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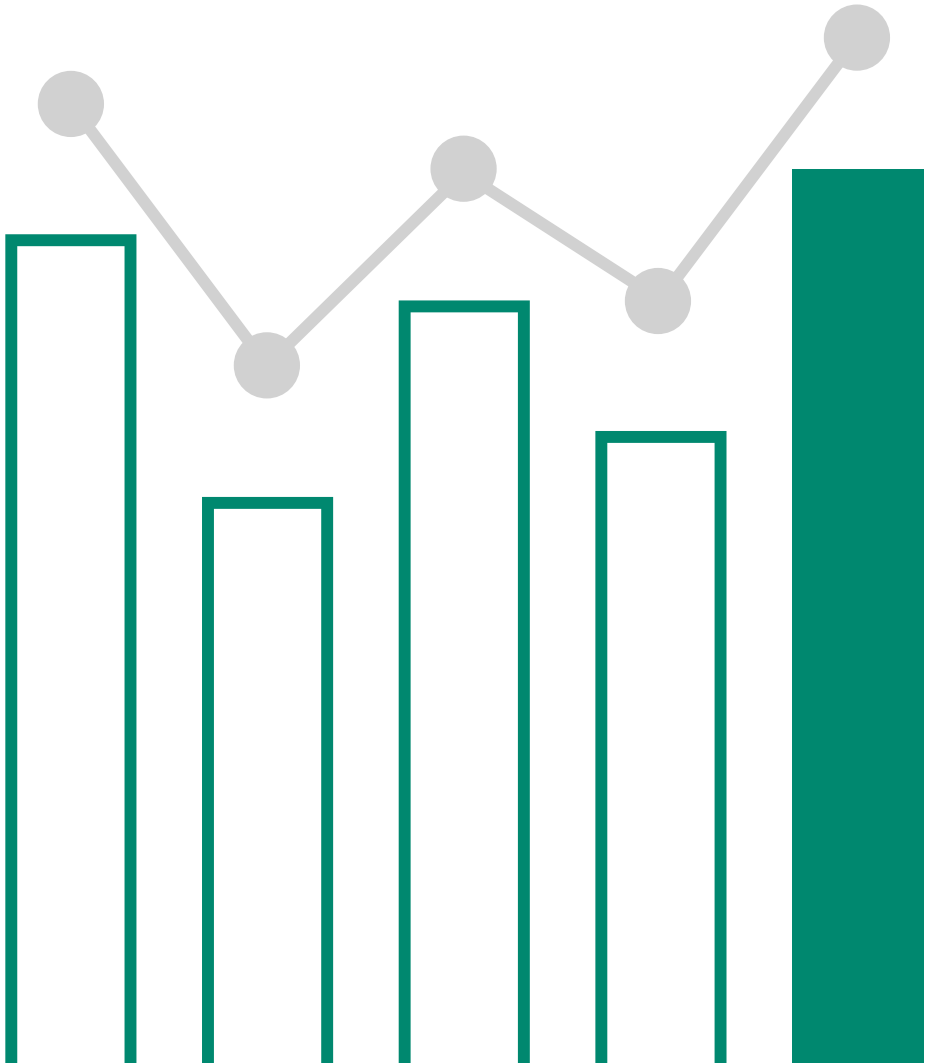
Case study collection

Optimizing the isolation of cell-free DNA from liquid biopsy samples



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Content

Introduction / foreword

Diagnostic tests are the bedrock of medicine. Analyzing the chemical makeup of saliva, blood, or urine can show if you have allergies, anemia, or even cancer. In fact, our very existence was most likely first confirmed through a pregnancy test, which is based on the principle of lateral flow.

Mounting clinical data show the advantages of testing blood or other bodily fluids to make a diagnosis. Liquid biopsies work by capturing and analyzing biomarkers, mostly cell-free DNA (cfDNA), and have been shown to give a more holistic disease profile and make it easier to measure a condition over time (1).

These tests have rapidly gained traction in recent years with applications in reproductive health, cancer, and transplant medicine, but there are still barriers to overcome for the technique to be adopted broadly. For example, with a liquid biopsy, you only have a limited amount of useful genetic material.

Cell-free DNA (cfDNA) is currently trending as a biomarker for liquid biopsy in several clinical applications, including oncology, organ and transplant medicine, and non-invasive prenatal testing (NIPT). Cell-free DNA comprises various forms of unencapsulated DNA freely circulating the bloodstream, including circulating tumor DNA (ctDNA) and cell-free fetal DNA (cffDNA). Due to the small amount of cfDNA found in circulation, there is a need to use efficient, highly sensitive technologies, such as NGS, to detect these biomarkers.

This collection of case studies highlights ways to optimize the isolation of cell-free DNA from a range of liquid biopsy sample inputs. therapeutic response in NSCLC using liquid biopsy

Reference

1. Bai Y, Zhao H. Liquid biopsy in tumors: Opportunities and challenges. *Ann Transl Med.* 2018;6(S1). doi:10.21037/atm.2018.11.31

Monitoring therapeutic response in NSCLC using liquid biopsy

Detecting EGFR mutations with Sera-Xtracta™ Cell-Free DNA Kit

By Monika Seidel PhD, Senior Development Scientist, Genomics and Cellular Research, Cytiva

Introduction

Personalized medicine relies on accurate and sensitive profiling of tumor genetic make-up for targeted therapy decisions. Clinicians now routinely implement this approach in patients with advanced non-small cell lung cancer (NSCLC). The patients undergo testing for the presence of genetic aberrations in the five most frequently mutated genes in NSCLC (EGFR, ALK, ROS1, BRAAF NTRK NTRK), against which there are approved targeted therapies (1).

Compared with standard chemotherapy, precision medicine offers prolonged progression-free survival and significant reduction in side-effects. Unfortunately, a large population of patients eventually develop acquired resistance leading to rapid disease progression. In some instances, the initial response to therapy is either limited or nonexistent. Early detection of resistance through real-time monitoring of changes in the tumor's genetic landscape is emerging as a new approach to facilitate prompt and informed implementation of the second-line therapy. Genetic profiling of spatial and temporal tumor evolution is becoming a key element in research aiming to identify novel molecular drivers of resistance for targeted drug development.

At the time of the diagnosis, the detection of actionable mutations is heavily dependent on the availability of tumor tissue, which remains a gold standard in clinical practice. The use of liquid biopsy for such profiling is only recommended in circumstances where a solid biopsy is not feasible. However, real-time monitoring of cancer genetic evolution is difficult or even impossible to achieve with solid tissue biopsy. Such an approach requires repeated surgery to obtain tumor tissue, with the potential need for patient hospitalization and the possibility of complications. At the advanced stage of the disease, a biopsy of a single lesion can be regarded as insufficient to obtain a full picture of tumor heterogeneity and to detect genetic alterations driving disease progression. In this context, real time monitoring of therapeutic response can only be achieved through liquid biopsy, an approach that is noninvasive and capable of capturing spatial and temporal differences in the cancer genetic landscape.

Study design

The purpose of the study was to evaluate the performance and suitability of Sera-Xtracta™ Cell-Free DNA Kit* for plasma based-monitoring of therapeutic response and resistance in advanced NSCLC in patients positive for epidermal growth factor receptor (EGFR) biomarker and undergoing treatment with tyrosine kinase inhibitors (TKI). We contracted with Biogazelle, of Gent, Belgium to conduct independent experiments and data analysis for our study.

*For research use only.

NSCLC is a leading cause of cancer mortality worldwide, mostly due to the fact that the diagnosis made at a very late stage with metastasis present and a five year survival rate lower than 15% (2). Mutations in the EGFR gene constitute a prevailing genetic cause of the disease. Over 90% of the EGFR mutations involve deletions in exon 19 or substitutions in exon 20 (L858R), both being predictive of positive response to first- and second-generation EGFR tyrosine kinase inhibitors (3). Unfortunately, after the first-line treatment, nearly all patients eventually develop disease progression either due to the acquired resistance or due to the presence of resistance mutations even before the treatment commenced. Although the mechanism for secondary resistance might involve activation of by-pass pathways or histological transformation, in most cases it can be attributed to secondary mutations in the EGFR gene, with the T790M SNV being the prevailing substitution found in around 60% of cases following secondary tissue biopsy (3). A third generation TKI therapy against this mutation is in clinical practice (1).

In the context of almost inevitable secondary resistance triggered by drug-induced clonal selection pressure, the real-time monitoring of patients' response to the therapy in advanced NSCLC appears to be a compelling practice for early detection of disease progression to facilitate a prompt change in the therapeutic approach. With an effective treatment, a gradual decline in plasma tumor burden would be expected to occur. This phenomenon would be reflected in the gradual drop of mutation allele frequency (MAF) of the primary driver mutation until a complete absence thereof. Detection of additional mutations absent at the start of the treatment would be suggestive of a secondary resistance, which indicates tumor evolution.

For our study, the plasma from three patients with advanced disease was collected in Cell-Free BCT tubes (Streck) at three consecutive time points starting from the first day (baseline) of targeted treatment (Fig 1). At the point of cancer diagnosis, all subjects were tested for mutations in primary cancer drivers, i.e., ALK, ROS, and EGFR, and for the presence of the histological marker programmed cell death (PD-1) using tissue biopsy. The presence of EGFR mutations was confirmed in all three patients and consequently all subjects were given tyrosine kinase inhibitors either as a standalone treatment or in combination with a chemotherapeutic agent (Table 1). Based on radiological follow-up, the treatment regimen was effective for Patient 1, whereas no benefit was observed for either Patient 2 or 3.

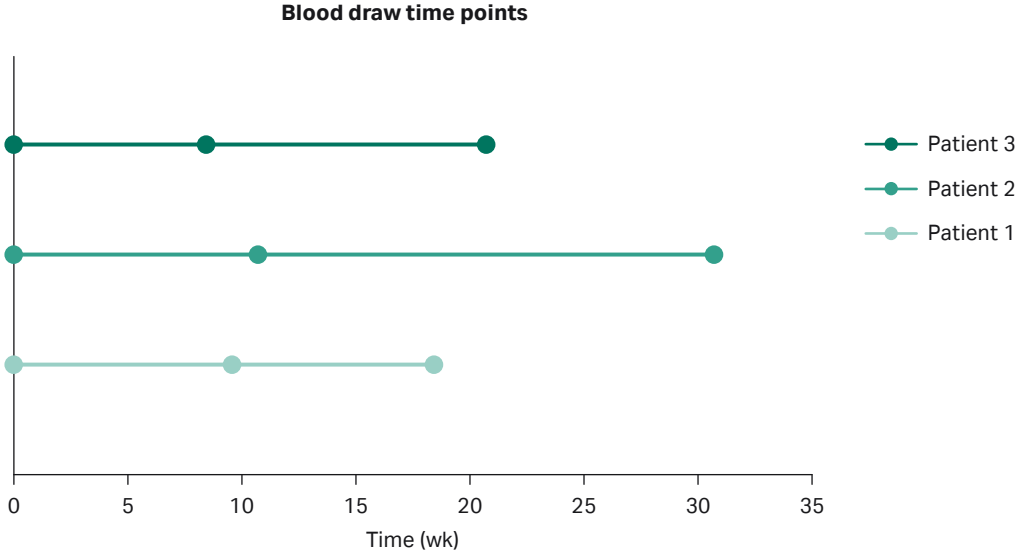

Fig 1. Blood draw time points; note that T0 represent the start of the targeted therapy.

