Development and analytical validation of a novel assay for HER2 assessment on circulating tumour cells using Parsortix® isolation and BioView imaging technologies

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Introduction

HER2 testing in tissue biopsies is extensively performed in breast cancer (BC) patients by immunohistochemistry methods (**Figure 1**), followed by In-Situ Hybridization for equivocal cases (2+). However, tissue biopsy is invasive and repeated examination is impractical. Additionally, HER2 overexpression in the primary tumour does not always match the overexpression status at the metastatic sites[1] and it has been demonstrated that there are cases of breast cancer where HER2 protein overexpression does not correlate with gene amplification[2]. In this study, we developed an end-to-end Research Use Only assay for evaluation of *HER2* gene amplification and HER2 protein overexpression in circulating tumor cells (CTCs) captured and harvested from one single blood sample.

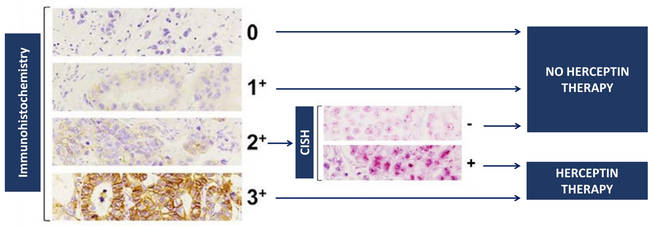


Figure 1. Schematic representation of HER2 diagnostic algorithm. Figure adapted from Fassan M et al., Pathologica, 2020.

For Research Use Only. Not For Use In Diagnostic Procedures.

Workflow

Blood samples from Healthy Volunteers (HVs) collected into Streck Cell-Free DNA Blood Collection tubes (BCTs) and spiked with cultured HER2-overexpressing (SKBR3) and HER2-low (Hs578T) breast cancer cell lines were processed as per workflow in **Figure 2** and used for analytical verification. The same workflow was tested in a cohort of 22 Metastatic Breast Cancer (MBC) patient samples.

CTCs were considered to overexpress HER2 protein if the Mean Fluorescence Intensity (MFI) value was above the established positivity threshold. In parallel, cells were considered to present *HER2* copy number amplification if the ratio of *HER2* foci to *CEN17* foci was ≥ 2.

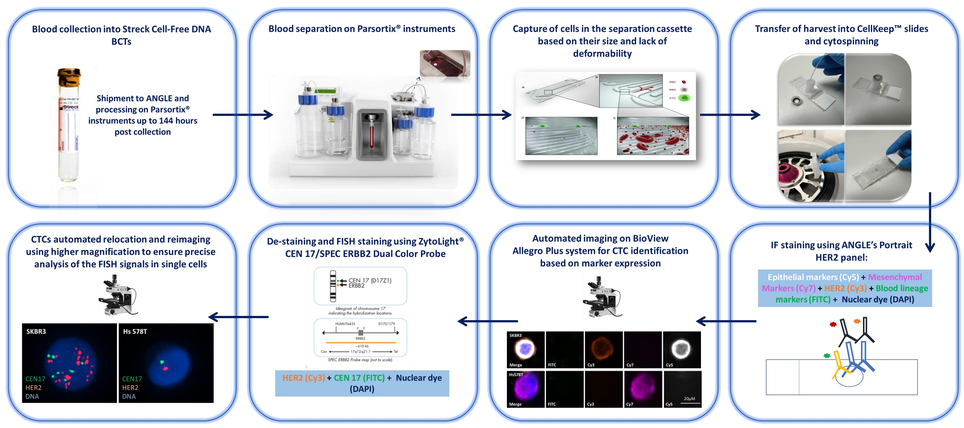


Figure 2. Schematic representation of the assay workflow. Peripheral blood was drawn into Streck Cell-Free DNA tubes and stored for up to 144 hours from collection before processing. Blood samples were processed on the Parsortix® instrument, a microfluidic device capable of capturing and harvesting CTCs from bodily fluids based on cell size and lack of deformability. Harvested CTCs were cytospun on ANGLE's CellKeep™ slides to maximize the retention. Slides were stained using ANGLE's IF-based Portrait® HER2 assay, comprising a nuclear dye (Hoechst) and antibodies against epithelial markers (Cy5), mesenchymal markers (Cy7), HER2 (Cy3) and blood lineage markers (FITC), including antigens expressed by blood cells (lymphocytes, macrophages, granulocytes, monocytes, fibroblasts, and cells of megakaryoblastic potential). Stained slides were imaged and then de-stained to be subjected to *HER2* FISH, using a commercially available kit (ZytoLight® CEN 17/SPEC ERBB2 Dual Color Probe). All slides were imaged twice using a BioView Allegro Plus system, a platform equipped with artificial intelligence for automated imaging, CTC candidate identification and reporting. First, an automated scan after IF was performed to identify CTCs based on marker expression and morphology profiles. Second, an automated relocation of the CTCs based on IF results and reimaging using higher magnification to ensure precise analysis of the FISH signals in single cells.

