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Multiplexed quantification of four neuroblastoma DNA targets in a single droplet digital PCR reaction

Constantin Peitz$^{1,10}$, Annika Sprüssel$^{1,10}$, Rasmus B. Linke$^{1,10}$, Kathy Astrahantseff$^4$, Maddalena Grimaldi$^{1,10}$, Karin Schmelz$^{1,3,4}$, Joern Toedling$^1$, Johannes H. Schulte$^{1,2,3,4}$, Matthias Fischer$^{5,6}$, Clemens Messerschmidt$^{7,8}$, Dieter Beule$^7$, Ulrich Keilholz$^9$, Angelika Eggert$^{1,2,3,4}$, Hedwig E. Deubzer$^{1,2,3,4,10^*}$ and Marco Lodrini$^{1,10^*}$

$^1$ Department of Pediatric Hematology and Oncology, Charité - Universitätsmedizin Berlin, Augustenburger Platz 1, 13353 Berlin, Germany
$^2$ Berliner Institut für Gesundheitsforschung (BIH), Anna-Louisa-Karsch-Straße 2, 10178 Berlin, Germany
$^3$ German Cancer Consortium (DKTK), partner site Berlin, Berlin, Germany
$^4$ German Cancer Research Center (DKFZ), Heidelberg, Germany
$^5$ Department of Experimental Pediatric Oncology, University Children's Hospital of Cologne, Kerpener Str. 62, 50937 Cologne, Germany
$^6$ Center for Molecular Medicine Cologne (CMMC), Robert-Koch-Str. 21, 50931 Cologne, Germany
$^7$ Core Unit Bioinformatics – CUBI, Berlin Institute of Health / Max-Delbrück Center for Molecular Medicine in the Helmholtz Association / Charité – Universitätsmedizin Berlin, Charitéplatz 1, 10117 Berlin, Germany
$^8$ Department of Computer Science, Humboldt-Universität zu Berlin, Unter den Linden 6, 10099 Berlin, Germany
$^9$ Charité Comprehensive Cancer Center, Virchowweg 23, 10117 Berlin, Germany
$^{10}$ Neuroblastoma Research Group, Experimental and Clinical Research Center (ECRC) of the Charité and the Max-Delbrück-Center for Molecular Medicine (MDC) in the Helmholtz Association, Lindenberger Weg 80, 13125 Berlin, Germany

* These authors contributed equally to this work.

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Corresponding author
PD Dr. Hedwig E. Deubzer, MD
Charité – Universitätsmedizin Berlin
Department of Pediatric Hematology and Oncology
Augustenburger Platz 1
13353 Berlin, Germany
E-mail: hedwig.deubzer@charite.de
ABSTRACT

Detection and characterization of cell-free DNA (cfDNA) in peripheral blood from neuroblastoma patients may serve as a minimally invasive liquid biopsy approach. Major challenges of cfDNA analysis purified from blood samples are small sample volumes and low cfDNA concentrations. Droplet digital PCR (ddPCR) is a suitable technology to analyze low levels of cfDNA. We here report two quadruplexed ddPCR assay protocols that (i) reliably quantify \textit{MYCN} and \textit{ALK} copy numbers in a single reaction together with the two reference genes, \textit{NAGK} and \textit{AFF3}, and (ii) accurately estimate \textit{ALK}^{F1174L} (3522, C>A) and \textit{ALK}^{R1275Q} (3824, G>A) mutant allele fractions using cfDNA as input. We optimized separation of positive and negative droplets to detect two targets in each ddPCR fluorescence channel by adjusting probe and primer concentrations for each target molecule. The quadruplexed assays were validated using a panel of 10 neuroblastoma cell lines and paired blood plasma and primary neuroblastoma samples from nine patients. Accuracy and sensitivity thresholds in quadruplexed assays corresponded well with those for the respective duplexed assays. We present two robust quadruplexed ddPCR protocols applicable in the routine clinical setting to assess \textit{MYCN} and \textit{ALK} oncogene status that require only minimal plasma volumes.
INTRODUCTION

Neuroblastoma, a neuroectodermally derived embryonic tumor and most common extracranial tumor of childhood, accounts for 11% of cancer-related deaths in children, mostly due to systemic and resistant relapses. It is characterized by a heterogeneous tumor biology and, hence, clinical variability ranging from spontaneous regression or localized, stable disease to rapid metastasizing progression with fatal outcome. The basic helix-loop-helix transcription factor, MYCN, regulates migration, proliferation, and differentiation of neural crest progenitor cells. MYCN amplification occurs in approximately 25% of primary human neuroblastomas to increase the rate of DNA synthesis, promote cell cycle progression and suppress differentiation. MYCN amplification is a strong predictive biomarker for unfavorable patient survival, and indirect approaches to target binding partners or downstream effectors of MYCN are yielding encouraging results. Recent data suggests MYCN amplification can exist at the (sub)clonal level, necessitating biosampling procedures and technologies capable of detecting these cell populations. Activating mutations in the anaplastic lymphoma kinase (ALK) gene, occur in ~10% of neuroblastomas, the most frequent causing the F1174L and R1275Q substitutes in the receptor tyrosine kinase domain. The resulting proteins are auto-hyperphosphorylated and cause uncontrolled proliferation. ALK-driven neuroblastomas, often relapses which may have expanded from a single ALK mutant clone, are frequently resistant to chemo- and radiotherapy. Activating ALK mutations or amplifications have become the first target in neuroblastomas that is directly druggable by small molecule inhibitors as a personalized medicine approach, necessitating continuous molecular monitoring in patients with neuroblastoma for potential (re)emergence of ALK mutant or amplified clones. Clinical testing for ALK variants is performed using a broad spectrum of methodologies including next-generation sequencing technologies, targeted panel sequencing or droplet digital PCR (ddPCR). Gold standards for ALK diagnostics in routine clinical care are expected to evolve within the framework of clinical trials and are likely to be...
composed of two complementary untargeted and targeted technologies, applied at least initially in partially overlapping analyses for longitudinal patient monitoring to keep costs affordable.

The invasive nature of surgical biopsies most often prevents their sequential application to monitor disease. Single biopsies also fail to reflect cancer dynamics, intratumor heterogeneity and drug sensitivities that most likely change during clonal evolution and under the selective pressure of therapy. Peripheral blood has several components that have been assessed for tumor-derived nucleic acid content.\textsuperscript{17-19} Thus, longitudinal patient monitoring using liquid biopsies may represent a promising strategy for patient care although we should be careful of generalizing from the limited data available at this time.

Droplet digital PCR (ddPCR) is a highly sensitive recently developed technology to quantify specific regions.\textsuperscript{20, 21} The ddPCR reaction reagents are partitioned into 20,000 droplets before allowing reactions to proceed to the end plateau in individual droplets, then droplets are assessed as positive or negative from their fluorescence signal intensity. We previously established duplex ddPCR protocols detecting $\textit{MYCN}$ and $\textit{ALK}$ copy number status in cell-free DNA (cfDNA) purified from patient plasma samples.\textsuperscript{22} Combaret and colleagues have presented ddPCR protocols to detect $\textit{ALK}^{F1174L}$ and $\textit{ALK}^{R1275Q}$ hotspot mutations, each together with the respective wildtype sequence in duplex reactions.\textsuperscript{23} Here, we aimed to minimize the blood sample volumes sequentially required from infants and young children, the patients most often affected by neuroblastoma, by extending multiplexing to create robust quadruplexed ddPCR protocols.

