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# Analytical validation of the Hevylite assays for M-protein quantification

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## Abstract

**Background:** The heavy/light chain (HLC) immunoassay quantifies the different heavy chain/light chain combinations of each immunoglobulin (Ig) class. This makes the HLC assay suited to quantify monoclonal immunoglobulins (M-protein) and for monitoring of patients with monoclonal gammopathies. This method is particularly advantageous for those samples in which electrophoretic quantification of the M-protein is not possible.

**Methods:** In this study we tested the analytical performance of the HLC assay in 166 routine clinical samples and in 27 samples derived from the Dutch external quality assessment (EQA) for M-protein diagnostics (74 participating laboratories). Analytical accuracy was assessed by verification that the sum of the HLC-pairs equaled total Ig concentration. Sensitivity of the HLC assay was determined in a direct method comparison with immunofixation electrophoresis (IFE).

**Results:** Comparison of HLC data with routine Ig diagnostics in 27 EQA samples showed very good correlation for both the quantification of polyclonal and monoclonal IgG, IgA and IgM (Pearson correlations [r] were 0.94, 0.99 and 0.99, respectively; slopes were 0.94, 1.07 and 0.98, respectively). The overall concordance between IFE and the HLC ratio was high (93%) with a Cohen  $\kappa$  coefficient of 0.84.

Discrepancies between both assays were mainly caused by the higher sensitivity of IFE to detect monoclonality.

**Conclusions:** We conclude that the HLC assay is an accurate method to quantify M-proteins that can improve monitoring of M-proteins in the beta fraction that cannot be quantified using electrophoretic techniques.

**Keywords:** heavy/light chain immunoassay; immunofixation electrophoresis; M-protein; monoclonal gammopathy; multiple myeloma; serum protein electrophoresis.

## Introduction

Monoclonal immunoglobulin (M-protein) detection and quantification in multiple myeloma patients is most commonly performed with serum protein electrophoresis (SPE), followed by immunofixation electrophoresis (IFE). Whilst SPE and IFE detect the majority of monoclonal intact immunoglobulins (Igs), additional immunochemical analysis of serum free light chains (FLCs) is recommended for the detection of monoclonal FLCs [1]. Monoclonal bands detected with SPE can be quantified using densitometry. However, in certain cases densitometry is not possible or not very precise, which may hamper monitoring of the M-protein. Cases in which densitometry will not suffice comprise (1) those M-proteins that migrate with other abundantly expressed proteins, such as M-proteins that run in the beta fraction. In this group an M-spike might not be possible [1]; and (2) those M-proteins that fail to resolve as a discrete band, such as M-proteins at low levels or M-proteins amidst high polyclonal background. In this group the variation in quantification is significant [2, 3]. In all patients, additional immunochemical assessment of total Igs is recommended [1].

The Hevylite® assay (heavy light chain [HLC]) is an alternative methodology for monoclonal protein assessment, giving an independent measure of Ig $\kappa$  and Ig $\lambda$  concentrations and is available for IgG $\kappa$ , IgG $\lambda$ , IgA $\kappa$ , IgA $\lambda$ , IgM $\kappa$  and IgM $\lambda$  [4]. As the antibodies bind to junctional regions between the  $\lambda$  or  $\kappa$  light chain and its respective heavy chain partner, the combination of  $\kappa$  and  $\lambda$  measurement allows for the accurate quantification

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of involved and uninvolved Igs. Clonality is assessed by calculating the ratio of involved Ig to background uninvolved Ig concentrations (e.g. IgA $\kappa$ /IgA $\lambda$  in an IgA $\kappa$  patient). As such, the HLC assay can be used for the prognostication and therapy response assessment in patients with multiple myeloma as well as other monoclonal gammopathies [5–7]. The HLC assay also measures levels of the uninvolved Ig chain that serves as parameter of suppression, which is an independent biomarker of clinical status [8]. The HLC assay is a quick, simple and sensitive laboratory serum test that can be performed by nephelometric or turbidimetric methods. In addition, the quantitative results avoid the more subjective interpretation of electrophoresis data.

In this study we set out to investigate the accuracy and sensitivity of the HLC assay in a Dutch cohort of patients with a monoclonal gammopathy and in samples derived from the Dutch external quality assessment (EQA) program for M-protein diagnostics.

