Review

David F. Keren* and Lee Schroeder

Challenges of measuring monoclonal proteins in serum

DOI 10.1515/cclm-2015-0862
Received September 3, 2015; accepted January 6, 2016; previously published online February 24, 2016

Abstract: The measurement of monoclonal protein (M-protein) is vital for stratifying risk and following individuals with a variety of monoclonal gammopathies. Direct measurement of the M-protein spike by electrophoresis and immunochemical measurements of specific isotypes or free light chains pairs has provided useful information about the quantity of M-protein. Nonetheless, both traditional electrophoresis and immunochemical methods give poor quantification with M-proteins smaller than 10 g/L (1 g/dL) when in the presence of polyclonal immunoglobulins that co-migrate with the M-protein. In addition, measurements by electrophoresis of M-proteins migrating in the β- and α-regions are contaminated by normal serum proteins in those regions. The most precise electrophoretic method to date for quantification involves exclusion of the polyclonal immunoglobulins by using the tangent skimming method on electropherograms, which provides a 10-fold improvement in precision. So far, however, tangent measurements are limited to γ migrating M-proteins. Another way to improve M-protein measurements is the use of capillary electrophoresis (CE). With CE, one can employ immunosubtraction to select a region of interest in the β region thereby excluding much of the normal proteins from the M-protein measurement. Recent development of an immunochemical method distinguishing heavy/light chain pairs (separately measuring IgGK and IgGL, IgAK and IgAL, and IgMK and IgML) provides measurements that could exclude polyclonal contaminants of the same heavy chain with the uninvolved light chain type. Yet, even heavy/light results contain an immeasurable quantity of polyclonal heavy/light chains of the involved isotype. Finally, use of liquid chromatography-tandem mass spectrometry (LC-MS/MS) looms on the horizon as a means to provide more consistent and sensitive measurements of M-proteins.

Keywords: free light chain; heavy/light chain measurement; immunosubtraction; mass spectrometry; monoclonal gammopathy of undetermined significance; M-protein; M-spike; multiple myeloma; protein electrophoresis; serum free light chains; serum protein electrophoresis.

***Corresponding author: David F. Keren,** MD, Department of Pathology, The University of Michigan, Ann Arbor, MI 48109, USA, E-mail: dkeren@med.umich.edu
Lee Schroeder: Department of Pathology, The University of Michigan, Ann Arbor, MI, USA

Introduction

"Measure something". Arnold Rice Rich, MD (Baxley Professor of Pathology, The Johns Hopkins Hospital 1947–1958, personal communication, Dr. J. H. Yardley). Sometimes it is easier to say than do. In this review, we chart the efforts to quantify monoclonal immunoglobulins for the past three-quarters of a century. While for many years we seemed to be approaching the matter asymptotically, the last decade has seen extraordinary advances placing us on the brink of establishing a precision in methodology that goes hand in hand with new therapies to improve the outcome of patients with the most malignant forms of monoclonal gammopathies.

The term, "monoclonal gammopathy" gathers a complex array of ailments that have in common clonal proliferations of plasma cells that can usually be detected by finding their products in serum or urine. The products are monoclonal immunoglobulin proteins (M-proteins) that may consist of intact immunoglobulin molecules and/or fragments such as free light chains (FLC). The detection, characterization, and measurement of M-proteins help in initial diagnosis of the disorders, stratification of risk progression, and monitoring response to therapy [1].

These disorders vary from asymptomatic monoclonal gammopathy of undetermined significance (MGUS) (a premalignant condition) to multiple myeloma (MM), an unrelenting malignancy with extensive bone marrow and systemic organ involvement. Virtually all cases of MM begin as an MGUS with an M-protein that may be present

in serum as early as a decade prior to diagnosis of MM [2]. MM comprises about 10% of hematologic malignancies while MGUS is vastly more prevalent; progressively increasing with age such that by age 50 it is found in 1:100 individuals and by age 70 in 1:20 [3]. Right now, MGUS is found by luck. The M-proteins in MGUS cases are detected by serum, or less commonly urine, protein electrophoresis screening performed on samples from patients who have findings suggestive of MM, such as anemia or elevated calcium, that are caused by other processes such as iron deficiency or hyperparathyroidism, not MGUS. While MM usually occurs in older individuals, it is useful to remember that about 2% of the MM cases are reported in individuals younger than 40 years of age. Therefore, the premalignant form (MGUS) of these individuals can begin as early as in their 20s [4].

Characterization of the M-protein by immunofixation, immunosubtraction (ISUB), or an immunochemical method (isotype class, free light chain, or heavy/light chain) is needed for prognosis and to follow patients after therapy. The isotype affects the likelihood of an MGUS progressing; individuals with non-IgG M-proteins have a greater likelihood of developing MM than those with IgG M-proteins [5, 6]. Further, information about isotype and electrophoretic migration position is needed when following response to therapy so that one may distinguish relapse of the original clone from the development of oligoclonal bands and/or a second (usually reactive) M-protein. Such additional M-proteins commonly occur after autologous stem cell transplants with rates as high as 73% being reported [7–10]. The development of oligoclonal bands and/or a second M-protein following autologous stem cell transplantation in MM has been reported to be a favorable prognostic sign for most patients [11]. A recent report of allogeneic stem cell transplants found an overall median overall survival of 115.3 months in patients who developed a second M-protein vs. 31.0 months in individuals not developing such proteins [12].

Beyond identification of isotype and electrophoretic position, key aspects of prognosis and follow-up of monoclonal gammopathies rely upon obtaining a reproducible, accurate quantification of M-proteins. For decades, these measurements have been used as surrogate markers of tumor burden [13–15].

Unfortunately, details of the electrophoretic method used to measure M-proteins are often omitted in peer-reviewed publications, even though such information is important in judging the precision and sensitivity of the reported information. Many reports do not describe how the M-protein spike itself is being demarcated. Further, the authors typically make no attempt to indicate how much co-migrating polyclonal material is included in the monoclonal peak. This likely reflects the fact that prior to the past decade, the perpendicular drop (measuring perpendicularly to the baseline at the points where the M-spike meets the polyclonal γ curve) was assumed to include such information, but the extent of the polyclonal immunoglobulins included was not readily demonstrable (Figure 1A and B). Questions that one may ask in looking at this literature could include: Was there adjustment made to try to compensate the measurement for the polyclonal material? Was other information, such as immunosubtraction pattern used to estimate the M-protein measurement in the α and β regions [16]? What did the authors do if there were two peaks present that overlap?

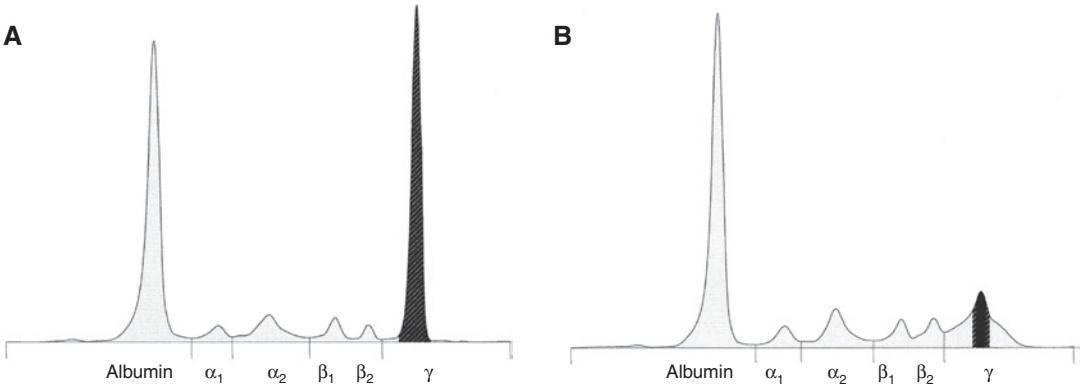
Currently, the most commonly used methods to measure M-proteins are those that measure the size of the M-protein spike itself by electrophoresis of serum and/or urine, or immunochemical measurement of the involved isotype.

Detection and measurement of M-proteins in serum by protein electrophoresis

In the past century, electrophoretic techniques have evolved from barely distinguishing three globulin fractions on moving boundary electrophoresis to high-resolution electrophoresis methods that permit one to see subtle differences in migration that suggest the presence of monoclonal proteins in α, β and γ regions.

With early moving boundary electrophoresis methods during the late 1930s, the Lamm "scale" method was a laborious photographic technique that was used for quantification by defining regions of interest [17, 18]. In 1939, Longsworth et al. used that method to demonstrate the large restricted band in three patients with MM [19]. They noted a unique migration of the band for each case. Two of the three cases gave exceptionally high globulin/albumin ratios, although the third case was in the normal range. Similar to present day problems of how to measure M-proteins, they noted that one must make a more or less arbitrary separation because boundaries of some normally occurring proteins overlap the M-proteins.

