Circulating Tumor Cells (CTCs) Enumeration Overcomes Limitations with other Blood-Based Cancer Detection Methods

The Genesis System Enables Sensitive CTC Enumeration

Liquid biopsies are rapidly gaining traction and becoming a standard of care diagnostic for cancer. It is an attractive alternative to invasive, painful, costly tissue biopsies and can facilitate real-time monitoring while providing insight into the disease state, even for tissues that are difficult to access, such as brain cancers. These assays target circulating tumor cells (CTCs) or circulating tumor DNA (ctDNA).

Limitations of ctDNA as a Marker for Disease Progression

While CTCs have been known to scientists for more than a century, ctDNA has received more attention in recent years due to advances and excitement surrounding the potential for next-generation sequencing (NGS) technologies. However, ctDNA concentrations in plasma are low and NGS analysis is complicated by the extreme scarcity of some mutations and by mutations that occur with increasing age that aren't necessarily oncogenic. Additionally, plasma can contain substantial contamination from cellular debris and genomic DNA that may not represent the current tumor state. Analysis of ctDNA is also subject to substantial variability as demonstrated in a study comparing four commercially available assays[1]. Results showed variation in both sensitivity and positive predictive value, mostly due to technical factors. As more sophisticated single-cell technologies have become available, there is a renewed interest in CTC enumeration and analysis as an alternative method of monitoring and detection.

CTC Enumeration: A Simple and Effective Predictive Biomarker

It is becoming clear the implementation of molecular and genomic characterization of CTCs can contribute to improving diagnosis and personalizing treatment selection[2]. CTCs are tumor cells shed by the primary tumor through the bloodstream and lymphatic systems (Figure 1). They are likely a main source of metastases[3-4], but they cannot be detected by CT or PET scans. The enumeration of CTCs has emerged as a simple and effective predictive biomarker with many applications in cancer prognosis and treatment[5-7].

A unique advantage of CTCs compared to other blood-based biomarkers is they represent a cancer-derived cell population, providing researchers a powerful tool to study tumor heterogeneity and progression of the disease at various stages.

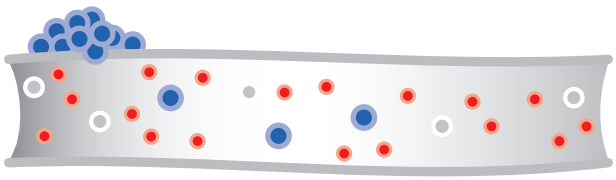


Figure 1: Circulating tumor cells are shed from the primary tumor and carried through the body in the blood.

CTC enumeration can be used to detect early development of (micro)metastases, assess therapeutic responses of advanced disease earlier than traditional imaging methods, and to select first-line treatment in various types of cancer (breast, prostate, colorectal, lung, bladder) from primary to metastatic states. For example, the decrease in CTC count after one cycle of chemotherapy is prognostic for tumor response, progression-free survival, and overall survival in stage III–IV cancer[8]. It is established that detecting minimally five or more CTCs in the peripheral blood in patients with metastatic breast cancer (MBC) is associated with a strong prediction of disease progression[9]. Mounting clinical evidence has prompted a group of international experts to recommend the use of CTC enumeration for staging of metastatic breast cancer and for disease stratification in prospective clinical trials[10]. The American Society of Clinical Oncology biomarker guidelines go on to note that CTCs may be used in the metastatic setting to monitor these patients during and after treatment.

Addressing the Limitations of CTC Detection

Techniques for extracting CTCs need to be highly sensitive. To be adopted clinically, analysis of CTCs must overcome a number of challenges, including their low abundance in the blood, contamination with leukocytes, loss of cell viability during purification, and tedious, low-throughput processes. Traditional techniques rely upon microfluidics, density gradient separation, and emulsion-based technologies which can lower the capture efficiencies even further. These techniques rely on either tumor- or epithelial-specific immune markers for the detection of the CTCs. Unfortunately, no single perfect marker can identify all CTCs present in the same tumor due to the inherent heterogeneity and genetic instability of cancer. The flexibility to use multiple markers or customize the markers for detection of specific CTC types enhances the accuracy and scope of capabilities for CTC enumeration.

