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Research paper

Development of a highly-sensitive multi-plex assay using monoclonal antibodies for the simultaneous measurement of kappa and lambda immunoglobulin free light chains in serum and urine

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ABSTRACT

Monoclonal κ and λ immunoglobulin free light chain (FLC) paraproteins in serum and urine are important markers in the diagnosis and monitoring of B cell dyscrasias. Current nephelometric and turbidimetric methods that use sheep polyclonal antisera to quantify serum FLC have a number of well-observed limitations. In this report, we describe an improved method using specific mouse anti-human FLC monoclonal antibodies (mAbs). Anti-κ and anti-λ FLC mAbs were, separately, covalently coupled to polystyrene Xmap® beads and assayed, simultaneously, in a multi-plex format by Luminex® (mAb assay). The mAbs displayed no cross-reactivity to bound LC, the alternate LC type, or other human proteins and had improved sensitivity and specificity over immunofixation electrophoresis (IFE) and Freelite™. The assay gives good linearity and sensitivity (<1 mg/L), and the competitive inhibition format gave a broad calibration curve up to 437.5 mg/L and prevented anomalous results for samples in antigen excess i.e. high FLC levels. The mAbs displayed good concordance with Freelite™ for the quantitation of normal polyclonal FLC in plasma from healthy donors (n = 249). The mAb assay identified all monoclonal FLC in serum from consecutive patient samples (n = 1000; 50.1% with monoclonal paraprotein by serum IFE), and all FLC in a large cohort of urine samples tested for Bence Jones proteins (n = 13090; 22.8% with monoclonal κ, 9.0% with monoclonal λ, and 0.8% with poly LC detected by urine IFE). Importantly this shows that the mAbs are at least close to the ideal of detecting FLC from all patients and neoplastic plasma cell clones. Given the overall effectiveness of the anti-FLC mAbs, further clinical validation is now warranted on serial samples from a range of patients with B cell disorders. Use of these mAbs on other assay platforms should also be investigated.

1. Introduction

The total of body plasma cells secretes about 1 g per day of kappa and lambda immunoglobulin free light chains (FLCs) into the extracellular fluids. These FLCs are cleared from the blood by glomerular filtration with a half-life of 2 to 6 h (Waldmann et al., 1972). A neoplastic clone of plasma cells must secrete up to 20 g of FLC per day to saturate FLC absorption in the proximal renal tubules of healthy kidneys and thus become detectable in urine (Drayson, 2012). Accordingly it would be preferable to detect and quantitate FLC in blood not urine but this is difficult because serum levels of FLC are mg/L compared to the one thousand-fold higher level of LC bound to whole immunoglobulin. Antibodies for routine clinical quantitation of serum FLC must have specificity for epitopes that are exposed on FLC and hidden on LC bound in whole immunoglobulin; further these antibodies must detect FLC from all patients and neoplastic plasma cell clones.

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Currently there is only one source of FDA approved serum FLC assays (Freelite™, the Binding Site Ltd., UK) (Bradwell et al., 2001). These immunoassays employ purified specific sheep polyclonal antisera adsorbed to render them specific for κ and λ FLCs, respectively, that are latex-enhanced for use in turbidimetric and nephelometric immunoassays. For the first time it has been possible to routinely measure serum FLCs from an array of patient groups that includes oligosecretory myeloma (Drayson et al., 2001), light chain only myeloma (Bradwell et al., 2003), light chain amyloidosis (Lachmann et al., 2003), monoclonal gammopathy of unknown significance (MGUS) (Rajkumar et al., 2004), healthy individuals (Katzmann et al., 2002), and others (Drayson, 2012). Dual measurement of serum κ and λ FLC levels has also highlighted the importance of the κ:λ ratio in the diagnosis and monitoring of B cell malignancies. The κ:λ ratio represents a sensitive balance between the two light chain types, whereby overproduction of one type by a malignant B cell clone leads to a perturbation of the normal κ:λ reference range (Freelite™ κ:λ ratio = 0.26–1.65 (Katzmann et al., 2002)). It is now possible to identify patients with a perturbed serum κ:λ ratio before disease has progressed to the extent that Bence Jones (BJ) protein appears in urine. The serum FLC ratio facilitates diagnosis and monitoring of oligosecretory myeloma and light chain amyloid where serum and urine immunofixation is negative, enables earlier diagnosis of active light chain only disease, reducing the risk of acute kidney damage (Hutchison et al., 2008) and gives prognostic information in all B cell dyscrasias and in healthy individuals (Dispenzieri et al., 2012).

These clinically significant developments are well established and international guidelines recommend the use of Freelite™ in diagnosis and management of a wide range of plasma cell dyscrasias (Dispenzieri et al., 2009). However, this first generation of serum FLC assays has technical limitations. A separate test for each κ and λ FLC measurement is required, introducing inter-test error and reducing the reliability of the κ:λ ratio result obtained. This variability is compounded further by the batch-to-batch differences observed in the polyclonal antisera produced from individual sheep (Tate et al., 2007, 2009). In clinical practise, it is important to detect both the elevation of one FLC type by secretion of malignant FLC and the reduction in levels of the alternate FLC by immunoparesis. Thus assays need to quantitate FLC levels ranging from 1 mg/L to >1000 mg/L. The latex-enhanced antisera have a calibration range of 3.7–56.2 mg/L for κ FLC and 5.6–74.8 mg/L for λ FLC, and are unreliable at the lower end. This can lead to an abnormal κ:λ ratio in healthy individuals and apparently significant changes in ratio between sequential samples from myeloma patients who are in fact still in remission. This problem is highlighted by 'gaps' above and below the working calibration range of the assay (Bradwell, 2008). The limited calibration range also requires that samples with high FLC be diluted several times. The assay is prone to antigen-excess (or "hook effect") which can cause false negative diagnoses in patients with grossly elevated FLC and false positive evidence of disease progression (Daval et al., 2007; Levinson, 2010a; Murata et al., 2010). Monoclonal FLC paraproteins tested on Freelite™ have been shown to be non-linear (Tate et al., 2007) meaning that dilutions could lead to inaccurate FLC quantitation. The polyclonal antisera in the assay are targeted against polyclonal FLC, as opposed to monoclonal FLC, potentiating the claim that the Freelite™ sensitivity to paraprotein levels slightly outside the normal reference range is negatively affected (Levinson, 2010b). Further, there are reports that the antisera are cross-reactive with bound κ and λ LC (Davern et al., 2008) leading to excessively high FLC results not representative of absolute FLC levels. A second generation of serum FLC tests is needed to overcome these problems. If monoclonal antibodies (mAbs) could be produced that specifically target human κ and λ FLCs, then they would provide a long term solution to the problems of the current polyclonal Freelite™ assay.

Development of FLC specific mAbs is difficult and complicated by the paucity of constant domain epitopes available for FLC recognition; which can be further reduced by polymerisation of FLC, particularly FLC λ, thus reducing the number of potential binding sites (Abraham et al., 2002; Bergen et al., 2004; Davern et al., 2008). Production of mAbs specific for FLC has been described previously (Abe et al., 1993, 1998; Nakano and Nagata, 2003; Davern et al., 2008) and these groups have demonstrated mAb specificity for epitopes that are exposed on FLC and hidden on LC bound in whole immunoglobulin. However these groups have either found that their mAbs did not detect FLC from all neoplastic plasma cell clones tested or have not tested sufficient clones to be confident that the mAbs would detect the FLC from at least 95% of neoplastic clones. Recently another group reported anti-FLC mAbs (te Velthuis et al., 2011; Hoedemakers et al., 2012) again, specificity with at least 95% of neoplastic FLC clones appears unlikely, especially for λ FLC (Drayson and Carr-Smith, 2012; Hutchison et al., 2012).

In the present study, we describe the development and initial validation of two anti-κ FLC and two anti-λ FLC mAbs in a competitive-inhibition multi-plex Luminex® assay (mAb assay). Whilst it is important that the new assay overcomes the problems with existing commercial assays, initial clinical validation must also demonstrate that the mAbs provide: (1) similar quantitation of polyclonal FLC from healthy donors to the Freelite™ assay; (2) appropriate sensitivity to reliably quantify low levels of FLC representative of immunosuppression or immunoparesis; and (3) by testing a large number of serum and urine samples it shows that the mAbs are at least close to the ideal of detecting FLC from all patients and neoplastic cell clones.

