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Double drop-off droplet digital PCR (DDO-ddPCR): A novel, versatile tool for mutation screening and residual disease monitoring in AML using cellular or cell-free DNA

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2-HG	2-hydroxyglutarate
alloSCT	allogenenic hematopoietic stem cell transplant
A.D.	Dr. Annika Dufour
AML	Acute myeloid leukemia
AML-MRC	AML with myelodysplasia related changes
BCL2	*B-cell lymphoma 2*
BHQ1	Black hole quencher 1
BM	Bone marrow
BRAF	*Proto-oncogene B-Raf*
C.R.	Christian Rausch
cDNA	Complementary DNA
CDS	Coding sequence
cfDNA	Circulating cell-free DNA
CNS	Central nervous system
CR	Complete remission
CSF	Cerebrospinal fluid
DDO-ddPCR	Double drop-off droplet digital PCR
ddPCR	Droplet digital PCR
DfN	Different from normal (immunophenotype)
DNMT3A	*DNA (cytosine-5)-methyltransferase 3A*
EMD	Extramedullary disease
F.Z.	Dr. med. Frank Ziemann
FAM	6-Carboxyfluorescein
FLT3	*Fms-like tyrosine kinase 3*
gDNA	Genomic DNA
GO	Gemtuzumab-ozogamicin
HEX	Hexachlorofluorescein
IC	Intensive chemotherapy
IDH1	*Isocitrate dehydrogenase isoform 1*
IDH2	*Isocitrate dehydrogenase isoform 2*
K.H.M.	Prof. Dr. med. Klaus H. Metzeler
K.S.	Prof. Dr. med. Karsten Spiekermann
KIT	*Proto-oncogene c-KIT*
LAIP	Leukemia-associated immunophenotype
LOD	Limit of detection
M.N.	Dr. Michaela Neusser
M.R-T.	Dr. Maja Rothenberg-Thurley
MDS	Myelodysplastic neoplasia
MNC	Mononuclear cell
MPC	Multiparameter flow cytometry
MRD	Measurable residual disease, formerly minimal residual disease
NGS	Next Generation Sequencing
NPM1	*Nucleophosmin 1*
NRAS	*Neuroblastoma RAS viral oncogene homolog*
pB	Peripheral Blood
qPCR	Quantitative PCR
RT-qPCR	Real-time quantitative PCR
S.A.B.	Simon A. Buerger
S.S.	Dr. Stephanie Schneider
S.T.	Sebastian Tschuri
SF3B1	*Splicing factor 3B subunit 1*
VAF	Variant allele frequency
WBC	White blood cell
wt	Wild type

2. German summary

Die akute myeloische Leukämie (AML) ist eine klonale Störung der Vorläuferzellen der myeloischen Blutzellreihe. Genetische Aberrationen, die sich in leukämischen Stammzellen ansammeln, definieren den Krankheitsverlauf. Daher werden sie auch zur Risikostratifikation herangezogen. Nach erfolgreicher Therapie der Erkrankung sollten keine AML-typischen genetischen oder immunphänotypischen Aberrationen mehr nachweisbar sein. Wenn jedoch einige leukämische Zellen persistieren, sind diese Veränderungen mit sehr sensitiven Assays weiterhin achweisbar. Dieses Phänomen wird als messbare Resterkrankung (MRD) bezeichnet. Die MRD hat eine hohe prognostische Bedeutung, da ihr Nachweis auf residuelle leukämische Zellen hinweist, welche Ausgangspunkt für ein Erkrankungsrezidiv sein können.

Daher sind für den klinischen Einsatz robuste, sensitive und schnelle diagnostische Assays zur MRD-Überwachung erforderlich. Die aktuellen Techniken zur Mutationsanalyse und -quantifizierung weisen Schwächen auf, die einen Bedarf an neuen diagnostischen Tests offenlassen.

Derzeit sind die gängigsten Methoden zum MRD-Monitoring die multiparametrische Durchflusszytometrie (MFC) und die quantitative Echtzeit-Polymerase-Kettenreaktion (RT-qPCR). Obwohl MFC weit verbreitet und in den meisten Fällen anwendbar ist, ist die Sensitivität im Allgemeinen geringer als bei molekulargenetischen Ansätzen. Während die qPCR derzeit die sensitivste Option darstellt, ist ihre Anwendung nur für wenige Ziele (mutierte Gene) gut evaluiert. Sie benötigt außerdem externe Referenzstandards zur Quantifizierung der MRD-Niveaus. Next-Generation-Sequencing (NGS) könnte potenziell breiter eingesetzt werden, ist jedoch in der Regel teurer und weniger sensitiv.

Ein Ansatz zur Überwindung dieser Einschränkungen ist die Digital-Droplet-PCR (ddPCR). Hier wird eine PCR-Reaktion in nanolitergroße Tröpfchen aufgeteilt, die einzeln die PCR durchlaufen und dann unter Verwendung von fluoreszierenden Reporter-Sonden jeweils auf die Amplifikation eines Ziels überprüft werden. Sie ist kostengünstiger und sensitiver als NGS-basierte Lösungen und beseitigt die Notwendigkeit externer Referenzstandards, die bei qPCR vorhanden ist. Kommerziell erhältliche ddPCR-Assays erfassen jedoch nur einzelne Nukleotidaustausche.

In dieser Arbeit, deren Resultate zu großen Teilen bereits publiziert werden konnten[1,3] wurden daher sogenannte „double drop-off digital droplet PCR (DDO-ddPCR) assays“ für Mutationen in den Genen *NPM1*, *IDH2* und *NRAS* entwickelt, die in der Lage sind, alle Alterationen, die an jeweils 2 benachbarten, häufig mutierten Positionen (sog., Hotspots) dieser Gene (*NPM1* c.863, c.877; *IDH2* c.140 c.172; *NRAS* c.12/13) vorkommen zu detektieren und zu quantifizieren. Diese Assays sind somit sowohl als Screening-Tools als auch zum seriellen Monitoring der Mutationslast zu gebrauchen. Die Assays wurden durch den Vergleich mit Next-Generation-Sequencing und existierenden qPCR-Assays validiert. Hierbei zeigte sich eine hohe Konkordanz mit existierenden Methoden, sowie eine mit konventionellen digitalen PCR-Assays vergleichbare Sensitivität.

Außerdem wurde in der vorliegenden Arbeit die Nutzbarkeit der Asssays zum Mutationsnachweis aus zell-freier DNA aus Blut und Liquor von AML-Patient:innen untersucht. Diese Untersuchungen weisen darauf hin, dass Detektion und Monitoring genetischer Alterationen mit dieser Methode eine vergleichbare Sensitivität wie die etablierte quantitative PCRs aus gDNA peripherer Leukozyten erreicht.

Zuletzt wurde die Anwendung der Assays in klinischen Szenarien überprüft. Hierbei konnten wir zeigen, dass langfristiges Monitoring zielgerichteter Therapien, frühzeitige Überprüfung des

Therapieanprechens bei intensiver Induktionstherapie, sowie die Detektion molekulargenetischer Veränderungen bei Patient:innen mit extramedullären Formen der AML mit unserer Methode möglich sind. Daher ist die cfDNA-basierte DDO-ddPCR eine wertvolle Erweiterung des diagnostischen Repertoires für Diagnose und Monitoring der AML

3. Abstract

Acute myeloid leukemia (AML) is a clonal disorder of precursor cells of the myeloid blood cell lineage. The disease course is defined by genetic aberrations accumulated in hematopoietic stem and progenitor cells. Therefore, these genetic changes can also be used for risk stratification at diagnosis. If antileukemic therapy successfully eradicated the leukemic cells, these aberrations should no longer be detectable. If some leukemic cells persist, however, these alterations are still detectable at a low level using very sensitive assays. This phenomenon is called measurable residual disease (MRD). It is highly prognostically significant, as its detection signals insufficient eradication of leukemic cells which often results in disease relapse. Therefore, robust, sensitive, and fast molecular genetic assays for MRD-monitoring are needed for clinical use.

Currently the most common methods for MRD-monitoring are multiparameter flow cytometry (MFC) and quantitative real-time PCR (RT-qPCR). While MFC is broadly established and applicable in most AML, its sensitivity is generally lower than that of molecular genetic approaches.

Though qPCR currently represents the most sensitive option, its use is only well evaluated for few targets, limiting its applicability. It also requires external reference standards for quantification of MRD levels. Next-generation-sequencing (NGS) could potentially be applied more broadly as variants in any leukemia-associated gene can potentially be detected ensuring a trackable target in virtually any patient but is generally more expensive and less sensitive.

One investigational approach to overcoming these limitations is droplet digital PCR (ddPCR). Here, a sample is split into nanoliter-sized droplets which undergo PCR individually and which can then each be assessed for amplification of a target using fluorescent reporter probes. It is cheaper and more sensitive than NGS-based solutions and abolishes the need for external reference standards present with qPCR. However, commercially available assays only detect individual nucleotide exchanges.

Therefore, we developed double drop-off digital droplet PCR (DDO-ddPCR) assays, which can quantitatively detect diverse alterations at two neighboring hotspot regions present in AML-associated genes (*NPM1*, *IDH2* and *NRAS*). These assays can be used for screening, quantification and monitoring. The assays were compared to existing ddPCR assays and next-generation sequencing and achieved high concordance as well as similar sensitivity as conventional ddPCR.

We also evaluated whether peripheral blood cell-free DNA (cfDNA) of AML patients was a viable substrate for MRD monitoring by DDO-ddPCR. We found that cfDNA-based analyses are as sensitive as conventional MRD-assays using qPCR of peripheral blood mononuclear cell cDNA. Furthermore, there are multiple clinical scenarios in which cfDNA-based mutation detection may be beneficial. Early response assessment during induction chemotherapy, long-term monitoring of targeted therapies, and detection of alterations found in extramedullary AML manifestations which are not easily amenable to biopsy are feasible using this approach. We were already able to publish these findings.[1,3] Thus, DDO-ddPCR based cfDNA analysis can complement routinely used molecular genetic assays for AML diagnostics.

4. Introduction

Acute myeloid leukemia (AML) is a clonal disorder of precursor cells of the myeloid blood cell lineage. Clinically, the disease usually becomes apparent due to the sequelae of cytopenias. The defining diagnostic criterion in most cases used to be the presence of at least 20% myeloid blasts in the peripheral blood or bone marrow.[4] However, newer classifications of myeloid disease assign more weight to recurrent AML-associated genetic aberrations. Risk stratification of the disease is performed in accordance with the European LeukemiaNet (ELN) 2022 criteria.[2,5,6] This classification stratifies AML into three risk groups (favorable, intermediate and adverse) based on recurrent genetic aberrations. The increasing importance of genetic markers has high clinical significance as fit young adults with core-binding-factor AML can be cured in about 75% of cases while patients with *TP53*mut AML achieve a 5y OS of <5%.[7-9] Accordingly, the three risk groups as defined by ELN have distinct outcomes. One large analysis of 1116 intensively treated AML patients found a 5y OS of 55%, 34%, and 15% respectively.[10]

In recent years, treatment algorithms have become more complex.[7,11,12] For fit patients, intensive induction chemotherapy with cytarabine and an anthracycline has long been the standard of care. Multiple large registry studies have shown that even in older patients and those with worse performance status (PS), intensive chemotherapy (IC) is beneficial.[13-16] In select populations, modifications of this regimen have brought incremental gains. Examples include the addition of midostaurin in patients with certain alterations to *FLT3*, the addition of the antibody-drug conjugate gemtuzumab-ozogamicin (GO) in low- or intermediate-risk AML with CD33-positive blasts and the use of a liposomal formulation of cytarabine and daunorubicin at a synergistic molar ratio of five to one within a liposome (CPX-351) in AML with myelodysplasia related changes (AML-MRC).[17-19]

Most patients who undergo such intensive treatment achieve complete remission (CR), which is considered the best early marker of favorable long-term outcomes after their initial therapy. Unfortunately, relapse rates remain high, even in patients with intermediate or favorable risk group assignment undergoing induction chemotherapy. [6,20]

However, a majority of patients is not fit enough to undergo such an intensive therapy. The combination of the hypomethylating agent Azacitidine and the bcl2 inhibitor Venetoclax has become the new standard therapy for those patients and is seeing increasing use in clinical practice. [21,22]

