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Harmonization of Reporting of ALK Genetic Alterations in Neuroblastoma

A SIOPEN Biology Study

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In high-risk neuroblastoma, identification of *ALK* activating genetic alterations is considered for clinical decision-making at relapse or more recently in frontline treatment. The accurate diagnosis of genetic alterations requires harmonization of molecular techniques and reporting, especially when these concern inclusion criteria for clinical trials. Analysis and validation of 14 DNA samples harboring distinct *ALK* alterations were performed across the 21 SIOPEN (International Society of Paediatric Oncology Europe Neuroblastoma) molecular diagnostic laboratories. These included *ALK* mutations at or outside hotspots in the tyrosine kinase domain with variant allele frequencies (VAFs) of 1% to 91%

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or *ALK* genomic amplification. Each laboratory used their own techniques: *ALK* amplifications were detected by pan-genomic copy number techniques or fluorescence *in situ* hybridization, and *ALK* mutations were characterized by next-generation sequencing techniques. All laboratories correctly identified high-level *ALK* amplification and *ALK* mutations within the known hotspots with VAF >5%, with the exception of two cases. Differences in interpretation and reporting were apparent for samples harboring mutations with a VAF <5% or outside known hotspots. These results highlight the importance of standard operating procedures, standardized reporting, and the robustness of *ALK* genetic testing in the SIOPEN laboratories, and the need for expert discussions regarding atypical *ALK* alterations, to validate eligibility for ALK targeted treatment in clinical trials. *(J Mol Diagn 2026, 28: 27—38; https://doi.org/10.1016/j.jmoldx.2025.09.007)*

Neuroblastoma (NB), the most common malignant extracranial solid tumor in young children, is characterized by important clinical and genetic heterogeneity. The outcome of patients with NB depends on risk groups defined by both clinical and biological features; patients with low- and intermediate-risk disease have an overall good outcome, whereas overall 5-year survival for patients with high-risk disease does not exceed 60% despite multimodal therapy approaches.[1—3] Important international efforts have focused on the identification of prognostic and predictive biomarkers to adapt treatment strategies.[4]

The most frequent genetic alterations in NB concern genomic copy number alteration.[5] Amplification of the oncogene *MYCN*, observed in 20% to 25% of all NB cases, is associated with a poor prognosis. Large-scale segmental chromosomal alterations, occurring most frequently as deletions of chromosome arms 1p, 3p, 4p, or 11q, and gains of chromosome arm 1q, 2p, or 17q, are also associated with a poorer outcome, whereas numerical chromosome alterations are associated with excellent survival.[6—8]

Gene-specific mutations are less frequent, with recurrent alterations targeting genes of the RAS—mitogen-activated protein kinase pathway or genes of chromatin remodeling, or affecting telomere maintenance through mechanisms activating telomerase or alternative lengthening of telomeres.[5,9—11] The most frequently altered gene is anaplastic lymphoma kinase (*ALK*), with genomic amplification or constitutively activating mutations in the tyrosine kinase domain (TKD) leading to active signaling of multiple downstream pathways.[12—15] Single base missense mutations are found at three major hotspots of the TKD at positions R1275, F1174, and F1245, accounting for 85% of NB *ALK* mutations.[16,17] *ALK* mutations are observed in approximately 10% of NB cases at diagnosis but in >25% at relapse, either due to expansion of subclonal *ALK* mutations or acquisition of new mutations upon relapse.[18,19]

ALK mutations exhibit differential sensitivity to ALK inhibitors.[16] With a limited sensitivity to the first-generation ALK inhibitor crizotinib, subsequent second- and third-generation ALK inhibitors were designed to overcome primary resistance.[20—23] Recent studies indicate single-agent response rates of 20% to ceritinib, of >30% to lorlatinib, and >60% to lorlatinib in combination with chemotherapy in a relapse setting. Integration of ALK targeted therapies in upfront treatment strategies is ongoing in different collaborative groups.

The common efforts of SIOPEN (International Society of Pediatric Oncology Europe Neuroblastoma Group, *www.siopen.org*, last accessed September 26, 2025) have led to the development of collaborative trials. These trials aim to improve outcomes for patients with low-, intermediate-, and high-risk NB, integrating clinical decision-making diagnostic and prognostic biomarkers such as *MYCN* amplification status and genomic copy number profiles.

With the participation of 21 laboratories in 16 countries, molecular analyses for patients with NB are performed within a network of SIOPEN reference laboratories.[1] Standards for determination of *MYCN* amplification status have been developed and harmonized within this extensive collaboration, based on technologies such as array-based comparative genomic hybridization, single nucleotide polymorphism array, or fluorescence *in situ* hybridization (FISH). The rapid recent development of next-generation sequencing (NGS), panel sequencing, whole-exome sequencing (WES), and whole-genome sequencing (WGS) provides a major breakthrough by enabling determination of the overall mutational burden and prognostic and predictive genetic alterations; important technological leaps enable molecular diagnostics even on small tumor samples or liquid biopsy specimens.[24] NGS techniques have therefore become an integral part of patient care in pediatric oncology.

However, the complexity of molecular diagnosis, with different technologies developed in different diagnostic laboratories, highlights the need for harmonization not only of molecular diagnostic techniques but especially the standardization of output between SIOPEN reference

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laboratories; the goal is to identify *ALK* genetic alterations when considering ALK targeted treatment either in the frontline or the relapse setting. To standardize molecular diagnostic and reporting results of the *ALK* genomic status (amplifications, mutations, and rearrangements) in NB samples, a SIOPEN cross-laboratory validation was performed. For this, samples were shared between participating SIOPEN tumor biology reference laboratories, and each laboratory used its own sample processing and analysis protocols. This report presents the results of inter-laboratory quality control testing for detection of *ALK* genetic alterations in NB samples conducted within the SIOPEN network. Recommendations for the optimal reporting of *ALK* genetic alterations in NB are provided based on these findings.

