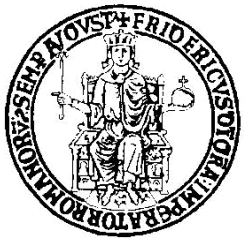
UNIVERSITA' DEGLI STUDI DI NAPOLI FEDERICO II



DIPARTIMENTO DI SANITA' PUBBLICA

DOTTORATO DI RICERCA IN SANITA' PUBBLICA E MEDICINA PREVENTIVA - XXXIII CICLO

"FULLY AUTOMATED REAL-TIME PCR IN MOLECULAR PATHOLOGY: NOVEL APLLICATIONS"

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Chapter 4

Introduction

A deeper knowledge of cancer biology, alongside advances in diagnostic technologies, has led to much more effective therapeutic strategies in Indeed, over the last decade or so, the incessant quest for new genetic alterations capable of predicting patient response to treatments, has given rise to the development of highly sophisticated molecular tests Accordingly, many sequencing platforms can nowadays detect multiple driver mutations simultaneously thanks to fully automated procedures which drastically reduce turnaround time and costs. A case in point is the *Idylla*™ system (Biocartis NV, Mechelen, Belgium. This system is a fully automated sample-to-result Real-Time PCR with all reagents integrated in a single-use cartridge; it consists of a console which displays results as either "mutation" or "no mutation" when DNA quality is adequate, or as "invalid" when it is. What is most striking about this platform is that the cartridge can run multiple sample types, including solid and liquid biopsies, yielding results within approximately two hours. Another advantage is its ease of use taking up only a couple of minutes of hands-on time to prepare samples and load the cartridge. This fascinating assay has been validated for the identification of many driver genes, both on tissue and on plasma specimens. In particular *Idylla*™ *EGFR*, *Idylla*™ *BRAF*, *Idylla*™ *KRAS*, *Idylla*™ *NRAS-BRAF*, and *Idylla*™ *MSI* have been validated on formalin fixed paraffin embedded (FFPE) samples; instead, *Idylla*™ *ctKRAS* and *Idylla*™ *ctNRAS-BRAF* have been validated on plasma samples. Many more are currently in the pipeline. The aim of my PhD project was to develop and validate novel clinical applications of the the *Idylla*™ system. I divided my thesis into four chapters.

The first chapter focuses on the performance of the *Idylla*™ *EGFR* Assay on cytological non-small cell lung cancer samples. This test was specifically designed to process formalin-fixed, paraffin-embedded sections without requiring preliminary DNA extraction. On the other hand, my PhD work has demonstrated that this assay can also be used to process archival smears from patients with NSCLC by scraping the stained cellular material directly into the cartridge.

The second chapter focuses on the viable application of *Idylla*™ *NRAS-BRAF* Assay to cytological thyroid fine-needle aspirates (FNAs) with undetermined morphology. Our research demonstrated that FNA needle rinses can be genotyped by the same cytopathologist who performs the FNA, a procedure that is commonly called rapid on-site molecular evaluation (ROME).

The third chapter, instead, discusses the feasibility of using *Idylla*™ to analyze liquid biopsy specimens. Such application is highly important, given the scant availability of tissue specimens in advanced NSCLC patients. Our laboratory results have indeed demonstrated the efficiency of

the *Idylla™ ctKRAS* Assay in detecting plasma the *KRAS* p.G12C mutation, a novel target in NSCLC patients.

Finally, in the fourth chapter, I explore the analytical and clinical performance of the *Idylla™* SARS-CoV-2 test on previously tested SARS-CoV-2 people by conventional RT-PCR based approach in different settings, including initial diagnosis and clinical follow-up. In this regard, I provide substantial evidence that this assay may represent a valid, fast, and highly sensitive and specific RT-PCR test for the identification of SARS-CoV-2 infection.

Chapter 1

Is the Idylla *EGFR* Mutation Assay feasible on archival stained cytological smears? A pilot study

Lung cancer is the leading cause of cancer death worldwide.[1] In the most advanced stages of the disease, several diverse genomic biomarkers are nowadays commonly assessed to guide targeted treatments.[2–5] In this regard, the recently updated guideline developed by the College of American Pathologists (CAP), the International Association for the Study of Lung Cancer (IASLC), and the Association for Molecular Pathology (AMP) identifies *EGFR*, *ALK* and *ROS1* as the 'must test genes'.[6] Therefore, depending on the resources and technologies locally available, different strategies can be adopted.[7] Ideally, multigene testing technologies, including next-generation sequencing (NGS), are preferable over single gene testing approaches, for they enable cytopathologists to detect large numbers of actionable mutations simultaneously.[8] However, not all institutions have the necessary expertise and sample workload required to implement NGS in their routine clinical practice. Moreover, because many laboratories commonly run sample batching, a longer turnaround time (TAT) is needed to collect test results. This scenario is, therefore, not always ideal for acute deteriorating patients, who would certainly benefit from receiving their laboratory test results in the shortest possible time. In this setting, fully automated testing platforms from sample-to-result, such as the *Idylla™* platform (Biocartis, Mechelen, Belgium), have been shown to be a viable option to reduce overall TAT.[9 10] The Idylla platform, which has been fully validated on unstained formalin-fixed and paraffin-embedded (FFPE) sections, entails the use of disposable cartridges in which microfluidic processing takes place with all reagents on board.[11] In particular, FFPE sections are inserted into the cartridge for DNA extraction and subsequent processing. Noticeably, the Idylla *EGFR* Mutation Test has been approved by the European Community (CE) as an in vitro diagnostic (IVD) assay.[12] It is based on real-time PCR and uses a

fluorophore-based detection system. In brief, microfluidic channels, embedded in the cartridge, transport the nucleic acids into five separate PCR chambers containing pre-deposited PCR reagents in dried form (i.e., primers, probes, and enzymes). These enable simultaneous analysis of the DNA sample and endogenous sample processing control.[12] Various research labs are currently investigating whether this system can also be applied to cytological smears, which are often the only specimens available to test lung cancer patients for actionable mutations. Not surprisingly, the American Society of Clinical Oncology has fully endorsed the recent CAP/IASLC/AMP guideline, which strongly recommends the use of cytological smears as the specimen of choice for lung cancer biomarker testing.[13] Usually, when cytological smears are processed for EGFR testing, the neoplastic cells are scraped off the slide and selected for DNA extraction. In a previous study, we showed that this approach can also be applied to the *Idylla*™ platform.

Indeed, a few years ago, my research team demonstrated that DNA extracted from stained smears can be directly pipetted into the *Idylla*™ cartridge, allowing for successful EGFR testing.[14] However, DNA extraction requires dedicated laboratory space, specific reagents, expensive equipment, and, not least, highly trained personnel. Thus, studies propose as an alternative , a sample-to-result procedure. With the goal of making the procedure more simple and feasible even for laboratories lacking expertise in DNA extraction, we hypothesized that the scraped cytological material could be directly inserted into the cartridge—a procedure that would avert, the risk of contamination and loss of material. Accordingly, one of my research objectives was to assess the applicability and performance of the *Idylla*™ *EGFR* Mutation Assay on cytological material scraped off stained smears and directly inserted into the cartridge without the need for DNA extraction.

Study Design

To assess the efficiency of *Idylla*™ *EGFR* Mutation Assay in detecting *EGFR* mutations on stained smears rather than on DNA extracts, we decided to divide the study into two parts. In the first part, we applied the *Idylla*™ *EGFR* Mutation Assay to evaluate a series of archival stained smears, whose *EGFR* mutational status had already been established. Cases showing discrepant results were further processed by NGS as previously described.[10 15] The raw data of every cartridge were also re-analyzed by TIBCO Spotfire application. In the second part, we evaluated whether the performance of *Idylla*™ *EGFR* Mutation Assay could be improved by de-staining the slides before testing. All information regarding human material was managed using anonymous numerical codes, and all

samples were handled in compliance with the Helsinki Declaration (http://www. wma. net/en/ 30publications/ 10policies/ b3/).

Idylla EGFR Mutation Assay on routine cytological specimens

The *Idylla™ EGFR* Mutation Assay, performed on the *Idylla™* platform, was carried out as previously described,[14] the only difference being that the scraped material rather than the extracted DNA was processed. The *Idylla™ EGFR* Mutation Assay (Research Use Only, RUO) and the *Idylla™ EGFR* Mutation Test (IVD) were used. The performance of the two tests displayed no intrinsic differences. Briefly, the *Idylla™ EGFR* Mutation Assay is fully automated, requiring only 2 min of hands-on time and no high technical expertise. The system covers 51 mutations in exons 18, 19, 20, and 21 in a single cartridge. The results appear on the platform console as mutation detected' or 'no mutation detected' if the sample is adequate for the analysis; otherwise, they appear as 'invalid' if the DNA quantity or quality is insufficient. The raw data of every cartridge were also re-analyzed by a TIBCO Spotfire application, which enabled us to visualize PCR curves and to estimate the fluorescence levels in channels 1–5.

Performance of the Idylla EGFR Mutation Assay on stained cytological smears

To assess whether the *Idylla™ EGFR* Mutation Assay could also be applied to stained cytological smears, we carried out a computerized search. Special care was taken to select cases in which the *EGFR* mutational status had been previously assessed on stained smears with DNA extraction and standard molecular methods.[16] Only those cases with availability of additional smears were selected; care was also taken to preserve at least one smear in the archives to keep a morphological record of the presence of diagnostic cells. Moreover, caution was taken to select cases that had been evaluated at least 5 years earlier to minimize the risk of using material potentially useful for further analysis. Every case was microscopically re-evaluated by experienced pathologists to confirm the original diagnostic pathological report and to assess the percentage of neoplastic cells. Morphological diagnoses included adenocarcinoma (ADC), favor ADC, carcinoma not otherwise specified (NOS), and squamous cell carcinoma (SqCC). The neoplastic cellularity was semi quantitatively graded according to the percentage of neoplastic cells on the total cellularity (5%–

24%; 25%–49%; ≥50%) (table 1). Overall, 39 NSCLC cases were selected for the study. In most cases (n=32), a single smear was used, whereas in seven cases, two or more smears were employed. The type of specimen preparation, for the most part obtained from fine-needle aspiration biopsy samples (33/39; 84.6%), is reported in Table 1. Since the smears were outsourced from different institutions, they were stained differently. In particular, 21 (53.8%) slides had been stained with H&E, 11 (28.2%) with Papanicolaou, 6 (15.4%) with Diff Quik and 1 (2.6%) with thyroid transcription factor-1 antibody. In all cases, the original diagnoses were confirmed on microscopic review. They included 22 (56.4%) ADC, 10 (25.6%) favor ADC, 6 (15.4%) NOS, and 1 (2.6%) SqCC. The percentage of neoplastic cells was ≥50% in 17 samples, between 25% and 49% in 5 samples, and between 5% and 24% in 17 samples, and <10% only in three cases. As reported above, care was taken to select smears that had been archived for at least 5 years. Back then, our laboratory carried out EGFR testing only for exon 19 deletions and p.L858R point mutation in exon 21 using fragment length assay and TaqMan assay, respectively.[16] Both laboratories developed tests that we had thoroughly validated and extensively used in our routine practice.16 Accordingly, in our study, 14 (35.9%) out of 39 selected cases harbored a mutation in either exon 19 (n=11;28.2%) or exon 21 (n=3; 7.7%) (Table 1).

Performance of EGFR testing on de-stained cytological Material

In the second part of the study, we set out to assess whether staining could negatively affect the performance of the *Idylla™ EGFR* Mutation Assay. In particular, we investigated whether dye residues from original cytological staining undermined the fluorescence intensity of the different channels. To this end, we carried out a computerized search to select 10 cases in which matched lung cancer smears and cell blocks (CBs) were available. In particular, the *Idylla™ EGFR* Mutation Assay level of fluorescence was assessed by using CB sections in four different modalities, in particular, by monitoring the background fluorescence in one of the channels (channel number 2). In brief, in the first modality, un-stained CB sections were inserted directly into the cartridge; in the second modality, CB sections were first stained with H&E and then inserted into the cartridge; in the third modality, CB sections were stained with H&E and then partially de-stained by decreasing concentrations of ethanol; in the fourth modality, the CB sections were first stained with H&E; then, the DNA was extracted by the Qiagen DNA Mini Kit (Qiagen, Hilden, Germany); finally, the extracts were directly pipetted into the cartridge.14 The results of the background fluorescence assessment were then compared with those generated from matched smears. The latter were de-stained, wholly scraped, and inserted into the cartridge. Lastly, the effect of de-staining on

fluorescent detection was investigated by re-analyzing the raw data with the TIBCO Spotfire application.

Results

Performance of the EGFR Idylla assay on archived stained Smears

To test whether the *Idylla™ EGFR* Mutation Assay could be successfully applied to cytological smears, we processed forty-nine 5-year-old archival stained smears from a total of 39 NSCLC cases. For each smear, a single cartridge was employed. Thirty-two cases were processed with a single smear, four cases with two smears, and three cases with three smears. Remarkably, only 6 out of 39 (15.4%) cases yielded invalid results. Moreover, although seven cases had been processed with more than one smear, the different cartridges always generated concordant results (Table 2). All invalid cases had been previously classified as wild-type (WT) by standard diagnostic methods (fragment length assay and TaqMan assay). Interestingly, whereas the standard protocols had assessed 19 cases, as EGFR WT cases, the *Idylla™* assay, instead, revealed the absence of *EGFR* mutations in 17/19 cases. Noteworthy, in the two remaining cases, the novel system validated *EGFR* p.G719X mutation in exon 18. This result was further confirmed by NGS analysis (Table 3). Overall, the *Idylla™ EGFR* Mutation Assay confirmed the presence of EGFR mutations in 11 (78.6%) out of 14 mutated cases. In particular, the *Idylla™ EGFR* Mutation Assay was more accurate in detecting *EGFR* exon 19 deletions than exon 21 p.L858R. In fact, it confirmed *EGFR* exon 19 deletion in 10 out of 11 cases, with a concordance rate of 90.9%. The single discordant case (case 6, Table 1) featured a very scant neoplastic component (5%). Conversely, the *Idylla™ EGFR* Mutation Assay missed most *EGFR* exon 21 p.L858R (2/3) mutations, as evidenced by the low concordance rate of 33.3%. Raw data analysis by the TIBCO Spotfire application consistently showed low background fluorescence in channels 1, 3, 4 and, 5. By contrast, a very high background fluorescence was observed in channel 2, where the reaction involving the detection of *EGFR* exon 21 p.L858R mutation took place (data not shown). In particular, background fluorescence was higher for H&E and for Papanicolaou than for Diff Quick stained smears (figure 1). In channel 2, the typical background fluorescence in unstained samples was below 50 arbitrary fluorescence units (AFU) (data not shown). Taken together, these comparative analyses suggest that the the Idylla™ EGFR Mutation Assay was not as efficient as the more conventional protocols.

