

# Development of a multi-parameter immunofluorescence assay for simultaneous detection of androgen receptor and androgen receptor variant 7 in prostate cancer circulating tumor cells

Yao Sun<sup>1</sup>, Daniel Campton<sup>1</sup>, Arturo Ramirez<sup>1</sup>, Tanisha Mojica<sup>2</sup>, Alisa Clein<sup>3</sup>, Celestia Higano<sup>3</sup>, Daniel Sabath<sup>3</sup>, Stephen Plymate<sup>3</sup>, Eric Kaldjian<sup>1</sup>  
<sup>1</sup>RareCyte, Inc., Seattle, WA; <sup>2</sup>Seattle Cancer Care Alliance, Seattle, WA; <sup>3</sup>University of Washington, Seattle, WA



## Background

Androgen receptor (AR) signaling is the primary driver of prostate cancer. Expression of the AR splice variant AR-V7, is predictive of resistance to anti-androgen therapies enzalutimide and abiraterone. There is great interest to non-invasively identify circulating tumor cells (CTCs) and investigate their expression of AR and AR-V7 and monitor them over time. RareCyte has developed a platform for automated visual identification of CTCs by immunofluorescence (IF) that allows up to 6-parameter assessment. We have developed a set of prototype assays using tyramide amplification to detect AR and AR-V7 individually and in combination with prostate membrane-specific antigen (PSMA). Using a novel method, we also developed an assay to identify AR and AR-V7 simultaneously.

## Methods

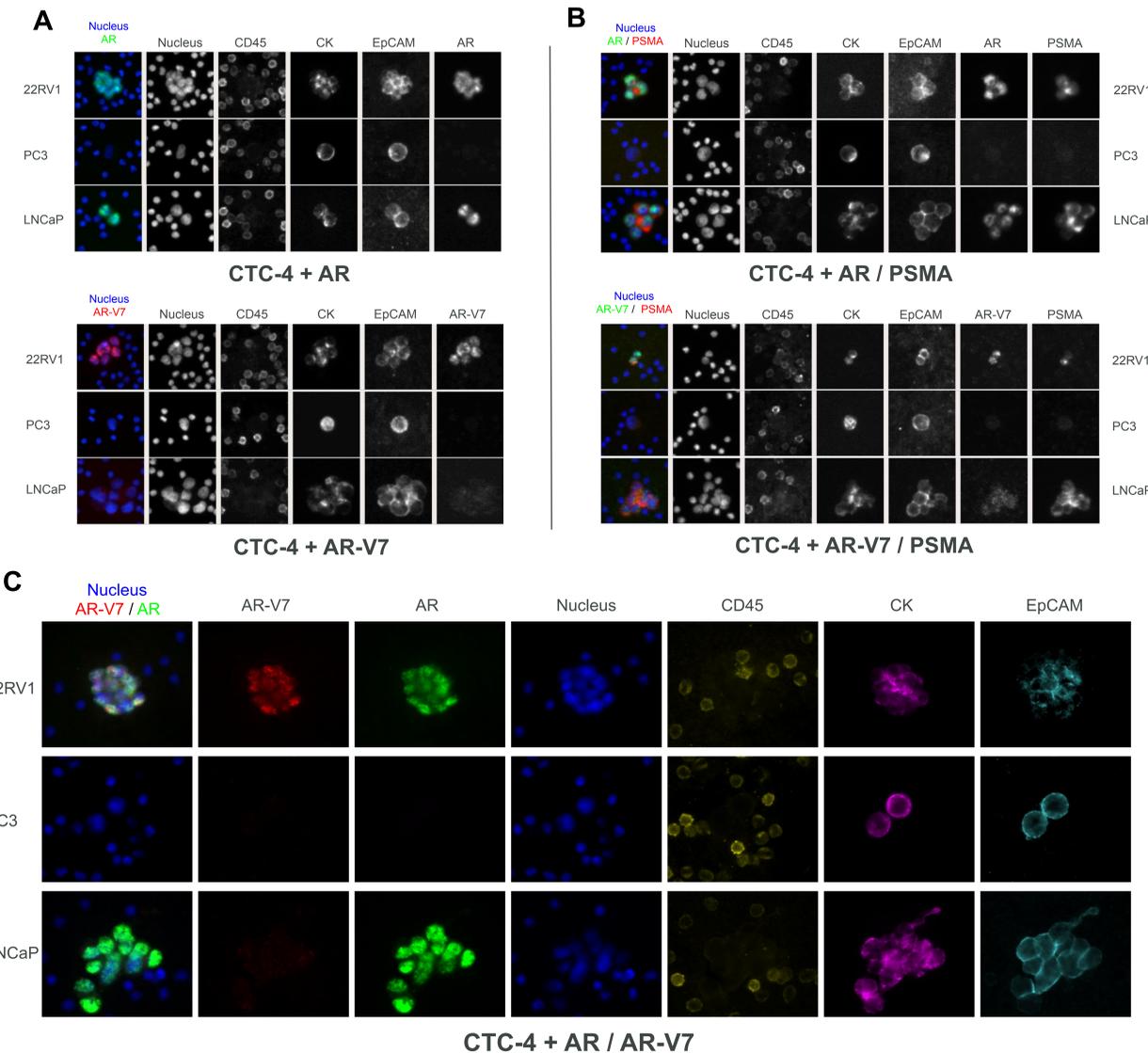
Normal human whole blood samples were spiked with prostate cancer lines serving as model CTCs (mCTCs). Prostate cancer blood from a patient with advanced and known high CTC count were collected under an IRB-approved protocol. Blood was processed onto microscope slides and stained on an automated stainer with CTC detection assays incorporating 4 core parameters (nuclear dye, CD45, pan-cytokeratin, EpCAM) plus the following markers: (1) AR; (2) AR-V7; (3) AR and PSMA; (4) AR-V7 and PSMA; (5) AR and AR-V7. Each assay was applied to the cancer cell lines PC3, LNCaP, and 22RV1 and to the patient sample. An antibody denaturing process was used to sequentially amplify two rabbit monoclonals for the assay containing both AR and AR-V7. No-primary (diluent only) staining controls were run to confirm success of the denaturing step.