Materials and Methods

An international inter-laboratory sample exchange was organized between 21 SIOPEN reference laboratories from 16 different countries: Australia ($n = 1$), Austria ($n = 1$), Belgium ($n = 1$), Czech Republic ($n = 1$), Denmark ($n = 1$), France ($n = 3$), Germany ($n = 2$), Ireland ($n = 1$), Israel ($n = 1$), Italy ($n = 1$), the Netherlands ($n = 1$), Norway ($n = 1$), Spain ($n = 2$), Sweden ($n = 1$), Switzerland ($n = 1$), and United Kingdom ($n = 2$) (Supplemental Table S1). All laboratories have expertise in the molecular diagnosis of NB and perform analysis in a clinical setting.

Biological Samples

For this inter-laboratory sample exchange, a series of 14 NB samples were selected by the lead institute (Institut Curie). Two established NB cell lines and frozen tissues from five patient-derived xenografts (PDXs) engrafted in mice were selected for their specific *ALK* mutation (Figure 1A) and/or copy number alteration, and one additional cell line was selected as an *ALK* wild-type and nonaltered sample.[25–27] The 14 samples were chosen to represent the most common genetic alterations of *ALK* in NB.[5,16]

Normal germline (blood) samples were chosen from individuals without NB for dilution and control samples. Patients or their parents/guardians provided consent for use of biological samples and for the establishment of PDXs. Experimental procedures and analyses for this study have been approved by the institutional review board of Institut Curie (DATA 250036).

Patient Germline DNA

Genomic DNA from patients without NB was extracted from the cell fraction of bone marrow or blood samples using a phenol-chloroform extraction procedure. Samples were collected after inclusion of patients in the national MICCHADO (Molecular and Immunological Characterisation of High Risk Childhood Cancer at Diagnosis) IC-2017-02 study. This study was authorized by the ethics committees "Comité de Protection des Personnes Sud-Est IV" (reference IC 2017-02) and "Comité de Protection des Personnes Ile de France" (reference AU 1388). Patients or their legal guardians signed a written informed consent form agreeing to the use of samples for research according to national law.

NB Cell Lines

Patient-derived NB cell lines used in this study (CLB-Ga, CLB-Ge, and CLB-Ber) have been previously described.[26] Cells were cultured in RPMI 1640 (Sigma-Aldrich, St. Louis, MO) supplemented with 10% v/v fetal calf serum (Gibco/Life Technologies Ltd., Paisley, UK), and checked routinely using PCR for the absence of *Mycoplasma*. When reaching 90% confluency, cells were detached by using TrypLE Express Enzyme (Thermo Fisher Scientific, Waltham, MA). For DNA extraction, cells were washed twice with phosphate-buffered saline and then lysed, and DNA was extracted by using the QIAamp DNA mini kit (Qiagen, Hilden, Germany).

PDX Models

PDX model GR-NB4 was provided by Dr. Birgit Geoerger (Gustave Roussy, Villejuif, France).[27] Models IC-pPDX-75 and IC-pPDX-112 were established at Institut Curie as described previously.[27] Models HSJD-NB-009, HSJD-NB-011, and HSJD-NB-012 were provided by Dr. Angel Carcaboso (Hospital Sant Joan de Déu, Barcelona, Spain).[25] Written informed consent was obtained for all patients from parents or guardians for all PDX models established. For DNA extraction, subcutaneous PDX tumors were dissected from immunodeficient mice upon reaching ethical size; they were then lysed and DNA extracted by using the QIAamp DNA mini kit.

Genomic DNA Preparation

Due to the limited DNA yield from patient tumor biopsies (typically 1 to 5 μg), genomic DNA was extracted from cell lines or PDX and, if relevant, further diluted with normal (germline) DNA, as indicated in Table 1. This approach ensures sufficient DNA quantity across all laboratories.

The quality and quantity of genomic DNA were validated by using routine laboratory protocols.[9,28] The expected *ALK* amplification status and *ALK* mutation variant allele frequency (VAF) in the samples was validated at Institut Curie by droplet digital PCR in all samples before shipment to the participating reference laboratories.[29] The test specimens were all prepared and stored under the same conditions. They were then packed and dispatched so that the 21 laboratories received 14 specimens each.