In addition, many genetically targeted treatment options are being evaluated both as monotherapies or in combination or sequence with the treatments detailed above. The most well-established therapeutic route is inhibition of FLT3 (FLT3i). Besides the use of midostaurin together with IC, midostaurin and other FLT3i are also approved for use together with chemotherapeutic consolidation therapy, as maintenance therapy after intensive chemotherapy (midostaurin), or as salvage therapy during relapse (gilteritinib).[23-26] In addition, sorafenib is used off-label as maintenance therapy after alloSCT.[27,28] It is expected that quizartinib will also be approved for first-line treatment together with chemotherapy and as maintenance therapy after alloSCT.[29]

Another option that is already in clinical use are inhibitors of mutated IDH enzymes (IDHi). In a subset of AML patients, mutations occur in the isocitrate dehydrogenase (*IDH*) isoforms *IDH1* or *IDH2*. These mutations lead to the production of an abnormal form of the IDH enzyme, which promotes the accumulation of an oncometabolite called 2-hydroxyglutarate (2-HG). This build-up of 2-HG leads to increased DNA methylation, changes in methylation patterns and other epigenetic changes which block cell differentiation. Inhibitors of these mutated IDH-isoforms (ivosidenib for IDH1 and enasidenib for IDH2) are already used as monotherapy in later therapeutic lines.[30,31]

While the European medicines agency (EMA) approved ivosidenib in combination with azacitidine for first-line treatment, enasidenib is currently not approved in Europe, due to lack of OS benefit in a phase three trial.[32] Combinations of these agents with other regimens in both the front-line and salvage setting are under investigation.[33]

Another approved frontline treatment option for less-fit patients is the hedgehog pathway inhibitor glasdegib. The hedgehog pathway is usually silenced in adults and aberrant hedgehog signaling enables leukemia stem cell survival and expansion.[34] In addition, an oral formulation of the hypomethylating agent decitabine has recently been approved in the EU as frontline treatment for those not eligible for intensice chemotherapy based on results of the Phase III ASCERTAIN-Study.[35] Numerous other therapies, including immunotherapeutic approaches such as the macrophage-checkpoint-inhibitor magrolimab, are currently under investigation.[36]

Regardless of what therapeutic regimen is used, monitoring therapeutic success is essential. This is especially important in the growing cohort of patients receiving continuous antileukemic therapies such as those mentioned above, where quicker detection of therapy failure might avoid unnecessary toxicities. Traditionally, maintenance of CR has been the primary marker of therapeutic success, but there has long been an effort to establish a more sensitive marker. Persistence of leukemic cells during and after therapy, i.e. measurable residual disease (MRD), has therefore become an important additional predictor of relapse risk.[37-42] Initially, presence of MRD has shown prognostic value primarily in the setting of intensive AML treatment. Those who still had MRD after induction chemotherapy or before alloSCT had noticeably higher chance of relapse as well as shorter survival.[43-46] Subsequently, the value of MRD has also been proven in relapsed AML.[47] More recently it has been shown that these findings also hold true in the setting of non-intensive AML therapy, as those with MRD levels of $<10^{-3}$ among a cohort of patients treated with Azacitidine and Venetoclax had significantly better outcomes than those with higher MRD levels.[48] In addition to predicting relapse, MRD-guided therapy may even prevent hematological relapse in AML.[49]

Currently, MFC and various molecular genetic assays are used for MRD assessment.[41] Advantages and disadvantages are detailed in table 1. MFC and quantitative PCR (qPCR) are the most widely used methods for MRD assessment. In MFC, multiple surface markers of leukemic cells are tagged with fluorophore antibodies and subsequently detected using light scatter in a flow cytometer. Based on the combination of surface markers, a leukemia specific immunophenotype (LAIP) or a “different from normal” immunophenotype (DfN) can be defined and then longitudinally tracked in subsequent patient samples. International collaborations such as the flow cytometry expert panel of the MRD working group of the ELN recommend a common set of fluorophores to standardize MRD-measurements. Nonetheless, there is a certain level of subjectivity inherent in the method, as fluorescence signals are manually gated.

The most sensitive method used in routine diagnostics today is qPCR. Here, a target gene locus is amplified using PCR, and the increase in fluorescent signal of a corresponding reporter probe is measured after every PCR-cycle. Absolute quantification of MRD levels by qPCR requires the use of external reference standards.[50] The ELN-guidelines recommend MRD-measurements at diagnosis, after 2 cycles of intensive treatment, at the end of treatment, and regularly during follow-up.[2,42] If molecular genetic assays are available, MRD-measurements from peripheral blood are allowed at intermediate timepoints, while for MFC sampling bone-marrow is recommended for all timepoints to combat the lower sensitivity of the method.

While the achievable sensitivities of up to 10^{-5} are very appealing, the method is not as widely applicable as MFC, as it depends on the presence of suitable targets (NPM1*mut,* RUNX1::RUNX1T1,CBFB::MYH11, …).[51]

Several methods which are not yet as widely established are under examination. Droplet digital PCR (ddPCR) has the advantage of not needing external reference standards for absolute MRD-quantification. However, its value is not as well established as that of qPCR-based assays, which is why the method is investigated in this work. More details on ddPCR and possible assay designs are given in the next section.

Next-generation sequencing (NGS)-based MRD-measurements are more broadly applicable than qPCR or ddPCR, as theoretically any altered region of the genome can be sequenced/analyzed. As virtually all AML carry leukemia-related somatic genetic alterations, sequencing the genome of a leukemic cell would guarantee finding a targetable MRD marker. However, most cancer centers use panel-sequencing approaches. Here, exons of a limited number of genes implicated in AML are sequenced, which drastically cuts the number of sequenced nucleotides per sample. Because sequencing cost correlates with the number of sequenced nucleotides, sequencing fewer nucleotides makes it affordable to sequence individual target areas more often, thus increasing so called “read depth”. This affords a higher sensitivity, as rare alterations are less likely to be missed if an area is covered more often. Regardless, currently NGS is less sensitive and more costly than qPCR.[52]

Method	Target	Sensitivity	Applicability (% of AML)	Turnaround time (h)	Cost	Limitations	Status
Drop-off Droplet digital PCR (DDO-ddPCR)	Specific hotspots	10^{-3} to 10^{-5}	~ 80	3-5	€€	Specific assay necessary for every hotspot	Exploratory
Droplet digital PCR (ddPCR)	Specific mutations	10^{-3} to 10^{-5}	~ 70	3-5	€€	Specific assay necessary for every mutation	Exploratory
Next generation sequencing (NGS)	Potentially any somatic mutation	10^{-2} to 10^{-4}	~ 100	5-10	€€€	Less sensitive, costly, technically challenging	Exploratory
Real-time quantitative PCR (RT-qPCR)	Robust data: *NPM1*, CBFB::MYH11, RUNX1::RUNX1T1 Less validated: KMT2A::MLLT3, DEK::NUP214, BCR::ABL1, *WT1*	10^{-4} to 10^{-5}	40-50	3-5	€	Limited applicability	Established
Multiparameter flow cytometry (MFC)	LAIP or DfN	10^{-3} to 10^{-4}	85-90	2-4	€	Less sensitive, more subjective analysis	Established

Table 1: Comparison of methods for MRD-asseessment. LAIP: leukemia-associated immunephenotype; DfN: different from normal; Adapted from Döhner et al, Blood, 2022.[2]

Emerging targeted therapies as well as the establishment of MRD-guided treatment strategies increase the utility of targeted molecular diagnostic assays that are sensitive, provide rapid turn-around times, and are inexpensive enough to allow for serial disease monitoring. Additionally, using peripheral blood (pB) samples causes less patient discomfort than to bone marrow (BM)-samples. An assay that is sensitive enough to already register any relevant MRD in a pB-sample may thus be better suited as a long-term disease monitoring tool.

Motivated by this need for versatile tools for sensitive monitoring of AML-related genetic alterations, we established digital droplet PCR (ddPCR)-based assays for several frequent AML driver mutations including *NPM1*mut, which are present in up to 40% of AML and are the most well established MRD-marker used in routine diagnostics today, as well as *IDH*mut, which are interesting because targeted agents against mutated IDH-proteins are already approved.[1]

When using ddPCR, a PCR reaction solution is automatically compartmentalized into microscopic droplets using oil immersion on a microfluidics cartridge. After undergoing the PCR itself, fluorescence measurement of each individual droplet allows for absolute quantification of variant alleles without the need for standard curves, and with high signal-to noise ratio (Fig. 1).

One area in which these advantages are particularly pronounced is the analysis of cell-free DNA (cfDNA), which – in the setting of malignancy – is also often referred to as cell free tumor DNA (ctDNA). Cell free DNA can be isolated from blood and other body fluids, and is thought to be a byproduct of cell deterioration, particularly necrotic cell death. In multiple solid tumors, analyses of cfDNA ('liquid biopsies') are already well established. Not only are they convenient as they circumvent the need for a (re-)biopsy, which – especially when the reason to harvest tumor tissue is a very well circumscribed question (e.g. detection of resistance mutations to targeted therapies) or when the tumor manifestations are not easily accessible – might save the patient unnecessary complications of biopsies or surgeries. But they have also shown to be more representative of intratumoral heterogeneity than biopsies of one individual metastasis, as clones in different localisations might undergo different evolutions and acquire different subsequent mutations. They might thus be more suited to predict therapy response, as they can represent a broader genetic context of the malignancy in question.[53-55]

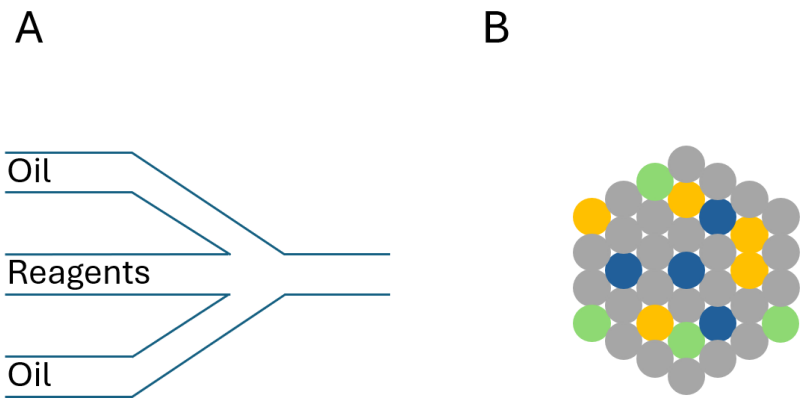

Figure 1: Schematic representation of ddPCR, adapted from Bio-Rad Bulletin 6407 (Bio-Rad, Hercules, CA). The reagents for a PCR-reaction, including fluorescent reporter probes specific to the region of interest, are emulsified in oil (A), creating ten- to twenty thousand nanoliter-sized droplets (B). Each droplet then undergoes ddPCR separately and can subsequently be analyzed separately. Droplets are excited with lasers and the fluorescence signal of each individual droplet is measured. As DNA templates are distributed stochastically into the droplets, the number of

droplets positive for FAM (blue), HEX (green) or both (orange) allows calculation of the absolute concentration of a target template.

Recent analyses in lymphomas and myelodysplastic neoplasias (MDS) proved that cfDNA analysis is also useful for genetic profiling and therapy monitoring of hematologic neoplasms.[56] Already in 1994, *NRAS*-mutations of patients with AML or MDS were detected in cfDNA.[57] Ten years later, chromosomal aberrations of AML patients could also be detected in cfDNA.[58] Subsequently, Yeh and colleagues analyzed the mutational profile and allelic burdens of cfDNA and BM of 12 patients with MDS treated on a phase I trial. Using a 55 gene panel-sequencing approach to detect relevant mutations and subsequently tracking them using PCR-based methods, they showed that mutational profile and mutant allele frequency correlated strongly between 83 matched cfDNA and bone marrow samples. Rising allele frequencies also were a predictor of therapy failure.[59] In addition, Nakamura and colleagues reported that relapse could be predicted for patients with high-risk MDS or AML after alloSCT by monitoring VAFs of disease-specific mutations on cfDNA using ddPCR.[40]

Beyond these initial studies, several other use cases for cfDNA-based disease monitoring exist in AML: First, as shown by the studies mentioned above, there is considerable intratumoral heterogeneity within AML, which cfDNA might represent better than a localized bone marrow biopsy. That is especially true in cases with extramedullary disease (EMD), where some mutations might only be found in the bone marrow and some only in the extramedullary site.[1] As serial biopsies of EMD manifestations are usually not possible or safe, detecting these mutations using cfDNA may greatly increase the information available about the clonal evolution and therapy response of an AML with EMD. Secondly, meningeosis leukemica, i.e. the presence of leukemic cells in the cerebrospinal fluid (CSF), is a form of EMD that is particularly suited to this technique. Not only can cfDNA from CSF be analyzed for the presence of AML-related mutations, due to the disruption of the blood-brain-barrier associated with meningeosis, cfDNA from the central nervous system (CNS) compartment may even be detectable in peripheral blood. And lastly, as cfDNA is considered a byproduct of cell destruction, cfDNA-kinetics during induction chemotherapy might serve as a marker for therapeutic response.

