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Research Article

Towards Diagnostic ctDNA Testing in Multiple Myeloma: How does Automated Magnetic Bead-Based Cell-Free DNA Extraction Compare to the Leading Silica Membrane-Based Manual Extraction?

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Abstract

Introduction: Diagnosis and monitoring of haematological malignancies rely heavily on bone marrow biopsies. Less invasive peripheral blood biopsies are an emerging alternative, involving isolation of cell-free DNA (cfDNA) and circulating tumour DNA (ctDNA) as a source of tumour information. To date, the Qiagen QIAamp Circulating Nucleic Acid kit is considered the gold standard for cfDNA isolation. However, it is time-consuming, labour intensive and relatively costly. To move ctDNA analysis into a diagnostic setting, standardisation by partially automating cfDNA extraction is highly desirable. This study revisited cfDNA extraction in multiple myeloma, a haematological cancer potentially shedding relatively high amounts of ctDNA. **Methods:** Four plasma samples from three myeloma patients carrying the NRASQ61R mutation and six healthy controls were used in this study. cfDNA was isolated from plasma with the manual method, Qiagen QIAamp Circulating Nucleic Acid kit and the automated Promega RSC Maxwell ccfDNA kit. The cfDNA yields were assessed and ctDNA (NRAS) detection compared by droplet digital PCR (ddPCR). **Results:** Although the Promega kit was more convenient, ctDNA detection was more sensitive with the Qiagen kit. Indeed, ddPCR successfully detected low NRAS mutant load in Qiagen extracts while only high NRAS mutant load was confidently detected in Promega extracts. **Conclusion:** The Promega kit is easier to use and more economical, but the Qiagen kit yielded significantly higher amounts of cfDNA. Moreover, critically low patient ctDNA concentration was only detectable using the Qiagen kit. This supports the superiority of the Qiagen kit for monitoring minimal residual disease.

Keywords: Multiple myeloma; Cell free DNA (cfDNA); Circulating tumour DNA (ctDNA); Droplet digital PCR (ddPCR); $NRAS^{Q61R}$

Introduction

Multiple Myeloma (MM) is an incurable blood cancer which involves the uncontrolled proliferation of plasma cells in the bone marrow and affects mostly the elderly (median age of 69 years at diagnosis). Bone marrow biopsy has so far been the gold standard in the histological diagnosis of MM while detection of structural chromosome abnormalities by fluorescence in situ hybridization or microarray on CD138-selected plasma cells is the standard of care.

In haematological malignancies, clinicians have easy access to tumour cells from peripheral blood and bone marrow samples. However, bone marrow biopsies have significant limitations. Bone marrow biopsies are invasive procedures that cause much stress and discomfort in patients. They require technical expertise and are considerably less accessible than peripheral blood. Another limitation of a bone marrow biopsy, pertinent to myeloma, is sampling bias. MM has a patchy distribution in the bone marrow with plasma cells growing in clusters or nodules throughout the skeleton. Therefore, a biopsy collected at a single site may not reflect the complete picture disease [1,2]. Indeed, within a single patient, MM is clonally and spatially heterogeneous with varying genetic abnormalities and malignant potential, thus challenging the monitoring of Minimal Residual Disease (MRD) [3].

For the analysis of myeloma biomarkers, blood biopsies are emerging as an alternative source of genetic information that could reduce the need for bone marrow biopsies [4]. Blood contains cell-free DNA (cfDNA) and within that a proportion is tumour cell derived and referred to as circulating tumour DNA (ctDNA). Although initially reported in late 1940 by Mandel and Métais [5], interest in cfDNA and ctDNA has most rapidly increased over the past two decades [6] and major work has been undertaken in solid tumours such as breast [7-9], colorectal [10-12] and lung [13-16] cancers.

Despite the fact that the main source of cfDNA are the hematopoietic cells [17-19], in MM, interest in liquid biopsies has only spiked in the second half of the last decade. In a haematological cancer such as MM, the over-proliferation of malignant plasma cells results in the release of high concentrations of ctDNA into the blood stream [4]. Therefore, ctDNA from blood biopsies could overcome the issues associated with bone marrow biopsies in clonally diverse MM. Most notably, some genetic aberrations are only detectable in cfDNA, demonstrating that cfDNA indeed better captures heterogeneity [20,21].

