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Screening Method for M-Proteins in Serum Using Nanobody Enrichment Coupled to MALDI-TOF Mass Spectrometry

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BACKGROUND: Current recommendations for screening for monoclonal gammopathies include serum protein electrophoresis (PEL), imunofixation electrophoresis (IFE), and free light chain (FLC) ratios to identify or rule out an M-protein. The aim of this study was to examine the feasibility of an assay based on immunoenrichment and MALDI-TOF-MS (MASS-SCREEN) to qualitatively screen for M-proteins.

METHODS: Serum from 556 patients previously screened for M-proteins by PEL and IFE were immunopurified using a κ/λ-specific nanobody bead mixture. Following purification, light chains (LC) were released from their heavy chains by reduction. MALDI-TOF analysis was performed and the mass-to-charge LC distributions were visually examined for the presence of an M-protein by both unblinded and blinded analysts.

RESULTS: In unblinded analysis, MASS-SCREEN detected 100% of the PEL-positive samples with an analytical sensitivity and specificity of 96% and 81% using IFE positivity as the standard. In a blinded analysis using 6 different laboratory personnel, consensus was reached in 92% of the samples. Overall analytical sensitivity and specificity were reduced to 92% and 80%, respectively. FLC ratios were found to be abnormal in 28% of MASS-SCREEN–negative samples, suggesting FLC measurements need to be considered in screening.

CONCLUSIONS: MASS-SCREEN could replace PEL in a panel that would include FLC measurements. Further studies and method development should be performed to validate the clinical sensitivity and specificity and to determine if this panel will suffice as a general screen for monoclonal proteins.

Monoclonal gammopathies are a group of plasma cell disorders (PCD)[4] that are defined by the presence of a serum monoclonal immunoglobulin (Ig; M-protein) expressed at a concentration above the polyclonal background. Because clinically significant disease can be associated with high or low M-protein concentrations, recommendations on screening for PCDs include the combination of 3 tests: protein electrophoresis (PEL), immunofixation electrophoresis (IFE), and free light chain (FLC) measurements to maximize the clinical sensitivity of screening *(1)*. In our clinical laboratory approximately 80% of the patients not being monitored for PCD are negative for an M-protein. If a cost-effective test to rule out the presence of an M-protein existed, it would markedly decrease the number of samples needing further electrophoretic testing.

As standalone serum tests, Katzmann demonstrated that IFE, PEL, and FLC had PCD screening sensitivities of 87%, 79%, and 74%, respectively *(2)*. Because IFE is the most costly and labor-intensive method of the 3 tests, the author suggested that a cost-effective approach to PCD screening could be a combination of PEL and FLC, offering an overall clinical sensitivity of 94%. Substitution of IFE for PEL increases clinical sensitivity from 94% to 97% and detects PCDs associated with lower concentrations of intact Igs. Attempts have been made to reduce the material cost of IFE screening by reducing the number of gel lanes per sample and by the use of antisera specific for all Ig isotypes (Sebia Penta Screen). Our laboratory has used this assay as a screen but experi-

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[4] Nonstandard abbreviations: PCD, plasma cell disorder; Ig, immunoglobulin; PEL, protein electrophoresis; IFE, immunofixation electrophoresis; FLC, free light chain; LC, light chain.

enced too many screen-positive samples that were subsequently negative by IFE, thus reducing the benefit of the screening.

