Development of a scoring system to classify HER2 protein expression in Circulating Tumor Cells through immunofluorescence following isolation using Parsortix® instruments

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Introduction

HER2 testing through immunohistochemistry (IHC) is routinely performed on tissue biopsy samples from metastatic breast cancer (MBC) patients, resulting in a HER2 score (**Figure 1**) which, in combination with In-Situ Hybridization (ISH) results in equivocal cases (2+), is used in patient stratification and treatment decisions. However, tissue biopsy is invasive, with little opportunity for re-examination, while liquid biopsy is minimally invasive and allows monitoring of disease progression through recurrent sample collection. Additionally, HER2 overexpression in the primary tumour and at metastatic sites are not always consistent[1] and it was previously shown that HER2 protein overexpression does not correlate with gene amplification in all cases[2]. This study aimed to establish an assay (Research Use Only) allowing scoring of HER2 protein expression in circulating tumor cells (CTCs), by immunofluorescence (IF) staining and evaluation of *HER2* gene amplification in the same CTCs harvested from a single blood sample.

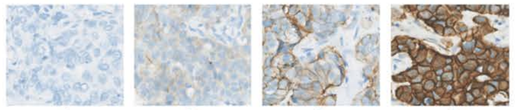


Figure 1. Representation of HER2 immunohistochemistry staining resulting in HER2 status designation. Figure adapted from van den Ende, NS et al., Scientific Reports, 2022.

Workflow

Cultured cancer cells with HER2 expression comparable to tissue samples designated as HER2 0, 1+, 2+ and 3+ by IHC testing (SKBR3 [3+], HCC1954 [3+], SKOV-3 [2+/3+], ZR-75-1 [2+], MCF-7 [0 – 1+] and Hs 578T [0]) were either spun onto CellKeep™ slides or spiked into blood collected from Healthy Volunteers (HV) and subjected to the workflow as described in **Figure 2.** The control slide samples were used to determine preliminary thresholds for scoring of HER2 protein expression, while spiked HV blood samples were used for analytical verification.

The same workflow was tested in two separate cohorts of MBC patient samples:

- One cohort of 38 MBC patients with known HER2 tissue status determined through IHC testing shortly before blood was drawn.
- One small cohort of 5 MBC patients with historical HER2 negative tissue biopsy results.

CTCs were considered to overexpress HER2 protein if the Mean Fluorescence Intensity (MFI) value was above the minimum positivity threshold and considered to present *HER2* gene amplification if the ratio of *HER2* foci to CEN17 foci was ≥ 2.

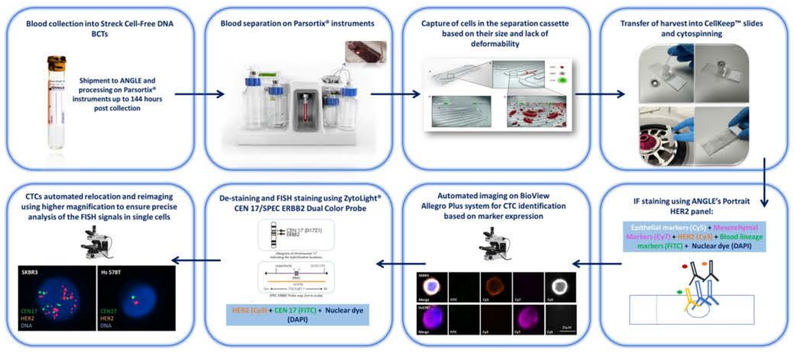


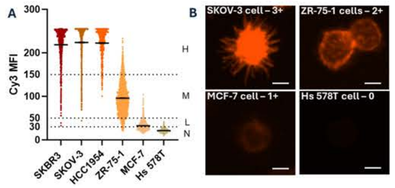
Figure 2. Schematic representation of the assay workflow. Peripheral blood was drawn into Streck Cell-Free DNA tubes and stored for up to 144 hours from collection before processing. Blood samples were processed on the Parsortix® instrument, a microfluidic device capable of capturing and harvesting CTCs from bodily fluids based on cell size and lack of deformability. Harvested CTCs were spun on ANGLE's CellKeep™ slides to maximize the retention. Slides were stained using ANGLE's IF-based 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 was performed on IF-stained samples 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).

Cultured cells loaded into control CellKeep™ slides and stained with the HER2 IF assay allowed the establishment of MFI thresholds representing a HER2 protein scoring system.

- 93%, 92% and 95% of SKBR3, HCC1954 and SKOV-3, respectively, fell in the High class, 83% of ZR-75-1 fell in the Medium class, 47% of MCF-7 fell in the Low class and 48% and 95% of MCF-7 and Hs 578T, respectively, fell in the Negative class.



