Cancer Letters 645 (2026) 218352

Contents lists available at ScienceDirect

Cancer Letters

journal homepage: www.elsevier.com/locate/canlet

Multiplexed biomarkers dynamically detect heterogeneous residual neuroblastoma cell clone activity in the bone marrow niche

Anna M. Schroeer [a], Annika Winkler [a], Marion Fillies [a], Charleen Kranig [a], Eliz Karaman [a], Felix von Stetten [b,c], Roland Zengerle [b,c], Michael Lehnert [b], Alexander Blume [d], Jan F. Hollander [a], Marco Lodrini [a], Angelika Eggert [a,e,f,g], Altuna Akalin [d], Kathy Astrahantseff [a], Cornelia Eckert [a], Hedwig E. Deubzer [a,f,g,h,1], Annabell Szymansky [a,*,1]

[a] Department of Pediatric Oncology and Hematology, Charité - Universitätsmedizin Berlin, Corporate Member of Freie Universität Berlin and Humboldt-Universität Berlin, 13353, Berlin, Germany
[b] Hahn-Schickard-Gesellschaft für angewandte Forschung e.V., 79110, Freiburg, Germany
[c] Laboratory of MEMS Applications, IMTEK – Department of Microsystems Engineering, University of Freiburg, 79110, Freiburg, Germany
[d] Bioinformatics & Omics Data Science Platform, Berlin Institute for Medical Systems Biology, Max-Delbrück-Center for Molecular Medicine Berlin, 13125, Berlin, Germany
[e] University Hospital Essen, University of Duisburg-Essen, 45147, Essen, Germany
[f] Berlin Institute of Health (BIH) at Charité – Universitätsmedizin Berlin, Berlin, Germany
[g] German Cancer Consortium (DKTK), Partner Site Berlin, and German Cancer Research Center (DKFZ), Germany
[h] National Center for Tumor Diseases (NCT), NCT Berlin, a Partnership between DKFZ, Charité – Universitätsmedizin Berlin, Berlin Institute of Health at Charité (BIH), Max Delbrück Center, Germany

ARTICLE INFO

Keywords:
Biomarker
Pediatric cancer
MYCN
Personalized medicine
MRD
MP-PCR

ABSTRACT

Monitoring MYCN-driven high-risk neuroblastoma presents challenges to capture dynamics of all tumor cell clones at their earliest divergence to current clinical course. Not all clones may enter the bone marrow, the most important monitoring site for minimal residual disease (MRD), causing relapse in ~50% of patients. We developed mediator-probe PCR assays to detect up to four multiplexed patient-individual genetic alterations in 37 longitudinally collected bone marrow aspirates from 8 patients with *MYCN*-amplified disease. Multiplexed biomarkers, including *MYCN* amplicon breakpoints, detected diverse neuroblastoma clones, surpassing conventional GD2 immunocytology accuracy. We provide proof-of-principle for clonally heterogeneous MRD biomarker detection (1 tumor: 10^6 reference cells). In selected patients, multiplexed biomarkers indicated divergent dynamics, suggesting individual tumor clones differ in their ability to disseminate to the bone marrow and escape therapy. Our pilot data support integrating multiplexed MRD detection in co-clinical trials to monitor the molecular remission state during therapy and follow-up.

1. Introduction

Approximately half of patients diagnosed, have high-risk neuroblastoma. Despite intensive multimodal therapy, minimal residual disease (MRD) limits event-free survival to ~50% [1]. MRD represents the lowest disease level detectable in patients by molecular technologies. The sensitivity of molecular methods surpasses standard-of-care histopathology or imaging approaches. Neuroblastoma cells can migrate to different niches throughout the body. Tumor cells are already disseminated to the bone marrow (BM) at initial diagnosis of 80% of high-risk cases [2]. Neuroblastoma cells can persist undetected in the BM microenvironment, and may enter a highly chemoresistant dormant state that eventually gives rise to relapse [3]. Assessing MRD in BM has been shown to be one of the most important diagnostic parameters to identify early responders and predict overall survival outcomes in patients with high-risk neuroblastoma [4]. BM is infiltrated in the majority

This article is part of a special issue entitled: MYCN Amplification published in Cancer Letters.

* Corresponding author. Department of Pediatric Oncology and Hematology, Charité - Universitätsmedizin Berlin, Corporate Member of Freie Universität Berlin and Humboldt-Universität Berlin, Augustenburger Platz 1, 13353, Berlin, Germany.
E-mail address: annabell.szymansky@charite.de (A. Szymansky).

[1] These senior authors contributed equally to this work.

https://doi.org/10.1016/j.canlet.2026.218352
Received 8 October 2025; Received in revised form 2 February 2026; Accepted 21 February 2026
Available online 23 February 2026
0304-3835/© 2026 The Authors. Published by Elsevier B.V. This is an open access article under the CC BY license (http://creativecommons.org/licenses/by/4.0/).

of relapsed high-risk cases, with studies reporting BM infiltration at recurrence in 50–60% of patients [1,5].

Tumor biopsies are routinely performed at initial neuroblastoma diagnosis and increasingly performed at any relapse, providing clinically accessible biomaterial for comprehensive molecular characterization. This enables applying deep next-generation sequencing techniques to identify patient-specific somatic variants, which form the basis for individualized, mutation-tailored rapid PCR assays [6]. *MYCN* amplification, first identified by Manfred Schwab et al. [7] and present in ~25% of primary neuroblastomas, has been used as an independent prognostic marker for high-risk disease since 1984 [8]. Recurrent *ALK* single-nucleotide variants (SNVs) occur in 10.5% to as high as 21.5% of neuroblastomas [9,10] activating ALK signaling. Drugs targeting ALK have entered clinical trials (crizotinib, NCT00939770; ceritinib, NCT01742286; lorlatinib, NANT2015-0, NCT03107988) in different treatment settings and are administered under compassionate use for patients with *ALK*-mutated neuroblastoma. *TERT* [12.5% [11]] and *ATRX* [19% [12]] rearrangements and alternative lengthening of telomeres [ALT, 13.8% [13]] in neuroblastomas have clinical significance, but diagnostics to monitor them remain in the preclinical development and validation phase. Relapsed neuroblastomas often acquire different genetic alterations beyond those in the primary tumor, including mutations in tyrosine kinase receptors [e.g., FGFR3 [14]], regulators of MDM2/TP53 signaling [e.g., MDM2, NBAS [15]] and ALK/RAS/MAPK signaling components [e.g., NF1 [16]]. Despite their potential clinical impact, these alterations are not yet included in routine clinical testing or trials. The ongoing European HR-NBL2 trial sequences a minimal consensus of 17 neuroblastoma-relevant genes [17,18] in tumor samples collected at initial and relapse diagnosis to better support targeted treatment decisions and investigate temporal and spatial heterogeneity. Current developments integrate the well-established *MYCN* and *ALK* copy number variations and SNVs into digital PCR-based MRD assays [19]. Multiplexing biomarkers for high-risk disease would enhance longitudinal monitoring of disease dynamics. The heterogeneity and emergence of distinct tumor cell clones during high-risk disease progression creates an unmet clinical need to develop multiplexed assays that simultaneously monitor multiple biomarkers.

The gold standard for neuroblastoma MRD detection currently relies on conventional BM immunocytology performed in specialized neuroblastoma reference centers. Recent advances in liquid biopsy-based quantitative digital PCR have entered the most recent clinical trials as accompanying research. Latest technology innovations have improved quantitative PCR methods, enabling advanced high-multiplexing approaches, such as mediator probe PCR [MP-PCR [20]]. This technique uses a two-stage hybridization and signal amplification system to improve simultaneous detection of multiple genetic targets, while minimizing cross-reactivity and sample input requirements [21]. A single universal reporter generates a fluorescent signal for multiple target sequences in MP-PCR [22]. MP-PCR could enhance diagnostic accuracy while optimizing sample efficiency, run times and costs, key factors for translation to the clinical routine.

We report the successful design and application of multiplexed MP-PCR assays incorporating up to four tumor-individual genomic alterations in a pilot cohort of eight patients with high-risk neuroblastoma to detect and quantify neuroblastoma cell infiltration in serially collected BM aspirates.

2. Methods

2.1. Patients and patient samples

Eight pediatric patients registered in the NB Registry 2016 (DRKS00023442) were treated for *MYCN*-amplified high-risk neuroblastoma at *Charité - Universitätsmedizin Berlin* between 2017 and 2025, comprising our retrospective study cohort (Suppl. Table S1). Two patients were enrolled in the RIST-rNB-2011 trial (NCT01467986). Tumor samples were available from all patients (initial diagnosis, n = 8; relapse, n = 2). BM aspirates were sequentially collected from four sites, whenever possible, according to national guidelines [23]. Biomaterials assessed in this study (EA2/055/17, EA2/011/23) were residuals obtained during routine patient care. Control DNA was obtained from peripheral blood mononuclear cells isolated from pooled blood samples from at least 10 healthy donors (buffy coat DNA) kindly provided by the MRD Molecular Genetics Laboratory for ALL-REZ BFM trials (EudraCT 2012-000810-12).

2.2. Mononuclear cell isolation from bone marrow

The mononuclear cell fraction was isolated from BM and blood samples using Ficoll density gradient centrifugation [24]. The mononuclear cell pellet was resuspended in 1 ml phosphate buffered saline, quantified and stored at −80 °C in aliquots of 1×10^6 to 4×10^6 cells.

2.3. Cell lines

The TR-14 neuroblastoma cell line (RRID:CVCL_B474), harboring four *MYCN* breakpoints, was used in MP-PCR assay development. The HeLa cervical adenocarcinoma cell line (RRID:CVCL_0030) was used as a non-neuroblastoma control. Both were cultured at 37 °C and 5% CO_2 in RPMI-1640 medium supplemented with 10% fetal calf serum and 1% penicillin-streptomycin. Cell lines were regularly monitored for mycoplasma infections and authenticated using high-throughput SNP-based assays [25,26].

