

# Fluorescence labeling of cytokeratin-negative circulating tumor cells in peripheral blood: A new paradigm for the study of cancer progression

Stephen D. Mikolajczyk, Lisa S. Millar, Maryam Zomorodi, Farideh Z. Bischoff, Jayne Scoggin, Tam Pham, Karina Wong, Tony J. Pircher  
Biocept Inc., San Diego, CA 92121

## ABSTRACT

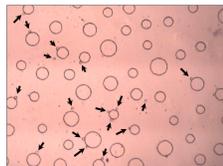
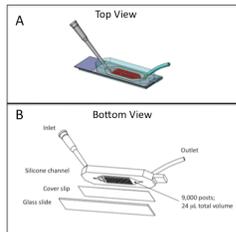
**Introduction** The measurement of circulating tumor cells (CTCs) in blood generally requires either anti-EpCAM for capture, anti-cytokeratin (CK) for detection, or both. However, EpCAM and CK are absent in some tumor cells, and both may be downregulated during neoplastic progression or during epithelial-to-mesenchymal transition (EMT). Here we show capture on a micro-fluidic system using single or multiple antibodies, and CTC detection with CEE-Enhanced™ (CE), a novel *in situ* staining method that fluorescently labels the capture antibodies bound to the CTCs.

**Methods** Buffy coat cells isolated from 8 mL of blood were pre-incubated with either anti-EpCAM alone or with a mixture of antibodies to epithelial and mesenchymal cell surface antigens. CTCs were captured on a micro-fluidic channel. Identification of CTCs was determined with anti-CK and with CE to detect cells retained on the channel with the capture antibodies bound to the cell surface antigens. All cells scored as positive for CK or CE were, by definition, CD45-negative and DAPI-positive.

**Results** Using anti-EpCAM alone for capture, significantly more CTCs were detected by CE staining than with anti-CK in breast and prostate cancers. This indicated that CK-negative CTCs were captured but not detected and that some EpCAM-positive CTCs were CK-negative. Results using capture antibody mixtures varied from sample to sample but gave up to 2-fold higher CK-positive cells than anti-EpCAM only, and as much as 4-fold higher CE-positive cells. Control blood from healthy donors was CK and CE-negative. All CK-positive cells co-stained with CE, as determined with different fluorescent labels. In a clinical study of stage IV breast cancer, CTCs were isolated with an antibody mixture and sequentially stained with CK and CE. Fifteen of 24 samples (63%) contained CK-positive cells (range 1-60 CTCs) while 24 of 24 samples (100%) contained additional CE-positive cells (range 1-41; median=11; Wilcoxon test,  $p=0.02$ ). The modest correlation coefficient ( $r = 0.57$ ,  $p=0.004$ ) for the number of CE-positive and CK-positive cells in each sample suggests that one or more different phenotypes of CTCs were being detected. Amplified HER2 was detected by FISH in isolated CK-positive CTCs, and also in CK-negative/CE-positive CTCs from HER2-positive patients, indicating these were tumor cells.

**Conclusions** The CEE-Enhanced™ staining technology enables the detection of CK-negative CTCs. This novel detection method, based on the *in situ* labeling of antibodies used for capture, greatly expands single and multi-antibody approaches to the study of rare circulating cells. Specifically, this allows the exploration and study of circulating cells not expressing CK such as highly de-differentiated tumor cells, stem cells or those tumor cells undergoing EMT. The clinical significance of these CK-negative CTCs is under investigation.

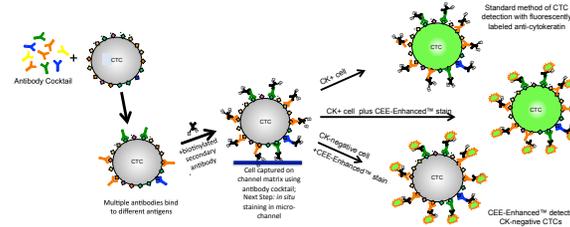
## CEE™ MICRO-CHANNEL



Visible light microscopic view of channel through the bottom cover slip showing the random array of posts and SKBR3 cells which have been captured (arrows).

