

# Detection of Rare Cells and Circulating Tumor Cells using Flow Cytometric Methods

## Authors

Lauren Jachimowicz,  
Ming Lei, Peifang Ye,  
and Garret Guenther  
Agilent Technologies, Inc.

## Abstract

Circulating tumor cells (CTC) are rare cells shed from a solid tumor or metastases that circulate through the bloodstream. Detection of CTC poses a challenge because they are present at extremely low concentrations, as few as a couple CTC in several milliliters of blood, which contain up to 3 to 6 million cells per mL. To determine the sensitivity and accuracy of rare cell detection on the Agilent NovoCyte Quanteon flow cytometer, a tumor cell spiking assay was used in which a small number of cells from a tumor cell line was added to whole blood and detected by a cell surface stain.

## Introduction

As a tumor grows, metastases can form in different organs, spreading the cancer. A subset of CTC are thought to be metastatic precursors, cells that detach from the primary tumor, circulate in the bloodstream, and initiate a new metastasis. Accumulating evidence shows the importance of CTC detection as a non-invasive method for early detection for cancer prognosis, also referred to as a “liquid biopsy”. The presence of CTC in peripheral blood has been associated with more aggressive disease and poorer outcomes, and therefore, enumeration of CTC has been shown to demonstrate the level of disease burden. CTC detection can also be used as a biomarker for metastatic cancer, therapeutic response monitoring, and drug discovery. However, detection of CTC is challenging because they are present at extremely low concentrations amongst other cell types. There is still much to be learned about the biological function and molecular characteristics of CTC.

Flow cytometry, capable of analyzing thousands of cells per second, can be useful for CTC studies and rare cell detection. Multiparameter flow cytometry of CTC also allows for simultaneous analysis of additional biomarkers. A tumor cell spiking assay is frequently used to evaluate the sensitivity, specificity, accuracy, and repeatability of the methods or systems for enumeration of rare cells. In these experiments, the NovoCyte Quanteon was employed to measure and quantify spiked human tumor cells of colon carcinoma (SW620) in PBMCs and whole blood, assessing the capability to detect rare cells amidst other types.

## Experimental

### Materials

- Agilent NovoCyte Quanteon flow cytometer
- SW620 cell line
- Whole human blood and isolated PBMCs
- Ficoll Paque Plus (GE Healthcare, GE17-440-02)
- Antibodies: Anti-CD45 FITC (Biolegend #304005), Anti-EpCAM APC (Biolegend #324207)
- EasySep human EpCAM positive selection (STEMCELL Technologies #18356)

### Methods

#### Assessment of EpCAM expression on SW620 and blood cells

SW620 cells were spiked into blood at 1,000 cells in 2 mL of blood. PBMCs were then isolated from blood of a healthy donor or SW620-spiked blood. Cells were stained with anti-CD45 FITC and anti-EpCAM APC antibodies, or isotype control and analyzed on the NovoCyte Quanteon.

#### Rare cell detection spiking assay

Anti-EpCAM APC antibody stained SW620 cells were serially diluted from 0 to 512 cells in 20  $\mu$ L and spiked into  $2 \times 10^6$  PBMCs in 180  $\mu$ L. 100  $\mu$ L of the spiked samples were analyzed on the NovoCyte Quanteon or a competitor flow cytometer.

#### SW620 tumor cell recovery analysis

SW620 tumor cells were serially diluted (0 to 2,000 cells) and spiked into 2 mL of whole blood. PBMCs and spiked tumor cells were isolated using Ficoll gradient centrifugation, followed by EpCAM-positive enrichment using EasySep human EpCAM positive selection kit. The enriched cells were then stained with anti-CD45 FITC and anti-EpCAM APC antibodies and resuspended in 200  $\mu$ L buffer. 100  $\mu$ L of sample was analyzed on the NovoCyte Quanteon.

