Label-Free Enrichment and Molecular Characterization of Viable Circulating Tumor Cells from Diagnostic Leukapheresis Products.

Running head: CTCs from DLA for culture and molecular analysis.

André Franken [1], Christiane Driemel [2], Bianca Behrens [2], Franziska Meier-Stiegen [1], Volker Endris [3], Albrecht Stenzinger [3], Dieter Niederacher [1], Johannes C. Fischer [4], Nikolas H. Stoecklein [2], Eugen Ruckhaeberle [1], Tanja Fehm [1], Hans Neubauer [1]

1 Department of Obstetrics and Gynecology, University Hospital and Medical Faculty of the Heinrich Heine University Düsseldorf

2 General, Visceral and Pediatric Surgery, University Hospital and Medical Faculty of the Heinrich Heine University Düsseldorf

3 Institute of Pathology, University Hospital Heidelberg

4 Institute for Transplantation Diagnostics and Cell Therapeutics, University Hospital and Medical Faculty of the Heinrich Heine University Düsseldorf

Corresponding author:

Hans Neubauer, Forschungslabore der Frauenklinik des Universitätsklinikums Düsseldorf, Life Science Center, Merowingerplatz 1A, 40225 Düsseldorf, Germany, Tel.: +49211385428118, Fax: +49211385428160, E-mail: hans.neubauer@med.uni-duesseldorf.de

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Abbreviations:

aCGH, array comparative genome hybridization

CTC, circulating tumor cell

CK, cytokeratin

DLA, diagnostic leukapheresis

ER, estrogen receptor

PB, peripheral blood

HER2, HER2/neu

MNC, mononuclear cells

WGA, whole genome amplification

List of human genes:

AKT1: AKT serine/threonine kinase 1

Abstract

Introduction: Circulating tumor cells (CTCs) may be used to improve cancer diagnosis, prognosis and treatment. However, because knowledge regarding CTC biology is limited and the numbers of CTCs and CTC-positive cancer patients are low, progress in this field is slow. We addressed this by combining diagnostic leukapheresis (DLA) and microfluidic enrichment to obtain large numbers of viable CTCs from metastasized breast cancer patients.

Methods: DLA was applied to nine patients, and 7.5 ml peripheral blood was drawn. CTCs were enriched with the Parsortix™ system. The quality of CTCs from fresh and cryopreerved DLA products was tested, and CTCs were cultured *in vitro*. Single uncultured and cultured CTCs were isolated by micromanipulation to determine different parameters, such as genomic aberrations and mutation profiles of selected tumor-associated genes. Expression levels of estrogen receptor and HER2/neu were monitored during *in vitro* culture.

Results: Viable CTCs from peripheral blood and fresh or frozen DLA products could be enriched. DLA increased the likelihood of successful CTC culture. Cryopreserved DLA products could be stored with minimal CTC loss and no overt reduction in the tumor cell quality and viability during an observation period of up to three years. The analyzed parameters did not change during *in vitro* culture. DLA samples with high CTC numbers and lower ratios of apoptotic CTCs were more likely to grow in culture.

Conclusion: The increased CTC numbers from fresh or cryopreserved DLA products facilitate multiple functional and molecular analyses and, thus, could improve our knowledge of their biology.

Introduction

Solid tumors and metastases release circulating tumor cells (CTCs) into the circulatory system. These CTCs can genotypically and phenotypically differ from the primary tumor (1,2). Most CTCs are either eliminated quickly, do not proliferate or perish due to a shortage of nutrients (3). However, some CTCs survive and may extravasate into different tissues where they may persist and eventually initiate metastatic growth. Theoretically, CTCs can be easily obtained by drawing peripheral blood (PB). Thus, CTCs have a promising potential to improve diagnosis and treatment accuracy as a "liquid biopsy" (4,5). CTC quantities in 7.5 ml PB determined with the FDA-approved CellSearch® assay are correlated with decreased progression-free and overall survival in primary (6) and metastasized breast cancer patients (7,8). In addition to the pure quantification of CTCs, their molecular analysis could provide information about their origin and biology, which could be used for drug selection. However, to date, this predictive utility of CTCs has not been demonstrated (9).

In particular, viable CTCs could be highly beneficial for obtaining further insight into their source and may increase the clinical utility of liquid biopsies. They could be used in drug testing and to investigate the functional properties and mechanisms of metastasis formation (10). Although a few CTC cell lines have already been established from different cancer entities, culturing CTCs still remains a challenging task, and culture success has mainly been limited to CTCs from a very few patients carrying remarkably high CTC numbers (11–15).

For the routine clinical use of cultured CTCs, improving the CTC yields per patient is necessary and could broaden the number of patients from which CTC cultures can be successfully derived. This major challenge can be solved by the implementation of diagnostic leukapheresis (DLA) in the CTC detection workflow. Leukapheresis is a standard procedure routinely used in the clinic to enrich mononuclear cells (MNCs) from blood for various applications, including stem cell harvest. During DLA, MNCs are continuously and extracorporeally separated by density from other blood components using constant centrifugation. By enlarging the analyzed blood volume up to several liters, DLA increases the CTC number obtained per patient and the patients' CTC positivity rates (16).

The primary aim of this project was to assess the feasibility of culturing CTCs from fresh and frozen DLA products compared to PB. CTC enrichment from DLA product with the microfluidic Parsortix™ system was established and optimized with MCF7 cells to maintain

96 the viability of enriched tumor cells for further culture approaches. Furthermore, molecular
97 analyses of cultured and uncultured CTCs were performed.

98

Materials and methods

Further protocols can be found in the online **Supplemental Methods**.

Diagnostic leukapheresis

DLA was performed as previously described for one hour (16). This study was carried out in accordance with the Good Clinical Practice guidelines and was approved by the Ethics Committee of the Medical Faculty of the Heinrich Heine University Düsseldorf (Ref-No: 3460). All patients enrolled in this study provided written informed consent. The clinical patient data are presented in online **Supplemental Table 1**. Aliquots of the DLA product were used for CTC enumeration and CTC culture or frozen and stored for future use.

For cryopreservation, 10^8 MNCs from the DLA samples were frozen in 2 ml freezing medium consisting of 45% RMPI 1640 medium (Thermo Fisher Scientific Inc.), 45% HSA (Octapharma AG) and 10% DMSO (Sigma-Aldrich Corporation) after platelet washing at 200 × g for 10 min and then stored in liquid nitrogen.

To determine whether long-term storage affects tumor cell recovery and quality, 5,000 KYSE30 cells were spiked into DLA products from healthy donors and cryopreserved for over five years.

Frozen DLA samples were rapidly thawed in a 37°C water bath and filtered through a 100 µm sieve before enriching CTCs with CellSearch® (Menarini Group) or Parsortix™ system (Angle plc).

Enumeration of CTCs in PB and DLA products

The enrichment and enumeration of the CTCs was performed using the CellSearch® Circulating Epithelial Cell Kit (Menarini) according to the manufacturer's instructions.

Enrichment of viable CTCs from peripheral blood or DLA products

Viable CTCs were enriched from 7.5 ml PB or DLA product adjusted to 2×10^8 MNCs using the Parsortix™ system. The system was operated according to the manufacturer's instructions after a sterilization step with 70% ethanol before sample analysis. Parsortix™ cassettes with a

6.5-micron gap were used, and 100 mbar pressure was applied. Prior to the Parsortix™ enrichment, DLA product was diluted in PBS (1:20).

To determine the capturing and harvesting rates of the Parsortix™ system, 100 MCF7 cells were prelabeled with CellTracker™ green (Thermo Fisher Scientific) and spiked into fresh or frozen CTC-negative DLA product using a FACS system (MoFlo XDP sorter, Beckman Coulter Inc.). Subsequently, the number of prelabeled cells captured in the Parsortix™ cassette during enrichment and the number of released cells after harvesting were counted by fluorescence microscopy.

The efficiency of the Parsortix™ enrichment of patients' CTCs was compared to the performance of CellSearch® Profile Kit. Then, the enriched cell fractions were stained in parallel for DAPI, CK, EpCAM and CD45 to identify and enumerate the enriched CTCs.

CTC culture

CTCs were grown in Tumor Sphere Medium (13). Following this protocol, CTCs were cultured in low attachment plates (Corning Inc.) with RPMI 1640 medium supplemented with 1x B27 (Thermo Fisher Scientific), 20 ng/ml hEGF (Sigma-Aldrich), 20 ng/ml FGF (Sigma-Aldrich), and 1% Penicillin-Streptomycin (Thermo Fisher Scientific) in a humidified atmosphere with 5% CO_2 and 4% O_2.

Results

DLA yields higher CTC numbers

During twelve DLAs of metastasized breast cancer patients, MNCs of a mean volume of 3.41 ± 0.91 l blood were enriched. Compared to the number of MNCs obtained in 7.5 ml PB, DLA led to increased numbers of MNCs, which could be screened for CTCs. In PB, a mean of $6.67\times10^6 \pm 2.40\times10^6$ WBCs per ml was observed, whereas the DLA products contained a mean of $108.28\times10^6 \pm 49.36\times10^6$ WBCs per ml (**Figure 1 A**).

CTCs were detected in eleven PB and matching DLA product samples using CellSearch® analysis. CTC numbers per ml were increased by a mean of 20.7 ± 9.9-fold in DLA products. One patient whose CTC numbers exceeded the CellSearch® detection range was excluded from this calculation. Additionally, in two DLA samples with corresponding CTC-negative PB samples, at least one CTC was detected (**Figure 1 B**). Of note, no significant difference was observed between CTC enrichment and WBC enrichment (*P*-value = 0.558, determined by paired two-tailed *t*-test).

Parsortix™ system enables tumor cell enrichment from DLA products

Subsequently, the ability to culture CTCs enriched from DLA products and patient-matched PB samples was compared using the Parsortix™ system. In this system, CTCs are captured based on their less deformable nature and larger size compared to those of other cellular blood components.

