A New Device for Rapid Isolation by Size and Characterization of Rare Circulating Tumor Cells

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Abstract. *Background: Circulating tumor cells (CTCs) likely derive from clones in the primary tumor, suggesting that they can be used for all biological tests applying to the primary cells. Materials and Methods: The ScreenCell® devices are single-use and low-cost innovative devices that use a filter to isolate and sort tumor cells by size. Results: The ScreenCell® Cyto device is able to isolate rare, fixed, tumor cells, with a high recovery rate. Cells are well preserved morphologically. Immunocytochemistry and FISH assays can be performed directly on the filter. The ScreenCell® CC device allows isolation of live cells able to grow in culture. High quality genetic materials can be obtained directly from tumor cells isolated on the ScreenCell® MB device filter. Conclusion: Due to their reduced size, versatility, and capacity to isolate CTCs within minutes, the ScreenCell® devices may be able to simplify and improve non-invasive access to tumor cells.*

Circulating tumor cells (CTCs) can be used as a surrogate for primary tumor cells when obtaining material non-invasively for the monitoring of tumor phenotypes. Indeed, CTCs are likely derived from clones in the primary tumor (1), suggesting that they can be used for all biological tests applying to the primary cells they represent. Several methods have been developed to detect and isolate CTCs in the peripheral blood of patients with cancer (2-13). Such methods rely mainly on cytometric/immunological characteristics (14, 15), although CTCs may also be isolated by size (16). Any useful method for isolation of CTCs must allow: (i) their identification and enumeration and (ii) their characterization through immunocytochemistry, fluorescence *in situ* hybridization (FISH) assays and all relevant molecular techniques using optimal quality DNA/RNA. However, it is as yet unknown to what extent different methods compare in terms of sensitivity, specificity and reproducibility.

Quantification of CTCs can be performed through the use of magnetic bead-conjugated antibodies against epithelial-cell adhesion molecule (EpCAM). However, its use in affecting treatment decisions remains a point of discussion (17-19). Indeed, isolation of an adequate number of CTCs in a reproducible manner and their use for molecular studies has been limited due to their extreme rarity (around 1 per 10^9 cells in peripheral blood of patients with metastatic cancer) and methodological limitations (20). It is also important to note that the concept of EpCAM-dependent assays is based upon the assumption that the presence of epithelial cells in peripheral blood indicates the presence of tumor cells. However, epithelial cells may be found in healthy donors (21), with their apparent prevalence varying according to: (i) the detection procedure used (9, 22); (ii) the 'cut-off' of reference (9), and (iii) morphological assessment of circulating cells by different investigators, which is a major source of error (23). Furthermore, EpCAM-based assays are not able to detect normal-like breast tumor cells (24). This, combined with the fact that certain tumor types such as melanoma are not of epithelial origin, suggests that EpCAM-based assays may be of limited use. Moreover, a precise cytological analysis of isolated cells is not possible due to technological limitations, therefore limiting the capacity to assess the true tumoral nature of individual cells and microclusters. Indeed, through different

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operators, a number of CK$^+$ DAPI$^+$ cells were not classified as CTCs (9). Finally, some data suggest that epithelial antigen may be lost on CTCs due to the epithelial–mesenchymal transition (EMT), which is considered to be a crucial event in the metastatic process (24-26).

Altogether, these results strongly suggest that morphological analysis including nucleo-cytoplasmic ratio, nuclear details and size of nucleoli, must be correlated with enumeration and immunocytochemical methods for identifying the tumoral origin of circulating cells. Furthermore, CTCs must be isolated alive for testing their potential capacity to initiate tumor formation in animal models and must become easily accessible to a large range of molecular biological analysis.

This study describes a newly developed filtration mini-device, the ScreenCell®, which can isolate, quantify, and analyze circulating tumor cells from a blood sample. In the ScreenCell® device, blood flow passes through a microporous membrane filter allowing size-selective isolation of rare tumor cells under fully reproducible and standardized conditions. The ScreenCell® device has been designed as a low-cost innovative technology with the aim of achieving isolation of tumor cells without the requirement for large and expensive apparatus. This device is fully accessible so that tumor cells isolated onto the filter can be analyzed using all relevant cellular and molecular biological techniques pertinent to the identification and characterization of CTCs and their potential genetic abnormalities. The device can isolate living cells, allowing further tissue culture experiments. Furthermore, tumor cells can be isolated without using an antibody-based assay, suggesting that the ScreenCell® device can be used for the isolation of a large spectrum of tumor cells, including cells of non-epithelial origin.

Materials and Methods

Cell culture and cell spiking. The NCI-H2030 and -H1975 cell lines (derived from non-small cell lung cancer), as well as the HT29 cell line (derived from colorectal adenocarcinoma), were cultured according to the suppliers instructions (CRL-5914, CRL-5908, and HTB-38; American Type Culture Collection (ATCC), Manassas, VA, USA). Following culture, cells were harvested using 0.05% trypsin. Cell suspensions were only used when their viability exceeded 90% as assessed by trypan blue exclusion. For accuracy, linearity, and sensitivity experiments, the spiked cell numbers were estimated to be two and five in 1 ml of peripheral blood from healthy donors. Blood samples from patients with melanoma and colorectal carcinoma were also filtered and analyzed for cytomorphology and immunocytochemistry.

Devices and buffers. The ScreenCell® filtration devices were developed in order to isolate CTCs by size on a microporous membrane filter. These devices are 19 cm long and designed for isolation of: (i) fixed cells for cytological studies (ScreenCell® Cyto); (ii) live cells for culture (ScreenCell® CC) and (iii) molecular biology (ScreenCell® MB). The filtration devices comprise a filtration tank, a filter capped by a removable nozzle/holder (Figure 1Aa and Ba) which, after removal of a protective membrane (Figure 1Ab and Bb), allows insertion and guidance of a collection tube (Figures 1Ac and Bc). The circular track-etched filter, which is composed of polycarbonate (it4ip, Belgium) material, is 18 μm thick with a smooth flat and hydrophilic surface. Circular pores are calibrated (7.5±0.36 μm or 6.5±0.33 μm for isolation of fixed or live cells, respectively) and randomly distributed throughout the filter (1×10^5 pores/cm^2). Before filtration and in order to lyse red blood cells (RBCs), 1 ml blood samples must be diluted in 7 or 8 ml of a specific dilution buffer for fixed or live cells, respectively. Following filtration of fixed cells and for better cytological studies, an additional 1 ml of PBS is filtered for removing RBC debris from the filter. Filtration is usually completed within approximately 50 s (a sample must be considered as micro-coagulated when filtration exceeds 60 s). At the end of filtration, the nozzle/holder of the ScreenCell® device is unclipped and removed from the filtration tank (Figures 1A and 1B).

ScreenCell® Cyto and CC devices (Figure 1A) are devoted to cytological studies and cell culture, respectively. The filter allows a fast and regular filtration, preserving the CTC morphology and microcluster structures. Blood samples are diluted with the ScreenCell® FC or ScreenCell® LC/CC dilution buffers for fixed or live cells, respectively. At the end of filtration, the ScreenCell® CC filter is released into a well of a 24-well tissue culture plate, by pushing down a rod located at the bottom part of the filtration device (Figure 1Ae). Adequate tissue culture medium and growth factors are added into the well. The multiwell plate is then closed and incubated under defined conditions. Similarly, the ScreenCell® Cyto filter is released onto a standard microscopy glass slide by pushing down a rod located at the bottom part of the filtration device; a 7 mm circular coverslip (Cat# CB00070RA1; Menzel-Gläser, Braunschweig, Germany) can be laid down on the filter (Figure 1Af) with the appropriate mounting medium. Cytological studies including staining, cell enumeration, immunocytochemistry, FISH assays, can then be conducted directly on the filter.

For both the ScreenCell® Cyto and CC device filters, the filtration area is delimited by an O ring made of surgical inox with a bar code to insure traceability of the filtered samples.

ScreenCell® MB device is nuclease-free and devoted to molecular biological studies before or after the cell culture (Figure 1B). CTCs are isolated on a circular filter clipped at the bottom part of a capsule (Figure 1Ba); a bar code ensures traceability of the filtered sample.

Before filtration, the blood sample is diluted in the ScreenCell® LC dilution buffer. At the end of filtration, the capsule-filter is then ejected and either inserted inside the upper inner part of an nuclease-free Eppendorf® tube (Figure 1Be) or into a well of a 24-well tissue culture plate (Figure 1Bf).The ScreenCell® MB filtration unit allows extraction of either DNA or RNA directly from cells isolated on the filter before or after cell culture (in this case, the capsule-filter containing the cultured cells is transferred with nuclease-free forceps into a nuclease-free Eppendorf® tube) (Figure 1Be and f). An adequate volume of lysis buffer is added into the capsule-filter which is then closed with the Eppendorf® tube cap (Figure 1Be). Following incubation at the appropriate temperature, the Eppendorf® tube containing the capsule-filter is centrifuged for 1 min at 12,000 ×g (Figure 1Be), and the capsule-filter removed and discarded. The flow-through is either stored in the closed Eppendorf® tube or used immediately to conduct further molecular biological procedures.