Microflow liquid chromatography coupled with electrospray ionization and quadrupole TOF mass spectrometry (microLC-ESI-Q-TOF-MS) can be used to identify and monitor an M-protein in patient serum and urine *(3, 4)*. The basic principle of the method leverages the unique mass resulting from LC Ig gene rearrangement in B-cells. The LC mass serves as a marker of B-cell clonality whereas the signal intensity is correlated to M-protein concentration *(3)*. Because of the high numbers of unique clones in a properly functioning immune system, the mass distribution of each LC is gaussian. In a patient with a PCD, the LC distribution can be inspected for an overabundant clone in a similar manner as PEL. We have termed the technique monoclonal Ig rapid accurate mass measurement (miRAMM). Given the accuracy, resolution, and analytical sensitivity of modern mass spectrometers, miRAMM has demonstrated the ability to detect M-proteins with increased analytical specificity and sensitivity compared to gel methods *(3)*. The increased analytical sensitivity of the miRAMM method has the potential to rule out the presence of an M-protein in a single test. However, the chromatography portion of the method required an acquisition time of 25 min, making it unsuitable for a high-throughput clinical laboratory.

An alternative to microLC-ESI-Q-TOF-MS is MALDI-TOF-MS, in which the sample is prepared as a dried mixture that is placed on a sample target and then introduced into the mass spectrometer. Automated sample acquisition available on current MALDI-TOF-MS instruments enables acquisition times on the order of seconds. In a clinical setting, the most notable application of MALDI-TOF-MS has been for the identification of bacteria *(5, 6)*. The use of MALDI-TOF-MS as a substitute for gel and capillary electrophoresis has been demonstrated by characterizing hemoglobin in the detection of hemoglobinopathies *(7, 8)*. The feasibility of using MALDI-TOF-MS to detect M-proteins was recently demonstrated for both serum and urine *(9)*, but the preanalytical method of Ig isolation (Melon Gel) was not analytically sensitive enough to detect lower concentrations of IgA and IgM M-proteins.

Our goal was to examine the feasibility of using miRAMM with nanobody Ig immunoenrichment and MALDI-TOF-MS (MASS-SCREEN) to provide a rapid and cost-effective method for qualitative (positive/negative) detection of M-Proteins.

Methods

SAMPLES

All patient samples and data were accessed in compliance with the Mayo Clinic Institutional Review Board. Waste serum samples previously tested by PEL and IFE in the Clinical Immunology Laboratory at Mayo Clinic were used to evaluate the analytical sensitivity and specificity of a MALDI-TOF-MS method. A total of 556 samples were tested. The cohort was biased toward positive patient samples with 421 gel positive sera (including 257 PEL positive M-proteins and 164 M-proteins detectable by IFE only) and 135 IFE negative patient sera. The cohort was selected to contain a comprehensive survey of M-proteins seen in our practice, covering all isotypes (IgG, IgA, IgM, and IgD), FLC, biclonal, and heavy chain M-proteins. The normal serum to which each patient was compared was pooled, delipidated, charcoal stripped serum (SeraCare Life Sciences).

PEL AND IFE ANALYSIS

All assays were performed according to protocols in the Clinical Immunology Laboratory. PEL was performed on the SPIFE SPE (serum protein electrophoresis) system (Helena Laboratories) and IFE on Hydrasys 9IF gels (Sebia). The total protein concentration was determined by colorimetric assay using biuret reagents on an Advia 1200 chemistry analyzer system (Siemens Healthcare). In our laboratory, PEL is considered positive when the M-protein is confirmed by IFE and there is a distinct restriction (band) within the polyclonal background. This determination can vary depending on the migration pattern (β vs γ) but in all cases the M-protein concentration is above 0.2 g/dL. Small unfractionable PEL questionable abnormalities or normal PEL patterns that require reflex to IFE for positive identification are considered PEL negative.

NANOBODY ENRICHMENT

For immunoaffinity purification of serum for Ig, 2.5 μL of each sample was added to a 20-μL mixture of agarose beads (50% beads + 50% 1× PBS) coupled to single-domain antibody fragments (nanobody) targeting the κ and λ constant region domains (Thermo Fisher). After a 45-min incubation at room temperature, the beads were washed twice with 500 μL PBS and twice with 500 μL water. Bound proteins were then eluted and reduced with 40 μL 50 mmol/L tris(2-carboxyethyl)phosphine in 10% formic acid followed by incubation for 15 min at room temperature.