\section*{MATERIALS AND METHODS}

\subsection*{Patient samples}

Paired blood plasma and fresh-frozen primary tumor samples were collected from patients treated at the Charité or provided from the German Neuroblastoma Biobank (Cologne).
patients were registered within the German NB2004 Trial or NB 2016 Registry, and informed patient/parent consent was obtained during trial participation. Peripheral blood was centrifuged at 1,900 x g for 7min at the Charité and at 1,000 x g for 10min in the German Neuroblastoma Biobank to separate plasma. After centrifugation at 3,250 x g for 10 min to remove cell debris, plasma was stored at -80°C. The ALK copy number in tumor samples was determined as a routine diagnostic method using FISH.

Cell culture

The BE(2)-C cell line was obtained from the ECACC (Salisbury, UK) and the Kelly and SH-SY5Y cell lines from the DSMZ (Braunschweig, Germany). CLB-GA (established in the Centre Léon BERARD (CLB), Lyon, France), IMR-5, LAN-5 and LAN-6 were kindly provided by J. Schulte (Charité, Berlin, Germany), NB-1 by I. Oehme (DKFZ, Heidelberg, Germany), and SH-EP and SK-N-AS by L. Savelyeva (DKFZ). Cell lines were authenticated by high-throughput SNP-based assays. Genomic cell line characteristics are summarized in Supplemental Table S1. BE(2)-C, CLB-GA, SH-SY5Y and SK-N-AS cell lines were maintained in DMEM (Lonza, Cologne, Germany) supplemented with 10% fetal calf serum and 1% non-essential amino acids. The LAN-6 cell line was cultured in DMEM supplemented with 20% fetal calf serum. IMR-5, Kelly, LAN-5, NB-1 and SH-EP cell lines were cultured in RPMI medium (Lonza) supplemented with 10% fetal calf serum and 1% non-essential amino acids. All cell lines were maintained at 37°C and 5% CO2, and continuous culture was avoided to maintain low passage numbers and reduce the risk of long-term culture-induced genomic alterations. Cells for experiments were grown in short-term culture from low-passage stock aliquots maintained in liquid nitrogen. All cell lines were regularly monitored for Acholeplasma laidlawii, mycoplasma species and squirrel monkey retrovirus infections using high-throughput, multiplexed testing.
Genomic and cell-free DNA preparation

Genomic DNA was extracted from tumor tissues and cell lines using the Qiagen Puregene Core kit A (Qiagen) or the QIAamp DNA Mini kit (Qiagen) according to the manufacturer’s instructions. Genomic DNA from cell lines was fragmented by sonication before ddPCR. Tumor DNA could not be sonicated because of the small sample volumes (20µl), and fragmentation was achieved by adding 5U of HindIII restriction enzyme (New England Biolabs, Frankfurt/Main, Germany) to each ddPCR reaction. Thawed plasma samples were centrifuged at 2000 × g for 5min to clear debris, then supernatants were centrifuged at 20,000 × g for 5min. Cell-free DNA was purified from a minimum of 200µl stored plasma samples using the QIAamp Circulating Nucleic Acid kit (Qiagen), then concentrated to 50µl using the DNA Clean and Concentrator-5 kit (Zymo Research, Freiburg, Germany), both according to the manufacturers’ directions. Cell-free DNA did not require fragmentation prior to ddPCR. Extracted DNA samples were quantified on a Qubit 2.0 fluorometer (Life Technologies, Darmstadt, Germany). The Cell-free DNA ScreenTape assay (Agilent, Santa Clara, CA) and the Agilent 4200 TapeStation System were used for DNA quality sample control according to the manufacturer’s instructions.

Droplet digital PCR

The QX200 Droplet Digital PCR System (Bio-Rad Laboratories, Munich, Germany) was used to analyze MYCN (2p24.3), ALK (2p23.2-2p23.1), NAGK (2p13.3) and AFF3 (2q11.2) copy number status and detect ALK F1174L (3522, C>A) and R1275Q (3824, G>A) hotspot mutations with their corresponding wildtype sequences. TaqMan ddPCR reaction mixtures contained the 2x ddPCR Supermix for Probes (no dUTP) (Bio-Rad Laboratories) and optimized primer and probe concentrations (see Table 1 for CNVs and Table 2 for ALK hotspot mutations) in a total volume of 20 µL. Primer3 software was used for primer and probe design. To confirm specificity of the probes used for the detection of $ALK^{F1174L}$ (3522,
C>A) and ALK<sup>R1275Q</sup> (3824, G>A), four double-stranded synthetic ALK templates with the following sequences were generated (Metabion, Planegg, Germany): ALK<sup>F1174L</sup> (3522, C>A, TTC>TTA), 5’-GCC CAG ACT CAG CTC AGT TAA TTT TGG TTA CAT CCC TCT CTG CTC TGC AGC AAA TTA AAC CAC CAG AAC ATT GTT CGC TGC ATT GGG G-3’; ALK<sup>F1174L</sup> (3522, C>G, TTC>TTG), 5’-GCC CAG ACT CAG CTC AGT TAA TTT TGG TTA CAT CCC TCT CTG CTC TGC AGC AAA TTA AAC CAC CAG AAC ATT GTT CGC TGC ATT GGG G-3’; ALK<sup>R1275Q</sup> (3824, G>A, CGA>CAA), 5’-GTC CAG GCC CTG GAA GAG TGG CCA AGA TTG GAG ACT TCG GGA TGG CCC AAG ACA TCT ACA GGT GAG TAA AGA CTG CCT CAC CCC-3’; and ALK<sup>R1275L</sup> (3824, G>T, CGA>CTA), 5’-GTC CAG GCC CTG GAA GAG TGG CCA AGA TTG GAG ACT TCG GGA TGG CCC AAG ACA TCT ACA GGT GAG TAA AGA CTG CCT CAC CCC-3’. The probe established for the detection of ALK<sup>F1174L</sup> (3522, C>A) reliably detected this but not the ALK<sup>F1174L</sup> (3522, C>G) mutation. The probe established for the detection of ALK<sup>R1275Q</sup> (3824, G>A) only detected this but not the ALK<sup>R1275L</sup> (3824, G>T) mutation (Supplemental Figure S1), altogether confirming specificity of the probes selected. Reaction mixtures were loaded into Bio-Rad droplet generator cartridges together with 70 µL Droplet Generation Oil (Bio-Rad). Droplets were generated in the QX200 Droplet generator, and manually transferred into a 96-well PCR plate (Eppendorf, Hamburg, Germany) according to manufacturer’s recommendations. The PCR plate was heat-sealed with the PX1 Plate Sealer (Bio-Rad), and PCR reactions were performed on a T100 Thermo Cycler (Bio-Rad) with the following programs for (i) CNV: denaturation at 95°C for 10min, 40 cycles of 30sec at 94°C and 1min at 58°C and final denaturation for 10min 98°C and (ii) ALK hotspot mutation analysis: denaturation at 95°C for 10min, 40 cycles of 30sec at 94°C and 1min at 62.5°C and final denaturation for 10min 98°C. Droplet reaction endpoints were assessed in the QX200 ddPCR Droplet Reader. Target gene copy number and mutant allele fraction were analyzed using the QuantaSoft Analysis software, version 1.7.4.0917 and the QuantaSoft
Analysis Pro software, version 1.0.596 (Bio-Rad). All multiplex ddPCR assays contained appropriate non-template, positive and negative controls in each run to allow the software programs to generate specific thresholds.