Table 1. Patients' clinical details

Patient	Gender	Morphology/stage	Treatment classification	Therapeutic agent #1	Therapeutic agent #2	Biomarker 1	Biomarker 2	Treatment response
1	M	Adenocarcinoma, IV	non-specific KI + chemotherapy	Nintedanib	Docetaxel	EGFR: del exon 19 (E746_T751del)	PD-1 positive	Partial remission
2	F	Acinar cell adenocarcinoma, IV	first generation EGFR KI	Erlotinib		EGFR L858R		Progressive disease
3	F	Adenocarcinoma, IV	first generation EGFR KI	Gefitinib		EGFR L858R		Progressive disease

Methods

All plasma samples were purchased from Indivumed GmbH (plasma obtained through double spin from blood collected in Cell-Free DNA BCT tubes). Cell-Free DNA (cfDNA) was extracted from 1 mL of plasma using Sera-Xtracta™ Cell-Free DNA Kit and two market leading competitor's kits, MagMAX Cell-Free DNA Isolation Kit (Thermo Fisher Scientific) and QIAamp Circulating Nucleic Acid Kit (Qiagen) following the manufacturers' protocols.

For T0 time point, two replicates of 1 mL of plasma were extracted with each kit, one replicate per kit was used for the remaining two time points (i.e., T1 and T2). All samples were processed at Biogazelle (note that no carrier RNA was added to buffer ACL in the Qiagen kit workflow as per the service provider's standard operating procedure).

The yield and fragment distribution of cfDNA extracted from plasma collected at T0 was analyzed using Qubit fluorometer (Thermo Fisher Scientific) and capillary electrophoresis on HS DNA Chip using Bioanalyzer (Agilent) prior to sequencing. Targeted next-generation sequencing (NGS) was performed on S5XL platform (Ion Torrent) using AmpliSeq technology CP-Alpha v3 panel (Thermo Fisher Scientific) at Bio.be (Institute of Pathology and Genetics). Libraries were prepared with maximum available input for all samples. For panel coverage, see Table 2. Data were analyzed using NextGENe (SoftGenetics) software; assay limit of detection is 1% (for samples with minimum coverage > 10 000 reads and read balance > 0.25). Only calls that fulfilled these criteria were considered positive.

Table 2. CP-Alpha v3 panel coverage

Gene	Details
EGFR	exons 18, 19, 20 and 21
KRAS et NRAS	codons 12, 13, 59 à 61, 117 and 146
BRAF	exons 11 and15
PIK3CA	exons 10 and 21
ALK	exons 22, 23 and 25
DDR2	codon 768
PTEN	codon 233
AKT1	codon 17
MEK1	codons 56 and 67
ERBB2	exon 20
cKIT	exons 9, 11, 13, 14, 17, 18 and junction intro 10/exon 11
PDGFRa	exons 12, 14 and 18
IDH1	exon 4
IDH2	exon 4
H3F3A	exon 2
HRAS	exon 2
GNAS	exons 8 and 9
ESR1	exons 8 and 9
GNAS	exons 8 and 9
ESR1	exons 8 and 9

Extracted cfDNA from the second replicate representing T0 time point and the samples from the remaining two time points (i.e., T1 and T2) were quantified using NanoDrop (Thermo Fisher Scientific) and Qubit (Thermo Fisher Scientific) and subjected to digital droplet PCR (ddPCR) using IDEGFR(b) SENSI-v3-50 Kit (ID Solutions) on QX200TM Droplet Digital PCR system (Bio-Rad). The kit is a multiplex quantification system that allows for the simultaneous detection of the EGFR most common primary driver mutations (Tables 3 and 4). All samples were run in duplicates. Maximum available input (5 µL per replicate) was used in ddPCR unless the upper input limit for the assay was exceeded (16 ng).

Table 3. Targeted mutations and alterations detected by IDEGFR(b) SENSI-v3-50 Kit

Exon	Mutations	Base Change	Cosmic ID
Exon 19	p.E746_A750del (1)	c.2235_2249del15	COSM6223
	p.E746_A750del (2)	c.2236_2250del15	COSM6225
	p.L747_P753>S	c.2240_2257del18	COSM12370
	p.E746_T751>I	c.2235_2252>AAT (complex)	COSM13551
	p.E746_T751del	c.2236_2253del18	COSM12728
	p.E746_T751>A	c.2237_2251del15	COSM12678
	p.E746_S752>A	c.2237_2254del18	COSM12367
	p.E746_S752>V	c.2237_2255>T(complex)	COSM12384
	p.E746_S752>D	c.2238_2255del18	COSM6220
	p.L747_A750>P	c.2238_2248>GC (complex)	COSM12422
	p.L747_T751>Q	c.2238_2252>GCA (complex)	COSM12419
	p.L747_E749del	c.2239_2247del9TTAAGAGAA	COSM6218
	p.L747_S752del	c.2239_2256del18	COSM6255
	p.L747_A750>P	c.2239_2248TTAAGAGAAG>C(complex)	COSM12382
	p.L747_P753>Q	c.2239_2258>CA (complex)	COSM12387
	p.L747_T751>S	c.2240_2251del12	COSM6210
	p.L747_T751del	c.2240_2254del15	COSM12369
	p.L747_T751>P	c.2239_2251>C(complex)	COSM12383
	p.L747_T751del	c.2238_2252del15	COSM23571
	p.L747_S752>Q	c.2239_2256>CAA	COSM12403
	p.E746_T751>V	c.2237_2252>T	COSM12386
	p.E746_T751>T	c.2236_2253> ACG	/
	p.L747_A750>P	c.2239_2250>CCC	/
	p.L747_K754>QL	c.2239_2261>CAATT	/
	p.E746_K754>EQHL	c.2238_2261>GCAACATCT	/
	p.E746_S752>EQ	c.2238_2256>GCAA	/
	p.E746_A750>QP	c.2236_2248>CAAC	COSM13557
	p.E746_T751>Q	c.2236_2253>CAA	COSM22999
Exon 21	p.L858R	COSM6224	c.2573-2574TG>GT
		c.2573T>G	COSM12979
	p. L861Q	c.2582T>A	COSM6213

Table 4. Limit of Blank (LoBkO) at 95% Confidence Interval for the ARM-IDEGFR(b) sensi-v3 defined for samples of circulating DNA.

Targets	Number of replicates	LoB95% (Total positive droplets)	Considered positive (Total positive droplets)
L861Q	Single	0	2
	Duplicate	0	2
	Triplicate	0	2
L858R/Del19	Single	0	2
	Duplicate	0	2
	Triplicate	0	2

Results

Cell-free DNA yield and fragment size distribution

The yield of DNA extracted at each time point with all three cfDNA extraction kits was estimated using Qubit (Fig 2). There was no statistically significant difference in the performance between Sera-Xtracta™ Cell-Free DNA Kit and MagMAX Cell-Free DNA Isolation Kit (based on one-way Anova with Dunnett's post hoc test). The performance of the Qiagen kit was significantly worse ($p < 0.05$). We must emphasize, however, that an alternative protocol suggested by the manufacturer was used and no carrier RNA was added. This use of the alternative protocol was to avoid overestimation of cfDNA yield when using absorbance at 260 nm. Although Biogazelle, our service provider, has successfully implemented and routinely used this approach in the extraction of nucleic acid, the absence of carrier RNA likely contributed to the observed low cfDNA recovery in samples processed with QIAamp Circulating Nucleic Acid Kit.