ID Sample	Type of preparation	Staining	Diagnosis	%NC	Reference methods	Idylla EGFR Mutation Assay
1	PE	Pap	ADC	5-24	p.E746_A750del	Del 19
2	FNA	Pap	Favour ADC	5-24	p.E746_A750del	Del 19
3	FNA	Pap	ADC	5-24	p.L858R	ND
4	FNA	H&E	ADC	50	p.LREA	Del 19
5	FNA	H&E	Favour ADC	5-24	p.E746_A750del	Del 19
6	FNA	IHC	ADC	5	p.E746_A750del	ND
7	FNA	H&E	ADC	≥50	WT	ND
8	FNA	H&E	ADC	≥50	p.E746_A750del	Del 19
9	FNA	H&E	ADC	≥50	WT	p.G719X
10	FNA	Pap	Favour ADC	5-24	WT	Invalid
11	FNA	H&E	Favour ADC	≥50	WT	Invalid
12	FNA	H&E	ADC	5-24	WT	ND
13	BB	Pap	Favour ADC	5-24	p.E746_A750del	Del ex 19
14	FNA	Pap	ADC	≥50	WT	ND
15	FNA	Diff Quik	ADC	5-24	WT	ND
16	PE	H&E	ADC	5-24	WT	ND
17	FNA	H&E	Favour ADC	5	WT	Invalid
18	PE	H&E	ADC	≥50	WT	ND
19	FNA	Pap	ADC	5-24	WT	Invalid
20	FNA	H&E	ADC	5-24	WT	ND
21	FNA	Pap	ADC	≥50	WT	ND
22	FNA	H&E	ADC	5-24	WT	ND
23	FNA	Diff Quik	ADC	≥50	p.L858R	p.L858R
24	FNA	H&E	ADC	25-49	WT	ND
25	FNA	H&E	Favour ADC	5-24	WT	p.G719X
26	FNA	Pap	Favour ADC	≥50	WT	Invalid
27	FNA	H&E	ADC	5	WT	ND
28	FNA	Pap	ADC	25-49	WT	ND
29	FNA	H&E	ADC	5-24	WT	ND
30	PE	Diff Quik	ADC	25-49	WT	ND
31	FNA	Diff Quik	ADC	≥50	WT	Invalid
32	FNA	H&E	Favour ADC	≥50	WT	ND
33	FNA	Diff Quik	ADC	≥50	p.E746_A750del	Del 19
34	FNA	Diff Quik	ADC	≥50	p.E746_A750del	Del 19
35	PE	H&E	ADC	≥50	p.E746_A750del	Del 19
36	FNA	H&E	ADC	≥50	WT	ND
37	FNA	H&E	ADC	≥50	p.E746_A750del	Del 19
38	FNA	Pap	Favour ADC	25-49	p.L858R	ND
39	FNA	H&E	ADC	25-49	WT	ND

Table 1 Study series including ID sample, type of preparation and staining, morphological diagnosis, percentage of neoplastic cells, reference methods, and Idylla *EGFR* Mutation Assay outcome

ADC, adenocarcinoma; BB, bronchial brushing; FNA, fine-needle aspiration;IHC, immunohistochemistry; NC, neoplastic cellularity; ND, no mutation detected; NOS, not otherwise specified; PE, pleuric effusion; Pap, Papanicolaou; SqCC, squamous cell carcinoma; WT, wild-type.

Performance of EGFR testing on de-stained cytological material

The findings of the first part of the study highlighted a less than optimal performance of the *Idylla™ EGFR* Mutation Assay. We hypothesized that this shortcoming was due to the high levels of background fluorescence generated in channel 2 by the interference of the residual dyes. Thus, in the second part of the study, we attempted to overcome this problem by simply de-staining the cytological material before inserting it into the cartridge. As described in the Materials and Methods section, CB sections were processed in four different manners. Although the level of fluorescence was highly variable between the different cases (ranging from 15 AFU to 1300 AFU) (Figure 2), we observed a very strong unspecific signal in two cases (cases 1 and 3) (Figure 2). Overall, in channel 2, background fluorescence was much higher in stained cytological material than in de-stained or un-stained samples. Intriguingly, we did not expect to see that even after de-staining, the background fluorescence in cases 1 and 3 remained above 100 AFU, that is, twofold higher than the background florescence typically observed in unstained samples.

Table 2 Cases featuring more than one smear are reported; results generated by different cartridges were always concordant

ID Sample	Idylla EGFR Mutation Assay		
	1st smear	2nd smear	3rd smear
2	Del 19	Del 19	Del 19
9	p.G719X	p.G719X	p.G719X
11	Invalid	Invalid	NA
34	Del 19	Del 19	NA
35	Del 19	Del 19	Del 19
37	Del 19	Del 19	NA
38	ND	ND	NA

NA, not available; ND, no mutation detected

Discussion

Lung cancer still remains one of the most frequent and aggressive cancer types worldwide. Unfortunately, being mostly asymptomatic in the early stages, it is often diagnosed in the advanced stages, leaving patients with grim prognosis and very few treatment options. Therefore, detecting the presence of genomic cancer biomarkers, in particular EGFR mutations, in the advanced stages of the disease is crucial to help physicians choose the best targeted treatment options

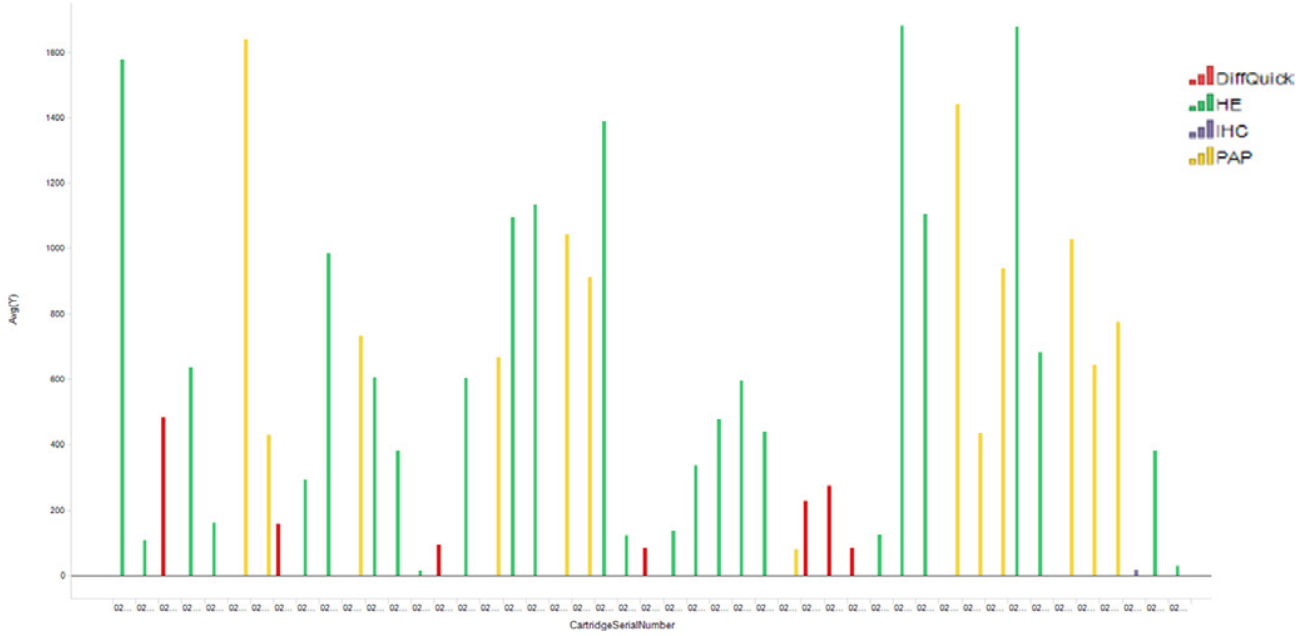

Figure 1 RAW data analysis by the TIBCO Spotfire application showing background level of fluorescence in channel 2 in relation to the type of staining. H&E and Papanicolaou (PAP) stained smears featured higher levels of background fluorescence than Diff Quik preparation.IHC, immunohistochemistry.

for their patients.[2–5] Accordingly, to enable physicians to select larger numbers of patients for treatment and to ward off the risk of leaving behind patients with acute deteriorating clinical conditions, much of my research work was dedicated to developing simple, rapid, and accurate *EGFR* mutation assays. As we pointed out in a previous study, *EGFR* testing should, ideally, be performed in the same institution where the cytological material is microscopically assessed for pathological diagnosis.[7] However, owing to infrastructural constraints and a lack of high technical expertise, many laboratories have no other choice but to outsource their cytological samples to external laboratories, leading to much longer turnaround time (TAT), especially when cytological smears require de-coverslipping.[7] Moreover, the fact that DNA extraction requires dedicated

facilities strongly limits the possibility of performing in-house *EGFR* testing. Conversely, we maintain that processing smears by wholly scraping the cellular material directly into the *Idylla*™ *EGFR* Mutation Assay cartridge could enable many more laboratories to perform in-house *EGFR* testing. Therefore, we strove to further extend previous investigations on the applicability of the *Idylla*™ platform to *EGFR* testing by assessing the performance of the *Idylla*™ *EGFR* Mutation Assay on cytological samples rather than on preliminary DNA extracts. To this purpose, we selected 5-year-old NSCLC archival smears to compare the efficiency of the *Idylla*™ *EGFR* Mutation Assay with standard techniques (fragment length assay and TaqMan assay) in detecting *EGFR* mutations. When we began this study, we were fully aware of the fact that the information generated by archival smears is very precious since cytological slides are irreproducible and irreplaceable. Although the ethical and legal challenges pertaining to the use of archival smears can be mitigated by digitally scanning smears to record the cytomorphology of representative diagnostic microscopic fields, it is reasonable, for research purposes, to select only those cases with several smears. Taking into account

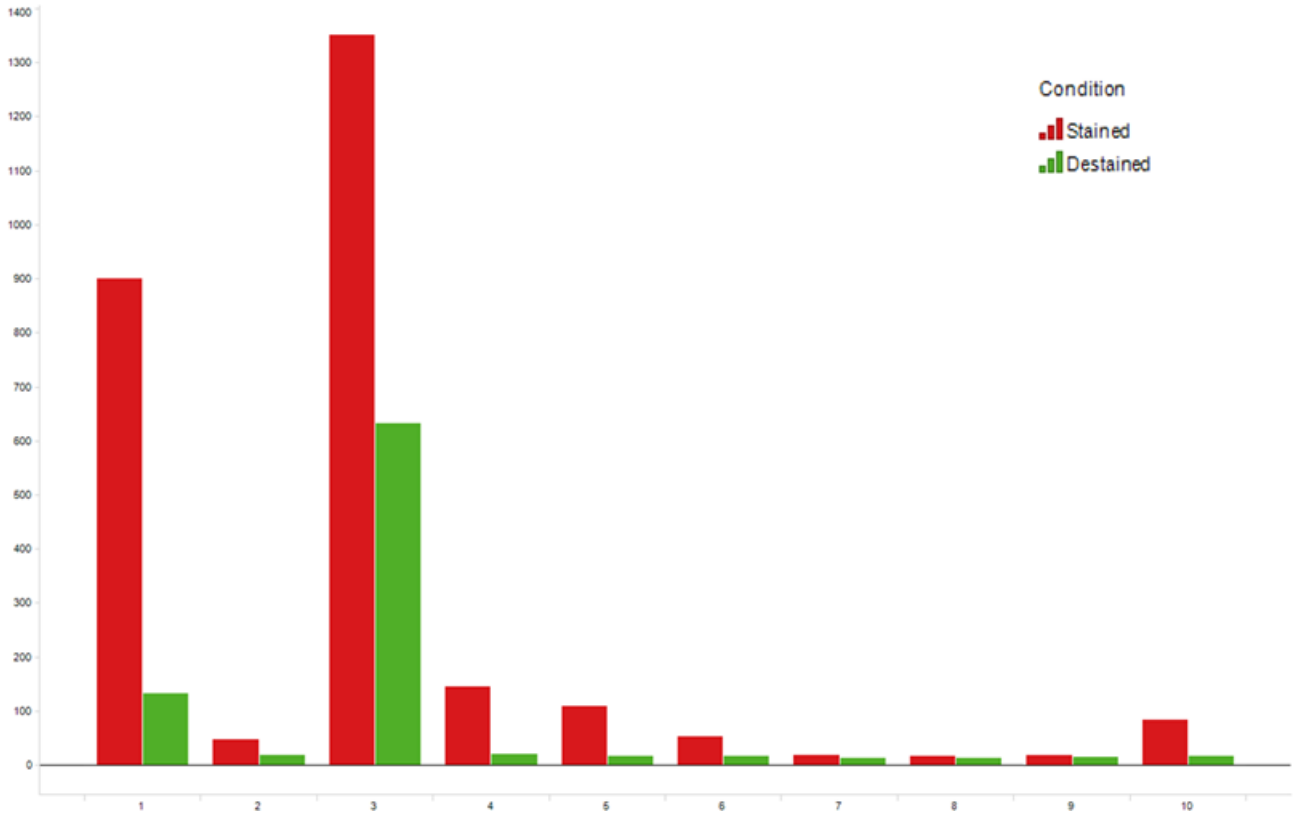

Figure 2 Background level of fluorescence in channel two for stained (red) and de-stained (green) cell block sections. In particular, a very strong unspecific signal was observed in two cases (case 1 and case 3).

all these limitations, we managed to investigate a considerable number of cases (n=39). This was

quite an achievement considering that only multicentric studies can provide a larger number of cases.[17] Overall, our data showed that, in most cases, the *Idylla™ EGFR* Mutation Assay can be successfully performed without preliminary DNA extraction. In fact, only 6 out of 39 (15.4%) cases were invalid. We hypothesized that these few invalid cases, which should not by any means be ignored, may have been due to either an abundant mucinous component or a prolonged fixation which, in turn, might have led to staining retention. Noteworthy, the *Idylla™ EGFR* Mutation Assay did not generate any false-positive results. In fact, it reconfirmed the absence of mutations in all exon 19 and 21 WT cases (table 3). Furthermore, a very high degree of concordance was obtained for exon 19 deletions. Notably, 10 out of 11 cases (90.9%) showed overlapping results. The only case showing a discordant result featured a very scant neoplastic cellularity (5%), a finding in line with previous evidence showing that the *Idylla™ EGFR* Mutation Assay requires at least 10% of neoplastic cells.[18–20] We did realize that this prerequisite could represent a slight drawback to implementing this system in routine clinical practice given that the quantity of neoplastic cells may vary even in smears from the same patients. Nonetheless, our research work showed that, the *Idylla™ EGFR* Mutation Assay generated reproducible results even when we processed different smears from the same patient. Although the data on the detection of EGFR exon 19 deletions were robust, those on *EGFR* exon 21 p.L858R were less so. We hypothesized that this discrepancy was attributable to the challenge of using clinical cytological material for research purposes. In fact, our series included only three cases with *EGFR* exon 21 p.L858R mutation. In this regard, the *Idylla™ EGFR* Mutation Assay was able to detect the *EGFR* exon 21 p.L858R on DNA extracts in two out of three cases, whereas it failed to do so on stained smears. This less-than-optimal result made us realize that the *Idylla™ EGFR* Mutation Assay may not be as effective on stained material. Indeed, raw data analysis by TIBCO Spotfire application revealed that in

ID Sample	Reference methods	Idylla EGFR Mutation Assay	NGS Analysis
9	WT	p.G719X	p.G719A
25	WT	p.G719X	p.G719A

Table 3 Cases showing discrepant results between standard methods and the Idylla EGFR Mutation Assay

channel 2, where the reaction involving the *EGFR* exon 21 p.L858R mutation detection occurred, there was a high level of background fluorescence. We attributed this phenomenon to the interference generated by the dye residues used to stain the material. This theory led us to examine

whether de-staining of the cytological material before insertion in the cartridge could reduce or eliminate the excessive amount of background fluorescence. Sure enough, the fluorescence level in channel 2 revealed by the TIBCO Spotfire application was greatly reduced, dropping to the same levels as those observed in un-stained cellular material. In conclusion, our data, although preliminary, showed that the *Idylla™ EGFR* Mutation Assay can be directly applied to direct smears. However, further investigation is warranted to reduce the level of background fluorescence on stained smears. Despite the few limitations mentioned above, our results clearly indicate that the implementation of the *Idylla™ EGFR* Mutation Assay in clinical practice to analyze cytological material from advanced lung cancer patients represents a valuable option to more conventional techniques. Indeed, we are adamant that by optimizing fully automated detection of *EGFR* mutations in cytological material without the need for preliminary DNA extraction[21] could enable laboratories, lacking the necessary expertise and infrastructures, to carry out in-house sample processing. Consequently, the costly practice of outsourcing cytological samples for *EGFR* testing would no longer be necessary—a scenario that would drastically reduce the TAT of test results, especially for acute deteriorating patients for whom rapid test results are critical to receiving highly effective personalized treatments.