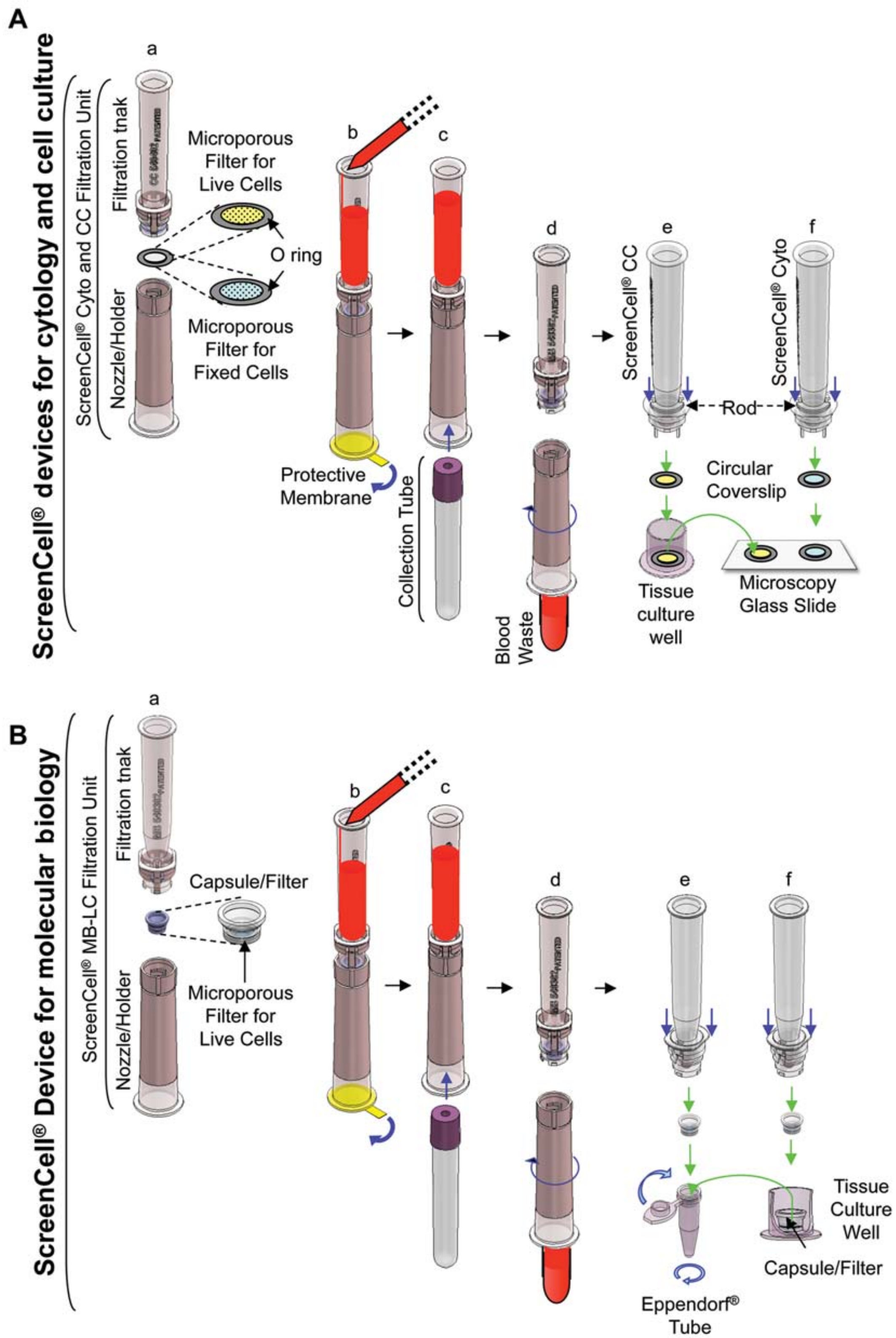

Figure 1. *The ScreenCell® Device. A, ScreenCell® Cyto and CC devices for cytology and cell culture. B, ScreenCell® MB device for molecular biology.*

Sensitivity tests. Two and five cultured NCI-H2030 cells were collected by micropippeting under a microscope and spiked in 1 ml of peripheral blood from a healthy donor. The whole blood was collected in EDTA and filtrated within three hours of collection. Blood spiked with the tumor cells was mixed with a dilution buffer for fixed cells and transferred into the ScreenCell® Cyto device for filtration. Cells isolated on the filter of the device were stained with hematoxylin and eosin. The filters were then mounted on a glass slide with Faramount mounting Medium (S3025, Dakocytomation, Glostrup, Denmark) and a circular glass coverslip. Cell enumeration was performed using a NIKON eclipse 80i fluorescence microscope integrated with cooled CCD camera system and NIS-Elements BR2.30 imaging software (NIKON, France).

qRT-PCR analysis. Lysate obtained from cells isolated on the capsule-filter was recovered into an Eppendorf tube by a one minute centrifugation at 12,000 ×g (10,500 rpm). Total RNA was then extracted and purified using the RNeasy FFPE kit (Qiagen GmbH, Hilden, Germany) according to the manufacturer's instructions with minor modifications. Purified RNA was eluted with 14 µl nuclease-free water (Ambion, NY, USA). RNA (10 µl) was reverse transcribed for 60 min at 37˚C followed by 5 min of RT inactivation at 95˚C using 2.5 µl of 20X RT Enzyme Mix and 25 µl of 2× RT Buffer in 50µl of TaqMan® PreAmp Cells-to-Ct™ Kit (Ambion) according to the manufacturer's instructions. Sequences of interest in the cDNA were then pre-amplified through 14 cycles using 12.5 µl of 1:200 diluted pool of primers and probes (20× Taqman Gene Expression Assays EGFR -Hs00193306_m1- and TaqMan Genotyping Assays; Applied Biosystems, Foster City, USA) and 25 µl of TaqMan PreAmp Master Mix (Ambion) in a final volume of 50 µl. Cycling conditions were as follows: 10 min of denaturing at 95˚C and 14 cycles of 95˚C for 15 s and annealing at 60˚C for 4 min.

For *EGFR* gene expression assay, 10 µl of the 1:10 diluted pre-amplification product was amplified at a final volume of 40 µl by qRT-PCR with 20 µl of 2× Taqman Gene expression Master Mix, 2 µl of 20× Taqman Gene Expression Assays EGFR primers and probes and 8 µl of 2.5 µg/µl BSA (Ambion). The qRT-PCR reactions were run using the ABI PRISM 7300 apparatus (Applied Biosystems). The cycling conditions were as follow: 2 min of UDG incubation at 50˚C, 10 min of enzyme activation at 95˚C and 40 cycles of 95˚C for 15 s and annealing at 60˚C for 1 min.

For detection of the *EGFR* exon 19 deletion, 1 ml of whole peripheral blood spiked with PC9 cells (20 and 100 cells), which harbor an exon 19 deletion (E746-A750), was lysed with 7 ml of ScreenCell® MB dilution buffer at room temperature for 2 min and then passed through ScreenCell® MB filters. Genomic DNA was extracted according to manufacturer's suggestion using QIAamp DNA Micro Kit (Qiagen Cat# 56304), and then amplified in a PCR reaction for exon 19 of *EGFR* (29). The PCR primers for amplification of the *EGFR* exon 19 were designed to hybridize to intron sequences flanking the exon. The sequences are as follows: *EGFR*-EX19F 5'-GTGGCACCATCTCACAATTGCC-3', *EGFR*-EX19R 5'-GGGCC TGAGGTTCAGAGCCAT-3'. The amplicon generated was 203 bp. The PCR reactions were as follows: 200 µM dNTP mix, 300 nM forward primer, 300 nM reverse primer, 1 µl genomic DNA, 0.25 µl JumpStart Taq (Sigma Cat#D4184), 1× JumpStart Buffer, 2.5 mM $MgCl_2$, and water up to 25 µl. Touchdown PCR conditions were as follows: 95˚C for 5 min, followed by 14 cycles of 95˚C for 20 s, 69˚C-62˚C for 20 s, and 72˚C for 40 s, where temperature was reduced by 0.5°C in each successive round of hybridization. Then 30 more cycles of the following conditions: 95˚C for 20 s, 62˚C for 20 s and 72˚C for 40 s, followed by a final extension of 72˚C for 5 min. Samples were then denatured and slowly renatured in order to form heteroduplexes using the following conditions: 95˚C for 2 min followed by a step decrease in temperature of 0.5˚C for 15 s per step, until the temperature reached 45˚C. The PCR products were analyzed on an Agilent 2100 BioAnalyzer. Genomic DNA extracted from cell lines A549 and PC9 was included as wild-type and deletion mutation-positive cells respectively. Two independent filtrations and PCR assays were performed.

For *EGFR* 21 L858R exon point mutation, detection was assessed by allelic discrimination using two sets of primers-probes designed to hybridize to the mutated and the wild-type sequence as follows: *EGFR*-EX21F 5'-GCAGCATGTCAAGATCACAGATTT-3', *EGFR*-EX21R 5'-CCTCCTTCTGCATGGTATTCTTTCT-3', *EGFR*-EX21 VIC 5'–CAGTTTGGCCAGCCCA-3', and *EGFR*-EX21 FAM 5'-CAGTTTGGCCCGCCCA-3'. The targets of interest (L858R mutation and wild-type *EGFR*) in the cDNA were pre-amplified for 14 cycles. Amplification was performed on an ABI PRISM 7300 apparatus (Applied Biosystems) in a 25 µl volume composed of 5 µl of a 1:20 dilution of the pre-amplified product, 12.5 µl of 2xTaqMan® GTXpress™ Master Mix (Applied Biosystems), 0.625 µl of 40x Assay Mix, 5.6 µl of 2.5 µg/µl BSA (Ambion), and 1.275 µl nuclease-free water. The reaction was performed with a preliminary step for 20 s at 95˚C, followed by 60 cycles of two steps at 95˚C for 1 s for denaturing, and 60˚C for 27 s for annealing. The results were analyzed using the allelic discrimination assay program as provided by the constructor.

