



A Novel Approach to Estimating M-Protein Concentration: Capillary Electrophoresis Quantitative Immunosubtraction

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Introduction: M-protein quantification is routinely performed by demarcating serum protein electrophoresis (SPE) regions. However, quantification of β -migrating M-protein is hindered by overlapping nonimmunoglobulin protein. Immunosubtraction (ISUB) on capillary electrophoresis is a method currently used qualitatively to subtract out (and therefore highlight) immunoglobulin isotypes in serum, thus reducing the masking effect of normal serum proteins. This study expands on traditional ISUB by developing a quantitative immunosubtraction (qIS) methodology.

Methods: qIS is achieved by estimating the monoclonal class-specific immunoglobulin contribution to the SPE region containing the M-protein. We conducted a recovery study by use of serial dilutions from 3 patients with β -region M-proteins ($n = 22$), performing SPE and ISUB on each dilution. We visualized the difference between the ISUB electrophoresis trace and the involved ISUB isotype-subtracted trace to distinguish M-protein and background polyclonal immunoglobulins, which was demarcated independently by 3 pathologists. The M-protein contribution to the β -region was calculated and applied to the β -region protein concentration producing the quantitative M-protein concentration, while minimizing contamination by nonimmunoglobulin or polyclonal immunoglobulin proteins.

Results: Using a quality target of 25% error, we determined that our analytical measurable range spanned the maximum concentration tested (0.81 g/dL) to 0.05 g/dL. Passing-Bablok regression between qIS and the expected M-protein produced a slope of 1.04 (95% CI, 0.94–1.09), $r = 0.99$. Total CV was 4.8% and intraclass correlation between pathologists was 0.998.

Discussion: qIS promises quantification of β -migrating M-proteins at concentrations an order of magnitude lower than traditional SPE methodology, allowing earlier detection of increasing or decreasing M-protein.

IMPACT STATEMENT

Current multiple myeloma guidelines consider M-proteins under 1 g/dL to be unmeasurable and, due to masking by nonimmunoglobulin proteins, recommend nephelometric IgA measurements for IgA M-proteins migrating in the β -region. This inability to accurately quantify low-concentration M-protein presents a barrier to early identification of relapse and subsequent treatment adjustment. In this manuscript, we show proof of principle for a method of quantitative immunosubtraction, which improves M-protein measurement by reducing the masking effects of both polyclonal immunoglobulin and nonimmunoglobulin proteins. This pilot shows the method can reduce the limit of M-protein quantification below 0.1 g/dL in the difficult-to-measure β -region.

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International guidelines recommend quantifying M-proteins by isolating the region of a serum protein electrophoresis (SPE)³ trace containing an M spike through placement of vertical brackets at the M spike's anodal and cathodal limits and integrating the total area under the curve (1). This "perpendicular drop" can drastically overestimate small M-proteins by including polyclonal immunoglobulins. Improvements can be achieved by "tangent skimming," (2) in which comigrating polyclonal immunoglobulins are distinguished from the overlying M-protein by drawing a line connecting the M-protein's anodal and cathodal deflection points and integrating only the area above that line. Unfortunately, neither method can reliably quantify β -migrating M-proteins because of nonimmunoglobulin interference. Consequently, some forgo reporting β -migrating M-protein concentration unless total β -region concentration surpasses 2 g/dL (3), and recent International Myeloma Working Group (IMWG) guidelines (4) recommend following β -migrating IgA M-proteins by use of total nephelometric IgA levels rather than electrophoresis-generated estimates.

Capillary zone electrophoresis immunosubtraction (ISUB) is currently used in clinical laboratories to subtract out each immunoglobulin isotype from a total SPE trace, thus reducing the masking effect of normal serum proteins and allowing M-protein isotype characterization without immunofixation. ISUB is an effective qualitative assay, but does not quantify M-proteins. This study demonstrates a new method, quantitative immunosubtraction (qIS), which combines the qualitative capabilities of ISUB with the quantitative precision of tangent skimming to quantify β -migrating M-proteins.

METHOD

Routine diagnostic samples from 3 patients with β -migrating M-proteins were identified through convenience sampling (2 IgA κ , 1 free λ). All experiments were conducted with approval of the University of Michigan Institutional Review Board.

Patient samples were evaluated with capillary zone electrophoresis SPE and ISUB performed on the Capillarys 2™ (Sebia; note that Sebia refers to immunosubtraction as "immunotyping") with use of the manufacturer's standard reagents and guidelines as described previously (5). While capillary zone electrophoresis SPE produces a single trace, ISUB produces 6: a protein electrophoresis trace (ELP; functionally equivalent to SPE) plus 5 immunosubtracted (IS) traces, one for each heavy or light chain antiserum.

