HIGH-SENSITIVITY FREE LIGHT CHAIN DETECTION FOR MINIMAL RESIDUAL DISEASE ANALYSIS IN MULTIPLE MYELOMA

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Abstract

Purpose: To overcome the various limitations of bone marrow-based minimal residual disease (MRD) measurement in patients with multiple myeloma (MM), the clinical Immunology service (CIS) at the University of Birmingham has developed a non-invasive, serum-based technique named hsFLC, which is capable of MRD detection via high-sensitive measurement of serum free light chains (FLCs). We compared MRD results from hsFLC analysis with those produced using multi-parameter flow cytometry (MFC). We also assessed the potential application of hsFLC for the diagnosis and monitoring of non-secretory (NS) and low-level FLC secretory MM patients.

Methods: The hsFLC assay combines isoelectric focussing with immunoblotting, and uses highly- specific mouse monoclonal antibodies targeted to FLCs. A total of 110 MM patient samples from the national Myeloma X & XI trials who had all undergone autologous stem cell transplants, were retrospectively analysed by hsFLC for the presence of MRD. From the selected patients, 54 had m-proteins with involved Kappa FLCs and 56 had involved Lambda FLCs at diagnosis. The original MRD results by MFC were available for 80 of these patients. A total of 29 MM patients clinically classified as NS from the national myeloma IX and XI clinical trials were also analysed by hsFLC for the presence of monoclonal FLCs. A small analysis of light-chain only MM (LCMM) and oligo-secretory MM (OSMM) patients was also conducted using hsFLC.

Results: Monoclonal FLCs were detected by hsFLC in all of the primary diagnostic samples tested, including the NSMM, LCMM and OSMM patients. MRD was detected in around double the number of post-transplant patients as those that were MRD-positive by MFC.

Retrospective analysis of disease relapsed patients by hsFLC, demonstrated monoclonal FLCs several months before conventional methods detected any disease.

Conclusions: The hsFLC assay is a highly-sensitive and non-invasive alternative to bone marrow-based techniques for the systemic measurement of disease in almost all MM patients.

1 Contents

1 Introduction

1.1 Multiple myeloma

1.1.1 Incidence, Presentation, and symptoms

Multiple myeloma (MM) arises from the malignant proliferation of a single clone of plasma cells (PC) in the bone marrow (BM) and accounts for ~10% of all haematological malignancies. In the United Kingdom, more than 5800 new patients are diagnosed with MM each year and there are over 3000 MM related deaths per year (1). MM accounts for 2% of all cancer related mortalities in the UK (1). MM seems to be slightly more common amongst males in the UK; 57% compared to 43% who are females (1). The median age of onset is 66 years. Only 2% of patients are less than 40 years old at onset (2). MM is more common in the black population than Caucasians (1).

In the UK, around one third of MM patients are initially diagnosed at a hospital emergency department following either GP referral or self-admission (3). The two most common presenting features of MM are fatigue and bone pain (4). The main contributor towards these initial symptoms is usually anaemia arising from the failure of haematopoiesis as clonal PCs infiltrate the BM (5). Another consequence of tumour-cell BM infiltration is immunoparesis, which is the suppression of the production of normal immunoglobulins (IMGs). Patients with immune paresis can become more susceptible to infection, including pneumonia (6). Around 80% of patients with active MM have osteolytic skeletal lesions, frequently found in the spine and ribs (4). Thus, presentation can range from bone pain and fractures to collapse of vertebrae (2). The increased breakdown of bone results in hypercalcaemia which is associated with confusion, drowsiness, nausea, abdominal pain and constipation (4). Impaired renal function is also a common presenting symptom associated with hypercalcaemia along with renal deposition of monoclonal free light chain complexes (4).

1.1.2 Aetiology & pathogenesis

Normal B cell development is terminated with the production of PCs, which are terminally differentiated, non-dividing cells which secrete antigen-specific antibodies (7). Upon exposure to antigen, naive B cells and follicular B cells initially secrete low-affinity antibodies that are short-lived and undergo apoptosis. Subsequent antigenic exposure and T-helper cell intervention promotes surviving naive B cells located within lymphoid germinal centres to undergo proliferation and somatic hypermutation of IMG gene segments. This ultimately leads to the production of memory B cells and high-affinity antibody secreting long-lived PCs (8). Long-lived PCs can survive for months to years within the bone marrow (9). MM tumour cells represent the malignant equivalent of and are phenotypically similar to long-lived post-germinal centre PCs (5). However, the exact origins and identity of MM cells in relation to PC development remain unclear, with evidence that they may even develop from a totally heterogeneous myeloma stem cell (5, 10). Nevertheless, there are several indications that the first oncogenic events in MM cells may occur during the isotype class switching and somatic hypermutation stage in the germinal centre of lymph nodes (11). Further post -germinal centre mutations within the BM are thought to be necessary for progression of these PCs to malignancy (12). In any case, MM cells seem to demonstrate widespread somatic hypermutation of IMG genes (13).

There is strong evidence to suggest that MM nearly always develops from a pre-malignant PC dyscrasia known as monoclonal gammopathy of undetermined significance (MGUS). Landgren et al. demonstrated prior MGUS in all 71 patients who developed MM in their studies, while Weiss et al, reported MGUS in at least 90% of cases tested prior to MM (13-15). There is also potential for MGUS to develop into an asymptomatic condition known as smouldering multiple myeloma (SMM), which in turn can develop into MM. Progression is very slow and the risk of progression from MGUS to MM is about 1% per year (10). It is interesting to note that MGUS patients also carry the same initial mutations as described for MM tumour cells (12). It follows therefore, that late oncogenic events within the BM are possibly linked to transformation of MGUS to MM (16).

1.1.3 Diagnostic work-up for MM

MM is characterised the presence of monoclonal antibodies (m-protein) in blood which serves as a unique biomarker because monoclonal IMGs and or free light chains (FLCs) are released by their corresponding clonal tumour cells. Capillary zone electrophoresis (CZE) can initially single out an m-protein in 82% of MM patients (2) at a sensitivity of about 1000mg/L (17). A major pitfall of screening for MM using CZE is the inability to detect non-secretory MM (NSMM) and Light-chain only MM (LCMM) which account for 2-3% and 18-20% of all MM cases, respectively (2). If an abnormality is detected using CZE, usually in the gamma region (sometimes also in the beta and very rarely alpha region), then Immunofixation electrophoresis (IFE) is performed (2). IFE is used to characterise the class of the m-protein detected. This is achieved using antisera to IgG, IgA, IgM, total Kappa (κ) and total Lambda (λ) light chains (LCs) which bind to any corresponding proteins in the sample, and are subsequently visualised by staining. The frequency of each type of m-protein observed in MM patients is 50% IgG, 20% IgA, 20 % light chain only, 2% IgD and 0.5% IgM (2). IFE has an improved sensitivity of about 100mg/L compared to CZE alone (17).

In healthy individuals, PCs produce around 500mg of FLCs in excess of the corresponding heavy chains, each day (18). This excess is rapidly cleared via the kidneys to facilitate a serum FLC (sFLC) half-life of only 2-6 hours. The half-life of intact IMGs on the other hand, ranges from 2-3 days for IgE and up to 28 days for IgG (19, 20). Free κ LCs circulate as monomers, whereas, free λ LCs circulate as covalently-bound dimers (20). The quantification of monoclonal sFLCs produced by malignant cells provides a real-time snapshot of disease activity as compared to the quantification of IMGs, such as IgG (21). FLC immunoassays use antisera directed to specific epitopes that are only exposed when FLCs are not bound to heavy chains i.e. free. The antisera bind to FLCs with a 10,000 fold greater avidity than to bound LCs within IMGs (21). In this manner, FLCs can be confidently quantified even in the presence of a large concentration of polyclonal serum IMGs. Both κ and λ LCs are measured individually. Then, a ratio of the two (κ/λ) is calculated and compared to a standard ratio to identify any unbalanced synthesis. A separate reference ratio is required to monitor patients with kidney

injury which is adjusted to allow for impaired clearance . Abnormal κ/λ ratios are found in 100% of LCMM, 90-95% of MM patients with intact m-proteins and 60-70% of patients with NSMM (22, 23).

Around 30% of MM patients present with hypercalcaemia associated with bone pain and weakness (2). When the initial laboratory findings of a raised calcium are suspected to be osteolytic in origin, then this necessitates the next step of patient investigation, which is bone imaging. Skeletal radiographs, computed tomography (CT), magnetic resonance imaging (MRI) and 18F-fluorodeoxyglucose-positron emission tomography (FDG-PET) and FDG-PET-CT scans are used to assess bone disease in MM (24). MRI is particularly useful to detect focal bone lesions throughout the entire body when MM diagnosis is uncertain or where SMM may be present. Spinal examination using MRI is also important to assess vertebral disease (25).

MM diagnosis is confirmed by light microscopy if PC infiltration of the BM is observed following BM aspirate and biopsy (Figure 1). Biopsy is also necessary to rule out MGUS and an isolated plasmacytoma, though it is an invasive technique (26).

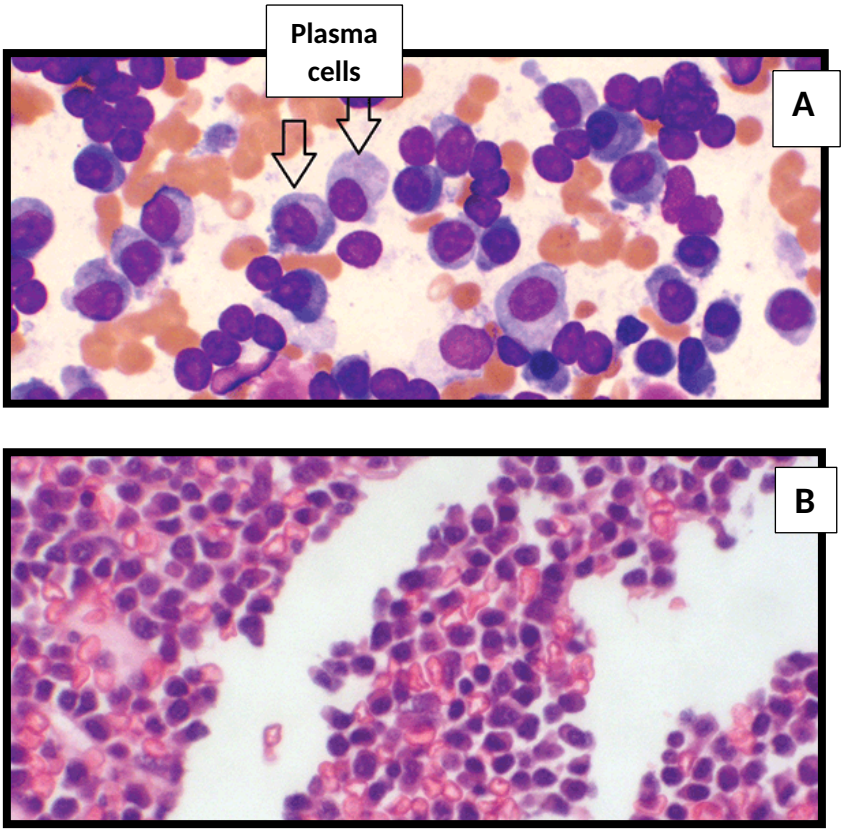


Figure 1. *A: BM aspirate showing increased number of PCs. B: Trephine biopsy showing PC infiltration of the BM. Reference: Eslick R, et al. Australian family physician vol. 42, no. 10, October 2013.*

1.1.4 IMWG diagnostic criteria and revisions to present

The revised International Myeloma Working Group (IMWG) criteria (2014) state that the diagnosis of MM requires the presence of one or more myeloma defining events (MDE). This is in addition to evidence of either ≥ 10% clonal PCs on bone marrow examination or a biopsy-proven plasmacytoma. MDE consists of established **CRAB** features-hyper**calcaemia** (**C**), **renal** failure (**R**), **anaemia** (**A**), or lytic **bone** lesions (**B**). In addition to these, there are 3 other specific biomarkers (**SLIM**): clonal BM PCs ≥ **60%** (**S**), serum free **light** chain (**LI**) ratio ≥ 100, provided involved FLC level is ≥ 100 mg/L, and more than one focal lesion on **MRI** (**M**) (Table 1). Each of the new biomarkers is associated with an approximately 80% risk of progression to symptomatic end-organ damage. Thus, the updated criteria allows for early diagnosis and initiation of therapy before end-organ damage (27). The changes in these criteria over time also reflect advances in therapeutics and technology.

Table 1. *IMWG revised criteria 2014 for the diagnosis of plasma cell disorders (26).*

Plasma cell disorder	Definition
MGUS	The following ***three*** criteria must be met: 1. Serum m-protein of <3 g/dL 2. Clonal BM PCs of <10% 3. Absence of end organ damage using the CRAB criteria (hyper**calcaemia**, **renal** impairment, **anaemia** and **bone** lesions)
SMM	The following ***two*** criteria must be met: 1. Serum m-protein (IgG or IgA) of ≥3 g/dL, or urinary monoclonal protein of ≥500 mg/24 h and clonal BM PCs of 10–60% 2. Absence of myeloma-defining events (MDEs) or amyloidosis
MM	The following ***two*** criteria must be met: 1. Clonal BM PCs of ≥10% or biopsy-proven bony or extramedullary plasmacytoma 2. Evidence of end organ damage involving one or more of the following myeloma-defining events (MDEs): • Hyper**calcaemia (C)**: serum calcium of >0.25 mmol/L higher than the upper limit of normal or >2.75 mmol/L • **Renal (R)** insufficiency: creatinine clearance of <40 mL/min or serum creatinine of >177 µmol/L • **Anaemia (A)**: Hb of >2 g/dL below the lower limit of normal or a Hb value of <10 g/dL • **Bone (B)** lesions: one or more osteolytic lesions on skeletal XR, CT or PET-CT • Clonal BM PCs of ≥**60% (S)** • Involved: uninvolved serum free **light (Li)** chain ratio of ≥100 (involved free light chain level must be ≥100 mg/L) • More than one focal lesion on **MRI (M)** studies (at least 5 mm in size)

MGUS*: monoclonal gammopathy of unknown significance*, ***MM****: Multiple Myeloma*, ***SMM****: smouldering Multiple Myeloma*, ***XR****: X-ray*, ***CT****: computed tomography*, ***PET-CT****: positron emission tomography/computed tomography*, ***MRI****: magnetic resonance imaging*.

1.1.5 Treatment

The impressive improvement in MM treatment has mainly been associated with novel drugs, autologous stem cell transplantation (ASCT) and enhanced post-induction supportive care (6). Corticosteroids form a fundamental part of most MM treatment regimes. Dexamethasone, prednisolone and glucocorticoids are used for their anti-inflammatory and immunosuppressive properties (28). Chemotherapy usually includes alkylating agents (such as melphalan) and anthracyclines (such as doxorubicin) to target malignant cell DNA. Unfortunately, normal cells are also adversely affected by this therapy, albeit to a lesser extent (6). Myeloma Cell signalling can be disrupted or even inactivated using proteasome inhibitors, such as bortezomib, carfilzomib and ixazomib (29). Immunomodulatory drugs, such as thalidomide, lenalidomide and pomalidomide, are used to target protein degradation pathways in order to counter MM progression (30). The use of monoclonal antibodies has revolutionised MM treatment for their cytotoxic effect on malignant cells. Daratumumab targets CD38 receptors found on malignant cells. When it is used in conjunction with other agents mentioned previously, it has a very significant impact on response rates (31). Elotuzumab and isatuximab are novel therapeutic antibodies now also approved for MM treatment (32, 33).

MM patients who have evidence of end-organ damage will be enrolled onto one of the aforementioned treatment regimens immediately. Asymptomatic SMM patients are closely monitored and may also be enrolled onto treatment if they are considered at high risk of developing symptoms. High-risk features include evidence of either ≥60% BM infiltration, a serum-free light chain ratio of ≥100 (provided involved FLC level is ≥100 mg/L) or >1 focal lesion on MRI (27). Depending on factors, such as age and comorbidities, MM patients may be eligible for ASCT. Prior to transplant, a phase of induction therapy is administered to such patients to reduce disease morbidity. In some countries, post-transplant maintenance therapy is also administered. ASCT patients have improved responses to treatment and prolonged remission times of up to 18 months

on average (4). Despite improvement in treatment, MM remains an incurable disease. The level of response and length of remission usually decreases after each relapse. Infection is the most often cited end-stage cause of death. Therefore, supportive treatment, such as prophylaxis, is essential for disease management alongside active treatments (6)

1.1.6 Response assessment

Response to therapy and progression of disease is monitored using BM infiltration studies, bone imaging and biochemical markers, such as m-protein and FLCs. Response assessment is usually carried out every 3 months and every 4 weeks in clinical trials (34). Following treatment, a patient is said to have gone into complete remission (CR), if the m-protein (serum &/or urine) is no longer detectable by IFE, there is no sign of any soft tissue plasmacytoma and ≤5% PCs are found upon BM aspiration (Table 2). On the other hand, the term disease progression refers to those patients who have a detectable increasing disease burden after having a low-level of persistent residual baseline disease. It follows then, that the definition of relapse is the reappearance of myeloma (and associated markers) after a patient has previously gone into CR. The standard IMWG criteria state that for disease progression or relapse to be defined in any patient the minimum absolute increase in the m-protein must be 0.5 g/dL and a 25% increase from the baseline (35).

***Table 2**. Standard IMWG response criteria (35).*

Response category	Criteria
Complete response (CR)	1.Negative serum and urine IFE. 2.Disappearance of any soft tissue plasmacytomas 3.<5% PCs in BM aspirates.
Stringent complete response (sCR)	1.CR plus normal FLC ratio 2.Absence of clonal cells in BM biopsy by immunohistochemistry.
Very good partial response (vgPR)	1.Serum and urine m-protein detectable by IFE but not on electrophoresis **or** 2. ≥90% reduction in serum m-protein plus urine m-protein level <100 mg per 24 h
Partial response (PR)	1.≥50% reduction of serum m-protein plus reduction in 24 h urinary m-protein by ≥90% or to <200 mg per 24 h; • *If the serum and urine m-protein are unmeasurable:* 2.a ≥50% decrease in the difference between involved and uninvolved FLCs in place of the m-protein criteria; • *If serum and urine m-protein are unmeasurable, and serum-FLCs also unmeasurable:* 3.≥50% reduction in PCs is required in place of m-protein, provided baseline BM PCs was ≥30%. • *In addition to these criteria, if present at baseline,* 4.a ≥50% reduction in the size of soft tissue plasmacytomas is also required
Minimal response	1.≥25% but ≤49% reduction of serum m-protein and reduction in 24-h urine m-protein by 50–89%. 2.Also, if present at baseline, a ≥50% reduction in the size of soft tissue plasmacytomas.

1.1.7 Depth of response and outcome

In a meta-analysis-involving nearly 5000 MM patients-responding to treatment, eight out of ten studies showed an important correlation between achievement of a CR and overall survival (OS) (36). In a study of 445 patients who underwent ASCT, improved long-term outcomes were related to the achievement of stringent CR (sCR) compared to patients with lesser degrees of responses (37). For this reason, CR was conventionally considered as the deepest level of response following any treatment and a target endpoint for achievement of progression-free survival (PFS) or at least OS (36, 38, 39). However, the correlation between CR and OS has not always been found to be completely convincing, most likely owing to differing disease biology between patients (40). Moreover, it has become apparent that the confusion may also be a result of different sensitivities between methods used to define CR and their varying abilities to accurately define the true level of response (41). The implementation of new high-sensitive techniques, such as next generation flow cytometry (NGF), has revealed the presence of subclinical **minimal residual disease (MRD)** in patients who had previously achieved CR by conventional methods (42). The demonstration of the prognostic significance of MRD positivity even in CR level patients (43-47) led to the inclusion of MRD negatively in the IMWG response criteria (2016 onwards), as detailed in table 3 (34, 35)

Table 3. *Updated IMWG MRD response criteria 2016.*

MRD response category	Criteria
Flow MRD-negative	• Absence of phenotypically aberrant clonal PCs by NGF on BM aspirates using the EuroFlow standard operation procedure for MRD in MM (or validated equivalent method) with a minimum sensitivity of 1 in 10^5 nucleated cells or higher
Sequencing MRD-negative	• Absence of clonal PCs by NGS in BM aspirates in which presence of a clone is defined as < two identical sequencing reads obtained after DNA sequencing of BM aspirates using the LymphoSIGHT platform (or validated equivalent method) with a minimum sensitivity of 1 in 10^5 nucleated cells or higher
Imaging plus MRD-negative	• MRD negativity by NGF or NGS **plus** a)disappearance of every area of increased tracer uptake found at baseline or a preceding PET/CT **or** b)decrease to less than mediastinal blood pool SUV **or** c)decrease to less than that of surrounding normal tissue
Sustained MRD-negative	• MRD negativity in the marrow (NGF or NGS, or both) and by imaging as defined below, confirmed minimum of 1 year apart. Subsequent evaluations can be used to further specify the duration of negativity (e.g., MRD-negative at 5 years

NGS*: next-generation sequencing*, ***NGF****: next-generation Flow cytometry*, ***SUV****: standardised uptake values.*

1.2 Minimal residual disease (MRD)

In 2016, the IMWG defined MRD in CR patients as the persistence or re-emergence of very low levels of cancer cells down to about one tumour cell in at least 10^5 normal cells (35). This is regardless of the diagnostic method used and the treatment to achieve this level of response. Since then, results from many clinical trials involving novel treatments and refined next-generation diagnostics has led to suggestions that the definition of MRD should be refined even further down to one tumour cell in 10^6 normal cells. Any patient with such a low level of tumour cells would be proposedly reclassified as having achieved a 'stringent-MRD negative' response (48)

1.2.1 The gathering evidence for MRD assessment and its relevance for clinical practice

A whole host of studies have since emerged supporting the importance of MRD assessment in place of the conventional criteria for the improved management of MM. For instance, Paiva et al. reported poor survival rates in patients who relapsed within one year of having had ASCT and high dose therapy (HDT), despite achieving CR. Interestingly, retrospective analysis of these patients using high sensitive methods showed they actually had underlying MRD positivity concurrently to CR (49). In fact, some studies have even shown a worse outcome for patients with such 'unsustained' CR, when compared to patients who didn't even achieve a CR in the first place (50, 51). In a pooled analysis of three clinical trials, CR patients-who were MRD positive-had no better PFS and OS compared to those with a near-CR or partial response, regardless of the treatment or patient risk group (52). In another retrospective study of 78 patients with CR, the achievement of MRD negativity was independently associated with a 2 year longer PFS than those who were MRD positive (53). A prospective analysis of 295 newly diagnosed MM patients, showed that PFS and OS were longer in MRD negative versus MRD positive patients at day 100 after ASCT (43). This study also showed that patients who were MRD negative, though positive by serum IFE had a significantly raised PFS and OS compared to patients who were MRD positive and IFE negative. The delayed degradation or long half-life of intact

IMGs may be one plausible reason for the surprising observation of IFE positivity despite MRD negativity based on next generation methods used in these studies (54). It has also been observed that many patients who have only achieved a VGPR by conventional criteria, have demonstrated MRD negativity in the BM even down to the 10^{-6} threshold using the most advanced diagnostic technologies (55). Again, this observation may be due to the fact that complete clearance of serum m-protein can take several months even after all tumour cells are eradicated, especially of the IgG-type, due to its long half-life.

MRD can be measured at various time points during therapy, including, post-induction, at 100 days post-ASCT, post-consolidation, pre-maintenance and during maintenance. In a pooled analysis of different MRD time points, 27-30% of MRD-positive patients were simply converted to MRD-negative status during the course of maintenance treatment alone (56). Indeed, there is now ample evidence to suggest that intensifying treatment increases the percentage of MRD-negative patients. However, there is still a great need to clarify how long that treatment should continue in order to sustain MRD-negativity.

Although not currently standard practise in the UK, the possibility to adapt therapy according to risk/MRD response status is being keenly researched. MRD is now used as an endpoint in most randomised controlled trials (RCT) worldwide. Indeed, the currently recruiting UK national trial for patients with newly diagnosed transplant-eligible myeloma (RADAR-UKMRA XV) randomises patients to continue or discontinue maintenance therapy according to MRD status.

1.2.2 Current techniques for MRD assessment and limitations

Table 4 below summarises the current techniques for MRD assessment. It is followed by a discussion on the limitation of these techniques.

Table 4. *A summary and comparison of current techniques used for MRD assessment.*

MRD technique	Principle of test	Sensitivity	Applicability	Availability	Turnaround time	Relative Cost (Scale of 1-5)	Standardisation	Sample requirement (baseline sample?)	Special points
MFC/NGF	Characterise/distinguish between normal and malignant PCs in a BM aspirate based on Immunophenotyping.	10^{-5} 10^{-6}NGF	Nearly 100%	Many labs (MFC) Specialist labs (NGF)	Hours	1-3	Yes (EuroFlow)	BM aspirate	Fresh sample required
ASO-PCR	DNA analysis of PCs in BM samples to detect gene rearrangements within the IMG gene found in MM.	10^{-5} to 10^{-6}	40-70%	Average	Days	2-3	Yes (EuroMRD)	BM aspirate, baseline sample for follow-up	Patient specific primers required
NGS	High-throughput DNA sequencing to detect gene rearrangements in MM (IgH VDJ, IgK and IgL) and their frequency (Clonality).	10^{-6}	80-90%	Specialist labs only	≥1 Week	4-5	No	BM/PB sample, baseline sample for follow-up	Can detect clonal heterogeneity
PET/CT	Imaging of metabolic activity of radiotracers incorporated into cell surface receptors and surface antibodies (ImmunoPET)	Not determined	100%	Average	Hours	4-5	No	NA	Can detect extra-medullary disease

***MFC**: multi-parameter flow cytometry, **NGF**: next-generation flow-cytometry, **ASO**: allele-specific oligonucleotide, **PCR**: polymerase chain reaction, **NGS**: next-generation sequencing, **PET**: positron emission tomography, **CT**: computed tomography.*

1.2.2.1 Limitations

MM is a clonally (57) and spatially heterogeneous disease (58). In fact, it has been demonstrated that MM can have an unequal distribution of intra and extra-medullary disease (59). Not only does the BM microenvironment vary from host to host but it also has a spatiotemporal distribution within a single individual (10). A BM biopsy will only represent a single area of the BM at the particular time of sampling. Therefore, biopsy sampling may give false negatives. Yet in other patients, there is the spread and development of extra-medullary disease which can only be detected by additional expensive radiography (PET-CT). Radiography is subject to availability and accuracy of results can be affected by the presence of inflammation and infection. Furthermore, the BM sampling procedure can cause significant physical discomfort and mental distress to patients. The procedure requires administration of local anaesthetic to the hip and needle insertion to extract the BM. Some patients require distraction techniques (talking to a specialist nurse or listening to music) during the procedure or a general sedative beforehand. The procedure is performed in hospital – requiring patients to undertake a separate hospital visit if they are an outpatient. Both the actual procedure and the mental build-up after receiving the appointment prior to the procedure can be extremely stressful for patients. A BM aspirate alone costs around £800 and requires a lot of special resources to perform. This includes a highly skilled nurse or doctor, nurse/doctor time, use of room, equipment for biopsy and sample transport. Then there is the laboratory cost. For example, MFC is an additional £150, with a total cost of around £950. If using NGS (rather than MFC), the total cost is even higher (around £2000). BM specimens must be analysed within 24-48 hours of aspiration to avoid PC degradation and internalisation of CD138 (60). It is therefore necessary to establish a collaboration between the ward and the laboratory beforehand to ensure that this is possible for a given patient.

1.3 The high-sensitive FLC test (hsFLC) - a novel method developed at the CIS

To overcome the various limitations of BM-based MRD measurement, the clinical Immunology service (CIS) at the University of Birmingham has developed a non-invasive, serum-based technique named hsFLC, which is capable of MRD detection via high-sensitive measurement of sFLCs. The test has the following key benefits:

1) Sensitivity

As discussed previously, the presence of monoclonal FLCs are currently screened for qualitatively by IFE which has a sensitivity of 100mg/l. Serum κ and λ FLC quantification by Immuno-turbidimetric methods provides a κ to λ ratio which if abnormal serves as a surrogate m-protein marker to a sensitivity of 20mg/l. The limit of FLC detection for Matrix-assisted laser desorption/ionization-time of flight-mass spectrometry (MALDI-TOF-MS) is around <10mg/l. Preliminary testing demonstrates that the new hsFLC test has a sensitivity of <0.01 mg/l and can detect both κ and λ monoclonal FLCs, simultaneously. Targeting FLCs, is not only sensitive but provides a real time measurement of MRD due to the short half-life of FLCs. This should enable MRD assessments earlier in the course of therapy.

2) Accuracy

Measuring monoclonal FLCs in blood provides a systemic reflection of medullary as well as extra-medullary disease, overcoming the issue of patchy disease and false negatives associated with BM biopsy. As described in detail before, BM disease can be patchy and thus misrepresented by a single biopsy.

3) Repurposes an established technique.

HsFLC has been adapted from an established clinical technique used for the detection of oligoclonal bands (OCBs) in Multiple Sclerosis (MS). The principle of the test is isoelectric focussing (IEF). Many

clinical laboratories already have the expertise and equipment to perform protein separation using IEF.

4) Patient and healthcare benefits

The hsFLC test is performed on serum acquired from a routine blood collection in line with most routine clinical laboratory tests. No special patient preparation is required beforehand, and the pre-analytical sample handling, transport and storage requirements are the same as for any routine blood test; 5ml of clotted blood in a plain or SST gel tube. Compared to BM biopsy, blood sampling is less invasive, easier, faster, and a cheaper procedure preferable to the patient and healthcare providers alike. The hsFLC serum test is at least 80% cheaper than BM-MRD (CIS figures).

The increased ease of MRD monitoring would allow its more frequent use throughout the various stages of MM. Increased frequency of MRD measurement and information on status could improve patient monitoring and guide more personalised application of therapy. For example, by identifying those eligible for dose intensification, therapy switch, or cessation of therapy to enable its best utilisation.

1.4 Objectives

The objectives of this research were to:

1) Further develop and optimise the technique.

Adapt the technique currently used for IgG-OCBs so that FLCs could be detected instead at subclinical levels such as those found in MRD. All stages of the established OCBs protocol would to be tested in isolation and optimised to this end.

2) Validate its use for MRD.

Firstly, to retrospectively analyse a group of MM patients that had already been tested for MRD previously by a clinically validated technique; BM-based MFC. Archived serum from these patients would be analysed by hsFLC to identify any monoclonal FLCs indicating the presence of MM related MRD. Once the technical/conceptual validation was successful, then the target was to pursue clinical validation of hsFLC as a test for MRD in routine clinical practice. The CIS would be the first centre to offer the test nationally. The long-term goal was to attract established biotechnological firms to invest in producing CE marked hsFLC kits and instruments to allow many more laboratories to measure MRD themselves.

3) Validate its potential for application in other areas of MM.

The high-sensitivity of hsFLC makes it applicable for FLC measurement in almost all MM patients and beyond. For example, hsFLC may be able to detect very low levels of FLC in the first instance in non-secretory myeloma patients, light-chain amyloid and other renal FLC deposition diseases. Not only can it serve as a new diagnostic test in these rare subsets of patients, but it could also be continually employed at regular intervals to replace invasive and costly CT-PET/WB MRI scans to monitor disease remission and relapse. To investigate its application outside of MRD, a preliminary clinical validation would be carried out in a rare subset of NSMM patients.

2 Methods

The methods section is split into three sections:

1. Section 2.1- The principles of the hsFLC test, and an example of a clinically validated application of the fundamental method for the detection of OCBs in MS.
2. Section 2.2- Optimisation & refinement experiments undertaken to adapt hsFLC from the OCB method to the assessment of disease in MM patients.
3. Section 2.3- Three lines of clinical validation experiments that were performed to assess the application of hsFLC in MM.

2.1 The principle of the hsFLC test; IEF and Immunoblotting.

The hsFLC test is based on the combination of two well-known analytical techniques; IEF and Immunoblotting (IB). IEF is a method of separating proteins or charged molecules on the basis of their isoelectric point (pI). IEF can be performed using a polyacrylamide gel. However, the agarose-based equivalent is non-toxic and easier to handle. IEF gels are infused with charged molecules called carrier ampholytes to enhance resolution. When an electrical field is applied to an IEF gel preloaded with samples, each molecule is separated in a continuous pH gradient until it reaches its pI; the pH at which it has no net charge. The pH gradient of the gel can be adjusted by using various mixtures of carrier ampholytes. After separation of proteins by IEF, the western blotting technique can be used to transfer the proteins onto a membrane, usually nitrocellulose (NCM) or polyvinylidene fluoride (PVDF). Once all the migrating proteins have been immobilised onto the membrane, the specific proteins of interest can be 'probed' using corresponding antibodies. The antibodies used are usually pre-conjugated with an enzyme, such as horseradish peroxidase. After a period of antibody incubation, all unbound antibody-conjugate is washed away leaving only the bound antibody-protein complexes on the surface of the blot. An appropriate substrate for the bound enzyme is added to the blot leading to the formation a product that can be chemically stained. Proteins can exhibit unique

and highly reproducible patterns of staining known as spectrotypes. When IEF is combined with IB, protein detection can go down to below 1mg/L (61).