Early application of zone electrophoresis by filter paper and later with cellulose acetate techniques measured the major protein fractions by time-consuming methods that involved cutting the protein dye-stained bands apart, then eluting and measuring the dye



Total protein: 9.7 g/dL (6.0–8.3)
A/G: 0.83

Fractions	%	g/dL		Ref. g/dL
Albumin	45.4	4.40		3.43–4.84
α_1	3.4	0.33		0.21–0.44
α_2	8.4	0.81		0.54–0.97
$\beta_{1\,\&\,2}$	6.2	0.60	L	0.65–1.03
γ	36.6	3.55	H	0.70–1.47
M-Spike	35.7	3.46		

Total protein: 7.1 g/dL (6.0–8.3)
A/G: 1.12

Fractions	%	g/dL	Ref. g/dL
Albumin	52.9	3.76	3.43–4.84
α_1	4.8	0.34	0.21–0.44
α_2	11.2	0.80	0.54–0.97
$\beta_{1\,\&\,2}$	11.5	0.82	0.65–1.03
γ	19.6	1.39	0.70–1.47
M-Spike	8.3	0.59	

Figure 1: Serum protein CE electropherograms.
(A) This serum protein CE electropherogram (Sebia Capillarys) demonstrates a γ-region migrating M-protein (shaded) measuring 34.6 g/L (3.46 g/dL) using the standard perpendicular drop. There is suppression of the polyclonal immunoglobulins in this case, so the M-protein measurement is only minimally contaminated with co-migrating polyclonal immunoglobulins. (B) This serum protein CE electropherogram (Sebia Capillarys) demonstrates a γ-region migrating M-protein (shading) measuring 5.9 g/L (0.59 g/dL) using the standard perpendicular drop (shaded). However, about two-thirds of the measured quantity of the standard perpendicular drop is due to polyclonal immunoglobulins that co-migrate with the M-protein and falsely increase the M-protein measurement.

concentration with in a standard spectrophotometer [20, 21]. As the use of filter paper as support medium for zone electrophoresis become more common, Reiner and Stern noted the heterogeneity of M-protein location and also the fact that as many as 22% of MM patients lacked sharp abnormal peaks on SPEP, though more subtle abnormalities such as hypogammaglobulinemia were often present [22]. Kyle et al. used a densitometer to plot and measure the area under the curve of normal and abnormal bands on graph paper by using filter paper electrophoresis stained with Amido black [23]. They measured the height and width (at the midpoint of the height) of the M-protein peaks, and used a 4:1 height:width ratio to distinguish M-protein peaks in MM patients from broader peaks having a lower height:width ratio found in conditions with polyclonal increases in γ globulin. However, this was not an absolute cut-off. When the ratio was less than this 4:1 in patients with MM, they used other key factors such as a β zone or β-γ zone location of the band to suggest that it was a product of the MM cells since polyclonal increases in chronic inflammatory processes are overwhelming present in the γ region. Similar to Reiner and Stern's report, Kyle et al. reported that 24% of their cases of MM did not have diagnostic SPEP patterns. Because some of these cases had hypogammaglobulinemia, they likely represented cases of light chain MM as the majority of them had monoclonal free light chain (Bence Jones) proteinuria.

While the electrophoresis techniques by 1964 were rapid and visualization of the serum protein patterns and the M-protein deflection (M-spike) were provided by the use of densitometric scanning equipment, final calculations of protein fractions and measurement of M-spikes were arduous, requiring the operator to perform manual counts and slide rule calculations to determine the percentage of protein in each fraction generated by the densitometric scan [24]. In 1969, Winkelman and Wybenga described the use of a serum protein electrophoresis system with a Disc integrator, an automatic digital readout that when combined with a computer provided a highly efficient and accurate measurement of protein fractions [25]. At this time, Lotito et al. introduced an analog system where the operator would examine the final electrophoresis strip and punch a narrow slot between the major bands, or the M-protein peak [26]. A light beam passing through the slot signaled a computer unit to begin to quantify data on that peak which terminated with the next punched slot. Clearly, this was still heavily dependent on the operator's

input both for selecting the point to begin measuring each peak and in a second phase where the operator was required to manually adjust a switch to a "sum" position and alter the gain of the summing amplifier.

Computers decreased both the time required to perform measurements and also reduced errors involved in manual counting by the operator [26, 27]. Because of the high cost of computer equipment in 1970, Sax and Moore further refined their process by recording the densitometric readings on a key-punch form that they sent to their hospital's data processing department. This off-line use of a computer further improved turnaround times, reduced errors from manual miscounting or miscalculation and improved the accuracy of quantification of hypogammaglobulinemic patterns [24].

The other major change that occurred in the late 1960s was deployment of a new high-resolution agarose system for electrophoresis that dramatically improved the ability to detect subtle M-proteins and genetic variants [28–30]. These advances were soon followed by the development of capillary zone electrophoretic (CE) techniques that provided excellent resolution and which offered the opportunity to perform immunosubtraction (ISUB) [31–35]. The sensitivity of CE for detecting M-proteins was reported to be 95% compared to 91% for agarose gel electrophoresis with specificity of 99% for both techniques [36, 37]. While basically comparable in their resolution, gel and CE systems differ in several respects that have resulted in discrepancies being reported of fractions such as α_1 as well as in measurement of large M-protein. A relevant difference was the saturation effect of protein dye staining on gels in the presence of large M-proteins [38]. However, this could be remedied by increasing the dilution of samples with large M-proteins prior to testing [39]. Following patients with M-proteins, subtle differences in the systems should be considered when switching from one technique to another [40].

Currently available instruments contain sophisticated computers that automatically demarcate major zones, permitting manual adjustment in cases with M-proteins or other unusual bands. Gravimetric measurement, however, still is based on applying these percentages to the total protein concentration of the fluid obtained by a standard technique such as the Biuret method for serum or the pyrogallol red method for urine [41].

Recognition of the improved detection and resolution of serum protein bands prompted a College of American Pathologists (CAP) Guideline Conference to recommend using techniques for the evaluation of M-proteins that provide high-resolution electrophoresis (either gel- or capillary-based) when screening for M-proteins [42, 43]. For purposes of screening, high-resolution electrophoresis was defined as a method that provided crisp separation of the β_1 (transferrin) and β_2 (C3) bands [44]. Low-resolution systems cannot separate those bands making it difficult to measure β-migrating M-protein spikes. While one may add the use of immunofixation (IFE) to lower resolution methods to improve their sensitivity, this comes at the cost of losing quantitative results required for monitoring patients.

Despite these advances, the actual measurement of the M-protein spike itself remains a subjective method with suboptimal measurements on small M-proteins and even ones up to 20 g/L (2 g/dL) that migrate in the β region. A standard perpendicular drop demarcation that is subjectively applied by the operator has been used for decades to measure M-proteins using densitometric scans of gels and, more recently, using electropherograms of capillary electrophoresis patterns [44, 45] (Figure 1A and B).

Most M-proteins migrate in the γ region so measurement of the M-spike by the perpendicular drop necessarily captures polyclonal immunoglobulins that are present at the same position. At the time of diagnosis of MM, the M-protein spike is often accompanied by suppression of the normal polyclonal immunoglobulins, making the measurement using the perpendicular drop both sufficiently accurate and reproducible to provide a useful surrogate measurement of tumor burden (Figure 1A). However, in the case of modest-sized and small M-proteins, such as those found in most patients with MGUS, or when following the response to therapy in MM patients, the presence of co-migrating polyclonal immunoglobulins by the perpendicular drop measurement creates a significant overestimation of the M-protein (Figure 1B) [46].

Given a constant amount of polyclonal immunoglobulins, the smaller the overlying M-protein in the γ region, the greater the overestimation of the M-protein by the perpendicular drop technique. Using dilution studies with gel electrophoresis, Bergón et al. reported that three M-proteins (IgG, IgA and IgM) gave linear results down to the range of 10–15 g/L (1.0–1.5 g/dL). The limit of qualitative visual detection was 0.43, 0.89 and 0.33 g/L (43, 89 and 33 mg/dL), respectively, but quantification overestimated these amounts by 78%, 22% and 130%, respectively. due to inclusion of co-migrating polyclonal material. Further, since the authors used a hypogammaglobulinemic polyclonal serum pool, their studies likely underestimated the effect of polyclonal immunoglobulins that would occur in most MGUS patients early in their course when they have normal quantities of uninvolved immunoglobulins [45].