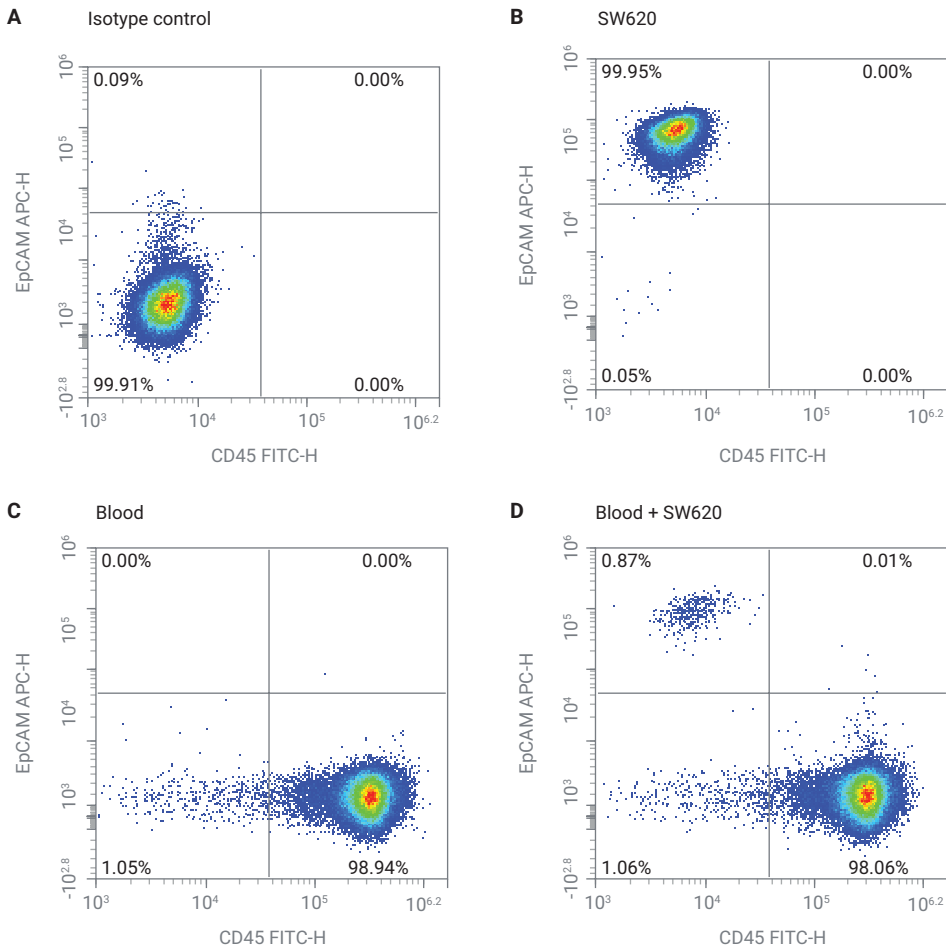
## Results and discussion

### EpCAM is highly expressed on SW620 but not on blood cells

CTC are commonly detected by differentiated CTC biomarkers that are not expressed on normal hematologic cells. Many cancers have epithelial cell phenotypes and therefore express high levels of epithelial cell surface antigens and cytokeratin, another common marker of epithelial cell differentiation. Therefore, epithelial cell surface markers, such as epithelial cell adhesion molecule (EpCAM), can be used to identify cancer cells in mixed populations of cells. In this study, two cell surface markers were used to distinguish cancer cells from PBMC or blood cells: EpCAM and CD45, respectively. To ensure that these markers can easily distinguish the two populations, human tumor cells of colon carcinoma (SW620) were spiked into whole blood and stained with anti-CD45 FITC and anti-EpCAM PE antibodies (Figure 1). SW620 cells exclusively express EpCAM and have no detectable expression of CD45. Therefore, SW620 and blood cells can be easily identified by flow cytometry.