IEF plus IB can be used to separate IMGs in human serum by probing with anti-IMG antibodies. Healthy patient samples usually demonstrate an IMG spectrotype that resembles a smear; or a normal distribution of polyclonal IMGs (see A, figure 2). The smear-like trace is actually composed of multiple tightly packed monoclonal protein bands that line up adjacently and only differ from one another by a single unit of charge. Such subtle differences in charge between proteins relates to post-translational changes in glycosylation and deamidation of asparagine/glutamine residues or modification of other amino acid side chains (62).

A serum sample containing an m-protein-from an MM patient for example-produces an IMG spectrotype that appears like a group of regularly spaced bands either within or without a background smear of polyclonal IMGs. This pattern is sometimes described as the rungs in a ladder spectrotype. The number of bands in these patterns and its complexity depends on the IMG class, assuming that a standard level of protein is applied to the gel each time. An IgG m-protein is usually the most straightforward to identify; they typically appear as about six regularly spaced bands (D & E, figure 2). An IgA m-protein doesn't resolve as clearly, mainly owing to the excess of carbohydrate residues; they may appear as numerous much more closely packed lines (see B & C, figure 2). IgM m-proteins exhibit variable migratory behaviours related to their complex structure and therefore have no typical presentation on IEF (61). Anti-FLCs antibodies can also be used in IB to identify monoclonal FLC spectrotypes. Small differences in charge between FLCs manifest as larger gaps in the IEF spectrotype compared to IMGs due to the smaller molecular weight of FLCs. The classical monoclonal FLC pattern is one major band plus one or two minor bands (See F & G, figure 2). FLC bands sometimes appear bow-shaped under the influence of the strong electric field. When samples

containing monoclonal protein are analysed by conventional electrophoresis, a single unresolved monoclonal band is observed regardless of the IMG isotype. (Figure 3).

Another commonly observed IMG spectrotype in human serum is OCBs. Serum OCBs represent infection or systemic inflammation of multiple causes. One of the key features of these inflammatory responses is that they usually involve activation of a select number of B cell clones each secreting specific IMGs. As a result, the corresponding IEF pattern appears as two or more randomly spaced IMG bands. Several bands can also appear in groups that may or may not overlap (see H, figure 2). An M-protein can sometimes be observed together with an OCB pattern on a single trace (See I, figure 2). The m-protein can usually be distinguished from OCBs by the following three features: uniform spacing between bands, a decrease in staining intensity from the most cathodic to the most anodic band and restriction of bands to a small area of the trace. For example, in figure 2 (trace I), the m-protein is restricted to the anodal end (left) while the OCBs are in the centre and spread out towards the cathodic end of the trace. The m-protein in this example was confirmed by IFE (figure 4).

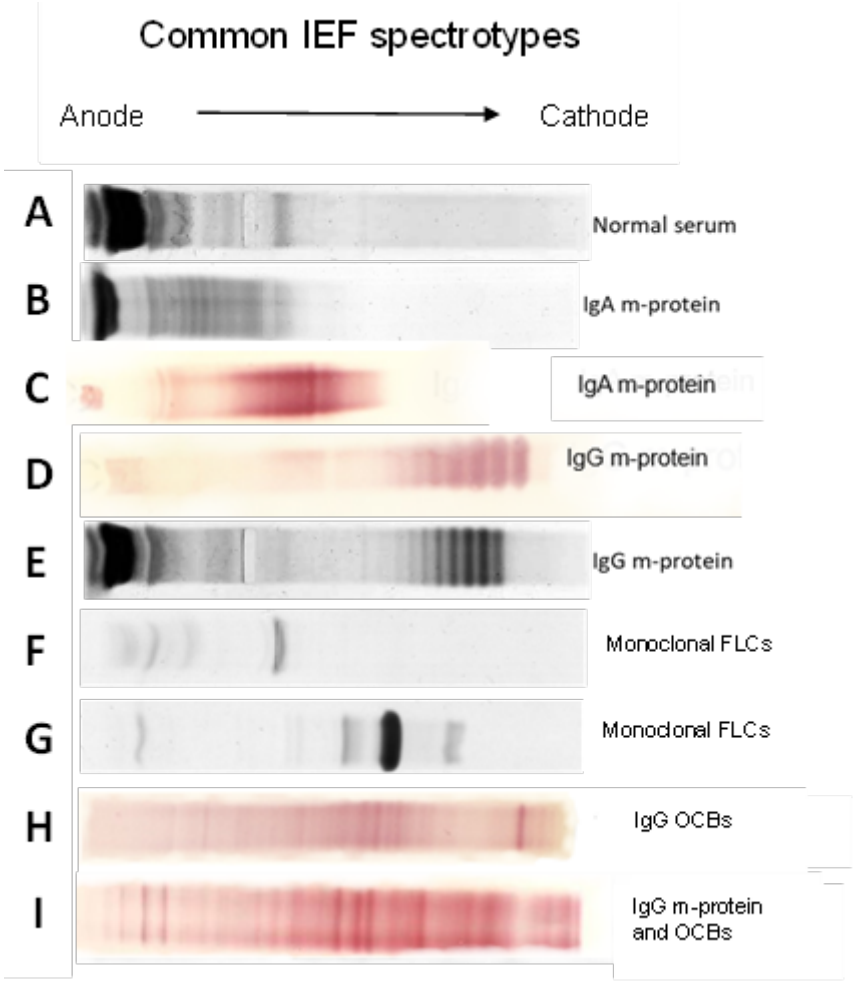


Figure 2. *Common IMG spectrotypes observed following IEF of human serum. The effective pH range of separation used in these examples was 4-10 for the detection of both IMGs and FLCs. The direction of protein migration is from the Anodal end (left) to the cathodal end (right). Images A, B, E, F, and G are taken from the work of Findley Cornell (Clin Biochem. Rev. 2009 Aug; 30(3): 123–130). Images C, D, H & I are from samples analysed by the CIS.*

Two examples of IFE results

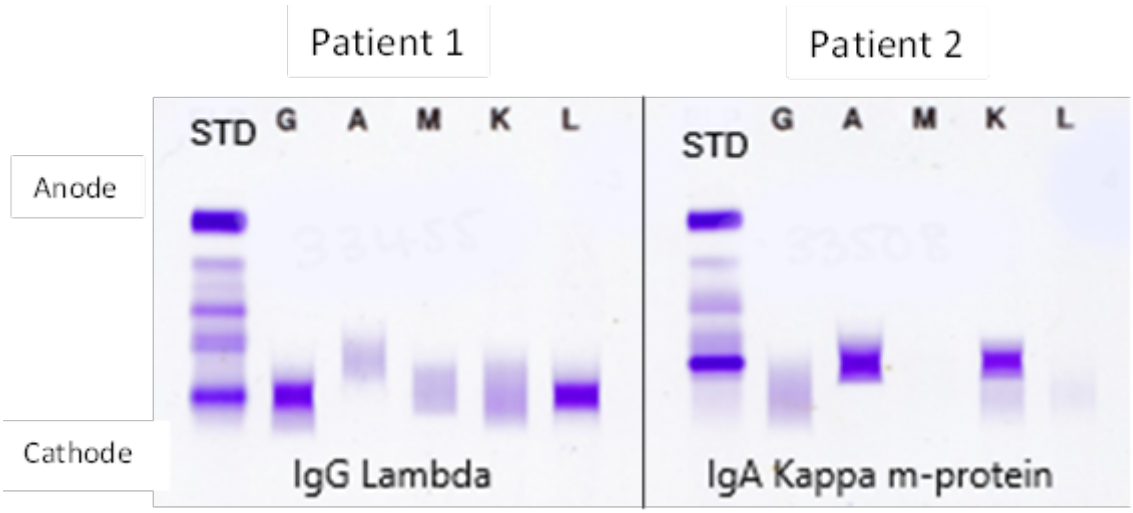


Figure 3. *Serum IFE results for two MM patient samples analysed at the CIS. Patient 1 has an IgG-λ m-protein (left) and patient 2 has an IgA-κ m-protein (right). Each IFE gel has six stained protein lanes from left to right; reference protein lane (STD), IgG, IgA, IgM IMGs, κ and λ FLCs. IMGs usually migrate towards the cathodic end of the gel.*

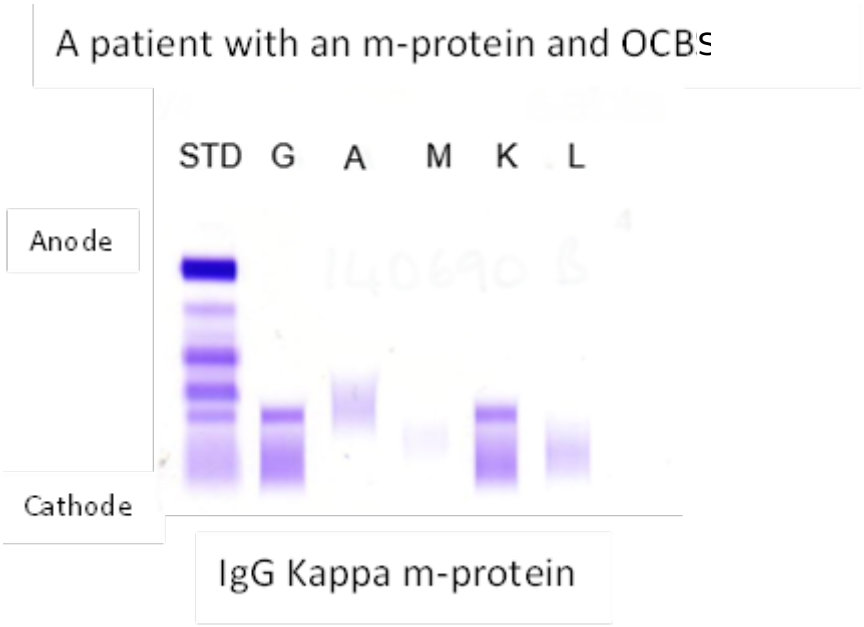


Figure 4*.Serum IFE results from a patient who had an IgG m-protein and OCBs detected by IEF/IB (See image I, figure 2). The presence of an anodic-biased IgG-κ m-protein was confirmed by immunofixation in the form of a single band in the G and the κ anti-sera lanes, respectively. However, the IgG-OCBs cannot be clearly distinguished at the cathodal end and the staining pattern appears as a polyclonal smear.*

IgG-OCBs are clinically relevant for the diagnosis of several central nervous system (CNS) disorders, such as MS and viral encephalitis. The CIS offers a clinically validated test based on IEF/IB that measures OCBs in serum and cerebrospinal fluid (CSF). When IgG-OCBs are found in a patients CSF but not in the corresponding serum sample then the IgG is said to be intrathecally or intracranially synthesized. More than 95% of patients with clinically defined MS have IgG-OCBS detectable in CSF (63).

***Figure 5**. IEF/IB results for a pair of serum and CSF samples taken around the same time from a patient suspected of MS. IgG OCBs can be seen in the CSF sample only. This indicates intrathecal IgG-synthesis in response to a CNS inflammatory process, such as MS.*

2.2 Adapting the CIS OCBs method to detect FLCs in serum

The following key areas of the OCB method were evaluated to optimise it for the detection of FLCs:

1. Gel type
2. Gel agarose concentration
3. Choice of carrier ampholytes
4. Sample diluent
5. Sample application and volume
6. Blotting membrane
7. Assay running conditions (concentration and volume of key reagents)

Details of all of these optimisation experiments that led to the final hsFLC protocol are given in the appendices (section 6.1). The only optimisation work included in this section are the experiments that demonstrated the maximum analytical sensitivity of the hsFLC method (section 2.2.2). This is followed by a step-by-step description of the fully optimised hsFLC method protocol (sections 2.2.3). The final part of the methods section (section 2.3) describes experiments performed to evaluate the clinical application of the hsFLC assay. Details of the equipment and reagents used throughout all of these experiments is given below first.

2.2.1 Equipment and reagents

Table 5. *Details of all the equipment used for the optimisation and the clinical validation of the hsFLC test.*

Name/description	Manufacturer	Serial number
Multiphor II Electrophoresis unit.	GE healthcare Biosciences LTD	56304683
LTC2 cooling unit	Grant instruments	R2153004
Electrophoresis power supply EPS 3500 XL	Pharmacia	56115553
QBT2 hot block (37°C)	Grant instruments	709517010
Vortex Whirlimixer	Fisons	16458
Mikro22 centrifuge	Hettich	1844
Temperature controlled water bath	Grant instruments	94226
Gel casting plate & frame	Pharmacia	N/A
Microwave for gel preparation	Moulinex	MCNNB67
Hairdryer 230V for heating gel plate	Babyliss	1118SE
Samples and reagents fridge (4°C)	Panasonic	17100546

Table 6. *Details of the reagents used for the optimisation and the clinical validation of the hsFLC test.*

Name/description	Source/ manufacturer	Storage temperature
Phosphate buffered saline (Dulbecco A) PH 7.3 at 25°C	OXOID LTD	Room temp (18 -22°C)
Elix 35 MilliQ Type-2 pure analytical grade H_2O	Merck Millipore	Room temp (18 -25°C)
D-sorbitol	Sigma-Aldrich	Room temp (18 -22°C)
Agarose IEF	GE Healthcare	Room temp (18 -22°C)
Gel bond film	LONZA Ltd	Room temp (18 -22°C)
High grain filter paper	Whatmann 3MM	Room temp (18 -22°C)
Nitrocellulose membrane (0.22µm pore size)	Ultra-Cruz/Santa Cruz Biotech.	Room temp (18 -22°C)
Pharmalyte, broad range pH 3-10	GE Healthcare	+2-8°C
Pharmalyte, broad range pH 8-10.5	GE Healthcare	+2-8°C
Dried skimmed milk powder	Marvel	Room temp (18 -22°C)
Glycerol	Sigma-Aldrich	Room temp (18 -22°C)
Methanol	VWR Chemicals	Room temp (18 -22°C)
Hydrogen peroxide 30% (w/w) in H_2O	Sigma-Aldrich	+2-8°C
3M Sodium Acetate buffer	Sigma-Aldrich	Room temp (18 -22°C)
3- Amino-9-ethylcarbazole (AEC) tablets	Sigma-Aldrich	Room temp (18 -22°C)
Anode (+) solution (0.05M sulphuric acid)	Reagecon	Room temp (18 -22°C)
Cathode (-) solution (12.5M NaoH)	Sigma Aldrich	Room temp (18 -22°C)

Table 7. *Details of the controls and antibodies used for the optimisation and clinical validation of the hsFLC test.*

Name/description	Source/ manufacturer	Storage temperature
1) Internal quality controls		
a. κ FLC control (Urine 1g/ml)	CIS	+2-8°C
b. λ FLC control (Urine 1g/ml)	CIS	+2-8°C
c. IgG κ control (Serum, 4.2g/l)	CIS	+2-8°C
2) Primary Mouse anti-human FLC Monoclonal Antibodies		
a. BUCIS 01 (κ, 1.15 mg/ml)	CIS	+2-8°C
b. BUCIS 04 (κ, 1.25mg/ml)	CIS	+2-8°C
c. BUCIS 03 (λ, 2.0 mg/ml)	CIS	+2-8°C
d. BUCIS 09 (λ, 1.0 mg/ml)	CIS	+2-8°C
3) Primary Mouse anti-human Total LC antibodies (Bound and free)		
a. BUCIS 14 (κ, 1.0 mg/ml)	CIS	+2-8°C
b. BUCIS 19 (λ, 1.0 mg/ml)	CIS	+2-8°C
4) Primary Mouse anti-human monoclonal IMG antibodies		
a. Anti-IgG-RIO (1.0mg/mL)	CIS	+2-8°C
b. Anti-IgA-MG156 (0.95 mg/ml)	CIS	+2-8°C
5) Secondary polyclonal Antibody-conjugate		
a. Goat anti-mouse IgG Human ads-HRP conjugate	Southern Biotech	+2-8°C
b. Goat anti-Mouse IgG, Human ads-AF647 fluorescent conjugate	Southern Biotech	+2-8°C

2.2.2 Sensitivity of the final method

An integrated description of the sensitivity experiments (methods plus results) is given in this section demonstrating the maximum analytical sensitivity of the final hsFLC protocol. Sensitivity of detection for the following four classes of m-proteins was investigated:

a) IgG

b) IgA

c) Free κ LCs

d) Free λ LCs

Patient samples with m-proteins from the above mentioned four categories were selected. The m-protein concentrations were confirmed by a combination of CZE and nephelometry. Each patient sample was serially diluted into normal human serum (NHS) to ascertain the highest dilution at which the m-protein was still detectable in the polyclonal background of NHS. The reason for using NHS as diluent instead of 2% BSA/PBS-as used in other optimisation experiments (see appendix, section 6.1.4)-was to test the method in a polyclonal IMG background representative of that found in MM patient's recovering after induction therapy. The standard IEF/IB protocol was used to analyse each patent sample separately. The following corresponding antibodies (listed in Table 7, section 2.2.1) were used to probe for the particular class of m-protein found in each patient.

a. Anti-IgG-RIO (table 7, 4a) against IgG.

b. Anti-IgA-MG156 (table 7, 4b) against IgA.

c. BUCIS 01 and BUCIS 04 (table 7, 2a & 2b) against κ FLCs

d. BUCIS 03 & BUCIS 09 (table 7, 2c & 2d) against λ FLCs

Figure 6 below shows the results of the sensitivity experiments. A single example is given for each class of m-protein.

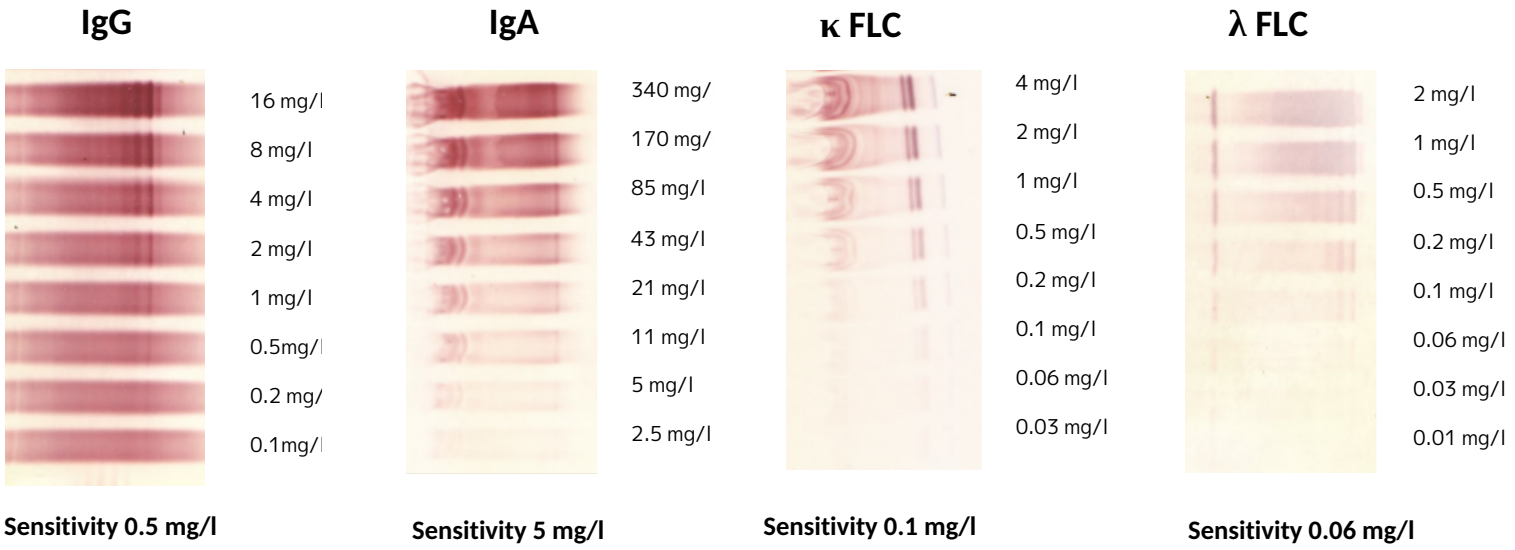


***Figure 6**. Scanned images of the IBs produced using the final protocol to investigate the sensitivity of detection for four classes of m-protein: IgG, IgA, κ FLCs and λ FLCs. The observed sensitivity for each class of m-protein is stated below each image in bold font. The method sensitivity for whole IMGs is ≤ 6 g/L and < 1 mg/L for FLC.*

The final method demonstrated a significantly greater sensitivity compared to conventional IFE (~150 mg/L) and also a superior sensitivity to those reported for MS (< 10 mg/L for whole m-protein)(64).

2.2.3 Final method protocol for the hsFLC test

In this section, a step-by-step description of the final method protocol for the hsFLC test is given and summarised afterwards in Figure 7. This protocol was applied to all of the clinical evaluation experiments that follow this section. A list of the equipment & reagents used was already given at the beginning of this section.

1. **Agarose-gel preparation**

a. The gel mixture

- A mixture containing 3.6g of D-Sorbitol, 0.3g of Agarose and 27ml of 10% v/v glycerol was heated in a Schott glass using a microwave oven until fully clear and dissolved.
- This mixture was then transferred to a water bath set at 75°C for 15 minutes.

b. Assembling the gel casting frame

- A small amount of solution containing 50% methanol (in deionised water) was applied to the surface of a gel casting plate.
- A sheet of gel bond film (hydrophilic side facing upwards) was placed onto the casting plate.
- A clean paper towel was placed on top of the bond film and a roller was used to ensure good contact between film and plate and to remove any air bubbles.
- A Perspex casting frame was placed on top of the gel bond and secured with clips.
- A spirit level was used to ensure the gel bond surface was completely level across its entire length.
- The gel bond surface was warmed for five minutes with a hair drier positioned above the assembly using a clamp stand.

c. Casting the gel

- Once the temperature of the agarose mixture was at 75ºC, 2ml of Pharmalyte (pH 3-10) and 0.5ml of Pharmalyte (pH 8-10.5) were slowly injected into the molten agarose. The solution was mixed and incubated for a further 5 minutes in the water bath at 75°C.
- Thereafter, the molten gel solution was poured onto the pre-warmed gel bond surface and spread evenly over the gel bond film. The gel was left to set for 10 minutes.
- Finally, the gel was placed in a clean moist chamber and stored in the fridge at +2-8°C overnight, ready to use the following day.

2. **Preparation of the IEF electrophoresis unit and gel prior to sample loading**

- The IEF unit was precooled to 10°C.
- A small amount of 50% aqueous methanol was applied to the cooling plate of the IEF unit.
- Prior to sample application, a single sheet of NCM was used to blot the surface of the gel to remove excess surface liquid.
- The gel was positioned onto the wet cooling plate ensuring no air bubbles were trapped beneath.
- Whatmann Filter paper (3mm) was used to prepare two electrode wicks. These were 10mm wide and cut to the exact length of the gel. One was soaked in 0.05M sulphuric acid (H_2SO_4) and the other in 1M sodium hydroxide (NaOH). Thereafter, wicks were blotted dry to remove surplus solution.
- The wick soaked in NaOH was placed at the cathodal (-) end of the gel and the H_2SO_4-soaked wick on the anodal (+) side, approximately 7cm apart.
- Two sheets of folded tissue paper (5cm x 25cm) were positioned over the outer edges of the gel (~5mm in) to drain away excess fluid during electrophoresis.
- Finally, the sample application strip was positioned approximately, 5mm in from the anodic wick and about 2.5cm from the top of the gel.

3. **Sample preparation and application**

- The primary disease presenting samples for each patient were run initially. Any patients with monoclonal components were selected for further analysis. In subsequent runs, presentation and follow up samples were run together in a series.
- Each gel had the capacity to run 30 samples at a time.
- All Samples were lightly vortexed before application and 5µl of sample was applied to each well in an assigned order.
- For quality control, standards prepared at the CIS containing either κ or λ FLCs (see table 7, 1a & 1b) were used. Both standards were diluted in PBS (1/100) before application.

4. **IEF procedure** (temperature regulated by cooling unit at 10°C)

1. The power unit was put through a conductivity test prior to each run and the settings were programmed as follows:
   A. Voltage- 1250V
   B. Current- 100mA
   C. Power- 20W
2. Electrophoresis was paused approximately every 50vh, to wipe away excess fluid on the glass plate, the wicks, the electrodes and around the gel bond.

5. **Transfer and press** (Western blot steps after IEF at 18-22°C).

- At the end of each run (1000vh), the gel was removed from the cooling plate and the outer edges including the wicks were cut away.

- The surface of the gel was blotted briefly with NCM (7.5cm x 23cm) to remove cross-reacting surface proteins and contaminants.
- A second piece of 0.4 µM NCM (7.5cm x 23cm) was placed shiny side-down onto the gel ensuring complete contact. The edges of the NCM were marked to indicate sample positions.
- Several pieces of filter paper (Whatman No 1), a Perspex plate and a 3kg weight were placed on top of the NCM. The blot was pressed in this way for up to one hour to transfer the separated proteins from the gel to the NCM by capillary diffusion.
- After one hour, the NCM was peeled away from the agarose gel, placed protein-side upwards into an incubating container and dried slowly with a hair dryer until the NCM returned to a smooth texture.

6. **Blocking** (temperature 18-22°C)

- The unoccupied sites on the NCM sheet were blocked for 60 minutes with 100ml of a 0.05g/ml milk protein solution (marvel in PBS) with gentle agitation on a rocker.

7. **Wash 1**

- After blocking was complete, the membrane was rinsed multiple times with water.

8. **Primary Antibody incubation** (temperature 18-22°C)

- The NCM was fully immersed in 20ml of a 0.002g/ml milk protein solution (marvel in PBS).
- Twenty-five µl each of the primary monoclonal antibodies (Kappa; BUCIS 01, 04 and Lambda BUCIS 03, 09; see section 2.2.1, table 7, 2a-d) were added to achieve a 1/800 antibody dilution. This was left to incubate for 60 minutes with gentle agitation on the rocker.

9. **Wash 2**

- After antibody incubation was complete, the blot was washed several times (20) under tap water followed by three 5-minute washes in PBS.

10. **Secondary antibody incubation** (temperature 18-22°C)

- Another 20ml of the 0.002g/ml marvel solution was poured onto the NCM in the incubating container before adding 40 µl of the secondary antibody-conjugate (Goat anti-mouse IgG Human-ads HRP conjugate; see section 2.2.1, table 7, 5a) at a 1/500 dilution. This was left to incubate for a further 60 minutes with gentle agitation on the rocker.

11. **Wash 3**

- After secondary antibody incubation was complete, the blot was washed several times (20 times) under tap water followed by three 5-minute washes in PBS.

12. **Development** (temperature 18-22°C)

- A single tablet of 3, amino-9-ethylcarbazole (AEC) was dissolved in 2.5ml of 100% methanol.
- A 50mM Sodium acetate (NaOAc) working solution was prepared by diluting 833µl of 3M NaOAc into a final volume of 50ml with deionised H_2O.
- The 50mM NaOAc solution, the AEC solution and 33µl of 30% w/w Hydrogen peroxide solution were thoroughly mixed and added to the NCM blot in the development container and left for 15 minutes- in the dark- with gentle agitation.

13. **Stop, wash and dry**

- On completion, the enzymatic reaction was stopped by washing the blot in several changes of tap water, followed by a final rinse in deionised water. The blot was dried, labelled and scanned into an electronic file.

14. **Interpretation**

- Two experienced readers with IEF expertise interpreted the spectrotypes for each sample based on known patterns (see methods 2.1, figure 2)

Figure 7 below summarises all of the steps mentioned above in the final hsFLC protocol.

hsFLC protocol steps	Description
1. Gel preparation	Incubate gel mixture containing carrier ampholytes, then cast gel using template and frame (**30 min**).
2. IEF preparation	Load gel onto cooled IEF platform (10°C). Place anodal & cathodal electrode wicks onto either side of the gel (**5 min**).
3. Sample loading	Position sample applicator onto gel & apply 5ul of each sample (**5 min**)
4. IEF	Perform electrophoresis at 1250V, 100mA & 20W, until 1000 volt/hour is reached (**75 min**).
5. Transfer and press	Remove gel, clean surface with NCM1. Apply NCM2, filter paper and press with a 3kg weight (**30 min**).
6. Blocking	Remove the NCM from the gel, heat dry and then incubate in a 5% milk/PBS solution (**30 min**).
7. Wash	Rinse away blocking solution with clean water (**2 min**)
8. Antibody 1 incubation	Incubate blot with mouse monoclonal antibodies at a 1:800 dilution in 0.2% milk/PBS solution (**60 min**).
9. Wash	Rinse blot in copious amounts of water several times. Incubate with PBS for 5 minutes. (**7 min**)
10. Antibody 2 incubation	Incubate blot with anti-mouse goat antibody-conjugate at a 1:500 dilution in 0.2% milk/PBS solution (**60 min**).
11. Wash	Rinse blot in copious amounts of water several times. Incubate with PBS for 5 minutes (**7 min**).
12. Development	Place NCM into development solution, add substrate and allow red colour to develop (**15 min**, protect from light)
13. Stop, wash and dry	Stop enzyme-substrate reaction by rinsing blot in water several times. Dry and store in dark (**5 min**)
14. Interpretation	See methods section 2.1- figure2, for common IEF spectrotypes (**5-10 min**)

***Figure 7**. A summary of all the steps of the final hsFLC protocol. The approximate operator time for each step is also given in brackets. The total operator time is around 5 hours.*

2.3 Clinical evaluation.

Three lines of clinical validation experiments are described here which were performed to assess the application of hsFLC as follows:

1. Section 2.3.1- Analysis of MRD in post-transplant MM patients.
2. Section 2.3.2- Analysis of LCMM and OSMM patients.
3. Section 2.3.3- The diagnosis and monitoring of NSMM patients.

2.3.1 Post-transplant MRD experiments

The UK National Cancer Research Institute (NCRI) Myeloma XI study was the largest trial ever conducted for first line MM treatment, involving 4420 patients (ISRCTN Registry number: ISRCTN49407852, recruitment from 25/05/2010 to 24/02/2016). A total of 110 MM patient samples from the national Myeloma X & XI trials (CIS sample archives) were retrospectively analysed by hsFLC for the presence of MRD. From the selected patients, 54 had m-proteins with involved κ FLCs and 56 had involved λ FLCs at diagnosis.

Out of the 54 patients tested with κ related m-proteins:

1. 33 patients were male and 21 patients were female (gender-specific analysis was not conducted in this study but is mentioned here for reference).
2. 45 patients had heavy chain related m-proteins; IgG (31 patients), IgA (13 patients) and IgD (1 patient).
3. 9 patients had monoclonal κ FLCs only.

Out of the 56 patients tested with λ related m-proteins:

1. 34 patients were male and 22 patients were female.

2. 45 patients had heavy chain related m-proteins; IgG (29 patients), IgA (14 patients) and IgD (2 patients).
3. 11 patients had monoclonal λ FLCs only.

For each patient, the disease presentation sample (primary sample at diagnosis) and the day-100 post-autologous stem-cell transplant (D100-PASCT) sample were analysed according to the hsFLC protocol given in section 2.2.3). All samples were tested neat (undiluted). The κ and λ patients were tested separately using the corresponding mouse monoclonal antibodies for primary incubation; BUCIS 01 & 04 for κ samples and BUCIS 03 & 9 for the λ samples (see section 2.2.1, table 7, 2a-d). Following analysis, the IBs were interpreted independently by two experienced readers. For each patient, the presentation sample trace was firstly scrutinised for the presence of FLC m-protein bands (see methods section 2.1, figure 2 for monoclonal FLC spectrotypes). The D100-PASCT sample trace was then assessed to see if the same m-protein bands (in terms of migratory position) were still detectable following treatment. If the same bands or at least the major band was detectable, then the MRD result by hsFLC was recorded as positive. Even if the major band was only partially detectable, it was still deemed indicative of residual disease for the purposes of demonstration. If the original m-protein bands were no longer detectable in the D100-PASCT sample, then the MRD result was recorded as negative. The presence or absence of new FLC OCBs in all of the post induction samples was also recorded separately as either positive or negative, to assess immune recovery. Any unusual spectrotypes were recorded and assessed separately.

