



Comprehensive Assessment of M-Proteins Using Nanobody Enrichment Coupled to MALDI-TOF Mass Spectrometry

John R. Mills,¹ Mindy C. Kohlhagen,¹ Surendra Dasari,² Patrick M. Vanderboom,³ Robert A. Kyle,⁴ Jerry A. Katzmann,¹ Maria A.V. Willrich,¹ David R. Barnidge,¹ Angela Dispenzieri,⁴ and David L. Murray^{1*}

BACKGROUND: Electrophoretic separation of serum and urine proteins has played a central role in diagnosing and monitoring plasma cell disorders. Despite limitations in resolution and analytical sensitivity, plus the necessity for adjunct methods, protein gel electrophoresis and immunofixation electrophoresis (IFE) remain front-line tests.

METHODS: We developed a MALDI mass spectrometry-based assay that was simple to perform, automatable, analytically sensitive, and applicable to analyzing the wide variety of monoclonal proteins (M-proteins) encountered clinically. This assay, called MASS-FIX, used the unique molecular mass signatures of the different Ig isotypes in combination with nanobody immunoenrichment to generate information-rich mass spectra from which M-proteins could be identified, isotyped, and quantified. The performance of MASS-FIX was compared to current gel-based electrophoresis assays.

RESULTS: MASS-FIX detected all M-proteins that were detectable by urine or serum protein electrophoresis. In serial dilution studies, MASS-FIX was more analytically sensitive than IFE. For patient samples, MASS-FIX provided the same primary isotype information for 98% of serum M-proteins ($n = 152$) and 95% of urine M-proteins ($n = 55$). MASS-FIX accurately quantified M-protein to <1 g/dL, with reduced bias as compared to protein electrophoresis. Intraassay and interassay CVs were $<20\%$ across all samples having M-protein concentrations >0.045 g/dL, with the ability to detect M-proteins <0.01 g/dL. In addition, MASS-FIX could simultaneously measure $\kappa:\lambda$ light chain ratios for IgG, IgA, and IgM. Retrospective serial monitoring of patients with myeloma posttreatment demonstrated that MASS-

FIX provided equivalent quantitative information to either protein electrophoresis or the Hevylite™ assay.

CONCLUSIONS: MASS-FIX can advance how plasma cell disorders are screened, diagnosed, and monitored.

© 2016 American Association for Clinical Chemistry

Plasma cell disorders (PCDs)⁵ are characterized by expansion of clonal PCs, which results in an overabundance of a monoclonal Ig, referred to as the monoclonal protein (M-protein). The M-protein is a surrogate marker of the PC clone, and as such, the diagnosis and management of PCDs depends on accurate identification, characterization, and quantification of M-proteins. Depending on the clinical indication (screening, monitoring, or assessing treatment response), different combinations of serum and urine protein electrophoresis (SPEP and UPEP), serum free light chain (FLC) assays, Ig quantification, and the Hevylite™ assay are used to detect and measure M-proteins (1–5). The heterogeneity of M-proteins associated with different PCDs and the limitations of each assay necessitates the use of multiple tests.

SPEP is used to screen and quantify M-proteins; however, given its limited analytical sensitivity and inability to differentiate comigrating proteins, additional reflex testing is often necessary (6–8). IFE, in addition to providing isotype information, is analytically more sensitive and specific than SPEP. IFE has a role in detection of minimal residual disease but is hindered by low resolution, variations in interpretation across institutions (9), posttreatment oligoclonal responses (10), and interfer-

¹ Departments of Laboratory Medicine and Pathology and ² Health Sciences Research; ³ Medical Genome Facility-Proteomics Core; and ⁴ Division of Hematology, Department of Medicine, Mayo Clinic, Rochester, MN.

* Address correspondence to this author at: Protein Immunology Laboratory, Division of Cellular and Department of Laboratory Medicine and Pathology, Mayo Clinic, 200 First St. SW, Rochester, MN, 55905. Fax 1-507-284-5000, e-mail murray.david@mayo.edu. Received December 30, 2015; accepted July 18, 2016.

Previously published online at DOI: 10.1373/clinchem.2015.253740

© 2016 American Association for Clinical Chemistry

⁵ Nonstandard abbreviations: PCD, plasma cell disorder; PC, plasma cell; M-protein, monoclonal-protein; SPEP, serum protein electrophoresis; UPEP, urine protein electro-

phoresis; IFE, immunofixation electrophoresis; FLC, free light chain; CE, capillary electrophoresis; IT, immunotyping; MS, mass spectrometry; miRAMM, monoclonal Ig rapid accurate mass measurement; NB, nanobody; LC, light chain; HC, heavy chain; TCEP, [2-carboxyethyl] phosphine; ACHCA, α -cyano-4-hydroxycinnamic acid; ACN, acetonitrile; FWHM, full-width at half-maximum height; AUC, area under the curve; HLC, heavy and light chain; M-spike, monoclonal-spike; NHS, normal human serum; EMR, electronic medical record; ESI-QTOF, electrospray ionization quadrupole TOF; MGUS, monoclonal gammopathies of undetermined significance.

ences from monoclonal therapeutics (11–13). Furthermore, IFE is not quantitative and remains one of the most manual laboratory assays used for identification of PCDs. Capillary electrophoresis (CE) is an alternative to gel-based methods (SPEP, IFE). CE has improved resolution and is more automatable but still encounters difficulties with limited analytical sensitivity and comigrating substances (14, 15). CE-based isotyping is accomplished using immunotyping (IT) techniques, but these methods are analytically less sensitive than IFE (16).

To improve the analytical sensitivity and specificity of detecting M-proteins, mass spectrometry (MS)-based methods are available to measure M-proteins in serum and urine (17–20). This technology, known collectively as monoclonal Ig rapid accurate mass measurement (miRAMM), is based on the fact that each monoclonal Ig has a conserved amino acid sequence and therefore a conserved molecular mass that can be measured with high accuracy and analytical sensitivity using MS.

Here, we report the development of a variant of this technology, called MASS-FIX, which uses isotope-specific nanobody (NB) enrichment coupled to MALDI-TOF MS. This assay is capable of screening, isotyping, measuring Ig class-specific κ : λ light chain (LC) ratios and the quantification of M-proteins. The aims of this feasibility study were to compare MASS-FIX performance with (a) SPEP/IFE for detecting M-proteins; (b) IFE for isotyping; (c) SPEP and the Hevylite assay for M-protein quantification; and (d) SPEP and the Hevylite assay for disease monitoring.

Methods

NANOBODY ENRICHMENT

Each sample (serum or urine) being analyzed underwent 5 unique NB enrichments. Ig enrichment was performed using camelid-derived NBs directed against the heavy chain (HC) constant domains of IgG, IgA, and IgM or the LC constant domains of κ and λ Igs (Thermo Fisher Scientific). Briefly, 10 μ L of beads were incubated with 20 μ L of serum diluted into 180 μ L of PBS for 45 min at ambient temperature. Alternatively, 10 μ L of beads were mixed with 1 mL of urine and 1 mL of 2 \times PBS and incubated at room temperature for 30 min. Subsequently, the supernatant was removed and the beads were washed 3 times with 200 μ L of PBS and then twice with 200 μ L of water. Samples were eluted with 40 μ L of 5% acetic acid containing 50 mmol/L Tris [2-carboxyethyl] phosphine (TCEP), which was sufficient to disassociate Igs into separated LC and HC components.

