Custom biomarker investigation of circulating tumor cells using RarePlex® Developer Kits

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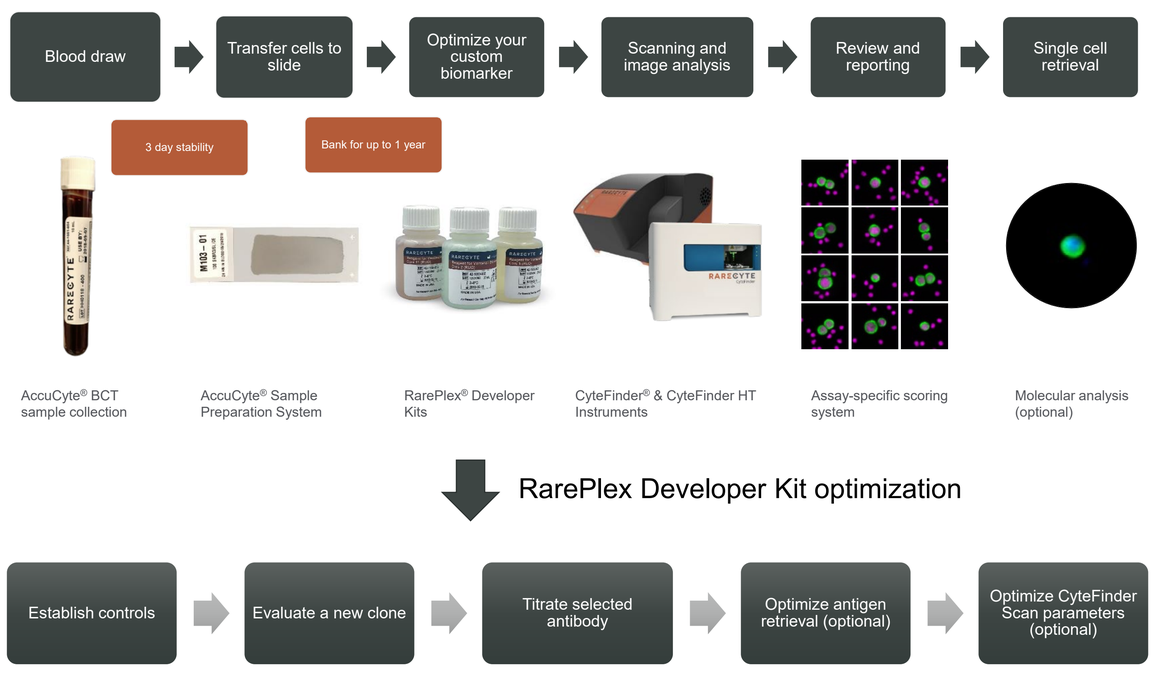
BACKGROUND

There is considerable interest in investigating circulating tumor cells (CTCs) for biomarkers that provide information on drug target expression, response to therapy, and metastatic potential. Currently, options for investigating biomarkers on CTCs are limited due to challenges of developing multiplexed assays. The RareCyte platform combines CTC sample preparation, multiparameter fluorescence staining, imaging, and single cell retrieval. For custom biomarker exploration, RareCyte's Developer Kit technology enables the simple addition of biomarkers of interest to a base epithelial CTC Panel Kit.

METHODS

Blood samples spiked with positive and negative model circulating tumor cells (mCTC-positive and mCTC-negative) for each investigative biomarker were processed using AccuCyte® Sample Preparation System. Slides were auto-stained with the RarePlex® CTC Panel Kit utilizing a three-channel CTC detection base: a nuclear dye, anti-CD45 antibody to exclude white blood cells, and cocktailed antibodies to cytokeratin (CK) and epithelial cell adhesion molecule (EpCAM). RarePlex Developer Kits were used to test biomarker expression of additional markers: HER2, ER, PR, EGFR, Ki67, AR, ARv7, and PSMA under various optimized conditions (antibody concentration, incubation time and temperature). Stained slides were imaged with a CyteFinder® Instrument. CTCs were identified using machine learning-based algorithms and confirmed by user review in CyteHub® software. Biomarker analysis was performed by visual observation and mean fluorescence intensity measurements on confirmed CTCs. Developer Kits were applied to clinical samples from prostate and breast cancer patients.

