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Evaluating on-wire lysis of cells captured by a functionalized medical device using RT-qPCR

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Graz, October 2017

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Date

Signature

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1. Abstract

Prostate cancer (PCa) is the second most common cancer disease among men, with a median age of 66 at diagnosis. For early detection and, thus, an early start of cancer treatment, development and evaluation of meaningful biomarker are necessary. Circulating tumor cells (CTC's) have emerged as a new biomarker in cancer research as their number within a certain volume of peripheral blood is associated with poor prognosis for cancer patients. The golden standard for CTC enumeration is the CellSearch system, but it is limited by the rare number of CTC's in the volume of peripheral blood used for the analysis. The CellCollector 01 (DC01), a medical wire functionalized within antibodies, allows immune-based isolation of CTC's by its application directly in the patient circumventing bulk sampling. The DC01 is already tested for clinical applications in lung, breast and prostate cancer. However, enumeration of CTC's does not take advantage of the information the CTC's provide with their DNA and RNA, thus, cancer research recently focused on the molecular diagnosis of CTC's.

Here we optimized the bulk analysis of cells isolated with DC01 using different genes like *CK18* and *ARv7* in HT-29 and VCaP cell line cells. First, the protocol was evaluated under conditions which can occur in clinical applications (i.e. co-isolation of leukocytes and erythrocytes) and then on-wire lysis of cells attached to the DC01 was optimized using different lysis solutions and lysis protocols.

Our data show that co-isolated erythrocytes did not lead to an inhibition of the PCR, but increasing numbers of background cells [i.e. > 100 mononuclear cells (MNCs)] caused a decrease of yield in target specific PCR.

Concentrations of guanidine thiocyanate (GTC) higher than 0.08 M lead to an inhibition of the PCR, so dilution of the high concentration for cell lysis was necessary. The need for dilution limits the use of the GTC lysis application to samples containing highly expressed target genes. For the lowly expressed genes GTC-based lysis needs to be changed to the BSA/Triton lysis solution-based protocol.

To achieve the highest yield with the DC01 and BSA/Triton protocol we evaluated multiple conditions. The most effective procedure was:

Incubation of 20 µl of a 1000 cells/µl suspension on the DC01 for 10 min allowed sufficient cells to attach to the wire. The lysis was performed with 20 µl of the BSA/Triton lysis solution by freezing the solution directly in the Pasteur pipette at -80° over night. The next day the lysis solution was thawed and the DC01 twisted before being removed from the lysis solution. Then, the cell lysate was mixed by pipetting in and out and recovered from the Pasteur pipette. Analysis of the lysed samples was done with RT-

qPCR. Analysis of patient samples by means of on-wire lysis and subsequent RT-qPCR based on this protocol will allow its applicability for detecting and characterizing tumor specific cells, which is a much sought-after aim in personalized cancer treatment.

2. Zusammenfassung

Prostatakrebs ist die zweithäufigste Krebskrankheit bei Männern, das durchschnittliche Alter bei der Diagnose liegt bei 66 Jahren. Für eine frühe Detektion und somit einem frühen Behandlungsstart ist die Entdeckung und Evaluierung von Biomarkern notwendig. Zirkulierende Tumorzellen (CTC's) haben sich als neuer potentieller Biomarker in der Krebsforschung herausgetellt. Der Grund dafür ist, dass die Anzahl von CTC's im peripheren Blut mit der Diagnose von Krebspatienten zusammenhängt.

Das CellSearch System ist der „goldene Standard" für die Anreicherung von CTC's, aber es ist limitiert durch die geringe Anzahl an CTC's im peripheren Blut, welches für die Analyse verwendet wird. Der CellCollector 01 (DC01) ist ein mit Antikörper funktionalisierter, medizinischer Draht und erlaubt durch die direkte Anwendung im Patienten eine immunbasierte Isolierung der CTC's. Der DC01 wird bereits für Lungen-, Brust- und Prostatakrebs medizinisch angewendet. Durch die Anreicherung der CTC's erhält man keine näheren Informationen, welche in RNA und DNA der CTC's steckt, deshalb beschäftigt sich die Forschung immer mehr mit der molekularen Diagnose der zirkulierenden Tumorzellen.

Hier wurde die Bulkanalyse von Zellen, welche mit dem DC01 isoliert wurden, durch die Verwendung unterschiedlicher Gene, wie *CK18* und *Arv7* in HT-29 und VCaP Zellen optimiert. Als erstes wurde evaluiert welche Bedingungen in der klinischen Anwendung vorkommen können (co-isolierte Erythrozyten und Leukozyten) und dann wurde die direkte Lyse der Zellen am DC01 optimiert, wobei unterschiedliche Lyselösungen und Lyseprotokolle ausprobiert wurden.

Unsere Daten konnten zeigen, dass co-isolierte Erytrozyten nicht zu einer Inhibierung der PCR führen, jedoch eine erhöhte Anzahl an Zellen im Hintergrund [d.h. > 100 mononukleäre Zellen (MNCs)] zu einer Verringerung der Ausbeute in der PCR führen.

Weiters führt eine GTC Konzentration von 0.08 M zu einer Inhibierung der PCR, daher ist eine Verdünnung notwendig, bevor GTC für die Zelllyse verwendet werden kann.

Aufgrund dieser Verdünnung kann die GTC Lyselösung nur für Proben mit hoch exprimierten Markergenen verwendet werden. Für die schwach exprimierten Gene muss die BSA/Triton Lyselösung verwendet weden.

Um mit dem DC01 und dem BSA/Triton Protokoll die höchste Ausbeute zu erreichen wurden unterschiedliche Konditionen ausgetested.

Als effektivste Prozedur hat sich folgende herausgestellt:

Inkubation von 20 µl einer Zellsuspension (1000 Zellen/µl) am DC01 für 10 min, erlaubt dass sich die Zellen ausreichend am Draht anheften. Die Lyse wurde mit 20 µl der BSA/Triton Lyselösung durchgeführt und die Lösung direkt in der Pasteurpipette bei -80° über Nacht gefroren. Am nächsten Tag wurde die Lyselösung aufgetaut, der DC01 darin

gedreht und aus der Lyselösung entfernt. Dann wurde das Zelllysat durch Aus- und Einpipettieren gemischt und aus der Pasteurpipette geerntet. Die Analyse der lysierten Proben erfolgte mittels RT-qPCR. Die Verwendung dieses Protokolls für die Analyse von Patientenproben mittels direkter Lyse am Draht und anschließender RT-qPCR, macht die Detektion und Charakterisierung von tumorspezifischen Zellen möglich, was ein begehrtes Ziel in der personalisierten Krebstherapie ist.

3. Introduction

Prostate cancer (PCa) is the second most common cancer disease among men, with a median age of 66 at diagnosis [1]. At the early stage of the disease the symptoms are infrequent and with the growth of the tumor more symptoms appear. When the tumor has matastasized a complete cure is unlikely [2]. Therefore, an early detection is neccessary.

3.1. Therapy of prostate cancer

The standard therapy for prostate cancer treatment is the androgen deprivation therapy (ADT). Androgen hormones, such as testosterone, are necessary for the growth of prostate cancer cells. ADT leads to a reduction of androgen hormone levels, through medical or surgical castration [3]. It is successful in > 90% of patients, but some patients develop resistance to this therapy resulting castration-resistant PCa's (CRPC) [3]. This is mostly associated with a mutation, due to the androgen receptor (AR) is always active and can not be blocked through ADT [4,5]. Enzalutamide and abiraterone are used for the treatment of CRPC [6,7]. Enzalutamide inhibits the androgen receptor (AR) signaling through binding to the ligand-binding domain and displacing the natural ligand of the AR (such as testosterone) [7,8]. Abiraterone leads to a reduction of the adrenal and intratumoral androgen synthesis through inhibition of cytochrome P450 17A1 (CYP17A1) [9,10]. Some CRPC patients show also resistance during this treatment (using enzalutamide and abiraterone), this resistance is attributable to AR splice variants like the androgen receptor variant 7 (ARv7), which lost the C-terminal ligand-binding domain, but are constitutively active as transcription factors and ligand independent [7,11,12]. The resistance can be explained by this, that enzalutamide cannot bind on the ligand binding domain and abiraterone cannot inhibit the activity of the ARv7 [11]. Taxane therapy (chemotherapy) leads to an inhibition of the cell division and seems to be more effective for patients, which are ARv7 positive [13,14]. Galeterone is developed, as a potential drug to target ARv7, because it is an androgen receptor antagonist and a CYP17A1 inhibitor [15]. For men, who are ARv7 negative taxan chemotherapy and the AR-targeting drugs (abiraterone, enzalutamide) show the same results [14].

To know if a patient is expressing the ARv7 would be a huge benefit for cancer therapy.

3.2. Circulating tumor cells (CTC's) and the metastatic cascade

Circulating tumor cells (CTC's) were detected for the first time 1869 in cancer patients and are the connection between primary tumors and forming metastasis [16,17]. Up to 90% of cancer-associated deaths are caused by metastasis and not by the primary tumor [18]. To form metastases the cancer cells translocate from the primary tumor to a distant organ and colonize within that organ [17]. First of all the cancer cells within the primary

tumor transform into an invasive phenotype, transmigrate into the surrounding matrix and to the blood vessels, where they enter the bloodstream to distant organs (especially lung and liver). These cells are called circulating tumor cells (CTC's). When the CTC's reach the distant organ, they leave the bloodstream and enter the microenvironment of the foreign tissue, where they have to survive the innate immune response. At least they adapt to the microenvironment and start proliferation to form metastases (Figure 1, adapted from Chaffer *et al.* 2011 [17]) [17].

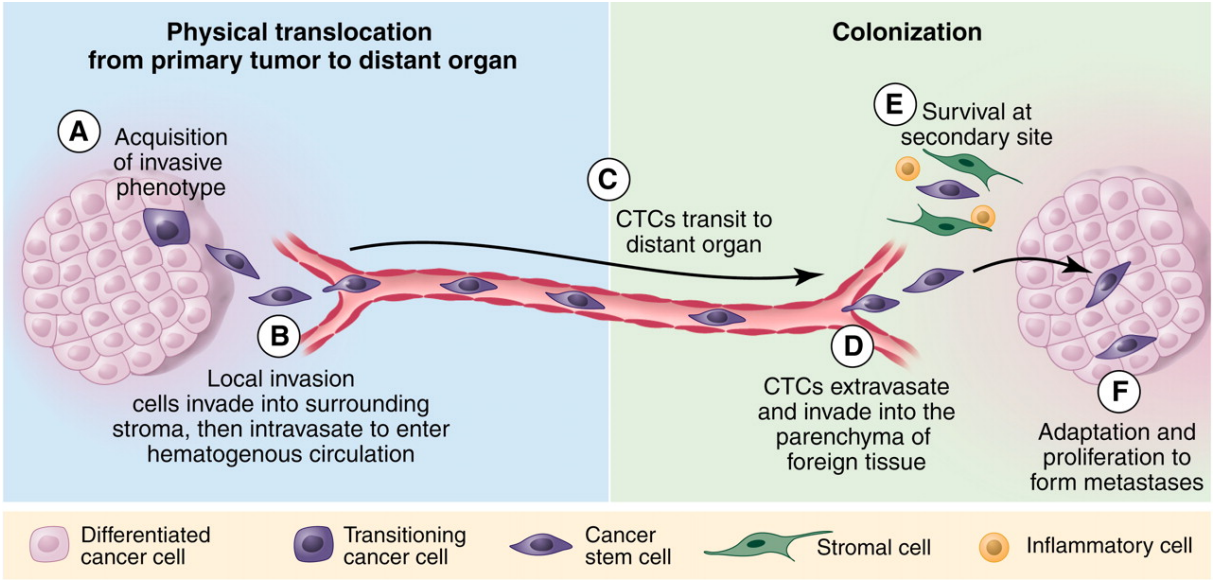

Figure 1: Physical translocation from the primary tumor to a distant organ.

CTC's have turned out as a new biomarker in cancer research. It is easy to isolate them from blood samples ("liquid biopsy"), so peripheral blood and CTC's might be the perfect source for cancer diagnosis [19]. "Liquid biopsy" with following molecular characterization has the potential to find out more about tumor metastasis, to discover novel drug targets and offer the possibility for a personalized cancer treatment [20].

3.3. CTC enrichment and detection

3.3.1. CellSearch system and CellCollector DC01

The problem of CTC detection is the low number of CTC's compared to the high number of blood cells [9]. Several methods [20,22–27] are already developed for the characterization of CTC's, mostly combined with enrichment procedures, like the use of epithelial markers. The epithelial cell adhesion molecule (EpCAM) is the most common antibody used to detect or isolate tumor cells [28]. The golden standard for the *ex vivo* CTC isolation is the semi-automated CellSearch system [20]. Peripheral blood (7.5 ml) is mixed with magnetic iron nanoparticles coated with the anti-EpCAM antibody to isolate EpCAM-positive cells. EpCAM-positive cells are labeled with an antibody cocktail against cytokeratin (CK) and CD45 allowing detection of epithelial cells and leukocytes, respectively. Additional the

fluorescent dye 4',6- diamidino-2-phenylindole (DAPI) is used to stain the nuclei. So the cells have to be EpCAM positive, cytokeratin positive, CD45 negative and DAPI positive to be identified as CTC's [29]. All *ex vivo* methods have one limitation in common, they have a low sensitivity, because of the small blood volume (7.5 ml) which is analysed [20]. To avoid this limitation new *in vivo* technologies are necessary and one ray of hope is the CellCollector 01 (DC01).

The DC01 is a 160 mm long stainless steel wire with a diameter of 0.5 mm. The functional part, the first 20 mm, is coated with a 2 µm thick gold layer and this gold layer is covered with a hydrogel layer consisting of a linear, synthetic polycarboxylate. The antibody (chiHEA 125, GILUPI) directed against EpCAM is attached to this hydrogel (Figure 2, adapted from Theil *et al.* 2016 [30]) [20].

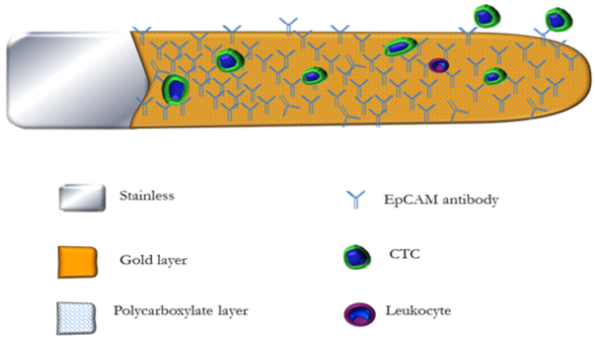

Figure 2: Structure of the DC01.

The DC01 is already used for *in vivo* application to isolate CTC's from peripheral blood of cancer patients. The DC01 is inserted into the cubital vein through a standard venous cannula and remains there for 30 min (Figure 3, adapted from Saucedo-Zeni *et al*. 2012 [20]). The most important thing is that the functional part is exposed to the blood flow. During this 30 min approximately 1500 ml of blood pass by [20].

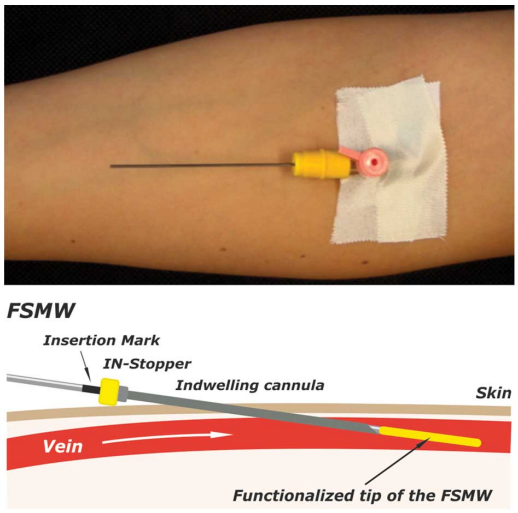

Figure 3: The DC01 inserted in the cubital vein, through a standard venous cannula.

3.3.2. CTC detection limitation - EMT

The DC01 and the CellSearch system are based on EpCAM enrichment and cytokeratin staining. The enrichment using EpCAM comes with bias because all CTC's, which undergo an epithelial-to-mesenchymal transition (EMT), cannot be isolated. During tumor progression the tumor cells can lose their epithelial characteristics and therefore it comes to a downregulation of the EpCAM and CK expression [28]. Cells, which undergo this process have a mesenchymal-like phenotype and enter the bloodstream, migrate to distant organs and form metastases (Figure 4 red, adapted from Pantel *et al*. 2010 [19]). When these disseminated tumor cells (DTCs) enter the distant organ they need to undergo a mesenchymal-to-epithelial transition (MET) to get back the epithelial characteristics, before they can form metastases. CTC's, which did not undergo EMT, can also circulate through the bloodstream, but cannot form metastases (Figure 4 blue, adapted from Pantel *et al*. 2010 [19]). This cells are detected with the different CTC isolation methods (using EpCAM) [19].

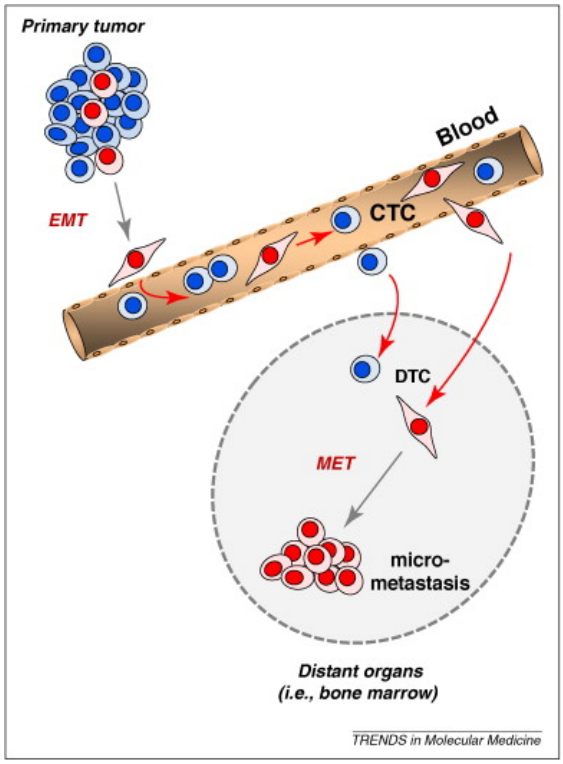

Figure 4: Epithelial-to-mesenchymal (EMT) and mesenchymal-to-epithelial transition (MET).

3.3.3. Comparison of the sensitivity of the CellSearch system and CellCollector (DC01)

The CellSearch system and the CellCollector 01 were compared in non-metastatic high-risk prostate carcinoma patients (Figure 5, data not published).

The study from Chen *et al.* shows that the performance of the DC01 reported twice as many patients to be CTC-positive as compared to the CellSearch system. Furthermore, the CellCollector detected higher numbers of CTC's per patient than the CellSearch system. This data shows that the DC01 has a higher sensitivity for detection in patients than the CellSearch system (data not published).

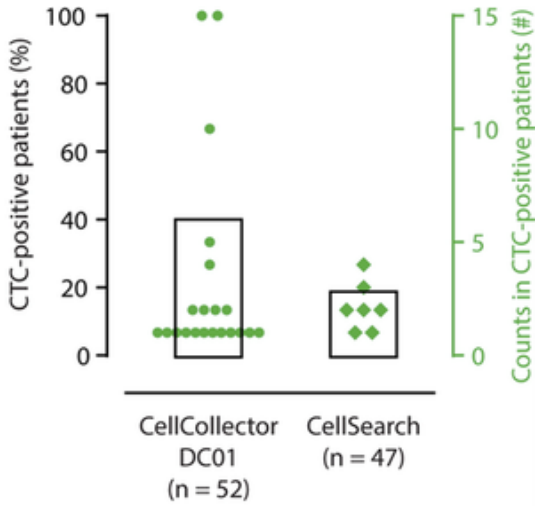

Figure 5: Comparison of the sensitivity from the DC01 and the CellSearch system.

The high sensitivity of the DC01 allows an *in vivo* application for the CTC isolation. After the CTC isolation it is possible to make a single cell (Catch&Release[31]) or a bulk analysis of all cells (direct on-wire lysis).

Chen *et al.* revealed first data for the direct lysis on the DC01. First BSA (1 mg/ml in H_2O) was used as direct lysis solution, but without a freeze/thaw cycle the hypotonic solution was not sufficient to break up the cells. After this knowledge guanidine thiocyanate (GTC; lytic effect) was used in combination with BSA and triton. Different concentrations were tested and so the GTC lysis solution was generated (0.3 M GTC, 0.5% Triton X-100 and 1 mg/ml BSA) (data not published).

The aim of this master thesis was to optimize the bulk analysis of cells isolated with the CellCollector 01, an *in vivo* enrichment device. First the protocol was evaluated under conditions, which can occur in clinical applications (co-isolated leukocytes and erythrocytes) and then the handling with the DC01 was optimized (using GTC lysis solution and BSA/Triton lysis solution with freeze/thaw cycles).

4. Material and Methods

Table 1: Material for cell culture

Material	Company
FBS	Gibco by Life Technologies
DMSO	Lactan
Accutase	Biowest – The Serum Specialist
1x PBS	Gibco by Life Technologies
Trypan blue	Sigma - Aldrich
McCoy's 5A Medium	Gibco by Life Technologies
DMEM (high glucose)	Gibco by Life Technologies
HEPES	PAA Labaratories GmbH
L-Glutamine	Gibco by Life Technologies
Penicillin/Streptomycin	PAA Laboratories

Table 2: Cell lines and used medium

Name	Cell line	Medium
HT-29	Human colorectal adenocarcinoma Cell line (ATCC)	McCoy supplemented with: 10% FCS 1% P/S L-Glutamine HEPES
VCaP	Human prostate cancer cell line	DMEM (high glucose) supplemented with: 10% FCS, 1% P/S

Table 3: Material for density gradient centrifugation

Material	Company
Lymphoprep	NYCOMED PHARMA AS
1x PBS	Gibco by Life Technologies
Blood	Institute of Hygiene, Microbiology and Environmental Medicine, Graz

Table 4: Material for RNA-Isolation

Material	Company
70% EtOH	AustrAlco
peqGOLD Total RNA Kit	peqLab – Biotochnologie GmbH

Table 5: Material for Reverse Transcription and Real-Time PCR

Material	Company
RNase Away	Lactan
TATAA GrandScript cDNA synthesis kit	TATAA Biocenter
TATAA Universal RNA spike I	TATAA Biocenter
dNTP (10 mM)	Sigma-Aldrich
Oligo-dT_{15}	Sigma-Aldrich
Random hexamers	Sigma-Aldrich
5x SuperScript II first-strand buffer	Thermo Fisher Scientific
DTT	Thermo Fisher Scientific
RNaseOUT Recombinant Ribonuclease Inhibitor	Thermo Fisher Scientific
SuperScript III Reverse Transcriptase	Thermo Fisher Scientific
TATAA SYBR GrandMaster Mix	TATAA Biocenter
AR (Human Single Assay), 100 rxn, Cat. nr.: qA-01-0364	TATAA Biocenter
ARv7 (Human Single Assay), 100 rxn, Cat. nr.: qA-01-0368	TATAA Biocenter

Table 6: Material for on wire lysis

Material	Company
GTC	Sigma-Aldrich
BSA (20 mg/ml)	Thermo Fisher Scientific
Triton X-100	Sigma-Aldrich
1x PBS	Gibco by Life Technologies
Distilled water	Gibco by Life Technologies
Hoechst 33342 (10 mg/ml)	Invitrogen

Table 7: PCR-Primer for Real-Timer PCR

Gene name	Primer sequence	Primer sequence (5'-3')
GAPDH	GAPDHv2_fwd	CCCACTCCTCCACCTTTGAC
GAPDH	GAPDHv2_rev	GCCAAATTCGTTGTCATACCAGG
RPS10	RPS10v2_fwd	AGCCGCAGAGATGTTGATG
RPS10	RPS10v2_rev	CCTCGGGACTTGAGAGACTG
EpCAM	EpCAM_fwd	CAGGAAGAATGTGTCTGTGAAAACT
EpCAM	EpCAM_rev	TTCATTTCTGCCTTCATCACC
CK18	CK18_fwd	GCTGGAAGATGGCGAGGACTTT
CK18	CK18_rev	TGGTCTCAGACACCACTTTGCC
CK8	CK8_fwd	GAGCCCCCTTGTCCT
CK8	CK8_rev	CCAGCATCTTGTTCTGC
CD45	CD45v1_fwd	CTTCAGTGGTCCCATTGTGGTG
CD45	CD45v1_rev	CCACTTTGTTCTCGGCTTCCAG
CD45	CD45v2_fwd	AACTCTTGGCATTTGGCTTTG
CD45	CD45v2_rev	GTGGAACACTGGGCATCTTT
CD45	CD45v3_fwd	CGAACTCTTGGCATTTGGCTTT
CD45	CD45v3_rev	CGTGGAACACTGGGCATCTTT

Table 8: Equipment

Equipment	Company
NanoDrop ND-1000 Spectrophotometer	peqLab – Biotochnologie GmbH
UV^3 HEPA workstation	Analytik Jena
MSC- Advantage – Safety bench	Thermo Scientific
Heracell 150i – CO_2 Incubator	Thermo Scientific
Waterbath	GFL
Mikroscope CH30	Olympus
Centrifuge (Hareus Megafuge 40R)	Thermo Scientific
Hettich Universal 32	DJB Labcare
Rotilabo-Mini-Centrifuge	Carl Roth
DNA Engine Dyad, Peltier Thermal Cyclers	Bio Rad
CFX96 Real-Time PCR Detection System	Bio Rad
CFX384 Real-Time PCR Detection System	Bio Rad
IKA MS1 Minishaker	Sigma Aldrich
Roller Mixer SRT6D	Stuart
UV Stratalinker 1800	Stratagene

Axio Observer Z1	Zeiss
Axiovert 200M	Zeiss
CellTram Vario	Eppendorf
Transfer Tip; diameter 20 µM	Eppendorf
„Neubauer“ counting chamber	Hecht-Assistent
Glass cover clip	Hecht-Assistent
50 ml centrifuge cubes	VWR
15 ml centrifuge cubes	VWR
2 ml Corning Cryogenetic vials	Sigma-Aldrich
Nunc™ Easy Flask, 25 cm^2	Thermo Scientific
Sempercare nitrile skin²	Sempermed®
Pasteur pipettes, 230 mm	Carl Roth
Pasteur pipette, 150 mm	Volac
5, 10, 25 ml serological pipette	Sigma Aldrich
Pipetus	Hirsch-Laborgeräte
Multi pipette	Eppendorf
Multichannel pipette	Eppendorf
DC01	Gilupi GmbH
1.5 ml reaction tubes	Eppendorf
0.2 ml reaction tubes	Eppendorf
Syringe	BD
Syringe Filter	Sigma-Aldrich
Corning gel loading pipette tips	Sigma-Aldrich
Micropipette tips	Eppendorf
Dualfilter tips	Eppendorf
FisherbrandTM Pipet Tips	Fisher Scientific
Combitips advanced	Eppendorf
Microseal ‚B’ Adhesive Seals	Bio-Rad
Absolut Seal	TATAA Biocenter
96 Well PCR Plate, colorless	Biozym
Hard Shell PCR Plates	Bio-Rad

Table 9: Used programs

Programs
ND-1000 V3.5.2 (Nanodrop)
BioRad CFX Manager
GenEx Version 6
Excel 2013
GraphPad – Prism 7
Inkscape
PalmRobo 4.5 (Axio Observer Z1)
ZEN 2 (blue edition; ZEISS) (Axio Observer Z1)
PalmRobo 3.0 (Axiovert M200)

Analysis based on Cq-values. Cq-values were converted into relative transcripts (using GenEx Version 6).

Table 10: Used statistical tests in GraphPad Prism

Statistical tests
Shapiro-Wilk normality test
Mann-Whitney test (non-parametric)
Unpaired t-test (parametric)

4.1. Cell culture

The HT-29 and the VCaP cell line were stored in liquid nitrogen. After thawing the cells, 2 x 10^4 cells/ml were seeded in a 25 cm^2 cell culture flask and cultivated in the incubator at 37°C, 5% CO_2 atmosphere and 95% humidity. The medium was changed 2-3 times per week. When the cells reached a confluency of 80-100% they were harvested with accutase, resuspended in 1x PBS and stored on ice/at 4° until further use. Cells not used in experiments were splitted for further culture in 25 cm^2 cell culture flasks. Occasionally, cells were harvested and stored in liquid nitrogen. The detailed protocols can be found at the end of this thesis (see apppenix 9.1. Cell culture)

4.2. Density gradient centrifugation

The density gradient centrifugation is a physical separation method based on sedimentation of cells in a density gradient. The density gradient solution (Lymphoprep: 1.119 g/ml) is covered with blood (diluted with 1x PBS). During centrifugation (800 x g without breaks for 30 min) the cells are separated based on their density. The ("polynucleated") granulocytes and the erythrocytes sediment through the solvent layer, because of the high density and the mononuclear cells remain at the interface of the solvent and blood plasma [32,33].

The buffy coat (mononuclear cells) and the pellet (erythrocytes and polynucleated cells) (Figure 6, adapted from Rubier *et al.* 2012 [34]) were harvested and transferred into separate tubes. The recovered cell suspensions were washed, diluted with 1x PBS and used for fluorescence activated cell sorting (FACS; for the exact protocol see appendix 9.4. Density gradient centrifugation)

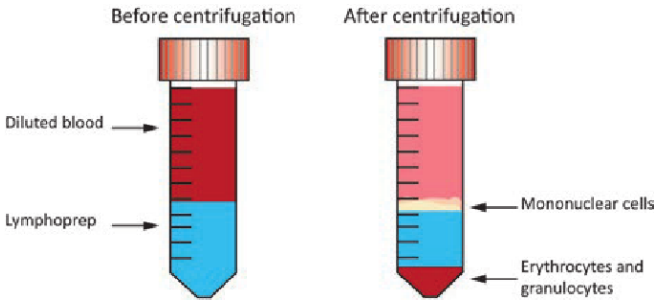

Figure 6: Schematic representation of a density gradient centrifugation.

