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## Introduction

HER2 overexpression in the primary tumour does not always match the overexpression status at the metastatic sites<sup>1</sup> and it has been demonstrated that there are cases of breast cancer where HER2 protein overexpression does not correlate with gene amplification<sup>2</sup>. Concomitant evaluation of *HER2* gene amplification and protein overexpression in Circulating Tumour Cells (CTCs) opens the possibility for minimally-invasive personalized monitoring of patients under treatment and improved stratification of patients that can benefit from HER2-targeted treatments.

Traditionally, HER2 protein expression is evaluated by immunohistochemistry methods (Figure 1). ANGLE developed a Research Use Only (RUO) workflow\* comprising the use of the Parsortix® System, an epitope-independent microfluidic device that isolates and harvests rare cells from blood based on size and deformability, followed by two downstream assays: ANGLE's Portrait Flex Immunofluorescence (IF) assay for epithelial, mesenchymal and Epithelial-to-Mesenchymal transitioning (EMT) CTC detection with the inclusion of HER2 antibody for protein detection, and an ANGLE's Portrait Flex assay combined with *HER2* FISH for evaluation of CTCs' *HER2* amplification status.

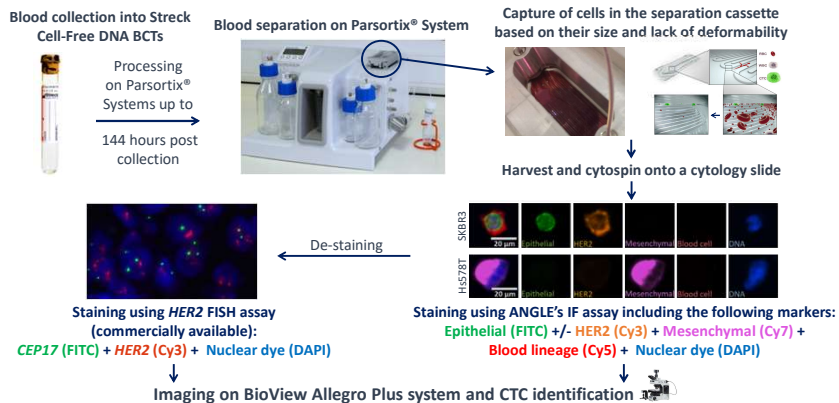
\*For Research Use Only. Not for use in diagnostic procedures.

## Workflow

Blood samples from 12 Healthy Volunteers (HVs) collected into Streck Cell-Free DNA Blood Collection tubes (BCTs) and spiked with cultured HER2-overexpressing (SKBR3, HCC1954) and HER2-negative (MCF-7, Hs578T) breast cancer cell lines were used for analytical verification. Samples were processed on Parsortix® PC1 Clinical Systems or Parsortix® PR1 Research Systems as per workflow in Figure 2 and used to assess analytical sensitivity and specificity (Figures 3, 4).

Clinical samples from 16 Metastatic Breast Cancer (MBC) patients were collected into Streck Cell-Free DNA BCTs, processed at 72-144 hours post-draw using Parsortix® PC1 Clinical System and stained using ANGLE's Portrait Flex assay combined with HER2 antibody as per workflow in Figure 2 (excluding FISH staining).

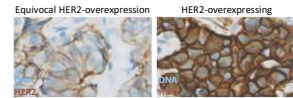
Cells were considered to show *HER2* amplification if the ratio of *HER2* foci to *CEP17* foci was  $\geq 2$ . Parallely, cells were considered to overexpress HER2 protein if their Mean Fluorescence Intensity (MFI) value was above the established positivity threshold.



**Figure 2. Schematic representation of the assay workflow.** Peripheral blood (7.5 mL) was drawn into Streck Cell-Free DNA BCTs from HVs and MBC patients and processed on Parsortix® Systems between 72- and 144-hours post collection. Contrived samples from HVs were prepared with cultured cancer cells. Cancer cells (CTCs or cultured) were captured in the Parsortix® cell separation cassettes (due to their larger size and lower compressibility compared to other blood components) and cytopspin onto slides. Slides were stained with ANGLE's IF assay for detection of epithelial, mesenchymal, and EMT transitioning cancer cells (including HER2 antibody in the first analytical study and in the clinical study, and excluding the HER2 antibody in the second analytical study) followed by *HER2* FISH assay (analytical samples) for detection of *HER2* amplification. Slides were imaged using BioView Allegro Plus automatic imaging system. CTCs and cultured cancer cells were characterized as epithelial (FITC+, Cy7-, Cy5-, DAPI+), mesenchymal (FITC-, Cy7+, Cy5-, DAPI+), or Epithelial-to-Mesenchymal transitioning (FITC+, Cy7+, Cy5-, DAPI+). *HER2* amplification and protein expression were evaluated as mentioned above.

## References

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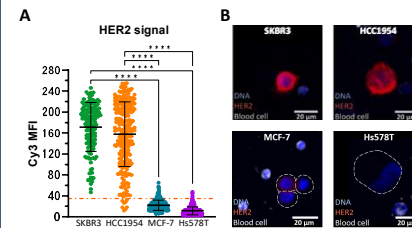


**Figure 1. Determination of HER2 overexpression by immunohistochemistry in a tissue section from a solid breast tumor<sup>2</sup>.**

## 1. IF assay for HER2 protein evaluation

Evaluation of HER2 signal in circulating cancer cells from contrived samples showed that mean HER2 MFI signal of HER2-positive cells (SKBR3 and HCC1954) was significantly higher than that of HER2-negative cells (MCF-7 and Hs578T) (Figure 3 A, B).