MALDI-TOF MS

Samples were spotted onto a 96-well microScout polished steel Bruker target plate (Bruker Daltonics) using

the sandwich matrix application method (21). Each spot was first prespotted with 0.6 μ L of the matrix α -cyano-4-hydroxycinnamic acid (ACHCA) (10 g/L) in 0.1% trifluoroacetic acid in a 50:50 acetonitrile (ACN):water solution and allowed to dry. Then, 0.6 μ L of NB eluent (5% acetic acid containing 50 mmol/L TCEP) was applied over the dried matrix. After the sample dried, an additional 0.6 μ L of ACHCA matrix was layered on top of each spot and allowed to dry for at least 5 min under ambient conditions. Mass analysis was performed in positive ion mode with summation of 500 laser shots using a MALDI-TOF mass spectrometer (Bruker Microflex LT). Both LC and HC Ig components were ionized into multiply charged ions, and the m/z distributions of the LC and HC ions were measured. Mass spectra were generated from each NB enrichment (IgG, IgA, IgM, κ , and λ) and overlaid for analysis. Mass spectra were generated corresponding to an m/z range of 9000–32000. Data acquisition for each mass spectrum took <10 s.

MASS SPECTRA INTERPRETATIONS

Peak modeling software was developed to automatically measure the m/z range, center, and peak full-width at half-maximum height (FWHM) of each healthy donor polyclonal LC and HC ion distribution. The software also calculated the area under the curve (AUC) of each LC ion distribution associated with IgG, IgA, or IgM HCs, enabling the heavy and light chain (HLC) pair ratio to be calculated. The use of this software was limited to characterizing normal donors and was not used for detection, isotyping, or quantification of M-proteins.

Detection and isotyping of M-proteins [or monoclonal-spikes (M-spikes)] was performed by visual inspection of the overlaid mass spectra generated for each sample. M-proteins were detected by the presence of abnormal nongaussian peak(s) present in 1 or more of the NB enrichment mass spectra, which were distinct from the normal, healthy polyclonal background. If an abnormal peak was present, the reviewer considered both the m/z of the abnormality as well as which NB enrichment mass spectra the abnormality was restricted to. This information was used to determine if the abnormality corresponded to an M-protein and to determine the isotype of the M-protein. A more detailed description of how isotype assignments were made is available in the Supplemental Methods file that accompanies the online version of article at <http://www.clinchem.org/content/vol62/issue10>.

When an M-protein was detected the HC NB enrichment mass spectra containing the abnormality was used for quantification of the M-protein. Raw mass spectra were loaded into an open-source MS analysis tool (mMass.org). Abnormal peaks corresponding to the monoclonal LC $[M+2H]^{2+}$ ions of the M-protein were selected for quantification. An operator empirically selected the locations for gates flanking the monoclonal LC

ions. Gates were also placed at m/z 11 100 and 12 500 for each sample, which corresponded to all of the LC $[M+2H]^{2+}$ ions (monoclonal and polyclonal) present in the HC-specific NB enrichment. mMass software was then used to calculate the total signal within each of these gates. The fraction of total LC ion $[M+2H]^{2+}$ signal arising from only the monoclonal LC $[M+2H]^{2+}$ ions was then calculated. This fraction was then multiplied by the corresponding HC isotype (total IgG, IgA, or IgM) concentration as measured by nephelometry to generate the M-protein concentration. More detailed descriptions of the MASS-FIX methodology, including peak modeling, interpretation of overlaid isotope specific mass spectra, isotope peak assignment, and quantitative results are provided in the online Supplemental Methods file that accompanies this article.

SPEP AND IFE ANALYSIS

SPEP and UPEP were performed on the SPIFE 3000 electrophoresis analyzer (Helena Laboratories). Serum IFE was performed with Hydrasys 9IF gels (Sebia). Urine IFE was performed with SPIFE IFE-15 gels (Helena Laboratories). Urine samples were concentrated between 10 and 200 times using Vivaspin Concentrators (Vivaproducts) based on the total protein concentration. Total serum protein concentration was measured by a colorimetric assay with biuret reagents on a Siemens Advia 1200 chemistry analyzer. M-spikes on SPEP and UPEP were gated manually and quantification was performed using the Helena QuickScan Touch system to perform the perpendicular drop method. Laboratory-specific procedures for interpretation and quantification are provided in the online Supplemental Methods.

PATIENT COHORTS

Distinct patient samples were divided into 3 cohorts based on their role in assessing assay performance. All patient samples and data were accessed in compliance with the Mayo Clinic Institutional Review Board.

HEALTHY DONORS: COHORT 1

Healthy adult donor control serum samples ($n = 113$) were obtained from the Mayo Clinic donor bank. These samples were used to define the LC and HC m/z distributions in healthy adults and to generate reference mass spectra to compare to those generated from patients with known M-proteins of varying concentration and isotope.

METHOD EVALUATION: COHORT 2

This cohort was selected from serum and urine samples submitted to the Protein Immunology Laboratory at the Mayo Clinic in Rochester, MN, for routine clinical testing that included SPEP/UPEP and IFE. These samples were used to compare the performance of MASS-FIX

compared to clinical gel-based assays. In total, this included 407 unique patient serum samples and 88 unique patient urine samples, which were divided into distinct subcohorts used for analyses of the assay characteristics below.

ANALYTICAL SENSITIVITY

M-protein positive patient serum samples (27 mol/L) (14 IgG, 5 IgA, 4 IgM, and 4 LC-only) were serially diluted (neat, 1:10, 1:100, and 1:200) into pooled normal human serum (NHS) (Cohort 2a). Each sample was tested by IFE and MASS-FIX. Two blinded reviewers who each had multiple years of experience reading IFE gels independently interpreted the raw results (IFE gels and PDFs of mass spectra). The reviewers were presented unlabeled IFE results one by one in a randomized order. On a different day, unlabeled PDFs of overlaid mass spectra were presented to reviewers one by one in a randomized order. No clinical information or prior testing results were provided to reviewers.

DIAGNOSTIC SENSITIVITY

Serum samples from 182 unique patients and urine samples from 88 unique patients with available physician-ordered SPEP/UPEP and IFE results as part of routine clinical practice were measured by MASS-FIX (Cohort 2b). SPEP/UPEP and IFE results were retrieved from the electronic medical record (EMR). MASS-FIX results (PDFs of overlaid mass spectra) were independently reviewed by 4 blinded analysts who each had >1 year of experience reading SPEP and IFE gels. Reviewers were asked to make a positive or negative call and, if positive, to identify the isotope. All serum MASS-FIX spectra were provided to each reviewer at once in a randomized order. Once all reviewers were finished with their reviews (approximately 2 days) all reviewer calls were compiled into a master data file that was then provided to each reviewer. A call was made when consensus was achieved, defined as at least 3 of 4 reviewers being in agreement. IFE was considered the gold standard as a surrogate diagnosis. Urine samples were analyzed in the same way after serum studies were completed. Of note, urine specimens were not concentrated before MASS-FIX analysis. In contrast to results pulled from the EMR, neither prior laboratory results nor clinical histories were provided to reviewers during analysis of MASS-FIX results.

M-PROTEIN QUANTIFICATION: LINEARITY

Fourteen patient serum samples with known M-proteins (9 IgG, 3 IgA, and 2 IgM; range 0.5–8 g/dL), each with varying amounts of polyclonal background, were diluted into NHS (neat, 1:2, 1:10, 1:20, 1:100, and 1:200) and quantified by both SPEP and MASS-FIX (6 samples per patient; 84 total samples) (Cohort 2c). SPEP quantification was performed in accordance with routine clinical

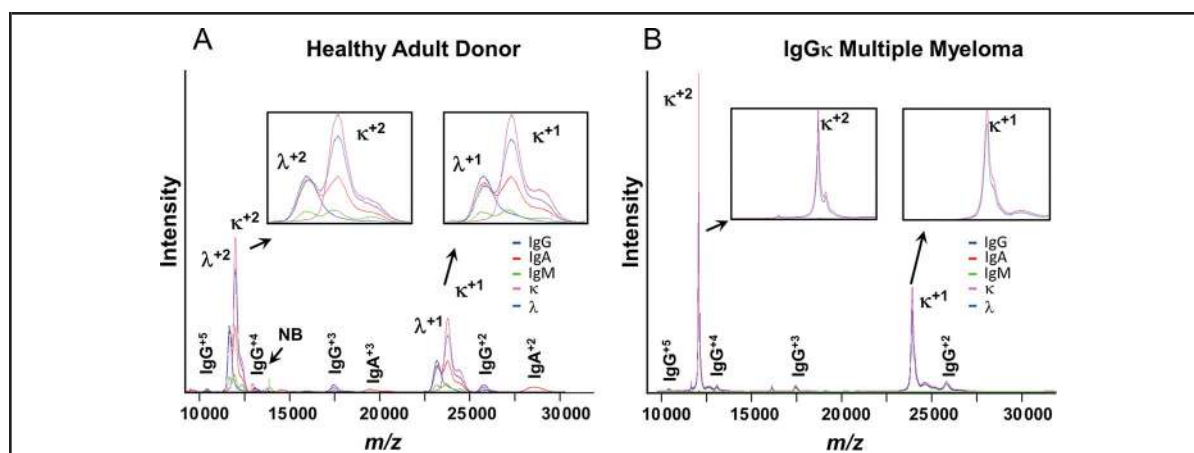


Fig. 1. (A), Mass spectra from a healthy donor.

