TCEP in 0.1% TFA was used with 30 minutes of incubation time at room temperature. Samples were loaded on a 96 well plate with CHCA sandwich method and run MALDI-TOF using AutoeXecute.

For method comparison, IFE was performed on HYDRASIS 2 (Sebia, Fulda, German). PEP was performed by Capillarys 3 Tera (Sebia, Fulda, German) was performed to quantify M-protein.

Results

1. High resolution MS analysis

Daratumumab, cetuximab, and human albumin were investigated under high resolution MS (Table 1). The calculated molecular mass of light chain from known amino acid sequence of daratumumab was 23384.02, which is consistent with mass from high resolution MS after deconvolution. In the same way, the calculated molecular mass of cetuximab was 23426.95, which is also consistent with the mass detected in high resolution MS.

In a stock solution of the drugs reduced only by DTT, the calculated molecular mass was consistent with the mass detected by high resolution mass after deconvolution. The difference was less than 1 Da in the high-resolution MS analysis. The m/z difference around 50 Da was shown in the Melon-MALDI-TOF (Table1).

On the other hand, the DTT treated with IAA showed 40 to 50 Da difference in t-mAb drugs due to the mass effect. The mass-shift effect of IAA also observed in Melon IgG purification kit combined with MALDI-TOF (Melon-MALDI-TOF). Since m/z ratio is critical

in MALDI-TOF MS analysis, IAA treatment was avoided to reduce the m/z difference.

In serum samples, there was approximately 100 Da of m/z difference in MALDI-TOF while it was less than 1 Da difference I high resolution MS (Table 2). Difference of m/z ratio appeared in all the preparation methods, including Melon kit with C4 ZIPTIP, MB, and NB.

Table 1. Results of drug samples performed with high resolution MS and MALDI-TOF with or without IAA.

Sample	Average molecular mass (calculated)	Mass of high resolution MS (DTT)	Mass of high resolution MS (DTT-IAA)	1+ LC of Melon-MALDI-TOF (DTT)	1+ LC of Melon-MALDI-TOF (DTT-IAA)
Daratumumab	23384.02	23384.40	23441.80	23380.36	23531.91
Cetuximab	23426.95	23427.40	23483.40	23350.73	23378.85

Abbreviation: m/z ratio, mass to charge ratio; Melon-MALDI-TOF, Melon with C4 ZIPTIP combined with MALDI-TOF; DTT, Dithiothreitol; DTT-IAA, Dithiothreitol with Iodoacetamide treatment; 1+ LC, single charged light chain.

Table 2. Difference of m/z ratio between high resolution MS and MALDI-TOF using serum samples.

Sample	Mass of high resolution MS (DTT)	1+ LC of Melon-MALDI-TOF (DTT)	1+ LC of NB-MALDI-TOF (DTT)	1+ LC of MB-MALDI-TOF (DTT)
Daratumumab interference reported serum	23385.20	23293.20	QNS	QNS
Daratumumab (1g/dL) spiked serum	N/A	23286.86	23289.44	23234.94

Abbreviation: m/z ratio, mass to charge ratio; Melon-MALDI-TOF, Melon with C4 ZIPTIP combined with MALDI-TOF; DTT, Dithiothreitol; 1+ LC, single charged light chain; N/A, not available; QNS, quantity not sufficient.

2. Analytical performance of MALDI-TOF

Range of m/z ratio and difference of five replicates for five days of a normal sample and an abnormal (IgG/kappa) sample prepared by NB, Melon kit with C4 ZIPTIP, and MB are shown in Table 3.

Coefficient of variation (CV) of five replications in a day resulted in 0.03% to 0.09% for Melon kit with C4 ZIPTIP, 0.04% to 0.07% for MB, and 0.06 to 0.12% for NB (Table 4).

Results of CVs for five days were 0.1% to 0.2% for single charged light chains, while they were 0.6 to 0.7% for double charged light chains (Table 5).

Limit of detection of IgG affinity beads was 0.1 g/dL, monoclonal peak at daratumumab concentration, which was the lowest among three methods (Figure 1). Melon kit with C4 ZIPTIP was showed monoclonal peak in 0.2 to 0.5 g/dL, while magnetic beads showed monoclonal peak in 0.5 g/dL (Table 6).

Table 3. Range and difference of m/z ratio in precision test.

Preparation methods	Sample	Charge of LC	m/z range		Difference
Melon+C4 ZIPTIP	N	1+	23271.8	23356.1	84.3
	AbN		23052.6	23098.7	46.1
	N	2+	11495.5	11699.6	204.1
	AbN		11390.4	11596.6	206.2
MB	N	1+	23305.3	23387.9	82.6
	AbN		23282.7	23394.7	112
	N	2+	11415.9	11749.2	333.3
	AbN		11412.2	11791.2	379
NB	N	1+	23277.3	23444.6	167.3
	AbN		23052.6	23171.9	119.3
	N	2+	11480.1	11728.4	248.3
	AbN		11389.1	11610.8	221.7

Abbreviation: LC, light chain; NB, nanobody affinity beads; MB, magnetic beads; N, normal; AbN, abnormal; m/z ratio, mass to charge ratio.

Table 4. Coefficient of variation (CV) of five replications in a day.

Charge of LC	Melon+C4 ZIPTIP		MB		NB	
1+	N	0.09%	N	0.05%	N	0.08%
	AbN	0.07%	AbN	0.04%	AbN	0.06%
2+	N	0.08%	N	0.07%	N	0.09%
	AbN	0.03%	AbN	0.04%	AbN	0.12%

Abbreviation: LC, light chain; NB, nanobody affinity beads; MB, magnetic beads; N, normal; AbN, abnormal; m/z ratio, mass to charge ratio; S/N ratio, signal to noise ratio.

Table 5. Results of five replicates for five days of a normal sample and an abnormal sample.

Charge of LC	Sample	NB		Melon + C4 ZIPTIP		MB	
1+	N	Ave.	23342.06	Ave.	23340.29	Ave.	23319.02
		SD	20.69	SD	32.97	SD	24.17
		CV (%)	0.10%	CV (%)	0.10%	CV (%)	0.10%
	AbN	Ave.	23322.4	Ave.	23095.72	Ave.	23079.06
		SD	36.46	SD	23.46	SD	13.6
		CV (%)	0.20%	CV (%)	0.10%	CV (%)	0.10%
2+	N	Ave.	11640.54	Ave.	11611.21	Ave.	11606.24
		SD	110.9	SD	76.95	SD	65.38
		CV (%)	1.00%	CV (%)	0.70%	CV (%)	0.60%
	AbN	Ave.	11634.58	Ave.	11497.15	Ave.	11495.83
		SD	113.24	SD	72.36	SD	66.95
		CV (%)	1.00%	CV (%)	0.60%	CV (%)	0.60%

Abbreviation: LC, light chain; NB, nanobody affinity beads; MB, magnetic beads; N, normal; AbN, abnormal; m/z ratio, mass to charge ratio; Ave., average; SD, standard deviation;, coefficient of variation.

Table 6. Limit of detection (LoD) of three IgG purification methods combined with MALDI-TOF MS.

Sample prepared methods	Limit of detection (g/dL)
NB	0.1
Melon kit with C4 ZIPTIP	0.2 ~ 0.5
MB	0.5

Abbreviations: NB, nanobody affinity beads; MB, magnetic beads.

