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Detection of minimal residual disease in childhood acute lymphoblastic leukemia by digital droplet PCR

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„Es ist besser, ein Licht zu entzünden,
als auf die Dunkelheit zu schimpfen.“

Konfuzius

Index

Abbreviations:

ALL	Acute lymphoid leukemia
AML	Acute myeloid leukemia
ASO	Allele specific oligonucleotide
BM	Bone marrow
CAR-T cells	Chimeric antigen receptor cells
CR	Complete remission
Ct	Cycle threshold
ddPCR	Digital droplet polymerase chain reaction
EFS	Event-free survival
ESG-MRD	European study group on minimal residual disease
FACS	Fluorescence-activated cell sorting
FU	Follow up
gDNA	Genomic deoxyribonucleic acid
HLA	Human leukocyte antigen
HSCT	Hematopoietic stem-cell transplantation
Ig	Immunoglobulin
LOD	Limit of detection
MFC	Multiparameter flow cytometry
MNC	Mononuclear cells
MRD	Minimal/measurable residual disease
NGF	Next generation flow cytometry
NGS	Next generation sequencing
NR	Non-remission
OAS	Over-all survival
PCR	Polymerase chain reaction
PB	Peripheral blood
PNQ	Positive-non-quantifiable
PR	Partial remission
RCT	Randomized controlled trial
RFU	Relative fluorescence units
qRT-PCR	Quantitative real-time polymerase chain reaction
STR	Short tandem repeats
TcR	T-cell receptor

1. Introduction:

1.1 Acute lymphoblastic leukemia:

Acute lymphoblastic leukemia (ALL) still remains the most common cancer of childhood that makes a stake of approximately 25% of all reported cases in children (Bhojwani et al., 2015). The incidence peaks between the age of one to four years and slightly increases again among adults older than 50 years (Katz et al., 2015).

Even though, some genetic factors are related to a higher risk of ALL, for instance trisomy 21, most affected children do not have inherited factors identified so far. Increased exposure to radiation and chemicals explains a small number of cases as well but usually a specific trigger of disease onset remains unexplained (Hunger and Mullighan, 2015).

A maturation block in the early phase of leukemic cell differentiation leads to abnormal proliferation and accumulation of immature leukemic cells hampers physiologic hemopoiesis and causes various reactions inside the patient´s organism. Thus, clinical manifestation and prognosis is heterogenous (Della Starza et al., 2019). Palor and fatigue caused by anemia, increased infections due to neutropenia, hematoma or bleeding because of thrombocytopenia, restricted organ functions after leukemic cell infiltration or extramedullary manifestations can be presenting symptoms (Hunger and Mullighan, 2015).

With reference to the immunophenotype of leukemic blasts, ALL can be grouped into B-lineage-ALL (B-ALL) and T-lineage-ALL (T-ALL) with a probability of occurrence of around 85% and 10-15% respectively (Bhojwani et al., 2015).

Pui et al. reviewed the development and results of several study groups during the past two decades and concluded that long-term survival of treated childhood ALL approaches 90% in many developed countries (Pui et al., 2015).

Relative survival decreases progressively with increasing age at diagnosis, varying from more than 90% relative five-year survival for small children to <30% among adults of 50 years age and older (Katz et al., 2015).

It has been historically poor for children with T-ALL as they frequently represent unfavorable characteristics such like central nervous system involvement and

experience more therapy failures and extramedullary relapses in comparison to B-ALL equivalents. Therefore, to improve a patient´s survival and clinical outcome, childhood T-ALL has to be medicated more intensely (Matloub et al., 2016).

To decide on the therapeutic steps and to estimate prognosis, a stratification regarding age, leukocyte count, immunophenotype, cytogenetics and defined biologically distinctive subgroups has to be done but also race, socioeconomic status and treatment adherence have to be considered (Vrooman and Silverman, 2016). Nevertheless, the both most important factors of influence are the genetic leukemia characteristics and therapy response of the patient (Iacobucci and Mullighan, 2017, Kruse et al., 2020).

According to risk stratification, different therapy stages are available. These include in succession chemotherapy, tyrosine kinase inhibitors for Philadelphia chromosome-positive ALL, furthermore hematopoietic stem cell transplantation or, in case of further non-remission or relapse, immunotherapy such like CD19 antibodies and the recently implemented Chimeric antigen receptor T-cell (CAR-T) cell approach (Vrooman and Silverman, 2016, Hunger and Raetz, 2020, Schlegel et al., 2014). Blinatumomab for instance was used in the MRD cohort of this project and is a bispecific T-cell engager antibody construct. It binds both CD3+ cytotoxic T-cells and CD19+ B-cells. Helping the patient´s T-cells to eliminate CD19+ B-lineage ALL blasts results in significantly longer over-all-survival (OAS) than chemotherapy alone in case of relapsed or refractory B-cell precursor ALL (Kantarjian et al., 2017). It was the first antibody approved for treatment of refractory ALL and minimal residual disease (MRD) positive patients (Della Starza et al., 2019).

1.2 General concepts:

Certain categories to evaluate treatment response have been defined for Acute myeloid leukemia (AML) and can be used to talk with slight adjustments about ALL as well (Döhner et al., 2010):

Complete remission (CR):	Bone marrow (BM) blasts <5%, independence of red cell transfusions, absolute neutrophil count >1.000/µl, platelet count >100.000/µl, no extramedullary manifestation
Partial remission (PR):	hematologic criteria for CR fulfilled, decrease of BM blast percentage to 5-20% but at least 50% to blast percentage before treatment
Molecular CR (CRm):	depending on molecular target, no standard definition
Non-remission (NR):	also called Resistant disease, failure to achieve CR or PR after completion of initial treatment
Relapse:	Reappearance of BM blasts >5% or in the peripheral blood (PB), development of extramedullary disease.

1.3 Relapsed leukemia and its treatment:

Despite all efforts, there is still a proportion of about 16-20% patients with diagnosed childhood ALL that will experience a relapse. Besides aftereffects of long-term treatment, relapse itself is the main cause of cancer-related mortality in children (Vrooman and Silverman, 2016, Tuong et al., 2020).

While summarizing findings of several studies on relapsed ALL, Bhojwani et al. showed that blast origin can vary a lot. It can either be a minor subclone already present at the initial diagnosis which multiplied uncontrolled, a clone with slightly or completely different genetic alterations or even an independent second malignancy. Despite different biological origins, leukemic blasts at relapse are more resistant to numerous chemotherapeutics compared to initial treatment (Bhojwani et al., 2015).

A short duration of first remission, T-cell immunophenotype, bcr-abl fusion transcript or an isolated BM relapse are seen as particularly unfavorable conditions (Einsiedel et al., 2005). Risk stratification and decision on treatment is often more complicated and less standardized. Relapse is defined by the Children's Oncology Group as "early" when occurring within 18 months of initial diagnosis, "intermediate" in between and "late" when occurring after 36 months of initial diagnosis (Nguyen et al., 2008).

In comparison, the Berlin-Frankfurt-Münster-Group defines early relapse as relapse during therapy or within 6 months after cessation of initial treatment (Einsiedel et al., 2005).

After BM relapse, typical reinduction involves a multi-drug combination of vincristine, glucocorticoid, asparaginase and anthracyclines in individual doses and times of delivery (Bailey et al., 2008, Lejman et al., 2021). The treatment opportunities for children with relapsed ALL expanded during the past decade as several promising immunotherapeutic and molecular approaches have been developed. To be named again, there are blinatumomab (antiCD3/19), inotuzumab ozogamicin (immunoconjugate antiCD22) and CAR-T cells (Hunger and Raetz, 2020).

Even tough higher therapy-related toxicity is accepted, leukemia-free-survival in second remission is very low. If feasible, a myeloablative chemotherapy followed by Hematopoietic stem-cell transplantation (HSCT) has to be considered additionally in high-risk groups (Bailey et al., 2008). However, a Human leucocyte antigen (HLA) matched family donor (MFD) is only available in 25-30% of patients considered for HSCT. Hoping for a HLA matched unrelated donor (MUD) can take long with potential relapse or death meanwhile (Bailey et al., 2008). But even if there is no matched donor available, HSCT can be pursued with a curative intent as haploidentical donors yield comparable outcomes in high risk leukemia patients (Leung et al., 2011). By regarding all current options on finding a HSCT donor and their similar outcomes, therapeutic HSCT can be conducted timely in almost all cases. (Algeri et al., 2021)

1.4 Minimal residual disease:

Minimal residual disease (MRD) is the common abbreviation for minimal or even more precise for measurable residual disease. It detects the post-therapeutic presence of leukemic blasts in up to 1:1,000 (10^{-4}) to 1:1,000,000 (10^{-6}) white blood cells. (Schuurhuis et al., 2018, Della Starza et al., 2019).

In many hematologic malignancies such like ALL, AML, chronic myeloid leukemia, non-Hodgkin lymphoma and multiple myeloma MRD detection is already quite common in clinical practice for a couple of years. (van der Velden

et al., 2003, Deeren et al., 2020). Early response to treatment measured with minimal residual disease assessment is a powerful predictor of outcome as well. Seemingly, patients with slow or no response to initial treatment have a poorer prognosis (Vrooman and Silverman, 2016).
Regarding this, it has been summarized that children with no detectable MRD at the end of induction treatment have an excellent prognosis and shall not be burdened with treatment intensification, such like HSCT (Szczepański, 2007).
But besides front-line therapy stratification, MRD information is also crucial for patients undergoing HSCT and those who experience a relapse. Especially, when high MRD levels remain during consolidation treatment, there is an urgent necessity for treatment augmentation or even for further novel treatment approaches (Szczepański, 2007, Lejman et al., 2021). Even before HSCT, maximal reduction of MRD is an inevitable requirement for successful long-term results (Szczepański, 2007).

There are different technical methods to measure MRD which are mentioned in the following part:
Multiparameter flow cytometry MRD (MFC-MRD) has the lowest sensitivity of all methods mentioned but is applicable in almost all patients. In general, it is based on two immunohistochemical methods. The first one is the Leukemia Associated Phenotype (LAIP) way which identifies leukemic blast immunophenotypes and monitors them from diagnosis to later follow-up time points. The other approach is called the Different from normal (DfN) one, where leukemic cells are distinguished from normal cells on the basis of their immunophenotypes (Buldini et al., 2019).
After HSCT, molecular chimerism analysis can help to determine the proportion of donor cells to recipient cells. Therefore, leukocyte subpopulations are separated, their DNA is extracted and Short-Tandem Repeats (STR) or small nucleotide polymorphisms are studied. Thereafter, results are expressed in % donor cells and give an assessment of graft engraftment (Clemente et al., 2017). Currently, the molecular polymerase chain reaction (PCR) approach is considered as the gold standard (Schuurhuis et al., 2018).

Thereby, unique rearrangement patterns such like fusion gene transcripts or immunoglobulin (Ig) and T-cell receptor (TcR) gene variations are used as MRD targets (Kruse et al., 2020). Allele-specific oligonucleotide (ASO) primers are designed complementary to individual junctional region sequences to further increase sensitivity. Fluorescently labeled probes also contribute to more reliant results. Thanks to European collaboration, additional targets and sequences are published and 90-95% of ALL-patients can be monitored with two or more suitable MRD targets (van Dongen et al., 2015). Yet, the applicability for AML is limited, as only around 40% of AML patients offer at least one suitable MRD-target (Schuurhuis et al., 2018). Digital droplet PCR (ddPCR) is an efficient, sensitive and accurate further development of conventional real-time quantitative PCR (qRT-PCR) and has the potential to further optimize the widespread use of PCR MRD assessment (Della Starza et al., 2018).

For the next-generation-sequencing (NGS) method, first of all, a consensus set of primers to amplify all possible rearranged TcR and Ig gene sequences is used before high-throughput sequencing is conducted. Bioinformatic knowledge and specifically designed algorithms are needed to specify and monitor clonal gene rearrangements (Faham et al., 2012).

With special sample preparation and innovative antibody combinations Next generation flow cytometry (NGF-)MRD enables to define the degree of immunophenotypic deviation of ALL cells from normal and also from regenerating BM cells. This improved characterization of cell composition helps to display and understand the complex immunophenotypic shifts during treatment, follow-up and potential relapse (Della Starza et al., 2019).

The AIEOP-BFM ALL 2000 study was the first standardized international assessment of quantitative MRD based on specific PCR targets at defined time points and reported MRD detection after treatment as highly predictive in childhood precursor B-ALL (Conter et al., 2010) and T-ALL (Schrappe et al., 2011). To name one more, the UKALL 2003 trial showed that if MRD is 0.01% at the end of remission therapy, an intensification of post remission treatment will

lead to a significant better 5-year-survival despite more therapy-related adverse effects (Vora et al., 2014).
MRD-based risk stratification was claimed to be even more meaningful compared to other clinically relevant risk factors, including age at disease onset, immunophenotype and blast count at diagnosis (van Dongen et al., 1998).
Nowadays, the absoluteness of this statement has been somewhat weakened, yet MRD remains an absolutely clinically applied decision-making and prognostic factor. (Pui et al., 2017)

1.5 Quantitative real-time PCR:

As early as 1998, the first report of quantitative real-time polymerase chain reaction (qRT-PCR) for MRD determination with TcR and Ig rearrangements was published (Pongers-Willemse et al., 1998) and since then followed up and improved (Pongers-Willemse et al., 1999, van der Velden et al., 2007a, van der Velden et al., 2003, van Dongen et al., 1999, van Dongen et al., 2015).
Highly specific markers to discriminate leukemia cells and normal cells are needed to ensure an accurate and sensitive detection of scattered tumor cells amidst thousands of normal cells. With oligonucleotide primers complementary to specific junctional sequences a theoretically sensitivity of 1:10,000 (10^{-4}) to 1:100,000 (10^{-5}) can be reached (van Dongen et al., 2015).

According to van der Velden et al. 2003, main MRD target categories are:

- <u>rearrangements of Ig and/or TCR genes</u>
  Germline variable (V), diversity (D) and joining (J) gene segments arrange during early B- and T- lymphocyte differentiation and thus every B- and T-lymphocyte receives a unique V-D-J segment combination. Even though they are not necessarily pathological nor directly linked to the oncogenic process (Pongers-Willemse et al., 1998), those combinations can be seen as DNA-fingerprints of leukemia cells.
- <u>breakpoint fusion genes</u>
  If breakpoint fusion regions are relatively small, PCR primers at the opposite sides of those regions can be chosen as a patient-specific MRD-

target as well. The submicroscopic 1p32 deletion (*TAL1*) in some T-ALL patients is an example for this. In contrast to Ig/TcR gene rearrangements, they are stable throughout the disease, directly involved into the ontogenetic process and less prone to false-positive results due to contamination and physiological rearrangements.

- tumor-specific fusion genes

  Similar to breakpoint fusion genes, tumor-specific fusion genes such like bcr-abl provide reliable leukemia markers as well.

With the use of allele-specific fluorescently labeled probes this ASO-PCR was further improved (Pongers-Willemse et al., 1998). However, this improvement goes hand in hand with the fact that a specially adapted probe has to be designed and ordered for every specific MRD-target (van der Velden et al., 2003).

As somatic hypermutations can lead to the loss of a primer binding site and oligoclonality at diagnosis brings uncertainty which subclone may multiply at relapse, preferably two or more leukemia targets per patient should be used to prevent false-negative PCR findings (van der Velden et al., 2003).

During the exponential phase of the qRT-PCR process the fluorescence signal is exponentially rising as well. With consideration of unspecific background signal, a threshold line can be drawn. The cycle where the fluorescence of a sample exceeds the threshold line for the first time is defined as crossing point or cycle threshold (C_t). It is inversely proportional to the target amount in the PCR sample (van der Velden et al., 2003).

International collaborations and efforts have taken place to standardize and optimize MRD detection: to be mentioned is the BIOMED-1 Concerted Action which set up standardized primer sets for defined leukemia targets and a standardized qRT-PCR-Protocol (van Dongen et al., 1999). To ensure good DNA sample quality and quantity a control gene, for instance albumin, has to be included (van der Velden et al., 2003). Dilution experiments have to be performed to evaluate the sensitivity of the qRT-PCR MRD detection. It is recommended to dilute patient DNA from diagnosis in healthy DNA and then plotting the logarithmic dilution figure against the associated C_t value (van der Velden et al., 2003).

Since 2002, the European Study Group on MRD detection in ALL (ESG-MRD-ALL) consisting of several MRD-laboratories worldwide ensures the quality and standardization of MRD assessment and also deals with further improvements of MRD detection (van der Velden et al., 2007a).

Despite many advantages and its accuracy, the MRD calibration curves for qRT-PCR are labor intensive and time-consuming. In-depth knowledge about immunobiology of lymphocytes and primer design is needed (van der Velden et al., 2003). Identification of suitable leukemia markers and designing of well-fitting ASO-primers can last three to four weeks and analysis of follow-up samples up to one week (van Dongen et al., 2015). Moreover, non-specific amplification of Ig- or TcR rearrangements can scarcely be distinguished from low level positive cases. As a consequence, the intrinsic risk of false positive results arises (Della Starza et al., 2019). One main defiance is the need of a dilution series from neoplastic DNA obtained at the onset of the disease to create a standard curve for classifying the leukemia cell ratio at every single follow-up (FU) point. A lack of sufficient diagnostic material from prognosis can thus restrict the number of FU measurements (Della Starza et al., 2019).

Therefore, efforts are made to further improve the molecular approach of MRD detection.

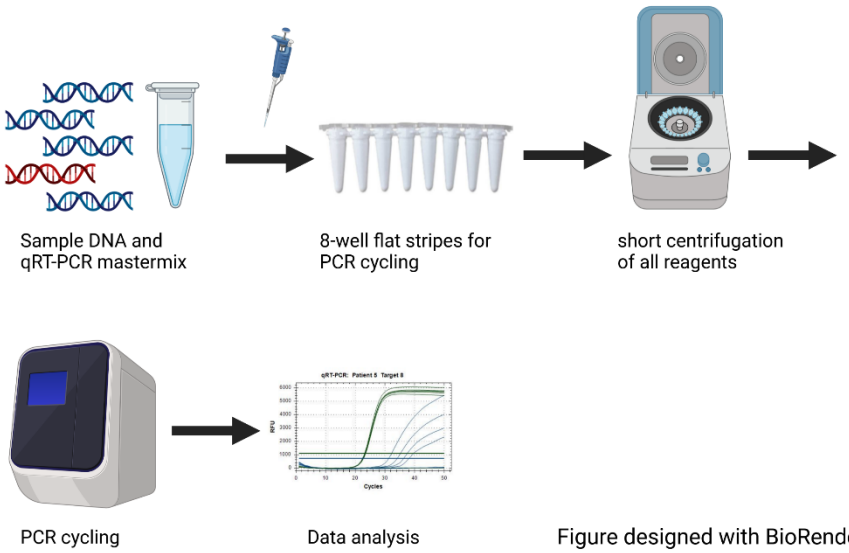


Fig. 1: Workflow for qRT-PCR Sample DNA and qRT-PCR mastermix are pipetted in the respective wells and centrifuged down shortly. During the amplification of the PCR products, fluorescence signals are measured and data analysis can be done immediately after the run.

