Multiplex Molecular Analysis of CTCs

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Abstract

Beyond enumeration, CTC characterization is expected to help guide therapeutic selection for personalized care of cancer patients. Different approaches may be used to simultaneously identify multiple CTC-specific markers for biological characterization; yet awareness of associated pitfalls is also important. We have focused this chapter on molecular profiling of CTCs following enrichment. We describe the MagSweeper technology that was specifically developed to isolate live and highly purified CTCs for pooled or single cell or pooled cell molecular analyses or for CTC growth in vitro or in vivo. However, most of what is discussed will apply to any multiplex analysis of CTCs, irrespective of the enrichment method.

Contents

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1 Introduction

Personalized cancer care depends on providing therapy that will be of most benefit and of least toxicity to an individual patient during their disease process. Chemotherapy is given to eradicate systemically shed tumor cells that not only travel to, but also possess the capability of surviving and replicating in distant sites, such as bone, lung, liver, and brain. However, primary breast tumors are heterogeneous, containing tumor cell populations that may differ in metastatic potential, in mechanisms governing metastatic homing to different organs [1–4], and in sensitivity to different chemotherapeutic agents [5]. Among CTCs, which are tumor cells that have migrated into and survived shear forces present within the circulatory system, there are likely subsets of circulating cells responsible for seeding and reseeding metastases. Thus, biological characterization of CTCs should impact both our understanding of metastatic disease progression and our choice of pharmaceutical agents at different times in the course of disease.

With limited number of CTCs present in a tube of blood, one of the challenges in the field is to assess as much information from as little material as possible. Thus, multiplex analyses offer great opportunities for discovering CTC biology. Given the tiny amount of material, such an approach may require some kind of pre-amplification. And for this, special care must be taken to ensure that after such an amplification step, the material still reflects the original composition. Moreover, vast numbers of neighboring leukocytes (10^6 white blood cells, WBCs, in 1 ml of blood) can potentially contaminate samples and confound molecular assays, and must be taken into account during molecular analyses.

2 CTC Enrichment Using the MagSweeper Technology

The MagSweeper was developed by our multidisciplinary team at Stanford to provide highly purified live CTCs [6] suitable for pooled or single cell analyses and for in vitro or in vivo investigations [7]. In brief, patient blood is drawn into a 10cc EDTA-coated tube to prevent coagulation, then labelled with magnetic beads functionalized with the human BerEP4 monoclonal antibody to epithelial cell adhesion molecule (EpCAM, also designated as tumor-associated calcium signal transducer 1, TACSTD1). Although most of our initial work was done with magnetic beads coated with EpCAM antibodies, labelling CTCs in blood with magnetic beads attached to other CTC cell surface antigens is feasible. Magnetic rods covered by plastic sheaths sweep through the labelled blood at a specified speed, generating a shear force that captures labelled cells while partially

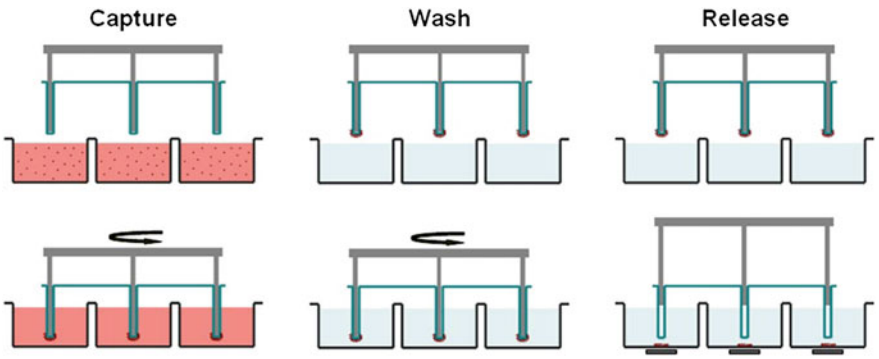

Fig. 1 Schematic showing the MagSweeper process of CTC capture, wash, and release using internal magnetic rods covered with plastic sheaths. The covered rods first sweep through a sample of immunomagnetically labelled unfractionated whole blood in concentric circular loops, then similarly sweep through a wash solution to remove loosely bound contaminating cells. In the capture solution, the magnetic rods are disengaged from their plastic sheaths and external magnets under the capture well facilitate release of CTCs and excess magnetic particles. An additional round of capture-wash-release eliminates the majority of remaining contaminant cells entrapped by excess magnetic particles. Permission from Proceedings National Academy of Sciences pending [6]