Immunocytochemistry. Prior to the immunocytochemical analysis, cells isolated on the circular filter of the ScreenCell® Cyto device were dried overnight at room temperature and then hydrated with tris-buffered saline (TBS). When needed, the antigens were retrieved with target retrieval solution at 95-99˚C for 20 min and rinsed with TBS. For detection of intracellular proteins, isolated cells were treated 5 min at room temperature with a permeabilizing buffer, and incubated 30 min at room temperature with a peroxidase-blocking solution. Filters were incubated with a monoclonal antibody against human cytokeratins (clone KL1; Abcam, Cambridge, UK or clone AE1/AE3; Dakocytomation, Glostrup, Denmark), or against human CD45 (clone 2B11 + PD7/26) (Dakocytomation). A standard HRP-peroxidase complex method (EnVision + Dual link system-HRP, DAB+, kit; Dakocytomation) was used for immunocytochemical detection directly on the ScreenCell® Cyto filters which were then incubated with 3,3'-diaminobenzidine chromogen solution (Dakocytomation). After a final wash with distilled water, the filter was counterstained with hematoxylin for 5 min at room temperature. The filter was then dried at room temperature and mounted on a glass slide with Faramount mounting medium (Dakocytomation) and a glass circular coverslip.

Fluorescence in situ hybridization. FISH was applied directly on the filter with the EGFR/CEN-7 FISH Probe Mix and Histology FISH Accessory kit (Dako Denmark, Germany), according to the manufacturer's protocol with minor modifications. In brief, the filter was hydrated with the kit washing buffer and 250 µl of cold (2-8˚C) pepsin were added for 15 min at 37˚C on each area of the filter inside the O ring. The filter was soaked twice for 2 min at room temperature in the kit washing buffer. It was then dehydrated through a graded ethanol series (70%, 90% and 100%) for 2 min at

room temperature and dried at room temperature. Following a 5 min denaturation step at 82 (±2)˚C, the *EGFR* and chromosome 7 centromere locus were directly labeled overnight at 45˚C with 10 µl EGFR/CEN-7 PNA probe mix in a Hybridizer (Dako Denmark). The filter was washed for 10 min at 65˚C with the stringent washing buffer and twice for 3 min at room temperature with the washing buffer. Dehydration was then conducted through an ethanol series and the filter was dried at room temperature and mounted on a slide with fluorescence mounting medium containing DAPI and a glass coverslip. The FISH analysis was performed using a NIKON eclipse 80i fluorescence microscope integrated with a cooled CCD camera system and NIS-Elements BR 2.30 imaging software (NIKON).

Statistics. *P*-values of unpaired unilateral/bilateral Student's *t*-test and Fisher's exact test were calculated to assess the sensitivity of the ScreenCell® Cyto device. It is usually considered that a *p*-value above 0.05 implies that there is no significant difference between compared groups.

Results

Sensitivity of the ScreenCell® Cyto device for CTC detection. Twenty-five independent experiments were conducted with two and five fixed H2030 cells (Tables IA to ID). These cells were micropipetted after being released from the tissue culture flask following standard trypsinisation. After fixation cells were spiked into whole peripheral blood drawn from a healthy donor, and filtered through the ScreenCell® Cyto device. The average filtration time was 50 s. The expected numbers of H2030 cells spiked into the blood sample plotted against the actual number of H2030 cells observed in the sample are shown in Tables IA and IB. The average percentage of H2030 cells recovered was 91.2% and 74% with five and two spiked cells respectively. In the samples spiked with five cells, no fewer than three cells were detected in all 25 samples. For two and five spiked H2030 cells, the average numbers of cells recovered were 1.48 (standard deviation: 0.71) and 4.56 (standard deviation: 0.71), respectively.

To verify whether the percentage of cell loss was related to the filtration device, two and five H2030 cells were harvested as indicated above. Once fixed, cells were micropipetted and collected directly into an Eppendorf tube. Under these conditions, the mean percentage of recovery was 82% and 88% for two and five cells, respectively (Tables IC and ID). For two and five cells, the average numbers of cells recovered were 1.64 (standard deviation 0.57) and 4.40 (standard deviation 0.71), respectively. The relative sensitivity of the ScreenCell® Cyto device *versus* direct cell collection was assessed through *P*-values calculated for unpaired unilateral Student's *t*-test (0.19 and 0.20 for two and five spiked cells respectively), unpaired bilateral Student's *t*-test (0.39 and 0.41 for two and five cells, respectively) and Fisher's exact test (0.14 and 0.34 for two and five cells, respectively). These tests showed that collection of two and five spiked tumor cells through the ScreenCell® Cyto device or by direct collection of the micropipetted cells directly into an Eppendorf tube resulted in similar sensitivities. Through the different series of tests using the ScreenCell® Cyto device and direct collection, similar numbers of cells were lost after 25 independent collections of two and five spiked tumor cells. Indeed, the percentage of cells lost through the ScreenCell® Cyto device was 26% (standard deviation 0.71, with an average of 0.52 cells lost), and 9% (standard deviation 0.65, with an average of 0.44 cells lost) for two and five spiked H2030 cells, respectively, while it was 18% (standard deviation 0.57, with an average of 0.36 cells lost) and 12% (standard deviation 0.71, with an average of 0.60 cells lost) through direct collection for two and five cells, respectively. The *P*-value for unpaired unilateral Student's *t*-test indicated similar numbers of lost cells using the ScreenCell® Cyto device or by direct collection with two or five tumor cells.

No significant differences were found when using the *P*-value for unpaired unilateral Student's *t*-test to compare the results obtained with two *versus* five spiked tumor cells through the ScreenCell® Cyto device or by direct collection (0.19 *versus* 0.20 for two and five spiked tumor cells, respectively). Furthermore, no significant differences were found when using the *P*-value for unpaired unilateral Student's *t*-test to compare the results obtained with two *versus* five spiked tumor cells through the ScreenCell® Cyto device (0.34) or by direct collection (0.10). Altogether, these results strongly suggest that cells were lost essentially through the micropipetting procedure and that the recovery rate of the ScreenCell® Cyto device was close to 100%.

Viability and culture of live H2030 cells following filtration through a ScreenCell® CC device. Five independent experiments were conducted to assess the viability of tumor cells after filtration through the ScreenCell® CC device. In each experiment, live H2030 cells were trypsinised and 50 cells were filtrated through the device. Viable cells were counted immediately after filtration using a standard trypan blue exclusion test; the mean was 0.85±0.09.

The capacity of isolated H2030 cells to grow in tissue culture was further tested through eight independent experiments. In each case, isolated cells were able to grow and expand directly onto the filter under adequate tissue culture conditions (Figures 2A and B).

Morphological and immunocytochemical analysis of fixed tumor cells after filtration through a ScreenCell® Cyto device. Cultured H2030 cells isolated onto ScreenCell® filters after spiking into blood exhibited a well-preserved morphology, with intact nuclear and cytoplasmic contents and membranes (Figure 3A). Isolated H2030 cells which were immunostained

5 cells spiked in 1 ml of blood per filter: isolation using the ScreenCell Cyto device

A

		Exp #1		Exp #2		Exp #3		Exp #4		Exp #5		Total
Number of cells spiked in 1 ml of blood per filter		5		5		5		5		5		25
Number and Percent of Cells Recovered per Filter	Filter #1	4	80 %	5	100 %	4	80 %	5	100 %	4	80 %	22
	Filter #2	5	100 %	5	100 %	5	100 %	3	60 %	5	100 %	23
	Filter #3	3	60 %	4	80 %	5	100 %	5	100 %	5	100 %	22
	Filter #4	5	100 %	4	80 %	4	80 %	5	100 %	5	100 %	23
	Filter #5	4	80 %	5	100 %	5	100 %	5	100 %	5	100 %	24
Total number of spiked cells		25		25		25		25		25		125
Total number of cells isolated on the 5 filters		21		23		23		23		24		114
Average % recovery per filter			84 %		92 %		92 %		92 %		96 %	91.2 %

2 cells spiked in 1 ml of blood per filter: isolation using the ScreenCell Cyto device

B

		Exp #1		Exp #2		Exp #3		Exp #4		Exp #5		Total
Number of cells spiked in 1 ml of blood per filter		2		2		2		2		2		10
Number and Percent of Cells Recovered per Filter	Filter #1	2	100 %	1	50 %	2	100 %	1	50 %	2	100 %	8
	Filter #2	2	100 %	1	50 %	0	0 %	2	100 %	2	100 %	7
	Filter #3	0	0 %	2	100 %	2	100 %	1	50 %	2	100 %	7
	Filter #4	2	100 %	2	100 %	1	50 %	0	0 %	2	100 %	7
	Filter #5	2	100 %	1	50 %	2	100 %	1	50 %	2	100 %	8
Total number of spiked cells		10		10		10		10		10		50
Total number of cells isolated on the 5 filters		8		7		7		5		10		37
Average % recovery per filter			80 %		70 %		70 %		50 %		100 %	74 %

Table 1. *continued*

5 cells spiked directly into the filtration buffer

C

		Exp #1		Exp #2		Exp #3		Exp #4		Exp #5		Total
Number of cells spiked into buffer		5		5		5		5		5		25
Number and Percent of Cells Recovered per Filter	Tube #1	4	80 %	4	80 %	3	60 %	5	100 %	5	100 %	21
	Tube #2	5	100 %	5	100 %	4	80 %	4	80 %	5	100 %	23
	Tube #3	5	100 %	5	100 %	4	80 %	4	80 %	4	80 %	22
	Tube #4	5	100 %	3	60 %	4	80 %	3	60 %	5	100 %	20
	Tube #5	5	100 %	4	80 %	5	100 %	5	100 %	5	100 %	24
Total number of spiked cells		25		25		25		25		25		125
Total number of cells recovered		24		21		20		21		24		114
Average % recovery			96 %		84%		80 %		84 %		96 %	88 %