Diagram of the CEE™ micro-channel. A) Top view of the channel showing the inlet where sample is loaded and the outlet that is attached to a syringe pump to draw sample through the channel. B) Bottom view shows the area where 9,000 posts are located in the silicone block and the channel sealed with the bottom cover slip. The total volume of the micro-channel is 24  $\mu$ L. The microscope slide is added for stability during handling but is removed to visualize cells. The micro-channel is inverted on a microscope and the captured cells viewed through the coverslip.

## CAPTURE AND STAINING OF CTCs



Buffy coat cells are prepared from blood using a density gradient. Capture antibodies are then incubated with the buffy coat cells, followed by biotinylated secondary antibody prior to application onto the streptavidin-coated CEE™ micro-channel. Staining takes place in the micro-channel, using either fluorescently labeled anti-cytokeratin, CEE-Enhanced™, or both in combination. CEE-Enhanced™ is used for universal *in situ* detection of CTCs containing bound capture antibodies, regardless of whether the cells express cytokeratin.

## RESULTS

Tumor Type	Anti-EpCAM only	Antibody Mix
breast	0	1
prostate	37	33
breast	8	25
lung	0	0
breast	8	12
breast	94	115
breast	0	1
prostate	57	97
prostate	0	0
Colorectal	0	1
breast	6	16
lung	1	5
breast	13	22
breast	54	72
breast	0	0
breast	0	1

Comparing CTC capture using EpCAM-only and an antibody mixture on duplicate blood samples. All cells were CK+/CD45-/DAPI-. Mean: 18.5 vs 26.5; Wilcoxon paired median: 6 vs 12 CTCs,  $p=0.02$ . Antibody mixture captures additional CK+ CTCs that don't express EpCAM.

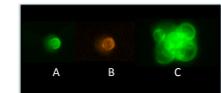
Cancer Type	Cytokeratin Stain		Additional CTCs detected with CEE-Enhanced™ stain	
	Anti EpCAM	Antibody Mix	Anti-EpCAM	Antibody Mix
Small Cell Lung Cancer	24	47	+151	+148
Prostate	127	200 (162) <sup>a</sup>	+49	+61
Prostate	2	5 (7) <sup>a</sup>	+11	+27
Prostate	12	10	+2	+25
Colorectal	ND	1	ND	+4

Additional CTCs are detected using CEE-Enhanced™ that are not detected with anti-CK stain alone. Greater numbers of CEE-Enhanced™ CTCs are detected when using an antibody mixture compared to EpCAM alone.  
<sup>a</sup> Antibody mixture without EpCAM. Note higher CTC numbers than for anti-EpCAM alone, suggesting capture of EpCAM-negative CTCs.

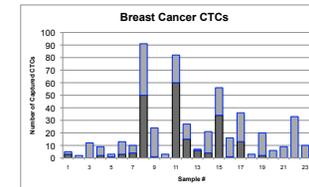


The use of CEE-Enhanced™ (CE) to improve detection of CK-positive cells on the micro-channel.

- A clinical breast cancer CTC stained for CK and nuclear-stained with DAPI. This cell is weakly CK-positive (and CD45-negative).
- The same cell after subsequent stain using CE with the same AlexaFluor-488 fluorophore shows enhanced stain intensity.



Staining clinical lung cancer CTCs with anti-CK and CEE-Enhanced™. A) CTC on the micro-channel stained with anti-CK (green). B) The same CTC co-stained with CEE-Enhanced™ (AlexaFluor-546, orange). C) Cluster of CTCs stained with anti-CK.



Clinical breast cancer samples sequentially stained with anti-CK and CEE-Enhanced™ (CE). An antibody mixture was used to capture CTCs. CK-positive cells were detected in a sequential series of stage IV breast cancer samples. The locations of these cells were recorded and then the channel was re-stained with CE. The stacked upper bars represent the new CTCs detected with CE. All cells detected as label-positive were CD45-negative and DAPI-positive.

## CONCLUSIONS

- Antibody mixtures improve the recovery of cancer cells, including CTCs not captured with anti-EpCAM alone
- CEE-Enhanced™ can be used to stain CTCs that do not stain with anti-cytokeratin
- CEE™ technology allows multiple screening and staining strategies for the analysis of CTCs