2.4. Neuroblastoma hybrid capture sequencing panel assay application to tumor samples

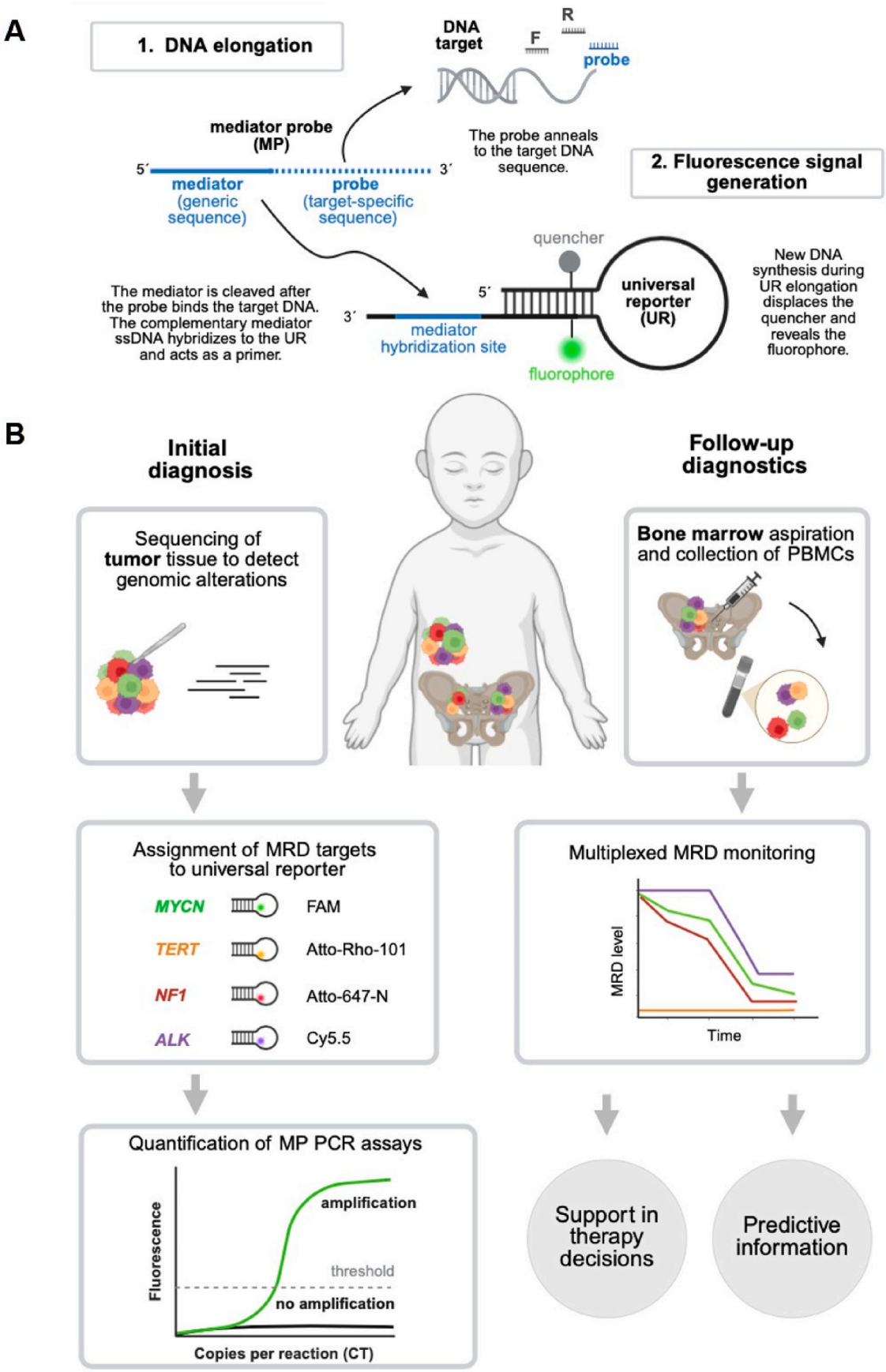
Targeted panel sequencing was performed on formalin-fixed paraffin-embedded (FFPE) tumor slices as previously described using a hybrid-capture assay (SureSelect^{XT}, Agilent) targeting 55 neuroblastoma-relevant genes [18]. Raw data reads from paired-end sequencing were aligned to the GRCh37 human reference genome (hg19), before extracting discordant read pairs and exporting mutation information [18]. The variant allele frequency (VAF) was set to ≥ 5% for SNV detection. Genomic breakpoints from rearrangements were required to have at least three high-quality spanning and encompassing reads.

2.5. DNA preparation for MP-PCR

Genomic DNA was isolated from snap-frozen tumor material, mononuclear cells and cell lines using the NucleoSpin Tissue Kit (Macherey-Nagel GmbH & Co. KG, Düren, Germany), and from FFPE tumor tissue using the QiAamp DNA FFPE Tissue Kit (Qiagen N. V., Hilden, Germany). DNA concentration was assessed by NanoDrop™ 2000 spectrophometer (Thermo Fischer Scientific, Waltham, MA, USA). DNA quality was controlled by 4200 TapeStation System (Agilent Technologies, Inc., Santa Clara, CA, USA) according to manufacturer's instructions.

2.6. MP-PCR primer and mediator probe design, production and initial testing

The semi-automated AssayManager design tool [GNWI mbH, Germany [27]] was used for primer and mediator probe oligonucleotide design. Assays were validated using conventional PCR (product sizing on the QIAxcel Advanced capillary electrophoresis system analyzed with ScreenGel v1.4.0 software) before integrating fluorogenic universal reporters for multiplexing. Sanger sequencing confirmed PCR product sequences. Final assays incorporated four previously optimized [22] fluorogenic universal reporters [UR02 (FAM), UR04 (Atto-Rho-101), UR05 (Atto-647-N), UR06 (Cy5.5); Fig. 1A, Table 1]. MP-PCR primer,



(caption on next page)

Fig. 1. Patient-specific multiplexed MP-PCR assays detect genomic alterations and reveal tumor cells in the bone marrow niche. A, Schematic view of a mediator-probe PCR (MP-PCR) reaction cycle: (1.) DNA elongation leads to cleavage of the mediator that binds the universal reporter (UR) where it acts as a primer. (2.) Subsequent elongation of the UR generates a fluorescence signal. **B,** Schematic view of the diagnostic procedure. At initial diagnosis neuroblastoma tumor tissue is sequenced (left panel, upper box). Based on the detected genomic alterations, patient-specific MP-PCR assays with up to four targets per assay are designed (left panel, middle box) and quantified (left panel, lower box). At follow-up diagnostics, bone marrow aspirates are (right panel, upper box) utilized to determine the level of residual neuroblastoma cells (right panel, lower box). This approach enables longitudinal minimal residual disease (MRD) assessment to guide therapeutic decisions and provide predictive information. PBMC, peripheral blood mononuclear cells.

Table 1
Universal reporters applied in MP-PCR assays.

Name	Fluorophore	Quencher	Detection channel	Absorption spectrum	Emission spectrum
UR02	FAM	BHQ1-NHS	*green*	470 nm	510 nm
UR04	Atto-Rho-101	BHQ2-amidit	*orange*	587 nm	609 nm
UR05	Atto-647-N	BHQ2-amidit	*red*	646 nm	664 nm
UR06	Cy5.5	BHQ2-amidit	*crimson*	684 nm	710 nm

Universal reporters (UR) with the corresponding fluorophores and quencher labels are detected at different emission and absorption spectra: UR02 (FAM, *green*); UR04 (Atto-Rho-101, *orange*); UR05 (Atto-647-N, *red*) and UR06 (Cy5.5, *crimson*).

mediator probe and universal reporter oligonucleotides were synthesized by biomers.net GmbH (Ulm, Germany) with high-performance liquid chromatography purification, supplemented by custom-ordered primers (Eurofins Genomics, Ebersberg, Germany). Principles described by Kipf et al. [27] were employed in MP-PCR assay design. Mediator probes were positioned ≥5 nt upstream of the breakpoint to avoid structural microhomologies. For each breakpoint, 1–3 primer pairs with varying lengths and distances were evaluated. Optimal pairs were selected for real-time MP-PCR quantification (Suppl. Fig. S1). Two distinct MP-PCR design strategies were tested in single-plex SNV reactions (Suppl. Fig. S2A): (i) the 5′ mediator probe end was located directly on the SNV with the forward primer directly adjacent (upstream) to the mediator probe [Suppl. Fig. S2B, previously used in Ref. [21]] and (ii) the mediator probe spanned the SNV with the forward primer not directly adjacent to the mediator probe (Suppl. Fig. S2C). SNV detection sensitivity varied according to background DNA content, base changes in the targeted SNV and PCR amplicon length.

2.7. Multiplexed MP-PCR analysis

Tumor-specific DNA was detected in BM-derived mononuclear cells using our MP-PCR assays on a 5-plex Rotor-Gene Q device (Qiagen) with the cycling protocol (1 × 2min 95 °C ramp, 50x cycles of 5 s at 95 °C following 30 s at 60 °C). Individual targets were detected via fluorescence readout after each amplification step in each color channel detecting a fluorogenic universal reporter [*green* (FAM), *orange* (Atto-Rho-101), *red* (Atto-647-N), *crimson* (Cy5.5); Table 1]. Testing the range, 55-68 °C in 2 °C increments, identified 60 °C as the optimal annealing temperature for assays on the Rotor-Gene Q device. The QuantiNova Multiplex PCR Kit (Qiagen) was used at 1.3-fold concentration. The 15 µl PCR reaction mixture (in the 20-µl total volume) contained 2 µl Dnase/Rnase-free ddH_2O, 6.5 µl 4x QuantiNova Multiplex PCR Master Mix (Qiagen), 1.5 µl 20 mg/mL bovine serum albumin (New England Biolabs GmbH, Frankfurt am Main, Germany), 1 µl universal reporter mix (final concentration 100 nm) and 1 µl primer/mediator probe mixture per target (50 nm forward primer, 75 nm reverse primer, 200 nm mediator probe in the final reaction volume). Sample DNA (5 µl volume, 100-0.001 ng/µl concentration range) was separately added to each reaction. Input concentrations ranged from 1:10 to 1:100 of the sample DNA stock solutions (sample quality-dependent). All samples in a given run were diluted equally when possible. Each run included TR-14 neuroblastoma and HeLa non-neuroblastoma DNA to control for background noise and no-template negative controls. A standard curve was generated for each patient-specific assay using serially diluted tumor DNA [28] in a background of control DNA from healthy controls to maintain the same total DNA level in each reaction. Technical duplicates were performed for each data point.

2.8. Multiplexed MP-PCR data pre-processing

MP-PCR data were exported from Rotor-Gene Q Software and visualized as amplification plots (fluorescence signal from experimental reaction minus baseline, ΔRn, versus cycle number). Manual threshold settings were set in Rotor-Gene Q software to define the baseline and detect target amplification in the exponential assay phase. Thresholds were optimized for each assay and then applied consistently across all samples in the same run. Pre-processed RQ-PCR data was exported to Excel for further analysis.

