A multiparameter assay for HER2 protein detection on circulating tumor cells in non-small cell lung cancer

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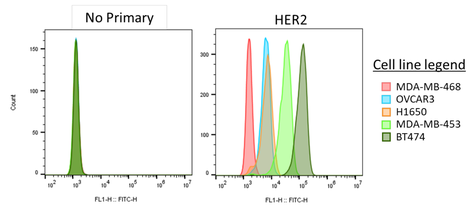
Abstract
Lung cancer biopsy can be difficult to perform successfully; it has a 30% adverse event risk and often provides insufficient material to test. Therefore, a liquid biopsy method to analyze the tumor is advantageous. Analysis of circulating tumor cells (CTCs) by multiparameter immunofluorescence (IF) microscopy allows non-invasive characterization of cancer cell biomarker expression. HER2 is a well-known therapeutic target in breast cancer and studies have shown that HER2 status on CTCs may provide prognostic information about response to anti-HER2 therapies. Less is understood about its frequency and clinical importance in non-small cell lung cancer (NSCLC). The RareCyte platform is uniquely suited for CTC identification and phenotypic characterization with a sensitive, accurate, simple and repeatable workflow from blood collection to CTC characterization and single-cell isolation.

Methods
We developed an assay for detecting HER2 expression on lung CTCs using the RareCyte platform and validated the assay on model CTC (mCTC) spike-in samples using cell lines characterized for HER2 expression by NanoString analysis to measure mRNA, and meso scale discovery and Quantibrite assays to measure protein levels in the cell lines. The final cell line panel demonstrated a range of HER2 expression with high (BT-474), medium (MDA-MB-453), low (H1650 and OVCAR-3) and absent (MDA-MB-468) HER2 protein levels. Blood was drawn into AccuCyte® Blood Collection Tubes and processed to 8 slides each using the AccuCyte Sample Preparation System and stained with an anti-HER2 antibody together with the RarePlex® CTC Panel Kit for CTC identification (nuclear dye, anti-CD45 to exclude WBC, and anti-cytokeratin/EpCAM for CTCs). Scanning of slides was performed on a CyteFinder® Instrument and CyteMapper® software was used to automatically detect CTCs and extract HER2 expression level on a per-cell basis. Files were analyzed by a trained reviewer. HER2 status was determined by MFI threshold.

Results
Spike-in samples showed the expected trend in per-cell HER2 mean fluorescence intensity (MFI) values. The percentage of HER2-positive cells was > 90% for the HER2-high, medium and low cell lines, and < 5% for the HER2-negative cell line. The assay was applied to 10 advanced stage (III or IV) post-treatment NSCLC patient samples. At least 1 CTC was found in 6 samples (range 0-2043) and HER2 expression was confirmed in 5 out of the 6 samples.

Conclusion
We have developed and analytically validated an immunofluorescence assay to measure HER2 protein expression on CTCs. The assay was used to characterize HER2 protein expression in NSCLC patient samples. A larger clinical sample set is needed to determine optimal clinical MFI thresholds.



Spike-in mCTC cell Line	Rank	Designation	ECL ELISA (MSD units)	RNA (nCounter units)	Flow Cytometry (S:B*)	Quantibrite (receptors per cell)
MDA-MB-468	0	Negative (N)	54	269	1.2	Not Detectable
OVCAR-3	1	Low level 2 (L2)	1069	423	4.7	7,903
H1650	2	Low level 1 (L1)	2409	920	5.3	8, 597
MDA-MB-453	3	Medium (M)	18006	4160	24.3	48,814
BT474	4	High (H)	ND	ND	91.3	ND

Figure 1. Verification of HER2 expression using cell line controls.
HER2 RNA and protein expression of the five cell line controls were measured using the methods indicated in the table. Flow cytometry data is show in the two histogram overlays (left: no primary control; right: HER2 primary). Results show expected expression level on each cell line.

