APPLICATION NOTE

MagMAX Cell-Free DNA Isolation Kit

A complete next-generation sequencing workflow for circulating cell-free DNA isolation and analysis

Abstract

Circulating cell-free DNA (cfDNA) has been shown to have potential as a noninvasive substrate for the detection and monitoring of tumor cells. As circulating tumor DNA is often present at low frequencies within cfDNA, targeted sequencing is an optimal tool for mutation detection. To support advancement of cfDNA research, we demonstrate a complete workflow providing: (1) isolation of cfDNA from plasma using the Applied Biosystems™ MagMAX™ Cell-Free DNA Isolation Kit either manually with a magnetic stand or automatically on the Thermo Scientific™ KingFisher™ Flex or KingFisher™ Duo Prime Magnetic Particle Processor, followed by (2) molecular characterization of isolated cfDNA using the multiplexing capabilities of Ion AmpliSeq™ technology and the Ion PGM™ System.

cfDNA isolated with the MagMAX Cell-Free DNA Isolation Kit and amplified with the Ion AmpliSeq™ Cancer Hotspot Panel v2 demonstrates reproducible amplicon representation and variant detection across 50 genes of interest, covering 2,800 COSMIC mutations. The performance is comparable whether cfDNA is isolated with the MagMAX Cell-Free DNA Isolation Kit or a column-based protocol. Importantly, performance is also comparable when using less than half the volume of plasma with the MagMAX Cell-Free DNA Isolation Kit than with column-based protocols. Through saturation studies and subsampling, the limit of detection of hotspots in cfDNA on the Ion PGM System is determined to be below 1%. This robust workflow for cfDNA analysis through targeted sequencing combines simple sample preparation with the ease of Ion AmpliSeq technology and the rapid turnaround time of the Ion PGM System.

Introduction

cfDNA has been shown to have potential as a noninvasive substrate for the detection and monitoring of tumor cells in published literature [1, 2]. As circulating tumor DNA is often present at low frequencies within cfDNA, targeted sequencing is an optimal tool for mutation detection. cfDNA in plasma is widely used for basic and clinical research, including oncology studies. However, some commercially available cfDNA isolation kits have lengthy protocols, use many reagents, and often require heating for Proteinase K treatment and DNA elution, which can make the process difficult to implement.

Advances in next-generation sequencing (NGS) technology now enable the use of cfDNA as a biomarker for liquid biopsy research applications. Successful use of cfDNA as an oncology research biomarker requires an easily implemented protocol to access the template and efficient DNA extraction from large volumes of plasma (1–10 mL) owing to the low concentration of cfDNA (10–1,000 copies/mL) in blood. The ability to concentrate and analyze the limited cfDNA contained in these large plasma volumes with technologies such as NGS enables researchers to obtain more information more quickly than with established methods. The accessibility of blood samples suggests that cfDNA extraction and analysis could be beneficial in cancer research for potential detection and monitoring of tumor cell progression in the future.

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Materials and methods

Circulating cfDNA isolation and quantification

cfDNA was isolated from the cell-free plasma of 4 normal samples to study reproducibility and scalability of cfDNA recovery, as well as enrichment of cfDNA. Extractions of 4 mL and 10 mL plasma were prepared from each sample using the MagMAX Cell-Free DNA Isolation Kit and the KingFisher Flex Magnetic Particle Processor with 24 Deep-Well Head. Quantitation was performed with the Agilent™ High Sensitivity DNA Kit to assess the cfDNA fraction and the Invitrogen™ Qubit™ dsDNA HS (High Sensitivity) Assay Kit to quantify total DNA yield.

To verify compatibility with NGS analysis, 4 non-small cell lung cancer (NSCLC) cfDNA samples were extracted from archived plasma using either the MagMAX Cell-Free DNA Isolation Kit or a commercially available column-based kit (kit Q). cfDNA was isolated from 1 mL of each plasma sample using the published protocol for kit Q. For extraction using the MagMAX Cell-Free DNA Isolation Kit, cfDNA was isolated from 1 mL for two of the plasma samples, while the sample input was substantially less for the other two plasma samples (0.580 mL and 0.381 mL).