Technical considerations are important to develop strategies to translate ctDNA analysis in MM into a diagnostic and clinical setting. To date there are a number of commercial kits available for the isolation of cfDNA. The strong interest in ctDNA analysis is exemplified by several studies that have tested many commercial kits and mostly agree on one clear frontrunner: the QIAamp Circulating Nucleic Acid Kit [15,22-24]. However, cfDNA extraction with this kit is relatively costly and labour intensive. A more automated approach to cfDNA extraction is desirable for implementation in a diagnostic laboratory. The Maxwell® RSC Instrument offers an automated approach as it is supplied with a pre-programmed purification protocol for isolation of cfDNA using pre-dispensed reagent cartridges. Therefore, we decided to revisit kit comparison to evaluate the potentially less labour intensive and scalable to medium throughput Maxwell® RSC Instrument/ccfDNA Plasma Kit (Kit P) and the generally used, manual method, QIAamp Circulating Nucleic Acid Kit (Kit Q). The focus on MM in this study is due to our clinical and research interest in this cancer. Additionally, unlike the leukemic blasts, myeloma cells are mostly confined to the bone marrow, with few cells escaping into the blood stream depending on disease stage or to invade other areas of the bone marrow to create these patchy niches characteristic of MM. Therefore, the application of liquid biopsy in MM has high potential to overcome sampling bias and heterogeneity issues.

Materials and Methods

Patients

This comparison study is part of a larger liquid biopsy feasibility study in multiple myeloma, which has been approved by the South Western Sydney Local Health District's Research and Ethics Office (Project number 13/097, HREC/13/LPOOL/158). Written consent was obtained prior to peripheral blood collection from MM patients and healthy participants. Four plasma samples from three MM patients and six healthy participants were used. The MM patients had previously been screened for and shown to carry the NM_002524.5(NRAS):c.182A>G (p.Gln61Arg) variant, referred to as $NRAS^{Q61R}$ in this study. The MM patients in this cohort were at different disease stages at the time of sample collection, with one newly diagnosed patient blood drawn prior to commencement of any therapy, one in remission following autologous bone marrow transplantation, and one relapsed patient initiated on a new treatment regimen. There were two consecutive samples collected from the relapsed patient, 4 weeks apart.

Sample collection

Nine mL of peripheral blood was collected in EDTA vacutainers. Plasma was separated within four hours of collection using a two-step centrifugation protocol as follows: initial centrifugation at 800 x g (10 min, 4°C), followed by centrifugation at 16 000 rpm (5 min, 4°C). Extracted plasma was stored frozen at -80°C until cfDNA isolation and thawed at room temperature

before cfDNA isolation.

Cell-free DNA extraction using the Qiagen Circulating Nucleic Acid Kit (Qiagen, Australia; product no.: 55114)

One to three mL of frozen plasma was thawed and cfDNA extracted according to the manufacturer's protocol on a QIAVac 24 manifold (Qiagen, Australia). cfDNA was collected via double elution from the column in 35µL elution buffer for each sample.

Cell-free DNA extraction using the Maxwell RSC ccfDNA Plasma Kit (Promega Corporation, USA; product no.: AS1480)

One mL of thawed plasma was used for cfDNA extraction on the Maxwell RSC Instrument (Promega, Australia) following the manufacturer's protocol. cfDNA was eluted in 60 µL of the elution buffer.

Quantitation of cfDNA

cfDNA concentrations were measured in 3µL of eluates using the Qubit dsDNA HS Assay kit on a Qubit 3.0 Fluorometer (Life Technologies, Australia) according to the manufacturer's instructions. cfDNA concentrations in ng/µL were normalised to 1 mL of input plasma for comparison of extraction yields. Extracted cfDNA samples were stored at 4^{o}C until ddPCR analysis.

Droplet Digital PCR

Detection of the NRASQ61R variant was performed using the QX200 Droplet Digital PCR platform (Bio-Rad, Australia). The primer and probe sequences used for the ddPCR assay were previously generated in our lab and nucleic acids purchased (IDT Inc., Australia), sequences are presented in Table 1.

Nucleotide variant	c.182A>G
Forward primer	5'-ggtgaaacctgtttgttggacatac-3'
Reverse primer	5'-tggtctctcatggcactgtact-3'
Wildtype probe	[hex]-acagctggacaagaag- [bhq1]
Mutant probe	[6fam]-acagctggacgagaag-[bhq1]

Table 1: ddPCR assay details.