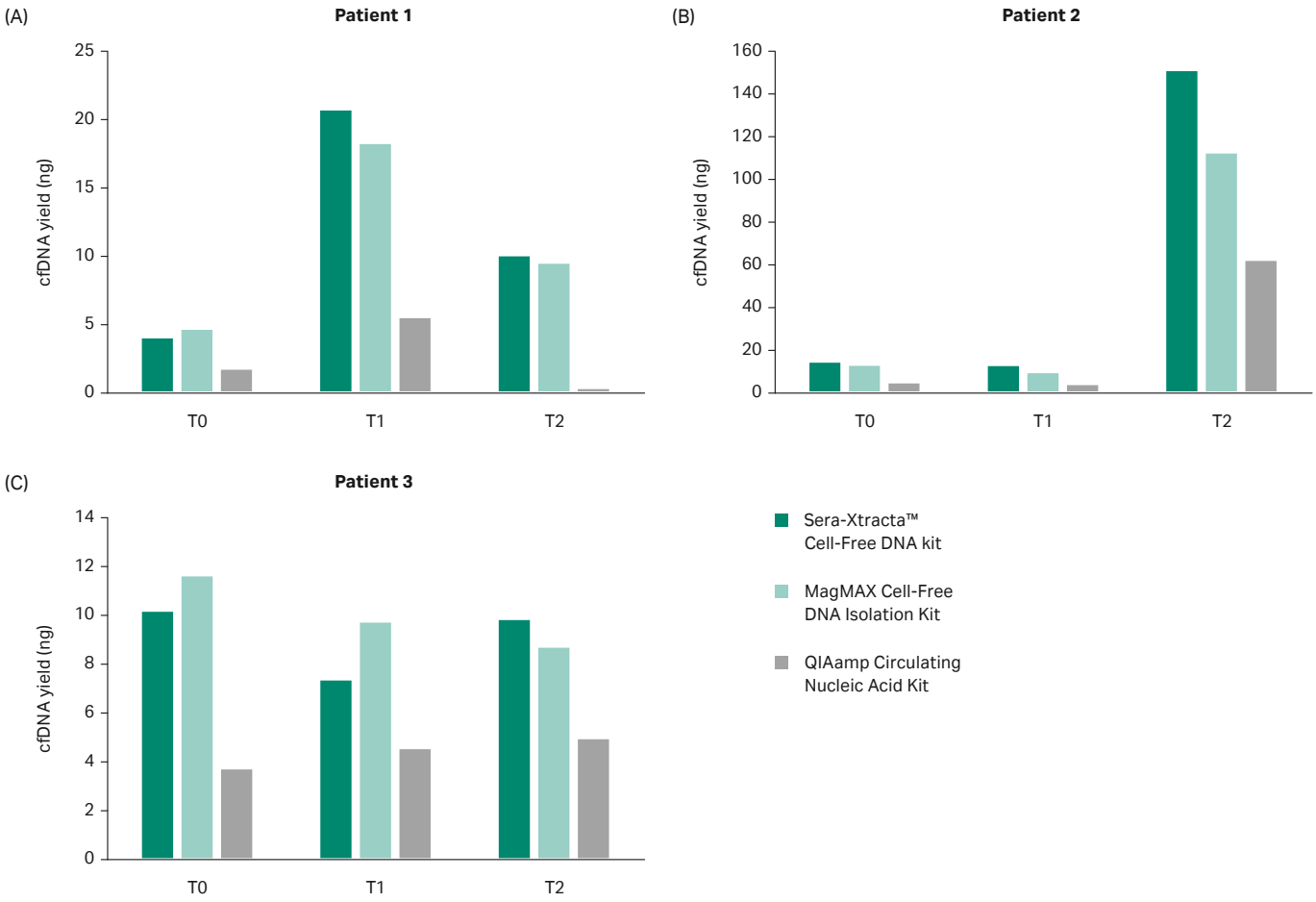

Fig 2. Total DNA yield, quantified using Qubit, at T0-T2: bar chart summarizing total DNA yield obtained from 1 mL of plasma processed with three extraction kits: Sera-Xtracta™ Cell-Free DNA Kit, MagMAX Cell-Free DNA Isolation Kit, and QIAamp Circulating Nucleic Acid Kit.

Fragment size profile for samples subjected to NGS is presented in Figure 3. Because all blood specimens were collected in cfDNA stabilizing tubes and processed using double centrifugation protocol, the level of gDNA release was expected to be minimal. This result is indeed the case for Patients 1 and 2, where no gDNA carry-over is present regardless of the kit used. The plasma sample obtained from Patient 3 appears to contain gDNA as shown in the electropherograms from samples processed with MagMAX Cell-Free DNA Isolation Kit and QIAamp Circulating Nucleic Acid Kit (additional peaks >> 500 bp) but absent in the sample extracted with Cytiva kit (Fig 3).

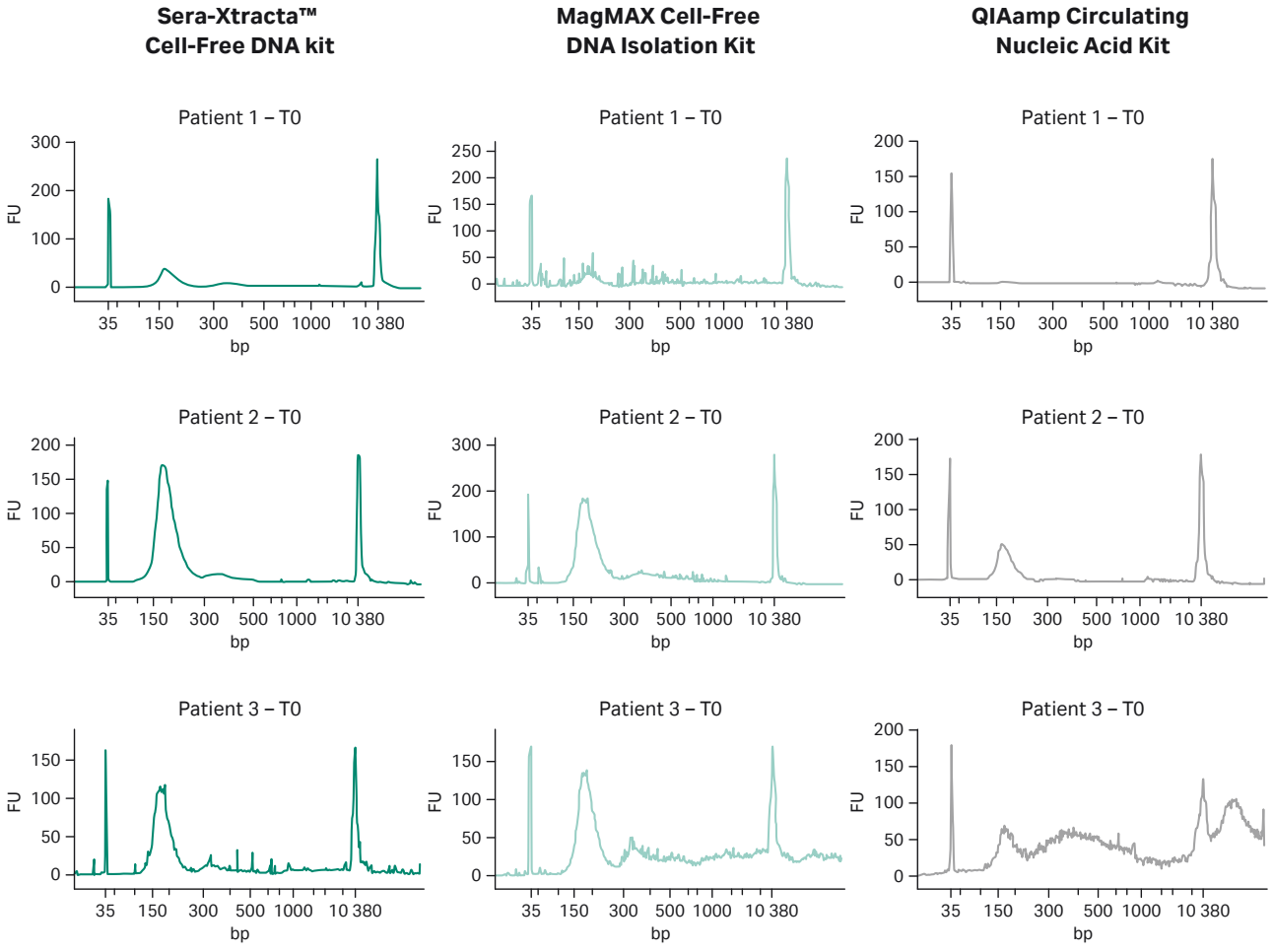

Fig 3. Cell-free DNA recovery and size distribution from plasma at T0: representative electropherograms showing DNA fragment distribution obtained from plasma at T0 (baseline). A 1 mL volume of plasma was independently processed with three extraction kits: Sera-Xtracta™ Cell-Free DNA Kit and MagMAX Cell-Free DNA Isolation Kit, and QIAamp Circulating Nucleic Acid Kit following the manufacturers' protocols (note that no carrier RNA was added to buffer ACL in the Qiagen workflow). Extracted samples were analyzed on 2100 Bioanalyzer using High-Sensitivity DNA Chip.

Targeted NGS results and concordance between tissue biopsy and plasma

Targeted NGS was used to assess the mutation profile in plasma for all three patients at the start of the kinase inhibitor therapy and compare biomarker profile to tissue biopsy. All patients included in our study had been diagnosed with advanced stage cancer, where tumor burden in the blood is expected to reach high levels, which enables confident detection of cancer associated mutations. Samples processed with Sera-Xtracta™ Cell-Free DNA Kit showed 100% concordance with tissue biopsy results, and the presence of EGFR driver mutation was confirmed in all patients. However, this was not the case when using alternative cfDNA extraction kits: the main driver mutation was only confirmed in one out of three patients (Table 5). No additional cancer biomarkers tested were detected in any of the samples.

Table 5. Biomarkers, MAF and sequencing depth detected at T0 in plasma samples in targeted NGS

Sample	Mutation	AA change	Sera-Xtracta™ Cell-Free DNA		MagMAX cfDNA Isolation Kit		QIAamp Circulating Nucleic Acid Kit	
			MAF	Sequencing Depth	MAF	Sequencing Depth	MAF	Sequencing Depth
Patient 1	EGFR del exon 19	Leu747_Pro753del	3%	22500	not detected	16273		not reached
Patient 2	EGFR del exon 19	E746_T751del	5%	14413		not reached	6.5%	15241
Patient 3	EGFR SNV	Leu858Arg	1%	15018	2%	26900	not detected	17309

Validation of targeted NGS by digital droplet PCR

Biogazelle used digital droplet PCR to validate targeted NGS results from plasma samples collected at T0 (Table 6). The presence of EGFR mutation was successfully confirmed in all samples processed with Sera-Xtracta™ Cell-Free DNA Kit, with excellent correlation in mutation frequency between the two detection methods (Pearson r = 0.98) for the Cytiva kit (Fig 4). There was no positive correlation between NGS results and ddPCR results for MagMAX Cell-Free DNA Isolation Kit (Pearson r = -0.75). A high concordance between ddPCR and NGS results obtained for QIAamp Circulating Nucleic Acid Kit (Pearson r = 0.91) can be explained by the fact that neither ddPCR nor NGS was able to confirm the presence of EGFR mutation in Patient 3.

Table 6. Biomarkers and MAF detected at T0 in plasma samples in ddPCR and targeted NGS at T0

Sample	Mutation	AA change	Sera-Xtracta™ Cell-Free DNA		MagMAX Cell-Free Isolation DNA Kit		QIAamp Circulating Nucleic Acid Kit	
			MAF by NGS	MAF by ddPCR	MAF by NGS	MAF by ddPCR	MAF by NGS	MAF by ddPCR
Patient 1	EGFR del exon 19	Leu747_Pro753del	3%	2.18%	0%	1.75%	0%	2.59%
Patient 2	EGFR del exon 19	E746_T751del	5%	4.51%	0%	5.43%	6.5%	6.13%
Patient 3	EGFR SNV	Leu858Arg	1%	0.99	2%	0	0%	0%

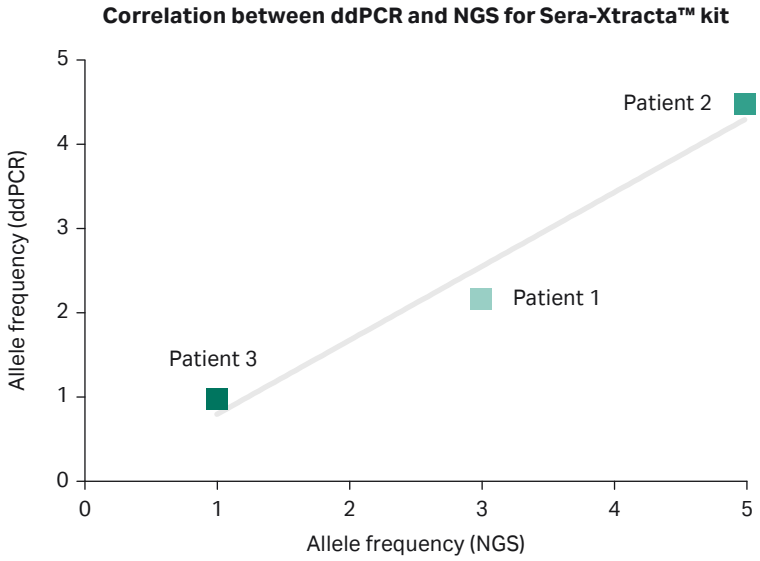

Fig 4. Comparison between targeted NGS and ddPCR for Sera-Xtracta™ cfDNA Kit at T0: mutation allele frequency obtained using targeted NGS is (*x*-axis) compared to allele frequencies estimated during ddPCR (*y*-axis) for identical genomic positions. The solid line represents a linear model.

