Custom Gene Expression Profiling of Circulating Tumor Cells via Amplitude-Based Multiplex digital PCR

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Introduction

Liquid biopsies provide a minimally invasive approach for cancer monitoring. Among them, circulating tumour cells (CTCs) offer unique advantages for longitudinal assessment, as they enable analysis of several analytes such as RNA and DNA. This makes CTC analysis a promising tool for patient stratification and potentially for guiding tailored therapeutic interventions (1).

In this study, we evaluated an amplitude-based digital PCR (dPCR) method for gene expression analysis from CTCs isolated using ANGLE's Parsortix® system (Research Use Only). This approach increases the multiplexing capability of dPCR and can be customized to investigate gene expression profiles across different cancer types.

We designed a panel of 6 genes, including KRT19, FN1 and PTPRC as epithelial, epithelial–mesenchymal transition (EMT) and Peripheral Blood Mononuclear Cells biomarkers, and HER2, ESR1 as breast cancer biomarkers and MKI67, a cell proliferation marker. These markers are currently used for breast cancer subtyping in several commercially available IVD kits. Here, we demonstrate the ability to detect differential expression of these biomarkers in blood samples spiked with as few as two cancer cells, enriched using ANGLE's proprietary Parsortix system. Furthermore, we tested our end-to-end workflow (Figure 1) in breast cancer patient samples, confirming its ability to detect gene overexpression in a clinical setting.

The results presented are for research purposes only and are not intended for use in diagnostic procedures.

Materials and methods

Analytical performance of the end-to-end workflow (Figure 1) was evaluated using contrived samples consisting of cultured cancer cell lines (MCF7, SKBR3 and Hs-578T) spiked into healthy donor blood collected in K2EDTA tubes (BD). Cell lines were selected based on the expression of the genes under investigation to assess linearity and repeatability of the workflow following enrichment with the Parsortix® system. Expression of the following genes was assessed by using predesigned TaqMan™ Gene Expression Assays (ThermoFisher): KRT19, FN1, PTPRC, HER2, ESR1, and MKI67.

mRNA was extracted using the Dynabeads® mRNA DIRECT Micro Kit (ThermoFisher). cDNA synthesis was performed using Superscript IV VILO Master Mix (ThermoFisher), and 1 µL of cDNA was used for downstream analysis. Digital PCR was carried out on a QIAcuity Four instrument using the QIAcuity High Multiplex Probe PCR Kit, with data analysis performed in the QIAcuity Software Suite using the amplitude multiplexing option.

For specificity assessment, blank (unspiked) samples were included. After initial assessment of the end-to-end workflow, the same approach was applied to blood samples obtained from 20 breast cancer patients.