Prostate CTC assays					
CTC-4 core epithelial cell identification panel				Marker 1	Marker 2
Nucleus	CD45	Cytokeratin (CK)	EpCAM	AR	--
				AR-V7	--
				AR	PSMA
				AR-V7	PSMA
				AR	AR-V7

**Table 1. Prostate CTC assays built on the RareCyte CTC-4 panel framework.** The CTC-4 panel identifies epithelial CTCs using four fluorescence channels. Two additional fluorescence channels are used for assessment of investigational biomarkers on the identified CTCs. Prostate cancer assays used in this study are shown.

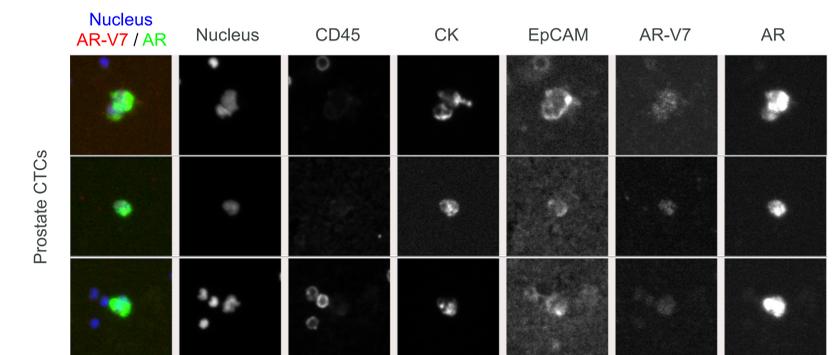
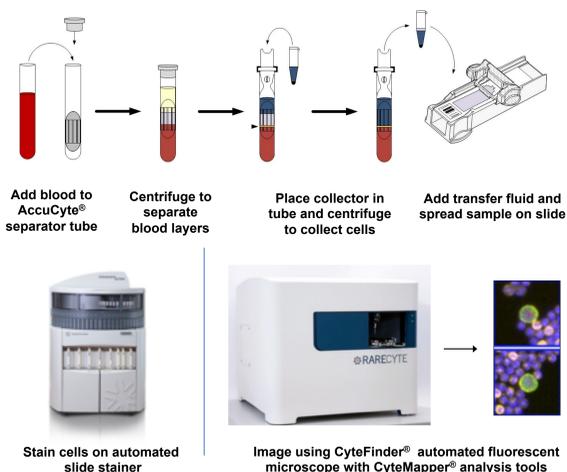
Assay	CTC Count	AR+ CTCs	AR-V7+ CTCs
CTC-4 + AR	43	37 (86%)	--
CTC-4 + AR-V7	47	--	12 (26%)
CTC-4 + AR / PSMA	33	20 (61%)	--
CTC-4 + AR-V7 / PSMA	18	--	7 (39%)
CTC-4 + AR / AR-V7	38	25 (66%)*	10 (26%)*
Mean percent biomarker-positive CTCs:		70%	30%

\* All AR-V7+ CTCs were AR+.



**Figure 1. Multi-parameter prostate CTC assays using CTC-4 panel framework.** Individual channel and composite images of the indicated prostate cancer cell lines are shown. (A) 5-parameter assays for CTC investigation of AR (upper) and AR-V7 (lower). (B) 6-parameter assays for CTC investigation of AR / PSMA (upper) and AR-V7 / PSMA (lower). (C) 6-parameter assay for CTC investigation of AR / AR-V7. Note that 22RV1 expresses both AR and AR-V7. LNCaP expresses AR but not AR-V7. PC3 expresses neither AR nor AR-V7.

## Sample preparation and analysis workflow



**Figure 2. Investigation of advanced prostate cancer CTCs using multi-parameter assays.** A blood sample from a patient with high CTC count was used to prepare slides that were used to compare results from the five multi-parameter assays. Expression of AR or AR-V7 was determined by visual interrogation of identified CTCs. The mean percentage of AR+ CTCs and AR-V7+ CTCs was consistent across the three assays that assessed each biomarker (table above). Lower panel shows individual channel images of CTCs investigated with the 6-parameter CTC-4 + AR / AR-V7 assay. AR-V7 fluorescence intensity is lower than AR expression. All CTCs that were AR-V7+ were also AR+.

## Results

Staining of spike-in CTC models confirmed the reported AR and AR-V7 phenotype of the cell lines, supporting the specificity of the assays. When applied to an equivalent volume of the patient sample, the assays identified a mean (SD) of 36 (11) CTCs. In the assays staining AR, the mean percent of AR+ CTCs was 70% (range 61 - 86) and for the assays staining AR-V7, the percent of AR-V7+ CTCs was 30% (range 26 - 39). In the assay staining both AR and AR-V7, 10 of 38 CTCs were ARV7+ (26%); all of these were AR+; 15 CTCs were only AR+ (39%).

## Conclusions

We have developed multi-parameter IF assays for detection of AR and AR-V7 in prostate CTCs, including the first assay we are aware of to simultaneously visualize AR and AR-V7. It has been validated in prostate cancer CTC models. The percentages of AR and AR-V7-positive CTCs identified in a patient sample with the assay are consistent with the percentages observed when AR and AR-V7 are detected in independent assays, supporting the accuracy of the simultaneous detection assay. All AR-V7-positive CTCs were also AR-positive, supporting specificity of AR-V7 staining.