Polyclonal HC and LC ions are labelled with isotype and charge. Each NB-enriched mass spectrum is a different color. NB ions are labelled "NB." The insets are focused on the m/z regions containing LC ions. (B), Mass spectra from a patient with an IgG κ M-protein. The y axis scale is relative intensity.

practice. Laboratory staff was blinded to the purpose of this study. For MASS-FIX, a single operator who was blinded to SPEP results performed gating and quantification with Mass software using the perpendicular drop method of gating and quantification. Each sample was gated independently.

M-PROTEIN QUANTIFICATION: METHOD COMPARISON

175 patient serum samples with previously quantified M-proteins (range 0.2–6.2 g/dL) of known isotype (62 IgG, 36 IgA, and 77 IgM) were pulled from the Mayo Clinic Dysproteinemia Biobank (Cohort 2d). Each sample was quantified by MASS-FIX. A single blinded operator performed gating and quantification. Nephelometry results used for M-protein concentration calculations were pulled from the EMR. Passing-Bablok analysis was used to compare MASS-FIX to SPEP.

M-PROTEIN QUANTIFICATION: IMPRECISION

To assess interassay imprecision, a set of 5 patient serum samples with quantitated M-proteins (2 IgG, 2 IgA, and 1 IgM) were diluted with NHS to 25 different concentrations (range 0.009–3.3 g/dL) as measured by MASS-FIX (Cohort 2e). The samples were quantified in duplicate over 10 days (a total of 20 measurements per sample). To assess intraassay imprecision, a set of 4 patients (2 IgG, 1 IgA, and 1 IgM) were each diluted with NHS to 2 different concentrations (0.04–2.3 g/dL). Twenty replicates of each sample were included per run. For these studies, each mass spectrum was gated independently by operators who were not blinded to the dilutions being analyzed.

DISEASE MONITORING IN MYELOMA PATIENTS: COHORT 3

For evaluation of diagnostic sensitivity and disease monitoring, 112 unique serum samples were identified in the Mayo Dysproteinemia Biobank for 40 unique IgG and IgA myeloma patients that had available SPEP, IFE, and Hevlyte results. Clinical history and laboratory results were used to confirm the diagnosis of myeloma. MASS-FIX detection and isotyping was performed on all samples. A subset of 15 of these patients with diagnostic plus 4 posttreatment samples available were quantified by MASS-FIX. For these studies, MASS-FIX mass spectra were visually inspected, gated, and quantified by a single reviewer who was blinded to prior laboratory results.

Results

MASS-FIX couples multiple-bead immunoenrichment and MALDI-TOF MS to detect, isotype, and quantify M-proteins (see online Supplemental Fig. 1). Characteristic overlaid mass spectra of each NB enrichment from a healthy donor serum are shown in Fig. 1A. The inset shows the overlapping gaussian m/z distributions of ions corresponding to the λ and κ polyclonal LCs. The κ LC ions also contained a less-abundant polyclonal population of similarly charged κ LC ions of heavier mass (22), consistent with earlier work using electrospray ionization–quadrupole TOF (ESI-QTOF) (23). Polyclonal LC ions (κ or λ) were identified in the mass spectra generated from LC-specific NBs (total LCs) and from HC-specific NB enrichments (LCs associated with HCs). Polyclonal IgG and IgA HC ions are identifiable

in the mass spectra generated with HC-specific NBs (total HCs) and LC-specific NB enrichments (HCs associated with LCs). IgM HC ions were not consistently detected by this method (see Discussion).

MASS-FIX ANALYSIS OF HEALTHY DONORS: COHORT 1

To characterize reference polyclonal HC and LC m/z distributions, a cohort of 113 healthy donors were analyzed by MASS-FIX. Peak modeling software was used to define the range, center, and FWHM of each polyclonal m/z LC distribution for each NB enrichment (see online Supplemental Tables 1 and 2). A representative result of peak fitting is shown for a normal donor (see online Supplemental Fig. 2). Normal reference LC m/z signals were fitted with gaussian distributions with minimal error. The software was also used to calculate the AUC of each LC m/z population (κ and λ) from mass spectra generated using HC-specific NB enrichment. This data was then used to calculate κ to λ LC ratios for each HC. The 95th percentile reference intervals for each ratio (IgG κ :IgG λ , IgA κ :IgA λ , and IgM κ :IgM λ) were calculated using the robust method (24) and compared to those reported for the Hevylite assay (6) (see online Supplemental Fig. 3). To visually demonstrate the interindividual variability of the polyclonal LC and HC m/z distributions in the healthy adult population, mass spectra from 40 healthy donors were overlaid (see online Supplemental Fig. 4, A–E). For all subsequent cohort analysis and characterization of MASS-FIX, electronic PDFs of the raw overlaid mass spectra were interpreted visually by reviewers without the use of peak modeling software. The peak statistics from the normal cohort served as reference for reviewer interpretation.

MASS-FIX PERFORMANCE EVALUATION: COHORT 2

Samples with M-proteins had qualitative features that can be visually distinguished from the mass spectra from the healthy adult population (Fig. 1B). These include nongaussian m/z distributions of LC and HC ions that were leptokurtic, with narrower widths and shifted apexes as compared to normal donors. These changes were restricted to the mass spectra corresponding to the isotype of the M-protein. In Fig. 1B, the mass spectra from the IgG HC and κ LC NB enrichments have m/z distributions that are nongaussian and appear as spikes illustrative of an IgG κ M-protein and reminiscent of M-spikes, as found by SPEP. Currently, M-proteins are more readily detected by their monoclonal LC as compared to their HC due to lack of glycosylation of LCs and their ability to ionize more readily (18). Representative examples of mass spectra corresponding to various commonly encountered M-protein isotypes are provided in online Supplemental Fig. 5, A–L.

ANALYTICAL SENSITIVITY

The analytical sensitivity of M-protein detection for MASS-FIX, IFE, and SPEP depend on the polyclonal background intensity and relative position of M-protein in the polyclonal distribution. Therefore, a variety of distinct M-protein-positive patient samples were used to assess analytical sensitivity. Because IFE is considered the most analytically sensitive gel-based method for detecting M-proteins, MASS-FIX was compared to IFE. Serum samples ($n = 27$) with M-proteins detectable by IFE (Cohort 2a) were serially diluted into NHS. These samples were then analyzed by both IFE and MASS-FIX. MASS-FIX outperformed IFE by identifying an M-protein in a higher percentage of samples at every dilution (Fig. 2A). An example is shown in Fig. 2B, in which an IgM κ M-protein was detected by reviewers in the 1:200 dilution by MASS-FIX but not IFE.