2.9. Quantifying disease biomarker burden from multiplexed MP-PCR assay data

Low-level tumor DNA burden was quantified using a procedure adapted from EuroMRD guidelines originally developed to monitor Ig/TCR rearrangements in samples from patients with acute lymphoblastic leukemia [28]. These criteria were tailored to the requirements for our multiplexed MP-PCR assays (Suppl. Table S2). Quantitative ranges were defined by the highest dilution step (lowest target DNA concentration) with (i) specific target sequence amplification, (ii) a reproducible signal with a ΔCT <1.5 across technical replicates and (iii) CT values at least 3.0 cycles below the lowest CT value observed in the mutation-negative DNA control (limit of quantification). A sample was considered positive at the assay limit of detection if at least one replicate yielded a CT value ≥ 1.0 cycle below the lowest CT of the mutation-negative control and fell within 4.0 cycles of the assay's upper quantifiable limit. Assays were considered negative if (i) no amplification occurred, (ii) all CT values were within 1.0 cycle of the background threshold or (iii) all replicates had CT values > 4.0 cycles above the upper quantification threshold. Samples yielding positive but non-reproducible results (ΔCT >1.5 between replicates) were deemed *positive but outside the quantifiable range*. The neuroblastoma-specific multiplexed PCR guidelines applied stricter thresholds [regression coefficient (R^2) > 0.99, CT < 29 at the 10^{-1} dilution step with the dilution series extended to 10^{-6}] than standard EuroMRD criteria. Both the EuroMRD-adapted and neuroblastoma-specific assays used 500 ng input DNA (corresponding to ~75,000 cell equivalents) and normalized their results using

hemoglobin subunit beta (*HBB*) as a reference.

2.10. Statistics

Associations between categorical variables were assessed using Fisher's exact test. Scatter plots visualizing results were generated in GraphPad Prism (v7.00, GraphPad Software Inc., San Diego, CA, USA). Values below the quantification limit and negative results were scaled to 1×10^{-6} and 1×10^{-7}, respectively, to enhance clarity. Reproducibility was evaluated via intra- and inter-assay coefficient of variation. Statistical analyses were conducted using the IBM SPSS Statistics package (v25, IBM Corp., Armonk, NY, USA).

2.11. Data availability

Targeted sequencing data from tumors are deposited in the European Genome-phenome Archive (http://ega-archive.org/studies/, accession number: study ID: 2476, EGAS50000001581). Targeted sequencing data from TR-14 cells are in the NCBI Sequence Read Archive (http://www.ncbi.nlm.nih.gov/Traces/sra; BioProject: PRJNA1301554; submission ID: SUB15515512). Original RQ-PCR data are available upon request.

3. Results

3.1. Integrating tumor genomic alterations into patient-individualized multiplexed PCR assays to sensitively detect multiple residual neuroblastoma clones

To enable individualized molecular monitoring of high-risk neuroblastoma, we developed a workflow using tumor-specific genomic alterations identified in tumor biopsies to design individualized multiplexed MP-PCR assays detecting and tracking MRD in BM. This workflow was optimized and validated using the TR14 neuroblastoma cell line and a BM specimen from *Patient 1*. MP-PCR assays were developed based on *MYCN* breakpoint sequences (Table 1; Suppl. Table S3), adapting a validated hydrolysis probe design [29] to MP-PCR (Suppl. Fig. S1). Optimal assay performance was observed when the mediator probe was positioned adjacent and downstream to the forward primer located within the specific target sequence upstream of the breakpoint. Mediator probes were placed $\geq$5 nt upstream of breakpoints. Assay sensitivity reached $10^{-5.0}$ to $10^{-6.0}$ (TR14) and $10^{-3.3}$ to $10^{-6.0}$ (BM; *Patient 1*; Fig. 1B; Suppl. Fig. S3, Suppl. Table S5).

As expected, targeted tumor sequencing revealed a diverse spectrum of somatic alterations in our patient cohort including *MYCN* breakpoints, *TERT* rearrangements and *ALK* and *PIK3CB* SNVs (Fig. 2, Suppl. Tables S3–4). In total, we developed individualized MP-PCR assays for 23 tumor-specific genomic breakpoints. A total of 96 primer pairs were screened to develop these assays, with 25% of assays requiring redesign before application. For each assay, several primer positions were tested to identify the most efficient design. Assays targeting *MYCN* amplicon breakpoints required the least optimization, likely attributable to the high coverage achieved by the neuroblastoma-specific panel sequencing combined with the elevated copy number, thereby enabling greater nucleotide-level accuracy and confidence. The complex rearrangements and repetitive sequences in other genomic region breakpoints (especially *TERT* and *NF1*) posed more design challenges. Breakpoints detected in tumor biopsies with higher variant read support ($\geq$10 reads) were more likely to yield successful PCR assays.

To develop multiplexed MP-PCR assays detecting tumor-specific SNVs, we tested two different SNV MP-PCR design approaches in single-plex (Suppl. Fig. S2). The universal reporter labeled with Cy5.5 BHQ2-amidit performed best for SNV detection. Each design approach accounted for SNV position relative to the probe and primers, and aimed to minimize unspecific background amplification (Suppl. Figs. S4–5). Both SNV assay design strategies performed comparably. Robust MP-PCR assays were developed for 2 SNVs in total for use in both single- and multi-plex settings. Collectively, all developed assays (4x 2-plex, 3x 4-plex, 1x 5-plex) enabled simultaneous detection of up to 5 structural variant rearrangements or SNV mutations per patient. The developed MP-PCR workflow enables reliable, patient-specific MRD detection by integrating genomic breakpoint and SNV identification, assay multiplexing and quantitative evaluation.

3.2. Multiplexed MP-PCR reliably quantifies neuroblastoma metastasis in bone marrow

To evaluate early neuroblastoma clonal dynamics in the BM niche, we performed individualized MP-PCR on longitudinal BM samples. The 4 *MYCN* breakpoints monitored by the 4-plex MP-PCR assay for *Patient 1* were detected at very high levels at induction chemotherapy start (Fig. 3A). All 4 markers declined after induction therapy and surgery, and were below the detection limit during consolidation therapy (Fig. 3A). In comparison, standard BM cytomorphology/immunocytology diagnostics for *Patient 1* only detected very low-level neuroblastoma infiltration at diagnosis and during induction chemotherapy and no tumor cells following induction or during consolidation therapy (Fig. 3A–Suppl. Table S6). Both standard BM diagnostics and MP-PCR for the 4 multiplexed markers were negative during follow-up. These findings demonstrate the higher sensitivity of individualized multiplexed MRD profiling using MP-PCR compared to the current standard. Negative values for all 4 *MYCN* markers in mononuclear BM cells from *Patient 1* aligned with durable remission, highlighting the potential of this approach to robustly reflect disease clearance by grasping different disease clones. To assess whether patterns of early marker decline and eventual clearance are consistent in other patients with favorable outcomes, we applied individualized MP-PCR assays to BM mononuclear cells from *Patient 2*. Two MP-PCR assays were created to monitor a total of 5 genomic breakpoints (3 *MYCN*, 1 *ACE2* on chromosome X, 1 intergenic upstream of *LOX* on chromosome 5). Assay sensitivity in BM mononuclear cells from *Patient 2* ranged from $10^{-3.3}$ to $10^{-5.0}$, with a quantitative individual breakpoint detection range from $10^{-3.3}$ to $10^{-4.3}$ (Suppl. Table S5). None of the 5 MP-PCR biomarkers were detected in the BM at diagnosis (Fig. 3B–Suppl. Table S6), suggesting none of the primary tumor clones infiltrated the BM. *Patients 1* and *2* responded favorably to induction therapy and remained in clinical remission at the conclusion of this study. Our findings demonstrate that multiplexed MP-PCR monitoring sensitively detects neuroblastoma cells infiltrating the BM, and can detect clonal disease dynamics. Detection of heterogeneous tumor clones is improved by combining multiple genetic markers. Monitoring robustness is also enhanced compared to conventional single-marker assays. Multiplexed MP-PCR monitoring supports a more accurate assessment of disease activity.

3.3. Multiplexed MP-PCR detects residual persisting neuroblastoma cell clones

We also explored clinical performance of individualized multiplexed MP-PCR assays in patients with persisting disease. An *ALK* SNV and 3 genomic breakpoints were monitored in the 4-plex assay for *Patient 3* (Suppl. Tables S3–4). Assay sensitivity ranged from $10^{-2.0}$ to $10^{-4.3}$, with a quantitative individual breakpoint detection range from $10^{-2.0}$ to $10^{-4.0}$ (Suppl. Table S5). All 4 biomarkers were detected at very high levels in samples from all 6 time points during multimodal therapy (Fig. 4A). Contrastingly, standard cytomorphology and GD2 immunocytology detected no neuroblastoma cells in BM from *Patient 3* (Fig. 4A–Suppl. Table S6), who experienced a central nervous system recurrence 11 months after diagnosis. The 2-plex assay for *Patient 4* assessed 2 breakpoints in *MYCN* and *GPHN* with individual breakpoint sensitivity ranging from $10^{-4.0}$ to $10^{-4.3}$ (Suppl. Table S5). Similar to results for *Patient 3*, we detected high biomarker levels throughout disease course (Fig. 4B), indicating the disease clone or clones persisted after induction chemotherapy. Conventional BM cytomorphology/