First, the Parsortix™ system was adapted to process DLA products. The efficacy of enriching tumor cells from DLA products was determined with CellTracker™ green-labeled MCF7 breast cancer cells that had been spiked into DLA products. A capturing rate of 67.7 ± 11.3% and a harvesting rate of 65.3 ± 9.7% were determined, which led to a total recovery rate of 44.2% of spiked MCF7 cells (**Figure 2 A**).

Subsequently, the CTC enrichment rate of the Parsortix™ system using DLA samples from four patients was compared to the performance of the CTC enrichment approach using CellSearch®. Using the Parsortix™ system, CTCs were enriched by 0.2- to 4-fold compared to those using CellSearch® (**Figure 2 B**). Immunofluorescence analysis of the Parsortix™-enriched samples exceeding the CTC numbers obtained using CellSearch® confirmed the

presence of CTCs with low EpCAM expression (**Supplemental Figure 1**). Notably, the Parsortix™-enriched CTCs had a significantly smaller mean size of 10.01 ± 1.96 µm than the mean size of 12.55 ± 1.36 µm observed with CellSearch® (*P*-value = 0.0015, determined by two-tailed *t*-test; **Figure 2 C**, **Supplemental Figure 2 A**). The same finding was observed with spiked MCF7 cells. This effect was independent of the different pressures used to process the samples in the Parsortix™ system (**Supplemental Figure 2 B**).

CTCs from DLA products can be isolated by the Parsortix™ system for subsequent culturing

After enriching CTCs from DLA products using the Parsortix™ system, we aimed to determine whether the CTC viability was affected and whether these cells could be used for further functional studies. First, the effect of different pressures on the viability of enriched MCF7 breast cancer cells was determined by measuring the mitochondrial reductase activity (MTT assay). Reducing the pressure from 100 to 50 mbar did not increase the viability of the enriched tumor cells (**Supplemental Figure 2 C**). Then, CTCs enriched and harvested as described above were tested for *in vitro* cultivation. Growing cells were isolated and stained for neoplastic and hematopoietic markers after one week. Cells displaying a nuclear DAPI signal, CK-positivity (CK 4, 5, 6, 8, 10, 13 or 18) and CD45-negativity by immunofluorescence microscopy were identified as CTCs. Cultivable CTCs could be obtained in two of the seven DLA samples classified as "CTC-positive" by CellSearch®. In sample #3, growing CTCs were obtained from both PB and the DLA product. In sample #1, we obtained growing CTCs from the DLA product but not the PB. These two samples had the highest CTC counts according to CellSearch® analysis (**Table 1**). Cultured CTCs formed clusters and could be cultured and expanded for several months for sample #3 (**Supplemental Figure 3 A**). Notably, despite EpCAM-independent enrichment, immunofluorescence analysis revealed EpCAM positivity of all cultured CTCs (**Supplemental Figure 3 B**). Short tandem repeat analyses confirmed patient origin of the cultured CTCs (**Supplemental Figure 3 C**). Furthermore, array comparative genome hybridization (aCGH) analyses of amplified genomes from single isolated CTCs cultured for three months were performed. Several genomic aberrations, such as smaller gains and larger losses, especially in chromosomes 1p, 6, 16q and 17p, were detected in the cultured CTCs, confirming their malignant origin (**Figure 3**). It is worth mentioning that without previous Parsortix™ enrichment blood cells rapidly and massively overgrew the CTC cultures.

Cryopreservation maintains CTC quality and viability

In total, a mean yield of 51.7 ml (maximum yield: 122 ml; minimum yield 35 ml) was achieved per DLA. Only a small portion of the total volume is used for routine analyses, and the remaining DLA product can be cryopreserved after platelet washing and stored for future analysis and cross-validation experiments. Platelet washing was required for successful processing of thawed DLA products using the Parsortix™ system. Without platelet washing, processing of thawed DLA products with Parsortix™ resulted in clogging.

We aimed to (a) determine the CTC loss due to cryopreservation, (b) compare the quality of CellSearch®-enriched CTCs from fresh and cryopreserved DLA products and (c) investigate whether CTCs enriched from cryopreserved DLA samples could be cultured.

First, the enrichment of tumor cells from cryopreserved DLA products was tested with EpCAM-positive KYSE30 cells that had been spiked into DLA products from healthy donors more than five years ago and have been cryopreserved since. The recovery rate using CellSearch® was 81.2 ± 4.9%. Then, CTC count and morphology of cryopreserved patient samples were compared to those of matched samples that had been analyzed immediately after the DLA procedure by CellSearch® analysis. The morphology of detected CTCs was compared by evaluating DAPI and cytokeratin (CK) signals, and the CTCs were grouped into four classes according to whether the nuclei and cytoplasm were intact or fragmented (**Supplemental Figure 4**). A mean total CTC loss of 16.6 ± 11.2% was observed. However, the number of CTCs with an intact nucleus and intact cytoplasm was reduced by a mean of only 7.6 ± 4.8% (**Figure 4 A**).

The genomic integrity of single KYSE30 cells and CTCs from cryopreserved DLA products was analyzed by whole genome amplification (WGA) and following Ampli1™ quality control. The analyzed KYSE30 cells showed a mean GII of 3.77. Furthermore, no reduction in GII of amplified genomic DNA of the CTCs was observed upon cryopreservation (**Figure 4 B**).

Upon thawing 86.2% ± 7.3% of all MNCs from cryopreserved DLA products from breast cancer patients were viable determined by propidium iodide staining. To compare cultivation performance of CTCs enriched from cryopreserved and fresh DLA products, nine samples of cryopreserved DLA product containing more than 5 CTCs per 1×10^8 MNCs as determined by CellSearch® analysis were chosen. Analysis of the corresponding fresh samples had been

performed in five samples. We observed no differences in Parsortix™ enrichment of thawed cryopreserved DLA products. CTCs originating from three of these nine samples were successfully cultured. As described above for the other two samples, the third frozen sample containing cultivable CTCs also had particularly high CTC numbers in the DLA product (**Table 1**). These results were consistent with the culture success using fresh samples and confirmed viability of cryopreserved CTCs. Of note, no differences in growth morphology between cultured CTCs from fresh or cryopreserved DLA products were observed.

To compare culture success to CTC quality, CTCs that had been enriched with the same DLA products using the CellSearch® Profile Kit were analyzed for caspase-cleaved CK as a surrogate for apoptosis by immunofluorescence analysis. First, specificity of the antibody (M30) was tested using MCF7 cells that had been treated with apoptosis-inducing bisphosphonate zoledronic acid (**Supplemental Figure 5 A**). Three apoptotic states were defined according to the intensity of the M30 signal and the appearance of intact or fragmented cytoplasm as determined by the CK pattern (**Supplemental Figure 5 B**). Applying this classification to CTCs from the DLA products, heterogeneous degrees of apoptosis were observed as follows: 1.6–100% of the CTCs exhibited signs of apoptosis. As expected, the DLA samples with cultivable CTCs contained higher ratios of nonapoptotic CTCs, whereas the samples with comparatively high CTC numbers that were not cultivable showed higher ratios of apoptotic CTCs (**Figure 4 C**).

Cultured CTCs maintain their phenotype and genotype

If cultured CTCs are to be used for further characterization, e.g., chemosensitivity assays, it is critical for these CTCs to maintain their phenotypic and genotypic characteristics and heterogeneity during culture. Thus, we analyzed chromosomal aberrations and mutations in 50 major oncogenes and tumor suppressor genes in the cultured CTCs from sample #3 after three months of expansion. Analysis of chromosomal aberrations by aCGH analysis revealed largely identical aberrations. In all cultured and uncultured CTCs, the same prominent losses in chromosomes 1p, 6, 16q and 17p were detected (**Figure 5 A**, **Supplemental Figure 6**). Targeted panel sequencing revealed that mutation profiles of the cultured and uncultured CTCs from this patient were identical (**Supplemental Table 2**).

In addition, the expression of estrogen receptor (ER) and HER2/neu (HER2) was determined over four weeks of culture by CellSearch® analysis. These CTCs were compared to uncultured

CTCs isolated directly from the DLA product. Heterogeneous ER expression was observed in single cells, and the percentage of ER positivity was unaltered during culture. Specifically, two-thirds of both uncultured and cultured CTCs were determined to be ER-positive (**Figure 5 B** and **C**). While the primary tumor was HER2 negative, 90% of the uncultured CTCs showed expression of HER2. During culture, the proportion of HER2-positive CTCs slightly increased to 95% and remained stable (**Figure 5 D** and **E**).

Discussion

Low CTC numbers in PB detected using different technologies are challenging for CTC analyses in general and CTC culture in particular. In contrast, DLA of larger blood volumes provides considerably more MNCs and by this much higher numbers of CTCs per cancer patient. In addition, DLA results in higher CTC-positivity rates (16). These advantages of DLA may enable to overcome the diagnostic deficiencies of PB, which is crucial for the successful implementation of CTC analyses in clinical diagnostics and patient management (17). Culturing CTCs from blood is still mainly limited to cancer patients with remarkably high CTC numbers (11,12). Due to the higher numbers of CTCs obtained from DLA products, we attempted to culture CTCs from DLA products.

While DLA provides higher CTC numbers, it is only a pre-enrichment step. In our selected cases, CTCs were not enriched relative to WBCs during DLA. Thus, a CTC enrichment technology is required after DLA to separate the CTCs from other blood-borne cells for further characterization (16), which is especially important for *in vitro* culture since these blood cells would outgrow the rare CTC populations. Viable CTCs can be enriched based on their surface protein expression by positive or negative selection, based on their physical properties or based on their functional properties (10). Among several available technologies, we chose the label-free Parsortix™ system, which separates CTCs from other blood components by size and deformability while keeping the tumor cells viable (18,19). Hence, the tumor cells are enriched regardless of the expression of specific surface markers, also enabling the capture of EpCAM-negative cells (20). Here, we observed enrichment of EpCAM-negative CTCs from DLA products, which could play a role in successfully deriving CTC cultures (21). However, we did not observe growing EpCAM-negative CTC clones despite the coseeding of both EpCAM-positive and EpCAM-low or EpCAM-negative CTCs. In our experiments, we showed that CTC enrichment using the Parsortix™ system not only keeps the captured CTCs viable but also enables subsequent *in vitro* culture by effectively depleting other blood components. Our experiments also demonstrate that CTCs can retain their viability and capacity to grow *in vitro* during the DLA procedure. Furthermore, our observations in sample #1, in which growing CTCs could only be derived from DLA product but not from PB, indicate that using the DLA product might be advantageous for CTC culture; this is most likely due to higher CTC numbers in the DLA product, which may be critical for obtaining enough of the few CTCs that are capable of *in vitro* culture.