Figure 2. The Genesis System

The Genesis System: A Single-Cell Analysis Platform Designed for Clinical Research

Single-cell analysis is critical for unraveling cancer heterogeneity; however, researchers have very limited access to these cells for conducting downstream characterization. With many technologies there is sample loss during the enrichment process, and the remaining contaminating white blood cells (WBCs) interfere with the results, making interpretation difficult. Very few systems have the resolution and precision required to visualize and analyze single cells.

The lack of reproducibility and sensitivity with existing technologies has limited the clinical adoption of CTCs in cancer diagnostics. The Genesis System (Figure 2) was developed by Celsee to address the shortcomings of single-cell analysis including CTC isolation and enumeration. This platform offers a robust solution that can accelerate clinical research and the acceptance of CTCs as a routine biomarker.

Automated Cell Enumeration Technology

One of the technologies the Genesis System supports, the Celselect™ Slide, utilizes patented microfluidics paired with 56,400 microchambers (Figure 3) to capture and isolate individual CTCs or other rare cells based on their size (>8 μm). Isolated WBCs are quickly identified by using the WBC-specific marker CD45 and excluded from analysis. Many of the traditional challenges for CTC enumeration are overcome by the Genesis System (Table 1).

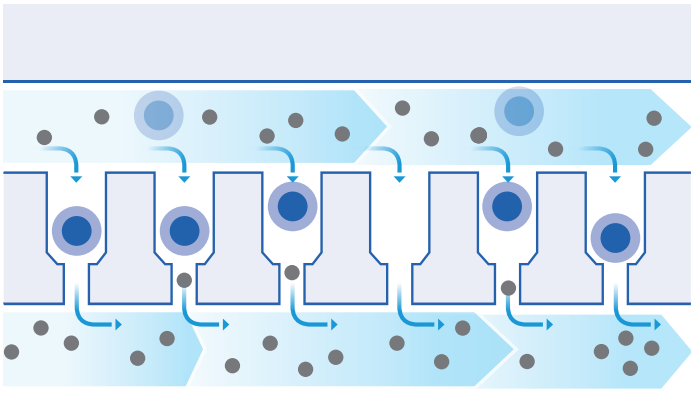


Figure 3: Size-based enrichment of CTCs. A schematic of the microfluidic chambers used to capture CTCs based on their size.

Current CTC Detection Limitations	The Genesis System Advantage
Legacy systems have low capture efficiency	High capture efficiency, reliably detects 1 in 1,000,000 cells per mL of blood[11]
Emulsion technologies suffer WBC contamination	WBCs are easily excluded from analysis using Celselect Technology
Harsh processing methods negatively affect cell viability	Viable cell isolation
Greater sample input required (7.5–10 mL)	Smaller volumes of sample (4 mL)
Labor intensive with limited automation	Fully-automated cell capture and staining
Solely dependent upon EpCAM-staining and only detect epithelial CTCs	Customizable staining to detect epithelial and mesenchymal CTCs for improved specificity
Designed only for enumeration of CTCs	Automated workflows for IHC, FISH, rare-cell enrichment and single-cell genomic applications

Table 1: Advantages of the Genesis System over legacy CTC detection technologies

The entire enumeration workflow (Figure 4) is automated, from loading blood sample through the microfluidic slide (Figure 5) to reagent dispensing for on-slide post-purification molecular analyses (immunohistochemistry, DNA FISH, RNA FISH) and cell counting. The Genesis System can be paired with an automated fluorescent imager for imaging and enumeration.