However, using ddPCR in these indications has one significant drawback. Commercially available assays rely on mutation-specific probes that detect only one particular mutation (Fig. 2 A). This unfortunately means that a high number of assays must be established and stocked in a laboratory to cover all possible nucleotide changes at common mutational hotspots that are recurrently altered in AML.

So-called drop-off assays were developed to address the problem. The drop-off ddPCR principle works as follows: One fluorophore-marked probe (reference) binds within the amplicon, at a location that is close to the targeted hotspot, while a second probe binds the wild-type (wt)-sequence of said hotspot within the same amplicon. Thus, a double-positive fluorescence signal from both probes indicates the presence of wt DNA, whereas a fluorescence signal for only the reference probe indicates the presence of a variant that inhibits the second probe from binding to its target sequence (Fig. 2B).[60-62] However, we have noted that some AML-related oncogenes or tumor-suppressor genes have two hotspots in close proximity. Therefore, we built on the drop-off assay principle to be able to seek out mutations at two mutational hotspots co-located within a region that could be covered by one PCR amplicon. In these DDO assays, which we've previously

published, one probe binds the wt DNA of one sequence hotspot area (eg, *IDH2* codon p.R140), whereas the other probe binds the wt sequence of another hotspot close by (eg, *IDH2* codon p.R172).[1] Thus, both probes serve as reporter probe for one and as reference probe for another hotspot. In the presence of wt DNA, both probes will bind, leading to a double-positive fluorescence signal. A single-positive fluorescence signal indicates that the hotspot covered by the non-binding probe is in some way altered (Fig. 2C).

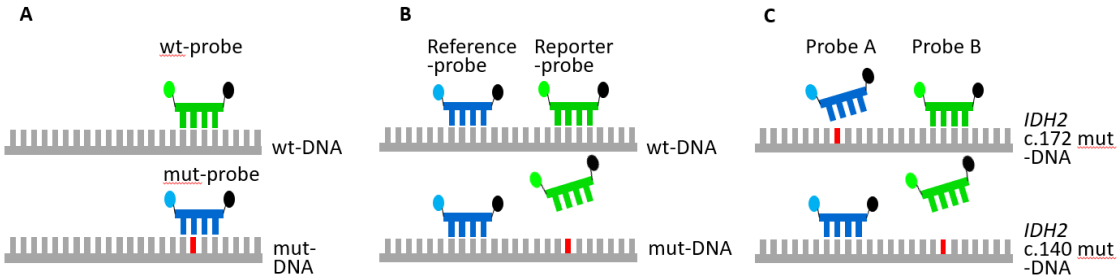

Figure two: Figure reprinted with permission from Rausch et al: "Principle of droplet digital PCR (ddPCR) assay designs. A: Standard variant-specific ddPCR assay. In the variant-specific assay, the reference probe (green) binds the wild-type DNA sequence in a known hotspot region, whereas the reporter probe (blue) binds a particular mutant DNA sequence (red) in the same hotspot. Other mutations in the same hotspot will not be detected. B: Conventional (reporter and reference probe) drop-off ddPCR assay. The reference probe binds to the DNA template of interest outside the hotspot region, whereas the reporter probe binds to the wild-type DNA sequence at the hotspot of interest. Presence of any sequence variant in the region covered by the reporter probe will be detected by a loss of the reporter signal. C: Double drop-off (DDO) ddPCR assay. In a DDO assay, one probe (probe A) binds to the wild-type sequence of one hotspot of interest, and the other (probe B) binds to another nearby wild-type sequence of a hotspot of interest. Thus, when a variant occurs in either of these hotspots, one probe will drop off, whereas the other will still bind to the template of interest and serve as reference. Thus, mutations in two different hotspot regions can be detected in one assay."[1]

Below we report key characteristics of the DDO-ddPCR assays we have developed. We also compare their sensitivity to other assays already in use in routine diagnostics. In addition, we demonstrate feasibility and clinical utility of cfDNA-based disease monitoring in AML.

5. Patients and methods

5.1 Patients and samples

For initial testing of newly designed primers, cell lines with known mutational profiles were used (OCI-AML3, MOLM-13). Cell lines are profiled in table 2. After this initial step, clinical validation was performed using BM genomic DNA (gDNA) of primary patient samples of patients with AML. These samples were available in the sample collection of the Laboratory for Leukemia Diagnostics (LMU Munich, Germany). DNA for our experiments was isolated from cell lines and patient samples by Simon A. Buerger (S.A.B.) and Sebastian Tschuri (S.T.). This DNA was subsequently analyzed (NGS, qPCR) by Dr. Annika Dufour (A.D.), Dr. Maja Rothenberg-Thurley (M.R-T.), and Dr. Michaela Neusser (M.N.).

As we have published, we also prospectively evaluated the assays using liquid biopsies.[1] For the liquid biopsies, we collected whole blood in Cell-Free DNA BCT blood collection tubes (Streck, La Vista, NE, USA) at several timepoints during the treatment course of AML patients subsequently treated at our center. Patients were recruited at first diagnosis of AML, and the first samples were collected before initiation of antineoplastic therapy. Follow-up samples were initially collected twice weekly to evaluate cfDNA kinetics under induction chemotherapy. After induction, cfDNA was collected prior to every cycle of consolidation chemotherapy, and at every regularly scheduled follow-up visit thereafter. Logistics for sample collection were designed by Dr. med. Frank Ziemann (F.Z.). Samples were collected by the doctoral candidate (C.R.), but individual samples were processed by F.Z. in the absence of C.R. At diagnosis, DNA extracted from BM samples of patients was analyzed using an NGS-panel of 68 genes commonly altered in AML, as published previously.[63] These NGS-Analyses were analyzed by A.D, M.R-T., and M.N. For the clinical characterization of the genetic profile and therapeutic outcomes of patients analyzed in section 6.3, further diagnostics (cytomorphology, cytogenetics) were needed. These were performed by Prof. Dr. Karsten Spiekermann (K.S.), Dr. Stephanie Schneider (S.S.), and K.H.M. Subsequently, data that were pertinent to the project were curated by C.R.

Our study was approved by the appropriate ethics committee (LMU Munich, approval number: 18-539, obtained by F.Z. and K.H.M.). All patients provided written and informed consent. All experiments were conducted in adherence to the Declaration of Helsinki (7th revision) and other applicable regulations.

Cell line	**OCI-AML3[64]**	**MOLM-13[65]**
Origin	pB of a 57 yo male with AML-M4	pB of a 20 yo male with AML-M5a at relapse after initial myelodysplastic syndrome (MDS, refractory anemia with excess of blasts, RAEB)
Karyotype	48,X,-Y,+1,der(1;7)(p11;q22) ,+5,i(5)(p10),+8,del(13)(q13;q21), dup(17)(q21;q25)[6]	49,XY,+6,+8,+13, ins(11;9)(q23;p22;p23), del(14)(q23.3q31.3)[25]
Gene Mutations	*DNMT3A* R822C	*FLT3*-ITD

	NPM1 Type A *NRAS* Q61L	*KMT2A-MLLT3*-Fusion
Immunopheno-type	Positive for CD4, CD13, CD15, cyCD68 and HLA-DR; negative for CD3, CD14, and CD19.	Positive for CD4, CD15, CD33, and cyCD68; negative for CD3, CD14, CD19, CD34 and HLA-DR.

Table 2: Characteristics of cell lines used in this work.

5.2 Assay design

Initially, we evaluated mutation-specific assays for *IDH1* R132H, *IDH2* R140Q, *IDH2* R172K and *DNMT3A* R882H from Bio-Rad (Hercules, CA, US).

During the course of the project, we moved toward using the more versatile drop-off and double drop-off assay designs, based largely on input from C.R. For the *NPM1* drop-off assay, we used previously published primers.[40] All other primers and all probes utilized in these assays were designed using Primer3 Plus Version 2.4.2.[66] We tried to achieve amplicon lengths of less than 200 nucleotides as short amplicons are described as beneficial given the nanoliter-sized droplets in which the reaction occurs (Bio-Rad Bulletin 6407). For primers, we aimed for a length of around 20nt, and a GC content of 30-80%. Primer specificity for the desired transcripts was evaluated using Primer Blast.[67]

Primer specificity and annealing temperature were evaluated using PCR temperature gradients. Primers and samples harboring the mutation of interest were cycled at temperatures around the recommended target annealing temperature of 60°C. PCR products were then transferred to 1% agarose gels, which were prepared in Horizon 11 gel chambers (Life technologies, Carlsbad, CA) using Agarose and 10X TBE Buffer (both by Invitrogen, Waltham, MA). 24µl GelRed Nucleic Acid Gel Stain (Biotium, Fremont, CA) was added to make DNA bands visible under UV-light. Gels were loaded with 7µl 10x Blue-Juice Gel Loading Buffer (Invitrogen, Waltham, MA) per well. 8µl of Molecular Weight Size Marker VI (Roche Diagnostics, Basel, CH) was used to estimate product size in the electrophoresis.

We then confirmed the primer sequences and annealing temperature that led to amplification of only one amplicon by confirming the presence of only one band under UV-light (321 nm) after electrophoresis.

Probes were designed to have an optimal length of only 15nt, as shorter probes are better at discriminating between single nucleotide variants (Fig. 3). As advised by the manufacturer of the ddPCR system we used (Bio-Rad Bulletin 6407), we strove for about 5°C higher annealing temperatures of ddPCR probes compared to ddPCR primers. This difference serves to decrease background fluorescence. As the probes are considerably shorter than the primers they have to undergo PCR with, we added locked nucleic acid (LNA) nucleotides to increase their melting temperature. These are artificial nucleotides in which the 2' oxygen and 4' carbon atom of the ribose-ring are connected, thus forcing the molecule to remain in the ideal configuration for Watson-Crick binding, which increases the melting temperature of LNA-containing nucleic acid oligomers. Melting temperatures were calculated using the Exiquon oligo analyzer tool (https://www.exiqon.com/ls/Pages/ExiqonTMPredictionTool.aspx). This tool was also used to evaluate primers and probes for auto-dimerization. We then optimized ddPCR conditions using ddPCR temperature gradients, finding the optimal annealing temperature for each assay. All

oligonucleotides were acquired from TIB MolBiol (Berlin, Germany). Detailed characteristics of all oligonucleotides are shown in table 5. In-silico assay-design was performed by C.R., after he had been instructed on how to use the tools detailed in this section by M.R-T., F.Z., and K.H.M. In-vitro assay optimization and validation was performed by C.R., after having been instructed on the principles of assay-validation by M.R-T.

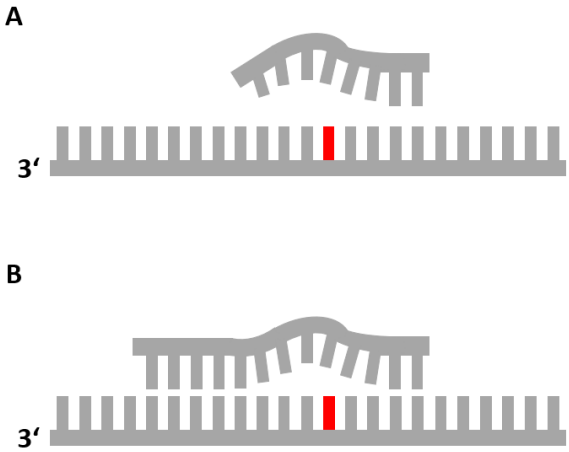

Figure 3: Schematic representation of different probe lengths coping with a mismatched nucleotide (red). A shorter probe (Panel A) might drop off due to one mismatch as one mismatch represents a larger proportion of the probe. Longer probes (Panel B) might not drop off despite a mismatch, as a relatively longer part of the probe can still bind matching nucleotides.

