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Comparing Sequential Steps For Detection Of Circulating Tumor Cells: More Specific Or Just Less Sensitive?

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The corresponding author Dr. Pachmann holds a patent 7615358 protecting the MAINTRAC method outside of Europe and is partner in the SIMFO GmbH a company dedicated at developing new tests but which does not sell tests to patients. Tests are performed for physicians and patients on request as medically indicated and supervised tests by the

transfusion-medical laboratory Dr. Ulrich Pachmann.

Comparing Sequential Steps For Detection Of Circulating Tumor Cells: More Specific Or Just Less Sensitive?

Abstract

Background: We compared two surface epithelial antigen (EpCAM)-based approaches for the detection of breast cancer cells present in the circulation.

Methods: Blood from 20 breast cancer patients was drawn into standard blood collection tubes (SBCT) and of 7 of these additionally into CellSave® tubes. After erythrocyte lysis of the samples from both systems, cells were stained with FITC-anti-EpCAM and propidium iodide, quantified with an automated microscope and intact cells counted.

Results: EpCAM-positive events from 1ml of blood ranged between 2051 and 28875/ml and from SBCT (MAINTRAC® approach) as compared to between 97 and 2343/ml from the CellSave® tubes, indicating a more than 10-fold reduction in EpCAM accessibility by the preservative. Duplicate cell preparations showed a high correlation of R^2=0.89 (MAINTRAC®) from SBCT vs. a moderate correlation of R^2=0.81 from CellSave® tubes, but a good correlation (R^2=0.91) between the events detected from both systems.

Between 1/2 and 1/5 of the positive events were viable cells in the MAINTRAC® approach with unequivocal morphology, and a good (R^2=0.89) correlation to total events; by contrast, 1/10 to less than 1/100 of the events in the CellSave® tubes were perhaps cells with equivocal morphology no correlation to total events most positive events being non-recognizable cells. Still 30 to 100-fold more cells were recovered than with the CellTrack® Analyzer.

Conclusions: The approach without fixative detects considerably more EpCAM-positive events with good cell morphology as compared to the CellSave® fixation where cell morphology is poor. Magnetic bead enrichment further reduces the number of retrieved cells.

Background

Solid malignant tumors are one of the most frequent causes of death in the developed world. Yet it is rarely the primary tumor which determines the fate of the patient; rather, it is the development of metastases arising from cells that must have left the tumor and reached their final destination via the peripheral blood. The dissemination of epithelial tumor cells from the primary tumor to secondary sites is one of several critical steps in cancer progression and the dissociation of tumor cells from the primary lesion is driven by different factors, including accumulated multiple genetic and epigenetic changes underlying the disorganization of tissue morphology and uncontrolled growth [1]. Additional genomic events may increase invasiveness of the tumor cells, which can subsequently progress to form metastases [2].

The presence of circulating tumor cells in the bloodstream of cancer patients was recognized over a century ago in autopsies of patients who died from a high tumor load [3]. Later, in animals implanted with experimental tumors, cells were seen to be released into the blood [4], and it was shown that trauma produces an increased release of tumor cells and an increase in metastases [5]. $1x10^5$ cells were calculated to be released per day in a highly metastatic tumor system chosen because it exhibited rapid hematogenous spread and a reproducible pattern of growth and development of metastases in about twelve days. Cell release may, however, be considerably lower in slower growing natural tumors. Subsequently, cells seeded into the circulation have been detected by many different groups in different tumors using nucleic acid-based methods [6,7,8,9,10] and cytometric methods [11,12,13,14,15,16,17] as reviewed by Seung Il Kim, Hyo-Il Jung [18], but the number of cells detected by different methods and at different stages of tumor development is still a matter of debate.

Due to the epithelial nature of most solid tumors, circulating tumor cells can be enriched/identified in peripheral blood using the expression of EpCAM, a molecule that is expressed on normal epithelial cells and shows a high level of expression on a variety of carcinomas [19]. Because of their easy accessibility, these cells would be an ideal tool for disease

surveillance. Here we analyze in detail the different steps of two methods, the CellSearch® system and the MAINTRAC® approach, in which the detection of epithelial cells in blood is based on the expression of the adhesion receptor EpCAM on cells from tumor patients circulating in peripheral blood. In the CellSearch® system, cells are aspirated into proprietary designed tubes containing a non-specified stabilizer and subsequently anti-EpCAM magnetic beads binding to surface EpCam epitopes are used for cell enrichment. In the MAINTRAC® approach, cells suspect of tumor origin are detected only by EpCAM expression using fluorochrome tagged anti-EpCAM and quantified.

