# Enhancing Multiplexing Capabilities in Digital PCR for Circulating Tumour Cell Analysis Using Amplitude-Based methodology

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## Introduction

Liquid biopsies represent a revolutionary approach in cancer diagnostics and monitoring, offering a non-invasive alternative to traditional tissue biopsies. While tissue biopsies provide a static snapshot of tumour characteristics, circulating tumour cells (CTCs) enable real-time monitoring of tumour evolution and heterogeneity. Despite their clinical significance, CTC analysis faces technical challenges due to their rarity in blood (1-10 CTCs per millilitre) and heterogeneous nature.

The molecular characterization of CTCs is particularly valuable in metastatic disease, where tissue biopsies may be challenging to obtain or may not represent the current state of the disease. Recent advances in digital PCR (dPCR) technology, combined with multiplexing capabilities, have enhanced our ability to perform precise molecular characterization of CTCs from limited cellular material. This technological progress opens new possibilities for comprehensive CTC analysis, potentially revolutionizing personalized cancer treatment approaches through more accurate monitoring of disease progression and treatment response.

## Workflow

Blood samples collected from healthy volunteers (n=6) were spiked with SKBR3 and Hs 578T breast cancer cells. Cancer cells were enriched using Parsortix® system and extracted cells were used for mRNA analysis on dPCR QIAcuity platform using amplitude-based multiplexing (Figure 1). Data analysis was performed using QIAcuity Software Suite with automatic amplitude-based signal classification. Positive detection was defined by ≥3 positive partitions per sample, with a minimum total droplet count of 10,000 per well. The same workflow has been used to process ten metastatic breast cancer patients.

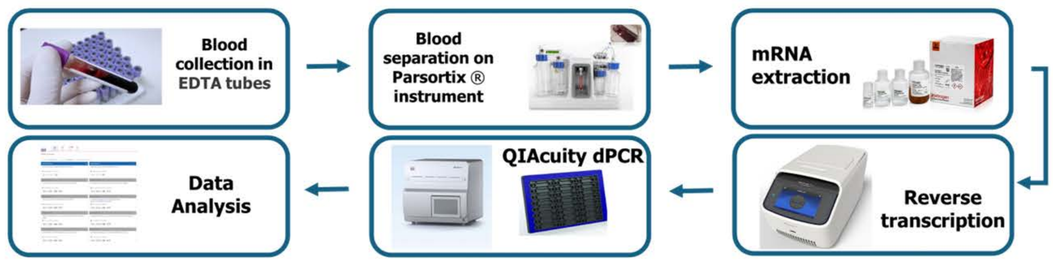


***Figure 1:*** *Experimental workflow showing sequential steps from blood collection through CTC enrichment to digital PCR analysis using amplitude-based multiplexing.*

**Method**: Amplitude-based multiplexing approach where multiple targets are detected in the same fluorescent channel (FAM or VIC) by varying probe concentrations. Each target was assigned a specific probe concentration to generate distinct fluorescence amplitude clusters, enabling simultaneous detection of up to 4 targets per channel.

## Assay Design

Eight gene targets were selected to create a comprehensive cancer panel: epithelial markers (*KRT19*, *EpCAM*, *KRT7*), oncogenes (*ERBB2*, *EGFR*), hormone receptor (*ESR1*), and EMT-associated genes (*FN1*, *CDH1*, *AGR2*). Amplitude-based multiplexing was established by systematically optimizing TaqMan probe concentrations (0.4-1.6 µM) across FAM and VIC channels. The assay development progressed from single-plex testing through duplex combinations, culminating in quadruplex validation using reference RNA and spiked blood samples (Figure 2). This design enabled analysis of all eight targets in just two dPCR reactions while maintaining sensitivity. Step 1 shows consistent amplitudes across different probe concentrations in single-plex reactions. Step 2 confirms successful separation of two targets in FAM duplex reactions. Step 3 demonstrates quadruplex capability using reference RNA, while Step 4 validates the approach in spiked blood samples, showing clear population separation for all four targets.