Recognition of the inaccuracies of perpendicular drop measurements below the level of 10 g/L (1.0 g/dL)

is consistent with the International Myeloma Working Group (IMWG) guidelines. These guidelines define a "measurable" M-spike as one having at least one of the following three measurements: serum M-protein ≥10 g/L (1 g/dL), urine M-protein ≥200 mg/24 h, or serum involved free light chain (FLC) level ≥100 mg/L (10 mg/dL) (only if the serum FLC ratio is abnormal) [47, 48]. Nonetheless, even with this requirement, "measurable disease" M-proteins with values between 10–20 g/L (1.0–2.0 g/dL) may overestimate the M-protein value as much as 50% due to interference by polyclonal immunoglobulins [46]. Unfortunately, the alternative use of total isotype immunoglobulin measurements by nephelometry or turbidimetry also is contaminated by polyclonal immunoglobulins. Katzmann et al. reported the total variation (biological variation as well as preanalytical and analytical error) of IgG M-protein spikes in clinically stable patients who were not undergoing chemotherapy, by comparing sequential serum protein M-spikes by gel electrophoresis and immunochemical methods (Table 1) [41]. Assuming this total variation, they calculated the percent changes in various biomarkers that would be required to represent a clinically significant change in M-spike (and not merely represent error or biological variation). To obtain a difference with 95% chance of representing a clinically significant change in a measurable >10 g/L (>1.0 g/dL) M-protein, the subsequent measurement would need to increase or decrease by 20.1% from the previous sample. However, when looking at all M-spikes [including those <10 g/L (<1.0 g/dL)], a 27.5% difference would be required. As shown by Katzmann et al., the M-spike CVs for unmeasurable M-proteins [<10 g/L (<1.0 g/dL)] are greater than those for measurable M-proteins. This may in part reflect the additional biological variation of the underlying polyclonal proteins that are included in the SPEP measurement and constitute a greater proportion of that measurement in patients with smaller M-proteins.

There are no strict guidelines as to when an M-spike is too small relative to the underlying polyclonal immunoglobulins to permit accurate measurement by the perpendicular drop method. A rough frame of reference is to measure M-proteins when a visual estimate shows that they account for at least 1/4 to 1/3 of the underlying polyclonal base [49]. M-proteins smaller than this have been termed IFE-M-proteins by Murray et al. who demonstrated that individuals with such processes develop malignant plasma cell and B cell lymphoproliferative disorders at a rate just slightly lower than the 1% annually for measureable MGUS [50]. And similar to classic MGUS cases, non-IgG isotypes are more likely to progress that those of the IgG isotype.

Table 1: Percentage decrease in M-proteins measurements to achieve 95% confidence.

Test	n	Biological and analytical CV	% Difference to achieve p=0.95
Serum M-spike	158	11.6	27.5
Measureable serum M-spike[a]	90	8.1	20.1
Involved Immunoglobulin (IgG)	148	13.0	30.3

[a]Measurable values defined as >10g/L (1 g/dL). Data from Table 3 of Katzmann et al. [41].

Recognizing the problem of including polyclonal immunoglobulins by the perpendicular drop measurement (Figure 2A), our laboratory has used a corrected perpendicular drop (c-perpendicular) that narrows the area measured using a visual estimation to account for polyclonal immunoglobulins (Figure 2B). However, either using a 1/3–1/4 cutoff, or estimating the polyclonal immunoglobulins included, are subjective and would be expected to yield wide variation in interpretation in common usage.

A more objective approach to measure M-proteins that migrate in the γ region was reported by Schild et al. [46]. They separated the M-spike from the underlying polyclonal immunoglobulins by using a tangent correction feature on the Sebia Capillarys CE instrument. This feature creates a tangent skimming line connecting the points where the M-spike meets the underlying polyclonal immunoglobulin (Figure 2C). As shown in these three examples, the c-perpendicular drop improves the measurement, but is not as accurate and was not as reproducible as the tangent skimming method. However, as discussed below, because the tangent skimming method does not work well in the β-γ or β regions, a c-perpendicular drop adjusted by immunosubtraction findings is still favored in our laboratory for that situation. By measuring the area of the spike above the tangent line and excluding the area below accounting for the polyclonal antibodies, Schild et al. demonstrated that dilutions of known M-proteins were linear down to 1.5 g/L (0.15 g/dL) while the perpendicular drop method on the same dilutions was linear only to 15 g/L (1.5 g/dL) (Figure 3). However, in his laboratory, Schild does not report data lower than 3.0 g/L (0.3 g/dL) because he found a coefficient of variation above 15% in comparing the four individuals who performed the measurement (personal communication Dr. C. Schild). Adherence to the tangent method markedly improved bias of the measurements for γ-migrating M-proteins. When Schild et al. performed linear regression analysis on 71 sera from patients with MM, they found that the perpendicular drop was higher than tangent measurements at all levels

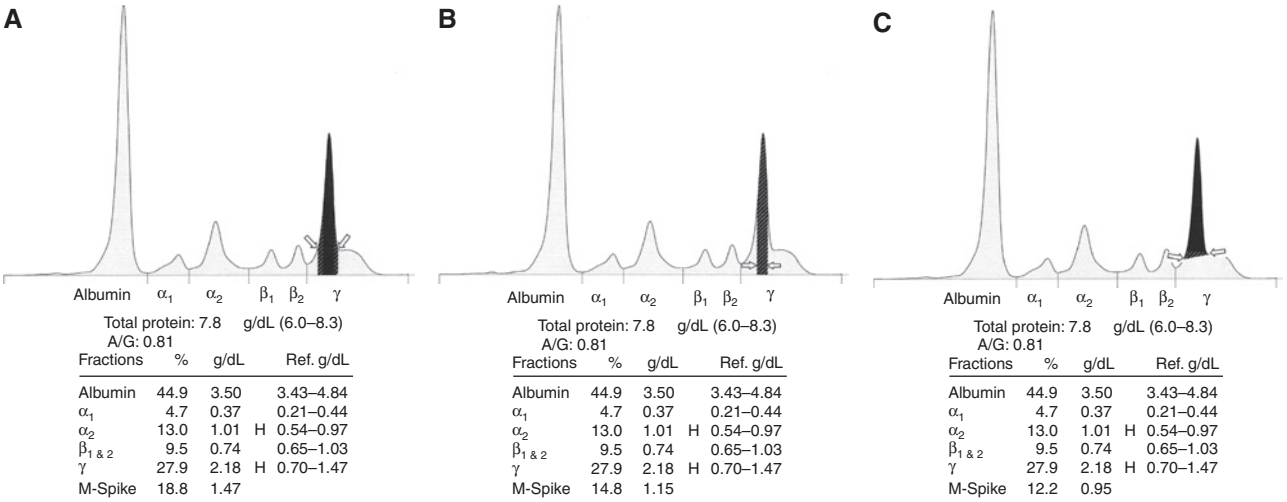


Figure 2: Serum protein CE electropherograms (Sebia Capillarys) showing three methods of measuring M-protein peaks.
(A) Standard perpendicular drop method (shaded) where the demarcation begins where the M-spike meets the polyclonal region below it (arrows) and then proceeds straight down to the baseline including all the area above and below. This measures 14.7 g/L (1.47 g/dL) and is the most frequently used technique currently. (B) Corrected perpendicular drop where an attempt is made to narrow the area measured to compensate for the inclusion of the polyclonal area below. The same M-spike here measures 11.5 g/L (1.15 g/dL). (C) Tangent skimming method described by Schild et al. on the Sebia Capillarys [46]. As shown by the arrows, this method attempts to cut off only the area above the peak. The M-protein now measures 9.5 g/L (0.95 g/dL). It works well in the γ region.

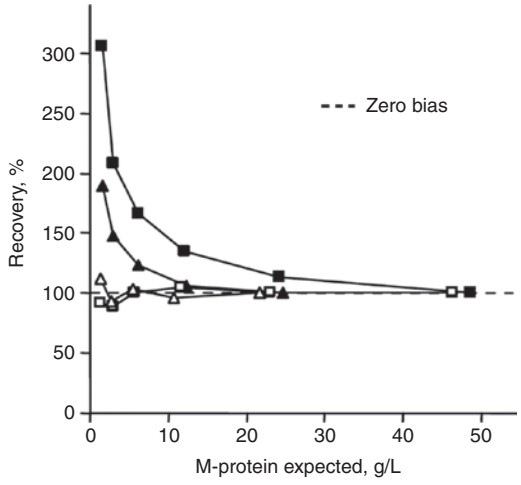


Figure 3: Schild et al. diluted an IgG M-protein and an IgA M-protein in normal serum and measured the M-protein spike by perpendicular drop for IgG (shaded squares) and IgA (shaded triangles) and by the tangent method for IgG (unshaded squares) and for IgA (unshaded triangles).
The perpendicular drops were linear to 15 g/L (1.5 g/dL) whereas the tangent measurements were linear to 1.5 g/L (0.15 g/dL). Figure from Schild et al. [46] used with permission.