Analytical specificity and sensitivity of the assay were over 90% (Figure 3 A).



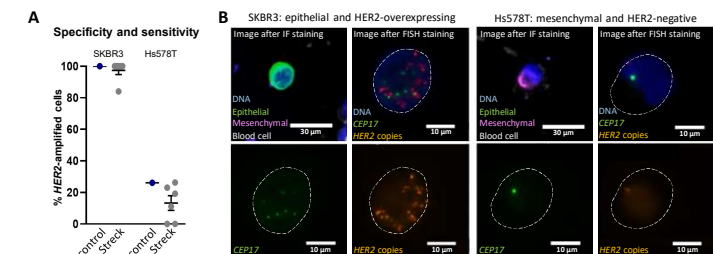
**Figure 3. Evaluation of HER2 protein signal in HER2-positive and HER2-negative cancer cells from contrived samples separated on Parsortix® PC1 Clinical System.** (A) Dot plot showing the mean  $\pm$  SEM HER2 MFI value for each cancer cell line tested where HER2 is shown in red (Cy3), blood cell lineage markers in white (Cy5), and DNA in blue (DAPI): SKBR3 (top-left), HCC1954 (top-right), MCF-7 (bottom-left), Hs578T (bottom-right). Data was statistically compared by running a one-way ANOVA followed by Tukey's multiple comparisons test (\*\*\*\* $p < 0.0001$ ) ( $n = 6$  HVs).

## Analytical results

### 2. IF for CTC identification combined with *HER2* FISH assay

Evaluation of *HER2* amplification by *HER2* FISH assay in circulating cancer cells from contrived samples previously stained with ANGLE's Portrait Flex assay probed to be highly sensitive and specific: ~100% of HER2-overexpressing cancer cells showed *HER2* amplification and ~80% of HER2-negative cancer cells did not show *HER2* amplification (calculated as the percentage of *HER2*-amplified cancer cells over the total number of cancer cells found in the slide), as previously reported in literature<sup>4</sup> (Figure 3).

The percentage recovery of cancer cells found on slides from contrived samples after combined IF and FISH staining is comparable to that of slides stained with *HER2* FISH assay only (data not shown).

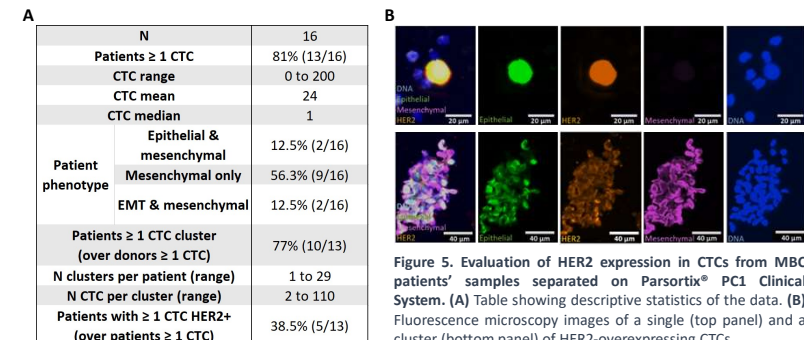


**Figure 4. Evaluation of *HER2* amplification in HER2-overexpressing (SKBR3) and HER2-negative (Hs578T) cancer cells from contrived samples separated on Parsortix® PR1 Research System.** (A) Dot plot showing the mean  $\pm$  SEM of the percentage of HER2-overexpressing cancer cells found on slides after IF and FISH combined staining ( $n = 6$  HVs) (control: slides prepared with cultured cells not spiked into blood; Streck: slides from Streck Cell-Free DNA samples separated on Parsortix® PR1 Research System). (B) Representative images of cancer cell lines tested as part of the assay combining IF and FISH staining. For each cell shown, the image on the top left was acquired after IF staining and shows epithelial markers in green (FITC), mesenchymal markers in purple (Cy7), and DNA in blue (DAPI); the other images show the same cell after IF de-staining and FISH staining and show *CEP17* in green (FITC) and *HER2* in orange (Cy3).

## Clinical results

Samples from 16 metastatic breast cancer patients were processed using ANGLE's Parsortix® PC1 Clinical System separation and IF assay workflow (presented in Workflow Section).

CTCs were identified in 81% of the patients, with 38.5% of the CTC-positive patients having  $\geq 1$  CTC with high HER2 levels (Figure 5 A, B). 100% of the patients with CTCs had  $\geq 1$  mesenchymal CTC. Approximately half of the patients with CTCs showed a mesenchymal only phenotype; while the others showed a mixed phenotype (epithelial and mesenchymal or EMT and mesenchymal CTCs) (Figure 5 A). 77% of CTC-positive patients had  $\geq 1$  CTC cluster, ranging from 1 to 29 CTCs.



## Conclusions

This study demonstrates that Streck Cell-Free DNA BCTs are suitable for collection and storage of blood samples processed for IF and/or *HER2* FISH assays, with the advantage of allowing longer blood sample storage and facilitating batch shipping and processing of clinical samples.

This study demonstrates the feasibility of combining IF staining for epithelial, mesenchymal, and EMT phenotype characterisation of CTCs with *HER2* FISH staining for gene amplification assessment, on blood separated using the Parsortix® Systems as part of ANGLE's workflow. This combined assay allows extraction of multiple information from each target cell in each sample, without significantly affecting cancer cell loss.