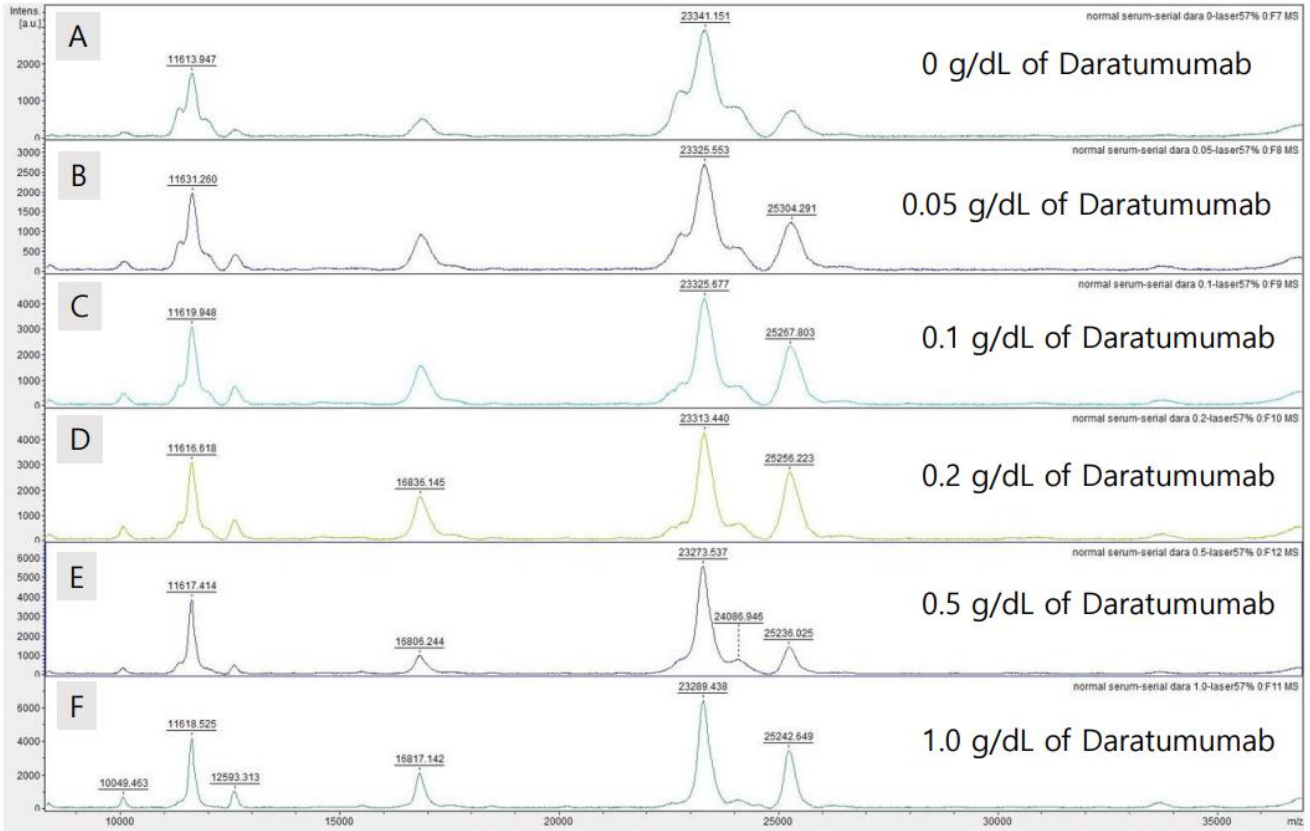


Figure 1. Result of limit of detection (LoD) using NB-MALDI-TOF for IgG purification. (A) Normal serum sample (B ~ F) daratumumab spiked sample at the concentration of 0.05, 0.1, 0.2, 0.5, 1.0 g/dL

3. Determination of normal and abnormal peaks

Reduced light chains with single and double charge of purified IgG, IgA, IgM, kappa and lambda were observed in mass spectrum range within 22,000−24,500 Da and 11,000−12,500 Da, respectively.

To identify M−proteins, the presence of monoclonal peaks is closely monitored and peak shapes were compared to negative controls. Relatively broad, rounded polyclonal peaks were observed in normal patients, while narrow and sharp monoclonal peaks were observed in abnormal patients (Figure 2). They were distinguishable in small amounts of M−protein, regardless of its low intensity.

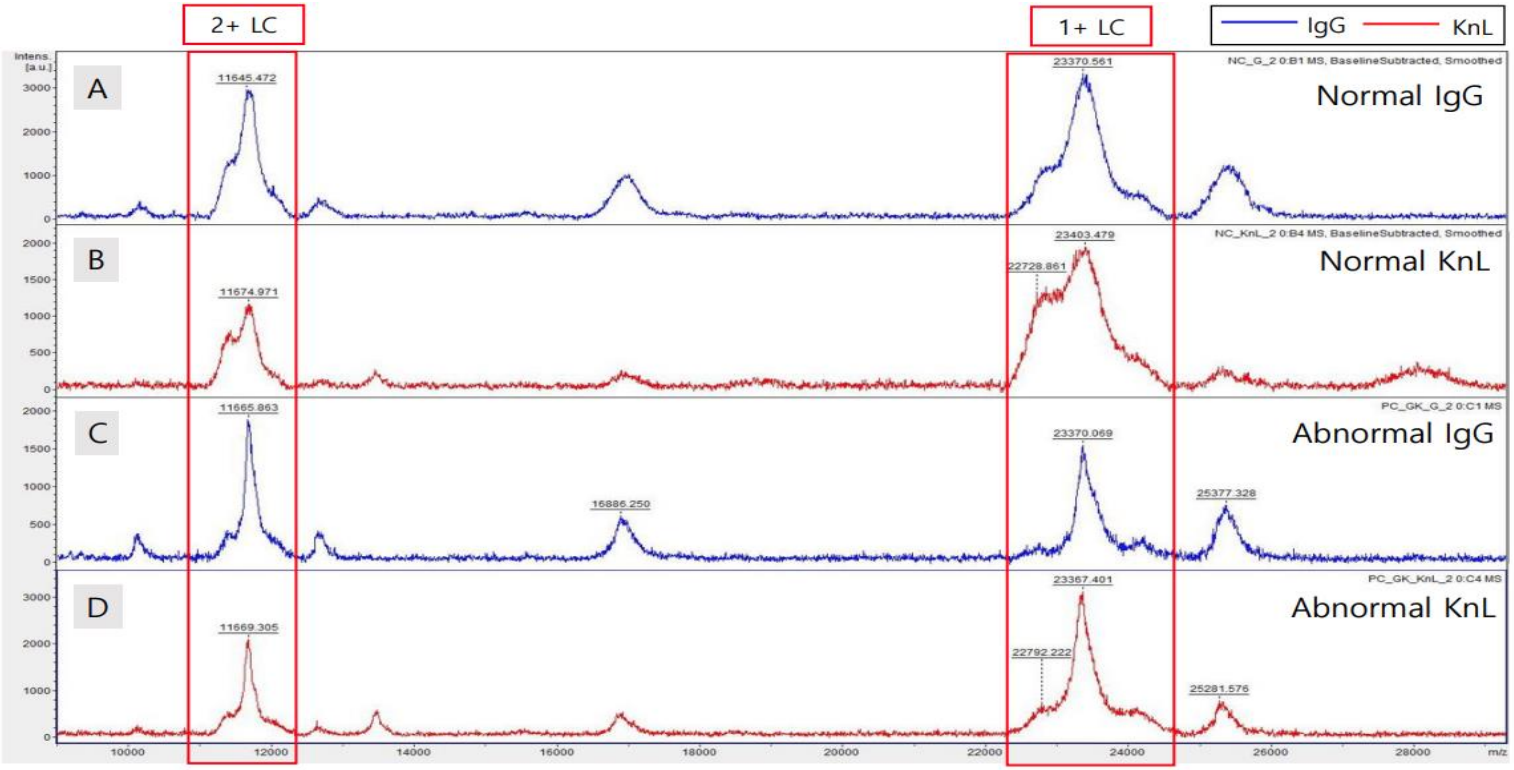


Figure 2. Example of normal and abnormal sample using NB-MALDI-TOF. (A) polyclonal peaks observed in IgG beads, (B) polyclonal peaks observed in kappa and lambda mixed beads, (C) monoclonal peaks observed in IgG beads,(D) monoclonal peaks observed in abnormal IFE sample identified IgG/kappa subtype. Abbreviations: 1+ LC, single charged light chain; 2+ LC, double charged light chain

4. NB-MALDI-TOF results in Patient Samples

Twenty-five normal and twenty-five abnormal IFE samples performed with NB-MALDI-TOF. For twenty-five abnormal IFE samples, different subtypes were included. Eight of IgG/kappa, six of IgG/lambda, two of IgA/kappa, three of IgA/lambda, three of IgM/kappa, one of IgM/lambda, one of lambda free light chain, one of biclonal (IgG/lambda, IgG/kappa) subtypes was included in this study.