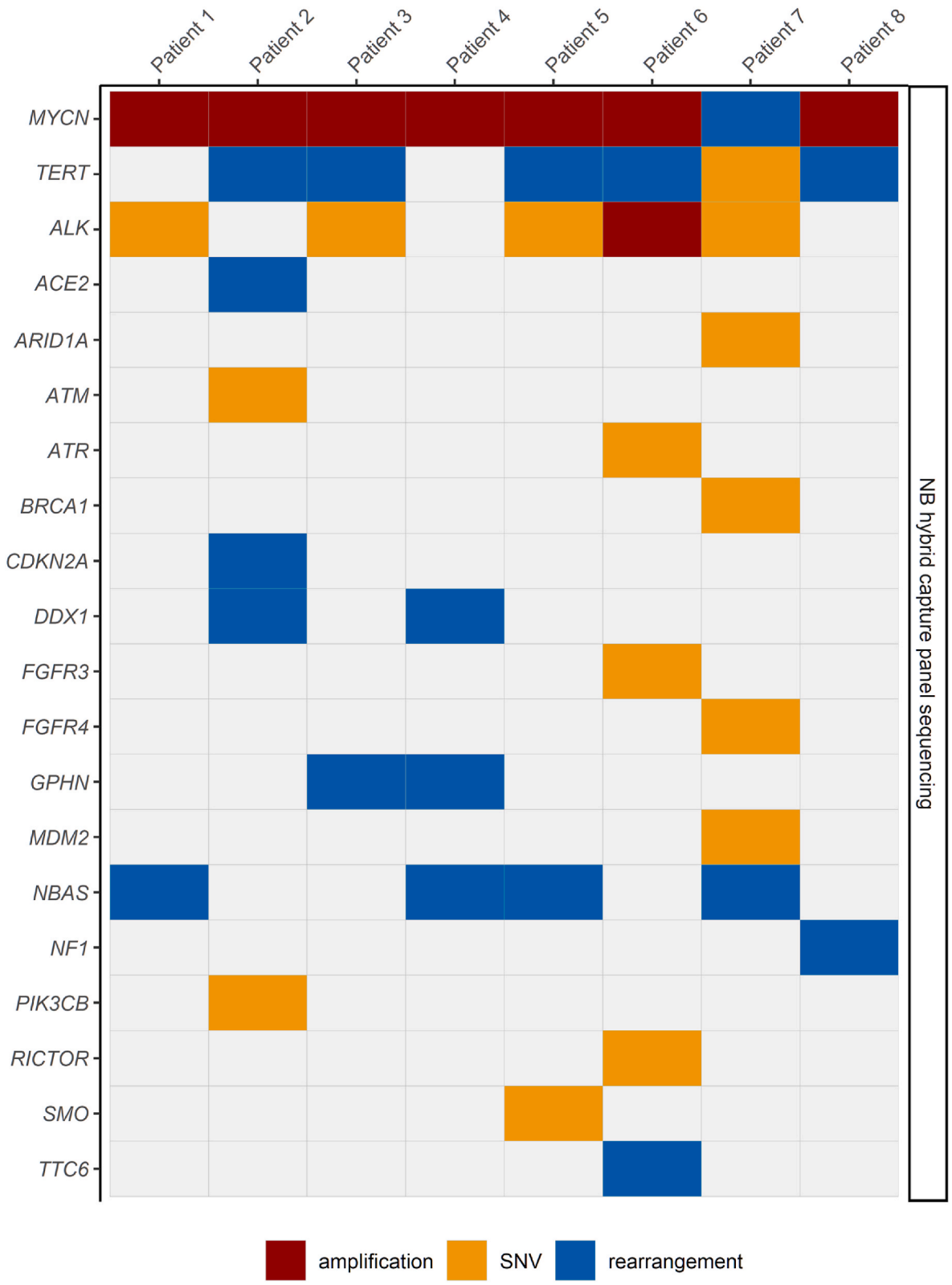


Fig. 2. Oncoprint displaying genomic alterations in tumor samples from our patient cohort. Genomic amplifications (red), rearrangements (blue) and single-nucleotide variants (SNVs, yellow) were detected using Neuroblastoma (NB) hybrid capture panel sequencing. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

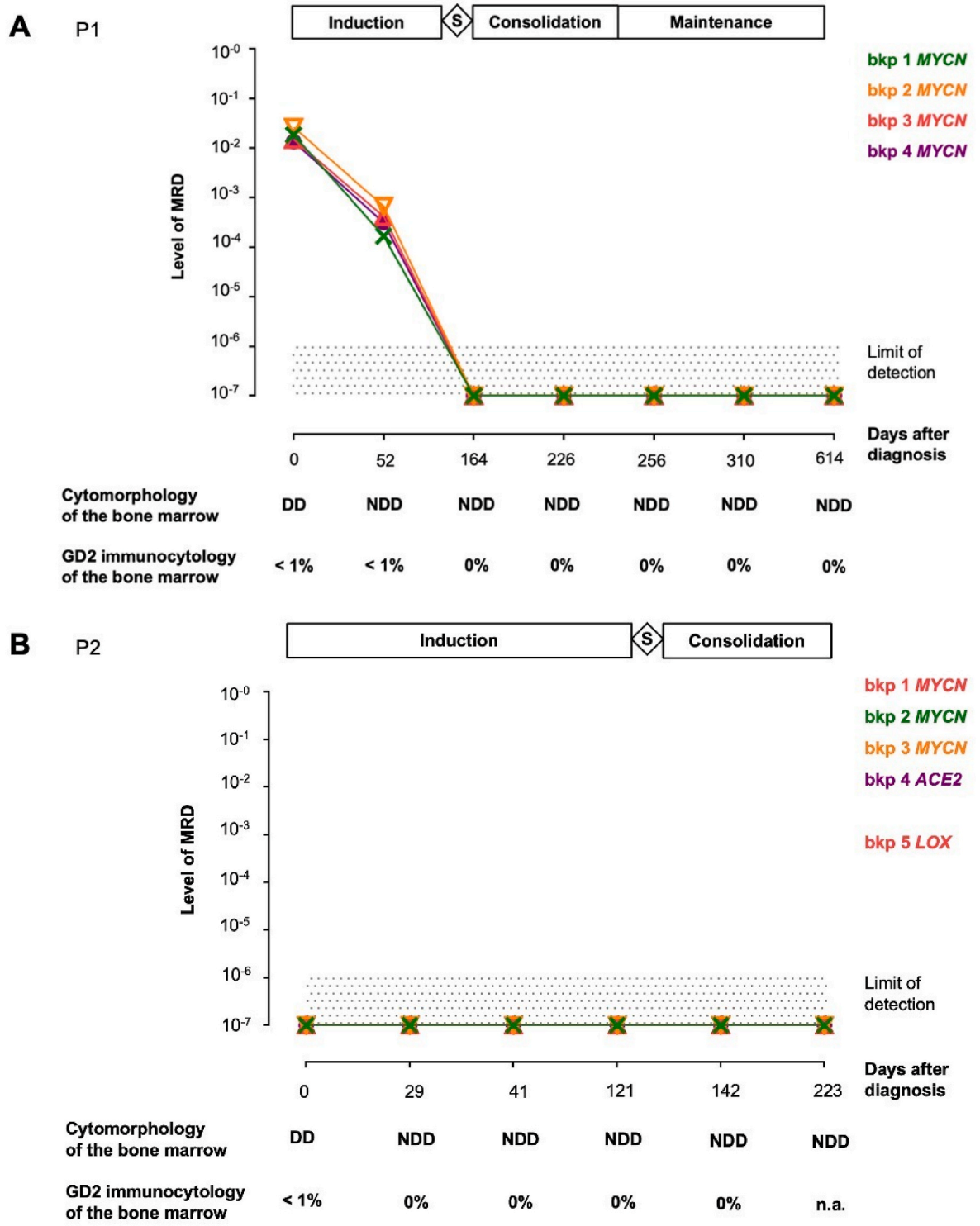


Fig. 3. MP-PCR identifies neuroblastoma remission in the bone marrow microenvironment in *Patients 1* and *2*. A, Bone marrow MRD levels were detected for *Patient 1* over the course of therapy. MRD levels were assessed throughout induction, consolidation and maintenance therapy over a time period of 614 days. The MP-PCR assay detected 4 *MYCN* breakpoints. **B,** Bone marrow MRD levels were detected for *Patient 2* over the course of therapy. MRD levels were assessed throughout induction and consolidation therapy over a time period of 223 days. Two MP-PCR assays detected 3 *MYCN* breakpoints and single breakpoints each in *LOX* and *ACE2*. Color coding represents the fluorescence color channels in which the respective genetic alterations were detected (*green*, FAM; *orange*, Atto-Rho-101; *red*, Atto-647-N; *crimson*, Cy5.5). Standard bone marrow diagnostics included cytomorphology and GD2 immunocytology performed on the mononuclear fraction from bone marrow aspirates. bkp, breakpoint; DD, detectable disease; MRD, minimal residual disease; n.a., not analyzed; NDD, no detectable disease; P1, *Patient 1*; P2, *Patient 2*; S, surgery. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