2. Materials and methods

2.1. Ethical approval

Ethical approval for development and validation of the FLC assay using residual, end-of-diagnostic use of patient serum and urine was granted by the Life and Health Sciences Ethical Review Committee of the University of Birmingham, UK. Financial support for the study was provided by the Clinical Immunology Service, University of Birmingham, UK.

2.2. Preparation of anti-FLC mAbs

Anti-FLC mAbs were prepared using standard methods (Galfre and Milstein, 1981). Briefly, BALB/c mice were immunised with κ or λ FLC purified from human urine

containing BJ Protein or immunoglobulin fragments. Spleens from immunised mice were dispersed into single cell suspensions, mixed with immortal mouse plasmacytoma cells (NSI, NSO) and fusions of cells facilitated with polyethylene glycol (PEG). The cell mixture was plated out in 96 well plates with selection being facilitated with hypoxanthine, thymidine, and methotrexate. Supernatants from wells containing clones were assayed for production of antibodies specific for κ or λ FLC. Anti-FLC mAbs were selected based on reactivity with a large panel of κ or λ FLC antigens containing a variety of variable and constant regions, and shown to be negative for alternate FLC, light chains bound to immunoglobulins of all classes and subclasses, albumin and other selected human serum proteins. Initial assays were performed in haemagglutination and haemagglutination inhibition assays where sheep red blood cells were coupled to purified FLC from individual patients (Ling et al., 1977). Ascites cells were adapted to in vitro culture, and were expanded in a mini-perm bioreactor. Bioreactor supernatants (MiniPerm, Sarstedt) containing anti-FLC mAbs were purified using protein G or SpA chromatography (GE Healthcare). Purified mAb collections were diluted 1/100 and quantified by spectrophotometry (Eppendorf) at 280 nm for protein concentration, with 1.43 extinction coefficient (Hay et al., 2002). Initially, anti-FLC mAbs were selected based on reactivity with all κ or λ FLC antigens in a panel of different BJ proteins, and minimal cross-reactivity to a panel of purified whole immunoglobulins. Specificity was established by covalently coupling mAbs to Luminex® Xmap® beads (Bio-Rad, UK) and quantifying polyclonal light chains from dithiothreitol treated immunoglobulin infusate (Gammagard Liquid), which was then reduced and/or acetylated and separated on a G100 column in the presence of proprionic acid, and quantified using Freelite™. In addition, specificity was established on the Luminex® against: (a) a panel of serum samples from patients with elevated polyclonal light chains and myeloma; and, (b) a panel of urine samples containing BJ proteins. From this process, two anti-κ (BUCIS 01 and BUCIS 04) and two anti-λ (BUCIS 03 and BUCIS 09) FLC mAbs were chosen for further development and initial validation in the mAb assay (Serascience, UK).

2.3. Preparation of calibrator material

Individual urines containing a high level of BJ protein were centrifuged and 0.2 μm filtered. Purity assessment was conducted by SDS Page and those identified as showing a single band of monomeric FLC and/or single band of dimeric FLC, indicating that there were no other proteins visible, were dialysed against deionised water with several changes of water. Each preparation was passed over activated charcoal, concentrated by vacuum dialysis, and freeze-dried on a vacuum dryer and protein stored at 4 °C. Calibrator material was made by combining four sources of purified BJ λ protein and five sources of BJ κ protein. 105 mg of each FLC protein was dissolved in 15 mL saline, overnight at 4 °C. The supernatants were 0.2 μm filtered before measuring the concentration by spectrophotometry at 280 Å at a dilution of 1/100 and extinction coefficient of 11.8 (Hay et al., 2002). Equal amounts of each BJ κ or λ protein were combined and the volumes of the two preparations were adjusted with sterile PBS to a concentration of 7 mg/mL. Sodium azide was added from a 0.2 μm filtered preparation of 9.9% w/v in deionised water to give a final concentration of 0.099%. The preparations were aliquoted into 1 mL volume and stored at −80 °C.

2.4. Biotinylation of immunoglobulin free light chains

For the purposes of a competitive inhibition assay, individual κ and λ FLC mixes were biotinylated for later binding to streptavidin-PE in the assay phase, as follows. Purified κ and λ FLC calibrator materials were separately incubated with biotinamidohexanoyl-6-aminohexanoic acid, N-hydroxysuccinimide ester in dimethyl sulfoxide overnight (all Sigma Aldrich). Biotinylated light chains were then separated using NAP™ 5 Columns (Sephadex G-25 DNA grade; GE Healthcare), eluted in PBS, and the concentration of the eluate was measured by spectrophotometry. After the addition of 0.099% sodium azide, biotinylated light chains were stored at 4 °C, until required.

2.5. Luminex® bead—mAb couplings

Prior to assaying, each of the anti-κ FLC and anti-λ FLC mAbs was covalently coupled to four different 5.5 μm polystyrene Xmap® beads (Bio-Rad UK) using a two step carbodiimide reaction protocol. Specifically, the different bead regions used were #27 (BUCIS 01), #28 (BUCIS 04), #29 (BUCIS 03), and #30 (BUCIS 09). After sonication and vortexing, beads were incubated with N-hydroxysuccinimide (NHS) and 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide hydrochloride (EDC) for 30 min in activation buffer (0.1 M PBS, pH 6.2). Following two wash procedures, the bead pellet was incubated for 3 h with the relevant mAbs (100 μL at 1 mg/mL) on a rotor, in the dark, at room temperature. Beads were then washed twice, and finally, the pellet was resuspended in blocking/storage buffer (0.1 M PBS, 0.05% Tween20, 1% BSA, 0.05% NaN_3, pH 7.2). The beads were enumerated using a haemocytometer to ensure consistency between conjugations. Labelled beads were stored in blocking/storage buffer in the dark at 4 °C until required in the assay; the beads were found to be stable for at least 6 months in these conditions (data not shown).

The efficiency of mAb conjugation to each beadset was determined in a one-step assay by incubating each beadset with goat anti-mouse IgG labelled with phycoerythrin (PE; Southern Biotech, USA), and subsequent measurement by Luminex®. The effectiveness of each mAb-bead complex at detecting κ and λ FLCs was tested in a two-step assay by (1) incubating beads with biotinylated purified κ and λ FLCs, and (2) incubation with streptavidin-PE (Invitrogen, UK), and subsequent measurement by Luminex®. A minimum median fluorescent intensity (mfi) was set at >10,000 mfi units for mAb conjugation to beads and >5000 mfi units for detection of κ and λ FLCs, as this provided calibration curves with the most sustained linearity, and, hence, more reliable and reproducible results on patient samples.

2.6. mAb assay for measurement of κ and λ FLCs

Human κ and λ FLCs were measured in a multi-plex competitive inhibition format. 40 μl of biotinylated FLC

diluted 1 in 400 in FLC buffer (PBS, 12.5% 2 M Tris, 1% BSA, 0.099% NaN_3, 0.05% Tween20) was added to each well in a 1.2 μm MV Multiscreen (Millipore, UK) 96-well filter plate, followed by 40 μl of each sample, calibrators or controls, and then incubated for 30 min with mAb coupled beads. Each sample was diluted 1 in 5 in assay buffer (PBS, 1% BSA, 0.05% NaN_3) prior to addition to the filter plate. A seven-point calibration curve with a five parameter logistical curve fitting was used (BioPlex Manager 6.0, BioRad UK). The calibration material was generated by mixing an equal amount of the stock κ and λ FLC material, and then diluting this 1 in 8 in FLC buffer to give the starting calibration point (437.5 mg/L). The top calibrator was then serially diluted 4-fold in FLC buffer to 0.1 mg/L, in duplicate. In-house quality controls were used on all assay plates to monitor assay performance and reproducibility. Following incubation for 30 min, filter plates were washed three times using assay buffer and aspirated using a manifold pump. 50 μl streptavidin-PE (diluted 1 in 500 in assay buffer) was added to all wells and incubated for 30 min. After further washing, plates were analysed on a Luminex® 100 system (Luminex Corp., USA). A minimum of 100 beads per bead region, per well of the filter plate, were counted on the Luminex®. Samples exhibiting a high FLC concentration above the initial working range of the calibration curve at a 1 in 5 dilution, were repeated at a 1 in 100 dilution in assay buffer, to avoid extrapolation and ensure reliable quantitation of samples on the linear sectors of the standard curves (see Fig. 1 for representative calibration curves).