MALDI-TOF-MS ANALYSIS

For each sample elution, 0.6 μL was spotted on an individual well of a microScout 96-well polished steel target plate (Bruker Daltonics) using a sandwich matrix application method. Each well had previously been spotted with 0.6 μL of matrix (α-cyano-4-hydroxycinnamic acid, 10 g/L in 50% acetonitrile + 0.1% trifluoroacetic acid), and an additional 0.6 μL of matrix was spotted on top of each sample preparation. Mass spectra were ac-

Table 1. Light chain mass/charge (*m/z*) ranges used to detect M-proteins for both $[M+H]^+$ and $[M+2H]^{2+}$ charge states.

LC	*m/z* (Da)	Charge state
κ	11 550–12 300	$[M+2H]^{2+}$
	23 100–24 600	$[M+H]^+$
λ	11 100–11 550	$[M+2H]^{2+}$
	22 200–23 100	$[M+H]^+$

quired in positive ion mode with delayed extraction and summation of 500 laser shots using a MALDI-TOF mass spectrometer (Microflex LT, Bruker).

A mass/charge (*m/z*) range of 9000–32000 was acquired. The total LC *m/z* spectrum for each sample was superimposed onto a normal total LC distribution. The *m/z* distribution was visually inspected for the presence of a peak that was distinct from the polyclonal background in both the $[M+H]^+$ and $[M+2H]^{2+}$ LC mass ranges *(10)* listed in Table 1. A first analyst who was not blinded to the PEL and IFE results interpreted each spectrum. Each spectrum was classified as positive or negative for the presence of an M-protein. A selection of cases was chosen as a training set to educate a second set of 6 analysts, including laboratory technologists, laboratory directors, and a trained mass spectrometrist. After brief training, the analysts were given the spectra to interpret, but were blinded to the gel results. Each blinded analyst was asked to assign the spectra into 1 of 3 categories (positive, negative, or repeat/undetermined). Each spectrum was assigned a result based on majority consensus among the blinded analysts.

FLC ANALYSIS

Free κ and λ were measured using FreeLite™ reagents (Binding Site) on a BNII Nephelometer (Siemens Healthcare). The κ/λ ratio was measured and compared to the diagnostic reference range *(11)*.

Results

The κ and λ molecular mass distributions observed using MALDI ionization were similar to those previously observed for deconvoluted ESI mass spectra *(3)*. Fig. 1 displays the typical spectrum for a negative/normal patient. The view of the entire acquisition range is displayed, along with approximate κ and λ mass ranges defined for demonstration purposes. In the absence of an M-protein, 2 gaussian distributions were distinguishable, corresponding to the majority of κ and λ LC masses as described earlier *(10)*. When the spectrum from a patient with an M-protein was overlaid onto the normal spec-

