Quantitative analysis of HER2, HER3, and γH2AX expression on circulating tumor cells for pharmacodynamic measurement in clinical trials

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BACKGROUND

Circulating tumor cells (CTCs) can provide quantitative biomarker expression of the tumor independent of tissue biopsies, making them an attractive target for pharmacodynamic measurements in clinical trials. The increasing challenge to obtain tissue samples continues to focus attention on non-invasive liquid biopsy approaches. The RareCyte platform offers a highly sensitive method for CTC enumeration and analysis of protein expression on CTCs. In this study, we describe the development of assays to quantify HER2, HER3, and γH2AX expression on CTCs. Each of the single biomarker assays allow for the inclusion of an additional biomarker of interest to be measured on each CTC.

METHODS

Cell lines with known positive or negative expression for each biomarker were spiked into healthy donor blood to generate CTCs. Nucleated cells were collected from these samples using the AccuCyte® sample preparation kit and spread onto microscope slides. Slides were fixed and stained with antibodies targeting cytokeratin, EpCAM, and CD45 to detect CTCs and exclude white blood cells (WBCs), as well as primary antibodies against HER2, HER3, or γH2AX to evaluate protein expression on identified CTCs. Slides were imaged with the CyteFinder® automated immunofluorescence scanning microscope to quantitatively measure Mean Fluorescence Intensity (MFI) of biomarkers to assess protein expression on individual CTCs. Assays were designed and optimized to maximize the signal-to-background ratio. The final assays were run on replicate slides of positive and negative cell lines over 3 independent days to evaluate the analytic performance of each assay. Thresholds for biomarker positivity were established based on MFI measurements from positive and negative control slides.

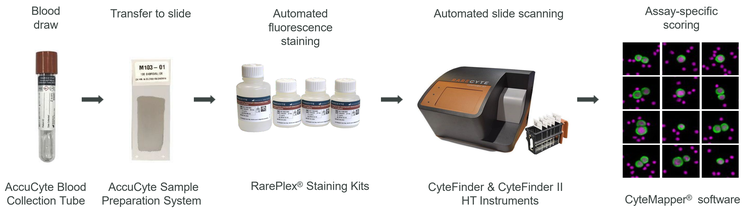

Figure 1. RareCyte platform workflow. Blood is collected into AccuCyte Blood Collection Tubes (BCTs). Nucleated blood cells are processed to slides using the AccuCyte Sample Preparation System. Slides are stained with assay-specific RareCyte staining kits. Slides are scanned using the CyteFinder Instrument and images are analyzed using CyteMapper software and analysis tools. Cells are analyzed by a trained reviewer, and cell biomarker status is determined with a fluorescence intensity threshold.

RESULTS

The accuracy of all three biomarker assays was >90%. Both repeatability (intra-run precision) and inter-run precision, assessed by the Coefficient of Variations (CV), were <20% for all three assays. The HER2 biomarker assay was tested on 16 cell lines with varying levels of HER2 expression, demonstrating its ability to confirm HER2 protein levels and distinguish between cell lines with known HER2 expression (0, 1+, 2+, 3+).

Biomarker	HER2	HER3	γH2AX
Accuracy (%)	99.9	92.1	94.3
Signal to Background (S:B)	96.6	28.2	35.0
Inter-stainer run Precision (CV%)	8.0	13.9	18.4
Intra-run Repeatability (CV%)	9.7-12.1	4.9-7.6	3.8-8.1
Sensitivity	100.0	88.1	91.9
Specificity	97.7	96.0	96.7

Table 1. Assay Performance Summaries. Accuracy was determined by setting a threshold that maximizes the discrimination between the MFI distributions of positive and negative control cells. S:B is the ratio of the median biomarker MFI of the positive control cells to the median biomarker MFI of the negative control cells. Inter-stainer run precision is the Coefficient of Variation (CV%) around the median biomarker MFI on positive control cells for 3 independent stainer runs. Intra-run repeatability is the CV% of the median biomarker MFI across 5 positive control slides within a single run. Sensitivity is the number of positive cells found by the assay compared to the number of total positive cells. Specificity is the number of negative cells found by the assay compared to the number of total negative cells.

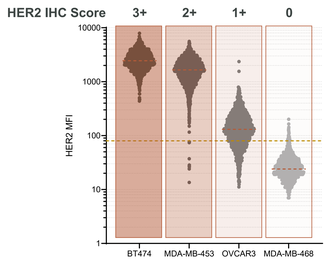

Figure 2. HER2 Assay Accuracy. MFI distribution of HER2 staining on BT474 (HER2 high, 3+ IHC score), MDA-MB-453 (HER2 medium, 2+ IHC score), OVCAR-3 (HER2 low, 1+ IHC score) and MDA-MB-468 (HER2 negative, 0 IHC score) spike-in cells. Each group represents 15 slides of each cell type run over 3 days. Each dot represents one cell. Dashed copper line indicates population median, gold dashed line represents threshold for maximum Accuracy (99.9%) based on model cell line data.

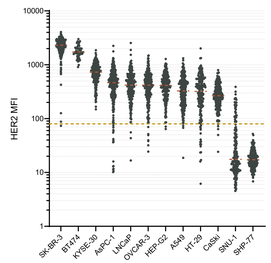

Figure 3. HER2 Assay on Additional Cancer Cell Lines. MFI distribution of HER2 staining on multiple cell lines with differing amount of HER2 expression. Each dot represents one cell. Dashed copper line indicates population median for each cell line. Gold dashed line represents positivity threshold for maximum Accuracy based on model cell line data during validation.