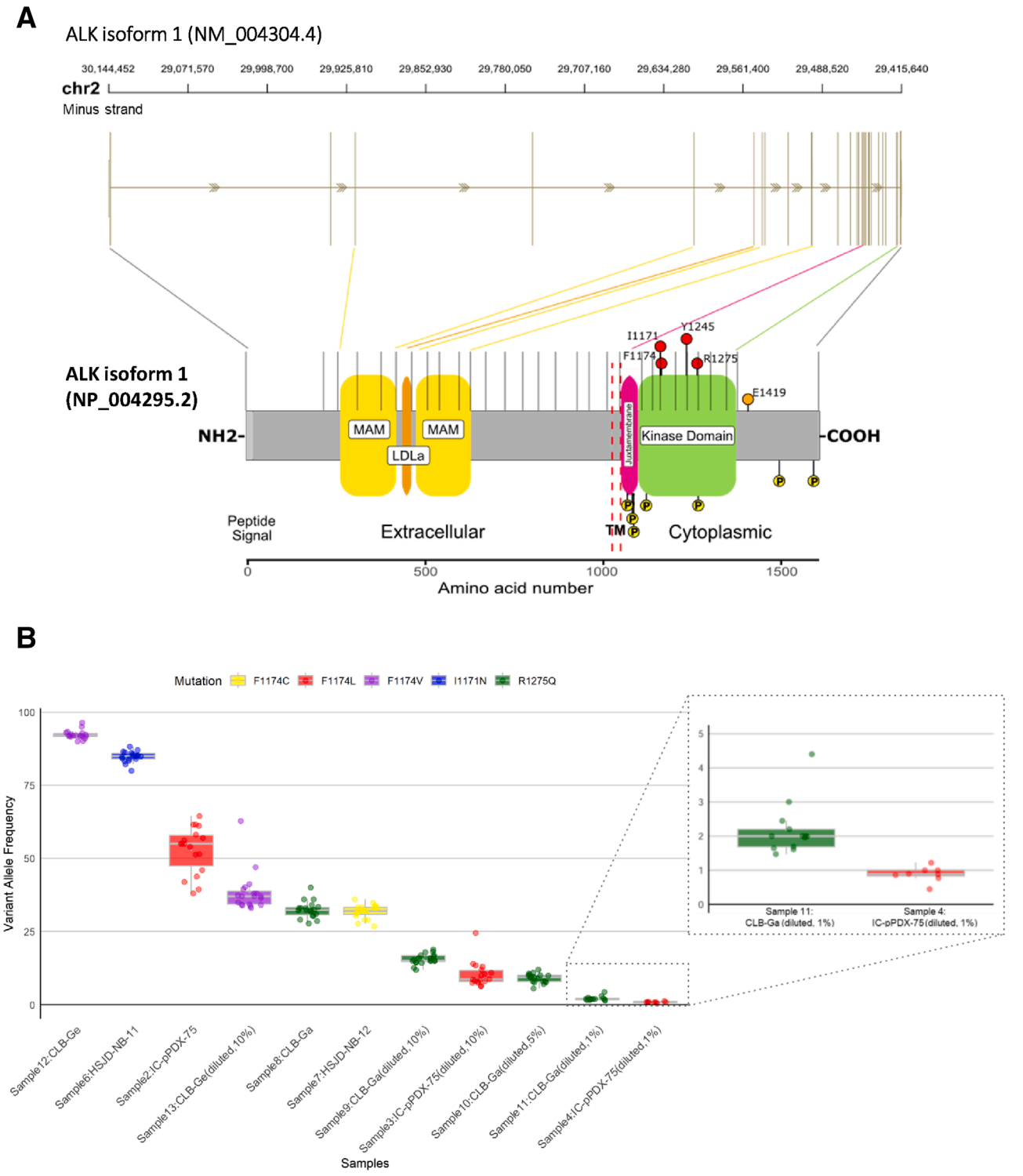

Figure 1 **A:** Sequence structure of the *ALK* gene on chromosome 2p (29415640-30144452 bp, minus strand). The positions of the most frequent activating mutations in the tyrosine kinase domain are indicated. **B:** Variant allele frequencies of *ALK* mutations detected in 11 samples identified at each test site. Data are visualized as boxplots, upper and lower quartiles, interquartile range, and mean, with a zoom-in version of samples 11 and 4. Whiskers indicate outliers. $n = 19$ (**B**).

Samples were labeled "1 to 14" for the recipient laboratories, without an indication of the expected *ALK* alterations. Only the sample number (1 to 14) and sample type (cell line or PDX) were provided to the recipient laboratory. *ALK* status of the 14 samples is summarized in Table 1. In addition, for six participating laboratories, formalin-fixed, paraffin-embedded tissue from two different PDX models were also sent upon request for FISH analyses (IC-pPDX-112 and GR-NB4).

Molecular Analyses

Molecular analyses were performed in each of the 21 participating laboratories according to local procedures, while respecting the requirements described in *Analysis of Copy Number and Single Nucletotide Variant*.

Analysis of Copy Number and Single Nucleotide Variant

The analysis of a genomic copy number profile was performed by using the following: (i) high-resolution (resolution <30 kb) array-based comparative genomic hybridization or single nucleotide polymorphism array; or (ii) NGS-based approaches, including targeted sequencing panel (>100× depth) or WES (>80×) or WGS (<80×). An important requirement was that genome-wide coverage and analytical resolution had to be sufficient to allow detection of focal amplification of *ALK* relative to the copy number of chromosome 2. Importantly, coverage should be adjusted to enable detection of VAF of at least 5%. Sequencing of *ALK* should cover at least exons 20 to 29, harboring the ALK TKD with amino acid residues 1116 to 1392, and particularly the *ALK* regions of interest (exon 23: chr2:29443647-29443776; exon 24: chr2:29436830-29436935; exon 25: chr2:29432603-29432704; coordinates are based on the hg19 reference genome) containing the *ALK* mutational hotspots F1174 (exon 23), F1245 (exon 24), and R1275 (exon 25).

The analysis of *ALK* amplification status was performed by using FISH techniques or genomic copy number analyses. *ALK* gene amplification was defined by FISH as more than a fourfold increase of *ALK* signals in relation to numbers of chromosome 2 reference probe (centromere or 2q arm), or by genomic copy number techniques as a focal genomic event with >10 copies of the gene.[30] Extended chromosome 2p gain (encompassing the *ALK* and/or *MYCN* genes) was not considered to be amplification.

Data Analyses

Each participant applied their expertise and institutional experience to analyze the data according to their established workflow. Each participant used their own bioinformatics pipeline for calling of single nucleotide variants and copy number alteration. A return form was provided to the participants to gather results and conclusions, which were subsequently compiled at the Institut Curie. The form included the criteria defined for *ALK* targeted treatment in a future amendment of the HR-NBL2 protocol (NCT04221035; ClinicalTrials.gov, *http://clinicaltrials.gov/study/NCT04221035*, last accessed September 26, 2025).