All breast cancer patients with cultivable CTCs had high CTC numbers. Their primary tumors were of luminal subtype, and the patients showed metastatic spread to the bone. Notably, the optimal culture conditions were determined using CTCs from patient VIII, and therefore, these conditions might be particularly suitable for CTCs derived from tumors of a similar subtype. Of note, similar observations were reported by Yu *et al.*, who developed the Tumor Sphere Medium for CTC culture (13). However, a primary tumor of luminal subtype and bone metastasis also correlate with a higher rate of CTC positivity (7,22).

Reasonably, a lower proportion of apoptotic CTCs in DLA product was found to predict culture success. This is not in contradiction to the clinical situation in which high numbers or even high rates of apoptotic CTCs correlate with poor, rather than good, prognoses (23–25). One explanation for this observation may be that in the first case, the viability of CTCs is a requisite for their *in vitro* growth. In the second scenario, the presence of apoptotic CTCs is most likely a by-product of fast-growing metastasis, which influences prognosis (26). This points towards a different relevance of CTCs depending on the readouts with which they are associated.

Despite our DLA approach, only CTCs from a few patients could be successfully cultured. However, to further understand the biology of CTCs, it is critical to combine data from more cultivable CTCs derived from a larger patient cohort. Thus, cryopreservation becomes an important requirement. Cryopreservation of leukapheresis products is widely used for preserving hematopoietic stem cells for future use (27,28). We tested the storage of cryopreserved DLA products and observed that more than 80% of spiked cell line cells could be recovered using the workflow described here after banking for over five years. Furthermore, we observed a particularly low loss of tumor cells with higher quality. Moreover, the morphology and quality were unaffected by cryopreservation, and CTCs from the same fresh and cryopreserved patient samples could be cultured *in vitro*. Thus, cryopreservation allows long-term storage and easy distribution of uncultured primary CTCs, e.g., between different clinical and research sites.

In one sample followed as an example for three months, the cultured CTCs were remarkably stable with no differences in the distribution of chromosomal aberrations or mutations compared to their uncultured counterparts. The *AKT1* E17K mutation, which has been hypothesized to be a disease driver in certain breast cancer patients (29), has been observed in most primary and cultured CTCs. Although only few genomic aberrations were found in this case, detected losses have previously been associated with breast cancer (30–32). Furthermore, the ratios of HER2- and ER-expressing CTCs remain stable during culture.

We acknowledge that some of our data resulted from the in-depth analysis of CTCs obtained from one patient suffering from extremely high numbers of CTCs. Therefore, our data may not be applicable to all CTCs or CTCs from all breast cancer subtypes. However, such cases provide an opportunity for investigations that are still in their initial phase and must be complemented by data obtained from breast cancer and other tumor patients.

In summary, here, we present data suggesting that DLA is an alternative source of higher numbers of CTCs for further *in vitro* culturing. Successful CTC culturing was observed even in one case in which no CTCs could be cultivated from PB. Viable CTCs from both PB and fresh or frozen DLA products can be enriched with the Parsortix™ system. Cryopreservation of DLA products enables their long-term storage with a low CTC loss and no impact on the tumor cell quality or viability. Culturing CTCs from DLA has the potential to promote the power of functional studies for a better understanding of the biology of CTCs and increasing the number of patients who could benefit from *ex vivo* drug testing.

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References

1. Yu M, Stott S, Toner M, Maheswaran S, Haber DA. Circulating tumor cells: Approaches to isolation and characterization. J Cell Biol 2011;192:373–82.

2. Allard WJ, Matera J, Miller MC, Repollet M, Connelly MC, Rao C, et al. Tumor Cells Circulate in the Peripheral Blood of All Major Carcinomas but not in Healthy Subjects or Patients With Nonmalignant Diseases. Clin Cancer Res 2005;10:6897–904.

3. Vanharanta S, Massagué J. Origins of Metastatic Traits. Cancer Cell 2013;24(4):410-21.

4. Pantel K, Alix-Panabières C. Circulating tumour cells in cancer patients: Challenges and perspectives. Trends Mol Med 2010;16:398–406.

5. Barradas AMC, Terstappen LWMM. Towards the biological understanding of CTC: Capture technologies, definitions and potential to create metastasis. Cancers 2013;5:1619–42.

6. Janni WJ, Rack B, Terstappen LWMM, Pierga J-Y, Taran F-A, Fehm T, et al. Pooled Analysis of the Prognostic Relevance of Circulating Tumor Cells in Primary Breast Cancer. Clin Cancer Res 2016;22:2583–93.

7. Bidard FC, Peeters DJ, Fehm T, Nolé 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;15:406–14.

8. Cristofanilli M, Hayes DF, Budd GT, Ellis MJ, Stopeck A, Reuben JM, et al. Circulating tumor cells: A novel prognostic factor for newly diagnosed metastatic breast cancer. J Clin Oncol 2005;23:1420–30.

9. Banys-Paluchowski M, Krawczyk N, Fehm T. Potential Role of Circulating Tumor Cell Detection and Monitoring in Breast Cancer: A Review of Current Evidence. Front Oncol 2016;6:1–9.

10. Pantel K, Alix-Panabieres C. Functional Studies on Viable Circulating Tumor Cells. Clin Chem 2016;62:328–34.

11. Baccelli I, Schneeweiss A, Riethdorf S, Stenzinger A, Schillert A, Vogel V, et al. Identification of a population of blood circulating tumor cells from breast cancer patients that initiates metastasis in a xenograft assay. Nat Biotechnol 2013;31:539–44.

12. Cayrefourcq L, Mazard T, Joosse S, Solassol J, Ramos J, Assenat E, et al. Establishment and characterization of a cell line from human Circulating colon cancer cells. Cancer Res 2015;75:892–901.

13. Yu M, Bardia A, Aceto N, Bersani F, Madden MW, Donaldson MC, et al. Ex vivo culture of circulating breast tumor cells for individualized testing of drug susceptibility. Science 2014;345:216–20.

14. Zhang Z, Shiratsuchi H, Lin J, Chen G, Reddy RM, Azizi E, et al. Expansion of CTCs from early stage lung cancer patients using a microfluidic co-culture model. Oncotarget 2014;5:12383–97.

15. Gao D, Vela I, Sboner A, Iaquinta PJ, Wouter R, Arora VK, et al. Organoid cultures derived from patients with advanced prostate cancer. Cell 2014;159:176–87.

16. Fischer JC, Niederacher D, Topp SA, Honisch E, Schumacher S, Schmitz N, et al. Diagnostic leukapheresis enables reliable detection of circulating tumor cells of nonmetastatic cancer patients. Proc Natl Acad Sci 2013;110:16580–5.

17. Stoecklein NH, Fischer JC, Niederacher D, Terstappen LWMM. Challenges for CTC-based liquid biopsies: Low CTC frequency and diagnostic leukapheresis as a potential solution. Expert Rev Mol Diagn 2016;16:147–64.

18. Xu L, Mao X, Imrali A, Syed F, Mutsvangwa K, Berney D, et al. Optimization and evaluation of a novel size based circulating tumor cell isolation system. PLoS One 2015;10:1–23.

19. Hvichia GE, Parveen Z, Wagner C, Janning M, Quidde J, Stein A, et al. A novel microfluidic platform for size and deformability based separation and the subsequent molecular characterization of viable circulating tumor cells. Int J Cancer 2016;138:2894–904.

20. Lampignano R, Yang L, Neumann MHD, Franken A, Fehm T, Niederacher D, et al. A novel workflow to enrich and isolate patient-matched EpCAMhighand EpCAMlow/negativeCTCs enables the comparative characterization of the PIK3CA status in metastatic breast cancer. Int J Mol Sci 2017;18.

21. Zhang L, Ridgway LD, Wetzel M a, Ngo J, Yin W, Kumar D, et al. The identification and characterization of breast cancer CTCs competent for brain metastasis. Sci Transl Med 2013;5.

22. De Gregorio A, Friedl TWP, Huober J, Scholz C, De Gregorio N, Rack B, et al. Discordance in Human Epidermal Growth Factor Receptor 2 (HER2) Phenotype Between Primary Tumor and Circulating Tumor Cells in Women With HER2-Negative Metastatic Breast Cancer. JCO Precis Oncol 2017;2:1–12.

23. Smerage JB, Budd GT, Doyle G V, Brown M, Paoletti C, Muniz M, et al. Monitoring apoptosis and Bcl-2 on circulating tumor cells in patients with metastatic breast cancer. Mol Oncol 2013;7:680–92.

24. Deutsch TM, Riethdorf S, Nees J, Hartkopf AD, Schönfisch B, Domschke C, et al. Impact of apoptotic circulating tumor cells (aCTC) in metastatic breast cancer. Breast Cancer Res Treat 2016;160:277–90.

25. Jansson S, Bendahl P-O, Larsson A-M, Aaltonen KE, Rydén L. Prognostic impact of circulating tumor cell apoptosis and clusters in serial blood samples from patients with metastatic breast cancer in a prospective observational cohort. BMC Cancer 2016;16:433.