separating them from surrounding unlabeled leukocytes. The cells are then transferred to a washing well and finally released into a third well after the plastic sheath is disengaged from the magnetic rod (Fig. 1). A second capture/wash/release cycle produces highly purified cells that can be individually transferred via micromanipulation into a tube for single cell transcriptional analysis, mutation detection, or growth in culture.

3 Sensitive Nucleic Acid and Protein Isolation Techniques

If one aims to characterize multiple markers, it is imperative that all available material from the sample is isolated as purely as possible. This is even more important if it concerns isolation of material from just a few cells, as is often the case in the CTC field. Methods will therefore be preferable that allow separate analysis of genomic DNA, mRNA, microRNAs (miRNAs), and proteins.

New technologies with increased sensitivity are continuously being commercialized, so labs must remain up to date. But once an isolation method for the molecular compartment of interest has been chosen and lysates are used for downstream processing to characterize markers, either at the uniplex or multiplex level, it is sometimes difficult to switch to a different method. This is because any method will have inherent biases, either toward the nucleic acid or the protein side. Which procedure to follow may therefore depend upon the specific research question being studied.

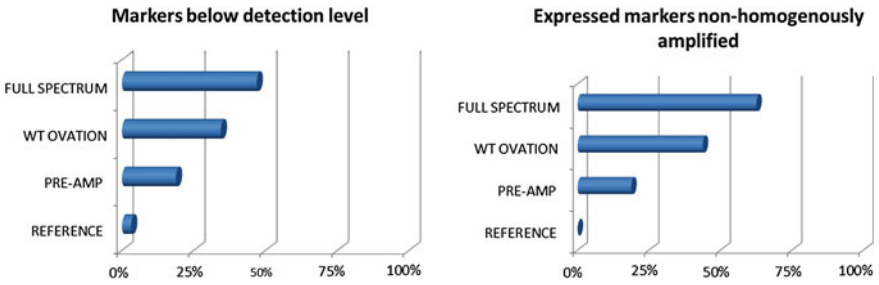

Fig. 2 Sensitivity and specificity of pre-amplification methods. Three different linear pre-amplification methods (TaqMan PreAmp from Applied BioSystems/Life technologies, suitable for multiplexing up to 100 gene expression targets; Whole Transcriptome Ovation RNA Amplification from NuGEN; Full spectrum RNA Amplification from System Bioscience) were utilized according to the manufacturer's instructions on RNA isolated from two epithelial breast cancer cells. The resulting pre-amplified cDNA preparations were analyzed by real-time PCR in a 20 µl reaction volume in an Mx3000P Real-Time PCR System (Agilent), and compared with expression levels measured in unamplified reference cDNA using TaqMan Gene Expression Assays in combination with TaqMan Universal PCR Master Mix No AmpErase UNG according to the manufacturer's instructions

4 Pre-amplification Methods

The next challenge is to find a method that will enable measurement of multiple markers in material isolated from as little as one cell in a linear and homogeneous (unbiased) manner. In view of the ultimate goal to characterize CTCs at the multiple marker level, any marker assay showing as a non-homogeneously amplified outlier in these tests cannot be used for further analysis because the data will not be truly representative of the original sample. Therefore, criteria require high sensitivity combined with a minimum number of non-homogeneously amplified marker assays.

An example of how to address this important issue is given in Fig. 2, which shows that, especially at the level of whole genome amplification techniques such as used by the WT Ovation and Full Spectrum RNA amplification methods, lack of sensitivity (Fig. 2, left panel), as well as homogeneous amplification (Fig. 2, right panel) might present a problem for certain markers [8].