1.6 Digital droplet PCR:

The main distinctive mark of digital PCR is the division of a reaction volume into partitions, either chambers in chamber and microfluidics-based digital PCR or droplets in droplet digital PCR (ddPCR) (Kosir et al., 2017). Using ddPCR for quantitation of DNA targets overcomes the need for calibration curves. It is a further development of qRT-PCR and allows an absolute quantitation of copy numbers (Della Starza et al., 2019).

When the idea first came up, many replicate reactions at limiting dilutions were used to split up individual analyte molecules into around one molecule per well and huge multiwell plates per sample have been analyzed (Sykes et al., 1992).

As its technology has been improved, digital PCR has become practical for routine use and commercially available (Hindson et al., 2013).

Modern high throughput ddPCR mostly uses the water-in-oil droplets approach. With the use of microfluidic circuits and surfactant chemistries Hindson and colleagues were able to divide 20µl of sample mixture into approximately 20,000 droplets. Therefore, they loaded template, ddPCR Mastermix and TaqMan reagents in the respective sample well of a single-use injection molded cartridge and droplet generator oil in the associated other well. Through vacuum, sample and oil were drawn through the tubules, 1nL droplets were generated and formed a clear layer due to density differences between the oil and aqueous phase (Hindson et al., 2011). Target DNA molecules are distributed at a level where most of the droplets or respectively partitions contain one template copy. Other partitions do not contain one or on the other hand count two or even more template copies per droplet. After amplification, partitions with one or more target templates will give a fluorescence signal whereas partitions without a template will not give a luminous sign. With regard to Poisson´s law and the fraction of positive end-point signals, the number of target copies per well can be directly determined (Hindson et al., 2011).

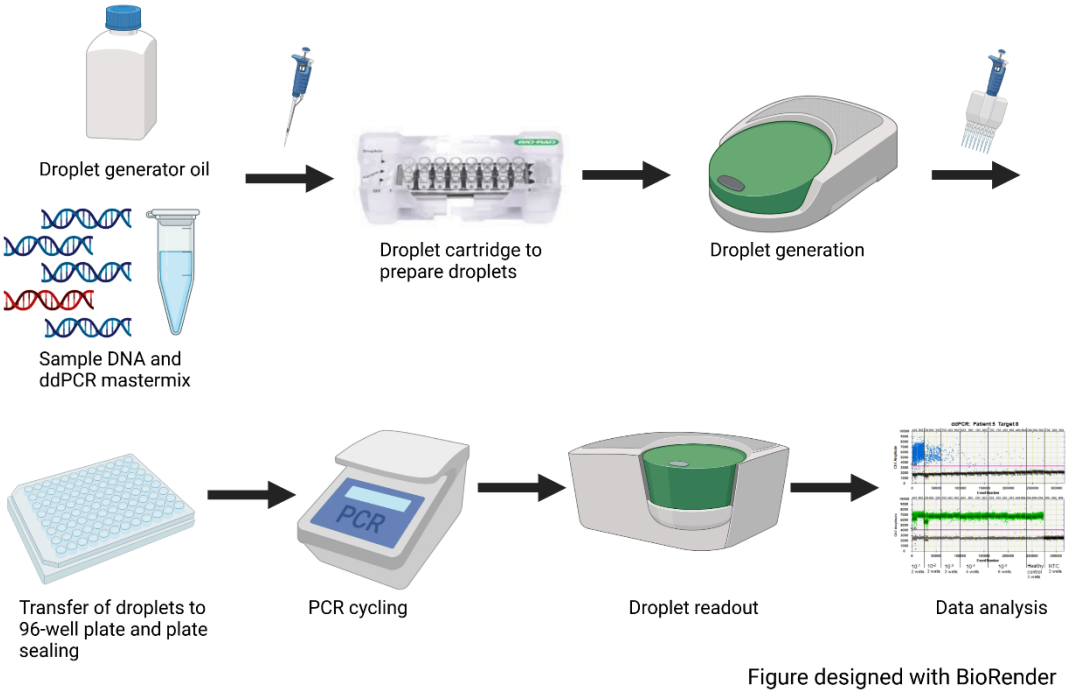


Fig. 2: Workflow for ddPCR Sample DNA and ddPCR mastermix are pipetted in one row of the droplet cartridge and droplet generator oil in the other respective row of wells. Droplets are generated and transferred to a 96-well plate. After covering the plate, the reagents are cycled in a PCR cycler. To read out their fluorescence signals, the single droplets are measured in a droplet reader and data analysis can be done afterwards.

Areas of application that have to be mentioned are the determination of copy number variations (CNV), rare mutation detection (RMD) and absolute DNA quantitation (Hindson et al., 2011). ddPCR is more and more applied for MRD determination of ALL as well as for AML patients (Voso et al., 2019).

As well as for qRT-PCR, patient-specific Ig or TcR gene rearrangements or leukemia specific fusion gene transcripts can be used as ddPCR MRD targets (Della Starza et al., 2019). So far, no standardized guidelines for ddPCR MRD analysis and interpretation for childhood ALL and AML have been published but the Euro MRD Consortium is working on it (Della Starza et al., 2019).

In several studies, a comparable accuracy and sensitivity of ddPCR to qRT-PCR was confirmed with high concordance between both methods (Della Starza et al.,

2016, Cavalli et al., 2017) . DdPCR is even said to reduce grey-zone positive non-quantifiable results (PNQ) (Drandi et al., 2020).

Recently, the first paper dealing with the detection of MRD levels using ddPCR in pediatric ALL was published. It is dedicated to the questions whether ddPCR is more sensitive at certain FU points and whether PNQ results obtained with qRT-PCR can be quantified with ddPCR in pediatric ALL as well. Major findings in this study were that ddPCR MRD measurement provides more accurate possibilities and potentials that qRT-PCR cannot technically offer. Especially for later FU points patient´s risk stratification was claimed to be more precise with ddPCR MRD measuremen(Della Starza et al., 2021)
It is very important that MRD evaluation is accurate and reliable as it is not only for prognostic risk evaluation within a given treatment protocol anymore, but also to plan and determine an individual patient´s therapy (Drandi et al., 2020).

The aim of this thesis was to gain basic knowledge and experience in the field of MRD diagnostics. It was started with qRT-PCR MRD diagnostics to approach the practical implementation and then a reliable strategy for performing MRD diagnostics with ddPCR was developed. By optimizing our PCR protocol, the hope was to reach at least the sensitivity of qRT-PCR by using ddPCR.

2. Material and methods:

This experimental work was approved by the Ethics Committee of the University of Tübingen by the end of 2019 with the number 922/2019BO2.

2.1 Laboratory equipment	Manufacturer
Centrifuges	Biozym, Sprout Mini Centrifuge Hettich, Rotixa 50 RS Carl Roth, Mini Centrifuge Hettich, Mikro 22 R
Multi Block Heater	Lab-Line
NanoDrop 2000	Thermo scientific
PCR-Cyclers	BioRad, CFX Connect Real-time system BioRad, C1000 Thermal Touch cycler
Pipettes	Eppendorf, research Brand, Transferpette Thermo Fisher, S1 Pipet filler HTL, Abimed Multimate
PX1 PCR Plate Sealer	BioRad
QX 200 Droplet Generator	BioRad
QX 200 Droplet Reader	BioRad
Vortexers	Heidolph, REAX 2000 Scientific industries, Vortex genie 2
Water bath	Memmert

2.2 Consumabels	Manufacturer
Pipett tips	Costar, 10ml Eppendorf, biopur Thermo Scientific, Art Tips Biozym, SurPhob Peqlab, Safeguard Sarstedt, Pipette Tips
DNA-Ex	innoTrain
Falcons	Greiner bio-one, cellstar tubes 15ml
Tubes	Eppendorf, tubes 0.5, 1.5 and 2.0ml
Droplet generation	BioRad, Cartridge holder BioRad, DG8 Cartridges BioRad, Droplet generator DG8 Gasket
ddPCR plate	Bio-Rad, ddPCR plates 96-well, semi-skirted Bio-Rad, pierceable foil heat seal
Control blood draw	Sarstedt, S-Monovette 9ml EDTA Sarstedt, Safety-Multifly-Set

2.3 Chemicals, reagents and kits	Manufacturer
qRT-PCR Supermix	PeqLab, KAPA Probe Fast Universal
DNA extraction kits	Qiagen, QIAamp DNA Mini Kit 50 Qiagen, QIAamp DNA Blood Midi Kit 100
RPMI medium	Gibco, RPMI Medium 1640 (1x)
ddPCR Supermix	BioRad, ddPCR Multiplex Supermix
Ethanol (absolute)	Supelco EMSURE
Water	VWR CHROMANORM, Water for HPLC Thermo Fisher scientific, Invitrogen RT-PCR grade water
Primers and Probes	Eurofins Metabion
Droplet generation	BioRad, Droplet generator oil for probes
Droplet readout	BioRad, Droplet reader oil

2.4 Software	Version
CFX Maestro	4.1.2433.1219
QuantaSoft	1.7.4.0917

2.5 Primer Sequences for MRD-Project:

2.5.1 ASO primers:

Name	Sequence (5´- 3´)
5617P13-F01	TGCCTTGTGGGAGAGGTT
5617P08-2F02	GCACAAGATCCTCCTGAGT
5082P12-F01	GGGCCTTTCCTAAGGGT
5082P03-4F01	GATGGTGGTTAGCTACGACA
5895MLL-fw	CAGTAATAACATGTCC
4686P01-5F01	GTACCAGCTGCCAGTACTG
4686P03-4F02	AGAGTGGGAGCTACTCCATAT
4766TRGV8JP2F02	ACCTGGGATGGGAGGTA
4766IGV4D4F01	GACACCCCCGAAGAGGAT
4938IGV23-6F02	CGAAGGCTTCTTAGGACGA
4938P20-1F01	GGGAGGGTTCGTGTCT
4808P01-6F01	TATTACTGTGCGAGAACGTAAAGA
4808P20-F01	TACTGTGCCTGTGTGGGT
4859DH7-5F01	ACCACTGTGCTAACTATAACT
4859P23-F01	TCATTGTGCCTTCCTAACAAACGCA
5014JaS8R1	CCCCTCTCGGCGGT
5014P14/2	TTGTGGGAGCCTCTTCAC

2.5.2 Consensus primers:

Target	Sequence (5´- 3´)
R-JG1.1/2.1	TATACTGAGGCCAGGAATGTGACATA
R-JG1.3/2.3	CTGCCTTCCCTCTATTACCT
R-DD3	TTGCCCCTGCAGTTTTTGTAC
Fw-VD2	TGGCCCTGGTTTCAAAGAC
R-JH 4	CAGAGTTAAAGCAGGAGAGAGGTTGT
R-JH 5	AGAGAGGGGGTGGTGAGGACT
R-JH 6	GCAGAAAACAAAGGCCCTAGAGT
R-KDE	TACAGACAGGTCCTCAGAGGTCAG
5895MLL-rv	CCATAATCTAAACTAACAACCATGC

2.5.3 Probes:

Target	Sequence (5´- 3´)
T-JG1.1/2.1	ACCAGGTGAAGTTACTATGAGCTTAGTCCCTTCAG
T-JG1.3/2.3	TGTCACAGGTAAGTATCGGAAGAATACAACATTTCC
T-DD3	ATACGCACAGTGCTACAAAACCTACAGAGACCT
T-VD2	ATTTCCAAGGTGACATTGATATTGCAAAGAACC
T-JH 1,2,4,5	CCCTGGTCACCGTCTCCTCAGGTG
T-JH 6	CACGGTCACCGTCTCCTCAGGTAAGAA
T-KDE	AGCTGCATTTTTGCCATATCCACTATTTGGAGT
5895MLL-T	CATATCACTGAGTGAAAAGAGCAGGTTAC

2.5.4 Primerassignment:

Patient	Target	ASO - Primer	Consensus Primer	Probe
1	TCRGV1-Jg1.3/2.3	5617P13-F01	R-JG1.3/2.3	T-JG1.3/2.3
1	Vkll-Kde	5617P08-2F02	R-KDE	T-KDE
2	TCRGV1-Jg1.1/2.1	5082P12-F01	R-JG1.1/2.1	T-JG1.1/2.1
2	IgHVH3-JH4	5082P03-4F01	R-JH 4	T-JH 1,2,4,5
3	MLL-Translocation	5895MLL-fw	5895MLL-rv	5895MLL-T
4	IgHVH1-JH5	4686P01-5F01	R-JH 5	T-JH 1,2,4,5
4	IgHVH3-JH4	4686P03-4F02	R-JH 4	T-JH 1,2,4,5
5	TCRGV1-Jg1.1/2.1	4766TRGV8JP2F02	R-JG1.1/2.1	T-JG1.1/2.1
5	IgHVH4-JH4	4766IGV4D4F01	R-JH 4	T-JH 1,2,4,5
6	IgHVH2-JH6	4938IGV23-6F02	R-JH 6	T-JH 6

6	TCRDV2-DD3	4938P20-1F01	R-DD3	T-DD3
7	IgHVH1-JH6	4808P01-6F01	R-JH 6	T-JH 6
7	TCRDV2-DD3	4808P20-F01	R-DD3	T-DD3
8	IgH-DH7-JH5	4859DH7-5F01	R-JH 5	T-JH 1,2,4,5
8	TCRDD2-DD3	4859P23-F01	R-DD3	T-DD3
9	TCRVd2-Ja58	5014JaS8R1	Fw-VD2	T-VD2
9	TCRGV9-Jg1.1/2.1	5014P14/2	R-JG1.1/2.1	T-JG1.1/2.1

Patient specific sequences designed and provided by Dr. H. Kreyenberg, University Hospital Frankfurt. All other sequences provided to us by him as well.

2.5.5 Housekeeping gene:

Target	**Sequence (5´- 3´)**
Fw-primer *Albumin*	TGAAACATACGTTCCCAAAGAGTTT
Rv-primer *Albumin*	CTCTCCTTCTCAGAAAGTGTGCATAT
Probe *Albumin*	TGCTGAAACATTCACCTTCCATGCAGA

Based on (Pongers-Willemse et al., 1998) and sequences published by (Taira et al., 2012) provided to us by Dr. H. Kreyenberg.

2.6 Cell washing / Sample preparation for DNA extraction:

The corresponding number of falcons was filled with 10ml RPMI medium. As soon as the cell pilots were thawed, their volume was added to the RPMI medium already prepared. 1ml RPMI was used to rinse the pilot tube. Afterwards the falcons were centrifuged at 400xg for 5 minutes. With an electric aspirator the supernatant was removed with attention not to remove any of the cells on the ground. The remaining cells were resuspended in 10ml RPMI and were again centrifuged as described above. The supernatant was discarded likewise. After this second washing step the cells were resuspended in the remaining volume and transferred to a 1.5ml Eppendorf cup.

Once again centrifuged, the supernatant was removed as careful as possible before the remaining cells and cell media were processed in the same cup to perform DNA isolation.

2.7 DNA-Isolation from leukemia blasts:

The cells were resuspended in 200µl Buffer T1. 25µl Proteinase K as well as 200µl Buffer B3 were added. After vortexing, the mix was incubated at 70°C for approximately 15 minutes. DNA binding conditions were adjusted by adding 210µl of ethanol (100%). All following centrifugation steps were performed at room temperature.

The NucleoSpin columns were placed in their collection tubes and loaded with the prepared samples. At 11000xg, the column and collection tube were centrifuged for 1 minute and the flow-through was discarded. For the first wash, 500µl Buffer BW was added and again centrifuged at 11000xg for 1 minute. The flow-through was discarded and a second wash with 600µl Buffer B5 was performed as previously described above. To remove the remaining ethanol the column with the collection tube was centrifuged as before.

To elute the DNA in a final step, the NucleoSpin column was placed into a 1.5ml microcentrifugation tube and 50µl of warm pure water were added. After 1 minute of incubation time at room temperature, the DNA was eluted by centrifuging for 1 minute at 11000xg.

2.8 DNA concentration determination:

After cleaning the measurement section on the arm of the NanoDrop, routine wavelength verification had to be waited before blanking with the elution medium pure water was possible.

Afterwards samples were vortexed and 1µl drops were measured one by one with wipe dry of the measurement section in between.

2.9 DNA-Isolation from whole blood:

To dilute leukemic DNA from diagnosis, pooled healthy DNA from eight healthy donors was used. Most of the other groups used DNA isolated from Mononuclear cells (MNC) only (Flohr et al., 2008, van der Velden et al., 2007a). Due to material, equipment and kit availability DNA was isolated directly from healthy whole blood in this project.

200µl QIAGEN Protease was pipetted into a 15ml centrifuge tube. 2ml blood were added and mixed shortly. After adding 2.4ml Buffer AL, the mix was inverted and vortexed thoroughly before an incubation time of 10 minutes at 70°C. Then, 2ml ethanol (100%) were added and mixed as well. Afterwards, one half of the solution was transferred into a column placed in a 15 ml centrifuge tube. The column and tube were centrifuged at 1850xg for 3 minutes. All centrifugation steps were performed at room temperature.

After discarding the filtrate and replacing the column, the remaining solution was loaded onto the column and centrifuged likewise. The flow-through was discarded again and the first washing step was done with 2ml Buffer AW1 and following centrifugation at 4500xg for 1 minute.

Without the need of discarding the flow-through, 2ml Buffer AW2 were loaded on the membrane of the column and centrifuged at 4500xg for 15 minutes.

Meanwhile, clean 15ml centrifugation tubes were labeled and the columns were placed onto them after centrifugation. The other collection tubes containing the filtrate were discarded. 300µl water at room temperature was pipetted onto the membrane and incubated for 5 minutes at room temperature. With a centrifugation speed of 4500xg for 2 minutes the DNA was eluted. To gain maximum concentration, the eluate was reloaded onto the membrane and centrifuged at 4500xg for 2 minutes again.

2.10 qRT-PCR for MRD diagnostic:

This part of the project was supported with knowledge and experience from Dr. H. Kreyenberg, responsible for MRD diagnostics at the university hospital in Frankfurt. Identification of MRD targets suitable for evaluation and primer designing was done in Frankfurt based on the summarized recommendations.

