ORIGINAL ARTICLE

Ultrafast Gene Fusion Assessment for Nonsquamous NSCLC

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ABSTRACT

Introduction: Gene fusion testing of *ALK*, *ROS1*, *RET*, *NTRK*, and *MET* exon 14 skipping mutations is guideline recommended in nonsquamous NSCLC (NS-NSCLC). Nevertheless, assessment is often hindered by the limited availability of tissue and prolonged next-generation sequencing (NGS) testing, which can protract the initiation of a targeted therapy. Therefore, the development of faster gene fusion assessment is critical for optimal clinical decision-making. Here, we compared two ultrafast gene fusion assays (UFGFAs) using NGS (Genexus, Oncomine Precision Assay, Thermo Fisher Scientific) and a multiplex reverse-transcriptase polymerase chain reaction (Idylla, GeneFusion Assay, Biocartis) approach at diagnosis in a retrospective series of 195 NS-NSCLC cases and five extrapulmonary tumors with a known *NTRK* fusion.

Methods: A total of 195 NS-NSCLC cases (113 known gene fusions and 82 wild-type tumors) were included retrospectively. To validate the detection of a *NTRK* fusion, we added five *NTRK*-positive extrathoracic tumors. The diagnostic performance of the two UFGFAs and standard procedures was compared.

Results: The accuracy was 92.3% and 93.1% for Idylla and Genexus, respectively. Both systems improved the sensitivity for detection by including a 5′-3′ imbalance analysis. Although detection of *ROS1*, *MET* exon 14 skipping, and *RET* was excellent with both systems, *ALK* fusion detection was reduced with sensitivities of 87% and 88%, respectively. Idylla had a limited sensitivity of 67% for *NTRK* fusions, in which only an imbalance assessment was used.

Conclusions: UFGFA using NGS and reverse-transcriptase polymerase chain reaction approaches had an equal level of detection of gene fusion but with some technique-specific limitations. Nevertheless, UFGFA detection in routine clinical care is feasible with both systems allowing faster initiation of therapy and a broad degree of screening.

Keywords: Gene fusion; Non–small cell lung carcinoma; Next generation sequencing; RT-PCR

Introduction

Therapeutic strategies based on targeted therapies and immunotherapy have increased tremendously the overall survival of nonsquamous NSCLC (NS-NSCLC).[1] Consequently, the number of actionable genomic alterations has expanded rapidly.[2,3] International guidelines now highlight mandatory testing of *EGFR*, *ALK*, *ROS1*, *BRAF*, *NTRK*, *RET*, and *MET* at diagnosis of advanced NS-NSCLC.[2–4] In addition, the programmed death-ligand 1 (PD-L1)–positive tumor cell status must be determined using immunohistochemistry (IHC).[3] Importantly, careful clinical decision-making requires the reporting of the above-mentioned molecular alterations immediately because in the absence of a full report treatments such as chemotherapy with or without immunotherapy are initiated while waiting for the results. This leads to suboptimal treatment as the response to treatment is often limited in patients with actionable alterations, such as *EGFR* mutations or gene fusions, particularly in *ALK*, *ROS*, *RET*, *MET*, or *NTRK*, in which targeted treatments were found to have impressive clinical benefit.[5]

Owing to the extensive requirements of biomarker testing for NS-NSCLC, next-generation sequencing (NGS) has become the optimal approach for parallel assessment of gene alterations.[4,6,7] Nevertheless, NGS can be difficult to master and can lead to a long turnaround time (TAT) to receive the results, which is often not compatible with international guidelines.[8,9] Therefore, many laboratories still rely on single gene and sequential approaches (such as IHC, fluorescence *in situ* hybridization [FISH], and targeted sequencing) to obtain rapid results in daily practice. Nevertheless, this latter strategy can be difficult to perform owing to the small sample size and, importantly, owing to the increased number of druggable genomic alterations in NS-NSCLC, notably gene fusions.[10,11]