Another challenge in any multiplex approach is to ensure that the multiplexing does not affect the efficiency and specificity of detection. One such example which forced us to change our strategy concerned the measurement of *hsa-miR*-22 located on chromosome 17 and which had attracted our attention based on its putative regulation of, amongst others, BCAR1 and ERBB3. For reasons still unknown, we were unable to get a quantification cycle (Cq) value for this particular microRNA if measured in a multiplex cDNA reaction that included 30 other microRNAs of our interest, while measurement of this particular microRNA

in a uniplex reaction resulted in very nice amplification curves with Cq values in the range of 20–30 and an efficiency close to 100%.

These data demonstrate that—besides reproducibility—reliability must be checked beforehand for each individual marker assay. For now it appears the chance of successful amplification is still higher for target-specific amplification methods such as the PreAmp method from Applied BioSystems/Life Technologies. The disadvantage is that these kinds of methods are restricted to a pre-selected set of markers. To include any new markers will require a new CTC enrichment and downstream processing procedure.

Fortunately, the field of single cell analysis is growing rapidly, and we therefore anticipate that unbiased whole genome amplification will soon—if not already is, such as for example, the PCR-based SMART^{TM} technology (Switching Mechanism At the 5′ end of RNA Transcript) from ClonTech—be possible at the one-cell level.

5 Estimating the Contribution by Remaining Leukocytes

Although most CTC enrichment systems allow capture of CTCs in cancer patient blood by selectively isolating, for example, EpCAM-positive cells followed by quantification of DAPI-and CK-8/18/19-positive cells, there may still be considerable quantities remaining of contaminating leukocytes (DAPI+/CD45+). For the MagSweeper device, contaminating leukocytes range between zero and one per CTC isolated [6]; however, other CTC systems may typically capture both CTCs and about 500–1,000 contaminating leukocytes after enrichment [8]. Thus leukocyte contamination, together with the fact that CTCs appear to occur in small numbers in humans (often fewer than five CTCs per 7.5 ml blood in metastatic breast cancer patients [9]), form pitfalls that cannot be ignored when one is interested in a multiplexed characterization specific for CTCs, no matter what system is used for CTC enrichment.

6 Selection of CTC-Specific Molecular Markers

Different approaches are possible to identify markers that are CTC specific. These include screening markers of interest against publicly available databases such as SAGE (http://cgap.nci.nih.gov/SAGE/AnatomicViewer). An example of this is shown in Table 1, where we illustrate the results of screening 20 putatively interesting gene markers for the expression levels measured in white blood cells, normal breast tissues, and breast cancer tissues. Based on the data presented in this table, one might decide to discard the lower eight markers based on their low expression in breast (cancer) tissue with probably no discrimination possible between leukocyte and breast tissue-derived expression levels. The upper eight markers on the other hand appear promising with levels differentially expressed between leukocytes (low) and breast cancer tissue (higher).

Table 1 Selection of CTC-specific markers

Gene Symbol	Database	Normal white blood cells [CD45+]	Normal breast	Breast cancer	Examples of differential range in breast subtypes	
KRT19	refseq_p5s				P1H12 DCIS	CD24+ META
MUC1	refseq_p5s				CD45+ IDC	CD24+ META
S100A16	refseq_p5s				CD24+ IDC	CD24+ META
CLDN3	refseq_p5s				CD24+ META	BerEP4+ DCIS
ERBB2	fl_p5s				CD45+ IDC	CD24+ IDC
SPDEF	mgc_p5s				CD45+ DCIS	CD24+ META
TACSTD1	refseq_p5s				CD24+ META	CD24+ IDC
AGR2	refseq_p5s				BerEP4+ IDC	CD24+ META
ESR1	refseq_sr				CD44+ META	CD24+ META
CCNE1	refseq_p5s				CD24+ META	CD45+ IDC
MELK	refseq_p5s				CD24+ META	CD44+ META
FGFR3	fl_p5s				CD45+ IDC	BerEP4+ IDC
FGFR4	refseq_p5s				CD45+ DCIS	BerEP4+ NB
PGR	refseq_sr				CD45+ IDC	BerEP4+ IDC
MKI67	fl_p5s				BerEP4+ IDC	CD24+ META
EGFR	fl_p5r				P1H12 DCIS	CD10+ DCIS
ERBB4	refseq_p5s				BerEP4+ IDC	BerEP4+ DCIS
FGFR2	fl_p5s				CD44+ META	CD24+ META
ALDH1A1	fl_p5s				CD44+ IDC	BerEP4+ NB
ALDH2	refseq_p5s				CD44+ META	BerEP4+ NB