2 cells spiked directly into the filtration buffer

D

		Exp #1		Exp #2		Exp #3		Exp #4		Exp #5		Total
Number of cells spiked into buffer		2		2		2		2		2		10
Number and Percent of Cells Recovered	Tube #1	2	100 %	2	100 %	2	100 %	2	100 %	1	50 %	9
	Tube #2	2	100 %	1	50 %	0	0 %	2	100 %	2	100 %	7
	Tube #3	1	50 %	1	50 %	2	100 %	2	100 %	2	100 %	8
	Tube #4	1	50 %	2	100 %	2	100 %	1	50 %	2	100 %	8
	Tube #5	2	100 %	2	100 %	2	100 %	2	100 %	1	50 %	9
Total number of spiked cells		10		10		10		10		10		50
Total number of cells recovered		8		8		8		9		8		41
Average % recovery			80 %		80 %		80 %		90 %		80 %	82 %

Table 1. *Sensitivity of the ScreenCell® Cyto device for tumor cell detection. Twenty-five independent experiments were conducted with five and two fixed H2030 cells (A to D). These cells were micropipetted after being released from the tissue culture flask following standard trypsinisation. After fixation, five and two cells were spiked into whole peripheral blood drawn from a healthy donor, and filtered through the ScreenCell® device (Tables A and B, respectively), or were collected directly into filtration buffer in an Eppendorf tube (Tables C and D, respectively). In each series of experiments, the expected numbers of H2030 cells (five and two) spiked into the blood sample was plotted against the actual number of H2030 cells observed in the sample.*

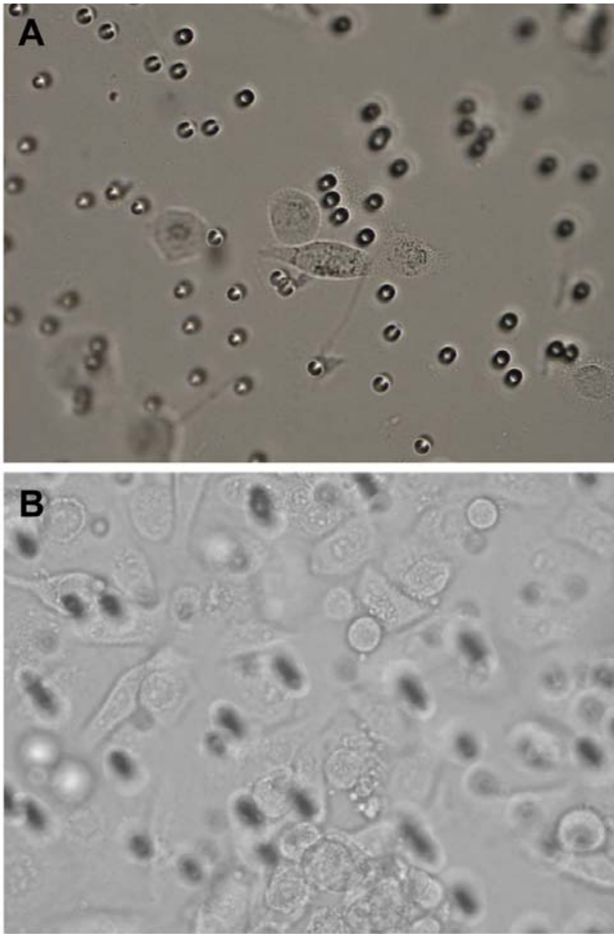

Figure 2. *Viability and culture of live H2030 cells following filtration through a ScreenCell® CC device. Viable H2030 cells were counted following filtration through a ScreenCell® CC device and filter, with isolated cells being cultured directly on the device filter. A: Adherent cells after four days in culture. B: Cells isolated in A were observed ten days after filtration. Cultures in A and B are representative of eight independent experiments; cells were observed by microscopy (×40).*

with an anti-pankeratin (KL1) antibody directly onto the filter on which they were isolated exhibited a dense brown granular cytoplasmic staining, while lymphocytes or monocytes were negative (Figures 3A and 3B). CD45-positive cells were also identified on the filter (~50 CD45-positive cells, with a large variation among donors) together with CD45-negative spiked tumor cells, with a minimal presence of RBCs (Figure 3C). Circulating tumor cells from patients with solid tumors were also analyzed morphologically. Figure 4 shows CTCs from patients with melanoma and colorectal carcinoma. Cell clusters with anisocaryosis and high nucleo-cytoplasmic ratio and hyperbasophilic nucleus were observed. After immunolabeling, a fine and granular cytoplasmic expression of the AE1/AE3 cytokeratins was detected on CTCs from the patient with colorectal cancer.

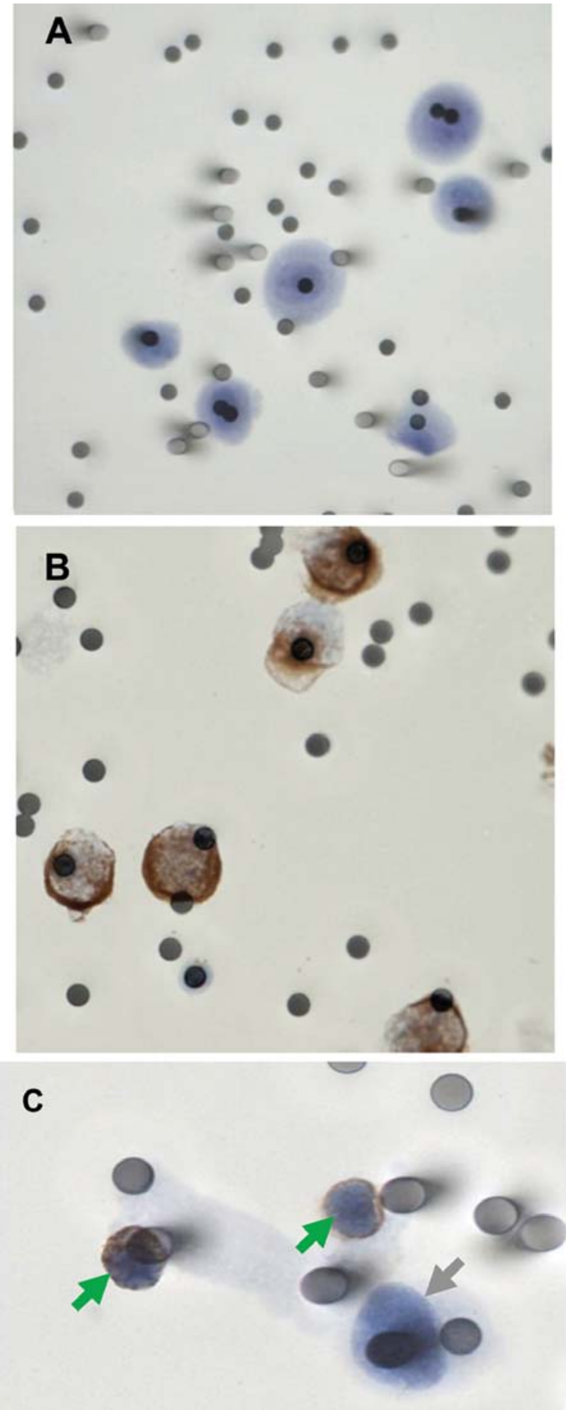

Figure 3. *Morphological and immunocytochemical analysis of fixed H2030 tumor cells after filtration through a ScreenCell® Cyto device. A: Detection of KL1 protein expression on isolated cells by immunolabeling with an anti-KL1 antibody, followed by counterstaining with hematoxylin & eosin. B: Negative control without the anti-KL1 antibody; C: Detection of CD45-positive cells (green arrows) by immunocytochemical analysis after filtration of CD45-negative H2030 cells (gray arrow) spiked into healthy donor blood through a ScreenCell® Cyto device. In A, B and C, H2030 cells were spiked into whole peripheral blood of a healthy donor, filtrated, and then observed under a microscope (×40 for A and B, and ×60 for C).*

