Comparative Molecular Analysis of CTCs and ctDNA Detection using ANGLE's Parsortix® System

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Introduction

Liquid biopsies offer a minimally invasive approach for cancer monitoring through the analysis of circulating tumour cells (CTCs) and circulating tumour DNA (ctDNA). The ability to detect tumour mutations in liquid biopsies may provide a powerful, non-invasive tool for real-time monitoring of tumour dynamics, which may enable rapid adjustments to clinical strategies. ANGLE's Parsortix® system is a microfluidic technology enabling the capture of viable CTCs from patient blood for downstream analysis. DNA analysis of both CTCs and cfDNA concurrently can provide a more comprehensive view of the genetic alterations present in a patient's cancer, as CTCs may harbour additional mutations not detected in cfDNA alone.

This study aims to provide Proof-Of-Concept demonstrating capabilities in detecting complementary information on genomic alterations between CTC and cfDNA in cancer patient samples.

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Workflow

Initial testing of the Illumina cfDNA prep with enrichment was conducted using cfDNA from the Mimix™ Multiplex I, cfDNA in Synthetic Matrix II Reference Standard. Replicates of three allelic frequencies (0.1%, 1% and 5%) were run preliminarily assess the repeatability and the accuracy of the assay.

Analytical performance of the assay was established using contrived samples consisting of cultured cancer cells spiked into healthy volunteer blood samples, drawn into PAXgene Blood DNA or EDTA blood collection tubes (BCTs). SK-MEL-28 and H1975 cells; known to harbour the following mutations – BRAF V600E, CDK4 R24C, PIK3CA G118D, PTEN T167A, TP53 R273H, EGFR L858R and T790M, were spiked to assess the degree of linearity and repeatability of the Illumina cfDNA Prep with Enrichment kit when processing samples through the Parsortix® system, by evaluating the detection of seven clinically relevant mutations.

After initial assessment of the assay, the same workflow was applied to samples obtained from 27 lung cancer patients. Mutation status of the tissue biopsy was unknown for all patients. Plasma was isolated by centrifugation and stored, while the cellular fraction was processed using the Parsortix system to enrich CTCs.

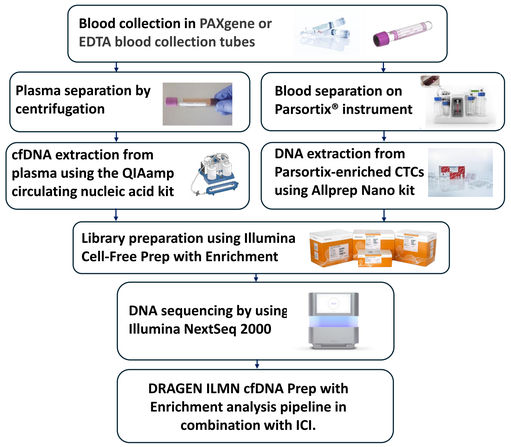


Figure 1. Schematic representation of the workflow. 7.5mL of peripheral blood was drawn into EDTA or PAXgene DNA tubes and stored up to 24 hours and 72 hours from collection before processing, respectively. 4 mL of Plasma was removed from each sample through centrifugation and replaced by PBS. The diluted cellular fractions were processed using the Parsortix® instrument, a microfluidic device capable of capturing and harvesting CTCs from bodily fluids based on cell size and lack of deformability. DNA was extracted from harvested CTCs and plasma (cfDNA) samples. All samples were subjected to enrichment-based sequencing using the Illumina Cell-Free Prep with Enrichment kit, which enriches for 79 clinically relevant lung cancer associated genes. Samples were sequenced on the NextSeq 2000 using the NEXT-LEAP P4 300 cycles flow cell. Sequencing data was analysed using the DRAGEN ILMN cfDNA Prep with Enrichment pipeline on basespace. Variant allele frequency (VAF%) data per variant was reported. Illumina connected insights (ICI) was utilised for analysis.

References

Cuadras, M. A, *et al.* Illumina Cell-Free DNA Prep with Enrichment: A custom enrichment assay that provides high sensitivity for somatic variants detection. (2024).