Monitoring disease progression through evaluation of the tumor burden in plasma (correlation between liquid biopsy findings and clinical response to therapy)

We further evaluated whether the presence and the relative level of primary driver mutation can be used to monitor the response to therapy and as such constitute a method of clinical utility for early detection of therapy resistance. Digital droplet PCR was used to estimate the level of MAF of primary driver mutation at two consecutive time points during targeted therapy.

In line with clinical findings showing partial remission in Patient 1, no EGFR mutation was detected at the time points investigated (nine weeks and 18 weeks) (Fig 5). All extraction kits performed comparably (Table 7).

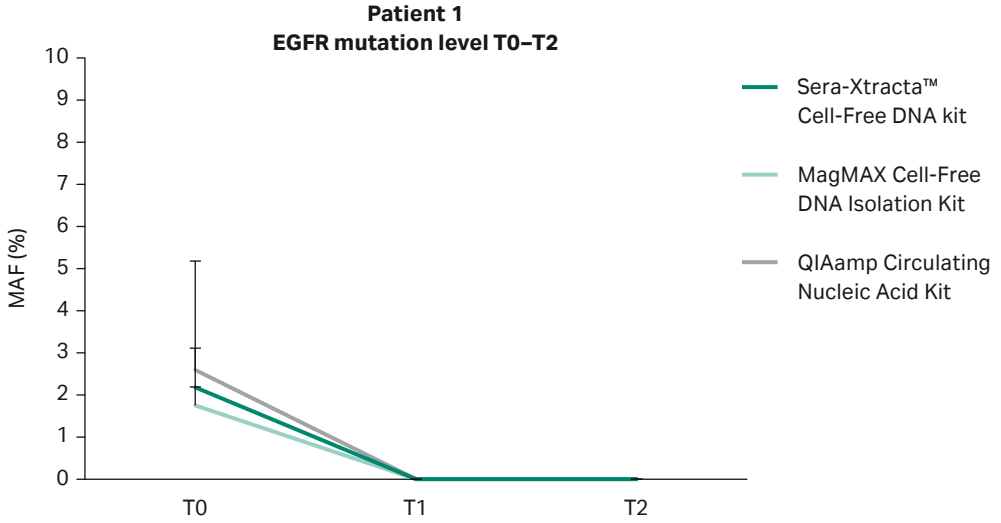

Fig 5. Monitoring tumor burden in response to targeted therapy in Patient 1: temporal changes in the level of primary driver mutation (EGFR del exon 19) in plasma evaluated with ddPCR at three consecutive time points (0, 9, and 18 weeks). A 1 mL volume of plasma was processed with three cfDNA extraction kits as described earlier.

Table 7. Temporal changes in MAF during targeted therapy (ddPCR) in plasma of Patient 1

Patient 1 (partial remission)	Mutation	Sera-Xtracta™ Cell-Free DNA	MagMAX Cell-Free Isolation DNA Kit	QIAamp Circulating Nucleic Acid Kit
		MAF	MAF	MAF
T0	EGFR del exon 19	2.18%	1.75%	2.59%
T1		0%	0%	0%
T1		0%	0%	0%

Based on clinical data provided, Patient 2 did not respond to targeted therapy and presented with progressive disease. This result is in agreement with the findings from plasma: in all samples extracted with the Sera-Xtracta™ Cell-Free DNA Kit, the presence of primary EGFR driver mutation was confirmed at all time points investigated albeit at different levels (Table 8). At baseline (T0) MAF was estimated to be around 5%. Although the level of MAF decreased to 1% at 11 weeks indicative of a decline in tumor burden, the treatment was not effective, and the disease progressed rapidly (MAF at 37% at T2; 31 weeks) (Fig 6).

The presence of cancer biomarker in plasma at T1 shows a clear utility of cfDNA-based analysis to track the disease progression before clinically recognized symptoms appear. Liquid biopsy monitoring provides an opportunity for additional testing to identify actionable biomarkers of tumor evolution and resistance and allows for a prompt re-adjustment of therapeutic approach. This approach is only viable with analytical methods that provide the highest possible sensitivity. In this context, only the Sera-Xtracta™ Cell-Free DNA Kit enabled detection of primary driver mutation at T1 (Table 8).

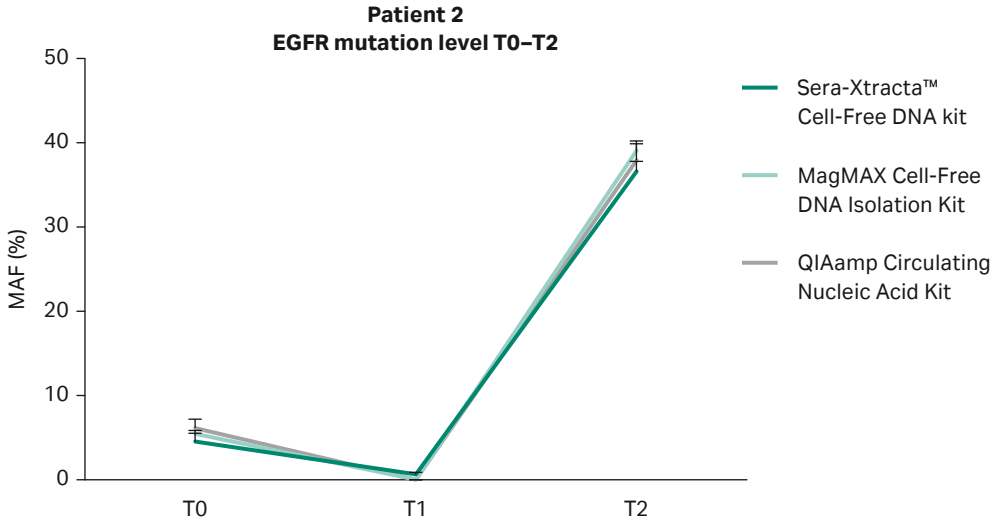

Fig 6. Monitoring of tumor burden in response to targeted therapy in Patient 2: temporal changes in the level of primary driver mutation (EGFR del exon 19) in plasma evaluated with ddPCR at three consecutive time points (0, 11, and 31 weeks). A 1 mL volume of plasma was processed with three cfDNA extraction kits as described earlier.

Table 8. Temporal changes in MAF during targeted therapy (ddPCR) in plasma of Patient 2

Patient 2 (non-responder)	Mutation	Sera-Xtracta™ Cell-Free DNA	MagMAX Cell-Free Isolation DNA Kit	QIAamp Circulating Nucleic Acid Kit
		MAF	MAF	MAF
T0	EGFR del exon 19	4.51%	5.43%	6.13%
T1		1%	0%	0%
T2		37%	39%	38%

Based on clinical data, Patient 3 presented symptoms of progressive disease despite a targeted treatment regime. This result is again reflected in the ddPCR results obtained from plasma samples processed with Sera-Xtracta™ Cell-Free DNA Kit (Fig 7, Table 9). At baseline, the MAF of primary driver mutation was estimated to be 1%. Two months later the mutation was undetectable in plasma, suggesting a decline in tumor burden beyond the level of assay sensitivity. However, at 21 weeks, MAF returned to baseline indicating therapy resistance. Significantly, no mutation was detected at baseline (T0) with any of the alternative kits (Table 9).

This outcome has important clinical implications. NSCLC is routinely diagnosed at a very advanced stage where tissue biopsy becomes unfeasible. In these circumstances, a liquid biopsy is considered a clinically relevant alternative for biomarker profiling (1). Based on the presence of an actionable biomarker, a treatment regimen which involves targeted therapy can be undertaken. However, in the absence of such a biomarker, treatment options become limited and usually involve chemotherapy. One could imagine a likely scenario in which a liquid biopsy would constitute the only available sample type for Patient 3, and the choice between different extraction kits could determine the therapeutic approach and ultimately influence clinical outcome.

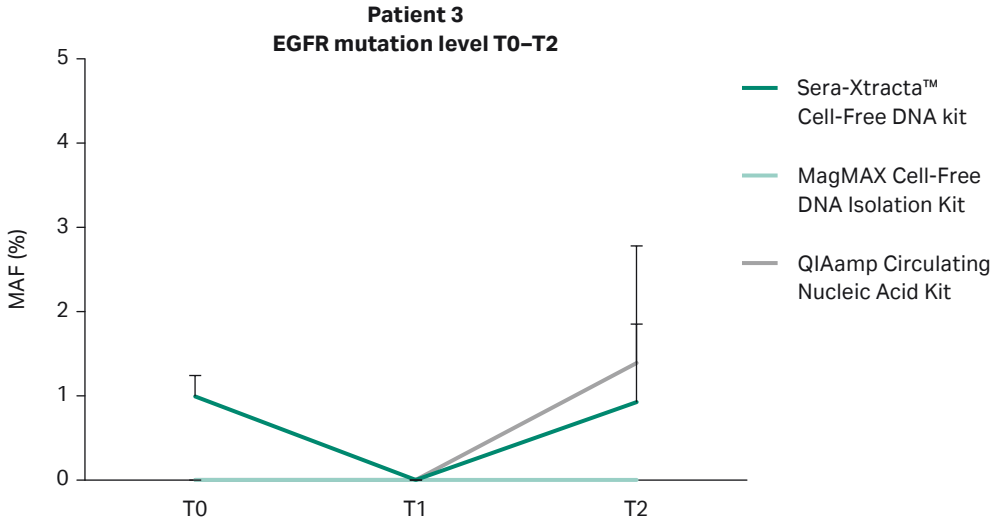

Fig 7. Monitoring tumor burden in response to targeted therapy in Patient 3: temporal changes in the level of primary driver mutation (EGFR del exon 19) in plasma evaluated with ddPCR at three consecutive time points (0, 8, and 21 weeks). A 1 mL volume of plasma was processed with three cfDNA extraction kits as described earlier.