## Materials and methods

### Samples

Samples from patients with a monoclonal gammopathy, all proven positive by immunofixation (IFE), were retrospectively selected from the Radboud University Medical Center in Nijmegen and the OLVG in Amsterdam. Sample selection was focused on M-proteins that migrate in the  $\alpha_2$ - and  $\beta$ -region with SPE which causes quantification problems. This is particularly apparent for monoclonal IgA. In total 166 samples derived from 123 individual patients were collected. From 21 of these patients follow-up samples were available, varying from one to four additional samples per patient. Nineteen out of the 166 samples were obtained from newly diagnosed patients.

In addition, samples from the Dutch M-protein EQA program from the Stichting Kwaliteit Medische Laboratoria (SKML) were used. Seventy-four labs in the Netherlands and Belgium participate in this M-protein EQA program. For this study 27 sera from the M-protein EQA program 2014–2016 were available. All samples were coded and

anonymized prior to analysis. The study was performed in accordance with the Helsinki Declaration and was approved by the authors' Institutional Review Board (#2016–2356).

### M-proteins and antibody analysis

For M-protein characterization, agarose gel electrophoresis and IFE were performed on the Hydrasys (Product numbers 4140 and 4881, respectively; Sebia, Evry, France) using reagents from Sebia according to the manufacturer's protocol. M-proteins were quantified using peak fractionation with a perpendicular drop gate and scanning densitometry using the Hydrasys 2 scan. IFE is considered positive if the monoclonal component identified at diagnosis is still present [9]. Serum IgG, IgA, and IgM were either measured on an Immage 800 analyzer (Beckman Coulter, Fullerton, CA, USA) with a nephelometric technique, or using a turbidimetric method on the COBAS8000 (Roche Diagnostics, Basel, Switzerland), both according to the manufacturer's protocols.

### Hevylite

Hevylite tests (IgG $\kappa$ , IgG $\lambda$ , IgA $\kappa$ , IgA $\lambda$ , IgM $\kappa$  and IgM $\lambda$ ; The Binding Site, Birmingham, UK) were run according to the instructions of the manufacturer on the SPAPLUS<sup>®</sup> turbidimeter. In Table 1, the specifications of the six Hevylite tests are depicted. Coefficient of variation (CV) values for the six Hevylite tests in our institute ranged from 2.2% to 7.4%. Reference ranges indicating normal HLC-ratios are as followed: IgG $\kappa$ / $\lambda$ -ratio 1.12–3.21; IgA $\kappa$ / $\lambda$ -ratio 0.78–1.94; IgM $\kappa$ / $\lambda$ -ratio 1.18–2.74.

For serum samples, in case where results were out of the measuring range, another pre-dilution was made and measured. The dilution factor was used in calculating the end results.

### Statistics

Deming regression analysis was performed and Pearson's correlation coefficient  $r$  was calculated to evaluate the correlation between the HLC assay and routine diagnostics. To evaluate qualitative concordance of the HLC assay with routine diagnostics, the Cohen kappa ( $\kappa$ ) coefficient was calculated. Complete agreement was defined as

**Table 1:** Specifications Hevylite kits for SPAPLUS.

Kit and product code	Measuring range	Reproducibility (TBS) CV% from insert	Reproducibility (Sanquin) CV%
Hevylite IgG- $\kappa$ NK621.S	0.09–160 g/L	5.7	3.8
Hevylite IgG- $\lambda$ NK622.S	0.046–118 g/L	8.7	7.4
Hevylite IgA- $\kappa$ NK623.S	0.018–67.2 g/L	11.1	3.1
Hevylite IgA- $\lambda$ NK624.S	0.016–62.4 g/L	6.9	2.5
Hevylite IgM- $\kappa$ NK625.S	0.02–45 g/L	4.9	2.8
Hevylite IgM- $\lambda$ NK626.S	0.018–40.5 g/L	5.4	2.2

CV, coefficient of variation.