The QuantaSoft Analysis software used for duplex ddPCR assays determined the copy number by calculating the ratio of target molecule concentration, A (copies/µL), to the reference molecule concentration, B (copies/µL), times the number of reference species copies, \(N_B\), in the human genome (\(copy\ number = \frac{A}{B} \times N_B\)). The QuantaSoft Analysis Pro software used triplex and quadruplex ddPCR assays determined the copy number by calculating the ratio of the target molecule concentration, A, to the geometric mean of the reference molecule concentrations, B (copies/µl) and C (copies/µl), times the number of reference species copies, \(N_B\), (\(copy\ number = \frac{A}{\text{geomean}(B,C)} \times N_B\)). MYCN and ALK amplifications were designated as the detection of \(\geq 8.01\) copies, gain as detection of 2.74 to 8.00 copies and normal diploid status as detection of 1.50 to 2.73 copies.\(^{22}\) False positive rate (FPR) and limit of detection for point mutation analyses were calculated with Bio-Rad lookup tables in line with the model by Armbruster and Pry.\(^{50}\) In principle, FPR calculation was based on two parameters: The number of false positive droplets and the minimally required concentration of mutant target molecules (copies/µl) for each point mutation protocol were defined by analyzing genomic DNA from cell lines with the wildtype sequence for the respective mutation. We scored a sample as positive if both the number of droplets detecting the mutation and concentration of mutant target molecules (copies/µL) were above the set thresholds. The limit of detection was determined for each assay by measuring an 1:10 to 1:100,000 mix of DNA derived from cell lines with (SH-EP, CLB-GA) or without (SK-N-AS) the respective mutations. Thresholds were separately calculated for 0.5, 2, 10, 20, 80 and 130 ng input DNA in duplex and quadruplex format.
Whole-exome sequencing of matched cfDNA and tumor samples

Whole-exome libraries were prepared with Agilent SureSelect XT v6 according to the manufacturer’s protocol. For sequencing, 4-5 libraries were pooled on one lane Illumina HiSeq 4000 (100 bp paired-end) flowcell version 2.5 with a median of 84 million fragments per sample. Standard quality control was performed using FastQC (http://bioinformatics.babraham.ac.uk/projects/fastqc/; date of last access: February 20, 2020). Reads were aligned to the GRCh37 reference sequence using BWA-MEM v0.7.15. Separate read groups were assigned for all reads from one lane, and duplicates were masked using Samblaster v0.1.24. MYCN and ALK copy numbers were estimated using CNVkit on each WES library without a normal control as described in their manual, respectively. Digital copy numbers for tumor samples were computed with Eq. 1: DCN = \((2 \times 2^{\log2FC} - 2 + 2 \times p) / p\). The log2 fold-change of a given gene was estimated to a diploid baseline by Copywriter, and p was the tumor cell content. Tumor samples had at least 70% tumor cells. Copy numbers derived from cfDNA analysis were calculated by setting the tumor cell content to 1.

Whole-exosome sequencing of neuroblastoma cell lines

DNA was extracted from the human neuroblastoma cell lines BE(2)-C, Kelly, LAN-6 and SH-SY5Y using the Nucleospin Tissue kit (Macherey-Nagel, Düren, Germany) according to the manufacturer’s instructions. Libraries for whole-exome sequencing were prepared using the SureSelect Human All Exon V7 kit (Agilent, Santa Clara, CA.) and the Illumina TruSeq Exome kit (Illumina, San Diego, CA). Libraries were sequenced on Illumina HiSeq 4000 and Illumina NovaSeq 6000 sequencers. Read sequences and base quality scores were demultiplexed and stored in Fastq format using the Illumina bcl2fastq software. Adapter remnants and low-quality read ends were trimmed off using custom scripts. The quality of the sequence reads was assessed using the FastQC software. Reads were aligned to the human genome, assembly GRCh38, using the bwa mem software, and duplicate read alignments
were removed using samblaster\textsuperscript{52}. Copy-number alterations were determined using cnvkit\textsuperscript{53}. Single-nucleotide variants were identified using strelka\textsuperscript{254}. Potential germline variants were filtered out by excluding all single nucleotide variants that had also been observed in at least 1% of samples in cohorts of healthy individuals, namely the 1000 Genomes Project\textsuperscript{55} and the NHLBI GO Exome Sequencing Project\textsuperscript{56} cohorts.

**Statistical analysis**

Correlation analyses were performed using GraphPad Prism, version 6.00 (GraphPad Software, San Diego, CA). P-values below 0.05 were considered significant.

**RESULTS**

**Developing quadruplexing to absolutely quantify *MYCN* and *ALK* copy numbers in a single ddPCR assay**

We previously reported duplex ddPCR assay conditions to assess *MYCN* and *ALK* copy numbers in genomic and cell-free DNA using the N-acetylglucosamine kinase (*NAGK*) gene as a reference gene.\textsuperscript{22} To save sample volume, which is substantially restricted in infancy and early childhood, we aimed to establish a quadruplex ddPCR assay to reliably measure both gene copy numbers in a single reaction together with two reference genes to increase robustness for normalization. We first established assay conditions for a triplex reaction, in which *MYCN* or *ALK* were measured in parallel in channel 1 while *NAGK* and the second reference gene, *AFF3*, which is frequently used as a control for fluorescence in situ hybridization directed against *MYCN*\textsuperscript{57}, were both detected in channel 2. To accurately discriminate between fluorescence amplitudes from negative, single- and double-positive droplet clusters for *AFF3* and *NAGK* in channel 2, we performed a uniplex reaction using fragmented DNA from the SK-N-AS neuroblastoma cell line as a template, in which we tested *AFF3* and *NAGK* probe concentration series ranging from 100 to 400nM together with
a fixed primer concentration of 900nM for both genes. We used optimal separation of \textit{AFF3} and \textit{NAGK}-positive droplet clusters and identical fluorescence amplitude of \textit{AFF3} and \textit{NAGK}-negative droplet clusters as the desired result to select probe concentrations of 125nM (\textit{AFF3}) and 350nM (\textit{NAGK}) for further testing in triplex reactions with these settings (Supplemental Figure S2). Using fragmented DNA from SK-N-AS cells as a template and the previously established primer and probe concentrations for \textit{ALK} and \textit{MYCN} for duplex reactions (Table 1), we performed triplex reactions (\textit{ALK}/\textit{AFF3}/\textit{NAGK}; \textit{MYCN}/\textit{AFF3}/\textit{NAGK}) and observed an accurate separation of negative, single-, double- and triple-positive droplet clusters with \textit{ALK} or \textit{MYCN} in channel 1 and both \textit{AFF3} and \textit{NAGK} in channel 2 (Figure 1A-B).