### Assessment of rare cell detection sensitivity on the NovoCyte Quanteon

To determine the capacity of flow cytometry to detect rare cells at a very low concentration in a mixed population of cells, a cell spiking assay was performed and evaluated on the NovoCyte Quanteon. SW620 cells were stained with anti-EpCAM APC antibody and added to 2 million PBMCs ranging from 0 to 512 spiked cells. Half of the SW620 spiked sample was analyzed on the NovoCyte Quanteon and the other half on a competitor flow cytometer. The number of detected SW620 cells was then compared to the expected cell number to determine the sensitivity and



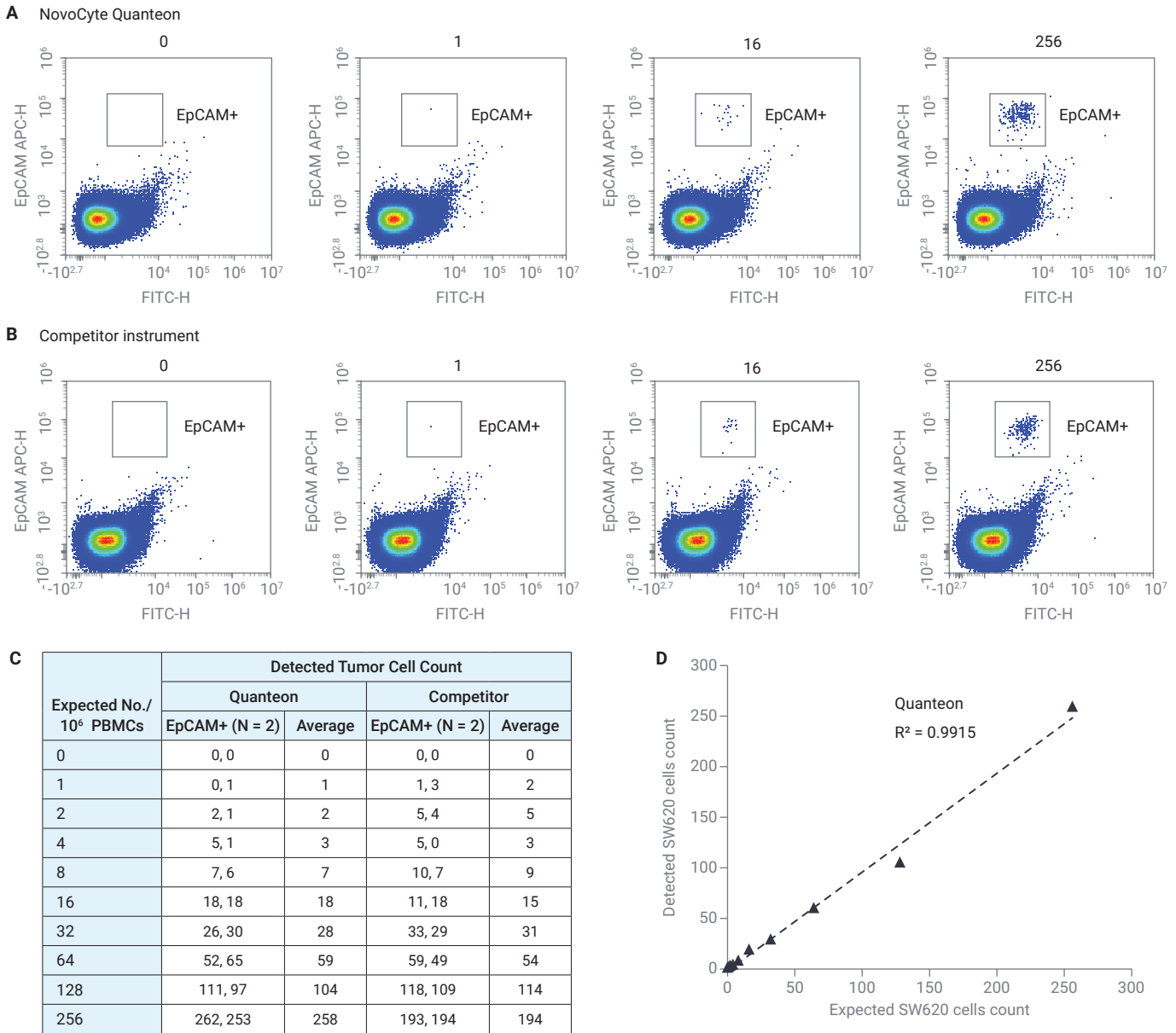
**Figure 1.** EpCAM is highly expressed on SW620 but not normal blood cells. SW620 cells (B), isolated PBMC from blood (C) or isolated PBMC from SW620-spiked blood (D) were labeled by anti-CD45 FITC and anti-EpCAM APC antibodies, or isotype control (A) and analyzed on the Agilent NovoCyte Quanteon.