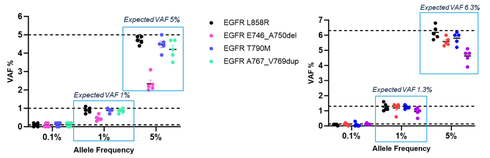
Analytical results

Accuracy and Repeatability

A

Variant	EGFR L858R			EGFR T790M			KRAS G12D		
VAF%	0.1%	1%	5%	0.1%	1%	5%	**0.13%**	**1.3%**	**6.3%**
Median	0.10	0.90	4.70	0.20	0.90	4.50	0.10	1.10	6.10
Mean	0.10	0.90	4.70	0.12	0.88	4.48	0.08	1.24	6.18
SD	0.10	0.16	0.21	0.11	0.11	0.40	0.04	0.25	0.43
%CV	100.00	17.57	4.51	91.29	12.45	8.84	55.90	20.24	7.00
Variant	EGFR E746_A750del			EGFR A767_V769dup			NRAS Q61K		
VAF%	0.1%	1%	5%	0.1%	1%	5%	**0.13%**	**1.3%**	**6.3%**
Median	0.00	0.40	2.20	0.20	0.90	4.20	0.10	1.30	5.50
Mean	0.08	0.46	2.34	0.12	0.86	4.20	0.10	1.18	5.58
SD	0.11	0.15	0.44	0.11	0.11	0.52	0.07	0.33	0.28
%CV	136.90	32.97	18.77	91.29	13.26	12.37	70.71	28.36	4.97
Variant	NRAS A59T			PIK3CA E545K					
VAF%	**0.13%**	**1.3%**	**6.3%**	**0.13%**	**1.3%**	**6.3%**			
Median	0.00	1.20	6.00	0.10	1.00	4.80			
Mean	0.08	1.24	5.80	0.12	0.94	4.62			
SD	0.13	0.11	0.40	0.04	0.27	0.46			
%CV	163.00	9.20	6.90	37.27	28.74	9.85			

B



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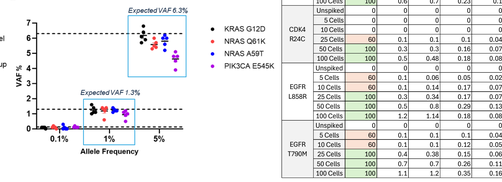
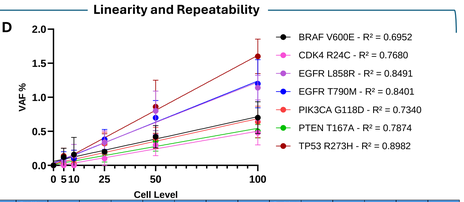


Linearity and Repeatability

D



E

Variant	Cell Level	Positive Call %	Median	Mean	SD	SE	%CV	Variant	Cell Level	Positive Call %	Median	Mean	SD	SE	%CV
BRAF V600E	Unspiked	0	0	0	0	0	0	PIK3CA G118D	Unspiked	0	0	0	0	0	0
	5 Cells	60	0.1	0.12	0.13	0.05	108.7		5 Cells	20	0	0.02	0.04	0.02	223.6
	10 Cells	60	0.1	0.16	0.25	0.11	156.9		10 Cells	20	0	0.02	0.04	0.02	223.6
	25 Cells	100	0.2	0.2	0	0	0		25 Cells	100	0.4	0.32	0.16	0.07	51.35
	50 Cells	100	0.3	0.42	0.16	0.07	39.12		50 Cells	100	0.4	0.4	0.16	0.07	39.53
	100 Cells	100	0.6	0.7	0.23	0.1	33.5		100 Cells	100	0.6	0.64	0.23	0.1	35.97
CDK4 R24C	Unspiked	0	0	0	0	0	0	PTEN T167A	Unspiked	0	0	0	0	0	0
	5 Cells	0	0	0	0	0	0		5 Cells	20	0	0.02	0.04	0.02	223.6
	10 Cells	0	0	0	0	0	0		10 Cells	20	0	0.02	0.04	0.02	223.6
	25 Cells	60	0.1	0.1	0.1	0.04	100		25 Cells	80	0.2	0.18	0.13	0.06	72.44
	50 Cells	100	0.3	0.3	0.16	0.07	52.7		50 Cells	100	0.4	0.36	0.15	0.07	42.13
	100 Cells	100	0.5	0.48	0.18	0.08	37.27		100 Cells	100	0.5	0.5	0.1	0.04	20
EGFR L858R	Unspiked	0	0	0	0	0	0	TP53 R273H	Unspiked	0	0	0	0	0	0
	5 Cells	60	0.1	0.06	0.05	0.02	91.29		5 Cells	80	0.1	0.14	0.11	0.05	81.44
	10 Cells	60	0.1	0.14	0.17	0.07	119.5		10 Cells	60	0.1	0.1	0.12	0.05	122.5
	25 Cells	100	0.3	0.34	0.17	0.07	49.22		25 Cells	100	0.4	0.38	0.08	0.03	22.02
	50 Cells	100	0.5	0.8	0.29	0.13	36.44		50 Cells	100	0.8	0.86	0.39	0.17	45.46
	100 Cells	100	1.2	1.14	0.18	0.08	15.94		100 Cells	100	1.6	1.6	0.25	0.11	15.93
EGFR T790M	Unspiked	0	0	0	0	0	0								
	5 Cells	60	0.1	0.1	0.1	0.04	100								
	10 Cells	60	0.1	0.1	0.12	0.05	122.5								
	25 Cells	100	0.4	0.38	0.15	0.06	39.03								
	50 Cells	100	0.7	0.7	0.26	0.11	36.42								
	100 Cells	100	1.1	1.2	0.35	0.16	29.46								