References

1. Ridge C, McErlean A, Ginsberg M. Epidemiology of lung cancer. Semin Intervent Radiol 2013;30:093–8.
2. Mok TS, Wu Y-L, Thongprasert S, et al. Gefitinib or carboplatin-paclitaxel in pulmonary adenocarcinoma. N Engl J Med 2009;361:947–57.
3. Rosell R, Carcereny E, Gervais R, et al. Erlotinib versus standard chemotherapy as first-line treatment for European patients with advanced EGFR mutation-positive nonsmall-cell lung cancer (EURTAC): a multicentre, open-label, randomised phase 3 trial. Lancet Oncol 2012;13:239–46.
4. Yang JC-H, Wu Y-L, Schuler M, et al. Afatinib versus cisplatin-based chemotherapy for EGFR mutation-positive lung adenocarcinoma (LUX-Lung 3 and LUX-Lung 6): analysis of overall survival data from two randomised, phase 3 trials. Lancet Oncol 2015;16:141–51.
5. Ramalingam SS, Yang JC-H, Lee CK, et al. Osimertinib as first-line treatment of EGFR mutation-positive advanced non-small-cell lung cancer. J Clin Oncol 2018;36:841–9.
6. Lindeman NI, Cagle PT, Aisner DL, et al. Updated molecular testing guideline for the selection of lung cancer patients for treatment with targeted tyrosine kinase inhibitors: guideline from the College of American Pathologists, the International Association for the Study of Lung Cancer, and the Association for Molecular Pathology. Arch Pathol Lab Med 2018;142:321–46.
7. Vigliar E, Malapelle U, Bellevicine C, et al. Outsourcing cytological samples to a referral laboratory for EGFR testing in non-small cell lung cancer: does theory meet practice? Cytopathology 2015;26:312–7.
8. Kamps R, Brandão R, Bosch B, et al. Next-generation sequencing in oncology: genetic diagnosis, risk prediction and cancer classification. IJMS 2017;18.
9. Janku F, Claes B, Huang HJ, et al. BRAF mutation testing with a rapid, fully integrated molecular diagnostics system. Oncotarget 2015;6:26886–94.
10. De Luca C, Rappa AG, Gragnano G, et al. Idylla assay and next generation sequencing: an integrated EGFR mutational testing algorithm. J Clin Pathol 2018;71:745–50.
11. Uguen A, Troncone G. A review on the Idylla platform: towards the assessment of actionable genomic alterations in one day. J Clin Pathol 2018;71:757–62.
12. Lambros L, Caumont C, Guibourg B, et al. Evaluation of a fast and fully automated platform to diagnose EGFR and KRAS mutations in formalin-fixed and paraffinembedded non-small cell lung cancer samples in less than one day. J Clin Pathol 2017;70:544–9.
13. Kalemkerian GP, Narula N, Kennedy EB, et al. Molecular testing guideline for the selection of patients with lung cancer for treatment with targeted tyrosine kinase inhibitors: American Society of Clinical Oncology endorsement of the College of American Pathologists/International Association for the Study of Lung Cancer/Association for Molecular Pathology clinical practice guideline update. J Clin Oncol2018;36:911–9.
14. De Luca C, Gragnano G, Pisapia P, et al. EGFR mutation detection on lung cancer cytological specimens by the novel fully automated PCR-based Idylla EGFR Mutation Assay. J Clin Pathol 2017;70:295–300.
15. Pepe F, De Luca C, Smeraglio R, et al. Performance analysis of SiRe next-generation sequencing panel in diagnostic setting: focus on NSCLC routine samples. J Clin Pathol 2019;72:38–45.
16. Malapelle U, Bellevicine C, De Luca C, et al. EGFR mutations detected on cytology samples by a centralized laboratory reliably predict response to gefitinib in non-small cell lung carcinoma patients. Cancer Cytopathol 2013;121:552–60.
17. Clark DP. Seize the opportunity: underutilization of fine-needle aspiration biopsy to inform targeted cancer therapy decisions. Cancer 2009;117:289–97.
18. Ilie M, Butori C, Lassalle S, et al. Optimization of EGFR mutation detection by the fullyautomated qPCR-based Idylla system on tumor tissue from patients with non-small cell lung cancer. Oncotarget 2017;8:103055–62.
19. Weyn C, Van Raemdonck S, Dendooven R, et al. Clinical performance evaluation of a sensitive, rapid low-throughput test for KRAS mutation analysis using formalin-fixed, paraffin-embedded tissue samples. BMC Cancer 2017;17.
20. Colling R, Bancroft H, Langman G, et al. Fully automated real-time PCR for EGFR testing in non-small cell lung carcinoma. Virchows Arch 2019;474:187–92.
21. Bellevicine C, Malapelle U, Vigliar E, et al. How to prepare cytological samples for molecular testing. J

Clin Pathol 2017;70:819–26.

Chapter 2

Rapid On-site Molecular Evaluation in thyroid cytopathology: A one-day cytological and molecular diagnosis

Fine-needle aspiration (FNA) biopsy, a minimally invasive and cost-effective procedure, can reliably diagnose the majority of thyroid nodules.[1] Our laboratory experience in FNA has repeatedly highlighted the important role played by cytopathologists in ensuring careful sample handling a crucial step to obtain well smeared, fixed, and stained cytopreparations.[2] After the introduction of ultrasound (US) screening for sub-clinical thyroid nodules, an increasing number of cytopathologists began to perform FNA under US-guidance by themselves, leading to the new professional figure of the interventional cytopathologists.[2] Nowadays, having developed expertise in US-guided FNA and rapid-on site evaluation (ROSE) of aspirated material, interventional cytopathologists can carry out on the same day physical and US examination.[2] Moreover, when ROSE yields an undetermined diagnosis, cytopathologists can readily and adequately collect the cytological material for the molecular analysis needed to refine the risk of malignancy (ROM).[3-8] This integrated approach provides all the relevant factors to fully exploit the potential of thyroid cytopathology. Building on this research, the second objective of my PhD was to further extend, the cytopathologist's role in an FNA clinic. Indeed, without the need of molecular biologists, we hypothesized that interventional cytopathologists could provide both a morphological and molecular diagnosis in a single day, thereby relieving the, anxiety arising in patients from delayed diagnoses and uncertain clinical management[9]. Several studies, previously carried out at our clinic, evidenced that the disposable *Idylla*™ (Biocartis, Mechelen, Belgium) cartridge included all reagents needed to detect the most frequent mutations occurring in thyroid neoplasms, specifically, V-Raf Murine Sarcoma Viral Oncogene Homolog B1 (*BRAF*) p.V600E and Neuroblastoma *RAS* Viral (V-Ras) Oncogene Homolog (*NRAS*) gene mutations.[10-14,15]. The ease of use of this platform was described in a diagnostic accuracy study by Colling et al. In brief, the cytopathologist's job is to simply insert the needle rinse into the cartridge, which is then loaded into the *Idylla*™ platform. Next, an integrated console auto-analyzes the real time polymerase chain reaction (RT-PCR) amplification curves, revealing the final results on a computerized console as either "No mutation detected" or "BRAF or NRAS mutation detected" in less than 2 hours.[16] These results are then rapidly integrated into the final cytological report.[9] My colleagues and I set out to validate this

novel procedure, which we termed rapid on site molecular evaluation (ROME) on thyroid cytological samples.

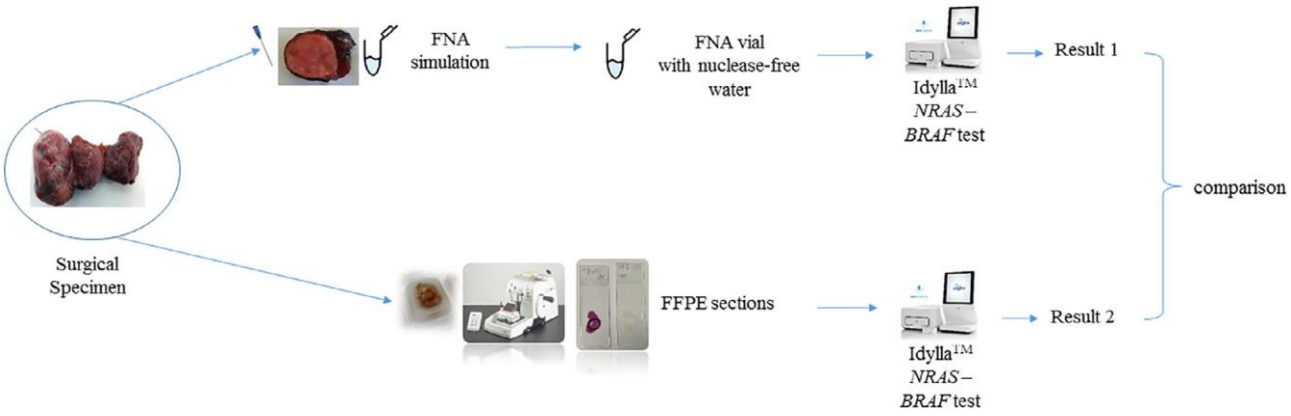

FIGURE 1 Study design: first part on ex-vivo cytological samples. In the gross room, simulated (bench-top) fine-needle aspirates (FNAs) were performed by a cytopathologist. The material yielded from the simulated FNA was collected into a vial containing 350 µL nuclease-free water (Invitrogen Ambion; Thermo Fisher, Waltham, Massachusetts). This solution was directly inserted into the Idylla NRAS-BRAF cartridge to perform automated genotyping.Simultaneously, corresponding formalin fixed paraffin embedded (FFPE) sections were microdissected and inserted into the Idylla cartridge and processed. BRAF and NRAS genotyping results were compared.

Study Design

The use of a combined *BRAF* and *NRAS Idylla*™ cartridge has only been described in colorectal cancer (CRC) and in melanoma.[15,17] Conversely, in thyroid cancer, a single *BRAF* cartridge has only been used to genotype formalin-fixed and paraffin embedded (FFPE) sections obtained from surgical samples.[18] As of today, however, no data are available on the use of the *Idylla*™ technology on thyroid cytological samples. This study was divided into two parts (Figures 1 and 2). The first part aimed to assess whether readily obtainable needle rinses, from thyroid FNAs, instead of FFPE sections, could be reliably processed by the *NRAS-BRAF Idylla*™ cartridge. The second part aimed to assess the *NRAS-BRAF Idylla*™ test performance parameters, in daily cytological practice. The first part was carried out on ex-vivo cytological samples. In a gross room, simulated (bench-top) FNAs were performed by a cytopathologist on a total of 25 fresh thyroid surgical specimens. Care was taken to sample surgical specimens featuring only a single distinct nodular lesion. In particular, samples whose macroscopic features were likely to be associated with colloid or regressive goiter were excluded. Only lesions whose size was larger than 2 cm were sampled in order to avoid any interference with standard histological evaluation. The material yielded from the simulated FNA

procedure was partially smeared and immediately stained by the Diff Quik method to ensure the presence of follicular cells, and partially collected into a vial containing 350 µL nuclease-free water (Invitrogen Ambion; Thermo Fisher, Waltham, Massachusetts).

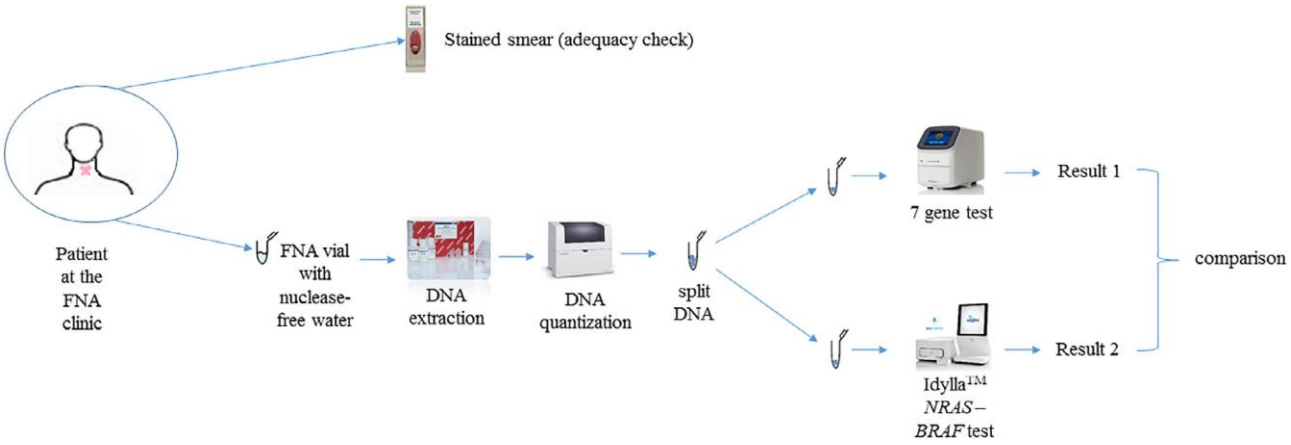

FIGURE 2 Study design: second part in real diagnostic practice. We prospectively collected 25 fine-needle aspirates (FNAs). Part of the aspirated material was smeared for microscopic diagnosis and adequacy check (rapid on-site evaluation [ROSE]). The material yielded from the FNA was collected into a vial containing 350 µL nuclease-free water (Invitrogen Ambion; Thermo Fisher, Waltham, Massachusetts). After DNA extraction and quantization, the results of the Idylla assay were compared with those obtained by standard real time polymerase chain reaction (RT-PCR)

This solution was directly inserted into the *Idylla™ NRAS-BRAF* cartridge for the automated genotyping. The surgical specimen was then fixed for 24 hours in buffered formalin and histologically processed to obtain tissue blocks that were microscopically evaluated. Most cases (24/25, 96.0%) were neoplastic lesions. Malignant lesions included 13 classic papillary thyroid carcinomas (PTCs), two follicular variant of PTCs (FVPTCs), and one follicular carcinoma (FC). Eight cases showed noninvasive neoplasms, including four follicular adenomas (FAs), three Hurtle cell adenomas (HCAs), and one noninvasive follicular thyroid neoplasm with papillary-like nuclear features (NIFTP). One case showed no malignancy, as the histological evaluation was consistent with an adenomatoid (hyperplastic) goiter. In every case, the tissue block, representative of the neoplasm, was selected for the *Idylla™* assay. The tissue area with the highest percentage of neoplastic cells (Table 1) was marked on a hematoxylin and eosin (H&E) stained slide and micro dissected on a corresponding FFPE section (10 µm thickness). Then the micro dissected area was inserted into the *Idylla™* cartridge and processed as previously described.[10,15-20] *BRAF* and *NRAS* genotyping results were compared with those obtained by processing the simulated FNA (Table 1). The second part of the study was prospectively carried out on 25 patients evaluated for a thyroid nodule at our FNA clinic. Nodule sampling, ROSE, cytological processing, microscopic evaluation, and reporting were performed by two staff cytopathologists as previously described.[2] Only cases

suspicious for malignancy (class V sec. The Bethesda System for Reporting Thyroid Cytology [TBSRTC]) or malignant (class VI sec. TBSRTC) were processed,[21] since these categories most likely harbor *BRAF* or *NRAS* mutations.[21-24] To this end, ROSE was performed at the time of the FNA to select cases showing nuclear features which were either suspicious (n = 2) or fully diagnostic (n = 23) of PTC. In all study cases, the cytopathologist rinsed the needle into a vial of nuclease-free water. The extracted DNA was used to compare the performance of the *Idylla*™ assay against a standard, manual RT-PCR procedure an assay that we have been using in our laboratory for 3 years to process undetermined thyroid FNAs (Table 2).[24,25] Written informed consent was obtained from all patients and documented in accordance with the general authorization to process personal data for scientific research purposes from "The Italian Data Protection Authority" (http://www.garanteprivacy.it/web/guest/home/docweb/-/docwebdisplay/export/2485392). All information regarding human material was managed using anonymous numerical codes, and all samples were handled in compliance with the Helsinki Declaration (http://www.wma.net/en/30publications/10policies/b3/). This study was approved by the Ethics Committee "Carlo Romano," of the University of Naples Federico II (protocol 155/15/ES1).