In twenty-five normal IFE samples, all the samples showed negative on PEP while two of them showed positive result in NB-MADLI-TOF (Table 7). One detected IgG/lambda and the other one was suspected as IgA/lambda subtype. Among twenty-five abnormal IFE samples, two samples showed negative in NB-MALDI-TOF (Table 8).

There were four cases of discrepancies between IFE and NB-MALDI-TOF methods. Negative results of serum IFE and NB-MALDI-TOF for identifying subtypes of immunoglobulins, including IgG, IgA, IgM, kappa, lambda were used as a negative control (Figure 2). IgG/kappa subtype sample in IFE showed a negative result in NB-MALDI-TOF. Kappa/lambda ratio was normal with increased kappa chain (Figure 3). A suspicious IgG/kappa subtype

and lambda type sample in IFE showed negative in NB-MALDI-TOF. Kappa/lambda ratio was increased with markedly increased kappa chain (Figure 4). A negative sample in IFE showed IgG/lambda result in NB-MALDI-TOF. Kappa/lambda ratio was normal with normal kappa and lambda chain (Figure 5). A negative sample in IFE showed a suspicious result of IgA/lambda subtype in NB-MALDI-TOF. Kappa/lambda ratio was normal with normal kappa and lambda chain (Figure 6).

In addition, there were two cases found that NB-MALDI-TOF was helpful to confirm IFE results. IgG/lambda subtype was suspected in IFE with an ambiguous result, peaks indicating IgG/lambda subtype were found in NB-MALDI-TOF. Kappa/lambda ratio was normal with increased kappa and lambda chain (Figure 7). IgM/kappa subtype was suspected in IFE result with dim band, while IgM/kappa peaks were found in NB-MALDI-TOF. Kappa/lambda ratio was normal with increased kappa chain (Figure 8).

Overall, NB-MALDI-TOF results showed 92% concordance with IFE results (Table 9). It resulted in 92% sensitivity, and 92% specificity.

Table 7. Results of normal serum samples from IFE, compared with MALDI-TOF with NB and PEP.

Sample	IFE	NB-MALDI-TOF
1	Negative	Negative
2	Negative	Negative
3	Negative	IgG/lambda
4	Negative	s/o IgA/lambda
5	Negative	Negative
6	Negative	Negative
7	Negative	Negative
8	Negative	Negative
9	Negative	Negative
10	Negative	Negative
11	Negative	Negative
12	Negative	Negative
13	Negative	Negative
14	Negative	Negative
15	Negative	Negative
16	Negative	Negative
17	Negative	Negative
18	Negative	Negative
19	Negative	Negative
20	Negative	Negative
21	Negative	Negative
22	Negative	Negative
23	Negative	Negative
24	Negative	Negative
25	Negative	Negative

Abbreviation: IFE, immunofixation electrophoresis; NB-MALDI-TOF, nanobody affinity beads combined with matrix-assisted laser desorption/ionization time-of-flight; PEP, protein electrophoresis; s/o, suspicious of.

Table 8. Results of abnormal samples from IFE, including NB-MALDI-TOF, PEP and kappa lambda ratio.

Sample	IFE	NB-MALDI-TOF	PEP	Amount of M-protein (g/dL)	Amount of M-protein (%)	K/L ratio (0.26~1.65)	kappa (3.3~19.4 mg/L)	lambda (5.71~26.3 mg/L)
1	IgG/kappa	IgG/kappa	Positive	0.03	0.5	N/A	<0.54	<1.40
2	IgG/kappa	IgG/kappa	Positive	1.14	14.2	4.92	452.53	92.06
3	IgG/kappa	IgG/kappa	Positive	0.3	5.2	1.12	21.19	19
4	IgG/kappa	IgG/kappa	Positive	0.26	4.3	1.1	18.25	16.52
5	IgG/kappa	IgG/kappa	Positive	0.08	1.3	1.57	4.53	2.88
6	IgG/kappa	Negative	Positive	0.17	2.5	1.56	25.39	16.25
7	IgG/kappa	IgG/kappa	Positive	0.88	12.4	2.21	47.47	21.52
8	s/o IgG/kappa	Negative	Positive	0.18	2.9	34.16	496.39	14.53
9	s/o IgG/lambda	IgG/lambda	Positive	0.6	7.2	1.28	40.52	31.6
10	IgG/lambda	IgG/lambda	Positive	1.36	18.2	0.40	19.13	47.67
11	IgG/lambda	IgG/lambda	Positive	0.72	11	0.22	27.34	124.43
12	IgG/lambda	IgG/lambda	Positive	0.28	4	1.15	37.14	32.25
13	IgG/lambda	IgG/lambda	Positive	1.51	24.8	0.02	6.00	256.49
14	IgG/lambda	IgG/lambda	Positive	0.8	10.9	10.95	357.89	32.69
15	IgA/kappa	IgA/kappa	Positive	0.63	9.4	26.72	196.12	7.34
16	IgA/kappa	IgA/kappa	Positive	0.3	4.36	1.78	11.23	6.31
17	IgA/lambda	IgA/lambda	Positive	4.49	49.9	0.02	4.02	221.48
18	IgA/lambda	IgA/lambda	Positive	0.31	4.3	0.98	55.73	56.89
19	IgA/lambda	IgA/lambda	Positive	0.4	6.4	0.50	97.23	195.41
20	IgM/kappa	IgM/kappa	Positive	0.58	8.6	3.63	24.47	6.75
21	IgM/kappa	IgM/kappa	Positive	0.63	9.5	5.02	26.39	5.26
22	s/o IgM/kappa	IgM/kappa	Negative	N/D	N/D	1.41	21.24	15.05
23	IgM/lambda	IgM/lambda	Negative	N/D	N/D	2.09	54.93	26.27
24	Lambda	Lambda	Positive	0.08	1.7	0.01	24.69	2050.12
25	IgG/lambda + IgG/kappa (s/o daratumumab)	IgG/lambda + IgG/kappa (s/o daratumumab)	Positive	0.38	5.9	N/A	<0.54	<1.40

Abbreviation: IFE, immunofixation electrophoresis; NB-MALDI-TOF, nanobody affinity beads combined with matrix-assisted laser desorption/ionization time-of-flight; PEP, protein electrophoresis; s/o, suspicious of; N/A, not detected; N/A, not available.

Table 9. Contingency table of IFE and NB-MALDI-TOF.

IFE / NB-MALDI-TOF	Positive	Negative	Total
Positive	23	2	25
Negative	2	23	25
Total	25	25	50

Abbreviation: IFE, immunofixation electrophoresis; NB-MALDI-TOF, nanobody affinity beads combined with matrix-assisted laser desorption/ionization time-of-flight.