Methods

From 7 patients 7.5 ml of blood were collected into proprietary designed tubes containing a non-specified stabilizer (CellSearch®) and from the same 7 patients and from 13 further patients into 2.5ml standard blood collection tubes (SBCT) according to ethics committee approval, and analyzed using the previously described microfluorimetric method. The assay method, stability of the sample and reproducibility has been described extensively [15]. In short, in order to compensate for shipping delays 1ml of each sample was subjected to red blood cell lysis on day 2 after blood collection (with usually 95% viability) using 10 ml of erythrocyte lysis solution (Qiagen, Hilden, Germany) for 10 minutes in the cold, spun down at 700 g and re-diluted in 1 ml of PBS. 10 µl of fluorescein isothiocyanate (FITC)-conjugated mouse anti-human epithelial antibody (EpCAM or synonym HEA) (Milteny, Bergisch Gladbach Germany) and 1 µl of Propidium Iodide (PI) were added to 100 µl of cell suspension, incubated for 15 minutes in the dark, readjusted to 1 ml and a defined volume of the cell suspension was applied to wells of ELISA plates and cells were measured using image analysis in the ScanR (Olympus, Munich, Germany) collecting the FITC-antiEpCAM and the PI fluorescence. Values are displayed in scatter grams and histograms and enable the user to locate cells contained within the positive population for visual examination and to take photos and fluoromicrographs. Illustration 1 depicts an example of the procedure. Cells were then visually inspected looking for nuclear propidium iodide (PI) and EpCAM staining in cells from the CellSave® tubes and for PI exclusion (PI entering dying cells due to membrane permeability), and exclusive surface EpCAM staining in cells from the MAINTRAC® approach. Statistical analyses were performed using the SPSS program, version 16.1.

Results

First, we investigated the amount of EpCAM epitopes accessible on unfixed cells as compared to the cells fixed in the CellSave® tubes using FITC-anti-EpCAM antibodies. From each patient blood sample two separate preparations were performed and compared. Samples aspirated into tubes containing the cell stabilizing reagent (CellSave®) were stained after red blood cell lysis in the same way as the cells from normal blood count tubes and the number of FITC-positive events was determined. 20 patients were studied from unfixed samples. The number of positive events detected was 10-20-fold higher (range 2051 to 28875/ml for the five patients used for direct comparison) than in the samples aspirated into CellSave® tubes (5pts) (range 97 to 2343) indicating an extensive loss of accessible epitopes (Illustration 2).

The correlation between duplicate preparations from the blood count tubes was considerably higher (R^2 = 0.89, slope 1.09x) (Illustration 3a) than between duplicate preparations from the same patients from CellSave® tubes (R^2 = 0.81, slope 0.4x) (Illustration 3b)) the slope indicating that numbers were highly concordant for duplicate samples from SBCT (the MAINTRAC® approach) but less so for samples from the CellSave® tubes. Positive events retrieved from individual patients showed, however, a good correlation (R^2 = 0.91) between SBCT and CellSave® tubes, indicating that comparable structures are detected in both approaches (Illustration 4).

The positive events in both approaches were subsequently visually inspected and categorized into discernible cells and cell debris among the events from CellSave® tubes and into live cells without nuclear stain and dead cells (positive nuclear staining due to permeable membrane) and debris among the positive signals from SBCT. Typical galleries generated according to size and staining from duplicate samples of one patient provided by the automated microscope from both approaches are shown in Illustration 5. 21 of 29 events from the live gate of the first preparation of the MAINTRAC® approach were viable cells (35 more events were gated in the dead cell gate), whereas none of the three events from the same patient from the CellSave® tube can be regarded as a cell. 26 of 36 events of the second preparation of the same patient from the MAINTRAC® approach defined by the automated microscope as live gate clearly are live cells, whereas the two events from the panel defined as cell-like

elements according to size and FITC and PI staining (permeable cell membrane due to fixation) from the second preparation from the CellSave® tube might be categorized as cells but with the same poor morphology as shown in the official press release from the company.

Viable cells from the MAINTRAC® approach amounted to 1/2 to 1/5 of all FITC-positive events, with the rest being mostly dead cells or some cell debris. The good correlation between all positive events and live cells (R^2 = 0.95) (Illustration 6a) indicates that in this approach a high proportion of the detected events are, indeed, cells. In contrast, the number of events definable as cells from the CellSave® tubes varied between non-detectable and 97 cells/ml and they amounted to between 1/10 and less than 1/100 of the total FITC-positive events. There was a very poor correlation between all events and discernable cells (Illustration 6b), indicating that most FITC-positive events are not intact cells. Determination of live cells from the duplicate preparation from the MAINTRAC® approach resulted in a higher variation than total events but the correlation between the duplicate analyses was still fairly good (Illustration 7a) (R^2= 0.88); by contrast, in the duplicate analyses from CellSave® tubes only in one patient with high values did both analyses yield comparably high numbers of cells (Illustration 7b). The number of cellular elements recovered from the CellSave® tubes was less than 1/100 from that recovered from the SBCT (Illustration 8a). If the number of live EpCAM-positive cells as determined from the SBCT tubes and the visually identifiable cells from the CellSave® tubes from individual patients were compared, there was no correlation between these two values (Illustration 8b).