5.3 cfDNA isolation

cfDNA tubes were collected at the timepoints indicated above. Within 14 days after collection in cfDNA tubes, plasma was separated by centrifuging whole blood specimens for 10 minutes at 1600 g. The plasma was transferred to a new tube and centrifuged another 10 minutes at 16100 g to remove any leftover cells. The supernatant was stored at -80 °C if it was not immediately processed further. CfDNA was isolated using the QIAamp Circulating Nucleic Acid Kit (Qiagen, Hilden, Germany) according to manufacturer's instructions. These steps were usually performed by C.R., after he had been introduced to the applied methods by S.T. and M.R-T, When C.R. was absent, this work was occasionally taken over by F.Z., S.A.B. or S.T.

5.4 DNA quantification and ddPCR

Genomic DNA was quantified with the Invitrogen Qubit dsDNA HS Assay Kit (Paisley, UK), while cfDNA samples were analyzed for both fragment size distribution and concentration using the Agilent High Sensitivity DNA Kit (Santa Clara, CA, US) on a Agilent 2100 Bioanalyzer-machine, according to the manufacturers' instructions. This was usually (>90% of samples) done by C.R., after instruction by M.R-T, and S.T. Occasionally, cfDNA quantification was performed and analyzed by F.Z., S.A.B. or S.T.

The Bio-Rad QX200 droplet digital PCR System (Bio-Rad), which consists of a droplet generator, a droplet reader and the accompanying software was used for ddPCR. ddPCR-experiments were usually performed by C.R. However, some initial ddPCR-runs were performed by M.R-T, and S.T., as they were introducing C.R. to this method. ddPCR reactions were prepared as follows: First, a master-mix was created, which includes all reagents necessary for the ddPCR to occur.

These were mixed in a 1.5 ml Eppendorf tube at the quantities and concentrations detailed in table 3:

	Amount in µl (per planned well)	Plus 10% pipeting margin	Stock concentration	Final concentration (in 20 µl well)
2xddPCR supermix	10	11	2x	1x
Hind III (restrictase)	0.5	0.55	10 U/µl	0.25 U/µl
Left primer	0.9	0.99	9,99 µM	450nM
Right primer	0.9	0.99	9,99 µM	450nM
Probe 1	0.5	0.55	10 µM	250 nM
Probe 2	0.5	0.55	10 µM	250nM
TOTAL	13.3	14.63	-	

Table 3: Amounts and concentrations of all reagents comprising the Master-mix used in our ddPCR-experiments. For specific primers and probes used refer to table 5.

Next, samples were pre-loaded into PCR-strips. Enough DNA solution to achieve the desired quantity of DNA was first pipetted into each tube. Then, sterile nuclease-free water was used to bring all tubes to a volume of 7,37 µl. To this volume, 14,63 µl of the master-mix was added, bringing each tube to 22 µl. Exactly 20 µl from each tube were then transferred into a well of a droplet generation cartridge. Per sample, 70 µl of droplet generation oil were pipetted into a different well, as recommended. Until this step, all reagents except the droplet generation oil were kept on ice, as recommended. The cartridges were then closed with a gasket and loaded into the droplet generator. After generation of the oil-emulsed droplets characteristic of ddPCR, emulsion from each well was transferred to a well of a 96-well PCR-plate. The plate was subsequently sealed and placed in a thermocycler for PCR. PCR cycling conditions are detailed in table 4:

Step	**Temperature**	**Time**	**Iterations**	**Ramp rate**
Initial denaturation	95°C	10 min	1	2°C/s
Denaturation	94°C	30 s	40	
Annealing and amplification	Varies by assay, see table 5	60 s	40	
Inactivation	98°C	10 min	1	
Pause	4°C	∞	1	1°C/s

Table 4: Cycling conditions used for our ddPCR-experiments. For annealing-temperatures refer to table 5.

Subsequently, the PCR-plates were transferred to the droplet reader. There, droplets from each well are aspirated through a microfluidic channel in single file. This allows for two lasers, whose wavelengths are suited to excite the fluorophores mentioned above to be shot at each individual droplet. Thus, each droplet can be evaluated individually for the presence of a fluorescence signal of either or both fluorescent probes. All droplets are then plotted on a graph displaying the amplitudes of both signals on the x- and y- axis by the QX manager software. This allows for differentiation of four clusters: droplets which are positive for neither signal have low values on both axes, putting them in the lower left quadrant of the plot. Those positive for either of the signals have a high value on the corresponding axis and a low value on the other, putting them on the upper left or lower right quadrant of the plot. Lastly, those positive for both signals will display a high value on both axes and end up in the upper right quadrant. Thus, each fluorescence signal can be evaluated for each individual droplet, and each droplet can be grouped into one of the clusters.

In analogy to flow cytometry, this process of assigning the droplets to clusters based on their fluorescence signal amplitudes is called gating.

5.5 Double drop-off ddPCR assay design

The ddPCR assays we procured from Bio-Rad use fluorescent probe pairs specific to one particular single nucleotide variant. These fluorescent reporter probes are so called hydrolysis probes. They are connected to both a fluorescent dye (FAM or HEX) and a quencher (BHQ1), which extinguishes light emissions from the fluorophore while both molecules are in close proximity. Once the probe binds its target sequence and the next PCR-amplification commences, DNA polymerase will recognize PCR primers and start synthesizing a DNA strand complementary to the probes target. As it reaches the probe, it hydrolyzes the probe to make way for the strand it is synthesizing. Thus, the fluorophore will no longer be connected to the quencher by the probe and can emit fluorescence once the sample is agitated by light of the appropriate wavelength. In the typical Bio-Rad assay design, the two probes bind the wt and mutant sequence of one amplicon, (Fig. 2A). As the probes are designed to discriminate between two defined nucleotide sequences at a specific DNA position, other sequence alterations at the same position will not be bound by either probe, and thus go unnoticed.

To address this limitation, the ‘drop-off‘-assays mentioned above were designed. [62] These assays use one probe that anneals to a DNA sequence in close proximity of the target position, which will bind regardless of the presence of any mutations at the target position. This probe serves as a marker for the presence of amplifiable DNA of a relevant sequence. A second probe binds the wt (unmutated) sequence at the target position. This probe serves as signal that the amplified DNA does not carry any mutation at the target position (Fig. 2B). If both fluorescent signals are present, the targeted DNA sequence is present and does not carry any mutation. If only the reference probe is binding, the DNA sequence is also present, but the hotspot must carry a mutation, since the wt-specific probe cannot bind. If neither probe binds, the target sequence is not present.

Many oncogenes have several mutational hotspots in close proximity. This should come as no surprise, since a typical mechanism for a proto-oncogene to become an oncogene is a mutation which alters the functional domain of an enzyme, which - in its mutant form - confers a growth advantage onto the cell. Therefore, mutations cluster in these domains. [68] One enzyme for which this is the case is IDH2. The gene encoding for it has two well-known mutational hotspots in close proximity to each other (*IDH2* codon p.R140, *IDH2* codon p.R172). However, they are too far apart for one reporter probe to be able to cover both hotspots. We therefore expanded on the drop-off assay principle by developing double drop-off assays. Regardless of the exact changes in DNA sequence, our ‘double drop-off’ (DDO) approach detects all possible mutations at both hotspots using a single assay: While one probe anneals to the wt DNA sequence of the first hotspot area (i.e., *IDH2* codon p.R140), the other one anneals to the wt sequence of a second hot-spot (i.e., *IDH2* codon p.R172) in close proximity. Thus, both probes serve as reporter probe for one and as reference probe for the other hotspot at the same time. Amplification of wt DNA will produce a double-positive fluorescence signal, as both hotspots contain wt-DNA which can be bound by both wt-complimentary probes. If the assay is only positive for one fluorescence signal, an alteration of the hotspot covered by the non-binding probe must be present, abrogating its annealing (Fig. 2C). If, for example, only the HEX-signal is detected, only the HEX-marked probe has bound its target sequence and thus undergone hydrolysis. It can thus be inferred that there must be wt-DNA at the spot which the HEX-marked probe binds (thus enabling the probe

to bind and be hydrolyzed) and non-wt-DNA at the other hotspot (thus preventing the probe which is complimentary to the wt-sequence of this hotspot from binding and being hydrolyzed). How the nucleotide sequence of this spot changes is irrelevant, as the non-binding of the probe is facilitated by the hotspots DNA not being the wt-sequence and not by any particular mutant sequence. Thus, a mutation abrogated binding of the wt-complimentary probe and can be detected due to the absence of a fluorescence signal corresponding to said probe.

5.6 Data analysis and statistics

ddPCR results were initially analyzed using version 1.7.4 of the the QuantaSoft-software (Bio-Rad). We manually gated all droplet clusters. After evaluating several gating strategies for droplets that could not clearly be assigned to one cluster, we decided to assign these droplets to the closest identifiable cluster. Wells were excluded according to manufacturer's instructions (Droplet Digital PCR Applications Guide, Bio-Rad). The data generated using this analytic approach were published in Rausch et al[1].

At the time of the publication of this data in Rausch et al[1], a drop-off or even double drop-off assay-design for a ddPCR-assay was a novel idea. Since then, the concept has broadly been accepted, with many laboratories using the approach and commercial assays made available by BioRad. In the wake of this development, BioRad has also developed a new software for analysis of ddPCR-assays, which can be set up specifically to analyze DO- and DDO-assays (QX Manager Software, Version 2.2). As the use of the DO-/DDO-design leads to a different distribution of droplets compared to the conventional assay-design, this development allowed us to refine the gating of our droplet clusters: In the conventional assay, there are four clusters of droplets: A cluster without DNA-templates complementary to either probe, which will not have a high amplitude for the fluorescence signal of either probes fluorophore (-/-), a cluster where either probe can bind because either mutant or wild-type DNA is present (-/+ or +/-) and a cluster of droplets in which both templates are present (+/+). However, in a drop-off assay the double-positive cluster represents droplets containing only wildtype-DNA, which is bound by both probes, and only one of the single-fluorescence-positive-clusters exists, representing mutated DNA. In addition, there is another cluster between those two clusters, which represents droplets containing both mutant and wild-type DNA. This difference in droplet clustering made it impossible to use automated or threshold-based gating in the QuantaSoft software. Droplets were therefore gated using a "lasso", i.e. by manually drawing a free form around all droplets which should go into one cluster. This approach limits replicability. Therefore, we looked for a software that could automatically gate at least some of the samples. As the new software solution QX Manager (Bio-Rad) represents the currently accepted best practice in analyzing DO- and DDO-ddPCR data, we have re-analyzed the wild-type samples from our cohort using automated gating with the "drop-off" preset of the program. This leads to slight changes to the data reported in table 6, compared to the results reported previously in Rausch et al. These changes thus represent an updated analysis strategy, not a correction of prior erroneous data, and do not affect the interpretation of the data as presented in the published manuscript. In addition, we hope this approach increases replicability.

Limits of detection (LOD) were defined as the lowest value at which a true positive measurement could reliably be discerned from a false positive. For this, there are two potential approaches: First, we calculated the median number of false-positive droplets occurring during the measurement of known-negative samples plus two times the standard deviation of the same median (LOD by droplet). We also calculated the ratio of the median number of false-positive droplets to the

median number of negative droplets measured during these experiments (LOD by ratio). Analytical specificity was defined as 1 – this ratio.

We defined analytical sensitivity as the lowest VAF of mutated DNA above the LOD that could still be detected in a serial dilution experiment. When measuring known negative samples, we observed significantly more variation in the number of droplets correctly identified as negative, than in the number of false-positive droplets. We have therefore chosen the LOD by droplet as cutoff for analytical sensitivity. Dilution curves were plotted on a logarithmic scale to allow for a more precise comparison between expected and measured VAFs across a wide range of VAFs. The LOD by ratio – though not the limit we ended up using as cutoff – was added to the figure visualizing the dilution curves for orientation, as the limit by droplet could not be plotted in these graphs.