DIAGNOSTIC SENSITIVITY (DETECTION AND ISOTYPING)

The ability of MASS-FIX to detect M-proteins was evaluated on a large cohort of patient samples with physician-ordered SPEP and IFE, which included a wide variety of M-proteins encountered in our clinical practice (Cohort 2b; see online Supplemental Table 3). MASS-FIX detected M-proteins in 100% of samples that were positive by both SPEP and IFE ($n = 84$) and in 97% of samples positive by IFE but negative by SPEP (68 of 70; Fig. 3A) with 100% consensus among blinded reviewers. In samples that were negative by both SPEP and IFE ($n = 28$), MASS-FIX identified 1 positive case. Similarly, MASS-FIX detected 100% of urine samples that were positive by both UPEP and IFE (29 of 29) and 90% of urine samples that were negative by UPEP but positive by IFE (26 of 29, Fig. 4A) with blinded reviewers unable to reach consensus for 1 sample. In urine samples that were negative by both UPEP and IFE ($n = 30$), MASS-FIX identified 2 additional positive cases, and for 2 cases, blinded reviewers did not reach consensus (2 of 4 reviewers detected an M-protein).

In this same cohort, the MASS-FIX isotype as assigned by 4 blinded reviewers was compared to IFE calls reported in the EMR. For the 152 MASS-FIX and IFE positive samples, reviewer consensus was reached for 151 of 152 samples. Isotype concordance with IFE for these samples was 91% (138 of 151; Fig. 3B). In 11 of the 13 discordant cases, MASS-FIX and IFE agreed on the major M-protein isotype, but there was disagreement on the presence/isotype of additional less-abundant clones. Only 2 cases were absolute isotyping discrepancies—thus, overall consensus agreement of the primary isotype call was 98% (149 of 152). A similar analysis using 55 urine samples was performed. The LC isotype identified by each assay agreed in 95% of cases (52 of 55; Fig. 3C).

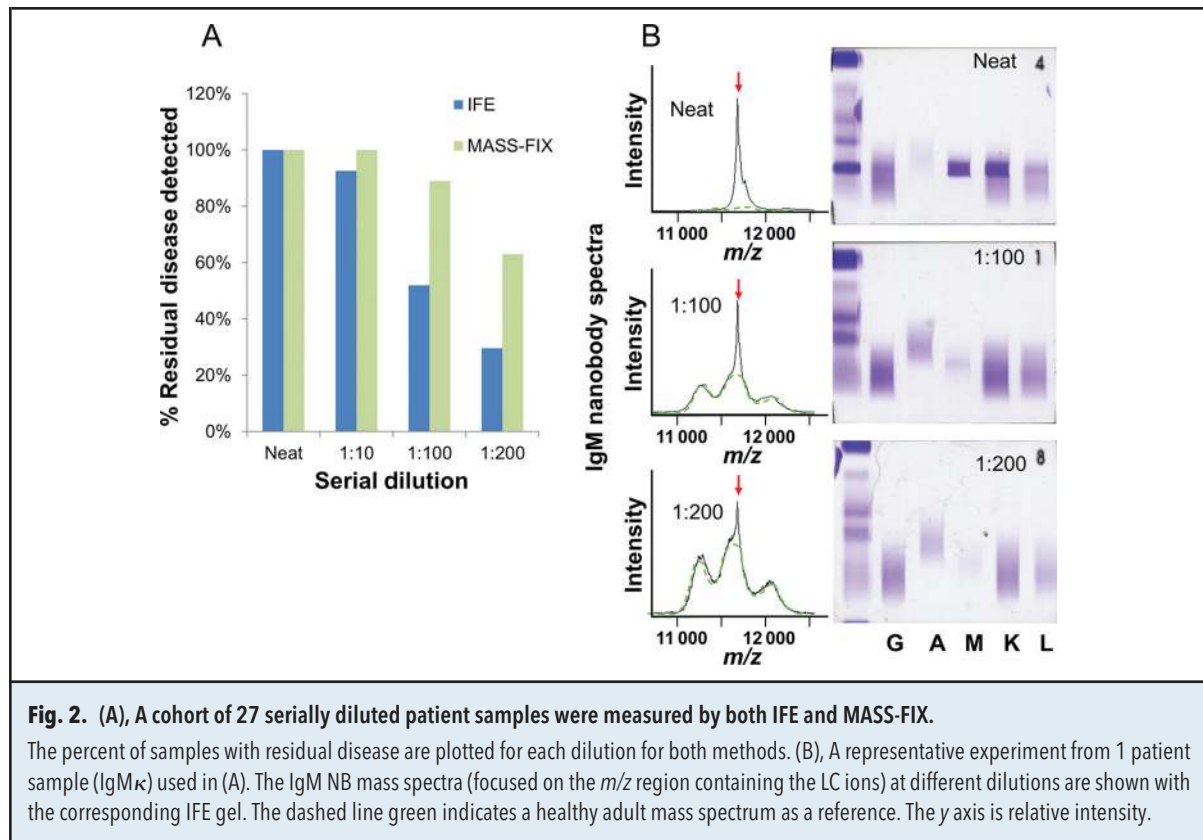


Fig. 2. (A), A cohort of 27 serially diluted patient samples were measured by both IFE and MASS-FIX.

The percent of samples with residual disease are plotted for each dilution for both methods. (B), A representative experiment from 1 patient sample (IgM κ) used in (A). The IgM NB mass spectra (focused on the m/z region containing the LC ions) at different dilutions are shown with the corresponding IFE gel. The dashed line green indicates a healthy adult mass spectrum as a reference. The y axis is relative intensity.

M-PROTEIN QUANTIFICATION: LINEARITY

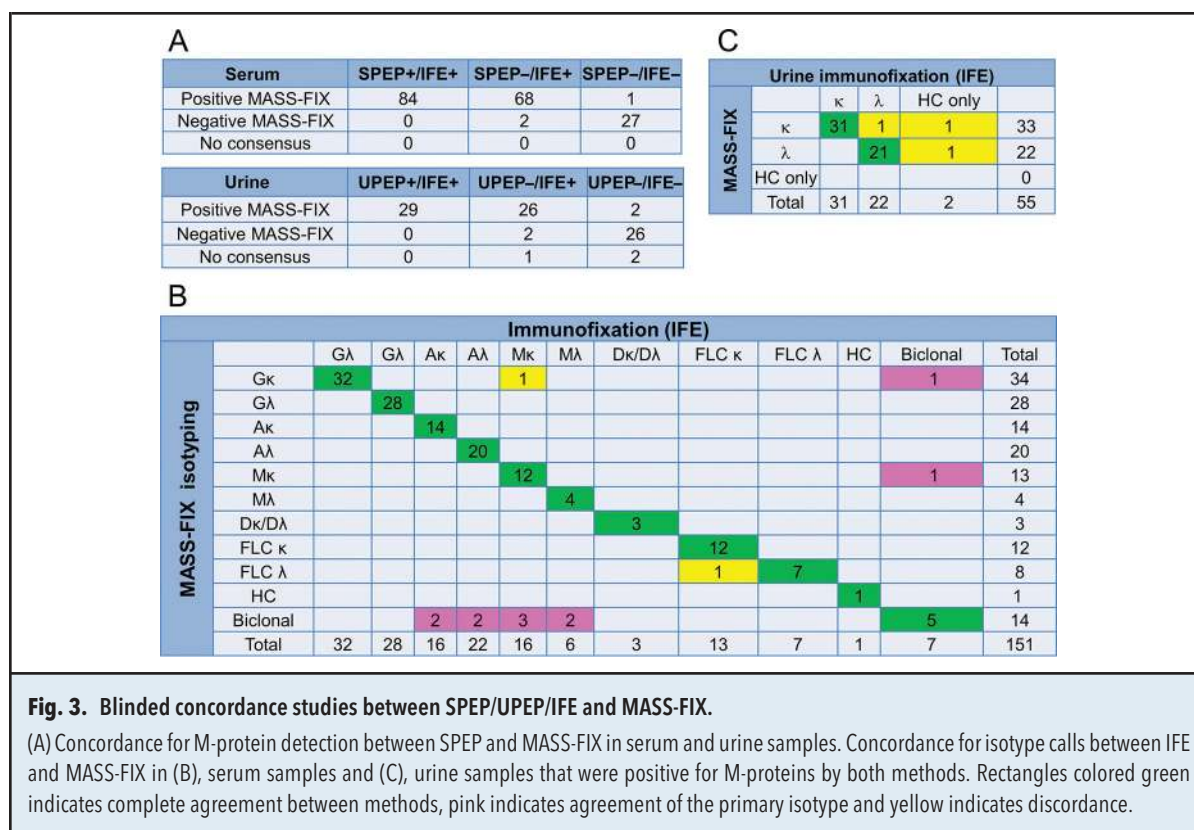
M-proteins were quantified by MASS-FIX as shown in Fig. 4A and described further in the online Supplemental Methods. To evaluate the dilutional linearity, 14 patients (Cohort 2c) were serially diluted into NHS (6 dilutions per patient; 84 total samples) and quantified by both SPEP and MASS-FIX. Nonlinearity analysis using the Hsieh-Lui method (25) confirmed the absence of statistically significant nonlinear response for MASS-FIX quantification across the range of measured concentrations (lack of fit; $F = 0.15$, P value = 1.000). Evaluating only dilutions with SPEP-quantifiable M-proteins (53 of 84), as determined by blinded laboratory staff following standard laboratory practice, the measured M-protein concentration for both SPEP and MASS-FIX were in excellent agreement with expected concentrations ($R^2 = 0.99$; Fig. 4B). However, difference plots indicated that SPEP overestimated the M-protein concentration when they were <1 g/dL (see Fig. 4B and online Supplemental Fig. 6). Unlike SPEP, MASS-FIX could quantify M-proteins in all serial dilutions, which included those that were expected to be <0.01 g/dL. Importantly, MASS-FIX quantification was linear down to at least 0.02 g/dL in all samples (see online Supplemental Fig. 7).