The MRD results obtained by hsFLC for these patients were retrospectively compared to previous MRD results from MFC analysis. MFC was originally performed on BM aspirates (at the point of sampling) at presentation and D100-PASCT by the haematological malignancy diagnostic service, Leeds, using the FACS CantoII 8-colour flow cytometer. The immunophenotype of both normal and abnormal PCs were measured in each aspirate using a six-colour panel of monoclonal antibodies

against specific MM-related cell markers. Abnormal PCs were classified as those with strong CD56 expression, weak CD27 (and/or CD45) expression and a lack of CD19 expression. MRD diagnosis was made on the basis of the detection of ≥ 50 abnormal clonal PCs per 500,000 in the BM. MRD results by MFC were reported accordingly at the same centre (44). All results recorded as either MRD positive or negative were accessed using the CIS trials database.

Serology results for each patient sample were also obtained from the clinical records to aid with the comparison. These included serum IMG and FLC levels, measured using the Roche Cobas 6000 Immunochemistry platform. The Binding site's Freelite turbidimetric method was used for all sFLC measurements. M-protein data was also gathered; typing was performed by IFE and quantification by a combination of CZE and nephelometry (to measure total protein). Following ASCT, the response status for each patient based on IMWG response criteria (see section 1.1.6, table 2) was also obtained.

2.3.1.1 *Methods for statistical analysis of MRD results*

The κ and λ related MRD results were analysed separately first to identify any FLC-specific bias in hsFLC results. Then, a combined analysis was performed to double the data set from which overall conclusions about the assay could be made. Both the raw hsfLC results and the MRD results by MFC, were qualitative. Therefore, method comparison was performed in the context of frequency of patients with positive and or negative values in relation to one another and also to various other parameters detailed below. Finally, concordance plots and the Cohen's Kappa Coefficient was used to measure inter-method agreement.

The first major parameter by which results were assessed was:

1. M-protein type at diagnosis; IgG, IgA, IgD and FLC-only.

The post-treatment response category (SCR, VGPR etc.) and the MRD results by both methods in relation to each m-protein type was analysed. The purpose of this was double-fold. Firstly, it would expose any m-protein-specific bias in the post-treatment response categories achieved by each patient. In the unlikely event of an m-protein specific finding, for example, if a particular m-protein type always failed to achieve an SCR post-ASCT, then MRD measurement may not be useful in these patients initially, until they demonstrate an SCR. Such a finding would require verification though, with a much larger data set. Secondly, any heavy chain-associated bias using either method of MRD measurement could be identified. If for example, certain m-protein categories were always MRD negative by a particular method, then the test may not be sufficiently useful (i.e. sensitive) for that subgroup. In principle, no bias was expected as the method measures FLCs in all cases. Nevertheless, the effect of the presence or absence of intact m-proteins on the assay was assessed by also measuring MRD in FLC-only secretory patients.

The second parameter assessed was:

2. The D100-PASCT response category.

It was hypothesised that the majority of patients who achieved an SCR post-transplant, would also be MRD negative considering that they should all have a normal κ/λFLC ratio. On the other hand, it was assumed that patients with other post-treatment responses besides SCR, would be MRD positive in association with an abnormal κ/λFLC ratio.

The final parameter was the:

3. The relationship between the D100-PASCT κ/λ FLC ratio and the MRD results.

The purpose of this was to directly investigate the assumption that patients with a normal κ/λFLC ratio (0.26–1.65) will be MRD negative, while patients with an abnormal κ/λFLC ratio will be MRD positive. Furthermore, it was expected that MRD results by hsFLC would be more in line with this assumption than MFC, considering it is an FLC based assay.

2.3.2 LCMM and OSMM experiments

A total of nineteen MM patients from the national Myeloma IX and XI trials were selected for retrospective analysis.

A. Eleven of these patients were clinically classified-at the point of first diagnosis- as LCMM by conventional IFE (using FLC antisera). Seven were identified as monoclonal κ-FLC secretory and the four were monoclonal λ-FLC secretors.
B. The other eight patients all tested negative for m-protein by IFE, and were classified as OSMM based on sFLC concentrations of around 25-80mg/L, quantified at the time by the binding site's Freelite method. Five of these patients were low level secretory (<50mg/L) OSMM patients. Six out of eight of the OSMM patients had an abnormal κ/λ FLC ratio and two had a normal κ/λ FLC ratio.

All nineteen samples had FLC levels quantified at diagnosis by the Binding site's Freelite method. These results were recovered from the CIS clinical records. All of the samples were analysed neat (undiluted) initially according to the hsFLC protocol given in section 2.2.3. Following IEF, the blots were probed using both anti-κ (BUCIS 01 & 04) and anti-λ (BUCIS 03 & 9) mouse monoclonal antibodies combined in order to identify the presence of any monoclonal FLC bands and therefore confirm the original diagnosis (see section 2.2.1, table 7, 2a-d for antibodies).

Six LCMM samples (3 κ and 3 λ secretors with sFLC concentrations ranging from around 250 to 800 mg/l of the involved LC), were then retested- by the same protocol and combination of antibodies- at a 1 in 4 and also at a 1 in 16 dilution (2% BSA in PBS diluent). These were loaded onto a single gel and positioned adjacently to one another in a patient wise manner for clear comparison. The rationale for the dilutions was to establish the ability and range of hsFLC to monitor post-treatment response in this cohort, i.e. to assess whether or not monoclonal FLC bands were still detectable after a 75% reduction (1/4 dilution) of the m-protein resembling a partial response (PR) to treatment and also if

bands could still be detected following a >90% reduction of m protein (1/16 dilution) resembling at least a very good partial response (VGPR) to treatment.

In order to achieve the highest clarity of signal, the second blot (with the diluted samples) was probed & tagged with a fluorescent conjugate and upon completion the fluorescence was measured accordingly (see section 2.2.1, table 7, 5b for antibody). The same blot was then stripped, probed again with the usual secondary antibody-conjugate and measured colorimetrically. Following analysis, the IBs were interpreted independently by two experienced readers. The presence of FLC monoclonal bands was recorded as positive. Otherwise, the result was recorded as negative.

2.3.3 Diagnosis and monitoring of NSMM patients

A total of 29 patients originally classified as NS from the national myeloma IX and XI clinical trials were selected for re-analysis by hsFLC. NSMM patients are technically defined as those that are negative for m-protein by conventional IFE and at the same time those that have none or barely any detectable FLC at diagnosis. Although the 29 NS patients chosen for this study were all negative for m -protein by IFE, the range of measured sFLCs (Binding site's Freelite method) ranged from around 0.1 to 50 mg/L.

- Eight patients had sFLCs <5mg/L,
- Eleven patients had sFLCs between 5-10 mg/L,
- Seven patients had sFLCs between 10-25 mg/L,
- Three patients had sFLCs from around 25 to 50 mg/L.

The last three patients at least could technically be described as low-level OSMM patients rather than true NS patients. In any case, only four patients in total had very slightly elevated κ/λ ratios and only 4 in total had very slightly supressed ratios. Otherwise, there was no other laboratory indication of monoclonal gammopathy.

A two-stage analytical strategy was devised for this rare cohort of patients with a view of applying the strategy to new NS patients if it was found successful. In the first part of the screening process, the samples were probed (according to the standard hsFLC protocol) using a combination of κ and λ antibodies to total LCs (free and bound) to achieve maximum sensitivity considering it was expected that these patients would have very low levels of FLCs. (antibodies used were BUCIS 14 & BUCIS 19; see reagents section 2.2.1, table 7, 3a and 3b). The screen blot was also repeated with the samples prediluted 1/100 in 2%BSA/PBS in order to remove some of the polyclonal background that might hinder the visual clarity of the monoclonal bands.

In the second stage which was applied only to samples that were positive for monoclonal FLCs, the utility of hsFLC to monitor treatment responses in NS patients was assessed. For this reason, the NS patient presentation samples and any available post-treatment follow-up samples that could be found, were run side by side to compare the changing trace in response to treatment. The follow up samples chosen-where available- were the first sample after induction therapy, any disease-remission and or disease-relapse samples. Each set of samples were analysed twice in separate assays using mouse anti-κ and anti-λ monoclonal antibodies. (See section 2.2.1, table 7, 2a-d for antibodies). The IBs were all interpreted independently by two experienced readers. The presence of FLC monoclonal bands was recorded as positive. Otherwise the result recorded was negative.

3 Results

The results of the three clinical validation experiments are presented here as follows:

1. Section 3.1- Analysis of MRD in post-transplant MM patients.
2. Section 3.2- Analysis of LCMM and OSMM patients
3. Section 3.3- The application of hsFLC to diagnose and monitor NSMM patients.

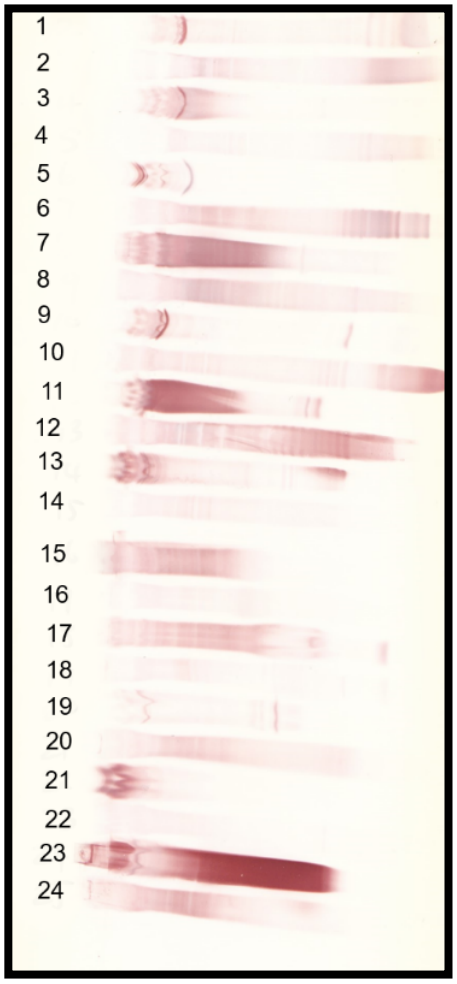
3.1 Post-transplant MRD results

The MRD results are split into two sections; κ (3.1.1) and λ results 3.1.2). For each set of patients, images of the blots produced by hsFLC are given firstly, alongside the raw results data for each blot. These are followed by tabulated data which include serology results, post treatment response statuses, the original MRD results (by MFC) for each patient and the MRD results achieved by hsFLC following interpretation of the blots shown. Finally, statistical analysis for each set of the results are given.

3.1.1 Kappa patients

Figure 8 below shows scanned images of the IBs produced by hsfLC for 54 MM patients with involved κ FLCs. For each patient, there are two sets of results (protein traces) adjacent to one another; the presentation sample trace followed by the D100-PASCT sample trace. Therefore, there are a total of 108 sample traces shown. The key to the right of each blot shows the raw hsfLC results data for each patient. The blots were incubated using mouse anti-κ FLC monoclonal antibodies.

Samples 1 to 24



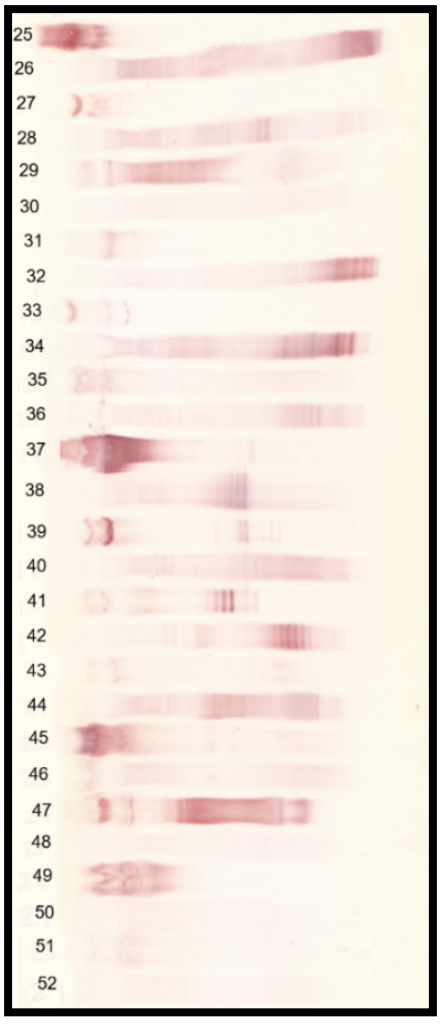
Sample key and raw hsFLC results data

Sample No.	Patient ID	Sample type	Monoclonal FLCs?	New OCBs?	Note
1	1	PRES	P	-	
2		DHAST	N	N	
3	2	PRES	P	-	
4		DHAST	N	P	
5	3	PRES	P	-	
6		DHAST	N	P	L
7	4	PRES	P	-	
8		DHAST	P	P	
9	5	PRES	P	-	
10		DHAST	P	P	L
11	6	PRES	P	-	
12		DHAST	P	P	L
13	7	PRES	P	-	
14		DHAST	N	P	
15	8	PRES	P	-	
16		DHAST	P	N	
17	9	PRES	P	-	
18		DHAST	P	N	
19	10	PRES	P	-	
20		DHAST	P	N	
21	11	PRES	P	-	
22		DHAST	N	N	
23	12	PRES	P	-	
24		DHAST	P	N	

PRES: *presentation sample,*
DHAST: *day-100 post-autologous stem cell transplant sample,*
P*: positive,* ***N****: negative,* ***L****: ladder pattern.*

Figure 8*. Part 1/4*

Samples 25 to 52

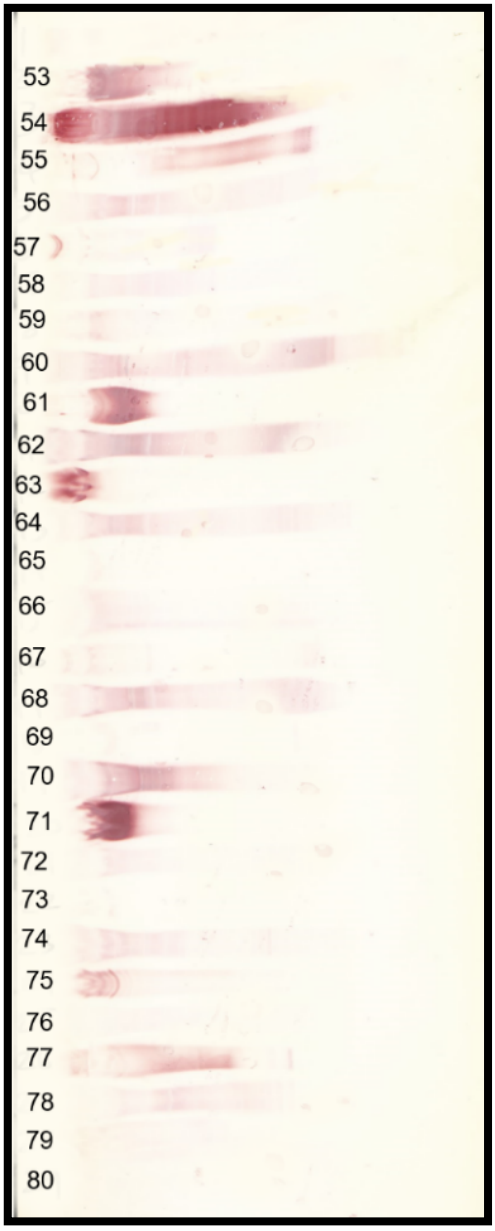


Sample key and raw hsFLC results data

Sample No.	Patient ID	Sample type	Monoclonal FLCs?	New OCBs?	Note
25	13	PRES	P	-	
26		DHAST	N	N	
27	14	PRES	P	-	
28		DHAST	N	P	L
29	15	PRES	P	-	
30		DHAST	N	N	
31	16	PRES	P	-	
32		DHAST	N	P	
33	17	PRES	P	-	
34		DHAST	N	P	
35	18	PRES	P	-	
36		DHAST	P	P	L
37	19	PRES	P	-	
38		DHAST	N	P	L
39	20	PRES	P	-	L
40		DHAST	N	P	
41	21	PRES	P	-	L
42		DHAST	N	P	L (new)
43	22	PRES	P	-	
44		DHAST	N	P	
45	23	PRES	P	-	
46		DHAST	P	N	
47	24	PRES	P	-	
48		DHAST	N	N	
49	25	PRES	P	-	
50		DHAST	N	N	
51	26	PRES	P	-	
52		DHAST	N	N	

Figure 8. *Part 2/4*

Samples 53 to 80

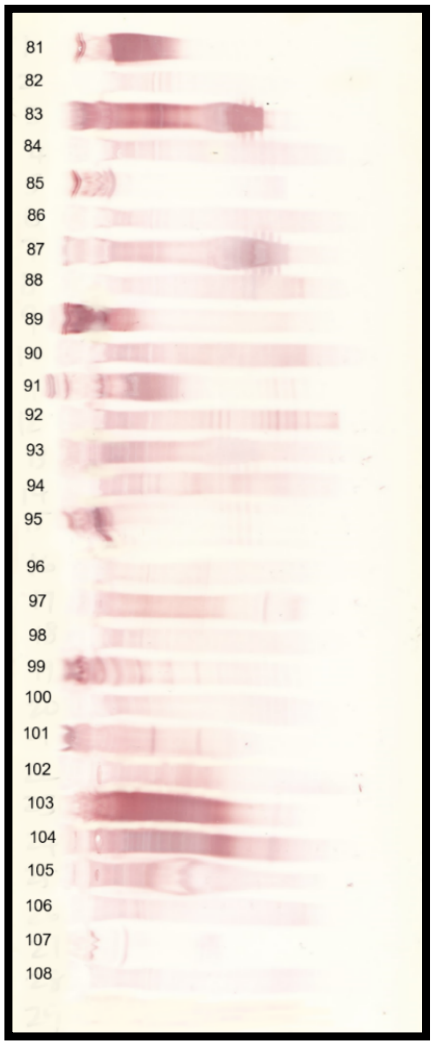


Sample key and raw hsFLC results data

Sample No.	Patient ID	Sample type	Monoclonal FLCs?	New OCBs?	Note
53	27	PRES	P	-	
54		DHAST	P	P	
55	28	PRES	P	-	L
56		DHAST	N	N	
57	29	PRES	P	-	
58		DHAST	N	N	
59	30	PRES	P	-	
60		DHAST	P	P	L
61	31	PRES	P	-	
62		DHAST	P	N	
63	32	PRES	P	-	
64		DHAST	P	P	L
65	33	PRES	P	-	
66		DHAST	P	N	
67	34	PRES	P	-	
68		DHAST	P	N	
69	35	PRES	P	-	
70		DHAST	P	P	
71	36	PRES	P	-	
72		DHAST	P	N	
73	37	PRES	P	-	
74		DHAST	P	P	
75	38	PRES	P	-	
76		DHAST	N	N	
77	39	PRES	P	-	
78		DHAST	P	N	
79	40	PRES	P	-	
80		DHAST	N	N	

Figure 8. *Part 3/4*

Samples 81 to 108



Sample key and raw hsfLC results data

Sample No.	Patient ID	Sample type	Monoclonal FLCs?	New OCBs?	Note
81	41	PRES	P	-	
82		DHAST	N	P	L
83	42	PRES	P	-	L
84		DHAST	P	P	
85	43	PRES	P	-	
86		DHAST	P	P	
87	44	PRES	P	-	L
88		DHAST	P	P	L (same)
89	45	PRES	P	-	
90		DHAST	N	P	
91	46	PRES	P	-	
92		DHAST	P	P	L
93	47	PRES	P	-	L
94		DHAST	P	N	
95	48	PRES	P	-	L
96		DHAST	P	N	
97	49	PRES	P	-	
98		DHAST	N	N	
99	50	PRES	P	-	
100		DHAST	P	N	
101	51	PRES	P	-	
102		DHAST	P	N	
103	52	PRES	P	-	
104		DHAST	P	N	
105	53	PRES	P	-	
106		DHAST	P	N	
107	54	PRES	P	-	
108		DHAST	N	N	

Figure 8. *Part 4/4. Scanned images of the IBs produced by hsfLC for 54 patient samples w*
The key to the right of each image shows the raw hsFLC results data associated with each

The actual IBs were used for recording results; the band clarity is not as obvious
In many cases, the major band from the presentation sample was only partially d
PASCT sample, yet still deemed positive for residual disease. Some of the sample
83 & 103) had a high protein content; a high background of polyclonal FLCs impa
monoclonal band detection. Such samples could be diluted 1/5 in 2% BSA/PBS be
in a clinical setting. See below for a summary of results.

Summary of the raw hsFLC results data for κ pateints

1. Presentation samples:
    a. Monoclonal κ FLCs were detected in all 54 of the presentation samples.
    b. 5 pateints demonstrated an unusual uniform laddering spectrotype exclusively in the presentation sample.

2. D100-PASCT samples:
    a. In 29 pateints, the same monoclonal κ FLC bands found in the presentation sample were also detectable in the respective D100-PASCT sample.
    b. New κ FLC OCBs were observed in 25 pateints (44%); 11 of these pateints also simultaneously had monoclonal FLCs detected (20%)
    c. 10 patients showed an unusual uniform laddering spectrotype exclusively in the D100-PASCT sample.

3. Pateint 21 (sample 41 & 42):
    - Both the presentation and the D100-PASCT samples demonstrated an unusual uniform laddering spectrotype. However, the postion of the laddering bands in the D100-PASCT was different to the presentation sample.

4. Pateint 44 (samples 87 & 88):
    - The spectrotypes of both the presentation and the D100-PASCT demonstrated the same unusual uniform laddering pattern.

The raw hsFLC results data from figure 8 above are presented below in the context of MRD (table 8). For each patient, there are two sets of results adjacent to one another; the presentation sample results followed by the D100-PASCT sample results. Serology results for both the presentation and the D100-PASCT are given where available. These include serum IMG, sFLC, m-protein typing and quantification results. For each D100 PASCT sample, the post-treatment response status defined by the IMWG response criteria is given, firstly. Then the MRD results previously reported by MFC are shown and finally, the new MRD results by hsFLC.

Table 8. *Presentation and D100-PASCT results for 54 kappa patients. The serology, treatment response status and MRD results (hsFLC vs MFC) are given for each patient.*

Sample No.	Patient No.	M/F	Sample type	m-protein type	[m-protein] (g/L)	IgG (g/L)	IgA (g/L)	IgM (g/L)	κ FLC (mg/L)	λ FLC (mg/L)	FLC Ratio	Response	MRD by MFC	MRD by hsFLC
1	1	M	Presentation	GK	51	65.4	0.1	0.1	255	1.2	209	-	-	-
2	1		D100-PASCT	–	0	6.6	0.6	0.2	7.6	8.7	0.87	SCR	P	N
3	2	F	Presentation	AK x2	27	3.2	27.8	0.1	1014	1.2	859	-	-	-
4	2		D100-PASCT	–	0	5.7	0.2	0.1	3.7	7.8	0.47	SCR	N	N
5	3	F	Presentation	FK	n/a	2.2	0.2	0.1	6352	1.0	6686	-	-	-
6	3		D100-PASCT	–	n/a	8.2	0.7	0.4	7.9	6.4	1.23	SCR	P	N
7	4	M	Presentation	AK	46	3.6	46.1	0.1	206	1.4	144	-	-	-
8	4		D100-PASCT	–	1	7.2	2.5	0.4	27.3	12.7	2.2	VGPR	n/a	P
9	5	M	Presentation	GK	28	33.9	0.1	0.1	958	0.7	1388	-	-	-
10	5		D100-PASCT	–	7	11.3	0.6	0.1	57.8	6.7	8.7	n/a	n/a	P
11	6	M	Presentation	AK	41	3.5	50.4	0.0	4421	8.3	536	-	-	-
12	6		D100-PASCT	–	2	5.5	2.2	0.1	37.2	6.9	5.39	VGPR	P	P
13	7	F	Presentation	GK	9	8.9	0.4	0.3	1548	6.9	226	-	-	-
14	7		D100-PASCT	–	0	3.3	0.1	0.0	6.6	6.6	1.0	SCR	n/a	N
15	8	M	Presentation	GK	24	24.9	0.5	0.3	190	6.1	31.1	-	-	-
16	8		D100-PASCT	–	0	3.4	0.5	0.3	5.7	6.9	0.83	SCR	N	P
17	9	F	Presentation	GK	33	40.4	0.1	0.1	216	0.8	266	-	-	-
18	9		D100-PASCT	–	12	13.1	0.0	0.3	29.3	5.7	5.14	PR	P	P
19	10	M	Presentation	GK	44	62.2	0.2	0.1	602	1.0	602	-	-	-
20	10		D100-PASCT	–	0	6.8	0.5	0.2	8.9	7.6	1	SCR	n/a	P
21	11	F	Presentation	GK+FK	28	32.3	0.2	0.2	1910	6.1	314	-	-	-
22	11		D100-PASCT	–	0	4.0	0.6	0.4	26.5	12.9	2.1	VGPR	n/a	N
23	12	M	Presentation	GK	50	57.7	0.4	0.2	991	0.7	1479	-	-	-
24	12		D100-PASCT	–	0	10.7	0.7	0.2	11.7	8.2	1.43	SCR	N	P
25	13	F	Presentation	FK	n/a	4.1	0.2	0.1	6923	1.3	5205	-	-	-
26	13		D100-PASCT	–	n/a	8.3	1.2	0.3	11.5	10.7	1.07	SCR	N	N

Sample No.	Patient No.	M/F	Sample type	m-protein type	[m-protein] (g/L)	IgG (g/L)	IgA (g/L)	IgM (g/L)	κ FLC (mg/L)	λ FLC (mg/L)	FLC Ratio	Response	MRD by MFC	MRD by hsFLC
27	14	M	Presentation	GK + FK	3	6.0	0.5	0.3	3891	1.3	2948	-	–	-
28	14		D100-PASCT	–	0	11.0	0.6	0.3	17.0	12.4	1.37	SCR	P	N
29	15	F	Presentation	GK	46	45.0	0.1	0.1	140	0.9	162	-	-	-
30	15		D100-PASCT	–	0	4.6	0.7	0.2	3.7	8.3	0.4	SCR	n/a	N
31	16	M	Presentation	FK	n/a	4.1	0.7	0.1	2501	0.8	3335	-	-	-
32	16		D100-PASCT	–	n/a	5.7	0.6	0.3	6.3	8.3	0.8	SCR	n/a	N
33	17	M	Presentation	FK	n/a	5.5	0.2	0.2	5039	0.9	5726	-	-	-
34	17		D100-PASCT	–	n/a	8.2	1.1	0.4	21.5	12.1	1.78	VGPR	N	N
35	18	M	Presentation	FK	n/a	6.6	1.0	0.2	590	8.5	69.8	-	-	-
36	18		D100-PASCT	–	n/a	6.9	0.4	0.2	4.2	8.7	0.48	SCR	N	P
37	19	F	Presentation	AK +FK	43	2.3	43.7	0.1	422	0.9	496	-	-	-
38	19		D100-PASCT	–	0	4.4	0.4	0.1	4.3	0.7	6.14	SCR	P	N
39	20	M	Presentation	FK	n/a	2.3	0.1	0.1	9820	0.7	13831	-	-	-
40	20		D100-PASCT	–	n/a	6.9	0.5	0.3	7.0	7.3	1.0	SCR	n/a	N
41	21	F	Presentation	AK	0.1	3.3	0.6	0.2	3427	1.5	2347	-	-	-
42	21		D100-PASCT	–	0	4.0	0.1	0.4	5.5	7.6	0.72	SCR	N	N
43	22	M	Presentation	GK	40	52.5	0.1	0.3	472	2.6	288	-	-	-
44	22		D100-PASCT	–	3	8.6	0.4	0.4	16.6	8.9	1.87	VGPR	N	N
45	23	F	Presentation	FK	n/a	4.1	0.2	0.1	520	1.4	371	-	-	-
46	23		D100-PASCT	–	n/a	4.4	0.3	0.2	9.8	7.1	1.5	SCR	n/a	P
47	24	F	Presentation	GK	41	54.8	0.1	0.0	938	0.7	1421	-	-	-
48	24		D100-PASCT	–	0	4.7	0.3	0.2	9.5	6.6	1.44	SCR	N	N
49	25	M	Presentation	DK	3	3.0	0.2	0.0	1114	5.8	193	-	-	-
50	25		D100-PASCT	–	0	5.2	0.8	0.4	9.3	9.1	1.02	SCR	N	N
51	26	M	Presentation	GK	30	34.2	0.8	0.4	476	1.4	350	-	-	-
52	26		D100-PASCT	–	0	3.7	0.4	0.3	8.0	6.4	1.25	SCR	N	N
53	27	M	Presentation	AK x2	41	3.5	41.5	0.1	368	1.3	281	-	-	-

Sample No.	Patient No.	M/F	Sample type	m-protein type	[m-protein] (g/L)	IgG (g/L)	IgA (g/L)	IgM (g/L)	κ FLC (mg/L)	λ FLC (mg/L)	FLC Ratio	Response	MRD by MFC	MRD by hsFLC
54	27		D100-PASCT	–	0	8.4	0.7	0.3	7.8	8.5	0.92	SCR	N	P
55	28	M	Presentation	GK+FK	47	64.1	0.5	0.2	424	6.9	61	-	-	-
56	28		D100-PASCT	–	0	4.8	0.9	0.4	6.1	1.3	4.69	SCR	N	N
57	29	F	Presentation	GK + FK	30	39.4	0.2	0.7	486	6.6	73.7	-	-	-
58	29		D100-PASCT	–	2	4.1	0.3	0.4	5.0	6.0	0.83	VGPR	P	N
59	30	M	Presentation	GK	18	21.4	0.7	0.5	120	13.0	9.3	-	-	-
60	30		D100-PASCT	–	0	12.6	0.4	0.4	35.4	21.0	1.69	SCR	P	P
61	31	M	Presentation	AK	45	3.8	45.6	0.2	367	8.2	45.0	-	-	-
62	31		D100-PASCT	–	2	6.9	2.6	0.2	8.0	9.6	0.83	VGPR	N	P
63	32	F	Presentation	FK	n/a	4.7	0.4	0.1	4264	1.1	3912	-	-	-
64	32		D100-PASCT	–	n/a	5.1	0.3	0.1	1.2	7.3	0	SCR	n/a	P
65	33	M	Presentation	GK	21	22.9	0.3	0.2	853	0.6	1422	-	-	-
66	33		D100-PASCT	–	2	5.5	0.4	0.2	14.0	6.5	2.1	VGPR	n/a	P
67	34	M	Presentation	GK	31	37.7	1.4	0.8	781	6.2	125	-	-	-
68	34		D100-PASCT	–	8	10.5	1.0	0.2	31.6	1.5	20.9	PR	n/a	P
69	35	F	Presentation	GK + FK	2	5.5	0.3	0.1	826	6.2	134	-	-	-
70	35		D100-PASCT	–	0	2.3	2.8	0.1	17.7	9.9	1.79	SCR	N	P
71	36	M	Presentation	AK	26	4.6	26.6	0.3	137	6.5	21.3	-	-	-
72	36		D100-PASCT	–	0	3.7	0.5	0.1	12.3	7.2	1.71	SCR	N	P
73	37	M	Presentation	FK	n/a	6.9	0.8	0.3	2753	1.0	2838	-	-	-
74	37		D100-PASCT	–	n/a	4.9	0.6	0.1	8.5	1.3	6.54	SCR	N	P
75	38	M	Presentation	GK + AK	48	64.6	1.3	0.6	100	6.1	16.5	-	-	-
76	38		D100-PASCT	–	0	4.4	0.3	0.7	1.4	9.5	0.15	SCR	P	N
77	39	M	Presentation	AK x2	59	1.4	59.4	0.0	2187	0.7	3038	-	-	-
78	39		D100-PASCT	–	1	1.8	1.4	0.1	12.0	0.9	13.33	VGPR	P	P
79	40	F	Presentation	GK	35	37.1	0.3	0.2	140	1.2	115	-	-	-
80	40		D100-PASCT	–	0	11.7	0.4	0.2	5.1	11.4	0.45	SCR	N	N

Sample No.	Patient No.	M/F	Sample type	m-protein type	[m-protein] (g/L)	IgG (g/L)	IgA (g/L)	IgM (g/L)	κ FLC (mg/L)	λ FLC (mg/L)	FLC Ratio	Response	MRD by MFC	MRD by hsFLC
81	41	M	Presentation	AK	42	2.1	42.3	0.1	1635	0.5	3144	-	-	-
82	41		D100-PASCT	–	0	0.9	0.3	0.3	4.7	1.5	3.13	VGPR	N	N
83	42	M	Presentation	GK	25	31.1	0.2	0.1	175	8.7	20.1	-	-	-
84	42		D100-PASCT	–	0	6.6	0.1	0.2	16.1	9.6	1.7	n/a	N	P
85	43	M	Presentation	GK+FK	36	47.8	0.2	0.1	4336	6.1	710	-	-	-
86	43		D100-PASCT	–	0	5.4	0.6	0.2	13.2	9.4	1.40	SCR	N	P
87	44	M	Presentation	GK	33	40.3	1.0	0.2	100	9.7	10.3	-	-	-
88	44		D100-PASCT	–	0	6.7	0.5	0.5	19.6	12.4	1.6	SCR	n/a	P
89	45	M	Presentation	AK	34	6.1	34.7	0.8	435	9.3	47.0	-	-	-
90	45		D100-PASCT	–	0	10.9	1.4	0.5	25.6	11.9	2.2	CR	n/a	N
91	46	F	Presentation	GK	43	45.8	0.1	0.1	405	1.2	346	-	-	-
92	46		D100-PASCT	–	0	7.6	1.1	0.3	16.5	8.6	1.92	SCR	N	P
93	47	M	Presentation	GK	11	14.7	1.1	1.0	143	16.1	8.9	-	-	-
94	47		D100-PASCT	–	0	8.5	0.5	0.8	20.1	12.2	1.7	SCR	n/a	P
95	48	F	Presentation	AK	16	5.8	16.4	0.1	2209	6.2	357	-	-	-
96	48		D100-PASCT	–	0	5.3	0.0	0.1	6.4	6.0	1.07	SCR	N	P
97	49	F	Presentation	GK	25	36.5	0.5	0.3	292	1.1	256	-	-	-
98	49		D100-PASCT	–	0	4.9	0.4	0.1	7.4	1.4	5.29	VGPR	P	N
99	50	F	Presentation	GK	33	32.7	0.8	0.4	2012	1.4	1407	-	-	-
100	50		D100-PASCT	–	0	7.3	1.2	0.4	11.2	8.0	1.4	SCR	n/a	P
101	51	F	Presentation	GK	25	33.9	0.7	0.4	165	1.1	153	-	-	-
102	51		D100-PASCT	–	0	9.6	0.9	0.6	14.7	7.2	2.04	VGPR	N	P
103	52	F	Presentation	AK	38	3.4	38.5	0.1	651	1.0	671	-	-	-
104	52		D100-PASCT	–	6	6.9	6.3	0.2	99.1	6.0	16.7	PR	n/a	P
105	53	M	Presentation	GK	35	49.9	1.0	0.3	90.4	7.9	11.5	-	-	-
106	53		D100-PASCT	–	0	9.7	1.3	0.1	15.6	8.6	1.8	n/a	N	P
107	54	M	Presentation	GK	45	63.4	0.6	0.1	1817	1.5	1211	-	-	-

Sample No.	Patient No.	M/F	Sample type	m-protein type	[m-protein] (g/L)	IgG (g/L)	IgA (g/L)	IgM (g/L)	κ FLC (mg/L)	λ FLC (mg/L)	FLC Ratio	Response	MRD by MFC	MRD by hsFLC
108	54		D100-PASCT	–	2	8.4	0.9	0.4	14.5	9.6	1.51	VGPR	N	N

D100-PASCT: *day-100 post-autologous stem cell transplant,* ***GK:*** *IgG Kappa,* ***AK x2:*** *two clones of IgA Kappa,* ***FK****: free Kappa light chains detected,* ***DK:*** *IgD Kappa-type paraprotein.* ***CR:*** *complete response,* ***SCR:*** *stringent complete response,* ***VGPR:*** *very good partial response,* ***PR:*** *partial response,* ***n/a:*** *results not available,* ***MP:*** *M-protein. Normal range for κ FLC: 3.30-19.40 mg/L, λ FLC: 5.71-26.30 mg/L, FLC ratio: 0.26–1.65.*

The λ results are presented next in the same format. Then, statistical analysis of all the results are given at the end of the section. However, before all of that, a special analysis (not part of the original methods protocol) was conducted for those patients who demonstrated unusual OCB spectrotypes by hsFLC in the D100-PASCT samples. A uniform laddering pattern was observed in these patients which was potentially linked to monoclonal FLC production. Therefore, data from the follow up samples for these patients ranging from around 6 months to 1-year post transplant was collected and is also shown below in table 9 for each patient.

