Integrating isolation using label-independent microfluidics and advanced staining for comprehensive Circulating Tumour Cell Analysis

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EBCC 14 — 20-22 March 2024 MILAN, ITALY — 14TH EUROPEAN BREAST CANCER CONFERENCE

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Introduction

In the realm of cancer diagnostics, liquid biopsy emerges as a transformative tool, enabling routine and repetitive characterization of cancer at genetic, transcriptional, and protein levels. Notably, the isolation of Circulating Tumor Cells (CTCs) from blood samples holds immense potential for cancer screening, and monitoring. However, the prevalent reliance on epitope-dependent CTC capture systems, primarily focused on epithelial markers, poses limitations in detecting mesenchymal and epithelial-to-mesenchymal transitioning (EMT) CTCs. Tumor cells undergoing EMT (**Figure 1**) during metastasis remain elusive with traditional platforms. Addressing this gap, our research introduces the use of ANGLE's Portrait®+ CTC Staining Kit leveraging epithelial and mesenchymal markers for identification and phenotyping of CTC isolated through the Parsortix® instrument.

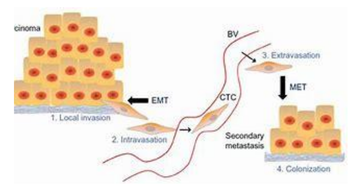

Figure 1. Epithelial to mesenchymal transition. Figure adapted from Guttilla Reed I., *Cell Health and Cytoskeleton*. 2015

Workflow

Analytical performance was established using Healthy Volunteer's (HV) blood samples spiked with either epithelial, mesenchymal or EMT cancer cell lines. The research method was then applied on blood samples from Metastatic Breast Cancer (MBC) patients and un-spiked HV donors.

In both cases (**Figure 2**):

- Peripheral blood was drawn into Streck Cell-Free DNA tubes and stored for up to 144 hours from collection before processing.
- Blood samples were processed on Parsortix® instruments, a microfluidic device capable of capturing and harvesting CTCs from bodily fluids based on cell size and lack of deformability, employing a separation cassette (GEN3P6.5) comprising a series of steps leading to a smaller critical gap. During the processing, most of the common blood cells and components pass across the critical gap, while CTCs are retained in the separation cassette due to their size and rigidity.
- Harvested CTCs were spun on ANGLE's CellKeep™ slides to maximize the retention.
- Slides were stained using ANGLE's Portrait®+ CTC staining kit, a freeze-dried antibody mixture, comprising a nuclear dye (Hoechst) and antibodies against epithelial markers (FITC), mesenchymal markers (Cy3), and blood lineage markers (Cy5), including antigens expressed by blood cells such as lymphocytes, macrophages, granulocytes, monocytes, fibroblasts, and cells of megakaryoblastic potential.
- Stained slides were imaged using a BioView DeNovo imaging system.

