Detection of EpCAM-negative and Cytokeratin-negative Circulating Tumor Cells in Peripheral Blood

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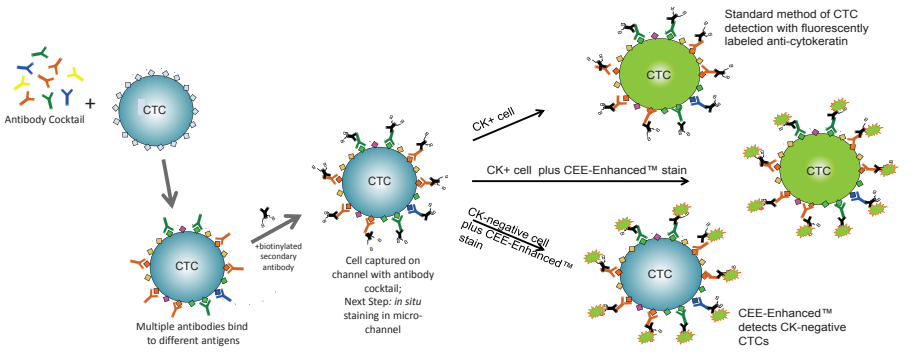
ABSTRACT

Enrichment of rare circulating tumor cells (CTCs) in blood is typically achieved using anti-epithelial cell adhesion molecule (EpCAM), with anti-cytokeratin (CK) used to identify CTCs. However, EpCAM and CK are absent in some tumor cells, and both may be down-regulated during epithelial-to-mesenchymal transition (EMT). A micro-fluidic system, CEE™, was developed using single or multiple antibodies for enrichment of CTCs followed by detection using CEE-Enhanced™ (CE), a novel in situ staining method that fluorescently labels the capture antibodies bound to CTCs.

Higher recovery of CK-positive CTCs was demonstrated on multiple cancer types using antibody mixtures compared to anti-EpCAM alone (median 12 vs 6 CTCs per 10 mL blood, respectively, p=0.02). In addition, CTCs were isolated from metastatic breast cancer samples using an antibody mixture, and sequentially stained with anti-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). All cells scored as positive for CK or CE were, by definition, CD45-negative and DAPI-positive. Control blood from healthy donors was CK and CE-negative. All CK-positive cells co-stained with CE, as determined with different fluorescent labels. 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.

In conclusion, antibody mixtures against a range of cell surface antigens enables capture of additional CTCs including EpCAM-negative cells, and the CEE-Enhanced™ staining technology enables the in situ detection of CK-negative CTCs on the micro-channel. This technology will promote the exploration and study of additional types of circulating tumor cells. The clinical significance of these results is under investigation.

CAPTURE AND STAINING OF CTCs



Buffy coat cells are prepared from blood using a density gradient. 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

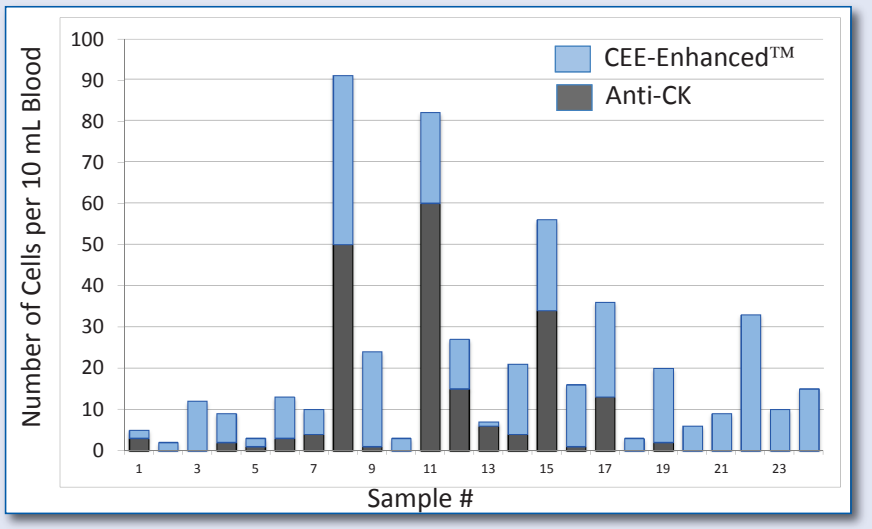
Tumor Type	Anti-EpCAM only	Antibody Mix
breast	0	1
prostate	37	33
breast	8	25
lung	0	0
breast	8	12
breast	94	115
breast	0	1
prostate	57	97
prostate	0	0
Colorectal	0	1
breast	6	16
lung	1	2
breast	13	22
breast	54	72
breast	0	0
breast	0	1