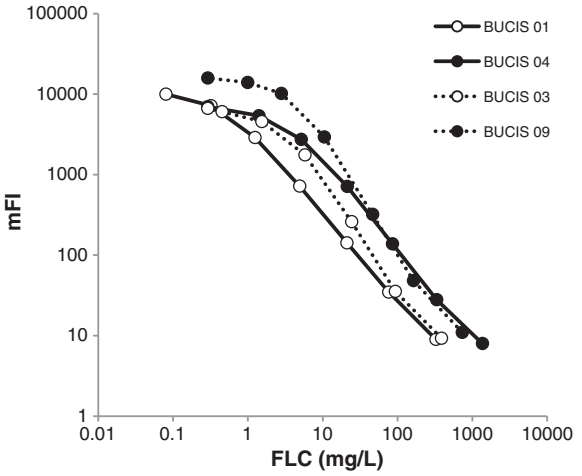


Fig. 1. Representative calibration curves showing the median fluorescent intensity (mFI) and FLC concentration (mg/L) generated by the mAb assay for each anti-κ FLC mAb (BUCIS 01 and BUCIS 04) and each anti-λ FLC mAb (BUCIS 03 and BUCIS 09). These representative curves included a calibration coefficient that was applied to each mAb, as described in Section 2.7. Briefly, the process of applying a calibration coefficient began by conducting an initial method comparison between each anti-FLC mAb and Freelite™ on 249 healthy donor plasma samples, to investigate whether each anti-FLC mAbs provided a similar quantitation of polyclonal FLC. From this process, it was clear that each anti-κ FLC mAb provided different results for polyclonal FLC, and each provided different results to Freelite™; the same was found for each anti-λ FLC mAb. Hence, it was necessary to use different calibration coefficients for each mAb to provide similar quantitation of polyclonal FLCs to Freelite™. Therefore, a calibration coefficient was applied to the calibrator material (mix of monoclonal FLC from the urine of myeloma patients) results obtained by spectrophotometry for κ FLC (437.5 mg/L) and λ FLC (437.5 mg/L), and these were: BUCIS 01 = 0.731X, BUCIS 04 = 3.086X, BUCIS 03 = 0.869X, BUCIS 09 = 1.600X (where X is equal to the spectrophotometry result).

2.7. Assignment of a calibration coefficient to the mAb assay

To establish if each anti-κ FLC mAb provided a similar quantitation of polyclonal κ FLC, and each anti-λ FLC mAb provided a similar quantitation of polyclonal λ FLC, an initial method comparison of each mAb was conducted using 249 donor plasma samples from the UK NHSBT. From this process, it became clear that each anti-κ FLC mAb provided different results for polyclonal FLC, and subsequent analyses found that each provided different results to Freelite™; the same was found for each anti-λ FLC mAb (data not shown). Hence, it was necessary to use different calibration coefficients for each mAb to provide similar quantitation of polyclonal FLCs to each other, and to Freelite™. Final calibration coefficients were derived by a method comparison (Krouwer et al., 2010) to the Freelite™ assay for polyclonal FLC (Katzmann et al., 2002). Calibration traceability to Freelite™ was preferred because there is no recognised international standard for FLC, and to ensure that the guidelines issued by the International Working Group on Multiple Myeloma (Dispenzieri et al., 2009) are transferable to the mAb assay, as discussed elsewhere (te Velthuis et al., 2011). Accordingly, a calibration coefficient was applied to the calibrator material result obtained by spectrophotometry for κ FLC (437.5 mg/L) and λ FLC (437.5 mg/L). For each anti-FLC mAb, the following calibration coefficients were applied to the calibrator material: BUCIS 01 = 0.731X, BUCIS 04 = 3.086X, BUCIS 03 = 0.869X, BUCIS 09 = 1.600X; where X is equal to the calibrator result by spectrophotometry. Representative calibration curves are displayed in Fig. 1.

2.8. Assay dynamics

Assay limit of detection was measured by spiking a pool of normal plasma samples with no more than 20% volume of purified BJ κ or λ protein from patient urine. These spiked samples were serially diluted 1 in 4 in assay buffer and measured on the mAb assay. Serial dilutions were replicated five times within the same plate, and the limit of detection for each mAb was then assessed. The limit of detection for each mAb was determined by selecting the lowest concentration detected by the mAb assay above the blank well containing only assay buffer (no BJ protein).

Assay linearity was assessed by serially diluting three serum samples containing elevated levels of monoclonal κ FLC and three samples containing elevated levels of monoclonal λ FLC, two-fold in assay buffer. These six samples were serially diluted nine times with three replicates of each dilution conducted within the same plate. Linearity of the mAb assay was then assessed on the ten sample dilutions. Because competitive inhibition assays are inherently non-linear, a strategy for demonstrating linearity was conducted by comparing the expected results against the acquired results from the serial dilutions.

Assay batch-to-batch variability was assessed by analysing fifty serum samples with varying FLC levels once, on separate assay days, using three consecutive batches of anti-FLC mAbs, calibrators and other appropriate assay reagents.

Assay imprecision was estimated by calculating the intra-assay coefficient of variation percentage (CV%) and the inter-assay CV%. For these tests, pools of samples with low, medium and high levels of κ and λ FLCs were used. All samples were analysed in duplicate, every morning, for ten working consecutive days, and all tests were conducted in accordance with the Clinical and Laboratory Standards Institute (CLSI) guideline EP5-A2 (Tholen et al., 2004).

The susceptibility of the mAb assay to interference was measured by adding known quantities of interference agents to a pool of National Health Service Blood and Transplant Service (NHSBT) plasma samples containing normal κ (11.12 mg/L) and λ (7.62 mg/L) FLC levels. Individual aliquots of the plasma pool were spiked with purified IgG-κ (3.5 g/L), IgG-λ (3.6 g/L), IgA-κ (1.5 g/L), IgA-λ (3.2 g/L), IgM-κ (6.5 g/L), IgM-λ (3.7 g/L), haemoglobin (4 g/L), bilirubin (0.2 g/L), cholesterol (2 g/L), triglyceride (5 g/L), as well as κ FLC (2 g/L) or λ FLC (2 g/L). Interference testing was conducted in accordance with CLSI guidelines EP7-A2 (McEnroe et al., 2005).

For all tests on assay dynamics, except mAb limit of detection, the maximal value obtained from each anti-κ FLC mAb (BUCIS 01 or BUCIS 04) was used as the final κ result, and the same approach was used for each anti-λ FLC mAb (BUCIS 03 and BUCIS 09) for λ FLC results.

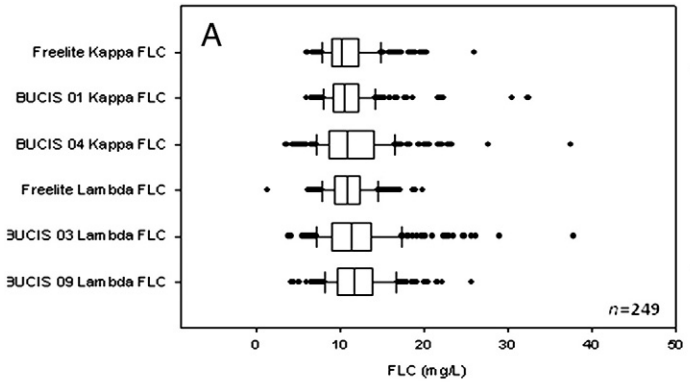
2.9. Reference values

250 plasma samples obtained from healthy random donors (NHSBT UK) were measured using the Freelite™ and mAb assays; results obtained by all four anti-FLC mAbs were used for these analyses. All samples were first screened for paraproteins by routine serum immunofixation electrophoresis (IFE). Results were presented as the 95% reference populations, as well as the 100% reference range, so as to allow a comparison to the Freelite™ assay (Katzmann et al., 2002). To generate the 95% reference results, extreme outliers three times the size of the inter-quartile range were removed. Due to a positively skewed distribution after outlier removal, results were ranked by z-scores to identify the central 95% intervals.