4.3. Single-cell handling using FACS

The sorting was carried out at the Core Facility Imaging/Team Flow, Center of Medical Research (ZMF).

The preparations of the samples were done at the Institute of Cell Biology, Histology and Embryology (Medical University of Graz). Two direct lysis solutions (Guanidine thiocyanate and BSA/Triton lysis solution) were prepared according to the protocols given in appendix 9.3. Solutions. All wells of a 96-well plate were loaded with 5 ml of direct lysis solution and chilled on ice. Preloaded plates and cells isolated from whole blood and cell culture (see chapter 4.1. Cell Culture and 4.2. Density gradient centrifugation) were transferred to the labs of the FACS Core Facility. The cells were sorted into direct lysis solutions, transferred to dry ice and stored at -80° until forwarded to reverse transcription (see chapter 4.12. Reverse Transcription and Appendix 9.5. Single-cell handling using FACS).

4.4. Cytospin

Cytospin is an easy method to transfer cells by centrifugation onto a slide. For later use with lasermicrodissection (LMD) the cell suspensions need to be transferred onto membrane-coated slides. To increase the “stickiness” of the membrane and to remove RNases and DNases membrane-coated slides are treated with UV-light. Generally, 1 x 10^5 cells per 300 µl 1x PBS were cytocentrifuged at Rtemp onto 120 mm^2 at 300 x g for 5 min. After removing the supernatant, the slides were dry-spin at 1140 x g for 1 min and transferred to the microscope capable of LMD for isolating cells (see next chapter).

When cells were to be forwarded to micromanipulation, cytocentrifugation was done at 370 x g for 3 min omitting the dry-spin. After this cytocentrifugation step the cells were transferred to the micromanipulation unit for harvesting cells (see chapter 4.6. Micromanipulation and appendix 9.6. Cytospin).

4.5. Lasermicrodissection

Using lasermicrodissection, single cells can be isolated from membrane-coated slides. I therefore used the PALM system (Zeiss Observer Z1 equipped with a laser and a motorized stage). Single cells were microdissected and catapulted into 5 µl lysis solution (1% BSA/0.2% Triton) placed in the cap of a 0.2 ml PCR tube, which was situated directly above the sample. The recovering lysis solution was checked for the presence of microdissected cells (successful LMD). Immediately thereafter the PCR-tube was put on dry ice and then stored at -80°C until reverse transcription (see chapter 4.12. Reverse Transcription and appendix 9.7. Lasermicrodissection).

4.6. Micromanipulation

Micromanipulation is another method to isolate single cells from a cell suspension.

A glass capillary, which is attached to a manually controlled pneumatic drive is used to soak up cells from a medium. Harvested cells are washed in 1x PBS and soaked up again. 1 µl of the cell suspension were transferred into 5 µl direct lysis downstream analysis (see appendix 9.8. Micromanipulation).

4.7. Attaching cells to DC01

The HT-29 cells were attached to the DC01 we obtained from Gilupi. The functional part of the DC01 was incubated with the cell suspension (with the desired cell density) for 10 min in a Pasteur pipette (Figure 7), then removed and washed in 1x PBS until the cell lysis was performed (for the exact protocol see appendix 9.10. Attaching cells to DC01).

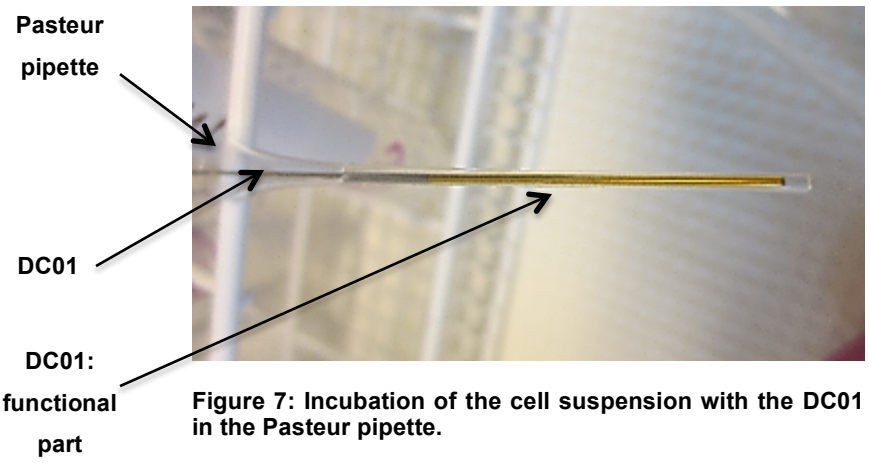

Figure 7: Incubation of the cell suspension with the DC01 in the Pasteur pipette.

4.8. Cell staining

HT-29 cells were stained using the fluorescent dye hoechst 33342 (0.001 mg/ml) was used, which dyes DNA. The cell pellet was resuspended with the fluorescent dye and incubated for 10 min at 37°C. After centrifugation at 300 x g, for 3 min and at Rtemp the cell pellet was diluted with 1x PBS to the desired cell density and placed on ice until further use (for the ptorocol see appendix 9.9. Cell staining).

4.9. Counting of the cells on the DC01

After the stained HT-29 cells were loaded on the DC01, they were counted under the fluorescence microscope (Observer Z1) using the DAPI filter (for the exact protocol see appendix 9.11. Counting of the cells on the DC01).

4.10. Cell lysis on the DC01

The cell lysis was performed with two different lysis solutions. One consists of 0.3 M GTC, 1 mg/ml BSA and 0.5% Triton X-100 whereas the other contained only 1% BSA and 0.2% Triton X-100. Different conditions were performed to optimize the direct cell lysis on the DC01. The detailed protocols can be found at the end of this thesis (see appendix 9.12. Lysis on the DC01 using GTC lysis solution, 9.13. Lysis on the DC01 using the BSA/Triton lysis solution with a subsequent freeze/thaw cycle, 9.14. Lysis on the DC01 using BSA/Triton – freeze over night, 9.15. Lysis on the DC01 using BSA/Triton an an RNA inhibitor – freeze over night).

4.11. RNA-Isolation and concentration specifications

Total RNA was isolated from harvested HT-29 and VCaP cell line cells and used as a positive control in RT-qPCR. The RNA isolation was performed using the peqGOLD Total RNA Kit according to the manufacturers's recommendations. The RNA concentration was determined with NanoDrop Spectrophotometer and then stored at -80°C until later use (see appendix 9.16. Nanodrop).

4.12. Reverse Transcription

For obtaining complement DNA (cDNA) total RNA was subjected to reverse transcription using the enzyme reverse transcriptase. Random hexamers and oligo-dTs (hybridized with poly-A) are used to synthesize cDNA. The cDNA is used as a template for the qPCR [35].

Different Kits were used for reverse transcription. Reverse transcription volume was 10 µl.

The TATAA Kit contains NF-H_2O, 5x TATAA GrandScript RT Reaction Mix and a 20x TATAA GrandScript RT Enzyme. Occasionally, 10^7 copies/µl TATAA Universal RNA Spike I were added to the reverse transcription mix.

The RT was performed in a thermal cycler at 22°C for 5 min, 42°C for 30 min, 85°C for 5 min and a hold at 4°C.

With the SuperScript III Kit additional a hybridisation step was performed. The hybridation mix contains NF-H_2O, 500 µM dNTPs, 2.5 µM oligo-dT_{15} and 2.5 µM random hexamers. The hybridisation was also performed in a thermal cycler at 65°C for 5 min and a hold at 4°C. After the hybridisation the RT master mix was added and RT was performed at 25°C for 5 min, 50°C for 60 min, 55°C for 15 min, 70°C for 15 min and a hold at 4°C. The cDNA was stored at -20°C until it was used for qPCR. The detailed protocols can be found at the end of this thesis [see appendix 9.17. Reverse Transcription using TATAA

Kit, 9.18. Reverse Transcription using TATAA Kit (RNA-spike in), 9.19. Reverse Transcription using SuperScript Kit].

4.13. Real-Time quantitative PCR with SYBR Green

The Real-Time or quantitative PCR (qPCR) is a method to monitore the amplification in real-time and to quantify the initial mRNA amount of the target gene. The fluorescence dye SYBR Green I intercalates into the DNA by binding into the minor groove of the double-stranded DNA. The higher the amount of DNA, the higher the fluorescence [36].

The qPCR master mix (sufficient for 6 µl and 10 µl rections volumes was also prepared in the PCR workstation. The components of the qPCR master mix are NF-H_2O, 10 µM assay mix and the ready-to-use 2x TATAA SYBR GrandMaster Mix. The ready-to-use TATAA SYBR GrandMaster Mix contains $MgCl_2$, dNTPs, Taq DNA polymerase, SYBR Green I dye and stabilisers. The qPCR was performed in a thermal cycler for 2 min at 95°C, followed by 50 cycles of 95°C for 5 sec, of 60°C for 20 sec and of 70°C for 20 sec. A melting curve was performed to check the quality of the PCR products [see appendix 9.20. Real Time Quantitative PCR (with undiluted cDNA), 9.21. Real Time Quantitative PCR (with diluted cDNA)].

5. Results

5.1. Evaluation of epithelial - (*CK18, EpCAM*) and leukocyte - specific (*CD45*) marker expression in blood and HT-29 cells

Initially, the expression of epithelial (*CK18, EpCAM*) and leukocyte-specific (*CD45*) transcripts in blood and HT-29 cells was evaluated. Replicates containing approximately 100 and 500 MNCs and HT-29 cell, respecticely, were lysed in 5 µl of 1 mg/ml BSA (5 biological replicates). Whole lysates were reverse transcribed and subsequently diluted with NF-H_2O (1:4). About 5% of the initial sample was analyzed by qPCR using *GAPDH, RPS10, CK18, CD45v1, CD45v2, CD45v3* and *EpCAM*.

All obtained Cq-values are listed in table 35 (see appendix 9.21. Raw data). PCR specific products with a Cq-value > 36 were set to 36 (representing 1 transcript). Negative qPCR reactions were set to Cq-values of 37 (Figure 8).

Expression of *GAPDH* and *RPS10* (reference genes) is generally higher in HT-29 cells (mean $Cq_{100\ cells,GAPDH}$ = 24.03, mean $Cq_{500\ cells,RPS10}$ = 24.09) than in MNCs (mean $Cq_{500\ cells,GAPDH}$ = 31.01, mean $Cq_{500\ cells,GAPDH}$ = 30.83). The epithelial markers *CK18* and *EpCAM* show a high expression in tumor cells (mean $Cq_{100\ cells,CK18}$ = 23.88, mean $Cq_{100\ cells,EpCAM}$ = 26.37) and are also found to be expressed in blood cells (mean $Cq_{100\ cells,CK18}$ = 38.26, $Cq_{100\ cells,EpCAM}$ = 33.62). As expected, the leukocyte marker *CD45* is expressed in blood cells (mean $Cq_{100\ cells,CD45v1}$ = 31.90) and, interestingly, also in one biological replicate of the 100 HT-29 cells albeit at low levels (Cq_{CD45v1} = 40.25) (Table 11).

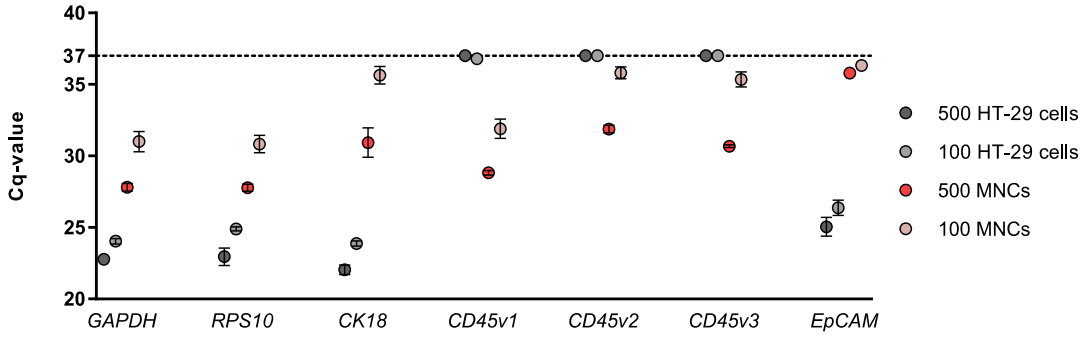

Figure 8: Evaluation of epithelial (*CK18, EpCAM*) and leukocytes specific (*CD45*) marker expression in blood and HT-29 cells. MNCs and HT-29 cells were lysed and the RNA was used for RTqPCR. Relative cDNA yields are presented as Cq-values. Data are shown as mean ± SD (n=5). Dashed line representing samples lacking transcripts. The epithelial markers (*CK18, EpCAM*) show a higher expression in the tumor cells (HT-29), as in the blood cells and *CD45* has been confirmed as a good marker for leukocytes.

Table 11: Mean of the Cq-value, standard diviation (SD) and the number of analysed replicates (n) of the expression analysis in HT-29 cells and MNCs. Missing data were excluded from analysis.

		500 HT-29	100 HT-29	500 MNCs	100 MNCs
GAPDH	**Mean**	22.77	24.03	27.81	31.01
	SD ±	0.1376	0.1928	0.2644	0.7102
	n	4 (5)	5	5	5
RPS10	**Mean**	22.95	24.90	27.78	30.83
	SD ±	0.6015	0.1384	0.2472	0.6086
	n	5	5	5	5
CK18	**Mean**	22.05	23.88	30.93	38.26
	SD ±	0.3374	0.1765	1.0280	3.8335
	n	5	5	5	5
CD45v1	**Mean**	-	-	28.82	31.90
	SD ±	-	-	0.1616	0.6729
	n	0	1	5	5
CD45v2	**Mean**	-	-	31.88	36.80
	SD ±	-	-	0.2701	1.1656
	n	0 (5)	0 (5)	5	5
CD45v3	**Mean**	-	-	30.68	35.38
	SD ±	-	-	0.0764	0.5215
	n	0 (5)	0 (5)	5	5
EpCAM	**Mean**	25.04	26.37	36.17	-
	SD ±	0.5889	0.4805	-	-
	n	5 (5)	5 (5)	2 (5)	1 (5)

5.2. Guanidine thiocyanate (GTC) lysis solution test

The Guanidine thiocyanate (GTC) lysis solution (0.3 M GTC, 0.5% Triton X-100, 1 mg/ml BSA) was evaluated as the best lysis solution for the direct lysis on the DC01 (data not published). GTC leads to an inhibition of the PCR, if the concentration is around 0.08 M [37]. In the following experiment, different concentrations of GTC were tested with respect to the qPCR inhibition. The GTC lysis solution (0.3 M GTC, 0.5% Triton X-100, 1 mg/ml BSA) was diluted with 1 mg/ml BSA (also used as reference).

The reverse transcription was performed using artificial RNA spike-in (10^7 copies). The cDNA was diluted with 30 µl NF-H_2O (1:4) and 30% per replicate was used for the qPCR (RNA spike-in), performing triplicates.

All obtained Cq-values are listed in table 36 (see appendix 9.22. Raw data). Cq-values were converted to relative transcripts (using GenEx). First, the mean of the qPCR replicates and then the average of the RT replicates was calculated. The average of the reference (1 mg/ml BSA) was set to 100%. Samples were tested for normal distribution using the Shapiro-Wilk normality test. Thereupon, an unpaired parametric t-test was performed against the reference (1 mg/ml BSA) (Figure 9).

Figure 9 shows that the efficiency of the qPCR is getting significant lower from 1 mg/ml BSA (100%) to 0.075 M GTC (41.86%) (unpaired t-test; * $p = 0.0317$; **** $p \leq 0.009$, $p = 0.0001$). Samples diluted to 0.01 M GTC do not significantly different to samples lacking GTC (1 mg/ml BSA only). qPCR is completely inhibited at GTC concentrations higher than 0.15 M (Table 12).

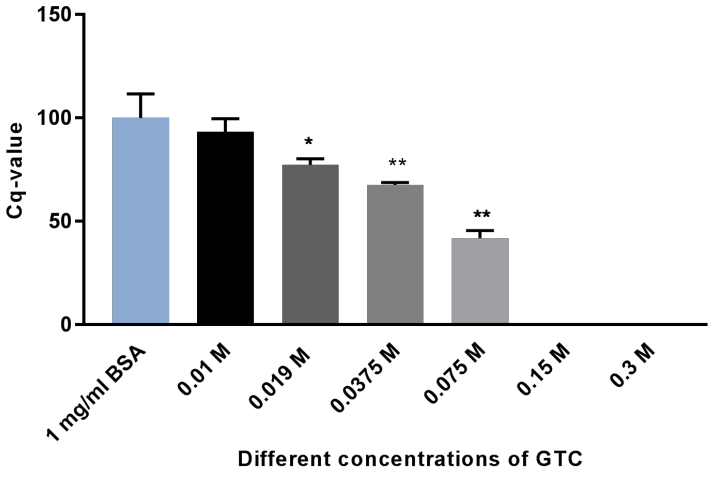

Figure 9: GTC lysis solution test with different concentrations of GTC. Different concentrations of GTC were tested in relation to qPCR inhibition of samples containing 10^7 copies of spike RNA. Data are shown as mean ± SD (n=3) (unpaired t-test; * $p = 0.0317$; **** $p \leq 0.009$, $p = 0.0001$).

Table 12: Mean (% of molecules), SD and number of replicates (n) from all concentrations of the GTC lysis solution test.

	Mean	SD ±	n
1 mg/ml BSA	100	11.73	3
0.01 M GTC	93.44	6.21	3
0.019 M GTC	77.41	2.86	3
0.0375 M GTC	67.68	1.18	3
0.075 M GTC	41.86	3.69	3
0.15 M GTC	0	0.00	3
0.3 M GTC	0	0.00	3

5.3. RT performance

For the RT performance, three different concentrations of BSA were tested as follows (total RT volume 10 µl): 500 pg RNA from HT-29 cells without BSA, in 0.5 mg/ml BSA and 0.75 mg/ml BSA. Analysis was performed on 5% of the initial cDNA using qPCR (*GAPDH, RPS10, CK18, CK8, EpCAM*).

All obtained Cq-values are listed in table 37 (see appendix 9.22. Raw data). The Cq-value was converted into relative transcripts (using GenEx) and the average of the reference (0.5 mg/ml BSA) was set to 100%. With *EpCAM* being an exception, the yield of PCR-specific products in samples containing 0.75 mg/ml BSA was higher compared to samples lacking BSA. On average, the yield in samples containing 0.75 mg/ml BSA dropped to 90.6% whereas RT lacking BSA resulted on average in a yield of 89% (Table 13). *CK18* and *CK8* show a significant lower expression without BSA (unpaired t-test compared to 0.5 mg/ml BSA; CK8 p = 0.0298; *CK18* p < 0.0001) (Figure 10).

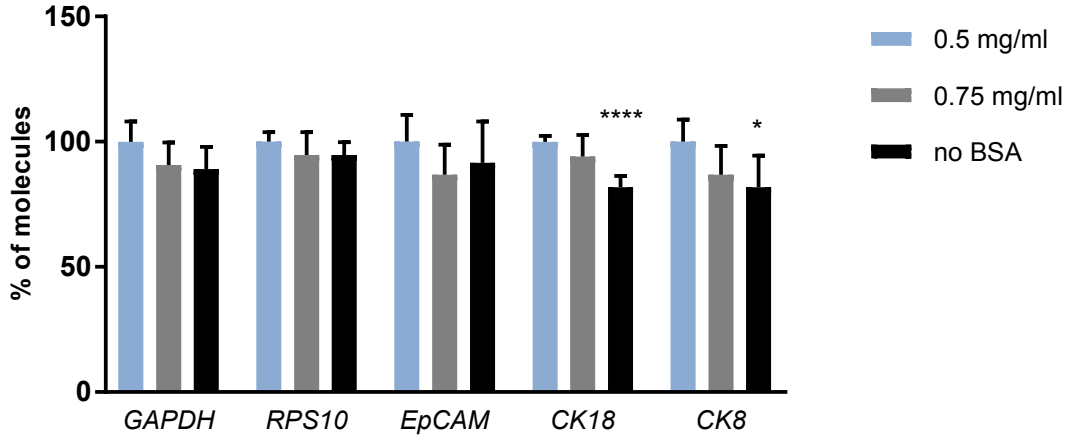

Figure 10: RT performance using different concentrations of BSA. The RNA of HT-29 cells was used in RTqPCR using different concentrations of BSA for the RT (0.5 mg/ml BSA, 0.75 mg/ml BSA, no BSA). The Cq-value was converted into relative transcripts and the average of the reference (0.5 mg/ml) was set to 100% (Table 13). Data are shown as mean ± SD (n=3). With the presence of BSA the RTqPCR performs best (depending on the marker gene). *CK18* and *CK8* show a significant lower expression without BSA (unpaired t-test compared to 0.5 mg/ml BSA; *CK8* p = 0.0298; CK18 p < 0.0001).

Table 13: Mean (% of molecules), SD and number of analysed replicates (n) from the RT performance test.

		Mean	SD ±	n
GAPDH	0.5 mg/ml BSA	100	8.09	5
	No BSA	89.02	8.88	5
	0.75 mg/ml BSA	90.69	9.04	5
RPS10	0.5 mg/ml BSA	100	3.82	5
	No BSA	94.73	5.12	5
	0.75 mg/ml BSA	94.69	9.12	5
EpCAM	0.5 mg/ml BSA	100	10.73	5
	No BSA	91.57	16.53	5
	0.75 mg/ml BSA	86.85	11.96	5
CK18	0.5 µg/ml BSA	100	2.29	5
	No BSA	81.76	4.59	5
	0.75 µg/ml BSA	94.06	8.58	5
CK8	0.5 mg/ml BSA	100	8.83	5
	No BSA	81.82	12.64	5
	0.75 mg/ml BSA	86.82	11.54	5

5.4. Sensitivity and Inhibition test

It is possible, that the CellCollector 01 is contaminated with blood, after it is extracted from the vein of a patient.

To see if hemoglobin has an effect on the RTqPCR some inhibition tests with different amounts of erythrocytes were performed. To test the sensitivity of the method a low number of tumor cells were sorted in a background of a high number of MNCs.

For the first inhibition test 0-15 HT-29 cells were sorted into a background of 0-1000 erythrocytes. The FACS was performed in 5 µl GTC lysis solution (0.3 M GTC, 0.5% Triton X-100, 1 mg/ml BSA). The lysate (5µl) was diluted with 1 mg/ml BSA (dilution factor = 12.5) and 8% were used for the reverse transcription. The qPCR was performed with 2% of the initial sample and four different marker genes (*GAPDH, RPS10, CK18, CK8*).

All obtained Cq-values are listed in table 38 (see appendix 9.22. Raw data). PCR specific products with a Cq-value > 36 were set to 36 (representing 1 transcript). Negative qPCR reactions were set to Cq-values of 37 (Figure 11).

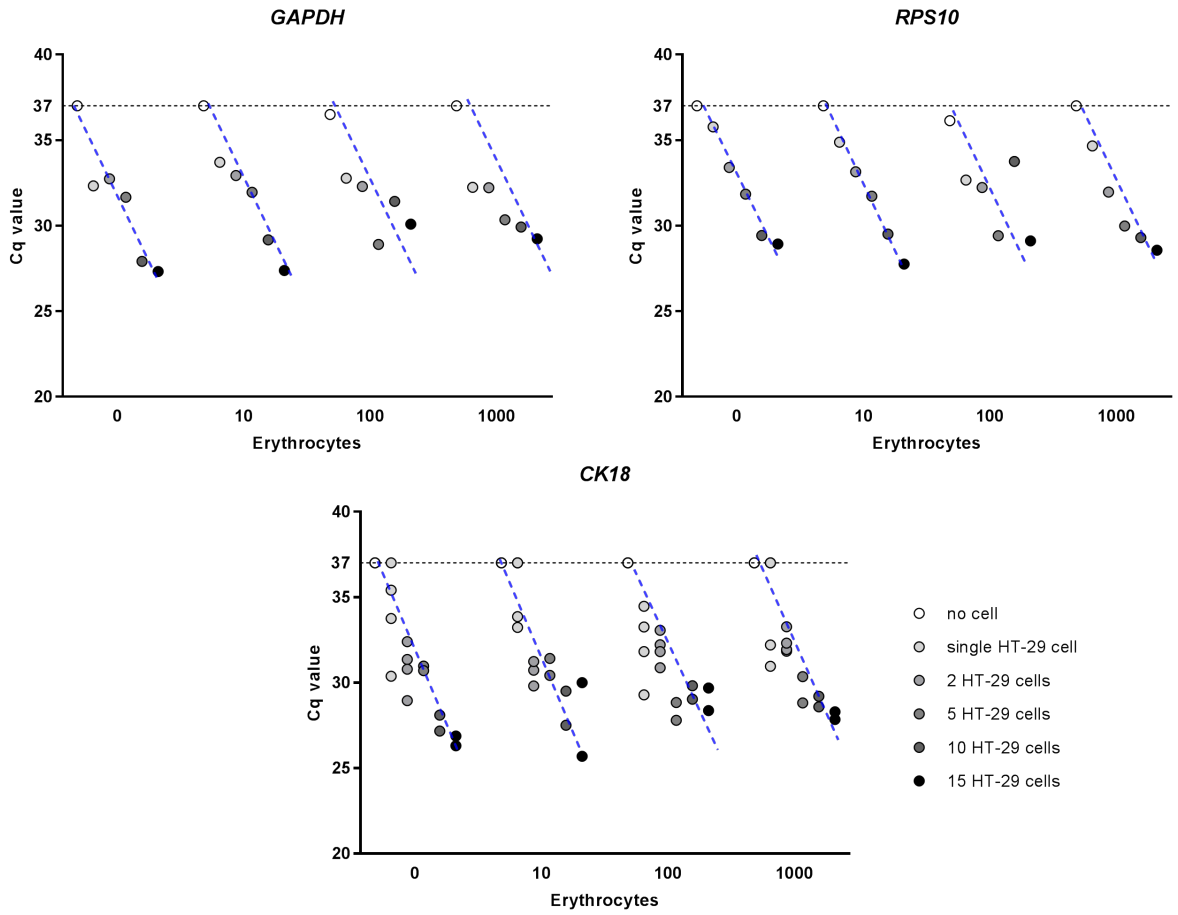

Figure 11: Inhibition test with 0-15 HT-29 cells in a background of 0-1000 erythrocytes using the GTC lysis solution. HT-29 cells were sorted in a background of erythrocytes and lysed using the GTC lysis solution. The lysate was used for RTqPCR. Relative cDNA yields are presented as Cq-values, PCR specific products with a Cq-value > 36 were set to 36 (representing 1 transcript). Negative qPCR reactions were set to Cq-values of 37. There is no influence on the RTqPCR, no matter how many erythrocytes are in the background (depending on the marker gene). Dashed blue lines are to guide eye only.

The Cq-value is getting lower from 1-15 HT-29 cells (mean $Cq_{0\ erythrocytes,GAPDH}$ = 31.58 vs. $Cq_{0\ erythrocytes,GAPDH}$ = 27.31). The dashed blue lines in figure 11 were drawn from 0 HT-29 cell to 15 HT-29 cells to guide eye only and show the tendency of the expression. All four lines are parallel and show that there is no inhibition of the RTqPCR, no matter how many erythrocytes are in the background.

Another inhibition test was performed with one and 30 HT-29 cells in a background of 0-1000 erythrocytes. The RT was performed as stated above and artificial RNA spike-in (10^7 copies) was used. The cDNA was diluted with NF-H_2O (1:1), and 1% of the initial samples were used for the qPCR (*GAPDH, RPS10, CK18, CK8*). *CK18* was used as cell type-specific marker.