Idylla™ NRAS-BRAF Test

The *Idylla*™ *NRAS-BRAF* Mutation Test is a fully automated test which requires less than 2 hours from sample collection to results. The operator hands on time are only 2 minutes and no specific expertise in molecular biology is required. More in detail, the *Idylla*™ *NRAS-BRAF* Mutation Test covers a wide range (n = 23) of clinically relevant mutations in *NRAS* and *BRAF*.[15] Briefly, the *NRAS* mutations detected by the *Idylla*™ *NRASBRAF* Mutation Test include those in exon 2 (p.G12D, p.G12C, p.G12S, p.G12A/V, p.G13D, p.G13R/V), exon 3 (p.A59T, p.Q61K, p.Q61R, p.Q61L, p.Q61H), and exon 4 (p.K117N, p.A146T/V). *BRAF* mutations, covered by the *Idylla*™ *NRAS-BRAF* Mutation Test include the most common alterations of exon 15 (p.V600E/D and p.V600K/R).[15] As I was mentioned before, the results appear on the platform console as "mutation detected" or "no mutation detected." Conversely, an "invalid" result appears when the DNA quantity and quality are insufficient.

TABLE 1 First part of study series showing cases number, percentage of neoplastic cell, histological diagnosis, and NRAS-BRAF Idylla results on sFNAs and matched FFPE sections

N	% Neoplastic cell	Diagnosis	IDYLLA NRAS (SFNA)	IDYLLA BRAF (SFNA)	IDYLLA NRAS (FFPE)	IDYLLA BRAF (FFPE)
1	70	PTC	No mutation	p.V600E/D	No mutation	p.V600E/D
2	60	FVPTC	No mutation	p.V600E/D	No mutation	p.V600E/D
3	60	PTC	No mutation	p.V600E/D	No mutation	p.V600E/D
4	70	FC	No mutation	No mutation	No mutation	No mutation
5	70	FA	No mutation	No mutation	No mutation	No mutation
6	70	PTC	No mutation	p.V600E/D	No mutation	p.V600E/D
7	40	PTC	No mutation	p.V600E/D	No mutation	p.V600E/D
8	70	PTC	No mutation	p.V600E/D	No mutation	p.V600E/D
9	90	PTC	No mutation	p.V600E/D	No mutation	p.V600E/D
10	70	FVPTC	No mutation	No mutation	No mutation	No mutation
11	70	NIFTP	No mutation	No mutation	No mutation	No mutation
12	70	HCA	No mutation	No mutation	No mutation	No mutation
13	70	HCA	No mutation	No mutation	No mutation	No mutation
14	40	PTC	No mutation	p.V600E/D	No mutation	p.V600E/D
15	70	HCA	No mutation	No mutation	No mutation	No mutation
16	70	FA	p.Q61K	No mutation	p.Q61K	No mutation
17	40	PTC	No mutation	No mutation	No mutation	No mutation
18	70	FA	No mutation	No mutation	No mutation	No mutation
19	80	PTC	No mutation	No mutation	No mutation	No mutation
20	80	PTC	p.Q61R	No mutation	p.Q61R	No mutation
21	60	PTC	No mutation	No mutation	No mutation	No mutation
22	60	FA	No mutation	No mutation	No mutation	No mutation
23	80	PTC	No mutation	p.V600E/D	No mutation	p.V600E/D
24	60	PTC	No mutation	p.V600E/D	No mutation	p.V600E/D
25	NA	G	No mutation	No mutation	No mutation	No mutation

Abbreviations: BRAF, V-Raf Murine Sarcoma Viral Oncogene Homolog B1; FA, follicular adenoma; FC, follicular carcinoma; FFPE, formalin fixed paraffin embedded; FVPTC, follicular variant of papillary thyroid carcinoma; G, goiter; HCA, Hurthle cell adenoma; NA, not assessed; NIFTP, Noninvasive follicular thyroid neoplasm with papillary-like nuclear features; NRAS, Neuroblastoma RAS Viral (V-Ras) Oncogene Homolog; PTC, papillary thyroid carcinoma;sFNA, simulated (bench-top) fine needle aspiration.

BRAF and NRAS genotyping by RT-PCR

Needle rinses, obtained from prospectively collected thyroid FNAs, were processed to extract DNA by using the AllPrep DNA/RNA Kit (Qiagen, Hilden, Germany), following the manufacturer's instructions. Extracted DNA was eluted in 30 μL of nuclease-free water. DNA quantity and quality (in terms of DNA Integrity Number [DIN]) were assessed by the Genomic DNA screen-tape assay on the 4200 TapeStation system (Agilent Technologies, Santa Clara, California) with a proprietary software. The extracted DNA was genotyped by the fully automated Idylla NRAS-BRAF Mutation Test and by our standard RT-PCR-based procedure. The latter was performed on the QuantStudio 5 platform (Applied Biosystems; Thermo Fisher) with the EntroGen Thyroid Cancer Mutation Analysis Panel Kit (EntroGen Inc, Woodland Hills, California), as previously described (Table 2).[24,25]

RESULTS

Idylla NRAS-BRAF Mutation Test on needle rinses and matched FFPE sections

In all study cases, needle rinses from the simulated (bench-top) thyroid FNA and the matched FFPE sections were successfully processed by the *Idylla™ NRAS-BRAF* Mutation Test. Moreover, data showed a complete concordance between the results obtained by processing needle rinses and the matched FFPE sections. Out of 25 cases, 10 harbored a *BRAF* (p.V600E/D; 40.0%) mutation, and two harbored *NRAS* (p.Q61K/R) (8.0%). Briefly, histologically proven classic PTC cases (n = 13) showed *BRAF* mutant alleles in nine (9/13; 69.2%) instances, whereas only one case showed an *NRAS* gene mutation (1/13; 7.7%). A *BRAF* mutation occurred in one of the two FVPTC cases. The only FC case showed no alteration in both *BRAF* and *NRAS*. In the remaining eight cases (n = 4 FAs, n = 3 HCAs, and n = 1 NIFTP) only one FA showed an *NRAS* p.Q61K point mutation. The only non-neoplastic study case (hyperplastic goiter) featured no mutation in the tested genes (Table 1).

TABLE 2 Second part of study series showing the case number, TBSRTC category, DNA concentration (ng/µL), and Idylla™ *vs* standard RT-PCR

N	TBSRTC class	[Dna] ng/µl	IDYLLA™ NRAS	IDYLLA™ BRAF	RT PCR NRAS	RT PCR BRAF
1	VI	0,68	No mutation	No mutation	No mutation	No mutation
2	VI	1,05	No mutation	p.V600E/D	No mutation	p.V600E
3	VI	2,1	No mutation	p.V600E/D	No mutation	p.V600E
4	VI	0,92	No mutation	No mutation	No mutation	No mutation
5	VI	1,65	No mutation	p.V600E/D	No mutation	p.V600E
6	VI	98,6	No mutation	p.V600E/D	No mutation	p.V600E
7	VI	5,09	No mutation	p.V600E/D	No mutation	p.V600E
8	V	1,07	No mutation	No mutation	No mutation	p.V600E
9	VI	2,59	No mutation	p.V600E/D	No mutation	p.V600E
10	VI	3,71	No mutation	No mutation	No mutation	No mutation
11	VI	1,04	No mutation	p.V600E/D	No mutation	p.V600E
12	VI	0,95	No mutation	No mutation	No mutation	p.V600E
13	VI	1,06	No mutation	p.V600E/D	No mutation	p.V600E
14	VI	0,8	No mutation	No mutation	No mutation	No mutation
15	VI	1,4	No mutation	No mutation	No mutation	No mutation
16	VI	4,16	No mutation	p.V600E/D	No mutation	p.V600E
17	VI	1	No mutation	p.V600E/D	Failed	Failed
18	VI	7,36	No mutation	No mutation	No mutation	No mutation
19	VI	1,91	No mutation	p.V600E/D	No mutation	p.V600E
20	VI	23,2	No mutation	p.V600E/D	No mutation	p.V600E
21	V	2,04	p.Q61R	No mutation	p.Q61R	No mutation
22	VI	6,35	No mutation	p.V600E/D	No mutation	p.V600E
23	VI	1,86	No mutation	p.V600E/D	No mutation	p.V600E
24	VI	28,2	No mutation	p.V600E/D	No mutation	p.V600E
25	VI	1,71	No mutation	p.V600E/D	No mutation	p.V600E

Abbreviations: BRAF, V-Raf Murine Sarcoma Viral Oncogene Homolog B1; NA, not assessed; NRAS, Neuroblastoma RAS Viral (V-Ras) Oncogene Homolog; PTC, papillary thyroid carcinoma; RT-PCR, real time polymerase chain reaction; TBSRTC, The Bethesda System for Reporting Thyroid Cytopathology.

Performance of the Idylla™ NRAS-BRAF Mutation Test on routine FNAs

The DNA extracted from routine FNA needle rinses was quantified, as described in Section 2 (Table 2). The extracted DNA was aliquoted in order to run the *Idylla™ NRAS-BRAF* Mutation Test and the EntroGen Thyroid Cancer Mutation Analysis Panel Kit simultaneously. In particular, 12 μL of extracted DNA was directly inserted into the *Idylla™ NRAS-BRAF* Mutation Test cartridge, whereas another 12 μL was analyzed with the EntroGen Thyroid Cancer Mutation Analysis Panel Kit on the QuantStudio 5 platform (RT-PCR). Overall, all 25 cases were successfully analyzed by the *Idylla™ NRAS-BRAF* Mutation Test. Noteworthy, whereas RT-PCR yielded an inadequate result in one case (1/25; 4.0%), the *Idylla™ NRAS-BRAF* Mutation Test accurately detected *BRAF* p.V600E/D mutations in all cases. As for the successfully processed cases (n=24), both methods generated concordant results in the vast majority of cases (22/24; 91.7%). In particular, 15 *BRAF* p.V600E/D mutated cases belonged to class VI (malignant) of TBSRTC and one *NRAS* p.Q61R mutant case belonged to class V (suspicious for malignancy) of TBSRTC. Six cases, belonging to class VI (malignant) of TBSRTC, were assessed as negative by both techniques. Even though the *Idylla™* system did not yield any false positive results (Table 2), in two cases (n = 1 in class V; n = 1 in class VI) it overlooked a *BRAF* p.V600E mutation that was instead detected by RT-PCR (Table 2) Altogether, in comparison with the EntroGen Thyroid Cancer Mutation Analysis routinely employed in our laboratory as the gold standard, the *Idylla™ NRAS-BRAF* Mutation Test showed a sensitivity of 88.9%, and a specificity of 100.0%. Practically, this particular area of research generated three novel findings. First, we found that the *Idylla™ NRAS-BRAF* Mutation Test, which is generally used to genotype melanomas and CRCs,15,17 can also have a specific role in screening thyroid neoplasms for the most common mutations. Second, this test can be carried out on FNA needle rinse samples. Third, the *Idylla™ NRAS-BRAF* Mutation Test has a high diagnostic performance. Not surprisingly, in just a few years, the *Idylla™* technology, owing to its ease of use, cost effectiveness, and rapid turnaround time, has been advancing at a rapid pace. Indeed, many institutions worldwide are now using this approach in routine clinical to rapidly assess the most common actionable oncogenes in a wide range of human neoplasms.[10-20] Thanks to this technology, acute deteriorating patients can now be quickly diagnosed and receive targeted treatments, as predictive biomarker testing can be carried out even where equipment and expertise are not available.[26] For example, two recent studies reported on a recently developed combined *NRAS-BRAF* Mutation Test capable of predicting targeted treatment response in patients with colorectal cancer and melanoma.[15,17] A new indication is to refine uncertain diagnosis in thyroid cytopathology.[23-25,27] Many efforts are being made worldwide to develop molecular thyroid FNA

testing tools. For example, in North America, thanks to a well-resourced, reimbursement-based healthcare system, undetermined FNAs are outsourced to a few large centralized laboratories that perform proprietary molecular testing by next generation sequencing to comprehensively assess point mutations, gene fusions, copy number gains, and expression profiling of very large numbers of target genes.[28-30] Conversely, in the European universal healthcare system, the limited financial resources are only sufficient to reimburse a preliminary screening based on less comprehensive and simpler assays performed in numerous local laboratories.[28,31] In this setting, the *Idylla*™ *NRAS-BRAF* Mutation Test represents an optimal screening tool at the local level. However, outsourcing is still necessary for those FNA specimens presenting no *BRAF* and *NRAS* alterations. Undoubtedly, these patients would definitely benefit from a comprehensive genomic profiling able to detect less frequent genomic and transcriptomic alterations. Remarkably, in the first part of the study, we demonstrated that *Idylla*™ *NRAS-BRAF* Test is as efficient on FNA needle rinses as it is on on FFPE sections in detecting *NRAS-BRAF* mutations. This makes this assay a very advantageous screening tool in terms of time and costs. In fact, since cell block preparations and DNA extraction are no longer needed, it can drastically reduce the long turnaround time required by more conventional techniques. Moreover, given its ease of use, testing laboratories can process the material on their own, without needing to outsource it to specialized molecular laboratories staffed with trained personnel factors that highly impact the screening costs. Ideally, we propose that the cytopathologist, present on site at the time of the FNA, could combine two different activities. For example, on one hand, ROSE could identify cases most likely to remain undetermined after the complete and definitive microscopic evaluation. On the other hand, ROME can provide a fast *BRAF* and *NRAS* genotyping. This combined approach (ROSE plus ROME) could yield a modern and timely morpho-molecular report. Similar to our findings, a recent report has shown that the *Idylla*™ *KRAS* cartridge can directly genotype pancreatic cyst fluid shortly after endoscopic ultrasound FNA with no need for DNA extraction.[32] In the second part of the study, we demonstrated that in most instances (22/24; 91.7%) *Idylla*™ is concordant with the standard RT-PCR assay. Moreover, no invalid results were generated; notably, one case that failed by the standard RT-PCR assay was successfully processed by the *Idylla*™ assay. The only two discordant cases were controversial. In both cases, *BRAF* p.V600E mutations were detected by the standard RT-PCR, whereas they were missed by the *Idylla*™ software. However, the difference between the quantification cycle values (ΔCQ) was borderline when the raw data were evaluated by *Idylla*™ Explore.[33] This application is a web-based application that allows the analysis of PCR curves, in PCR chamber C, which contains the corresponding *BRAF* p. V600E probes (Figure 3). Although visual inspection by an experienced molecular biologist could increase the sensitivity of the assay, the ΔCQ threshold set by the

manufacturer ensures a high specificity. The high specificity was indeed confirmed in our study, where the *Idylla*™ assay generated no false positive results. Thus, this suggests that this fascinating automated technology should not be used as a stand-alone test, but should be integrated in a more complex diagnostic algorithm capable of reflecting the complexity of the thyroid cancer genomic landscape.[34] In conclusion, our data, although preliminary, have shown that the *Idylla*™ *NRAS-BRAF* Mutation Test is feasible on thyroid FNAs and can be exploited alongside ROME and the traditional morphological rapid on-site evaluation of smears (Figure 4). We are adamant that the implementation of this approach in routine clinical practice would reduce the long waiting times for test results in patients with undetermined FNA diagnosis, thereby reducing anxiety issues deriving from the uncertainty of their diagnoses. Equally important, it would allow patients to receive rapid and tailored treatments.[9] Further investigation is warranted to evaluate whether the interventional cytopathologists can act a liaison between local FNA clinics and specialized cancer genomic centers.