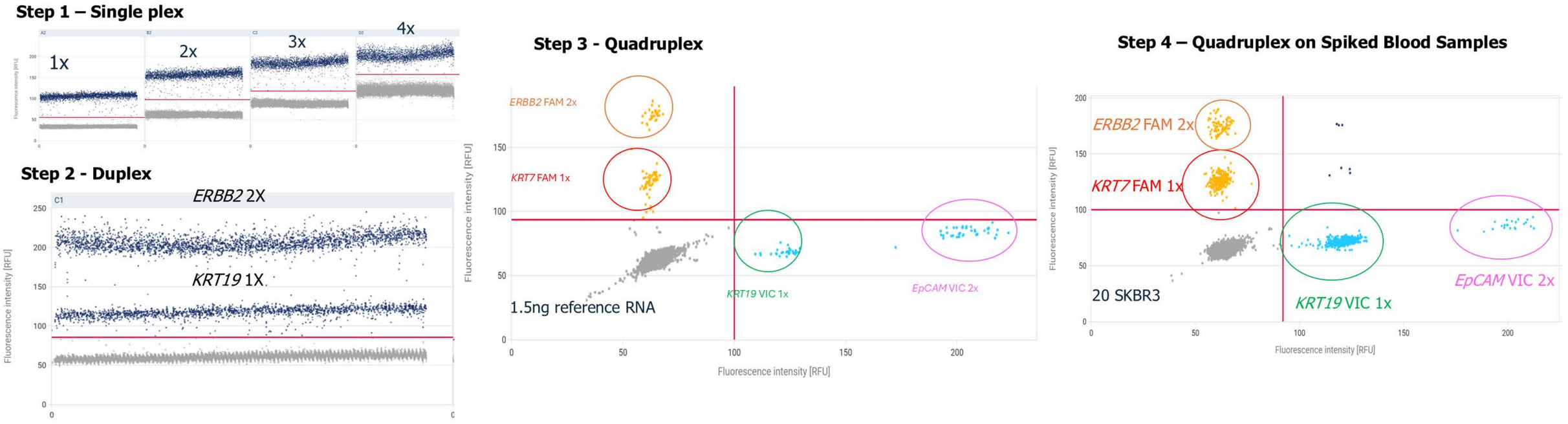


***Figure 2.*** *Development and validation of amplitude-based multiplexing showing progression from single target assessment through to quadruplex detection. The workflow demonstrates single-plex (Step 1), duplex (Step 2), triplex (Step 3), and quadruplex (Step 4) configurations using reference RNA and spiked blood samples).*

## Breast Cancer Panel and Patient Sample Results

### HV Samples

A panel of eight genes (two quadruplex dPCR reactions) known to be overexpressed in breast cancer cells was generated. Gene expression was detected in 6/8 genes when HV bloods spiked with only 5 SKBR3 and Hs 578T cancer cells, showing high assay sensitivity with detection ranging from 21-2334 copies/µL (*KRT7*: 545, *FN1*: 2334, *EGFR*: 21, *AGR2*: 39, *ERBB2*: 430, and *EpCAM*: *71* copies/µL).

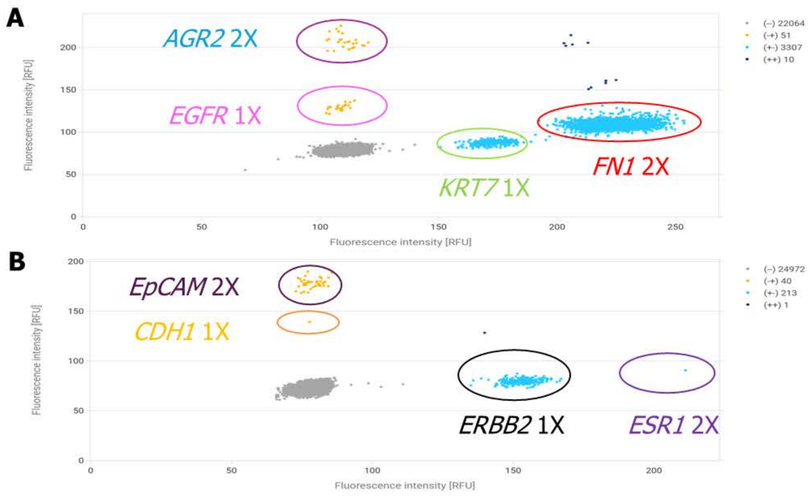


***Figure 3. (A)*** *Quadruplex reaction 1 showing distinct population separation for EGFR (1x FAM), AGR2 (2x FAM), KRT7 (1x VIC), and FN1 (2x VIC) in blood samples spiked with 5 SKBR3 cancer cells.* ***(B)*** *Quadruplex reaction 2 demonstrating clear amplitude separation between EpCAM (2x FAM), CDH1 (1x FAM), ERBB2 (1x VIC), and ESR1 (2x VIC) targets in spiked samples.*

### 10 MBC Patient Samples

Analysis of 10 metastatic breast cancer patient samples revealed varying expression patterns across the eight-gene panel. *ERBB2* showed the highest detection rate (8/10 patients), followed by *EpCAM* (4/10) and CDH1 (3/10). *AGR2*, *ESR1*, and *KRT7* were detected in 2/10 patients, while *EGFR* and *FN1* showed no expression. Two patients showed detection in 6/8 genes assessed. It is important to note that some genes, such as *ERBB2*, may be expressed in white blood cells and care is needed to establish appropriate thresholds to distinguish true positives.