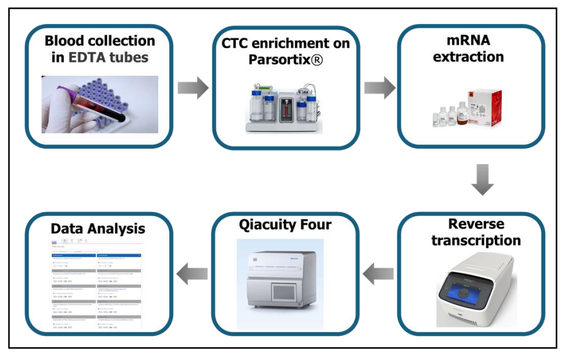
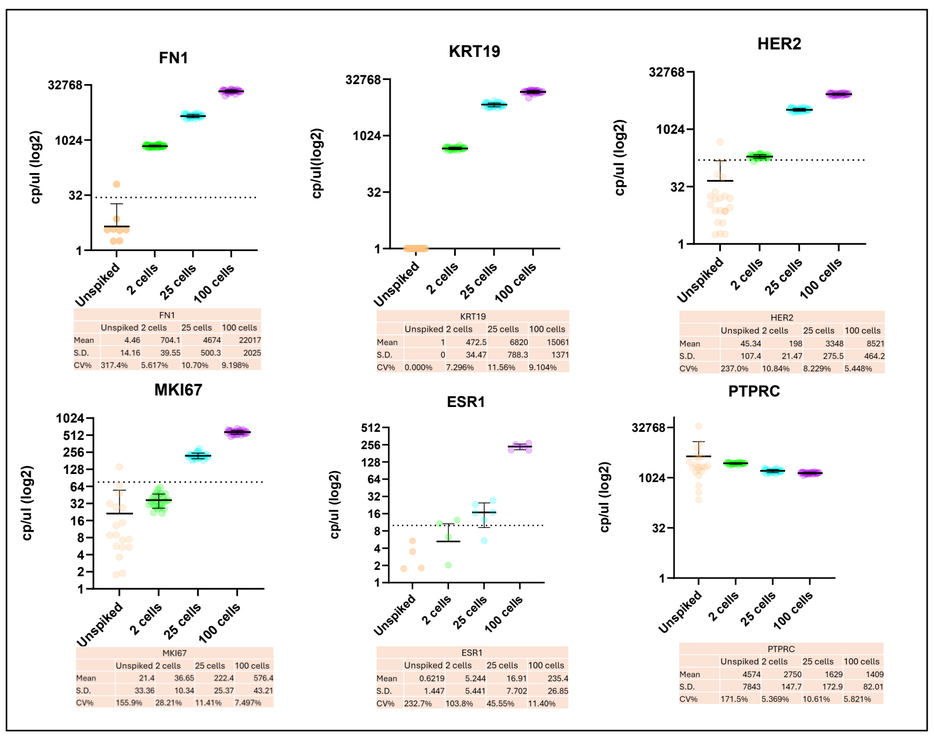


Fig. 1: Visual summary of the end-to-end workflow for gene expression analysis of CTCs

Analytical Results of the end-to-end workflow

Specificity and Repeatability



FN1	Unspiked 2 cells	25 cells	100 cells
Mean	4.46	704.1	4674	22017
S.D.	14.16	39.55	500.3	2025
CV%	317.4%	5.617%	10.70%	9.198%

KRT19	Unspiked 2 cells	25 cells	100 cells
Mean	1	472.5	6820	15061
S.D.	0	34.47	788.3	1371
CV%	0.000%	7.296%	11.56%	9.104%

HER2	Unspiked 2 cells	25 cells	100 cells
Mean	45.34	198	3348	8521
S.D.	107.4	21.47	275.5	464.2
CV%	237.0%	10.84%	8.229%	5.448%

MKI67	Unspiked 2 cells	25 cells	100 cells
Mean	21.4	36.65	222.4	576.4
S.D.	33.36	10.34	25.37	43.21
CV%	155.9%	28.21%	11.41%	7.497%

ESR1	Unspiked 2 cells	25 cells	100 cells
Mean	0.6219	5.244	16.91	235.4
S.D.	1.447	5.441	7.702	26.85
CV%	232.7%	103.8%	45.55%	11.40%

PTPRC	Unspiked 2 cells	25 cells	100 cells
Mean	4574	2750	1629	1409
S.D.	7843	147.7	172.9	82.01
CV%	171.5%	5.369%	10.61%	5.821%

Figure 1. Specificity and repeatability studies. Specificity results are shown here as unspiked for each target (n=20). Dotted line indicates the threshold between background signal and signal from spiked-in cells. Table at the bottom of each graph shows mean copies/ul, Standard Deviation and % Coefficient of Variation for each target at each spike level.