Linear correlation of VAFs measured by different methods was measured using Pearsons r^2.

Statistical analyses were performed in R version 3.5.1 (R Foundation for Statistical Computing, Vienna, Austria). Graphs were designed in GraphPad Prism Version 9.0.0 (GraphPad Software, San Diego, California, USA). All statistical analyses were performed by CR under supervision by M.R-T., F.Z. und K.H.M. All graphs and figures were drafted by C.R. under supervision by M.R-T., F.Z. und K.H.M.

Part of the work presented here has been published as a conference abstract and a full paper. [1,3] Both have been drafted by C.R. and revised by C.R. in accordance with feedback from F.Z. and K.H.M. Afterwards, the other co-authors also contributed feedback and revisions, which were worked into the articles by C.R. The final versions were approved by all coauthors.

6. Results

6.1 Characteristics of our DDO-ddPCR assays targeting mutations in common AML driver genes

As mentioned above, mutations in *IDH2* are clinically relevant as there is an approved inhibitor of the mutant protein (enasidenib), while *NPM1*mut are important for risk stratification and MRD-monitoring. In both genes, mutations cluster at two hotspots co-located within one exon. However, numerous different alterations occur at these hotspots. Using the standard ddPCR assay design, these nucleotide insertions (in *NPM1*) or exchanges (in *IDH2*) would each necessitate development of a new assay. To demonstrate the clinical utility of the DDO-ddPCR assay principle, which catches all mutations in one gene using a single assay, we developed DDO-ddPCR assays for *IDH2*mut and *NPM1*mut. While the targeted mutations of *IDH1/2* are usually single-nucleotide substitutions, mutations in *NPM1* are typically multi-nucleotide insertions. Within this work, we classify them in accordance with the established nomenclature used for example by Thiede et al and Ivey et al [69,70]. This nomenclature uses an alphabetic system to differentiate a multitude of small (4-16 nt) insertions at or close to the codons cited above. The most frequent of these are the so-called type A (c.863_864insTCTG), B (c.863_864insCATG) and D (c.863_864insCCTG) mutations, which together make up ~90% of *NPM1*-mutations. We also developed a drop-off assay for mutations in *NRAS* codons 12/13 (table 5). At one point, we have previously published their performance characteristics in Rausch et al.[1] However, owing to the advances in droplet gating outlined above, some of these characteristics have since been updated. In this dissertation, we show the assay characteristics as derived from the more recent gating strategy.

Assay	PCR target region (GRCh37/hg19)	Annealing Temperature (°C)	Left Primer	Right Primer	Probe 1	Probe 2
***NPM1* double drop-off**	5:170837474-170837645	59	5′ -TTGATGTC-TATGAAGTGTTGTGGTTC -3′	5′ -GGACAGCCAGA-TATCAACTGTTACA -3′	5′ -HEX-TTC+AGATCT+CT+G+GC-BHQ1-3′	5′ -FAM-TGG+A+G+GAAGTCTCTT-BHQ1-3′
IDH2 double drop-off	15:90631789-90631974	57	5′ -GTGGAAAAGTCCCAATGG -3′	5′ -AGGTCAGTGGATCCCCTCT -3′	5′ -HEX-C+CGGA+AC+AT+CC+TGG G-BHQ1-3′	5′ -FAM-CC+ATTGGCAG+GCACG-BHQ1-3′
NRAS drop-off	1:115258626-115258772	60	5′ -TAC-AAACTGGTGGTGGTTGG -3′	5′ -TGAGAGACAG-GATCAGGTCA -3′	5′ -HEX-CCCA+C+CATA+GAGGT-BHQ1-3′	5′ -FAM-CAG+GTGG+T+GTTGGG-BHQ1-3′

Table 5, reprinted with permission from Rausch et al[1]: Sequences and positions of the newly developed ddPCR assays. + represents locked nucleic acid nucleotides. BHQ1: Black hole quencher 1; FAM: 6-Carboxyfluorescein; HEX: Hexachloro-fluorescein

To establish the limit of detection (LOD) and define analytical specificity, we analyzed samples which had proven to be negative for the target gene variants using NGS. All assays achieved high analytical specificity (99.963% - 99.99%; table 6), as only very few false-positive droplets were detected.

Assay	Target site	Number of negative samples	False-positive droplets (median, range)	Negative droplets (median, range)	LOD by droplet	LOD by ratio	Analytical specificity
NPM1	c.863	19	0 (0-2)	8462	3	0.037%	99.963%
	c.877	19	1 (0-3)	8462	3	0.01%	99.99%
IDH2	c.140	21	1 (0-2)	7501	3	0.035%	99.965%
	c.172	21	3 (1-6)	7501	6	0.07%	99.93%
NRAS	c.12/13	17	1 (0-2)	7342	3	0.02%	99.98%

Table 6: Specificity and limit of detection (LOD) of each assay as determined by measuring negative samples for each mutation. Table adapted from Rausch et al.[1]

To ascertain the analytical sensitivity of our assays we first determined the VAF of DNA extracted from mutated primary patient samples using NGS. Subsequently, we spiked them into known wt-DNA to generate serial dilutions (table 7). Decisions on which dilution steps to perform were based on the initial allele frequency, and the expected assay characteristics. Most dilution steps were performed for the *NPM1*-assay as this assay was evaluated first and we had the least knowledge about expected assay performance in this case.

	Variant Allele Frequencies										
	1:1	**1:10**	**1:20**	**1:25**	**1:40**	**1:50**	**1:100**	**1:250**	**1:500**	**1:1000**	**1:5000**
NPM1 **codon 288**	0.5	0.05	NA	NA	NA	0.01	0.005	NA	0.001	0.0005	0.0001
IDH2 **codon 140**	0.484	NA	0.0242	NA	0.0121	NA	0.00484	NA	NA	0.000484	NA
IDH2 **codon 172**	0.5061	NA	NA	0.0202	NA	0.0101	0.00506	NA	NA	0.000506	NA
NRAS **codon 12/13**	0.34	0.034	NA	NA	NA	NA	0.0034	0.00136	0.00068	0.00034	NA

Table 7: Dilution steps for the serial dilution of gDNA from mutant primary patient samples in gDNA from unmutated primary patient Samples for each hotspot. NA = Not available.

Using the LOD established above, the DDO-ddPCR for *NPM1* achieved a sensitivity of 0.05% for mutations at c.863 (i.e., type A/B/D mutations; Fig. 4A). Repeating the same process with the DDO-assay for *IDH2*mut, an analytical sensitivity of 0.048% for codon p.R140 and 0.051% for codon p.R172 was defined (Fig. 4C/D). The conventional drop-off ddPCR assay we had

constructed for detection of mutations in *NRAS* c.12/c.13 achieved slightly worse analytical sensitivity of 0.068% (Fig. 4B). As the theoretical limit for the sensitivity of ddPCR-assays is about one in 10,000 for a single well, these data prove that our DDO-assays perform at an analytical sensitivity close to what the ddPCR-method can achieve (table 8).

Assay	Target site	Performed Replicates	Discordance between replicates	Lowest detected VAF above LOD (= Analytical Sensitivity)
NPM1	codon 288	2	No	0.05%
IDH2	codon140	2	No	0.048%
	codon172	2	No	0.051%
NRAS	codon12/13	2	No	0.068%

Table 8: Sensitivity as determined by dilution curve. Table adapted from Rausch et al.[1]

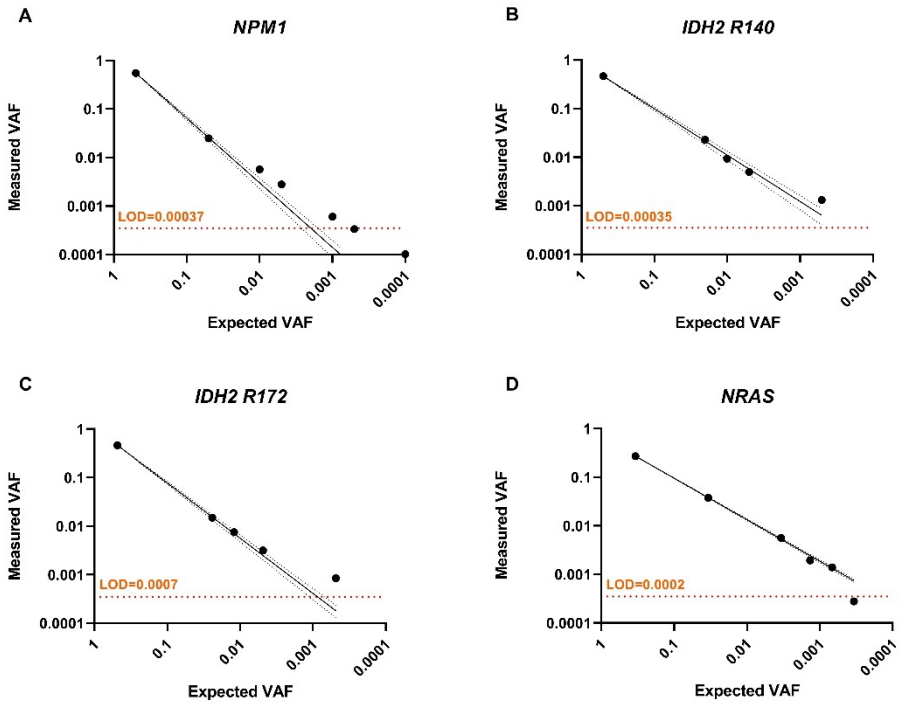

Figure 4: Figure adapted with permission from Rausch et al: "We generated dilution curves by spiking mutant DNA into wildtype DNA and diluting this sample in H_2O. The lowest detected VAFs above the previously specified LOD are 0.05% for NPM1 *(A), 0.048% for* IDH2 *p.R140 (B), 0.051% for* IDH2 *p.R172 (C) and 0.068% for* NRAS *(D). All results are shown on a logarithmic scale. The regression line (black lines) with its 95% confidence interval (dashed lines) and the limit of detection (red-dashed lines) are also shown. VAF: variant allele frequency; LOD: limit of detection by ratio."[1]*

We also wanted to ensure that VAFs detected by our assays were concordant to VAFs detected using NGS testing. To elucidate this, BM gDNA samples that harbored mutations at *NPM1* positions c.863 and c.877, *IDH2* codons p.R140 and p.R172 and *NRAS* codons p.G12 and p.G13 were measured using the NGS panel-sequencing method cited above and our own assays. In samples mutated in *NPM1* position c.863 (n=15), both methods detected the mutation at very similar VAFs (r2 = 0.95; 95% CI: 0.88-0.99; Fig. 5A). Samples carrying *NPM1*-mutations occurring at position c.877, which are rarer, correlated less well (types U, AB, AE, AF and AT; n=6, r^2 = 0.79, 95% CI: 0.69-0.89; Fig. 5A). Taking a closer look at this finding, we noticed that the VAF of bigger insertions (type AF, 12nt and type AE, 9nt) was often estimated lower by NGS than by our assays, possibly due to inefficient mapping and variant calling for these mutations. VAFs determined by DDO-assay and NGS-determined VAFs for *IDH2* mutations at c.140 (n=10, r^2 = 0.94, 95% CI: 0.88-0.99) and c.172 (n=11, r^2 = 0.94, 95% CI: 0.88-0.99; Figure 5B/C) were highly concordant. Similarly, eight samples carrying *NRAS* mutations also yielded highly correlated results using both NGS and our assays (r^2 = 0.86, 95% CI: 0.504-1.22, Figure 5D). Thus, VAFs as assessed by DDO-ddPCR were highly concordant to NGS-based measurements across all assays.