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

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

Additional CTCs are detected using CEE-Enhanced™ that are not detected with anti-CK stain. Greater numbers of CEE-Enhanced™ CTCs are detected when using an antibody mixture compared to anti-EpCAM alone.

[a] Antibody mixture without EpCAM. Note higher CTC numbers than for anti-EpCAM alone, suggesting capture of EpCAM-negative CTCs.



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 location of these cells was recorded and then the channel was re-stained with CE. The stacked upper bars represent the new CTCs detected with CE. All cells designated as positive were CD45-negative and DAPI-positive.

CEE™ MICRO-CHANNEL

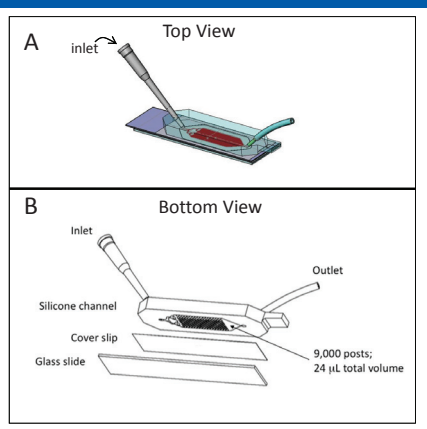
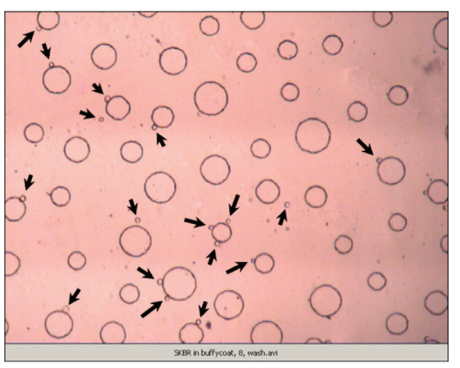
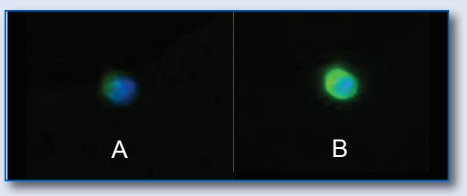


Diagram of the CEE™ micro-channel. A) Top view of the channel showing the inlet where sample is loaded and the outlet that is attached to a syringe pump to draw sample through the channel. 1B. Bottom view shows the area where 9,000 posts are located in the silicone block and the channel sealed with the bottom cover slip. The total volume of the micro-channel is 24µ mL. 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.



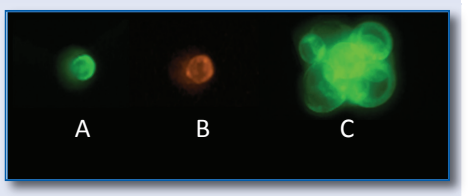
Visible light microscopic view of channel through coverslip showing the random array of posts and the SKBR3 cells which have been captured (arrows)



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

A. A clinical breast cancer CTC stained for CK and nuclear-stained with DAPI. This cell is weakly CK-positive (and CD45-negative)

B. The same cell after subsequent stain with CE labeled 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

CONCLUSIONS

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