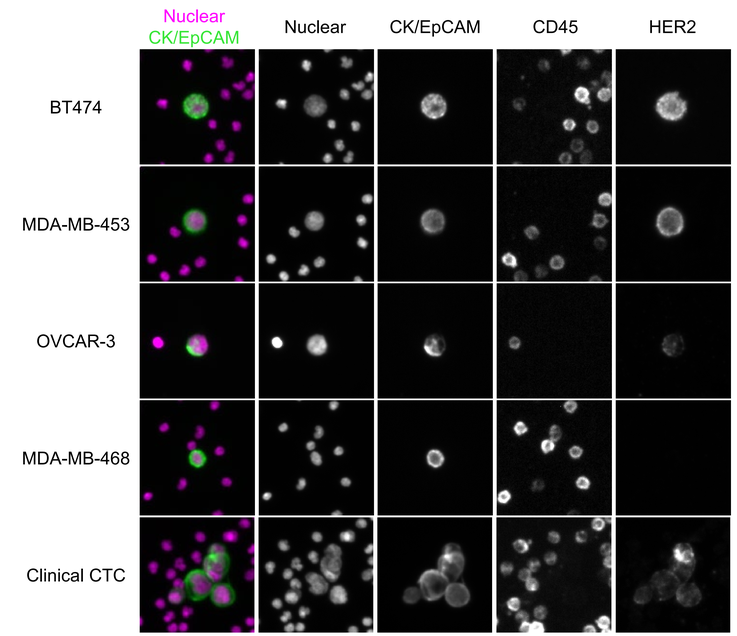

Figure 4. HER2 Assay Representative Images. High (BT474), medium (MDA-MB-453), low (OVCAR-3), and negative (MDA-MB-468) control cell lines for HER2 expression and example CTC from colorectal clinical sample tested with HER2 assay.

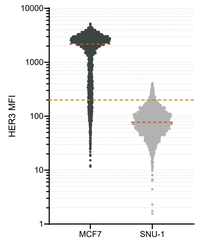

Figure 5. HER3 Assay Accuracy. MFI distribution of HER3 staining on MCF7 (dark grey, HER3 positive) and SNU-1 (light grey, HER3 negative) spike-in cells. Each group represents 15 slides of each cell type run over 3 days. Each dot represents one cell. Dashed copper line indicates population median, gold dashed line represents threshold for maximum Accuracy (92.1%) based on model cell line data.

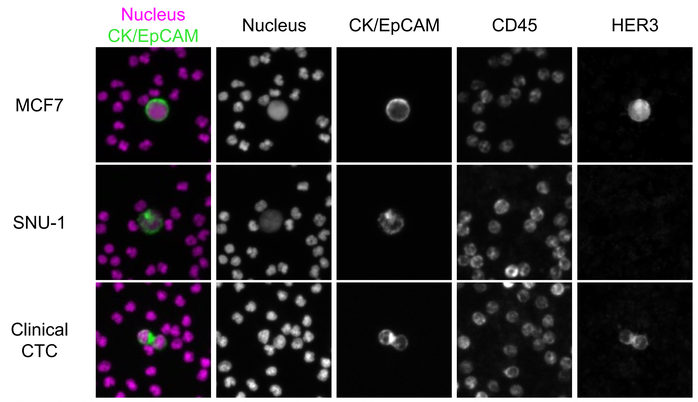

Figure 6. HER3 Assay Representative Images. Positive (MCF7) and negative (SNU-1) control cell lines for HER3 expression and example CTC from non-small cell lung cancer (NSCLC) clinical sample tested with HER3 assay.

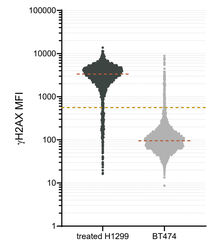

Figure 7. γH2AX Assay Accuracy. MFI distribution of γH2AX staining on topotecan treated H1299 (dark grey, γH2AX positive) and BT474 (light grey, γH2AX negative) spike-in cells. Each group represents 15 slides of each cell type run over 3 days. Each dot represents one cell. Dashed copper line indicates population median, gold dashed line represents threshold for maximum Accuracy (94.3%) based on model cell line data.

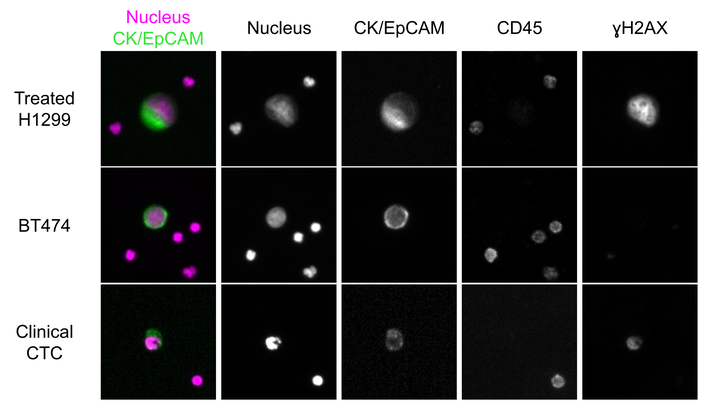

Figure 8. γH2AX Assay Representative Images. Positive (topotecan treated H1299) and negative (BT474) control cell lines for γH2AX expression and example CTC from prostate clinical sample tested with γH2AX assay.

CONCLUSIONS

- Accurate and reproducible assays were developed to assess HER2, HER3, and γH2AX expression on individual CTCs.
- All three assays successfully deployed in clinical trials providing a pharmacodynamic biomarker.
- Can be converted to dual biomarker assays by adding a custom biomarker of choice.
- Offers an exciting tool for patient selection and longitudinal, non-invasive monitoring of cancer patients in clinical trials.