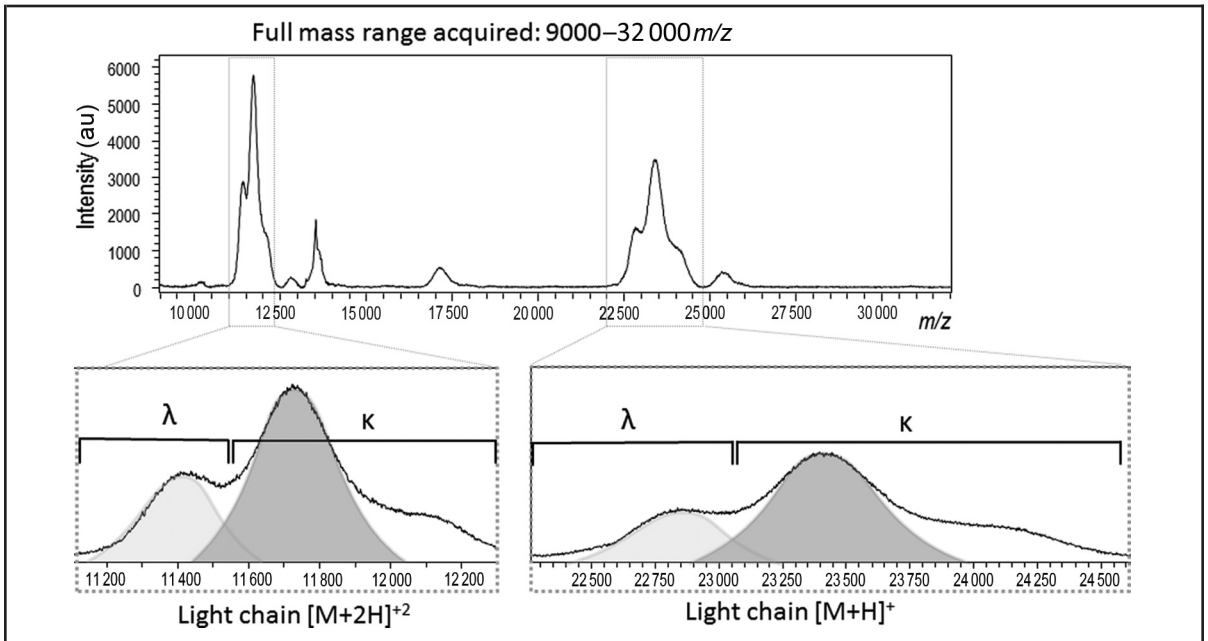

Fig. 1. Normal serum spectrum from 9000 to 32 000 *m/z* with expanded views of LC *m/z* distributions for both $[M+H]^+$ and $[M+2H]^{2+}$ charge states.

Approximate mass ranges for κ and λ are defined for each charge state. Gaussian distributions are observed for κ and λ light chain populations in both charge states. Au, arbitrary units.

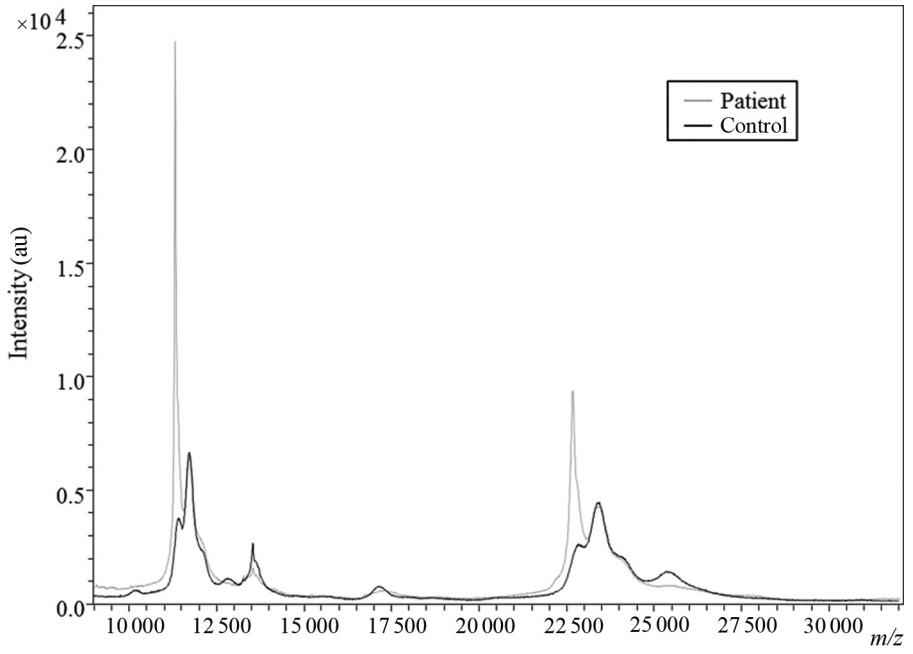

Fig. 2. Spectra from patient serum containing an M-protein (grey) superimposed onto a normal polyclonal background (black).