QUANTIFICATION: METHOD COMPARISON

To compare quantitative results generated by MASS-FIX to SPEP, a cohort of patient serum samples (Cohort 2d) with SPEP M-protein concentrations documented in the Mayo Clinic Dysproteinemia biobank were quantified by MASS-FIX. Quantification by SPEP and MASS-FIX agreed well for IgG and IgA M-proteins (IgG slope = 1.27 and $r^2 = 0.94$; IgA slope = 0.91 and $r^2 = 0.84$), but for IgM M-proteins at concentrations >3 g/dL, MASS-FIX resulted in higher M-protein concentrations in comparison with SPEP (see Fig. 5 and online Supplemental Table 4). For IgM M-proteins, there was both random error and systematic bias [slope = 1.50 95% CI, 1.35–1.62], intercept = -1.15 (95% CI, -1.45 to -0.81) and $r^2 = 0.84$].

QUANTIFICATION: METHOD IMPRECISION

Interassay imprecision was assessed using 5 patient samples with known M-protein concentrations (Cohort 2e), each diluted to 5 different concentrations with NHS. MASS-FIX quantification was performed over 10 days, with duplicate measurements on each sample (20 measurements per sample). CVs were calculated for the M-protein concentration (CV_{Total}) as well as for nephelometry ($CV_{\text{Nephelometry}}$) and the percentage of the



monoclonal LC signal measured by MASS-FIX (CV_{MALDI}) (see online Supplemental Table 5). The CV_{Total} was $<20\%$ in all samples with M-protein concentrations >0.05 g/dL. Of 14 samples evaluated with M-protein concentrations <0.1 g/dL, 12 of 14 had a $CV_{Total} <20\%$. Within-run precision was performed using 8 different samples of varying concentration and isotype. Twenty replicates were included per run, and the CV_{Total} was $<20\%$ for all samples tested (see online Supplemental Table 6).

DISEASE MONITORING IN MYELOMA PATIENTS: COHORT 3

For evaluation of diagnostic sensitivity and disease monitoring, 112 frozen sera were identified in the Mayo Dysproteinemia Biobank for 40 unique IgG and IgA myeloma patients that had SPEP, IFE, and Hevylite results available for every sample. In this cohort, 70% of samples were positive by SPEP, 75% were positive by the Hevylite assay, and 90% were positive by IFE (defined as the gold standard). 96% of these samples were called positive by MASS-FIX with isotypes that matched the original diagnosis implying that MASS-FIX has adequate sensitivity to detect residual disease. A subset of 15 of these patients had diagnostic plus 4 posttreatment samples available, which subsequently were quantified by MASS-FIX.

The relative percent change in M-protein concentration from the diagnostic sample was plotted over serial collections (Fig. 6) for each method. Pearson correlation coefficients of the time-series data indicated that the MASS-FIX results were in agreement with SPEP and/or the Hevylite assay (see online Supplemental Table 7). When discrepancies existed (see Fig. 6; patient nos. 1, 7–10), MASS-FIX was not the outlying assay. In 2 instances, both MASS-FIX and the Hevylite assay indicated a relapse of disease that was not yet detectable by SPEP (Fig. 6; patient nos. 7 and 8). In the first instance, patient no. 7 was noted to have progressive disease between visits 4 and 5 while receiving melphalan and prednisone. SPEP detected the M-protein 1 month after visit 5. In the second instance, patient no. 8 had a β -migrating IgA M-protein and received single-agent dexamethasone at visit 4 due to increasing disease burden detected by an increase in the total IgA concentration (175–575 mg/dL). By visit 5 (approximately 7 months later), the M-protein was detectable by SPEP and, due to progressive disease therapy, was switched to melphalan, prednisone, and thalidomide. Therefore, in these 2 cases, MASS-FIX demonstrated a quantitative increase in disease burden that proved to be clinically relevant earlier than SPEP.