A good and short description was given by Conter et al., 2010 as follows:

At first, DNA samples from diagnosis were screened using PCR amplification with the BIOMED-1 primer sets for Ig kappa deleting element gene rearrangements, complete and incomplete TcR delta and TcR gamma rearrangements (van Dongen et al., 1999, Pongers-Willemse et al., 1999).

IgH rearrangements (complete and incomplete) were identified using 5 VH and 7 DH family primers in combination with 1 JH consensus primer (Szczepański et al., 1999, Szczepański et al., 2001).

The BIOMED-2 multiplex PCR primer sets were used for incomplete and complete TCR beta and *IGK* rearrangements (van Dongen et al., 2003).

Junctional regions of clonal PCR products were sequenced and potential patient-specific junctional region MRD sequences were identified. Afterwards, Allele-specific oligonucleotide primers were designed and tested for sensitivity as well as for specificity (Flohr et al., 2008).

The objective is to identify at least 2 targets with a sensitivity of 10^{-4} or lower and a similar quantitative range (van der Velden et al., 2007a).

According to this workflow, Mr. H. Kreyenberg provided us two MRD-Targets per patient with respective primer and probe sequences.

To imitate different blast concentrations, we diluted leukemia blast DNA in pooled healthy DNA with a dilution factor of ten. Due to DNA shortage, only one sample per dilution step has been measured. The experimental set-up contained dilutions from 10^{-1} down to 10^{-6} with one healthy DNA control and a Non-template control (NTC).

First of all, a master mix was prepared depending on the number of samples that had to be analyzed. Calculations were conducted with a factor resulting of the number of samples plus 5-10%.

Super mix, master mix and DNA have been pipetted into an 8-well flat stripe according to the following table:

Table 10: Pipetting sheme qRT-PCR

Reagent		**Volume**	**Final concentration**
Primer fw (80µmol/µl)	**master mix**	0,1µl	0,4µmol/µl
Primer rv (80µmol/µl)		0,1µl	0,4µmol/µl
Probe (20µmol/µl)		0,2µl	0,1µmol/µl
Water		4,6µl	
Supermix		10µl	
DNA (100ng/µl)		5µl	25ng/µl

In case of preliminary experiments, DNA amount was reduced to 200ng per well. Usually, one well per dilution step, one well with pooled healthy control blood and a NTC were analyzed besides the actual dilution samples. The first dilution step (10^{-1}) was left out in case of DNA shortage.

The wells were closed with a flat stripe lid and the final volume of was centrifuged down with the butterfly centrifuge. If the PCR cycler was not vacant immediately, the prepared wells were kept at 4°C.
Afterwards they were put into the qRT-PCR cycler and cycled according to the following protocol:

Table 11: Cycling protocol qRT-PCR

PCR-step	Time	Temperature	Cycles	Ramp rate
Denaturation	10 min	95°C	1	1°C/s
Amplification	15 sec 60 sec	95°C 60°C	50	1°C/s
Denaturation	10 min	95°C	1	1°C/s
Cooling and Infinite hold		4°C		1°C/s

We used the qRT-PCR protocol for quantification of MRD used in Frankfurt for routine diagnostic which is based on former protocols (Pongers-Willemse et al., 1999, Pongers-Willemse et al., 1998) and was kindly provided to us by Dr. H. Kreyenberg as well.

After the run, data was analyzed with Bio-Rad CFX Maestro software and evaluated based on the guidelines developed within the European Study Group for MRD detection in ALL.

In 2007, international guidelines for the interpretation of qRT-PCR-based MRD data have been published by the European Study Group on MRD detection in ALL (ESG-MRD-ALL). They specify experimental set-up, quantitative range and sensitivity, MRD-positivity and MRD-negativity as well as quantitation of follow up samples (van der Velden et al., 2007a).

All the following subitems are taken from these guidelines (van der Velden et al., 2007a) and adjusted to our own possibilities and experimental setup:

- We consulted FACS data of the original samples to verify that the 10^{-1} dilution contains 10% of leukemia blasts. If no information on blast count of our DNA samples had been available, we performed an absolute quantitation per ddPCR and approached to the blast percentage by the ratio between leukemia target and albumin gene occurrence.
- Whenever possible, we used 500ng DNA per well and reduced it to 200ng in case of patient DNA shortage.
- By serial dilution (10^{-1} - 10^{-6}) of the patient´s DNA in pooled DNA obtained from eight healthy donors, a standard curve has been created. An additional 5×10^{-4} step and duplicates are favored, which we had to skip.
- The standard curve must have a slope between −3.1 and −3.9 and a correlation coefficient $\geqslant$0.98 between different replicates.
- The threshold was set in the region of exponential amplification across all amplification plots.
- Background is described as nonspecific amplification in normal cells in which the lowest Ct value of nonspecific amplification is defined as the highest background signal. Therefore, our healthy control consisting of pooled healthy DNA also used for dilution and was taken as the reference for background signals.
- No template controls (NTC) have been included too.
- With a housekeeping gene (e.x. *Albumin*) a constant amount of total DNA per well is controlled
- The quantitative range describes the section of the standard curve where MRD values can be determined reproducibly, exactly and sufficiently far from the background.
- Sensitivity, in contrast, reflects the lowest MRD level to be measured, even though the signal may not be reproducible and accurate. The signal must be $\geqslant$1.0 Ct lower than the Ct value of the background.
- MRD-positivity and negativity in FU samples is assessed depending on the aim to reduce or intensify therapy. For our analysis we judged dilution

samples to be positive if they gave a signal matching to the previous dilution steps with a Ct of ⩾3 apart from the background signal. As we did not run replicates, we could not differentiate between maximum sensitivity and quantitative range and decided to look at our results by only using the expression “sensitivity”.

For reasons of completeness, the consensus document from the European LeukemiaNet MRD Working Party for AML shall be mentioned as well, but technical recommendations for MRD detection in AML rarely differ from those for ALL (Schuurhuis et al., 2018).

2.11 ddPCR for MRD diagnostic:

To establish MRD diagnostic from qRT-PCR to ddPCR, the qRT-PCR protocol from Frankfurt was used as a general orientation. Equally to qRT-PCR we measured dilution series in steps of 10 of leukemic DNA available from initial diagnosis in healthy pooled DNA.

The master mixes and remaining reactants were prepared and mixed as shown below:

Table 12: Pipetting sheme ddPCR

Reagent		Volume	Final concentration
Primer ASO-Target (80µmol/µl)		0,28µl	1,0µmol/µl
Primer fw-Albumin (80µmol/µl)		0,28µl	1,0µmol/µl
Primer rv-Target (80µmol/µl)		0,28µl	1,0µmol/µl
Primer rv-Albumin (80µmol/µl)	**master mix**	0,28µl	1,0µmol/µl
Probe Target (20µmol/µl)		0,33µl	0,3µmol/µl
Probe Albumin (20µmol/µl)		0,33µl	0,3µmol/µl
Supermix 2x		5,5µl	
Water		9,22µl	
DNA (100ng/µl)		5,5µl	25ng/µl

Depending on the number of replicates per dilution step, the respective volume of master mix (7,28µl per replicate) was pipetted in 0.5ml tubes and water and sample DNA were added.

To prepare droplets, a cartridge was placed in the holder. It had to be planned in eight sample formats as droplets could only be produced in multiples of eight. Without generating any bubbles by touching the bottom at a 20° angle and only going to the first stop of the pipet, 20µl of reaction mix were pipetted into the according cartridge well. After all samples of a row had been pipetted, 70µl of Droplet Generator Oil for Probes were pipetted similarly by avoiding any bubbles into their foreseen wells. The prepared cartridge was covered with a red gasket and then put into the Droplet Generator.

By running through the small tubules, droplets were generated and collected in the upper row of wells. From there, 42µl were slowly aspirated with a multichannel pipet and transferred to a 96-well plate where they were released slowly to not destroy the droplets. Those steps are repeated according to the number of rows being filled with the samples.

After sealing the 96 well plate in the Plate sealer, it is placed into the PCR cycler and cycled after the following protocol:

Table 13: Cycling protocol ddPCR

PCR-step	Time	Temperature	Cycles	Ramp rate
Denaturation	10 min	95°C	1	2°C/s
Amplification	20 sec 30 sec 60 sec	95°C 60°C 72°C	45	2°C/s
Denaturation	10 min	98°C	1	2°C/s
Cooling and Infinite hold		12°C		2°C/s

Afterwards the 96 well plate was put in the Droplet Reader to read out the fluorescent signals of every single droplet amplicon.

Data analysis was conducted consistent with current recommendations.

During the data acquisition of this project, a statement for ddPCR workflows, standard protocol and guidelines has been published within the Euro-MRD consortium regarding MRD analysis in mature B lymphoid malignancies (Drandi et al., 2020).

Based on these recommendations and the Bio-Rad´s application guide we adjusted and took over the following principles for our work:

- 500ng of DNA were contained in the final reaction mix for the target gene.
- With a housekeeping gene (e.x. *Albumin*) a constant amount of total DNA per well is controlled.
- Blackhole quencher have to be used for Probes that there is no interference in fluorescence signals measured afterwards.
- At least 2-3 sample replicates, 3 negative replicates from pooled DNA from eight healthy donors and 2 non-template control (NTC) replicates have been tested.
- The threshold was set manually below the positive cluster, as close as possible to the background signal, but nevertheless with a sufficient distance from background signals to ensure reliable sensitivity and specificity.
- Only replicates with a number of droplets ≥8 000 were accepted. And at least 2 replicates with >8000 droplets have been available for analysis.
- A sample was considered as positive, if the merge of positive target events ⩾3 in all summarized replicates of the corresponding sample.
- A sample was considered positive non-quantifiable (PNQ) sometimes also called below quantifiable range (BQR), if the merge of positive target events equaled 2 in all summarized replicates of the corresponding sample.
- A sample was considered negative, if there was only one or even no positive target event in all summarized replicates of the corresponding sample.

3. Results:

In this project nine pediatric patients with relapsed B-ALL were analyzed. For eight of them two patient-specific MRD targets were provided and in total it was possible to measure 17 different leukemia targets.

All of them have been transplanted one or several times here at Children's Hospital Tübingen and received a CD19 antibody as one part of their treatment. In cooperation with Dr. H. Kreyenberg from the University hospital in Frankfurt, responsible for chimerism analysis and MRD detection, we approached MRD diagnostics via qRT-PCR first as well as with ddPCR thereafter in the following steps:

First of all, dilution series of leukemic blasts have been measured with qRT-PCR. If Ct-results were reliable and fulfilled the expectations mentioned by the ESG-MRD, we started measuring dilution series of the same target with higher replicate numbers in ddPCR. After optimization of detecting MRD in our dilution series with both PCR methods, conclusive and reproducible values could be measured.

3.1 Quantitative Real-Time PCR results:

The FAM-labeled patient specific MRD target showed increasing Ct-values depending on the dilution factor of leukemic blasts. A dilution of 10^{-1} should correspond with 10% of leukemic blasts and gives a clear signal in qRT-PCR as long as there are no technical restrictions. The higher the dilution factors, the higher the Ct-values with a theoretical increase of 3.3 cycles per 1:10 dilution. This follows from the simple consideration that usually one cycle is needed for doubling a PCR product. If PCR efficiency is hampered, the slope of the standard curve can be lower than -3.9. The slope may be less steep than -3.1 in case of increased amplification efficiency, not entirely accurate dilution or primer binding. The last signal that can be clearly distinguished from background signals represents the highest sensitivity, each in regard to a specific patient target. In the meanwhile, the corresponding HEX-labeled housekeeping gene proves a constant total DNA content per well. The threshold line for each probe was set manually in the exponential part of amplification with sufficient distance to any

unspecific background signals. In this way, cycle thresholds were displayed with exact numerical values for each respective dilution.

These results were used to draw a standard curve linking Ct values with the underlying dilution of leukemia blasts. If the curve fulfills the criteria already mentioned in the methods section, this standard curve can be used to recalculate the blast count of a probe where the Ct-value is known. A detailed illustration of the standard curves of our qRT-PCR experiments can be found in the appendix (see 7. Appendix Fig.7).

The qRT-PCR results of patient 3 target 5 (Fig. 3, **E**) show exemplarily how the dilutions from 10^{-1} to 10^{-2} to 10^{-3} and 10^{-4} have approximately equal Ct value distances when regarding the graphic representation of the FAM fluorescence signals per dilution step. The fluorescence signal of 10^{-5} crosses the Ct value with a wider interval than before, but is still clearly above the Ct value and thus to be regarded as a positive signal. The signal of 10^{-6} is not detected anymore, it is below the sensitivity of this PCR reaction. Healthy control and NTC do also not give a signal, which makes contamination or non-specific amplification unlikely.

Since the HEX signal indicates a constant amount of DNA in all samples, this experiment is coherent and reliable and the sensitivity is 10^{-5}.

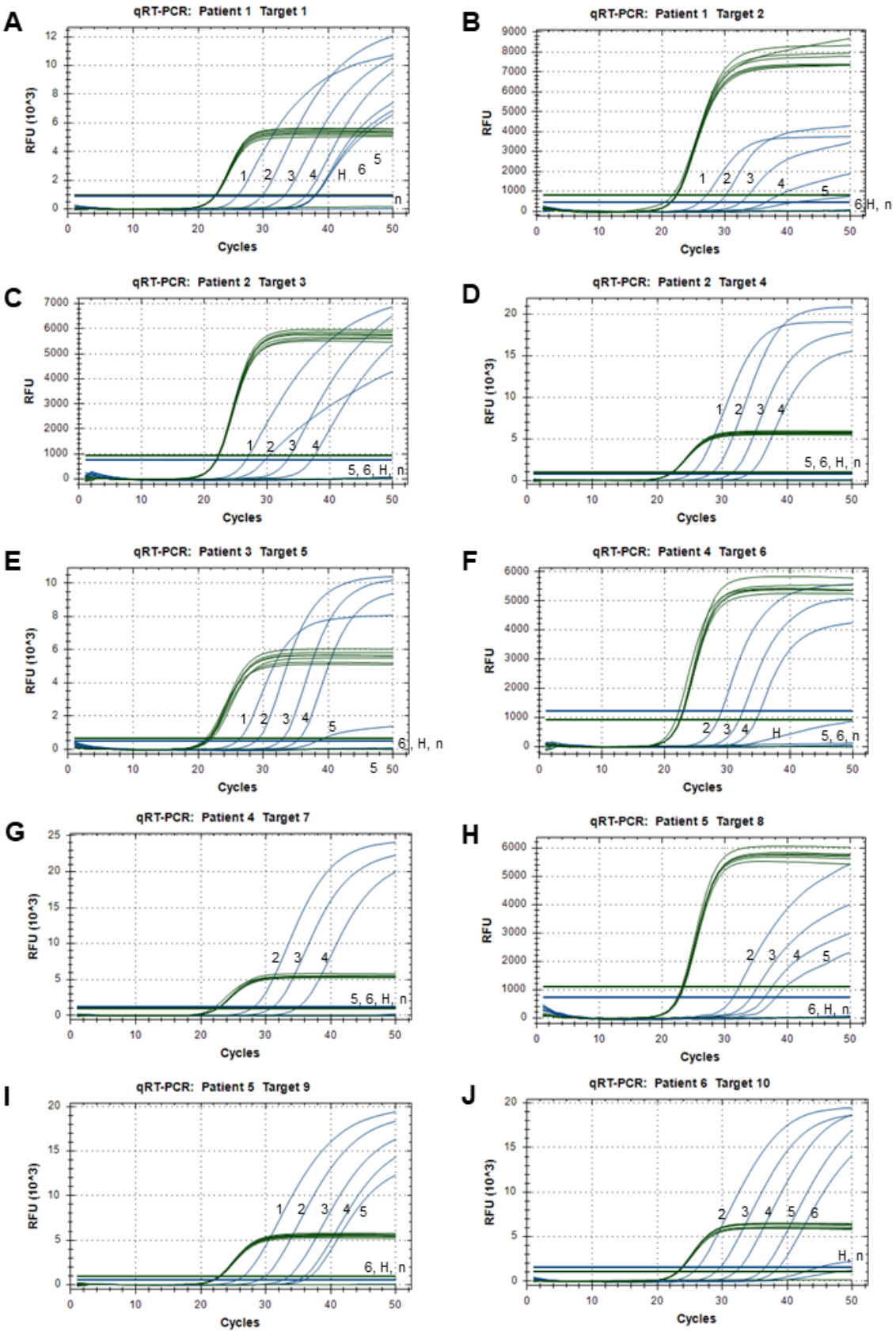
The same clear results were also obtained for the majority of the other experiments and targets:

A sensitivity of 10^{-5} was reached by six more targets (Fig. 3, **B, H, I, K, M, O**) whereby here, too, the last positive signal could be clearly distinguished from the background.

A sensitivity of 10^{-4} was reached by seven targets (Fig. 3, **C, D, F, G, N, P, Q**). The signal of 10^{-4} was still clearly pronounced and from 10^{-5} onwards no more detection could be shown.

Two targets (Fig. 3, **J, L**) reached even a clearly positive signal for 10^{-6}.

One target (Fig. 3, **A**) showed reproducible signals in the healthy control in similar magnitude of the 10^{-5} and 10^{-6} dilution. To guarantee sufficient Ct value distance from unspecific amplification, the sensitivity of this target and experiment lies only at 10^{-4}.