Analysis of cfDNA

Libraries were constructed using the Ion AmpliSeq™ Library Kit 2.0 and the Ion AmpliSeq Cancer Hotspot Panel v2 using 20 cycles of PCR. Template preparation was performed on the Ion Chef™ System followed by sequencing on the Ion PGM System. Variant detection was performed by the Torrent Variant Caller plugin on Torrent Suite™ Software or Ion Reporter™ Software.

Results

Protocol overview

Faster processing of samples can be achieved with automated isolation using the MagMAX Cell-Free DNA Isolation Kit on the KingFisher Flex or KingFisher Duo Prime system. Alternatively, the samples can be processed manually with a magnetic stand. This is followed by a targeted sequencing approach in which library and template are prepared from the enriched cfDNA and sequenced on the Ion PGM System (Figure 1).

Figure 1. A scalable and reproducible workflow to successfully analyze cfDNA from large volumes of plasma via NGS.

Recovery and reproducibility

Circulating cfDNA is highly fragmented, with the major fraction around 170 bp. To examine DNA size-dependent recovery, 50 µL of 50 bp DNA ladder was spiked into 4 mL of commercially available plasma that was depleted of endogenous cfDNA. The MagMAX Cell-Free DNA Isolation Kit was then used to purify the DNA ladder. Following analysis on the Agilent™ 2100 Bioanalyzer™ system, approximately 100% of cfDNA ranging from 100 bp to 750 bp was recovered, while less-efficient elution of DNA ≥800 bp was observed (Figure 2). This efficient recovery of double-stranded DNA (dsDNA) <800 bp yields eluted DNA samples specifically enriched for cfDNA.

To examine reproducibility, 4 plasma samples were processed in duplicate using the MagMAX Cell-Free DNA Isolation Kit. The extracted DNA was analyzed with a 2% Invitrogen™ E-Gel™ EX Agarose Gel (Figure 3). Each replicate set showed a similar yield of cfDNA with the major fraction clearly visible at ~170 bp.

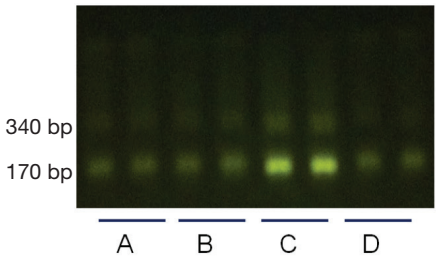


Figure 3. Reproducibility of cfDNA recovery. Plasma samples from 4 donors were processed in duplicate using the MagMAX Cell-Free DNA Isolation Kit. cfDNA was extracted from 4 mL plasma and eluted in 50 µL elution solution. 20 µL of eluted DNA was run on a 2% E-Gel EX Agarose Gel for analysis.

Protein contamination

Protein contamination in DNA extracted with the MagMAX Cell-Free DNA Isolation Kit and a commercially available column-based kit (kit Q) was quantified with the Invitrogen™ Qubit™ Protein Assay Kit. The samples processed with the MagMAX Cell-Free DNA Isolation Kit show considerably less protein carryover as compared to the competitor kit even though the MagMAX kit protocol does not utilize Proteinase K (Figure 4). The protocol for kit Q starts with a 30-minute Proteinase K digestion at 60°C.