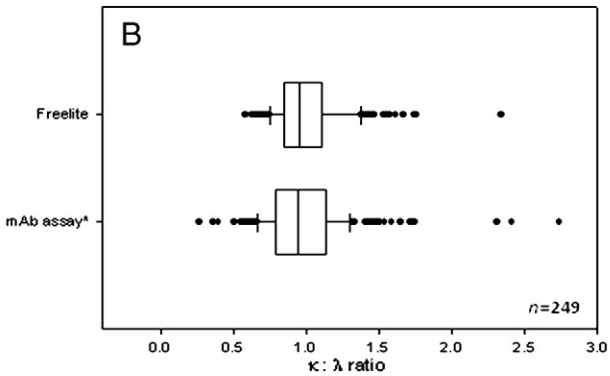
2.10. Clinical specificity on patient serum samples and urine samples

1000 consecutive serum samples received by the Clinical Immunology Service (CIS) for routine clinical measurement of κ and λ FLCs (on Freelite™) were analysed simultaneously on the mAb assay. This exercise served three purposes: to establish the specificity of each mAb at detecting FLC levels in patients with a wide range of disease conditions; to make a comparison with Freelite™; and to serve as a preliminary assessment of the mAb assay in a clinical setting. 209 samples were from patients enrolled in myeloma trials. Of the 791 non-trial patient samples, 292 had a known serum paraprotein, 106 had no paraprotein, and no admission diagnosis was available for the remaining 393 samples. In addition, 289 samples had a matched urine sample and 711 samples had no matched urine. Samples were collected chronologically throughout July and August 2011 as they arrived at the Clinical Immunology Service, and inclusion criteria required the sample volume be greater than 500 μL; no other inclusion/exclusion criteria were set. Results generated by each mAb were compared to the results obtained by Freelite™. Experimenters were blind to the original Freelite™ result and patient diagnosis. Any discrepant results between Freelite™ and the mAb assay were repeated on both platforms to exclude the possibility of user/instrument error. A discrepancy was defined as any sample with an abnormal κ:λ FLC ratio on one assay but not the other, or, an elevated FLC concentration outside the normal 95% reference range on one assay but not the other (see Fig. 2 for reference ranges). To exclude the possibility that anti-FLC mAbs 'missed' any monoclonal FLC, any discrepant samples were further investigated by routine serum IFE analysis, IFE and mAb assay analysis of urine, and patient history, if available.

The specificity of the anti-FLC mAbs at measuring FLC levels and ability to detect FLC from all patients was further tested in a large cohort of urine samples. An initial comparison was made between the mAb assay and commercially available radial immunodiffusion assays (RID; the Binding Site, UK). Correlations between the two assays were good (results not shown) and a further comparison was made between the mAb assay and densitometric scanning of protein electrophoresis, regarded as the "gold standard" in urine FLC paraprotein quantitation. Individual concentrated urine (30× concentrated; Zeba) was analysed by densitometry according to the manufacturer's instructions (Interlab, Italy), total protein (Total Protein Gen.2, Roche) was estimated on a Roche Hitachi Analyser, and for comparison, FLCs were also analysed on the mAb assay. Results demonstrated that the mAb assay correlated well with densitometry (representative data in Fig. 3). Given the success of the mAb assay at detecting FLC in urine, the clinical utility of the mAb assay was then assessed in 13,090 unconcentrated urine samples sent to the laboratory for routine FLC analysis between April 2008 and Nov 2010. All samples were also analysed by urine IFE (the gold standard for presence of LC in urine) to assess the specificity of the mAb assay, and to ensure that the mAb assay detected all FLC paraproteins. All samples were analysed as they arrived in the laboratory. After initial routine analyses, samples were stored at −20 °C. 2995 samples (22.8%) had monoclonal κ, 1180 samples (9.0%) had monoclonal λ, and 105 samples (0.8%) had poly LC, as detected by IFE. 12,242 of these samples were from patients who had a known immunoglobulin paraprotein in serum by IFE (93.5%), 641 samples had no paraprotein in matched serum, and 207 had no serum IFE diagnosis or no serum available. 3806 samples were received from patients enrolled in myeloma trials and the remaining 9284 samples were non-trial samples. Because two anti-κ FLC and two anti-λ FLC mAbs were used in each test, the maximal concentration detected by each anti-κ (BUCIS 01 or BUCIS 04) and each anti-λ mAb (BUCIS 03 or BUCIS 09) was chosen as the final urine FLC result. As a means of determining the specificity of the mAb assay in urine, any results that were immunofixation positive and mAb assay negative (recorded clinically as <10 mg/L), were classed as discrepant. To ensure that each of the anti-FLC mAbs targeted all FLC epitopes, all discrepant samples were re-tested on the mAb assay and by urine IFE. If a discrepancy remained, a full urine IFE was conducted to exclude the presence of whole paraprotein because initial IFE used anti-sera against LC free and bound. Further investigation of matched serum



	n	95% median (±SD) mg/L	95% reference range mg/L
Freelite FLC κ	234	10.12 (± 2.11)	6.00 – 15.91
BUCIS 01	233	10.45 (± 1.95)	6.46 – 15.10
BUCIS 04	235	10.62 (± 3.26)	4.35 – 19.44
Freelite FLC λ	239	10.77 (± 2.16)	6.26 – 16.02
BUCIS 03	232	11.03 (± 3.03)	4.13 – 19.18
BUCIS 09	233	11.55 (± 2.80)	5.19 – 18.87



	n	95% median (±SD)	95% reference range
Freelite κ:λ ratio	247	0.96 (± 0.23)	0.58 – 1.76
mAb assay κ:λ ratio*	239	0.93 (± 0.24)	0.40 – 1.59

Fig. 2. A. κ and λ FLCs diagnostic ranges of Freelite™ and the mAb assay derived from the analysis of 249 plasma samples from healthy donors. Whisker boxplots illustrate the 5%, 25%, median, 75% and 95% intervals and extreme values (<5% & >95% intervals) are dotted. The full diagnostic ranges represent 100% of the healthy donors (n = 249) after IFE screen. The adjacent table details the κ and λ FLCs 95% median, standard deviation and reference ranges derived from the same samples; due to a positively skewed distribution after removal of extreme outliers, z-scores were used to identify the central 95% median and reference ranges. 2B. κ:λ FLC ratio diagnostic ranges derived from Freelite™ and the mAb assay. As above, the full diagnostic ranges represent 100% of the healthy donors (n = 249) after IFE screen. The adjacent table details the κ:λ FLC ratio 95% reference ranges derived from these samples and, as above, after removal of outliers these were calculated using z-scores to identify the central 95% median, standard deviation and reference ranges. *The ratio obtained by BUCIS 04 and BUCIS 09 was used for these calculations.

and patient history was conducted where necessary and available.

2.11. Routine laboratory assays

Freelite™ κ and λ FLC assays were conducted on a Roche Hitachi Modular analyser using manufacturer's instructions. The reported working range of Freelite™ on this instrument from the manufacturer was 3.7–56.2 mg/L for κ FLC and 5.6–74.8 mg/L for λ FLC (Bradwell, 2008).

Urine and serum IFE was performed using Hydragel IF 2/4 gels on a Hydrasys analyser according to manufacturer's instructions (all antisera from Sebia, France). Routine serum IFE comprised a panel of antisera against: bound and free κ and λ LC, IgA, IgM, and IgG. Where necessary, antisera against IgD, IgE, and κ and λ FLCs were also used. Routine IFE on unconcentrated urine comprised a panel of antisera against bound and free κ and λ LC. Where necessary, antisera against IgA, IgD, IgE, IgG, IgM and κ and λ FLCs were used. In-house detection limits for κ and λ FLCs in unconcentrated urine using bound and free κ and λ antisera was approximately 10 mg/L; not replicating the sensitivity of IFE in concentrated urine (Palladini et al., 2009). The sensitivity of bound and free κ and λ LC antisera in serum was approximately 100 mg/L (representative images not shown).