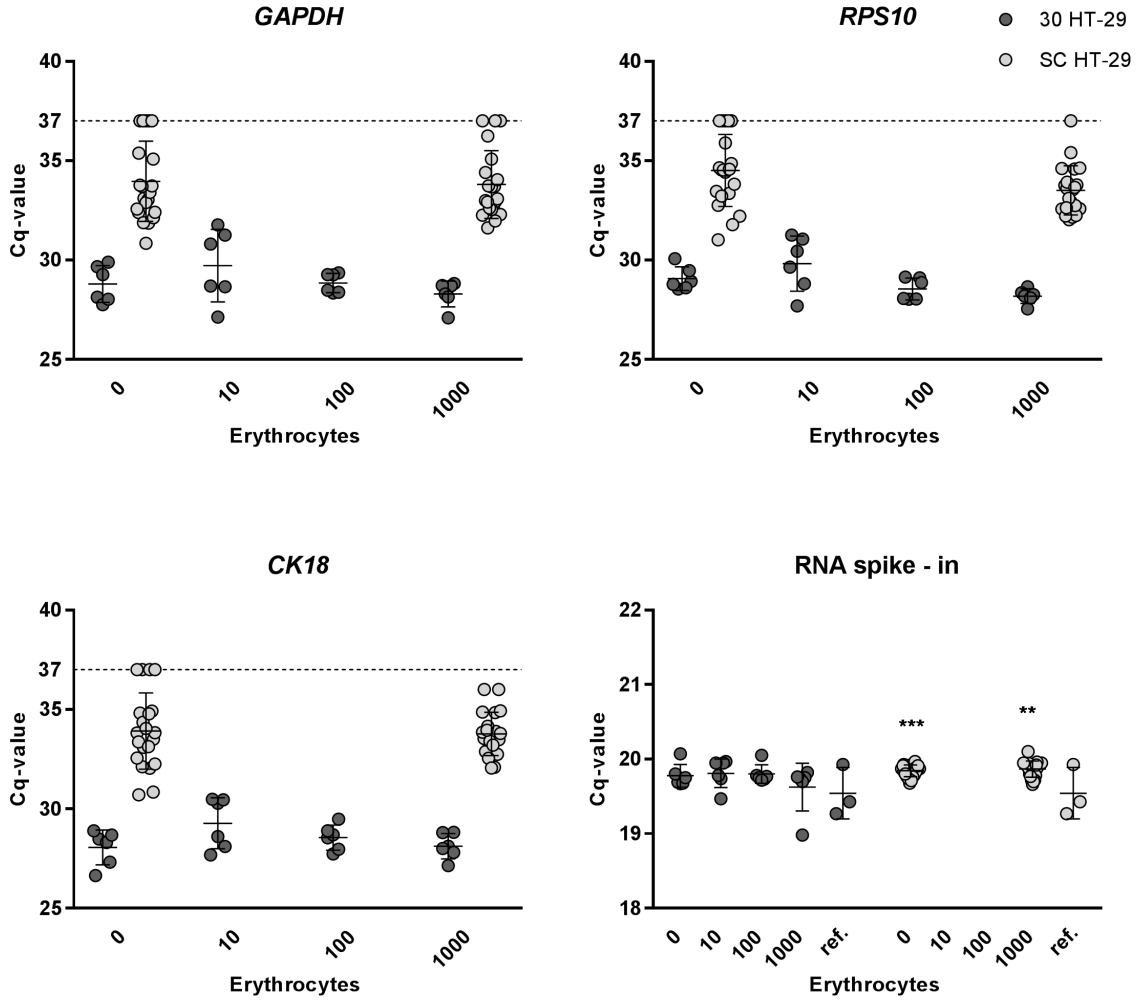

Figure 12: **Inhibition test with 30 and single HT-29 cells in a background of 0-1000 erythrocytes using the GTC lysis solution.** HT-29 cells were sorted into a background of erythrocytes and lysed using the GTC lysis solution. The lysate was used for RTqPCR performing a spike-in test with 10^7 copies. Relative cDNA yields are presented as Cq-values, PCR specific products with a Cq-value > 36 were set to 36 (representing 1 transcript). Negative qPCR reactions were set to Cq-values of 37. *CK18, GAPDH* and *RPS10* show the same expression no matter irrespective of the number of erythrocytes present. The RNA spike-in test shows that the different numbers of erythrocytes in the background of 30 HT-29 cells are not significantly different compared to the reference (1 mg/ml BSA). With a single HT-29 cell and zero and 1000 erythrocytes in the background the RNA spike-in test shows a significant difference compared to the reference (unpaired t-test; ** p = 0.002, *** p = 0.001).

All obtained Cq-values are listed in table 39 and table 40 (see appendix 9.22. Raw data). PCR specific products with a Cq-value > 36 were set to 36 (representing 1 transcript). Negative qPCR reactions were set to Cq-values of 37 (Figure 12).

Cq-values of *GAPDH* and *RPS10* in 30 HT-29 cells are lower than in single cells (mean $Cq_{1000\ cells,GAPDH}$ of 28.30 vs. 33.27 and mean $Cq_{1000\ cells,RPS10}$ 28.18 vs. 33.34, respectively). The difference in Cq-values for *GAPDH* (deltaCq = 4.97 equaling 31.34-fold difference in yield) and *RPS10* (deltaCq = 5.16 equaling 35.75-fold difference in yield) nicely correspond to the difference in analyzed cells (30 cells vs. single cell). Single cell $Cq\text{-values}_{0\ erythrocytes,GAPDH}$ range from Cq 30.84 to Cq 35.08. There is no

inhibition of the RTqPCR no matter how many erythrocytes are in the background (mean $Cq_{0\ cells,CK18}$ = 28.06 vs. $Cq_{1000\ cells,CK18}$ = 28.11). The RNA spike-in test shows that the different numbers of erythrocytes in the background of 30 HT-29 cells are not significantly different compared to the reference (1 mg/ml BSA). With a single HT-29 cell and zero and 1000 erythrocytes in the background the RNA spike-in test shows a significant difference compared to the reference (1 mg/ml BSA; unpaired t-test; ** p = 0.002, *** p = 0.001) (Table 14).

Table 14: Mean (Cq-value), SD and number of analysed replicates (n) from all samples of the inhibition test with GTC.

	Number of erythrocytes	Number of HT-29 cells	Mean	SD ±	n
GAPDH	0	1	33.01	1.174	16 (21)
	1000	1	33.27	1.152	18 (21)
	0	30	28.79	0.9262	6
	10	30	29.72	1.8250	6
	100	30	28.85	0.4907	6
	1000	30	28.30	0.6488	6
RPS10	0	1	33.72	1.8130	16 (21)
	1000	1	33.34	0.969	20 (21)
	0	30	29.07	0.5942	6
	10	30	29.82	1.3830	6
	100	30	28.54	0.5502	6
	1000	30	28.18	0.3707	6
CK18	0	1	33.19	1.29	17 (21)
	1000	1	33.77	1.0900	21
	0	30	28.06	0.8786	6
	10	30	29.27	1.2810	6
	100	30	28.55	0.6339	6
	1000	30	28.11	0.6371	6
RNA spike - in	0	1	19.84	0.0778	21
	1000	1	19.87	0.1087	21
	Reference	1	19.54	0.3443	3
	0	30	19.78	0.1520	6
	10	30	19.81	0.1930	6
	100	30	19.80	0.1246	6
	1000	30	19.63	0.3184	6
	Reference	30	19.54	0.3443	3

For the sensitivity test 0-15 HT-29 cells and 0-1000 MNCs were sorted into 5 µl GTC lysis solution. The lysate was diluted with 57.5 µl of 1 mg/ml BSA (dilution factor = 12.5). For the reverse transcription into cDNA 8% of the lysate was used and with 2% of the initial sample a qPCR was performed (*GAPDH, CK18* and *RPS10)*.

All obtained Cq-values are listed in table 41 (see appendix 9.22. Raw data). PCR specific products with a Cq-value > 36 were set to 36 (representing 1 transcript). Negative qPCR reactions were set to Cq-values of 37 (Fig. 13).

The Cq-value is getting lower from 1-15 HT-29 cells (mean $Cq_{0\ MNCs,GAPDH}$ = 35.01 vs. $Cq_{0\ MNCs,GAPDH}$ = 30.24). The dashed green lines in figure 13 were drawn from 0 HT-29 cell to 15 HT-29 cells to guide eye only and show the tendency of the expression. It comes to an inhibition of the RTqPCR when 250 or more MNCs are in the background (Figure 13).

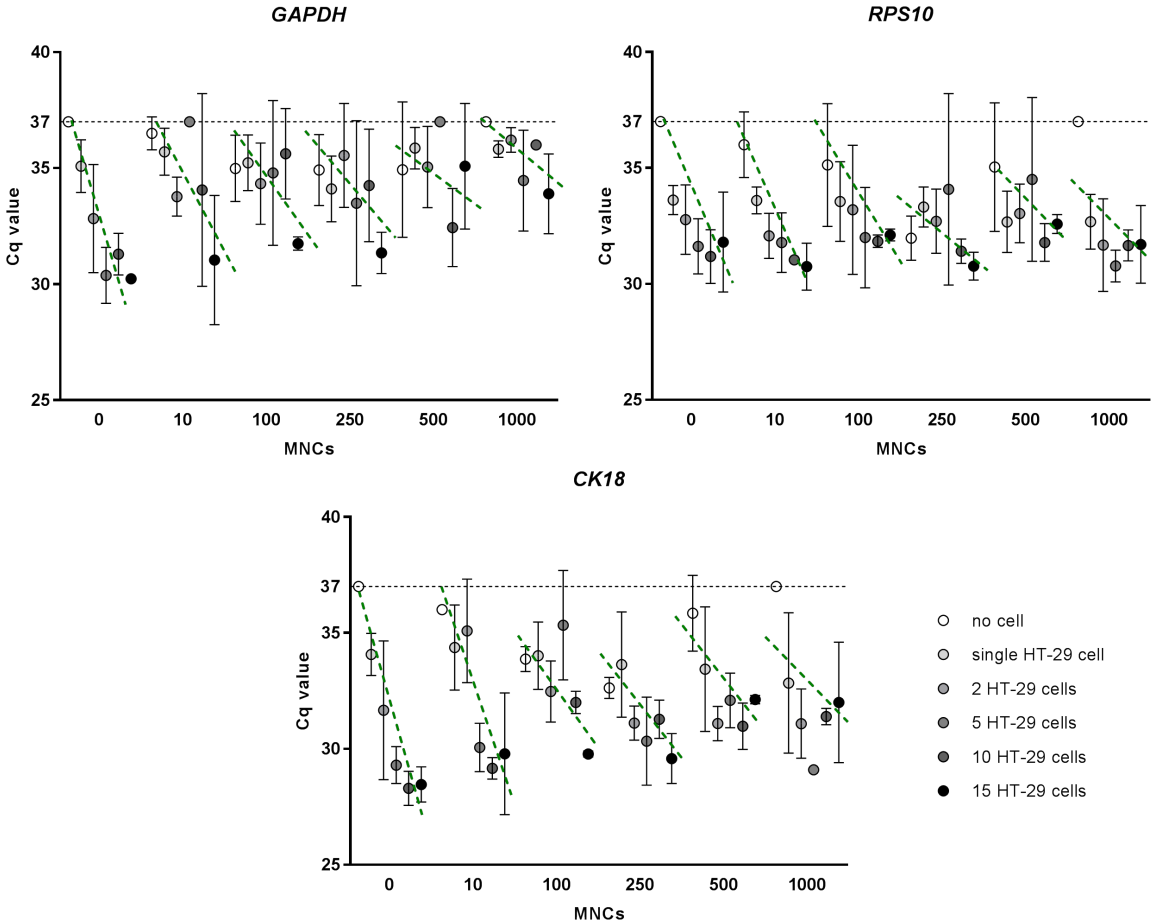

Figure 13: Sensitivity test with 0-15 HT-29 cells in a background of 0-1000 MNCs using the GTC lysis solution. HT-29 cells were sorted in a background of 0-1000 MNCs and lysed. Relative cDNA yields are presented as Cq-values, PCR specific products with a Cq-value > 36 were set to 36 (representing 1 transcript). Negative qPCR reactions were set to Cq-values of 37. It comes to an inhibition of the RTqPCR when 250 or more MNCs are in the background. Dashed green lines are to guide eye only.

Another sensitivity test was performed with the GTC lysis solution and zero ond one HT-29 cell in a background of 0-1000 MNCs. The lysate was diluted with 57.5 µl of 1 mg/ml BSA (dilution factor = 12.5). For the reverse transcription into cDNA 8% of the lysate was used, the cDNA was diluted with NF-H_2O (1:1) and with 1% of the initial sample a qPCR was performed (*GAPDH, CK18* and RNA spike-in*)*. For the RNA spike-in test 10^7 copies were used.

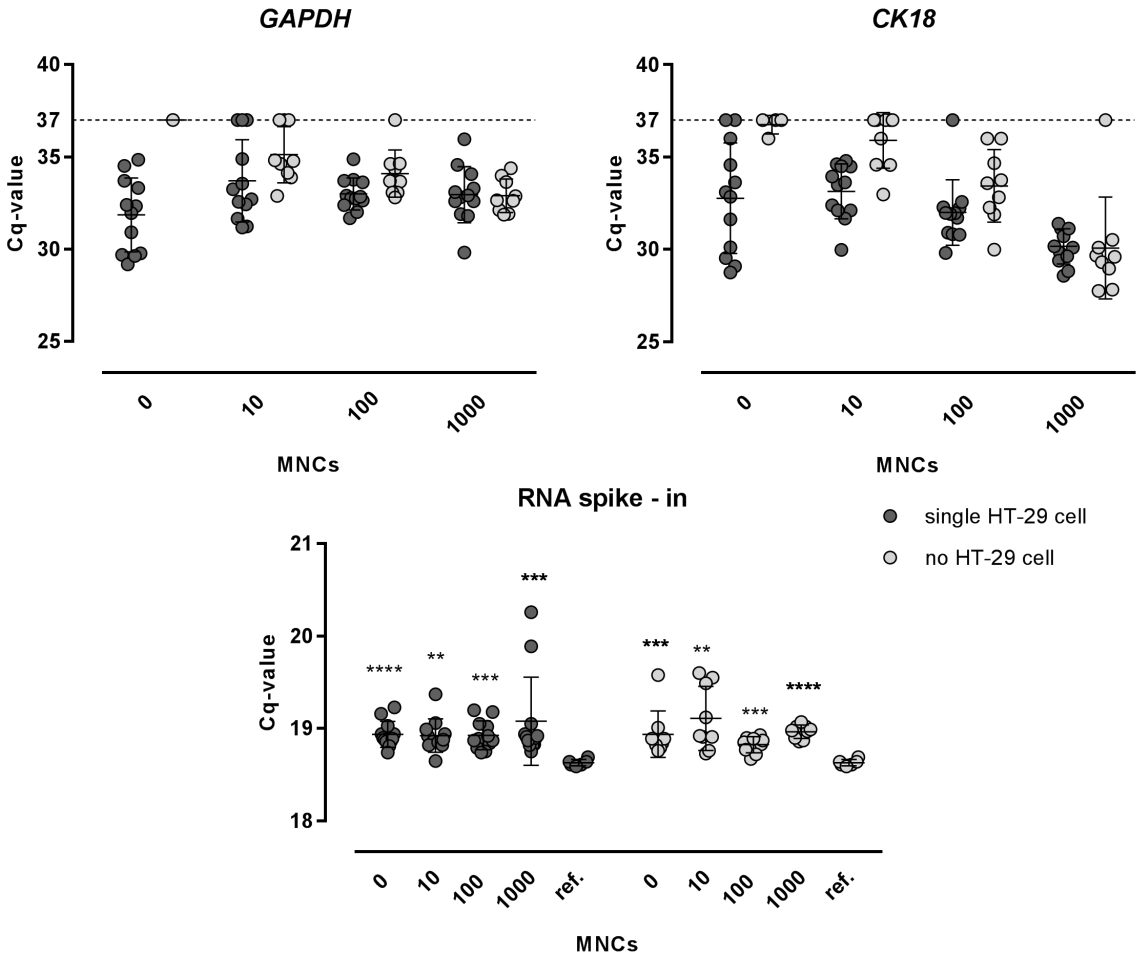

Figure 14: Sensitivity test with the GTC lysis solution and HT-29 cells (single and zero) in a background of 0-1000 MNCs. The HT-29 cells were sorted in a background of 0-1000 MNCs and lysed using the GTC lysis solution. Relative cDNA yields are presented as Cq-values, PCR specific products with a Cq-value > 36 were set to 36 (representing 1 transcript). Negative qPCR reactions were set to Cq-values of 37. According to the RNA spike-in test the expression of the MNCs is significant lower compared to the reference (1 mg/ml BSA) (Table 16).

All obtained Cq-values are listed in table 42 and table 43 (see appendix 9.22. Raw data). PCR specific products with a Cq-value > 36 were set to 36 (representing 1 transcript). Negative qPCR reactions were set to Cq-values of 37 (Figure 14).

Single cell HT-29 (0 MNCs) Cq-values range from Cq_{GAPDH} 29.19 to Cq_{GAPDH} 34.85. The MNC samples lacking HT-29 cells show increasing expression of *GAPDH* and *CK18* from 0 - 1000 cells (mean $Cq_{10\ cells,GAPDH}$ = 34.60 vs. $Cq_{1000\ cells,GAPDH}$ = 32.90, mean $Cq_{10\ cells,CK18}$ = 34.54 vs. $Cq_{1000\ cells,CK18}$ = 29.22). On the contrary, the expression of *GAPDH* in HT-29 cells decreases with inceasing numbers of MNCs in the sample ($Cq_{10\ cells}$ = 32.62 vs. $Cq_{10\ cells}$ = 32.96). Spiked artificial RNA reveals an increase of Cq-values in the presence of MNCs. Yield is significantly higher compared to the reference (1 mg/ml BSA) sample containing RNA spike-in only ($Cq_{single\ HT\text{-}29\ cells,MNCs}$ = 18.97 vs. $Cq_{single\ HT\text{-}29\ cells,ref.}$ = 18.63; Table 15, Table 16).

Table 15: Mean (Cq-value), SD and number of analysed replicates (n) from the sensitivity test using the GTC lysis solution.

	Number of MNCs	Number of HT-29 cells	Mean	SD ±	n
GAPDH	0	1	31.86	2.0010	12
	10	1	32.62	1.1940	9 (12)
	100	1	33.00	0.8705	12
	1000	1	32.96	1.5250	12
	0	0	-	-	-
	10	0	34.60	1.2560	7 (9)
	100	0	33.74	0.7311	8 (9)
	1000	0	32.90	0.9135	9
CK18	0	1	32.78	2.9970	12
	10	1	33.15	1.4900	12
	100	1	31.55	0.8438	11 (12)
	1000	1	30.17	0.9483	12
	0	0	36.00	-	1 (9)
	10	0	34.54	1.2340	4 (9)
	100	0	33.44	1.9620	9
	1000	0	29.22	1.0020	8
RNA spike - in	0	1	18.94	0.1407	12
	10	1	18.92	0.1817	11 (12)
	100	1	18.93	0.1546	12
	1000	1	19.08	0.4774	12
	Reference	1	18.63	0.0352	6
	0	0	18.94	0.2509	9
	10	0	19.11	0.3466	9
	100	0	18.83	0.0878	9
	1000	0	18.96	0.0727	9
	Reference	0	18.63	0.0352	6

Table 16: Data of the used statistical tests from the sensitivity test with RNA spike-in

Number of MNCs	Number of HT-29 cells	Statistical test	p-value
0	1	unpaired t-test	<0.0001
10	1	unpaired t-test	0.0015
100	1	unpaired t-test	0.0003
1000	1	Mann-Whitney	0.0001
0	0	Mann-Whitney	0.0004
10	0	unpaired t-test	0.0053
100	0	unpaired t-test	0.0002
1000	0	unaired t-test	<0.0001

For the sensitivity test single VCaP cells were sorted into 5 µl of GTC lysis solution. The lysate was diluted with 57.5 µl of 1 mg/ml BSA (dilution factor = 12.5). The RT was performed as before and 1% of the initial samples were used in qPCR (*RPS10, GAPDH, ARv7, CD45*) and additional a positive control (VCaP RNA) was used.

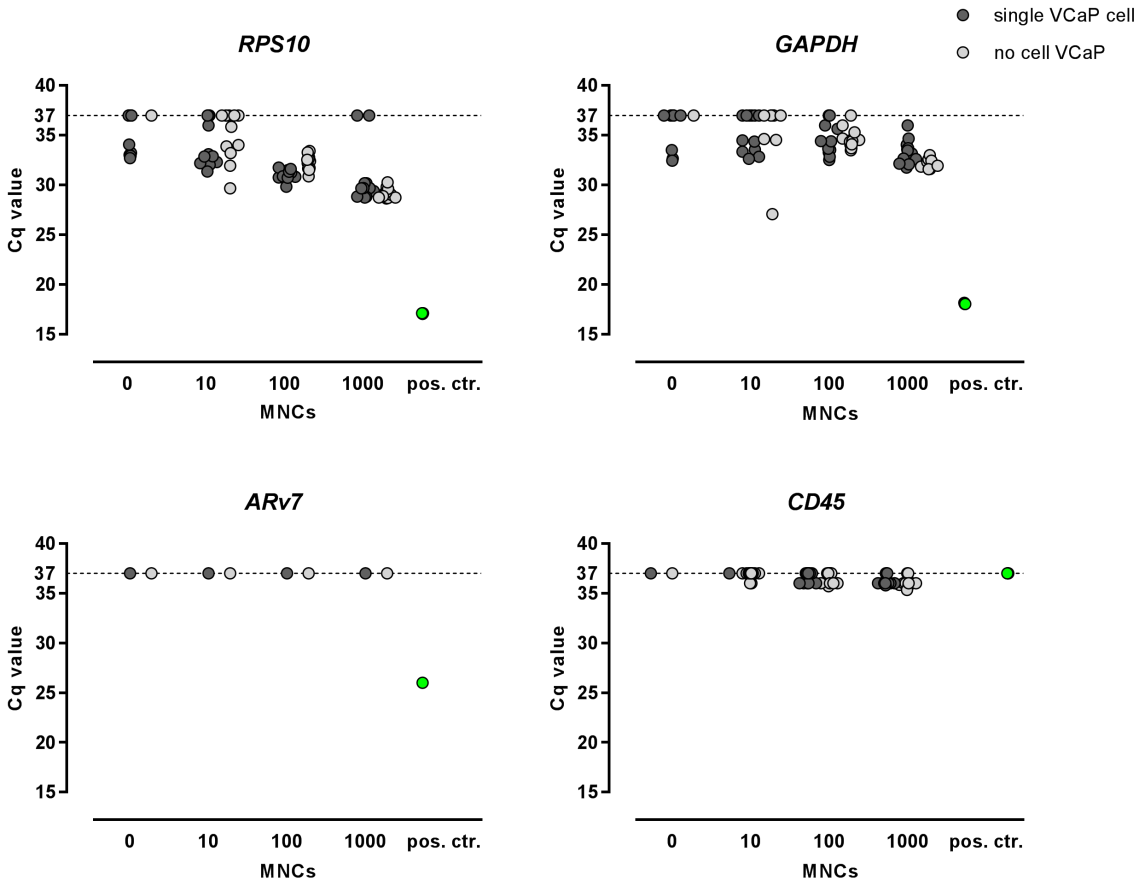

Figure 15: Sensitivity test with VCaP (single and zero) cells in a background of 0-1000 MNCs using the GTC lysis solution. The VCaP cells were sorted in a background of 0-1000 MNCs and lysed with the GTC lysis solution. Relative cDNA yields are presented as Cq-values, PCR specific products with a Cq-value > 36 were set to 36 (representing 1 transcript). Negative qPCR reactions were set to Cq-values of 37. The *ARv7* could not be detected in VCaP cells.

All obtained Cq-values are listed in table 44 and table 45 (see appendix Raw data). PCR specific products with a Cq-value > 36 were set to 36 (representing 1 transcript). Negative qPCR reactions were set to Cq-values of 37 (Figure 15).
The positive control shows, that the expression of the *ARv7* is lower than the expression of the other reference genes (mean Cq_{ARv7} = 26.01 vs. Cq_{GAPDH} = 18.11, Cq_{RPS10} = 17.12). The *ARv7* could not be detected in VCaP nor in MNCs, but the positive control shows a signal. No *CD45* transcripts were detected in the positive control and *CD45* shows just a low expression (Cq = 36) from 10-1000 MNCs (Figure 15).

The sensitivity test was repeated with single and no HT-29 cells in a background of 0-1000 MNCs and with the BSA/Triton lysis solution (0.2% Triton X-100, 1 mg/ml BSA), where no dilution of the lysate is necessary. The reverse transcription was performed with the SuperScript Kit and 10% (*GAPDH, CD45, AR*) or 30% (*ARv7*) of the cDNA (total sample) were used for the qPCR.
All obtained Cq-values are listed in table 46 and table 47 (see appendix 9.22. Raw data). PCR specific products with a Cq-value > 36 were set to 36 (representing 1 transcript). Negative qPCR reactions were set to Cq-values of 37 (Figure 16).
Additional the Cq-value was converted into relative transcripts and log2 normalized (Figure 17).
Single cell Cq-values range from Cq_{GAPDH} 26.8 to Cq_{GAPDH} 30.64. In MNCs the *AR* and *ARv7* could not be detected. *CD45* is the only gene, which shows a higher expression with 500 MNCs in the background (mean $Cq_{0\ MNCs,single\ VCaP}$ = 33.91 vs. $Cq_{500\ MNCs,single\ VCaP}$ = 31.52). *CD45* is also expressed in VCaP cells (mean $Cq_{0\ MNCs,single\ VCaP}$ = 33.91). Only in 29 of 42 samples the *AR* and in 26 of 42 the *ARv7* could be detected (with no MNC cells in the background) (Table 17). The higher the number of MNCs is in the background, the lower is the expression of the *AR* (mean $Cq_{0\ MNCs,single\ VCaP}$ = 30.93 vs. $Cq_{500\ MNCs,single\ VCaP}$ = 33.89) and the *ARv7* (mean $Cq_{0\ MNCs,single\ VCaP}$ = 35.98 vs. $Cq_{500\ MNCs,single\ VCaP}$ = 39.72; Figure 16, Figure 17). With 500 MNCs in the background only 5 of 42 (AR) and 1 of 42 (ARv7) samples could be detected and with 1000 MNCs in the background no detection is possible (Table 17).

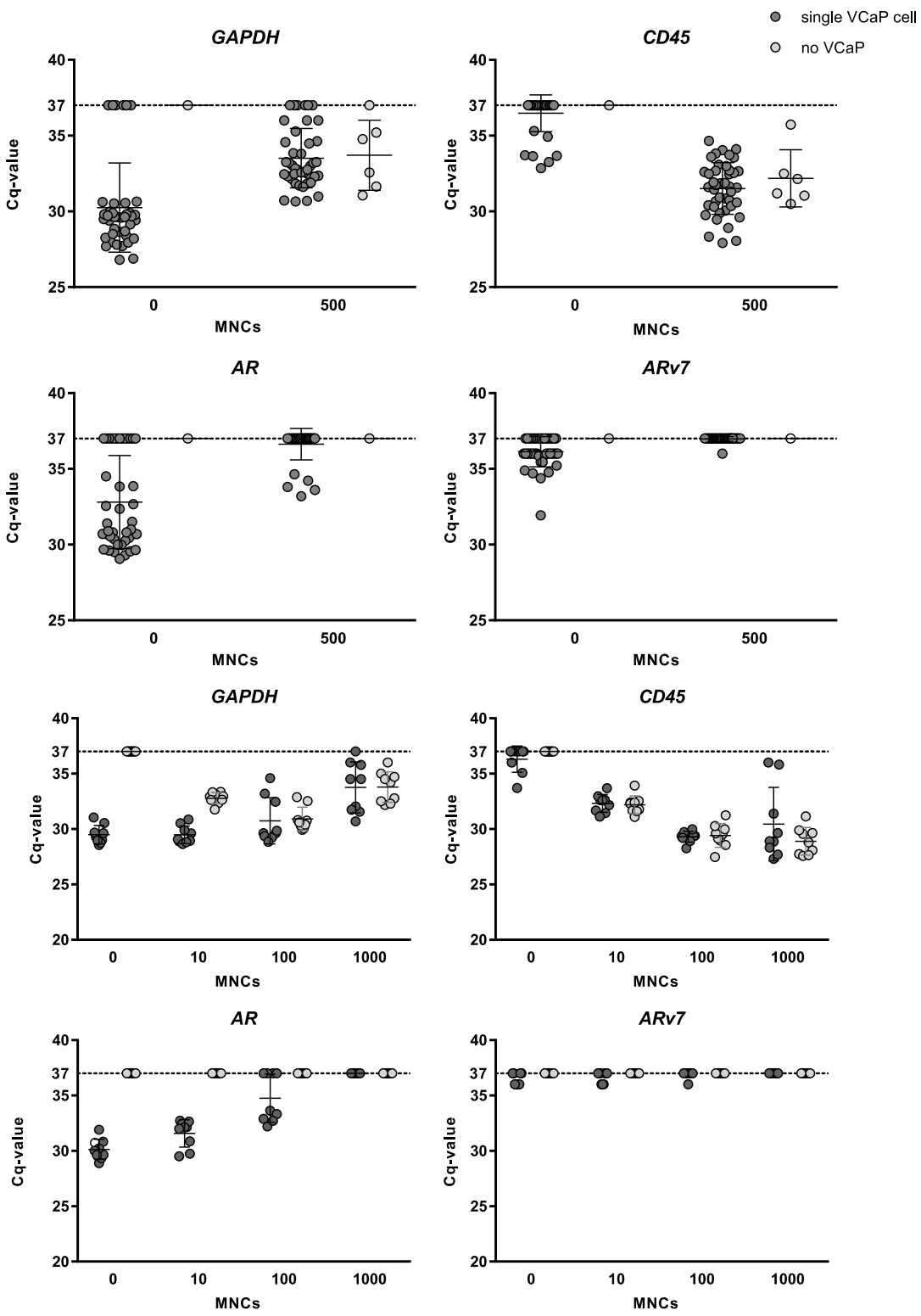

Figure 16: Sensitivity test with the BSA/Triton lysis solution and VCaP cells in a background of 0-1000 MNCs. VCaP cells were sorted in a background of 0-1000 MNCs and lysed in the BSA/Triton lysis solution. Relative cDNA yields are presented as Cq-values, PCR specific products with a Cq-value > 36 were set to 36 (representing 1 transcript). Negative qPCR reactions were set to Cq-values of 37. *AR* and *ARv7* are not expressed in MNCs. Increasing numbers of MNCs cause decrease of ARv7 yield. In the background of 1000 MNCs ARv7 was no longer detected.