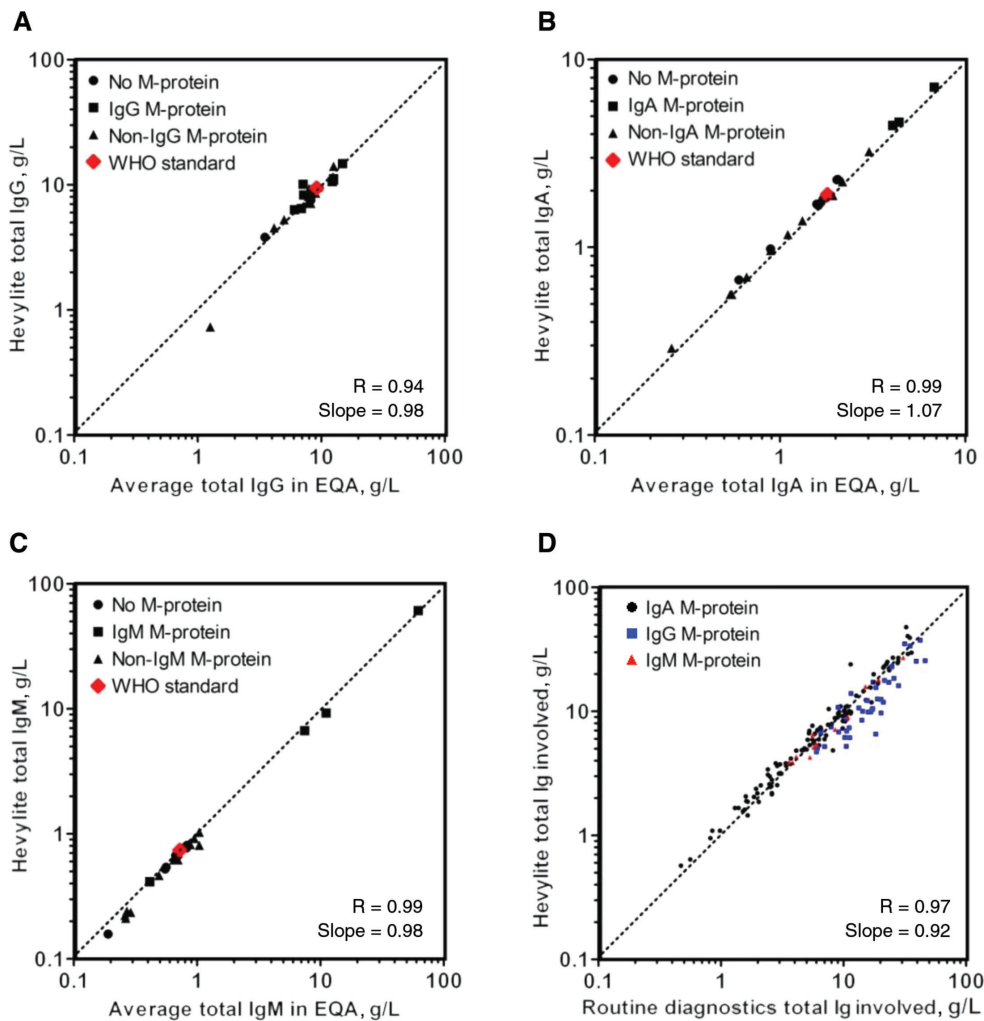
$\kappa$  coefficient = 1, high agreement as  $0.8 \leq \kappa$  coefficient < 1, good agreement as  $0.6 \leq \kappa$  coefficient < 0.8, moderate agreement as  $0.4 \leq \kappa$  coefficient < 0.6, and low agreement as  $\kappa$  coefficient < 0.4. Because of the low number of patients included for serial analysis, statistical analysis for correlation during follow-up was not performed. Statistical analysis was performed using GraphPad Prism 5.03.

## Results

### Analytical performance

Reproducibility of the HLC assay has been validated at the Sanquin diagnostics laboratory (Table 1). In the current

study, we additionally investigated the analytical performance of the HLC assay to quantify Igs. For this purpose, the data of the Dutch M-protein EQA program were used. Seventy four labs participate in this EQA program and report total IgG, IgM and IgA, M-protein detection, characterization and quantification in cases where an M-protein is detected. Twenty-seven samples from the previous 3 years were available. All six Hevylite tests were performed on these samples. In addition, the WHO certified reference material ERM-DA470k/IFCC was included as a serum antibody reference [10]. In Figure 1 the correlation between the routine diagnostic IgA, IgM and IgG measurements of these labs are set out against the HLC results. The data for the 27 samples obtained by these 74 laboratories