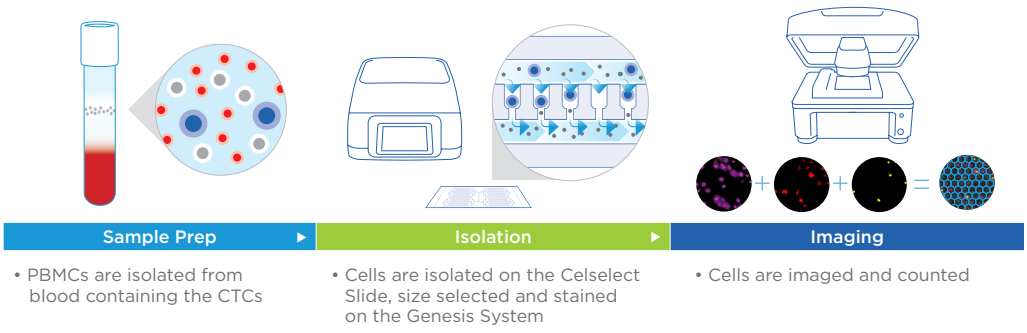


Figure 4. Cell Enumeration Workflow. Blood is directly added to the sample inlet; CTCs are isolated in the Celselect Slide while the smaller RBCs and WBCs pass through to the waste. CTCs are processed and immunostained directly in the slide. Subsequently, CTCs can be visualized and counted on the slides using an automated fluorescent imager. Contamination from larger WBCs can be excluded through image analysis.

The Genesis System enables fast analysis without compromising cell-capture efficiency. It has been validated with several human cancer cell lines: MCF7 (breast), SKBR3 (breast)[12], LnCAP (prostate), PC3 (prostate) and HT29 (colorectal), and capture efficiency was found to be greater than 80%. Celselect Technology captured both epithelial cancer cells, MCF7 and SKBR3, and mesenchymal cells, MDA-MB-231[13] in as little as 4 mL of blood.

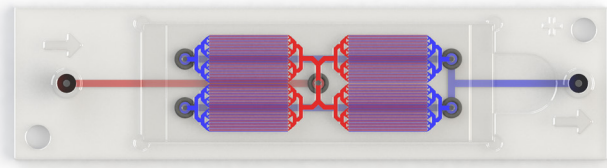
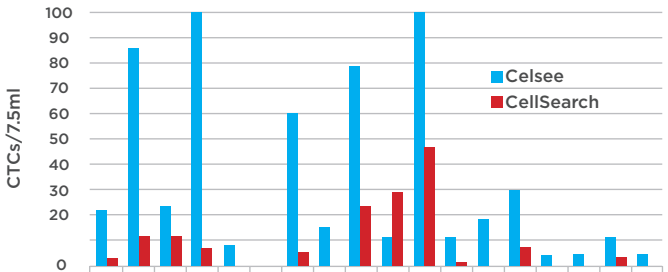


Figure 5. A schematic representation of the Celselect Slide. Blood sample (red, left) flows through the microchambers in the Celselect Slide and exits to the waste port (blue, right)

Validated Rare Cell Analysis and CTC Enumeration

A clinical study conducted at the Sidney Kimmel Cancer Center at Thomas Jefferson University revealed CTC detection in prostate cancer is more sensitive with the Genesis System than with the FDA-approved CellSearch® System (Silicon Biosystems)[13]. Analysis of 18 blood samples from patients with metastatic prostate cancer, showed the Genesis System detected CTCs in 17/18 samples (94%) whereas the CellSearch System detected CTCs in only 11/18 samples (61%). CTC counts were significantly higher using the Genesis system, implying greater sensitivity for CTC detection (Figure 6). The Celselect Technology captured a very low level of leukocytes, and these cells were easily discriminated from CTCs with differential immunostaining using cell type-specific antibodies.



	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18
Celsee	22	86	23	120	8	0	60	15	79	11	375	11	19	30	4	4	11	4
CellSearch	3	12	6	7	0	0	5	0	23	29	47	1	0	7	0	0	3	0

Figure 6: Comparison of CTC counts using the Genesis System with Celselect Technology versus CellSearch. Number of CTCs determined using either the Genesis System or CellSearch were normalized to CTC-count per 7.5 mL of blood[11].

While the CellSearch system is limited to EpCAM detection, the Celselect Technology allowed for the use of additional markers including anti-vimentin for the detection of mesenchymal cells.

High levels of vimentin were observed in some cells, demonstrating that the Genesis System captured both epithelial and mesenchymal CTCs. The cells were further analyzed and found to be prostate-specific antigen (PSA)-positive and nucleated, demonstrating they were CTCs from the prostate tumor. Not only was the Genesis System able to capture and detect more CTCs than the CellSearch System, it was able to detect subpopulations of CTCs missed by other technologies, making it a more reliable tool for monitoring.