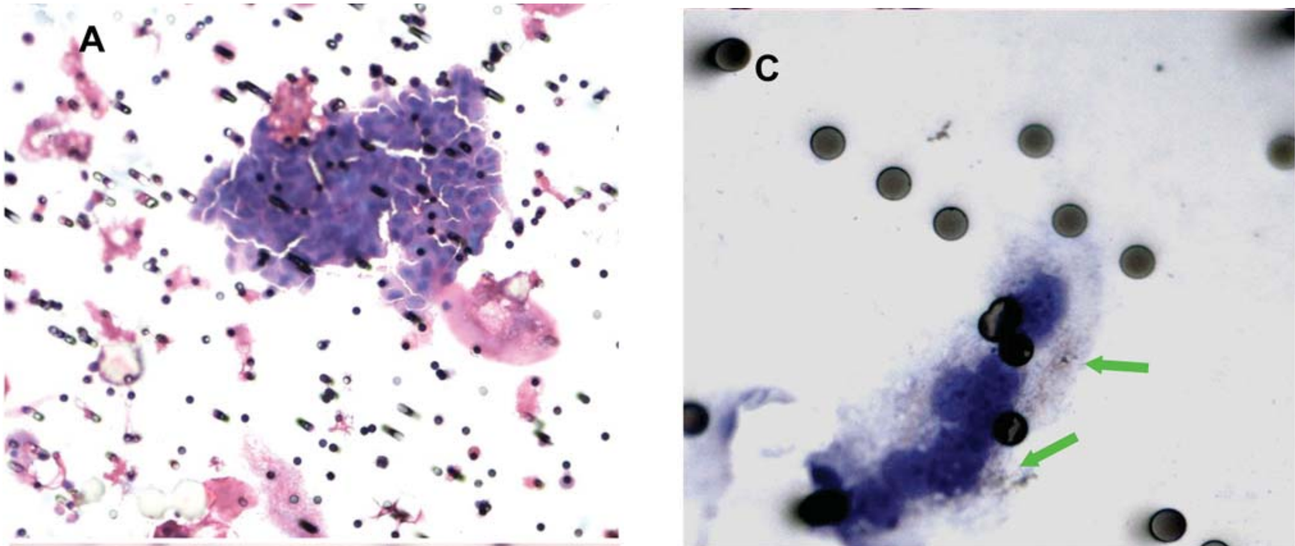

Figure 4. *Morphological and immunocytochemical analysis of fixed cells from peripheral blood isolated through a ScreenCell® Cyto device, then stained with hematoxylin-eosin. A: Cell cluster from a patient with skin melanoma before surgery. Cell diameter was approximately twice that of the filter pore, with anisocaryosis, high nucleo-cytoplasmic ratio and hyperbasophilic nucleus. B: Cell cluster from a patient with colorectal cancer (T3N0M0). Cells formed a nest arranged as a glandular-like structure. Anisocaryosis and high nucleo-cytoplasmic ratio suggested a microembolus of tumor cells. C: Immunocytochemical analysis of CTCs from the patient (see above in B) with colorectal cancer. AE1/AE3 cytokeratin expression in an isolated cell after immunolabeling with anti-AE1/AE3 antibody, followed by counterstaining with hematoxylin. The positive intracytoplasmic staining was fine and granular. Positive and negative controls were obtained by incubating HT29 cells with or without primary antibody, respectively (data not shown). In A, B and C, cells were observed at ×40 magnification.*

Detection of the epidermal growth factor receptor (EGFR) gene by FISH assay in isolated HT29 cells. Diluted HT29 cells were filtrated through the ScreenCell® Cyto device and a FISH assay was conducted directly on the filter using an EGFR/CEN-7 FISH probe mix (EGFR with a red fluorophore specific to the Red Texas spectrum and CEN7 with a green fluorophore specific to the FITC spectrum). After hybridization, the filters were counterstained with DAPI and the *EGFR* and *CEN7* copy numbers were evaluated by FISH using fluorescence microscopy at ×60 magnification (Figure 5). *EGFR* gene amplification was attested to by the detection in the nuclei of more than two *EGFR* signals, as well as a high level of incidence of polysomy 7.

Detection of EGFR expression in isolated H2030 cells. Quantitative real-time RT-PCR was used for the detection of gene copy number changes of *EGFR*. Two, five and ten

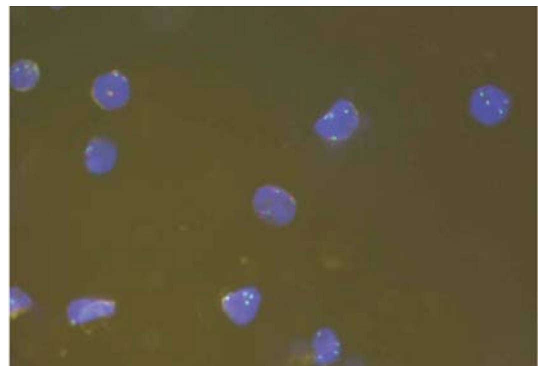

Figure 5. *A dual-colour FISH assay was used for EGFR detection in HT-29 cells. HT29 cells were spiked into healthy donor blood and isolated on a filter following filtration through a ScreenCell® Cyto device; EGFR (red) and chromosome-7 centromere (CEP7, green) were used as probes. Nuclei showed gene amplification represented by more than two red signals, as well as a high level of polysomy 7, represented by green signals.*

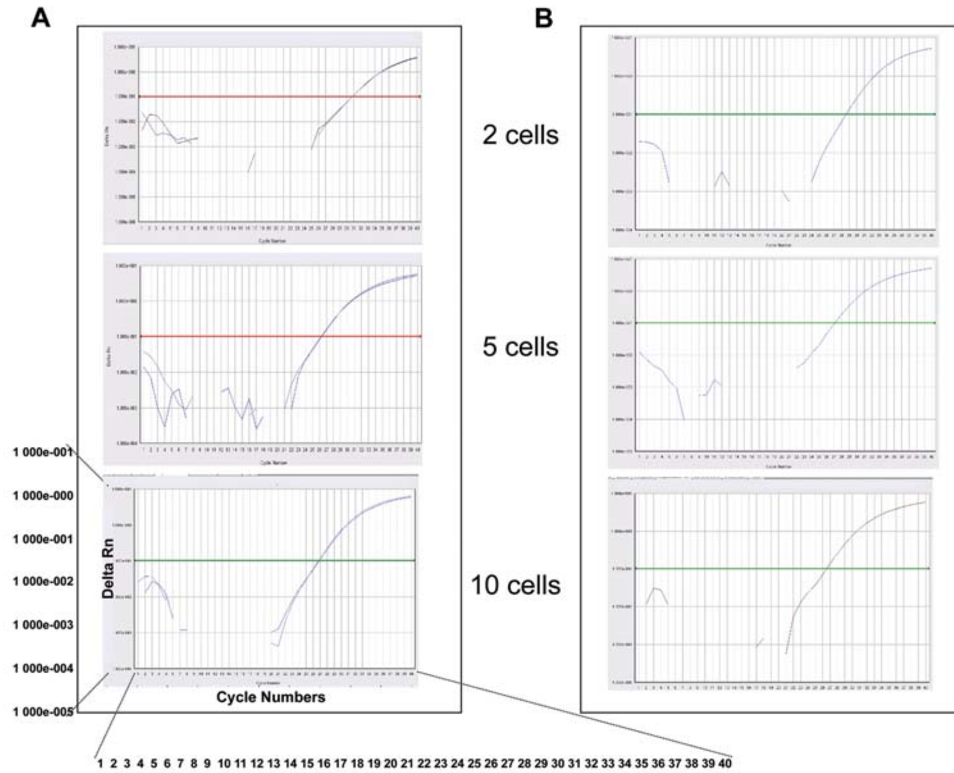

Figure 6. *Detection of EGFR expression in spiked H2030 cells. A: Two, five and ten H2030 cells were spiked into whole peripheral blood and filtrated through a ScreenCell® MB device (A), or directly lysed without filtration (B). RT, preamplification and PCR were then conducted using an EGFR probe. Each curve is a representative of five independent experiments with duplicates in (A). As indicated in the lower part of (A), the axis for each experiment represent the delta Rn (normalized reporter) values i.e. the Rn value of an each reaction minus the Rn value of the baseline signal generated by the instrument.*

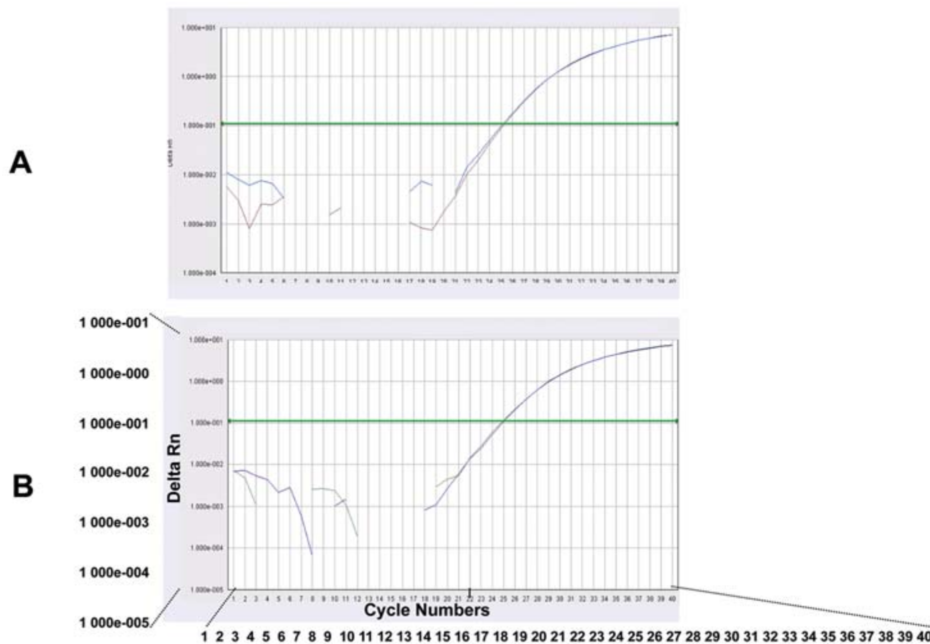

Figure 7. *Material accumulation after two filtrations through the same ScreenCell® MB filter. H2030 cells were spiked into healthy donor blood and filtered through one ScreenCell® MB device. RT, preamplification and PCR were then conducted using an EGFR probe. A: Ten H2030 cells filtered once through one filter. B: Two sets of five H2030 cells were filtered through the same filter. Axis on (B) is also valid for (A).*

