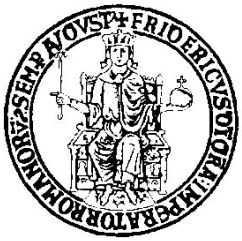
UNIVERSITA' DEGLI STUDI DI NAPOLI FEDERICO II



DIPARTIMENTO DI SANITA' PUBBLICA

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

"VALIDATION OF AN AUTOMATIC SYSTEM REAL-TIME PCR TO PERFORM PREDICTIVE MOLECULAR TESTS ON CYTOLOGICAL SAMPLES"

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Introduction

During the last years, treatment strategies in oncology have undergone considerable improvements due to a better understanding of tumour biology. In particular, a new branch of pathology, the so-called predictive molecular pathology, aims to define a better therapeutic strategy applying the basic principle "the most appropriate drug for any single patient". Thus, tumour tissue samples are analysed in molecular pathological laboratories to detect actionable genomic alterations. Thus, in each laboratory a wide range of different techniques is developed and validated, also taking care to assess continuously the quality of testing.

Technology is advancing at a rapid pace and fully automated platform are emerging for rapid molecular testing. In particular the *Idylla™* (Biocartis NV, Mechelen, Belgium) system is a fascinating technology. The system relies on a molecular diagnostic device for detection of genetic mutations based on automated quantitative allele-specific RT-PCR.[1-3] The apparatus features a sample preparation module integrated with a combined PCR thermocycling and fluorescence detection module connected to the computer-console. All consumables required to perform sample preparation and RT-PCR detection are provided in disposable cartridges that are loaded onto the *Idylla™* system to enable the simultaneous detection of up to 30 molecular targets from a variety of solid and liquid sample types, including DNA preparations from routine cytological specimens. Through microfluidic channels in the cartridge, nucleic acids are transported into five separate PCR chambers, which contain predeposited PCR reagents in dried form (i.e., primers, probes, enzymes). Closing of the cartridge after inserting the sample avoids cross contamination. The *Idylla™* method is very simple and rapid, with a time around testing approximatively two hours.

There are many types of cartridge on the market developed for many gene mutational test, already being widely adopted on histological materials.

In routine practice, molecular testing in predictive pathology laboratories is often performed on cytological, rather than on histological samples. Fine Needle Aspiration (FNA) samples are obtained by minimally invasive procedures and represent the best option in patients with advanced metastatic diseases to guide targeted therapy. The aim of this Doctorate Thesis is therefore, to validate the *Idylla™* technology on cytological samples of a number of different neoplasms such as lung, colon and pancreatic cancers. Briefly, this Thesis will focus on the validation two different *Idylla™* molecular assays on cytological samples. Cytology is versatile approach to the diagnostic evaluation of both morphology and genomic alterations of neoplastic cells. In fact, different modalities of fixation, cytopreparation and staining enable cytology to address a large number of different applications. Although versatility is a remarkable feature of cytology, the variability of different specimens types may be an issue when validating a novel molecular approach. In this Thesis, special care what taken to validate both EGFR and KRAS *Idylla™* assay on a large number of different cytological sample types, including direct smears, cytospins, liquid based cytology preparations, and cell blocks. A constant effort was spent to provide an effective and well addressed research aimed to address a relevant and important issue, the validation of the *Idylla™* assays on routine cytological samples.

The first chapter will focus on lung cancer predictive testing. Recent guidelines from the College of American Pathologists (CAP), International Association for the Study of Lung Cancer (IASLC) and the Association for Molecular Pathology (AMP) recommend epidermal growth factor (*EGFR*) testing in patients with lung adenocarcinoma, regardless of sex, race, smoking history, histological grade or other clinical risk factors.[4] If *EGFR* demonstrates an activating (sensitizing) mutation, the first-generation tyrosine kinase inhibitors (TKI) Gefitinib (Iressa®, AstraZeneca, London, UK) and Erlotinib (Tarceva®, F. Hoffmann-La Roche, Basel, Switzerland), or the second-generation TKI Afatinib (Giotrif®, Boehringer Ingelheim, Ingelheim, Germany) should be given as first-line therapy. To date, *EGFR* testing is often centralized in large laboratories, with longer testing times. In this scenario, *Idylla™* may represent a viable option to widespread *EGFR* testing even in less experienced and equipped laboratories. As it is detailed in chapter 1, this Thesis provides a large body of evidences demonstrating that *Idylla™* testing can reliably be applied to Non Small Cell Lung Cancer

(NSCLC) cytological samples, with a short time around testing and high cost-effective results. In the second chapter of this Thesis, we aimed to validate on cytological samples the *KRAS Idylla™* assay. Previous studies showed that the *Idylla™* technology is suitable for paraffin histological blocks, which is relevant since *KRAS* mutations have a remarkable clinical significance in a large number of human neoplasms. In particular, *KRAS* testing can provide both diagnostic and predictive information. As an example, the diagnosis of pancreatic cancer may be aided by the demonstration of an oncogenic *KRAS* mutation to differentiate between mucinous from non mucinous lesions.[5] Since cytological sample are often used to diagnose pancreatic masses, investigation focusing on the application of novel technologies on pancreatic cytology is relevant. In this Thesis, we demonstrated that *KRAS Idylla™* assay on may be well performed with useful results on pancreatic FNA samples.

Besides its diagnostic relevance, the detection of *KRAS* mutations have also remarkable predictive significance. In particular, *KRAS* testing is mandatory to select colon cancer patients for the therapy with Monoclonal Antibody (MoAbs) against *EGFR*, such as *Cetuximab* or *Panitumumab.* In the routine clinical setting, *KRAS* testing is usually performed on resected primary histological tumor samples of colon cancer, but the cytological sampling of the metastatic sites can be useful when there is complete response to neoadjuvant in patients with rectal cancer[6] and to serially monitor for the emergence of mutant treatment resistant clones.[7] [8] In this setting, *KRAS* testing on cytological samples is an useful opportunity and, in this Thesis, *Idylla KRAS* testing has been validated also for this application, as detailed in chapter 2.

Chapter 1

1.1 Idylla EGFR testing

The *Idylla EGFR* Mutation Assay is a single-use cartridge-based test. Via microfluidic channels in the cartridge nucleic acids are transported into five separate PCR chambers, which contain predeposited PCR reagents in dried form (ie, primers, probes, enzymes) designed for the qualitative detection of 18 types of genetic changes for which 53 different mutations have been validated. These include three different point mutations: p.G719S (c. G2155A), p.G719C (c. G2155T), p.G719A (c.G2156C) in exon 18; six different families of deletions ranging from 9 to 24 bp in exon 19; five different insertions (p.InsASV9, p.InsASV11, p.InsSVD, p.InsG, p.InsH) and two point mutations p.S768I (c.G2303T) and p.T790M (c.C2369T) in exon 20 and two point mutations p.L858R (c.T2573G) and p.L861Q (c.T2582A) in exon 21. Detection of these specific targets is performed using fluorescently labelled probes. The analytic time required to perform the results is approximately 2.5 hours, with a hands-on time of less than 2 min.

1.2 *Idylla EGFR* Mutation Assay on lung cancer cytological specimens

Testing for Epidermal Growth Factor Receptor (*EGFR*) mutations is part of the current standard of care in advanced non-small cell lung cancer.[4] The recent guidelines from the College of American Pathologists (CAP), International Association for the Study of Lung Cancer (IASLC) and the Association for Molecular Pathology (AMP) recommend *EGFR* testing in patients with lung adenocarcinoma, regardless of sex, race, smoking history, histological grade or other clinical risk factors.[4] Cytological samples are suitable for *EGFR* testing, which is crucial, since the large majority of patients present in advanced disease stages.[4 9]

In patients with acute deterioration, *EGFR* testing results should be available as soon as possible, as administration of *EGFR* antagonists as second-line agents is less efficient than their use in first-line therapy.[4] As a general recommendation, tumour specimens should be sent to testing laboratories within three working days of receiving oncologist's requests.[4] However, in a previous survey, we showed the period of time between test request and delivery of the sample is in routine practice nearly double the recommended time.[10] Laboratories may use any validated *EGFR* testing method that is able to detect mutations in cytological specimens with as little as 10% tumour cells.[4] As a matter of the fact, the competence needed to validate molecular diagnostic assays is beyond most cytopathology laboratories and, even if there was expertise, a larger number of cases is required to run in-house testing cost-effectively.[11] Therefore, the current practice is often external centralised testing. Unfortunately, this is fraught with a higher rate of inadequate samples than in-house testing;[12] in fact, the primary cytopathologist is often reluctant 'to sacrifice' the morphology of malignant cells for DNA extraction, and the smear sent to centralised laboratories is often

paucicellular.[13] Thus, ideally, *EGFR* testing should be carried out in the same centre where the patient is diagnosed.

To this end, automated allele-specific real-time PCR (RT-PCR) technology is advancing at rapid pace. In particular, the fully automated molecular diagnostics system *Idylla*™ (Biocartis, Mechelen, Belgium) is a fascinating technology.[1-3] Sample preparation is combined with PCR thermocycling and fluorescence detection of target sequences. Without need in highly skilled staff, within approximately 90 min, the European Community (CE)-in vitro diagnostic use (IVD) marked *Idylla™* mutational tests can genotype V-Raf murine sarcoma viral oncogene homolog B (*BRAF*) and Kirsten rat sarcoma viral oncogene homolog (*KRAS*) with 1% detection limit.[1-3] More recently, Biocartis developed an *Idylla EGFR* test prototype. Although, the *Idylla™* tests were designed for use with formalin-fixed paraffin-embedded (FFPE) sections, the *Idylla™* system can also process DNA preparations from cytological samples, as previously shown.[14] The aim of the present study was to assess the *Idylla™ EGFR* test performance on lung cancer cytological specimens.