***Table 9**. Follow up sample results for the 12 patients with distinct laddering of OCBs in the D100-PASCT samples.*

No.	Sample No.	Patient No.	m-protein at diagnosis	[m-protein] (g/L)	κ FLC (mg/L)	λ FLC (mg/L)	FLC Ratio	Response	6m-1yr follow up relapse check			
									[m-protein] (g/L)	κ FLC (mg/L)	FLC Ratio	Comments
1	6	3	FK	n/a	7.9	6.4	1.2	SCR	n/a	13.9	1.8	No more data
2	10	5	GK	7	57.8	6.7	8.7	n/a	11.2	224.0	25.0	Relapse
3	12	6	AK	2	37.2	6.9	5.4	VGPR	n/a	262.1	19.5	Relapse
4	28	14	GK + FK	0	17.0	12.4	1.4	SCR	<0.1	2772.0	381.0	Relapse
5	36	18	FK	n/a	4.2	8.7	0.5	SCR	n/a	71.4	7.1	Relapse
6	38	19	AK + FK	0	4.3	0.7	6.6	SCR	2.27	37.5	5.0	Relapse/ IFE positive
7	42	21*	AK	0	5.5	7.6	0.7	SCR	<0.1	17.0	1.2	No more data
8	60	30	GK	0	35.4	21.0	1.7	SCR	8.6	52.1	8.0	Relapse / IFE Positive
9	64	32	FK	n/a	1.2	7.3	0	SCR	n/a	268.4	32.5	Relapse
10	82	41	AK	0	4.7	1.5	3.1	VGPR	n/a	11.0	1.2	No more data
11	88	44*	GK	0	19.6	12.4	1.6	SCR	<0.1	35.0	2.9	Relapse/ IFE Positive
12	92	46	GK	0	16.5	8.6	1.9	SCR	<0.1	30.0	1.5	Stable

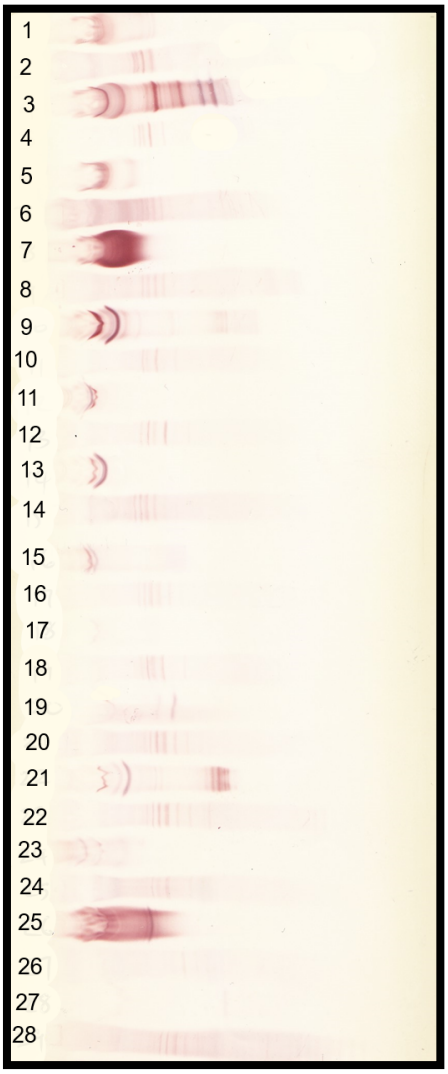
**Patient 21*- *Laddering in D100-PASCT sample different to presentation sample.*
**Pateint 44*- *Laddering in D100-PASCT sample same as presentation sample.*
Normal range for κ FLC: 3.30-19.40 mg/L, λ FLC: 5.71-26.30 mg/L, FLC ratio: 0.26–1.65.

Out of the twelve patients that demonstrated the uniform OCB spectrotype in the D100-PASCT samples, at least eight demonstrated a relapse of disease in the follow up samples as evidenced by the abnormal κ FLCs and κ/λ ratios. Patients 19, 30 and 44 even demonstrated reappearance of m-protein by conventional IFE. Three patients had no further data available to check for relapse.

3.1.2 Lambda patients

Figure 9 below shows scanned images of the IBs produced by hsfLC for 56 MM patients with involved λ FLCs. For each patient, there are two sets of results (protein traces) adjacent to one another; the presentation sample trace followed by the D100-PASCT sample trace. Therefore, there are a total of 112 sample traces shown. The key to the right of each blot shows the raw hsfLC results data for each patient. The blots were incubated using mouse anti-λ FLC monoclonal antibodies.

Samples 1 to 28



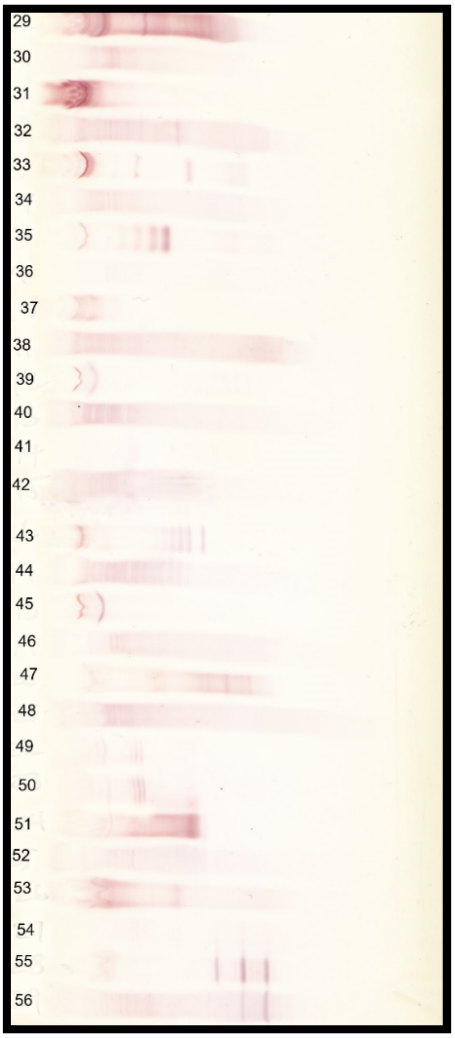
Sample key and raw hsFLC results data

Sample No.	Patient ID	Sample type	Monoclonal FLCs?	New OCBs?
1	1	PRES	P	-
2		DHAST	P	P
3	2	PRES	P	-
4		DHAST	P	P
5	3	PRES	P	-
6		DHAST	P	P
7	4	PRES	P	-
8		DHAST	N	P
9	5	PRES	P	-
10		DHAST	N	P
11	6	PRES	P	-
12		DHAST	N	P
13	7	PRES	P	-
14		DHAST	P	P
15	8	PRES	P	-
16		DHAST	N	P
17	9	PRES	P	-
18		DHAST	N	P
19	10	PRES	P	-
20		DHAST	P	P
21	11	PRES	P	-
22		DHAST	P	P
23	12	PRES	P	-
24		DHAST	P	P
25	13	PRES	P	-
26		DHAST	N	P
27	14	PRES	P	-
28		DHAST	N	P

PRES: *presentation sample,*
DHAST: *day-100 post-autologous stem cell transplant sample,*
P: *positive,* ***N:*** *negative.*

Figure 9*. Part 1/4*

Samples 29 to 56



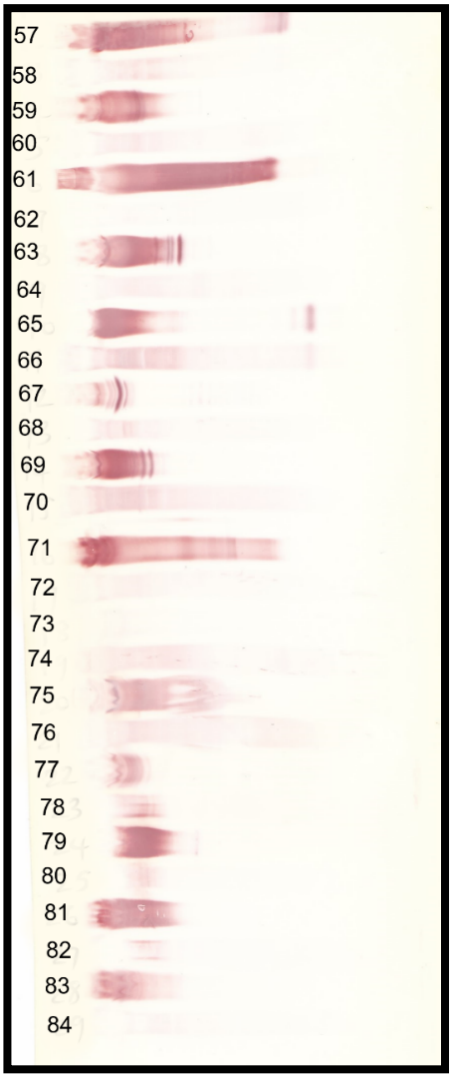
Sample key and raw hsFLC results data.

Sample No.	Patient ID	Sample type	Monoclonal FLCs?	New OCBs?	Note
29	15	PRES	P	-	
30		DHAST	P	N	
31	16	PRES	P	-	
32		DHAST	P	P	
33	17	PRES	P	-	
34		DHAST	N	N	
35	18	PRES	P	-	L
36		DHAST	N	N	
37	19	PRES	P	-	
38		DHAST	P	N	
39	20	PRES	P	-	
40		DHAST	N	N	
41	21	PRES	P	-	
42		DHAST	P	N	
43	22	PRES	P	-	L
44		DHAST	P	P	L (same)
45	23	PRES	P	-	
46		DHAST	N	P	
47	24	PRES	P	-	
48		DHAST	N	N	
49	25	PRES	P	-	L
50		DHAST	P	N	L (same)
51	26	PRES	P	-	
52		DHAST	N	P	
53	27	PRES	P	-	
54		DHAST	N	N	
55	28	PRES	P	-	
56		DHAST	P	N	

***L**: ladder pattern*

***Figure 9**. Part 2/4*

Samples 57 to 84

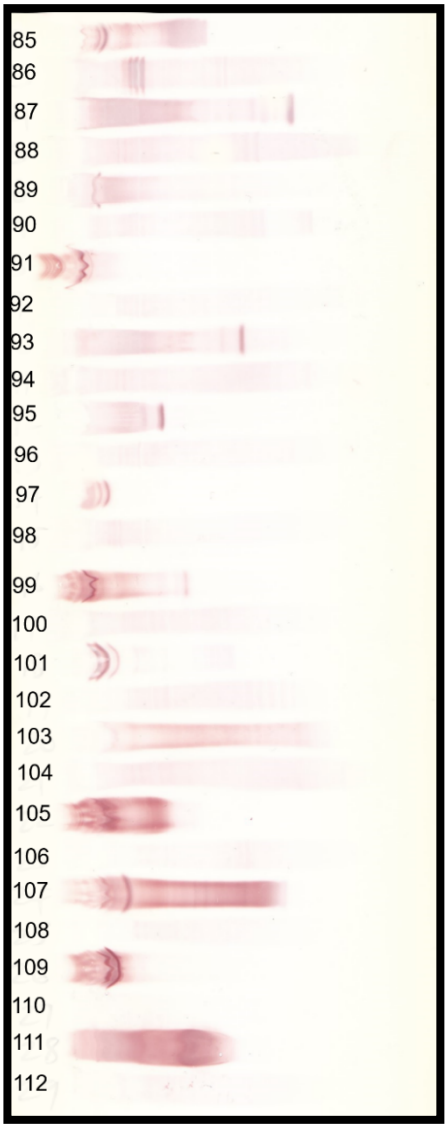


Sample key and raw hsFLC results data.

Sample No.	Patient ID	Sample type	Monoclonal FLCs?	New OCBs?	Note
57	29	PRES	P	-	
58		DHAST	N	N	
59	30	PRES	P	-	
60		DHAST	P	N	
61	31	PRES	P	-	
62		DHAST	N	N	
63	32	PRES	P	-	
64		DHAST	N	N	
65	33	PRES	P	-	
66		DHAST	P	N	
67	34	PRES	P	-	
68		DHAST	P	N	
69	35	PRES	P	-	L
70		DHAST	P	N	
71	36	PRES	P	-	
72		DHAST	N	N	
73	37	PRES	P	-	
74		DHAST	N	N	
75	38	PRES	P	-	
76		DHAST	P	N	
77	39	PRES	P	-	
78		DHAST	P	N	
79	40	PRES	P	-	
80		DHAST	N	N	
81	41	PRES	P	-	
82		DHAST	N	N	
83	42	PRES	P	-	
84		DHAST	P	N	

***Figure 9**. Part 3/4*

Samples 85 to 112



Sample key and raw hsFLC results data

Sample No.	Patient ID	Sample type	Monoclonal FLCs?	New OCBs?	Note
85	43	PRES	P	-	
86		DHAST	P	P	L
87	44	PRES	P	-	
88		DHAST	P	P	
89	45	PRES	P	-	
90		DHAST	P	P	
91	46	PRES	P	-	
92		DHAST	N	P	
93	47	PRES	P	-	
94		DHAST	P	P	
95	48	PRES	P	-	
96		DHAST	N	P	
97	49	PRES	P	-	
98		DHAST	N	P	
99	50	PRES	P	-	
100		DHAST	P	N	
101	51	PRES	P	-	
102		DHAST	N	P	
103	52	PRES	P	-	
104		DHAST	N	P	
105	53	PRES	P	-	
106		DHAST	N	N	
107	54	PRES	P	-	
108		DHAST	N	N	
109	55	PRES	P	-	
110		DHAST	N	N	
111	56	PRES	P	-	
112		DHAST	N	N	

Figure 9. *Part 4/4. Scanned images of the IBs produced by hsfLC for 56 patient samples with λ related m-proteins. The key to the right of each image shows the raw hsFLC results data associated with each trace.*

The actual IBs were used for recording results; the band clarity is not as obvious on the scanned images. In many cases, the major band from the presentation sample was only partially detectable in the D100-PASCT sample, yet still deemed positive for residual disease. Some of the samples (e.g., samples 7, 61 and 111) had a high protein content; a high background of polyclonal FLCs impaired the clarity of monoclonal band detection. Such samples could be diluted 1/5 in 2% BSA/PBS before confirming results in a clinical setting. See below for a summary of results.

Summary of the raw hsFLC results data for λ pateints

1. Presentation samples:
    c. Monoclonal λ FLCs were detected in all 56 of the presentation samples.
    d. 2 pateints deomonstrated an unusual uniform laddering spectrotype exclusively in the presentation sample.

2. D100-PASCT samples:
    a. In 26 pateints, the same monoclonal λ FLCs found in the presentation sample were also detectable in the respective D100-PASCT sample.
    b. New λ FLC OCBs were observed in 27 pateints (48%); 13 of these pateints also simultaneously had monoclonal FLCs detected (23%)
    c. 1 patients showed an unusual uniform laddering spectrotype exclusively in the D100-PASCT sample.

3. Pateint 22 (samples 43 & 44):
    a. the spectrotypes of both the presentation and the D100-PASCT demonstrated the same unusual uniform laddering pattern.

4. Pateint 25 (sample 49 & 50):
    a. the spectrotypes of both the presentation and the D100-PASCT demonstrated the same unusual uniform laddering pattern.

The raw hsFLC results data from figure 9 above are presented below in the context of MRD (table 10). For each patient, there are two sets of results adjacent to one another; the presentation sample results followed by the D100-PASCT sample results. Serology results for both the presentation and the D100-PASCT are given where available. These include serum IMG, sFLC, m-protein typing and quantification results. For each D100 PASCT sample, the post-treatment response status defined by the IMWG response criteria is given, firstly. Then the MRD results previously reported by MFC are shown and finally, the new MRD results by hsFLC.

***Table 10**. Presentation and D100-PASCT results for 56 Lambda patients. The serology, treatment response status and MRD results (hsFLC vs MFC) are given for each patient.*

Sample No.	Patient No.	M/F	Sample type	MP type	[MP] (g/L)	IgG (g/L)	IgA (g/L)	IgM (g/L)	κ FLC (mg/L)	λ FLC (mg/L)	FLC Ratio	Response	MRD by MFC	MRD by hsFLC
1	1	M	Presentation	GL+FL	7	8.4	0.1	0.1	6.6	8515	0.00	–	–	–
2	1		D100-PASCT	GL+FL	0	3.8	0.1	0.1	2.1	8.2	0.26	SCR	N	P
3	2	F	Presentation	GL	25	27.1	0.0	0.1	0.8	1221	0.00	–	–	–
4	2		D100-PASCT	GL	1	2.0	0.0	0.1	0.6	0.8	0.80	VGPR	N	P
5	3	M	Presentation	GL	18	5.5	18.6	0.1	1.6	1452	0.00	–	–	–
6	3		D100-PASCT	GL	0	8.8	1.3	0.5	13.2	40.8	0.32	n/a	P	P
7	4	M	Presentation	AL	50	2.2	50.9	0.1	0.4	992	0.00	–	–	–
8	4		D100-PASCT	AL	0	10.2	0.9	0.5	8.1	9.3	0.87	SCR	N	N
9	5	M	Presentation	GL	30	36.8	0.7	0.4	11.6	762	0.02	–	–	–
10	5		D100-PASCT	GL	0	6.9	0.6	0.2	13.0	8.8	1.48	SCR	N	N
11	6	M	Presentation	FL	n/a	8.0	0.5	0.4	5.6	3207	0.00	–	–	–
12	6		D100-PASCT	FL	n/a	6.8	0.4	0.2	5.2	10.2	0.51	CR	n/a	N
13	7	M	Presentation	FL	n/a	4.7	0.4	0.3	4.1	1159	0.00	–	–	–
14	7		D100-PASCT	FL	n/a	10.4	1.1	0.2	9.8	12.8	0.76	SCR	N	P
15	8	M	Presentation	GL	35	48.8	0.5	0.4	4.7	232	0.02	–	–	–
16	8		D100-PASCT	GL	0	4.3	0.3	0.1	4.3	8.3	0.52	SCR	N	N
17	9	M	Presentation	GL	26	26.2	0.2	0.2	5.1	749	0.01	–	–	–
18	9		D100-PASCT	GL	4	8.1	0.2	0.1	0.5	41.9	0.01	PR	P	N
19	10	M	Presentation	GL	4	5.9	0.2	0.2	0.6	788	0.00	–	–	–
20	10		D100-PASCT	GL	0	5.2	0.5	0.7	3.7	10	0.37	SCR	N	P
21	11	M	Presentation	GL	36	36.9	0.4	0.2	5.1	126	0.04	–	–	–
22	11		D100-PASCT	GL	2	8.7	0.9	0.3	7.8	9	0.86	VGPR	P	P
23	12	M	Presentation	FL	n/a	4.4	0.3	0.1	6.5	10631	0.00	–	–	–
24	12		D100-PASCT	FL	n/a	10.4	0.5	0.2	20.3	12.4	1.64	SCR	N	P
25	13	F	Presentation	AL	15	3.5	15.1	0.2	3.8	967	0.00	–	–	–

Sample No.	Patient No.	M/F	Sample type	MP type	[MP] (g/L)	IgG (g/L)	IgA (g/L)	IgM (g/L)	κ FLC (mg/L)	λ FLC (mg/L)	FLC Ratio	Response	MRD by MFC	MRD by hsFLC
26	13		D100-PASCT	AL	0	5.5	0.5	0.3	9.1	11.9	0.76	SCR	P	N
27	14	M	Presentation	GL	23	22.8	0.2	0.1	4.3	550	0.01	–	–	–
28	14		D100-PASCT	GL	0	11.4	0.6	0.2	11.7	20	0.59	SCR	N	N
29	15	F	Presentation	GL	46	54.9	0.2	0.2	7.2	105	0.07	–	–	–
30	15		D100-PASCT	GL	0	2.8	0.3	0.1	5.3	7.8	0.68	SCR	N	P
31	16	M	Presentation	FL	n/a	6.6	0.8	0.5	13.4	3314	0.00	–	–	–
32	16		D100-PASCT	FL	n/a	5.4	0.9	0.5	13.8	21.8	0.63	VGPR	n/a	P
33	17	M	Presentation	GL+FL	46	69.5	0.2	0.1	6.4	1379	0.01	–	–	–
34	17		D100-PASCT	GL+FL	3	9.5	0.4	0.1	13.4	10	1.34	VGPR	n/a	N
35	18	F	Presentation	GL+FL	23	22.8	0.3	0.1	1.0	465	0.00	–	–	–
36	18		D100-PASCT	GL+FL	0	1.6	0.1	0.1	4.2	0.9	4.67	VGPR	n/a	N
37	19	M	Presentation	AL+FL	6	4.0	6.8	0.1	3.6	1675	0.00	–	–	–
38	19		D100-PASCT	AL+FL	0	8.6	0.2	0.1	9.9	8.9	1.11	SCR	N	P
39	20	F	Presentation	GL+FL	40	48.9	1.7	1.2	8.6	1571	0.01	–	–	–
40	20		D100-PASCT	GL+FL	0	6.0	1.1	0.3	7.8	8.1	0.96	SCR	N	N
41	21	F	Presentation	GL+FL	72	97.2	0.1	0.1	7.6	1935	0.00	–	–	–
42	21		D100-PASCT	GL+FL	23	29.9	0.2	0.2	7.4	892	0.01	PR	n/a	P
43	22	F	Presentation	GL	28	38.4	0.7	0.3	5.7	173	0.03	–	–	–
44	22		D100-PASCT	GL	2	3.5	0.8	0.3	5.0	6.2	0.81	VGPR	N	P
45	23	F	Presentation	FL	n/a	8.9	1.2	0.9	12.6	283	0.05	–	–	–
46	23		D100-PASCT	FL	n/a	5.9	0.4	0.1	21.6	7	3.09	VGPR	n/a	N
47	24	F	Presentation	GL	90	105.4	0.3	0.5	11.5	1162	0.01	–	–	–
48	24		D100-PASCT	GL	0	10.6	1.3	0.5	12.7	13.2	0.96	SCR	N	N
49	25	F	Presentation	GL	25	30.7	0.6	0.8	5.2	319	0.02	–	–	–
50	25		D100-PASCT	GL	0	6.4	1.4	0.3	12.6	8	1.58	SCR	N	P
51	26	F	Presentation	GL	60	84.6	0.1	0.1	0.5	688	0.00	–	–	–

Sample No.	Patient No.	M/F	Sample type	MP type	[MP] (g/L)	IgG (g/L)	IgA (g/L)	IgM (g/L)	κ FLC (mg/L)	λ FLC (mg/L)	FLC Ratio	Response	MRD by MFC	MRD by hsFLC
52	26		D100-PASCT	GL	11	14.1	0.3	0.5	7.6	24.2	0.31	PR	n/a	N
53	27	F	Presentation	GL+FL	58	74.4	0.1	0.1	4.3	5782	0.00	–	–	–
54	27		D100-PASCT	GL+FL	2	2.7	0.0	0.1	5.5	1.7	3.24	VGPR	N	N
55	28	M	Presentation	GL	64	80.2	0.1	0.3	7.9	2844	0.00	–	–	–
56	28		D100-PASCT	GL	5	9.3	0.8	0.1	8.1	18.8	0.43	VGPR	P	P
57	29	M	Presentation	DL	11	2.1	0.1	0.0	0.9	117	0.01	–	–	–
58	29		D100-PASCT	DL	0	3.3	0.5	0.1	3.8	7	0.54	SCR	P	N
59	30	M	Presentation	AL	25	5.2	25.3	0.1	5.2	5042	0.00	–	–	–
60	30		D100-PASCT	AL	0	5.7	1.1	0.5	18.1	17.5	1.03	SCR	n/a	P
61	31	M	Presentation	AL x2	68	1.6	68.8	0.1	1.2	451	0.00	–	–	–
62	31		D100-PASCT	AL x2	0	3.8	0.7	0.3	6.5	1.6	4.06	VGPR	N	N
63	32	M	Presentation	AL+MK	27	3.9	27.1	0.1	3.8	909	0.00	–	–	–
64	32		D100-PASCT	AL+MK	0	3.5	0.5	0.3	7.3	7.9	0.92	SCR	N	N
65	33	M	Presentation	AL x2	44	2.6	44.2	0.0	1.1	420	0.00	–	–	–
66	33		D100-PASCT	AL x2	1	6.1	1.3	0.2	13.3	9.6	1.39	VGPR	N	P
67	34	F	Presentation	GL+FL	32	48.2	0.1	0.0	5.9	9224	0.00	–	–	–
68	34		D100-PASCT	GL+FL	0	3.8	0.4	0.3	8.4	8.8	0.95	SCR	n/a	P
69	35	M	Presentation	DL	7	5.0	0.4	0.1	7.6	835	0.01	–	–	–
70	35		D100-PASCT	DL	0	6.2	0.7	0.2	5.8	12	0.48	SCR	P	P
71	36	F	Presentation	GL	28	31.2	0.2	0.0	1.0	792	0.00	–	–	–
72	36		D100-PASCT	GL	0	5.9	0.2	0.4	1.3	7.2	0.18	VGPR	N	N
73	37	F	Presentation	FL	n/a	5.9	0.3	0.4	8.6	279	0.03	–	–	–
74	37		D100-PASCT	FL	n/a	n/a	n/a	n/a	8.6	6.9	1.25	SCR	P	N
75	38	M	Presentation	GL+FL	67	96.4	0.0	0.0	1.2	107	0.01	–	–	–
76	38		D100-PASCT	GL+FL	4	7.4	0.5	0.5	2.0	6.5	0.31	VGPR	P	P
77	39	M	Presentation	FL	n/a	3.6	0.1	0.0	1.2	7020	0.00	–	–	–

Sample No.	Patient No.	M/F	Sample type	MP type	[MP] (g/L)	IgG (g/L)	IgA (g/L)	IgM (g/L)	κ FLC (mg/L)	λ FLC (mg/L)	FLC Ratio	Response	MRD by MFC	MRD by hsFLC
78	39		D100-PASCT	FL	n/a	2.3	0.1	0.1	9.0	7.8	1.15	SCR	N	P
79	40	F	Presentation	AL	65	2.8	65.1	0.2	4.9	603	0.01	–	–	–
80	40		D100-PASCT	AL	0	2.5	0.3	0.1	9.9	1.5	6.60	VGPR	P	N
81	41	M	Presentation	AL	34	5.2	34.1	0.1	5.4	887	0.01	–	–	–
82	41		D100-PASCT	AL	0	8.7	0.5	0.1	6.5	6.9	0.94	SCR	N	N
83	42	M	Presentation	FL	n/a	8.2	3.1	0.5	14.7	1142	0.01	–	–	–
84	42		D100-PASCT	FL	n/a	5.4	1.0	0.3	9.6	8	1.20	SCR	N	P
85	43	F	Presentation	GL	25	30.7	0.6	0.8	5.2	319	0.02	–	–	–
86	43		D100-PASCT	GL	0	6.4	1.4	0.3	12.6	8.1	1.56	SCR	N	P
87	44	M	Presentation	AL	18	4.2	18.0	0.1	1.7	37.4	0.05	–	–	–
88	44		D100-PASCT	AL	0	12.7	2.0	0.5	23.9	17.2	1.39	SCR	n/a	P
89	45	M	Presentation	AL+GL	36	5.1	36.2	1.9	6.2	40.9	0.15	–	–	–
90	45		D100-PASCT	AL+GL	0	4.0	0.5	0.7	7.6	7.2	1.06	VGPR	N	P
91	46	F	Presentation	FL	n/a	7.0	1.9	0.7	6.6	1515	0.00	–	–	–
92	46		D100-PASCT	FL	n/a	7.5	0.6	0.2	9.4	7.2	1.31	SCR	N	N
93	47	F	Presentation	AL	7	3.8	7.6	0.2	7.6	29.7	0.25	–	–	–
94	47		D100-PASCT	AL	0	14.7	1.4	1.5	55.1	35.2	1.57	SCR	N	P
95	48	M	Presentation	AL	58	2.7	58.9	0.2	1.9	174	0.01	–	–	–
96	48		D100-PASCT	AL	0	5.4	0.1	0.3	4.7	5.8	0.81	SCR	N	N
97	49	M	Presentation	AL	21	4.1	21.2	0.4	1.0	1237	0.00	–	–	–
98	49		D100-PASCT	AL	0	5.7	0.5	0.3	3.6	7.7	0.47	SCR	N	N
99	50	F	Presentation	FL	n/a	5.1	0.4	0.3	7.3	717	0.01	–	–	–
100	50		D100-PASCT	FL	n/a	5.1	0.5	0.2	17.4	10.1	1.72	SCR	n/a	P
101	51	M	Presentation	GL+FL	45	64.8	0.0	0.1	1.0	690	0.00	–	–	–
102	51		D100-PASCT	GL+FL	3	5.8	0.1	0.2	4.7	8.3	0.57	VGPR	P	N
103	52	M	Presentation	GL	28	37.3	4.0	0.4	16.2	348	0.05	–	–	–

Sample No.	Patient No.	M/F	Sample type	MP type	[MP] (g/L)	IgG (g/L)	IgA (g/L)	IgM (g/L)	κ FLC (mg/L)	λ FLC (mg/L)	FLC Ratio	Response	MRD by MFC	MRD by hsFLC
104	52		D100-PASCT	GL	0	12.6	2.2	0.3	26.3	16.3	1.61	SCR	N	N
105	53	M	Presentation	GL+FL	26	34.3	0.3	0.1	7.9	3800	0.00	–	–	–
106	53		D100-PASCT	GL+FL	0	7.7	0.7	0.3	25.6	7.8	3.28	VGPR	n/a	N
107	54	F	Presentation	GL+FL	57	81.7	0.4	0.1	12.0	2020	0.01	–	–	–
108	54		D100-PASCT	GL+FL	0	3.0	0.2	0.1	5.9	1.7	3.47	VGPR	N	N
109	55	F	Presentation	FL	n/a	5.9	0.4	1.0	8.1	1542	0.01	–	–	–
110	55		D100-PASCT	FL	n/a	2.9	0.3	0.2	12.4	7.9	1.57	SCR	N	N
111	56	M	Presentation	GL	86	96.0	0.2	0.1	5.2	53.2	0.10	–	–	–
112	56		D100-PASCT	GL	0	9.0	0.9	0.3	10.2	8.1	1.26	SCR	n/a	N

D100-PASCT: *day-100 post-autologous stem cell transplant,* ***GL:*** *IgG lambda,* ***AL x2:*** *two clones of IgA Lambda,* ***FL:*** *free Lambda light chains,* ***DL****: IgD Lambda,* ***MK:*** *IgM Kappa m-protein,* ***CR:*** *complete response,* ***SCR:*** *stringent complete response,* ***VGPR:*** *very good partial response,* **PR:** *partial response,* ***n/a:*** *results not available,* ***MP:*** *M-protein. Normal range for κ FLC: 3.30-19.40 mg/L, λ FLC: 5.71-26.30 mg/L, FLC ratio: 0.26–1.65.*

Statistical analysis of all the results are given next. However, before that, a special analysis (not part of the original methods protocol) was conducted for those patients who demonstrated unusual OCB spectrotypes by hsFLC in the D100-PASCT samples. A uniform laddering pattern was observed in these patients which was potentially linked to monoclonal FLC production. Therefore, data from the follow up samples for these patients ranging from around 6 months to 1-year post transplant was collected and is also shown below in table 11 for each patient.