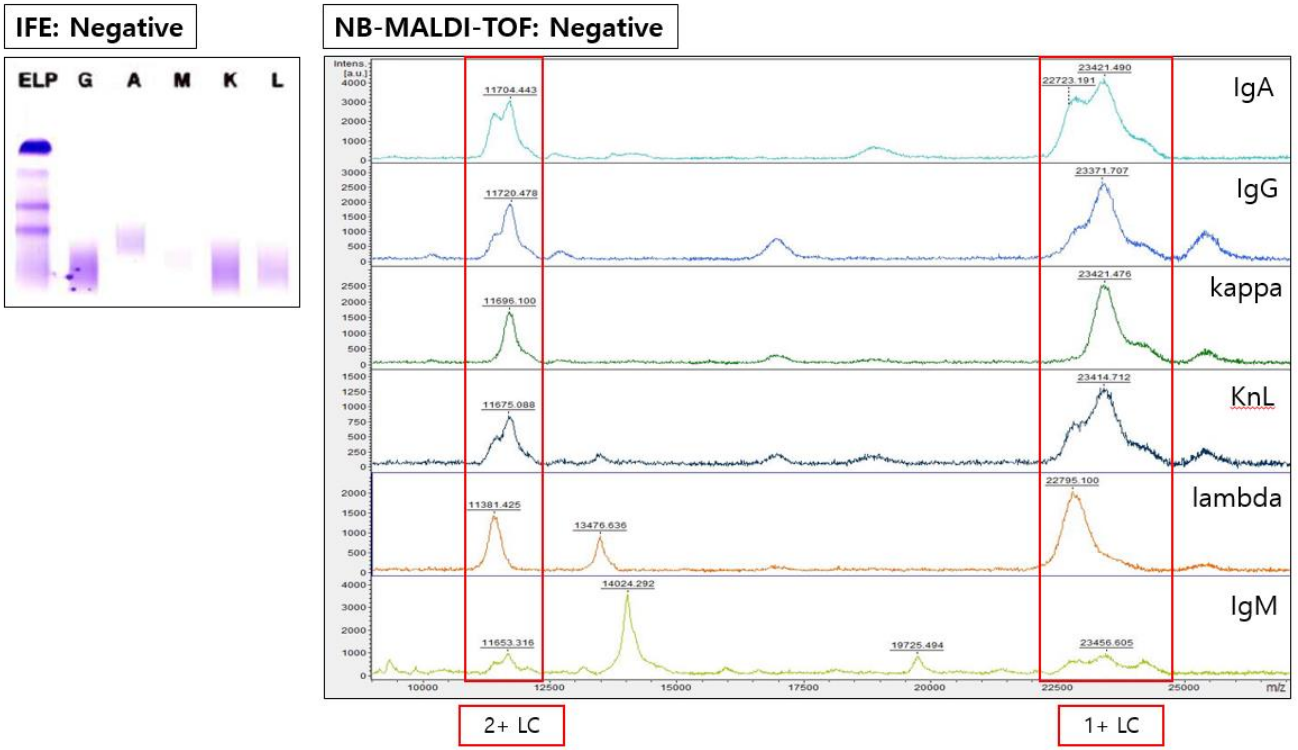
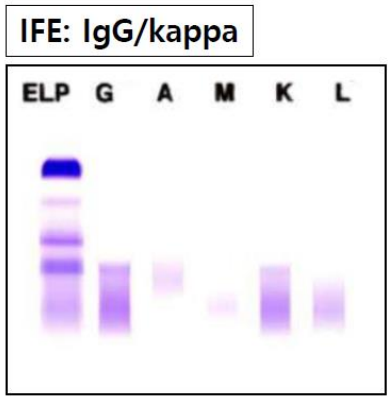


Figure 3. Negative controls of NB-MALDI-TOF and IFE. Single and double charged light chain ranges of m/z ratio are presented in red boxes. Abbreviations: IFE, immunofixation electrophoresis; NB-MALDI-TOF, nanobody affinity beads combined with matrix-assisted laser desorption/ionization time-of-flight; KnL: mixed kappa and lambda beads; PEP, protein electrophoresis; s/o, suspicious of; 1+ LC, single charged light chain; 2+ LC, double charged light chain; 2+ HC, double charged heavy chain.



Kappa/lambda ratio

K/L ratio (0.26~1.65)	Kappa (3.3~19.4)	Lambda (5.7~26.3)
1.56	25.4▲	16.3

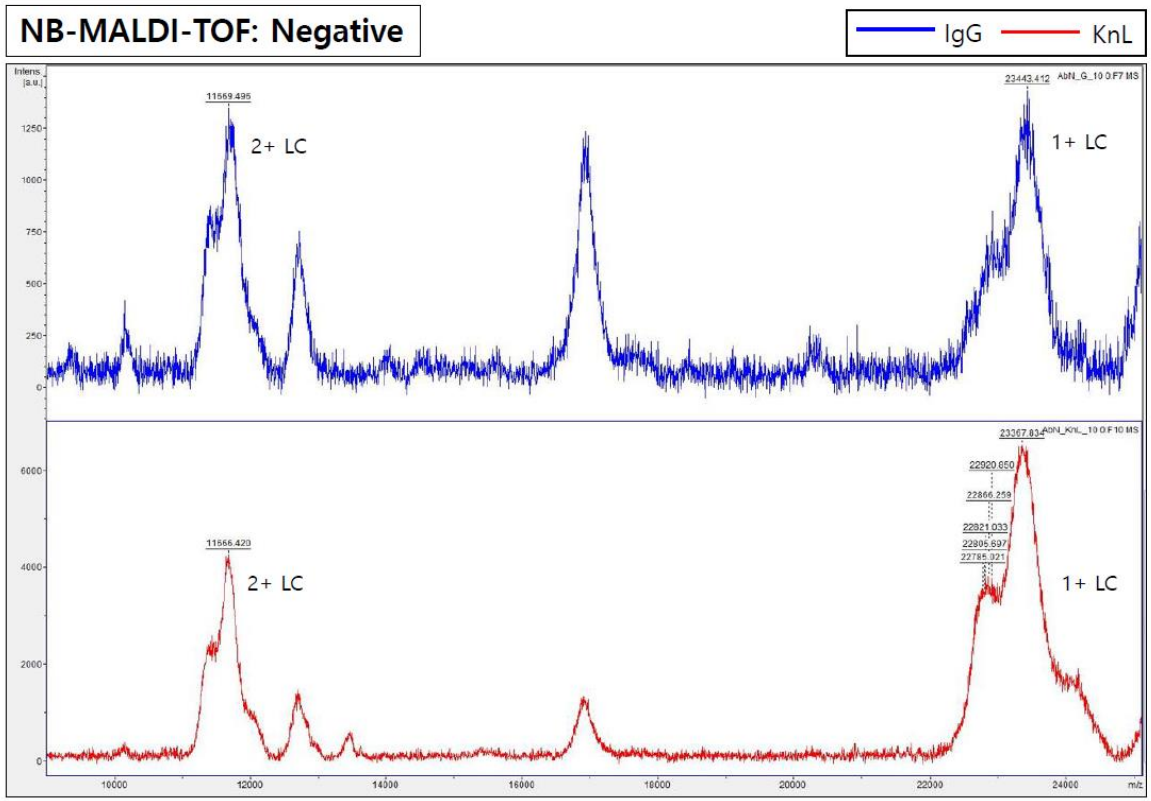
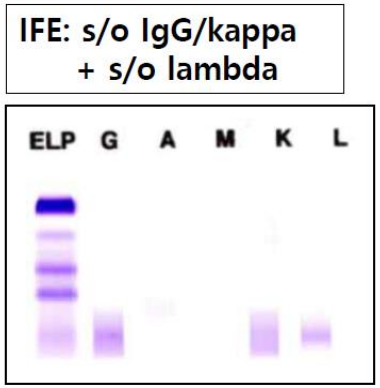


Figure 4. First case of discrepancies beteween NB-MALDI-TOF and abnormal IFE. IgG/kappa subtype in IFE (No. 6) showed negative in NB-MALDI-TOF. Kappa/lambda ratio was normal with increased kappa chain. Abbreviations: see Figure 2.