RareCyte workflow for CTC analysis



RESULTS

RareCyte Developer Kits were successfully tested on a broad range of biomarkers on cell line control samples with default antigen retrieval and fixation conditions. For each biomarker, fluorescence intensity cut-offs that segregated negative and positive cell lines were statistically defined. When applied to clinical samples of appropriate type, staining with the expected localization was observed.

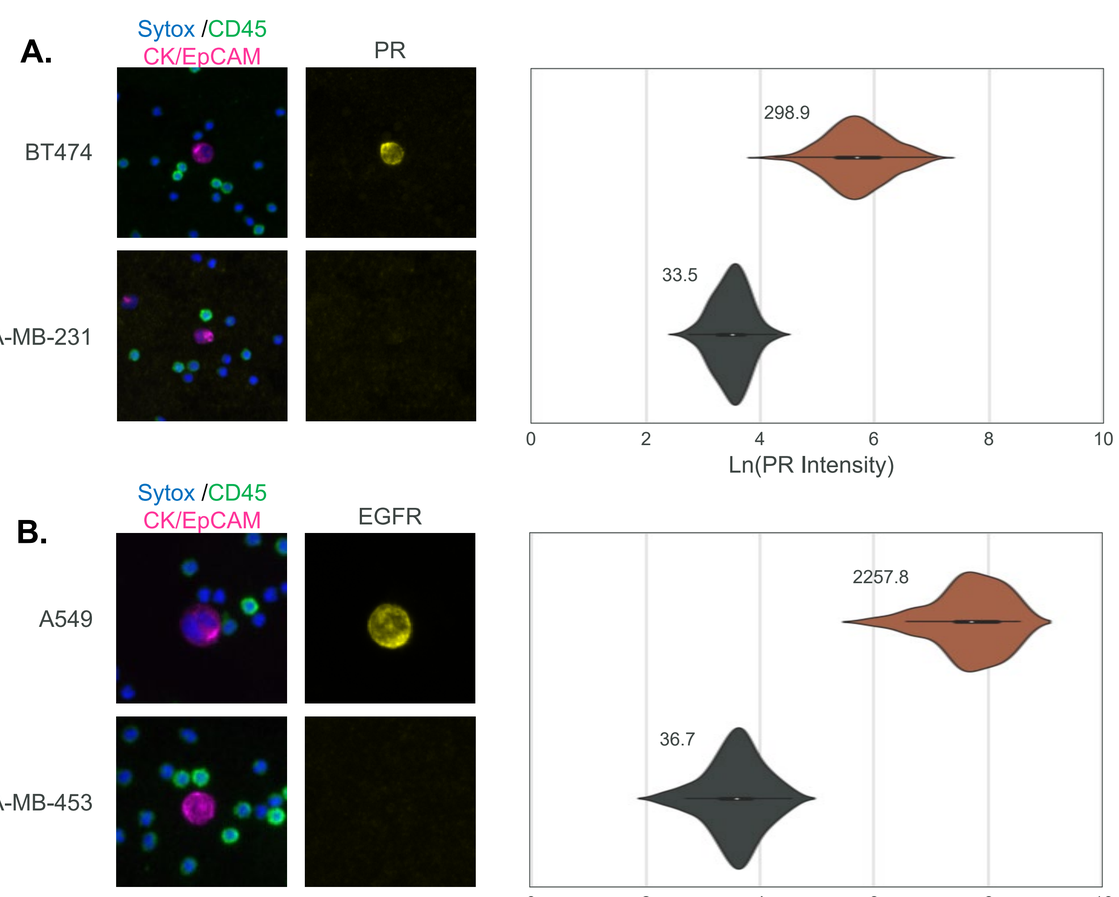
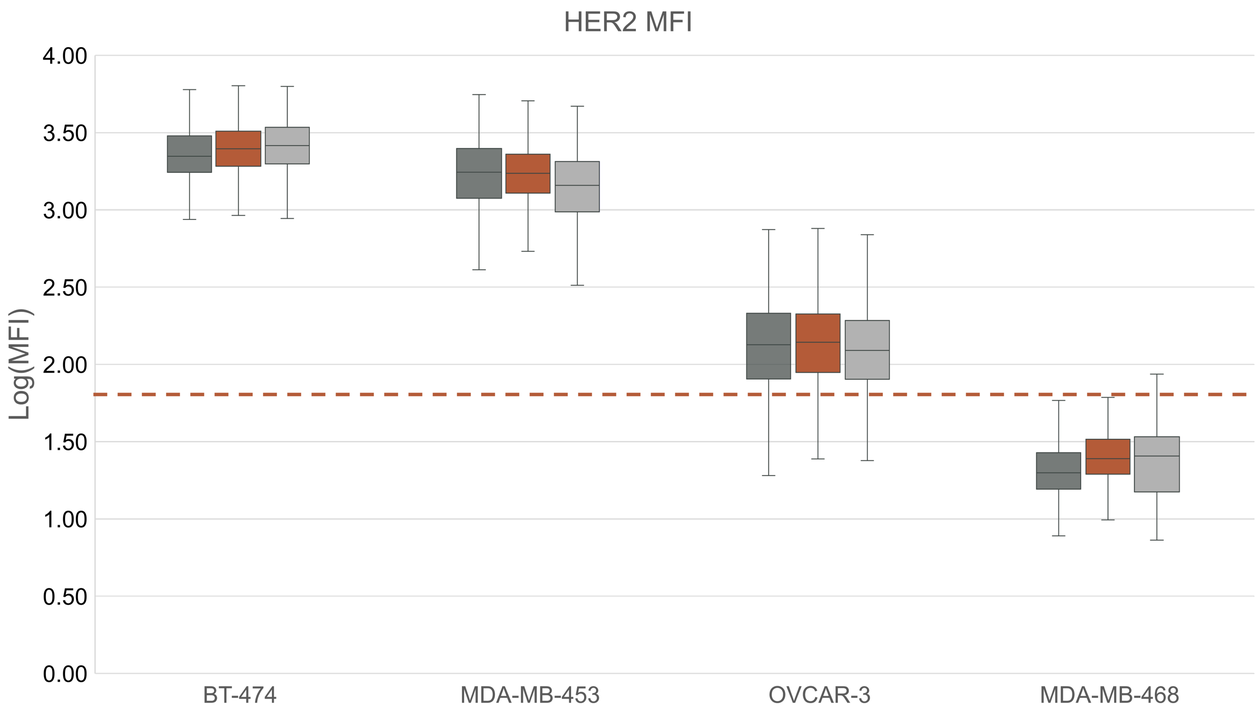