trum, the M-protein was identified as a sharp peak above the polyclonal (normal) background (Fig. 2). The LC $[M+2H]^{2+}$ distribution was noted to be more visually sensitive to the presence of an M-Protein than the $[M+H]^{+}$ distribution. To establish the approximate, qualitative sensitivity of MASS-SCREEN relative to PEL and IFE, 8 different PEL positive M-proteins were diluted into negative sera and reevaluated by each method. (spectral example, see Fig. 1 in the Data Supplement that accompanies the online version of this article at http://www.clinchem.org/content/vol62/issue10). The limit of detection for each M-protein sample was defined by the last dilution in which a visual spike (PEL and MASS-SCREEN $[M+2H]^{2+}$) or band restriction (IFE) was deemed to be present by a blinded reader. Based on the initial value of the serum M-spike, analytical limit of detection was between 0.01 and 0.05 g/dL for MASS-SCREEN, between 0.01 and 0.25 g/dL for IFE, and between 0.05 and .50 g/dL for PEL depending on the migration pattern. The analytical sensitivity of the MASS-SCREEN was on the same order of magnitude as IFE.

MASS-SCREEN results for the entire cohort of 556 patients were visually examined for the presence of an M-protein. The overall analytical sensitivity and specificity of MASS-SCREEN for the unblinded comparison was 96% and 81% respectively, compared to IFE (Fig. 3A). If the data was limited to M-protein positive PEL results, the agreement was 100%, indicating that discrepancies were limited to those smaller M-protein concentrations identified by IFE only. The comparison of the MASS-SCREEN and PEL/IFE by isotype is shown in Fig. 3B. Among the PEL negative/IFE positive samples, MASS-SCREEN identified an additional 149 IFE-only positive samples, lending support to the higher analytical sensitivity for MASS-SCREEN compared to PEL found during dilution studies. Among the negative samples, MASS-SCREEN would have identified another 25 (4%) of cases to reflex for PEL and IFE confirmation. The IFE positive/MASS-SCREEN negative cases were limited to IgG and IgM proteins, which were detected by IFE but not PEL. Our study included 1 heavy chain disease patient; MASS-SCREEN was not able to detect the heavy chain positive patient.

To assess the feasibility of MASS-SCREEN alone to rule out monoclonal gammopathies, samples that were negative by MASS-SCREEN were also screened for abnormal FLC ratios. Of 125 MASS-SCREEN negative samples, 120 had enough volume to be analyzed for FLCs. Normal FLC ratios (0.26–1.65) were measured in 87 (72.5%) of samples, whereas ratios for 3 (2.5%) samples were below the reference range and 30 (25%) samples had ratios above the reference range (see online Supplemental Fig. 2), suggesting that MASS-SCREEN may not be sufficient in ruling out monoclonal gammopathies by itself. Only 2 samples with positive FLC ratios were called positive by IFE (and negative by PEL):

A: Overall analytical sensitivity and specificity between MASS-SCREEN and gel methods

	MASS-SCREEN positive	MASS-SCREEN negative
PEL+/IFE+	257	0
PEL−/IFE+	149	15
PEL−/IFE−	25	110

B: Specificity by isotype

Isotype (IFE)	N	MASS-SCREEN positive	MASS-SCREEN negative	Agreement
IgG	217	211	6	97%
IgA	105	105	0	100%
IgM	73	64	9	88%
IgD	5	5	0	100%
FLC	11	11	0	100%
Biclonal	10	10	0	100%
Neg/CRO	135	25	110	81%

Fig. 3. Results from the unblinded analysis.

(A), Overall analytical sensitivity and specificity of MASS-SCREEN was 96% and 81%, respectively when compared to IFE positive/negative results. (B), When broken down by isotype of the M-protein, disagreements between MASS-SCREEN and gel methods were restricted to IgG and IgM isotypes. CRO, cannot rule out presence of M-protein.