Finally, an analysis done on samples of the same patients from a commercial laboratory using the CellSearch® system retrieved 1, 3 and 18/7.5 ml cells in three of the 5 samples whereas in the two other samples no cells were detected.. Therefore, even if no enrichment procedures and only staining with anti-EpCAM had been used, cellular elements from only 1ml of blood with morphology quite comparable to that shown as typical cells by the CellSearch® system (CellSearch™ Circulating Tumor Cell (CTC) Test) would have been detected. This is still about 10-fold more than what was detected using the CellSearch® approach.

The increasing loss of information during the different steps is depicted in Illustration 9, showing the loss was significant in both the fixation and the analysis step but was highest from the total events to the definition of cellular elements.

Conclusions

The question regarding the number of cells dissociating from solid tumors over time [20], their potential to survive in the bloodstream [5,21] or in remote loci [17,22] has not yet been solved. This is due to the considerable differences in the numbers detected by different approaches. Different pre-analytical and analytical influences must be considered.

It has been shown that in the pre-analytic phase, time and temperature of storing the samples of blood or bone marrow may play a role [23]. In previous studies we have shown that the number of epithelial antigen-positive cells remains constant even when the samples are stored up to 7 days at room temperature [15]. However, because of increasing deterioration and the loss of white blood cells, samples were processed no later than after 48 hrs of storage in standard blood count tubes without preservative in order to compensate for shipping delay. At that time white blood cells still were <90% viable.

Components of blood, such as proteins and platelets, may influence the retrieval of cells from whole blood. Thus tumor cell spiking into isolated white blood cell buffy coat resulted in reasonable variation in RT-PCR detection, whereas spiking into blood samples resulted in a considerable quantitative and qualitative variation between laboratories [24].

Separation of the cells in question from the remnant blood components is another critical point. Density gradient separation has been shown to reduce the detection of circulating tumor cells as compared to magnetic bead enrichment [25] or cell filtration [26].

Separation methods based on EpCAM expression distinguishing tumor cells derived from epithelial tumors from blood cells are dependent on the amount of EpCAM present on the cell surface. Preservatives used to stabilize the cells may influence the retrieval rate because most fixatives have been shown to either reduce the accessibility of surface antigens or destroy antigenic epitopes [27,28].

In the present work, we have compared different steps of two methods based on EpCAM expression but differing substantially in results: the CellSearch® and MAINTRAC® approaches.

In the MAINTRAC® approach, blood is drawn into SBCT containing EDTA (ethylendiamine tetra acid) as an anticoagulant and no other preservative. In contrast, in the CellSearch® system cells are aspirated into proprietary designed tubes containing a non-specified

stabilizer. We could clearly show that in samples from CellSave® tubes the number of events staining positive for EpCAM was more than tenfold reduced as compared to samples from standard blood count tubes from the same patients stained with the same anti-EpCAM antibody. Reproducibility of duplicate preparations from the same sample was higher from standard blood count tubes (CV < 10%, R^2 = 0.89) as compared to CellSave® tubes (CV about 90%, R^2= 0.81); however, with a slope indicating a high concordance of values in the MAINTRAC® approach but less well conformance in duplicate preparations from the CellSave® tubes. The correlation between positive events detected by both methods remained high and this correlation between the positive events in both approaches indicates that comparable events are detected. Thus, even if the comparison is based only on a low number of patients´ samples this signifies that results will not change even in a comparison of a higher number of patients. The stabilizer included in the CellSave® tubes obviously leads to a reduction in the accessibility of the respective epitopes. In earlier publications by the same research group before using the stabilizer, frequencies of circulating tumor cells were reported [21,29,30] which were higher than presently with the CellSave® tubes [31]..

Not all positive events are, however, intact cells. Therefore, the automated microscope was programmed to retrieve events of cell size. Only vital cells were counted from the MAINTRAC® approach, whereas all cell-like elements were analyzed from the CellSave® tubes. In the MAINTRAC® approach there was a high correlation between positive events and vital cells. The fraction of EpCAM-positive events that are dying cells or cell debris is obviously dependent upon the current therapy of the patient. Live and dead cells could be clearly distinguished and in transmitted light a nucleus could unequivocally be allocated to every vital cell. In contrast, a very high fraction of the positive events from the CellSave® tubes were particles no longer identifiable as cells. This indicates that in addition to reducing the accessibility of the antigen the preservative also destroys cell morphology and this is now also recognized by the developer of this technique [32]. And, although there was still a good correlation between both methods with respect to positive events, this correlation was largely lost when comparing positive events to discernable cells from the CellSave® tubes as well as between the MAINTRAC® and the CellSave® approach.