Consequently, ultrafast testing strategies that allow the rapid assessment of genomic alterations have become increasingly important to ensure optimal clinical decision-making, especially for gene fusions in which a plethora of different methods are currently being used.[12] Here, we evaluated the workflows of two ultrafast gene fusion assays (UFGFAs) used as reflex and point-of-care testing in routine clinical practice for NS-NSCLC. The Idylla GeneFusion assay is a fully automated cartridge-based quantitative polymerase chain reaction (qPCR) system that assesses gene fusions in *ALK*, *ROS1*, *RET*, *MET* exon 14 skipping, and *NTRK*1/2/3. The Ion Torrent Genexus NGS system is a fully integrated sequencing device that allows the assessment of 50 genes from both DNA and RNA, including gene fusions in *ALK*, *ROS1*, *RET*,

MET, and *NTRK*1/2/3. We hypothesized that both systems are able to rapidly deliver results into gene fusions without compromising diagnostic accuracy. We report on 195 NS-NSCLC cases, including 113 cases with known gene arrangements in *ALK*, *ROS1*, *RET*, *MET*, and *NTRK* and five *NTRK*-positive extrathoracic tumors and 82 wild-type (WT) tumors, as defined by the standard procedures that are used routinely.

Material and Methods

In total, 195 patients diagnosed between 2005 and 2022 were retrospectively included. The different samples were selected from centers in five academic hospitals in France (Department of Pathology, Hôpital Haut-Lévêque, CHU of Bordeaux; Department of Pathology, Institut Universitaire du Cancer, CHU of Toulouse; Department of Pathology, CHU of Rouen; Department of Pathology, Hospices Civils de Lyon; Laboratory of Clinical and Experimental Pathology, Pasteur Hospital, CHU of Nice). The main epidemiologic, clinical, and pathologic data are found in Table 1. Histologic classification was made according to the 2021 WHO classification of thoracic tumors, using the terminology for resected specimens, small biopsies, and cytology specimens.[13] Using the standard procedures routinely used in daily practice in the different centers, 113 gene fusion-positive tumors were selected: *ALK* (62 of 113 cases, 55%), *ROS1* (24 of 113 cases, 21%), *RET* (12 of 113 cases, 10.6%), *NTRK* (two of 113 cases, 1.7%), and *MET* exon 14 skipping alterations (13 of 113 cases, 11.5%). We selected five additional *NTRK*-positive tumors (four thyroid carcinomas and one inflammatory myofibroblastic tumor of the orbit) owing to the scarcity of *NTRK* rearrangements in NS-NSCLC. Standard procedures included *ALK* IHC (D5F3 clone, Ventana, Tucson, AZ); *ALK* FISH (Vysis ALK Break Apart FISH Probe Kit, Abbott Molecular, Des Plaines, IL); *ROS1* IHC (D4D6 clone, Cell Signaling Technology, Danvers, MA); *ROS1* FISH (Poseidon ROS1 Break Probe Kit, Kreatech Inc., Durham, NC); Oncomine Focus Assay (OFA NGS, Thermo Fisher Scientific, Waltham, MA); and nCounter Gene Fusion Panel (NanoString Technologies, Seattle, WA). An additional 82 NS-NSCLCs were found to be WT for the genes cited previously when using the standard procedures (Table 1).

Cases were analyzed using two workflows for multiplex reverse-transcriptase polymerase chain reaction and NGS analyses. The percentage of tumor cells and the tumor surfaces were noted, and a macrodissection or whole sections were obtained after selection of the tumor area by an expert thoracic pathologist (VH, CB, SL, EL, MI, and PH). Selected paraffin tissue sections were processed using an Idylla platform and the GeneFusion Assay (Biocartis) (ref A0121/6, Biocartis NV, Malines, Belgium). Selected paraffin tissue sections were processed for NGS analyses using a Genexus system (Thermo Fisher Scientific) after nucleic acid extraction (Maxwell RSC Instrument, Promega Corporation, Madison, WI). Quantification and quality assessment of the extracted nucleic acid were first evaluated using a Qubit (Qubit 2.0 Fluorometer, Thermo Fisher Scientific [Bourgoin-Jallieu, France]; Qubit dsDNA HS Assay Kit [ref Q32851 and Qubit RNA HS Assay Kit ref Q32852]) and a nanodrop (Nanodrop One, Ozyme [Saint-Cyr-l'Ecole, France]) instrument. The Oncomine Precision Assay that assessed parallel alterations in RNA and DNA was used according to the manufacturer's recommendations (Thermo Fisher Scientific). The standard procedure used at the LPCE (Nice, France) for *EGFR* status assessment was performed using the Idylla *EGFR* test (Biocartis; ref A0060/6, Biocartis NV, Mechelen, Belgium). All the samples were considered as routine samples, and molecular testing was completed within working days. The study was performed in accordance to the guideline of