A
qRT-PCR: Patient 1 Target 1
RFU (10^3)
Cycles
1 2 3 4 H 6 5 n
B
qRT-PCR: Patient 1 Target 2
RFU
Cycles
1 2 3 4 5 6, H, n
C
qRT-PCR: Patient 2 Target 3
RFU
Cycles
1 2 3 4 5, 6, H, n
D
qRT-PCR: Patient 2 Target 4
RFU (10^3)
Cycles
1 2 3 4 5, 6, H, n
E
qRT-PCR: Patient 3 Target 5
RFU (10^3)
Cycles
1 2 3 4 5 6, H, n
F
qRT-PCR: Patient 4 Target 6
RFU
Cycles
2 3 4 H 5, 6, n
G
qRT-PCR: Patient 4 Target 7
RFU (10^3)
Cycles
2 3 4 5, 6, H, n
H
qRT-PCR: Patient 5 Target 8
RFU
Cycles
2 3 4 5 6, H, n
I
qRT-PCR: Patient 5 Target 9
RFU (10^3)
Cycles
1 2 3 4 5 6, H, n
J
qRT-PCR: Patient 6 Target 10
RFU (10^3)
Cycles
2 3 4 5 6 H, n

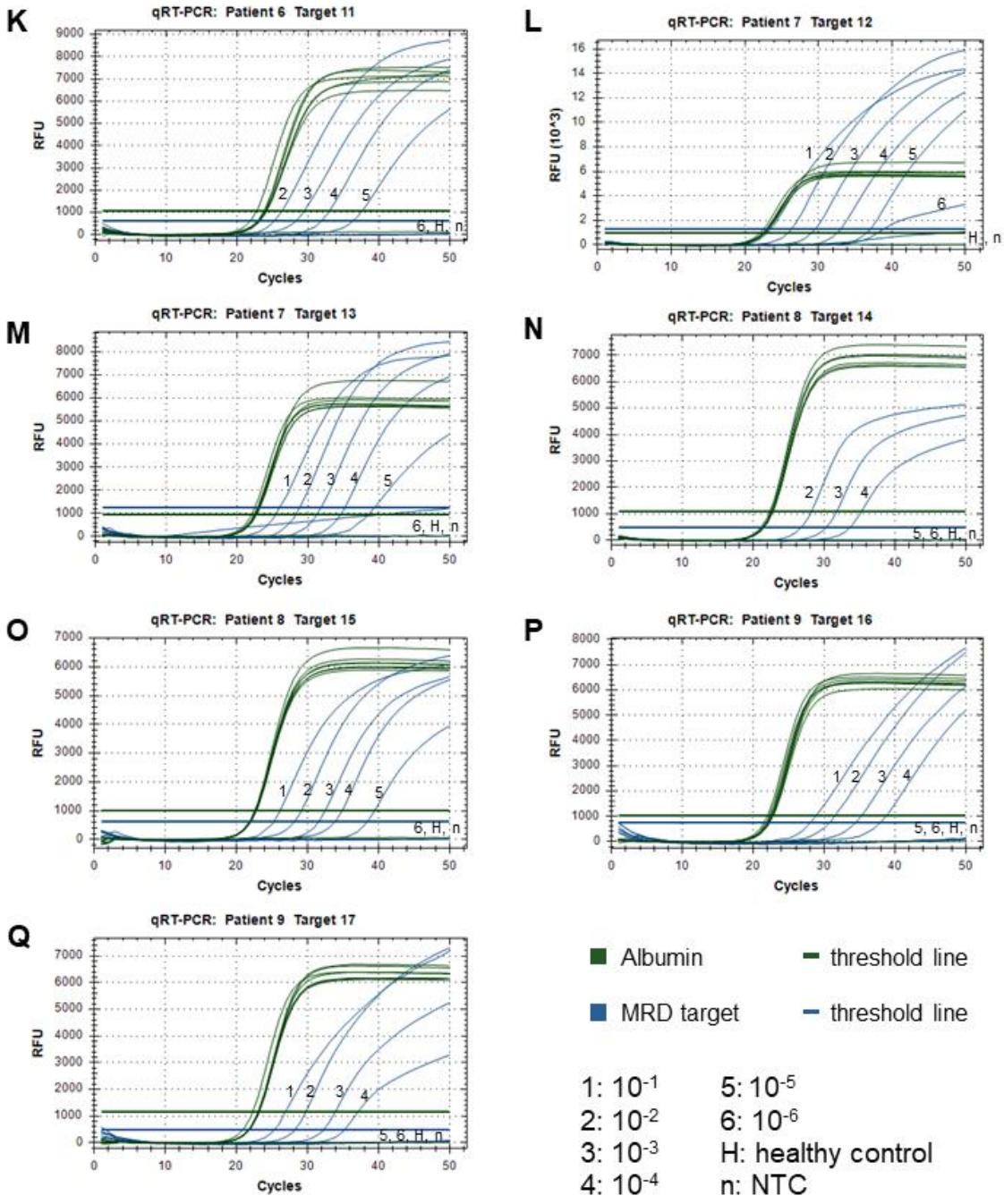


Fig. 3: qRT-PCR results for all 17 MRD targets Amplification of the housekeeping gene *Albumin* is shown in green (HEX) and serves as a reference for an equally high total DNA content per well. In blue, ascending dilutions with their steadily decreasing fluorescence signal (FAM) depending on the blast concentration from left to right can be seen. In some cases, no fluorescence signals can be detected for higher dilutions. Occasionally, a background signal has been detected in healthy DNA. The threshold lines for both probes were set manually in the range of exponential increases of the signals. Ct-values are generated when the MRD target fluorescence signal of one well exceeds the background signal for the first time. The higher the target concentration, the earlier the exponential amplification onset and the lower the Ct value.
The qRT-PCR sensitivity for our MRD targets goes down from 10^{-4} to 10^{-6}.

Repeatedly, positive signals also appeared in patient 4 target 6 and patient 6 target 10 (Fig. 3, **F, J**) in the healthy controls, but these were considered weak compared with the last positive signal detected.

To conclude, in this part of the project, the MRD targets produced reliable and reproducible signals and sensitivities of qRT-PCR MRD detection ranged from 10^{-4} up to 10^{-6} in our experiments.

Based on the on the experience thus gained, MRD quantification was conducted using ddPCR too with comparable results and sensitivity.

3.2 Digital droplet-PCR results:

ddPCR results are shown in droplet clusters of positive and negative droplets. The more specific the probe, the more clearly the positive droplets can be distinguished from those without a fluorescence signal or those with altered fluorescence intensity. Positive clusters of the patient specific MRD target show a decrease in count of positive droplets depending on the dilution factor. While still a lot of positive droplets can be seen at dilutions as 10^{-1} and 10^{-2}, it decreases with higher percentages of healthy, non-leukemic DNA. If leukemic DNA is diluted by factors like 10^{-4} or even 10^{-5} to 10^{-6}, only occasional positive MRD signals have been reported.

No unspecific amplification or contamination was shown in healthy control and NTC. In case of positive droplets in healthy donor DNA contamination had to be excluded and an increased non-specific background signal had to be assumed and taken into account for the evaluation.

The size of the negative droplet cluster behaves in an inversely proportional manner. In terms of DNA content, the housekeeping gene *Albumin* gives a high and stable number of positive droplets per well, indicating an approximately constant DNA content despite declining leukemic blast percentages.

The results of patient 3 target 5 (Fig. 4, **E**) also show exemplarily for ddPCR, how the number of positive FAM droplets declines with increasing dilution factors.

Both wells for 10^{-1} and 10^{-2} each show a big cluster of positive target counts. At 10^{-3} the single droplets can already be seen and at 10^{-4} they can already be counted by hand. All merged six wells of the 10^{-5} dilution count two positive droplets and thus end up in a grey area between negative and positive results. The dilution of 10^{-6} is not included anymore. Healthy control and NTC do also not give a signal, which makes contamination or non-specific amplification unlikely.

Since the droplet cluster of the housekeeping gene signal indicates a constant amount of DNA in all samples, this experiment is coherent and reliable and the sensitivity is 10^{-5}.

Similar grev zone results were also obtained for one other experiment and target (Fig. 4, **G**) where the sensitivity was 10^{-5} as well.

A sensitivity of 10^{-5} with clearly positive signals was reached by six more targets (Fig. 4, **D, I, K, L, O, Q**).

One target even reached a sensitivity of 10^{-6} (Fig. 4, **J**).

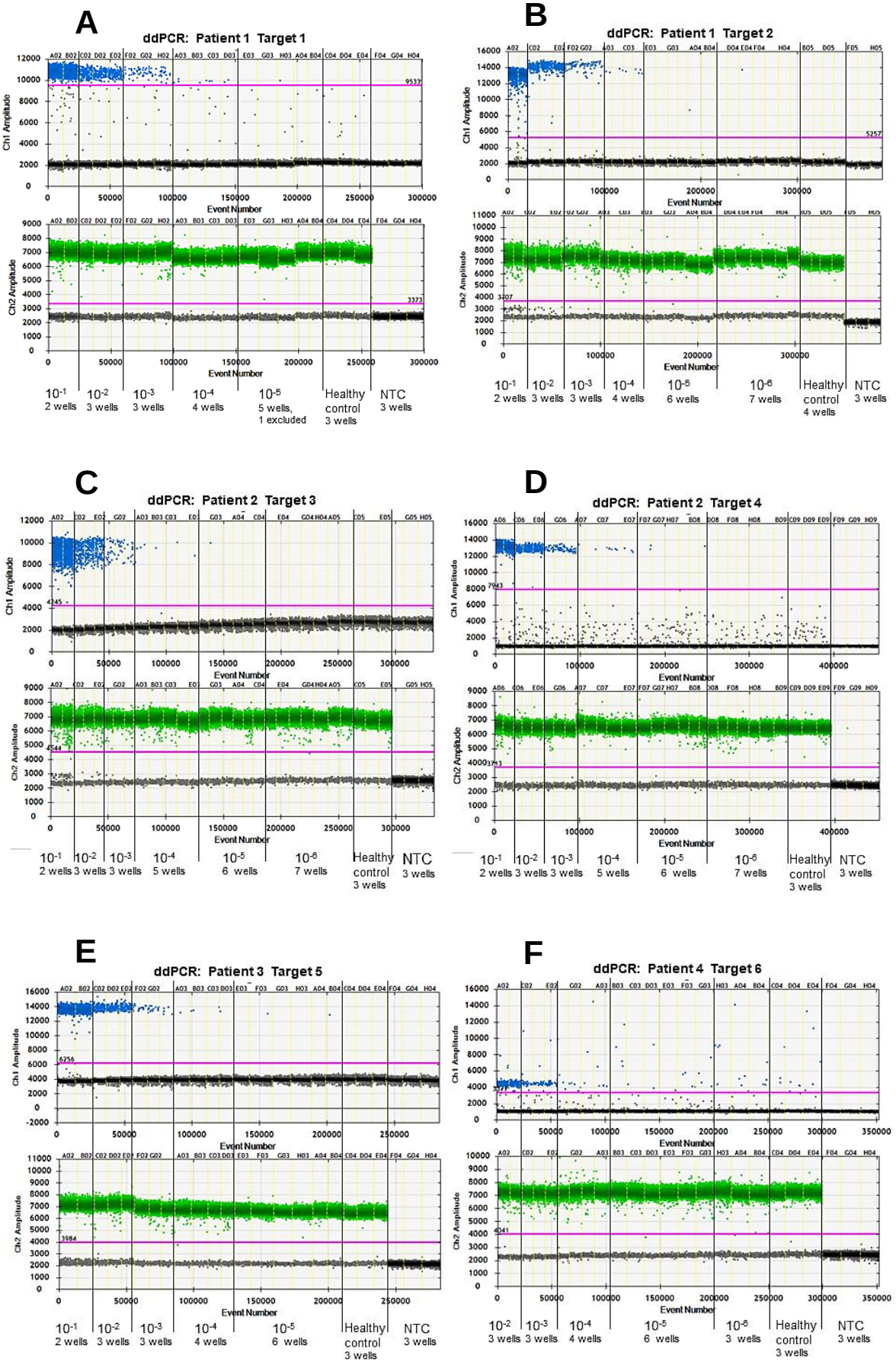
A sensitivity of 10^{-4} was reached by five targets (Fig. 4, **A, B, C, M, P**). For patient 1 target 1 (Fig. 4, **A**) it was possible to adjust the height of the threshold so that the non-specific amplification with its different signal pattern could be separated from the target signals.

Reproducible signals in the healthy control were found in three targets (Fig. 4, **F, H, N**) and thus partly hindered detection of low level positive target signals.

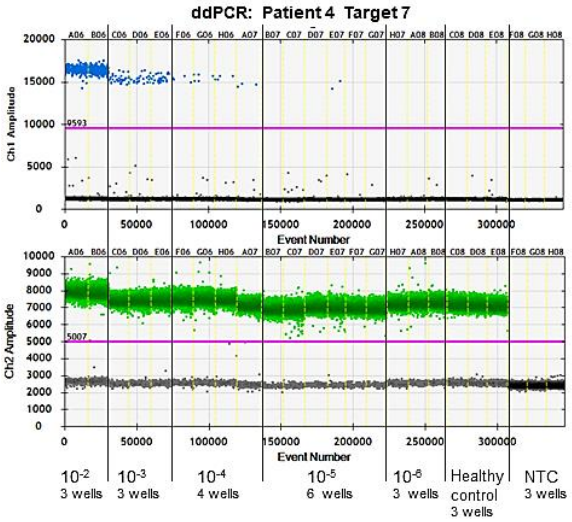
For patient 4 target 6 (Fig. 4, **F**) no clear distinction between healthy control and 10^{-6} was possible but the positive droplet count of 10^{-5} was three droplets away from the healthy control, fulfilling formally the sensitivity of 10^{-5}.

Similar applies to patient 8 target 14 (Fig. 4, **N**) where a sensitivity of 10^{-5} was possible, whereas 10^{-6} is masked by the positive droplet count of the healthy control.

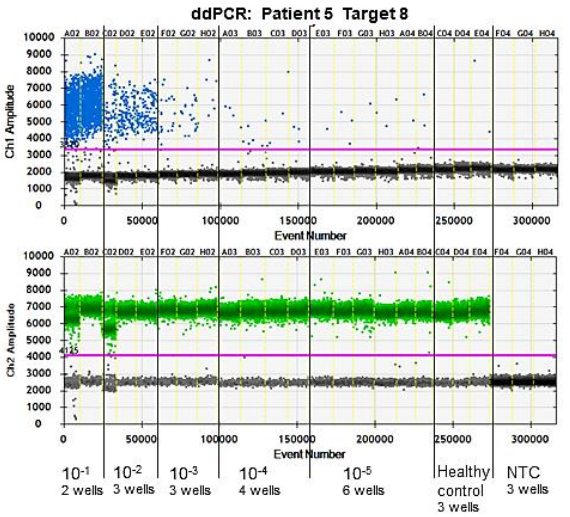
A clear distance to the number of positive target droplets in the 10^{-5} dilution was ensured despite two positive droplets in the healthy control for patient 5 target 8 (Fig. 4, **H**).


A
ddPCR: Patient 1 Target 1
Ch1 Amplitude
Ch2 Amplitude
Event Number
9533
3373
10-1 2 wells
10-2 3 wells
10-3 3 wells
10-4 4 wells
10-5 5 wells, 1 excluded
Healthy control 3 wells
NTC 3 wells
B
ddPCR: Patient 1 Target 2
5257
3207
10-1 2 wells
10-2 3 wells
10-3 3 wells
10-4 4 wells
10-5 6 wells
10-6 7 wells
Healthy control 4 wells
NTC 3 wells
C
ddPCR: Patient 2 Target 3
4145
4544
10-1 2 wells
10-2 3 wells
10-3 3 wells
10-4 5 wells
10-5 6 wells
10-6 7 wells
Healthy control 3 wells
NTC 3 wells
D
ddPCR: Patient 2 Target 4
7943
3743
10-1 2 wells
10-2 3 wells
10-3 3 wells
10-4 5 wells
10-5 6 wells
10-6 7 wells
Healthy control 3 wells
NTC 3 wells
E
ddPCR: Patient 3 Target 5
6256
3984
10-1 2 wells
10-2 3 wells
10-3 3 wells
10-4 4 wells
10-5 6 wells
Healthy control 3 wells
NTC 3 wells
F
ddPCR: Patient 4 Target 6
4041
10-2 3 wells
10-3 3 wells
10-4 4 wells
10-5 6 wells
10-6 3 wells
Healthy control 3 wells
NTC 3 wells

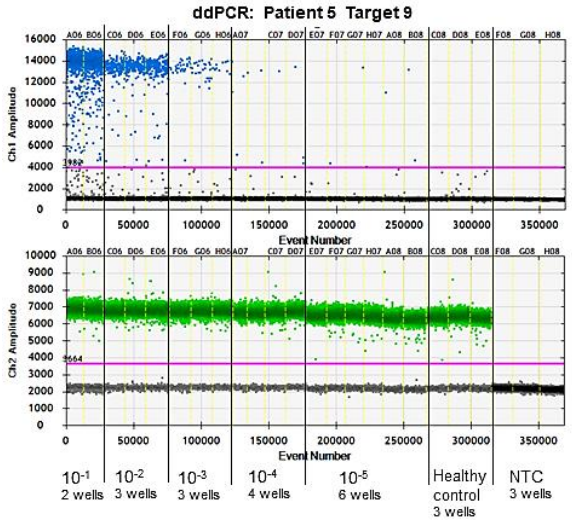
G


ddPCR: Patient 4 Target 7
Ch1 Amplitude
Ch2 Amplitude
Event Number
10^{-2} 3 wells
10^{-3} 3 wells
10^{-4} 4 wells
10^{-5} 6 wells
10^{-6} 3 wells
Healthy control 3 wells
NTC 3 wells

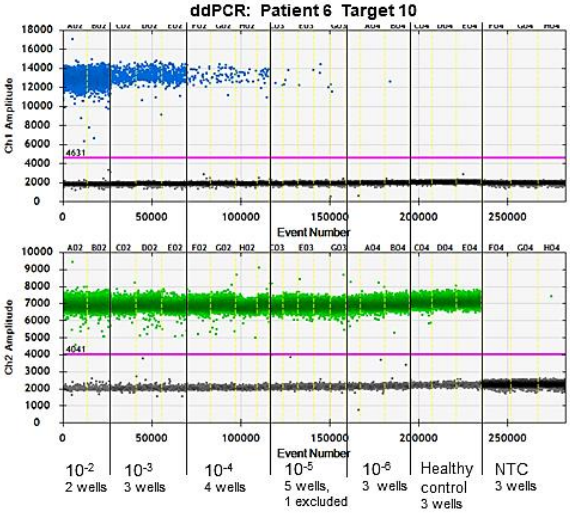
H


ddPCR: Patient 5 Target 8
Ch1 Amplitude
Ch2 Amplitude
Event Number
10^{-1} 2 wells
10^{-2} 3 wells
10^{-3} 3 wells
10^{-4} 4 wells
10^{-5} 6 wells
Healthy control 3 wells
NTC 3 wells

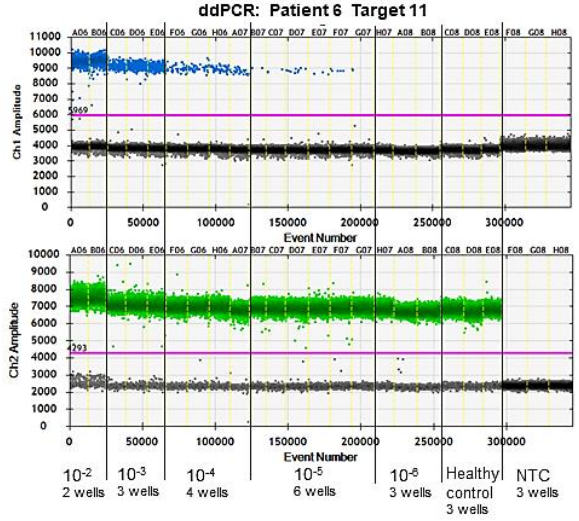
I


ddPCR: Patient 5 Target 9
Ch1 Amplitude
Ch2 Amplitude
Event Number
10^{-1} 2 wells
10^{-2} 3 wells
10^{-3} 3 wells
10^{-4} 4 wells
10^{-5} 6 wells
Healthy control 3 wells
NTC 3 wells