Additional Information

The conclusions of this study are integrated in Standard Operating Procedures for Tumor Molecular Diagnostics of Neuroblastoma by the SIOPEN Biology committee. This document is accessible online via the SIOPEN website (*https://www.siopen.org/specialty-committees/biology*, last accessed September 26, 2025) and can be provided by authors upon request.

Results

A total of 14 genomic DNA samples (NB cell lines or NB PDX models), labeled from 1 to 14, were distributed to 21 participating laboratories by the Institut Curie for *ALK* genomic testing. Analysis was performed in a blinded manner (ie, expected results of *ALK* genomic testing in the 14 DNA samples was not communicated to the laboratories) (Supplemental Table S1).

Techniques for Detection of *ALK* Mutations

Twenty of the 21 SIOPEN laboratories analyzed the distributed samples. In a first step, information on techniques for the detection of *ALK* activating mutation were compiled. Two participating laboratories used WGS techniques, two others used WES techniques, and 16 used the NGS gene panel (Supplemental Figure S1). Sequencing libraries were constructed based on standard procedures and according to manufacturer recommendations. For WES, DNA sequences were captured by using SureSelect V6 (Agilent Technologies, Santa Clara, CA; one laboratory), or KAPA HyperExome (Roche, Indianapolis, IN; one laboratory). For WGS, libraries were made using TruSeq Nano DNA (Illumina, San Diego, CA; one laboratory) or DNA PCR-Free Library Prep, Tagmentation (one laboratory; Illumina). Sequencing was performed by using Illumina NovaSeq sequencers for both techniques. For NGS panel sequencing, commercial or specific in-house NGS panel approaches were used. The most used sequencing manufacturers were Agilent Technologies (6 laboratories), Roche (3 laboratories), Thermo Fisher Scientific (3 laboratories), Agena Bioscience (San Diego, CA; one laboratory), Illumina (one laboratory), SiMSen-seq (one laboratory), and Twist Bioscience (South San Francisco, CA; one laboratory). Twelve participants used the Illumina sequencer [NextSeq (5 laboratories), NovaSeq (4 laboratories), or MiSeq (3 laboratories)], three used the Ion Torrent (Thermo Fisher Scientific), and one used the MassARRAY (Agena Bioscience, San Diego, CA). All sites used commonly available software tools that were already implemented in the routine clinical workflow for variant calling and annotation.

Results of *ALK* Mutation Detection

Two samples (sample 1 and sample 14) did not carry any *ALK* gene mutations, and 100% of participant feedback indicated the absence of activating mutations (Figure 1B). In nine samples (samples 2, 3, 6, 7, 8, 9, 10, 12, and 13) harboring mutations in the *ALK* TKD and with a VAF >5% (range, 5% to 95%), all mutations were successfully

Table 1 Description of ALK Genomic Alterations per Sample Tested by 21 Participating SIOPEN Reference Laboratories

Sample	Sample type	Sample name	Sample condition	*ALK* cDNA change NM_004304.4*	*ALK* amino acid change	*ALK* variant allele frequency	*ALK* copy number status	*MYCN* status	Chromosome 2p status
1	PDX	IC-pPDX-112	Not diluted		Wild type		Amplified (Intron 3)	Amplified	Gain
2	PDX	IC-pPDX-75	Not diluted	c.3522C > A	F1174L	32%	Nonamplified	Nonamplified	Gain
3	PDX	IC-pPDX-75	Diluted	c.3522C > A	F1174L	10%	Nonamplified	Nonamplified	Gain
4	PDX	IC-pPDX-75	Diluted	c.3522C > A	F1174L	1%	Nonamplified	Nonamplified	Normal
5	PDX	HSJD-NB-009	Not diluted	c.4255G > A	E1419K	57%	Nonamplified	Nonamplified	Gain
6	PDX	HSJD-NB-011	Not diluted	c.3512T > A	I1171N	87%	Nonamplified/ rearranged	Amplified	Gain (rearranged)
7	PDX	HSJD-NB-012	Not diluted	c.3521T > G	F1174C	31%	Nonamplified	Amplified	Normal
8	Cell line	CLB-Ga	Not diluted	c.3824G > A	R1275Q	34%	Gain of ALK (3 copies)	Nonamplified	Gain
9	Cell line	CLB-Ga	Diluted	c.3824G > A	R1275Q	10%	Gain of ALK (3 copies)	Nonamplified	Gain
10	Cell line	CLB-Ga	Diluted	c.3824G > A	R1275Q	5%	Gain of ALK (3 copies)	Nonamplified	Normal
11	Cell line	CLB-Ga	Diluted	c.3824G > A	R1275Q	1%	Gain of ALK (3 copies)	Nonamplified	Normal
12	Cell line	CLB-Ge	Not diluted	c.3520T > G	F1174V	91%	Amplified	Nonamplified	Normal
13	Cell line	CLB-Ge	Diluted	c.3520T > G	F1174V	10%	Amplified	Nonamplified	Normal
14	Cell line	CLB-Ber	Not diluted		Wild type		Nonamplified	Nonamplified	Normal
AQ	PDX	IC-pPDX-112	FFPE		Wild type		Amplified (Intron 3)	Amplified	Normal
AP	PDX	GR-NB4	FFPE		Wild type		Amplified	Amplified	Normal

*Accession number is available via the National Center for Biotechnology Information Nucleotide repository (*https://www.ncbi.nlm.nih.gov/nuccore/NM_004304.4*).