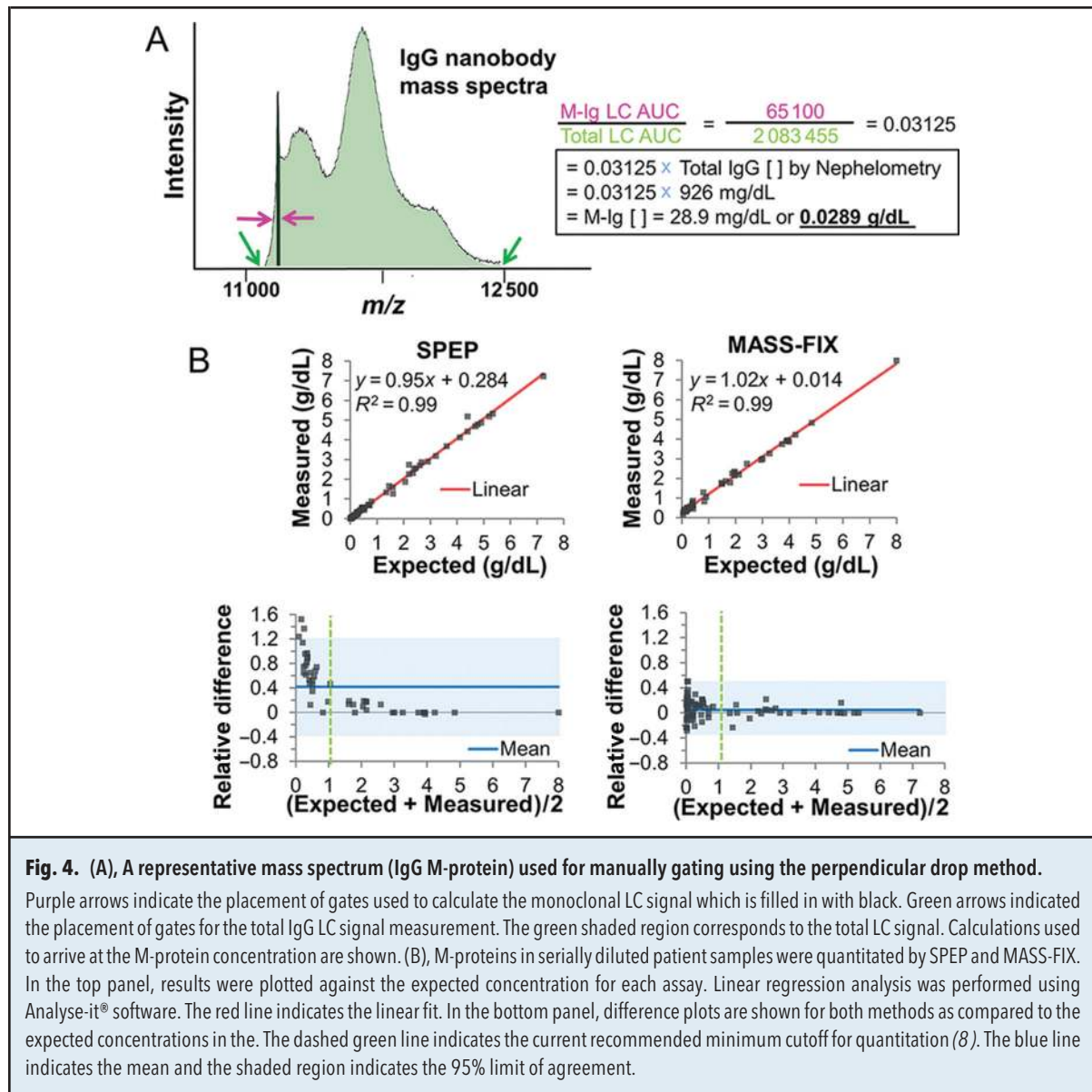


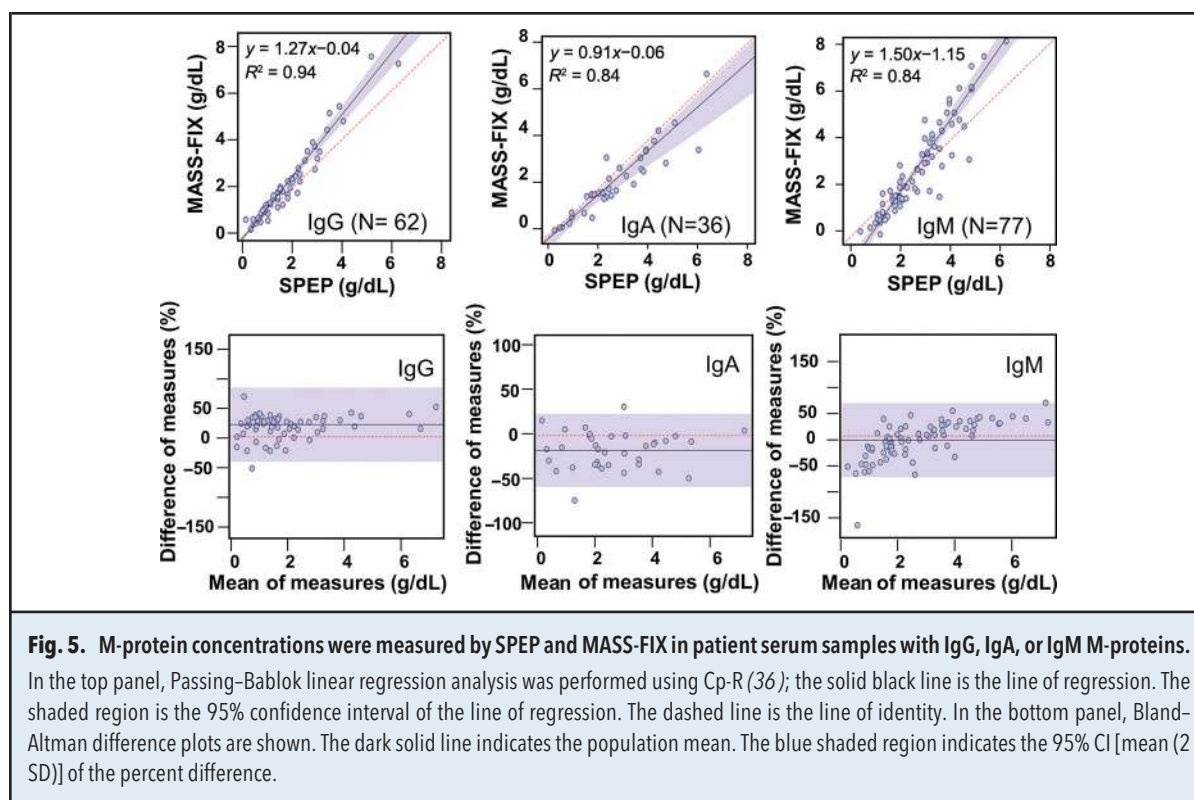
Fig. 4. (A), A representative mass spectrum (IgG M-protein) used for manually gating using the perpendicular drop method.

Purple arrows indicate the placement of gates used to calculate the monoclonal LC signal which is filled in with black. Green arrows indicated the placement of gates for the total IgG LC signal measurement. The green shaded region corresponds to the total LC signal. Calculations used to arrive at the M-protein concentration are shown. (B), M-proteins in serially diluted patient samples were quantitated by SPEP and MASS-FIX. In the top panel, results were plotted against the expected concentration for each assay. Linear regression analysis was performed using Analyse-it® software. The red line indicates the linear fit. In the bottom panel, difference plots are shown for both methods as compared to the expected concentrations in the. The dashed green line indicates the current recommended minimum cutoff for quantitation (8). The blue line indicates the mean and the shaded region indicates the 95% limit of agreement.

Discussion

Electrophoretic separation of serum and urine proteins remains the standard approach for screening, diagnosis, and monitoring of PCDs. However, limitations in resolution and analytical sensitivity require the use of supplemental testing. This can lead to increased cost, increased testing complexity, and poorer test utilization. This study demonstrated that MASS-FIX has the potential to be more informative than current methodologies providing M-protein detection, isotype, HLC ratios, and quantitative information with equivocal or improved analytical characteristics.

As a screening assay, MASS-FIX identified an M-protein in every case that was positive by SPEP or UPEP. In samples in which M-proteins were only detectable by IFE, MASS-FIX identified 97% of serum M-proteins and 90% of urine M-proteins. MASS-FIX did not detect an M-protein in 2 serum samples out of 154 (1%) that were positive by IFE. In 1 case (IgM λ), there was a detectable peak in the LC m/z region in the IgM mass spectrum, but it was absent from the corresponding λ -NB mass spectrum. The second discrepant case was a small monoclonal λ . In 1 case, an M-protein was detected by MASS-FIX but not by IFE; however, additional samples were not available for follow-up stud-