Probe and primer concentrations to simultaneously detect \textit{ALK} and \textit{MYCN} in channel 1 were next established using fragmented SK-N-AS genomic DNA as a template. Probes for \textit{ALK} and \textit{MYCN} were each tested in the range of 100 – 400nM in combination with the standard primer concentration (900nM) for duplex and triplex assays (Table 1) in an uniplex assay. The \textit{ALK} probe concentration of 400nM and two \textit{MYCN} probe concentrations (125 and 250nM) were further tested in \textit{MYCN}/\textit{AFF3} and \textit{ALK}/\textit{NAGK} duplex reactions (Supplemental Figure S3A). We focused on finalizing conditions to absolutely quantify \textit{MYCN} copy number, and combined the two probe concentrations with primer concentrations varying between 225 and 900nM. Positive and negative droplet clusters were optimally separated using probe and primer concentrations of 125 and 450nM, respectively, as i) the bandwidth of both clusters was comparatively smallest, ii) the distance between both clusters was comparatively largest and iii) the fluorescence amplitudes of \textit{MYCN} and \textit{ALK} negative droplet clusters were almost identical (Supplemental Figure S3B). The assay conditions were then optimized to absolutely quantify \textit{ALK} copy numbers. The probe range of 350 – 400nM was tested with 300 or 900nM primers in the \textit{MYCN}/\textit{AFF3} and \textit{ALK}/\textit{NAGK} duplex reactions. While \textit{ALK} positive and negative droplet clusters were clearly separable with each of the probe/primer combinations
tested, 300nM primer with probe concentrations between 360 and 380nM were most suited for simultaneous ALK and MYCN detection in channel 1 (Supplemental Figure S4A). All three ALK-specific probe concentrations produced separation of negative, single-positive, double-positive, triple-positive and quadruple-positive clusters in the ALK/MYCN/AFF3/NAGK quadruplex reaction, but with varying degree of “droplet rain” between the ALK and MYCN double-positive cluster. The ALK single-positive cluster in channel 1 was smallest with 360nM ALK probe, prompting its selection as the optimized concentration (Supplemental Figure S4B-D). QuantaSoft Analysis Pro software accurately distinguished a total of 16 different droplet clusters including negative and single-, double-, triple- and quadruple-positive droplets from the 2-dimensional (2D) plot (Figure 2A, Table 1). Findings were confirmed for channels 1 and 2 in 1D plots (Figure 2B-C) and histograms (Figure 2D-E).

The accuracy and sensitivity of the quadruplex ddPCR reaction was compared to the triplex and previously published duplex reactions using fragmented SK-N-AS genomic DNA as a template in a dilution series from 0.5 to 100ng. Detected and assigned MYCN, ALK, NAGK and AFF3 concentrations significantly and comparably correlated in all assay types (Pearson’s correlation coefficient \( r > 0.99 \) for all four DNA targets in all assay types; Supplemental Figure S5A-D). These data demonstrate that the quadruplex reactions maintain the same linearity as triplex and duplex reactions within the range 0.5 to 100ng template for the absolute quantification of MYCN and ALK copy numbers using AFF3 and NAGK as reference genes.

**Quadruplex ddPCR can quantify MYCN and ALK copy numbers in cell lines**

Eight previously analyzed neuroblastoma cell lines known to harbor MYCN and/or ALK amplifications, gains or normal diploid chromosomal complements for comparative re-analysis in duplex, triplex and quadruplex reactions were selected. In cell lines known to harbor MYCN amplifications, quadruplex ddPCR quantified MYCN copy numbers ranging
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from 109.9 to 504.8 copies, which corresponded well with copy numbers quantified in triplex (109.3 to 542.6) and duplex (106.2 – 530.8) assays (Figure 3A). Absolute MYCN copy numbers for the Kelly cell line were higher in quadruplex (521.3) and triplex (596.6) reactions than in duplex reactions (385.4, Figure 3A), which is most likely due to the modified normalization procedure introduced by the second reference gene in quadruplex and triplex reactions. Quadruplex ddPCR confirmed MYCN gains in SH-SY5Y (3.13) and LAN-6 cells (3.18), and copy numbers corresponded well with MYCN copy numbers assessed by triplex and duplex reactions (Figure 3A). Quadruplex (1.99 ± 0.11), triplex (1.73 ± 0.11) and duplex (1.73 ± 0.08) ddPCR reliably detected the normal diploid MYCN status in the SK-N-AS cell line (Figure 3A). Quadruplex (87.8), triplex (85.1) and duplex (87.4) ddPCR also quantified the ALK amplification in the NB-1 cell line, and ALK gains in the BE(2)-C, Kelly, SH-SY5Y and LAN-6 cell lines (Figure 3B). ALK copy numbers ranged between 2.74 to 4.05 in the quadruplex format, between 2.92 to 4.49 in triplex format and between 2.92 to 5.35 in the duplex format. Absolute ALK copy numbers in the Kelly cell line were, with 4.32 and 4.27 in the quadruplex and triplex assays, slightly higher than the 3.0 measured in the duplex assay (Figure 3B), most likely again reflecting the more robust normalization in the quadruplex and triplex assay design. Normal ALK diploid status was detected in LAN-5, IMR-5 and SK-N-AS cell lines (Figure 3B). Copy numbers ranged from 1.99 ± 0.13 to 2.10 ± 0.05 using the quadruplex protocol, which corresponded well with those determined using triplex (1.93 ± 0.24 to 2.13 ± 0.15) and duplex (1.85 ± 0.37 to 2.00 ± 0.25) protocols. For comparison, we generated whole-exome sequencing (WES) data for the BE(2)-C, Kelly, SH-SY5Y and LAN-6 cell lines and determined copy number alterations on the p-arm of chromosome 2. This analysis showed a strong focal amplification of the MYCN gene locus in BE(2)-C and Kelly cells (Supplemental Figure S6). Further, the Kelly, SH-SY5Y and LAN-6 cells showed widespread albeit weaker copy number gains, up to 4 copies in total, in the first 50 Mb of chromosome 2 that include the ALK gene locus (Supplemental Figure S6), thus validating the
ddPCR results. Interestingly, the WES data showed a loss of heterozygosity in the \textit{AFF3} gene in the Kelly cell line, which explains the different \textit{MYCN} and \textit{ALK} copy number results obtained in those ddPCR assays that included \textit{AFF3} as a second reference gene in the calculation. Our data clearly demonstrate robust \textit{MYCN} and \textit{ALK} copy number quantification that clearly distinguishes between amplification, gain and diploid allele status by the quadruplex ddPCR assay.

\textbf{Quadruplex ddPCR can quantify \textit{MYCN} and \textit{ALK} copy numbers from blood plasma or neuroblastoma samples from patients}

We next evaluated quadruplex ddPCR-based \textit{MYCN} and \textit{ALK} copy number assessment in blood plasma samples paired with genomic DNA from the corresponding primary neuroblastoma from three patients. Results were compared with copy numbers determined by duplex and triplex ddPCR and re-analyzed from WES data, which have been deposited at the European Genome-phenome Archive (https://www.ebi.ac.uk/ega/studies, accession number EGAS00001004275, date of last access: March 2, 2020). Quadruplex ddPCR of tumor DNA revealed high-level \textit{MYCN} amplifications in samples from patients 1 and 2, which were confirmed by duplex and triplex ddPCR reactions and re-analysis of WES tumor data (Table 3). All multiplexed ddPCR assays detected the \textit{MYCN} amplification using cell-free DNA in plasma from patients 1 and 2, although the copy numbers estimated were consistently lower than direct assessment of tumor DNA (Table 3). Findings were confirmed through re-analysis of WES data from these cfDNA samples (Table 3). The lower copy numbers detected in circulating cfDNA may stem from a dilution effect caused by handling-induced damage to white blood cells in the samples (these blood samples were previously collected for a different purpose) from tumor heterogeneity or cfDNA derived from non-tumor cells. Therefore, the DNA quality was evaluated in plasma samples from patients 1 and 2 using the Agilent 4200 Tapestation System. The cfDNA content amounted to 69.8\% and 81.1\% in these samples,
thus pointing either to tumor heterogeneity or the presence of cfDNA derived from non-tumor cells (Table 3). Diploid \textit{MYCN} allele status was detected by quadruplex ddPCR using either tumor or cell-free DNA from patient 3, and confirmed by all other assays (Table 3). Quadruplex ddPCR detected an \textit{ALK} gain using either tumor or cell-free DNA from patient 1 and diploid \textit{ALK} allele status in patients 2 and 3 using either DNA source (Table 3). While the \textit{ALK} gain could not be clearly identified in the WES data, all other results were confirmed in all other assays (Table 3). \textit{ALK} FISH was performed on interphase nuclei of a tumor section from patient 1 and detected three \textit{ALK} copies (Supplemental Figure S7), thus confirming our ddPCR data and a false negative result in the WES data. Our data consistently demonstrate that absolute \textit{MYCN} and \textit{ALK} copy numbers can be quantified from DNA derived from either tumor or plasma samples from patients, and that quadruplexed ddPCR using two diploid reference genes for normalization is a feasible and sensitive alternative to reduce sample volumes.