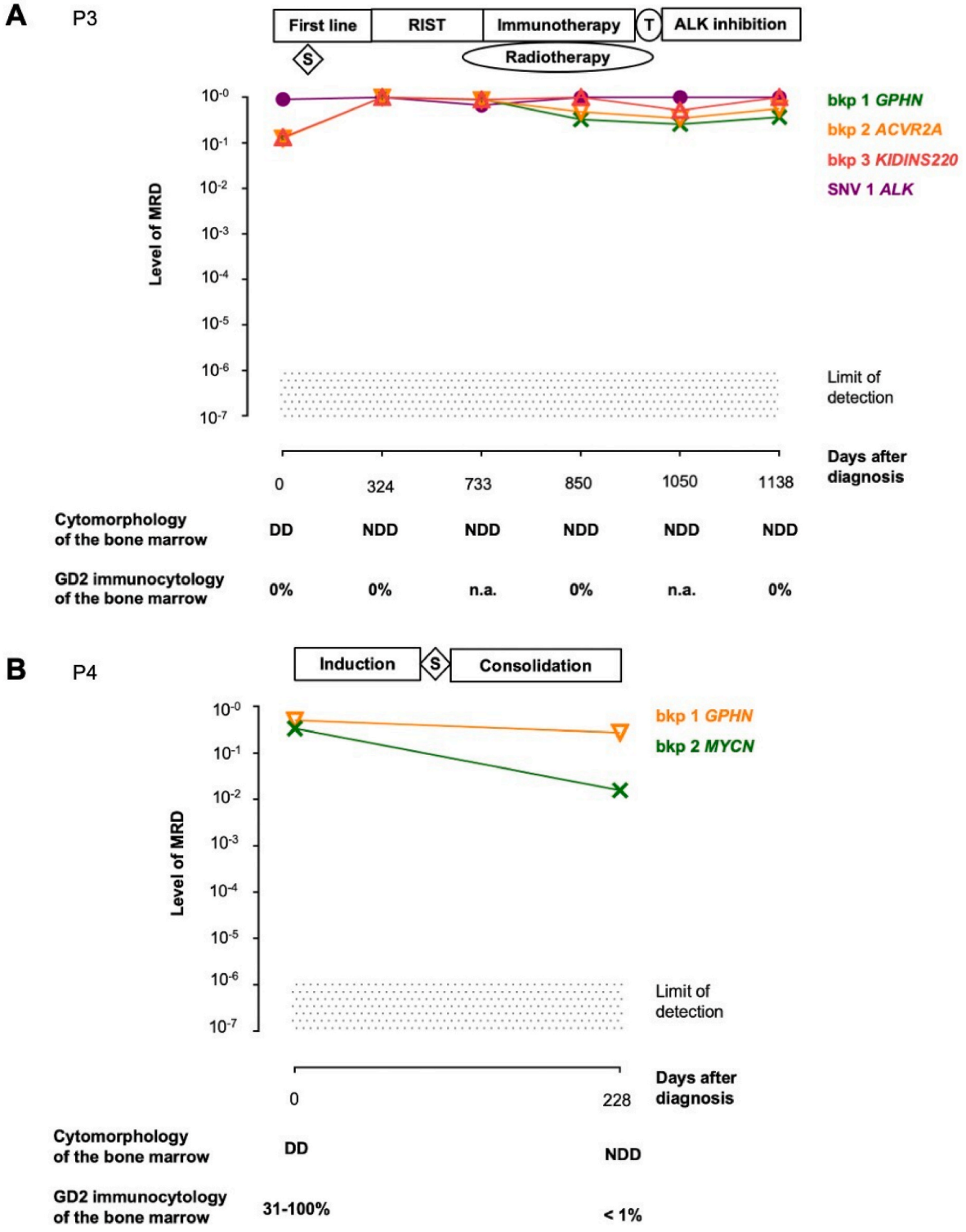


Fig. 4. MP-PCR identifies residual neuroblastoma clones following induction therapy in *Patients 3* and *4*. A, Bone marrow MRD levels were detected for *Patient 3* over the course of therapy. MRD levels were assessed over a time period of 1138 days throughout first-line polychemotherapy, second-line therapy according to RIST-rNB-2011, immunotherapy with the dinutoximab beta GD2 antibody, radiotherapy and ALK inhibition with lorlatinib. The MP-PCR assay detected breakpoints in *GPHN*, *ACVR2A*, *KIDINS220* and one SNV in *ALK*. **B,** Bone marrow MRD levels were detected for *Patient 4* over the course of therapy. MRD levels were assessed over a time period of 228 days throughout induction and consolidation therapy. The MP-PCR assay detected breakpoints in *MYCN* and *GPHN*. Color coding represents the fluorescence color channels in which the respective genetic alterations were detected (*green*, FAM; *orange*, Atto-Rho-101; *red*, Atto-647-N; *crimson*, Cy5.5). Standard bone marrow diagnostics included cytomorphology and GD2 immunocytology performed on the mononuclear cell fraction from bone marrow aspirates. Bkp, breakpoint; DD, detectable disease; MRD, minimal residual disease; n.a., not analyzed; NDD, no detectable disease; P3, *Patient 3*, P4, *Patient 4*; S, surgery; T, haploidentical stem cell transplantation. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

immunocytology showed early biomarker decline that remained negative after induction therapy in *Patient 4* (Fig. 4B–Suppl. Table S6). In patients with poor outcomes (*Patients 3 & 4*), biomarker levels remained high throughout therapy. Despite consistently high overall biomarker levels in BM from *Patient 3*, longitudinal profiling revealed distinct fluctuations in individual biomarker dynamics over time, as well as variability between different biomarkers at the same sampling time point (Fig. 4A). These findings demonstrate that sustained biomarker positivity detected by multiplexed genetic marker profiling can also capture clonal heterogeneity and residual disease activity in the BM niche.

3.4. Longitudinal multiplexed genetic marker profiling reveals clonal dynamics

To investigate the value of individual markers in multiplexed detection of clonal heterogeneity, we applied MP-PCR to longitudinally monitor multiple tumor-specific breakpoints in 2 patients. The 4-plex assay for *Patient 5* monitored 3 different *MYCN* breakpoints and 1 *TERT* breakpoint (Suppl. Table S5). MP-PCR detected only 2 of the *MYCN* breakpoints in BM specimens collected at diagnosis (Fig. 5A), although all breakpoints were detected in the primary tumor biopsy. In the following, none of the 2 initially positive MP-PCR markers were detected in BM, indicating therapy had eradicated both *MYCN* breakpoint clones. These findings demonstrate that multiplexed MP-PCR can detect and monitor divergent clonal responses to therapy. The complexity of residual disease monitoring is also demonstrated in a further patient. The 2-plex assay for *Patient 6* monitored 1 *MYCN* breakpoint and 1 *TTC6* breakpoint (Suppl. Table S5). High biomarker levels were detected at relapse in BM from *Patient 6* (Fig. 5B). MP-PCR no longer detected the *MYCN* breakpoint in BM during RIST-rNB-2011 treatment, but detected the *TTC6* breakpoint throughout the complete course of therapy (Fig. 5B; Suppl. Table S6). Thus, *Patient 6* never achieved molecular remission. These findings highlight that individual MRD biomarkers can exhibit distinct longitudinal patterns, with some dropping below the detection limit early during therapy and others persisting throughout disease course. Such clonal fluctuations underscore the underlying tumor heterogeneity and demonstrate that no single biomarker can reliably capture residual disease dynamics. Comprehensive multiplexed MRD monitoring improves accounting for clonal complexity to provide more accurate disease surveillance.

3.5. Multiplexed biomarker detection provides superior prognostic accuracy to conventional immunocytology

To better understand the molecular significance of persistent MRD signals undetected by conventional diagnostics, we closely examined two cases with fatal outcomes, comparing individualized multiplexed MP-PCR biomarker trajectories against standard follow-up methods across longitudinal BM sampling. The 2-plex MP-PCR assay designed for *Patient 7* monitored 1 *MYCN* breakpoint and 1 *BRCA1* SNV (Suppl. Tables S3–5). Both genetic biomarkers were detected in all 5 longitudinal sampling points at high quantitative levels (Fig. 6A). No neuroblastoma cells were detected in routine BM cytomorphology diagnostics from the third sampling point onward. GD2 immunocytology detected strong neuroblastoma infiltration of BM samples at initial diagnosis, which dropped to <1% infiltration from sampling day 25 of induction therapy onward (Fig. 6A; Suppl. Table S6). Our findings from *Patient 7* prompted continued investigation into clonal biomarker dynamics to better understand the relationship between heterogeneity in mediator-probe PCR-based multiplexed MRD biomarker detection and the current state-of-the-art BM diagnostic standard. The 2-plex assay designed for *Patient 8* monitored 1 *MYCN* breakpoint and 1 *NF1* breakpoint (Suppl. Table S5). Both biomarkers were quantified at 10^1 at initial diagnosis, in concordance with GD2 immunocytology and confirming the strong initial BM infiltration (Fig. 6B; Suppl. Table S6). During induction therapy, *NF1* breakpoint detection remained high, while *MYCN* breakpoint detection dimished. In contrast, standard-of-care BM diagnostics showed no or very low neuroblastoma cell infiltration during induction therapy (Fig. 6B; Suppl. Table S6). Biomarker fluctuations did not collectively always mirror clinical remission or relapse timing based on gold-standard cytology, as demonstrated for *Patient 3* (Fig. 4A), *Patient 6* (Fig. 5B) and *Patient 7* (Fig. 6A), where MRD remained detectable by multiplexed MP-PCR throughout therapy despite negative immunocytology. This highlights that molecular MRD assays can capture low-level disease that escapes conventional BM diagnostics. For one case (*Patient 6*), MP-PCR identified early molecular signs of relapse, which were later clinically confirmed. Finally, genetic markers for disease drivers were detected by sequencing in the tumor biopsies for *Patient 2* and *Patient* 5 that were not captured in the infiltrated BM aspirates (Figs. 3B and 5A). These findings demonstrate that multiplexed biomarker detection provides superior diagnostic accuracy to conventional BM cytomorphology and GD2 immunocytology. Altogether, our findings strongly support the value of a multiplexed PCR approach to serially monitor the bone marrow niche in patients with high-risk neuroblastoma.

4. Discussion

We demonstrate the clinical utility of patient-specific multiplexed MP-PCR assays to sensitively monitor minimal residual disease in the bone marrow niche in a pilot cohort of eight children with high-risk neuroblastoma. Individualized genetic biomarkers, structural variants and single-nucleotide variants can be robustly detected in the mononuclear cell fraction from bone marrow aspirates at levels below detection limits of conventional cytomorphology and GD2 immunocytology. MP-PCR detects MRD across a range of mutational profiles with high specificity and sensitivity, allowing precise individualized disease monitoring. Underscoring the limitations of current clinical tools to capture the full extent of disease persistence, MRD levels remained markedly elevated throughout treatment in selected cases despite negative or low-level detection by standard-of-care diagnostics. Our findings reveal considerable molecular heterogeneity and dynamic shifts in clonal biomarker profiles during disease course, reflecting the complex biology of treatment-resistant neuroblastoma and difficulty to comprehensively monitor MRD. Our findings emphasize the added value of patient-specific, multiplexed approaches to improve disease monitoring.

MRD assessment offers quantitative insights into tumor cell burden and its dynamic changes over time [4]. High MRD levels serve as a strong predictor of poor outcome. MRD below the threshold has been correlated with improved event-free and overall survival in pediatric [28] and adult patients with ALL for over 20 years [30]. The BM is a key site for MRD assessment for both leukemias, where disease originates, and high-risk neuroblastomas, a common site of distant metastasis. We developed patient-specific multiplexed MP-PCR assays that simultaneously detect up to four different tumor-related genomic breakpoints or SNVs in mononuclear cells from BM aspirates for neuroblastoma MRD detection. Each target was detected with a sensitivity of up to 10^{-6}, detecting a single tumor cell in a pool of one million reference cells. Our findings support the use of MP-PCR assays as a sensitive tool to molecularly monitor neuroblastoma MRD and inform future personalized diagnostic strategies.

DNA breakpoint sequences can serve as excellent biomarkers to reliably monitor cancer in patients [31]. Incorporating patient-specific genomic breakpoints into personalized assays presents a promising strategy for sensitive molecular monitoring in high-risk neuroblastoma. *MYCN* amplification is a defining feature of aggressive biology in approximately 40% of high-risk neuroblastomas [17,32], giving breakpoints located within *MYCN* amplicons particular clinical relevance. *MYCN* breakpoints are absent from healthy cells and clonally stable over time [29], and in our experience, were highly specific to the tumor