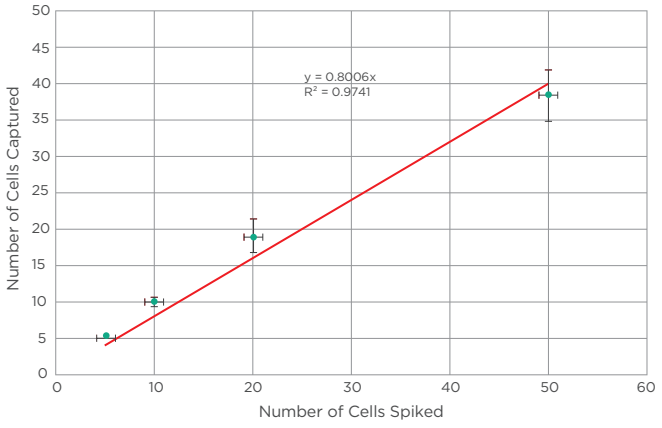
CTC and Expression of Cancer and Immune Markers

In a collaborative study, between Celsee, IncellDx, Cytek Biosciences, and Qognit, single-cell immune and cancer marker profiling of primary tumor cells was studied to investigate possible signatures to predict the presence or absence of CTCs in the blood[11]. This low-cost screening tool could be used to select patients for ongoing CTC monitoring for disease progression and response to therapy. A comprehensive study on 10 Non-Small Cell Lung Cancer (NSCLC) was performed on patient tissue samples with paired blood.

As part of the study, the lower limits of the reproducible detection (LOD) of the Genesis System was evaluated. As few as 5 PD-L1+ CTCs were reproducibly detected in 4 mL of whole blood. (The PD-1/PD-L1 pathway is a target for NSCLC immunotherapy.) This represents a capture rate as low as 1 in 1,000,000 cells (Figure 7), which is much higher than the 50–60% capture rate observed with legacy systems.

The Genesis System detection of CTCs was reproducible and sensitive demonstrating its utility for monitoring PD-L1⁺ CTCs in NSCLC patients undergoing immunotherapy.

A



B

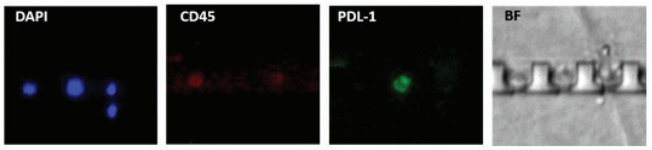
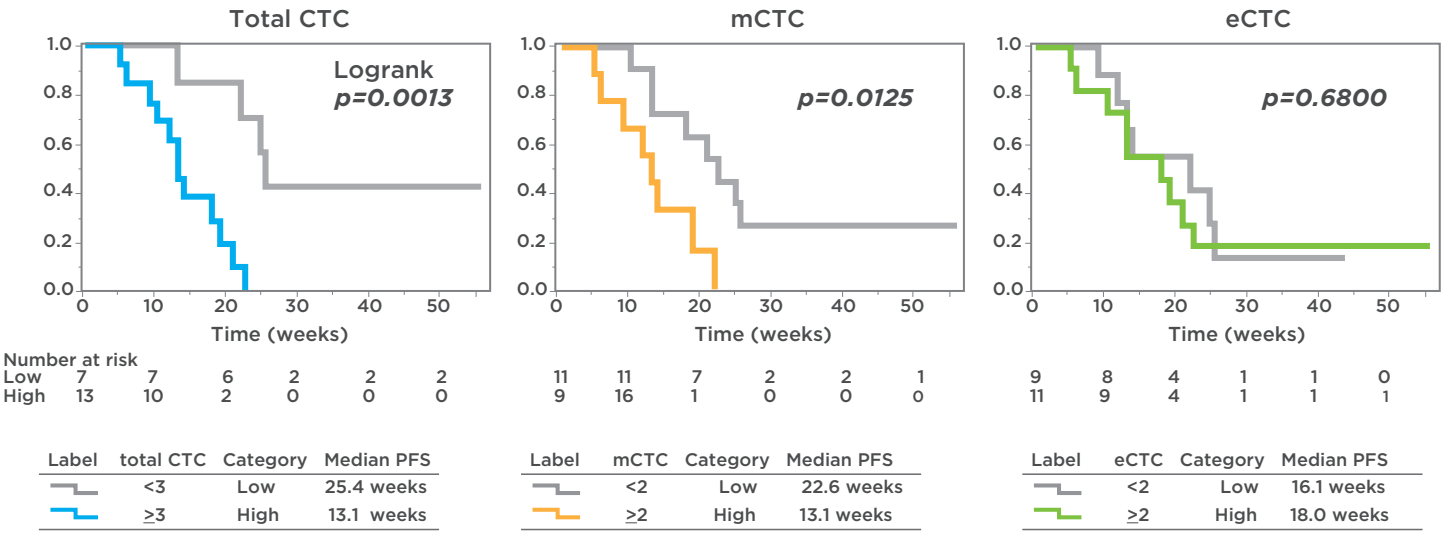