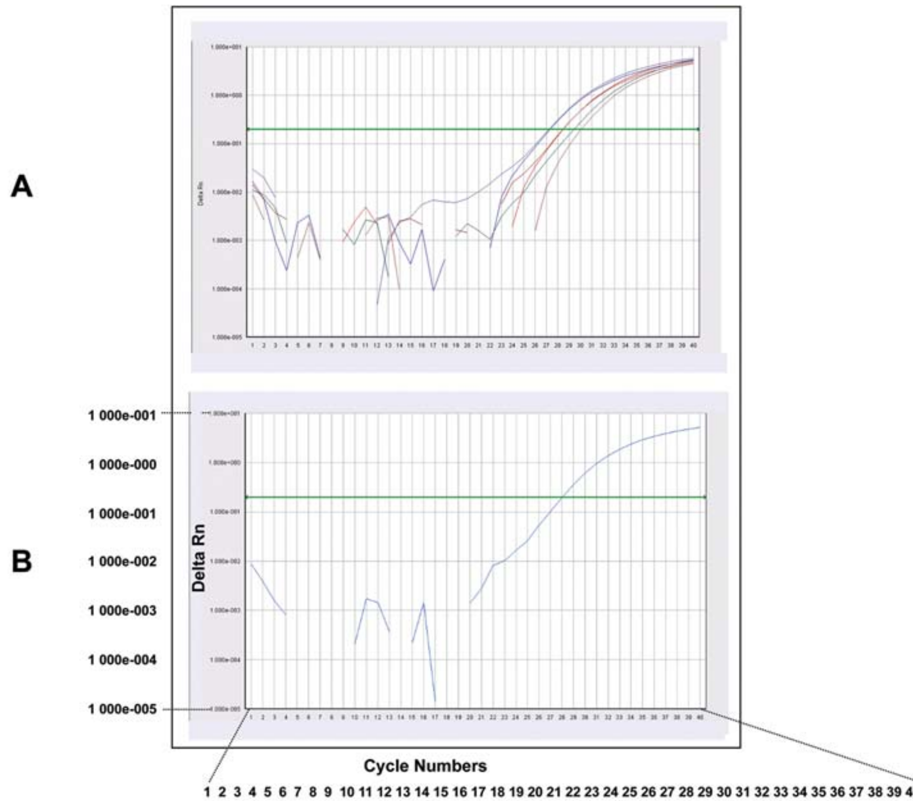

Figure 8. *Reproducibility of RNA extraction and amplification. Five H2030 cells were spiked into whole blood and isolated through the ScreenCell® MB device. A: EGFR expression was detected following RNA extraction; duplicates are from 3 independent samples. B: Positive control experiment. Axis on (B) is also valid for (A).*

H2030 cells were spiked into whole peripheral blood from a healthy donor and filtered through the ScreenCell® MB device. Five independent filtrations were conducted. Isolated tumor cells were lysed directly on the capsule filter and mRNA was extracted. Reverse transcription was performed followed by a pre-amplification step, and PCR. The number of PCR cycles (Ct) required for the 6-carboxyfluorescein (FAM) intensities to exceed a threshold just above background was calculated using 7300 Real-Time PCR System software (Applied Biosystems). *EGFR* expression was detected in all samples of two, five and ten cells (Figure 6A). No significant differences were observed when two, five and ten H2030 cells were micropipetted and directly collected (without filtration) into an Eppendorf tube, suggesting that most spiked tumor cells were recovered through the ScreenCell® MB device (Figure 6Aa *vs*. B). For ten filtered H2030 cells, *EGFR* was detected after 26.5 cycles, while 30.8 cycles were necessary to amplify only two cells.

For material accumulation, it was possible to conduct a second filtration through the same ScreenCell® MB filter. Figure 7 shows *EGFR* detection for a single ten-cell filtration *versus* two five-cell filtrations through the same filter. Similar Cts were observed.

Reproducibility of RNA extraction and amplification was verified using five H2030 cells spiked into whole blood from a healthy donor and isolated through the ScreenCell® MB device (Figure 8).

Detection of EGFR exon 19 deletion and exon 21 L858R EGFR mutation in cells spiked into healthy donor blood. Full length PCR product (204 bp) was amplified from the DNA from A549 cells. A mixture of full-length product and deletion product (188 bp) was amplified from the DNA of PC9 cells, as well as DNA extracted from PC9-spiked blood samples that went through the filters (Figure 9A). Sequencing analysis confirmed that the ScreenCell® MB filters successfully captured PC9 cells which carry the *EGFR* exon 19 deletion mutation (Figure 9B). *EGFR* exon 21 L858R EGFR point mutation was detected in two, five, ten and thirty H1975 cells containing the heterozygous mutation, while, as expected, wild-type *EGFR* was detected in H2030 cells (Figure 9C).

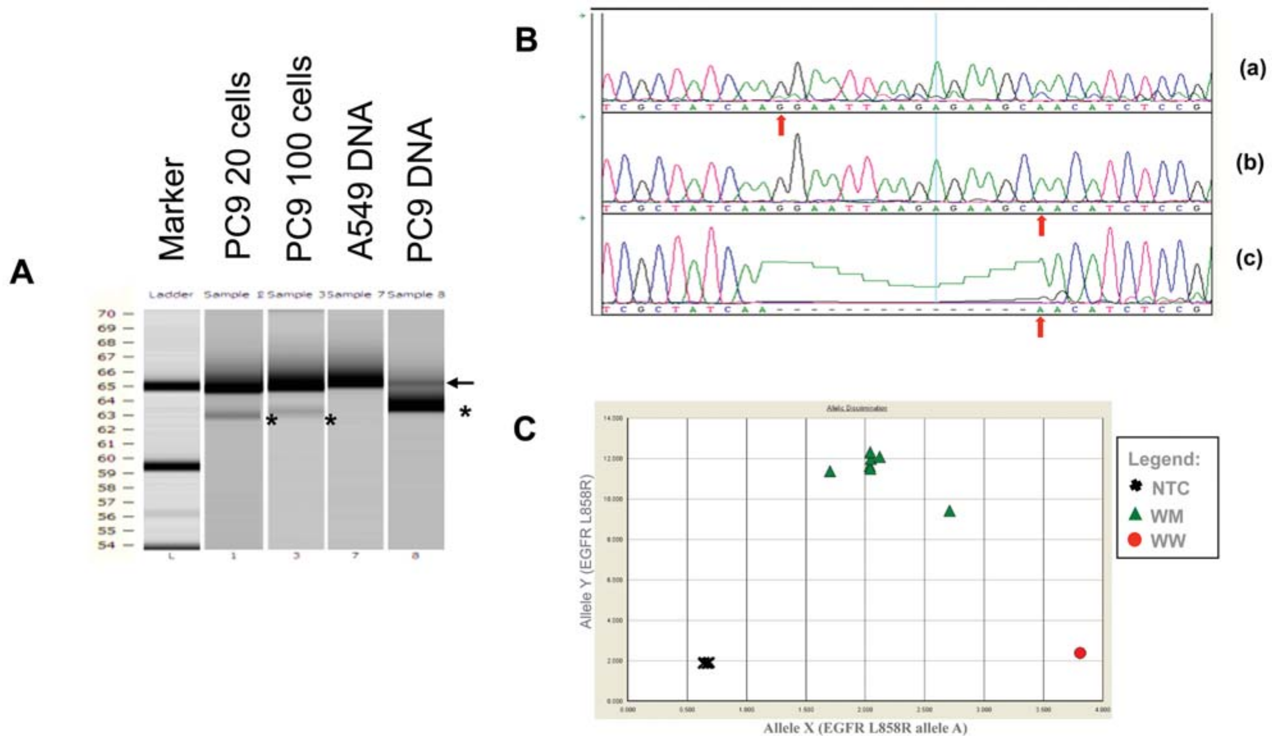

Figure 9. *Use of ScreenCell® MB filter for EGFR mutations. For detection of exon 19 deletion, A549 and PC9 cells were spiked into normal healthy blood and filtered through one ScreenCell® MB device. A: Full-length PCR product (204 bp) and a mixture of full-length product and deletion product (188 bp) were amplified from A549 and PC9 cells DNA respectively, as well as DNA extracted from PC9-spiked blood samples that went through the filters. The arrow represents the position of full length product (204 bp). Asterisks indicate the 188 bp deletion products. B: Sequencing analysis were conducted as a confirmation that the ScreenCell® MB filters successfully captured PC9 cells which carry the EGFR exon 19 deletion mutation. Right hand side a, b, and c represent 200 PC9 cells spiked in blood and filtered, A549 DNA (EGFR wild-type), and PC9 DNA (EGFR exon 19 deletion), respectively. Red arrows under the PC9 cell sequence indicate the beginning of a minor sequence in PC9 cells starting at AAC which is superimposed with the wild-type dominant sequence, GGA) and is found 15 bp downstream in both the A549 wild-type DNA and the PC9 DNA (see red arrows under each sequence), indicating that the minor sequence in PC9 cells corresponds to the mutant one. C: Allelic discrimination plot of fluorescence for EGFR exon 21 L858R point mutation detection. Allelic discrimination assay was conducted using total RNA extracted from NCI-H2030 and -H1975 cells spiked into 1 ml of healthy donor blood and filtered through a ScreenCell® MB device. Reporter fluorophores for the probes were VIC (Applied Biosystems) for detecting the wild-type allele (W), and 6-carboxyfluorescein (FAM) for the mutant allele (M). Each point in the clusters represents a sample corresponding to a particular genotype or no amplification. For exon 21 genotyping, the sample is typed WW if there is fluorescence from the reporter (VIC) for the homozygous wild-type allele (DNAs extracted from wild-type homozygous H2030 cells). Fluorescence from both reporters represents the heterozygous mutated population typed WM, showing detection for 30, 10, 5, and 2 H1975 cells spiked in healthy donor blood, as well as for 10 unspiked H1975 cells (Preamp+, Lysis+, and PCR+). NTC stands for no template control (dilution buffer Lysis–, RT–, and PCR–).*

Discussion

As a proof of principle, this study describes a new filtration device that allows the isolation of CTCs. While very easy to use and not requiring any large equipment, the ScreenCell® device allows CTC isolation with high sensitivity and specificity, with the capability of identifying tumor cells and assessing their specific genotypes. It is not limited to the isolation of CTCs of epithelial origin (28) and the device allows isolation of live cells which may be used for further tissue culture experiments and potentially for testing their capacity to grow in animal models.