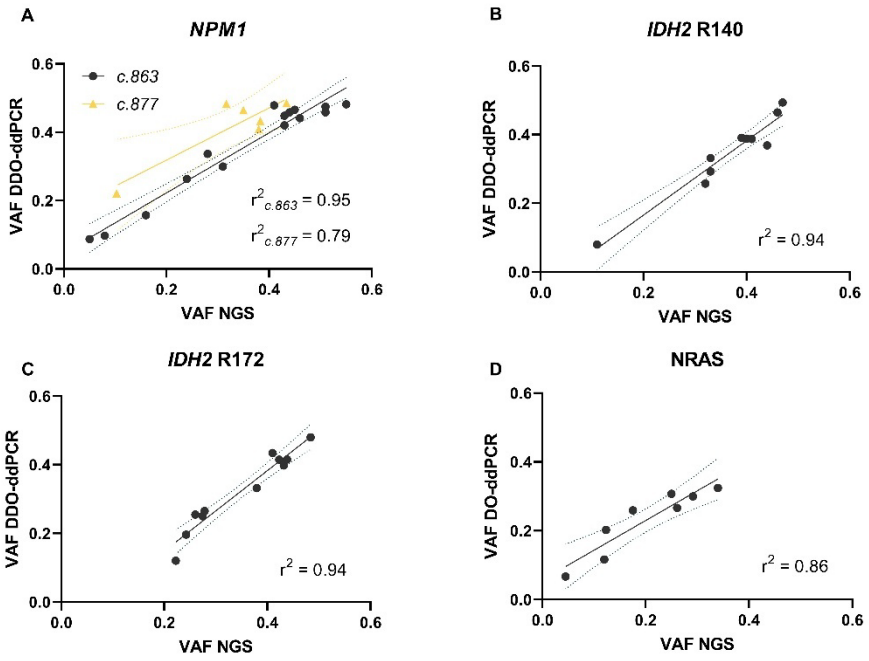

Figure 5: figure adapted with permission from Rausch et al: Comparison of VAFs detected by ddPCR (DO-assay for NRAS and DDO-assays for IDH2 and NPM1) or NGS panel sequencing. There is high concordance between both methods for all assays as determined by Pearsons r^2 (A: NPM1 *c.863, r^2=0.95,* NPM1 *c.877, r^2=0.79; B:* IDH2 *p.R140Q, r^2=0.94 C:* IDH2 *p.R172K, r^2=0.94; D:* NRAS*, r^2=0.86). Dashed lines represent 95% confidence interval. Abbreviations: VAF: variant allele frequency; DDO: double drop-off; ddPCR: digital droplet PCR; DO: drop-off; NGS: next generation sequencing.*[1]

6.2 Comparison of cfDNA analysis to routine RT-qPCR-based MRD monitoring

As we have laid out above, using DDO-ddPCR assays for MRD monitoring is intuitive. The use of ddPCR with its high signal-to-noise ratio allows MRD detection at very low allele frequencies. A less intuitive application however, is monitoring MRD not only in pB or BM specimens, but also in cfDNA. ddPCR is well suited to this due to its short amplicon sizes, which can handle the fragmentation of the cfDNA well. In an ideal scenario, this might even spare the patient serial BM examinations, as it might be hypothesized that cfDNA-based MRD monitoring might be more sensitive than pB-cell-based MRD monitoring, as cfDNA should represent cell death of any compartment and not just that of circulating cells. Thus, the results of a standard cDNA-based RT-qPCR test used on pB and BM specimens in routine patient care were compared to the results of our DDO-ddPCR assay for *NPM1*mut used on cfDNA, as we've previously published.[1]

Samples of nine *NPM1*mut patients were selected. Their characteristics are shown in table 9. In total, we gathered fifty-seven pB cfDNA samples at time points when BM and/or pB samples were also obtained. Results of DDO-ddPCR of pB-cfDNA was compared to qPCR results from pB and BM mononuclear cells (MNC).

Corresponding results of pB MNC DNA and cfDNA were available for 28 time points (Fig. 6). At most time points, cfDNA-ddPCR and pB MNC DNA qPCR were concordant. However, cfDNA was positive at two timepoints at which pB MNC did not present with a mutation. Unfortunately, a corresponding BM sample was only available for one of the two cases, where it carried the mutation also detected in cfDNA. Conversely, for three pairs of samples, pB qPCR was positive while cfDNA ddPCR was negative. In one case, no BM sample was available to weigh in on the discordant results. In the other two cases, the BM qPCR agreed with the DDO-ddPCR-measurement in one and the pB qPCR in the other case.

There were fifty-three sample pairs of cfDNA and BM MNC gDNA (Figure 6). At 40 time points (75%) cfDNA-ddPCR and BM gDNA qPCR yielded the same result. In a significant number of cases (n=13, 25%) of paired follow-up samples however, the *NPM1* mutation which was present at diagnosis remained measurable in BM but not in cfDNA.

All three possible measurements (qPCR on BM and pB cells and cfDNA-ddPCR) were available on 24 occasions. Here, cfDNA-ddPCR and pB cell-based qPCR were equally accurate in detecting positive samples, as 18 triplets were fully concordant while samples where the BM remained positive did not show as positive in pB and cfDNA in three cases while showing as positive in either cfDNA or pB in one case each. In one case, only the pB was positive (Figure 6). In summary, cfDNA based *NPM1*mut-MRD-measurements are less sensitive than BM gDNA qPCR-based measurements while performing at least as well if not better than pB-based qPCR.

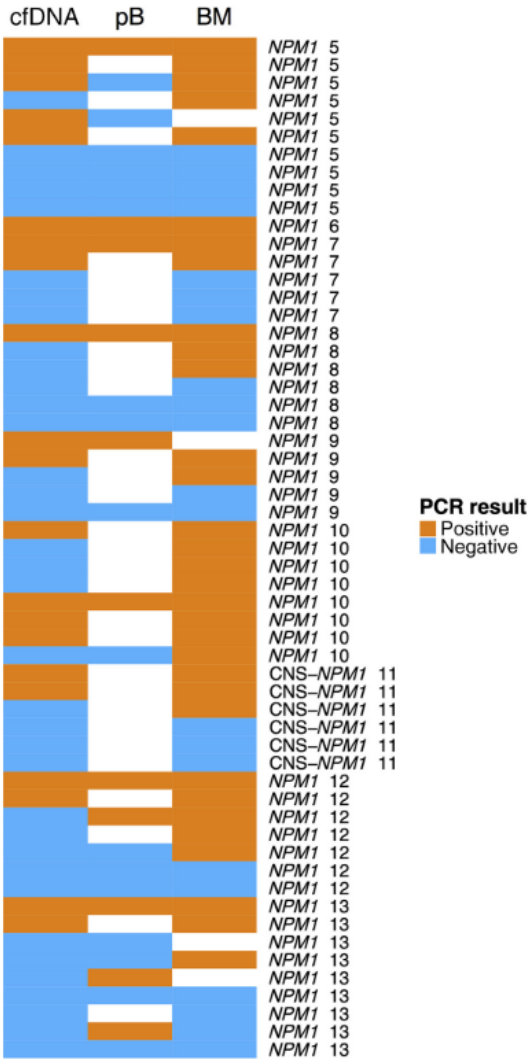

Figure 6: Figure reprinted with permission from Rausch et al.: Sensitivity of DDO-ddPCR for NPM1 *mutations is comparable to routine testing by RT-qPCR. Comparison of cfDNA-based DDO-ddPCR and pB-cell cDNA-based qPCR from 9 patients (see table 9) harboring an* NPM1 *mutation. Heatmap showing positive (orange) and negative (blue) results of cfDNA based DDO-ddPCR, pB-cell cDNA-based qPCR and BM-cell cDNA-based qPCR (gold standard). Matched samples were collected within ± 5 days. While qPCR from BM shows a higher sensitivity, leading to fewer false negative results, sensitivity of cfDNA-based DDO-ddPCR was equal to sensitivity achieved by qPCR from pB. Abbreviations: pB: peripheral blood; cDNA: complementary DNA; cfDNA: cell-free DNA; DDO-ddPCR: double drop-off digital droplet PCR; qPCR: quantitative PCR; BM: bone marrow.*[1]

6.3 Clinical use cases of cfDNA-based mutation detection in AML: Serial disease monitoring and characterization of extramedullary disease

Finally, we explored several scenarios in which cfDNA-based digital PCR analyses might add clinical value by performing exploratory case studies. All case studies have previously been published by us.[1] Clinical characteristics of all examined patients are listed in table 9.

ID	IDH2_1	NPM1_2	EMD_3	NPM1_5	NPM1_6	NPM1_7	NPM1_8	NPM1_9	NPM1_10	CNS-NPM1_11	NPM1_12	NPM1_13
Sex	female	female	male	female	male	female	female	female	male	male	female	male
Age	74	68	54	52	76	35	45	27	60	32	32	52
Diagnosis	relapsed sAML	newly diag-nosed tAML	myelosar-coma	newly diag-nosed AML	newly diag-nosed AML	newly diag-nosed AML	newly diag-nosed AML	newly diag-nosed AML	newly diag-nosed AML	newly diag-nosed AML	newly diag-nosed AML	newly diag-nosed AML
ELN 2017 risk group	adverse	favorable	adverse	favorable	adverse	favorable	intermediate	intermediate	favorable	intermediate	intermediate	favorable
Treatment	Enasidenib, AMG-673	HU, decita-bine	sHAM, radia-tion, alloSCT	7+3 + mido, 4x HiDAC + mido, mido	HU	2x 7+3, 2x HiDAC	HU, 2x 7+3 + mido, HiDAC + mido allo-SCT	HU, 7+3 + mido, HiDAC + mido, allo-SCT	7+3 + mido, HiDAC + mido	7+3 + mido, HiDAC + mido, allo-SCT	7+3 + mido, HiDAC + mido, allo-SCT	HU, 2x 7+3 + midostaurin, 4x HiDAC + midostaurin, midostaurin
Blast BM (%)	64	62	n/a	54	89	63	76	95	37	81	69	72
Blasts pB (%)	6	17	0	5	83	24	78	96	3	47	43	15
Leukocytes (G/l)	1.8	54.1	4.73	16.3	114	6.66	71.2	270	1.63	135	25.8	40.2
Known mutations	*ASXL1, IDH2*	*NPM1, IDH1, FLT3*-TKD	*IDH2, CBFB-MYH11, KIT*	*NPM1, FLT3*-ITD, *KRAS, DNMT3A*	*NPM1, FLT3*-ITD *DNMT3A, TET2*	*NPM1, NRAS*	*NPM1, FLT3*-ITD, *DNMT3A, ASXL1, SH2B3*	*NPM1, FLT3*-ITD, *TET2, ETV6, GATA2*	*NPM1, DNMT3A, IDH1, FLT3*-TKD	*NPM1, FLT3*-ITD, *DNMT3A, TET2*	*NPM1, IDH2, FLT3*-ITD	*NPM1, DNMT3A, FLT3*-ITD, *PTPN11*
Karyotype	46,XX,del(5)(q14q35)	46,XX, der(6)t(6;13)	45,XY,-8, inv(16)(p13.1q22), der(17)	46,XX	46,XY	46,XX,del(9)(q13q22)	46,XX	46,XX	47,XY,+13	46,XY	46,XX	46,XY

Table 9: Clinical characteristics of AML patients. alloSCT = Allogeneic hematopoietic stem cell transplant; HiDAC = high-dose Cytarabine; HU = Hydroxyurea; Mido = Midostaurin; sAML = secondary AML; tAML = therapy-related AML; Table adapted from Rausch et al.[1]

For our first scenario, we looked at a patient with IDH2 p.R172K mutated AML who was not fit enough to undergo intensive therapy, and who was therefore selected to be treated with the IDH2-inhibitor enasidenib. She could remain on this treatment for more than 400 days, the entirety of which was accompanied by cfDNA-based IDH2mut detection (table 9, patient ID: IDH2_1). After almost 120 days on treatment, the patient achieved a complete remission. During this initial phase, both the total cfDNA concentration and the IDH2 p.R172K VAF measured by ddPCR in pB cfDNA and in pB and BM MNC gDNA slowly decreased. On day 155, ddPCR failed to detect the mutation in pB MNCs while pB cfDNA remained positive. On day 168 the mutation was no longer detectable in either material. However, after 223 days of treatment the IDH2mut was detected in cfDNA once again, and total pB cfDNA concentration rose again. At this time pB MNC DNA remained negative for the mutation. On day 306, pB MNC DNA turned positive and the next BM-sample at day 330 was positive as well, thus confirming the molecular relapse first detected in cfDNA. After confirmation of a molecular relapse, the dose of the IDH-inhibitor was doubled, temporarily leading to a second decline in IDHmut-VAF and overall cfDNA-load. Unfortunately, the effect was temporary and once again an increase of IDH2 p.R172K VAF in pB cfDNA and BM indicated a relapse. On day 407, enasidenib was discontinued due to clinically apparent refractory disease (Fig. 7).