Figure 7. CTC enumeration and analysis. **A.** Spike in analysis for CTC recovery. NCI-441 cells (PD-L1 positive lung cancer cell line) were spiked into normal blood sample. Cell recovery was reproducible down to 1 CTC in a million cells. **B.** Representative image showing CD45 negative and PD-L1 positive cell



Label	total CTC	Category	Median PFS
	<3	Low	25.4 weeks
	≥3	High	13.1 weeks

Label	mCTC	Category	Median PFS
	<2	Low	22.6 weeks
	≥2	High	13.1 weeks

Label	eCTC	Category	Median PFS
	<2	Low	16.1 weeks
	≥2	High	18.0 weeks

Figure 8. Kaplan–Meier curves of PFS relative to CTC-count. The log-rank test was applied for comparisons of the survival distributions of the groups. Total CTCs including eCTCs and mCTCs was the most predictive for PFS[14].

CTC Enumeration as a Possible Predictor of Treatment Efficacy

A study conducted at Juntendo University School of Medicine evaluated the use of CTC enumeration to predict Eribulin treatment efficacy in MBC patients[14]. Previous CTC enumeration systems have relied solely on EpCAM staining of CTCs to detect epithelial CTCs (eCTCs), but not all CTCs are EpCAM positive. A sub-population of CTCs with decreased levels of epithelial markers escape EpCAM-based detection. Mesenchymal CTCs (mCTCs) are not EpCAM positive. To evaluate how CTCs could be used to predict Eribulin efficacy, three populations of CTCs were analyzed: eCTCs, mCTCs and total CTCs. The ability to customize stains on the Genesis System to detect mCTCs made this study possible.

Blood samples were collected from 22 patients before and during treatment. Progression-free survival (PFS) and CTCs counts were monitored by CTC type. The results demonstrate the total CTCs including eCTCs and mCTCs were the most predictive for PFS over eCTCs or mCTCs alone (Figure 8). Since legacy systems rely solely on detection of EpCAM positive CTCs (eCTCs), Eribulin therapy monitoring with these systems would not be possible.

The Genesis Systems has high capture efficiency and the ability to customize which fluorescent stains are used to enable the detection of CTC sub-populations and offers a significant improvement upon legacy systems.

Versatility of the Genesis System: The Celsingle Slide Technology

The Genesis System supports two distinct slide technologies (Figure 9) that enable multiple applications. The Celsingle Slide enables single-cell genomic and proteomic applications and the Celselect Slide enables CTC enumeration and rare-cell enrichment.