Kappa/lambda ratio

K/L ratio (0.26~1.65)	Kappa (3.3~19.4)	Lambda (5.7~26.3)
34.16 ▲	496.4 ▲	14.5

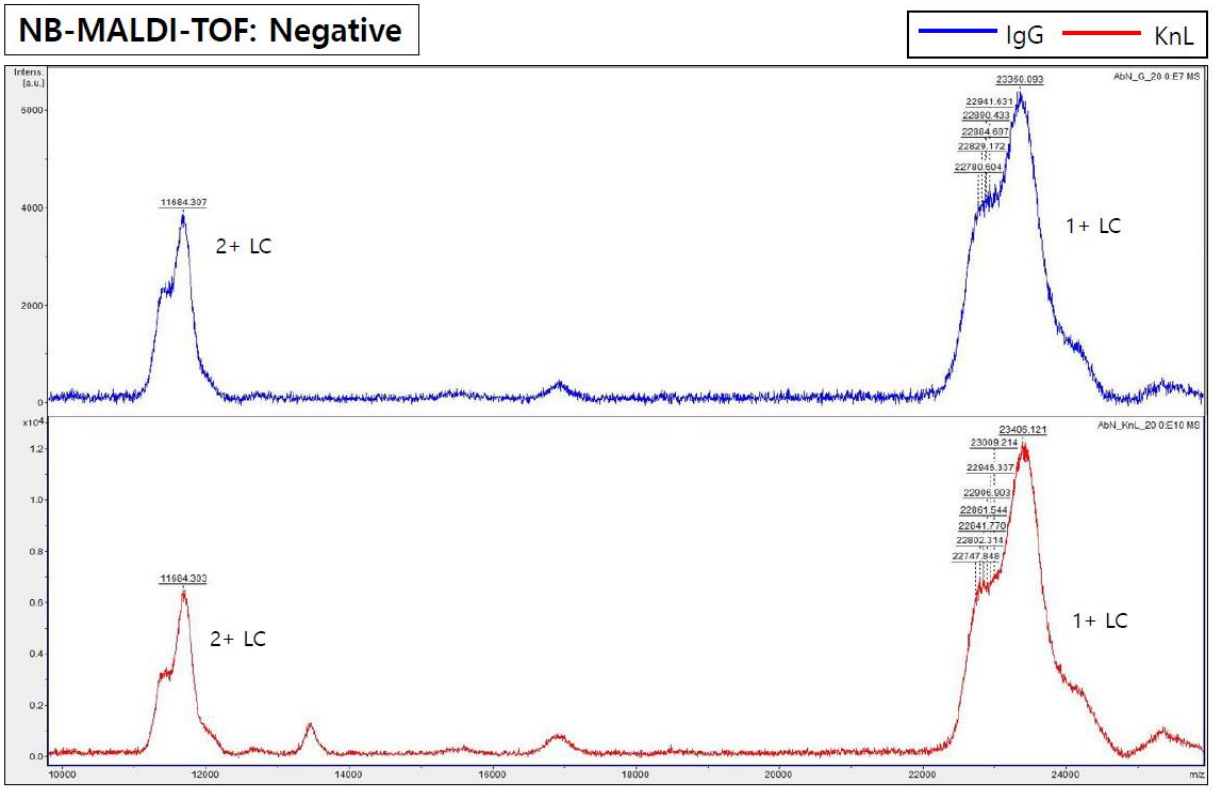
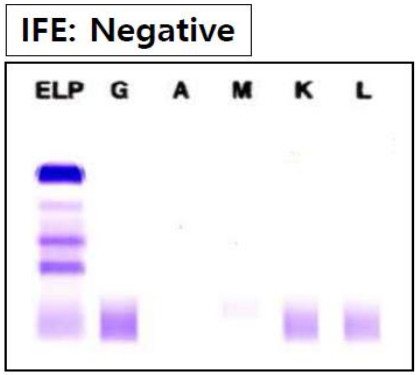


Figure 5. Second case of discrepancies beteween NB-MALDI-TOF and abnormal IFE. Suspicous of IgG/kappa subtype and lambda type sample in IFE (No. 8) showed negative in NB-MALDI-TOF. Kappa/lambda ratio was increased with markedly increased kappa chain. Abbreviations: see Figure 2.



Kappa/lambda ratio

K/L ratio (0.26~1.65)	Kappa (3.3~19.4)	Lambda (5.7~26.3)
0.99	6.9	7.0

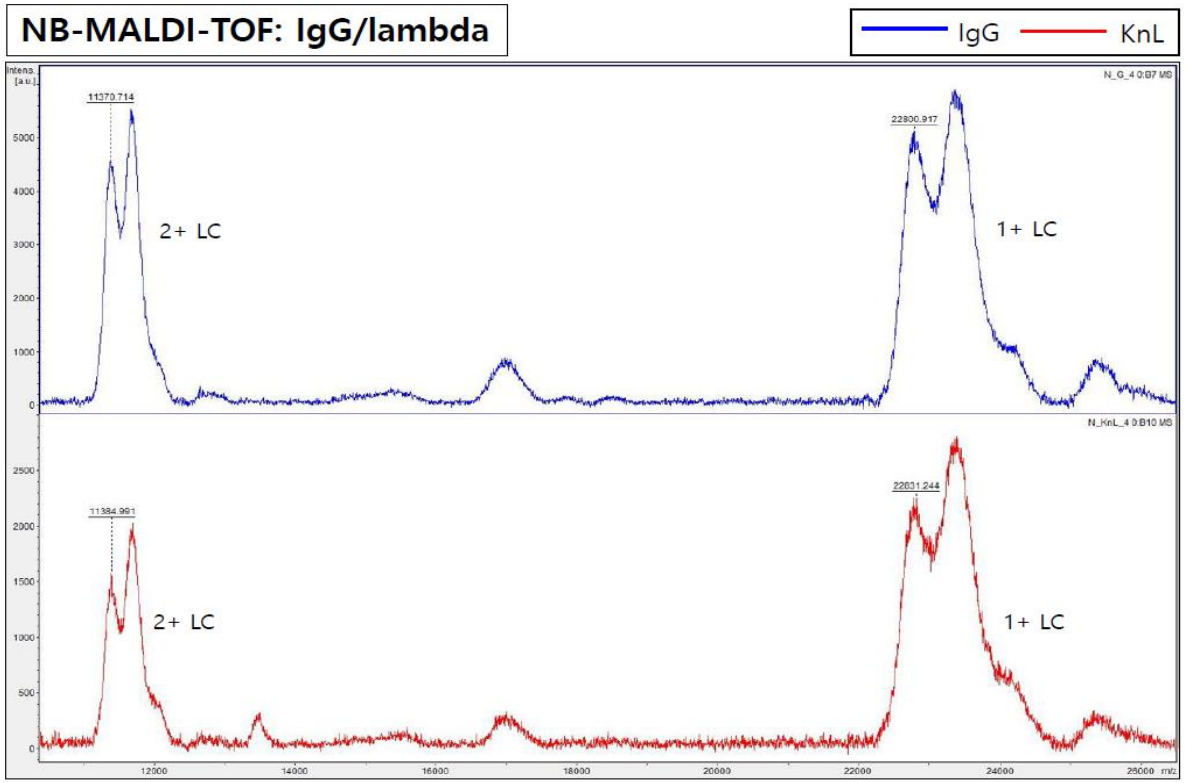


Figure 6. First case of discrepancies beteween NB-MALDI-TOF and normal IFE Negative sample in IFE (No. 4) showed IgG/lambda result in NB-MALDI-TOF. Kappa/lambda ratio was normal with normal kappa and lambda chain. Abbreviations: see Figure 2.