Table 9. Temporal changes in MAF during targeted therapy (ddPCR) in plasma of Patient 3

Patient 3 (non-responder)	Mutation	Sera-Xtracta™ Cell-Free DNA	MagMAX Cell-Free Isolation DNA Kit	QIAamp Circulating Nucleic Acid Kit
		MAF	MAF	MAF
T0	EGFR Leu858Arg	1.0%	0.0%	0.0%
T1	0.0%	0.0%	0.0%	
T2	0.9%	0.0%	1.4%	

Concluding remarks

Personalized medicine is revolutionizing cancer treatment with unprecedented results in molecularly defined patient subgroups. This development has led to a dramatic shift in cancer treatment from using cytotoxic chemotherapy to patient specific biomarker-driven therapeutic approaches, resulting in significantly improved survival and considerable reduction in systemic side-effects. The implementation of precision medicine relies on the availability of a tumor sample for actionable biomarker profiling. In many circumstances, a solid tissue biopsy is not available. Thankfully for patients, liquid biopsy has become a clinically recognized sample type in cases where no alternative is available.

With the limited amount of total cfDNA and significantly lower amount of tumor derived fraction, the major factor that prevents wider use of liquid biopsies in biomarker testing is limited sensitivity of detection. The variables that affect detection sensitivity include both pre-analytical and analytical methods such as blood collection and sample processing, choice of cfDNA extraction method, and end-point detection assays (NGS versus qPCR versus ddPCR). In addition, the ultimate outcome is also affected by the cancer biology itself, including cancer type, stage, and tumor location, all of which influence the amount of tumor-derived fraction in the bloodstream. For these reasons, liquid biopsy profiling is recommended as an alternative to tissue biopsy only in advanced disease when tumor burden is expected to be high enough to enable confident detection of cancer biomarkers.

This study evaluated the use of cfDNA for biomarker profiling in a clinically relevant setting and highlighted the impact that the cfDNA extraction method can have on the sensitivity of biomarker detection and ultimately on patient outcomes. From three extraction methods tested, only samples processed with Sera-Xtracta™ Cell-Free DNA Kit showed 100% concordance with tissue biopsy results, allowing for confirmation of the presence of EGFR driver mutation in all patients at baseline. This report further demonstrated that with the Sera-Xtracta™ Cell-Free DNA Kit, the same sensitivity can be achieved when using an alternative detection method (ddPCR) with an extremely high correlation in MAF between NGS and ddPCR.

The suitability of cfDNA-based analysis for monitoring disease progression is an exciting application of liquid biopsy that has no alternative in solid tissue sampling. The clinical utility of cfDNA-based analysis for spatial and temporal tracking of tumor evolution for monitoring therapy response and emergence of secondary resistance in NSCLC has been confirmed in a number of studies (4, 5, 6, 7, 8). Of the extraction methods tested, only samples processed with Sera-Xtracta™ Cell-Free DNA Kit allowed for unequivocal detection of driver mutations at baseline in all patients using ddPCR, as well as early detection of therapy resistance in Patient 2. The results of this study also demonstrate that monitoring tumor burden through the analysis of the primary driver mutation level correlates well with clinical manifestation of disease progression and might constitute an important early indicator of resistance before radiologically visible changes can be detected.

This data is based on three independent experiments with the equal number of replicates in each experiment. All samples tested were treated equally (with the number of replicates being the same for all products tested in the comparison) and according to manufacturers' protocols and recommendations. Cell-free DNA extraction and ddPCR were carried out Biogazelle, Technologiepark-Zwijnaarde 82, 9052 Gent, Belgium in February 2020 and September-December 2020, respectively, and data are held at this location. NGS library prep, NGS, and data analysis were performed at Bio.be SA – groupe IPG, Avenue Georges Lemaître, 25, 6041 Gosselies, Belgium during April-July 2020, and data are held at this location.

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Detection of cancer-associated mutations in liquid biopsies for the identification of therapeutic targets

The effective capture of highly fragmented cfDNA and minimized co-purification of higher molecular weight DNA increases the probability of detecting mutations that constitute a minor proportion of genetic alterations present in the tumor.

Introduction

Personalized medicine relies on accurate and sensitive profiling of tumor genetic make-up for targeted therapy decisions, monitoring the response to treatment and detecting changes in tumor molecular landscape that result in secondary resistance. Successful implementation of targeted therapy is dependent on both the availability of tumor tissue and on comprehensive biomarker profiling that reflects tumor heterogeneity. Liquid biopsies that rely on the presence of the genetic material released by cells into biofluids such as blood and urine are now entering routine clinical practice (1). As DNA present in the bloodstream represents a pool of genetic material released by all cells, these approaches are non-invasive, allow for molecular profiling in circumstances when tumor tissue biopsy is not feasible, and, most importantly, have the potential to fully uncover tumor heterogeneity and detect genetic alterations associated with tumor development.

Characteristics of cell-free DNA

The predominant size of cell-free DNA (cfDNA) circulating in the blood corresponds to the size of DNA wrapped around a histone octamer, i.e. ~ 170 bp. It is believed that the size of cfDNA is highly dependent on its source, with tumor-derived cfDNA exhibiting significant fragmentation and lower size profile when compared to healthy individuals (2, 3). Based on those characteristics, any system that allows for enrichment of smaller, more degraded DNA fragments should be beneficial in detecting low-frequency mutations associated with tumor evolution and therapy induced resistance. Similarly, any method of cfDNA extraction that effectively eliminates the contamination of high molecular weight (HMW) genomic DNA (gDNA) originating from white blood cells would be advantageous, removing the background signal that further dilutes tumor-derived cfDNA.

Sera-Xtracta™ Cell-Free DNA extraction kit

Sera-Xtracta™ Cell-Free DNA Kit* has been designed to effectively capture highly fragmented cfDNA and to minimize co-purification of higher molecular weight DNA at the same time. This unique formulation increases the probability of detecting mutations that constitute a minor proportion of genetic alterations present in the tumor. Identification of these mutations is critical for undertaking a fully informed decision regarding therapy profile. It also facilitates the detection of early resistance variants that call for a prompt change in the therapeutic approach to increase the chances of a successful outcome.

*For research use only.

Cancer biomarker detection and mutation allele frequency in targeted next-generation sequencing (NGS)

Non-small cell lung cancer (NSCLC) is a leading cause of cancer mortality worldwide, mostly due to the fact that the diagnosis is made at the very late stage with metastasis present. The five-year survival rate is lower than 15% (4). Current guidelines advocate the use of molecular profiling in the evaluation of genetic drivers in NSCLC and support the use of cfDNA-based profiling from patients with insufficient tissue (5). Endothelial growth factor receptor (EGFR) is the most well-established mutation with both prognostic and predicted value and with the approved Food and Drug Administration (FDA) therapy (6). Other well recognized genomic alterations associated with NSCLC, for which a targeted therapy approach is available, include rearrangements in anaplastic lymphoma kinase (ALK), ROS proto-oncogene 1 (ROS1), and ret proto-oncogene (RET) genes; B-Raf proto-oncogene (BRAF) mutation; and met proto-oncogene (MET) amplification (7).

We have evaluated the performance of Sera-Xtracta™ Cell-Free DNA Kit using plasma from stage IV NSCLC patient samples.

Cell-free DNA yield and size profile

A 1 mL volume of plasma per patient obtained from blood collected in standard ethylenediaminetetraacetic acid (EDTA) and Heparin blood collection tubes was processed using Sera-Xtracta™ Cell-Free DNA Kit, MagMAX cfDNA Isolation kit (all but patient 1) (Thermo Fisher Scientific) and QIAamp MinElute ccfDNA kit (Qiagen) following the manufacturers' protocols. The results presented in Figure 1 show overall comparable performance for cfDNA recovery for all kits tested. No statistically significant difference was detected using mixed-effects model with Dunnett's multiple comparison test.

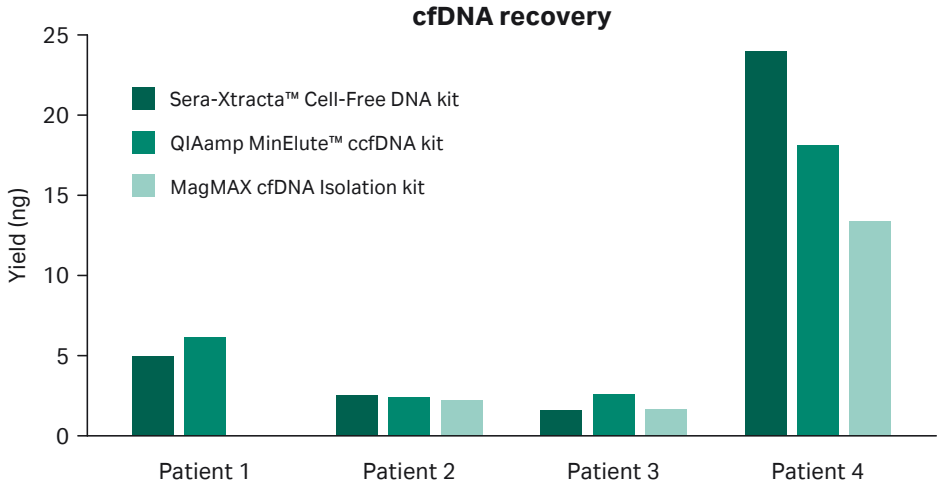

Fig 1. Cell-free DNA recovery from plasma following extraction with three different kits as described in the figure legend. Relative cfDNA yield and gDNA carry-over was calculated using smear analysis tool (2100 Expert software, Agilent) for fragments between 100–270 bp.

Data presented in Figure 2 indicate that Sera-Xtracta™ Cell-Free DNA Kit reduces the amount of gDNA carry-over in circumstances where a significant amount of white blood cell genetic material is released prior to plasma processing, as apparent for Patient 4. Note that no statistically significant difference was detected using mixed-effects model with Dunnett's multiple comparison test.