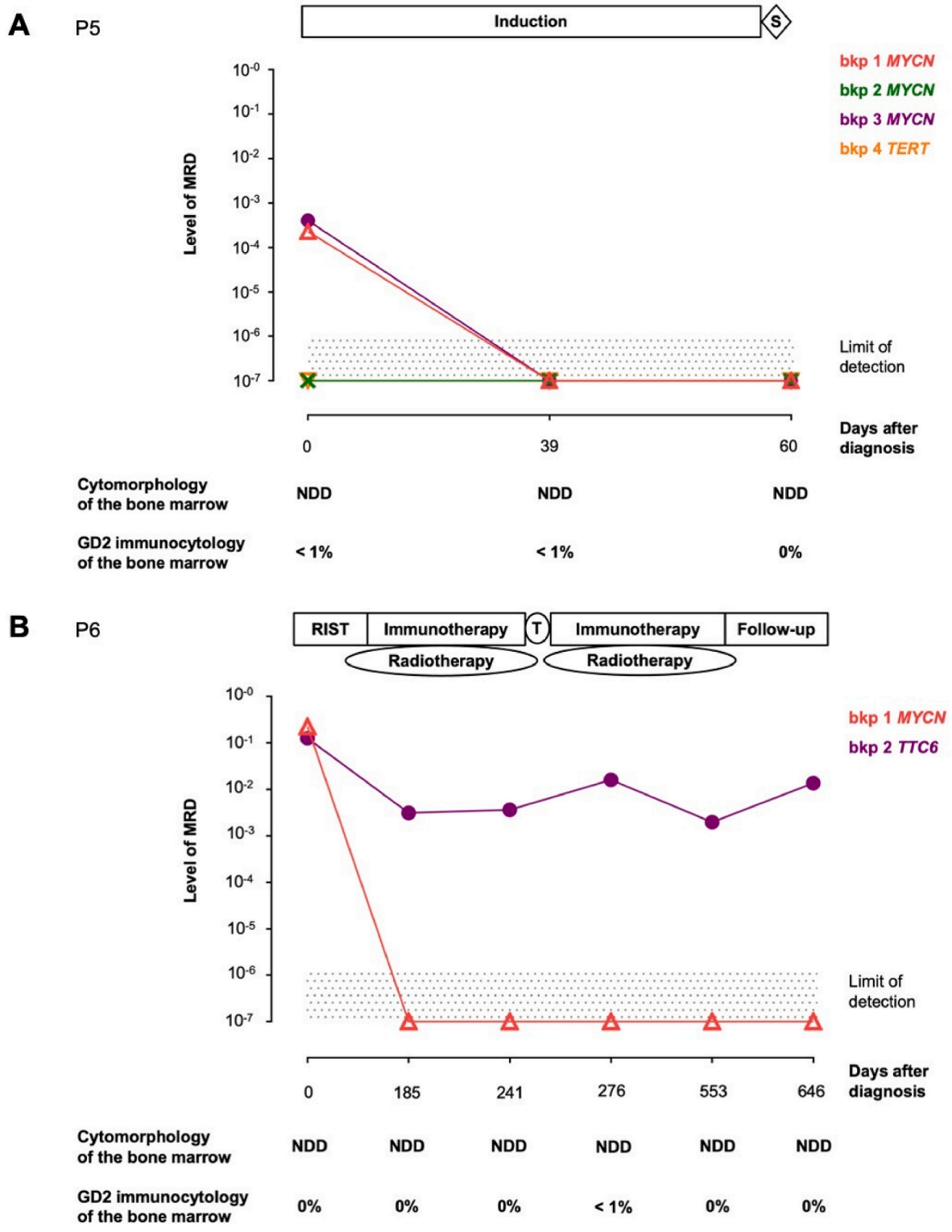


Fig. 5. Longitudinal assessment of multiplexed genetic biomarkers uncovers clonal diversity and dynamics in the bone marrow from *Patients 5* and *6*. A, Bone marrow MRD levels were detected for *Patient 5* over the course of therapy. MRD levels were assessed over a time period of 60 days throughout induction therapy. The MP-PCR assay detected four breakpoints in *MYCN* and *TERT*. **B,** Bone marrow MRD levels were detected for *Patient 6* over the course of therapy. MRD levels were assessed over a time period of 646 days throughout relapse therapy including chemotherapy according to RIST-rNB-2011, immunotherapy with the dinutuximab beta GD2 antibody and follow-up. The assay detected 1 breakpoint each in *MYCN* and *TTC6*. Color coding represents the fluorescence color channels in which the respective genetic alterations were detected (*green*, FAM; *orange*, Atto-Rho-101; *red*, Atto-647-N; *crimson*, Cy5.5). Standard bone marrow diagnostics included cytomorphology and GD2 immunocytology performed on the mononuclear cell fraction from bone marrow aspirates.bkp, breakpoint; DD, detectable disease; MRD, minimal residual disease; n.a., not analyzed; NDD, no detectable disease; P5, *Patient 5*; P6, *Patient 6*; S, surgery. T, haploidentical stem cell transplantation. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

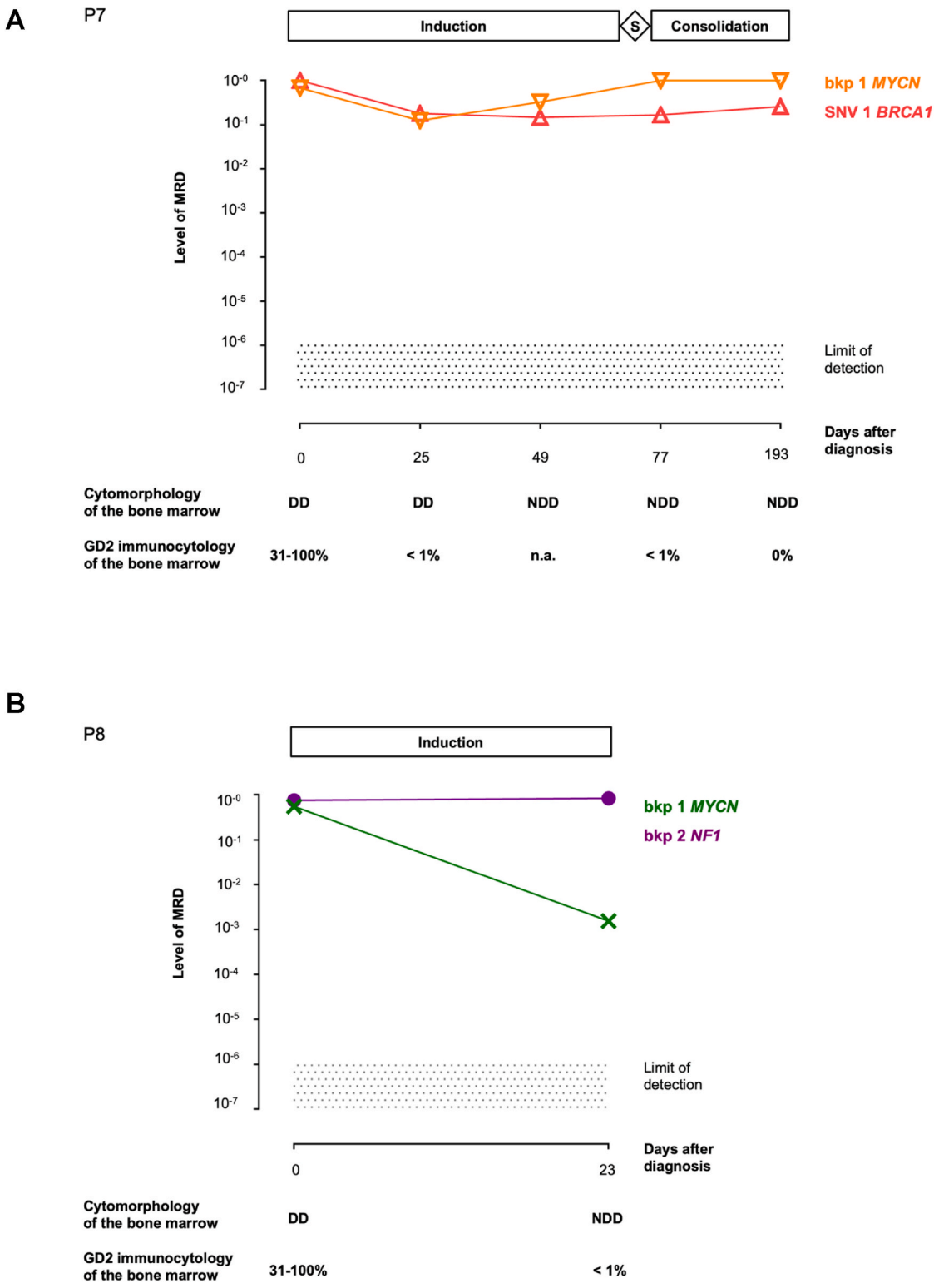


Fig. 6. Multiplexing neuroblastoma biomarkers enhance prognostic accuracy over conventional neuroblastoma follow-up diagnostics in *Patients 7* and *8*. A, Bone marrow MRD levels were detected for *Patient 7* over the course of therapy. MRD levels were assessed over a time period of 193 days throughout induction and consolidation therapy. The PCR assay detected one breakpoint in *MYCN* and one SNV in *BRCA1*. **B,** Bone marrow MRD levels were detected for *Patient 8* over the course of therapy. MRD levels were assessed over a time period of 23 days throughout induction therapy. The assay detected one breakpoint each in *MYCN* and *NF1*. Color coding represents the fluorescence color channels in which the respective genetic alterations were detected (*green*, FAM; *orange*, Atto-Rho-101; *red*, Atto-647-N; *crimson*, Cy5.5). Standard bone marrow diagnostics included cytomorphology and GD2 immunocytology performed on the mononuclear cell fraction from bone marrow aspirates. bkp, breakpoint; DD, detectable disease; MRD, minimal residual disease; n.a., not analyzed; NDD, no detectable disease; S, surgery; T, haploidentical stem cell transplantation. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

genome, making them excellent targets for molecular monitoring. Genomic breakpoints have several advantages over SNVs as targets. Their tumor specificity and stability enable highly sensitive detection, reaching as low as 10^{-6} in our MP-PCR assays. Background amplification is lower and reproducibility improved in PCR-based assays targeting breakpoints compared to SNVs, which often suffer from suboptimal allele frequency and specificity. The limited prevalence of recurrent alterations (e.g., *ALK* mutations, *MYCN* amplifications, *TERT* rearrangements) cause considerable inter-patient heterogeneity in high-risk neuroblastoma cases [33] and make personalized approaches necessary to molecularly monitor disease. Logistic and technical issues make implementing individualized assays in clinical workflows challenging. Our multiplexed MP-PCR assay design represents a feasible and efficient compromise for design, providing flexibility to integrate both broadly informative (e.g., *ALK* SNVs) and patient-specific (e.g. genomic breakpoints) targets. We demonstrate that combining one common alteration with three individualized breakpoints enables detection across a wider clonal population in the patient while preserving assay sensitivity and specificity. Multiplexing allows simultaneous detection of multiple tumor-associated signals from minimal sample input, which is particularly relevant in pediatric settings with limited biosample volume. Tailoring assay design to each patient's molecular profile supports refined therapeutic decision-making and longitudinal disease tracking throughout treatment and follow-up.