The PCR was performed using the 2X ddPCR Supermix for probes with no dUPT (Bio-Rad, Australia), 20µM of forward primer, 20 µM of reverse primer, 10µM of NRAS wild type probe, 10µM of NRAS^{Q61R} mutant probe and 8.8 µL of each cfDNA sample was used per reaction irrespective of the cfDNA concentration. Nuclease-free water was added to a total of 22µL per reaction. The reaction was partitioned into droplets using the QX100 Droplet Generator (Bio-Rad, Australia) before endpoint-PCR was performed with a C1000 Touch Thermal Cycler with 96-deep well reaction module (Bio-Rad, Australia). The PCR conditions were as follows: initial denaturation at 95^{o}C for 10 min, 41 cycles of 94^{o}C for 30s, 53^{o}C for 60s and final denaturation at 98^{o}C for 10 min. Post-PCR data acquisition and analysis were performed with the QX200 Droplet Reader (Bio-Rad, Australia) and QuantaSoft version 1.7.4. The NRAS^{Q61R} mutant genomic DNA used as control in this study was extracted from the melanoma cell line MelRM [25].

Statistical Analysis

Statistical analysis was performed using Graph Pad Prism version 8.2.1. The cfDNA concentrations and total events (mutant + wild type) generated by ddPCR from both extraction kits were compared using Wilcoxon matched-pairs signed rank test.

Results

Comparison of the Qiagen QIAamp Circulating Nucleic Acid Kit (Kit Q) and the Promega Maxwell RSC ccfDNA Plasma Kit (Kit P)

Kit Q consists of a four-step protocol (lyse, bind, wash, elute), carried out using QIAamp mini columns and a QIAVac 24 vacuum manifold, while Kit P is used on the Promega Maxwell RSC Instrument, which provides a fully automated, cartridge-based extraction system for cfDNA. Table 2 summarises the key differences between the cfDNA extraction kits.

Kit features	Kit Q	Kit P
Kit's full name	QIAmp circulating nucleic acids (Qiagen)	Maxwell® RSC ccfDNA Plasma Kit (Promega)
Extraction principle	Silica membrane	Paramagnetic particles
Automated	Partially (the vacuum)	Fully
Preparation and pre-processing	• Thaw plasma if frozen • Buffers and reagents need to be prepared prior to first use of the kit • Proteinase K and Buffer ACL incubation required (30 minutes)	Thaw plasma if frozen
Equipment required	• Heated water bath or heating block at 60°C compatible with 50 mL tubes • Heating block at 56°C compatible with 2 mL collection tubes • Benchtop centrifuge for 2 mL tubes • Vacuum	The Maxwell® RSC Instrument
Maximum number of samples per run	24	16
Run time (manufacturer protocol)	120 minutes for 24 samples	70 minutes for 1-16 samples
Actual run time (in our hands)	2.5 hours for 12 samples (including plasma thawing, heating up water bath and heating block, 30 minutes incubation of the Proteinase K/ ACL buffer/plasma sample mixture	80 mins (including plasma thawing, pipetting plasma into the cartridges and elution buffer into elution tube and shut down of instrument)
Elution volume (µL)	From 20 µL to 150 µL	60 uL loaded into elution tube and 45-50 uL approximately after the run ends
No. of preps per kit	50	48
Cost per sample (list price)	1896 AUD/50 preps = 37.92 AUD	785 AUD/48 preps = 16.35 AUD

Table 2: Comparison of the Kit Q and Kit P for cfDNA extraction from plasma samples.

Quantitation using the Qubit dsDNA HS Assay kit

Extracted cfDNA yields (in ng/µL) are presented in the Supplemental Table 1. The amount of isolated cfDNA varied between the kits and between the samples, ranging from as high as 13.4 ng/µL per 1 mL of plasma for one MM patient to as low as 0.0567 ng/µL per 1 mL of plasma for one healthy control. As expected, the newly diagnosed MM patient had the highest amount of isolated cfDNA per mL of plasma. The other MM samples in this comparison study were from post-treatment patients (either transplant or chemotherapy) and had lower amounts of isolated cfDNA per mL of plasma, regardless of the kit used. The Wilcoxon matched-pairs test showed that the cfDNA yields (ng/µL per mL) were statistically higher with Kit Q ($p=0.019$, Figure 1).