1 IgGλ IFE and 1 IgMκ IFE+, with FLC ratios of 1.76 and 2.81, respectively.

The spectra were reevaluated by 6 blinded analysts (authors D.L. Murray, J.A. Katzmann, D.I. Lofgren, J.R. Mills, D.R. Barnidge, A.M. Liptac) with different laboratory roles. All analysts except one had multiple years' experience reading traditional gel methods. Each analyst was given a training set and was trained by the unblinded analyst (M.C. Kohlhagen). The analysts were only given access to static copies of the full spectra with a normal LC *m/z* distribution superimposed onto the spectra (Fig. 2). The final result was recorded for each spectrum based on consensus agreement. (i.e., greater or equal to 4/6 agreement). Consensus was reached in 92% (513/556) of samples. In 2.5% of cases, consensus was to repeat the sample. The 2 main reasons for repeat were inadequate signal or presence of albumin (Fig. 4B). The identity of the albumin was confirmed by spiking albumin into immunoenriched samples. Subsequently, we learned that by increasing the number of bead washes to 6 total washes (adding 1 wash with phosphate buffered saline and 1 with water), albumin peaks could be eliminated (data not shown). The overall analytical sensitivity and specificity dropped compared to the unblinded results (Table 2). All abnormalities missed by the blinded readers were M-proteins <0.9 g/dL. In examining the cases that were missed, 3 categories were identified. The first category (7 of 513) was Ig κ M-proteins that had *m/z* in the middle of the κ *m/z* distribution (Fig. 4A). These represented the most clinically important misses because this category contained 4 PEL positive samples ranging from 0.3 to 0.9 g/dL. In the second category (7 of 513) are small abnormalities that were difficult to see because of the overlying normal distribution (Fig. 4C). In all these cases, the blinded readers reversed the call upon seeing the expanded spectra, demonstrating the importance of having access to spectra control. The third category of misses (6 of 513) was due to LC clones that were outside the expected κ and λ *m/z* ranges.

Discussion

The data from this cohort indicates that if MASS-SCREEN results were used to triage these samples to PEL for quantification and IFE for isotypying, MASS-SCREEN has the potential to triage 100% (257 of 257) of PEL and IFE positive samples and 91% (149 of 164) of PEL-negative and IFE-positive samples while accurately ruling out an M-protein in 88% (110 of 125) of PEL- and IFE-negative samples. MASS-SCREEN's overall unblinded analytical sensitivity and specificity using IFE as the standard was 96% and 81%, respectively, and equal to or superior to that of other studies comparing the analytical sensitivity of capillary electrophoresis and agarose gels *(12–16)*. Previous data have demonstrated that when PEL is used as a screen, small, unfractionated PEL abnormalities are IFE positive in approximately 30% of cases *(17)*, whereas 83% of blinded MASS-SCREEN–positive/PEL-negative samples had a positive IFE result. As for the blinded MASS-SCREEN analyses, the reduction in analytical sensitivity can be attributed to 2 causes: a lack of experience with the new technology and a limited ability for analysts to expand the mass distribution on the paper copy. As an example of lack of experience, 6 of the IFE-positive/MASS-SCREEN–negative cases had an M-protein LC outside the expected κ and λ mass distribution. The training set used to teach the readers did not contain examples of these types of cases and hence readers were reluctant to call spikes outside the training set mass windows. Also, when the clonal κ LC was in the middle of normal κ distribution, readers had difficultly detecting the spike. For these cases, it may be possible to either calculate the κ/λ ratio by dividing their respective peak areas and reflexing cases substantially higher than those in the normal healthy population (similar to FLC) or to use peak shape parameters to detect the nongaussian LC mass distributions with narrower widths (in *m/z* units) and shifted apexes (Fig. 4A insert) *(18)*.