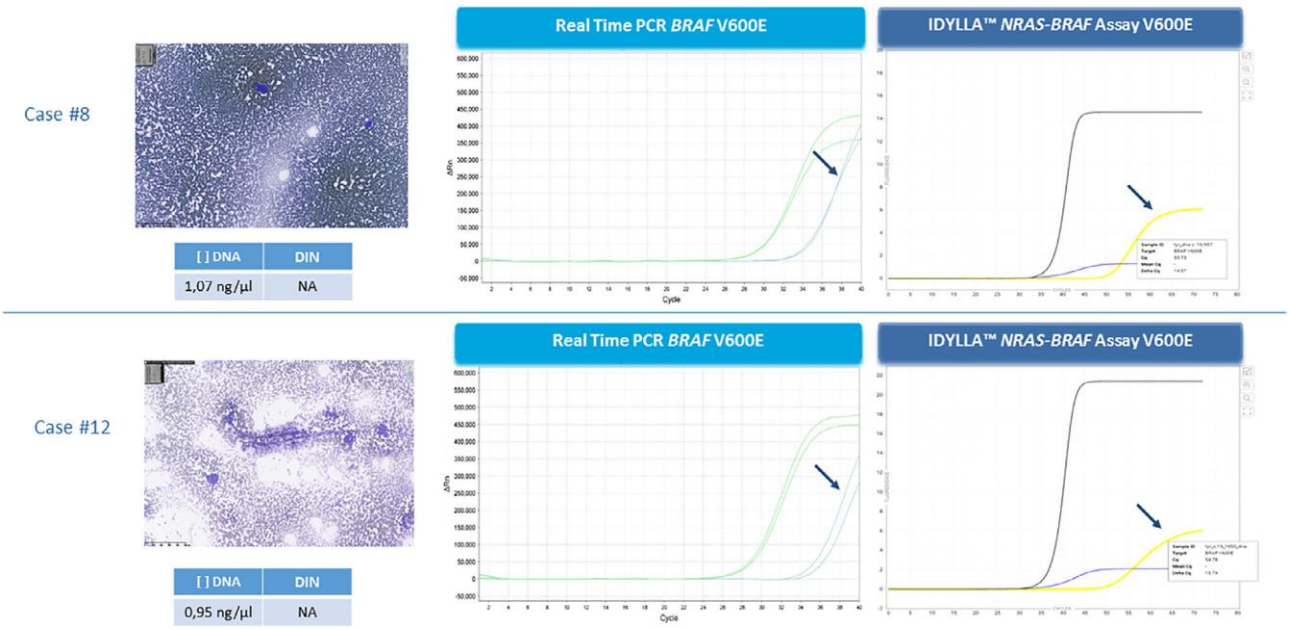

FIGURE 3 Two discordant cases between the Idylla assay and standard real time polymerase chain reaction (RT-PCR). In both cases, both BRAF p.V600E mutations were detected by the standard RT-PCR (blue curves, arrows), whereas they were missed by the Idylla software. However, when raw data were evaluated by Idylla Explore, the interpretation was unclear (yellow curves, arrows)

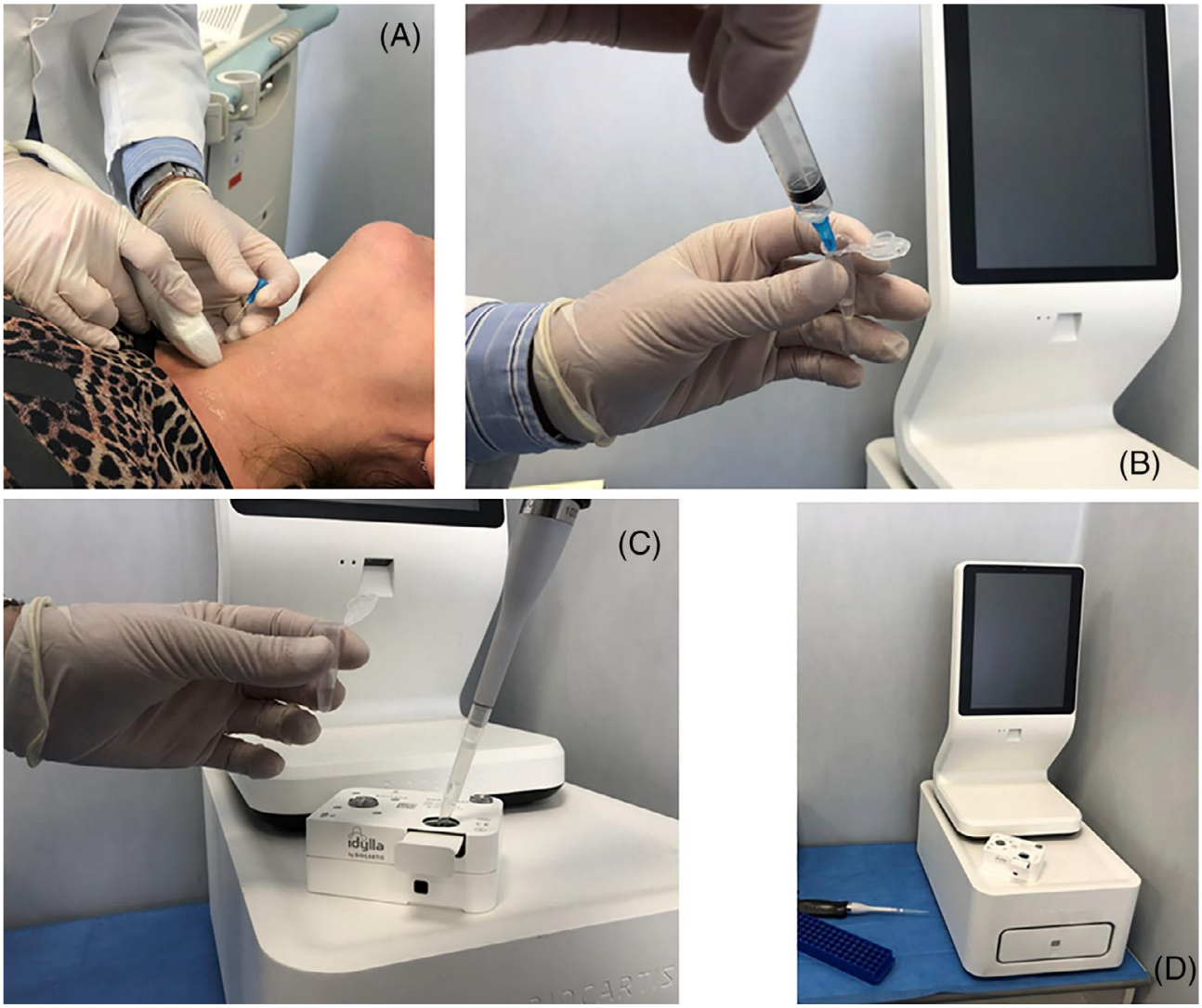

FIGURE 4 Rapid on-site molecular evaluation (ROME) workflow. A, The interventional cytopathologist performs thyroid US guided fine-needle aspirate (FNA). B, The needle rinse is collected in 350 µL nuclease-free water (Invitrogen Ambion; Thermo Fisher, Waltham, Massachusetts). C, This solution is directly pipetted into the Idylla NRAS-BRAF cartridge. D, The genotype analysis may be carried out in the same room where the FNA is performed

References

1. Allen L, Al Afif A, Rigby MH, et al. The role of repeat fine needle aspiration in managing indeterminate thyroid nodules. J Otolaryngol Head Neck Surg. 2019;48:16.
2. Bellevicine C, Vigliar E, Malapelle U, et al. Cytopathologists can reliably perform ultrasound-guided thyroid fine needle aspiration: a 1-year audit on 3715 consecutive cases. Cytopathology. 2016;27:115-121.
3. Jo VY, Stelow EB, Dustin SM, Hanley KZ. Malignancy risk for fine-needle aspiration of thyroid lesions according to the Bethesda System for Reporting Thyroid Cytopathology. Am J Clin Pathol. 2010;134:450-456.
4. Pisapia P, Bellevicine C, Malapelle U, et al. Bird's eye view of modern cytopathology: report from the seventh international Molecular Cytopathology Meeting in Naples, Italy, 2018. Cancer Cytopathol. 2019.[Epub ahead of print].
5. Onder S, Firat P, Ates D. The Bethesda system for reporting thyroid cytopathology: an institutional experience of the outcome of indeterminate categories. Cytopathology. 2014;25:177-184
6. Ho AS, Sarti EE, Jain KS, et al. Malignancy rate in thyroid nodules classified as Bethesda category III (AUS/FLUS). Thyroid. 2014;24:832-839.
7. Straccia P, Rossi ED, Bizzarro T, et al. A meta-analytic review of the Bethesda System for Reporting Thyroid Cytopathology: has the rate of malignancy in indeterminate lesions been underestimated? Cancer Cytopathol. 2015;123:713-722.
8. Bongiovanni M, Giovanella L, Romanelli F, Trimboli P. Cytological diagnoses associated with noninvasive follicular thyroid neoplasms with papillary-like nuclear features according to the Bethesda system for reporting thyroid cytopathology: a systematic review and metaanalysis. Thyroid. 2019;29:222-228.
9. Lodewijk L, Vriens MR, Vorselaars WM, et al. Same-day fine-needle aspiration cytology diagnosis for thyroid nodules achieves rapid anxiety decrease and high diagnostic accuracy. Endocr Pract. 2016;22:561-566.
10. De Luca C, Conticelli F, Leone A, et al. Is the Idylla EGFR mutation assay feasible on archival stained cytological smears? A pilot study. J Clin Pathol. 2019;72:609-614.
11. De Luca C, Vigliar E, d'Anna M, et al. KRAS detection on archival cytological smears by the novel fully automated polymerase chain reaction-based Idylla mutation test. Cytojournal. 2017;14:5.
12. De Luca C, Gragnano G, Pisapia P, et al. EGFR mutation detection on lung cancer cytological specimens by the novel fully automated PCRbased Idylla EGFR mutation assay. J Clin Pathol. 2017;70:295-300.
13. de Biase D, de Luca C, Gragnano G, et al. Fully automated PCR detection of KRAS mutations on pancreatic endoscopic ultrasound fineneedle aspirates. J Clin Pathol. 2016;69:986-991.
14. De Luca C, Rappa AG, Gragnano G, et al. Idylla assay and next generation sequencing: an integrated EGFR mutational testing algorithm. J Clin Pathol. 2018;71:745-750.
15. Prieto-Potin I, Montagut C, Bellosillo B, et al. Multicenter evaluation of the Idylla NRAS-BRAF mutation test in metastatic colorectal cancer. J Mol Diagn. 2018;20:664-676.
16. Colling R, Wang LM, Soilleux E. Automated PCR detection of BRAF mutations in colorectal adenocarcinoma: a diagnostic test accuracy study. J Clin Pathol. 2016;69:398-402.
17. Vallée A, Denis-Musquer M, Herbreteau G, Théoleyre S, Bossard C, Denis MG. Prospective evaluation of two screening methods for molecular testing of metastatic melanoma: diagnostic performance of BRAF V600E immunohistochemistry and of a NRAS-BRAF fully automated real-time PCR-based assay. PLoS One. 2019;14:e0221123.
18. Yeo MK, Jung MK, Lee SY, Lee YM, Hur GM, Kim JM. The usefulness of a novel fully automated PCR-based Idylla test for detection of the BRAF V600E mutation in thyroid tissue: comparison with PNAclamping PCR, real-time PCR and pyrosequencing. J Clin Pathol. 2017; 70:260-265.
19. Melchior L, Grauslund M, Bellosillo B, et al. Multi-center evaluation of the novel fully-automated PCR-based Idylla™ BRAF mutation test on formalin-fixed paraffin-embedded tissue of malignant melanoma. Exp Mol Pathol. 2015;99:485-491.
20. Schiefer AI, Parlow L, Gabler L, et al. Multicenter evaluation of a novel automated rapid detection

system of BRAF status in formalin-fixed, paraffin-embedded tissues. J Mol Diagn. 2016;18:370-377.
21. Cibas ES, Ali SZ. The 2017 Bethesda system for reporting thyroid cytopathology. Thyroid. 2017;27:1341-1346.
22. Elisei R, Ugolini C, Viola D, et al. BRAF(V600E) mutation and outcome of patients with papillary thyroid carcinoma: a 15-year median followup study. J Clin Endocrinol Metab. 2008;93:3943-3949.
23. Bae JS, Choi SK, Jeon S, et al. Impact of NRAS mutations on the diagnosis of follicular neoplasm of the thyroid. Int J Endocrinol. 2014;2014:289834.
24. Bellevicine C, Migliatico I, Sgariglia R, et al. Evaluation of BRAF, RAS, RET/PTC, and PAX8/PPARg alterations in different Bethesda diagnostic categories: a multicentric prospective study on the validity of the 7-gene panel test in 1,172 thyroid FNAs deriving from different hospitals in South Italy. Cancer Cytopathol. 2019. https://doi.org/10.1002/cncy.22217. [Epub ahead of print].
25. Bellevicine C, Sgariglia R, Migliatico I, et al. Different qualifiers of AUS/FLUS thyroid FNA have distinct BRAF, RAS, RET/PTC, and PAX8/PPARg alterations. Cancer Cytopathol. 2018;126:317-325.
26. Uguen A, Troncone G. A review on the Idylla platform: towards the assessment of actionable genomic alterations in one day. J Clin Pathol.2018;71:757-762.
27. Bose S, Sacks W, Walts AE. Update on molecular testing for cytologically indeterminate thyroid nodules. Adv Anat Pathol. 2019;26:114-123.
28. Hynes SO, Pang B, James JA, Maxwell P, Salto-Tellez M. Tissue-based next generation sequencing: application in a universal healthcare system. Br J Cancer. 2017;116:553-560.
29. Nikiforova MN, Wald AI, Roy S, Durso MB, Nikiforov YE. Targeted next-generation sequencing panel (ThyroSeq) for detection of mutations in thyroid cancer. J Clin EndocrinolMetab. 2013;98:E1852-E1860.
30. Nikiforov YE, Baloch ZW. Clinical validation of the ThyroSeq v3 genomic classifier in thyroid nodules with indeterminate FNA cytology. Cancer Cytopathol. 2019;127:225-230.
31. Bellevicine C, Sgariglia R, Malapelle U, et al. Young investigator challenge: can the Ion AmpliSeq Cancer Hotspot Panel v2 be used for next-generation sequencing of thyroid FNA samples? Cancer Cytopathol. 2016;124:776-784.
32. Al-Turkmani MR, Gordon SR, Smith KD, Tsongalis GJ, Liu X. Pancreatic cyst fluid harboring a KRAS mutation. Cold Spring Harb Mol Case Stud. 2019;5:a003830.
33. Evrard SM, Taranchon-Clermont E, Rouquette I, et al. Multicenter evaluation of the fully automated PCR-based Idylla EGFR mutation assay on formalin-fixed, paraffin-embedded tissue of human lung cancer. J Mol Diagn. 2019;21:1010-1024.
34. Cancer Genome Atlas Research Network. Integrated genomic characterization of papillary thyroid carcinoma. Cell. 2014;159:676-690.