FFPE, formalin-fixed, paraffin-embedded; PDX, patient-derived xenograft.

detected at the expected VAF by all participating laboratories, with the exception of two cases. One of the undetected cases contained the F1174L mutation with a VAF of 10% (sample 3) not reported by one laboratory using WES, and another case harbored R1275Q with a VAF of 5% (sample 10) not reported by another laboratory using NGS panel sequencing; there was no correlation between an undetected *ALK* TKD mutation and any specific technique (Figure 1B).

At one testing site, sample 2 contained DNA of insufficient quality. Overall, these results indicate a high concordance rate for the results obtained among the participating test sites for samples harboring an *ALK* mutation in the TKD, at the known hotspots, and with a VAF >5%. The most challenging samples, samples 4 and 11, harbored an *ALK* mutation in the TKD and both were diluted with genomic DNA to achieve a VAF <5%. In these two cases, differences in the reported results were observed. For sample 11 (R1275Q; with a VAF of 1%), 13 sites reported this alteration (techniques used: 11 NGS panel, 1 WGS, and 1 WES) while seven did not (techniques used: 5 NGS panel, 1 WGS, and 1 WES). For sample 4 (F1174L; with a VAF of 1%), this mutation was reported by eight of 20 centers performing the analysis (techniques used: 7 NGS panels and 1 WES), while 12 did not (techniques used: 9 NGS panels, 2 WGS, and 1 WES). These results highlight the difficulty in detection and reporting of mutations at lower VAF by WGS, WES, or NGS and to correctly distinguish these from background noise. The most challenging samples, samples 4 and 11, harbored an *ALK* mutation in the TKD, and both were diluted with genomic DNA to achieve a VAF <5%. In these two cases, differences in the reported results were observed. A sample with an *ALK* mutation outside the TKD (sample 5; E1419K, VAF 57%) was also analyzed. For this case, nine laboratories identified the alteration, and 11 did not (Supplemental Figure S2).

Techniques for Detection of *ALK* Amplification

Techniques for calling *ALK* copy number status included DNA genomic profiling such as array-based comparative genomic hybridization or single nucleotide polymorphism array (nine laboratories), gene panel sequencing (six laboratories), WES (one laboratory), low coverage WGS, or deep WGS (three laboratories). Six laboratories performed analysis by FISH on formalin-fixed, paraffin-embedded sections (four in parallel with sequencing techniques and two only by FISH); the most frequently used probe was the SPEC ALK/2q11 dual color probe ZytoLight (ZytoVision GmbH, Bremerhaven, Germany) in three cases and an *ALK* break-apart probe in three cases.