1.2.1 Material and Methods

Study design

Study design is graphically shown in figure 1. Preliminarily, the *Idylla EGFR* Mutation Assay analytical sensitivity was assessed on DNA derived from cell lines. Then, *Idylla EGFR* Mutation Assay was carried out on DNA preparations previously extracted from routine cytological samples (n=76) quantified (ng/µL) and tested for clinical reporting by in-house PCR-based methods (fragment length and TaqMan assays) as previously described.[15] In cases of discrepancies, next generation sequencing was used as an orthogonal technique, following our previously validated protocol.[16] In 17 cases, an additional direct smear was available and *Idylla EGFR* Mutation Test was also carried out directly on cytological material exploiting the whole *Idylla™* system workflow, as explained below.

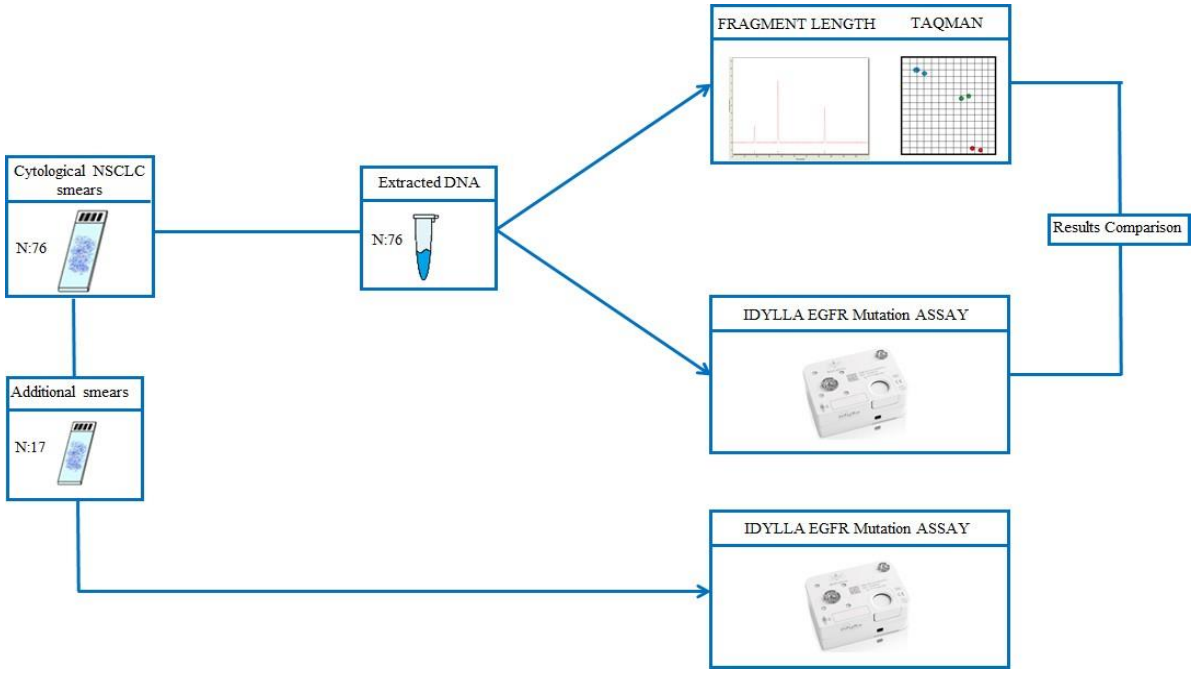


Figure 1. Study design: *Idylla EGFR* Mutation Assay was carried out on DNA preparations previously extracted from routine cytological samples (n=76) and compared with in-house validated PCR-based methods (fragment length and TaqMan assays), In 17 cases, an additional direct smear was available and *Idylla EGFR* Mutation Test was also carried out directly on cytological material exploiting the whole *Idylla*™ system workflow. NSCLC, non-small cell lung cancer.

Analytical sensitivity assessment

The limit of detection of the *Idylla EGFR* prototype assay was assessed by cell line dilution studies. The mutated PC9 (harbouring *EGFR* p.E746-A750del) and H1975 (carrying L858R point mutation) and the A549 (*EGFR* wt) cell lines were serially mixed at dilutions of 25%, 10% and 1%.

Sample series

We retrospectively selected from our archive 32 patient DNA samples by the following criteria: (1) the presence of *EGFR* exon 19 deletion or exon 21 L858R mutation

assessed by our laboratory validated assay (our laboratory is registered in the Italian Society of Pathology *EGFR* external quality control scheme, http://www.egfrquality.it) and (2) the availability of a sufficient (10 ng) amount of stored genomic DNA. Overall, 25 cases harboured an exon 19 deletion, whereas 7 cases carried the L858R point mutation. To ensure specificity of the *Idylla EGFR* prototype assay, a total of 44 additional *EGFR* wild-type DNA samples extracted from routine cytological specimens were selected. Overall, a total of 65 cases had ≥20% of neoplastic cells, whereas 11 cases had <20% of cancer cells (table 1).

Case	% Neoplastic cells	DNA (ng/µL)	Reference method	Idylla
1	80	60	WT	WT
2	60	60	L858R	L858R
3	40	60	WT	WT
4	50	7.15	DEL19	DEL15
5	20	6.9	DEL19	DEL15
6	70	60	WT	WT
7	30	4.94	DEL19	DEL18/21b/24
8	10	1.75	WT	WT
9	80	60	WT	WT
10	10	6.56	WT	WT
11	50	5.69	WT	WT
12	40	16.2	L858R	L858R
13	30	2.3	WT	WT
14	15	12.9	WT	WT
15	25	6.33	DEL19	DEL15
16	50	16	WT	WT
17	80	8.8	WT	WT
18	25	5.71	WT	WT
19	40	16.9	DEL19	DEL15
20	5	26.3	WT	WT
21	60	11.3	DEL19	DEL15
22	25	4.79	WT	WT
23	25	4.96	WT	WT
24	23	21.2	WT	WT
25	60	26.1	L858R	L858R
26	50	8.3	DEL19	DEL18/21b/24
27	30	7.05	WT	WT
28	50	1.99	WT	WT
29	25	5.86	WT	WT
30	25	0.5	WT	INVALID
31	50	15.3	DEL19	DEL15
32	30	10.9	WT	G719A
33	50	16.8	WT	WT
34	5	3.5	WT	DEL15
35	50	5.21	DEL19	DEL9/21 a
36	50	6.14	WT	WT
37	60	13.7	WT	WT
38	25	3.71	WT	WT
39	25	1.58	L858R	L858R
40	10	4.08	WT	DEL15

Continued

Table 1 Continued

Case	% Neoplastic cells	DNA (ng/µL)	Reference method	Idylla
40	10	4.08	WT	DEL15
41	30	8.61	WT	WT
42	20	7	WT	WT
43	50	4.57	WT	WT
44	5	14.7	WT	WT
45	5	0.28	WT	WT
46	20	1.21	WT	WT
47	20	2.65	WT	WT
48	50	3	DEL19	DEL15
49	70	6.5	DEL19	DEL15
50	25	9	L858R	L858R
51	50	5.19	DEL19	DEL15
52	50	10	DEL19	DEL15
53	70	5.7	DEL19	DEL9/21 a
54	30	2.6	DEL19	DEL12
55	30	2.7	DEL19	DEL15
56	50	0.2	L858R	L858R
57	30	5.2	DEL19	DEL15
58	5	1.21	DEL19	DEL15
59	20	1.65	WT	WT
60	60	9.07	WT	INVALID
61	50	36.4	WT	WT
62	30	2.87	WT	G719C
63	30	1.29	DEL19	DEL15
64	5	3.02	DEL19	DEL15
65	50	20.8	DEL19	DEL15
66	40	22.4	L858R	L858R
67	50	2.63	DEL19	DEL9/21 a
68	20	5	DEL19	DEL9/21 a
69	20	60	WT	WT
70	25	4.97	WT	WT
71	50	5.15	DEL19	DEL15
72	25	8	DEL19	DEL15
73	40	60	WT	WT
74	30	20.8	WT	WT
75	50	0.5	WT	WT
76	10	5.4	WT	WT

Table 1. *Idylla EGFR* Mutation Assay performance on archival extracted DNA.
A series of 76 archival DNAs extracted from lung adenocarcinoma cytological slides is reported. In all cases, the neoplastic cell percentage and the DNA concentration (ng/µL) were assessed. EGFR mutational status, previously tested by our laboratory reference method (fragment length and TaqMan assays), was re-evaluated by Idylla.
DEL15, DEL18/21b/24, DEL9.21a: Idylla result reporting the different families of EGFR exon 19 deletions.

These latter smears had been wholly scraped, since featured a stochastic distribution of malignant and non malignant cells, which precluded tumour component enrichment by manual microdissection. Of these, 59 were Papanicolaou and 17 were Diff-Quik. Archived DNA was processed by the *Idylla EGFR* Prototype Mutation Test between November 2015 and March 2016. As previously shown, we have directly pipetted 10 ng of extracted genomic DNA in the prototype *EGFR* cartridge. A total of 17/76 study cases featured either an air-dried Diff-Quik or a Papanicolaou stained cytological slide, in addition to slides that were previously used for DNA extraction. Any cytological slide was microscopically reviewed, annotating the neoplastic cell percentage and incubated in xylene for 3 days to allow coverslip removing. Each slide was dried and wholly scraped into an Idylla *EGFR* cartridge.