Linearity

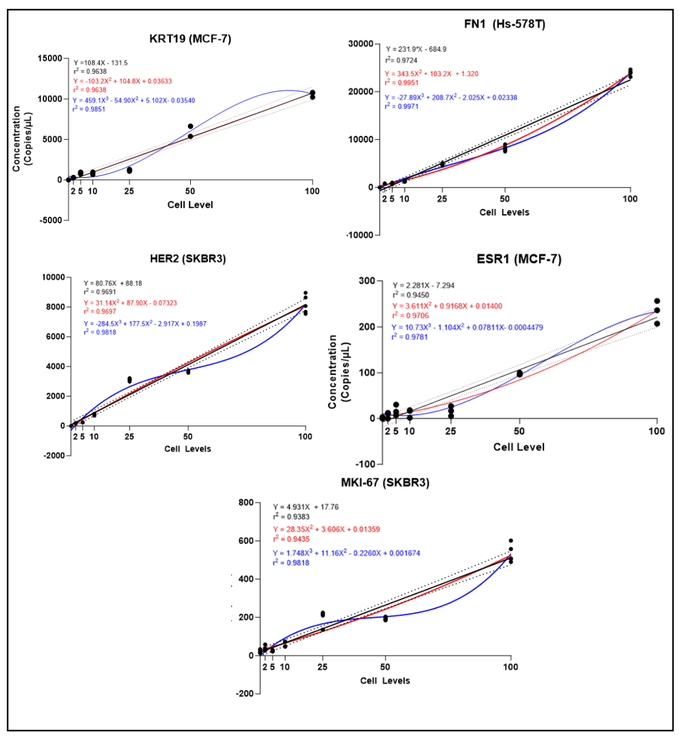


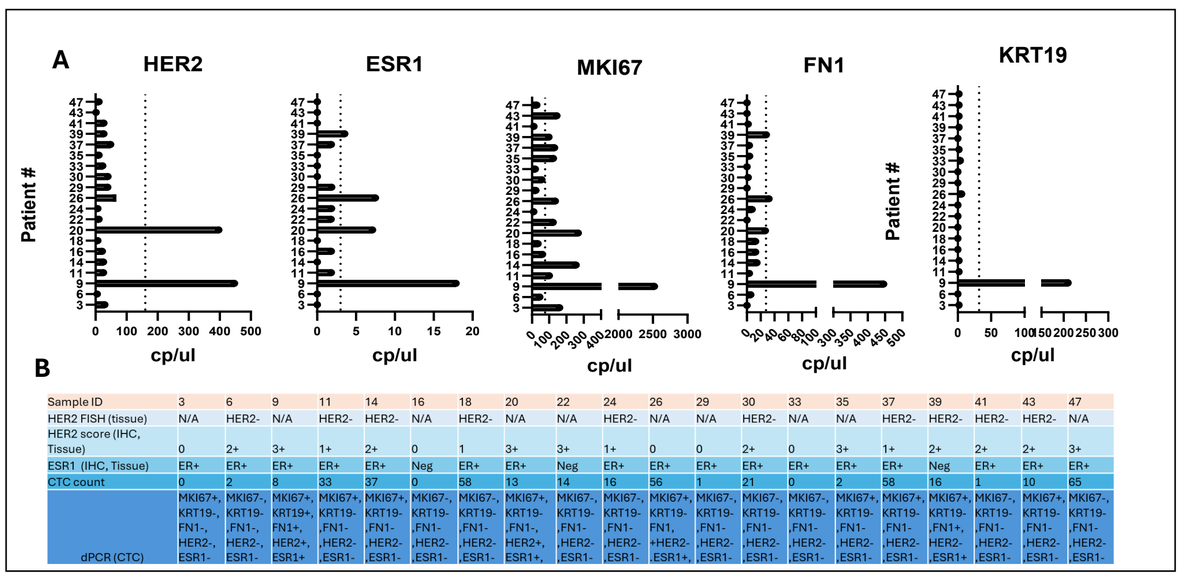
Figure 2. Linearity of the end-to-end workflow. 1st, 2nd and 3rd degree linearity was calculated for each target by spiking whole blood of healthy volunteers with varying cell numbers (2–100). Each degree of linearity is represented respectively in black, red and blue. The assay demonstrated linear performance across all markers within this range.

Specificity: The workflow shows high specificity and enables clear discrimination between signal from spiked-in cells and background (**Figure 1**).

Repeatability: The end-to-end workflow is highly repeatable for all biomarkers at 3 different levels of spiked cells (**Figure 1**).

Linearity: The assay produced linear results through the end-to-end workflow across the range of 0 – 100 cells (R^2 = 0.938– 0.972) for 5 biomarkers (**Figure 2**). 100% detection across three replicates was reported when 2 cancer cells were spiked for KRT19, FN1, and HER2, 5 cancer cells for ESR1 and 25 cancer cells for MKI-67.