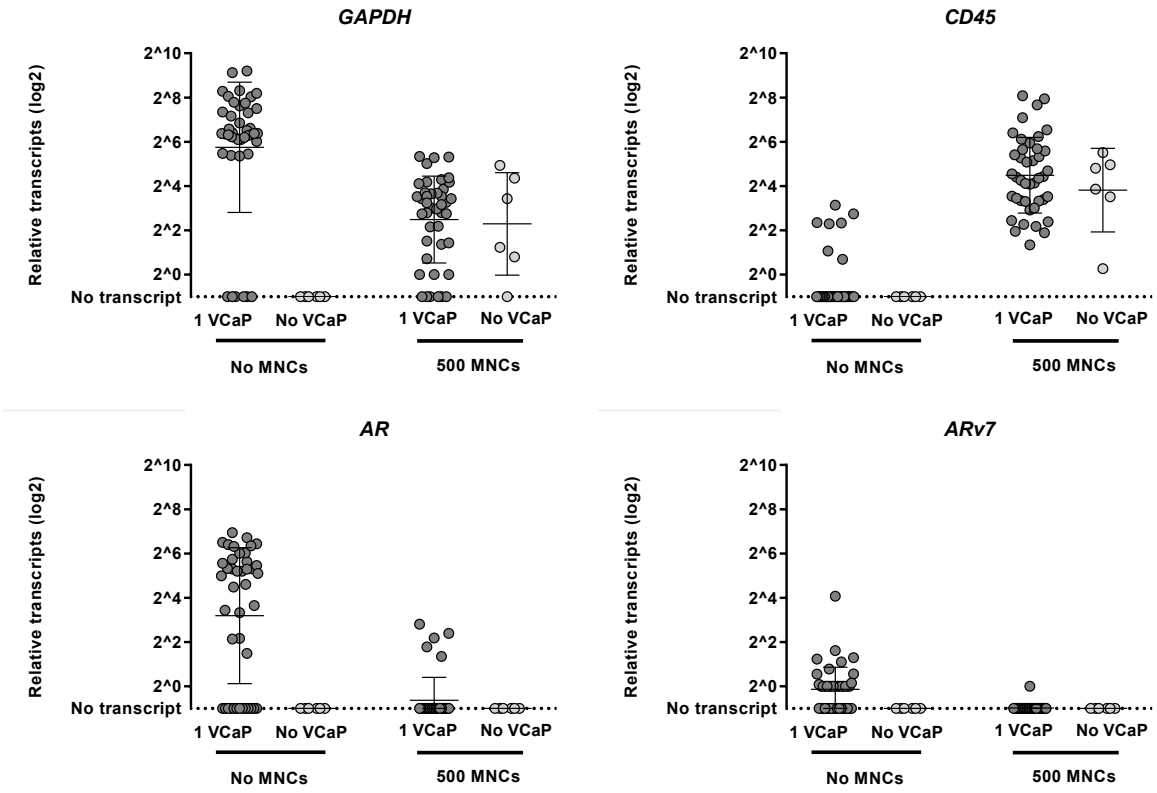

Figure 17: Relative transcripts from the sensitivity test with the BSA/Triton lysis solution and VCaP cells in the background of MNCs. VCaP cells were sorted into a background of 0-500 MNCs and lysed in the BSA/Triton lysis solution. Cq-values were converted to relative transcripts and log2 normalized. *AR* and *ARv7* are not expressed in MNCs. Increasing numbers of MNCs cause decrease of ARv7 yield. In the background of 1000 MNCs ARv7 was no longer detected.

Table 17: Mean (log2), SD (log2) and number of analysed replicates (n) of the sensitivity test with the BSA/Triton lysis solution.

			Mean (log2)	SD ± (log2)	n
GAPDH	**single VCaP cell**	**0 MNCs**	6.876	1.017	36 (42)
		500 MNCs	3.07	1.435	36 (42)
	no VCaP cell	**0 MNCs**	-	-	0 (6)
		500 MNCs	2.952	1.855	5 (6)
CD45	**single VCaP cell**	**0 MNCs**	2.089	0.8846	7 (42)
		500 MNCs	4.488	1.709	42 (42)
	no VCaP cell	**0 MNCs**			0 (6)
		500 MNCs	3.82	1.889	6 (6)
AR	**single VCaP cell**	**0 MNCs**	5.073	1.419	29 (42)
		500 MNCs	2.106	0.5628	5 (42)
	no VCaP cell	**0 MNCs**	-	-	0 (6)
		500 MNCs	-	-	0 (6)
ARv7	**single VCaP cell**	**0 MNCs**	0.4019	0.9307	26 (42)
		500 MNCs	-	-	1 (42)
	no VCaP cell	**0 MNCs**	-	-	0 (6)
		500 MNCs	-	-	0 (6)

5.5. VCaP dilution series

5.5.1. FACS

VCaP cell dilution series were sorted into 5 µl BSA/Triton lysis solution. The reverse transcription of the total cell lysate was performed with the SuperScript Kit and 10% of the initial sample was used for the qPCR (*GAPDH, AR* and *ARv7*). The number of replicates is listed in table 16.

All obtained Cq-values are listed in table 48 (see appendix 9.22. Raw data). PCR specific products with a Cq-value > 36 were set to 36 (representing 1 transcript). Negative qPCR reactions were set to Cq-values of 37 (Figure 18).

When the number of cells is doubled, the Cq is reduced approximately by one (mean $Cq_{\text{single VCaP cell},GAPDH}$ = 29.02 vs. $Cq_{2\text{ VCaP cells},GAPDH}$ = 27.80, mean $Cq_{\text{single VCaP cell},AR}$ = 30.34 vs. $Cq_{2\text{ VCaP cells},AR}$ = 28.53). With a low number of VCaP cells in the sample it was not always possible to detect the *ARv7*. Only 13 of 30 samples containing single VCaP cell show *Arv7* expression (Table 18). In general the expression of the *ARv7* is lower than the expression of the *AR*.

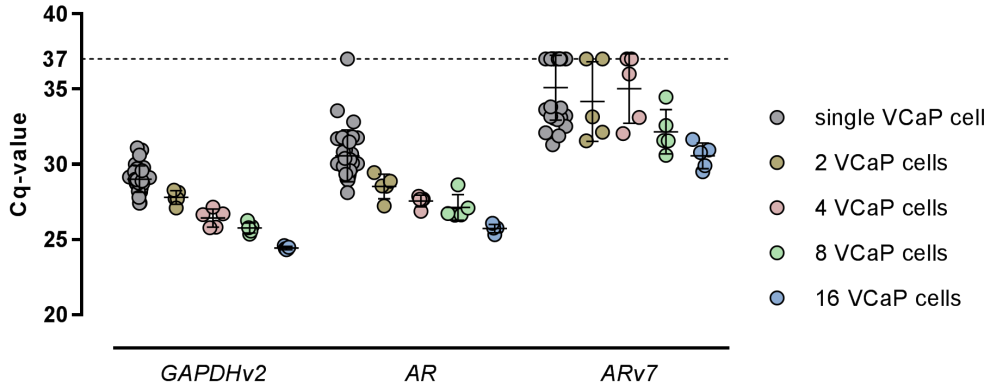

Figure 18: FACSed VCaP dilution series. A VCaP cell dilution series from 1-16 cells were sorted into BSA/Triton lysis solution and the lysate was used for RTqPCR. The sorting with the BSA/Triton lysis solution was successful, but only in 13 of 30 samples containing single VCaP cells the Arv7 could be detected (Table 18).

Table 18: Mean (Cq-value), SD and number of analysed replicates from the FACSed VCaP dilution series.

	Number of VCaP cells	Mean	SD ±	n
GAPDH	1	29.02	0.8827	30
	2	27.80	0.4587	5
	4	26.43	0.6014	5
	8	25.77	0.3410	5
	16	24.44	0.1121	5
AR	1	30.34	1.2375	29 (30)
	2	28.53	0.8140	5
	4	27.56	0.3876	5
	8	27.15	0.8529	5
	16	25.73	0.2730	5
ARv7	1	33.86	3.6273	13 (30)
	2	32.28	0.8074	3 (5)
	4	34.83	3.9299	3 (5)
	8	32.16	1.4687	5 (5)
	16	30.56	0.8568	5

5.5.2. Lasermicrodissection

A VCaP cell dilution series from 1-15 VCaP cells was laser microdissected into 5 µl Triton/BSA lysis solution and stored at -80°. The whole cell lysate was used for reverse transcription and the qPCR was performed with 30% of the initial sample. *GAPDH* and *ARv7* expression were tested and additional a positive control was inserted (VCaP cDNA).

All obtained Cq-values are listed in table 49 (see appendix 9.22. Raw data). PCR specific products with a Cq-value > 36 were set to 36 (representing 1 transcript). Negative qPCR reactions were set to Cq-values of 37 (Figure 19).

The reference gene *GAPDH* shows that the expression is getting higher from 1-15 VCaP cells ($Cq_{\text{single VCaP cell}}$ 28.14 = vs. $Cq_{\text{15 VCaP cells}}$ = 24.23). Only 5 of 12 samples containing 1-4 VCaP cells show ARv7 expression (Figure 19).

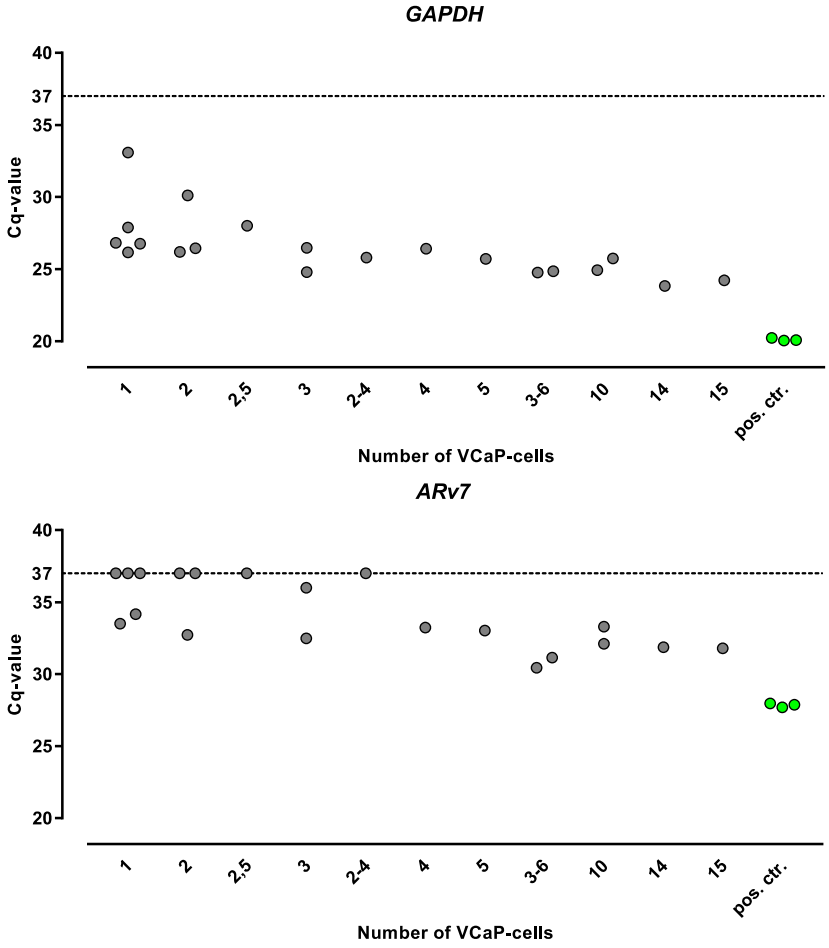

Figure 19: Lasermicrodissection of VCaP cells into the BSA/Triton lysis solution. After the micromanipulation the lysate was used for RTqPCR. Relative cDNA yields are presented as Cq-values, PCR specific products with a Cq-value > 36 were set to 36 (representing 1 transcript). Negative qPCR reactions were set to Cq-values of 37. A detection of the *ARv7* is possible after sorting the VCaP cells with microdissection.

5.5.3. Micromanipulation

Finally VCaP cells (range 1-15) were aspirated from single cell suspension and transferred into 5 µl BSA/Triton lysis solution and stored at -80°. Total cell lysate was reverse transcripted into cDNA and 30% of the initial sample was used for the qPCR. The qPCR was performed with *GAPDH, ARv7* and RNA spike–in as well as with an additional positive control (VCaP cDNA).

All obtained Cq-values are listed in table 50 (see appendix 9.22. Raw data). PCR specific products with a Cq-value > 36 were set to 36 (representing 1 transcript). Negative qPCR reactions were set to Cq-values of 37 (Figure 20).

The positive control shows that the *ARv7* is less expressed in VCaP cells than *GAPDH* (Cq_{ARv7} = 27.9 vs. Cq_{GAPDH} = 20). Using micromanipulation no VCaP cell shows an expression of *ARv7*. Also with *GAPDH* just a low number of cells (6 of 12) show an expression. The spike-in test without VCaP cells (Cq = 21.1, Cq = 21.28) shows similar Cq-values as the spike-in test with VCaP cells (mean Cq = 23.63) (Figure 20).

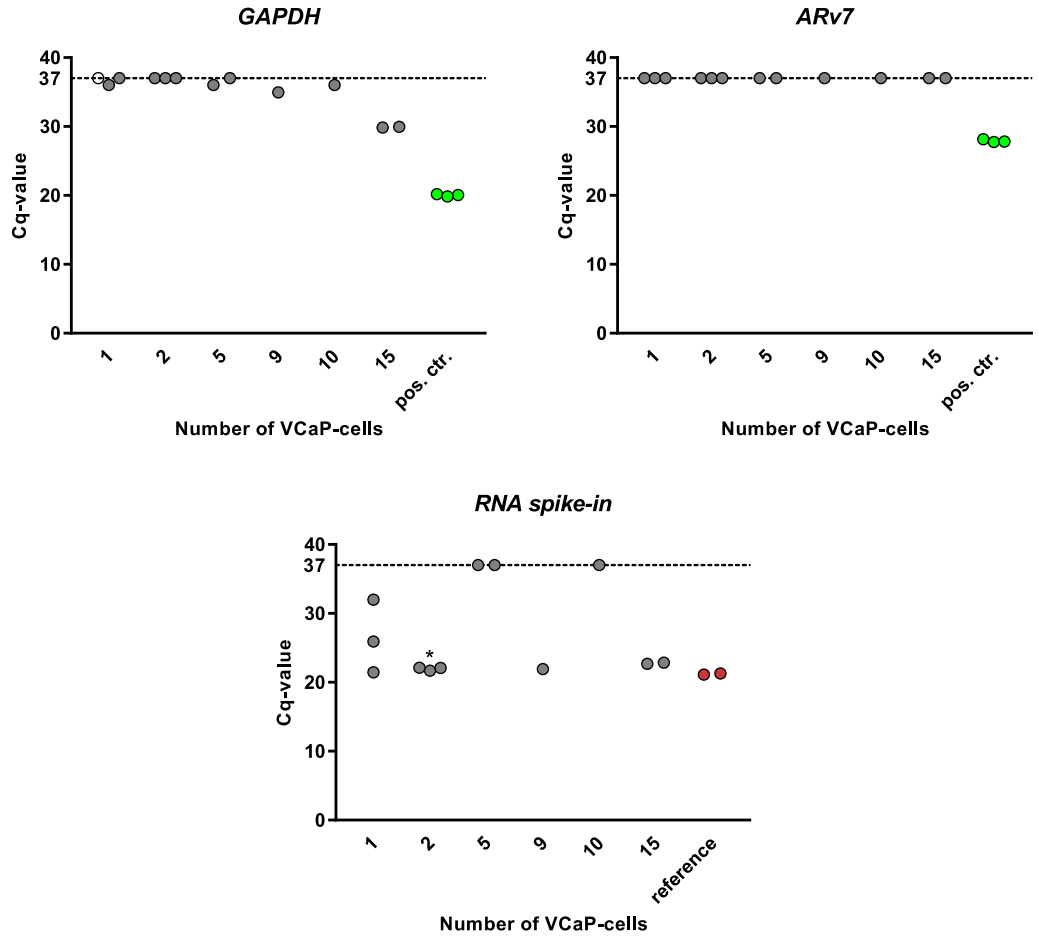

Figure 20: Micromanipulation from VCaP cells into BSA/Triton. After the micromanipulation the lysate was freezed and then used for RTqPCR. Relative cDNA yields are presented as Cq-values, Cq 37 represents no transcripts and Cq-values higher than 36 are modified to 36. With micromanipulation the *ARv7* could not be detected in VCaP cells.

5.5.4. Comparison of FACS, Microdissection and Micromanipulation

The VCaP dilution series was performed with three different methods: FACS, microdissection and micromanipulation. All three methods are compared in figure 21. The Cq value was converted to relative transcripts (with GenEx) and plotted against the number of VCaP cells. Regression analysis (GraphPad) shows that the cell sorting performed better (R^2=0.9873) than microdissection (R^2=0.8403) and micromanipulation (R^2=0.5536) (Figure 21).

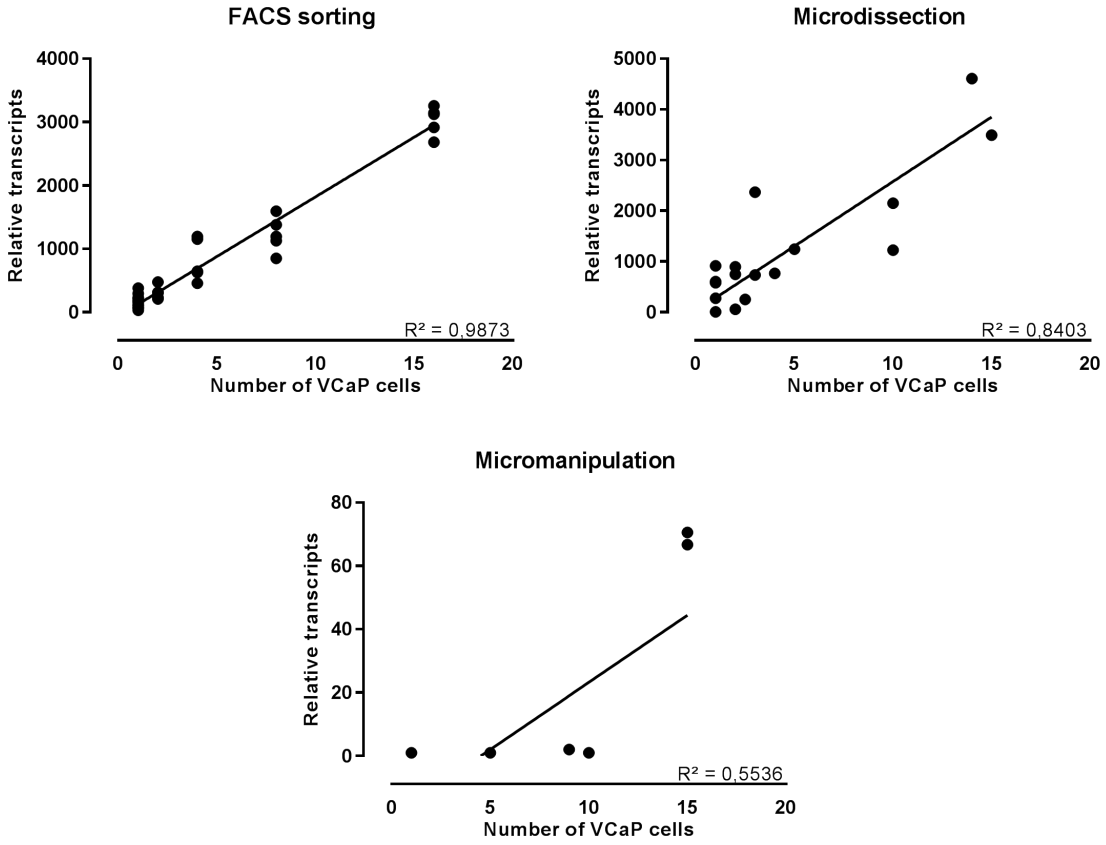

Figure 21: Comparison of FACS, micromanipulation and microdissection. A regression line was created with the relative transcripts of the Cq-value. FACS is the best method for sorting cells into the BSA/Triton lysis solution (R^2=0.9873).

5.6. Optimization of the on wire lysis protocol

After all preliminary tests were done, the optimization of the on wire lysis protocol started.

5.6.1. Lysis on the DC01 using GTC lysis solution

20 µl of a cell suspension containing 1000 HT-29 cells/µl were loaded on the CellCollector DC01.

For the lysis (protocol see appendix 9.22. Raw data) the functional part of the wire was incubated with 20 µl of the GTC lysis solution (0.3 M GTC, 0.5% Triton X-100, 1 mg/ml BSA). The lysis solution was pipetted in and out and then the lysate was collected. The functional part was rinsed additionally with 1 mg/ml BSA, which was mixed with the lysate and diluted to a total volume of 250 µl. The lysis step was repeated five times per wire and two wires were used. The RNA lysate was freezed and thawed and three aliquots of the lysate (5 µl each) were reverse transcribed into cDNA (total volume of 10 µl). The cDNA was diluted to 40 µl with NF-H_20 (1:4) and 3 µl (representing 0.15% of the initial sample) were used for the qPCR (*GAPDH, RPS10, CK18, EpCAM*).

All obtained Cq-values are listed in table 51 (see appendix 9.22. Raw data). PCR specific products with a Cq-value > 36 were set to 36 (representing 1 transcript). Negative qPCR reactions were set to Cq-values of 37 (Figure 22A).

Figure 22A shows the trend, that the yield is getting lower from the first lysis to the fifth lysis step ($Cq_{wire\#1,GAPDH}$ = 27.88 vs. $Cq_{wire\#1,GAPDH}$ = 32). Yield of *CK8* is only Cq 32 in the first lysis step.

All Cq-values were converted into relative transcripts (using GenEx), the average of all genes was calculated and the five lysis steps per wire were summed up as 100% (Figure 22B). For the schematic representation of the analysis see figure 28 (appendix 9.22. Raw data). With the GTC lysis solution and 10 min incubation 55.31% of the total yield is collected in the first lysis step and the yield is getting constantly lower (Table 19).

Table 19: Mean (% of molecules), SD and number of replicates (n) of the on wire lysis using the GTC lysis solution.

	Mean	SD ±	n
1. lysis	55.31	8.05	3
2. lysis	24.04	3.17	3
3. lysis	10.18	5.59	3
4. lysis	7.47	3.70	3
5. lysis	3.01	1.20	3

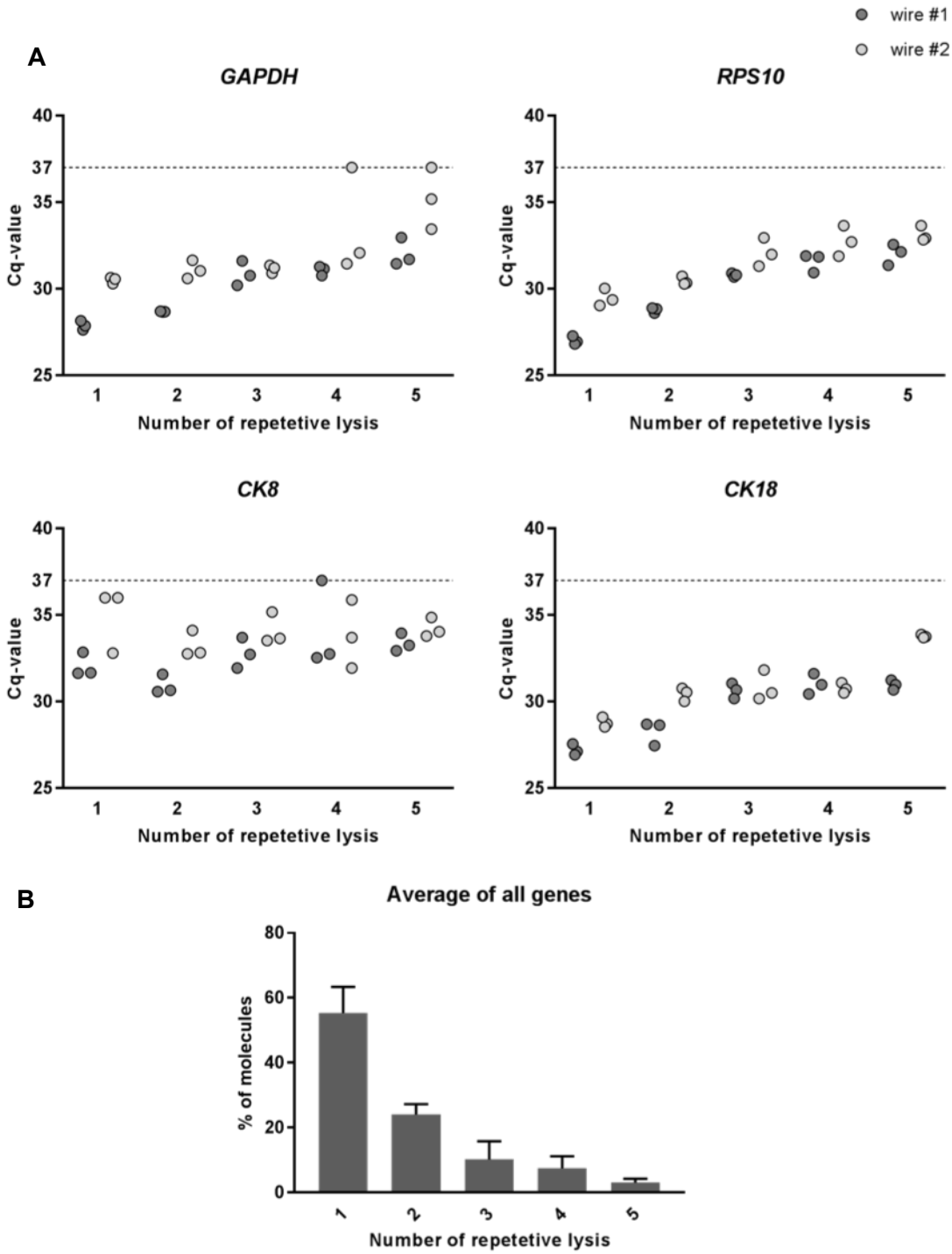

Figure 22: Direct lysis on the DC01 using the GTC lysis solution. 1000 cells/µl (in total 20 µl) were loaded on the DC01 and lysed directly on the wire with the GTC lysis solution. 5 lysis steps, two wires and technical triplicates were performed. The lysate was frozen and thawed and used for RTqPCR. **(A)** Relative cDNA yields are presented as Cq-values, PCR specific products with a Cq-value > 36 were set to 36 (representing 1 transcript). Negative qPCR reactions were set to Cq-values of 37. The expression is getting lower from the first to the fifth lysis step. **(B)**The Cq-values were converted into relative transcripts, all genes (exception of *CK8*) were averaged and all lysis steps were summed up as 100%. Approximately 55% of the total yield is collected in the first lysis step (Table 19).

5.6.2. Lysis on the DC01 using the BSA/Triton lysis solution with a subsequent freeze/thaw cycle

The sensitivity test with GTC shows that the GTC lysis solution cannot be used for low expressed genes like the *AR*. Instead of the GTC lysis solution the BSA/Triton lysis solution (0.2% Triton X-100, 1 mg/ml BSA) was used for the following experiments.

20 µl of a cell suspension containing 1000 HT-29 cells/µl were loaded on the DC01. The on wire lysis (protocol see appendix 9.13. Lysis on the DC01 using the BSA/Triton lysis solution with a subsequent freeze/thaw cycle) was performed by freezing the lysis solution (20 µl) directly in the Pasteur pipette on dry ice for approximately 1 min. After thawing and in/out pipetting the lysis solution was collected. Five lysis steps per wire on three wires were performed. After each lysis step additionally a wash step with lysis solution (20 µl) was done. Three aliquots of the lysate (4 µl each) were reverse transcribed into cDNA (total volume of 10 µl). The cDNA was diluted to 40 µl with NF-H_20 (1:4) and 2 µl (representing 1.7% of the initial sample) were used for the qPCR (*GAPDH, RPS10, CK18, EpCAM*).

All obtained Cq-values are listed in table 52 (see appendix 9.22. Raw data). PCR specific products with a Cq-value > 36 were set to 36 (representing 1 transcript). Negative qPCR reactions were set to Cq-values of 37 (Fig. 23A).

All genes show the trend that the expression is getting lower from the first to the last lysis and wash step (Figure 23A). A few replicates do not show a signal, especially with the low expressed *EpCAM* gene (Figure 23A).

The Cq-value was converted into relative transcrips (using GenEx), the average of all replicates and genes (exception of *EpCAM*) was calculated and the five lysis steps and wash steps per wire were summed up as 100% (Fig. 23B). For the schematic representation of the analysis see figure 28 (appendix 9.22. Raw data).

Analysis across all genes (Figure 23B) show that BSA/Triton lysis treatment with a subsequent freezing step (1 min) allows collecting 45.68% of the total yield in the first lysis step (Table 20). The additional wash step shows always a lower yield (16.50%) compared to the lysis step (45.68%) (Table 20).