Figure 1: Clone specificity evaluation by mCTC-positive and mCTC-negative cell lines. Representative images of marker-positive and negative cells are shown on the left and violin plots of all cell MFIs are shown on the right. Positive and negative cell lines showed the expected localization and expression, confirmed by fluorescence intensity values. **A.** BT474 cells display the expected PR nucleo-cytoplasmic distribution while MDA-MB-231 shows no PR staining. **B**. A549 cells display the expected EGFR cell surface ring distribution while MDA-MB-453 shows no EGFR staining. **C.** BT474 cells display the expected HER2 cell surface ring while MDA-MB-231 shows the expected lack of HER2 staining. Positive and negative images were scaled to the same display settings.



Overall % HER2 Positivity			
BT474	MDA-MB-453	OVCAR3	MDA-MB-468
100.0	99.6	77.0	3.0

Figure 2: MFI comparison for mCTCs with a range of HER2 expression. Four cell lines with known HER2 expression levels were compared: BT-474 (High), MDA-MB-453 (Medium), OVCAR-3 (Low) and MDA-MB-468 (Negative). Each cell line was spiked in to three separate healthy normal donors (indicated by bar color). MDA-MB-468 cells (marker-negative) were used to establish an MFI cut-off for marker positivity and applied to the other cell lines (dashed line) to determine the percentage of cells positive for HER2, as listed in the table. Whiskers are set to 1.5xInter Quartile Range.

