

# Validation of a dual-marker ARv7/SYP assay for CTC characterization

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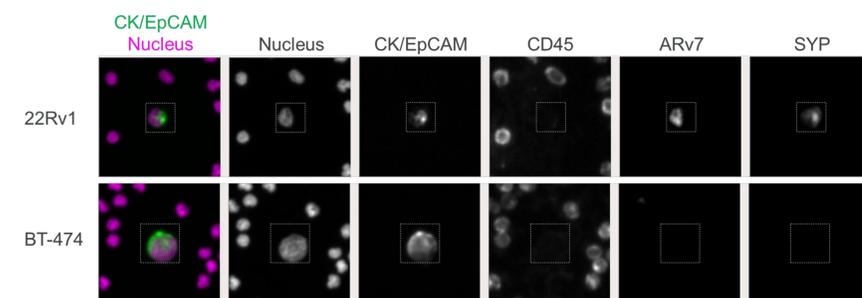
## ABSTRACT

Analysis of circulating tumor cells (CTCs) by multiparameter immunofluorescence (IF) microscopy allows non-invasive characterization of cancer cell biomarker expression in real time. This information can be helpful in prognosis, treatment selection, or disease classification. In prostate cancer, protein expression of androgen receptor variant 7 (ARv7) and synaptophysin (SYP) can provide predictive and prognostic information. ARv7 expression has been associated with resistance to second-generation anti-androgen treatments and synaptophysin expression indicates a tumor has undergone differentiation from an epithelial to neuroendocrine subtype. Expression of either marker is correlated with less favorable prognosis.

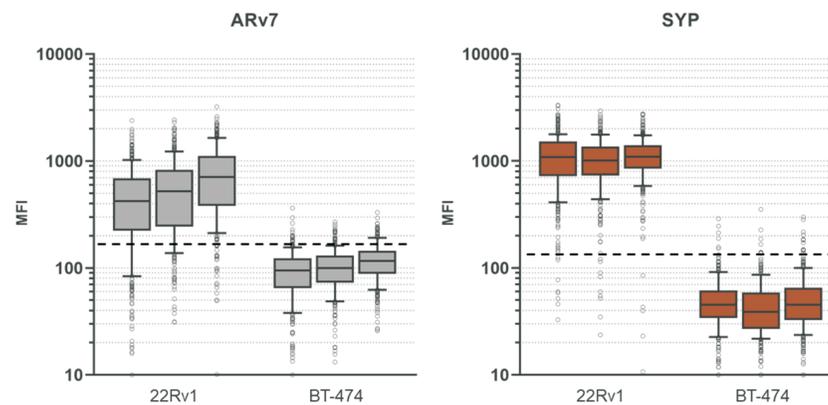
Here we describe the validation of a dual biomarker IF assay for enumeration of CTCs and characterization of their ARv7 and SYP expression. The 0919-LB ARv7/SYP CTC assay workflow includes processing blood samples to slides (AccuCyte® Sample Preparation System), staining slides with a panel of fluorescent markers (RarePlex® Staining Kit and Leica® BOND® RX immunostaining system), and multiparameter imaging and analysis (CyteFinder® Instrument). The panel consists of a nuclear dye, and antibodies to SYP and ARv7, cytokeratin and EpCAM (to identify epithelial CTCs), and CD45 (to exclude white blood cells). Both clinical and spike-in samples were used for the validation. The model CTCs used to generate spike-in samples included 22Rv1 (ARv7 and SYP high) and BT-474 (ARv7 and SYP low). Performance metrics for the ARv7/SYP assay included accuracy, sensitivity, specificity, repeatability, and intermediate precision. Single cell ARv7 and SYP mean fluorescence intensities (MFI) were analyzed to determine expression levels.

Using MFI thresholds for ARv7 accuracy and precision, the assay correctly classified ARv7 for 89% of the cells and SYP for 97% of the cells with an Inter-stainer Run coefficient of variation (CV) of <10%. The ARv7/SYP assay was also applied to clinical breast, colon and prostate cancer samples from patients known to have CTCs. In these samples, subsets of CTCs were found to be ARv7 or SYP positive with the expected nuclear ARv7 localization and cytoplasmic SYP localization. Overall, this assay provides an analytically sensitive and specific method to find CTCs and characterize their ARv7 and SYP expression.