Table 11. *Follow up sample results for the 3 λ patients with distinct laddering of OCBs in the D100-PASCT samples.*

No.	Sample No.	Patient No.	m-protein at diagnosis	[m-protein] (g/L)	κ FLC (mg/L)	λ FLC (mg/L)	FLC Ratio	Response	6m-1yr follow up relapse check			
									[m-protein] (g/L)	κ FLC (mg/L)	FLC Ratio	Comments
1	44	22*	GL	2	5.0	6.2	0.8	VGPR	n/a	n/a	n/a	n/a
2	50	25*	GL	0	12.6	8.0	1.6	SCR	n/a	n/a	n/a	n/a
3	86	43	GL	0	12.6	8.1	1.6	SCR	n/a	n/a	n/a	n/a

*****Patient 22 and patient 25****- Laddering in D100-PASCT sample same as presentation sample.*

No post-transplant follow-up data was available for the three λ patients to check for relapse. However, samples from the Myeloma XI trial continue to be collected and archived by the CIS. These three patients may have stored samples beyond 1-year post-transplant that could be tested for relapse.

3.1.3 *Statistical analysis of κ results*

The presence of any κ FLC-specific trends in the hsFLC results was investigated, to begin with. An analysis of the MRD results in the κ FLC group and the four post-treatment response categories is presented in relation to the m-protein type at diagnosis (four categories; IgG, IgA, IgD and free κ FLCs).

Table 12. *Response categories and MRD results specific to each type of m-protein tested in the κ patient group.*

M-protein type	No. of patients	Response category					MRD by MFC			MRD by hsFLC	
		SCR	CR	VGPR	PR	n/a	P	N	n/a	P	N
IgG	31	19	0	7	2	3	7	14	10	17	14
IgA	13	6	1	5	1	0	3	7	3	8	5
IgD	1	1	0	0	0	0	0	1	0	0	1
FK	9	8	0	1	0	0	1	4	4	4	5
Total	54	34	1	13	3	3	11	26	17	29	25

n/a: *missing data*

Figure 10 below summarises the key findings from table 12.

M-protein type	Key results
IgG	• 61% of patients had an SCR post-treatment. • 33% were MRD positive by MFC (excluding patients with n/a for MFC). • 55% were MRD positive by hsFLC.
IgA	• 46% of patients had an SCR post-treatment. • 30% were MRD positive by MFC (excluding patients with n/a for MFC). • 62% were MRD positive by hsFLC.
IgD	• Single patient had an SCR post-treatment. • MRD Negative by both methods.
FK	• 89% of patients had an SCR post-treatment. • 20% were MRD positive by MFC (excluding patients with n/a for MFC). • 44% were MRD positive by hsFLC.
Overall: • 63% of all patients achieved an SCR, 2% had a CR, 24% had a VGPR and 6% had a PR post-transplant (confirmed by conventional methods i.e., CZE and IFE). • Out of all patients tested by MFC, 30% were MRD positive. • Out of all patients tested by HsFLC, 54% were MRD positive.	

Figure 10. *Summary of results in table 12.*

A common finding across all the m-protein types tested was that a SCR was achieved most frequently by patients, followed by a VGPR. Almost 90% of FK-only secretory patients achieved a SCR, though only a small number of them were tested overall.

Both sets of possible MRD results (positive and negative) were observed (using hsFLC) in all of the various m-protein categories. This was also observed when MFC was used. The presence or absence of heavy chains seemed to have no effect on results either. The only exception to these findings, was the single patient with an IgD m-protein who was MRD negative by both methods. A look back at the raw hsFLC results for this patient in figure 8 (patient 25, trace 49), shows that monoclonal FLCs were detected using hsFLC in the presentation sample. Otherwise, the frequency of MRD positive results was higher using hsfLC compared to MFC in each m-protein category and generally overall.

More than 60% of the kappa group overall, achieved a SCR following treatment; it was expected that most of these patients would also be MRD negative (at least by hsFLC) considering that they should all have a normal κ/λFLC ratio. On the other hand, it was assumed that all patients with non-SCR post treatment responses would be MRD positive, in association with an abnormal κ/λFLC ratio. This was assessed next in table 13 which shows the MRD results by both methods in relation to each of the four post-transplant response categories: SCR, CR, VGPR and PR.

Table 13. *Comparison of MRD results by MFC vs hsFLC related to each response category achieved by the κ patients.*

Response category	Total No. of patients	MRD by MFC			MRD by hsFLC		Adjusted hsFLC*	
		POS	NEG	n/a	POS	NEG	POS	NEG
SCR	34	6	18	10	17	17	11	13
CR	1	0	0	1	0	1	0	0
VGPR	13	4	6	3	6	7	4	6
PR	3	1	0	2	3	0	1	0
n/a	3	0	2	1	3	0	2	0
Total	54	11	26	17	29	25	18	19

** **Adjusted hsFLC**: recalculated by excluding 17 patients with missing MFC data.*

Figure 11 below summarises the key findings from table 13.

Response category	Key results
SCR	• 25% were MRD positive by MFC (excluding patients with n/a for MFC). • 50% were MRD positive by hsFLC (46% by corrected hsFLC).
CR	• Single patient was MRD negative by hsFLC (not tested by MFC)
VGPR	• 40% were MRD positive by MFC (excluding patients with n/a for MFC). • 46% were MRD positive by hsFLC (40% by corrected hsFLC).
PR	• Single patient tested by MFC was MRD positive • 100% were MRD positive by hsFLC and corrected hsFLC).
Overall: • Out of all patients tested by MFC, 30% were MRD positive. • Out of all patients tested by HsFLC, 54% were MRD positive (49% by corrected hsFLC)	

Figure 11*. Summary of results in table 13.*

It was found that a quarter of SCR patients tested by MFC were actually MRD positive and around half of those tested by hsFLC were still MRD positive. On the other hand, more than half of the VGPR patients were MRD negative by both methods. The most striking finding was that the single CR patient was MRD negative by hsfLC. As expected, the PR patients were all MRD positive by both methods.

A closer look at the MRD results in light of the actual κ/λFLC ratios post-ASCT, follows further on. Before that, a direct patient by patient comparison of MRD results was made between MFC and hsFLC, in relation to each response category in table 14 below and also in the concordance plot in figure 12 that follows.

***Table 14**. Concordance & discordance between the MRD results obtained by MFC vs hsFLC in relation to each response category for the κ patient group.*

		Comparison of MRD results between methods			
Response category	**Total Patients***	**Patients with MRD concordance**	**Patients with MRD discordance**	**% Concordance**	**% Discordance**
SCR	24	9	15	38	63
VGPR	10	6	4	60	40
PR	1	1	0	100	0
n/a	2	0	2	0	100
Total	37	16	21	-	-

**Total patients excluding 17 patients with missing MFC data.*
***n/a**: missing data.*

The results show a very significant discordance between the MRD results obtained by each method in respect of two main response categories; SCR and VGPR. Moreover, the greatest discordance in MRD results between methods is found in the SCR category (>60%). The overall patient by patient concordance and discordance of MRD results between methods is demonstrated in the concordance plot below.

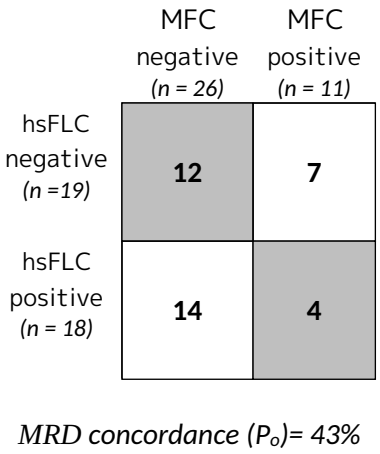


Figure 12. *A concordance plot showing the overall agreement between hsFLC and MFC for the classification of MRD in the κ patient group.*

The Grey shaded boxes in figure 12 indicate the number of patients in agreement between methods in terms of MRD positive and MRD negative for n = 37 (17 patients excluded with missing MFC data). The overall concordance between methods for MRD classification post-ASCT was 43% for patients in the κ patient group which leaves a very significant overall MRD discordance of 57% between methods.

In terms of inter-method reliability analysis by Cohen's kappa, the MRD concordance value can be described as the P_o (**Observed** agreement between methods) which is compared to the *Pe* value (**Expected** probability of chance agreement) as follows:

$$Cohen'sKappa = \frac{Po - Pe}{1 - Pe}$$

$$Pe = \left(\frac{19}{37} \times \frac{26}{37}\right) + \left(\frac{18}{37} \times \frac{11}{37}\right)$$

$$= (0.51 \times 0.70) + (0.49 \times 0.30)$$

$$= 0.36 + 0.15$$

$$Pe = 0.51$$

$$\kappa = \frac{0.43 - 0.51}{1 - 0.51}$$

$$= \frac{-0.08}{0.49}$$

$$Cohen's\ Kappa\ score = -0.16$$

***Table 15**. Interpretation of Cohen's kappa result*

Cohen' Kappa statistic (κ)	Strength of agreement
<0.00	Poor
0.00-0.20	Slight
0.21-0.40	Fair
0.41-0.60	Moderate
0.61-0.80	Substantial
0.81-0.99	Almost complete

Adapted from McHugh 2012 (65)

According to Cohen's kappa score interpretations shown in table 15 above, the strength of agreement between MRD results by MFC and hsFLC was **poor**.

Next, the expected relationship between the κ/λFLC ratio at D100-PASCT and the MRD result was assessed. i.e. It was expected that patients with a normal κ/λFLC ratio (0.26–1.65) will be MRD negative, while patients with an abnormal κ/λFLC ratio will be MRD positive (See Figure 13 below).

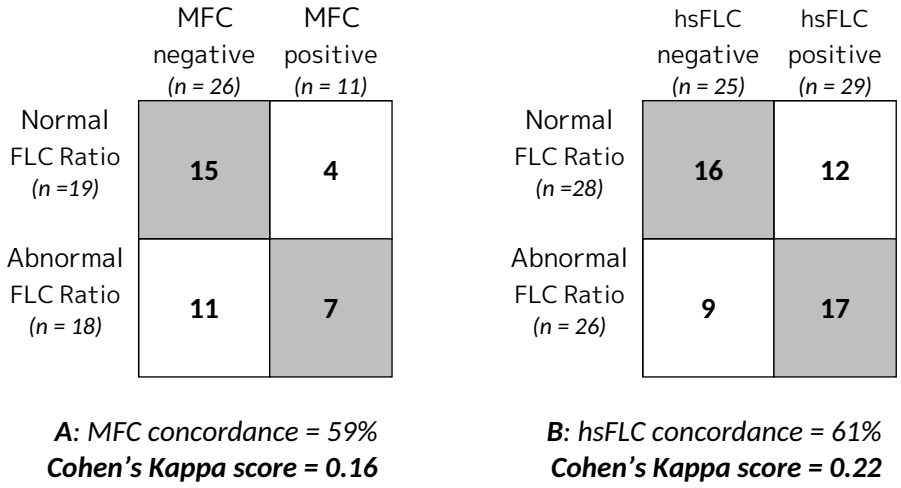


Figure 13. *Two concordance plots showing the agreement between MRD results (A: MFC; left and B: hsFLC; right) and the normal/abnormal κ/λ FLC ratios). The calculated Cohen's kappa score is also shown accordingly (Calculation steps not shown)*

The Grey shaded boxes in figure 13 indicate the number of patients in agreement between the κ/λ FLCratio result and the expected MRD result.

A: The Concordance between the MRD results by **MFC** and the κ/λFLC ratio was **59%** (n = 37). The strength of agreement according to the calculated Cohen's Kappa score is only **slight** (see table 15)

B: The Concordance between the MRD results by **hsFLC** and the κ/λFLC ratio was **61%** (n = 54). The strength of agreement according to the calculated Cohen's Kappa score is **fair** (see table 15)

Both methods demonstrated similar and significant discordance between the κ/λ FLC ratio and the expected MRD results. Though, hsFLC results were slightly more in agreement with the ratio than MFC results.

3.1.4 *Statistical analysis of λ results*

The presence of any λ FLC-specific trends in the hsFLC results was investigated, likewise. Firstly, an analysis of MRD results and the four post-treatment response categories is presented in relation to the m-protein type at diagnosis (four categories; IgG, IgA, IgD and free λ FLCs).

***Table 16**. Response categories and MRD results specific to each type of m-protein tested in the λ patient group.*

M-protein type	No. of patients	Response category					MRD by MFC			MRD by hsFLC	
		SCR	CR	VGPR	PR	n/a	P	N	n/a	P	N
IgG	29	13	0	12	3	1	6	16	7	13	16
IgA	14	10	0	4	0	0	2	10	2	6	8
IgD	2	2	0	0	0	0	2	0	0	1	1
FL	11	8	1	2	0	0	1	6	4	6	5
Total	56	33	1	18	3	1	11	32	13	26	30

***n/a**: missing data*

Figure 14 below summarises the key findings from table 16.

M-protein type	Key results
IgG	• 45% of patients had an SCR post-treatment. • 27% were MRD positive by MFC (excluding patients with n/a for MFC). • 45% were MRD positive by hsFLC.
IgA	• 71% of patients had an SCR post-treatment. • 17% were MRD positive by MFC (excluding patients with n/a for MFC). • 43% were MRD positive by hsFLC.
IgD	• 2 patients: Both had an SCR post-treatment. • 2 patients: both MRD positive by MFC, only 1 was positive by hsfLC
FL	• 73% of patients had an SCR post-treatment. • 14% were MRD positive by MFC (excluding patients with n/a for MFC). • 55% were MRD positive by hsFLC.
Overall: • Around 59% of all patients achieved an SCR, 2% had a CR, 32% had a VGPR and 5% had a PR post-transplant (confirmed by conventional methods i.e., CZE and IFE). • Out of all patients tested by MFC, 26% were MRD positive. • Out of all patients tested by hsFLC, 46% were MRD positive.	

***Figure 14**. Summary of results in table 16.*

In line with the κ results presented previously, a common finding across all the m-protein types tested in the λ group was that a SCR was achieved most frequently by patients, followed by a VGPR. Once again, the highest frequency of SCR achieved was found in the FLC-only secretory group of patients (though, only a small number were tested). However, this time, more than 70% of IgA-λ secretory patients also achieved a SCR, refuting to some extent any conclusion of superior responses to treatment in FLC-only-secretory patients.

Once again, both sets of possible MRD results (positive and negative) were observed (using hsFLC) in the various m-protein categories, including the two IgD patients. The same was observed when MFC was used, except in the two IgD patients who were both positive by MFC. A look back at the raw hsFLC results in figure 9 for the IgD secretory patient who was judged to be MRD positive (patient 35), show that the same diagnostic monoclonal FLCs found in the presentation sample (trace 69) can also be clearly seen in the D100 PASCT sample (trace 70). The presence or absence of heavy chains-regardless of type-seemed to have no effect on hsFLC results in the λ set of patients. As found in the κ group, the frequency of MRD positive results was higher using hsfLC compared to MFC in each m-protein category and generally overall.

Around 60% of the λ group overall, achieved a SCR following treatment. Again, it was expected that most of these would also be MRD negative (at least by hsFLC). This was assessed next in table 17 which shows the MRD results by both methods in relation to each of the four post-transplant response categories: SCR, CR, VGPR and PR.

Table 17. Comparison of MRD results by MFC vs hsFLC related to each response category achieved by the λ patients.

Response category	Total No. of patients	MRD by MFC			MRD by hsFLC		Adjusted hsFLC*	
		POS	NEG	n/a	POS	NEG	POS	NEG
SCR	33	4	24	5	16	17	12	16
CR	1	0	0	1	0	1	0	0
VGPR	18	5	8	5	8	10	7	6
PR	3	1	0	2	1	2	0	1
n/a	1	1	0	0	1	0	1	0
Total	56	11	32	13	26	30	20	23

* ***Adjusted hsFLC**: excluding 13 patients with missing MFC data.*

Figure 15 below summarises the key findings from table 17.

Response category	Key results
SCR	• 14% were MRD positive by MFC. • 48% were MRD positive by hsFLC (43% by corrected hsFLC).
CR	• Single patient was MRD negative by hsFLC (not tested by MFC)
VGPR	• 38% were MRD positive by MFC. • 33% were MRD positive by hsFLC (54% by corrected hsFLC).
PR	• One patient MRD positive by MFC but negative by corrected hsFLC. • Two PR patients with missing MFC data: 1 was positive and 1 was negative for MRD by hsFLC
Overall: • Out of all patients tested by MFC, 26% were MRD positive. • Out of all patients tested by HsFLC, 46% were MRD positive (47% by corrected hsFLC)	

***Figure 15**. A summary of findings from table 17.*

Although, only around 14% of the λ set of SCR patients were MRD positive by MFC, almost half of the SCR group was judged as MRD positive by hsFLC. On the other hand, more than half of the VGPR patients were MRD negative by both methods. As in the kappa group, the single CR patient in the λ group was MRD negative by hsfLC. As opposed to the κ group findings, two out the three PR patients were MRD negative by hsFLC.

A closer look at the MRD results in light of the actual κ/λFLC ratios post-ASCT, follows further on. Before that, a direct patient by patient comparison of MRD results was made between MFC and hsFLC, in relation to each response category in table 18 below and also in the concordance plot in figure 16 that follows.

***Table 18**. Concordance & discordance between the MRD results obtained by MFC vs hsFLC in relation to each response category for the λ patient group.*

		Comparison of MRD results between methods			
Response category	**Total Patients***	**Patients with MRD Concordance**	**Patients with MRD Discordance**	**% Concordance**	**% Discordance**
SCR	28	14	14	50	50
VGPR	13	7	6	54	46
PR	1	0	1	0	100
n/a	1	1	0	100	0
Total	**43**	**22**	**21**	**-**	**-**

**Excluding 13 patients with missing MFC data.*

The results show a very significant discordance between the MRD results obtained by each method in respect of two main response categories; SCR and VGPR. The overall patient by patient concordance and discordance of MRD results between methods is demonstrated in the concordance plot below.

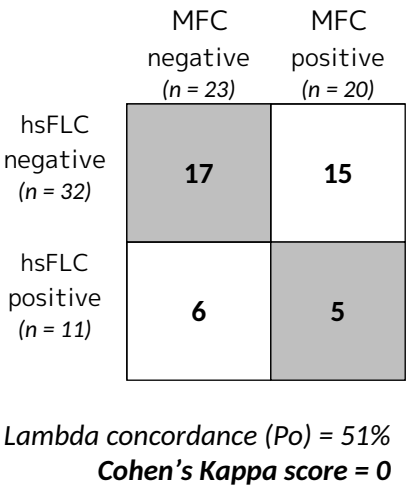


***Figure 16**. A concordance plot showing the overall agreement between hsFLC and MFC for the classification of MRD in the λ patient group.*

The Grey shaded boxes in figure 16 indicate the number of patients in agreement between methods in terms of MRD positive and MRD negative for n = 43 (13 patients excluded with missing MFC data). The overall concordance between methods for MRD classification post-ASCT was 51% for patients in the λ patient group which leaves a very significant overall MRD discordance of 49% between methods. According to Cohen's kappa score interpretations shown in table 15, the strength of agreement between MRD results by MFC and hsFLC was **slight**.

Next, the expected relationship between the κ/λ FLC ratio at D100-PASCT and the MRD result was assessed. i.e. It was expected that patients with a normal κ/λ FLC ratio (0.26–1.65) will be MRD negative, while patients with an abnormal κ/λ FLC ratio will be MRD positive (See Figure 17 below).

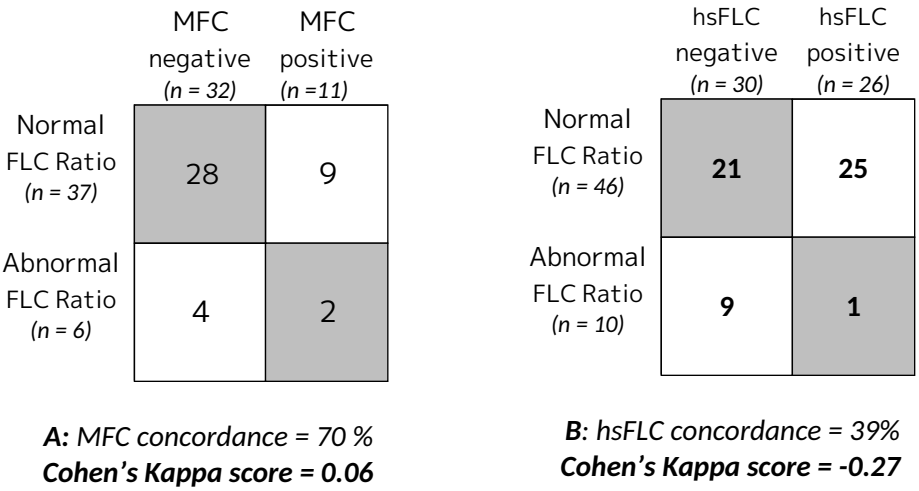


Figure 17. *Two concordance plots showing the agreement between MRD results (A: MFC; left and B: hsFLC; right) and the normal/abnormal κ/λ FLC ratios). The calculated Cohen's kappa score is also shown accordingly.*

The Grey shaded boxes in figure 17 indicate the number of patients in agreement between the κ/λ FLC ratio result and the expected MRD result.

A: The Concordance between the MRD results by **MFC** and the κ/λFLC ratio was **70%** (n = 43). The strength of agreement according to the calculated Cohen's Kappa score is only **slight** (see table 15)

B: The Concordance between the MRD results by **hsFLC** and the κ/λFLC ratio was **39%** (n = 56). The strength of agreement according to the calculated Cohen's Kappa score is **poor** (see table 15)

The MRD results by MFC demonstrated a slightly superior concordance this time with the κ/λ FLC ratio than the MRD results by hsFLC. However, the Cohens kappa score indicates that this difference was very small considering the greater number of patients that were measured overall for the hsFLC result (13 patients with missing MFC data were not measured).

3.1.5 Combined overall results.

The results of the κ and λ group of patients were combined into a single data set and the overall results were investigated in the same way as before. In-depth statistical analysis of the relationship between m-protein categories and the response to treatment was not pursued as this was not the focus of this project. Instead, the focus was to demonstrate hsFLC method applicability amongst all of the m-protein and response categories tested as outlined in section 2.3.1.1.

An analysis of the MRD results overall and the four post-treatment response categories is presented in relation to the m-protein type at diagnosis (four categories; IgG, IgA, IgD and FLC-secretory only).

***Table 19**. Response categories and MRD results (MFC vs hsFLC) related to each type of m-protein tested in total.*

M-protein type	No. of patients	Response category					MRD by MFC			MRD by hsFLC	
		SCR	CR	VGPR	PR	n/a	P	N	n/a	P	N
IgG	60	32	0	19	5	4	13	30	17	30	30
IgA	27	16	1	9	1	0	5	17	5	14	13
IgD	3	3	0	0	0	0	2	1	0	1	2
FLC only	20	16	1	3	0	0	2	10	8	10	10
Total	**110**	**67**	**2**	**31**	**6**	4	**22**	**58**	**30**	**55**	**55**

***n/a**: missing data*

Figure 18 below summarises the key findings from table 19.

M-protein type	Key results
IgG	• 53% of patients had an SCR post-treatment. • 30% were MRD positive by MFC (excluding patients with n/a for MFC). • 50% were MRD positive by hsFLC.
IgA	• 59% of patients had an SCR post-treatment. • 23% were MRD positive by MFC (excluding patients with n/a for MFC). • 52% were MRD positive by hsFLC.
IgD	• All three patients had an SCR post-treatment. • Two were MRD positive and one was MRD Negative by MFC. • One was MRD positive and two were MRD Negative by hsFLC.
FLC only	• 80% of patients had an SCR post-treatment. • 17% were MRD positive by MFC (excluding patients with n/a for MFC). • 50% were MRD positive by hsFLC.
Overall: • 61% of all patients achieved an SCR, 28% had a VGPR, 5% had a PR *and* 2% had a CR post-transplant (confirmed by conventional methods i.e., CZE and IFE). • Out of all patients tested by MFC, 28% were MRD positive. • Out of all patients tested by HsFLC, 50% were MRD positive.	

***Figure 18**. Summary of results in table 19.*

A common finding overall across all the m-protein types tested was that a SCR was achieved most frequently by patients post-transplant, followed by a VGPR. A higher proportion of the FLC-only secretory group of patients, 80% specifically, achieved a SCR compared to patients in the other m-protein categories. Besides the three IgD patients all exclusively achieving a SCR, no other m-protein specific result concerning the response categories achieved was found which was exclusive to any particular m-protein category.

Both sets of possible MRD results (positive and negative) were observed (using hsFLC) in the various m-protein categories. This was also observed when MFC was used. Both positive and negative MRD results were observed in the presence and also in the absence of heavy chains. The frequency of MRD positive results was higher using hsfLC compared to MFC in each m-protein category and generally overall.

More than 60% of all patients achieved a SCR following treatment. The proposition that most of the SCR patients would also be MRD negative (at least by hsFLC) was investigated. Table 20 below shows the MRD results by both methods in relation to each of the four post-transplant response categories.

***Table 20**. Comparison of total MRD results by MFC vs hsFLC for each response category.*

Response category	Total No. of patients	MRD by MFC			MRD by hsFLC		Adjusted hsFLC*	
		POS	NEG	n/a	POS	NEG	POS	NEG
SCR	67	10	42	15	33	34	23	29
CR	2	0	0	2	0	2	0	0
VGPR	31	9	14	8	14	17	11	12
PR	6	2	0	4	4	2	1	1
n/a	4	1	2	1	4	0	3	0
Total	110	22	58	30	55	55	38	42

**Adjusted hsFLC: excluding 30 patients with missing MFC data.*

Figure 19 below summarises the key findings from table 20.

Response category	Key results
SCR	• 19% were MRD positive by MFC (excluding patients with n/a for MFC). • 49% were MRD positive by hsFLC (44% by corrected hsFLC).
CR	• Both patients were MRD negative by hsFLC (not tested by MFC)
VGPR	• 39% were MRD positive by MFC (excluding patients with n/a for MFC). • 45% were MRD positive by hsFLC (48% by corrected hsFLC).
PR	• Both patients tested by MFC were MRD positive. • 67% were MRD positive by hsFLC (50% by corrected hsFLC).
Overall: • Out of all patients tested by MFC, 28% were MRD positive. • Out of all patients tested by HsFLC, 50% were MRD positive (48% by corrected hsFLC)	

***Figure 19**. Summary of key findings from table 20.*

Out of all the SCR patients tested in this study, 20% were MRD positive by MFC, compared to 50% by hsFLC. Less than half of all VGPR patients were found to be MRD positive by either method. Both the CR patients measured exclusively by hsFLC were MRD negative. Two out of six PR patients were found to be MRD negative by hsFLC.

A closer look at the MRD results in light of the actual κ/λFLC ratios post-ASCT, follows further on. Before that, a direct patient by patient comparison of MRD results was made between MFC and hsFLC, in relation to each response category in table 21 below and also in the concordance plot in figure 20 that follows.

Table 21. *Overall concordance & discordance between the MRD results obtained by MFC vs hsFLC in relation to each response category.*

		Comparison of MRD results between methods			
Response category	**Total Patients***	**Patients with MRD Concordance**	**Patients with MRD Discordance**	**% Concordance**	**% Discordance**
SCR	52	23	29	44	56
VGPR	23	13	10	57	43
PR	2	1	1	50	50
n/a	3	1	2	33	67
Total	80	38	42	-	-

**Excluding 30 patients with missing MFC data.*
***n/a**: missing response data*

The results show a very significant discordance between the MRD results obtained by each method in respect of all the response categories shown. The overall patient by patient concordance and discordance of MRD results between methods is demonstrated in the concordance plot below.

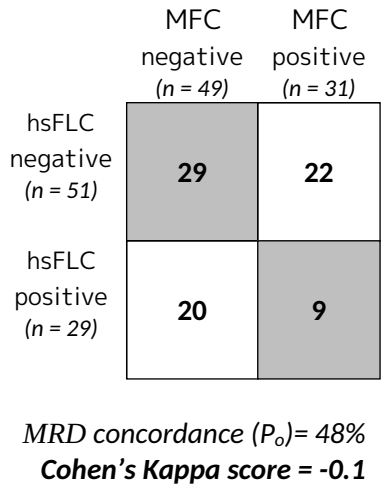


Figure 20. *Overall MRD Concordance between MFC and hsFLC for the classification of MRD in MM.*

The Grey shaded boxes in figure 20 indicate the number of patients in agreement between methods in terms of MRD positive and MRD negative for n = 80 (30 patients excluded with missing MFC data). The overall concordance between methods for MRD classification post-ASCT was 48% which leaves a very significant overall MRD discordance of more than 50% between methods. According to Cohen's kappa score interpretations in table 15, the strength of agreement between MRD results by MFC and hsFLC was **poor**.

Next, the expected relationship between the κ/λFLC ratio at D100-PASCT and the MRD result was assessed. i.e. It was expected that patients with a normal κ/λFLC ratio (0.26–1.65) will be MRD negative, while patients with an abnormal κ/λFLC ratio will be MRD positive (See Figure 21 below).

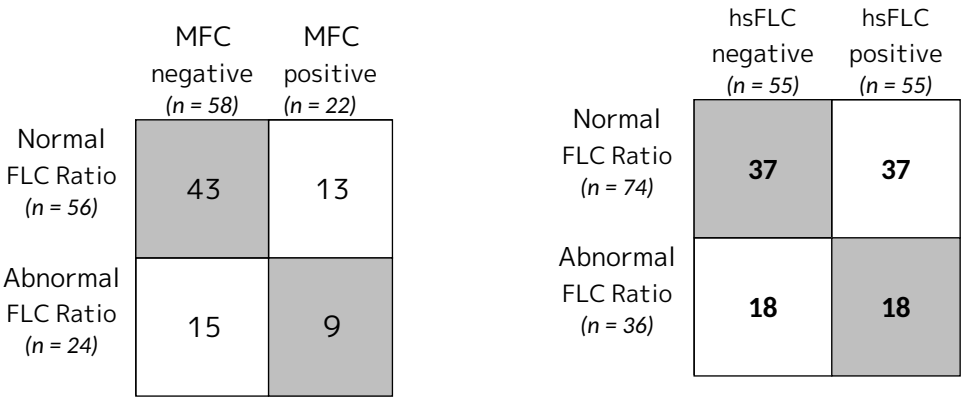


***Figure 21**. Two concordance plots showing the agreement between MRD results (A: MFC; left and B: hsFLC; right) and the normal/abnormal κ/λ FLC ratios). The calculated Cohen's kappa score is also shown accordingly.*

The Grey shaded boxes in figure 21 indicate the number of patients in agreement between the κ/λ FLC ratio result and the expected MRD result.