Thus it seems that destruction of cell morphology is a pivotal step leading to poor retrieval of epithelial tumor cells from blood by the CellSearch® approach.

In the CellSearch® system then follows the magnetic bead enrichment. Cell capture is dependent upon expression and accessibility of the target antigens [33], and the spiking of tumor cell line cells used as model systems to determine the sensitivity of different methods may not provide an adequate comparison since cell line cells may have much higher surface antigen expression and differ considerably in size, density and stability from circulating tumor cells. Another reason for ineffective retrieval of circulating tumor cells by magnetic bead enrichment in patients may be low surface expression of the target epithelial antigen on circulating tumor cells [34] as compared to primary tissue or cell line cells which is additionally reduced by the fixation process. EpCAM is reported to be frequently downregulated in circulating tumor cells [35]. Indeed, the cells we detected had only part of the cell surface staining positive for EpCAM frequently appearing as a “cap”, possibly due to epithelial/mesenchymal transition processes postulated to be a basic trait of metastasis formation [36] or masking of the relevant epitopes in blood for example due to differential glycosylation [37,38]. Comparing non-enriched samples and those with positive magnetic enrichment revealed an additional significant loss of events [39]. Negative enrichment [40] results in higher numbers of tumor cells and a higher frequency of positive results than positive immunomagnetic selection [41].

Therefore, the retrieval of cells from epithelial tumors from the peripheral blood of patients is dependent upon methodological conditions and an optimal approach will be the one with the least interference with the composition of cell populations under investigation. In the future, only the recovery of a high proportion of the tumor cells present in the circulation will enable determination of the heterogeneity of these cells and their molecular properties, thereby providing the opportunity to further investigate the preconditions necessary for metastasis formation.

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Illustrations

Illustration 1

Flow chart of the approach for detection of live and dead circulating epithelial tumor cells.

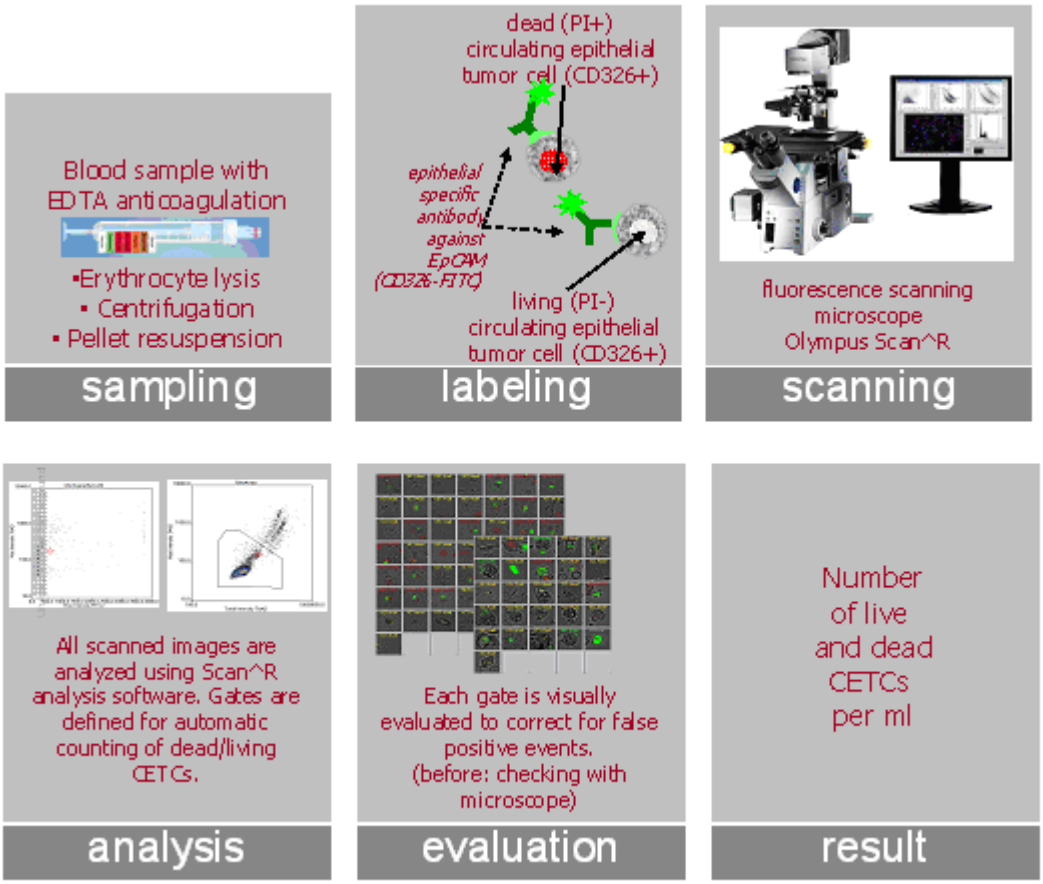


Illustration 2

Box plot of the range of numbers of EpCAM positive events detected from the SBCT and the CellSaveÂ® tubes.