Table 1. Characteristics of the NS-NSCLC Patient Cohort

Features		n
Age [median (range)]		67 (25-90)
Sex	Female	108
	Male	87
Histology	Acinar adenocarcinoma	68
	Papillary adenocarcinoma	39
	Solid adenocarcinoma	32
	Micropapillary adenocarcinoma	14
	Invasive mucinous adenocarcinoma	12
	Lepidic adenocarcinoma	10
	Non-small cell carcinoma, NOS	5
	Non-small cell carcinoma, favoring adenocarcinoma	4
	Large cell neuroendocrine carcinoma	4
	Adenosquamous carcinoma	3
	Minimally invasive adenocarcinoma	2
	Adenocarcinoma *in situ*	1
	Unclassified malignant tumor	1
Stage	I	76
	II	31
	III	33
	IV	51
	NA	4
Gene altered	*ALK*	62
	MET	13
	NTRK	2
	RET	12
	ROS1	24
	WT	82

NA, not available; NOS, not otherwise specified; NS-NSCLC, nonsquamous NSCLC; WT, wild-type.

the Declaration of Helsinki, approved by the local ethics committee, and all patients provided written informed consent.

The results were compared for accuracy, sensitivity, and specificity according to each of the two UFGFA workflows and to the standard procedures. In addition, positive and negative predictive values were estimated on the basis of an estimated prevalence of *ALK* (4%), *ROS1* (2%), *MET* (3%), *RET* (2%), and *NTRK* (0.2%).

Results

Patient Cohort

In total, we included 200 patient cases, of which 118 were known to harbor gene fusions and 82 WT control samples. Among these, 195 samples were from NS-NSCLC and 5 from extrapulmonary tumors with known *NTRK* fusions, which have been included to improve the analysis of the gene fusion owing to the scarcity of this fusion type in NSCLC. In total, 200 were tested using the Idylla qPCR system (196 successful, 98%) and 188 using the Genexus NGS system (188 successful, 100%) (Fig. 1). A small proportion (18 of 218, 8%) of the screening cohort of specimens were not used because they were unsuitable for testing owing to insufficient amount of tumor material.

Diagnostic Accuracy

The accuracy, sensitivity, and specificity were assessed for each of the two techniques. The negative and positive predictive values were calculated on the basis of an estimated prevalence of 4%, 2%, 3%, 2%, and 0.2% for *ALK*, *ROS1*, *MET*, *RET*, and *NTRK*, respectively,[14,15] and are summarized in Table 2. Both systems not only report the detection of gene fusions but also allow the detection of gene fusion events by using 5′-3′ imbalance, which indicates a genomic rearrangement without further specifying the gene fusion partner. Consequently, the diagnostic accuracy is highlighted for both systems with and without imbalance assessment (Table 2).

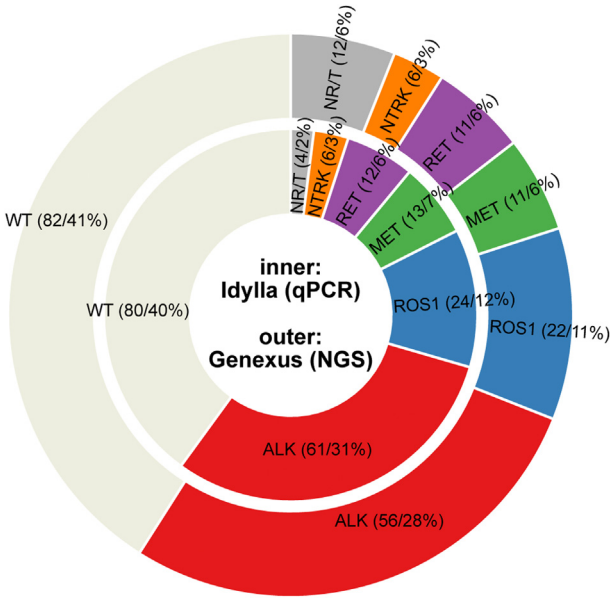

Figure 1. Testing of different populations using the Idylla qPCR system (inner ring) and the Genexus NGS system (outer ring). The numbers in parentheses are the case numbers with the alterations or percentages. qPCR, quantitative polymerase chain reaction; NGS, next-generation sequencing.