Table 20: Mean (% of molecules), SD and number of replicates (n) from the direct lysis on the DC01 by freezing for 1 min using Triton/BSA

	Mean	SD ±	n
1. lysis	45.68	12.4700	3
1. wash	16.50	13.0400	3
2. lysis	10.88	11.0900	3
2. wash	3.11	3.1250	3
3. lysis	4.81	2.4080	3
3. wash	2.48	0.9824	3
4. lysis	6.09	3.8130	3
4. wash	2.74	1.1210	3
5. lysis	3.74	2.4480	3
5. wash	3.97	2.2380	3

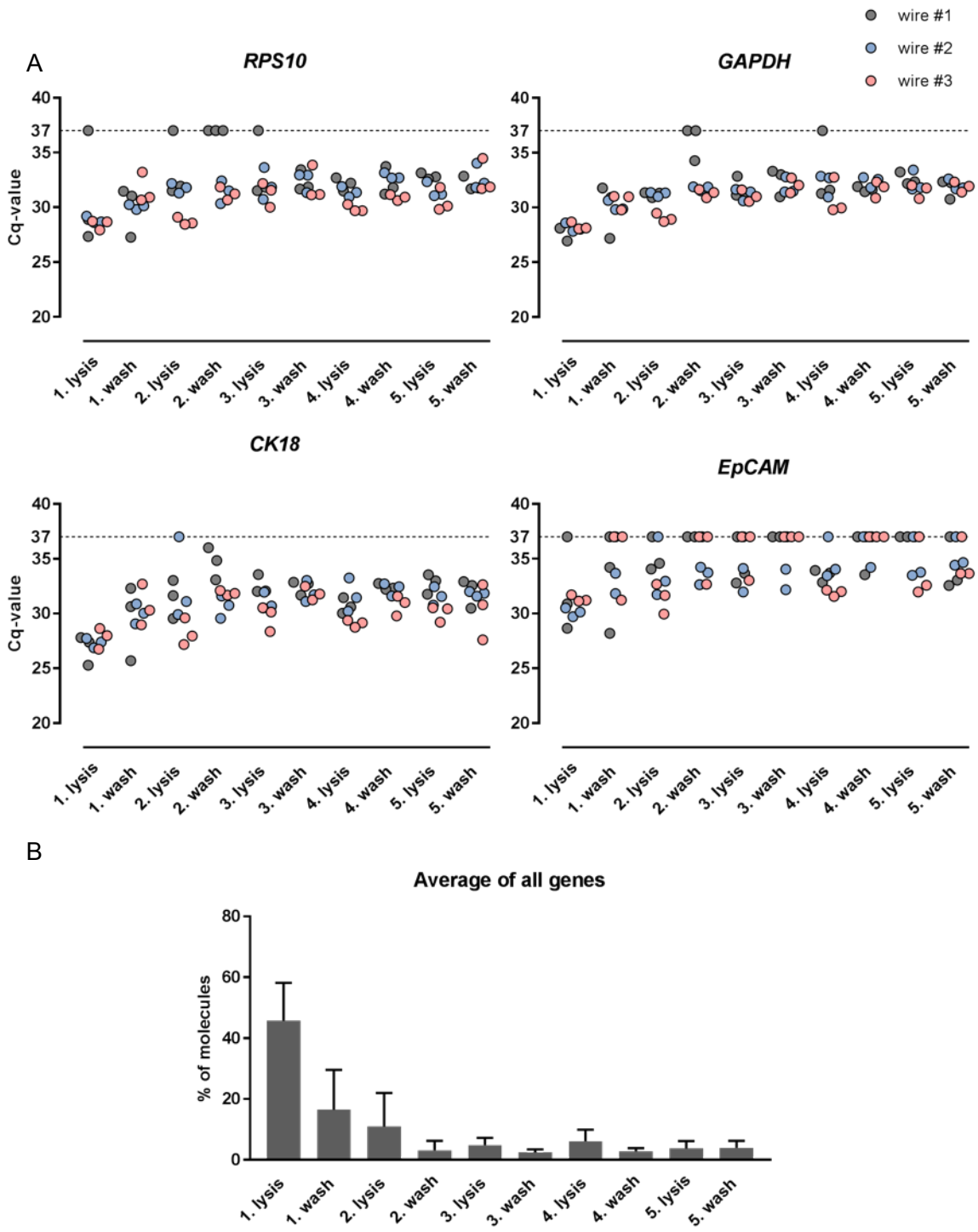

Figure 23: Direct lysis on the DC01 by freezing 1 min using the BSA/Triton lysis solution. 1000 cells/µl (20 µl in total) were loaded on the DC01 and lysed by freezing 1 min with the BSA/Triton lysis solution. 5 lysis steps and wash steps, three wires and technical triplicates were performed. The thawed lysate was lysate and the wash fraction was used for RTqPCR. **(A)** Relative cDNA yields are presented as Cq-values, PCR specific products with a Cq-value > 36 were set to 36 (representing 1 transcript). Negative qPCR reactions were set to Cq-values of 37. The expression is getting lower from the first to the fifth lysis step. **(B)** Cq-values were converted into relative transcripts, all genes were averaged and all lysis and wash steps were summed up as 100%. Approximately 46% of the total yield is collected in the first lysis step (Table 20).

5.6.3. Lysis on the DC01 using the BSA/Triton lysis solution - freeze over night

To evaluate the most effective cell density of HT-29, for the lysis on the DC01, different densities were tested in 20 µl total volume (1000 cells/µl, 500 cells/µl, 250 cells/µl 0 cells/µl). Stained cells (Hoechst 33342) were loaded on the DC01 and only the first wire (1000 cells/µl) was assessed under the fluorescence microscope (100 cells were counted).

The lysis solution (20 µl) was frozen over night at -80°C directly in the Pasteur pipette. Before the lysate collection the wire was not twisted and the lysis solution was not pipetted in and out, but before the wash step the wire was twisted and the lysis solution was pipetted in and out. Two lysis and wash steps were performed for each density/wire. The whole lysate was reverse transcribed (20 µl), three aliquots per cDNA (1 µl each, representing 3.3% of the initial sample) were used in the qPCR (*GAPDH, RPS10, CK18, EpCAM)*.

All obtained Cq-values are listed in table 53 (see appendix 9.22. Raw data). PCR specific products with a Cq-value > 36 were set to 36 (representing 1 transcript). Negative qPCR reactions were set to Cq-values of 37 (Figure 24A).

The negative control wire (containing no cells) shows some signals with the genes *RPS10* (Cq = 35.38) and *EpCAM* (Cq = 37.91, Cq = 34.26, Cq = 37.33). The DC01, which was incubated for 10 min with 1000 cells/µl shows higher yields (mean Cq_{GAPDH} = 26.45) compared to the DC01, which was incubated under the same conditions, but with only 250 cells/µl (mean Cq_{GAPDH} = 29.56). The expression after the first lysis and wash step is mostly the highest, depending on the condition (Figure 24A).

The Cq-value was converted into relative transcripts (using GenEx), the average of all replicates and genes was calculated and the two lysis and wash steps per condition were summed up as 100% (Figure 24B). For the schematic representation of the analysis see figure 28 (appendix 9.22. Raw data).

The highest yield was achieved with the 1000 cells/µl. Approximately 90% of the total yield was collected in the first step (combined yield of first lysis and wash step) (Table 21).

Table 21: Mean (% of molecules), SD and number of replicates (n) from the direct lysis on the DC01, evaluating the best cell density using Triton/BSA

		Mean	SD ±	n
1000 cell/µl	1. lysis	44.82	5.2490	3
	1. wash	47.58	7.5590	3
	2. lysis	5.42	1.4850	3
	2. wash	2.18	0.8741	3

500 cells/µl	1. lysis	22.66	0.8439	3
	1. wash	63.14	2.5070	3
	2. lysis	7.64	0.1021	3
	2. wash	6.56	1.8770	3
250 cells/µ	1. lysis	40.29	9.7900	3
	1. wash	21.44	3.4320	3
	2. lysis	30.10	7.1050	3
	2. wash	8.17	3.0660	3

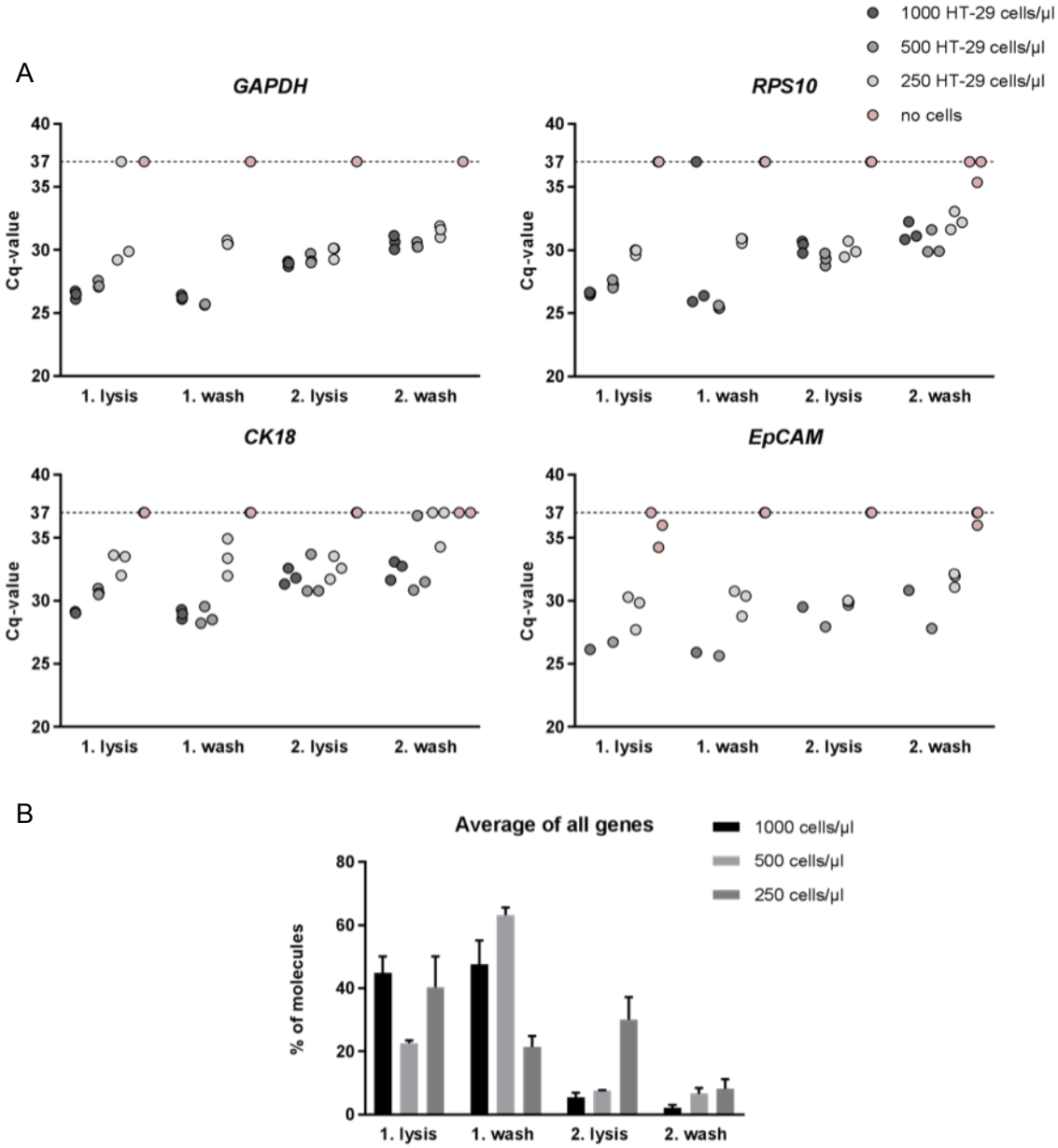

Figure 24: Evaluating the most effective cell density for the direct lysis on the DC01 by freezing over night using the BSA/Triton lysis solution. 1000, 500 and 250 cells/µl (20 µl in total) were loaded on the DC01 and lysed by freezing over night with the BSA/Triton lysis solution. Two lysis and wash steps were performed per cell density and with the thawed lysate an RTqPCR was performed. (A) Relative cDNA yields are presented as Cq-values, PCR specific products with a Cq-value > 36 were set to 36 (representing 1 transcript). Negative qPCR reactions were set to Cq-values of 37. The expression is getting lower from the first to the second lysis step (depending on the condition). **(B)** Cq-values were converted into relative transcripts, all replicates and genes were averaged and all lysis and wash steps were summed up as 100%. The cell density with 1000 cells/µl shows the most effective results, approximately 90% of the total yield was collected in the first step (combined yield of first lysis and wash step) (Table 21).

5.6.4. Evaluating the most effective (yield) wash condition for the lysis on the DC01 using the BSA/Triton lysis solution and RNAse out- freeze over night

20 µl of a cell suspension containing 1000 HT-29 cells/µl were loaded on the wire and the lysis and the wash steps were united. Additionally RNAse out was added to the BSA/Triton lysis solution. 4 different conditions for the wash step were tested: washing the cells with 5 µl lysis soltution and twisting in the lysis solution (5 µl wash and twist), washing with 20 µl but without twisting (20 µl wash and no twist), washing with 20 µl and twisting (20 µl wash and twist) and no washing, but twisting (20 µl twist) in the lysate. Additional a negative control wire (containing no cells) was done. Two lysis steps were performed, the whole lysate was reverse transcribed into cDNA and three aliquots (1 µl each, representing 3.3% of the initial sample) were used performing a qPCR (*GAPDH, RPS10, CK18, EpCAM)*.

All obtained Cq-values are listed in table 54 (see appendix 9.22. Raw data). PCR specific products with a Cq-value > 36 were set to 36 (representing 1 transcript). Negative qPCR reactions were set to Cq-values of 37 (Figure 25A).

The Cq-value was converted into relative transcripts (using GenEx), the average of all genes was calculated and the two lysis steps per condition were summed up as 100% (Figure 25B). For the schematic representation of the analysis see figure 28 (appendix 9.22. Raw data). Twisting the wire in the lysis solution without a following wash step shows the most effective results. Almost 80% of the total yield is collected in the first lysis step. The other conditions are reaching not more than 60% in the first lysis step (Table 22).

Table 22: Mean, SD and n from direct lysis on the DC01, evaluating the most effectice wash conditions with BSA/Triton

		Mean	SD	n
5 µl (wash and twist)	**1. lysis**	49.31	1.7770	3
	2. lysis	50.69	1.7770	3
20 µl (wash)	**1. lysis**	60.46	2.4300	3
	2. lysis	39.54	2.4300	3
20 µl (wash and twist)	**1. lysis**	57.36	3.1400	3
	2. lysis	42.64	3.1400	3
20 µl (twist)	**1. lysis**	76.67	3.2530	3
	2. lysis	23.33	3.2530	3

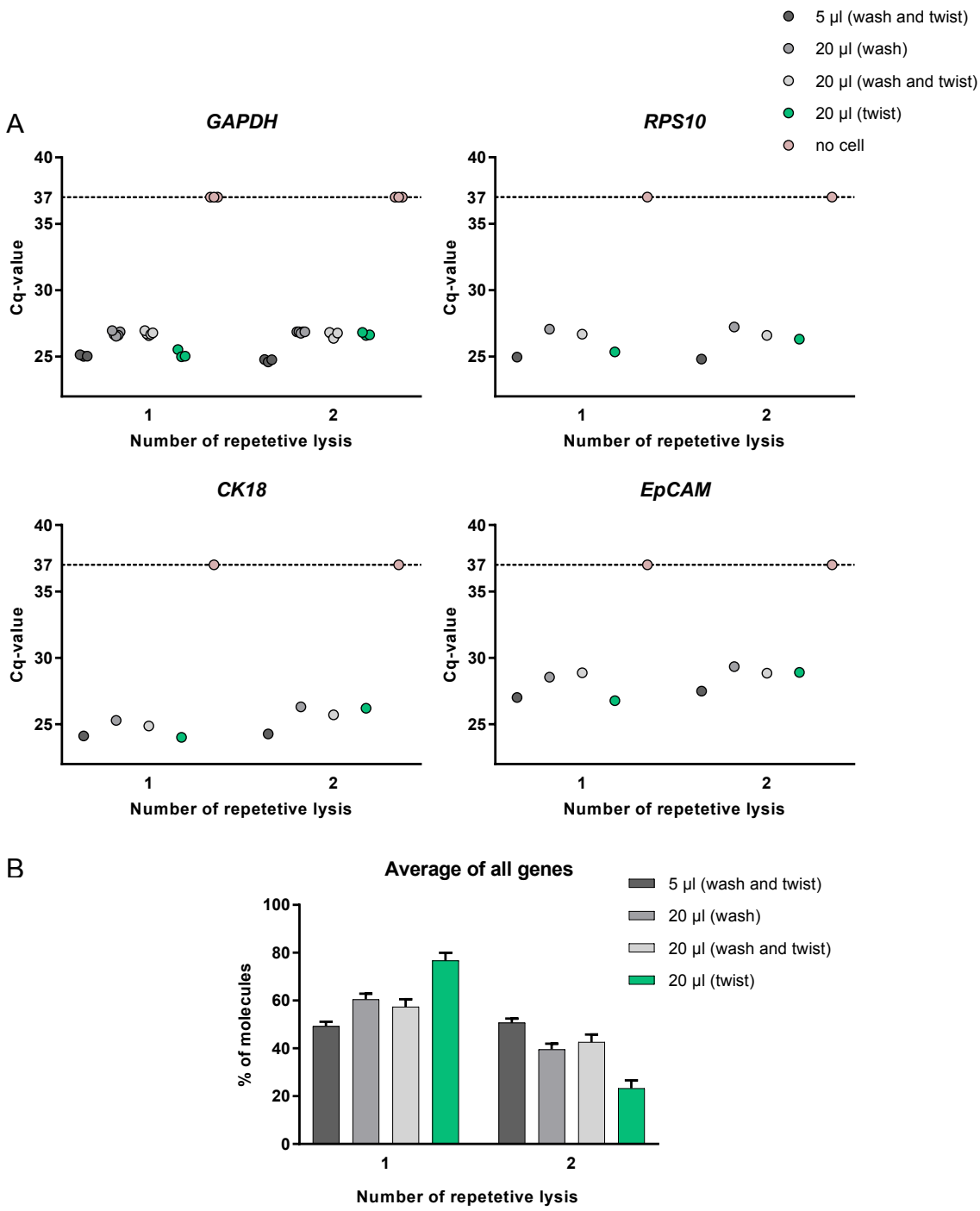

Figure 25: Evaluating the most effectice (yield) wash condition for the direct lysis on the DC01 by freezing over night using the BSA/Triton lysis solution. 1000 cells/µl (20 µl in total) were loaded on the DC01 and lysed by freezing over night with the BSA/Triton lysis solution. Four different conditions for the wash step were tested (5 µl wash and twist, 20 µl wash, 20 µl wash and twist, twist) and additional a negative control was performed (no cell). Two lysis and wash steps were performed, united and the thawed lysate was used for RTqPCR. **(A)** Relative cDNA yields are presented as Cq-values, PCR specific products with a Cq-value > 36 were set to 36 (representing 1 transcript). Negative qPCR reactions were set to Cq-values of 37. The expression is getting lower from the first to the second lysis step. **(B)** Cq-values were converted into relative transcripts, all genes were averaged and all lysis and wash steps were summed up as 100% (Table 22). The condition without a wash step shows the most effective results. Almost 80% of the total yield is collected in the first lysis step (Table 22).

5.6.5. Lysis on the DC01 using the BSA/Triton lysis solution in the presence of an RNase inhihitor (RNAse out, no wash step) - freeze over night

After evaluating the most effective (yield) condition for the lysis on the wire (performing no wash step), this condition was performed again with 5 different wires and additional a negative control (no cells). RNAse out was added again to the BSA/Triton lysis solution. Three lysis steps were performed with freezing over night at -80°C directly in the Pasteur pipette. After thawing the wire was twisted a few times in the lysis solution, the solution was pipetted in and out and then collected. The whole lysate was reverse transcribed into cDNA and three aliquots (1µl each, representing 3.3% of the initial sample) were used for qPCR (*GAPDH, RPS10, CK18, EpCAM)*.

All obtained Cq-values are listed in table 55 (see appendix 9.22. Raw data). PCR specific products with a Cq-value > 36 were set to 36 (representing 1 transcript). Negative qPCR reactions were set to Cq-values of 37 (Figure 26A).

The negative control (no cells) shows a signal with *CK18* (Cq = 32.73, Cq = 44.92, Cq = 36.07; Figure 26A*)*.

The Cq-value was converted into relative transcripts (using GenEx), then the average of all genes was calculated and the two lysis steps were summed up as 100% (Figure 26B). For the schematic representation of the analysis see figure 28 (appendix 9.22. Raw data). Wire #3 was excluded, because of handling mistakes.

Twisting the wire in the lysis solution without a following wash step leads to 56.74% of the total yield in the first lysis step (Table 23).

Table 23: Mean (% of molecules), SD and number of replicates (n) of the averaged genes from the lysis on the DC01 with BSA/Triton and no additional wash step.

	Mean	SD ±	n
1. lysis	56.74	16.81	4
2. lysis	29.82	11.33	4
3. lysis	13.44	6.41	4

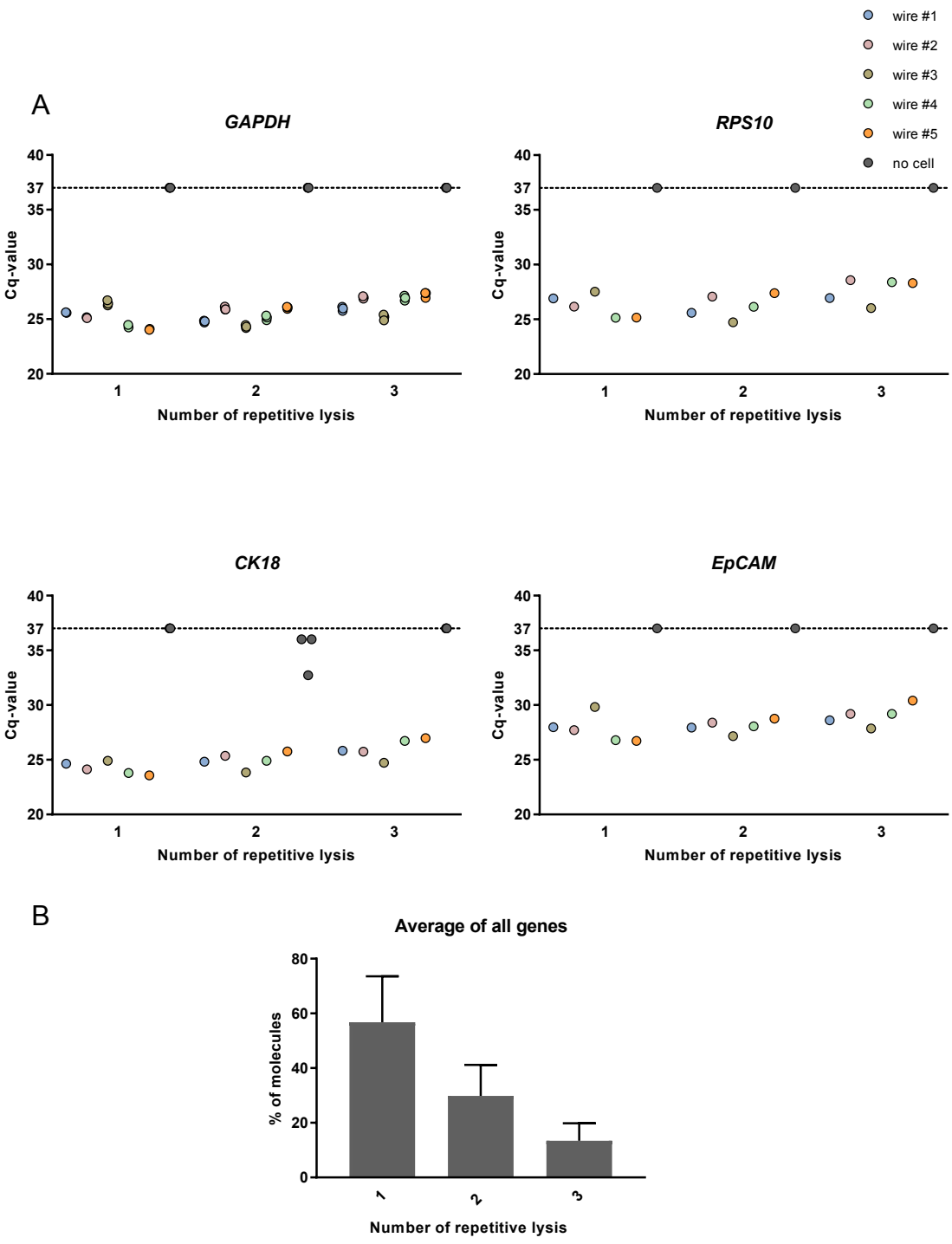

Figure 26: Direct lysis on the DC01 by freezing over night using the BSA/Triton lysis solution, without an additional wash step. 1000 cells/µl (20 µl in total) were loaded on the DC01 and lysed by freezing over night with the BSA/Triton lysis solution. No additional wash step was performed, but a negative control (no cells) was used. Three lysis steps were done and the thawed lysate was used for RTqPCR. **(A)** Relative cDNA yields are presented as Cq-values, Cq 37 represents no transcripts and Cq-values higher than 36 are modified to 36. The expression is getting lower from the first to the third lysis step. **(B)** Cq-values were converted into relative transcripts, all genes were averaged and all lysis and wash steps were summed up as 100% (Table 23). Twisting the wire in the lysis solution without a following wash step leads to 56.74% of the total yield in the first lysis step.

6. Discussion

6.1. Evaluation of epithelial - (*CK18, EpCAM*) and leukocyte - specific (*CD45*) marker expression in blood and HT-29 cells

First step was to evaluat the expression of *EpCAM* and *CK18*, which are marker genes for CTC's, and the expression of the leukocyte specific marker gene *CD45*. The evaluation should show if we could use these genes for further analysis.

As expected epithelial markers (*EpCAM, CK18*) are higher expressed in the human colon adenocarcinoma cell line HT-29, than in the blood cells. One replicate of the blood cells shows an expression of the epithelial marker *EpCAM*. This could be attributable to a crosscontamination in this replicate or to the heterogeneity of the single cells. The epithelial marker *CK18* shows also an expression in the blood cells (Figure 8). Here a contamination is unlikely, because each replicate of the 100 and 500 MNCs shows a signal (n=5) and also the qPCR negative control is negative. Probably the blood cells express also *CK18,* but than *CK18* is not a good specific epithelial marker. The expression of blood cells is in general lower than the expression of tumor cells (Cq_{GAPDH} = 27.81 vs. Cq_{GAPDH} = 22.77). *CD45* is a leukocyte specific gene marker and is only expressed in the leukocytes, only one replicate of the HT-29 cells shows a signal. This is also attributable to a crosscontamination or the HT-29 also express *CD45*.

In summary, our data show, that *EpCAM, CK18* and *CD45* are good marker genes, but not always on single cell level.

6.2. RT performance

With the RT performance test we wanted to know what effect BSA has on the PCR. Svec *et al.* already revealed that BSA performs best compared to other PCR additives [37]. BSA is an enhancer in PCR, it also binds inhibitors and leads to a reduction of them in the PCR [38,39].

In this work different solutions for the lysate dilution were tested (0.5 mg/ml BSA, 0.75 mg/ml BSA, no BSA) and it shows that the RTqPCR performed best with the presence of BSA.

6.3. GTC lysis solution test

Chen *et al*. tested different lysis solutions for the direct lysis on the DC01. The GTC lysis solution (0.3 M GTC, 0.5% Triton X-100, 1 mg/ml BSA) was evaluated as the most effective lysis solution for the direct lysis on the DC01. With GTC as an additive the lysis could be performed more effecticely. However, increased levels of GTC (80 mM) lead to PCR inhibition [37]. The lysis solution has to be diluted with a dilution factor of 12.5 to avoid

a loss of the PCR efficiency (Chen *et al.*, data not published). Here also different concentrations of GTC in RTqPCR were tested. The higher the concentration of GTC in RTqPCR, the lower is the PCR efficiency (Figure 9). Samples diluted to 0.01 M GTC (yield = 93.44% in PCR) do not significantly different to samples lacking GTC (1 mg/ml BSA only; yield = 100% in PCR). With a concentration of 0.3 M and 0.15 M GTC the qPCR is completely inhibited (Figure 9).

In summary, our data show that the GTC lysis solution has to be diluted to avoid an inhibition of the PCR.

6.4. Inhibition test: HT-29 cells sorted in a background of erythrocytes

It is possible, that the CellCollector 01 is contaminated with blood, after it is extracted from the vein of a patient and with the inhibition test we wanted to show if the erythrocytes have an effect on the PCR.

Al-Soud *et al.* revealed that content of the erythrocytes lead to an inhibition of the PCR. The main inhibitor in erythrocytes is hemoglobin. It is a ferrous protein complex and the iron ions reduce the PCR efficiency [40]. With the addition of BSA the inhibition of DNA amplification can be reduced. This is possibly attributable to the high binding efficiency [41].

For the inhibition test with erythrocytes the GTC lysis solution was used, which contains 1 mg/ml BSA. The data show that with BSA in the solution the erythrocytes seem to have no influence on the DNA amplification (mean $Cq_{0\ cells,CK18}$ = 28.06 vs. $Cq_{1000\ cells,CK18}$ = 28.11). It makes no difference if there are no or 1000 erythrocytes in the background, the efficiency of the RTqPCR remains the same.

6.5. Sensitivity test: HT-29 and VCaP cells sorted in a background of MNCs

We also performed a sensitivity test to see what effect leukocytes have on the PCR.

It is already known, that the leukocytes inhibit the PCR. A main cause for the PCR inhibition with MNCs in the background are ribonucleases from leukocytes, like the eosinophil-derived neurotoxin (EDN) [42]. Beside the fact that leukocytes lead to an inhibition of the PCR the detection of a single cell can be reduced by the presence of a too high number of other cells in the background [43]. This would also explain why it comes to an inhibition with the genes *GAPDH, AR* and *ARv7*, but not with the leukocytes specific marker gene (*CD45*) (Figure 16).

Our data show that less than 100 MNCs in the background seem to have no influence on the PCR (Figure 16). According to previous experiments in patients 0-500 CD45 positive cells (in rare cases up to 1000 CD45+ cells) were counted on the DC01, but mostly less than 100 (not published, informations are available at Chen Shukun and Kroneis

Thomas). If less than 100 CD45 positive cells would be on the wire it would not influence our on wire lysis experiment.

In the sensitivity test we used high (*CK18*) and low *(ARv7*) expressed marker genes.