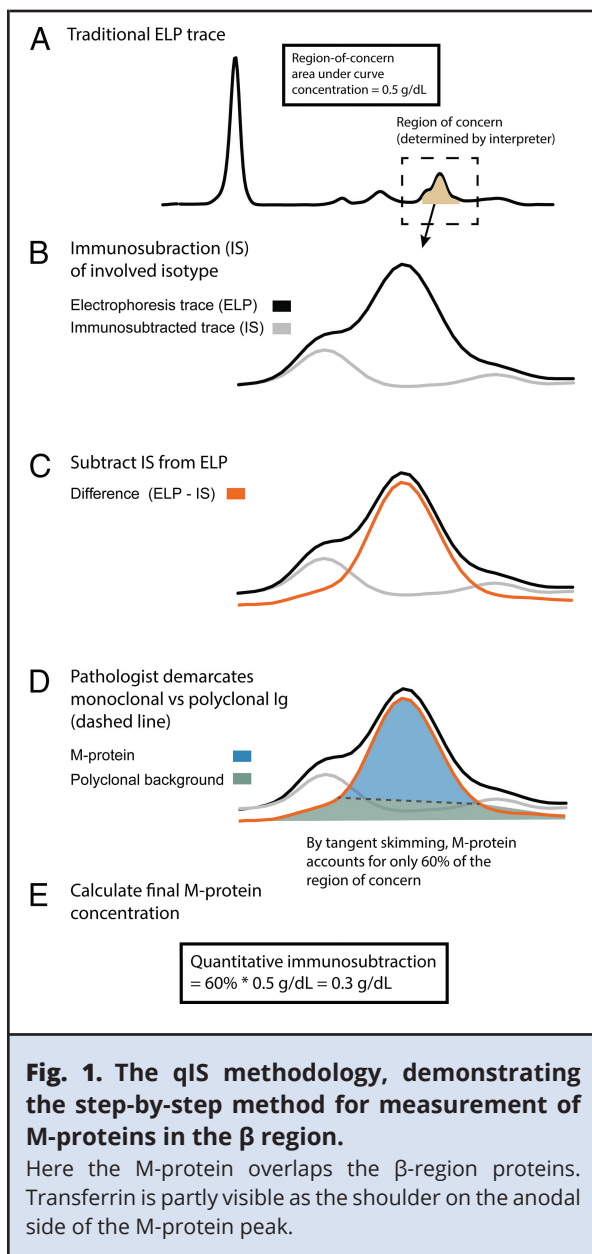
Dilutions (down to 1/256) of patient samples were performed with pooled normal serum. As β -region M-protein concentration is confounded by nonimmunoglobulin proteins, establishing the true M-protein concentration is not straightforward. Therefore, we used qIS to estimate the highest M-protein concentration per patient, and these values were used to calculate expected concentrations of subsequent dilutions. Only these subsequent dilutions were included in the accuracy evaluation.

To quantify immunosubtraction, we exported ISUB traces (ELP and IS traces) and SPE traces from the Capillarys 2 software Phoresis™ into a PostgreSQL file. Traces were imported via SQL query into the R statistical environment (6). M-protein quantification was achieved through 5 steps (Fig. 1): (a) The operator demarcated a region of concern for a suspected M-protein in both the

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³ **Nonstandard abbreviations:** SPE, serum protein electrophoresis; IMWG, International Myeloma Working Group; ISUB, immunosubtraction; qIS, quantitative immunosubtraction; ELP, protein electrophoresis trace; IS, immunosubtracted.



SPE and ELP traces, typically including the entire β region plus any extension of M-protein beyond the β region. Total protein concentration was calculated for this region by use of the SPE trace and knowledge of total protein in the sample. (b) ELP and involved IS traces were overlaid to aid the operator in further localizing M-proteins. (c) The involved IS trace was subtracted from the ELP trace

(ELP-IS) to distinguish M-protein from polyclonal immunoglobulins of the same isotype. (d) The operator performed tangent skimming, from inflection point to inflection point, to demarcate the region in the ELP-IS trace that contained the M-protein rather than polyclonal background. The area of this M-protein region was then divided by the ELP region of concern from step 1 to establish the fraction of that region attributable to M-protein. (e) The M-protein fraction was applied to the SPE region-of-concern protein concentration to obtain a final M-protein concentration with minimal contamination by nonimmunoglobulin or polyclonal immunoglobulin proteins. All steps, except the demarcations in steps 1 and 4, were automatically performed by custom functions written in the R statistical environment.