Sample preparation and analysis workflow

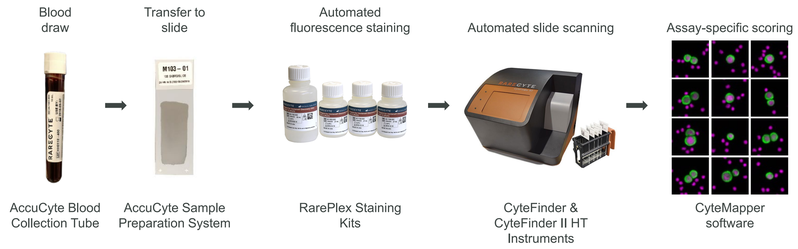
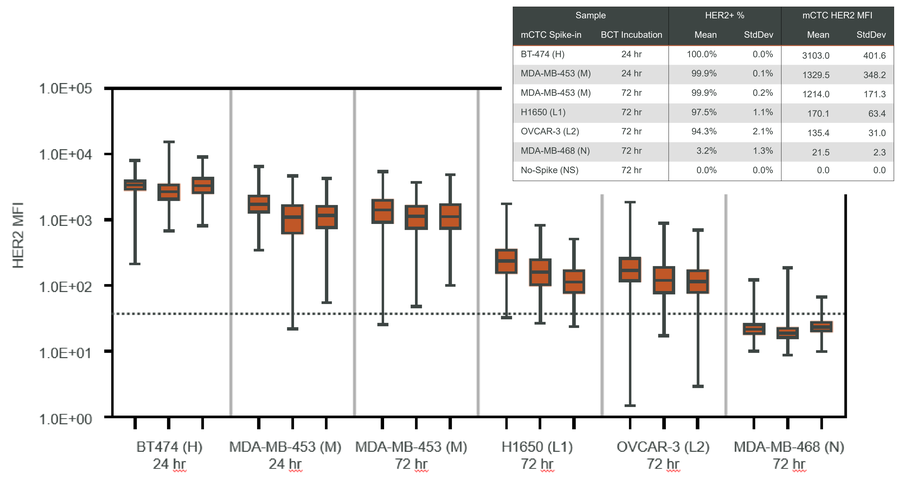


Figure 2. The RareCyte CTC assay workflow.
Blood was collected into AccuCyte Blood Collection Tubes. Nucleated blood cells were processed to slides using the density-based AccuCyte Sample Preparation System. Slides were stained with the RarePlex HER2 CTC Staining Kit using the Ventana® DISCOVERY® ULTRA automated slide staining system. Slides were scanned using the CyteFinder Instrument and images were analyzed using CyteMapper software and analysis tools. CTCs were analyzed by a trained reviewer, and CTC HER2 status was determined with a fluorescence intensity threshold.



Sample		HER2+ %		mCTC HER2 MFI	
mCTC Spike-in	BCT Incubation	Mean	StdDev	Mean	StdDev
BT-474 (H)	24 hr	100.0%	0.0%	3103.0	401.6
MDA-MB-453 (M)	24 hr	99.9%	0.1%	1329.5	348.2
MDA-MB-453 (M)	72 hr	99.9%	0.2%	1214.0	171.3
H1650 (L1)	72 hr	97.5%	1.1%	170.1	63.4
OVCAR-3 (L2)	72 hr	94.3%	2.1%	135.4	31.0
MDA-MB-468 (N)	72 hr	3.2%	1.3%	21.5	2.3
No-Spike (NS)	72 hr	0.0%	0.0%	0.0	0.0

Figure 3. HER2 expression on mCTC cell lines measured using the RarePlex HER2 CTC assay.
The indicated cell lines were spiked into blood and incubated at room temperature for the indicated times followed by analysis with the RarePlex HER2 CTC assay. Four slides per mCTC line for 3 stainer runs are shown. Threshold dotted line at MFI=41 is used to determine HER2 status on a per-cell basis. The threshold was set to 2 standard deviations above the mean HER2 MFI value for the negative cell line. Boxes represent the 25th, 50th, 75th percentiles, and whiskers represent min/max values. Statistics summarized in table at top.

	HER2- cells	HER2+ Cells			
	MDA-MB-468 (N)	BT-474 (H)	MDA-MB-453 (M)	H1650 (L1)	OVCAR-3 (L2)
Test Positive (MFI > 41)	32	1000	999	975	944
Test Negative (MFI < 41)	968	0	1	25	56
Specificity	0.968				
Sensitivity		1.000	0.999	0.975	0.944
Accuracy		0.984	0.984	0.972	0.956

Figure 4. HER2 sensitivity, specificity and accuracy.
Sensitivity, Specificity, and Accuracy determined from HER2-negative (MDA-MB-468) and each HER2+ mCTC line. The number of cells for each cell line is normalized to 1000 to balance the accuracy equation. The actual number of cells tested per cell line: MDA-MB-468 (2187); BT-474 (580); MDA-MB-453 (1481); H1650 (1219); OVCAR-3 (2183).