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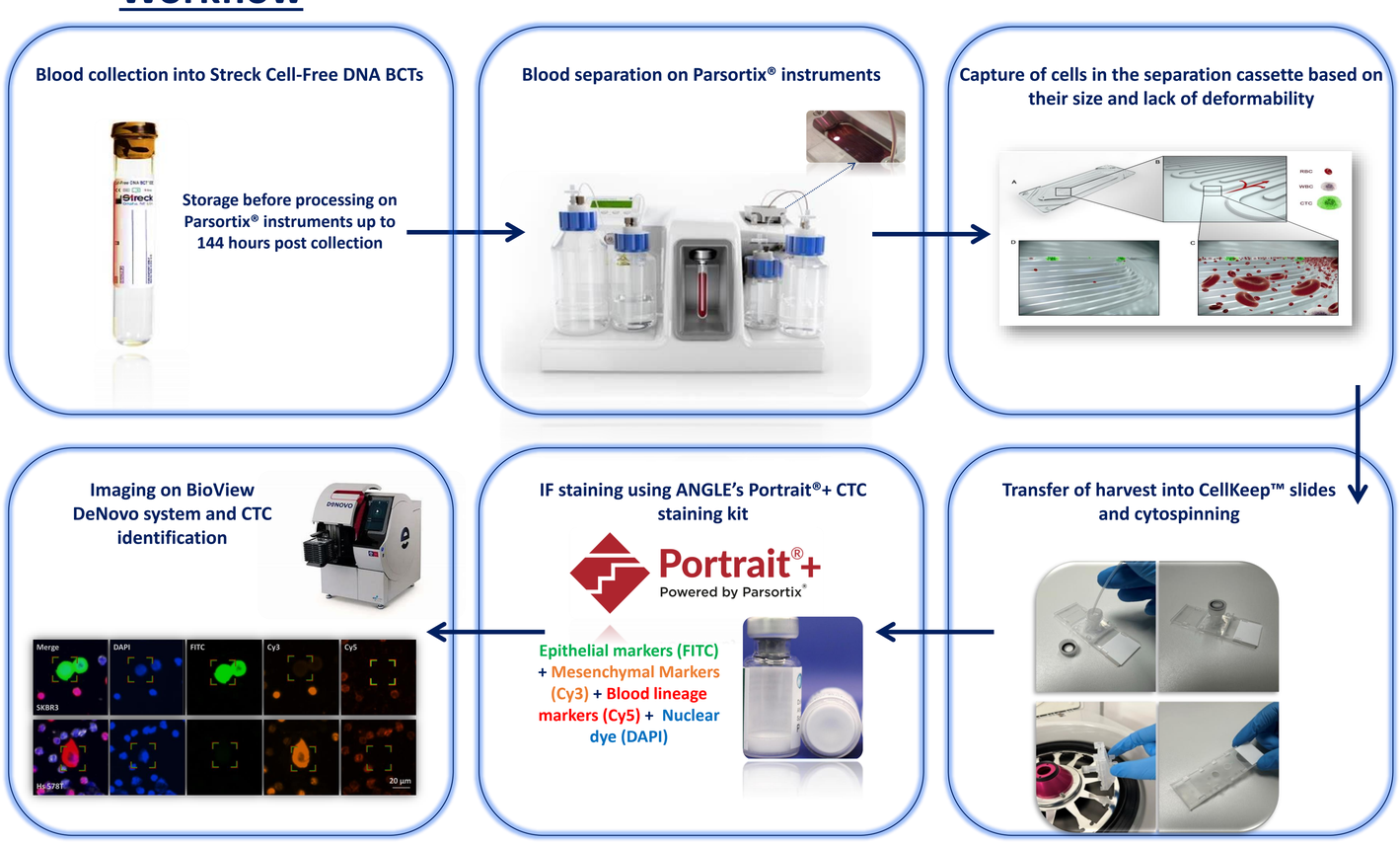


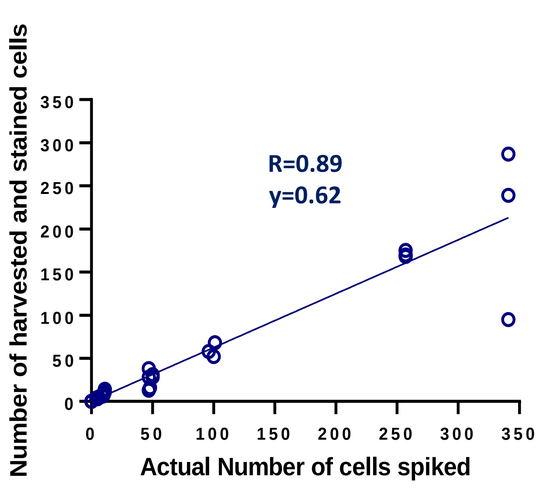
Figure 2. Schematic representation of the assay workflow.

Analytical results

Linearity, Accuracy and Precision were established by assessing the quantitative recovery of 0 to ~350 H226 cells spiked into 7.5 mL of HVs' blood drawn in Streck Cell-Free DNA BCTs:

- **Linearity**: a linear relationship between the number of H226 cells spiked and the number of cells harvested and stained was confirmed, with R^2=0.89, slope = 0.62 (**Figure 3A**).
- **Accuracy** refers to the proximity of the number of harvested and stained cells to the number of spiked cells in blood. Mean accuracy across six donors for the end-to-end workflow was 68% (**Figure 3B**).
- **Precision** relates to the reproducibility of the experiment and indicates how close or dispersed the percentage of harvested and stained cells is between samples as a measure of variability. Coefficient of variation (CV%) of mean percentage harvest across samples of different donors spiked with the same level of cells was <37% (**Figure 3C**).

A



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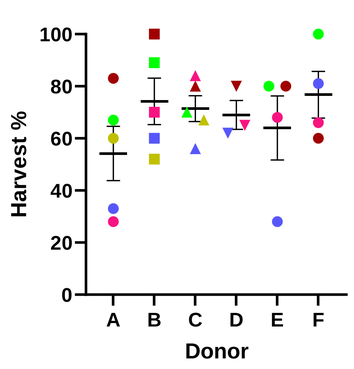


C

Number spiked cells	5	10	50	250
N	6	5	6	6
Mean	80.5	81.2	53.33	63.5
CV (%)	15.79%	16.78%	37.00%	29.47%

Figure 3. Quantitative analytical performance evaluation. (A) Graph shows actual number of H226 cells spiked (x-axis) vs. the actual number of harvested and stained cells (y-axis) across all spike levels for six donors. **(B)** Dot plot shows Mean ± Standard Error of the Mean (SEM) for the percentage of harvested and stained H226 cells per donor at each spike level. **(C)** Table shows number of samples (N), mean % harvested cells and CV% by spiking level.