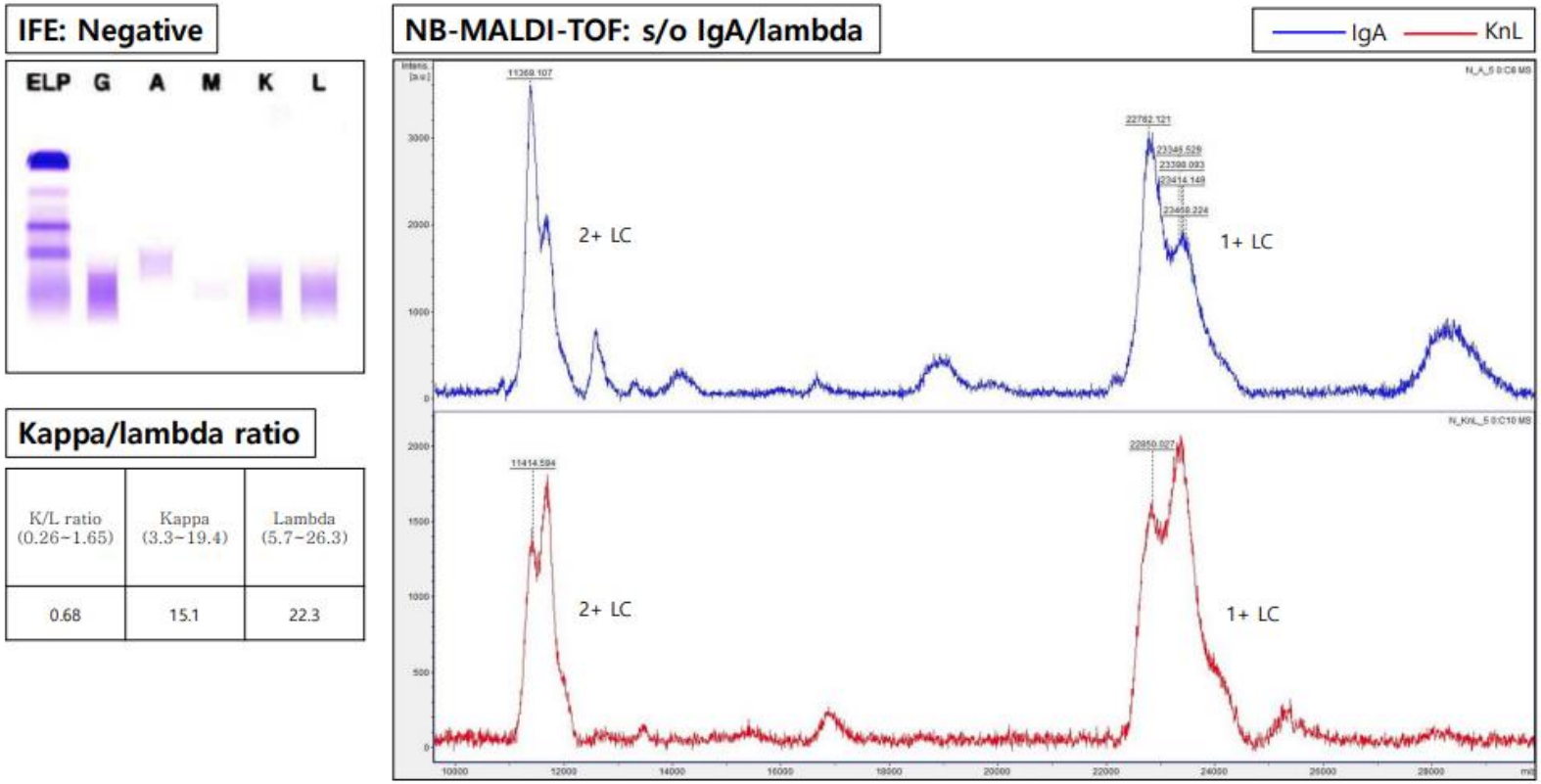
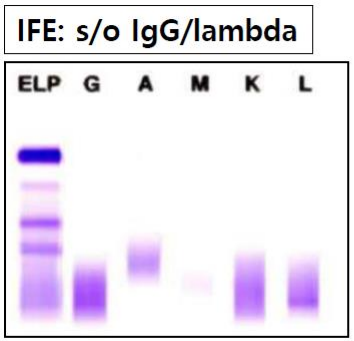


Figure 7. Second case of discrepancies beteween NB-MALDI-TOF and normal IFE. Negative sample in IFE (No. 5) showed suspicous result of IgA/lambda subtype in NB-MALDI-TOF. Kappa/lambda ratio was normal with normal kappa and lambda chain.



Kappa/lambda ratio

K/L ratio (0.26~1.65)	Kappa (3.3~19.4)	Lambda (5.7~26.3)
1.28	40.5▲	31.6▲

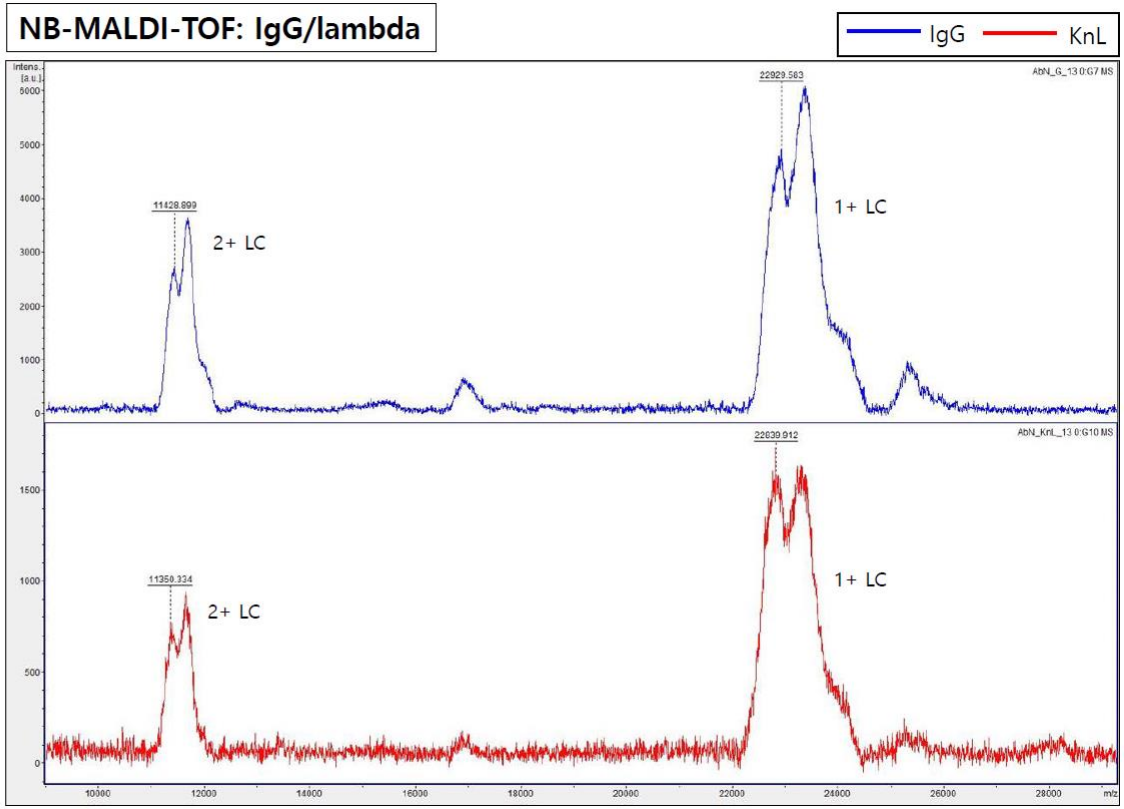


Figure 8. First case of supportive result of NB-MALDI-TOF for abnormal IFE sample. IgG/lambda subtype was suspected in IFE (No. 13) with ambiguous result, peaks indicating IgG/lambda subtype were found in NB-MALDI-TOF. Kappa/lambda ratio was normal with increased kappa and lambda chain. Abbreviations: see Figure 2.