The key performance parameters including the accuracy, sensitivity, and specificity were higher for the Genexus NGS assay versus the Idylla qPCR assay (Table 2). Although there were minimal differences between most of the fusions tested, the Idylla qPCR system had a low sensitivity for *NTRK* fusions (67%) whereas the NGS assay had 100% sensitivity in this limited sample set. For both systems, the imbalance analysis not only increased the sensitivity but also decreased the specificity owing to the presence of false-positive calls (Table 2).

We further analyzed factors contributing to the accuracy of the assays with emphasis on *ALK* gene fusions when the performance was reduced in both assays.

The different tumor stages for each gene are highlighted in Supplementary Figure 1. Tumor stages have been balanced across the different groups tested and did not influence the results. Nevertheless, lower sensitivities were found in early stage (I, II) *ALK* cases for samples tested with the Idylla without imbalance, whereas staging did not affect the sensitivity using the NGS system (Supplementary Fig. 2). In contrast, the Genexus system yielded a slightly lower sensitivity with small samples (bronchial and transthoracic biopsies or cytologic specimen), which did not influence the results on the Idylla system (Supplementary Figs. 3 and 4).

Furthermore, we analyzed how the tumor cell content influenced the detection of gene fusions using the respective assays. The tumor cell content did not differ significantly between the cohorts (Supplementary Fig. 5), and analysis of the association of the tumor cell content with detection of *ALK* gene fusions using receiver operating characteristics highlighted the limited effect of the tumor cell content on sensitivity (Supplementary Fig. 6).

Fusion Partner and Detection

The detection of gene fusion events is illustrated in Figure 2*A*. As previously found, the introduction of detection of gene fusions by imbalance not only increased the sensitivity but also led to false-positive calls. Although false-positive calls with the Idylla

Table 2. Diagnostic Performance Summary

Performance According to Biomarkers	Idylla	Idylla (No Imbalance)	Genexus	Genexus (No Imbalance)
Accuracy (95% CI)	0.923 (0.88-0.96)	0.867 (0.81-0.91)	0.931 (0.89-0.96)	0.931 (0.89-0.96)
Sensitivity (95% CI)	0.914 (0.86-0.97)	0.793 (0.72-0.87)	0.934 (0.89-0.98)	0.877 (0.82-0.94)
ALK	0.87	0.72	0.88	0.80
ROS1	1.00	1.00	1.00	1.00
MET	1.00	1.00	1.00	1.00
RET	1.00	0.92	1.00	1.00
NTRK	0.67	0.000	1.00	0.67
Specificity (95% CI)	0.951 (0.91-1.0)	0.988 (0.96-1.0)	0.927 (0.87-0.98)	1.0 (1.0-1.0)
ALK	0.99	1.00	0.95	1.00
ROS1	0.99	0.99	1.00	1.00
MET	1.00	1.00	1.00	1.00
RET	0.99	0.99	1.00	1.00
NTRK	0.99	1.00	1.00	1.00
NPV[a]				
ALK (4%)	0.99	0.99	0.99	0.99
ROS1 (2%)	1.00	1.00	1.00	1.00
MET (3%)	1.00	1.00	1.00	1.00
RET (2%)	1.00	0.77	1.00	1.00
NTRK (0.2%)	1.00	1.00	1.00	1.00
PPV[a]				
ALK (4%)	0.71	1.00	0.45	1.00
ROS1 (2%)	0.78	0.78	1.00	1.00
MET (3%)	1.00	1.00	1.00	1.00
RET (2%)	0.79	0.77	1.00	1.00
NTRK (0.2%)	0.20	NA	1.00	1.00