Color Code										
Tags per 200,000	<2	<4	<8	<16	<32	<64	<128	<256	<512	>512

Despite these promising screening data for eight and possibly 12 out of the 20 markers, this unfortunately does not guarantee a successful CTC-specific multiplex analysis. For that, the actual levels need to be measured in a clinical cohort that consists of patients that presented themselves with and without CTCs, as well as a decent cohort of healthy blood donors without evidence of disease. Furthermore, it is at this stage important to screen these markers following well-defined standard operating protocols. Not only for the blood sampling, but also for the downstream processing and the performance of these markers in the actual multiplex protocol, which should then include all markers of interest.

Alternatively, one might consider skipping the in silico pre-screen and immediately perform analyses according to well-defined Standard Operating Procedures (SOPs) with all markers of interest included, and discarding markers that in the end do not fulfill the pre-defined criteria. But be aware that this approach still requires a sufficiently large enough control cohort and although more likely to identify additional markers, might in the end be the more expensive option.

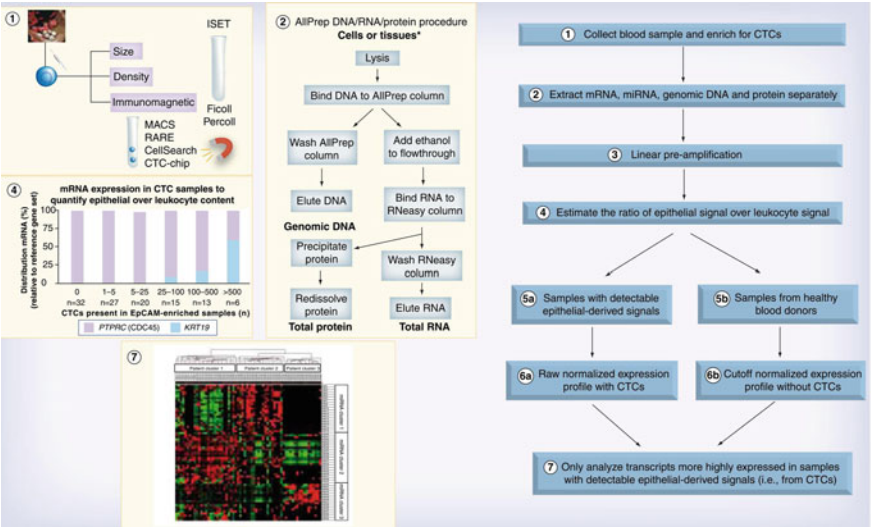

Fig. 3 Step-by-step schematic for reliable measurement of CTC-associated transcripts. After collecting blood samples in EDTA tubes to preserve RNA integrity and enrichment for CTCs (*1*), a sensitive isolation technique—preferably one that is able to isolate genomic DNA, mRNA, miRNA and protein in separate fractions (*2*, an example from qiagen.com)—and linear pre-amplification steps (*3*) are needed to enable detection of molecules in material from as little as one cell. Next, CTC- and leukocyte-specific signals are used to estimate the ratio of the tumor cell-specific signal over leukocyte-derived signal (*4*). Now, samples can be grouped into those with detectable epithelial-derived signals (*5a*) and those without detectable epithelial signals (*5b*), with the latter group comprising both patient samples without detectable epithelial signals and samples from healthy donors. Until consensus has been established on a robust reference miRNA set, normalizing on the mean expression of all expressed miRNAs in both groups (*6a* and *6b*) is probably the optimal method when multiple miRNA transcripts are measured at the same time. Finally, to ensure epithelial tumor cell-specific gene expression profiling of CTCs, levels measured in the samples without detectable epithelial signals (*6b*) are used as cut-off for the samples with detectable epithelial signals (*6a*) to calculate the remaining CTC-specific signals (*7*). Reproduced from Expert Review of Molecular Diagnostics 11(3), 259–275 (2011) [10] with permission of Expert Reviews Ltd

7 Data Analysis and Validation

In Fig. 3, we summarize the necessary steps to ensure epithelial tumor cell-specific transcript profiling of CTCs [10].