**Figure 1:** Method comparison of HLC assay in EQA samples and routine clinical sera.

The sum of  $\kappa$  HLC plus  $\lambda$  HLC was compared to the average total Ig in 27 sera samples measured as part of the Dutch M-protein EQA program (74 participants). All three HLC-pairs are depicted and shown as IgG (A), IgA (B), and IgM (C). The red diamond indicates the ERM-DA470k/IFCC reference material. Panel (D) illustrates the method comparison of the summated  $\kappa$  HLC plus  $\lambda$  HLC concentration for each of the involved Igs with the nephelometric total involved Ig in 155 sera with an M-protein. Symbols in different colors represent the M-protein isotype. The diagonal dotted lines in each panel indicates the  $x=y$  correlation. Deming regression analysis was performed and the slope and Pearson's correlation ( $r$ ) are shown.

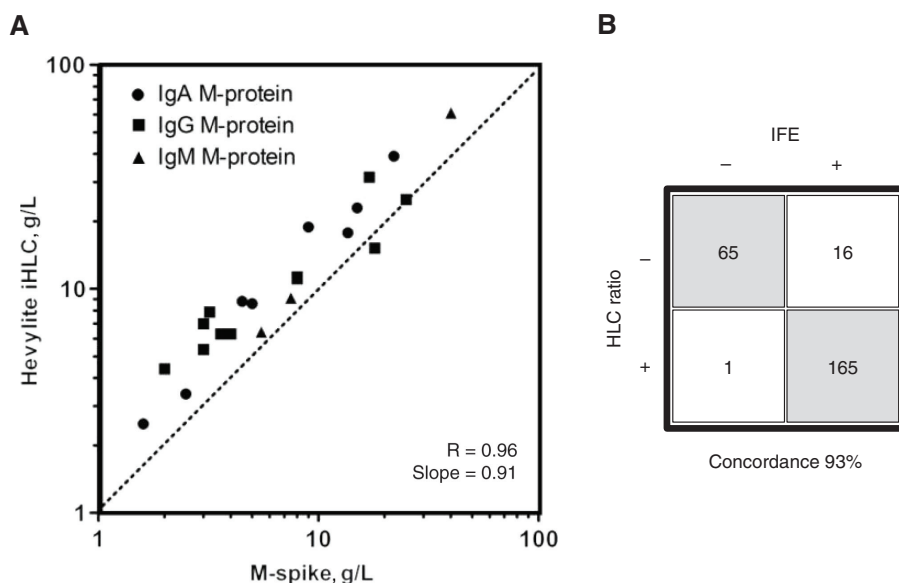
were averaged and depicted on the x-axes. HLC results are depicted on the y-axes (Figure 1A–C). It was shown that the sum of HLC- $\kappa$  plus  $\lambda$  correlated well with the nephelometric and turbidimetric total Ig concentrations obtained in the EQA program. The Pearson correlations ( $r$ ) of IgG, IgA and IgM were 0.94, 0.99 and 0.99, respectively. The slopes of the Deming regression of this method comparison were for IgG, IgA and IgM 0.94, 1.07 and 0.98, respectively. Good correlations were observed in sera with both polyclonal and M-protein. The same trend was observed when the method comparison of the involved Ig was performed in routine clinical sera with a M-protein (Figure 1D).

### Hevylite quantification compared to M-spike quantification

Among the EQA samples and the routine clinical samples, 23 samples had an M-spike. These were further analyzed to compare the involved HLC quantification to the M-spike quantification. The Deming regression of this method comparison had a slope of 0.91 and the Pearson correlation was 0.96 (Figure 2A). In all but one sample, the HLC assay concentration was higher than the M-spike concentration. On average the concentration of the involved HLC was 63% higher than the M-protein concentration obtained with an M-spike ( $p=0.0005$ ).