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Cell Line	Percentage of cells in proposed classification			
	High	Medium	Low	Negative
SKBR3	92.6	7.2	0.1	0
HCC1954	91.5	8.4	0.1	0
SKOV-3	94.6	5.4	0	0
ZR-75-1	8.8	83.4	6.3	1.5
MCF-7	0	5.1	47	47.9
Hs 578T	0	0	4.9	95.1

Figure 3. HER2 protein grading. (A) Dot plot showing mean ± SEM of the HER2 MFI value in Cy3 channel in SKBR3, SKOV-3, HCC1954, ZR-75-1, MCF-7 and Hs 578T cells. **(B)** Representative 10× images of cancer cells lines (Top left = SKOV-3; Top right = ZR-75-1; Bottom left = MCF-7; Bottom right = Hs 578T), HER2 protein in orange (Cy3) Micron bar = 10 µm. **(C)** Table shows percentage of cells from each cultured cell line which fell into each proposed HER2 protein grading classification based on the Cy3 MFI value of each cell.

Analytical results

Analytical specificity and sensitivity of the IF panel were calculated as the percentage of harvested cancer cells known to express or not express each marker that displayed MFI above/below established MFI thresholds per marker, respectively.

- Analytical specificity and sensitivity of the HER2 IF assay were above 90% for all markers (**Figure 4.A**).

Specificity and sensitivity of the FISH assay was calculated as the percentage of SKBR3 and MCF-7 cells, previously stained with the HER2 IF panel, which had a *HER2*/CEN17 foci ratio ≥ 2.

- Specificity and sensitivity of the HER2 FISH assay were both 100% when evaluated in SKBR3 and MCF-7 cells.

A

	Specificity	Sensitivity
Epithelial	98.2%	99.7%
Mesenchymal	99.5%	97.4%
HER2	98.8%	99.8%
Blood Lineage	96.5%	99.8%
HER2 FISH	100%	100%

B

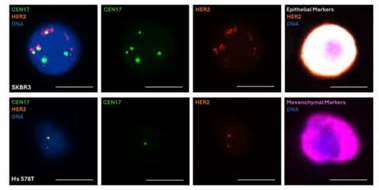

Figure 4. Assay analytical sensitivity and specificity. (A) Table shows mean percentage analytical specificity and sensitivity based on MFI thresholds for each IF channel, in positive and negative cells, and percentage analytical specificity and sensitivity of the FISH assay based on expected positivity of SKBR3 (*HER2* amplified) and MCF-7 (*HER2* non-amplified) cells. **(B)** 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

A

	All MBC	HER2 0 Donors	HER2 1+ Donors	HER2 2+ Donors	HER2 3+ Donors	Historical HER2 NegativeSubjects
N	43	11	6	8	13	5
≥1 CTC	38 (88.4%)	8 (72.7%)	6 (100%)	7 (87.5%)	12 (93.3%)	5 (100%)
Mean CTCs	62	82	140	20	37	50
Median CTCs	26	7	115	20	22	4
Range	1 - 474	1 - 474	32 - 338	1 - 73	1 - 152	0 - 213
N Donors with Ep CTCs only	4 (10.5%)	0 (0%)	0 (0%)	0 (0%)	2 (16.7%)	2 (40%)
N Donors with Ep & Mez CTCs and/or EMT CTCs	6 (15.8%)	0 (0%)	2 (33.3%)	1 (14.3%)	3 (25%)	0 (0%)
N Donors with Mez CTCs only	28 (73.7%)	8 (100%)	4 (66.7%)	6 (85.7%)	7 (58.3%)	3 (60%)
N Donors with ≥1 *HER2*+ CTC (IF)	6 (15.8%)	0 (0%)	0 (0%)	0 (0%)	4 (33.3%)	2 (40%)
N Donors with ≥1 *HER2*+ CTC (FISH)	13 (34.2%)	3 (37.5%)	0 (0%)	0 (0%)	7 (58.3%)	3 (60%)
Tissue / CTC HER2 protein concordance	28 (73.7%)	100%	100%	100%	33.3%	60%
Tissue / CTC *HER2* gene concordance	27 (71.1%)	63.5%	100%	100%	58.3%	40%