Figure 2. Analytical results of synthetic cfDNA and spiked-in CTC samples. (A, B, C) VAF% reported when using the Mimix™ Multiplex I, cfDNA in Synthetic Matrix II Reference Standard. (**A**) The descriptive statistics: median, mean, standard deviation of the mean (SD) and coefficient of variation (%CV). (**B, C**) Scatter plot showing the mean VAF% ± Standard Error of the Mean (SEM) reported for each of the six expected variants (EGFR L858R, , EGFR T790M, EGFR E746_A750del, EGFR A767_V769dup, KRAS G12D, NRAS Q61K, NRAS A59T and PIK3CA E545K), at the following three allele frequencies: 0.1%, 1% and 5%. (**D, E**) VAF% reported when accessing Parsortix® harvest enriched samples spiked will cancer cell lines. (**D**) Linear regression analysis for the seven expected variants. (**E**) Descriptive statistics: median, mean, SD, SEM, %CV for each expected variant.

Accuracy and Repeatability

- The assay was accurate and repeatable across the five technical replicates at 1% and 5% allelic frequencies (**Figures 2A, B, C**).
- Assay has good sensitivity for cfDNA, 100% of variants were positive at 1% allelic frequency and 6/8 variants were positive in 3/5 of the 0.1% allelic frequencies were at the expected value (**Figures 2A,B**).

Linearity and Repeatability

- Specificity of the expected variants was 100% (**Figure 2E**).
- There is a positive correlation observed when processing contrived samples through the NGS workflow. Increased VAF% were observed across the range of 0 – 100 cells (**Figure 2D**).
- 100% detection across five replicates was reported when ~ 25 cancer cells were spiked-in, for 5/7 of the expected variants, indicating good repeatability (**Figure 2E**).
- Positive VAF% were reported for samples spiked with only 5 or 10 cancer cells, indicating great sensitivity (**Figures 2D, E**).

Patients' results

A total of 27 lung cancer patient samples (total draws 39) were included in this study, with a CTC-DNA and cfDNA sample per draw.

- The number of variants detected in CTC-DNA-only or cfDNA-only samples, across the 39 samples ranged from between 8 – 987 and 8 - 50 variants respectively (**Figure.3A**).
- Across all patient samples, a higher percentage of variants were detected exclusively in CTC-only samples in comparison to cfDNA (**Figure. 3B**).
- The complementary percentages show significant variation across patient samples, ranging from 70.73% to 99.60%, reflecting substantial heterogeneity and highlighting the added value of integrating both CTC and cfDNA analysis to achieve a more comprehensive mutational profile (**Figure. 3C**).

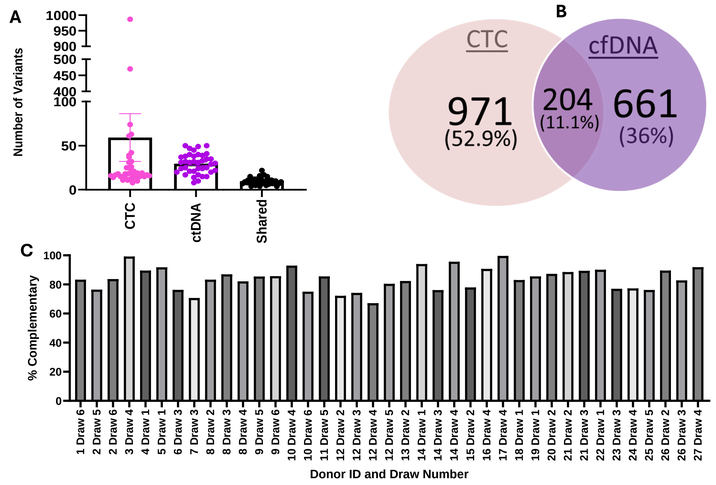
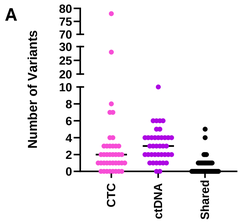


Figure 3. Positive variant calls on CTC and cfDNA. (A) A comparison of positive variant calls per donor for CTC-DNA, cfDNA and the number of variants shared between the two analytes. **(B)** Venn diagram summarizing the percentage of variant positive calls exclusive to each sample type and the percentage of shared variants. (**C**) % complementary variants identified for CTC-DNA and cfDNA samples per donor, the % missing is where the same variant is identified in both analytes.