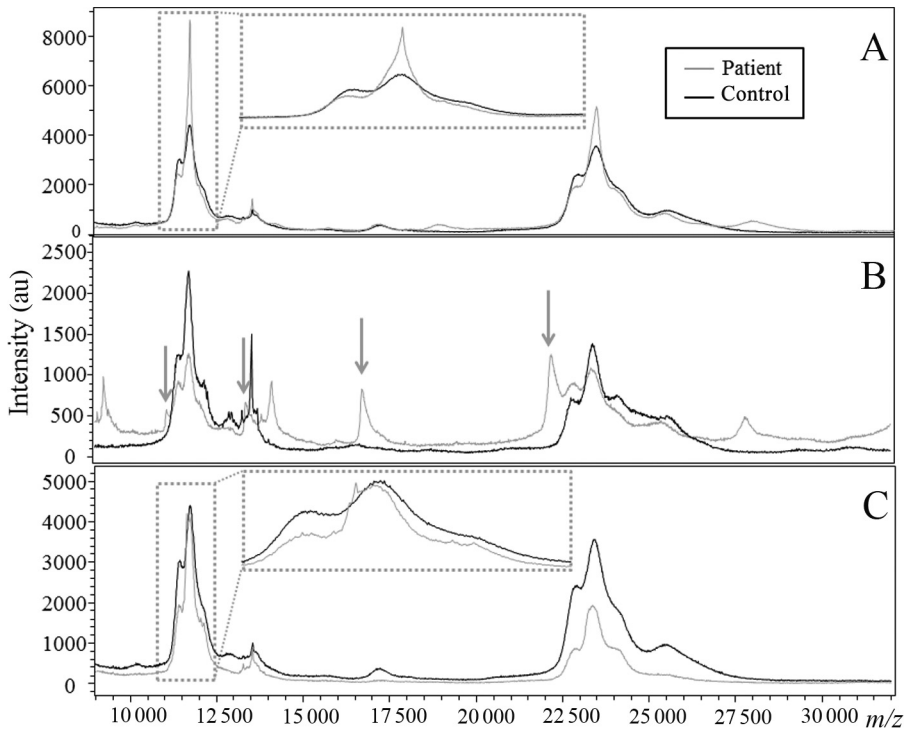

Fig. 4. Examples of difficult-to-interpret patterns encountered in this study.
(A), Mass of an IgGκ M-protein falling in the center of the κ distribution. (B), Albumin interference in the spectrum (arrows). (C), A small abnormality detected only when zooming in on the x-axis of the spectra.

Our choice to use static copies of the spectra was done to standardize the data presentation to the reader. This resulted in instances in which small abnormalities were overlooked or obscured. In the majority of the cases, when presented with an expanded view of the LC $[M+2H]^{2+}$ mass distribution, the abnormalities became more apparent. Regardless, the results of the blinded analysis demonstrate that for a laboratory to adopt MASS-SCREEN, substantial effort will be needed to educate the laboratory personnel on the proper reading of the spectra. Further studies will be needed to determine if analytical sensitivity will improve as the technologists/laboratory directors become more familiar with the technology or software enhancements can be made to aid in detection of abnormalities.

Table 2. Results from blinded MASS-SCREEN analysis compared to PEL and IFE methods.[a]

	MASS-SCREEN positive	MASS-SCREEN negative	Repeat	No consensus
PEL+/IFE+	239	4	6	8
PEL−/IFE+	118	29	4	13
PEL−/IFE−	24	99	4	8

[a] Results were determined based on consensus (at least 4/6 agreement) among 6 blinded analysts.