[a]Estimate based on highlighted predicted prevalence. The percentages in the parentheses are frequencies of molecular alterations.
CI, confidence interval; NPV, negative predictive value; PPV, positive predictive value.

system occurred in almost all cohorts, false-positives for the Genexus system were exclusively limited to *ALK* using imbalance detection. We found that reads were particularly low in these cases and assumed that there were problems with the software algorithm rather than underlying problems with the biochemical limitations of the assay. On discussion with the manufacturer who recommended an improved and more stringent analysis, we analyzed a second time the false-positive samples and two true-positive *ALK* samples on the basis of imbalance (Supplementary Fig. 7). Indeed, the improved algorithm removed all false-positive calls but seemed to reduce overall sensitivity.

Specific gene fusion partners can also be identified using the NGS system and are highlighted in Figure 2*B*. The predominant fusion partner in *ALK* was EML4 with different exon junctions, but non-EML4 partners, EPS15, KIF5B, and STRN, were also detected. ROS1 fusions, in contrast, were more diverse with multiple defined gene fusion partners with EZR and CD74 as predominant ones, whereas only KIF5B-RET fusions have been detected. NTRK fusions were predominantly ETV6-NTRK3 with one LMNA-NTRK1 fusion, though the sample size was limiting (Fig. 2*B*). Importantly, less gene fusions can be detected using the Idylla system compared with the Genexus NGS system; fusions that are not covered by the system are highlighted in bold.

Discussion

This study revealed good concordance between the two UFGFAs, a multiplex reverse-transcriptase polymerase chain reaction (Idylla) and an NGS (Genexus) assay for the assessment of gene fusions in NS-NSCLC. Although both systems had comparable performance and accuracy, sensitivity was higher with the Genexus NGS system. Nevertheless, both systems had limitations. Not including cytologic samples is a limitation of this study because it is the only type of material available in a proportion of routine cases. The Idylla system had a lower sensitivity especially for the *NTRK* fusion but also yielded false-positive gene fusion calls across multiple genes. The performance of the Idylla assay was in line with recently published data.[16,17] Notably, the assay was designed to detect *NTRK* fusions using only imbalance and consequently may yield a lower sensitivity compared with the targeted approach used for other genes.[18] The Genexus system reported ALK rearrangements owing to imbalance for several patients with ALK WT. Careful assessment highlighted a minimal number of ALK reads and consequently a bioinformatic limitation.