Without knowledge of calculated M-protein concentrations, 3 pathologists independently performed the 5-step process described above to quantify immunosubtraction. Three rules were followed: (a) Limit the region of evaluation in demarcating monoclonal from polyclonal immunoglobulins in step 4 by use of the traces from step 2. This rule was established to avoid confusion from small deflections in the ELP-IS trace due to minor misalignment and offset baselines between the ELP and IS traces. (b) Use the ELP-IS trace to determine inflection points demarcating monoclonal from polyclonal immunoglobulins, as is commonly performed in the γ region by Phoresis built-in functionality (i.e., tangent skimming; 2). (c) Only attempt measurement when an M-protein was visible in steps 1 and 2.

There are no international guidelines on acceptable error for M-protein concentration measurements. IMWG defines minimal response or progressive disease starting at 25% change from baseline, in which measurable M-protein are those with levels ≥1 g/dL (7). The 1 g/dL used by IMWG may reflect the uncertainty of measurements below this level when performed by the “perpendicular drop” method. IMWG also requires

>0.5 g/dL change for progression, since large relative differences can be seen with small absolute differences at low concentrations. Recognizing we are measuring values well below 0.5 g/dL, we chose 25% as the quality target. Therefore, our analytic measurable range was defined as the contiguous range of concentrations in which qIS produced results <25% from expected values. We depicted the data with respect to this quality target in a relative difference plot comparing measured to expected M-protein concentration. Performance of qIS within the analytic measurable range was evaluated via Passing-Bablok regression and total CV. We computed total CV (including intra- and interpathologist variation) as the ANOVA mean square computed on logarithm-transformed data. A single fit was performed for all measurements made, i.e., for all dilutions within the analytic measurable range for all pathologists. The resulting variance (mean square) directly estimates the CV of the original (untransformed) data (8, 9). Interpathologist consistency was calculated as the intraclass correlation coefficient with the R package "irr," using random effects on columns and rows.

RESULTS

Three pathologists performed qIS measurements on the samples prepared through serial dilutions, resulting in 62 measurements on 22 samples. The samples with the highest concentration per patient were estimated by qIS at 0.67, 1.13, and 1.62 g/dL, and these values were used for calculating expected concentrations for the remaining samples and not used directly to evaluate the method. Therefore, the range of M-protein concentrations used to evaluate qIS from 0.81 g/dL down to near-zero concentration. The absolute differences between qIS estimates and expected concentrations were always <0.1 g/dL. A relative difference plot (Fig. 2) demonstrated qIS estimates of M-protein concentration to be within

the quality target of 25% from the highest concentration (i.e., 0.81 g/dL) down to 0.05 g/dL, with minimal bias. Below 0.05 g/dL qIS estimates were outside of quality goals due to increasing random error and slight negative bias, such that some estimates were above 25% and some below -25%. For measurements on samples with expected M-protein concentrations between 0.05 g/dL and 0.81 g/dL (12 samples, 36 measurements), Passing-Bablok regression produced a slope of 1.04 (95% CI, 0.94-1.09), an offset of -0.01 g/dL (95% CI, -0.02-0.01), and a Pearson $r = 0.99$. Total CV (estimating both inter- and intrapathologist variation) as calculated by ANOVA mean square was 4.8%. The intraclass correlation coefficient between the 3 pathologists was 0.998 (95% CI, 0.996-0.999).

DISCUSSION

qIS achieves quantification of β -migrating M-proteins an order of magnitude lower than traditional SPE methodology, allowing earlier detection of increasing M-protein concentration. IMWG guidelines call for β -migrating IgA M-proteins to be measured by total IgA due to difficulties in quantifying β -region M-proteins. This policy impairs detection of changes in small M-proteins and requires multiple reporting procedures dependent on the involved heavy chain. More accurate M-protein measurements would allow IMWG the opportunity to review this issue. Furthermore, IMWG currently defines "measurable M-protein" as M-protein >1 g/dL. However, with tangent skimming, Schild et al. have shown that accurate measurements can be made at much lower concentrations (2). Also, MGUS cases, which by definition have lower quantities of M-protein, may be able to be more precisely categorized than in current practice. For instance, samples >1.5 g/dL are considered higher risk, but this does not account for underlying polyclonal immunoglobulins (10). Likewise, improved methodologies may allow a more refined