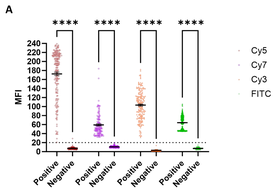
References

1. Zidan J, Dashkovsky I, Stayerman C, *et al*. Comparison of HER-2 overexpression in primary breast cancer and metastatic sites and its effect on biological targeting therapy of metastatic disease. *Br J Cancer* 93, 552–556 (2005).
2. Koo JS, Jung W, Yang WI. HER-2 protein overexpressing breast cancer without gene amplification shows higher hormone receptor expression than HER-2 protein overexpressing breast cancer with gene amplification. *Int. J. Surg. Pathol*.19:425–432 (2011).
3. Grushko TA, Blackwood MA, Schumm PL, *et al*. Molecular-cytogenetic analysis of *HER-2/neu* gene in *BRCA1*-associated breast cancers. *Cancer Res* 62 (5): 1481-1488 (2002).

Analytical results

- Analytical sensitivity and specificity of the immunofluorescence (IF) panel were calculated by assessing the percentage of harvested cancer cells known to express/not express a marker that had MFI above/below the established thresholds for that marker (**Figure 3A**), respectively.
  → Analytical specificity and sensitivity of the Portrait® HER IF assay were above 90% for all markers (**Figure 3B**).

- Positivity rate of the FISH assay was established by calculating the percentage of SKBR3 and Hs578T cells (previously IF-stained with ANGLE's Portrait HER2 IF assay) that had a *HER2/CEN17* foci ratio ≥2 (**Figure 3C-D**).
  → Mean positivity rate was 96% in the cancer cell line known to have high *HER2* amplification (SKBR3) and 12% in Hs 578T cells, reported in the literature to present low HER2 amplification[3], highlighting the accuracy of the Portrait® *HER2* FISH assay (**Figure 3C**).



B

	Cy5 (Epithelial Markers)		Cy7 (Mesenchymal Markers)		Cy3 (HER2)		FITC (Blood cells markers)	
	+	-	+	-	+	-	+	-
Mean MFI	171	7	59	11	103	3	64	7
Median MFI	191	7	52	10	103	3	65	7
Dynamic range	23		6		36		9	
IF Sensitivity (%)	100		100		100		100	
IF Specificity (%)	99		100		100		100	

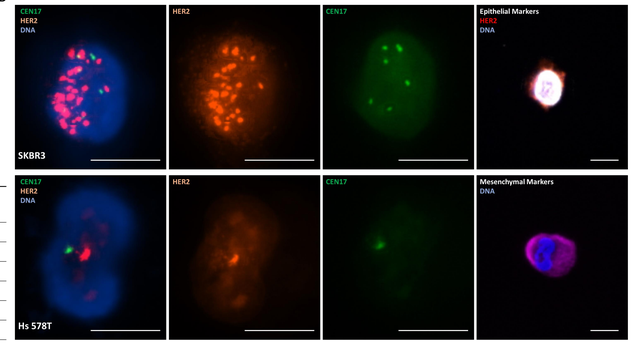
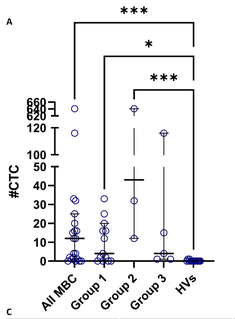


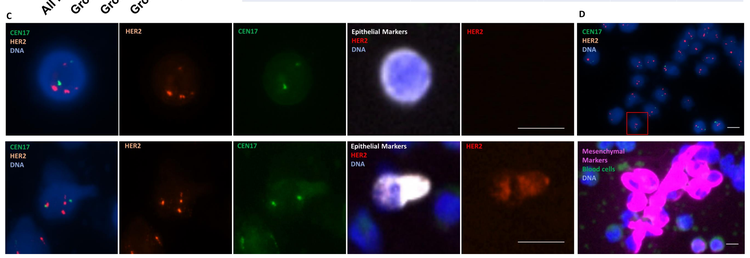
Figure 3. Assay analytical sensitivity and specificity. (A) Dot plot showing mean ± SEM of the MFI value in each IF channel in positive and negative cells (2-way ANOVA with Sidak's multiple comparisons test, *p****<0.0001*); **(B)** Table shows mean MFI, median MFI, dynamic range (calculated as a ratio of mean MFI between positive and negative cells) and percentage analytical sensitivity and specificity based on MFI thresholds for each IF channel, in positive and negative cells; **(C)** Dot plot showing mean ± SEM of the percentage of harvested cancer cells considered *HER2*-positive by FISH after IF and FISH combined staining (paired t-test, *p****<0.0001*); **(D)** Representative images of cancer cell lines (Top= SKBR3; Bottom= Hs 578T) tested as part of the assay combining IF and FISH staining. From left to right: 60× merge FISH staining, *HER2* foci in orange (Cy3), *CEN17* foci in green (FITC), 10× IF image of the same cell before FISH, showing epithelial markers in white (Cy5), mesenchymal markers in purple (Cy7), HER2 protein in orange (Cy3), DNA in blue (DAPI), blood lineage markers in green (FITC). Micron bar = 20 µm.