Sample	Disease status	Paraprotein levels (g/L)	Normalised cfDNA concentration (ng/mL plasma) - Kit P	Normalised cfDNA concentration (ng/mL plasma) - Kit Q
S 1	Newly diagnosed MM stage III	11	12.3	13.4
S 2	MM post autologous bone marrow transplant	Not available	0.257	1.99
S 3*	Relapsed myeloma on a new treatment regimen line of treatment	29.	0.321	0.553
S 4*	Relapsed myeloma on a new treatment regimen line of treatment	25	0.26	0.8
HC 1	Healthy control	Not tested	0.159	0.347
HC 2	Healthy control	Not tested	0.192	0.341
HC 3	Healthy control	Not tested	0.088	0.159
HC 4	Healthy control	Not tested	0.0781	0.0567
HC 5	Healthy control	Not tested	0.0372	0.211
HC 6	Healthy control	Not tested	0.161	2.86
S: patient sample, HC healthy control * same patient one month apart				

Supplementary Table 1: Concentrations of plasma cell-free DNA (cfDNA) extracted using the Kit Q and the Kit P 3* and 4* are from same patient, one month apart.

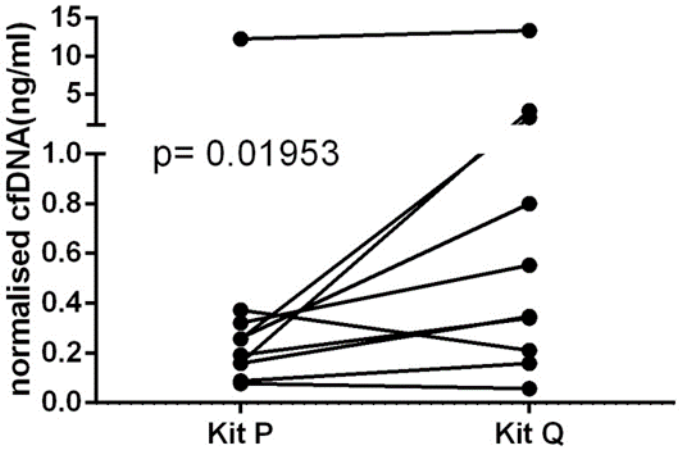

Figure 1: Concentrations of plasma cell-free DNA (cfDNA) extracted using the Kit Q and the Kit P.

ddPCR for NRAS^{Q61R}

The ddPCR plots for the NRASQ61R mutant are presented in Figure 2. To ensure specificity, the ddPCR threshold for positive mutant call was set at 3500 (amplitude), based on the data from a heterozygous NRASQ61R mutant control cell line MelRM19 (Figure 2A). No NRAS mutations were detected in the six healthy cfDNA samples used in this study, assuring specificity of our assay. For the patient samples, the newly diagnosed patient had detectable ctDNA using either kit, as expected. Similarly, the post-transplant patient that was in remission had undetectable ctDNA using both kits. However, for the two consecutive samples collected from the relapsed patient who had been initiated new treatment regimen, Kit Q clearly detected NRASQ61R ctDNA (≥3 events), while this was either undetectable or marginally detectable (one ddPCR event only) for Kit P (Figure 2B).