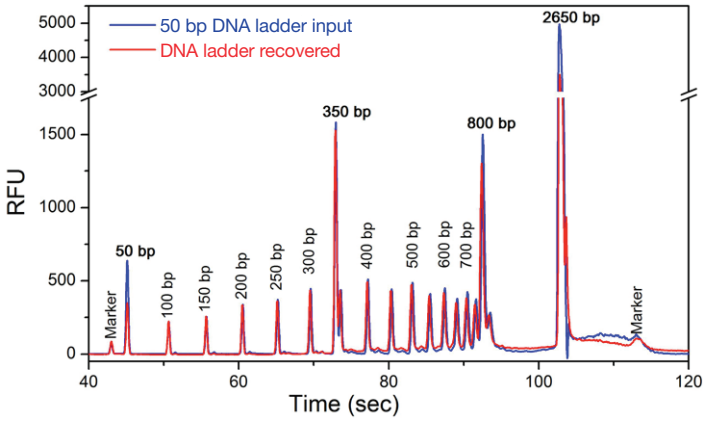


Figure 2. Efficiency of short DNA recovery. 60 µL of MagMAX™ Cell-Free DNA Magnetic Beads was added to 4 mL of cfDNA-depleted plasma that was spiked with 50 µL of 50 bp DNA ladder (2 ng/µL). Bound DNA was washed and eluted in 50 µL elution solution. To assess recovery efficiency of the MagMAX Cell-Free DNA Isolation Kit, 1 µL of ladder and 1 µL of the extracted DNA sample were run on the Agilent 2100 Bioanalyzer system using the High Sensitivity DNA Chip.

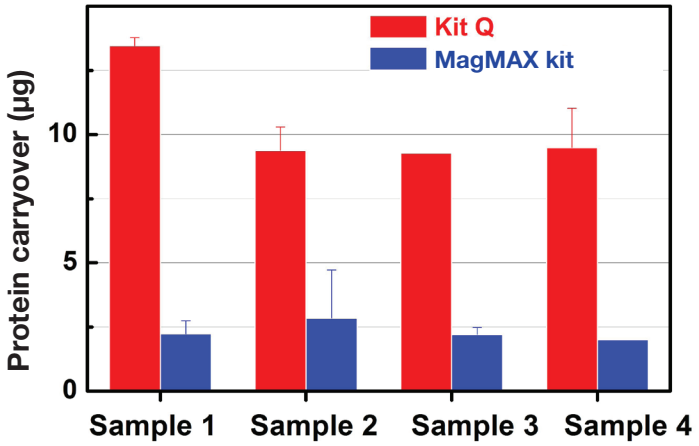


Figure 4. Protein carryover after DNA isolation. Protein was quantified using the Qubit Protein Assay Kit following DNA isolation with the MagMAX Cell-Free DNA Isolation Kit or kit Q, a column-based kit.

Enrichment of cfDNA

Some high molecular weight cellular DNA may be released from white blood cells after blood draw and during plasma separation. cfDNA is highly fragmented and co-migrates with ribosomal RNA with a major peak at ~170 bp. By design, the MagMAX Cell-Free DNA Isolation Kit efficiently recovers only DNA shorter than 800 bp (Figure 2), and thus has the potential to enrich for cfDNA. This was confirmed by comparing cfDNA enrichment with the MagMAX Cell-Free DNA Isolation Kit to enrichment with kit Q using various plasma samples (Figure 5). Compared to kit Q, the MagMAX Cell-Free DNA Isolation Kit demonstrates equivalent cfDNA (<700 bp fraction) yield but lower cellular DNA (>700 bp fraction) yield. Thus, the cfDNA fraction as a percentage of total extracted DNA was higher (i.e., cfDNA was enriched) for the MagMAX Cell-Free DNA Isolation Kit.

Scalability and automation

The sample volume processed and DNA recovered are highly scalable using the MagMAX Cell-Free DNA Isolation Kit. dsDNA of 120 bp and 170 bp in length were spiked into plasma samples at varying input amounts ranging from 10 ng to 200 ng. Consistent, efficient recovery of both DNA spikes is observed over the input range (Figure 6A). To demonstrate sample volume scalability, cfDNA from 4 mL and 10 mL plasma were isolated from the same sample. As expected, DNA yield from 10 mL plasma was ~2.5x greater than that from the 4 mL plasma sample (Figure 6B and C), while total protein carryover only marginally increased (Figure 6D).

Processing up to 5 mL of plasma can be automated with the KingFisher Flex (24 samples per run) or the KingFisher Duo Prime (6 samples per run) system. Enriched cfDNA samples are ready in about 40 minutes after reagents and plasma samples are loaded into the plates. To demonstrate the performance of both systems, 4 mL per sample was processed from 4 plasma samples using the MagMAX Cell-Free DNA Isolation Kit. The eluted DNA was loaded onto a High Sensitivity DNA Chip and run on the Agilent Bioanalyzer 2100 system. The yield and size profile are approximately equivalent for all 4 samples, indicating similar recovery performance (Figure 7).