As shown in figure 8 the evaluation of epithelial specific (*CK18, EpCAM*) marker genes were not exclusively expressed in the tested cancer cell line cells. *CK18* and *EpCAM* were also expressed in leukocytes. So we decided to use an additional marker gene, with a lower expression and a higher specifity, the *ARv7,* which is expressed in the human prostate cancer cell line VCaP. Although initial tests (Figure 15), the analysis of one single VCaP cell in the background of 0-1000 MNCs failed to obtain *ARv7* when using the GTC lysis solution procedure. This failure is attributable to the high dilution (dilution factor = 12.5), which is necessary with the GTC lysis solution. Thus, we switched back to the Triton/BSA lysis solution to circumvent this drawback and implemented an on-wire freeze/thaw cycle allowing us to use the lysate in the RT reaction without further treatment.

6.6. Comparison of FACS, Micromanipulation and Microdissection

After we emphasized that the *ARv7* is too low expressed for the GTC lysis solution we compared three different single cell isolation methods (FACS, micromanipulation and microdissection) to ensure, that during FACS no degradation of the RNA happens. We evaluated FACS as the most effective method for spiking single VCaP cells in Triton/BSA lysis solution (R^2 = 0.9873). Only 13 of 30 samples containing single VCaP cell show *Arv7* expression (Figure 18), this is attributable to the low expression of the marker gene. The microdissection showed also good data (R^2 = 0.8403), but the micromanipulation has turned out as not suitable (R^2 = 0.5536). A lot of cells did not survive the micromanipulation. Additionally the micromanipulation was only performed ones and maybe something went wrong with the handling.

6.7. Evaluation of the direct lysis on the DC01

The evaluation of the direct lysis on the DC01 was performed considering all previous tests.

Based on previous data obtained by Shukun Chen the GTC lysis solution for the direct lysis on the DC01 was used and we aimed at reproducing 80% of the total yield in the first lysis step (data not published).

We lysed only approximately 55% of total yield in the first lysis step. The difference between these two experiments could be explained by the different number of cells in the cell suspensions. The more cells are attached to the wire, the higher is the difference between first and second lysis step.

Data from the sensitivity test showed that low expressed transcripts such as *ARv7* in VCaP cells (Figure 15) escape detection when using GTC lysis solution. To detect something in the PCR with the GTC lysis solution 1500 transcripts per sample are necessary. One VCaP cell line cell has only 1.2 *Arv7* transcripts, which is why a detection wih the GTC lysis solution is not possible [44]. Thus, lowly expressed genes long for a more sensitive method and so we used the BSA/Triton lysis solution approach for the direct lysis on the DC01. To optimize the lysis efficiency of the BSA/Triton lysis solution we had to implement an on wire freeze step performed directly in the Pasteur pipette.

With freezing the lysis solution for 1 min we harvested approximately 45% of total yield in the first lysis step. To optimize this yield we started an experiment, where we froze the lysis solution on dry ice and subsequently stored the wire/Pasteur pipette assembly at -80° over night. Additionally we evaluated the most suitable cell density for evaluating lysis efficiency and RNA recovery of the different lysis approaches. From our data we conclude that applying 15 – 30 µl of cell suspensions containing 1000 cells/µl results in sufficiently high numbers of cells being attached to the wire. Applications of lower cell densities results in a lower number of cells attached to the wires and, thus, reduced RNA yield. In the *CK8* data (Figure 22) we saw that an initially low number of transcripts cause low percentages of transcripts in the first lysis step as compared to transcripts starting from initially high numbers of transcripts (e.g. *CK18*; Figure 22). Therefore, *CK8* assay was excluded from analysis.

With this protocol we figured out, that the 1000 cells/µl (20 µl in total) performed best. With this cell density approximately 100 cells bind on the wire. We did not count the wire with the cell density of 250 cell/µl. For the sake of convience we assume that cell attachment rates do not differ. Thus, the number of attached cells would be approximately 25. Only 3.3% of the sample ends up in the qPCR.

This low cell density and the following low number of cells attached to the wire could be the cause for the bad performance (Figure 24).

With 1000 cells on the wire around 45% of total yield was lysed in the first lysis and wash step. The cause for the similar yields in the lysis and wash step could be the missing mechanical movement (DC01 was not twisted in the lysis step). Taken together yield recovery of both steps (lysis and wash) sums up to 80% of the total transcript. 2 of 3 control wires (7 of 96 samples; no cells loaded; negative control) showed expression (Cq 32 – Cq 45) of Gene *CK18, RPS10* and *EpCAM* (7 of 96 samples), but the qPCR negative control is negative. The cause for the contamination could be a crosscontamination, which happened during opening the 96-well plate with the lysate.

According to these results we decided to go on with this “freezing over night” protocol, but added an RNAse inhibitor to avoid possible degradation of RNA after cell lysis.

For further optimization of the lysis procedure we evaluated different approaches to perform the lysis and washing procedure of the DC01. 4 different conditions for the wash step were tested: washing the cells with 5 µl lysis solution and twisting in the lysis solution (5 µl wash and twist), washing with 20 µl but without twisting (20 µl wash and no twist), washing with 20 µl and twisting (20 µl wash and twist) and no washing, but twisting (20 µl twist) in the lysate. According to our data the lysis without washing the DC01 showed the best results ($Cq_{EpCAM,20\ µl\ twist,\ 1.\ lysis}$ = 25.87 vs. $Cq_{EpCAM,20\ µl\ twist\ and\ wash,\ 1.\ lysis}$ = 28.76, $Cq_{EpCAM,20\ µl\ wash,\ 1.\ lysis}$ = 28.05, $Cq_{EpCAM,5\ µl\ wash\ and\ twist,\ 1.\ lysis}$ = 25.93; Figure 25). Nearly 80% of the total yield was lysed in the first lysis step, corresponding to the 80% we wanted to achieve with the GTC lysis solution. The other conditions are reaching not more than 60% in the first lysis step (Table 22).
Loading 20 µl of 1000 cells/µl on the DC01, snap-freezing, O/N storage at -80° recovery of the lysate without rinsing the wire was evaluated as the most effective protocol for the direct lysis on the DC01. Thus, we performed another five experiments to confirm the initial data. One wire excluded from analysis due to fact that the added volume of NF-H_2O in the RT was not noted. Averaging the data of the remaining four wires and all genes in the recovery of 60% of the total yield. In summary, the GTC lysis solution can only be used for high expressed genes and for low expressed genes the BSA/Triton lysis solution must be used.

The CellCollector is already used for *in vivo* applications in lung, breast and prostate cancer and shows better results as the CellSearch system, which is known as the golden standard for CTC isolation from peripheral blood [20]. Until yet no further downstream analysis is possible in clinical applications. A ray of hope is the direct lysis on the DC01, where the cells are lysed directly on the wire and used for further RTqPCR. The detection of *ARv7* in patients could indicate, that chemotherapy would be more effective than a hormonal cancer therapy (enzulutamid and abiraterone) [11].

With this master thesis it was possible to optimize the protocol for the direct lysis on the DC01 and to bring it one step closer to clinical applications. It is necessary to differentiate between high and low expressed marker genes, because the protocol using the GTC lysis solution is not suitable for low expressed genes.
Further steps would include evaluation of the direct lysis protocol in metastasized prostate cancer patients. The purpose of this clinical device is to allow personalized cancer treatment, in return more targets for therapy relevant transcripts should be identified, like the *ARv7* or *ARv9* [45] in prostate cancer.

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8. Shortcut Index

ADT	androgen deprivation therapy
AR	androgen receptor
Arv7	AR variant 7
BSA	bovine serum albumin
C	Celsius
cDNA	complementary DNA
CTC	circulating tumor cells
CK	cytokeratin
cm^2	square centimeter
CO_2	carbon dioxide
Cq	quantification cycle
CRPC	castration-resistant PCa's
CYP17A1	cytochrome P450 17A1
DAPI	4',6-Diamidin-2-phenylindol
DC01	detector CANCER01
dsDNA	double stranded DNA
DMEM	dulbecco's modified eagle medium
DMSO	dimethylsulfoxid
DNA	deoxyribinucleic acid
dNTP	deoxynucleosidtriphosphate
DTC	disseminated tumor cells
DTT	dithiothreitol
EDN	eosinophil-derived neurotoxin
EMT	epithelial-to-mesenchymal transition
EpCAM	epithelial cell adhesion molecule
EtOH	ethanol
FACS	fluorescence-activated cell sorting
FBS	fetal bovine serum
GAPDH	glycerinaldehyde-3-phosphate
GLT	gel loading tip
GTC	guanidine thiocyanate
h	hour
HEPES	2-(4-(2-Hydroxyethyl)-1-piperazinyl)-ethansulfonsäure
HT-29	human colorectal adenocarcinoma cell line
HYB	hybridisation
M	molare Masse

MET	mesenchymal-to-epithelial transition
mg	milligram
min	minutes
ml	milliliter
mm	millimeter
MM	master mix
MNCs	mononuclear cells
NF-H_2O	nuclease free water
PBS	phophate buffered saline
PCa	prostate cancer
PCR	polymerase chain reaction
µl	microliter
µm	micrometer
qPCR	quantitative real-time PCR
RNA	ribonucleic acid
RPS10	ribosomal protein S10 variant 2
RT	reverse transcription
Rtemp	room temperature
U	Units
UV	ultraviolet light
VCaP	human protate cancer cell line

9. Appendix

9.1. Cell culture – HT-29 cell line

9.1.1. Thawing of the cells

- Switch on the laminar flow
- Clean everything in the working bench with 70% EtOH
- Fill 10 ml of cold medium into a 50 ml centrifuge tube
- Pre-warm the rest of the medium 20 min in the water bath (37°C)
- Take the cryovial with the correct cell line out of the liquid nitrogen and thaw in the water bath by shaking for 1-2 min, until just a bit of the ice is left
- Clean the cryovial with 70% EtOH
- Transfer the cell suspension into the pre-filled 50 ml centrifuge tube, resuspend and centrifuge (1200 x g, Rtemp, 5 min)
- Fill 5 ml of the pre-warmed medium into a 25 cm_2 cell culture flask (lable the flask with cell name, passage number, date and name)
- When the centrifuge is ready, remove the supernatant with a sterile Pasteur pipette and loose the pellet with the fingers
- Add 2 ml of pre-warmed medium to the pellet and resuspend 20-30 times (always with the 1 ml pipette)
- Transfer the whole cell suspension into the pre-filled cell culture flask, shake a little bit and check the cells under the microscope
- Put the cells into the incubator (37°C)
- Change the medium on the next day to remove the DMSO residual (DMSO is toxic at Rtemp)
- Split the cells when they are 80-100% confluent

9.1.2. Splitting of the cells

- Switch on the laminar flow
- Clean everything in the working bench with 70% EtOH
- Pre-warm a aliquot of the medium and a aliquot of the 1x PBS 20 min in the water bath (37°C)
- Put a aliquot of the accutase at Rtemp and pre-warm 1-2 min in the waterbath (the accutase is getting inactive after 30 min at 37°C)
- Remove the medium with a sterile Pasteur pipette
- Wash the cells with 5 ml 1x PBS
- Remove the 1x PBS with a sterile Pasteur pipette

- Add 1.5 ml of the accutase
- Incubate the cells in the incubator (37°C) until the cells are detached from the ground of the cell culture flask (10-15 min)
- In the meantime fill 5 ml of the pre-warmed medium into a new cell culture flask (lable the flask with cell name, passage number, date and name)
- Add 3.5 ml of the pre-warmed medium to the detached cells
- Transfer the cell suspension into a new 50 ml centrifuge tube and resuspend 20-30 times
- Transfer 1 ml (or less) of the cell suspension to the pre-filled cell culture flask
- When the splitting rate is 1:5 the cells are confluent after 2 days
- Change the medium 2-3 times per week

9.1.3. Freezing of the cells

- Swith on the laminar flow
- Clean everything in the working bench with EtOH
- Prepare the freeze medium: 10% DMSO + 90% FBS
- Filter the freeze medium with a 0.2 µm filter
- Dissociate the cells with accutase
- Count the cells
- Centrifuge the cell suspension (1200 x g, Rtemp, 5 min)
- Resuspend the pellet in the correct volume of the freeze medium
- Transfer 1 ml of the cells suspension and the freeze medium into a cryovial (label the cryovial with the cell name, passage number, date, cell amount and name)
- 2 x 10^6 cells/ml
- Store the cryovial in a CoolCell for 24-72h
- Transfer the cryovial into the liquid nitrogen

9.1.4. Cell counting

- Clean the „neubauer“ counting chamber and one glass cover slip with 70% EtOH
- Wet the counting chamber a little bit, put the glass cover slip on it and press firmly
- You can see „newton rings“, when the glass cover slip fits correctly
- Mix 10 µl of the cell suspension with 10 µl trypan blue (trypan blue dyes dead cells)
- Incubate 2-3 min

- Resuspend and transfer 10 µl of the cell suspension and the trypan blue directly under the glass cover slip
- Put the counting chamber under the light microscope
- The counting chamber consists of 9 big squares (area = 1 mm^2) and the 4 squares at the corner are subdivided in 16 small squares. This 4 squares at the corner are counted.
- Calculation:
  Calculate the average of the 4 squares.
  Dilution factor = 2 (cell suspension and trypan bue)
  Average x 2 x 10^4 = cell number/ml
  Cell number/ml x volume of the whole cell suspension

9.2. Cell culture – VCaP cell line

The protocol is the same as in chapter 9.1. Cell culture - HT29 cell line.

Deviations:

Splitting of the cells:

- Count the cells
- Spitting rate: 20 x 10^5 – 40 x 10^5 cells/cm^2
- Cells are confluent after one week

9.3. Solutions

- GTC lysis solution

Table 24: Components of the GTC lysis solution

Reagents	Volume [µl]	Final concentration
Distilled H_2O	4475	
GTC (6 M)	250	0.3 M
BSA (20 mg/ml)	250	1 mg/ml
Triton X-100	25	0.5%
total	**2500**	

- BSA/Triton lysis solution

Table 25: Components of the BSA/Triton lysis solution

Reagents	Volume [µl]	Final concentration
Distilled H_2O	4740	
BSA (20 mg/ml)	250	1 mg/ml
Triton X-100	10	0.2%
total	**5000**	

- 1% BSA for the lysate dilution

Table 26: Components of 1% BSA

Reagents	Volume [µl]	Final concentration
Distilled H_2O	4750	
BSA (20 mg/ml)	250	1 mg/ml
Total	**5000**	

9.4. Density gradient centrifugation

- Resuspend the blood and note the volume
- Dilute the blood 1:1 with 1x PBS
- Pipette the density gradient solution in 50 ml tubes (the same volume as blood at the beginning)
- Overlay the density gradient solution with blood and 1x PBS slowly: keep the tube at an angle and let the blood run off the edge (using long blue tips)
- Centrifugation (30 min, Rtemp, 800 x g, without breaks)
- Remove MNCs (buffy coat) and the erythrocytes (pellet)
- Transfer each into separate 15 ml tubes
- Fill up the tube with 1x PBS (10 ml)
- Centrifugation (10 min, 300 x g, Rtemp, full breaks)
- Remove supernatant and resuspend with 1x PBS
- Resuspend the erythrocytes in 10 ml and the MNCs in 1 ml 1x PBS
- Count the MNCs and fill up to 10 ml 1x PBS
- Centrifugation (10 min, 300 x g, Rtemp, full breaks)
- Dilute the MNCs with 1x PBS to 5 x 10^6 cells/ml
- Resuspend the erythrocytes in 10 ml 1x PBS (no need to count the cells)

9.5. Single–cell handling using FACS

Preparations:

- Prepare the lysis solution (GTC or BSA/Triton) one day before
- Harvest the cells
- Centrifuge the cell suspension (1200 x g, 5 min, Rtemp)
- Remove supernatant and resuspend in 5 ml 1x PBS
- Count the cells
- Centrifuge the cell suspension again (1200 x g, 5 min, Rtemp)
- Dilute the cells with 1 x PBS to 5 x 10^6 cells/ml
- Prepare the 96 well plates: fill 5 µl lysis solution in each well and seal with a film
- Store at 4°C
- Density gradient centrifugation with the blood (see 9.3. Density gradient centrifugation)

The FACS was carried out at the Core Facility Imaging/Team Flow, Center of Medical Research (ZMF).

- After the FACS the samples were stored immediately on dry ice and then at -80 °C for further downstream analysis.

Note: when the GTC lysis solution is used it is necessary to dilute the lysate (dilution factor = 12.5) with 1 mg/ml BSA before performing an RTqPCR.

9.6. Cytospin

- Beam the object carrier with UV-light (for following lasermicrodissection only)
- Put the object carrier into a chipboard, place the correct filter paper on top, place the cyto chamber on this and fix everything with a clamping ring (size of the chamber: 120 mm^2)
- Count the cells
- 1 x 10^5 cells per 300 µl 1x PBS are centrifuged on the glass slides
- First centrifugation step: 300 x g, 5min, Rtemp
- Take up the supernatant
- Remove the clamping ring and the cyto chamber
- Tilt the chipboard with the object carrier, that the filter paper can soak up the liquid
- Second centrifugation step: 1140 x g, 1min, Rtemp

- For micromanipulation just one centrifugation step is necessary (no filter paper to soak up the liquid): 370 x g, 3 min, Rtemp

9.7. Lasermicrodissection

- Prepare the lysis solution (1% BSA/0.2% Triton) one day before
- Switch on the microscope (Observer Z1) and the computer
- Open the program PalmRobo 4.5
- Harvest the cells and make a cytospin with the cell suspension
- Put the slide with the cell suspension under the microscope
- Fill 5 µl of the lysis solution into the cap of a 0.2 ml PCR tube
- Put the PCR tube in the holder, provided for it
- Cut out the desired number of cells
- Transfer the cut-out cells into the lysis solution
- Check the liquid for the presence of microdissected cells
- Store on dry ice and later at -80°C for further downstream analysis

9.8. Micromanipulation

- Prepare the lysis solution (1% BSA/0.2% Triton) one day before
- Switch on the computer and the microscope (Axiovert M200)
- Open the program Palm Robo 3.0
- Fill 5 µl lysis solution in a 0.2 ml PCR tube
- Change the capillary from the micromanipulator
- Harvest the cells and make a cytospin with the cell suspension (without soaking up the liquid)
- Use a grease pen to make a rectangle area beside the cell suspension
- Place the slide under the microscope
- Add 100 µl 1x PBS into the rectantgle area
- Wash the capillary with FBS to prevent that the cells stick on it
- Focus the capillary and get the right position
- Soak up 10-15 cells from the cell suspension
- Wash in 1x PBS
- Soak up the desired cell number and micromanipulate 1 µl into 5 µl lysis solution
- Put on dry ice
- Store at -80°C for later use

9.9. Cell staining

- Harvest the cells with accutase
- Wash the cell pellet once
- Resuspend the pellet in 1 ml Hoechst 33342
  (stock 1:100 diluted in 1x PBS; work dilution = 1:10.000; final concentration = 0.001 mg/ml)
- Transfer into a 1.5 ml tube
- 10 min, 37°C (water bath)
- 300x g, 3 min, Rtemp
- Resuspend the pellet in 1ml 1x PBS and transfer into a 15 ml tube
- Add 4 ml 1x PBS and thoroughly mix by pipetting
- Cell counting
- 300x g, 3 min, Rtemp
- Pellet resuspension in 1x PBS to adjust the cell density

9.10. Attaching cells to DC01

- Harvest the HT-29 cells and adjust the cell density in 1x PBS
- Store on ice
- Fix the Pasteur pipette (150 mm, Volac) onto a rack with plastic tape
- Insert the DC01 to the Pasteur pipette, leave 1 mm space between the two tip ends (to avoid pressure)
- Adjust the other end to make sure that the yellow stopper does not close the pipette tightly to avoid inner pressure
- Resuspend the cell suspension
- Using gel loading tips (GLT) to load 20 µl cell suspension from the sharp tip of the pipette and incubate for 10 min
- During incubation, twist the DC01 a little bit and make sure that the whole length of functional part is covered with suspension
- Remove the DC01 from the pipette and wash the tip with 5 ml of 1x PBS
- Keep the DC01 in 1x PBS before further steps

9.11. Counting of the cells on the DC01

- Switch on the computer and the microscope (Axio Observer Z1)
- Switch on the fluorescence light and open the program Zen 2 (blue editon)
- Use a grease pen to make a rectangle area on a cover slide
- Add 200 µl of 1x PBS

- Insert the functional part of the DC01 into 1x PBS and fix the non-functional part with a double-faced adhesive tape
- Use the DAPI filter to count the cells
- When the first side of the wire is counted, rotate the wire 90 degrees and count again

Note:
Do not let the wire expose to dry conditions.

9.12. Lysis on the DC01 using GTC lysis solution

- Prepare the GTC lysis solution and 1% BSA for the lysate dilution one day before
- Insert the DC01 to a new Pasteur pipette, adjust the position again on a rack with plastic tape
- Loading 20 µl of lysis solution from the tip of the pipette and incubate for 10 min
- Twist the wire a few times during incubation
- After 10 min, carefully pipette-in and -out for ~5 times, and collect the lysate (12-15 µl) to a 0.2 ml PCR tube (or a 96 well plate) and place the tube immediately on ice
- Using 20 µl of 1 mg/ml BSA to rinse the functional part and collect the rinsing solution into the PCR tube (on ice)
- Repeat the rinse step for additional 2 times and add 170 µl of 1 mg/ml BSA to the tube (final volume: 250 µl, dilution factor is 12.5); mix the samples thoroughly and place the sample immediately on dry ice.
- Repeat the lysis step a few times with a new Pasteur pipette WITHOUT washing in between.
- Store the samples at -80 at least overnight for RT-qPCR.

Note:
Do not let the wire expose to dry condition.

9.13. Lysis on the DC01 using the BSA/Triton lysis solution with a subsequent freeze/thaw cycle

- Prepare the BSA/Triton lysis solution one day before

Lysis step:

- Insert the DC01 to a new Pasteur pipette, adjust the position again on a rack with plastic tape
- Load 20 µl of the lysis solution from the tip of the pipette, twist the wire and put the Pasteur pipette with the wire on the freezing metal rack

- After freezing (lasts less than 1 min), put the Pasteur pipette again on a rack with plastic tape and wait until the lysis solution is thawed
- Carefully pipette-in and -out (with GLT) for ~5 times
- Collect the lysate (12-15 µl) to a well of a 96 - well plate and put it on normal ice
- Tilt the Pasteur pipette/wire construct and remove the wire to the back. Lead the tip along the pipette glas and try to peel of the residual liquid on the glass. Take up the residual liquid at the tip of the Pasteur pipette (with GLT) and add to the same well (-> take up the residual liquid also after the wash step)

Wash step - to see how many cells remains on the DC01 and in the Pasteur pipette:

- Insert the DC01 into the same Pasteur pipette and load 20 µl of the lysis solution from the tip of the pipette
- Twist the wire
- Carefully pipette-in and -out for ~5 times, and collect the lysate (12-15 µl) to a new well
- Repeat the lysis step and wash step a few times with a new Pasteur pipette WITHOUT washing in between
- Store the samples at -80 overnight for RT-qPCR.

Note:
Do not let the wire expose to dry condition.

9.14. Lysis on the DC01 using BSA/Triton - freeze over night

- Prepare the BSA/Triton lysis solution one day before

First day

1. lysis step:

- Insert the DC01 to a new Pasteur pipette, adjust the position on a rack with plastic tape
- Load 20 µl of the lysis solution from the tip of the pipette, twist the wire and put the Pasteur pipette with the wire on the freezing metal rack
- After freezing (lasts less than 1 min), store the Pasteur pipette immediately into a box with dry ice and then at -80 °C (over night).

Second day

Harvest the cells:

- Put the Pasteur pipette again on a rack with plastic tape and wait until the lysis solution is thawed
- Carefully put the DC01 out of the Pasteur pipette, pipette-out (with GLT), collect the lysate (12-15 µl) to a well of a 96 - well plate and put it on normal ice

Wash the DC01:

- Load 20 µl of lysis solution in the same Pasteur pipette. Insert the DC01 into the Pasteur pipette, twist a little bit, carefully pipette-in and –out for ~5 times, and collect the wash fraction (12-15 µl) to a new well of the 96 – well plate

2. lysis step:

- Insert the DC01 to a new Pasteur pipette, adjust the position on a rack with plastic tape
- Load 20 µl of the lysis solution from the tip of the pipette, twist the wire and put the Pasteur pipette with the wire on the freezing metal rack
- After freezing (lasts less than 1 min), store the Pasteur pipette immediately into a box with dry ice and then at -80 °C (over night).
- Store also the 96 well plate with the first lysate at -80 °C

Third day

- Harvest the lysate and wash the DC01 like the day before
- Store the samples at -80 or use immediately for RT-qPCR

ALWAYS:

Put up the residual liquid after every wash and lysis step (see 9.13. Lysis on the DC01 using the Triton/BSA lysis solution with a subsequent freeze/thaw cycle)

Note:

Do not let the wire expose to dry condition.

9.15. Lysis on the DC01 using BSA/Triton and an RNA inhibitor- freeze over night

The Protocol is the same as in chapter 9.14. Lysis on the DC01 using Triton/BSA - freeze over night.

Deviations:

- Add 0.7 µl RNAse out per 20 µl lysis solution > make a MM (fresh)
- Collect the lysate and the wash fraction together in one well of a 96 well plate
- 4 different szenarios for the wash step (lysis step is the same):

  #1: 5 µl > wash and twist

  #2: 20 µl > wash (without twisting)

  #3: 20 µl > wash and twist

  #4: no wash step, but additional twisting the DC01 in the lysate and pipette in and out (~5 times)

  Treat the negative control (without cells) the same way as #4

- Repeat for additional lysis and wash steps

9.16. NanoDrop

- Switch on the computer
- Open the program ND-1000
- Set on RNA or DNA
- Initialize and measure the blank (follow on the introduction of the NanoDrop): pipette 1.5 µl of NF-H_2O on the measurement point
- Click blank
- Clean the measurement point
- Pipette 1.5 µl NF-H2O again on the measurement point (as reference)
- Name the sample
- Click measure
- Measure the other samples the same way as the reference
- The results are given in ng/µl
- When each sample is measured, clean the measurement point with NF-H_2O
- Print the results

9.17. Reverse Transcription using TATAA Kit

- Thaw the RNA lysate on ice
- Thoroughly mix the lysate
- Short spin down the lysate
- Store on ice
- Clean everything in the PCR workstation with RNAse Away
- Prepare the Master mix in the PCR workstation

Table 27: Components for the 10 µl reaction RT mix using the TATAA Kit

Reagents	1 reaction [µl]	Comments
NF-H_2O	2.5	
TATAA GrandScript RT Reaction mix (5x)	2	Invert and short spin
TATAA GrandScript RT Enzyme (20x)	0.5	Invert and short spin
Master mix in total	**5**	
RNA lysate	5	

- Vortex and short spin the MM
- Lay before 5 µl of the MM
- Close with a film
- Short spin
- Template loading area:
  add 5 µl of the lysate, a negative control and if necessary a positive control
- Close with a film
- Short spin
- Run RT in a thermal cycler

Table 28: Conditions for the RT with the TATAA Kit.

22°C	5 min
42°C	30 min
85°C	5 min
4°C hold	

- Store the cDNA at -20°C or use immediately for qPCR

9.18. Reverse Transcription using TATAA Kit (RNA spike-in)

The protocol is the same as for the normal RT, but additional spike-in RNA is added to the MM:

Table 29: Componets for the 10 µl reaction RT mix using the TATAA Kit and RNA spike-in

Reagents	1 reaction [µl]	Comments
NF-H_2O	1.5	
TATAA GrandScript RT Reaction mix (5x)	2	Invert and short spin
TATAA GrandScript RT Enzyme (20x)	0.5	Invert and short spin
Spike-in RNA	1	Always on ice
Master mix in total	**5**	
RNA lysate	5	

1 µl spike-in RNA consists of 10^7 copies.

9.19. Reverse Transcription using SuperScript Kit

- Thaw the RNA lysate on ice
- Thoroughly mix the lysate by pipetting
- Short spin down the lysate
- Store on ice
- Clean everything in the PCR workstation with RNAse Away
- Prepare the Master mix in the PCR workstation

Table 30: Components for the HYB mix

Reagents	**Final concentration in RT volume (10 µl)**	**1 reaction [µl]**	**Comments**
NF-H_2O	-	0.50	
dNTP (10mM)	500 µM	0.50	Vortex and short spin
Oligo-dT_{15} (100 µM)	2.5 µM	0.25	Always on ice – vortex and short spin
Random hexamers (100 µM)	2.5 µM	0.25	Always on ice – vortex and short spin
Total		**1.50**	

Table 31: Components for the RT mix using the SuperScript kit

Reagents	Final concentration in RT volume (10 µl)	1 reaction [µl]	comments
NF-H_2O	-	0.50	
5x SuperScript II first-strand buffer	1x	2.00	Vortex and short spin
DTT (100 mM)	5 µM	0.50	Vortex and short spin
RNAseOUT (40 U/µl)	10 U	0.25	Always on ice – flick and short spin
SuperScript III reverse transcription (200 U/µl)	50 U	0.25	Always on ice- flick and short spin
Total		**3.50**	**Always on ice- flick and short spin**

- Vortex and short spin
- Add 1.5 µl of the HYB mix directly to 5 µl lysate
- Short spin
- Run HYB in a thermal cycler (using heated lid) at 65°C for 5 min followed by a hold at 4°C
- Place samples always on ice
- Short spin
- Add 3.5 µl of the RT mix
- Short spin
- Run RT in a thermal cycler (using heated lid)
-

Table 32: Conditions for the RT with the SuperScript Kit.

25°C	5 min
50°C	60 min
55°C	15 min
70°C	15 min
4°C hold	

It is recommended to start the RT and pause when the cycler reaches 25°C, room temperature could be too high for the first step!