Analysis of collected fluorescent signals

Conversely to the *Idylla BRAF* and *KRAS* mutation tests*, where Idylla™* console auto-analyses the PCR curve to determine the presence or absence of a mutation and the results are presented on screen as either 'no mutation detected' or 'mutation detected',[1-3] the raw data obtained by the *Idylla EGFR* prototype on both DNA preparations and scraped cells were centrally analysed at Biocartis; this analysis was blind to the results previously obtained by our standard reference method. In principle, a cycle value (Cq) was calculated by *Idylla* beta version software for every PCR curve. The presence of a mutant genotype was determined by calculating the difference between the control and sample Cq (ΔCq). The mutant signal is considered valid if the ΔCq is within a validated range. All samples with a valid wild-type signal but a ΔCq value outside the validated range were characterised as *EGFR* mutation negative. Cases showing invalid results were further investigated to assess the reason behind the failure by evaluating DNA quality by a microfluidic platform based on electrophoretic system 4200 TapeStation (Agilent, Santa Clara, California, USA).

1.2.2 Results

Idylla EGFR prototype sensitivity was evaluated on cell line-derived DNA. As reported in the methods section, we created dilutions down to 1% for both exon 19 and 21 *EGFR* mutations in wild-type DNA background. In any of the dilutions tested, the presence of *EGFR* mutant alleles was detected by the *EGFR Idylla* prototype. The *Idylla™* results relative to the analysis of 76 cytology samples derived archival DNAs were compared with those obtained by our laboratory validated assays and reported in table 1 (figure 2).

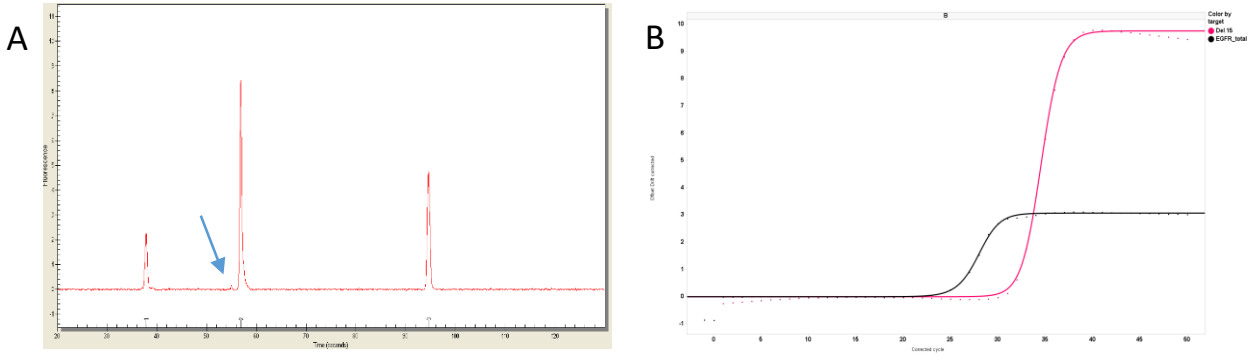
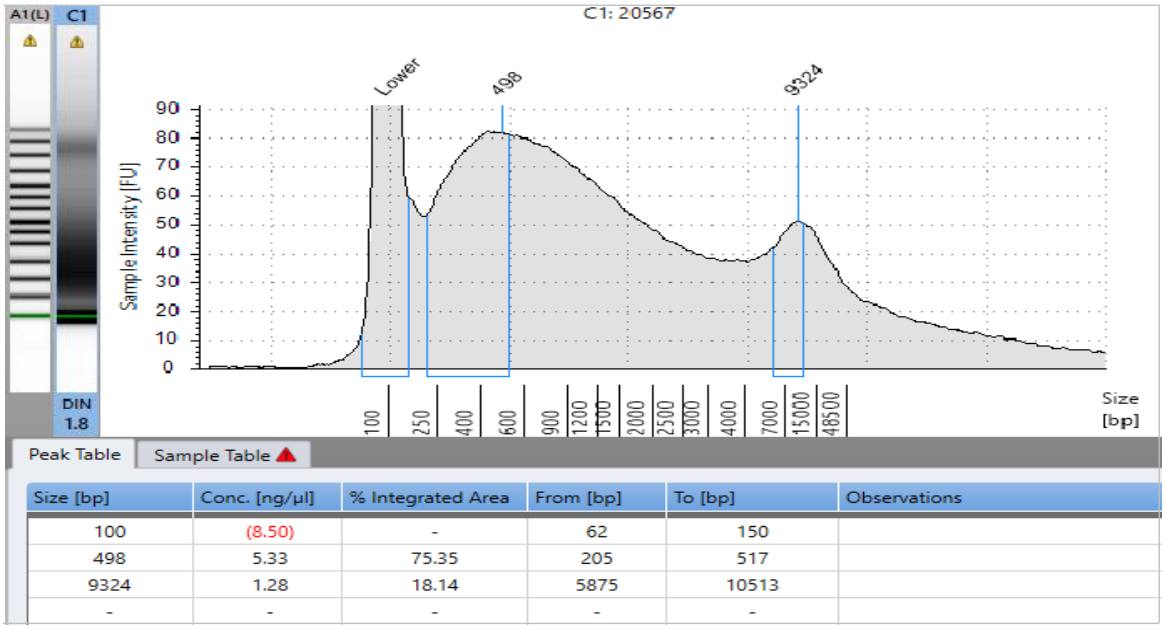


Figure 2. Discordance between standard reference method and *Idylla*™ relative to epidermal growth factor receptor exon 19. Case #40. (A) Fragment length electropherogram showing a clear wild-type allele peak and an additional very low peak of uncertain significance (B), Del15 mutation was detected by using the Idylla™ assay; note that two curves are detectable, one corresponding to wild-type allele and the other one to Del15-mutated allele.

Briefly, *Idylla™* yielded valid results in 74/76 (97.3%) samples in a first run. The two invalid samples, showing highly degraded DNA (figure 3), were excluded from the analysis.



Size [bp]	Conc. [ng/µl]	% Integrated Area	From [bp]	To [bp]	Observations
100	(8.50)	-	62	150	
498	5.33	75.35	205	517	
9324	1.28	18.14	5875	10513	
-	-	-	-	-	

Figure 3. Archival DNA from a case showing epidermal growth factor receptor *Idylla*™ invalid result (#60) further investigated by a microfluidic platform based on electrophoretic system (4200 TapeStation, Agilent) to assess the reason behind the failure. Note that the electrophoretic profile features several peaks due to DNA fragmentation.

All 32 mutant cases were confirmed by *Idylla™*, showing a sensitivity of 100%. In addition, in the group of 44 wild-type samples by our standard techniques, *Idylla™* gave a discordant result in four cases. In particular, *EGFR Idylla* prototype revealed in cases 34 and 40 an *EGFR* exon 19 deletion, whereas cases 32 and 62 featured a G719X exon 18 point mutation, not covered by our standard reference method. Only these two latter cases could be further investigated by Next Generation Sequencing (NGS), as cases 34 and 40 did not have sufficient residual DNA. In cases 32 and 62, the presence of G719A and G719C mutations was confirmed by NGS analysis. In 17/76 cases, an additional air-dried Diff-Quik stained cytological slide was available, and the *EGFR Idylla* prototype was carried out also by scraping cytological material directly in the cartridge. When the results were compared with those obtained on matched archival DNA, most of the cases (15/17) gave concordant results. Only two cases showed a discordancy, featuring an exon 19 deletion (case n. 15) and an L858R (case n. 25) point mutation detected only by *EGFR Idylla* prototype on archival DNA. Since *Idylla™* system does, at the moment, not allow the withdrawal of extracted DNA from the cartridge, the discrepancies between archival DNA and directly scraped cytological material could not be further assessed.

1.2.3 Discussion

Our data demonstrate that the *Idylla EGFR Mutation Assay* is a highly sensitive method that can consistently detect *EGFR* mutations with as little as 1% mutant DNA in a wild-type background, as shown in cell line studies. Thus, the *Idylla EGFR Mutation Assay* meets the CAP/IASLC/AMP guideline strong recommendation to use methodologies able to detect *EGFR* mutations in specimens with only 10% cancer cells.[4] This requirement is particularly appropriate when testing cytological samples that often feature a stochastic distribution of malignant and non-malignant cells, which precludes tumour component enrichment by manual microdissection.[9] In our series, the *EGFR Idylla* prototype showed a good performance, enabling in 100% of cases (32/32) the *EGFR* mutational status confirmation. In addition, this assay was able to resolve cases with low intensity peaks on the fragment assay electropherograms of the uncertain technical interpretation but suggestive of clinical significance. In particular, cases n. 34 and 40, reported as negative by our reference methods were defined as mutant by *EGFR Idylla* prototype, as illustrated in figure 2, probably reflecting a higher sensitivity level. In addition, while these latter techniques cover only exon 19 deletions and L858R mutation, *EGFR Idylla* prototype has a broader reference range, as shown by cases 32 and 62, in which *Idylla™* detected G719X exon 18 point mutations, also confirmed by our validated NGS approach. Noteworthy, the CAP/IASLC/AMP guideline strongly recommends to extend the *EGFR* mutational assessment also to exons 18 and 20 for the most common mutations[4] and for lower prevalence variants, whose response to *EGFR* antagonists may differ.[17]

Besides a high analytical sensitivity and a large reference range, the main advantage of the *Idylla* system is the possibility to extend the feasibility of *EGFR* testing to smaller laboratories, fully exploiting the automated workflow, which reduces the time required to genotype DNA for EGFR mutational status assessment in approximately 2.5 hours, compensating for the high cost of a single cartridge (~ €200). Until recently, the automated *Idylla* tests for mutation detection in predictive molecular pathology were limited to

histological samples. This reflected the manufacturer design to process FFPE sections. We here showed that the *Idylla EGFR* prototype test was able to analyze also cytological samples, which is crucial since these specimens represent the most common available material to select patients for targeted treatments in clinical practice. In addition, we also showed that only 10 ng of archival DNA, directly pipetted into the cartridge, is sufficient to obtain results in most samples (74/76). Only two cases showing marked DNA degradation (figure 3) yielded an invalid result.