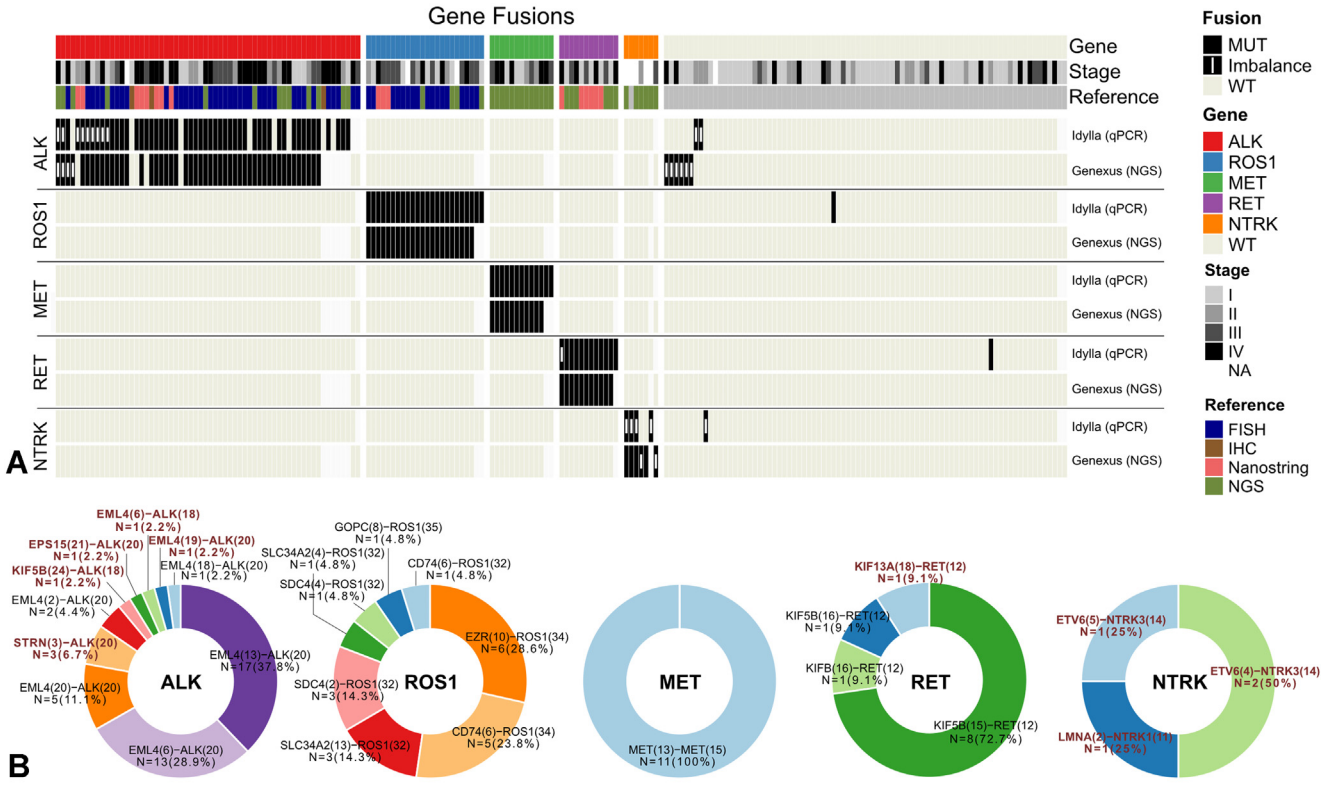

Figure 2. Detection of gene fusions using ultrafast gene fusion assays. (*A*) The result based on standard procedure is highlighted on the top. Each column represents one case, and each result for the respective genes analyzed using both systems is highlighted. (*B*) Description of gene fusion events. Results from the Genexus NGS system on the distribution of gene fusion events for each of the respective genes. Gene fusions that are not part of the Idylla design and consequently cannot be detected by the respective devices are highlighted in bold. The numbers in parentheses are the case numbers with the alterations or percentages. FISH, fluorescence in situ hybridization; IHC, immunohistochemistry; qPCR, quantitative polymerase chain reaction; NGS, next-generation sequencing.

Nevertheless, improved algorithms for analysis may improve the assay, and consequently current users are encouraged to consider updating the bioinformatics to improve the specificity of the assay with imbalance assessment. Importantly, both manufacturers highlight the importance of secondary testing in case of a positive imbalance, and on the basis of our results, we strongly suggest confirming the results of these cases with another test, such as FISH or IHC. Nevertheless, introducing the detection of gene fusions by 5′-3′ imbalance improved the sensitivity and is therefore a promising addition to the detection of gene arrangements in routine clinical care.

The detection of ALK gene fusions, however, remained particularly challenging for both systems. This leads to low positive predictive values and consequently the need for an additional method of testing (FISH or IHC) to confirm the presence of the ALK rearrangement. Other NGS assays have already highlighted some of the limitations of the detection of ALK rearrangements,[19,20] in particular gene-specific limitations for the alterations confirming the persistent need of ALK IHC or FISH. In this context, notably for very small sample sizes, it is useful to obtain two tissue sections before nucleic acid extraction for NGS assays. Moreover, reflex ALK IHC testing in all advanced NS-NSCLC should be considered as a valid option to speed up and facilitate the diagnostic process.[21]

Importantly, both systems are able to deliver the promise of ultrafast gene assessment to enable quick clinical decision-making. The Idylla qPCR assay uses single-use cartridges for each patient and is thus not limited by batching. The highly automated process only requires the addition of a tissue section and has been established in many laboratories for rapid implementation without the need of additional training. The TAT from adding a formalin-fixed, paraffin-embedded sample to a report is approximately 3 hours and thus allows same-day reporting. In contrast, the Genexus NGS system requires batching of samples to initiate a run, but the minimal number of four samples allows quick initialization even in low to medium throughput laboratories.[22] The TAT is approximately 24 hours, which often allows next-day reporting. Both systems consequently offer a

considerable improvement compared with other NGS assays, which often require several days to weeks.[23] Nevertheless, high-throughput centers that can conduct the analysis daily might achieve a TAT as rapid as 24 hours for the Genexus NGS technology, although this would not be cost-efficient in centers with lower throughput.