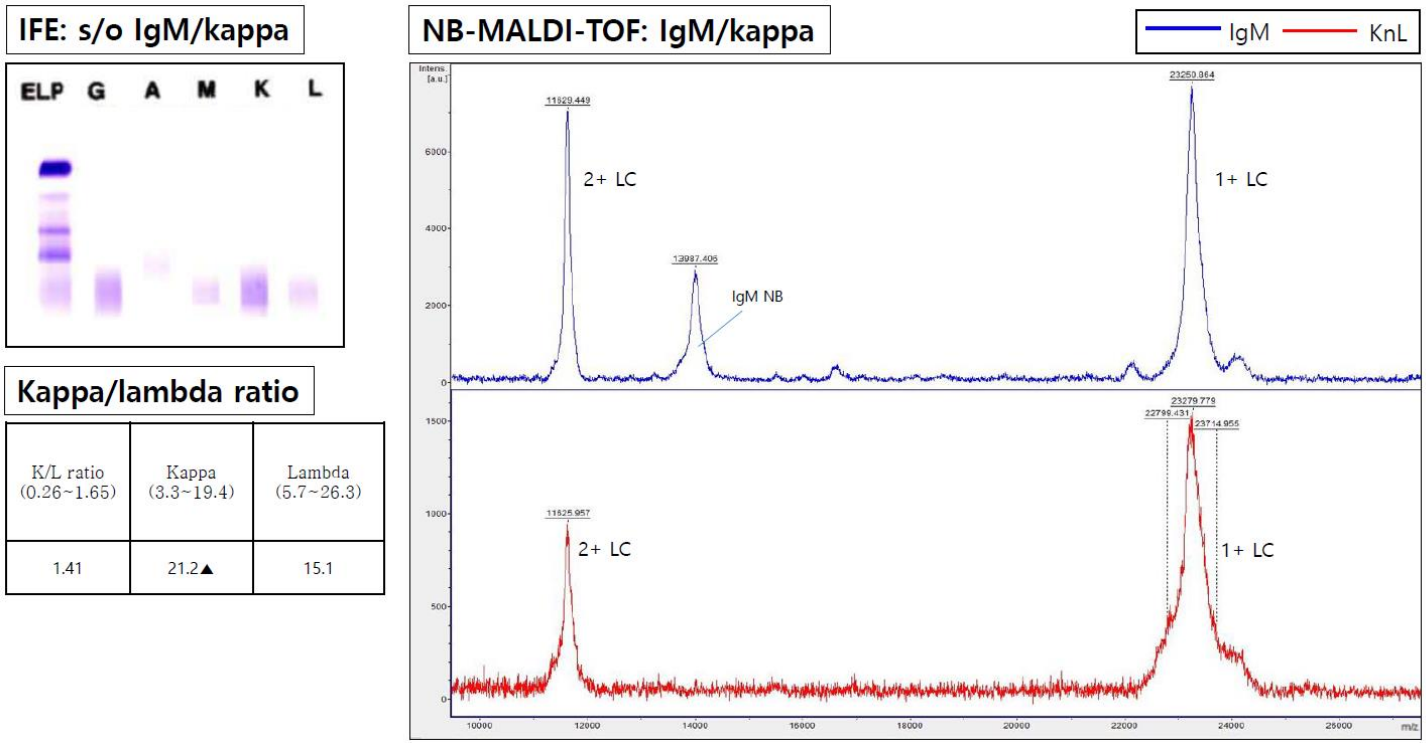


Figure 9. Second case of supportive result of NB-MALDI-TOF for abnormal IFE sample. IgM/kappa subtype was suspected in IFE (No. 22) result with dim band, while IgM/kappa peaks were found in NB-MALDI-TOF. Kappa/lambda ratio was normal with increased kappa chain. Abbreviations: see Figure 2.

Discussion

Mass spectrometry (MS) based M-protein detection has emerged as a sensitive method, with many possible approaches based on mass spectrometry [5]. As the use of t-mAb is emerging, the paradigm of M-protein work-up methods and treatment is changing [3] . A sensitive and more efficient method is required in the clinical aspect of monitoring MRD [9]. The use of MS based methods for M-protein detection is a promising alternative method to conventional methods that has the potential to revolutionize the way MRD is detected and monitored in patients with PCDs [10]. Here, considerations of feasibility and optimization of MALDI-TOF MS based method in clinical laboratory are made.

There are several different types of MS that can be used, such as Matrix-Assisted Laser Desorption/Ionization Time-of-Flight (MALDI-TOF), Quadrupole Time-of-Flight (qTOF), Liquid Chromatography-Mass Spectrometry (LC-MS), and Quadrupole-Orbitrap Mass Spectrometer (QE Orbitrap). Each of these has its own advantages and disadvantages, and the choice of method will depend on factors such as sensitivity, cost, and complexity [11]. Among many possible modalities, MALDI-TOF MS selected for demonstrating cost-effectiveness, simplicity, and speed.

Sample preparation is a critical step in developing a MALDI-TOF MS-based method for detecting M-protein[12]. Proper sample preparation ensures accurate and reliable results in mass spectrometry-based analysis. There were three possible methods to consider combining with MALDI-TOF, one method was NB which could purify IgG, IgA, IgM, kappa, and lambda subtypes. Nanobodies are single-domain antigen-binding fragments derived from heavy-chain antibodies found in camelids [13]. The second choice was using affinity liquid chromatography [13, 14]. The third choice was the development of novel antibody that specifically bind to IgG, IgA, IgM, kappa, and lambda [15]. NBs were selected for being cost-effective and for being validated in previous studies [16]. Comparing the conditions for optimizing MALDI-TOF MS by using various conditions including NB, most of results showed agreement to previous reports [16, 17].

In analytical sensitivity, NB showed the lowest LoD value at 0.1g/dL. Melon kit with C4 ZIPTIP showed 0.2 to 0.5 g/dL, and MB showed 0.5 g/dL. For MRD patients or patients under treatment of t-mAb such as daratumumab, M-proteins below 0.5 g/dL are commonly detected [18, 19]. A sensitive method is required for MRD patients with a LoD below 0.5 g/dL to ensure that low levels

of residual M-protein can be accurately detected and monitored [20]. Therefore, it is clinically important to find a way to achieve LoD below 0.5 g/dL. However, the LoD at 0.01 g/dL previously reported in MALDI-TOF based method to detect M-protein [5]. 2 μL of serum were used with 20 μL of beads in this study, which was assumed to be the minimum amount to perform the test. Refining the amounts of samples and beads may yield even greater sensitivity.

In this study, kappa and lambda beads were mixed at 50:50 ratio. Besides kappa and lambda beads, sample preparation was additionally conducted by using kappa and lambda mixed (KnL) beads. As a qualitative method, monoclonal peaks were readily found in the mass spectrum of KnL. Meanwhile, individual kappa and lambda beads were used to match the m/z value of kappa and lambda. When the positive results were found in the KnL mass spectrum, abnormal subtype of kappa or lambda was 100% agreed with abnormal IFE in this study. Consequently, using kappa and lambda mixed beads is a useful technique for screening the presence of monoclonal peaks.

Washing steps for NB protocol over five times is an important procedure to reduce the peaks with low signal to noise ratio. As it

takes over three hours for thirty samples, it is time consuming and labor intensive. It would possible to prepare many samples consistently and accurately if it is automated by liquid handler[16]. In addition, this study lacked the database or data analysis software for high mass range, which might be developed in further study. Therefore, it would be more efficient if it is fully automated and validated with proper database and software.

Despite all the efforts to optimize the methods, there were several limitations which were inevitable. NBs were limited to that of IgG, IgM, IgA. Therefore, IgD and IgE were not included. Small number of patients in IgM and IgA were involved due to their rarity. Serum samples were reported only in this study, while urine samples were not included.

In conclusion, NB-MALDI-TOF MS is a method comparable to IFE for detecting the presence of M-protein. NB-MALDI-TOF is recommended as an additional method to IFE that can provide additional information.