A: The Concordance between the MRD results by **MFC** and the κ/λFLC ratio was **65%** (n = 80). The strength of agreement according to the calculated Cohen's Kappa score is **slight** (see table 15)

B: The Concordance between the MRD results by **hsFLC** and the κ/λFLC ratio was **50%** (n = 110). The strength of agreement according to the calculated Cohen's Kappa score is **poor** (see table 15)

The MRD results by MFC demonstrated a slightly superior concordance with the κ/λ FLC ratio than the MRD results by hsFLC. However, the Cohens kappa score indicates that this difference was very small considering the greater number of patients that were measured overall for the hsFLC result (30 patients with missing MFC data were not measured).

3.2 LCMM and OSMM patients.

Figure 22 below shows the scanned image of the IB produced by hsfLC for a total of 19 samples taken at the point of disease presentation. Eleven of these samples were from LCMM patients and 8 were from OSMM patients. The samples were probed for monoclonal FLCs using a combination of κ and λ mouse monoclonal antibodies.

LCMM & OSMM patients

Samples 1 to 19

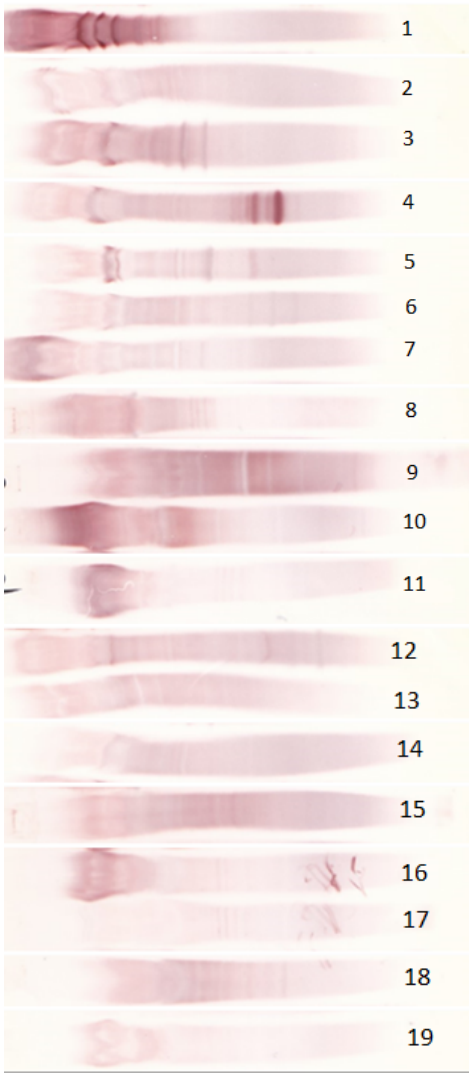


***Figure 22**. A scanned image of the IB produced by hsFLC showing results for 11 LCMM patient samples (1-11) and also 8 OSMM patient samples (12-19) taken at disease presentation. Each numbered protein trace corresponds to the patients listed in order of table 22 below.*

The interpretation of results from figure 22 above are recorded below in table 22, alongside the sFLC concentrations for each patient.

***Table 22**. Serology and hsFLC results for 11 LCMM patients (1-11) & 8 OSMM patients (12-19 in grey) at disease presentation. All eight of the OSMM samples were negative for m-protein by serum IFE.*

Patient	Secretory type	κ FLC* (mg/L)	λ FLC* (mg/L)	FLC Ratio	m-protein by hsFLC
1	λ-LCMM	5.0	640	0.0	P
2	κ- LCMM	458	13.4	34.1	P
3	λ- LCMM	5.0	255	0.0	P
4	κ- LCMM	419	9.0	46.6	P
5	κ- LCMM	577	7.8	74.0	P
6	κ- LCMM	564	8.6	65.7	P
7	κ- LCMM	823	1.4	580	P
8	λ- LCMM	0.7	288	0.0	P
9	κ- LCMM	744	12.2	60.8	P
10	κ- LCMM	610	9.2	66.3	P
11	λ- LCMM	147	8.7	16.9	P
12	LLOS	0.6	41.0	0.0	P
13	LLOS	7.1	24.7	0.3*	N
14	OSMM	40.3	51.6	0.8*	P
15	OSMM	71.3	17.6	4.1	P
16	LLOS	33.1	6.9	4.8	P
17	LLOS	48.0	8.0	6.0	P
18	OSMM	79.4	23.0	3.5	P
19	LLOS	25.8	6.2	4.2	P

***LCMM**: light-chain only secretory MM, **OSMM:** oligosecretory MM, **LLOS:** low-level OS, **P**: positive, **N**: negative.*
**Samples with normal κ/λ FLC ratio (0.26–1.65)*

Monoclonal FLCs can be seen very clearly as expected in all of the LCMM patient traces, in figure 22. Patients 1 (λ), 3 (λ) and 4 (κ) seem to have the most distinctly stained bands. Though, there are other samples with higher FLC concentrations in which banding is not as distinct.

Monoclonal Bands are also visible in almost all of the OSMM traces, with the exception of trace 13. Patient 13 was one of the low-level OSMM patients and had a normal κ/λ FLC ratio. Patient 14 is the only other patient with a normal κ/λ FLC ratio, yet a monoclonal band can just be seen in trace 14.

In summary, hsFLC confirmed the original diagnosis of LCMM in 100% of the patients tested in that category and also confirmed the diagnosis in 7 out of 8 (88%) patients classified as OSMM.

In the follow up experiment to see whether or not hsFLC could be used for monitoring these LCMM patients post-treatment, six LCMM samples (3 κ and 3 λ secretors) were selected from above:

1. Patient 1 (λ secretor),
2. Patient 3 (λ secretor),
3. Patient 4 (κ secretor),
4. Patient 5 (κ secretor),
5. Patient 6 (κ secretor),
6. Patient 8 (λ secretor),

The sFLC concentrations ranged from around 250 to 800 mg/l of the involved LC. Each patient was run at a 1 in 4 and also at a 1 in 16 dilution. The IB produced was probed with a fluorescent antibody conjugate firstly (see section 2.2.1, table 7, 5b for antibody). It was then stripped and re-probed with the standard conjugate to produce the usual red staining. Figure 23 below shows the results using both probing methods.

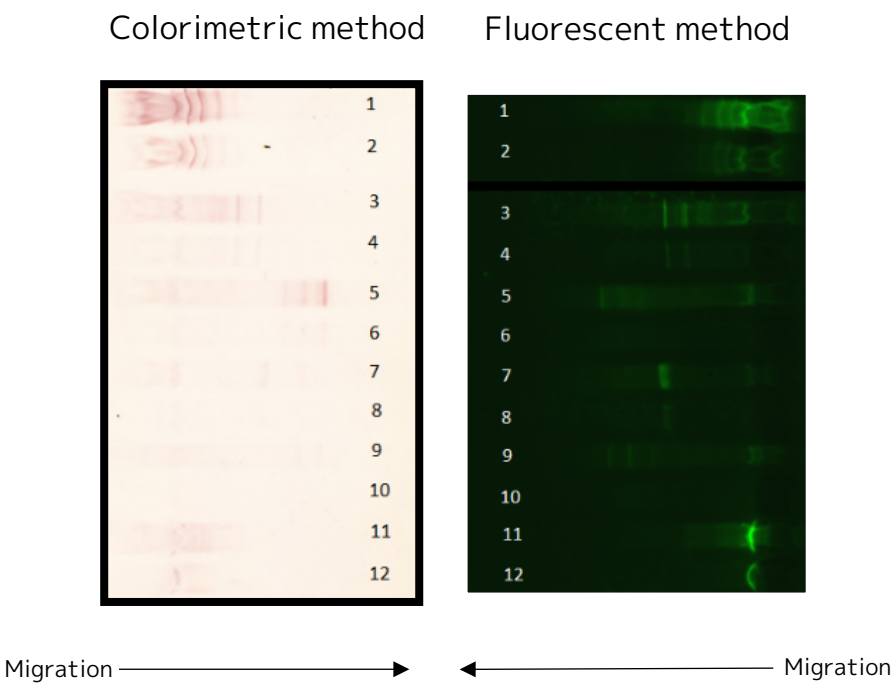


***Figure 23**. A scanned image of IEF results showing 6 LCMM patients; each run at 1/4 and 1/16 dilutions consecutively. The image of the IB probed using fluorescence is shown on the right and the image on the left shows when the standard colorimetric method was used on the same blot.*

Table 23 below shows the order and the dilution level of each of the samples that were loaded onto the blot shown in figure 23 above. The results which were interpreted by two independent readers are also shown.

***Table 23**. hsFLC results for six LCMM patients chosen from table 22. Each sample was tested at two dilutions; 1/4 and 1/16. Samples were interpreted by the standard colorimetric staining protocol and also the fluorescence conjugated protocol.*

Trace No.	Patient No. from Table 22	LCMM type	Dilution	m-protein by colorimetric method	m-protein by fluorescence method
1	1	λ	1/4	P	P
2	1	λ	1/16	P	P
3	3	λ	1/4	P	P
4	3	λ	1/16	P	P
5	4	κ	1/4	P	P
6	4	κ	1/16	P	WP
7	5	κ	1/4	P	P
8	5	κ	1/16	W	P
9	6	κ	1/4	WP	P
10	6	κ	1/16	N	N
11	8	λ	1/4	P	P
12	8	λ	1/16	P	P

***P**: positive, **WP**: weak positive, **N**: negative.*

Both types of Monoclonal FLCs (κ and λ) were still detectable in all six of the LCMM patients at the 1/4 dilution using both methods of antibody probing. The 1/4 dilution represented a 75% reduction in m-protein, resembling a partial response (PR) to treatment (see response category criteria, section 1.1.6, table 2).

Monoclonal FLCS were also still detectable at the 1/16 dilution using both methods for all patients, except patient 6 (κ-secretor, trace 10). The 1/16 dilution represented a greater than 90% reduction in m-protein following treatment, resembling at least a very good partial response (VGPR) to treatment.

In two patients, (patient 5; trace 7 and 8 and patient 8; trace 11 and 12) band clarity seemed to be superior using the fluorescent method. Otherwise, there was no consistent advantage of using fluorescence over the standard colorimetric method.

3.3 Non-secretory experiments

The NSMM experiments were split into two parts as described in the methods. The results for the first part of the screening process are shown in figure 24 below. Twenty-nine patients were probed using total (bound and free) anti-κ and λ LC antibodies in order to identify any monoclonal LCs. The results of the duplicate IB are also shown in which the samples were pre-diluted 1/100 before analysis.

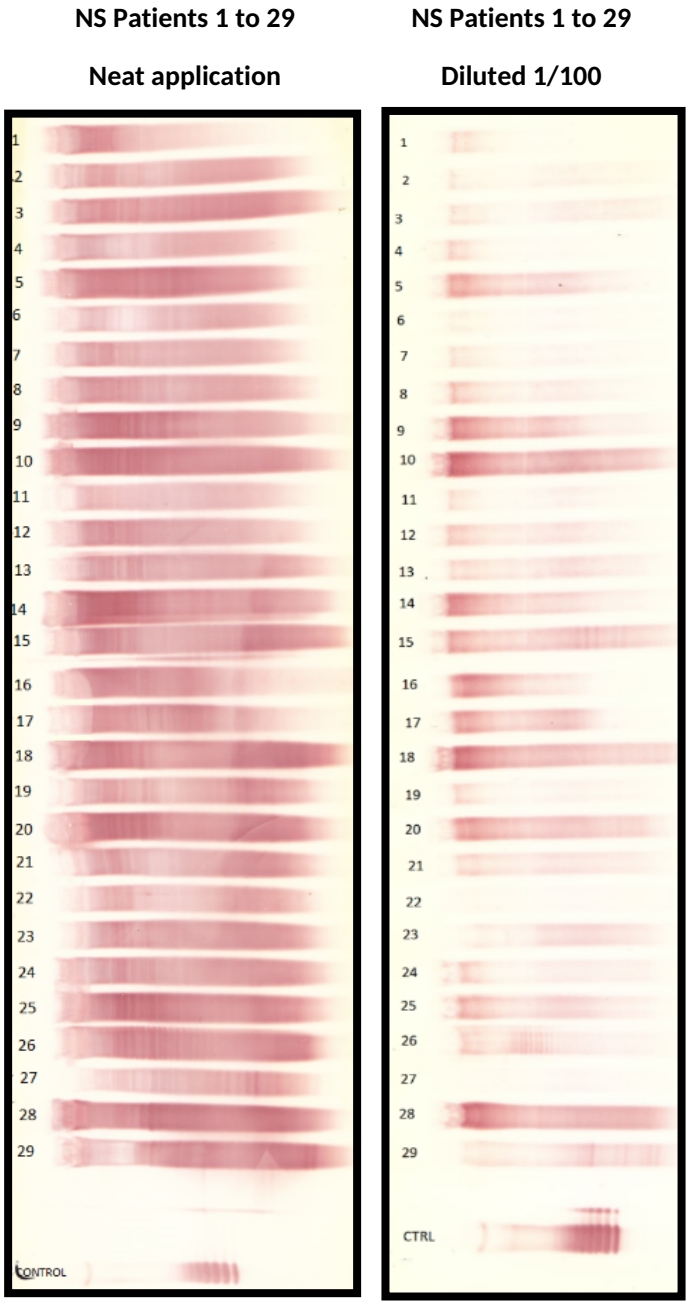


Figure 24. *A scanned image of an IB showing 29 NS patient samples at disease presentation probed with anti-total LC (bound and free) antibodies. Each numbered protein trace corresponds to the listed patients in order of Table 24 below. The blot on the left is produced using neat application of the samples. The blot on the right was produced using 1/100 dilution of the same samples. The control sample (IgG Kappa m-protein, see 2.2.1, Table 7, 1c) at the bottom end of the blots demonstrates both bound and free LCs in each blot.*

The interpretation of the blots in figure 24 are given below in table 24, alongside serology results for each patient. Although monoclonal bands were visible in all of the traces, the use of anti-total-LCs antibodies also produced a lot of polyclonal FLC background staining. The monoclonal bands are more easily distinguishable in the second blot in which the samples were diluted 1/100. Though in some traces, especially patient 22 and 27, monoclonal FLCs were undetectable at the higher dilution. The optimal dilution for the detection of monoclonal FLCs with minimal background staining would require further verification.

***Table 24**. Serology and hsFLC results for 29 NS patients tested using anti-total LC (bound and free) antibody. All patients were negative for m-protein by conventional IFE.*

Patient	IgG (g/L)	IgA (g/L)	IgM (g/L)	κ FLC* (mg/L)	λ FLC* (mg/L)	FLC Ratio	M-protein by hsFLC
1	1.8	0.4	0.0	7.1	24.7	0.29	P
2	2.6	0.1	0.1	5.1	8.0	0.64	P
3	4.0	0.1	0.0	5.3	7.5	0.71	P
4	1.7	0.3	0.2	3.7	1.6	**2.31**	P
5	7.0	1.2	1.1	9.2	36.8	0.25	P
6	2.5	0.1	0.1	0.4	11.2	**0.04**	WP
7	3.1	0.1	0.1	0.8	1.1	0.73	P
8	3.6	0.3	0.2	3.8	25.1	**0.15**	P
9	6.0	1.7	1.1	10.0	10.0	1.00	P
10	11.2	3.4	0.8	40.3	51.6	0.78	P
11	2.1	0.1	0.0	0.7	1.2	0.58	WP
12	3.4	0.2	0.1	8.5	2.2	**3.86**	P
13	4.0	0.1	0.2	4.3	1.7	**2.53**	WP
14	3.8	1.4	0.8	18.4	12.1	1.52	P
15	6.3	0.4	0.1	7.9	7.3	1.08	P
16	5.0	2.3	0.5	8.4	5.8	1.45	P
17	4.2	0.8	0.6	5.2	6.8	0.76	P
18	10.5	2.6	1.0	25.7	17.3	1.49	P
19	2.4	0.3	0.1	4.8	1.3	**3.69**	P
20	7.3	1.1	0.7	10.5	13.3	0.79	P
21	4.0	0.8	0.1	7.2	7.3	0.99	P
22	1.9	0.1	0.1	0.3	0.5	0.60	P
23	5.6	0.1	0.1	0.1	0.6	**0.17**	P
24	5.0	0.4	0.1	6.0	8.6	0.70	P
25	6.0	0.6	0.5	4.0	21.2	**0.19**	P
26	4.0	0.6	0.2	4.2	7.1	0.59	P
27	2.3	0.0	0.1	1.2	0.9	1.33	WP
28	14.0	3.3	0.7	21.2	14.3	1.48	P
29	6.2	0.3	0.8	10.5	11.3	0.93	P

***P:** positive, **WP:** weak positive.*
Normal range: κ FLC 3.30-19.40 mg/L; λ FLC: 5.71-26.30 mg/L; κ/λ ratio: 0.26-1.65.
*Abnormal κ/λ ratio results are **underlined** and in **bold** font.*

Table 24 above shows that only eight out of the 29 patients had abnormal κ/λ ratios at presentation. The serology results for the other 21 patients gave no indication at all of any monoclonal gammopathy, though hsFLC was able to demonstrate monoclonal FLCs in all 29 patients.

Three patients from table 24 above were chosen as examples for the next stage of analysis. In this final part of the analysis, the utility of hsFLC to monitor treatment responses in NS patients was assessed. For this reason, the NS patient presentation samples and any available post-treatment follow-up samples that could be found, were run side by side to compare the changing trace in response to treatment. The follow up samples chosen-where available- were first after induction, remission and any relapse samples. Each set of samples were analysed twice in separate assays using anti-κ FLC firstly, and then anti-λ FLC monoclonal antibodies.

In the first example, shown below in figure 25, three samples for patient 5 were analysed;

A. Patient 5

1) Presentation sample,

2) 6-month post-induction treatment (6MPIT) sample,

3) 9-months post-induction treatment (9MPIT) sample.

Samples probed with kappa are labelled K1, K2 and K3. Samples probed separately with Lambda antibodies are labelled L1 to L3.

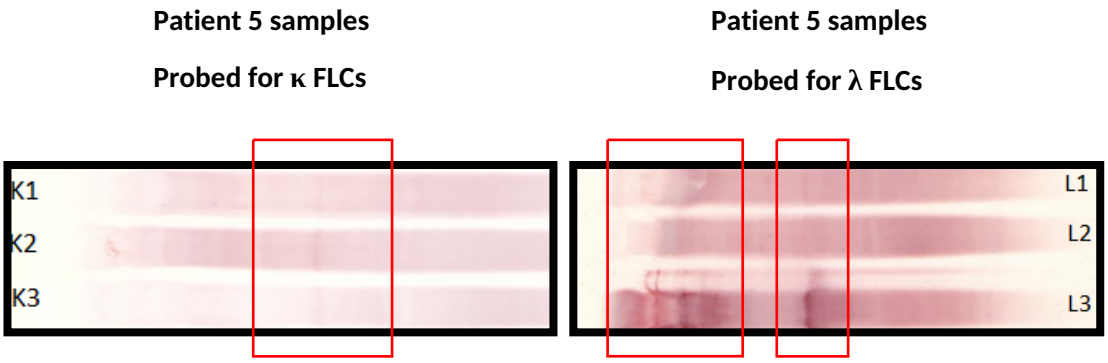


***Figure 25**. Scanned images of the hsFLC results for patient 5 presentation and follow-up samples. Samples probed with anti-kappa FLC antibodies are labelled K1, K2 and K3. Samples probed with anti-Lambda FLC antibodies are labelled L1 to L3.*

The presentation sample for this patient demonstrates monoclonal Lambda bands to the left and also to the middle of the trace (L1). The bands are not as distinct in the 6MPIT sample (L2). After 9 months PIT, a dramatic relapse ensues which is confirmed by all methods and the patient is no longer classified as non-secretory. Some corresponding monoclonal bands are also seen in the middle of all three kappa traces (K1-K3). Table 25 below shows the serology results accompanying each of the three samples.

Table 25. Serology and hsfLC results for the presentation and follow up samples for patient 5.

Sample No.	Sample ID	IgG (g/L)	IgA (g/L)	IgM (g/L)	κ FLC (mg/L)	λ FLC (mg/L)	FLC Ratio	IFE (S & U)	κ result	λ result
1	PRES	7.0	1.2	1.1	9.2	36.8	0.3	N	P	P
2	6MPIT	n/a	n/a	n/a	18.2	26.0	0.7	N	P	P
3	9MPIT	5.6	0.6	0.4	1.5	909	0.0	AL + FLU*	P	P

***S & U:** serum & urine, **MPIT:** months post induction treatment, **n/a:** no results available, **P:** positive.*
***AL + FLU:** IgA λ m-protein detected in serum and free λ LCs in urine.*

Although the κ/λ ratio was normal in the presentation sample, the λ sFLCs were raised (normal range 5.71-26.30 mg/L). These fell back into the normal range 6 months PIT. After 9 months PIT, they increased so much so that the κ/λ ratio became abnormal and IgA λ m-proteins were detectable in the serum and λ FLCs were detectable in the urine by IFE. Even in the 6MPIT, which appeared disease free by IFE and sFLC quantification, hsFLC was able to demonstrate the presence of residual disease. Upon close inspection of the hsFLC traces, κ and λ monoclonal FLC bands seem to appear in corresponding positons in all three samples as found in extremely rare cases of bi-phenotypic PC myeloma. Special immunohistochemistry, in-situ hybridization and MFC analysis would be required to confirm whether or not these m-proteins originate from the same or different PC clones (66).

The second example shown below in figure 26, is four samples for:

B. Patient 8

1) Presentation sample
2) 12 months PIT
3) 17 months PIT
4) 21 months PIT

Samples probed with κ are labelled K1 to K4. Samples probed separately with λ antibodies are labelled L1 to L4.

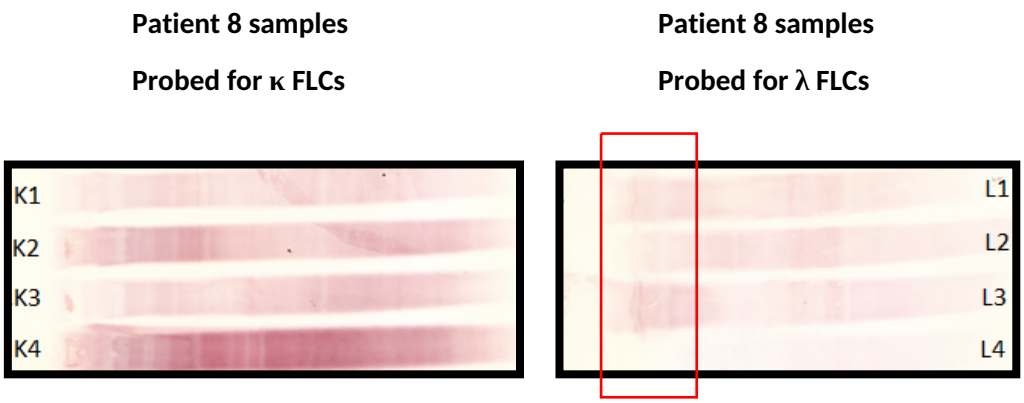


***Figure 26**. Scanned ima*
with kappa are labelled

Monoclonal λ FLCs were just visible in the presentation sample (L1). In the post-induction sample (L2), the Lambda monoclonal bands disappear. The presentation Lambda monoclonal bands reappear in the sample clinically described in the patient records as indicating disease progression 17 months PIT (L3). Kappa OCBs are apparent in all four samples (K1-4). Following another treatment cycle 21 months PIT, the bands are no longer visible. Table 26 below shows the serology results accompanying each of the four samples.

Table 26. Serology and hsfLC results for the presentation and follow up samples for patient 8.

Sample No.	Sample ID	IgG (g/L)	IgA (g/L)	IgM (g/L)	κ FLC (mg/L)	λ FLC (mg/L)	FLC Ratio	IFE (S & U)	κ result	λ result
1	PRES	3.6	0.3	0.2	3.8	25.1	0.15	N	N	P
2	12MPIT	5.0	n/a	n/a	13.2	12.8	1.03	N	N	N
3	17MPIT	3.3	n/a	n/a	4.5	161	0.03	N	N	P
4	21MPIT	2.2	n/a	n/a	5.4	1.6	3.34	N	N	N

S & U: *serum & urine,* ***MPIT:*** *months post induction treatment,* ***n/a****: no results available.*
N*: negative,* ***P:*** *positive.*

The κ/λ ratio in the presentation sample was slightly lower than the normal range supporting the finding of the monoclonal lambda band by hsFLC in sample L1, figure 26. The ratio was in the normal range 12MPIT. No monoclonal bands were detected by hsFLC in the corresponding sample L2. The ratio was clearly abnormal in the 17MPIT sample which is reflected by the lambda bands detected in sample L3 shown in figure 26. IFE results were negative throughout.

The final example shown below in figure 27, is five samples for:

C. Patient 16

1) Presentation sample,
2) 4 months PIT,
3) 10months PIT,
4) 34 months PIT,
5) 38 months PIT.

Samples probed with kappa are labelled K1 to K5. Samples probed separately with Lambda antibodies are labelled L1 to L5.

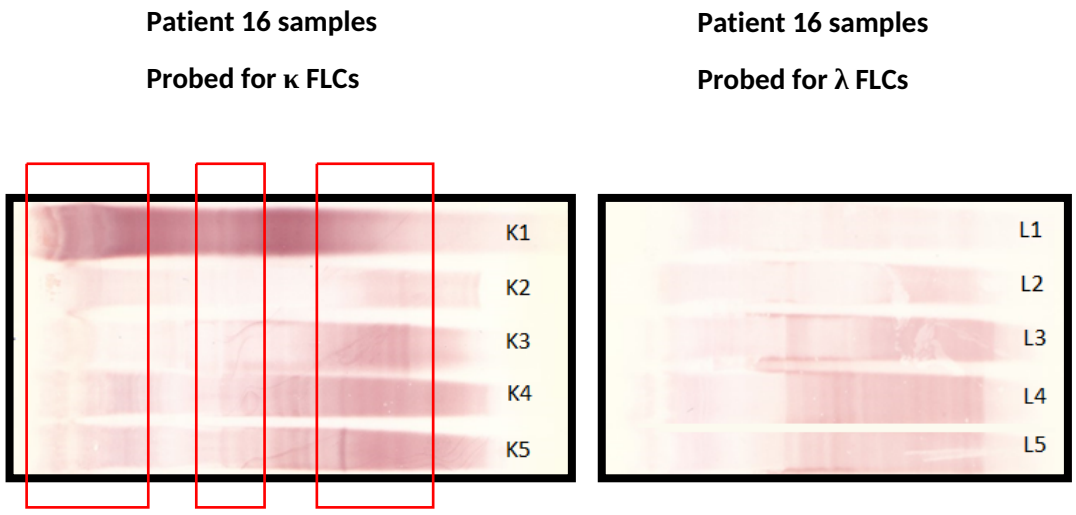


***Figure 27**. Scanned images of the hsFLC results for patient 16 presentation and follow-up samples. Samples probed with kappa are labelled K1 to K5. Samples probed with Lambda antibodies are labelled L1 to L5.*

A strong monoclonal kappa band (far left) is immediately noticeable in the presentation sample (K1). This band disappears post induction whilst new OCBs appear (far right, K2). In the follow-up sample three years later (K4), the re-emergence of monoclonal Kappa LCs is apparent in both highlighted boxes; left and middle. Indeed, these are more noticeable in the 38-month PIT sample (K5). A fresh m -protein also appeared to have emerged in K3 taking the appearance of the classic ladder pattern seen in intact m-proteins (right highlighted box). A single band from the ladder intensifies into K5 corresponding to a relapse in Kappa m-protein. Table 27 below shows the serology results accompanying each of the four samples.

Table 27. *Serology and hsfLC results for the presentation and follow up samples for patient 16*

Sample No.	Sample ID	IgG (g/L)	IgA (g/L)	IgM (g/L)	κ FLC (mg/L)	λ FLC (mg/L)	FLC Ratio	IFE (S & U)	κ result	λ result
1	PRES	5.0	2.3	0.5	8.4	5.8	1.45	N	P	N
2	4MPIT	n/a	n/a	n/a	0.6	0.8	0.75	N	N	N
3	10MPIT	n/a	n/a	n/a	5.4	8.9	0.61	N	P	N
4	34MPIT	n/a	n/a	n/a	19.5	13.4	1.45	N	P	N
5	38MPIT	n/a	n/a	n/a	121	12.2	9.92	FKU	P	N

S & U: *serum & urine,* ***MPIT:*** *months post induction treatment.*
N*: negative,* ***P:*** *positive,* ***n/a****: no results available.*
FKU: *free κ LCs detected in urine*

Although, hsFLC detected a strong monoclonal κ band in the presentation sample (K1), the κ/λ ratio was normal. No other laboratory findings supported monoclonal gammopathy at that point. Therefore, NS classification was given to explain clinical findings. In the follow-up sample three years later (sample 4), a normal κ/λ ratio was still maintained despite the reappearance of monoclonal bands in sample K4 (figure 27). The κ/λ ratio was clearly abnormal 38MPIT by which time monoclonal kappa LCs were even detectable in the urine by IFE and an updated diagnosis of Light chain escape was given in the patient records.

Along with the three examples given above, a summary of follow up sample results for 13 other NS patients is given below in table 28.

***Table 28**. Summary of main findings from the follow up analysis of 16 NS patient samples.*

No.	Patient No. from table 24	Summary of main findings
1	2	λ relapse detected by hsfLC 18-months PIT. IFE still negative throughout.
2	4	Transient Min IgM λ detected 4months PIT but not at 9 months PIT by IFE. HsFLC was negative in both follow ups.
3	5	Big relapse as AL & MONLU positive 9 months PIT by IFE but not at 6 months PIT. hsFLC was positive for m-protein throughout.
4	6	Clear monoclonal λ bands in presentation sample by hsFLC (& low κ/λ ratio), which disappear in remission. IFE negative throughout.
5	8	hsFLC shows reappearance of diagnostic λ m-protein in 17-month PIT sample (& low κ/λ ratio). Clinical records state disease relapse & progression. Bands disappear again following next cycle.
6	9	κ m-protein detected at presentation despite normal IFE and ratio, which disappears into remission.
7	11	κ m-protein detected at presentation despite normal IFE and ratio, which disappears into remission.
8	12	Both κ & λ m-proteins detected at presentation by hsfLC. λ disappear 2 months PIT but κ bands still visible. No more follow ups.
9	13	Same κ monoclonal bands visible in all four samples; presentation into remission 47 months PIT, but disappears thereafter. κ/λ ratio also raised in the presentation sample.
10	14	Both κ and λ m-proteins visible at presentation (normal κ/λ ratio). Only one sample after cycle 1 available; same κ banding as presentation
11	16	Strong κ m-protein at presentation by hsflc only (normal ratio). hsFLC detected relapse 10 months PIT. IFE shows MONKU first at 38 months PIT (high ratio also)
12	17	κ monoclonal FLCs visible in presentation sample and in 2 months PIT sample. κ/λ ratio normal throughout. No other follow-up samples.
13	23	κ monoclonal bands just visible at presentation & 3-months PIT. Other bands possibly from therapeutic monoclonal antibody.
14	24	Same monoclonal λ bands visible throughout; presentation to 11 months sample. 11-month bands too weak to be called positive.
15	25	Same λ monoclonal bands visible throughout; presentation to post 17 months PIT. Strong λ diagnostic band disappears following therapy.
16	26	Multiple λ bands at presentation also seen in the follow up sample.

***AL & MONLU**: IgA λ in serum and monoclonal λ in urine*
***MONKU: m**onoclonal κ in urine.*

hsFLC detected relapse in four out of sixteen patients tested post induction. In two of these cases, IFE was completely negative (patient 2 & 8, summary table 24) and in the two other cases, IFE only detected disease many months later (Patient 5 & 16). In fact, patient 16 showed relapse 10 months

PIT by hsFLC, as compared to 38 months PIT by IFE. In the remaining twelve patients, five patients still showed the presence of PIT disease by hsFLC in the follow up samples that were available and seven patient's hsFLC results changed from positive to negative reflecting disease remission.