The demonstration that *Idylla™* reliably processes extracted DNA suits cytological practice, where often only a single slide is available. In this study, we also investigated the possibility to directly scrape cytological material from de-coverslipped archival smears into the cartridge; in fact, the sample preparation module of *Idylla™* system uses high-frequency intensity focused ultrasound technology to enable automated DNA extraction. This enables performing mutation testing even without the basic expertise and equipment to perform DNA extraction and purification. While previous studies showed that a full concordance can be obtained between FFPE sections and extracted DNA, in this study, when scraping cytological material directly into the cartridge, cases 15 and 25 gave a false-negative result. Since in both cases the scraped material contained a tumour cell percentage sufficient for *EGFR* mutational status assessment (table 2), it is conceivable that the original cytological staining might have interfered with the detection of the fluorescence being developed during target amplification.

Case	% Neoplastic Cells	Idylla
15	25	WT
20	5	WT
22	25	WT
23	30	WT
24	25	DEL 9/21a
25	50	WT
27	20	WT
33	25	WT
35	10	DEL 9/21a
40	10	DEL 15
41	40	WT
42	20	WT
43	50	WT
44	5	WT
45	5	WT
46	25	WT
47	15	WT

Table 2. *Idylla EGFR* Mutation Assay performance on scraped cytological samples.
A group of 17 study cases featured an archival cytological slide, in addition to slides previously used for DNA extraction. Any cytological slide was microscopically reviewed, annotating the neoplastic cell percentage and wholly scraped into an *Idylla EGFR* cartridge.

Thus, further technological refinements are needed to better adapt the automated extraction modalities to stained cytological material. However, the possibility to use directly the extracted DNA may overcome the limitations in number of smears and abundance of material, inherent to cytological specimens. Furthermore, aliquoting of the extracted DNA may offer the opportunity to perform additional biomarker testing and to run an orthogonal technique for result confirmation.

In conclusion, we showed that the fully automated molecular diagnostics system *Idylla™* represents a promising option for *EGFR* mutation testing on cytological samples of lung cancer to enable taking, also in less experienced laboratories, very rapid treatment decisions with high sensitivity, large reference range and ease of use.

Chapter 2

2.1 *Idylla KRAS* testing

The *Idylla™ KRAS* mutation test is a single-use cartridge-based test designed for the qualitative detection of 21 *KRAS* mutations and including *KRAS* total (amplification region off the mutational *hot-spot* acting as sample processing control). Seven mutations are targeted in exon 2: p.G12C (c.34G>T), p.G12R (c.34G>C), p.G12S (c.34G>A), p.G12A (c.35G>C), p.G12D (c.35G>A), p.G12V (c.35G>T) and p.G13D (c.38G>A). Nine mutations can be detected in exon 3: p.A59E (c.176C>A), p.A59G (c.176C>G), p.A59T (c.175G>A), p.Q61K (c.181C>A; c.180_181TC>AA), p.Q61 L (c.182A>T), p.Q61R (c.182A>G), and p.Q61H (c.183A>C; c.183A>T); whereas five mutations are targeted in exon 4: p.K117N (c.351A>C; c.351A>T), p.A146P (c.436G>C), p.A146T (c.436G>A), and p.A146V (c.437C>T). The assay has been designed by the manufacturer to process 5–10 µm formalin-fixed paraffin-embedded (FFPE) tissue sections or FFPE slices. The *Idylla KRAS* mutation test approved for *in vitro* diagnostic use by the European Community (CE-IVD) marked can genotype *KRAS* with 5% detection limit (LOD). The fully automated analysis required approximately 2 h. The *Idylla™* console autoanalyzed the PCR curve to determine the presence or absence of a *KRAS* mutation and the results were presented on screen as either “no mutation detected” or “*KRAS* mutation detected”, indicating the specific mutation.

2.2 *Idylla KRAS* Mutation test on pancreatic aspirates

Endoscopic ultrasound-guided fine-needle aspiration (EUS-FNA) plays a crucial role in the management of pancreatic neoplastic lesions.[18] More than 85% of pancreatic adenocarcinoma (PDAC) are diagnosed by EUS-FNA in an advanced stage when surgical pathological examination is precluded.[19] As a general rule, EUS-FNA is a rapid, safe and cost-effective procedure.[20] However, in up to 20% of cases, well-differentiated PDAC cannot reliably be distinguished from benign diseases such as chronic pancreatitis (CP) especially in its pseudo-tumoral form.[20] In particular, EUS-FNA is less accurate, when PDAC is small, well vascularised or desmoplastic.[21] Similarly, in patients with pancreatic cysts presenting for EUS evaluation, cyst fluid cytological examination has high specificity for malignancy but lacks sensitivity,[22] not always being able to distinguish between benign cysts and premalignant or malignant mucinous cysts.[5]

KRAS mutations represent an early genetic event in PDAC pathogenesis.[23] Even if molecular biology cannot replace cytology, the presence of a *KRAS* mutation in an inconclusive EUS-FNA specimen taken from a solid lesion suggests malignancy, reducing the false-negative (FN) rate by 55.6%, according to a recent meta-analysis;[24] conversely, the presence of wild-type *KRAS* may be evocative of benignity.[24 –27] However, the presence of *KRAS* mutations is not entirely specific, occurring in a minority of patients with CP[27] and in preinvasive dysplastic lesions.[28] Since EUS-FNA smears often feature contaminating benign gastrointestinal cells, *KRAS* testing requires the use of a high analytically sensitive molecular technique.

To date, real-time PCR (RT-PCR) assays have mostly been designed to target only exon 2 'hot-spot' mutations[25 29] Conversely, next-generation sequencing (NGS) ensures analytical sensitivity similar to that of mutation-specific assays, allowing for the detection of common and uncommon mutations, including those of *KRAS* exons 3 and 4.[29] However, the NGS procedure requires a complex validation procedure,[30] being cost-effective only in large volume centralised laboratories.[31] The *Idylla KRAS* Mutation Test (Biocartis, Mechelen,

Belgium) recently received CE-IVD certification for the detection of 21 mutations in codons 12 and 13 (exon 2), 59 and 61 (exon 3) and 117 and 146 (exon 4) of the *KRAS* gene, with a validated 5% limit of detection. This test, based on allele-specific RT-PCR and performed on the fully automated *Idylla™* platform, provides sample to result functionality, enabling method standardization even in those diagnostic units without molecular expertise and infrastructure.[1] [3] The aim of this study was to validate this novel technology on indeterminate pancreatic EUS-FNA.

2.2.1 Materials and Methods

Selection of cases

In this retrospective study, the clinical performance of the *Idylla KRAS* test was assessed on archival DNA from a well-characterized series of EUS-FNAs that had already been tested for *KRAS* mutational status in a prior study by using three different techniques —Sanger sequencing, Allele Specific Locked Nucleic Acid PCR (ASLNAqPCR) and 454 Next Generation Sequencing (454-NGS)—as previously reported.[29] Details regarding the modality of EUS-FNA sample collection and specimen handling and preparation for microscopic observation have been described.[29] Briefly, in any single case an aliquot of the aspirated material, besides microscopic slide preparation, had directly been collected in a tube containing 100% ethanol for *KRAS* analysis ('direct' EUS-FNA material). Specimens were stored at room temperature for a period of up to two weeks and, then, DNA was extracted, as previously described.[29] The criterion for including samples in this study, aiming to assess *Idylla KRAS* test performance, was the availability of at least 20 mL of archival DNA. Overall, a total of 52 cases were selected to undergo *Idylla KRAS* test. Results were compared with those previously obtained by Sanger sequencing, ASLNAqPCR and 454-NGS. Archived cases were processed with *Idylla KRAS* Mutation Test between October and December 2015.

The assay has been designed by the manufacturer to process 5–10 µm formalin-fixed paraffin-embedded (FFPE) tissue sections or FFPE slices. However, for other applications disposable cartridges have been applied to liquid sample types, including swab, blood, urine,

stool, sputum and tissue.[32] To perform the analysis on the archival EUS-FNA DNA, 3 µL (corresponding to 1/10th of a DNA preparation from the aspirate sample) of extracted genomic DNA was directly pipetted inside an *Idylla KRAS* Mutation Test cartridge. The cartridge was loaded onto the *Idylla™* system for processing. Via microfluidic channels in the cartridge, nucleic acids are transported into five separate PCR chambers, which contain pre-deposited PCR reagents in dried form (i.e., primers, probes, enzymes) for the analysis of the sample DNA that includes *KRAS* Total (wild-type gene acting as Sample Processing Control). Detection of these specific targets is performed using fluorescently labelled probes. The *Idylla™* console auto-analyses the PCR curve to determine the presence or absence of a *KRAS* mutation and the results are presented on screen as either 'no mutation detected' or '*KRAS* mutation detected'.[33] A quantification cycle value (Cq) value is calculated by *Idylla™* software for every valid PCR curve. The presence of a mutant genotype is determined by calculating the ΔCq, that is, the difference between the *KRAS* wild-type Cq and the cut-offs for individual mutations. Mutant signal is considered valid if the ΔCq is within a validated range, and the sample will then be characterised as *KRAS* mutation positive, indicating the specific mutation. Noteworthy, the *Idylla KRAS* Mutation Test does not report double mutants, and in these cases, only the mutation with smallest ΔCq is called. All samples with a valid wild-type signal but a ΔCq value outside the validated range are characterized as 'no mutation detected'.