2.12. Statistical analysis software

All statistical analyses were conducted using PASW Statistics Version 18 (IBM, USA) with the exception of assay linearity, batch-to-batch variability and mAb correlations with Freelite™, which were assessed using the Microsoft Excel Add-in Analyse-it (version 2.26, www.analyse-it.com). Spearman correlations were ranked as 'good' between 0.75 and 0.90, and 'excellent' above 0.90. All figures were produced using SigmaPlot version 11.0 (Systat Software Inc., USA).

3. Results

3.1. Assay reference ranges

250 plasma samples from healthy donors were analysed for κ and λ FLCs using the mAb assay and a comparison was made between each of the anti-FLC mAbs, and to Freelite™. All samples were pre-screened for paraproteins by routine serum IFE analysis. IFE revealed that one sample had an IgG λ paraprotein with λ FLC, and the sample was excluded from

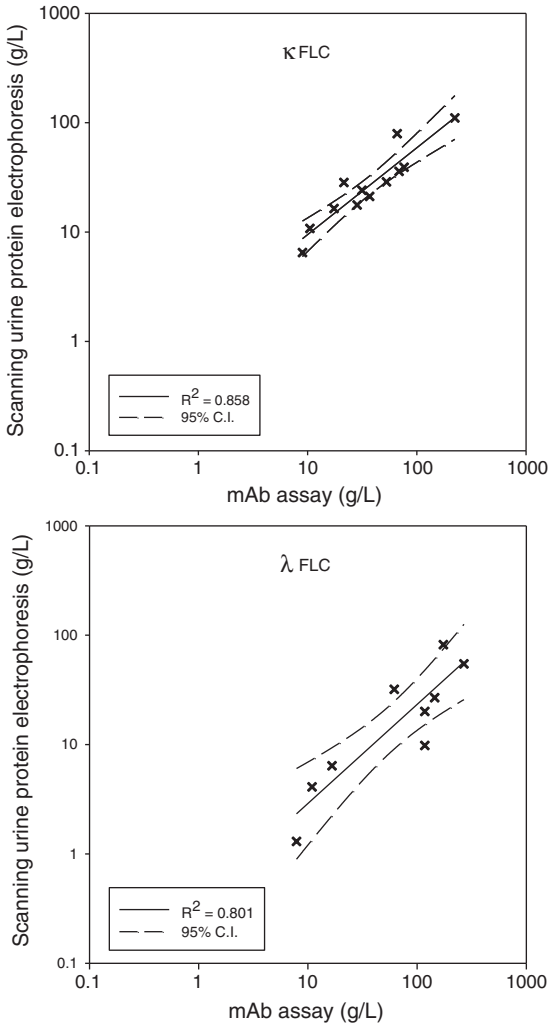


Fig. 3. Representative data from 12 concentrated urines containing κ FLC paraproteins (top) and 9 concentrated urines containing λ FLC paraproteins (bottom), quantitated on the mAb assay and by densitometric scanning of urine protein electrophoresis. For each sample on the mAb assay, the maximum value obtained by either BUCIS 01 or BUCIS 04 was used for the for κ FLC result, and the maximum value obtained by either BUCIS 03 or BUCIS 09 was used for the λ FLC result. Linear regression (R^2) and 95% confidence intervals are displayed in each figure.

further analyses; both the mAb assay and Freelite™ assays identified elevated λ FLC and an abnormal κ:λ FLC ratio in this sample. This finding accords with expected prevalence of MGUS in the general population (Kyle et al., 2006). Reference ranges for each anti-FLC mAb were similar to Freelite™ for the remaining 249 samples (Fig. 2). The two anti-κ mAbs also had similar reference ranges to each other, with BUCIS 04 having a slightly broader reference range than BUCIS 01 (BUCIS 01: 6.46–15.10 mg/L; BUCIS 04: 4.35–19.44 mg/L). The two anti-λ mAbs were also similar, with BUCIS 03 having a slightly broader reference range than BUCIS 09 (BUCIS 03: 4.13–19.18 mg/L; BUCIS 09: 5.19–18.87 mg/L). In terms of the κ:λ ratio, the mAb assay had a similar range to Freelite™ (mAb assay: 0.40–1.59; Freelite™: 0.58–1.76).

3.2. Assay specificity — serum samples

1000 consecutive serum samples, selected as they arrived in the CIS for routine serum FLC analysis, were analysed using the mAb assay and Freelite™ (Fig. 4). Overall, each anti-FLC mAb showed good or excellent Spearman correlations with Freelite™: anti-κ BUCIS 01 ($R^2 = 0.79$, 95% CI 0.76–0.81), anti-κ BUCIS 04 ($R^2 = 0.92$, 95% CI 0.91–0.93), anti-λ BUCIS 03 ($R^2 = 0.87$, 95% CI 0.85–0.88) and anti-λ BUCIS 09 ($R^2 = 0.85$, 95% CI 0.84–0.87). Compared to each other, BUCIS 01 and BUCIS 04 mAbs provided a good correlation for κ FLC ($R^2 = 0.78$, 95% CI 0.76–0.80) and BUCIS 03 and BUCIS 09 mAbs provided an excellent correlation for λ FLC ($R^2 = 0.97$, 95% CI 0.97–0.98). In terms of the κ:λ ratio (Fig. 5), both Freelite™ and the mAb assay demonstrated a good correlation ($R^2 = 0.85$, 95% CI 0.83–0.86).

Individual results from each assay were then compared to identify any discrepancies between the mAb assay and Freelite™. For this initial clinical validation of the mAb assay, the mean κ FLC results generated by BUCIS 01 and BUCIS 04 mAbs were used, and the λ FLC results obtained by BUCIS 03 and BUCIS 09 mAbs were used. From this process, 61 discrepancies were identified (6.1% of total samples tested), of which there were 41 discrepant κ FLC results and 20 discrepant λ FLC results. Further investigations on these discrepant samples revealed that elevated levels of FLC, or an elevated FLC ratio, on the Freelite™ assay was not supported by the mAb assay or serum IFE, and may have reflected cross-reactivity on the Freelite™ assay with whole immunoglobulin paraprotein or hindrance from the Freelite 'gaps' (see Fig. 6); for detailed assessment, see supplementary results. Results from the mAb assay were supported by serum IFE, as well as investigations on matched urine and analysis of patient history, where available. In summary, results indicated that all serum samples with abnormal FLC levels, or an abnormal κ:λ ratio, were detected by the mAb assay from 1000 consecutive serum samples. Further investigations revealed that both anti-κ FLC mAbs (BUCIS 01 and BUCIS 04) were diagnostically similar, and either could be used to indicate a sample containing an abnormal κ FLC level. Similarly, both anti-λ FLC mAbs (BUCIS 03 and BUCIS 09) were diagnostically similar, and either could be used to indicate a sample containing an abnormal λ FLC level.

3.3. Assay specificity — urine samples

Results from 13,090 urine samples analysed routinely by the CIS on the mAb assay and on urine IFE were compared to assess the specificity and sensitivity of the mAbs at detecting FLC in urine. Urine IFE was conducted using antisera against κ and λ LCs that did not distinguish between free and bound LC. Of the 13,090 urines, 12,242 samples were from patients who had a known serum paraprotein, 641 samples had no paraprotein present, and 207 had an unknown admission diagnosis. After initial comparisons between the mAb assay and IFE, 199 discrepancies were identified (1.52% of all samples tested). 143 of these samples had polyclonal LC by IFE but <10 mg/L of the relevant FLC on the mAb assay. The other 56 samples had monoclonal LC present in the urine, but <10 mg/L of the relevant FLC on the mAb assay. These samples were re-tested on the mAb assay to exclude the