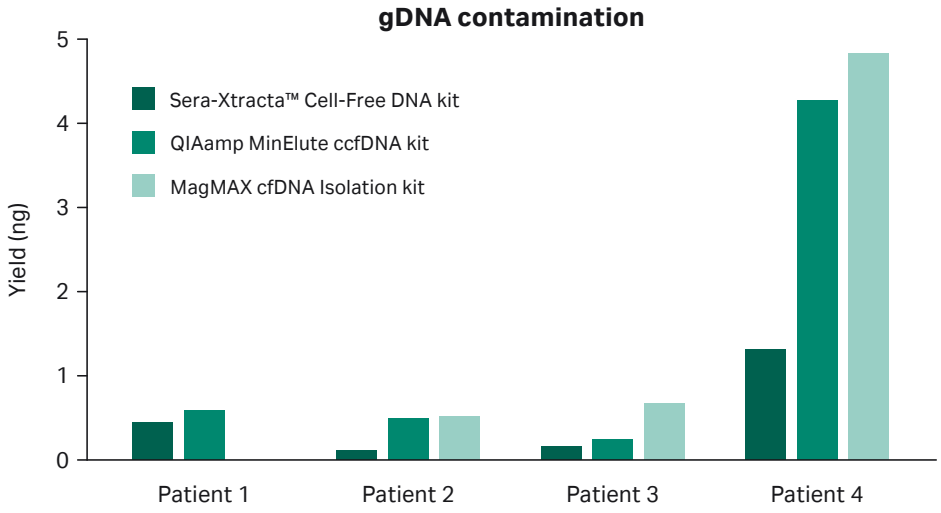

Fig 2. Carry-over of HMW DNA from plasma following extraction with three different kits as described in the figure legend. Relative HMW DNA yield was calculated using smear analysis tool (2100 Expert software, Agilent) for fragments above 700 bp.

Cancer biomarker detection and mutation allele frequency in targeted NGS

Extracted cfDNA was concentrated to 10.4 µL to allow for maximum input into library preparation. Samples were tested using the Target Selector NGS Lung Panel service (Biocept) and run alongside positive controls (Table 1). The panel allows for preparation of amplicon-based NGS libraries to detect somatic alterations in 12 clinically relevant lung cancer genes. Panel list and limit of detection (LOD) can be found in Table 2 and Table 3 respectively.

Table 1. Target Selector NGS Lung Panel positive controls run alongside clinical samples

Sample ID	SNVs and INDELs			CNVs			Fusions	
	Gene	MAF %	AA Chg	Gene	Gain / Loss	CNV ratio	Variant (exons)	Mol Cov. Mutant
Positive control	NRAS	0.5249	p.Q61R	MET	Gain	1.34	not determined	
	ALK	0.4327	p.G1202R					
	ALK	0.5446	p.F1174L					
	PIK3CA	0.8436	p.H1047R					
	EGFR	0.887	p.L747_P753delinsS					
	EGFR	0.2545	p.T790M					
	EGFR	0.536	p.L858R					
	BRAF	0.4318	p.V600E					
	KRAS	0.3642	p.Q61H					
	KRAS	0.7926	p.G12D					
	ERBB2	1.3353	p.A771_Y772insYVMA					

Table 2. Target Selector NGS Lung Panel gene list. Note that genes indicated in bold are referenced in National Comprehensive Cancer Network (NCCN) Guidelines and are targeted by FDA-approved therapy.

Target selector NGS Lung Panel gene list

Hotspot genes			CNVs	Fusions	Exon variants
ALK	KRAS	PIK3CA		ALK	
BRAF	MAP2K1	ROS1	MET	RET	MET exon 14 skipping
EGFR	MET	TP53		ROS1	
ERBB2	NRAS				

Table 3. Target Selector NGS Lung Panel LOD

Target selector NGS Lung Panel content

Assay input	DNA + RNA
Hotspot SNV/indel LOD	0.1% MAF
De novo LOD	0.5% MAF
CNV LOD	1.12X
Fusion/exon skipping LOD	3 molecular counts

The results presented in Table 4 show that samples extracted with Sera-Xtracta™ Cell-Free DNA Kit exhibit higher mutation allele frequency (MAF) in all samples tested, which is indicative of enrichment in tumor-derived fraction.

Table 4. The summary of MAF detected using Target Selector NGS Lung Panel for NCSC samples. Note that Patient 3 plasma yielded a suboptimal level of cfDNA for the NGS library prep that might account for the discrepancy in allele variant and MAF (i.e. not detected for MagMAX cfDNA Isolation kit).

	Patient 1 TP53 G245A	Patient 2 BRAF V600E	Patient 3 TP53	Patient 4 BRAF V600E
Sera-Xtracta™ Cell-Free DNA Kit	2.14%	2.15%	0.61% (Variant ID: R282W)	0.64%
QIAamp MinElute ccfDNA Kit	1.46%	0.26%	0.35% (Variant ID: C277Y)	0.27%
MagMAX cfDNA Isolation Kit	N/A	0.32%	Not detected	0.27%

Patients 2 and 4 were both identified as positive for mutation in BRAF gene at position 600, in which substitution of valine by glutamic acid leads to constitutively active Raf kinase and uncontrolled growth (6). The mutation is consistently detected in all samples processed with all kits; however, MAF, which is indicative of the relative content of tumor-derived cfDNA fraction, is consistently higher in samples processed with Sera-Xtracta™ Cell-Free DNA Kit. The advantage of minimizing gDNA carry-over is particularly evident for Patient 2, where the relative recovery of cfDNA (Fig 1) is similar between all kits tested but the amount of HMW contamination is considerably lower for samples extracted with the Sera-Xtracta™ kit. This results in at least 6.7x higher frequency of mutated allele detected.

Patients 1 and 3 were identified positive for mutation in tumor protein 53 (TP53), which is one of the most frequently mutated genes in human cancers and encodes tumor suppressor protein p53. Alterations in TP53 gene are found in 35% to 60% of NSCLC patients, more frequently in squamous cell carcinomas and patients with a smoking history (8). P53 is composed of three distinct domains with the DNA binding domain being key in tumor-suppressing function of the protein and the one representing mutation hotspot. Approximately 90% of point mutations occur in highly conservative sites including 175, 245, 248, 249, 273, and 282 (8). Two mutations in this hotspot region were identified in cfDNA extracted with the Sera-Xtracta™ kit (G245A and R282W in Patient 1 and Patient 3, respectively). Surprisingly, the results for Patient 3 were inconsistent

when comparing samples processed with competitor kits: the sample extracted with MagMax cfDNA Isolation kit failed to yield a positive signal for any of the cancer-associated mutations tested, while the sample processed with QIAamp MinElute ccfDNA kit detected an alternative TP52 mutation, i.e. C277Y. This mutation is not recognized as a hotspot single-nucleotide variant (SNV) and lies below the LOD of Target Selector NGS Lung Panel specified for de novo SNV (> 0.5%). As such, it would be interpreted as an inconclusive result.

Concluding remarks

The data presented strongly suggests that size selection-based extraction of cfDNA offers a distinct advantage in clinically relevant scenarios. Enrichment of tumor-derived fraction allows for detection of low frequency mutations that otherwise might be missed with standard extraction methods. The clear advantage of the Sera-Xtracta™ technology can be attributed to the three key features: efficient extraction of the main cfDNA peak, reduction in gDNA carry-over, and increased recovery of small fragments.

These data are based on three independent experiments with the equal number of replicates in each experiment and a fourth experiment with two replicates. All samples tested were treated equally (with the number of replicates being the same for all products tested in the comparison) and according to manufacturers' protocols and recommendations. Cell-free DNA extraction was carried out at Cytiva, The Maynard Centre, Forrest Farm Ind. Estate, Longwood Drive, Cardiff CF14 7YT (R&D Laboratory) during August 2019. NGS library prep, NGS, and data analysis were performed at Biocept Inc. 5810 Nancy Ridge Dr # 150, San Diego, CA 92121, United States (R&D Laboratory) during December 2019, and data are held at this location.

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Isolation of cfDNA in serum using Sera-Xtracta™ Cell-Free DNA Kit

Performance evaluation for Sera-Xtracta™ Cell-Free DNA Kit in a targeted NGS biomarker discovery workflow, using serum from stage IV non-small cell lung cancer (NSCLC) and colorectal cancer (CLC) patient samples.

By Monika Seidel PhD, Senior Development Scientist, Genomics and Cellular Research, Cytiva

Introduction

Liquid biopsy-based diagnostics using blood-based analysis of circulating cell-free DNA (cfDNA) holds great promise for early cancer screening, detection of minimal residual disease (MRD), and evaluating response to treatment.

For blood-based cfDNA analysis, both plasma and serum have been shown to be compatible with molecular profiling applications. However, plasma has become the sample of choice for cfDNA-based biomarker detection. This trend is well reflected in the number of scientific reports, the fast-growing market of specialized anticlotting blood collection tubes, and current workflows implemented by diagnostic laboratories.

The preferential use of plasma can be predominantly attributed to the requirement for allowing the blood to clot in serum processing. Blood clotting is associated with considerable release of genomic DNA (gDNA) from blood cells, which can result in significant loss in mutation detection sensitivity. Additionally, the clotting requirement is difficult to standardize.

The Sera-Xtracta™ Cell-Free DNA Kit* (Cytiva) has been designed to effectively capture cfDNA with minimal co-purification of higher molecular weight DNA. This kit has already been demonstrated to give a distinct advantage in cfDNA analysis when using plasma samples collected in both standard blood collection tubes and specialized cfDNA blood collection tubes. This application note presents results from research we made to verify the suitability of the Sera-Xtracta™ cfDNA Kit for efficient extraction of cfDNA with concomitant reduction in genomic DNA from serum samples.

*Sera-Xtracta™ cfDNA Kit is for research use only.

Biorepositories worldwide contain huge numbers of serum samples that are indispensable for retrospective large cohort studies for new cancer biomarker discovery. The identification of new mutations driving cancer development and progression constitutes the foundation of personalized medicine. This field relies on the discovery of novel targets for the development of targeted therapies that inhibit the mutated version of a given protein involved in cancer An efficient extraction of cfDNA from serum with concomitant reduction in gDNA is a critical tool for facilitating use of these serum samples in cancer-related research.

Extraction of cfDNA from serum of healthy donors

We collected 1 mL of serum from two healthy donors, used the samples for extraction with the Sera-Xtracta™ cfDNA Kit, and compared the results with those obtained using the MagMAX Cell-Free DNA Isolation Kit (Thermo Fisher Scientific). Fragment distribution of extracted sample(s) was analyzed using capillary electrophoresis on the 2100 Bioanalyzer (Agilent), with the High-Sensitivity DNA Kit (Agilent) (Fig 1A). For cfDNA recovery estimation, samples were run on the 7500 DNA kit (Agilent), and the DNA concentration within the main cfDNA peak was calculated with 2100 software smear analysis tool for fragments between 130 bp and260 bp (Fig 1B).

In agreement with the reported high level of gDNA in serum samples, representative electropherograms in Figure 1(A) show a significant amount of high molecular weight (HMW) DNA present in the eluted fraction for MagMAX Cell-Free DNA Isolation Kit, while the amount of gDNA carry-over is substantially reduced in the sample processed with Sera-Xtracta™ Cell-Free DNA Kit. More importantly, the yield of cfDNA is consistently and significantly higher ($p < 0.01$ based on paired t-test) in samples processed with the Cytiva product, indicating the superior performance of Sera-Xtracta™ Cell-Free DNA Kit in serum samples.