The data analysis may prove to be the most difficult part and greatly depends on the question being asked. If the interest is in identifying specific markers for use in monitoring prognosis or therapy response (i.e., if multiplex analysis was set up for screening purposes), a selection can be made from a subset of markers that were identified in the screening phase to be CTC specific and related to response. However, it is necessary that any such marker or set of markers be tested in an

independent cohort and according to a protocol that only includes the final selection of markers.

8 Examples of Multiplex and Other Molecular Analyses of CTCs

The amount of RNA in a single CTC is in the picogram range and currently insufficient for affordable and reproducible whole genome microarray analysis. Thus, we have been measuring the expression of up to 96 genes in single cells using a microfluidic qRT-PCR dynamic array from Fluidigm, finding different subpopulations of CTCs within one blood sample. Which genes are selected for expression measurement by the Fluidigm chip will impact the transcriptional profiles and the resulting phenotypic groupings of CTC subpopulations. Cells that express leukocyte-specific genes, such as *PTPRC* encoding for CD45, are eliminated from analyses.

We have also measured specific mutations—such as exon-specific *PIK3CA* mutations—in single CTCs isolated from cancer patient blood by the MagSweeper. We have found that within one tube of blood from a single blood draw, there may coexist diverse subpopulations of CTCs, some that are mutant or others that are wild-type. This is consistent with the findings of Dupont Jensen and colleagues [11] who have shown that within neighboring areas of an invasive breast cancer on a single hematoxylin and eosin slide were tumor cells with a *PIK3CA* exon 9 mutation, other tumor cells with a *PIK3CA* exon 20 mutation, and a third cluster of tumor cells that were wild-type. They also showed significant mutation discordance between primary tumors and distant metastases.

We have used the MagSweeper to isolate viable CTCs from a mouse xenograft model that contained profound regions of hypoxia and anoxia and produced lung metastases in all mice [7]. Using this model, we isolated CTCs from mouse blood and studied their response to hypoxia by growing the CTCs in culture under normoxic (room air, 21% oxygen) and hypoxic (<0.1% oxygen) conditions. We observed that CTCs had an altered response to hypoxia compared to parental tumor cells as well as distinctive expression and/or induction of anoxia-induced factors and target genes, including those involved in adaptation to nutrition deprivation (as might be present in tumor areas with poor blood supply) and the endoplasmic reticulum stress response. We observed that chronic hypoxia markedly increased colony formation in CTCs compared to parental tumor cells. Moreover, when CTCs were implanted into mouse mammary fat pads, the resulting CTC xenograft tumors showed a more aggressive phenotype, producing larger tumors that developed lung metastases twice as fast as xenografts generated from primary parental tumor cells. Together, the data showing that CTCs have a distinct response to hypoxia in vitro and greater aggression in vivo support the claim that CTCs have a different phenotype than the primary parental tumor cells from which they were derived. Our results suggest that CTCs may be selected for

Table 2A Examples of multiplexed analyses from the literature

Study	Enrichment method	Device name	Enrichment antigen	Molecular targets	Pre-amplification method	CTC-specific marker selection	Detection method	References
1	Flow Cytometry	FACSCalibur equipped with a second red-diode laser	CD45 + CD133 [negative selection], P1H12 and CD31 [positive selection for CEC/CEP]	CDH5, TEK, KDR, PROM1, ACTB	none	clinical relevance	flow cytometry, qRT-PCR	[18, 19]
2	Immuno-magnetically	CellSearch (Veridex)	EpCam	37 mRNA transcripts	WGA [MessageAmp Ambion]	cancer and CTC specific	qRT-PCR	[20]
3	Gradient density centrifugation + immuno-magnetically enrichment + culture of remaining viable single cells	EPISPOT assay	CD45 [negative selection] and CXCR4 + or -	MUC1, CATH-D (as secreted proteins), CXCR4 (for the sorting)	culture of viable cells	clinical relevance	colorimetric EPISPOT assay	[21]
4	Immuno-magnetically	?	EpCAM	PLAUR, ERBB2	none	clinical relevance	FISH	[22]
5	Micro-manipulation	N/A	none	whole genome	PCR-based global amplification	N/A	large-scale oligonucleotide array	[14]
6	Immuno-magnetically	in-house method with Dynabeads	EpCAM	KRT19, MUC1	none	CTC-specificity	RT-PCR	[23, 24]
7	None	N/A	N/A	KRT19, SCGB2A2, ERBB2, GAPDH	none	clinical relevance	real-time PCR, RT-PCR, IF	[25]
8	Immuno-magnetically	AdnaTest (AdnaGen)	EpCAM and MUC1	EpCAM, MUC1, ERBB2, ESR1, PGR, ACTB	none	clinical relevance	qRT-PCR	[26]