26. Rupa JD, De Bruïne AP, Gerbers AJ, Leers MPG, Nap M, Kessels AGH, et al. Simultaneous detection of apoptosis and proliferation in colorectal carcinoma by multiparameter flow cytometry allows separation of high and low-turnover tumors with distinct clinical outcome. Cancer 2003;97:2404–11.

27. Fisher V, Khuu H, David-Ocampo V, Byrne K, Pavletic S, Bishop M, et al. Analysis of the recovery of cryopreserved and thawed CD34+ and CD3+ cells collected for hematopoietic transplantation. Transfusion 2014;54:1088–92.

28. Lecchi L, Giovanelli S, Gagliardi B, Pezzali I, Ratti I, Marconi M. An update on methods for cryopreservation and thawing of hemopoietic stem cells. Transfus Apher Sci 2016;54(3):324-36.

29. Rudolph M, Anzeneder T, Schulz A, Beckmann G, Byrne AT, Jeffers M, et al. AKT1E17K mutation profiling in breast cancer: Prevalence, concurrent oncogenic alterations, and blood-based detection. BMC Cancer 2016;16:1–12.

30. Noviello CC, Courjal F. Loss of Heterozygosity on the Long Arm of Chromosome 6 in Breast Cancer: Possibly Four Regions of Deletion. Clin Cancer Res 1996;2:1601–6.

31. Brunelli M, Nottegar A, Bogina G, Caliò A, Cima L, Eccher A, et al. Monosomy of chromosome 17 in breast cancer during interpretation of HER2 gene amplification. Am J Cancer Res 2015;5:2212–21.

32. Rakha EA, Green AR, Powe DG, Roylance R, Ellis IO. Chromosome 16 tumor-suppressor genes in breast cancer. Genes Chromosomes Cancer 2006;45:527-35.

Table 1: CTC positivity rates in PB and corresponding DLA products and the success of CTC culture.

Culture success of CTCs obtained from PB and fresh or frozen DLA products was compared. Samples with cultivable CTCs are marked with "+". CTCs were enumerated by CellSearch® analysis of a sample with the same volume in parallel.

*CTC numbers exceeded the detection range of CellSearch®.

	PB		DLA Product		
				fresh	cryopreserved
Sample ID	CTC Count	Culture Success	CTC Count	Culture Success	Culture Success
#1	34	-	320	+	+
#2	0	-	1	-	nd
#3	*	+	*	+	+
#4	11	-	50	-	-
#5	0	-	2	-	nd
#6	2	-	14	-	-
#7	0	-	0	-	nd
#8	3	-	11	-	-
#9	291	nd	2913	nd	+
#10	63	nd	223	nd	-
#11	83	nd	296	nd	-
#12	4	nd	6	nd	-

Figure 1: DLA yields greater CTC numbers.

[A] WBC numbers per ml of PB and DLA products were compared. Horizontal lines indicate the mean. [B] CTC count per ml of PB and DLA products was determined by CellSearch® analysis.

*CTC numbers exceeded the detection range of CellSearch®.

Figure 2: CTCs from DLA products can be enriched with the Parsortix™ system.

[A] CellTracker green-labeled MCF7 cells were spiked in DLA product and enriched with the Parsortix™ system to determine the capturing and harvesting rates. [B] The numbers of enriched CTCs using the CellSearch® and Parsortix™ system were compared. Samples of four patients were processed with CellSearch® profile kit and Parsortix™ system and stained in parallel. The number of Parsortix™-enriched CTCs was normalized to the number of CellSearch®-enriched CTCs. [C] CTCs were identified by immunofluorescence analysis of CK and CD45 and nuclear staining using DAPI. Representative CTCs are shown. A magnification of 40x was used.

Figure 3: Cultured CTCs harbor chromosomal aberrations.

Single cultured CTCs from sample #3 were isolated by micromanipulation. Genomic DNA was amplified by WGA, and chromosomal aberrations were detected by aCGH analysis.

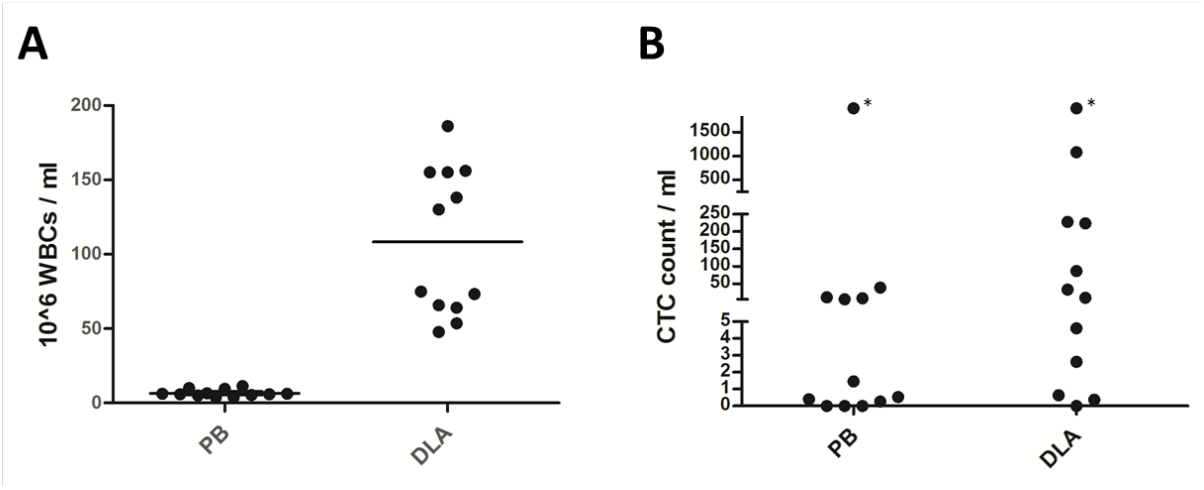
Figure 4: Cryopreservation of DLA products only slightly impairs CTC quantity and quality.

[A] CTCs from fresh and cryopreserved DLA products were enumerated by CellSearch® analysis. Numbers of CTCs in thawed DLA products were normalized to CTC numbers in fresh samples. Morphology of nuclei and cytoplasm was analyzed, and the cells were grouped into four categories (**Supplemental Figure 4**). [B] Genomes of single tumor cells – either spiked KYSE30 cells or patient CTCs – isolated from fresh and cryopreserved DLA products were amplified by WGA. GII was determined by Ampli1™ QC. *P*-values were determined by two-

tailed *t*-test. No significant difference was observed in GII between the fresh and cryopreserved tumor cells. In all cases, very few numbers of 0-band samples were obtained most likely due to cell loss during the single cell isolation process and are, therefore, not shown. [C] CTCs enriched with the CellSearch® Profile Kit from thawed DLA product were stained by immunofluorescence analysis for caspase-cleaved CK to determine apoptosis. CTCs were grouped into three "apoptotic categories" according to the detected nuclear staining and caspase-cleaved CK pattern (**Supplemental Figure 5**).

Figure 5: Cultured CTCs maintain their genotype and phenotype.

[A] Single uncultured CTCs from sample #3 were isolated by micromanipulation. Genomic DNA was amplified by WGA, and chromosomal aberrations were detected by aCGH analysis. [B] ER expression was determined by CellSearch® analysis. Representative pictures of an ER-positive and -negative CTC are shown. [C] Ratio of ER-positive cells was analyzed over four weeks of culture. [D] HER2 expression was determined by CellSearch® analysis. Representative pictures of HER2-positive and HER2-negative CTCs are shown. [E] Ratio of HER2-positive cells was analyzed over four weeks of culture.



508

509 **Figure 1**

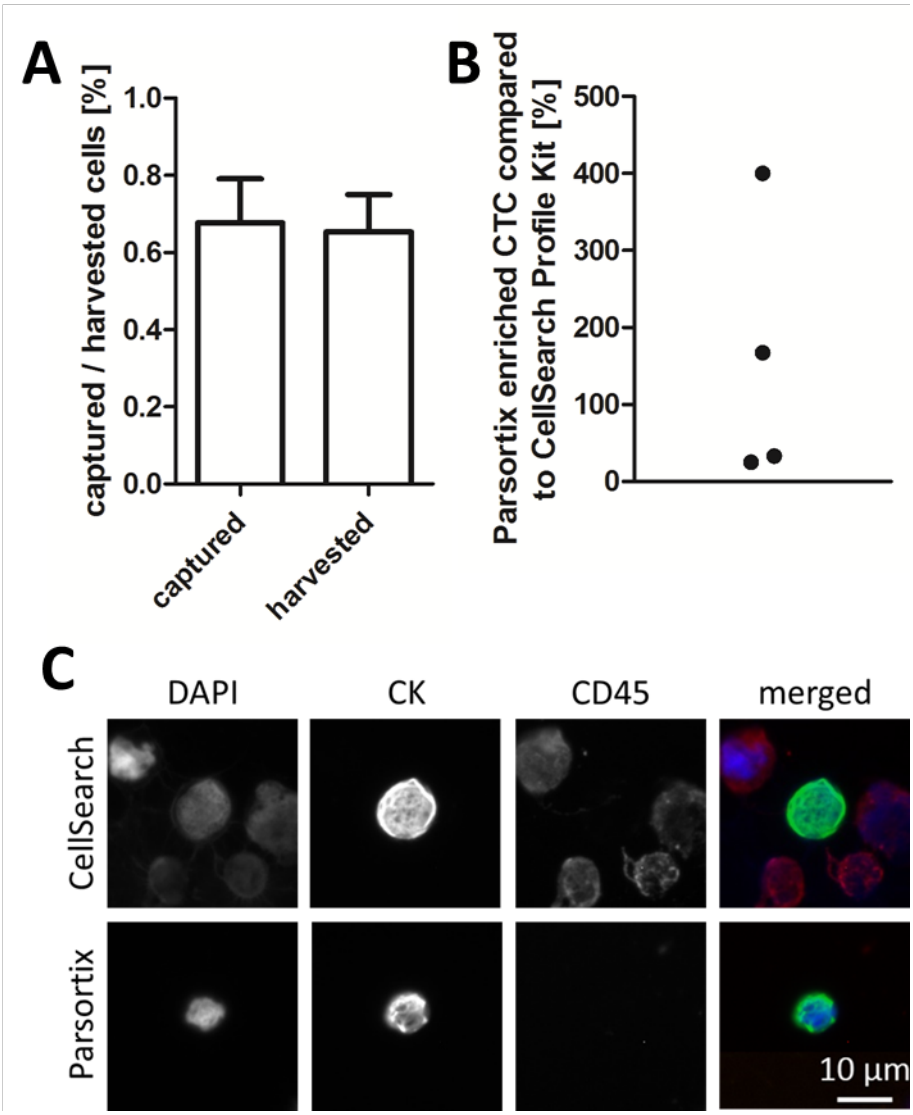

A
captured / harvested cells [%]
1.0
0.8
0.6
0.4
0.2
0.0
captured
harvested
B
Parsortix enriched CTC compared to CellSearch Profile Kit [%]
500
400
300
200
100
0
C
DAPI
CK
CD45
merged
CellSearch
Parsortix
10 µm