**Developing quadruplex ddPCR-based detection of \textit{ALK}^{F1174L} and \textit{ALK}^{R1275Q} hotspot mutations**

The \textit{ALK} hotspot mutations at F1174 (C>A conversion at nucleotide 3522 in exon 23) and R1275 (G>A conversion at nucleotide 3824 in exon 25) occurring in neuroblastomas can be detected by duplex ddPCR.\textsuperscript{23} We hypothesized that simultaneously detecting both \textit{ALK} hotspot mutations in a quadruplex ddPCR reaction is technically feasible. As template providing both hotspot mutations and the wildtype \textit{ALK} sequences to develop the quadruplex assay, DNA was extracted from the SH-EP neuroblastoma cell line (harboring a heterozygous \textit{ALK}^{F1174L} mutation) and mixed 1:1 with DNA extracted from the CLB-GA neuroblastoma cell line (harboring a heterozygous \textit{ALK}^{R1275Q} mutation). Uniplex and duplex assays determined that 900nM and 600nM primer with 350nM and 400nM probe concentrations were optimal to detect \textit{ALK}^{F1174L} and \textit{ALK}^{R1275Q} target molecules, respectively, in channel 1.
Both mutation sites in the wildtype sequence were targeted (\(ALK^{1174}\) and \(ALK^{1275}\)) to detect wildtype \(ALK\), with both clearly detected in channel 2 using 900nM and 600nM primers and 50nM and 150nM probe, respectively (Table 2). Combining \(ALK^{F1174L}\) and \(ALK^{R1275Q}\) detection in channel 1 with \(ALK^{1174}\) and \(ALK^{1275}\) detection in channel 2 in a quadruplex reaction allowed no clear identification of individual droplet clusters (data not shown), prompting us to apply the concept of an inverted ddPCR protocol described by Alcaide et al.\(^58\) The HEX fluorophore on the \(ALK^{1174}\) probe was replaced by a FAM fluorophore, and the FAM fluorophore on the \(ALK^{R1275Q}\) probe was replaced by a HEX fluorophore to simultaneously detect \(ALK^{F1174L}\) and \(ALK^{1174}\) in channel 1 and \(ALK^{R1275Q}\) and \(ALK^{1275}\) in channel 2. This approach produced accurate separation of negative, single-positive and double-positive droplet clusters including \(ALK^{F1174L}/ALK^{1275}\), \(ALK^{1174}/ALK^{1275}\), \(ALK^{F1174L}/ALK^{R1275Q}\) and \(ALK^{1174}/ALK^{R1275Q}\) (Figure 4A). In line with previous reports on other inverted ddPCR approaches,\(^57\) some double-double positive clusters (\(ALK^{F1174L}/ALK^{1174}\) in channel 1 and \(ALK^{R1275Q}/ALK^{1275}\) in channel 2) as well triple- and quadruple-positive droplets were not clearly separable from other droplet clusters (Figure 4A). Findings were confirmed for channels 1 and 2 in 1D-plots (Figure 4B-C) and histograms (Figure 4D-E). Our data demonstrate that an inverted quadruplex ddPCR approach can be used to analyze the neuroblastoma-specific \(ALK^{F1174L}\) and \(ALK^{R1275Q}\) hotspot mutations in a single reaction.

We next assessed the dynamic range of the \(ALK^{F1174L}/ALK^{R1275Q}\) quadruplex ddPCR detection using serially diluted DNA template (0.5 to 130ng) from SK-N-AS, SH-EP or CLB-GA cell lines. The detected target molecule (\(ALK^{F1174L}\), \(ALK^{R1275Q}\), \(ALK^{1174}\) and \(ALK^{1275}\)) concentrations significantly correlated with the theoretically assigned concentrations (Pearson’s correlation coefficient \(r\) ranged from 0.9942-0.9981) and strongly resembled the concentrations detected in duplex reactions (\(ALK^{F1174L}/ALK^{1174}\) and \(ALK^{R1275Q}/ALK^{1275}\)) with a Pearson’s correlation coefficient \(r\) ranging from 0.9871-0.9981 (Supplemental Figure S8). Our
data demonstrate that quadruplex $\text{ALK}^{F1174L}$ and $\text{ALK}^{R1275Q}$ hotspot mutation detection remains linear within the range of 0.5 to 130ng input DNA.

Sensitivity thresholds for $\text{ALK}$ hotspot mutation detection in quadruplex reactions were also defined and compared to sensitivity in duplex detection. DNA from cell lines harboring either $\text{ALK}$ hotspot mutation was mixed 1:10 – 1:100,000 with DNA from cell lines with homozygous wildtype $\text{ALK}$. Mutant allele fractions (MAFs) were designated 0% in cells with wildtype $\text{ALK}$. A neuroblastoma cell was categorized as harboring an $\text{ALK}^{F1174L}$ mutation using the following thresholds: $\geq 4$ positive droplets and at least 0.31 copies/µl for up to 10ng input DNA and $\geq 5$ positive droplets and at least 0.57 copies/µl for higher input DNA amounts. These thresholds were very close to those defined for the duplex reaction ($\geq 4$ positive droplets, at least 0.33 copies/µL). The thresholds for categorizing a neuroblastoma cell as harboring an $\text{ALK}^{R1275Q}$ mutation varied according to input DNA amounts in both assays (quadruplex reaction: $\geq 5$ to $\geq 15$ positive droplets, 0.42 to 1.34 copies/µL; duplex reaction: $\geq 4$ to $\geq 17$ positive droplets, 0.32 to 1.31 copies/µL). Altogether, sensitivity thresholds to simultaneously detect both neuroblastoma-specific $\text{ALK}$ hotspot mutations in quadruplex ddPCR were close to those for the respective duplex ddPCR reactions.