[46]. As the IMWG guidelines recommend quantitative monitoring when M-proteins are above 10 g/L (1.0 g/dL), it was instructive that Schild et al. found discrepancies of up to 58% between the tangent measurement and the perpendicular drop in some sera with M-protein spikes in the range between 10 and 20 g/L (1.0 and 2.0 g/dL).

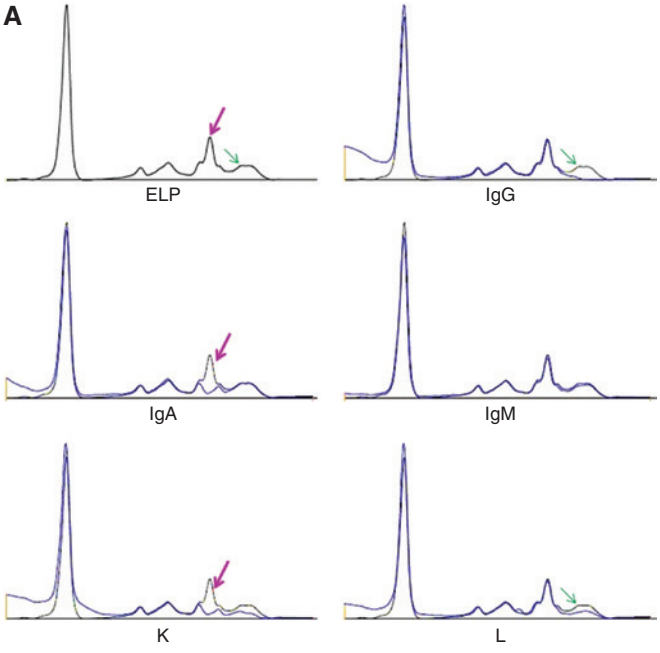
Unfortunately, while the tangent method works well in the γ region, M-proteins can migrate anywhere in the electrophoretic pattern [51]. When they are occur in the α-, β- or β-γ region, the presence of other serum proteins such as transferrin and C3 alter the shape of the trace where the M-spike would have met polyclonal immunoglobulin and instead the intersection of monoclonal and polyclonal immunoglobulin is hidden under non-immunoglobulin protein. In these cases, the tangent method is not reliable. These problems mainly concern IgA due to its typical migration in the β region. However, other isotypes occasionally migrate in the β or α regions and also present difficulties in measurement by the tangent method. Because of the variabilities involved in measuring the β migrating IgA M-spikes when using relatively low resolution gel techniques, Katzmann et al. do not measure symmetric IgA β M-spikes smaller than 20 g/L (2.0 g/dL) [52]. Recognizing these issues, the 2014 IMWG guidelines recommend measurement of total IgA rather than the M-protein spike to follow patients with β-migrating IgA M-proteins [53].

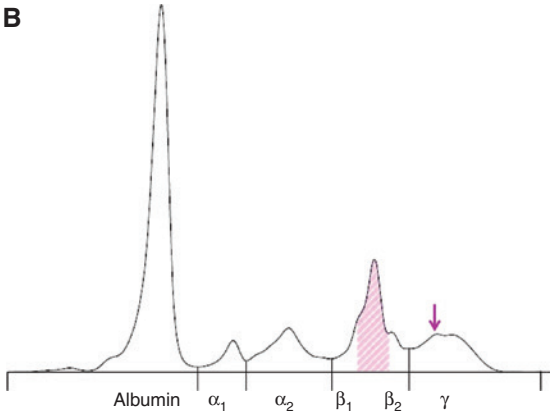
An alternative to measuring the β-migrating IgA M-spike could be, nephelometric measurement of the heavy-light chain pairs for IgA (IgAK separately from IgAL), which Katzmann et al. have shown to provide superior results to those from the standard perpendicular drop measurements of gel electrophoretic traces in the β region [52].

Another alternative for measuring M-proteins in the β region is using a CE method that provides crisp separation of transferrin and C3 where one can obtain

reproducible measurement of M-spikes by performing a corrected perpendicular measurement guided by results from an immunosubtraction (ISUB) pattern on the same specimen [40, 49]. ISUB patterns reveal the precise area that has been removed and clearly identifies the amount of the normal underlying constituents, such as transferrin and C3, or polyclonal immunoglobulin, in the β region (Figure 4). For instance, in Figure 4A, when either IgA or κ light chain is subtracted, the normal β-region bands (transferrin and C3) are readily identified (Figure 4A). This permits one to estimate the amount of M-protein and can inform the corrected perpendicular drops as shown in Figure 4B. While such methods are subjective and are correctly termed estimates, the immunochemical measurements of total immunoglobulin of the involved isotype as suggested by Ludwig et al., or even heavy-light chain pairs as performed by Katzmann et al. [52], also include incalculable amounts of polyclonal immunoglobulins of that isotype or heavy-light chain pair. As with the perpendicular drop, quantification of M-protein using these methods is more substantially distorted the more the M-protein declines and normal clones recover after therapy. Another complicating factor is when two or three M-protein bands are seen. Some of these are truly bi- or triclonal gammopathies. But many reflect the presence of posttranslational modifications and/or polymerization. IgA and IgM M-proteins are particularly prone to polymerize [51].

Finally, improvements in the measurement of M-proteins have been proposed by two workers very recently. At the 2015 AACC meeting, Wunsch [54] reported a method that mathematically fits the γ region polyclonal immunoglobulin curve and subtracts estimates of polyclonal immunoglobulin in the β region on CE analysis allowing accurate measurement of transferrin and C3. At the University of Michigan we have been developing a quantitative ISUB method on CE that involves integrating





Total protein: 7.2 g/dL (6.0–8.3)

A/G: 1.12

Fractions	%	Ref. %	g/dL	Ref. g/dL
Albumin	50.5	52.9–66.9	3.6 L	3.7–4.9
α_1	4.3	3.0–5.8	0.3	0.2–0.4
α_2	11.8	7.5–13.4	0.8	0.5–0.9
β	20.5	8.5–13.7	1.5 H	0.6–1.0
γ	12.9	8.8–19.2	0.9	0.6–1.4

Figure 4: (A) This ISUB (Sebia Capillarys) of serum shows six electropherogram images of serum from a patient being treated for IgAK multiple myeloma. The top left image is the protein electrophoresis (ELP) which has a black line indicating the electropherogram of this serum. The other five images show the result of subtracting the indicated immunoglobulin by preincubating the serum with specific antiserum prior to performing the ISUB. There are two lines that largely superimpose on these five images. The black line is the ELP pattern repeated on each image. However, a blue line is also present on these five images that shows what has happened following the immunosubtraction. Where the two lines overlap, one sees only the blue line. But when there is a difference, the difference between the blue and black lines shows the area that has been subtracted. This patient has a modest M-spike in the β region on the ELP image. A pink arrow points to this area in the ELP, IgA and K images. Under these arrows in the IgA and K images note that the blue lines go down to the two normal β region proteins (transferrin and C3) while the original black line remains above them. This indicates that the M-spike is composed of IgAK and the amount subtracted corresponds to the amount of the M-protein. Note also that a tiny restriction can be seen in the γ region (green arrows). This area is not subtracted by IgA or κ, but is subtracted by IgG and λ. It represents a small oligoclonal band commonly seen following autologous stem cell transplantation. (B) The measured area in the β region in this CE electropherogram from the same serum as in 4A is shaded in pink (the corrected perpendicular drop). It was estimated by observing the area subtracted in the IgA image of Figure 4A. The arrow in the γ region indicates the tiny band noted above that likely reflects a reactive oligoclonal process. It is too small to measure.

the immunosubtracted traces in a region defined by the pathologist. Using this method, linearity of M-protein measurements in the β region is comparable to that obtained by Schild et al. for measuring M-protein spikes in the γ region, even with small M-proteins approaching 1.0 g/L (0.1 g/dL) (Dr. Lee Schroeder, personal communication).

Detection and measurement of M-proteins in serum by immunochemical methods

Radial immunodiffusion

Immunochemical methods to measure immunoglobulin isotypes have been used for more than a half-century. Early work combined visual gel technology with isotype specific antibodies to measure immunoglobulins. Radial immunodiffusion (RID) was a single-immunodiffusion technique that was used in the mid-1960s to measure immunoglobulins. For the technique, polyclonal antibodies against a specific isotype was added to warm (about 50 °C), liquid agarose to form a mixture with a consistent concentration of the reagent antibody. This mixture was allowed to solidify at room temperature on a solid surface such as a slide or Petri dish [55]. Wells of a precise diameter were sharply punched out of the antibody-agarose gel. Each well received a standardized volume of either a control solution containing a known concentration of the specific antibody isotype or the same volume of an unknown serum or other fluid to be tested. These samples were placed in a moisture chamber (to prevent dehydration of the gel), usually at room temperature, and allowed to diffuse for a set period of time (typically 16–48 h). As the immunoglobulins diffused radially from the well, their concentrations decreased. Large molecules such as IgM diffuse more slowly than smaller molecules such as IgG. When the isotype of immunoglobulins reached a concentration that was equivalent to that of the embedded isotype specific antisera, a precipitin band formed that was readily visualized. The diameter of the band was measured and the square of the diameter was graphed against the known concentration of the standard. The diameter of the unknown samples was measured and its gravimetric value in mg/dL was determined from the standard curve [56, 57]. To improve the speed of the reaction from the slow diffusion step of the RID, Laurell supplied electrophoresis to propel the unknown antibody solution into the gel creating precipitin bands that resembled rockets. This advance allowed the work to be completed in a few of hours [58, 59].