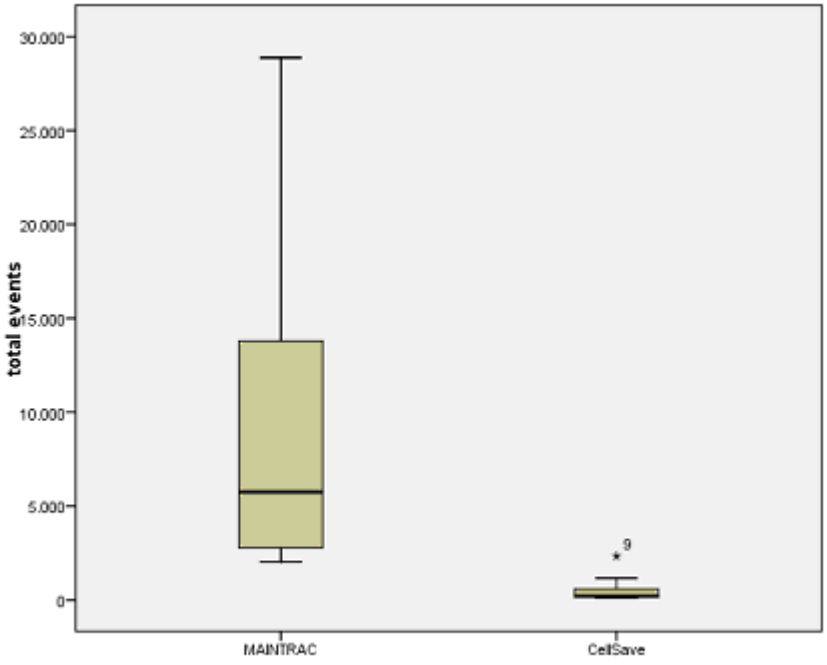


Illustration 3

Correlation between total EpCAM positive events from duplicate preparations from blood drawn either into SBCT or CellSaveÃ‚Â® tubes. a) Duplicate preparations according to the MAINTRACÃ‚Â® approach show a high correlation (R2 = 0.89) and a high consistency (y = 1,09) whereas b) the correlation between duplicate preparations from the CellSaveÃ‚Â® tubes is still high (R2 = 0.81) but less consistent (y = 0.4).

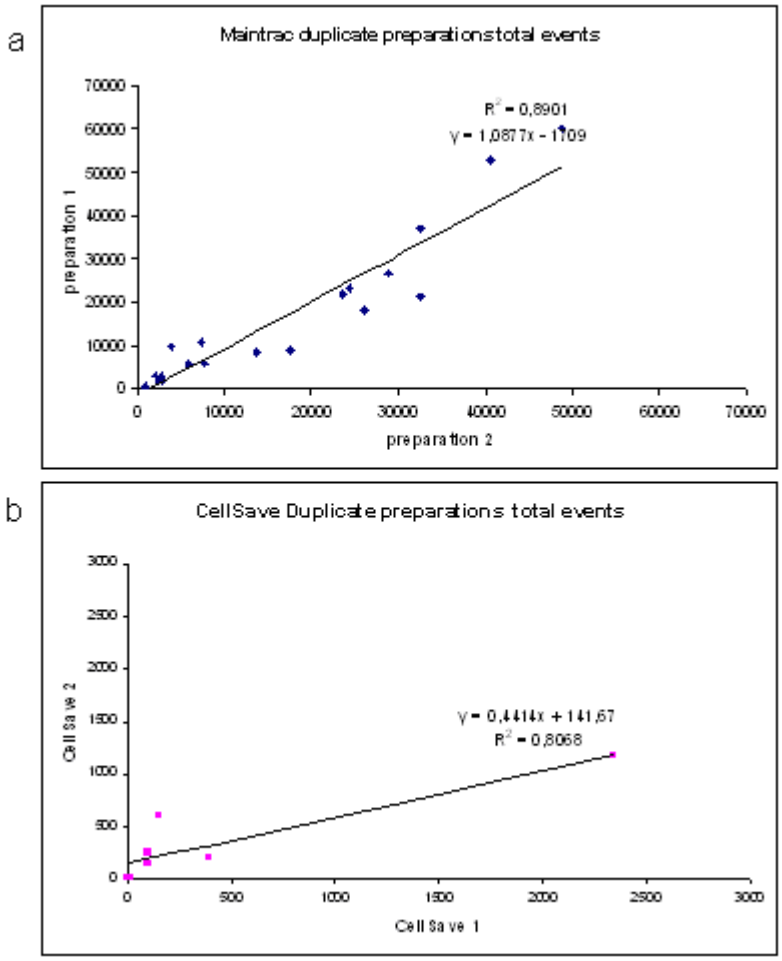


Illustration 4

Correlation between total events detected from the SBCT and the CellSaveÂ® tubes; there is a high correlation between both approaches (R2 = 0.92) but with a tenfold lower detection rate from the CellSaveÂ® tubes.