**Quadruplex ddPCR correctly assesses $\text{ALK}^{F1174L}$ and $\text{ALK}^{R1275Q}$ MAFs in cell lines**

The quadruplex ddPCR assay for $\text{ALK}$ hotspot mutations was tested in a panel of six neuroblastoma cell lines harboring the $\text{ALK}^{F1174L}$ mutation (SH-EP, Kelly$^{12, 34}$), the $\text{ALK}^{R1275Q}$ mutation (CLB-GA, LAN-5$^{10, 11}$) or wildtype $\text{ALK}$ (SK-N-AS, BE(2)-C$^{12}$). The $\text{ALK}^{F1174L}$ mutation was correctly detected in SH-EP and Kelly cells, and MAFs determined using quadruplex ddPCR corresponded well those determined by duplex ddPCR (Table 4). Both quadruplex and duplex ddPCR correctly detected MAFs indicating that only one allele harbored the mutation. Wildtype $\text{ALK}$ in both alleles was correctly detected in CLB-GA, LAN-5, SK-N-AS and BE(2)-C cells using the $\text{ALK}^{F1174L}$ target in quadruplex ddPCR (Table 4).
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The monoallelic $\text{ALK}^{R1275Q}$ mutation was similarly detected in CLB-GA and LAN-5 cells with strongly corresponding MAFs detected by quadruplex and duplex ddPCR assays (Table 4). The single wildtype $\text{ALK}$ allele in SH-EP and Kelly cells and the biallelic wildtype $\text{ALK}$ status in SK-N-AS and BE(2)-C cells were correctly detected using the $\text{ALK}^{1275}$ target in quadruplex ddPCR (Table 4). Quadruplex ddPCR-based detection of wildtype $\text{ALK}$ and $\text{ALK}$ hotspot mutations performs well in neuroblastoma cell lines as an assay validating step.

**Quadruplex ddPCR detects $\text{ALK}^{F1174L}$ and $\text{ALK}^{R1275Q}$ MAFs in blood plasma and neuroblastoma samples from patients**

After validating quadruplex ddPCR-based detection of neuroblastoma-specific $\text{ALK}$ hotspot mutations in cell lines, we applied our assay to paired blood plasma and tumor samples from six patients with neuroblastoma to test its accuracy and sensitivity in comparison to the established duplex ddPCR reactions using both standard tumor biopsies and liquid biopsies suited to longitudinal patient monitoring. The quadruplex ddPCR correctly detected an $\text{ALK}^{F1174L}$ mutation in the tumor and plasma samples from patients 4 and 5 with MAFs indicating that a varying proportion of tumor cells harbored the mutation on one allele (although 14% higher in quadruplex detection for cfDNA from patient 4; Table 5). No $\text{ALK}^{F1174L}$ mutation was detected in matched tumor-derived DNA / cfDNA samples from patients 6 through 9 by either assay (Table 5). The quadruplex ddPCR assay correctly demonstrated an $\text{ALK}^{R1275Q}$ mutation in tumor-derived DNA and plasma-derived cfDNA from patients 6 to 8, and the MAFs obtained by the two assays very closely resembled each other in all cases except one, which was a 10% higher MAF measured in tumor-derived DNA from patient 8 by quadruplex ddPCR (Table 5). No $\text{ALK}^{R1275Q}$ mutation was detected in matched tumor / blood plasma samples from patients 4, 5 and 9 by either assay (Table 5). The higher MAFs in cfDNA from patient 4 and tumor-derived DNA from patient 8 as determined by quadruplex ddPCR compared to duplex ddPCR prompted us to test the influence of input...
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quantity on MAF measurement, as the two respective samples contained higher DNA amounts than all other samples analyzed. We turned to our cell line model and serially diluted fragmented DNA from an $ALK^{F1174L}$ mutant (SH-EP) and an $ALK^{R1275Q}$ mutant cell line (CLB-GA) in H$_2$O to obtain 0.5, 2, 10, 20, 80 and 130ng of DNA. $ALK^{F1174L}$ MAF assessment based on 0.5 to 20ng of total input DNA resulted in similar findings in quadruplex and duplex reactions. Using DNA input amounts of 80 ng and 130ng resulted in comparatively higher MAF results in the quadruplex reaction (Supplemental Figure S9A). Similar findings were obtained when analyzing the $ALK^{R1275Q}$ MAF with varying DNA input amounts. Using 0.5 to 20ng of DNA input, MAF results were similar in the quadruplex and duplex reactions, but were higher in the quadruplex reaction for total input DNA amounts of 80 and 130ng (Supplemental Figure S9B). This observation is likely due to the underestimation of an increasing amount of wildtype and mutant double-positives droplets in higher DNA input samples in inverted ddPCR protocols because the fluorescence signals of double-positive droplets have a similar amplitude as single mutant-positive droplets and might be obscured in this droplet cluster.\textsuperscript{58} Taken together, the data demonstrate that the quadruplex ddPCR protocol accurately distinguishes between $ALK^{F1174L}$, $ALK^{R1275Q}$ and wildtype $ALK$ in plasma-derived cfDNA and tumor DNA.

DISCUSSION

One of the challenges in infants and children with a body weight less than 10kg is to sequentially obtain sufficient blood volumes for molecular analyses without considerably lowering hemoglobin levels and, ultimately, necessitating an iatrogenically induced blood transfusion. Volumes for blood sampling are therefore ethically restricted to 1ml in infants and a maximum of 3mL in children. Our own experiences with blood samples from neuroblastoma patients indicate that, on average, 10 to 130ng of cfDNA diluted in 50µL elution buffer are available after purification for further analysis. Previously published duplex
ddPCR protocols assessing MYCN and ALK copy numbers using NAGK as a single reference gene each require 5µL of cell-free DNA extract. Droplet digital PCR is a rapid, highly sensitive, less expensive and more accessible tool for many laboratories compared to next-generation sequencing approaches. It enables targeted analyses of known copy number variations and mutations, while next-generation sequencing enables an unbiased approach, but needs longer time to obtain, process and analyze data with the need of bioinformatics expertise. Increasing evidence suggests that characterizing the cfDNA in plasma from neuroblastoma patients with targeted and untargeted approaches such as ddPCR, qRT-PCR, shallow whole-genome sequencing, OncoScan arrays or WES contributes to our understanding of molecular risk factors as well as spatial and temporal heterogeneity in neuroblastoma.

Technical feasibility of multiplexing DNA targets by ddPCR beyond single and duplex reactions has been exemplarily shown by quantifying recurrent somatic mutations in diffuse large B-cell and follicular lymphoma tissue sections and scoring PD-L1 in non-small cell lung cancer biopsies. To optimize the amplitude of a droplet cluster in higher-order multiplexing assays, i) probe concentrations, ii) primer concentrations and iii) annealing temperature in the PCR cycler program can be adapted. Following optimization of the first two parameters, simultaneous copy number assessment of two of the major oncogenic drivers in neuroblastoma, MYCN and ALK, was shown to be technically feasible using NAGK and AFF3 as two normal diploid reference genes. Quality assessment of linearity and limit of detection showed highly comparable results between the respective duplex and triplex reactions, saving precious sample volume. While we show that the quadruplexed ddPCR protocol is a highly sensitive and robust analytical tool to exactly assess copy number status, it maintains a targeted analysis by nature, which is limited to the analysis of a short range of DNA base pairs, often less than 100 nucleotides. This is reflected by a higher ALK amplification status in the NB-1 cell line and misdetection of a partial ALK amplification in
the IMR-5 cell line due to the use of different primer pairs than used for previously reported data. Another example of the targeted nature of the ddPCR technology is the observation that only the WES technology but none of the ALK ddPCR assays were able to discriminate between a specific ALK gain and copy number gains within the first 50 Mb of chromosome 2p including the ALK gene in the neuroblastoma cell lines investigated. The WES data reported in our study are in line with previous studies summarized in Supplemental Table S1. The well-known intrinsic ddPCR assay limitation justifies the combination of this targeted analysis applicable in routine clinical testing at sequential time points for monitoring disease status and emergence of new potentially druggable targets such as ALK or activating RAS-MAPK pathway mutations in combination with unbiased next-generation sequencing approaches at a defined time point such as initial or relapse diagnosis. Other molecular characteristics used in neuroblastoma risk stratification including DNA ploidy and segmental copy number alterations are not detected with our targeted MYCN/ALK quadruplexing ddPCR approach. The ddPCR technology could nevertheless impact clinical decision-making by considerably shortening the time needed to switch to a potentially promising alternative targeted therapy, and may therefore, have considerable potential with regard to its translation into daily clinical practice in the near future. While the number of studies reporting ddPCR applications in oncology has rapidly increased over recent years, additional prospective studies in larger patients cohorts are needed for further validation.