Smith and Thompson compared RID results for IgG, IgA and IgM to measurement of the M-protein by densitometric estimations in 186 serum samples from 11 patients with MM or Waldenström macroglobulinemia [60]. By electrophoresis, the total protein was measured by Biuret technique, and gels stained by Amido Black had the M-protein spike measured by perpendicular drop densitometry. They found good correlation overall (correlation coefficient 0.83) with a slope of 0.88 for IgG. However, the slopes for IgA and IgM were 0.62 and 0.76, respectively. Not surprisingly, the values were higher by RID as they included all of the polyclonal immunoglobulins while the perpendicular measurement of the M-protein spike by densitometry included only the polyclonal immunoglobulins that migrated in the same region as the spike, though also any non-immunoglobulin protein. In addition, they found that values by the two systems correlated much better in some patients than in others. They recommended using both methods to follow patients with MM because, in their hands, neither gave clearly superior performance. Interestingly, considering the recent development of heavy/light chain antisera, Smith and Thompson also suggested that the ideal method might employ an anti-idiotype reagent (clearly not anti-heavy/light, but unique antisera that reacts with the binding site of the monoclonal protein that then had recently been described by Hopper and Nisonoff) [61].

Automated immune precipitin (AIP)

These gel based antibody tests were slow, limited in their accuracy and required considerable manual attention. Killingsworth and Savory pioneered the automated immune precipitin (AIP) method by using the Technicon AutoAnalyzer for measurement of human IgG, IgA and IgM [62]. By combining isotype-specific antisera reagents with serum in a liquid suspension, antibody-antigen immune complexes formed that could be measured by their scattering of light at an acute or right angle to the incident light. By measuring this at end-point, or by calculating the changes in the light scatter with time, one could measure the concentration of the specific isotype. Adding reagents, such as polyethylene glycol that enhanced the formation of the immune complexes, improved both the sensitivity and speed of the reaction. Alternatively, one could obtain similar results with turbidimetry by measuring the decrease in light passing through the mixture of complexes. These assays

dramatically reduced the technical expertise and time required to make measurements of immunoglobulins and paved the way for subsequent studies that automated the measurement of IgG, IgA, IgM as well as antibodies to κ or λ light chains (whether free or attached to heavy chains) [63–66].

By using the measurement of IgG, IgA, IgM, κ, λ, and the ratio of κ/λ (attached or unattached to heavy chains) one could determine the heavy and light chain type of most M-proteins [67–71]. This was useful in the mid-1980s because the immunoelectrophoresis (IEP) and IFE techniques available at that time were slow, laborious and expensive, while the immunochemical measurements could be done in minutes. However, small or subtle M-proteins could not be characterized by these methods. So, as inexpensive automated immunofixation techniques became available, the use of immunochemical typing of serum M-proteins went out of vogue.

As with RID measurements, higher estimations of M-protein concentrations compared to densitometric scans of SPEP gels occurred with immunoturbudimetric and nephelometric assays because those techniques also included a disproportionately larger amount of polyclonal antibodies of the involved isotype [72]. Furthermore, as with RID techniques, because the reagent antisera are made against polyclonal immunoglobulins, the M-protein being measured may differ considerably in antigenic makeup and thus provide widely varying results [68, 73]. While IgG and IgA values by RID and immunoturbidity tend to be comparable (considering the polyclonal overestimation by AIP technique), a study by Sinclair et al. on 82 patients with MM and 25 with Waldenström macroglobulinemia found an unacceptably large overestimation of monoclonal IgM by immunoturbidity. In dilution studies, they found that SPEP IgM M-protein values overestimated the M-protein concentration slightly at low levels, but was more reliable at high concentrations. Conversely, immunoturbidity overestimated IgM at high levels, and was more reliable at low levels (Table 2).

Nephelometry and turbidimetry for IgG, IgA and IgM

Riches et al. conducted a comparative study of three systems, one using rate nephelometry, one with endpoint nephelometry, and a third with rate turbidimetry on a large number of sera from patients followed over a period of several years. They reported an overestimation of the M-protein by all instruments with all classes of immunoglobulins. But similar to Sinclair et al., the overestimation was the most problematic with IgM M-proteins and with rate nephelometry [74]. The rate nephelometry gave significantly ($p<0.001$) higher results for all three analytes, but the percentage increase was greater for IgM (Table 3). The authors suggested this could reflect a two stage reaction with an anomalous behavior in the early part of the IgM reaction [74].

A similar result was reported more recently by Murray et al. in their comparison of the Dade Behring BNII with SPEP densitometry from a Helena SPIFE system [14]. In addition to documenting the disproportionate increase in IgM found by Sinclair et al., Murray et al. suggested that it could be due to the presence of low molecular weight forms of IgM. They also reported that IgG measurement by SPEP was nonlinear possibly due to dye saturation as suggested by earlier workers [39].

Table 2: Recovery of purified monoclonal immunoglobulin added to normal human serum and quantitated by electrophoresis followed by densitometry (SPEP) and immunoturbidimetry (IT).

IgG added, g/L	IgG by SPEP, g/L	SPEP % recovery	IgG M-protein+poly IgG, g/L	IgG by IT, g/L	IT % recovery
25	25.1	100	35	35	100
12.5	13.5	108	22.5	25.4	113
6.25	7.8	124	16.3	18.6	114
3.12	4.7	152	13.1	14.5	111
1.6	3.28	206	11.6	12.9	111
IgM added, g/L	**IgM by SPEP, g/L**	**SPEP % recovery**	**IgM M-protein+poly IgM, g/L**	**IgM by IT, g/L**	**IT % recovery**
40	42	105	40.4	59	146
20	21	105	20.4	32	157
10	12	120	10.4	12	115
5	5.4	108	5.4	6.9	128
2.5	2.9	116	2.9	3.0	103

The normal serum used as base material had polyclonal IgG and IgM concentrations of 10.0 g/L and 0.4 g/L. Table from Sinclair et al. [72].

Table 3: Mean M-protein measurement by SPEP, or mean concentration of involved M-protein isotype by rate nephelometry.

M-protein Isotype	n	SPEP, g/L (1SD)	Nephelometry, g/L (1SD)
IgG	260	27.9 (18.69)	36.9 (26.7)
IgA	71	32.1 (19.3)	40.15 (29.2)
IgM	120	21.4 (35.1)	38.9 (35.1)

Data from Riches et al. [74].

Nephelometry and turbidimetry for serum free light chains (FLC)

Nephelometric technologies and antisera continue to evolve creating novel ways to measure M-proteins. In 2001, Bradwell et al. developed polyclonal antisera that reacted only with free light chains, but not light chains that were bound to heavy chains [75]. The reagent antibodies were directed against antigenic determinants that were cloaked in the interior of intact immunoglobulin molecules. With these highly specific and sensitive reagents, Bradwell et al. could detect circulating monoclonal free light chains by measuring the ratio of free κ/free λ (FLC K/L). Katzmann et al. further improved the utility of this assay; establishing a diagnostic range for the FLC K/L of 0.26–1.65 by requiring that an abnormal value would fall out of the measurements occupied by 100% of the normal population they tested [76]. By using the diagnostic range rather than the typical 95% confidence intervals, the specificity of the test was so strong that that ratio is still used in screening tests today.

The availability of the serum FLC test during the past 15 years has provided us with an objective quantitative test for initial detection as well as prognostic information about monoclonal gammopathies [75, 77, 78]. Indeed, sensitivity and specificity of the serum FLC test are so strong that for the initial detection of monoclonal free light chains a urine sample is no longer needed as long as one also has performed SPEP, serum immunofixation, and serum FLC [79]. Current IMWG guidelines recommend using the serum detection of M-protein to follow MM, yet, serum FLC measurements has been used to document stringent complete remission as long as free light chain is produced by the malignant cells [80]. While serum FLC has been an important assay for detection and prognosis, urine studies are still recommended by the IMWG to follow patients with light chain MM because an aliquot of a 24-h urine specimen provides a good surrogate marker of tumor burden to follow disease [81]. Serum FLC are considered less effective for detecting intact/fragmented immunoglobulin in urine, features that correlates with impaired renal function and reduced survival in MM [82], as well antigen excess artifacts are occasionally experienced.