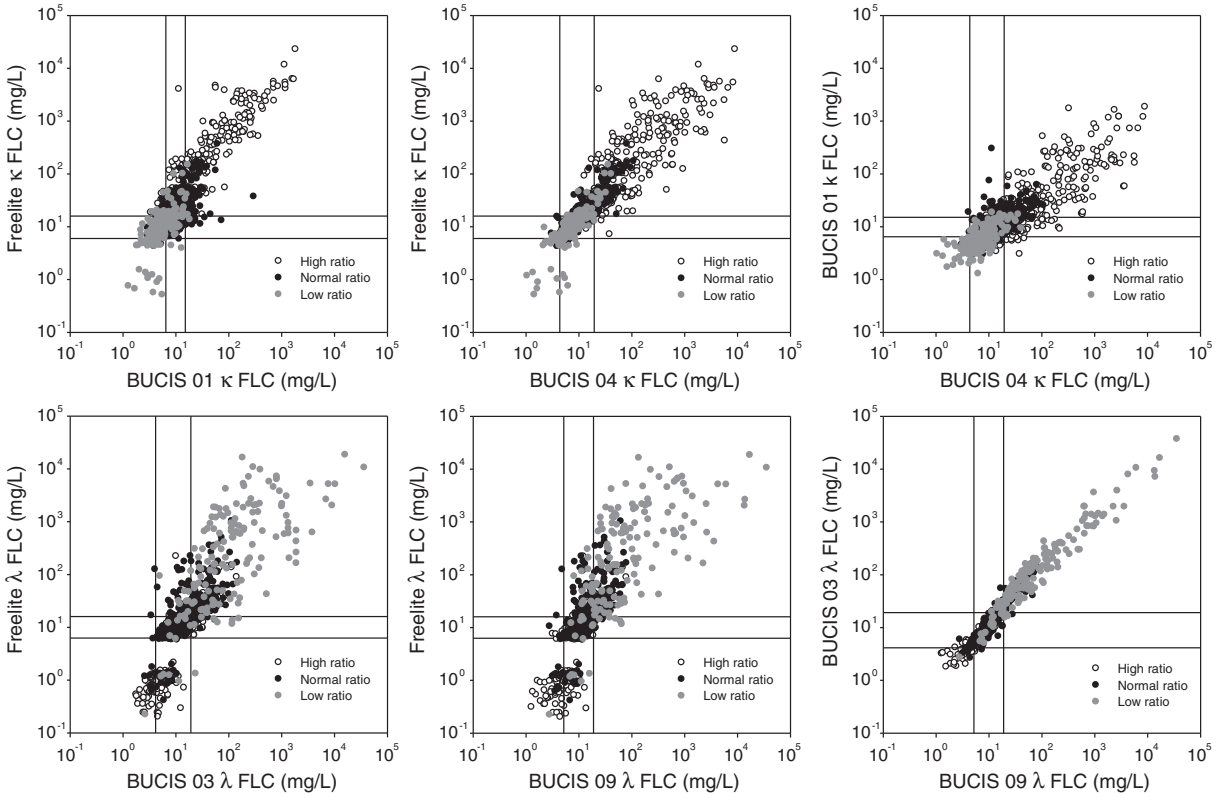


Fig. 4. 1000 consecutive routine clinical serum samples analysed for κ and λ FLCs on the mAb assay and Freelite™. Results comparing the anti-κ FLC mAbs (BUCIS 01 and BUCIS 04) and Freelite™ κ FLC are located at the top, and located at the bottom are the results obtained by the anti-λ FLC mAbs (BUCIS 03 and BUCIS 09) and Freelite™ λ FLC. In each of the figures above, the results were trichotomised into groups based on whether the sample had a high, normal or low κ:λ FLC ratio. For these assessments, the κ:λ FLC ratio was generated by the results obtained by BUCIS 04 and BUCIS 09 and the ratio boundaries were set using the 95% reference ranges from Fig. 2. Lines on each figure represent the 95% reference ranges for each of the anti-FLC mAbs and Freelite™, also derived from the 95% reference ranges in Fig. 2.

possibility of user error, and all samples were re-analysed by full IFE analysis to distinguish between FLC and LC bound to whole immunoglobulin. Results from this process revealed that all samples with FLC detected by IFE were also detected by the mAb assay. Complementary analyses of matched serums and patient history also supported these findings. In summary, the mAbs detected FLC in all 2995 urine samples containing monoclonal κ FLC and all 1180 urine samples containing monoclonal λ FLC, as detected by IFE specific for FLC.

3.4. Assay dynamics

Assay imprecision, or CV%, was measured in pools of serum samples with low, medium and high κ or λ FLC levels. For κ FLC, at 8.00 mg/L, 16.85 mg/L, and 238.94 mg/L, the intra-assay CV% was 4.46%, 4.69%, and 4.85%, respectively; and the inter-assay CV% was 6.45%, 6.50%, and 5.31%, respectively. For λ FLC, at 7.27 mg/L, 10.38 mg/L, and 91.13 mg/L, the intra-assay CV% was 5.69%, 4.86%, and 2.84%, respectively; and the inter-assay CV% was 9.19%, 7.99%, and 6.96%, respectively.

Assay batch-to-batch variability was assessed by analysing 50 serum samples with varying FLC levels (κ range 3.42–329.88 mg/L; λ range 1.09–130.51 mg/L) and the results are displayed in Fig. 7. All samples were analysed once, on separate assay days, using three consecutive batches of anti-FLC mAbs, calibrators and other appropriate assay reagents. Passing and Bablok regression analysis gave slopes between 0.93–1.01 for κ FLC and 0.86–1.05 for λ FLC. Spearman correlation coefficients for κ FLC were ≥0.99 and for λ FLC were ≥0.96.

Representative assay linearity results are displayed in Fig. 8. Serum samples containing high levels of either κ (581.36, 416.37, and 256.97 mg/L) or λ (485.04, 379.41and 370.56 mg/L) FLC paraproteins were serially diluted in assay buffer. Results indicated that assay linearity was maintained on the monoclonal κ FLC samples between 7.61 mg/L and 568.01 mg/L, 1.94 mg/L and 410.36 mg/L, and, 6.32 mg/L and 260.78 mg/L, respectively. For the λ monoclonal FLC samples, linearity was maintained between 1.38 mg/L and 476.1 mg/L, 1.78 mg/L and 361.72 mg/L, and, 4.45 mg/L and 381.62 mg/L, respectively. For κ FLC, below 10 mg/L no more than 1.45 mg/L non-linearity was found, and above 10 mg/L no more than 16.37% non-linearity was observed. For λ FLC, below 10 mg/L no more than 2.03 mg/L non-linearity was found, and above 10 mg/L no more than 19.0% non-linearity was found.

The assay limit of detection for each mAb was assessed by measuring each against a κ or λ BJ protein, firstly mixed with normal serum, and then serially diluted in assay buffer. Limit

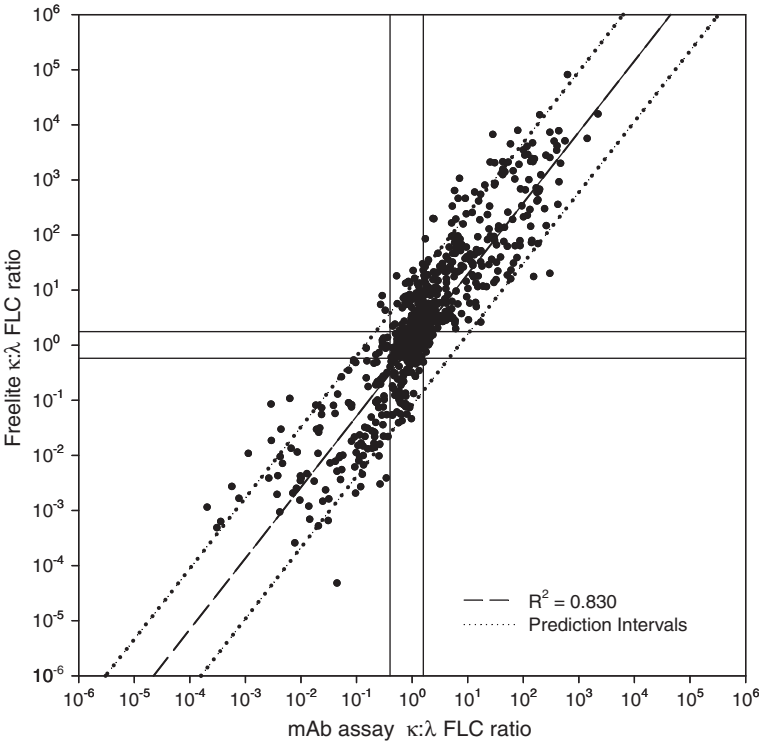


Fig. 5. Illustrated are the κ:λ FLC ratio results from 1000 consecutive routine clinical serum samples analysed on Freelite™ and the mAb assay. For the mAb assay results, for each sample, the κ:λ FLC ratio was generated by using the κ FLC result obtained by BUCIS 04 and the λ FLC result obtained by BUCIS 09. Linear regression (R^2) and prediction intervals are displayed as dashed and dotted lines. Intact lines represent the reference ranges for each assay derived from the analysis 249 plasma samples from healthy donors.

of detection for BUCIS 01 was 0.63 mg/L, BUCIS 04 was 0.86 mg/L, BUCIS 03 was 0.72 mg/L, and BUCIS 09 was 0.52 mg/L.