Chapter 3

Performance evaluation of a fully closed real-time PCR platform for the detection of KRAS p.G12C mutations in liquid biopsy of patients with non-small cell lung cancer

Over the past few years, several clinical trials have established that the molecular characterization of predictive biomarkers is key to improving progression free survival, overall survival, and quality of life in patients with advanced-stage non-small cell lung cancer (NSCLC). More recently, novel genomic biomarkers are being evaluated as positive predictive biomarkers. For example, recent clinical trials have demonstrated that the Kirsten rat sarcoma viral oncogene homolog (*KRAS*) p.G12C mutation, which occurs in 13% of patients with NSCLC,2 responds well to small molecule *KRAS* inhibitors like AMG510 (Amgen, Thousand Oaks, California, USA) and MTRX894 (Mirati Therapeutics, San Diego, California, USA). However, the identification and evaluation of new predictive biomarkers in patients with NSCLC may often be hindered by insufficient or unavailable tissue material for molecular testing, especially when cancer diagnosis is based on small biopsy specimens. Fortunately, with the advent of molecular cytopathology, non-invasive techniques are nowadays being successfully employed to identify and evaluate relevant predictive biomarkers in advanced cancers. In particular, liquid biopsy is constantly gaining momentum in evaluating the mutational status of clinically relevant genes in cell-free DNA (cfDNA). Recently, Biocartis, a Belgium biotechnology company, developed a cartridge for analyzing *KRAS* mutations in liquid biopsy.[14–16] However, no study has ever investigated the performance of this cartridge in liquid biopsy routine samples previously analyzed by NGS. Hence, one of the aims of my research project was to to define for the first time the efficiency of *Idylla*™ in detecting *KRAS* p.G12C in a series of NSCLC liquid biopsies previously characterized by NGS in our institution.

Study Samples

This retrospective study was designed to assess the relative performance of the *Idylla*™ *KRAS* liquid biopsy assay in a series of NSCLC liquid biopsies previously characterized by NGS. To this end, we collected archived liquid biopsy samples previously processed by our custom NGS SiRe panel in the last 12 months of our clinical practice. All samples had to contain at least 10 µL of

archival extracted residual cfDNA and/or 2 mL of plasma. On the basis of this prerequisite, a total of 33 NSCLC plasma samples were selected for the study; in 30 cases, a paired archival cfDNA sample was available (table 1). Briefly, in all cases, plasma was isolated by double centrifugation at 2300 r.p.m. for 10 min. Then, the supernatant was aliquoted and used immediately for cfDNA isolation and stored at −80°C. Next, cfDNA was purified from the plasma samples of each NSCLC patient (1.2 mL) with the QIAsymphony robot (Qiagen) and QIAsymphony DSPVirus/Pathogen Midi Kit, according to the manufacturer's instructions. Finally, cfDNA was eluted in a final volume of 30 µL.

Table 1 KRAS mutational status of NSCLC liquid biopsy archival samples (plasma and cfDNA) characterized by NGS SiRe panel and KRAS liquid biopsy Idylla assay

Number	KRAS(AF)	Plasma	cfDNA
1	*p.G12C*(1,3%)	Failed	G12C
2	*p.G12C*(0,6%)	*p.G12C*	WT
3	*p.G12C*(5,6%)	Failed	G12C
4	*p.G12C*(3,3%)	*p.G12C*	G12C
5	*p.G12C*(24%)	*p.G12C*	G12C
6	*p.G12C*(0,9%)	*p.G12C*	WT
7	*p.G12C*(4,6%)	*p.G12C*	Failed
8	*p.G12C*(46,8%)	*p.G12C*	G12C
9	*p.G12C*(3,9%)	*p.G12C*	G12C
10	*p.G12C*(4,7%)	*p.G12C*	G12C
11	*p.G12C*(8,8%)	*p.G12C*	G12C
12	*p.G12C*(8,8%)	*p.G12C*	NA
13	*p.G12C*(24%)	*p.G12C*	NA
14	*p.G12C*(0,9%)	*p.G12C*	NA
15	WT	WT	Failed
16	WT	WT	WT

17	WT	WT	WT
18	WT	WT	WT
19	WT	WT	WT
20	WT	WT	WT
21	WT	WT	WT
22	WT	WT	WT
23	WT	WT	WT
24	WT	WT	WT
25	WT	WT	WT
26	WT	WT	WT
27	WT	WT	WT
28	WT	*p.G12C*	WT
29	WT	Failed	WT
30	WT	WT	WT
31	WT	WT	WT
32	WT	WT	WT
33	WT	WT	WT

AF, allelic fraction; cfDNA, cell-free DNA; KRAS, Kirsten rat sarcoma viral oncogene homolog; NA, not available; NGS, next generation sequencing; NSCLC, non-small cell lung cancer; WT, wild-type.

In particular, our research team retrieved n=14 *KRAS* p.G12C, with a mutant allelic fraction ranging from 0.6% to 46.8%, and n=19 exon 2 wild-type (WT) cases (Table 1).

NSCLC KRAS Liquid Biopsy Idylla Assay

Each sample was retested by the *KRAS* NSCLC liquid biopsy *Idylla*™ assay. Although the manufacturer's instructions generally recommend processing plasma directly into the cartridge, we decided to pipette DNA extracted from archival plasma samples directly into the cartridge, as done in one of our previous studies. Thus, both cfDNA and paired plasma samples were tested. Cross-contamination was avoided by closing the cartridge immediately after the insertion of cfDNA or plasma. Microfluidic channels embedded in the cartridge transported cfDNA into five separate PCR chambers. These chambers, which contain pre-deposited PCR reagents in dried form (i.e., primers, probes, and enzymes) are specifically designed for qualitative detection of clinically relevant *KRAS* variants, including, *KRAS* p.G12C mutation. After a 120-min run, final reports were directly available on the Idylla console. The results, which appeared on the screen as either 'no mutation detected' or 'KRAS mutation detected', were compared with those previously generated by NGS.

Results

Overall, the study series, which comprised a total of 33 NSCLC plasma samples and 30 paired archival cfDNA previously analyzed by our in-house SiRe NGS panel, were retested with the *KRAS Idylla*™ liquid biopsy assay. Of these, 30/33 (91%) plasma and 28/30 (93%) cfDNA samples showed valid results and were thus compared with the NGS results. Failure rates were 7% and 9% for cfDNA and plasma samples, respectively. Notably, *Idylla*™ confirmed the NGS results in 29/30 (96.7%) *KRAS* p.G12C mutant plasma samples and 26/28 (93%) paired cfDNA. Only one sample (1/30 (3%)) in the NGS WT plasma population showed a false positive result. In contrast, all NGS WT cfDNA results were confirmed by *Idylla*™ (table 1). In addition, the *Idylla*™ concordance rate between cfDNA and paired plasma samples was 22/25 (88%); that is, only 3/25 (12%) cases showed discrepant results (Figure 1).

Discussion

Detection of the *KRAS* p.G12C variant in NSCLC is clinically important to select patients for AMG510 and MTRX849 treatments. Liquid based biopsy samples for molecular analysis are a valid alternative to histological specimen, especially in hard-to-reach tumors like advanced NSCLC. In this retrospective study, NSCLC routine liquid biopsy samples, previously characterized by NGS, were retested with the more user-friendly, fully automated *Idylla*™ platform. Our data clearly demonstrated the technical feasibility of the NSCLC *KRAS Idylla*™ cartridge to detect the *KRAS* p.G12C mutation in patients with advanced NSCLC. Whereas the routine clinical use of liquid biopsy samples for *EGFR* detection in patients with advanced-stage NSCLC has been well documented, .[7 8] clinical procedures to detect the novel actionable *KRAS* exon 2 biomarker still need to be refined. Generally, *KRAS* testing in patients with advanced-stage NSCLC is done either to provide prognostic information or to rule out less common driver alterations.[17 18] However, since AMG510 and MTRX849 clinical trial programs have shown promising results, especially against *KRAS* exon 2 p.G12C mutations, it is now considered as a positive predictive biomarker.[19] In our clinical practice experience as a referral center for predictive molecular pathology, 17% of tissue specimens from

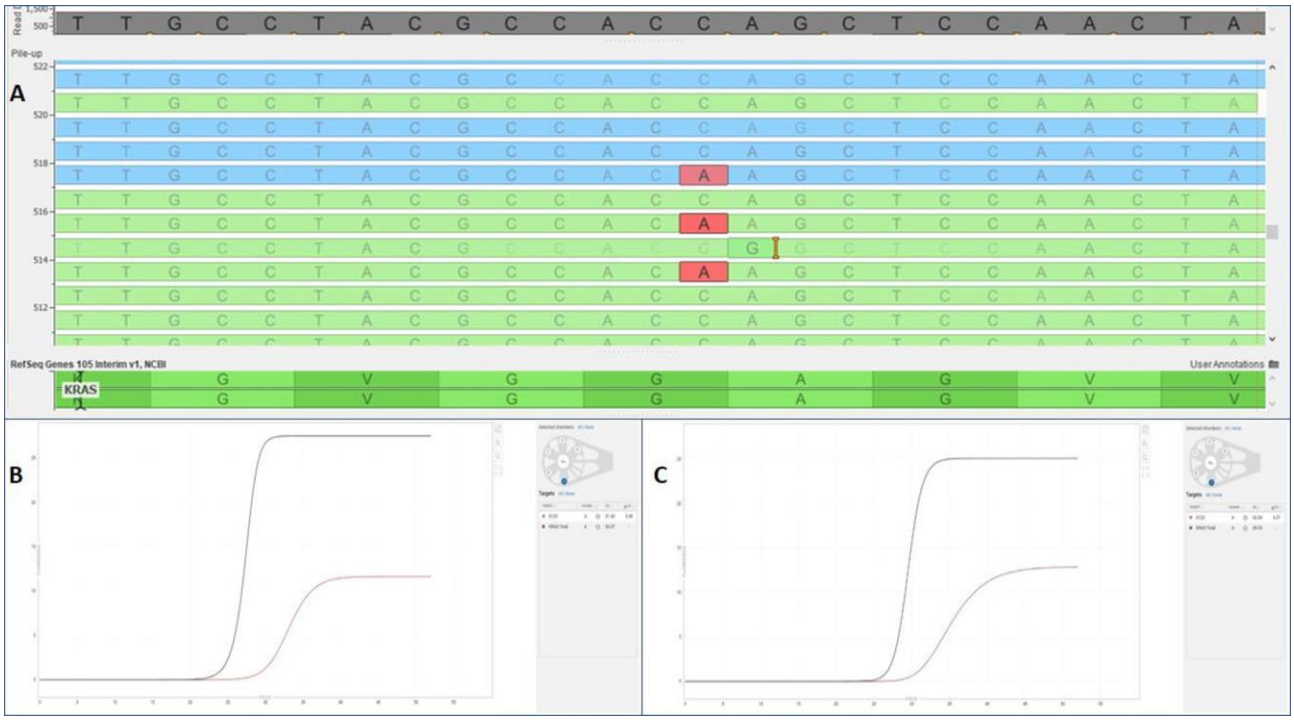

Figure 1 An exemplificative p.G12C concordant case between NGS (A) and NSCLC KRAS liquid biopsy Idylla assay on plasma (B) and paired archival cfDNA (C). cfDNA, cell-free DNA; KRAS, Kirsten rat sarcoma viral oncogenehomolog; NGS, next generation sequencing; NSCLC, non-small cell lung cancer.

patients with advanced-stage NSCLC,20 are insufficient for ancillary *EGFR* genomic testing.[9] For this reason, cytological samples applied to NGS platforms oftentimes are an excellent alternative to histological specimens. Among these preparations, cfDNA from liquid biopsies has proven helpful in managing patients with NSCLC, as evidenced by researchers in our laboratory and in other groups. Consequently, the possibility of adopting liquid-based biopsy for *KRAS* testing is highly conceivable. Undoubtedly, we do realize that sample multiplexing by NGS represents the best approach. However, despite its invaluable clinical versatility, it does require significant technical and bioinformatical skills, hence being cost-effective only in large-volume laboratories. Conversely, in low-volume laboratories, we believe that an easy-to-use, fully automated RT-PCR platform, such as the *Idylla*™ system, could be far more useful and economically convenient than NGS to provide rapid assessment of KRAS p.G12C mutations. Noticeably, Biocartis, a Belgian biotechnology firm, has recently developed an NSCLC *KRAS* liquid biopsy *Idylla*™ assay, which, as my dissertation data indicate, is highly efficient when applied to cfDNA and plasma samples. Indeed, as evidenced by the results reported in my PhD work, *Idylla*™ failed to detect *KRAS* p.G12C mutations only in 7% of cfDNA and in 9% of plasma samples, thereby confirming its reliable performance. In addition, *Idylla*™ confirmed previous NGS results in 29/30 (96.7%) *KRAS* p.G12C cfDNA and in 26/28 (93%) plasma samples. Concerning NGS WT sample population, only 1/30 plasma samples (3%) showed a false positive result, whereas all NGS WT cfDNA results were confirmed by *Idylla*™. Lacking additional archival material, we were unable to carry out additional investigation. Overall, *Idylla*™ successfully analyzed most of the samples. Indeed, it yielded a high concordance rate between cfDNA and paired plasma samples in 22/25 (88%). We hypothesize that the 12% discordance rate seen in two *Idylla*™ WT cfDNA results was probably due to the serial withdrawal from the same archived residual material. The main limitations of our study were most likely attributable to its retrospective design and to a lack of corresponding tissue samples. However, the nearly complete concordance rate we obtained between *Idylla*™ and our standard NGS assays clearly points to the feasibility of applying the newly developed NSCLC *KRAS* liquid biopsy *Idylla*™ assay in routine clinical practice. In conclusion, the results from this study underline the technical feasibility of the new *Idylla*™ NSCLC *KRAS* liquid biopsy assay as an alternative tool to NGS for the *KRAS* p.G12C mutation detection on ctDNA in low-volume testing laboratories. Further and prospective investigations are however warranted to assess the clinical performance of this newly developed diagnostic tool.