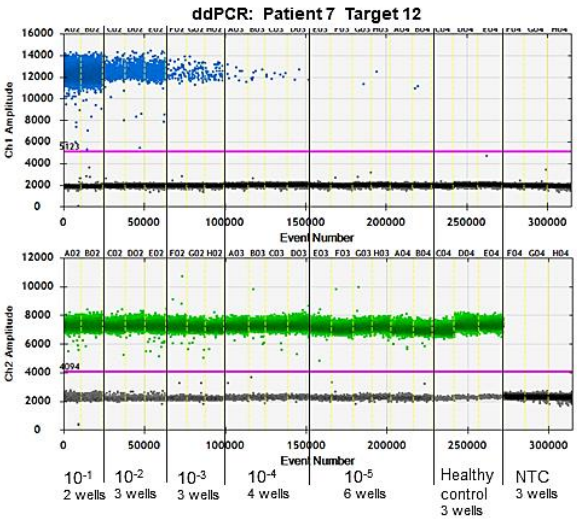
J


ddPCR: Patient 6 Target 10
Ch1 Amplitude
Ch2 Amplitude
Event Number
10^{-2} 2 wells
10^{-3} 3 wells
10^{-4} 4 wells
10^{-5} 5 wells, 1 excluded
10^{-6} 3 wells
Healthy control 3 wells
NTC 3 wells

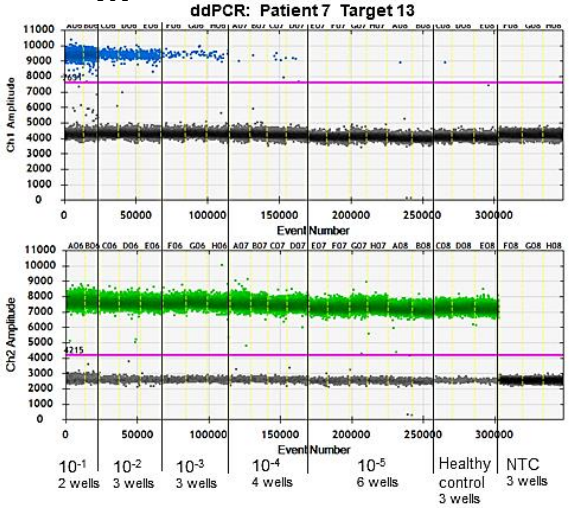
K


ddPCR: Patient 6 Target 11
Ch1 Amplitude
Ch2 Amplitude
Event Number
10^{-2} 2 wells
10^{-3} 3 wells
10^{-4} 4 wells
10^{-5} 6 wells
10^{-6} 3 wells
Healthy control 3 wells
NTC 3 wells

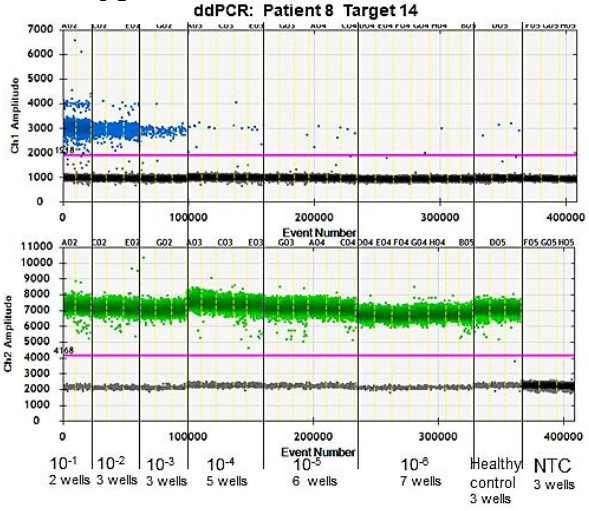
L


ddPCR: Patient 7 Target 12
Ch1 Amplitude
Ch2 Amplitude
Event Number
10^{-1} 2 wells
10^{-2} 3 wells
10^{-3} 3 wells
10^{-4} 4 wells
10^{-5} 6 wells
Healthy control 3 wells
NTC 3 wells

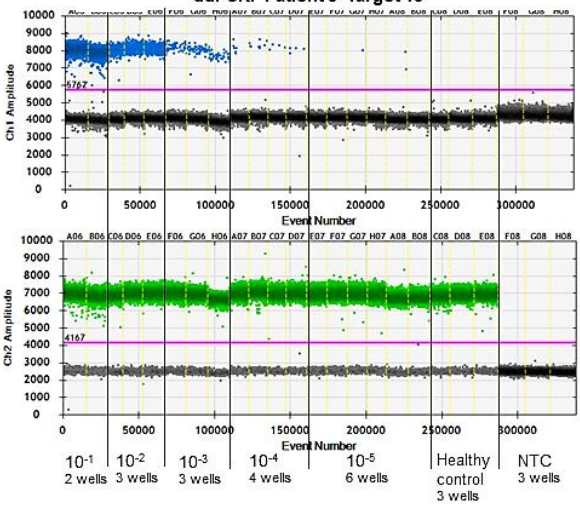
M


ddPCR: Patient 7 Target 13
Ch1 Amplitude
Ch2 Amplitude
Event Number
10-1 2 wells
10-2 3 wells
10-3 3 wells
10-4 4 wells
10-5 6 wells
Healthy control 3 wells
NTC 3 wells

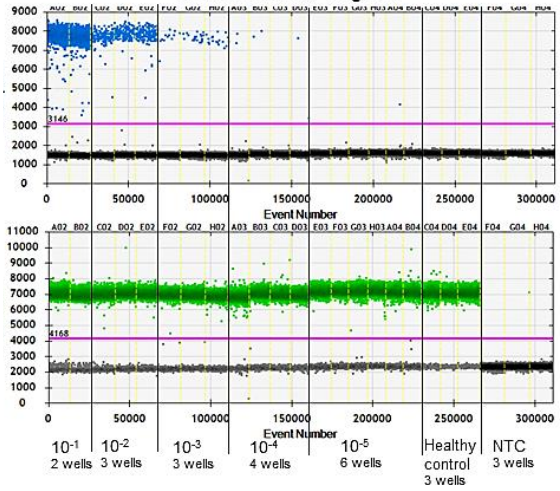
N


ddPCR: Patient 8 Target 14
Ch1 Amplitude
Ch2 Amplitude
Event Number
10-1 2 wells
10-2 3 wells
10-3 3 wells
10-4 5 wells
10-5 6 wells
10-6 7 wells
Healthy control 3 wells
NTC 3 wells

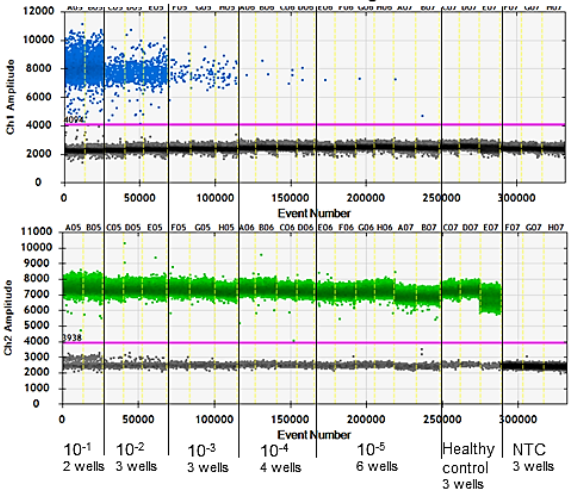
O


ddPCR: Patient 8 Target 15
Ch1 Amplitude
Ch2 Amplitude
Event Number
10-1 2 wells
10-2 3 wells
10-3 3 wells
10-4 4 wells
10-5 6 wells
Healthy control 3 wells
NTC 3 wells

P


ddPCR: Patient 9 Target 16
Event Number
10-1 2 wells
10-2 3 wells
10-3 3 wells
10-4 4 wells
10-5 6 wells
Healthy control 3 wells
NTC 3 wells

Q


ddPCR: Patient 9 Target 17
Ch1 Amplitude
Ch2 Amplitude
Event Number
10-1 2 wells
10-2 3 wells
10-3 3 wells
10-4 4 wells
10-5 6 wells
Healthy control 3 wells
NTC 3 wells

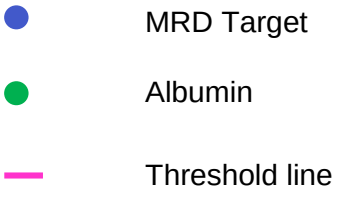

MRD Target
Albumin
Threshold line

Fig. 4: ddPCR results for all 17 MRD targets Ch 1 shows positive droplets in blue for the FAM marked patient specific MRD target. Positive droplets can be clearly separated from negative droplets at the bottom of the respective graph. Threshold lines vary in height depending on the distinctiveness of the individual droplet clusters. With increasing dilution factors the count of positive MRD signals declines, whereas the signal of the housekeeping gene *Albumin* shown in Ch 2 indicates the unchanging amount of DNA per well. Three or more positive droplets in total for the respective replicates are considered as a positive sample, two positive droplets constitute a grey area and one droplet or less are negative by definition.
The ddPCR sensitivity for these target ranges between 10^{-4} and 10^{-5} but goes down to 10^{-6} in one case as well.

The threshold line for each dye was set manually below the positive droplet cluster and at sufficient distance to any unspecific background signals. In this way, number of positive droplets for the examined target, resulting copies per µl and fractional abundance of leukemia blasts were displayed. A complete listing of the mentioned values for each separate target can be found in the appendix (see 7. Appendix Fig. 8).
The number of positive droplets per dilution step has to be interpreted with regard to the number of merged wells per dilution step. Thus, the calculated number of copies per µl shows more vividly the dilutions of leukemic blasts in steps of ten.
The fractional abundance provides us a direct quantification of leukemic blast percentages. It is calculated from the ratio of the leukemia target in relation to *Albumin*. Even though in theory percentages of 10% at a 10^{-1} dilution were expected, percentages around 3 or 4% were observed. Accordingly, the subsequent values were also reduced. Nevertheless, the blast percentages ranged within the approximate order of magnitude that was calculated before. It becomes clear as well that the expected and the measured blast percentage values diverge more the higher dilutions of blasts are which hinders the reliable absolute determination of especially low MRD values.

The sensitivity of ddPCR was between 10^{-4} and 10^{-6} and thus exactly in the same range as qRT-PCR.
For patient 1 target 1, ddPCR was able to overcome the non-specific amplification in qRT-PCR by adjusting the threshold line. In the case of patient 4 target 6, there

were non-specific signals in both methods. Both patient 5 target 8 and patient 8 target 14 showed a slight signal in the healthy DNA in ddPCR, which was not detected in qRT-PCR.

3.3 Concordance of both methods

For this question, only matched dilution samples were evaluated and showed an overall concordance of 87.36% (76/87) in deciding whether a sample is still positive or not. Minor disconcordances where one method considered a sample as PNQ whereas it was either positive or negative with the other method occurred in 6.90% (6/87). Mayor disconcordances where one method could still quantify a sample whilst the other considered it to be negative happened in 5.75% (5/87).

3.4 Comparative listing of the sensitivities achieved

Despite small differences and individual particularities of each target, however, the major trends were clearly to recognize and reproducible.

To finally compare the sensitivity between both PCR methods, two graphs showing a summary of all MRD measurements were created:

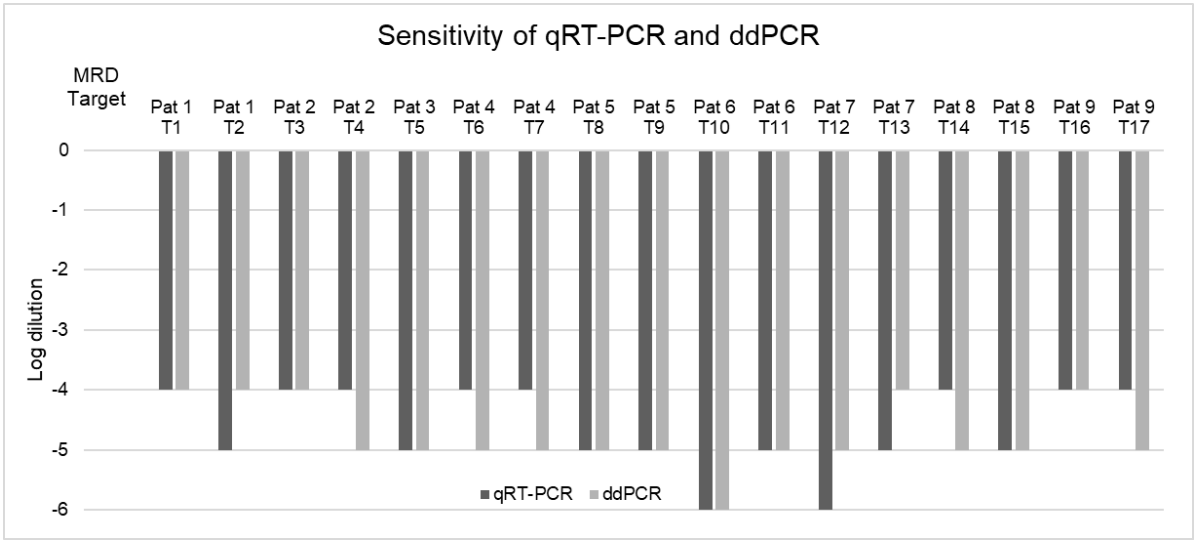


Fig. 5: Comparison of sensitivity of qRT-PCR and ddPCR in MRD diagnostics
All our targets reached a sensitivity between 10^{-4} and 10^{-6} measured with both PCR methods. In the majority of the cases (9/17) qRT-PCR and ddPCR had exactly the same sensitivity. In 3/17 cases qRT-PCR was more sensitive and in 5/17 cases ddPCR was more precise about detecting high dilutions of leukemic blasts.

It became clear that in most cases qRT-PCR and ddPCR were equivalent in their sensitivity (52,9%; 9/17). In fewer, qRT-PCR was better (17,7%; 3/17) and in the remaining, ddPCR was superior in sensitivity (29,4%; 5/17).

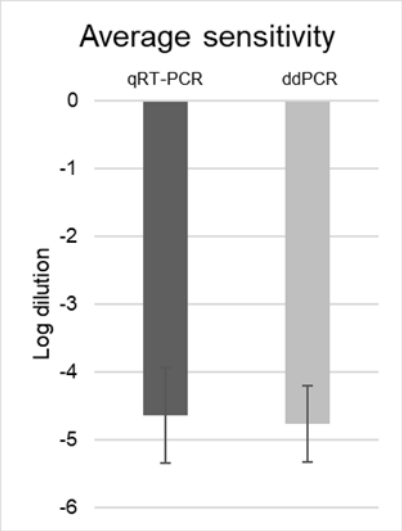


Fig. 6: Average sensitivity of qRT-PCR and ddPCR in MRD diagnostics
The average sensitivity of qRT-PCR and ddPCR did barely differ in this project. DdPCR was shown to be at least as sensitive as qRT-PCR.

Taking together all the 17 MRD targets measured with qRT-PCR and ddPCR the sensitivity of both methods is equally good. The average sensitivity of qRT-PCR was 10 to the power of -4,65 (±0,70) and the one of ddPCR 10 to the power of -4,76 (±0,56). A double sided, unpaired t-test showed a t-test value of 0,593. ddPCR was therefore shown to be at least as sensitive as qRT-PCR.

4. Discussion:

Besides the optimization of childhood leukemia treatment, the optimization of reliable and practical monitoring throughout the therapy and also afterwards is an important cornerstone. Even with the use of higher drug doses and intensified chemotherapy doses and or the usage of HSCT, therapy cures of relapsed childhood leukemia fluctuate between 10-50%. Therefore, it is exceedingly important to understand the molecular biology and to detect relapse as fast as possible (Bailey et al., 2008, Asare et al., 2021).

4.1 Technical aspects for qRT-PCR:

The usage of qRT-PCR is widely common. Overall, it is possible to perform qRT-PCR-based MRD assessments in about 90-95% of ALL patients (Della Starza et al., 2019). Our patients were already predefined through the cooperation with Mr. Kreyenberg, but here the specific targets showed good applicability to the patients' DNA.

Throughout the years, stable and international accepted protocols and guidelines for data interpretation have been established and have been an important basis of this thesis (Pongers-Willemse et al., 1999, Gabert et al., 2003, Schuurhuis et al., 2018, van der Velden et al., 2007a, van Dongen et al., 2003).

With the support of Dr. Kreyenberg from Frankfurt and in-depth research we were able to approach the topic of MRD detection. Our starting position regarding DNA availability hindered us from executing MRD detection exactly as recommended by the ESG-MRD-ALL but we tried to approach the standards as good as possible. In our experiments, we used targets that were patient specific, sensitive (at least up to 10^{-4}), applicable to the majority of patients, standardized within our cooperating clinical center and gave reliable, reproducible quantification of MRD levels as also recommended by others (Szczepański, 2007, van Dongen et al., 2015). Furthermore, we used the recommendations for qRT-PCR and self-gained experience as a starting point and wanted to put the main focus on establishing and improving ddPCR-MRD detection.

Nevertheless, most of our qRT-PCR assay standard curves had a slope of around −3.1 to −3.9, fulfilling the requirements of the ESG-MRD-ALL (van der Velden et al., 2007a) and would have qualified as standard curves for further MRD determination of FU samples. Deviations could be explained with a deviating amplification efficiency of the leukemia targets or inaccuracies of the single, non-duplicated dilution series for example through slight inaccuracies when pipetting which could not be minimized by replicates.

By examining our 17 different MRD-targets, it was already visible that the amplification efficiency, fluorescence intensity and sensitivity varies between the different primer and DNA combinations but the basic tendency of dilution was still always well recognizable.

4.2 Technical aspects for ddPCR:

DdPCR seems to be more precise than qRT-PCR because of the partition of samples into droplets and their individual readout, so even small changes in fluorescence intensity can be detected. Moreover, the ratio between target DNA molecules to PCR reagents is considerably higher (Della Starza et al., 2016).