Patients' results



B

	All MBC	Group 1	Group 2	Group 3	HVs
N	22	13	4	5	15
≥1 CTC	18 (82%)	9 (69%)	4 (100%)	5 (100%)	2 (13%)
Mean CTCs	46	10	185	27	0
Median CTCs	12	4	43	4	0
Range	0-641	0-33	0-641	0-116	0-1
N donors with Ep CTCs only (≥1)	3 (17%)	1 (11%)	0 (0%)	2 (40%)	0 (0%)
N donors with Ep + Mz CTCs and/or ≥1 EMT CTC	2 (11%)	1 (11%)	1 (25%)	0 (0%)	0 (0%)
N donors with Mz CTCs only	13 (72%)	7 (78%)	3 (75%)	3 (60%)	2 (100%)
N Donors with ≥1 HER+ CTC (IF)	3	1 (EMT donor)	0	2 (both Ep donors)	0
N Donors with ≥1 HER+ CTC (FISH)	6	1 (Ep donor)	2 (Mz and EMT donor)	3 (2 Ep and 1 Mz donor)	0
Tissue / HER2 Protein Concordance	22%	11%	0%	60%	N/A
Tissue / HER2 Gene Concordance	28%	11%	50%	40%	N/A



- Samples from 22 MBC patients and 11 HVs were processed as per **Workflow Section**. Of the MBC patients, 13 were diagnosed with a HER2+ primary tumour by tissue biopsy and have received HER2 targeted therapy (Group 1), 4 were diagnosed with a HER2+ primary tumour not undergoing HER2 targeted therapy (Group 2) and 5 were diagnosed with a HER2- tumour. Results are summarized in **Figure 5**.
- CTCs positivity rate and overall number of CTCs (mean, median and range) was higher in Groups 2 and 3 compared to Group 1, however, no statistically significant difference was identified.
- CTCs positivity rate and number of CTCs were significantly lower in HVs compared to MBC patients with 2/15 HVs presenting only 1 mesenchymal, HER2-negative cell each.
- While in all groups most of the donors presented exclusively mesenchymal CTCs, the HER2- group had a higher percentage of donors with an epithelial only phenotype compared to the other two groups.
- Epithelial phenotype correlated with HER2 protein positivity, while *HER2* gene amplification was observed across all CTCs phenotypes.
- Only two donors had matched HER2 positivity in both FISH and IF and these were both donors presenting epithelial only CTCs.
- Concordance between reported tissue biopsy results and HER2 status in CTC by IF and/or FISH was low in all groups highlighting the heterogeneity of tumour cells.

Figure 5. HER2 protein expression and gene amplification in CTCs from MBC patients' and HVs' samples separated on Parsortix® instruments. (A) Dot plot shows median ±95% CI of the total number of CTC isolated in MBC patients by group and in HVs (1-way-ANOVA with Kruskal-Wallis, **p<0.05, ***p<0.001)*; **(B)** Table shows number (N) of donors in each group, N and % donors with ≥1 CTC, mean, median and range of the number of CTCs, donors phenotype based on the CTCs' EMT status and HER2 protein/gene positivity, HER2 concordance between tissue and CTCs; **(C)** Representative images of a single epithelial CTC positive for HER2 by FISH (*HER2/CEN17* ratio = 2) only (top panel) and one positive by both HER2 FISH (*HER2/CEN17* ratio = 2) and IF (bottom panel). From left to right: 60× merge FISH staining, *HER2* foci in orange (Cy3), *CEN17* foci in green (FITC), 10× IF merge image of the same cell before FISH, 10× IF HER2 image (Cy3); **(D)** Representative images of a cluster of mesenchymal CTCs (top= FISH, bottom= IF) with mixed HER2 FISH genotype. Red square highlights HER2 positive cell (*HER2/CEN17* ratio = 4). Micron bar = 20 µm.

Conclusions

This study demonstrates the possibility of combining IF staining for CTCs identification based on epithelial and mesenchymal markers and HER2 protein assessment on those CTCs with *HER2* FISH for evaluation of *HER2* copy numbers amplification. Overall, CTCs and primary tumour concordance was low in this study, highlighting the potential of liquid biopsy and concomitant evaluation of HER2 gene copy number and protein level on CTCs for minimally-invasive personalized monitoring of patients under treatment and more accurate stratification of patients that may benefit HER2 targeted therapy.