References

1. Pisapia P, Pepe F, Troncone G, et al. Predictive biomarkers for molecular pathology in lung cancer. Biomark Med 2020;14:253–7.
2. Román M, Baraibar I, López I, et al. Kras oncogene in non-small cell lung cancer: clinical perspectives on the treatment of an old target. Mol Cancer 2018;17:33.
3. Hong DS, Fakih MG, Strickler JH, et al. KRASG12C Inhibition with Sotorasib in Advanced Solid Tumors. N Engl J Med 2020;383:1207–17.
4. Jänne PA, Rybkin II, Spira AI, et al. KRYSTAL-1: activity and safety of Adagrasib (MRTX849) in Advanced/ metastatic Non–Small-Cell lung cancer (NSCLC) harboring KRAS G12C mutation. Eur J Cancer 2020;138:S12.
5. Barbareschi M, Barberis M, Buttitta F, et al. Predictive markers in lung cancer: a fewhints for the practicing pathologist. Pathologica 2018;110:29–38.
6. Pisapia P, Malapelle U, Troncone G. Liquid biopsy and lung cancer. Acta Cytol 2019;63:489–96.
7. Rolfo C, Mack PC, Scagliotti GV, et al. Liquid Biopsy for Advanced Non-Small Cell Lung Cancer (NSCLC): A Statement Paper from the IASLC. J Thorac Oncol 2018;13:1248–68.
8. Rolfo C, Cardona AF, Cristofanilli M, et al. Challenges and opportunities of cfDNA analysis implementation in clinical practice: perspective of the International Society of liquid biopsy (ISLB). Crit Rev Oncol Hematol 2020;151:102978.
9. Nacchio M, Sgariglia R, Gristina V, et al. KRAS mutations testing in non-small cell lung cancer: the role of Liquid biopsy in the basal setting. J Thorac Dis 2020;12:3836–43.
10. De Luca C, Conticelli F, Leone A, et al. Is the Idylla EGFR Mutation Assay feasible on archival stained cytological smears? A pilot study. J Clin Pathol 2019;72:609–14.
11. Uguen A, Troncone G. A review on the Idylla platform: towards the assessment of actionable genomic alterations in one day. J Clin Pathol 2018;71:757–62.
12. Barel F, Guibourg B, Lambros L, et al. Evaluation of a rapid, fully automated platform for detection of BRAF and NRAS mutations in melanoma. Acta Derm Venereol 2018;98:44–9.
13. Lambros L, Caumont C, Guibourg B, et al. Evaluation of a fast and fully automated platform to diagnose EGFR and KRAS mutations in formalin-fixed and paraffin-embedded non-small cell lung cancer samples in less than one day. J Clin Pathol 2017;70:544–9.
14. Vivancos A, Aranda E, Benavides M, et al. Comparison of the clinical sensitivity of the Idylla platform and the OncoBEAM Ras CRC assay for KRAS mutation detection in liquid biopsy samples. Sci Rep 2019;9:8976.
15. Vessies DCL, Greuter MJE, van Rooijen KL, et al. Performance of four platforms for KRAS mutation detection in plasma cell-free DNA: ddPCR, Idylla, COBAS z480 and beaming. Sci Rep 2020;10:8122.
16. Holm M, Andersson E, Osterlund E, et al. Detection of KRAS mutations in liquid biopsies from metastatic colorectal cancer patients using droplet digital PCR, Idylla, and next generation sequencing. PLoS One 2020;15:e0239819.
17. Yuan M, Huang L-L, Chen J-H, et al. The emerging treatment landscape of targeted therapy in non-small-Cell lung cancer. Signal Transduct Target Ther 2019;4:61.
18. Martin P, Leighl NB, Tsao M-S, et al. Kras mutations as prognostic and predictive markers in non-small cell lung cancer. J Thorac Oncol 2013;8:530–42.
19. Uras IZ, Moll HP, Casanova E. Targeting KRAS Mutant Non-Small-Cell Lung Cancer: Past, Present and Future. Int J Mol Sci 2020;21:4325.
20. Malapelle U, Bellevicine C, De Luca C, et al. Egfr mutations detected on cytology samples by a centralized laboratory reliably predict response to gefitinib in non-small cell lung carcinoma patients. Cancer Cytopathol 2013;121:552–60.

Chapter 4

Evaluation of a fully closed real time PCR platform for the detection of SARS-CoV-2 in nasopharyngeal swabs: a pilot study.

The COVID-19 pandemic caused by SARS-CoV-2 was first identified in Wuhan (Hubei region, China) at the end of 2019.[1] [2] The infection, which continues to affect populations worldwide despite the ongoing mass vaccination campaigns, was determined a pandemic by the WHO on 11 March 2020.[3] At the time of my writing (12 February 2021), more than 107 million people have been infected by SARS-CoV-2 with more than 2 million deaths, worldwide.[4] As it is widely know by now thanks to the publication of several seminal studies, SARS-CoV-2 is a beta-coronavirus, enveloped, positive-sense, single stranded RNA virus from the Coronaviridae family.[5] [6] The viral genome, composed of about 30,000 nucleotides, contains genes encoding for nucleocapsid (N), envelop (E), membrane (M), internal (I) and spike (S) structural proteins and two open reading frame genes (ORF1a and ORF1b) encoding for 16 non-structural proteins including the RNA-dependent RNA polymerase.[7] The 'gold standard' for the diagnosis of COVID-19 is reverse transcriptase PCR (RT-PCR) on nasopharyngeal swabs.[8] [9] However, despite the high sensitivity and specificity, the performance of this diagnostic tool in routine practice seems to be affected and, at times, compromised by several factors. In particular, besides requiring highly trained personnel, a complex infrastructure is mandatory to manage hundreds or thousands of daily testing while trying to reduce to a minimum the biological risk of staff exposure to the virus. Finally, it is highly challenging to strike a balance between the urgency of batching a minimum number of testing samples while giving the results as quickly as possible (within 24 hours). To overcome these limitations, several authors have endeavored to develop new diagnostic tools which would be easy to use, rapid, and reliably robust, and which would require minimal hands-on time requirements.[10] In this setting, our research laboratory hypothesized that the fully automated RT-PCR *Idylla*™ platform (Biocartis NV, Mechelen, Belgium) could be a valuable solution. The rationale behind this ideas was that, compared to more conventional technologies, this platform could represent a rapid, robust, sensitive and specific approach to reduce the risk of sample cross-contamination and personnel exposure to high risk specimens.[11] As I previously reported, the *Idylla*™ platform has been successfully adopted by our molecular predictive laboratory at the Department of Public Health of the University of Naples Federico II for predictive purposes in advanced stage solid tumor patients during the healthcare emergency.[12] Interestingly, during the height of the pandemic, Biocartis, a major Belgian molecular diagnostics company, dedicated its highly innovative research

efforts to developing a novel cartridge (Biocartis NV.1) (see the Materials and methods section) capable of assessing the infectious SARS-CoV-2 status in a much more rapid and cost-effective way. Thus, in the hope of making this new tool readily available to the community, we decided to invest our time and efforts to evaluate the analytical and clinical performance of this novel disposable cartridge on previously tested SARS-CoV-2 people by conventional RT-PCR based approach in different settings, including initial diagnosis and clinical follow-up.

Study design

This study was designed to evaluate the efficiency of *Idylla*™ SARS-CoV-2 Test to identify SARS-CoV-2 viral RNA. To this end, we retrieved from the archive of the Clinical Pathology Laboratory at the Department of Translational Medical Sciences, University of Naples Federico II, 55 nasopharyngeal swabs that had been collected and preserved in sterile viral medium Universal Transport Medium (UTM, Copan Diagnostic, Brescia, Italy) from symptomatic patients or from people who had been in close contact with COVID-19 positive cases. These latter, had previously been tested by a fully validated assay (Real-Time SARS-CoV-2 kit, #09N77-095, Abbott Laboratories, Chicago,Illinois, USA) which detects the RdRP and the N gene of SARS-CoV-2; this assay, which was approved by the Food and Drug Administration for emergency use, can run a complete molecular analysis in about six hours. To assess the sensitivity and specificity of the *Idylla*™ SARS-CoV-2 Test against the Abbott Real-Time SARS-CoV-2 kit, samples, including positive (n=35) and negative (n=20) nasopharyngeal swabs, were retested by the *Idylla*™ SARS-CoV-2 Test. Cases showing discrepant results and lack of agreement between the *Idylla*™ SARS-CoV-2 Test and Abbott Real-Time SARS-CoV-2 kit, were further analyzed by a third technique (RealStar SARS-CoV-2 RT-PCR Kit, Altona Diagnostics GmbH, Hamburg, Germany). In addition, we also evaluated whether the *Idylla*™ SARS-CoV-2 Test could also be used to yield fast and accurate results to end ahead of time the 21-day precautionary quarantine of suspected Covid-19 cases. To this aim, we retrieved a second subset of 14 nasopharyngeal swab samples with uncertain results (cycle threshold (Ct) between 37 and 40) from patients with viral infection beyond day 21 by using Abbott Real-Time SARS-CoV-2 assay. In all these instances, a third methodology (RealStar SARS-CoV-2 RT-PCR Kit) was also adopted.

Abbott Real-Time SARS-CoV-2 kit

At the Clinical Pathology Laboratory at the Department of Translational Medical Sciences, University of Naples Federico II, nasopharyngeal swabs are routinely analyzed by using Abbott Real-Time SARS-CoV-2 kit, as described in the manufacturer's instructions, on the Alinity platform. Briefly, 800 μL of UTM was collected. Results were considered as positive when the Ct value on N and/or on ORF1b genes was equal or less than 37.[14]

Idylla™ SARS-CoV-2 Test

In this paragraph, I will succinctly describe how we ran the *Idylla*™ SARS-CoV-2 Test. The *Idylla*™ SARS-CoV-2 Test is a fully automated RT-PCR system that adopts disposable cartridges. In particular, 200 μL of UTM was directly pipetted into the SARS-CoV-2 Test cartridge. Via microfluidic channels, RNA was extracted after cell lysis performed by a combination of HIFU, enzymatic/chemical digestion, and heat. The extracted RNA was first converted into complementary DNA (cDNA) by the RT enzyme and then transported into five PCR chambers for amplification. These chambers generally contain dried primers and probes designed to detect two N and three ORFb1 target regions. This fluorescent-based assay, which allows cytopathologists to detect two SARS-CoV-2 RNA targets such as N gene (nucleocapsid phosphoprotein gene) and ORF1b region, is covered with five PCR targets (two N and three ORF1b targets). In addition, for each amplification chamber, the amplification of MS2 Bacteriophage is adopted as an internal control to monitor the correct execution of RNA extraction and amplification steps in the cartridge. After a 90-min run, the final report is displayed on the *Idylla*™ console as 'positive', 'negative' or 'not valid'. As a general rule, a positive result requires at least two N amplified targets (with a Ct value ≤41.9) and/or at least one or more ORF1b amplified targets (in this case, a Ct value cut-off is not required owing to, the very high specificity of this gene amplification). Negative results indicate the absence of SARS-CoV-2 target amplification.

		Idylla SARS-CoV-2 Test		
		Positive	**Negative**	**Total**
Abbott Real-Time SARS-CoV-2	**Positive**	31	2	33
	Negative	0	20	20
	Total	31	22	53

Table 1 Comparison between the results of the Idylla SARS-CoV-2 Test and the results of routine Abbott Real-Time SARS-CoV-2 assay

RealStar SARS-CoV-2 RT-PCR Kit

The RealStar SARS-CoV-2 RT-PCR Kit, an RT-PCR based technology that enables qualitative detection of target regions in SARS-Cov-2 E and S genes, was adopted according to the manufacturer's instructions.

RESULTS

Overall, the *Idylla*™ assay generated valid results in almost all analyzed samples (96.4%, 53/55). Interestingly, among successfully analyzed cases, we observed a 96.2% concordance rate (51/53) between the *Idylla*™ SARS-CoV-2 Test and the Real-Time SARS-CoV-2 kit. Of note, the *Idylla*™ SARS-CoV-2 Test yielded no false positive results (specificity 100.0%) (Table 1). Conversely, only in two cases (6.1%, 2/33) did *Idylla*™ miss SARS-CoV-2 (Ct 34.73 and 36.11 with the gold standard technology), indicative of a false negative result (Figure 1) (online supplemental Table 1). The possibility of a false negative was also suggested by the detection of the virus by the Altona assay. In addition, in five cases (#1, #16, #25, #30 and #31) with a high discrepancy between the standard technology and the *Idylla*™ SARS-CoV-2 Test in terms of Ct and quantification cycle (Cq), respectively, the (Altona assay) confirmed a positive result in all instances (online supplemental table 2). Of note, in the supplementary 14 nasopharyngeal swab samples taken after 21 days from the first positive nasopharyngeal swab showing uncertain results with Abbott Real-Time SARS-CoV-2 assay, only five (35.7%) featured a positive result according to the *Idylla*™ SARS-CoV-2 Test (online supplemental table 3). Interestingly, in the vast majority of the analyzed samples (11/14, 78.6%) the adoption of a third methodology (RealStar SARS-CoV-2 RT-PCR Kit) confirmed the *Idylla*™ SARS-CoV-2 Test results (online supplemental table 3). Conversely, three positive *Idylla*™ SARS-CoV-2 Test cases were classified as 'uncertain'(n=2) or 'negative' (n=1) with the third methodology (online supplemental table 3).

DISCUSSION

Our research demonstrated that the *Idylla*™ Test is a sensitive, specific, easy to use, and rapid assay for SARS-CoV-2 detection in nasopharyngeal swabs. In particular, our experience showed that the *Idylla*™ SARS-CoV-2 Test is able to reach a specificity of 100.0% and a sensitivity of 93.9% as compared with the results of a reliable reference technology. Only two cases showed discrepant results, with *Idylla*™ assay being negative. In effect, we suspect that these discrepancies were actually true false negative cases although we cannot rule-out that technical issues related to the thawing of the archival frozen nasopharyngeal swabs might have skewed the results. Despite these few setbacks, we have demonstrated the usefulness of this platform, especially during a health emergency. In the difficult time of the COVID-19 pandemic, there is an urgent need of rapid and automated tests for the detection of SARS-CoV-2 infection. The main problem posed by the conventional RT-PCR technology is that it requires several dozen or even hundreds of samples to be grouped in batches which must be be tested in parallel—a process that is time consuming, prolongs TAT, and, ultimately, delays test results (6–24 hours). In a clinical scenario, these delays can compromise the safety of clinicians, for example, especially when having to perform life-saving surgeries, as in an emergency situation, or when having to manage vaginal deliveries. In this setting, the employment of rapid assays capable of characterizing the COVID-19 status of patients is key to reducing the risk of staff exposure to the virus. In fact, the possibility of having a technology able to process selected cases rapidly, efficiently, and inexpensively is what has led to the widespread adoption of the *Idylla*™ technology in clinical practice, in particular for mutational testing in oncological patients. In fact, this automated molecular technology, which is often present in molecular laboratories dealing with oncological procedures, could also offer the opportunity to identify patients with COVID-19. To date, several tests and technologies have been developed for the diagnosis of SARS-CoV-2 infection.[15–18] Particular attention has been given to developing technologies requiring minimum hands-on time and capable of reducing the risk of potential staff exposure to SARS-CoV-2 infected samples, as well as sample cross-contamination. In this setting, the fully automated *Idylla*™ platform may represent a valid diagnostic tool. Indeed, during the current COVID-19 pandemic, a technological shift towards fully automated platform has been highly advantageous especially to offset reduced laboratory personnel to prevent contagion. In a single and a multicenter experience coordinated by our Molecular Predictive Pathology Laboratory at the Department of Public Health of the University of Naples Federico II, the implementation of fully automated platforms, such as *Idylla*™, has significantly simplified predictive molecular testing while ensuring safety and cost-effective management of laboratory staff.[12 19] Recently, the novel *Idylla*™ SARS-CoV-2 Test has been developed to further simplify

Covid-19 sample processing.[13] Generally, the *Idylla*™ system adopts computer that can be associated with up to eight separately operating instruments. Strikingly, each independent instrument can process disposable cartridges able to perform viral RNA extraction, retro transcription, amplification, and data analysis in about 90 min.[13] Interestingly, this assay has recently obtained the CE-IVD mark with a lower limit of detection for viral genomic of 500 copies/mL.[13] Remarkably, in our experience, the *Idylla*™ SARS-CoV-2 Test generated only 2 false negative results out of 53 successfully analyzed cases. Overall, these cases were further analyzed by another RT-PCR assay, specifically the Altona RealStar SARS-CoV-2 RT-PCR Kit.[20]