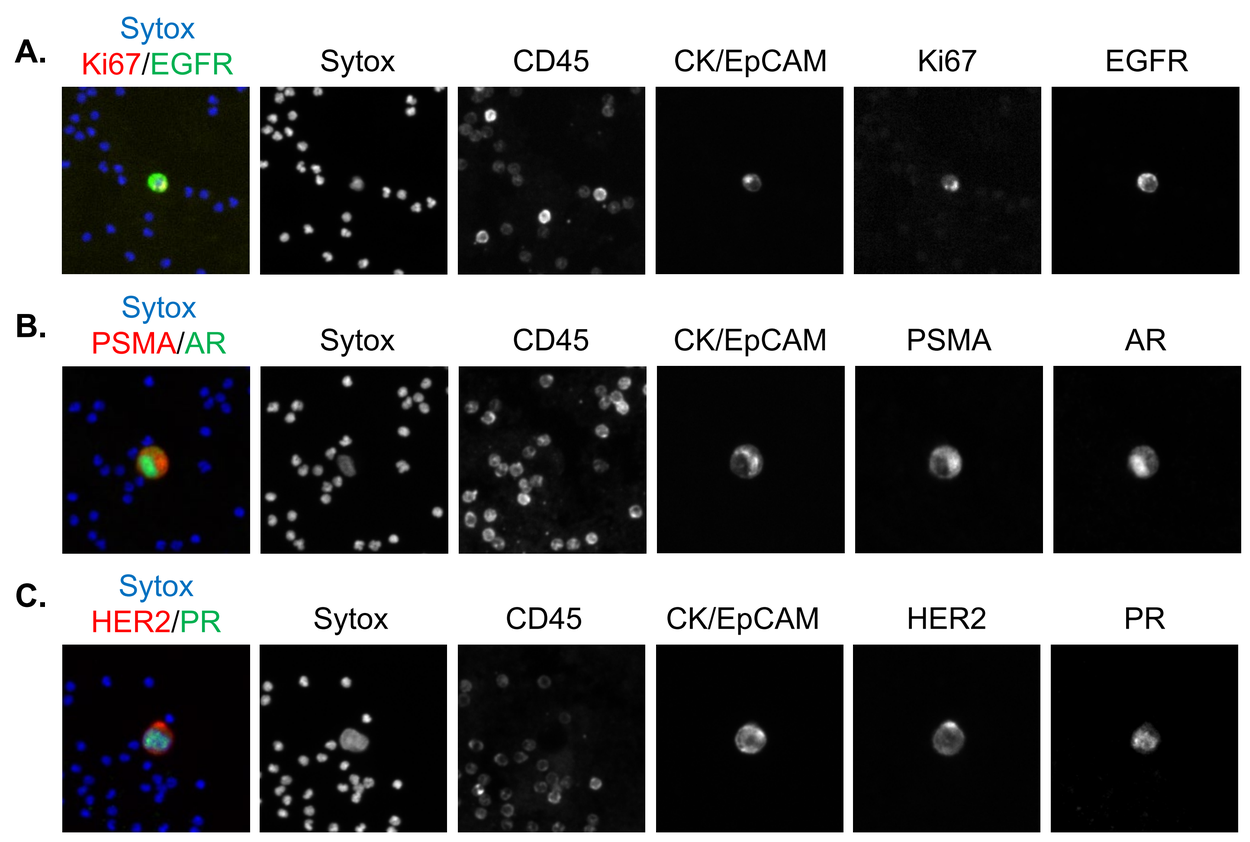
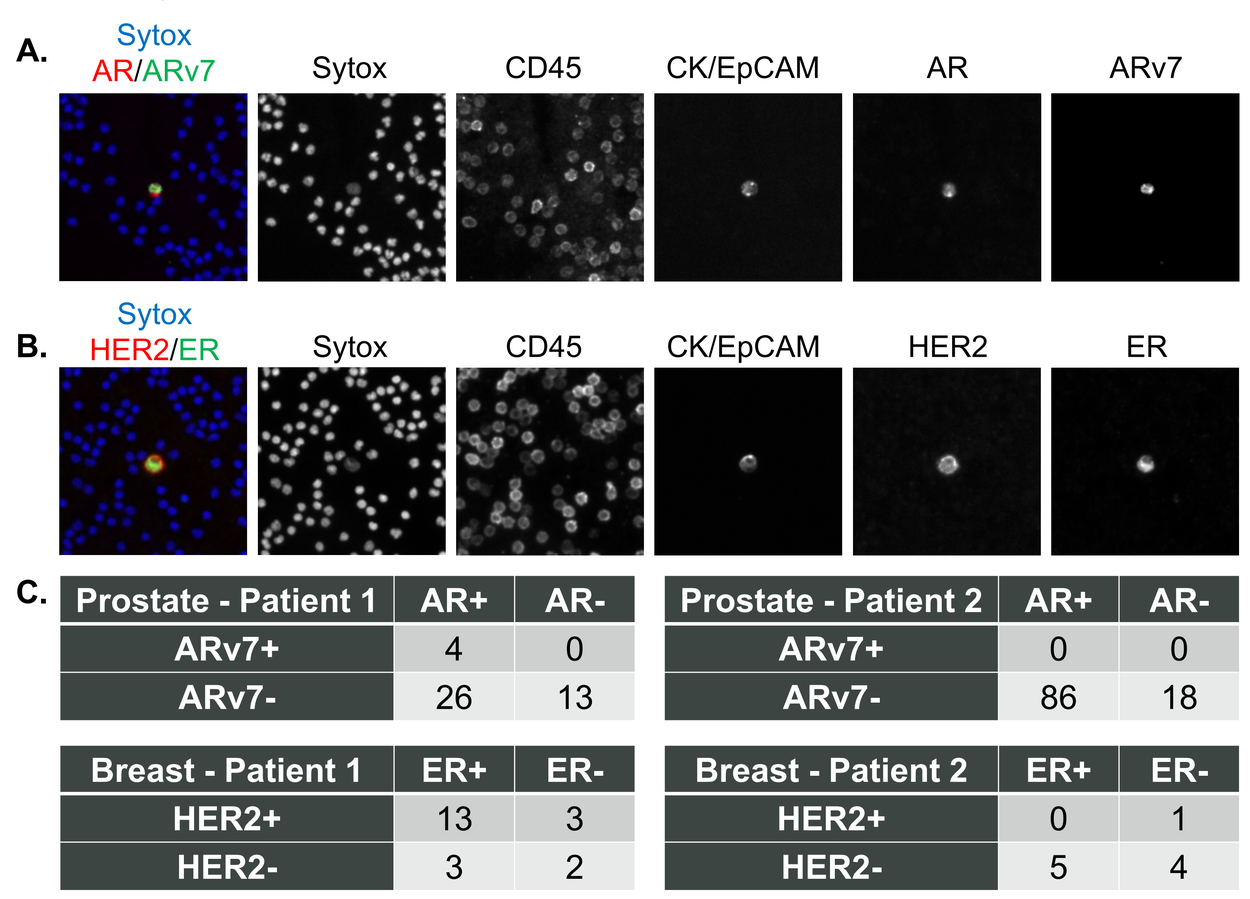