(continued)

Table 2A (continued)

Study	Enrichment method	Device name	Enrichment antigen	Molecular targets	Pre-amplification method	CTC-specific marker selection	Detection method	References
9	Immuno-magnetically	Microfluidic module (MIRACLE)	EpCAM and MUC1	20 mRNA transcripts	gene specific	literature-based and clinical relevance	electrochemical detection of MLPA fragments	[27]
10	Immuno-magnetically / CTC-chip	CellSearch (Veridex), and OncoCEE microchannel (Biocept Inc) and CELLective/On-Q-ity CTC-chips	EpCAM	12 mRNA and gene transcripts, KRAS mutation	yes, for KRAS	Subtype specific and clinical relevance	Taqman low density array (TLDA), IF, FISH, Taqman genotyping assay	[28]
11	Affinity-based	microfluidic "CTC-chip"	EpCAM	PSA, Ki-67, TMPRSS2-ERG fusion [negative marker: CD45)	WTA1	clinical relevance	CTCs: IF, RT-PCR, nucleotide sequencing	[29]
12	None	N/A	N/A	microRNA-10b, -34a, -141, -155 and-16	gene-specific	clinical relevance	qRT-PCR	[30]
13	Immuno-magnetically	CellSearch (Veridex)	EpCAM	55 mRNA and 10 microRNA transcripts	gene-specific	literature-based and clinical relevance	qRT-PCR	[31]
14	Gradient density centrifugation + immuno-magnetically	Ficoll-Paque PLUS (GE Healthcare BioSciences) + CELLection Epithelial Enrich (Invitrogen)	EpCAM	KRT19, ERBB2, MAGEA3, SCGB2A2, TWIST1, HMBS	none	clinical relevance	Liquid Bead Array Hybridization (Luminex)	[32]
15	Size filtration	ScreenCell MB	none	wild-type EGFR and mutations	gene-specific	clinical relevance	qRT-PCR, sequencing	[33]

(Continued)

Table 2B Examples of multiplexed analyses from the literature, (continued).

Study	Patient material	# Patients	mL blood	Negative controls**	Positive controls	Claimed sensitivity	Claimed specificity	Data analysis	Validation in independent cohort	References
1	cancer patients	84	100,000 cells/ blood sample	14 HBDs	cell lines	25 copies for CDH5	CDH5 solely in lympho-monocyte, not in granulocyte-like, cell population	standard calibration curve prepared from plasmids containing the respective target sequences relative to ACTB	no	[18, 19]
2	metastatic colorectal, prostate and breast cancer	30 31 13	7.5 mL	50 HBDs	74 metastatic cancer patients with at least 1 enumerated CTC [parallel study]	?	82%	standard calibration curve prepared from gene-specific RT-PCR products with known concentrations relative to RPS27A	no	[20]
3	metastatic breast cancer	22	10 mL	11 HBDs	cell lines	1 cell	100%	visual count of number of spots, defined by minimal size/area	no	[21]
4	advanced recurrent breast carcinoma	46	5 mL	leukocytes of same patient	cell lines	?	?	visual count over ref chromosome markers	no	[22]
5	none	0	?	?	cell lines	1 cell	N/A	GeneSpring software, normalized using Lowess and GAPDH	no	[14]
6	different stages of breast cancer	94	10 mL	28 patients with benign disease	cell line	?	100%	positive/negative at fixed threshold Cq	no	[23, 24]