As is the case with all screening assays, the overall clinical sensitivity of the assay is important. The cases in which unblinded MASS-SCREEN was negative and IFE was positive were the least common disagreement (15 out of 556) and were limited to unfractionated M-spikes. The disagreement was greater in a blinded analysis of MASS-SCREEN, for which 33 out of 390 samples (8.5%) were positive by IFE and negative by MASS-SCREEN. Because disease can be associated with low M-protein concentrations, these missed cases are concerning. Interestingly, these cases were limited to IgG and IgM. Because this method uses a combination of anti-κ and -λ antibodies, the decreased detection of IgM by MASS-SCREEN could be due to the inability to detect these clones when competing with polycloncal LCs from the background IgG, whereas IFE has higher analytical sensitivity for IgM when the polyclonal IgG and IgA are effectively removed during immunofixation.

In the absence of treatment, monoclonal gammopathies typically are persistent over time. In a study looking

at long-term prognosis for monoclonal gammopathies of unknown significance (MGUS), the M-protein persisted in 99.6% of patients *(19)*. However, if the cohort is limited to the majority of discrepancies in this study, (i.e., PEL negative and IFE positive or "IFE-only" MGUS), M-proteins persist in about 70% of patients, with 3.2% eventually progressing to clinically significant disease. On the other hand, 16% of IFE-only MGUS were not found on subsequent testing and had no known intervention to explain the loss of the M-protein *(20)*. These cases could represent immune responses to transient infections or overcalls by IFE readers. In this present study, 15 of our 421 IFE-positive patients were negative by MASS-SCREEN and 25 of our 135 IFE-negative patients were MASS-SCREEN positive. The clinical details for these patients are not available and further clinical studies will be needed to determine the outcome of these types of samples. The fact that 28% of MASS-SCREEN–negative patients had FLC ratios outside of the normal reference range suggests that FLCs are still needed in a screening panel.

MASS-SCREEN offers several benefits over traditional gel methods for screening for PCDs. First, the acquisition time is markedly reduced. The MALDI-TOF-MS spectra were collected at a rate of approximately 96 patients per 20 min. This exceeds the acquisition rate for PEL and IFE significantly. The material cost of screening with MASS-SCREEN is higher than PEL but less than IFE. With automation, labor costs could become substantially less. Therefore, in our large volume setting, MASS-SCREEN is cost competitive, offers improved analytical sensitivity and specificity compared to PEL, and has a limit of detection in the range of IFE, all of which are beneficial for a screening assay. MASS-SCREEN uses an immunoenrichment step to remove non-Ig serum proteins and relies on *m/z* for protein separation, thus making the assay less prone to some of the common analytical interferences that are problematic with PEL, such as fibrinogen or β-migrating proteins. For example, even if the immunoenrichment step fails to remove all of fibrinogen from a plasma sample, the high mass of fibrinogen (approximately 350 kDa) would not interfere with the mass range for Ig LCs. Albumin was one notable exception in which nonspecific adsorption onto the nanobody resin resulted in a peak in the shoulder of the LC mass region. Further development should be focused minimizing albumin contamination in the assay.

MASS-SCREEN has some notable limitations. This screening assay is not designed to detect Heavy Chain disease. To adopt MASS-SCREEN, an Immunology laboratory would need to purchase a MALDI-TOF mass spectrometer or use instruments already in the clinical laboratory. The work performed in the study reported here was done on the same MALDI-TOF mass spectrometer used for bacterial identification in our microbiology laboratory, and institutions that have adopted this technique could easily explore MASS-SCREEN to determine suitability for their practice.

In our clinical practice, after removing patients with a known history of PCDs, approximately 80% of our results are negative for an M-protein. When used as a qualitative screen, MASS SCREEN could reduce the number of samples needing follow-up PEL/IFE testing. A follow-up study of this method in an unbiased cohort not enriched for positive cases and including patients with detailed clinical history is warranted. In addition, the FLC ratio should be included for all samples to evaluate the combination of MASS-SCREEN plus FLC for increased screening sensitivity.

In summary, our study demonstrates the feasibility of using MASS-SCREEN and FLC as a screen for the detection of serum M-proteins, as opposed to screening with PEL and FLC. Further studies and method development will be necessary to determine if the clinical sensitivity and specificity can be improved.

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