4 Discussion

4.1 MRD

Even before considering the post-transplant MRD results, the first major finding was that monoclonal FLCs were detected in the disease presentation samples of all 110 patients tested. This was completely in line with the conventional methods used to arrive at the original diagnosis, namely CZE, IFE and sFLC quantification. Even the three IgD patient diagnostic samples added to the study, showed clear monoclonal FLC bands. The intact IgD m-protein concentration in these three patient samples was around 3 to 11 g/L, measured originally by Ouchterlony immunodiffusion. Surprisingly, the diagnostic trace for one of the IgD-λ patients (figure 9, patient 35, trace 69), very distinctly appeared to resemble the spectrotype seen in intact IgA m-proteins. There were also examples of other patients in which the FLC diagnostic sample spectrotype appeared to resemble the pattern for intact IgG and IgA m-proteins (both κ and λ) e.g. patient 21 and 44 in figure 8). The intact m-protein concentration in these other patients was around 30g/L. This was not particularly high relative to the rest of the patients in the study, who demonstrated the expected monoclonal FLC spectrotype in the presentation samples. It has been mentioned in the optimisation experiments (section 6.1.9), that the anti-FLC antibodies used in this study have been verified to bind FLCs with a many thousand-fold greater avidity than to bound light chains within IMGs. Therefore, there may be some other unknown explanation for these exceptional patterns of staining requiring further investigation. The only other major class of m-proteins not tested in this study was IgM. The separation of these IMGs by electrophoresis is known to be more complex associated with its unique structure. However, the demonstration of monoclonal FLCs in one hundred percent of the presentation samples in this study would likely extend the applicability of hsFLC to all MM patients, regardless of the presence, the absence or the subtype of intact m-proteins. This would be the subject of further studies.

In terms of the post-ASCT results, no findings were entirely exclusive to a particular m-protein class in the post-treatment response categories achieved by each patient in either the κ or the λ group. One of the possibilities considered at the outset, was that if a particular m-protein type always failed to achieve an SCR post-ASCT, then MRD measurement may not be useful in these patients initially, until they demonstrate an SCR. However, a common finding overall across all the m-protein types (in both FLC groups) tested was that a SCR was achieved most frequently by patients post-transplant, followed by a VGPR. Out of all the m-protein categories, the LCMM secretory group of patients (regardless of LC type) seemed to achieve an SCR in the most cases. Neither was there any IMG heavy chain-associated exclusive finding, in the two methods of MRD measurement; MFC and hsFLC. Both sets of possible MRD results (positive and negative) were observed using hsFLC (and MFC) between the various m-protein types, i.e. the sensitivity of monoclonal FLC measurement was apparently unaffected by the presence/absence or type of intact m-protein. Although, the frequency of MRD positive results was higher using hsfLC compared to MFC in each m-protein category and generally overall.

In relation to the 76 patients with complete results in this study that were tested for MRD, around 61% overall achieved an SCR at 100 days following PASCT. In line with the latest criteria, it would follow that all these patients should all have had a normal κ/λ FLC ratio post-ASCT. However, this was not the case and the reasons for this are unclear. It is possible that the assigned response levels were made using more conventional criteria or that the ratio was overlooked in some cases as it is known to be unreliable post-ASCT. Nevertheless, at that time of sampling, the clinical laboratory confirmed that around 20% of these patients were still MRD positive by MFC. When the same 76 follow up samples were retested by hsFLC, around 44% were found to be MRD positive instead. At first glance, one could conclude that this reflected the higher diagnostic sensitivity of the hsFLC method related to its high analytical sensitivity (see hsFLC sensitivity data in methods section, 2.2.2) However, a direct patient by patient comparison of MRD status given by the two methods revealed quite a

significant overall discordance of around 50% between MFC and hsFLC results. This not only nullified the identification of a common fraction of MRD positive patients between methods but also raised a question over the accuracy of the MRD results obtained by either method.

Another rather crude point of reference in order to compare the diagnostic specificities of each method for MRD detection, was the FLC ratio. Though the κ/λ ratio is known to be unreliable following therapy and so the difference between involved and uninvolved FLCs is used instead. Nevertheless, MRD negativity would at least in theory be supported by a normal ratio while MRD positivity by a relatively abnormal ratio. In this regard, it was found that MRD negativity by hsFLC was supported by a normal FLC ratio in 34% of all patients tested by hsFLC, whilst MFC negativity corroborated with a normal ratio in 54% of cases tested by this method suggesting that MFC had a superior diagnostic specificity. MRD positivity on the other hand was not as closely aligned to an abnormal FLC ratio for either method; 16% for hsFLC and 11% for MFC, respectively. The overall strength of agreement between the MRD results and the κ/λ FLC ratio-according to the expected relationship- was rated as poor for both methods by the Cohens Kappa coefficient.

The MRD concordance between methods for VGPR patients was 57%. MRD was undetectable in more than 50 % of VGPR patients and negative even in some PR patients by both methods. The most likely explanation for these findings is the delayed degradation of intact IMGs which renders them detectable in the serum and/or urine by IFE long after they are first secreted by PCs. Complete clearance of m-protein may occur several months after all the tumour cells have been eradicated. These findings highlight a major flaw in using intact IMGs as tumour markers and demonstrates the advantage of using FLCs instead to give a more real-time measurement, owing to their much shorter half-life.

There are a number of plausible explanations that may account for the observed differences in diagnostic specificity and sensitivity between the two MRD methods themselves and also in their

relationship with the measured sFLC concentrations (and calculated κ/λ ratio). Firstly, hsFLC is a blood-based test and is therefore a measure of systemic health; medullary as well as extra-medullary, akin to commercial FLC immunoassays that are used to deduce the κ/λ FLC ratios. MFC on the other hand, measures MRD in a single BM biopsy sample obtained from a single site within the BM pool. Not only does it overlook extra-medullary disease but also fails to consider the well documented intra-medullary heterogeneous distribution and spread of MM disease. Consequently, MFC analysis is more prone to underestimate disease burden and to include false MRD-negative patients.

The apparently higher diagnostic sensitivity of hsFLC for MRD measurement over other methods is not surprising considering the greater scope of a single hsFLC measurement to detect spatiotemporally distributed MM within the BM and beyond. Though, 34% of MRD positive patients by hsFLC had normal FLC ratios. This finding may be related to the limited performance of quantitative FLC immunoassays to distinguish monoclonal from polyclonal FLCs at the lower limit of detection. hsFLC on the other hand, has been shown to detect m-proteins in samples which have very low or barely any detectable FLCs. This is discussed in the next section on OSMM and NSMM samples. A third MRD assessment on these samples by another method, such as Mass spectrometry, would shed more light on these findings.

Although, MFC is a clinically validated and established method for MRD measurement, there are some well-known potential sources of variation that must be considered when making a judgement over the accuracy of MFC results. In clinical laboratory practice, this assessment is expressed as a measurement of uncertainty. Potential sources of error or variation in MFC results include sample age (altered levels of antigen expression & internalisation of CD138 (68)), sample quality (especially haemodilution) and operator error (e.g., manual pipetting of antibody and cell suspension volumes). Even with the Euroflow standardisation protocol in place, MFC remains very labour intensive

requiring special pre-lysis of red cells, a large pooling of antigen markers, double the reagents and processing time (for two-tube panels). In contrast, hsFLC only requires a minimal serum sample (~0.5ml) and the simplicity of the protocol itself excludes or at least reduces many of the potential sources of errors associated with MFC.

Fifteen patients in total (12 Kappa and 3 Lambda) demonstrated new OCBs (by hsFLC) in their respective D100-PASCT samples that resembled a uniform laddering spectrotype often seen in intact IMG m-proteins. The follow up data for six of these patients was not available. Out of the remaining 9 patients, eight demonstrated a relapse within a year post-ASCT. In those patients for whom a relapse was not proven, the uniform banding at the very least may represent a secondary MGUS (67). A closer study of such atypical post-induction banding is required to arrive at a firm conclusion; a monoclonal protein pattern needs to be clearly identifiable from OCBs that may simply represent a rejuvenated immune response. A separate retrospective study looking back at the post-induction samples of relapsed patients would help to decipher whether or not certain monoclonal banding patterns by hsFLC could predict relapse. Henry and Clegg have also previously presented a related case report highlighting the need for this investigation (68). In any case, it is certainly fair to say that the high sensitivity of hsFLC should aid the detection of relapse much earlier than conventional methods.

It is worth mentioning, that are other non-BM based MRD strategies currently gaining interest besides hsFLC. For example, there is the so-called liquid biopsy. Using this technique, circulating tumour cells (CTCs) released from the BM can be isolated in peripheral blood (PB) throughout the various stages of MM and their levels (measured by special instruments) have been shown to correlate with remission and also long-term survival (69). Circulating cell-free tumour DNA (ctDNA) can also be isolated from the PB and then measured by NGS to investigate gene rearrangements and also to monitor single nucleotide variants between samples (70, 71). However, the technology

currently gaining most interest for MRD application is Mass spectrometry (MS). MS can measure the unique mass of each constituent peptide found within IMGs and can therefore be used to detect m-proteins isolated from PB samples. The two most prevalent MS methods currently under scrutiny for MRD detection are matrix-assisted laser desorption ionization time-of-flight MS (MALDI-TOF-MS) and liquid chromatography (LC) quadrupole time-of-flight MS (LC-MS). In a recent study conducted by Derman et al, LC-MS in the PB was found to be at least as sensitive as NGS (at 10^{-6}) in the BM for the detection of MRD (72). Limitations of MS and the aforementioned techniques, include lack of consensus between the different methods, the lack of availability and of course high-cost.

4.2 LCMM & OSMM

Every one of the disease presentation LCMM patient samples demonstrated monoclonal FLC bands by hsFLC, which was wholly consistent with the abnormal κ/λ ratios associated with each patient. Abnormal κ/λ ratios are usually found in 100% of LCMM patients (32, 33).

From the LCMM patients chosen for dilutions, all were still positive at a ¼ dilution that represented a seventy-five percent reduction in m-protein and all but one were positive at the 1/16 dilution representing a >90 % reduction in m-protein. This dilution strategy could be applied to real patient samples as follows: The presentation (diagnostic) sample for each patient would be tested and used as the baseline for any diagnostic monoclonal FLCs present. The presentation sample could then be diluted according to the strategy to provide traces resembling a 75% or more reduction in m-protein. The diluted traces (or images of them) could be used as references to judge the level of reduction in m-protein in follow-up samples. In this manner, a semi-quantitative result based on the reduction in m-protein relative to the presentation sample could be given and a real-time response status may be assigned to these patients each time.

In the OSMM disease presentation samples in which involved/uninvolved FLCs were only around 25-80mg/L, hsFLC confirmed diagnosis in all but one patient by demonstrating monoclonal bands, even in one of the patients with a normal K/L ratio, who therefore had no other laboratory indicator of disease. The one LLOS patient that was negative by hsFLC, had very low FLCs (<25mg/L) by the Freelite assay and the κ/λ ratio was normal. These initial results provided the impetus for the NS part of the project in this study.

Although, the use of fluorescent conjugates appeared to bolster sensitivity of m-protein identification in some cases, the cost effectiveness of the standard method alongside its good sensitivity makes it the better all-round choice.

4.3 Non-secretors

Firstly, this study was unique in the sense that the CIS had an archive of so many NSMM patient samples to analyse. In fact, as interest in hsFLC gathered pace, local centres began to send new NS patient samples to the CIS for trial testing as there is currently no laboratory assay for the diagnosis and monitoring of this very rare subset of patients. Indeed, the initial hsFLC diagnostic screen testing of this rare cohort, demonstrated positive monoclonal FLCs in all 29 of the disease-presentation samples even though of all these samples were originally negative by IFE and only eight patients had abnormal κ/λ ratios.

In terms of disease monitoring, hsFLC detected relapse in four out of sixteen patients tested post induction. In two of these cases, IFE was completely negative throughout (patient 2 & 8, summary table 28) and in the two other cases, IFE only detected disease many months later (Patient 5 & 16). In fact, patient 16 showed relapse 10 months PIT by hsFLC, as compared to 38 months PIT by IFE. In the remaining twelve patients, five patients showed the presence of PIT disease by hsFLC in the follow up samples that were available and seven patient's hsFLC results changed from positive to negative reflecting disease remission.

In a clinical setting, the diagnostic NS protocol by hsFLC would include a preliminary screen to confirm the absence of any intact m-proteins in the presentation samples by probing the samples with anti-IgG and anti-IgA antibodies. Especially, as the original NS classification would be based upon results from conventional IFE which has a much lower sensitivity than IEF. This precautionary screen was suggested following the finding of intact-m proteins (IgG and IgA) by hsFLC in certain patients that were originally classified as NSMM. Such exceptional findings from supplementary investigations were not documented here to maintain brevity and focus.

4.4 Strengths of the HsFLC assay

HsFLC has many key advantages over alternative methods at each stage of analysis. There is virtually no special patient preparation required prior to sample collection and the collection procedure itself is minimally invasive compared to BM aspiration. This point alone is conducive to more frequent sampling for the close monitoring of disease progression and the early identification of relapse. Moreover, both deep and sustained responses to treatment can also be more easily identified in this way if ever these become treatment objectives as the literature suggests. The analytical HsFLC procedure itself can be performed at a fraction of the cost (around £6.00 per sample) of alternative methods owing to relatively simple instrumentation and cost-effective reagents. Both MFC and MS on the other hand, cost around £250-400 per sample to perform (CIS figures). HsFLC is therefore accessible to a much wider community of patients. Extended coverage is further bolstered by the number of laboratories that can easily perform hsFLC themselves owing to the straightforward procedure.

Preliminary results show that hsFLC is at least as diagnostically sensitive and specific, if not more so than alternative methods for MRD detection. Although the assay is essentially qualitative, the results can be semi-quantified by using a dilution strategy which compares the percentage reduction of monoclonal bands relative to the disease presentation sample. In this way, a treatment-response category can be assigned to patients using hsFLC. Even at its infancy, hsFLC can already be presented for clinical validation in relation to the diagnosis and monitoring of OSMM and NSMM patients because there is currently no alternative procedure available for these rare subsets.

4.5 Limitations

The interpretation of these preliminary hsFLC results was challenging for two main reasons. Firstly, the results are qualitative and therefore subjective. Experienced readers are required for interpretation. Quantitative scanning software that is able to measure the intensity of staining would help overcome this issue. Secondly, there is no other validated laboratory technique currently in use for monitoring NSMM patients to compare these results with. It is anticipated that Mass spectrometry will be performed on these same samples allowing a retrospective comparison with these results. Another limitation of hsFLC is that a baseline presentation sample is required as reference to assess bands in follow-up samples. Archived images of the diagnostic bands may help to overcome the need for analysing the baseline sample each time.

The focus of this work was to test a novel concept in MRD analysis and to develop a functional method accordingly. Though, it was greatly satisfying for all involved to ultimately present visual traces of MRD in patients tested by hsFLC, the project itself-if it were to be repeated-would benefit from an increase in patients from the various treatment response categories, especially CR and PR. Patients with IgM m-proteins (and even the extremely rare IgE, stored in CIS archives) would be included, along with more IgD patients. Ideally, all patients in the study would have complete serology and MFC results and there would also be complete clinical justification of the assigned treatment response levels. In this way, a much more thorough and all-encompassing statistical analysis of the hsFLC method could be conducted in relation to MRD by both MFC and MS. Until this time, BM biopsies will continue to be required in routine practice each time an MRD assessment is made. If the accuracy of MRD by hsFLC can be verified according to clinical regulatory standards then one may at least see a decrease in the number of BM biopsies required for monitoring MRD.

4.6 Future research

A through clinical validation of the hsFLC assay to national regulatory standards would require a much larger data set of patients at least for MRD and if possible, a larger set of NSMM pateints. MRD by hsFLC could be compared on a much larger scale with MFC and MS results. The outcomes of this clinical validation would be to verify:

- MRD by hsFLC as a prognostic marker for PFS and OS.
- hsFLC as a tool to identify relapse, especially in NSMM before development into secretory MM.

Finally, there is great potential for the hsFLC procedure to be optimised even further in a professional biotechnological setting to increase sensitivity and reproducibility of results. The main motive for attracting commercial intervention (& CE marking etc.) would be to achieve standardisation especially in relation to results interpretation across all centres providing the assay.

4.7 Conclusions

The hsFLC assay demonstrated proof of concept as a low-cost, non-invasive, blood-based technique, for the measurement of MRD and the diagnosis and monitoring of non-secretory disease.

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6 Appendices

6.1 Optimisation experiments supplement

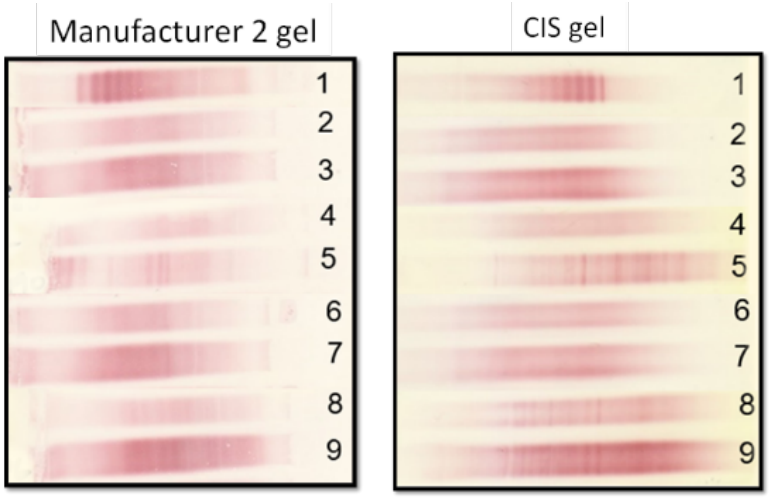
Details of all of the experiments performed to optimise the CIS OCB method for the detection of FLCs -leading to the final hsFLC protocol- are given in this section.

6.1.1 CIS in-house prepared gel vs commercially available gels

The first investigation sought to compare the performance of the IEF gel used in the existing OCB method to other commercially available gels. The CIS OCB method uses an agarose-based gel (prepared in-house) for IEF on the Multiphor 2 platform (see equipment section 2.2.1, table 5). Serum & CSF samples from four different patients that had already been clinically tested for IgG OCBs -using the in-house gel-were rerun using two commercially manufactured gels:

- **Manufacturer 1 gel:** This was an agarose-based gel containing undisclosed amounts of ampholytes and additives. The size of the gel was about a third of the length of the CIS gel and at least three times thinner. Approximately, 18 samples in total could be loaded onto the gel compared to 33 on the CIS gel. Due to size constraints, IEF was performed on the instrument provided by the supplier of the gel (normally used for immunofixation) instead of the Multiphor 2 platform. The complete manufacture IEF protocol was followed using reagents provided by the manufacturer. Following IEF, IB was performed using CIS protocol and reagents. Preliminary experiments using this gel were unsuccessful and so it was excluded outright from the final comparison.
- **Manufacturer 2 gel**: This was also an agarose-based gel containing ampholytes suitable for establishing a gradient of pH 3-10. The size and thickness of the gel was more comparable to the CIS gel. A total of 20 samples could be loaded onto it. IEF was performed on this gel using the Multiphor 2 platform and then IB using CIS protocol and reagents.

Figure 28 below shows the scanned images of blots produced using the CIS and the manufacturer 2 gel. The samples labelled 1 to 9 are detailed in the key below the images.



Sample key

Position No.	Sample ID & type	Related clinical details
1	Control serum	MM patient with an IgG m-protein
2	Patient 1- serum	Healthy patient
3	Patient 1- CSF	
4	Patient 2- serum	MS patient
5	Patient 2- CSF	
6	Patient 3- serum	Healthy patient
7	Patient 3- CSF	
8	Patient 4- serum	Infection/Inflammation
9	Patient 4- CSF	

Figure 28*. Scanned images of the IEF/IB blots produced using two different gels for the detection of IgG-OCBs, A diluted control sample containing an IgG m-protein was tested in position 1 (see table 7, 1c). Thereafter, a pair of serum and CSF samples taken from 4 different patients were tested.*

For patient 1, no OCBs are seen in the serum or the CSF samples on either of the two gels (Positions 2 & 3). For patient 2, OCBs can be seen in the CSF sample only (position 5) and not in the corresponding patient's serum (position 4) in both gels. For patient 3, no OCBs are seen in the serum or the CSF samples on either of the two gels (Positions 6 & 7). For patient 4, the same OCBs can be seen in both the serum and the CSF sample (position 8 & 9) in the CIS gel only. The equivalent OCBs are not so obvious in the manufacturer 2 gel. See table 29 for summary.

Table 29 below summarises the IgG-spectrotypes observed in figure 28 for each gel.

***Table 29**. A comparison of IgG- spectrotypes observed using the CIS gel and manufacturer 2 gel.*

Sample No.	Sample ID & type	CIS result	Manufacturer 2 result
1	Control serum	P	P
2	Patient 1- serum	N	N
3	Patient 1- CSF	N	N
4	Patient 2- serum	N	N
5	Patient 2- CSF	OCBs	OCBs
6	Patient 3- serum	N	N
7	Patient 3- CSF	N	N
8	Patient 4- serum	OCBs	N
9	Patient 4- CSF	OCBs	N

***P**: positive*, ***N**: negative*, ***OCBs**: oligoclonal bands.*

Overall, and particularly patient 2 & 4 results, demonstrate a much better resolution of monoclonal bands using the CIS gels as compared to manufacturer 2 gels. Manufacturer 1 gels were already excluded following preliminary investigations. It was decided accordingly that all subsequent experiments would use the CIS produced gels for the following advantages:

a. Almost double the samples could be loaded onto it compared to either of other manufacturer's gels.
b. CIS gels are simple, quick and cheap to produce. The commercial gels (1 & 2) were part of a kit costing £1250 and £778, respectively.
c. The size of the CIS gel allows for well-spaced lanes and improved resolution of bands. The smaller & thinner commercial gels are more sensitive to damage (e.g. drying and cracking) and artefacts.
d. CIS gels could easily be produced commercially and optimised further.

6.1.2 The composition of the agarose-based gel.

The OCB method employs a 1% agarose gel that is suitable for the migration of IgG IMGs which are around 150 kDa in size. It was hypothesized that increasing the agarose concentration to 2% would result in a denser gel with smaller pore size throughout. This would be more suited to the migration of smaller FLC molecules which vary in size from 22 kDa (monomer) to 44 kDa (dimer) and higher, depending on the degree of polymerisation or aggregation (73, 74). The smaller pore size would also potentially hinder the migration of larger non-specific migrating species thereby negating them as an interfering factor.

To test this theory, two diluted quality control samples used at the CIS, one containing κ and the other λ FLCs (see section 2.2.1, table 7, 1a & 1b), were both tested on two different gels containing 1% agarose and 2 % agarose, respectively. Whilst all other reagents remained the same as for the OCB method, the probing and tagging antibodies obviously had to be changed to detect monoclonal FLCs instead of whole IgG. A two-step antibody incubation was used to increase sensitivity instead of the one-step conjugated antibody incubation used in the OCB method. (See Table 7, primary antibodies; 2a to d and secondary antibody; 5a). Figure 29 below shows the results of these experiments.

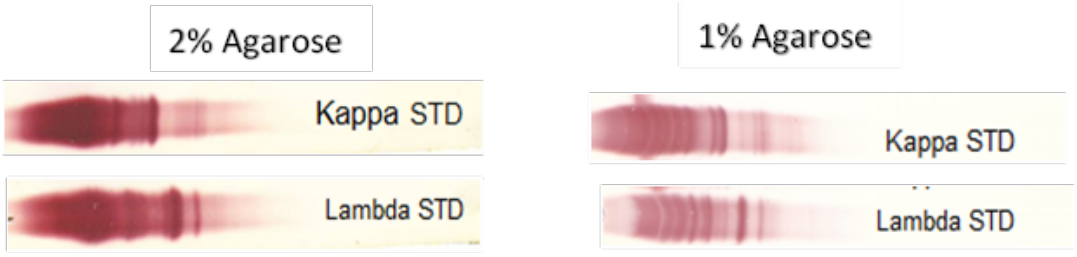


***Figure 29**. Scanned images of the IBs produced following IEF of quality control samples containing FLCs. Two different gels were used; 1% agarose (right) and 2% agarose (left). STD= standard.*

The migration and separation of Kappa and Lambda FLC bands appears to be better using the 1% agarose gel.

Even before analysis of the results, it was found that the 2% agarose gel was much more difficult to pour and spread compared to the standard 1% OCB gel. Any further increase in the level of agarose would make the gel very difficult to spread evenly, especially when produced in-house. The images in figure 29 show that the 2% gel appears to hinder migration of FLCs so that the bands are not fully separated and discreet as compared to the 1% agarose-based gel. As a result, the bands seem over-focused on the 2% gel. The 1% agarose gel was selected as standard for all further experiments.

6.1.3 Choice of ampholytes to establish a pH gradient.

The use of various ampholytes were explored to establish an optimal pH gradient in the CIS gel for precise separation and focussing of monoclonal FLCs. Carrier ampholytes are mixtures of around 600-700 low molecular weight (<1kDa) amphoteric compounds, such as amino acids, with a range of isoelectric points between pH 3-10. They possess a high buffering capacity at their respective isoelectric points and when an electric field is applied to them, they form a pH gradient. Within a protein electrophoresis gel system, they serve to improve resolution by 'carrying' migrating species to their respective points of neutrality. However, they do not bind to migrating protein because they are highly hydrophilic (75). In this manner, they can help to separate protein bands that are only 0.1 pH units apart. Ampholine® and Pharmalyte® are commercially available mixtures of carrier ampholytes differing slightly in their chemistries of production. Mixtures with narrow pH intervals are available for even higher resolution and one may select them according to the known range of isoelectric points of the proteins to be separated by IEF.

The isoelectric point of monoclonal FLCs can range from pH ~4.5 to 8.5 (73, 76). In one study, the mean pI for myeloma related FLCs was found to be ~6.2 and amyloid FLC was pH 4.8 (74). However, a review of the literature suggests that the pI range coverage in order to capture all possible migrating monoclonal FLCs (including free, dimers & aggregates) is pH 3-10 (77, 78).

In order to find the optimal ampholyte mixture, experiments were conducted using the following samples:

- Two MM patient samples-with involved FLCs of around 160-180mg/L-were selected from the CIS clinical archive. Sample 1 had an IgG λ (Gλ) m-protein. and sample 2 had an IgG κ (Gκ) m-protein The total κ, λ and IgG concentrations in both samples were quantified by turbidimetry on the Roche Cobas 6000 chemistry platform (see table 30 below).

***Table 30**. Serology results obtained using the Roche Cobas 6000 analyser for two MM samples chosen for the ampholyte experiments.*

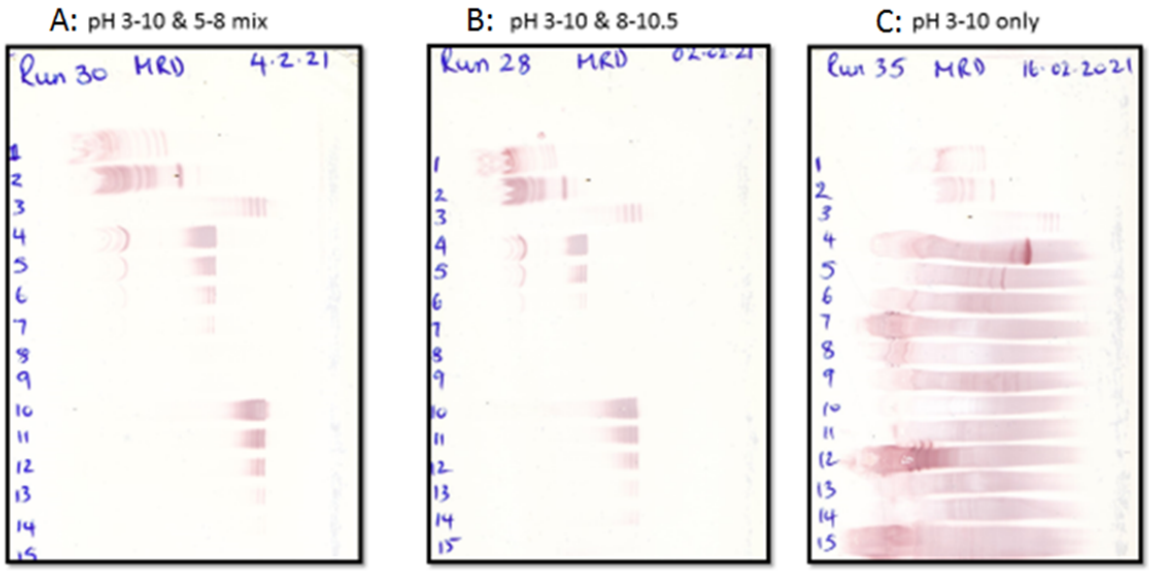
Sample No.	m-protein type	Free κ (mg/l)	Free λ (mg/l)	IgG (g/l)
1	Gλ	9	180	36
2	Gκ	158	11	29

- From the above mentioned two samples, a 1/100 dilution was made first for each. From the 1/100 dilutions, 1/3 serial dilutions were made ranging from 1/300 to 1/24,300 (Diluent: 2% BSA in PBS).
- Three diluted quality control samples, with known m-protein levels, were also selected; FLC controls 1a, 1b (see table 7), and also a Gκ control (1c).

In total, fifteen samples (six dilutions of sample 1, six dilutions of sample 2 and three controls) were applied to each of the following three IEF gels infused with:

A. A mixture containing primarily pH 3-10 (2 ml) with pH 5-8 (0.5 ml) Pharmalyte,
B. A mixture containing primarily pH 3-10 (2 ml) plus pH 8-10.5 (0.5 ml) Pharmalyte (as used in the OCB method).
C. Pharmalyte pH 3-10 alone,

The normal OCB IEF/IB protocol was applied to each gel except that each gel was probed with a combination of anti-free κ & λ LC antibodies (2a-d, table 7) before secondary antibody (5a, table 7) incubation. See figure 30 below for results.



Sample key

Position No.	Sample ID
1	κ FLC control
2	λ FLC control
3	Gκ control
4 **Sample 1**	1/100
5	1/300
6	1/900
7	1/2700
8	1/8700
9	1/24300
10 **Sample 2**	1/100
11	1/300
12	1/900
13	1/2700
14	1/8700
15	1/24300

***Figure 30**. Scanned images of the IBs produced after the same 15 samples were applied to three different gels (A, B & C) infused with various mixtures of ampholytes as stated above each gel. The sample key below the images shows the order and ID of each protein trace labelled 1 to 15.*

Sample 1 was still detectable at the 1/2700 in gel A (position 7) but is only detectable until the 1/900 dilution in gel B (position 6). Sample 2 was detectable down to the 1/2700 dilution and in gel A (position 13) but was detectable down to 1/8700 in gel B (position 14).

The blots pictured in figure 30 show that when using pH 3-10 ampholytes alone in the gel preparation (gel C), protein separation is incomplete and focussing of monoclonal bands is relatively poor. Resolution was much better and comparable between gels A and B. Gel A had a superior sensitivity for sample 1; monoclonal λ FLCs were detectable down to around a concentration of 0.06 mg/L (1/2700 dilution of 180mg/L) in gel A, compared to 0.2 mg/L in gel B. However, gel B had a superior sensitivity for sample 2; monoclonal κ FLCs were detectable down to around 0.06 mg/L (1/2700 of 158 mg/L neat) in gel A, compared to 0.02 mg/L in gel B. Protein banding was more visible and discreet overall in gel A compared to gel B (See positions no. 1, 2, 4 and 11).

Although, mixture A demonstrated superior focussing overall and a higher sensitivity at least for the λ FLCs in sample 1, it was decided that mixture B would be chosen instead for all subsequent experiments working up to the final hsFLC protocol. The reason for this was partly due to the proven track record of mixture B in the OCB method and also with a view of maintaining standardisation of reagents across assays. Mixture A would potentially be more suited to resolving & focussing known monoclonal FLCs that migrate within a narrow range of 5-8 pH units. Whereas one of the primary objectives of the hsFLC test was to screen for a wide spectrum of all possible monoclonal FLCs found in MM patients.

6.1.4 Dilution of sample into analytical grade water vs 2% BSA (PBS)

Protein is known to bind non-specifically to hydrophobic surfaces and protein loss during storage is especially significant when working with very small concentrations (<0.1mg/L), such as in MRD. Various approaches to minimise protein loss during storage have been described in the literature. These include using tubes coated with polyethylene glycol (PEG) or siliconizing agents (79). A much more direct and cost-effective approach is to consider the liquid medium in which the samples are to be stored. In this respect, the constituents of an ideal diluent would non-specifically bind (coat) the walls of the sample container but interfere minimally with the sample itself. A solution containing 2% Bovine-serum albumin (BSA) in phosphate buffered saline (PBS) can be used as a sample diluent to minimise non-specific aggregation of FLCs and absorption loss to the walls of plastic sample containers (79, 80).