To increase robustness of our ddPCR assay assessing MYCN and ALK copy number status, we included a second normal diploid reference gene on the 2q arm (AFF3 at 2q11.2). Incorporation of a second reference gene enables the internal control between both reference genes to detect the unlikely but non-excludable event of a potential copy number aberration in a well-established reference gene in individual patient samples. For example, the approximately 30% higher MYCN and ALK copy numbers detected by triplex and quadruplex reactions in Kelly cells are explained by a loss of heterozygosity in AFF3. ALK copy number
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assessment in cfDNA purified from plasma samples will enable monitoring in patients with neuroblastoma for the targetable ALK amplification in the future. This is important because ALK inhibitor treatment of ALK-amplified neuroblastoma cell lines was shown to potently suppress ALK downstream signaling and trigger an apoptotic response \textit{in vitro}.\textsuperscript{28, 69} ALK amplification is emerging as a potential biomarker associated with response to targeted inhibition in neuroblastoma models.\textsuperscript{15, 16}

The quadruplexed protocol detecting the ALK hotspot mutations, \(ALK^{F1174L}\) (3522, C>A) and \(ALK^{R1275Q}\) (3824, G>A), is based on the previously reported “inverted” ddPCR approach\textsuperscript{58} and allows successful detection and quantification of either or both mutations in a simultaneous reaction in samples with low DNA input by changing the labeled probes and thereby measuring \(ALK^{F1174L}\) (3522, C>A) together with the respective \(ALK^{\text{wildtype}}\) sequence in channel 1 and \(ALK^{R1275Q}\) (3824, G>A) with this corresponding \(ALK^{\text{wildtype}}\) sequence in channel 2. In this setting, the respective mutant and wildtype sequences are amplified with the same primer pair, the reactions compete against each other for the existing resources in the droplet, diminishing the fluorescence signal from double-positive clusters. This well-known phenomenon from duplex reactions\textsuperscript{68} leads to a shift of the double-positive clusters in one channel to the upper single-positive clusters. While the occurrence of double-positive droplets is comparatively low in samples with low-DNA input, it increases in high-DNA input samples causing MAF overestimation for the respective mutation in the calculation. While mutant and wildtype sequences are detected in separate channels in duplex reactions and can, thereby, clearly be distinguished from each other, the natural limitation of the inverted ddPCR approach used here necessitates that DNA input be restricted to maximally 20ng, making it also well-suited to analyze cfDNA. Here we present two novel quadruplexed ddPCR protocols that enable, with high analytical sensitivity and low cost, the assessment of copy number variations and hotspot mutations crucial for monitoring and treating children with neuroblastoma in routine clinical settings.
ACKNOWLEDGEMENTS

The authors thank Daniela Tiburtius and Aleixandria McGearey for technical assistance, the German Neuroblastoma Biobank for providing blood plasma samples paired with genomic DNA from the corresponding primary tumor and the DKFZ High Throughput Sequencing Unit for providing sequencing services.
REFERENCES


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LEGENDS TO FIGURES

Figure 1. Representative 2-D-plots of triplex ddPCR assays assessing ALK and MYCN copy number status. Genomic DNA was extracted from SK-N-AS cells and fragmented by sonication before ddPCR reactions using a total of 20 ng as input material. Channel 1 fluorescence (FAM) was plotted against channel 2 fluorescence (HEX) for each droplet in the triplex reactions. ALK (A) and MYCN copy numbers (B) were detected in channel 1, and the normal diploid reference genes, AFF3 and NAGK, were measured in channel 2. Circles indicate individual droplet clusters.

Figure 2. Representative plots and histograms of quadruplex ddPCR assay assessing ALK and MYCN copy number status. Genomic DNA was extracted from SK-N-AS cells and fragmented by sonication before ddPCR reactions using a total of 10 ng as input material. A. Representative 2-D-plot of droplet fluorescence in the quadruplex reaction. MYCN and ALK droplets were plotted in channel 1 (FAM) against AFF3 and NAGK droplets in channel 2 (HEX). Pink lines indicate thresholds for negative as well as single-, double-, triple- and quadruple-positive droplet clusters. B-C. Shown are one-dimensional plots of droplet fluorescence in channel 1 for MYCN and ALK (B) and in channel 2 for AFF3 and NAGK (C). Plots documented optimal separation of negative, single-positive and double-positive droplets. Positive droplets, blue (channel 1) and green (channel 2); negative droplets, grey. D-E. One-dimensional fluorescence amplitude histograms indicate the droplet frequency at specific fluorescence amplitudes in channel 1 (D; MYCN and ALK) and in channel 2 (E; AFF3 and NAGK). Again, droplet frequencies for negative, single-positive and double-positive clustered were clearly distinguishable.

Figure 3. Comparison of absolute MYCN and ALK copy numbers determined by multiplexed ddPCR assays for neuroblastoma cell lines. Genomic DNA was extracted and
fragmented by sonication before ddPCR reactions to quantify MYCN (A) and ALK (B) copy numbers (mean±SD; n≥3). A total of 2 ng DNA was used as input material.

**Figure 4. Quadruplex ddPCR assay for the detection of the neuroblastoma-specific hotspot mutations ALK<sup>F1174L</sup> and ALK<sup>R1275Q</sup>.** For assay design, DNA was extracted from SH-EP (ALK<sup>F1174L</sup>) and CLB-GA (ALK<sup>R1275Q</sup>) cells, fragmented by sonication and mixed 1:1 before ddPCR reactions to generate a DNA mixture comprising both mutations. A total of 20 ng of this DNA mixture was used as input material. A. Representative 2-D-plot of droplet fluorescence. ALK<sup>F1174L</sup> and ALK<sup>L1174</sup> droplets were plotted in channel 1 (FAM) against ALK<sup>R1275Q</sup> and ALK<sup>L1275</sup> droplets in channel 2 (HEX). Shown are negative, single-positive and double-positive clusters. B-C. Shown are one-dimensional plots of droplet fluorescence for ALK<sup>F1174L</sup> and ALK<sup>L1174</sup> in channel 1 (B) and for ALK<sup>R1275Q</sup> and ALK<sup>L1275</sup> in channel 2 (C). Plots documented optimal separation of negative and single-positive droplets. Positive droplets, blue (channel 1) and green (channel 2); negative droplets, grey. D-E. One-dimensional fluorescence amplitude histograms indicate the droplet frequency at specific fluorescence amplitudes in channel 1 (D; ALK<sup>F1174L</sup> and ALK<sup>L1174</sup>) and in channel 2 (E; ALK<sup>R1275Q</sup> and ALK<sup>L1275</sup>). Droplet frequencies were clearly distinguishable.
### TABLES

Table 1. Sequences and concentrations of primers and probes used in multiplexed ddPCR protocols to assess *ALK* and *MYCN* copy number status.