Figure 2

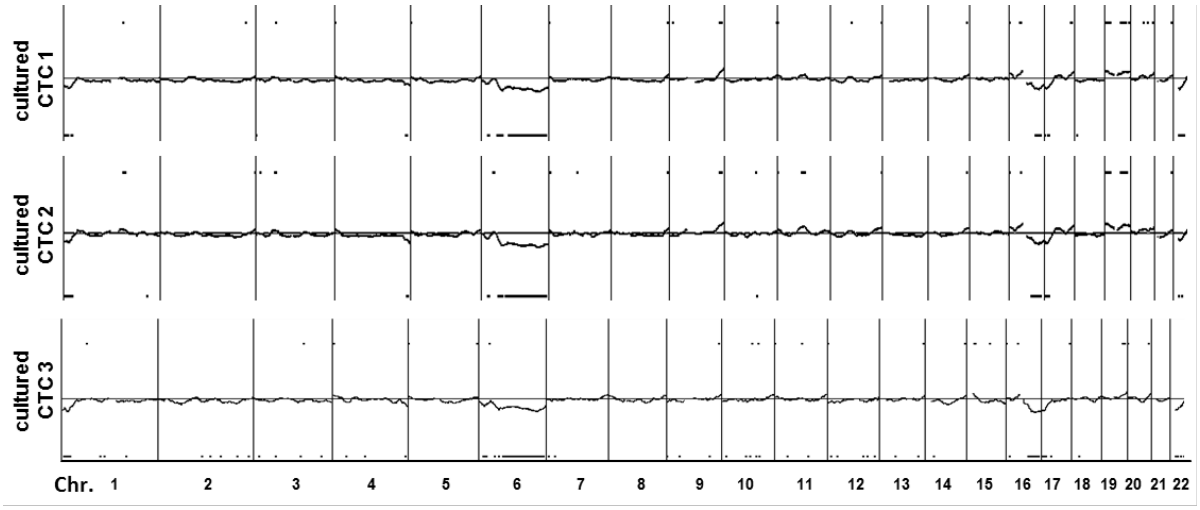
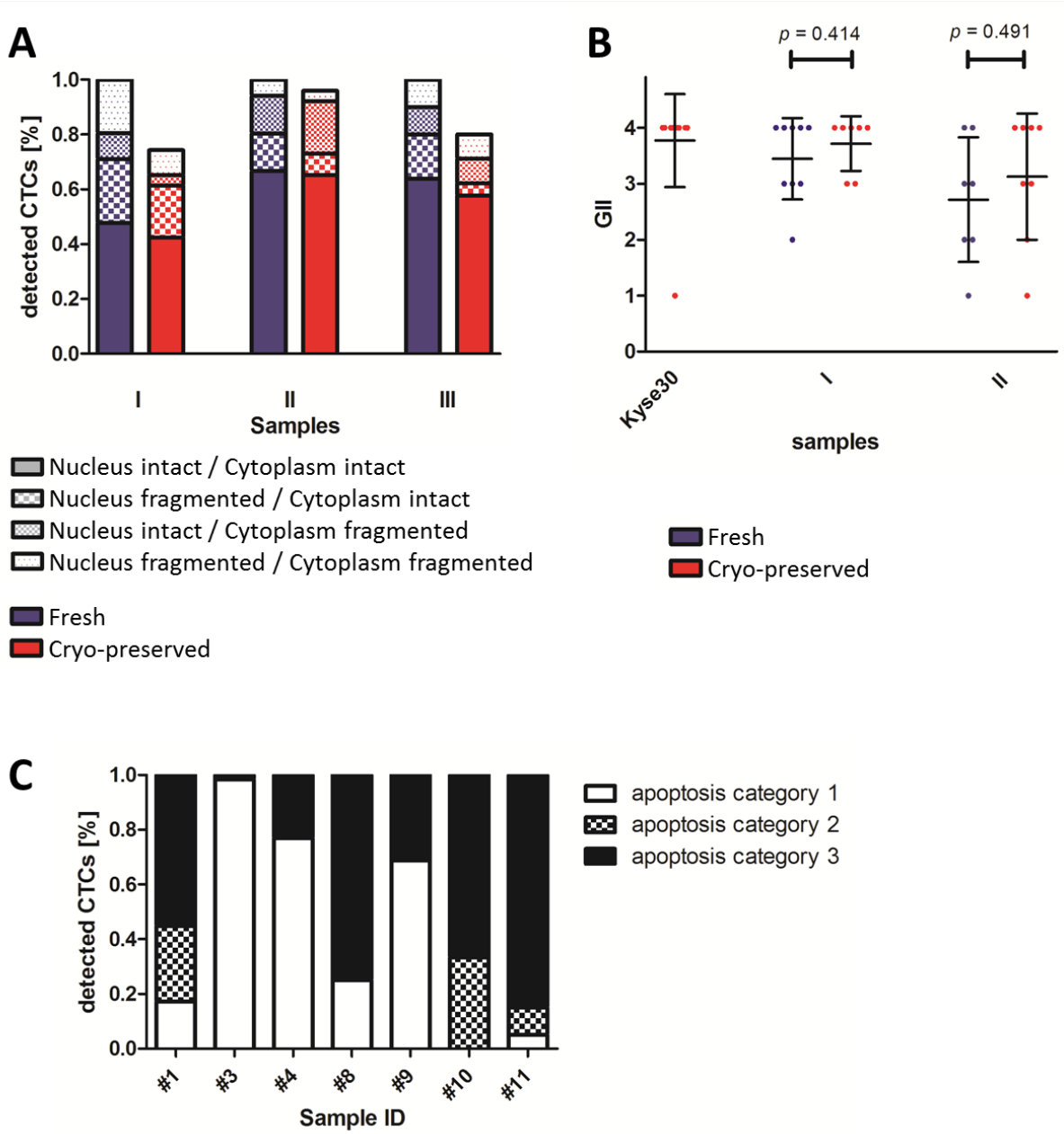

cultured CTC 1
cultured CTC 2
cultured CTC 3
Chr. 1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20 21 22