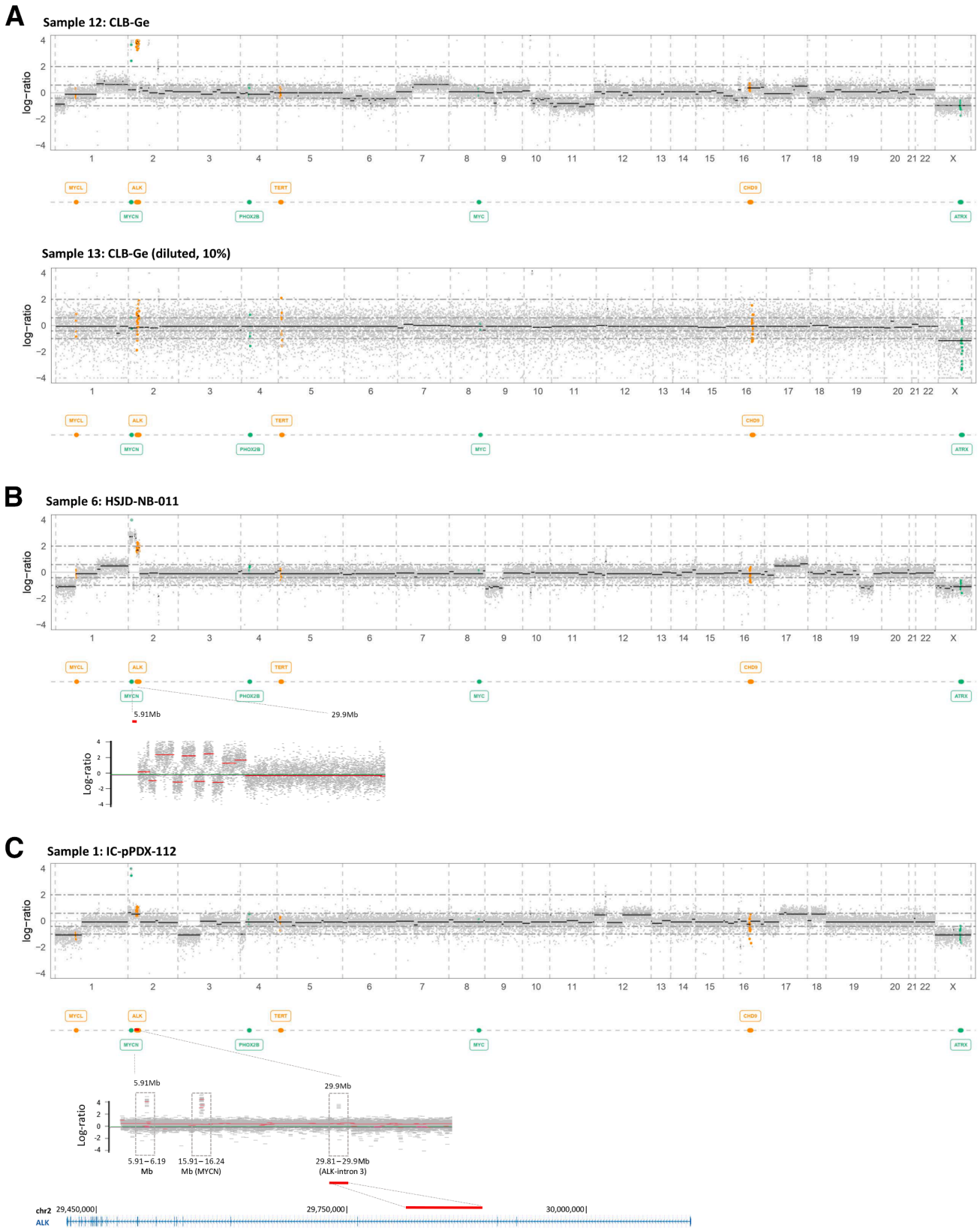

Figure 2 Copy number profile of genomic DNA from the neuroblastoma cell lines CLB-Ge in a DNA sample extracted from 100% tumor cells and a dilution to 10% tumor cells analyzed using an array-based comparative genomic hybridization (**A**). Copy number profile of genomic DNA of HSJD-NB-011, with a zoom on the chromosome 2p region (**B**). Copy number profile of genomic DNA of IC-pPDX-112, indicating gains of chromosome 2p (tel-46.84 Mb); with additional interstitial amplifications (zoom) of 2p (5.91-6.19 Mb); 2p (15.91-16.24 Mb) harboring *MYCN*; and 2p (29.81-29.90 Mb) harboring the intron 3 of *ALK* (**C**).

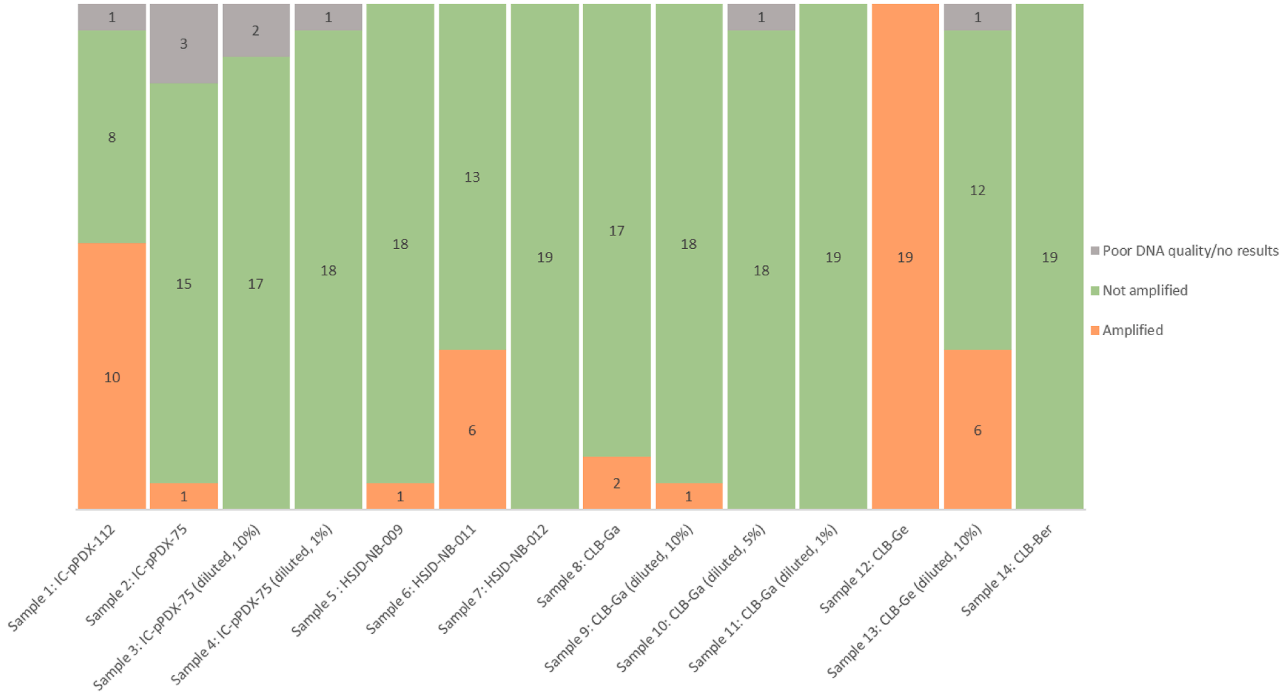

Figure 3 Detection of *ALK* amplification in 14 samples at each SIOPEN biology reference laboratory.

Results of *ALK* Amplification Detection

All SIOPEN laboratories ($N = 21$) analyzed the distributed samples. Among the 14 samples, 11 did not harbor an *ALK* amplification (samples 2 to 11 and 14). Among these, 10 were unequivocally identified as not amplified by the majority of the laboratories (Figures 2 and 3, and Supplemental Figure S3). An *ALK* amplification was called in four of these 10 cases by one or two laboratories, for samples 2, 5, 8, and 9. Sample 6, harboring a complex genomic structure, was called amplified by six laboratories and not amplified by 13. Regarding identification of the *ALK* amplification, on the other hand, sample 12 was correctly identified as harboring an *ALK* amplification by all 19 laboratories providing copy number profiling results. After dilution of this DNA (sample 13) to a genomic content of 10%, *ALK* amplification was identified by six laboratories performing this analysis. Finally, in sample 1, harboring an unusual copy number profile with an amplification encompassing only intron 3 of *ALK*, this small interstitial amplification was detected by 10 of 18 laboratories (Figures 2 and 3). However, only one laboratory considered this small interstitial genomic amplification as a criterion for ALK targeted treatment, whereas three other laboratories highlighted the importance of further discussions to document its role as a potential predictive biomarker.

All laboratories using the FISH technique correctly identified the amplified and nonamplified samples (Supplemental Figure S4).