The challenge of this work was to achieve the same sensitivity as in qRT-PCR wherefore we changed some of the parameters:

The Limit of detection in ddPCR can mainly be adjusted by modifying the amount of input template per well, by varying the number of replicates and optimized PCR handling (Brunetti et al., 2017).

We did increase the amount of input gDNA to 500ng per droplet generation well and could not observe a negative impact on reaction performance. Similar findings have been described by Coccaro et al. where scaling up to even 750ng of copy DNA did not have a negative impact on the reaction performance (Coccaro et al., 2018).

gDNA can be more viscous depending on the extraction protocol which can negatively affect droplet formation. In case of sticky DNA and excessively low DNA amounts after droplet generation flow through, restriction enzymes to decrease the size of the input DNA have to be used. 2U/µL of restriction enzyme

(HINFI) directly added into the prepared reaction mix are enough for improved ddPCR performance and proper droplet generation. Anyhow, it must be verified in advance that target sequences will not be cutted (Drandi et al., 2018).
In this project, ddPCR performance worked out well with 500ng of input gDNA and the use of a restriction enzyme was not necessarily needed.

It has to be considered that even if the starting amount of DNA is 500ng per well for both methods, just a part of it will finally be analyzed in ddPCR. Only 42µl from the sum of 20µl reaction mix and 70µl oil will be aspirated at the end. We did these evaluations for our results as well, but similar thoughts have also been depicted by other colleagues (Cavalli et al., 2017, Drandi et al., 2020).
With the final amount of DNA and PCR-procedure, this project reached approximately 4,000 copies of albumin per µl. If this number is multiplied with 20µl, we do have 80,000 copies of albumin per well. Estimating 3.3pg/copy *(BioRad, Rare mutation detection best practices guidelines, Bulletin 6628, p.33)* the true final DNA amount per ddPCR well was around 264ng and therefore almost only half of that of qRT-PCR.

With several replicates the over-all amount of analyzed DNA can be raised again as the replicates sum up *(BioRad, Rare mutation detection best practices guidelines, Bulletin 6628, p.9).*
Of course, it raises sensitivity to analyze more DNA per follow-up sample in form of several replicates, but it has to be clear that this signifies the need of a higher amount of DNA that has to be available as well (Coccaro et al., 2018). So, for both methods a sufficient amount of DNA has to be available, as already mentioned in former papers too (van Dongen et al., 2015). For qRT-PCR large amounts of diagnostic DNA have to be available, whereas ddPCR requires more DNA input of the current sample due to loss during droplet generation.

To briefly interrupt all thoughts on increasing sensitivity here, it must be asked to what extent a further increase in the amount of DNA has an effect clinically too.

For 45 PNQ DNA samples in childhood ALL, ddPCR performed by Della Starza with 1.5µg DNA (triplicates à 500ng per well) revealed that 13 were positive and quantifiable, 16 remained PNQ and 16 were found to be negative. When 3.0µg (sixplicates à 500ng) of DNA were used, 41 samples could still be analyzed due to material availability, 12 of them were positive and quantifiable, 19 PNQ and 10 were negative. Conclusively, the use of 3.0 µg of DNA instead of 1.5 µg was only slightly reducing MRD negative cases, but did not substantially modify the risk stratification. (Della Starza et al., 2021)

Away from the required DNA quantity to further increase ddPCR-MRD sensitivity, the number of partitions has to be raised. The dynamic range for absolute quantitation in 20.000 droplets stretches from a single copy up to 100.000 copies (Hindson et al., 2011). Therefore, we optimized the handling and droplet generation process and could achieve improved quantities of droplets to be analyzed.

The Limit-of-detection which was reached with ddPCR within this project is comparable with the results from literature, where the detection limit of blasts ranges around 0,01%-0,0001% (10^{-4} – 10^{-6}) as well (Coccaro et al., 2018).

Excellent reproducibility between laboratories using the same ddPCR protocols has been shown as well, which is important for widely applied routine use in the future (Drandi et al., 2020). We could only compare results within this single MRD project.

The more, ddPCR makes data interpretation less complicated and leads to the possibility of immediate and relatively intuitive data interpretation (Brunetti et al., 2017). We were able to benefit from this as well.

4.3 Concordance of qRT-PCR and ddPCR results:

A research group on MRD in early stage follicular lymphoma published overall ddPCR/qRT-PCR concordance of 81,9% (113/138 samples). In case of quantifiable disease (qRT-PCR≥10^{-5}) concordance between both methods even reached 97,5%. No discrepancy ≥1 log was observed in the samples that were quantifiable by both detection methods (Cavalli et al., 2017). Another comparative

project showed concordant results between qRT-PCR and ddPCR in 117 FU samples from Ph+ ALL patients in 73% of cases (Coccaro et al., 2018).
In the case of 504 FU samples from patients with several hematological malignancies, MRD detection was concordantly positive or negative in 78%. Most discordances occurred in FU samples with a low level of disease and did not cluster in specific disease subsets (Della Starza et al., 2019). The same group investigated 141 FU cases of 50 adult ALL cases as well and reported a concordance of 88% regardless of the time point analyzed (Della Starza et al., 2016).
The concordance of 87.36% calculated in this project ranges within the values that can be expected. As this project was conducted with dilution series of leukemic blasts only instead of follow-up samples, we could just assess the distinction between positive and negative and could not check our measured values for quantitative consistency at all. Therefore, the mentioned value must be evaluated considering the different methodology and lower amount of comparison samples used and is certainly less representative.

Cavalli and colleagues characterized “minor discordances” as MRD differences between PNQ in one method and negative or slightly positive in the other and they defined “major discordances” as a positive or negative MRD finding in one method and exactly the other result using the other PCR method (Cavalli et al., 2017). This definition was used for this work as well. Drandi and colleagues defined discordances in terms of positivity versus negativity as “qualitative discordances” and discrepancies >1 log, as “quantitative discordances” (Drandi et al., 2015).

4.4 Comparison between qRT-PCR and ddPCR MRD detection:

The main disadvantages of qRT-PCR to overcome are the need for a serial dilution as a basis for relative quantification and an imprecise MRD quantification in between the quantitative range of the assay and its maximum sensitivity (Brunetti et al., 2017, van der Velden et al., 2007b).

The opportunity to analyze FU samples with ddPCR without a standard curve offers a great possibility for high-risk patients, which might need repeated MRD monitoring throughout their disease and treatment (Della Starza et al., 2021). Our findings also show that it is possible with ddPCR to make a quantitative statement about blast proportions even without a reference curve. The percentages we measured were not exactly as expected, but within a range of one log level. For low-percentage MRD ranges, however, ddPCR also showed limitations in accuracy. There is still space for optimization here, so that ddPCR can certainly become even more reliable in means of absolute quantification.
Another aspect is that multiplexing set-up is easier and more reliable in ddPCR (Zhong et al., 2011). Even though descriptions of multiplex approaches in qRT-PCR have been published (Viehmann et al., 1999), we had less clear and distinct MRD-fluorescence signals when multiplexing with qRT-PCR during a few pre-experiments. In accordance with the already described consensus from ESG-MRD we preferred singleplex PCR for qRT-PCR. When multiplexing with ddPCR we observed less mutual influences of our fluorescence signals.

The more, qRT-PCR efficiency is said to be more affected by the presence of inhibitors such as anticoagulants, residual reagents from DNA extraction e.g. alcohol or blood components e.g. heme, in comparison to ddPCR (Yang et al., 2014, Drandi et al., 2015). As ddPCR bases on binary output data – the droplet endpoint can either be positive or negative – wide variations in amplification efficiency can be accepted without negatively affecting the copy number estimation (Hindson et al., 2011). Good efficiency and comparable or even better sensitivity of ddPCR in comparison to nested PCR and qRT-PCR was also published for MRD detection in acute promyelocytic leukemia (Brunetti et al., 2017). We can report good efficiency for both PCRs.

With both methods it is crucial to use clean supplies and to work as neat as possible as these sensitive methods are vulnerable to contamination. Some even recommend separate workspaces for different experimental steps (Drandi et al.,

2018). Here, we could not agree more and have once again increased emphasis on avoiding contamination when carrying out the experiments.

At this point the discussion if PB or BM should be used as a source material for MRD diagnostics, has to be mentioned. Whether qRT-PCR or ddPCR is used makes no difference for this question because nowadays it has become clear that the scenario is different between B-lineage and T-lineage ALL. In B-ALL, MRD levels tend to be 1–3 logs lower in PB than in BM, whereas in T-ALL MRD levels are similar in both materials. So, PB is claimed to be a reliable source in T-ALL as well, nevertheless MRD assessments are routinely carried out on BM samples for both methods (van Dongen et al., 2015).

A major challenge is that non-specific amplification of physiological Ig/TcR rearrangements is hardly distinguishable from low-level positive cases (Della Starza et al., 2019). At this point, we found that ddPCR was able to overcome the non-specific amplification in qRT-PCR in one case but showed slight signals in healthy DNA in some other samples. To conclude, non-specific amplification can represent a problem for both PCR methods.

It is said that the use of ddPCR significantly reduces the incidence of grey-zone PNQ results compared with qRT-PCR (Della Starza et al., 2021). PNQ-qRT-PCR samples have been revealed as approximately 40% positive, 50% negative and 10% borderline (PNQ) (Drandi et al., 2020).
As we did not have a representative number of grey-zone results in our experiments, we cannot make a statement on this.
Importantly, it must be claimed that an increase in sensitivity can, but does not have to always directly translate into a significant prognostic impact (Della Starza et al., 2021).

4.5 Pitfalls and limitations:

The most important, but difficult question to be clarified is where the relapse comes from. Relapse can either arise from residual leukemia cells being already

present and monitored since diagnostic, from another minor subclone or from completely new mutations in leukemic cells (Bailey et al., 2008, Bhojwani et al., 2015). MRD detected shortly after induction is phenotypically often more alike to the specimen at diagnosis. The remaining subclone sometimes becomes undetectable after postinduction therapy and can reoccur as early relapse (Bailey et al., 2008). A late relapse is more likely to originate from a de novo mutation (Bailey et al., 2008). In cases with oligoclonal rearrangements, clonal evolution of a particular Ig/TcR rearrangement can lead to false negative MRD results (Della Starza et al., 2019). Fusion transcript markers are more stable throughout the disease and therefore reliable MRD targets: Within B-lineage ALL, bcr-abl -also called Philadelphia chromosome- is the most common translocation in adult cases and the most common chimeric transcript in pediatric patients is represented by *ETV6-RUNX (*25–30% of childhood ALL). In T-ALL, *TAL1* deletions can be found in about 20% of patients (Della Starza et al., 2019).
But the demarcation of physiological states and non-specific bindings is also a challenge. Some Ig/TCR gene rearrangement junctions will still remain inevitable stumbling blocks as even though they are unique for a patient, nonspecific amplification due to sequence similarity to normal Ig-/ TcR repertoire cannot be excluded (Szczepański, 2007).
Non-specific amplification by ddPCR was defined by a sum of more than 3 positive droplets within the healthy donor DNA replicates. However, in the case of unspecific amplification, an alternative and specific Ig or TcR target or ASO primer for analysis should be preferred (Drandi et al., 2020).

Cellularity limits of BM samples, as well as the purely arithmetical fact of DNA from 100,000 cells per well analyzed in a few replicates still stays multiples of 100,000 has to be accepted (van Dongen et al., 2015). Considering this mathematical calculation, MRD levels of 10^{-7} should be interpreted with extreme caution (Szczepański, 2007). If a negative MRD result is obtained, it is of high importance to know and consider the Limit of detection (LOD) of the measurement (Schuurhuis et al., 2018).

When analyzing the results, from a technical point of view, discrepancies between the droplet volume assigned by the manufacturer and the true droplet volume can represent a falsification of the estimated copy number concentration. Significant differences in droplet volume have been shown when different super mixes or ddPCR systems had been used with throughout lower volumes than stated by the manufacturer (Kosir et al., 2017).

Apart from technical expertise, it has to be admitted that MRD laboratories need extensive biological and immunogenetical knowledge, especially for initial MRD-target determination and primer designing (van Dongen et al., 2003).
Thoughts have been raised to limit the number of laboratories preferably to one laboratory per 10–14 million inhabitants in order to ensure and maintain a sufficiently detailed level of experience (van der Velden et al., 2007b).

If an event-free survival (EFS) is long despite sustainable MRD levels, one has to reflect about the heterogeneity of tumors and their hosts. And if EFS lasts shortly despite MRD negativity, it may indicate an actively growing tumor or may question the sensitivity of the assay. Although MRD directly measures the burden of the disease, it cannot detect all impacting factors causing a relapse, which has to be considered during all further treatment decisions (Berry et al., 2017).
The limitations of MRD determination with both qRT-PCR and ddPCR must always be taken into account when evaluating the results.

4.6 Clinical importance:

There is consensus that the threat of clinical relapse increases with the amount of MRD (Bailey et al., 2008, van Dongen et al., 1998, Szczepański, 2007, Schuurhuis et al., 2018, Pui et al., 2017). The European Organization for Research and Treatment of Cancer–Childhood Leukemia Cooperative Group demonstrated that the presence or absence as well as the level of MRD were significantly ($p<0.001$) correlated with the risk of relapse. Patients with MRD levels $\geq 10^{-2}$ after completed induction therapy and those with $\geq 10^{-3}$ residual blasts at later time points were found to be at high risk for relapse. The risk of

death was increased by a factor of approximately 25 in case of MRD levels ≥10^{-3} at any time point. MRD is the most influential independent prognostic factor (Cavé et al., 1998).

Other data on ALL children are showing a 5-year EFS of 94.6% with negative MRD status (Limit of detection (LOD): 10^{-4}) on day 33 of chemotherapy compared with 76.1% for children with positive MRD status (Jovanovska et al., 2019).

Another recent meta-analysis for pediatric and adult ALL confirmed that MRD negativity is clearly related to a much better long-term outcome as well. 10-year EFS for MRD negativity (LOD: 10^{-3}) was 77% versus 32% for detectable MRD in childhood ALL (Berry et al., 2017).

The impact of MRD detection on clinical outcome was coherent across different therapy strategies, methods of MRD assessment, selected MRD determination time points, cutoff levels and also leukemia subtypes (Berry et al., 2017).

The AIEOP-BFM ALL 2000 study classified 3184 patients with precursor B-ALL (Conter et al., 2010):

- MRD standard risk: MRD already negative at day 33, 5-year EFS was 92.3%
- MRD intermediate risk: MRD levels in between 10^{-4} and 10^{-3}, 5-year EFS was 77.6%
- MRD high risk: MRD 10^{-3} or more at day 78, 5-year EFS was 50.1%%

All patients have been analyzed by two markers, with a sensitivity of at least 10^{-4} (Conter et al., 2010). But even if there is only one sensitive PCR-MRD marker the study claims that it may thus be adequate and may allow risk-stratification of 90% or more of the patients (Conter et al., 2010).

The same study for childhood T-ALL emphasized the differences between both immunophenotypes (Schrappe et al., 2011):

- Overall, the clearance of leukemic blasts in T-ALL is slower than in B-ALL
- Therefore, MRD detection at a later time point just like day 78 instead of an earlier evaluation is more suited to define risk of relapse

- high levels of MRD in T-ALL do not only carry the risk of medullar relapses but also extramedullary relapses

Besides the MRD values, risk stratification at first relapse includes the time to and site of relapse as well as the cell immunophenotype (Bhojwani and Pui, 2013) . Early BM relapse within 24 months after induction therapy correlates with poor survival, whereas late relapse after 36 months of remission correlates with better cure rates (Bailey et al., 2008).
T-cell lineage ALL goes along with a higher risk of relapse in comparison to B-lineage counterparts. Additional risk factors just like MLL-rearrangements and bcr-abl translocations have to be considered as well (Bailey et al., 2008, Bhojwani et al., 2015). There are many more (age, sex, central nervous system involvement, …) but these are used less clinically (Bhojwani and Pui, 2013).

In case of relapse and second morphologic remission, achieving negative MRD is more difficult and its meaningfulness less clear. 54% of ALL patients in second remission after their first relapse had $\geqslant$0.01% leukemic cells identifiable by flow cytometry in the BM. These findings stand in contrast to 75% MRD negative patients at the end of first remission induction. Levels of residual leukemia among MRD-positive patients in second remission were found to be higher compared to the levels during first remission (Coustan-Smith et al., 2004). The 2-year cumulative incidence of relapse in patients with relapsed ALL is around 70% for MRD positive findings after second remission therapy and around 28% for MRD negative patients (Coustan-Smith et al., 2004).

To address another aspect, ddPCR providing quantitative MRD levels can assist clinicians to decide which patient needs to be monitored within which time intervals and for which duration. Therefore, also MRD measuring points could be set suiting to a patient´s needs and risks (Brunetti et al., 2017).

Even more meaningful than adopting the time points of MRD measurement to a patient´s need is adopting the personalized treatment to the therapy-response

and hence MRD levels. It improves the ALL outcome if patients who can be successfully managed with low-intensity and low-toxicity regimens, are distinguished from those who need intensified treatment to prevent relapse (Pui et al., 2017). In Europe and the United States treatment stratification is commonly accomplished based on postinduction or post consolidation MRD levels. In cases of persistent or recurring MRD, therapy efforts will be increased or will be reduced in case of MDR negativity and clinical or molecular changes will be observed attentively (Berry et al., 2017). A research group recommends that patients with undetectable MRD after two weeks of remission induction therapy and without high-risk constellations shall receive a de-intensified therapy, while novel therapies should be applied for patients with high levels of MRD at the end of induction therapy (Pui et al., 2017). Adopting a patient´s therapy to his or her MRD level at certain time points, improves 5-year OAS (Vora et al., 2014).

Besides all scientific efforts to improve the limit of detection of MRD methods, it remains to be determined in large scale clinical studies whether the enhanced sensitivity between 10^{-4} to 10^{-6} makes a significant difference in a patient´s clinical outcome (Faham et al., 2012). Promising results were recently shown for ddPCR: To investigate whether ddPCR could improve patients' risk definition, PNQ MRD samples determined by qRT-PCR in 209 pediatric B-lineage ALL cases in the AIEOP-BFM ALL2000 trial have been determined again by ddPCR. Most relapses occurred in patients with quantifiable MRD in ddPCR, while ddPCR negativity or ddPCR PNQ patients had a significantly better outcome ($p< 0.001$) (Della Starza et al., 2021).

ALL-patients with positive MRD prior to HSCT had a significantly higher rate of relapse and decreased EFS and OAS compared to those with negative MRD findings. As it was to be expected, non-relapse mortality was not influenced by MRD levels (Shen et al., 2018). Therefore, MRD evaluation before HSCT is immensely useful for treatment intensification because nowadays we do have the possibility to use immunotherapeutic compounds such as blinatumomab,

inotuzumab and CAR-T cells in addition to chemotherapy to obtain a negative MRD status (Della Starza et al., 2019).