Antigen excess is a well-recognized phenomenon in a wide variety of immunological techniques. Because most immunoglobulin molecules have only two binding sites, to form an optimum precipitation with an antigen of a similar size, the ratio of antibody to antigen should be approximately 1:1. This permits formation of a huge latticework structure that readily precipitates. However, if antigen is present in great excess, the typical antibody molecule will only be bound to a single antigen, creating a complex that is too small to precipitate [40]. With serum FLC, there are relatively few epitopes, and so with a large number of molecules found in light chain multiple myeloma may overwhelm the ability of relatively small amounts of antibodies optimized to precipitate with the tiny amount of FLC usually present in serum [83, 84].

Nephelometry and turbidimetry for heavy-light chain combinations

A further advance in measuring M-proteins occurred in 2009 when Bradwell et al. described the development of reagent antisera that was able to detect antigenic determinants that are created by the link between heavy and light chain constant regions of immunoglobulins [85]. The antisera developed distinguished IgGK from IgGL, IgAK from IgAL, and IgMK from IgML. Bradwell et al. demonstrated high sensitivity and specificity similar to their original studies on antisera against free κ and free λ chains nearly a decade before [75]. They established a normal range for each heavy light chain pair, and a normal ratio for each specific heavy chain K/specific heavy chain L using samples from healthy blood bank donor sera. Using a 95% confidence interval for their initial studies, their heavy-light chain (HLC) ratio measurement could reasonably match the performance of a relatively low-resolution (5-band pattern) gel for detecting M-proteins. They did have a few samples with abnormal HLC ratios but no M-proteins. But, since the test used a 95% confidence interval finding 1 out of 20 false positives would be expected [86].

In a subsequent work, Ludwig et al. reported that use of the HLC assay allowed measurement of M-proteins that were not accurately measurable by SPEP or nephelometry against the isotype of the involved M-protein [87]. For these studies they defined HLC ratios outside of the 95% reference range to be considered as a clonal process. However, the false negatives by immunofixation had an IgA HLC of 2.23 (RI 0.78–1.94) in one case and 3.05 in

the other. This finding suggests that the 95% range was too narrow as a diagnostic reference range. Once again, Katzmann et al. developed a confidence interval (99%) that improved the specificity of this assay [52]. If one were to apply the 99% confidence interval of 0.53–2.52 to the study of Ludwig et al., only one of the two cases would have been positive, but that sample had a total IgAK (the involved heavy/light chain pair) measuring only 1.25 g/L (125 mg/dL), well within the normal range of 0.43–2.36 g/L (43–236 mg/dL) [87].

The use of the ratios such as the serum FLC ratio (rFLC) is primarily for initial detection not for monitoring following treatment. That concept has the logic of a process with an M-protein superimposed on either a normal set of immunoglobulins or a suppressed set. However, to follow therapy with serial measurements in conditions such as oligosecretory MM with light chain production, the IMWG guidelines recommends using the involved light chain iFLC or the difference (dFLC) between the involved FLC and the uninvolved FLC rather than rFLC [77]. The rFLC was not recommended for serial measurements after therapy because of the marked distortion of the ratio that could occur with therapeutic suppression of both the involved and uninvolved FLC. Furthermore, after therapy such as autologous stem cell transplants we often see recovery of immunoglobulin production with oligoclonal bands and second M-proteins that can alter free light chain ratios up or down and may not reflect a recurrence. These are lessons learned from serum free light chains. Until the use of HLC ratios is validated using a more rigorous 99% cut-off, we do not recommend that such data be used to change the status of a patient.

Katzmann et al.'s study recommended use of the IgA heavy/light measurement for β-migrating IgA M-proteins, however, they did not see an advantage to using the IgG heavy/light measurements in preference to the M-spike measurement for these β region bands [52]. One would suspect that β-migrating IgG or IgM M-spikes would also benefit from the more precise heavy/light chain measurement. As noted above, for β-region migrating IgA M-proteins, we have used a c-perpendicular drop based upon correcting the measurement with the use of data from an ISUB pattern, and this may prove to be competitive with HLC. Further, although it should be possible to use the ISUB pattern itself to measure the subtracted area directly, the manufacturers currently do not support that use of their product

Mass spectrometry

A strong hint of what testing for M-proteins will be in the near future has come from a team at the Mayo Clinic in the past 2 years [88–90]. They have shown that mass spectrometry is an exquisitely sensitive and specific technique that offers great promise to change the entire manner in which M-proteins are characterized and measured [88–90]. Mills et al. reported using immunochemical techniques to purify immunoglobulins from other proteins and reduction to separate the heavy and

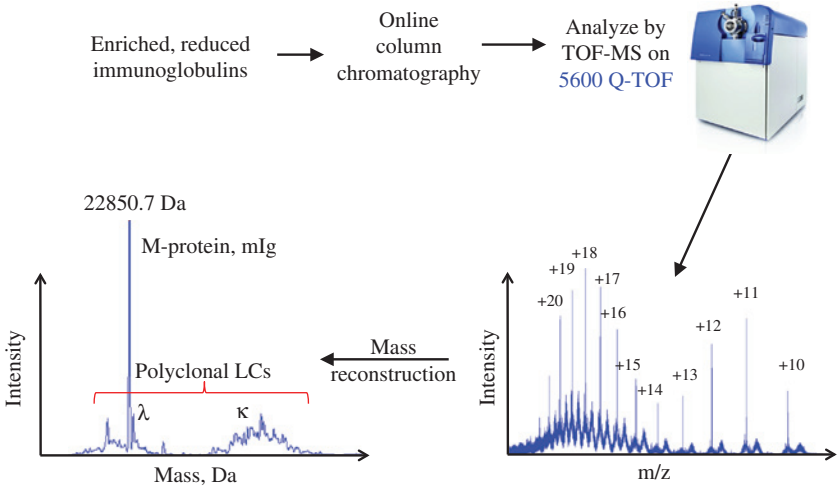
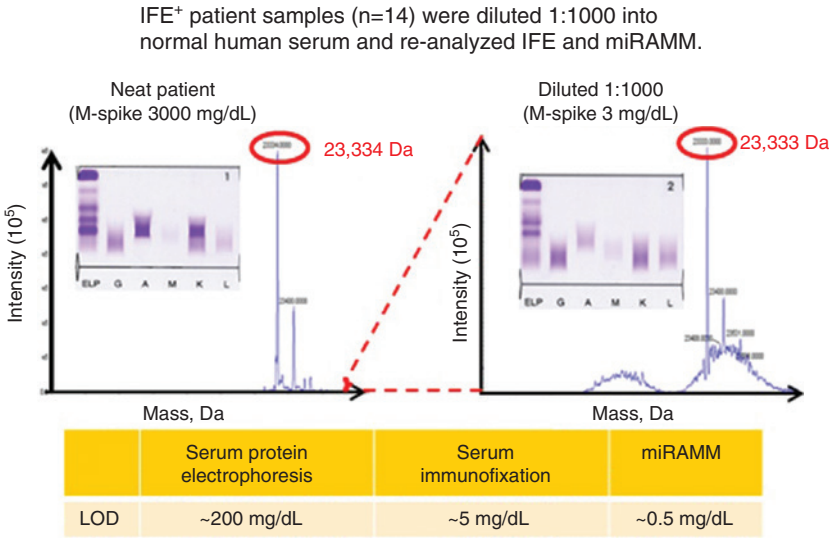


Figure 5: The method to purify and prepare immunoglobulins, followed by microflow liquid chromatography and electrospray ionization with quadropole time of flight mass spectrometry.
In the enriched, reduced step, light chains are purified. The monoclonal protein is represented by the single large peak seen in the bottom left panel in the λ region. This method has been termed "monoclonal immunoglobulin rapid accurate mass measurement" (miRAMM) [90]. Image provided by and used with permission from Drs. John R. Mills, David R. Barnidge and David L. Murray.



	Serum protein electrophoresis	Serum immunofixation	miRAMM
LOD	~200 mg/dL	~5 mg/dL	~0.5 mg/dL

Figure 6: The miRAMM technology has an order of magnitude lower limit of detection for M-proteins compared to immunofixation. This example shows an M-protein (visible on the immunofixation of neat serum) by when diluted 1000 times in normal human serum it is detected and measurable by mRAMM but invisible to immunofixation (on the right). Image provided by and used with permission from Drs. John R. Mills, David R. Barnidge and David L. Murray.

light chains. They showed that microflow liquid chromatography used together with electrospray ionization and quadropole time of flight mass spectrometry could detect unique charge/mass ratios to both identify and measure M-proteins (Figures 5 and 6). They have termed the method "monoclonal immunoglobulin Rapid Accurate Mass Measurement" (miRAMM) [90]. They use the light chains because they are also monoclonal and being smaller, they are more readily ionized [90]. In addition, light chains undergo much less posttranslational modification than heavy chains [91]. The exquisite sensitivity of this method may be the best way to detect minimal residual disease. At the present time, however, available instruments would be impractical for screening large numbers of samples. However, these elegant studies are certain to provide strong impetus for manufacturers to develop instruments that can provide such information with rapid throughput in a timely and efficient manner that will allow us to "measure something" with aplomb.