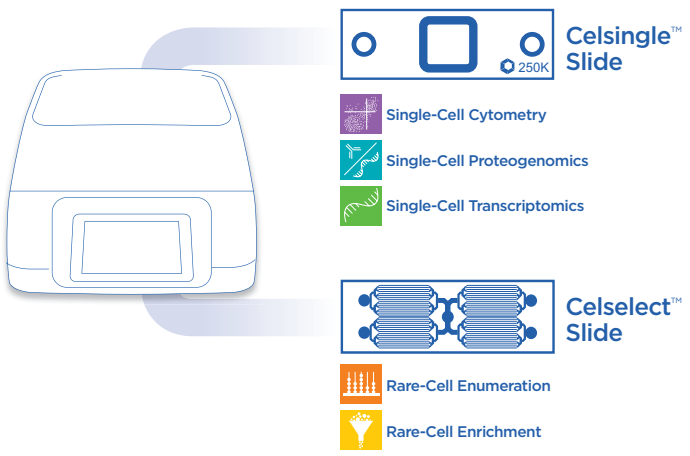


Figure 9. Celsingle Slides and the Celselect Slides

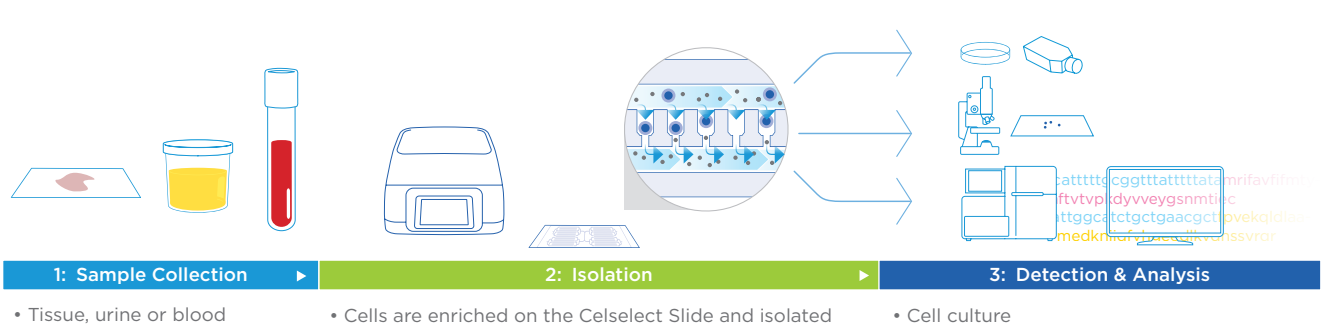


Figure 10. Cell Enrichment Workflow

Celselect™ Enrichment Technology

CTCs and other rare cells from common liquid samples can be enriched for use in a variety of downstream applications. Cells isolated on slide can be collected from the microchambers for purification, culture, and downstream analyses such as PCR and NGS (Figure 10).

Common samples such as peripheral blood, urine, or tissue sample can be processed. Cells are isolated with Celselect Slide Technology on the Genesis System and then recovered for various downstream uses like cell culture, microscopic analysis, or sequencing.

Celsingle™ Slide Technology

While most commercially available CTC systems only perform enrichment and/or enumeration of CTCs, they do not enable further downstream molecular analysis of the cells. In addition to the cell enumeration workflow, the Genesis System can perform single-cell analyses using Celsingle™ Technology.

With Celsingle Slides, the Genesis System captures and isolates single-cells with a gentle, gravity-based approach. Discrete microwells allow a single-cell to be paired with a unique cellular barcode and unique molecular identifier for desired applications, such as gene expression or protein quantification. The Genesis System enables scientists to analyze and interpret cellular heterogeneity and previously inaccessible single-cell-based information.

Throughput is flexible across one or two Celsingle Slides per run, supporting hundreds to millions of cells per experiment. The Genesis System automates steps in single-cell library preparation, from lysis through reverse transcription. The Celsingle Slide design enables visualization and quality control of cells prior to lysis, as well as treatment or perturbation of cells. The scalability of this approach is unmatched and has the potential to provide a 10-fold increase in cell throughput compared with other technologies. The Genesis System software also includes a flexible protocol-building tool that supports method development and optimization for single-cell applications.

Summary

The examples described in the three studies discussed above, demonstrate the Genesis System overcomes the limitations of current CTC enumeration systems as summarized in Table 1.

The Celselect technology addresses the challenges of CTC isolation and analysis by providing the following benefits:

- **Capture of CTCs with high efficiency from as low as 4 mL of whole blood**
- **Remove 99 percent of RBCs and WBCs**
- **Automated workflow**
- **On-slide immunostaining, IHC and FISH analysis**
- **High-sensitivity CTC enumeration to understand tumor progression and response to therapy**

The Genesis System offers an automated easy-to-use, robust, and precise approach to CTC enumeration and single-cell analysis that has the potential to implement and accelerate clinical adoption of CTCs as a biomarker in cancer diagnostics.