- Store the cDNA at -20°C

9.20. Real Time Quantitative PCR (with undiluted cDNA)

- Thaw the cDNA on ice
- Vortex and short spin
- Store on ice
- Clean everything in the PCR workstation with RNAse Away
- Prepare the master mix in the PCR workstation

Table 33: Components of the 6 µl and 10 µl reaction qPCR mix, if the cDNA is undiluted.

Reagents	6 reaction [µl]	Comments
TATAA SYBR® GrandMaster® Mix (2x)	3	Invert and short spin
Distilled H_2O	1.76	
10 µM Assay mix	0.24	Vortex and short spin
total	**5**	
Undiluted cDNA	1	
Reagents	**10 reaction [µl]**	**Comments**
TATAA SYBR® GrandMaster® Mix (2x)	5	Invert and short spin
Distilled H_2O	1.6	
10 µM Assay mix	0.4	Vortex and short spin
total	**7**	
Undiluted cDNA	3	

- Vortex and short spin the MM
- Pipette the MM
- Short spin
- Template loading area:
  add the cDNA, a negative control and if necessary a positive control
- Short spin
- Run the qPCR in a thermal cycler

Figure 27: Conditions for the qPCR run in the thermal cycler

95°C	2 min	
95°C 60°C 72°C	5 sec 20 sec 20 sec	50 cycles
Melting curve		

9.21. Real Time Quantitative PCR (with diluted cDNA)

The protocol is the same as for the qPCR with undiluted cDNA, but the volume of the MM is different.

Diviations:

- Dilute the cDNA with 30 µl NF-H_2O (1:4)

Table 34: Components of the 6 µl and 10 µl reaction qPCR mix, if the cDNA is diluted.

Reagents	6 µl reaction [µl]	Comments
TATAA SYBR® GrandMaster® Mix (2x)	3	Invert and short spin
Distilled H_2O	0.76	
10 µM Assay mix	0.24	Vortex and short spin
total	**4**	
Diluted cDNA	2	
Reagents	**10 µl reaction [µl]**	**Comments**
TATAA SYBR® GrandMaster® Mix (2x)	5	Invert and short spin
Distilled H_2O	3.6	
10 µM Assay mix	0.4	Vortex and short spin
total	**5**	
Diluted cDNA	1	

9.22. Raw data

Table 35: All measured Cq-values from the evaluation of epithelial specific and leukocytes specific marker expression in blood and HT-29 cells. * = primer dimer

GAPDH	**500 HT-29**	24.23*	22.66	22.81	22.67	22.95
	100 HT-29	24.23	24.04	23.80	23.88	24.22
	500 MNCs	28.00	27.79	28.10	27.74	27.41
	100 MNCs	30.84	30.34	30.93	30.72	32.21
RPS10	**500 HT-29**	23.92	22.60	22.56	22.51	23.17
	100 HT-29	24.77	25.10	24.84	24.80	24.98
	500 MNCs	27.53	27.96	27.94	27.96	27.48
	100 MNCs	30.11	30.55	31.04	30.71	31.74
CK18	**500 HT-29**	22.63	21.87	21.80	21.91	22.02
	100 HT-29	23.70	24.04	23.72	23.84	24.08
	500 MNCs	30.54	30.82	31.72	32.09	29.48
	100 MNCs	42.50	35.63	42.31	34.58	36.26
CD45v1	**500 HT-29**	32.85*	n.a.	n.a.	n.a.	n.a.
	100 HT-29	42.86*	40.25	44.33*	37.79*	n.a.
	500 MNCs	28.90	28.82	28.73	29.04	28.62
	100 MNCs	31.52	31.33	31.51	32.18	32.95
CD45v2	**500 HT-29**	n.a.	n.a.	n.a.	n.a.	n.a.
	100 HT-29	46.41*	n.a.	n.a.	n.a.	n.a.
	500 MNCs	31.65	32.34	31.85	31.77	31.77
	100 MNCs	36.66	35.06	37.22	36.80	38.28
CD45v3	**500 HT-29**	n.a.	n.a.	n.a.	n.a.	n.a.
	100 HT-29	n.a.	n.a.	n.a.	n.a.	n.a.

EpCAM	**500 MNCs**	30.67	30.66	30.64	30.83	30.61
	100 MNCs	35.58	36.16	34.81	35.55	34.79
	500 HT-29	25.94	24.63	24.63	24.47	25.55
	100 HT-29	26.96	26.95	25.91	25.92	26.13
	500 MNCs	40.38	n.a.	40.18*	n.a.	31.97
	100 MNCs	33.62	n.a.	n.a.	n.a.	n.a.

Table 36: All measured Cq-values of the different concentrations of GTC in the GTC lysis solution. * = primer dimer

1 mg/ml BSA	18.24	18.21	18.08	18.40	18.35	18.26	18.77	18.28	21.34*
0.01 M GTC	18.40	18.32	18.30	18.36	18.46	18.53	18.49	18.71	18.39
0.019 M GTC	18.52	18.77	18.65	18.64	18.83	18.71	18.74	18.77	18.74
0.0375 M GTC	18.95	18.93	18.77	18.94	18.88	18.87	18.96	18.93	18.91
0.075 M GTC	19.36	19.62	19.40	19.54	19.79	19.59	19.75	19.85	19.51
0.15 M GTC	34.80	33.70	34.76	33.73	33.72	33.60	33.50	34.08	34.28
0.3 M GTC	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.

Table 37: All measured Cq- values from the RT performance test.

GAPDH	**0.5 mg/ml BSA**	22.70	22.73	22.70	23.10	22.93	22.88	23.01	22.93	23.09	22.93
	No BSA	22.90	22.89	22.97	22.96	22.93	23.24	23.05	23.24	23.27	23.25
	0.75 mg/ml BSA	22.74	22.94	22.88	23.15	23.39	22.77	23.03	23.05	23.10	23.38
RPS10	**0.5 mg/ml BSA**	23.48	23.59	23.61	23.57	23.41	23.60	23.57	23.58	23.74	23.56
	No BSA	23.61	23.55	23.57	23.62	23.61	23.75	23.58	23.66	23.76	23.79
	0.75 mg/ml BSA	23.30	23.60	23.61	23.59	23.96	23.55	23.66	23.69	23.69	23.89
EpCAM	**0.5 mg/ml BSA**	25.34	25.16	n.a.	25.53	25.33	25.02	25.07	25.17	25.48	24.84
	No BSA	25.54	25.51	25.55	25.66	25.73	24.95	25.02	24.95	25.31	25.62
	0.75 mg/ml BSA	25.32	25.62	25.59	25.49	26.11	25.31	25.36	25.00	25.17	25.57
CK18	**0.5 mg/ml BSA**	22.04	22.16	22.00	22.25	22.26	21.99	22.03	22.06	22.15	22.05
	No BSA	22.48	22.19	22.35	22.37	22.53	22.41	22.19	22.42	22.45	22.52
	0.75 mg/ml BSA	21.90	22.22	22.20	22.13	22.60	21.93	22.05	22.12	22.20	22.57
CK8	**0.5 mg/ml BSA**	23.93	24.30	24.07	24.17	23.94	24.54	24.07	24.49	24.43	24.43
	No BSA	24.40	24.04	24.44	24.37	24.60	24.71	24.56	24.86	24.89	24.48
	0.75 mg/ml BSA	24.04	24.42	24.19	24.52	24.78	24.25	24.38	24.38	24.56	24.95

Table 38: All measured Cq-values of the inhibition test with GTC and 0-15 HT-29 cells in a background of 0-1000 erythrocytes. * = primer dimer

GAPDH	**HT-29 cells**	**0 erythrocytes**				**10 erythrocytes**			
	0	-	-	-	-	41.35*	n.a.	-	-
	1	33.48	30.25	33.12	32.46	32.96	29.90	34.13*	34.97
	2	29.04	31.25*	32.85	32.07	32.98*	30.28	33.12	31.30
	5	32.33	30.98	-	-	31.96	31.94	-	-
	10	27.42	28.39	-	-	28.26	30.06	-	-
	15	27.68	26.94	-	-	26.21	28.55	-	-
		100 erythrocytes				**1000 erythrocytes**			
	0	n.a.	36.35	-	-	n.a.	n.a.	-	-

	1	34.82	33.64	33.76	28.88	32.95	33.19	30.94	31.87
	2	32.43	31.90	32.70	32.10	31.11	32.91	33.02	31.84
	5	28.69	29.12	-	-	29.99	30.69	-	-
	10	31.55	31.27	-	-	29.86	29.96	-	-
	15	29.06	31.10	-	-	29.36	29.09	-	-
RPS10		**0 erythrocytes**				**10 erythrocytes**			
	0	-	-	-	-	n.a.	n.a.	-	-
	1	35.65	33.42	n.a.	n.a.	35.24*	32.00	n.a.	33.52
	2	31.21	33.50	n.a.	31.90	32.88*	30.84	32.59	32.13
	5	32.73	30.94	-	-	31.96	31.48	-	-
	10	29.39	29.46	-	-	29.54	29.46	-	-
	15	29.01	28.84	-	-	26.58	28.91	-	-
		100 erythrocytes				**1000 erythrocytes**			
	0	n.a.	35.26	-	-	43.66*	n.a.	-	-
	1	34.42	33.59	32.63	29.99	33.89	36.74*	31.91	32.72
	2	32.95	32.26	32.64	31.06	31.39	32.69	31.01	32.75
	5	28.89	29.92	-	-	29.83	30.11	-	-
	10	32.5*	30.49	-	-	29.66	28.94	-	-
	15	28.58	29.64	-	-	28.95	28.16	-	-
CK18		**0 erythrocytes**				**10 erythrocytes**			
	0	-	-	-	-	n.a.	35.82*	-	-
	1	40.59*	30.37	35.41	33.75	32.81*	31.32*	33.86	33.23
	2	28.95	31.35	30.79	32.39	30.72	29.81	31.24	31.24
	5	30.97	30.70	-	-	31.41	30.42	-	-
	10	27.17	28.10	-	-	27.51	29.50	-	-
	15	26.31	26.88	-	-	25.69	30.00	-	-
		100 erythrocytes				**1000 erythrocytes**			
	0	n.a.	n.a.	-	-	41.72*	n.a.	-	-
	1	33.25	34.46	31.82	29.29	42.14*	n.a.	30.95	32.20
	2	30.87	32.22	33.06	31.82	31.84	31.93	33.27	32.31
	5	27.80	28.84	-	-	28.82	30.35	-	-
	10	29.03	29.82	-	-	28.59	29.20	-	-
	15	28.37	29.69	-	-	28.29	27.85	-	-
CK8		**0 erythrocytes**				**10 erythrocytes**			
	0	-	-	-	-	n.a.	n.a.	-	-
	1	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.
	2	n.a.	n.a.	n.a.	n.a.	43.21	n.a.	n.a.	n.a.
	5	n.a.	n.a.	-	-	n.a.	n.a.	-	-
	10	n.a.	n.a.	-	-	n.a.	n.a.	-	-
	15	45.04	n.a.	-	-	50.78	n.a.	-	-
		100 erythrocytes				**1000 erythrocytes**			
	0	n.a.	n.a.	-	-	n.a.	n.a.	-	-
	1	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.
	2	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.
	5	n.a.	n.a.	-	-	n.a.	n.a.	-	-
	10	n.a.	n.a.	-	-	n.a.	n.a.	-	-

	15	n.a.	n.a.	-	-	n.a.	n.a.	-	-

Table 39: All measured Cq-values from the inhibition test with GTC and 30 HT-29 in a background of 0-1000 erythrocytes. * = primer dimer

	0 erythrocyes	10 erythrocytes	100 erythrocytes	1000 erythrocytes	
GAPDH	29.67	27.13	28.34	28.82	
	28.13	31.77	28.38	28.71	
	29.27	28.69	29.35	27.09	
	27.76	28.66	28.48	28.72	
	28.03	31.26	29.25	28.29	
	29.89	30.81	29.27	28.14	
RPS10	30.07	27.7	28.03	28.66	
	28.54	31.06	28.07	28.36	
	28.93	28.8	29.1	27.54	
	28.6	29.65	28.04	28.19	
	28.78	30.44	29.14	28.26	
	29.47	31.26	28.87	28.09	
CK18	28.48	27.68	27.73	28.82	
	28.3	30.28	27.97	28.8	
	28.9	28.11	29.48	27.14	
	26.65	28.61	28.54	28.12	
	27.32	30.46	28.69	28	
	28.68	30.48	28.89	27.8	**Reference**
RNA spike-in	19.67	19.47	19.73	19.82	19.93
	19.69	19.97	19.77	19.75	19.27
	19.8	19.74	19.78	18.98	19.43
	19.68	19.78	19.75	19.74	
	19.75	19.95	19.72	19.7	
	20.07	19.95	20.05	19.76	

Table 40: **All measured Cq-values from the inhibition test with GTC and one single HT-29 cell in a background of 0 and 1000 erythrocytes.** * = primer dimer

GAPDH		***RPS10***		***CK18***		**RNA spike-in**	
0 erys	**1000 erys**	**0 erys**	**1000 erys**	**0 erys**	**1000 erys**	**0 erys**	**1000 erys**
32.4	33.71	34.85	32.03	33.09	32.88	19.87	19.94
33.1	32.72	32.2	33.78	34.82	33.45	19.87	19.8
31.86	36.24	31.78	34.58	34.35	33.44	19.79	19.87
31.88	31.62	32.75	35.41	33.82	34.87	19.92	19.91
35.38	34.41	34.42	32.16	34.92	33.89	19.87	19.9
33.03	32.29	33.36	32.58	30.71	33.48	19.93	19.72
32.13	33.68	34.55	32.94	34.81*	33.55	19.86	19.88
30.84	32.94	31.02	33.76	32.04	34.16	19.91	19.94
35.08	32.26	34.53	32.24	30.84	32.09	19.77	19.89
32.9	32.99	33.45	32.57	32.12	36	19.89	19.91
32.41	33.08	34.64	33.54	33.37	32.74	19.97	19.95
33.41	34.05*	33.22	32.74	32.56	34.84	19.84	19.96

33.73	33.73	34.53	33.61	34.78	34.93	19.88	20.1
32.57	35.08	35.89	34.63	32.25	33.86	19.83	19.78
33.71	32.55	33.82	33.92	33.5	32.54	19.76	19.72
33.77	32.62	34.57	34.61	33.83	33.41	19.91	19.94
	34.05		33.66	34.04	33.2	19.68	19.66
	31.97		32.22	33.13	36	19.74	19.95
	32.92		33.11	42.46*	32.06	19.71	19.7
			32.63		33.95	19.8	19.77
					33.79	19.91	19.92

Table 41: All measured Cq-values from the sensitivity test with GTC and 0-15 HT-29 cells in a background of 0-1000 MNCs. * = primer dimer

GAPDH	**HT-29**	**0 MNCs**				**10MNCs**				**100 MNCs**			
	0	-	-	-	-	37.93	n.a.	-	-	36.08	33.98	-	-
	1	34.91	33.77	36.53	35.13	36.95	35.04	46.71	34.78	37.77	33.48	35.45	36.21
	2	35.73	31.35	30.58	33.65	33.51	34.01	34.78	32.79*	35.02	31.89	34.43	36.18
	5	31.23	29.52	-	-	32.98*	31.91*	-	-	n.a.	32.59	-	-
	10	31.93	30.66	-	-	31.12	n.a.	-	-	29.98*	34.24		
	15	30.20	30.27	-	-	33.01	29.07	-	-	31.95	31.55	-	-
		250 MNCs				**500 MNCs**				**1000 MNCs**			
	0	36.08	33.98	-	-	32.87	n.a.	-	-	-	-	-	-
	1	32.31	35.76	34.31	34.03	40.83	34.87	35.58	44.96	35.29	38.63	39.73	40.40
	2	32.27	35.12*	35.93	39.95*	40.06	33.23	34.13*	33.97	39.85	35.86	38.22	31.06*
	5	38.31	30.97	-	-	31.97*	34.23*	-	-	32.93	38.26	-	-
	10	35.97	32.54	-	-	33.63	31.25	-	-	38.94	41.30	-	-
	15	31.98	30.72	-	-	37.77*	33.17	-	-	32.68	35.11	-	-
RPS10		**0 MNCs**				**10MNCs**				**100 MNCs**			
	0	-	-	-	-	35.00	n.a.	-	-	33.26	n.a.	-	-
	1	34.26	32.90	34.00	33.31	33.58	34.22	32.83	33.78	36.11	32.48	32.76	32.86
	2	34.84	31.92	31.46	32.88	32.04	32.09	33.27	30.89	n.a.	32.86	30.32	32.61
	5	32.46	30.78	-	-	32.69	30.87	-	-	33.53	30.47	-	-
	10	30.36	32.00	-	-	30.91	31.16	-	-	32.03	31.65	-	-
	15	30.28	33.33	-	-	31.46	30.03	-	-	32.29	31.93	-	-
		250 MNCs				**500 MNCs**				**1000 MNCs**			
	0	31.30	32.65	-	-	33.08	n.a.	-	-	-	-	-	-
	1	33.17	34.54	33.02	32.53	31.04	32.50	32.91	34.25	33.60	33.78	31.47	31.87
	2	30.84	33.96	32.49	33.51	34.71	31.63	32.97	32.84	33.93	31.24	32.34	29.18
	5	n.a.	31.16	-	-	n.a.	32.01	-	-	30.29	31.26	-	-
	10	31.78	31.04	-	-	31.21	32.36	-	-	31.19	32.13	-	-
	15	31.19	30.34	-	-	32.29	32.87	-	-	30.52	32.89	-	-
CK18		**0 MNCs**				**10MNCs**				**100 MNCs**			
	0	-	-	-	-	41.61	43.61	-	-	34.25	33.49	-	-
	1	34.31	32.84	35.02	34.10	34.06*	33.22	33.01	34.24	33.29	35.09	35.35	32.32
	2	34.92	29.60	28.69	33.44	32.75*	32.79	33.55	33.78*	33.92	30.73	32.51	32.74
	5	29.86	28.74	-	-	30.80	29.32	-	-	33.66	30.81*	-	-

	10	27.78	28.82	-	-	29.49	28.83	-	-	31.66	32.34	-	-
	15	27.93	29.00	-	-	31.64	27.93	-	-	29.87	29.69	-	-
		250 MNCs				**500 MNCs**				**1000 MNCs**			
	0	32.31	32.95	-	-	34.69	n.a.	-	-	-	-	-	-
	1	30.31*	32.65	32.05	32.83	30.19*	33.56	30.57	32.61	29.75	32.28*	32.25	32.36
	2	30.74	30.36	31.29	32.06	30.24	30.81	31.35	31.97	31.43	32.16	31.87	28.89
	5	31.67	28.99	-	-	31.26	32.92	-	-	29.22	28.98	-	-
	10	31.86	30.69	-	-	31.68	30.27	-	-	31.14	31.64	-	-
	15	30.34	28.82	-	-	32.25	32.00	-	-	30.17	33.84	-	-
CK8		**0 MNCs**				**10MNCs**				**100 MNCs**			
	0	-	-	-	-	n.a.	n.a.	-	-	n.a.	n.a.	-	-
	1	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.
	2	n.a.	43.27	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	45.14	49.36	n.a.
	5	n.a.	43.03	-	-	n.a.	n.a.	-	-	n.a.	n.a.	-	-
	10	n.a.	50.00	-	-	50.28	44.59	-	-	50.00	n.a.	-	-
	15	n.a.	n.a.	-	-	n.a.	49.49	-	-	50.43	49.52	-	-
		250 MNCs				**500 MNCs**				**1000 MNCs**			
	0	n.a.	n.a.	-	-	n.a.	n.a.	-	-	-	-	-	-
	1	49.91	49.54	n.a.	n.a.	50.62	n.a.	49.17		n.a.	n.a.	50.20	n.a.
	2	n.a.	n.a.	n.a.	n.a.	50.00	50.26	n.a.	n.a.	49.40	n.a.	n.a.	47.14
	5	43.24	n.a.	-	-	49.60	n.a.	-	-	50.00	44.02	-	-
	10	n.a.	50.38	-	-	n.a.	n.a.	-	-	n.a.	n.a.	-	-
	15	43.46	n.a.	-	-	n.a.	n.a.	-	-	47.29	n.a.	-	-

Table 42: All measured Cq-values from the sensitivity test with GTC and one single HT-29 cells in a background of 0-1000 MNCs. * = primer dimer

	0 MNCs	**10 MNCs**	**100 MNCs**	**1000 MNCs**
GAPDH	32.34	33.26	32.91	33.30
	30.92	32.72	31.69	35.96*
	34.52	31.66*	33.77	29.82
	34.85	34.90	33.71	32.61
	33.33	31.24	32.78	34.08
	29.70	31.66	32.02	31.91
	31.97	33.56	32.64	32.82
	29.78	31.19	34.88	32.60
	29.19	32.44	32.40	34.58
	33.71	33.86*	32.75	31.81
	29.65	n.a.	33.63	32.92
	32.41	32.58	32.85	33.11
CK18	45.23	34.62	32.24	31.13
	31.63	32.12	30.91	31.09
	n.a.	34.49	31.71	31.4
	33.63	34.8	30.83*	29.83
	33.1	33.49	30.82	30.72
	29.53	29.98	31.96	30.11

	32.86	33.63	32.29	29.4	
	29.09	31.68	29.81	28.57	
	28.76	32.39	31.93	31.13	
	34.58	34.51	30.8	30.17	
	30.12	32.12	32.57	29.63	
	44.68*	33.95	31.98	28.82	**Reference**
RNA spike-in	18.94	19.06	19.18	18.83	18.61
	18.9	n.a.	19.2	18.94	18.69
	18.86	18.92	19.02	18.91	18.61
	18.88	18.86	18.84	18.82	18.64
	18.94	18.99	18.89	19.89	18.64
	18.86	18.82	18.87	20.26	18.59
	18.89	18.94	18.79	18.75	
	18.81	18.65	18.75	18.82	
	18.74	18.85	18.74	19.05	
	19.03	18.82	19.05	18.9	
	19.16	18.88	18.92	18.87	
	19.23	19.37	18.87	18.92	

Table 43: All measured Cq-values from the sensitivity test with GTC and no HT-29 cell in a background of 0-1000 MNCs. * = primer dimer

	0 MNCs	**10 MNCs**	**100 MNCs**	**1000 MNCs**	
GAPDH	39.54*	34.63	33.68	34.39	
	n.a.	n.a.	34.79*	33.99	
	n.a.	34.78	33.07	32.13	
	n.a.	32.89	33.7	31.93	
	n.a.	33.9	33.07	32.88	
	n.a.	34.83	34.35	32.63	
	n.a.	n.a.	32.81	33.65	
	n.a.	n.a.	34.63	31.88	
	n.a.	34.14	34.64	32.66	
CK18	36.82	34.57	33.76	29.67	
	n.a.	n.a.	33.59	27.82	
	n.a.	44.69*	31.89	29.31	
	n.a.	49*	32.27	n.a.	
	36.75*	34.59	32.83	29.6	
	46.87*	42.62*	29.99	30.11	
	45.36*	38.89	39.58	28.96	
	n.a.	47.99*	34.67	27.75	
	n.a.	32.98	37.51	30.52	**Reference**
RNA spike-in	18.87	18.73	18.9	18.86	18.61
	18.84	18.76	18.93	19.02	18.69
	18.79	18.91	18.83	18.87	18.61
	18.89*	18.92	18.87	19.02	18.64
	18.82	18.91	18.67	18.97	18.64
	18.89	19.12	18.72	18.99	18.59

	18.76	19.6	18.85	18.9	
	19.01	19.55	18.89	19.07	
	19.58	19.49	18.77	18.98	

Table 44: All measured Cq-values from the sensitivits test with one single VCaP cell in a background of 0-1000 MNCs using the GTC lysis solution. * = primer dimer

	0 MNCs	10 MNCs	100 MNCs	1000 MNCs	Pos. Ctr.
RPS10	36.54*	33.78*	31.38	29.74	17.13
	33.26	32.33	30.85	32.59*	17.08
	33.04	32.04	31.3	30.35*	17.15
	n.a.	n.a.	31.27	30.19	
	n.a.	33.12	30.78	29.42	
	n.a.	n.a.	31.51	29.72	
	34.09	37.15	31.78	30.19	
	32.96	n.a.	29.85	28.82	
	32.72	32.21	30.9	28.77	
	34.5*	32.91	30.75	29.7	
	n.a.	31.37	31.35	28.86	
	n.a.	32.88	31.63	29.69	
GAPDH	33.34*	33.58	38.78	34.06	18.18
	32.67	33.36	32.54	33.19	18.07
	33.53	32.85	40.23*	34.68	18.07
	n.a.	44*	33.3	33.61	
	n.a.	34.33*	33.56	31.76	
	n.a.	32.63*	35.67	33.71	
	n.a.	34.39	32.87	33.48	
	n.a.	32.64	33.66	32.62	
	32.47	34.51	34.41	32.67	
		n.a.	34.43	37.13	
	38.02*	n.a.	n.a.	32.09	
	n.a.	n.a.	n.a.	32.17	
ARv7	n.a.	n.a.	n.a.	n.a.	25.99
	n.a.	n.a.	n.a.	n.a.	26.07
	n.a.	n.a.	n.a.	n.a.	25.97
	n.a.	n.a.	n.a.	n.a.	
	n.a.	n.a.	n.a.	n.a.	
	n.a.	n.a.	n.a.	n.a.	
	n.a.	n.a.	n.a.	n.a.	
	n.a.	n.a.	n.a.	n.a.	
	n.a.	n.a.	n.a.	n.a.	
	n.a.	n.a.	n.a.	n.a.	
	n.a.	n.a.	n.a.	n.a.	
	n.a.	n.a.	n.a.	n.a.	
	n.a.	n.a.	n.a.	n.a.	
CD45	n.a.	1.19	39.44	38.32	n.a.
	n.a.	n.a.	n.a.	36.81	n.a.
	n.a.	n.a.	n.a.	36.45	n.a.
	n.a.	n.a.	37.77	n.a.	

	n.a.	n.a.	37.78	35.94	
	n.a.	n.a.	n.a.	36.01	
	n.a.	n.a.	n.a.	37.46	
	n.a.	n.a.	37.32	38.99	
	n.a.	n.a.	n.a.	35.81	
	n.a.	n.a.	n.a.	n.a.	
	n.a.	n.a.	37.67	n.a.	
	n.a.	n.a.	n.a.	37.36	

Table 45: All measured Cq-values from the sensitivity test with no VCaP cell in a background of 0-1000 MNCs using the GTC lysis solution. * = primer dimer

	0 MNCs	10 MNCs	100 MNCs	1000 MNCs	Pos. Ctr.
RPS10	n.a.	31.95	30.89	29.26	17.13
	n.a.	33.9	32.21	29.25	17.08
	n.a.	31.99	32.43	28.67	17.15
	n.a.	n.a.	31.72	28.72	
	n.a.	n.a.	31.67	29.03	
	n.a.	n.a.	31.53	29.83	
	n.a.	35.86	32.77	28.76	
	n.a.	37	33.44	29.27	
	n.a.	29.69	32.78	29.64	
	n.a.	33.24	32.62	30.28	
	n.a.	n.a.	33.29	28.9	
	n.a.	34.05	32.55	28.75	
GAPDH	n.a.	49*	33.5	32.67	18.18
	n.a.	n.a.	n.a.	32.03	18.07
	n.a.	n.a.	34.08	32.63	18.07
	n.a.	34.63	37.01	31.86	
	n.a.	n.a.	34.49	31.64	
	n.a.	34.55	34.69	32.36	
	n.a.	27.09	34.37	32.6	
	n.a.	45.3*	34.56	33.01	
	n.a.	n.a.	34.44	32.11	
	n.a.	n.a.	33.71	32.47	
	n.a.	n.a.	34.12	31.61	
	n.a.	n.a.	35.31	31.96	
ARv7	n.a.	n.a.	n.a.	n.a.	25.99
	n.a.	n.a.	n.a.	n.a.	26.07
	n.a.	n.a.	n.a.	n.a.	25.97
	n.a.	n.a.	n.a.	n.a.	
	n.a.	n.a.	n.a.	n.a.	
	n.a.	n.a.	n.a.	n.a.	
	n.a.	n.a.	n.a.	n.a.	
	n.a.	n.a.	n.a.	n.a.	
	n.a.	n.a.	n.a.	n.a.	
	n.a.	n.a.	n.a.	n.a.	
	n.a.	n.a.	n.a.	n.a.	

	n.a.	n.a.	n.a.	n.a.	
CD45	n.a.	37.79	38.54	37.17	n.a.
	n.a.	n.a.	n.a.	n.a.	n.a.
	n.a.	38.58	n.a.	n.a.	n.a.
	n.a.	n.a.	37.87	35.85	
	n.a.	n.a.	n.a.	38.01	
	n.a.	n.a.	47.97*	36.2	
	n.a.	n.a.	35.7	36.6	
	n.a.	n.a.	36.79	36.55	
	n.a.	n.a.	n.a.	35.75	
	n.a.	n.a.	n.a.	37.65	
	n.a.	n.a.	38.54	35.34	
	n.a.	n.a.	36.02	36.89	

Table 46: All Cq-values from the sensitivity test with VCaP cells in a background of 0-500 MNCs using the BSA/Triton lysis solution. * = primer dimer

	single VCaP cell		**no cell**			**single VCaP cell**		**no cell**	
	0 MNCs	**500 MNCs**	**0 MNCs**	**500 MNCs**		**0 MNCs**	**500 MNCs**	**0 MNCs**	**500 MNCs**
GAPDH	26.80	32.15	n.a.	34.77	***CD45***	40.42*	31.60	n.a.	30.49
	27.97	33.84	n.a.	35.2		n.a.	27.92	n.a.	31.04
	29.98	33.00	n.a.	31.64		n.a.	31.45	n.a.	35.73
	27.69	32.73	n.a.	32.57		n.a.	29.76	n.a.	32.49
	27.72	32.47	n.a.	31.06		34.93	30.85	n.a.	32.14
	26.88	37.90		35.24*		n.a.	30.06		31.19
	29.39	42.90				n.a.	30.67		
	29.49	33.20				n.a.	31.86		
	30.62	34.63				n.a.	31.57		
	29.62	33.04				n.a.	32.66		
	29.80	35.28				46.64*	34.04		
	27.81	33.25				35.31	32.61		
	29.68	30.98				33.70	31.64		
	29.64	31.82				n.a.	30.73		
	28.65	32.58				n.a.	34.11		
	30.55	32.32				n.a.	33.08		
	30.52	33.21				46.6*	30.41		
	30.64	32.83				n.a.	34.66		
	28.38	32.48				n.a.	30.35		
	29.59	31.83				n.a.	31.74		
	29.63	33.26				n.a.	32.46		
	29.42	32.35				n.a.	30.92		
	28.25	32.30				n.a.	33.82		
	29.89	34.57				n.a.	31.92		
	29.72	n.a.				33.26	29.88		
	29.90	n.a.				33.65	30.31		
	29.77	n.a.				32.86	30.59		
	28.84	n.a.				n.a.	28.06		
	28.50	n.a.				n.a.	31.31		

	27.95	n.a.				33.67	33.61		
	n.a.	30.72				n.a.	32.55		
	n.a.	32.57				n.a.	32.70		
	n.a.	31.89				n.a.	32.68		
	n.a.	34.48				n.a.	32.99		
	n.a.	32.46				n.a.	33.56		
	n.a.	32.76				n.a.	31.88		
	28.21	30.66				n.a.	29.46		
	29.15	30.69				n.a.	32.48		
	29.68	31.71				n.a.	29.6		
	29.80	33.81				n.a.	28.33		
	29.63	36.33				n.a.	28.91		
	28.69	31.62				n.a.	33.73		
	single VCaP cell		**no cell**			**single VCaP cell**		**no cell**	
	0 MNCs	**500 MNCs**	**0 MNCs**	**500 MNCs**		**0 MNCs**	**500 MNCs**	**0 MNCs**	**500 MNCs**
AR	33.83	33.51*	n.a.	29.05*	***ARv7***	36.94	37.57*	n.a.	45.41*
	30.7	n.a.	n.a.	46.44*		36.12	n.a.	n.a.	n.a.
	30.68	n.a.	n.a.	n.a.		n.a.	n.a.	n.a.	n.a.
	29.5	44.79*	n.a.	n.a.		35.91	n.a.	n.a.	n.a.
	31.51	42.59*	n.a.	n.a.		36.4	n.a.	n.a.	n.a.
	29.29	n.a.	n.a.	n.a.		35.44	n.a.	n.a.	n.a.
	30.44	n.a.				n.a.	n.a.		
	30.69	n.a.				36.51	n.a.		
	30.8	34.65				n.a.	n.a.		
	30.37	28.52*				34.77	n.a.		
	30.26	n.a.				36.34	n.a.		
	31.39	n.a.				34.9	n.a.		
	29.06	n.a.				37.17	n.a.		
	31.01	33.19				n.a.	n.a.		
	29.97	n.a.				36.96	n.a.		
	32.67	n.a.				n.a.	39.72		
	29.6	n.a.				36.63	n.a.		
	29.99	34.22				36.63	n.a.		
	32.35	n.a.				31.93	n.a.		
	34.51	46.85*				n.a.	n.a.		
	29.56	n.a.				n.a.	n.a.		
	30.8	46.39*				n.a.	n.a.		
	29.65	n.a.				34.7	35.04*		
	32.56	n.a.				37.05	n.a.		
	n.a.	n.a.				47.81*	n.a.		
	n.a.	n.a.				n.a.	n.a.		
	n.a.	n.a.				36.98	n.a.		
	n.a.	n.a.				36.27	n.a.		
	n.a.	n.a.				35.22	n.a.		
	n.a.	n.a.				n.a.	n.a.		
	n.a.	33.81				n.a.	n.a.		
	30.54	n.a.				35.85	n.a.		