accuracy of the instruments (Figure 2). The NovoCyte Quanteon had a sensitivity of 0.0001%, which translates to the ability to detect one tumor cell in one million PBMCs. The number of predicted and detected tumor cells held an excellent linear relationship, with an  $R^2$  value of 0.991. These data demonstrate that the NovoCyte Quanteon can detect one single cell in a mixed population of a million cells, which is critical for detection of rare cell types such as CTC.

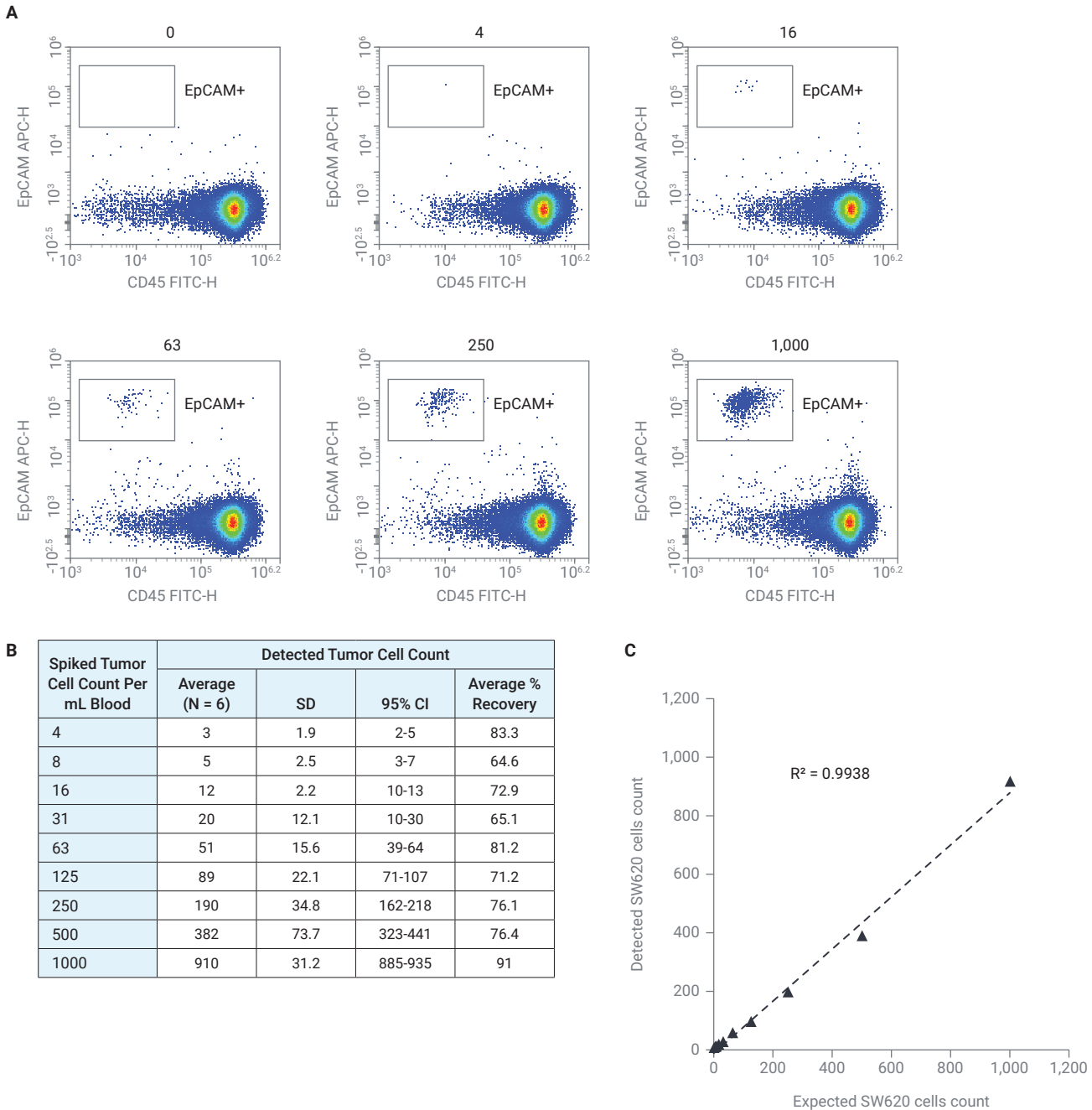
### SW620 tumor cell recovery analysis

CTC are present at extremely low concentrations within a larger population of cells and therefore are difficult to detect. By enriching the cells for the target cells, either by eliminating unwanted cells or positively selecting the cells of interest, you increase the efficiency of cell detection and decrease the total number of cells that need to be analyzed to detect the cells of interest. Techniques for the enrichment of CTC are based on biological and/or

physical properties that distinguish CTC from normal blood cells. Affinity-based enrichment is by far the most commonly employed strategy to separate CTC from blood cells. Affinity techniques take advantage of distinctive antigens expressed either by CTC and not blood cells (EpCAM) or by blood cells but not CTCs (CD45). An affinity-based tumor cell enrichment and analysis was tested on the NovoCyte Quanteon. SW620 cells were serially diluted from 2,000 to 0 cells into 2 mL of whole blood. PBMCs and spiked tumor cells were then isolated using Ficoll gradient centrifugation, and CTC were then enriched by their expression of EpCAM on the cell surface using the EasySep Human EpCAM positive selection kit. The number of CTC was determined in each sample by flow cytometry to count the number of recovered cells (Figure 3). The numbers of detected SW620 cells were then compared to the expected number, which showed a close linear relationship with an  $R^2$  value of 0.994. The % recovery was also calculated and ranged from 64 to 91% depending on the specific sample. Although the recovery rate only reaches over 90% with a greater number of spiked cells in the initial sample, the data demonstrates that the majority of spiked cells were recovered through the enrichment process, and as little as