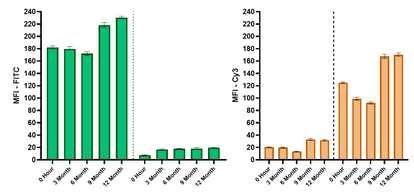
Analytical Sensitivity and **Specificity** refer to the percentage of harvested cells known to express/not express a marker that had a mean fluorescence intensity (MFI) above/below the established thresholds for that marker, respectively (**Figure 4**).

→ Analytical Specificity and Sensitivity were above 90% for all markers, apart from sensitivity of mesenchymal markers (83%), due to unavailability of purely mesenchymal control cell lines.

Stability was established by comparing the intensity of fluorescence for each panel of markers on cells known to express or not express those markers, at different timepoints up to 12 months post kit manufacturing (**Figure 5**).

→ Portrait®+ CTC staining Kit is stable up to 12 months from lyophilization.

A



B

Analytical Specificity			Analytical Sensitivity		
FITC	Cy3	Cy5	FITC	Cy3	Cy5
95%	92%	100%	97%	83%	99%

Figure 4. Qualitative analytical performance evaluation. (A) Representative images of (left) an epithelial cancer cell line and (right) a mesenchymal cancer cell line (top) and a white blood cell (bottom). Merge colours: Epithelial markers (FITC) in green, Mesenchymal markers (Cy3) in red, Blood lineage markers (Cy5) in white, Nuclear dye (DAPI) in blue. **(B)** Analytical sensitivity: proportion of harvested cells known to express the marker(s) of interest which were positive. Analytical specificity = proportion of harvested cells known to not express the markers of interest which were marker negative in the assay.

A

B

Analytical Specificity					Analytical Sensitivity				
Timepoint	0 hrs	3 mo	6 mo	9 mo	12 mo	0 hrs	3 mo	6 mo	9 mo	12 mo
FITC	98	94	94	89	96	99	100	96	100	100
Cy3	99	97	100	91	93	97	86	92	98	100

Figure 5. Stability evaluation. (A) Histograms show mean ± SEM of the Mean Fluoresce Intensity (MFI) of epithelial (FITC) and mesenchymal markers (Cy3) on epithelial (SKBR3) and mesenchymal (Hs 578T) breast cancer cell lines at different timepoints. **(B)** Analytical sensitivity: proportion of harvested cells known to express the marker(s) of interest which were positive. Analytical specificity = proportion of harvested cells known to not express the markers of interest which were marker negative in the assay.

Patients' results

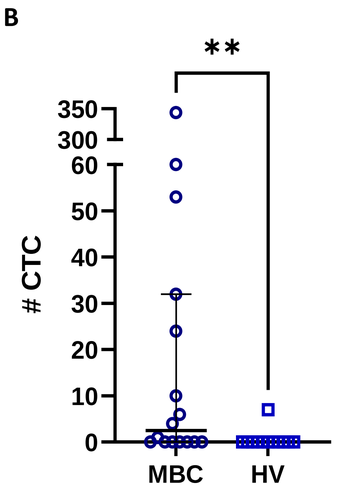
The workflow was performed on blood samples collected from 16 MBC patients and 12 HVs to assess the number of epithelial, mesenchymal and EMT CTCs and CTCs clusters captured (**Figure 6**):

- 56% (9/16) of the MBC patients included in this study had ≥1 CTC identified and CTC clusters (consisting of 2 to 66 CTCs per cluster) were observed in 67% (6/9) of the CTC positive patients.
- Phenotypically, more than half of the donors had only mesenchymal CTCs identified (55%), 33% had a combination of epithelial and mesenchymal CTCs and/or EMT CTCs and 11% had exclusively epithelial CTCs.
- Only one cluster of 7 mesenchymal CTCs was identified in 1/12 (8%) HVs.