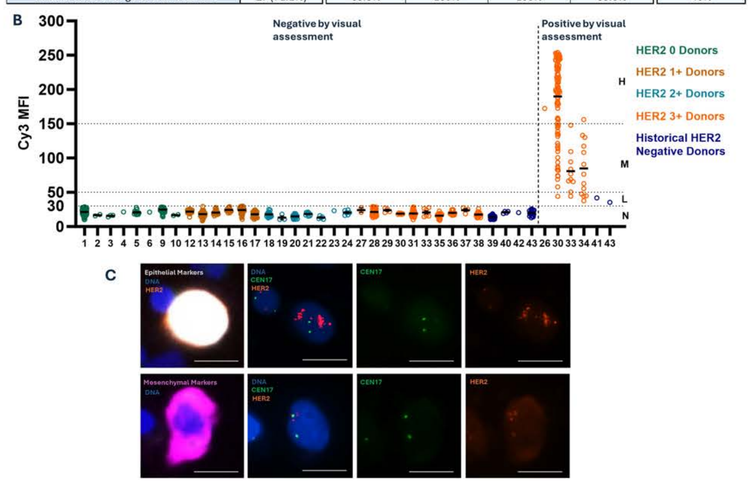

Figure 5. HER2 protein expression and gene amplification in CTCs from MBC patients' samples separated on Parsortix® instruments. (A) Table shows number (N) of subjects in each group, N and % subjects with ≥1 CTC, mean, median and range of the number of CTCs, donors phenotype based on the CTCs' EMT status and HER2 protein/gene positivity, HER2 concordance between tissue and CTCs. Numbers of CTCs are given per 15 mL of blood; **(B)** Dot plot shows MFI in the Cy3 channel representing bound anti-HER2 antibodies, of each CTC identified in samples produced from MBC patient blood samples separated on Parsortix instruments, which were deemed HER2 negative or positive by visual inspection, compared to the protein grading thresholds previously established. Dotted lines at MFI = 30, MFI = 50 and MFI = 150 represent thresholds for Low, Medium and High HER2 protein expression respectively; **(C)** Representative images of a single epithelial CTC positive for HER2 by both IF and FISH (*HER2/CEN17* ratio = 7) (top panel) and a single mesenchymal CTC positive for HER2 by FISH only (*HER2/CEN17* ratio = 2.5) (bottom panel). From left to right: 10× IF merge image, 40× merge FISH staining, *CEN17* foci in green (FITC), *HER2* foci in orange (Cy3). Micron bar = 20 µm.

Samples from 43 MBC patients were processed as per **Workflow section**. Of the patients, 38 were treatment naïve with recent tissue biopsy IHC tests, of which; 11 were HER2 0, 6 were HER2 1+, 8 were HER2 2+ (FISH negative) and 13 were HER2 3+. The 5 remaining patients had historical HER2 negative tissue biopsies of unknown IHC scores. Results are summarised in **Figure 5**.

- CTC (≥1) positivity rate was consistently high across groups, with an overall positivity of 88.4%.
- Across all groups, 73.7% of subjects presented only mesenchymal CTCs. Exclusively epithelial subjects were only observed in the HER2 3+ (16.7%) and historical HER2 Negative groups (40%).
- No CTCs with HER2 protein expression were observed in HER2 0, 1+ or 2+ groups (0%), while 33.3% and 40% of subjects in the HER2 3+ and historical HER2 Negative groups displayed ≥1 HER2 positive CTC, respectively.
- Epithelial phenotype correlated with HER2 protein positivity within CTCs, while HER2 gene amplification was observed across both epithelial and mesenchymal CTCs.
- Concordance between reported tissue biopsy results and CTC HER2 status by IF was 100% in HER2 0, 1+ and 2+ subjects compared to 40% in Historical HER2 Negative subjects, showing the assay's specificity when used close to time of tissue biopsy and the possible application of the assay for monitoring of disease progression. Low concordance in HER2 3+ (33.3%) may have been influenced by mesenchymal CTCs not showing HER2 expression as previously noted.
- Concordance between tissue biopsy results and CTC HER2 status by FISH were 63.5%, 58.3% and 40% in HER2 0, 3+ and historical HER2 negative groups respectively, showing that either one of *HER2* amplification or HER2 protein expression may be observed in cells singularly.
- Percentage of subjects HER2 positive by IF and FISH were similar between the HER2 3+ and Historical HER2 Negative groups, indicating HER2 status of the tumors in the latter group may have changed since tissue biopsy.
- All CTCs deemed HER2 negative or positive (before establishment of thresholds) by visual assessment were of the same HER2 status when protein grading thresholds were applied, resulting in 100% concordance of CTC HER2 status by visual assessment and assessment using thresholds, demonstrating robustness of the protein grading system.
- In historical HER2 Negative subjects, 100% of HER2 positive CTCs by IF fell into the Low protein grade, whereas in HER2 3+ subjects 4.9%, 32.8% and 62.3% fell into the Low, Medium and High grades respectively.

Conclusions

This study demonstrates the possibility of combining IF staining for CTCs identification based on epithelial and mesenchymal markers and HER2 protein with *HER2* FISH for evaluation of *HER2* amplification, and the possibility of establishing a robust HER2 protein grading system. High concordance of HER2 status between CTCs and primary tumour in patients with recent biopsies highlights the specificity of this assay, while discordance in historical patient samples highlights the potential of liquid biopsy and concomitant evaluation of HER2 protein expression and gene amplification in CTCs, for minimally-invasive disease monitoring of patients undergoing treatment for metastatic breast cancer, to enable more accurate stratification of patients that may benefit from HER2 targeted therapy.