## RESULTS

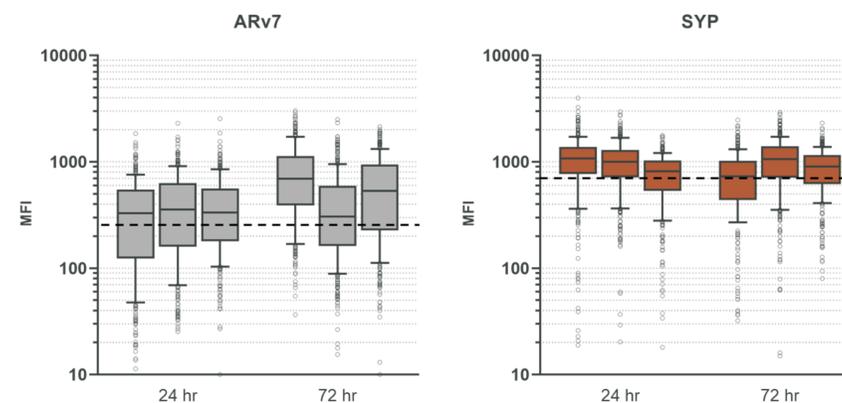


**Figure 1. Representative images of the indicated mCTC obtained by processing slides with the ARv7/SYP assay.** ARv7 and SYP protein is expressed in 22Rv1 mCTC but not in BT-474 mCTC. ARv7 is localized to the nucleus and SYP is localized to the cytoplasm. Image display properties within a channel are set to the same values for both cell types.

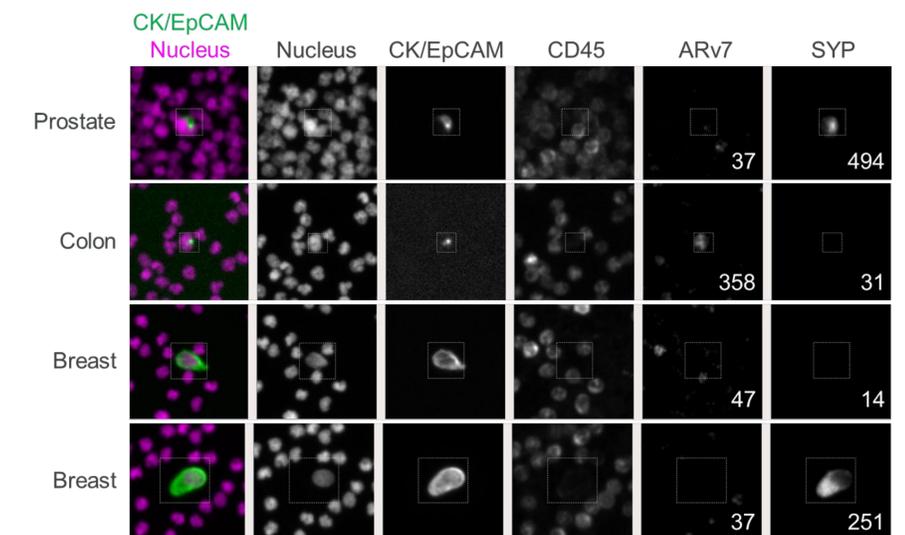


ALL 3 STAINER RUNS	ARv7- cells BT-474	ARv7+ Cells 22Rv1	ALL 3 STAINER RUNS	SYP- cells BT-474	SYP+ Cells 22Rv1
Test Positive (MFI > 180)	80	852	Test Positive (MFI > 120)	38	982
Test Negative (MFI < 180)	920	148	Test Negative (MFI < 120)	962	18
<b>Specificity</b>	<b>0.920</b>		<b>Specificity</b>	<b>0.962</b>	
<b>Sensitivity</b>		<b>0.852</b>	<b>Sensitivity</b>		<b>0.982</b>
<b>Accuracy</b>		<b>0.886</b>	<b>Accuracy</b>		<b>0.972</b>