(continued)

Table 2B (continued)

Study	Patient material	# Patients	mL blood	Negative controls**	Positive controls	Claimed sensitivity	Claimed specificity	Data analysis	Validation in independent cohort	References
7	stage I to III breast cancer	175	20 mL	31 HBDs	cell lines	1 CTC / 10^6 PMBC	97%	relative to MCF-7 equivalents/5 ug of total RNA in GAPDH positive cases	no	[25]
8	primary breast cancer	431	2 x 5 mL	106 HBDs [literature-based]	?	2 cells / mL blood	95%	evaluation of PCR fragments in actin-positive samples and use of cut-off concentration values [Agilent 2100 Bioanalyzer]	no	[26]
9	none	0	5–7.5 mL	none	(spiked) cell lines	2-5 MCF7 cells / 5 mL blood	?	standard calibration curve prepared from gene-specific RT-PCR products with known concentrations relative to ACTB	no	[27]
10	stage IV metastatic breast cancer and non-small-cell-lung cancer	38 BC [HER2] 34 LC [KRAS]	7.5 mL, extrapolated to 7.5 mL on CTC-chip	wild type DNA	cell lines	HER2 by IF: >3 CTCs KRAS Taqman genotyping assay: ≥10 mutant positive tumor cells Taqman gene expression (TLDA): ≥10 tumor cells	?	hierarchical clustering, visual count over ref chromosome markers, ratio mutant over wild-type	no	[28]
11	localized and metastatic prostate cancer	19 36	2–3 mL	17 HBDs and cell lines	cell lines	Detection rate in localized patients: 42%, in metastatic patients: 64%	100%	Automated image processing with subsequent visual evaluation	no	[29]
12	primary and metastatic breast cancer	89	400 uL serum	29 HBDs	cell lines	?	?	delta Cq over miR16	no	[30]

(continued)

Table 2B (continued)

Study	Patient material	# Patients	mL blood	Negative controls**	Positive controls	Claimed sensitivity	Claimed specificity	Data analysis	Validation in independent cohort	References
13	advanced recurrent breast carcinoma	50	7.5 mL	53 HBDs	cell lines	1 CTC / mL blood	93%	delta Cq ref genes and CTC-specific cut-offs	no	[31]
14	operable and metastatic breast cancer	84	20 mL	corresponding PMBC fractions and 17 HBDs	cell lines	1 cell	100%	fluorescence intensity per gene-specific bead in HMBS positive cases	no	[32]
15	none	0	3 mL	x? HBD	cell lines	2 cells /mL blood	~50 CD45+ events/mL blood	Cq values and ratio mutant over wild-type	no	[33]

by a combination of tumor hypoxia and nutritional deprivation and/or endoplasmic reticulum stress response [7]. Our study also shows that CTCs captured by the MagSweeper are indeed live cancer cells that produce metastasizing tumors in vivo.

9 Additional Examples of Multiplex Analysis of CTCs

In this last part of our whys, dos, and donts when setting up a multiplex molecular analysis for CTCs, we by no means intend to completely cover the large body of literature currently available on this subject. We have therefore decided to restrict the selection of articles to those published by the co-authors of this book. The examples depicted in Table 2 are merely to give an overview of how researchers started, learned, and with increased knowledge and novel equipment and molecular techniques available, have set out to get as much information as possible from CTCs.

10 Concluding Remarks

Tissues, and therefore CTCs, are rarely homogeneous. Therefore, any expression profile based on pooled CTCs will blend the true expression profiles of its constituent cells to identify the CTCs that are ultimately responsible for the development of a distant metastasis. Single cell analysis rather than analysis of cell populations may be more informative. Indeed, single cell methods have been developed for both microarrays [12–14] and, recently, RNA-Seq [15–17]. Although still too expensive and with questionable reproducibility for cost-effective and accurate clinical use, as whole genome single cell analysis technology develops further, these methods may become preferable for the analysis of small numbers of single cells, and may in particular be useful to study cells that are difficult to obtain in large numbers, such as CTCs.

While the field of multiplexed CTC analyses is now being explored extensively, validation and application to clinical context requires further study in independent clinical cohorts.

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