Discussion

In NB, the discovery of *ALK* activating genetic alterations has led to a major breakthrough in therapeutic strategies, with the possibility of proposing ALK targeted treatment in the presence of *ALK* activating genetic alterations.[23] Early clinical trials have investigated the efficacy of ALK targeted treatment for patients with NB at relapse. Although the first-generation ALK inhibitor crizotinib did not show convincing efficacy in NB patients with relapse, recent studies using second- and third-generation ALK inhibitors, including ceritinib or lorlatinib, have confirmed the benefit of this treatment, particularly in association with chemotherapy.[20–22,31] Ongoing trials are exploring the possibility and efficacy of such treatment in first-line therapy. Importantly, previous studies have emphasized the importance of identifying predictive biomarkers for patient selection of treatment, and the identification of genetic alterations leading to constitutive activation of ALK activity and downstream cellular signaling should be considered as predictive biomarkers. In NB, such genetic alterations consist of activating mutations in the TKD or high-level genomic amplifications. Rearrangements leading to ALK activation are rare in NB.[32] In addition, the predictive value of *ALK* overexpression remains controversial.[33]

In this context, precise identification of genetic alterations as biomarkers for patient selection for ALK targeted treatment is essential. The development of clinical research networks, associated with molecular diagnostic laboratories performing molecular diagnosis on tumor samples of patients with cancer,

underlines the importance of performing quality control for the identification of such predictive biomarkers for the selection of patients for biomarker-driven clinical trials. SIOPEN has developed clinical trials aiming to improve the outcome of patients with NB. In this context, SIOPEN has established a firm network of national molecular diagnostic laboratories for patients with NB, as well as laboratories contributing to translational and fundamental research in NB.[1,34] Within this network, previous work focused on standardization and common reporting for *MYCN* amplification, a major prognostic factor for NB, with quality control for the detection of genetic alterations.

The importance of definition standardization to define *MYCN* amplification in the context of NB has been highlighted in previous studies.[1] Important features of *MYCN* amplification in NB include the variability of genomic amplification, ranging from >10 to >100 copies within one cell. Challenges for *MYCN* molecular diagnostics concern the small size of tumor samples from patients with NB, the frequency of variable or often low tumor content in a given sample, and the possibility of intra-tumor heterogeneous *MYCN* amplification. SIOPEN has explored the importance of harmonized and standardized definitions of *MYCN* amplification across SIOPEN reference laboratories, and these definitions are fully integrated into the International Neuroblastoma Risk Group Classification System.[2]

In the current study, building on previous experience, SIOPEN biology reference laboratories have sought to establish standard operating procedures and common definitions for the calling of activating *ALK* genetic alterations, including *ALK* mutations and genomic amplifications. This study underlines the importance of a common nomenclature; alignment of the calling of genomic regions, both amplification and mutation, and the calling of pathogenic or likely pathogenic variants/mutations versus variants of unknown significance requires standard definitions.

In the context of *ALK* activating mutations, somatic mutations occur most frequently at three main hotspots across the TKD in exons 23, 24 and 25, with substitutions leading to amino acid changes with functional impact. Single nucleotide variants outside the known hotspots can also be observed but occur more rarely.[17] In NB, an *ALK* activating mutation might be considered for a non-synonymous substitution leading to a proven, or highly likely, activation of the TKD based on either known *in vitro*/*in vivo* validation or based on prediction of its functional impact using strong bioinformatics tools.

The oncogenic role of *ALK* activating mutations occurring at the most frequent hotspots F1174, F1245, and R1275 has been firmly documented based on functional assays *in vitro* and its driver role in particular in synergy with *MYCN* in different animal models.[35–38] Variants occurring outside the hotspots are described more and more frequently. However, in the absence of functional data, their potential impact must be carefully considered with regard to activity and downstream signaling. In this study, mutations occurring at the known hotspots were identified unequivocally. Importantly, an alteration occurring outside the known hotspot was also identified by most laboratories at position E1419; however, its functional impact remains elusive, albeit reported in other cancer types. Consideration for ALK targeted treatment based on the presence of this mutation should be discussed between experts based on more recent data.

The current study highlights the importance of defining detection limits for *ALK* mutations. Sanger sequencing techniques enable detection of single nucleotide variants down to VAFs of 10%. When performing NGS techniques, although low VAF below 1% or even below 0.1% have been documented depending on the sequencing depth, clinical grade detection limits are frequently defined around a VAF of 5%. In NB, variable tumor cell purity is often observed due to contamination with normal stromal tissue. A lower VAF might be observed due to contamination of normal tissue or due to the occurrence of subclonal mutations, or intra-tumor heterogeneity, which occur more rarely.[17,28,39] The current study underlines the difficulty of clear identification across different laboratories of mutations observed at VAF <5%, justifying a cutoff of 5% when selecting patients for targeted treatment within a clinical trial. Thus, it is suggested that for patient inclusion in clinical trials, identification of a mutation with a VAF >5% should be considered as inclusion criteria, while encouraging the development of techniques with higher sensitivity. Future studies will enable determination of the prognostic and predictive value of ALK mutations with very low VAF in these heterogeneous tumors.[40]

Based on our findings and the challenges associated with detecting low-frequency VAF mutation, targeted NGS panels with high coverage and low detection limits (<1%) are recommended for an enhanced sensitivity of ALK TKD mutation detection. Similarly, the detection limit for genomic *ALK* amplifications can equally be affected by contamination with normal tissue. In this study, the interpretation of an *ALK* amplification after dilution in normal DNA is shown and further underlines the importance of central review when considering predictive biomarkers.

Considering tumor heterogeneity and assay resolution, the FISH method is recommended for the detection of ALK amplification due to its more consistent performance across laboratories, with genomic copy number techniques for ALK copy number reporting considered less robust in the context of tumor heterogeneity. For the identification of ALK mutations and other alterations, sequencing assays remain the preferred approach despite some variability in concordance. This dual strategy ensures robust and comprehensive molecular characterization.