Statistical measures of clinical performance

Idylla™ clinical performance was evaluated according to the final end point. This was represented by cytological, histological or, in cases of inoperable neoplasms, by clinical features. On this basis, we distinguished three different categories of lesions: (i) benign lesions, including non-neoplastic or benign cysts and a pancreatitis; (ii) adenocarcinomatous lesions or precursor lesions of adenocarcinoma, including PDAC, intraductal papillary mucinous neoplasms (IPMNs) and inoperable neoplasias with overt malignant clinical features; (iii) not adenocarcinomatous lesions, including pancreatic neuroendocrine tumour (pNET) and solid pseudopapillary tumours (SPPTs). *Idylla* clinical performance was assessed by evaluating its clinical sensitivity, specificity, negative predictive value (NPV), positive predictive value and accuracy also in comparison to Sanger sequencing, ASLNAqPCR and

454-NGS performance. True positives were cases that showed a mutation in *KRAS* and that were PDAC, inoperable neoplasias or IPMNs according to final end point. False positives were cases in which a mutation was found but with a 'benign' end point or else diagnosed as SPPT or pNET. True negatives were cases with a wild-type *KRAS* result and a 'benign' end point or with an end point of neuroendocrine or pseudopapillary neoplasia. FNs were cases with a wild-type *KRAS* but were PDAC/inoperable neoplasias or IPMN at the final end point. Comparisons between clinical sensitivities were performed according to recommendations previously described.

2.2.2 Results

A total of 52 archival DNA from EUS-FNA pancreatic samples was tested at the University of Naples Federico II with the *Idylla KRAS* Mutation Test. Representative examples of PCR curves and the corresponding Cq values for different *Idylla™* analysis results are shown in figure 4.

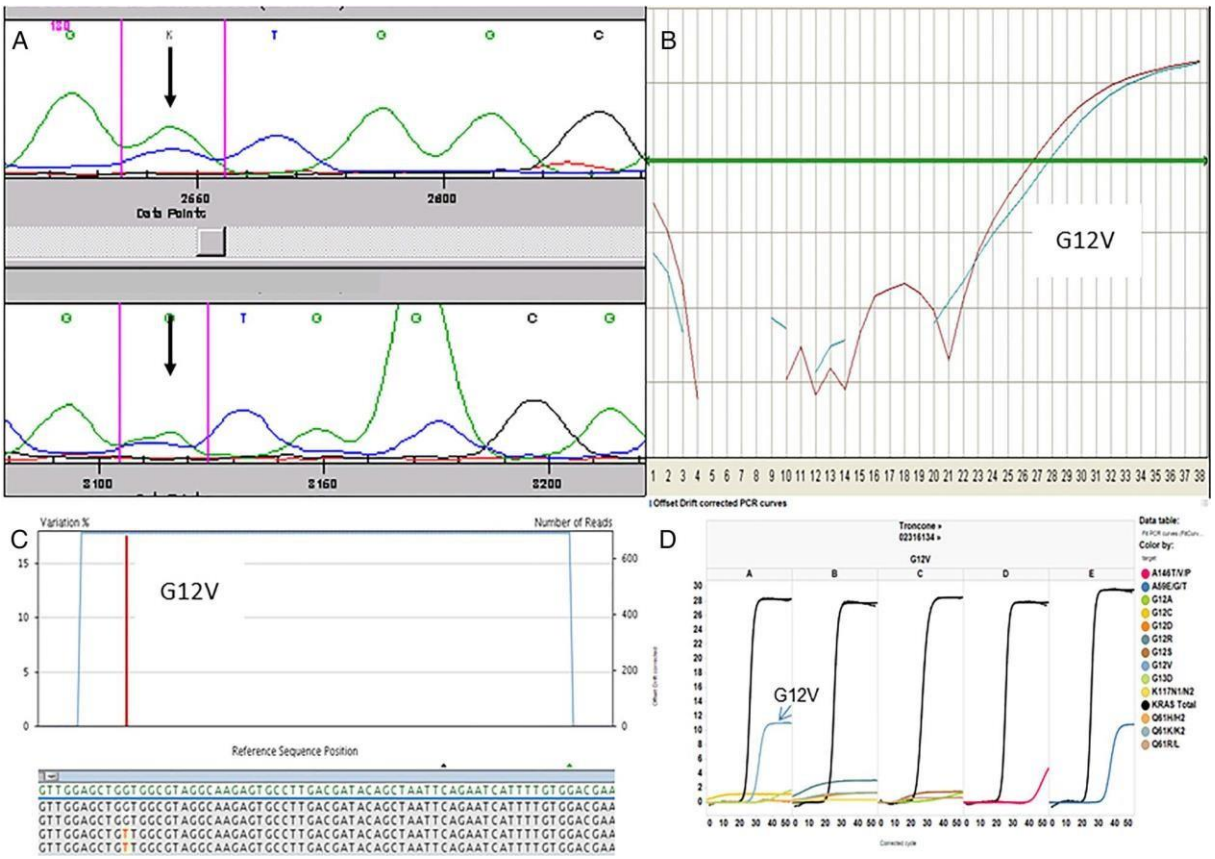


Figure 4. Example of concordant molecular results in a *KRAS* exon 2 mutated sample.
(A) Electropherogram obtained using Sanger sequencing. The *KRAS*-G12V mutation (c.35G>T) is pointed by an arrow. (B) *KRAS*-G12V mutation was also detected using Allele Specific Locked Nucleic Acid PCR assay: two curves are detectable, one corresponding to wild-type allele and the other one to G12V-mutated allele. (C) Profile obtained using 454-Next Generation Sequencing. The *KRAS*-G12V mutation is identified by the vertical red bar. The percentage of mutated alleles is indicated on the left y-axis while the total number of reads on the right one. (D) Representative examples of *Idylla*™ analysis result showing a *KRAS*-G12V mutation detection call.

The results were compared with the original assessments made by Sanger sequencing, ASLNAqPCR and 454-NGS at the University of Bologna (table 3).

N°	Cytological diagnosis	Final diagnosis (Cytology+hystology)	Age	Gender	454-NGS	%	ASLNAq PCR	SANGER	IDYLLA
1	C5	PDAC	54	M	WT		WT	WT	WT
2	C5	PDAC	84	M	G12R	22.0	G12R	G12R	G12R
3	C5	Inoperable neoplasm	54	F	WT		WT	WT	WT
4	C5	PDAC	76	F	G12D/G12V	28.5 / 1	G12D/G12V	G12D	G12D
5	C5	PDAC	76	M	G12D/G12V	21 / 15	G12D/G12V	G12D/G12V	G12V
6	C5	PDAC	75	M	G12V/Q61H	19.2 / 11.3	G12V	G12V	Q61H
7	C5	pNET	66	M	WT		WT	WT	WT
8	C5	PDAC	61	M	G12D	19.3	G12D	WT	G12D
9	C5	PDAC	47	F	Q61H	15.0	WT	WT	Q61H
10	NA	IPMN	68	F	G12V	1.0	G12V	WT	WT
11	C5	PDAC	58	M	G12V	32	G12V	G12V	G12V
12	C5	Inoperable neoplasm	71	M	G12V	21.2	G12V	G12V	G12V
13	C5	Inoperable neoplasm	62	M	G12D	3.0	G12D	WT	G12D
14	C5	pNET	79	M	WT		WT	WT	WT
15	C5	pNET	58	F	WT		WT	WT	WT
16	C5	pNET	46	F	WT		WT	WT	WT
17	C5	pNET	61	M	WT		WT	WT	WT
18	C4	PDAC	68	F	WT		WT	WT	WT
19	C1c	IPMN	63	F	Q61R	1.1	WT	WT	WT
20	C5	PDAC	75	F	G12V	3.7	G12V	WT	G12V
21	C1c	Benign cyst	46	F	WT		WT	WT	WT
22	NA	IPMN	60	F	Q61H	1.4	WT	WT	WT
23	NA	NA	64	F	G12C/G12V	2.6 / 2	G12C/G12V	WT	G12C
24	C1c	NA	72	F	G12V	27	G12V	G12V	G12V
25	C2	Pancreatitis	43	M	WT		WT	WT	WT
26	C1c	IPMN	71	M	G12V	20.0	G12V	G12V	G12V
27	C1c	IPMN	71	M	Q61H	4.3	WT	WT	WT
28	C1c	Benign cyst	80	M	WT		WT	WT	WT
29	C1c	Benign cyst	81	F	WT		WT	WT	WT
30	C1c	Benign cyst	49	F	WT		WT	WT	WT
31	C1c	Benign cyst	64	F	WT		WT	WT	WT
32	C1s	Inoperable neoplasm	84	F	WT		WT	WT	WT
33	C1c	IPMN	73	M	Q61H	2.0	WT	Q61H	Q61H
34	C1c	IPMN	76	M	G12V	23.0	G12V	G12V	G12V
35	C3	Inoperable neoplasm	78	F	G12R	23.0	G12R	G12R	G12R
36	C1c	NA	75	F	G12V	2.7	G12V	WT	WT
37	C2	Benign cyst	73	M	WT		WT	WT	WT
38	C2	Benign cyst	53	M	WT		WT	WT	WT
39	C2	Benign cyst	73	F	WT		WT	WT	WT
40	C5	SPPT	47	F	WT		WT	WT	WT
41	C5	SPPT	17	M	WT		WT	WT	WT
42	C4	PDAC	78	F	Q61L	32.0	WT	Q61L	Q61R/L
43	C4	PDAC	69	F	WT		WT	WT	WT
44	C4	Inoperable neoplasm	66	F	G12D	33.9	G12D	G12D	WT
45	C4	PDAC	72	M	G12D	4.1	G12D	WT	WT
46	C1c	IPMN	72	F	WT		WT	WT	WT
47	NA	NA	41	F	WT		WT	WT	WT
48	C4	PDAC	67	F	G12R	12.0	G12R	WT	G12R
49	C3	PDAC	50	F	WT		WT	WT	WT

Table 3. Series of 49 endoscopic ultrasound-guided fine-needle aspiration tested for *KRAS* mutational status by using four different techniques: Allele Specific Locked Nucleic Acid PCR, 454 Next Generation Sequencing (454-NGS), Sanger sequencing and *Idylla KRAS* test. % indicates percentage of mutant allele by 454-NGS. Cytological evaluation is reported as unsatisfactory (C1), negative for malignancy (C2), atypical cells present (C3), suspicious for malignancy (C4) or positive for malignancy (C5), (NA) not available.