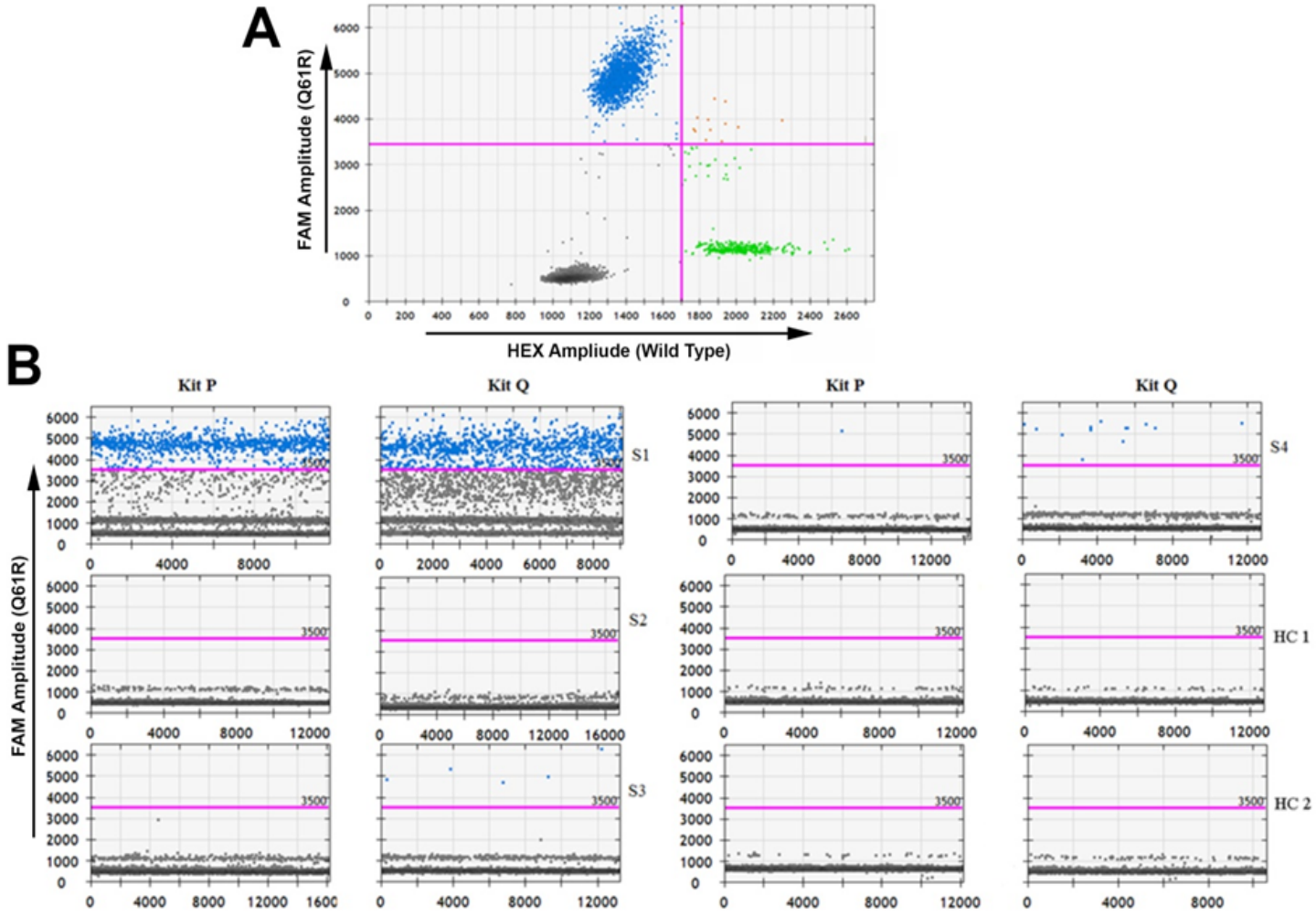

Figure 2: Detection of NRAS^{Q61R} in cfDNA in MM by ddPCR.

A. 2-D plot of the NRAS^{Q61R} positive genomic DNA was extracted from the melanoma cell line MelRM. The blue dots represent the droplets with mutant NRAS, the green dots represent the droplets with wild type NRAS, the brown dots represent the droplets with both mutant and wild type DNA and the grey dots represent the empty droplets. B. 1-D plots of the mutant events for the MM samples (S1 - S4) and the healthy controls. Data for two healthy controls (HC1, HC2) are included. S1: new diagnosed MM, S2: post autologous transplant MM, S3 ad S4: relapsed myeloma on a new treatment regimen with PB collected one month apart.

Total cfDNA concentration by ddPCR detection of wild type and mutant copies

To further assess the efficiency of cfDNA extraction for both kits we evaluated the wild type NRAS events detected by ddPCR. The concentration (copies/mL plasma) of mutant and wild type cfDNA for each sample and healthy controls was normalised based on the volume of plasma used for cfDNA isolation, elution volume and the cfDNA input into the ddPCR reaction as previously described [15]. Normalising the total events detected by the NRAS ddPCR assay corrects for any variation in plasma input. The normalised copy numbers for wild type and mutant DNA for the patient samples were compared (Figure 3). While there was a tendency for higher total events with Kit Q, given the small patient cohort, significance could not be reached.