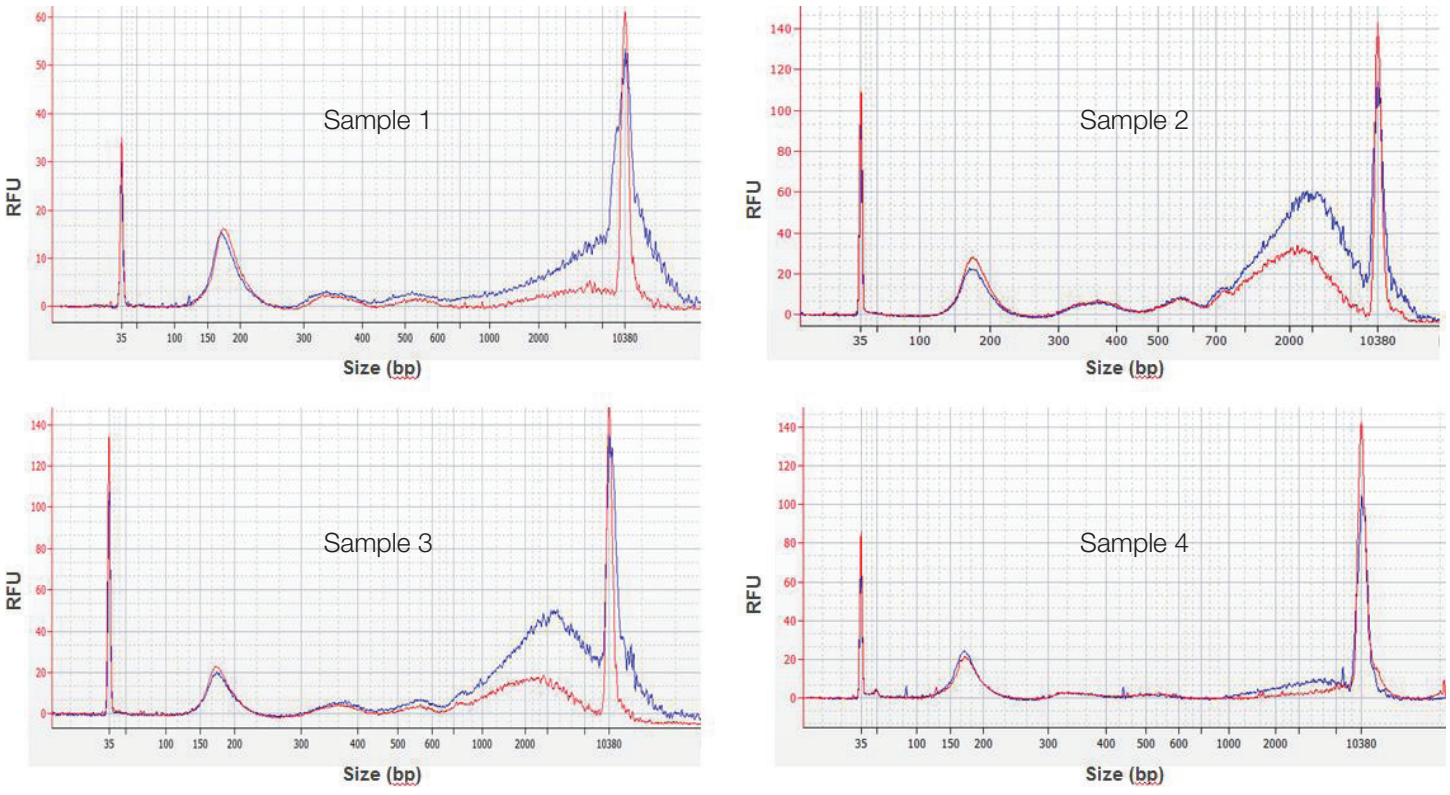


Figure 5. Enrichment of cfDNA following extraction from plasma samples. Cell-free plasma was separated from 4 normal blood samples by centrifugation for 20 minutes at 2,000 x *g*, then for 30 minutes at 6,000 x *g*. DNA was extracted from 4 mL plasma using either the MagMAX Cell-Free DNA Isolation Kit (red trace) or kit Q (blue trace). The eluted DNA was loaded onto a High Sensitivity DNA Chip and run on the Agilent Bioanalyzer 2100 system.