### HLC ratio versus IFE

IFE analysis of monoclonal bands is currently the most sensitive method to detect intact M-proteins [11]. To determine the sensitivity of the HLC ratio to assess Ig-monoclonality, we compared in both the routine clinical samples ( $n=166$ ) and the EQA samples ( $n=27$ ) the HLC assay with the IFE results. The HLC ratio was in concordance with the IFE result in 93% of the analyses. Most discrepancies were observed in samples that were positive in the IFE and had a normal HLC ratio (16 out of 181 IFE-positive samples) as illustrated in Figure 2B. Relatively most of these discrepancies were observed in samples with an IgG M-protein. In line with the literature [6], we did, however, also observe the opposite in one patient during follow-up of an IgA M-protein whose IFE turned normal and the HLC ratio was still abnormal. The overall concordance between IFE and the HLC ratio was high with a Cohen  $\kappa$  coefficient of 0.84 (95% confidence interval 0.76–0.91). Most discrepancies between both assays were observed in samples with faint IFE bands and borderline normal HLC ratio. In 19 sera derived from patients with newly diagnosed monoclonal gammopathy, we observed 100% concordance between the HLC assay and IFE. These samples were mostly from newly diagnosed multiple myeloma and had on average higher M-protein concentrations as they were all sampled prior to treatment.



**Figure 2:** HLC accuracy and concordance.

(A) Method comparison of the involved HLC with M-spike values of 23 sera with an M-protein that could be quantified with SPE. Deming regression was performed with a slope of 0.91 and a Pearson correlation of 0.96. (B) Concordance analysis of the clinical interpretation of the HLC ratio ( $n=247$ ) and IFE analysis. IFE can either be positive (+) or negative (-) and the HLC ratio is either abnormal (+) or normal (-). The gray boxes indicate agreement between both assays. The overall concordance between the HLC ratios and IFE analysis was 93% and the calculated Cohen  $\kappa$  coefficient was 0.84.

## Follow-up using HLC assay

One of the potential diagnostic applications of the HLC assay is the quantitative monitoring of M-proteins that migrate with other abundantly expressed proteins, such as M-proteins that run in the beta fraction [12]. To evaluate the monitoring capacity of the HLC assay in these patients, four patients with an M-protein that migrates in the beta fraction and at least three sequential serum samples were measured using both the HLC assay and nephelometric total Ig. Figure 3 illustrates the follow-up of the HLC involved Ig and the nephelometric total Ig of two patients with an IgG M-protein (A) and two patients with an IgA M-protein (B). Each patient has a different initial M-protein concentration with unique M-protein kinetics during follow-up. It can be concluded that both assays showed a parallel evolution at all sampling points, indicating that both assays can be used for monitoring the M-protein. At all sampling points the iHLC concentration is lower than the total Ig concentration. This can be explained by the fact that the HLC assay specifically monitors the involved HLC, and therefore measures relatively less polyclonal background. In that respect, the involved HLC assay more accurately represents the M-protein concentration.

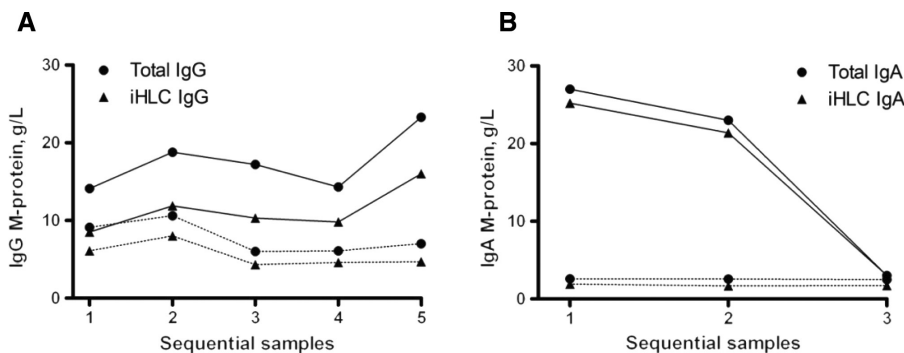
## Discussion

The HLC immunoassay was launched in 2009 to quantify the different heavy chain/light chain combinations of each Ig class [4]. This makes the HLC assay suited to quantify and monitor M-proteins of patients with different types of monoclonal gammopathies. This method is particularly advantageous for those samples in which

electrophoretic quantification of the M-protein is not possible [5, 7].

For analytical validation of novel methods it would be preferential to perform a method comparison using accepted reference materials or samples that are directly traceable to such standards. For M-proteins, no such internationally accepted reference material is available [13]. For a robust analytical validation we obtained samples from the Dutch M-protein EQA program in which 74 different laboratories participate in addition to 166 routine clinical samples. Comparison of the HLC-data with routine Ig diagnostics in these samples showed very good correlation for both the quantification of polyclonal and monoclonal IgG, IgA and IgM (Pearson correlations [ $r$ ] were 0.94, 0.99 and 0.99, respectively; slopes were 0.98, 1.07 and 0.98, respectively). The ERM-DA470k/IFCC, a WHO certified reference material for polyclonal IgG, IgA and IgM [10], was included in this study and was exactly on target with the HLC assay.