	30.71	n.a.		n.a.	n.a.
	33.86	n.a.		37.1	n.a.
	30.9	33.6		n.a.	n.a.
	29.68	n.a.		36.76	n.a.
	n.a.	46.43*		n.a.	45.44*
	n.a.	46.32*		37.06	n.a.
	n.a.	46.06*		n.a.	n.a.
	n.a.	47.92*		n.a.	n.a.
	n.a.	42.52*		35.45	n.a.
	n.a.	33.04*		34.38	n.a.

Table 47: All Cq-values from the sensitivity test with VCaP cells in a background of 0-1000 MNCs using the BSA/Triton lysis solution. * = primer dimer

	single VCaP cell				no cell			
	0 MNCs	**10 MNCs**	**100 MNCs**	**1000 MNCs**	**0 MNCs**	**10 MNCs**	**100 MNCs**	**1000 MNCs**
GAPDH	31.04	28.89	29.63	31.55	n.a.	33.35	29.93	34.2
	29.25	29.07	29.35	35.8	n.a.	33.08	30.04	35
	28.55	29.6	29.6	34.5	n.a.	32.92	30.75	32.18
	29	28.94	29.23	32.04	n.a.	33.33	30.31	32.77
	29.67	28.64	32.45	37.82	n.a.	32.96	30.39	36.71
	29.62	30.86	29.84	34.51	n.a.	32.34	32.87	34.7
	30.54	30.53	28.84	36.59*	n.a.	31.76	32.52	32.3
	28.75	29.9	34.59	30.69	n.a.	32.65	30.81	34.5
	28.96	28.81	33.19	31.74	n.a.	32.63	30.59	32.51
CD45	39.49	32.16	29.6	38.15	n.a.	32.55	28.95	31.15
	35.07	32.61	29.39	31.39	n.a.	32.41	29.52	29.55
	n.a.	32.64	29.36	35.83	n.a.	32.29	31.24	29.64
	n.a.	32.95	28.25	29.64	n.a.	33.92	29.14	28.79
	33.7	31.44	29.35	28.85	n.a.	31.67	29.6	27.74
	n.a.	31.67	29.72	28.91	n.a.	31.09	30.28	28.08
	n.a.	33.69	28.91	27.3	n.a.	31.78	29.98	27.57
	n.a.	31.12	29.21	27.7	n.a.	32.4	28.55	27.64
	n.a.	32.6	29.98	28.33	n.a.	31.61	27.48	29.9
AR	31.91	30.86	32.21	48.23*	n.a.	n.a.	49*	45.17*
	28.89	29.74	39.86*	n.a.	n.a.	n.a.	n.a.	n.a.
	29.34	32.72	32.89	n.a.	n.a.	n.a.	n.a.	40.71*
	29.65	32.46	33.62	n.a.	n.a.	n.a.	n.a.	n.a.
	30.82	29.5	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.
	30.22	32.14	33.33	n.a.	n.a.	n.a.	n.a.	47.55*
	30.73	32.64	32.71	46.72*	n.a.	n.a.	n.a.	44.55*
	30.01	32.12	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.
	29.62	31.99	33.88*	n.a.	n.a.	n.a.	n.a.	n.a.
ARv7	45.57*	37.39	39.07	47.72*	n.a.	n.a.	n.a.	n.a.
	43.05	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.
	37.42	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.
	43.14*	37.04	n.a.	35.11*	n.a.	n.a.	n.a.	n.a.
	42.97*	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.

	n.a.	43.24*	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.
	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.
	40.15	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.
	38.91	n.a.	36.04*	n.a.	n.a.	n.a.	n.a.	n.a.

Table 48: All measured Cq- values of the FACSed VCaP dilution series with BSA/Triton. * = primer dimer

GAPDH					***AR***					***ARv7***				
1 V.	**2 V.**	**4 V.**	**8 V.**	**16 V.**	**1 V.**	**2 V.**	**4 V.**	**8 V.**	**16 V.**	**1 V.**	**2 V.**	**4 V.**	**8 V.**	**16 V.**
28.76	28.14	26.71	25.86	24.61	n.a.	28.54	27.70	26.62	25.77	n.a.	33.15	33.12	34.47	30.95
28.18	27.74	25.83	25.36	24.39	29.52	28.56	27.65	26.64	25.32	33.24	31.56	n.a.	31.58	29.50
29.79	27.71	25.78	26.27	24.38	29.77	29.46	26.88	28.63	25.77	33.11	n.a.	32.04	31.57	29.92
30	28.29	26.66	25.57	24.33	30.17	28.88	27.88	27.10	25.72	n.a.	32.14	39.32	30.60	30.79
28.58	27.11	27.16	25.78	24.49	30.70	27.24	27.67	26.74	26.09	n.a.	n.a.	n.a.	32.59	31.66
29.32					29.16					31.30				
29.15					30.97					n.a.				
27.43					30.05					n.a.				
29.72					31.76					43.67*				
30.95					31.82					33.63				
28.98					29.31					n.a.				
29.51					33.56					33.75				
28.75					31.71					n.a.				
28.65					30.11					47.45*				
29.12					29.04					n.a.				
28.42					30.32					n.a.				
28.74					29.74					n.a.				
27.41					29.56					32.52				
28.71					29.32					33.06				
28.69					28.95					31.89				
28.22					30.05					n.a.				
29.01					29.59					32.11				
28.76					29.88					32.98				
28.97					28.12					33.18				
28.89					31.79					n.a.				
29.58					29.35					n.a.				
31.1					32.83					45.68				
27.8					30.82					n.a.				
30.62					31.49					33.82				
28.68					30.40					n.a.				

Table 49: All measured Cq-values from the microdissection of the VCaP cells.

	Number of VCaP cells					
GAPDH	1	26.16	26.76	26.82	33.08	27.89
	2	26.19	26.45	30.12		
	2.5	28.01				
	3	24.79	26.48			
	2-4	25.81				
	4	26.42				
	5	25.72				
	3-6	24.86	24.76			
	10	25.74	24.93			
	14	23.83				
	15	24.23				
	pos. ctr.	20.23	20.09	20.05		
ARv7	1	34.17	n.a.	33.51	n.a.	n.a.
	2	n.a.	32.72	n.a.		
	2.5	n.a.				
	3	32.49	36.97			
	2-4	n.a.				
	4	33.24				
	5	33.02				
	3-6	30.45	31.15			
	10	33.29	32.11			
	14	31.87				
	15	31.8				
	pos. ctr.	27.7	27.96	27.88		

Table 50: All measured Cq-values from the micromanipulation of the VCaP cells. * = primer dimer

	Number of VCaP cells			
GAPDH	1	40.22	44.55*	n.a.
	2	35.9*	n.a.	n.a.
	5	40.24	n.a.	
	9	34.95		
	10	37.92		
	15	29.86	29.94	
	pos. ctr.	20.17	20.05	19.85
ARv7	1	47.45*	n.a.	n.a.
	2	n.a.	n.a.	n.a.
	5	n.a.	n.a.	
	9	n.a.		
	10	n.a.		
	15	39.42*	n.a.	
	pos. ctr.	28.14	27.74	27.8
RNA spike-in	1	31.99	25.91	21.44
	2	21.69	22.12	22.08
	5	n.a.	n.a.	

	9	21.93	
	10	n.a.	
	15	22.68	22.86
	reference	21.1	21.28

Table 51: All measured Cq-values from the direct lysis on the DC01 using the GTC lysis solution.

	Number of repetitive lysis	Wire #1			Wire #2		
GAPDH	1	27.62	27.86	28.15	30.63	30.29	30.56
	2	28.66	28.67	28.70	31.03	30.59	31.64
	3	30.76	30.19	31.60	31.34	30.89	31.21
	4	31.15	31.27	30.76	n.a.	31.45	32.07
	5	32.96	31.70	31.45	33.44	n.a.	35.18
RPS10	1	26.93	26.80	27.27	29.35	29.03	30.02
	2	28.59	28.83	28.88	30.72	30.33	30.28
	3	30.90	30.68	30.79	32.94	31.30	31.98
	4	31.84	31.89	30.93	31.88	33.64	32.71
	5	32.56	32.13	31.36	32.92	33.64	32.83
CK18	1	31.67	31.65	32.85	32.81	36.14	37.78
	2	30.66	30.58	31.58	32.77	34.12	32.83
	3	31.95	32.73	33.71	35.18	33.66	33.53
	4	n.a.	32.75	32.55	33.71	31.95	35.88
	5	32.94	33.25	33.95	34.87	33.79	34.04
CK8	1	27.12	26.93	27.56	28.72	28.54	29.10
	2	28.70	27.46	28.65	30.77	30.54	30.02
	3	31.06	30.68	30.18	31.83	30.51	30.18
	4	30.98	30.45	31.62	31.09	30.73	30.51
	5	31.24	30.98	30.68	33.88	33.75	33.71

Table 52: All measured Cq-values from the direct lysis on the DC01 by freezing for 1 min and using the BSA/Triton lysis solution.

		Wire #1			Wire #2			Wire #3		
GAPDH	**1. lysis**	28.11	27.97	26.94	28.58	27.83	28.00	28.05	28.68	28.12
	2. lysis	30.89	31.35	31.30	31.37	31.33	30.99	28.91	29.48	28.71
	3. lysis	31.12	32.84	31.28	31.41	31.64	30.60	30.55	30.99	31.60
	4. lysis	30.84*	31.26	31.58	32.84	32.70	30.96	29.96	29.79	32.75
	5. lysis	32.34	32.17	33.23	33.41	31.68	31.81	32.00	31.76	30.81
	1. wash	30.90	27.18	31.76	29.80	30.63	29.86	31.00	30.98	29.77
	2. wash	n.a.	n.a.	34.26	31.53	31.87	31.88	31.64	30.88	31.36
	3. wash	32.99	33.30	30.98	31.42	31.47	32.78	32.69	32.03	31.32
	4. wash	31.62	31.89	31.43	31.81	32.74	32.57	32.32	31.88	30.87
	5. wash	32.25	32.35	30.75	31.69	32.61	31.79	31.94	32.33	31.40
CK18	**1. lysis**	27.81	27.38	25.29	27.74	26.88	27.39	26.75	28.63	27.99
	2. lysis	29.55	33.02	31.63	32.42*	29.92	31.10	27.95	29.60	27.18
	3. lysis	32.06	33.57	32.02	31.96	30.69	30.51	28.37	30.52	30.13
	4. lysis	30.02	31.45	30.60	33.25	31.46	30.22	29.16	28.75	29.38

	5. lysis	32.99	31.77	33.55	31.57	30.76	32.46	30.53	30.44	29.22
	1. wash	32.30	25.70	30.63	29.07	30.88	30.03	30.30	32.70	28.98
	2. wash	33.09	34.85	42.15	29.56	30.75	31.61	31.67	31.84	32.10
	3. wash	32.73	32.87	31.67	33.02	31.72	31.11	31.25	32.49	31.78
	4. wash	32.33	32.22	32.75	32.71	32.45	31.61	31.00	31.58	29.78
	5. wash	32.57	32.92	30.49	32.00	31.86	31.58	30.80	32.64	27.61
EpCAM	**1. lysis**	n.a.	30.88	28.66	29.72	30.14	30.50	31.21	31.14	31.72
	2. lysis	n.a.	34.07	34.58	31.74	32.95	n.a.	32.67	31.66	29.95
	3. lysis	33.67	32.79	n.a.	34.12	31.97	n.a.	33.02	n.a.	n.a.
	4. lysis	32.85	33.69	33.93	34.04	n.a.	33.46	32.01	31.57	32.13
	5. lysis	n.a.	n.a.	n.a.	33.76	33.49	n.a.	31.98	32.59	n.a.
	1. wash	28.20	34.22	n.a.	31.83	n.a.	33.68	31.24	n.a.	n.a.
	2. wash	n.a.	n.a.	n.a.	33.73	34.23	32.64	32.67	n.a.	n.a.
	3. wash	n.a.	n.a.	n.a.	n.a.	34.04	32.18	n.a.	n.a.	n.a.
	4. wash	33.55	n.a.	n.a.	n.a.	n.a.	34.21	n.a.	n.a.	n.a.
	5. wash	33.04	n.a.	32.56	34.42	34.66	n.a.	33.66	33.65	n.a.

Table 53: All measured Cq-values from the direct lysis on the DC01 with different cell densities, using the BSA/Triton lysis solution.

		1000 cells/µl			**500 cells/µl**			**250 cells/µl**			**No cells**		
GAPDH	**1. lysis**	26.73	26.11	26.50	27.59	27.07	27.14	n.a.	29.22	29.90	n.a.	n.a.	n.a.
	2. lysis	28.71	29.11	28.98	29.72	29.12	29.00	30.12	30.14	29.25	n.a.	n.a.	n.a.
	1. wash	26.45	26.09	26.22	25.64	25.71	25.76	30.51	30.77	30.45	n.a.	n.a.	n.a.
	2. wash	30.64	31.14	30.06	30.64	30.25	30.26	31.91	31.00	31.62	n.a.	n.a.	n.a.
RPS10	**1. lysis**	26.45	26.62	26.66	27.25	27.02	27.66	30.03	29.60	30.01	n.a.	n.a.	n.a.
	2. lysis	30.70	30.47	29.78	28.77	29.33	29.75	29.90	29.47	30.72	n.a.	n.a.	n.a.
	1. wash	26.40	25.93	n.a.	25.39	25.43	25.62	30.90	30.55	30.93	n.a.	n.a.	n.a.
	2. wash	32.25	31.12	30.85	29.90	31.62	29.93	32.21	31.65	33.08	n.a.	35.38	n.a.
CK18	**1. lysis**	29.14	29.03	29.05	30.97	30.62	30.49	32.00	33.53	33.64	n.a.	n.a.	n.a.
	2. lysis	31.33	32.59	31.80	33.71	30.79	30.78	31.71	32.59	33.56	n.a.	n.a.	n.a.
	1. wash	29.29	28.56	28.96	29.54	28.23	28.50	33.39	34.94	31.97	n.a.	n.a.	n.a.
	2. wash	33.11	32.76	31.64	31.49	36.78	30.84	n.a.	34.29	n.a.	n.a.	n.a.	n.a.
EpCAM	**1. lysis**	26.15	26.26	26.03	26.63	26.80	26.73	27.71	29.84	30.29	n.a.	37.91	34.26
	2. lysis	29.69	29.73	29.12	27.85	27.97	28.02	29.68	29.91	30.03	n.a.	n.a.	n.a.
	1. wash	25.90	25.69	26.14	25.49	25.72	25.70	28.77	30.75	30.37	n.a.	n.a.	n.a.
	2. wash	30.83	30.94	30.69	28.66	27.22	27.57	31.95	32.14	31.07	n.a.	37.33	n.a.

Table 54: All measured Cq-values from the evaluation of the different wash conditions of the direct lysis on the DC01, using the BSA/Triton lysis solution.

GAPDH	**1. lysis**	**5 µl (wash and twist)**	25.02	25.14	25.03		
		20 µl (wash)	26.87	26.61	26.66	26.54	26.96
		20 µl (wash and twist)	26.58	26.71	26.96	26.69	26.79
		20 µl (no wash step,twist)	25.53	24.99	25.04		
		no cell	n.a.	n.a.	n.a.		
	2. lysis	**5 µl (wash and twist)**	24.79	24.60	24.76		
		20 µl (wash)	26.86	26.87	26.87	26.76	26.86

		20 µl (wash and twist)	26.65	26.70	26.82	26.37	26.78
		20 µl (no wash step,twist)	26.60	26.64	26.82		
		no cell	n.a.	n.a.	n.a.		
RPS10	**1. lysis**	**5 µl (wash and twist)**	25.04	25.00	24.86		
		20 µl (wash)	26.49	26.98	27.33	27.25	27.28
		20 µl (wash and twist)	27.30	26.80	26.65	25.65	27.07
		20 µl (no wash step,twist)	25.71	25.15	25.20		
		no cell	n.a.	n.a.	n.a.		
	2. lysis	**5 µl (wash and twist)**	24.39	24.84	25.20		
		20 µl (wash)	26.96	27.28	27.28	27.15	27.49
		20 µl (wash and twist)	26.79	26.50	26.55	26.68	26.48
		20 µl (no wash step,twist)	26.39	26.27	26.30		
		no cell	n.a.	n.a.	n.a.		
CK18	**1. lysis**	**5 µl (wash and twist)**	24.09	24.10	24.19		
		20 µl (wash)	25.12	25.39	25.16	25.48	25.31
		20 µl (wash and twist)	24.87	24.75	24.92	24.89	24.91
		20 µl (no wash step,twist)	24.42	24.03	23.59		
		no cell	n.a.	n.a.	n.a.		
	2. lysis	**5 µl (wash and twist)**	24.08	24.44	24.30		
		20 µl (wash)	26.43	26.09	26.29	26.34	26.39
		20 µl (wash and twist)	25.57	25.89	25.86	25.79	25.46
		20 µl (no wash step,twist)	26.19	26.30	26.13		
		no cell	n.a.	n.a.	n.a.		
EpCAM	**1. lysis**	**5 µl (wash and twist)**	25.93	27.80	27.31		
		20 µl (wash)	28.05	28.88	28.39	28.72	28.69
		20 µl (wash and twist)	28.76	28.91	28.92	28.65	29.14
		20 µl (no wash step,twist)	25.87	27.46	27.00		
		no cell	n.a.	n.a.	n.a.		
	2. lysis	**5 µl (wash and twist)**	27.58	27.21	27.70		
		20 µl (wash)	29.41	29.72	29.61	28.68	29.28
		20 µl (wash and twist)	28.76	29.52	28.33	28.89	28.74
		20 µl (no wash step,twist)	28.80	28.90	29.04		
		no cell	n.a.	n.a.	n.a.		

Table 55: All measured Cq-values from the direct lysis on the DC01 without a wash step, using the BSA/Triton lysis solution.

GAPDH	**1. lysis**	**Wire #1**	25.58	25.62	25.51	***CK18***	**1. lysis**	**Wire #1**	24.32	24.75	24.84
		Wire #2	25.17	25.09	25.09			**Wire #2**	24.10	24.13	24.16
		Wire #3	26.25	26.43	26.72			**Wire #3**	24.98	24.88	24.85
		Wire #4	24.24	24.47	24.42			**Wire #4**	23.76	23.76	23.85
		Wire #5	24.11	24.13	24.02			**Wire #5**	23.40	23.69	23.63
		No cell	n.a.	n.a.	n.a.			**No cell**	n.a.	n.a.	n.a.
	2. lysis	**Wire #1**	24.69	24.85	24.81		**2. lysis**	**Wire #1**	24.78	24.86	24.86
		Wire #2	26.14	25.87	25.90			**Wire #2**	25.59	25.28	25.22
		Wire #3	24.19	24.46	24.31			**Wire #3**	23.82	23.88	23.81
		Wire #4	24.91	25.16	25.32			**Wire #4**	24.77	24.91	25.05
		Wire #5	25.95	26.07	26.12			**Wire #5**	25.79	25.72	25.78
		No cell	n.a.	n.a.	n.a.			**No cell**	32.73	44.92	36.07
	3. lysis	**Wire #1**	25.75	26.13	25.97		**3. lysis**	**Wire #1**	25.96	25.80	25.71
		Wire #2	26.97	26.87	27.09			**Wire #2**	25.71	25.76	25.77
		Wire #3	25.21	25.41	24.89			**Wire #3**	24.68	24.67	24.82
		Wire #4	26.70	27.15	26.95			**Wire #4**	26.78	26.68	26.69
		Wire #5	26.94	27.37	27.39			**Wire #5**	26.76	27.11	27.05
		No cell	n.a.	n.a.	n.a.			**No cell**	n.a.	n.a.	n.a.
RPS10	**1. lysis**	**Wire #1**	27.22	26.78	26.72	***EpCAM***	**1. lysis**	**Wire #1**	28.20	28.07	27.66
		Wire #2	25.46	26.40	26.59			**Wire #2**	27.69	27.66	27.78
		Wire #3	27.27	27.65	27.68			**Wire #3**	29.62	30.15	29.70
		Wire #4	25.81	25.72	23.87			**Wire #4**	26.82	26.63	26.91
		Wire #5	25.06	25.28	25.13			**Wire #5**	26.66	26.81	26.72
		No cell	n.a.	n.a.	n.a.			**No cell**	n.a.	n.a.	n.a.
	2. lysis	**Wire #1**	25.58	25.71	25.49		**2. lysis**	**Wire #1**	27.54	28.53	27.77
		Wire #2	26.98	26.89	27.36			**Wire #2**	28.56	28.01	28.61
		Wire #3	24.69	24.99	24.49			**Wire #3**	27.00	27.26	27.21
		Wire #4	25.92	26.50	26.00			**Wire #4**	27.75	27.84	28.57
		Wire #5	26.98	27.28	27.90			**Wire #5**	28.91	28.78	28.57
		No cell	n.a.	n.a.	n.a.			**No cell**	33.09*	n.a.	n.a.
	3. lysis	**Wire #1**	26.78	27.04	26.99		**3. lysis**	**Wire #1**	28.65	28.63	28.55
		Wire #2	27.63	27.79	30.33			**Wire #2**	29.47	29.38	28.73
		Wire #3	26.21	26.18	25.67			**Wire #3**	28.16	27.84	27.58
		Wire #4	28.06	28.46	28.66			**Wire #4**	29.19	29.30	29.06
		Wire #5	28.64	28.12	28.18			**Wire #5**	30.14	30.80	30.28
		No cell	n.a.	n.a.	n.a.			**No cell**	30.83*	n.a.	n.a.

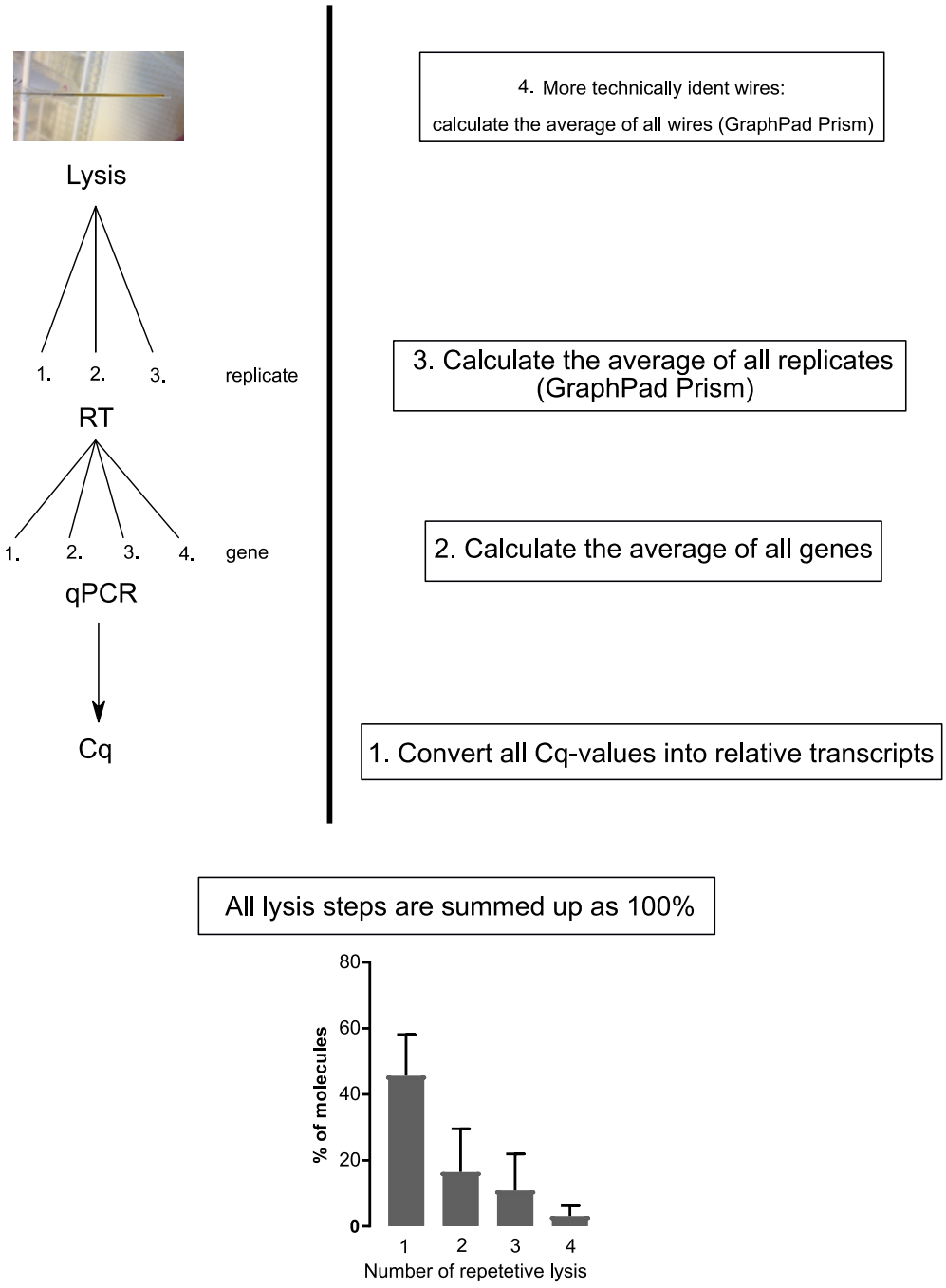

Figure 28: Schematic representation of the analysis from the direct lysis experiments. After the direct lysis on the DC01 was performed the lysate was used for RTqPCR. Three technical replicates were performed in the RT and the cDNA of each replicate was used for qPCR (mostly four marker genes). The measured Cq-values were converted into relative transcripts (using GenEx), the average of all genes was calculated and then the average of all replicates were calculated in GraphPad Prism. When more than one technical ident wire was analysed, the average of all replicates was calculated in Excel and the average of the wires was calculated in GraphPad Prism.