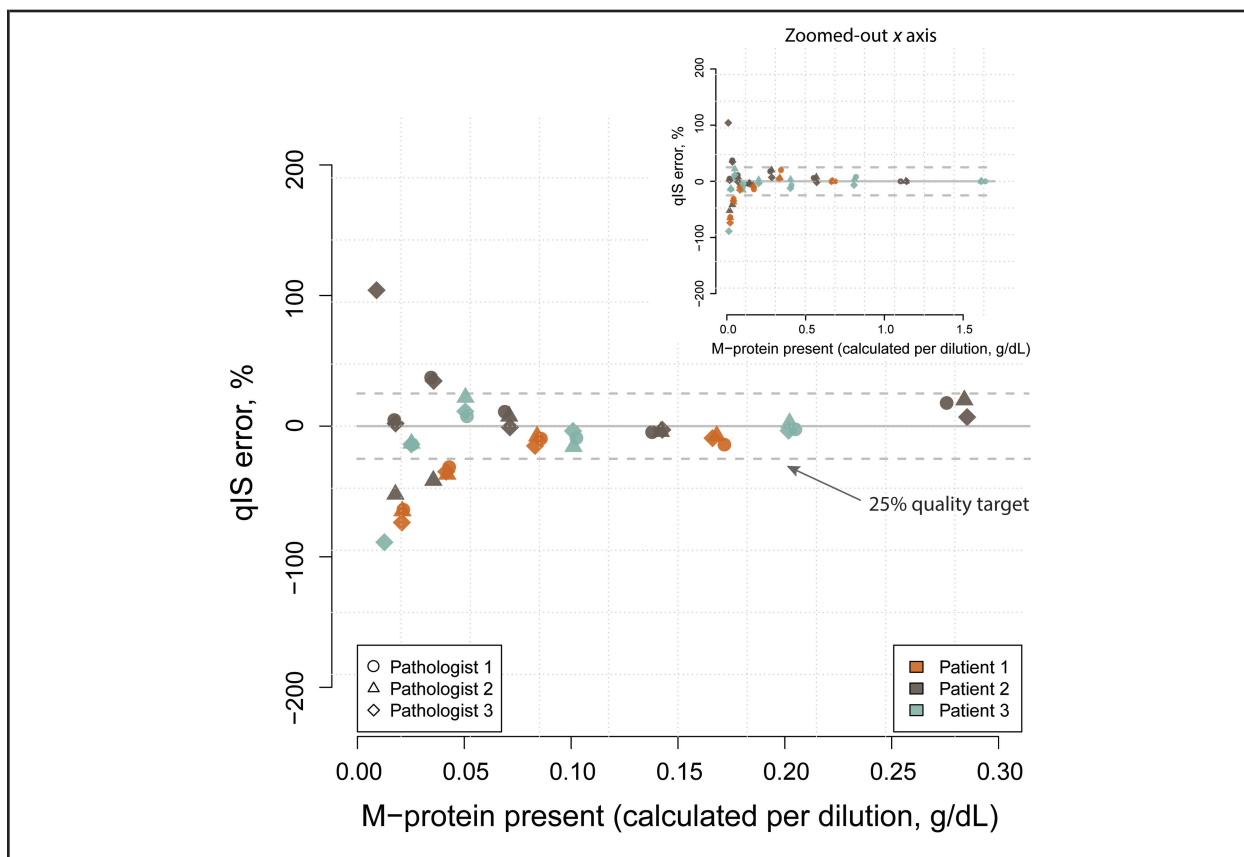


Fig. 2. Relative difference plot between qIS and expected M-protein recovery for samples with expected concentrations less than 0.3 g/dL. Pathologists are coded by shape and patients are coded by color. The dashed lines represent the 25% quality target. The inset shows a zoomed-out view of all concentrations.

definition of the 3 g/dL smoldering multiple myeloma standard. Currently, a patient sample measured by perpendicular drop that contains 1.5 g/dL of M-protein located on top of an additional 1.5 g/dL polyclonal background (or nonimmunoglobulin proteins) could be categorized as having smoldering myeloma. However, this patient's risk is likely different than that of the patient with 3 g/dL of M-protein without significant polyclonal immunoglobulins included in the measurement.

Study limitations include a small sample size with only 3 patients and 22 samples. It is possible that infrequent technical problems may not

have been encountered, perhaps due to patient-specific interferences. Furthermore, we lacked a gold standard estimate for the M-protein concentrations used to calculate expected recoveries and therefore relied on qIS. This may have masked a bias to the method. However, while random error is difficult to improve, bias can often be addressed with calibration. Future studies with enriched samples of known quantities of M-protein will be needed. Finally, improvements might be achieved through horizontal and vertical shifting or stretching of the IS traces to account for slight differences in elution times between instrument capillaries.

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