Compared to electrophoretic techniques, we observed that the concentration of the iHLC strongly correlates to the SPE M-spike ( $r=0.96$ ). The iHLC concentration was on average 63% higher than the M-spike concentration which. This systematic bias can in our opinion be explained by the fact that the iHLC fraction also contains polyclonal Ig of that specific heavy/light-chain class. In addition, the HLC assay is a turbidimetric assay. In a large cohort of patients, Murray et al. [3] have previously shown that there is a systematic higher quantification of M-proteins by nephelometry/turbidimetry than by SPE. Because of differences between the M-protein quantification methods, SPE and the HLC assay cannot be used interchangeably for monitoring response. When a laboratory considers adopting the HLC assay to monitor a specific patient, it is imperative to run both assays for a



**Figure 3:** M-protein follow-up of sequential serum samples.

Sequential serum samples are shown for two patients with an IgG M-protein (A) and two patients with an IgA M-protein (B). The dotted line and the solid line in both panels each represent two different patients. The M-protein concentration of each sequential serum sample is measured both by nephelometric total Ig (circles) and involved HLC (triangles).



transition period in which a new M-protein baseline can be established for each individual patient monitored.

The overall concordance between IFE and the HLC ratio was high (93%) with a Cohen  $\kappa$  coefficient of 0.84. Discrepancies between both assays were mainly caused by the higher sensitivity of IFE to detect monoclonality, which is in line with previous observations [5, 12, 14, 15]. In our cohort we observed relatively most of these discrepancies in samples with an IgG M-protein, and is caused by the relatively high concentration of polyclonal IgG in these samples. After normalization of the HLC ratio during follow-up of an individual patient, IFE and sFLC measurements are therefore needed to assess whether a patient serologically achieved a stringent complete remission [16]. Moreover, the HLC may not have added value over conventional M-protein diagnostic methods in specific patients such as those with biclonal gammopathies and in patients that suffer from disease relapse caused by FLC escape [17].

Our validation data in EQA samples and monitoring data of individual patients demonstrate that the HLC assay is an accurate method to quantify monoclonal intact Igs, which can improve monitoring of M-proteins migrating in the  $\beta$  fraction of SPE and cannot be quantified using electrophoretic techniques. This supports the statement in the 2016 International Myeloma Working Group (IMWG) consensus criteria for response assessment in multiple myeloma [18], which states that Hevylite can overcome the limitations associated with monitoring  $\beta$ -migrating monoclonal IgA by electrophoresis. The impact on response assessment could not be performed in this study, because the results of analyses of serum FLCs, assessment of percentage clonal cells in bone marrow and radiographic studies were not available for all time points [18]. Several groups have found the HLC ratio to be an independent risk factor for survival in multiple myeloma patients, both at diagnosis and during response [6, 14, 19].

An additional advantage of the HLC assay over current techniques for M-protein assessment is that it measures the suppression of uninvolved Ig, which most likely reflects both treatment related toxicity and tumor related inhibition of Ig production [20]. Suppression of uninvolved Igs is shown to be an important risk factor for progression of MGUS patients and predictor of progression-free survival for patients with multiple myeloma and Waldenström's macroglobulinemia [8, 21–23]. As such, the prognostic significance of HLC responses might partly depend on the patients' ability to recover their immune system, as determined by normalization of the HLC measurements.

As an abnormal HLC ratio may be driven by suppression of the uninvolved HLC, it might indicate the

presence of residual M-protein in cases with low concentrations of the involved HLC [6]. The SPE quantification of low-concentration M-proteins is challenging and has high % CVs [24]. Considering the low % CVs that we have observed here with Hevylite, the HLC assays are likely to be beneficial for quantification of patients with low M-protein concentrations, this however warrants further investigation.

Overall, we conclude that the HLC assay is an accurate method to quantify M-proteins and can improve the monitoring of  $\beta$ -migrating M-proteins that cannot be quantified using electrophoretic techniques.

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