In the OCB protocol, serum samples are pre-diluted to bring the total protein down to around the level of that usually found in CSF (~0.5 g/L). The diluent used is ultrapure (Type 2), analytical grade (miliQ®) water. An investigation was carried out to ascertain whether this diluent was also suitable for the analysis of very low levels of FLCs; a comparison of IEF results was made between serum diluted in the following two diluents:

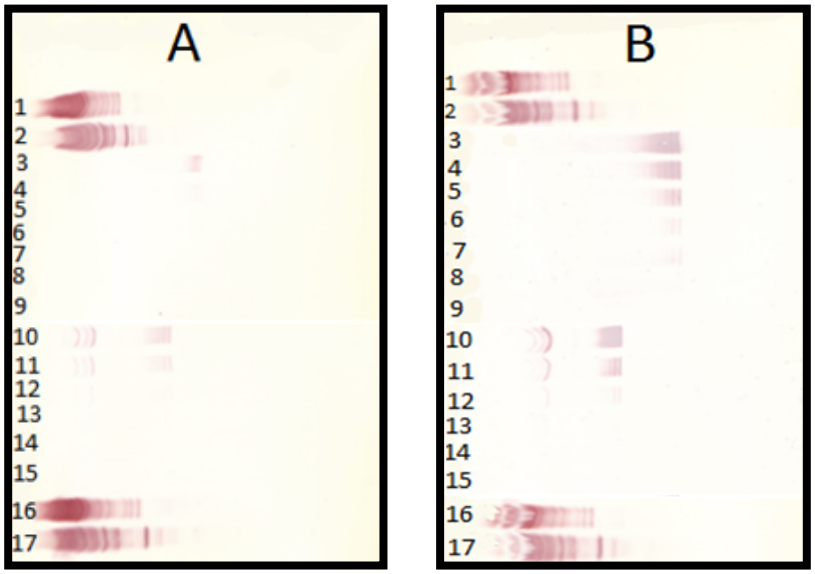
A. Ultrapure, Type 2, analytical grade (miliQ®) H_2O

B. 2% BSA in PBS.

The same two patient samples from the ampholyte experiments (section 6.1.3) were selected. i.e., Sample 1 (Gλ) and sample 2(Gκ). Two identical sets of dilutions were made from these using diluent A firstly, and then diluent B. The dilutions were made in the same manner as before; six dilutions ranging from 1/100 to 1/24300. FLC controls 1a and 1b (see section 2.2.1, table 7) were also used to check performance. The normal OCB IEF/IB protocol was applied to each gel except that each gel was

probed with a combination of anti-free κ & λ LC antibodies (2a-d, table 7) before secondary antibody (5a, table 7) incubation. Figure 31 below shows the differences in results between samples diluted in H2O (Gel A) and 2% BSA (Gel B).

A: Analytical grade H_2O B: 2% BSA/PBS



Sample key

Position No.	Sample ID
1	κ FLC control
2	λ FLC control
3 **Sample 1**	1/100
4	1/300
5	1/900
6	1/2700
7	1/8700
8	1/24300
9	BLANK
10 **Sample 2**	1/100
11	1/300
12	1/900
13	1/2700
14	1/8700
15	1/24300
16	κ FLC control
17	λ FLC control

Figure 31. *Scanned images of the IBs produced when using two different sample diluents; A: H2O & B: 2% BSA/PBS to make up six dilutions each of sample 1 and sample 2. FLC control samples were also run at the top and bottom of each gel. A total of 17 samples were run in the order shown in the sample key on the right of the images.*

Sample 1 can only be seen at the 1/100 dilution in gel A (position 3) but is still detectable down to the 1/8700 dilution in gel B (position 7). Sample 2 is detectable at 1/300 in gel A (position 11) but is still detectable down to the 1/900 dilution in gel B (position 12).

The FLC bands in the control samples (top and bottom) appear more distinctly in gel B. (top; position 1 and 2, bottom; 16 and 17)

The results from figure 31 demonstrated that the dilution of samples into 2% BSA (PBS) appeared to minimise protein loss (during sample storage) when compared to using H_2O as a sample diluent. Sample 1 was only detectable down to around 1.8 mg/L in gel A, whilst it remained detectable down to around 0.06 mg/L in gel B. Thus, in the context of MRD analysis, there appeared to be significant loss of protein when using H_2O as a diluent. The results also show that the BSA diluent seemed to have had a stabilising effect on the migration of the control samples. In gel A, the control sample traces (both top and bottom) appear compressed compared to control results in gel B. A closer look at gel A in isolation, reveals differences between the corresponding control traces at either end of the gel. Whereas in gel B, the control traces appear more uniform at both ends. It was therefore decided that all subsequent experiments would be performed using 2% BSA in PBS as the sample diluent.

6.1.5 Sample application and loading volume.

The standard applicator template (used to apply samples to the gel for the CIS OCB method) allows 33 samples to be loaded onto the gel. Each well is 3 mm^2 in size and approximately 0.5 mm in depth. Each well can hold up to 10 µl of sample and the distance between each well is 3 mm. The difference between loading 5 µl of sample to each well versus 10 µL of sample was assessed.

A trial applicator was also tested that had wells much closer together and therefore allowed up to 45 samples to be loaded onto the CIS gel. The size of each well was 5 mm in length and 2 mm wide. The depth of each well was approximately 2 mm and could hold up to 20 µl of sample. Using this applicator, various experiments were performed to assess the difference between using 5, 10 and 20 µl of sample and the results were compared with the standard applicator mentioned above for any qualitative advantage.

The same two samples (Gκ& Gλ) from section 6.1.3, were diluted at 1/100 using 2% BSA (in PBS). From the 1/100 dilutions, serial 1/3 dilutions were made down to 1/24300 and then tested on two gels. On one gel the standard OCB sample applicator (A) was used and on the other gel, the trial applicator (B) was used. Different sample volumes were used on each to identify the optimum volume of sample loading. The normal OCB IEF/IB protocol was applied to each gel thereafter except that each gel was probed with a combination of anti-free κ & λ LC antibodies (2a-d, table 7) before secondary antibody (5a, table 7) incubation. See Figure 32 below for results.

Sample key for gel A

A: Standard applicator

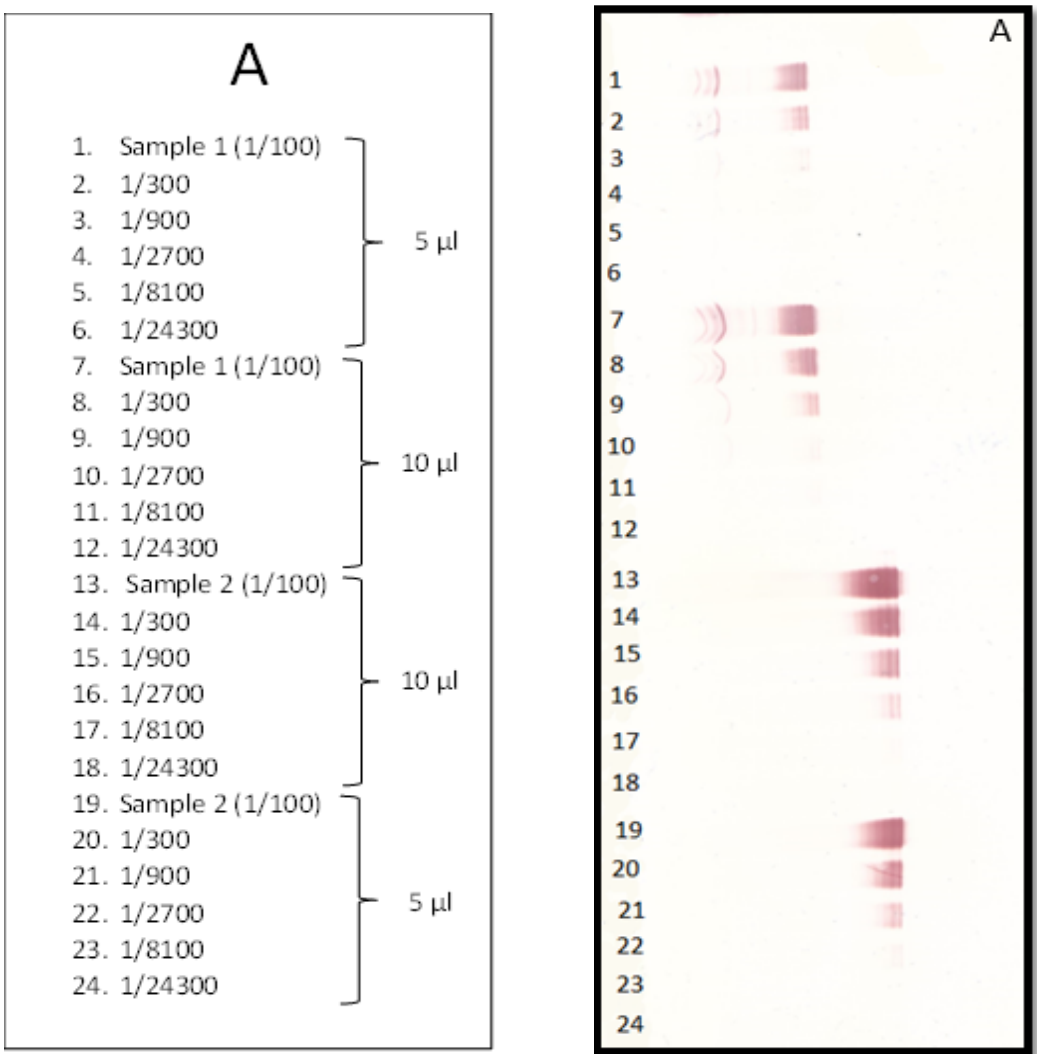
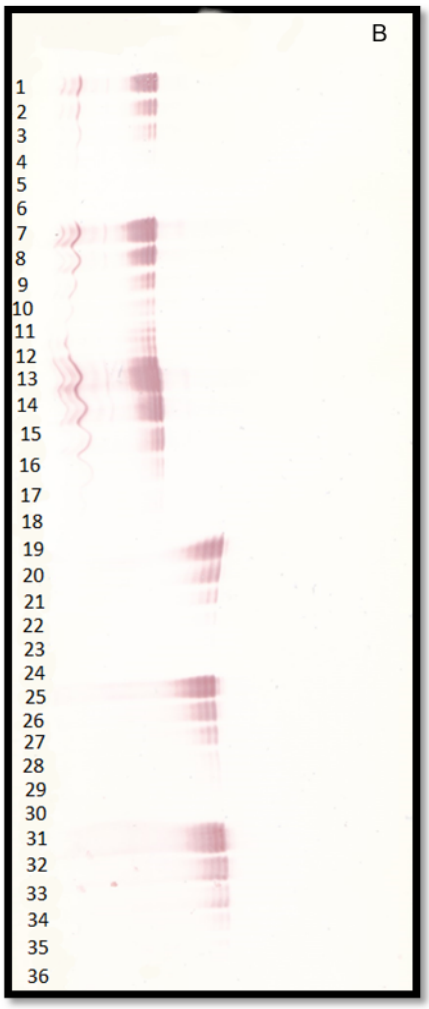


Figure 32 *(1/2).*

B: Trial applicator



Sample key for gel B

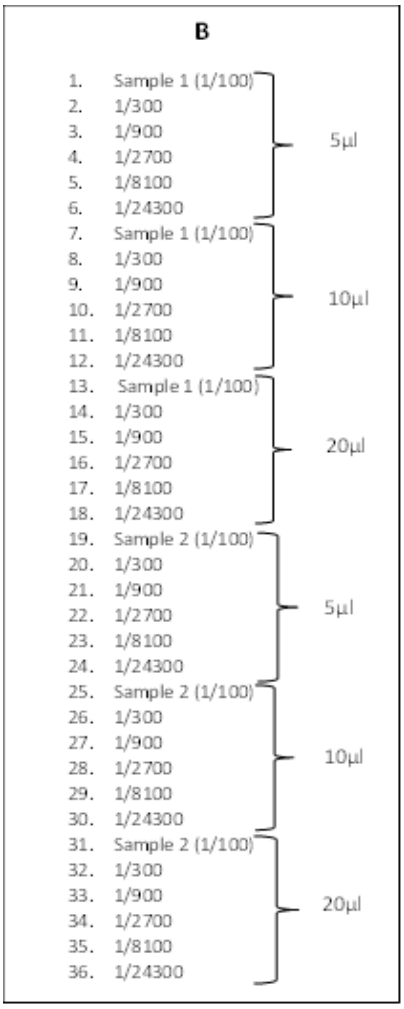


***Figure 32** (2/2). Scanned images of the IBs produced using two different sample applicators: A: standard applicator and B: trial applicator. Various sample volumes from 5-20µL were also tested. 24 samples were run on gel A in the order shown in the sample key on the right of the image. 36 samples were run on gel B in the order shown in the sample key to the left of the image.*

In gel A (standard applicator): Sample 1 was detectable at around 1/900 (position 3) when using 5µl but was still detectable at around 1/2700 (Position 10) when using 10µL. Sample 2 was detectable at round 1/900 (position 21) when using 5µl but was still detectable at around 1/2700 (Position 16) when using 10µL.

In gel B (trial applicator): Sample 1 was detectable at around 1/900 (position 3) when using 5µl but was still detectable at around 1/2700 (Position 10) when using 10µL and 1/2700 (Position 16) when using 20µL. Sample 2 was detectable at around 1/900 (position 21) when using 5µl but was still detectable at around 1/2700 (Position 28) when using 10µL and 1/2700 (Position 34) when using 20µL.

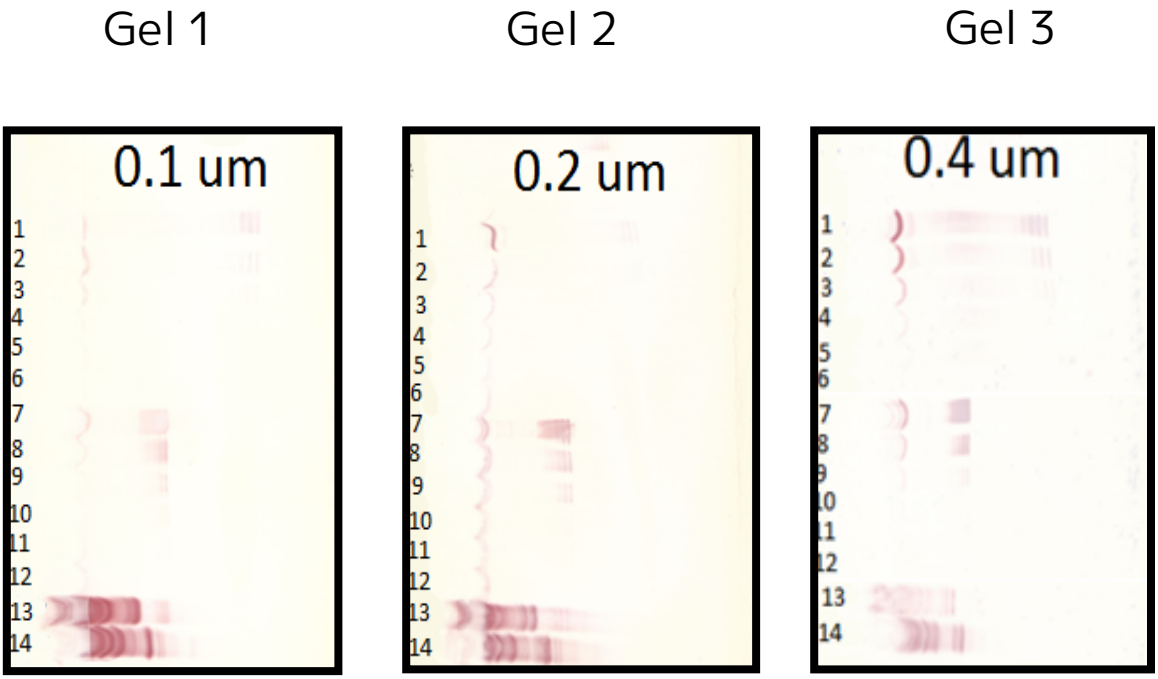
The results from both gels A and B shown in figure 32 demonstrated that increasing the sample volume from 5 to 10µl increased the sensitivity of detection as expected. A further increase to 20µl sample volume in gel B did not seem to improve sensitivity significantly. Instead, there seemed to be an increased tendency of samples to leak into co-migrating lanes (Positions 11 to 15) when using 20µl, especially as the wells were a lot closer together in applicator B. Subsequently, it was decided that Applicator B was surplus to requirements. In other similar experiments (not detailed here), using 10µL on the standard applicator seemed to be linked with increased sample cross-over between lanes during migration. It was therefore decided that 5µl would be the standard volume of application for all subsequent experiments.

6.1.6 Choice of blotting membrane and pressing time - Nitrocellulose versus PVDF

In the OCB method, nitrocellulose membrane (NCM) is used to extract the proteins from the gel following IEF. The pore size of the NCM used is 0.4 µm. This is well suited to large proteins, such as IgG which is approximately 150 kDa in molecular weight. Monomeric FLCs are around ~22 kDa in size. They can form dimers (~44 kDa) and they can also polymerise or aggregate to form even larger molecules (73). Transfer membranes of various pore sizes were assessed to establish the best size for maximal transfer and retention of FLCs. The optimal size would also limit the transfer of undesirable non-specific proteins. The following pore-sizes were investigated:

a) 0.1 µm
b) 0.2 µm
c) 0.4 µm

The same two samples (Gκ& Gλ) from section 6.1.3, were diluted at 1/100 using 2% BSA (in PBS). From the 1/100 dilutions, serial 1/3 dilutions were made down to 1/24300 and then tested using the three different NCM membranes mentioned above. FLC controls 1a and 1b (see table 7) were also used to check performance. Besides the differences in NCM used for transfer, the normal OCB IEF/IB protocol was applied to each gel except that each gel was probed with a combination of anti-free κ & λ LC antibodies (2a-d, table 7) before secondary antibody (5a, table 7) incubation. See Figure 33 below for results.



Sample key

Position No.	Sample ID
1 **Sample 1**	1/100
2	1/300
3	1/900
4	1/2700
5	1/8700
6	1/24300
7 **Sample 2**	1/100
8	1/300
9	1/900
10	1/2700
11	1/8700
12	1/24300
13	κ FLC control
14	λ FLC control

Figure 33*. Scanned images of the IBs produced when using NCM with three different pore sizes: Gel 1- 0.1µm, gel 2- 0.2 µm and gel 3- 0.4µm. A total of 14 samples were run on each gel in the order shown in the sample key below the images.*

Sample 1 is detectable at: 1/900 (position 3) in gel 1, at 1/100 (position 1) in gel 2 and at 1/2700 (position 4) in gel 3.

Sample 2 is detectable at: 1/900 (Position 9) in gel 1, at 1/900 (position 9) in gel 2 and at 1/900 (position 9) in gel 3.

Figure 33 shows how optimal sensitivity and band resolution was achieved using 0.4 µm NCM as compared to the other pore sizes. The FLC controls also seemed to be over focussed on the other membranes possibly due to hindered migration.

In other membrane related experiments (only summarised here), pure Nitrocellulose membranes from two different manufactures were tested. One of them was pre-infused with an inert polymer for improved handing. No significant difference between NCM produced by different manufacturers was found. The effect of moistening the membrane prior to transfer was also assessed. The literature mentions using 10% methanol and deionised water to moisten the membrane before transfer. It was found that, pre-wetting the membrane before protein transfer seemed to encourage bubble formation during the pressing step resulting in poor protein transfer. Polyvinylidene difluoride (PVDF) membrane was also assessed to see if it had any advantage over nitrocellulose. Results from PVDF experiments demonstrated that PVDF membrane was very difficult to handle, more sensitive to damage and transfer was very poor as compared to NCM. After all these investigations, the transfer membrane agreed upon for all further experiments was 0.4 µm NCM.

6.1.7 Blocking methods

Blocking non-specific sites on the transfer membrane is an essential step in western blotting to minimise loss of the probing antibody to unwanted sites and to avoid a high background signal. Any protein solution can be used as a blocking agent provided it does not have significant affinity for the target antigen or the probing antibody. The concentration of blocking agent used, and the blocking time are also both important; too little will result in a high background and too much will interfere with the probing antibody to target antigen complex formation. Excessive blocking protein can also result in displacement of the blotted protein from the membrane. Displacement it this way can also be minimised by adjusting the pH of the blocking solution. For instance, an acidic pH is reported to be most ideal when using milk protein as a blocking solution (81). The CIS currently uses a 5% non-fat milk solution (in PBS)-for one hour-as blocking agent in the OCB method because it contains an ideal level of protein, is inexpensive and very easy to prepare. The effect of decreasing the pH on FLC binding to NCM is yet to be verified.

Following blocking, it is still important to use some milk protein in the antibody incubation steps. This is so because non-specifically bound blocking protein tends to dissociate easily from the NCM exposing non-specific sites once again. A small amount of milk protein is therefore necessary to keep the antibody in solution long enough to bind to the specific target antigenic sites. It was anticipated that the same blocking protocol from the OCB method would be used for hsFLC as the lack of background interference achieved by it was already very satisfactory. Nevertheless, four experiments were carried out to investigate the effects of decreasing the protein level from 5% and also the effect of using 2% BSA instead of milk.

The following samples were used for each experiment:

a) A control sample with an IgG κ m-protein (See table 7, 1c) diluted 1 in 4 into 2% BSA (in PBS).

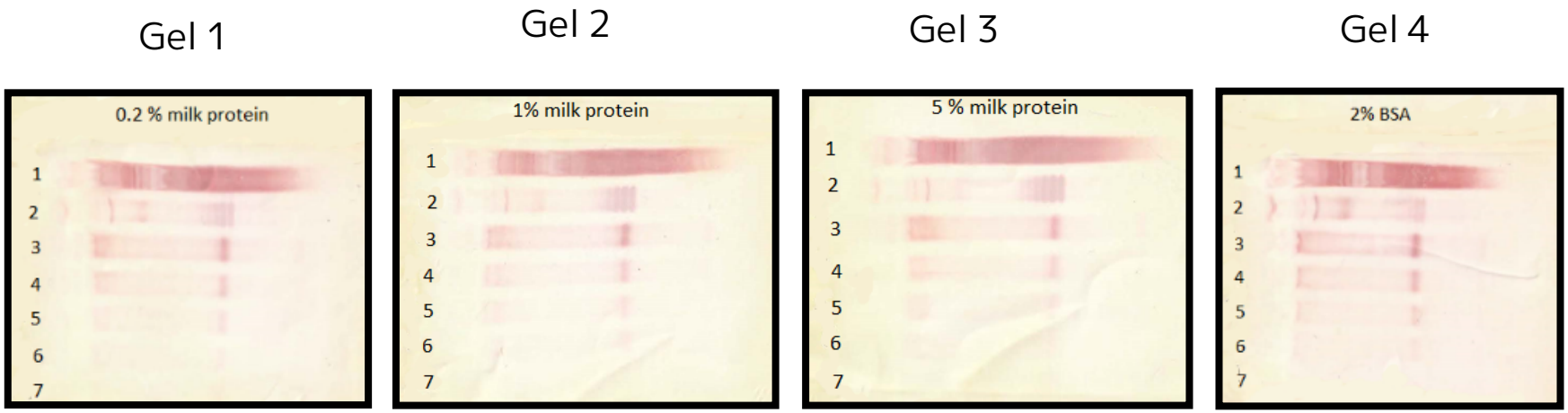
b) The same stock κ control (1c) diluted 1 in 4 into normal human serum (NHS). The reason for diluting into NHS was to reduce the m-protein level to a suitably detectable level whilst maintaining a normal representative polyclonal background. In this way, the loss of polyclonal FLCs in various blocking solutions could also be assessed.

c) Sample A (containing a free κ LC-only m-protein at 128 mg/l) diluted out (in 2% BSA) in 5 serial dilutions ranging from 1/256 (0.5 mg/l) to 1/4096 (0.03 mg/l) L

Four IEF/IB experiments were conducted using the following four blocking solutions:

1. 0.2% milk protein
2. 1 % milk protein
3. 5 % milk protein
4. 2% BSA

The protocol was the same otherwise as for the OCB method except that all blots were probed with FLC κ antibodies, (See table 7, 2a-b,) then secondary antibody (see table 7, 5a).



Sample key

Position No.	Sample ID
1	GK control (in NHS)
2	GK control (in BSA)
3 **Sample A**	1/256
4	1/512
5	1/1024
6	1/2048
7	1/4096

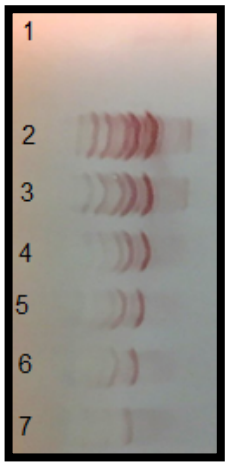
Figure 34. *Scanned images of the IBs produced when using four different blocking solutions; 0.2 % milk protein (gel 1), 1% milk protein (gel 2, 5 % milk protein (gel 3) and 2% BSA (gel 4). A total of 7 samples were run on each gel in the order shown in the sample key below the images.*

The GK control diluted in NHS (position 1) cannot be seen clearly due to the high polyclonal background. However, when diluted in BSA (position 2), monoclonal bands are visible, most clearly in gel 3. Sample A is detectable down to the 1/2048 dilution (position 6) in gels 1-4.

Looking at figure 34, the level of polyclonal background was far too high in position 1. NHS would need to be pre-diluted itself in 2% BSA to reduce the polyclonal background before using it as a diluent in subsequent experiments. It was suggested that around 5mg/l of polyclonal FLCs would be sufficiently representative of the background level normally found in treated MM patients. In this way, the detectability of monoclonal bands upon a polyclonal background could be assessed. In position 2, free κ LC monoclonal bands were easily seen in on all gels in the absence of a strong polyclonal background. However, on gel 4 blocked with BSA, less monoclonal bands could be seen in position 2 compared to the other 3 gels that were all blocked using milk protein. For sample A, there was not really any significant difference in sensitivities achieved between the four blots. The impact of reducing the volume of blocking agent could have also been assessed for example, from 100ml as in the OCB method to 50 ml, as a strategy to minimise displacement of the target protein from the blot. In the meanwhile, it was decided to continue using 100ml of 5% milk protein in PBS as the blocking agent to ensure minimal background staining.

6.1.8 Theoretical disintegration of polyclonal IMGs

One concern or doubt about using the IEF method to detect monoclonal FLCs was the potential for polyclonal IMGs to disintegrate into FLCs owing to the high electromotive force applied during migration. This would potentially manifest as artefactual bands. Theoretically, even if this did occur, the FLCs should still migrate as a polyclonal smear rather than focussed bands owing to their heterogeneity. In any case, to dispel this doubt, NHS was tested alone by IEF and retested after spiking it (1:1) with a sample containing monoclonal λ FLCs (sample X) at 20mg/L (quantified by turbidimetry on the Roche Cobas 6000). NHS was tested again with five doubling dilutions of Sample X from ¼ to 1/24. The IEF/IB protocol was the same as for the OCB method except that the blot was probed with FLC λ antibodies only, (See table7, 2c-d) then secondary antibody (see table 7, 5a). The results are shown below in figure 35.



Sample key

Position No.	Sample ID	λ FLC (mg/L)
1	NHS	10.0
2	NHS (Plus sample X)	20.0
3	**Sample X** 1/4 (plus NHS)	5.0
4	1/8 (plus NHS)	1.2
5	1/16 (plus NHS)	0.3
6	1/20 (plus NHS)	0.08
7	1/24 (plus NHS)	0.02

***Figure 35**. A scanned image of the IB produced following IEF of NHS and NHS spiked with m-protein (sample X). Sample X was then serially diluted 5 times in 2% BSA and each dilution was added to NHS (1:1). The sample key shows the λ FLC concentration of each sample quantified using the Roche Cobas 6000.*

A polyclonal smear can just be seen in the NHS sample trace (position 1, right; less visible on the scanned image). The same polyclonal NHS trace is visible in all subsequent positions 2 to 7. The λ monoclonal bands of sample X are still visible at the 1/24 dilution (0.02mg/L).

Figure 35 shows no that no artefactual monoclonal bands were seen in the NHS trace. Yet, when the same sample was pooled with another sample that had monoclonal lambda m-protein, clear discreet bands were seen across all the dilutions even down to 0.02mg/L of m-protein. (Lane 2 to 7). The same polyclonal smear is visible in all 7 samples owing to NHS. Other similar experiments also showed that the IEF procedure was not fragmenting whole immunoglobulins into FLCs by virtue of the high voltage of separation.

6.1.9 Concentration of mouse monoclonal antibodies, conjugates and substrate etc.

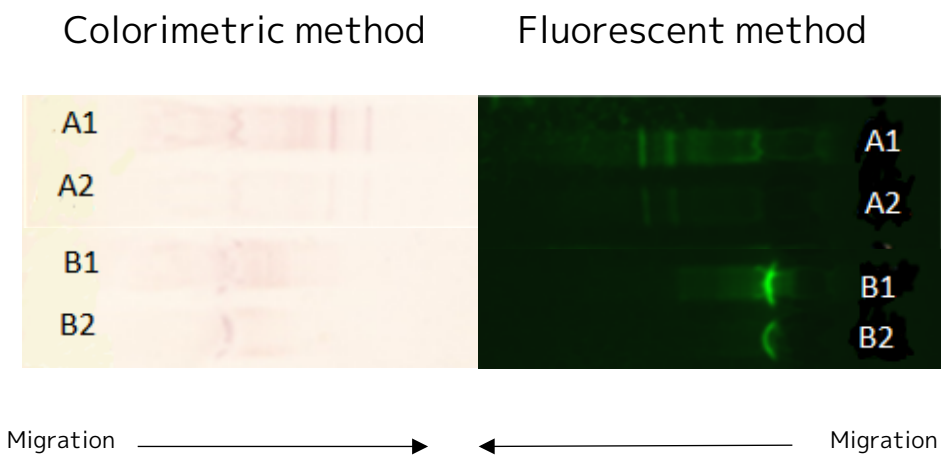
Extensive work has already been carried out within the CIS related to the optimisation of the selected mouse monoclonal antibodies used for the detection of SFLCs (see table 7, 2a-d). Indeed, much data has already been published demonstrating the clinical utility of these specific antibodies (82). In fact, these antibodies have already been analytically validated for real clinical and commercial applications (83, 84). The acceptability of the combined use of κ and λ specific antibodies in a single assay can also be traced back to these publications.

The objective henceforth was simply to find the optimal concentration of the monoclonals to achieve maximum sensitivity for the hsFLC method. The secondary antibody used throughout was a polyclonal goat anti-mouse-HRP conjugate (See table 7, 5a). The use of alternate enzyme conjugates and substrates (for example, to produce blue staining as opposed to red) could still be investigated in future ultra-refinements of the protocol.

The availability of modern next generation western blot improvements is well publicised (85). Advanced imaging and interpretation software would easily allow quantification of hsFLC results. However, the focus of this work to begin with was to take advantage of the sheer number of samples from various cohorts possessed by the CIS as an MM clinical trials unit to test the basic application of the test principle. Collaboration with commercial companies is ongoing nevertheless, as they are more suited to investigate technological refinements once the method receives expert acceptance.

In a single experiment, the use of fluorescent conjugates instead of colorimetric markers was briefly investigated. Two samples were selected: one with λ FLC only (sample A) and the other with κ FLC only m-protein (sample B). Two dilutions were made of each sample; the ¼ dilutions were called sample A1 and B1, respectively and the 1/16 dilutions were called sample A2 and B2, respectively. The normal IEF/IB OCB protocol was used to analyse them except that the blot was probed with a

combination of anti-free κ & λ LC antibodies (2a-d, table 7). Then after primary antibody incubation, a fluorescent probe was used (see section 2.2.1, table 7, 5b for antibody). Once the fluorescence was measured (using a special fluorescence reader), the blot was stripped and incubated with secondary antibody-HRP conjugate (5a, table 7) and measured colorimetrically in the usual way. See figure 36 below for results.



Sample key

Sample ID	Dilution	M-protein
A1	1/4	λ FLCs
A2	1/16	
B1	1/4	κ FLCs
B2	1/16	

***Figure 36**. Scanned images of the same IB stained by the standard colorimetric method (left) and by the fluorescent method (right). The key below the images shows how samples A and B were diluted. The direction of migration is in opposite directions in the two images.*

Sample A results are very similar by both methods. The monoclonal bands in Sample B are more distinct using fluorescence.

Figure 36 shows good reproducibility of results between the colorimetric method and the fluorescent method. In fact, the fluorescent conjugate appeared to enhance the visibility of the bands even further in most cases (only two examples are shown above) and could also be considered in the future. However, the colorimetric method was chosen for the final protocol because it was more cost effective.