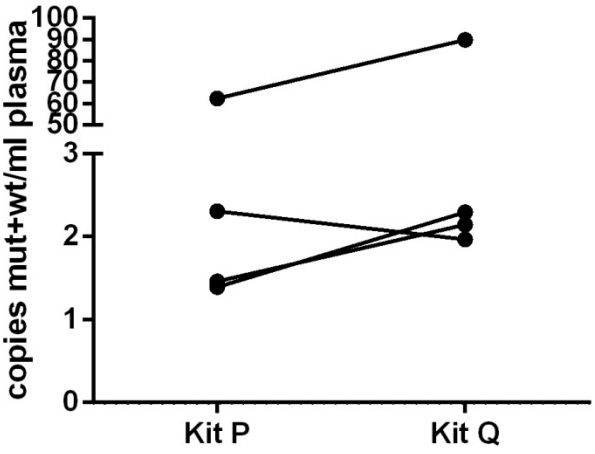

Figure 3: Total mutant and wildtype events for MM patient samples from both kits. Lines indicate matched samples.

Discussion

The use of cfDNA as a screening tool is an attractive alternative to invasive bone marrow biopsies which are the current gold standard for MM diagnosis and monitoring. Moreover, several studies have reported that the main source of cfDNA was the hematopoietic system [17,18,26], making cfDNA an ideal biomarker for haematological malignancies. This study compared the performance of two commercially available cfDNA extraction kits. We and others have previously compared these kits, especially Kit Q (QIAamp) with other extraction methods for plasma derived from patients with solid cancers or using DNA spiked into blood, and the QIAamp emerged as a clear front runner in these studies [15,19,23,24]. As a result, we consider the Qiagen kit to be the "goldstandard" reference method for any cfDNA extraction kit evaluation.

Whilst Kit Q (QIAamp) has been demonstrated to be superior in numerous studies, the performance of the Maxwell® RSC ccfDNA Plasma Kit and the QIAamp kit has not been directly compared in the setting of haematological malignancies. Therefore, we decided to evaluate the ccfDNA Plasma Kit in direct comparison to the QIAamp kit for MM. The Promega kit was chosen because of the availability of the Maxwell® RSC instrument in our diagnostic haematology lab where it is routinely used for genomic DNA (gDNA) extractions. As previously mentioned, myeloma cells circulate in peripheral blood in limited numbers associated with disease stage in comparison to other blood cancer cells, making MM a great "test subject" for the evaluation of cfDNA isolation methods. While likely more challenging than other blood cancers, relatively high ctDNA are expected compared to solid cancers [4].

The primary goal of this study was to compare the ease of use and yield of cfDNA isolated with these kits from plasma of MM patients. Despite the small sample size, the yield of cfDNA isolated from peripheral blood was significantly higher with Kit Q than with Kit P. We believe the differences in detectability of the yield of cfDNA between kits in our study are due to the differences in the sample pre-processing of both kits. Kit Q involves a 30 minutes proteinase K digestion, which had demonstrated to greatly improve the yield of cfDNA in serum and plasma [27] and removes protein contaminants, which might inhibit downstream PCR applications [28]. Malignant plasma cells in the bone marrow of MM patients produce large amounts of monoclonal proteins [29] (paraprotein) which can be released into blood circulation and urine. Therefore, it is possible that the statistically lower cfDNA yields with Kit P may be due to lack of the proteinase K digestion and resultant decreased yield. However, testing this idea is beyond the scope of this study, while potentially worth addressing by the kit manufacturer.

To evaluate the detectability of ctDNA by these two kits, we used a ddPCR assay for NRASQ61R. As expected ctDNA, defined here as the detection of NRASQ61R, was readily detectable in plasma from a newly diagnosed MM patient and the yield of cfDNA and ctDNA was extremely high using both kits for cfDNA extraction, as previously reported [30]. The other patient samples analysed in our comparison study were generally expected to have lower ctDNA levels as they were obtained post-treatment, potentially challenging ctDNA analysis in detecting minimal residual disease. Sample 2 was from a MM patient who previously tested positive for the $NRAS^{Q61R}$ mutation at diagnosis. However, the sample used in this study was collected a few weeks following autologous bone marrow transplant, where the patient's own healthy bone marrow cells are used to replace the bone marrow after intensive chemotherapy. Therefore, the expected levels of ctDNA post-transplant are relatively low in the case of continued residual disease or in theory, undetectable. In this study, neither