<table>
<thead>
<tr>
<th>Primer/Probe</th>
<th>Sequence</th>
<th>Concentration [nM]</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Duplex ddPCR</td>
</tr>
<tr>
<td><em>AFF3</em>-for</td>
<td>5’-CACCTAGCATGTGTGGCATTTT-3’</td>
<td>900</td>
</tr>
<tr>
<td><em>AFF3</em>-rev</td>
<td>5’-GCAGATCCAGGTCGTTGAAG-3’</td>
<td>900</td>
</tr>
<tr>
<td><em>AFF3</em>-probe</td>
<td>5’-HEX-AACAACCTCCTTTCTGCCCCCT-BHQ1-3’</td>
<td>250</td>
</tr>
<tr>
<td><em>ALK</em>-for</td>
<td>5’-CTTGCTCatTGACATCTTCACG-3’</td>
<td>900</td>
</tr>
<tr>
<td><em>ALK</em>-rev</td>
<td>5’-CAAGACTCCAGAATGAGCG-3’</td>
<td>900</td>
</tr>
<tr>
<td><em>ALK</em>-probe</td>
<td>5’-FAM-TCACAGCTCCAGAATGCTTG-BHQ1-3’</td>
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<td><em>MYCN</em>-for*</td>
<td>5’-GTGCTCTGACATCTACCTCG-3’</td>
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<tr>
<td><em>MYCN</em>-rev*</td>
<td>5’-GATGGCATCAGAGAGGGGA-3’</td>
<td>900</td>
</tr>
<tr>
<td><em>MYCN</em>-probe*</td>
<td>5’-FAM-CACTCAAGCTTTCTCCACCTCCTTBHQ1-3’</td>
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<tr>
<td><em>NAGK</em>-for*</td>
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<tr>
<td><em>NAGK</em>-rev*</td>
<td>5’-CACCTTCATCCACCTCAAC-3’</td>
<td>900</td>
</tr>
<tr>
<td><em>NAGK</em>-probe*</td>
<td>5’-HEX-TGTGGCCAGATGACCCGTT-BHQ1-3’</td>
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</table>

*Gotoh et al., 2005.*
Table 2. Sequences and concentrations of primers and probes used in multiplexed ddPCR protocols to detect the neuroblastoma-specific $ALK^{F1174L}$ and $ALK^{R1275Q}$ hotspot mutations.

<table>
<thead>
<tr>
<th>Primer/Probe</th>
<th>Sequence*</th>
<th>Concentration [nM]</th>
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<td>Duplex ddPCR</td>
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<td>5’-GCCCAAGACTCAGCTCAGT-3’</td>
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<td>$ALK^{F1174}$-rev</td>
<td>5’-CCCAATGCAGCGAACAAT-3’</td>
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<td>5’-FAM-TCTCTGCTCTGCAGCAAATTAAC-BHQ1-3’</td>
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<td>$ALK^{R1275}$-rev</td>
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<td>$ALK^{R1275Q}$ probe, HEX</td>
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<tr>
<td>$ALK^{R1275}$ probe, HEX</td>
<td>5’-HEX-TTCGGGATGGCCGAACGACAT-BHQ1-3’</td>
<td>250</td>
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*Adapted from: Combaret et al., 2015.23
Table 3. Comparatively determined \textit{MYCN} and \textit{ALK} copy numbers in patient samples.

<table>
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<tr>
<th>Patient</th>
<th>Disease stage</th>
<th>Sample</th>
<th>Tumor cell content (%)</th>
<th>cfDNA content (%)</th>
<th>\textit{MYCN} copy number</th>
<th>\textit{ALK} copy number</th>
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<td></td>
<td></td>
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<td>Quadruplex ddPCR*</td>
<td>Triplex ddPCR*</td>
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<td>1</td>
<td>M</td>
<td>gDNA</td>
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<td>cfDNA</td>
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<tr>
<td>2</td>
<td>M</td>
<td>gDNA</td>
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<td>cfDNA</td>
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<td>81.1</td>
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<td>3</td>
<td>M</td>
<td>gDNA</td>
<td>90</td>
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<td>1.86</td>
<td>1.82</td>
</tr>
<tr>
<td></td>
<td></td>
<td>cfDNA</td>
<td></td>
<td>n.a.</td>
<td>2.23</td>
<td>2.68</td>
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</tbody>
</table>

gDNA, genomic DNA; cfDNA, cell-free DNA; ddPCR, droplet digital PCR; WES, whole-exome sequencing; n.a., not analyzed.
* DNA input for ddPCR reactions ranged from 1 to 2 ng.
† DNA input for WES ranged from 8 to 110 ng.
Table 4. Comparison of multiplexed ddPCR assays for detecting $ALK^{F1174L}$ and $ALK^{R1275Q}$ mutant allele fractions in neuroblastoma cell lines.

<table>
<thead>
<tr>
<th>Cell line*</th>
<th>$ALK^{F1174L}$ (3522, C&gt;A)†</th>
<th>$ALK^{R1275Q}$ (3824, G&gt;A)†</th>
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<tbody>
<tr>
<td></td>
<td>Quadruplex ddPCR</td>
<td>Duplex ddPCR</td>
</tr>
<tr>
<td>SH-EP</td>
<td>45.82 ± 0.87</td>
<td>48.64 ± 0.31</td>
</tr>
<tr>
<td>Kelly</td>
<td>31.58 ± 0.69</td>
<td>31.20 ± 0.52</td>
</tr>
<tr>
<td>CLB-GA</td>
<td>0</td>
<td>0</td>
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<td>LAN-5</td>
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<tr>
<td>SK-N-AS</td>
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<tr>
<td>BE(2)-C</td>
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dPCR, droplet digital PCR.

*DNA input: 10 ng.

†Mutant allele fraction is shown in % ± SD for n ≥ 3 replicates; Mutant allele fraction of 0% indicates wildtype $ALK$. 
Table 5. Comparatively determined $ALK^{F1174L}$ and $ALK^{R1275Q}$ mutant allele fractions in paired blood plasma and neuroblastoma samples from patients.

<table>
<thead>
<tr>
<th>Patient</th>
<th>Sample*</th>
<th>$ALK^{F1174L}$ (3522, C&gt;A)†</th>
<th>$ALK^{R1275Q}$ (3824, G&gt;A)†</th>
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<td>Duplex ddPCR</td>
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<td>4</td>
<td>gDNA</td>
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gDNA, genomic DNA; cfDNA, cell-free DNA; ddPCR, droplet digital PCR.

*Cell-free DNA input material ranged between 6.9 and 72.6 ng, and gDNA input material ranged between 6.9 and 98.5 ng.

†Mutant allele fraction of 0% indicates wildtype $ALK$. 

Table 5. Comparatively determined $ALK^{F1174L}$ and $ALK^{R1275Q}$ mutant allele fractions in paired blood plasma and neuroblastoma samples from patients.
Figure 1

A

Channel 1 amplitude

12000

10000

8000

6000

4000

2000

0

negative

AFF3

NAGK

NAGK AFF3

ALK

ALK AFF3

ALK NAGK

ALK NAGK AFF3

B

Channel 1 amplitude

12000

10000

8000

6000

4000

2000

0

negative

AFF3

NAGK

NAGK AFF3

MYCN

MYCN AFF3

MYCN NAGK

MYCN NAGK AFF3

Channel 2 amplitude
Figure 2

Peitz et al.

A

B

C

D

E

[Diagram images of plots and graphs related to Peitz et al.]