Assay interference tests showed minimal assay cross-reactivity to alternate κ or λ FLC or intact immunoglobulins, bilirubin, haemoglobin, cholesterol or triglyceride (Fig. 9, in supplementary data). Results demonstrated that no more than a median 2.7 mg/L change was observed for the anti-κ FLC mAbs, and no more than a median 3.7 mg/L change for the anti-λ FLC mAbs.

4. Discussion

This study describes the development of four mouse anti-human κ:λ FLC mAbs and their initial validation in a multi-plex Luminex® immunoassay. Each of the anti-FLC mAbs exhibited: excellent sensitivity (<1 mg/L); low batch variation; sustained assay linearity; specificity and minimal cross-reactivity to bound LC, or alternate FLC isotype. Each of the mAbs provided good quantitative concordance with the Freelite™ assay in the measurement of polyclonal FLC in plasma from 249 healthy donors, and FLC levels in serum from 1000 consecutive samples. Specificity and sensitivity were further illustrated in the measurement of FLC in 13,090 urine samples tested for BJ proteins. In this set of urine samples the mAbs detected FLC in all 2995 samples containing monoclonal κ FLC and all 1180 samples containing monoclonal λ FLC, as detected by IFE. This is important because previously described mAbs against FLC have not been shown to detect FLC from substantially all neoplastic plasma cell clones (Abe et al., 1993, 1998; Nakano and Nagata, 2003; Davern et al., 2008; te Velthuis et al., 2011; Hoedemakers et al., 2012); many of the mAbs that we have produced against FLC do not bind FLC from up to a quarter of individual myeloma patients. The extent of FLC structural diversity is reflected in the LC gene structure. Thus, the κ immunoglobulin gene family contains 81 genes located on chromosome 2, of which, at least 40 functional genes are responsible for V region variability, giving rise to at least 4 major V region types (Vκ1, Vκ2, Vκ3, and Vκ4) (Sitnikova and Nei, 1998; Davern et al., 2008). Further, there are 5 genes responsible for encoding the J region, and 1 constant region gene expressing 1 of 3 allotypic forms (κm^1, κm^2, κm^3) (Sitnikova and Nei, 1998; Davern et al., 2008; Jefferis and Lefranc, 2009). The λ immunoglobulin gene family appears to support more diversity, in that there are at least 40 functional genes responsible for V region variability that results in at least 5 major V region types (Vλ1, Vλ2, Vλ3, Vλ6, and Vλ8). Further, there are at least 5 genes responsible for encoding the J region, and up to 7 genes for the C region that gives rise to at least 3 C region isotypes ($C\lambda^1$, $C\lambda^{2/3}$, $C\lambda^7$) (Solomon and Weiss, 1995; Davern et al., 2008). FLC diversity is extended by somatic mutations in the encoding genes and post-translational modifications of FLC. Given this multiplicity of human FLC structures, it is not surprising that it is difficult to produce mAbs that would detect the FLC from substantially all patients and neoplastic plasma cell clones. To be clinically reliable any new assay for FLC should be tested against a large number of serum and urine samples to show that the mAbs are

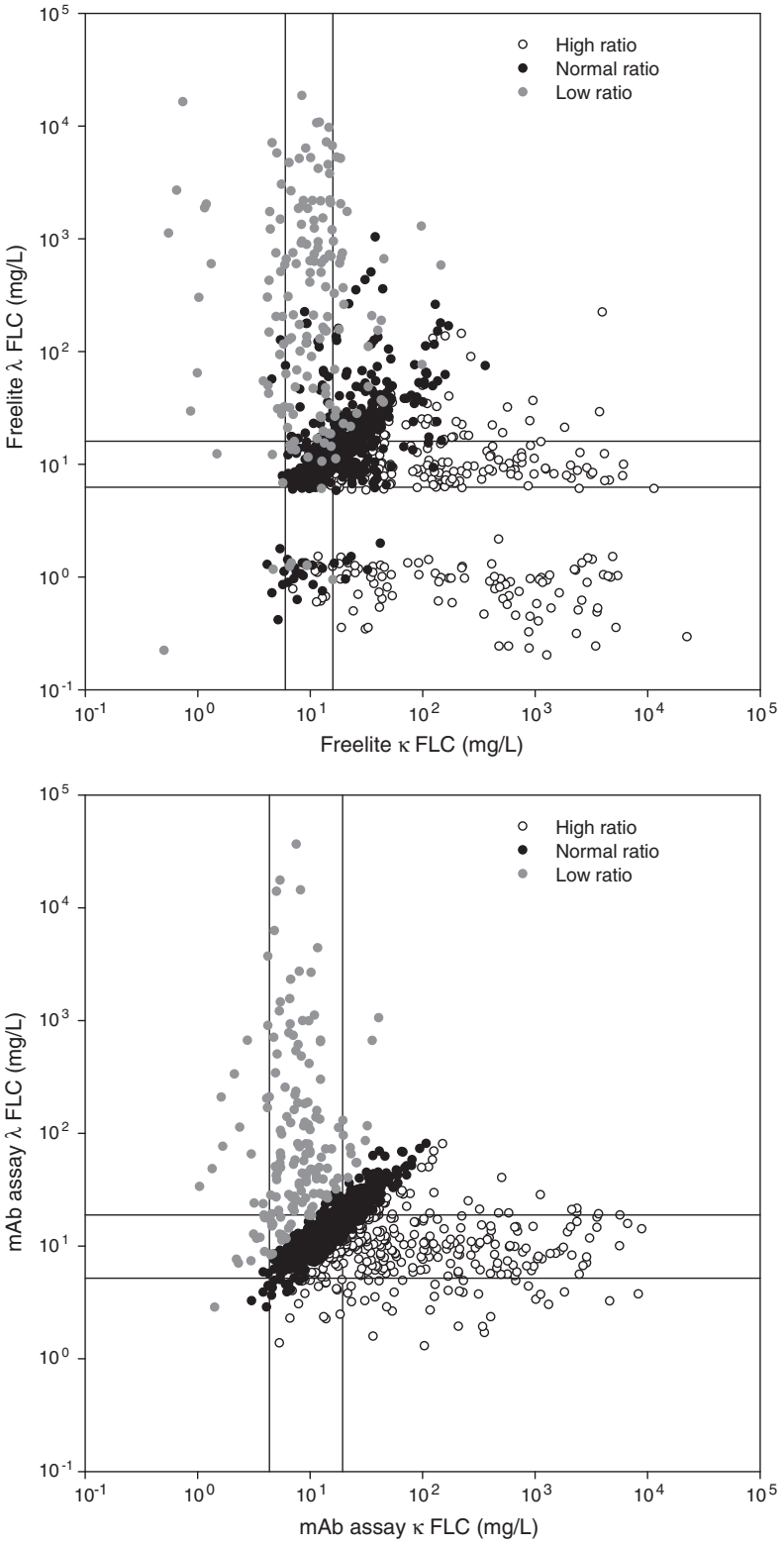


Fig. 6. κ and λ FLC levels in 1000 consecutive routine clinical serum samples analysed on the Freelite™ assay (top), with the so-called 'gaps' in Freelite™ quantitation of FLC clearly visible (Bradwell, 2008). Illustrated, bottom, are results from the same consecutive samples generated by the mAb assay using the BUCIS 04 (anti-κ FLC) and BUCIS 09 (anti-λ FLC) mAbs. No quantitative 'gaps' were observed in the results generated by these mAbs, or the other BUCIS 01 and BUCIS 03 anti-FLC mAbs (not illustrated). In both figures above, the results were trichotomised into groups based on whether the sample had a high, normal or low κ:λ FLC ratio generated by BUCIS 04 and BUCIS 09; for this calculation, the ratio limits were determined using the BUCIS 04 and BUCIS 09 95% reference ranges in Fig. 2.