Author contributions: All the authors have accepted responsibility for the entire content of this submitted manuscript and approved submission.
Research funding: None declared.
Employment or leadership: None declared.
Honorarium: None declared.
Competing interests: The funding organization(s) played no role in the study design; in the collection, analysis, and interpretation of data; in the writing of the report; or in the decision to submit the report for publication.

References

1. Katzmann JA. Screening panels for monoclonal gammopathies: time to change. Clin Biochem Rev 2009;30:105–11.
2. Landgren O, Kyle RA, Pfeiffer RM, Katzmann JA, Caporaso NE, Hayes RB, et al. Monoclonal gammopathy of undetermined significance (MGUS) consistently precedes multiple myeloma: a prospective study. Blood 2009;113:5412–7.
3. Kyle RA, Therneau TM, Rajkumar SV, Larson DR, Plevak, MF, Offord JR, et al. Prevalence of monoclonal gammopathy of undetermined significance. N Engl J Med 2006;354:1362–9.
4. Kyle RA, Gertz MA, Witzig TE, Lust JA, Lacy MQ, Dispenzieri A, et al. Review of 1027 patients with newly diagnosed multiple myeloma. Mayo Clin Proc 2003;78:21–33.
5. Rajkumar SV, Kyle RA, Therneau TM, Melton LJ, Bradwell AR, Clark RJ, et al. Serum free light chain ratio is an independent risk factor for progression in monoclonal gammopathy of undetermined significance. Blood 2005;106:812–7.
6. Rajkumar SV, Kyle RA, Buadi FK. Advances in the diagnosis, classification, risk stratification, and management of monoclonal gammopathy of undetermined significance: implications for recategorizing disease entities in the presence of evolving scientific evidence. Mayo Clin Proc 2010;85:945–8.
7. Wadhera RK, Kyle FA, Larson DR, Dispenzieri A, Kumar S, Lazarus HM, et al. Incidence, clinical course, and prognosis of secondary monoclonal gammopathy of undetermined significance in patients with multiple myeloma. Blood 2011;118:2985–7.
8. Alejandre ME, Madalena LB, Pavlovsky MA, Facio ML, Corrado C, Milone G, et al. Oligoclonal bands and immunoglobulin isotype switch during monitoring of patients with multiple myeloma and autologous hematopoietic cell transplantation: a 16-year experience. Clin Chem Lab Med 2010;48:727–31.
9. Jo JC, Yoon DH, Kim S, Lee K, Kang EH, Jang S, et al. Clinical significance of the appearance of abnormal protein bands in patients with multiple myeloma. Ann Hematol 2014;93:463–9.

10. Tovar N, de Larrea CF, Arostegui JI, Cibeira MT, Rosinol L, Rovira M, et al. Natural history and prognostic impact of oligoclonal humoral response in patients with multiple myeloma after autologous stem cell transplantation: long-term results from a single institution. Haematologica 2013;98:1142–61.
11. Manson GV, Campagnaro E, Balog A, Kaplan D, Sommmers SR, Fu P, et al. Secondary MGUS after autologous hematopoietic progenitor cell transplantation in plasma cell myeloma: a matter of undetermined significance. Bone Marrow Transplant 2012;47:1212–6.
12. Schmitz MF, Otten HG, Franssen LE, van Dorp S, Stroolsma T, Lokhorst HM, et al. Secondary monoclonal gammopathy of undetermined significance after allogeneic stem cell transplantation in multiple myeloma. Haematologica 2014;99:1846–53.
13. Guideline. CADTH Rapid Response Reports. Canadian Agency for Drugs and Technologies in Health 2015.
14. Murray DL, Ryu Euijung R, Snyder MR, Katzmann JA. Quantitation of serum monoclonal proteins: relationship between agarose gel electrophoresis and immunonephelometry. Clin Chem 2009;55:1523–9.
15. Smith A, Wisloff F, Samson D. Guidelines on the diagnosis and management of multiple myeloma. Brit J Haematol 2006;132:410–51.
16. Keren DF. Chapter 9. Protein electrophoresis in clinical diagnosis. Chicago: ASCP Press, 2012.
17. Tiselius A, Horsfall FL. Mixed molecules of hemocyanins from two different species. J Exp Med 1939;69:83–101.
18. Tiselius A, Kabat EA. An electrophoretic study of immune sera and purified antibody preparations. J Exp Med 1939;69: 119–31.
19. Longsworth LG, Shedlovsky T, MacInnes DA. Electrophoretic patterns of normal and pathological human blood serum and plasma. J Exp Med 1939;70:399–413.
20. Kunkel HG, Tiselius A. Electrophoresis of proteins on filter paper. J Gen Physiol 1951;35:89–118.
21. Dirstine PH, MacCallum DB, Anson JH, Mohammed A. Optimum clinical application of serum protein electrophoresis. Clin Chem 1964;10:853–61.
22. Reiner M, Stern KG. Electrophoretic studies on protein distribution in serum of multiple myeloma patients. Acta Haematol 1953;9:19–29.
23. Kyle RA, Bayrd ED, McKenzie BF, Heck FJ. Diagnostic criteria for electrophoretic patterns of serum and urinary proteins in multiple myeloma. J Am Med Assoc 1960;174:107–13.
24. Sax SM, Moore JJ. Computer calculation of serum protein electrophoresis, based on peak height measurements. Clin Chem 1970;16:760–2.
25. Winkelman J, Wydenga DR. Automatic calculation of densitometer scans of electrophoretic strups. Clin Chem 1969;15:708–11.
26. Lotito LA, McKay DK, Seligson D. Analog computation of electrophoresis patterns. Clin Chem 1965;11:386–94.
27. Vanzetti G, Palatucci F, Cosci G. Automatic analysis of electrophoresis strips by means of a cyclic electronic scanner. Clin Chim Acta 1968;20:215–25.
28. Wieme R. Agar gel electrophporesis. Amsterdam: Elsevier, 1965.
29. Rosenfeld L. Serum protein electrophoresis. A comparison of the use of thin-layer agarose gel and cellulose acetate. Am J Clin Pathol 1974;62:702–6.
30. Heremans JF, Masson PL. Specific analysis of immunoglobulins. Techniques and clinical value. Clin Chem 1973;19:294–300.
31. Jorgenson JW, Lukacs KD. Capillary zone electrophoresis. Science 1983;222:366–8.
32. Chen FA, Liu CM, Hsieh YZ, Sternberg JC. Capillary electrophoresis-a new clinical tool. Clin Chem 1991;37:14–19.
33. Jenkins MA, Kulinskaya E, Martin HD, Guerin MD. Evaluation of serum protein separation by capillary electrophoresis: prospective analysis of 1000 specimens. J Chromatogr B Biomed Appl 1995;672:241–51.
34. Jenkins MA, Guerin MD. Optimization of serum protein separation by capillary electrophoresis. Clin Chem 1996;42:1886–6.
35. Keren DF. Capillary zone electrophoresis in the evaluation of serum protein abnormalities. Am J Clin Pathol 1998;110:248–52.
36. Katzmann JA, Clark S, Sanders E, Landers JP, Kyle RA. Prospective study of serum protein capillary zone electrophoresis and immunotyping of monoclonal proteins by immunosubtraction. Am J Clin Pathol 1998;110:503–9.
37. Bossuyt X. Detection and classification of paraproteins by capillary immunofixation/subtraction. Clin Chem 1998;44:760–4.
38. Henskens Y, de Winter J, Pekelharing M, Ponjee G. Detection and identification of monoclonal gammopathies by capillary electrophoresis. Clin Chem 1998;44:1184–90.
39. Keren DF, DiSante AC, Bordine. Densitometric scanning of high-resolution electrophoresis of serum: methodology and clinical applications. Am J Clin Pathol 1986;85:348–52.
40. Keren DF. Protein Electrophoresis in Clinical Diagosis. Chapter 3. Chicago: ASCP Press, 2012.
41. Katzmann JA, Snyder MR, Rajkumar SV, Kyle RA, Therneau TM, Benson JT, et al. 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.
42. Keren DF, Alexanian R, Goeken JA, Gorevic PD, Kyle RA, Tomar RH. Guidelines for clinical and laboratory evaluation of patients with monoclonal gammopathies. Arch Pathol Lab Med 1999;123:106–7.
43. Keren, DF. Procedures for the evaluation of monoclonal immunoglobulins. Arch Pathol Lab Med 1999;123:126–32.
44. Mussap M, Pietrogrande F, Ponchia S, Stefani PM, Sartori R, Piebani M. Measurement of serum monoclonal components: comparison between densitometry and capillary zone electrophoresis. Clin Chem Lab Med 2006;44:609–11.
45. Bergón E, Miranda I, Miravalles E. Linearity and detection limit in the measurement of serum M-protein with the capillary zone electrophoresis system Capillarys. Clin Chem Lab Med. 2005;43:721–3.
46. 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.
47. 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.
48. Rajkumar SV, Harousseau JL, Durie B, Anderson KC, Dimopoulos M, Kyle RA, et al. Consensus recommendations for the uniform reporting of clinical trials: report of the International Myeloma Workshop Consensus Panel 1. Blood 2011;117:4691–5.
49. Katzman JA, Keren DF. Strategy for detecting and following monoclonal gammopathies. In: Deitrick B, Hamilton R, Folds J, editors. Manual of molecular and clinical immunology. Washington, DC: ASM Press, In Press 2016.