**A**

| Patient INF | *EpCAM* Copies/ µL | *CDH1* Copies/ µL | *ERBB2* Copies/ µL | *ESR1* Copies/ µL | *KRT7* Copies/ µL | *EGFR* Copies/ µL | *ARG2* Copies/ µL | *FN1* Copies/ µL |
|---|---|---|---|---|---|---|---|---|
| A | 0 | 0 | 39.96 | 4.24 | 2.08 | 2.08 | 0 | 3.84 |
| B | 64.4 | 34.16 | 44.96 | 6.4 | 81.24 | 0 | 32.04 | 0 |
| C | 10.76 | 10.84 | 432.4 | 4.32 | 0 | 0 | 0 | 3.84 |
| D | 142.96 | 124.12 | 80.16 | 45.76 | 404.8 | 0 | 102.4 | 0 |
| E | 0 | 2.2 | 28.04 | 0 | 0 | 2.16 | 0 | 0 |
| F | 0 | 0 | 17.36 | 2.2 | 0 | 0 | 0 | 0 |
| G | 8.64 | 0 | 2.16 | 4.36 | 0 | 0 | 0 | 0 |
| H | 2.12 | 0 | 12.68 | 0 | 4.2 | 2.12 | 0 | 1.92 |
| I | 0 | 4.52 | 4.24 | 0 | 0 | 0 | 0 | 0 |
| J | 0 | 2.24 | 4.44 | 0 | 0 | 2.24 | 0 | 1.92 |
| Number of positive samples | 4 | 3 | 8 | 2 | 2 | 0 | 2 | 0 |

**B**

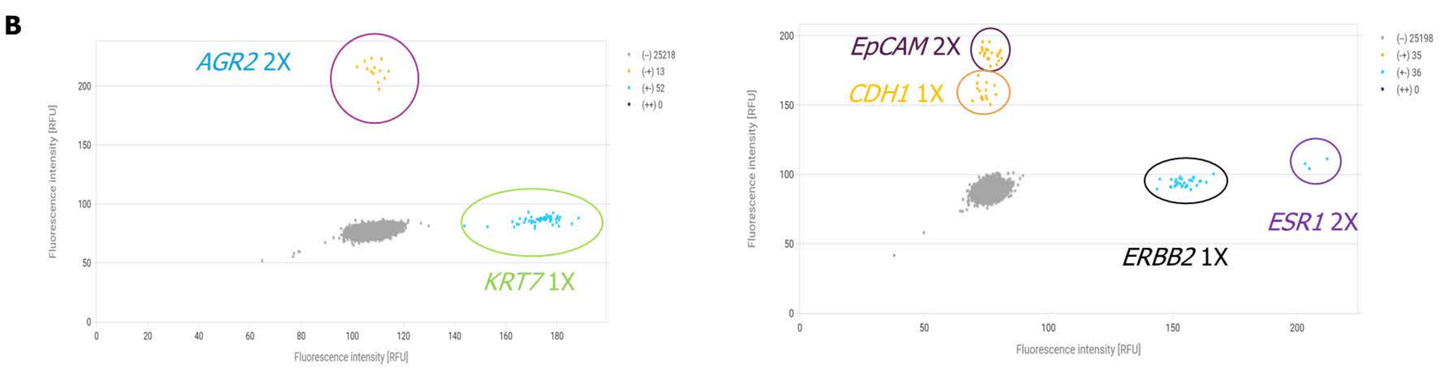


***Figure 4. (A)*** *Gene expression analysis across 10 MBC patient samples showing detection frequencies and expression levels for eight targets, pending threshold optimization.* ***(B)*** *Representative 2D amplitude plots showing population separation in clinical samples, requiring threshold determination for specific CTC detection.*

## Conclusions

- Amplitude-based multiplexing can be successfully performed on the QIAcuity dPCR. No impact on assay performance is observed when combining multiple assays labelled with the same fluorophore in a single reaction, compared to the single plex reactions. Currently up to four assays can be effectively multiplexed in a single reaction, allowing for visualisation and quantification of the four populations of positive droplets.
- We demonstrated that amplitude-based multiplexing can increase the number of genes assessed simultaneously in a CTC sample enriched with Parsortix system.
- This proof of concept demonstrates that dPCR can be used to significantly improve the ability to report a wider range of genetic markers from a single sample leading to a more comprehensive view into tumour biology.