Patients' Results



Sample ID	3	6	9	11	14	16	18	20	22	24	26	29	30	33	35	37	39	41	43	47
HER2 FISH (tissue)	N/A	HER2-	N/A	HER2-	HER2-	N/A	HER2-	N/A	N/A	HER2-	N/A	N/A	HER2-	N/A	N/A	HER2-	HER2-	HER2-	HER2-	N/A
HER2 score (IHC, Tissue)	0	2+	3+	1+	2+	0	1	3+	3+	1+	0	0	2+	0	3+	1+	2+	2+	2+	3+
ESR1 (IHC, Tissue)	ER+	ER+	ER+	ER+	ER+	Neg	ER+	ER+	Neg	ER+	ER+	ER+	ER+	ER+	ER+	ER+	Neg	ER+	ER+	ER+
CTC count	0	2	8	33	37	0	58	13	14	16	56	1	21	0	2	58	16	1	10	65
dPCR (CTC)	MKI67+, KRT19-, FN1-, HER2-, ESR1-	MKI67-, KRT19-, FN1-, HER2-, ESR1-	MKI67+, KRT19+, FN1+, HER2+, ESR1+	MKI67+, KRT19-, FN1-, HER2-, ESR1-	MKI67+, KRT19-, FN1-, HER2-, ESR1-	MKI67-, KRT19-, FN1-, HER2-, ESR1-	MKI67-, KRT19-, FN1-, HER2-, ESR1-	MKI67+, KRT19-, FN1-, HER2+, ESR1+,	MKI67-, KRT19-, FN1-, HER2-, ESR1-	MKI67-, KRT19-, FN1-, HER2-, ESR1-	MKI67+, KRT19-, FN1, +HER2-, ESR1+,	MKI67-, KRT19-, FN1-, HER2-, ESR1-	MKI67-, KRT19-, FN1-, HER2-, ESR1-	MKI67-, KRT19-, FN1-, HER2-, ESR1-	MKI67+, KRT19-, FN1-, HER2-, ESR1-	MKI67+, KRT19-, FN1-, HER2-, ESR1-	MKI67+, KRT19-, FN1+, HER2-, ESR1+	MKI67-, KRT19-, FN1-, HER2-, ESR1-	MKI67+, KRT19-, FN1-, HER2-, ESR1-	MKI67-, KRT19-, FN1-, HER2-, ESR1-

Figure 3. Analysis of 20 patient samples using the end-to-end workflow. (A) Dotted line indicates the positivity threshold. (B) Table shows Sample ID, CTC count, HER2 status in tissue biopsy by IHC and IF, ESR1 status and dPCR results for CTCs. CTC count was performed using ANGLE CTC enumeration workflow by IF (2).

- Our end-to-end workflow successfully detected differential expression of breast cancer biomarkers in patient samples (**Figure 3**).
- dPCR results were compared with CTC counts, immunofluorescence, and FISH analysis from tissue biopsies (**Figure 3**).
- dPCR-based CTC analysis showed a positive correlation in 18/20 cases with HER2 status determined by tissue biopsy (**Figure 3**) when both FISH and IHC staining are considered.
- In one case (sample 39), a patient classified as ER-negative by tissue biopsy was identified as ER-positive in CTCs (**Figure 3**).

Conclusions

- This proof-of-concept study demonstrates that our proposed workflow enables the highly specific and sensitive detection of CTCs, enriched from breast cancer patient blood sample, processed with Parsortix system, paired with Qiacuity platform.
- Analysis of clinical samples shows that CTC profiling correlates with tissue biopsy results, while also showing potential for real-time monitoring of tumor evolution for timely intervention.
- This workflow is readily expandable to investigate additional actionable genes across different tumor types.

References: (1) Fehm T., *et al.* Efficacy of Lapatinib in Patients with HER2-Negative Metastatic Breast Cancer and HER2-Positive Circulating Tumor Cells-The DETECT III Clinical Trial Clin Chem . 2024 Jan 4;70(1):307-318
(2) Ciccioli M. *et al.* Identification of circulating tumor cells captured by the FDA-cleared Parsortix® PC1 system from the peripheral blood of metastatic breast cancer patients using immunofluorescence and cytopathological evaluations. *J Exp Clin Cancer Res* **43**, 240 (2024).

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