four tumor cells in 2 mL of whole blood can be detected. Since whole blood contains anywhere from 4 to 6 million cells per mL of blood, this equates to a detection limit of one CTC in about 2.5 million cells. This is a 2.5-fold increase in rare cell detection than what was determined in the previous tumor spiking experiment. Overall, tumor cell enrichment is an effective way to increase the capacity to detect CTC by flow cytometry.



**Figure 2.** Assessment of rare cell detection sensitivity with a spiking assay. Anti-EpCAM APC antibody stained SW620 cells were serially diluted from 0 to 512 cells in isolated PBMCs. Spiked samples were analyzed on the Agilent NovoCyte Quanteon or a competitor flow cytometer. (A,B) Detection of spiked tumor cells by a NovoCyte Quanteon flow cytometer and a competitor flow cytometer; (C) table with detailed data of numbers of spiked SW620 cells detected in samples; (D) linearity of tumor cell detection. Plot with the numbers of spiked and detected SW620 cells were plotted on the X- and Y-axis, respectively. The correlation coefficient was given on the graph (N = 2).



**Figure 3.** SW620 tumor cell recovery analysis. SW620 cell were serially diluted and spiked into whole blood. Cells were then isolated using Ficoll gradient centrifugation, followed by EpCAM-positive enrichment. Cells were then stained with anti-CD45 FITC and anti-EpCAM APC antibodies and analyzed on the Agilent NovoCyte Quanteon. (A) Gating of serially diluted EpCAM+ tumor cells; (B) table with detailed data, numbers of recovered SW620 cells in enriched samples; (C) linearity analysis. Numbers of expected and detected SW620 cells were plotted on the X- and Y-axis, respectively. The correlation coefficient was given on the graph (N = 6).

## Conclusion

Flow cytometry is a valuable tool for rare cell detection, immunotherapy, and cancer research. Detection of CTC is a noninvasive technique for real-time monitoring and CTC detection. The NovoCyte Quanteon can detect as few as one CTC in a million blood cells and can also be coupled with EpCAM affinity-based enriched methods for increased detection. Through flow cytometry, additional surface markers can be added to further characterize CTC populations in addition to counting of CTCs.

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