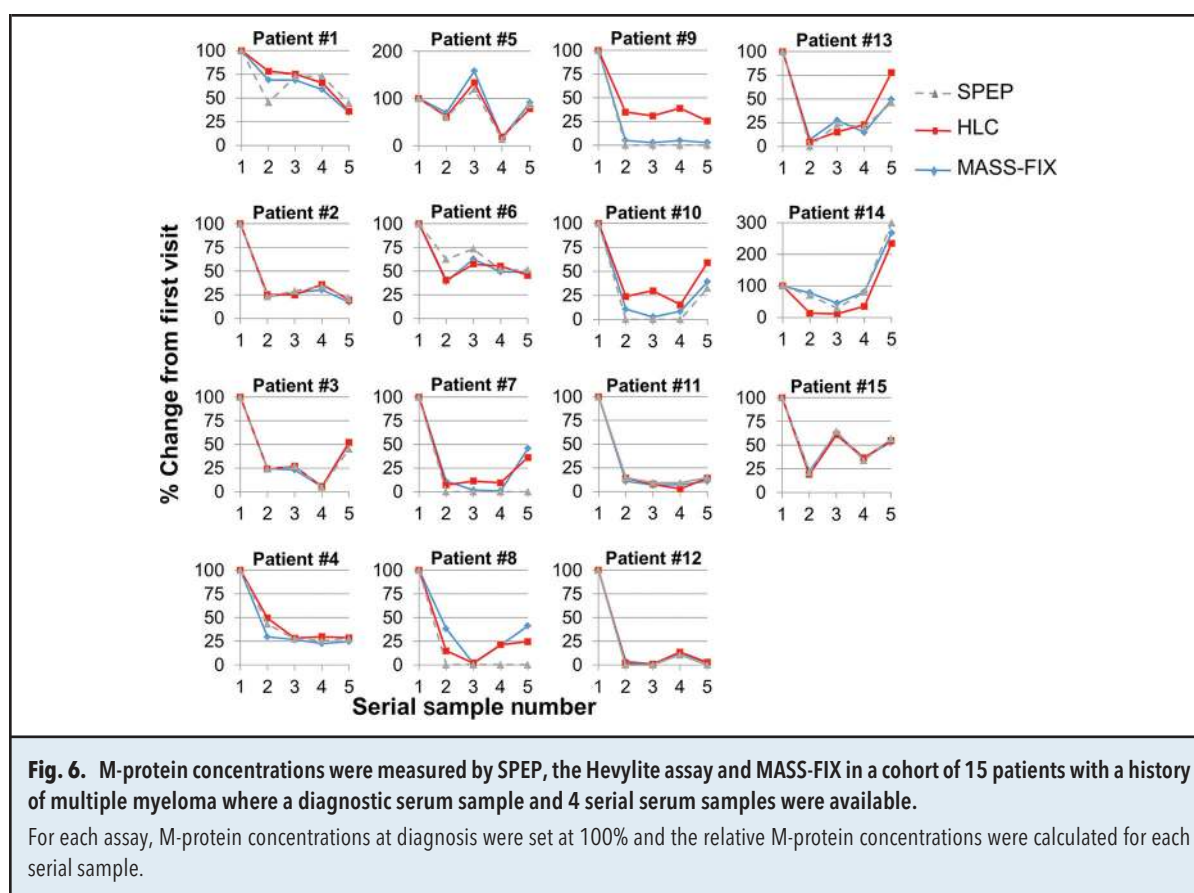
ies to determine which method better reflected the clinical picture.

Protein electrophoresis coupled with measurement of total protein has traditionally been used to measure M-protein concentrations. However, these approaches can be inaccurate due to dye-saturation, failure to exclude the polyclonal background from the measurement and the presence of comigrating non-Ig proteins (26, 27). The most commonly applied method to calculate the M-protein concentration is to measure the contribution of the M-protein to the total protein concentration using the perpendicular drop method (28). This approach can be imprecise and inaccurate when the M-protein is <1 g/dL due to the poor resolving power of electrophoresis and the increasing proportion of the measurement being derived from the polyclonal background (30). In contrast to electrophoretic methods, MASS-FIX quantification first fractionates the Igs by NB enrichment and subsequently isolates the monoclonal portion using the higher resolution of MS, eliminating more of the polyclonal background from the measurement, thus improving the accuracy of the perpendicular drop method at lower M-protein concentrations. An alternative to the perpendicular drop method is the tangent skimming method, which reduces the amount of the polyclonal background in the measurement, thereby

improving the accuracy of quantification of M-proteins <1 g/dL (28, 29). Incorporating the principles of the tangent skimming method into MASS-FIX could further improve the analytical performance of this technology.

A limitation of current electrophoretic-based quantitative assays is that they cannot reliably quantify M-proteins migrating outside of the γ -region. Importantly, MASS-FIX is not impacted by the migration pattern of the M-protein and thus does not require additional reflex testing for β -migrating IgA M-proteins (6). IgA M-proteins are found in approximately 11% of monoclonal gammopathies of undetermined significance (MGUS) and approximately 20% of cases of myeloma of which >40% migrate within the β -region suggesting that approximately 5%–10% of all M-proteins may require reflex testing (30, 31).

Evaluation of the analytical sensitivity of MASS-FIX indicates that MASS-FIX may have a greater analytical sensitivity than SPEP, IFE, and the Hevlyte assay. MASS-FIX has the ability to quantitatively track concentrations of M-proteins at lower concentrations than SPEP and may aid in studies aimed at better understanding the implications of obtaining deeper treatment responses with novel therapies (32). Furthermore, MASS-



FIX quantification provided equivalent results to the other methodologies for monitoring disease.

The MASS-FIX method described here has some limitations. MASS-FIX quantification uses nephelometric measurements, which overestimate IgM M-proteins due to their pentameric structure (33). Consistent with this phenomenon, in method comparison studies, a slope of 1.5 between SPEP and MASS-FIX IgM quantification was noted, consistent with previous studies comparing SPEP and nephelometric IgM quantification of M-proteins (7). In addition, nephelometry can be prone to hook effects caused by antigen excess, although it is likely a false negative result due to antigen excess, which would be spotted by MASS-FIX due to discordance between the mass spectra and the nephelometric measurement (34, 35). Quantification in this study was limited to intact M-proteins. However, it is conceivable that total serum κ and λ nephelometry quantification could be used in a similar manner as IgG, IgA, and IgM to quantify LC-only M-proteins, although this study did not evaluate this. Quantification of M-proteins using this methodology assumes that each LC will have equivalent ionization efficiency relative to the polyclonal background, which could contribute error to the measurement.

In the MASS-FIX imprecision studies, it was noted that for M-proteins <0.045 g/dL the CVs are $>20\%$ for some M-proteins, particularly IgG M-proteins. This likely relates to a combination of the limitations of the perpendicular drop method, operator variability, and the presence of residual polyclonal background, which is more abundant for IgG relative to IgA and IgM. Also, whereas IgD M-proteins can be detected by the presence of the IgD HC m/z signature; the ability of MASS-FIX to detect IgE HCs has not been fully evaluated. This limitation can be overcome by either triaging all new cases of LC-only disease to IFE for IgE analysis or using an IgE-specific NB. Lastly, IgM HCs are heterogeneously glycosylated and therefore are poorly resolved by this method. Therefore, while IgM M-proteins are readily identifiable by their monoclonal LC m/z signal after enrichment with IgM HC-specific NBs, detecting the monoclonal IgM HC m/z signal requires further optimization. Lastly, to date, a comparison of the analytical sensitivity of FLC and MASS-FIX assays has not been performed.

Author Contributions: All authors confirmed they have contributed to the intellectual content of this paper and have met the following 3 requirements: (a) significant contributions to the conception and design, acquisi-

tion of data, or analysis and interpretation of data; (b) drafting or revising the article for intellectual content; and (c) final approval of the published article.

Authors' Disclosures or Potential Conflicts of Interest: Upon manuscript submission, all authors completed the author disclosure form. Disclosures and/or potential conflicts of interest:

Employment or Leadership: None declared.

Consultant or Advisory Role: None declared.

Stock Ownership: None declared.

Honoraria: None declared.

Research Funding: None declared.

Expert Testimony: None declared.

Patents: J.R. Mills, patent no. PCT/US2015/24379; S. Dasari, patent no. PCT/US2015/24379; D.R. Barnidge, US provisional application no. 61/975,524; D.L. Murray, patent no. PCT/US2014/022475.

Role of Sponsor: No sponsor was declared.