Importantly, the guidelines include the mandatory testing of certain gene fusions but most importantly also common druggable mutations, including mutations in *EGFR*, *BRAF*, and *KRAS*, but also other emerging biomarkers, such as *STK11*, are often assessed for clinical decision-making.[24] Consequently, the ultrafast assessment of gene fusions needs to be integrated into the other recommended biomarkers in NS-NSCLC and PD-L1 IHC. This is facilitated by the use of the Genexus NGS system, which includes both gene fusion detection and DNA mutation calling, and thus allows a full report on recommended molecular assessment in NS-NSCLC, with the exception of PD-L1. Notably, some emerging markers such as *KEAP1*, a negative predictor to the response to immunotherapy,[25] have been included in the latest IVD version of the Genexus panel (Oncomine Dx Express Test Ref. A54103). In contrast, the Idylla cartridge is specific for detection of gene fusions, and thus an additional analytical run needs to be performed to analyze the remaining mutations. Although additional cartridges for the assessment of these genes can be used, additional tissue is needed, which is often limiting, and parallel assessment requires the installation of additional devices. Nevertheless, the combination of the automated qPCR system with subsequent NGS analysis for broader biomarker assessment has already been found to be useful,[26] and such a hybrid strategy might be useful with the inclusion of the UFGFA. This scenario may be limited to urgent cases depending on the epidemiologic profile of the patient (e.g., young, nonsmoker).

In summary, the ultrafast detection of gene fusions in NS-NSCLC in routine clinical care for rapid clinical decision-making is feasible and yields a sufficient level of performance. Both the Idylla qPCR and the Genexus NGS systems are able to provide reliable data with limitations primarily on the basis of their underlying technologies. Importantly, this study reveals that the need for shorter TAT in clinical diagnosis can be addressed without compromising reliability.

CRediT Authorship Contribution Statement

Véronique Hofman: Conceptualization, original draft preparation, data curation, writing—reviewing, final approval of the manuscript.

Simon Heeke: Conceptualization, methodology, investigation, original draft preparation, data curation, writing—reviewing, final approval of the manuscript.

Christophe Bontoux: Writing—reviewing, resources, final approval of the manuscript.

Lara Chalabreysse: Writing—reviewing, resources, final approval of the manuscript.

Marc Barritault: Writing—reviewing, resources, final approval of the manuscript.

Pierre Paul Bringuier: Writing—reviewing, resources, final approval of the manuscript.

Tanguy Fenouil: Writing—reviewing, resources, final approval of the manuscript.

Nazim Benzerdjeb: Writing—reviewing, resources, final approval of the manuscript.

Hugues Begueret: Writing—reviewing, resources, final approval of the manuscript.

Jean Philippe Merlio: Writing—reviewing, resources, final approval of the manuscript.

Charline Caumont: Writing—reviewing, resources, final approval of the manuscript.

Nicolas Piton: Writing—reviewing, resources, final approval of the manuscript.

Jean-Christophe Sabourin: Writing—reviewing, resources, final approval of the manuscript.

Solène Evrard: Writing—reviewing, resources, final approval of the manuscript.

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Jonathan Benzaquen: Writing—reviewing, resources, final approval of the manuscript.

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Charles-Hugo Marquette: Writing—reviewing, validation, final approval of the manuscript.

Marius Ilié: Writing—reviewing, validation, final approval of the manuscript.

Paul Hofman: Supervision, project administration, conceptualization, validation, original draft preparation, writing—reviewing, final approval of the manuscript.

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Supplementary Data

Note: To access the supplementary material accompanying this article, visit the online version of the *JTO Clinical and Research Reports* at www.jtocrr.org and at 10.1016/j.jtocrr.2022.100457.

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