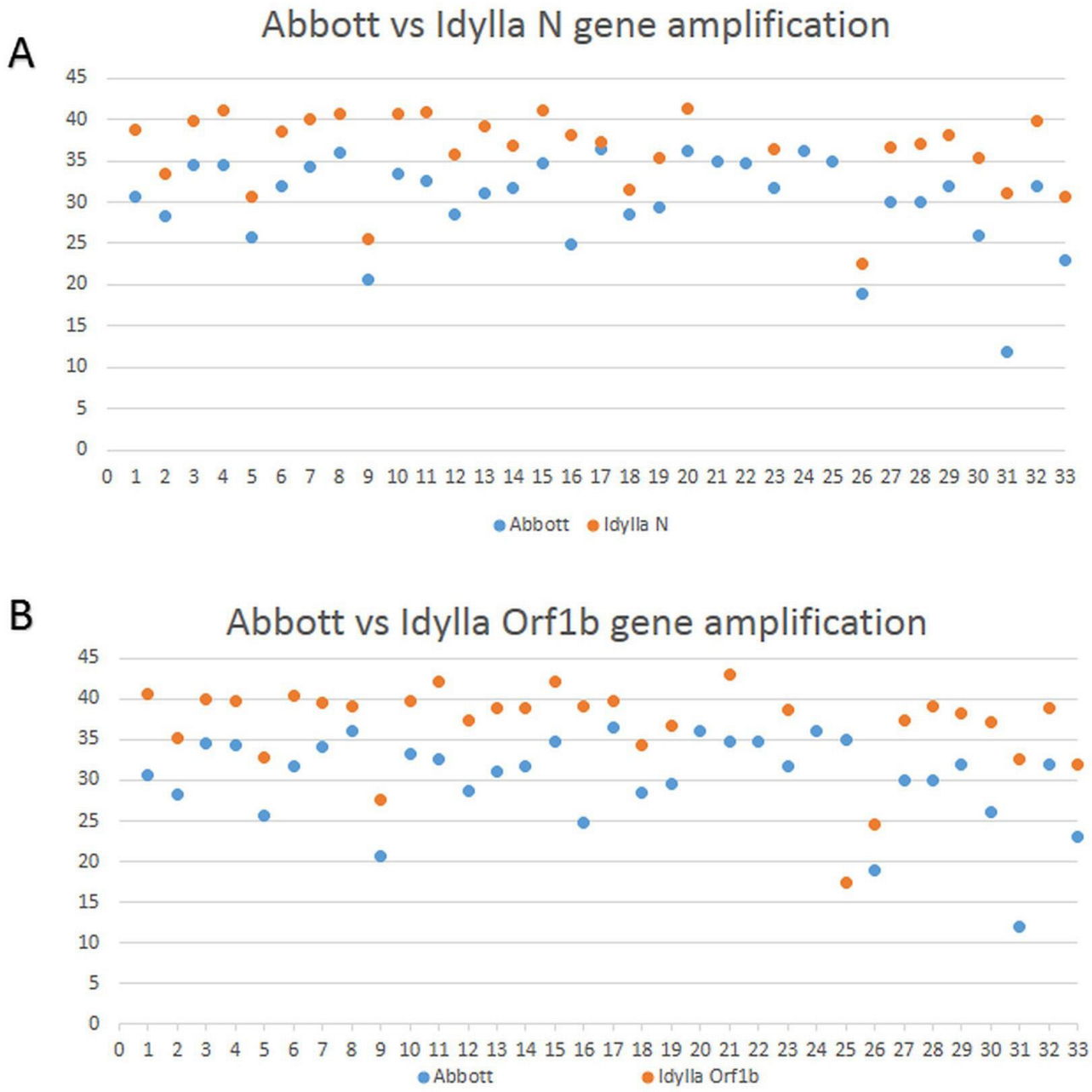

Figure 1 Graphical comparison between cycle threshold (Ct) (Abbott) versus quantification cycle (Cq) (Idylla) for N and ORF1b gene amplification.

This latter was able to analyze target regions in E and S genes. Interestingly, with this RT-PCR approach negative and undetermined results were reported (online supplemental table 1). In addition, the Altona assay confirmed a positive result in five cases (#1, #16, #25, #30 and #31) with a high discrepancy between the standard technology and the *Idylla*™ SARS-CoV-2 Test in terms of Ct and Cq, respectively (online supplemental table 2). We hypothesized that these discrepancies may be related to the fluorescence detection thresholds used by Abbott technology, whereas the *Idylla*™ SARS-CoV-2 calculates the Cq on the basis of normalized amplification curves. In this

particular study, we selected only positive cases within a Ct equal or less than 37. Interestingly, a recent study reported that Covid-19 patients with positive amplification with a Ct value >37 beyond day 21are no longer contagious.[21] Thus, we tested the *Idylla*™ SARS-CoV-2 Test could be exploited to end the precautionary quarantine of patients who still tested positive after 21 days. To this end, we analyzed 14 nasopharyngeal swabs belonging to patients with viral infection beyond day 21 who tested as uncertain according to the Abbott Real-Time SARS-CoV-2 kit. Interestingly, only 5 (35.7%) out of 14 nasopharyngeal swabs featured a positive result by the *Idylla*™ SARS-CoV-2 Test, thereby confirming the validity of this automated approach to end self-isolation periods. Of note, in the vast majority of these samples (11/14, 78.6%) the adoption of a third methodology (RealStar SARS-CoV-2 RT-PCR Kit) confirmed the *Idylla*™ SARS-CoV-2 Test results (online supplemental table 3). Conversely, only three positive *Idylla*™ SARS-CoV-2 Test cases were classified as 'uncertain' (n=2) or 'negative' (n=1) with the third methodology (online supplemental table 3). However, the major limitation of this pilot study was the extremely limited sample size. Thus, further investigation is required to assess whether the *Idylla*™ SARS-CoV-2 Test may be useful to analyze cases with undetermined and unclear results (Ct between 37 and 40) requiring rapid evaluation. In these cases, the *Idylla*™ SARS-CoV-2 Test may be considered a component of a diagnostic algorithm that exploits both conventional and automated RT-PCR platforms. In conclusion, despite the small sample size, our results are encouraging and clearly suggest that the *Idylla*™ SARS-CoV-2 Test may represent a valid, fast, highly sensitive and specific RT-PCR test for the identification of SARS-CoV-2 infection.

ID sample	**Abbott Real-Time PCR**		**Idylla SARS-CoV-2 Test**					
	Ct value	Result	Ct value (N)		Ct value (Orf1b)			Result
1	30,54	Positive	39,18	38,41	41,06	39,51	41,54	Positive
2	28,28	Positive	33,46	33,49	35,22	34,73	35,55	Positive
3	34,55	Positive	39,74	39,82	39,95			Positive
4	34,43	Positive	42,5	41,08		39,73		Positive
5	25,75	Positive	30,71	30,64	32,76	32,77	32,95	Positive
6	31,82	Positive	38,77	38,3	41,81	39,91	39,82	Positive
7	34,22	Positive	40,2	40,02	39,68	39,48		Positive
8	35,96	Positive	39,62	41,58			39,16	Positive
9	20,62	Positive	25,56	25,55	27,49	27,61	27,84	Positive
10	33,34	Positive	40,41	40,83		39,67		Positive
11	32,49	Positive	40,5	41,11	43,05		41,33	Positive
12	28,6	Positive	35,71	35,67	37,22	37,81	37,37	Positive
13	31,17	Positive	38,84	39,42	39,14	38,75	38,52	Positive
14	31,62	Positive	36,78	36,86	39,05	38,28	39,27	Positive
15	34,77	Positive	40,41	41,75		42,11		Positive
16	24,83	Positive	38,28	38,13	38,37	39,81	39,29	Positive
17	36,5	Positive	37,26	37,4	38,79	40,22	40,4	Positive
18	28,54	Positive	31,4	31,5	34,19	34,28	34,39	Positive
19	29,46	Positive	35,5	35,36	36,67	36,59	36,89	Positive
20	36,09	Positive	40,77	41,85				Positive
21	34,84	Positive					43,03	Positive
22	34,73	Positive						Negative
23	31,62	Positive	36,71	36,14	38,52	38,57	38,89	Positive
24	36,11	Positive						Negative
25	35	Positive					17,44	Positive
26	19	Positive	22,54	22,46	24,61	24,65	24,76	Positive
27	30	Positive	36,57	36,52	37,65	37,21	36,98	Positive
28	30	Positive	37,06	36,96	39,16	38,87	39,47	Positive
29	32	Positive	37,81	38,26	38,83	38,18	37,91	Positive
30	26	Positive	35,1	35,56	37,31	36,65	37,32	Positive
31	12	Positive	31,06	30,98	32,67	32,7	32,71	Positive
32	32	Positive	39,89	39,64	38,6	39,08		Positive
33	23	Positive	30,66	30,65	31,83	31,82	32,04	Positive
34		Negative						Negative
35		Negative						Negative
36		Negative						Negative
37		Negative						Negative
38		Negative						Negative
39		Negative						Negative
40		Negative						Negative
41		Negative						Negative
42		Negative						Negative

43		Negative						Negative
44		Negative						Negative
45		Negative						Negative
46		Negative						Negative
47		Negative						Negative
48		Negative						Negative
49		Negative						Negative
50		Negative						Negative
51		Negative						Negative
52		Negative						Negative
53		Negative						Negative

Supplementary Table 1 Idylla SARS-CoV-2 and Abbott Real-Time PCR comparison with Ct values <37.

ID	Abbott Real-Time PCR		Idylla SARS-Cov-2 Test						RealStar SARS-CoV-2 Real-Time PCR		
Sample	Ct value	Result	Cq value (N)		Cq value(Orf1b)			Result	Ct value(E)	Ct value (S)	Result
1	30,54	Positive	39,18	38,41	41,06	39,51	41,54	Positive	31,46	31,67	Positive
16	24,83	Positive	38,28	38,13	38,37	39,81	39,29	Positive	28,12	30,45	Positive
25	35	Positive					17,44	Positive	25,78	26,31	Positive
30	26	Positive	35,1	35,56	37,31	36,65	37,32	Positive	29,84	27,78	Positive
31	12	Positive	31,06	30,98	32,67	32,7	32,71	Positive	14,24	22,69	Positive

Supplementary Table 2 High discrepant results between Idylla SARS-CoV-2 and Abbott Real-Time PCR were further analyzed by a third technique (RealStar SARS-CoV-2 Real-time PCR).

ID sample	Abbott Real-Time PCR		Idylla SARS-CoV-2 Test					
	Ct value	Result	Ct value (N)		Ct value (Orf1b)			Result
1	37,7	Uncertain	43,02	42,68	40,97		40,96	Positive
2	38,95	Uncertain	42,47					Negative
3	38,86	Uncertain						Negative
4	37,95	Uncertain	41,15	41,36		40,17		Positive
5	37,37	Uncertain	41,26	41,82	41,43			Positive
6	38,88	Uncertain						Negative
7	38,76	Uncertain	42,25	42,66			43,4	Positive
8	39,38	Uncertain		42,15				Negative
9	38,98	Uncertain						Negative
10	39,64	Uncertain						Negative
11	37,94	Uncertain		42,67	41,45			Positive
12	38,28	Uncertain		43,45				Negative
13	37,83	Uncertain						Negative
14	37,9	Uncertain	41,84	42,07				Negative

Supplementary Table 3 Idylla SARS-CoV-2 and Abbott Real-Time PCR comparison with Ct values >37.

References

1. Zhu N, Zhang D, Wang W, et al. A novel coronavirus from patients with pneumonia in China, 2019. N Engl J Med 2020;382:727–33.
2. Lai C-C, Shih T-P, Ko W-C, et al. Severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) and coronavirus disease-2019 (COVID-19): the epidemic and the challenges. Int J Antimicrob Agents 2020;55:105924.
3. World Health Organization (WHO). Coronavirus disease (COVID-19). Available:https://www. who. int/ dg/ speeches/ detail/ who- director- general- s- opening- remarks- atthe-media- briefing- on-covid- 19- 11- march- 2020
4. Gisand Data. COVID-19 Dashboard by the center for systems science and engineering (CSSE) at Johns Hopkins University (JHU). Available: https:// gisanddata. maps. arcgis.com/ apps/ opsdashboard/ index. html#/ bda7 5947 40fd 4029 9423 467b 48e9ecf6[Accessed 11 Feb 2021].
5. Paules CI, Marston HD, Fauci AS. Coronavirus Infections-More than just the common cold. JAMA 2020;323:707–8.
6. Coronaviridae Study Group of the International Committee on Taxonomy of Viruses. The species severe acute respiratory syndrome-related coronavirus: classifying 2019-nCoV and naming it SARS-CoV-2. Nat Microbiol 2020;5:536–44.
7. Kim D, Lee J-Y, Yang J-S, et al. The architecture of SARS-CoV-2 transcriptome. Cell 2020;181:914–21.
8. Benzigar MR, Bhattacharjee R, Baharfar M, et al. Current methods for diagnosis of human coronaviruses: pros and cons. Anal Bioanal Chem 2021;413:2311–30.
9. Böger B, Fachi MM, Vilhena RO, et al. Systematic review with meta-analysis of the accuracy of diagnostic tests for COVID-19. Am J Infect Control 2021;49:21–9.
10. Allam M, Cai S, Ganesh S, et al. COVID-19 diagnostics, tools, and prevention. Diagnostics 2020;10:409.
11. Uguen A, Troncone G. A review on the Idylla platform: towards the assessment of actionable genomic alterations in one day. J Clin Pathol 2018;71:757–62.
12. Malapelle U, Pisapia P, Iaccarino A, et al. Predictive molecular pathology in the time of coronavirus disease (COVID-19) in Europe. J Clin Pathol 2020. doi:10.1136/ jclinpath-2020-206957. [Epub ahead of print: 31 Jul 2020].
13. Biocartis. IdyllaTM SARS-CoV-2 test. Available: https://www. biocartis. com/ en/ meetidylla/idylla-infectious- disease- assays/ idylla- sars- cov- 2- test
14. Wang J, Cai K, Zhang R, et al. Novel one-step single-tube nested quantitative real-time PCR assay for highly sensitive detection of SARS-CoV-2. Anal Chem2020;92:9399–404.
15. Li C, Zhao C, Bao J, et al. Laboratory diagnosis of coronavirus disease-2019 (COVID-19). Clin Chim Acta 2020;510:35–46.
16. Lippi G, Horvath AR, Adeli K, Editorial AK. Editorial and executive summary: IFCC interim guidelines on clinical laboratory testing during the COVID-19 pandemic. Clin Chem Lab Med 2020;58:1965–9.
17. Thompson S, Bohn MK, Mancini N, et al. IFCC interim guidelines on Biochemical/Hematological monitoring of COVID-19 patients. Clin Chem Lab Med 2020;58:2009–16.
18. Vandenberg O, Martiny D, Rochas O, et al. Considerations for diagnostic COVID-19 tests. Nat Rev Microbiol 2021;19:171–83.
19. Malapelle U, De Luca C, Iaccarino A, et al. Predictive molecular pathology in the time of COVID-19. J Clin Pathol 2021;74:234–7.
20. RealStar® SARS-CoV-2 RT-PCR Kit RUO. Altona diagnostics GmbH. Available: https://altona-diagnostics. com/ en/ products/ reagents- 140/ reagents/ realstar- real- time- pcrreagents/realstar-sars- cov- 2- rt- pcr- kit- ruo.html
21. Cevik M, Tate M, Lloyd O, et al. SARS-CoV-2, SARS-CoV, and MERS-CoV viral load dynamics, duration of viral shedding, and infectiousness: a systematic review and meta-analysis. Lancet Microbe 2021;2:e13–22.