Liquid biopsies are increasingly being used for analysis of cell-free DNA and its circulating tumor DNA component. In NB, high levels of cell-free DNA and high circulating tumor DNA content are frequently observed.[41–43] These approaches also show the frequency of genetic heterogeneity and the possibility of subclonal mutations, the

predictive power of which remains to be documented. Future work will establish criteria for the calling of *ALK* mutations in cell-free DNA.

The current study used cell lines and PDXs, providing high and well-documented tumor DNA content (purity). However, when analyzing patient samples, it is crucial to consider tumor content, which can be evaluated based on pathology reports, bioinformatics analyses, or ideally both. Accurate assessment of tumor purity is essential for correct interpretation of VAFs. Moreover, spatial tumor heterogeneity remains a challenge in this context. Liquid biopsy analyses may represent a valuable approach for further assessments and for evaluations in the context of potential spatial tumoral heterogeneity in the future.

Genomic amplification encompassing the entire coding sequence of the *ALK* gene is the most common copy number alteration leading to ALK activation in NB.[14] Rearrangements or translocations only occur rarely in NB.[32] In this study, the occurrence of an amplification targeting only intron 3 of the *ALK* gene highlighted the difficulty of detecting such rare alterations and the importance of clear definitions of alterations to be considered as predictive biomarkers. In these rare cases, confirmation of ALK activation in functional analyses might be of interest.

Recent developments regarding integration of targeted therapies based on identification of specific biomarkers in different pathologies underline the importance of regulatory obligations of molecular diagnostic laboratories for performing molecular diagnostics for clinical decision-making. Accreditation is an important aspect of molecular diagnosis throughout cancer genetic laboratories, obtained either at the national level or in some instances corresponding to ISO15189. The European legislation on in vitro diagnostic medical devices (In Vitro Diagnostic Medical Devices; Regulation-2017/746-EN-Medical Device Regulation-EUR-Lex) and the College of American Pathologists/Clinical Laboratory Improvement Amendments regulations in the United States underscore the importance of guidelines for noncommercialized, so-called in-house–developed molecular tests used for clinical decision-making. This is particularly relevant for specialized diagnostics for rare diseases such as pediatric cancers.

This study has contributed to the establishment of SIOPEN Biology reference laboratory Standard Operating Procedures (*https://www.siopen.org/specialty-committees/biology*). Within the SIOPEN network, patients with NB might be eligible for *ALK* targeted treatment in case of identification of an activating genetic alteration of the *ALK* gene in a SIOPEN Biology Reference Laboratory, according to the following criteria: i) presence of a pathogenic or likely pathogenic *ALK* mutation in the *ALK* TKD at a VAF $\geq$5% detected by NGS, WES, or WGS (minimum coverage 80×); ii) high-level genomic *ALK* amplification (defined as a fourfold increase in ALK signal compared with chromosome 2 centromeric signal or signal on the q-arm of chromosome 2 by FISH, or focal amplification >10 copies by genomic copy number techniques); or iii) other (rare) genetic alterations leading to the unequivocal constitutive activation of *ALK* (confirmed after central review).

It is recommended that analysis of *ALK* copy number status be performed by using FISH or genomic copy number analyses. *ALK* gene amplification is defined, by FISH, as more than a fourfold increase of *ALK* signals in relation to numbers of chromosome 2, or by genomic copy number techniques, a focal genomic event with >10 copies of the gene, in alignment with the definition used for MYCN amplification, and as reported previously.[30] Extended chromosome 2p gain (encompassing the *ALK* and/or *MYCN* genes) is not considered to be amplification. It is recommended that ALK mutational status be determined by using NGS-based techniques such as panel sequencing approaches, including large, targeted panels (>100× depth) or WES (>80×) or whole genome–based approaches including high-coverage WGS. Importantly, coverage should be adjusted to enable detection of VAF> 5%. A mutation in the *ALK* TKD will be called in case of a modification of the DNA sequence with a VAF >5%. Lower VAF might be indicated in the detailed report but might not be taken into account as an inclusion criterion for *ALK* inhibitor treatment depending on the trial. If the sequence variation leads to a change in the amino acid sequence and is known to be of functional impact, this will then be reported as pathogenic or likely pathogenic. If the sequence variation leads to a change in the amino acid sequence that does not have well-established evidence for functional impact, the laboratory should assess these variants for pathogenicity. If deemed pathogenic or likely pathogenic, laboratories should then submit these variants to an expert committee for central review, including their evidence supporting their pathogenicity assessment, which may include *in silico* evidence. The results will be indicated using Human Genome Variation Society nomenclature, with annotations indicating the nucleic acid and amino acid change, c. and p., applied to the NM_004304.4 transcript isoform (ALK Transcript: NM_004304.4, *https://www.ncbi.nlm.nih.gov/nuccore/NM_004304.4*; ALK Protein: NP_004295.2, *https://www.ncbi.nlm.nih.gov/protein/29029632*; last accessed September 26, 2025). Importantly, it is recommended that the clinical decision-making conclusion of the *ALK* copy number status and mutational status be reported to the clinician responsible for a given patient within 21 days after tumor biopsy.

In summary, the current study led to the development of guidelines for the detection and nomenclature for *ALK* activating genetic alterations in NB. The results highlight the importance of molecular diagnosis within a network of national reference laboratories to enable discussion of rare molecular events in clinical decision-making.

Disclosure Statement

None declared.

Supplemental Data

Supplemental material for this article can be found at *https://doi.org/10.1016/j.jmoldx.2025.09.007*.

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