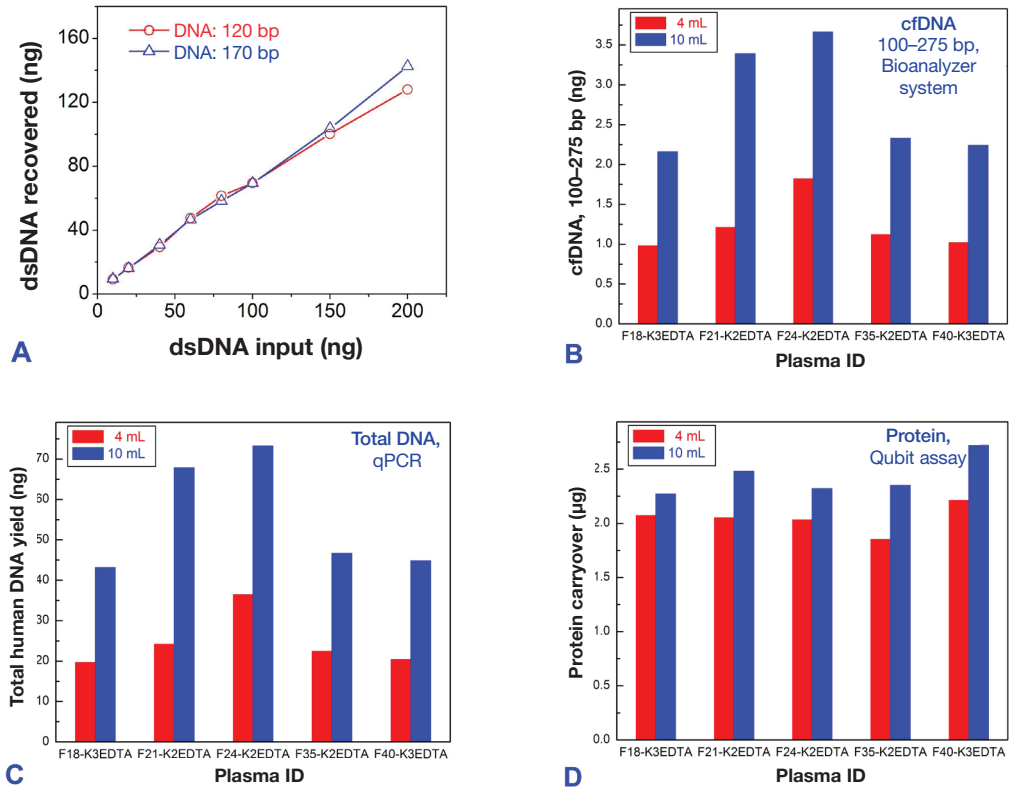


Figure 6. Scalability of cfDNA isolation. (A) 120 bp and 170 bp dsDNA were spiked into plasma samples at varying input amounts and recovered using the MagMAX Cell-Free DNA Isolation Kit. **(B)** cfDNA from 4 mL and 10 mL plasma quantified using the Agilent Bioanalyzer 2100 system. **(C)** cfDNA from 4 mL and 10 mL plasma quantified by qPCR. **(D)** Total protein carryover as measured using the Qubit Protein Assay Kit.