Accuracy = (TP + TN) / (total number of tests)
Sensitivity = TP / (TP + FN)
Specificity = TN / (TN + FP)

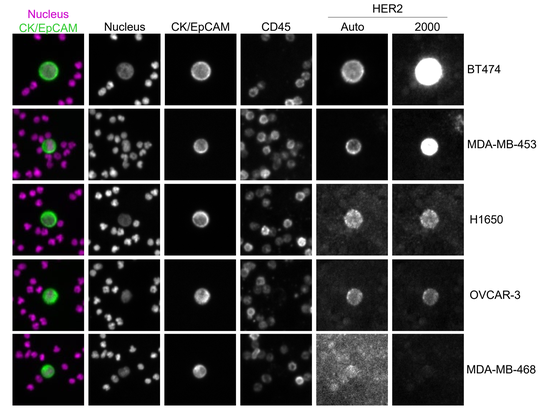


Figure 5. Representative images of mCTCs.
Each row shows the indicated representative images for a single mCTC. HER2 images were scaled in two different ways to accommodate the high dynamic range of HER2 expression across the cell lines. Autoscale was used to optimally visualize the HER2 signal on all mCTCs regardless of expression level and is achieved by using linear display scaling with the dimmest pixel displayed as black and the brightest pixel displayed as white. To perceive relative difference in HER2 intensity across the cell lines, we also used constant scaling with display saturation set to 2,000 intensity counts. All positive mCTCs shown display the expected HER2 membrane localization.

A

Sample	Diagnosis	Stage	Gender	Age	CTC/7.5 ml	HER2+ CTC	HER2+ CTC%	HER2 MFI
1	NSCLC	IVA	M	51	3	1	33	20, 45, 334
2	NSCLC	IIIB	F	65	0	NA	NA	NA
3*	NSCLC	IIIB	M	50	2043	511*	25*	18-2098*
4	NSCLC	IVB	F	53	1	0	0	26
5	NSCLC	IV	F	47	1	1	100	110
6	NSCLC	IIIA	F	61	0	NA	NA	NA
7	NSCLC	IIIA	M	67	1	1	100	402
8	NSCLC	IV	M	67	5	1	20	16, 29, 37, 59, 144
9	NSCLC	IV	F	66	0	NA	NA	NA
10	NSCLC	Unk	M	73	0	NA	NA	NA

B

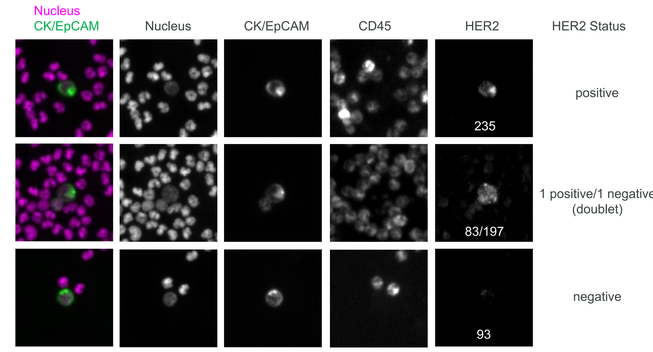


Figure 6. Testing HER2 assay on NSCLC samples.
Blood from ten NSCLC patients was collected and processed with the RarePlex HER2 CTC assay. A) CTC count and HER2 status (determined using an MFI threshold of 100) for each patient is indicated in the table. The increased threshold was used due to higher non-specific background staining found on clinical slides. (*) Patient 3 HER2 expression data was extrapolated from 1 slide. HER2 expression in patient 3 had mean MFI of 141 (interquartile range 75-153). CTC numbers in patient 3 are reflective of the clinical status of the patient (stage IIIB with disease progression). B) Four representative CTC images from patient 3 are shown, including a cluster of 2 CTCs in middle row.