Idylla™ yielded valid results in 47/52 samples in a first run. The five invalid samples underwent a second run by increasing the DNA input up to 6 µL. Two cases gave a valid call, for a total of 49/52 (94.2%) adequate *Idylla KRAS* Mutation tests. *KRAS* mutation detection by four different techniques according to preoperative cytology evaluation and clinicopathological end points is reported in table 4.

Cytology diagnosis (number of cases)	**454-NGS**	**End point of mutated samples**	**ASLNAqPCR**	**End point of mutated samples**	**Sanger**	**End point of mutated samples**	**Idylla**	**End point of mutated samples**
C1 (14)	7 (50%)	5 IPMN 2 NA	4 (28%)	2 IPMN 2 NA	4 (28%)	3 IPMN 1 NA	4 (28%)	3 IPMN 1 NA
C2(4)	0		0		0		0	
C3(2)	1(50%)	Inoperable neoplasms	1(50%)	Inoperable neoplasms	1(50%)	Inoperable neoplasms	1(50%)	Inoperable neoplasms
C4(6)	4(67%)	3 PDAC 1 inoperable neoplasm	3(50%)	2 PDAC 1 inoperable neoplasm	2(33%)	1 PDAC 1 inoperable neoplasm	2(33%)	2 PDAC
C5 (19)	10 (52%)	8 PDAC 2 inoperable neoplasms	9 (47%)	7 PDAC 2 inoperable neoplasms	6 (31%)	5 PDAC 1 inoperable neoplasm	10 (52%)	8 PDAC 2 inoperable neoplasms
NA (4) Total (60)	3 (75%)	2 IPMN 1 NA	2 (30%)	1 IPMN 1 NA	0		1 (25%)	1 NA

Table 4. *KRAS* mutation detection by four different techniques according to preoperative cytology evaluation and clinicopathological endpoints. Cytological evaluation is reported as unsatisfactory (C1), negative for malignancy (C2), atypical cells present (C3), suspicious for malignancy (C4) or positive for malignancy (C5), (NA) not available.

Briefly, *KRAS* mutations were found by *Idylla™* in the 4/14 (28.6%) of inadequate samples (C1), in one of the two cases (50.0%) with atypical cells (C3), in 2/6 (33.3%) of the cases suspect for malignant neoplasia (C4) and in the 10/19 (52.6%) of samples diagnosed as malignant (C5). None of the benign (C2) cases showed *KRAS* gene mutations, and they were benign cysts (three cases) or pancreatitis (one case) on follow-up. One of four cases without available material for cytological evaluation was mutated for *KRAS* by *Idylla KRAS* Mutation Test. Considering the final end point information available in 45 cases, we detected a *KRAS* mutation in 16/29 (55.2%) adenocarcinomatous and pre-neoplastic lesions (in 10/15 of PDAC, 3/8 of IPMNs and in the 3/6 of inoperable neoplasms), while no *KRAS* mutations were

observed in not adenocarcinomatous or in benign lesions. In 15/49 (30.6%) cases, discordant results in *KRAS* mutational status were obtained using at least one of the four different techniques, as reported in table 3.

Discordant KRAS results in relation to mutant allele abundance

To evaluate the reason for discordant results among different techniques (15/49), the rate of mutant allele (MA) yielded by 454-NGS was taken into account when evaluating methods performance. In five discordant cases (#8, #9, #19, #42, #44 and #48) the MA abundance, as detected by 454-NGS was >10%. In cases #8 (MA=19%) and #48 (MA=12%), *KRAS* exon 2 gene mutations were missed by Sanger sequencing. Cases #9 (MA=15%) and #42 (MA=32%) harboured a mutation in *KRAS* exon 3, which was undetectable by ASLNAqPCR and missed in one instance (case #9) by Sanger sequencing. In case #44 (MA=34%), *KRAS* G12D mutation was missed by *Idylla KRAS* Mutation Test. In 10 discordant cases (#10, #13, #19, #20, #22, #23, #27, #33, #36 and #45), <5% of MA was identified by 454-NGS. A representative example of discordant results (case #19) is shown in figure 5.

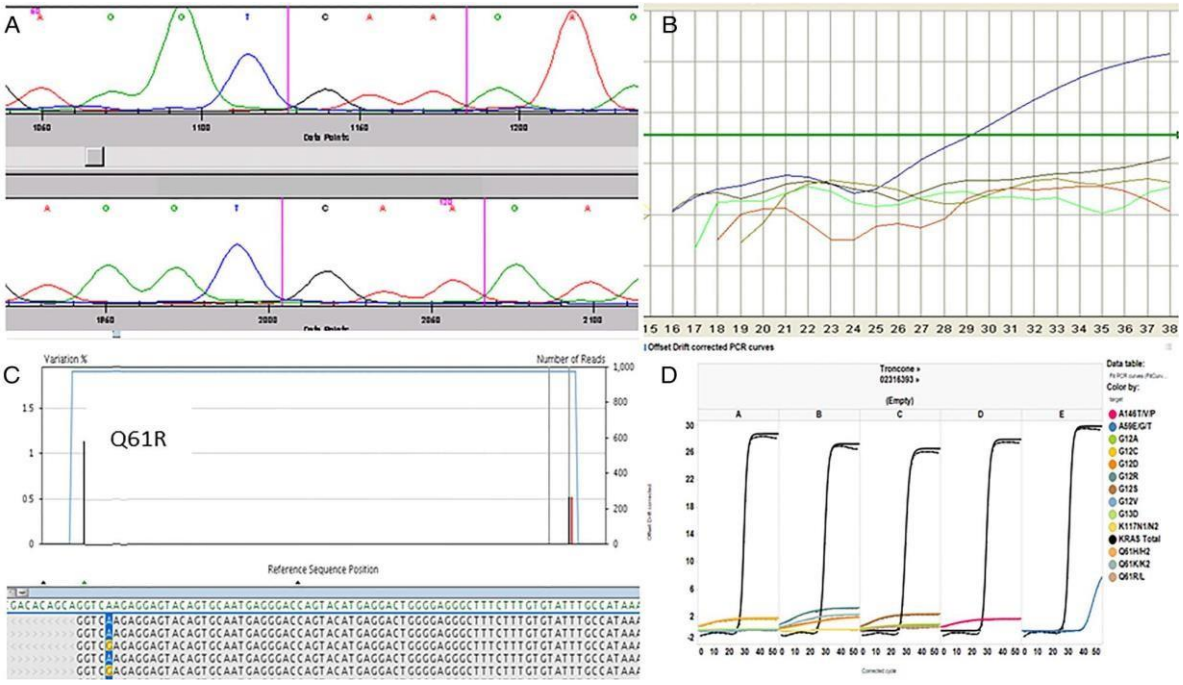


Figure 5. Example of discordant molecular results in a *KRAS* exon 3 mutated sample.
(A) Electropherogram obtained using Sanger sequencing, showing a native nucleotide sequence. Codon 61 is marked by two pink vertical bars. (B) No KRAS mutation (codons 12–13) was detected using Allele Specific Locked Nucleic Acid PCR assay: only the curve corresponding to the wild-type allele is visible. (C) Profile obtained using 454-Next Generation Sequencing. The KRAS-Q61R mutation is identified by the vertical black bar. The percentage of mutated alleles is indicated on the left y-axis while the total number of reads on the right one. (D) The KRAS-Q61R mutation was not detected by Idylla.

Only case #33 was detected by Sanger sequencing, whereas ASLNAqPCR confirmed 454-NGS results, not considering cases #19, #22, #27 and #33 harbouring an exon 3 *KRAS* mutation that was undetectable by this technique. Also, 4 of 10 low-abundant discordant cases were detected by *Idylla™*. In particular, in four cases that showed between 3% and 5% of MA (#13, #20, #27 and #45), two mutant cases (#13 and #45) were detected by *Idylla KRAS* Mutation Test. Out of two cases that showed between 2% and 3% of MA (#23 and #36), one mutant case (#23) was detected by *Idylla KRAS* Mutation Test. Among four cases that showed <2% of MA (#10, #19, #22 and #33), only one case (#33) was detected by *Idylla KRAS* Mutation Test. Statistical measures of performance *Idylla KRAS* Mutation Test had 100% specificity, a clinical sensitivity (55.1%) higher than Sanger sequencing (41.3%), and identical to that of ASLNAqPCR (55.1%). Clinical sensitivity (71.1%), NPV (69.6% vs 55.1%) and accuracy (82.546% vs 71.1%) of 454-NGS were higher than *Idylla KRAS* Mutation Test.