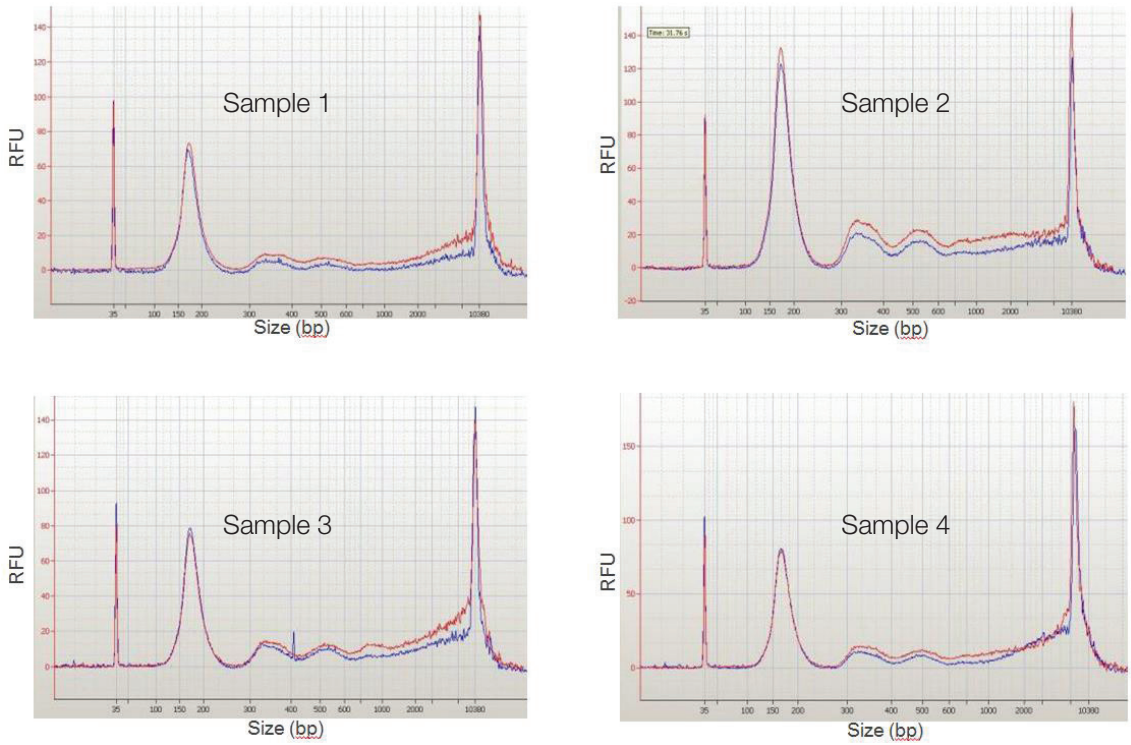


Figure 7. Automated cfDNA isolation using the KingFisher systems. Overlapping traces for cfDNA isolated with the KingFisher Flex (red) and KingFisher Duo Prime (blue) systems are shown.

Targeted sequencing of cfDNA

Four NSCLC cfDNA samples were isolated using the MagMAX Cell-Free DNA Isolation Kit or kit Q. For kit Q, each sample was isolated from 1 mL of plasma. For the MagMAX Cell-Free DNA Isolation Kit, samples 1 and 2 were isolated from 1 mL of plasma, while sample 3 was isolated from 0.580 mL of plasma and sample 4 was isolated from 0.381 mL of plasma. Ion AmpliSeq™ libraries were prepared with the Ion AmpliSeq Cancer Hotspot Panel v2, template was prepared on the Ion Chef System, and sequencing was performed on the Ion PGM System. The percent of sequencing reads that were on target were comparable between the two isolation methods, including when a lower volume of plasma was used for the MagMAX Cell-Free DNA Isolation Kit (Figure 8). The uniformity of the amplicons was comparable for all 4 samples.