Single-plex assays targeting a *MYCN* breakpoint consistently yielded the highest design success and required the least optimization, highlighting their robustness for use in multiplexed PCR. These points present *MYCN*-specific breakpoints as reliable biomarkers in multiplexed MP-PCR to monitor neuroblastoma MRD. Genomic alterations with rearrangements or breakpoints at highly fragmented genes with repetitive sequences complicated MP-PCR assay design. MP-PCR assay design for genomic breakpoints produced less (or no) background amplification (compared to SNVs), required fewer optimization steps and were more likely to yield successful results. Accordingly, genomic breakpoints predominated in all multiplexed MP-PCR assays developed for the 8 patients in our pilot cohort. Higher sensitivities were achieved using genomic breakpoints (10^{-5}, especially for *MYCN* amplicons) than SNVs (10^{-4}), possibly due to the elevated copy number. Our previous work showed that neuroblastomas have a broad SNV mutation pattern, particularly within the *ALK*, *BRCA1/2*, *FGFR3/4* and *PIK3CB* genes [18]. SNVs included in our multiplexed assays still scored deep sensitivities (up to 10^{-4}) even though lower average sensitivities result if wildtype gene frequency is high compared to the SNV. SNV presence alone has clinical relevance, since detecting therapeutically actionable mutations can guide treatment decisions (e.g., switching to or adding a targeted ALK inhibitor). Integrating SNVs into multiplexed MP-PCR assays for MRD also better captures disease heterogeneity in the patient, thereby increasing diagnostic accuracy. Recent advances in digital and quantitative PCR technologies have demonstrated the feasibility of high-level multiplexing, with assays simultaneously detecting more than six targets using generic reporter sets or colorimetric probe panels [34,35]. High-multiplexing PCR strategies allow broader genomic coverage without compromising sensitivity or specificity. Adopting highly multiplexed PCR formats for neuroblastoma MRD diagnostics could substantially improve detection of heterogeneous disease, and represents a crucial step toward more comprehensive and personalized molecular monitoring.

A first study by Kipf et al. successfully demonstrated proof-of-concept for MP-PCR application to detect TCR/Ig gene rearrangements, fusions or deletions in pediatric patients with relapsed acute lymphoblastic leukemia [27]. Target-specific fluorogenic hydrolysis TaqMan probes were used in two further published approaches to interrogate *MYCN* rearrangement breakpoints for neuroblastoma MRD detection [36,37], though both excluded complex genomic regions and used low-resolution or costly platforms. Both studies only achieved sensitivities of 10^{-4} to 10^{-5} in multiplexed settings [38]. Here we extend this proof-of-concept for MP-PCR application. High-risk neuroblastoma is highly heterogeneous within each patient [39] and evolves clonally through the course of disease [40]. No approach currently exists to predict which clones will prevail [41]. Analysis of different *MYCN* rearrangements raised the question whether they originated from a single tumor clone or from multiple subclones. Their synchronized longitudinal dynamics suggest they stemmed from the same clonal origin. This finding warrants confirmation in a large co-clinical trial.

To date, neuroblastoma-specific RNA targets, including *PHOX2B*, *TH* and *DCX* [31,42] have predominantly been used in multiplexed MRD monitoring approaches. RNA-based assays achieve high sensitivity for viable neuroblastoma cells, but depend on cell viability and target gene expression. This may lead to the underdetection of mesenchymal or dormant tumor cells with low transcriptional activity. Combining DNA- and RNA-based targets is currently under investigation in several laboratories.

Our data indicate that patient-specific assays based on genomic breakpoints or structural variants provide high MRD sensitivity, while being applicable for a variety of different genomic alterations. We demonstrate that monitoring minimally two, and preferably four, molecular targets per patient is necessary to effectively capture clonal evolution over the course of therapy and relapse. Patient-specific *MYCN* breakpoints emerged in our data as the most stable targets. Thus, we recommend including at least one *MYCN* breakpoint in the personalized multiplex panel for patients with *MYCN*-amplified neuroblastoma. A hybrid assay design combining two patient-specific markers (such as *MYCN or TERT* breakpoints) with two recurrent genomic targets (e.g., *ALK* SNVs, RAS signaling SNVs) could balance feasibility with diagnostic depth. Multiplexing is a relatively recent application within digital PCR technology and is becoming more popular for diagnostics because it allows the simultaneous quantification of multiple target and reference sequences within a single PCR reaction [43,44]. Although extensive optimization for multiplexed assays requires time and effort, it ultimately proves worthwhile when analyzing limited clinical material (e.g. from infants and young children), as it markedly improves reproducibility, data quality and mutation detection reliability [45,46]. We established a multiplexed workflow that efficiently screens multiple genomic targets from minimal sample input, maximizing information gain from each sample. Our findings underscore the potential of multiplexed biomarker monitoring to inform targeted therapy decisions and improve individualized disease management through a clearer picture of the molecular complexity of each patient's disease.

CRediT authorship contribution statement

Anna M. Schroeer: Writing – original draft, Visualization, Software, Methodology, Investigation, Formal analysis, Data curation. **Annika Winkler:** Writing – original draft, Visualization, Software, Methodology, Investigation, Data curation. **Marion Fillies:** Visualization, Software, Methodology, Investigation, Data curation. **Charleen Kranig:** Software, Methodology, Investigation. **Eliz Karaman:** Visualization, Software, Methodology, Investigation, Data curation. **Felix von Stetten:** Resources, Funding acquisition, Conceptualization. **Roland Zengerle:** Resources, Funding acquisition, Conceptualization. **Michael Lehnert:** Resources, Methodology, Funding acquisition, Conceptualization. **Alexander Blume:** Visualization, Software, Methodology, Formal analysis, Data curation. **Jan F. Hollander:** Visualization, Software, Methodology, Data curation. **Marco Lodrini:** Visualization, Supervision, Software, Data curation. **Angelika Eggert:** Resources, Project administration, Conceptualization. **Altuna Akalin:** Software, Resources, Project administration, Funding acquisition. **Kathy Astrahantseff:** Writing – original draft, Visualization. **Cornelia Eckert:** Writing – original draft, Visualization, Supervision, Resources, Project administration, Funding acquisition, Conceptualization. **Hedwig E. Deubzer:** Writing – original draft, Visualization, Supervision, Resources, Project administration, Funding acquisition, Conceptualization.

Annabell Szymansky: Writing – original draft, Visualization, Supervision, Software, Project administration, Methodology, Formal analysis, Data curation.

Financial support

This work was supported by the Deutsche Forschungsgemeinschaft (DFG, German Research Foundation) through the Collaborative Research Center "Decoding and Targeting Neuroblastoma Evolution" CRC1588 (project number 493872418) to A.E., M.L. and H.E.D.; by the European Union (EU) and German Federal Ministry of Research, Technology and Space (BMFTR) through the TRANSCAN-3 EXPLORE-NB consortium (01KT2401A) to A.A. and H.E.D.; by the former Federal Ministry of Education and Research (BMBF) project IRMA-4-ALL grant (FKZ 01 EK1508A,B,D); and the Ministry of Economic Affairs, Labor and Tourism Baden-Württemberg, Germany, project PRIMO grant (AZ 3-4332.62-HSG/84) to F.v.S., R.Z., M.L. and C.E.; by an individual scholarship (Promotionsstipendium) from the Kind-Philipp Foundation to A.M.S.; by an individual scholarship from Friedrich-Ebert-Stiftung to E.K., by the Junior Clinician Scientist Program by the CRC1588 to J.F.H., by the Charité-BIH Advanced Clinician Scientist Program to H.E.D.; and by the Charité Medical Scientist Program and the Lydia Rabinowitsch grant to A.S.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Acknowledgements

The authors thank the patients and their parents, who agreed to take part in this study, all physicians involved in biosample collection, our hematological laboratory technicians, Constanze Passenheim, Nadine Sachs and Simone Roβa, for their assistance in sample preparation; the CharLi Liquid Biopsy Laboratory technicians, Jasmin Wünschel, Robin Gottschalk and Franziska Seifert, for their technical assistance; Andrea Menne from the Pediatric Surgery Research for her valuable laboratory support and Aneka Laura Frietsch from Hahn-Schickard-Gesellschaft for her support with initial MP-PCR assay development.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.canlet.2026.218352.