Figure 3



514

515 **Figure 4**

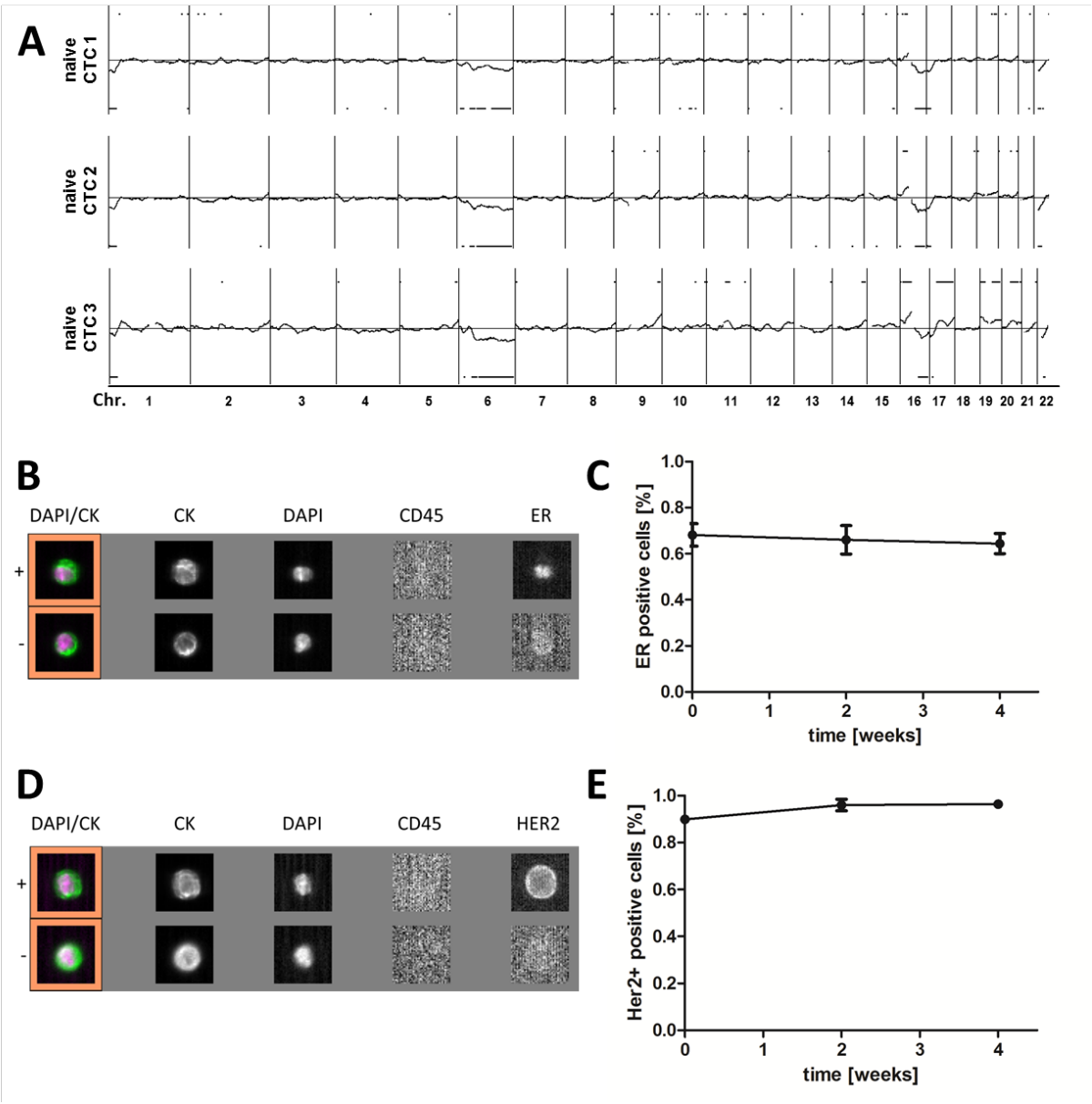

A
naive CTC1
naive CTC2
naive CTC3
Chr. 1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20 21 22
B
DAPI/CK
CK
DAPI
CD45
ER
+
-
C
ER positive cells [%]
1.0
0.8
0.6
0.4
0.2
0.0
0 1 2 3 4
time [weeks]
D
DAPI/CK
CK
DAPI
CD45
HER2
+
-
E
Her2+ positive cells [%]
1.0
0.8
0.6
0.4
0.2
0.0
0 1 2 3 4
time [weeks]

Figure 5

Supplemental methods

Cell lines and culture

The cell lines MCF-7 and MDA-MB 231 were purchased from the American Type Culture Collection (ATCC, Manassas, USA; catalog numbers: MCF7: HTB-22™ and MDA-MB 231: HTB-26™). The cell line KYSE30 was obtained from the German Collection of Microorganisms and Cell Cultures (DSMZ GmbH; catalog number ACC-351). The cell line GM14667 was purchased from Coriell Institute. The MCF-7 cells were cultured in RPMI 1640 containing 10% fetal calf serum, 25 mmol/L HEPES and 1% Penicillin-Streptomycin (all Thermo Fisher Scientific). Cells were grown at 37°C in a humidified atmosphere with 5% CO_2, were authenticated via short tandem repeat analysis, and regularly tested negative for *Mycoplasma*.

Enumeration of CTCs in PB and DLA products

CTCs were enriched and enumerated using the CellSearch® Circulating Epithelial Cell Kit (Menarini) according to the manufacturer's instructions. Briefly, 10 ml PB was collected into CellSave™ Preservative Tubes (Menarini) and processed within 96 hours. 7.5 ml PB was used for the enumeration of CTCs. Enrichment is based on immunomagnetic ferrofluid conjugated to an EpCAM-directed antibody. Subsequent characterization of the CTCs is performed using immunofluorescent staining directed against CK to identify CTCs, CD45 to exclude leucocytes and DAPI to confirm nucleo-morphological integrity (1). DLA products were processed as previously described as controls, and 2×10^8 MNCs per sample were processed (2).

Usage of the CellSearch® CXC Kit allows the detection of the additional markers ER and HER2, which are expressed at a low antigen density.

Enrichment of EpCAM-positive CTCs without automated subsequent immunofluorescent characterization was performed using the CellSearch® Epithelial Cell Profile Kit (Menarini) according to the manufacturer's instructions. DLA products were performed as controls (2), and 10^8 MNCs were processed per sample. CTCs were enriched in a total volume of approximately 900 µl and spun onto glass slides (Hettich Group) for subsequent characterization.

Immunofluorescence analysis

CTCs were identified by immunofluorescence analysis. The samples were fixed with CellSave™ reagent, permeabilized with 0.1% Triton X-100 (Merck, Darmstadt, Germany), and stained for nucleic acid (DAPI; F. Hoffmann-La Roche AG), CK (clone C11, Alexa Fluor® 488 conjugated, Cat#: GTX11212, GeneTex Inc. or clones C11/AE1/AE3, TRITC conjugated, Cat#: CKALLRMB000S, Aczon S.r.l.), CD45 (clone 35-ZS, Alexa Fluor® 647 conjugated, Cat#: sc-1178 AF647, Santa Cruz Biotechnology) and EpCAM (clone VU1D9, Alexa Fluor® 555 conjugated, Cat#: 5488S, Cell Signaling Technology Inc.).

Apoptotic cells were identified by staining for caspase-cleaved CK (M30 Cytodeath, FITC conjugated, Cat#: 10800, VLVbio).

Isolation of single cells

Single CTCs were isolated from culture after staining in suspension or from CellSearch® cartridge by micromanipulation using CellCelector™ (ALS, Jena, Germany). This semiautomated micromanipulator consists of an inverted fluorescence microscope (CKX41, Olympus K.K.) with a CCD camera system (XM10-IR, Olympus) and a robotic arm with a vertical glass capillary of 30 µm in diameter. Isolation was performed as previously described (3).

Whole genome amplification

For the genomic analysis, chromosomal DNA of single isolated cells was amplified by WGA with Ampli1™ WGA Kit (Menarini) according to the manufacturer's protocol. The cells were lysed overnight, and DNA was fragmented for three hours.

Subsequently, DNA integrity was determined with Ampli1™ QC Kit (Menarini). Only highest quality WGA products displaying a genome integrity index (GII) of 4 amplicons in the QC-PCR were used for further analysis.

Array comparative genome hybridization

WGA products were processed for aCGH as previously described (4,5). 1 µg WGA product was processed for aCGH. As reference, the WGA product of a single GM14667 cell, a normal B-lymphocyte cell line derived from a healthy male human, was used. For data analysis, the output image files were imported, normalized and fluorescence ratios for each probe were determined using Feature Extraction software (Agilent Technologies Inc.; version 10.7.3.1, Protocol CGH_1105_Oct09). Data were visualized and analyzed with the Genomic Workbench 6.5.0.18 software by applying the ADM-2 algorithm with a threshold of 6.0. The centralization algorithm was set to a threshold of 4.0 with a bin size of 10. To identify the copy number alterations, an aberration filter with a minimum $\log_2$ ratio of ±0.3 and a minimum of 100 consecutive probes was set.

Analysis of cell viability and proliferation

Optimal enrichment conditions for viable tumor cells were determined by operating Parsortix™ with 50,000 MCF7 cells. Harvested cells were seeded in 96-well plates, and after 2 days, cell viability and proliferation was analyzed by MTT assay. Briefly, the cells were incubated in 0.25 mg/ml thiazolyl blue tetrazolium bromide (Sigma-Aldrich) for 3 hours. Formazan crystals were dissolved in DMSO, and absorption was measured at 550 nm. Cell viability scores were calculated under different conditions by normalizing the obtained values of the cells that were processed with Parsortix™ to the values of the control cells that were not processed with Parsortix™.

The influence of cryopreservation on cell viability was determined by staining with propidium iodide (1 µg/ml, Thermo Fisher Scientific). Ratio of propidium iodide positive cells was measured by flow cytometry (CyAn ADP, Beckman Coulter).

Library preparation and semiconductor sequencing

Amplicon library preparation was performed with the Ion AmpliSeq™ Library Kit v2.0 using approximately 10 ng of DNA. Briefly, the DNA was mixed with the primer pool for the 315 amplicons and the AmpliSeq™ HiFi Master Mix prior to PCR with a BioRad Inc. PCR cycler. Subsequently, primer end sequences were partially digested using FuPa reagent, followed by the ligation of barcoded sequencing adapters (Ion Xpress Barcode Adapters, Thermo Fisher Scientific). The final library was purified using AMPure® XP magnetic beads (Beckman

Coulter) and quantified using qPCR (Ion Library Quantitation Kit, Thermo Fisher Scientific) on a StepOne qPCR machine (Thermo Fisher Scientific). The individual libraries were diluted to a final concentration of 100 pmol/L and processed to library amplification on Ion Spheres using Ion PGM™ Template OT2 200 Kit (Thermo Fisher Scientific). Unenriched libraries were quality-controlled using Ion Sphere quality control measurement on a QuBit instrument (Thermo Fisher Scientific). After library enrichment (Ion OneTouch ES), the library was processed for sequencing using the Ion Torrent 200 bp sequencing v2 chemistry and the barcoded libraries were loaded onto a chip. Our way of pooling eight samples on a 318v2 chip resulted in a mean coverage of 3000-fold per amplicon.

Short tandem repeat analysis

Short tandem repeat analysis was routinely performed regularly to confirm patient origin of the cultured CTCs and exclude contamination of WGA products, respectively. To that aim the Ampli1™ STR Kit (Menarini) was used according to the manufacturer's protocol.

1. Allard WJ, Matera J, Miller MC, Repollet M, Connelly MC, Rao C, et al. Tumor cells circulate in the peripheral blood of all major carcinomas but not in healthy subjects or patients with nonmalignant diseases. Clin Cancer Res 2005;10:6897–904.