A number of devices have been described for isolating CTCs. They use either direct methods, relying essentially on combining negative and positive immunological selection of non-epithelial and epithelial cells, respectively, or indirect methods, mainly the detection of epithelial-specific mRNA transcripts by RT-PCR. The development of the CellSearch™ system (Veridex, Warren, NJ, USA), has been instrumental in opening the field of CTCs to clinical trials. This semi-automated device, based on isolation of potential CTCs by immunomagnetic beads coated with antibodies against EpCAM, aims at identifying cytokeratin-positive cells with positive nuclear staining and CD45 negativity (10, 18, 29). The CellSearch system has been approved by the U.S. Food and Drug Administration as an aid in the monitoring of metastatic breast, colorectal cancer, and metastatic prostate cancer.

This immunological approach to detecting CTCs has given rise to various devices such as the commercially available AdnaTest BreastCancer™ device (AdnaGen AG, Langenhagen, Germany), which allows the isolation of potential CTCs by immunomagnetic beads coated with anti-MUC1 and -EpCAM antibodies. After isolation of the mRNA, transcripts of epithelial-specific markers such as GA 73.3, EpCAM and human epidermal growth factor receptor 2 (HER2) were amplified by a multiplex PCR (31-33).

A relevant marker must be useful to the monitoring of treatment and particularly of targeted therapy. Therefore, a main issue is to obtain cells and genetic material under the best conditions in order to assess whether the monitoring of tumor genotypes can be accomplished using CTCs as a surrogate for primary tumor cells. To this extent, Maheswaran *et al*. (34) have used a microfluidic device, a CTC chip, mediated by the interaction between CTCs and microposts coated with antibody against EpCAM under controlled laminar-flow conditions (35). As a proof of principle, these authors used such a device to isolate viable CTCs from 27 patients with metastatic non-small cell lung cancer (34) and to identify EGFR-activating mutations in 92% of patients with advanced stage disease. Furthermore, the T790M mutation, which confers drug resistance, was found in 55% of patients. That the authors claimed a sensitivity of 92%, together with the fact that in some patients lower CTC numbers correlated with a radiographic response to gefitinib, while increased CTC numbers correlated with an onset of additional *EGFR* mutations and clinical progression, suggested that a survey of the genotypic CTC status is relevant to monitoring tumor cells to assess whether the treatment is likely to reach its target efficiently.

Although this type of approach may allow real-time tumor genotyping, Maheswaran *et al*. indicated that optimization and automation of their device for high-throughput processing will be required to allow large-scale clinical trials. In a recent development of the CTC chip device, Stott *et al*. (36) described a high-throughput microfluidic mixing device, the herringbone-chip, or 'HB-Chip', which provides an enhanced platform for CTC isolation, allowing detection of microclusters of CTCs, previously unappreciated tumor cell aggregates which may contribute to the hematogenous dissemination of cancer.

Using DNA sequencing of tumor tissue as well as a pre-defined gene expression arrays to trace the presence of CTCs in peripheral blood of patients with metastatic colorectal cancer treated with cetuximab plus FOLFOX or FOLFIRI, Yen *et al*. (37) observed a strong correlation between the detection of *KRAS* mutations between circulating tumor cells and the primary malignant tissue. Furthermore, the majority of responders to the cetuximab-containing regimen had wild-type *KRAS* and experienced superior progression-free and overall survival than patients with mutant *KRAS*. This method suggests that CTCs harbor *KRAS* mutations representative of the primary tumor status. However, this methodology does not *per se* allow CTC isolation, and the necessity for a predefined expression array to trace the presence of CTCs appears of limited interest for monitoring a targeted therapy, since detection of a *KRAS* mutation indicates likely resistance to cetuximab (38).

Whether the technique described by Nagrath *et al*. (35) and Yen *et al*. (37) can be adapted to allow consistent detection and isolation of viable CTCs for molecular analysis of pathways other than that of EGFR and KRAS in different tumor types remains to be determined.

Although recent meeting reports disclosed concordance rates of 68% and 88% for data obtained with the CellSearch™ system and with the AdnaTest BreastCancer™ kit, it is difficult (39, 40) to know to what extent these methods may differ in terms of sensitivity, specificity and reproducibility.

Due to its reduced size, large versatility, and capacity to isolate and characterize large circulating cells within minutes, the ScreenCell® device is likely to simplify and amplify clinical access to CTCs considerably. This study conducted spiking experiments using tumor cell lines for assessing the sensitivity, specificity and reproducibility of the system in isolating and characterizing CTCs. The ScreenCell® filtration device is a technology able to capture isolated tumor cells and clusters from patient samples. Further investigations are needed to analyze whether such clusters may provide insight into the metastatic process. Moreover, the fact that the ScreenCell® CC device allows isolation of live cells may open the possibility of testing the presence of tumor-initiating cells among CTCs using highly permissive xenotransplantation conditions. Finally, the use of flat circular filters allows analysis of full microscopy laser scan images of CTCs (data not shown).

Through different devices, the clinical validation of using CTCs as surrogates of primary tumor cells for detecting mutations relevant to the monitoring of targeted therapies is urgently required. Indeed, once this critical point is clarified, a direct comparison between CTC enrichment techniques will be required which will be best conducted through prospective clinical trials.

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Author Contributions

YEC conceived and designed the study. NBF, BG, ID, YK, MY, LW, and JAB, contributed to designing protocols and performed experiments. JW contributed to cytological studies. PAJ and RJD contributed to designing the *EGFR* exon 19 deletion experiment. NBF, BG, ID, YEC, and RJD contributed to data analysis. YEC wrote the paper with contributions by NBF, BG, ID, JW, and RJD.

References

1 Fehm T, Sagalowsky A, Clifford E, Beitsch P, Saboorian H, Euhus D, Meng S, Morrison L, Tucker T, Lane N, Ghadimi BM, Heselmeyer-Haddad K, Ried T, Rao C and Uhr J: Cytogenetic evidence that circulating epithelial cells in patients with carcinoma are malignant. Clin Cancer Res *8*: 2073-2084, 2002.

2 Racila E, Euhus D, Weiss AJ, Rao C, McConnell J, Terstappen LW and Uhr JW: Detection and characterization of carcinoma cells in the blood. Proc Natl Acad Sci USA *95*: 4589-4594, 1998.

3 Ross AA, Cooper BW, Lazarus HM, Mackay W, Moss T J, Ciobanu N, Tallman M S, Kennedy MJ, Davidson NE and Sweet D: Detection and viability of tumor cells in peripheral blood stem cell collections from breast cancer patients using immunohistochemical and clonogenic assay techniques. Blood *82*: 2605-2610, 1993.

4 Naume B, Borgen E, Beiske K, Herstad TK, Ravnås G, Renolen A, Trachsel S, Thrane-Steen K, Funderud S and Kvalheim G: Immunomagnetic techniques for the enrichment and detection of isolated breast carcinoma cells in bone marrow and peripheral blood. J Hematother *6*: 103-114, 1997.

5 Hardingham JE, Kotasek D, Farmer B, Butler RN, Mi JX, Sage RE and Dobrovic A: Immunobead-PCR: a technique for the detection of circulating tumor cells using immunomagnetic beads and the polymerase chain reaction. Cancer Res *53*: 3455-3458, 1993.

6 Zigeuner RE, Riesenberg R, Pohla H, Hofstetter A and Oberneder R: Immunomagnetic cell enrichment detects more disseminated cancer cells than immunocytochemistry *in vitro*. J Urol *164*: 1834-1837, 2000.

7 Bilkenroth U, Taubert H, Riemann D, Rebmann U, Heynemann H and Meye A: Detection and enrichment of disseminated renal carcinoma cells from peripheral blood by immunomagnetic cell separation. Int J Cancer *92*: 577-582, 2001.

8 Kagan M, Howard D, Bendele T, Mayes J, Silvia J, Repollet M, Doyle J, Allard J, Tu N, Bui T, Russell T, Rao C, Hermann M, Rutner H and Terstappen LWMM: A sample preparation and analysis system for identification of circulating tumor cells. J Clin Ligand Assay *25*: 104-110, 2002.

9 Allard WJ, Matera J, Miller MC, Repollet M, Connelly MC, Rao C, Tibbe AGJ, Uhr JW and Terstappen LWMM: Tumor cells circulate in the peripheral blood of all major carcinomas but not in healthy subjects or patients with nonmalignant diseases. Clin Cancer Res *10*: 6897-6904, 2004.

10 Müller V, Stahmann N, Riethdorf S, Rau T, Zabel T, Goetz A, Jänicke F and Pantel K: Circulating tumor cells in breast cancer: correlation to bone marrow micrometastases, heterogeneous response to systemic therapy and low proliferative activity. Clin Cancer Res *11*: 3678-3685, 2005.

11 Wu CH, Lin SR, Yu FJ, Wu DC, Pan YS, Hsieh JS, Huang SY and Wang JY: Development of a high-throughput membrane-array method for molecular diagnosis of circulating tumor cells in patients with gastric cancers. Int J Cancer *119*: 373-379, 2006.