A

	MBC
N	16
Donors with ≥1 CTC	9/16 (56%)
Median	24
Mean	59
Range	1-344
Donors with ≥1 CTC Cluster	6/9 (67%)
Cluster size (range CTC)	2-66
Cluster per donor (range)	2-30
Donors with only Epithelial CTCs	1/9 (11%)
Donors with only Mesenchymal CTCs	5/9 (55%)
Donors with Epi, Mes and EMT CTCs	1/9 (11%)
Donors with Epi and Mes CTCs	2/9 (22%)

B



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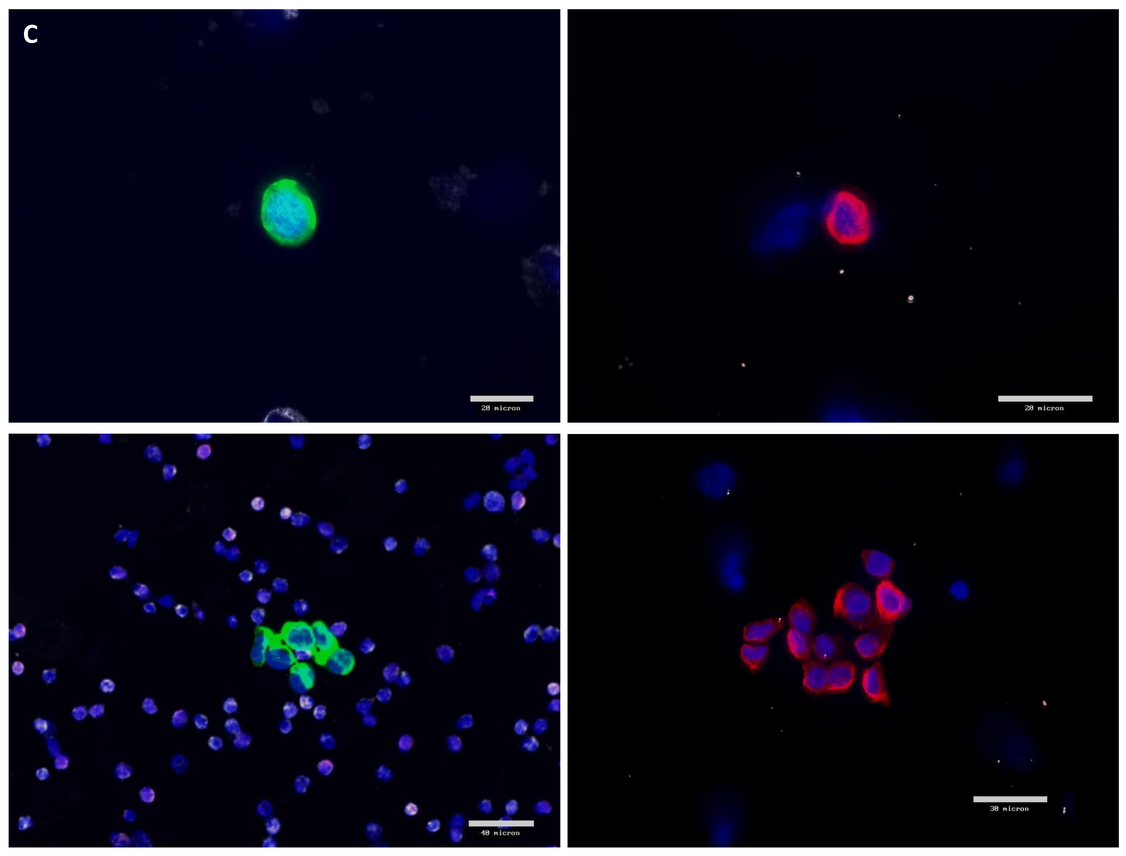

Figure 6. CTC identification and phenotyping in MBC patients and HVs. (A) Table showing number of donors included in each cohort (N), N and percentage (%) of donors with ≥1 CTC, range, mean and median of CTCs (without negative donors), number and percentage of donors with ≥1 CTC cluster, range of CTC clusters per donor, range of number of CTCs per cluster and donors' CTC phenotype; **(B)** Dot plot showing the number of CTCs (median ± 95% CI) found in each group, *p<0.01*, Mann-Whitney test; **(C)** Representative images of a single epithelial CTC (Top Left), a single mesenchymal CTC (Top Right), a cluster of epithelial CTCs (Bottom Left) and a cluster of mesenchymal CTCs (Bottom Right). Merge colors: Epithelial markers (FITC) in green, Mesenchymal markers (Cy3) in Red, Blood lineage markers (Cy5) in white, Nuclear dye (DAPI) in blue.

Conclusions

This research underscores the significance of incorporating mesenchymal markers in CTC characterization as most captured CTCs expressed mesenchymal markers, suggesting that an epithelial-only approach would have missed a substantial proportion of harvested CTCs. ANGLE's Portrait®+ CTC staining kit to detect and phenotype CTCs, combined with ANGLE's Parsortix® instrument, provides an efficient, easy-to-use and standardized solution for the harvesting and characterization of multiple CTC phenotypes. Additionally, the kit has the added advantage of being suitable for use with blood collected in Streck Cell-Free DNA BCTs, allowing processing up to 144 hours post collection, for the shipment of samples for centralised analysis in support of global clinical trials.