Performance in targeted next-generation sequencing (NGS): cancer biomarker detection in advanced stage cancer

Both non-small cell lung cancer (NSCLC) and colorectal cancer (CLC) are leading causes of mortality worldwide, with the five-year survival rate lower than 15% for the advanced stage (1, 2). NSCLC treatment has undergone tremendous progress reflected in current clinical practice, which involves routine testing for the presence of the genetic aberration in the five genes most frequently mutated in NSCLC, against which a targeted therapy has been approved (3). Unfortunately, targeted treatments for advanced CLC have not evolved as much and are currently restricted to vascular endothelial growth factor (VEGF) or epidermal growth factor (EGF) pathway inhibition, with decisions driven by the presence of additional predictive biomarkers (4).

Despite this significant shift from standard chemotherapy to precision medicine, which offers a prolonged progression-free survival and remarkable reduction in side-effects, there is still a significant number of patients who fail to respond to the first-line targeted therapy. Patients who do initially respond to targeted therapy eventually develop secondary resistance that allows the cancer to bypass drug-mediated inhibition, leading to rapid disease progression. The discovery of novel cancer biomarkers and secondary resistance mutations is critical to enable further progress in the field that will ultimately translate into successful outcomes in more patients.

To further demonstrate the suitability of Sera-Xtracta™ Cell-Free DNA Kit for serum samples, we have evaluated its performance in a NGS-based biomarker discovery workflow. For this purpose, we isolated cfDNA from 1 mL of serum from stage IV NSCLC and CLC patient samples with the Sera-Xtracta™ Cell-Free DNA Kit and the MagMAX Cell-Free DNA Isolation Kit and used them in targeted NGS.

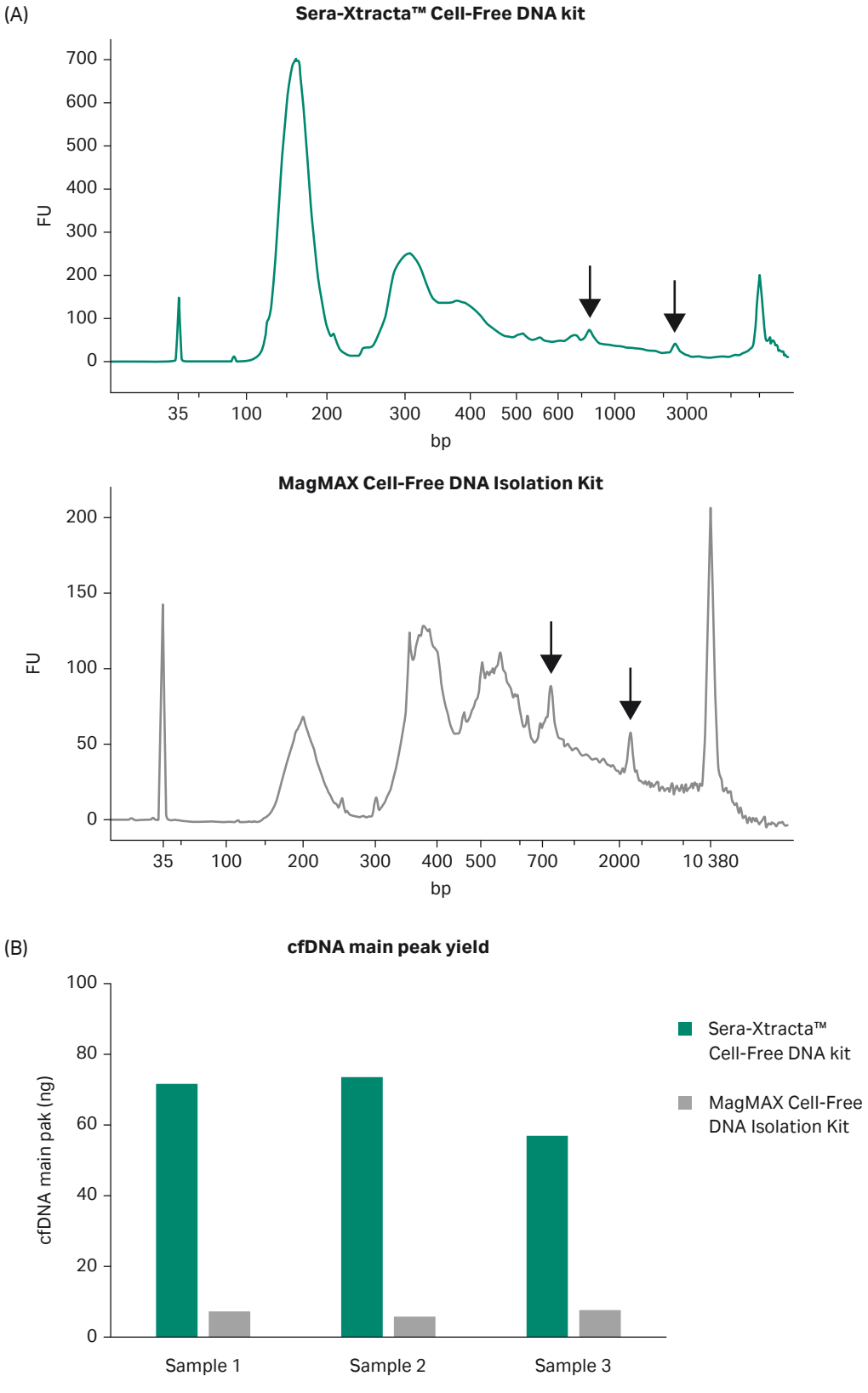

Fig 1. Extraction of cfDNA from serum samples from healthy donors. (A) Representative electropherograms showing DNA fragment distribution obtained from healthy donor serum following cfDNA extraction using Sera-Xtracta™ Cell-Free DNA Kit and MagMAX Cell-Free DNA Isolation kit. Extracted samples were analyzed on a 2100 Bioanalyzer using High-Sensitivity DNA Kit. Cell-free DNA is represented by a main peak centered around 170 bp, presence of high molecular weight (HMW) DNA represented by arrows. (B) Bar chart summarizing cfDNA recovery from serum calculated using smear analysis tool (2100 Expert software) for fragments between 130 and 260 bp following capillary electrophoresis on a 7500 DNA chip.

Cell-free DNA yield

We evaluated cell-free DNA yield as described above. Consistent with previous results, Sera-Xtracta™ Cell-Free DNA Kit outperformed MagMAX Cell-Free DNA Isolation Kit when used for extraction of cfDNA from serum (Fig 2).

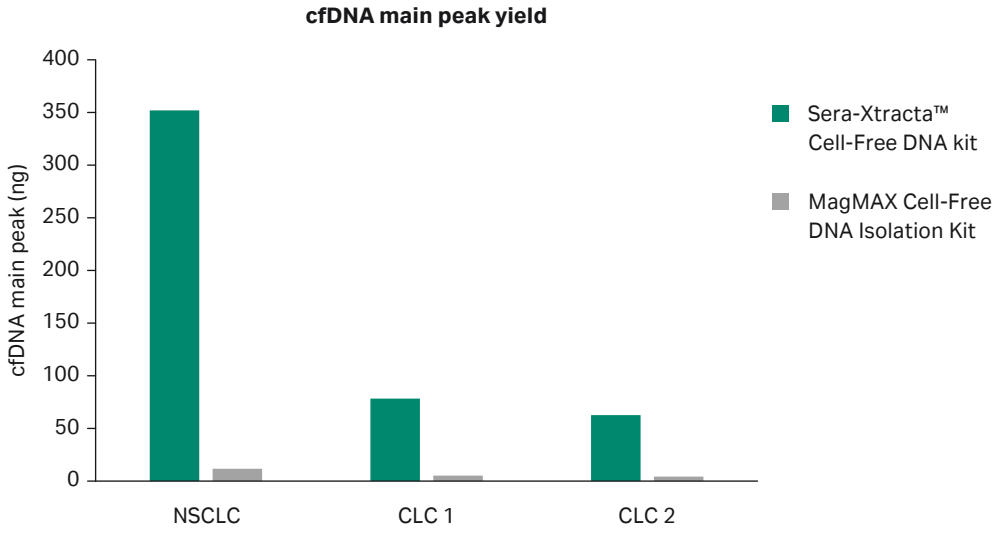

Fig 2. Extraction of cfDNA from serum samples from cancer patients. Bar chart summarizing cfDNA recovery following extraction with Sera-Xtracta™ Cell-Free DNA Kit and MagMAX Cell-Free DNA Isolation Kit. Cell-free DNA yield calculated using a smear analysis tool (2100 Expert software) for fragments between 120 and275 bp following capillary electrophoresis on a 2100 Bioanalyzer using the 7500 DNA Kit.

Targeted NGS library preparation

To prepare targeted NGS libraries using the hybridization-based approach, we used Trusight Tumor 170 RUO Kit (Illumina), which allows for the detection of somatic variants in 170 genes associated with cancer with > 95% sensitivity down to 5% mutation allele frequency (MAF). We used Qubit dsDNA HS Assay Kit (Invitrogen) to estimate total DNA concentration and adjusted DNA library input was according to manufacturer's recommendations (input range 30–120 ng of total DNA). For all samples processed with the MagMAX Cell-Free DNA Isolation Kit, we prepared libraries using total remaining volume of extracted samples (12 µL: with input ranging from 20–95 ng). For the Sera-Xtracta™ Cell-Free DNA Kit, an equivalent of 120 ng was used. All nine libraries were pooled following bead-based normalization and run on the NextSeq (Illumina) instrument using High-Output Kit (Illumina) with paired-end reads of 101 bp.

Data analysis

For data analysis we used Trusight Tumor 170 BaseSpace App version 2.0.0 (Illumina) using BWA-MEM alignment to the human hg19 genome. App output files were filtered for all single nucleotide variant (SNV) in the protein coding sequence. In the absence of matched normal tissues control (e.g. blood gDNA), germline mutations were filtered out assuming that any call with the frequency range between 45%–55% is highly likely to represent a germline heterozygote, and any call with MAF > 95% corresponds to a germline homozygote. The remaining SNVs, which fulfilled sensitivity criteria (i.e. > = 5% MAF with minimum of 250x coverage), were considered potential somatic mutations.