When the cases with <5% MA were excluded from the analysis, the *Idylla KRAS* Mutation Test clinical sensitivity increased up to 61.9% (with 100% specificity), higher than Sanger sequencing (52.3%) and ASLNAqPCR (57.1%). Clinical sensitivity (66.6% vs 61.9%), NPV (69.6% vs 66.6%) and accuracy (81.1% vs 78.3%) of 454-NGS were similar to *Idylla KRAS* Mutation Test.

2.2.3 Discussion

In both cystic and solid pancreatic lesions, the assessment of *KRAS* mutational status may be useful to refine the diagnosis of uncertain EUS-FNA samples;[21] while in solid lesions, to state malignancy is crucial, in cystic lesions, the approach is different and is nowadays more to differentiating mucinous from non-mucinous lesions. In any case, *KRAS* testing should have a fast turnaround testing, in line with the need of urgent clinical actions. Thus, instead of outsourcing suspicious pancreatic EUS-FNA to a small number of referral molecular pathology laboratories, *KRAS* testing may be carried out in the same centre where the patient is being diagnosed, enabling the most cellular slide to be easily selected from in-house collected cytological material. However, *KRAS* testing is difficult to be implemented in cytopathology laboratories, with little expertise in molecular biology procedures and in-house validation of a laboratory-developed methods.

The *Idylla KRAS* Mutation Test, a rapid and fully automated CE-IVD (European Conformity-In Vitro Diagnostic)-certified test, can represent a valid option for a wider number of pathological centres.[1-3] The assay has been designed by the manufacturer to process FFPE tissue sections using high-frequency intensity-focused ultrasound technology to obtain amplifiable DNA.[1-3] In this study, however, we have shown that the extracted DNA can directly be placed inside an *Idylla KRAS* Mutation Test cartridge. Usually the EUS-FNA sampling of a pancreatic lesion yields abundant contaminating gastrointestinal cells; thus, a highly analytical sensitive technique is required to detect *KRAS* mutation in a minority of neoplastic cells.[34] On the other side, a supersensitive test may detect early *KRAS* mutant clones even in small foci of pancreatic intraepithelial neoplasia, whose clinical significance is

questionable.[35] As stated by the manufacturer, the *Idylla KRAS* Mutation Test has a limit of detection of 5% (validated at the 95% CI) of MA, which seems to be well suited for clinical applications. Accordingly, in this study, the *Idylla KRAS* Mutation Test had a clinical sensitivity (55.1%) higher than Sanger sequencing (41.3%), but lower than 454-NGS (71.1%).In fact, 454-NGS had the capability to detect small mutant clones in 10 cases, which was achieved in only four cases by *Idylla™*. Noteworthy, low abundance mutant cases usually were IPMN; only case #55, featuring as little as 4% of MA and detected only by 454 NGS was an overt malignant neoplasm. When the low-abundant mutant cases were excluded from the analysis, the *Idylla KRAS* Mutation Test clinical sensitivity increased from 55.1% up to 61.9% approaching that of 454-NGS (66.6%). The allelic discrimination laboratory-developed technique can be applied to EUS-FNA with several advantages as it was very recently shown by Bournet et al,[25] who developed TaqMan probe sets to identify the most prevalent *KRAS* codon 12 mutations(p.G12R- c.34G>C; p.G12D- c.35G>A; p.G12V-c.35G>T). In their experience, based on the analysis of solid suspect pancreatic lesions, the sensitivity of cytopathology alone increased from 73% to 88%[25] Compared with a similar qRT-PCR-based approach, ASLNAqPCR, *Idylla KRAS* Mutation Test was less sensitive in low-abundant MA cases, but detected exon 3 mutations in four instances. Only one of these cases harbouring a double G12V/Q61H mutation was detected by ASLNAqPCR. No mutation were detected in exon 4, confirming that these are uncommon in pancreatic cancer.[29] Their inclusion in the reference range of the *Idylla KRAS* Mutation Test probably reflects its design for other common cancer types, such as colon cancer. Overall, six different mutation types (G12C, G12D, G12R, G12V, Q61H and Q61R/L) were detected by the *Idylla KRAS* Mutation Test, confirming that the whole spectrum of clinically relevant mutations for pancreatic cancer is covered.[21] The clinical specificity of *Idylla KRAS* Mutation Test was 100% and no mutant cases were detected in benign lesions; conversely, *Idylla KRAS* Mutation Test missed only one G12D mutation detected by ASLNAqPCR and confirmed by Sanger sequencing. It may be important to note that the *Idylla* tests were performed on archived DNA that had been stored for several years and shipped to Naples before testing, while the other methods used freshly prepared DNA tested directly in Bologna. To assess whether a confirmation may be needed to avoid any possibility of FN results, a prospective study is required. Additionally, the performance of the *Idylla KRAS* Mutation test may be further improved by prior cytological observation of the harvested material and tumour cell enrichment.[29] In

conclusion, this is the first study that applied the novel *Idylla KRAS* test to the clinical setting. In particular, this system can be easily implemented in the routine assessment of pancreatic EUS-FNA samples to quickly provide information on *KRAS* mutational status that can supplement cytological evaluation.

2.3 *Idylla KRAS* testing on archival cytological smears

Subsequently to the previous experience with *Idylla KRAS* testing on extracted DNA from fresh cells of pancreatic EUS-FNA, it was evaluated this assay performance on the DNA extracted from archival smears of pancreatic lesions and metastatic CRC. In routine practice, *KRAS* testing is usually performed on resected primary tumor samples of CRC, but the cytological sampling of the metastatic sites is useful when there is complete response to neoadjuvant in patients with rectal cancer[6] and to serially monitor for the emergence of mutant treatment resistant clones.[7 8]

In both pancreatic and colorectal cancer patients, *KRAS* testing should have a fast turnaround testing, in line with the need of urgent clinical actions. Thus, instead of outsourcing cytological samples to a small number of referral molecular pathology laboratories, *KRAS* testing may be carried out in the same center where the patient is diagnosed. Unfortunately, the competence needed to validate and run complex molecular diagnostic assays is beyond most cytopathology laboratories.[10] However, the automated allele-specific real-time polymerase chain reaction (RT-PCR) technology is advancing at rapid pace. In particular, the fully automated molecular diagnostics system *Idylla™* is a fascinating technology.[1-3] Sample preparation is combined with PCR thermocycling and fluorescence detection of target sequences. Without needing highly skilled staff, within approximately 90 min, the *Idylla™* mutational tests approved for *in vitro* diagnostic use by the European Community (CE-IVD) marked can genotype *KRAS* with 5% detection limit, enabling method standardization even in those diagnostic units without molecular infrastructures.[14] Although the *Idylla™* tests were designed for use with formalin-fixed paraffin-embedded (FFPE) sections, as we previously have shown the *Idylla™* system can also process DNA preparations obtained from fresh cells.[14] The aim of this study is to assess *Idylla™* performance on the DNA extracted from archivial smears of pancreatic and mCRC.

2.3.1 Materials and Methods

Study cases

The database of the Cytopathology Department at the University of Naples was searched to select archival smears of pancreatic cancer (*n* = 10) and metastatic adenocarcinoma of colorectal origin (*n* = 11). The smears were reviewed by two qualified pathologists, EV and MdA, and the diagnosis was confirmed, and in all cases, the purity of tumor cells was estimated as a percentage of malignant cells out of the total nucleated cells. Since the analytic sensitivity of the *Idylla™* test is high (5% of mutant allele detection), cases were selected without a specific requirement of neoplastic cellularity. Archival smears were processed between March and May 2016. A single slide was retrieved for each case. Each slide was incubated in xylene for 3 days to allow coverslip removing and air-dried; tumor cells were scraped off directly from the whole glass surface. Genomic DNA was extracted using the QIAamp DNA Mini Kit (Qiagen, Crawley, West Sussex, UK) and quantified (ng/µl) as previously described.[36] All cases were tested by *Idylla™* regardless from the DNA concentration. Since *KRAS* mutational analysis is part of the routine diagnostic workup of patients with pancreatic and colorectal lesions, the need for Ethic Committee's approval was not necessary for this study, in accordance with Medical Ethical Guidelines of the University Federico II Medical School. Accordingly to these guidelines, a comprehensive written informed consent was signed for the procedure (fine needle aspiration) that produced the tissue samples. All information regarding the human material was managed using anonymous numerical codes. All samples were handled in compliance with the Declaration of Helsinki (http://www.wma.net/ en/30publications/10policies/b3/).

To perform the analysis, 10 ng of extracted genomic DNA was directly pipetted inside an Idylla KRAS mutation test cartridge. This latter was, then, loaded into the *Idylla™* instrument. The fully automated analysis required approximately 2 h. The *Idylla™* console autoanalyzed the PCR curve to determine the presence or absence of a *KRAS* mutation and the results were presented on screen as either "no mutation detected" or "*KRAS* mutation detected," indicating the specific mutation. To assess the performance of *Idylla KRAS*

mutation test, in any single case, extracted DNA was analyzed by the CE-IVD marked Easy *KRAS* kit (Diatech Pharmacogenetics, Jesi, Italy) on a QuantStudio 5 instrument (Thermo Fisher, Monza, Italy) and the obtained results were compared.[37] The CE-IVD marked Easy *KRAS* kit detects all relevant mutations of exon 2, 3, and 4 of *KRAS* by standard RT-PCR.

2.3.2 Results

Idylla™ yielded valid results in 18/21 (85.7%) samples (Table 5) in a first run.