As MRD is a reliable and early marker for treatment response within established treatment protocols, it can be used as one primary end point of outcome (Berry et al., 2017). Blinatumomab as well as other antibody-targeted therapies as a more recent therapy approach have already proved to be effective in attaining MRD negativity (Paul et al., 2019). A study of CAR T-cell therapy (tisagenlecleucel) provided promisingly durable remission with negative MRD findings as well (Maude et al., 2018). Evidence about the rate of MRD and its relationship with EFS for upcoming novel therapies still has to be attentively examined (Berry et al., 2017).
Maybe, as a surrogate end point it could gain further importance in drug approval studies as well. (Schuurhuis et al., 2018)

As the importance of MRD is growing, research for new and appropriate targets still goes on and the reliability of each newly applied target is examined (Schuurhuis et al., 2018). It is not unrealistic that MRD measurement with defined rearrangements and especially specific fusion-genes could offer unexpected options for identifying new targets for therapy. Reference should be made to *EBF1-PDGFRB* or *NUP214-ABL1* fusion that respond to ABL1 tyrosine kinase inhibitors, whereas ALL patients with *BCR-JAK2* fusion or mutated *IL7R* showed response to JAK2 inhibitors as reviewed in Bhojwani et al. 2013 (Maude et al., 2012).

To summarize briefly, MRD does not only suit as an objective parameter for risk stratification and predictor of transplant outcome in clinical routine but also, it is influencing vital clinical decisions. Moreover, it allows a better surveillance during and after therapy to adapt therapy intensity, enables early intervention in case of relapse and may even present the possibility for new therapeutic and diagnostic approaches.

4.7 Economic analysis:

An economic analysis mentioned annually costs of approximately $341 for MRD testing with MFC. With those expenditures and the knowledge of gained Quality-adjusted-life-years an incremental cost-effectiveness ratio of $43.613/Quality-adjusted-life-year gained can be calculated. For future estimations costs below $100.000/ Quality-adjusted-life-year gained can be expected. So, the Health Quality Ontario concludes that MRD testing in patients with precursor B-cell ALL is reasonable and cost-effective compared with no testing from an economic point of view as well (Ontario, 2016).

A comparative cost analysis between qRT-PCR and ddPCR (96-well plate) showed two-time higher costs for ddPCR (1.5€ per well for qRT-PCR vs. 3.0€ for ddPCR). On the other hand, it must be seen, that ddPCR does not require dilution series standard curves for every follow-up measurement and thus spares precious diagnostic DNA, needs less wells per follow-up sample and saves time (Cavalli et al., 2017, Della Starza et al., 2016).

4.8 Other methods for MRD detection:

The fact that analysis of molecular chimerism in (Short tandem Repeats) STRs provides clinics that are not members of the ESG-MRD consortium a chance to evaluate the course of their patient's disease on their own, cannot be belittled. Hence, hematopoietic chimerism measurement after HSCT is remaining a useful tool for monitoring the engraftment of donor cells and increasing vigilance at times of possibly imminent relapse (Clemente et al., 2017). Nevertheless, it still cannot compete with the accuracy of other methods (Stahl et al., 2015).

At this point, ddPCR could find a new area of application and could be used for even more precise quantitative chimerism measurement as already started by other groups (Stahl et al., 2015).

Even though Multiparameter flow Cytometry (MFC) has a lower sensitivity than molecular methods, it will keep its importance as the applicability, especially in AML, is broader (Buldini et al., 2019).

Yet, an international consensus to conduct and interpret extended MFC-MRD detection in pediatric AML is still lacking but certainly needed (Buldini et al., 2019). Since data interpretation is highly subjective and strongly relies on the operator's skills, an automated analysis of MFC-MRD data is lately discussed to further strengthen its application in the clinical setting, especially with the prospect of increasingly extensive marker combinations (Buldini et al., 2019).
In terms of time, material has to be analyzed within 24 hours after sampling but therefore could provide a better insight into the composition and dynamic change of normal and aberrant cells (Della Starza et al., 2019, Flores-Montero et al., 2017). The novel EuroFlow-Next generation Flowcytometry approach is said to have a similar applicability as conventional flow-MRD and could reach a Limit of detection close to 10^{-6} (Flores-Montero et al., 2017).

The Limit of detection of Next generation sequencing (NGS) is said to be even lower than 10^{-6} and most importantly, NGS-MRD allows monitoring of all leukemic rearrangements regardless of their prevalence at diagnosis. As the sequencing assay utilizes a set of universal primers, there is no need for individualized procedures (Faham et al., 2012). But even though NGS-MRD detection has already achieved promising data with regard to sensitivity and prognostic value, large cohort studies and standardization of workflow, preanalytics and bioinformatics have been missing for some time (Kotrova et al., 2015). The major costs of this approach do also pose an obstacle to be mentioned (Della Starza et al., 2019). EuroClonality-NGS primer sets were successfully tested with reliable MRD results in five European laboratories and NGS is supposed to better display oligoclonality than other so far used methods (Brüggemann et al., 2019).
Besides potentially crucial mutations for patients' outcome, NGS-MRD can be misled by mutations belonging to physiological hematopoiesis that are not per se leading to a relapse (Voso et al., 2019). Interestingly, it was observed that patients with lower IgH repertoire diversity at later FU days were at higher risk of relapse and showed a significantly worse 5-year EFS. This finding raises the question if adjustment of MRD stratification criteria for childhood ALL will be needed in case of NGS routine use (Kotrova et al., 2015).

As assay optimizations, reliable standardization of work-flow and data interpretation and feasible modifications for further applications of Ig/TcR NGS are still ongoing, they will certainly provide a basis for further discussion and improvement (Brüggemann et al., 2019).

4.9 Conclusion:

To conclude, this project showed that ddPCR is a well applicable, reliable and sensitive method for absolute quantification of MRD levels and has potential for broad-based application.

In concordance with other studies already mentioned, we conclude that ddPCR has a sensitivity, reproducibility and accuracy comparable to qRT-PCR and can furthermore partly overcome some qRT-PCR specific disadvantages. Some pitfalls and limitations will nevertheless remain with this method and should not be lost of sight when evaluating the data and applying them clinically.

The technical application certainly needs some experience in the field of MRD and hopefully will be supported with generally applicable recommendations over the coming years.

Nowadays, MRD detection is already used for the assessment of initial treatment response and the following definition of MRD-level-based risk group stratification, for monitoring disease load in the setting of HSCT and an early marker of impending relapse. Efforts to make the detection of MRD as reliable, uncomplicated and accessible as possible should continue.

But even if improved MRD detection is a good basis of treatment adjustment, further understanding of subclone resistance and mechanisms of relapse as well as new therapeutic approaches have to be pursued as well.

All in all, the field of MRD and its treatment will remain an exciting and evolving area of research.

5. Summary

5.1 Summary in English

During the last years, the measurement of minimal residual disease (MRD) has become more and more accepted in the clinical and prognostic assessment of various malignant diseases. In childhood acute leukemic leukemia (ALL) as well, the determination of MRD burden during and after therapy is a crucial factor for the prognosis of an affected patient and further therapeutic steps. Although various methods can be used to measure MRD, the molecular approach, mainly using quantitative real-time polymerase chain reaction (qRT-PCT), is the current gold standard. Different target sequences, such as T-cell receptor and immunoglobulin rearrangements and specific fusion genes are used to monitor a specific blast subpopulation. Based on preferably two or more patient-specific target sequences, disease progression can be estimated.

For qRT-PCR, each follow-up bone marrow aspiration is compared to a standard curve of blasts obtained at diagnosis onset, allowing to calculate the current blast burden in the patients' bone marrow. The sensitivity of qRT-PCR ranges up to the detection of one leukemia-type DNA copy in up to 10,000 (10^{-4}) and in some cases even 1 in 1,000,000 (10^{-6}) healthy copies.

In this project, nine pediatric ALL patients and 17 different MRD targets have been investigated. By measuring dilution series of leukemic blast DNA in healthy DNA with dilution factors from 10^{-1} to 10^{-6} with qRT-PCR, reliable and reproducible MRD determination was possible. Eight targets reached a sensitivity of 10^{-5}, also eight a sensitivity of 10^{-4} and two targets even 10^{-6}. Subsequently, MRD measurements were established with digital droplet PCR (ddPCR) as well. Here, the sample to be measured is split into up to 20,000 tiny partitions, thus enabling precise detection and absolute quantification of a PCR product. Eleven of our targets reached a sensitivity of 10^{-5}, however, among them are two samples with grey-zone results at the dilution of 10^{-5}. Five targets reached a sensitivity of 10^{-4} and one target 10^{-6}. Unspecific background amplification occurred in both methods and hampered accurate sample determination in one but not the same target per method.

Within this project it was possible to visualize all MRD sequences of our nine exemplary patients well and to achieve the same sensitivities with ddPCR as with qRT-PCR.

ddPCR mainly offers the advantage to absolutely quantify blasts present at any later follow-up time point without the need for a dilution curve.

Further experience in our laboratory and international guidelines for MRD measurement by ddPCR are next necessary steps towards the clinical application of this method.

5.2 Summary in German

Während der letzten Jahre hat sich die Messung der Minimalen Resterkrankung (MRD) in der klinischen und prognostischen Einschätzung verschiedener maligner Krankheiten immer weiter durchsetzen können. Auch bei der Akuten leukämischen Leukämie (ALL) im Kindesalter ist die Bestimmung der MRD-Last während und nach Therapie ein entscheidender Faktor für die Prognose des betroffenen Kindes sowie die weiteren therapeutischen Schritte.

Obwohl verschiedene Methoden zur Messung der MRD genutzt werden können, ist der molekulare Ansatz -vornehmlich mittels quantitativer real-time Polymerasekettenreaktion (qRT-PCT) - der aktuelle Goldstandard. Es werden verschiedene Zielsequenzen, wie beispielsweise T-Zell-Rezeptor- und Immunglobulin-Rearrangements und spezifische Fusions-Gene genutzt, um eine bestimmte Blastensubpopulation zu überwachen. Ausgehend von möglichst zwei oder mehr patientenspezifischen Zielsequenzen kann der Verlauf der Krankheit eingeschätzt werden.

Für die qRT-PCR wird jede spätere Knochenmarkspunktion mit einer Standardkurve der zu Diagnosebeginn gewonnenen Blasten verglichen, womit die aktuelle Blastenlast im Knochenmark der Patienten ausgerechnet werden kann. Die Sensitivität der qRT-PCR reicht bis zur Detektion einer leukämietypischen Kopie DNA in bis zu 10 000 (10^{-4}) und teilweise sogar 1 zu 1 000 000 (10^{-6}) gesunden Kopien.

In diesem Projekt wurden neun ALL-Patienten und 17 verschiedene MRD-Targets untersucht. Durch Messung von Verdünnungsreihen leukämischer Blasten-DNA in gesunder DNA mit Verdünnungsfaktoren von 10^{-1} bis 10^{-6} mittels qRT-PCR war eine zuverlässige und reproduzierbare MRD-Bestimmung möglich. Acht Targets erreichten eine Sensitivität von 10^{-5}, ebenfalls acht eine Sensitivität von 10^{-4} und zwei Targets sogar 10^{-6}.

Anschließend wurden MRD-Messungen auch mit digitaler droplet PCR (ddPCR) durchgeführt. Hierbei wird die zu messende Probe in bis zu 20 000 winzige Partikel aufgeteilt, welche eine präzise Detektion und absolute Quantifizierung des PCR-Produkts ermöglichen. Elf unserer Targets erreichten eine Sensitivität von 10^{-5}, darunter waren allerdings auch zwei Proben mit Ergebnissen in einem

Graubereich zwischen positiv und negativ bei der Verdünnung von 10^{-5}. Fünf Targets erreichten eine Sensitivität von 10^{-5} und ein Target 10^{-6}. Unspezifische Hintergrundamplifikation trat bei beiden Methoden auf und behinderte die genaue Probenbestimmung bei jeweils einem, aber nicht demselben Target pro Methode. Es konnten somit alle MRD Sequenzen unserer neun exemplarischen Patienten gut darstellt werden und die gleichen Sensitivitäten wie bei Verwendung der qRT-PCR auch mit der ddPCR erreicht werden.

DdPCR bietet vor allem den Vorteil, vorhandene Leukämieblasten ohne eine Verdünnungskurve absolut zu quantifizieren.

Weitere Erfahrungen in unserem Labor und internationale Leitlinien für die MRD Messung mittels ddPCR sind die nächsten nötigen Schritte hin zur klinischen Anwendung dieser Methode in unserer Klinik.

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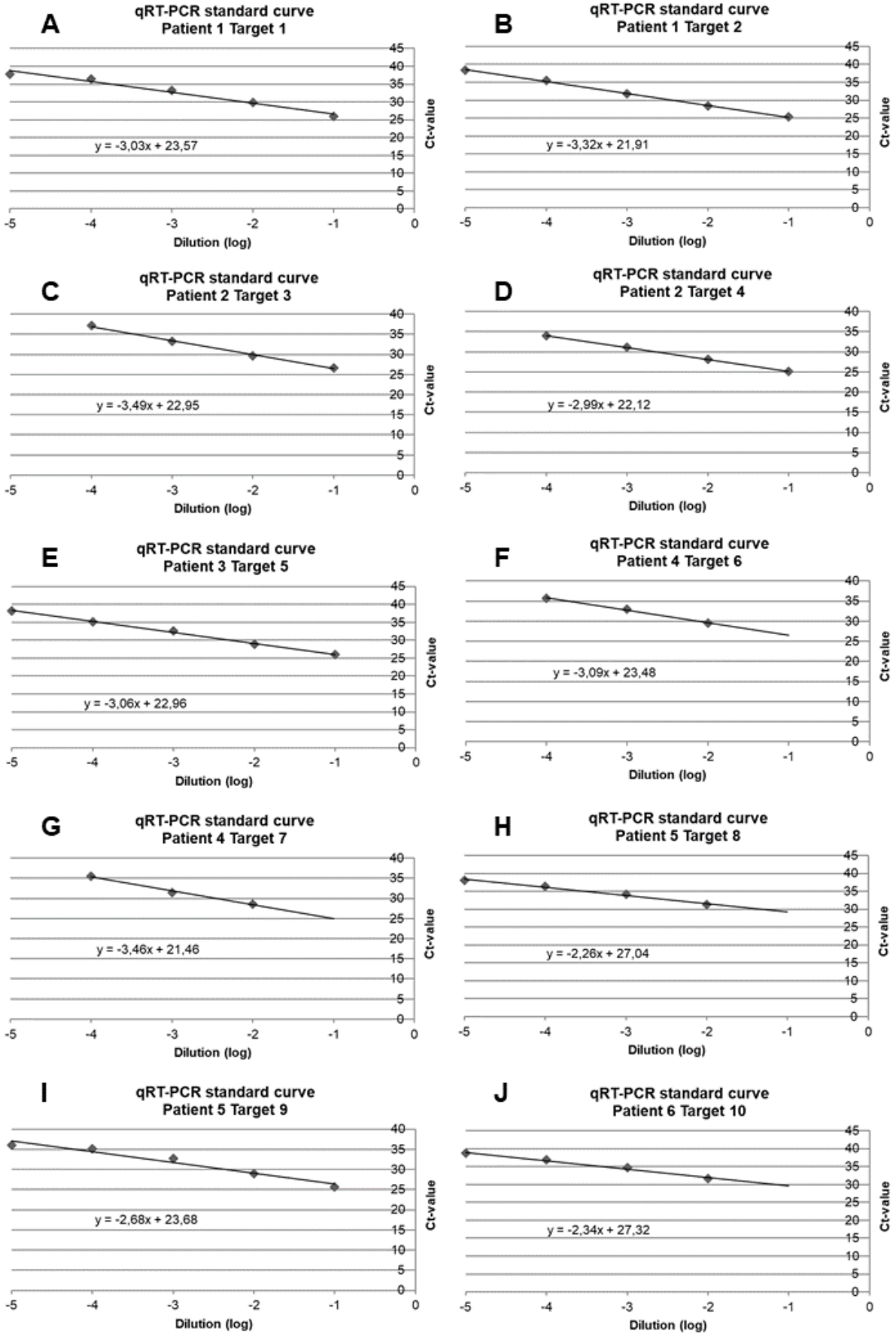
7. Appendix


A
qRT-PCR standard curve
Patient 1 Target 1
y = -3,03x + 23,57
B
qRT-PCR standard curve
Patient 1 Target 2
y = -3,32x + 21,91
C
qRT-PCR standard curve
Patient 2 Target 3
y = -3,49x + 22,95
D
qRT-PCR standard curve
Patient 2 Target 4
y = -2,99x + 22,12
E
qRT-PCR standard curve
Patient 3 Target 5
y = -3,06x + 22,96
F
qRT-PCR standard curve
Patient 4 Target 6
y = -3,09x + 23,48
G
qRT-PCR standard curve
Patient 4 Target 7
y = -3,46x + 21,46
H
qRT-PCR standard curve
Patient 5 Target 8
y = -2,26x + 27,04
I
qRT-PCR standard curve
Patient 5 Target 9
y = -2,68x + 23,68
J
qRT-PCR standard curve
Patient 6 Target 10
y = -2,34x + 27,32
Ct-value
Dilution (log)