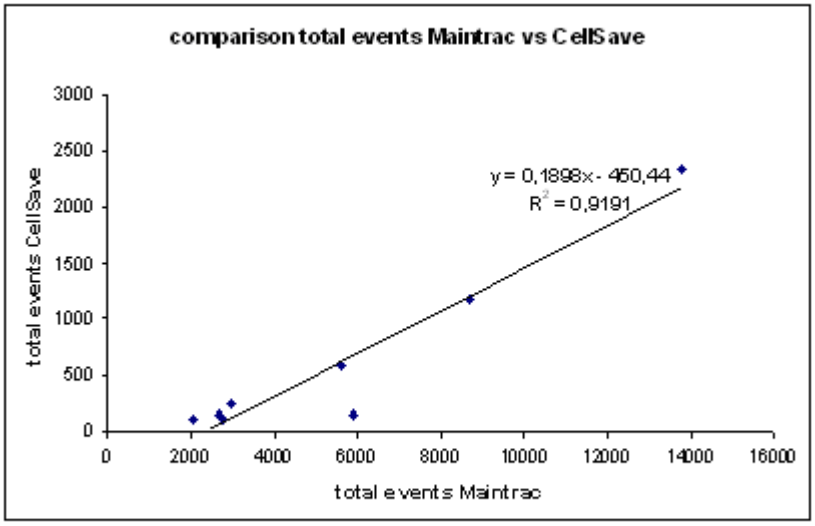


Illustration 5

Demonstration of a gallery of cells from two preparations of one patient using the MAINTRACÃ‚Â® approach (upper two panels) showing the highly conserved morphology of the cells and their different staining patterns and from the same patient cell-like events recovered from the CellSaveÃ‚Â® tubes.

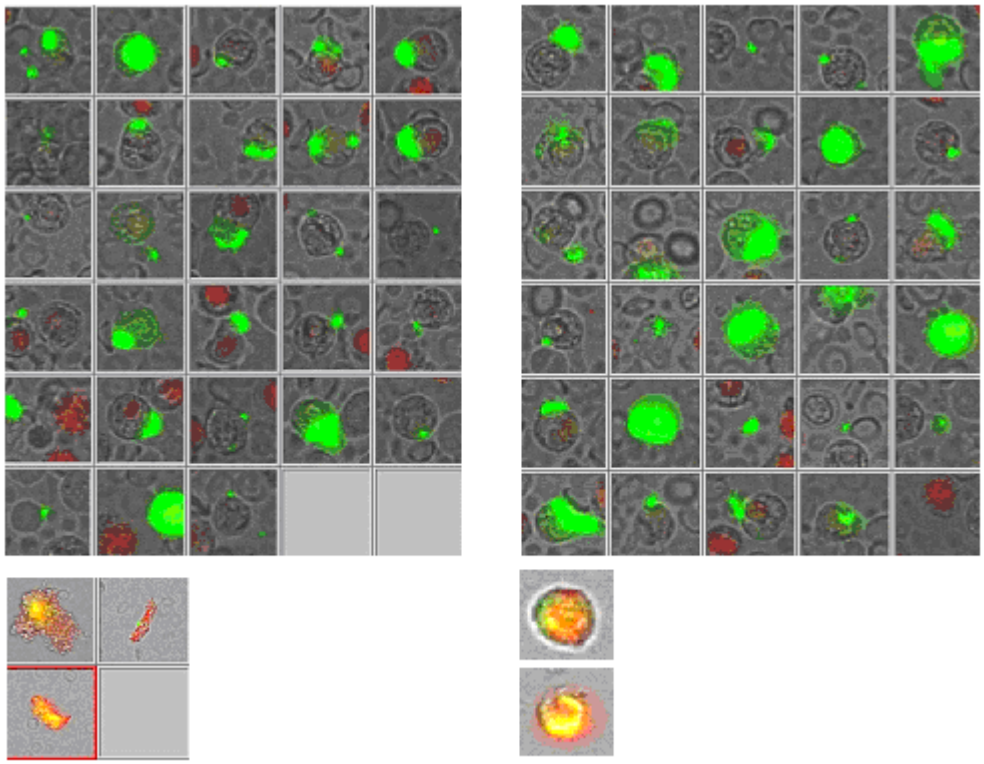
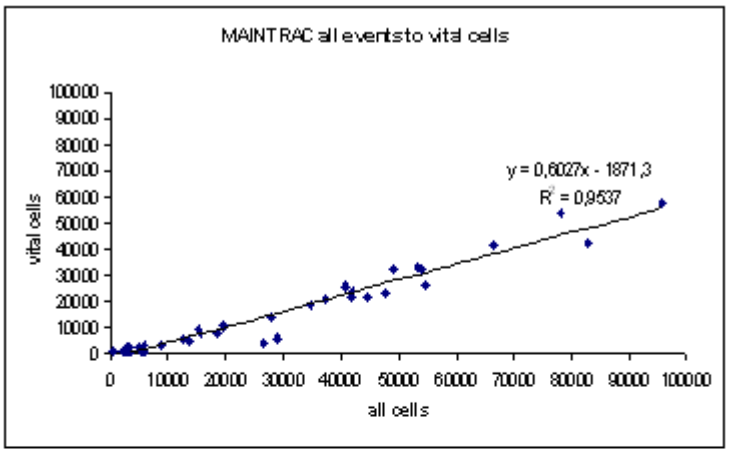