References

[1] K.S. Lin, S. Uemura, K.K.M. Thwin, N. Nakatani, T. Ishida, N. Yamamoto, et al., Minimal residual disease in high-risk neuroblastoma shows a dynamic and disease burden-dependent correlation between bone marrow and peripheral blood, Transl. Oncol. 14 (8) (2021 Aug 1) 101019.
[2] V.P. Tolbert, K.K. Matthay, Neuroblastoma: clinical and biological approach to risk stratification and treatment, Cell Tissue Res. 372 (2) (2018 May 1) 195.
[3] C. Hochheuser, L.J. Windt, N.Y. Kunze, DiL. De Vos, G.A.M. Tytgat, C. Voermans, et al., Mesenchymal stromal cells in neuroblastoma: exploring crosstalk and therapeutic implications, Stem Cell. Dev. 30 (2) (2021 Jan 15) 59.
[4] S. Uemura, T. Ishida, K.K.M. Thwin, N. Yamamoto, A. Tamura, K. Kishimoto, et al., Dynamics of minimal residual disease in neuroblastoma patients, Front. Oncol. 9 (JUN) (2019).
[5] S.A. Burchill, K. Beiske, H. Shimada, P.F. Ambros, R. Seeger, G.A.M. Tytgat, et al., Recommendations for the standardization of bone marrow disease assessment and reporting in children with neuroblastoma on behalf of the international neuroblastoma response criteria bone marrow working group, Cancer 123 (7) (2017 Apr 1) 1095–1105.
[6] M. Brüggemann, M. Kotrová, H. Knecht, J. Bartram, M. Boudjogrha, V. Bystry, et al., Standardized next-generation sequencing of immunoglobulin and T-cell receptor gene recombinations for MRD marker identification in acute lymphoblastic leukaemia; a EuroClonality-NGS validation study, Leukemia 33 (9) (2019 Sep 1) 2241–2253.
[7] M. Schwab, H.E. Varmus, J.M. Bishop, K.H. Grzeschik, S.L. Naylor, A.Y. Sakaguchi, et al., Chromosome localization in normal human cells and neuroblastomas of a gene related to c-myc, Nature 308 (5956) (1984 Mar) 288–291.
[8] G.M. Brodeur, R.C. Seeger, M. Schwab, H.E. Varmus, J. Michael Bishop, Amplification of N-myc in untreated human neuroblastomas correlates with advanced disease stage, Science 224 (4653) (1984 Jun 8) 1121–1124.
[9] C. Rosswog, J. Fassunke, A. Ernst, B. Schömig-Markiefka, S. Merkelbach-Bruse, C. Bartenhagen, et al., Genomic ALK alterations in primary and relapsed neuroblastoma, Br. J. Cancer 128 (8) (2023 Apr 12) 1559–1571.
[10] E.R. Berko, A. Naranjo, A.A. Daniels, S.N. McNulty, K. Krytska, T. Druley, et al., Frequency and clinical significance of clonal and subclonal driver mutations in high-risk neuroblastoma at diagnosis: a children's oncology group study, J. Clin. Oncol. 43 (14) (2025 May 10) 1673–1684.
[11] M. Peifer, F. Hertwig, F. Roels, D. Dreidax, M. Gartlgruber, R. Menon, et al., Telomerase activation by genomic rearrangements in high-risk neuroblastoma, Nature 526 (7575) (2015 Oct 29) 700–704.
[12] M. Zeineldin, S. Federico, X. Chen, Y. Fan, B. Xu, E. Stewart, et al., MYCN amplification and ATRX mutations are incompatible in neuroblastoma, Nat. Commun. 11 (1) (2020 Feb 14) 1–20.
[13] L. Werr, C. Rosswog, C. Bartenhagen, S.L. George, M. Fischer, Telomere maintenance mechanisms in neuroblastoma: new insights and translational implications, EJC Paediatr. Oncol. 3 (2024 Jun 1) 100156.
[14] O.N. Kostopoulou, S. Holzhauser, B.K.A. Lange, A. Ohmayer, T. Andonova, C. Bersani, et al., Analyses of FGFR3 and PIK3CA mutations in neuroblastomas and the effects of the corresponding inhibitors on neuroblastoma cell lines, Int. J. Oncol. 55 (6) (2019) 1372–1384.
[15] C.T. Storlazzi, A. Lonoce, M.C. Guastadisegni, D. Trombetta, P. D'Addabbo, G. Daniele, et al., Gene amplification as double minutes or homogeneously staining regions in solid tumors: origin and structure, Genome Res. 20 (9) (2010 Sep) 1198–1206.
[16] S. He, M.R. Mansour, M.W. Zimmerman, D.H. Ki, H.M. Layden, K. Akahane, et al., Synergy between loss of NF1 and overexpression of MYCN in neuroblastoma is mediated by the GAP-related domain, eLife 5 (2016 Apr 27).
[17] S. Ackermann, M. Cartolano, B. Hero, A. Welte, Y. Kahlert, A. Roderwieser, et al., A mechanistic classification of clinical phenotypes in neuroblastoma, Science 362 (6419) (2018) 1165–1170.
[18] A. Szymansky, L.M. Kruetzfeldt, L.C. Heukamp, F. Hertwig, J. Theissen, H. E. Deubzer, et al., Neuroblastoma risk assessment and treatment stratification with hybrid capture-based panel sequencing, J. Personalized Med. 11 (8) (2021) 691.
[19] M. Lodrini, J. Graef, T.M. Thole-Kliesch, K. Astrahantseff, A. Sprussel, M. Grimaldi, et al., Targeted analysis of cell-free circulating tumor DNA is suitable for early relapse and actionable target detection in patients with neuroblastoma, Clin. Cancer Res. 28 (9) (2022; May 1) 1809–1820.
[20] S. Wadle, S. Rubenwolf, M. Lehnert, B. Faltin, M. Weidmann, F. Hufert, et al., Mediator probe PCR: detection of real-time PCR by label-free probes and a universal fluorogenic reporter, Methods Mol. Biol. 1160 (2014) 55–73.
[21] F. Schlenker, E. Kipf, M. Deuter, I. Höffkes, M. Lehnert, R. Zengerle, et al., Stringent base specific and optimization-free multiplex mediator probe ddPCR for the quantification of point mutations in circulating tumor DNA, Cancers (Basel) 13 (22) (2021 Nov 1).
[22] M. Lehnert, E. Kipf, F. Schlenker, N. Borst, R. Zengerle, F. Von Stetten, Fluorescence signal-to-noise optimisation for real-time PCR using universal reporter oligonucleotides, Anal. Methods 10 (28) (2018 Jul 19) 3444–3454.
[23] T. Simon, T. Thole, S. Castelli, B. Timmermann, D. Jazmati, R. Schwarz, et al., GPOH guidelines for diagnosis and first-line treatment of patients with neuroblastic tumors, update 2025, Klin. Pädiatr. 237 (3) (2025 May 9) 117–140.
[24] C. Schwinghammer, Droplet Digital PCR Zur Quantifizierung Der Minimalen Resterkrankung (MRD) Bei Der Akuten Lymphoblastischen Leukämie. Doctoral Dissertation, Refubium, Freie Universität Berlin, 2024.
[25] F. Castro, W.G. Dirks, S. Fähnrich, A. Hotz-Wagenblatt, M. Pawlita, M. Schmitt, High-throughput SNP-based authentication of human cell lines, Int. J. Cancer 132 (2) (2013 Jan 15) 308–314.
[26] M. Schmitt, M. Pawlita, High-throughput detection and multiplex identification of cell contaminations, Nucleic Acids Res. 37 (18) (2009) e119.
[27] E. Kipf, F. Schlenker, N. Borst, M. Fillies, R. Kirschner-Schwabe, R. Zengerle, et al., Advanced minimal residual disease monitoring for acute lymphoblastic leukemia with multiplex mediator probe PCR, J. Mol. Diagn. 24 (1) (2022 Jan 1) 57–68.
[28] V.H.J. van der Velden, G. Cazzaniga, A. Schrauder, J. Hancock, P. Bader, E. R. Panzer-Grumayer, et al., Analysis of minimal residual disease by Ig/TCR gene rearrangements: guidelines for interpretation of real-time quantitative PCR data, Leukemia 21 (4) (2007) 604–611.
[29] A. Szymansky, F. Hertwig, A. Winkler, R. Chamorro González, K. Helmsauer, S. Fuchs, et al., Quantitative and sensitive neuroblastoma minimal residual disease detection using extrachromosomal DNA (ecDNA) MYCN breakpoints, in: Advances in Neuroblastoma Research Meeting - ANR2023, 2023. Amsterdam, Netherlands.
[30] V.H.J. van der Velden, A. Hochhaus, G. Cazzaniga, T. Szczepanski, J. Gabert, J.J. M. van Dongen, Detection of minimal residual disease in hematologic malignancies by real-time quantitative PCR: principles, approaches, and laboratory aspects, Leukemia 17 (6) (2003) 1013–1034.
[31] J. Stutterheim, A. Gerritsen, L. Zappeij-Kannegieter, B. Yalcin, R. Dee, M.M. Van Noesel, et al., Detecting minimal residual disease in neuroblastoma: the superiority of a panel of real-time quantitative PCR markers, Clin. Chem. 55 (7) (2009) 1316–1326.
[32] J.W. Lee, M.H. Son, H.W. Cho, Y.E. Ma, K.H. Yoo, K.W. Sung, et al., Clinical significance of MYCN amplification in patients with high-risk neuroblastoma, Pediatr. Blood Cancer 65 (10) (2018).

[33] K. Schmelz, J. Toedling, M. Huska, M.C. Cwikla, L.M. Kruetzfeldt, J. Proba, et al., Spatial and temporal intratumour heterogeneity has potential consequences for single biopsy-based neuroblastoma treatment decisions, Nat. Commun. 12 (1) (2021 Dec 1).
[34] G. Wils, L. Hamerlinck, W. Trypsteen, C. Van Den Eeckhaut, J. Weiss, A.A. Nour, et al., Digital PCR-based gene expression analysis using a highly multiplexed assay with universal detection probes to study induced pluripotent stem cell differentiation into cranial neural crest cells, Methods Mol. Biol. 2880 (2025) 17–47.
[35] M. Neugebauer, S. Calabrese, S. Müller, T.T. Truong, P. Juelg, N. Borst, et al., Generic reporter sets for colorimetric multiplex dPCR demonstrated with 6-Plex SNP quantification panels, Int. J. Mol. Sci. 25 (16) (2024).
[36] E.M. Van Wezel, D. Zwijnenburg, L. Zappeij-Kannegieter, E. Bus, M.M. Van Noesel, J.J. Molenaar, et al., Whole-genome sequencing identifies patient-specific DNA minimal residual disease markers in neuroblastoma, J. Mol. Diagn. 17 (1) (2015) 43–52.
[37] L.M.J. van Zogchel, N.S.M. Lak, N.U. Gelineau, I. Sergeeva, E. Stelloo, J. Swennenhuis, et al., Targeted locus amplification to develop robust patient-specific assays for liquid biopsies in pediatric solid tumors, Front. Oncol. 13 (2023) 1124737.
[38] C.C. Raggi, M.L. Bagnoni, G.P. Tonini, M. Maggi, G. Vona, P. Pinzani, et al., Real-time quantitative PCR for the measurement of MYCN amplification in human neuroblastoma with the TaqMan detection system, Clin. Chem. 45 (11) (1999) 1918–1924.
[39] N. Aygun, Biological and genetic features of neuroblastoma and their clinical importance, Curr. Pediatr. Rev. 14 (2) (2018) 73–90.
[40] J. Mora, N.K.V. Cheung, W.L. Gerald, Genetic heterogeneity and clonal evolution in neuroblastoma, Br. J. Cancer 85 (2) (2001) 182–189.
[41] M. Schulte, J. Köster, S. Rahmann, A. Schramm, Cancer evolution, mutations, and clonal selection in relapse neuroblastoma, Cell Tissue Res. 372 (2) (2018) 263–268.
[42] L.M.J. van Zogchel, L. Zappeij-Kannegieter, A. Javadi, M. Lugtigheid, N. U. Gelineau, N.S.M. Lak, et al., Specific and sensitive detection of neuroblastoma mRNA markers by multiplex RT-qPCR, Cancers (Basel) 13 (1) (2021 Jan 1) 1–12. A.
[43] V. Rowlands, A.J. Rutkowski, E. Meuser, T.H. Carr, E.A. Harrington, J.C. Barrett, Optimisation of robust singleplex and multiplex droplet digital PCR assays for high confidence mutation detection in circulating tumour DNA, Sci. Rep. 9 (1) (2019) 12620.
[44] M. Alcaide, M. Cheung, K. Bushell, S.E. Arthur, H.L. Wong, J. Karasinska, et al., A novel multiplex droplet digital PCR assay to identify and quantify KRAS mutations in clinical specimens, J. Mol. Diagn. 21 (2) (2019) 214–227.
[45] J. Parker, N. Fowler, M.L. Walmsley, T. Schmidt, J. Scharrer, J. Kowaleski, et al., Analytical sensitivity comparison between singleplex real-time PCR and a multiplex PCR platform for detecting respiratory viruses, PLoS One 10 (11) (2015 Nov 16) e0143164.
[46] E.M. Elnifro, A.M. Ashshi, R.J. Cooper, P.E. Klapper, Multiplex PCR: optimization and application in diagnostic virology, Clin. Microbiol. Rev. 13 (4) (2000 Oct) 559–570.