Oncogenic Mutational Status

The mutation calls for the 39 patient draws were filtered to visualise oncogenic mutation detected in both CTC and cfDNA samples, annotations were added by utilising Illumina's connective insights (ICI).

A

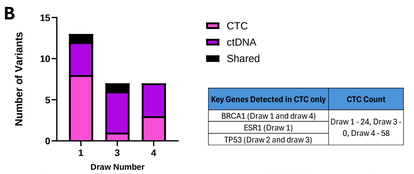


- Oncogenic variants were detected in 100% of patients.
- 22/39 (56.4%) draws demonstrated non-overlapping oncogenic mutations between CTC-DNA and cfDNA samples, highlighting the complementary nature of these two analytes in capturing the full picture of tumour heterogeneity (**Figure 4A**).
- The number of variants detected per CTC sample ranged from 0 – 93. With most samples for both CTC and cfDNA detecting ≤ 10 oncogenic variants (**Figure 4A**).
- In case study 1 **(Figure 4B)**, three sequential draws from the same donor revealed fluctuations in the number of oncogenic variants in both CTC-DNA and cfDNA samples. Notably, key variants (*TP53*) were absent in the most recent draw, reflecting the dynamic nature of tumour biology and the complementary insights provided by analysing both analytes over time.
- In Case Study 2 (**Figure 4C**), four sequential draws from the same donor revealed that the number of oncogenic variants in CTC samples from draws 2 and 4 exceeded those in the corresponding cfDNA samples. Additionally, key mutations such as *RET* were consistently detected across all four CTC samples but were absent in cfDNA, underscoring the complementary nature of CTC and cfDNA analyses in capturing additional oncogenic and potentially actionable information

Age	Sex	Cancer Type	Stage	TNM	Metastasis	Mutational Status
72	Female	Lung, Ovarian (High grade serous	4	Not specified	Abdomen, Ascites, Chest Wall, Liver, Lymph Node(s), Pleural effussion	P53 mutated

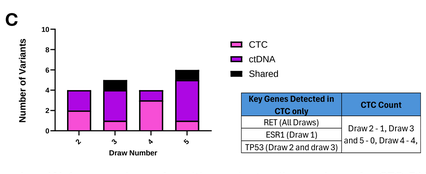
Age	Sex	Cancer Type	Stage	TNM	Metastasis	Mutational Status
81	Male	Lung Adenocarcinoma	Not specified	T4N3M1a	Small pleasal effussion	Unknown

B



Key Genes Detected in CTC only	CTC Count
BRCA1 (Draw 1 and draw 4)	Draw 1 - 24, Draw 3 - 0, Draw 4 - 58
ESR1 (Draw 1)	
TP53 (Draw 2 and draw 3)	

C



Key Genes Detected in CTC only	CTC Count
RET (All Draws)	Draw 2 - 1, Draw 3 and 5 - 0, Draw 4 - 4,
ESR1 (Draw 1)	
TP53 (Draw 2 and draw 3)	

Figure 4. Positive oncogenic variant calls on CTC-DNA and cfDNA samples. (A) A comparison of positive variant calls per donor for CTC-DNA, cfDNA and the number of variants shared between the analytes. **(B)** The table shows patients age, sex, cancer type, TNM, metastasis and mutational status. Plot shows the number of oncogenic variants reported per draw and analyte. Table shows CTC count per draw key variants reported across three draws from a single patient. **(C)** The table shows patients age, sex, cancer type, TNM, metastasis and mutational status. Plot shows the number of oncogenic variants reported per draw and analyte. Table shows CTC count per draw and key variants reported across four draws from a single patient.

Conclusions

- The analytical data showcases the ability of detecting mutations in Parsortix-enriched CTC samples, using Illumina cfDNA Prep with Enrichment, from spiked cancer cell lines with good sensitivity and specificity across multiple actionable variants.
- Actionable oncogenic variants were exclusively detected in the CTC-DNA and cfDNA samples, with a high number of exclusive mutations reported in CTC samples and not in cfDNA.
- The added value of CTC profiling using the Parsortix® systems ability to capture a comprehensive picture of the tumour's genetic heterogeneity. By interrogating both CTCs and cfDNA in parallel, this integrated approach holds promise for potentially guiding personalized cancer treatment selection, monitoring therapeutic responses, and improving overall clinical management strategies for patients.