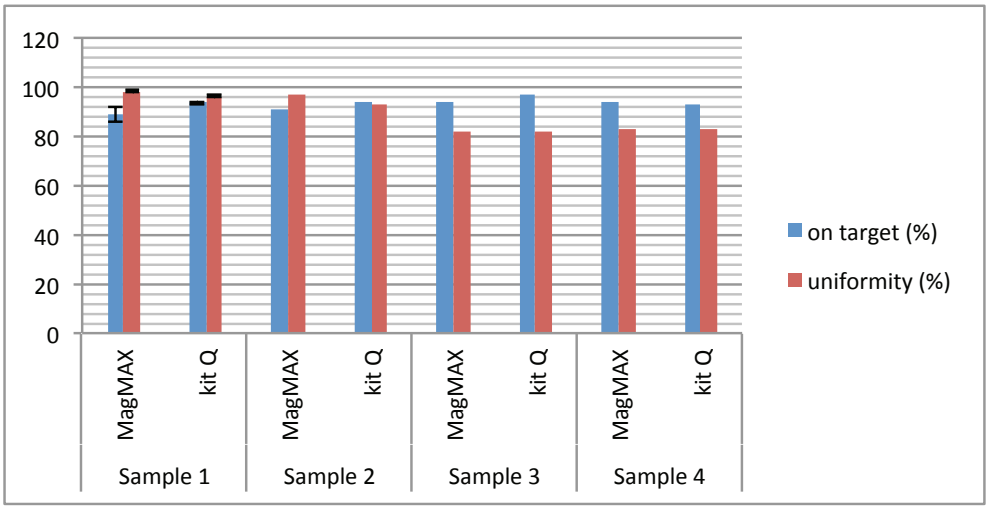
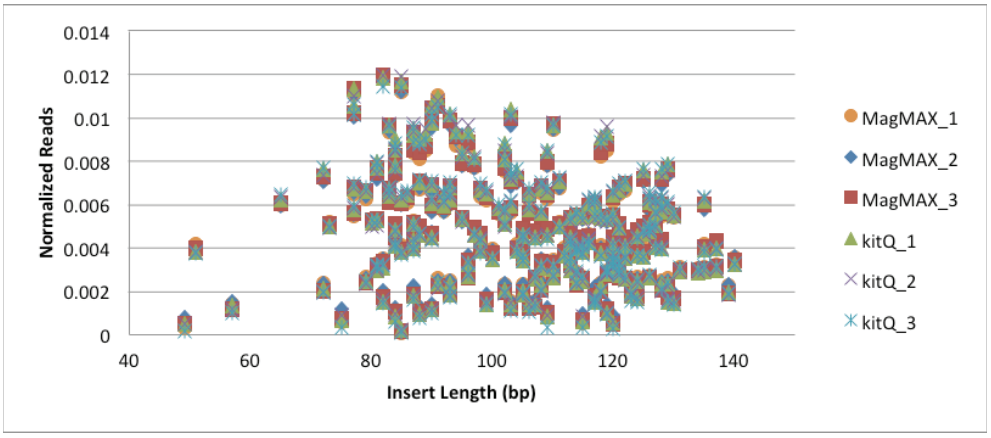


Figure 8. Amplicon performance of NSCLC samples. Samples 1–4 were isolated using the MagMAX Cell-Free DNA Isolation Kit or kit Q and sequenced on the Ion PGM System. The percent of on-target sequencing reads and uniformity are shown for both isolation methods.

Triplicate libraries were prepared using the Ion AmpliSeq Cancer Hotspot Panel v2 from NSCLC cfDNA isolated using the MagMAX Cell-Free DNA Isolation Kit or kit Q. Template preparation and sequencing was performed independently for each library. The insert length versus normalized amplicon coverage as reported by the Coverage Analysis plugin provided in Torrent Suite Software shows a high degree of reproducibility between the two cfDNA isolation protocols (Figure 9A). The percent GC content present in each amplicon versus the normalized amplicon coverage also shows reproducible results for the two cfDNA isolation protocols (Figure 9B).