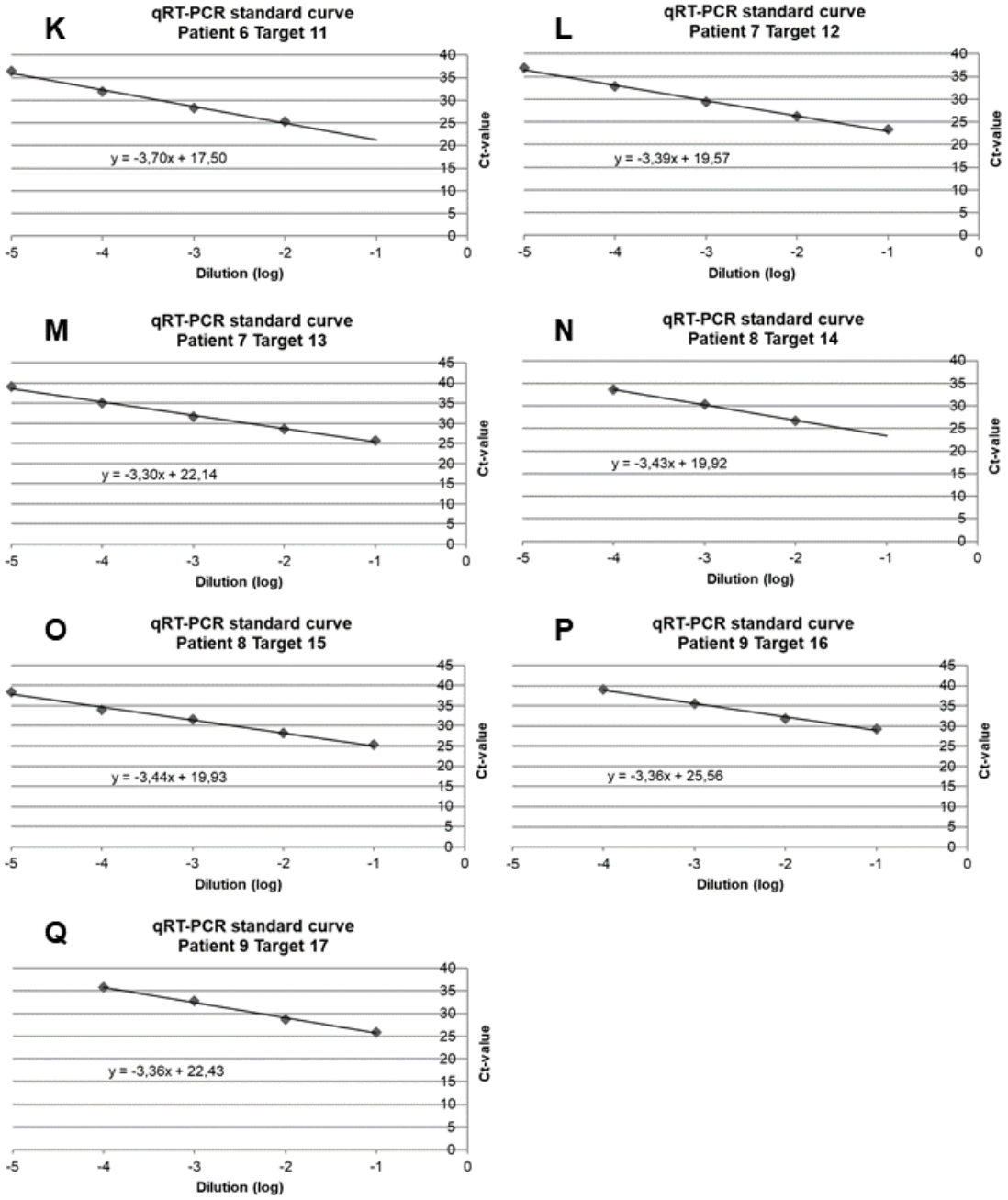


Fig. 7: qRT-PCR standard curves for all 17 MRD targets Increasing Ct-values due to the decrease of the fluorescence signal per dilution step were correlated with the underlying dilution of leukemia blasts to create a linear standard curve.
The slope should lie as far as possible between the range of -3.1 and -3.9 determined by the ESG-MRD. Most of our slope values also fulfil this criterion. Some are a bit lower, which could be caused, for example, by increased amplification efficiency due to non-specific amplifications or attenuated dilution effects.
Follow-up samples for the respective specific MRD targets can now be quantified with the knowledge of their Ct-value. With qRT-PCR a standard curve is inevitably necessary to quantify a sample during follow-up and has to be repeated at any time of a further MRD determination to guarantee equal calibration conditions to the actual measured value.

A

ddPCR: Patient 1 Target 1

Dilution (log)	Number of positive events	Copies of MRD targets/µl	Fractional abundance
-1	2057	102.7	3.68 %
-2	486	16.1	0.41 %
-3	75	2.3	0.056 %
-4	11	0.24	0.0056 %
-5	1	0.17	0.0004 %
Healthy control	0	0	-
NTC	0	0	-

B

ddPCR: Patient 1 Target 2

Dilution (log)	Number of positive events	Copies of MRD targets/µl	Fractional abundance
-1	3102	188	3.6 %
-2	620	18.6	0.353 %
-3	48	2.2	0.042 %
-4	5	0.19	0.0038 %
-5	1	0.016	0.0003 %
-6	1	0.013	0.00027 %
Healthy control	0	0	-
NTC	0	0	-

C

ddPCR: Patient 2 Target 3

Dilution (log)	Number of positive events	Copies of MRD targets/µl	Fractional abundance
-1	3053	195	3.8 %
-2	445	19.8	0.398 %
-3	55	2.5	0.052 %
-4	7	0.14	0.0029 %
-5	1	0.021	0.0004 %
-6	0	0	-
Healthy control	0	0	-
NTC	0	0	-

D

ddPCR: Patient 2 Target 4

Dilution (log)	Number of positive events	Copies of MRD targets/µl	Fractional abundance
-1	3876	213	4.23 %
-2	599	20.7	0.428 %
-3	82	2.5	0.054 %
-4	11	0.18	0.0038 %
-5	3	0.04	0.0009 %
-6	0	0	-
Healthy control	0	0	-
NTC	0	0	-

E

ddPCR: Patient 3 Target 5

Dilution (log)	Number of positive events	Copies of MRD targets/µl	Fractional abundance
-1	3191	151	3.19 %
-2	368	14.5	0.3 %
-3	34	1.35	0.028 %
-4	5	0.13	0.0027 %
-5	2	0.029	0.0006 %
-6			
Healthy control	0	0	-
NTC	0	0	-

F

ddPCR: Patient 4 Target 6

Dilution (log)	Number of positive events	Copies of MRD targets/µl	Fractional abundance
-2	457	23.9	0.524 %
-3	90	3.1	0.07 %
-4	17	0.42	0.0093 %
-5	16	0.2	0.0042 %
-6	13	0.3	0.0064 %
Healthy control	13	0.3	0.0073 %
NTC	0	0	-

G

ddPCR: Patient 4 Target 7

Dilution (log)	Number of positive events	Copies of MRD targets/µl	Fractional abundance
-2	533	20.6	0.52 %
-3	83	2.22	0.047 %
-4	16	0.3	0.0065 %
-5	2	0.027	0.0005 %
-6	0	0	-
Healthy control	0	0	-
NTC	0	0	-

H

ddPCR: Patient 5 Target 8

Dilution (log)	Number of positive events	Copies of MRD targets/µl	Fractional abundance
-1	2323	114.9	2.87 %
-2	338	11.1	0.262 %
-3	46	1.42	0.032 %
-4	18	0.36	0.0078 %
-5	12	0.18	0.005 %
Healthy control	2	0.06	0.0015 %
NTC	0	0	-

I

ddPCR: Patient 5 Target 9

Dilution (log)	Number of positive events	Copies of MRD targets/µl	Fractional abundance
-1	3555	158	3.94 %
-2	603	14.8	0.348 %
-3	71	1.75	0.038 %
-4	10	0.22	0.0048 %
-5	6	0.08	0.002 %
Healthy control	0	0	-
NTC	0	0	-

J

ddPCR: Patient 6 Target 10

Dilution (log)	Number of positive events	Copies of MRD targets/µl	Fractional abundance
-2	5437	264	4.68 %
-3	927	25.6	0.624 %
-4	113	2.9	0.072 %
-5	12	0.33	0.008 %
-6	1	0.03	0.0009 %
Healthy control	0	0	-
NTC	0	0	-

K ddPCR: Patient 6 Target 11

Dilution (log)	Number of positive events	Copies of MRD targets/µl	Fractional abundance
-2	4412	221	4.76 %
-3	911	26.7	0.637 %
-4	151	3.1	0.076 %
-5	24	0.33	0.0093 %
-6	0	0	-
Healthy control	0	0	-
NTC	0	0	-

L ddPCR: Patient 7 Target 12

Dilution (log)	Number of positive events	Copies of MRD targets/µl	Fractional abundance
-1	13975	976	21.91 %
-2	1801	55.6	1.34 %
-3	176	5.8	0.147 %
-4	25	0.55	0.013 %
-5	4	0.06	0.0017 %
Healthy control	0	0	-
NTC	0	0	-

M ddPCR: Patient 7 Target 13

Dilution (log)	Number of positive events	Copies of MRD targets/µl	Fractional abundance
-1	5968	340	8.67 %
-2	717	19.2	0.446 %
-3	73	1.81	0.044 %
-4	11	0.24	0.0058 %
-5	1	0.013	0.0004 %
Healthy control	1	0	0.0005 %
NTC	0	0	-

N ddPCR: Patient 8 Target 14

Dilution (log)	Number of positive events	Copies of MRD targets/µl	Fractional abundance
-1	7680	477	7.48 %
-2	1438	44	0.775 %
-3	119	3.8	0.065 %
-4	18	0.35	0.0067 %
-5	7	0.11	0.0019 %
-6	2	0.025	0.0004 %
Healthy control	4	0.12	0.0022 %
NTC	0	0	-

O ddPCR: Patient 8 Target 15

Dilution (log)	Number of positive events	Copies of MRD targets/µl	Fractional abundance
-1	9155	434	8.93 %
-2	1284	40.8	1.01 %
-3	142	3.9	0.102 %
-4	23	0.53	0.013 %
-5	3	0.04	0.0011 %
-6			
Healthy control	0	0	-
NTC	0	0	-

P ddPCR: Patient 9 Target 16

Dilution (log)	Number of positive events	Copies of MRD targets/µl	Fractional abundance
-1	3043	140	3.61 %
-2	465	13.5	0.362 %
-3	54	1.47	0.039 %
-4	7	0.17	0.0041 %
-5	1	0.017	0.0005 %
-6			
Healthy control	0	0	-
NTC	0	0	-

Q ddPCR: Patient 9 Target 17

Dilution (log)	Number of positive events	Copies of MRD targets/µl	Fractional abundance
-1	5659	278	6.76 %
-2	1028	29.1	0.704 %
-3	115	2.9	0.074 %
-4	8	0.18	0.0045 %
-5	4	0.06	0.0014 %
-6			
Healthy control	0	0	-
NTC	0	0	-

Fig. 8: Detailed ddPCR results for all 17 MRD targets By counting the number of positive leukemia target droplets per well and applying Poisson´s statistic to the measured volume the Quanta Soft software calculates the number of copies of the examined MRD target per well. By setting the amount of leukemic DNA in relation to the amount of albumin DNA, the fractional abundance of leukemic blasts to healthy blood cells is calculated. The absolute number of positive events must be seen in relation to the number of replicates added together.
The number of copies of MRD target per µl shows the dilution with a factor of 10 per step. It can be seen that blast concentrations represented by the fractional abundance are a little bit lower than in theory, but ranged within the order of magnitude expected to see.

No.	Disease	Target	Number of patients / Follow-up samples	Sample material	Dilution material / Negative control	House-keeping gene	Number of replicates	DNA amount per well (20µl)	Genomic DNA digestion	Final concentrations	PCR cycling protocol	Maximum sensitivity	Reference
1	Acute promyelocytic leukemia	*PML-RARA* fusion gene	21 patients, 48 FU samples	cDNA from BM	cDNA from HL-60 cells	*Glucuroni-dase β*	8 per sample, 1 positive control, 1 negative control, 1 NTC	200 ng (in sum 1.6 µg target RNA per sample)	no	Primers: 900nM, Probe: 250nM	10 min: 95°C, (30 sec: 94°C, 1 min: 60°C) 40x, 10 min: 98°C, Ramp rate 2°C/s	10^{-4}	(Brunetti et al., 2017)
2	Adult philadelphia positive acute lymphoblastic leukemia	*BCR-ABL* fusion transcript	26 patients, 117 FU samples	cDNA from BM	pooled RNA samples from 4 healthy donors	*Glucuroni-dase β*	4 to 9 per sample depending on the concentration, 21 negative replicates	50ng for sample at the onset, 750ng for FU, 100ng for housekeeping gene	no	Primers: 900nM, Probe: 250nM	10 min: 95°C, (30 sec: 94°C, 1 min: 60°C) 40x, 10 min: 98°C, Ramp rate 2°C/s	10^{-5}	(Coccaro et al., 2018)
3	Adult philadelphia positive acute lymphoblastic leukemia	*BCR-ABL* fusion transcript	40 patients, 98 samples	cDNA from BM	pooled MNC cDNA from healthy donors	*ABL1 gene*	3 per sample, 3 positive controls, 3 negative controls, 3 NTC	5µl of cDNA gained from 1µg of RNA	no	Primers: 900nM, Probe: 250nM	10 min: 95°C, (30 sec: 94°C, 1 min: 60°C) 40x, 10 min: 98°C, Ramp rate 2°C/s	10^{-5} to 5×10^{-6}	(Ansuinelli et al., 2021)
4	Adult acute lymphoblastic leukemia	Ig rearrangements, TcR rearrangements	50 patients, 141 FU samples	gDNA from BM	pooled MNC gDNA from 5 healthy donors	*Albumin*	3 per sample, 6 negative controls, 2 NTC	500ng	5 units Hinf1 per well	Primers: 500nM, Probe: 250nM	10 min: 95°C, (30 sec: 94°C, 1 min: 60°C) 40x, 10 min: 98°C, Ramp rate 2°C/s	10^{-5}	(Della Starza et al., 2016, Della Starza et al., 2019)
5	Pediatric B-acute lymphoblastic leukemia	IgH, IgK, TRG, TRD, and TRB rearrangements	209 patients, 397 samples	gDNA from BM	pooled MNC gDNA from 5 healthy donors	*Albumin*	3 per sample, 4 positive controls, 6 negative controls, 2 NTC	500ng	5 units Hinf1 per well	Primers: 500nM, Probe: 250nM	10 min: 95°C, (30 sec: 94°C, 1 min: 60°C) 40x, 10 min: 98°C, Ramp rate 2°C/s	10^{-5}	(Della Starza et al., 2021)
6	Pediatric B-Acute Lymphoblastic Leukemia	Ig rearrangements, TcR rearrangements, Patient specific target sequences	9 patients, 17 targets	gDNA from BM	pooled DNA from 8 healthy donors	*Albumin*	2 to 7 replicates depending on dilution factor and DNA availiability, 3 negative controls, 3 NTC	500ng	no	Primers: 1000nM, Probe: 300nM	10 min: 95°C, (20 sec: 95°C, 30 sec: 60°C, 1 min: 72°C) 45x, 10min: 98°C, Ramp rate 2°C/s	10^{-5} (10^{-6})	this project

7	Multiple myeloma, mantle cell lymphoma, follicular lymphoma	IgH rearrangements, B*CL1/IgH* fusion gene, *BCL2-MBR/IGH* fusion gene	69 patients, 225 samples	gDNA from BM or PB	pooled buffy coat gDNA from 10 healthy donors, gDNA from a BCL2-MBR/IgH-negative sample	*Albumin, RNAseP*	3 per sample, repetition with 6 replicates in case of unclear results, 1 positive control, 6 negative controls, 2 NTC	500ng	2.2 units Hinf1 per well	Primers: 500nM, Probe: 200nM	10min: 95°C, (30 sec: 94°C, 1 min: Tm°C) 40x, 10 min: 98°C Ramp rate 2.5°C/s, Tm according to the Tm of the ASO primers	10^{-5}	(Drandi et al., 2018, Drandi et al., 2015)
8	Early stage follicular lymphoma	*BCL2/IgH* gene rearrangement	67 patients	gDNA from MNC from BM and PB	K562 cell line DNA	N/A	3 per sample, repetition with 6 replicates in case of RT-PCR/ddPCR mismatch, 6 negative controls, 2 NTC	500 ng	5 units Hinf1 per well	Primers: 1000nM, Probe: 400nM	10min: 95°C, (30 sec: 94°C, 1 min: 55°C) 40x, 10 min: 98°C, Ramp rate 2°C/s	10^{-6}	(Cavalli et al., 2017)
9	Chronic myeloid leukemia	*BCR-ABL* fusion transcript	15 patients	cDNA from mRNA	N/A	N/A	no template control and a positive control (cDNA synthesized from K562 RNA)	40, 20 or 5 ng	no	Primers: 150nM, Probe: EvaGreen Supermix (1x)	10min: 95°C, (30 sec: 95°C, 1 min: 58°C) 40x, 5min: 4°C, 5min: 90°C, Ramp rate 2°C/s	N/A	(Park et al., 2019)
10	Chronic myeloid leukemia	*BCR-ABL* fusion transcript	10 patients	cDNA from MNC RNA from PB	N/A	N/A	1 per sample, 1 positive control (cDNA synthesized from K562 RNA), 1 NTC	200ng RNA	N/A	N/A	N/A	10^{-6}	(Wang et al., 2018)
11	Chronic myeloid leukemia	*BCR-ABL* fusion transcript	11 patients, 65 samples	cDNA from RNA from PB or BM	pooled RNA of samples without BCR–ABL translocation	*ABL1 gene*	3 per sample, 1 negative control	100ng RNA	no	Primers: 900nM, Probe: 250nM	10min: 95°C, (30 sec: 94°C, 1 min: 60°C) 40x, 10 min: 98°C, Ramp rate 2°C/s	10^{-5}	(Petiti et al., 2020)
12	Chronic myeloid leukemia	*BCR-ABL* fusion transcript	37 samples	RNA from MNC from PB	pooled RNA from 5 healthy donors	*ABL1 gene*	4 per sample	350ng	N/A	Primers: 450nM, Probe: 250nM	10min: 95°C, (30 sec: 94°C, 1 min: 60°C) 40x, 10 min: 98°C, Ramp rate 2°C/s	N/A	(Bochicchio et al., 2021)

Table 14: ddPCR for MRD measurement in different hematological diseases

8. List of figures and tables

9. Contribution statement:

This work was performed under the supervision of Dr. med Dr. rer. nat. Markus Mezger and Prof. Dr. med. Rupert Handgretinger in the Department for Pediatrics I of the University Hospital Tübingen.

It grew from a cooperation between our working group and Dr. rer. nat. H. Kreyenberg from the University Hospital in Frankfurt.
Half of the pediatric patient samples were provided to us by Mr. Kreyenberg and the remaining DNA samples were obtained from the study center of the Department for Pediatrics I of the University Hospital Tübingen by Prof. Dr. med. R. Handgretinger. FACS data were available via our hospital patient information system.
Mr. Kreyenberg provided us the ASO-, consensus- and Albumin-primer sequences as well as the respective probe sequences and the qRT-PCR pipetting and cycling protocol.

Based on this information, I was able to adapt the qRT-PCR method for my project and establish ddPCR by myself. The experimental strategies and planning as well as the performance of all experiments, collection and analysis of the data shown in this dissertation have been done by my own.
This thesis, including all figures and tables, was drafted and written by myself and any additional source of information has been cited.

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