References

- Katzmann JA, Kyle RA, Benson J, Larson DR, Snyder MR, Lust JA, et al. Screening panels for detection of monoclonal gammopathies. *Clin Chem* 2009;55:1517-22.
- Dispenzieri A, Kyle R, Merlini G, Miguel JS, Ludwig H, Hajek R, et al. International myeloma working group guidelines for serum-free light chain analysis in multiple myeloma and related disorders. *Leukemia* 2009;23:215-24.
- Kraj M. Immunoglobulin heavy chain/light chain pairs (HLC, Hevlyte™) assays for diagnosing and monitoring monoclonal gammopathies. *Adv Clin Exp Med* 2014;23:127-33.
- Palumbo A, Rajkumar SV, San Miguel JF, Larocca A, Niesvizky R, Morgan G, et al. International Myeloma Working Group consensus statement for the management, treatment, and supportive care of patients with myeloma not eligible for standard autologous stem-cell transplantation. *J Clin Oncol* 2014;32:587-600.
- Kyle RA, Durie BG, Rajkumar SV, Landgren O, Blade J, Merlini G, et al. Monoclonal gammopathy of undetermined significance (MGUS) and smoldering (asymptomatic) multiple myeloma: IMWG consensus perspectives risk factors for progression and guidelines for monitoring and management. *Leukemia* 2010;24:1121-7.
- Katzmann JA, Willrich MA, Kohlhagen MC, Kyle RA, Murray DL, Snyder MR, et al. Monitoring IgA multiple myeloma: immunoglobulin heavy/light chain assays. *Clin Chem* 2015;61:360-7.
- Murray DL, Ryu E, Snyder MR, Katzmann JA. Quantitation of serum monoclonal proteins: relationship between agarose gel electrophoresis and immunonephelometry. *Clin Chem* 2009;55:1523-9.
- Durie BG, Harousseau JL, Miguel JS, Blade J, Barlogie B, Anderson K, et al. International uniform response criteria for multiple myeloma. *Leukemia* 2006;20:1467-73.
- Caillon H, Dejoie T, Le Loupp AG, Azoulay-Fauconnier C, Masson D, Moreau P, et al. Difficulties in immunofixation analysis: a concordance study on the IFM 2007-02 trial. *Blood Cancer J* 2013;3:e154.
- Fernandez de Larrea C, Tovar N, Cibeira MT, Arostegui JI, Rosinol L, Elena M, et al. Emergence of oligoclonal bands in patients with multiple myeloma in complete remission after induction chemotherapy: association with the use of novel agents. *Haematologica* 2011;96:171-3.
- McCudden CR, Voorhees PM, Hainsworth SA, Whinna HC, Chapman JF, Hammett-Stabler CA, Willis MS. Interference of monoclonal antibody therapies with serum protein electrophoresis tests. *Clin Chem* 2010;56:1897-9.
- Laubach JP, Tai YT, Richardson PG, Anderson KC. Daratumumab granted breakthrough drug status. *Expert Opin Investig Drugs* 2014;23:445-52.
- Lonial S, Dimopoulos M, Palumbo A, White D, Grosicki S, Spicka I, et al. Elotuzumab therapy for relapsed or refractory multiple myeloma. *N Engl J Med* 2015;373:621-31.
- Litwin CM, Anderson SK, Philipps G, Martins TB, Jaskowski TD, Hill HR. Comparison of capillary zone and immunosubtraction with agarose gel and immunofixation electrophoresis for detecting and identifying monoclonal gammopathies. *Am J Clin Pathol* 1999;112:411-7.
- Bossuyt X, Marien G. False-negative results in detection of monoclonal proteins by capillary zone electrophoresis: a prospective study. *Clin Chem* 2001;47:1477-9.
- McCudden CR, Mathews SP, Hainsworth SA, Chapman JF, Hammett-Stabler CA, Willis MS, Grenache DG. Performance comparison of capillary and agarose gel electrophoresis for the identification and characterization of monoclonal immunoglobulins. *Am J Clin Pathol* 2008;129:451-8.
- Botz CM, Barnidge DR, Murray DL, Katzmann JA. Detecting monoclonal light chains in urine: microLC-ESI-Q-TOF mass spectrometry compared to immunofixation electrophoresis. *Br J Haematol* 2014;167:437-8.
- Barnidge DR, Dasari S, Botz CM, Murray DH, Snyder MR, Katzmann JA, et al. Using mass spectrometry to monitor monoclonal immunoglobulins in patients with a monoclonal gammopathy. *J Proteome Res* 2014;13:1419-27.
- Mills JR, Barnidge DR, Murray DL. Detecting monoclonal immunoglobulins in human serum using mass spectrometry. *Methods* 2015;81:56-65.
- Barnidge DR, Kirck TP, Griffin TJ, Murray DL. Using matrix-assisted laser desorption/ionization time-of-flight mass spectrometry to detect monoclonal immunoglobulin light chains in serum and urine. *Rapid Commun Mass Spectrom* 2015;29:2057-60.
- Kussmann M, Roepstorff P. Sample preparation techniques for peptides and proteins analyzed by MALDI-MS. *Methods Mol Biol* 2000;146:405-24.
- Barnidge DR, Lundstrom SL, Zhang B, Dasari S, Murray DL, Zubarev RA. Subset of kappa and lambda germline sequences result in light chains with a higher molecular mass phenotype. *J Proteome Res* 2015;14:5283-90.
- Barnidge DR, Dasari S, Ramirez-Alvarado M, Fontan A, Willrich MA, Tschumper RC, et al. Phenotyping polyclonal kappa and lambda light chain molecular mass distributions in patient serum using mass spectrometry. *J Proteome Res* 2014;13:5198-205.
- Horn PS, Pesce AJ, Copeland BE. A robust approach to reference interval estimation and evaluation. *Clin Chem* 1998;44:622-31.
- Hsieh E, Hsiao CF, Liu JP. Statistical methods for evaluating the linearity in assay validation. *J Chemometr* 2009;23:56-63.
- Snozek CL, Saenger AK, Greipp PR, Bryant SC, Kyle RA, Rajkumar SV, Katzmann JA. Comparison of bromocresol green and agarose protein electrophoresis for quantitation of serum albumin in multiple myeloma. *Clin Chem* 2007;53:1099-103.
- Katzmann JA, Snyder MR, Rajkumar SV, Kyle RA, Therneau TM, Benson JT, Dispenzieri A. Long-term biological variation of serum protein electrophoresis M-spike, urine M-spike, and monoclonal serum free light chain quantification: implications for monitoring monoclonal gammopathies. *Clin Chem* 2011;57:1687-92.
- Keren DF, Schroeder L. Challenges of measuring monoclonal proteins in serum. *Clin Chem Lab Med* 2016;54:947-61.
- Schild C, Wermuth B, Trapp-Chiappini D, Egger F, Nuoffer JM. Reliability of M protein quantification: comparison of two peak integration methods on Capillarys 2. *Clin Chem Lab Med* 2008;46:876-7.
- Kyle RA, Therneau TM, Rajkumar SV, Offord JR, Larson DR, Plevak MF, Melton LJ, 3rd. A long-term study of prognosis in monoclonal gammopathy of undetermined significance. *N Engl J Med* 2002;346:564-9.
- Boyle EM, Fouquet G, Guidez S, Bonnet S, Demarquette H, Dulery R, et al. IgA kappa/IgA lambda heavy/light chain assessment in the management of patients with IgA myeloma. *Cancer* 2014;120:3952-7.
- Kapoor P, Kumar SK, Dispenzieri A, Lacy MQ, Buadi F, Dingli D, et al. Importance of achieving stringent complete response after autologous stem-cell transplantation in multiple myeloma. *J Clin Oncol* 2013;31:4529-35.
- Sinclair D, Ballantyne F, Shanley S, Caine E, O'Reilly D, Shenkin A. Estimation of paraproteins by immunoturbidimetry and electrophoresis followed by scanning densitometry. *Ann Clin Biochem* 1990;27(Pt4):335-7.
- Daval S, Tridon A, Mazon N, Ristori JM, Evraud B. Risk of antigen excess in serum free light chain measurements. *Clin Chem* 2007;53:1985-6.
- McCudden CR, Voorhees PM, Hammett-Stabler CA. A case of hook effect in the serum free light chain assay using the Olympus AU400e. *Clin Biochem* 2009;42:121-4.
- Holmes DT. cp-R, an interface the R programming language for clinical laboratory method comparisons. *Clin Biochem* 2015;48:192-5.