Illustration 6

Correlation between a) total EpCAM positive events and vital cells from blood drawn into SBCT or b) positive events and cellular elements from blood drawn into CellSaveÂ® tubes.

a



b

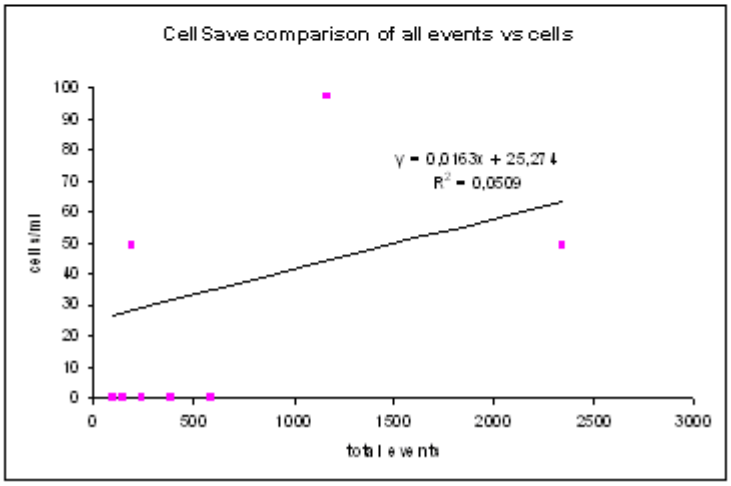
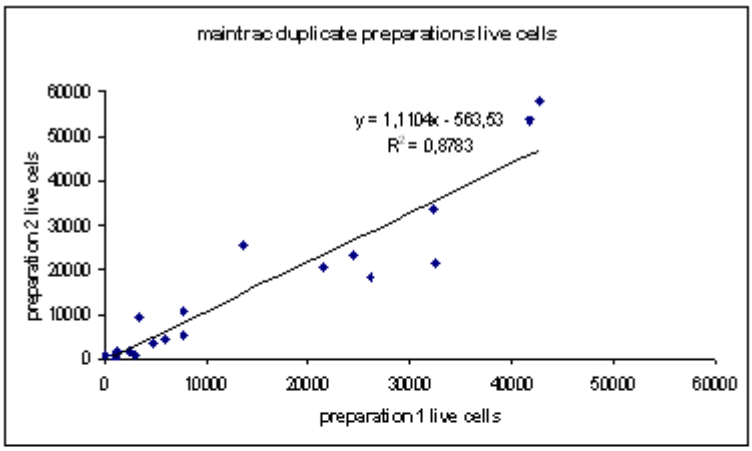


Illustration 7

Correlation between vital cells from duplicate preparations from blood drawn either into SBCT or CellSaveÂ® tubes.

a



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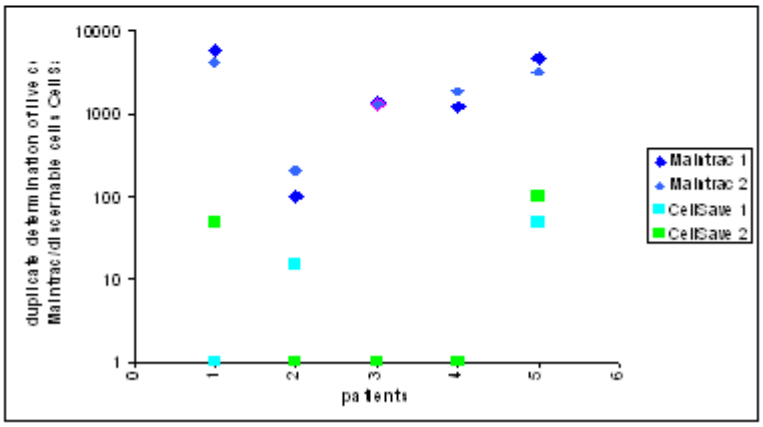
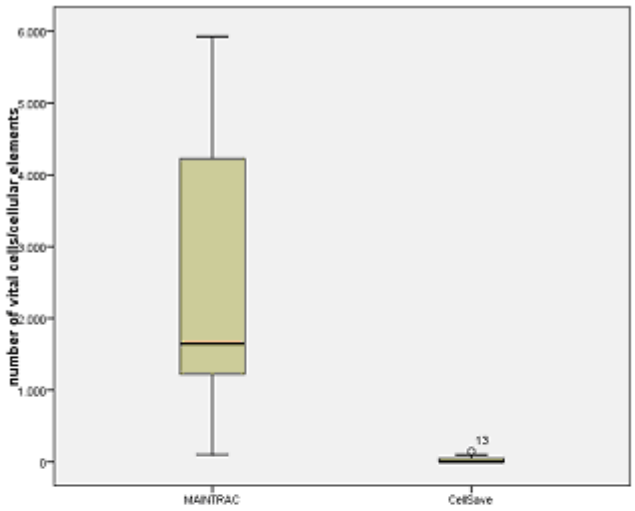