kit detected the NRASQ61R mutation in this sample confirming that the patient was in remission at the time of sample collection, contrast. Sorber et al. [24] found that the QIAamp and Maxwell RSC kits gave comparable yields (mutated KRAS copies/µL) and were able to detect KRAS mutations in all stages of pancreatic cancer. The reason for this discrepancy is unclear, but for our study we chose patients with expectedly easily detectable $NRAS^{Q61R}$ (newly diagnosed) as well as patients previously treated and without clinical signs of disease at the time of sample collection, to potentially challenge the sensitivity of ctDNA detection.

It has been reported that cfDNA concentration varies between different sample collection points throughout the course of a disease [31]. Two successive blood samples were collected for one patient (sample 3 and 4), four weeks apart, while undergoing a new line of therapy, enabling mutant load tracking in addition to monitoring of other clinical parameters. Kit Q convincingly captured the low amounts of ctDNA and confirmed minimal residual disease, which interestingly slowly increased between consecutive samples. Kit P, on the other hand, produced only a single detectable mutant ddPCR event, compared to the 13 mutant events produced with Kit Q. Even when taking into account the 0.7-fold input difference (due to different elution volumes between the kits), Kit Q more convincingly allows $NRAS^{Q61R}$ detection for both samples of this patient, suggesting the presence of residual disease despite the patient clinically responding to a new treatment regimen. On the other hand, it would be difficult to define this patient as having minimal residual disease based on the Kit P result.

The utility of liquid biopsies in a pathology laboratory for disease diagnosis and monitoring depends on the hands-on time, ease of use, cost per sample as well as the efficiency of cfDNA isolation and its utility for downstream assays to screen for ctDNA. A fully automated extraction system like the Maxwell RSC from Promega would offer the possibility of medium throughput on top of its technical and financial advantages over the QIAamp kit used with the QIAVac 24 system. The Maxwell RSC can automatically process 16 samples in 70 minutes. When compared to the time it takes to process 12 samples with the Qiagen QIAVac 24 system, 32 samples could be processed with the Maxwell RSC instrument. However, the elution volume of Kit Q can be adjusted between 20 µL and 150 µL to accommodate low or high concentration of cfDNA based on starting material whereas the elution volume of Kit P is recommended at 60 µL, with a loss of approximately 15 µL by the end of the extraction. This volume loss would impact the scalability of elution volumes as well as the standardisation of cfDNA concentrations eluted with the Maxwell® RSC instrument. More importantly, in our study the Maxwell® RSC ccfDNA Plasma Kit was not as sensitive as the Qiagen QIAamp Circulating Nucleic Acid kit in detecting ctDNA in MM patients. In a clinical setting, where detection of residual disease or early relapse and post-transplant monitoring are important for adequate patient management, assay sensitivity in detecting ctDNA reliably is ultimately most important.

Conclusion

In our hands, the QIAamp kit from Qiagen, proved to be more sensitive as it was able to confidently detect low NRAS mutant loads in cfDNA samples from multiple myeloma patients. The Maxwell RCS ccfDNA kit offers a more cost-effective, automated and time efficient method but was not capable of confidently detecting low NRAS mutant loads, which is an important aspect of monitoring treatment response, minimal residual disease and detecting early relapse.

In this study, manual extraction using the Qiagen QIAamp Circulating Nucleic Acid kit continues to demonstrate superiority over other kits for cfDNA extraction. Further development and refinement of automated extraction methods is required prior to the incorporation of ctDNA as a standard diagnostic biomarker in a clinical setting.

Author's Contribution

AV and SL organised blood sample collection and were involved in the management of the patients. AV, JM, ST and CR processed the blood samples, performed cfDNA isolation with kit Q. JM and CR performed cfDNA isolation with kit P. JM and CR performed the ddPCR for this study. ST and CR performed Qubit assays. JM, CR, YM, TR and TB performed data analysis and interpretation. JM wrote the first draft and final manuscript. TB co-wrote the manuscript. JP assisted with ethics approval, figures and critically reviewed the manuscript. All co-authors have contributed to the draft and approved the final version of the manuscript.

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