N. CASE	SITE	CYTOLOGICAL DIAGNOSIS	SMEAR STAINING	% NEOPLASTIC CELLS	[ng/µl]	REAL-TIME PCR	IDYLLA
1	Pancreatic body	PDAC	PAP	15	0,7	WT	WT
2	Pancreatic head	PDAC	PAP	5	3,62	WT	WT
3	Pancreatic body	PDAC	PAP	15	2,74	WT	WT
4	Pancreatic head	PDAC	PAP	60	3,59	WT	WT
5	Pancreatic head	PDAC	PAP	15	2,86	WT	WT
6	Pancreatic head-body	PDAC	DIFF QUIK	25	9,43	MT (G12D)	MT (G12D)
7	Pancreatic head-body	PDAC	PAP	40	2,61	MT (Q61X)	MT (Q61H)
8	Pancreatic head	PDAC	DIFF QUIK	50	3,57	MT (G12R)	MT (G12R)
9	Pancreatic head	PDAC	PAP	50	>60	MT (G12V)	MT (G12V)
10	Lung	mCRC	PAP	35	1,02	WT	WT
11	Abdominal wall	mCRC	DIFF QUIK	15	0,1	WT	WT
12	Peritoneal effusion	mCRC	PAP	35	2,49	MT (G12D)	MT(G12D)
13	Liver	mCRC	DIFF QUIK	45	40,7	MT (A59X)	MT(A59E/G/T)
14	Abdominal wall	mCRC	DIFF QUIK	30	>60	MT (G12D)	MT(G12D)
15	Lung	mCRC	PAP	50	7,97	MT (G12D)	MT(G12D)
16	Abdominal wall	mCRC	DIFF QUIK	50	1,6	MT (G13D)	MT(G13D)
17	Abdominal wall	mCRC	DIFF QUIK	60	2,74	MT (A59X)	MT(A59E/G/T)
18	Lung	mCRC	PAP	40	5,46	MT (G12V)	MT(G12V)

Table 5. *Idylla KRAS* performance on DNA extracted from 18 archival smears.
For any case the specimen site, the cytological diagnosis and staining, the neoplastic cell percentage and the DNA concentration (ng/µl) are shown *KRAS* mutational status was tested by *Idylla*™ and results were compared to standard RT-PCR.

Three samples, whose DNA concentration was <0.5 ng/µl, gave an invalid result. Only one of these cases was successfully amplified by the Easy *KRAS* kit, resulting wild type (WT). On the overall, the group of 18 cases, was equally composed (n = 9) by pancreatic and mCRC smears. Papanicolaou-stained smears were more frequent (n = 11) than Diff-Quik-stained smears (n = 7). A total of 11 cases showed a *KRAS* mutation, and a representative example is reported in Figure 6.

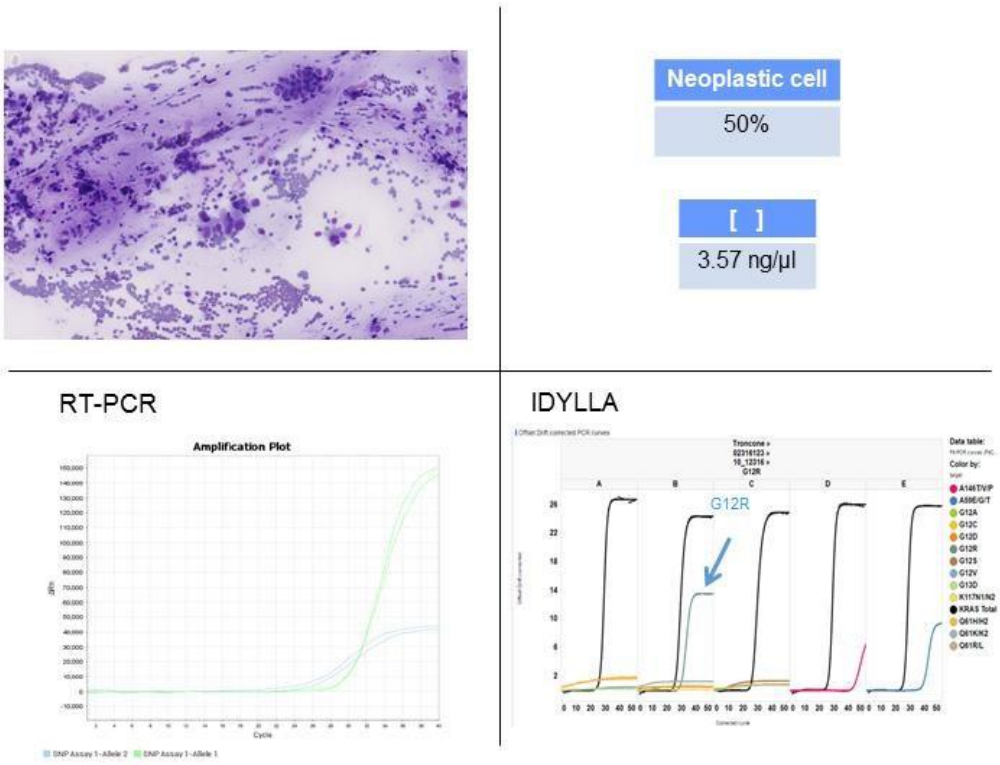


Figure 6. Example of concordant molecular results in a *KRAS* exon 2 mutated sample. (case #8, Table 5). Archival DNA was extracted from a Diff-Quik-stained smear of pancreatic cancer featuring 50% of neoplastic cells and a concentration of 3,57 ng/µl. Representative graphs of standard real-time polymerase chain reaction and *Idylla*™ are reported both showing G12R *KRAS* mutation.

In all cases, the Easy *KRAS* kit confirmed the obtained results (Table 5). In particular, the mutations detected by both techniques were the following: p.G12D (n = 4), p.G12V (n = 2), p.G12R (n = 1), p.G13D (n = 1), p.A59E/G/T (n = 2), p.Q61H (n = 1). Four mutant cases (p.G12D, p.G12R, p.G12V, and p.Q61H) were observed in the group of pancreatic smears, whereas seven mCRC smears harbored a *KRAS* mutation (n = 3 p.G12D; n = 2 p.A59E/G/T; n = 1 p.G12V; n = 1 p.G13D). The absence of mutations in five pancreatic and two mCRC smears was also confirmed by Easy *KRAS* kit. Noteworthy, most of the wild-type pancreatic cancer featured a low cellularity with the presence of <20% of cancer cells.

2.3.3 Discussion

On pancreatic fresh cells, collected by fine-needle aspiration and directly immersed in a tube with a preserving buffer, *Idylla KRAS* test showed a clinical sensitivity higher than Sanger sequencing.[14] Similar to that observed on pancreatic fresh cells, also on archival smears, the *Idylla KRAS* test performance is high and similar to that of Easy *KRAS* kit. In fact, the two methods showed a complete concordance, alleviating the concern of possible false negative results by *Idylla™*. According to manufacturer, the minimum tumor percentage to avoid false negative results is 10%. Thus, there was only onecase (#2) with really low tumor (5%) that does not rule out the possibility of false negative results by *Idylla™*. However, recent cell line dilution data showed that the *EGFR* L858R point mutation can be detected by *Idylla™* even at 1% dilution.[38]

Noteworthy, the rate of wild-type pancreatic adenocarcinoma was higher than the documented rates in most other published series.[34] This may reflect the limited number of cases tested but may also be explained by the low (<20%) neoplastic cellular content of most (4/5) cases. In addition, due to the stochastic distribution of benign and malignant cells, in these cases, manual microdissection to enrich for neoplastic cells was unfeasible, leading to

a further dilution of potentially present mutant alleles. These limitations may be overcome by the more sensitive next-generation sequencing,[29] whose implementation is cost-effective only in large volume centralized laboratories.[31] Conversely, we showed that *Idylla™* may be easily adopted by a large number of cytopathology laboratories.

Only three cases yielded an invalid result that was likely due to insufficient DNA (<0.5 ng/µl). Although this system has obtained the CE-IVD mark for FFPE material, the extracted DNA from routine smears can be directly pipetted into the cartridge, which may be automatically run as if an FFPE sample had been inserted. This off-label use of the *Idylla™* test should not be seen as limiting factor since it is the same use of direct smears that classifies the procedure as a laboratory developed test, requiring in-house validation and quality control monitoring.

The time required to genotype DNA for *KRAS* mutational status assessment is approximately 2 h, compensating for the high cost related to the CE-IVD mark of a single cartridge (~€150). Although the process of removing the coverslip of archival smears does not compromise the quality of the DNA, it is time-consuming. In this study, smears archived for at least 2 years were employed, requiring even 3 days, but to avoid any delay, rapid on-site evaluation, at the time of the FNA procedure, enables the best triage of the sample for diagnosis and ancillary studies and the selection of a representative slide, that it is maintained uncoverslipped for immediate DNA extraction and *Idylla™* processing.[34]

Our results, showing that mCRC smears can reliably be tested by Idylla for *KRAS* mutation, and the recent availability of *Idylla NRAS*/*BRAF* test can give the cytopathologist the opportunity to sample metastatic sites to perform molecular analysis on cytological smears when histological resections are not available or when the patient is monitored to early detect the arising of mutant-resistant clones.[7]

In conclusion, this study demonstrates that the fully automated molecular diagnostics system *Idylla™* is a promising opportunity for *KRAS* mutation testing on cytological samples. Even in less experienced laboratories, the cytopathologist may easily integrate morphological diagnostic report with accurate molecular information relevant for diagnostic and treatment decisions. However, it should be born in mind that long-term experiences are required to assess the feasibility of this automated molecular diagnostics system, and specific educational programs are required to enable pathologists to review and sign out clinical molecular genetic/molecular pathology results, and meanwhile, the relevance of a

staff board-certified molecular pathologist or clinical molecular geneticist cannot be overemphasized.

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