Illustration 8

a): Box plot of the range of numbers of vital cells (MAINTRACÂ®) and cellular elements (CellSaveÂ® tubes). b) Correlation between vital cells from blood drawn into SBCT or cellular elements from blood drawn into CellSaveÂ® tubes with a very poor correlation (R2 = 0.17) between both approaches.

a



b

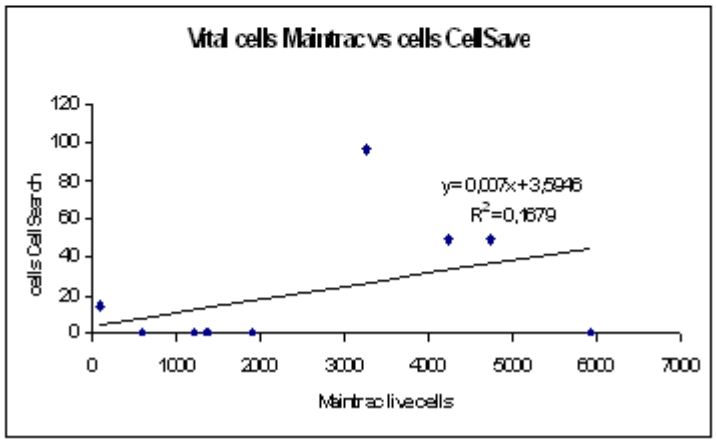


Illustration 9

Comparison between EpCAM positive events and cells detected from SBCT and CellSave® tubes.

Cell loss during cell preparation: comparison of MAINTRAC® and CellSearch® approach

	MAINTRAC®) lowest/ml	highest/ml	CellSearch® lowest/ml	highest/ml	Recovery %	T-Test 2sided
total events	2051	28875	97	2343		
mean	10002		542		5,41896492	p=0,014
vital cells/cell elements	101	5925	0	97		
mean	2474		20		0,80840744	p=0,003
			CellSearch®			
cells			0	2,4	magnetic bead enrichment	
mean			0,97777778		4,88888889	

Illustration 9

Reviews

Review 1

Review Title: Comparing Sequential Steps For Detection Of Circulating Tumor Cells: More Specific Or Just Less Sensitive?

Posted by Prof. Eman I El-Abd on 09 Feb 2011 04:17:26 PM GMT

1	Is the subject of the article within the scope of the subject category?	Yes
2	Are the interpretations / conclusions sound and justified by the data?	Yes
3	Is this a new and original contribution?	Yes
4	Does this paper exemplify an awareness of other research on the topic?	Yes
5	Are structure and length satisfactory?	Yes
6	Can you suggest brief additions or amendments or an introductory statement that will increase the value of this paper for an international audience?	Yes
7	Can you suggest any reductions in the paper, or deletions of parts?	No
8	Is the quality of the diction satisfactory?	Yes
9	Are the illustrations and tables necessary and acceptable?	No
10	Are the references adequate and are they all necessary?	Yes
11	Are the keywords and abstract or summary informative?	Yes

Rating: 7

Comment:
I suggest:

1. Adding more information about the clinical and pathological data of the patients since the number of CTCs depends on the stage of the tumor.
2. Using gold standard technique such as IHC to justify for the number of the detected tumor cells
3. Consider the presence of ~20% of EpCAM negative cells
4. Blood samples from normal volunteers, patients with benign breast lesions would be advantageous.

Competing interests: No

Invited by the author to make a review on this article? : No

Experience and credentials in the specific area of science:
I have worked on CTCs from body fluids in bladder and colorectal cancer. I also worked on circulating RNA in breast cancer.

Publications in the same or a related area of science: Yes

How to cite: El-Abd E.Comparing Sequential Steps For Detection Of Circulating Tumor Cells: More Specific Or Just Less Sensitive? [Review of the article 'Comparing Sequential Steps For Detection Of Circulating Tumor Cells: More Specific Or Just Less Sensitive? ' by].WebmedCentral 2011;2(2):WMCRW00451

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