References

1. Rajkumar, S.V., et al., *International Myeloma Working Group updated criteria for the diagnosis of multiple myeloma.* Lancet Oncol, 2014. 15(12): p. e538-48.
2. *Criteria for the classification of monoclonal gammopathies, multiple myeloma and related disorders: a report of the International Myeloma Working Group.* British Journal of Haematology, 2003. 121(5): p. 749-757.
3. Eveillard, M., et al., *Using MALDI-TOF mass spectrometry in peripheral blood for the follow up of newly diagnosed multiple myeloma patients treated with daratumumab-based combination therapy.* Clin Chim Acta, 2021. 516: p. 136-141.
4. O'Brien, A., F. O'Halloran, and V. Mykytiv, *Minimal Residual Disease in Multiple Myeloma: Potential for Blood-Based Methods to Monitor Disease.* Clin Lymphoma Myeloma Leuk, 2022. 22(1): p. e34-e40.
5. Giles, H.V., A. Wechalekar, and G. Pratt, *The potential role of mass spectrometry for the identification and monitoring of patients with plasma cell disorders: Where are we now and which questions remain unanswered?* Br J Haematol, 2022.

198(4): p. 641-653.

6. Chapman, J.R. and K.L. Thoren, *Tracking of low disease burden in multiple myeloma: Using mass spectrometry assays in peripheral blood.* Best Pract Res Clin Haematol, 2020. 33(1): p. 101142.

7. Ladwig, P.M., D.R. Barnidge, and M.A.V. Willrich, *Mass Spectrometry Approaches for Identification and Quantitation of Therapeutic Monoclonal Antibodies in the Clinical Laboratory.* Clin Vaccine Immunol, 2017. 24(5).

8. Milani, P., et al., *The utility of MASS-FIX to detect and monitor monoclonal proteins in the clinic.* Am J Hematol, 2017. 92(8): p. 772-779.

9. Bertamini, L., M. D'Agostino, and F. Gay, *MRD Assessment in Multiple Myeloma: Progress and Challenges.* Curr Hematol Malig Rep, 2021. 16(2): p. 162-171.

10. Zajec, M., et al., *Mass Spectrometry for Identification, Monitoring, and Minimal Residual Disease Detection of M-Proteins.* Clin Chem, 2020. 66(3): p. 421-433.

11. Murray, D.L., et al., *Mass spectrometry for the evaluation of monoclonal proteins in multiple myeloma and related disorders: an International Myeloma Working Group Mass*

Spectrometry Committee Report. Blood Cancer J, 2021. 11(2): p. 24.

12. Murray, D.L. and S. Dasari, *Clinical Mass Spectrometry Approaches to Myeloma and Amyloidosis.* Clin Lab Med, 2021. 41(2): p. 203-219.

13. Mills, J.R., D.R. Barnidge, and D.L. Murray, *Detecting monoclonal immunoglobulins in human serum using mass spectrometry.* Methods, 2015. 81: p. 56-65.

14. Li, Y., *Immunoglobulin-binding protein-based affinity chromatography in bispecific antibody purification: Functions beyond product capture.* Protein Expr Purif, 2021. 188: p. 105976.

15. Goulet, D.R. and W.M. Atkins, *Considerations for the Design of Antibody-Based Therapeutics.* J Pharm Sci, 2020. 109(1): p. 74-103.

16. Kohlhagen, M., et al., *Automation and validation of a MALDI-TOF MS (Mass-Fix) replacement of immunofixation electrophoresis in the clinical lab.* Clin Chem Lab Med, 2020. 59(1): p. 155-163.

17. Mills, J.R., et al., *Comprehensive Assessment of M-Proteins Using Nanobody Enrichment Coupled to MALDI-TOF Mass*

Spectrometry. Clin Chem, 2016. 62(10): p. 1334-44.

18. McCudden, C.R., et al., *Recognition and management of common, rare, and novel serum protein electrophoresis and immunofixation interferences.* Clin Biochem, 2018. 51: p. 72-79.

19. Tang, F., et al., *Interference of Therapeutic Monoclonal Antibodies With Routine Serum Protein Electrophoresis and Immunofixation in Patients With Myeloma: Frequency and Duration of Detection of Daratumumab and Elotuzumab.* Am J Clin Pathol, 2018. 150(2): p. 121-129.

20. Kumar, S., et al., *International Myeloma Working Group consensus criteria for response and minimal residual disease assessment in multiple myeloma.* Lancet Oncol, 2016. 17(8): p. e328-e346.

국문초록

서론: 단세포성 면역글로불린(단클론성 단백, M-단백)은 다발성골수종과 같은 형질세포질환에서 증가 소견이 관찰되며, 질병 활성도를 반영하는 것으로 알려져 있다. M-단백 검출을 위하여 기존의 검사 방법으로 단백전기영동검사(protein electrophoresis, PEP), 면역전기영동법(immuno-electrophoresis, IEP), 그리고 면역고정 전기영동법(immunofixation electrophoresis, IFE)의 검사를 시행한다. 최근 단클론성항체를 치료제의 사용으로, 소량의 M-단백이 남아 있는 미세잔존질환(minimal residual disease, MRD)에 대해 관심이 높아지고 있다. 질량분석기를 기반으로 M-단백을 검사하는 방법은 소량의 M-단백도 측정할 수 있는 민감도가 높은 방법으로, 임상적으로 질량분석기를 이용한 검사 가능성이 대두되고 있다. 이에 질량분석기를 이용하여 혈청 내에 존재하는 M-단백을 검출하고자 하였다.

방법: 서울대학교병원에서 IFE 검사를 요청한 환자를 대상으로, 요청된 검사를 시행 후 잔여 검체를 사용하였다. 잔여 약제인 다라투무맙(daratumumab)과 세툭시맙(cetuximab)을 사용하였다. 해당 약제는 기존에 아미노산 서열과 분자량이 알려진 단클론성약제로, 고분해능 질량분석기로 분자량을 확인하여 MALDI-TOF 결과와 비교하였다. 전처리 방법으로 Captureselect nanobody affinity beads (NB)를 사용하여 IgG, IgM, IgA, kappa and lambda (KnL), kappa, lambda로 분리하였고, Melon spin gel kit와 magnetic beads (MB) 방법으로 IgG 정제를 시행하였다. 위 세가지 방법으로 전처리를 시행한 검체는 a-Cyano-4-hydroxycinnamic acid (CHCA) matrix를 사용하여 1 μL씩 도포하였고, 말디토프 질량분석기(matrix-assisted laser desorption/Ionization-time of flight mass spectrometry, MALDI-TOF MS)로 측정 및 분석을 시행하였다. IFE 양성 25건, IFE 음성 25건의 환자 검체를 대상으로, NB 전처리 후 MALDI-TOF (NB-MALDI-TOF) 검사를 진행하였다.

결과: 검사 결과의 해석은 질량 스펙트럼에서 관찰되는 경쇄의 질량대전하비를 확인하였다. 2가의 경쇄는 11,000-12,500 Da, 1가의 경쇄는 22,000-24,500 Da의 질량 대 전하비를 보였다. 2가의 중쇄는 약 25,000 Da, 1가의 중쇄는 대략 50,000 Da 정도의 질량 대 전하비를 보였다. 정상의 경우 polyclonal peak가 관찰되는 반면, 비정상의 경우 monoclonal peak가 관찰되었다. NB로 전처리한 경우, 검출 한계가 0.1 g/dL로 가장 낮았다. Melon spin gel kit를 사용한 방법은 0.2 – 0.5 g/dL의 검출 한계를 보였고, MB 방법은 0.5 g/dL였다. NB-MALDI-TOF 결과 상 양성 23건, 음성 23건으로 IFE 검사와 92% 의 일치도를 보였다. NB-MALDI-TOF 검사의 민감도와 특이도는 각각 92%, 92%였다.

결론: NB-MALDI-TOF 검사법은 민감도가 높은 검사법으로 확인되었다. IFE 검사에 추가적으로 NB-MALDI-TOF 검사를 시행하는 경우, 정성검사로써 소량의 M단백 검출에 유용할 것으로 사료된다. 특히, IFE 결과가 음성이거나 판단하기가 어려운 경우에 M단백을 검출하는데 도움이 될 수 있을 것이다.

주요단어: 다발골수종, 형질세포질질환, 단세포군감마글로불린병증, 단클론성 단백, 말디토프 질량분석법
학번: 2019-26416