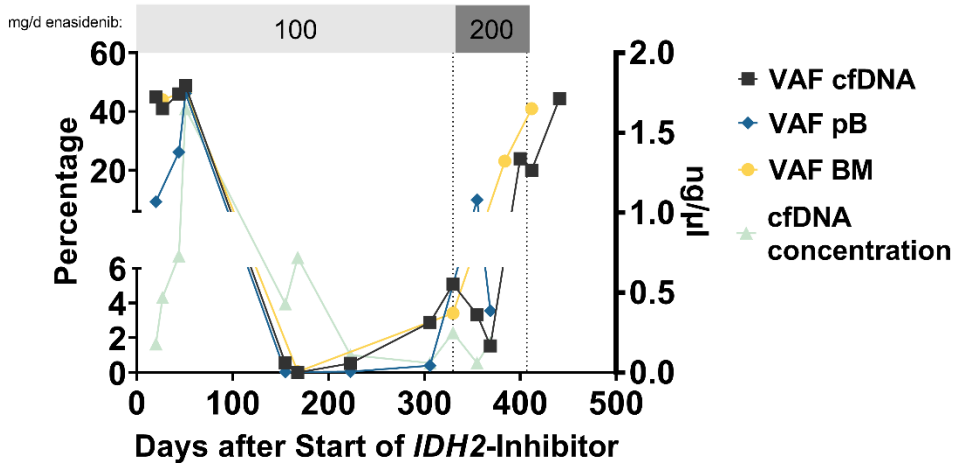

Figure 7: Figure reprinted with permission from Rausch et al.: "Use of ddPCR for serial disease monitoring during targeted therapy. Shown is the time course of one patient with an IDH2 *p.R172K mutation treated with an IDH2-inhibitor (enasidenib), from the beginning of targeted therapy until discontinuation due to refractory disease. We measured the absolute amount of cfDNA (green line), VAF of* IDH2 *p.R172K in BM (yellow line) and pB MNC (blue line), and in cfDNA from pB (black line). At day 324 (first dashed line), relapse was diagnosed morphologically, and the dose of enasidenib was doubled. At day 407 (second dashed line) the drug was discontinued due to refractory disease. Abbreviations: VAF: variant allele frequency; BM: bone marrow; pB: peripheral blood; LOD: limit of detection; MNC: mononuclear cells."*[1]

A second scenario where cfDNA-based sampling could prove useful due to the possibility of frequent sensitive VAF-measurements is early response assessment of patients starting a new line of treatment. To investigate this, we collected serial cfDNA-samples of patients carrying *NPM1*mut who were undergoing intensive induction chemotherapy (Figure 8). Firstly, we

measured total cfDNA concentration in these samples. Total cfDNA concentration seemed to correlate well with leukocyte production, but not necessarily with leukemic activity as it did not only rise during relapse, but also in patients whose WBC counts increased for other reasons, such as in patient NPM1_9 (Fig. 8C) where the steep rise in cfDNA concentration was due to fungal infection.

NPM1^{mut} cfDNA remained detectable during aplasia when WBC counts were low, while *NPM1*^{mut} VAF showed good correlation with BM blast count. However, *NPM1*mut-VAF detected in cfDNA correlated tightly with BM myeloblast counts, allowing us to differentiate between ctDNA and non-tumor-related cfDNA. To further underscore the reliability of our DDO-Assay we compared its measurements to a commercially available mutation-specific ddPCR assay for *IDH1* R132H in a patient carrying both mutations (Figure 8D). We observed highly similar VAF-measurements using both our assay and the commercial assay. The fact that the VAF of a *DNMT3A* mutation in the same patient did not follow the same decrease probably indicates persistence of preceding clonal hematopoiesis and can thus not be used for comparison.

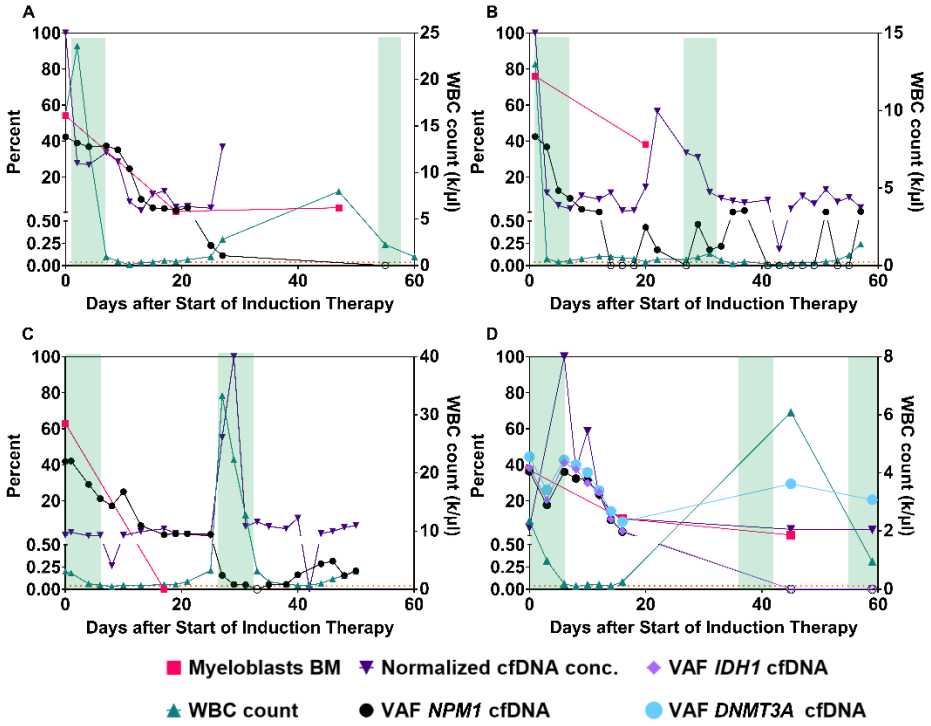

Figure 8: Figure reprinted with permission from Rausch et al. "Disease monitoring during intensive therapy. Four representative patients with an NPM1 *mutation who received intensive induction chemotherapy are shown. The VAF is shown for* NPM1 *(black line),* DNMT3A *(blue line) and* IDH1 *(light purple line). Additionally, the normalized cfDNA concentration (dark purple line), the myeloblast count in the BM (magenta line) and the WBC count (green line) are shown for each patient. Green backgrounds represent administration of therapy. The limit of detection is represented by an orange line. Open symbols indicate values below the limit of detection. BM: bone marrow; VAF: variant allele frequency; WBC: white blood cell."*[1]

Usually, BM aspiration is the gold standard diagnostic for AML. However, in some cases AML manifests outside the bone marrow (i.e. EMD). Particularly when bone marrow involvement is minimal or absent (i.e. isolated myelosarcoma), this makes AML diagnostics significantly more challenging, as EMD-tissue might not be as readily accessible. This led us to test whether pB

cfDNA analysis represents the genetic profile of EMD. For a patient with isolated myelosarcoma (table 9, patient ID: EMD_3), we analyzed an EMD biopsy (obtained from a paraaortic myelosarcoma), BM MNC DNA, and pB cfDNA (Figure 9A).

Panel sequencing of the EMD biopsy detected an *IDH2* p.R140Q mutation. Using the same sequencing assay, this mutation was not detected in a BM sample. However, our IDH2 DDO-ddPCR assay identified it in pB cfDNA. The measured VAF was similar to the NGS result in the EMD sample.

A frequent form of EMD is CNS-involvement of AML, which is also called meningeosis leukaemica. To figure out how cfDNA extracted from the cerebrospinal fluid (CSF) represented the mutational profile of intracranial AML, we studied serial paired blood cfDNA and CSF cfDNA samples of a patient with *NPM1*^{mut} AML (table 9, patient ID: CNS-NPM1_11). During his induction treatment, the suspicion of CSF involvement had arisen. A lumbar puncture was undertaken on day 9 of induction therapy, and intrathecal therapy was initiated. Cytology of the CSF samples revealed no leukemic cells. However, the known *NPM1*mut was repeatedly detected in CSF cfDNA. Initially, the VAF of the mutation was considerably higher in CSF cfDNA than in blood cfDNA samples, but over the course of the intrathecal therapy *NPM1*-VAFs in the CSF declined to the levels measured in pB. On d21, CSF cfDNA was negative for the *NPM1*mut and the patient's symptoms had resolved (Figure 9B).

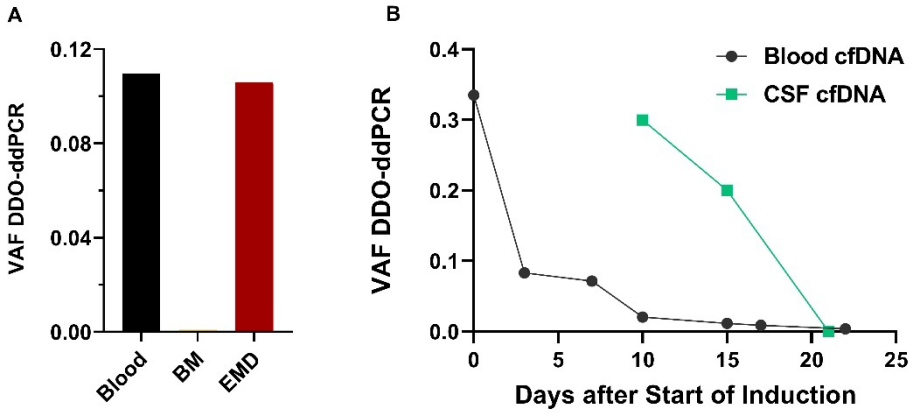

Figure 9: Figure adapted with permission from Rausch et al.: Detection of AML-associated gene mutations in patients with extramedullary disease. (A) In a patient with myelosarcoma, an IDH2 *p.R140Q Mutation was detected in the gDNA from an EMD biopsy specimen as well as by "liquid biopsy" using pB-derived cfDNA. Both compartments showed equal VAFs, while the mutation was not detected in a BM biopsy. Panel (B) shows the treatment course of one patient with* NPM1^{mut} *(Type A) AML who had CSF involvement. He received serial applications of intrathecal chemotherapy, and we serially studied cfDNA in the CSF. In the pB, the* NPM1 *VAF (black line) declined after induction therapy, while* NPM1 *VAF in the CSF (green line) decreased only after intrathecal chemotherapy. Abbreviations: BM: bone marrow; CNS: central nervous system; CSF: cerebrospinal fluid; EMD: extramedullary disease; pB: peripheral blood; VAF: variant allele frequency.*[1] *DDO-ddPCR: double drop-off digital droplet PCR.*

7. Discussion

The increasing importance of genetic alterations as MRD markers and therapeutic targets increases the relevance of molecular genetic assays that can diagnose them cheaply, quickly and at a high sensitivity. Here, we describe innovative DDO-ddPCR assays that can be a valuable addition to established methods.

One main advantage of the new assay design is versatility, as standard ddPCR assays require a fluorescent DNA reporter probe for any one alteration occurring at a mutational hotspot in the genomic sequence. For hotshots at which many different mutations occur, this would require establishing one assay for each one of these mutations, which is not resource efficient.[50]

Drop-off ddPCR assays address this problem. Such assays have previously been developed to detect mutations in *KRAS* and *BRAF*.[60,61] Within any hotspot short enough to be covered by one oligonucleotide reporter probe, they can theoretically detect most possible mutations. Here, we expand on this principle by developing the 'double drop-off' assay. By giving both fluorescent probes the role of reference and reporter at the same time, various mutations occurring at two hotspots that are close enough to lay within the same PCR amplicon can be covered using only one assay.

We demonstrated that assays developed using this design come close to matching the sensitivity and specificity commercially available ddPCR assays can reach and have performance characteristics close to the technical limitations of the method (Droplet Digital PCR Applications Guide, Bio-Rad). When validating the assay against NGS-measurements, detected mutations were fully concordant between both methods and VAFs measured with either method correlated tightly. In addition, others have proven the feasibility of the double drop-off design by reporting a DDO-ddPCR assay for the detection of *KIT* exon 11 mutations in gastrointestinal stromal tumors.[62] Taken together, these results allow as to conclude that DDO-ddPCR assays are a reliable, versatile tool for targeted molecular genetic analyses in AML as well as other neoplasias.