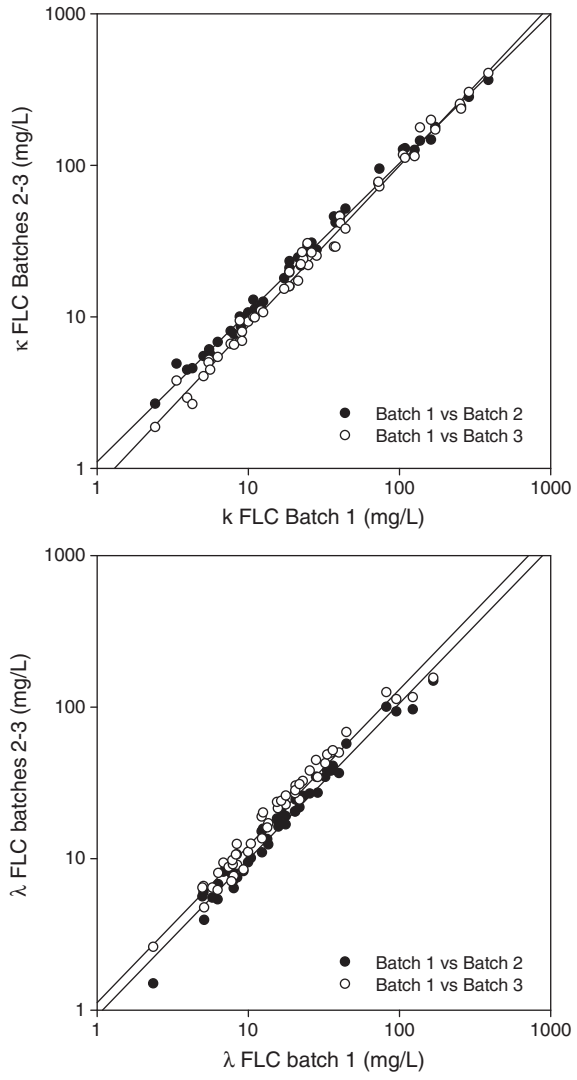


Fig. 7. Batch-to-batch variation from three independent lots of anti-FLC κ mAbs (top) and λ mAbs (bottom) and other assay materials, when measuring 50 serum samples containing varying levels of FLC.

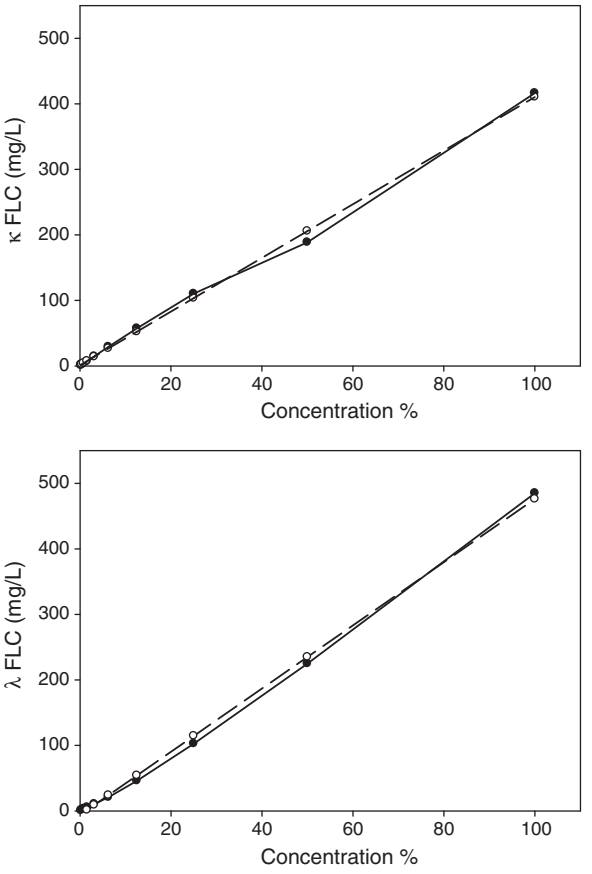


Fig. 8. Linearity examples of serial dilutions of serum containing either a κ FLC paraprotein (top) or λ FLC paraprotein (bottom); samples were serially diluted 2-fold in assay buffer. In each figure, comparative linear (predicted) values are represented by a dashed line and the acquired (non-linear) titrations are represented by a black continuous line.

at least close to the ideal of detecting FLC from all patients and neoplastic plasma cell clones.

In plasma samples containing normal polyclonal FLC, obtained from healthy donors, each of the mAbs provided similar quantitation of absolute FLC levels. These samples were obtained from UK blood donors, which include persons up to the age of 65 years. It is likely that some of these donors had MGUS, and indeed, one donor found to have an abnormal FLC ratio detected by both the mAb assay and Freelite™, had a 30 g/L IgG λ paraprotein. Similarly, we cannot exclude the possibility that some donors had a degree of renal impairment. For both polyclonal and monoclonal λ FLC in a thousand consecutive serum samples, the two anti-λ FLC mAbs exhibited excellent correlations with each other, and displayed good clinical concordance with Freelite™. The diversity in FLC repertoire may explain the more divergent correlations demonstrated in this study between the mAb assay and Freelite™ for highly elevated monoclonal λ FLC paraproteins (see Fig. 4). Similarly, the two anti-κ FLC mAbs displayed good clinical concordance with each other; there were some quantitative differences in samples with monoclonal FLC but each mAb identified an abnormal level of the relevant FLC, as well as an abnormal κ:λ FLC ratio in each of these samples. In addition, each mAb displayed good concordance with Freelite™.

The development of precise anti-FLC mAbs, as shown in this study, enables diversification away from existing assay platforms and may lead to improvements in FLC assay design. Current commercial tests, using turbidimetric and nephelometric formats (Bradwell et al., 2001) have a number of well observed limitations. Firstly, these systems are reliant on a separate test for each κ and λ FLC measurement. This introduces inter-test variability and reduces the precision of the κ:λ ratio. Simultaneous measurement of both κ and λ FLCs in our assay removes some of this inter-test variability and should thus provide a more reliable measure of the κ:λ ratio. To our knowledge, this is the first assay to adopt this configuration. From a practical perspective this format is also beneficial as a single test because it is more time and resource efficient, and the sample volume required (<10 μL) is much lower than typical turbidimetric and nephelometric requirements, thus preserving stock sample volume. A second

problem with the format of existing serum FLC tests is known as antigen excess or a 'hook-effect' and has been documented elsewhere (Daval et al., 2007; Levinson, 2010a). This phenomenon occurs when high levels of FLC analyte exceed the number of available antibody binding sites thus reducing or eliminating FLC-antibody aggregates, resulting in a false-negative signal output. Use of a competitive inhibition format in our assay overcomes this problem and such an improvement is likely to improve the reliability of patient diagnosis and monitoring. Indeed, we found no instances where the mAbs 'missed' elevated FLC above 100 mg/L (sensitivity of serum IFE), indicating that there were no instances of antigen excess using the mAb assay in serum or in the large numbers of urine samples tested. Our assay also provides a larger dynamic range, better sensitivity, and avoidance of 'gaps' seen in the current serum FLC assay in Figs. 4 and 6, and discussed elsewhere (Bradwell, 2008).

In conclusion, we have developed a new method of measuring urine and serum FLC using anti-κ and anti-λ FLC mAbs. This method offers improved sensitivity and reliability over existing methods that rely on sheep polyclonal antisera. Further, the mAbs used in this study demonstrated excellent specificity and identified FLC in 13,090 urine samples tested for the presence of BJ proteins, normal and abnormal FLC levels in 1000 consecutive serums samples, and normal levels of polyclonal FLC from healthy donors. This analysis served as an initial clinical assessment of the mAbs, and given the effective specificity displayed, further assessment of patients with known multiple myeloma, light chain only myeloma, non-secretory myeloma and other FLC-relevant disorders is now warranted. Utilisation of these mAbs in other assay platforms should also be investigated.

Supplementary data to this article can be found online at http://dx.doi.org/10.1016/j.jim.2013.01.014.

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