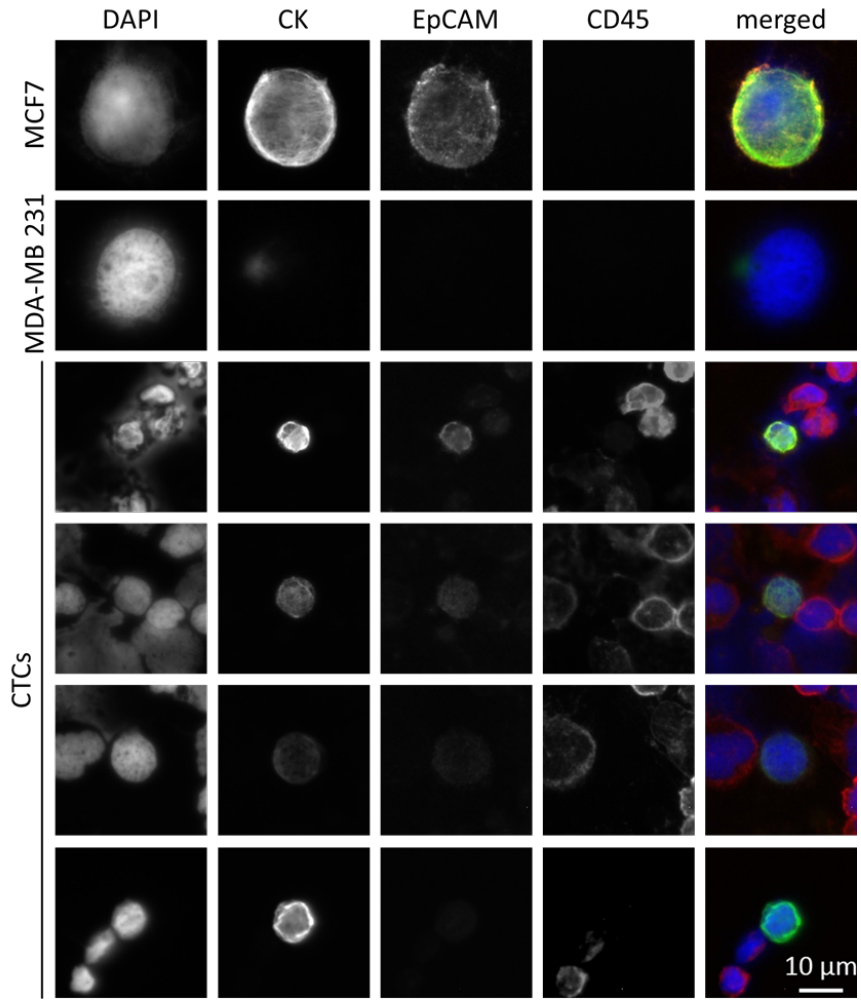
2. Fischer JC, Niederacher D, Topp SA, Honisch E, Schumacher S, Schmitz N, et al. Diagnostic leukapheresis enables reliable detection of circulating tumor cells of nonmetastatic cancer patients. Proc Natl Acad Sci 2013;110:16580–5.

3. Neumann MHD, Schneck H, Decker Y, Schömer S, Franken A, Endris V, et al. Isolation and characterization of circulating tumor cells using a novel workflow combining the CellSearch® system and the CellCelector™. Biotechnol Prog 2017;33:125–32.

4. Möhlendick B, Bartenhagen C, Behrens B, Honisch E, Raba K, Knoefel WT, et al. A Robust method to analyze copy number alterations of less than 100 kb in single cells using oligonucleotide array CGH. PLoS One 2013;8.

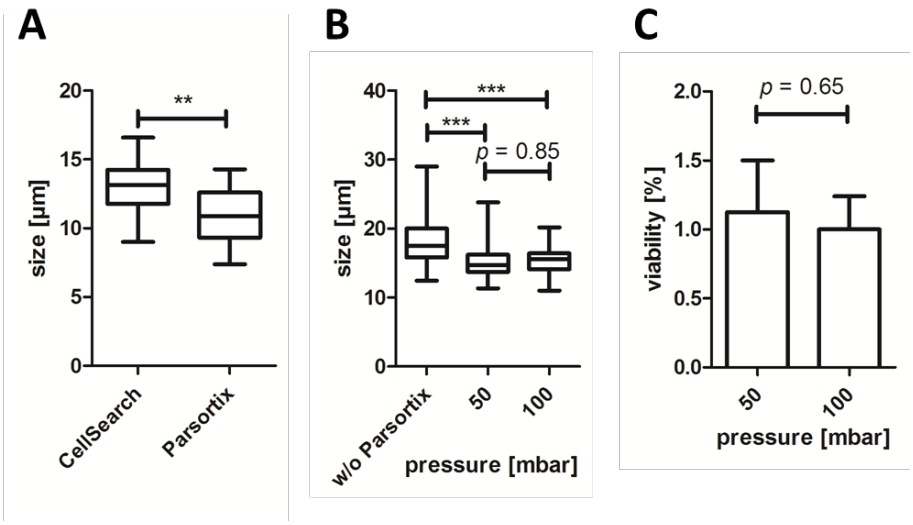
5. Neves RPL, Raba K, Schmidt O, Honisch E, Meier-Stiegen F, Behrens B, et al. Genomic high-resolution profiling of single CKpos/CD45neg flow-sorting purified circulating tumor cells from patients with metastatic breast cancer. Clin Chem 2014;1297.

Supplemental Figures



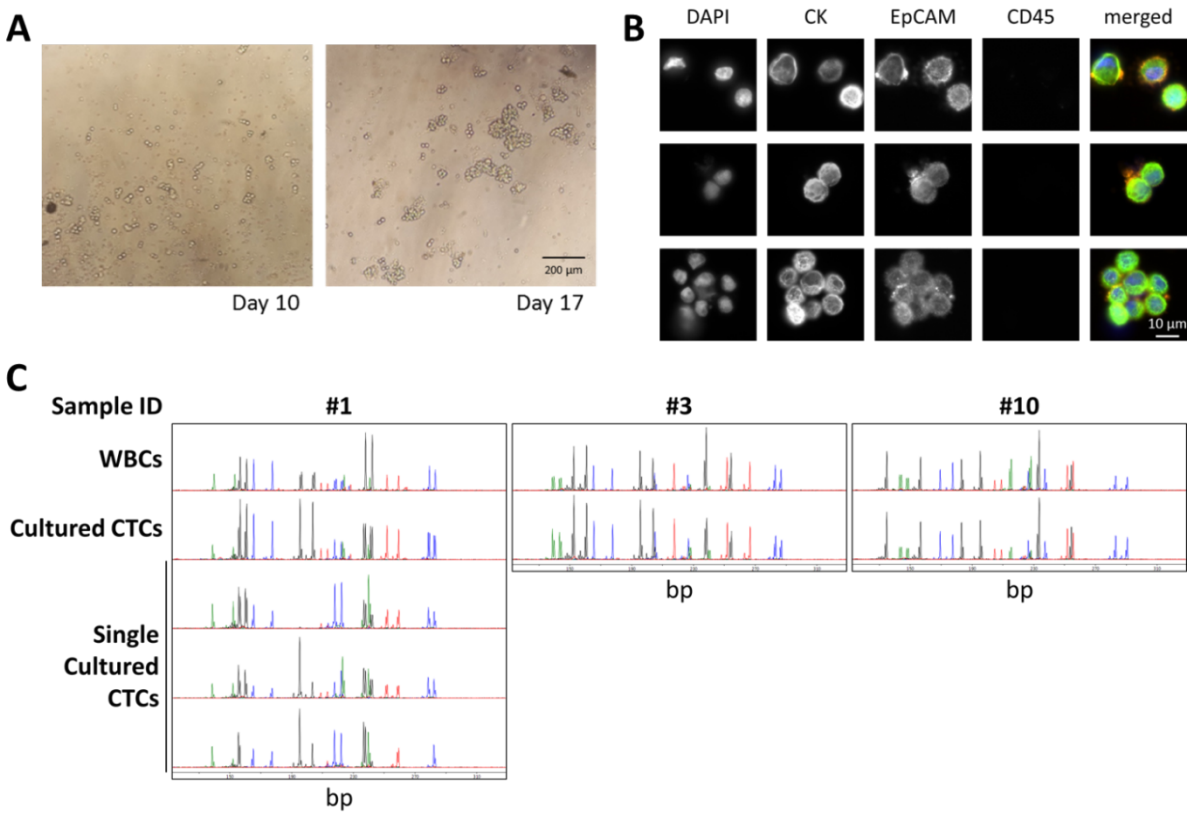
Supplemental Figure 1: Parsortix™ system-enriched CTCs

Parsortix™-enriched CTCs were stained by immunofluorescence analysis of CK, EpCAM and CD45 and for nuclear staining with DAPI. EpCAM expression ranged from high, i.e., comparable to the EpCAM-positive cell line MCF7, to low, i.e., comparable to the EpCAM-negative cell line MDA-MB 231. A magnification of 40x was used.



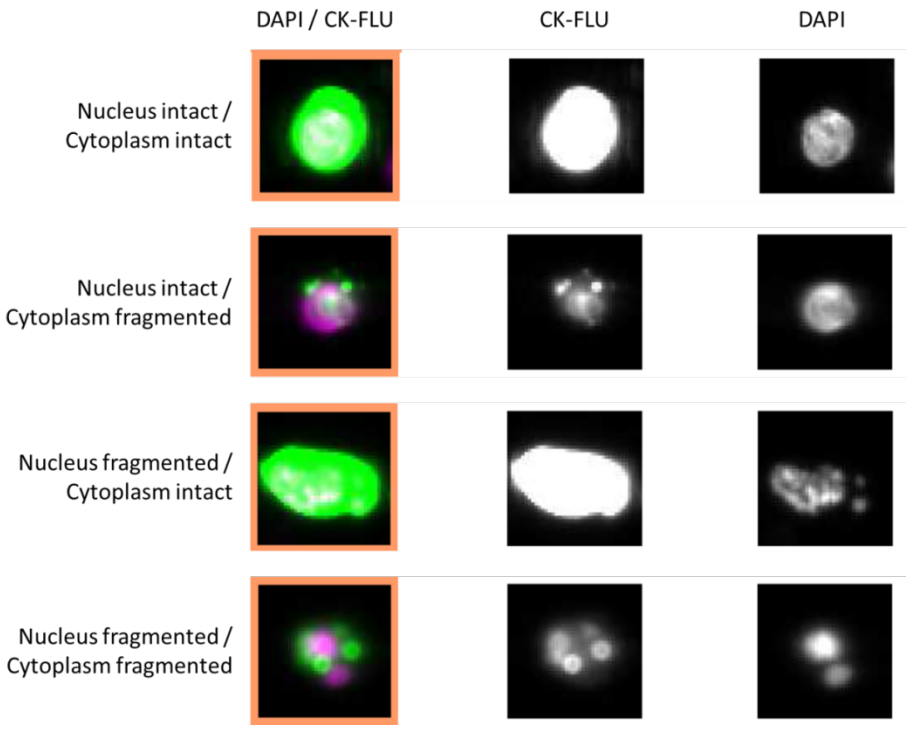
Supplemental Figure 2: Effects of different Parsortix™ protocols

[A] Sizes of CTCs enriched with CellSearch® and Parsortix™ system were measured. A *p*-value of 0.0015 was determined by two-tailed *t*-test. [B] The size of MCF7 cells captured with different Parsortix™ protocols was determined and compared to the size of cells without Parsortix™ enrichment. The *P*-values <0.001 were determined by two-tailed *t*-test. No significant difference was observed between a pressure of 50 and 100 mbar. [C] Effect of Parsortix™ enrichment with different pressures was determined using MCF7 cells. Harvested cells were grown for three days after harvesting and compared to cells without Parsortix™ enrichment by MTT assay. Obtained values are shown relative to viability after enrichment with 100 mbar. The *P*-value was determined by two-tailed *t*-test. No significant difference was observed.