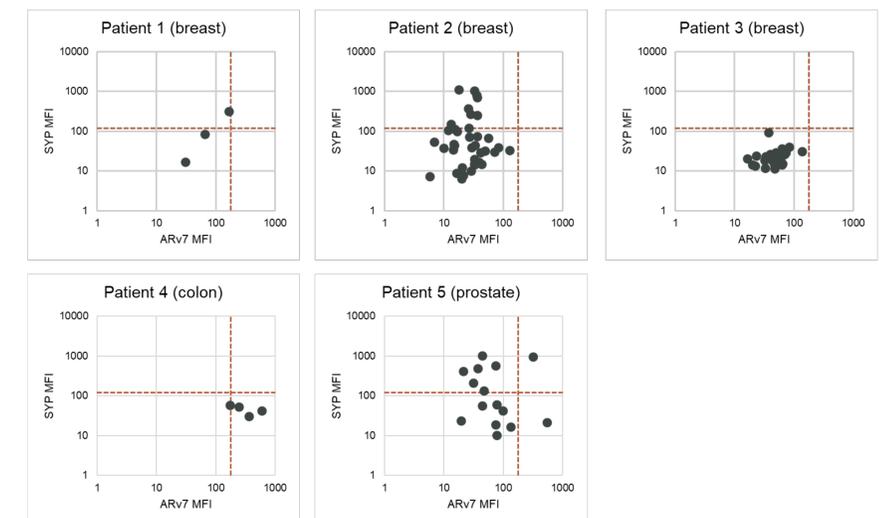
**Figure 2 and summary tables. Accuracy and Inter-stainer Run Intermediate Precision.** A) Distribution of ARv7 (left graph) and SYP (right graph) MFI for mCTC across 5 slide replicates per cell line for stainer run 1 (left), 2 (center), and 3 (right). Bottom and top of each box represent 1<sup>st</sup> and 3<sup>rd</sup> quartiles, middle line represents mean, and whiskers indicate the 10-90 percentile range. Dotted lines indicate thresholds (MFI=180 for ARv7 and MFI=120 for SYP) optimal for biomarker accuracy. Test positive and test negative values (normalized to 1000 cells per cell type) used to calculate Specificity, Sensitivity, and Accuracy. BT-474 are used as true negatives for expression for both markers and 22Rv1 are used as true positives for both markers. The actual number of cells tested per cell line: BT-474 (922); 22Rv1 (1084). The CVs for ARv7 and SYP Inter-stainer Run Intermediate Precision were 9.8% and 5.1%, respectively.



**Figure 3. Blood incubation time and Precision-Inter-donor.** Distribution of ARv7 (left) and SYP (right) MFI for 22Rv1 mCTC across 4 slide replicates per BCT incubation time for donor 1 (left), 2 (center), and 3 (right). Bottom and top of each box represent 1<sup>st</sup> and 3<sup>rd</sup> quartiles, middle line represents mean, and whiskers indicate the 10-90 percentile range. Dotted lines indicate thresholds (MFI=250 for ARv7 and MFI=700 for SYP) used to determine precision.



**Figure 4. Representative clinical CTCs.** Patient samples from prostate, colon, and breast tumors were stained with the ARv7/SYP assay. Image display properties within a channel are set to the same values for all cells shown. ARv7 and SYP MFIs are indicated in bottom right corner.



**Figure 5. Per-cell ARv7 and SYP MFI on clinical CTC.** Blood samples obtained from prostate, colon, or breast cancer patients were processed with the ARv7/SYP assay. Each dot represents a CTC. Dashed lines indicate MFI thresholds established for maximum Accuracy.

## CONCLUSIONS

These data demonstrate a highly specific, sensitive, reliable, and repeatable assay for use in RUO applications for characterizing ARv7 and SYP expression by CTCs.

## ACKNOWLEDGEMENTS

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