Since ddPCR assays can detect any genomic DNA molecule, they can be utilized to perform circulating cell-free DNA based liquid biopsies. While the value of liquid biopsies in solid tumors is well established, the value of cfDNA based profiling of hematologic malignancies as diverse as MDS, multiple myeloma and DLBCL has only recently been proven.[56,59,71] In AML however, prospective data on the utility of cfDNA-based analyses is limited to one analysis in the alloSCT context.[40]

While it might seem counterintuitive to use a 'liquid biopsy' to characterize a 'liquid tumor' such as AML, where neoplastic cells circulate in the bloodstream or are easily accessible via BM aspirate, the method has distinct advantages. One aspect pertains to sensitivity. Analyses comparing pB- and BM-based MRD monitoring have consistently shown that the sensitivity of BM-based tests is approximately one order of magnitude greater.[41] This fact explains why repeat BM sampling is still recommended to monitor MRD in AML. However, the procedure is not without risks, and causes relevant discomfort to patients. Thus, the interval between MRD-measurements is defined by the tradeoff between one less sensitive and one more invasive method of sampling. As the ultimate goal of MRD-monitoring is to detect molecular relapse before it develops into clinically apparent relapse, frequent monitoring remains key. It would therefore be ideal to have a method that combines higher sensitivity and lower invasiveness. One possible avenue to achieving this goal is the utilization of cfDNA. As cfDNA is a byproduct of - mostly necrotic - cell death, all dying tumor cells release it into the bloodstream, allowing the detection of AML-associated mutations even when no leukemic cells are circulating in the bloodstream, as would be the case

for a patient in CR. This was confirmed by the fact that we were able to detect *NPM1*mut in profoundly leukopenic patients undergoing induction chemotherapy, when most of the detected mutant cfDNA will have come out of the BM. Comparing our method to established assays, we show that DDOddPCR-based cfDNA testing is at least as sensitive as pB MNC-DNA based testing. Some of our results even point towards a potentially higher sensitivity. Thus, the addition of cfDNA-based MRD detection may complement existing MRD-diagnostics and potentially even reduce the needed frequency of BM aspirates.

Taken together, the experiments in this work elucidate a promising new set of tools for MRD-monitoring in AML. However, this dissertation has several limitations, which will be addressed in the following.

Firstly, there are some considerations regarding the assay design. The assays evaluated in this work use gDNA as their input. While this is advantageous because it allows for the analysis of cfDNA, in the context of *NPM1* specifically, it also comes at a tradeoff. When not analyzing cfDNA, but cellular samples, using cDNA instead of gDNA would potentially achieve even higher sensitivity, as *NPM1*-RNA-transcripts seem to be more abundant than DNA copies of *NPM1*.[72] For situations in which disease-monitoring is not performed as liquid biopsy it is therefore advisable to redesign the assay to also be usable with cDNA. We acknowledge that this will be difficult, as the *NPM1*-RNA is quite similar to that of *CLEC2D*. Furthermore, while this assay is more versatile than commercially available assays, it is still not able to detect all known alterations in the targeted hotspots, as some mutations (designated DD9-DD13 by Thiede et al[69]) are too far to the 5'-end of the gene to be covered by our probes. A redesigned assay should aim to also cover these bases.

Besides the assay design, the analysis of the assay results could also be improved. Gating of positive droplets has been performed manually, and at the discretion of the experimenter. A more objective evaluation could have been achieved by using a computer-assisted gating strategy. However, at the time of this work we were unaware of any tool that could reliably perform automated gating for our DDO-ddPCR assays, as the droplet distribution differs from what is expected for a standard ddPCR assay. Another possible way to address this limitation would have been blinded gating of ddPCR results by another person. However, we did not implicate such a strategy to ensure speedy analysis of the results to allow for the rapid planning of the next experiment. With improved computational assistance or improved manpower for blinded evaluations, further experiments could reduce bias in droplet analysis.

In addition, our assay validation could have been augmented. While the *NPM1*-assay presented here has been compared to RT-qPCR, which is broadly accepted as the gold-standard for MRD-monitoring in *NPM1*-positive AML, no reference assays exist for *IDH2* or *NRAS*. A comparison to other existing assays such as commercially available conventional ddPCR-assays for the most common *IDH2* hotspot-mutations might nevertheless have been another piece of supporting evidence for the utility of our assays. Besides the methodical validation, the clinical validation also needs further work. The case studies performed here give some anecdotal evidence to the utility of cfDNA-based MRD monitoring in AML, but a structured, prospective evaluation which ideally would include blinded analysis of ddPCR results is lacking so far.

These limitations notwithstanding, this work still adds an important contribution to the field. Some strengths of this contribution will be emphasized again here.

One crucial advantage our assay affords is its flexibility. Unlike the conventional ddPCR assay design, our assays can cheaply and quickly detect a host of common alterations in AML-

associated hotspots. As they are gDNA-based they allow for use in a host of different settings, using pB MNC DNA, BM MNC DNA or cfDNA as analyte, allowing for disease monitoring regardless of which patient sample is available.

Especially the use of cfDNA is not widely established yet but might grant some crucial advantages. On the one hand, there is an increasing number of therapeutic regimens which is given over long periods of time. Especially small-molecule inhibitors are usually given until progression, and - as their mechanism of action depends on a particular genetic lesion – are particularly amenable to molecular genetic disease monitoring techniques. Both to detect molecular relapse early and to keep a close eye on those who achieve disease control but not complete remission using these therapies, tight monitoring of therapeutic success is needed. Understandably, many patients prefer if said monitoring does not require frequent BM aspiration. Thus, the combination of high sensitivity and low invasiveness that pB-based cfDNA offers may be particularly valuable to patients on such continuous therapies. On the other hand, cfDNA based assays might aid management of isolated myelosarcoma. These manifestations of AML are not always readily accessible. In this case, cfDNA-based assays may allow the detection of therapeutic targets and monitoring of therapy response. This work has provided useful proof of concept for using cfDNA in these scenarios.

Besides the flexibility, another strength of this work is the meticulous optimization that the assays have gone through. Primers and probes have first been designed in silico based on guidance by the manufacturer of the ddPCR system and pre-existing literature. Then, primers were evaluated for specificity using conventional PCR. Subsequently, the ddPCR assays were tested at a host of different temperatures, with several different primer/probe concentrations, several restriction enzymes and various PCR cycling conditions. Suboptimal primers or probes were redesigned and underwent this process again. This has led to considerable improvements in sensitivity and specificity, despite the challenges posed by the fact that using ddPCR for the purpose of DDO-assays is a novel concept for which little support by the manufacturer or others was available to avoid a trial-and-error approach. This process has the added advantage that considerable expertise in ddPCR assay design has been developed within our laboratory, making future improvements of the assay as well as the future addition of other assays more convenient. Some of these next steps seem evident given the discussion of strengths and weaknesses above.

To improve the sensitivity of the *NPM1*-assay, a redesign of the assay using cDNA is currently underway. This assay will, of course, need to undergo the same analytical validation as our previous assay designs. Ideally, this validation would then be aided by blinded assay analysis, as this approach could lead to a more objective evaluation of assay performance. After completion of this step, a retrospective clinical validation of the assay could follow, where – analogous to the results we present here – the performance of our assay could be compared to the performance of the gold-standard RT-qPCR. Going beyond the present work, a larger cohort could not only give more clarity on the relative performance of our assay compared to the benchmark but could also elucidate whether MRD-monitoring using DDO-ddPCR identifies patient subsets with clinically meaningful distinct outcomes. Subsequently, a prospectively enrolled patient cohort could confirm that MRD-positivity as measured using our *NPM1*-DDO-ddPCR assay does indeed predict inferior outcomes.

The same clinical validation should also be undertaken for our other assays, which detect markers that are not as well established as meaningful markers of MRD as NPM1 is. As has been shown, some AML-associated mutations are more likely to persist during remission, and might only indicate the continued presence of a preleukemic clone, and not necessarily MRD.[73]

Correspondingly, a recent analysis found no predictive value in persistent detection of *IDH1*-mutations in AML prior to alloSCT.[74] Analyzing our *NRAS*- and *IDH2*-assays for clinical utility would therefore not only answer the question of whether they can sensitively detect their target throughout the disease course, but also how relevant the continued presence of that target is for patient outcomes. We hypothesize that some prognostic value can at least be derived from the dynamics of the allelic burden of a mutation, as an increasing VAF of *IDH2* predicted relapse in the case study we show above.

Going beyond the improvement of existing assays, the application of this assay design to other relevant myeloid malignancy associated hotspots as well as to other entities is a promising future avenue of research. Besides *IDH2*, a drop-off assay for *IDH1* would also be advantageous, as almost all mutations occur in one hotspot (codon R132) which, besides the most common mutation which a conventional ddPCR assay would also detect (R132H) also harbors multiple other variants that would either require separate assays or a drop-off design for detection. As is mentioned above this would be particularly relevant as a screening tool, as quick detection of *IDH*-variants would allow the use of established inhibitors. Having these two assays ready would also be relevant in the context of other entities, as *IDH*-mutations also play an important role in cholangiocellular carcinomas and gliomas. The proven applicability of our assay design in the liquid-biopsy setting would be particularly useful when branching out to solid tumors, as it is often challenging to acquire tumor tissue in these cancers.

cfDNA-based liquid biopsies have also shown promise as markers of MRD in other hematologic malignancies treated with curative intent. One analysis found cfDNA closely associated with tumor burden as well as relapse and survival in primary CNS lymphoma.[75] However, this analysis uses a cfDNA sequencing panel, which is methodologically more challenging than ddPCR, illuminating another potential future use-case for ddPCR-based cfDNA detection. A similar case can be made for other B-cellular malignancies, which are often curatively treated and where precise markers of therapeutic success might help stratify relapse risk after therapy.[76]

In conclusion, we developed of a novel, versatile digital PCR assay design, double drop-off (DDO) ddPCR, which proved useful for mutation detection and MRD monitoring of AML. Our assays have several distinct advantages: They can be used to rapidly screen for relevant prognostic markers (e.g. *NPM1*mut and therapeutic targets (e.g. IDH*mut*) due to their speedy turn-around time. Especially due to the increasing importance of IDH-inhibitors in earlier lines of therapy, this application is highly useful. In addition, they are much cheaper than NGS, potentially allowing their use in more resource-deprived health systems. Furthermore, the assays can also be utilized in MRD-monitoring, as they achieve sensitivities close to the technical limit of the highly sensitive ddPCR method. Finally, we have proven that DDO-ddPCR based monitoring of AML-associated mutations in cfDNA is feasible, which opens a new avenue for therapeutic monitoring of EMD. While we also proved feasibility of serial cfDNA-based VAF-measurements of AML-associated mutations in patients undergoing therapy, the prognostic value of these data needs to be clarified in larger cohorts and subsequently in a prospective study. Evaluation of the utility of this assay design in other disease settings might be an additional promising avenue of research.

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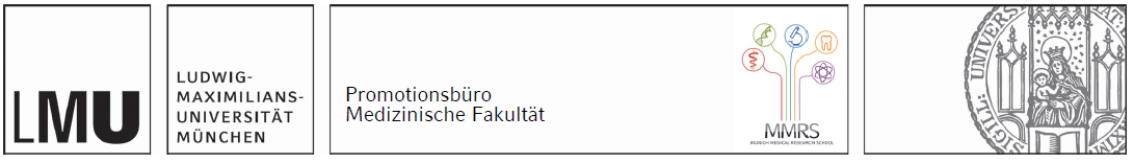
10. Affidavit



Eidesstattliche Versicherung

Rausch, Christian Georg
Name, Vorname

Ich erkläre hiermit an Eides statt, dass ich die vorliegende Dissertation mit dem Titel:

“Double drop off- droplet digital PCR (DDO-ddPCR): a novel, versatile tool for mutation screening and residual disease monitoring in AML using cellular or cell-free DNA.”

selbständig verfasst, mich außer der angegebenen keiner weiteren Hilfsmittel bedient und alle Erkenntnisse, die aus dem Schrifttum ganz oder annähernd übernommen sind, als solche kenntlich gemacht und nach ihrer Herkunft unter Bezeichnung der Fundstelle einzeln nachgewiesen habe.

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