Results

All samples were successfully sequenced; however, substantial differences in the quality of NGS data were evident, as shown in Table 1. Extracted cfDNA from Sera-Xtracta™ Cell-Free DNA Kit yielded libraries in which at least 98% of targets reached the minimum required sequencing depth (250x) for all samples, while target coverage in samples processed with the MagMAX Cell-Free DNA Isolation Kit was highly variable, and, in one instance, was as low as 28.5% ($p < 0.01$ for mean target coverage, as shown in Figure 3A and 3B.). Similarly, library complexity was consistently higher for all samples extracted with the Cytiva Kit ($p < 0.05$ for unique reads as shown in Figure 3C). Observed compromised library quality can be attributed to lower extraction efficiency obtained with the MagMAX Cell-Free DNA Isolation Kit. The cell-free DNA extraction method developed by Cytiva minimizes sample processing-induced DNA damage, a phenomenon that creates sequencing artifacts such as chimeric reads (5). Consequently, the presence of chimeric reads was only detected in samples processed with MagMAX cfDNA Isolation kit. The total absence of chimeric reads in samples processed with Sera-Xtracta™ cfDNA Kit provides users with a representative, unaltered source of nucleic acid material that adequately represents input sample.

Table 1. Sequencing metrics as reported by Trusight Tumor 170 BaseSpace App version 2.0.0 (Illumina)

	NSCLC		CLC1		CLC2	
	Sera-Xtracta™ Cell-Free DNA	**MagMAX cfDNA kit**	**Sera-Xtracta™ Cell-Free DNA**	**MagMAX cfDNA kit**	**Sera-Xtracta™ Cell-Free DNA**	**MagMAX cfDNA kit**
Chimeric reads (%)	0	0.1	0	0.1	0	0.1
Exon coverage at 250X (%)	98.4	87.6	98.8	31.1	98.9	98.8
Target coverage at 250X (%)	98.2	86.1	98.7	28.5	98.8	98.5
Mean target coverage	2519.3	498.8	2343.5	189.7	2730	1179.3
Unique reads (%)	38.5	13.3	38.5	14.3	36.7	22.2

Concordant somatic SNV with potential clinical significance were detected in the serum of two patients (i.e. NSCLC and CLC 2) with comparable mutation allele frequency for both extraction methods (Table 2). However, it should be noted that, due to the much lower sequencing depth consistently obtained for samples processed with the MagMAX Cell-Free DNA Isolation Kit, the confidence of detection in those samples is compromised. In addition, due to the low cfDNA yield observed with the MagMAX Cell-Free DNA Isolation Kit, the entire eluted sample volume from the extraction step was used for the library preparation. As a result, there was no more sample available for further confirmatory testing, if required.

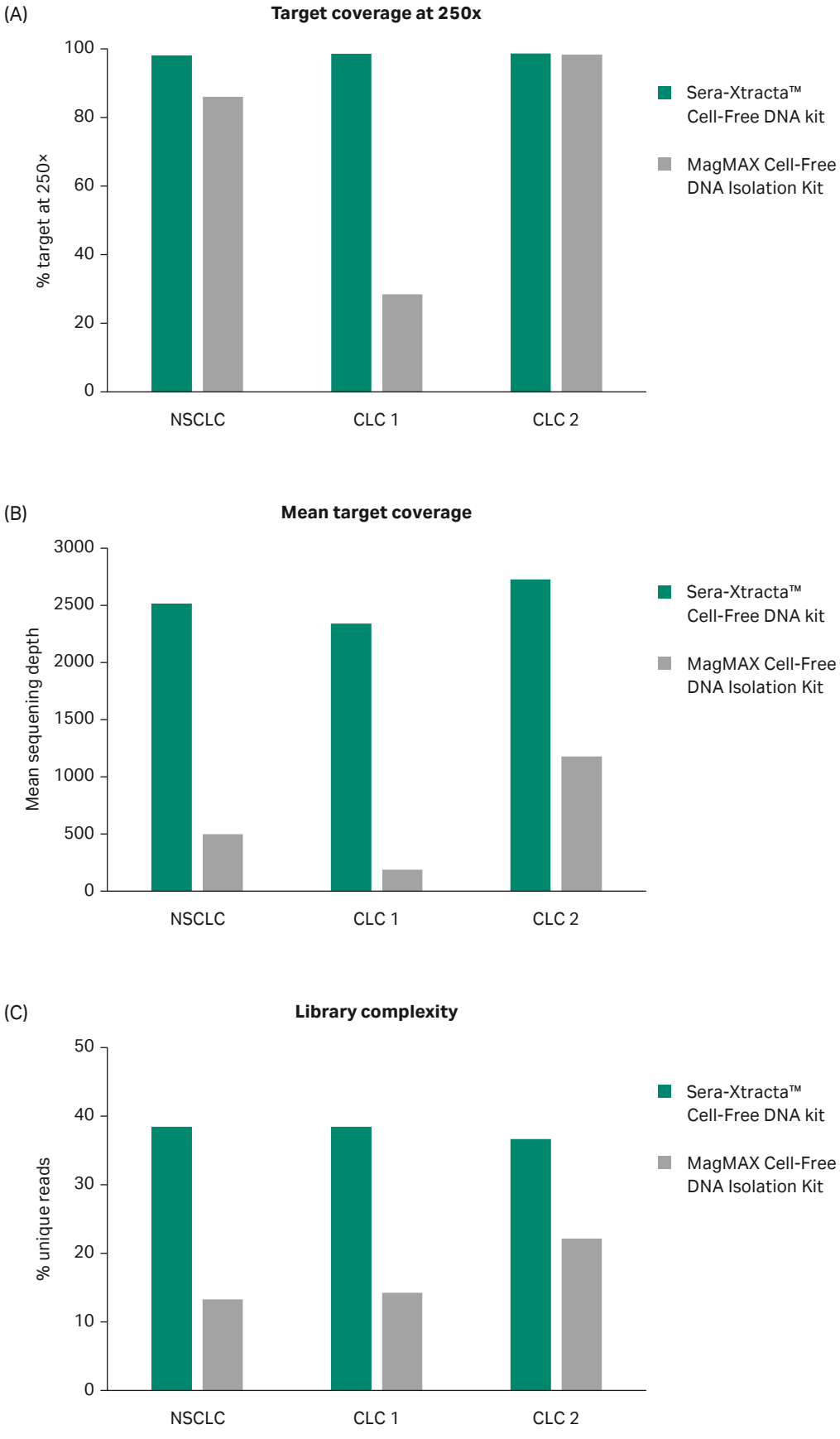

Fig 3. Summary of key NGS data metrics.

Table 2. Somatic SNV detected in serum. Reported mutant allele frequency (MAF) represents the percentage of calls at a specific genomic position that are mutated over a cumulative number of calls at this position [(mutant / (mutant + wildtype)].

			Sera-Xtracta™ Cell-Free DNA Kit		MagMAX cfDNA kit	
Sample	**Mutation**	**AA change**	**MAF**	**Sequencing depth**	**MAF**	**Sequencing depth**
NSCLC	JAK2	Val617Phe	13%	1725	15%	921
	NRG1	Met349Thr	33%	3479	35%	1751
CLC 2	IDH1	Arg132His	16%	2264	13%	342
	DNMT3A	Arg803Lys	17%	2328	12%	664

The V617F mutation in Janus kinase 2 (JAK2) gene identified in the serum of the patient with advanced NSCLC is well documented in myeloproliferative disorders and targeted by two FDA-approved drugs with proven clinical benefits for patients with advanced hematological malignancies (6). The role of JAK2 V617F mutation in NSCLC has been recognized only recently. It has been shown to predominantly coexist with other mutations at a substantially lower MAF level suggesting a sub-clonal origin and, as such, is a potential biomarker of tumor evolution (7). With growing evidence of the role JAK-STAT pathway in NSCLC, clinical trials of JAK inhibitors in patients with advanced NSCLCs are ongoing (https://clinicaltrials.gov).

The second mutation (NRG1: M349T) found in NSCLC serum samples has not been previously reported, and its pathogenicity and clinical utility remain unclear. Although rare, Neuregulin 1 (NRG1) mutations have been found in NSCLC; however, the predominant forms of genetic abnormalities involve gene fusions. The R132H mutation in isocitrate dehydrogenase (IDH1), identified in the patient with advanced CLC, is considered a direct driver of mutagenesis in glioma cancers and is suggested to be an important biomarker of clinical and prognostic significance (8). Although, only a few cases of IDH-1/2 mutations have been reported in CLC, IDH mutations are starting to be recognized as potential drug targets in patients with advanced stages of the disease (9). This recognition is reflected in ongoing clinical trials using IDH1 targeted inhibitors (NCT04584008, https://clinicaltrials.gov).

The second mutation identified in the serum of CLC patient (DNTM3A, R803K) is one of the most prevalent variants associated with acute myeloid leukemia (AML) and is considered of negative prognostic value (10). The role of this mutation in CLC is not well understood, but aberrant DNMT3A-mediated DNA methylation in CLC has been widely reported, indicative of the role of this enzyme in gastric cancers (11).

Concluding remarks

Personalized medicine has revolutionized cancer treatment with unprecedented results in molecularly defined patient subgroups. This development led to a dramatic shift in our approach to cancer treatment from cytotoxic chemotherapy targeting all rapidly dividing cells to patient specific biomarker driven therapeutic approaches with significantly improved survival and considerable reduction in systemic side-effects. The identification of novel mutations that might expose new therapeutic targets or prompt re-evaluation of already-approved drugs for new clinical indications is critical to drive progress in biomarker driven cancer therapy. In this respect, serum constitutes an important sample type that has great potential to facilitate the discovery of novel actionable biomarkers.

We have demonstrated that Sera-Xtracta™ Cell-Free DNA Kit efficiently extracts cfDNA from serum with superior extraction efficiency when compared to an alternative bead-based commercial product (MagMAX Cell-Free DNA Isolation Kit). It consistently yields cfDNA at a sufficient quantity for sensitive downstream applications such as next-generation sequencing with as little as 1 mL of serum input. In targeted NGS, the Sera-Xtracta™ Cell-Free DNA Kit outperforms MagMAX Cell-Free DNA Isolation Kit in the quality of cfDNA extracted. This result is reflected in the quality of the sequencing data such as higher library diversity, increased sequencing depth, and excellent target coverage, which are all necessary for confident SNV calls. In addition, in samples where a significant amount of gDNA is present, Sera-Xtracta™ cfDNA kit provides additional distinct advantage: it effectively reduces gDNA carry-over, ensuring maximum enrichment in cfDNA.

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Further information

Read more about liquid biopsy and cell-free DNA extraction in the following resources:

- Video: The Power of Liquid Biopsy
- Blog: Liquid biopsy in cancer diagnosis and treatment
- Blog: Liquid biopsy eases lung cancer testing for patients
- Liquid biopsy – a game changer
- Application webpage: Nuclei acid isolation
- Whitepaper: Investigating cell-free DNA in liquid biopsy
- Webinar: Detection of mutations from liquid biopsies – applications in cancer diagnostics
- Product webpage

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CY34334-08Feb23-CS