References

1. Stetson D, Ahmed A, Xu X, Nuttall BRB, Lubinski TJ, Johnson JH, et al. Orthogonal Comparison of Four Plasma NGS Tests with Tumor Suggests Technical Factors are a Major Source of Assay Discordance. JCO Precis Oncol. 2019 Mar 14;(3):1–9.

2. Pantel K, Alix-Panabieres C. (2010) Circulating tumour cells in cancer patients: challenges and perspectives. Trends Mol Med 16: 398–406. pmid:20667783

3. Maheswaran S, Haber DA. (2010) Circulating tumor cells: a window into cancer biology and metastasis. Curr Opin Genet Dev 20: 96–99. pmid:20071161

4. Hwang WL, Pleskow HM, Miyamoto DT. Molecular analysis of circulating tumors cells: Biomarkers beyond enumeration. Adv Drug Deliv Rev. 2018 Feb 1; 125:122–31.

5. Jiang Z, Li L, Zhang S, Wang T, Liu Y, Bian L, et al. Predictive value of CTCs biomarkers status in patients with metastatic breast cancer (MBC) receiving anti-HER2 therapy. J Clin Oncol. 2014 May 20;32(15_suppl):628–628.

6. Li J, Han X, Yu X, Xu Z, Yang G, Liu B, et al. Clinical applications of liquid biopsy as prognostic and predictive biomarkers in hepatocellular carcinoma: circulating tumor cells and circulating tumor DNA. J Exp Clin Cancer Res CR. 2018 Sep 3;37(1):213–213.

7. Toss A, Mu Z, Fernandez S, Cristofanilli M. CTC enumeration and characterization: moving toward personalized medicine. Ann Transl Med. 2014 Nov;2(11):108–108.

8. Liberko M, Kolostova K, Bobek V. Essentials of circulating tumor cells for clinical research and practice. Crit Rev Oncol Hematol. 2013 Nov 1;88(2):338–56.

9. Bidard F-C, Peeters DJ, Fehm T, Nole F, Gisbert-Criado R, Mavroudis D, et al. Clinical validity of circulating tumour cells in patients with metastatic breast cancer: a pooled analysis of individual patient data. Lancet Oncol. 2014 Apr;15(4):406–14.

10. Cristofanilli M, Pierga J-Y, Reuben J, Rademaker A, Davis AA, Peeters DJ, et al. The clinical use of circulating tumor cells (CTCs) enumeration for staging of metastatic breast cancer (MBC): International expert consensus paper. Crit Rev Oncol Hematol. 2019 Feb 1; 134:39–45.

11. Xiaoyang Wang, Pin-I Chen, Maria Jaimes, Humin Gu, Keith Shults, Santosh Putta, Fariba Fazeli, Janine Fernandez, Vishal Sharma, Will Chow, Priya Gogoi, Kalyan Handique, Bruce K. Patterson. Cell by Cell Immuno- and Cancer Marker Profiling of Non-Small Lung Cancer Tissue: Checkpoint Marker Expression on CD103+, CD4+ T-cells Correlates with Circulating Tumor Enumeration. 2019 Submitted.

12. Riahi R, Gogoi P, Sepehri S, Zhou Y, Handique K, Godsey J, et al. A novel microchannel-based device to capture and analyze circulating tumor cells (CTCs) of breast cancer. Int J Oncol. 2014 Jun;44(6):1870–8.

13. Gogoi P, Sepehri S, Zhou Y, Gorin MA, Paolillo C, Capoluongo E, et al. Development of an Automated and Sensitive Microfluidic Device for Capturing and Characterizing Circulating Tumor Cells (CTCs) from Clinical Blood Samples. PloS One. 2016;11(1):e0147400.

14. Horimoto Y, Tokuda E, Murakami F, Uomori T, Himuro T, Nakai K, et al. Analysis of circulating tumour cell and the epithelial mesenchymal transition (EMT) status during eribulin-based treatment in 22 patients with metastatic breast cancer: a pilot study. J Transl Med. 2018 Oct 20;16(1):287.

For research use only. Not for use in diagnostic procedures.