Figure 3: **mCTCs staining with expected subcellular localization using the base epithelial CTC detection assay and two additional biomarkers.** Individual channels and composite images of the indicated markers are shown. **A.** A549 cells stained positive for both Ki67 and EGFR, with the expected nuclear localization for Ki67 and cell surface distribution for EGFR. **B.** LnCAP cells stained positive for both PSMA in the cytoplasm and AR in the nucleus. **C.** BT474 cells stained positive for both HER2 on the cell membrane and PR in the nucleus.



C.

Prostate - Patient 1	AR+	AR-
ARv7+	4	0
ARv7-	26	13

Prostate - Patient 2	AR+	AR-
ARv7+	0	0
ARv7-	86	18

Breast - Patient 1	ER+	ER-
HER2+	13	3
HER2-	3	2

Breast - Patient 2	ER+	ER-
HER2+	0	1
HER2-	5	4

Figure 4: **Clinical samples stained using the base epithelial CTC detection assay and two additional biomarkers.** Individual channels and composite images of the indicated markers are shown. **A.** Prostate cancer patient showing positive staining for both AR and ARv7. As expected, ARv7 has exclusively nuclear staining while AR has nuclear and partial cytoplasmic staining. **B.** Breast cancer patient showing positive staining and expected localization for both HER2 and ER. **C.** Tables indicating marker expression on individual CTCs from 2 prostate and 2 breast cancer patients. Images from A and B correspond to Prostate – Patient 1 and Breast – Patient 1, respectively. Breast Patient 1 had ER+/HER2- and ER+/HER2+ tumors; this heterogeneity was also observed in the CTCs.

CONCLUSIONS

- RareCyte Developer Kits can be easily combined with a base epithelial CTC Panel Kit to stain an additional one or two user-selected markers with minimal staining optimization
- We have successfully applied the combined kits to 8 markers with default antigen retrieval conditions that have shown the expected sub-cellular localization
- Model circulating tumor cells are used to establish fluorescence intensity cut-offs to separate positive and negative marker expression
- Custom assays created by combining Developer Kits and Panel Kits have been applied to clinical samples to characterize the expression of biomarkers of interest on CTCs