50. Murray DL, Seningen JL, Dispenzieri A, Snyder MR, Kyle RA, Rajkumar SV, et al. Laboratory persistence and clinical progression of small monoclonal abnormalities. Am J Clin Pathol 2012;138:690–13.
51. Whicher JT, Calvin J, Riches P, Warren C. The laboratory investigaition of paraproteinemia. Ann Clin Biochem 1987;24:119–32.
52. Katzmann JA, Willrich MA, Kohllhagen MC, Kyle RA, Murray DL, Snyder MR, et al. Monitoring IgA multiple myeloma: immunoglobulin heavy/light chain assays. Clin Chem 2015;61:360–7.
53. Ludwig H, Miguel JS, Dimoopoulos MA, Palumbo A, Garcia Sanz R, Powles R, et al. International myeloma working group recommendations for global myeloma care. Leukemia 2014;28:981–92.
54. Wunsch C. Extending capillary zone electrophoresis (CZE) of serum proteins. Poster Annual AACC meeting 2015.
55. Mancini G, Carbonara AO, Heremans JF. Immunochemical quantitation of antigens by single radial immunodiffusion. Immunochem 1965;2:235–54.
56. Vaerman JP, Lebaco-Verheyden AM, Scolari L, Heremans JF. Further studies on single radial immunodiffusion. I. Direct proportionality between area of precipitate and reciprocal of antibody concentration. Immunochem 1969;6:279–85.
57. Vaerman JP, Lebaco-Verheyden AM, Scolari L, Heremans JF. Further studies on single radial immunodiffusion. II. The reversed system: diffusion of antibodies in antigen-containing gels. Immunochem 1969;6:287–93.
58. Laurell CB. Quantitative estimation of proteins by electrophoresis in agarose gel containing antibodies. Analyt Biochem 1966;15:45–52.
59. Cawley LP, Schneider D, Eberhardt L, Harrough J, Millsap G. A simple semi-automated method of immunoelectrophoresis. Clin Chim Acta 1965;12:105–10.
60. Smith AM, Thompson RA. Paraprotein estimation: a comparison of immunochemical and densitometric techniques. J Clin Pathol 1978;31:1156–60.
61. Hopper JE, Nisonoff A. Individual antigenic specificity of immunoglobulins. Adv Immunol 1971;13:57–99.
62. Killingsworth LM, Savory J. Nephelometric studies of the precipitin reaction: a model system for specific protein measurements. Clin Chem 1973;19:403–7.
63. Savory J, Buffone G, Reich R. Kinetics of the IgG-anti-IgG reaction, as evaluated by conventional and stopped-flow nephelometry. Clin Chem1974;20:1071–5.
64. Whicher JT, Price CP, Spencer K. Immunonephelometric and immunoturbidimetric assays for proteins. Crit Rev Clin Lab Sci 1983;18:213–60.
65. Ritchie RF, Alper CA, Graves J, Pearson N, Larson C. Automated quantititon of proteins in serum and other biologic fluids. Am J Clin Pathol 1973;59:151–9.
66. Keren DF, Frye RM, Datiles TB, Grindon AJ. A modification of the automated immune precipitin method for quantitation of human serum immunoglobulins. Am J Clin Pathol 1978;70:41–4.
67. Rees J, Ohrmundt J. The usefulness of quantifying kappa and lambda light chains. Clin Chem 1986;32:899.
68. Wallage MJ, Whicher JT. "Quantimetric" kappa:lambda ratio test. Clin Chem 1987;33:448–8.
69. Normansell DE. Use of kappa and lambda chain quantitation for the detection of immunoglobulin abnormalities in serum. Diagn Clin Immunol 1987;5:100–3.
70. Whicher JT, Wallage M, Fifield R. Use of immunoglobulin heavy- and light-chain measurements compared with existing techniques as a means of typing monoclonal immunoglobulins. Clin Chem 1987;33:1771–3.
71. Keren DF, Warren FS, Lowe JB. Strategy to diagnose monoclonal gammopathies in serum: high-resolution electrophoresis, immunofixation and kappa/lambda quantification. Clin Chem 1988;34:2196–201.
72. 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;27:335–7.
73. Whicher JT, Warren C, Chambers RE. Immunochemical assays for immunoglobulins. Ann Clin Biochem 1984;21:78–91.
74. Riches PG, Sheldon J, Smith AM, Hobbs JR. Overestimation of monoclonal immunoglobulin by immunochemical methods. Ann Clin Biochem 1991;28:253–9.
75. Bradwell AR, Carr-Smith HD, Mead GP, Tang LX, Showell PJ, Drayson MT, et al. Highly sensitive automated immunoassay for immunoglobulin free light chains in serum and urine. Clin Chem 2001;47:673–80.
76. Katzmann JA, Clark RJ, Abraham RS, Bryant S, Lymp JF, Bradwell AR, et al. Serum reference intervals and diagnostic ranges for free kappa and free lambda immunoglobulin light chains: relative sensitivity for detection of monoclonal light chains. Clin Chem 2002;48:1437–44.
77. 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.
78. Bakshi, NA, Gulbranson R, Garstka D, Bradwell AR, Keren DF. Serum free light chain (FLC) measurement can aid capillary zone electrophoresis in detecting subtle FLC-producing M-proteins. Am J Clin Pathol 2005;124:214–8.
79. Katzmann JA, Dispenzieri A, Kyle RA, Synder MR, Plevak MF, Larson DF. Elimination of the need for urine studies in the screening algorithm for monoclonal gammopathies by using serum immunofixation and free light chain assays. Mayo Clin Proc 2006;81;1575–8.
80. Mori S, Crawford BS, Roddy JVF, Phillips G, Elder P, Hofmeister CC, et al. Serum free light chains in myeloma patients with an intact M-protein by immunofixation: potential roles for response assessment and prognosis during induction therapy with novel agents. Hematol Oncol 2012;30: 156–62.
81. Kyle RA, San-Miguel JF, Mateos M-V, Rajkumar SV. Monoclonal gammopathy of undetermined significance and smoldering multiple myeloma. Hematol Oncol Clin N Am 2014;28:775–90.
82. Kraj M, Kruk B, Lech-Maranda E, Warzocha K, Prochorec-Sobieszek M. High incidence of intact or fragmented immunoglobulin in urine of patients with multiple myeloma. Leukemia Lymphoma 2015; Early Online:1–9, DOI: 10.3109/10428194.2015.1037753.
83. 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.
84. Murata K, Clark RJ, Lockington KS, Tostrud LJ, Greipp PR, Katzmann JA. Sharply increased serum free light-chain concentraitons after treatment for multiple myeloma. Clin Chem 2010;56:16–20.

85. Bradwell AR, Harding SJ, Fourrier NJ, Wallis GL, Drayson MT, Carr-Smith HD, et al. Assessment of monoclonal gammopathies by nelphelometric measurement of individual immunoglobulin kappa/lambda ratios. Clin Chem 2009;55:1646–55.
86. Keren DF. Heavy/Light-chain analysis of monoclonal gammopathies. Clin Chem 2009;55:1606–8.
87. Ludwig H, Milosavljevic D, Zojer N, Faint JM, Bradwell AR, Hübl W, et al. Immunoglobulin heavy/light chain ratios improve paraprotein detection and monitoring, identify residual disease and correlate with survival in multiple myeloma patients. Leukemia 2013;27:213–9.
88. 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.
89. 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.
90. Mills JR, Barnidge DR, Murray DL. Detecting monoclonal immunoglobulins in human serum using mass spectrometry. Methods 2015;81:56–65.
91. Tartakoff A, Vassalli P. Plasma cell immunoglobulin M molecules. Their biosynthesis, assembly and intracellular transport. J Cell Biol 1979;83:284–99.