12 Maheswaran S, Sequist LV, Nagrath S, Ulkus L, Brannigan B, Collura CV, Inserra E, Diederichs S, Iafrate AJ, Bell DW, Digumarthy S, Muzikansky A, Irimia D, Settleman J, Tompkins RG, Lynch TJ, Toner M and Haber DA: Detection of mutations in *EGFR* in circulating lung-cancer cells. N Engl J Med *359*: 366-377, 2008.

13 Fehm T, Hoffmann O, Aktas B, Becker S, Solomayer EF, Wallwiener D, Kimmig R and Kasimir-Bauer S: Detection and characterization of circulating tumor cells in blood of primary breast cancer patients by RT-PCR and comparison to status of bone marrow disseminated cells. Breast Cancer Research *11*: 109, 2009.

14 Zabaglo L, Ormerod MG, Parton M, Ring A, Smith IE and Dowsett M: Cell filtration-laser scanning cytometry for the characterisation of circulating breast cancer cells. Cytometry Part A *55*: 102-108, 2003.

15 Hosokawa M, Hayata T, Fukuda Y, Arakaki A, Yoshino T, Tanaka T and Matsunaga T: Size-selective microcavity array for rapid and efficient detection of circulating tumor cells. Anal Chem *82*: 6629-6635, 2010.

16 Vona G, Sabile A, Louha M, Sitruck V, Romana S, Schütze K, Capron, Franco D, Pazzagli M, Vekemans M, Lacour B, Bréchot C and Paterlini-Bréchot P: Isolation by size of epithelial tumor cells: a new method for the immunopharmological and molecular characterization of circulating tumor cells. Am J Pathol *156*: 57-63, 2000.

17 Braun S and Marth C: Circulating tumor cells in metastatic breast cancer – Toward individualized treatment? N Engl J Med *351*: 824-826, 2004.

18 Cristofanilli M, Budd GT, Ellis MJ, Stopeck A, Matera J, Miller MC, Reuben JM, Doyle GV, Allard WJ, Terstappen LW and Hayes DF: Circulating tumor cells, disease progression, and survival in metastatic breast cancer. N Engl J Med *351*: 781-791, 2004.

19 Cristofanilli M, Hayes DF, Budd GT, Ellis MJ, Stopeck A, Reuben JM, Doyle GV, Matera J, Allard WJ, Miller MC, Fritsche HA, Hortobagyi GN and Terstappen LW: Circulating tumor cells: a novel prognostic factor for newly diagnosed metastatic breast cancer. J Clin Oncol *23*: 1420-1430, 2005. [Erratum, J Clin Oncol 23:4808, 2005.]

20 Krivacic RT, Ladanyi A, Curry DN, Hsieh HB, Kuhn P, Bergsrud DE, Kepros JF, Barbera T, Ho MY, Chen LB, Lerner RA and Bruce RH: A rare-cell detector for cancer. Proc Natl Acad Sci USA *101*: 10501-10504, 2004.

21 Steinarsdottir M, Jónasson JG, Vi arson H, Júlíusdóttir H, Hauksdóttir H and Ögmundsdóttir HM: Cytogenetic changes in nonmalignant breast tissue. Genes Chromosomes and Cancer *41*: 47-55, 2004.

22 Sheu CC, Yu YP, Tsai JR, Chang MY, Lin SR, Hwang JJ and Chong IW: Development of a membrane array-based multimarker assay for detection of circulating cancer cells in patients with non-small cell lung cancer. Int J Cancer *119*: 1419-1426, 2006.

23 Tibbe AG, Miller J, M Craig M and Terstappen LWMM: Statistical considerations for enumeration of circulating tumor cells. Cytometry A *71*: 154-162, 2007.

24 Sieuwerts AM, Kraan J, Bolt J, van der Spoel P, Elstrodt F, Schutte M, Martens JWM, Gratama J-W, Sleijfer S and Foekens JA: Anti-epithelial cell adhesion molecule antibodies and the detection of circulating normal-like breast tumor cells. J Natl Cancer Inst *101*: 61-66, 2009.

25 Mego M, Mego M, Megà M, Mani S, Li C, Andreoupolou E, Tin S, Jackson S, Cohen E, Gao H, Cristofanilli M and Reuben J: Circulating tumor cells (CTCs) and epithelial-mesenchymal transition (EMT) in breast cancer: Describing the heterogeneity of microscopic disease. Cancer Res 69 (Meeting Abstract Supplement), 3011, December 15, 2009.

26 Thiery JP: Epithelial–mesenchymal transitions in tumour progression. Nat Rev Cancer *2*: 442-454, 2002.
27 Visvader JE and Lindeman GJ: Cancer stem cells in solid tumours: accumulating evidence and unresolved questions. Nat Rev Cancer *8*: 755-768, 2008.
28 James Bean J , Brennan C, Shih J-Y, Riely G, Viale A, Wang L, Chitale D, Motoi N, Szoke J, Broderick S, Balak M, Chang W-C, Yu C-J, Gazdar A, Pass H, Rusch V, Gerald W, Huang S-F, Yang P-C, Miller V, Ladanyi M, Yang C-H and Pao W: *MET* amplification occurs with or without *T790M* mutations in *EGFR* mutant lung tumors with acquired resistance to gefitinib or erlotinib. Proc Natl Acad Sci USA *104*: 20932-20937, 2007.
29 De Giorgi V, Pinzani P, Salvianti F, Panelos J, Paglierani M, Janowska A, Grazzini M, Wechsler J, Orlando C, Santucci M, Lotti T, Pazzagli M and Massi D: Application of a filtration- and isolation-by-size technique for the detection of circulating tumor cells in cutaneous melanoma. J Invest Dermato *130*: 2440-2447, 2010.
30 Cristofanilli M, Broglio KR, Guarneri V, Jackson S, Fritsche HA, Islam R, Dawood S, Reuben JM, Kau SW, Lara JM, Krishnamurthy S, Ueno NT, Hortobagyi GN and Valero V: Circulating tumor cells in metastatic breast cancer: biologic staging beyond tumor burden. Clin Breast Cancer *7*: 471-479, 2007.
31 Demel U, Tilz GP, Foeldes-Papp Z, Gutierrez B, Albert WH and Böcher O: Detection of tumour cells in the peripheral blood of patients with breast cancer. Development of a new sensitive and specific immunomolecular assay. J Exp Clin Cancer Res *23*: 465-468, 2004.
32 Zieglschmid V, Hollmann C, Gutierrez B, Albert W, Strothoff D, Gross E and Bocher O: Combination of immunomagnetic enrichment with multiplex RT-PCR analysis for the detection of disseminated tumor cells. Anticancer Res *25*: 1803-1810, 2005.
33 Lankiewicz S, Gutierrez B, Rivero B and Böcher O: Quantitative real-time RT-PCR of disseminated tumor cells in combination with immunomagnetic cell enrichment. Mol Biotechnol *34*: 15-28, 2006.
34 Maheswaran S, Sequist LV, Nagrath S, Ulkus L, Brannigan B, Collura CV, Inserra E, Diederichs S, Iafrate AJ, Bell DW, Digumarthy S, Muzikansky A, Irimia D, Settleman J, Tompkins RG, Lynch TJ, Toner M and Haber DA: Detection of mutations in *EGFR* in Circulating Lung Cancer Cells. N Engl J Med *359*: 366-377, 2008.
35 Nagrath S, Sequist LV, Maheswaran S, Bell DW, Irimia D, Ulkus L, Smith MR, Kwak EL, Digumarthy S, Muzikansky A, Ryan P, Balis UJ, Tompkins RG, Haber DA and Toner M: Isolation of rare circulating tumour cells in cancer patients by microchip technology. Nature *450*: 1235-1239, 2007.
36 Stott SL, Hsu CH, Tsukrov DI, Yu M, Miyamoto DT, Waltman BA, Rothenberg SM, Shah AM, Smas ME, Korir GK, Floyd FP Jr,Gilman AJ, Lord JB, Winokur D, Springer S, Irimia D, Nagrath S, Sequist LV, Lee RJ, Isselbacher KJ, Maheswaran S, Haber DA and Toner M: Isolation of circulating tumor cells using a microvortex-generating herringbone-chip. Proc Natl Acad Sci USA *107*: 18392-18397, 2010.
37 Yen LC, Yeh YS, Chen CW, Wang HM, Tsai HL, Lu CY, Chang YT, Chu KS, Lin SR and Wang JY: Detection of *KRAS* oncogene in peripheral blood as a predictor of the response to cetuximab plus chemotherapy in patients with metastatic colorectal cancer. Clin Cancer Res *15*: 4508-4513, 2009.
38 Lièvre A, Bachet JB, Le Corre D, Boige V, Landi B, Emile JF, Côté JF, Tomasic G, Penna C, Ducreux M, Rougier P, Penault-Llorca F and Laurent-Puig P: *KRAS* mutation status is predictive of response to cetuximab therapy in colorectal cancer. Cancer Res *66*: 3992-3995, 2006.
39 Andreopoulou E, Urbauer DL, Krishnamurthy S, Reuben JM, Valero V, Cristofanilli M and Fritsche H: Comparison of circulating tumor cells (CTCs) in metastatic breast cancer (MBC): AdnaTest breast cancer for detection and biological characterization. AACR Meeting Abstracts *2008*: A6, 2008.
40 Dirix LY, Elst H, Benoy I, Auw I Van der, Prové A, Wuyts H, Maes H, van Dam P, Huget P, Vermeulen P and Sint-Augustinus AZ: Circulating tumor cell detection: a direct comparison between the CellSearch system, the AdnaTest, and CK-19/mammaglobin RT-PCR in patients with metastatic breast cancer [abstract]. J Clin Oncol *27(Suppl)*: e22117, 2009.

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