A



B

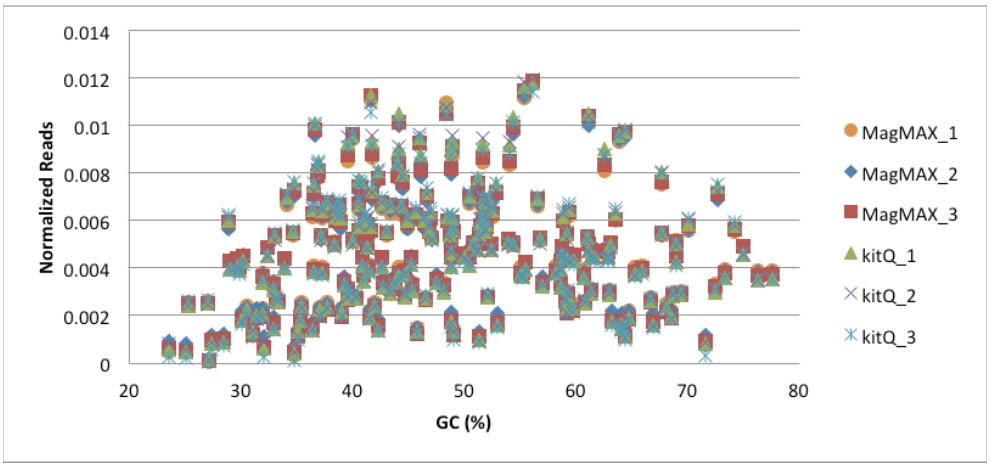


Figure 9. Reproducibility of amplicon coverage in NSCLC samples. Triplicate libraries were independently prepared and sequenced from cfDNA isolated using the MagMAX Cell-Free DNA Isolation Kit or kit Q. **(A)** Insert length plotted against normalized amplicon coverage. **(B)** Percent GC content in each amplicon plotted against normalized amplicon coverage.

There was good agreement in allelic frequencies using the Torrent Variant Caller plugin provided in Torrent Suite Software. Sample 1 had triplicate cfDNA libraries prepared from the MagMAX Cell-Free DNA Isolation Kit and triplicate cfDNA libraries prepared from kit Q (Table 1). All six libraries showed comparable variant detection results. Sample 3 had 0.381 mL of plasma used for the MagMAX Cell-Free DNA Isolation Kit versus 1 mL used for kit Q and showed comparable variant detection results.

Table 1. Allelic frequencies for hotspots in NSCLC samples.

Sample	Gene	COSMIC ID	FFPE	MagMAX Cell-Free DNA Isolation Kit	Kit Q
Sample 1	*MET*	710	ND	49.8	47.9
				46.8	50.1
				49.2	50
Sample 3	*MET*	710	63	47.9	46
	SMARCB1	1090	62.9	46	45.2
	PTEN	5915	62.5	49.6	46.6
	PDGFRA	22413	73.6	47.7	46.5
Sample 4	*MET*	710	31.8	49.1	47.2
	PTEN	5915	86.7	52.7	47.9
	HRAS	249860	99	99.8	96

Conclusions

We have developed a workflow with a set of reagents and tools for recovery and analysis of cfDNA from cell-free samples such as plasma or serum. The complete workflow includes automated isolation of cfDNA and sensitive, reproducible downstream analysis via targeted sequencing using the Ion AmpliSeq Cancer Hotspot Panel v2.

The Ion AmpliSeq Cancer Hotspot Panel v2 has reproducible amplicon performance and variants detected with cfDNA. The performance of Ion AmpliSeq technology is comparable when the cfDNA has been isolated with the MagMAX Cell-Free DNA Isolation Kit protocol or a column-based method, even when there is less than half the volume of plasma used with the MagMAX kit protocol than with the column-based method. This robust workflow for cfDNA isolation and targeted sequencing combines simple sample preparation with the ease of Ion AmpliSeq technology and the rapid turnaround time of the Ion PGM System.

References

1. Bettegowada C, Sausen M, Leary RJ et al. (2014) Detection of circulating tumor DNA in early- and late-stage human malignancies. *Sci Transl Med* 6:224ra24.
2. Lebofsky R, Decraene C, Bernard V et al. (2015) Circulating tumor DNA as a non-invasive substitute to metastasis biopsy for tumor genotyping and personalized medicine in a prospective trial across all tumor types. *Mol Oncol* 9:783–790.

Ordering information

Product	Cat. No.
MagMAX Cell-Free DNA Isolation Kit	A29319
KingFisher Duo Prime Magnetic Particle Processor	5400110
KingFisher Flex Magnetic Particle Processor with 96 Deep-Well Head (for plasma volumes less than 1 mL)	5400630
KingFisher Flex Magnetic Particle Processor with 24 Deep-Well Head (for plasma volumes less than 5 mL)	5400640
Qubit dsDNA HS Assay Kit	Q32854
Ion AmpliSeq Library Kit 2.0	4480442
Ion AmpliSeq Cancer Hotspot Panel v2	4475346

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