Supplemental Figure 3: Cultured Parsortix™-enriched CTCs

[A] Parsortix™-enriched CTCs start to expand in clusters. A magnification of 10x was used. [B] Cultured CTCs were identified by immunofluorescence analysis of CK, EpCAM and CD45 and nuclear staining using DAPI. A magnification of 40x was used. [C] The origin of cultured CTCs and amplified genomes of single cells was confirmed by short tandem repeat analysis. The following targets were analyzed: D21S1435 (Fam, 150-185 bp range), D21S11 (Fam, 210-245 bp range), HPRT (Fam 260-300 bp range), SRY (Fam 318 bp), D5S818 (Vic, 135-190 bp range), D12S391 (Vic, 210-260 bp range), D18S535 (Ned, 120-165 bp range), D13S137 (Ned, 170-215 bp range), D21S2039 (Ned, 225-260 bp range), D13S631 (Pet, 200-220 bp range), and D21S1442 (Pet 230-290 bp range). Dyes are shown in the following colors: Fam, blue; Vic, green; Ned, black; and Pet, red.

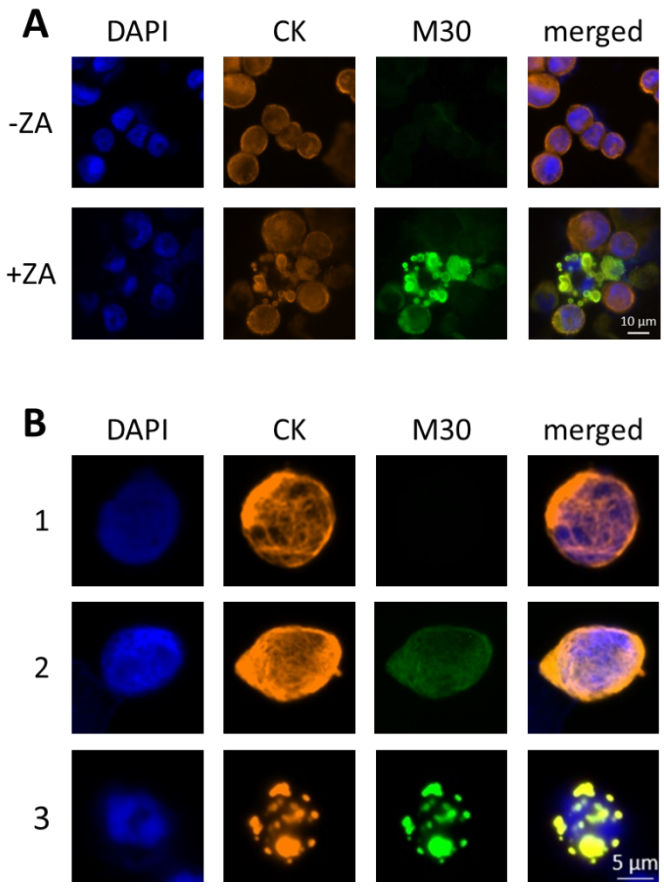


669

670 **Supplemental Figure 4: CTCs from cryopreserved DLA products**

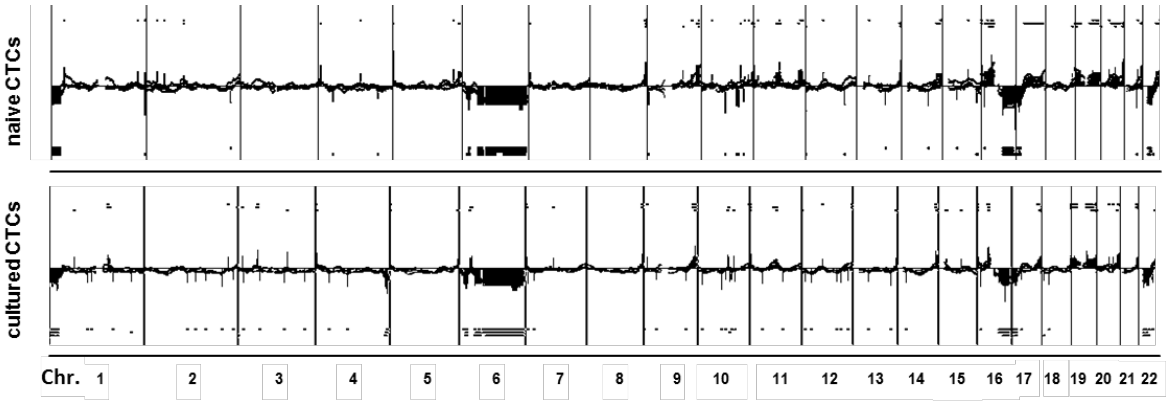
671 CellSearch®-enriched and -detected CTCs from fresh and cryopreserved DLA products were
672 classified into four groups according to their nuclear and cytoplasmic integrity as determined
673 by DAPI staining and immunofluorescence analysis of CKs.

674



Supplemental Figure 5: Analysis of apoptosis in CTCs

[A] MCF7 cells were treated with ZA for 72 hours. Cells were spun on glass slides and stained for caspase-cleaved CK (M30). [B] CTCs were identified by immunofluorescence analysis of CK and CD45 (not shown) and nuclear staining using DAPI. CTCs were classified into three categories by immunofluorescence analysis of caspase-cleaved CK (M30). Category one consisted of nonapoptotic CTCs and included cells without a M30 signal. Category 2 included CTCs with a detectable M30 signal but an unfragmented cytoplasm, and category 3 included CTCs with a detectable M30 signal and a fragmented cytoplasm. A magnification of 40x was used.



Supplemental Figure 6: Overlay of aCGH profiles

Supplemental Table 1: Clinical patient data

The following abbreviations are used: duc (ductal), lob (lobular), M (metastasis side(s)), Oss (bone metastasis), Vis (visceral metastasis), G (tumor grading), ER (estrogen receptor status), PR (progesterone receptor status), and AB (Antibody).

*No further information is available.

Patient ID	Sample IDs	Age at DLA	Type	M	G	ER	PR	HER2/neu	Chemotherapy	Radiation	Endocrine Therapy	AB Therapy
I	#9	52	Invasive-duc	OSS/Vis	2	+	-	-	+	+	+	-
II	#10	55	Invasive-duc	OSS/Vis	2	-	-	+	+	+	-	+
III	#12	71	Invasive-lob	OSS	2	+	+	-	-	-	+	
IV	#1, #11	50	Invasive *	OSS/Vis	3	+	+	-	+	-	+	-
V	#7	51	Invasive-duc	OSS/Vis	3	+	+	+	+	+	+	+
VI	#2, #5	48	Invasive-duc	Vis	2	+	+	+	+	-	+	+
VII	#8	51	Invasive-lob	OSS	2	+	+	-	-	+	+	-
VIII	#3, #4	74	Invasive-lob	OSS/Vis	2	+	+	-	+	+	+	-
IX	#6	72	Invasive-lob	OSS	2	+	+	-	+	+	+	-

Supplemental Table 2: Panel sequencing of whole genome-amplified DNA from single CTCs

Single uncultured and cultured CTCs from sample #3 were isolated by micromanipulation, and their genomic DNA was amplified by WGA. For the library preparation, the multiplex PCR-based Ion Torrent AmpliSeq™ technology (Life Technologies) with the Ampli1™ CHPCustom Beta panel was used. This panel was designed to target 315 amplicons covering mutations in the following 50 tumor suppressor genes and oncogenes: *ABL1*, *AKT1*, *ALK*, *APC*, *AR*, *ATM*, *BRAF*, *CDH1*, *CDKN2A*, *CSF1R*, *CTNNB1*, *DDR2*, *EGFR*, *ERBB2*, *ERBB4*, *EZH2*, *FBXW7*, *FGFR1*, *FGFR2*, *FGFR3*, *FLT3*, *GNA11*, *GNAS*, *HNF1A*, *IDH1*, *IDH2*, *JAK3*, *KDR*, *KIT*, *KRAS*, *MAP2K1*, *MET*, *MLH1*, *MPL*, *MYC*, *NRAS*, *NOTCH1*, *PDGFRA*, *PIK3CA*, *PTEN*, *PTPN11*, *SRC*, *SMAD4*, *SMARCB1*, *RB1*, *RET*, *STK11*, *SMO*, *TP53* and *VHL*. Mutation E17K in the *AKT1* gene was observed, and the frequency of the mutant allele is shown. During WGA of single cells, allelic dropouts can occur. Thus, the absence of alleles does not necessarily indicate that the allele was missing from the original cell.

Group	Cell-ID	*AKT1* E17K Mutant Allele Frequency
naive	CTC 1	16%
	CTC 2	100%
	CTC 3	no reads achieved
cultured	CTC 1	52%
	CTC 2	60%
	CTC 3	71%